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1
2 Original research article

3 # Embelin inhibits proliferation, induces apoptosis and alters gene
4 expression profiles in breast cancer cells

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ABSTRACT

Purpose: To investigate effect of embelin on proliferation, apoptosis and gene expression profile changes in breast cancer cells.
Methods: Cell viability was determined by MTT assay and apoptosis assayed using flow cytometry. Differential expression of 84 genes commonly involved in breast cancer carcinogenesis was assessed by real-time PCR using the Human Breast Cancer RT^2 Profiler PCR Array.
Results: MCF-7 and MDA-MB-231 cells were treated with embelin (0–25 μM) for 24 and 96 h. Embelin exhibited time and dose dependence in both cell lines and was more potent in inhibiting MDA-MB-231 cell proliferation compared to MCF-7 cells. IC_{50} for embelin in MDA-MB-231 cells was ~4.45 μM and 3.28 μM at 24 h and 96 h, respectively. In contrast, IC_{50} for embelin in MCF-7 cells was ~6.04 μM and 4.51 μM at 24 h and 96 h, respectively. Embelin (50 μM) induced apoptosis and activated caspase 3 activity in both cell lines when exposed for 72 h. Treatment of MDA-MB-231 cells with embelin (10 μM) for 24 h resulted in significant differential expression of 27 genes commonly involved in breast cancer carcinogenesis.
Conclusions: Our findings show that embelin inhibits cell proliferation, induces apoptosis and alters expression of breast cancer focused genes in MCF-7 and MDA-MB-231 cells. Based on RT^2-PCR array analysis, embelin down-regulated expression of pivotal oncogenes. This knowledge could be beneficial in the development of effective embelin-based therapies for treating breast cancer.
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9
10 ## Introduction

11 Q2 Breast cancer is a leading cause of cancer related mortality in
12 women in the United States [1]. An estimated 231,840 new
13 invasive cases are expected in 2015 making it the most common
14 cancer in American women and despite diagnostic improve-
15 ments, 40,290 fatalities are projected. Consequently, tremen-
16 dous effort has been devoted to developing potent anticancer
17 agents to treat breast cancer. However, most anticancer drugs
18 are not site-specific, are toxic to normal cells and exhibit high
19 toxicity to benefit ratios. Therefore, there is renewed interest in
20 molecules that have low toxicity profiles and can selectively
21 target cancer cells.

22 Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) (Fig. 1A),
23 a naturally occurring benzoquinone found in the fruit of the *Embelia*
24 *ribes* plant displays low toxicity and shows potent anticancer
25 properties either alone or in combination with other anticancer
26 agents. Numerous studies have attributed the anticancer properties
27 of embelin to its ability to inhibit the x-linked inhibitor of the
28 apoptosis protein (XIAP) and modulate NF-κB signaling pathways
29 [2–4]. Nonetheless, little is known about the effect of embelin on
30 breast cancer cells at the genomic level.

31 In this work, we investigated the effect of embelin on cell
32 proliferation and apoptosis in MCF-7 and MDA-MB-231 breast
33 cancer cells. We also surveyed the gene expression profile of a
34 focused panel of 84 breast cancer related genes following
35 exposure of MDA-MB-231 and MCF-7 cells to embelin. Better
36 understanding of key breast cancer genes impacted by exposure
37 of breast cancer cells to embelin is urgently needed since this
38 knowledge could potentially facilitate rationale development of
39 more potent embelin analogs or new effective embelin-based
40 combination therapy strategies for treating breast cancer. To the

Abbreviations: DAVID, Database for Annotation, Visualization and Integrated Discovery; EMT, Epithelial-Mesenchymal Transition; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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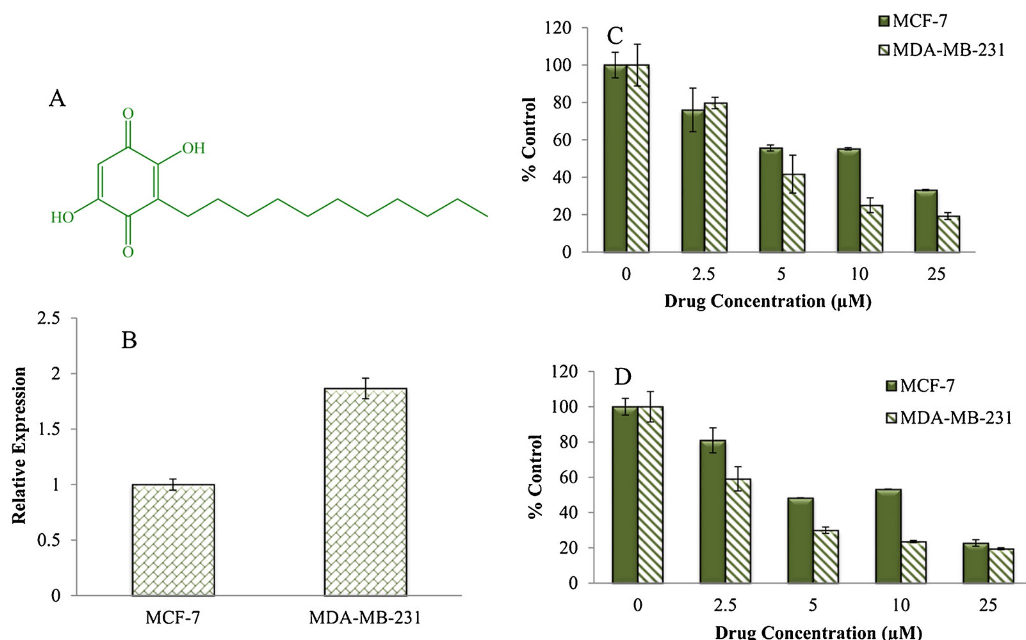

Fig. 1. Anticancer effect of embelin on breast cancer cells. (A) Chemical structure of embelin. (B) Endogenous expression of XIAP in MDA-MB-231 and MCF-7 human breast cancer cell lines. Cell viability of 2×10^3 MCF-7 and MDA-MB-231 cells treated with embelin for (C) 24 h and (D) 96 h. Cell viability measured using MTT assay.

41 best of our knowledge, this is the first study examining the effect
42 of embelin on gene expression in breast cancer cell lines using
43 real time PCR array.

44 Materials and methods

45 *Materials*

46 MDA-MB-231 and MCF-7 human breast cancer cell lines were
47 purchased from American Type Culture Collection (ATCC, Manassas,
48 VA, USA) and stored in liquid nitrogen. Dulbecco's Modified Eagle
49 Medium (DMEM), TrypLE Express and antibiotic-antimycotic were
50 obtained from Life Technologies (Carlsbad, CA, USA). Embelin was
51 purchased and all other reagents were obtained from Sigma–Aldrich
52 (St. Louis, MO, USA) and used as received unless otherwise stated.

53 *Cell proliferation assay*

54 The effect of embelin on MDA-MB-231 and MCF-7 breast
55 cancer cell lines was evaluated using MTT assay. Briefly, cells
56 were maintained in DMEM supplemented with 10% FBS and 1%
57 Antibiotic-Antimycotic and incubated in 5% CO_2 at 37 °C.
58 Subsequently, 2×10^4 viable cells per well were seeded in 96-
59 well plates, incubated for 24 h and treated with embelin at
60 various concentrations (0-25 μM) for 24 and 72 h. Following
61 incubation, 20 μL of MTT (5 mg/mL) was added to each well and
62 incubated for 3–4 h. The residual formazan crystals were
63 solubilized with 200 μL DMSO and analyzed using a microplate
64 reader recording absorbance values at a test wavelength of
65 560 nm. Cell viability for a given concentration was expressed as
66 a percentage of the intensity of controls. All measurements were
67 performed in triplicates.

68 *Cell migration assay*

69 MDA-MB-231 and MCF-7 cells were grown to 70% confluence in
70 six well plates and three parallel wounds made using pipette tip.
71 Micrographs of each wound were capture using an inverted
72 microscope and used as a reference point. Cells were treated with
73 embelin (0, 5 and 50 μM) for 72 h after which cells were washed
74 with ice cold 1× PBS and imaged under an inverted microscope.

75 *Apoptosis analysis using flow cytometry*

76 The Chromatin Condensation/Dead Cell Apoptosis kit with
77 Hoechst 33342 and PI (Invitrogen, Carlsbad, CA, USA) was used to
78 evaluate apoptosis. MDA-MB-23 and MCF-7 cells were harvested
79 using TrypLE Express following treatment of embelin (0, 10 and
80 50 μM). Cell density was adjusted to 1×10^6 cells/mL following
81 which 1 μL of Hoechst 33342 and 1 μL of propidium iodide (PI)
82 was added to each group and incubated on ice for 30 min. Stained
83 cells were then subjected to flow cytometry and subsequently
84 analyzed using FCS Express 5.

85 *Western blot*

86 MDA-MB-231 breast cancer cells were treated with embelin
87 (0, 5 and 50 μM) for 72 h. Afterwards, cells were lysed using
88 Mammalian Protein Extraction Reagent (M-PER) buffer (Pierce,
89 Rockford, IL, USA) and protein concentration measured with
90 bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL,
91 USA). Lysate was boiled for 5 min, subjected to a 15% SDS-PAGE
92 and transferred to a PVDF membrane using iBlot™ system
93 (Invitrogen, Carlsbad, CA, USA). Membranes were blocked with
94 Odyssey blocking buffer at room temperature and then
95 incubated with BCl-2 primary antibody at 4 °C overnight.
96 Subsequently, membranes were incubated with secondary
97 antibody conjugated with horseradish peroxidase (HRP) at
98 room temperature for 1 h. The signal of target proteins was
99 detected using Li-COR Odyssey® infrared imaging system (Li-
100 COR, Lincoln, NE, USA).

101 *ELISA for caspase 3*

102 MDA-MB-231 and MCF-7 cells were treated with embelin (0,
103 5 and 50 μM) for 72 h. Subsequently, cells were lysed and caspase

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104 3 concentration determined using Human Caspase 3 ELISA kit per
105 the manufacturer's protocol.

106 *RNA extraction*

107 Total RNA was extracted using RNeasy mini kit from Qiagen
108 (Valencia, CA, USA) per the manufacturer's protocol. The solution
109 containing extracted RNA was then subjected to DNase treatment
110 for further purification. Subsequently, RNA concentration and
111 quality was quantified using an Eppendorf BioPhotometer Plus
112 (Hauppauge, NY, USA).

113 *Human Breast Cancer RT^2 Profiler PCR Array*

114 cDNA synthesis was performed using RT^2 First Stand Kit
115 (Qiagen, Valencia, CA, USA) according to the manufacturer's
116 protocol. The procedure comprises two key steps. First, 10 μL of
117 the genomic DNA elimination mix was prepared in a sterile PCR
118 tube by mixing 2 μL Buffer GE and appropriate quantities of
119 RNase-free water and each RNA sample. The resulting solution
120 was gently by pipetted up and down, centrifuged briefly,
121 incubated for 5 min at 42 °C and then placed immediately on
122 ice for at least 1 min. Second, 10 μL reverse-transcription mix
123 was prepared by mixing 4 μL 5× Buffer BC3, 1 μL Control P2,
124 2 μL RE3 Reverse Transcriptase mix and 3 μL RNase-free water.
125 The 10 μL reverse-transcription mix was then added to each
126 tube containing genomic DNA elimination mix, mixed gently
127 and incubated at 42 °C for precisely 15 min using a BioRad
128 Thermal Cycler (Hercules, CA, USA). Afterwards, the reaction
129 was immediately stopped by incubating at 95 °C for 5 min.
130 RNase-free water (91 μL) was then added to each reaction and
131 mixed by pipetting up and down several times. The resulting
132 cDNA mixture was placed on ice or stored at -20 °C until used.
133 The Human Breast Cancer RT^2 Profiler PCR Array (Qiagen,
134 Valencia, CA, USA) consisting of 84 key genes commonly
135 involved in the dysregulation of signal transduction and other
136 normal biological processes in breast cancer was used to analyze
137 gene expression. Samples were prepared by mixing 1350 μL of
138 2× RT^2 SYBR Green mastermix, 102 μL cDNA reaction mixture
139 1248 μL of RNase-free water and pipetted into 96-well pcr array
140 plates to assess expression of pertinent breast cancer genes. RT^2
141 Profiler PCR Arrays were run on Eppendorf Mastercycler ep
142 realplex model 4 (Hauppauge, NY, USA) and the following
143 PCR cycling condition used: 95 °C for 10 min, 45 cycles of 95 °C
144 for 15 s and 60 °C for 1 min. Cycle thresholds from the real-time
145 PCR was exported to an excel file and analyzed using web-based
146 PCR Array Data Analysis Software available through SABios-
147 ciences at www.SABiosciences.com/pcrarraydataanalysis.
148 php. Relative gene expression was computed by comparing
149 $\Delta\Delta C_t$ determined for each gene in the embelin-treated to the
150 control array. A fold change equal to or greater than 2 was
151 considered significant.

152 *Gene functional annotation*

153 The Database for Annotation, Visualization and Integrated
154 Discovery (DAVID) gene annotation website (https://david.
155 ncifcrf.gov/) was used to classify genes whose expression levels
156 were changed by ≥2-fold. DAVID provides a comprehensive set
157 of functional annotation tools which facilitates biological
158 classification of genes based on their cellular function and the
159 pathways they are involved in. Analysis was done using the
160 official gene symbol to identify key biological pathways and
161 cellular processes affected following treatment of MDA-MB-231
162 cells with embelin. The Kyoto Encyclopedia of Genes and
163 Genomes (KEGG) database was used for pathway analysis.

164 **Results**

165 *XIAP basal expression in MCF-7 and MDA-MB-231 Cells*

166 Since embelin is a known XIAP inhibitor, we first evaluated the
167 endogenous mRNA expression of XIAP in MCF-7 and MDA-MB-231
168 breast cancer cells using real time RT-PCR. Our results reveal XIAP
169 expression to be 1.9 times more in MDA-MB-231 cells compared to
170 MCF-7 (Fig. 1B). XIAP expression has been shown to correlate with
171 disease progression [5–7]. Hence, it is not surprising that XIAP
172 expression is greater in MDA-MB-231 cells compared to MCF-7 cells.

173 *Effect of embelin on MCF-7 and MDA-MB-231 cell proliferation and*
174 *migration*

175 We next investigated the potency of embelin in treating breast
176 cancer by determining its IC_{50} values in MCF-7 and MDA-MB-231
177 breast cancer cells at concentrations ranging from 0 to 25 μM for
178 24 and 96 h. Specifically, IC_{50} for embelin in MDA-MB-231 cells
179 was ~ 4.45 μM and 3.28 μM at 24 h and 96 h, respectively. In
180 contrast, IC_{50} for embelin in MCF-7 cells was ~6.04 μM and
181 4.51 μM at 24 h and 96 h, respectively. Our results showed
182 embelin exhibited time and dose dependence in both cell lines and
183 was more potent in inhibiting MDA-MB-231 cell proliferation
184 compared to MCF-7 cells (Fig. 1C and D).
185 We also characterized the effect of embelin (0, 5 and 50 μM) on
186 the migration ability of MCF-7 and MDA-MB-231 cells 72 h post
187 treatment using the scratch wound assay. Our findings reveal
188 embelin inhibited cell migration in both cell lines and the ability of
189 embelin to inhibit cell migration appeared to be dose-dependent
190 (data not shown).

191 *Effect of embelin on apoptosis*

192 We next examined the effect of embelin on apoptosis.
193 Specifically, MCF-7 and MDA-MB-231 cells were treated with 0,
194 10 and 50 μM of embelin for 72 h and the extent of apoptosis
195 quantified using flow cytometry. From Figs. 2 and 3 there was no
196 significant increase in apoptotic rate in both cell lines following
197 treatment with 10 μM of embelin. In contrast, treatment with
198 50 μM embelin resulted in an apoptosis rate of ~30% in MCF-7
199 cells and ~23% in MDA-MB-231 cells. In both instances, apoptosis
200 observed post treatment with 50 μM embelin was three-fold more
201 compared to control.
202 To provide mechanistic insight into the effect of embelin on
203 apoptosis, we examined whether embelin modulates the expres-
204 sion of caspase 3 and Bcl-2 which are known to play a pivotal role
205 in the apoptotic pathway. Treatment of MCF-7 and MDA-MB-231
206 cells with embelin (0, 5 and 50 μM) for 72 h revealed caspase
207 3 activity was highest when cells were exposed to 50 μM embelin
208 (Fig. 4A). In both cell lines, caspase 3 activity following treatment
209 with 50 μM embelin was approximately two-fold that of control.
210 Furthermore, Bcl-2 expression in MDA-MB-231 cells was signifi-
211 cantly suppressed when exposed to 50 μM embelin for 72 h. In
212 contrast, no difference in Bcl-2 expression was observed following
213 treatment with 5 μM of embelin (Fig. 4B).

214 *Effect of embelin on gene expression in MCF-7 and MDA-MB-231*

215 To elucidate the effect of embelin on the genomic level, Human
216 Breast Cancer RT^2 Profiler PCR Array was performed on MCF-7 and
217 MDA-MB-231 cells to identify expression differences in breast
218 cancer associated genes following treatment with embelin
219 (10 μM) for 24 h. Expression analysis revealed significant changes
220 in 27 of the 84 genes studied in MDA-MB-231 cells and all 27 genes
221 were down-regulated (Table 1 and Fig. 5A). In contrast, no

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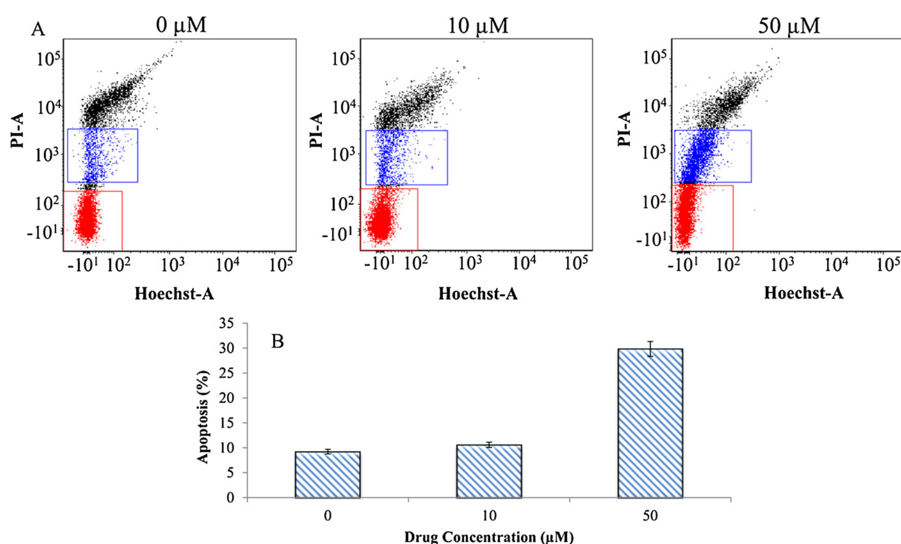

Fig. 2. Effect of embelin on apoptosis in MCF-7 human breast cancer cells. (A) Hoechst 33342/propidium iodide (PI) staining was used to determine apoptosis rate following treatment of cells. (B) Quantitative analysis of apoptotic rate (%).

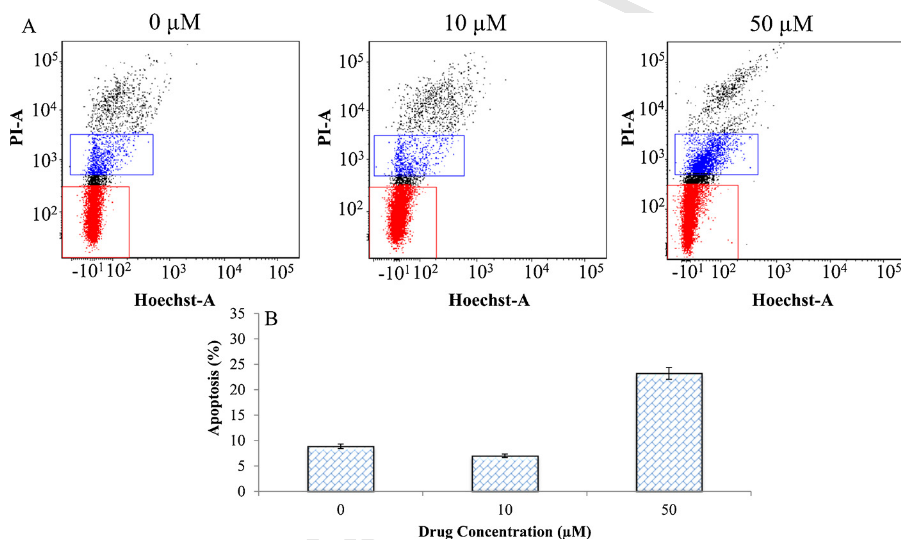

Fig. 3. Effect of embelin on apoptosis in MDA-MB-231 human breast cancer cells. (A) Hoechst 33342/propidium iodide (PI) staining was used to determine apoptosis rate following treatment of cells. (B) Quantitative analysis of apoptotic rate (%).

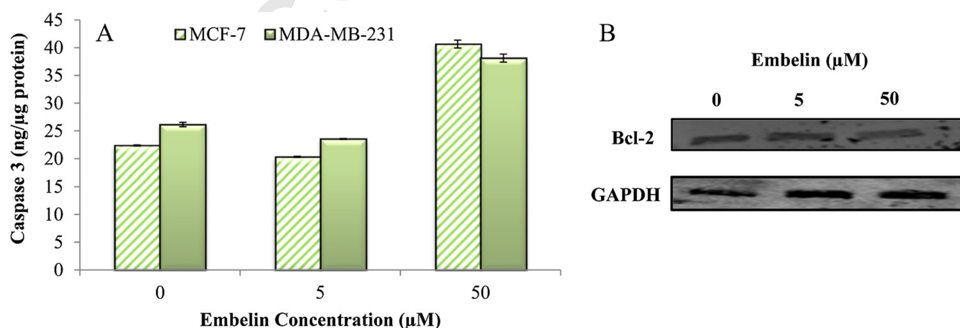

Fig. 4. Effect of embelin on caspase 3 and BCl-2 expression in human breast cancer cells. (A) 5×10^5 MCF-7 and MDA-MB-231 cells were treated with embelin for 72 h, protein isolated and caspase 3 expression at protein level determined using ELISA. (B) 1×10^6 MDA-MB-231 cells were treated with embelin for 72 h, protein isolated and BCl-2 expression at protein level assayed using Western blot.

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Table 1
Down-regulated genes in MDA-MB-231 cells.

Gene symbol	Fold regulation
AR	−2.1
CDK	−2.1
AKT1	−2.1
CCND1	−2.1
MAPK3	−2.1
SRC	−2.1
ERBB2	−2.3
KRT8	−2.3
RARB	−2.3
SFN	−2.4
ADAM23	−2.4
PLAU	−2.4
JUN	−2.4
SERPINE1	−2.6
CCNA1	−2.6
MKI67	−2.6
EGF	−2.6
CTNNB1	−2.7
BIRC5	−2.6
NOTCH1	−2.8
HIC1	−3.1
THBS1	−3.2
RASSF1	−3.3
CDH1	−3.3
TP73	−3.3
SLIT2	−3.3
GATA3	−3.6

Table 2
Gene ontology groups for down-regulated genes (≥2-fold) in MDA-MB-231 cells.

Category	Count	Genes
Regulation of cell proliferation	12	NOTCH1, CTNNB1, CCND1, CDK2, EGF, JUN, PLAU, RARB, SERPINE1, SFN, THBS1, ERBB2
Intracellular signaling cascade	12	RASSF1, AR, CTNNB1, CCND1, EGF, MAPK3, SFN, THBS1, TP73, AKT1, ERBB2, SRC
Regulation of apoptosis	11	NOTCH1, BIRC5, CDH1, JUN, RARB, SFN, THBS1, TP73, AKT1, ERBB2, SRC
Cell cycle	11	RASSF1, MKI67, BIRC5, CTNNB1, CCNA1, CCND1, CDK2, MAPK3, THBS1, TP73, AKT1
Positive regulation of biosynthetic processes	9	NOTCH1, AR, CTNNB1, CDK2, JUN, RARB, THBS1, TP73, AKT1
Blood vessel development	8	NOTCH1, CTNNB1, EGF, JUN, PLAU, SLIT2, THBS1, AKT1
Regulation of phosphorylation	8	CCND1, EGF, JUN, SFN, THBS1, TP73, AKT1, ERBB2
Positive regulation of gene expression	7	NOTCH1, AR, CTNNB1, CDK2, JUN, RARB, TP73

Table 3
Pathway analysis for down-regulated genes (≥2-fold) in MDA-MB-231 cells.

Pathway	Count	Genes
Pathways in cancer	14	RASSF1, AR, BIRC5, CDH1, CTNNB1, CCNA1, CCND1, CDK2, EGF, MAPK3, JUN, RARB, AKT1, ERBB2
p53 signaling pathway	6	CCND1, CDK2, SERPINE1, SFN, THBS1, TP73
ErbB signaling pathway	6	EGF, MAPK3, JUN, AKT1, ERBB2, SRC
MAPK signaling pathway	4	EGF, MAPK3, JUN, AKT1
VEGF signaling pathway	3	MAPK3, AKT1, SRC

significant changes in gene expression were observed in MCF-7 cells (Fig. 5B).

Gene ontology categories and pathway analysis of down-regulated genes are summarized in Tables 2 and 3. Our findings reveal overall changes in gene expression patterns following treatment of MDA-MB-231 cells with embelin (10 μM) for 24 h. Pathway analysis showed down-regulated genes participated in pathways in cancer and several signaling pathways including: p53, ErbB, MAPK and VEGF.

Discussion

Embelin has been reported to inhibit cell proliferation and induce apoptosis in a variety of human cancer cells. While embelin has been investigated extensively in general, there are very few studies examining its effect on breast cancer cells in the literature. In this study, we investigated the effect of embelin on inhibiting cell proliferation, inducing apoptosis and altering gene expression in MCF-7 (estrogen receptor positive (ER^+), progesterone receptor positive (PR^+) and human epidermal growth factor receptor 2 negative ($HER2^-$)) and the triple negative (ER^-, PR^-, $HER2^-$) MDA-MB-231 breast cancer cells. Our results showed that embelin inhibits cell proliferation, reduces cell migration and induces apoptosis in a dose- and time-dependent manner regardless of cell line. These findings are consistent with the work of Dhanjal et al. who reported slower migration of MCF-7 and MDA-MB-231 cells in the wound area when treated with 15 μM embelin and that of Li

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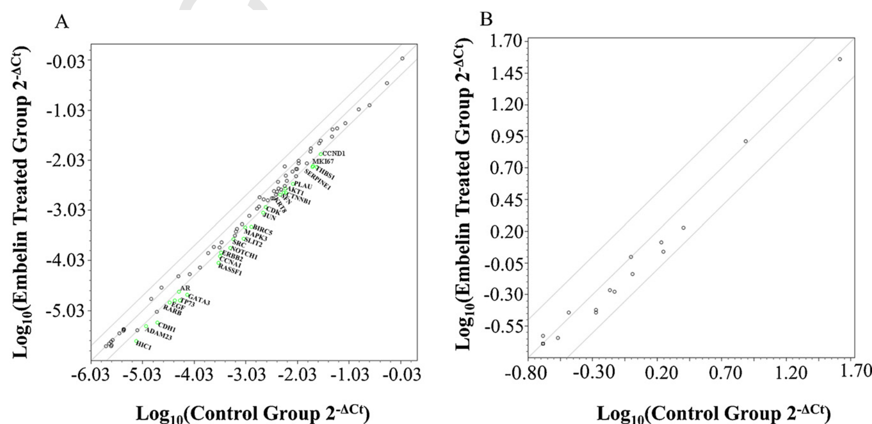

Fig. 5. Effect of embelin on expression levels of 84 key human breast cancer genes in (A) MDA-MB-231 cells and (B) MCF-7 cells.

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and coworkers who showed similar dose-dependent apoptotic effect [8,9]. However, the study by Li and coworkers only focused on MCF-7 cells [9].

To date very few studies have investigated gene expression profiles of solid tumors treated with embelin. In the current study, we examined the expression profile of 84 key genes involved in breast cancer carcinogenesis following treatment of breast cancer cells (MDA-MB-231 and MCF-7) with embelin. Our objective was to provide new data on genes that are significantly altered by embelin in both cell lines. Such information could be pertinent to designing and developing suitable embelin-based combination therapies for treating breast cancer.

Among the 84 genes examined, 27 were highly down-regulated in MDA-MB-231 cells while no change in gene expression was observed in MCF-7 cells following exposure to embelin (10 μM) for 24 h. We selected this concentration since based on our cell proliferation studies it is slightly higher than the IC_{50} of embelin in both MDA-MB-231 and MCF-7 cells after 24 h of treatment. Our finding of no changes in gene expression profiles in MCF-7 is interesting and warrants further investigation in the future. Presently it is not clear why this phenomenon occurs, however, it is conceivable that dose and exposure time may play a key role. Unlike MDA-MB-231 cells, MCF-7 cells were less responsive to embelin at 10 μM following exposure to the drug for 24 h. Although, it is possible that variation in replicates can prevent significant changes from emerging in the MCF-7 cells, it is unlikely the case in our studies. Since we were interested in the functional role of the differentially expressed genes, we grouped them as follows: Signal transduction, Epithelial-Mesenchymal Transition (EMT), Angiogenesis, Cell Adhesion Molecules, Proteolysis, Apoptosis, Cell Cycle, DNA Damage and transcription factors. Several signaling pathways affect signal transduction. Therefore, differentially expressed genes in the signal transduction group were further examined under the following sub-categories: Steroid Receptor-Mediated, Hedgehog, Glucocorticoid, Classical WNT, PI3 K/AKT, NOTCH and MAPK. Among these sub-categories, no glucocorticoid signaling genes were significantly upregulated or downregulated. In contrast, we observed two Steroid Receptor-Mediated sub-category genes to be significantly differentially expressed. *CTNNB1* and *AR* were downregulated. *AR* (Androgen Receptor) is an important transcription factor involved in breast cancer carcinogenesis which plays a pivotal role in signal transduction as a steroid receptor-mediated molecule. It has been shown to be expressed in majority of breast cancers and across the three main sub-types, with AR expression associated with well-differentiated breast cancers [10–13]. We found AR expression to be downregulated in MDA-MB-231 cells following treatment with embelin. This result is in close agreement with the findings of Danquah et al. In their study, AR expression was observed to be downregulated in C4-2 prostate cancer cells following treatment with embelin [3].

Notch signaling is abnormally activated in various cancers including breast cancers [14]. Therefore, we examined expression of two genes associated with Notch signaling (*NOTCH1* and *BIRC5*). *BIRC5* encodes survivin, a member of the inhibitor of apoptosis protein (IAP) family. Recently, Lee et al. have shown survivin to be a direct transcription target of *NOTCH1* [15]. Survivin is an antiapoptotic protein which functions by inhibiting caspase activation and is overexpressed in several solid tumors. Consequently, survivin has become an attractive drug target for treating cancer and numerous approaches have been exploited to down-regulate its expression in cancer [16]. *NOTCH1* is typically upregulated in several cancers. It is regarded as an oncogene since its overexpression inhibits apoptosis in certain cancers and is associated with poor prognosis in breast cancer [17–19]. Suman and coworkers showed inhibition of *NOTCH1* by psoralidin to result in growth arrest and inhibition of EMT in breast cancer cells and breast cancer stem cells [20]. Nakagawa's group has reported on the effect of EGFR inhibitors on survivin expression [21,22]. Their results show gefitinib decreased survivin expression in NSCLC cells while erlotinib had no effect on survivin expression in EGFR mutation positive NSCLC cells with PTEN loss. In our study, we found *NOTCH1* and *BIRC5* expression to be significantly down-regulated in MDA-MB-231 cells following treatment with embelin.

The role of EMT in breast cancer has been well studied. For instance, Hiscox et al. showed EMT triggered by EGFR signaling increased invasiveness and caused MCF-7 breast cancer cells to be resistant to tamoxifen [23]. We therefore investigated expression changes of five EMT regulators (*CTNNB1, NOTCH1, SRC, TGFβ1* and *TWIST1*) following exposure of MDA-MB-231 cells to embelin. In our study, only *CTNNB1* and *NOTCH1* exhibited altered expression post embelin treatment and both molecules were downregulated. As EMT regulators, it is not surprising that *CTNNB1* and *NOTCH1* are downregulated together. *CTNNB1* encodes the proto-oncogene Beta-Catenin which accompanies slug-induced EMT resulting from a series of cascading molecular events initiated by Notch activity [24].

The significant changes in gene expression profile in MDA-MB-231 cells following treatment with embelin involved three apoptosis regulatory genes: AKT1, JUN and TP73. All three genes were downregulated following treatment with embelin in our study. AKT1 and JUN are known to inhibit the apoptotic process and hence promote cancer cell survival. The observed decrease in AKT1 and JUN expression post embelin treatment may represent an effort to accelerate the apoptotic signaling pathway thereby promoting cell death. TP73 is a tumor suppressor which induces apoptosis. In this regard, our results of decreased TP73 expression in embelin treated MDA-MB-231 cells are rather unexpected and warrant further investigation.

Conclusions

In summary, this study identified 27 differentially expressed key genes involved in breast cancer carcinogenesis following exposure of the breast cancer cell line MDA-MB-231 to embelin using the Human Breast Cancer RT^2 Profiler PCR Array. The differentially expressed genes were subjected to pathway analysis and gene ontology using DAVID. Pathways identified confirmed the importance of p53, ErbB, MAPK and VEGF signaling in embelin therapy. Also, differentially expressed genes were primarily involved in regulation of cell proliferation, apoptosis and blood vessel development. Collectively, our results provide an initial yet universal view of potential molecular targets in MDA-MB-231 cells affected by embelin and this knowledge could aid in the design and development of effective embelin-based combination therapies for treating breast cancer.

Conflict of interest

The authors report no conflict of interest.

Funding body

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370 **References**

371 [1] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin
372 2015;65(1):5–29.
373 [2] Ahn KS, Sethi G, Aggarwal BB. Embelin, an inhibitor of X chromosome-linked
374 inhibitor-of-apoptosis protein, blocks nuclear factor-kappaB (NF-kappaB) sig-
375 naling pathway leading to suppression of NF-kappaB-regulated antiapoptotic
376 and metastatic gene products. Mol Pharmacol 2007;71(1):209–19.
377 [3] Danquah M, Duke 3rd CB, Patil R, Miller DD, Mahato RI. Combination therapy
378 of antiandrogen and XIAP inhibitor for treating advanced prostate cancer.
379 Pharm Res 2012;29(8):2079–91.
380 [4] Danquah M, Li F, Duke 3rd CB, Miller DD, Mahato RI. Micellar delivery of
381 bicalutamide and embelin for treating prostate cancer. Pharm Res
382 2009;26(9):2081–92.
383 [5] Flanagan L, Kehoe J, Fay J, Bacon O, Lindner AU, Kay EW, et al. High levels of X-
384 linked Inhibitor-of-Apoptosis Protein (XIAP) are indicative of radio chemo-
385 therapy resistance in rectal cancer. Radiat Oncol 2015;10:131.
386 [6] Pluta P, Jeziorski A, Cebula-Obrzut AP, Wierzbowska A, Piekarski J, Smolewski
387 P. Expression of IAP family proteins and its clinical importance in breast cancer
388 patients. Neoplasma 2015;62(4):666–73.
389 [7] Zhou S, Ye W, Shao Q, Qi Y, Zhang M, Liang J. Prognostic significance of XIAP and
390 NF-kappaB expression in esophageal carcinoma with postoperative radiother-
391 apy. World J Surg Oncol 2013;11:288.
392 [8] Dhanjal JK, Nigam N, Sharma S, Chaudhary A, Kaul SC, Grover A, et al. Embelin
393 inhibits TNF-alpha converting enzyme and cancer cell metastasis: molecular
394 dynamics and experimental evidence. BMC Cancer 2014;14:775.
395 [9] Li Y, Li D, Yuan S, Wang Z, Tang F, Nie R, et al. Embelin-induced MCF-7 breast
396 cancer cell apoptosis and blockade of MCF-7 cells in the G2/M phase via the
397 mitochondrial pathway. Oncol Lett 2013;5(3):1005–9.
398 [10] Cochrane DR, Bernales S, Jacobsen BM, Cittelly DM, Howe EN, D'Amato NC,
399 et al. Role of the androgen receptor in breast cancer and preclinical analysis of
400 enzalutamide. Breast Cancer Res 2014;16(1):R7.
401 [11] Hu R, Dawood S, Holmes MD, Collins LC, Schnitt SJ, Cole K, et al. Androgen
402 receptor expression and breast cancer survival in postmenopausal women.
403 Clin Cancer Res 2011;17(7):1867–74.
404 [12] Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al.
405 Identification of human triple-negative breast cancer subtypes and preclinical
406 models for selection of targeted therapies. J Clin Invest 2011;121(7):2750–67.
407 [13] Park S, Koo J, Park HS, Kim JH, Choi SY, Lee JH, et al. Expression of androgen
408 receptors in primary breast cancer. Ann Oncol 2010;21(3):488–92.
409 [14] Stylianou S, Clarke RB, Brennan K. Aberrant activation of notch signaling in
410 human breast cancer. Cancer Res 2006;66(3):1517–25.
411 [15] Lee CW, Raskett CM, Prudovsky I, Altieri DC. Molecular dependence of estrogen
412 receptor-negative breast cancer on a notch-survivin signaling axis. Cancer Res
413 2008;68(13):5273–81.
414 [16] Groner B, Weiss A. Targeting survivin in cancer: novel drug development
415 approaches. BioDrugs 2014;28(1):27–39.
416 [17] Jundt F, Anagnostopoulos I, Forster R, Mathas S, Stein H, Dorken B. Activated
417 Notch1 signaling promotes tumor cell proliferation and survival in Hodgkin
418 and anaplastic large cell lymphoma. Blood 2002;99(9):3398–403.
419 [18] Jundt F, Probsting KS, Anagnostopoulos I, Muehlinghaus G, Chatterjee M,
420 Mathas S, et al. Jagged1-induced Notch signaling drives proliferation of
421 multiple myeloma cells. Blood 2004;103(9):3511–5.
422 [19] Miele L. Notch signaling. Clin Cancer Res 2006;12(4):1074–9.
423 [20] Suman S, Das TP, Damodaran C. Silencing NOTCH signaling causes growth
424 arrest in both breast cancer stem cells and breast cancer cells. Br J Cancer
425 2013;109(10):2587–96.
426 [21] Okamoto K, Okamoto I, Hatashita E, Kuwata K, Yamaguchi H, Kita A, et al.
427 Overcoming erlotinib resistance in EGFR mutation-positive non-small cell
428 lung cancer cells by targeting survivin. Mol Cancer Ther 2012;11(1):
429 204–13.
430 [22] Okamoto K, Okamoto I, Okamoto W, Tanaka K, Takezawa K, Kuwata K, et al.
431 Role of survivin in EGFR inhibitor-induced apoptosis in non-small cell lung
432 cancers positive for EGFR mutations. Cancer Res 2010;70(24):10402–10.
433 [23] Hiscox S, Jiang WG, Obermeier K, Taylor K, Morgan L, Burmi R, et al. Tamoxifen
434 resistance in MCF7 cells promotes EMT-like behaviour and involves modula-
435 tion of beta-catenin phosphorylation. Int J Cancer 2006;118(2):290–301.
436 [24] Leong KG, Niessen K, Kulic I, Raouf A, Eaves C, Pollet I, et al. Jagged1-mediated
437 Notch activation induces epithelial-to-mesenchymal transition through Slug-
438 induced repression of E-cadherin. J Exp Med 2007;204(12):2935–48.

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