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Research Article

Metixene is an incomplete autophagy inducer in preclinical models of metastatic cancer and brain metastases

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ABSTRACT

A paucity of chemotherapeutic options for metastatic brain cancer limits patient survival and portends poor clinical outcomes. Using a central nervous system (CNS) small-molecule inhibitor library of 320 agents known to be blood-brain barrier permeable and approved by the U.S. Food and Drug Administration, breast cancer brain metastases vulnerabilities were interrogated to identify an effective agent. Metixene, an antiparkinsonian drug, was identified as a top therapeutic agent that was capable of decreasing cellular viability and inducing cell death across different metastatic breast cancer subtypes. This agent significantly reduced mammary tumor size in orthotopic xenograft assays and improved survival in an intracardiac model of multiorgan site metastases. Metixene further extended survival in mice bearing intracranial xenografts and in an intracarotid model of multiple brain metastases. Functional analysis revealed that metixene induced incomplete autophagy through N-Myc Downstream Regulated 1 (NDRG1) phosphorylation thereby leading to caspase-mediated apoptosis in both primary and brain metastatic cells, regardless of cancer subtype or origin. CRISPR Cas9 knockout of NDRG1 led to autophagy completion and reversal of the metixene apoptotic effect. Metixene is a promising therapeutic agent against metastatic brain cancer, with minimal reported side effects in humans, which merits consideration for clinical translation.

KEYWORDS: Autophagy; Brain metastasis; Breast cancer; NDRG1; Drug screening; Metixene.
INTRODUCTION

Metastasis is a hallmark of cancer that remains a primary cause of cancer-related deaths (1). Brain metastases are the most common type of central nervous system (CNS) malignancies. They often manifest with neurological impairment that portends poor quality of life and limits survival outcomes. It is estimated that 10-30% of all patients with cancer will develop brain metastases at some point in their disease (2). The incidence of peripheral cancer spread to the CNS may be increasing, though, as a result of improved diagnostics and better control of extracranial disease through systemic therapies (3, 4).

Breast cancer is one of the major causes of brain metastases (5). It is the most common cancer among women, impacting 2.3 million per year worldwide (6). It is also the most common cause of cancer-related deaths in women, with rates increasing in nearly every region globally (7). The incidence of brain metastases depends on the breast cancer molecular subtype, with human epidermal growth factor receptor 2 (HER2)-positive and triple-negative breast cancers having rates of brain metastases as high as 50% (8, 9). The introduction of the anti-HER2 monoclonal antibody, trastuzumab, for patients with HER2-positive breast cancer improved survival outcomes in systemic disease (10). Yet, HER2-positive breast cancers are at the greatest risk for intracranial-specific metastases. When metastatic cancer cells colonize the brain, they have developed resistance to trastuzumab (11, 12). In addition, the poor penetration of trastuzumab and other systemically administered drugs across the blood-brain barrier (BBB) limits their effectiveness against breast cancer brain metastases (BCBM) (13, 14).

A major limitation in treating patients with BCBM is the lack of clinical trials and new therapeutic options. With more than 10,000 registered clinical trials for breast cancer, less than 1
percent include patients with brain metastases (15). Moreover, out of the trials that include patients with brain metastases, less than 15% are being completed, and less than 25% publish their results (15). As such, novel, effective agents against the disease that can be swiftly translated to the clinic are urgently needed.

In this study, we conducted a CNS small-molecule inhibitor screen with agents that are known to be permeable through the BBB and have been approved by the Food and Drug Administration (FDA) on BCBM cell lines that are sensitive and resistant to trastuzumab. Metixene, an antiparkinsonian drug, was identified as a potent agent that induces caspase-mediated cell death in primary breast and brain metastatic cancer cells. *In vivo*, metixene significantly decreased tumor size in primary breast cancer and significantly increased survival in multiple preclinical models of metastatic breast cancer and brain metastases. The functional proteomic analysis highlighted signaling pathways that are involved in cellular stress response and macroautophagy. Macroautophagy involves the formation of double membrane autophagosomes that bind to lysosomes to form single membrane autolysosomes, where cargo can be degraded through lysosomal hydrolases (16). The accumulation of such autophagic structures can lead to autophagic stress that induces caspase-mediated apoptosis (17). Further exploration proved that metixene induced NDRG1-mediated incomplete autophagy in which accumulated autophagic vesicles do not degrade and trigger caspase-mediated apoptosis in HER2-positive and triple-negative metastatic breast cancer and brain metastases.
RESULTS

CNS small-molecule inhibitor screen identifies agents with anti-cancer effects in BCBM

A blinded screen was performed to identify potential hits for therapeutic intervention against BCBM using the Prestwick CNS Drug Library consisting of 320 structurally diverse drugs that are FDA approved, BBB permeable, and known for their pharmacological effects in the CNS. The first screen was on BT-474Br, a HER2-positive BCBM cell line, to prioritize cytotoxic agents at a standard concentration of 25 μM. The screen yielded five different therapeutic agents that caused a >95% reduction in cellular viability in vitro (Table 1). As HER2-positive BCBM are believed to be resistant to trastuzumab (18), the second screen was conducted on the HCC1954 metastatic breast cancer cell line that is inherently resistant to therapy (19) (Table 1). This revealed metixene, an antiparkinsonian agent, as a top candidate in both screens. To evaluate the effect of metixene on a panel of various metastatic breast cancer cell lines, the IC₅₀ was determined in two HER2-positive cell lines (BT-474Br and HCC1954) and five triple-negative breast cancer cell lines (MDA-MB-231Br, HCC1806, HS578T, HCC3153, and SUM159) after 72 hours of treatment. Metixene was shown to be a potent inhibitor of BCBM regardless of breast cancer subtype with an IC₅₀ ranging from 9.7 μM to 31.8 μM (Table 2 and Supplementary Figure 1). Following the determination of an appropriate range for treatment of BCBM cells, the anti-cancer activity of metixene in the brain metastatic HER2-positive BT-474Br and the triple-negative MDA-MB-231Br cell lines were evaluated using increasing concentrations of the drug at different time points. After both 24- and 48-hours of treatment, a dose response was evident in cellular viability assays for both cell lines (Figure 1A).
Metixene induces caspase-mediated apoptosis in cancer cells

Flow cytometry results indicated that metixene was inducing cell death in cancer cells (Supplementary Figure 2). To clarify the mechanism of cell death, the caspase cascade was assessed. A caspase 3/7 assay was performed to check for caspase-mediated apoptosis, which showed a significant increase in activity that was dependent on dose and time in the BT-474Br and MDA-MB-231Br cells (Figure 1B). Immunofluorescence was used to confirm that metixene induced caspase-3 cleavage in the two BCBM cell lines (Figure 1C and D). The caspase 8 and 9 assays were also used to measure the effect of metixene in BCBM cells. After 24 hours, caspase 9 activity was significantly elevated at 10 μM in the BT-474Br cells (P=0.0055), and at 15 μM in the BT-474Br cells (P<0.0001) and the MDA-MB-231Br cells (P<0.0001) (Figure 1E and F). This data indicates that the intrinsic pathway of apoptosis was activated as a result of metixene treatment.

Metixene decreases tumor size and improves survival in murine models of established metastatic breast cancer

To ascertain if metixene could exert in vivo therapeutic activity, HCC1954 cells were orthotopically implanted into the mammary fat pads of nude mice. When the tumors reached the size of 5 mm, the mice were randomized into three groups: control (25% captisol, n=12), metixene (0.1 mg/kg, n=8); and metixene (1.0 mg/kg, n=8) and treated intraperitoneally three times a week (Figure 2A). After 6 weeks, the fat pad tumors were collected and analyzed (Figure 2B). Tumor weight was significantly decreased with metixene treatment at 0.1 mg/kg (P<0.0001) and 1.0 mg/kg (P<0.0001) (Figure 2C). Tumor volume was also significantly reduced with metixene treatment at 0.1 mg/kg (P=0.0043) and 1.0 mg/kg (P=0.0004) (Figure
Cleaved caspase-3 staining of tumor sections showed a significant increase in percent of cleaved caspase-3-positive cells in metixene-treated groups at 0.1 mg/kg (P=0.001) and 1.0 mg/kg (P=0.0002) (Figure 2E and F).

In an experimental model of metastatic breast cancer involving multiple organ sites, triple-negative MDA-MB-231 cells were intracardially injected into the left ventricle of immunodeficient mice. Following a seven-day interval post-tumor cell inoculation, the mice were randomly assigned to two distinct cohorts: a control group (25% captisol, n=5) and a metixene-treated group (1.0 mg/kg, n=5). The treatment regimen involved intraperitoneal administration three times per week (Figure 2G). While control mice exhibited a median survival of 31 days, those receiving metixene demonstrated a significantly extended median survival of 38 days (P=0.0197) (Figure 2H). Histological examination of both control and metixene-treated mice revealed a reduction in tumor burden within the lungs and large intestines of the metixene-treated group. Furthermore, no discernible tumor formation was observed in the stomach and liver of these treated mice (Supplementary Figure 3).

To assess the pharmacokinetics and bioavailability of metixene, our study aimed to quantitatively analyze metixene concentrations in both blood plasma and brain tissue. Metixene was administered via intraperitoneal injection, and the mice were randomly divided into five distinct groups, each corresponding to a specific timepoint post-administration. At each designated timepoint, comprising a cohort of three mice, the animals were humanely sacrificed. Subsequently, 500 µl of blood was collected from each mouse, and their brain tissues were harvested for analytical purposes (Figure 2I). The analysis revealed a peak concentration of metixene in both blood plasma and brain tissue occurring approximately one-hour post-injection, reaching an average of 9.7 ng/ml in the plasma and 101.6 ng/mg in the brain tissue. Metixene
was completely cleared from the plasma within three hours post-administration and from brain tissue within a 12-hour timeframe (Figure 2J).

**Metixene improves in vivo survival in preclinical models of metastatic brain cancer**

To ascertain if metixene exerted therapeutic activity against breast cancer in the brain, HER2-positive BT-474Br cells were stereotactically implanted in the brains of nude mice. After 10 days, the mice were randomized into two groups: control (25% captisol, n=7) and metixene (1.0 mg/kg, n=8), and were treated intraperitoneally three times a week (Figure 3A). The metixene-treated mice had a 23% increase in median survival, with a median survival period of 64 days, in contrast to the median survival of control mice of 52 days (P=0.0008) (Figure 3B). This finding was consistent with the measurement of the luciferase signal in the brain when the BT-474Br cells were fluorescently labeled and treated with metixene relative to controls after six weeks of treatment (P=0.039) (Figure 3C). Immunohistochemistry staining of the brains of treated mice confirmed that metixene significantly induced caspase-3 cleavage in the established HER2-positive BCBM in vivo (Figure 3D and Supplementary Figure 4A).

In a multiple brain metastases model, triple-negative MDA-MB-231Br cells were injected into the carotid artery of nude mice. Ten days after injection of tumor cells, mice were randomized into two groups: control (25% captisol, n=5) and metixene (1.0 mg/kg, n=6), and treated intraperitoneally three times per week (Figure 3E). In comparison to controls that exhibited a median survival of 44 days, metixene-treated mice showed a median survival of 67 days. Therefore, metixene significantly increased survival by 52% in comparison to controls (P=0.037) (Figure 3F). Histological examination of the brains harvested from control-treated mice showed multiple metastases, vascular co-option, and micro-metastases formation (Figure
Histological comparison of brains of control and metixene treated mice showed decreased tumor burden in metixene-treated mice (Supplementary Figure 5). Immunohistochemistry staining showed that cleaved caspase-3 was significantly higher in the BCBM tissue of the metixene-treated mice in vivo (Figure 3H and Supplementary Figure 4B).

Toxicity studies conducted by treating nontumor bearing nude mice (n=4) with the same metixene regimen (1 mg/kg; intraperitoneally; three times weekly) for more than 3 months confirmed the absence of toxic or adverse effects when compared with untreated mice (n=4). Difference in body weight of treated and control mice was not significant (Supplementary Figure 6A). Evaluation of organ tissue, such as the brain, heart, lung, liver, pancreas, kidneys, spleen, stomach, and intestines revealed no differences in weight or anatomical architecture between the treated and untreated mice (Supplementary Figure 6B and C).

Metixene induces cellular stress and activates macroautophagy signaling pathways

To clarify the mechanism of action of metixene, an agent with known antimuscarinic and antihistaminic properties (20), the expression of muscarinic and histamine receptors on the breast cancer cell lines was determined and correlated with the IC$_{50}$. RNA levels of either muscarinic (M1-M5) or histamine (H3) receptors did not correlate with the induced cytotoxicity of metixene (Supplementary Figure 7), indicating that the anti-cancer activity of metixene is not a function of muscarinic or histaminic receptors. As such, to identify in an unbiased fashion the underlying mechanism of action of metixene, BT-474Br cells were treated with metixene and a reverse-phase protein array (RPPA) analysis was conducted to assess the changes in signaling pathways (Figure 4A). Four biological replicates for each condition (control, 12 hours, and 24 hours) were analyzed and revealed that autophagy is a primary biological mechanism regulated by metixene.
(P < 0.05) *(Figure 4B and Supplementary Figure 8)*. Among the proteins that exhibit significant changes (with p<0.001) in expression or phosphorylation, 52 and 26 proteins, respectively were significantly altered *(Figures 4C and D)*. These proteins implicated the MAPK, PI3K/Akt, apoptosis and autophagy, mTOR signaling pathways that are involved in the process of macroautophagy. Other proteins implicated in DNA damage and repair, P53, and cell cycle control pathways were also affected, alluding to cellular stress induction as a result of metixene treatment *(Figures 4E and F; Supplementary Table 1 and 2)*.

Next, MDA-MB-231Br cells were treated with metixene for different durations to further characterize the mechanisms of autophagic signaling in brain metastatic cells. A strategy for monitoring autophagy is the detection of LC3 conversion (LC3I to LC3II) by immunoblot since the amount of LC3II can be associated with the number of autophagosomes *(21, 22)*. Conversion of LC3I to LC3II was shown to increase as early as ten minutes after metixene treatment *(Figure 5A)*. Thereafter, autophagy flux changes were tested after ten minutes, one hour, and three hours of treatment. LC3II levels in untreated control cells were compared to cells treated with the autophagy inhibitor chloroquine (20 μM, an autophagy flux inducer), metixene (10 μM), or the combination of metixene and chloroquine. Cells treated with the combination of metixene and chloroquine had higher LC3II levels than that of untreated cells or cells treated with either agent alone *(Figure 5B)*. This finding was further confirmed with immunofluorescence staining of LC3 *(Figure 5C)*. Quantification of area of LC3 puncta per cell showed a significant increase (P<0.05) upon the addition of chloroquine to metixene in both BT-474Br and MDA-MB-231Br cells *(Figure 5D and E)*. Immunofluorescence staining of LC3 in metixene treated cells in a dose-dependent manner also showed a significant increase in the area of LC3 puncta per cell as the dose increased in two BCBM cell lines *(Supplementary Figure 9)*. The addition of
wortmannin, a PI3K inhibitor and an early inhibitor of autophagy flux (23), to metixene led to a decrease in LC3II expression (Supplementary Figure 10). Altogether, these data indicate that metixene is inducing autophagy signaling in cancer cells.

Metixene induces incomplete autophagy in cancer cells

Using a system in which LC3, the marker of autophagosomes, is fused to both GFP and mCherry, we found that metixene-treated BT-474Br and MDA-MB-231 cells contained numerous autophagosomes (yellow/green), which do not bind to lysosomes that degrade their contents (red) (Figure 6A). Electron microscopy of metixene-treated cells at 10 μM showed accumulation of double membranad autophagic vesicles in the cytoplasm of BT-474Br and MDA-MB-231Br cells (Figure 6B). Dose-dependent treatment of the two cell lines showed a significant increase in LC3II/I levels in BT-474Br (P<0.0001) and MDA-MB-231Br (P=0.0487). Similar results were confirmed in primary breast cancer cell lines (Supplementary Figure 11). Furthermore, levels of p62, an autophagy cargo protein that binds other proteins for selective autophagy, increased significantly in BT-474Br (P=0.0033) and MDA-MB-231Br (P=0.0043), indicating that that the content of autophagic vesicles is not being degraded and that autophagy is incomplete (Figure 6C). Immunohistochemistry staining of brain samples of control and metixene-treated mice showed that LC3B was significantly higher in metixene-treated samples in both BT-474Br intracranial models and MDA-MB-231Br intracarotid injection models (Figure 6D and E; Supplementary Figure 12).

NDRG1 regulates metixene-induced incomplete autophagy and caspase-mediated apoptosis

Upon further analysis of the RPPA data, the phosphorylation of N-Myc Downstream Regulated 1 (NDRG1) was noted to be relevant in four major pathways related to autophagy after 24 hours of
metixene treatment. The expression of NDRG1 and its phosphorylated form, pNDRG1, increased significantly in a dose-dependent manner in both BT-474Br and MDA-MB-231Br after treatment with metixene (Figure 7A and B). To confirm the role of NDRG1 in autophagy, we generated NDRG1 knockout (KO) cells using CRISPR Cas9 in MDA-MB-231Br cells. The cells were then treated with metixene in a dose-dependent manner and compared to cells transfected with a vector control (VC). Western blots showed that LC3II levels decreased significantly in NDRG1 KO cells at 10 μM (P=0.0009) and 15 μM (P=0.0001). Furthermore, the significant decrease in p62 levels in NDRG1 KO cells at 5 μM (P=0.0014), 10 μM (P<0.0001), and 15 μM (P=0.0073) indicated that autophagy is being completed in comparison to NDRG1 VC cells (Figure 7C). This showed that NDRG1 expression and phosphorylation regulate metixene-induced incomplete autophagy.

We then checked whether NDRG1 is further involved in the apoptotic cell death induced by metixene. We conducted a cell viability assay to check the effect of NDRG1 KO versus VC upon metixene dose-dependent treatment. Results showed that NDRG1 KO cells were significantly more viable than control cells at 20 μM (P<0.0001) (Figure 7D). A caspase 3/7 assay further confirmed that apoptosis was significantly reduced in the NDRG1 KO cells at 10 μM (P=0.0078) and 15 μM (P<0.0001) (Figure 7E). To check the cascade of events upon metixene administration, we treated BT-474Br and MDA-MB-231Br in a time-dependent manner. Incomplete autophagy, as indicated by LC3I-to-II conversion and accumulation of p62 precedes NDRG1 phosphorylation and eventual caspase-3 cleavage (Figure 7F). Altogether, this shows that metixene induces incomplete autophagy and intrinsic apoptosis through NDRG1 expression and phosphorylation. Upon NDRG1 KO, autophagy is completed, and the caspase-mediated pathway of apoptosis is not activated. Immunohistochemistry staining of pNDRG1 in
murine brain samples showed that metixene treatment led to significantly higher expression of pNDRG1 than in controls (Figure 7G and Supplementary Figure 13). This mechanism of action was consistent in other types of metastatic brain cancer, such as lung cancer and melanoma (Supplementary Figure 14).

NDRG1, a marker of cellular stress response, is known to be induced under conditions of cellular stress (24), including DNA damage. The other signaling pathways underscored by the RPPA analysis suggests a complex interplay between autophagy, DNA damage, and cell death. The downregulation of DNA damage repair and checkpoint proteins, such as ATRX, ATR, BAP1, and 53BP1, may exacerbate cellular stress induced by metixene, upregulating NDRG1 in the process and leading to incomplete autophagy. The accumulation of damaged DNA and organelles, in turn, may lead to cell cycle arrest or apoptosis. These mechanisms can also affect cancer stem cells, as they are particularly sensitive to genomic instability and cellular stress (25). Stem cell markers, such as SOX2 and OCT4, were downregulated after metixene treatment (Supplementary Figure 15).

DISCUSSION

The current management of patients with metastatic brain cancer typically include resection and/or radiation with median survival of less than 14 months (26). The development of novel therapeutics for patients with metastatic cancer that also have brain metastases is hindered by the slow pace of drug development, expense, and high attrition rates during clinical trials (27). Drug repositioning or repurposing in cancer is a strategy that aims to reuse existing medical agents developed for other diseases as anti-cancer treatments (28-30). The Repurposing Drugs in Oncology project was launched in 2014 to reposition reputable and well-characterized drugs as
new agents in oncology based on evidence from published literature (31). In brain metastases, novel anti-cancer therapies must have the ability to cross the BBB and to instigate cytotoxic effects against metastatic cancer cells that are often resistant to standard therapies. We conducted a screening on trastuzumab-sensitive and resistant brain metastatic cells using a library of agents that are known to cross the BBB, which identified metixene as a translational candidate. The dose at which survival in mice harboring established brain metastases was shown to be significant was 1 mg/kg given three times weekly. In humans, metixene can be administered at a starting dose of 2.5 mg TID and then increased gradually depending on clinical response to a total of 15-60 mg daily in divided doses (32). The human equivalent dose calculation based on body surface area would be 0.0813 mg/kg (33). In a person who weighs between 50-60 kg, a metixene dose of 4-5 mg per day should be tolerable and realistic. This may allow a truncated Phase 1 component since metixene has usually mild anti-muscarinic side effects (20).

Repositioning of antiparkinsonian drugs has shown to be effective against cancer metastasis. Specifically, Benztropine, a second-line drug for the treatment of Parkinson’s disease, significantly inhibited in vivo tumor growth, reduced the number of circulating tumor cells, and decreased the rate of metastasis in a tumor allograft model in mice (34). Carbidopa, another anti-parkinsonian medication, has also been shown to be effective against pancreatic cancer by the inhibition of indoleamine-2,3-dioxygenase-1 and the potentiation of aryl hydrocarbon receptor signaling (35). Epidemiological studies have shown that the relative risk of cancer development among patients treated with Parkinson’s disease was less than half that in the normal population (36, 37). A systematic review and meta-analysis of the literature exploring cancer risk among patients with Parkinson’s disease further showed that the aggregate risk for cancer in patients with Parkinson’s disease was 0.73 (38).
Metixene was found to be a modulator of autophagy signaling in metastatic breast cancer and brain metastases. Recent genetic investigations and emerging functional research demonstrate the existence of shared and intersecting pathways in both Parkinson's disease and cancer (39). Parkinson's disease is a protein-misfolding disease, where misfolded α-synuclein aggregates and accumulates in Lewy bodies, leading to neurodegeneration (40). In cancer, protein misfolding and aggregation can activate oncoproteins or inactivate tumor suppressors, leading to tumorigenesis (41). As such, the autophagy–lysosomal pathway can help degrade protein aggregates and organelles by autophagosome engulfment and fusion to lysosomes that contain hydrolases. Modulators of this pathway, such as 17-AAG and rapamycin have been shown to be effective in reducing α-synuclein in cells (42, 43). Furthermore, autophagy regulation in animal models has been shown to suppress tumor formation (44). Temsirolimus, a rapamycin derivative, was approved for the treatment of renal cell carcinoma (45).

Autophagy modulators have been used in the setting of brain metastases (46). The addition of chloroquine to whole-brain radiation significantly improved median progression-free survival and decreased mortality related to brain disease progression in comparison to controls, with no increase in toxicity (47). Nevertheless, this strategy did not control extracranial disease and did not significantly increase overall survival (47). A major limitation of autophagy modulators, such as chloroquine, in the brain tumor setting is low BBB permeability (48, 49). Higher concentrations are needed to achieve the desired autophagic effects, which can become toxic (50). As such, metixene – a CNS agent that is BBB permeable, FDA approved with a low toxicity profile – can be a promising therapeutic option in clinical settings of BCBM, as it has shown efficacy against primary and metastatic breast cancer cells in vitro and in vivo, intracranially and extracranially.
Our results demonstrated that metixene activates autophagy but suppresses autophagic degradation, a process known as incomplete autophagy. Incomplete autophagy is an impaired self-eating process of intracellular macromolecules in which generated and accumulated autophagic vesicles do not degrade, resulting in the blockage of autophagic flux (17). It has been reported that incomplete autophagy plays a crucial role in disrupting cellular homeostasis and promotes only cell death (17). Our results confirm that the elevation of LC3II upon metixene treatment with or without chloroquine is indicative of efficient induction of autophagosome formation (21, 22). Moreover, p62 accumulation proves autophagic vesicle accumulation and inhibition of autophagy completion (22). Most of the recognized compounds that affect autophagy typically act as either inducers or inhibitors of the process; few can do both functions. Drugs capable of modulating autophagy through dual mechanisms, both induction and inhibition, have emerged as a promising strategy in anticancer therapy (51-53). These compounds can elicit metabolic energy depletion, ultimately resulting in cellular stress and apoptosis (53). Furthermore, the accumulation of autophagic vesicles containing undegraded cargo, which cannot be effectively recycled, can exacerbate cellular stress (53).

NDRG1 has been reported to act as a metastasis suppressor in multiple human cancers (54-57), including breast cancer (58). Its upregulation can induce tumor differentiation and inhibit metastasis and cell proliferation (54, 59). Its potent anticancer effects have made it an important target for therapy. Iron chelators, such as Dp44mT and DpC, which exhibit selective antitumor activity were shown to upregulate NDRG1 and inhibit stress-induced autophagy in cancer cells (60, 61). In our work, we found that metixene upregulated NDRG1 expression and phosphorylation, which led to metastasis suppression through incomplete autophagy and apoptosis (Supplementary Figure 16).
The role of NDRG1 in human cancer has sparked controversy due to its dual nature. While it exhibits metastasis-suppressing properties in many cancer types, it has also been identified as a biomarker associated with metastasis, cancer recurrence, and poor prognosis in few others (62). In context of breast cancer, NDRG1 appears to be multifaceted, as it has been proposed to act as a promoter of tumor growth and brain metastasis in some cases of ER-negative, aggressive breast cancers (63, 64). Analysis of multiple independent datasets has shown that NDRG1 levels are statistically significantly higher in tumors with aggressive molecular subtypes, such as triple-negative breast cancers (65). In our work, we show that overexpressing NDRG1 with metixene beyond the baseline elevated levels in triple negative breast cancer cell lines and brain metastases, such as MDA-MB-231Br, induces incomplete autophagy, leading to decreased cell viability \textit{in vitro} and improvement of survival in multiple preclinical models \textit{in vivo}.

Further investigation and experimentation are needed to provide a comprehensive understanding of how the molecular mechanisms of metixene are interconnected in the context of cancer cell biology. Following cancer cell exposure to metixene, several coordinated processes unfold. Metixene induces substantial DNA damage while concurrently impairing DNA repair mechanisms, as evidenced by the downregulation of DNA damage repair proteins. The surge in DNA damage may increase cellular stress and induce the expression and phosphorylation of NDRG1, a marker of cellular stress response. Incomplete autophagy, driven by NDRG1 upregulation, further exacerbates the accumulation of damaged cellular constituents, overwhelming the cell and prompting its elimination via apoptosis. This process may further disrupt the self-renewal capabilities of cancer stem cells by accumulating damaged DNA or organelles, ultimately leading to their differentiation or death. This intricate network underscores
the efficacy of metixene in dismantling cancer cells by targeting DNA repair machinery, stem-like properties, and invoking cellular stress responses, providing a comprehensive and nuanced approach to its anticancer effect.

In conclusion, our study provides preclinical, translational evidence on the effectiveness of metixene as a therapeutic agent against metastatic cancer and brain metastases, while offering insights on its mechanism of action. Our findings warrant further exploration and potential translation of metixene onto the clinic.
MATERIALS AND METHODS

Cell Culture

HCC1954, HCC3153, and HCC1806 cells were cultured in RPMI-1640 medium (Corning) supplemented with 10% FBS (HyClone). MDA-MB-231, MDA-MB-231BrM2 (referred to as MDA-MB-231Br), BT-474, BT-474BrM3 (referred to as BT-474Br), and HS578T were cultured in DMEM medium (Corning) with 10% FBS. SUM159PT cells were maintained in F12 medium (Corning) supplemented with 5% FBS, 1μg/ml hydrocortisone, 10mM HEPES, and 5μg/mL insulin. All cells were supplemented with 1% penicillin/streptomycin (Invitrogen) and cultured at 37°C in a humidified (5%) CO₂ incubator. MDA-MB-231BrM2 and H2030Br cell lines were kindly provided by Dr. Joan Massagué (Memorial Sloan Kettering Cancer Center). BT-474 and BT-474BrM3 cell lines were kindly provided by Dr. Dihua Yu (MD Anderson Cancer Center). HCC1954, HCC3153, HCC1806, MDA-MB-231, and HS578T, cell lines were purchased from ATCC. SUM159PT cell line was purchased from BIOVT. WM3734 was purchased from Rockland. Cells were screened periodically for mycoplasma contamination. Metixene hydrochloride was obtained from MedChemExpress LLC.

CNS Small-Molecule Inhibitor Screening

The Prestwick CNS Drug Library of 320 CNS compounds was purchased from Prestwick Chemical Libraries for screening. Cells were seeded at a density of 5000 cells/well in clear, flat-bottomed, black-walled, 96-well plates coated with laminin and poly-d-lysine. Culture medium and differentiation factors were the same as in previous assays. Each compound from the library was added at a final concentration of 25μM per well 1 day after seeding of cells. The screens were run with concentrations ranging from 5 to 100 μM. The 5 μM concentration failed to
identify a sufficient number of meaningful hits. High concentrations (50 and 100 μM), on the other hand, identified numerous hits likely as a result of non-specific toxicity. As such, 25 μM was chosen as a moderate concentration for screening. After three days of treatment, cell viability was measured using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer’s instructions. Briefly, cells were incubated with an equal volume of Cell Titer Glo reagent and incubated for 10 minutes, then luminescence was measured using Cytation 5 according to manufacturer’s instructions.

**Cleaved Caspase 3/7 Assay**

Cells were plated in a 96-well plate. The next day, cells were treated with metixene at different concentrations (control, 5μM, 10μM, and 15μM) for 24 or 48 hours. Apoptosis was measured using the Caspase-Glo® 3/7 Assay System (Promega) according to manufacturer’s instructions. Briefly, cells were incubated with an equal volume of caspase 3/7 glo reagent and incubated for 10 minutes; then luminescence was measured using Cytation 5 according to the manufacturer’s instructions.

**Caspase 8 and 9 Assays**

Cells were plated in a 96-well plate. The next day, cells were treated with metixene at different concentrations (control, 5μM, 10μM, and 15μM) for 24 hours. Apoptosis was measured using the Caspase-Glo® 8 Assay System and the Caspase-Glo® 9 Assay System (Promega) according to manufacturer’s instructions. Briefly, cells were incubated with an equal volume of caspase 8 or 9 glo reagent and incubated for 20 minutes; then luminescence was measured using Cytation 5 according to the manufacturer’s instructions.
Western Blot

Western blotting was performed using standard protocols. Briefly, after treatment with metixene, chloroquine, and/or wortmannin, cells were lysed with M-PER Buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). After conducting electrophoresis, blots were blocked for 1 hour at room temperature with 5% milk in 10mM Tris-HCl pH 7.5, 150mM NaCl containing 0.1% Tween 20 (TBST) and incubated at room temperature for 1 hour with primary antibodies reactive to cleaved-caspase 3 (9661, Cell Signaling Technology), β-actin (4967, Cell Signaling Technology), LC3A/B (4108, Cell Signaling Technology), Phospho-NDRG1 (5482, Cell Signaling Technology), NDRG1 (5196, Cell Signaling Technology), SQSTM1/p62 (5114, Cell Signaling Technology), and GAPDH (2118, Cell Signaling Technology). After washing, blots were incubated with donkey anti-Rabbit IgG (H+L) cross-adsorbed secondary antibody, HRP (1:5000, 31458, Thermo Fisher Scientific) for 1 hour at room temperature. The antigen-antibody reaction was detected using ECL prime kit according to the manufacturer's instructions (Sigma).

Immunofluorescence

Cells were grown on coverslips for 48 hours until they reached 70% confluency. Then, adherent cells were washed twice with phosphate-buffered saline (PBS) and fixed with methanol for 15 minutes at -20°C for LC3, or in 2% paraformaldehyde (PFA) for 30 minutes in case of cleaved-caspase 3. Then, cells were permeabilized using 0.3% Triton X-100 for 10 minutes and blocked in 5% BSA for 1h at room temperature. Primary antibodies against cleaved caspase-3 (9661, Cell Signaling Technology) or LC3A/B (4108, Cell Signaling Technology) were incubated for 1 hour at room temperature. After three washes in PBS, cells were incubated with anti-rabbit Alexa
Fluor 568 conjugated secondary antibody for 1 hour. Cells were then washed in PBS thrice, and slides were mounted in DAPI mounting medium (Prolong Gold, Fisher, MA) and imaged with a Leica DMI8 epifluorescence microscope. For *in vivo* cleaved caspase-3 detection, brains from mice previously injected with brain metastatic breast cancer cells and receiving the indicated *in vivo* treatments were harvested, embedded in optimal cutting temperature (OCT), and sectioned. 5µm thick sections were washed in PBS, fixed with 4% PFA for 15 minutes, and permeabilized with 0.3% Triton X-100 (Sigma) for 10 minutes. Nonspecific binding sites were blocked using 5% BSA for 1 hour and then incubated overnight with anti-human cleaved caspase-3 (9661, Cell Signaling Technology). After intermittent washes in PBS, sections were incubated with anti-rabbit secondary antibody coupled to Alexa Fluor 555 for 1 hour at room temperature. After washing in PBS, the slides were mounted in DAPI mounting medium (Prolong Gold) and imaged with a Leica DMI8 epifluorescence microscope. The quantification of fluorescent areas was conducted using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (66).

**mCherry-GFP-LC3**

FUW mCherry-GFP-LC3 was a gift from Anne Brunet (Addgene plasmid # 110060; http://n2t.net/addgene:110060; RRID: Addgene_110060) (67). Cells at 70% confluency were transiently transfected with the plasmid using Lipofectamine 2000 (Invitrogen, ThermoFisher Scientific). Following 24 h of transfection, media was changed, and cells were sorted to isolate those that expressed mCherry-GFP configuration. Confocal fluorescent images were collected with a Nikon AXR point scanning confocal fluorescence microscope (Nikon Instruments, Melville NY, USA) using a Nikon Plan Apo λD 60x / NA 1.42 Type F oil immersion objective and GaAsP point detectors. All images were collected with a pinhole size of 32.6 µm corresponding to 1.0 AU at 561 nm excitation and a 4.0 µsec dwell time for all channels. DAPI
was imaged with 405 nm excitation and a 430 - 475 nm emission range, a detector gain factor of 34.0, and nominal laser power of 4.0%. GFP was imaged with 488 nm excitation and 499 - 530 nm emission, a detector gain factor of 34.0, and nominal laser power of 5.0%. mCherry was imaged with 561 nm excitation and 600 - 635 nm emission, a detector gain factor of 30.0, and nominal laser power of 4.0%. Volume Z stack series were collected over a total volume of 9.4 µm in depth, corresponding to 48 total frames of 512x512 pixels in each frame with a voxel size of 0.11x0.11x0.2 µm. All images were collected with a pinhole size of 32.6 µm corresponding to 1.0 AU at 561 nm excitation and a 4.0 µsec dwell time for all channels, using NIS Elements Software v. 5.41.

**Electron Microscopy**

Cell culture samples on Thermanox plastic in a 24 well plate were fixed in 0.1 M sodium cacodylate buffer pH7.35 containing 2% paraformaldehyde and 2.5% glutaraldehyde and post-fixed with 2% osmium tetroxide in unbuffered aqueous solution, rinsed with distilled water, en bloc stained with 3% uranyl acetate, rinsed with distilled water, dehydrated in ascending grades of ethanol, transitioned with 1:1 mixture of ethanol and resin, and embedded in resin mixture of Embed 812 kit, cured in a 60°C oven. Samples were sectioned on a Leica Ultracut UC6 ultramicrotome. 70 nm thin sections were collected on 200 mesh copper grids, post stained with 3% uranyl acetate and Reynolds lead citrate. Photos were obtained with The FEI Tecnai Spirit G2 transmission electron microscope at 120 kV.

**Immunohistochemistry**

Murine brain tissues were embedded in paraffin and cut into 4 µm-thick sections on positively charged slides. Dewaxed sections were routinely stained with hematoxylin and eosin (H&E)
dyes for histological evaluation and were also processed for chromogenic immunohistochemistry for the following primary rabbit antibodies: LC3B (Cat. # ab48394, Abcam; dilution: 1:600), cleaved caspase-3 (Cat # ab4051, Abcam; dilution: 1:200), and p-NDRG1 (Cat# 5482, Cell Signaling Technology; dilution: 1:500). An anti-rabbit antibody-horseradish peroxidase (HRP) polymer conjugate (MACH2, Biocare) was used in conjunction with the chromogenic substrate 3,3’-diaminobenzidine (DAB) to visualize the primary antibody sites. IHC slides were counterstained with hematoxylin. The quantification of stained areas was conducted using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (66).

**qRT-PCR**

RNA was extracted using RNAeasy kit from Qiagen according to the manufacturer’s protocol. cDNA was prepared from 1 µg RNA using iScrip cDNA synthesis kit according to the manufacturer’s protocol. The cDNA was then subjected to qRT-PCR in Biorad CFX-connect. The primer sequences can be found in the Supplementary Methods.

**Generation of NDRG1 knockout cells**

Single gene knockout (KO) clones were generated in lentiCRISPRv2 (one vector system). The vector backbone was purchased from Addgene (lentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid #52961; http://n2t.net/addgene:52961; RRID: Addgene_52961) (68). The protocol for guide cloning and generation of the virus was as described (68). The guide sequence for human NDRG1 KO was "CCTGCAAGAGTTTGATGTCC" and non-targeting control (VC) was "AATATTTGGCTCGGCTGCGC". The NDRG1 KO and control clones were selected using puromycin from Sigma (1 µg/ml) in MDA-MB-231Br cell line. The NDRG1 KO was confirmed using western blotting (NDRG1 antibody, 5196, Cell Signaling Technology).
Animal Experiments

All animal studies were completed per the National Institutes of Health guidelines on the care and use of laboratory animals for research purposes. The Institutional Committee on Animal Use at Northwestern University approved the protocols. Six- to eight-week-old athymic, immunodeficient (nu/nu) female mice were obtained from Charles River and maintained in a specific pathogen-free facility. Mice were anesthetized with an intraperitoneal injection of 100μl of a stock solution containing ketamine HCl (25 mg/ml) and xylazine (2.5 mg/ml).

For mammary gland injections, 1×10⁶ HCC1954 cells were diluted 1:1 with Matrigel Matrix (BD Biosciences) for a final volume of 40μl and injected in the inguinal mammary fat pad of nude female mice. Two weeks after tumor cell injection, mice were randomly divided into 3 groups, where treatment was given intraperitoneally: (i) vehicle (25% captisol), (ii) metixene (0.1 mg/kg), (iii) metixene (1.0 mg/kg). Higher doses of 5 and 10 mg/kg were toxic to the mice. Tumor growth was monitored once weekly by caliper measurement.

For the stereotactic intracranial injection, the surgical site was prepared with 70% ethyl alcohol. A midline incision was made, and a 1-mm-diameter parietal burr hole, centered 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture, was drilled. Mice were placed in a stereotactic frame, and 5×10⁵ BT-474Br cells previously resuspended in 5μl of sterile PBS were intracranially injected with a 26-gauge needle at a depth of 3mm. The needle was removed, and the skin was sutured with a 4-0 absorbable, synthetic braided suture. After ten days, mice were randomly divided among two treatment groups: (i) vehicle (25% captisol) through intraperitoneal injection, and (ii) metixene metixene (1.0 mg/kg) through intraperitoneal injection. Mice were treated 3 times per week and were monitored for 3 months or upon meeting the established
Institutional Animal Care and Use Committee (IACUC) criteria for euthanasia (69). Brain tissues were embedded in OCT freezing reagent, and sections with a thickness of 4 μm were cut, air-dried, and stained with H&E.

For the intracardiac injection of cancer cells, the surgical site was prepared with 70% ethyl alcohol to ensure aseptic conditions. Subsequently, the mice were positioned supinely, and a suspension containing $2.5 \times 10^5$ MDA-MB-231 cells, resuspended in 100 μl of sterile PBS, was loaded onto a 26-gauge needle for precise intracardiac delivery into the left ventricle of the heart. To verify the accurate placement of the needle within the left ventricle, 10-20 μl of fluid was aspirated, with confirmation indicated by the presence of bright red (arterial) blood. Once proper placement was confirmed, the cancer cell suspension was administered gradually over a 30-second interval. After a seven-day incubation period, the mice were randomly allocated to one of two treatment cohorts: (i) a vehicle group, receiving 25% captisol via intraperitoneal injection, and (ii) a metixene-treated group, subjected to intraperitoneal injections of metixene at a dosage of 1.0 mg/kg. Treatment was administered thrice weekly, and continuous monitoring of the mice ensued until they met the predetermined euthanasia criteria, as established by IACUC (69).

Following euthanasia, organ tissues were promptly embedded in 4% PFA reagent, followed by the preparation of 4 μm-thick sections. These sections were subsequently air-dried and subjected to H&E staining for histological examination.

**Intracarotid Artery Injections**

The animal studies were approved by the IACUC of Northwestern University, Chicago. The brain metastatic MDA-MB-231Br cells were harvested and passed through a 70μM strainer to obtain single cells. The cells were then centrifuged at 350g for 5 min and washed with cold PBS.
The cells were counted and aliquoted, with 1 million cells in 200μl of cold PBS. Six- to eight-week-old nude mice were anesthetized by intraperitoneal injection of ketamine/xylazine cocktail and positioned in dorsal recumbency. Eye lubricant was applied to the eyes to prevent corneal ulcer formation. The surgical field at the ventral side of the neck was draped and sterilized by applying povidone-iodine followed by 70% alcohol solution application. A 1-cm-long skin incision was created using a surgical blade. The underlying subcutaneous tissue was gently dissected, and the sternohyoid muscle was bluntly separated from the sternomastoid muscle to expose the left common carotid artery. Then, the digastric muscle was bluntly separated to expose the external and internal branches of the common carotid artery. Further, the vagus nerve was gently separated from the carotid artery using surgical forceps under a dissection microscope. A tight surgical knot was placed at the caudal end of the common carotid artery. A cotton ball dipped in sterile PBS was placed below the carotid artery to assist in injection and to maintain moisture over the artery. A second surgical knot was placed over the rostral end of the common carotid artery. Before injection, a hemoclip was placed over the external branch of the common carotid artery. A 31-gauge BD insulin syringe was inserted into the lumen of the artery followed by infusion of 100 μl of cells. Once the infusion of cells was completed, the common carotid artery was permanently ligated at its rostral end to prevent the leakage of cells. Previously bluntly separated muscles were moved to the original sites, and subcutaneous tissue and skin were closed with 6-0 size nylon surgical sutures in a simple interrupted fashion.

**Bioluminescence Imaging**

The brain-metastatic capabilities of BT-474BrM3-mCherry-Luc cells were monitored biweekly. Mice were administered Luciferin (15 mg/ml) (in sterile PBS) (GoldBio) through intraperitoneal
injection. The mice were sedated with isoflurane anesthesia and placed in the bioluminescence camera box of IVIS spectrum imager for about 10 min, and bioluminescence was measured.

**Pharmacokinetics and Bioavailability**

Sample preparation entailed a meticulous acetonitrile-based protein crash procedure, with the incorporation of the internal standard (Methadone) for precise quantification. Quantification was facilitated through a matrix-matched curve spanning a dynamic range from 1 to 1000 ng/mL in both plasma and brain homogenate samples. To ensure data accuracy, samples were analyzed in triplicates, and the reported results represent the average of these measurements.

Mass spectrometry data acquisition was conducted using the Agilent 6475 LC/TQ instrument, utilizing MassHunter v.12 acquisition software, and subsequent data processing was performed with MassHunter v.12 Quantitative Analysis Tool. The mass spectrometer was seamlessly integrated with the Agilent 1290 Infinity II UPLC system. During analysis, the samples were subjected to a gradient method, with a precisely controlled injection volume of 1 µL. Positive detection mode was employed, with methadone serving as the internal standard for calibration and quantification purposes. Chromatographic separation was meticulously executed utilizing the InfinityLab Poroshell 120 EC-C18 column, measuring 2.1 x 100 mm and featuring a 1.9-micron particle size. The mobile phase composition encompassed 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient method was characterized by a flow rate of 0.45 ml/min, which facilitated a transition from an initial 30% B composition to a final 100% B composition over a span of 6 minutes.
Reverse Phase Protein Array

A reverse-phase protein array (RPPA) was performed in the MDACC CCSG core as described at http://www.mdanderson.org/education-and-research/resources-for-professionals/scientific-resources/core-facilities-and-services/functional-proteomics-rppa-core/index.html.

Morpheus, https://software.broadinstitute.org/morpheus, a versatile matrix visualization and analysis software was used for generation of heat maps.

Statistics

Statistical analyses were performed using GraphPad Prism Version 8 for Windows (GraphPad Software, www.graphpad.com), SAS 9.4 (SAS Institute Inc.), and R version 3.5.2 (R Foundation for Statistical Computing). For continuous variables, data were reported as means ± SEM. For categorical variables, data were reported as numbers (percentage). For continuous variables, depending on whether the assumption of normality and the assumption of equal variance were met, a two-tailed Student’s unpaired t test (when both assumptions were met) or unpaired t test with Welch’s correction (when only the assumption of equal variance was not met) was used for two unpaired groups. One-way and two-way analysis of variance (ANOVA) test with Tukey’s or Dunnett’s multiple comparisons tests were performed for multiple group comparisons. For data with two independent variables, for example, treatment and time, a linear mixed model was fitted, and the differences among groups were evaluated using the least squares means method and adjusted by Sidak’s method or Dunnett’s method. Kaplan-Meier survival curves were plotted and compared using the log-rank test for proportional hazard rates or Renyi statistics for crossing hazard rates. Statistical significance is indicated as *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
Study Approval

The animal studies were approved by the IACUC Office at Northwestern University (Protocol #2559).

Data availability

All data generated or analyzed during this study were included in this article [and its supplementary information files]. Raw data for the manuscript are also available in the Supporting Data Values file.
**Author contributions:** Conceptualization, resources, supervision, validation, visualization, methodology, and project administration: JF and MSL. Data Curation and Formal Analysis: JF. Funding Acquisition: MSL. Writing–Original Draft: JF. Investigation and revising final draft: JF, EP, DK, CD, AC, JD, RY, MHS, PZ, AR, VAA, IU, AUA, JM, IVB, CDJ, AMS, ABH, MSL.

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Figure 1: Metixene induces apoptotic cell death in brain metastatic breast cancer cells. A, Cellular viability of BT-474Br and MDA-MB-231Br cells under treatment at different concentrations of metixene for 24h and 48h. B, Caspase 3/7 activity in BT-474Br and MDA-MB-231Br cells under treatment with different concentrations of metixene for 24h and 48h. C, Immunofluorescent staining of cleaved caspase-3 after metixene treatment in BT-474Br cells for two days. D, Immunofluorescent staining of cleaved caspase-3 after metixene treatment in MDA-MB-231Br cells for two days. E, Caspase 8 and 9 activities in BT-474Br under treatment with different concentrations of metixene for 24h. F, Caspase 8 and 9 activities in MDA-MB-231Br cells under treatment with different concentrations of metixene for 24h. The results in A, B, E, and F are representative of ≥3 technical replicates, and bar graphs represent means ± SEM. Statistical analysis was performed using one-way ANOVA with post hoc Dunnett’s test. ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05.
Figure 2: Metixene’s anti-cancer efficacy decreases the size of mammary fat pad tumors and improves survival in a metastatic breast cancer murine model. A, Experimental setup of mammary fat pad injections with HCC1954 cells and subsequent treatment with metixene. B, Metixene treatment at 0.1 mg/kg and 1.0 mg/kg decreased tumor sizes in comparison to control. C, Tumor weight at 0.1 mg/kg and 1.0 mg/kg of metixene treatment. D, Tumor volume at 0.1 mg/kg and 1.0 mg/kg of metixene treatment. E, H&E (scale bar = 250μm) and cleaved caspase-3 (scale bar = 50μm) staining of tumors treated with control, 0.1mg/kg and 1.0mg/kg of metixene; staining, red color indicates cleaved caspase 3, and blue color represents DAPI. F, Percent of cleaved caspase-3–positive cells, n = 3 per group. G, Experimental setup of intracardiac injections with MDA-MB-231 cells and subsequent treatment with metixene. H, Kaplan-Meier curve showing survival of metixene-treated mice versus controls upon intracardiac injection of MDA-MB-231 cells. I, Experimental setup to determine the bioavailability of metixene in blood and brain tissue. J, Metixene levels in plasma and brain tissue across time after intraperitoneal administration. Statistical analysis in C and F was performed using one-way ANOVA with post hoc Dunnett’s test. Statistical analysis in D was performed using two-way ANOVA with post hoc Tukey’s test. Survival curves were compared using a log-rank test. ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05.
**Figure 3: Metixene improves survival in preclinical models of metastatic brain cancer.** A, Experimental setup of stereotactic intracranial injection of BT-474Br in the brains of nude mice and subsequent treatment with metixene. B, Kaplan-Meier curve showing survival of metixene-treated mice versus controls upon intracranial injection of BT-474Br cells. C, Bioluminescent imaging of the two groups (control vs treated) across time. D, Immunohistochemistry staining of cleaved caspase-3 in brain samples of control and metixene treated mice, bearing BT-474Br tumors (quantification of stain area is in *Supplementary Figure 4A*). E, Experimental setup of intracarotid artery injection of MDA-MB-231Br cells in nude mice and subsequent treatment with metixene. F, Kaplan-Meier curve showing survival of metixene-treated mice versus controls upon intracarotid injection of MDA-MB-231Br cells. G, Histological section upon death of control mice confirmed growth of brain tumors in mice as a result of intracarotid injection of MDA-MB-231Br. H&E staining of mouse brain shows metastatic tumors (black box), the formation of multiple micrometastases (blue box), and vascular co-option (red box). H, Immunohistochemistry staining of cleaved caspase-3 in brain samples of control and metixene treated mice, bearing MDA-MB-231Br tumors (quantification of stain area is in *Supplementary Figure 4B*).

Survival curves were compared using a log-rank test. Linear mixed model was fitted for C, and the differences between the two groups for each time point were calculated using the least squares means method and adjusted using Sidak’s method. ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05
Figure 4: Reverse phase protein array highlights autophagy signaling in breast cancer brain metastatic cells. **A**, Experimental setup of RPPA in BT-474Br cells that underwent metixene treatment for 12h and 24h, respectively. **B**, Pathway analysis of changes in protein phosphorylation that were significant with a P<0.05 using the KEGG 2019 Human database. **C**, Heat map of protein phosphorylation changes that were significant with a P<0.001 at 12h. **D**, Heat map of protein phosphorylation changes that were significant with a P<0.001 at 24h. **E**, Biological processes that are significantly activated after 12h of metixene treatment. **F**, Biological processes that are significantly activated after 24h of metixene treatment. *Statistical analysis was done using a two-tailed Student’s t test to compare the means of protein/phosphorylation expression between control samples and metixene treated samples.*
Figure 5: Metixene induces autophagy signaling in metastatic brain cancer cells. A, LC3 protein expression analysis upon metixene treatment (10μM) in MDA-MB-231Br cells at the specified time points. B, Autophagy flux protein analysis in MDA-MB-231Br cells treated with metixene (M, 10μM) and/or chloroquine (CQ, 20μM) at different time points. C, Representative LC3 puncta immunofluorescence in BT-474Br and MDA-MB-231Br cells under metixene (10μM) for 48h, chloroquine (20μM) for 24h, and the combination of both metixene and chloroquine. D, Quantification of area of LC3 puncta per cell in BT-474Br cells upon treatment with control, metixene (M), chloroquine (CQ), or the combination of M+CQ. E, Quantification of area of LC3 puncta per cell in MDA-MB-231Br cells upon treatment with control, M, CQ, or M+CQ. The results are representative of three independent experiments. Statistical analysis was performed using one-way ANOVA with post hoc Dunnett’s test or Tukey’s test. ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05
Figure 6: Metixene induces incomplete autophagy in metastatic brain cancer cells. A, Control and metixene-treated BT-474Br and MDA-MB-231Br cells expressing mCherry-GFP-LC3. Autophagosomes display both GFP and mCherry fluorescence, appearing yellow/green. Autolysosomes showcase mCherry fluorescence only, as GFP is denatured by acidic lysosomes, appearing red. B, Electron microscopy of metixene-treated BT-474Br and MDA-MB-231Br cells (white arrows show double-membraned autophagosomes). C, LC3 and p62 protein expression in BT-474Br and MDA-MB-231Br cells treated with increasing concentrations of metixene for 48h (μM). D, Immunohistochemistry staining of LC3B in brain samples of control and metixene-treated mice, bearing BT-474Br tumors (quantification of stain area is in Supplementary Figure 12A). E, Immunohistochemistry staining of LC3B in brain samples of control and metixene-treated mice, bearing MDA-MB-231Br tumors (quantification of stain area is in Supplementary Figure 12B). The results are representative of three independent experiments. Statistical analysis was performed using one-way ANOVA with post hoc Dunnett's test. ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05
Figure 7: NDRG1-mediated incomplete autophagy induces apoptotic cell death in metastatic brain cancer cells. A, pNDRG1 and NDRG1 protein expression in BT-474Br cells treated with increasing concentrations of metixene (μM). B, pNDRG1 and NDRG1 protein expression in MDA-MB-231Br cells treated with increasing concentrations of metixene (μM). C, Protein expression of NDRG1 Knockout (KO) versus vector control (VC) in MDA-MB-231Br cells treated with increasing concentrations of metixene (μM). D, Cellular viability of NDRG1 KO versus VC in MDA-MB-231Br cells under treatment with different concentrations of metixene for 24h. E, Caspase 3/7 activity of NDRG1 KO versus VC in MDA-MB-231Br cells under treatment with different concentrations of metixene for 24h. F, Protein expression of LC3, p62, pNDRG1, and cleaved caspase-3 in BT-474Br and MDA-MB-231Br cells treated with metixene in a time-dependent manner. G, Immunohistochemistry staining of pNDRG1 in brain samples of control and metixene-treated mice, bearing BT-474Br tumors (quantification of stain area is in Supplementary Figure 13). The results in A, B, and C are representative of three independent experiments. The results in D are representative of eight technical replicates. The results in E are representative of four technical replicates. Statistical analysis was performed using two-way ANOVA with post hoc Sidak's test. ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05
**Table 1:** The top CNS agents that decreased cell viability in BT-474Br and HCC1954 cells

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<th>Viability (%)</th>
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<td>Flusipirilen</td>
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Table 2: IC$_{50}$ of metixene in different metastatic breast cancer cell lines after three days of treatment

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