High-Copy Overexpression Screening Reveals PDR5 as the Main Doxorubicin Resistance Gene in Yeast

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Abstract

Doxorubicin is one of the most potent anticancer drugs used in the treatment of various cancer types. The efficacy of doxorubicin is influenced by the drug resistance mechanisms and its cytotoxicity. In this study, we performed a high-copy screening analysis to find genes that play a role in doxorubicin resistance and found several genes (CUE5, AKL1, CAN1, YHR177W and PDR5) that provide resistance. Among these genes, overexpression of PDR5 provided a remarkable resistance, and deletion of it significantly rendered the tolerance level for the drug. Q-PCR analyses suggested that transcriptional regulation of these genes was not dependent on doxorubicin treatment. Additionally, we profiled the global expression pattern of cells in response to doxorubicin treatment and highlighted the genes and pathways that are important in doxorubicin tolerance/toxicity. Our results suggest that many efflux pumps and DNA metabolism genes are upregulated by the drug and required for doxorubicin tolerance.

Introduction

Doxorubicin is an anthracycline with a strong anticancer activity. It exerts its effects through different mechanisms, such as intercalation into DNA and inhibition of DNA/RNA biosynthesis, formation of free radicals, inhibition of topoisomerase II, changing membrane properties and inhibition of RNA helicase [1,2]. Unfortunately, these different modes of actions for doxorubicin bring along serious side effects. The most notable one is its cardiotoxicity [3]. Doxorubicin leads to iron accumulation and ROS production, which eventually damages mitochondria and leads to cardiac problems [4].

Multi-drug resistance (MDR) is believed to be an important cause of the treatment failure in metastatic cancer patients [5]. The efflux of the chemotherapeutics by membrane transporters is the main mechanism leading to MDR. Even though mechanisms of MDR have not been revealed exclusively, evading drug resistance and controlling MDR, have been a great issue in chemotherapy.

The mechanisms of doxorubicin resistance have been studied both in yeast and mammalian cells. In mammals, resistance mechanisms include primarily drug efflux from the cell via
upregulation of the P-glycoprotein [6], multidrug resistance protein (MRP) [7], anthracycline resistance associated protein MRP6 [8], breast cancer resistance protein (BCRP) [9], and lung resistance-related protein (LRP) [10]. Additionally, changes in the topoisomerase II expression [11], overexpression of glutathione S-transferase (GST) [12], and changes in ERK1/ERK2 proteins [13] provide resistance to doxorubicin.

The budding yeast *S. cerevisiae* is a valuable model to identify doxorubicin resistance and sensitivity genes. Screening of deletion collection strains for non-essential genes and studies with specific genes have revealed many genes and pathways in doxorubicin tolerance in yeast [14,15]. These include Ssl2 protein [16], Bsd2 protein [17], SUMO pathway [18], nascent polypeptide-associated complex activity in ribosomes [19], extracellular signal-regulated kinases ERK1 and ERK2 [13], endocytic Ark/Prk kinase [20], nitrogen permease regulator 2 (Npr2) [21], cytochrome oxidase subunit IV gene [22], and overexpression of CLN1, CLN2 and ERG13 [23]. Additionally, checkpoint and recombination functions in G1 and early S phase [14], as well as several proteins involved in DNA repair, RNA metabolism, chromatin remodeling, amino acid metabolism, and heat shock response [15], play roles in doxorubicin resistance.

Identification of new genes that play role in cancer drug resistance may provide further prognostic information, which in turn may help to improve the development of new chemotherapeutic agents and increase efficacy of chemotherapeutics. In this study, we intended to identify doxorubicin resistance mechanisms by performing a high copy genomic DNA library screening in the presence of doxorubicin. Several new genes were found to cause resistance against high level of doxorubicin (500μM). Among these genes, PDR5 had the most remarkable effect on doxorubicin resistance. We also profiled the expression pattern of yeast genome for doxorubicin treatment and highlighted the paths that played roles in resistance and detoxification for this drug.

**Materials and Methods**

**Yeast strains, cell growth and plasmids**

The BY4741 (MATa, his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and BY4743 (MATa, his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0) strains of the budding yeast *Saccharomyces cerevisiae* were used in this study. The high copy yeast genomic library (ATCC No. 37323) was used for genomic library screenings. Yeast transformations were performed by the standard LiAc method. Unless indicated otherwise, all experiments were performed on Yeast Nitrogen Base (YNB, 2% Glucose) media supplemented with appropriate amino acids and bases.

For yeast expression experiments, the genes that reside within the original YEp13 genomic clones that caused resistance against Doxorubicin, were each cloned separately into the pAG426-GPD plasmid (Addgene) and then expressed under control of the GPD promoter, except for PDR5 plasmid, which was obtained from Prof. Dr. Wenjun Guan (Zhejiang University, China). For plasmid isolations, yeast cells were predigested by lyticase (5u/ml) for 30 minutes in Tris-EDTA (TE) buffer before the isolation and plasmids were isolated from yeast cells by using GeneJET Plasmid Miniprep kit (Thermo-Molecular Biology) as described by the manufacturer. The isolated plasmids were amplified in *E. coli* DH5α cells and sequenced by using a pair of vector-specific primers at IzTech Biotechnology Center (İzmir). Doxorubicin was purchased from SABA pharmaceuticals (Cat No.: 8699511796063 /Turkey).

**Gradient spot assays**

Petri plates with a continuous gradient of a drug was described by Szybalski and Bryson (1952). Briefly, two layers of agar were poured into a square petri dish. The bottom layer
contained normal medium and the plate was propped up slightly for agar to cover the entire bottom. When the agar was solidified, the dish was placed in a horizontal position and doxorubicin harbouring medium (50 ml) was added on top of the plate. Downward diffusion of doxorubicin resulted in its dilution proportional to the thickness of the agar layers and established a concentration gradient changing from approximately 0 μM on one side to 500 μM on the other [24].

The WT BY4741 and BY4743 strains carrying the plasmids were grown overnight diluted by growth media and incubated 3h to obtain exponentially growing cells. Cells were washed with dH2O, diluted to OD600 0.02 and 5 μl of cell solution was transferred to each spot. Plates were photographed after three days of incubation at 30°C.

RNA isolation and real-time PCR analysis
Total RNA samples from exponentially growing yeast cells were isolated using the RNeasy Mini Kit (Qiagen). Genomic DNA contaminations were removed by DNase treatment (DNase RQ1, Promega). cDNA synthesis was performed using the First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer’s instructions. The cDNAs were used as templates to amplify internal parts of the selected genes. ACT1 gene was used as an internal control. Real-time PCR assays were performed with IQ5 real-time PCR system (BIO-RAD).

Microarray analysis
Concentrations and purity of RNA samples were determined by measuring their absorbances at 260/280nm, using a nanodrop spectrophotometer (Thermo Scientific), and the quality of RNAs were determined by Agilent RNA 6000 Nano Kit in Agilent Bioanalyzer.

Total RNA and spike-in mixes were prepared by mixing minimum 100ng of total RNA and spike-in kit for each qualified RNA sample. Total RNA sample was resuspended in nuclease-free water to obtain minimum of 50ng/μl RNA. The spike-in solution and T7 primer mix was added on diluted RNA samples and denaturation was performed by incubating the mix at 65°C for 10 minutes. cDNA master mix was prepared and added on each RNA sample mix. The mix was then incubated at 40°C for 2 hours; 70°C for 10 minutes and on ice for 5 minutes, respectively. The Cyanine-3-labeled and amplified RNA samples (cRNA) were purified and quantification was performed by using Nanodrop spectrophotometer (Thermo Scientific).

For hybridization, cRNA samples were incubated with a fragmentation buffer and gene expression blocking agent at 60°C for 30 minutes. The fragmented samples were loaded on arrays and the array slides were placed in a hybridization oven and the hybridization reaction was performed at 65°C for 18 hours. After the hybridization step, the samples were washed with a washing solution. The washing step was performed at room temperature for the first and the second washes. The third wash was performed at 37°C. After the washing step, the slides were scanned and signal intensities were obtained by a feature extraction program.

Statistical analysis
Student’s T-test was used for Real-time PCR analysis and fold-change analysis was used for microarray analysis data. P-value 0.05 was chosen as the significance level (p< 0.05) for statistical analysis.

Results and Discussion
Screening for the genes that show resistance to doxorubicin
In order to identify genes that confer resistance to doxorubicin, we first determined the toxic drug concentrations for our strains. We analyzed cell growth rates in different drug
concentrations in liquid media for both haploid (BY4741) and diploid (BY4743) wild type cells. As seen in Fig 1, the growth of both strains was completely inhibited in the presence of 200 μM or higher amounts of doxorubicin. Next, we transformed wild type (BY4741) cells with a 2μ-based genomic expression library and isolated 6 transformants that could grow in the presence of 500 μM of doxorubicin on solid media. By using a high concentration of doxorubicin for screening, we aimed to find genes that help cells tolerate very toxic levels of the drug. Plasmids from the transformants were isolated and amplified in E. coli and used for the re-

Fig 1. Growth curves. Wild type (A) haploid and (B) diploid yeast strains on 0 μM, 100 μM, 200 μM and 300 μM doxorubicin media. Error bars represent SD of the means for triplicate determinations.

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transformation of fresh wild type cells for confirmation purposes. All the isolated plasmids conferred resistance to the new cells, thus, we confirmed that the resistance observed in the original transformants were provided by the plasmids.

The nucleotide sequences of the expression cassettes in each plasmid were determined. Sequence analyses yielded nine intact genes (Table 1). In order to find out which one of these genes provided resistance, they were all cloned onto the pAG426-GDP plasmid individually and expressed in haploid (BY4741) and diploid (BY4743) wild type cells. As seen in Fig 2A and 2B, only expression of AKL1, CUE5, CAN1, YHR177w and PDR5 genes provided resistance to doxorubicin. Particularly, cells overexpressing PDR5 gene were highly resistant to the drug and able to grow in the presence of 2 mM of doxorubicin (Fig 2C), which is the highest dose that could be tolerated by yeast cells as of our knowledge.

As the next step, we analyzed the deletion mutants of AKL1, CUE5, CAN1, YHR177w and PDR5 genes in both haploid and diploid backgrounds to identify whether they were sensitive to doxorubicin (Fig 3A and 3B). In both backgrounds, pdr5Δ mutants were the most sensitive cells and their growth was inhibited by 50 μM of doxorubicin. In addition to pdr5Δ mutants, akl1Δ cells were also more sensitive to doxorubicin when compared to wild type cells, however the rest of the mutants (cue5Δ, can1Δ and yhr177wΔ) showed a growth pattern similar to that of wild type cells.

Pdr5p is a member of the ATP-binding cassette family of transporters and mediates resistance to many xenobiotics such as mutagens, antifungals and steroids [25,26]. In addition to drug stress, it is also involved in cation resistance [27], and lipid transport in yeast cells [28]. Pdr5 resembles (orthologous) mammalian MDR1 which is a major factor in tumor resistance [29]. Regulation of PDR5 is controlled by transcription factors PDR1 and PDR3 and deletion of these transcription factors leads to loss of PDR5 expression, while gain of function mutations in pdr1/3 leads to over expression of this protein [30–32].

Being an efflux pump, PDR5 gene has previously shown to be associated with doxorubicin resistance. Golin et. al. showed that pdr5Δ mutants could not grow at 50μM doxorubicin [33] and Rogers et. al. showed that pdr5Δ mutants are sensitive to doxorubicin [34]. Kolaczkowski et al. (1996) showed that pdr1-3 mutants, that overexpress PDR5, are doxorubicin resistant, while the pdr5Δ mutant is sensitive to the drug [35]. A more recent study has shown that pdr1-3 mutation leads to upregulation of about twenty-five other genes in addition to PDR5 [36], which may also affect the resistance level in pdr1-3 mutants. Here, we showed that not only pdr5Δ mutants were doxorubicin sensitive but also PDR5 overexpression from a plasmid increased doxorubicin resistance, which supports the findings from previous studies [33–35].

PDR5, SNQ2 and YOR1 are known to be major determinants of multidrug resistance in yeast and they all are controlled by PDR1 [30,37,38]. In order to check if PDR5 overexpression may lead to resistance to doxorubicin in the absence of these genes, we overexpressed PDR5 in yor1Δ, snq2Δ and pdr1Δ mutants and analyzed them by a spotting assay (Fig 4). In addition to doxorubicin, transformants were also tested on clotrimazole, an inhibitor of ergosterol synthesis in fungi [39], and cerulenin, an inhibitor of fatty acid synthesis [40], since both are known

| Colony No | Chromosome Information | Covered Genes |
|-----------|------------------------|---------------|
| 1         | Chromosome XV Coordinates 407059 bp to 411911 bp | GLO4, CUE5, part of WHI2 |
| 2         | Chromosome V Coordinates 30280 bp to 33605 bp | CAN1 |
| 3         | Chromosome II Coordinates 346000 bp to 366000 | TSC3, AKL1 |
| 4         | Chromosome VII Coordinates 445000 bp to 465000 bp | YHR177w |
| 5         | Chromosome XV Coordinates 607000 bp to 627000 bp | YOR152C, PDR5 |

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to be substrates of Pdr5 (S1 Fig). PDR5 overexpression made these cells resistant to all three drugs. The pdr1Δ strain was more sensitive to the drugs compared to the wild type cells, whereas snq2Δ and yor1Δ mutants showed no sensitivity. Cerulenin was very toxic to cells and only cells that carried PDR5 plasmid were able to survive in the presence of this drug even at the minimal concentrations (S1 Fig). We also noticed that PDR5 overexpressing pdr1Δ cells were less resistant to the drugs compared to PDR5 overexpressing wild type cells. On the other hand, yor1Δ mutants were previously shown to be sensitive to doxorubicin [34], however we did not observe this phenotype under our experimental conditions.

Another gene whose overexpression provided resistance and deletion rendered cells to doxorubicin was AKL1. Akl1, which is a serine-threonine protein kinase involved in endocytosis and actin cytoskeleton organization [41], has previously been linked to doxorubicin resistance by others [14]. Sla1/Pan1/End3 complex is involved in endocytosis and affected by Akl1
overexpression [20]. Akl1 phosphorylates Pan1p and leads to dissociation of the yeast Sla1/Pan1/End3 complex, which regulates the internalization step of endocytosis [42]. Dissociation
of the complex subsequently results in inhibition of endocytosis. Reduction in endocytosis did not affect doxorubicin accumulation significantly [20], and only very few drugs have been shown to enter cells through endocytosis [29]. Therefore, resistance to doxorubicin provided by Akl1p overexpression may be through mechanisms other than reduced endocytosis. However, reduction in endocytosis may have indirect effects on doxorubicin resistance, because end4 endocytosis mutants accumulate Pdr5p at the plasma membrane [35,43]. To test if doxorubicin resistance in AKL1 overexpressing strain is somehow affected by the presence of Pdr5, we overexpressed AKL1 in pdr5Δ mutants and spotted cells in the presence of doxorubicin (Fig 5) and other drugs (S2 Fig). Our results indicated that AKL1 overexpression in pdr5Δ mutants did not make cells resistant to doxorubicin or to other drugs, which suggested that PDR5 was required for Akl1 action. It is likely that AKL1 overexpression may act through Pdr5 stabilization in plasma membrane. Overexpression of AAK1, which is the human orthologue of AKL1, also causes doxorubicin resistance in Hela cells [20], suggesting that the role of AKL1 in doxorubicin resistance is conserved in higher eukaryotes, but the mechanisms of resistance are not clear yet.

CAN1, YHR177W and CUE5 are the other genes obtained from genomic library screening, of which their overexpression made cells resistance to doxorubicin, but their deletion mutants did not show any sensitivity. Can1p is a plasma membrane arginine permease, arginine-H+ symporter, which is exclusively associated with lipid rafts [44]. Possible doxorubicin resistance mechanism for CAN1 may be upregulation of filamentous growth due to its location in ergosterol-rich domains of the plasma membrane that harbors several proteins required for filamentous growth [45].

YHR177w is a putative transcription factor with a WOPR domain, of which its overexpression causes either cell cycle delay or arrest [46]. Proteins with WOPR domains are important in pathogenesis [46] and YHR177w overexpression leads to invasive growth (pseudohyphae formation) in yeast [47]. Therefore, YHR177w overexpression may also cause doxorubicin resistance through activation of invasive growth.

Cue5p functions as ubiquitin-Atg8p adaptor in ubiquitin-dependent autophagy [48,49] and it is role in drug resistance is not clear.

Expression patterns of resistance genes in response to doxorubicin treatment

Screening analyses showed that the overexpression of PDR5, AKL1, CAN1, YHR177W and CUE5 from a plasmid provided doxorubicin resistance, however it is not known whether these
genes are upregulated or not in the presence of doxorubicin. In order to study expression patterns of these genes, we incubated cells with a sublethal dose of doxorubicin (80 μM) for two hours and determined the transcript levels of each gene by a Real-Time PCR approach (Fig 6). Interestingly, expression of only AKL1 increased slightly (1.5-fold) (p = 0.25) and the rest of the genes did not physiologically respond to the drug. Expression analyses also showed that PDR1, a known activator of PDR5, expression was not activated by doxorubicin treatment. Thus, these genes were not transcriptionally responsive to doxorubicin and provided resistance only if they were expressed ectopically. On the other hand, doxorubicin sensitivity of pdr5Δ and akl1Δ mutants suggested a possible role for these genes in doxorubicin resistance with their basal expression levels.

Global expression profiling for doxorubicin response

To further evaluate the mechanisms of doxorubicin resistance and toxicity, we analyzed the changes in the global gene expression profile of yeast cells after doxorubicin exposure.

The genes showing more than 2-fold changes (p<0.05) are listed in S1 Table. Our microarray results also showed that doxorubicin resistance genes were not upregulated in response to the drug treatment and were consistent with the qPCR data. Out of approximately 6200 yeast genes, 211 of them were significantly upregulated, while 148 genes were downregulated. We categorized these genes by functional MIPS classification (Tables 2 and 3) to highlight the pathways that play role in doxorubicin tolerance and toxicity.

Funspec analysis [50] of the upregulated genes showed that paths related to transport dependent-detoxification systems and DNA metabolism such as DNA restriction, integration and recombination were upregulated in the presence of doxorubicin (Table 2).

Among the transporters (Table 2) polyamine transporter TPO2, azole resistance gene AZR1, drug:H(+) antiporter YHK8, pleotropic drug resistance gene PDR18 and membrane
proteins Pnt1 and Svs1 were upregulated in response to doxorubicin. Pdr18, besides being a membrane transporter, can affect the membrane sterol composition and lead to MDR phenotype [51]. Among the upregulated genes, MRE1 (meiotic recombination) and MMS4 (Methyl Methane Sulfonate Sensitivity) both function in DNA repair [52,53], and are related to doxorubicin sensitivity [15,54].

When we functionally categorized the down-regulated genes in response to doxorubicin treatment, only a small fraction of genes were clustered (Table 3).

Mainly, expression of iron transporters (FIT1, FIT2 and FIT3), sugar transporters (HXT15, HXT13, HXT16 and HXT17) and proton driven antiporters (KHA1 and ATO2) were inhibited by doxorubicin.

Yeast responds to iron limitations by activating Aft1 transcription factor and expressing FIT1, FIT2 and FIT3 genes. These genes encode for mannoproteins that are incorporated into the cell wall and play roles in retention of siderophore-iron in the cell wall [55]. Decreasing iron levels by inhibition of FIT1, FIT2 and FIT3 genes might be a good defense system for doxorubicin toxicity, since iron plays role in doxorubicin cytotoxicity by producing ROS [4]. Sugar transporters that were downregulated by doxorubicin are all low affinity hexose transporters and their roles in drug response is not known.

Ion transporters KHA1 and ATO2 play role as K(+) / H(+) antiporter and ammonia extruder, respectively [56,57]. Inhibition of KHA1 may lead to accumulation of K(+) and disruption of vacuole membrane potential, which might be important in doxorubicin transport/defense. However, resistance to doxorubicin decreases in kha1Δ mutants [54]. Thus, the exact role of KHA1 in doxorubicin resistance is not clear. Similarly, possible benefits that could be gained by lowering the transcript levels of ATO2, ammonia transporter, are not clear.

Our microarray and real-time PCR results were consistent with each other. They both confirmed that none of the genes obtained from genomic library screenings were upregulated by doxorubicin treatment. The genomic DNA library used in this work was a high copy number (2 μ) library and supposedly genes on the plasmids were expressed at high levels. We observed that PDR5 mRNA level was 7-fold higher than that of empty vector carrying transformants (S3 Fig) (p = 0.039) and that was apparently enough for cells to tolerate 2mM doxorubicin.

Seemingly, doxorubicin did not activate the transcriptional machinery required for the expression of PDR5, CAN1, YHR177W and CUE5 genes since their mRNA levels did not change much upon the treatment. A specific support for the transcriptional inertness of these genes was the unaffected level of PDR1, an activator of PDR5 gene (Fig 6). In addition to Pdr1, transcription factors Pdr3, Yap1, and Mig3 also play roles in expression of PDR5 in response to

| Category                        | p-value     | In Category from Cluster | k  | f  |
|---------------------------------|-------------|--------------------------|----|----|
| DNA restriction [10.01.09.03]    | 1.865e-05   | AI1 AI2 AI4 MMS4 MRE11   | 5  | 12 |
| detoxification [32.07]          | 0.003305    | ADH5 FZF1 TPO2 AZR1 YHK8 PDR18 PNT1 SVS1 | 8 | 80 |
| meiosis I [10.03.02.01]         | 0.006791    | REC104 MRE11 NDJ1        | 3  | 13 |
| DNA integration [10.01.05.03.05]| 0.009102    | AI1 AI4                  | 2  | 5  |

Table 3. MIPS functional classification of >2-fold downregulated genes in response to Doxorubicin treatment (p value cutoff: 0.01).

| Category                        | p-value     | In Category from Cluster | k  | f  |
|---------------------------------|-------------|--------------------------|----|----|
| ion transport [20.01.01]         | 0.0003473   | FIT1 FIT2 FIT3           | 3  | 7  |
| sugar transport [20.01.03.01]    | 0.004537    | HXT15 HXT13 HXT16 HXT17 | 4  | 31 |
| proton driven antiporter [20.03.02.03.01] | 0.00948 | KHA1 ATO2 | 2  | 7  |

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drug/chemical stress exposure [58], however, their mRNA levels were not increased significantly by the doxorubicin treatment (S1 Table). Thus, even though PDR5 played a major role in doxorubicin tolerance, the transcription factors that regulate it were not activated by doxorubicin treatment.

**Conclusion**

In this study, we screened a yeast genomic DNA library to identify genes that are responsible for doxorubicin resistance and found that PDR5 was the primary gene that played role in doxorubicin tolerance. Our screen also pointed out roles of other genes such as, AKL1, CAN1, YHR177W and CUE5 in protecting cells from doxorubicin toxicity. We also showed that transcriptional regulation of these genes was not dependent on doxorubicin treatment, however, overexpression of them make S. cerevisiae cells resistant to high doses of doxorubicin.

Additionally, we analyzed the global expression profile of yeast cells after doxorubicin treatment and highlighted the genes and paths that might be important in doxorubicin tolerance and toxicity. Our results showed that membrane transporters and DNA metabolism genes are upregulated in the presence of doxorubicin and these genes may function in doxorubicin detoxification/tolerance processes.

When we consider the genes whose overexpression caused doxorubicin resistance, excluding Cue5, their common effect seemed to be the change in membrane asymmetry. Thus, changing the composition of the cell membrane could be a common response of cells to high doxorubicin levels.

**Supporting Information**

**S1 Fig. Spotting assays for PDR5 overexpression.** PDR5 was cloned and expressed in wild type, yor1Δ, snq2Δ, pdr1Δ, and pdr5Δ cells. Spotting assays were performed on (A) clotrimazole and (B) cerulenin. (TIFF)

**S2 Fig. Spotting assays for AKL1 overexpression.** AKL1 was cloned and expressed in akl1Δ and pdr5Δ. Spotting assays were performed on (A) clotrimazole and (B) cerulenin. (TIFF)

**S3 Fig. Real-time PCR analyses for PDR5 overexpression.** PDR5 transcript analyses in haploid and diploid wild-type strains that overexpress PDR5. (TIFF)

**S1 Table.** List of the genes that were up or down-regulated by 2-fold or more in response to doxorubicin treatment. (XLSX)

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**Author Contributions**

Conceived and designed the experiments: AK. Performed the experiments: ABD. Analyzed the data: AK ABD. Contributed reagents/materials/analysis tools: AK ABD. Wrote the paper: AK ABD.
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