Sulfisoxazole inhibits the secretion of small extracellular vesicles by targeting the endothelin receptor A

Eun-Ju Im1, Chan-Hyeong Lee1, Pyong-Gon Moon1, Gunassekaran Gowri Rangaswamy2, Byunghan Lee2, Jae Man Lee2, Jae-Chul Lee3, Jun-Goo Jee4, Jong-Sup Bae4, Taeg-Kyu Kwon5, Keon-Wook Kang6, Myeong-Seon Jeong7,8, Joo-Eun Lee8, Hyun-Suk Jung8, Hyun-Joo Ro9, Sangmi Jun9, Wonku Kang10, Seung-Yong Seo10, Young-Eun Cho12, Byoung-Joon Song12 & Moon-Chang Baek1

Inhibitors of the secretion of cancer exosomes, which promote cancer progression and metastasis, may not only accelerate exosome biology research but also offer therapeutic benefits for cancer patients. Here we identify sulfisoxazole (SFX) as an inhibitor of small extracellular vesicles (sEV) secretion from breast cancer cells through interference with endothelin receptor A (ETA). SFX, an FDA-approved oral antibiotic, showed significant anti-tumor and anti-metastatic effects in mouse models of breast cancer xenografts, the reduced expression of proteins involved in biogenesis and secretion of sEV, and triggered co-localization of multivesicular endosomes with lysosomes for degradation. We demonstrate the important role of ETA, as target of SFX, by gain- and loss-of-function studies of the ETA protein, through a direct binding assay, and pharmacological and genetic approaches. These findings may provide a foundation for sEV-targeted cancer therapies and the mechanistic studies on sEV biology.
Metastasis is the main cause of mortality in cancer patients, but clinical options against advanced metastasis stage of cancer remain limited owing to high complexity of the biological events of metastasis, leading to inefficient drug development and poor treatment outcomes. Exosomes are 50–150 nm small extracellular vesicles (sEV) that harbor proteins, lipids, RNAs, and DNA, and thereby act as important mediators of cell–cell communications in various physiological and pathological pathways. Cancer-cell-derived sEV prepare a favorable microenvironment at future metastatic sites as well as the primary tumor. Hence, the clearance of these malicious sEV in circulating system has emerged as a novel and potentially useful therapeutic strategy for anti-metastatic drug development. Many reports have already demonstrated that the reduction of sEV secretion (or secreted sEV), achieved by using a chemical inhibitor, genetic engineering, or an antibody, can enhance the efficiency of cancer chemotherapy and inhibit cancer metastasis. However, further work is required to determine whether these inhibitors can affect the secretion of other EVs or soluble proteins, or the pathophysiological features of donor cells, as reviewed previously. Moreover, the underlying mechanisms of the already-identified inhibitors that have been demonstrated to control exosome biogenesis and secretion have still not been clearly elucidated while their safety/toxicity profiles are unknown.

Drug repurposing, the process of finding new indications for existing drugs, is a faster, cheaper, and safer drug development strategy. In this process, the new indication can be derived from the same target (on-target) or a newly-recognized target (off-target) of the original drug. A significant advantage of drug repurposing is that regulatory agency-approved drugs have already passed toxicity and safety tests in humans. One of major concerns for the development of a new drug to inhibit the secretion of sEV is the toxicity, probably caused by any partial or temporary inhibition of exosome secretion from normal cells when a drug candidate inhibits the secretion of sEV from cancer cells. We believe that drug repurposing could reduce the risk of failure by saving valuable time and efforts during the identification and development of a new inhibitor of sEV secretion as a novel anti-cancer therapeutic agent.

In this study, by screening the library of FDA-approved drugs, we identified sulfoxazole (SFX), an oral antibacterial drug, as a specific inhibitor of the biogenesis and secretion of sEV from breast cancer cells, resulting in the effective suppression of breast cancer growth and metastasis without significant toxicity. Furthermore, we found that endothelin receptor A (ETA), a member of GPCR family, is critically associated with sEV biogenesis and secretion in breast cancer cells, and that ETA is a newly-identified target (off-target) of SFX, as evidenced by gain- and loss-of-function studies of the ETA protein through pharmacological and genetic approaches. Our findings may provide a foundation for sEV-targeted cancer therapies and the mechanistic studies on sEV biology.

**Results**

**Discovery of a drug for inhibition of EV secretion.** To identify drugs that reduce sEV secretion, we developed cell-based high-throughput assay system with 1163 FDA-approved drugs, according to the flow chart for primary and secondary screenings (Fig. 1a). To accomplish this task, MDA-MB231 triple-negative human breast cancer cells were engineered to stably secrete sEV that contain CD63-GFP (MDA-MB231 CD63-GFP) and grown in 96-well plates (Supplementary Fig. 1a–h). Inhibitory effect on sEV secretion was determined by decreased fluorescence from the individual culture supernatant, which should contain sEV secreted from the cancer cells treated with each drug. During the initial primary screening, we tested drugs at 30 μM, and the top 26 drugs (that inhibited sEV secretion by up to 30%) were selected as potential candidates for the secondary assessment at 50 and 100 μM concentrations (Supplementary Fig. 2a, b). However, some drugs were not further studied based on a range of exclusion criteria, as described (Fig. 1a).

**SFX inhibits sEV secretion quantitatively and qualitatively.** We systematically analyzed the inhibitory effects of SFX on the secretion of sEV in three representative human breast cancer cell lines: MCF10A (normal), MCF7 (weakly invasive), and MDA-MB231 (highly invasive). Notably, SFX treatment quantitatively inhibited sEV secretion from MCF7 and MDA-MB231 cancer cells, as determined by the ultracentrifugation method (Supplementary Fig. 3a, b), in a dose-dependent manner without morphological change or cytotoxic effect (Fig. 1b, c and Supplementary Fig. 3e). However, no significant inhibition was observed in normal MCF10A cells, which rarely secrete sEV, suggesting that SFX shows a strong inhibitory effect on the secretion of sEV from breast cancer cells (Fig. 1b and Supplementary Fig. 3c). Consistent with this reduction of secreted sEV, the amounts of sEV proteins, such as CD9, filamentin-1, Alix, Tsg101, and CD63, were decreased (Supplementary Fig. 3f). Moreover, the number of sEV and amounts of sEV proteins were reduced in SK-MEL-28 human melanoma cancer cells, which secrete a large number of sEV (Supplementary Fig. 3g).

Next, we investigated whether SFX affects other secretion pathways, such as microvesicles (MVs) and various soluble protein secretions. SFX neither significantly affected the secretion of MVs from cells nor altered the activity of acidic sphingomyelinase (aSMase), which is involved in the formation of large MV (Fig. 1d and Supplementary Fig. 3h). In addition, the classical secretion pathway was not significantly affected by SFX treatment (Fig. 1e). Furthermore, we performed miRNA microarray (Supplementary Fig. 4a) and proteomics analyses (Supplementary Fig. 4b) of MDA-MB231 sEV, and confirmed that SFX affected the components of sEV cargo, including various miRNAs and proteins (EDIL3, HSP90, and GPC1), known to be present in sEV derived from MDA-MB231 cells (Fig. 1f, g).

**SFX inhibits breast cancer progression.** We also examined the anti-cancer effect of SFX on breast cancer cells because cancer-cell-derived sEV are known to play important roles in cancer progression and metastasis. SFX significantly halted cellular proliferation, colony formation, and cancer cell invasion/migration activities (Supplementary Fig. 5a–e), without obvious cytotoxicity (Supplementary Fig. 3c), compared with the control cells. Based on these in vitro data, we examined the anti-cancer effect of SFX by using two different mouse cancer xenograft models (Fig. 2a).

In our previous pharmacokinetic study, SFX showed excellent oral bioavailability and maintained desirable exposure levels at 200 mg kg$^{-1}$ day$^{-1}$ in mice after an oral administration. Before the validation of chemotherapeutic effect of SFX, we studied the subacute oral toxicity profile of SFX. Any pathological signs, including abnormal behaviors, body weight changes, and unexpected death, were evaluated after daily oral administrations of SFX (100, 300, and 900 mg kg$^{-1}$ day$^{-1}$) for 28 consecutive days.
Our subacute toxicity study revealed that mice appeared healthy and statistically significant differences in the body weights and various parameters of serum chemistry were not observed between SFX-treated mice and controls, suggesting that SFX up to 900 mg kg\(^{-1}\) daily administrations did not cause any obvious clinical indications in both males and females (Supplementary Fig. 6a, b).

Therefore, we evaluated the effect of SFX on the growth rates of MDAMB231 cells orthotopically implanted into female nude mice. Docetaxel (DOC), currently used intravenously as an anti-cancer agent, was used for control and combination therapy experiments. The growth of MDA-MB231 cells was significantly suppressed by oral administrations of SFX, compared to the vehicle-control group (Fig. 2b). SFX also effectively delayed the...
metastasis of mouse 4T1 breast cancer xenografts on day 29 relative to the vehicle group (Fig. 2c), thus survival rates were significantly increased (Fig. 2d). In addition, SFX treatment markedly reduced the countable colonies of metastatic foci of 4T1 cancer cells in the lung and liver (Fig. 2e) with massive growth of tumor nodules of the experimental mice, compared with those of control animals. To further study whether SFX can inhibit the secretion of sEV from transplanted cancer cells in vivo, we determined the amounts of circulating human cancer cells-derived sEV in the sera of host mice by using the specific antibody recognizing the human CD63 but not the mouse CD63 (ref. 12). Densitometric analysis revealed that the expression of human CD63 exosomal protein was significantly decreased in the SFX-treated animals and DOC-combination group compared to vehicle-exposed controls (Fig. 2f). To further validate whether the anti-cancer effect of SFX is mediated through the inhibition of
sEV secretion, we performed rescue experiments using two animal models. The anti-proliferation and anti-metastasis effects of SFX and DOC combination groups were significantly reduced by SFX treatment, suggesting that the anti-cancer effects of SFX are mostly mediated through the inhibition of sEV secretion (Supplementary Fig. 7a–e).

SFX influences ESCRT-dependent multivesicular endosome biogenesis and secretion. To investigate whether SFX inhibits sEV secretion through altered expression of the genes involved in sEV biogenesis, we performed a microarray analysis of mRNAs obtained from MDA-MB231 and SK-MEL-28 cells after SFX treatment. Strikingly, most genes down-regulated by SFX treatment in both cell lines encode the regulatory proteins associated with transport and small GTPase-mediated signal transduction (Fig. 3a, b and Supplementary Fig. 8a–c). This result strongly suggests that an upstream factor (a potentially newly-recognized off-target of SFX), which regulates the transcriptions of various genes associated with sEV biogenesis and secretion, is likely present in these cancer cells and that this upstream factor could be regulated by SFX, even though this drug was originally developed as an antibiotic to kill bacteria.

Several RABs (RAB5, RAB7, and RAB27A), VPS4B, and MITF genes were down-regulated by SFX treatment, as confirmed by both quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 3c) and western blotting (Fig. 3d). Greater amounts of RAB5 and RAB27A proteins were expressed in cancer cells, compared to normal cells (Supplementary Fig. 9a). Moreover, the expressed amounts of RAB5 and RAB27A in breast cancer cells (Fig. 3d) and SK-MEL-28 melanoma cells (Supplementary Fig. 9b) were significantly reduced after treatment with SFX in a dose-dependent manner. The ESCRT machinery is important in the multivesicular endosomes (MVE) maturation. VPS4B, an important ESCRT-related component to regulate intraluminal vesicles formation, and Alix, an ESCRTIII-binding partner, were decreased by SFX (Fig. 3d). Moreover, cellular CD63, ESCRT-dependent or -independent sEV biogenesis regulator, was also down-regulated (Fig. 3d). However, neutral sphingomyelinase (nSmase) enzyme activity, which is related to ceramide-regulated events and a target of GW4869 (ref. 26), was not affected by SFX (Fig. 3d and Supplementary Fig. 9c). In addition, SFX significantly suppressed the levels of a transcription factor MITF (Fig. 3d), which can increase the expression of late endosomal proteins, such as RAB7 and CD63, and a main sEV secretion regulator, RAB27a, in melanoma cells. Hence, the decreased levels of RAB7, CD63, and RAB27a might be due to the down-regulation of MITF by SFX. RAB-interacting lysosomal protein (RILP), which is the RAB7 effector required for transport to lysosomes, was markedly upregulated by SFX in a dose-dependent manner (Fig. 3c, d). SFX did not alter the intracellular calcium concentration when compared to dimethyl amiloride (DMA), an inhibitor of the secretion of exosome via reducing intracellular calcium levels, although sEV secretion can be regulated by a calcium-dependent mechanism (Supplementary Fig. 9d). These results suggest that SFX would influence both sEV biogenesis and secretion through the ESCRT-dependent mechanism.

SFX inhibits sEV secretion via ETA. Our data described above strongly indicate that a newly-recognized off-target of SFX exists in cancer cells to exhibit its inhibitory effect on the secretion of sEV. First, to determine whether the active site (the NH2-group on the N4 position) of SFX as an antibiotic entity is essential for the inhibition of sEV secretion, three SFX derivatives consisting of two mono-acetylated (N1-acetylated SFX, N1AS and N4-acetylated SFX, N4AS) and one diacetylated SFX (DAS) by modifying the two nitrogen atoms on SFX were synthesized (Fig. 4a). As expected, an antibiotic effect completely disappeared in two derivatives (N4AS and DAS) in which the active site (N4) was blocked by acetylation, as indicated by the minimum inhibitory concentration (MIC) against two microorganisms (MIC >512 in both Staphylococcus aureus and Escherichia coli) (Fig. 4b). Surprisingly, however, all three derivatives still reduced the sEV secretion (Fig. 4c) and the expression of Rab27a in MDA-MB231 (Fig. 4d), similar to the changes induced by SFX. These results strongly suggest that the NH2 group at the N4 site is not critical for inhibition of sEV secretion although this group is essential for antibiotic function.

To identify a direct protein target of SFX in cancer cells, we applied an in silico approach based on a Similarity Ensemble Approach (SEA), a method developed by our group. Using data from the BindingDB, SEA identified four proteins (Fig. 4e) as target candidates, which are: endothelin receptor type A (ETA), kynurenine 3-monooxygenase (KMO), carbonic anhydrase 13 (CA13), and angiotensin II type 1a receptor (AGTR1). To determine whether each candidate protein can bind directly to SFX, a pull-down assay was conducted using biotinylated SFX with the lysate or membrane fraction of MDA-MB231 cells. Our analysis revealed that only the antibody recognizing ETA showed a signal in elution parts from the membrane fraction but not the lysate (Fig. 4f). Consistent with these results, the expressions of Rab27a, Rab5, and Rab7 proteins were significantly decreased by siRNA-mediated knockdown of the ETA gene (Fig. 4g), but not by suppression of the other three genes, in MDA-MB231 cells (Fig. 4h). To demonstrate direct binding of SFX with ETA, radioactive binding assay was performed and an IC50 value of 11.0 μM SFX was subsequently calculated (Fig. 4i). This binding assay result was consistent with an earlier result reported over two decades ago, where SFX could be an ETA selective endothelin antagonist.

It has been reported that the ETA activation by its agonists (endothelin-1, ET1, and endothelin-2, ET2) promotes cancer progression through a network of cellular pathways and interactions with the tumor microenvironment. Consistently, we observed that ETA and its agonists were strongly expressed in highly invasive MDA-MB231, compared to MCF7 and MCF10A
cells (Fig. 4j). Small-molecule antagonists of ETA have been developed as anti-cancer drugs32; however, to our knowledge, there has been literally no report on the relationship between ETA and sEV secretion.

We therefore studied the underlying mechanisms by which ETA interferes with sEV secretion. First, to achieve this, MDA-MB231 cells were stably transfected with an ETA shRNA (Knockdown, K/D) or an ETA ORF (over-expression, OE). Similar to SFX treatment, ETA K/D significantly decreased the secretion of sEV (Fig. 5a), with a reduction of RAB27A, RAB5, and RAB7 proteins (Fig. 5b). Conversely, ETA-OE significantly increased the secretion of sEV (Fig. 5a), with elevated levels of RAB5 and RAB7 proteins (Fig. 5b). To further investigate the relationship between ETA and sEV secretion, we studied the
effects of several ETA-specific agonists ET1 and ET2 or antagonists (zibotentan, BQ123, and PD159701) on sEV secretion. ET2 significantly increased sEV secretion, which was slightly elevated by ET1, compared to control (Fig. 5c and Supplementary Fig. 10a). In contrast, the three antagonists tested in this study potently inhibited sEV secretion with respect to the number and the protein amounts of secreted sEV (Fig. 5c and Supplementary Fig. 10a). Moreover, the number and the protein amounts of the secreted sEV were still decreased by the combined treatment of SFX and ET1 or ET2 agonist (Fig. 5d and Supplementary Fig. 10b), indicating a potency of SFX in decreasing the sEV secretion despite the presence of ETA agonists. Similar to the SFX-treated cells (Fig. 1e), ETA antagonists did not significantly alter the classical cytokine secretion pathway (Fig. 5e). Consistently, the mRNA expression levels of RAB27a, RAB5, RAB7, RILP, MITF, and VPS4B, as shown in SFX-treated cells (Fig. 3c), were down-regulated by ETA antagonists (Supplementary Fig. 10c). Additionally, we confirmed the relationship between ETA and cancer progression in a breast cancer model. ETA antagonists, zibotentan, BQ123, and PD156707, significantly repressed the invasion and migration activities of cancer cells, MDA-MB231 and 4T1 in vitro (Supplementary Fig. 11a–e). Furthermore, the potent anti-proliferation effects of ETA antagonists through the inhibition of sEV secretion were observed in mice inoculated with MDA-MB231-ETA K/D cells or pretreated with ETA antagonists (Fig. 5f, g and Supplementary Fig. 11a). Taken together, these results suggest that ETA is one of the critical elements of the complex machinery for sEV secretion and constitutes an upstream protein for regulating sEV in MDA-MB231 cells.

**ETA antagonists increase fusion of MVE with lysosomes.** To further investigate morphological changes inside cancer cells after SFX treatment, we performed transmission electron microscopy (TEM) analysis of MDA-MB231 cells at 6, 12, and 24 h post-exposure. Interestingly, the structure of degraded autophagic vacuoles (AVs) following lysosomal fusion could be observed 12 h after SFX treatment (Fig. 6a). The most abundant type of vesicles occupying the cytosol of MDA-MB231 cells appeared to be “empty”. In addition, much larger structures occupied extensive areas of the cytosol of MDA-MB231 and MCF7 cells at 24 h after SFX treatment, but the control groups looked normal (Fig. 6a). More importantly, lysosomes or autolysosomes densely filled with multi-lamellar structures were also observed, while the number of these sub-organelle structures and the expression of lysosome-associated membrane proteins 1 (LAMP-1), which is critical in lysosome biogenesis and autophagy, were increased in SFX-treated cells (Fig. 6b).

It has been reported that the balance between autophagy and sEV biogenesis is important for the maintenance of cellular homeostasis. Hence, we investigated whether the relationship between MVE and lysosomes could be affected by SFX. We observed that lysosome activity was strongly increased by SFX (Fig. 6c, d) and the fusion of MVE with lysosomes was accelerated by SFX treatment relative to the control (Fig. 6e). Importantly, these observations have led to the hypothesis that SFX initially influences ESCRT-dependent MVE biogenesis, followed by the imperfect MVEs moving to the lysosomes for degradation.

Next, we investigated whether the ETA signaling pathway is related to the fusion of lysosomes and MVEs. Similar to the effects in SFX-treated cells, autolysosomes or AVs were detected in antagonist-treated MDA-MB231 cells (Fig. 7a). Moreover, confocal microscopy verified co-localization of MVEs with lysosomes, the increase in lysosomal activity, and the up-regulation of LAMP-1 in antagonist-treated or ETA-siRNA-transfected MDA-MB231 cells (Fig. 7b–e). These results strongly suggest that one of the major targets of SFX in MDA-MB231 cells is ETA, which could be a GPCR protein newly-recognized for the regulation of sEV biogenesis and secretion.

**Discussion**

Breast cancer is the most prevalent cancer in women and the leading cause of death worldwide, up to half-a-million deaths annually, despite surgical treatments combined with advanced radiotherapy and chemotherapy. Metastasis is a major cause for deaths of many patients with various types of cancer, including breast cancer. Metastatic dissemination of breast cancer can occur in a late stage of cancer progression as well as in preinvasive stages of tumor progression, by various systemic factors secreted from the tumors. The exosomes and/or sEV, emerging players of systemic factors, play an important role in establishing pre-metastatic niche in future metastatic organs and furthermore in dictating organotropic metastasis by integrin repertoire of sEV in breast cancer cells. Therefore, early detection as well as prevention of metastasis by conventional treatments and/or sEV secretion could become very important in reducing the cancer-related pathologies and deaths.

Despite significant advancement in exosome/sEV research in recent decades, no drugs targeting to inhibition of sEV secretion have been approved for human usage. In this study, we identified SFX as an inhibitor of the secretion of sEV by high-throughput screening of a library of FDA-approved drugs. SFX affects the expression of many genes associated with the pathways of sEV biogenesis and secretion in cancer cells (Fig. 3). Our results suggest that an upstream target, as a newly-recognized off-target, of this old drug might regulate the sEV-associated pathways in breast cancer cells. From our computational approach, ETA was selected as the prime target of SFX with the highest priority and was confirmed by a direct binding study, genetic and pharmacology approaches. Therefore, we showed that SFX exhibits a potential anti-cancer effect that is mediated through ETA dependent-inhibition of sEV biogenesis/secretion.

For physiologically relevance, we simply compared the concentration of SFX as an inhibitor of sEV secretion with that of SFX as an antibiotic in vitro and in vivo. As shown in Supplementary Fig. 11, each MIC of SFX was 16 and 32 μg ml−1 in *E. coli* and *S. aureus* in this study, converted to 60 and 120 μM, respectively, and thus the concentration (up to 100 μM in this...
Experiments were performed with 95% confidence in the inhibitory constant, and usage. Therefore, we believe that the concentrations of SFX used were 8 times lower than the maximal dosage as an antibiotic in human studies. The study can be physiologically relevant in humans, we used a formula for dose translation from animal to human studies. The minimal inhibitory concentration (MIC) of SFX and its derivatives against Staphylococcus aureus ATCC 29213 and Escherichia coli ATCC 25922. Measurement of the number of sEV and the amounts of sEV proteins. The number of sEV and the amounts of sEV proteins. Left, the number of sEV. Right, the amounts of sEV. The Gut microbiota is a critical factor for many pathophysiological conditions such as immune reaction and inflammatory processes. SFX, an antibiotic, may decrease gut microbiome density and/or modify its composition. Several bacteria phyla are shown in this study, as an inhibitor of sEV secretion in cancer cells and as an anti-cancer agent in two different mouse models of breast cancer xenografts, could be physiologically relevant.
Fig. 5 Identification of ETA as the novel regulator of sEV biogenesis. a Measurements of the number of sEV secreted (left) and the amounts of sEV proteins (right) from WT, ETA-overexpressed (ETA-OE), and ETA knockdown (K/D) MDA-MB231 cells. \( n = 3 \). b Immunoblot of various proteins in ETA-OE and ETA-K/D MDA-MB231 cells. \( n = 3 \). c Measurement of the number of sEV secreted and the amount of sEV proteins from ETA agonist- or antagonist-treated MDA-MB231 cells. \( n = 3 \). d Measurement of the number of sEV and the amounts of sEV proteins from MDA-MB231 cells treated with 10 nM ET1 or 10 nM ET2 in the presence of 100 μM SFX. \( n = 3 \). e Measurement of soluble proteins secreted from MDA-MB231 cells in the presence of PD156707 or zibotentan for 24 h. The unit of IL8, IL2, CXCL13, CCL2, and TGFβ1 is pg ml\(^{-1}\). The unit of CX3CL1 is ng ml\(^{-1}\). \( n = 3 \). f Tumor mass volume of in BALB/c nude female mice inoculated with MDA-MB231 or MDA-MB231 ETA K/D. Bar, 1 cm. \( n = 8 \) per group. g Left top, immunoblot of human CD63 in circulating cancer-cell-derived sEV from MDA-MB231 or MDA-MB231 ETA K/D-bearing mice (\( n = 8 \) per group) where similar amounts of protein/lane were verified by Ponceau S staining (Bottom). Right, densitometric analysis of the relative protein intensity of CD63. Experiments were performed with 95% confluent cells. Significance was determined using an unpaired two-tailed Student’s t-test. ***\( p < 0.001 \), **\( p < 0.005 \) and *\( p < 0.05 \). Error bar, SD. Source data (a–g) are provided as a Source Data file.
to critically important in cancer development during dysbiosis\textsuperscript{40,41}. Based on the recent reports, gut microbiota is considered as a holistic hub point for cancer development, thus antibiotics-mediated microbiome modulation can be a novel anti-cancer strategy\textsuperscript{42,43}. Antibiotics-mediated gut microbiome changes might be partially responsible for the anti-cancer effect of SFX. However, antagonists (not antibiotics) to ETA, a target of SFX, also showed the anti-proliferation effect through the inhibition of sEV secretion (Supplementary Fig. 5a). Therefore, these results suggested that the inhibitory effect of SFX on cancer cell
proliferation and metastasis mostly depends on the inhibition of sEV although its effect may come from other properties.

Recent reports suggested that the stimulation of GPCR by its agonists initiates the signaling cascade to regulate exosome formation and secretion. For instance, the activation of GPR143 by its ligand, L-DOPA, halted the secretion of exosomes for intercellular communication in the eye44. Activated group I mGluRs increase the secretion of exosomes by calcium release from the endoplasmic reticulum via the secondary messenger, IP345. The activation of histamine H1 receptor in HeLa cells also increases the secretion of exosomes through the promotion of MVB-PM fusion46. Some GPCRs, such as A2A receptors, can be transferred via exosomes from the source to the recipient target cells47.

Fig. 7 Antagonists against the endothelin receptor induce fusion of MVEs with lysosomes in MDA-MB231 cells. a TEM analysis of ETA-antagonist-treated MDA-MB231 cells. Scale bar, 5 μm. b Measurement of lysotracker (red) intensity in rapamycin-treated or antagonist (Zibotentan and PD156707)-treated MDA-MB231. Lysotracker intensity measurement >100 cells per group. c Top, Immunoblot of LAMP-1 protein in antagonist (Zibo, BQ-123, and PD156707)-treated MDA-MB231. Bottom, Immunoblot of LAMP-1 protein in ETA-siRNA-transfected MDA-MB231 and in ETA-K/D MDA-MB231. n = 3.

d Left, Image of MDA-MB231 treated by SFX. Green, CellLight-Lysosome-GFP (GFP-LAMP1). Red, CellLight-late endosome/MVE-RFP (RFP-RAB7). Scale bar, 2 μm. Right, Quantitative colocalization rates of lysosomes and MVE in MDA-MB231 treated with Zibotentan, PD156707, or BQ123. Colocalization puncta count >65 cells per group. e Images of ETA K/D MDA-MB231 or WT MDA-MB231 cells. Different colors represent the same markers as described in d. Scale bar, 2 μm. Experiments were performed with 95% confluent cells. Significance was determined using an unpaired two-tailed Student’s t-test. ***p < 0.001, **p < 0.005 and *p < 0.05. Error bar, SD. Source data (b, d) are provided as a Source Data file.
and angiogenesis. Thus, ETRs have emerged as key targets and angiogenesis. Thus, ETRs have emerged as key targets induced various effects in cancer cells, such as growth, metastasis, particularly, the activation of the ETA pathway, by ET1 or ET2, GPCRs, consist of two receptor subtypes, ETA and ETB. In endothelin receptors (ETRs), which are Family A (Class 1) with sEV biogenesis and secretion in breast cancer cells. The results suggested that ETA is related with autophagy regulation in H9C2 myoblasts. Therefore, we additionally studied whether the ETA antagonists can interfere with the ETA function, suppress the secretion of sEV, and change the components of sEV cargo from cancer cells, contributing to anti-cancer effects. Therefore, these ETA-related SFX results are novel and very important for clinical implications although we only tested the effects of SFX and ETA antagonists in breast cancer cells. We expect that these compounds can also beneficially affect other types of cancer cells possibly through preventing sEV biogenesis and secretion, although this needs to be confirmed by future experiments.

**Methods**

**Cells and cell culture.** All breast cancer and other cells were obtained from the American Type Culture Collection (ATCC) and grown at 37 °C under humidified atmosphere with 5% CO2, and 95% air using the recommended culture medium. MCF7, MDA-MB231, and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (HyClone) with 10% fetal bovine serum (FBS) and 1% antibiotics. SK-MEL-28 cells were cultured in Minimum Essential Medium with Earle’s Balanced Salts (MEM/EBSS; HyClone). For drug treatment experiments, cells were washed and incubated with FBS-free medium after cell cultures reached 90% confluence, except invasion, migration, soxazole (SFX, S6377), 5-(N,N-dimethyl)amino)urea hydrochloride (DMA, A4562), GW4869 (D1692), Rapamycin (R8781), FTY720 (SML0700), ET1 (E7764), BQ123 (B150), PD156707 (PZ0141), zibotentan (SML1550), and Rafinomycin (R1793). ET2 (#1164) was obtained from Tocris Bioscience. Docetaxel (DOC) was a gift from professor Keon-Wook Kang (Seoul National University).

**Chemicals.** An FDA-approved drug library was purchased from Selleckchem (L1300) and used for in vitro screening study. The following chemicals were purchased from Sigma Aldrich, including Sulforaphane (SFX, S6377), 5-((N,N-dimethyl)amino)urea hydrochloride (DMA, A4562), GW4869 (D1692), Rapamycin (R8781), FTY720 (SML0700), ET1 (E7764), BQ123 (B150), PD156707 (PZ0141), zibotentan (SML1550), and Rafinomycin (R1793). ET2 (#1164) was obtained from Tocris Bioscience. Docetaxel (DOC) was a gift from professor Keon-Wook Kang (Seoul National University).

In this study, we first demonstrated that ETA is associated with sEV biogenesis and secretion in breast cancer cells. The endothelin receptors (ETRs), which are Family A (Class 1) GPCRs, consist of two receptor subtypes, ETA and ETB. In particular, the activation of the ETA pathway, by ET1 or ET2, induces various effects in cancer cells, such as growth, metastasis, and angiogenesis. Thus, ETRs have emerged as key targets for cancer therapy, and small-molecule antagonists, including zibotentan and BQ123, have been tested in humans for cancer drug development.

The increased degradation of MVEs via the autophagy–lysosome pathway was demonstrated by ultrastructural analysis when breast cancer cells were treated with SFX or specific ETA antagonists (Figs. 6a and 7a). Another report showed that ETA is related with autophagy regulation in H9C2 myoblasts. Therefore, we additionally studied whether the inhibitory effect of SFX and ETA antagonists depend on the autophagy pathway, even if the elevation of autophagosome structures was not clearly observed in cancer cells by treatment of these drugs. Through the expression of LC-3BII protein, one of the critical markers in macroautophagy pathway, and the additional LC3B puncta study, SFX and ETA antagonists did not fully induce the LC3B-dependent autophagy pathway. Furthermore, these drugs still inhibited sEV release in LC3B K/D cells. These results suggested that the degradation of MVEs by SFX or ETA antagonists might not be related to the LC3B-dependent autophagy pathway. However, we could not exclude the possibility that other non-canonical autophagy pathways might function partially in cancer cells following treatment with ETA antagonists. Therefore, further work is required to identify the detailed mechanism of the degradation pathway via ETA antagonists for the reduction of sEV biogenesis and secretion.

Here, we found that ETA, selected as a newly-recognized off-target of SFX, is associated with sEV biogenesis and secretion, and this finding will accelerate the development of a novel class of drugs through mechanistic studies on the regulation of sEV biogenesis or secretion (Fig. 8). Furthermore, SFX and ETA antagonists can interfere with the ETA function, suppress the secretion of sEV, and change the components of sEV cargo from cancer cells, contributing to anti-cancer effects. Therefore, these ETA-related SFX results are novel and very important for clinical implications although we only tested the effects of SFX and ETA antagonists in breast cancer cells. We expect that these compounds can also beneficially affect other types of cancer cells possibly through preventing sEV biogenesis and secretion, although this needs to be confirmed by future experiments.
Fluorescence-based high-throughput screening assay. MDA-MB231 cells were transfected with pCt-C2D63-GFP (pCMV, ExosomeSecretome, C2D63 Tetraspanin and CD63 plasmid (System Bioscience) to construct MDA-MB231-C2D63-GFP stable cell line. For high-throughput screening assay, MDA-MB231-C2D63-GFP (+) cells (1 × 10^4/well) were seeded in 96-well culture plates and fluorescence from each supernatant was measured at 485 nm (excitation) and 538 nm (emission) using a microplate reader (GeminiEM; Molecular Devices, San Jose, CA).

FACS. For green fluorescence protein (GFP) detection, cells were re-suspended in FACS buffer (phosphate-buffered saline (PBS) with 5% fetal calf serum). The GFP intensities in MDA-MB231 were determined by FACS Calibur (BD Bioscience).

Proliferation and cytotoxicity assay. Concentration- and time-dependent effects on cell proliferation and cytotoxicity were measured using the MTT [3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide] assay. In the cytotoxicity assay, cells were seeded in a 24-well plate at a density of 2 × 10^4 cells/well and grown overnight. These cells were subsequently incubated with 1163 individual FDA-approved drugs (Selleckchem) for 24 h under serum-depleted condition. The culture supernatants were then transferred into 96-well black plates and fluorescence from each supernatant was measured at 485 nm (excitation) and 538 nm (emission) using a microplate reader (GeminiEM; Molecular Devices, San Jose, CA).

Isolation of MVs and sEV. The MVs were isolated by established methods described in the literature. Briefly, the individual supernatants from MCF7, MDA-MB231, and SK-MEL-28 cells were serially centrifuged at 300 × g for 6 min and 1000 × g for 5 min. The MV pellets were re-suspended with PBS and examined with the nanoparticle tracking analysis (NTA). To isolate sEV, individual supernatants obtained from the indicated cells were serially centrifuged at 300 × g for 3 min, 2500 × g for 20 min, and 10,000 × g for 30 min. Then the supernatants were centrifuged using a 0.2 μm syringe filter, and centrifuged at 100,000 × g for 90 min. The resulting supernatants were centrifuged at 200,000 × g for 90 min again. The MV pellets were re-suspended with PBS or SFM and centrifuged at 120,000 × g for 90 min. The purified sEV pellets were re-suspended with PBS or 1× RIPA buffer for further experiments.

For miRNA microarray assay, ExoQuick-TC exosome precipitation solution (System Bioscience) was used for sEV isolation with the method optimized by our group. Briefly, MDA-MB231 cells were treated with vehicle or SFX in FBS-free media, and incubated for 24 h. Then, conditioned media were serially centrifuged at 300 × g for 3 min, 2500 × g for 20 min, and 10,000 × g for 30 min. Then, conditioned media were filtered using 0.2 μm filter. These medium were incubated with ExoQuick-TC at 4 °C overnight. After centrifugation, sEVs were centrifuged at 1500 × g for 30 min and washed with PBS three times. Finally, sEVs were suspended in nuclease-free water (Promega) for total RNA extraction.

Proteomics. sEV proteins were digested by trypsin and the tryptic peptides were analyzed by nano-ultra-high-performance LC (UPLC) (Waters) and tandem mass spectrometry using a Q-Tof Premier (Waters) tandem mass spectrometer by using 0.2 μm syringe filter and centrifuged at 10,000 × g for 40 min. Then, the supernatants were centrifuged using a 0.8 μm syringe filter and centrifuged at 100,000 × g for 90 min. The resulting supernatants were centrifuged using a 0.2 μm syringe filter, and centrifuged at 100,000 × g for 90 min again. The purified sEV pellets were re-suspended with PBS or 1× RIPA buffer for further experiments.

The following primary antibodies were used: anti-CD63 (ab8418, 1:1000; Abcam), CD9 (ab2212, 1:1000; Abcam), CD81 (ab109201, 1:1000; Abcam), AXL (ab59632, 1:1000; Abcam), Tsg101 (ab36871, 1:1000; Abcam), Filotillin-1 (#2353, 1:2000; CST), LAMP-1 (ab25630, 1:1000; Abcam), RILP (ab128661, 1:1000; Abcam), Rab-7 (ab8922, 1:1000; Abcam), Rab-7 (ab215927, 1:1000; Abcam), Rab27A (ab50533, 1:1000; Abcam), EDIL3 (ab88667, 1:1000; Abcam), Glycopain-1 (PA5-20055, 1:1000, Thermofisher), HSP90a (#8165, 1:1000; CST), ASMAse (ab36871, 1:1000; CST), nSMase2 (ab68735, 1:1000; Abcam), LC3B (NB600-1384, 1:1000; Novus Biologicals), Endolfin receptor type A (ab117521, 1:2500; Abcam), Angiotensin II type I receptor (ab38807, 1:1000; Abcam), Rab5 (ab37811, 1:1000; Abcam), Rab7 (ab58533, 1:1000; Abcam), Rab8 (ab58538, 1:1000; Abcam), Rab11f2a (ab315956, 1:1000; Abcam), Kynurenine 3-monooxygenase (ab130959, 1:1000; Abcam), Endolfin-1 (ab2786, 1:1000; Abcam), Endolfin-2 (sc292348, 1:1000; SCBT), Carbonic anhydrases-13 (ab18801, 1:1000; Abcam). For the measurement of cytokine, the following ELISA kits from R&D systems were used: IL8 (D8008C, IL2 (D2020), IL6 (D6050), CXCL1 (DCX310), CXCL3 (DCX310), CCL2 (DCP00), and TGFβ1 (DR1008).

RNA extraction and qRT-PCR. Total RNA from different cells was extracted by using TRIzol reagent (#15596026; Invitrogen) according to the manufacturer’s recommendation. A total of 2 μg RNA was reverse-transcribed using the RT-primex (K2041; Bioneer). qRT-PCR gene expression analysis was performed in three biological replicates using gene-specific qRT-PCR oligonucleotides. RT-PCR reactions were monitored on an ABI stepOne Plus instrument (Applied Biosystems) using the SYBR premix (50 μl) in a 75 μl reaction with 10% raw data were converted into peak lists using Mascot® Power Tools (Matrix Science). Mass spectral data were analyzed using Mascot® software to identify proteins or peptides. The Mascot® results were further confirmed by in house BLAST search. The resulting protein identifications from Mascot® analysis were further confirmed using Gene Ontology (GO) and KEGG databases. The CEL files were imported into GeneSpring® (AgcCc) to perform statistical analysis. A threshold value of 1.5 was used to identify differentially expressed proteins. The data were assumed to be normally distributed and analyzed with Student’s T-test with p < 0.05. The results were further confirmed using Gene Ontology and KEGG databases. The CEL files were imported into GeneSpring® (AgcCc) to perform statistical analysis. A threshold value of 1.5 was used to identify differentially expressed proteins. The data were assumed to be normally distributed and analyzed with Student’s T-test with p < 0.05. The results were further confirmed using Gene Ontology and KEGG databases.
VP54B (ab102687;1:1000; Abcam), MITF (#12590, 1:1000, CST), and Lamin B (13435; 1:1000; CST). For the detection of specific human cell-derived sEV, the following antibodies were used: the anti-human CD63 (SH-EOX-M02, 1:1000; Cosmo Bio Co., Ltd).

**miRNA expression analysis.** sEV miRNA was extracted according to the manufacturer’s instruction (total exosome RNA isolation kit, #4477455; Thermofisher). Briefly, after ultracentrifugation, the sEV pellets were resuspended with the excess glycerol, and then aliquoted and stored at −80 °C. The total RNA-containing aqueous phase was then transferred to filter cartridge for miRNA isolation. Extracted miRNA was washed using the miRNA wash solution and eluted using the elution buffer. TagMan® MicroRNA system was used for miRNA expression analysis. First, RT master mix [extracted miRNA, dNTPs (with dTTP), reverse transcriptase and RNAse inhibitor] was loaded into the thermal cycler to perform reverse transcription for 1 h at 37 °C. Samples were then added with Ultra microtube UltraClear (200 μL) at 30 min at 42 °C, and 5 min at 4 °C. Then, the fluorescence signals of specific miRNAs by real-time PCR system were detected and recorded. The reaction mixture contained: TagMan® MicroRNA assay reagent (Thermofisher), RT product, and TagMan® universal PCR master mix (#4304437; Thermofisher). U6 siRNA was used as reference. miRNA sequence used for miRNA expression analysis is summarized in Supplementary Table 2.

**RNAi analysis.** Transient transfection was performed by using Lipofectamine 3000 (1:1000; Thermofisher) according to the manufacturer’s instructions. Briefly, cells were seeded in 60 mm dishes at 70% confluence, and then were transfected with 2.5 μg siRNA with Lipofectamine 3000 under serum-reduced condition. After 1 day, culture media were replaced with a serum-containing medium, and then with 2.5 μg plasmid (pMAX-MiR-11, pMAX-MiR-17, pMAX-MiR-21, pMAX-MiR-155, pMAX-MiR-122) after 48 h post-transfection. siRNAs of ETA, AGTR1, KMO, and CA13 were purchased from Dharmaco; negative control siRNA was purchased from Bioneer (AccuTarget™ Negativ Control siRNA, SN-1001-CFG). The siRNA sequence used for RNAi interference analysis is summarized in Supplementary Table 3.

**Phosphoglyceromine (sMas) activity measurements.** Acidic phosphoglyceromine activity (acidic sMas, ab189354; Abcam) and phosphoglyceromine activity (neutral sMas, ab138877; Abcam): acidic and neutral phosphoglyceromine activities were determined by using an assay kit. Briefly, cells were co-cultured with SFX for 24 h and then lysed with 1× mammalian lysis buffer. These samples were then reacted with the acidic sMas assay reagents according to the manufacturer’s recommended protocol. After incubation, fluorescence from each sample was obtained by using a microplate reader (GEMINI; Molecular Devices) at Ex/Em = 540/590 nm (the cut off was 570 nm). The fluorescence in a blank well was used as a negative control; GW4869 and FTY720 were used for positive controls of neutral SMas and acidic sMas, respectively.

**Intracellular calcium concentration measurements.** [Ca2+]i concentration was measured using fluo-3/AM (F1242; Invitrogen). For the experiments, MDA-MB231, MCF7, and SK-MEL-28 cells were loaded with 5 μM fluo-3/AM for 30 min at 16 °C. Subsequently, the cells were scanned using a microscope (HT7700; Hitachi) operated at 120 kV.

**Synthesis of a sulfisoxazole-based affinity probe.** First, we synthesized 4-((4-(N-(3,4-dimethoxysulfanyl)-3-ylsulfonyl)phenyl)amino)-4-oxobutanoic acid (SP-1). To a solution of SFX (557 mg, 2.08 mmol), Et3N (0.58 mL, 4.16 mmol) and DMAP (127 mg, 1.04 mmol) in CHCl3 (4 mL) were added triethylamine to methyl 4-chloro-4-oxobutan-2-amine (0.257 mL, 2.08 mmol) at 0 °C. The reaction mixture was then stirred at room temperature for 2 h and washed with 5% HCl and sat. NaHCO3 (2 × 50 mL). The reaction mixture was extracted three times with ethyl acetate and the combined organic phase was washed with brine, dried over anhydrous MgSO4, and concentrated under reduced pressure. The residue was purified by flash column chromatography on a silica gel (MeOH/CH2Cl2/ACO3 = 10:1:0.1) to yield the amide (310 mg, 39%). 1H-NMR (600 MHz, CDCl3), δ 8.49 (s, 1H), 7.66 (d, 2H, J = 8.4 Hz), 7.26 (d, 2H, J = 7.2 Hz), 2.68 (d, 2H, J = 7.2 Hz), 2.01 (s, 3H), 1.65 (s, 3H); HRMS (FAB+) - mass calculated for C6H11NO2S2 [M + H]+, 382.0995; found, 382.1072. Additionally, to the solution of the amide compound (105 mg) in tetrahydrofuran (10 mL), lithium hydroxide monohydrate (105 mg) in H2O (10 mL) was added at 0 °C. The reaction mixture was stirred for 48 h and concentrated under reduced pressure. The residue was purified by flash column chromatography on a silica gel (MeOH/CH2Cl2/ACO3 = 1:10:1) to yield the amide (401 mg, 92%). 1H-NMR (600 MHz, CDCl3), δ 8.49 (s, 1H), 7.66 (d, 2H, J = 8.4 Hz), 7.22–7.30 (m, 4H), 2.76 (t, 2H, J = 8.4 Hz), 2.18 (m, 2H, J = 7.2 Hz), 1.97 (m, 2H, J = 7.2 Hz), 1.65 (s, 3H); HRMS (FAB+) - mass calculated for C6H11NO2S2 [M + H]+, 382.0995; found, 382.1072. Additional 3,27

**Transmission electron microscopy (TEM) analysis.** For TEM analyses to evaluate cell morphologies and sub-organelle structures, drug-treated or control MDA-MB231 CD63 GFP fusion cells were pelleted by centrifugation, and pelleted cells were fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer. After washing several times with 0.1 M cacodylate buffer, cells were dehydrated by gradient series of ethanol (50%, 70%, 80%, 90% ethanol for 10 min each step, 100% ethanol was followed by propylene oxide for twice) and then embedded with Ultra microtome UltraClear (Leica) and stained with uranyl acetate and lead citrate. Sections were examined with energy filtering TEM (LEO-912AB OMEGA; Carl Zeiss) at the Korean Basic Science Institute Choncuheon.

For TEM analyses to detect sEV, MDA-MB231 and MCF7 cells were pelleted by serial ultracentrifugation, and the pellets were deposited on pure carbon-coated EM grids. After staining with 1% uranyl acetate, the grids were dried at room temperature and viewed at ×12,000 magnification using a Biotribion transmission electron microscope (HT7700; Hitachi) operated at 120 kV.

**Immunoﬂuorescence staining and confocal microscopy.** Cells were seeded onto glass coverslips at 2 × 105 cells/well in a six-well conical chamber overnight and were treated with SFX for 24 h. For the detection of lysosomal activity, 500 nM Lysotracker Red CMHR1 (CellLight Lysosomes-GFP, BacMam 2.0, C10596; Molecular Probes) and GFP-LAM-1 vector (CellLight lysosome-GFP, BacMam 2.0, C10596; Molecular Probes) and grown overnight. Fluorescence images were obtained with a Zeiss LSM5 laser scanning fluorescence confocal microscope. Colocalization puncta was measured by ImageJ.

**Radioligand binding assay.** Radioligand binding assay was performed in Eurofins Panlab Discovery Service Center (Taiwan). To evaluate the binding activity of SFX, assay was performed under the following conditions. Human recombinant CHO-K1 cells were used as the source, and ligand concentration was 0.030 nm [125I]-endothelin-1. IC50 values were determined by a non-linear, least-squares regression analysis using MathiQTM (ID Business Solutions Ltd, UK). The inhibition constants (Ki) were calculated by the equation of Cheng and Prusoff using the observed IC50 of the tested compound, the concentration of radioligand employed in the assay, and the historical values for the KD of the ligand (obtained experimentally at Eurofins Panlab, Ltd). The Hill coefficient (nH), defining the slope of the competitive binding curve, was calculated by using MathiQTM.

**ARTICLE NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-019-09387-4 | www.nature.com/naturecommunications**
concentrated under reduced pressure. The residue was purified by flash column chromatography on a silica gel (MeOH/CH2Cl2 = 1:10) to afford the sulfisoxazole affinity probe (4) (25 mg, 49%). 1H-NMR (600 MHz, CD3OD) δ 7.67 (d, 2H, J = 8.4 Hz, 7.67, 4.39–4.37 (m, 1H), 4.20 (m, 1H), 3.53–3.50 (m, 16H), 3.43 (d, 4H, J = 5.4 Hz), 3.27–3.25 (m, 4H), 3.10 (m, 1H), 2.83 (dd, 1H, J = 12.6 and 4.2 Hz), 2.62–2.59 (m, 3H), 2.49 (t, 2H, J = 6.6 Hz), 2.12 (t, 2H, J = 7.2 Hz), 2.04 (s, 3H), 1.67 (s, 3H), 1.33–1.35 (m, 7H), 1H (4H), 161.7, 153.9, 143.2, 134.7, 127.8, 118.9, 107.4, 70.1, 70.0, 69.8, 69.2, 61.9, 60.2, 55.6, 39.0, 38.9, 35.3, 31.6, 30.1, 28.3, 25.1, 9.2, 5.0, HRMS (FAB+): m/z calculated for C23H24N4O2S2 [M + H]^+ 856.3507; found, 856.3580. Chemical structure of sulfisoxazole-based affinity probe is summarized in Supplementary Fig. 13.

Target identification. To predict the functional targets of SFX, an in silico approach termed Similarity Ensemble Approach (SEA) was used. Of the data registered in BindingDB (56), only those measured through the protein-based assays were extracted. Tamocito coefficient (Tc) calculated based on Morgan circular fingerprint using the RD Kit (http://www.rdkit.org) was used to quantify the chemical similarity between a test molecule and SFX. The value of Tc was between 0 and 1, indicating that the two molecules with the value closer to 1 shared greater chemical similarity. After retrieval of the protein targets that were small-molecule modulators with the higher Tcs to SFX, SEA was used to compare the similarities. In SEA, the pairwise Tc values between two molecules are summed to form 2Tc. The value of the 2Tc in a test molecule and the distribution of 2Tc in a set of small molecules were drawn with a bar and histogram for comparison.

Biotin-based pull-down assay. The biotin-based pull-down assay was performed according to the manufacturer’s instructions (21115; Thermo Scientific). Briefly, a streptavidin ligand-bound agarose gel was immobilized on the biotin-tagged SFX, which was then washed away. Next, membrane proteins of MDA-MB231 cell lysates were incubated with biotin-tagged SFX-bound agarose gel to isolate the prey proteins that were selectively eluted with a low-pH elution buffer.

Antimicrobial susceptibility testing. The minimal inhibitory concentrations (MICs) of SFX and three structural derivatives were determined by microdilution in Mueller Hinton agar (22520; Difco Laboratories) according to the guidelines of the Clinical and Laboratory Standard Institute (CLSI, 2015). Staphylococcus aureus (ATCC 29213) and Escherichia coli (ATCC 25922) were used as quality control strains.

Toxicology study and blood chemistry. Seven-week-old male and female ICR mice were supplied by Hanabio Corporation (Seoul, Korea). Animals were bred under SPF conditions and maintained in barrier housing during the experiment. They were maintained in animal care facilities at Chung-Ang University (22 °C ± 1 °C, humidity 60% ± 10%, and a 12 h/12 h light/dark cycle). Nutritionally bred under SPF conditions and maintained in barrier housing during the experiment. Animals were supplied by Hanabio Corporation (Seoul, Korea). Animals were weighed every 2 days during the experiment. Individual body weight changes to remove cell debris. Each wound was imaged at 0 h, and again after 24 h.

Cellular assays. For wound healing assay, MDA-MB231 cells were seeded in a 24-well plate and grown to confluence overnight. The next day, the monolayer was wounded by repeated scratches with a 200 µl pipette tip, and the media was changed to remove cell debris. Each wound was imaged at 0 h, and again after 24 h. The average wound healing was assessed by the average of three measurements of the wound area.

A transwell migration assay was performed using the Costar transwell system (CLS3364; Corning). Briefly, MDA-MB231 cells (2 × 10^6) were suspended in 200 µl serum-free medium and seeded in the lower chamber and 500 µl medium was added to the lower chamber. At 4 h after the cells were seeded, media in both the upper insert and lower chambers were removed. Cells that migrated into the lower chamber through the 8-µm pore membrane were stained by crystal violet solution and then the migrated cells were visualized using a microscope (×4 magnification).

The cell invasion study was performed using a cell culture inserts chamber (CLS3364; Corning). Chamber was coated with the basement membrane Matrigel (100 µl of 20% matrigel/filter; #354248; Corning). The cancer cells were seeded at 10,000 cells/well into upper chamber, and invading cells were fixed and stained with crystal violet. The membranes were mounted on glass slides, and images of the cells were captured using a microscope (×4 magnification).

2D colony-forming assay. Cells were seeded in a six-well plate (1 × 10^5)well and treated with SFX immediately. Then, cells were washed using PBS and treated with the indicated concentrations of SFX every 24 h. After 6 days, cell colonies were fixed with glutaraldehyde (6.0% v/v) and stained with crystal violet (0.5% w/v). Stained colonies were dissolved in 25% methanol, incubated for 10 min, and then measured at 590 nm with a spectrometer (Multiskan^TM GO Microplate spectrometer; Thermofisher).

Cancer xenograft studies in mice. All animal research was performed in accordance with protocols approved by the Kyungpook National University (KNU) Institutional Animal Care and UCommittees (IACUCs). Approve number: 2017–0146. In the proliferation study, MDA-MB231-luci (+) cells suspended in PBS were orthotopically injected into the left fat pad of 5-week-old female BALB/c nu/nu mice. SFX (200 mg kg^-1 day^-1) was orally administered for 14 days or zibotentan (10 mg kg^-1 day^-1) was intraperitoneally administrated for 14 days. Docetaxel (8 mg kg^-1 week^-1) was intravenously administered once a week for two weeks. Tumor growth was measured every 2 days by using a caliper. After 14 days, mice were euthanized, and luciferase signals were measured using an IVIS imaging system. In rescue experiments, MDA-MB231-luci (+) were intravenously injected at the left fat pad of 5-week-old female BALB/c nude mice to monitor the cancer progression.

In the metastasis study, 4T1-luci (+) cells suspended in PBS were orthotopically injected into the left fat pad of 5-week-old female BALB/c wild-type mice; other procedures were as described for the proliferation study. Tumor attenuation was monitored every week for 5 weeks using an IVIS imaging system. In each group, five mice were used for the survival test. In addition, Zibotentan (10 mg kg^-1 day^-1) or BQ732 (5 mg kg^-1 day^-1) were intraperitoneally administrated for 21 days to validate the anti-cancer effect of ETA antagonists. Final attenuation of tumor metastasis was monitored using an IVIS imaging system. In rescue experiments, 4T1-luci (+)-derived SEV (5 µg) were intravenously injected once every 2 days for 11 times.

Statistical analysis. Unpaired two-tailed students t-test was used for experiments comparing two sets of data. The error bars in the graphical data represent means ± standard deviation. All in vitro experiments were performed in triplicates unless otherwise stated. p values less than 0.05 were considered to denote statistically significant differences. *, **, and *** denote p value of <0.05, 0.005 and 0.001, respectively. NS denotes not significant. Data were analyzed using PRISM 6 software (GraphPad Software, Inc.).

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability All microarray data that support the findings of this research have been deposited in the Gene Expression Omnibus (GEO) and are accessible through the GEO accession number GSE117991 (mRNA microarray) and GSE124320 (miRNA microarray). The proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE with the dataset identifier PXD012689. The source data underlying Figs. 5 (b–d), 2 (b–d), 3 (c, d), 4b–d, i), 5 (a–c, g), 6 (b, d), 7 (b, d) as well as those underlying Supplementary figs. 1 (b, d–h), 2 (a, b), 3 (c–e, h), 5 (a–c), (a, 7 (c–e), 9 (a–d), 10 (a–c), and 11 (b, c, e) are provided as a Source Data file. All other relevant data of this study are available from the corresponding authors upon reasonable request. A reporting summary is available as a Supplementary Information file.

Received: 21 August 2018 Accepted: 6 March 2019 Published online: 27 March 2019

References
1. Steeg, P. S. Targeting metastasis. Nat. Rev. Cancer 16, 201–218 (2016).
2. Wan, L., Pantel, K. & Kang, Y. Tumor metastasis: moving new biological insights into the clinic. Nat. Med. 19, 1450–1464 (2013).
3. Colombo, M., Raposo, G. & Thery, C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu. Rev. Cell Dev. Biol. 30, 255–289 (2014).
4. Costa-Silva, B. et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. Nat. Cell Biol. 17, 816–826 (2015).

5. Peinado, H. et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat. Med. 18, 883–891 (2012).

6. Kaiser, J. Malignant messengers. Science 352, 164–166 (2016).

7. Chen, G. et al. Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. Nature 560, 382–386 (2018).

8. E. L. Andaloussi, S., Mäger, L., Breakefield, X. O. & Wood, M. J. Extracellular vesicles: biology and emerging therapeutic opportunities. Nat. Rev. Drug Discov. 12, 347–357 (2013).

9. Datta, A. et al. Manumycin A suppresses exosome biogenesis and secretion via targeted inhibition of Ras/Raf/ERK1/2 signaling and hnRNP H1 in castration-resistant prostate cancer cells. Cancer Lett. 408, 73–81 (2017).

10. Datta, A. et al. High-throughput screening identified selective inhibitors of exosome biogenesis and secretion: a drug repurposing strategy for advanced cancer. Sci. Rep. 8, 8161 (2018).

11. Bobrie, A. et al. Rab27a supports exosome-dependent and -independent mechanisms that modify the tumor microenvironment and can promote tumor progression. Cancer Res. 72, 4920–4930 (2012).

12. Nishida-Aoki, N. et al. Disruption of circulating extracellular vesicles as a novel therapeutic strategy against cancer metastasis. Mol. Ther. 25, 181–191 (2017).

13. Tkach, M. & Thery, C. Communication by extracellular vesicles: where we are and where we need to go. Cell 164, 1226–1232 (2016).

14. Ashburn, T. T. & Thor, K. B. Drug repositioning: identifying and developing new uses for existing drugs. Nat. Rev. Drug Discov. 3, 673–683 (2004).

15. Hong, Y. L., Hossler, P. A., Calhoun, D. H. & Meshnick, S. R. Inhibition of recombinant Pneumocystis carinii dihydropteroate synthetase by sulfa drugs. Antimicrob. Agents Chemother. 39, 1756–1763 (1995).

16. Bianco, F. et al. Acid sphingomyelinase activity triggers microparticle release from glial cells. EMBO J. 28, 1043–1054 (2009).

17. Moon, P. G. et al. Identification of developmental endothelial locus-1 on circulating extracellular vesicles as a novel biomarker for early breast cancer detection. Clin. Cancer Res. 22, 1757–1766 (2016).

18. McCready, J., Sims, J. D., Chan, D. & Jay, D. G. Secretion of extracellular hsp90alpha via exosomes increases cancer cell motility: a role for plasminogen activation. BMC Cancer 10, 294 (2010).

19. Melo, S. A. et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. Nature 523, 177–182 (2015).

20. Sempere, L. F., Kato, J. & Fabbrri, M. Exosomal microRNAs in breast cancer towards diagnostic and therapeutic applications. Cancers (Basel) 9, pii: E71 (2017).

21. Oh, K., Baek, M. C. & Kang, W. Quantitative determination of sulfisinoloxide and its three N-acetylated metabolites using HPLC-MS/MS, and the saturable role of autophagy. J. Pharm. Biomed. Anal. 129, 32–38 (2016).

22. Henne, W. M., Buchkovich, N. J. & Emr, S. D. The ESCRT pathway. Dev. Cell 21, 77–91 (2011).

23. Adell, M. A. Y. et al. Recruitment dynamics of ESCRT-III and Vps4 to endosomes and implications for reverse membrane budding. Elife 6, e31652 (2017).

24. Hurley, J. H. & Odorizzi, G. Get on the exosome bus with ALIX. Nat. Cell Biol. 14, 654–665 (2012).

25. van Niel, G. et al. The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis. Dev. Cell 21, 708–721 (2011).

26. Trajkovic, K. et al. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science 319, 1244–1247 (2008).

27. Chiaverini, C. et al. Microphthalmia-associated transcription factor regulates RAB27A gene expression and controls melanoma transport. J. Biol. Chem. 283, 12635–12642 (2008).

28. Savina, A., Furlan, M., Vidal, M. & Colombo, M. I. Exosome release is antagonized by pharmacophore directed screening. EMBO J. 21, 708–721 (2002).

29. Kohan, D. E., Cleland, J. G., Rubin, L. J., Theodorescu, D. & Barton, M. Clinical trials with endothelin receptor antagonists: what went wrong and where we can improve? Life Sci. 91, 528–539 (2012).

30. Ge, Y. et al. Cardiomyocyte-specific deletion of endothelin receptor A rescues aging-associated cardiac hypertrophy and contractile dysfunction: role of autophagy. Basic Res. Cardiol. 108, 335 (2013).

31. Rosano, L. & Bagnato, A. Endothelin receptors as novel targets in tumor therapy. J. Transl. Med. 16, 708 (2018).

32. Sempere, L. F., Keto, J. & Fabbrri, M. Exosomal microRNAs in breast cancer towards diagnostic and therapeutic applications. Cancers (Basel) 9, pii: E71 (2017).

33. Cho, Y. E. et al. Increased liver-specific proteins in circulating extracellular vesicles as potential biomarkers for drug- and alcohol-induced liver injury. PLoS ONE 12, e0172463 (2017).

34. Cho, Y. E. et al. In-depth identification of pathways related to cisplatin-induced hepatotoxicity through an integrative method based on an informatics-assisted label free protein quantitation and microarray gene expression approach. Mol. Cell Proteomics 11, M111 010884 (2012).

35. Moon, P. G. et al. Proteomic analysis of urinary exosomes from patients of early IgA nephropathy and thin basement membrane nephropathy. Proteomics 11, 2459–2475 (2011).

36. Cheng, Y. & Prusoff, W. H. The relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108 (1973).

Acknowledgements

We thank David Lyden for comments and additional support; Stephen J. Gould for critical suggestions; we also thank members of this laboratory for helpful discussion and supports. This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) of Korea funded by the Ministry of Science & ICT (2017M3A9G8083382).

Author contributions

E.-J.L. designed, performed, and analyzed most experiments, and wrote the manuscript. C.-H.L. performed confocal analysis. P.-G.M. conducted ELISA and TEM analysis. G.R.G. and B.-H.L. conducted mouse work. J.-C.L. conducted MIC analysis. J.G.J. conducted in silico modeling. M.-S.J., J.-E.L., H.-S.J., H.-J.R., and S.I. conducted TEM analysis. W.K. conducted mouse toxicology study. S.-Y.S. synthesized chemical derivatives. J.-S.B., J.-M.L., T.-K.K., K.-W.K., Y.-E.C. discussed the hypothesis and contributed to data interpretation. B.-J.S. contributed to data interpretation and wrote the manuscript. M.-C.B. designed and analyzed experiments, wrote the manuscript, and conceived the idea and supervised the study.
