Abstract. Background/Aim: Triple-negative breast cancer (TNBC) is characterized by the absence of hormone receptors (estrogen, progesterone and human epidermal growth factor receptor-2) and a relatively poor prognosis due to inefficacy of hormone receptor-based chemotherapies. It is imperative that we continue to explore natural products with potential to impede growth and metastasis of TNBC. In this study, we screened over 1,000 natural products for capacity to induce cell death in TNBC (MDA-MB-231) cells. Materials and Methods: Frankincense (Boswellia serrata extract (BSE)) and 3-O-Acetyl-β-boswellic acid (3-OAβBA) were relatively potent, findings that corroborate the body of existing literature. The effects of BSE and 3-OAβBA on genetic parameters in MDA-MB-231 cells were evaluated by examining whole-transcriptomic influence on mRNAs, long intergenic non-coding RNA transcripts (lincRNA) and non-coding miRNAs. Results: Bio-statistical analysis demarcates the primary effect of both BSE/3-OAβBA on the up-regulation of PERK (protein kinase RNA-like endoplasmic reticulum kinase)-endoplasmic reticulum (ER)/unfolded protein response (UPR) pathways that are closely tied to activated programmed cell death (APCD). Global profiling confirms concomitant effects of BSE/3-OAβBA on upwardly expressed ER/UPR APCD key components PERK (EIF2AK3), XBP1, C/EBP homologous protein transcription factor (CHOP), ATF3 and DDIT3,4/DNA-damage-inducible transcript 3,4 (GADD34). Further, BSE and/or 3-OAβBA significantly down-regulated oncogenes (OG) which, heretofore, lack functional pathway mapping, but are capable of driving epithelial–mesenchymal transition (EMT), cell survival, proliferation, metastasis and drug resistance. Among these are cell migration-inducing protein hyaluronan binding (CEMIP) [-7.22]; transglutaminase 2 [-4.96], SRY box 9 (SOX9) [-4.09], inhibitor of DNA binding 1, dominant negative helix-loop-helix protein (ID1) [-6.56]; and endothelin 1 (EDN1, [-5.06]). Likewise, in the opposite manner, BSE and/or 3-OAβBA induced the robust overexpression of tumor suppressor genes (TSGs), including: glutathione-depleting ChaC glutathione-specific gamma-glutamylcyclotransferase 1 (CHAC1) [+21.67]; the mTOR inhibitors - strerin 2 (SESN2) [+16.4]; Tribbles homolog 3 (TRIB3) [+6.2], homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (HERPUD1) [+12.01]; and cystathionine gamma-lyase (CTh) [+11.12]. Conclusion: The anti-cancer effects of the historically used frankincense sap (BSE) appear to involve major impact on the ER/UPR response, concomitant to effecting multiple targets counter to the growth, proliferation and metastasis of TNBC cancer cells. The microarray data are available at Expression Omnibus GEO Series accession number GSE102891.

Frankincense has been used as a valuable multi-purpose natural product for over 5,000 years, where its medicinal form is derived from the tree sap resin of diverse species from the genus Boswellia/family Burseraceae. Its extended historical use reflects valuable insight about its properties from our ancestors who had a greater dependency on natural medicines. In the past century, with the rapid development of synthetic medicines, botanical therapeutics are perceived as menial compared to that of current medical treatment. Yet, at the same time, scientific literature continues to report Boswellia and its active component: boswellic acid can exert
dissolved in DMSO [5-20 mg/mL], where the crude herbs including 
*Boswellia serrata* and its constituents suppress NF-
κB, Bcl-2, Bcl-xl, Mcl-1, IAP-1, BIRC5, VEGF (2, 8, 9) 
mPGES-1, MMP-2,7,9, PGE2 (5) cyclin D1, PCNA, c-Myc 
(10), cyclin E, CDK 2 and 4 and retinoblastoma (Rb) (11). 
Central to these effects are control over STAT3 phosphorylation of Jak 2/Src or Akt/GSK3β signaling 
tantamount to triggering apoptotic pathways through caspase-
9, caspase-3, and cleaved PARP (12, 13). Other reported anti-
cancer attributes of *Boswellia* include its potential to block 
the development of chemically induced cancers such as that 
as azothemone (14), prevent multidrug resistance (15) and act 
as a chemo-sensitizing agent (4, 16). These effects are 
consistently observed both in *in vitro* and *in vivo* (10). 
With regards to triple negative breast cancer (TNBC), *Boswellia serrata* extract (BSE) and 3-O-Acetyl-β-boswellic acid (3-
OAβBA) are equally effective against its growth and that of 
other malignant breast tumor cell lines (8, 17, 18). 

Here, we further investigate precipitating transcriptome 
changes induced by *Boswellia serrata* extract and 3-OAβBA, 
in order to determine the major cause of cell death in TNBC 
breast cancer cells. These findings can serve as a general 
directive in future studies investigating the anti-cancer 
properties of frankincense. 

**Materials and Methods**

Hanks Balanced Salt Solution, (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) (HEPES), absolute ethanol ≥99.8%. 96 well plates, pipette tips, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin general reagents and supplies were all purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and VWR International (Radnor, PA, USA). Triple-
negative human breast tumor (MDA-MB-231) cells were obtained 
from the American Type Culture Collection (Rockville, MD, USA). 
*Boswellia serrata* was obtained from Starwest Botanicals 
(Sacramento, CA, USA) and 3-O-Acetyl-β-boswellic acid was 
purchased from Cayman Chemical (Ann Arbor, MI, USA). All 
microarray equipment, reagents and materials were purchased from 
Affymetrix/Thermo Fisher (Waltham, MA, USA).

All natural chemicals, reference drugs and (3-OAβBA) were 
dissolved in DMSO [5-20 mg/mL], where the crude herbs including 
*Boswellia serrata* were prepared in absolute ethanol [50 mg/mL] 
after being diced, macerated and powdered prior to being stored 
at −20°C. All dilutions were prepared in sterile HBSS + 5 mM HEPES, 
adjusted to a pH of 7.4, ensuring solvent concentration of DMSO 
or absolute ethanol at less than 0.5%.

**Cell culture.** MDA-MB-231 cells were cultured in 175 cm² 
flasks containing DMEM supplemented with 10% FBS and 100 U/ml 
penicillin G sodium/100 µg/ml streptomycin sulfate. Cells were 
grown at 37°C in 5% CO₂ atmosphere and sub-cultured every three 
to five days.

**Cell viability assay.** Alamar Blue cell viability assay was used to 
determine cytotoxicity. Viable cells are capable of reducing 
resazurin to resorufin (a detectable fluoroprobe). Briefly, 96-well 
plates were seeded with MDA-MB-231 cells at a density of 5x10⁶ 
cells/ml. Cells were treated with HBSS (control) and various 
concentrations of *Boswellia serrata* extract or 3-O-Acetyl-β-
boswellic acid for 24 h at 37°C, 5% CO₂ in atmosphere. Alamar 
blue (0.1 mg/ml in HBSS) was added at 15% v/v to each well, and 
the plates were incubated for 6-8 h. Quantitative analysis of dye 
conversion was measured on a Synergy™ HTX Multi-Mode 
microplate reader (BioTek, Winooski, VT, USA), 550nm /580nm 
(excitation/emission). The data were expressed as a percentage of 
untreated controls. 

**Fluorescence microscopy.** Live cell imaging was conducted using 
Fluorescein diacetate (FDA), which is a cell-permeable esterase 
substrate. The fluorescein molecule accumulates in cells that possess 
intact membranes, serving as a marker of cell viability. Briefly, FDA 
was dissolved in ethanol 4.2 mg/ml and subsequently prepared at 
20 μM in HBSS. After 30 min of incubation, samples were analyzed 
photographically using a fluorescent /inverted microscope, CCD 
camera and data acquisition by ToupTek View (ToupTek Photonics 
Co, Zhejiang, P.R. China).

**Microarray WT 2.1 human datasets.** After treatment, cells were 
washed three times in HBSS, rapidly frozen and stored at −80°C. Total 
RNA was isolated/ purified using the TRIzol/chloroform method, 
quality was assessed and concentration was equalized to 82 ng/μl in 
nuclease free water. Whole transcriptome analysis was conducted 
according to the GeneChip™ WT PLUS Reagent Manual for Whole 
Transcript (WT) Expression Arrays. Briefly, RNA was synthesized to 
first strand cDNA, second-strand cDNA, followed by transcription to 
cRNA. cRNA was purified and assessed for yield, prior to 2nd cycle 
single stranded cDNA. cDNA was then quantified for 
yield and equalized to 176 ng/ml. Subsequently, cDNA was 
fragmented, labeled and hybridized on to the arrays prior to being 
subjected to fluids and imaging using the Gene Atlas (Affymetrix, 
ThermoFisher Scientific, Waltham, MA, USA).

The array data quality control and initial processing from CEL 
to CHP files were conducted using expression console, prior to data 
evaluation using the Affymetrix transcriptome analysis console. 
Supportive analysis was accomplished using geneontology.org (19) 
and DAVID Bioinformatics Resources 6.8 National Institute of 
Allergy and Infectious Diseases (NIADD), NIH (20).

**Microarray miRNA 4.1 human datasets.** miRNA was isolated using 
the QIAzol reagent and miRNeasy Mini Kit (Qiagen, Germantown, 
MD). Briefly, after RNA purification, samples were labeled with a 
POLY A tail, and ligated using a flash tag ligation mix from the 
FlashTag™ Biotin HSR RNA kit (Affymetrix). Subsequently, labeled 
RNA was detected using streptavidin – EP and hybridized onto 
a Genechip miRNA 4.1 human array, prior to fluidsics and imaging by 
the Gene Atlas. The array data quality control and initial processing 
from CEL to CHP files were conducted using expression console, prior 
to data evaluation using the transcriptome analysis console provided
Supportive analysis was accomplished using geneontology.org (19) and DIANA miRPath tools (21, 22).

Data analysis. Statistical analysis was performed using Graph Pad Prism (version 3.0; Graph Pad Software Inc. San Diego, CA, USA) with significance of difference between the groups assessed using a one-way ANOVA, then followed by a Tukey post hoc means comparison test, or a Student’s t-test. LC50s were determined by regression analysis using Origin Software (Origin Lab, Northampton, MA, USA).
Results

A high throughput (HTP) screening module is routinely used in our facility to enable the preliminary evaluation of thousands of herbs and plant chemicals on selective targets, and in this case for relative capacity to induce cell death in MDA-MB-231 cells (Figure 1). Briefly, LC₅₀s were established, natural products were ranked for potency and lead compounds identified. Here we focus on the natural herb: *Boswellia serrata* (BSE), where we present fluorescence FDA staining showing a loss of viability over concentration (Figure 2) and corresponding cytotoxicity as determined by Alamar Blue (Figure 3). The LC₅₀s were determined (128.8 μg/ml) for BSE and its active component 3-OAβBA (46.32 μg/ml) (Figure 4).

For whole transcriptome microarray studies, the LC₅₀s of BSE and 3-OAβBA were applied to fully viable cells at Time 0 (zero minutes), and morphological changes were monitored every hour, to ensure no cell death was evident. At 6-8 h, the cells retained morphological shape, flask attachment and had no obvious signs of cell death. At this point, cells were rapidly washed in HBSS 3x, spun and frozen at –80°C. This time of acquisition was ascertained as appropriate to ensure capture of information on pivotal events elicited/precipitating cell death.

Using affymetrix human whole transcriptome arrays [GeneChip Human Gene Array 2.1], the data showed that of the 48226 transcripts tested, there were 300 differentially expressed genes (DEGs) for BSE (265 up-regulated/65 down-regulated) and for 3-OAβBA: 931 DEGs (391 up-regulated/540 down-regulated). An overview of the transcriptome data for BSE treatment are presented by a volcano plot (Figure 5) showing fold change (FC) vs. significance – then cross referenced to Table I, which presents the largest differentially expressed changes. An overview of microarray data for 3-OAβBA treatments are presented by a similar volcano plot (Figure 6) also cross referenced to Table II, showing the largest differentially expressed changes. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE102891 located at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102891.

The pathway of greatest impact for both BSE and 3-OAβBA elucidated by the Affymetrix transcriptome analysis console, sorted by greatest relevance was up-regulation of the photodynamic therapy unfolded protein response (Figure 7).
Using DAVID Functional Annotation Bioinformatics Microarray Analysis (20), we also found that the endoplasm reticulum was largely affected – up-regulated genes are shown on a KEGG overlay pathway map (Figure 8). Again, there was a very close overlay between BSE and 3-OAcβBA to elicit these ER mediated responses. In terms of dataset analysis for
functional biological relevance, we used geneontology.org enrichment analysis tool, which also confirms the findings from Affymetrix and David bio analytic tools, corroborating uniform up-regulation on the ER stress, unfolded protein responses as well as glucose depletion/starvation (Tables III and IV). Interestingly, very few changes were reported for the miRNAs, with reported pathways for hsa-miR-34b-3p (target of 14 genes) and hsa-miR-184 (target of 6 genes), as presented in Table V. These findings provide an overview of Boswellia seratta and its pharmacologically active compound, boswellic acid on the transcriptome of TNBCs.

Discussion

The data in this study suggest a primary mode of cell death by 3-OAβBA and BSE to involve ER stress leading to a UPR (unfolded protein response), this commonly associated with activated cell death. There has been a recent surge in research describing the importance of the ER/UPR in a variety of human pathologies, many of these relevant to cancer (23-25).

A literature review of the ER/UPR involvement in cancer unveils a scientific uncertainty and need for answers as to why activation of ER/UPR creates a double-edged sword. On the one hand, ER stress inducers (e.g. hypoxia, glucose, nutrient deprivation (26, 27) activate the ER/UPR which leads to tumor adaptation (a persistent elevation of pro-survival proteins, a resistance to chemotherapy, greater tumor progression, angiogenesis, invasion and thriving of dormant stem cells (28-30). Yet at the same time, activation of main pathways in the ER/UPR an also trigger programmed cell death (PCD) evidenced by many natural anti-cancer agents (31-41) alkylating/ platinum based drugs and anti-cancer steroids (42-47). Several articles have expressed the need for

Figure 5. WT changes in BSE treated cells using GeneChip™ Human Gene 2.1 ST Array. 48226 transcripts were tested, 300 differentially expressed genes (DEGs) were identified for (265 up-regulated/65 down-regulated). The data are presented by a volcano plot (fold-change by significance) for whole transcriptome changes in BSE treated MDA-MB-231 cells vs. controls, n=3. The left panel shows down-regulated genes (red)/right panel (green) shows up-regulated genes: highlighting some of the highest differential changes, also listed in Table I.
further understanding of the pro-survival/pro-death ER/UPR processes and the relevance timing on cancer initiation, progression and treatment (48). It is believed that if we can further understand control of ER stress regulators on cancer growth, we can successfully use this information to overcome acquired resistance to (49) and augment existing
Figure 6. WT changes in 3-OAβBA treated cells using GeneChip™ Human Gene 2.1 ST Array. 48226 transcripts tested: 931 DEGs were identified (391 up-regulated/540 down-regulated). The data are presented as a volcano Plot (fold change by significance) for whole transcriptome changes in 3-OAβBA treated MDA-MB-231 cells vs. controls, n=3. The left panel shows down-regulated genes (red)/right panel shows up-regulated genes (green): highlighting some of the top changes, also listed in Table II.

Table II. Largest differential shifts incurred by 3-OAβBA in MDA-MB-231 cells. The data represent signal, fold-change, p-value, gene symbol and gene description. Top mRNA Changes: Affymetrix Microarray WT Human 2.1 ST.

| Transcript | 3-OAβBA | Control | Fold | ANOVA | Gene | Gene Description |
|------------|---------|---------|------|-------|------|------------------|
| Cluster ID | Bi-weighted AVE Signal Log2 | Bi-weighted AVE Signal Log2 | Change | p-Value | Symbol | Description |
| 17016375   | 7.32    | 2.47    | 28.77 | 0.0001 | HIST1H1T | Histone cluster 1, H1t |
| 17114701   | 7.12    | 2.54    | 23.91 | 0.0038 | CDR1   | Cerebellar degeneration related protein 1 |
| 17083614   | 7.71    | 3.91    | 13.9  | 0.0002 | LURAPIL | Leucine rich adaptor protein 1-like |
| 17005573   | 8.39    | 4.6     | 13.88 | 0.0002 | HIST1H2BD | Histone cluster 1, H2bd |
| 16692603   | 7.32    | 3.53    | 13.77 | 0.0001 | HIST2H2BF | Histone cluster 2, H2bf |
| 17016379   | 7.07    | 3.35    | 13.17 | 0.0005 | HIST1H2BC | Histone cluster 1, H2bc |

Table II. Continued
### Table II. Continued

| Transcript ID | Bi-weighted AVE Signal Log2 | Bi-weighted AVE Signal Log2 | Change | p-Value | Symbol | Gene Description |
|---------------|-----------------------------|-----------------------------|--------|---------|--------|------------------|
| 16756310      | 8.34                        | 4.75                        | 12.02  | 0.0001  | TCP1L2  | t-complex 11, testis-specific-like 2 |
| 16691619      | 7.17                        | 3.68                        | 11.22  | 0.0006  | LINC00622 | Long intergenic non-protein coding RNA 622 |
| 16677278      | 7.12                        | 3.78                        | 10.17  | 0.0001  | ATF3    | Activating transcription factor 3 |
| 16756202      | 9.71                        | 6.41                        | 9.8    | 0.0002  | EID3    | EP300 interacting inhibitor of differentiation 3 |
| 16692611      | 6.16                        | 2.88                        | 9.73   | 0.0001  | HIST2H2BF | Histone cluster 2, H2bf |
| 17006863      | 11.42                       | 8.15                        | 12.17  | 0.0009  | PA2G4P4 | Proliferation-associated 2G4 pseudogene 4 |
| 17005862      | 5.04                        | 2.17                        | 7.28   | 0.0055  | HIST1H3H | Histone cluster 1, H3b |
| 16743222      | 9.02                        | 6.19                        | 12.17  | 0.0009  | CHORDC1 | Cysteine and histidine rich domain containing 1 |
| 17052425      | 6.04                        | 3.23                        | 7.04   | 0.0020  | MGAM    | Malate-glutamylase |
| 16889797      | 8.71                        | 5.9                         | 1.57   | 0.0009  | NABP1   | Nucleic acid binding protein 1 |
| 16705961      | 9.29                        | 6.49                        | 4.94   | 0.0002  | DDTT4   | DNA damage inducible transcript 4 |
| 16768738      | 8.84                        | 6.05                        | 4.71   | 0.0006  | NTN4    | Netrin 4 |
| 16924602      | 7.28                        | 4.52                        | 6.75   | 0.0046  | ADAMTS1 | ADAM metallopeptidase / thrombospondin type 1 M1 |
| 16766578      | 6.17                        | 3.47                        | 4.31   | 0.0017  | DDT3    | DNA-damage-inducible transcript 3 |
| 16967771      | 5.61                        | 2.96                        | 6.25   | 0.0014  | CXCL8   | Chemokine (C-X-C motif) ligand 8 |
| 16992467      | 6.59                        | 3.97                        | 6.17   | 0.0004  | CREB3    | CREB3 regulatory factor |
| 16703028      | 7.29                        | 4.97                        | 4.12   | 0.0003  | DNAJB9  | DnaJ (Hsp40) homolog, subfamily B, member 9 |
| 17661631      | 6.95                        | 4.46                        | 6.5    | 0.0051  | DUSP16  | Dual specificity phosphatase 16 |
| 16761430      | 7.94                        | 5.49                        | 2.55   | <0.0001 | LINC01004 | Long intergenic non-protein coding RNA 1004 |
| 17028116      | 5.24                        | 3.31                        | 1.01   | 0.0062  | MTRN2   | MicroRNA 4668 |
| 16703242      | 6.98                        | 4.6                         | 5.2    | 0.0005  | OTU1    | OTU deubiquitinase 1 |
| 17000518      | 6.13                        | 3.76                        | 6.54   | 0.0016  | HPA9    | Heat shock 70kDa protein 9 (mortalin) |
| 16903897      | 6.39                        | 4.05                        | 5.26   | <0.0001 | NR4A2   | Nuclear receptor subfamily 4, group A, member 2 |
| 16799739      | 7.08                        | 4.81                        | 4.36   | 0.0019  | CHAC1   | ChaC glutathione-specific gamma-glutamylcysteine transferase 1 |
| 17060061      | 7.9                         | 5.6                         | 4.42   | 0.0005  | ASNS    | Asparaginase synthetase (glutaminyl-hydrolyzing) |
| 16692632      | 7.89                        | 5.6                         | 3.42   | 0.0001  | HIST2H2BE | Histone cluster 2, H2be |
| 16890653      | 10.54                       | 8.3                         | 4.17   | <0.0001 | DNAJB1  | DnaJ (Hsp40) homolog, subfamily B, member 1 |
| 16951188      | 5.66                        | 3.44                        | 6.66   | 0.0018  | METTL6  | Methyltransferase like 6 |
| 16743432      | 5.34                        | 3.31                        | 2.03   | 0.0018  | SESP3   | Sestrin 3 |
| 16859763      | 5.88                        | 7.73                        | -6.67  | 0.0068  | IFI30   | Interferon, gamma-inducible protein 30 |
| 17075529      | 3.81                        | 5.67                        | -3.64  | 0.0002  | ENTPD4  | Ectonucleoside triphosphate diphosphohydrolase 4 |
| 16845657      | 8.02                        | 9.88                        | -3.64  | 0.0077  | SLC25A39 | Solute carrier family 25, member 39 |
| 17000208      | 3.79                        | 5.69                        | -1.73  | 0.0064  | VTRNA2-1 | Vault RNA 2-1 |
| 1685310       | 7.86                        | 9.79                        | -2.13  | 0.0368  | TFNRF512A | Tumor necrosis factor receptor superfamily, member 12A |
| 16925398      | 5.3                         | 7.23                        | -3.43  | 0.0664  | RUNXI-IT1 | RUNXI intronic transcript 1 |
| 16886946      | 7.43                        | 9.37                        | -2.54  | 0.0345  | TIMM13   | Translocase of inner mitochondrial mem 13 homolog (yeast) |
| 16902071      | 4.86                        | 6.81                        | -2.16  | 0.0039  | PMEPA1  | Prostate transmembrane protein, androgen induced 1 |
| 16921433      | 4.76                        | 6.71                        | -2.03  | 0.0049  | RG519    | Regulator of G-protein signaling 19 |
| 16837418      | 7.05                        | 9.07                        | -2.2    | 0.0048  | SRYx9   | SRY box 9 |
| 16726183      | 8.29                        | 10.33                       | -5.14  | 0.0175  | COX8A    | Cytochrome c oxidase subunit VIII A (ubiquitous) |
| 16816368      | 5.65                        | 7.75                        | -2.2    | 0.0047  | SEMA7A   | Semaphorin 7A, GPI membrane anchor |
| 16828886      | 4.26                        | 6.42                        | -2.47  | 0.0160  | GINS2    | GINS complex subunit 2 (Pof2 homolog) |
| 16919158      | 5.91                        | 8.22                        | -3.77  | 0.0032  | TGM2    | Transglutaminase 2 |
| 16819233      | 4.56                        | 6.92                        | -2.76  | 0.0066  | MT1A     | Metallothionein 1A |
| 16803754      | 4.95                        | 7.8                         | -3.27  | 0.0015  | CEMIP    | Cell migration inducing protein, hyaluronan binding |
cancer therapies (50). The data from this study show BSE and 3-OAβBA to impact several processes within the ER/UPR.

**ER/UPR.** If we take a look at the normal function of the ER under non-stress conditions, its main purpose is in the post-translational modification and folding of mature proteins using chaperones and foldases, which are then trafficked to the Golgi. Anything that impairs this system elicits ER stress and a UPR. This later response (ER/UPR) serves a primary means to reduce protein load by decreasing translation, and removing mis-folded proteins. This is accomplished by increasing the folding capability of the ER, and the degradation rate of damaged proteins through binding to glucose-regulated protein 78 (Bip/GRP78) (a pivotal event) in preparation for disposal through an endoplasmic reticulum-associated degradation pathway (ERAD) by the ubiquitin/proteasome pathway or alternatively, an autophagic/lysosomal pathway (51, 52).

Briefly, the ER/UPR main branches can all initiate pro-apoptotic events. These include:

[Pathway 1] protein kinase RNA-like endoplasmic reticulum kinase (PERK),
[Pathway 2] inositol-requiring enzyme-1 (IRE1), or
[Pathway 3] activating transcription factor-6 (ATF-6) (53, 54).

The effects of 3-OAβBA and BSE on the transcriptome suggests extensive up-regulation on many of these processes.

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Figure 7. Affymetrix Transcription Analysis Console/Wikipaths Correlation by Significance shows impact on Photodynamic therapy unfolded protein response by BSE and 3-3-OAβBA in MDA-MB-231 cells, with a high degree of overlap. The data represents relative fold change by intensity (green) up-regulation, (red) down-regulation with (!!!) filtered out as non-significant directional changes. Highlighted in yellow is the shift in DDIT3, with values presented.
Figure 8. DAVID Functional Annotation Bioinformatics Microarray Analysis. DAVID Bioinformatics Resources 6.8. KEGG Diagram Overlap of up-regulated transcripts in response by BSE and 3-O-Acetyl-β-boswellic acid in MDA-MB-231 cells, with a high degree of overlap. The data represents Protein Processing in the ER and up-regulated transcripts noted by an arrow.
Table III. Biological processes impacted by BSE-treated MDA-MB-231 cells. The data are derived from full dataset analysis using a relational database provided by geneontology.org. This service connects GO Enrichment Analysis to the analysis tool from the PANTHER Classification System, which is maintained up to date with GO annotations. The p-Value (Column 4) is the probability of seeing at least x number of genes (Column 2) out of the total n genes in the process annotated (Column 1) with greater fold enrichment score (Column 3) corresponding to relevant pathway significance impact.

Table IIIa: GO biological process: Up-regulated by BSE

| Biological Process | Homo sapiens - REFLIST (21002) | Genes | (fold Enrichment) | (p-Value) |
|--------------------|---------------------------------|-------|-------------------|-----------|
| PERK-mediated unfolded protein response | 12 | 6 | 44.31 | 0.0001 |
| Positive regulation of transcription from RNA polymerase | | | | |
| II promoter in response to ER stress | 12 | 5 | 36.92 | 0.0028 |
| Intrinsinc apoptotic signaling pathway in response to endoplasmic reticulum stress | 31 | 8 | 22.87 | <0.0001 |
| Positive regulation of transcription from RNA polymerase | | | | |
| II promoter in response to stress | 25 | 6 | 21.27 | 0.0045 |
| IRE1-mediated unfolded protein response | 58 | 12 | 18.33 | <0.0001 |
| ER-nucleus signaling pathway | 36 | 7 | 17.23 | 0.0020 |
| Endoplasmic reticulum unfolded protein response | 115 | 21 | 16.18 | <0.0001 |
| Response to unfolded protein | 160 | 29 | 16.06 | <0.0001 |
| Cellular response to unfolded protein | 121 | 21 | 15.38 | <0.0001 |
| Response to topologically incorrect protein | 180 | 30 | 14.77 | <0.0001 |
| Positive regulation of response to endoplasmic reticulum stress | 36 | 6 | 14.77 | 0.0359 |
| Serine family amino acid metabolic process | 42 | 7 | 14.77 | 0.0056 |
| Cellular response to glucose starvation | 37 | 6 | 14.37 | 0.0419 |
| Cellular response to topologically incorrect protein | 139 | 22 | 14.03 | <0.0001 |
| Regulation of response to endoplasmic reticulum stress | 75 | 11 | 13.0 | <0.0001 |
| Response to endoplasmic reticulum stress | 244 | 32 | 11.62 | <0.0001 |
| Regulation of transcription from RNA polymerase II promoter in response to stress | 115 | 13 | 10.02 | <0.0001 |
| Cell redox homeostasis | 73 | 8 | 9.71 | 0.0198 |
| Regulation of DNA-templated transcription in response to stress | 121 | 13 | 9.52 | <0.0001 |
| Intrinsic apoptotic signaling pathway | 156 | 12 | 6.82 | 0.0026 |
| Protein folding | 237 | 16 | 5.98 | 0.0002 |
| Response to starvation | 173 | 11 | 5.63 | 0.0487 |
| Response to toxic substance | 215 | 13 | 5.36 | 0.0119 |
| Cellular response to extracellular stimulus | 208 | 12 | 5.11 | 0.0486 |
| Cellular response to external stimulus | 281 | 16 | 5.05 | 0.0016 |
| Regulation of apoptotic signaling pathway | 383 | 20 | 4.63 | 0.0002 |
| Cellular amino acid metabolic process | 313 | 16 | 4.53 | 0.0065 |
| Apoptotic signaling pathway | 294 | 15 | 4.52 | 0.0147 |

Analysis Type: http://www.geneontology.org/PANTHER Overrepresentation Test (release 20170413)
Annotation Version and Release Date: GO Ontology database Released 2017-07-1
Analyzed List: Up-regulated by BSE in MDA-231 Cells
Reference List: Homo sapiens (all genes in database)

Table IIIb: GO biological process: Down-regulated by BSE

| Biological Process | Homo sapiens - REFLIST (21002) | Genes | (fold Enrichment) | (p-Value) |
|--------------------|---------------------------------|-------|-------------------|-----------|
| Negative regulation of epithelial cell differentiation | 39 | 4 | 44.88 | 0.019 |
| Mesenchymal cell differentiation | 132 | 6 | 19.89 | 0.005 |
| Regulation of ossification | 183 | 6 | 14.35 | 0.034 |
| Mesenchyme development | 194 | 6 | 13.53 | 0.047 |
| Animal organ morphogenesis | 880 | 11 | 5.47 | 0.032 |

Pathway I/PERK: Briefly, when proteins are misfolded in the ER, they bind to BiP/Grp78 which triggers X-box-binding protein 1 (XBP1) splicing, which then initiates PERK to phosphorylate +P (eIF2α). We found evidence of BSE not only up-regulating XBP1 +3.31, p=0.0003 (3-OAβBA), +3.04, p=0.0003 (BSE) but also PERK (EIF2AK3) +4.6 p=0.0003 (3-OAβBA) and +3.67, p<0.0003 (BSE). This active +PeIF2 α, is central to the control of downstream events which halting protein synthesis, cell cycle arrest in addition to activating ATF4, which in turn elevates ATG12, TRB3 (AKT/mTOR
Table IV: Biological processes impacted by 3-O-Acetyl-β-boswellic acid-treated MDA-MB-231 cells. The data are derived from full dataset analysis using a relational database provided by geneontology.org. This service connects GO Enrichment Analysis to the analysis tool from the PANTHER Classification System, which is maintained up to date with GO annotations. The p-Value (Column 4) is the probability of seeing at least x number of genes (Column 2) out of the total n genes in the process annotated (Column 1) with greater fold enrichment score (Column 3) corresponding to relevant pathway significance impact.

| Process                                                                 | Homo sapiens - GENES | (fold enrichment) | (p-Value) |
|------------------------------------------------------------------------|-----------------------|-------------------|-----------|
| PERK-mediated unfolded protein response                                 | 12                    | 7                 | 46.06     | <0.0001   |
| Positive regulation of transcription from RNA polymerase               | 12                    | 5                 | 32.9      | 0.0049    |
| II promoter in response to ER stress                                   | 75                    | 10                | 10.67     | 0.0005    |
| Regulation of response to endoplasmic reticulum stress                | 115                   | 15                | 10.3      | <0.0001   |
| ER-nucleus signaling pathway                                           | 115                   | 15                | 10.09     | <0.0001   |
| Response to unfolded protein                                           | 115                   | 12                | 8.24      | 0.0004    |
| Regulation of smooth muscle cell proliferation                         | 115                   | 12                | 8.24      | 0.0004    |
| Regulation of transcription from RNA polymerase                        | 115                   | 12                | 8.24      | 0.0004    |
| II promoter in response to stress                                      | 136                   | 14                | 8.13      | <0.0001   |
| Regulation of DNA-templated transcription in response to stress        | 121                   | 12                | 7.83      | 0.0006    |
| Regulation of fat cell differentiation                                 | 113                   | 11                | 7.69      | 0.0026    |
| Nucleosome assembly                                                    | 118                   | 11                | 7.36      | 0.0039    |
| Response to hydrogen peroxide                                          | 108                   | 10                | 7.31      | 0.0139    |
| Cellular response to nutrient levels                                   | 180                   | 16                | 7.02      | <0.0001   |
| Response to starvation                                                 | 173                   | 15                | 6.85      | 0.0001    |
| Cellular response to extracellular stimulus                            | 208                   | 18                | 6.83      | <0.0001   |
| Chromatin assembly                                                     | 134                   | 11                | 6.48      | 0.0133    |
| Response to endoplasmic reticulum stress                               | 244                   | 19                | 6.15      | <0.0001   |
| Nucleosome organization                                                | 147                   | 11                | 5.91      | 0.0319    |
| Cellular response to external stimulus                                 | 281                   | 21                | 5.9       | <0.0001   |
| Chromatin assembly or disassembly                                      | 154                   | 11                | 5.64      | 0.0492    |
| Response to reactive oxygen species                                    | 183                   | 12                | 5.18      | 0.0437    |

Analysis Type: http://www.geneontology.org/PANTHER Overrepresentation Test (release 20170413)
Annotation Version and Release Date: GO Ontology database Released 2017-07-21
Analyzed List: Up-regulated by 3-OAβBA in MDA-231 Cells
Reference List: Homo sapiens (all genes in database)

Table IVb: GO biological process: Down-regulated by 3-OAβBA

| Process                                                                 | Homo sapiens - GENES | (fold enrichment) | (p-Value) |
|------------------------------------------------------------------------|-----------------------|-------------------|-----------|
| Intrinsic apoptotic signaling pathway in response to DNA damage        | 70                    | 11                | 6.85      | 0.008     |
| Positive regulation of apoptotic signaling pathway                     | 181                   | 17                | 4.09      | 0.014     |
| Extracellular matrix organization                                      | 308                   | 22                | 3.11      | 0.036     |
| Extracellular structure organization                                   | 309                   | 22                | 3.1       | 0.038     |
| Response to oxidative stress                                           | 366                   | 25                | 2.98      | 0.018     |
| Regulation of endopeptidase activity                                   | 391                   | 26                | 2.9       | 0.018     |
| Regulation of apoptotic signaling pathway                              | 383                   | 25                | 2.84      | 0.038     |
| Regulation of peptidase activity                                       | 418                   | 27                | 2.81      | 0.019     |
| Negative regulation of protein metabolic process                       | 1105                  | 55                | 2.17      | 0.001     |
| Negative regulation of cellular protein metabolic process              | 1046                  | 51                | 2.12      | 0.004     |
| Negative regulation of molecular function                              | 1161                  | 55                | 2.06      | 0.004     |
| Regulation of cellular component organization                          | 2331                  | 87                | 1.63      | 0.035     |
inhibitor), triggering autophagy required for removal of unfolded proteins. These events are often simultaneous with the rise in C/EBP homologous protein transcription factor (CHOP)/DNA damage-inducible transcript 3, 4 or GADD153,GADD34, and ATF3 (triggering cell death) (55, 56).

The data in this study show mediated effects for TRB3 [+6.3 fold, \( p<0.0001 \) BSE/+3.68, \( p<0.0001 \) 3-OAβ[BA] ATF3 [+12.61 fold, \( p<0.0001 \) BSE/+2.9, \( p<0.0001 \) 3-OAβ[BA], DDT3 [+17.09 fold, \( p<0.0001 \) BSE/+8.03, \( p<0.0001 \) 3-OAβ[BA] and DDT4 [+8.41 fold, \( p<0.0001 \) BSE/+11.77, \( p<0.0001 \) 3-OAβ[BA]. If CHOP driven ER stress mediated apoptosis prevails, this would drive up-regulation of death molecules (BIM, BAX, PUMA), death receptors (Tnfrsf10b/Dr5) juxtaposed to a reduction of BLC2 (anti-apoptotic molecules) (55, 57), activation of JNK and apoptotic molecules) (55, 57), activation of JNK and death receptors (Tnfrsf10b/Dr5). In response to unfolded proteins, IRE1α; ERN1. The data in this study again, show consistent trends in downstream events including elevated levels of EGR-1, \( [+15.18 \text{ fold}, p<0.0001 \) BSE/+2.48, \( p<0.0001 \) 3-OAβ[BA], TRB3 [+6.3 fold, \( p<0.0001 \) BSE/+3.68, \( p<0.0001 \) 3-OAβ[BA] and ATF3 [+12.61 fold, \( p<0.0001 \) BSE/+2.9, \( p<0.0001 \) 3-OAβ[BA].

**Pathway 2/ IRE1α; ERN1.** In response to unfolded proteins, IRE1α; ERN1 is cleaved by endoribonuclease activity at the 26bp intron of XBP1 (involved with pathway 1 above), which facilitates the formation of transcription factor XBP1 mRNA, where IRE1-XBP1 can trigger recruitment of TRAF2 to the ER membrane (+ASK1 recruitment). TRAF2 is an activator of apoptosis signal-regulating kinase 1 (ASK1), which can lead to JNK mediated apoptosis. Also, this pathway can trigger ERO1α to activate the ER calcium channel Drosophila inositol-1,4,5-trisphosphate receptor 1 (IP3R1) enabling activate cAMP response elements (CREs).

**Pathway 3/ATF6:** Upon ER stress, ATF6 dissociates from GRP78/BiP – leaving it free to translocate to the Golgi, where it is cleaved by S1P and S2P, and its fragment released to the cytosol. ATF6 fragments can include the active 50kDa transcription factor (ATF6 p50) which translocate to the nucleus. There, ATF6 p50 and XBP1 bind ERSE promoters and up-regulate chaperones that are involved with unfolded protein response including GRP78.

**ER/UPR stress mediated apoptosis and cancer drugs.** Many natural products are being reported to impact the aforementioned, including a spiked rise in Grp78, CHOP with activated ER/UPR – PCD occurring through PERK, IRE1alpha and ATF6 pathways as in the case of cryptotanshinone (32) 2-(3,4-dihydroxyphenyl)ethanol (olive oil) (58) selenium (59) methylseleninic acid, sodium selenite (33) xanthohumol (hops), docosahexaenoic acid (34, 35) isohuachiolactone (Nan-Chai-Hu) (36) Shikonin (Lithospermum erythrorhizon) (37) chrysin (31) curcumin (40) silibinin (41) or whole herbs such as the Chinese herbal medicine *Tu Bei Mu* (39). A number of drugs also mediate similar effects, such as steroids, platins, taxol, alkylating agents, or cancer chemicals which on the one hand block the growth of diverse cancers, and on the other hand elevated ER/UPR – PCD, associated with up-regulation of GRP78, CHOP and three UPR-associated pathways, PERK, IRE1alpha, and ATF6 (42-44, 46,

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### Table V. Transcriptome miRNA changes in BSE treated MDA-MB-231 cells. The data represents cluster, signal, fold change, p-value, gene symbol, and gene targets.

| Transcript | BSE | Control | Fold | ANOVA | Gene |
|------------|-----|---------|------|-------|------|
| Cluster ID | Bi-weighted AVE Signal Log2 | Bi-weighted AVE Signal Log3 | Change | p-Value | Symbol |
| 20519591   | 2.11 | 1.09    | 2.03 | 0.00398 | hsa-miR-4740-3p |
| 20518919   | 5.7  | 6.92    | -2.34 | 0.004005 | hsa-miR-4521 |
| 20500722   | 3.85 | 5.3     | -2.72 | 0.009182 | hsa-miR-27b-5p |
| 20501169   | 0.7  | 2.2     | -2.82 | 0.020278 | hsa-miR-34b-3p |
| 20500786   | 0.96 | 2.28    | -2.49 | 0.032848 | hsa-miR-184 |
| 20538228   | 4.1  | 2.95    | 2.22  | 0.039779 | U70D |

**Gene targets**

| [miR-34b-3p] | [miR-184] |
|-------------|-----------|
| MET         | INPPL1    |
| CREB        | NEAT1     |
| CDK4        | AK T2     |
| c-MYC       | NEATC2    |
| BCL2        |           |
| CDK6        |           |
| MYC         |           |
| VEGFA       |           |
It is also believed that hydrogen peroxide tumor mediated cell death also corresponds to up-regulation of the PERK branch evident by +P eIF2α and the mRNA levels of activating transcription factor 4 (ATF4), C/EBP homologous (CHOP) and tribbles homolog 3 (TRB3)(61). The findings in this work, place 3-OA βBA and BSE in this category of anti-cancer agents.

While discussing all the changes in the transcriptome initiated by 3-OA βBA and BSE are beyond the scope of this paper, noteworthy is the rise in CHAC1, which is involved in the degradation of glutathione (62, 63) reported to occur in parallel to rise of ATF4-ATF3-CHOP PERK and the phosphorylation of EIF2α, where its rise creates vulnerability of cancer cells to the losses of glutathione associated with radiation and oxidative insult (64, 65) also rendering losses on glutathione detoxification systems (66).

In conclusion, we provide whole transcriptome data analysis of RNA from TNBC cells treated with 3-OA βBA and BSE. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE102891 located at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102891.The findings reflect a high probability of ER/UPR involvement through PERK phosphorylation of eIF2α, leading to up-regulation of ATF3, 4, TRB3, DNA damage-inducible transcript 3, 4 (CHOP) and rise in immediate early response genes. Future research will be required to determine the unique controlling factors in common between natural products and the ER/ UPR programmed death events in tumor cells.

Conflicts of Interest

The Authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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