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(54) Title: CYCLODEXTRIN COMPOSITIONS ENCAPSULATING A SELECTIVE ATP INHIBITOR AND USES THEREOF

(57) Abstract: The invention provides compositions comprising cyclodextrins encapsulating a selective ATP inhibitor, as well as uses thereof.



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## CYCLODEXTRIN COMPOSITIONS ENCAPSULATING A SELECTIVE ATP INHIBITOR AND USES THEREOF

### Related Applications

5           This application claims the benefit of U.S. Provisional Application No. 61/927,259, filed on January 14, 2014, and U.S. Provisional Application No. 61/992,572, filed on May 13, 2014; which are hereby incorporated by reference.

### Statement of Rights

10           This invention was made with government support under Grant Numbers R01 CA160771, P30 CA006973, and NCRR UL1 RR 025005 awarded by the National Institutes of Health. The U.S. government has certain rights in the invention. This statement is included solely to comply with 37 C.F.R. § 401.14(a)(f)(4) and should not be taken as an assertion or admission that the application discloses and/or claims only one invention.

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### Background of the Invention

          The knowledge that cancer cells rely on increased glycolysis rather than oxidative phosphorylation for survival is known as the “Warburg hypothesis” (Warburg (1956) *Science* 123:309-314). This concept constitutes the basis for using glycolysis and its  
20   associated enzymes as unique targets for the development of new anticancer therapeutic agents (Shaw (2006) *Curr. Opin. Cell Biol.* 18:598-608; Gatenby and Gillies (2007) *Biochem. Cell Biol.* 39:1358-1366). One such agent is 3-bromopyruvate (3-BrPA), a synthetic brominated derivative of pyruvic acid that acts as an irreversible glycolytic inhibitor (Ko *et al.* (2001) *Cancer Lett.* 173:83-91; Geschwind *et al.* (2002) *Cancer Res.*  
25   62:3909-3913). It disrupts energy metabolism by targeting the glycolytic enzyme, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) (Ganapathy-Kanniappan *et al.* (2009) *Anticancer Res.* 29:4909-4918). Further, the anticancer effects of 3-BrPA have been consistent and reproducible against multiple tumor models both *in vitro* and *in vivo*. A wide variety of tumors have been demonstrated to be sensitive to 3-BrPA treatment,  
30   including, for example, liver cancer (Geschwind *et al.* (2002) *Cancer Res.* 62:3909-30913; Vali *et al.* (2007) *J. Vasc. Interv. Radiol.* 18:95-101; and Ganapathy-Kanniappan *et al.* (2012) *Radiology* 262:834-845), pancreatic cancer (Cao *et al.* (2008) *Clin. Cancer Res.* 14:1831-1839; Bhardwaj *et al.* (2010) *Anticancer Res.* 30:743-749; and Ota *et al.* (2013)

*Target Oncol.* 8:145-151), brain tumor (El Sayed *et al.* (2012) *J. Bioenerg. Biomembr.* 44:61-79; Davidescu *et al.* (2012) *J. Bioenerg. Biomembr.* 44:51-60) and breast cancer (Buijs *et al.* (2013) *J. Vasc. Interv. Radiol.* 24:737-743). Together, the inhibition of GAPDH and the molecular specificity of 3-BrPA have established that targeting tumor glycolysis via 3-BrPA could be a viable strategy in treating cancer, especially solid malignancies (Ganapathy-Kanniappan *et al.* (2012) *Oncotarget* 3:940-95; Ganapathy-Kanniappan *et al.* (2013) *Anticancer Res.* 33:13-20).

Despite the potential of selective ATP inhibitors, such as 3-halopyruvates like 3-BrPA, for therapeutic use, however, there are several factors that have hampered development of systemic administration formulations. For example, the alkylation (chemical) properties of 3-halopyruvates and related compounds render them very reactive with electrophilic molecules that has generally required increases in dosing with the negative effect of increasing toxicity, especially increased alkylation near the injection site. In particular, the presence of water or any nucleophilic group, such as amino or sulfhydryl groups commonly found in proteins, chemically inactivates the compound. Also, the *in vivo* stability of such compounds is influenced by multiple factors including glutathione, NADH and other reducing molecules in the blood and circulatory system. Hence, it is critical that the compounds remain unaffected by such factors, at least until the first pass of circulation.

While recognizing that protecting selective ATP inhibitors, such as 3-halopyruvates like 3-BrPA, until they are delivered to organs or tissues is critical for their antitumor efficacy under systemic delivery, numerous approaches to achieve such protection, such as encapsulating them in liposomes, microspheres, nanospheres, nanoparticles, bubbles, and the like, have not been successful. For example, it is known that molecules such as 3-BrPA undesirably leach out rapidly from PEGylated liposomes or react with proteins such as albumin in albumin-based nanoparticles. Although sporadic reports have documented the intraperitoneal delivery of 3-BrPA in preclinical models, the efficacy and dosage regimen were very limited. Due to these failures, 3-BrPA therapies are currently relegated to loco-regional delivery (*e.g.*, percutaneous ablation, intra-arterial delivery, and intra-tumoral injections) as opposed to systemic delivery (Kunjithapatham *et al.* (2013) *BMC Res. Notes* 6:277).

Accordingly, there is a great need in the art to identify compositions of selective ATP inhibitors, such as 3-halopyruvates like 3-BrPA, suitable for systemic administration.



### Summary of the Invention

The present invention is based in part on the discovery that encapsulating selective inhibitors of ATP production, such as 3-halopyruvates (*e.g.*, 3-BrPA), within cyclodextrins both a) stabilizes the alkylating agent *in vivo* by protecting the halogen moiety away from aqueous and nucleophilic environments that would deactivate the compound and b) provides a steady release of the compound necessary to maintain a reasonable half-life of the compound *in vivo*.

In one aspect, a composition comprising a cyclodextrin and a pharmaceutical agent

represented in the general formula: 
$$\text{XH}_2\text{C}-\overset{\text{O}}{\overset{\parallel}{\text{C}}}-\overset{\text{O}}{\overset{\parallel}{\text{C}}}-\text{R}_1$$
 wherein, independently of each occurrence: X represents a halide, a sulfonate, a carboxylate, an alkoxide, or an amine oxide; R<sub>1</sub> represents OR, H, N(R'')<sub>2</sub>, C1-C6 alkyl, C6-C12 aryl, C1-C6 heteroalkyl, or C6-C12 heteroaryl; R'' represents H, C1-C6 alkyl, or C6-C12 aryl; R represents H, alkali metal, C1-C6 alkyl, C6-C12 aryl or C(O)R'; and R' represents H, C1-C20 alkyl or C6-C12 aryl, wherein the cyclodextrin encapsulates the pharmaceutical agent, is provided. In one embodiment, at least one  $\alpha$ -D-glucopyranoside unit of the cyclodextrin has at least one hydroxyl chemical group replaced with an ionizable chemical group. In another embodiment,

the at least one hydroxyl chemical group of the at least one  $\alpha$ -D-glucopyranoside unit is selected from the group consisting of C2, C3, and C6 hydroxyl chemical groups. In still another embodiment, the C2, C3, and C6 hydroxyl chemical groups of at least one  $\alpha$ -D-glucopyranoside unit of the cyclodextrin that are replaced with ionizable chemical groups. In yet another embodiment, the at least one  $\alpha$ -D-glucopyranoside unit of the cyclodextrin is selected from the group consisting of two, three, four, five, six, seven, eight, and all  $\alpha$ -D-glucopyranoside units of the cyclodextrin. In another embodiment, the ionizable chemical group is the same at all replaced positions. In still another embodiment, the ionizable chemical group is a weakly basic functional group or a weakly acidic functional group. For example, the weakly basic functional group (X) can have a pK<sub>a</sub> between 6.5 and 8.5 according to CH<sub>3</sub>-X<sup>-</sup> or the weakly acidic functional group (Y) can have a pK<sub>a</sub> between 4.0 and 6.5 according to CH<sub>3</sub>-Y. In yet another embodiment, the weakly basic or weakly acidic functional groups are selected from the group consisting of amino, ethylene diamino, dimethyl ethylene diamino, dimethyl anilino, dimethyl naphthylamino, succinyl, carboxyl, sulfonyl, and sulphate functional groups. In another embodiment, the cyclodextrin has a

pK<sub>a1</sub> of between 4.0 and 8.5. In still another embodiment, the composition is a liquid or solid pharmaceutical formulation. In yet another embodiment, the pharmaceutical agent is neutrally charged or hydrophobic. In another embodiment, the cyclodextrin is selected from the group consisting of  $\beta$ -cyclodextrin,  $\alpha$ -cyclodextrin, and  $\gamma$ -cyclodextrin. In still another embodiment, the cyclodextrin is  $\beta$ -cyclodextrin. In yet another embodiment, the pharmaceutical agent is 3-halopyruvate. In another embodiment, the pharmaceutical agent is 3-bromopyruvate. In still another embodiment, the composition is formulated for systemic administration. In yet another embodiment, the composition further comprises an anti-cancer therapeutic agent.

In another aspect, a kit comprising a composition described herein, and instructions for use, is provided.

In still another aspect, a method of treating a subject having a cancer comprising administering to the subject a therapeutically effective amount of a composition described herein, is provided. In one embodiment, the composition is administered systemically. In another embodiment, the systemic administration is selected from the group consisting of oral, intravenous, intraperitoneal, subcutaneous, and intramuscular administration. In still another embodiment, the subject is treated with at least one additional anti-cancer therapy. In yet another embodiment, the at least one additional anti-cancer therapy is radiation therapy. In another embodiment, the cancer is a solid tumor. In still another embodiment, the cancer is selected from the group consisting of liver cancer, pancreatic cancer, lung cancer and breast cancer. In yet another embodiment, the cancer is liver cancer. In another embodiment, the subject is a mammal. In still another embodiment, the mammal is a human.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The present disclosure provides a composition comprising a  $\beta$ -cyclodextrin and a pharmaceutical agent, wherein the pharmaceutical agent is a 3-halopyruvate, wherein at least one  $\alpha$ -D-glucopyranoside unit of the cyclodextrin has at least one hydroxyl chemical group replaced with an ionizable chemical group resulting in a negative charge and wherein the cyclodextrin encapsulates the pharmaceutical agent.

#### **Brief Description of Figures**

**Figure 1** shows the chemical structure and toroidal topology of beta-cyclodextrin molecule (see, for example Rasheed *et al.* (2008) *Sci. Pharm.* 76: 567-598).

**Figures 2A-2B** show the effects of 3-BrPA or Beta-CD-3-BrPA on MiaPaCa2 cells (Figure 2A) and Suit-2 cells (Figure 2B) after 24 hours of treatment.

**Figure 3** shows the effects of 3-BrPA or Beta-CD-3-BrPA on MiaPaCa2 cells after 72 hours of treatment.

**Figure 4** shows the effects of Beta-CD-3-BrPA on *in vivo* tumor growth.

**Figure 5** shows complete tumor response in 3 mice treated with Beta-CD-3-BrPA.

**Figure 6** shows the results of histopathological analysis of the orthotopic MiaPaCa-2 tumors treated as in Figures 4 and 5.

**Figure 7** shows the results of NMR spectroscopy. The magnified insert demonstrates the upfield shift of the methylene protons (0.1 ppm), which was observed upon complexation of  $\beta$ -CD-3-BrPA.

**Figure 8** shows kill curves of 2D and 3D organotypic cell cultures based on the luminescence-based cell viability in MiaPaCa-2 (upper row) and Suit-2 (middle row) cells. Cells were incubated under normoxic and hypoxic conditions for 72 hrs. prior to exposure to 3-bromopyruvate (3-BrPA), 1:1- $\beta$ -Cyclodextrine (CD)-3-BrPA, or  $\beta$ -CD only as a control, for 24 hrs. Cells were incubated for 24 hrs. before being treated with gemcitabine for 72 hrs. For the 3D organotypic cell cultures (lower row), *luc*MiaPaCa-2 cells were incubated under normoxic or hypoxic conditions for a total of 6 days. Single-time treatments with 3-BrPA or  $\beta$ -CD-3-BrPA were performed on day 5 for 24 hrs. Exposure to gemcitabine was initiated on day 3 for 72 hrs. Bioluminescence imaging was performed on day 6 to evaluate drug penetration and effects on cell viability. The lower-right box contains the immune-blots for HIF-1alpha to confirm that hypoxia was present.

**Figure 9** shows the effects of 3-bromopyruvate in 3D organotypic cell culture. Homogeneous embedding *luc*MiaPaCa-2 cells into the collagen I-matrix was confirmed by confocal light microscopy (day 1). 3D organotypic cell cultures were incubated under normoxic conditions and treated with 3-BrPA cumulatively three times on alternate days. Phase-contrast microscopy and bioluminescence imaging (the latter only for MiaPaCa-2 cells) were performed on day 6 to evaluate effects on cell morphology and viability. Immunofluorescence staining of F-actin and cleaved caspase-3 were done in cryosections of the 3D organotypic cell culture. DAPI was used as nucleic acid counterstain.

**Figure 10** shows further effects of 3-bromopyruvate in 3D organotypic cell culture. Homogeneous embedding of Suit-2 cells into the collagen I-matrix was confirmed by confocal light microscopy (day 1). 3D organotypic cell cultures were incubated under normoxic conditions and treated with 3-BrPA cumulatively three times on alternate days. Phase-contrast microscopy and bioluminescence imaging (the latter only for MiaPaCa-2 cells) were performed on day 6 to evaluate effects on cell morphology and viability. Immunofluorescence staining of F-actin and cleaved caspase-3 were done in cryosections of the 3D organotypic cell culture. DAPI was used as nucleic acid counterstain.

**Figures 11A-11D** show the effects of 3-bromopyruvate on cell invasiveness.

MiaPaCa-2 (Figure 11A) and Suit-2 (Figure 11B) cells were plated into a Boyden invasion chamber. Incubation overnight was followed by treatment with 3-bromopyruvate for 48 hrs. (MiaPaCa-2) or 72 hrs. (Suit-2). Invaded cells on the bottom side of the membrane of the invasion insert were stained using a Giemsa-like staining. Images show invaded cells at 4x, 10x, and 20x magnification. Relative quantification of invasion was calculated by measuring the area of stained cells in the entire field of view at 10x. MMP-9 activity and secretion were determined in the concentrated supernatant of MiaPaCa-2 and Suit-2 cells by zymography (Figure 11C) and Western Blot (Figure 11D). (\*) indicates statistical significant (p-value < 0.05).

**Figures 12A-12D** show *in vivo* efficacy of  $\beta$ -cyclodextrin-3-bromopyruvate. A total of 42 male nude mice were orthotopically implanted with a total of  $1.5 \times 10^6$  *luc*MiaPaCa-2 cells. After one week of xenograft growth, tumors were confirmed using bioluminescence imaging (BLI). A representative number of animals are shown in Figure 12A. Animals were randomized to receive  $\beta$ -CD-3-BrPA (N = 21), free 3-BrPA (N = 7), gemcitabine (N = 7), and  $\beta$ -CD (N = 7). Animals were imaged once per week over the course of 28 days. The overall progress of the signal is demonstrated in Figure 12B. According to Kaplan-Meier analysis, animals treated with free 3-BrPA showed excessive treatment-related toxicity leading to the loss of 5/7 animals at the end of the experiment, such that a statistically relevant number did not survive to be included in the final image analysis (Figure 12C). The vehicle-control ( $\beta$ -CD) did not demonstrate any treatment-related toxicity and was inert when given intraperitoneally (Figure 12C). Upon completion of the experiments, all animals were sacrificed and exploratory necropsies were performed in order to extract organs and to assess potential damage. No organ toxicity (tissue effects) was observed for  $\beta$ -CD-3-BrPA, when compared with the inert vehicle (Figure 12D).

**Figure 13** shows *ex vivo* pathological and immunohistochemical tumor analysis. The H&E staining of tumors treated with  $\beta$ -CD, gemcitabine, or  $\beta$ -CD-3-BrPA (3 representative tumors are shown) demonstrated the treatment effects of  $\beta$ -CD-3-BrPA. The squares within the H&E-stained whole-tumor overviews indicate the areas magnified for further analysis of the anti-tumoral effects of the drugs, which was confirmed by the staining for cleaved caspase-3 and Ki67. In addition, the marked reduction of GAPDH as the primary target of 3-BrPA, as well as MCT-1 as the specific transporter, was determined.

**Detailed Description of the Invention**

It has been determined herein that cyclodextrins can encapsulate selective inhibitors of ATP production such as 3-halopyruvates (*e.g.*, 3-BrPA), in order to stabilize the alkylating compound in an aqueous environment, as well as reduce the ability of nucleophilic entities in proteins to access it, thereby lowering its systemic toxicity and maintaining its alkylating ability. Such compositions are demonstrated herein in multiple *in vitro* cell lines, using different forms of cyclodextrins (*e.g.*, beta and alpha) and at different ratios of active agent encapsulation relative to the cyclodextrin, and in *in vivo* animal tumor models. For example, such compositions are demonstrated herein to maintain the functional characteristics of the selective inhibitors of ATP production to kill cancer cells both *in vitro* and *in vivo* such that their activity can be preserved and protected for systemic administration until it reaches the target tissue, organ, and/or tumor while minimizing toxicity. This determination was unexpected because cyclodextrins are known to have a destabilizing effect on many compounds through direct catalysis, particularly with increasing pH (Rasheed *et al.* (2008) *Sci. Pharm.* 76: 567-598). Although this catalytic effect of cyclodextrins would have been expected to be great for 3-halopyruvates since they are halogenated derivatives of pyruvic acid, it was surprisingly determined that cyclodextrins actually protected and stabilized 3-BrPA. It was further surprisingly determined that cyclodextrins modified to replace one or more hydroxyl groups on one or more of its  $\alpha$ -D-glucopyranoside units with ionizable groups resulting in negative charges (anions) stabilizes the 3-halopyruvates better than those having ionizable groups resulting in positive charges (cations) or unmodified cyclodextrins, such as unmodified alpha- or beta-cyclodextrin. Without being bound by theory, it is believed that anionic moieties on cyclodextrins force the halogen atom (*e.g.*, bromine) of a halopyruvate (*e.g.*, 3-BrPA) to sit in the cavity. It was also surprisingly determined that  $\beta$ -cyclodextrins encapsulate 3-BrPA in a form that protects and stabilizes 3-BrPA for *in vivo* efficacy especially and also *in vitro* efficacy significantly better than  $\alpha$ -cyclodextrins.

Thus, the present invention provides compositions and kits comprising such 3-halopyruvate compounds encapsulated within cyclodextrins, as well as methods of making and using such compositions and kits.

A. Definitions

In order for the present invention to be more readily understood, certain terms and phrases are defined below and throughout the specification.

5 The articles “a” and “an” are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

The term “3-bromopyruvate” or “3-BrPA” refers to 3-bromopyruvate, analogs and derivatives of 3-bromopyruvate, prodrugs of 3-bromopyruvate, metabolites of 3-bromopyruvate and salts thereof.

10 The term “administering” means providing a pharmaceutical agent or composition to a subject, and includes, but is not limited to, administering by a medical professional and self-administering.

The term “cancer” includes, but is not limited to, solid tumors and blood borne tumors. The term cancer includes diseases of the skin, tissues, organs, bone, cartilage,  
15 blood and vessels. The term “cancer” further encompasses primary and metastatic cancers.

The term “inhibit” or “inhibits” means to decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease, disorder, or condition, the activity of a biological pathway, or a biological activity, such as the growth of a solid malignancy, *e.g.*, by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%,  
20 99%, or even 100% compared to an untreated control subject, cell, biological pathway, or biological activity or compared to the target, such as a growth of a solid malignancy, in a subject before the subject is treated. By the term “decrease” is meant to inhibit, suppress, attenuate, diminish, arrest, or stabilize a symptom of a cancer disease, disorder, or condition. It will be appreciated that, although not precluded, treating a disease, disorder or  
25 condition does not require that the disease, disorder, condition or symptoms associated therewith be completely eliminated.

The term “modulation” refers to upregulation (*i.e.*, activation or stimulation), downregulation (*i.e.*, inhibition or suppression) of a response, or the two in combination or apart.

30 The term “pharmaceutically acceptable” is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and

animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The term “pharmaceutically-acceptable carrier” as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid  
5 filler, diluent, excipient, or solvent encapsulating material, involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be “acceptable” in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1)  
10 sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols,  
15 such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates and/or polyanhydrides; and (22)  
20 other non-toxic compatible substances employed in pharmaceutical formulations.

The term “pharmaceutically-acceptable salts” refers to the relatively non-toxic, inorganic and organic salts of compounds.

A “subject” can include a human subject for medical purposes, such as for the treatment of an existing disease, disorder, condition or the prophylactic treatment for  
25 preventing the onset of a disease, disorder, or condition or an animal subject for medical, veterinary purposes, or developmental purposes. Suitable animal subjects include mammals including, but not limited to, primates, e.g., humans, monkeys, apes, gibbons, chimpanzees, orangutans, macaques and the like; bovines, e.g., cattle, oxen, and the like; ovines, e.g., sheep and the like; caprines, e.g., goats and the like; porcines, e.g., pigs, hogs,  
30 and the like; equines, e.g., horses, donkeys, zebras, and the like; felines, including wild and domestic cats; canines, including dogs; lagomorphs, including rabbits, hares, and the like; and rodents, including mice, rats, guinea pigs, and the like. An animal may be a transgenic animal. In some embodiments, the subject is a human including, but not limited to, fetal,



neonatal, infant, juvenile, and adult subjects. Further, a “subject” can include a patient afflicted with or suspected of being afflicted with a disease, disorder, or condition. Thus, the terms “subject” and “patient” are used interchangeably herein. Subjects also include animal disease models (e.g., rats or mice used in experiments, and the like).

5           The terms “prevent,” “preventing,” “prevention,” “prophylactic treatment,” and the like refer to reducing the probability of developing a disease, disorder, or condition in a subject, who does not have, but is at risk of or susceptible to developing a disease, disorder, or condition.

10           The term “subject suspected of having” means a subject exhibiting one or more clinical indicators of a disease or condition. In certain embodiments, the disease or condition is cancer. In certain embodiments, the cancer is leukemia or lymphoma.

          The term “subject in need thereof” means a subject identified as in need of a therapy or treatment.

15           The terms “systemic administration,” “administered systemically,” “peripheral administration,” and “administered peripherally” mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the patient's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

20           The term “therapeutic agent” or “pharmaceutical agent” refers to an agent capable of having a desired biological effect on a host. Chemotherapeutic and genotoxic agents are examples of therapeutic agents that are generally known to be chemical in origin, as opposed to biological, or cause a therapeutic effect by a particular mechanism of action, respectively. Examples of therapeutic agents of biological origin include growth factors, hormones, and cytokines. A variety of therapeutic agents is known in the art and may be  
25           identified by their effects. Certain therapeutic agents are capable of regulating red cell proliferation and differentiation. Examples include chemotherapeutic nucleotides, drugs, hormones, non-specific (*e.g.* non-antibody) proteins, oligonucleotides (*e.g.*, antisense oligonucleotides that bind to a target nucleic acid sequence (*e.g.*, mRNA sequence)), peptides, and peptidomimetics.

30           The term “therapeutic effect” refers to a local or systemic effect in animals, particularly mammals, and more particularly humans, caused by a pharmacologically active substance. The term thus means any substance intended for use in the diagnosis, cure, mitigation, treatment or prevention of disease or in the enhancement of desirable physical

or mental development and conditions in an animal or human. The phrase “therapeutically-effective amount” means that amount of such a substance that produces some desired local or systemic effect at a reasonable benefit/risk ratio applicable to any treatment. In certain embodiments, a therapeutically effective amount of a compound will depend on its  
5 therapeutic index, solubility, and the like. For example, certain compounds discovered by the methods of the present invention may be administered in a sufficient amount to produce a reasonable benefit/risk ratio applicable to such treatment.

The terms “therapeutically-effective amount” and “effective amount” as used herein means that amount of a compound, material, or composition comprising a compound of the  
10 present invention which is effective for producing some desired therapeutic effect in at least a sub-population of cells in an animal at a reasonable benefit/risk ratio applicable to any medical treatment.

The term “treating” a disease in a subject or “treating” a subject having a disease refers to subjecting the subject to a pharmaceutical treatment, *e.g.*, the administration of a  
15 drug, such that at least one symptom of the disease is decreased or prevented from worsening.

The terms “tumor,” “solid malignancy,” or “neoplasm” refer to a lesion that is formed by an abnormal or unregulated growth of cells. Preferably, the tumor is malignant, such as that formed by a cancer.

20 B. Cyclodextrins

The term “cyclodextrin” refers to a family of cyclic oligosaccharides composed of 5 or more  $\alpha$ -D-glucopyranoside units linked together by C1-C4 bonds having a toroidal topological structure, wherein the larger and the smaller openings of the toroid expose certain hydroxyl groups of the  $\alpha$ -D-glucopyranoside units to the surrounding environment  
25 (*e.g.*, solvent) (see, for examples, Figure 1). The term “inert cyclodextrin” refers to a cyclodextrin containing  $\alpha$ -D-glucopyranoside units having the basic formula  $C_6H_{12}O_6$  and glucose structure without any additional chemical substitutions (*e.g.*,  $\alpha$ -cyclodextrin having 6 glucose monomers,  $\beta$ -cyclodextrin having 7 glucose monomers, and  $\gamma$ -cyclodextrin having 8 glucose monomers). The term “cyclodextrin internal phase” refers to the  
30 relatively less hydrophilic region enclosed within (*i.e.*, encapsulated by) the toroid topology of the cyclodextrin structure. The term “cyclodextrin external phase” refers to the region not enclosed by the toroid topology of the cyclodextrin structure and can include, for example, the aqueous environment present during systemic administration *in vivo* or to the

internal phase of a structure that itself encapsulates the selective ATP production inhibitor/cyclodextrin complex. Cyclodextrins are useful for solubilizing hydrophobic compositions (see, for example, Albers and Muller (1995) *Crit. Rev. Therap. Drug Carrier Syst.* 12:311-337; Zhang and Ma (2013) *Adv. Drug Delivery Rev.* 65:1215-1233; Laza-Knoerr *et al.* (2010) *J. Drug Targ.* 18:645-656; Challa *et al.* (2005) *AAPS PharmSci. Tech.* 6:E329-357; Uekama *et al.* (1998) *Chem. Rev.* 98:2045-2076; Szejtli (1998) *Chem. Rev.* 98:1743-1754; Stella and He (2008) *Toxicol. Pathol.* 36:30-42; Rajewski and Stella (1996) *J. Pharm. Sci.* 85:1142-1169; Thompson (1997) *Crit. Rev. Therap. Drug Carrier Sys.* 14:1-104; and Irie and Uekama (1997) *J. Pharm. Sci.* 86:147-162). Any substance located within the cyclodextrin internal phase is said to be “encapsulated.”

As used herein, a cyclodextrin is useful according to the present invention so long as the cyclodextrins can encapsulate a selective ATP production inhibitor. In some embodiments, the cyclodextrin further bears ionizable (*e.g.*, weakly basic and/or weakly acidic) functional groups to enhance the stabilization of the selective ATP production inhibitor. By protecting the stability of the selective ATP production inhibitor, it is meant that the selective ATP production inhibitor /cyclodextrin complex makes the selective ATP production inhibitor molecule more stable as seen by photo stability, shelf life stability, thermal stability, stability against intramolecular cyclization, stability to acid hydrolysis, stability against general degradation, and the like, as compared to the stability of a selective ATP production inhibitor molecule that is not in a complex with cyclodextrin.

For encapsulating a desired therapeutic agent, cyclodextrins can be selected and/or chemically modified according to the characteristics of the desired therapeutic agent and parameters for efficient, high-concentration loading therein. For example, it is preferable that the cyclodextrin itself have high solubility in water in order to facilitate loading of a therapeutic agent, such as a 3-halopyruvate. In some embodiments, the water solubility of the cyclodextrin is at least 10 mg/mL, 20 mg/mL, 30 mg/mL, 40 mg/mL, 50 mg/mL, 60 mg/mL, 70 mg/mL, 80 mg/mL, 90 mg/mL, 100 mg/mL or higher. Methods for achieving such enhanced water solubility are well known in the art.

In some embodiments, a large association constant with the therapeutic agent is preferable and can be obtained by selecting the number of glucose units in the cyclodextrin based on the size of the therapeutic agent (see, for example, Albers and Muller (1995) *Crit. Rev. Therap. Drug Carrier Syst.* 12:311-337; Stella and He (2008) *Toxicol. Pathol.* 36:30-42; and Rajewski and Stella (1996) *J. Pharm. Sci.* 85:1142-1169). As a result, the

solubility (nominal solubility) of the therapeutic agent in the presence of cyclodextrin can be further improved. For example, the association constant of the cyclodextrin with the therapeutic agent can be 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, or higher.

Derivatives formed by reaction with cyclodextrin hydroxyl groups (*e.g.*, those lining the upper and lower ridges of the toroid of an inert cyclodextrin) are readily prepared and offer a means of modifying the physicochemical properties of the parent (inert) cyclodextrin. In some embodiments, the physicochemical properties of the inert cyclodextrin molecule or cyclodextrin molecule that is not complexed with a selective ATP production inhibitor differ from the properties of a cyclodextrin molecule complexed with the selective ATP production inhibitor. Accordingly, the selective ATP production inhibitor molecules complexed with cyclodextrin can be characterized by observing changes in solubility, chemical reactivity, UV/VIS absorbance, drug retention, chemical stability, and the like. For example, it has been determined herein that modifying hydroxyl groups, such as those facing away from the cyclodextrin interior phase, can be replaced with ionizable chemical groups to facilitate loading of therapeutic agents, such as poorly soluble or hydrophobic agents, within the modified cyclodextrins and stabilization thereof. In one embodiment, a modified cyclodextrin having at least one hydroxyl group substituted with an ionizable chemical group will result in a charged moiety under certain solvent (*e.g.*, pH) conditions. The term “charged cyclodextrin” refers to a cyclodextrin having one or more of its hydroxyl groups substituted with a charged moiety and the moiety bearing a charge. Such a moiety can itself be a charged group or it can comprise an organic moiety (*e.g.*, a  $\text{C}_1\text{-C}_6$  alkyl or  $\text{C}_1\text{-C}_6$  alkyl ether moiety) substituted with one or more charged moieties.

In one embodiment, the “ionizable” or “charged” moieties are weakly ionizable. Weakly ionizable moieties are those that are either weakly basic or weakly acidic. Weakly basic functional groups (X) have a  $\text{pK}_a$  of between about 6.0-9.0, 6.5-8.5, 7.0-8.0, 7.5-8.0, and any range in between inclusive according to  $\text{CH}_3\text{-X}$ . Similarly, weakly acidic functional groups (Y) have a log dissociation constant ( $\text{pK}_a$ ) of between about 3.0-7.0, 4.0-6.5, 4.5-6.5, 5.0-6.0, 5.0-5.5, and any range in between inclusive according to  $\text{CH}_3\text{-Y}$ . The  $\text{pK}_a$  parameter is a well-known measurement of acid/base properties of a substance and methods for  $\text{pK}_a$  determination are conventional and routine in the art. For example, the  $\text{pK}_a$  values for many weak acids are tabulated in reference books of chemistry and pharmacology. See, for example, IUPAC Handbook of Pharmaceutical Salts, ed. by P. H.

Stahl and C. G Wermuth, Wiley-VCH, 2002; CRC Handbook of Chemistry and Physics, 82nd Edition, ed. by D. R. Lide, CRC Press, Florida, 2001, p. 8-44 to 8-56. Since cyclodextrins with more than one ionizable group have pKa of the second and subsequent groups each denoted with a subscript.

5           Representative anionic moieties include, without any limitation, succinyl, carboxylate, carboxymethyl, sulfonyl, phosphate, sulfoalkyl ether, sulphate carbonate, thiocarbonate, thiocarbonate, phosphate, phosphonate, sulfonate, nitrate, and borate groups.

          Representative cationic moieties include, without limitation, amino, guanidine, and quaternary ammonium groups.

10           In another embodiment, the modified cyclodextrin is a “polyanion” or “polycation.” A polyanion is a modified cyclodextrin having more than one negatively charged group resulting in net negative ionic charge of more than two units. A polycation is a modified cyclodextrin having more than one positively charged group resulting in net positive ionic charge of more than two units.

15           In another embodiment, the modified cyclodextrin is a “chargeable amphiphile.” By “chargeable” is meant that the amphiphile has a pK in the range pH 4 to pH 8 or 8.5. A chargeable amphiphile may therefore be a weak acid or base. By “amphoteric” herein is meant a modified cyclodextrin having a ionizable groups of both anionic and cationic character wherein: 1) at least one, and optionally both, of the cation and anionic  
20 amphiphiles is chargeable, having at least one charged group with a pK between 4 and 8 to 8.5, 2) the cationic charge prevails at pH 4, and 3) the anionic charge prevails at pH 8 to 8.5.

          In some embodiments, the “ionizable” or “charged” cyclodextrins as a whole, whether polyionic, amphiphilic, or otherwise, are weakly ionizable (*i.e.*, have a pKa<sub>1</sub> of  
25 between about 4.0-8.5, 4.5-8.0, 5.0-7.5, 5.5-7.0, 6.0-6.5, and any range in between inclusive).

          Any one, some, or all hydroxyl groups of any one, some or all  $\alpha$ -D-glucopyranoside units of a cyclodextrin can be modified to an ionizable chemical group as described herein. Since each cyclodextrin hydroxyl group differs in chemical reactivity, reaction with a  
30 modifying moiety can produce an amorphous mixture of positional and optical isomers. Alternatively, certain chemistry can allow for pre-modified  $\alpha$ -D-glucopyranoside units to be reacted to form uniform products.

The aggregate substitution that occurs is described by a term called the degree of substitution. For example, a 6-ethylenediamino- $\beta$ -cyclodextrin with a degree of substitution of seven would be composed of a distribution of isomers of 6-ethylenediamino- $\beta$ -cyclodextrin in which the average number of ethylenediamino groups per 6-ethylenediamino- $\beta$ -cyclodextrin molecule is seven. Degree of substitution can be determined by mass spectrometry or nuclear magnetic resonance spectroscopy. Theoretically, the maximum degree of substitution is 18 for  $\alpha$ -cyclodextrin, 21 for  $\beta$ , and 24 for  $\gamma$ -cyclodextrin, however, substituents themselves having hydroxyl groups present the possibility for additional hydroxylalkylations. The degree of substitution can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or more and can encompass complete substitution.

Another parameter is the stereochemical location of a given hydroxyl substitution. In one embodiment, at least one hydroxyl facing away from the cyclodextrin interior is substituted with an ionizable chemical group. For example, the C2, C3, C6, C2 and C3, C2 and C6, C3 and C6, and all three of C2-C3-C6 hydroxyls of at least one  $\alpha$ -D-glucopyranoside unit are substituted with an ionizable chemical group. Such carbon positions are well known in the art. For example, the CH<sub>2</sub>OH moiety shown in Figure 1 of each  $\alpha$ -D-glucopyranoside unit represents the C6 carbon. Any such combination of hydroxyls can similarly be combined with at least two, three, four, five, six, seven, eight, nine, ten, eleven, up to all of the  $\alpha$ -D-glucopyranoside units in the modified cyclodextrin as well as in combination with any degree of substitution described herein.

It is also acceptable to combine one or more of the cyclodextrins described herein.

#### C. Selective Inhibitors of ATP Production and Related Compounds

Some embodiments of the present invention relate to the encapsulation of selective inhibitors of ATP production within cyclodextrins. The term “selective inhibitors of ATP production” refers to anti-metabolite agents that inhibit ATP production by interfering with the enzymatic process of generating ATP (*e.g.*, GAPDH inhibitors such as 3-halopyruvates like 3-bromopyruvate). In some embodiments, the selective inhibitor of ATP production is an “antineoplastic alkylating agent,” which refers to an agent used in cancer treatment that causes replacement of hydrogen by an alkyl group. As used herein the term “alkyl” refers to C<sub>1-20</sub> inclusive, linear (*i.e.*, “straight-chain”), branched, or cyclic, saturated or at least partially and in some cases fully unsaturated (*i.e.*, alkenyl and alkynyl) hydrocarbon

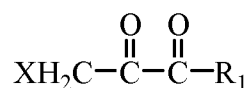
radicals derived from a hydrocarbon moiety containing between one and twenty carbon atoms by removal of a single hydrogen atom. Representative alkyl groups include, but are not limited to, methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, sec-pentyl, iso-pentyl, neopentyl, n-hexyl, sec-hexyl, n-heptyl, n-octyl, n-decyl, n-undecyl, dodecyl, and the like, ethenyl, propenyl, butenyl, pentenyl, hexenyl, octenyl, butadienyl, propynyl, butynyl, pentynyl, hexynyl, heptynyl, and allenyl groups.

“Branched” refers to an alkyl group in which a lower alkyl group, such as methyl, ethyl or propyl, is attached to a linear alkyl chain. “Lower alkyl” refers to an alkyl group having 1 to about 8 carbon atoms (i.e., a C<sub>1-8</sub> alkyl), e.g., 1, 2, 3, 4, 5, 6, 7, or 8 carbon atoms.

“Higher alkyl” refers to an alkyl group having about 10 to about 20 carbon atoms, e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 carbon atoms. In certain embodiments, “alkyl” refers, in particular, to C<sub>1-8</sub> straight-chain alkyls. In other embodiments, “alkyl” refers, in particular, to C<sub>1-8</sub> branched-chain alkyls.

Alkyl groups can optionally be substituted (a “substituted alkyl”) with one or more alkyl group substituents, which can be the same or different. The term “alkyl group substituent” includes but is not limited to alkyl, substituted alkyl, halo, arylamino, acyl, hydroxyl, aryloxy, alkoxy, alkylthio, arylthio, aralkyloxy, aralkylthio, carboxyl, alkoxy carbonyl, oxo, and cycloalkyl. There can be optionally inserted along the alkyl chain one or more oxygen, sulfur or substituted or unsubstituted nitrogen atoms, wherein the nitrogen substituent is hydrogen, lower alkyl (also referred to herein as “alkylaminoalkyl”), or aryl. Thus, as used herein, the term “substituted alkyl” includes alkyl groups, as defined herein, in which one or more atoms or functional groups of the alkyl group are replaced with another atom or functional group, including for example, alkyl, substituted alkyl, halogen, aryl, substituted aryl, alkoxy, hydroxyl, nitro, amino, alkylamino, dialkylamino, sulfate, and mercapto.

In one embodiment, selective inhibitors of ATP production are generally represented by the formula:



wherein X represents a halide, a sulfonate, a carboxylate, an alkoxide, or an amine oxide. In certain embodiments, X is a halide selected from the group consisting of: fluoride, bromide, chloride, and iodide. In one embodiment, the inhibitor is a 3-halopyruvate. In certain other embodiments, the 3-halopyruvate is selected from the group

consisting of: 3-fluoropyruvate, 3-chloropyruvate, 3-bromopyruvate and 3-iodopyruvate. In one embodiment, the 3-halopyruvate is 3-bromopyruvate. In other embodiments, X is a sulfonate and may be selected from the group consisting of: triflate, mesylate and tosylate. In yet another embodiment, X is an amine oxide is dimethylamine oxide. In certain  
5       embodiments R<sub>1</sub> represents OR, H, N(R'')<sub>2</sub>, C1-C6 alkyl, C6-C12 aryl, C1-C6 heteroalkyl, or a C6-C12 heteroaryl. Independently, in other embodiments, R'' represents H, C1-C6 alkyl, or C6-C12 aryl. Independently, in still other embodiments, R represents H, alkali metal, C1-C6 alkyl, C6-C12 aryl or C(O)R'; and R' represents H, C1-C20 alkyl or C6-C12 aryl.

10       In a preferred embodiment, the invention further provides inhibitors of ATP production represented by general formula:



wherein X represents a halide, a sulfonate, a carboxylate, an alkoxide, or an amine oxide. In certain embodiments, X is a halide and may be selected from the group consisting  
15       of: fluoride, bromide, chloride, and iodide. In one embodiment, the inhibitor is 3-halopyruvate. In certain embodiments, the 3-halopyruvate is selected from the group consisting of: 3-fluoropyruvate, 3-chloropyruvate, 3-bromopyruvate and 3-iodopyruvate. In one embodiment, the 3-halopyruvate is 3-bromopyruvate. In other embodiments, X is a sulfonate selected from the group consisting of: triflate, mesylate and tosylate. In yet  
20       another embodiment, X is an amine oxide is dimethylamine oxide.

Other analogs, derivatives, prodrugs, metabolites and salts thereof of 3-bromopyruvate can also be used, provided that these compounds or compositions have an anticancer effect that is statistically similar to that of 3-bromopyruvate. When referring herein to a treatment using 3-bromopyruvate, it should be understood that the treatment  
25       may also be conducted with analogs, derivatives, prodrugs, metabolites and salts of 3-bromopyruvate, where applicable.

#### D. Cyclodextrin/ATP Inhibitor Compositions

The present invention provides pharmaceutical compositions comprising selective  
30       inhibitors of ATP production described above encapsulated within inert and/or modified cyclodextrins. Such complexes are referred to herein as cyclodextrin/ATP inhibitor compositions. The ratio of selective inhibitor of ATP production to cyclodextrin may be



1:1 such that one inhibitor molecule forms a complex with one cyclodextrin molecule. Alternatively, the ratio can be 2:1, 3:1, 4:1, 5:1, or more.

In one aspect, the present invention provides pharmaceutically acceptable compositions which comprise a therapeutically-effective amount of one or more such cyclodextrin/ATP inhibitors described above, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. In another aspect the compositions can be administered as such or in admixtures with pharmaceutically acceptable carriers and can also be administered in conjunction with other anti-cancer therapies, such as chemotherapeutic agents, scavenger compounds, radiation therapy, biologic therapy, and the like. Conjunctive therapy thus includes sequential, simultaneous and separate, or co-administration of the composition, wherein the therapeutic effects of the first administered has not entirely disappeared when the subsequent compound is administered.

As described in detail below, the pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, *e.g.*, those targeted for buccal, sublingual, and systemic absorption, boluses, powders, granules, pastes for application to the tongue; (2) parenteral administration, for example, by subcutaneous, intramuscular, intravenous or epidural injection as, for example, a sterile solution or suspension, or sustained-release formulation; (3) topical application, for example, as a cream, ointment, or a controlled-release patch or spray applied to the skin; (4) intravaginally or intrarectally, for example, as a pessary, cream or foam; (5) sublingually; (6) ocularly; (7) transdermally; or (8) nasally.

As set out above, certain embodiments of the selective ATP inhibitors or cyclodextrin/ATP inhibitor compositions may contain a basic functional group, such as amino or alkylamino, and are, thus, capable of forming pharmaceutically-acceptable salts with pharmaceutically-acceptable acids. These salts can be prepared *in situ* in the administration vehicle or the dosage form manufacturing process, or by separately reacting a purified compound of the invention in its free base form with a suitable organic or inorganic acid, and isolating the salt thus formed during subsequent purification. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, phosphate, nitrate, acetate, valerate, oleate, palmitate, stearate, laurate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphthylate, mesylate,

glucoheptonate, lactobionate, and laurylsulphonate salts and the like (see, for example, Berge et al. (1977) "Pharmaceutical Salts", *J. Pharm. Sci.* 66:1-19).

The pharmaceutically acceptable salts of the subject compounds include the conventional nontoxic salts or quaternary ammonium salts of the compounds, *e.g.*, from  
5 non-toxic organic or inorganic acids. For example, such conventional nontoxic salts include those derived from inorganic acids such as hydrochloride, hydrobromic, sulfuric, sulfamic, phosphoric, nitric, and the like; and the salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, palmitic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicylic, sulfanilic, 2-  
10 acetoxybenzoic, fumaric, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isothionic, and the like.

In other cases, the selective ATP inhibitors or cyclodextrin/ATP inhibitor compositions of the present invention may contain one or more acidic functional groups and, thus, are capable of forming pharmaceutically-acceptable salts with pharmaceutically-  
15 acceptable bases. These salts can likewise be prepared *in situ* in the administration vehicle or the dosage form manufacturing process, or by separately reacting the purified compound in its free acid form with a suitable base, such as the hydroxide, carbonate or bicarbonate of a pharmaceutically-acceptable metal cation, with ammonia, or with a pharmaceutically-acceptable organic primary, secondary or tertiary amine. Representative alkali or alkaline  
20 earth salts include the lithium, sodium, potassium, calcium, magnesium, and aluminum salts and the like. Representative organic amines useful for the formation of base addition salts include ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine and the like (see, for example, Berge et al., *supra*).

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

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

acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Cyclodextrin/ATP inhibitor composition formulations include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect.

In certain embodiments, a formulation of cyclodextrin/ATP inhibitor compositions can comprise other carriers to allow more stability, to allow more stability, different releasing properties *in vivo*, targeting to a specific site, or any other desired characteristic that will allow more effective delivery of the complex to a subject or a target in a subject, such as, without limitation, liposomes, microspheres, nanospheres, nanoparticles, bubbles, micelle forming agents, *e.g.*, bile acids, and polymeric carriers, *e.g.*, polyesters and polyanhydrides. In certain embodiments, an aforementioned formulation renders orally bioavailable a compound of the present invention.

Liquid dosage formulations of cyclodextrin/ATP inhibitor compositions include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

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

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan

esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

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

In solid dosage forms (*e.g.*, capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically-acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid;  
15 (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents,  
20 such as, for example, cetyl alcohol, glycerol monostearate, and non-ionic surfactants; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the compositions may also comprise buffering agents. Solid compositions of a similar type  
25 may also be employed as fillers in soft and hard-shelled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

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

suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

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

Formulations for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the invention with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active compound.

Formulations which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

Dosage forms for the topical or transdermal administration of a cyclodextrin/ATP inhibitor composition of the present invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically-acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

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

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

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

15           Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this invention.

            Pharmaceutical compositions suitable for parenteral administration can comprise sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions  
20 just prior to use, which may contain sugars, alcohols, antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

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

30           In certain embodiments, the above-described pharmaceutical compositions can be combined with other pharmacologically active compounds ("second active agents") known in the art according to the methods and compositions provided herein. Second active agents can be large molecules (*e.g.*, proteins) or small molecules (*e.g.*, synthetic inorganic,

organometallic, or organic molecules). In one embodiment, second active agents independently or synergistically help to treat cancer.

For example, chemotherapeutic agents are anti-cancer agents. The term chemotherapeutic agent includes, without limitation, platinum-based agents, such as carboplatin and cisplatin; nitrogen mustard alkylating agents; nitrosourea alkylating agents, such as carmustine (BCNU) and other alkylating agents; antimetabolites, such as methotrexate; purine analog antimetabolites; pyrimidine analog antimetabolites, such as fluorouracil (5-FU) and gemcitabine; hormonal antineoplastics, such as goserelin, leuprolide, and tamoxifen; natural antineoplastics, such as taxanes (*e.g.*, docetaxel and paclitaxel), aldesleukin, interleukin-2, etoposide (VP-16), interferon alfa, and tretinoin (ATRA); antibiotic natural antineoplastics, such as bleomycin, dactinomycin, daunorubicin, doxorubicin, and mitomycin; and vinca alkaloid natural antineoplastics, such as vinblastine and vincristine.

Further, the following drugs may also be used in combination with an antineoplastic agent, even if not considered antineoplastic agents themselves: dactinomycin; daunorubicin HCl; docetaxel; doxorubicin HCl; epoetin alfa; etoposide (VP-16); ganciclovir sodium; gentamicin sulfate; interferon alfa; leuprolide acetate; meperidine HCl; methadone HCl; ranitidine HCl; vinblastin sulfate; and zidovudine (AZT). For example, fluorouracil has recently been formulated in conjunction with epinephrine and bovine collagen to form a particularly effective combination.

Still further, the following listing of amino acids, peptides, polypeptides, proteins, polysaccharides, and other large molecules may also be used: interleukins 1 through 18, including mutants and analogues; interferons or cytokines, such as interferons  $\alpha$ ,  $\beta$ , and  $\gamma$ ; hormones, such as luteinizing hormone releasing hormone (LHRH) and analogues and, gonadotropin releasing hormone (GnRH); growth factors, such as transforming growth factor- $\beta$  (TGF- $\beta$ ), fibroblast growth factor (FGF), nerve growth factor (NGF), growth hormone releasing factor (GHRF), epidermal growth factor (EGF), fibroblast growth factor homologous factor (FGFHF), hepatocyte growth factor (HGF), and insulin growth factor (IGF); tumor necrosis factor- $\alpha$  &  $\beta$  (TNF- $\alpha$  &  $\beta$ ); invasion inhibiting factor-2 (IIF-2); bone morphogenetic proteins 1-7 (BMP 1-7); somatostatin; thymosin- $\alpha$  -1;  $\gamma$ -globulin; superoxide dismutase (SOD); complement factors; anti-angiogenesis factors; antigenic materials; and pro-drugs.

Chemotherapeutic agents for use with the compositions and methods of treatment described herein include, but are not limited to alkylating agents such as thiotepa and cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gammall and calicheamicin omegall; dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores, aclacinomysins, actinomycin, authrarnycin, azaserine, bleomycins, cactinomycin, carabycin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiothane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil;



amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex);  
 5 razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, *e.g.*,  
 10 paclitaxel and doxetaxel; chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum coordination complexes such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (*e.g.*, CPT-11); topoisomerase inhibitor RFS 2000;  
 15 difluoromethylomithine (DMFO); retinoids such as retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

In another embodiment, the composition of the invention may comprise other biologically active substances, including therapeutic drugs or pro-drugs, for example, other chemotherapeutic agents, scavenger compounds, antibiotics, anti-virals, anti-fungals, anti-  
 20 inflammatories, vasoconstrictors and anticoagulants, antigens useful for cancer vaccine applications or corresponding pro-drugs.

Exemplary scavenger compounds include, but are not limited to thiol-containing compounds such as glutathione, thiourea, and cysteine; alcohols such as mannitol, substituted phenols; quinones, substituted phenols, aryl amines and nitro compounds.

25 Various forms of the chemotherapeutic agents and/or other biologically active agents may be used. These include, without limitation, such forms as uncharged molecules, molecular complexes, salts, ethers, esters, amides, and the like, which are biologically active.

### 30 E. Methods of Making Cyclodextrin/ATP Inhibitor Compositions

Methods of preparing cyclodextrin/ATP inhibitor compositions and formulations thereof include the step of bringing into association a compound of the present invention with the carrier and, optionally, one or more accessory ingredients. In general, the

formulations are prepared by uniformly and intimately bringing into association a selective inhibitor of ATP production described herein with a cyclodextrin. Generally, such complexes can be obtained by agitating and mixing the cyclodextrin (*e.g.*, a solution containing the cyclodextrin) upon dropwise addition of the therapeutic agent (*e.g.*, a solution containing the selective inhibitor of ATP production) or *vice versa*. Many mixing means are known in the art to aid in combining the inhibitor and cyclodextrin for example, without limitation, sonication, vortexing, stirring, heating, co-precipitation, neutralization, slurrying, kneading, grinding, and the like. It is possible to use a substance dissolved in a solvent or a solid substance as the therapeutic agent according to the physical properties of the therapeutic agent. There are no particular limitations on the solvent, and one can use, for example, a substance identical to the cyclodextrin external phase. The amount of the therapeutic agent that is mixed with the cyclodextrin can be equimolar quantities or in different ratios depending on the desired level of incorporation. In some embodiments, absolute amounts of the selective inhibitor of ATP production can range between 0.001 to 10 mol equivalents, 0.01 to 1 mol equivalent, or any range inclusive relative to the amount of cyclodextrin. Also, there are no particular limitations on the heating temperature. For example, 5°C or higher, room temperature or higher (*e.g.*, 20°C or higher is also preferable), are all acceptable.

Well-known methods exist for removing any undesired or unincorporated complexes or compositions, such as therapeutic agent not encapsulated by cyclodextrins or therapeutic agent cyclodextrin complexes not encapsulated by liposomes. Representative examples include, without limitation, dialysis, centrifugal separation, and gel filtration. Dialysis can be conducted, for example, using a dialysis membrane. As a dialysis membrane, one may cite a membrane with molecular weight cut-off such as a cellulose tube or Spectra/Por. With respect to centrifugal separation, centrifugal acceleration may be conducted preferably at 100,000 g or higher, and more preferably at 300,000 g or higher. Gel filtration may be carried out, for example, by conducting fractionation based on molecular weight using a column such as Sephadex or Sepharose.

In some cases, in order to prolong the effect of a drug, it is desirable to modify (*e.g.*, slow) the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form.

Alternatively, delayed absorption of a parenterally-administered drug form can be accomplished by dissolving or suspending the drug in an oil vehicle. In some embodiments, the cyclodextrin-encapsulated selective ATP inhibitor compositions described herein can be loaded into liposomes.

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

#### F.     Therapeutic Methods

          The present invention further provides novel therapeutic methods of preventing, delaying, reducing, and/or treating a cancer, including a cancerous tumor. In one  
15       embodiment, a method of treatment comprises administering to a subject (*e.g.*, a subject in need thereof), an effective amount of a cyclodextrin/selective ATP production inhibitor composition. A subject in need thereof may include, for example, a subject who has been diagnosed with a tumor, including a pre-cancerous tumor, a cancer, or a subject who has been treated, including subjects that have been refractory to the previous treatment.

20           The term “effective amount,” as in “a therapeutically effective amount,” of a therapeutic agent refers to the amount of the agent necessary to elicit the desired biological response. As will be appreciated by those of ordinary skill in this art, the effective amount of an agent may vary depending on such factors as the desired biological endpoint, the agent to be delivered, the composition of the pharmaceutical composition, the target tissue  
25       or cell, and the like. More particularly, the term “effective amount” refers to an amount sufficient to produce the desired effect, *e.g.*, to reduce or ameliorate the severity, duration, progression, or onset of a disease, disorder, or condition, or one or more symptoms thereof; prevent the advancement of a disease, disorder, or condition, cause the regression of a disease, disorder, or condition; prevent the recurrence, development, onset or progression of  
30       a symptom associated with a disease, disorder, or condition, or enhance or improve the prophylactic or therapeutic effect(s) of another therapy.

          The methods of the present invention may be used to treat any cancerous or pre-cancerous tumor. In certain embodiments, the cancerous tumor has a highly glycolytic

phenotype. For example, highly glycolytic tumors may be located in a tissue selected from brain, colon, urogenital, lung, renal, prostate, pancreas, liver, esophagus, stomach, hematopoietic, breast, thymus, testis, ovarian, skin, bone marrow and/or uterine tissue. In some embodiments, methods and compositions of the present invention may be used to

5 treat any cancer. Cancers that may be treated by methods and compositions of the invention include, but are not limited to, cancer cells from the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestinal, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. In addition, the cancer may specifically be of the following histological type, though it is not limited to

10 these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and

15 cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; bronchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma;

20 follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometrioid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary

cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma;

25 mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; and rhabdomyosarcoma, malignant; sertoli cell carcinoma; leydig

30 cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malignant melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma;

fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma;  
 leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar  
 rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor;  
 nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner  
 5 tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant;  
 dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant;  
 choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma,  
 malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma;  
 osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant;  
 10 mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic  
 tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic  
 fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma;  
 astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma;  
 glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal;  
 15 cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory  
 neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant;  
 granular cell tumor, malignant; malignant lymphoma; Hodgkin's disease; Hodgkin's  
 lymphoma; paraganuloma; malignant lymphoma, small lymphocytic; malignant  
 lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other  
 20 specified non-Hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell  
 sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia;  
 plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia;  
 basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia;  
 megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

25 The compositions described herein may be delivered by any suitable route of  
 administration, including orally, nasally, transmucosally, ocularly, rectally, intravaginally,  
 parenterally, including intramuscular, subcutaneous, intramedullary injections, as well as  
 intrathecal, direct intraventricular, intravenous, intra-articular, intra-sternal, intra-synovial,  
 intra-hepatic, intralesional, intracranial, intraperitoneal, intranasal, or intraocular injections,  
 30 intracisternally, topically, as by powders, ointments or drops (including eyedrops),  
 including buccally and sublingually, transdermally, through an inhalation spray, or other  
 modes of delivery known in the art.

The terms “systemic administration,” “administered systemically,” “peripheral administration,” and “administered peripherally” as used herein mean the administration of the selective ATP production inhibitor/cyclodextrin complex such that it enters the patient's system and, thus, is subject to metabolism and other like processes.

5       The terms “parenteral administration” and “administered parenterally” as used herein mean modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intarterial, intrathecal, intracapsular, intraorbital, intraocular, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid,  
10   intrapinal and intrasternal injection and infusion.

      In certain embodiments the pharmaceutical compositions are delivered generally ( *e.g.*, via oral or parenteral administration). In certain other embodiments the pharmaceutical compositions are delivered locally through direct injection into a tumor or direct injection into the tumor's blood supply (*e.g.*, arterial or venous blood supply). In  
15   some embodiments, the pharmaceutical compositions are delivered by both a general and a local administration. For example, a subject with a tumor may be treated through direct injection of a composition containing a composition described herein into the tumor or the tumor's blood supply in combination with oral administration of a pharmaceutical composition of the present invention. If both local and general administration is used, local  
20   administration can occur before, concurrently with and/or after general administration.

      In certain embodiments, the methods of treatment of the present invention, including treating a cancerous or pre-cancerous tumor comprise administering compositions described herein in combination with a second agent and/or therapy to the subject. By “in combination with” is meant the administration of the selective ATP production  
25   inhibitor/cyclodextrin complexes with one or more therapeutic agents either simultaneously, sequentially, or a combination thereof. Therefore, a subject administered a combination of the selective ATP production inhibitor/cyclodextrin complexes and/or therapeutic agents, can receive the selective ATP production inhibitor/cyclodextrin complexes as described herein, and one or more therapeutic agents at the same time (*i.e.*,  
30   simultaneously) or at different times (*i.e.*, sequentially, in either order, on the same day or on different days), so long as the effect of the combination of both agents is achieved in the subject. When administered sequentially, the agents can be administered within 1, 5, 10, 30, 60, 120, 180, 240 mins. or longer of one another. In other embodiments, agents

administered sequentially, can be administered within 1, 5, 10, 15, 20 or more days of one another.

When administered in combination, the effective concentration of each of the agents to elicit a particular biological response may be less than the effective concentration of each agent when administered alone, thereby allowing a reduction in the dose of one or more of the agents relative to the dose that would be needed if the agent was administered as a single agent. The effects of multiple agents may, but need not be, additive or synergistic. The agents may be administered multiple times. In such combination therapies, the therapeutic effect of the first administered agent is not diminished by the sequential, simultaneous or separate administration of the subsequent agent(s).

Such methods in certain embodiments comprise administering pharmaceutical compositions comprising compositions described herein in conjunction with one or more chemotherapeutic agents and/or scavenger compounds, including chemotherapeutic agents described herein, as well as other agents known in the art. Conjunctive therapy includes sequential, simultaneous and separate, or co-administration of the composition in a way that the therapeutic effects of the first selective ATP inhibitor administered have not entirely disappeared when the subsequent compound is administered. In one embodiment, the second agent is a chemotherapeutic agent. In another embodiment, the second agent is a scavenger compound. In another embodiment, the second agent is radiation therapy. In a further embodiment, radiation therapy may be administered in addition to the composition. In certain embodiments, the second agent may be co-formulated in the separate pharmaceutical composition.

In some embodiments, the subject pharmaceutical compositions of the present invention will incorporate the substance or substances to be delivered in an amount sufficient to deliver to a patient a therapeutically effective amount of an incorporated therapeutic agent or other material as part of a prophylactic or therapeutic treatment. The desired concentration of the active compound in the particle will depend on absorption, inactivation, and excretion rates of the drug as well as the delivery rate of the compound. It is to be noted that dosage values may also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions. Typically, dosing will be determined using techniques known to one skilled in the art.

Dosage may be based on the amount of the composition or active compound thereof (*e.g.*, selective inhibitor of ATP production) per kg body weight of the patient. For example, a range of amounts of compositions or compound encapsulated therein are contemplated, including about 0.001, 0.01, 0.1, 0.5, 1, 10, 15, 20, 25, 50, 75, 100, 150, 200  
5 or 250 mg or more of such compositions per kg body weight of the patient. Other amounts will be known to those of skill in the art and readily determined.

In certain embodiments, the dosage of the composition or active compound thereof (*e.g.*, selective inhibitor of ATP production) will generally be in the range of about 0.001 mg to about 250 mg per kg body weight, specifically in the range of about 50 mg to about  
10 200 mg per kg, and more specifically in the range of about 100 mg to about 200 mg per kg. In one embodiment, the dosage is in the range of about 150 mg to about 250 mg per kg. In another embodiment, the dosage is about 200 mg per kg.

In some embodiments the molar concentration of the composition or active compound thereof (*e.g.*, selective inhibitor of ATP production) in a pharmaceutical  
15 composition will be less than or equal to about 2.5 M, 2.4 M, 2.3 M, 2.2 M, 2.1 M, 2 M, 1.9 M, 1.8 M, 1.7 M, 1.6 M, 1.5 M, 1.4 M, 1.3 M, 1.2 M, 1.1 M, 1 M, 0.9 M, 0.8 M, 0.7 M, 0.6 M, 0.5 M, 0.4 M, 0.3 M or 0.2 M. In some embodiments the concentration of the composition or active compound thereof (*e.g.*, selective inhibitor of ATP production) will be less than or equal to about 0.10 mg/ml, 0.09 mg/ml, 0.08 mg/ml, 0.07 mg/ml, 0.06 mg/ml,  
20 0.05 mg/ml, 0.04 mg/ml, 0.03 mg/ml or 0.02 mg/ml.

Alternatively, the dosage may be determined by reference to the plasma concentrations of the composition or active compound thereof (*e.g.*, selective inhibitor of ATP production). For example, the maximum plasma concentration ( $C_{\max}$ ) and the area under the plasma concentration-time curve from time 0 to infinity (AUC (0-4)) may be  
25 used. Dosages for the present invention include those that produce the above values for  $C_{\max}$  and AUC (0-4) and other dosages resulting in larger or smaller values for those parameters.

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

The selected dosage level will depend upon a variety of factors including the activity of the particular therapeutic agent in the formulation employed, or the ester, salt or



amide thereof, the route of administration, the time of administration, the rate of excretion or metabolism of the particular therapeutic agent being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compound employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could prescribe and/or administer doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

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

If desired, the effective daily dose of the active compound may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms.

The precise time of administration and amount of any particular compound that will yield the most effective treatment in a given patient will depend upon the activity, pharmacokinetics, and bioavailability of a particular compound, physiological condition of the patient (including age, sex, disease type and stage, general physical condition, responsiveness to a given dosage and type of medication), route of administration, and the like. The guidelines presented herein may be used to optimize the treatment, *e.g.*, determining the optimum time and/or amount of administration, which will require no more than routine experimentation consisting of monitoring the subject and adjusting the dosage and/or timing.

While the subject is being treated, the health of the patient may be monitored by measuring one or more of the relevant indices at predetermined times during a 24-hour period. All aspects of the treatment, including supplements, amounts, times of administration and formulation, may be optimized according to the results of such monitoring. The patient may be periodically reevaluated to determine the extent of improvement by measuring the same parameters, the first such reevaluation typically occurring at the end of four weeks from the onset of therapy, and subsequent reevaluations

occurring every four to eight weeks during therapy and then every three months thereafter. Therapy may continue for several months or even years, with a minimum of one month being a typical length of therapy for humans. Adjustments, for example, to the amount(s) of agent administered and to the time of administration may be made based on these  
5 reevaluations.

Treatment may be initiated with smaller dosages which are less than the optimum dose of the compound. Thereafter, the dosage may be increased by small increments until the optimum therapeutic effect is attained.

As described above, the composition or active compound thereof (*e.g.*, selective  
10 inhibitor of ATP production) may be administered in combination with radiation therapy. An optimized dose of radiation therapy may be given to a subject as a daily dose. Optimized daily doses of radiation therapy may be, for example, from about 0.25 to 0.5 Gy, about 0.5 to 1.0 Gy, about 1.0 to 1.5 Gy, about 1.5 to 2.0 Gy, about 2.0 to 2.5 Gy, and about 2.5 to 3.0 Gy. An exemplary daily dose may be, for example, from about 2.0 to 3.0 Gy. A  
15 higher dose of radiation may be administered, for example, if a tumor is resistant to lower doses of radiation. High doses of radiation may reach, for example, 4 Gy. Further, the total dose of radiation administered over the course of treatment may, for example, range from about 50 to 200 Gy. In an exemplary embodiment, the total dose of radiation administered over the course of treatment ranges, for example, from about 50 to 80 Gy. In certain  
20 embodiments, a dose of radiation may be given over a time interval of, for example, 1, 2, 3, 4, or 5 mins., wherein the amount of time is dependent on the dose rate of the radiation source.

In certain embodiments, a daily dose of optimized radiation may be administered, for example, 4 or 5 days a week, for approximately 4 to 8 weeks. In an alternate  
25 embodiment, a daily dose of optimized radiation may be administered daily seven days a week, for approximately 4 to 8 weeks. In certain embodiments, a daily dose of radiation may be given a single dose. Alternately, a daily dose of radiation may be given as a plurality of doses. In a further embodiment, the optimized dose of radiation may be a higher dose of radiation than can be tolerated by the patient on a daily base. As such, high  
30 doses of radiation may be administered to a patient, but in a less frequent dosing regimen.

The types of radiation that may be used in cancer treatment are well known in the art and include electron beams, high-energy photons from a linear accelerator or from

radioactive sources such as cobalt or cesium, protons, and neutrons. An exemplary ionizing radiation is an x-ray radiation.

Methods of administering radiation are well known in the art. Exemplary methods include, but are not limited to, external beam radiation, internal beam radiation, and radiopharmaceuticals. In external beam radiation, a linear accelerator is used to deliver high-energy x-rays to the area of the body affected by cancer. Since the source of radiation originates outside of the body, external beam radiation can be used to treat large areas of the body with a uniform dose of radiation. Internal radiation therapy, also known as brachytherapy, involves delivery of a high dose of radiation to a specific site in the body. The two main types of internal radiation therapy include interstitial radiation, wherein a source of radiation is placed in the effected tissue, and intracavity radiation, wherein the source of radiation is placed in an internal body cavity a short distance from the affected area. Radioactive material may also be delivered to tumor cells by attachment to tumor-specific antibodies. The radioactive material used in internal radiation therapy is typically contained in a small capsule, pellet, wire, tube, or implant. In contrast, radiopharmaceuticals are unsealed sources of radiation that may be given orally, intravenously or directly into a body cavity.

Radiation therapy may also include stereotactic surgery or stereotactic radiation therapy, wherein a precise amount of radiation can be delivered to a small tumor area using a linear accelerator or gamma knife and three dimensional conformal radiation therapy (3DCRT), which is a computer assisted therapy to map the location of the tumor prior to radiation treatment.

Toxicity and therapeutic efficacy of subject compounds may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD<sub>50</sub> and the ED<sub>50</sub>. Compositions that exhibit large therapeutic indices are preferred. In some embodiments, the LD<sub>50</sub> (lethal dosage) can be measured and can be, for example, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more reduced for the cyclodextrin-encapsulated selective ATP inhibitor compositions described herein relative to the selective ATP inhibitor without any cyclodextrin encapsulation. Similarly, the ED<sub>50</sub> (*i.e.*, the concentration which achieves a half-maximal inhibition of symptoms) can be measured and can be, for example, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more increased for the

cyclodextrin-encapsulated selective ATP inhibitor compositions described herein relative to the selective ATP inhibitor without any cyclodextrin encapsulation. Also, Similarly, the  $IC_{50}$  (*i.e.*, the concentration which achieves half-maximal cytotoxic or cytostatic effect on cancer cells) can be measured and can be, for example, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more increased for the cyclodextrin-encapsulated selective ATP inhibitor compositions described herein relative to the selective ATP inhibitor without any cyclodextrin encapsulation. Although compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets the compounds to the desired site in order to reduce side effects.

In some embodiments, the presently disclosed methods produce at least about a 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or even 100% inhibition of cancer cell growth in an assay.

In any of the above-described methods, the administering of the selective ATP production inhibitor/cyclodextrin complexes can result in at least about a 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or even 100% decrease in a solid malignancy in a subject, compared to the solid malignancy before administration of the selective ATP production inhibitor/cyclodextrin complexes.

In some embodiments, the therapeutically effective amount of a complex of a selective ATP production inhibitor/cyclodextrin is administered prophylactically to prevent a solid malignancy from forming in the subject.

In some embodiments, the subject is human. In other embodiments, the subject is non-human, such as a mammal.

The data obtained from the cell culture assays and animal studies may be used in formulating a range of dosage for use in humans. The dosage of any supplement, or alternatively of any components therein, lies preferably within a range of circulating concentrations that include the  $ED_{50}$  with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For agents of the present invention, the therapeutically effective dose may be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the  $IC_{50}$  as determined in cell culture. Such information may be used to more accurately determine useful doses in

humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

G. Kit

5           The selective ATP production inhibitor/cyclodextrin complexes and compositions described herein can be assembled into kits or pharmaceutical systems for use in treating or preventing a disease, such as cancer. In some embodiments, the 3-BrPA-cyclodextrin complex and compositions can be used to prevent or treat solid malignancies caused by a cancer. In general, a presently disclosed kit contains some or all of the components,  
10   reagents, supplies, and the like to practice a method according to the presently disclosed subject matter. The kit typically comprises an effective amount of complex to prevent, delay, reduce, or treat an unwanted disease (*e.g.*, a solid malignancy). In one embodiment, a kit comprises at least one container (*e.g.*, a carton, bottle, vial, tube, or ampoule) comprising a selective ATP production inhibitor/cyclodextrin complex and/or compositions  
15   thereof described herein. Typically, the complex and/or compositions will be supplied in one or more container, each container containing an effective amount of complex to allow a solid malignancy to regress, slow, or be arrested.

          Accordingly, in some embodiments, the presently disclosed subject matter provides a kit comprising at least one selective ATP production inhibitor encapsulated within at least  
20   one cyclodextrin carrier. In other embodiments, the kit further comprises a set of instructions for using the at least one selective ATP production inhibitor encapsulated within the at least one cyclodextrin carrier.

          It may be desirable to store the selective ATP production inhibitor and cyclodextrin separately and then combine them before use. Accordingly, in still other embodiments, the  
25   kit comprises at least one selective ATP production inhibitor in one container and at least one cyclodextrin carrier in another container.

**Exemplification**

          The following Examples have been included to provide guidance to one of ordinary  
30   skill in the art for practicing representative embodiments of the presently disclosed subject matter. In light of the present disclosure and the general level of skill in the art, those of skill can appreciate that the following Examples are intended to be exemplary only and that numerous changes, modifications, and alterations can be employed without departing from

the scope of the presently disclosed subject matter. The following Examples are offered by way of illustration and not by way of limitation.

Example 1: Materials and Methods for Examples 2-3

5           A.       General method for synthesis of modified  $\beta$ -cyclodextrins

Succinyl- $\beta$ -cyclodextrins were purchased from Sigma Chemical (St. Louis, MO, USA; Catalog No. 85990). Unmodified  $\beta$ -cyclodextrin and  $\alpha$ -cyclodextrin were purchased (Sigma-Aldrich, St. Louis, MO).

10           However, succinylated cyclodextrins can also be synthesized. For example,  $\beta$ -cyclodextrin (Sigma-Aldrich, St. Louis, MO) was mono-tosylated with 0.9 molar equivalent of tosyl chloride in pyridine at the primary 6' hydroxyl group to afford the corresponding tosylate, which was converted to the iodo-derivative by treatment with sodium iodide in acetone. The iodo derivative was converted to the desired 6' aminated cyclodextrin by heating at 80°C for 8-12 hours with the appropriate amine (Tang and Ng (2008) *Nat. Protocol.* 3:691-697). 6' mono-succinyl- $\beta$ -cyclodextrin was synthesized by treatment of  
15           parent  $\beta$ -cyclodextrin with 0.9 equivalents of succinic anhydride in DMF (Cucinotta *et al.* (2005) *J. Pharmaceut. Biomed. Anal.* 37:1009-1014). The product was precipitated in acetone and purified by HPLC before use.

The pH range with optimal stability is pH 4-9.

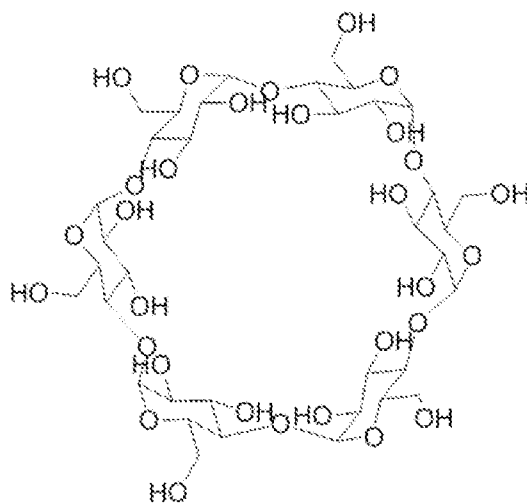
20           B.       General procedure of preparation of encapsulated complexes

A 1:1 ratio of 3-BrPA encapsulated within succinyl- $\beta$ -cyclodextrins was prepared. 3-BrPA (150 mg, 1 mmol) was added in small portions (10 mg each) to a stirring solution of succinyl-beta-cyclodextrin (1,500 mg in distilled water). After complete addition, the solution was sonicated for 1 hour at room temperature. The sonicated solution was then  
25           allowed to shake overnight on a thermomixer at 25°C, flash frozen in a dry ice-acetone bath, and lyophilized.

Similarly, a 2:1 ratio of 3-BrPA encapsulated within succinyl- $\beta$ -cyclodextrins was prepared. 3-BrPA (166 mg, 1 mmol) was added in small portions (10 mg each) to a stirring solution of succinyl-beta-cyclodextrin (918 mg in 20 ml distilled water). After complete  
30           addition, the solution was sonicated for 1 hour at room temperature. The sonicated solution was then allowed to shake overnight on a thermomixer at 25°C, flash frozen in a dry ice-acetone bath, and lyophilized.

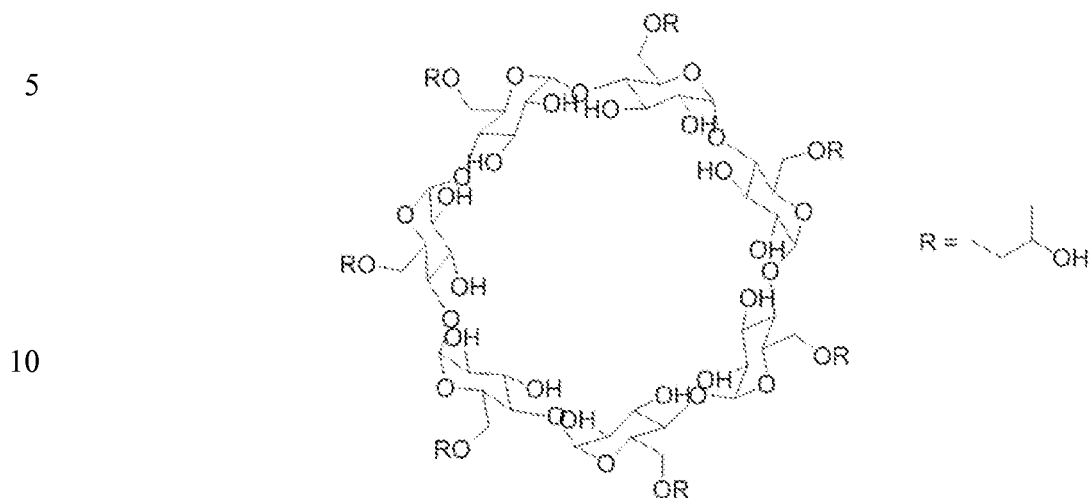
In addition, a 1:1 ratio of 3-BrPA encapsulated within  $\alpha$ -cyclodextrins (see structure below) was prepared. 3-BrPA (166 mg, 1 mmol) was added in small portions (10 mg each) to a stirring solution of alpha-cyclodextrin (972 mg, 1 mmol in 10 ml distilled water). After complete addition, the solution was sonicated for 1 hour at room temperature. The sonicated solution was then allowed to shake overnight on a thermomixer at 25°C, flash frozen in a dry ice-acetone bath, and lyophilized. Non-GRAS and GRAS versions were used with similar results.

$\alpha$ -cyclodextrin structure



It was surprisingly determined that cyclodextrins modified to replace one or more hydroxyl groups on one or more of its  $\alpha$ -D-glucopyranoside units with ionizable groups resulting in negative charges (anions) stabilizes the 3-halopyruvates better than those having ionizable groups resulting in positive charges (cations) or unmodified cyclodextrins, such as unmodified alpha- or beta-cyclodextrin. It was also surprisingly determined that  $\beta$ -cyclodextrins encapsulate 3-BrPA in a form that protects and stabilizes 3-BrPA for *in vivo* efficacy especially and also *in vitro* efficacy significantly better than  $\alpha$ -cyclodextrins.

In addition, *in vitro* cell culture and *in vivo* mouse treatments were prepared and performed as described above and below for succinyl- $\beta$ -cyclodextrins encapsulating 3-BrPA, using generally recognized as safe (GRAS) versions of  $\beta$ -cyclodextrins (*e.g.*, hydroxypropyl- $\beta$ -cyclodextrin having a level of substitution of 3-5 such as that shown in chemical form below) encapsulating 3-BrPA and the results were similar to those described for succinyl- $\beta$ -cyclodextrins encapsulating 3-BrPA.

GRAS hydroxypropyl- $\beta$ -cyclodextrin structureC. *In vitro* cell culture

3-BrPA and  $\beta$ -cyclodextrin (vehicle) were purchased from Sigma Chemical (St. Louis, MO, USA). For the viability assay, MiaPaCa-2 and Suit-2 cells were seeded in triplicates in 96-well plates at a density of  $5 \times 10^3$  cells per well. After 12 hours, cells were treated with increasing concentrations of 3-BrPA, CD-3BrPA (0-150  $\mu$ m) and the vehicle. Intracellular ATP levels were measured using a Cell Titer-Glo Luminescence Cell Viability assay kit (Promega, Durham, NC, USA) according to the manufacturer's protocol. The measurements were performed at 24 hours and 72 hours after the treatment.

D. *In vivo* mouse treatment

A total of 15 animals were randomized to receive daily injections with 5 mg/kg Beta-CD-3BrPA (in a 1:1 ratio) (N=10) or vehicle control (N=5). Baseline bioluminescence imaging confirmed tumor growth in all animals (five representative animals shown in Figure 4 and 5). After two weeks of intra-peritoneal injections, all animals were subjected to follow-up imaging. Animals treated with the vehicle demonstrated a strong increase of the bioluminescence signal, representing tumor progression.

Male athymic nude mice (20-25 g, 4 weeks old, Crl:Nu-Nu, Charles River Laboratories, Wilmington, MA, USA) were used in accordance with the institutional guidelines under approved protocols. Mice were maintained in laminar flow rooms at constant temperature and humidity with food and water given ad libitum. The MiaPaCa-2 cell line, stably transfected with the luciferase-aminoglycoside phosphotransferase fusion



gene under the control of the elongation factor 1 alpha promoter was used. Mice were anesthetized by isoflurane inhalation anesthesia before surgery and treatment. A small left abdominal flank incision was made and the pancreas was exteriorized. Orthotopic pancreatic tumors were generated by injection of  $1-2 \times 10^6$  MiaPaCa-2 cells into the tail of the pancreas. A successful subcapsular intrapancreatic injection of the tumor cells was identified by the appearance of a fluid bleb without intraperitoneal leakage.

For bioluminescence imaging (BLI), anesthetized mice bearing orthotopic tumors were injected intraperitoneally with 150 mg/kg of D-Luciferin (Gold Biotechnology, St Louis, MO, USA) and optically imaged after 5 mins. using the IVIS 100 (Xenogen Corp, Alameda, CA, USA). The pseudocolor image which represented the spatial distribution of detected photons was overlaid on a grayscale photographic image. Signal intensity was quantified with ROIs (p/s/cm<sup>2</sup>/Sr) after a 10-second exposure using Living Image software (Xenogen Corp.). Imaging was performed on day 7, 14, 21, 28 and 35 after tumor implantation.

Following the confirmation of tumor growth in each animal using BLI one week after tumor implantation, all animals were randomized in 3 groups to receive either 3-BrPA, CD-3BrPA or the vehicle via daily intra-peritoneal injections (injection volume, 500  $\mu$ l / mouse / day; dose, 5 mg / kg). The injection solution was prepared by dissolving the chemicals in phosphate buffered saline, adjusted to a pH of 7.4. Animals were observed once per hour during the initial injections and every 4-6 hours after every follow-up injection. Any changes in the overall clinical condition were noted for all treatment groups.

Within 24 hours after the last BLI imaging, animals were sacrificed using cervical dislocation. The entire abdomen was opened and tumors were obtained using en-bloc extraction with the spleen and pancreas. Tumor specimens were fixed using 4% paraformaldehyde for 72 hours, paraffin embedded, and sectioned. Histological sections were hematoxylin and eosin (H&E) stained and interpreted in consultation with a pathologist.

#### Example 2: *In Vitro* Effects of Cyclodextrins Encapsulating 3-BrPA on Human Pancreatic Cancer Cell Lines

Two cell lines of human pancreatic cancer, namely MiapaCa-2 and Suit-2, were tested for their response to 3-BrPA and CD-3-BrPA. MiaPaCa-2 is derived from a locally

invasive human adenocarcinoma and forms typical solid nodules within the pancreas. It is known to show a pronounced resistance to several standard-of-care anticancer agents, including gemcitabine. Suit-2 is derived from a highly aggressive pancreatic tumor that has been isolated from a metastatic liver mass. It has highly aggressive phenotypic properties, such as invasion and migration. The cellular response or the cell viability was assayed using the standard ATP viability assay. Each experiment was repeated at least twice, with triplicate biological samples.

Figures 2A-2B show the effects of 3-BrPA (conventional 3-BrPA in phosphate-buffered saline without any cyclodextrin encapsulation or complex formation) or beta-CD-3-BrPA (succinyl- $\beta$ -cyclodextrins encapsulating 3-BrPA in phosphate-buffered saline) on MiaPaCa-2 cells (Figure 2A) and Suit-2 cells (Figure 2B) after 24 hours of treatment. The data show a dose dependent decrease in viability of cells treated with beta-CD-3-BrPA compared to 3-BrPA treated cells. In addition, the multi-drug resistant MiaPaCa2 cells were found to be more sensitive to 3-BrPA/CD-3-BrPA than Suit2 cells. The vehicle ( $\beta$ -cyclodextrin in phosphate-buffered saline without any 3-BrPA) by itself did not contribute to any toxicity.

Figure 3 shows the effects of 3-BrPA (conventional 3-BrPA in phosphate-buffered saline without any cyclodextrin encapsulation or complex formation) or beta-CD-3-BrPA (succinyl- $\beta$ -cyclodextrins encapsulating BrPA in phosphate-buffered saline) on MiaPaCa2 cells after 72 hours of treatment. MiaPaCa-2 cells show a significant loss of viability even at ~50 uM concentration of CD-3-BrPA at 72 hours of treatment, whereas at the same concentration at 24 hours (Figure 2A), there was no significant loss of viability. Thus, with longer duration (48 hrs.) of treatment, CD-3-BrPA at fairly low concentration (50 uM) is sufficient to initiate cell death (~50% death) (Figure 3).

### Example 3: Materials and *In Vivo* Effects of Cyclodextrins Encapsulating 3-BrPA in a Mouse Model of Human Pancreatic Cancer

An athymic mouse model of human pancreatic cancer was used for *in vivo* studies. The human pancreatic cancer cell line, MiaPaca-2, stably expressing the luciferase gene, was orthotopically implanted onto the pancreas. Tumor growth and response were monitored by bioluminescence imaging.

Table1 describes the clinical signs or symptoms observed in tumor-bearing animals treated with a high-dose of 3-BrPA or CD-3-BrPA. These symptoms were recorded in an

unbiased and blind-study fashion. These symptoms were observed at a dose of >5 mg/kg body weight and at a concentration of ~3.5 mM (higher than the recommended therapeutic dose). Undesirable clinical signs or symptoms were seen in the mice treated with 3-BrPA and these signs or symptoms were significantly less in the mice treated with CD-3-BrPA, indicating that the cyclodextrin carrier protects the subject from the side effects of the 3-BrPA molecule.

**Table 1**

Clinical Signs/ Conditions	3-BrPA treatment	Succinyl- $\beta$ -cyclodextrins-3-BrPA treatment
Seizures/Shiver/Spasms	+++	+/-
Salivation	+++	+/-
Shortness of Breath	+++	+/-
Abnormal Behavior ( <i>e.g.</i> , inactive/unresponsive)	+++	+/-
Hypothermia	+++	+/-

Animals treated with succinyl- $\beta$ -cyclodextrin-3-BrPA showed complete or almost-complete tumor response on bioluminescence imaging (Figures 4 and 5). Subsequently, animals were sacrificed to confirm the bioluminescence results with necropsy (Figure 4). The results indicate that CD-3BrPA preserves its anticancer activity even after complex formation with CD.

Histopathological analysis of the orthotopic MiaPaCa-2 tumors was performed. Figure 6 shows that hematoxylin and eosin (H&E)-stained tumors showed no changes in the control group, while tumors harvested from treated animals show extensive central necrosis as well as areas of dissociating tumor tissue.

Thus, it has been determined that cyclodextrin complex formation does not affect the anticancer properties of 3-BrPA, as evident from both *in vitro* and *in vivo* data. Also, the activity of 3-BrPA can be preserved or protected by CD until it is delivered or distributed to the target organ or tumor. In addition, CD-3-BrPA administration to animals results in lesser toxicity or related-clinical signs compared to 3-BrPA alone.

Example 4: Materials and Methods for Examples 5-6

The experiments described in Examples 2-3 were expanded in order to advance the results obtained therefrom using the following materials and methods. For example, pancreatic ductal adenocarcinomas (PDAC) rank as the fourth most common cause for cancer related death in the world (Siegel *et al.* (2014) *CA Canc. J. Clin.* 64:9-29). As the majority of patients are diagnosed at advanced stages, therapeutic options remain limited and the prognosis is dismal with a 5-year survival rate of less than 5% (Hidalgo (2010) *New Engl. J. Med.* 362:1605-1617). The last two decades brought significant advances in the understanding of tumorigenesis and disease progression in pancreatic cancer, which can now be seen as a diverse and multifactorial neoplastic process (Hidalgo (2010) *New Engl. J. Med.* 362:1605-1617; Hanahan and Weinberg (2011) *Cell* 144:646-674). Pancreatic tumor tissue is composed of several distinctive, cellular and non-cellular elements including a collagen-rich, poorly vascularized and highly hypoxic, non-neoplastic stroma (Chu *et al.* (2007) *J. Cell. Biochem.* 101:887-907; Mahadevan and Von Hoff (2007) *Mol. Canc. Therapeut.* 6:1186-1197). These characteristics are associated with profound chemoresistance to the most commonly used systemically applicable anti-cancer agents, such as gemcitabine (Muerkoster *et al.* (2004) *Cancer Res.* 64:1331-1337; Yokoi and Fidler (2004) *Clin. Canc. Res.* 10:2299-2306). Notably, altered energy metabolism has been recently added to the organizing principles of tumor microenvironment and can now be seen as a “hallmark” of pancreatic cancer (Hanahan and Weinberg (2011) *Cell* 144:646-674; Guillaumond *et al.* (2014) *Arch. Biochem. Biophys* 545:69-73. The oxygen-independent reliance on glycolysis as the main axis of energy supply for cancer cells has long been known as the “Warburg effect”; however, this circumstance has not yet been clinically exploited for therapeutic purposes (Warburg *et al.* (1927) *J. Gen. Physiol.* 8:519-530; Ganapathy-Kanniappan and Geschwind (2013) *Mol. Cancer* 12:152). 3-bromopyruvate (3-BrPA), a highly potent small-molecular inhibitor of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is the only available anti-glycolytic drug candidate that is able to enter cancer cells selectively through the monocarboxylate transporter 1 (MCT1) (Ganapathy-Kanniappan *et al.* (2009) *Anticancer Res.* 29:4909-4918; Birsoy *et al.* (2013) *Nature Genet.* 45:104-108). The anti-tumoral effects of 3-BrPA have been extensively studied and confirmed in several murine tumor models in the setting of loco-regional therapy, delivered either through tumor-feeding arteries or with direct intra-tumoral injections (Ota *et al.* (2013) *Target. Oncol.* 8:145-151; Geschwind *et al.* (2002)

*Canc. Res.* 62:3909-3913). However, due to its alkylating properties, 3-BrPA has demonstrated significant toxicity when delivered systemically in therapeutic doses, which in return could impede the clinical development and use of this drug in cancer patients (Chang *et al.* (2007) *Acad. Radiol.* 14:85-92; Cao *et al.* (2008) *Clin. Canc. Res.* 14:1831-1839).

#### A. Antibodies, reagents, and kits

The following primary antibodies were used: rabbit anti-MMP-9 polyclonal antibody (pAB) #3852 (Cell Signaling), DAPI #D1306 (Invitrogen), Alexa Fluor 568 Phalloidin #12380 (Life Technologies), GAPDH (14C10) monoclonal AB (mAB) Alexa Fluor 488 Conjugate #3906 (Cell Signaling), GAPDH pAB #sc-47724 (Santa Cruz), cleaved caspase-3 pAB #9661 (Cell Signaling), MCT-1 pAB #sc50324 (Santa Cruz), and a Ki-67 kit / antibody (Dako Inc.). The following secondary antibodies were used: goat anti-rabbit IgG HRP-conjugated #7074 (Santa Cruz), anti-rabbit IgG (H+L), F(ab')<sub>2</sub> fragment PE conjugate #8885 (Cell Signaling), and goat anti-mouse IgG-FITC #sc2010. The following chemicals were used: 3-bromopyruvatic acid (3-BrPA, Sigma Aldrich), gemcitabine hydrochloride salt (LC Laboratories), succinyl- $\beta$ -cyclodextrin ( $\beta$ -CD, Sigma Aldrich), and D-luciferin potassium salt (Gold Biotechnology, St Louis, MO, USA). The following cell culture reagents were used: RPMI-1640 (Life Technologies), MEM (Life Technologies), fetal bovine serum (FBS, Thermo Scientific), penicillin/streptomycin (Sigma Aldrich), collagen I rat tail (BD Biosciences, #354326), and controlled atmosphere chamber (Plas. Labs). The following invasion assay reagents were used: matrigel basement membrane matrix (BD Biosciences) and matrigel invasion chamber transwell polycarbonate membrane inserts (Corning). The following kits were used: CellTiter-Glo luminescence cell viability assay kit (Promega), dual-luciferase reporter assay kit (Promega), 2D quant kit (GE Healthcare), histostain plus 3rd gen ICH detection kit (Invitrogen), and diff quik stain kit (Polysciences Inc.). The following imaging equipment was used: Zeiss 700 LSM confocal microscope, Olympus IX81 inverted microscope, Eclipse TS100 inverted microscope (Nikon), and IVIS200 (Xenogen Corp., Alameda, CA)

#### B. Complex preparation and nuclear magnetic resonance (NMR) spectroscopy

To prepare 3-BrPA encapsulated in  $\beta$ -CD, 3-BrPA (166 mg, 1 mM) was added in small portions (10 mg each) to a stirring solution of  $\beta$ -CD (918 mg in 20 ml DI water). After completing the addition, the solution was sonicated for 1 hour at 50°C. The sonicated

solution was then allowed to shake overnight on a thermomixer at 25°C, flash frozen in a dry ice-acetone bath and lyophilized. The lyophilized complex was used as such for further biological and biophysical studies. <sup>1</sup>H NMR experiments were performed at 400 MHz on a Bruker Avance spectrometer. The NMR spectra were recorded in 99.9% D<sub>2</sub>O and are reported in parts per million downfield relative to tetramethylsilane (TMS). Ten mM solutions of β-CD alone, 3-BrPA alone, or the complex of 3-BrPA and β-CD, were prepared with 1% DSS (3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt; Sigma Aldrich) as an internal standard. Spectra were recorded at 25 °C with 32 scans. An upfield shift of the methylene protons (0.1 ppm) was observed upon complexation (see Figure 7).

#### C. Monolayer cell culture and viability assay

Two human pancreatic adenocarcinoma cell lines, *lucMiaPaCa-2* (stably transfected with the luciferase-aminoglycoside phosphotransferase fusion gene, kindly provided by Dr. Phuoc T. Tran) and Suit-2 (kindly provided by Dr. Shinichi Ota, Japan) were cultured in RPMI or MEM media, respectively, both supplemented with 10% FBS and 1% Penicillin-Streptomycin. The effects of different drugs on cell viability were determined by quantifying intracellular adenosine triphosphate (ATP) levels using a luminescence-based kit (CellTiter-Glo, Promega) and a multilable 96-well plate (Costar). The accuracy and reproducibility of viability measurements using this luminescence-based kit in *lucMiaPaCa-2* cells was confirmed using the Dual-Reporter assay kit (Promega). In brief, 5 x 10<sup>3</sup> cells were seeded in triplicate and incubated for 72 hrs. under normoxic or hypoxic (1% O<sub>2</sub>-level, balanced with CO<sub>2</sub> and nitrogen within a controlled atmosphere chamber) conditions. Indicated amounts of free 3-BrPA, 1:1-β-CD-3-BrPA or β-CD as a control were dissolved in PBS and added to the medium for 24 hrs. of treatment. For the experiments with gemcitabine, cells were incubated for 24 hrs. prior to a 72 hrs. exposure to the drug. Cell viability was determined following the manufacturer's protocol.

#### D. 3D organotypic cell culture, imaging, and immunofluorescence

A collagen 1-based 3D organotypic cell culture was used to mimick an extracellular-matrix (ECM)-rich environment and to test the effects of 3-BrPA on tumor invasion (Cheung *et al.* (2013) *Cell* 155:1639-1651; Nguyen-Ngoc and Ewald (2013) *J. Microscop.* 251:212-223). Specifically, a collagen solution which initially consisted of 25 μl of 10x DMEM and 217 μl of collagen I (3.83 mg/ml) was prepared on ice. The pH value was adjusted by dropwise addition of sodium hydroxide (Sigma Aldrich) to reach pH = 7.0.

The collagen I was then diluted using DMEM F12/GlutaMAX (Life Technologies) to a final concentration of 3 mg/ml. An underlayer was created on the bottom of each well of an uncovered glass-bottom 24-well plate (InVitroScientific) using 15  $\mu$ l of the collagen solution, which was then allowed to polymerize at 37°C for at least 1 hr. The remaining collagen solution was kept on ice for 3-5 hrs. to allow initial polymerization. A total of 65 x 10<sup>3</sup> *lucMiaPaCa-2* or 45 x 10<sup>3</sup> *Suit-2* cells were resuspended in a volume of 150  $\mu$ l collagen solution. By creating a drop with the height of 0.5 cm, the cell suspension was placed on top of the pre-warmed underlayer. The collagen-cell suspension was allowed to polymerize for 1h at 37°C and subsequently covered with cell culture medium (Nguyen-Ngoc and Ewald (2013) *J. Microscop.* 251:212-223).

3D organoids were treated either once or sequentially. For single treatments, embedded cells were incubated for 5 days under normoxic or hypoxic (1% O<sub>2</sub>-level within a controlled atmosphere chamber) conditions prior to treatment. On day 5, medium was replaced by 1:1- $\beta$ -CD-3-BrPA / 3-BrPA /  $\beta$ -CD-containing medium and the cells were incubated for 24 hrs. with the respective concentrations of the drug. For experiments with gemcitabine, cells were allowed to grow for 48 hrs. before being treated and incubated with the drug for another 72 hrs. Initial experiments with gemcitabine did not demonstrate any efficacy after 24 hrs., and it was decided to follow the most commonly reported incubation times of 72 hrs. Sequential treatment with 3-BrPA was performed on alternate days for one week with the respective doses and evaluated by bright field microscopy (Olympus) at 40x magnification with a 1.3 NA oil objective. A Hamamatsu Photonics C9100-02 EMCCD camera was used to acquire the images with the SlideBook 5.0 program.

Microscopic observations were compared with the quantification of cell viability as seen on *in vitro* bioluminescence imaging (BLI). For the latter measurements, the cell culture medium covering the 3D organotypic cell culture was replaced by 500  $\mu$ l of a luciferase substrate (D-luciferin, potassium salt, Life Technologies, 20 mg/ml) in PBS. After 5 mins. of exposure, the plate was positioned and images were acquired (Xenogen Ivis Imaging System 100). Signal intensity was determined by the photon emission (in counts) and measured within a region of interest (ROI) which enclosed the entire 3D organoids (Living Image Software, PerkinElmer).

The microscopic and BLI findings were verified using immunofluorescence microscopy. 3D organoids were fixed using 4% formaldehyde and cryofixed with OCT Compound (Tissue Tek) at -80°C. The samples were cut into sections of 100  $\mu$ m thickness

at -20°C. OCT was washed off using PBS twice for 10 mins. Each. Prior to staining, sections were permeabilized with 0.5 % Trizol 100 in PBS for 30 mins. and washed twice with PBS for 10 mins. each. After blocking with 10% FBS in PBS for 2 hrs., samples were incubated with primary antibodies (Alexa Fluor 568 Phalloidin, Invitrogen, 1:100; GAPDH  
5 Alexa Fluor 488 conjugate, Cell Signalling, 1:800; cleaved caspase-3, Cell Signalling, 1:500; HIF-1 alpha 1:50) for 1 hr. at room temperature (RT) under light protection. For non-conjugated primary antibodies, additional incubation with a phycoerythrin (PE)- or fluoresceine isothiocyanate (FITC)-conjugated secondary antibody for 1 hr. at RT was used. This was followed by two washings with PBS for 10 mins. each. DAPI was used as  
10 a counter stain at a concentration of 300 ng/ml and added to the specimen simultaneously with the conjugated antibody. Specimens were sealed with an antifading mountant and covered with a coverslip. Confocal fluorescence microscopy was performed at 40x magnification with a 1.4 NA oil objective and 63x with a 1.4 NA oil objective. Images were analyzed with Zen2012 software (Carl Zeiss). Excitation and emission wavelengths  
15 were those recommended by the conjugate manufacturers. For example, 555 nm was used to excite for phalloidin and PE-conjugates, 488 nm for Alexa Fluor 488, as well as FITC-conjugates and 405 nm for DAPI. Emission was detected between 555 and 1000 nm for red fluorescence and 490 nm and above for green fluorescence. Emission of DAPI was captured below 490 nm or below 529 nm when imaged with red or green fluorescence,  
20 respectively.

#### E. Matrigel invasion assay, zymography, and immunoblotting

The ability of 3-BrPA to inhibit tumor invasion was studied using a matrigel invasion assay, as well as gelatin zymography (Hu and Beeton (2010) *J. Visual. Exp.*  
25 45:2445; Scott *et al.* (2011) *J. Visual. Exp.* 58:e3525). For the matrigel invasion assays, a coating buffer containing 0.01 M Tris and 0.7% sodium chloride was prepared and used to dilute the matrigel basement membrane (BD Biosciences, #356234) to 300 ug/ml. Subsequently, Boyden chambers (Transwell, Corning; 6.5 mm-diameter, 8 um pore size) were coated with 100 µl matrigel solution and stored at 37°C for 2 hrs. to allow for  
30 gelatination. Approximately  $7.5 \times 10^4$  cells were resuspended in 500 µl FBS-free medium and plated into the upper chamber of the insert, which was then placed into a 24-well plate containing 750 µl of FBS-containing medium. After overnight incubation at 37°C, various amounts of 3-BrPA dissolved in PBS were added to the upper chamber. Forty-eight hours



later, non-invasive cells were removed from the matrigel with a cotton swab. Invaded cells adherent to the bottom side of the permeable insert were fixed and stained with the Diff Quik Stain Kit (Polysciences Inc.). Light microscopy was performed using a colored inverted microscope (Eclipse TS 100) at 4x, 10x, and 20x magnification. Invasion of cells was quantified by measuring the area of stained cells after treatment compared to untreated samples at 10x magnification.

Zymography assays were performed to determine the activity of secreted MMP-9. Accordingly,  $4 \times 10^6$  Suit-2 cells and  $2.5 \times 10^6$  *lucMiaPaCa-2* cells were seeded in 75 cm<sup>2</sup>-flasks and incubated overnight at 37°C under normoxic conditions. The following day, fresh FBS-free medium containing different concentrations of 3-BrPA was added and cells were incubated for an additional 24 hrs. Subsequently, supernatants were collected, filtered, and the final protein concentrations were determined using the 2D Quant Kit (GE Healthcare). After adjustment for concentration, each sample was loaded onto two 10% gelatin zymography gels (Novex, Invitrogen). Following electrophoresis, proteins in one of the two gels were renaturated and enzymatic digestion was allowed overnight at 37°C in a developing buffer. The gel was stained with 4 parts 0.1% Coomassie Brilliant Blue in 1 part 100% methanol for 24 hrs. and washed with distilled (DI) water until digested areas were detectable as white bands. Western Blot analysis was performed using the duplicate gel. Proteins were blotted onto a PVDF-Membrane and blocked using 5% skimmed milk in 1x TBS and 0.1% Tween in DI (TBST). Primary anti-MMP antibody (Cell Signaling) was used in a 1:1000 dilution and incubated at 4°C overnight, followed by an HRP-conjugated secondary antibody (Santa Cruz) incubation for 1 hr. at room temperature. The HRP provided an electrochemiluminescence signal (ECL Kit, GE Healthcare), which was analyzed with ImageJ 1.46r software (Wayne Rasband, National Institute of Health) and used to quantify signal intensity by comparing line integrals.

#### F. Orthotopic animal xenografts

Male athymic nude mice (body weight, 20-25 g; 4 weeks old; Crl:NU (NCr)-Foxn1<sup>nu</sup>; Charles River Laboratory, Germantown, MD, USA) were used in accordance with institutional guidelines under approved Animal Care and Use Committee protocols. Mice were maintained in laminar flow rooms at constant temperature and humidity with food and water given *ad libitum*. Orthotopic xenograft tumors were generated by implantation of  $1.5 \times 10^6$  *lucMiaPaCa-2*, suspended in 50 µl PBS, into the tail of the pancreas in anesthetized

mice. To accomplish this, mice were placed into a clean anesthesia induction chamber (oxygen flow rate, 1 liter/minute; isoflurane concentration of 3-4%). Upon loss of the righting reflex, animals were placed on the surgical procedure surface, where a nose cone was used to maintain anesthesia (oxygen flow, 0.8 liters/minute; Isoflurane concentration, 1.5-2%). A small, left abdominal flank incision was made, and the pancreas was exteriorized to inject the cell solution using a 30G Hamilton syringe. A successful subcapsular intrapancreatic injection was identified by the appearance of a fluid bleb without intraperitoneal leakage. The abdominal cavity was closed with a double-layer of non-absorbable suture material (Kim *et al.* (2009) *Nat. Protocol.* 4:1670-1680).

#### G. Bioluminescence imaging and ultrasound imaging

Tumor viability was confirmed via *in vivo* bioluminescence imaging (BLI) on day 7 after the surgical implantation. Anesthetized tumor-bearing mice were injected intraperitoneally with D-luciferin 150 mg/kg and optically imaged 5 minutes later using the IVIS 200 system (Xenogen). The pseudocolor image representing the spatial distribution of photons was overlaid on a previously acquired grayscale photographic image. A region of interest (ROI) was created to include the optical tumor image. Signal intensity was quantified within the ROI in photons/second/squared centimeter/steradian (p/s/cm<sup>2</sup>/Sr) after a 10-second exposure using Living Image software (Xenogen). Additionally, orthotopic growth of the tumors was confirmed prior to treatment using small-animal ultrasound imaging (USI). In brief, anesthetized mice were subjected to examination using the VEVO2100 (Visual Sonics Inc., Toronto, Canada, kindly provided by Dr. Harry C. Dietz) by applying a MS-550D MicroScan transducer probe with 40 MHz (broadband with 22-55 MHz). Tumor localization was confirmed using the cranial tip of the left kidney and the caudal tip of the spleen as anatomic landmarks (Ota *et al.* (2013) *Target. Oncol.* 8:145-151; Tuli *et al.* (2012) *Translat. Oncol.* 5:77-84).

#### H. Treatment regimen and imaging follow-up

Animals with tumors, as confirmed by both LI and USI, were randomized into four groups: group 1 (N = 21 animals) received daily intraperitoneal injections of the  $\beta$ -CD-3-BrPA complex (in a 1:1 molecular ratio, 5 mg/kg 3-BrPA in 26 mg/kg  $\beta$ -CD, dissolved in 500  $\mu$ l saline), group 2 (N = 7 animals) received intraperitoneal injections of gemcitabine (150 mg/kg dissolved in 200  $\mu$ l saline, three injections / week as commonly reported in literature, such as Liau and Whang (2008) *Clin. Canc. Res.* 14:1470-1477; Larbouret *et al.*

(2010) *Annal. Oncol.* 21:98-103), group 3 (N = 7) received daily intraperitoneal injections of  $\beta$ -CD (26 mg/kg  $\beta$ -CD, dissolved in 500  $\mu$ l saline), and group 4 (N = 7 animals) received daily intraperitoneal injections of 3-BrPA alone (5 mg/kg dissolved in 500  $\mu$ l saline). All animals were treated without interruptions for a period of four weeks. BLI was acquired on  
 5 day 7, 14, 21, 28 after the first injection. Animals were sacrificed on day 28 after the last imaging session or when moribund.

### I. Immunohistochemistry

Upon sacrificing the animals, tumors were obtained, fixed with a 4% formaldehyde  
 10 solution for a period of at least 72 hrs., and embedded in paraffin for immunohistochemical analysis. Hematoxylin and eosin (H&E) staining of the slides was performed according to standard protocols, such as those described in Casadonte and Caprioli (2011) *Nat. Protocol.* 6:1695-1709. Eighteen  $\mu$ m thick tumor sections were stained for the following targets: GAPDH, MCT-1, cleaved caspase-3, and Ki-67 using the Histostain Plus 3rd Gen IHC  
 15 Detection Kit (Invitrogen), as well as the Ki-67 kit (Dako Inc.). Specifically, specimens were deparaffinized using xylene and rehydrated using a descending ethanol dilution series. After washing with deionized water, samples were permeabilized in boiling retrieval solution containing citrate (Dako) for 40 mins. at 95°. Specimens were cooled down to RT and incubated with 2 drops (~ 100  $\mu$ l total) of peroxidase quenching solution for 5 min. and  
 20 blocked for 20 mins. Incubation with primary antibodies (GAPDH, 1:500; MCT-1, 1:250; Ki-67 and HIF-1 $\alpha$ ; 1:50, cleaved caspase-3, 1:1,500; in PBS) occurred at RT in a moist chamber for 60 mins. Biotinylated secondary antibody and streptavidin-peroxidase conjugate were added to the samples in sequence for 10 min. each. 26.5  $\mu$ l of 3,3'-diaminobenzidine (DAB) chromogen were mixed well with 1 ml of DAB substrate buffer  
 25 and 100  $\mu$ l were added to each specimen for 5 mins. Hematoxylin was used as a counterstain. Incubation steps were followed by washing with distilled water and twice PBS for 2 mins. each. Samples were sealed using antifading mountant and covered with a coverslip. All slides were scanned and digitalized at a 20x magnification using a high-resolution Aperio<sup>®</sup> scanner system (Vista, California, USA). The digitalized slides were  
 30 then assessed using the Aperio ImageScope<sup>®</sup> software. For the Ki-67-stained tissue sections, a total of 5-10 fields were viewed at 10x, and the number of Ki-67-positive cells, as well as the total number of cells were recorded to calculate the Ki-67 labeling index (formula: Index [%] = [number of positive cells / total cell number] x 100).

#### J. Statistical data analysis

All experiments were performed independently and repeated at least three times. Data from the experiments were summarized with means  $\pm$  standard error of the mean.

- 5 Statistical comparisons of data sets were carried out by the Student's t-test as well as the one-way ANOVA test. Reported BLI signal intensities were normalized among the animals and reported as multiples based on the baseline value.

#### Example 5: $\beta$ -CD-3-BrPA Shows Strong Anti-Cancer Effects in 2D and 3D Cell Culture and Targeting Metabolism Reduces Invasive Potential of Cancer Cells

- 10 Upon NMR-spectroscopic confirmation of the complexation between 3-BrPA and  $\beta$ -CD (Figure 7), the microencapsulated formulation of the drug was used for further experiments. In order to assess the efficacy of the microencapsulated 3-BrPA ( $\beta$ -CD-3-BrPA) to achieve dose-dependent ATP depletion and cell death, two human pancreatic
- 15 cancer cell lines were employed. MiaPaCa-2 was derived from a primary pancreatic adenocarcinoma (PDAC) and Suit-2 was derived from a metastatic primary pancreatic adenocarcinoma from a different patient (Kitamura *et al.* (2000) *Clin. Exp. Metast.* 18:561-571). The dose-dependent effects of  $\beta$ -CD-3-BrPA were compared with free 3-BrPA, as well as gemcitabine, and  $\beta$ -CD was used as a control. As hypoxia is often associated with
- 20 chemoresistance in PDACs, hypoxic exposure was added to the experimental design (Yokoi and Fidler (2004) *Clin. Canc. Res.* 10:2299-2306; Kasuya *et al.* (2011) *Oncol. Rep.* 26:1399-1406; Onozuka *et al.* (2011) *Canc. Sci.* 102:975-982; Zhao *et al.* (2014) *Canc. Res.* 74:2455-2464)). It was found that  $\beta$ -CD-3-BrPA and free 3-BrPA demonstrated similar cytotoxicity profiles under normoxic (50-75  $\mu$ M), as well as hypoxic (12.5-25  $\mu$ M),
- 25 conditions and, interestingly, were more sensitive to the drugs when hypoxic (Figure 8). Cell lines treated with  $\beta$ -CD alone were perfectly viable throughout the experiment, even when exposed to very high concentrations. Similar results were observed for Suit-2 cells but with less pronounced differences between normoxic and hypoxic conditions (Figure 8). When assessing the efficacy of gemcitabine, IC<sub>50</sub> in MiaPaCa-2 and Suit-2 cells was barely
- 30 achieved under normoxic conditions (0.1  $\mu$ M), no concentration achieved a complete kill, and hypoxia seemed to increase the resistance towards the drug.

In order to test the efficacy of  $\beta$ -CD-3-BrPA in an ECM-rich environment, *luc*MiaPaCa-2 cells were cultured in a 3D Collagen 1 matrix and treated with a single dose

of either  $\beta$ -CD-3-BrPA, free 3-BrPA or  $\beta$ -CD (as a control). BLI quantification showed that both drug formulations had equivalent potencies in normoxic conditions ( $IC_{50}$ , 25-50  $\mu$ M) (Figure 8). Under hypoxic conditions, MiaPaCa-2 cells were slightly more sensitive to free 3-BrPA than to  $\beta$ -CD-3-BrPA (Figure 8). The cells cultured in 3D were treated sequentially with the drugs, as described in Example 4. Morphological, BLI, and immunofluorescence-based analysis confirmed the ability of 3-BrPA to penetrate an ECM-rich matrix and to inhibit cell proliferation, as well as to induce apoptosis (Figures 9-10). As such, untreated MiaPaCa-2 cells proliferated and formed “grape”-like structures within the collagen 1 matrix, while Suit-2 cells demonstrated a more invasive growth pattern with cellular protrusions visible after 6 days of growth (Figure 10). When treated with 3-BrPA, proliferation in both cell lines was inhibited with a marked reduction of cell protrusions in Suit-2 cells (Figure 10). In addition, immunofluorescence imaging confirmed a dose-dependent induction of apoptosis by 3-BrPA.

In addition, the ability of 3-BrPA to inhibit the invasiveness of pancreatic cancer cells in sub-lethal drug concentrations was tested using a matrigel invasion assay. As shown in Figures 11A-11B, both the locally invasive MiaPaCa-2 cells and the metastatic Suit-2 cells showed a reduction in invasion at drug concentrations as low as 12.5  $\mu$ M. In addition, the effect of sub-lethal doses of 3-BrPA on the secretion of the matrix-metalloproteinase 9 (MMP-9), a well-described marker for the invasive potential of pancreatic cancer cells, was tested using gelatin zymography and immunoblotting (Jones *et al.* (1999) *Annal. N. Y. Acad. Sci.* 880:288-307; Merdad *et al.* (2014) *Anticanc. Res.* 34:1355-1366; Yang *et al.* (2001) *J. Surg. Res.* 98:33-39). Accordingly, a marked reduction in the secretion of MMP-9 was detected in both cell lines. This effect was observed beginning with a 3-BrPA concentration of 6.25  $\mu$ M, which is a dose that did not induce apoptosis or reduce cell viability, and an earlier onset in the more metastatic Suit-2 cell line (Figure 11C-11D).

Example 6: Systemic Delivery of  $\beta$ -CD-3-BrPA Achieves Strong Anti-Cancer Effects *In Vivo*

The anti-cancer efficacy of systemically delivered  $\beta$ -CD-3-BrPA was tested using a xenograft model of human pancreatic cancer in athymic nude mice. Prior to choosing the therapeutic dose for more detailed studies, comparative dose escalation studies in non-tumor-bearing animals were performed for both  $\beta$ -CD-3-BrPA and free 3-BrPA.

Accordingly, 20 mg/kg of  $\beta$ -CD-3-BrPA and 10 mg/kg free 3-BrPA were identified as the median lethal doses ( $LD_{50}$ ) after a single injection and 5 mg/kg  $\beta$ -CD-3-BrPA was identified as a safe dose that did not cause any toxicity when given systemically and daily over the course of 7 days. A total of 42 animals with orthotopically-implanted and BLI- and USI-confirmed MiaPaCa-2 tumors were then randomized to receive intraperitoneal (i.p.) injections of  $\beta$ -CD-3-BrPA (N = 21), gemcitabine (N = 7), or  $\beta$ -CD (N = 7). An additional group of animals with orthotopic implants (N = 7) was treated with free 3-BrPA. Animals treated with daily intraperitoneal (i.p.) injections of free 3-BrPA (5 mg/kg in 500  $\mu$ l saline) demonstrated high treatment-related toxicity and 3/7 (43%) animals died before the acquisition of the first follow-up BLI (Figure 12C). At the end of the experiment (Day 28), only 2/7 animals (28%) treated with the free drug were still alive (Figure 12C). No such mortality rate was observed for any of the remaining groups. Daily i.p. injections of  $\beta$ -CD-3-BrPA (5 mg/kg in 500  $\mu$ l saline) demonstrated strong anti-cancer effects with early effects visible on day 14 after the first injection (Figure 12B). After four weeks of treatment, a comparison of BLI signal intensity between the groups was performed. Animals treated with the  $\beta$ -CD control demonstrated a 140-fold signal increase as compared to baseline. A moderate deceleration of tumor growth was observed in gemcitabine-treated animals with a 60-fold signal increase over time. Most importantly, animals treated with  $\beta$ -CD-3-BrPA showed minimal or no progression of the signal as compared to gemcitabine and control groups (Figure 12). After achieving this endpoint, animals were sacrificed and tumors were harvested for further analysis. All animals were subjected to necropsies and organs (brain, heart, lungs, bowel, liver, and kidneys) were harvested for the analysis of potential tissue damage. No organ toxicities or tissue damage was observed in animals treated with  $\beta$ -CD-3-BrPA (Figure 12D). The analysis of tumor pathology demonstrated vast tumor destruction with central areas of colliquative necrosis in animals treated with  $\beta$ -CD-3-BrPA (Figure 13). Tumor regions with intact cell junctions demonstrated a high expression of cleaved caspase-3, indicating fulminant tumor apoptosis. Animals treated with  $\beta$ -CD-3-BrPA demonstrated a significant reduction in proliferation as assessed with Ki67 immunohistochemistry with a mean of 17% and 51%, respectively (Figure 13). In addition, animals treated with  $\beta$ -CD-3-BrPA demonstrated lower expression levels of MCT1 and GAPDH within the treated tumors as compared to the  $\beta$ -CD or gemcitabine groups.

These results indicate that systemically delivered  $\beta$ -CD-3-BrPA achieved strong anti-tumoral effects *in vivo* while causing much less toxicity in therapeutic doses when compared to the free drug. Furthermore, microencapsulation of 3-BrPA did not alter the efficacy of the drug against pancreatic cancer cells *in vitro*, which was demonstrated using 2D, as well as ECM-rich 3D cell cultures, both under normoxic and hypoxic conditions. The abilities of 3-BrPA to inhibit the secretion of MMP-9 and to reduce the invasiveness of pancreatic cancer cells in sublethal doses further indicates the anti-metastatic potential of this drug.

Selectively targeting tumor metabolism has long been considered as a desirable therapeutic option, but has yet not been translated into clinical practice. The primary limitation in reaching the milestone of systemic deliverability with 3-BrPA is the reported toxicity due to its alkylating properties (Ganapathy-Kanniappan and Geschwind (2013) *Mol. Cancer* 12:152; Chang *et al.* (2007) *Acad. Radiol.* 14:85-92; Kunjithapatham *et al.* (2013) *BMC Res. Not.* 6:277). As a result, local image-guided delivery of the drug has been explored as an alternative therapeutic option; however, the practical use of these approaches is limited to treating localized disease (Ota *et al.* (2013) *Target. Oncol.* 8:145-151; Geschwind *et al.* (2002) *Canc. Res.* 62:3909-3913). The results described herein clearly demonstrate that the drug, when appropriately formulated for systemic delivery, was extremely effective, thereby expanding the use of this compound to virtually any cancer. These results contrast to those of the only other study where the drug was used systemically in its free form to treat solid tumors. In that study, free 3-BrPA failed to elicit any meaningful tumor response at the dose used in the experiments described herein (Cao *et al.* (2008) *Clin. Canc. Res.* 14:1831-1839; Schaefer *et al.* (2012) *Translat. Res.* 159:51-57). Specifically, a study, which explored the systemic delivery of free 3-BrPA in combination with an HSP90 inhibitor in subcutaneous pancreatic cancer xenografts, did not report any significant efficacy for 3-BrPA alone in a dose of 5 mg/kg, given twice per week out of safety considerations (Cao *et al.* (2008) *Clin. Canc. Res.* 14:1831-1839). A possible explanation for this unfavorable efficacy profile of the free drug is the rapid inactivation of 3-BrPA through unspecific interaction with serum proteins, which is known to occur *in vivo* as early as 2-3 minutes after systemic administration (Kunjithapatham *et al.* (2013) *BMC Res. Not.* 6:277). Some efficacy was observed at these doses, but excessive toxicity, with treatment-related deaths in most animals, was the predominant result. Hence, it is believed that systemic administration of free 3-BrPA may not be effective and may promote

undesirable toxicities. It is also believed that in the microencapsulated formulation, 3-BrPA is more bioavailable for uptake into tumor cells and less available to the normal cells that apparently mediate its toxicity (Birsoy *et al.* (2013) *Nature Genet.* 45:104-108; Zhang and Ma (2013) *Advanc. Drug Deliv. Rev.* 65:1215-1233; Heidel and Schluep (2012) *J. Drug Deliv.* 2012:262731).

A characteristic feature of pancreatic tumor tissue is the excessive accumulation of dense ECM which limits oxygen diffusion and creates a highly hypoxic, ill-perfused tumor microenvironment known for its profound chemoresistance and increased invasiveness (Yokoi and Fidler (2004) *Clin. Canc. Res.* 10:2299-2306; Yang *et al.* (2001) *J. Surg. Res.* 98:33-39). Published studies confirmed that more than 30% of pancreatic tumor cells are located in hypoxic tumor compartments, thereby escaping the effects of conventional chemotherapy. These cells then go on to re-form a tumor that has become even more aggressive and resistant to chemotherapy (Guillaumond *et al.* (2013) *Proc. Natl. Acad. Sci. U.S.A.* 110:3919-3924). The results described herein demonstrate that 3-BrPA is able to effectively block tumor glycolysis even when it is exacerbated under hypoxic conditions. On the contrary, the inability of gemcitabine, even at the highest dose, to cope with hypoxia in pancreatic cancer cells was confirmed. So far, conflicting data have been reported for the oxygen dependency of 3-BrPA in cancer cells (Cao *et al.* (2008) *Canc. Chemother. Pharmacol.* 62:985-994; Xiao *et al.* (2013) *Oncol. Rep.* 29:329-334). However, there is significant evidence in support of the ability of 3-BrPA to overcome hypoxia as a key mechanism of drug resistance (Xu *et al.* (2005) *Canc. Res.* 65:613-621). Specifically, more recent studies established the link between hypoxia and the expression of MCT-1, which was shown to be overexpressed in hypoxic cells and tumor regions, thus providing the functional explanation for as the increased sensitivity of hypoxic tumor tissue towards 3-BrPA (Matsumoto *et al.* (2013) *Magnet. Res. Med.* 69:1443-1450). Of note, combining gemcitabine and 3-BrPA in order to potentially achieve an increase of efficacy has been explored *in vitro*; however, no combination effects were identified and, accordingly, no respective *in vivo* experiments were performed.

Furthermore, the use of the collagen 1-rich 3D organotypic cell culture as a model for an ECM-rich tumor microenvironment has demonstrated the ability of 3-BrPA to successfully penetrate the matrix without any measurable reduction of efficacy as compared to the monolayer cell culture. The 3D cell culture model used in the studies described herein can be seen as relatively specific primarily because it is composed of a matrix, which



mimics the collagen 1-rich ECM as seen in human *ex vivo* samples (Mollenhauer *et al.* (1987) *Pancreas* 2:14-24). While the benefits of such *in vitro* models for the purpose of drug testing are increasingly recognized, mimicking these conditions *in vivo* represents a greater challenge (Longati *et al.* (2013) *BMC Canc.* 13:95). When designing this study, different animal models were considered. On the one hand, using a widely recognized orthotopic xenograft model brings about important advantages, such as reproducibility, predictable tumor growth dynamics, as well as allowing for genomic modification of tumor cells to express specific and imageable reporter genes (Kim *et al.* (2009) *Nat. Protocol.* 4:1670-1680). On the other hand, the degree to which these models reflect the tumor microenvironment in human lesions remains unknown. Although several well defined mouse tumor models are able to mimic the ECM-component and tumor hypoxia more realistically, these models seem as less suitable for the purpose of standardized drug testing (Guillaumond *et al.* (2013) *Proc. Natl. Acad. Sci. U.S.A.* 110:3919-3924). In light of the demonstrated ability of 3-BrPA to inhibit cell invasiveness *in vitro*, the use of a metastatic Suit-2 xenograft model was considered. However, orthotopic implantation of Suit-2 xenografts resulted in complications (*i.e.*, bloody ascites) and loss of a majority of animals within 14 days after implantation, which facilitated the selection of MiaPaCa-2 xenografts as a practicable alternative.

An additional unexpected result was observed in the immunohistochemical analysis of treated tumor tissues: next to the anticipated and previously reported depletion of GAPDH as the molecular target of 3-BrPA, the amount of MCT-1 as the specific transporter for 3-BrPA was significantly reduced in treated samples (Ganapathy-Kanniappan *et al.* (2012) *Radiol.* 262:834-845). No evidence has heretofore existed for the presence of MCT-1 as a potential target of 3-BrPA. Yet, this lactate transporter has been repeatedly identified as a suitable molecular target of cancer therapy (Schneiderhan *et al.* (2009) *Gut* 58:1391-1398; Shih *et al.* (2012) *Oncotarget* 3:1401-1415; Sonveaux *et al.* (2012) *PloS One* 7:e33418).

Thus, the results described herein identified microencapsulation of 3-BrPA as a promising advance towards finally achieving the goal of systemically deliverable anti-glycolytic tumor therapy. The strong anti-cancer effects of  $\beta$ -CD-3-BrPA and the favorable toxicity profile pave the way towards clinical trials in patients with pancreatic cancer and potentially other malignancies.

**Incorporation by Reference**

The contents of all references, patent applications, patents, and published patent applications, as well as the Figures and the Sequence Listing, cited throughout this application are hereby incorporated by reference. It will be understood that, although a  
5 number of patent applications, patents, and other references are referred to herein, such reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art.

**Equivalents**

10 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed:

1. A composition comprising a  $\beta$ -cyclodextrin and a pharmaceutical agent, wherein the pharmaceutical agent is a 3-halopyruvate, wherein at least one  $\alpha$ -D-glucopyranoside unit of the cyclodextrin has at least one hydroxyl chemical group replaced with an ionizable chemical group resulting in a negative charge and wherein the cyclodextrin encapsulates the pharmaceutical agent.
2. The composition of claim 1, wherein the at least one hydroxyl chemical group of the at least one  $\alpha$ -D-glucopyranoside unit is selected from the group consisting of C2, C3, and C6 hydroxyl chemical groups.
3. The composition of claim 2, wherein the C2, C3, and C6 hydroxyl chemical groups of at least one  $\alpha$ -D-glucopyranoside unit of the cyclodextrin are replaced with ionizable chemical groups.
4. The composition of any one of claims 1 to 3, wherein the at least one  $\alpha$ -D-glucopyranoside unit of the cyclodextrin is selected from the group consisting of two, three, four, five, six, seven, eight, and all  $\alpha$ -D-glucopyranoside units of the cyclodextrin.
5. The composition of any one of claims 1 to 4, wherein the ionizable chemical group is the same at all replaced positions.
6. The composition of any one of claims 1 to 5, wherein the ionizable chemical group is a weakly basic functional group or a weakly acidic functional group.
7. The composition of claim 6, wherein the weakly basic functional group (X) has a  $pK_a$  between 6.5 and 8.5 according to  $CH_3-X^-$ .
8. The composition of claim 6, wherein the weakly acidic functional group (Y) has a  $pK_a$  between 4.0 and 6.5 according to  $CH_3-Y$ .
9. The composition of claim 6, wherein the weakly basic or weakly acidic functional groups are selected from the group consisting of amino, ethylene diamino, dimethyl

ethylene diamino, dimethyl anilino, dimethyl naphthylamino, succinyl, carboxyl, sulfonyl, and sulphate functional groups.

10. The composition of any one of claims 1 to 9, wherein the cyclodextrin has a  $pK_{a1}$  of between 4.0 and 8.5.

11. The composition of any one of claims 1 to 10, wherein the composition is a liquid or solid pharmaceutical formulation.

12. The composition of any one of claims 1 to 11, wherein the pharmaceutical agent is neutrally charged or hydrophobic.

13. The composition of any one of claims 1 to 12, wherein the cyclodextrin is  $\beta$ -cyclodextrin selected from the group consisting of 6' modified  $\beta$ -cyclodextrin, 6' mono-succinyl  $\beta$ -cyclodextrin, hydroxypropyl- $\beta$ -cyclodextrin, and succinyl- $\beta$ -cyclodextrin.

14. The composition of any one of claims 1 to 13, wherein the pharmaceutical agent is 3-bromopyruvate.

15. The composition of any one of claims 1 to 14, wherein the composition is formulated for systemic administration.

16. The composition of any one of claims 1 to 15, further comprising an anti-cancer therapeutic agent.

17. A kit comprising a composition of any one of claims 1 to 16, and instructions for use.

18. A method of treating a subject having a cancer comprising administering to the subject a therapeutically effective amount of a composition of any one of claims 1 to 17.

19. The method of claim 18, wherein the composition is administered systemically.

20. The method of claim 19, wherein the systemic administration is selected from the group consisting of oral, intravenous, intraperitoneal, subcutaneous, and intramuscular administration.

21. The method of any one of claims 18 to 20, wherein the subject is treated with at least one additional anti-cancer therapy.
22. The method of claim 21, wherein the at least one additional anti-cancer therapy is radiation therapy.
23. The method of any one of claims 18 to 22, wherein the cancer is a solid tumor.
24. The method of any one of claims 18 to 23, wherein the cancer is selected from the group consisting of liver cancer, pancreatic cancer, lung cancer and breast cancer.
25. The method of claim 24, wherein the cancer is liver cancer.
26. The method of any one of claims 18 to 25, wherein the subject is a mammal.
27. The method of claim 26, wherein the mammal is a human.

Figure 1

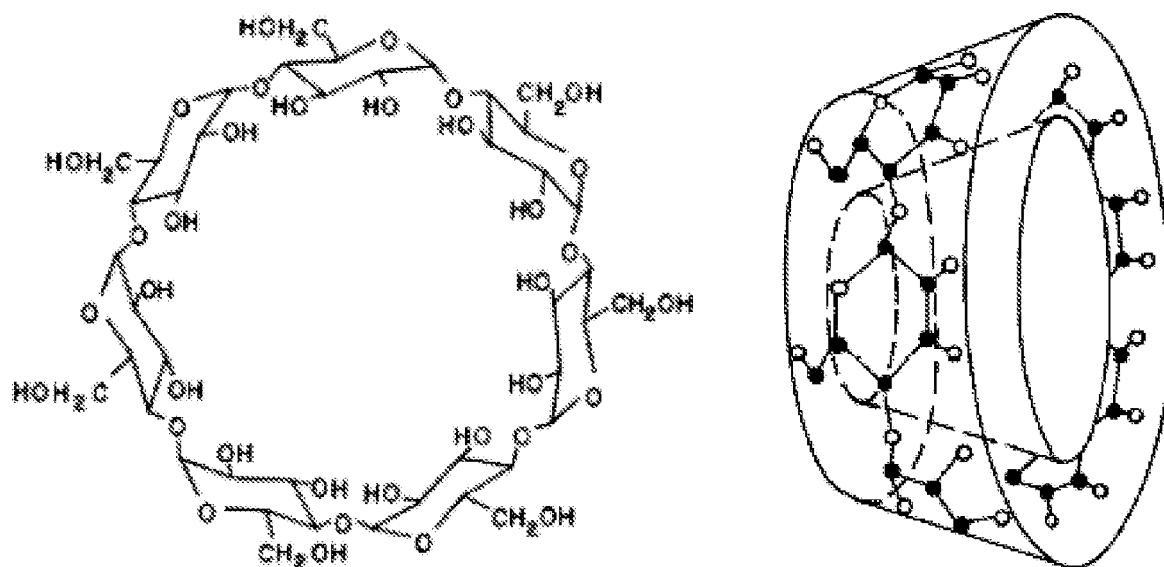


Figure 2

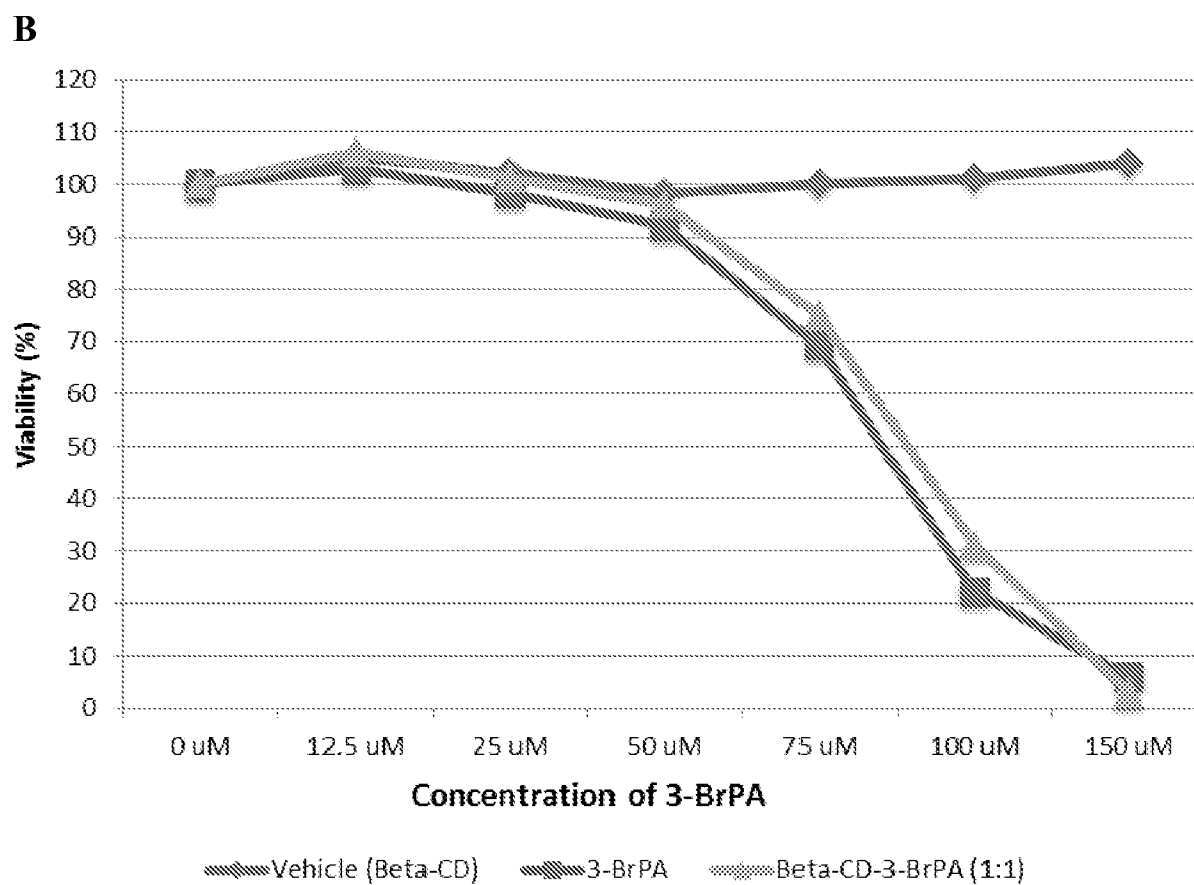
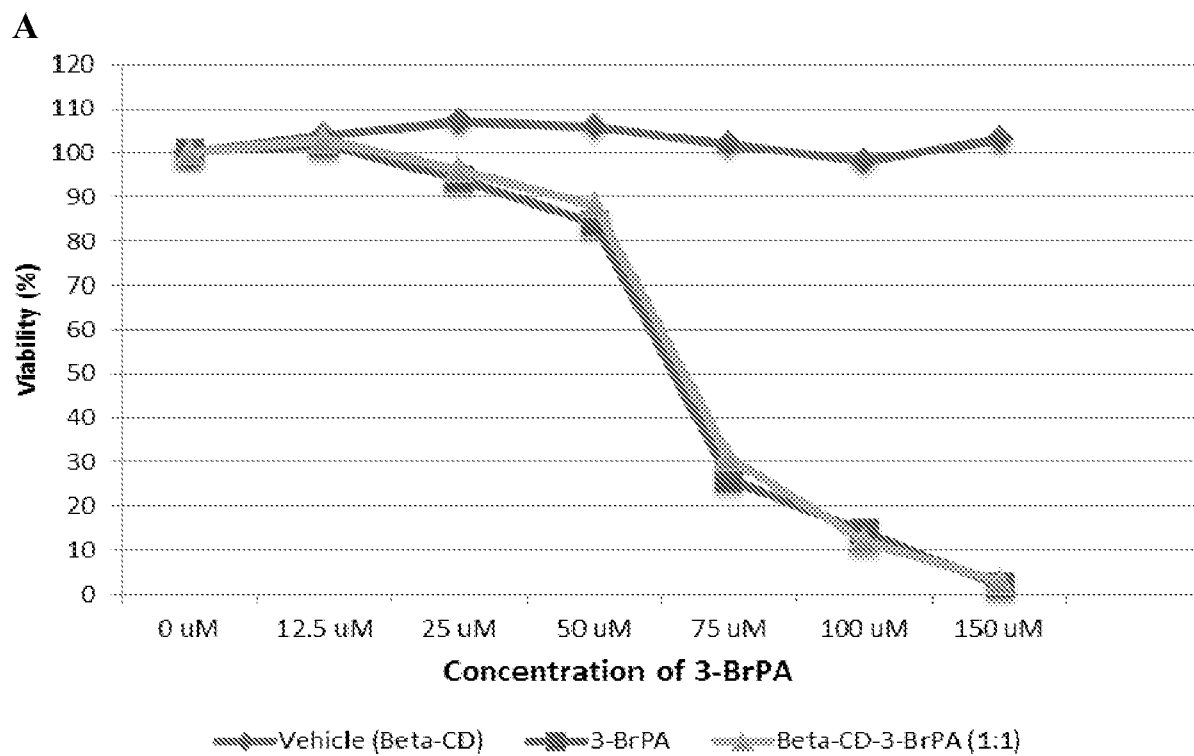
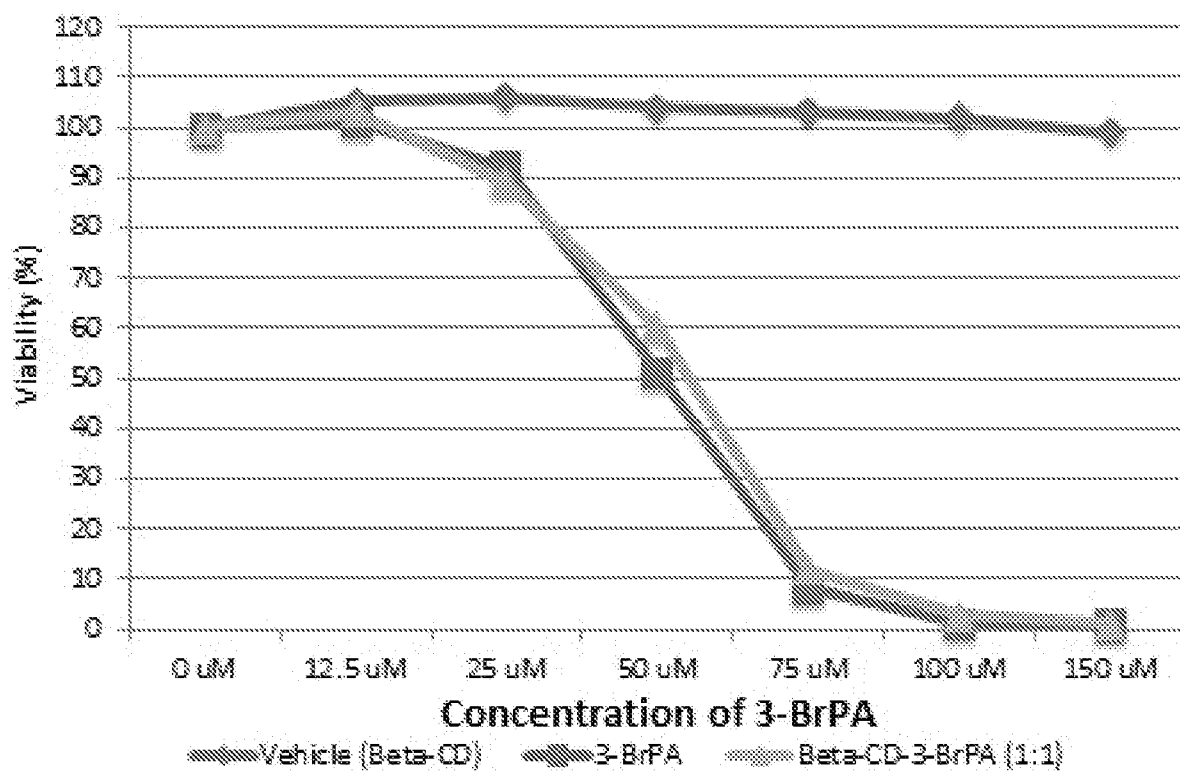


Figure 3





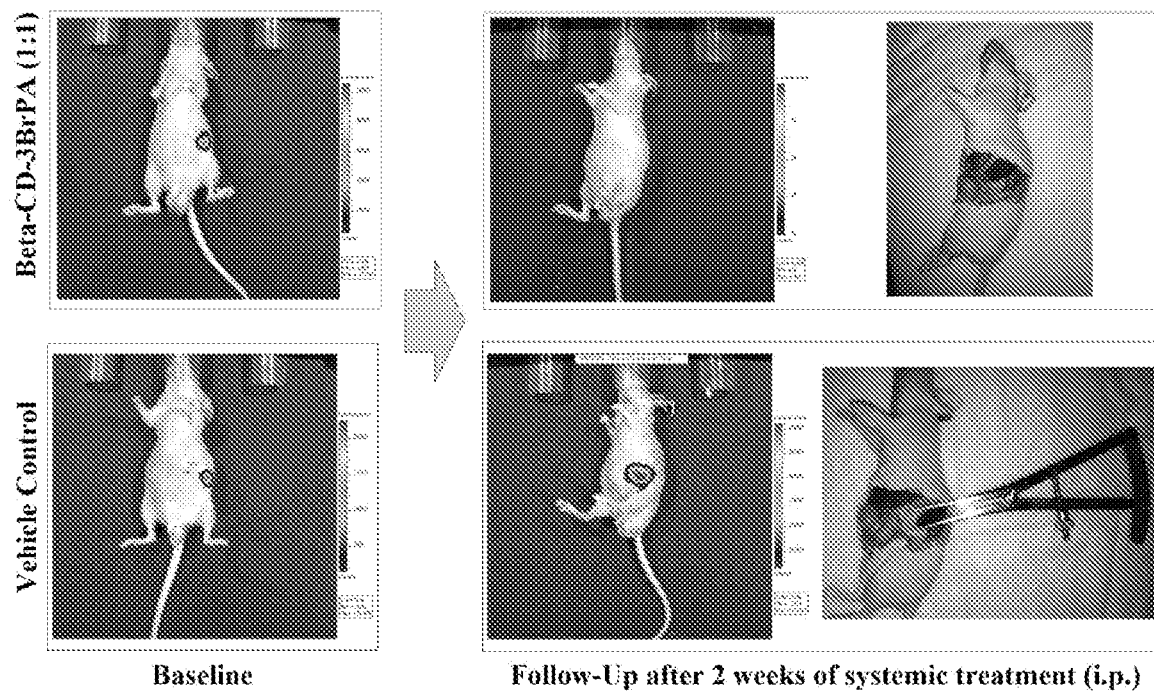
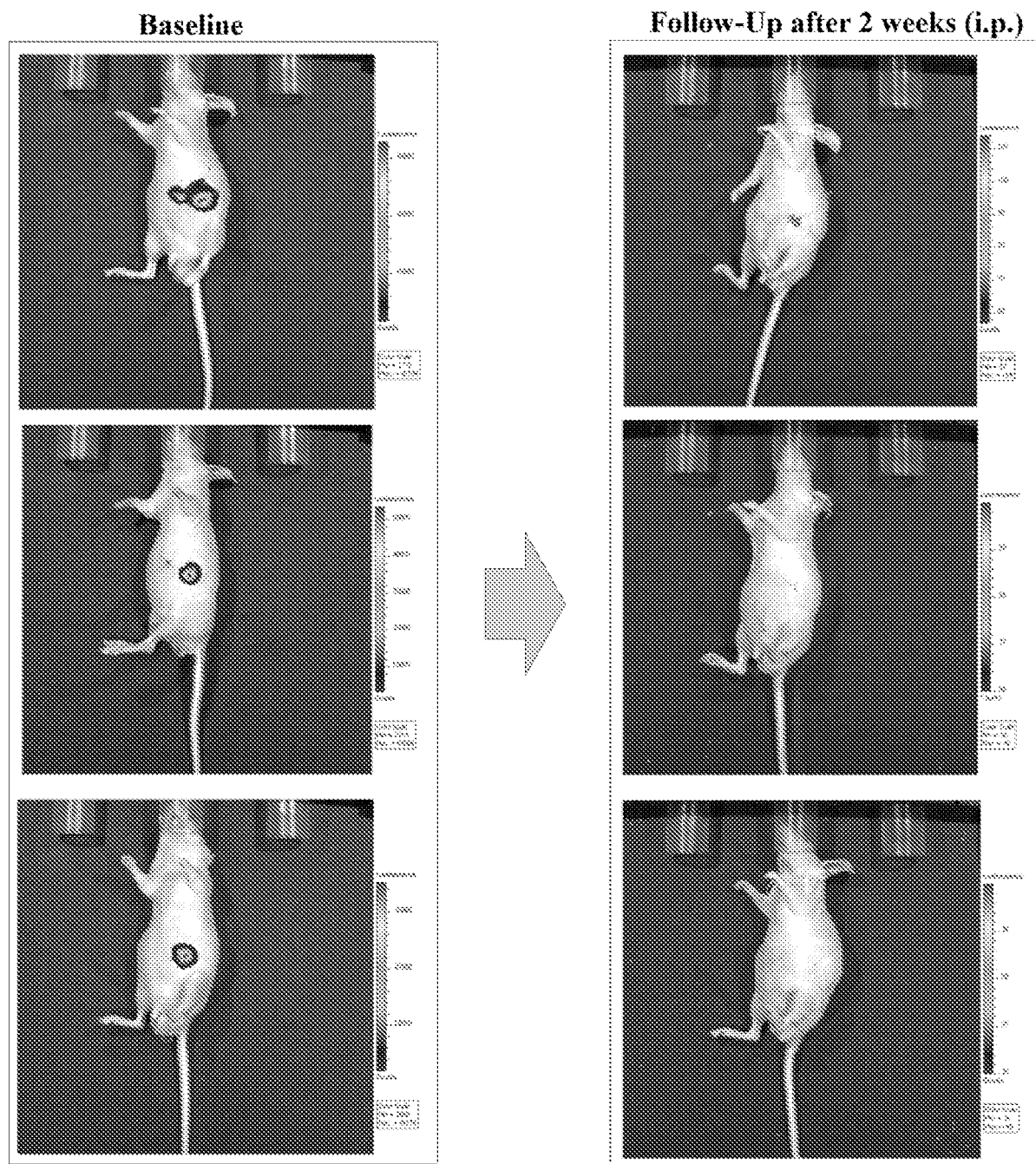
**Figure 4**

Figure 5



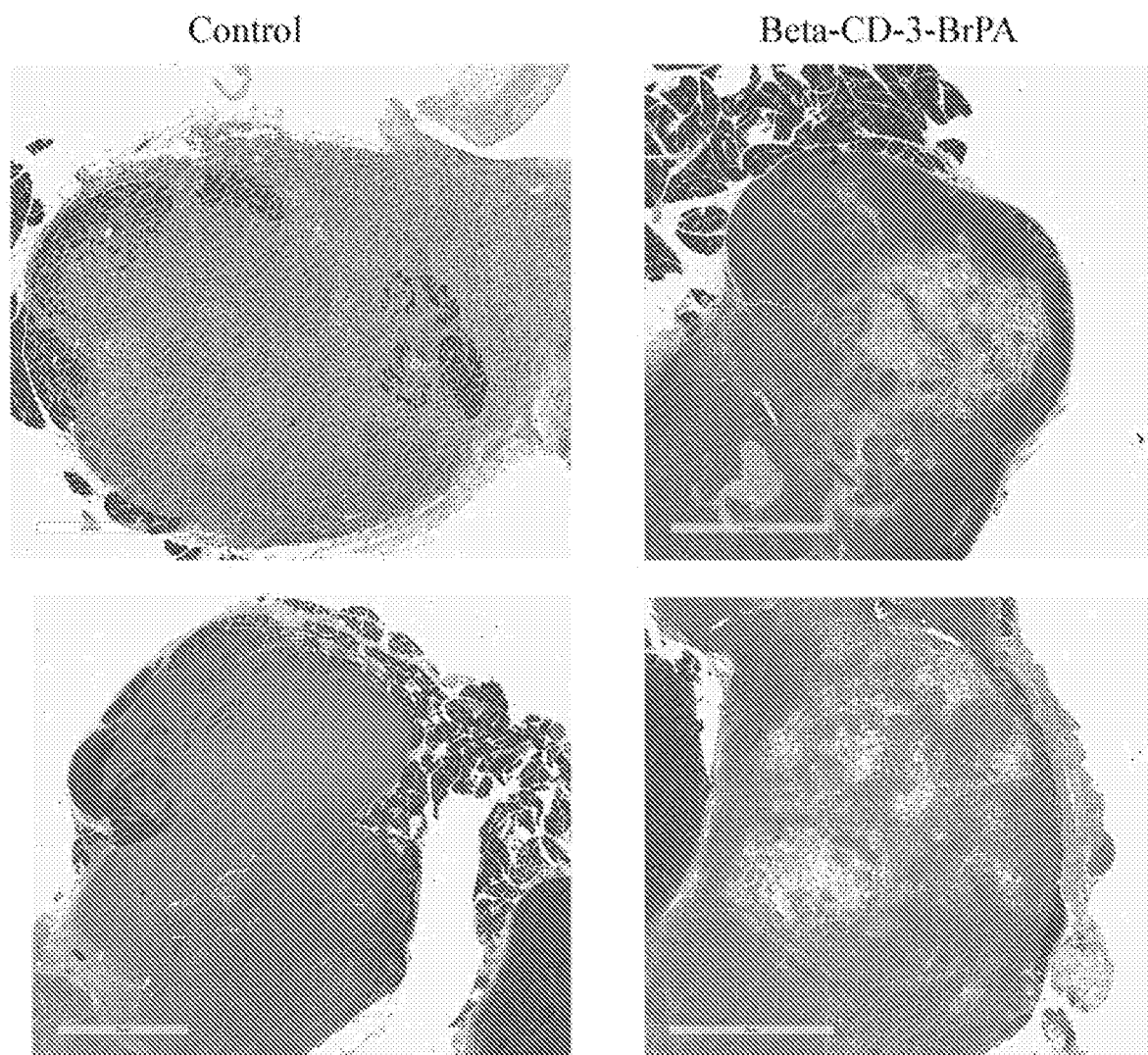
**Figure 6**

Figure 7

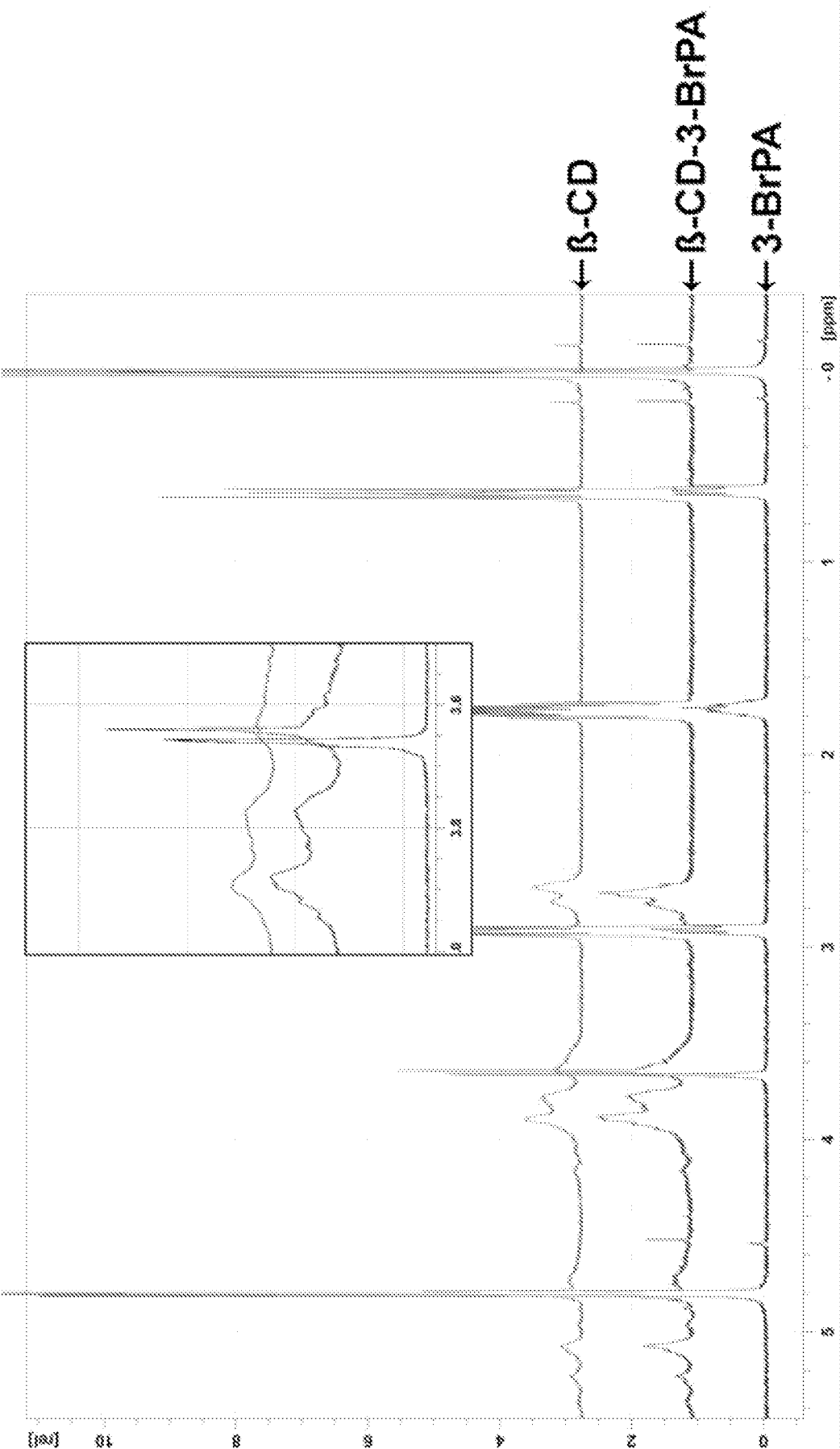


Figure 8

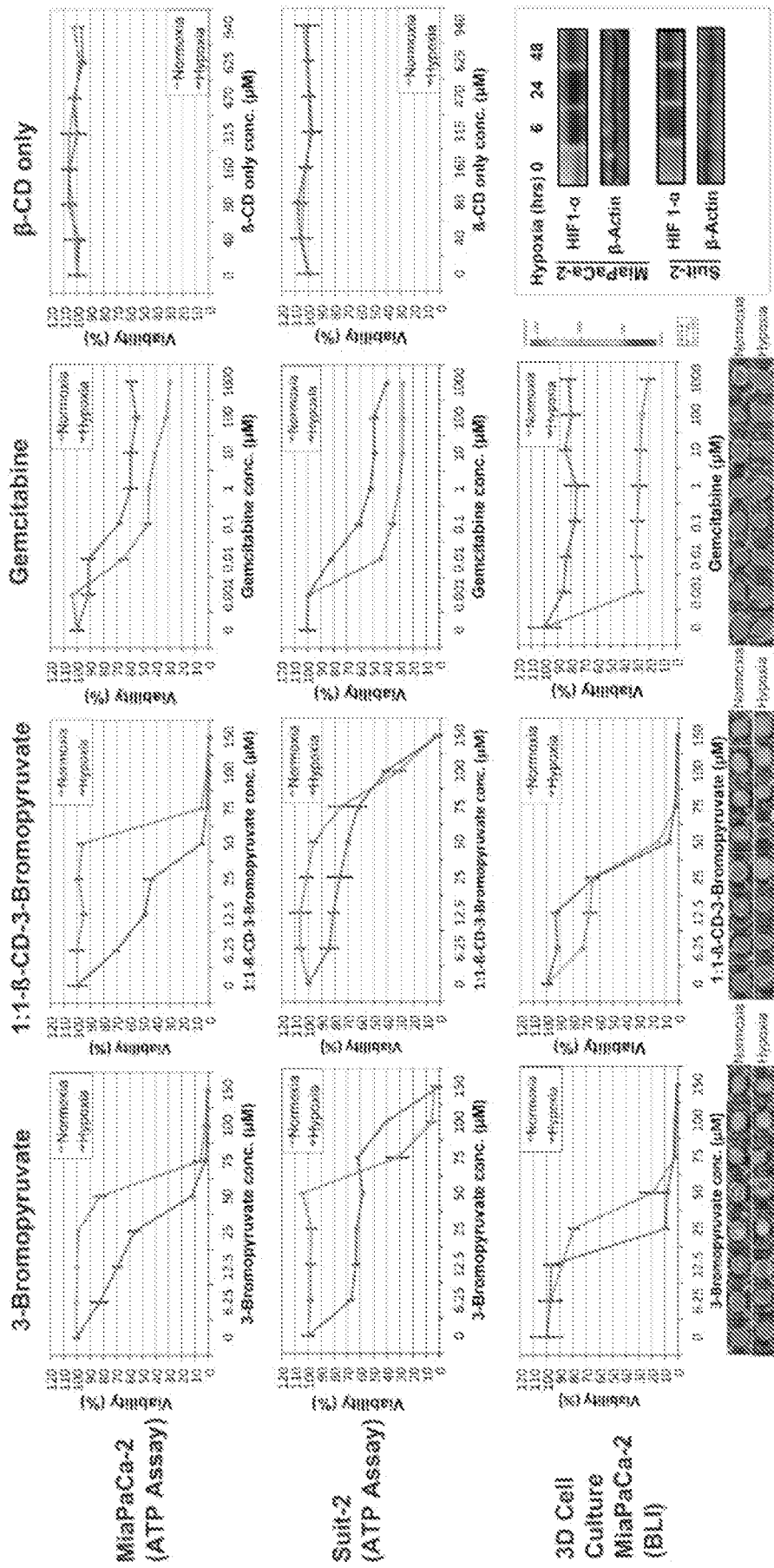


Figure 9

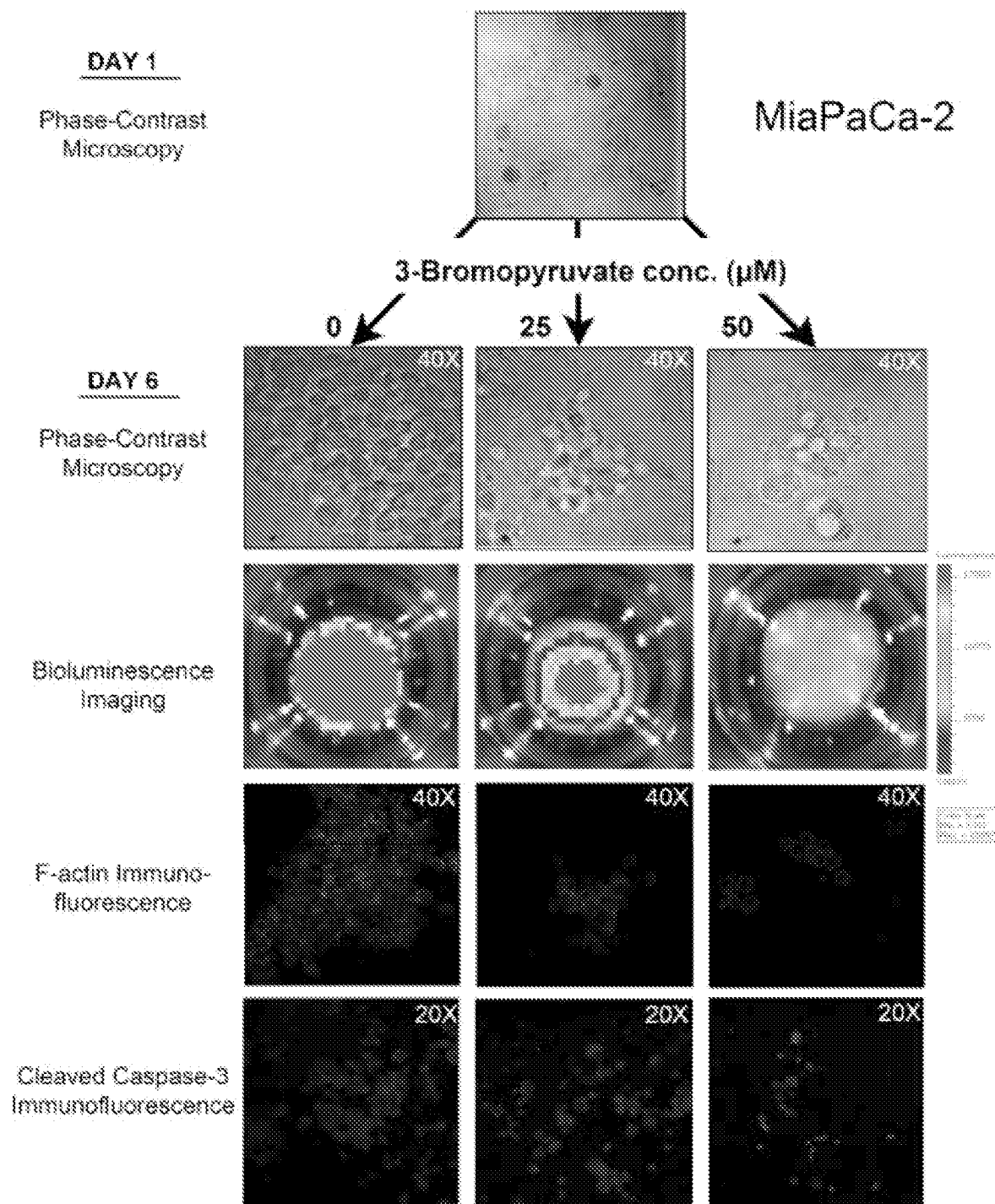


Figure 10

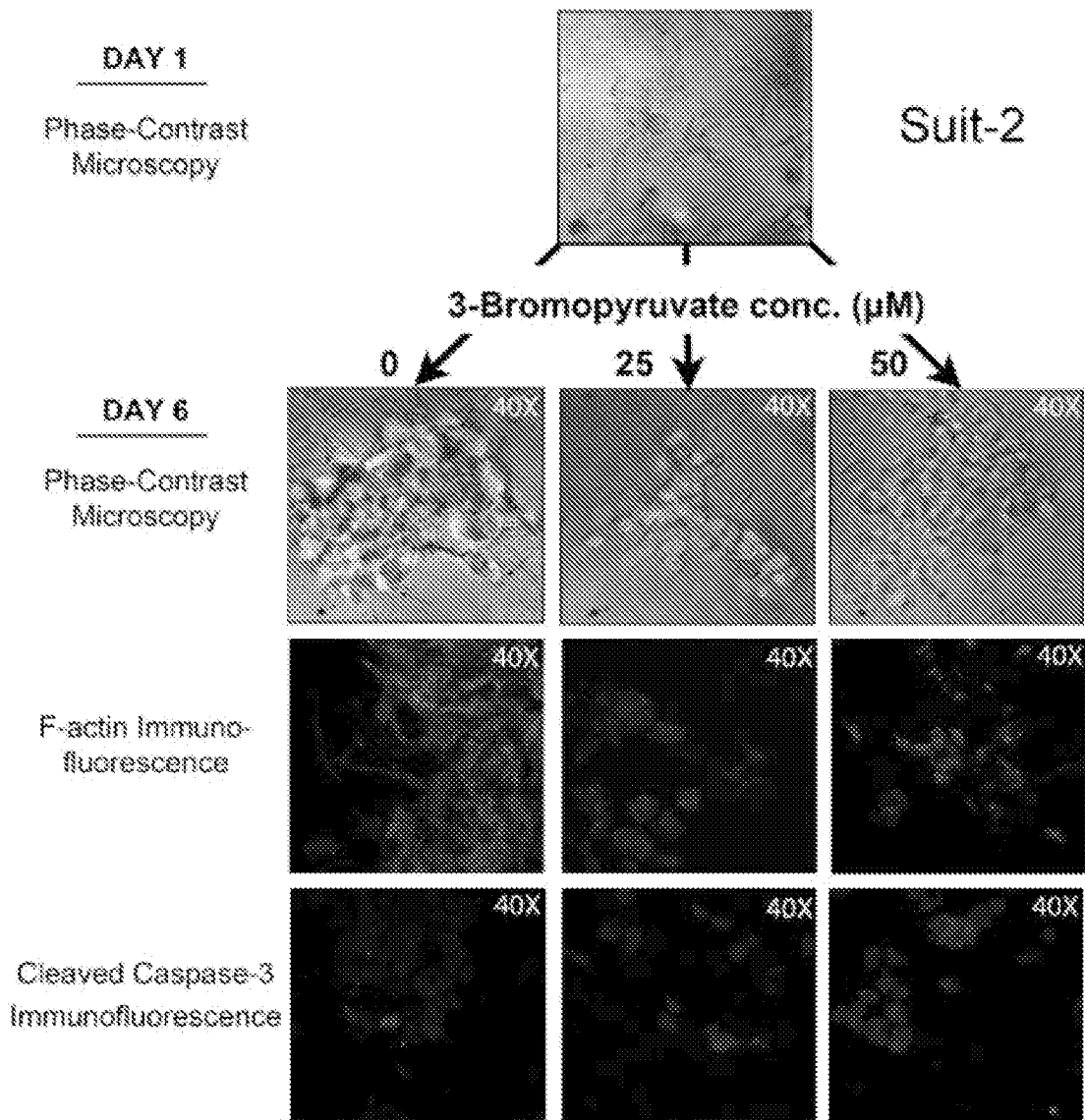


Figure 11

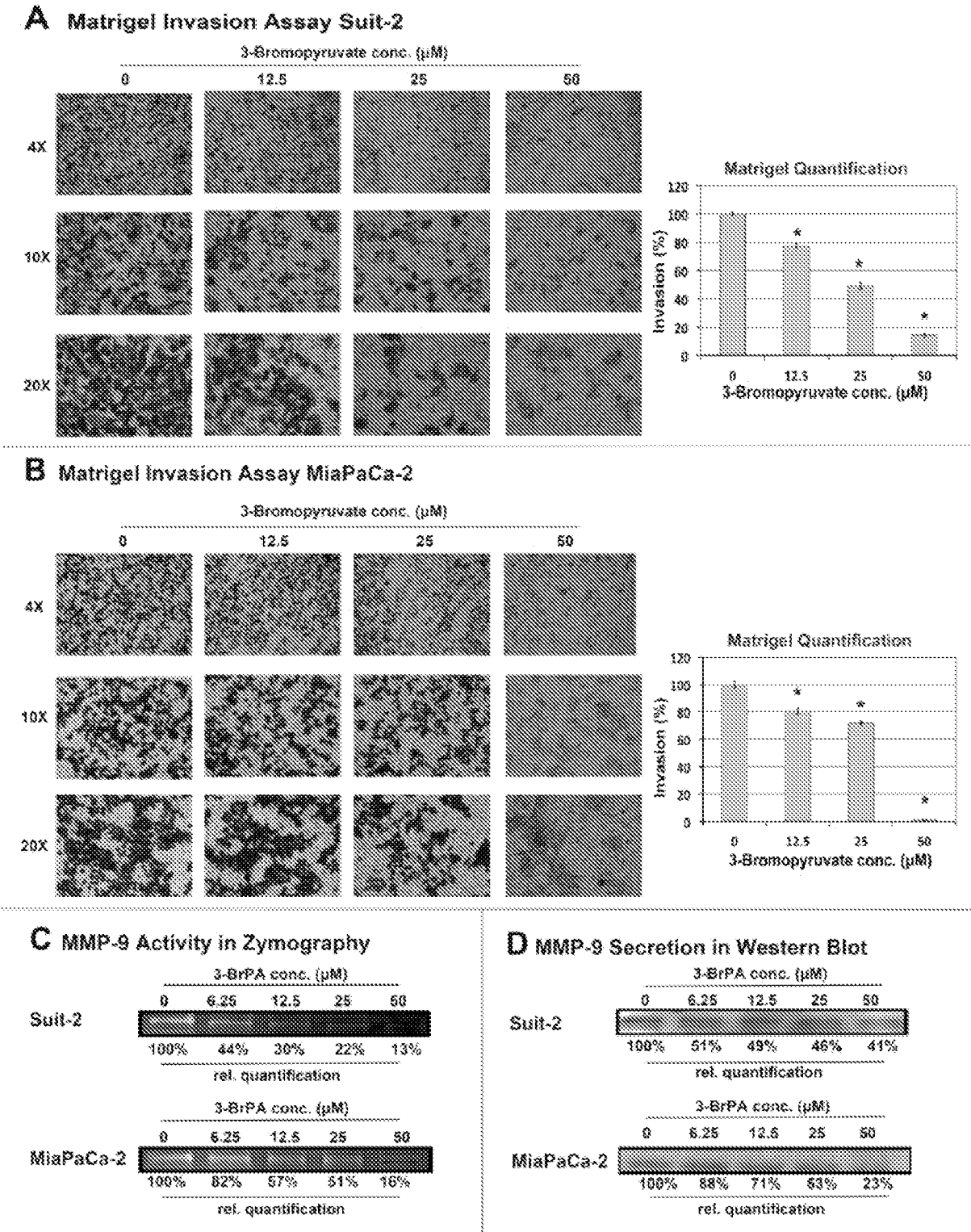




Figure 12

A

Orthotopically Implanted *lucMiaPaCa-2* Xenografts (N=42)

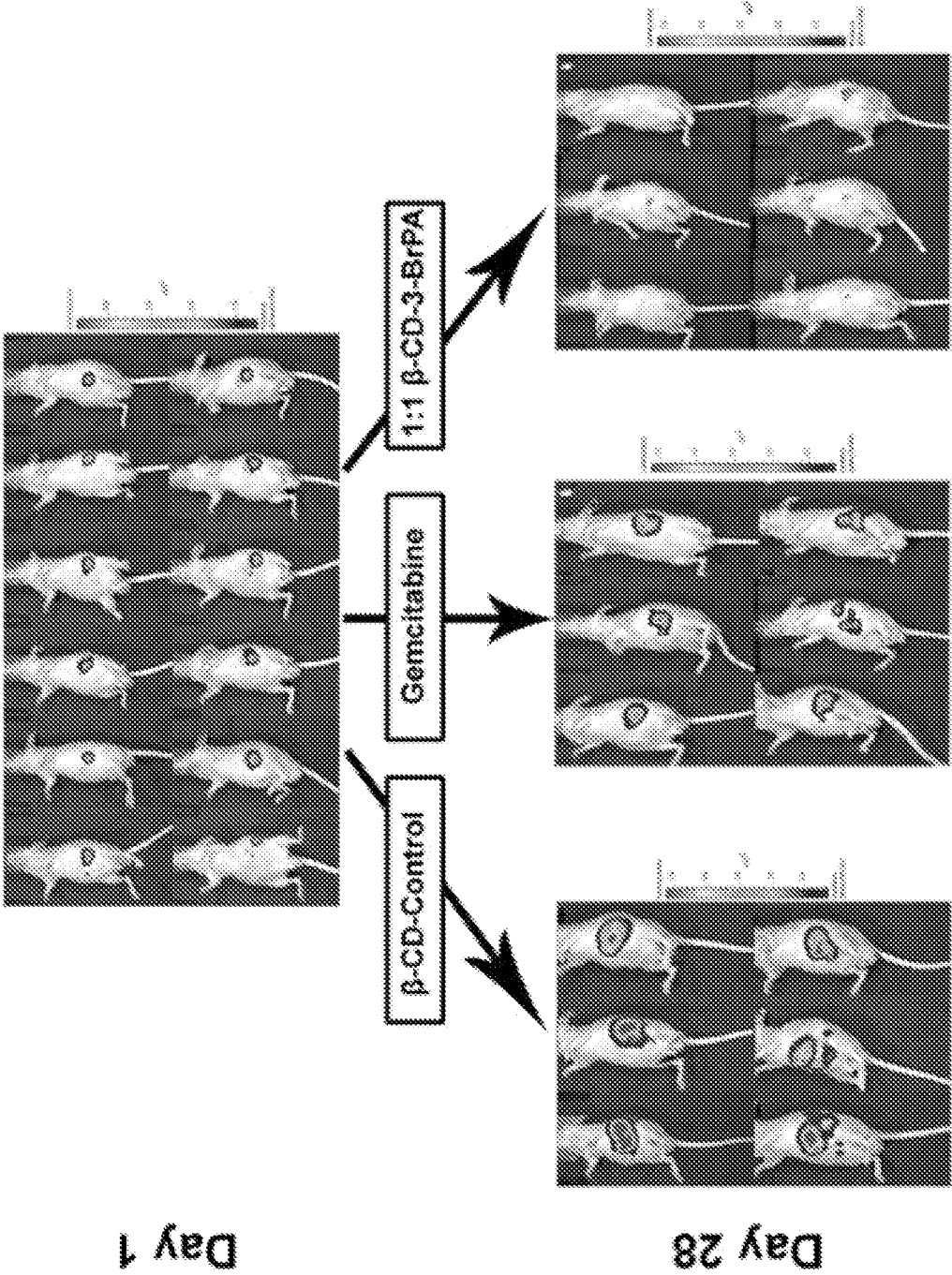
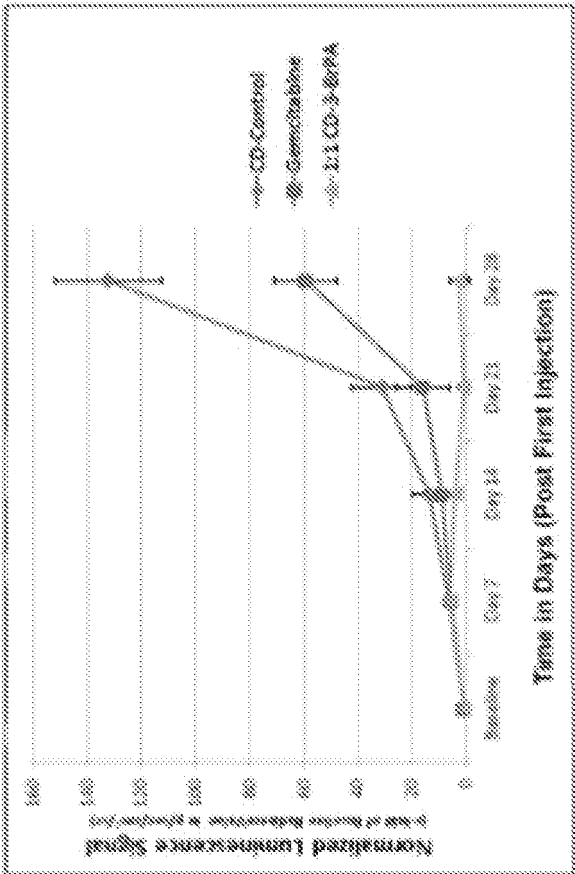


Figure 12 (cont.)

**B**

Treatment Monitoring using Bioluminescence Imaging



**C**

Treatment-related Mortality

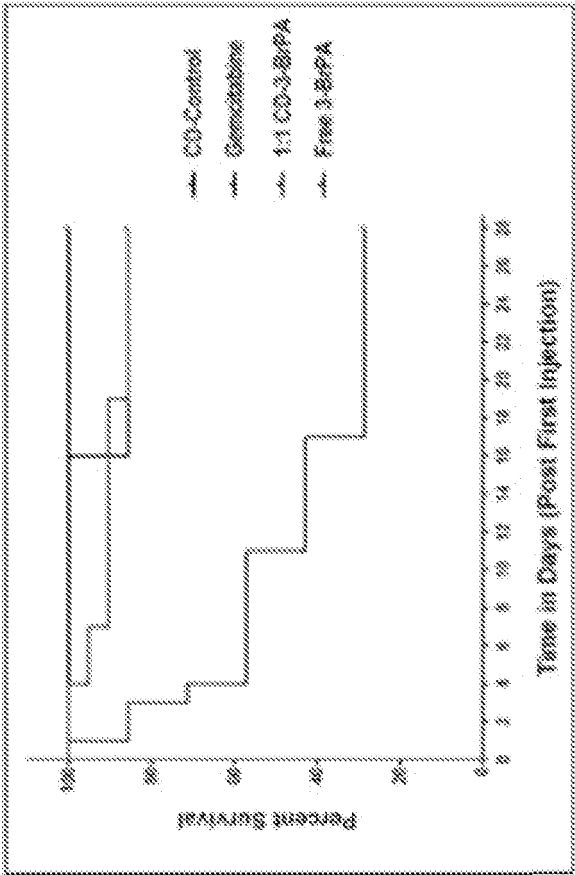


Figure 12 (cont.)

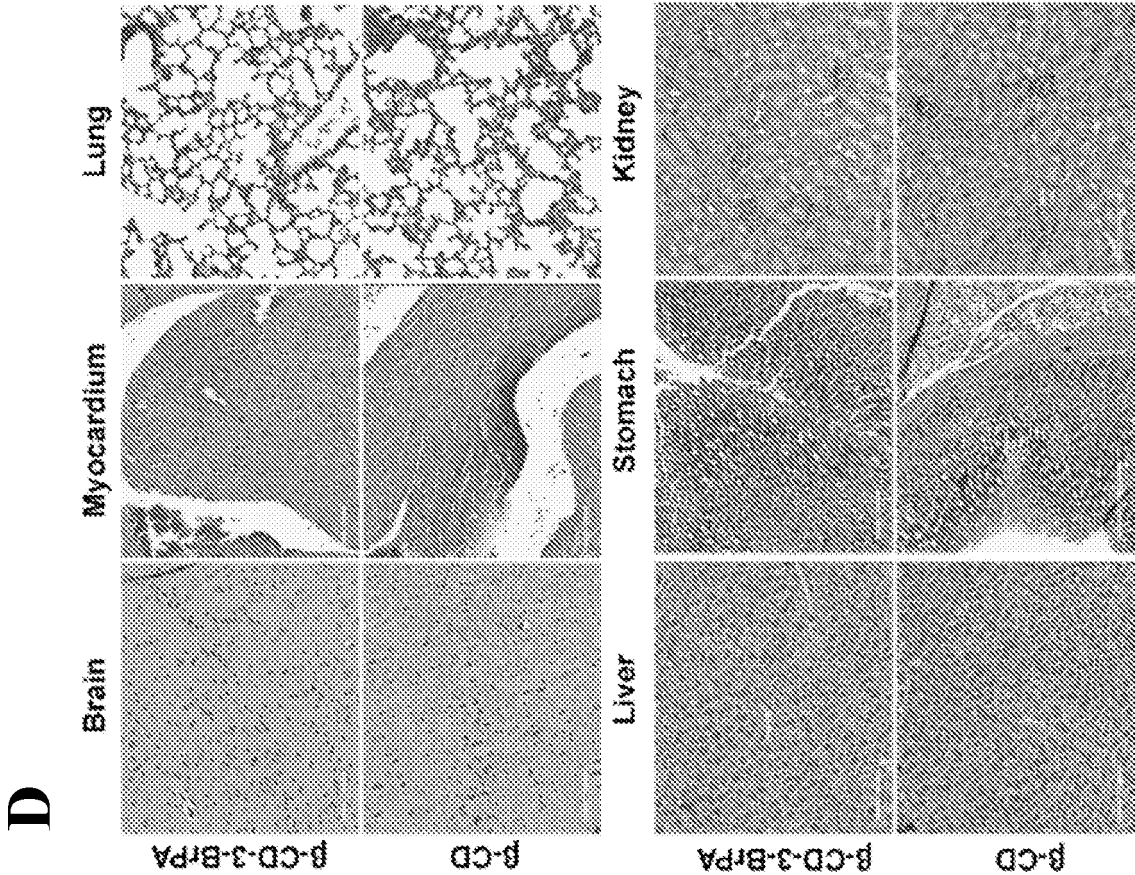


Figure 13

