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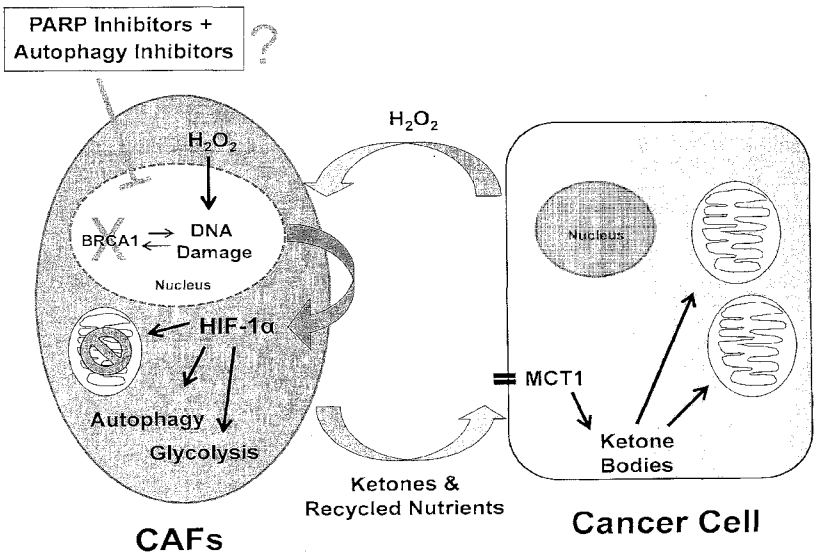

(57) Abstract: This invention provides a method for treating a subject afflicted with breast cancer, comprising concurrently administering to the subject (i) a PARP inhibitor and (ii) an autophagy inhibitor, wherein the amounts of the PARP inhibitor and autophagy inhibitor, when concurrently administered, are therapeutically effective. This invention also provides a related method for inducing the death of a breast cancer cell, as well as a composition comprising (i) a PARP inhibitor, (ii) an autophagy inhibitor, and (iii) a pharmaceutically acceptable carrier.

USE OF PARP INHIBITORS TO TREAT BREAST CANCER

This application claims priority of U.S. Provisional Application No. 61/706,450, filed September 27, 2012, the contents of which are incorporated herein by reference.

Throughout this application, various publications are cited. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.

Background of the Invention

In 1889, Dr. Stephen Paget proposed the "seed and soil" hypothesis to describe the supporting role of the tumor microenvironment (the soil) in promoting the growth and survival of metastatic cancer cells (the seeds). However, most previous studies have focused solely on the role of cancer cells in tumor progression.

Recently, several authors have illustrated the adverse role of the tumor stroma in carcinogenesis [1-3]. To recognize lethal cancer stroma, various authors have demonstrated that loss of caveolin-1 (Cav-1) in the tumor stroma is a biomarker that correlates with poor prognosis in breast, prostate, and melanoma cancer patients [4-10].

Molecular profiling of Cav-1-deficient cancer stroma revealed an elevation of autophagy and glycolysis pathways [11]. Also, previous studies show that the tumor stroma provides metabolic support directly to cancer cells [12]. Tumor initiation begins when cancer cells recruit the surrounding stromal cells by producing reactive-oxygen species (ROS), and inducing oxidative stress. Consequently, cancer-associated fibroblasts (CAFs) undergo DNA damage, which initiates several catabolic pathways, such as autophagy and mitophagy (mitochondrial degradation) [13]. Lacking functional mitochondria, CAFs then shift their metabolism towards glycolysis, producing energy-rich molecules, such as L-lactate, ketone bodies, and glutamine.

Eventually, neighboring cancer cells take these recycled nutrients and "burn" them via oxidative mitochondrial metabolism (OXPHOS) [14]. This vicious cycle has been called the "Reverse Warburg Effect" and, more recently, "Two-Compartment Tumor Metabolism."

Breast cancer type 1 susceptibility protein (BRCA1) is a tumor suppressor that is mutated in 45% of hereditary breast cancers and down-regulated in sporadic breast cancers [15-17]. Approximately 85-90% of hereditary BRCA1 mutation carriers develop breast cancer [15]. BRCA1 is involved in several signaling pathways and cellular processes, such as the DNA damage response, the regulation cell cycle progression, apoptosis, and ubiquitination [15]. Most tumor suppressors, such as Beclin-1, p53, PTEN, and LKB, are autophagy inducers [18-21]. However, BRCA1 has been described recently as an autophagy inhibitor [22, 23]. Esteve *et al.* observed that starved cells down-regulate BRCA1 expression and display an elevation of ROS and autophagy. Conversely, BRCA1 was found to induce several anti-oxidant genes that are responsible for ROS inhibition [22-24]. Unfortunately, all previous BRCA1 studies focused only on the role of BRCA1 in epithelial cancer cells, and not the tumor stroma.

Summary of the Invention

This invention provides a method for treating a subject afflicted with breast cancer, comprising concurrently administering to the subject (i) a PARP inhibitor and (ii) an autophagy inhibitor, wherein the amounts of the PARP inhibitor and autophagy inhibitor, when concurrently administered, are therapeutically effective.

This invention also provides a method for inducing the death of a breast cancer cell, comprising concurrently contacting the cell with (i) a PARP inhibitor and (ii) an autophagy inhibitor, wherein the amounts of the PARP inhibitor and autophagy inhibitor, when concurrently contacted with the cell, are effective to induce the cell's death.

Finally, this invention provides a composition comprising (i) a PARP inhibitor, (ii) an autophagy inhibitor, and (iii) a pharmaceutically acceptable carrier.

Brief Description of the Figures

Figure 1: Generating BRCA1 knock-down fibroblasts. Using shRNA targeted against BRCA1, a BRCA1 knock-down was attempted in hTERT-immortalized human fibroblasts using four different constructs. Construct number 2 displayed the most significant inhibition of BRCA1 expression. Therefore, all subsequent experiments used only this fibroblast cell line.

Figure 2: Increased proliferation in shBRCA1 fibroblasts. shBRCA1 fibroblasts showed a higher rate of cell proliferation, as compared to control cells. Note that after 10 days, the shBRCA1 fibroblast cell count was 5-fold higher than control cells. P-values are as shown on the graph. Error bars = the standard error for n = 3.

Figure 3: BRCA1 knock-down induces autophagy and mitophagy. (A) Immuno-blotting shows an increase in the expression of markers for autophagy (LC3) and mitophagy (BNIP3) in shBRCA1 fibroblasts. (B) Increased autophagy in shBRCA1 fibroblasts when co-cultured with breast cancer cells. Immuno-fluorescence shows that shBRCA1 fibroblasts demonstrate higher expression of the mitophagy marker (BNIP3, red color, top panel) when co-cultured with triple negative breast cancer cell line MDA-MB-231-GFP (green color). Also, shBRCA1 fibroblasts displayed an elevation of the autophagy marker (LAMP1, red color, low panel). Scale bar, 20 μm.

Figure 4: BRCA1 knock-down induces HIF1 and mitochondrial dysfunction. (A) Knock-down of BRCA1 induces HIF-1α expression. shBRCA1 fibroblasts were incubated with 10 μM of MG132 (proteasome inhibitor) plus 1mM of DMOG (HIF-1 α stabilizer) for 5 hours. Pseudohypoxic shBRCA1 fibroblasts demonstrate an elevation of HIF-1α expression. Immuno-blotting for LC3 shows an increase in activated LC3 (LC3 II) in pseudo-hypoxic shBRCA1 fibroblasts. Beta-actin is shown as a loading control. (B) Down-regulation of SDHB in BRCA1-deficient fibroblasts. Immuno-blot analysis displays a decrease of SDHB expression in shBRCA1 fibroblasts. SDHB down-regulation will abrogate mitochondrial oxidative phosphorylation coupling and allow excess succinate to stabilize HIF-1α, which will mediate the mitophagy pathway. Beta-actin is shown as a loading control.

Figure 5: Knock-down of BRCA1 induces Akt-activation. shBRCA1 fibroblasts were incubated with 10 μM of MG132 (proteasome inhibitor) plus 1 mM of DMOG (HIF-1 α stabilizer) for 5 hrs. shBRCA1 fibroblasts demonstrate an increased activation of the Akt pathway, as judged by the elevated phosphorylation of Akt and mTOR. β-actin is shown as a loading control.

Figure 6: Ketone body production and metabolic reprogramming of cancer cells towards oxidative mitochondrial metabolism. (A) Knock-down of BRCA1 induces elevated ketone body production. To evaluate if BRCA1 knock-down induces mitochondrial dysfunction, ketone body generation was measured in the condition media of control and shBRCA1 fibroblasts. It is worth noting that ketone body generation is increased up to 5.5-fold in shBRCA1 fibroblasts, relative to control cells. (B) Mitochondrial activity is induced in cancer cells when co-cultured with shBRCA1 fibroblasts. MDA-MB-231-GFP cancer cells were co-cultured with shBRCA1 fibroblasts in DMEM supplemented with 10% Nu serum for 2 days. Flow-cytometry study shows a 1.4-fold increase in mitochondrial activity of cancer cells, when co-cultured with shBRCA1 fibroblasts.

Figure 7: shBRCA1 fibroblasts induce tumor growth when co-injected with cancer cells. Fibroblasts were subcutaneously co-injected with MDA-MB-231 cells into athymic nude mice. After 4 weeks, mice were sacrificed and tumors were extracted and weighed. shBRCA1 tumors were ~2.2-fold larger than control tumors. N = 10 per experimental group. The P-value is as shown on the graph.

Figure 8: Stromal DNA damage and the "Autophagic Tumor Stroma Model of Cancer Metabolism." Cancer cells release ROS (such as H_2O_2) into the tumor micro-environment, thereby affecting neighboring stromal cells. Oxidative stress and starvation induces DNA damage in stromal cells. Consequently, an activated tumor stroma initiates autophagy and mitophagy related signaling pathways, which will switch fibroblast metabolism towards glycolysis. Moreover, oxidative stress stabilizes HIF-1α expression, which will up-regulate glycolytic molecules and induce mitophagy. High-energy mitochondrial fuels (such as ketones) are secreted into the tumor micro-environment and are then transferred by MCT1

(monocarboxylate transporter) into cancer cells and are consumed by cancer cell mitochondria, via OXPHOS. PARP inhibitors alone can induce autophagy in tumor stroma, which can be detrimental for breast cancer patients. Thus, by combining autophagy inhibitors with PARP inhibitors, cancer cells can be deprived from utilizing stromal autophagy as a fuel source.

Detailed Description of the Invention

Definitions

As used herein, "breast cancer" can be any type of breast cancer, such as noninvasive (in situ) breast cancer and invasive breast cancer. For example, breast cancer includes a breast tumor that is Luminal A, Luminal B, triple negative/basal-like or HER2-type, as well as ductal carcinoma *in situ* (DCIS). Breast cancer also includes, for example, neoplasms having a subtype of ER(+), PR(+), HER2(+), triple negative (ER(-)/PR(-)/HER2(-)), ER(-), PR(-), all tumor and nodal stages, and all tumor grades.

As used herein, "concurrently administering" a first and second agent to a subject means, in one embodiment, administering the first agent according to a first regimen, and administering the second agent according to a second regimen, whereby the first and second regimens overlap in time. For example, a first and second agent are concurrently administered to a subject if, beginning on the first day of treatment, the first agent is administered once per week for 10 weeks, and the second agent is administered daily for the first, third, fifth and seventh weeks. In another embodiment, a first and a second agent are concurrently administered to a subject if they are administered in the form of a single composition containing both agents.

As used herein, "PARP inhibitor" (i.e., an inhibitor of poly ADP ribose polymerase) shall mean an agent that inhibits PARP more than it inhibits any other polymerase. In one embodiment, the PARP inhibitor inhibits PARP at least two-fold more than it inhibits any other polymerase. In another embodiment, the PARP inhibitor inhibits PARP at least 10-fold more than it inhibits any other polymerase. In a third embodiment, the PARP inhibitor inhibits PARP more than it inhibits any other enzyme.

A pharmaceutical composition typically comprises an active agent and a "pharmaceutically acceptable carrier." Pharmaceutical compositions can be in the form of a liquid (e.g., an injectable), a solid (e.g., a tablet), or an emulsion, for example. Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, 0.01-0.1 M

and preferably 0.05 M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers can be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions and suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as Ringer's dextrose, those based on Ringer's dextrose, and the like. Fluids used commonly for i.v. administration are found, for example, in Remington: The Science and Practice of Pharmacy, 20th Ed., p. 808, Lippincott Williams & Wilkins (2000). Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases, and the like. Tablets typically contain excipients such as magnesium stearate, binders such as gelatin or sorbitol, HPMC, carboxymethylcellulose, fillers and lubricants, all known in the art.

As used herein, "subject" shall mean any animal, such as a human, non-human primate, mouse, rat, guinea pig or rabbit.

As used herein, "treating" a subject afflicted with breast cancer shall mean slowing, stopping or reversing the cancer's progression. For example, treating a subject afflicted with breast cancer can mean increasing the subject's progression-free survival ("PFS") by a factor of 1.1, 1.2, 1.5, 2, or greater than 2. In the preferred embodiment, treating a subject afflicted with breast cancer means reversing the cancer's progression, ideally to the point of eliminating the cancer.

Therapeutic agents can be administered by any known means including, for example, orally, intravenously, intramuscularly, topically (such as by injection into, or other direct contact with, the tumor) and subcutaneously. The two agents administered, i.e., the PARP inhibitor and autophagy inhibitor, can be administered by the same route or different routes.

Embodiments of the Invention

This invention provides a method for treating a subject, preferably human, afflicted with breast cancer, comprising concurrently administering to the subject (i) a PARP inhibitor and (ii) an autophagy inhibitor, wherein the amounts of the PARP inhibitor and autophagy inhibitor, when concurrently administered, are therapeutically effective.

In one embodiment, the amount of PARP inhibitor administered would be therapeutically effective even if it were administered without the autophagy inhibitor. In another embodiment, the amount of autophagy inhibitor administered would be therapeutically effective even if it were administered without the PARP inhibitor. In a further embodiment, the amount of PARP inhibitor administered would not be therapeutically effective if it were administered without the autophagy inhibitor. In yet a further embodiment, the amount of autophagy inhibitor administered would not be therapeutically effective if it were administered without the PARP inhibitor.

The PARP inhibitor can be any agent that inhibits PARP. In one embodiment, the PARP inhibitor is olaparib, rucaparib, veliparib, CEP 9722, MK 4827, BMN-673 or 3-aminobenzamide. The PARP inhibitor can be administered, for example, according to any dosing regimen, known or otherwise, that would be appropriate for breast cancer treatment in conjunction with an autophagy inhibitor. In one example, the PARP inhibitor is administered once or twice weekly, for one or more cycles of three weeks or more. In another example, the PARP inhibitor is administered once or twice daily, for one or more cycles of three weeks or more.

The autophagy inhibitor can be any agent that inhibits autophagy. In one embodiment, the autophagy inhibitor is chloroquine, N-acetyl-L-cysteine, L-asparagine, bafilomycin A1, catalase, DBeQ, E-64d protease inhibitor, or GMX1778. Preferably, the autophagy inhibitor is chloroquine, which is preferably administered orally.

The autophagy inhibitor can be administered, for example, according to any dosing regimen, known or otherwise, that would be appropriate for breast cancer treatment in conjunction with a PARP inhibitor. In one example, chloroquine is administered in tablet form at the dosing regimen prescribed for malarial suppression or for treating an acute malarial attack. In another embodiment, the chloroquine is administered according to one of the following dosing regimens: (i) 500mg chloroquine phosphate once per week; (ii) 5mg chloroquine phosphate per kg body weight per week; and (iii) 5mg chloroquine phosphate per kg body weight every 12 hours for a cycle of at least two days, three days, four days, five days, six days, seven days, or more.

The inhibitors can be administered in the form of one or more pharmaceutical compositions. In one embodiment, the inhibitors are administered in the form of one pharmaceutical composition. In another embodiment, the inhibitors are administered in the form of two pharmaceutical compositions, each with its own dosing regimen.

This invention also provides a method for inducing the death of a breast cancer cell (preferably a human cell), comprising concurrently contacting the cell with (i) a PARP inhibitor and (ii) an autophagy inhibitor, wherein the amounts of the PARP inhibitor and autophagy inhibitor, when concurrently contacted with the cell, are effective to induce the cell's death. The meaning of "concurrently" as defined above in conjunction with "administering" applies, *mutatis mutandis*, to "contacting" in the context of this method. In that regard, contacting may occur *in vivo* or *in vitro*.

The PARP and autophagy inhibitors can be any agents that inhibit PARP or autophagy, respectively. In one embodiment, the PARP inhibitor is olaparib, rucaparib, veliparib, CEP 9722, MK 4827, BMN-673, or 3-aminobenzamide, and the autophagy inhibitor is chloroquine, N-acetyl-L-cysteine, L-asparagine, bafilomycin A1, catalase, DBeQ, E-64d protease inhibitor, or GMX1778. Preferably, the autophagy inhibitor is chloroquine.

The inhibitors can be contacted with the breast cancer cell in the form of one or more pharmaceutical compositions. In one embodiment, the inhibitors are contacted with the breast cancer cell in the form of one pharmaceutical composition. In another embodiment, the

inhibitors are contacted with the breast cancer cell in the form of two pharmaceutical compositions.

Finally, this invention provides a composition comprising (i) a PARP inhibitor, (ii) an autophagy inhibitor, and (iii) a pharmaceutically acceptable carrier.

The PARP and autophagy inhibitors can be any agents that inhibit PARP or autophagy, respectively. In one embodiment, the PARP inhibitor is olaparib, rucaparib, veliparib, CEP 9722, MK 4827, BMN-673, or 3-aminobenzamide, and the autophagy inhibitor is chloroquine, N-acetyl-L-cysteine, L-asparagine, bafilomycin A1, catalase, DBeQ, E-64d protease inhibitor, or GMX1778. Preferably, the autophagy inhibitor is chloroquine.

By way of example, in the subject methods and compositions, the following combinations of PARP inhibitor and autophagy inhibitor are specifically envisioned, as set forth in Table 1.

Table 1

olaparib + chloroquine	Rucaparib + chloroquine	Veliparib + chloroquine	CEP 9722 + chloroquine	MK 4827 + chloroquine	BMN-673 + chloroquine	3-ABZ + chloroquine
olaparib + NAC	Rucaparib + NAC	Veliparib + NAC	CEP 9722 + NAC	MK 4827 + NAC	BMN-673 + NAC	3-ABZ + NAC
Olaparib + L-Asp	Rucaparib + L-Asp	Veliparib + L-Asp	CEP 9722 + L-Asp	MK 4827 + L-Asp	BMN-673 + L-Asp	3-ABZ + L-Asp
Olaparib + Baf	Rucaparib + Baf	Veliparib + Baf	CEP 9722 + Baf	MK 4827 + Baf	BMN-673 + Baf	3-ABZ + Baf
Olaparib + catalase	Rucaparib + catalase	Veliparib + catalase	CEP 9722 + catalase	MK 4827 + catalase	BMN-673 + catalase	3-ABZ + catalase
Olaparib + DBeQ	Rucaparib + DBeQ	Veliparib + DBeQ	CEP 9722 + DBeQ	MK 4827 + DBeQ	BMN-673 + DBeQ	3-ABZ + DBeQ
Olaparib + E-64d	Rucaparib + E-64d	Veliparib + E-64d	CEP 9722 + E-64d	MK 4827 + E-64d	BMN-673 + E-64d	3-ABZ + E-64d
Olaparib + GMX1778	Rucaparib + GMX1778	Veliparib + GMX1778	CEP 9722 + GMX1778	MK 4827 + GMX1778	BMN-673 + GMX1778	3-ABZ + GMX1778

PARP inhibitors = olaparib (AZD-2281), rucaparib (AG014699, PF-01367338), veliparib (ABT-888), CEP 9722, MK 4827, BMN-673, and 3-aminobenzamide (ABZ).

Autophagy inhibitors = chloroquine, N-acetyl-L-cysteine (NAC), L-asparagine (L-Asp), bafilomycin A1 (Baf), catalase, DBeQ, E-64d protease inhibitor (E-64d), and GMX1778.

As an additional embodiment of this invention, iniparib is envisioned for use in lieu of olaparib, *mutatis mutandis*, in each of the subject methods and compositions.

This invention will be better understood by reference to the experimental details which follow, but those skilled in the art will readily appreciate that those experimental details are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

A. Introduction

In the current study, it is demonstrated that the knock-down of BRCA1 in cancer-associated stromal fibroblasts is able to significantly promote tumor growth.

First, BRCA1 expression was stably knocked-down in hTERT-immortalized human fibroblasts by using a targeted short hairpin RNA against BRCA1 (shBRCA1). shBRCA1 fibroblasts showed a significant increase in cell proliferation and an elevation of both autophagy and mitophagy markers. Under chemical pseudo-hypoxia, shBRCA1 fibroblasts expressed elevated levels of HIF-1α. This was accompanied by a decrease in succinate dehydrogenase B (SDHB) expression levels. Using flow-cytometry, the co-culture of shBRCA1 fibroblasts and a breast cancer cell line (MDA-MB-231 cells) showed an increase in cancer cell mitochondrial activity. Finally, shBRCA1 fibroblasts dramatically enhanced tumor growth (by >2-fold), when subcutaneously co-injected with a breast cancer cell line into nude mice.

B. Results

Generating BRCA1 knock-down stromal fibroblasts.

Several previous studies have suggested that DNA-damage and chromosomal instability in the tumor stroma are potently tumorigenic [1, 25, 26]. To investigate the possible tumor promoting effects of a defective DNA-damage response in the tumor stroma, hTERT-immortalized human fibroblasts (hTERT-BJ1 cells) were stably transduced with 4 different constructs of short hairpin RNA's targeting BRCA1 (shBRCA1).

Figure 1 shows that only one shBRCA1 construct (designated as #2) effectively knocked-down BRCA1 expression. Thus, all subsequent studies used this cell line transduced with shBRCA1 construct #2.

BRCA1 knock-down accelerates fibroblasts proliferation.

Previous studies have shown BRCA1 knock-down increases the epithelial cancer cell proliferation rate [27]. To validate this result in fibroblasts, shBRCA1 fibroblasts were plated in a 12-well dish. For 10 days, cells were trypsinized and counted using a hemocytometer chamber. At day 10, shBRCA1 fibroblasts demonstrated a 5-fold increase in cell number, as compared to control fibroblasts (Figure 2).

shBRCA1 fibroblasts display higher levels of autophagy and mitophagy markers.

Several studies indicate that knocking-down BRCA1 induces autophagy [22-24]. Using immuno-blot analysis, shBRCA1 fibroblasts displayed an increase in the autophagy marker (LC3) and the mitophagy marker (BNIP3) (Figure 3A). Moreover, immuno-fluorescence shows that shBRCA1 fibroblasts overexpressed BNIP3 and LAMP1 (autophagy marker), when co-cultured with a triple negative breast cancer cell line (namely, MDA-MB-231 cells) (Figure 3B).

shBRCA1 fibroblasts express HIF-1α, under chemical pseudo-hypoxia.

Stable overexpression of HIF-1α mediates mitophagy by triggering over-expression of BNIP3 [28]. As shBRCA1 fibroblasts displayed an elevation of BNIP3 expression, it was desirable to examine HIF-1α expression levels in these cells. Interestingly, shBRCA1 fibroblasts showed an increase of HIF-1α after 5 hours of chemically-induced pseudo-hypoxia (Figure 4A). Concurrently, pseudohypoxic fibroblasts demonstrated an elevation in an autophagy marker (LC3) (Figure 4A).

Knocking-down BRCA1 down-regulates SDHB.

While BRCA1 functions mainly as a DNA guardian, it is also important in maintaining the integrity of mitochondria [22, 29]. Although BRCA1 is mainly present in the cell nucleus, phosphorylated BRCA1 has also been found to be localized in the mitochondrial matrix [30]. Interestingly, in BRCA1-mutated cancers, succinate dehydrogenase B (SDHB, mitochondrial

complex II subunit) was found to be a hot spot gene that frequently showed loss of heterozygosity and allelic instability in cancer stroma [31]. Notably, shBRCA1 fibroblast lysates showed a down-regulation of SDHB expression (Figure 4B).

Complex II is critical in mitochondrial metabolism; upon down-regulation, it disturbs both the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). Consequently, it is likely that disrupted mitochondrial metabolism allowed succinate accumulation and elevated ROS levels to stabilize HIF-1α expression [32]. Moreover, Figure 5 shows that a loss of BRCA1 induces the activation of the Akt-mTOR signaling pathway, likely as a compensatory mechanism to survive the induction of autophagy.

shBRCA1 fibroblasts display increased ketone body generation.

To evaluate if BRCA1 knock-down induces mitochondrial dysfunction, ketone body generation was measured in the conditioned media of control and shBRCA1 fibroblasts. Figure 6A shows that ketone body generation is increased up to ~5.5-fold in shBRCA1 fibroblasts, relative to control cells.

Elevated mitochondrial activity in cancer cells co-cultured with shBRCA1 fibroblasts.

In previous studies, it was shown that autophagic fibroblasts, when co-cultured with cancer cells, induce cancer cell mitochondrial activity [33]. This is due to the production of L-lactate and ketone bodies by autophagic fibroblasts, which are then metabolized by cancer cell mitochondria [33]. Moreover, it has been shown that BRCA1 knock-down decreases mitochondrial activity, which shifts metabolism toward glycolysis [22]. As shBRCA1 fibroblasts show an elevation of mitophagy, shBRCA1 fibroblasts were expected to induce mitochondrial activity, when co-cultured with breast cancer cells. The mitochondrial activity of MDA-MB-231 cells was significantly elevated by ~1.4-fold when they were co-cultured with shBRCA1 fibroblasts (Figure 6B).

shBRCA1 fibroblasts promote xenograft tumor growth.

It was hypothesized that a BRCA1-deficient cancer stroma can promote tumor growth. To test this hypothesis, a pre-clinical model was used in which shBRCA1 fibroblasts were subcutaneously co-injected with MDA-MB-231 cancer cells into the flanks of nude mice. After 31 days, the tumors were excised and weighed.

Importantly, shBRCA1 fibroblasts markedly increased tumor growth by ~2.2-fold, as compared with control fibroblasts (Figure 7). This study highlights the potential lethality of a BRCA1-deficient tumor stroma.

C. Discussion

BRCA1 and the tumor micro-environment

Here, BRCA1 expression was successfully knocked-down using a lentiviral shRNA approach in hTERT-immortalized fibroblasts. shBRCA1 fibroblasts displayed increased proliferation. Moreover, immuno-blot and immuno-fluorescence studies demonstrate that shBRCA1 fibroblasts display an increase in markers of autophagy (LAMP1 and LC3) and mitophagy (BNIP3). Interestingly, shBRCA1 fibroblasts show an increase in HIF-1α expression, when treated with chemical proteasome inhibitors to inhibit HIF-1 α degradation. Furthermore, a decrease in succinate dehydrogenase subunit B (SDHB, a complex II subunit) was demonstrated in shBRCA1 fibroblasts. Under normoxia, prolyl-4-hydroxylase (PHD) promotes the ability of von Hippel-Lindau (VHL) to target HIF-1α for proteasomal-degradation [34]. Guzy et al. showed that knock-down of SDHB allowed excess succinate to migrate to the cytoplasm and inhibit PHD oxidation of HIF-1α, resulting in the stabilization of HIF-1α [32]. Alternatively, stabilized HIF-1α mediates the expression of BNIP3, which is responsible for mitochondrial autophagy [35]. Therefore, the relationship between BRCA1 and mitochondrial integrity is unclear [22, 30]. Also, when shBRCA1 fibroblasts were co-cultured with breast cancer cells, the cancer cells displayed an increase in mitochondrial activity. Similar results were obtained when glycolytic fibroblasts were co-cultured with cancer cells, as the fibroblasts secrete ketone bodies, which are utilized in cancer cell mitochondria [33]. Interestingly, shBRCA1 fibroblasts

increased tumor growth by ~2.2-fold when co-injected with MDA-MD-231 human breast cancer cells.

For the first time, this current study demonstrates the potential lethal effects of a BRCA1-deficient tumor stroma on breast cancer tumor growth.

In previous studies, evidence was provided for the pro-tumorigenic effects of an autophagic and glycolytic tumor stroma [5, 11]. To better explain epithelial-stromal interactions in cancer progression, a new paradigm was suggested to describe this co-evolution termed the "Autophagic Tumor Stroma Model of Cancer Metabolism" (Figure 8) [13]. In this model, it is hypothesized that the first step is the release of hydrogen peroxide (H_2O_2) by cancer cells, which then activates neighboring stromal cells, thereby inducing more oxidative stress [13]. Accordingly, several authors have indicated that an activated stroma displays higher DNA damage than cancer cells. Following DNA damage, several molecules trigger autophagy and mitophagy pathways [36]. As a result, CAFs switch their metabolism towards glycolysis and secrete energy-rich molecules (such as L-lactate and ketone bodies) into the tumor micro-environment. Moreover, elevated ROS or mitochondrial dysfunction stabilizes HIF-1α, which will act as a feed-forward mechanism for more mitophagy and glycolysis [32, 37]. In turn, cancer cells over-express MCT1 to import stromally-derived nutrients (L-lactate and ketone bodies) and to employ them in oxidative mitochondrial metabolism [38]. This vicious cycle provides a steady-stream of high-energy metabolites that directly supports cancer cell growth.

PARP inhibitors in clinical trials.

BRCA1 is a homologous recombination (HR) protein that is involved in the repair of DNA double strand breaks (DSB's). When BRCA1 is down-regulated by mutation or methylation, HR fails to repair the DSB's, which will be subsequently repaired by non-homologous end joining (NHEJ). As NHEJ fixes DSB's randomly, it induces chromosomal instability, which can lead to cancer development or cell death [39].

The process of random repair in BRCA1-deficient cancer cells led to the idea to employ poly (ADP-ribose) polymerase (PARP) inhibitors, in treating familial BRCA1 cancers or triple negative breast cancers (TNBC) [39]. The PARP enzyme is responsible for base excision repair (BER) in single strand breaks (SSB's). BRCA1-mutation carriers have heterozygous BRCA1 mutations in normal tissue, while tumor cells display homozygous BRCA1 mutations after loss of heterozygosity (LOH) [31]. Hence, when BRCA1-deficient cancer cells are treated with PARP inhibitors, SSB's develop DSB's, and then NHEJ haphazardly repairs the damaged DNA. Increased DNA repair errors selectively kill BRCA1-deficient cancer cells [39]. This "synthetic lethality" theory was promising for the treatment of lethal TNBC. Thus, several pharmaceutical companies conducted phase I/II trials using PARP inhibitors to treat BRCA1 tumors. Unfortunately, a phase III trial of Iniparib (originally thought to be a PARP inhibitor, and produced by Sanofi-Aventis) failed to offer any significant benefits for the treatment of metastatic TNBC patients, when combined with cytotoxic agents [40]. This drawback discouraged other companies, such as AstraZeneca, from performing a phase III trial for the PARP inhibitor, olaparib. It is speculated that this failure is due to the fact that PARP inhibitors not only target epithelial cancer cells, but will also target the BRCA1-deficient tumor stroma [31]. Inhibiting PARP in the BRCA1-deficient tumor stroma can elevate or activate the autophagy pathway [41]. According to the subject study, inducing autophagy in the tumor stroma will promote the production of recycled nutrients that increase cancer cell growth.

In view of the subject discoveries discussed above, it is expected that PARP inhibitors will benefit breast cancer patients when combined with autophagy inhibitors such as chloroquine.

D. Materials and Methods

Materials

Antibodies used in this paper were purchased from commercial sources: anti-BRCA1 (Santa Cruz, C-20, SC-642), anti-BNIP3 (ab10433, Abcam), anti-LC3A/B (Ab58610, Abcam), anti-HIF-1 α (NB100-123, Novus), anti-beta actin (A5441, Sigma), anti-SDHB (NBP-54154, Novus), and anti-LAMP1 (Santa Cruz, SC-17768). Other reagents were purchased as follows; Hoechst-

33258 nuclear stain was from Sigma, Anti-fade reagent (S2828) was from Invitrogen, MG-132 purchased from Calbiochem and DMOG was from Cayman Chemical.

Cell culture and transfection

MDA-MB-231 cells (a human triple-negative breast cancer cell line) and hTERT-immortalized human fibroblasts (hTERT-BJ1) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum in a 37°C humidified atmosphere containing 5% CO_2, unless otherwise noted. Lentiviral particles were produced after 48 hours of transfecting GeneCopoeia 293Ta lentiviral packaging cell line with lentiviral plasmids (CSHCTR001-HIVmH1) (empty vector) or shBRCA1-1 (HSH017713-1-HIVmH1(OS224289)) or shBRCA1-2 (HSH017713-2-HIVmH1(OS224290)) or shBRCA1-3 (HSH017713-3-HIVmH1(OS224291)) or shBRCA1-4 (HSH017713-4-HIVmH1(OS224292)) (all obtained from GeneCopoeia, Inc.), using the Lenti-Pac HIV Expression Packing Kit (GeneCopoeia, Inc.), according to manufacturer's recommendation. hTERT-immortalized fibroblasts were transduced by lentivirus particles in the presence of 5 μg/ml of Polybrene (Santa Cruz Biotech). Transduced cells were then selected with 1.5 μg/ml of puromycin.

Growth curves

Two thousand fibroblast per well were plated in a 12-well tissue-culture dish. For each time point, the cells were trypsinized, stained with trypan blue, and then counted using a hemocytometer. The average of each cell count was then calculated from three independent representative wells.

Immuno-blot analysis

For chemically-induced pseudo-hypoxia, one million fibroblasts were plated in 10 cm dishes. The next day, the media was discarded and replaced with complete DMEM, supplemented with 10 μM MG-132 (a proteasome inhibitor) plus 1mM dimethyloxallyl glycine (DMOG, a stabilizer of HIF-1 α) for 5 hours. Then, the cells were lysed by scraping into lysis-buffer (10 mM Tris,

pH 7.5, 150 mM NaCl, 1% Triton X-100 and 60 mM n-octylglucoside), containing protease inhibitors (Boehringer Mannheim, Indianapolis, IN). Samples were incubated on a rotating platform at 4°C and were then centrifuged at 12,000x g for 10 min (at 4°C) to remove insoluble debris. Protein concentrations were analyzed using the BCA reagent (Pierce, Rockford, IL).

Samples were then separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose. All subsequent wash buffers contained 10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20, which was supplemented with 5% nonfat dry milk (Carnation) for the blocking solution and 1% bovine serum albumin (Sigma) for the antibody diluent. Horseradish peroxidase-conjugated secondary antibodies were used to visualize bound primary antibodies with an ECL detection kit (Pierce). (Pierce, Rockford, IL).

Immuno-fluorescence

MDA-MB-231 cells and hTERT-fibroblasts were plated on coverslips in 12-well plates for 48 hours in DMEM supplemented with 10% Nu serum. Then, the cells were rinsed with PBS with 0.1 mM CaCl2 and 1mM MgCl2 (PBS/CM), and fixed with 2% paraformaldehyde in PBS/CM for 30 minutes. After fixation, cells were washed three times with PBS/CM, and permeablized with IF buffer (PBS/CM with 0.1% Triton-X100 and 0.2% bovine serum albumin) for 10 minutes. Then, cells were quenched with 50 mM NH4Cl in PBS/CM for 10 minutes, rinsed and incubated with anti-BNIP3 or anti-LAMP1 antibodies for 1 hour at room temperature. Cells were washed with IF buffer and incubated with fluorescent secondary antibodies (Molecular Probes) for 30 minutes. After washing, cells were incubated with Hoechst-33258 for nuclear staining, rinsed, and mounted with ProLong Gold anti-fade (Molecular Probes). Images were acquired with a Zeiss LSM510 Meta confocal microscope system and analyzed with Zeiss LSM Browser (3,5,0,359; Zeiss).

Mitochondrial activity studies

Cell lines were stained with MitoTracker Orange (CMTMRos; cat #M7510, Invitrogen, Inc.) to measure mitochondrial activity, as the stain's accumulation is dependent upon an intact

mitochondrial membrane potential. Fibroblasts and cancer cells were cultured for 2 days in DMEM supplemented with 10% Nu serum. Then, after 2 days, the cells were incubated with pre-warmed MitoTracker staining solution (diluted in serum-free DMEM to a final concentration of 25 nM) for 10 min at 37°C. All subsequent steps were performed in the dark. Cells were washed in PBS, trypsinized and re-suspended in 300 μL of Binding Buffer (BD Biosciences). Cells were then analyzed by FACS. Data analysis was performed using FlowJo 8.8 software.

Animal studies

All studied animals were maintained in a pathogen free environment/barrier facility at the Kimmel Cancer Center at Thomas Jefferson University under National Institutes of Health (NIH) guidelines. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC). hTERT-BJ1 fibroblasts (3×10^5 cells) with tumor cells (MDA-MB-231; 1×10^6 cells) in 100 μl of sterile PBS were co-injected into the flanks of athymic NCr nude mice (NCRNU; Taconic Farms; 6–8 weeks of age). Mice were then sacrificed at 31 days post-injection; tumors were excised to determine their weight.

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What is claimed is:

1. A method for treating a subject afflicted with breast cancer, comprising concurrently administering to the subject (i) a PARP inhibitor and (ii) an autophagy inhibitor, wherein the amounts of the PARP inhibitor and autophagy inhibitor, when concurrently administered, are therapeutically effective.

2. The method of claim 1, wherein the PARP inhibitor is selected from the group consisting of olaparib, rucaparib, veliparib, CEP 9722, MK 4827, BMN-673, and 3-aminobenzamide.

3. The method of claim 1, wherein the autophagy inhibitor is selected from the group consisting of chloroquine, N-acetyl-L-cysteine, L-asparagine, bafilomycin A1, catalase, DBeQ, E-64d protease inhibitor, and GMX1778.

4. The method of claim 3, wherein the autophagy inhibitor is chloroquine.

5. The method of claim 1, wherein the subject is human.

6. The method of claim 1, wherein the inhibitors are administered in the form of one or more pharmaceutical compositions.

7. A method for inducing the death of a breast cancer cell, comprising concurrently contacting the cell with (i) a PARP inhibitor and (ii) an autophagy inhibitor, wherein the amounts of the PARP inhibitor and autophagy inhibitor, when concurrently contacted with the cell, are effective to induce the cell's death.

8. The method of claim 7, wherein the PARP inhibitor is selected from the group consisting of olaparib, rucaparib, veliparib, CEP 9722, MK 4827, BMN-673, and 3-aminobenzamide.

9. The method of claim 7, wherein the autophagy inhibitor is selected from the group consisting of chloroquine, N-acetyl-L-cysteine, L-asparagine, bafilomycin A1, catalase, DBeQ, E-64d protease inhibitor, and GMX1778.

10. The method of claim 9, wherein the autophagy inhibitor is chloroquine.

11. The method of claim 7, wherein the breast cancer cell is a human breast cancer cell.

12. The method of claim 7, wherein the inhibitors are contacted with the breast cancer cell in the form of one or more pharmaceutical compositions.

13. A composition comprising (i) a PARP inhibitor, (ii) an autophagy inhibitor, and (iii) a pharmaceutically acceptable carrier.

14. The composition of claim 13, wherein the PARP inhibitor is selected from the group consisting of olaparib, rucaparib, veliparib, CEP 9722, MK 4827, BMN-673, and 3-aminobenzamide.

15. The composition of claim 13, wherein the autophagy inhibitor is selected from the group consisting of chloroquine, N-acetyl-L-cysteine, L-asparagine, bafilomycin A1, catalase, DBeQ, E-64d protease inhibitor, and GMX1778.

16. The composition of claim 15, wherein the autophagy inhibitor is chloroquine.

FIGURE 1

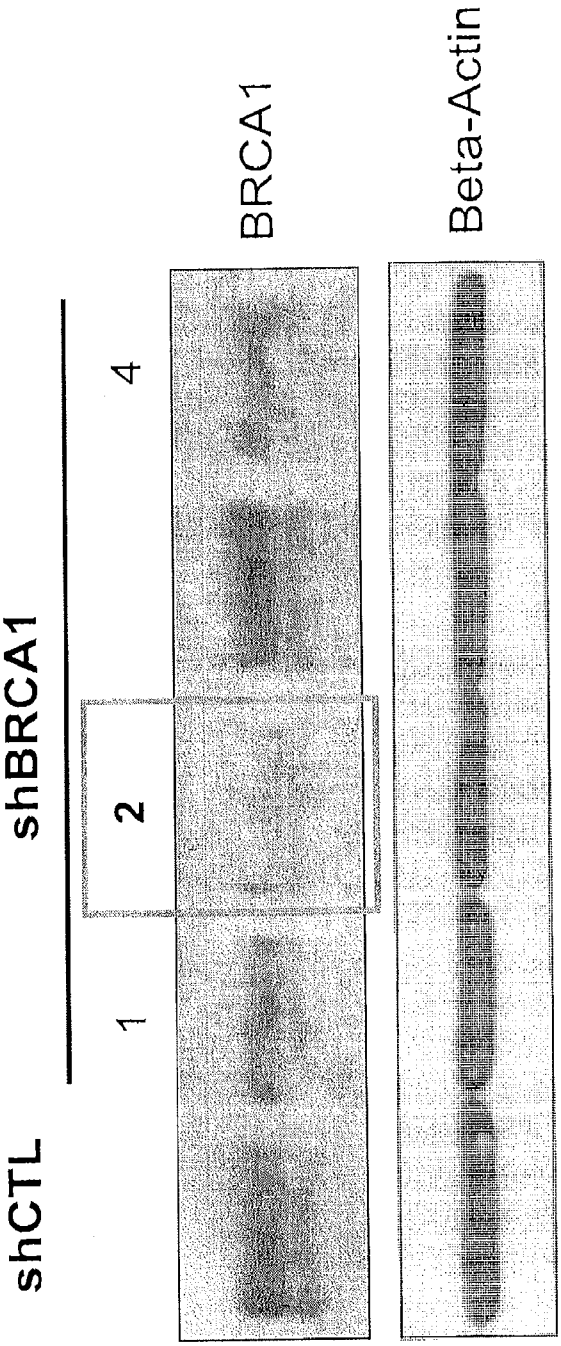


FIGURE 2

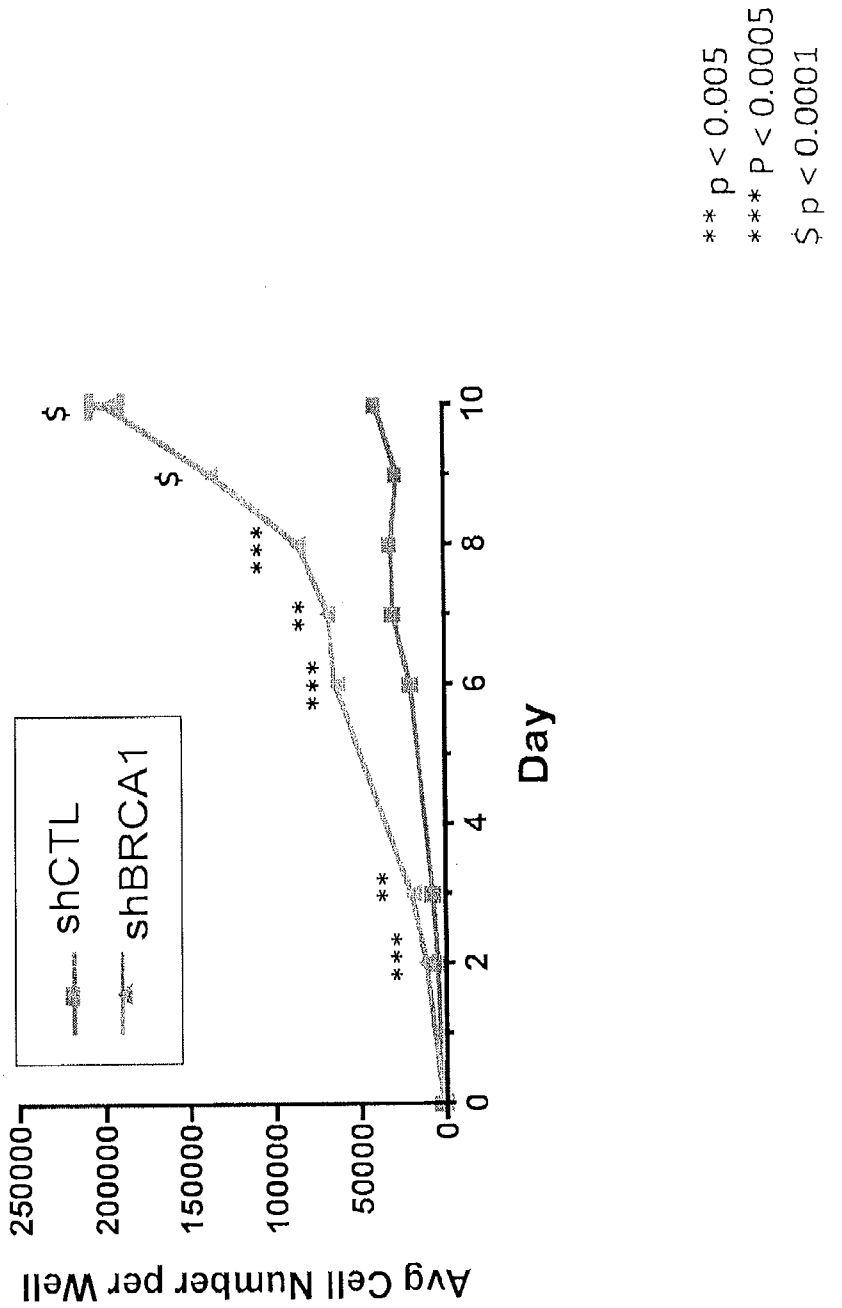

** p < 0.005
*** P < 0.0005
$ p < 0.0001
Avg Cell Number per Well
250000
200000
150000
100000
50000
0
0
2
4
6
8
10
Day
shCTL
shBRCA1
$
$
***
**
***
**
***
**

FIGURE 3A

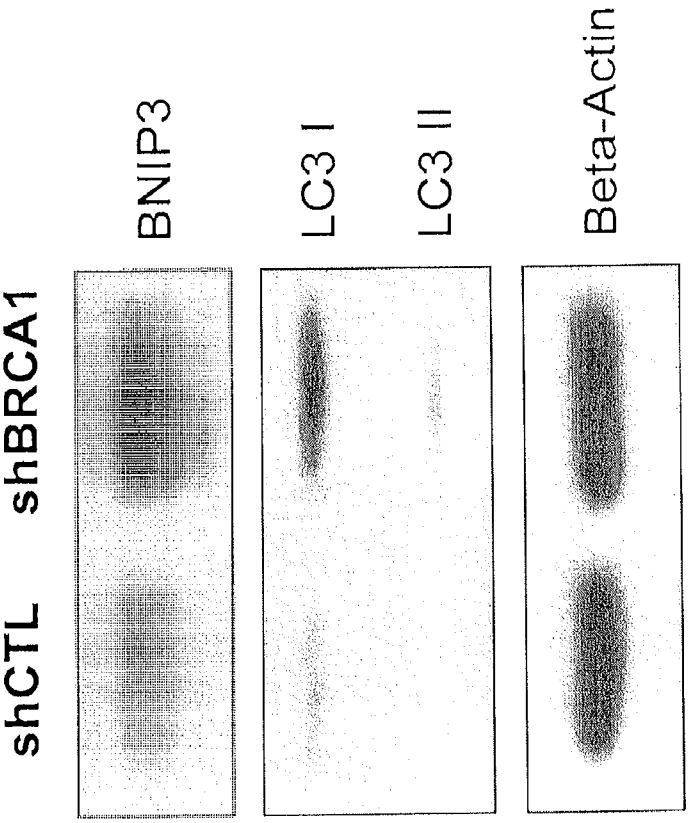


FIGURE 3B

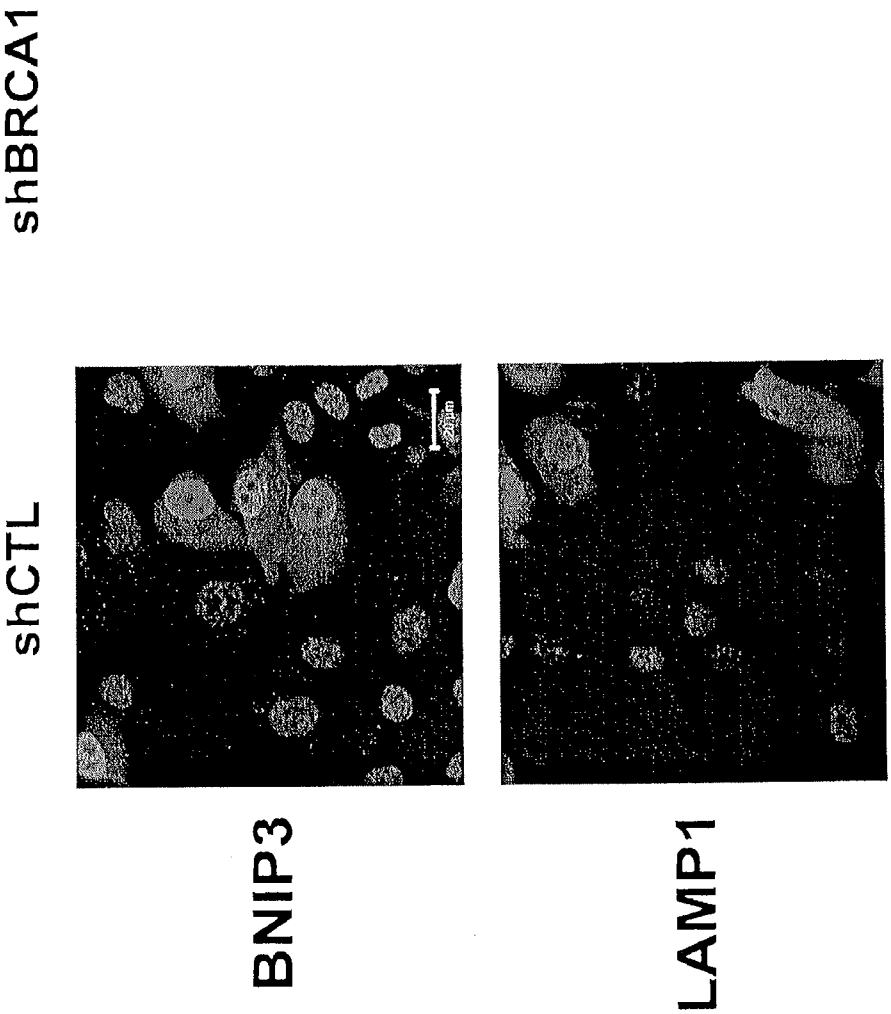


FIGURE 4A

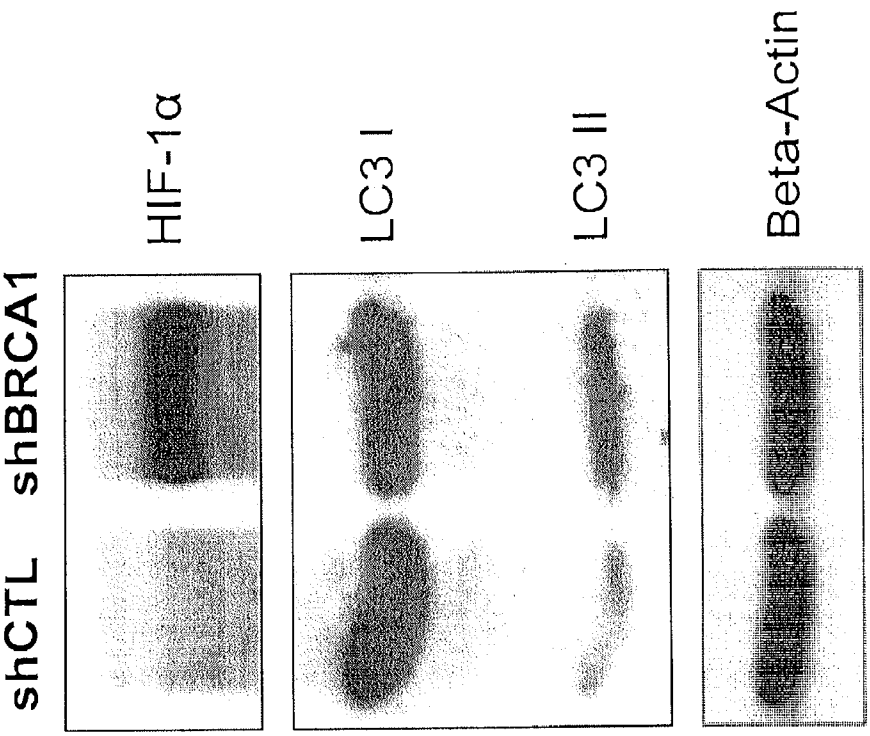


FIGURE 4B

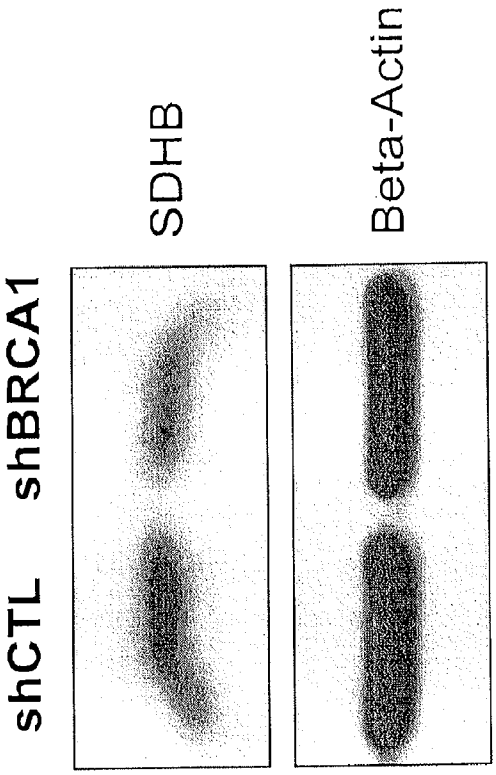


FIGURE 5

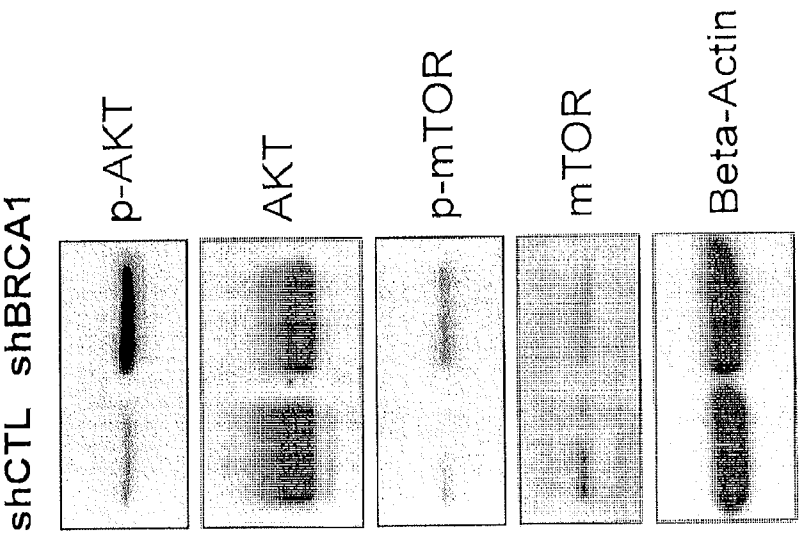


FIGURE 6A

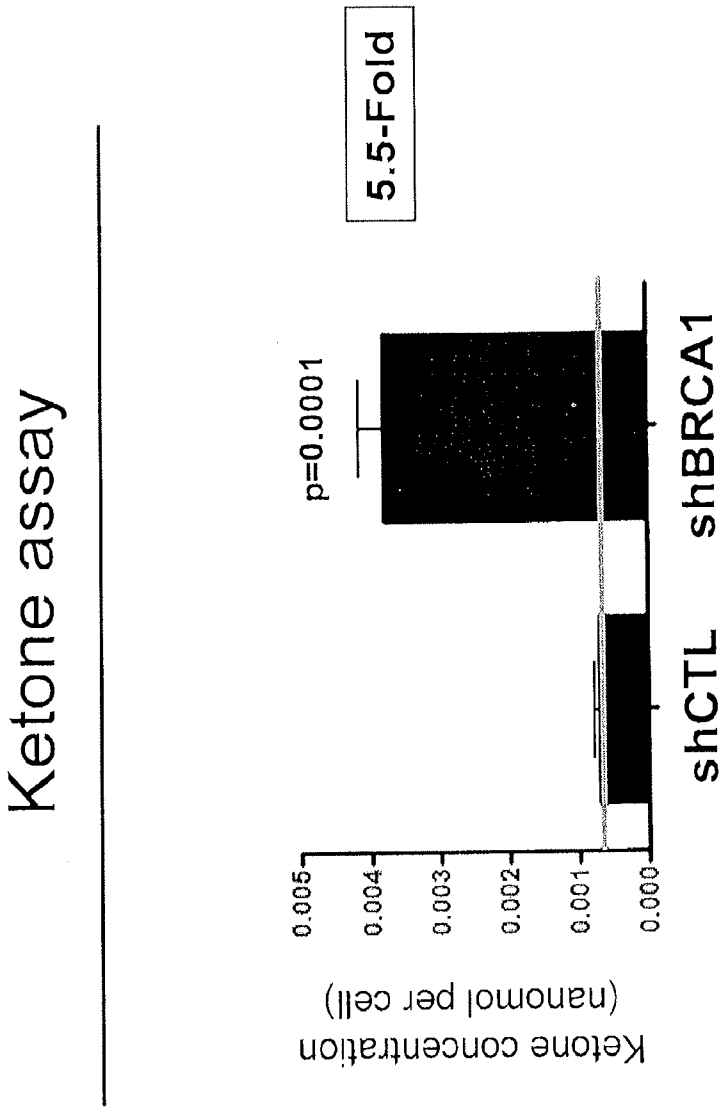

Ketone assay
5.5-Fold
p=0.0001
0.005
0.004
0.003
0.002
0.001
0.000
Ketone concentration
(nanomol per cell)
shCTL
shBRCA1

FIGURE 6B

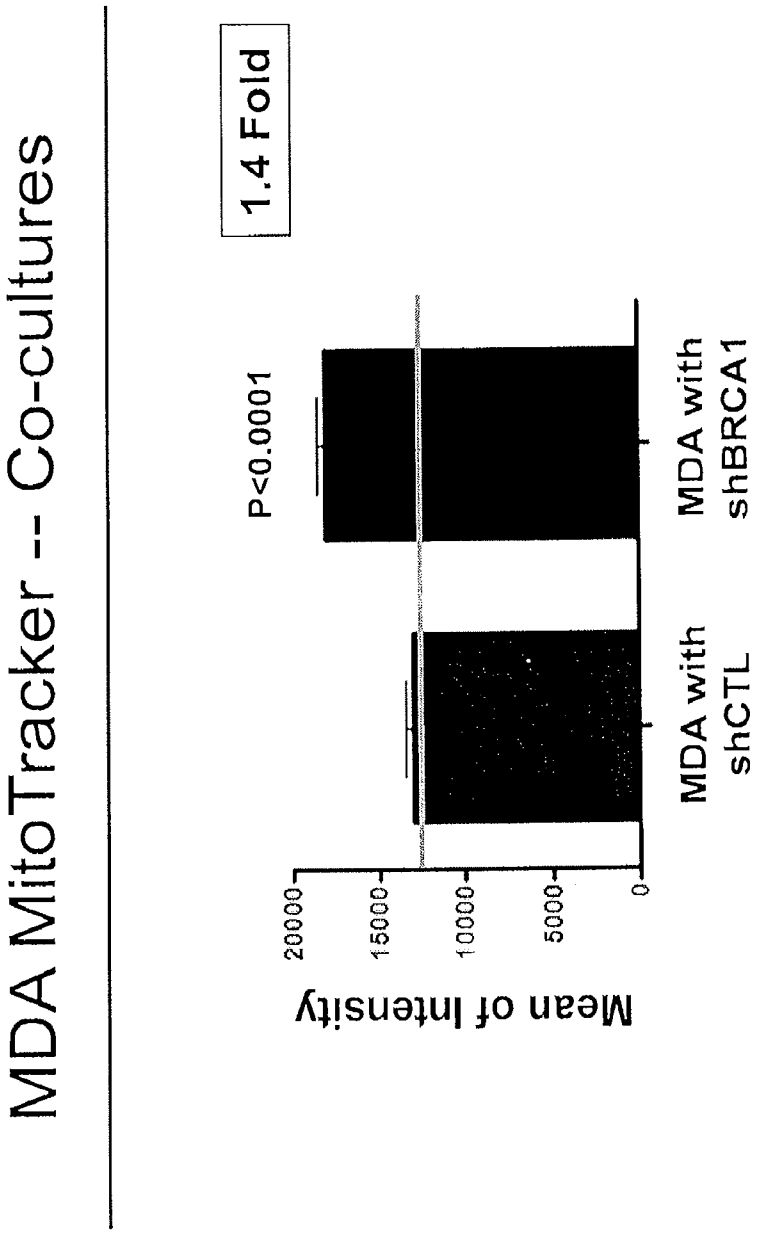

MDA MitoTracker -- Co-cultures
1.4 Fold
P<0.0001
Mean of Intensity
20000
15000
10000
5000
0
MDA with shCTL
MDA with shBRCA1

FIGURE 7

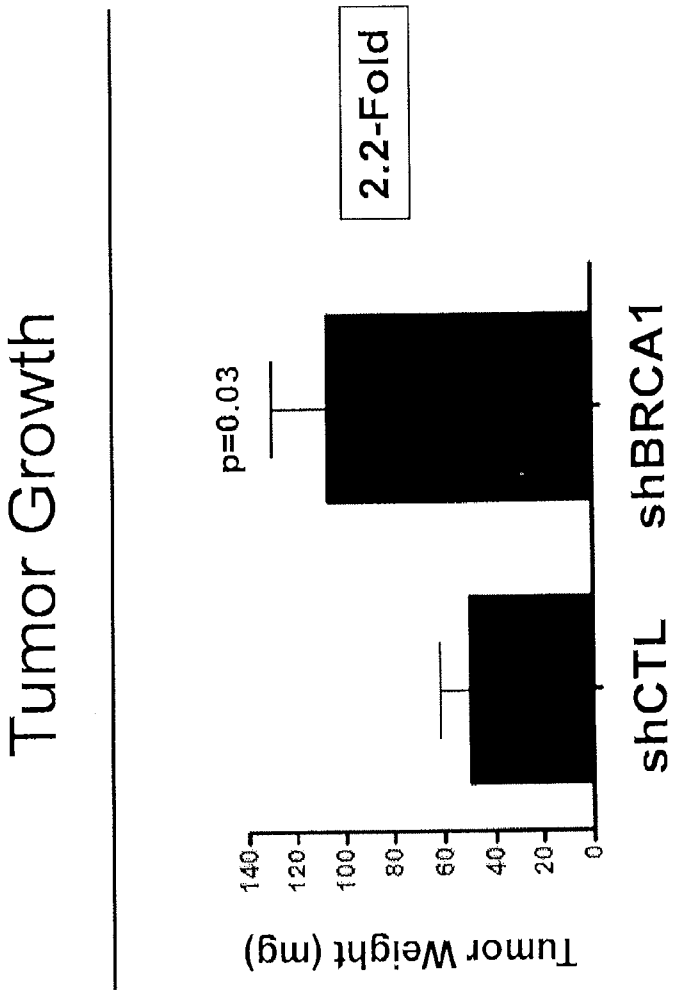
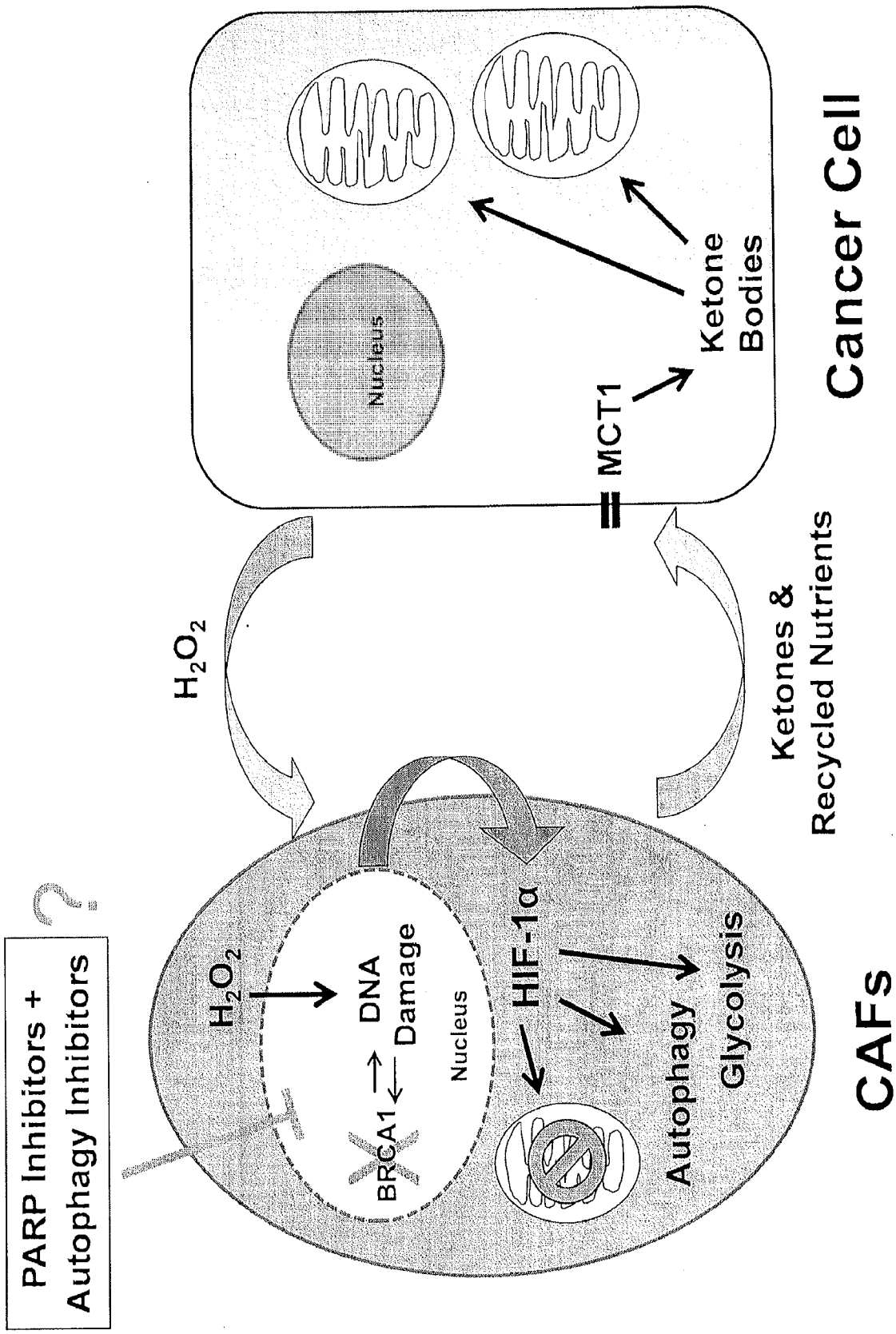

Tumor Growth
2.2-Fold
p=0.03
Tumor Weight (mg)
140
120
100
80
60
40
20
0
shCTL
shBRCA1

FIGURE 8


PARP Inhibitors +
Autophagy Inhibitors
?
H_2O_2
DNA
Damage
BRCA1
Nucleus
HIF-1α
Autophagy
Glycolysis
CAFs
H_2O_2
Ketones &
Recycled Nutrients
MCT1
Ketone
Bodies
Nucleus
Cancer Cell

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/61862

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/166, 45/06; A61P 35/00 (2014.01)
USPC - 435/184, 375; 514/044
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 31/166, 31/282, 31/433, 45/06; A61P 35/00; C07D 237/26, 285/14; C12N 9/99 (2014.01)
USPC - 435/184, 375; 514/044, 249, 361, 619; 544/235; 548/126

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google Scholar; PubMed; IP.com; phagocytic inhibitor, PARP*1 inhibitor; olaparib, rucaparib, 3-aminobenzamide;chloroquine, N-acetyl-L-cysteine, bafilomycin; breast cancer, carcinoma NEAR5 breast, apoptosis, programmed cell death

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 2002/013826 A1 (KI-CHANG, K et al.) February 21, 2002; page 7, lines 15-16; page 8, lines 20-26; page 9, lines 1-10; page 11, lines 1-11, lines 20-25; page 12, lines 9-17, lines 22-33; page 13, lines 12-16	1, 3-7, 9-12 ----- 2, 8, 13-16
Y	US 2012/0190565 A1 (LISANTI, MP et al.) July 26, 2012; paragraphs [0166]-[0167]	2, 8, 13-16
Y	ZAREMBA, T et al. "Doxorubicin-Induced Suppression of Poly(ADP-ribose) Polymerase-1 (PARP-1) Activity and Expression and Its Implication for PARP Inhibitors in Clinical Trials". Cancer Chemotherapy Pharmacology. Vol. 66, pages 807-812; May 21, 2010; page 810, column 2	1-16
A	WO 2010/051043 A1 (REN, P et al.) May 6, 2010; entire document	1-16

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 January 2014 (21.01.2014)	06 FEB 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)