Title: Repurposing the anti-psychotic trifluoperazine as an anti-metastasis agent

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Abbreviations: 3-D - three dimensional, 2-D - two dimensional, TFP – trifluoperazine, AKT - Protein Kinase B, DRD2 - dopamine receptor D2, NCI-DTP National Cancer Institute Developmental Therapeutics Program, FDA - Food and Drug Administration, MMP- matrix metalloproteinase, CAM - chorioallantoic membrane, VEGF - vascular endothelial growth factor, TCF-LEF - T-cell factor and lymphocyte enhancer factor
ABSTRACT

Since cancer cell invasion is a critical determinant of metastasis, targeting invasion is a viable approach to prevent metastasis. Utilizing a novel three-dimensional high throughput invasion assay, we screened a National Cancer Institute compound library and discovered compounds demonstrating inhibitory effects on cancer cell invasion. One hit, trifluoperazine, suppresses invasion of human cancer cell lines while displaying a limited cytotoxicity profile. This inhibition is due to the interference with cancer cell migratory ability, but not proteolytic activity. Treatment of cancer cells with trifluoperazine significantly reduces angiogenesis and prevents cancer cell invasion through a chorioallantoic basement membrane. Mechanistically, treatment results in decreased phosphorylated AKT (Ser\textsuperscript{473} and Thr\textsuperscript{308}) and β-catenin (Ser\textsuperscript{552}). Lack of phosphorylation of Ser\textsuperscript{552} of β-catenin prevents β-catenin nuclear relocation resulting in decreased expression of vascular endothelial growth factor, likely mediated through dopamine receptor D2. Taken together, we have demonstrated that trifluoperazine is responsible for reducing the angiogenic and invasive potential of aggressive cancer cells through dopamine receptor D2 to modulate the β-catenin pathway and propose that trifluoperazine may be used as an anti-metastasis chemotherapeutic.
INTRODUCTION

Malignant tumors are largely considered incurable and those without evidence of dissemination will frequently relapse (Redig and McAllister, 2013), as they often harbor micrometastases that are not apparent at the time of diagnosis (Talmadge and Fidler, 2010). Accordingly, patients have a high mortality rate and approximately 90% of cancer patients succumb to the disease because of metastasis, even with early detection (Leaf and Burke, 2004). Treatments designed to target primary tumors, such as surgery, radiotherapy, and chemotherapy in refractory types of cancer are ineffective in eradicating disseminated cancers (Norton and Massague, 2006). Hence, there is an unmet need to identify agents capable of inhibiting metastasis.

A strategy to combat metastasis includes pursuing drug development for compounds that can attenuate cell invasion, as it is a critical early step in the complex metastatic cascade (Hanahan and Weinberg, 2011). The metastatic process was long considered a unidirectional event, with cells leaving the primary tumor at early stages of disease and colonizing distant organs. The course of metastasis has been redefined to include not only the distribution of cancer cells to secondary organs, but also the re-seeding and perpetuation of the established tumors by circulating cancer cells (Comen and Norton, 2012; Norton and Massague, 2006). Because circulating tumor cells that have escaped from the primary tumor can reseed tumor cells at the primary site and contribute to secondary lesions (Kim et al., 2009), identifying novel compounds capable of inhibiting cancer invasion provides an effective strategy for all stages of the disease.

Although it is clear that metastasis is often the reason for treatment failure, it is generally not an endpoint that is taken into consideration when initially evaluating anti-cancer drugs.
Cancer drug development has traditionally focused on identifying anti-mitotic and cytotoxic compounds; drugs with selective anti-metastatic effects have not been identified. This is mostly due to the fact that cancer metastasis is a complex cascade and there has been a lack of effective tools for studying metastasis. For many types of solid cancers, drugs that have been developed to suppress cancer cell proliferation are capable of prolonging patient survival, but are not likely to improve a person’s chances of survival. The key to improving patient survival is stopping metastases, which accounts for 90% of treatment failure among all cancers (Leaf and Burke, 2004).

In order to address the lack of effective drugs targeting cancer metastasis, we employed our novel three-dimensional (3-D) high-throughput assay (Evensen et al., 2013) to identify potential agents targeting cancer cell invasion. Screening drugs in vitro against cancer cells has historically been done in a two-dimensional (2-D) format, assaying cells on a flat surface for proliferation and/or cytotoxicity. Monitoring the effects of anti-cancer agents on cancer cells in 2-D cultures, however, may lead to false positives. It has been demonstrated that the phenotype of a cell tethered to a 2-D surface as compared to being embedded in a 3-D matrix can be vastly different (Anders et al., 2003; Bissell and Radisky, 2001; Bissell et al., 1999). These differences lie in receptor expression, extracellular matrix deposition, and metabolism and contribute to altered responses of cells to treatment. Over 700 different genes have documented changes in expression in 3-D culture when compared to 2-D culture (Horning et al., 2008). Cells grown in 3-D cultures also demonstrate a decrease in drug uptake and an extended time to drug uptake from the environment, leading to the conclusion that tumor architecture influences drug efficacy (Horning et al., 2008). Three-dimensional culture better recapitulates tumor structure; therefore,
employing a 3-D high-throughput assay facilitates identification of drugs more likely to be effective *in vivo* (Horning et al., 2008).

Herein, we utilized our 3-D high-throughput assay to screen an NCI Diversity compound library against invasive cancer cells. One of the hits, a clinically used anti-depressant drug trifluoperazine [TFP], was found to reduce cancer cell invasion in the 3-D environment without notable cytotoxicity. TFP reduced levels of phosphorylated AKT and β-catenin and the reduction in the activity of these proteins results in decreased invasive behavior and angiogenic potential. Knockdown of the TFP target dopamine receptor D2 [DRD2], recapitulates the reduction in p-β-catenin and p-AKT, suggesting that the ability of TFP to reduce the invasive capacity of cancer cells lies in its ability to target the dopamine receptor. Our study demonstrates for the first time that TFP may have a new use in preventing cancer metastasis.
MATERIALS AND METHODS

Materials

Collagen Type I (acetic acid-extracted native type I collagen from rat tail tendon), Matrigel, and propidium iodide (PI) were obtained from BD Bioscience Discovery Labware. Diversity Set II compound library was obtained from the Developmental Therapeutics Program in the NCI/NIH (Bethesda, MD). Hoechst nuclear stain was purchased from Invitrogen (Grand Island, NY). Trifluoperazine hydrochloride and haloperidol were acquired from Sigma-Aldrich. Rabbit anti-p-β-cateninSer552, anti-β-catenin (total), anti-p-AKTSer473, anti-p-AKThr308, and anti-AKT (total) were all purchased from Cell Signaling Technology (Davers, MA). Anti-DRD2 antibody was purchased from EMD Biosciences (Billerica, MA). Mouse anti-actin and anti-α/β-tubulin antibodies were purchased from Cell Signaling Technology (Davers MA). Horseradish peroxidase [HRP] conjugated anti-rabbit and anti-mouse antibodies were obtained from Rockland Immunochemicals (Gilbertsville, PA). Alexa Fluor 568 anti-rabbit antibodies were purchased from Molecular Probes, Life Technologies (Grand Island, NY).

Cell Lines and Treatment of Cells

Cell lines were obtained from the ATCC (Manassas, VA). Cell lines utilized include human prostate cancer PC3 and human metastatic prostate cancer C4-2b, both of which were maintained in RPMI 1640 medium (Corning, Mediatech). Human fibrosarcoma HT1080 and murine fibroblasts NIH/3T3 were maintained in DMEM-high glucose medium (Corning, Mediatech); all media were supplemented with 10% FBS and 1% penicillin/streptomycin. Human umbilical vein endothelial cells (HUVEC) were maintained in human endothelial SFM (Gibco, Life Technologies, Norwalk, CT) supplemented with 20% FBS, 20 ng/mL basic fibroblast growth factor (bFGF) and 20 ng/mL epidermal growth factor (EGF) (R&D Systems,
Minneapolis, MN). HUVEC were cultured in tissue culture dishes coated with sterile 5% gelatin. All cells were maintained in a humidified environment at 37°C and 5% CO2 for all experiments.

3-D High-Throughput Invasion Assay

The 3-D high-throughput invasion assay was performed as previously described (Evensen et al., 2013). Briefly, cells were mixed with 3 mg/mL neutralized type I collagen (1.5 mg/mL final concentration of type I collagen). The mixture was dotted into each well of a 96-well plate and allowed to solidify at 37°C. The cell-matrix dot was then covered with a layer of 1.5 mg/mL neutralized type I collagen. After solidification of cover collagen at 37°C, compounds from the NCI library were added at 10 μM and DMSO was used as a control in complete medium. Incubation was carried out overnight at 37°C and cells were stained with PI and Hoechst.

Cell Viability Assay

HT1080 and NIH/3T3 cells were cultured in complete media with or without TFP. Media and drugs were changed daily and cell viability was monitored by MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Promega, Madison, WI). Each day, cells were exposed to MTT and incubated at 37°C for four hours. The reaction was stopped and formazan crystals were solubilized; the resultant solution was subject to colorimetric spectrophotometry and read at a wavelength of 570 nm.

Protease Assay

Total proteolytic activity was detected by employing the Fluorescent Detection Kit (Sigma, St. Louis, MO). Cell lysates were incubated overnight at 37°C with a fluorescein-isothiocyanate [FITC]-labeled casein substrate and subsequently precipitated with trichloroacetic acid [TCA]. The supernatant was assessed for fluorescence intensity after dilution in assay buffer. Fluorescence was measured using excitation and emission wavelengths of 485 nm and
535 nm, respectively, on a SpectraMax Gemini EM (Molecular Devices, Sunnyvale, CA) fluorescent plate reader.

**Chorioallantoic Membrane Angiogenesis and Invasion Assay**

The CAM assay was performed as previously described (Deryugina and Quigley, 2008). Fertilized white chicken eggs (SPF Premium, Charles River Laboratory, CT) were incubated at 37°C in 70% humidity for three days. The embryos were then incubated *ex ovo* in a sterile Petri dish for seven days. Gelatin sponges adsorbed with HT1080 cells treated with or without TFP were implanted on the CAM surface (Ribatti et al., 2006) and neovasculature was counted on day four post-implantation. For histochemical analysis of the chorioallantoic membrane, embryos were treated as for the angiogenesis assay, except at day 10 the embryos were inoculated with pretreated HT1080 cells in a sterile 2mm ring. After a four day incubation, CAM segments containing the ring were formalin fixed and sectioned by microtome into 5 μm sections after paraffin embedding. Sections were then stained with hematoxylin and counterstained with eosin.

**Enzyme-linked immunosorbent assay for vascular endothelial growth factor**

Secreted VEGF was assayed using the R&D Systems Human VEGF DuoSet ELISA kit (R&D Systems,). Briefly, HT1080 cells were incubated overnight in the presence or absence of TFP in complete media and the media was then incubated in a 96-well plate that had previously been coated in the VEGF capture antibody and blocked with filtered 1% BSA and washed in phosphate buffered saline-tween (PBS-T [0.05% Tween]). Subsequently, the samples were removed and the plate incubated with the VEGF detection antibody and streptavidin-HRP at the concentrations suggested by the manufacturer, with PBS-T washes between each buffer change.
3, 3',5,5'-tetramethylbenzidine [TMB] was utilized to develop the ELISA and the optical density was read at 450 nm with a correction wavelength of 540 nm.

**Endothelial Cell Network Formation**

HUVEC cells were trypsinized and collected. Equivalent numbers of cells were resuspended in media with either DMSO or 2.5 μM TFP. The isolated cells were then placed over a solidified Matrigel coating within the wells of a 48-well tissue culture dish (70,000 cells/well). The cells were allowed to incubate overnight before bright-field imaging using the Nikon Eclipse TE2000-S equipped with a Sutter Instruments SmartShutter System and a QiClick QImaging camera.

**Kinex Antibody Array**

Cell lysates from HT1080 cells treated with TFP or with a DMSO control were obtained according to the protocol suggested by the Kinexus Bioinformatics Corporation [KBC] (Vancouver, BC, Canada), which manufactures the Kinex antibody microarray. This assay contains over 700 antibodies against both phospho- and pan-specific antibodies. The detailed protocols can be found at the KBC website (www.kinexus.ca).

**Dual Luciferase Assay**

To study β-catenin promoter activity, HT1080 cells were transiently transfected with either TOPflash or FOPflash promoter constructs (Upstate Biotechnology) along with the Renilla luciferase reporter gene using polyethyleneimine [PEI, MW: 250kD, Polysciences] after a 30 minute incubation of DNA and PEI at room temperature. Medium was changed 18 hours after transfection. Forty-eight hours after transfection and following drug treatment, luciferase activity of both firefly and Renilla luciferase were measured using the Promega Dual-Glo Luciferase System with the SpectraMaxL (Molecular Devices).
Transwell Migration

Transwell migration assays were performed as described previously (Dufour et al., 2008), except nuclei were stained in Hoechst/PBS (1:2000) for 20 minutes and imaged using a Nikon Eclipse TE2000-S equipped with a Sutter Instruments SmartShutter System and a QiClick QImaging camera. Migrated cells were counted with the assistance of the Nikon Elements Basic Research Software analysis tools.

Scratch Wound Migration Assay

Cells were grown to confluence in a 12-well plate and serum starved to induce cell cycle synchronization with or without TFP overnight under standard tissue culture conditions. A scratch wound was made in each well the following morning and cells were washed twice with 1X PBS and supplemented with complete media containing drugs or vehicle. Cells were allowed to migrate over eight hours, with bright field images being taken at time 0 and time 8 hours. Area for time 0 and time 8 hours were calculated using the Nikon Elements Basic Research Software analysis tools and percent change was calculated.

2-D dot migration assay

A collagen-cell mixture was dotted in a 96-well dish in a similar fashion to the 3-D invasion assay. Following collagen solidification, cell-matrix dots were overlaid with complete media. Cells were allowed to migrate up to 8 hours. Cells were then stained in Hoechst/PBS (1:2000) and images were captured using the previously described microscope and camera system. Migration was then quantified by counting nuclei using the Nikon Elements Basic Research Software analysis tools.

Gelatin zymography
Gelatin zymography was performed as described (Zucker et al., 1995). After electrophoresis, the gels were incubated in Triton X-100 to replace SDS followed by incubation in a Tris-based buffer overnight at 37°C. Staining was accomplished using Coomassie Brilliant Blue and cleared areas were indicative of gelatinolytic activity.

Immunoblotting and Immunofluorescent staining

Immunoblotting was done according to previously published methods and developed on a BioRad ChemiDoc (Cao et al., 1996). Immunofluorescent staining began by fixing treated cells in 4% paraformaldehyde in PBS at 4°C, followed by permeabilization in 0.2% Triton X-100 at room temperature for 10 minutes. Blocking solution was composed of 3% bovine serum albumin/5% normal goat serum in PBS. After one hour blocking at room temperature, cells were exposed to anti-p-β-cateninSer552 antibody (Cell Signaling Technology), visualized using the complementary secondary fluorescent antibody (anti-rabbit Alexa Fluor 568), counterstained with Hoechst, and imaged on the Nikon microscope previously described.

Knockdown of DRD2

HT1080 cells were transiently transfected with aiRNA directed against DRD2 (Boston Biomedical, Inc.) (Sun et al., 2008). Briefly, aiRNA and RNAiMax (Life Technologies) were incubated together at room temperature in serum free DMEM. Transfection mixture was added to HT1080 cells and incubated overnight under standard tissue culture conditions. Four separate aiRNA were tested and the most efficient aiRNAs were identified by real-time RT-PCR and chosen to complete downstream experiments.

Real-Time RT-PCR

Cellular RNA was isolated from the target cells with the Qiagen RNeasy kit. cDNA was generated using random hexamers and the iScript reverse transcriptase (BioRad). The resultant
cDNA was used for real-time RT-PCR to detect DRD2 transcript levels. cDNA was subject to real-time PCR using primers generated specifically identifying DRD2 (F: 5’- CGGACAGACCCCATACAA -3’, R: 5’- CCTGCTGAATTTCACACTCACC -3’) using the BioRad MyiQ Single-color real-time PCR thermocycler. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a normalization control. Results were analyzed using the BioRad software, MyiQ 2.0.

**Statistical Analysis**

Data is expressed as the standard error of the mean, as each experiment was repeated three times. Student’s T-test was used to determine significant differences; any P<0.05 was considered significant.
RESULTS

Identification of compounds capable of inhibiting cancer cell invasion

To identify small molecule compounds capable of inhibiting cancer cell invasion, a novel 3-D high-throughput invasion assay was utilized to screen the National Cancer Institute’s Developmental Therapeutics Program [NCI-DTP] compound library (Diversity Set II) against aggressive, androgen-independent PC3 human prostate cancer cells. This particular compound library includes 1,974 compounds and covers a wide variety of chemical structures. After incubating compounds (10 μM) with PC3 cells assembled in the 3-D invasion assay with type I collagen in a 96-well plate for 18 hours, invaded cells were quantified based on the number of cells that were able to escape the original cell-matrix dot and invade through the adjacent cell-free collagen. The positive hits were defined as any compound that impeded 50% of invasion compared to the DMSO vehicle control. Of the 1974 compounds screened, 84 compounds were found to inhibit invasion. To segregate the inhibitory hits from potentially cytotoxic compounds, we analyzed the available GI₅₀ data of those positive compounds from the NCI-60 human cell line screening dataset. We defined that compounds with a half maximal inhibitory concentration (GI₅₀) of more than 1 μM to be of interest and 56 compounds that we identified fit within this parameter (Figure 1A). One of the hits, TFP, is a Food and Drug Administration [FDA] approved antipsychotic drug. Since this hit is already clinically used as a well-tolerated, first line drug for patients in the acute phase of schizophrenia (Marques et al., 2004) (suggesting low systemic toxicity) and potentially has a novel function in inhibition of cancer cell invasion, the ability of TFP to impede cancer cell invasion and the working mechanism of this drug were further evaluated.
Validating TFP as an anti-invasion drug

To evaluate the potency of TFP, the dose dependent effects of TFP on cancer cell invasion were assessed. To determine if inhibition of cancer cell invasion by TFP is not relegated to prostate cancer cells, an additional aggressive and highly invasive cancer cell line, human fibrosarcoma HT1080, was assessed. The cancer cells were assembled in a 3-D collagen matrix similar to the PC3 cells and exposed to increasing doses of TFP up to 10 \( \mu \text{M} \). The HT1080 cells exposed to TFP demonstrated a dose-dependent decrease in invasive behavior as compared to vehicle control cells (Figure 1B and 1C). We continued to use HT1080 for the remainder of these experiments because of their highly aggressive, invasive nature.

To ensure that the inhibition of invasive behavior was not due to toxicity from TFP exposure, both normal fibroblasts (murine NIH/3T3) and HT1080 cells were analyzed for chronic (repeated exposures for 3 days) toxicity in the presence of increasing doses of TFP using an MTT viability analysis. The chronic cytotoxicity assay did not show any significant cell death (Figure 1D). Given the consideration that cells behave differently in two-dimensional (2-D) culture as opposed to 3-D culture in response to cytotoxic agents (Peng et al., 2013), cytotoxicity of TFP was also determined in the 3-D culture system in the presence or absence of TFP for 24 hours, followed by imaging-based determination of cytotoxicity using propidium iodide (PI). As negative and positive controls for cell death, cells embedded in collagen were also treated with DMSO alone or 1 \( \mu \text{M} \) staurosporine (STS), an apoptosis inducer, respectively. Induction of cell death by STS resulted in intense PI staining, while the vehicle control group displayed minimal PI positive staining. Consistent with the 2-D MTT cytotoxicity assays, TFP did not cause any notable cell death in the 3-D milieu (Figure 1E). Collectively these data indicate that the
reduction in the invasive capacity of cancer cells observed is not a result of cell death, but rather inhibition of the drivers of invasion.

**Inhibition of cancer cell migration by TFP without affecting global protease activity**

Cancer cell migratory ability and proteolytic activity are two critical determinants of cancer cell invasion (Friedl and Alexander, 2011). To determine which pathological process of cell invasion is interfered with by TFP, both cell migratory ability examined by transwell migration assays, scratch wound assays, and proteolytic activity examined by substrate degradation assays were assessed. PC3 cells and C4-2b cells are inhibited from migrating by TFP treatment based on transwell migration assay analyses (Figure 2A). HT1080 cells treated with TFP had significantly reduced migratory ability as compared to vehicle control (Figure 2B and 2C). Importantly, TFP had no effect on the migratory ability of the immortalized fibroblastic cell line NIH/3T3 (Figure 2B and 2C). In order to rule out the possibility that TFP interferes with the global proteolytic activity of the cells, a protease assay was employed to monitor enzymatic activity using a fluorescently-labeled general protease substrate. The cell lysate from HT1080 cells treated with increasing doses of TFP were incubated with fluorescently-labeled general protease substrate followed by analyzing substrate cleavage on a fluorescence spectrophotometer. Treatment of HT1080 cells with TFP had no significant effects on global protease activity at any dose as compared to DMSO control (Figure 2D). It has been demonstrated that HT1080 cells express a variety of matrix metalloproteinases, specifically MMP-2, -9, and -14, which have been correlated with migratory behavior (Dufour et al., 2008; Zarrabi et al., 2011). Gelatin zymography of conditioned media from HT1080 cells treated with TFP shows no changes in MMP-2 or MMP-9 gelatinolytic activity (Figure 2E). Since MMP-14 is a physiological activator of proMMP-2, our results suggest that TFP does not affect MMP-14...
activity. Taken together, these data imply that reduction of cancer cell invasion under 3-D conditions by TFP is via inhibition of cell migratory ability, rather than protease activity.

Effects on angiogenesis and basement membrane invasion by TFP in vivo

Cell migration is essential to angiogenesis and invasion (Tonini et al., 2003). We next sought to determine if exposure to TFP of invasive cancer cells results in reduced angiogenesis and invasion through underlying basement membrane in vivo, by using the chicken chorioallantoic membrane [CAM] assay. HT1080 cells pre-treated with TFP were adsorbed to an inert sponge and implanted on the surface of the CAM of chicken embryos. After a four day incubation, neovascularization induced by HT1080 cells significantly decreased in the presence of TFP as compared to DMSO controls (Figure 3A and 3B). This result may be due to TFP inhibiting endothelial cell migration and/or reducing HT1080 cells’ ability to coordinate angiogenesis.

In an effort to determine the mechanism of the TFP mediated reduction in angiogenesis, the effect of TFP on secretion of vascular endothelial growth factor [VEGF], a major soluble protein involved in vascular dynamics (Ferrara, 2004), was examined. Conditioned media from HT1080 cells treated with or without TFP were evaluated by enzyme-linked immunosorbent assay [ELISA] to quantify secretion of VEGF. The conditioned media collected from HT1080 cells treated with TFP demonstrate a decreased amount of soluble VEGF in a dose responsive fashion (Figure 3C). This data suggests that TFP is able to inhibit the production of VEGF, leading to a decrease in angiogenesis in vivo. In order to further confirm that the effects on angiogenesis were mediated through modification of the cancer cell behavior and not an effect of TFP modulating the surrounding endothelial cells directly, human umbilical vein endothelial
cells (HUVEC) were monitored for network formation when laid on solidified Matrigel in the presence or absence of TFP. HUVEC exposed to 2.5 μM TFP were not impaired in forming a network similar in morphology to vehicle treated HUVEC (Figure 3D), suggesting that TFP exerts its anti-angiogenic effect directly on the responsive cancer cells.

To directly examine if TFP is capable of inhibition of cancer cell invasion through basement membrane in vivo, the CAM invasion assay was employed. The CAM consists of the chorionic epithelium and underlying allantoic membrane that is primarily made of type IV collagen (Ribatti et al., 2001), which simulates the basement membrane of human epithelium (LeBleu et al., 2007). Invasion of cancer cells through the epithelium and basement membrane of the upper CAM into connective tissue was examined by Hematoxylin and Eosin [H&E] staining. DMSO-treated HT1080 cells that were loaded into 2 mm diameter plastic rings over the CAM invaded into the connective tissues through the breached basement membrane. In contrast, TFP-treated HT1080 cells failed to cross through the basement membrane. Instead, the TFP-treated cells only grew on the top of the CAM (Figure 3E). This reduction in invasion, along with the decrease in angiogenesis, suggests that TFP is effective at inhibiting invasion in vivo.

Dissection of the mechanism of TFP-inhibited cell migration via a cascade of DRD2, AKT, and β-catenin

To aid in determining the molecular mechanism by which TFP reduces cancer cell invasive ability, antibody microarrays (Kinexus) were employed using HT1080 cells treated with or without TFP. These arrays simultaneously detected the presence and relative quantities of over 500 pan-specific and over 300 phospho-site specific antibodies. The data from the antibody array
was analyzed using the DAVID bioinformatics program (Huang da et al., 2009; Huang et al., 2008). Input of the top hits from the antibody array into the DAVID program identified potential pathways involved, including the focal adhesion kinase pathway and the β-catenin pathway. Given the ability of each of these pathways to influence cell migration, we attempted to validate each pathway to determine the mechanism of action of TFP in inhibition of cancer cell migration. Upon further analysis, we found that treatment with TFP reduces the phosphorylation of β-catenin at Ser\(^{552}\) in HT1080 cells, while NIH/3T3 cells, which do not respond to TFP treatment in terms of cell migratory ability, exhibit no change in p-β-catenin\(^{552}\) (Figure 4A). Ser\(^{552}\) phosphorylation of β-catenin is associated with β-catenin stabilization and subsequent translocation to the nucleus for transcription of downstream targets, some of which are associated with invasive behavior and angiogenesis (Fang et al., 2007; Zhang et al., 2001). Accordingly, immunofluorescent staining of HT1080 cells with anti-p-Ser\(^{552}\)-β-catenin antibody resulted in significant nuclear staining of p-β-catenin in HT1080 cells treated with DMSO control. In contrast, HT1080 cells treated with TFP displayed decreased p-Ser\(^{552}\)-β-catenin in the cytoplasm and nucleus (Figure 4D). This decreased phosphorylated β-catenin was accompanied by reduced β-catenin activity examined by a TOPflash reporter luciferase assay (Figure 4B).

Since active AKT has been reported to phosphorylate β-catenin at Ser\(^{552}\) for nuclear translocation and subsequent interaction with the T-cell factor and lymphocyte enhancer factor [TCF/LEF] transcription factors (Fang et al., 2007), AKT phosphorylation at Ser\(^{473}\) and Thr\(^{308}\) was examined by Western blotting using anti-p-AKT\(^{473}\) and anti-p-AKT\(^{308}\) antibodies, respectively. When HT1080 cells were treated with TFP for 24 hours, AKT phosphorylation at both phospho-sites was decreased, suggesting that TFP may suppress activity of AKT and therefore was unable to activate β-catenin through its kinase activity (Figure 4C).
TFP effectiveness as an antipsychotic drug derives from its ability to abrogate dopamine receptor D2 (DRD2) activity (Marques et al., 2004). To determine if TFP-reduced cell migration is working through DRD2, we first surveyed DRD2 expression in cell lines that differentially respond to TFP treatment. Employing a real-time RT-PCR approach, we observed that HT1080 cells demonstrate higher expression of DRD2 as compared to NIH/3T3 cells (Figure 5A). Interestingly, NIH/3T3 cells do not respond to TFP treatment in terms of inhibition of cell migration (Figure 2A). To further extend and determine whether inhibition of cell migration by TFP is through antagonistic effects on DRD2, we used an aiRNA interference approach (Sun et al., 2008) to downregulate DRD2 expression in HT1080 cells. Effective downregulation of DRD2, achieved using DRD2 aiRNA in HT1080 cells, was demonstrated both at the mRNA and protein levels (Figure 5B and 5C). By the functional study in terms of cell migration, silencing DRD2 in HT1080 cells results in a decrease in cell migration as compared to controls (Figure 5F). Since TFP affects protein phosphorylation status of \( \beta \)-catenin\(^{\text{Ser552}} \) and AKT\(^{\text{Thr308}} \) (Fig 4A and 4C), we additionally measured these protein phosphorylation status in DRD2-silenced cells. By Western blotting using corresponding antibodies, those HT1080 cells with knockdown of DRD2 demonstrate a reduced level of p-\( \beta \)-catenin\(^{\text{Ser552}} \) and p-AKT\(^{\text{Thr308}} \) (Figure 5D and 5E), further confirming that TFP-reduced cell migration is through an antagonistic effect on DRD2.

To further support the idea that DRD2 antagonism is the cause of these molecular changes, we treated HT1080 cells with haloperidol, another FDA approved antipsychotic medication that is a known DRD2 inhibitor, followed by Western blotting analysis. Consistent with TFP treatment, cells treated with haloperidol display reduced p-\( \beta \)-catenin\(^{\text{Ser552}} \) (Figure 5G). In addition, HT1080 cells treated with haloperidol demonstrated reduced migration when assessed via scratch wound, similar to those treated with TFP (Figure 5H and 5I). These data
reinforce our conclusion that TFP-reduced cell migration is primarily through antagonistic effects on DRD2, while inhibition of alternate targets of TFP, such as calmodulin or multi-drug resistance [MDR] gene products, may not be the primary cause of a reduction in p-β-catenin$_{\text{Ser552}}$, p-AKT$_{\text{Thr308}}$, and p-AKT$_{\text{Ser473}}$. Collectively, our data shows that DRD2 is likely the direct target of TFP and inhibition of cell migration by TFP is via a DRD2-AKT-β-catenin network that ultimately modifies migratory behavior.

**DISCUSSION**

Unlike conventional high throughput screening assays for anti-cancer invasion drug discovery that used biochemical assays for targeting proteolytic activity or various 2-D migration assays for targeting cancer cell migratory machinery, our unbiased 3-D screening platform has the capacity to mimic *in vivo* conditions of cancer invasion for identification of compounds that efficiently inhibit cancer cell invasion. Utilizing this novel 3-D high throughput invasion assay, we identified TFP, an FDA approved antipsychotic drug, which was efficient at attenuating cancer cell invasion through a DRD2-AKT-β-catenin network without causing significant cell death, as a drug for preventing cancer metastasis. Our data highlight a new potential treatment strategy by use of an old drug against cancer progression.

The cancer drug discovery process has traditionally focused on identifying cytotoxic agents in lieu of compounds that reduce the mobile phenotype of invasive cancer cells. While highly effective in some hematologic malignancies, the tumor-shrinkage paradigm is not curative for most advanced solid cancers. This important point indicates that a phenotype based screening program targeting cancer invasion has the potential to yield effective, less-toxic drugs. Utilizing
the high-throughput 3-D invasion assay developed in our laboratory (Evensen et al., 2013),
Diversity Set II from the NCI collection was screened for compounds that target cancer cell
invasion, a critical determinant of cancer metastasis. Based on our initial screening, TFP is
found to be effective at reducing invasion without notable cytotoxicity, as evidenced not only by
the *in vitro* data presented here, but also by its wide clinical use and tolerable side effects.

Our findings may have implications for a novel treatment strategy in patients with cancer.
Not only is TFP safe when administered alone to patients, but it has been shown to be safe in
combination with some traditional chemotherapies and may even potentiate the cytotoxic actions
of these therapies (Polischouk et al., 2007; Sangodkar et al., 2012; Sullivan et al., 2002; Zhelev
et al., 2004). We currently propose that TFP is a valuable tool specifically for prevention of
metastases. In support of this idea, a retrospective study indicates that cancer patients
incidentally taking calcium channel antagonists, including TFP, for several months during their
treatments had delayed disease progression and increased survival rates (Zacharski et al., 1990).
The work presented here suggests that TFP has great potential because of its ability to reduce
cancer cell invasion and tumor angiogenesis as a single agent, not necessarily related to
potentiating chemotherapeutic effects or reversal of multidrug resistance or calmodulin inhibition.

The angiogenesis data presented here demonstrate that TFP can have far-reaching effects,
even in an *in vivo* context. Treatment of HT1080 cells with TFP reduced their angiogenic
potential, demonstrated by the significant decrease in neovascularization and reduction in VEGF
production. This reduction is likely not due to an effect on the surrounding endothelial cells, as
demonstrated by the network of HUVEC cells that form in the presence or absence of TFP,
indicating that the endothelial cells are not directly responding to TFP, but are responding to the
changing signals from TFP-treated HT1080 cells.
Our use of 25 μM concentration of TFP for the in vivo invasion assay utilizing the CAM is justified in that the volume that contained TFP and tumor cells was small compared to the volume of the entire chicken embryo and there is an assumption that diffusion through the fluids in the chicken embryo reduces local concentrations of TFP during a four day incubation period. Furthermore, the TFP concentration in the CAM assay shows no sign of cytotoxicity, as there is a mass of cells that grow atop the basement membrane during the length of the experiment even with higher concentration TFP exposure. Finally, there are reports that identify patient plasma levels of TFP in the range of 2-36 μM (Zhelev et al., 2004), placing our chosen higher concentration within a clinically relevant range.

Previous studies have demonstrated that the effects of TFP on neurological diseases is via antagonism of DRD2 (Marques et al., 2004). We postulate based on the data herein that the reduction in phosphorylated β-catenin and AKT is a result of inhibition of DRD2. DRD2 is a seven transmembrane domain, G-protein coupled receptor that is highly expressed in certain parts of the central nervous system, particularly the striatum, and in vasculature and the retina, amongst other organs (Beaulieu and Gainetdinov, 2011). Over several years, evidence that dopamine receptors are expressed in cancer cells outside the nervous system has been increasing. DRD2 expression has been found in abnormally proliferating Jurkat cells, prostate cancer lines (LnCAP), and lung cancer stem cells (Arvigo et al., 2010; Basu et al., 2010; Yeh et al., 2012).

Our data specifically indicates that TFP treatment of HT1080 cells leads to a decrease in phosphorylated β-catenin. Not only does inhibition of DRD2 result in this decrease, but knockdown of DRD2 in HT1080 cells recapitulates the molecular phenotype of TFP treatment. Further, we have demonstrated that β-catenin inhibition by TFP, potentially via inhibition of AKT, results in a decrease in β-catenin transcriptional activity and therefore a downregulation of
downstream targets, specifically VEGF. The antibody array indicated that proteins such as cyclin D1, c-met, and connexin were downregulated by TFP, which is relevant, considering these molecules have been tied to β-catenin activity (Ai et al., 2000; Boon et al., 2002; Tetsu and McCormick, 1999).

The phenotype assessed in this work, namely cancer cell invasion, can be considerably influenced by β-catenin activity. Several past reports have associated β-catenin activity with enhanced cell migration and invasion, primarily by upregulation of molecules that initiate the epithelial-to-mesenchymal transition (EMT), a contributing factor to the initial cell invasion step of metastasis (Thakur and Mishra, 2013). ZEB1, Twist1, and Brachyury are all examples of EMT-associated transcription factors that are direct targets of β-catenin (Arnold et al., 2000; Fernando et al., 2010; Howe et al., 2003; Sanchez-Tillo et al., 2011). These transcription factors have all been associated with a reduction in epithelial characteristics, such as a loss of E-cadherin, and an increase in mesenchymal features, such as an increase in the intermediate filament vimentin (Aigner et al., 2007; Fernando et al., 2010; Yang et al., 2004). Ultimately, reducing the stability of β-catenin can exert a great effect on the ability of cells to become motile due to the relationship between β-catenin activity and an increase in EMT. While β-catenin is a crucial molecule to cancer development and progression, this protein has traditionally been considered difficult to target because of the nature of its interacting site participating in several different interactions (Takada et al., 2012). A number of recent reports have focused on identifying specific β-catenin inhibitors, with various strategies being taken to effectively and specifically disrupt β-catenin signaling.

Interfering with β-catenin activity can also reduce products such as IL-10 that influence the ability of cancer cells to act in an immunosuppressive fashion (Yaguchi et al., 2012).
Additionally, it has been reported that cancer stem-like cells [CSCs] rely on the β-catenin pathway for self-renewal (Hsieh et al., 2013); since this particular cell type found in the heterogeneous milieu of tumors are thought to be responsible for cancer metastasis and relapse, it would be beneficial to target the pathways on which they are reliant. A recent publication that focused on lung CSCs found that TFP treatment results in a decrease in the β-catenin pathway and loss of some stem-like phenotypes (Yeh et al., 2012). In additional support of our findings here, a screening of compounds by Sachlos et al. to identify anti-CSC therapies revealed that dopamine receptors are found on CSC surfaces as well as patient breast cancer cells and antipsychotic drugs may be promising avenues of treatment (Sachlos et al., 2012). Therefore, there exists potential for agents such as TFP that reduce β-catenin target gene products to influence multiple aspects of cancer cell biology, including migration and invasion, angiogenesis, local immunosuppression, and reduction in clonogenic CSCs. Since the Wnt/β-catenin pathway is frequently dysregulated in cancer and mediates some cancer stem cell-like properties, it is desirable to inhibit this pathway (Barker and Clevers, 2006; Takahashi-Yanaga and Kahn, 2010). TFP’s ability to inhibit β-catenin signaling is valuable for a broad spectrum of cancers. We have shown that cancer cell lines of various lineages have functional responses to TFP, specifically those of fibrosarcoma and prostate origin.

Our observation of this old drug as an anti-invasive agent opens up the possibility of using an FDA-approved, clinically-used drug “off-label” for patients with cancer. The FDA approval associated with TFP implies that it is a safe drug to use in large populations and has tolerable or manageable side-effects. It has been reported that only 5% of oncology drugs that reach clinical trials will succeed to approval, but there exists hope in improving these statistics by re-examining known drugs that might have secondary applications [for review, see (Gupta et
al., 2013)]. Repurposing known small molecules is a strategy to combat the decline in introduction of new molecular entities to the market (Paul et al., 2010). Our work clearly identifies a relatively safe, known drug (TFP) as a potential anti-invasion cancer drug in addition to its traditional role as an anti-psychotic.

We have identified TFP as a potential novel therapeutic agent for invasion and metastasis via a reduction in phosphorylation of AKT and β-catenin, reducing target gene expression and invasive behavior. This drug has potential immediate translational value, as this compound has a relatively low cost and has been used extensively in other clinical situations. We have identified the potential molecular basis for the anti-invasive activity of TFP, but based on effects on other properties of cancer cells (e.g. CSC, calcium channels), the clinical effectiveness of this type of drug use in cancer, remains to be determined. Future studies are needed to determine whether the cellular and molecular changes that we demonstrated in prostate cancer and fibrosarcoma cells are recapitulated in a variety of cancer cell types.
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REFERENCES


Footnotes

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FIGURE LEGENDS

Fig. 1: Identification of the new role of TFP on inhibition of cancer cell invasion. A) Classification of the compound library according to invasive ability. Of the 1974 compounds assessed, 63 of compounds were found to inhibit cancer cell invasion at 10 μM concentration, while inhibition of cancer cell invasion by another 21 compounds was due to cytotoxicity of the compounds. B) Representative bright field images of HT1080 cells in a 3-D invasion assay demonstrates TFP reduces cell invasion at 2.5 μM concentration as compared to vehicle control. Cells in the invasion zone were compared. C) Dose dependent inhibition of HT1080 cell invasion examined by the 3-D invasion assay in the presence of different doses of TFP for 18 hours. D) No significant effect by TFP on HT1080 (left panel) and NIH/3T3 (right panel) cell growth examined in a 2-D platform by MTT assay in the presence of different concentrations of TFP up to 72 hours E) TFP at 2.5 μM concentration does not cause HT1080 cell death as examined by propidium iodide staining in the 3-D invasion assay. Staurosporine (STS) serves as a positive control for cell death.

Fig. 2: TFP inhibits cell migration without affecting protease activity. A) Transwell migration assays reveal inhibition of cancer cell migration among PC3 cells and C4-2b by TFP at 10 μM concentration. B and C) Treatment of HT1080 cells with TFP results in a significant decrease in cell migration based on wound healing analysis, while exhibiting no effect by TFP on NIH/3T3 wound closure. NIS-Elements imaging software was used for wound closure analysis. Representative images are shown. D) TFP has no effect on HT1080 cell proteolytic activities as examined by a fluorescent substrate degradation assay. E) TFP treatment of HT1080 cells does
not influence the expression of MMP-2 and MMP-9 and does not influence the activation of either of the gelatinases as observed in gelatin zymography.

**Fig. 3: TFP reduces angiogenesis and invasion examined by a chicken chorioallantoic membrane (CAM) assay.** A) Representative image of a CAM assay demonstrates that treatment of HT1080 cells with TFP reduces the ability of those cells to induce angiogenesis. B) Quantification of neovasculature induced by HT1080 cells in the CAM assay. 2.5 μM treatment results in a significant decrease in angiogenesis. C) TFP treatment reduces soluble VEGF. HT1080 cells treated with various doses of TFP demonstrate a reduction in VEGF expression as determined by VEGF ELISA. D) TFP treatment does not induce changes in HUVEC network formation on a 3-D Matrigel platform. HUVEC cells treated with 2.5 μM TFP do not have impaired network formation or change in morphology as compared to the vehicle treated endothelial cells. Bar = 100 μm E) H&E staining of a tissue section collected from the CAM assay shows that HT1080 cells implanted on the CAM surface can invade through the underlying membrane whereas TFP treatment reduces the ability of these cells to invade, but continue to grow.

**Fig. 4: TFP treatment of HT1080 cells results in a decrease in phosphorylated β-cateninSer552, AKTSer473, and AKTThr308.** A) Western blot analysis of HT1080 lysates show that TFP treatment results in a decrease in p-β-cateninSer552, while there are no changes in TFP-treated NIH/3T3 cells. Total β-catenin and α/β tubulin were used as controls. B) TFP treatment of HT1080 cells reduces the transcriptional activity of β-catenin as assessed by TOPflash/FOPflash luciferase activity, expressed in relative luciferase units (RLUs). C) Decreases of phosphorylated AKT in HT1080 cells treated with TFP (2.5 μM) for 18 hours examined by Western blot using anti-phospho-AKTSer473 and AKTThr308 antibodies, respectively. Total AKT
and α/β-tubulin were used as controls. D) Immunofluorescent staining of HT1080 cells treated with TFP shows decreased nuclear p-β-catenin^{Ser552} staining as compared to vehicle control using anti-phospho-β-catenin antibody. Nuclei were stained by Hoechst. β-catenin staining and nuclear staining were superimposed (Overlay) Bar = 50 μm. Enlarged representative images are shown in the inserts. Bar = 20 μm

**Fig. 5:** Knockdown of DRD2 results in decreased phosphorylated β-catenin^{Ser552} and AKT^{Ser473} and reduced cancer cell migration. A) Real-time RT-PCR analysis of NIH/3T3 and HT1080 cells reveals that HT1080 cells express a relatively high level of DRD2 mRNA as compared to NIH/3T3. The HPRT housekeeping gene was used as a normalization control. The experiment was repeated three times. B) Silencing of DRD2 by validated aiRNAs in HT1080 cells examined by real-time RT-PCR. The HPRT housekeeping gene was used as a normalization control. The experiment was repeated three times. C) aiRNA knockdown of DRD2 results in less DRD2 expression examined by western blot using an anti-DRD2 antibody. Mouse brain lysate was used as a positive control and non specific bands were used as loading control. D) Knockdown of DRD2 results in decreased p-β-catenin^{Ser552} as demonstrated by western blot analysis using an anti-p-β-catenin^{Ser552} antibody. Total β-catenin and β-actin were used as controls. E) p-AKT^{Thr308} is reduced when DRD2 is knocked down in HT1080 cells examined by western blotting using anti-p-AKT^{Thr308} antibody. Total AKT and actin were used as controls. F) Knockdown of DRD2 results in reduced cancer cell migration in HT1080 cells examined by a 2-D dot cell migration assay. G) Haloperidol, as well as TFP reduced p-β-catenin^{Ser552}, examined by western blotting analysis using an anti-p-β-catenin^{Ser552} antibody. Total β-catenin and β-actin were used as controls. H) TFP and haloperidol decrease HT1080 cell migration as demonstrated by a wound healing migration assay. There are no effects on NIH/3T3 cells. The experiment was
repeated three times. I) Representative images of wound healing in HT1080 and NIH/3T3 untreated, TFP-treated, and haloperidol-treated cells.