Upregulation of endogenous TRAIL-elicited apoptosis is essential for metformin-mediated antitumor activity against TNBC and NSCLC

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) shows promising antitumor activity in preclinical studies. However, the efficacy of recombinant TRAIL in clinical trials is compromised by its short serum half-life and low in vivo stability. Induction of endogenous TRAIL may overcome the limitations and become a new strategy for cancer treatment. Here, we discovered that metformin increased TRAIL expression and induced apoptosis in triple-negative breast cancer (TNBC) and non-small cell lung cancer (NSCLC) cells. Metformin did not alter the expression of TRAIL receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5). Metformin-upregulated TRAIL was secreted into conditioned medium (CM) and found to be functional, since the CM promoted TNBC cells undergoing apoptosis, which was abrogated by a recombinant TRAIL-R2-Fc chimera. Moreover, blockade of TRAIL binding to DR4/DR5 or specific knockdown of TRAIL expression significantly attenuated metformin-induced apoptosis. Studies with a tumor xenograft model revealed that metformin not only significantly inhibited tumor growth but also elicited apoptosis and enhanced TRAIL expression in vivo. Collectively, we have demonstrated that upregulation of TRAIL and activation of death receptor signaling are pivotal for metformin-induced apoptosis in TNBC and NSCLC cells. Our studies identify a novel mechanism of action of metformin exhibiting potent antitumor activity via induction of endogenous TRAIL.

INTRODUCTION

Breast cancer and lung cancer are among the leading cancer types for estimated new cases and deaths in the United States.1 Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype that represents a significant clinical challenge, as patients with TNBC have a poor prognosis and account for a disproportionate number of breast cancer deaths.2,3 Unlike hormone-receptor-positive or HER2-positive breast cancers, in which targeted therapy has improved patient survival, TNBC lacks effective therapeutic targets and results in poor overall survival.4,5 Also, non-small cell lung cancer (NSCLC), accounting for approximately 80%–85% of all lung cancers, is another highly lethal cancer type with limited therapeutics available to date.6 Recent advances in the development of targeted therapies against driver mutations in epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), other genes,7 and immunotherapy,8,9 have greatly improved the survival of NSCLC patients. However, the patients’ 5-year overall survival rate remains poor at 19%.1,10 Despite their distinct tissues of origin, TNBC and NSCLC possess several similarities based on gene mutations and pathway activation. The EGFR pathway is excessively activated in both TNBC and NSCLC,11,12 and once activated, EGFR can initiate the PI-3K/Akt and MEK/ERK signaling pathways to enhance cancer cell proliferation and survival.13 Liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK) and the insulin-like growth factor-1 receptor (IGF-1R) pathways, which act as oncogenic signals promoting tumorigenesis and causing therapeutic resistance, are commonly dysregulated in TNBC and NSCLC.14–16 Years of investigation on the dysregulated signaling has made substantial progress in the development of effective therapies with numerous promising drugs that have entered clinical trials.2,17 However, the efficacy of current treatments for TNBC and NSCLC is far from satisfactory. Novel molecular targets and therapeutic strategies are in urgent need to improve the survival of patients with TNBC and NSCLC.

Dysregulation of apoptosis is associated with tumorigenesis, making it an attractive target for cancer treatment. Conventional therapy may activate apoptotic signaling in cancer cells, but the lack of cancer cell selectivity often causes significant toxicity. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) binds to TRAIL

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receptor 1 (TRAIL-R1, also known as death receptor 4 [DR4]) or TRAIL receptor 2 (TRAIL-R2, or death receptor 5 [DR5]). This triggers extrinsic apoptosis selectively in cancer cells while sparing normal cells. This unique property leads to the development of recombinant human TRAIL and agonists of DR4 or DR5 for clinical use. Dulanermin is a recombinant non-tagged TRAIL, which shows potent antitumor activity and is well tolerated in both in vitro and in vivo models of solid tumors, including breast cancer and NSCLC. Despite its encouraging preclinical results, dulanermin has failed to demonstrate significant efficacy in clinical trials. This failure is in part due to dulanermin’s short half-life in vivo and weak activity to induce clustering of TRAIL-Rs. In addition, recombinant TRAIL, including dulanermin, has the potential to develop anti-drug antibody (ADA) responses, which may be responsible for liver toxicity. Thus, induction of endogenous TRAIL is believed to be able to overcome the limitations, and it has become a new strategy to harness the TRAIL-TRAIL-R system for identifying more effective treatments for human cancers. Our previous studies have reported that induction of endogenous TRAIL triggers tumor-selective death and sensitizes cancer cells to chemotherapy in various human cancers.

Metformin, a safe and commonly prescribed drug for type II diabetes, possesses promising therapeutic activity in various human cancers, including TNBC and NSCLC. Nonetheless, the mechanism of action of metformin in suppressing tumor growth remains elusive. We have reported that metformin selectively induces apoptosis in TNBC cells, likely through a caspase-8-initiated caspase cascade, suggesting that metformin might trigger extrinsic apoptosis signaling in TNBC cells. In the current study, we have explored the capability of metformin to enhance endogenous TRAIL expression in TNBC and NSCLC cells and investigated whether TRAIL-induced apoptosis plays a critical role in metformin-mediated antitumor activity.

RESULTS

Metformin inhibited viability of TNBC and NSCLC cells via induction of apoptosis.

To investigate whether metformin would exhibit a similar anti-proliferative/anti-survival effect on NSCLC cells, as we observed in TNBC cells, both short-term cell proliferation (MTS) and long-term clonogenic assays were performed in three NSCLC cell lines (H460, H1650, and A549) treated with different concentrations of metformin. First, we confirmed our previous results showing that metformin potently inhibited cell proliferation and colony formation in TNBC cells (Figure S1). Then, we found that metformin significantly inhibited proliferation of NSCLC cells in a dose-dependent manner (Figure 1A). In clonogenic assays, metformin dramatically suppressed NSCLC colony formation at concentrations as low as 0.5 mM. Colony numbers decreased upon metformin treatment in a dose-dependent manner (Figure 1B). Therefore, TNBC and NSCLC cells seemed to show a similar sensitivity to metformin-mediated inhibition regarding cell viability.

Next, we wondered whether metformin might induce apoptosis in NSCLC cells, as we have reported in TNBC cells. Thus, we treated both TNBC (HCC70, MDA-MB-468, and BT549) and NSCLC (H460, H1650, and A549) cells with increasing concentrations of metformin. An apoptosis-specific enzyme-linked immunosorbent assay (ELISA) showed that metformin promoted apoptosis in both TNBC and NSCLC cells in a dose-dependent manner (Figure 2A). Western blot assays confirmed the metformin induced apoptosis, evidenced by enhanced PARP cleavage, a hallmark of apoptosis, and increased active caspase-8 and caspase-3 (Figure 2B). These data were not only in agreement with our previous findings but they also indicated that metformin triggered a caspase-cascade-dependent apoptosis. Collectively, our studies supported the notion that metformin profoundly inhibited cell viability via induction of apoptosis in both TNBC and NSCLC cells.

Metformin enhanced expression of TRAIL, which exhibited its biological function to trigger apoptosis in TNBC and NSCLC cells

Our previous studies showed that a specific inhibitor of caspase-8 was more effective than a caspase-9 inhibitor to abrogate metformin-induced apoptosis in TNBC cells. This suggested that caspase-8-initiated extrinsic apoptosis signaling was crucial for metformin to elicit apoptosis. Thus, we investigated if the TRAIL-death receptor pathway might be involved in metformin-induced apoptosis in both TNBC and NSCLC cells. Western blot analyses revealed that metformin, in a dose-dependent manner, increased the protein levels of TRAIL in all TNBC and NSCLC cell lines tested (Figure 3A). However, metformin did not alter the expression of DR4 and DR5 (Figure 3B). Because only cell membrane DR4 and DR5 were able to bind to TRAIL to trigger extrinsic apoptosis, we then examined the membrane-bound DR4 and DR5 by flow cytometry analyses. The presence of these receptors on the cell membrane was indicated by a right shift of the peak compared to the isotype control. Downregulation or upregulation of the receptors with metformin treatment would be indicated by a left or right shift of the peak, respectively, compared to the untreated control. DR5 was detected on the membranes of TNBC (HCC70 and BT549) and NSCLC (H460 and H1650) cells, whereas DR4 was moderately present on the membrane of HCC70 cells, rarely on that of H460 and BT549 cells, and not present on H1650 cell membranes (Figure 3C). The inconsistency between DR4 total protein levels and cell surface presentation might be due to the DR4 endocytosis mechanism, which has been previously reported. Overall, metformin treatment had little effect on total DR4/DR5 expression and did not alter the receptors’ membrane presentation in all TNBC and NSCLC cells tested (Figure 3D). Our data suggest that regulation of DR4/DR5 expression is not involved in metformin-induced apoptosis.

Next, we wondered if metformin-upregulated TRAIL in TNBC and NSCLC cells was secreted into the culture medium and if the soluble TRAIL retained its biological function of binding to DR4/DR5, thereby forming an autocrine stimulation to trigger apoptosis. To this end, we first collected conditioned medium (CM) of TNBC and NSCLC cells, untreated (control) or treated with metformin. After centrifugation, the CM was concentrated 50-fold through an
ultrafiltration filter with a cutoff at molecular weight of 10 kDa (Figure 4A). Then, TRAIL levels were assessed in the CM concentrates through western blot assays and a specific ELISA. Meanwhile, we utilized the CM concentrates to determine if CM was able to promote apoptosis in the TNBC (MDA-MB-231) cells. After exposure to each CM concentrate, apoptosis was measured via LIVE/DEAD Cell Imaging and apoptosis ELISA. Metformin treatment increased TRAIL levels in the concentrated CM of MDA-MB-468 and H460 cells (Figure 4B, left), as well as that of HCC70 and H1650 cells (Figure S2). In addition, a specific ELISA detected a significant increase of TRAIL levels in the concentrated CM of metformin-treated MDA-MB-468 cells (Figure 4B, right). To determine whether the soluble CM TRAIL was biologically active, concentrated CM obtained from H460 cells that were cultured with medium only or treated with metformin was used to treat MDA-MB-231 cells. While treatment with metformin at the concentration of 5 mM had no cytotoxicity effect on MDA-MB-231 cells, the CM obtained from H460 cells treated with the same amount of metformin profoundly elicited apoptotic cell death detected by both the LIVE/DEAD Cell Imaging and apoptosis ELISA. This did not occur with the CM obtained from H460 cells treated with medium only (Figures 4C and 4D). Moreover, the apoptosis was largely abrogated by a recombinant human TRAIL-R2 Fc chimera protein, which contains the extracellular domain and a truncated intracellular domain of DR5 as well as the Fc fragment of the human immunoglobulin G1 (IgG1). This fusion protein neutralized the ability of TRAIL to induce apoptosis. Similar results were also observed with the CM obtained from MDA-MB-468 cells-treated with metformin. That is, the CM of metformin-treated MDA-MB-468 cells was weakened from inducing apoptosis in MDA-MB-231 by the TRAIL-R2 Fc chimera (Figure S3).

Together, our data demonstrate that metformin enhances the expression of endogenous TRAIL in TNBC and NSCLC cells and that upregulated TRAIL is secreted into CM, where it retains its bioactivity to trigger an autocrine stimulation and death-receptor-mediated apoptosis.

Inhibition of TRAIL function or expression significantly attenuated metformin-induced apoptosis in TNBC and NSCLC cells

To determine whether the induction of TRAIL was responsible for metformin-induced apoptosis in TNBC and NSCLC cells, we took advantage of two kinds of strategies. First, the recombinant TRAIL-R2-Fc chimera protein was used to block TRAIL’s bioactivity in
metformin-treated cells. TNBC and NSCLC cells treated with metformin alone or in combination with the TRAIL-R2-Fc chimera were subjected to apoptosis ELISA and western blot assays. We discovered that the chimera significantly attenuated metformin-induced apoptosis in a dose-dependent manner (Figure 5A). Blockade of TRAIL function by the chimera markedly diminished metformin-mediated PARP cleavage and activation of caspase-8 and caspase-3 (Figure 5B). These data indicate that enhanced activation of the TRAIL-death receptor pathway is essential for metformin-induced apoptosis in TNBC and NSCLC cells. Then, we used a genetic approach with targeted gene silencing. A lentiviral vector, carrying a nonspecific scramble short hairpin RNA (shRNA) (sh-scr) or specific shRNA targeting TRAIL mRNA (sh-1 or sh-2), was used to generate stable clone pools. Both TRAIL sh-1 and sh-2 effectively repressed TRAIL expression in both TNBC and NSCLC cells untreated or treated with metformin (Figure 6A). Importantly, specific knockdown of TRAIL dramatically reduced metformin-mediated PARP cleavage and activation of caspase-8 and caspase-3 in all TNBC and NSCLC cells tested (Figure 6A). Moreover, downregulation of TRAIL with the shRNAs significantly decreased metformin-induced DNA fragmentation (Figure 6B). Collectively, our data demonstrate that upregulation of TRAIL is required for metformin to promote apoptosis in TNBC and NSCLC cells.

Metformin suppressed tumor growth and induced endogenous TRAIL expression and apoptosis in vivo

To determine the antitumor activity of metformin in vivo, we took advantage of a tumor xenograft model established from H460 cells. When tumors reached ~100 mm³, tumor-bearing mice were treated daily with either sterile water (control) or the same volume of water containing metformin (350 mg/kg) by oral gavage for 14 days. We monitored the progression of tumor proliferation and discovered that tumor growth in metformin-treated mice was significantly slower than that in control mice (Figure 7A). Metformin inhibition of tumor growth was also evidenced by a marked reduction in tumor size (Figure 7B; Figure S4A) and weight (Figure 7C). There was no difference in mouse body weight between the two groups (Figure S4B), suggesting that metformin at this dosage had few side effects.

We next examined whether metformin elicited apoptosis and upregulated endogenous TRAIL expression in vivo. To this end, we collected...
mouse serum and tumors at the end of our animal experiments. In both control and metformin-treated mice, the serum levels of TRAIL were undetectable by a specific ELISA (data not shown). However, western blot assays showed that metformin clearly enhanced expression of TRAIL but not that of DR4 and DR5 in the tumors (Figure 7D). Metformin treatment also decreased the levels of full-length PARP, caspase-8, and caspase-3, and increased cleaved caspase-3 (Figure 7E), which were consistent with our in vitro results. Moreover, immuno-

Figure 3. Metformin upregulated TRAIL protein levels without altering expression of DR4 and DR5 in TNBC and NSCLC cells

(A) TNBC and NSCLC cells were treated with different concentrations of metformin in 5% FBS medium for 48 h. Cells were collected and subjected to western blot analysis of TRAIL expression. The densitometry analyses of TRAIL signals were shown underneath, and the arbitrary numbers indicated the intensities of each cell line relative to controls, defined as 1.0. (B) Western blot analysis of TRAIL receptors DR5 and DR4 expression in NSCLC and TNBC cells treated with metformin for 48 h. (C) Flow cytometric analysis of membrane DR5 and DR4 in NSCLC and TNBC cells with or without metformin treatment. Cells were stained with APC-conjugated DR5 and PE-conjugated DR4 antibodies (tinted histograms) or with the isotype-matched IgG control (shadowed histogram). Data show a representative of three independent experiments with similar results. (D) The mean fluorescent intensity (MFI) of DR4 and DR5 was quantified. Values were expressed as the mean ± SD of three independent experiments. ns, not significant.

HISTOCHEMISTRY (IHC) analyses confirmed that metformin significantly reduced expression of Ki67, a typical cell proliferation marker, upregulated TRAIL, and increased the number of tumor cells with positive staining for cleaved caspase-3 (Figure 7F). Collectively, our data indicated that metformin exerted potent anti-tumor activity against NSCLC, likely via its capability of inducing endogenous TRAIL expression and apoptosis in vivo.

DISCUSSION

Numerous cohort studies and meta-analyses have documented a correlation of reduced cancer risk and increased cancer survival with metformin use in diabetic patients.38-41 The appeal of metformin as an anti-cancer agent also lies in its low cost and reassuring safety profile. Metformin has been shown to enhance TRAIL-based treatments in various cancers and sensitize TNBC cells to TRAIL receptor agonist-induced apoptosis via decrease in X-linked inhibitor of apoptosis protein (XIAP) expression.42 Metformin promotes Mcl-1 degradation to potentiate TRAIL-induced apoptosis in colorectal cancer cells43 and enhances TRAIL-induced apoptosis in bladder cancer cells and TRAIL-resistant lung cancer cells via reduction of c-FLIP.44,45 However, none of these studies has investigated if metformin alters expression of endogenous TRAIL in cancer cells. Herein, for the first time, we have shown that metformin is able to induce expression of endogenous TRAIL, correlated with increased apoptosis in TNBC and NSCLC cells. The upregulation of TRAIL is
required for metformin to elicit apoptosis, as this effect is significantly attenuated when the TRAIL-mediated apoptotic pathway is inhibited, through either blockade of TRAIL’s binding to DR4/DR5 or specific knockdown of TRAIL. Interestingly, we found that metformin had little effect on the expression of total DR4/DR5 or membrane DR4/DR5 in all TNBC and NSCLC cell lines tested. Our data are consistent with the results from the studies of bladder cancer cells but inconsistent with those of pancreatic cancer cells, suggesting that metformin’s effect on DR4/DR5 might be cell type dependent.

TRAIL is thought to be a great antitumor agent because of its selective cytotoxicity against cancer cells but not normal cells. However, TRAIL alone may not be as effective as metformin to induce apoptosis in some TNBC and NSCLC cells, because TRAIL resistance frequently occurs due to enhanced survival signaling or upregulation of inhibitor of apoptosis proteins (IAPs) in the cancer cells. It has been shown that activation of the PI-3K/Akt signaling and/or the signal transducer and activator of transcription-3 (STAT3) results in resistance to TRAIL-mediated apoptosis. We, as well as others, have reported that metformin inhibits PI-3K/Akt signaling and STAT3 activity and decreases XIAP expression in breast and/or lung cancer cells. Here, we discover that metformin can also upregulate endogenous TRAIL in both TNBC and NSCLC cells. Thus, metformin, on one hand, inhibits cell survival signaling and reduces expression of anti-apoptosis proteins; on the other hand, it proactively triggers apoptosis via induction of TRAIL. We believe that, because of its simultaneous effects on suppression of survival signaling and autocrine stimulation of the extrinsic apoptotic pathway, metformin will be an excellent therapeutic agent against TNBC and NSCLC.
It is worth emphasizing that metformin-induced TRAIL can be secreted into the cancer cells’ CM, and that soluble TRAIL retains its bioactivity as it effectively induces apoptosis in MDA-MB-231 cells (Figure 4; Figure S3). These findings have significant clinical implications. In the development of TRAIL-based strategies against human cancers, TRAIL gene transfection exerts potent antitumor activity in part due to its "bystander effect." Since both TNBC and NSCLC are highly heterogeneous, one clone within a given tumor may be sensitive to metformin upregulation of TRAIL expression, whereas other clones may not. The increased TRAIL by one clone can be secreted into the local microenvironment, thus giving the soluble TRAIL an opportunity to exhibit its "bystander effect" to trigger apoptosis in the otherwise metformin-insensitive clones. This hypothesis seems to be supported by our in vivo animal studies, showing massive apoptosis occurring in the tumors, evidenced by the presence of substantially increased cleaved caspase-3 upon metformin treatment (Figures 7E and 7F). Further investigations are underway to determine whether metformin-induced upregulation of endogenous TRAIL may also be able to promote apoptosis in metastatic tumors of TNBC and/or NSCLC.

The concentrations of metformin used in our in vitro experiments are high, which may raise some translational concerns of metformin’s antitumor activity. The millimolar (mM) levels of metformin are required to explore the mechanism of its action in TNBC and NSCLC cells. This may be attributed to the nature of the in vitro experiments, applying one-time, short-term exposure (24–48 h) to metformin. Repeated administration and longer exposure to metformin, as is the case in the clinic, should lower the threshold for metformin-mediated inhibitory effects to micromolar (µM) range. In addition, rich nutrients in cell culture media, including high levels of glucose, will reduce the efficacy of metformin in in vitro experiments. Having considered these limitations, we performed in vivo animal experiments with orally given metformin daily, which mimic the clinical use of metformin. Our in vivo data confirmed the findings of our cell culture studies, demonstrating that metformin was able to enhance TRAIL expression and induce apoptosis both in vitro and in vivo. Recent studies examined the in vivo levels of metformin and found that the plasma levels of metformin in the mouse
tumor models given by intraperitoneal injection or orally were in the μM range, which was comparable to the levels of diabetic patients receiving a standard dose of metformin. Moreover, Chandel et al. propose that when assessing the efficacy of metformin, both pharmacokinetic and tumor-related biologic factors need to be considered. A number of intrinsic tumor-related factors, such as the status of oncogenes or tumor suppressors, as well as critical drug transporters will certainly alter metformin’s accumulation in cancer cells and its antitumor activity. A recent study showed that metformin preferentially accumulated in metastatic colorectal cancer (mCRC) cells with Kras mutations and exerted potent antitumor activity against Kras mutant mCRC. Collectively, these studies along with ours support the notion that further investigations of the pharmacokinetics and mechanism of action of metformin are warranted to determine its therapeutic efficacy in human cancers.

In summary, we demonstrated that metformin induced TRAIL expression to trigger apoptosis in TNBC and NSCLC cells. Inhibition of TRAIL function or specific knockdown of TRAIL expression significantly attenuated metformin-induced apoptosis, indicating that induction of TRAIL and activation of the TRAIL-death receptor signaling were essential for metformin to promote TNBC and NSCLC cells undergoing apoptosis. Our studies identified metformin as a novel agent capable of inducing endogenous TRAIL expression and uncovered a new mechanism of action of metformin exhibiting its antitumor activity against TNBC and NSCLC.

MATERIALS AND METHODS
Reagents and antibodies
Metformin (1,1-dimethyl biguanide hydrochloride) was purchased from MP Biomedicals (Solon, OH, USA) and dissolved in sterile water to make a 1 M stock solution. Recombinant human TRAIL-R2/TNFRSF10B Fc chimera protein from R&D Systems (Minneapolis, MN, USA) was reconstituted at 100 μg/mL in PBS. Antibodies for western blot analyses were from the following sources: PARP rabbit mAb (46D11), caspase-8 mouse mAb (1C12), caspase-3 rabbit mAb (8G10), DR4 rabbit mAb (D9S1R), DR5 rabbit mAb (D4E9) (Cell Signaling Technology, Beverly, MA, USA); TRAIL
mouse mAb (B35-1) (R&D Systems); and β-actin mouse mAb (clone AC-75) (Sigma Chemical, St. Louis, MO, USA). Antibodies for flow cytometric analyses were from Biolegend (San Diego, CA, USA): PE anti-human CD261 (DR4) Ab (307206); APC anti-human CD262 (DR5) Ab (307408); APC Mouse IgG1, κ Isotype control Ab (400120); and PE Mouse IgG1, κ Isotype control Ab (400112). All other reagents were from Sigma unless otherwise specified.

**Cells and culture condition**

Human TNBC cell lines (HCC70, MDA-MB-468, MDA-MB-231, and BT549) and NSCLC cell lines (H460, H1650, and A549) were from the American Type Culture Collection (ATCC, Manassas, VA, USA). TNBC cells were maintained in DMEM/F-12 (1:1) medium supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA). NSCLC cells were maintained in RPMI 1640 medium supplemented with 10% FBS. Cells were authenticated with DNA profiling by short tandem repeat (STR) analysis in 2016–2018. Cells were free of mycoplasma contamination, determined by the MycoAlert Mycoplasma Detection Kit (Lonza Group, Basel, Switzerland) once every 6 months. All cells were cultured in a 37°C humidified atmosphere containing 95% air and 5% CO2 and were split twice a week.

**Cell proliferation assay**

The CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to determine cell viability as described. Brieﬂy, cells were plated onto 96-well plates with medium containing 10% FBS. After 24 h, the culture medium was replaced with fresh medium containing 5% FBS (control), or the same medium containing different concentrations of metformin. After 48 h, MTS reagent was added into the cell culture. Cells were then incubated at 37°C for an additional 1 h, and the absorbance was measured by a Synergy LX Multi-Mode Reader (Biotek, Winooski, VT, USA).

**Colony-formation assay**

Colony-formation assays were performed as we described previously. The images of colonies were taken by a digital camera. Colony numbers were counted using the ImageJ software.

**Figure 7. Metformin inhibited tumor growth and induced apoptosis and TRAIL expression in a tumor xenograft model**

(A) Tumor growth curves were plotted using average tumor volume within each group at the indicated time points. A two-tailed Student’s t test was used for statistical analysis (p < 0.01, **p < 0.003). Bars: SD. (B and C) At the end of treatment, tumor-bearing mice from control group and metformin-treated group were sacrificed. The tumors were dissected, imaged as indicated (B), and measured for weight (C). (D and E) Cell lysis from the tumor tissues was prepared. Western blot assays were performed to examine the expression of TRAIL, DR4, DR5, and apoptotic markers PARP, caspase-8, and caspase-3. β-actin was used as an internal control. The densitometry analyses of TRAIL signals are shown underneath, and the arbitrary numbers indicate the intensities of each tumor relative to control 1, deﬁned as 1.0. (F) Formalin-fixed paraffin-embedded sections of xenograft tumors were analyzed with H&E staining, IHC staining for Ki67, TRAIL, and cleaved caspase-3. Scale bar, 210 μm. Quantification of IHC staining with ImageJ and ImageJ plugin IHC profiler are shown underneath.
Quantification of apoptosis
An apoptotic ELISA kit (Roche Diagnostics, Indianapolis, IN, USA) was used to quantitatively measure cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) as previously described.57

Western blot assay
Cells were lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail and sonicated at 4°C. Equal amounts of total cell lysates or CM of cell culture were subjected to western blot assays as we described previously.35,56,58 to measure protein expression and/or activation (cleavage).

LIVE/DEAD Cell Imaging assay
Cells were stained with a LIVE/DEAD Cell Imaging Kit (488/570) (Life Technologies Carlsbad, CA, USA) as described previously.58 After incubation at room temperature in the dark, the cells were observed under EVOS FLoid Cell Imaging Station (Life Technologies) and measured for live cells (green) with green-light channel and dead cells (red) with red-light channel.

Flow cytometric analysis
Flow cytometric analyses were performed to define the presentation of cell membrane DR4 and DR5. In brief, cells grown in culture were harvested by trypsinization and resuspended in PBS (1 × 10^7 cells/mL). Then, 100 μl cell suspension was incubated with 5 μL antibodies (APC-DR5, PE-DR4 or the relative isotype controls) on ice in the dark for 30 min. Flow cytometric analyses were performed with a BD FACSymphony flow cytometer (San Jose, CA, USA), and the mean fluorescent intensity of DR4 and DR5 was calculated by the FlowJo software (FlowJo, Ashland, OR, USA).

Preparation and measurement of supernatant TRAIL in the CM
One million NSCLC cells (H460 and H1650) or TNBC cells (MDA-MB-468 and HCC70) were cultured with complete medium (10% FBS). The following day, the cells were untreated or treated with metformin in the same medium containing 0.5% FBS (H460 and H1650: 30 h; MDA-MB-468 and HCC70: 48 h). The CM was collected, centrifuged, and concentrated 50-fold by a centrifugal column (UFC901008, MWCO 10 kDa) (Millipore, Billerica, MA, USA). TRAIL levels in the CM were determined by a quantitation ELISA (R&D Systems).

Lentivirus production and transduction of target cells
Lentiviral pLKO.1 vector containing a shRNA specifically targeting human TRAIL (sh-1 or sh-2) or a scrambled control (sh-scr) that does not target any human genes was obtained from Sigma. Clone IDs of TRAIL-targeting shRNAs were TRCN0000005927 and TRCN0000005928. The production of lentivirus in HEK293T cells and transduction of targeted cells were carried out as described previously.59

IHC assay
IHC assays were performed as we described previously.56,57,60 In brief, 5-μm-thick paraffin sections were deparaffinized; antigens were unmasked and immunohistochemically stained for Ki67 rabbit mAb (Cell Signaling Technology, cat. no. 9027), 1:400 dilution; cleaved caspase-3 rabbit pAb (Cell Signaling Technology, cat. no. 9661), 1:400 dilution; and TRAIL (R&D Systems, cat. no. MAB687) mouse pAb (15 μg/mL). The slides were blocked with a blocking sniper (Biocare Medical, Pacheco, CA, USA) and then incubated with a primary Ab at room temperature for 1 h. After washing with Tris-buffered saline (pH 8.0), the slides were incubated with a MACH 1 HRP Polymer detection kit (Biocare Medical) according to the manufacturer’s instructions. The staining colors were developed with a DAB Chromogen Kit (Biocare Medical). Finally, all sections were counterstained in Mayer’s hematoxylin, nuclei blued in 1% ammonium hydroxide (v/v), dehydrated, and then mounted with permanent aqueous mounting medium (Bio-Rad).

Quantification of IHC analysis
ImageJ and Image) plugin IHC profiler were applied for quantification of IHC staining analysis as reported.57 After importing images into the software, IHC profiler was used for color deconvolution, by which DAB brown stain was separated from Mayer’s hematoxylin counterstain. Then, images were changed to 8-bit grayscale type and inverted under the “Edit” menu of ImageJ. After inversion, the DAB-stained areas are bright, and unstained areas are dark. The mean intensity was measured using the “Measure” function of ImageJ. Three fields of each group were assessed.

Tumor xenograft model
Athymic nu/nu mice (Charles River Laboratories, Wilmington, MA, USA) were maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) procedures and guidelines. Two × 10^6 H460 were suspended in 100 μL of PBS, mixed with Matrigel (BD Biosciences), and injected subcutaneously into the right flank of female athymic mice. Tumor volume and mouse body weight were measured every other day. The tumor volume was calculated by the formula: volume = (length × width^2)/2, where length was the longest axis and width the measurement at a right angle to the length. When tumors reached ~100 mm^3, mice were randomly assigned into two groups (n = 5) and treated daily with sterile water (control) or 350 mg/kg of metformin by oral gavage for 14 days. The tumor growth curves were plotted using average tumor volume and followed by statistical analysis, as we described previously.56,57,60 At the end of treatment, mice were sacrificed and imaged; the tumors were dissected and measured for weight. All tumors and mouse serum were collected for further analysis.

Statistical analysis
Statistical analyses of the experimental data were performed using the two-sided Student’s t test. Data were presented as means ± SD from at least three independent experiments. Significance was set at a p value < 0.05. All statistical analyses were conducted with the GraphPad Prism (v.5.0).

SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS

Conceptualization, A.D.T., Z.H., and B.L.; methodology, S.L., E.V.P., and B.L.; supervision, A.D.T., Z.H., and B.L. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Siegel, R.L., Miller, K.D., and Jemal, A. (2020). Cancer statistics, 2020. CA Cancer J. Clin. 70, 7–30.
2. Garrido-Castro, A.C., Lin, N.U., and Polyak, K. (2019). Insights into Molecular Classifications of Triple-Negative Breast Cancer: Improving Patient Selection for Treatment. Cancer Discov. 9, 176–198.
3. Yam, C., Mani, S.A., and Moulder, S.L. (2017). Targeting the Molecular Subtypes of Triple Negative Breast Cancer: Understanding the Diversity to Progress the Field. Oncologist 22, 1086–1093.
4. Brouckaert, O., Wildiers, H., Floris, G., and Neven, P. (2012). Update on triple-negative breast cancer: prognosis and management strategies. Int. J. Womens Health 4, 511–520.
5. Engebretsen, O., Vollan, H.K.M., and Børresen-Dale, A.L. (2013). Triple-negative breast cancer and the need for new therapeutic targets. Am. J. Pathol. 183, 1064–1074.
6. Herbst, R.S., Morgenstern, D., and Boshoff, C. (2018). The biology and management of non-small cell lung cancer. Nature 553, 446–454.
7. Chu, Q.S. (2020). Targeting non-small cell lung cancer: driver mutation beyond epidermal growth factor mutation and anaplastic lymphoma kinase fusion. Ther. Adv. Med. Oncol. 12, 175883591985756.
8. Marinilli, A., Novello, S., Scaglotti, G.V., and Ramalingam, S.S. (2020). Double immune checkpoint blockade in advanced NSCLC. Crit. Rev. Oncol. Hematol. 152, 102980.
9. Upetty, D., Mandrekar, S.I., Wigger, D., Roden, A.C., and Adeji, A.A. (2020). Neoadjuvant Immunotherapy for NSCLC: Current Concepts and Future Approaches. J. Thorac. Oncol. 15, 1281–1297.
10. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. 68, 394–424.
11. Gumerov, A., Alper, M., Hucumunoglu, S., Altundag, K., Uner, A., and Guler, G. (2010). EGER expression and gene copy number in triple-negative breast carcinoma. Cancer Genet. Cytogenet. 203, 222–229.
12. Lynch, T.J., Bell, D.W., Sordella, R., Gurubhagavatula, S., Okimoto, R.A., Brannigan, B.W., Harris, P.L., Haselrat, S.M., Supko, J.G., Halinka, F.G., et al. (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N. Engl. J. Med. 350, 2129–2139.
13. Scaldriti, M., and Basela, J. (2006). The epidermal growth factor receptor pathway: a model for targeted therapy, Clin. Cancer Res. 12, 5268–5272.
14. Cao, W., Li, J., Hao, Q., Vadgama, J.V., and Wu, Y. (2019). AMP-activated protein kinase: a potential therapeutic target for triple-negative breast cancer. Breast Cancer Res. 21, 29.
15. Ciccarese, F., Zulato, E., and Indraccolo, S. (2019). LKB1/AMPK Pathway and Drug Response in Cancer: A Therapeutic Perspective. Oxid. Med. Cell. Longev. 2019, 8730816.
16. Iams, W.T., and Lovly, C.M. (2015). Molecular Pathways: Clinical Applications and Future Direction of Insulin-like Growth Factor-1 Receptor Pathway Blockade. Clin. Cancer Res. 21, 4270–4277.
17. de Miguel, D., Lemke, J., Anel, A., Walczak, H., and Martinez-Lostao, I. (2016). Onto better TRAILS for cancer treatment. Cell Death Differ. 23, 733–747.
18. Yuan, X., Gajan, A., Chu, Q., Xiong, H., Wu, K., and Wu, G.S. (2018). Developing TRAIL/TRAIL death receptor-based cancer therapies. Cancer Metastasis Rev. 37, 733–748.
19. Wong, S.H.M., Kong, W.Y., Fang, C.M., Loh, H.S., Chua, L.H., Abdullah, S., and Ngai, S.C. (2019). The TRAIL to cancer therapy: Hindrances and potential solutions. Crit. Rev. Oncol. Hematol. 143, 81–94.
20. Kretz, A.L., Trauzold, A., Hällenbrand, U., Knippuch, U., Henne-Bruns, D., von Karstedt, S., and Lemke, J. (2019). TRAILblazing Strategies for Cancer Treatment. Cancers (Basel) 11, 456.
21. Ashkenazi, A., Pai, R.C., Fong, S., Leung, S., Lawrence, D.A., Marsters, S.A., Blackie, C., Chang, L., McMurtrey, A.E., Hebert, A., et al. (1999). Safety and antitumor activity of recombinant soluble Apo2 ligand. J. Clin. Invest. 104, 155–162.
22. Herbst, R.S., Eckhardt, S.G., Kurzrock, R., Ebhinge, S., O’Dwyer, P.J., Gordon, M.S., Novotny, W., Goldwasser, M.A., Tohnya, T.M., Lum, B.L., et al. (2010). Phase I dose escalation study of recombinant human Apo2L/TRAIL, a dual pro-apoptotic receptor agonist, in patients with advanced cancer. J. Clin. Oncol. 28, 2839–2846.
23. Ouyang, X., Shi, M., Jie, F., Bai, Y., Shen, F., Yu, Z., Wang, X., Huang, C., Tao, M., Wang, Z., et al. (2018). Phase II study of dulanermin (recombinant human tumor necrosis factor-related apoptosis-inducing ligand/apo2 ligand) combined with vinorelbine and cisplatin in patients with advanced non-small-cell lung cancer. Invest. New Drugs 36, 315–322.
24. Graves, J.D., Kordich, J.I., Huang, T.H., Pisecki, J., Bush, T.L., Sullivan, T., Foltz, I.N., Chang, W., Douangpanya, H., Dang, T., et al. (2014). Apo2L/TRAIL and the death receptor 5 agonist antibody AMG 655 cooperate to promote receptor clustering and antitumor activity. Cancer Cell 26, 177–189.
25. Kelley, S.K., Harris, L.A., Xie, D., Deforge, L., Totpal, K., Bussiere, J., and Fox, J.A. (2001). Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of in vivo efficacy, pharmacokinetics, and safety. J. Pharmacol. Exp. Ther. 299, 31–38.
26. von Karstedt, S., Montinano, A., and Walczak, H. (2017). Exploring the TRAILS less travelled: TRAIL in cancer biology and therapy. Nat. Rev. Cancer 17, 352–366.
27. Zuch de Zafra, C.L., Ashkenazi, A., Darbonne, W.C., Cheu, M., Totpal, K., Ortega, S., Flores, H., Walker, M.D., Kaballof, B., Lum, B.L., et al. (2016). Antiangiogenic therapy-mediated hepatotoxicity of recombinant human Apo2L/TRAIL in the cynomolgus monkey. Cell Death Dis. 7, e2338.
28. Nebbioso, A., Clarke, N., Volte, E., Germain, E., Ambrozino, C., Bontempi, P., Alvarez, R., Schiavone, E.M., Ferrara, F., Bresciani, F., et al. (2005). Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells. Nat. Med. 11, 77–84.
29. Xu, J., Zhou, J.Y., Tainsky, M.A., and Wu, G.S. (2007). Evidence that tumor necrosis factor-related apoptosis-inducing ligand induction by 5-Aza-2’- deoxycytidine sensitizes human breast cancer cells to adriamycin. Cancer Res. 67, 1203–1211.
30. Xu, J., Zhou, J.Y., and Wu, G.S. (2006). Tumor necrosis factor-related apoptosis-inducing ligand is required for tumor necrosis factor alpha-mediated sensitization of human breast cancer cells to chemotherapy. Cancer Res. 66, 10092–10099.
31. Lee, J.O., Kang, M.J., Byun, W.S., Kim, S.A., Seo, I.H., Han, J.A., Moon, J.W., Kim, J.H., Kim, S.J., Lee, E.J., et al. (2019). Metformin overcomes resistance to cisplatin in triple-negative breast cancer (TNBC) cells by targeting RAD51. Breast Cancer Res. 21, 115.
32. Marrone, K.A., Zhou, X., Forde, P.M., Partell, M., Brahmer, J.R., Hann, C.L., Kelly, R.J., Coleman, B., Gabrielson, E., Rosner, G.L., and Ettinger, D.S. (2018). A Randomized Phase II Study of Metformin plus Paclitaxel/Carboplatin/Bevacizumab in Patients with Chemotherapy-Naive Advanced or Metastatic NonSquamous Non-Small Cell Lung Cancer. Oncologist 23, 859–865.

33. Chen, K., Li, Y., Guo, Z., Zeng, Y., Zhang, W., and Wang, H. (2020). Metformin: current clinical applications in nondiabetic patients with cancer. Aging (Albany NY) 12, 3993–4009.

34. Zhao, B., Luo, J., Yu, T., Zhou, L., Lv, H., and Shang, P. (2020). Anticancer mechanisms of metformin: A review of the current evidence. Life Sci. 254, 117717.

35. Liu, B., Fan, Z., Edgerton, S.M., Deng, X.S., Ailinova, I.N., Lind, S.E., and Thor, A.D. (2009). Metformin induces unique biological and molecular responses in triple negative breast cancer cells. Cell Cycle 8, 2031–2040.

36. Zhang, Y., and Zhang, B. (2018). TRAIL resistance of breast cancer cells is associated with constitutive endocytosis of death receptors 4 and 5. Mol. Cancer Res. 6, 1861–1871.

37. Walczak, H., Degli-Esposti, M.A., Johnson, R.S., Smolak, P.J., Waugh, J.Y., Boiani, N., Walczak, H., Degli-Esposti, M.A., Johnson, R.S., Smolak, P.J., Waugh, J.Y., Boiani, N., Timour, M.S., Gerhart, M.I., Schooley, R.A., Smith, C.A., et al. (1997). TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J. 16, 5386–5397.

38. Arrieta, O., Varela-Santoyo, E., Soto-Perez-de-Celis, E., Sánchez-Reyes, R., De la Torre-Vallejo, M., Mutü, Hernández, S., and Cardona, A.F. (2016). Metformin use and its effect on survival in diabetic patients with advanced non-small cell lung cancer. BMC Cancer 16, 633.

39. Hou, G., Zhang, S., Zhang, X., Wang, P., Hao, X., and Zhang, J. (2013). Clinical pathologic characteristics and prognostic analysis of 1,013 breast cancer patients with diabetes. Breast Cancer Res. Treat. 137, 807–816.

40. Tan, B.X., Yao, W.X., Ge, J., Peng, X.C., Du, X.B., Zhang, R., Yao, B., Xie, K., Li, L.H., Dong, H., et al. (2011). Prognostic influence of metformin as first-line chemotherapy for advanced nonsmall cell lung cancer in patients with type 2 diabetes. Cancer 117, 5103–5111.

41. Xu, H., Chen, K., Jia, X., Tian, Y., Dai, Y., Li, D., Xie, J., Tao, M., and Mao, Y. (2015). Metformin Use Is Associated With Better Survival of Breast Cancer Patients With Diabetes: A Meta-Analysis. Oncology 20, 1226–1244.

42. Strekalova, E., Malin, D., Rajanala, H., and Cyrns, V.L. (2017). Metformin sensitizes triple-negative breast cancer to prosaptopotic TRAIL receptor signaling by suppressing XIAP expression. Breast Cancer Res. Treat. 163, 435–447.

43. Park, S.H., Lee, D.H., Kim, J.L., Kim, B.R., Na, Y.J., Jo, M.J., Jeong, Y.A., Lee, S.Y., Lee, S.I., Lee, Y.Y., and Oh, S.C. (2016). Metformin enhances TRAIL-induced apoptosis by Mcl-1 degradation via Mule in colorectal cancer cells. Oncotarget 7, 59503–59518.

44. Nazim, U.M., Moon, J.H., Lee, J.H., Lee, Y.J., Seol, J.W., Eo, S.K., Lee, J.H., and Park, S.Y. (2016). Activation of autophagy flux by metformin downregulates cellular FLICE-like inhibitory protein and enhances TRAIL-induced apoptosis. Oncotarget 7, 23468–23481.

45. Zhang, T., Wang, X., He, D., Jin, X., and Guo, P. (2014). Metformin sensitizes human bladder cancer cells to TRAIL-induced apoptosis through mTOR/S6K1-mediated downregulation of c-FLIP. Oncotarget Drugs 25, 887–899.

46. Tanaka, R., Tomosugi, M., Horinaka, M., Sowa, Y., and Sakai, T. (2015). Metformin Causes G1-Phase Arrest via Down-Regulation of MiR-221 and Enhances TRAIL Sensitivity through DR5 Up-Regulation in Pancreatic Cancer Cells. PLoS ONE 10, e0125779.

47. Dong, Y., Yin, S., Li, J., Jiang, C., Ye, M., and Hu, H. (2011). Bufadienolide compounds sensitize human breast cancer cells to TRAIL-induced apoptosis via inhibition of STAT3/Mcl-1 pathway. Apoptosis 16, 394–403.

48. Shankar, E., Sivaprasad, U., and Basu, A. (2008). Protein kinase C epsilon confers resistance of MCF-7 cells to TRAIL by Akt-dependent activation of Hdm2 and down-regulation of p53. Oncogene 27, 3957–3966.

49. Ko, E., Baek, S., Kim, J., Park, D., and Lee, Y. (2020). Antitumor Activity of Combination Therapy with Metformin and Trametinib in Non-Small Cell Lung Cancer Cells. Dev. Reprod. 24, 113–123.

50. Deng, X.S., Wang, S., Deng, A., Liu, B., Edgerton, S.M., Lind, S.E., Wahdan-Alaswad, R., and Thor, A.D. (2012). Metformin targets Stat3 to inhibit cell growth and induce apoptosis in triple-negative breast cancers. Cell Cycle 11, 367–376.

51. Kagawa, S., He, C., Gu, J., Koch, P., Rha, S.J., Roth, J.A., Curley, S.A., Stephens, L.C., and Fang, B. (2001). Antitumor activity and bystander effects of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene. Cancer Res. 61, 3330–3338.

52. Seol, J.Y., Park, K.H., Hwang, C.L., Park, W.Y., Yao, C.G., Kim, Y.W., Han, S.K., Shim, Y.S., and Lee, C.T. (2003). Adenovirus-TRAIL can overcome TRAIL resistance and induce a bystander effect. Cancer Gene Ther. 10, 540–548.

53. Wahdan-Alaswad, R., Fan, Z., Edgerton, S.M., Liu, B., Deng, X.S., Arnadottir, S.S., Richer, J.K., Anderson, S.M., and Thor, A.D. (2013). Glucose promotes breast cancer aggression and reduces metformin efficacy. Cell Cycle 12, 3759–3769.

54. Chandel, N.S., Avizondis, D., Recek, C.R., Weinberg, S.E., Menz, S., Neuhaus, R., Christian, S., Haeghebarth, A., Aligres, C., and Pollak, M. (2016). Are Metformin Doses Used in Murine Cancer Models Clinically Relevant? Cell Metab. 23, 569–579.

55. Xie, J., Xia, L., Xiang, W., He, W., Yin, H., Wang, F., Gao, T., Qi, W., Yang, Z., Yang, X., et al. (2020). Metformin selectively inhibits metastatic colorectal cancer with the KRAS mutation by intracellular accumulation through silencing MATE1. Proc. Natl. Acad. Sci. USA 117, 13012–13022.

56. Huang, J., Wang, S., Lyu, H., Cai, B., Yang, X., Wang, J., and Liu, B. (2013). The anti-erbB3 antibody MM-121/SAR256212 in combination with trastuzumab exerts potent antitumor activity against trastuzumab-resistant breast cancer cells. Mol. Cancer 12, 134.

57. Lyu, H., Wang, S., Huang, J., Wang, B., He, Z., and Liu, B. (2018). Survivin-targeting miR-542-3p overcomes HER3 signaling-induced chemoresistance and enhances the antitumor activity of paclitaxel against HER2-overexpressing breast cancer. Cancer Lett. 420, 97–108.

58. Lyu, H., Yang, X.H., Edgerton, S.M., Thor, A.D., Wu, X., He, Z., and Liu, B. (2016). The erbB3- and IGF-1 receptor-initiated signaling pathways exhibit distinct effects on lapatinib sensitivity against trastuzumab-resistant breast cancer cells. Oncotarget 7, 2921–2935.

59. Wang, S., Zhu, L., Zuo, W., Zeng, Z., Huang, L., Lin, F., Lin, R., Wang, J., Lu, J., Wang, Q., et al. (2016). MicroRNA-mediated epigenetic targeting of Survivin significantly enhances the antitumor activity of paclitaxel against non-small cell lung cancer. Oncotarget 7, 37693–37713.

60. Wang, S., Huang, J., Lyu, H., Cai, B., Yang, X., Li, F., Tan, J., Edgerton, S.M., Thor, A.D., Lee, C.K., and Liu, B. (2013). Therapeutic targeting of erbB3 with MM-121/SAR256212 enhances antitumor activity of paclitaxel against erbB2-overexpressing breast cancer. Breast Cancer Res. 15, R101.

61. Zheng, J., Zhao, S., Yu, X., Huang, S., and Liu, H.Y. (2017). Simultaneous targeting of CD44 and EpCAM with a bispecific aptamer effectively inhibits intraperitoneal ovarian cancer growth. Theranostics 7, 1373–1388.