Original Article

Oxidative stress and breast cancer biomarkers: the case of the cytochrome P450 2E1

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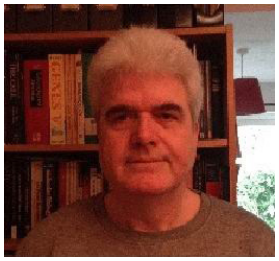

Dr. Demonacos joined the University of Manchester, Manchester Pharmacy School in 2003 where he is involved in the investigation of the role of ROS in cellular energy metabolism and breast carcinogenesis. In addition, Dr. Demonacos' laboratory explores the signaling events that facilitate cancer cells to evade immunosurveillance.

ABSTRACT

Aim: The aim of the study is to investigate the impact of the cytochrome P450 2E1, which is the most efficient CYP450 family member in generating reactive oxygen species (ROS), on cellular energy metabolism of breast cancer cells and therefore the effects of CYP2E1 on breast carcinogenesis. **Methods:** The estrogen receptor positive MCF-7 and the triple negative MDA-MB-231 breast cancer cells were used as experimental system to estimate ROS generation in these cells overexpressing CYP2E1 and treated with the glycolytic inhibitors 3-bromopyruvate or 2-deoxyglucose in the presence or absence of the CYP2E1 inhibitor chlormethiazole. Adenosine triphosphate (ATP) assay was used to measure ATP production and lactate assay to quantify the efflux of lactic acid in breast cancer cells treated with the CYP2E1 inhibitor chlormethiazole, the mitochondrial membrane potential and cell viability assays were employed to assess the pathway of cellular energy production and cellular death respectively after treatment of MCF-7 and MDA-MB-231 with the CYP2E1 activator acetaminophen or the CYP2E1 inhibitor chlormethiazole. **Results:** The results indicated increased ROS generation in breast cancer cells overexpressing CYP2E1. ROS generation was differentially regulated in breast cancer cells upon treatment with the CYP2E1 inhibitor chlormethiazole. Chlormethiazole treated MCF-7 cells exhibited reduced lactate efflux implying that CYP2E1 directly or indirectly regulates the glycolytic rate in these cells. Furthermore the mitochondrial membrane potential of both MCF-7 and MDA-MB-231 cells was differentially affected by the CYP2E1 activator acetaminophen versus the CYP2E1 inhibitor chlormethiazole providing additional support for the involvement of CYP2E1 in energy metabolic pathways in breast cancer. **Conclusion:** Results presented in this study provide evidence to suggest that CYP2E1 regulates cellular energy metabolism of breast cancer cells in a manner dependent on cell type and potentially on the clinical staging of the disease therefore CYP2E1 is a possible breast cancer biomarker.

Key words: Reactive oxygen species; cytochrome P450 2E1; glycolysis; breast cancer

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide, hydroxyl radical, and hydrogen peroxide are metabolic by-products leaking from the complexes I and III of the mitochondrial respiratory chain.[1] Generation of high ROS levels is detrimental for the cells as it can lead to DNA damage and oxidation of proteins and lipids changing their functions.[2] Accumulating evidence indicates that apart from their

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(Lonza, Slough, UK), based on the bioluminescent measurement of ATP present in cells. ATP monitoring reagent (AMR plus) was prepared by adding assay buffer into the vial containing the lyophilized AMR and incubated at room temperature for 15 min for complete rehydration. Cells were lysed in 50 µL of cell lysis reagent for 10 min. A total volume of 100 µL of cell lysate was added to a luminometer plate and 100 µL of AMR plus was added to the appropriate well. The plate was then incubated at room temperature for 2 min and values were obtained from the luminometer.

Lactate assay

To measure the lactate efflux MCF-7 and MDA-MB-231 breast cancer cells were grown in 6 well plates and left untreated or treated with CYP2E1 specific inhibitor CMZ. Media was collected in a 96 well plate after treatment. Two microlitre of this media was mixed with 60 µL of lactate reagent and incubated at room temperature for 15 min and the absorbance was recorded at 540 nm. Lactic acid standard solutions (Trinity Biotech, Ireland) were used to plot the standard curve and the concentration of lactic acid present in the media was calculated accordingly. Lactate production rates were expressed as mmol/L.

Mitochondrial membrane potential

Mitochondrial transmembrane potential (Δψm) was measured using the cationic dye JC- 1 (5, 5, 6, 6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide) (ChemoMetec, Allerod, Denmark) using the NucleoCounter® NC-3000™ system. Cells were grown in 6-well plates and treated with the CYP2E1 activator APAP

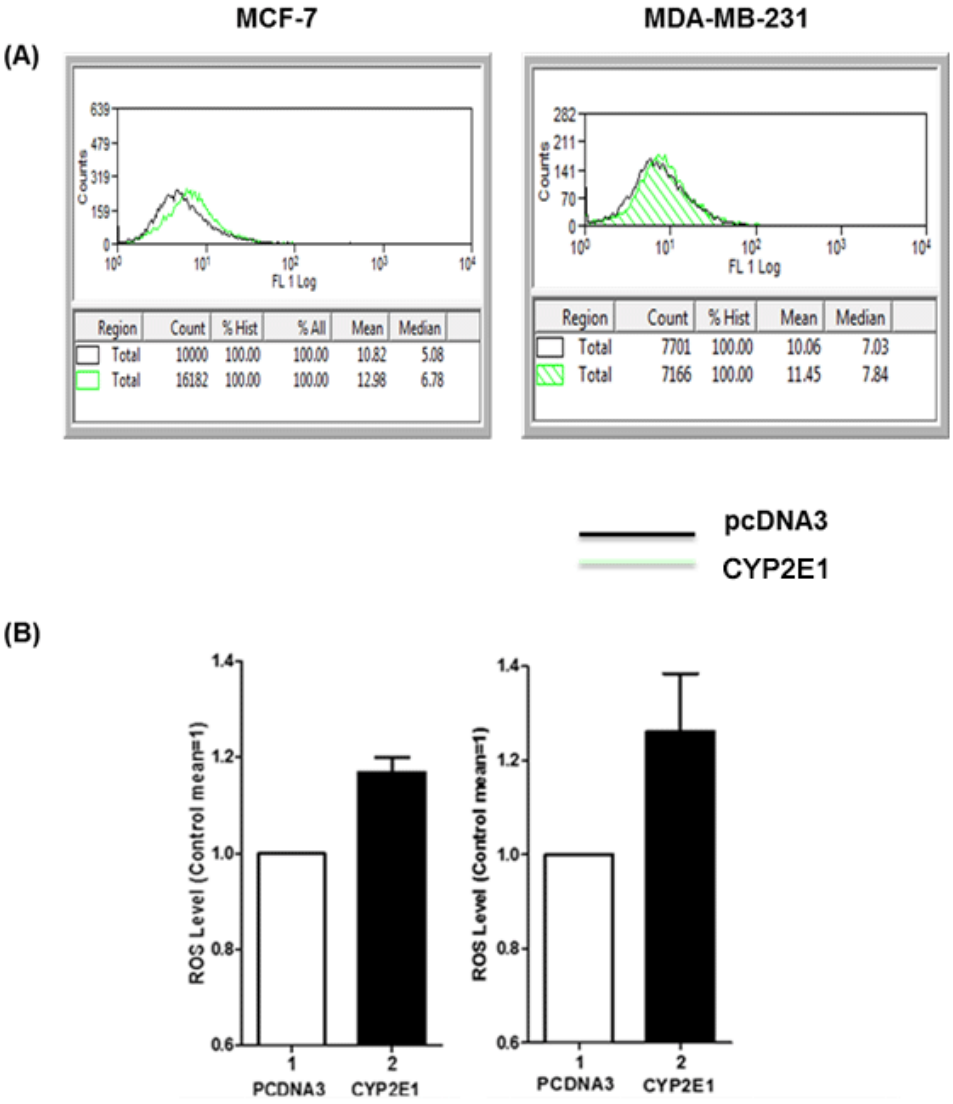

Figure 1: ROS generation in MCF-7 and MDA-MB-231 cells ectopically expressing CYP2E1. MCF-7 and MDA-MB-231 cells were transiently transfected with a CYP2E1 expressing or the empty vector PCDNA3. ROS levels were determined using H2DCFDA fluorescent dye and flow cytometry only in the cells ectopically expressing CYP2E1 (co-transfected with CD20). FACS data were analyzed using Beckman Coulter Summit 4.1 software. (A) Histograms displaying ROS levels after transient transfection of CYP2E1 or pcDNA3 as indicated. Green coloured histograms represent ROS levels in cells transfected with CYP2E1 and black histograms represent ROS levels in cells transfected with PCDNA3; (B) bar graphs representing ROS levels generated in cells transfected with PCDNA3 and CYP2E1 as indicated. Data are average of three independent experiments. ROS: reactive oxygen species; CYP: cytochrome P450

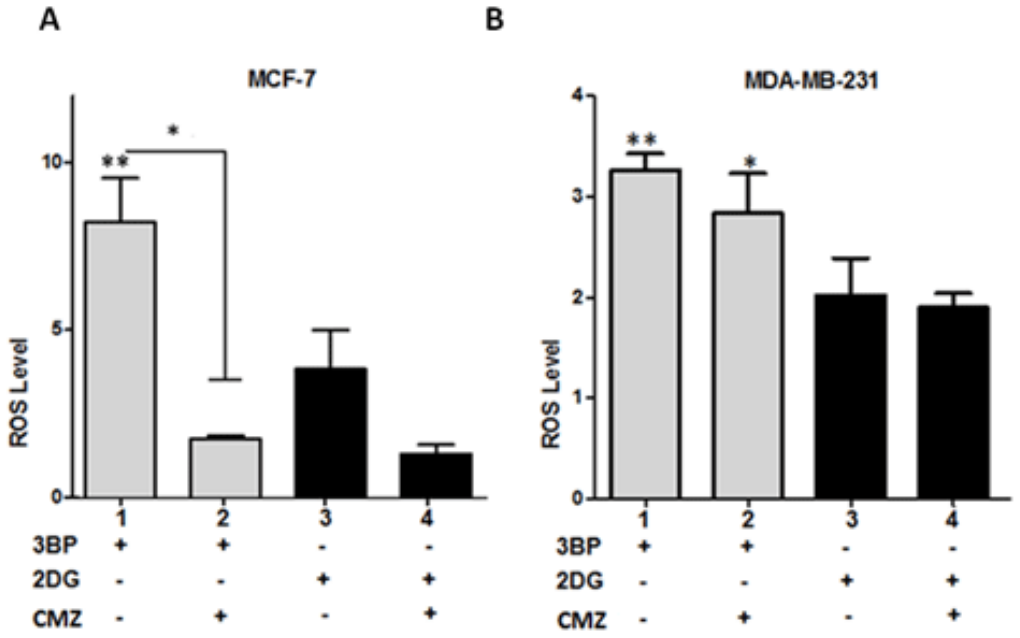

Figure 2: CYP2E1 mediated ROS generation in breast cancer cells under diverse stress conditions. Graph indicating ROS levels generated in 3BP, 2DG and CMZ treated MCF-7 (A) and MDA-MB-231 (B) cells. Error bars represent mean ± SEM from three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey post hoc for multiple pair-wise comparisons. One asterisk indicates $P < 0.05$ and two asterisks $P < 0.005$. ROS: reactive oxygen species; CMZ: chlormethiazole

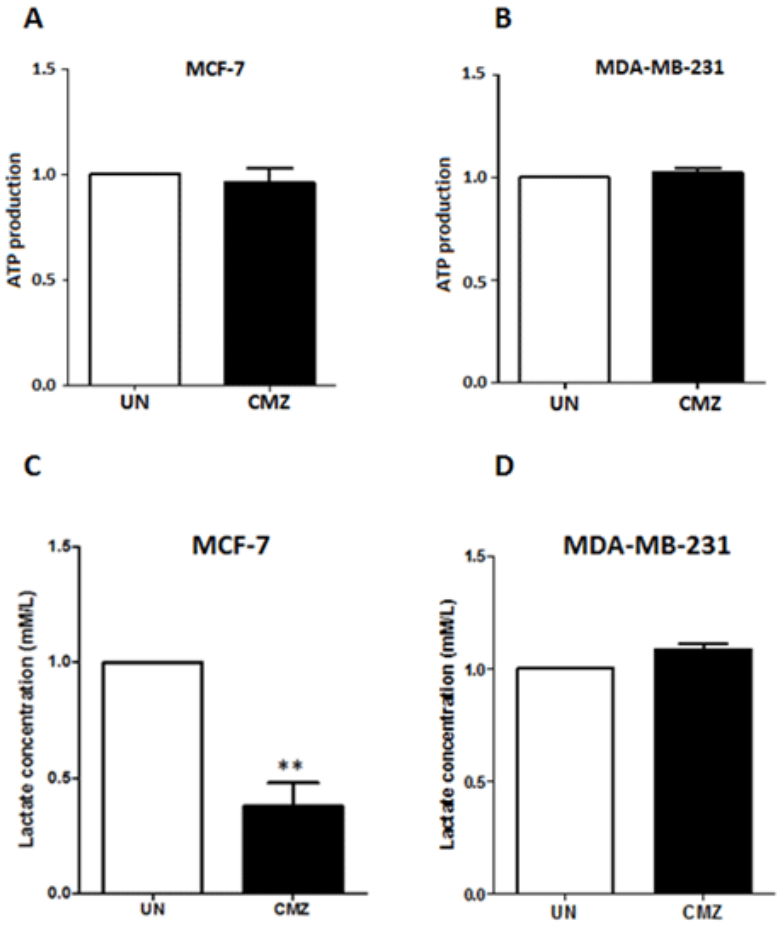

Figure 3: ATP production and lactate efflux in MCF-7 and MDA-MB-231 cells treated with the CYP2E1 inhibitor CMZ. MCF-7 and MDA-MB-231 cells were either left untreated or treated with the CYP2E1 specific inhibitor CMZ. ATP production (A and B) was determined using the ViaLight™ plus kit (Lonza, Slough, UK) and lactate efflux (C and D) using the lactate reagent (Trinity Biotech, Dublin, Ireland). Data are average of three independent experiments ± SEM; $^{**}P < 0.005$. ATP: adenosine triphosphate; CMZ: chlormethiazole

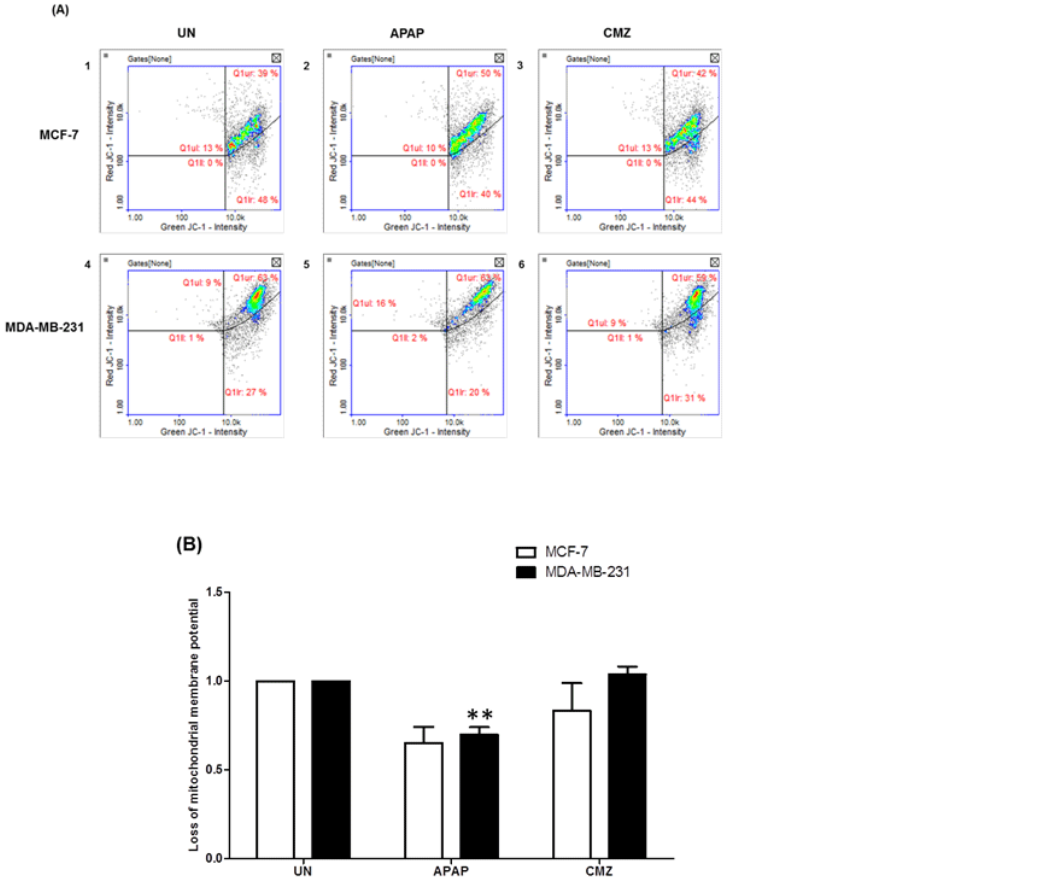

Figure 4: Mitochondrial membrane potential (Δψ) in breast cancer cells treated with the CYP2E1 activator APAP or the CYP2E1 inhibitor CMZ. Breast cancer cells were left untreated or treated with either the CYP2E1 inducer (APAP) or the CYP2E1 inhibitor (CMZ). Mitochondrial membrane potential (Δψ) was determined using JC-1 and DAPI fluorescent dye (ChemoMetec, Allerod, Denmark) and the NucleoCounter NC3000. Data were analyzed using NucleoView software. (A) Histograms representing the mitochondrial membrane potential (Δψ) in breast cancer cells under different stress conditions; (B) bar graphs representing the effect of APAP and CMZ treatments on mitochondrial membrane potential (Δψ) in breast cancer cells. Error bars represent mean ± SEM from three independent experiments. Two asterisks indicate $P < 0.005$. APAP: acetaminophen; CMZ: chlormethiazole

or the CYP2E1 inhibitor CMZ. After treatment, cells were stained with JC-1 and DAPI (ChemoMetec, Allerod, Denmark). Cellular JC-1 monomers and aggregates are detected as green and red fluorescence, respectively. Mitochondrial depolarization and apoptosis are revealed as a decrease in the red/green fluorescence intensity ratio. Necrotic and late apoptotic cells are detected as blue fluorescent (DAPI) cells. After staining cells were loaded on an 8-chamber NC-Slide A8™ and samples were analysed using the NC-3000™ system and the amount of blue, green and red fluorescence of the individual cells was quantified.

Cell viability assay

Cell viability was measured using the NucleoCounter® NC-3000™ system. Cell viability assay was used to detect changes in the intracellular level of (reduced) thiols. Cells were seeded in 6 well plates and cultured until they reached 80% confluence prior to different treatments. After the treatments, cell culture medium was aspirated and 500 µL of cell dissociation buffer was added to cells for dissociation from culture plates. Five hundred microlitre of complete culture medium was added to quench the toxicity of dissociation buffer after cell dissociation. Then cells were stained with solution 5 as described by the manufacturer. Solution 5 (ChemoMetec, Allerod, Denmark) contains three different stains, each one of them staining either all nucleated cells (DAPI), dead cells (Propidium iodide) or viable cells (VB-48) (ChemoMetec, Allerod, Denmark) and the intensity of the stain depends on the GSH level. After staining, cells were loaded into an 8-chanmber NC-slide. Samples were analysed using the NC-3000™ system.

RESULTS

The role of CYP2E1 in ROS generation in breast cancer cells has been investigated by our and other groups indicating that overexpression of this cytochrome P450 family member in breast cancer cells coincides with elevated ROS levels implying that CYP2E1 is one of the intracellular sources of ROS.[29,34] To confirm that this is the case in the triple positive MCF-7 and the triple negative MDA-MB-231 cells CYP2E1 expressing vectors were transiently transfected and the ROS levels in mock and ectopically expressing CYP2E1 cells were followed as described in Materials and Methods. Increased ROS levels were recorded in both cell lines ectopically expressing

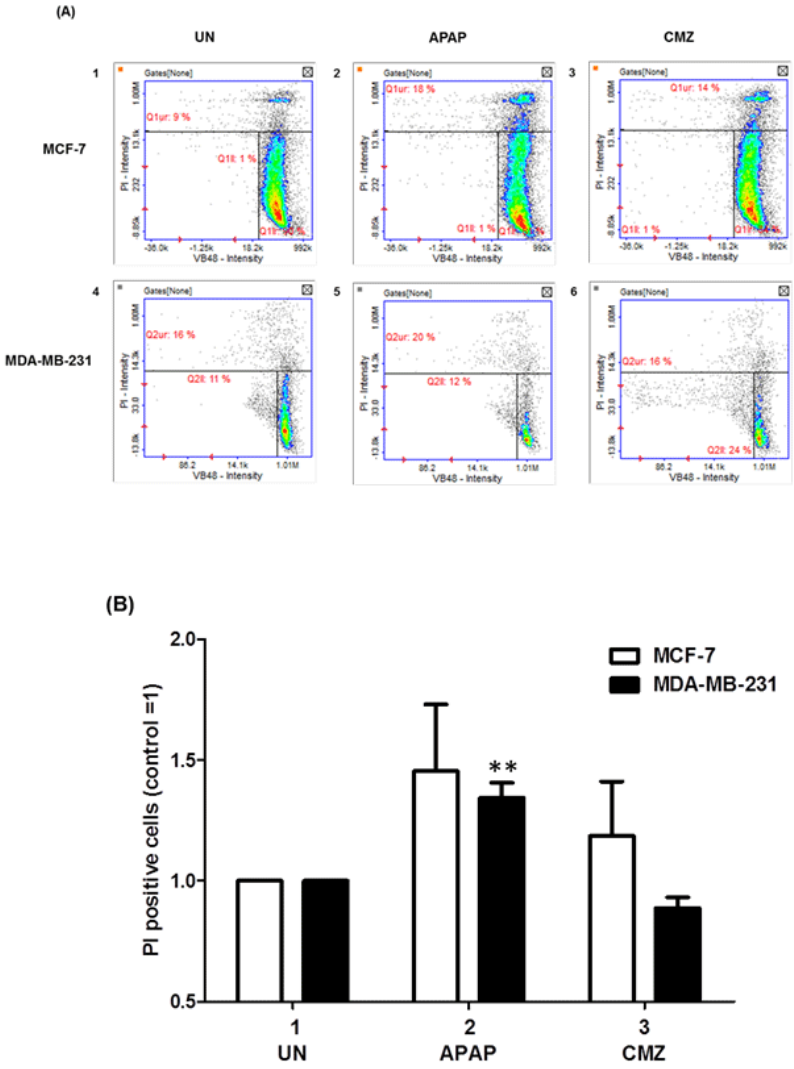

Figure 5: Cell viability of breast cancer cells treated with the CYP2E1 activator APAP or the CYP2E1 inhibitor CMZ. Breast cancer cells were left untreated or treated with either the CYP2E1 activator APAP or the CYP2E1 inhibitor CMZ as indicated. Cell viability was calculated using the Vitality kit Assay (ChemoMetec, Allerod, Denmark). (A) Histogram representing cell viability under different stress conditions. Dead cells stained with PI are shown in the Q1ur gates; (B) bar graph representing the PI positive breast cancer cells treated with either APAP or CMZ. Error bars represent mean ± SEM from three independent experiments. Two asterisks indicate $P < 0.005$. APAP: acetaminophen; CMZ: chlormethiazole

CYP2E1 compared to mock transfected cells [Figure 1B, compare bars 2 to bars 1 respectively].

To explore further the effects of CYP2E1 on the glycolytic pathway of energy production the glycolytic inhibitors 3BP and 2DG were used to inhibit glycolysis in MCF-7 and MDA-MB-231 cells either individually or in combination with the CYP2E1 inhibitor CMZ and the ROS generated under these conditions were monitored as described in Materials and Methods. Treatment of MCF-7 cells with 3BP generated higher ROS levels compared to MCF-7 cells treated with 2DG [Figure 2A, compare bar 1 to bar 3]. Combination of 3BP or 2DG treatment with CMZ resulted in dramatic decrease of ROS levels in MCF-7 cells [Figure 2A, compare bar 2 to bar 1 and bar 4 to bar 3]. In contrast, in MDA-MB-231 cells CMZ had marginal effect on that observed when cells were treated with the glycolytic inhibitors 3BP and 2DG alone [Figure 2B, compare bars 2 and 4 to bars 1 and 3 respectively] providing additional evidence that CYP2E1 exerts cell type dependent effects in ROS generation in a manner dependent on the genetic background and potentially their invasive potential.

Accumulating evidence supports the notion that ROS generation is associated with cellular energy production.[31,32,35] Results shown in Figure 1 indicate that CYP2E1 overexpression led to elevation of ROS in MCF-7 and MDA-MB-231 breast cancer cells implying a potential role of CYP2E1 in cellular energy metabolism. To test this hypothesis MCF-7 and MDA-MB-231 cells were treated with the CYP2E1 inhibitor CMZ and the levels of ATP produced under these conditions were determined as described in Methods. CMZ treatment of both MCF-7 and MDA-MB-231 cells did not have any significant effect on the ATP produced under these conditions [Figure 3A and 3B]. To test whether the ROS levels' profile observed in breast cancer cells was related to lactate production, MCF-7 and MDA-MB-231 cells were treated with the CYP2E1