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Metformin and an insulin/IGF1-receptor inhibitor are synergistic in blocking growth of triple-negative breast cancer

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Abstract

Purpose: Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer with poor survival outcomes. Metformin has been shown to have anti-tumor effects by lowering serum levels of the mitogen insulin and having pleiotropic effects on cancer cell signaling pathways. BMS-754807 is a potent and reversible inhibitor of both insulin-like growth factor 1 receptor (IGF-1R) and insulin receptor (IR). Both drugs have been reported to have some efficacy in TNBC. However, it is unclear whether the combination of the two drugs is more effective than single drug treatment in TNBC.

Methods: We treated a panel of TNBC cell lines with metformin and BMS-754807 alone and in combination and tested cell viability using MTS assays. We used the *CompuSyn* software to analyze for additivity, synergism, or antagonism. We also examined the molecular mechanism by performing reverse phase protein assay (RPPA) to detect the candidate pathways altered by single drugs and the drug combination and used Western blotting to verify and expand the findings.

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Results: The combination of metformin and BMS-754807 showed synergy in 11 out of 13 TNBC cell lines tested (85%). RPPA analysis detected significant alterations by the drug combination of multiple proteins known to regulate cell cycle and tumor growth. In particular, the drug combination significantly increased levels of total and phosphorylated forms of the cell cycle inhibitor $p27^{Kip1}$ and decreased the level of the $p27^{Kip1}$ E3 ligase SCF^{Skp2}.

Conclusions: We conclude that the combination of metformin and BMS-754807 is more effective than either drug alone in inhibiting cell proliferation in the majority of TNBC cell lines, and that one important mechanism may be suppression of SCF^{Skp2} and subsequent stabilization of the cell cycle inhibitor $p27^{Kip1}$. This combination treatment may represent an effective targeted therapy for a significant subset of TNBC cases and should be further evaluated.

Keywords

Triple-negative breast cancer (TNBC); Metformin; BMS-754807; IGF-1R inhibitor; insulin receptor inhibitor

Introduction

Breast cancer is the most prevalent invasive cancer in women [1]. Triple-negative breast cancer (TNBC) is an aggressive subtype that lacks expression of estrogen receptor (ER) and progesterone receptor (PR) and does not show amplification of the gene encoding human epidermal growth factor receptor 2 (HER2) [2]. Compared with other subtypes of breast cancer, TNBC has a worse prognosis and lacks effective targeted therapy. At present, chemotherapy is the mainstay of treatment for early stage and advanced TNBC. Developing efficacious targeted therapeutics is crucial for improving survival in patients with TNBC.

Insulin-like growth factor-1 receptor (IGF-1R) is a transmembrane tyrosine kinase growth factor receptor that plays a key role in the establishment and maintenance of the transformed phenotype [3]. Suppression of IGF-1R activity can prevent and treat breast cancer [3,4]. Insulin receptor (IR) is closely related to IGF-1R and is also increased in breast cancer [5]. High expression levels of total IR as well as insulin are correlated with recurrence and poor survival in breast cancer patients [6–8]. Activation of IGF-1R or IR activates PI3-Kinase, Akt, and Ras, potent oncoproteins that are deregulated in many cancers [9,10]. BMS-754807, a pyrrolotriazine that is a reversible dual inhibitor of IGF-1R/IR [11,12], has antitumor activity in a broad range of tumor types and enhances the antitumor response of other therapeutic agents [13–16]. BMS-754807 has been reported to suppress TNBC in vitro and in tumor xenografts [17]. It has been investigated in several phase I and phase II clinical trials as an anti-cancer drug [18,19] including in ER+ breast cancer patients and HER2+ patients, but it has not been tested in TNBC patients[20,21].

Metformin is a biguanide that is widely used as a front-line therapeutic for type 2 diabetes since it can lower blood glucose levels and alleviate insulin resistance in these patients [22,23]. The primary mechanism is the inhibition of complex I of mitochondrial electron transport, resulting in decreased ATP levels, increased AMP levels and subsequent activation of the AMP-activated protein kinase (AMPK) [24]. AMPK suppresses hepatic gluconeogenesis and improves glucose uptake in muscle and liver [25]. Activation of AMPK

inhibits the growth of cancer cells [26]. Epidemiological studies have found that metformin decreases the incidence of cancer in diabetic patients [27,28] although others did not [24]. Some but not all studies have suggested that metformin can enhance the response to neoadjuvant treatment in breast cancer patients [29,30]. Metformin has been shown to inhibit TNBC cell proliferation/survival in vitro and in mouse models [31–34]. Anticancer molecular mechanisms of metformin have been reported to include systemic effects in controlling hyperinsulinemia and blood glucose levels, indirect effects on inflammation and body weight, AMPK-dependent effects in cancer cells (such as mTOR and mRNA translation suppression [35,36]), and AMPK-independent impact in cancer cells (such as inhibition of RAG GTPase and reactive oxygen species as a result of inhibition of mitochondrial complex I [24]).

Though several studies have investigated the response of TNBC cells to metformin or BMS754807 as single agents, no studies have examined the combination of these two drugs. In our work, we found that combining BMS-754807 with metformin in TNBC cell lines led to better therapeutic efficacy in TNBC cell lines than either drug alone. We also utilized reverse phase protein array (RPPA), a platform that allows for the broad-scale and quantitative measurement of the level and activation/phosphorylation state of hundreds of proteins [37], to investigate potential molecular mechanisms underlying the synergistic effects of BMS-754807 and metformin.

Materials and methods

Cell lines and culture conditions

Breast cancer cell lines as listed in Table 1 were obtained from American Type Culture Collection (ATCC) and cultured using the recommended culture conditions. Briefly, BT-549, HCC1937, HCC38, HCC1806, HCC70 and HCC1395 were cultured in RPMI-1640 medium (ATCC) supplemented with 10% fetal bovine serum (FBS). MDA-MB-231, MDA-MB-436, BT-20, MDA-MB-453, Hs 578T, MDA-MB-157 and MDA-MB-468 were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS). Cultures were maintained at 37°C in a 5% CO_2 atmosphere using a tissue culture incubator. Cells were carried not more than 10 passages and not more than 3 months before using new frozen stocks.

Drugs

BMS-754807 was purchased form Selleck Chemicals (Houston, TX), dissolved in DMSO (Sigma-Aldrich, Oakville, ON), and stored at −20°C. Metformin (1,1-Dimethylbiguanide-hydrochloride) was obtained from Sigma-Aldrich, dissolved in water, and stored at −20°C.

Cell viability and synergy assay

Cells were seeded in 96-well tissue culture plates at 2,000–4,000 cells/well depending on the growth characteristic of the cell line so that each was in growth phase at the time of assay (60%−80% confluency). After 24 hours seeding, cells were treated in triplicate with single drugs individually, as well as their constant-ratio combination over a 5-point range centered on the single agent concentrations that inhibited viability by 50% (IC_{50}). Cells were further

incubated at 37°C for 72 hours, and cell proliferation was measured using a methanethiosulfonate-based viability assay (CellTiter96 Aqueous One Solution Reagent, Promega Corporation, Madison, WI) according to the manufacturer's instructions and then read by microplate reader (Biotek, SYNERGY HTX, Vermont, USA). Results were expressed as percentage of vehicle DMSO-treated cells. Results presented are mean ± SD from three separate experiments done in triplicate. The CI (combination index) value was calculated by software *CompuSyn* following the manufacturer's guidance. A CI of <1, =1, or >1 indicates synergism, an additive effect, and antagonism, respectively [38].

Reverse phase protein lysate microarray (RPPA)

HCC1806 cells were seeded for 24 hours and then treated with vehicle (DMSO), metformin (5 mM), BMS-754807(15 μM), and the combination of two drugs (Metformin 5mM and BMS-754807 15μM) for 48 hours. Protein lysates were prepared from cultured cells with modified Tissue Protein Extraction Reagent (TPER; Pierce) and a cocktail of protease and phosphatase inhibitors (Roche Life Science). Lysates were spun at 14,000xg for 15min at 4°C, and the supernatants were transferred to fresh tubes. The centrifugation was repeated until the supernatants were clear. Protein concentration was determined by BCA assay (Pierce™). Lysates of 0.5mg/ml were denatured in 2x SDS sample buffer with 2.5% 2-mercaptoethanol at 100°C for 8 min. RPPA was performed by the Antibody-based Proteomics Core Facility at Baylor College of Medicine and analyzed as previously described [39]. Samples were probed with 216 antibodies.

Immunoblot analyses

HCC1806 cells were treated with vehicle (DMSO), BMS-754807 (15 μM), metformin (5 mM) and the combination of the two drugs for 48 hours and cells were lysed in RIPA buffer containing protease inhibitors and phosphatase inhibitors. Cell lysates were boiled at 95°C for 5 mins and loaded on 15% polyacrylamide gels. Proteins were separated by electrophoresis, transferred to nitrocellulose membranes. The membranes were then blocked in 5% non–fat milk in TBST (w/v) for 1 h at room temperature, incubated with a primary antibody, washed 4 times in TBST, incubated with a secondary antibody for 1 h, washed with TBST, and imaged by the Odyssey System (Li-Cor Biosciences, Lincoln, NE, USA). The relative intensity of a band to the β-actin control was measured using ImageJ software (NIH). One-way ANOVA and multiple comparisons were performed using Prism6. Antibody against SKP2 (2652, 1:1000) was purchased from Cell Signaling Technology (Danvers, MA). Antibody against Phospho-p27Kip1 (Thr187) (71–7700, 1:1000) was from Invitrogen (Grand Island, NY). Anti-p27 KIP1 antibody [Y236] (ab32034, 1:1000) was from Abcam (Cambridge, UK).

Statistical analysis

Error bars represent standard deviations from the mean of at least three replicates, unless otherwise indicated. The normalized data (Norm) were used for RPPA data analysis. The normalized data were log2 transformed for hypothesis testing. There are 4 groups (untreated, metformin treatment, BMS-754807 treatment and the drug combination treatment) and each group has 3 biological replicates. For each sample, the median value of the three technical replicates was used for statistical analysis. Two-way ANOVA was used to test main effect of

different treatment and their interaction and then diverse contrasts were performed. p value < 0.05 was considered statistically significant.

Results

The majority of TNBC cell lines are sensitive to combined treatment with metformin and BMS-754807

Antiproliferative effects of metformin and BMS-754807 were tested via an MTS assay in a panel of TNBC cell lines [17]. The combination index (CI) was calculated using *CompuSyn* software to determine whether the drug combination demonstrated synergism, additivity, or antagonism (CI <1, =1, and >1, respectively) (Table 1). We found that 11 out of 13 cell lines (85%) showed synergy towards the combination of metformin and BMS-754807. These cell lines represent 6 of the 7 known TNBC subtypes [40] (Table 1). Among these synergistic cell lines, BT-20, HCC1806, MDA-MB-436, HCC70 and MDA-MB-468 were most sensitive to the drug combination.

Representative examples of the synergistic effect of metformin and BMS-754807 are shown in Fig 1. The cell viability of HCC1806 and BT-20 after treatment with increasing concentrations of the two drugs for 72 hours was measured by the MTS assay. At all concentrations tested, the combination of the two drugs enhanced the inhibition of cell proliferation compared with single agent treatment. Overall, our MTS assay results revealed the synergistic effects of metformin and BMS-754807 in the great majority of TNBC cell lines.

Reverse-phase protein array identifies proteins differentially regulated by metformin and BMS-754807 alone and in combination

To discover protein profile changes caused by metformin or BMS-754807 or their combination, we performed a reverse-phase protein array (RPPA) analysis on HCC1806 cell lysates using 216 different cancer-related antibodies. These lysates were prepared from cells treated with vehicle (DMSO), metformin (5 mM), BMS-754807 (15μM), and the combination of the two drugs for 48 hours. From our analysis, we considered the significantly changed proteins between experimental groups by employing Tukey's pairwise T-test (significant for p value < 0.05). We first compared each of the single drug treatment groups to the vehicle group to detect protein changes caused by treatment with metformin and BMS-754807 individually. We found that metformin altered 35 proteins while BMS-754807 changed 118 proteins in HCC1806 cells (Supplementary Table 1). Among those proteins significantly changed by single drug treatment, 19 were shared (Fig 2a). Examples include decreased levels of p-EGFR(Y845; Y1045), p-FAK(Y576/577; Y397), p-ALK(Y1604), p-HER2/ErbB2(Y877), c-Myc, Ezh2, RRM2, HIF-1α, and LRP6 (Table 2) and increased levels of p-p27(T187). These results confirm that these two agents individually have impacts on a number of cancer-related signaling pathways in HCC1806, reflecting our observation that HCC1806 was sensitive to each drug individually.

To detect the biological pathways altered by the drug combination, we compared protein expression of cells receiving combination treatment vs. single drug treatment. Adding

metformin to BMS-754807 increased the effects of BMS-754807 alone by 51 proteins, while adding BMS-754807 to metformin enhanced the effects of metformin by 121 proteins (Fig 3a, Supplementary Table 2). 35 proteins are shared in these two comparisons (Fig 3 and Table 3). One of these proteins, the cell proliferation marker Ki-67, was dramatically reduced by the combination compared to either drug alone, consistent with the synergistic inhibition of cell proliferation seen in the MTS assays. Other shared proteins may include targets that are crucial to the synergistic inhibition of tumor cell growth. For example, the expression level of cleaved caspase-7 was raised by the combination compared to either drug alone (Table 3 vs. Supplementary Table 1), implying that metformin and BMS-754807 together but not as single agents induce apoptosis in HCC1806. Aurora A, a mitotic serine/threonine kinase that plays a crucial role in mitosis, was significantly suppressed by the combination compared with BMS-754807 only ($p < 0.0001$, fold change = −2.13) or metformin only ($p < 0.0001$, fold change = −4.56). Other notable proteins that were downregulated include p-EGFR(Y1068), p-SHC(Y317), BRCA1, ATM, ILK1, Notch1, and STAT3 (Table 3). These proteins are known to promote cell proliferation and tumor growth. The phosphorylated (inactive) form of Rb was reduced by approximately 53% in the combination treatment vs. BMS-754807 alone, whereas the total Rb was reduced by approximately 14% (Table 3), indicating that the fraction of unphosphorylated (active) Rb, which can bind E2F and thereby inhibit cell cycle progression, was increased by the combination treatment.

Metformin and BMS-754807 combination treatment enhances p27 protein levels and reduces Skp2 protein levels in HCC1806

Among the proteins that are upregulated by the combination treatment, the important cell cycle inhibitor $p27^{KIP1}$ and its phosphorylated form stood out (Fig 3 and Table 3). We performed Western blotting to verify the expression changes of p-p27(T187) in HCC1806 in the presence of single drugs and the combination. As shown in Fig 4, metformin alone did not appear to have a significant impact on p27 and p-p27(T187) expression levels, BMS-754807 alone led to an increase in both total and phosphorylated levels of p27, and the drug combination caused a further increase of the total and phosphorylated levels of p27, consistent with the findings from RPPA (Fig 3b). p27, a well-recognized inhibitor of the cyclin/cyclin-dependent kinase (CDK) complex, is phosphorylated at Thr187, ubiquitinated and degraded in the late G1 phase of the cell cycle [41,42]. The responsible E3 ligase is SCF^{Skp2}, which targets p-p27(T187) for proteasome-mediated degradation [43–45]. To test whether this abnormal accumulation of p-p27(T187) as well as total p27 was due to depleted levels of Skp2, we quantified Skp2 levels in these cells. We found that BMS-754807 treatment led to a reduced level of Skp2, and that the combination treatment caused a further reduction of Skp2 (Fig 4b). Together, these data suggest that combination treatment with BMS-754807 and metformin leads to diminished levels of Skp2, therefore allowing p27 to accumulate and suppress cell cycle progression in TNBC cells.

Discussion

We demonstrate that treatment with the combination of metformin and BMS-754807 is synergistic in inhibiting cell proliferation in 11 out of 13 (85%) TNBC cell lines tested,

representing 6 of the 7 known subtypes of TNBC [40]. We found that treatment with either drug alone led to changes of many proteins, but that the combination treatment led to changes of additional proteins and to increased intensities of changes. Notably, the expression levels of both total p27 and p-p27(T187) were increased more when the two drugs were combined. p27 plays a crucial role in suppressing cell cycle progression by blocking the activity of Cyclin/CDK complexes [46,47]. Although rarely mutated, p27 protein levels are commonly decreased in breast cancer and other types of cancer, and low p27 levels are often correlated with poor prognosis [48].

In progressing cancer, the p27 protein level is kept low primarily by CDK-mediated phosphorylation at Thr187 which then can be recognized and ubiquitinated by the Skp2 E3 ligase [49]. Combination treatment with metformin and BMS-754807 led to increased levels of both total and p-p27(T187), excluding inhibition of phosphorylation as the underlying reason for increased levels of total p27. Rather, Skp2 levels were found to be decreased. Downregulation of Skp2, with associated accumulation of p27, has been reported to drive cells into quiescence [50]. On the other hand, overexpression of Skp2 is frequently observed in human cancer and metastases [51]. Therefore, it is likely that the drug-induced suppression of Skp2 protein levels allowed p27 and p-p27 to accumulate in the treated cells. However, it is yet to be determined how Skp2 is reduced by BMS-754807 and further diminished by combination treatment with metformin and BMS-754807.

It also remains to be determined whether increased levels of phosphorylated p27 are entirely due to blockage of the degradation process mediated by Skp2. Enhanced kinase activities may also play a role. CDKs are the primary kinase that phosphorylate p27. AKT, a key downstream component of IGF signaling, has also been reported to phosphorylate p27 (at Thr157/Thr157) and exclude it from the nucleus [52–54]. Src has been reported to phosphorylate p27 (at Y78/88) and target it for degradation [55,56]. But our RPPA analysis did not show an increase in any of these kinases, although we found a decrease of p-Src with an inhibitory phosphorylation at Y527 in cells treated with the drug combination (Table 3). These data suggest that increased levels of p27 phosphorylation are primarily the outcome of blockaded degradation.

Increased levels of p27 may explain why the proportion of unphosphorylated Rb is increased in HCC1806 treated with these two drugs (Table 3). p27 binds to the complex of Cyclin D/CDK4, prevents CDK4 from phosphorylating pRb protein, and increases the fraction of unphosphorylated Rb. Unphosphorylated Rb is the form that is able to bind to and sequester the transcription factor E2F, thereby blocking cell cycle progression [57]. Therefore, this p27-Rb signaling axis may be another mechanism underlying the therapeutic effect of the drug combination.

There are likely other mechanisms by which the combination treatment slows cell growth. In particular, the Wnt signaling pathway appears to be downregulated by the drug combination: the Wnt co-receptor LRP6, p-β-Catenin(S33/37/T41), a classic Wnt signaling target c-Myc and its phosphorylated form p-c-Myc(T58), were all downregulated and they were in the same sub-cluster of the heatmap (Fig 3b), suggesting that they are coordinately controlled. Wnt signaling is known to play a critical role in TNBC formation, progression and

therapeutic resistance [58]. Besides the Wnt signaling pathway, other notable proteins that may contribute to the growth inhibitory effects of the drug combination include p-EGFR(Y1068), Aurora, and RRM2 (Ribonucleoside-diphosphate reductase subunit M2). EGFR is amplified and overexpressed in TNBC and is a known core player of TNBC growth and progression [59]. Aurora A dysregulation occurs frequently in many human cancers [60], and inhibition of this kinase has been reported to inhibit TNBC growth [61]. RRM2 high expression predicts worse prognosis in TNBC [62].

In conclusion, we discovered that the combination of metformin and an insulin/IGF-1 inhibitor are synergistic in blocking the proliferation of the majority of the TNBC cell lines tested. One probable major mechanism of this synergy is the significant impact of the drug combination on the Skp2-p27 signaling axis and other proteins regulating the cell cycle and cell proliferation, leading to blocked cell growth. Although chemotherapy remains the mainstay of treatment for TNBC, research into targeted therapies has recently led to the addition of novel agents such as PARP inhibitors and immune checkpoint inhibitors as treatment options for a subset of TNBC patients [63–65]. Our in vitro data call for robust in vivo preclinical evaluation of the combination of metformin and an insulin/IGF-1 inhibitor in treating TNBC, which will provide critical data for a possible clinical trial to test this drug combination in TNBC patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TNBC	Triple-negative breast cancer
IGF-1R	Insulin-like growth factor 1 receptor
IGF	Insulin-like growth factor
IR	Insulin receptor
ER	Estrogen receptor
PR	Progesterone receptor
HER2	Human epidermal growth factor receptor 2
RPPA	Reverse phase protein array

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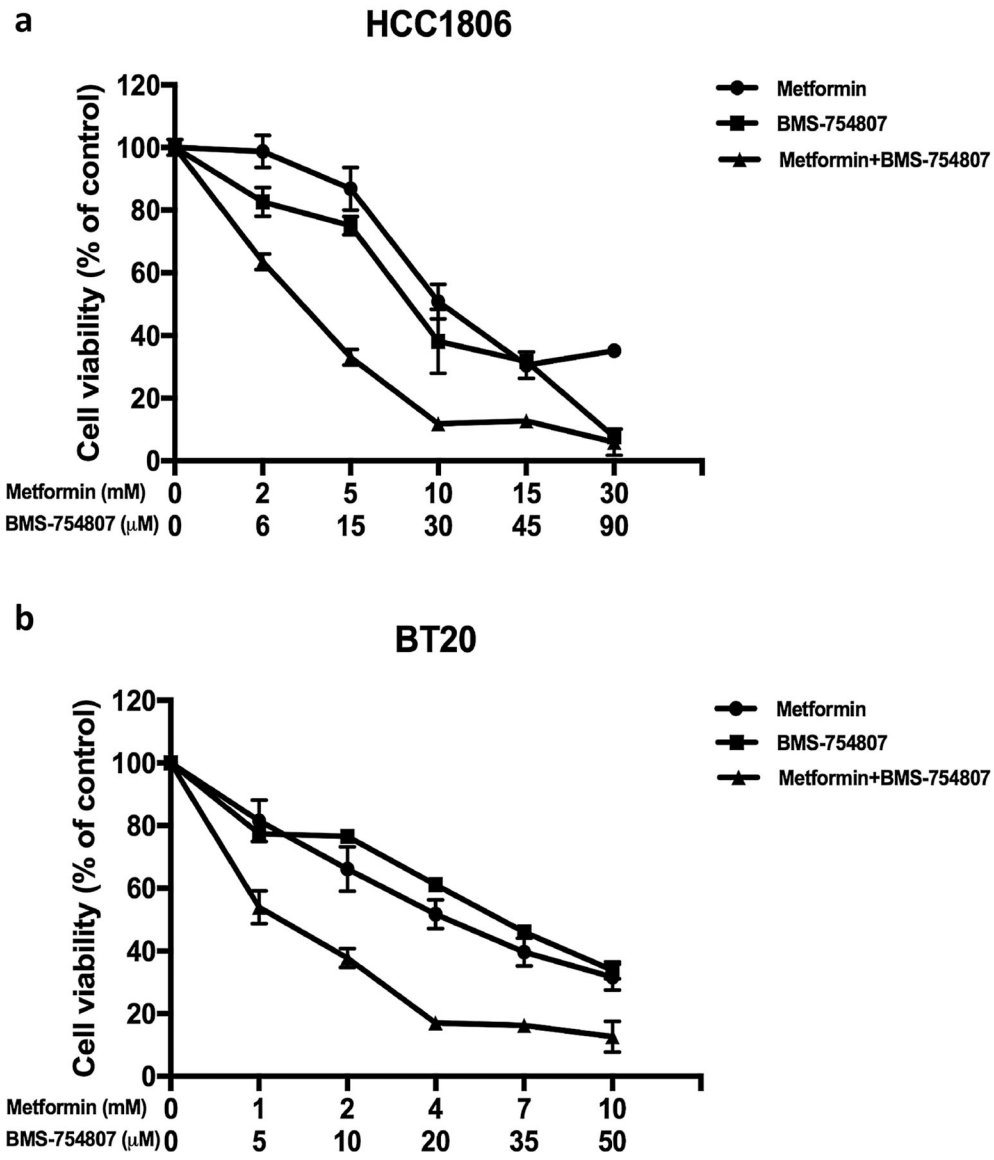

Fig. 1. The majority of TNBC cell lines are sensitive to combined treatment with metformin and BMS-754807

Proliferation assay of HCC1806 and BT-20 cells treated with metformin, BMS-754807 and their combination with different concentrations. Both HCC1806 and BT-20 cells were plated onto a 96-well plate (2000 – 5000 cells per well) in regular growth medium. After 24 hours of incubation, cells were treated with single agent or combined agents for 72 hours and then assayed for percent proliferation using MTS and normalized to control treatment. Experiments are representative of triplicate assays. Data are representative of averages of triplicate determinations ± SE.

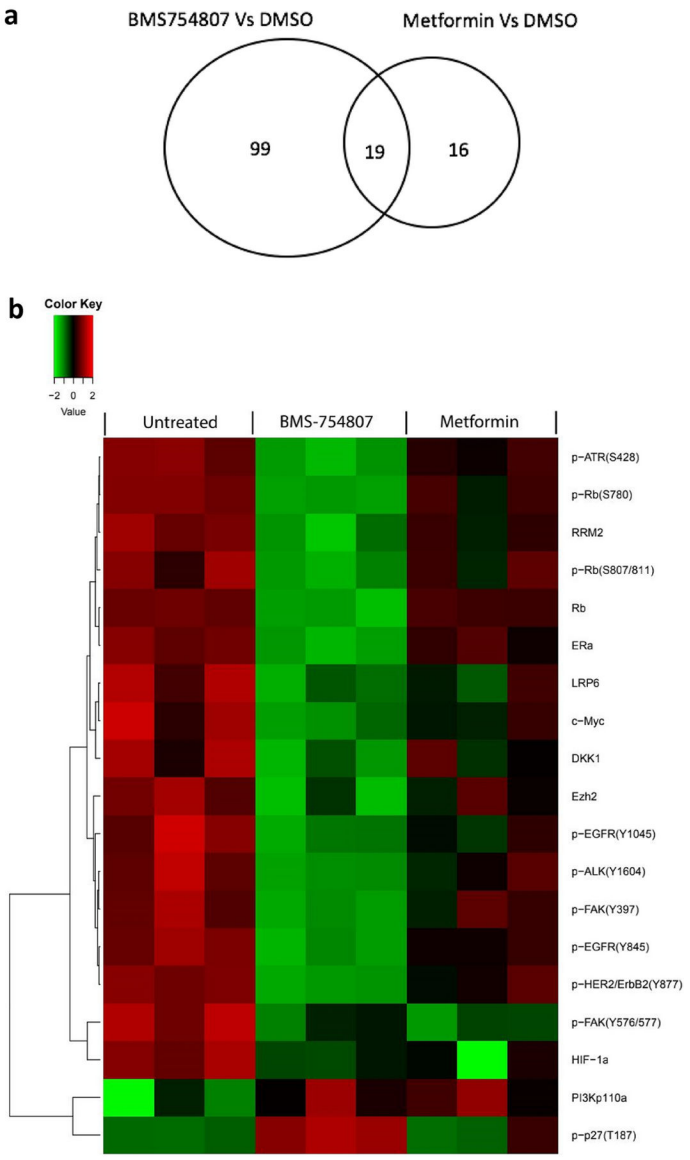

Fig. 2. Protein changes as a result of treatment with BMS-754807 or metformin as a single agent

(a) Venn diagram showing the numbers of proteins in the listed comparisons. (b) Heatmap from RPPA analysis showing proteins/phosphoproteins differentially expressed in HCC1806 following treatment with DMSO, metformin alone, and BMS-754807 alone. The normalized data were log2 transformed. Each group has 3 biological replicates. For each sample, the median value of three technical replicates was used for statistical analysis. Two-way ANOVA was used to test the main effects of the different treatments and their interactions. A p value < 0.05 was considered statistically significant. Red indicates higher intensity while green indicates lower intensity.

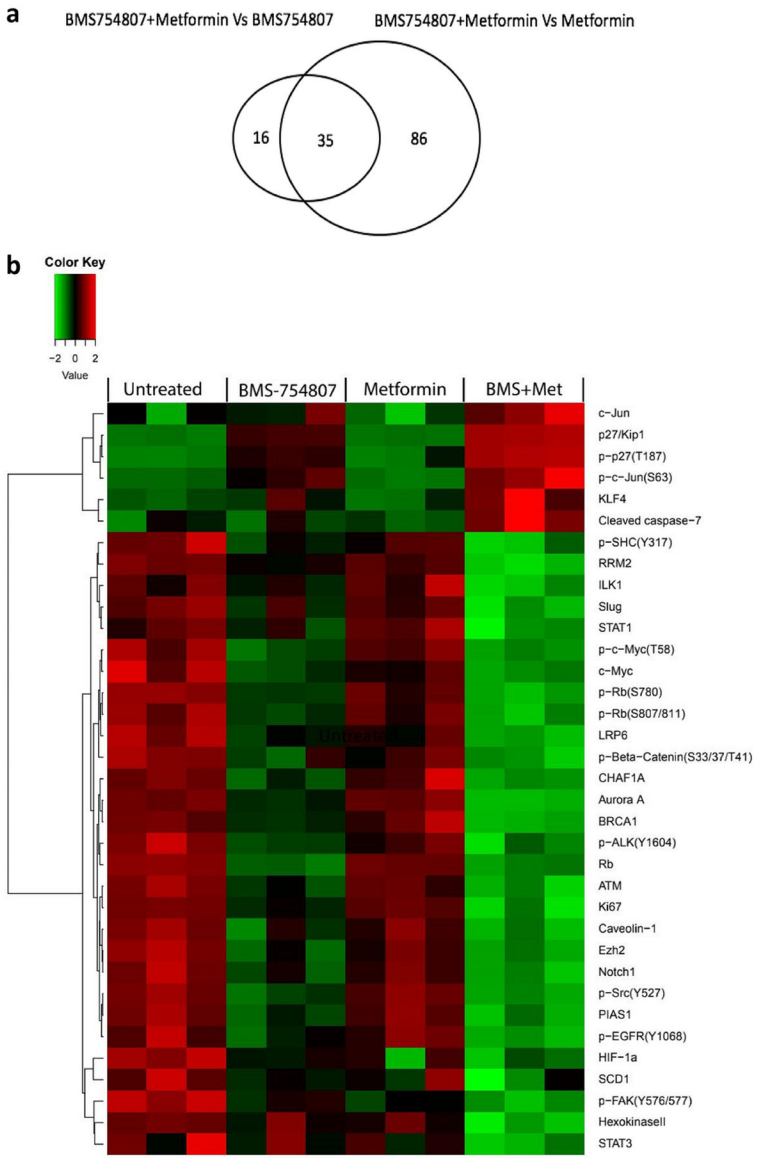

Fig. 3. Protein changes as a result of treatment with BMS-754807 and metformin combination

(a) Venn diagram showing the numbers of proteins in the listed comparison. (b) Heatmap from RPPA analysis showing proteins/phosphoproteins differentially expressed in HCC1806 following treatment with DMSO, metformin alone, BMS-754807 alone, and the drug combination. The normalized data were log2 transformed. There are 4 groups and each group has 3 biological replicates. For each sample, the median value of the three technical replicates was used for statistical analysis. Two-way ANOVA was used to test the main effects of the different treatments and their interactions. A p value < 0.05 was considered statistically significant. Red indicates higher intensity while green which indicates lower intensity.

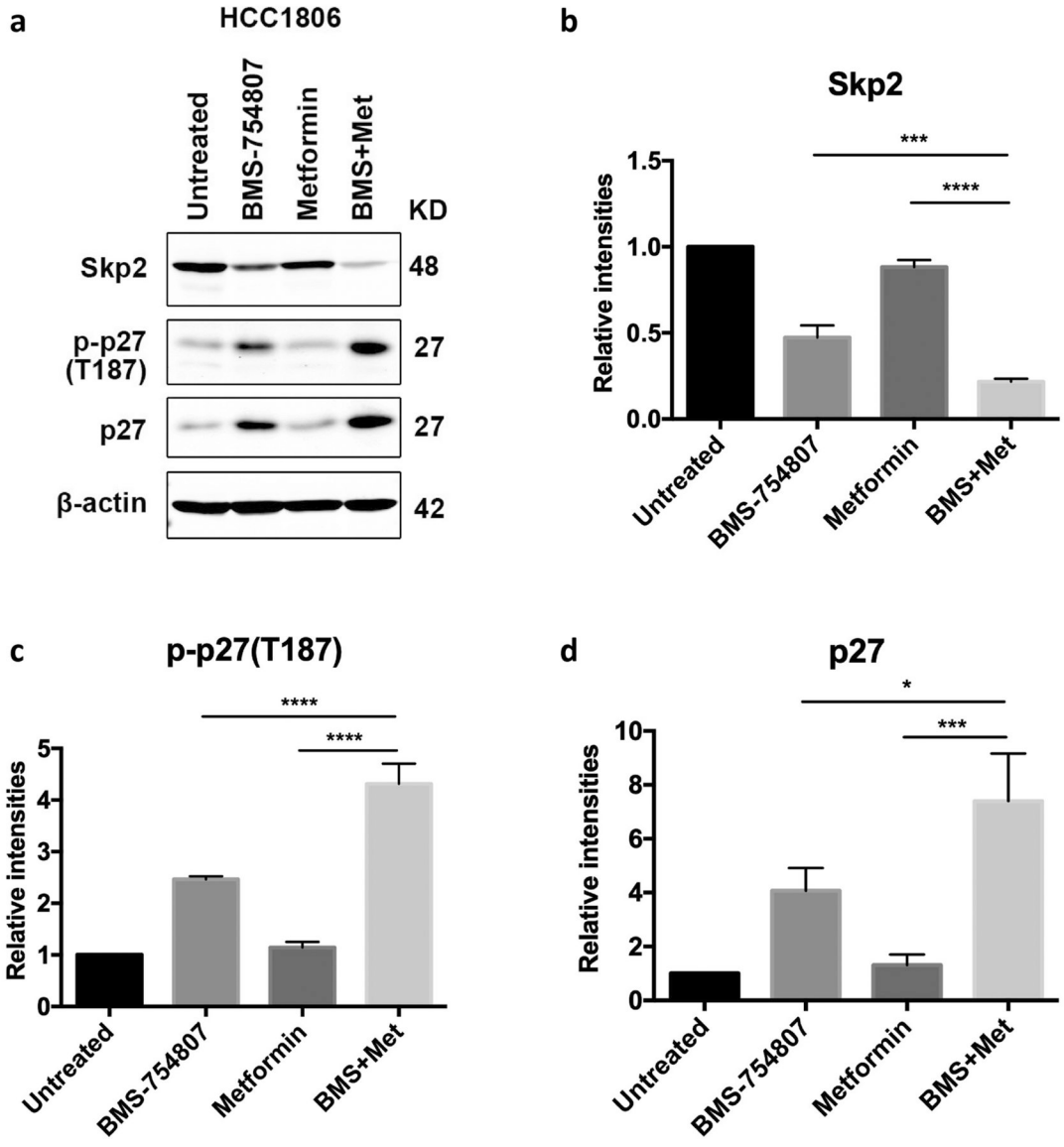

Fig. 4. Combined treatment with metformin and BMS-754807 synergistically enhances p27 and p-p27(T187) but reduces Skp2

Immunoblots (a) and quantification (b-d) of the indicated proteins in the HCC1806 cell line. Cells were treated for 48 hours with metformin (5mM), BMS-754807 (15μM), or the drug combination.

Table 1

The majority of TNBC cell lines are sensitive to combined treatment with metformin and BMS-754807

Cell line	TNBC subtype	ED50 BMS (μM)	ED50 Metformin (mM)	CI Value	Effect
BT-20	LAR Unclassified	5.41	1.08	0.43	Synergism
MDA-MB-453	LAR LAR	45.9	0.9	0.43	Synergism
HCC1806	Basal-like BL2	7.69	2.56	0.49	Synergism
MDA-MB-436	Mesenchymal-like MSL	2.34	0.23	0.56	Synergism
HCC70	Basal-like BL2	1.16	5.82	0.59	Synergism
MDA-MB-231	Mesenchymal-like MSL	3	15.33	0.63	Synergism
MDA-MB-468	Basal-like BL1	0.39	0.16	0.63	Synergism
HCC1937	Basal-like BL1	10.42	2.08	0.75	Synergism
HCC38	Basal-like BL1	0.95	1.9	0.75	Synergism
MDA-MB-157	Mesenchymal-like MSL	3.29	0.55	0.92	Synergism
BT-549	Mesenchymal-like M	5.09	12.73	0.93	Synergism
Hs 578T	Mesenchymal-like MSL	4.32	11.52	1.03	Additive
HCC1395	LAR Unclassified	29.5	31.1	1.28	Antagonism

All the cells were cultured in regular growth medium for 24 hours and then treated with increasing concentrations of either a single agent or both agents for 72 hours. CI values were calculated by software *CompuSyn* to determine the anticancer effect of drug combination. The concentration of each drug at ED50 were given by the report from the software *CompuSyn*.

Table 2

Proteins whose levels are altered by single agent treatment with metformin and with BMS-754807

Proteins	BMS-754807 vs. DMSO		Metformin vs. DMSO	
	p value	Fold Change	*p* value	Fold Change
p-p27(T187)	<0.0001	2.96	0.0404	1.32
PI3Kp110a	0.0318	1.14	0.0239	1.15
Rb	<0.0001	−3.61	0.0118	−1.20
p-Rb(S807/811)	<0.0001	−3.48	0.0261	−1.40
p-Rb(S780)	<0.0001	−3.17	0.0002	−1.42
p-EGFR(Y845)	<0.0001	−2.71	0.0062	−1.42
ERa	<0.0001	−2.44	0.0387	−1.23
DKK1	<0.0001	−2.28	0.0188	−1.40
p-FAK(Y397)	<0.0001	−2.12	0.0236	−1.23
p-ALK(Y1604)	<0.0001	−2.09	0.0358	−1.32
Ezh2	<0.0001	−2.04	0.0321	−1.30
p-HER2/ErbB2(Y877)	<0.0001	−2.01	0.0181	−1.25
RRM2	<0.0001	−1.97	0.0063	−1.29
c-Myc	<0.0001	−1.84	0.0013	−1.36
HIF-1a	0.0004	−1.80	0.0001	−1.80
LRP6	0.0007	−1.67	0.0330	−1.35
p-EGFR(Y1045)	0.0002	−1.62	0.0330	−1.30
p-ATR(S428)	<0.0001	−1.53	0.0159	−1.13
p-FAK(Y576/577)	<0.0001	−1.32	<0.0001	−1.38

For each sample, the median value of three technical replicates was used for statistical analysis. Two-way ANOVA was used to test the main effects of the different treatments and their interactions. p value < 0.05 was considered statistically significant.

Table 3

Proteins whose levels are significantly altered by combination treatment with metformin and BMS-754807 compared with single agent treatment

Proteins	BMS-754807 + Metformin vs. BMS-754807		BMS- 754807 + Metformin vs. Metformin	
	p value	Fold Change	*p* value	Fold Change
p27/KIP1	<0.0001	2.60	<0.0001	13.81
p-p27(T187)	<0.0001	2.27	<0.0001	5.11
KLF4	0.0010	1.71	<0.0001	2.32
c-Jun	0.0145	1.60	<0.0001	2.65
p-c-Jun(S63)	0.0001	1.53	<0.0001	2.73
Cleaved caspase-7	<0.0001	1.45	<0.0001	1.52
RRM2	<0.0001	−3.94	<0.0001	−6.05
Ki-67	<0.0001	−3.31	<0.0001	−8.22
Slug	<0.0001	−2.43	<0.0001	−3.51
Aurora A	<0.0001	−2.13	<0.0001	−4.56
p-Rb(S807/811)	<0.0001	−1.91	<0.0001	−4.73
CHAF1A	0.0122	−1.88	<0.0001	−9.76
p-Rb(S780)	<0.0001	−1.87	<0.0001	−4.16
HexokinaseII	<0.0001	−1.82	<0.0001	−1.84
BRCA1	<0.0001	−1.70	<0.0001	−3.28
Caveolin-1	0.0353	−1.65	<0.0001	−2.76
HIF-1a	0.0087	−1.49	0.0246	−1.49
LRP6	0.0101	−1.46	0.0002	−1.80
PIAS1	0.0211	−1.44	<0.0001	−2.81
p-Src(Y527)	0.0054	−1.43	<0.0001	−3.33
ATM	0.0002	−1.42	<0.0001	−2.06
p-EGFR(Y1068)	0.0117	−1.38	<0.0001	−1.98
p-SHC(Y317)	0.0032	−1.36	<0.0001	−1.70
Ezh2	0.0351	−1.32	<0.0001	−2.07
p-ALK(Y1604)	0.0370	−1.30	<0.0001	−2.06
p-FAK(Y576/577)	<0.0001	−1.29	0.0006	−1.24
STAT3	0.0008	−1.28	0.0012	−1.26
Notch1	0.0151	−1.26	<0.0001	−1.63
p-Beta-Catenin(S33/37/T41)	0.0069	−1.25	<0.0001	−1.49
ILK1	0.0015	−1.23	<0.0001	−1.42
STAT1	0.0042	−1.21	<0.0001	−1.43
c-Myc	0.0352	−1.21	<0.0001	−1.63
p-c-Myc(T58)	0.0396	−1.21	<0.0001	−2.21
Rb	0.0287	−1.17	<0.0001	−3.51
SCD1	0.0410	−1.17	0.0041	−1.26

The normalized data were log2 transformed for hypothesis testing. For each sample, the median value of three technical replicates was used for statistical analysis. Two-way ANOVA was used to test main effects of the different treatments and their interactions. p value < 0.05 was considered statistically significant.