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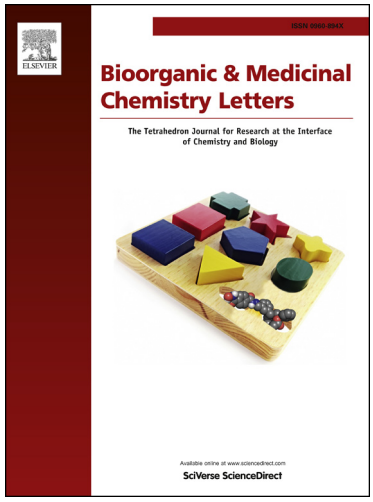

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PII: S0960-894X(13)01067-6
DOI: http://dx.doi.org/10.1016/j.bmcl.2013.09.004
Reference: BMCL 20856

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 4 June 2013
Revised Date: 10 August 2013
Accepted Date: 3 September 2013

Please cite this article as: Sangthong, S., Ha, H., Teerawattananon, T., Ngamrojanavanich, N., Neamati, N., Muangsin, N., Overcoming doxorubicin-resistance in the NCI/ADR-RES model cancer cell line bynovel anthracene-9,10-dione derivatives, *Bioorganic & Medicinal Chemistry Letters* (2013), doi: http://dx.doi.org/10.1016/j.bmcl.2013.09.004

Title:

Overcoming doxorubicin-resistance in the NCI/ADR-RES model cancer cell line by novel anthracene-9,10-dione derivatives

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ABSTRACT

Overcoming drug resistance with remarkable cytotoxic activity by anthracene-9,10-dione derivatives would offer a potential therapeutic strategy. In this study, we report the synthesis and the cytotoxicity of a novel set of anthraquninones. *(4-(4-Aminobenzylamino)-9,10-dioxo-9,10-dihydroanthracen-1-yl-4-methylbenzenesulfonate)* (**3**) has excellent *in vitro* cytotoxicity against doxorubicin-resistant cancer cell line (IC_{50} = 0.8 μM), 20-fold higher than doxorubicin. The cytotoxic effect via G2/M arrest does not appear to be ROS.

Keywords: doxorubicin-resistance, cytotoxic mechanism, anthraquinone derivatives

Doxorubicin, or hydroxyl daunorubicin (Fig. 1a) is a broad-spectrum chemotherapeutic agent that has been widely used in the clinics for various cancers.[1] As with many other anthraquinone derivatives, doxorubicin inhibits DNA synthesis by intercalating DNA, inducing p53 and oxidative stress.[2-4] Unfortunately, many cancer cell types that have high expression of drug efflux transporters, such as P-glycoprotein are resistant to doxorubicin and many of its analogues.[5] Although increasing the drug dosage is an effective strategy to overcome drug resistance, it typically leads to increased side effects including hypotension and cardiac arrhythmia.[6]

Since the development of drug resistance is a major impediment towards the successful treatment of various cancers, it has encouraged the development of novel chemotherapeutics that can overcome chemoresistance to drugs such as doxorubicin. Anthraquinones (Fig. 1b) have been commonly used as a pharmacophore for the treatment of various diseases including cancer. The anthraquinone derivative, emodin (Fig. 1c), has been reported to suppress the proliferation of various cancer cells.[7-9] Co-treatment of emodin with a number of anticancer drugs was shown to be effective against a multidrug resistance cell line, working as a reactive oxygen species (ROS) generator.[10-13] In addition to ROS generation, emodin targets a number of cancer pathways and molecular targets such as tyrosine kinases, phosphoinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and NF-kappaB.[7] Moreover, novel anthraquinone platinum derivatives have shown improved *in vitro* cytotoxic activity in tumor cell lines representative of human breast (A2780 and A2780cisR) and breast cancers (T47D).[14]

Considering the antitumor effects of anthraquinones and drug resistance of cancer cells to anthraquinone-based chemotherapies such as doxorubicin, we sought to design and discover new anthraquinone derivatives that show anti-proliferative properties in chemo-resistant cancer cells. In our study, we present a novel series of anthraquinones that can overcome drug resistance. One of these new derivatives, *4-(4-aminobenzylamino)-9,10-dioxo-9,10-dihydroanthracen-1-yl 4-methylbenzenesulfonate* (compound **3**) inhibits cell proliferation by arresting G2/M cell cycle in doxorubicin-resistant cell (NCI/ADR-RES).

Figure 1

To investigate the effects of both asymmetric and symmetric substituents at the 1- and/or 4-position on cytotoxicity and anticancer properties, 1,4-dihydroxy anthraquinone was modified according to the procedures shown in Scheme 1. Two hydroxyl groups of 1,4-dihydroxy anthraquinone were substituted with tosyl chloride (TsCl) to obtain compound **1** as an intermediate.[21] The 4-position of compound **1** was substituted with either a series of benzylamine or glycols. The modifications were made based on the aromatic/aliphatic substituent groups (i) a group of aromatic ring including benzylamines, bearing -Cl, -NH_2 and –OMe at 4-position of the bezylamine ring to provide **2**-**4** and (ii) a group of aliphatic chain of di, tri, tetra-ethylene glycol subsituents to provide **5**-**7**, respectively. Finally, the 1-position of compound **5** & **6** was attached with di-ethylene (**8**) and tri-ethylene glycol (**9**),

respectively in dry acetonitrile followed by treatment of K_2CO_3. The purity and structural assignments of synthesized compounds were determined by ^{1}H-NMR, ^{13}C-NMR, and MS spectral analysis (shown in the Supplementary data A).

Scheme 1

To assess the anti-proliferative properties of our newly synthesized compounds, a panel of human cancer cell lines derived from different tumor origins was treated with 20 μM of compounds **2**-**9** and doxorubicin as a reference. Cell proliferation was measured using MTT as reported.[22] The panel of cancer cell lines include pancreatic (BxPC3, MiaPaCa-2), prostate (DU145, PC-3, LNCaP), ovarian (CAOV3, OVCAR-8), breast (NCI/ADR-RES), osteosarcoma (U2OS), and colorectal (HCT116) cancer cell lines.

Table 1

Doxorubicin at 20 μM showed excellent cell proliferation inhibition in all cell lines except in NCI/ADR-RES (41% inhibition). NCI/ADR-RES is a resistant breast cancer cell that over-express multidrug resistance transporter, MDR1, conferring resistance to various chemotherapies including doxorubicin.[15] Among the eight new anthraquinone derivatives, only three asymmetric subsituents bearing the substituted benzylamine (**2** (-Cl), **3** (-NH_2), and **4** (-OCH_3)) at 1-position of anthraquinone inhibited cell proliferation more than 50% at 20 μM in all cancer cell lines tested except PC-3 for **4**. Especially, in the NCI/ADR-RES cell line was inhibited nearly 80% (Table 1). However, another asymmetric or symmetric subsituents (**5-9**) that were substituted with long glycol chains at the only 1-position or both 1,4-positions were not as active as the benzylamine containing compounds, inhibiting less than 50% cell proliferation in most cell lines tested. These data demonstrates that the benzylamine substituents at the 4-position of the quinone core is important for activity, while compounds **5-9** with flexible long glycol chain were inactive perhaps due to steric effect.

In comparison, asymmetric anthraquinones (**2-4**) with different substituent groups on the 4-position of benzylamine, namely Cl, NH_2, and OCH_3 showed similar activity; compound **3** inhibited cell proliferations with IC_{50} values within 0.4-6.5 μM and compounds **2** and **4** also inhibited cell proliferation with IC_{50} values within 0.6-8.0 μM against almost all cell lines except PC-3 for compound **2** and H1299 for compound **4** (Table 2, Figure 2B). This data suggests that not only the benzylamine at 4-position of anthraquinone play an important role in cytotoxic activity, but the substituent groups (-Cl, -NH_2, -OCH_3) at 4-position of the bezylamine does not seem to affect the compounds' activity.

We also investigated the level of colony formation which was proportional to assess the effects of cytotoxic agents on the NCI/ADR-RES cancer cell growth *in vitro* as descripted.[20,23] The colony numbers were suppressed after treatment of compounds **2-4** at 24 h as dose dependence (Figure 2A). Surprisingly, compound **3** at concentration of 1μM significantly inhibited the colony-

forming abilities (Figure 2C) with IC_{50} ranging from 0.5-1 μM compared to its level of inhibition of untreated control culture. The colony forming result was correlated with MTT (IC_{50} = 0.8 μM). Our result indicates that compound **3** affects the cell's reproduction ability to form progenies in NCI/ADR-RES cell leading cell death. The advancements of inhibiting colony formation assay has been made for benefit for cancer therapy in recent years with combination of previous individual modalities such as radiation and chemotherapy.[28] Thus compound **3** as the most active compound was chosen for the future experiment.

Figure 2 and Table 2

To test the hypothesis that compound **3**-induced inhibition of colony formation is mediated by modulation of the expression of cell cycle. Flow cytometry was used to examine the effects of compound **3** on cell cycle progression in NCI/ADR-RES cell. The analysis of DNA profiles is classified into three phases based on linear fluorescence intensity after staining with propidium iodide; the large initial peak (left) represent cell in G0/G1, the intervening area represent cells in S phase and the final tail/small peak (right) represent cell in G2/M. The percentage of cells in G0/G1 phase decreased from 51% to 30%, while in G2/M increased from 35% to 55% in NCI/ADR-RES after treatment of compound **3** at 10 μM as shown in Figure 3A. In our observation Compound **3** inhibited cell proliferation by arresting G2/M phase**.** This data is consistent with previously reported anthraquinone derivatives such as an emodin that also arrest cancer cells in the G2/M phase.[16-17]

Glutatione is an important pathway by which the dominant pro-oxidant molecule such as hydrogen peroxide, detoxified. Some transporters for substrate transport including multidrug resistant proteins (MRPs)[30] involved with the biotransformation and detoxification of cells develop resistance to chemotherapeutic drugs.[31] Reduced glutathione becomes the most abundant intracellular thiol which acts as a major antioxidant by protecting the cells against the damaging effects of free radicals and reactive oxygen species (ROS).[32] Anthraquinone, including emodin and doxorubicin, has been reported to induce ROS-mediated apoptosis.[11-13] Therefore; we sought to determine the role of ROS on our compounds' activity. NCI/ADR-RES cells were pretreated with antioxidants, N-acetylcysteine (NAC) or glutathione (GSH) at 5 mM for 2 hours, prior to the addition of compounds **2**-**4** at 20 μM based on %inhibition of DOX as positive control. Pretreatment with NAC or GSH decreased the effectiveness of doxorubicin (from 32% to 13% and 21%, respectively, Figure 4) while compounds **2**-**4** still showed cytotoxicity more than 60%ihibition. Similar results also observed in OVACAR-8 as the DOX-sensitive cells after treatment with compounds **2-4**, while DOX's activity was higher than un-pretreatment with antioxidants (Supplementary data B). Our observation is that pretreatment with antioxidants did not decrease the activity of compounds **2**-**4**. This result indicates that the anthraquinones's mechanism may interfere with glutathione transferase activity leading to death of NCI/ADR-RES[33] or it may not be ROS-mediated as observed with doxorubicin.

Figure 3

Since doxorubicin-induced apoptosis in response to DNA damage is p53-mediated, we also assess whether the cytotoxic effects of compound **3** is also p53-mediated.[18] Because OVACAR-8 and NCI/ADR-RES are carrying a p53 mutant,[26-27] we assess caspase activation since it is downstream of p53 induction in response to DNA damage using LNCaP with wild-type p53 as a representative cell. Using Western blot,[25] we assessed the protein levels of p53 and caspase 9 upon treatment of doxorubicin and compound **3** using GAPDH as a loading control. Doxorubicin significantly up-regulated p53 and cleaved caspase-9 at 3 μM, thereby activating it, in LnCaP cells, while compound **3** at the same concentration did not alter p53 but slightly activated caspase-9, suggesting the cytotoxic effects of compound **3** is not p53-mediated (Figure 4). This is further supported by our MTT results that show no comparable difference in activity in p53 wild type (U2OS) and p53 null (PC3 and H1299) cell lines (Table 1).

Figure 4

We have discovered a new class of anthraquinone derivatives that inhibit cancer cell proliferation in the low micromolar range. We have shown compound **3** can overcome drug resistance in NCI/ADR-RES, with IC_{50}'s 20 times greater than doxorubicin. Interestingly, compound **3**'s cytotoxic effect does not appear to be ROS or p53-mediated suggesting its mechanism of action may be different from previously reported doxorubicin and emodin. Further studies are required to further assess the precise mechanism of action, perhaps targeting potential new anticancer targets. Additionally, these new compounds may be beneficial for the treatment of resistant cancer or used in adjuvant therapy with other anticancer agents.

Acknowledgements

We thank the Royal Golden Jubilee Ph.D. Program Fellowship (PHD/0075/2553) to S.S., and the Advanced Materials, Chulalongkorn University, the Integrated Innovation Academic Center: IIAC Chulalongkorn University Centernary Academic Development Project (CU56-FW10) to N.M. and This work was partially supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (FW657B), and the Center of Innovative Nanotechnology (CIN) for funding

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22. Cells were maintained as monolayer cultures in RPMI 1640 supplemented with 10% fetal bovine serum (Gemino-Bioproducts, Woodland, CA) and 2 mM L-glutamine at 37 °C in a humidified atmosphere of 5% CO_2. To remove the adherent cells from the flask for passaging and counting, cells were washed with phosphate buffered saline (PBS) without calcium or magnesium, incubated with a small volume of 0.25% (w/v) trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO) for 5-10 minutes, then washed with culture medium and centrifuged. All experiments were performed using cells in exponential growth. Cells were routinely checked for *Mycoplasma* contamination using a reporter based assay (Invivogen, San Diego, CA).
23. Cells were plated in 12-well plates at a density of 100 cells / well and allowed to attach. The next day, serial dilutions of the corresponding compounds were added and allowed to incubate for 24 h. After exposure, cells were washed in PBS and cultured in free media until colonies were formed (8-10 days). Cells were subsequently washed, fixed in 1% (w/w) glutaraldehyde for 30 min, stained with 2% (w/v) crystal violet for 30 min, and then thoroughly washed with water. Colonies were imaged on the Versa Doc Imaging System (Bio-Rad) and counted using the Quantity One software package (Bio-Rad). The data reported represent means of at least three independent experiments.
24. Cell cycle perturbations were analyzed by propidium iodide DNA staining. Briefly, exponentially growing cells were treated with different doses of 3 for 24 h. At the end of each treatment time, cells were collected and washed with PBS after a gentle centrifugation at 3,000 rpm for 5 mins.

Cells were thoroughly resuspended in 0.5 mL of PBS and fixed in 70% ethanol overnight at -20 C. Ethanol-resuspended cells were then centrigued at 3,000 rpm for 5 min and washed twice in PBS to remove residual ethanol. For cell cycle analysis, the pellets were resuspended in 1 mL of PBS containing 0.02 mg/mL of propidium idodide, 0.05 mg/mL of DNase-free RNase A and incubated for 2 h in dark condition. Cell cycle profiles were obtained using BD LSRII flow cytometer (BD Bioscience, San Jose, CA, USA).

25. Cells were plated in 6 well plates, treated with DOX at 5 µM and **3** at 0.1, 1.0 2.5 and 5 µM for 24 h before cells were lysed in 50 µL of RIPA lysis solution. Equal amounts of protein were electrophoresed on 12% SDS-polyacrylamide gel and transferred to a PVDF membrane. The membrane was incubated for 1 h in blocking buffer (5% skim milk powder in TBS containing 0.1% Tween) and then incubated with the mouse antibody against human p53 and caspase-9 (Cell Signaling Technology, Beverly, MA) at 4 °C overnight. Membranes were washed with TBST and incubated with mouse secondary antibody (SantaCruz Biotechnology, CA) before detection by enhanced chemiluminescence (ECL) system on the ChemiDoc system (Biorad. Hercules, CA).

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List of Figures

Figure 1. Chemical structures of (A) doxorubicin, (B) anthraquinone , and (C) emodin.

Figure 2. Compounds **2-4** inhibit cell proliferation and colony formation. (A) Compounds **2-4** inhibit colony formation in NCI/ADR-RES cells. (B) Dose response of compounds **2-4** in MTT cell proliferation assay. (C) Representative colony formation of compound **3** in NCI/ADR-RES cell line.

Figure 3. Compound **3** arrest cells in G2/M of NCI/ADR-RES cells treated with compound **3** at 10 μM for 24 h. Control cells were untreated.

Figure 4. Antioxidants have no effect on compound **3**-mediated cytotoxicity. NCI/ADR-RES cells were pretreated with NAC or GSH prior to 72 h treatment with compound **3**.

Figure 5. Compound **3** does not up-regulate p53 in LNCaP was treated at doses within the range of 0.1-5 μM for 24 h. Thirty microgram of total protein from each sample was analyzed by Western blot.

List of Tables

Table 1. Antiproliferative activities of compounds **2-9**

Compound (20 μM)	Cell Proliferation (% Inhibition)									
	BXPC3	**MIAPaCa-2**	**NCI/ADR-RES**	**CAOV3**	**OVCAR-8**	**PC-3**	**DU145**	**LNCaP**	**H1229**	**U20S**
2	85.2±3.9	80.6±3.1	80.4±1.6	84.6±1.0	64.4±3.5	81.2±2.9	69.1±2.7	79.9±1.0	63.5±1.0	66.1±1.0
3	94.0±1.0	91.1±2.3	80.5±1.3	85.7±1.0	85.4±1.0	75.8±5.2	87.2±3.3	88.9±2.5	75.1±1.6	92.3±1.0
4	91.5±7.4	81.4±3.5	77.8±1.0	83.1±2.8	58.2± 3.2	46.2±5.5	62.2±4.5	78.7±4.9	53.3±1.0	59.8±2.0
5	14.5±9.8	35.9±5.0	15.3±1..5	42.7±8.9	8.5±7.3	9.9±4.1	9.0±1.9	26.7±7.4	31.4±5.7	43.0±6.1
6	15.3±3.2	53.4±8.9	20.4±7.1	47.3±10.0	13.1±4.4	16.6±1.82	16.8±4.3	24.5±1.6	46.9±6.9	44.2±2.3
7	11.8±1.2	29.5±1.2	1.7±1.0	48.2±5.4	5.5±0.4	23.7±3.9	21.6±3.2	40.3±6.8	47.8±2.8	41.9±7.3
8	23.1±2.5	31.1±0.4	46.4±3.6	7.3±6.8	3.4±1.6	10.6±2.9	10.0±1.0	46.9±5.2	44.3±6.3	32.4±2.0
9	17.5±6.9	36.1±0.8	41.5±6.2	16.3±6.7	37.4±0.8	16.4±1.0	17.7±4.5	38.2±2.0	6.4±6.1	11.1±1.2
DOX	96.2±2.3	88.1±1.0	41.4±1.0	92.6±1.0	86.0± 3.4	6.9±2.3	80.6±3.5	85.8±1.3	83.5±1.0	90.0±1.0

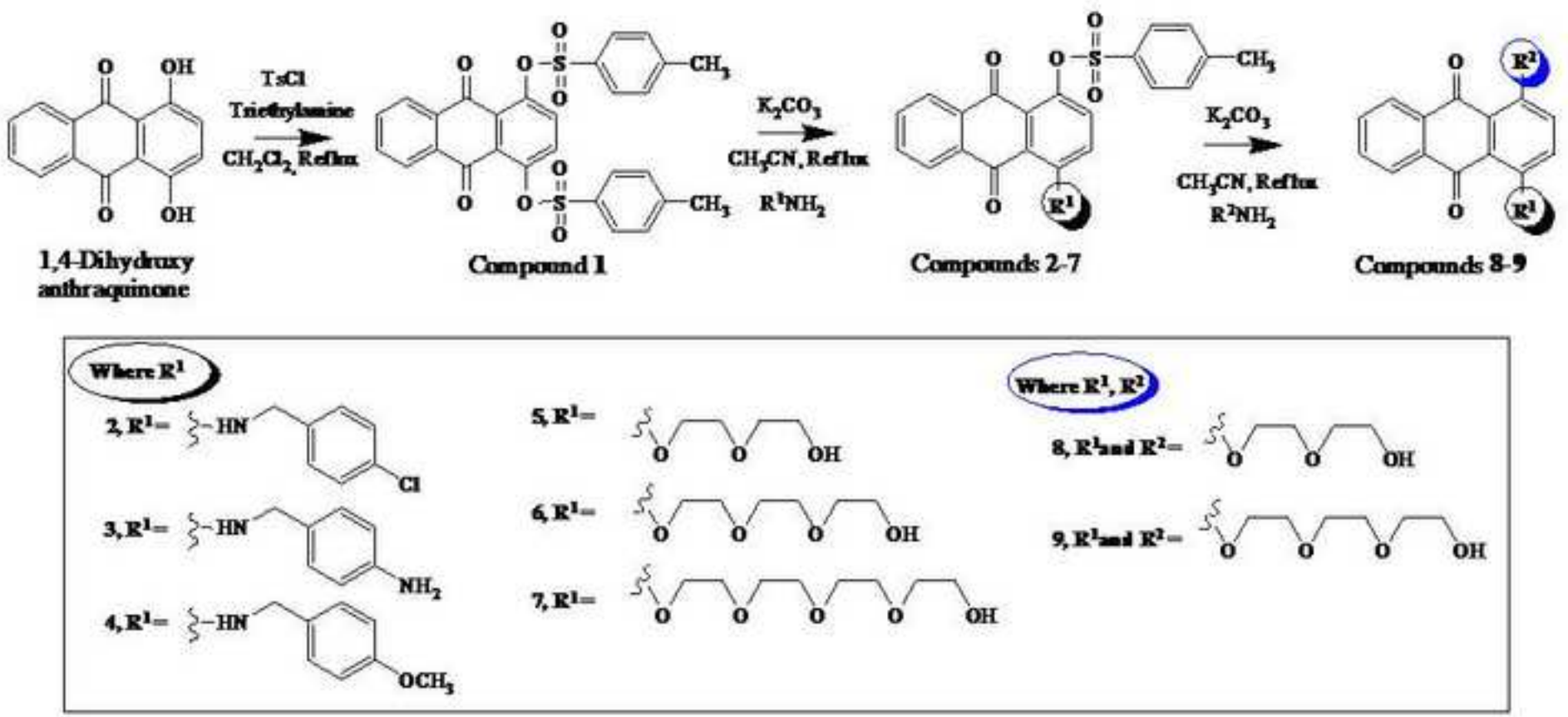
Dox = Doxorubicin. Cytotoxicity was derived after 74 h exposure to the compound from the MTT assay as a surrogate measure of the number of viable cells and is relative to that of the control (no treatment) culture. Data are shown as the mean ± SD derived from three independent experiments.

Table 2. Antiproliferative activities of active compounds **2-4**

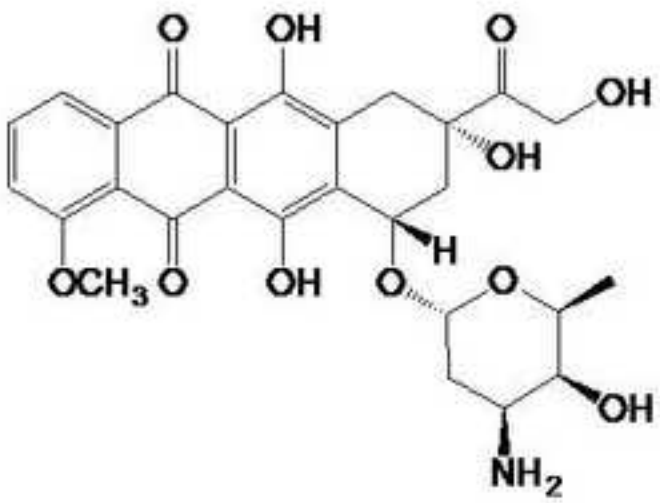
Compound	Cell Proliferation Inhibition, IC_{50} (μM)[a]									
	BXP3	**MIAPaCa-2**	**NCI/ADR-RES**	**CAOV3**	**OVCAR-8**	**PC-3**	**DU145**	**LNCaP**	**H1229**	**U20S**
2	2.4 ± 0.3	4.5 ± 0.4	2.2 ± 0.3	2.5 ± 1.2	4.4 ± 0.6	> 20	6.5 ± 1.5	2.4 ± 0.3	4.0 ± 0.8	4.8 ± 1.0
3	1.8 ± 0.3	2.7 ± 0.3	0.8 ± 0.2	2.5 ± 0.3	3.5 ± 0.3	6.5 ± 0.7	5.4 ± 1.1	0.4 ± 0.1	3.8 ± 0.3	2.8 ± 0.3
4	0.6 ± 0.1	1.9 ± 0.3	1.2 ± 0.1	1.8 ± 0.7	8.0 ± 1.1	8.0 ± 0.5	2.2 ± 0.2	0.9 ± 0.3	> 20	4.5 ± 0.3
DOX	0.4 ± 0.1	< 0.1	> 20	< 0.1	0.6 ± 0.1	1.0 ± 0.1	0.2 ± 0.0	0.2± 0.0	1.5 ± 0.1	0.3 ± 0.1

[a]IC_{50}, is the drug concentration that caused a 50% decrease in the cell viability as determined using the MTT assay. Data are shown as the mean ± SD.

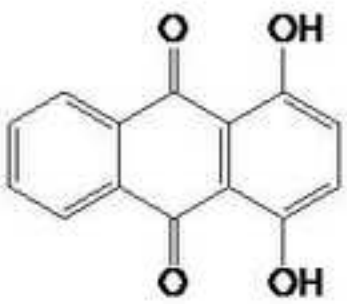
Scheme.jpg



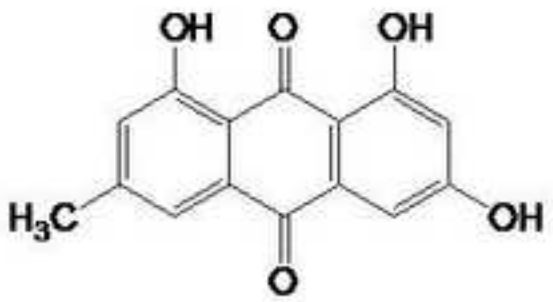
Scheme 1.



(a) Doxorubicin



(b) Anthraquinone



(c) Emodin

Figure. 1

Figure 2.jpg

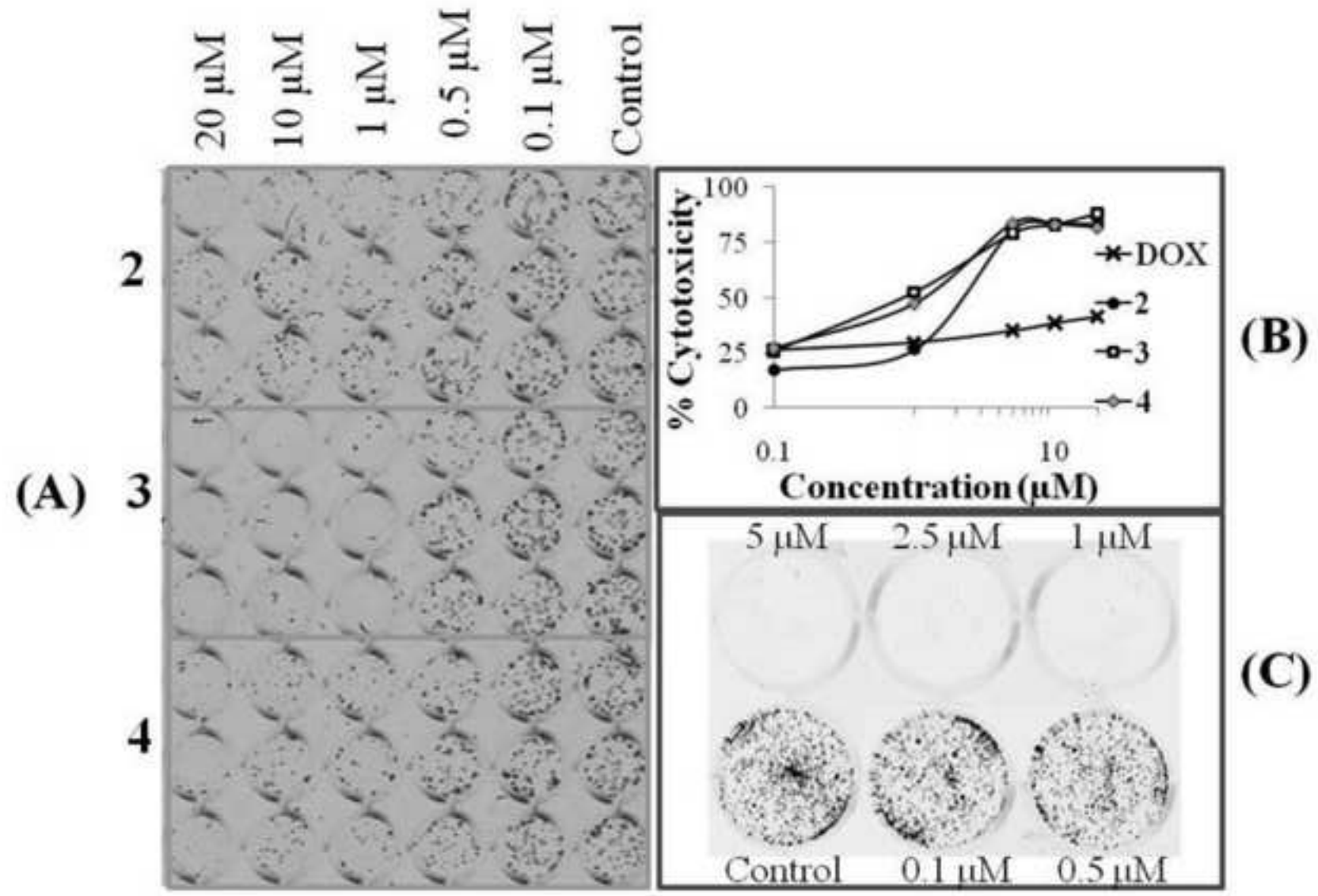


Figure 2

Figure 3.jpg

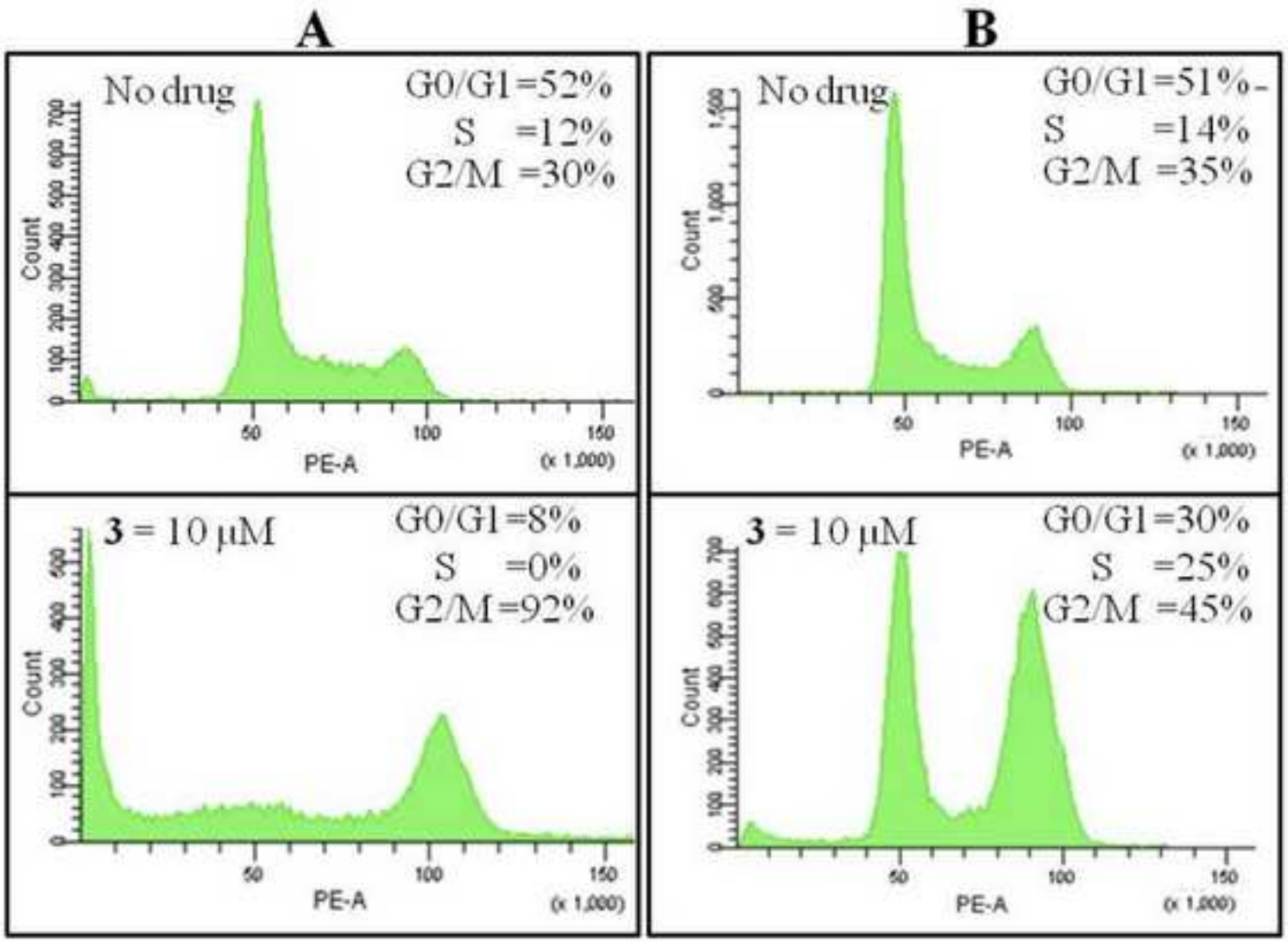


Figure 3

Figure 4.jpg

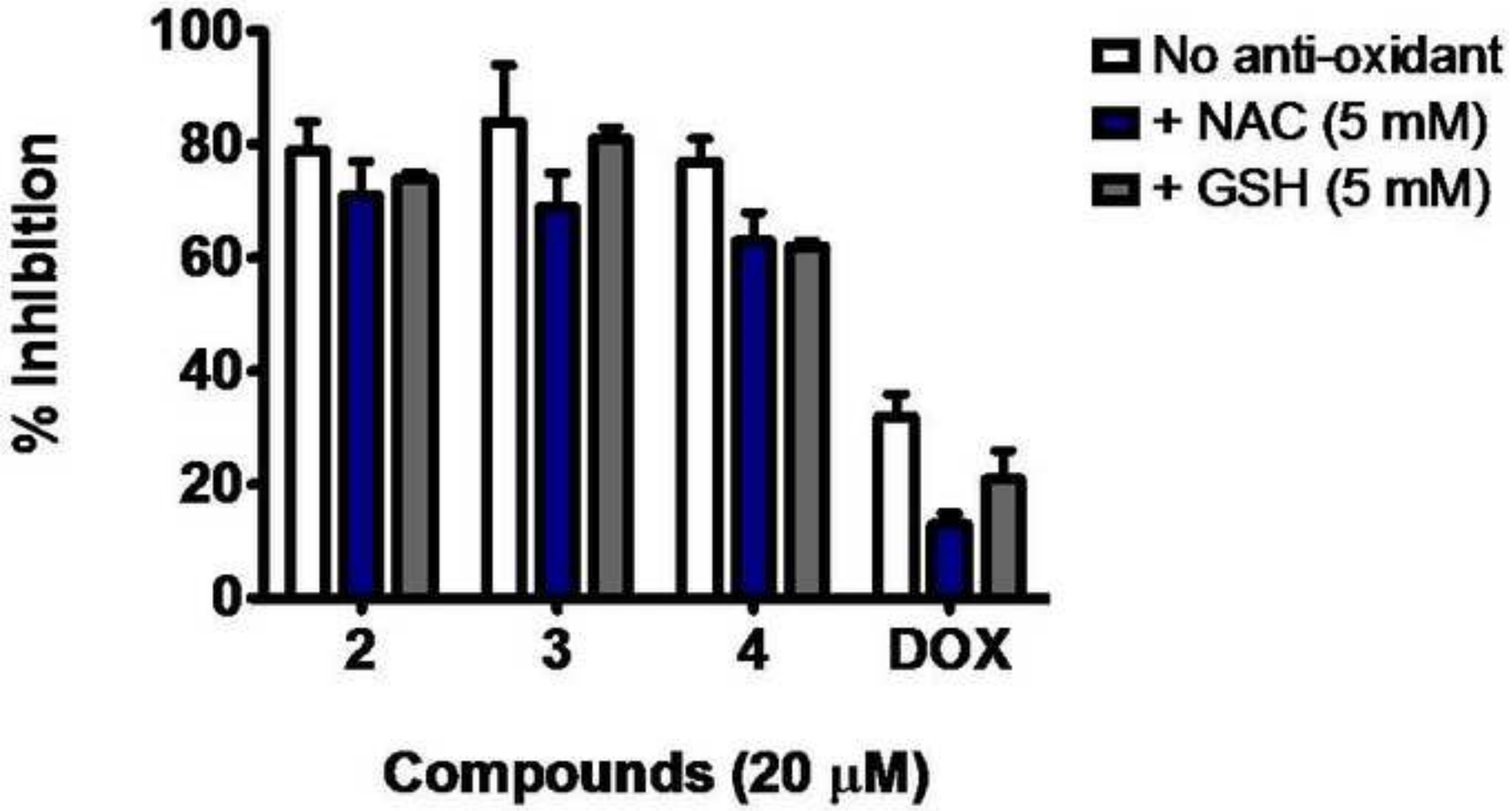


Figure 4

Figure 5.jpg

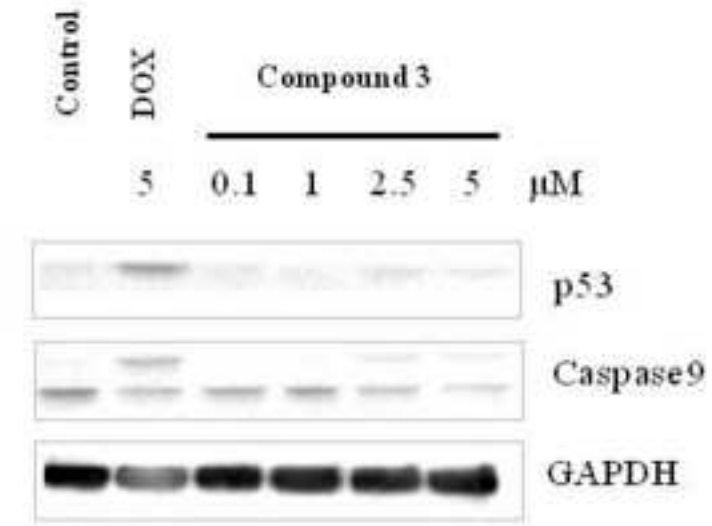
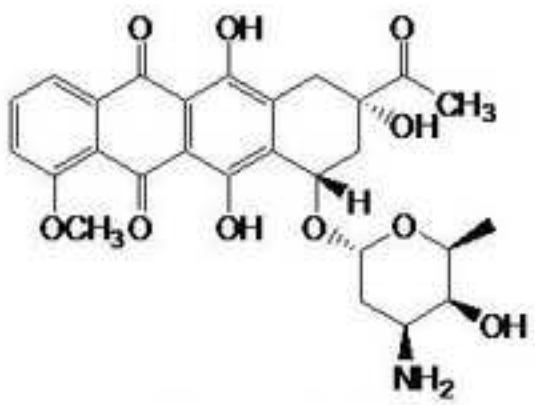
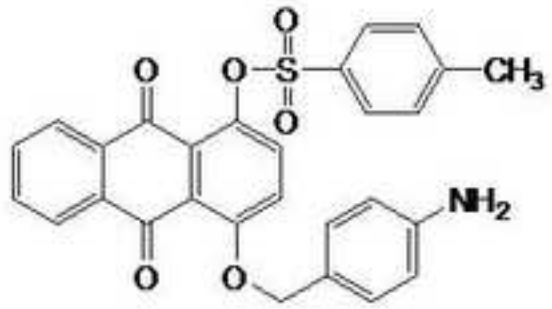


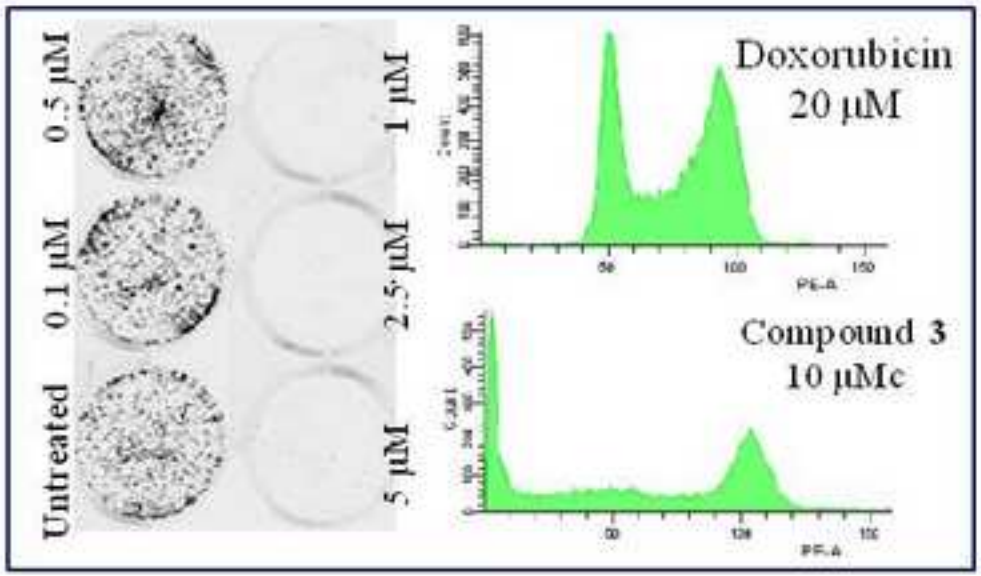
Figure 5.



Doxorubicin
$IC_{50} > 20\ \mu M$



Compound 3
$IC_{50} = 0.8\ \mu M$



Graphic abstract