

Simultaneous CK2/TNIK/Dyrk1 inhibition by 108600 suppresses triple negative breast cancer stem cells and chemotherapy-resistant disease

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Abstract

Triple negative breast cancer (TNBC) remains clinically challenging as patients have heterogeneous responses to current standard of care therapies. Chemotherapy sensitivity is a strong predictor of long-term outcomes for patients, and incomplete response of early stage disease to chemotherapy treatment is associated with a much higher risk of disease relapse and metastatic progression, often occurring within a short time from initial diagnosis. Therefore, treatment strategies that target chemotherapy-resistant TNBC and/or enhance chemosensitivity would improve outcomes for these high-risk patients. Breast cancer stem cell-like cells (BCSC) have been proposed to represent a chemotherapy-resistant subpopulation within the tumor which are also responsible for tumor initiation, progression and metastases. Targeting this population could lead to improved TNBC disease control. We have identified a novel multi-kinase inhibitor 108600 from a screen for inhibitors of this TNBC BCSC population. 108600 treatment suppresses growth, colony and mammosphere forming capacity of the BCSC population. Treatment with 108600 induces G2M arrest and eventual apoptosis of TNBC cells *in vitro* and of TNBC xenografts *in vivo*, and overcomes chemotherapy (paclitaxel) resistance of triple negative patient-derived xenografts (PDX). Finally, treatment with 108600 and chemotherapy suppressed the growth of already established TNBC metastases, providing additional support for the clinical translation of this agent to clinical trials.

Introduction

Triple Negative Breast Cancers (TNBCs), defined as tumors that lack expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) represent approximately 15% of diagnosed breast cancers¹. While some patients do well with standard of care multi-modality therapy that includes chemotherapy, many patients have suboptimal response. Chemotherapy resistance is a negative prognostic factor with poorer survival^{2,3}. Chemotherapy resistance of TNBC is thought to be due to the presence of breast cancer stem cells (BCSCs), a subpopulation of cells that resides within the tumor^{4,5}. These cells are relatively resistant to standard chemotherapeutic agents, and have the ability to renew, grow as mammospheres *in vitro* and initiate tumor development in mouse models. At a molecular level, BCSCs are generally defined as CD44^{high}CD24^{low}EpCAM⁺ and/or have high levels of aldehyde dehydrogenase-1 (ALDH1^{high}) enzyme activity^{6,7}. Several studies have shown that sorted human breast cancer CD44^{high}/CD24^{low}EpCAM⁺ cells have increased tumorigenic potential in mouse xenograft assays⁶. Like normal stem cells, BCSCs divide asymmetrically, generating additional BCSCs that display the same characteristics as the parental cell (CD44^{high}/CD24^{low}) as well as differentiate into daughter cells (CD44^{low}CD24^{low}).

Metastatic and invasive properties of TNBC is attributed to CD44 which is thought to mediate invasion via urokinase-type plasminogen activators (uPAs)^{8,9}. Furthermore, ALDH1, a BCSC marker, is statistically correlated with decreased survival and poor prognosis^{10,11}. Together, these studies strongly suggest that targeting these (CD44^{high}/CD24^{low}/ALDH1⁺) cells is likely to result in a successful therapeutic strategy.

Identification of new proteins and pathways in tumor cells from RNAi screens has presented new opportunities for drug development, both in terms of using RNAi as a therapeutic agent and identifying new targets. However, a common finding from these screens is that many genes are required for the survival of tumor cells, making it difficult to choose a single target for drug development^{12,13}. This complexity of cancer has led to recent interest in targeted polypharmacological approaches for developing multikinase inhibitors with low toxicity profiles¹⁴

In an attempt to understand the molecular basis of TNBC growth and to develop a targeted therapy for TNBCs, we designed an approach where we combined the identification of “oncogenic kinases” with drug discovery. Because growth of most tumors is dictated by the activation of kinase cascades, we designed a strategy to identify possible oncogenic kinases upon which a breast cancer stem cell might be dependent. This was made possible because of our library of approximately 4,000 novel compounds, which consists of approximately 2,000 ATP-mimetics which can function as kinase inhibitors. We used this group of compounds to determine if any of these compounds can induce the death of CD44^{high}/CD24^{low} BCSCs purified from TNBC cells. This approach allowed two important aspects of drug discovery to occur simultaneously: the identification of new therapeutic targets and the development of novel pharmaceuticals that can inhibit these targets. This strategy identified 108600, which shows remarkable efficacy against triple negative breast tumor cells, including the BCSC population, with minimal toxicity towards normal cells. Our studies presented here show that 108600 is a potent inhibitor of TNBC growth *in vitro* and *in vivo*, including chemotherapy-resistant and metastatic disease. Our data support clinical translation of 108600 as a complement to standard chemotherapy for high risk, aggressive TNBC to improve patient outcomes.

Results

Derivation of a small molecule inhibitor of TNBC growth.

In this study, we used phenotypic screening of TNBCs as an approach to identify the nature of signal transduction pathways activated in these cells and simultaneously identify compounds that can target these pathways. To accomplish this goal, we used four TNBC cell lines MDA-MB-231, MDA-MB-468, Hs-578T and BT-20 as well as CD44^{high}/CD24^{low} BCSCs purified cell population derived from these cell lines to screen a compound library synthesized in our laboratory to identify compounds that induce apoptosis of these cells. This strategy identified 108600 (Fig. 1A), which showed remarkable toxicity towards these tumor cells and the purified BCSC population with little toxicity to normal cells. This compound was assayed against a panel of 285 functional kinases by Reaction Biology Corporation (RBC), which revealed that 108600 is a multi-kinase inhibitor and inhibits CK2α1, α2, DYRK1A, 1B, and DYRK2 and TNIK at low nanomolar concentration (Fig. 1B). We independently tested the inhibitory activity in *in vitro* kinase assays using recombinant CK2α1 and CK2α2 as well as DYRK1A and DYRK1B kinases (data not shown). Our results showed that 108600 is a potent inhibitor of all four kinases, thus validating the analysis by RBC.

108600 inhibited endogenous CK2 and DYRK activity toward its substrates in a dose-dependent manner in triple negative breast cancer cells. (Fig. 1C). We evaluated the effect of 108600 treatment on established substrates of these kinases. CK2 has been shown to phosphorylate AKT1 at serine (S) 129, which promotes a hyper-activated state of AKT1^{15,16}. Additionally, threonine (Thr) 286 of CYCLIN D1 is a known phosphorylation site for DYRK1A¹⁷. Treatment with 108600 led to decreased phosphorylation at both of these sites, indicating that 108600 treatment suppresses endogenous CK2 α and DYRK1A activities in TNBC cells.

108600 inhibits viability of TNBC CD44^{high}/CD24^{low} breast cancer stem cells.

We evaluated the effect of 108600 treatment on growth of an expanded panel of breast cancer cell lines, as well as five normal and non-transformed cells. The kinases targeted by 108600 are robustly expressed in most TNBC cell lines with relatively lower expression in normal cells (fibroblasts, immortalized human mesenchymal stem cells) (Supplementary Fig. 1). 108600 potently inhibited viability of all triple negative and other breast tumor cell lines with little or no toxicity against normal cells, including normal mammary epithelial cell lines derived from 2 different donors (Fig. 1D). The growth suppression of TNBC cells (MDA-MB231) observed with 108600 treatment was phenocopied by simultaneous transfection of MDA-MB-231 cells with three siRNA pools targeting CK2, DYRK1 and TNIK, all of which are targets of 108600 (Fig. 1E). Transfection of MDA-MB-231 cells with single siRNA pools targeting CK2, DYRK1, or TNIK individually had no or modest effect on growth, supporting the need for simultaneous multi-kinase targeting (Supplementary Fig. 2).

To specifically examine the effect of 108600 treatment on the BCSC population, we purified CD44^{high}/CD24^{low} cells from two TNBC cell lines (MDA-MB-231 and BT-20) by FACS and examined the effects of 108600 treatment on colony and mammosphere forming capabilities of this CD44^{high}/CD24^{low} fraction. 108600 potently inhibited colony growth (Fig. 1F). We also examined the effects of 108600 on patient derived TNBC cells in 2D and 3D cultures using commercially available breast cancer stem cells (XLC 479) (CD44^{high}/CD24^{low}/ALDH+) derived from a TNBC patient (Creative Bioarray, Shirley, NY). 108600 potently inhibited mammosphere formation by these cells in *in vitro* 2D and 3D cultures (Fig. 1F and Supplemental Fig. 3), which is in agreement with the data obtained from sorted populations isolated from established TNBC cell lines.

Structure of CK2 α 1 in complex with 108600

To gain an understanding of the mechanisms by which 108600 inhibits CK2 kinases, we expressed, purified, crystallized, and determined the structure of the human CK2 α 1 catalytic domain in complex with 108600 (Supplemental Fig. 4). The structure was solved by molecular replacement and refined to 1.80 Å resolution. The structure of CK2 α 1 has the familiar bilobal architecture of a protein kinase, composed of N-terminal lobe followed by a larger C-terminal lobe (Fig. 2B). 108600 binds in the cleft between the two domains, where ATP would normally be accommodated (Fig. 2A and 2B). The drug adopts a conformation where the NO₂ group and the aromatic ring are coplanar. The density for 2,6-dichlorobenzyl

ring of the drug is disordered due to the conformational flexibility of this aromatic moiety in the solvent-exposed region of the active site (Fig. 2A-2D).

Earlier work has shown that CK2 is able to use both, ATP and GTP as phosphoryl donors with similar efficiencies. The crystal structure of the human CK2 α 1 in complex with the ATP non-hydrolysable analogue AMP-PNP has been determined at a 1.3 Å resolution (Fig. 2E) ¹⁸. The CK2 α 1 (Zea mays) complex structure with GMP-PNP resolved at 2.2 Å showed that GMP-PNP and water molecules mimic AMP-PNP in the active site of protein kinase CK2 ¹⁹. Our studies show that the O3 and N4 of 108600 take the positions of the O6 and N1 atoms of GMP-PNP, respectively, and maintain the same interactions with the backbone atoms of His115 and Val 116 (Fig. 2D and 2F). Moreover, the NO₂ group of the drug is near the position that is occupied by the Pa group of AMP-PNP or GMP-PNP and establishes a hydrogen bond with the side chain of Lys68 (Fig. 2D, 2E and 2F). In addition, the central benzothiazinone and aromatic rings are involved in numerous hydrophobic contacts with residues such as Leu45, Val66, Ile95, Phe113, and Ile174 that line the ATP/GTP binding cavity. The carbonyl group of the thiazine ring interacts with the main chain NH group of His115 and the NH proton of the ring establishes a hydrogen bond with the main chain carbonyl group of Val116. Importantly, a water molecule (wat 1) in the drug complex forms hydrogen bonds with the OH group of the drug and the side chain of Glu81 (Fig. 2D). Wat1 is also observed in the complex with AMP-PNP (Fig. 2E). Moreover, the drug covers the area where wat 2, wat 3 and wat 4 are bound in the AMP-PNP/ CK2 α 1 complex (Fig. 2E) and wat 7 in the GMP-PNP/ CK2 α 1 complex (Fig. 2F). To compare the binding modes of 108600 and AMP-PNP/GMP-PNP, the coordinates for all complexes were superimposed (Fig. 2G). Significantly, the drug mimics not only the shape and electrostatics of AMP-PNP/GMP-PNP, but also their hydration patterns in the CK2 α 1 active site pocket (Fig. 2D, 2E, 2F and 2G).

108600 induces a conformational change in CK2 α 1 which is not conducive to holoenzyme formation

The CK2 holoenzyme consists of two catalytic subunits (CK2 α 1 or CK2 α 2), which interact with a CK2 β dimer forming a transient complex ²⁰. Interestingly, the CK2 holoenzyme has been shown to bind substrates and activate them in a kinase-independent manner ²¹. CK2 α 1-AMP-PNP either alone or integrated into the holoenzyme has the loop connecting β 4 and β 5 sheets in the “open” conformation (Fig. 2H) conducive to integration with CK2 β subunit (Fig. 2I and 2J). In the absence of AMP-PNP, the β 4- β 5 loop is in the “closed” conformation (Fig. 2H). Intriguingly, in the presence of 108600, the β 4- β 5 loop adopts the “closed” conformation that is obstructive to holoenzyme formation (Fig. 2H, 2I and 2J). Hence, 108600 binding to CK2 α 1 appears to induce a conformational change, which represents a state that is not conducive to holoenzyme formation (Fig. 2I and 2J). 108600 may thus inhibit CK2 as a both a competitive inhibitor of ATP/GTP and as an allosteric inhibitor that weakens the interaction between C2Ka and C2Kb *in vivo*.

108600 inhibits tubulin polymerization and causes abnormal mitosis

Small interfering RNAs directed against CK2 α and β subunits exhibit mitotic abnormalities ranging from defects in centrosome duplication, anaphase spindle elongation and chromosome mis-segregation²². CK2 holoenzyme is an established Microtubule Associated Protein (MAP) that aids in tubulin polymerization and regulates microtubule cytoskeletal reorganization^{23,24}. Since 108600 induces a conformational change in the CK2 α subunit potentially interfering with its ability to bind to the β -subunit, we examined the effects 108600 on CK2-holoenzyme mediated tubulin dynamics *in vitro* and assessed for effects on mitosis. We adapted and employed a published protocol²⁴, which measures CK2 holoenzyme mediated de novo tubulin polymerization. We incubated MAP-free tubulin preparations with either recombinant purified CK2 α , CK2 β -His-Sumo or pre-assembled (CK2 α -CK2 β) holoenzyme respectively and measured the extent of tubulin polymerization. We did not observe any tubulin polymerization in the presence of individual CK2 subunits (CK2 α or CK2 β), even after a prolonged incubation (Fig. 3A and B). However, addition of CK2 holoenzyme to tubulins resulted in enhanced polymerization, in accordance to the published observation that CK2 holoenzyme promotes tubulin polymerization. When we pre-treated the CK2 holoenzyme with 108600, we observed a decrease in CK2-holoenzyme-mediated tubulin polymerization (Fig. 3A and B). These effects suggest that 108600 inhibits CK2 holoenzyme formation and hence tubulin dynamics.

To study the mitotic phenotype induced by 108600, cells on collagen coated glass coverslips were treated with either DMSO or 108600 for varying periods of time. The cells were stained for α -tubulin and the centrosomal marker pericentrin. While DMSO-treated mitotic cells displayed normal bipolar spindle architecture, the cells treated with 108600 formed multipolar spindles and multiple centrosomes (Fig. 3C). These effects are similar to those observed with CK2 subunit inhibition reported by Bettencourt-Dias et al²², further supporting the fact that 108600 inhibits CK2-dependent functions.

108600 treatment induces cell cycle arrest and apoptosis of TNBC cells

Given the potent inhibition of TNBC cell growth observed with 108600 treatment (Fig. 1) and effects on tubulin polymerization and mitosis (Fig. 3), we assessed cell cycle progression in triple negative MDA-MB-231, BT-474 and Hs578T cells treated with increasing concentrations of 108600 for varying periods of time. Treatment of all three cell lines with 108600 resulted in a potent, dose and time-dependent G2/M arrest (Fig. 4A and Supplemental Fig. 5) as assessed by flow cytometric analysis. The percentages of cells in the G2/M phase were clearly increased with 108600 treatment, while the fraction of cells in the G1 or S phases concomitantly decreased. Notably, 108600 treatment increased the percentage of cells in the sub-G1 fraction, indicating apoptotic cells, as well as increasing the percentage of polyploid or aneuploid cells.

Similar results were observed with organoid cultures derived from triple negative patient-derived xenograft (PDX) that express the targets of 108600 (CK2, DYRK, TNIK) (Fig. 4B, Supplemental Fig. 6). These observations strongly suggest that treatment of TNBC with 108600 leads to prolonged G2/M arrest and eventually induces cell death. To more specifically determine whether 108600 treatment induces apoptosis, we examined levels of cleaved PARP and cleaved Caspase 3 in cells treated with

increasing doses of 108600 for 24-48 hours. Increased cleaved PARP was detected in both MDA-MB-231 cells and TNBC PDX organoids (Fig. 4C and D), supporting the induction of apoptosis by 108600 treatment. Interestingly, when we performed these studies with normal proliferating cells such as hMSC-hTERT (immortalized human-mesenchymal stem) or MCF-10A cells (human mammary stem cells) which express very low levels of CK2, we observed minimal or no mitotic arrest at concentrations of 1 μ M or higher of 108600 (data not shown). The majority of these cells remained in the G1 phase, with a modest increase in the G2/M population. Taken together, these results suggest that 108600 does not induce mitotic arrest of normal cells, which might explain its selective toxicity to tumor cells.

Chemotherapy-Resistant TNBCs are Sensitive to 108600 Treatment

Patients with TNBC are often treated with anthracycline and taxane-based chemotherapy as standard of care. However, as response to these chemotherapies is heterogenous, we determined whether 108600 treatment would also be effective in suppressing viability and inducing apoptosis of chemotherapy-resistant TNBC cells *in vitro*. We generated paclitaxel-resistant variants of the MDA-MB-231 and BT-20 cell lines by exposing them to increasing concentrations of paclitaxel (PTX). Resistance was validated by cell viability, colony formation and apoptosis assays. After continuous exposure and passaging in PTX-containing medium, we obtained MDA-MB-231 and BT-20 cells (MDA-MB-231-TR and BT-20-TR), which exhibited greater than 250-fold resistance to PTX as compared to the parental cell lines (Fig. 5B).

We investigated whether cells with the identified paclitaxel-resistant phenotype were associated with increased expression of the CD44^{high} CD24^{-/low} stem-cell like population, similar to those of patients who are refractory to taxane therapy²⁵. Interestingly, protein expression analysis of parental and MDA-MB-231-TR cells showed an increase in the level of CD44 expression relative to the parental cell line, which is similar to that observed with the BCSC population (Fig. 5C). In addition, several target kinases of 108600 were also upregulated in the PTX-R variant cell line (Fig. 5C).

When parental and PTX-R MDA-MB-231 and BT-20 cells were treated with varying concentrations of 108600 for 72-96 hours, we observed that 108600 potently inhibited growth and colony formation of both parental and PTX-resistant cells with the same efficiency (Fig. 5D), suggesting that 108600 effectively overrides PTX-resistance of the MDA-MB-231-TR and BT-20-TR variant cell lines. When we performed mammosphere assays with PTX-resistant cells, we again observed that while PTX had little or no inhibitory effect on mammosphere formation, 108600 inhibited mammosphere formation in a concentration-dependent manner (data not shown).

Pilot toxicity, pharmacokinetics and *in vivo* efficacy studies.

We next performed studies to determine the acute maximum tolerated dose of 108600 in mice. Three sets of CD-1 female mice at 10-12 weeks of age (n=3 per group) were injected intraperitoneally (ip) with a single dose of 100, 200 or 400 mg/kg of 108600. Viability, signs of toxicity and body weight were monitored over the next 7 days. The survival rate was 100%, and we did not observe any signs of toxicity or weight loss. We next administered 100mg/kg of 108600 (ip) each day for 7 days and again, did not

observe any signs of toxicity. We also conducted a preliminary pharmacokinetic study where we determined 108600 plasma level concentrations where female CD-1 mice were treated with 108600 (Phosphate salt) (50mg/kg) dissolved in water via ip injection). Mice were euthanized at 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and 8 hours post-administration and blood was collected. Plasma samples were analyzed by liquid chromatography/mass spectrometry (LC/MS) (Agilent 1200 HPLC /Agilent Triple Quad). Area Under the Curve (AUC) was calculated according to the trapezoidal rule: $(1/2)(C_1+C_2)(t_1-t_2)$. n=2 per time point. LC/MS studies showed that the area under the curve (AUC) values were 5.96 µg/ml/hr in mice which reflects a concentration of 11.09 µM/ml/hr in the plasma. Significant concentrations of 108600 were found in the heart, kidney, liver and mammary tissues of mice up to eight hours of dosing (data not shown).

108600 inhibits the growth of TNBC *in vivo*

To determine the efficacy of 108600 against TNBC growth *in vivo*, MDA-MB-231 cells were injected subcutaneously into the mammary fat pads of female athymic (NCR-nu/nu) mice and the tumors allowed to grow for 10-14 days to a size of 100-150 mm³. The mice were then grouped (n=8) and treated every other day with 50 mg/kg or 100 mg/kg of 108600 or an equal volume of placebo. Treatment doses were chosen based on toxicity data and efficacy with respect to suppression of 108600 target kinases (Fig. 6A and Supplemental Fig. 7). Tumor measurements and body weights were determined every alternate day. 108600 is a potent inhibitor of TNBC tumor growth as judged by the inhibition of tumor growth (Fig. 6B). The study had to be terminated at the end of 22 days due to the large size of tumors in the control group, which began to undergo necrosis. Measurement of body weights and gross pathological examination of drug-treated animals showed lack of any detectable toxicity (data not shown).

Following the termination of the experiment, H&E staining and immunofluorescence was performed on tumor tissues (Fig. 6C). 108600-treated tumors showed massive apoptosis with very few tumor cells that stained for CD44. Immunofluorescence staining for cleaved caspase-3 showed significant activation of apoptosis in 108600-treated tumor tissues, confirming our *in vitro* findings (Fig. 4) and supporting apoptosis as a mechanism underlying the decreased growth of the tumor xenografts.

To expand the clinical relevance of 108600 as a therapeutic for TNBC, we also examined the effect of 108600 on the growth of a TNBC patient-derived xenograft (PDX), which, like the parental MDA-MB-231 cell line, is sensitive to taxane therapy. Tumor-bearing nod/scid/gamma (NSG) mice were randomly assigned to 2 groups and treated with 108600 (100mg/kg) or an equal volume of placebo as described above. The results presented in Figure 6 show that treatment with 108600 suppressed the growth of this tumor *in vivo* without adverse effects on animals' body weights (Supplemental Fig. 7)

108600 suppresses aggressive TNBC PDX growth in combination with chemotherapy.

To further evaluate the efficacy of 108600 in suppressing growth of chemotherapy-resistant TNBC *in vivo*, we utilized clinically annotated PDX models of drug-resistant TNBC that we have been developing as part of an ongoing co-clinical study. We prioritized models based on relative chemotherapy resistance

of the original patient cancer from which the PDX model was derived, as well as levels of expression of 108600 targets (CK2, DYRK and TNIK kinases, Supplemental Fig. 6). After establishing that 100mg/kg q2D dosing of 108600 is sufficient to suppress established targets of CK2, specifically pAKT S129 (Fig. 6A and B), we propagated two PDX models (CTG1883 and CTG2397) for efficacy studies based on robust expression of 108600 targets. CTG1883 is derived from a primary TNBC with intrinsic resistance to anthracycline and taxane-based chemotherapy, and CTG2397 is derived from a metastatic TNBC that recurred despite anthracycline and taxane-based chemotherapy. For these studies, mice bearing PDX tumor grafts were treated with either vehicle control, PTX (10mg/kg q4D), 108600 (100mg/kg q2D) or a combination of PTX/108600, and tumor growth was monitored over a period of 12-24 days. While paclitaxel had a moderate (CTG1883) or minimal inhibitory effect (CTG2397) on tumor growth, single agent 108600 significantly suppressed tumor growth in both models. However, the combination of 108600 and paclitaxel showed near complete suppression of tumor growth in both chemotherapy-resistant PDX models, as assessed by caliper measurement and endpoint tumor weight (Fig. 6E) without adverse effects on body weights of the treated animals (Supplemental Fig. 7).

We next evaluated the effect of 108600 on the stem-cell like population in these PDX tumors by assessing ALDH activity in organoids derived from these PDX models (Fig. 6F). 108600 treatment suppressed the ALDH-high fraction in CTG1883 organoid cultures in a dose-dependent manner. These results collectively support 108600's efficacy against chemotherapy-resistant TNBC by suppressing the BCSC population and enhancing sensitivity to chemotherapy.

108600 suppresses growth of pre-established TNBC metastases

The results from the above *in vivo* studies encouraged us to investigate the efficacy of 108600 treatment against already established TNBC metastatic disease. This is particularly important as most novel therapeutics are first evaluated in the context of advanced, metastatic disease that has already been exposed to prior chemotherapy. For these studies, MDA-MB231 LM2-4/mCherry Luc cells (LM2-4/mCherry), a luciferase and mCherry-expressing derivative of MDA-MB-231 that readily disseminates from the primary site to form metastases in lung and liver, was used for modeling metastatic breast cancer²⁶. For these studies, LM2-4/mCherry Luc cells were implanted into the mammary fat pads of NSG mice and the primary tumors removed prior to evidence of metastasis (11 days post-implantation). Serial *in vivo* imaging (IVIS) was performed to monitor metastases development and once metastases were detected in all mice (primarily in the lung), the mice were randomized into four treatment groups: 1) DMSO vehicle control; 2) 108600 (100mg/kg q2D); 3) PTX (10mg/kg q4D); or 4) a combination of 108600 and PTX. Metastases were serially imaged on days 18, 23, 28 and 32 by IVIS (Figure 7A, B), using the same acquisition settings for all treatment groups. Acquisition timing after luciferin injection (15 minutes), exposure time (5 minutes), lung gates (3.3 mm horizontal x 3.3 mm vertical), were all kept constant for all treatment groups and time points to enable comparisons. At baseline, there was no significant difference in metastatic disease burden among the four groups (Fig. 7C). We also determined the ratio of luciferase signal from lung metastases at different treatment timepoints (2nd, 3rd 4th IVIS) relative to the signal obtained at time of randomization (1st IVIS) (Figure 7D). While treatment with

paclitaxel had little or no effect on metastatic progression, treatment with 108600 alone had an early suppressive effect on metastatic burden but the effect was not sustained. However, combined treatment with 108600 and PTX significantly and stably suppressed growth of already established lung metastases. (Fig. 7D). These promising results show that 108600 synergizes with chemotherapy to suppress TNBC metastatic growth, and support clinical translation of combined 108600/chemotherapy treatment for stage 4 TNBC.

Discussion

Chemotherapy resistance remains a critical problem facing patients diagnosed with triple negative breast cancer, especially since suboptimal response to chemotherapy is a strong predictor of long-term poor prognosis. Multiple mechanisms have been proposed to contribute to chemotherapy resistance, among which is the presence of a subpopulation of cells within the tumor that has stem-cell like properties with tumor initiation and self-renewal capacity^{27, 28}. This population has been identified by CD44^{high}/CD24^{low} expression^{29, 30}, as well as by high ALDH activity³¹. It is associated with chemotherapy resistance and worse prognosis for multiple tumor types, and may contribute to tumor progression and metastasis^{7, 32, 33, 34, 35}. Therapeutically targeting this population could enhance chemosensitivity and improve outcomes for patients. Here, we have identified a novel compound, 108600, identified in a screen for novel therapeutics that suppress the BCSC-like population. 108600 synergizes with chemotherapy to inhibit growth of aggressive TNBC that is resistant to standard of care chemotherapy.

The efficacy of 108600 lies in its potent simultaneous inhibition of multiple signaling pathways active in CSCs and required for their maintenance and/or propagation. Its main targets, CK2, Dyrk and TNIK kinases, collectively activate signaling molecules like STAT3^{36, 37}, Wnt/ β -catenin^{38, 39, 40}, PI3K/Akt^{15, 16}, hedgehog^{41, 42} and Notch1⁴³. These signaling molecules have been reported to be active in the CSC population and required for stem cell maintenance⁴⁴. They also contribute to drug resistance and metastasis^{27, 28}. CK2, while ubiquitously expressed, seems to be especially critical for growth and survival of cancer cells^{45, 46, 47}. CK2 activation is associated with aggressive tumor behavior with higher levels associated with poorer prognosis across multiple tumor types^{26, 48, 49, 50, 51, 52}. CK2 promotes activation of Akt^{15, 16} and β -catenin³⁹ by direct phosphorylation, and NF- κ B signaling by phosphorylation and degradation of I κ B α ^{53, 54, 55, 56}. Among the relevant substrates of CK2 that could impact chemotherapy sensitivity are ABC proteins (P-gp/MDR and MRP1) that are pumps responsible for efflux of chemotherapeutic agents⁵⁷, and XRCC1/XRCC4 which regulate DNA single and double-stranded break repair⁵⁸. Inhibition of CK2 kinase activity or expression inhibited growth and induced apoptosis of multiple cancer cell types in vitro^{59, 60, 61, 62} and suppressed the stem-like tumor initiating cells in glioblastomas^{63, 64}.

Dyrk kinases (class I) also have diverse functions in cell proliferation and differentiation by regulating the degradation of key cell cycle regulators such as Cyclin D1 and p27Kip1 via direct phosphorylation^{17, 65}. Dyrk1 kinases regulate gene expression through direct effects on transcription factors and chromatin modification. For example, Dyrk1A activates transcription factors critical for stem cell maintenance such as Gli1, a transcriptional effector of Hedgehog signaling, and Stat3^{66, 67}. TNIK activates Wnt/ β -catenin signaling^{68, 69} in addition to JNK and NF- κ B⁷⁰. TNIK is part of the β -catenin/TCF4 complex responsible for transcriptional programs activated by Wnt signaling⁶⁹.

Small kinase inhibitors that individually target CK2, Dyrk1 or TNIK kinase are in various stages of pre-clinical development. Treatment with NC-1, an oral inhibitor of TNIK, was reported to suppress the ALDH positive colon cancer CSC population, as well as growth of tumor xenografts in pre-clinical models⁷¹. An orally available CK2 inhibitor (CX-4945) has already been evaluated in Phase 1 clinical trials for solid tumors. While it was found to be generally well tolerated, no complete or partial response was observed^{72, 73}. These findings support the need for simultaneous inhibition of multiple signaling pathways to overcome drug resistance in advanced cancers. These are also consistent with our results that individual downregulation of CK2, Dyrk1A or TNIK had negligible effect in inhibiting TNBC growth and simultaneous downregulation of all three kinases was required to phenocopy the effects of 108600 treatment. Despite potent multi-kinase inhibition, 108600 treatment does not induce cell death of normal cells and toxicities were not observed in our *in vivo* studies.

Multiple mechanisms are responsible for the growth inhibition and apoptosis induced by 108600 treatment. CK2 localizes to the mitotic apparatus and regulates levels of core cell cycle proteins^{46, 74}. Inhibition of tubulin polymerization, chromosome missegregation and potent G2/M arrest observed with short-term 108600 treatment trigger apoptosis⁷⁵. Our crystallographic studies show that these effects are likely mediated in part by direct inhibition of CK2 holoenzyme complex formation. Moreover, 108600-treated cells are likely sensitized to apoptotic triggers due to inhibition of multiple CK2/Dyrk/TNIK-dependent survival signaling pathways (Akt, NF κ B, Stat), activation of caspases and direct suppression of anti-apoptotic Bcl2 family members (Bclxl, survivin)^{40, 55, 76}. Finally, suppression of cells with ALDH-high, CSC-like characteristics, as we observed in organoid cultures derived from a chemotherapy-resistant TNBC PDX model treated with 108600, may be critical for sensitizing cancer cells to standard of care chemotherapy and eliminating a drug-resistant population.

Our cell biological studies show that 108600 is very potent in suppressing the growth of both CD44^{high}/CD24^{low} and CD44^{low}/CD24^{low} TNBCs. Moreover, 108600 induces apoptosis of paclitaxel-resistant TNBCs *in vitro*. We also examined the efficacy of 108600 using MDA-MB-231 xenografts as well as two different PDX models of TNBC, that are both resistant to paclitaxel. 108600 works synergistically with paclitaxel in both PDX models, even when the tumor is non-responsive to paclitaxel. This is particularly significant since chemotherapy resistance remains a critical problem facing patients diagnosed with triple negative breast cancer. Another major problem encountered with triple negative breast cancer patients is the highly metastatic nature of this disease, which is the primary cause of

patient mortality. Our studies with LM2-4 cells which is a highly metastatic variant of MDA-MB-231 cells show that 108600 is an effective suppressor of establish metastases in combination with paclitaxel. This property of 108600 may prove to be highly advantageous to patients with advanced TNBC whose cancers are resistant to chemotherapy.

In addition to direct effects on the tumor cell compartment, it is possible that 108600 induces favorable changes in the immune microenvironment of TNBC tumors that promote effective anti-tumor immunity. Recently, it was reported that CK2 inhibition suppressed the differentiation of myeloid-derived suppressor cells and tumor associated macrophages and synergized with immune checkpoint inhibitors to suppress tumor growth⁷⁷. Therefore, 108600 could have immune microenvironmental effects that also contribute to its potent anti-tumor effects and raises the possibility of synergies with immunotherapies.

In summary, we have identified a potent multi-kinase inhibitor of CSC that suppresses TNBC growth and overcomes chemotherapy resistance. Its efficacy against established metastatic disease and its favorable toxicity profile make 108600 a promising therapeutic for clinical translation. Effective inhibition of chemotherapy resistant TNBC, including the CSC-subpopulation could lead to dramatic improvements in morbidity and mortality associated with TNBC.

Methods

Cell lines

Established cell lines were purchased from ATCC. LM2-4/mCherry Luc cells (LM2-4/mCherry) were a gift from Dr. Robert Kerbel (Sunnybrook). MDA-MB-231 and LM2-4/mCherry cells were maintained with 10% FBS containing DMEM and Roswell Park Memorial Institute (RPMI) medium (ThermoFisher Scientific), respectively. All cell lines were maintained at 37°C under humidified conditions and 5% CO₂.

PDX organoid cultures were maintained in F12/DMEM (GE healthcare, Chicago, IL) containing 5% FBS (ThermoFisher Scientific), 10 mM HEPES (ThermoFisher Scientific), 1 µg/ml bovine serum albumin (BSA) (Sigma-aldrich, St. Louis, MO), 1x insulin-transferrin-selenium (ThermoFisher Scientific), 0.5 µg/ml hydrocortisone (Sigma-aldrich), 2.5 µg/ml Fungizone (ThermoFisher Scientific), 50 µg/ml Gentamicin (ThermoFisher Scientific), and 5 µM ROCK inhibitor (Y27632) (Selleck Chemicals, Houston, TX).

Animals

CD-1 and nu/nu mice were purchased from Charles River Laboratories or Taconic. NCr-nu/nu, NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) and TNBC PDX (TM00098)-bearing NSG mice were purchased from the Jackson Laboratory. All animal experiments were performed under protocols approved by the Icahn School of Medicine at Mount Sinai's Institutional Animal Care and Use Committee according to federal and institutional guidelines and regulations.

Patient Samples

Samples from patients treated at the Mount Sinai Hospital were collected for PDX generation and expansion. Written informed consent was obtained for all subjects on Icahn School of Medicine at Mount Sinai (ISMMS) Institutional Review Board (IRB)–approved protocol (HSM# 14-00330) prior to the procedure at which the specimen was obtained. The studies were conducted in accordance with the Belmont Report and U.S. Common Rule and approved by the ISMMS IRB.

Reagents:

Antibodies. Primary antibodies were purchased from Cell Signaling Technologies: phospho-AKT1 (Ser129) (#13461), DYRK1A (#2771), DYRK1B (#2703), AKT1 (#2967), phospho-CYCLIN D1 (Thr286) (#3300), CYCLIN D1 (#2926), TNIK (#32712), cleaved PARP (#9541), PARP (#9532), cleaved Caspase 3 (#9661), CD44 (#3507), MDR-1 (#13342); Abcam: CK2a1 (ab76040); Santa Cruz Biotechnology: GAPDH (sc-365062), TNIK (sc-136103), CK2a2 (sc-9030), EGFR (sc-53274); Sigma-Aldrich: tubulin (T9026); BD Biosciences: CD44 (561858), CD24 (560992); Invitrogen: b-catenin (AH00462). Secondary antibodies were purchased from Cell Signaling Technologies (horse radish peroxidase [HRP] conjugated anti-rabbit IgG [#7074]) or LICOR Biosciences (926-32220, 926-32211, 926-68070, 926-32210).

Chemicals. Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK, Sigma Aldrich) was dissolved into DMSO, and then used as inhibitor of apoptosis. Paclitaxel (Sigma-Aldrich) was dissolved into 1:1 mixture of ethanol and Cremophore EL (GE healthcare) for *in vivo* experiments. Four volumes of phosphate buffer saline without Ca^{2+} and Mg^{2+} (PBS(-)) was added to the paclitaxel:cremophore mix immediately before every injection, and the concentration of paclitaxel was adjusted to 10 mg/kg. 108600 was dissolved into dimethyl sulfoxide (DMSO) (Sigma-aldrich) immediately before every injection.

Western blotting analysis

The protein concentrations of whole cell lysates were measured using the Lowry or bicinchoninic acid (BCA) assay. Lysates were resolved by SDS-PAGE, transferred onto an Immobilon PVDF or nitrocellulose membrane and incubated in primary antibody overnight at 4°C. After secondary antibody incubation, the membranes were developed using ECL Western Blotting Substrate (ThermoFisher Scientific or MiliporeSigma) or an Odyssey CLx imaging system (LICOR Biosciences).

For *in vivo* tumor lysates, tumors were harvested 3 hours after final treatment and immediately snap frozen in liquid nitrogen. Frozen tumors were ground using mortar and pestle and immediately lysed at 4°C using radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling, Denver, MA). The lysates were centrifuged at 15,000 rpm for 20 min, and the clarified supernatant was collected for Western analysis.

Immunoprecipitation

Whole cell lysates were harvested with RIPA buffer (Cell signaling) and the concentrations measured using the BCA assay. Fifteen µg of whole cell lysate was incubated with anti-TNIK antibody (Santa Cruz

Biotechnology Inc., sc-136103) or its isotype control antibody (Santa Cruz Biotechnology Inc., sc-3877) for 2 hours at 4°C. Subsequently, these lysates were incubated with agarose conjugated Protein A/G (Santa Cruz Biotech., sc-2003) for 1 hour at 4°C, and then were centrifuged with 1000 xG for 5 min. to collect the precipitates. The precipitations were washed 4 times with RIPA buffer and the precipitates denatured using 20 µL of 1x SDS sample buffer. An identical concentration of whole cell lysates that was used as input control. Western blotting using anti-TNIK (Cell Signaling Technologies, #32712) and anti-GAPDH(Santa Cruz Biotechnology Inc., sc-365062) were performed to measure TNIK expression.

CK2α1 protein expression, purification and co-crystallization with 108600

The CK2α1 catalytic domain was expressed and purified as described previously ⁷⁸. Briefly, a construct consisting of residues 3-339 encompassing the CK2α1 catalytic domain was expressed in *E. coli* as His6-SUMO (small ubiquitin-like modifier) N-terminally tagged fusion protein. The His6-SUMO tag was removed by cleavage with Ulp1 protease after Ni-NTA affinity chromatography and the protein was further purified by Ni-NTA, heparin and size-exclusion gel-filtration column chromatography (Supplemental Fig. 4). The crystals of CK2α1-108600 complex were obtained by the vapor diffusion method at 20°C and grew as thin plates. To set up a crystallization drop, 1 µL of 10 mg/mL CK2α1 protein was mixed with 1 µL of the reservoir solution containing 100 mM Tris-HCl pH 7.5, 200 mM ammonium sulfate and 21% PEG5000 on a cover slide. 0.2 µL of 10 mM 108600 solution was then added to the drop. 108600 was dissolved in 25% Cremophor EL, 24.5% ethanol, 40% PEG 400, 5 mM Tris-HCL pH 8.0. The use of Cremophor EL-PEG 400-buffer mixture for 108600 solution preparation was critical for co-crystallization since 108600 dissolved in DMSO resulted in heavy precipitation in the crystallization drop and the absence of crystal growth. The CK2α1-108600 complex crystals diffracted to high 1.80 Å resolution at X25 synchrotron beamline at Brookhaven National Laboratory. The structure was solved by molecular replacement with CK2α1 catalytic domain (PDB ID: 3OWJ) ⁷⁹ as a search model. The final model was refined to 1.80 Å resolution and R_{work}/R_{free} 18.4/22.4 (Supplemental Table S3 within Supplemental Fig. 4).

Tubulin Polymerization Assay

Microtubules were assembled *in vitro* from the purified Microtubule Associated Protein MAP-free tubulin (Cytoskeleton Cat#TL238c) at 2 mg/ml in PEM buffer supplemented with 1mM GTP at 35°C, and the turbidity of the solutions was monitored at 340nm. DMSO or ON108600 at varying concentrations was preincubated with recombinant purified CK2α1 for 30 mins and recombinant in-house purified CK2β was added to the mixture. This mixture was then added to the MAP-free tubulin to initiate CK2 holoenzyme dependent polymerization. The rate of tubulin polymerization was measured using densitometry at 2 minute intervals.

siRNA transfection

siRNA transfections were performed using Oligofectamine (ThermoFisher Scientific, Inc.). siGENOME SMART pools (Horizon Discovery) for CSNK2A1 (M-003475-03-0005), DYRK1A (M-00485-01-005), and

TNIK (M-004542-03-0005), and non-targeting control siRNAs (D-001210-05-50) were transfected according to the manufacturer's protocol. Briefly, the culture medium was changed to Opti-MEM (ThermoFisher Scientific, Inc.) before transfection, and the mixture of Oligofectamine and 30 nM of each siRNA pool were added to the cells. Media containing 3X serum was added a five-hour incubation. The cells were harvested for analysis 96 hours post-transfection.

Flow cytometry

Cell cycle analysis. Organoids were washed and harvested with PBS (-) and Tryp-LE (ThermoFisher Scientific), and then fixed with ice cold 95% ethanol for a minimum of 24 hours. The cells were then washed twice with PBS(-) and stained with PI/RNase staining buffer (Becton Dickinson, Franklin Lakes, NJ) for 15 min. at room temperature. Flow cytometric analysis was performed using an LSRII Fortessa (Becton Dickinson) and the data analyzed using FCS Express 6.0 (De Novo Software, Glendale, CA).

Aldeflour assay. Aldeflour kit (Stemcell Technologies, Vancouver, Canada) was used according to manufacturer's instructions. Organoids were washed twice with PBS(-) and then incubated with digestion buffer, (1 mg/ml Collagenase IV, 100 units/ml hyaluronidase ,and 100 µg/ml DNase I in PBS(-)), with shaking at 200 rpm. for 15 min at 37°C. Organoids were incubated with Aldeflour solution at 37 °C for 60 min. DEAB (Stemcell technologies) treatment was used as negative controls for this assay.

Isolation of BCSC populations. Single-cell suspensions were prepared in phenol red-free RPMI supplemented with 2% heat-inactivated FBS. Defined numbers of cells were stained on ice for 1 hr, washed, and then subjected to flow cytometric analysis. Antibodies used were as follows: anti-CD44 PE (BD Biosciences Cat#561858) and anti-CD24 FITC (BD Biosciences Cat#560992). DAPI was used at a concentration of 1µg/ml as a measurement of viability. Flow cytometric sorting was performed by the Flow Cytometry Shared Resource Facility at the Icahn School of Medicine at Mount Sinai using an Arial II (BD Biosciences).

Immunofluorescence and Confocal Microscopy

Exponentially growing cells were trypsinized and then seeded at a density of at 0.1×10^5 cells/ml in 100 mm dishes containing sterilized glass microscope cover slips and allowed to transfix overnight. The next day, cells were treated with DMSO or 108600 for 12 hours. Coverslips were gently washed with PBS, fixed with 4% paraformaldehyde for 15 minutes and washed three times with PBS for 5 mins. Cells were then permeabilized using 0.1 % Triton X-100 in PBS for 15 mins at room temperature. After another PBS wash, coverslips were incubated in blocking buffer (1% BSA in PBST) for 1 hour at 37 °C. The cells were incubated with anti-α-tubulin-FITC (Sigma Cat# 2168) and anti-pericentrin rabbit antibody (Abcam, #4448) diluted 1:200 in blocking buffer for 1 hour at 37°C. The coverslips were again washed three times with PBS and stained with Texas red goat anti-rabbit secondary antibody (BD Biosciences, T2767). Coverslips were washed again with PBS and incubated in 1 µg/mL DAPI solution (Southernbiotech 0100-20) for 5 minutes before being washed 2 times with PBS. Finally, coverslips were mounted onto glass

slides using ProLong® Gold Antifade Reagent (Thermofisher Scientific, P36930) and dried overnight in the dark before being imaged.

Viability assays and GI₅₀ value determination

2.5x10³ cells were seeded into each well of a black, clear-bottom 96-well plate. The cells were treated with 108600 24 hours post-plating. Fluorescence was measured by excitation at 579 nm and an emission at 594 nm after a 96-hour incubation using CellTiter-Blue reagent (Promega) and the GI₅₀ value calculated by normalizing fluorescence values to those of DMSO-treated controls after background subtraction.

***In vivo* tumor efficacy studies**

For MDA-MB-231 xenografts, 1x10⁷ cells in 0.2ml of sterile PBS were subcutaneously injected into the mammary fat pads of Ncr-nu/nu mice. PDX tumor grafts were propagated as follows: 4-5 mm frozen tumor fragments were subcutaneously transplanted into the right flank of NSG mice. Approximately 1 month after primary transplantation, the growing tumors were harvested, cut into 5 mm tumor fragments, and re-transplanted into cohorts of NSG mice. When tumor grafts reached approximately 50-100 mm³ in size, treatment was initiated with intraperitoneal injections of paclitaxel or 108600 injected every 4 or 2 days, respectively. DMSO was used as control. Tumors were measured by calipers every 2 or 3 days and volume estimated using the formula: $\frac{1}{2} (\text{width}^2 \times \text{length})$. Where indicated, pre-engrafted, PDX-bearing NSG mice purchased from the Jackson Laboratory were used for efficacy studies that followed the above dosing and monitoring protocols

***In vivo* imaging (IVIS)**

5x10⁵ LM2-4/mCherry cells were injected into the right inguinal mammary fat pad of NSG mice. 11 days after injection, the primary tumor and the mammary fat pad were surgically removed. NSG mice were anesthetized using inflowing isoflurane (2-2.5%) in 2-2.5 L/min oxygen, and placed into the imaging chamber, and background luminescence was acquired. 10 µL/g mouse body weight of luciferin (PerkinElmer Inc., Waltham, MA) was intraperitoneally injected. 1 minute after the injection of luciferin, luminescence was acquired to check for presence of metastasis or residual tumor. To block luminescence from any residual primary tumor, the area around the inguinal mammary fat pad was covered with black aluminum foil and clay. The luciferin signal was acquired 5, 15, and 25 min. after luciferin injection. The luminescence intensity at 15 min. was used for the quantitative analysis. To analyze the lung metastasis, a 3.3 x 3.3 cm window was set as region of interest (ROI) prior to measuring radiance.

Mice with metastases were selected based on the luminescence signal at the first IVIS imaging following primary tumor removal, and each mouse was randomly assigned to DMSO, 108600, paclitaxel or 108600/paclitaxel combination treatment groups. DMSO and 108600 were intraperitoneally injected every 2 days and paclitaxel was injected every 4 days for 2 weeks. The luminescence intensity at the first IVIS imaging was used as a baseline intensity for each mouse, and the ratio of luminescence between

each IVIS imaging and baseline was used for comparison. Living Image (version 4.7.2, PerkinElmer Inc.) was used for acquisition and analysis of luminescence intensity.

Preparation of organoid cultures

Frozen small tumor fragments in Cellbanker 1 (Nippon Zenyaku Kogyo) at -80 °C were used for making organoids. After thawing, the fragments were cut, minced and incubated in digestion buffer at 37°C with shaking. After digestion, the cell suspension was filtered (100 µm) and centrifuged. Pellets were collected and re-suspended in human breast epithelial cell (HBEC) medium (5 % FBS, 10 mM HEPES, BSA, 0.5 µg/ml Hydrocortisone, 50 µg/ml gentamycin, 2.5 µg/ml Fungizone, 1x insulin-transferrin-selenium, and 5 µM ROCK inhibitor (Y27632) (Selleck Chemicals) in DMEM/F12⁸⁰. Ficoll density gradient centrifugation was performed and the live cell fraction was collected. After washing with PBS, cells were seeded on non-adherent 24 well plate. After 3 days, non-viable cells were removed by density gradient centrifugation.

Colony-formation assays

Sorted cells were plated into 6 well plates at 750 cells/well and allowed to adhere overnight. 108600 was then added at varying concentrations for a 72 hr period. Drug-containing medium was replaced with fresh medium and the cells were incubated for an additional 8-10 days with media changes made every 3 days. Cells were fixed in methanol (-20°C) and stained with 5% crystal violet. Colonies were counted using ImageJ.

Mammosphere-formation assays

TNBC cell lines, sorted BCSCs from cell lines and patient-derived human breast cancer stem cells (XLC479, Creative Bioarray) were cultured in suspension in serum free DMEM/F12 1:1 supplemented with EGF (20ng/ml, BD Biosciences), B27 (1:50, Invitrogen), 0.4% bovine serum albumin (Sigma) and 4 µg/ml insulin (Sigma) on Costar ultra-low attachment multi-well plates (Corning Cat#3471). Cells were grown in DMSO or 108600 (200nM) for 72 hours. Drug-containing medium was replaced with fresh medium and the cells were further incubated for 1-2 weeks to induce mammosphere formation.

Declarations

Declearation of interets

The authors declare no competing interests.

Author contributions:

K.S., A.P., S.B., S.C., O.R., D.S., J.D., E.P. conducted the experiments; A.A., M.R., H.I., E.R. designed the experiments and K.S., A.P., S.B., O.R., E.P., A.A., H.I. and E.R. wrote the manuscript.

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Figures

Figure 1

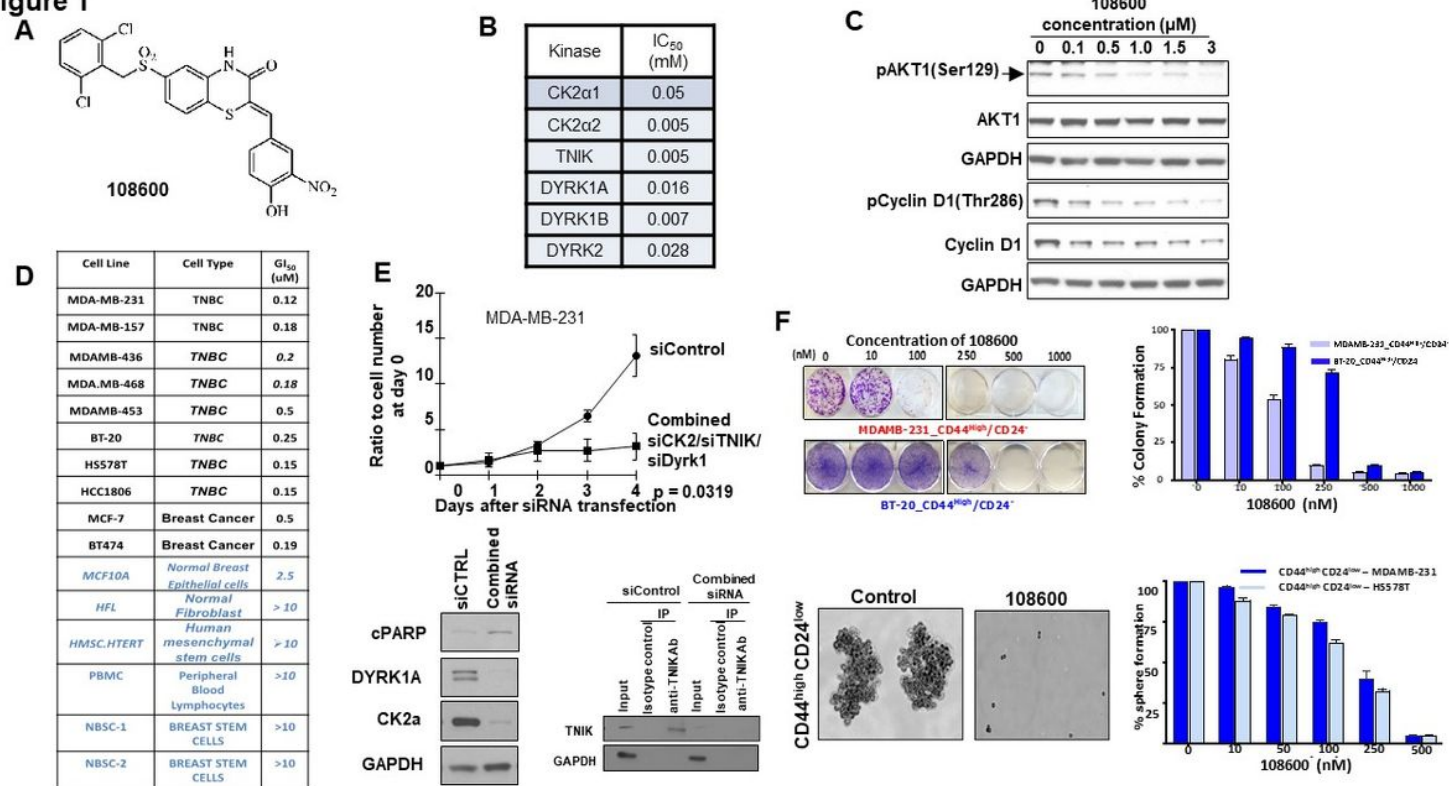


Figure 1

108600 suppresses TNBC stem cells. (A) Chemical structure of 108600; (B) IC₅₀ value for inhibition of 108600 target kinases; (C) 108600 inhibits phosphorylation of CK2 and DYRK1 substrates. Lysates of MDA-MB-231 cells treated for 24 hours with 108600 at the indicated concentrations were probed for expression of phospho-AKT1 (Ser129) and phospho-CYCLIN D1 (Thr286); (D) GI₅₀ values for growth inhibition of TNBC and normal cells treated with 108600 (E) Simultaneous siRNA-mediated knockdown of CK2, DYRK1A and TNIK suppresses proliferation of MDA-MB-231 cells. The protein expression of TNIK was checked by IP-WB. Statistical difference between the target and non-target siRNA treated group was tested using 2-way ANOVA. (F) 108600 treatment suppresses colony and mammosphere formation of CD44^{high}/CD24^{low} stem cell fraction of MDA-MB-231 and BT-20 cells. CD44^{high}/CD24^{low} cells were purified by FACS, treated with various concentrations of 108600 for 72 hours, washed and allowed to grow for 7-14 days in drug-free growth medium. The cells were fixed with 4% paraformaldehyde in PBS, stained with crystal violet solution and colonies quantified microscopically. All data points represent mean value ± standard deviation.

Figure 2

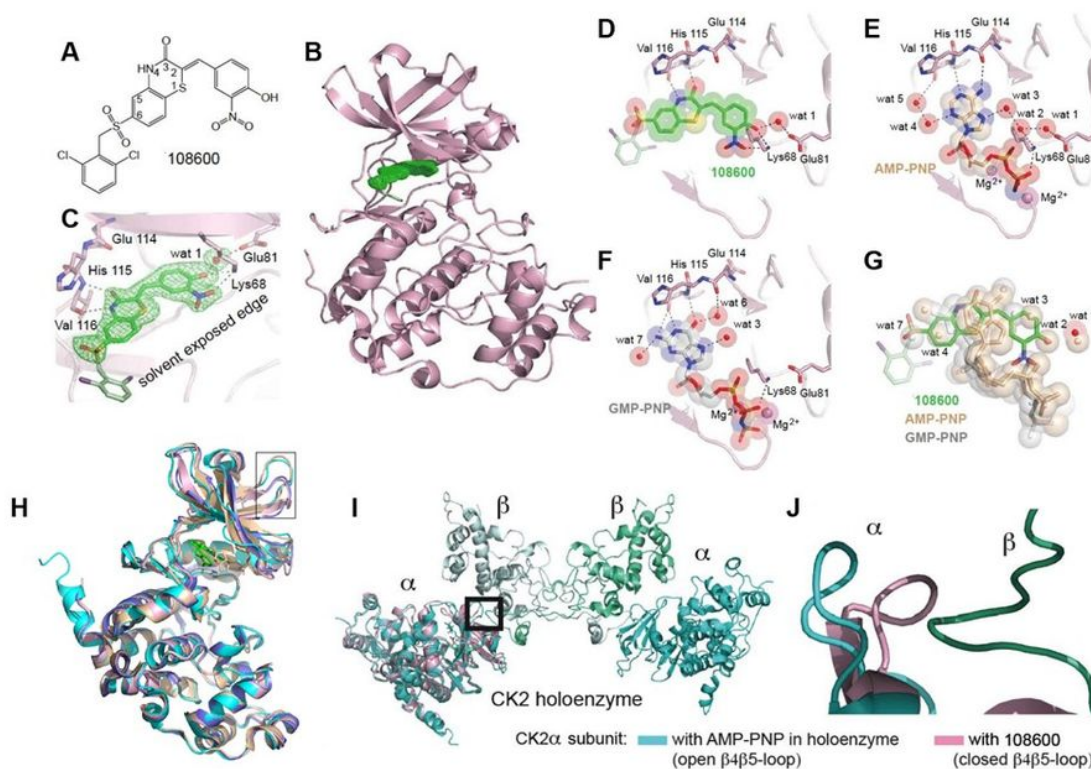


Figure 2

Crystal structure of 108600 in complex with CK2α. (A) Chemical structure of 108600. (B) Overall structure of the CK2α1-108600 complex. CK2α1 protein is shown in cartoon representation and colored in pink. 108600 is shown in sticks with its carbon atoms colored in green, oxygen atoms in red, nitrogen atoms in blue and sulfur atoms in yellow. A simulated annealing Fo-Fc omit electron density map for 108600 is contoured at 3.0 σ -level at 1.80 Å resolution and is colored in green. (C) Zoom-in view of 108600 interactions with CK2α1. Hydrogen bonds are indicated by dashes. The water molecule (wat1) is shown as a red sphere. Modeling of the 3D structure of 108600 drug by itself predicted several conformations including the one where the NO2 group and the aromatic ring of the drug are coplanar. This is due to an intermolecular bond between the oxygen atom of NO2 and the H of the adjacent OH group (A) (NO2 group is, usually, perpendicular to the aromatic ring plane in the absence of other interactions). The clear simulated annealing Fo-Fc omit electron for the drug indicates this coplanar conformation. The electron density for the 2,6-dichlorobenzyl ring of the drug is disordered due to the conformational flexibility of this aromatic moiety in the solvent-exposed region of the active site; (D) A close-up view of 108600 interactions with CK2α1 depicting a surface view of the drug. (E&F) AMP-PNP (PDB ID: 3NSZ) 18 and GMP-PNP (PDB ID: 1DAY) 19 accommodations within the active site of CK2α1. The water-mediated ligand-protein interactions are also shown. CK2α1 is able to use both ATP and GTP as phosphoryl donors with almost similar efficiencies. Interestingly, the O3 and N4 groups of the drug take the positions of the O6 and N1 groups of GMP-PNP, respectively, and maintain similar interactions with the backbone atoms of His115 and Val116. Moreover, the NO2 group of the drug is near the position that is occupied by the Pa group of AMP-PNP or GMP-PNP and interacts with the side chain of Lys68. (G) 108600, AMP-PNP and

GMP-PNP in surface representation in the active site of CK2 α 1. The structures are superpositioned by the CK2 α 1 protein residues. Significantly, the drug mimics not only the shape and electrostatics of AMP-PNP and GMP-PNP, but also their hydration patterns. A water molecule (wat1) in the drug complex forms hydrogen bonds with the OH group of the drug and the side chain of Glu81. Wat1 is also observed in the complex with AMP-PNP. Moreover, the drug covers the area where wat2, wat3 and wat4 are bound in the AMP-PNP/CK2 α 1 complex and wat7 in the GMP-PNP/CK2 α 1 complex; (H, I&J) 108600 induces change in the conformation of the β 4- β 5 loop, which interacts with the regulatory CK2 β subunit in the holoenzyme complex. (H) Superposition of CK2 α 1 structures with AMP-PNP alone (PDB ID: 3NSZ, colored in beige) and integrated in the holoenzyme complex with CK2 β regulatory subunit dimer (PDB ID: 1JWH, colored in cyan) 20, CK2 α 1 in the absence of ligand (PDB ID: 1NAZ, colored in blue) and CK2 α 1 with 108600 (pink). Black frame highlights the loop connecting sheets β 4 and β 5 (β 4- β 5 loop). CK2 α 1-AMP-PNP either alone (beige) or integrated into the holoenzyme (cyan) has the β 4- β 5 loop in the “open” conformation. In the absence of AMP-PNP (blue) and in the presence of 108600 (pink), the β 4- β 5 loop is in the “closed” conformation. (I) CK2 kinase holoenzyme (PDB ID: 1JWH) and CK2 α 1-108600 complex. The two CK2 α 1 subunits of the holoenzyme are colored in cyan, the two CK2 β regulatory subunits are colored in green. CK2 α 1 subunit of the holoenzyme contains AMP-PNP. The CK2 α 1 β 4- β 5 loop region and its interaction with the loop of the CK2 β subunit are highlighted by a thick black frame. (I) The close-up view of the interactions between the CK2 α 1 and CK2 β subunits of the CK2 holoenzyme. The “closed” conformation of the β 4- β 5 loop in the CK2 α 1/108600 complex (pink) appears to interfere with interactions with the loop of the CK2 β subunit (green).

Figure 3

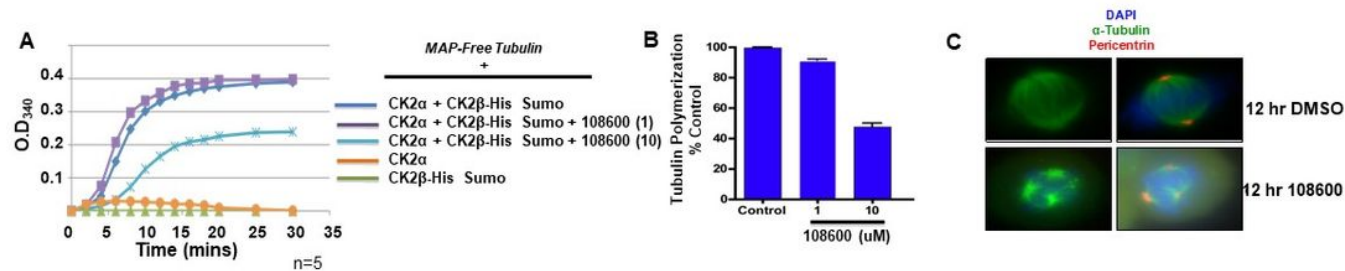


Figure 3

108600 suppresses tubulin polymerization. (A) Recombinant CK2 or CK2 or CK2+CK2 holoenzyme preparations were pretreated with either DMSO or 108600 for 5 mins and the mixture was then added to MAP-free tubulins in buffer containing 1mM GTP. The reaction mixtures were transferred to a heated spectrophotometer and tubulin polymerization was evaluated by monitoring absorbance at 340 nm over a period of 40 mins (n=5); (B) Graphical representation of % inhibition of polymerization by 108600; (C) Exponentially growing HeLa cells were treated with vehicle (DMSO) or 108600 for 12hrs, fixed and stained with α -tubulin and pericentrin antibodies and DAPI. Images obtained using confocal microscopy show abnormal mitotic spindle formation induced by 108600 (n=2).

Figure 4

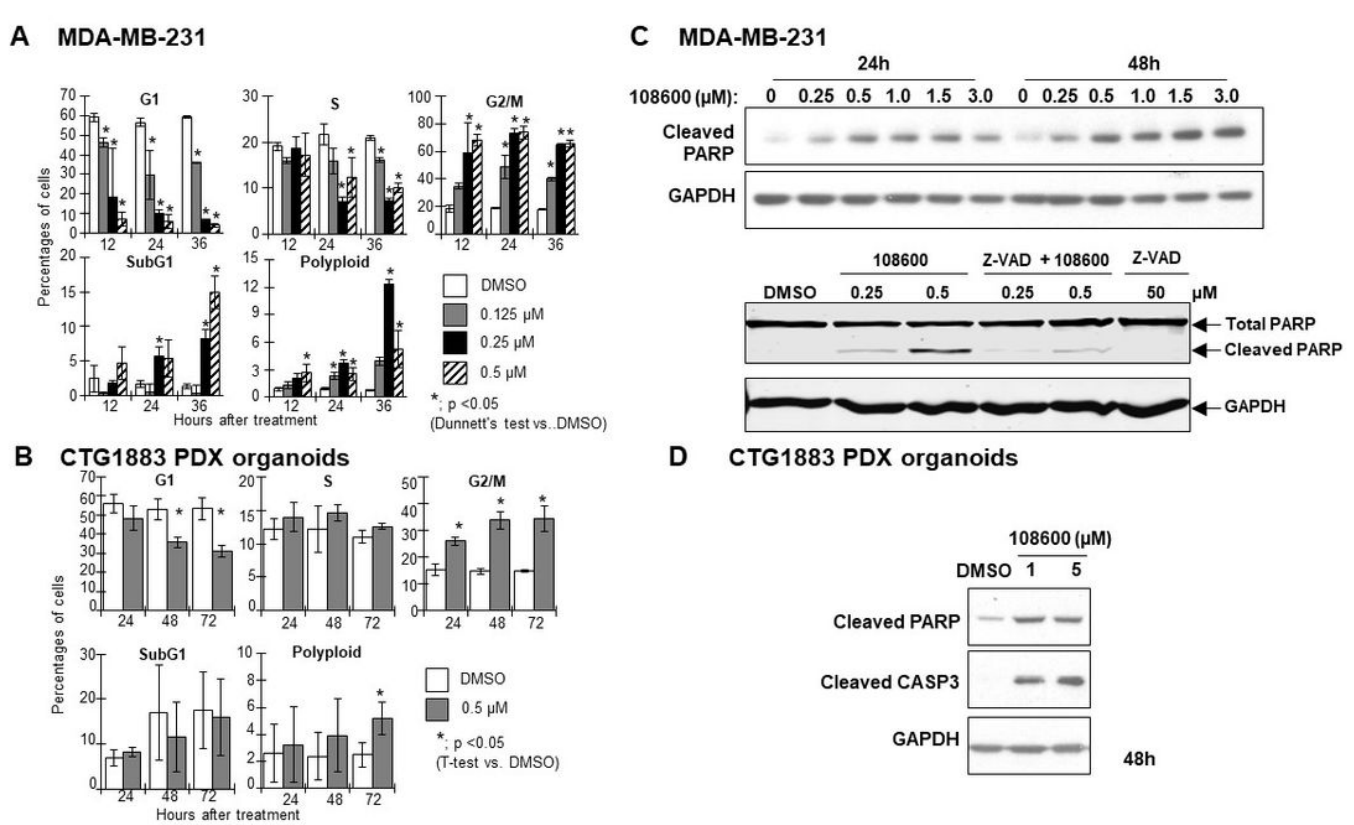


Figure 4

108600 induces G2/M arrest and apoptosis of TNBC cells and organoids. (A) MDA-MB-231 cells were treated with the indicated concentrations of 108600, stained with propidium iodide (PI) and analyzed by flow cytometry. (B) CTG1883 TNBC organoids were treated with 108600 (0.5 μ M), stained with PI and analyzed by flow cytometry. All data shown are mean values $\pm$ standard deviation. The statistical difference was tested using Dunnett's test. (C) MDA-MB 231 cells were treated with 108600 for 24 or 48 hrs. Lysates were probed for the expression of cleaved PARP (top), which is inhibited by the Z-VAD (50 μ M) Caspase inhibitor (bottom). (D) CTG1883 organoids were treated with 108600 for 48hrs. and lysed. Lysates were probed for the expression of cleaved PARP and cleaved caspase 3. The blots are representative of three independent experiments.

Figure 5

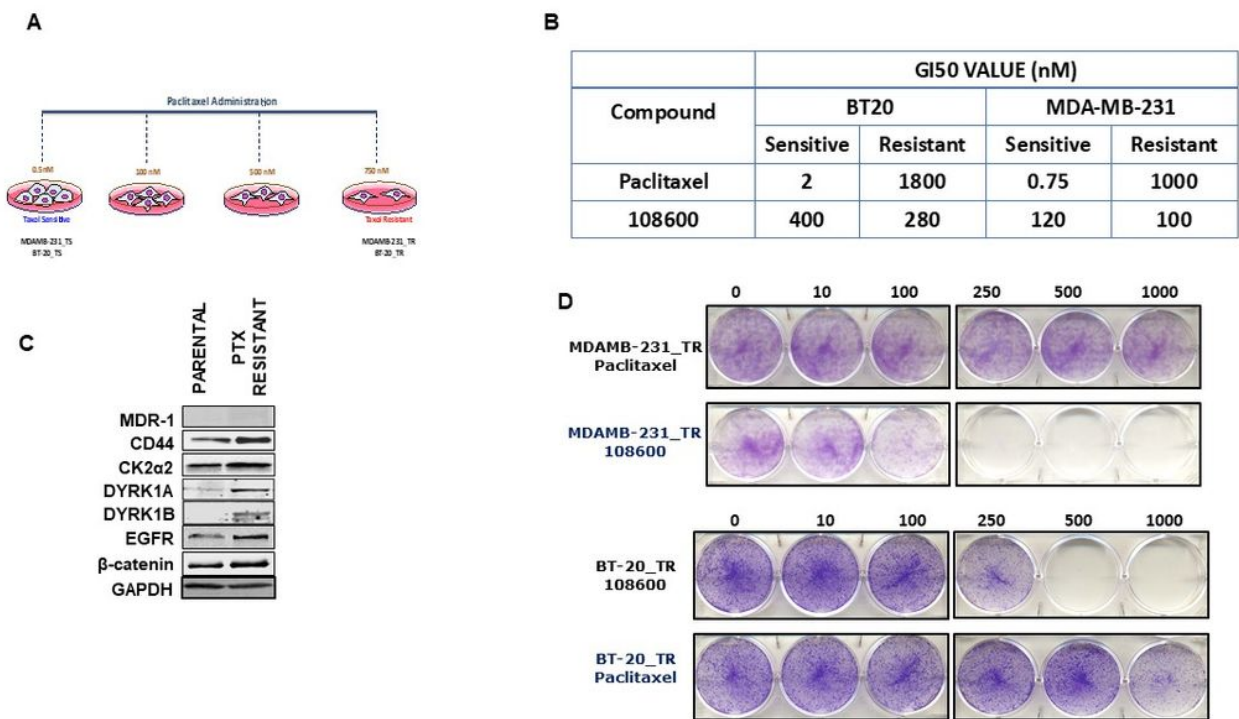


Figure 5

108600 suppresses chemotherapy-resistant TNBC cells. (A) Schematic diagram showing the strategy used for the generation of paclitaxel-resistant TNBC cells. MDAMB-231 or BT-20 cells were grown in increasing concentrations of paclitaxel and GI50 values were determined after every passage. Each subsequent passage was maintained in the highest possible concentration of paclitaxel-containing medium. Selection was completed when resistance reached > 200-fold; (B) GI50 values for paclitaxel and 108600 for parental (TS) and paclitaxel-resistant (TR) MDA-MB-231 and BT-20 using CellTiter Blue in conjunction with clonogenic assays. (C) Western blot analysis of cell lysates derived from paclitaxel-sensitive and resistant MDA-MB-231 cells for MDR-1, CD44, CK2, DYRK1A, DYRK1B, EGFR, b-Catenin and GAPDH (D) Paclitaxel-resistant MDA-MB-231 cells were treated with increasing concentrations of paclitaxel or 108600 for 72 hrs, washed and allowed to grow for 7 days in drug-free growth medium. The colonies were stained with crystal violet.

Figure 6

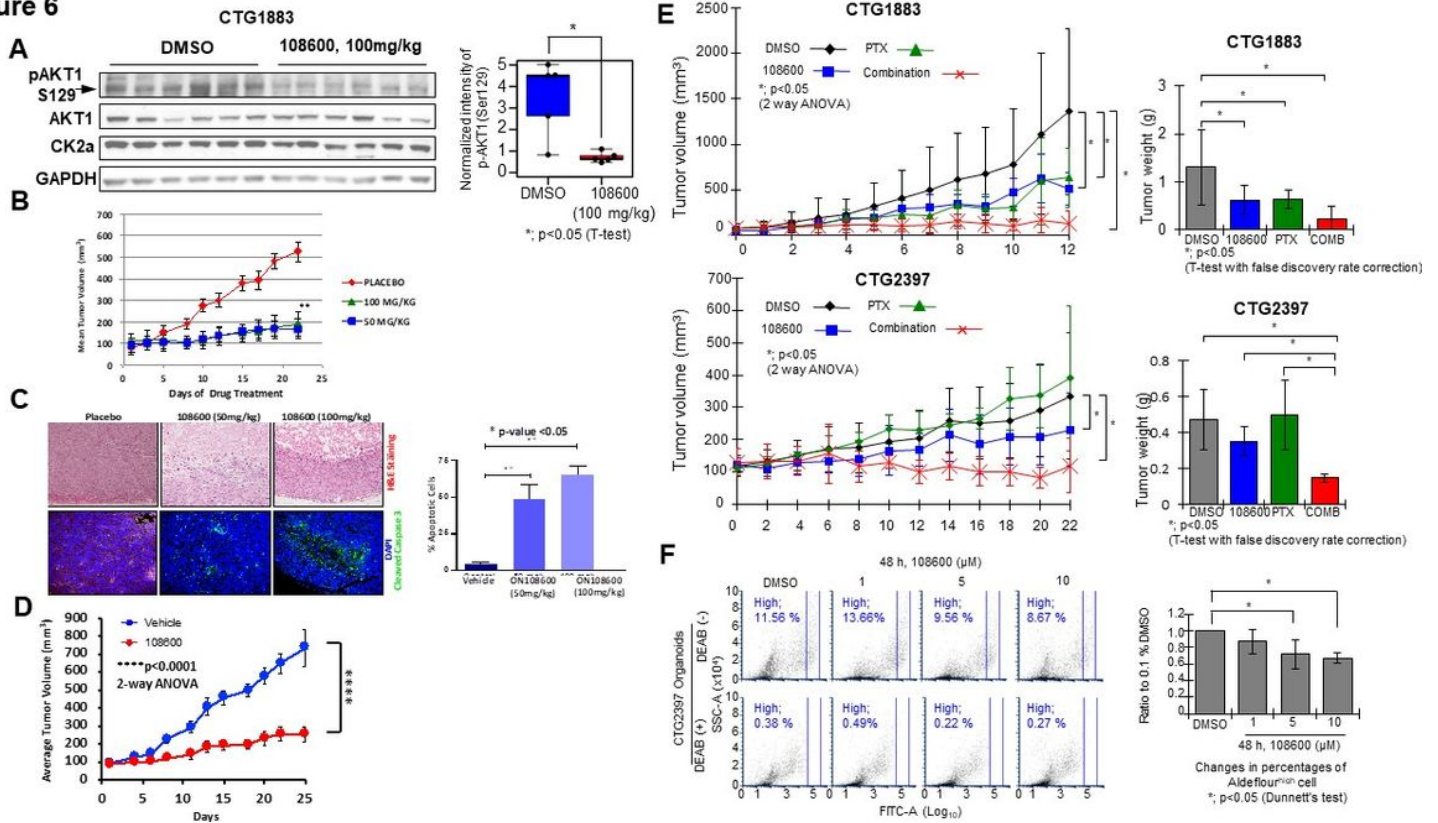


Figure 6

108600 suppresses the growth of TNBC cell lines and PDX tumorgrafts in vivo. (A) 108600 suppresses phosphorylation of CK2 substrates in vivo. NSG mice harboring CTG1883 PDX tumorgrafts were treated with 108600 (100mg/kg) or DMSO for 5 days (n=6/group). Lysates of harvested tumors were probed for the expression of phospho-AKTt1 (Ser129). The box plot shows levels of phospho-AKT1 normalized to those of total AKT1 and GAPDH. Variance in each group was tested with an F-test, and then the indicated p-value was calculated using a t-test. (B) MDA-MB-231 cells were orthotopically implanted into the mammary fat pads of 6-8 week old female nude mice and the mice treated intraperitoneally with vehicle control or 108600 (50mg/kg or 100mg/kg) daily for 21 days beginning when average tumor volume was 100 mm³. Tumor volumes were measured every 2 days. N=8/treatment group. (C) Tumor tissues derived from mice at the end of the 21-day treatment were fixed and stained with hematoxylin and eosin. Frozen sections were also stained with DAPI and anti-cleaved caspase 3 and imaged by immunofluorescence. Quantitation of cleaved caspase 3 staining levels are provided. (D) 108600 suppresses the growth of TNBC PDX tumors. Mice bearing pre-engrafted TNBC PDX tumors (TM00098) were purchased from The Jackson Laboratory. Mice were randomly assigned to the treatment groups when the tumor volumes reached a volume of 50 mm³ and treated with vehicle (n=4) or 108600 (100mg/kg q2D) (n=5) for a 25-day period. Error bars represent mean $\pm$ SEM. The statistical difference in the effect between the treatment groups was assessed by 2-way ANOVA. (E) PDX tumor growth suppression by 108600 treatment. Chemotherapy-resistant TNBC PDX models (CTG1883, CTG2397) were propagated and treated with DMSO, paclitaxel (10 mg/kg), 108600 (100 mg/kg) or a combination of paclitaxel and 108600 (n=5-7/treatment group). Paclitaxel and 108600 were administered q4d and q2d, respectively. The statistical

difference in the effect between the treatment groups was assessed by 2-way ANOVA. Weights of tumors harvested from each treatment group at the study endpoint are shown on the right. The data represent mean + standard deviation. Statistical differences were assessed by Dunnett's and Tukey's test. The synergistic effects of paclitaxel and 108600 treatment were tested with assessment of interactions between single and combination treatment by means of 2- way ANOVA. (F) 108600 decreased the ALDH^{high} fraction in CTG2397 organoids. CTG2397 organoids were treated with 1, 5, or 10 μ M 108600 for 48 hours and ALDH activity was measured using the ALDEFLOUR assay. Diethylaminobenzaldehyde (DEAB) treated organoids were used as negative controls to set the gating for the ALDH^{high} fraction. Mean values and standard deviations from 3 independent experiments are shown. The statistical differences between the ALDH^{high} fractions were tested using one-way ANOVA and Dunnett's test. A p-value of less than 0.05 was considered as statistically significant

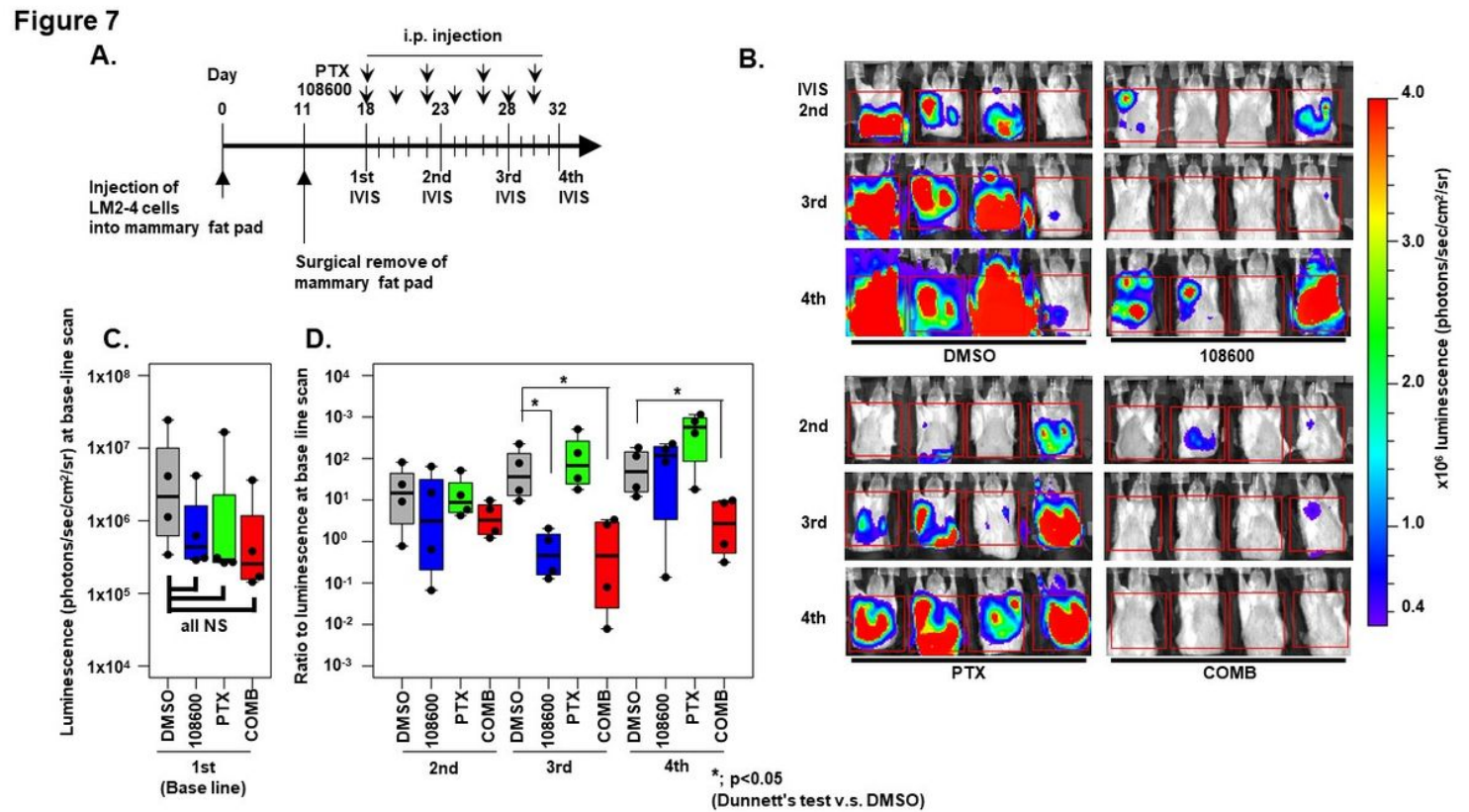


Figure 7

108600 and Paclitaxel synergize to inhibit established TNBC metastases. MDA-MB-231/LM2-4 mCherry cells were used to generate tumors in the mammary fat pad of 7 week old NSG mice. Primary tumor removal surgery was performed and all mice were imaged by IVIS for the presence of metastases. (A) Schema of experiment. Mice with metastases were randomized into four treatment groups (n=5/group) and metastatic disease was monitored by serial IVIS imaging. (B) Serial IVIS imaging of mice in the four treatment groups. (C) Total luminescence in the region-of-interest (lung) window for the four groups at time of treatment randomization (1st IVIS), showing no statistically significant difference in lung metastases burden (Dunnett's test). (D) The ratio of luminescence intensity compared to base-line luminescence (C) for each treatment group at serial time points indicated in the schema. The ratio was

calculated at each IVIS imaging. The p-values were calculated with Dunnett's test in comparison to the value for the DMSO treated group.

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