Garcinol sensitizes human pancreatic adenocarcinoma cells to gemcitabine in association with microRNA signatures

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Background: Alterations in microRNA (miRNA/miR) genes are of biological importance in the pathophysiology of cancers, including pancreatic cancer (PaCa). Although growing evidence supports the role of miRNA in cancer, their response to dietary phytochemicals is less known. Previously, we showed that garcinol induces PaCa cell growth arrest and apoptosis in vitro. The present study, discusses chemo-sensitization by garcinol in synergism with first-line PaCa drug, gemcitabine. The miRNA expression profile of gemcitabine-resistant Panc-1 cells treated with garcinol and/or gemcitabine was also evaluated.

Methods and results: Garcinol synergizes with gemcitabine to inhibit cell proliferation and induce apoptosis in PaCa cells with significant modulation of key cancer regulators including PARP, VEGF, MMPs, ILs, caspases, and NF-κB. In addition, biostatistical analyses, quantitative reverse transcription PCR data, and in silico modeling using TargetScan5, PicTar, and DNA intelligent analysis, microT-V.B4 database showed that these two agents modulated a number of microRNAs (miR-21, miR-196a, miR-495, miR-605, miR-638, and miR-453) linked to various canonical oncogenic signaling pathways.

Conclusion: We identified garcinol-specific miRNA biomarkers that sensitize PaCa cells to gemcitabine treatment, thus attenuating the drug-resistance phenotype. These results prompt further interest in garcinol and gemcitabine combination strategy as a drug modality to improve treatment outcome in patients diagnosed with PaCa.

Keywords: Chemosensitization / Garcinol / Gemcitabine / MicroRNA / Pancreatic cancer

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1 Introduction

Pancreatic cancer (PaCa) is the fourth leading cause of cancer death in the United States with an overall survival rate of around 5% for all tumors. Of the estimated 43,920 new PaCa patients diagnosed in the United States, 37,390 death will occur due to this disease in 2012 [1]. Gemcitabine, the standard clinical chemotherapeutic agent available for advanced PaCa treatment offers a tumor response rate of approximately 12% with a median survival time of 5 months, providing some relief to patients [2]. However, there has been limited advantage due to dose-limiting toxicity to normal tissues and acquired resistance to therapy. An adjuvant therapy in synergism with other agents targeting chemoresistance-related multifactorial events might restore sensitivity, providing long-term benefits. One of the several approaches is to use dietary agents as a neoadjuvant with a standard chemotherapeutic drug.
Recently, we reported the beneficial effects of garcinol, a polyisoprenylated benzophenone, against PaCa cells harboring different molecular signatures [3,4].

Deregulation of multiple pathways leads to tumor formation. In recent years, microRNAs (miRNAs/miRs) have been implicated in several cancers, including PaCa [5–7]. These small, nongenic RNA sequences modulate cellular mechanisms through posttranscriptional regulation by either degrading or repressing the expression of messenger RNAs (mRNAs) that would otherwise code for critical proteins involved in cancer development and progression [8]. Since miRNAs can regulate hundreds of genes simultaneously, it is important to investigate the alterations in miRNA profile in PaCa and their effects under the influence of therapeutic interventions. Recently, miRNAs have also been reported to be altered by various dietary agents in various cancers [9–12].

In the present study, we investigated the synergistic effects of these agents, garcinol and gemcitabine on cell viability, apoptosis, and identified miRNA signatures specific to the chemoprotective effects of garcinol alone and in combination with gemcitabine in PaCa cells. Using an in silico approach, we identified regulators of multiple drug resistance and other cancer promoting pathways as key targets of these miRNA biomarkers. We further validated our findings using biochemical assays to confirm anti-tumorigenic effects of these agents by evaluating key molecules regulating these events in the cell. Overall, these biomarkers may be further explored and used as therapeutic targets to sensitize PaCa cells to chemotherapeutics.

2 Materials and methods

2.1 Cell culture

Human pancreatic adenocarcinoma cell lines, BxPC-3 and Panc-1 were obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in continuous exponential growth by twice a week passage in DMEM (Cellgro Manassas, VA) and supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 10 mg/mL streptomycin (Sigma Chemical Co., St. Louis, MO) in a humidified incubator containing 5% CO₂ in air at 37°C. Cells were harvested regularly before attaining 70–80% confluence.

2.2 Antibodies and reagents

Garcinol (≥95% [TLC], Biomol International, USA) was dissolved in DMSO to make 20 mM stock solution. Gemcitabine (El Lilly) was dissolved in sterile 1X PBS to make 100 µM stock solution. Dilutions were made as per the treatment requirement. Antibodies were obtained from the following commercial sources: anti-Bcl-xL antibody from Trevigen Inc. (Gaithersburg, MD); anti-PARP antibody from Biomol Research (Plymouth, PA) and anti-β-actin antibody from Sigma Chemical Co. (St. Louis, MO).

2.3 Cell viability

Cells were seeded at a density of 3 × 10⁴ cells/well in 96-well micro-titer culture plates. After overnight incubation the medium was replaced with fresh medium containing different concentrations of garcinol (0–20 µM) and/or gemcitabine (0–500 nM). At the end of 48 h, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt) solution was added to each well and incubated further for 1 h. Color development was measured spectrophotometrically at 595 nm on a plate reader (BIO-TEK Instruments) and quantified as per the manufacturer’s protocol (Promega, USA). Cell viability has been expressed as a percentage, for each treatment group relative to untreated control PaCa cells.

2.4 Quantification of apoptosis

The Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA) was used to detect apoptosis according to manufacturer’s protocol. Briefly, after 48-h treatment of BxPC-3 and Panc-1 cells with different concentrations of garcinol (0–20 µM) and/or gemcitabine (0–500 nM), the cytoplasmic histone/DNA fragments from cells were extracted and bound to immobilized anti-histone antibody. Subsequently, a peroxidase-conjugated anti-DNA antibody was used for detection of immobilized histone/DNA fragments. After addition of the peroxidase substrate, the absorbance by the samples was determined at 405 nm with an ULTRA Multifunctional Microplate Reader (BIO-TEK Instruments). Enrichment of apoptotic bodies was measured as a ratio of treated cells relative to untreated control PaCa cells.

2.5 Analysis of cytotoxic synergy

BxPC-3 and Panc-1 cells were plated as described above and allowed to attach overnight. The culture medium was replaced with fresh medium containing different concentrations of garcinol (0–40 µM) and/or gemcitabine (0–1000 nM) in ratios of 50:1, 100:1, and 200:1. The effect on cell growth was observed by the MTS assay as described above. Cytotoxic synergy induced by garcinol and gemcitabine was analyzed by CalcuSyn software (Biosoft, Cambridge, UK) that is based on the model developed by Chou and Talalay [13]. Briefly, isobologram analysis yields combination index (CI) values that are a measure of synergism. A CI = 1 indicates additive effect, < 1 indicates synergistic effect and > 1 suggests antagonistic effect [14].
2.6 Morphological changes

Morphological changes characteristic of apoptosis were determined by DAPI (4', 6-diamidino-2-phenylindole) staining as per manufacturer’s protocol (Invitrogen, USA). Briefly, 5 × 10⁵ cells (BxPC-3 and Panc-1) were seeded into 6-well plates containing 1–2 mL medium. Upon 60–70% confluency, cells were treated with different concentrations of garcinol (0–20 μM) and/or gemcitabine (0–500 nM) and incubated for another 48 h. Cells were harvested by trypsinization, washed with 1X PBS and subsequently incubated with DAPI at room temperature in dark for 30 min. Cells were stained with Prolong Gold Antifade reagent and visualized under Fluorescence Microscope (Nikon Eclipse, 80i) with an Excitation maximum at 358 nm and an Emission maximum at 461 nm and scored.

2.7 MiRNA extraction

Adhered human PaCa Panc-1 cells were treated with 10 μM garcinol and/or 100 nM gemcitabine for 36 h, harvested, washed with 1X PBS and homogenized on ice with QIAzol lysis buffer (miRNase kit, Qiagen, Valencia, CA, USA). Cell homogenates were flash-frozen and miRNA isolation was performed as per the manufacturer’s instructions. Briefly, cell homogenates were centrifuged with chloroform for 15 min at 12,000 × g at 4°C for phase separation followed by ethanol treatment and a series of washes using wash buffers provided in the kit. Samples were eluted in water and RNA quality and integrity was evaluated from the absorbance at 260 and 280 nm (260/280 ratio > 1.9).

2.8 MiRNA microarrays

Microarray analyses were performed at LC Sciences (Houston, TX). Briefly, the assay started from 2 μg total RNA (quantified by 260 nm/280 nm ratio followed by bioanalyzer), 3'-extended with a poly(A) tail using poly(A) polymerase. Hybridization was performed overnight on a μParaflo microfluidic chip probed with a chemically modified nucleotide coding segment complementary to miRNA transcripts listed in Sanger miRBase Release 7.1 (http://miRBase.org). Fluorescence images were collected using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA) and digitized using Array-Pro image analysis software (Media Cybernetics, Bethesda, MD). Cluster plots were generated using software from The Institute for Genomic Research (San Diego, CA, USA).

2.9 Data analysis

The differentially expressed transcript signal values were derived by background subtraction from raw data, log transformed, normalized using a locally weighted regression filter, and analyzed with a p-value < 0.05. All the experiments were done in triplicate for each of the observations and data generated were compared among the various experimental groups by volcano scatter plot analysis [15]. Representative miRNAs from each treatment group were selected based on fold variations, and statistical significance (p < 0.05) for further analysis. For quantitative reverse transcription PCR (qRT-PCR) data analysis, each of the dataset represents the mean ± SD from three separate experiments. To analyze the statistical significance of the differences found in the data, analysis of variance was done.

2.10 MiRNA (qRT-PCR)

Representative miRNA expressions were validated by qRT-PCR using TaqMan MicroRNA Assay kit (Applied Biosystems) following the manufacturer’s protocol. Briefly, reverse transcription master mix was prepared by mixing 100 mM dNTP mix, reverse transcriptase, 10 × RT buffer, RNase inhibitor and nuclease-free water. Each 15 μL reaction consisted of 7 μL of Mastermix prepared above, 3 μL respective primer (Supporting Information Table 1) and 5 μL total RNA sample (5 ng/15 μL reaction). The thermocycler was programmed as per the manufacturer’s protocol. The PCR program was initiated by 10 min at 95°C before 40 thermal cycles; each for 15 s at 95°C and 1 min at 60°C. Data was analyzed according to the comparative Ct method and normalized to RNU6B expression for each sample.

2.11 Target analyses

The candidate miRNAs from each treatment group were chosen for target prediction and analyzed using target prediction tools, TargetScan software (http://www.targetscan.org/), PicTar software (http://picTar.mdc-berlin.de/), and the DNA Intelligent Analysis, microT-4.0–beta version (http://diana.cslab.ece.ntua.gr/). These algorithms are based on the principle of comparative genome hybridization and help in identification of targets based on comparative sequence analysis, seed match complementation and Z-score for assigned untranslated regions (UTR). Human genes related to cancer pathways were selected using the above-mentioned tools. Finally, genes predicted as targets of candidate miRNAs were aligned and matched for confirmation using the Sanger database.

2.12 Caspase activity

Cell death was measured in whole-cell lysates prepared from samples treated with different concentrations of garcinol (0–10 μM) or gemcitabine (0–500 nM), using commercially available assay kit (R&D Assay System, Minneapolis, MN).
according to manufacturer’s instruction. Briefly for both the assays, cells were treated for 48 h and cell pellets were collected using extraction buffer and diluted using the calibrator diluents provided with the kit. Sample or standard were added to the plate, covered and incubated at room temperature for 2 h. After a series of washing with wash buffers provided, samples were incubated with Caspase 3/9 conjugates followed by 30-min incubation in dark with the substrate solution. The reaction was stopped using stop solution and mixed by gentle tapping. Optical density was determined using a microplate reader at 450 nm with wavelength correction set at 540 nm (BIO-TEK Instruments).

2.13 Protein extraction and Western blot analysis

Panc-1 cells were plated in 100 mm dish and allowed to attach for 36 h followed by treatment with 10 μM garcinol and/or 100 nM gemcitabine and incubated for 48 h. Control cells were incubated in the medium with equivalent concentration of DMSO. After the incubation period, the cells were harvested in sterile 1X PBS. Cellular lysates were prepared by suspending the cells in 200 μL of lysis buffer (150 mM NaCl, 1 mM EGTA, 0.1% Triton X-100; 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/mL leupeptin, 2 μg/mL aprotinin). The cells were disrupted by sonication and extracted at 4°C for 30 min at maximal microfuge speed to remove debris [16]. For Western blot analysis, each extract equivalent to 35 μg total protein was separated on SDS-PAGE, electro-transferred onto nitrocellulose membranes and probed with specific antibodies. Detection of specific proteins (PARP, Bcl-xL, and β-actin) was carried out with an enhanced chemiluminescence Western blotting kit according to manufacturer’s instructions (Pierce Biotechnology, USA).

2.14 Electrophoretic mobility shift assay

Nuclear extracts were prepared according to the method described by Chaturvedi et al. [17]. Briefly, the cells were washed with cold 1X PBS and suspended in 0.15 mL of lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.5 mg/mL benzamidine]. The cells were allowed to swell on ice for 20 min followed by addition of 4.8 μL of 10% Nonidet P-40. The nuclear pellet was resuspended in nuclear extraction buffer [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.5 mg/mL benzamidine] and incubated on ice. The tubes were centrifuged for 5 min in a microfuge at 4°C, and supernatant (nuclear extract) was collected in a cold eppendorf tube and stored at –70°C for later use. The protein content was measured by BCA method. EMSA was performed by incubating 10 μg of nuclear extract with IRDyeTM–700 labeled NF-κB oligonucleotide. The incubation mixture included 2 μg of poly (dI-dC) in a binding buffer. The DNA-protein complex formed was separated from free oligonucleotide on 8.0% native polyacrylamide gel using buffer containing 50 mM Tris, 200 mM glycine, pH 8.5, and 1 mM EDTA, and then visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1.

2.15 VEGF, MMP-9, IL-8 assay

VEGF, MMP-9, and IL-8 levels were measured in conditioned media collected from 1 × 10⁶ cells treated for 48 h with different concentrations of garcinol (0–20 μM) or gemcitabine (0–500 nM), using a commercially available assay kit (R&D Assay System, Minneapolis, MN) according to manufacturer’s instruction. Briefly, cell culture supernates were collected and particulates removed by centrifugation. Samples were stored at –20°C until further use. VEGF and IL-8: Each well was soaked with the assay diluent followed by addition of standard, control or sample solution and incubated for 2 h at room temperature. After a series of washing steps, the conjugate solution was added to each well, covered and incubated at room temperature for 2 h. This was followed by addition of substrate solution and stop solution and incubation. The plate was read at 450 nm using a correction wavelength of 570 nm with the help of a microplate reader (BIO-TEK Instruments). MMP-9: Each well was soaked with the assay diluent followed by addition of standard, control or sample solution and incubated for 2 h at room temperature on a horizontal orbital microplate shaker set at 500 ± 50 rpm. Following a series of washing steps, p-aminophenylmercuric acetate solution was added and plates were incubated for 2 h at 37°C in a humidified environment. Substrate solution was added and plate was incubated for 17–20 h at 37°C in a dark, humidified environment. The relative fluorescence units of each well were determined using a fluorescence plate reader: excitation wavelength set to 320 nm and emission wavelength set to 405 nm; endpoint mode; 1 × 20 mS integration time; plate speed = 6.

2.16 Clonogenic assay

To test the survival of cells treated with garcinol and/or gemcitabine Panc-1 cells were plated (50 000–100 000 per well) in a 6-well plate and incubated overnight at 37°C. After 72-h exposure to either 10 μM garcinol and/or 100 nM gemcitabine, the cells were trypsinized, and viable cells were counted (trypsin blue exclusion) and plated in 100 mm petri dishes in a range of 100–1000 cells to determine the plating efficiency and assess the effects of treatment on clonogenic survival. The cells were then incubated for 12 days at 37°C in a 5% CO₂/5% O₂/90% N₂ incubator. The colonies were stained with 2% crystal violet and counted. The surviving fraction was
normalized to untreated control cells with respect to clonogenic efficiency [16].

2.17 Cell adhesion assay

Ninety-six-well plates were coated with Laminin-1 (10 μg/mL) at 4°C overnight. After two washes with washing buffer (0.1% BSA in DMEM medium), the plates were blocked with 0.5% BSA in DMEM medium and kept in an incubator at 37°C for 1 h. Plates were washed with washing buffer and chilled on ice. A 50 μL of garcinol (0–20 μM) and/or gemcitabine (0–500 nM) -treated Panc-1 cells (4 × 10⁵ cells/mL) were added to each well and incubated in an incubator at 37°C for 30 min. The plates were centrifuged at 2000 rpm for 15 s and washed with washing buffer thrice before fixing it with 4% paraformaldehyde and incubated at room temperature for 15 min. The plates were washed with washing buffer and stained for 10 min with crystal violet stain, rinsed with water and drained off to get dry plates. The plates were incubated with 2% SDS for 30 min and read at 550 nm.

2.18 Wound healing assay

Cells were cultured in 35 mM cell culture dishes and maintained until 90% confluency was achieved. A uniformly thick scratch was made on the dish using a sterile pipette tip to create a “wound” through the cells treated with different concentrations of garcinol (0–10 μM) or gemcitabine (0–500 nM). Microphotographic images were captured at the beginning (0 h) and at regular intervals (every hour) up to 24 h, to observe cell migration for wound closure. The images were compared to determine the migration abilities of cells.

3 Results

3.1 Garcinol and gemcitabine inhibit cell viability and induce apoptosis

PaCa cells were treated with increasing concentrations of garcinol (0–20 μM) and/or gemcitabine (0–1000 nM) for up to 72 h. As reported previously by us, garcinol reduced cell viability in a dose-dependent manner in both Panc-1 and BxPC-3 cells [3]. As shown in Fig. 1A (BxPC-3 cells) and 1B (Panc-1 cells), gemcitabine treatment for 48 h inhibited cell viability in a dose-dependent manner in both PaCa cell lines investigated, with 50% loss of cell viability with approximately 50 nM and approximately 250 nM gemcitabine treatment in BxPC-3 and Panc-1 cells, respectively. Garcinol in combination with gemcitabine at varying doses reduced cell viability to different extents, suggesting a potent inhibitory pattern in both PaCa cell lines. Further, to confirm whether the loss in viability is due to cell death by apoptosis, quantification of Histone-DNA ELISA and alterations in cellular morphological features were evaluated. As depicted in Fig. 1C (BxPC-3 cells) and 1D (Panc-1 cells), a significant increase (p < 0.05) in apoptotic cells following combinatorial regimen of garcinol and gemcitabine in both investigated cell lines was observed. In addition, morphological alterations closely mimicking the loss of viable cells, is evident (Fig. 2A and B). This data demonstrates chemosensitization potential of garcinol in PaCa therapy.

3.2 Garcinol and gemcitabine exhibit synergistic relation in PaCa therapy

Isobologram analysis generated the CI values for different ratios of treatment in BxPC-3 and Panc-1 cells. In BxPC-3 cells the CI values for ED₇₅ (effective dose for 75% inhibition) when gemcitabine and garcinol were administered in the ratios of 1:50, 1:100, and 1:200 were 1.02, 0.88, and 0.76, respectively (Fig. 2C). Three combination treatments were tested for each ratio. Similarly in Panc-1 cells treated with same ratios, the CI values for ED₇₅ were 1.07, 0.65, and 0.57, respectively (Fig. 2D). These foregoing results clearly show that garcinol and gemcitabine have a synergistic effect in reducing PaCa cell viability.

3.3 Garcinol and/or gemcitabine treatment alters miRNA profile in PaCa cells

Heat maps (Fig. 3A, B, and C) generated by miRNA microarray analysis revealed expression of several miRNAs that are dysregulated in Panc-1 upon treatment. Figure 3D shows a volcano scatter plot with relative expression of miRNAs in all three treatment groups normalized to that of untreated control cells. A threshold of p < 0.05 (dotted line) was set as a selection criterion. Since garcinol showed a more potent effect in combination with gemcitabine in Panc-1 cells, we used this cell line as a model for further evaluation.

Signal intensities of miRNA expressions between the two groups were analyzed for statistical significance using one-way analysis of variance. Comparison of miRNA expression between untreated control cells (G1) and treated Panc-1 cells (G2) was compared using log₂ (G2/G1) ratio, which represents the fold-change in specific miRNA upon garcinol treatment (Fig. 3D, red square). Garcinol treatment downregulated the expression of miR-21 (0.56-fold), miR-495 (1.14-fold), miR-494 (0.97-fold), and miR-1977 (0.80-fold) as compared to untreated control cells; implying the oncogenic role of these miRNAs in PaCa. Interestingly, garcinol upregulated the expression of miR-453 (4.69-fold), miR-128 (1.82-fold), miR-1280 (0.46-fold), and miR-720 (0.43-fold) compared to control; suggesting tumor suppressor activity of these miRNAs. Gemcitabine treatment resulted in alteration of a few miRNAs; with a significant (p < 0.001) downregulation in expression of miR-605 as compared to untreated control cells (Fig. 3D, blue diamond). Combination of garcinol and gemcitabine resulted in a downregulation in expression of
Figure 1. Effect of garcinol and gemcitabine on PaCa cell viability and apoptosis. Significant dose-dependent reduction in cell viability and induction of apoptosis upon treatment with different concentrations of garcinol (Gar) (0–20 μM) and/or gemcitabine (Gz) (0–500 nM) for 48 h was observed. (A) On treatment of BxPC-3 cells with gemcitabine, IC50 = 50 nM was observed and (B) Panc-1 cells, IC50 = 250 nM gemcitabine concentration was observed suggesting its gemcitabine resistant phenotype. Suboptimal doses of gemcitabine were combined with varying concentrations of garcinol to achieve enhanced loss in cell viability in both PaCa cell lines. Induction of apoptosis was measured by estimation of enrichment of cytosolic nucleosomes in (C) BxPC-3 and (D) Panc-1 cells on treatment with either agent alone or in combination. *p < 0.05 relative to control was considered as significant.

3.4 Key cancer pathway signaling molecules targeted by miRNAs altered upon treatment with garcinol and/or gemcitabine in PaCa cells

The biological functions of the miRNAs expression were affected by garcinol and/or gemcitabine in Panc-1 cells and were scrutinized by taking into account the context of the present study. Using in silico methods, data were inferred by selecting miRNA target genes with a context score > 0.5, as reported in TargetScan (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/), and the DNA Intelligent Analysis, microT–4.0-beta version (http://diana.cslab.ece.ntua.gr/), along with the pertinent literature available on PubMed. These algorithms, based on the principle of comparative genome hybridization helped in identification of targets based on comparative sequence analysis, seed match complementation, and Z-score for assigned UTR regions. The results of these functional analyses are plotted in Fig. 5, where the x-axis represents an equivalent negative natural logarithm of the p-value [p < 0.05 = –ln (p-value) < 0.13], which is used as a more intuitive measure for analysis, and the y-axis represents...
Figure 2. Synergistic cytotoxicity. In PaCa cells, BxPC-3 cells (A and C) and Panc-1 cells (B and D). (A and B) Apoptotic morphological changes were observed due to single agent garcinol and/or gemcitabine (Gz) treatment using DAPI stain. (C and D) Isobologram analyses performed to evaluate combination index values on treatment with garcinol and gemcitabine in combination resulted in synergism (CI < 1) in higher ratios of 100:1 and 200:1 in PaCa cells. CI value is suggestive of the potential of garcinol in sensitizing PaCa cells to effects of gemcitabine.

potential-targeted cellular pathways. Finally, genes predicted as targets of candidate miRNAs were aligned and matched for confirmation using the Sanger database to eliminate false-positive analyses.

The target analysis revealed the biological roles of the affected miRNAs and an overlapping multitude of cellular and physiological functions affected by treatments. In PaCa cells, miR-21 is involved in inducing gemcitabine resistance [18]. A downregulation in signal intensity of miR-21 by 32, 1, and 22% was noted on treatment with garcinol, gemcitabine, and combination, respectively, compared to untreated control. A significant reduction in miR-21 expression on treatment with garcinol alone as compared to untreated control cells was observed. Interestingly, previous reports suggesting a role of miRNA-21 in clinical drug resistance paralleled our findings on treatment with gemcitabine alone and in combination with garcinol. The in silico data reported herein show that miR-21 gene targets involve molecules in various cancer pathways including cytokine receptor, TGF-β signaling, MAPK signaling, Jak-STAT signaling, apoptosis, VEGF, Notch, mTOR signaling, and Focal adhesion pathways (Fig. 5). Recent study provided evidence of positive correlation between miRNA-21 and mRNA expression of angiogenic factors such as VEGF, MMP-9, and others [18]. Thus treatment with garcinol can be a potent strategy to inhibit miR-21 and overcome the effect of clinical drug resistance in PaCa.

Another gastrointestinal cancer-specific oncogenic miRNA is miR-196a [19]. Its expression was downregulated by garcinol (25%), gemcitabine (33%), and combinatorial (43%) treatment compared to untreated Panc-1 control cells. A gradual reduction in signal was observed on treatment with single agent; however, the combination treatment
Figure 3. Alteration in miRNA profile on treatment with garcinol alone or in combination with gemcitabine. Panc-1 cells treated with (A) 10 μM garcinol (Gar), (B) 100 nM gemcitabine (Gz), and (C) combination (Com) of above treatments was subjected to microarray analysis. Effect of each of these therapeutic doses on miRNA profile was compared to untreated control cells (p < 0.01). (D) A volcano scatter plot to screen the expression of miRNAs altered between the untreated control to garcinol (red), gemcitabine (Gz; blue), or combination (green) based on fold change and statistical significance. MiRNAs were selected for further investigation based on fold-change on either side of the axis.

(p-value: 5.75E-03) reduced miR-196a signal intensity by almost half suggesting a potent therapeutic effect on PaCa cells. Molecular targets identified using different database tools include, molecules associated with ECM receptor interactions, focal adhesion pathway, cell communication, Jak-STAT signaling, mTOR, actin cytoskeleton regulation, and cell-cycle regulation (Fig. 5). Ninety percent of PaCa cases have K-ras mutation [20]. In our study, the role of miR-495 on treatment with both agents was evaluated in Panc-1 cells since it is known to be overexpressed (>10 folds) in K-ras positive tumors [21]. Expression of miR-495 was downregulated by 55, 15, and 59% on treatment with garcinol, gemcitabine, and combination, respectively. Garcinol alone or in combination with gemcitabine showed significant (p-value: 1.45E-04) reduction as compared to individual treatment with the drug. Target analysis revealed its association with pathways involving Insulin signaling, mTOR regulation, VEGF, cell cycle, Wnt, and MAPK molecules (Fig. 5). Since K-ras mutation is commonly associated with PaCa, targeting miR-495 by garcinol alone or in combination would be a key step in therapeutics.

Certain miRNAs are known to regulate carcinogenesis via a tumor suppressive mechanism. miR-638 was downregulated in gastric cancers suggesting a tumor suppressor activity [22]. We compared the expression data for miR-638 in all treatment groups. Garcinol treatment caused upregulation in miR-638 expression by 31%; gemcitabine by 7%, and combination treatment by 57%, relative to control cells. Our results clearly suggest that garcinol in combination with gemcitabine could induce the tumor suppressor
activity of miR-638 more potently (p-value: 5.14E-03) than either agent alone. Also, gemcitabine by itself was not very efficient in upregulating miR-638 levels, but a synergistic effect on combining with garcinol is possible as suggested by the signal intensity values. Probable targets of miR-638 are molecules of glucose metabolism, ABC transporters, ErbB pathway, purine metabolism, and ubiquitin-mediated proteolytic activities (Fig. 5). It is possible that miR-638 might be downregulating cancer-promoting molecules via some yet unknown mechanisms. It would thus be interesting to explore the role of garcinol in mediating induction of miR-638 as a tumor suppressor miRNA.

The exact function of miRNA-605 and miR-453 in gastrointestinal cancers is not well documented. However, garcinol treatment resulted in approximately 1.81-fold increase in miR-605 expression as compared to approximately 1.46-fold (p-value: 1.90E-03) and approximately 2.42-fold (p-value: 4.18E-04) downregulation on treatment with gemcitabine and combination, respectively. One possibility is that miR-605 is a tumor suppressor due to its observed upregulation on treatment with a natural agent as compared to downregulation by a chemotherapeutic drug. Also, gene sequence and database analyses indicate that this miRNA is targeting Wnt signaling, MAPK signaling, EGF signaling, Hedgehog signaling, and cell-cycle regulation (Fig. 5). Limited literature on miR-605 suggests it is a non-conserved miRNA, influencing the mRNA expression though its 5'UTR region that is specific to the 3' end of mRNA [23]. However, its exact role in PaCa is not very clear at this point.

Expression of miR-453 was observed to increase (p-value: 2.27E-04) by several folds (~25–26-fold) on treatment with garcinol alone and in combination with gemcitabine; however, there was no change observed on treatment with gemcitabine alone. Target analysis revealed that miRNA-453 targeted several pathways such as EGFR signaling, amino acid biosynthesis, regulation of actin cytoskeleton and Wnt signaling (Fig. 5). The role of miR-453 is not known in PaCa but a recent report has documented the role of miR-453 in breast carcinogenesis [24].

3.5 Garcinol in synergism with gemcitabine modulates key miRNA target signaling pathways in PaCa cells

Caspase-3 (Fig. 6A) and caspase-9 (Fig. 6B) activity was assessed in Panc-1 cells and while garcinol or gemcitabine treatment alone at the investigated low dose did not cause a significant increase in caspase-3 or 9 relative to control, the combination regimen significantly increased their activity by 1.5–2-folds relative to control (p < 0.05). The chemosensitization effects of garcinol via induction of apoptosis was assessed by evaluating the molecular expression of key apoptotic (PARP cleavage) and antiapoptotic (Bcl-XL) proteins by Western immunobloting (Fig. 6C). Our data show that while treatment with garcinol or gemcitabine alone show no appearance of the band for cleaved PARP, their combination showed strong cleaved PARP bands indicative of apoptosis induction. Consistent with the results obtained with PARP cleavage, significant downregulation of the antiapoptotic Bcl-XL in the combination treatment group was also noted indicating that garcinol indeed sensitizes PaCa cells to the cytotoxic effect of gemcitabine. In addition, a reduction in NF-κB-binding activity was observed in gemcitabine resistant Panc-1 cells on treatment with 10 μM garcinol in combination with 100 nM gemcitabine (Fig. 6D). With the aim of targeting other cancer
pathways targeted by the miRNAs under investigation, angiogenic markers were next investigated using suboptimal dose of gemcitabine. Our results show that garcinol alone, even at low doses is a strong inhibitor of VEGF, MMP-9, and IL-8 angiogenic factors. In combination the two agents proved to be even more potent than the single agents \((p < 0.05)\) (Fig. 6E–G).

Cancer cells exhibit the tendency to grow and metastasize by forming colonies. The effect of treatment on inhibition of cell growth was also assessed by clonogenic assay. Based on our results showing apoptosis, the clonogenic survival of cells treated with garcinol was moderately reduced relative to control, while gemcitabine treatment lead to a significant decrease in clonogenic survival of Panc-1 cells. Most interestingly, the combination group showing more cytotoxicity resulted in fewer clones compared to control and single agent-treatment alone \((p < 0.05;\) Fig. 7A). Interplay between multiple and diverse cell adhesive molecules are crucial for metastatic cancer. A quantitative analysis to check for effect of garcinol and gemcitabine on cell adhesion ability was performed based on our computational analysis. In Panc-1 cells, cell adhesion reduced by half in the combination (e.g. 100 nM gemcitabine and 10 \(\mu\) M garcinol) treatment with respect to individual treatments (Fig. 7B). A qualitative wound-healing

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**Figure 5.** Garcinol and gemcitabine treatment alters miRNAs targeting cancer-signaling pathways in PaCa cells. In silico analyses identified potential cancer signaling target pathways of representative miRNAs from all treatment groups (garcinol and/or gemcitabine). An equivalent negative natural logarithm of the \(p\)-value was used as a more intuitive measure for analysis.
assay suggested an expansion in the wound on treatment with either agent after 24 h as compared to a closed wound in untreated control cells (Fig. 7C).

4 Discussion

An approximate 55% increase in new PaCa cases is expected by year 2030 [25], the reasons being late diagnosis, lack of better treatment modalities, and variability in patient outcome. As discussed previously, gemcitabine remains the standard care for PaCa patients today; however, multiple side effects and development of chemoresistance is a major hurdle in developing successful strategy to handle this disease. One of the several approaches to improve prognosis of PaCa patients is the development of chemo-sensitization strategies by using dietary agent as an adjuvant with standard chemotherapeutic drugs. We demonstrated that garcinol individually, and in combination with gemcitabine can modulate miRNA signatures of multiple drug resistance and other cancer-promoting pathways. Garcinol in a signal context-dependent manner enhances therapeutic efficacy of gemcitabine in human PaCa cell lines, BxPC-3, and Panc-1. Of the two cell lines used, Panc-1 is a poorly differentiated PaCa cell line that possesses mutated K-ras and p53 mutation, while BxPC-3 cells represent a moderately differentiated cell line with wild-type K-ras and p53 mutation. Thus, our results illustrate that despite functional heterogeneity in terms of genetic signatures (K-ras status) garcinol can mitigate biological activities including cellular growth, and survival by stimulating cells to undergo apoptosis.

In silico analysis indicates that because a strong interconnected “network grid” of miRNAs, regulate several oncogenic, and tumor suppressor pathways simultaneously,
miRNAs can be a richer source of pathological evidence in tumor profiling as compared to mRNA expressions. Several recent studies have reported the deregulation of miRNA expression in pancreatic tumors. This information might be useful in differential diagnosis of PaCa from other tumors [26]. For example, miR-375 and miR-376 expressions were significantly high in the mouse pancreas and pancreatic islet cells as compared to brain, heart, and liver tissue [27]. Studies have documented the significant upregulation of miR-21 expression in PaCa [18, 28, 29]. Another advanced study using QuantIMir system analyzed the differential expression of 95 miRNAs in 10 PaCa cell lines and 17 pairs of PaCa/normal tissues. A significant upregulation of miR-196a, miR-190, miR-186, miR-221, miR-222, miR-200b, miR-15b, and miR-95 in most PaCa tissues and cell lines was reported. The upregulation of these eight miRNAs ranged from 70 to 100% between normal control and tumor cells or tissues [19]. Accumulating evidence also supports a role of miRNAs in the development of chemotherapeutic drug resistance [30, 31]. For example, miR-200, which is considered as a tumor suppressor miRNA, is found to be involved in drug resistance mechanisms in PaCa [32]. Overexpression of miR-200 resulted in enhanced sensitivity to gemcitabine in PaCa by modulation of several associated gene targets and other downstream targets.

Yekta et al. first described miR-196a-directed cleavage of specific homebox genes (HoxB8, HoxC8, HoxD8, and HoxA7) in mouse embryos and mammalian cells thus promoting tumor progression by cell detachment and tumor cell dissemination in vitro via activation of Akt pathway [33]. A recent study describing the role of miR-196a in colorectal cancer suggested that this miRNA was mainly involved in enhanced cell detachment, migration, invasion, and chemosensitivity toward platin drugs [34]. Also, studies using miRNA ChIP analyses in PaCa found that 75% of tumors expressed high levels of miR-196a [35, 36]. This initial finding of chemosensitization of PaCa cells with a dietary agent by modulating miR-196a expression could be an important lead in therapeutic strategy. Chemoprotective role of curcumin was

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**Figure 7.** Garcinol and/or gemcitabine alter migratory and adhesive properties of PaCa cells. (A) Clonogenic assay was performed to assess cell proliferation and migration abilities in Panc-1 cells and resulted in fewer clones in combination treatment relative to monotherapy, suggesting a potent synergistic effect. Fraction of cell survival is shown in the bottom panel. (B) Treatment effect on cell adhesion was demonstrated using laminin-coated plates. Gemcitabine (Gz) individually and in combination restricted cell adhesion more potently than garcinol (Gar) treatment. *p < 0.05 relative to control. (C) Microphotograph of wound healing assay on treatment of PaCa cells depicts the ability of these agents in restricting cell migration and proliferation.
studied in PaCa cells, with evidence for upregulation of 11 miRNAs and downregulation of 18 miRNAs. Specifically, miR-22 was upregulated and miR-196, an oncogenic miRNA, was reported to be significantly downregulated upon curcumin treatment [37]. Similarly, expression of tumor suppressor miRNAs, miR-200, and let-7, which are downregulated in gemcitabine-resistant PaCa cells, are increased upon treatment with isoflavones [32].

Here, we observe that our proposed treatment strategies altered several miRNAs; however, an overlapping trend in their probable mRNA targets was seen. Some of these miRNAs might be influencing these known cancer-signaling pathways via indirect mechanisms. The nuclear transcription factor NF-κB serves as a prototype example for defining chemoresistance. Clinical studies have shown that overexpression of NF-κB correlates with gemcitabine-resistance leading to markedly negative clinical outcome. We hypothesized that garcinol and gemcitabine may act synergistically as a novel chemosensitizing combination by downregulation of NF-κB DNA-binding activity, resulting in inhibition of multiple downstream survival factors in PaCa. Two major mechanisms by which chemotherapeutic drugs generally mediate their effect is by loss of cell viability and induction of apoptotic cell death. Treatment of PaCa cells with garcinol in synergism with gemcitabine resulted in a dose-dependent increase in levels of cleaved caspase-3 and -9 as well as cleaved PARP band as compared to individual treatments in both PaCa cell lines, suggesting the involvement of the mitochondrial pathway in promoting apoptosis.

Complex regulatory networks are responsible for the expression of genes involved in maintaining homeostasis in normal cells. Carcinogenesis is a result of perturbations in these networks. Pancreatic tumors are highly vascular and produce multiple proangiogenic factors and cytokines such as VEGF, MMP-9, and IL-8 that help in invasion and progression of cancer cells [38–41]. VEGF is involved in angiogenesis and tumor growth and is overexpressed in a variety of cancers. Thus, agents that can inhibit VEGF are of benefit in cancer therapy. MMPs are critically involved in tumor cell invasion and metastasis. Specifically, MMP-9 is commonly overexpressed in PaCa patients and is associated with poor survival. Interleukins are a family of proinflammatory cytokines that assist in the process of invasion and angiogenesis. Although blocking cytokines will not kill tumor cells, such agents behave as tumorostatic agents and work best in adjunct therapy that includes tumorcidal drugs. Epidemiological evidence shows that overexpression of VEGF in PaCa patients correlates with poor prognosis. We also demonstrated loss of adherence and lack of proliferating abilities in cells treated with garcinol and/or gemcitabine by using simple experiments like cell adhesion assay and wound healing assay in PaCa cell lines.

Taken together, these results provide preliminary evidence supporting the potential antitumor activity of the combination treatment garcinol with gemcitabine in PaCa cells. Overall, the miRNA biomarkers and their downstream mRNA targets may be further explored and used as therapeutic targets to sensitize PaCa cells to chemotherapeutics. Since these findings result from analysis in in vitro models, the potential of this approach and its ultimate value toward translation to a clinical environment needs to be evaluated and further confirmed in relevant animal models. Based on our experiments, there is substantial evidence to suggest that from the point of personalized medicine and cancer therapy, miRNAs may prove to be useful diagnostic and prognostic markers for cancer treatment and this will be a promising strategy for treatment of PaCa.

The authors have declared no conflict of interest.

5 References

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