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Highly synergistic effect of sequential treatment with epigenetic and anticancer drugs to overcome drug resistance in breast cancer cells is mediated via activation of *p21* gene expression leading to G2/M cycle arrest

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Abstract

Epigenetic alterations such as aberrant DNA methylation and histone modifications contribute substantially to both the cause and maintenance of drug resistance. These epigenetic changes lead to silencing of tumor suppressor genes involved in key DNA damage-response pathways, making drug-resistant cancer cells nonresponsive to conventional anticancer drug therapies. Our hypothesis is that treating drug-resistant cells with epigenetic drugs could restore the sensitivity to anticancer drugs by reactivating previously silenced genes. To test our hypothesis, we used drug-resistant breast cancer cells (MCF-7/ADR) and two epigenetic drugs that act via different mechanisms—5-aza-2' deoxycytidine (Decitabine, DAC), a demethylating agent and suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor—in combination with doxorubicin. We show that the sequential treatment of resistant cells, first with an epigenetic drug (DAC), and then with doxorubicin, induces a highly synergistic effect, thus reducing the IC₅₀ of doxorubicin by several thousand folds. The sequential treatment caused over 90% resistant cells to undergo G2/M cell cycle arrest, determined to be due to upregulation of *p21^{WAF1/CIP1}* expression, which is responsible for cell-cycle regulation. The induction of *p21^{WAF1/CIP1}* correlated well with the depletion of DNA methyltransferase 1 (DNMT1), an enzyme that promotes methylation of DNA, suggesting that the *p21^{WAF1/CIP1}* gene may have been methylated and hence is inactive in MCF-7/ADR cells. Microarray analysis shows expression of several tumor suppressor genes and downregulation of tumor promoter genes, particularly in sequentially treated resistant cells. Sequential treatment was found to be significantly more effective than simultaneous treatment, and DAC was more effective than SAHA in overcoming doxorubicin resistance. Synergistic effect with sequential treatment was also seen in drug-sensitive breast cancer cells, but the effect was significantly more pronounced in resistant cells. In conclusion, the sequential treatment of an epigenetic drug in combination with doxorubicin induces a highly synergistic effect that overcomes doxorubicin resistance in breast cancer cells.

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SUPPORTING INFORMATION (SI) Heat map showing the expression of house keeping genes following treatment of MCF-7/ADR cells with DAC alone, doxorubicin alone, and sequentially with DAC and doxorubicin for 48 h and 72 h. Red indicates relatively high expression relative to the sample mean and blue indicates relatively low expression. Results show that the expression of housekeeping genes is not significantly altered following different treatments. This material is available free of charge via the Internet at <http://pubs.acs.org>

Keywords

Epigenetic drugs; Drug resistance; Breast cancer; Combination therapy; Cell-cycle analysis; Gene expression

INTRODUCTION

Epigenetic aberrations play a crucial role in cancer initiation and progression.^{1, 2} The most common epigenetic alterations in cancer are DNA methylation and histone modifications.^{3, 4} These epigenetic changes lead to silencing of tumor suppressor genes involved in key DNA damage-response pathways, such as cell-cycle control, apoptosis and DNA repair.^{5, 6} Epigenetic silencing of genes influences tumorigenesis and tumor response to drug therapy and is also the main cause of drug resistance.^{7, 8} The prevalence of epigenetic gene silencing in cancer initiation and drug resistance makes this characteristic an attractive target for epigenetic drug therapy.^{3, 9} Since epigenetic modifications do not mutate the DNA sequence of a gene,² strategies to reverse epigenetic abnormalities are considered useful in reversing drug resistance in cancer therapy.

Anthracyclines (doxorubicin, daunorubicin, epirubicin, etc.) are the most important class of anticancer drugs used in treating breast cancer patients.^{10, 11} However, the therapeutic advantages of these drugs are significantly impaired by potentially life-threatening cardiotoxicity and other lethal side effects.¹² Cardiotoxicity caused by doxorubicin, the most commonly used drug in breast cancer therapy, is dose dependent and cumulative;¹³ and the repeated administration of doxorubicin results in the selection of drug-resistant cells within the tumor.¹⁴ Once cancer cells develop drug resistance, these drugs are rendered ineffective at the usual tolerable doses. These concerns have led investigators to explore new approaches to reducing doses of doxorubicin while enhancing its efficacy.

One strategy currently being explored is to formulate doxorubicin in nanocarriers that can alter the pharmacodynamics of drug distribution to reduce its accumulation in the heart.¹⁵ Doxil, a liposomal formulation of doxorubicin used to treat breast cancer patients, is an example of such an approach. However, Doxil is not effective in treating drug-resistant breast cancer because of the impaired endocytic function that inhibits effective intracellular drug delivery.¹⁶ Several actively targeted nanocarrier systems are being investigated to enhance drug uptake and retention in tumor tissue to address the above problem of drug delivery and efficacy using nanocarrier systems.¹⁷

Resistance against doxorubicin at the cellular level might arise due to epigenetic abnormalities of genes, which results in alteration of the drug's target and response. Doxorubicin mediates its cytotoxicity by inhibiting topoisomerase II (Topo II), a nuclear enzyme essential for DNA replication.¹⁸ Topo II levels are reported to be lower in doxorubicin-resistant tumors from breast cancer patients, unlike drug-sensitive tumors, which express high levels of the enzyme.¹⁹ It has been shown that this enzyme is methylation silenced in drug-resistant cells to gain a survival advantage.^{20, 21} Methylation silencing of genes also limits intracellular drug levels by increasing drug efflux and decreasing drug influx.^{22–24} One or all of these events lead to impaired drug delivery and hence efficacy in resistant cells.

Therapeutic strategies without addressing the root cause of drug resistance in breast cancer cells, (i.e., epigenetic dysregulation) may not be effective. Certain compounds, such as 5-aza-2'-deoxycytidine (DAC), a DNA hypomethylating agent, and suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, can reactivate the expression of

transcriptionally silenced genes.^{25, 26} By reversing the hypermethylation state of DNA, DAC can reduce DNA methyl transferase (DNMT) activity and the expression of multidrug resistance-1 (mdr1) efflux protein, thus enhancing intracellular doxorubicin levels.^{22, 23} Doxorubicin sensitivity could also depend on the state of the cell's chromatin. Highly condensed chromatin, a form observed in many cancers, restricts the toxicity of doxorubicin by limiting the accessibility of both the drug and its substrate (Topo II) to DNA.²⁷

Our hypothesis is that treating drug-resistant cells with epigenetic drugs could restore sensitivity to doxorubicin by reactivating previously silenced genes and could thereby overcome drug resistance. Here we show that the sequential treatment of resistant cells, first with epigenetic drug (DAC or SAHA) followed by doxorubicin, demonstrates a highly synergistic effect in overcoming drug resistance in breast cancer cells.

EXPERIMENTAL SECTION

Materials

5-aza-2'-deoxycytidine (DAC, generic name decitabine) and suberoylanilide hydroxamic acid (SAHA, generic name vorinostat) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Doxorubicin hydrochloride was purchased from Drug Source Co. LLC (Westchester, IL). Cell culture media, DPBS, penicillin and streptomycin were purchased from the Central Cell Services' Media Laboratory of our institution. MTS reagent was purchased from Promega (Madison, WI).

Cell Culture

MCF-7/ADR cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (Gibco BRL, Grand Island, NY) and 100 µg/mL penicillin G and 100 µg/mL streptomycin. MCF-7, MDA-MB 231 and BT-459 were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics at 37 °C in a humidified and 5% CO₂ atmosphere. To maintain drug resistance, MCF-7/ADR cells were cultured in a medium containing 100 ng/mL of doxorubicin. Resistant cells were maintained in drug-free media in the last two passages prior to any experiments. Sensitive cell lines were cultured in drug-free cell culture media. Culturing conditions for resistant cells were optimized; resistant cells require a higher serum concentration than sensitive cells for proper cell growth.

Cell Viability—Cell viability following different treatments was determined using a standard MTS assay (CellTiter 96 Aqueous, Promega, Madison, WI). In brief, to each well, 20 µL of reagent was added, and the plates were incubated for 2 h at 37 °C in cell culture incubator. Color intensity was measured at 490 nm using a microplate reader (Bio-Tek Instrument, Winooski, VT). The effect of the drug on cell proliferation was calculated as percentage growth of cells relative to respective controls.

Sequential versus Simultaneous Treatments—We first tested the efficacy of pretreatment of DAC and SAHA in MCF-7/ADR in time- and dose-response studies for each drug's ability to sensitize cells to the cytotoxic effect of doxorubicin. For this step, drug-resistant cells were seeded at 3,000 cells per well/0.1 mL culture medium in 96-well plates (BD Biosciences, San Jose, CA). At 24 h post seeding, cells were treated with different concentrations of DAC for 24 h or 48 h; then cells were washed with 1× DPBS prior to measuring cell viability using an MTS assay.

The cells pretreated with DAC or SAHA at different doses were tested with doxorubicin in a dose-response study. For this, the DAC- or SAHA-treated cells were incubated with

different doses of doxorubicin for 48 h. Cells were washed with 1× DPBS, and then replaced with drug-free cell culture media and incubated for an additional 48 h.

To determine the efficacy of simultaneous treatment of DAC and doxorubicin vs. sequential treatment as described above, cells were treated with DAC at the dose (50 ng/mL) that demonstrated a highly synergistic effect in sequential treatment; that dose was mixed with different doses of doxorubicin. Cells were incubated with the combination of DAC and doxorubicin for 72 h and cell viability was determined using MTS assay as mentioned above. Identical protocol was followed for sequential treatment of SAHA in combination with doxorubicin, except that SAHA was incubated with cells for a shorter time than with DAC. This step was necessary because with long-term treatment, SAHA is known to induce cell-cycle arrest in cancer cells. Doxorubicin requires dividing cells to execute its cytotoxicity, and hence it may not be effective in cells that are in the cell-cycle arrest phase. To find an optimal time point for SAHA pretreatment and to increase the synergy between SAHA and doxorubicin, cells were incubated with SAHA for different time points. Doses of DAC or SAHA for pretreatment were selected in such a way that they did not have their own significant antiproliferative effect at that concentration.

In addition to the studies in drug-resistant cells, the efficacy of sequential therapy of DAC and doxorubicin was tested in drug sensitive breast cancer cells (MCF-7, MDA-MB 231 and BT-459). Since DAC at its half the IC₅₀ dose (50 ng/mL) showed synergy with all the doses of doxorubicin tested in resistant cells, sensitive cells were first treated with the above dose of DAC followed by different doses of doxorubicin.

Calculation of Combination Index—The synergistic or antagonistic effects of the various drug combinations (DAC + doxorubicin or SAHA + doxorubicin) and the treatment protocols were analyzed using the CalcuSyn software program (Biosoft, Ferguson, MO). The MTS data from the above experiments were entered into the software to calculate the combination index value (CI). From the CI values, the synergistic action of drugs can be analyzed. A CI of 1 indicates an additive effect, CI <1, a synergistic effect, and CI >1, antagonistic activity.²⁸

Cell-Cycle Analysis

To understand the mechanism of efficacy of the combination treatments, cells were analyzed for cell cycle via flow cytometry using the optimized protocol from the above set of experiments. For this, cells were seeded at 300,000 cells/3 mL culture medium/well in 6-well plates (BD Biosciences) and allowed to attach for 24 h. Cells following different treatments were trypsinized and centrifuged at 1,300 rpm (Sorvall Legend RT centrifuge, Thermo Electron Corp., Waltham, MA) for 3 min at 4 °C. The cell pellet was washed twice with ice cold 1× DPBS (pH 7.4) and then resuspended in propidium iodide-(PI) solution (12.5 mg PI, 250 mg sodium-citrate and 250 µL Triton X-100 in 250 mL of water). Cells were then incubated in an ice bucket, kept in a cold room for 2 h, and the DNA content was determined by flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). ModFit LT software (Verity Software House, Inc., Topsham, ME) was used for data analysis.

Microarray Analysis and Validation of p21 Expression

Gene array was carried out to determine the genes that may have been activated and involved in G2/M arrest following treatment of resistant cells with DAC alone or sequentially DAC (50 ng/mL) and doxorubicin (1 ng/mL). Briefly, cells were seeded and treated as mentioned above for the flow cytometry protocol. Following treatment, total RNA was isolated from cells using the SV Total RNA Isolation System (Promega, Madison, WI).

The isolated RNA was stored at -80°C in nuclease free water at the concentration of 100 ng/ μL until used for gene array analysis. Approximately 5 μg of total RNA was subjected to gene expression array study using an Illumina human 6v2 chip (Illumina, Inc. San Diego, CA). The array-hybridization and scanning of array images were performed at the Cleveland Clinic Genomics Core.

Statistical and functional analyses of gene expression data were performed by the Cleveland Clinic Bioinformatics Core facility. The raw gene expression datasets were log2 transformed and processed with background correction and quantile normalization. Quality control analyses were applied to detect the outlier samples. No outliers were observed. The expression signals with an Illumina detection threshold < 0.05 across all the samples were used. To detect changes in expression between sensitive and resistant cells at two time points, the differential P value (Diff Pval) and false discovery rate (FDR) p value²⁹ were calculated using the Illumina custom error model (Genome Studio Gene Expression Module v3.3 software, Illumina Inc.).

To show the differentially expressed genes that satisfy both the statistical (negative log p-values) and biological (fold change) criteria, volcano plots were generated using R statistical package. Hierarchical clustering and heat maps were also generated using the same statistical package. Genes that satisfied the FDR p-value threshold of < 0.05 or raw p-value of < 0.001 and absolute fold change threshold of 2.0 were identified as significant for the functional pathway and network analyses.

Functional profiling of differentially affected biological processes, functions, and pathways in resistant cells treated with DAC or sequentially with DAC followed by doxorubicin either for 48 h and 72 h were evaluated using a suite of publicly available tools (such as GO::TermFinder, GO Term Mapper, Reactome) and commercial pathway analysis databases such as Ingenuity Pathway Analysis (IPA., from Ingenuity Systems, Inc., Redwood city, CA) and Metacore™ (GeneGo, Inc., St. Joseph, MI). Direct interaction network of genes was generated using Metacore™ analytical suite version 5.2 build 17389 (GeneGo, Inc.)^{30, 31}

Quantitative polymerase chain reaction (qPCR) was carried out to validate p21 gene expression. Briefly, RNA isolated from cell treated with either drugs alone (DAC or doxorubicin) or in combination (DAC + doxorubicin) and untreated cells were converted into cDNA using Mo-MLV reverse transcriptase (Promega Inc., Madison, WI) according to manufacturers' instructions. Taqman expression primers (Applied Biosystems Inc., Carlsbad, CA) and ABI PRISM Sequence Detection Instrument 7700 (Applied Biosystems Inc.) were used for real-time reverse transcription-PCR (RT-PCR). Since housekeeping genes remain unaltered following DAC treatment in resistant cells (Supporting Information), the fold change in target genes was calculated about the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western blot

Resistant cells were seeded (5×10^6 cells/dish) in 100-cm² cell culture dishes (BD Biosciences) and incubated for 24 h in CO₂ incubator for attachment. Cells were treated as mentioned above for the flow cytometry protocol to analyze p21 expression. To determine the effect of DAC treatment on DNMT levels, cells were cultured in cell culture dishes as above and treated with DAC (50 ng/mL) for 1, 2, 3 or 5 days. Following treatment, cell lysates were prepared by lysing 1×10^6 cells with RIPA buffer (Sigma-Aldrich) containing $1 \times$ protease inhibitor cocktail (Calbiochem, Gibbstown, NJ). Each cell lysate was centrifuged at 14,000 rpm for 15 min. Protein concentration in cell lysates was determined using bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL). About 20–40 μg proteins

of cell lysates were electrophoresed through 13.5% SDS-polyacrylamide gel or 4–15 % linear precast polyacrylamide gradient gel (Bio-Rad Laboratories, Hercules, CA) and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare Biosciences, Piscataway, NJ). The blots were probed for mouse monoclonal p21 (Cell Signaling Technology, Inc., Danvers, MA), mouse monoclonal DNA methyltransferase1 (DNMT1; Abcam Inc., Cambridge, MA) and mouse monoclonal anti-actin (Sigma-Aldrich). For detection of bound antibody, PVDF membrane was incubated with horseradish-tagged goat anti-mouse antibody. After incubation, membrane was washed Tris-buffered saline with 0.5% Tween 20 (TBST) and stained with enhanced chemiluminescence (ECL) reagent or ECL plus reagent (GE Healthcare Biosciences, Piscataway, NJ) according to the manufacturer's protocol.

Statistical Analysis

Data are expressed as mean $\pm$ standard error of mean (s.e.m.). Statistical analyses were performed using Student's *t* test. Differences were considered significant for $p < 0.05$.

RESULTS

Cytotoxic Effect of DAC in Combination with Doxorubicin

Doxorubicin demonstrated sigmoidal dose-dependent antiproliferative activity in both drug-resistant (MCF-7/ADR) and drug-sensitive (MCF-7) cells; however the IC₅₀ dose of doxorubicin for resistant cells was significantly greater than for sensitive cells (7,385 ng/mL vs. 21 ng/mL; Table 1). Resistant cells treated with DAC showed a dose-response antiproliferative effect with IC₅₀ = 108 ng/mL (Figure 1a); however, sensitive cells (MCF-7) did not show the same dose-dependent antiproliferative effect (Figure 2a). Pretreatment of resistant cells with DAC followed by with doxorubicin at all the combinations tested demonstrated a synergistic effect (Figure 1b and c). For example, resistant cells pretreated with half the IC₅₀ dose of DAC (50 ng/mL) showed significant cytotoxicity following doxorubicin treatment; even a 1 ng/mL doxorubicin concentration caused over 50% cell death, which otherwise without pretreatment requires 7,385 ng/mL doxorubicin to induce 50% cell killing (Figure 1a; Table 1). This is an almost 7,000-fold enhancement in drug efficacy of doxorubicin based on IC₅₀, with a highly synergistic effect (CI = 0.2; Table 1). We also demonstrated that the sequential treatment showed greater synergistic effect than simultaneous treatment, which was mostly an additive effect (Figure 1b and c).

Sequential treatment using DAC and doxorubicin also showed a synergistic effect in sensitive breast cancer cells, but the extent of this synergistic effect was cell-line dependent. Based on the IC₅₀ values, cytotoxicity of doxorubicin increased by 2.8 fold in MCF-7, 4.4 fold in MDA-MB-231 and 4 fold in BT-459 cells (Figure 2a–c). MCF-7 and MDA-MB 231 cells showed a highly synergistic effect at lower doses of doxorubicin, whereas BT-459 cells only showed this effect at higher doses of doxorubicin. The synergistic effect of sequential treatment was significantly more pronounced in resistant cells than in sensitive cells which is evident from relative CI values (Table 1).

Effect of SAHA and Doxorubicin

Unlike DAC treatment, which showed significantly higher sensitivity in resistant cells (MCF-7/ADR) than in sensitive cells (MCF-7), the response of SAHA treatment was almost similar in both the cell lines (Figure 3a). Although sequential treatment with SAHA and doxorubicin showed synergistic activity in both the resistant and sensitive cells, the synergistic effect of SAHA was highly dependent on pretreatment time. Resistant cells pretreated with SAHA for 4 and 8 hrs followed by doxorubicin showed a synergistic effect; in contrast, the cells pretreated with SAHA for 24 h showed a highly antagonistic effect

(Figure 3b). This result is evident from the CI values, which showed reduced synergism in resistant cells with increased incubation time of SAHA with cells prior to doxorubicin treatment (Figure 3c). In sensitive cells, SAHA showed a synergistic effect at all the pretreatment times tested, but the synergic effect diminished with increased pretreatment time (Figure 3d and e).

Cell-Cycle Analysis

Since cells pretreated with 50 ng/ml of DAC for 24 h and then with 1 ng/ml of doxorubicin showed a highly synergistic effect in sequential treatment in breast cancer cells, the above protocol was used to evaluate cells for cell-cycle analysis. Resistant (MCF-7/ADR) and sensitive cells (MCF-7) cells treated with doxorubicin alone at 1 ng/ml dose showed no significant effect on the number of cells going into the G2/M cell-cycle phase. However, treating cells with DAC alone increased the number of cells going into cell-cycle arrest phase with respect to control (Figure 4). Interestingly, DAC treatment had no effect on the number of resistant cells going into cell-cycle arrest phase with treatment time (48 h vs. 72 h; Figure 4b), whereas sensitive cells showed more cells going into the cell-cycle arrest phase with treatment time (Figure 4d). The sequential treatment of both resistant and sensitive cells caused over 90% cells going into the cell-cycle arrest phase, but the difference was that resistant cells required 72 h treatment time to reach this level of cell-cycle arrest phase, whereas sensitive cells required only 48 h treatment time (Figure 4b and d).

Microarray Analysis

The results of microarray analysis showed marked changes in gene expression ($\approx 2 \times$) in resistant cells following treatment with DAC alone or sequential with DAC and doxorubicin (Figure 5a–e). The number genes either overexpressed or repressed increased with increased treatment time (48 h vs. 72 h) (Fig 5a and b). Several genes including *p19*, *p21* and *PAK3*, involved in cell-cycle regulation, were upregulated. In addition, transcription factors activating *p21* expression such as *EGR1*, *HES6* and *SMAD3* were also upregulated following DAC treatment (Figure 5f, Table 2). Further, several tumor suppressor genes were activated following DAC treatment (Table 3). The pathway analysis shows direct and indirect interaction of transcription factors and tumor suppressor genes with *p21* (Figure 5f). Treating cells with doxorubicin alone did not cause significant changes in gene expression pattern (Figure 5e). Further, treatment time (48 h vs. 72 h) influence differential gene expression in DAC alone or sequentially treated cells with DAC and doxorubicin (Figure 5). Most noticeable observation was that in sequential treated cells for 72 h, more number of tumor promoter genes were downregulated in comparison to cells treated with DAC alone (Table 4).

Western Blot and Gene Expression

To validate our microarray results and to confirm the induction of p21, a critical regulator of the cell cycle, semiquantitative RT-PCR analyses were carried out using TaqMan probes. The expression level of p16, another cell-cycle regulator, whose expression is not altered by DAC, was used as a negative control. Consistent with our microarray results, DAC alone or its combination with doxorubicin in sequential treatment markedly increased the expression of p21 by >4 fold at 48 h and >10 fold at 72 h. Under these conditions, the expression of p16 was unaltered (Figure 6a). Furthermore, our immunoblotting results showed a significant increase in p21 protein expression in both DAC alone and in combination with doxorubicin in sequential treatment (Figure 6b and c).

DNMT1 Depletion

Treating drug-resistant cells with DAC caused depletion of DNMT1 at 24 h post treatment, but the effect was transient; the DNMT1 level returned to the level present in untreated cells at 2 days post treatment (Figure 7).

DISCUSSION

Chemotherapeutic strategies using a combination of anthracyclines with other cytotoxic anticancer drugs, acting via different pathways – so-called drug cocktails– have widely been used in clinical settings for cancer therapy. However, most such combination therapies produce additive rather than synergistic effects and also increase toxicity.^{70, 71} In addition, the risk that tumors will develop drug resistance and the difficulty in treating them once they do so remain pressing issues in cancer chemotherapy. Therefore, new and effective strategies to overcome drug resistance and to reduce drug-induced side effects are urgently needed. In this study, we show that epigenetic drugs, when used sequentially in combination with doxorubicin, induce a highly synergistic effect and overcome drug resistance of cells to a significant extent.

An interesting observation was the selective response of DAC to resistant MCF-7/ADR cells rather than sensitive MCF-7 cells. The resistant cells demonstrated a dose-response antiproliferative effect, but the sensitive cells were not significantly responsive to DAC treatment (Figure 1a vs. Figure 2a). This difference in response could be due to the hypermethylated state of DNA in resistant cells, suppressing the expression of tumor suppressor genes, which control many cellular functions, including proliferation. Following treatment with the epigenetic drug, these genes are re-expressed in resistant cells, and hence the effect of the epigenetic drug is more selective in resistant cells than in sensitive cells. For the same reason, the synergistic effect was much more pronounced in resistant cells than in sensitive cells (Table 1), signifying the role of epigenetic alternations in resistant cells and response to cancer chemotherapy.

The sequential treatment of DAC and doxorubicin was more effective than simultaneous treatment in resistant cells (Figure 1b and c). This difference could be due to the interference of doxorubicin on DNA polymerase activity⁷² when cells are treated simultaneously, thus inhibiting the incorporation of DAC into the DNA²⁵ and hence re-expression of tumor suppressor genes. This may not happen when DAC and doxorubicin are given sequentially. Recently, we demonstrated that DAC treatment alters the resistant cell membrane lipid composition and biophysical properties to favor increased doxorubicin influx and a cytotoxic effect.⁷³ For such an effect, resistant cells require pretreatment with DAC to induce changes in lipid biosynthesis, which may not occur in simultaneous treatment due to interference of doxorubicin in re-expression of genes, some of which may be responsible for the altered lipid biosynthesis.

Unlike DAC, which demonstrated a greater antiproliferative effect in resistant cells than in sensitive cells, SAHA treatment caused almost the same antiproliferative effect in both the resistant (MCF-7/ADR) and sensitive (MCF-7 cells; Figure 1a vs. 3a). The selective action of DAC in resistant cells thus indicates the role of DNA methylation in drug resistance.

A second interesting observation was the synergistic effect seen in resistant cells which had undergone sequential treatment, with SAHA first, followed by doxorubicin. In this case, treating resistant cells with SAHA for a shorter period of time (4 h) prior to doxorubicin treatment was more effective than prolonged pretreatment of cells with SAHA (Figure 3b and c). It is known that through histone acetylation, SAHA induces p21 expression and causes cells to undergo cell-cycle arrest.⁷⁴ Incubation of cells with SAHA for a brief period

of time could have relaxed the chromatin so that doxorubicin and its substrate Topo II might have better access to DNA than when increasing number cells undergo cell-cycle arrest with prolonged incubation time with SAHA. The other possibility could be that the chromatin relaxation could increase the accessibility of Topo I to the DNA to induce DNA replication^{27, 75}, thus counteracting the effect of doxorubicin. Sensitive cells demonstrated a synergistic effect, but the effect diminished with increased pretreatment time with SAHA. The resistant and sensitive cells thus showed the same pattern of reduced synergistic effect with increased pretreatment time with SAHA, but the effect was much more pronounced in resistant cells than in sensitive cells (Figure 3 d and e). It is clear that further studies are needed to understand the above difference in SAHA pretreatment in resistant and sensitive cells, particularly the difference in the histone acetylation patterns and genes reactivated following SAHA treatment in these cells.

DAC pretreatment overcomes drug resistance in breast cancer cells by depleting DNMT1 protein which is reported to relieve transcriptional repression of *p21*, thereby increasing its expression⁷⁶(Figure 7). DNMT1 depletion also indirectly plays a role in activation of *p21* via re-expression of methylation silenced transcription factors such as *EGR1*, *SMAD3* and *HES6* in resistant cells that directly interact with *p21* promoter^{41, 52, 76} and increase its expression in cancer cells. This suggests that *p21* might be a methylation-suppressed gene in drug-resistant cells. In addition to reactivation of *p21*, *EGR1* and *SMAD3* were also found to transcriptionally activate various genes which could negatively regulate the growth of cancer cells (Figure 5f). Induction of *p21* expression in cells treated with DAC (Figure 6a–c) cause cells to undergo G2/M arrest (Figure 4a–d). Topo II α levels are reported to be high in the G2/M phase,⁷⁷ therefore the arrest of cells in G2/M might result in increased accumulation of Topo II α . Doxorubicin utilizes Topo II α to induce DNA damage and achieve its cytotoxic effect.^{78, 79} This may explain the synergistic effect seen in our study following sequential treatment with DAC and doxorubicin. In addition, our data show sequential treatment causes downregulation of several tumor promoter genes (Table 4).

Despite the synergistic efficacy of sequential treatment of DAC and doxorubicin, we found that about 20% of resistant cells were not responsive to the sequential treatment even at higher doses of DAC and doxorubicin (Figure 1a). It is possible that these cells may be at different stages of the cell cycle than the proliferative phase (S-phase). DAC is a cytosine analog; hence it is recognized as a nucleotide by DNA polymerase and is incorporated in DNA strands during S-phase.²⁵ We intentionally did not synchronize cells to S-phase because in tumors, cells are at different stages of the cell cycle. The other possibility could be the instability of DAC in culture medium (half-life in culture medium = 17.5 h⁸⁰), which may have prevented a fraction of the slowly dividing cells from being exposed to DAC at doses and for the time period that is effective to cause epigenetic alterations.

The western blot results demonstrate that DNMT1 levels revert back to normal levels at day 2 post treatment with DAC solution (Figure 7), suggesting its transient effect due to its rapid degradation in culture medium, as mentioned above.⁸⁰ This reversion also raises the issue of translation of such epigenetic drugs for the treatment of solid tumors. Because of their short half-life, DAC and SAHA are not effective in providing enough of a therapeutic dose to solid tumors to induce expression of epigenetically silenced tumor suppressor genes. In addition to addressing the issue of their stability *in vivo* without causing toxicity,^{81, 82} the other key issue would be how to achieve precise sequential delivery of the drugs used. Although investigators are working on developing more stable analogs of the currently used epigenetic drugs or developing new epigenetic drugs that are more stable *in vivo*, the other option could be to develop an appropriate nanocarrier system that can target epigenetic drugs directly to the tumor. One could also potentially develop a nanocarrier system that can deliver the epigenetic drug and an anticancer agent in sequential order to achieve a

synergistic effect. We have previously shown that resistant cells treated with DAC restore endocytic functions,⁷³ which is critical for the efficacy of nanoparticle-based drug delivery system in resistant cells.

Despite the challenges, sequential treatment with an epigenetic drug followed by doxorubicin provides a highly promising approach for the treatment of breast cancer. Another advantage of the highly synergistic effect achieved is that one may need significantly lower doses of doxorubicin that can reduce its cardiac toxicity. More importantly, the sequential treatment can provide effective therapy for treating doxorubicin-resistant breast cancer.

It would be interesting to further analyze the effect of SAHA in resistant and sensitive cells, particularly the changes in gene expression profile and determine whether two epigenetic drugs in combination (DAC and SAHA) for pretreatment could be more effective in overcoming doxorubicin resistance than either DAC or SAHA. We have tested the efficacy of sequential treatment in drug-resistant breast cancer, but the issue also exists in other cancers that develop drug resistance. Drug resistance could be due to other reasons including genetic changes in cells, hence it is necessary to analyze tumors to ascertain that the drug resistance is due to epigenetic alterations and accordingly design a therapeutic strategy of sequential treatment to overcome drug resistance. This method may mean developing personalized therapy to effectively treat cancer patients.

CONCLUSIONS

The sequential treatment of epigenetic drug plus doxorubicin shows a highly synergistic effect in overcoming drug resistance in tumor cells. The synergistic effect depends on the epigenetic drug as well as duration of treatment and cell line. In general, the synergistic effect was more pronounced in resistant cells than in sensitive cells and DAC was more effective than SAHA in overcoming drug resistance. The combination therapy with the epigenetic drug and doxorubicin, when given in sequential order at optimal doses and duration, could provide an effective therapeutic strategy to overcome drug resistance that can potentially minimize doxorubicin-induced cardiotoxicity because lower doses of doxorubicin may be needed to achieve tumor regression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BCA	Bicinchoninic acid
CI	Combination Index
DAC	Decitabine (trade name Dacogen)
DMEM	Dulbecco's Modified Eagle's Medium
DNMT	DNA methyl transferase

Dox	Doxorubicin in solution
Doxil	Trade name of a liposome form of doxorubicin
DPBS	Dulbecco's phosphate-buffered saline
ECL	Electrochemiluminescence
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IC₅₀	Inhibitory concentration required for 50% cell death
PBS	Phosphate-buffered saline
PI	Propidium Iodide
PVDF	Polyvinylidene difluoride
SAHA	Suberoylanilide hydroxamic acid (generic name vorinostat)
TBST	Tris-buffered saline with 0.5% Tween 20
Topo I	Topoisomerase I
Topo II	Topoisomerase II

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Figure 1. Synergistic effect of sequential treatment with DAC and Dox in drug-resistant breast cancer cells

a) Effect of DAC alone treatment and sequential treatment with different doses of DAC and Dox on cell growth in resistant breast cancer (MCF-7/ADR) cells. For sequential treatment, resistant cells were treated with different doses of DAC for 24 h prior to treatment with different doses of Dox. Data points in Figure 1a: Black, Dox alone; Orange, DAC alone (arrow). Sequential treatments: Red; cytotoxicity of Dox at different doses in cells pretreated with DAC at 10 ng/mL; Green 25 ng/mL; Blue 50 ng/mL, Sky blue 75 ng/mL and Magenta 100 ng/mL. **b)** Comparison of sequential and simultaneous treatment of DAC and Dox in MCF-7/ADR cells. For sequential treatment, cells were pretreated with DAC (50 ng/mL) for 24 h and then with varying doses of Dox for 48 h. For simultaneous treatment, cells were treated with DAC (50 ng/mL) and different doses of Dox mixed together. **c)** Combination index (CI) for sequential and simultaneous treatments in MCF-7/ADR cells. CI Value: < 1 Synergistic; = 1 Additive; and > 1 Antagonistic. Data are expressed as mean $\pm$ s.e.m., $n = 6$. Error bars are within symbols for certain data points.

Figure 2. Synergistic effect of sequential treatment with DAC and Dox in drug-sensitive breast cancer cells

Effect of sequential treatment in **a)** MDA-MB 231; **b)** BT-459; **c)** MCF-7. **d)** Combination index for sequentially treated drug-sensitive breast cancer cells. The sequential treatment DAC and Dox demonstrates the synergistic effect in MDA-MB 231 and MCF-7 cells, but additive to moderate synergism in BT-459 cells. Data are expressed as mean $\pm$ s.e.m., $n = 6$. For certain data points, error bars are smaller than symbol size, hence are not visible.

Figure 3. Synergistic effect of sequential therapy with SAHA and Dox in drug-resistant and -sensitive breast cancer cells

a) Dose-response effect of cytotoxicity of SAHA in drug-resistant (MCF-7/ADR) and -sensitive (MCF-7) cells. IC_{50} of SAHA in MCF-7/ADR and MCF-7 was $2 \mu\text{M}$ and $0.75 \mu\text{M}$, respectively **b)** Effect of pretreatment time with SAHA on IC_{50} of Dox in MCF-7/ADR cells. Cells were pretreated with SAHA ($0.5 \mu\text{M}$) for different time points prior to Dox treatment. **c)** Combination index for sequential treatment with SAHA and Dox in MCF-7/ADR cells. **d)** Effect of pretreatment time with SAHA on IC_{50} of doxorubicin in MCF-7. Cells were pretreated with SAHA ($0.25 \mu\text{M}$) for different time points prior to Dox treatment **e)** Combination index for sequential treatment with SAHA and Dox in MCF-7 cells. Data are expressed as mean $\pm$ s.e.m., $n = 6$.

Figure 4. Cell-cycle analysis of sequentially treated drug-resistant and -sensitive cells
a) Cell-cycle analysis of MCF-7/ADR cells treated with DAC alone, Dox alone, and sequentially treated, first with DAC for 24 h followed by with doxorubicin for 48 or 72 hrs. DAC alone or its combination with Dox showed an increase in cells arrested at the G2/M phase as compared to cells left untreated or treated with Dox alone. Dose of DAC= 50 ng/mL, dose of Dox = 1 ng/mL. **b)** Quantification of MCF-7/ADR cells arrested in the G2/M phase of the cell cycle using ModFit LT software. **c)** Cell-cycle analysis of MCF-7 cells treated with DAC alone, Dox alone and sequentially with DAC and Dox. **d)** Quantification of MCF-7 cells arrested at the G2/M phase using ModFit LT software. Representative histograms from three independent experiments.

Figure 5. Microarray analysis of sequentially treated drug-resistant MCF-7/ADR cells

The RNA isolated from cells sequentially treated with DAC (50 ng/mL) and doxorubicin (Dox) (1 ng/mL) or from its respective controls treated for 48 or 72 h were subjected to microarray analysis. Volcano plots for each contrast was computed using R statistical package. Volcano plots show significantly expressed genes vs. fold change of the pair-wise comparisons made between different groups. Negative log₁₀ p value on y axis indicates the significance of each gene and the fold change (log base2) mean expression difference on x axis. The dashed and solid lines is the significance threshold for selecting the differentially expressed genes at FDR p of 0.05 and raw p value of 0.001. In each volcano plot, genes downregulated were plotted on negative x axis scale and the upregulated genes were plotted on the positive side of the x axis scale. Genes that satisfy the statistical significance is shown in red, rest of the genes were shown as blue circles. **a)** Volcano plot showing differentially expressed genes in resistant cells treated with DAC in comparison to untreated cells for **a)** 48 h **b)** 72 h. Comparison of gene expression in resistant cells treated with DAC or DAC + Dox for **d)** 48 h. **d)** 72 h. **e)** The heat map shows the effect of DAC alone, Dox alone and sequentially treated with DAC and Dox for 48 h or 72 h on global gene expression in resistant breast cancer cells. Intensity of red indicates relatively high expression relative to the sample mean and blue indicates relatively low expression. Comparison of DAC vs. DAC + Dox shows similar gene expression pattern. Dox and untreated cells shows similar expression pattern. **f)** Network showing the direct and indirect interaction of p21, DNMT1 with other tumor suppressor genes. Network also shows downregulation of some of the genes which could positively play a role in cell proliferation. Red and blue color circles indicate genes upregulated and downregulated, respectively.

Figure 6. Semiquantitative RT-PCR analyses of p21

Expression of p16 was used as a negative control. **a)** DAC alone or its combination with Dox increased the expression of p21 mRNA. Data are expressed as mean $\pm$ s.e.m, $n = 4$ $**p < 0.0005$, $*p < 0.005$ untreated vs. DAC or DAC + Dox. **b)** Expression of p21 protein analyzed by western blot in drug-resistant cells in different treatment groups. p21 showed increased expression in cells treated with DAC alone and sequentially with DAC and Dox. Actin served as a loading control. Representative results from two separate experiments. **c)** Quantification of western blot data using Image J analysis software.

Figure 7. Effect of DAC treatment on DNMT1 protein expression in drug-resistant cells
a) Analysis of DNMT1 protein expression by western blot in MCF-7/ADR cells treated with DAC. **b)** Quantification of western blot data using Image J analysis software. DAC depleted DNMT1 for 24 h. No difference in DNMT1 levels was observed with respect to untreated cells in the samples collected at 2, 3 and 5 days post treatment with DAC.

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Table 1
Cytotoxicity of doxorubicin and combination treatment with decitabine and Doxorubicin.

Human breast cancer cell line	IC ₅₀ (ng/mL)			
	Dox		DAC + Dox	
	Sequential		Simultaneous	
MCF-7/ADR	7385.2 ± 698	<1	1825.8 ± 62.8	0.2 ± 0.1
MCF-7	20.7 ± 0.8	7.4 ± 1.3	ND	0.8 ± 0.1
MDAMB -231	19.3 ± 1.9	4.4 ± 0.1	ND	0.3 ± 0.1
BT 459	102.2 ± 4.7	25.0 ± 5.5	ND	0.6 ± 0.1

Data are expressed as mean ± s.e.m. from three individual experiments.
Decitabine concentration in sequential and simultaneous treatment = 50 ng/mL.
ND = Not done, Dox=Doxorubicin, DAC=Decitabine.

Table 2

Differentially expressed genes involved in key pathways/processes in resistant breast cancer (MCF-7/DR) cells following treatment with decitabine for different time periods.

Differentially expressed genes in resistant cells treated with DAC		
Key pathways/ Processes		
	48 h	72 h
APOPTOSIS	<i>CDKN1A, 1BCL2, CRR9, CTSL, H2-ALPHA, LOC112714, MAEL, MGMT, PLAUR, PNMA5, PRKCA, TUBA2, UBB</i>	<i>CCK, CDKN1A, CDKN2C, CDKN2D, CIDEA, CRR9, CTSL, FOLR1, GADD45B, G1P3, HIST1H1C, HIST1H2BD, H2-ALPHA, LOC112714, LGALS7, MAEL, MGMT, MMP9, NES, PHLDA2, PLAUR, PNMA5, RAC1, RHOB, SGK, TEK, TUBA2, UBB, VAV2</i>
CELL ADHESION	<i>CCL20, CELSR3, CST6, FZD9, H2-ALPHA, LOC112714, MAG, PRKCA, TUBA2, TUBB3, WNT5A</i>	<i>CCL20, CD99L2, CELSR3, CLDN23, CST6, FZD9, H2-ALPHA, HMMR, LOC112714, LGALS7, MAG, PRKCA, RAC1, RHOB, TEK, TUBA2, TUBB3, VWF, WNT5A, WNT5B</i>
CELL CYCLE	<i>CDKN1A, DPEP3, FES, H2-ALPHA, HSPA2, LOC112714, MAEL, PRKCA, RAGE, TUBA2, TUBB3, UBB, ZFP42</i>	<i>CTAG1A, CDKN1A, CDKN2C, CDKN2D, DPEP3, EGRI, FBXO6, FES, GADD45B, HSPA2, HIST1H1C, H2-ALPHA, LOC112714, MAEL, MYH3, PIWIL2, PRKCA, RAGE, RHOB, SMAD3, TEX15, TUBA2, TUBB3, UBB, ZFP42</i>
CELL CYCLE ARREST AT G2M PHASE	<i>CDKN1A, HSPA2, HES6</i>	<i>CDKN1A, CDKN2C, CDKN2D, HSPA2, HES6, NES, SMAD3</i>
CELL DIFFERENTIATION	<i>BCL2, CELSR3, DAZL, FZD9, HES6, HSPA2, IL11, KRT14, KRT17, MAEL, MGMT, MMP9, NIBP, NNAT, PLAUR, S100A4, SOX15, SOX18, SPANXB1, TAF7L, WNT5A, ZFP42</i>	<i>ATF5, BAMBI, C9ORF157, CCK, CDKN2C, CELSR3, DAZL, CSRP2, EFN3, EGRI, FGFR3, FZD9, GADD45B, GALR2, LGALS7, HEG1, HES6, HSPA2, IFITM1, KRT17, MAEL, MGMT, MMP9, NIBP, NNAT, OTP, PAX8, PIWIL2, PLAUR, RAC1, RHOB, SERPINE2, SOHLH1, SOX15, S100A4, SLC7A5, SOX18, SPANXB1, TAF7L, TDRD9, TEK, VAMP5, VAV2, ZFP42, WNT5A</i>
CELL MOTILITY	<i>BMPR2, CELSR3, CST6, MMP1, PRKCA, S100P, SOX18</i>	<i>BAMBI, CCK, CELSR3, CST6, IFITM1, MMP1, MMP9, PS G2, RAC1, S100P, SGK, SLC7A5, SOX18, TEK, TFF2, VAV2</i>
NEGATIVE REGULATION OF CELL PROLIFERATION	<i>CDKN1A, CST6, IL6, PRKCA, SOCS1</i>	<i>ALDH1A2, CDKN1A, CDKN2C, CDKN2D, FGFR3, FGFBP1, IGFBP6, IFITM1, IL6, PRKCA, PTGES, SMAD3, SERPINE2, SOCS1, WARS</i>
NOTCH SIGNALING	<i>CDKN1A, HES6, FZD9, MIB2, MFNG, MFAP5, MIB2, UBB, WNT5A</i>	<i>CDKN1A, MFAP5, MIB2, SMAD3, UBB, WNT5A, WNT5B</i>
IMMUNE RESPONSE	<i>CCL20, CDKN1A, CTS L, FZD9, HSPA2, IL6, IL11, KCNN4, MMP1, PRKCA, UBB</i>	<i>C4BPB, CCL20, CD79B, CDKN1A, CTSL, EGRI, FZD9, G1P2, G1P3, HLA-B, IFI30, IFITM1, IL6, IRF7, MMP1, NCR3, PRKCA, PYCARD, RAC1, SLC7A5, TFF2, UBB, ULBP1</i>
RESPONSE TO STRESS	<i>BMPR2, CCL20, CDKN1A, CTSL, GAGE8, HSPA2, IL11, KCNN4, MAEL, MC1R, MGMT, MMP1, PAGE1, PLAUR, PRKCA, SOX15, UBB</i>	<i>C1ORF24, C4BPB, CBARA1, CCK, CCL20, CDKN2D, CIDEA, CTSL, EGRI, G1P2, G1P3, GADD45B, GAGE8, FAM129A, HCP5, HIST1H2BK, HSPA2, HSPB6, IDH2, IFI30, IFITM1, MAEL, MC1R, MGMT, MMP1, MMP9, NCR3, PAGE1, PLAUR, RAC1, RHOB, SGK, SLC7A5, TEK, TEX15, TFF2, UBB, VAV2, VWF</i>
SIGNAL TRANSDUCTION	<i>BMPR2, CCL20, CDKN1A, CELSR3, CENTG2, FZD9, IL11, KCNN4, KRT17, MC1R, PLAUR, PRKCA, RASD1, UBB, SOCS1, KCNH3, KCNS1, NNAT</i>	<i>ADM2, BAMBI, CCK, CCL20, CD79B, CELSR3, CIDEA, DEFL1, DUSP5, EFN3, EGRI, FGFBP1, FZD9, G1P2, G1P3, GADD45B, GALR2, IFI30, IFITM1, KCNS1, KRT17, MC1R, NNAT, OTEX, PLAUR, RAC1, RASL11B, RHOB, S100A4, SOCS1, TEK, TFF2, UBB, VAV2</i>
DNA DAMAGE CHECKPOINT	<i>CDKN1A, TAF7L, UBB</i>	<i>GADD45B, TAF7L, UBB, CDKN1A</i>

Table 3

Tumor suppressor genes upregulated following decitabine treatment for 48 or 72 h in resistant breast cancer (MCF-7/ADR) cells.

No	Gene Symbol	Entrez Gene I.D	Description	Sub cellular location	Function	Comments
1	<i>CD19</i>	930	CD19 molecule	Plasma Membrane	Assembles with antigen receptor of B Lymphocytes	Expression leads to growth inhibition and reduced tumorigenicity in human multiple myeloma ²⁹
2	<i>CSR2</i>	1466	Cysteine and glycine rich protein-2	Nucleus	Development and cell differentiation	Acts as a repressor for NF-kappa B to suppress tumorigenesis and angiogenesis ³⁰
3	<i>CST6</i>	1474	Cystatin E/M	Extracellular, secreted protein	Physiological inhibitors of lysosomal cysteine proteases	Significantly reduces cell proliferation and migration ^{31,32}
4	<i>CRMP3</i>	10570	Dihydropyrimidinase-like 4	Cytoplasm	Cell signaling and cytoskeleton remodeling	Direct downstream target of p53 on apoptosis. Apoptosis inducible factor in response to DNA damage ³³
5	<i>DUSP4/MKP2</i>	1846	Dual specificity phosphatase 4	Nucleus	Negatively regulates Mitogen activated protein (MAP) kinase	Inhibits MAP kinase signaling and thereby cell proliferation. Tumor suppressor in gliomas ³⁴
6	<i>DUSP5</i>	1847	Dual specificity phosphatase 5	Nucleus	Negatively regulates Mitogen activated protein (MAP) kinase	Direct transcriptional target of p53 to deactivate MAP - kinase pathway and thereby inhibits cell proliferation ³⁵
7	<i>ECRG4/C2orf 40</i>	84417	Chromosome 2 open reading frame 40	Secreted protein	Probable hormone	Putative tumor suppressor gene in various cancers including breast cancer ³⁶
8	<i>EGR1</i>	1958	Early growth response 1	Nucleus	Transcriptional regulator	Functions as tumor suppressor via regulation of p53 in skin carcinoma (ref). Direct regulator of multiple tumor suppressors including TGFbeta1, p53, p21 and PTEN ³⁷
9	<i>HES6</i>	55502	Hairy and enhancer of split 6	Nucleus	cell differentiation	Inhibits cell proliferation through induction of p21 ³⁸
10	<i>H19</i>	283120	H19, imprinted maternally expressed transcript (non-protein coding)	Nucleus and Cytoplasm	Acts as translational repressor	Functions as a tumor suppressor in various cancers ³⁹
11	<i>IBP6</i>	3489	Insulin like growth factor binding protein 6	Secreted	Binds to IGF-I and -II with high affinity and specificity	Activates programmed cell death in non-small cell lung cancer cells both in vitro and in vivo ⁴⁰

No	Gene Symbol	Entrez Gene I.D	Description	Sub cellular location	Function	Comments
12	<i>IF127</i>	3429	Interferon, alpha-inducible protein 27	Plasma membrane and mitochondria	Promotes cell death	Mediates interferon induced apoptosis characterized by release of cytochrome c, and activation of BAX and caspases - 2,3,6,8 and 9. Also sensitizes cells to other stimulus such as etoposide ⁴¹
13	<i>LGALS7</i>	3963	Lectin, galactoside-binding, soluble, 7	Cytoplasm and nucleus	Involved in cell- cell and/or cell-matrix interactions	Suppresses angiogenesis and tumor growth ⁴²
14	<i>LXN</i>	56925	Latexin	Cytoplasm	Endogenous carboxypeptidase inhibitor	Inhibits lymphoma cell proliferation and proliferation of gastric carcinoma in vitro and in vivo ^{43,44}
15	<i>MTIF</i>	4494	Metallothionein 1F	Cytoplasm and nucleus	Binds various heavy metals	Putative tumor suppressor gene in colon carcinogenesis ⁴⁵
16	<i>MT1G</i>	4495	Metallothionein 1G	Cytoplasm and nucleus	Binds various heavy metals	Reported as tumor suppressor gene in hepatocellular carcinoma ⁴⁶
17	<i>p21</i>	1026	Cyclin-dependent kinase inhibitor 1A	Cytoplasm and nucleus	Binds to and inhibits cyclin-dependent kinase activity	Tumor suppressor, induces cell cycle arrest in cancer cells ⁴⁷
18	<i>RASD1</i>	51655	RAS, dexamethasone-induced 1	Plasma membrane	Regulates cell growth and cell differentiation	Inhibits cell proliferation of G418 resistant MCF 7 breast cancer cells, lung adenocarcinoma A549 and murine fibroblasts (NIH 3T3) cells in vitro ⁴⁸
19	<i>SMAD3</i>	4088	SMAD family member 3	Cytoplasm and nucleus	Signal transducer and transcriptional regulator	In gastric cancer cells, SMAD3 expression restored the expression of TGF- β , p21 and p15, thereby inhibits cancer growth ⁴⁹
20	<i>SOC31</i>	8651	suppressor of cytokine signaling 1	Intracellular, nucleus	Protein kinase inhibitor, negative regulator of JAK/STAT pathway	Functions as tumor suppressor gene in various cancers including breast carcinomas ⁵⁰⁻⁵²
21	<i>THY1</i>	7070	Thy-1 cell surface antigen	Plasma membrane	Cell- cell adhesion and cytoskeletal organization	Suppresses the growth of human ovarian cancer and nasopharyngeal carcinoma ^{53, 54}

Table 4

Tumor promoter genes downregulated in resistant breast cancer (MCF-7/ADR) cells following sequential treatment with decitabine and doxorubicin with reference to decitabine alone treated cells for 72 h.

No	Gene Symbol	Entrez Gene ID	Description	Sub cellular location	Function	Comments
1	<i>CCL20</i>	6364	Chemokine (C-C motif) ligand 20	Secreted	Chemoattractant of CCR6 bearing dendritic cells	Promote cell proliferation and metastasis. Also plays a role in organ specific metastasis of colorectal cancer ⁵⁵
2	<i>CTSL</i>	1514	Cathepsin L1	Lysosome and extracellular	Protein degradation and extracellular matrix (ECM) degradation	Due its ability to degrade ECM over a wide range of pH, it plays a key role in metastasis of tumor cells ⁵⁶
3	<i>HDGFL1</i>	154150	Hepatoma derived growth factor like 1	Secreted	Exogenous mitogen	Involved in anchorage independent growth, invasion and angiogenesis of non-small cell lung cancer cells ⁵⁷
4	<i>HSPA2</i>	3306	Heat shock 70kDa protein 2	Cytoplasm and nucleus	Molecular chaperone for CDC2	Promotes tumor promotion, migration and invasion ⁵⁸
5	<i>LOC342897</i>	342897	Non-specific cytotoxic cell receptor protein 1 homolog (zebra fish)	Intracellular	Ubiquitin ligase	Enhances cell proliferation of various cancers ⁵⁹
6	<i>MAP7</i>	9053	Microtubule-associated protein 7	Cytoplasm	Stabilize microtubules	Higher expression of MAP7 was associated with poor prognosis. Plays a role in cell division, motility and cell shape ⁶⁰
7	<i>MMP9</i>	4318	Matrix metalloproteinase 9	Extracellular, secreted protein	Proteolysis of ECM	Tumor invasion and metastasis ⁶¹
8	<i>NNAT</i>	4826	Neuronatin	Endoplasmic reticulum	Regulates intracellular calcium levels in dendrites	Increases cell proliferation and decreases patient survival in Glioblastoma Multiforme ⁶²
9	<i>NMB</i>	4828	Neuromedin B	Extracellular, secreted protein	Involved in various physiological functions: exocrine and endocrine secretions, smooth muscle contraction	Angiogenesis and growth of malignant tumor cells ⁶³
10	<i>RPL10L</i>	140801	Ribosomal protein L10-like	Intracellular, cytoplasm and ribosome	Could play a role in sperm differentiation and maturation	Possibly enhance ribosomal activity and/or translational activity in ovarian cancer ⁶⁴
11	<i>STMN3</i>	100174566	Stathmin-like 3	Golgi apparatus	Microtubule depolymerization	Induce cell proliferation, migration and matrix invasion of adenocarcinoma and squamous cell carcinoma ⁶⁵
12	<i>TKTL1</i>	8277	Transketolase-like 1	Cytoplasm and nucleus	Transketolase metabolism	Enhances oxygen independent glucose metabolism and lactate based matrix degradation to favor tumor invasion and metastasis ⁶⁶