Hierarchical clustering method identifies adaptation of mitochondrial signaling to the treatment of mitochondrial blockers that regulates the expression of cell cycle genes.

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ABSTRACT:

The cross-talk between the cell nucleus and mitochondria appears to control hormone-induced signaling involved in the apoptosis, proliferation, and differentiation of both normal and malignant cells. Some pharmacological agents such as tamoxifen; antibiotics such as tetracycline or chloramphenicol (inhibitors of the mitochondrial protein synthesis); and other pharmacological agents exert their effects on cell growth by their influence on mitochondria. We propose to investigate how mitochondrial signaling pathways in breast cancer cells may contribute to molecular mechanisms of mitochondrial inhibitor resistance. We have examined the innovative hypothesis that mitochondria through its signaling to the nucleus control the expression of cell cycle genes. Mitochondrial transcription/replication was impaired in MCF7 cells by culturing cells in routine growth medium supplemented with a low dose of ethidium bromide (50 ng/ml), pyruvate (1 mM), and uridine (50 µg/ml) for 20 days. An alternative approach to blocking mitochondrial transcription/replication was to use rhodamine 6G (R6G) that inhibits oxidative phosphorylation. Acute (1 to 2 h) exposure of cells to inhibitors of oxidative phosphorylation was also given prior to 17β-estradiol (E2) treatment. Gene expression was measured using the Non-radioactive GEArray Q Series Kit for human cell cycle , genes. MCF7 cells and ethidium bromide (EtBr) or rhodamine 6G (R6G) treated MCF7 cells were exposed to E2 (100pg/ml) for 6 and 24 h. Data were analyzed using the hierarchical clustering method. This method performs a hierarchical cluster analysis using a set of dissimilarities for the gene profiles being clustered. Initially, each object is assigned to its own cluster and then the algorithm proceeds iteratively, at each stage joining the two most similar clusters, continuing until there is just a single cluster. At each stage distances between clusters are recomputed by the Lance-Williams dissimilarity update formula according to the particular clustering method being used. Both of the mitochondrial inhibitors attenuated the expression of early cell cycle genes. Acute treatment of MCF7 cells with R6G produced a drastic inhibition of cell cycle genes; whereas a sub-chronic treatment with EtBr showed a recovery of cell cycle expression compared to R6G. Similar effects were observed in E2 co-treated MCF7 cells. These findings suggest that mitochondria may play an important role in the progression of the breast cancer cell cycle through altering the expression of cell cycle genes and breast cancer cells may adapt to the mitochondrial stress produced by sub-chronic or chronic treatment with inhibitors. In conclusion, the use of mitochondrial inhibitors to study signal transduction networks requires attention to the altered expression of nuclear genes as well as adaptive responses that are attributed to acute versus chronic exposure.

INTRODUCTION:

Mitochondria are unique structures in mammalian cells because gene products from two physically separated genomes: one contained within an organelle and the other within the nucleus; are involved in the biogenesis and function of mitochondria. Intergenomic communication between the nucleus and mitochondria occurs to maintain the activity of the mitochondrial respiratory chain and hence the mitochondrial capacity to generate ATP. Although mitochondrial activity depends on nuclear transcription activators and co-activators, the role that mitochondria play in controlling the nuclear expression of genes not related to mitochondria function is not clearly understood. Recently, we have reported that mitochondria are an important early target of estrogen action. The cross-talk between the cell nucleus and the mitochondria appears to control estrogen-induced signaling involved in cell growth of both normal and malignant cells (1). 17β-estradiol (E2) induced mitochondrial (mt) reactive oxygen species (ROS) act as signal-transducing messengers that control the G_1/S transition of G_0-

arrested estrogen dependent cells (2, 3). It is known that ROS act as signaling molecules that can affect nuclear gene expression. Since mitochondrial electron transport is the principal source of intracellular ROS in epithelial cells, we hypothesize that mitochondrial function can control the expression of nuclear cell cycle genes in adenocarcinoma cells. Specific inhibitors of mitochondrial function such as rhodamine 6G (R6G) and ethidium bromide (EtBr) are commonly used to study mitochondrial signaling to the nucleus, however, little is known about the effect of these inhibitors on nuclear gene expression in human cells. Thus, we postulate that mitochondrial inhibitors alter the expression of nuclear genes and are a potential confounder in the mechanistic study of cell signaling pathways.

MATERIALS AND METHODS:

Cell culture. MCF7 cells (ATCC) were propagated in DMEM/F12 medium without phenol red supplemented with 10% FBS and 1X antibiotic-antimycotic. Serum deprivation was used to synchronize the majority of MCF7 cells in the G_0/G_1 phase of the cell cycle.

Mitochondrial blockers. Mitochondrial transcription/replication was impaired by culturing cells in routine growth medium supplemented with a low dose of ethidium bromide (50 ng/ml), pyruvate (1 mM), and uridine (50 µg/ml) for 20 days (4, 5). An alternative approach to blocking mitochondrial transcription/replication was to block mitochondrial protein synthesis by prolonged treatment with chloramphenciol (100 µg/ml) for 2 days prior to experiments (6-8). Acute (1 to 2 h) exposure with blockers of oxidative phosphorylation: rhodamine 6G (R6G), rotenone, antimycin A, cyclosporin, and oligomycin B. R6G blocks oxidative phosphorylation and is considered to be the most potent agent to impair mitochondrial transcription/replication in cells (9). Rotenone and antimycin A block electron transport in mitochondrial respiratory complex I and III, respectively, while oligomycin B binds to ATP synthase F0 subunit and blocks the flow of protons through the channel (10).

Cell growth. Sulforhodamine B (SRB) assay was used to measure cell growth (11). Dye intensity was measured at 560 nm on a Tecan Genios plate reader.

BrdU assay. MCF7 cells were grown (2500 cells/well) in 96-well plates until 50% confluent in 10% FBS DMEM/F12, serum starved for 2 days followed by treatments as described in the figures for 24 h to 48 h. A colorimetric BrdU cell proliferation assay was performed according to the manufacturer's instructions. Absorbance of the samples was measured in a Tecan Genios plate reader at 450 nm.

Flow cytometry. Cells were fixed in 70% ethanol, washed in PBS, and resuspended at 10^6 cells/ml. Cells were incubated with RNase for 30 min. and stained with propidium iodide (10 µg/ml). Samples were analyzed with a Becton Dickinson FACStar. Data were analyzed by ModFitLt® V2.0.

Measurement of AP-1 activation. Activation of transcription factor AP-1 in nuclear extracts was measured with the TransAM AP-1 assay (Active Motif). The binding of AP-1 consensus sequence was detected using a primary anti-phospho-c-jun (Ser 73) followed by a secondary Ab conjugated to HRP. Signal was detected by Tecan Genios plate reader at 450 nm.
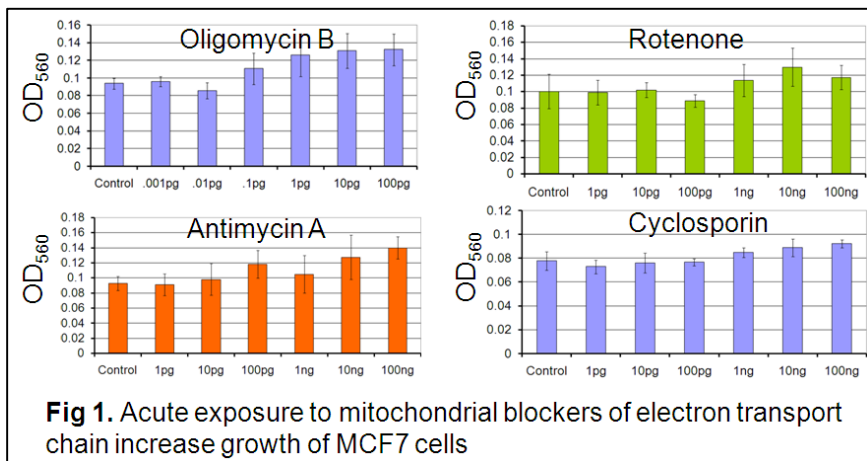
Electrophoretic mobility shift assay (EMSA). EMSA was performed with DIG-11-ddUTP 3'-end labeled probes. The AP-1 consensus sequence [5'-AAAAAAAA<u>TGAGTCA</u>GAATGGAGATCAC-3'(sense) and 3'-TTTT<u>ACTCAGT</u>CTTACCTCTAGTGACAA-5'(antisense)]; was taken from the human cyclin D1 promoter region (12). Probe labeling and binding reactions were performed following the protocols provided by the DIG Gel Shift Kit manufacturer (Roche).

Cell cycle cDNA array. Gene expression was measured using the GEArray Q Series Kit (SuperArray, Inc., Bethesda MD) for human cell cycle genes, MAP kinase pathway genes, and Ca^{2+}/cAMP pathway genes. MCF7 cells and EtBr treated MCF7 cells were treated with E2 (100pg/ml) and R6G

(0.5 µg/ml) for 6 and 24 h. The GEArray membrane was hybridized with denatured cDNA probe of the samples overnight and gene expression was detected by ECL (BioRad). The intensity value of the genes was obtained using the image analysis program Scanalyze (Michael Eisen, Stanford). Each gene was normalized to ribosomal protein L13a (RPL13A) using the GEArrayAnalyzer software.

RESULTS AND DISCUSSION:

Recently, we have shown that mitochondria are a major source of E2-induced ROS in breast cancer cells. To evaluate whether E2-induced mitochondrial ROS signal the growth of breast cancer cells, we first tested the effect of mitochondrial blockers alone on cell growth. Cells were treated for 1 to 2 h with blockers of oxidative phosphorylation: rotenone, antimycin A, cyclosporin, and oligomycin B. As shown in **Fig. 1**, MCF7 cells showed an increase in cell growth at the higher doses mitochondrial inhibitors. Since this was only an acute exposure, the cells response to these inhibitors may be attributed to a survival response. Based on our previous studies (data not shown); MCF7 cells exposed acutely (48h) to the inhibitor EtBr showed an increase of cells in the S phase when compared cells exposed for (20 days). Therefore, we investigated the effect of the inhibitors:

Fig 1. Acute exposure to mitochondrial blockers of electron transport chain increase growth of MCF7 cells

rotenone, R6G, and EtBr. Higher doses of rotenone showed a 30% decrease in DNA synthesis (**Fig. 2**). R6G showed a severe inhibition of DNA synthesis which is not surprising since this chemical is a cation which is attracted to the negative membrane potential of the mitochondria. In this case, mitochondria transcription/replication is severely inhibited. Consequently, we postulate that mitochondria signals to the nucleus were dysregulated and this inhibited activation of cell cycle genes involved in DNA synthesis. Chronic EtBr exposure to MCF7 showed a 20% decrease in DNA synthesis.

Fig 2. Acute exposure to inhibitors of mitochondria biogenesis decrease DNA synthesis of MCF7 cells
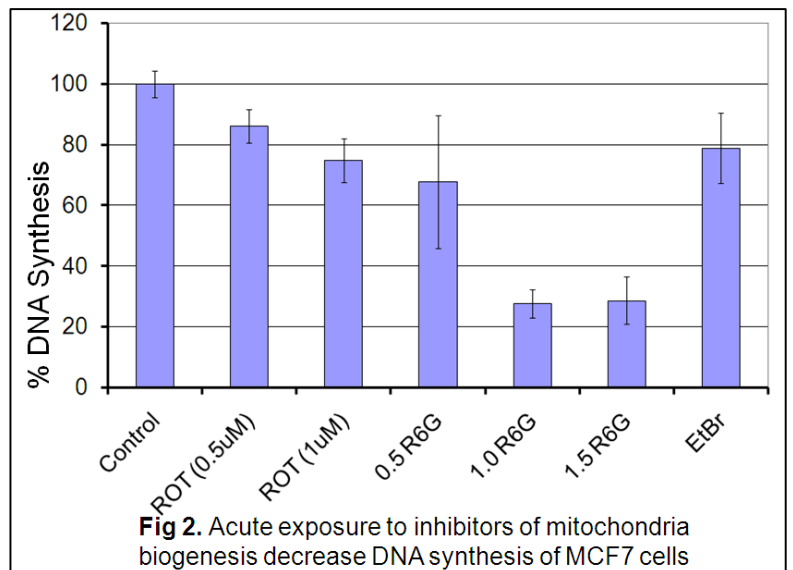
Since our data suggested that DNA synthesis is impaired by mitochondrial inhibitors, we further evaluated the fate of cells within the cell c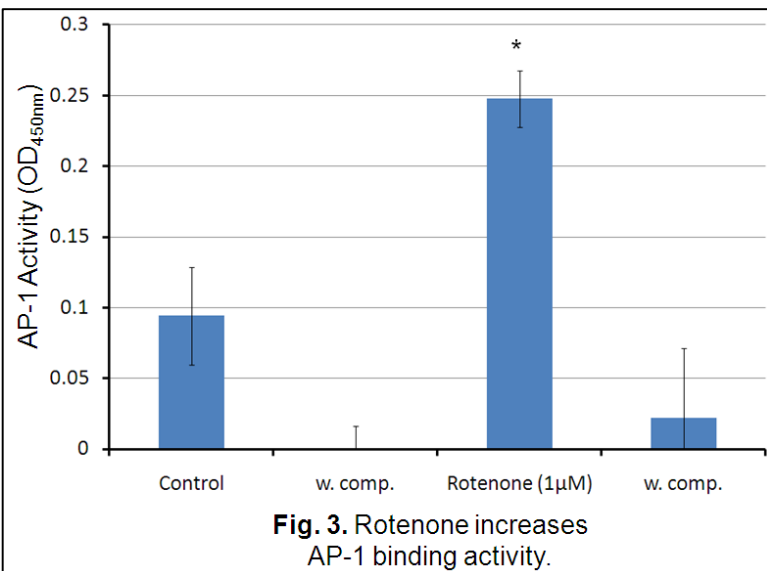ycle in the presence of various mitochondrial blockers. FACS analysis of DNA content was performed on cells stained with propidium iodide. FACS data are from three separate experiments and results are summarized in **Table 1**. We first examined the effect of the antibiotic chloramphenicol. Treatment with the antibiotic chloramphenicol was used as an alternate approach to

	% DNA		
	G0/G1	S	G2/M
Control	87.7	6.5	5.8
Chloramphenicol (50µg/ml)	85.7	9.9	4.4
Chloramphenicol (150µg/ml)	86.9	10.4	2.7
Oligomycin B (25 pg/ml)	88.5	5.8	5.7
Rotenone (25 ng/ml)	91.8	2.4	5.8

Table 1. Flow cytometry analysis of the effect of mitochondrial blockers

study the effect of blocking mitochondrial transcription/replication on cell cycle progression because it is an inhibitor of mitochondrial protein synthesis. The percentage of DNA content in S phase was 6.5% in the control, which increased up to 10.4% in the 48 h chloramphenicol treated cells. Again, we observed in the acute (48h) exposure to chloramphenicol that cells showed an increase in S Phase while chronic (7 day) exposures showed a decrease of cells in the S Phase (data not shown). Our final approach consisted of acute treatments with blockers of mitochondrial function. MCF7 cells were treated for 2 h with specific blockers of respiratory complex I (rotenone) and the ATP synthase blocker, oligomycin B. Compared to the control, both inhibitors decreased DNA content in S phase to 2.4% and 5.8 %, respectively. The FACS data shows that the effects of mitochondrial inhibitors on cell cycle progression can either increase MCF7 entry into S phase or arrest cells in G_0/G_1 phase depending on the type of mitochondrial inhibitor as well as the amount of time cells are exposed to the inhibitor. Since chloramphenicol inhibits mitochondrial protein synthesis versus rotenone or oligomycin B which directly act on mitochondrial respiratory protein complexes; we postulate that chloramphenicol treated cells have time to adapt to the mitochondrial inhibitor. The adaptive response is indicated by the increase in the amount of cells in S Phase which in turn, promotes MCF7 cell survival when exposed acutely to chloramphenicol.

Several studies have implicated cyclin D1 involvement in estrogen-dependent growth of both normal and malignant mammary epithelial cells. For instance, hormone-dependent growth of breast epithelium is impaired in cyclin D1 deficient mice (13) and in MCF7 cells cyclin D1 is a well defined target of E2 action (14). Since these studies suggest a direct regulation of cyclin D1 expression by E2 in the process of cell proliferation, we selected cyclin D1 to investigate the effect of mitoch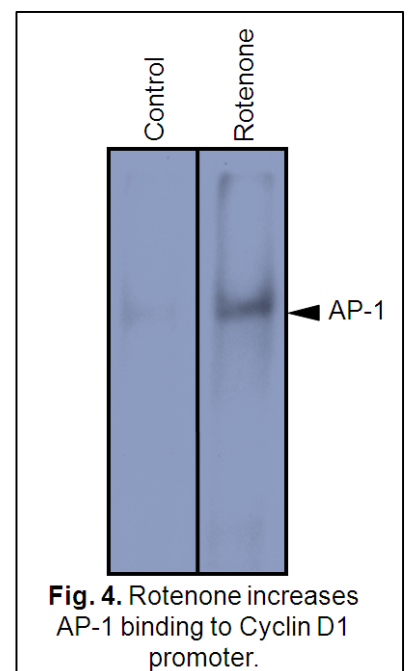ondrial inhibitors on cell cycle gene expression. ROS are known to stimulate the phosphorylation of transcription factor AP-1 which consists of dimmers of either c-jun or c-fos; therefore we tested whether mitochondrial inhibitors modified the AP-1 binding activity. The TransAM AP-1 c-jun assay was used to determine

Fig. 3. Rotenone increases AP-1 binding activity.

whether the respiratory complex I inhibitor, rotenone, modulates AP-1 binding to the TPA-response element (TRE). As shown in **Fig. 3**, rotenone exposed MCF7 cells showed a significant 2-fold increase in AP-1 binding. Therefore, we performed an electrophoretic mobility shift assay (EMSA) to determine whether rotenone increased AP-1 binding to its consensus sequence found in the human cyclin D1 promoter region. As shown in **Fig. 4**, rotenone treatment significantly increased AP-1 binding. Based on these results, we postulated that other cell cycle genes besides cyclin D1 could be modulated by mitochondrial inhibitors.

Gene expression was screened using the human cell cycle GEArray Q Series Kit (SuperArray, Inc) to determine whether the effects of mitochondrial inhibitors were due to changes at the nuclear level of gene expression. MCF7 cells and ethidium bromide (EtBr) or rhodamine 6G (R6G) treated MCF7 cells were exposed to E2

Fig. 4. Rotenone increases AP-1 binding to Cyclin D1 promoter.

(100pg/ml) for 6 and 24 h. Data were analyzed using the hierarchical clustering method. This method performs a hierarchical cluster analysis using a set of dissimilarities for the gene profiles being clustered. Initially, each object is assigned to its own cluster and then the algorithm proceeds iteratively, at each stage joining the two most similar clusters, continuing until there is just a single cluster. At each stage distances between clusters are recomputed by the Lance-Williams dissimilarity
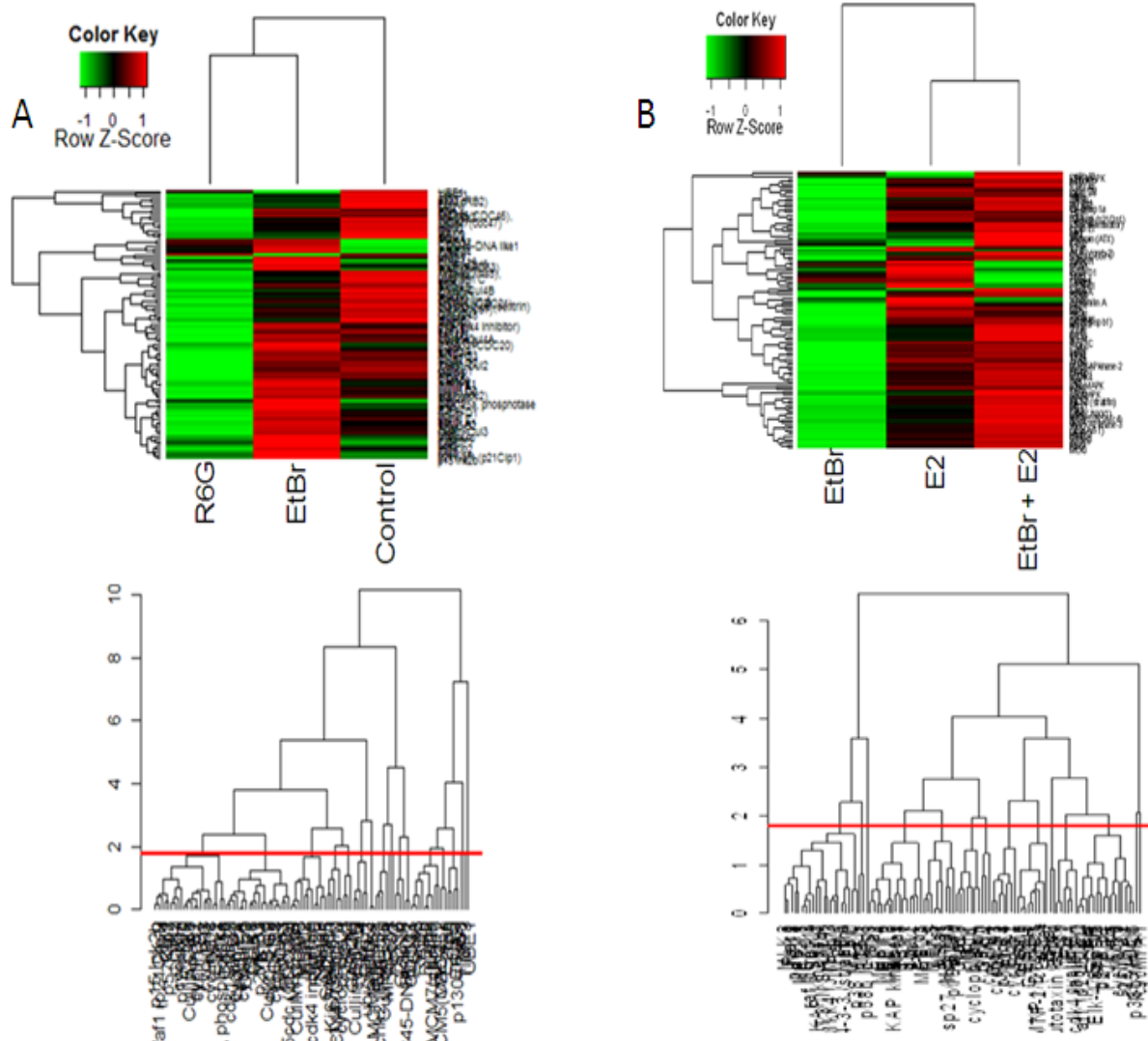

Fig 5. Hierarchical cluster analysis of cell cycle genes from R6G and EtBr exposed MCF7 cells

update formula according to the particular clustering method being used. Both of the mitochondrial inhibitors attenuated the expression of early cell cycle genes. Both of the mitochondrial inhibitors attenuated the expression of early cell cycle genes. Acute treatment of MCF7 cells with R6G produced a drastic inhibition of cell cycle genes; whereas a sub-chronic treatment with EtBr showed a recovery of cell cycle expression compared to R6G (**Fig. 5A**). Similar effects were observed in E2 co-treated MCF7 cells (**Fig. 5B**). These findings suggest that mitochondria may play an important role in the progression of the breast cancer cell cycle through altering the expression of cell cycle genes and breast cancer cells may adapt to the mitochondrial stress produced by sub-chronic or chronic treatment with inhibitors.

We further determined the genomic effects of mitochondrial blockers on genes involved in cAMP/Ca2+ pathway because the mitochondria is known to buffer intracellular calcium. We screened

cells with the nonradioactive human cAMP/Ca^{2+} PathwayFinder gene array (SuperArray). MCF7 cells were treated for 6h with either R6G or EtBr. The human cAMP/Ca^{2+} PathwayFinder gene array that contains three groups of Ca^{2+} responsive target genes (96 in total): i) target genes with SRE; ii) target genes with CRE; iii) target genes with undefined elements. We observed that mitochondrial blockers R6G and EtBr also modulate these genes (**Fig. 6**).
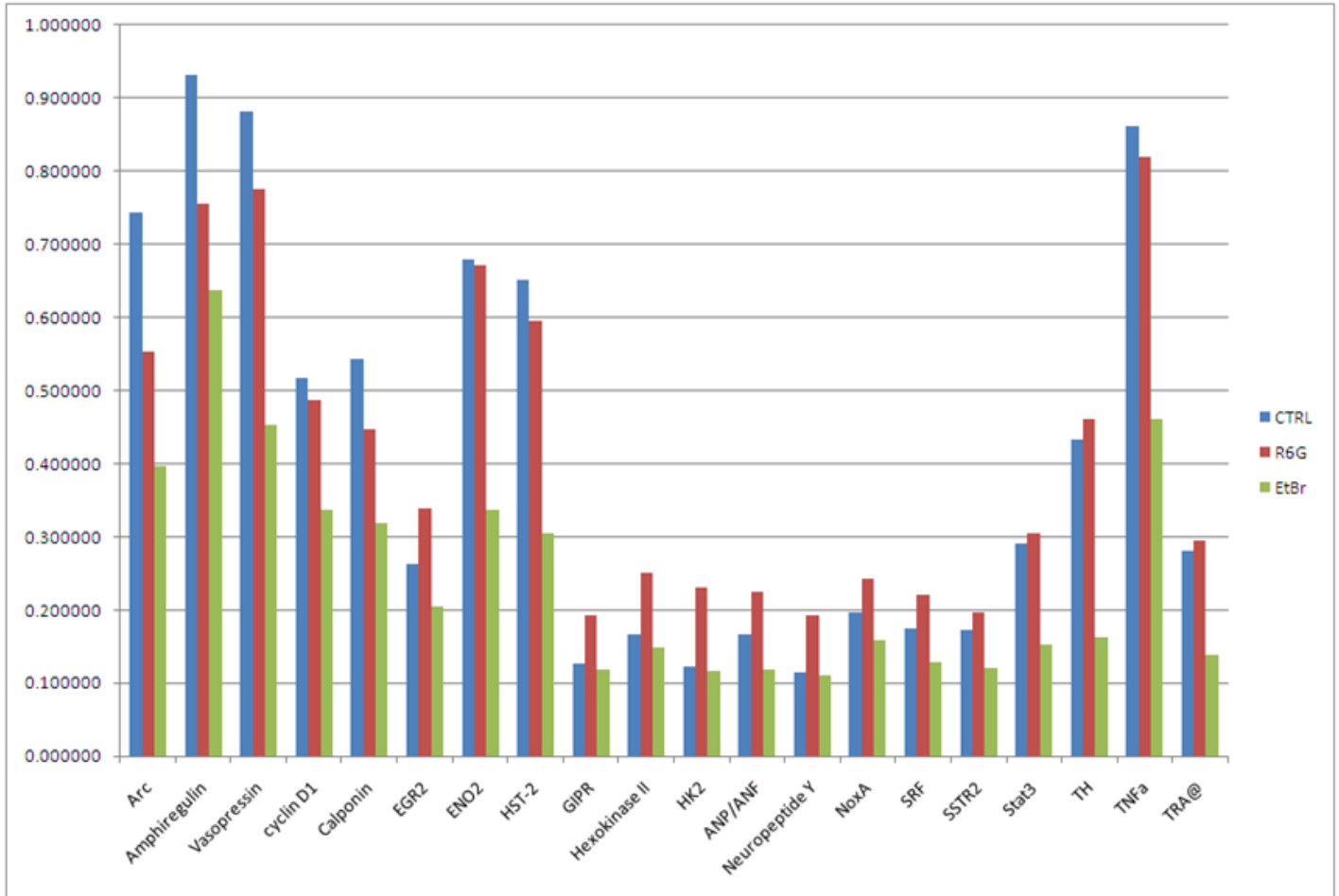
Fig. 6. Mitochondrial blockers R6G and EtBr also modulate cAMP/Ca2+ pathway genes

In summary, inhibitors of mitochondria-chloramphenicol, rhodamine 6 G, and ethidium bromide were used to study the influence of mitochondria on cell growth, cell proliferation, cell cycle progression, and cell cycle genes. Our results show that: 1) chemical inhibitors may increase MCF7 entry into S phase or arrest cells in G_0/G_1 phase depending on the type of mitochondrial inhibitor as well as the exposure time. 2) Hierarchical clustering method identified adaptation of mitochondrial signaling to the treatment of mitochondrial blockers at the level of cell cycle genes as well as genes involved in cAMP/Ca2+ pathways. Therefore, the use of mitochondrial inhibitors to study signal transduction networks requires attention to the altered expression of nuclear genes as well as adaptive responses that are attributed to acute versus chronic exposure.

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