Published in final edited form as:

Pharmacol Res. 2016 September ; 111: 757–766. doi:10.1016/j.phrs.2016.07.013.

Selective GPR55 antagonism reduces chemoresistance in cancer cells

Nagendra S. Singh^a, Michel Bernier^{b,*}, and Irving W. Wainer^{a,c}

^a Laboratory of Clinical Investigation, National Institute on Aging, National Institutes of Health (NIH), Baltimore, MD 21224, USA

^b Translational Gerontology Branch, National Institute on Aging, (NIH), Baltimore, MD 21224, USA

^c Mitchell Woods Pharmaceuticals, Shelton, CT 06484, USA

Abstract

G protein-coupled receptor 55 (GPR55) possesses pro-oncogenic activity and its function can be competitively inhibited with (*R,R*′)-4′-methoxy-1-naphthylfenoterol (MNF) through poorly defined signaling pathways. Here, the anti-tumorigenic effect of MNF was investigated in the human pancreatic cancer cell line, PANC-1, by focusing on the expression of known cancer biomarkers and the expression and function of multidrug resistance (MDR) exporters such as P-glycoprotein (Pgp) and breast cancer resistance protein (BCRP). Incubation of PANC1 cells with MNF (1μM) for 24 h significantly decreased EGF receptor, pyruvate kinase M2 (PKM2), and β-catenin protein levels and was accompanied by significant reduction in nuclear accumulation of HIF-1α and the phospho-active forms of PKM2 and β-catenin. Inhibition of GPR55 with either MNF or the GPR55 antagonist CID 16020046 lowered the amount of MDR proteins in total cellular extracts while diminishing the nuclear expression of Pgp and BCRP. There was significant nuclear accumulation of doxorubicin in PANC-1 cells treated with MNF and the pre-incubation with MNF increased the cytotoxicity of doxorubicin and gemcitabine in these cells. Potentiation of doxorubicin cytotoxicity by MNF was also observed in MDA-MB-231 breast cancer cells and

* Corresponding author at: Translational Gerontology Branch, National Institute on Aging, Biomedical Research Center, 251 Bayview Boulevard, Suite 100, Baltimore, MD 21224, USA. Tel.: +1 (410) 558-8199; fax: +1 (410) 558-8381. Bernierm@mail.nih.gov (M. Bernier).
Email Addresses: nagendrasingh1980@gmail.com (N.S. Singh), iwwainer@mitchellwoods.com or iwwainer@gmail.com (I.W. Wainer).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure of Potential Conflicts of Interest

Drs. Bernier and Wainer are listed as co-inventors on an issued patent for (*R,R*′)-MNF and other fenoterol derivatives and in submitted patents for their use in glioblastomas and astrocytomas. Drs. Bernier and Wainer have assigned their rights in the patents to the US government, but will receive a percentage of any royalties that may be received by the government. Dr. Wainer is currently Chief Scientific Officer of Mitchell Woods Pharmaceuticals, which has an exclusive license for the use of (*R,R*′)-MNF and related compounds in the treatment of pancreatic, liver and brain cancers. The remaining author states no conflict of interest.

Authorship Contributions

Participated in research design: Singh, Bernier, Wainer

Conducted experiments and data acquisition: Singh,

Performed data analysis and interpretation: Singh, Bernier, Wainer

Wrote or contributed to the writing of the manuscript: Singh, Bernier, Wainer

Study supervision: Bernier, Wainer

U87MG glioblastoma cells, which express high levels of GPR55. The data suggest that inhibition of GPR55 activity produces antitumor effects via attenuation of the MEK/ERK and PI3K-AKT pathways leading to a reduction in the expression and function of MDR proteins.

Graphical abstract

Keywords

GPR55; Multidrug resistance (MDR); Pyruvate kinase M2 (PKM2); β -catenin; hypoxia-inducible factor 1 α (HIF-1 α); Cancer biomarkers

1. Introduction

While there have been significant advances in the treatment of cancer, a number of tumors are resistant to frontline chemotherapeutic agents and patients who receive chemotherapy often relapse with the development of multidrug resistance (MDR) [1, 2]. MDR is a complex interrelated phenomenon, which is dependent, in part, upon increased expression and function of ABC transporters such as P-glycoprotein (P-gp, MDR1/ABCB1), multidrug resistance protein 1-7 (MRP1-7, ABCC1-7) and breast cancer resistance protein (BCRP, ABCG2) [1, 2]. For example, pancreatic adenocarcinoma (PancCa) is the fourth leading cause of cancer death in the U.S. and has one of the worst clinical prognosis as only 6% of PancCa patients will survive beyond 5 years post diagnosis [3]. MDR contributes to the poor response to frontline treatment of PancCa [3], as enhanced expression of MRP-1, MRP-3 and MRP-5 has been associated with resistance to gemcitabine and 5-fluorouracil [4]. In the treatment of breast cancer, over 80% of the breast cancer patients who receive chemotherapy will relapse with the eventual development of MDR disease primarily associated with overexpression of Pgp, MRP1 and BCRP [5, 6].

The expression of the MDR phenotype in tumors has been linked to activation of the epidermal growth factor receptor (EGFR) and the subsequent downstream increase in ERK1/2 phosphorylation (p-ERK), which contributes to increased MDR-related protein

expression [5, 7, 8]. Cellular proliferation and MDR have also been associated with increased expression of β -catenin and hypoxia-inducible factor 1 α (HIF-1 α) [9-11]. Another key player implicated in the upregulation of genes linked to tumor proliferation is pyruvate kinase M2 (PKM2) [12, 13], whose phosphorylation by ERK2 enables its nuclear translocation [14, 15] and association with β -catenin to enhance transactivation of target genes, including cyclin D1 (CCND1) [13, 14]. Activated Wnt/ β -catenin pathway activity has been found to enhance Pgp expression in chronic myeloid leukemia [9] and cholangiocarcinoma [10], while the knockdown of β -catenin decreases BCRP expression and augments the antiproliferative effects of 5-fluorouracil in MDA-MB-468 breast cancer cells [11] and downregulates Pgp expression in glioblastoma stem cells [16]. In response to ERK-mediated phosphorylation, PKM2 binds with nuclear HIF1 α to upregulate HIF-1 α -dependent transcription [13]. Notably, HIF-1 α transactivates Pgp gene expression in breast, colon, and stomach tumors and raises MRP-2 levels in breast cancer cell lines [17]. However, the contribution of PKM2 to the expression and function of MDR proteins through its association with β -catenin and HIF-1 α pathways remains unclear.

The relationship between increased activity of the MEK/ERK and PI3K-AKT pathways with oncogenesis and elevated MDR protein expression makes these pathways important targets for drug development. One potential approach is through the inhibition of GPR55, a G protein-coupled receptor whose activation is linked to tumorigenesis via increased activity of the MEK/ERK and PI3K-AKT pathways [18-21]. Elevated expression of GPR55 mRNA has been linked to aggressiveness in human PancCa and glioblastoma tumors, and GPR55 siRNA knockdown reduced growth of a T98G glioblastoma tumor maintained as a subcutaneous tumor in mice [18]. GPR55 expression was detected in MDA-MB-231 breast cancer cell lines and incubation with the endogenous GPR55 agonist 1- α -lysophosphatidylinositol (LPI) increased cellular migration, orientation and polarization [22]. In prostate and ovarian tumor cells, LPI activation of GPR55 increased p-ERK and p-AKT concentrations, which was blocked by pre-incubation with the GPR55 antagonist cannabidiol [19]. In addition, siRNA-mediated GPR55 knockdown and treatment with the GPR55 antagonist CID 16020046 (CID) effectively blocked the LPI-mediated ovarian cancer-induced angiogenesis in an *in vivo* chicken chorioallantoic assay [23].

We recently reported that the human-derived PancCa cell line PANC-1 and the human-derived hepatocellular HepG2 cell line express GPR55 mRNA and protein [21]. In PANC-1 and HepG2 cells, knockdown of GPR55 with specific siRNAs abrogates cellular uptake of Tocrifluor 1117, a selective GPR55 ligand [21]. We have also demonstrated that treatment of these cell lines with (*R,R'*)-4'-methoxy-1-naphthylfenoterol (MNF) inhibits cellular uptake of Tocrifluor 1117 in a concentration-dependent manner and has a negative impact on GPR55 signaling, as evidenced by the significant impairment in ligand-inducible changes in p-ERK levels, cell morphology, and migration using scratch wound healing assay [21]. In addition, incubation of HepG2 cells with MNF decreased signaling through the MEK/ERK and PI3K/AKT pathways to produce cell cycle arrest and apoptosis [20].

The ability of MNF to block the inducible expression of EGFR protein by a GPR55 ligand and significantly lower GPR55-mediated activation of the EGFR-MEK/ERK cascade in PANC-1 cells [21] led us to an expanded investigation of the effect of MNF on cellular

signaling and expression of key tumor biomarkers such as PKM2 and MDR expression and function. We now report that the pharmacological inhibition of GPR55 is a viable approach to the attenuation in PKM2 nuclear accumulation and MDR protein expression in PANC-1 cells. The reduction in both the expression and function of MDR proteins results in increased doxorubicin and gemcitabine cytotoxicity. Incubation with MNF also increased the cytotoxicity of doxorubicin in MDA-MB-231 and U87MG glioblastoma cell lines. The results suggest that in addition to having anti-mitogenic properties, pharmacological inhibitors of GPR55 may be useful in the treatment of MDR disease.

2. Materials and Methods

2.1. Materials

(*R,R'*)-4-methoxy-1-naphthylfenoterol (MNF) was synthesized as described previously [24]. Radiolabeled [³H]-thymidine (10 Ci/mmol) was purchased from PerkinElmer Life Science (Waltham, MA); human recombinant insulin, AM251, doxorubicin, gemcitabine, and CID 16020046 were from Sigma-Aldrich (St-Louis, MO), while the Nuclear and Cytoplasmic Extraction Reagents (NE-PER Kit) was obtained from Thermo Fisher Scientific (Waltham, MA). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, Eagle's Minimum Essential Medium (EMEM), trypsin solution, phosphate-buffered saline (PBS), fetal bovine serum (FBS), 100X solutions of sodium pyruvate (100 mM), L-glutamine (200 mM), and penicillin/streptomycin (a mixture of 10,000 units/ml penicillin and 10,000 µg/ml streptomycin) were obtained from Quality Biological (Gaithersburg, MD).

2.2. Cell Culture

The human PancCa cell line PANC-1, glioblastoma cell line U-87 MG (U87MG), and breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from ATCC (Manassas, VA). Upon receipt of the cell lines, cells were expanded for a few passages to enable the generation of new frozen stocks. Cells were resuscitated as needed and used for fewer than 6 months after resuscitation (no more than 10 passages). ATCC performs thorough cell line authentication utilizing Short Tandem Repeat (STR) profiling.

PANC-1 and U87MG cells were maintained in DMEM with L-glutamine supplemented with 10% FBS (Hyclone, Logan, UT) and 1% penicillin/streptomycin. MDA-MB-231 cells were maintained in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin and MCF-7 cells were maintained in EMEM with L-glutamine supplemented with 10% FBS and 0.01 mg/ml human recombinant insulin. Cells were maintained in a controlled environment (37 °C under humidified 5% CO₂ in air), and the medium was replaced every 2-3 days. Prior to experiments, cells were seeded on 100 × 20 mm tissue culture plates and grown to ~70% confluency unless stated otherwise.

2.3. [³H]-Thymidine Incorporation Studies

Cells were seeded in 24-well plates at 2.5×10^4 cells per well and grown for 24 h at 37°C. The effect of MNF (0-10 µM) on [³H]-thymidine incorporation into PANC-1, MDA-MB-231, and MCF-7 cells was investigated following previously described procedures [20, 21]. The effect of MNF on doxorubicin cytotoxicity in PANC-1 cells was measured using

[³H]-thymidine incorporation. In these studies, the cells were incubated with either fresh medium containing vehicle (0.01% DMSO) or MNF (1 μ M) for 24 h. The spent media was removed and replaced with fresh media supplemented with doxorubicin (0 - 10 μ M) and the cells incubated for an additional 24 h. [³H]-Thymidine (1 μ Ci per well) incorporation into DNA was then performed for 16 h. Cells were washed with PBS, lysed in 600 μ L of 0.1 N NaOH, and the radioactivity was measured by liquid scintillation counting. Three independent experiments were conducted on separate days. Similar experiments were conducted to investigate the effect of MNF on the cytotoxicity of gemcitabine (0-10 μ M) in PANC-1 cells.

The effects of MNF (1 μ M) on the cytotoxicity of doxorubicin in MDA-MB-231 cells and of MNF (0.01 μ M) on the cytotoxicity of doxorubicin in U87MG cells were investigated using the protocol described above.

2.4. Western Blot Studies

2.4.1. Cytosolic Proteins—In the first series of experiments, PANC-1 cells were incubated with media containing vehicle (0.01% DMSO) or MNF (10, 100, 500 and 1000 nM) for 24 h. The medium was removed, and cells were collected and processed for immunoblot analysis. In a second series of experiments, PANC-1 cells were pretreated with vehicle, 1 μ M MNF or 1 μ M CID (GPR55 antagonist) for 30 min followed by the addition of 1 μ M AM251 (GPR55 agonist) for 24 h. Cell lysates were processed for immunoblot analysis, focusing on the expression of selected cancer cell biomarkers. In the third series of experiments, serum-starved PANC-1 cells were pretreated with vehicle (0.01% DMSO), 1 μ M MNF, or 1 μ M CID for 10 min followed by a 20-min incubation with and without 1 μ M AM251. Cell lysates were processed for immunoblot analysis and the levels of phospho-active (Ser473) and total forms of Akt were determined. All experiments were repeated three times on three separate days, unless stated otherwise.

2.4.2. Constitutive and Inducible Nuclear Translocation of Select Cancer Biomarkers—PANC-1 cells were serum starved for 3 h prior to treatment with vehicle, 1 μ M MNF, or 1 μ M CID for 10 min followed by a 20-min incubation with and without 1 μ M AM251. Nuclear protein extraction was carried out using a NE-PER Kit followed by the determination of the levels of HIF1 α and phospho-active forms of β -catenin (Tyr333) and PKM2 (Ser37) by immunoblotting. The same experiment was repeated three times on three separate days.

2.4.3. Western Blot Analysis—Cells were lysed in radioimmunoprecipitation buffer containing EGTA and EDTA (Boston BioProducts, Ashland, MA) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Protein concentration in clarified lysates was determined using the bicinchoninic acid reagent (Thermo Fisher Scientific). Proteins (20 μ g/well) were separated on 4-12% precast gels (Invitrogen, Carlsbad, CA) using SDS-polyacrylamide gel electrophoresis under reducing conditions and then electrophoretically transferred onto polyvinylidene fluoride membrane (Invitrogen). Western blots were performed according to standard methods, which involved a blocking step in Tris-buffered saline/0.1% Tween-20 (TBS-T) supplemented with 5% non-fat milk and incubation with

primary antibodies of interest. All membrane-bound primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, TX) and visualized by enhanced chemiluminescence (ECL Plus, GE Healthcare, Piscataway, NJ). Quantification of the protein bands was performed by volume densitometry using ImageJ software (National Institutes of Health, Bethesda, MD) and normalization to β -actin or Lamin A/C. The primary antibodies used in this study were raised against EGFR (sc-03), BCRP (sc-58222), MRP1 (sc-7773), MRP5 (sc-5780), cyclin D1 (sc-8396), β -catenin (sc-7199), and lamin A/C (sc-20681) (Santa Cruz Biotechnology); Pgp (ab129450), PKM2 (ab38237), HIF1 α (ab51608), and β -actin (ab6276) (Abcam, Cambridge, MA); phospho-Ser473 AKT (4060) and AKT (4685) (Cell Signaling Technology, Beverly, MA); phospho-Tyr333 β -catenin (#11574) and phospho-Ser37 PKM2 (#11456) antibodies were purchased from Signalway Antibody, Co. (College Park, MD). The antibodies were used at the dilution recommended by the manufacturers.

2.5. Measurement of Nuclear phospho-PKM2

PANC-1 cells were seeded on Lab-Tek II CC2 chamber slides (Thermo Scientific Nunc, Rochester, NY) and grown until they reached >70% confluence. The medium was removed and cells were incubated with serum-free medium for 3 h, after which vehicle, 1 μ M MNF or 1 μ M CID was added for 10 min. The GPR55 agonist AM251 (1 μ M) was added for 20 min where indicated. Cells were washed twice with PBS, fixed with 4% (v/v) paraformaldehyde (Sigma-Aldrich) in PBS for 20 min at room temperature, followed by 3 $\times$ PBS washes and permeabilization with 0.1% Triton X-100 (Sigma-Aldrich) for 15 min. Next, the cells were incubated with 10% goat serum for 1 h, followed by overnight incubation with rabbit polyclonal anti-phospho-PKM2 antibody (Ser37, 1:200) at 4 $^{\circ}$ C. After 3 $\times$ PBS washes, Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen; 1:1000) was added to the slides for 2 h at room temperature. The slides were washed with PBS and mounted in Prolong Gold antifade mounting medium containing DAPI (4',6-diamidino-2-phenylindole) (Invitrogen) for nuclear counterstaining. The slides were cured for 24 h at room temperature in the dark, after which images were acquired with a Zeiss LSM 710 confocal microscope using Carl Zeiss LSM software (Thornwood, NY). ImageJ software was used to quantify the changes in the nuclear pool of phospho-PKM2 levels.

2.6. Intracellular Uptake of Doxorubicin

PANC-1 and MDA-MB-231 cells were seeded on Lab-Tek II CC2 chamber slides and grown until they reached >70% confluence. The original media was removed and cells were incubated in fresh media supplemented with vehicle (0.01% DMSO) or 1 μ M MNF for 24 h, after which doxorubicin (0.125 μ M) was added for an additional 2 h. The cells were washed with PBS and the images were acquired with a Zeiss axiovert 200 microscope using standard FITC filter (#09 filter set) and Carl Zeiss LSM software. ImageJ software was used to quantify the changes in the nuclear accumulation of doxorubicin defined as ring-shaped regions of interest (ROI).

2.7. Statistical Analysis

Sigmoidal dose-response curves (IC₅₀ curves) were determined using the 'nonlinear regression (curve fit)' model contained within the Prism 4 software package (GraphPad

Software, Inc., La Jolla, CA) running on a personal computer. For immunoblot analyses, statistical comparisons between treated and control groups were performed using unpaired Student's t-tests. Data are expressed as relative fold change $\pm$ standard deviation. *P* values 0.05 were considered significant.

3. Results

3.1. MNF Inhibits Proliferation and Attenuates the Expression of Cancer Biomarkers in PANC-1 Cells

The effect of MNF on the proliferation of PANC-1 cells was assessed using a [³H]-thymidine incorporation assay. MNF produced a significant dose-dependent inhibition in cell growth (maximum of 42% reduction, *p*<0.001) with an IC₅₀ of 0.65 ± 0.25 μ M (Fig. 1). To assess whether this loss in cell growth correlated with altered expression of proteins that have pro-oncogenic activity, PANC-1 cell lysates were analyzed using immunoblotting with specific primary antibodies. A significant dose-dependent reduction in EGFR, β -catenin, PKM2, and cyclin D1 protein levels was observed at 24 h after MNF treatment (Fig. 2A and B). A significant attenuation in endogenous levels of MDR proteins, such as Pgp, BCRP, MRP1, and MRP5, was also noted in MNF-treated PANC-1 cells (Fig. 2C and D).

3.2. MNF Exerts Antitumorigenic Signaling through Inhibition of GPR55

To confirm that the inhibition of GPR55 activity was involved in the antitumorigenic effects of MNF, PANC-1 cells were pretreated with CID, a characterized pharmacological inhibitor of GPR55 [23], and then incubated with AM251, a potent GPR55 agonist [21, 25]. In a pattern similar to the one produced by MNF, cell treatment with CID significantly reduced the basal levels of EGFR, BCRP, and PKM2 proteins and blocked the induction of these cancer biomarkers by AM251 (Fig. 3A and B). Upon exposure to MNF and CID, the activating phosphorylation of AKT was significantly decreased both basally and after cell treatment with AM251 (Fig. 3C and D). These results are compatible with our previous findings showing a reduction in phosphoactive ERK levels together with attenuated cancer cell motility in response to MNF treatment and specific siRNA-mediated GPR55 knockdown [21]. Moreover, constitutive and AM251-induced nuclear accumulation of HIF-1 α and phosphoactive forms of PKM2 (Ser37) and β -catenin (Tyr333) was significantly abrogated in PANC-1 cells after 10 min of MNF and CID treatment (Fig. 3E and F). We have previously reported that treatment of PANC-1 cells with AM251 increases both EGFR mRNA and protein levels [26], although only the transcriptional effects are found to be MNF-dependent. Hence, the ability of MNF to downregulate the expression of cancer biomarkers in GPR55-expressing cancer cells may stem from inhibition of the transcriptional activity of PKM2, β -catenin and/or HIF-1 α . Of significance, immunofluorescence confocal microscopy experiments established that acute treatment with either MNF or CID markedly reduced the nuclear pool of phosphoactive form of PKM2 both in basal conditions and following cell stimulation with the GPR55 agonist, AM251 (Fig. 4A). Quantitative analysis of the immunofluorescence data is presented in Fig. 4B.

3.3. MNF Increases the Cytotoxicity of Chemotherapeutic Drugs in PANC-1 cells by Suppressing Expression of Multidrug Resistance Proteins

To determine if the MNF-associated reduction of the endogenous expression of Pgp, BCRP, MPR1 and MRP5 in PANC-1 cells (Fig. 2C and D) had functional consequences, the effect of MNF pretreatment on the anti-proliferative response of doxorubicin was assessed using a [³H]-thymidine incorporation assay. Doxorubicin is a prototypical substrate of Pgp and MDR1-5 transporters and a key probe of the MDR phenotype [27, 28]. In order to control for competitive inhibition of MDR transport by MNF, the PANC-1 cells were incubated with MNF for 24 h and the spent media was then replaced with fresh culture medium containing only doxorubicin. The data demonstrate that MNF pretreatment significantly enhanced doxorubicin cytotoxicity resulting in ~4-fold decrease in the calculated IC₅₀ value from $0.193 \pm 0.004 \mu\text{M}$ to $0.051 \pm 0.002 \mu\text{M}$ (Fig. 5A, $p < 0.001$). Resistance to the cytotoxic effects of gemcitabine, a frontline chemotherapeutic agent in PancCa treatment, has been associated with MRP5-mediated export of the drug [29]. The ability of MNF to reduce MRP5 expression in PANC-1 cells (Fig. 2C and D) led us to investigate the cytotoxic response of gemcitabine +/- MNF using the same protocol utilized with doxorubicin. The results indicate that MNF pretreatment produced a ~3-fold decrease in the calculated IC₅₀ value of gemcitabine from $0.487 \pm 0.003 \mu\text{M}$ to $0.153 \pm 0.003 \mu\text{M}$ (Fig. 5B, $p < 0.001$).

The intrinsic fluorescence of doxorubicin led us to evaluate its cellular distribution using fluorescence imaging microscopy. The results indicated an enhancement in the uptake and nuclear retention of doxorubicin in MNF-treated PANC-1 cells compared to control cells (Fig. 5C). Immunoblots of nuclear extracts revealed a marked reduction in the nuclear pool of Pgp and BCRP, two MDR-related proteins, following a 24-h treatment of PANC-1 cells with MNF (Fig. 5D).

3.4. Pretreatment with MNF Increases Doxorubicin Cytotoxicity in MDA-MB-231 Breast Cancer Cells and U87MG Glioblastoma Cells

The effect of MNF pretreatment on doxorubicin cytotoxicity was examined in two additional human tumor-derived cell lines using MDA-MB-231 breast cancer cells and U87MG glioblastoma cells. The MDA-MB-231 cell line was chosen based on previous data demonstrating a high expression of functional GPR55 [18, 22]. We also used human breast cancer cells MCF-7 that express low levels of GPR55 [22]. The effect of MNF on the proliferation of MDA-MB-231 and MCF-7 cells was assessed using a [³H]-thymidine incorporation assay. MNF incubation with MDA-MB-231 cells produced a 50% dose-dependent inhibition in cell growth with an IC₅₀ of $0.106 \pm 0.003 \mu\text{M}$, but it had no significant effect on the growth of MCF-7 cells (Fig. 6A). As observed with PANC-1 cells, MNF pretreatment resulted in an enhanced uptake and nuclear retention of doxorubicin in MDA-MB-231 cells compared to control cells (Fig. 6B and C). Consistent with this observation, incubation of MDA-MB-231 cells with MNF for 24 h followed by replacement with fresh culture medium containing only doxorubicin produced a significant enhancement of doxorubicin cytotoxicity as the calculated IC₅₀ value decreased from $0.570 \pm 0.014 \mu\text{M}$ to $0.255 \pm 0.013 \mu\text{M}$ (Fig. 6D, $p < 0.05$).

The U87MG cell line expresses GPR55 [18] and previous studies in our laboratory have established the antimetogenic effect of MNF in these cells [20]. In this study, incubation of U87MG cells with MNF for 24 h followed by replacement with fresh culture medium containing only doxorubicin, produced a significant enhancement of doxorubicin cytotoxicity as the calculated IC₅₀ value decreased from $0.0060 \pm 0.0008 \mu\text{M}$ to $0.0011 \pm 0.0005 \mu\text{M}$ (Fig. 6E, $p < 0.001$).

4. Discussion

In the current study, we demonstrate that GPR55 antagonism decreased the expression of Pgp, BCRP, MRP1 and MRP5 in a human-derived PancCa cell line, PANC-1. Moreover, treatment of PANC-1 cells with the GPR55 agonist AM251 produced a significant increase in BCRP expression, which was blocked by the two GPR55 antagonists tested, MNF and CID (Fig. 4A). Recent reports have indicated a role for basal GPR55 expression and activity on ERK1/2/MAPK signaling and the ability of GPR55 antagonists in blocking agonist-induced increases in the levels of phospho-active ERK [18, 19]. Additionally, we have previously reported that MNF-mediated inhibition of ERK1/2 phosphorylation in PANC-1 cells is specifically modulated by GPR55 antagonism [21]. These findings clearly indicate a link between GPR55 activity and MDR protein expression via the MEK/ERK pathway, which is consistent with the previously identified link between activation of the EGFR/MEK/ERK pathway and increased MDR [7].

Here, PKM2 has been found to be a key factor in linking the GPR55-MEK/ERK nexus with the expression levels of MDR proteins. PKM2 plays a key transcriptional role in metabolic regulation and enhancement in the expression of proteins associated with cellular replication and survival through β -catenin-, HIF1 α - and c-Myc-mediated transcription [13, 14]. The non-receptor tyrosine kinase c-Src mediates β -catenin tyrosine phosphorylation [14] and nuclear PKM2 interacts with tyrosine-phosphorylated β -catenin to enhance its transcriptional activity toward cyclin D1, EGFR, and Pgp [13]. Furthermore, inducible PKM2-HIF1 α interaction in the nucleus leads to higher HIF-1 α -dependent expression of Pgp, MRP1 and MRP2 [30, 31], EGFR and PKM2 [13]. PKM2 phosphorylates histone H3, which leads to the dissociation of pre-bound histone deacetylase 3 (HDAC3) accompanied by subsequent monoacetylation of histone H3 at lysine 9 and the transactivation of *CCND1* and *MYC*. Amplification of c-Myc levels is associated with increased expression of MRP1, MRP2 and BCRP [32].

Our results demonstrate that incubation of PANC-1 cells with MNF and CID led to significant reduction in nuclear PKM2 levels both under basal conditions and upon GPR55 stimulation. This effect is consistent with the GPR55/MEK/ERK pathway resulting in the ERK-mediated phosphorylation of PKM2 at Ser 37, which is necessary for PKM2 nuclear translocation [15]. It follows that the impaired formation of multiprotein complexes involving nuclear PKM2 with tyrosine-phosphorylated β -catenin and/or HIF1 α combined with lower phosphorylation of histone H3 may be responsible for the downregulation in the expression of tumor biomarkers, including MDR proteins, EGFR, and cyclin D1 as well as PKM2. Although cyclin D1 is a target gene of β -catenin (Fig. 1A), the effect of MNF on cyclin D1 was more sensitive than β -catenin. We surmise that small changes either in

posttranslational modification of β -catenin, its nuclear translocation and/or association with other transcription factors will have profound effects on its ability to induce target gene expression.

The manuscript provides mechanistic insights into the suppression of EGFR, BCRP, PKM2, and other cancer biomarkers in response to pharmacological inhibition of GPR55 with MNF and CID (Fig. 7). Firstly, inactivation of ERK has been previously demonstrated following treatment of PANC-1 cells with MNF [21], consistent with the suppression of the upstream c-Raf/MEK cascade. Our earlier work has established that GPR55 is a key target of MNF in PANC-1 cells and the human hepatocarcinoma HepG2 cells [21]. The fact that the effects of MNF can be replicated by CID further supports the notion that GPR55 is indeed a major player in the antitumorigenic actions of MNF. Thus, pharmacological inhibition of GPR55 has a clear negative impact on the MEK/ERK pathway-dependent phosphorylation and nuclear translocation of PKM2, thereby reducing pro-oncogenic gene expression. Our data also identifies a feed-forward loop in which GPR55 activation leads to increased EGFR and PKM2 expression, thus potentiating the EGFR-MEK/ERK-PKM2 effects on proliferation and MDR (Fig. 7) [14, 15].

Secondly, the proposed EGFR-MEK/ERK-PKM2 pathway does not completely explain the data obtained in this study as GPR55 antagonism with MNF and CID caused also a significant attenuation of the constitutive and GPR55-inducible increases in phosphoactive AKT levels. Sustained activation of the PI3K/AKT pathway is required for enhanced β -catenin transcriptional activity through phosphorylation of β -catenin by protein kinase A at Ser-522 [33] and the inactivation of GSK-3 β via Ser-9 phosphorylation [34, 35]. AKT-mediated inhibition of GSK-3 β activity leads to the upregulation of Pgp, MRP-4 and BCRP expression [8, 36]; conversely, impaired AKT activity (e.g., lower pAKT levels) promotes the ability of active, unphosphorylated GSK-3 β to phosphorylate β -catenin at Ser33, a modification that leads to the ubiquitination and subsequent proteolytic degradation of β -catenin [36]. The PI3K/AKT pathway has also been associated with the induction of HIF-1 α [17]. Here, the pharmacological inhibition of GPR55 was associated with impaired activation of the PI3K/AKT signaling pathway, which led to the attenuated expression and nuclear accumulation of β -catenin and HIF-1 α (Fig. 7).

The effectiveness of chemotherapy in a broad spectrum of tumors is limited by a multitude of factors including transporter-mediated MDR [1-3]. For example, incubation of human-derived PancCa tumor cell lines with standard of care agents such as gemcitabine, 5-fluorouracil, and cisplatin results in an elevation of MDR protein expression in surviving cells [4] and MDR-based resistance to frontline agents in breast cancer therapy is a well-established fact [5, 6]. One clinical strategy to overcome MDR is to co-administer inhibitors of MDR transport with the objective of increasing intracellular concentrations of the chemotherapeutic agents and achieving required therapeutic exposure [27]. An alternative strategy is to modulate MDR protein expression by targeting select tyrosine kinases such as EGFR and HER2 [37] or the Wnt-3a/GS3K/ β -catenin pathway [16]. The data from this study demonstrate that GPR55 inhibition may be an additional approach to the clinical treatment of MDR. In addition, while MDR proteins are generally assumed to be localized in the cellular membrane, MDR protein expression has been identified at subcellular sites

like mitochondria [38, 39], the perinuclear region [40] and in the nuclear membranes of glioblastoma cell lines and biopsy tissues [41, 42]. It is of interest to note that incubation with MNF and CID reduces the total and nuclear expression of MDR proteins and that the reduction of the nuclear expression of Pgp and MRP1 in PANC-1 cells is associated with increased nuclear accumulation of doxorubicin and increased cytotoxicity of doxorubicin and gemcitabine. The MNF-associated increase in cellular and nuclear accumulation of doxorubicin was also observed in MDA-MB-231 breast cancer cells as was the increase in doxorubicin cytotoxicity in MDA-MB-231 and U87MG glioblastoma cells. Both of these cell lines express GPR55 and it is reasonable to assume that the mechanisms associated with the observed effects of MNF and CID on MDR protein expression and function also contribute to increased cytotoxicity in these cell lines.

We have previously established the pharmacokinetic profile and effective dose of MNF in the rat [43] and demonstrated the effectiveness of MNF as an anticancer drug in cells and xenograft models [44]. Moreover, MNF was found to effectively block GPR55 signaling in tumor cells [21]. The compound CID inhibits agonist-induced Ca^{2+} signaling and ERK1/2 phosphorylation in HEK cells overexpressing GPR55, and blocks GPR55-mediated activation and nuclear translocation of NFAT and NF- κ B transcription factors in these cells [45]. More recently, CID was reported to reduce tumorigenic properties of ovarian and colon cancer cell lines and in *in vivo* tumor models [23, 46]. These results indicate that both MNF and CID have the potential to become effective therapeutic agents against cancers where GPR55 plays a significant role. Since PANC-1 cells are widely used in the development of pancreatic cancer therapies, the data from this study suggest that GPR55 antagonism represent a promising new approach to the treatment of pancreatic cancer. We are currently exploring the extent of this effect across multiple pancreatic cancer cell lines and patient-derived xenograft models and the results will be published elsewhere.

In conclusion, GPR55 antagonists offer a rational therapeutic approach to reversing the MDR phenotype. On the basis of our findings, we provide a bimodal model for the activity of GPR55 antagonists in PancCa cells (Fig. 7), which appears to also be applicable to other GPR55-expressing tumors. In this model, GPR55 antagonism attenuates both the constitutive and ligand-inducible activity of the MEK/ERK and PI3K/AKT pathways, which results in reduced phosphorylation and nuclear translocation of PKM2 and concomitant decrease in HIF-1 α and β -catenin levels. This, in turn, significantly downregulates MDR protein expression and disrupts feed-forward loops responsible for chemoresistance and survival in tumor cells.

Acknowledgments

This work was supported by funds from the Intramural Research Program of the National Institute on Aging at NIH. This paper is subject to the NIH Public Access Policy.

List of nonstandard abbreviations

GPR55	G protein-coupled receptor 55
MNF	(<i>R,R'</i>)-4'-methoxy-1-naphthylfenoterol

Pgp	P-glycoprotein
BCRP	breast cancer resistance protein
PancCa	pancreatic adenocarcinoma
MRP	multidrug resistance-related protein
AM251	1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide
EGFR	epidermal growth factor receptor
CID 16020046	4-[4,6-Dihydro-4-(3-hydroxyphenyl)-3-(4-methylphenyl)-6-oxopyrrolo[3,4- c]pyrazol-5(1H)-yl]-benzoic acid

References

- [1]. Persidis A. Cancer multidrug resistance. *Nat. Biotechnol.* 1999; 17:94–95. [PubMed: 9920278]
- [2]. Chang G. Multidrug resistance ABC transporters. *FEBS Lett.* 2003; 555:102–105. [PubMed: 14630327]
- [3]. Chiorean EG, Covelev AL. Pancreatic cancer: optimizing treatment options, new, and emerging targeted therapies. *Drug Des. Devel. Ther.* 2015; 9:3529–3545.
- [4]. Hagmann W, Jesnowski R, Faissner R, Guo C, Lohr JM. ATP-binding cassette C transporters in human pancreatic carcinoma cell lines. *Pancreatol.* 2009; 9:136–144. [PubMed: 19077464]
- [5]. Xu JW, Li QQ, Tao LL, Cheng YY, Yu J, Chen Q, Liu XP, Xu ZD. Involvement of EGFR in the promotion of malignant properties in multidrug resistant breast cancer cells. *Int. J. Oncol.* 2011; 39:1501–1509. [PubMed: 21805028]
- [6]. Wind NS, Holen I. Multidrug resistance in breast cancer: from in vitro models to clinical studies. *Int. J. Breast Cancer.* 2011; 2011 DOI: 10.4061/2011/967419.
- [7]. Hoffman K, Xiao Z, Franz C, Mohr E, Serba S, Büchler MW, Schemmer P. Involvement of the epidermal growth factor receptor in the modulation of multidrug resistance in human hepatocellular carcinoma cells in vitro. *Cancer Cell Int.* 2011; 11:40. [PubMed: 22088142]
- [8]. Katayama K, Noguchi K, Sugimoto Y. Regulation of P-glycoprotein/ ABCB1/MRD1 in human cancer cells. *New J. Sci.* 2014; 2014 DOI: 10.1155/2014/476974.
- [9]. Correa S, Binato R, Du Rocher B, Castelo-Branco MTL, Pizzatti L, Abdelhay E. Wnt/ β -catenin pathway regulates ABCB1 transcription in chronic myeloid leukemia. *BMC Cancer.* 2012; 12:303. [PubMed: 22823957]
- [10]. Shen D-Y, Zhang W, Zeng X, Liu C-Q. Inhibition of Wnt/ β -catenin signaling downregulates P-glycoprotein and reverses multi-drug resistance of cholangiocarcinoma. *Cancer Sci.* 2013; 104:1303–1308. [PubMed: 23822562]
- [11]. Lv X, Pang X, Jin X, Song Y, Li H. β -Catenin knockdown enhances the effects of fluorouracil in breast cancer cell line MDA-MB-468. *Biomed. Rep.* 2014; 2:910–914. [PubMed: 25279168]
- [12]. Harris I, McCracken S, Mak TW. PKM2: a gatekeeper between growth and survival. *Cell Res.* 2012; 22:447–449. [PubMed: 22184007]
- [13]. Tamada M, Suematsu M, Saya H. Pyruvate kinase M2: multiple facets for conferring benefits on cancer cells. *Clin. Cancer Res.* 2012; 18:5554–5561. [PubMed: 23071357]
- [14]. Yang W, Xia Y, Ji H, Zheng Y, Liang J, Huang W, Gao X, Aldape K, Lu Z. Nuclear PKM2 regulates β -catenin translocation upon EGFR activation. *Nature.* 2011; 480:118–122. [PubMed: 22056988]

- [15]. Yang W, Zheng Y, Xia Y, Ji H, Chen X, Guo F, Lyssiotis CA, Aldape K, Cantley LC, Lu Z. ERK1/2-dependent phosphorylation and nuclear translocation of PKM2 promotes the Warburg effect. *Nat. Cell Biol.* 2012; 14:1295–1304. [PubMed: 23178880]
- [16]. Riganti C, Salaroglio IC, Caldera V, Campia I, Kopecka J, Mellai M, Annovazzi L, Bosia A, Ghigo D, Schiffer D. Temozolomide downregulates P-glycoprotein expression in glioblastoma stem cells by interfering with the Wnt3a/glycogen synthase-3 kinase/ β -catenin pathway. *Neuro-Oncology.* 2013; 15:1502–1517. [PubMed: 23897632]
- [17]. Karakashev SV, Reginato MJ. Progress toward overcoming hypoxia-induced resistance to solid tumor therapy. *Cancer Manag. Res.* 2015; 7:253–264. [PubMed: 26316817]
- [18]. Andradas C, Caffarel MM, Pérez-Gómez E, Salazar M, Lorente M, Velasco G, Guzmán M, Sánchez C. The orphan G protein-coupled receptor GPR55 promotes cancer cell proliferation via ERK. *Oncogene.* 2011; 30:245–252. [PubMed: 20818416]
- [19]. Piñeiro R, Maffucci T, Falasca M. The putative cannabinoid receptor GPR55 defines a novel autocrine loop in cancer cell proliferation. *Oncogene.* 2011; 30:142–152. [PubMed: 20838378]
- [20]. Paul RK, Ramamoorthy A, Scheers J, Wersto RP, Toll L, Jimenez L, Bernier M, Wainer IW. Cannabinoid receptor activation correlates with the proapoptotic action of the β 2-adrenergic agonist (R,R')-4-methoxy-1-naphthylfenoterol in HepG2 hepatocarcinoma cells. *J. Pharmacol. Exp. Ther.* 2012; 343:157–166. [PubMed: 22776956]
- [21]. Paul RK, Wnorowski A, Gonzalez-Mariscal I, Nayak SK, Pajak K, Moaddel R, Indig FE, Bernier M, Wainer IW. (R,R')-4'-methoxy-1-naphthylfenoterol targets GPR55-mediated ligand internalization and impairs cancer cell motility. *Biochem. Pharmacol.* 2014; 87:547–561. [PubMed: 24355564]
- [22]. Ford LA, Roelofs AJ, Anavi-Goffer S, Mowat L, Simpson DG, Irving AJ, Rogers MJ, Rajnicek AM, Ross RA. A role for L- α -lysophosphatidylinositol and GPR55 in the modulation of migration, orientation and polarization of human breast cancer cells. *Br. J. Pharmacol.* 2010; 160:762–771. [PubMed: 20590578]
- [23]. Hofmann NA, Yang J, Trauger SA, Nakayama H, Huang L, Strunk D, Moses MA, Klagsbrun M, Bischoff J, Graier WF. The GPR55 agonist, L- α -lysophosphatidylinositol, mediates ovarian carcinoma cell-induced angiogenesis. *Br. J. Pharmacol.* 2015; 172:4107–4118. [PubMed: 25989290]
- [24]. Jozwiak K, Khalid C, Tanga MJ, Berzetei-Gurske I, Jimenez L, Kozocas JA, Woo A, Zhu W, Xiao RP, Abernethy DR, Wainer IW. Comparative molecular field analysis of the binding of the stereoisomers of fenoterol and fenoterol derivatives to the β 2-adrenergic receptor. *J. Med. Chem.* 2007; 50:2903–2915. [PubMed: 17506540]
- [25]. Kapur A, Zhao P, Sharir H, Bai Y, Caron MG, Barak LS, Abood ME. Atypical responsiveness of the orphan receptor GPR55 to cannabinoid ligands. *J. Biol. Chem.* 2009; 284:29817–29827. [PubMed: 19723626]
- [26]. Fiori JL, Sanghvi M, O'Connell MP, Krzysik-Walker SM, Moaddel R, Bernier M. The cannabinoid receptor inverse agonist AM251 regulates the expression of the EGF receptor and its ligands via destabilization of oestrogen-related receptor G protein. *Br. J. Pharmacol.* 2011; 164:1026–1040. [PubMed: 21449913]
- [27]. Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. *J. Nat. Cancer Inst.* 2000; 92:1295–1302. [PubMed: 10944550]
- [28]. Pérez-Tomás R. Multidrug resistance: retrospect and prospects in anti-cancer drug treatment. *Curr. Med. Chem.* 2006; 13:1859–1876. [PubMed: 16842198]
- [29]. Hagmann W, Jesnowski R, Löhr JM. Interdependence of Gemcitabine Treatment, Transporter Expression, and Resistance in Human Pancreatic Carcinoma Cells. *Neoplasia.* 2010; 12:740–747. [PubMed: 20824050]
- [30]. Zhu H, Chen XP, Luo SF, Guan J, Zhang WG, Zhang BX. Involvement of hypoxia-inducible factor-1- α in multidrug resistance induced by hypoxia in HepG2 cells. *J. Exp. Clin. Cancer Res.* 2005; 24:565–574. [PubMed: 16471319]
- [31]. Chen M, Huang SL, Zhang XQ, Zhang B, Zhu H, Yang VW, Zou XP. Reversal effects of pantoprazole on multidrug resistance in human gastric adenocarcinoma cells by down-regulating

the V-ATPases/mTOR/HIF-1 μ P-gp and MRP1 signaling pathway in vitro and in vivo. *J. Cell. Biochem.* 2012; 113:2474–2487. [PubMed: 22396185]

- [32]. Wook KW, Im YB, Go WJ, Han HK. cMyc amplification altered the gene expression of ABC- and SLC-transporters in human breast epithelial cells. *Mol. Pharm.* 2009; 6:627–633. [PubMed: 19239227]
- [33]. Fang D, Hawke D, Zheng Y, Xia Y, Meisenhelder J, Nika H, Mills GB, Kobayashi R, Hunter T, Lu Z. Phosphorylation of β -catenin by AKT promotes β -catenin transcriptional activity. *J. Biol. Chem.* 2007; 282:1122–1129.
- [34]. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated protein kinase B. *Nature.* 1995; 378:785–789. [PubMed: 8524413]
- [35]. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3 β regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.* 1998; 12:3499–3511. [PubMed: 9832503]
- [36]. Lim JC, Kania KD, Wijesuriya H, Chawla S, Sethi JK, Pulaski L, Romero IA, Couraud PO, Weksler BB, Hladky SB, Barrand MA. Activation of β -catenin signaling by GSK-3 increases p-glycoprotein expression in brain endothelial cells. *J. Neurochem.* 2008; 106:1855–1865. [PubMed: 18624906]
- [37]. Zhang Y-K, Wang Y-J, Gupta P, Chen Z-S. Multidrug resistance proteins (MRPs) and cancer therapy. *Amer. Assn. Pharmaceut. Sci. J.* 2015; 17 doi: 10.1208/s12248-015-9757-1.
- [38]. Takada T, Suzuki H, Gotoh Y, Sugiyama Y. Regulation of the cell surface expression of human BCRP/ABCG2 by the phosphorylation state of Akt in polarized cells. *Drug Metab. Dispos.* 2005; 33:905–909. [PubMed: 15843490]
- [39]. Solazzo M, Fantappiè O, D'Amico M, Sassoli C, Tani A, Cipriani G, Bogani C, Formigli L, Mazzanti R. Mitochondrial expression and functional activity of breast cancer resistance protein in different multiple drug-resistant cell lines. *Cancer Res.* 2009; 69:7235–7242. [PubMed: 19706772]
- [40]. Lemos C, Kathmann I, Giovannetti E, Calhau C, Jansen G, Peters PG. Impact of cellular folate status and epidermal growth factor receptor expression on BCRP/ABCG2-mediated resistance to gefitinib and erlotinib. *Br. J. Cancer.* 2009; 100:1120–1127. [PubMed: 19277036]
- [41]. Bhatia P, Bernier M, Sanghvi M, Moaddel R, Schwarting R, Ramamoorthy A, Wainer IW. Breast cancer resistance protein (BCRP/ABCG2) localizes to the nucleus in glioblastoma multiforme cells. *Xenobiotica.* 2012; 42:748–755. [PubMed: 22401348]
- [42]. Habicht KL, Frazier C, Singh NS, Shimmo R, Wainer IW, Moaddel R. The synthesis and characterization of a nuclear membrane affinity chromatography column for the study of human breast cancer resistant protein (BCRP) using nuclear membranes obtained from the LN-229 cells. *J. Pharm. Biomed. Anal.* 2013; 72:159–162. [PubMed: 23146242]
- [43]. Siluk D, Mager DE, Kim HS, Wang Y, Furimsky AM, Ta A, Iyer LV, Green CE, Wainer IW. Pharmacokinetics and metabolism of (R,R)- methoxyfenoterol in rat. *Xenobiotica.* 2010; 40:195–206. [PubMed: 20039779]
- [44]. Bernier M, Paul RK, Dossou KS, Wnorowski A, Ramamoorthy A, Paris A, Moaddel R, Cloix JF, Wainer IW. Antitumor activity of (R,R')-4-methoxy-1-naphthylfenoterol in a rat C6 glioma xenograft model in the mouse. *Pharmacol. Res. Perspect.* 2013; 1:e00010. [PubMed: 25505565]
- [45]. Kargl J, Andersen L, Hasenöhl C, Feuersinger D, Stan i A, Fauland A, Magnes C, El-Heliebi A, Lax S, Uranitsch S, Haybaeck J, Heinemann A, Schicho R. GPR55 promotes migration and adhesion of colon cancer cells indicating a role in metastasis. *Br. J. Pharmacol.* 2016; 173:142–154. [PubMed: 26436760]
- [46]. Kargl J, Brown AJ, Andersen L, Dorn G, Schicho R, Waldhoer M, Heinemann A. A selective antagonist reveals a potential role of G protein-coupled receptor 55 in platelet and endothelial cell function. *J. Pharmacol. Exp. Ther.* 2013; 346:54–66. [PubMed: 23639801]

Figure 1. MNF treatment reduces PANC-1 cell proliferation. Thymidine incorporation was assessed into PANC-1 cells treated with increasing concentrations of MNF (0-1 μ M) for 24 h. Data are expressed as mean $\pm$ SD from 3 independent experiments.

Figure 2.

MNF dose-dependently reduces expression of cancer biomarkers in PANC-1 tumor cells.

(A) Representative immunoblot showing protein levels of EGFR, β -catenin, PKM2, cyclin D1, and β -actin in PANC-1 cells at 24 h after MNF treatment. (B) Protein bands were quantified by densitometry and the data normalized to β -actin levels and plotted relative to cells treated with 0.01% DMSO as control. (C) Representative experiment showing protein levels of PgP, BCRP, MRP1, MRP5, and β -actin in PANC-1 cells at 24 h after MNF treatment. (D) Densitometric analysis of the protein bands was performed as in (B). Data are expressed as mean $\pm$ SD from 3 independent experiments. *, **, *** $P < 0.05$, < 0.01 , and < 0.001 vs. control.

Figure 3.

GPR55 promotes tumorigenesis by upregulating expression of cancer biomarkers. (A) Representative immunoblot depicting protein levels of EGFR, BCRP, PKM2, and β -actin in PANC-1 cell lysates at 24 h after cell pretreatment with MNF (1 μ M) or the GPR55 antagonist CID 16020046 (CID, 1 μ M) for 30 min followed by treatment without or with AM251 (1 μ M). (B) Protein bands were quantified by densitometry and the data normalized to β -actin levels and plotted relative to cells treated with 0.01% DMSO as control. (C) Representative immunoblot depicting phosphoactive (pSer) and total forms of AKT in PANC-1 cells after pretreatment with MNF or CID for 10 min followed by a 20-min incubation with and without AM251. (D) Densitometric analysis of the protein bands was performed and the ratio pSer-AKT/total AKT calculated and plotted relative to cells treated with 0.01% DMSO as control. (E) Representative immunoblot depicting protein levels of phospho-active forms of PKM2 (pPKM2) and β -catenin (p β -catenin), and those of HIF-1 α and lamin A/C in PANC-1 nuclear extracts after cell pretreatment with MNF or CID for 10 min, followed by a 20-min treatment with and without AM251. (F) Densitometric analysis was performed as in (B) and the data normalized to lamin A/C levels. Data are expressed as mean $\pm$ SD from 3 independent experiments. *, **, *** $P < 0.05$, <0.01 , <0.001 vs. control.

Figure 4.

GPR55 targets phospho-active PKM2 to the nucleus in PANC-1 cells. (A) Immunofluorescence confocal images depicting nuclear pool of phospho-active PKM2 (p-PKM2) after cell pretreatment with MNF (1 μ M) or CID (1 μ M) for 10 min followed by a 20-min treatment without (control) and with AM251 (1 μ M). White arrows indicate the accumulation of nuclear p-PKM2 or its lack thereof in response to various treatments. (B) Densitometric analysis indicating the nuclear levels of p-PKM2 normalized to control samples. Data are expressed as mean $\pm$ SD from 3 independent experiments. *** $P < 0.001$ vs. control.

Figure 5.

MNF increases the cytotoxicity of chemotherapeutic drugs by suppressing expression of multidrug resistance proteins. (A) Thymidine incorporation into PANC-1 cells treated with MNF (1 μM) for 24 h and followed with 0-10 μM of doxorubicin for 24 h. (B) Thymidine incorporation into PANC-1 cells as in (A) but with 0-10 μM of gemcitabine for 24 h. Values were normalized to PANC-1 cells treated with 0.01% DMSO as control. Data are expressed as mean ± SD from 3 independent experiments. (C) Fluorescence imaging of intracellular doxorubicin in PANC-1 cells pretreated with MNF (1 μM) for 24 h and followed with 0.125 μM of doxorubicin treatment for 2 h. (D) (*upper panel*), Representative immunoblot depicting protein levels of Pgp, BCRP, and lamin A/C in PANC-1 nuclear extracts after cell treatment with 0.5 and 1 μM of MNF for 24 h; (*lower panel*), protein bands were quantified by densitometry and the data normalized to lamin A/C levels and plotted relative to cells treated with 0.01% DMSO as control.

Figure 6. MNF lowers mitogenesis and reduces chemoresistance in cancer cell lines

(A) Thymidine incorporation was carried out in human MCF-7 and MDA-MB-231 breast cancer cell lines after a 24-h incubation with increasing concentrations of MNF. Bars represent the mean $\pm$ SD (n=3 triplicate dishes) of a representative experiment. (B) Fluorescence imaging of intracellular doxorubicin in MDA-MB-231 cells pretreated with vehicle or MNF (1 μM) for 24 h, followed with 0.125 μM of doxorubicin treatment for 2 h. (C) Quantitative determination of fluorescence intensity from three independent experiments, *** P < 0.001. (D) Thymidine incorporation into MDA-MB-231 cells treated without or with MNF (1 μM) for 24 h and followed with 0-10 μM of doxorubicin for 24 h. Data points represent the mean $\pm$ SD (n=3 triplicate dishes) of a representative experiment. (E) Thymidine incorporation into human U87MG glioma cells treated without or with MNF (0.01 μM) for 24 h, followed with the indicated concentrations of doxorubicin for 24 h. Data points represent the mean $\pm$ SD (n=3 triplicate dishes) of a representative experiment

Figure 7.

Diagram depicting the anti-tumorigenic function of GPR55 antagonists and consequent reduction in multidrug resistance. Upon agonist engagement of GPR55 with L- α -lysophosphatidylinositol (LPI) or AM251, there is increase in PI3K/AKT and c-Raf/MEK/ERK signaling pathways, which are responsible for the activation and nuclear translocation of β -catenin and PKM2, and formation of transcriptionally competent complexes on the promoters of pro-oncogenic target genes. The inhibition of GSK3 β by AKT stabilizes β -catenin by preventing its phosphorylation-coupled proteolytic degradation. Pharmacological inhibition of GPR55 with MNF and CID 16020046 (CID) promotes the dephosphorylation of both signaling pathways with subsequent impairment in PKM2, β -catenin and HIF-1 α -dependent regulation of PanCa cell chemoresistance and behavior.