CHAPTER V
RESULTS AND DISCUSSION

Part I Anthracene, 9-10-dione derivatives with specially-inhibiting HPV type 16

Since, we have already reported that addition of side chain to planar molecule is an important role on activity, especially the benzylamine-containg molecule [Supranee *at al.*, 2011]. In this research, we are interested in the planar molecule, 1,4-hydroxy anthracene, 9-10-dione (compound 1) because it is found to be one part of commercial drug such as mitoxantrone and doxorubicin (Cheng and Zee-Cheng, 1983). Compound 1 was modified according to the procedures shown in Scheme 1 to investigate the effects of different asymmetric substituents at the 1- and 4- positions in order to enhance the activity. One position of 1, 4-dihydroxy anthracene, 9-10-dione was replaced by a tosyl group, whilst the other position was introduced a series of amine substituents. Briefly, the di-hydroxy groups of compound 1 were reacted with tosyl by tosylation to give compound 2 as a key intermediate. After that, one of the two resultant tosyl groups of the intermediate was removed by substituted amines using K_2CO_3 in dry acetonitrile. Compounds 3-7 with very good yields were obtained. The derivatives was divided into three groups based on the types of amine side chains, a ring containing morpholine (3-4), benzylamines (5-6) and a branch chain of amine (7). The chemical structures of the synthesized compounds were determined by ^1H, ^{13}C-NMR and MS spectral, and X-ray structural analysis. Selected crystallographic data of compounds 3, 5 and 7 are shown in Table 1.

Crystal data and refinement for compounds 3, 5 and 7 are given in Table 1. The ORTEP structures of these compounds are represented with the labeling numbers and in the side view are shown in Figure 7.

Table 1. Selected crystallographic data for compounds 3, 5 and 7.

Compound	3	5	7
Empirical formula	$C_{25}H_{21}NO_6S$	$C_{28}H_{21}NO_5S$	$C_{25}H_{23}NO_5S$
Molecular weight (g/mol)	463.49	483.51	449.50
Wavelength (Å)	0.71073	0.71073	0.71073
Crystal system	Triclinic	Triclinic	Monoclinic
Space group	P(-1)	P(-1)	$P2_1/n$
Unit cell dimensions			
a(Å)	7.8320(3)	9.4790(6)	7.3950(10)
b(Å)	11.7455(6)	10.2212(8)	23.936(4)
c(Å)	11.7750(6)	12.4670(9)	21.360(2)
α (°)	90(2)	90(3)	90
β(°)	94(10)	91(3)	91(8)
γ (°)	91(10)	90(3)	90
θ(°)	1.73<θ <28.29	1.73<θ<29.65	2.88<θ<27.12
V(Å^3)	1078.84(9)	1130.01(14)	2186.9(6)
Z	2	2	4
D (calc) (g/cm^3)	1.427	1.421	1.365
F (000)	484	504	944
Final *R* indices [$I>2\ \delta(I)$]	R_1=0.0448, R_2=0.1342	R_1=0.0512, R_2=0.1357	R_1=0.1020, R_2=0.3131
R indices (all data)	R_1=0.0685, R_2=0.1668	R_1=0.0785, R_2=0.1542	R_1=0.1427, R_2=0.3458
Largest difference peak and hole (e Å^{-3})	0.311 and -0.297	0.464 and -0.526	1.036 and -0.348
CCDC number	826207	826206	820953

[a] $R_1 = \sum||Fo|-|Fc||/\sum|Fo|$, $wR_2 = [\sum w(Fo^2 - Fc^2)^2/\sum w(Fo^2)^2]^{1/2}$

The molecules consist of an anthraquinone plane, one tosyl and one amine substituent groups at 1- and 4-positions. The anthraquinone core of compounds 3 and 7 are highly distorted from the least-square plane consisting of 16 atoms with the r.m.s of 0.3244 Å and 0.3526 Å, respectively. The anthraquinone oxygen atoms are bent in the opposite direction of the 1,4-dihydroxy anthraquinone plane with the deviation distances of 0.424(1) Å and 0.383(1) for O1, 0.667(1) Å and 0.707(1) Å for O2 in compounds 3 and 5, respectively. The tosyl group of both compounds is twisted and stacked over the anthraquinone plane to participate π-π stacking with the aromatic ring system of the anthraquinone, existing as a *U*-shaped form, with the shortest distance between C10...C11 of 3.314(1) Å and 3.350(1) Å for 3 and 7, respectively. The diethylamine and morpholine least-square planes are bent from the anthraquinone plane with the deviation of 55.43° and 66.10°, respectively. There is no classical hydrogen bonding in the molecule. In contrast to compounds 3 and 7, the anthraquinone plane of compound 5 is nearly planar with the r.m.s. of 0.064 Å (defined by 16 atoms). The anthraquinone oxygen atoms are in the plane of 1,4-dihydroxy anthraquinone (0.094 and 0.140 Å). The tosyl substituent group is nearly in the plane with the anthraquinone core and form π-π interaction with adjacent tosyl group of the neighboring molecule.

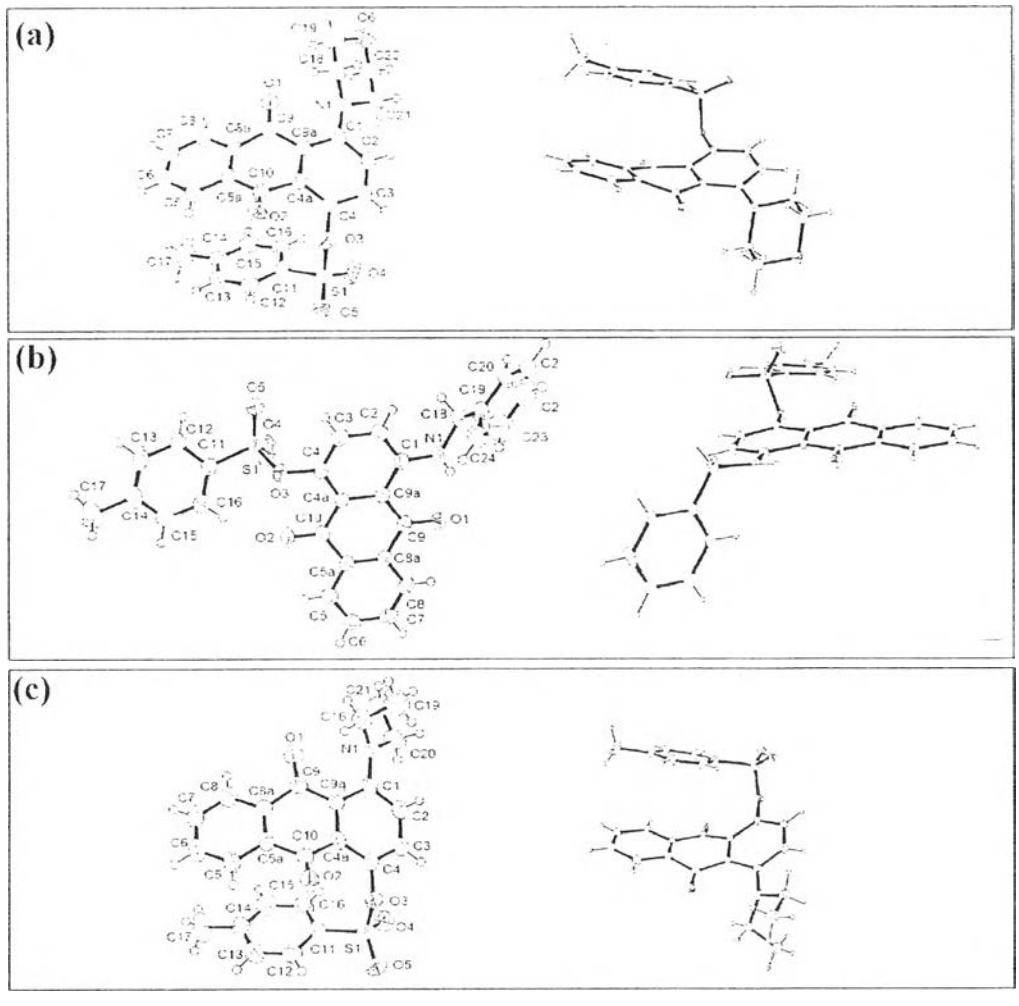


Figure 7 Crystallgriphic structures of (a) compound 3 (b) compound 5 and (c) compound 7

The activity of anthraquinone derivatives was evaluated for their *in vitro* ability to inhibit the growth of five cell lines derived from five human cancers; colon (SW620), gastric (KATO-III), lung (CHAGO), hepato (HCC), cervix (Ca Ski) using the MTT assay (Table 2). The ability to inhibit with the cancer cell lines will be compared with cisplatin. Because cisplatin has been reported for treatment of lung and MCF-7 cancer, but inactive for Ca Ski (Scanlon et al., 1991) and (Chu et al., 1994). Thus, this research used cisplatin as a control in order to find out the mechanism for overcoming Ca Ski.

.Table 2 %Screening inhibition of anthraquinone derivatives (1-7) compared to cisplatin

Compound (10 μM)	Cell mortality (%) in each cell line after 72 h				
	HCC	Ca Ski	Kato	Chago	SW620
1	5.6±1.7	48.6±9.1	38.4±8.6	12.3±1.1	21.1±2.4
2	11.9±1.8	36.4±9.4	31.1±5.5	5.8±0.7	22.2±2.7
3	6.1±1.9	43.8±6.4	35.3±7.2	11.3±7.1	22.3±1.4
4	11.1±1.8	42.8±10.1	29.3±5.1	4.9±0.7	19.4±1.7
5	40.5±2.2	84.5±3.4	41.5±2.2	39.8±7.7	49.1±9.4
6	8.3±0.9	27.4±6.6	42.5±2.7	13.3±9.0	49.5±2.4
7	35.6±3.7	50.0±2.1	39.9±9.3	43.9±20.4	32.6±4.4
Cisplatin	68.7±3.8	85.5±2.9	59±7.8	56.0±5.5	33.0±18.6

Cytotoxicity was derived after 72 h exposure to the compound using the MTT assay. Data are shown as the mean ± 1SD and are derived from two independent trials. Means followed by a different letter are significantly different ($p < 0.05$; Duncan's Multiple Means Test after log transformation.

From the result in Table 2, almost anthracene-9, 10-dione derivatives showed %screening inhibition less than 50% in the tested cell lines, except compound 5 inhibited Ca Ski with 85%inhibition compared to cisplatin having %inhibition ranging from 56-86% against HCC, Ca Ski, Kato and Chago. Compound 5 exhibited strong cytotoxic activity against human cervical cancer cell line (Ca Ski) with IC_{50} = 0.3 µM, compared with the un-modified compound 1 (IC_{50} > 10.0 µM) and the reference (cisplatin, IC_{50} = 8.0 µM) as shown in Table 3, Figure 8c and 8d. Different anthraquinone derivatives were found to exhibit different *in vitro* cytoxicity levels against the different human cancer derived cell lines, depending on their substituents. For example, the analogues (2-4 and 6-7) exhibited no activity on all five tested cell lines. Surprisingly, compound 5 (bezylamine) specifically showed activity against only Ca Ski cells with IC_{50} = 0.3 µM (Table 3) compared to the positive control (Cisplatin) showing good activity in Kato, Ca Ski and HCC cell lines with IC_{50} ranging from 5.6-8.0 µM, while had IC_{50} > 10 µM for Chago and SW620. Moreover, compound 5 exhibited low cytotoxicity against WI-38 (IC_{50} > 30 µM) as shown in Figure 9 and Table 3. The data is consistent with our previous reports on bezylamine-containing asymmetric planarity that showed excellent cytotoxic activity against cancer cell lines with negligible effects on normal cells (Supranee, *at al*., 2013).

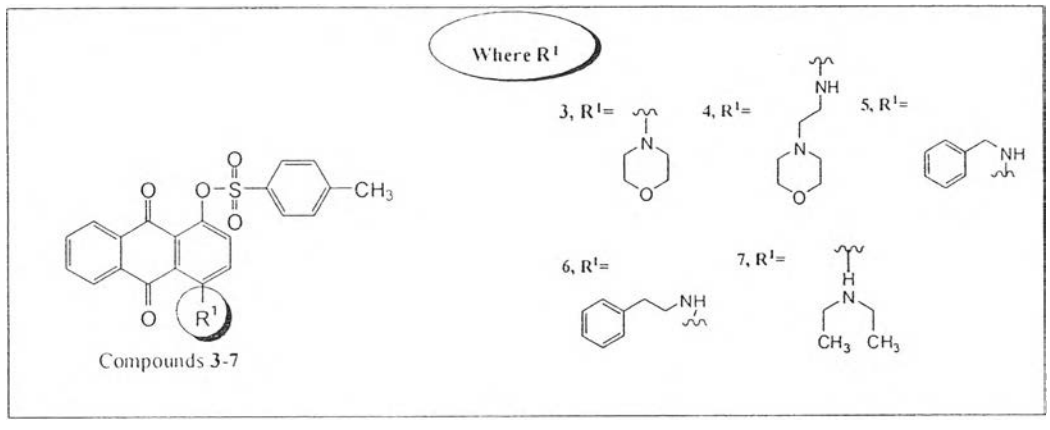


Table 3 IC_{50} of compound 5 and cisplatin on five cancer cells and lung normal (WI-38) cells

Compound	Cytotoxicity, as the IC_{50} (μM) value, of each compound on the respective cell lines					
	HCC	Ca Ski	Kato	Chago	SW620	WI-38
5	>10	0.3±0.1	>10	>10	>10	>30
Cisplatin	7.5±1.5	8.0±1.0	5.6±3.3	>10	>10	>30

Cytotoxicity was derived after 72 h exposure to the compound using the MTT assay. Data are shown as the mean ± 1SD and are derived from two independent trials. Means followed by a different letter are significantly different ($p < 0.05$; Duncan's Multiple Means Test after log transformation.

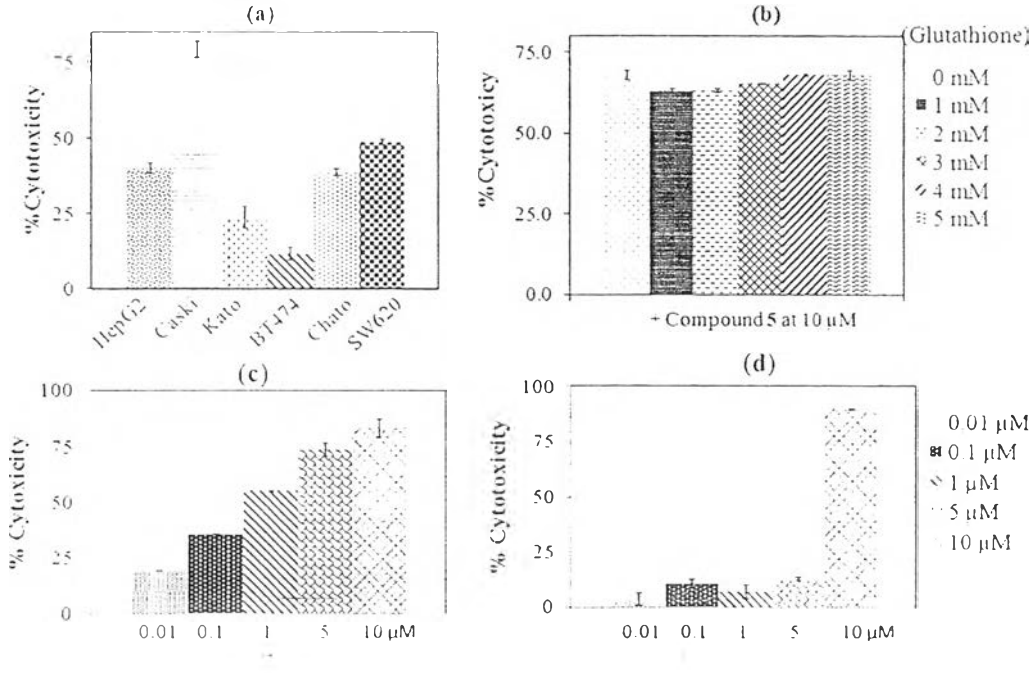


Figure 8 (a) % Cytotoxicity in the screening of compound 5 (10 μM) using six human carcinoma cell lines (b) No effect of glutathione on compound 5's activity at 10 μM and (c) %cytotoxicity of compound 5 (IC_{50}= 0.3 μM)and (d) cisplatin as dose dependences against Ca Ski cells, and profiles are shown representative from three independent trials.

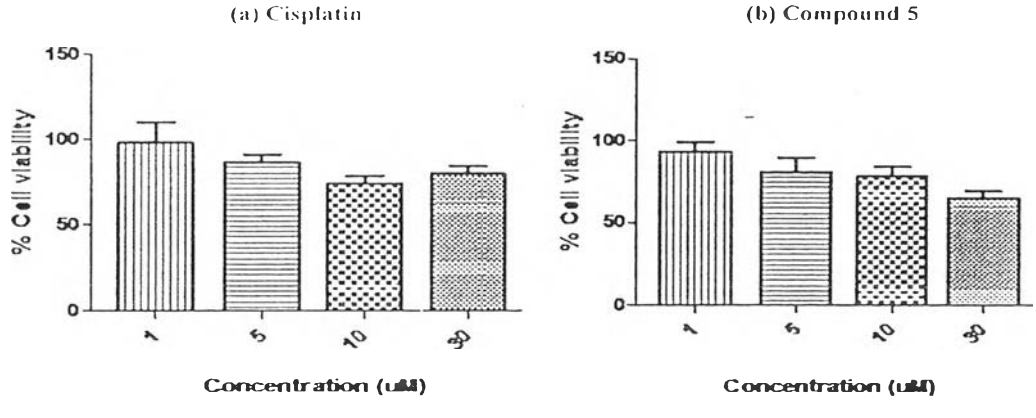


Figure 9. % Cell viability upon treatment of WI-38 normal cells with (a) cispatin and (b) compound 5 at 30, 10, 5, 1 μM, and profiles are shown representative from three independent trials

Since, anthraquinone analogues including doxorubicin (Figure 3) and emodin (Figure 4b) have been reported to induce ROS-mediated apoptosis and the affinity of these compounds are blocked by glutathione or GSH (Agbandje, 1992 and McKnight, 2004). In comparison with compound 5, the structures are similar with containing quinone, thus, compound 5 possibly shows the same anticancer mechanism. This research, we sought to determine the role of ROS in our compounds' activity. After Ca Ski cells were pretreated with glutathione as an antioxidant at 0, 1, 2, 3, 4, 5 mM for 2 hours, prior to the addition of compound 5 at 10 μM. The activity showed that pretreatment with GSH yielded similar results with untreated group (Figure 8b). We concluded that GSH did not have an effect of compound 5's activity in Ca Ski cell line. This indicates that the activities of compound 5 may not be ROS-mediated or may induce apoptosis via another pathway such as caspase activity (Perdomo, 2013). Moreover and surprisingly, compound 5 at 5 μM completely blocked the formation of colony in Ca Ski cells compared to cisplatin treatment group at 5 and 10 μM as shown in Figure 10a. This suggests that compound 5 is selectively and highly cytotoxic against Ca Ski cells. Thus, compound 5 will be studied in the future work.

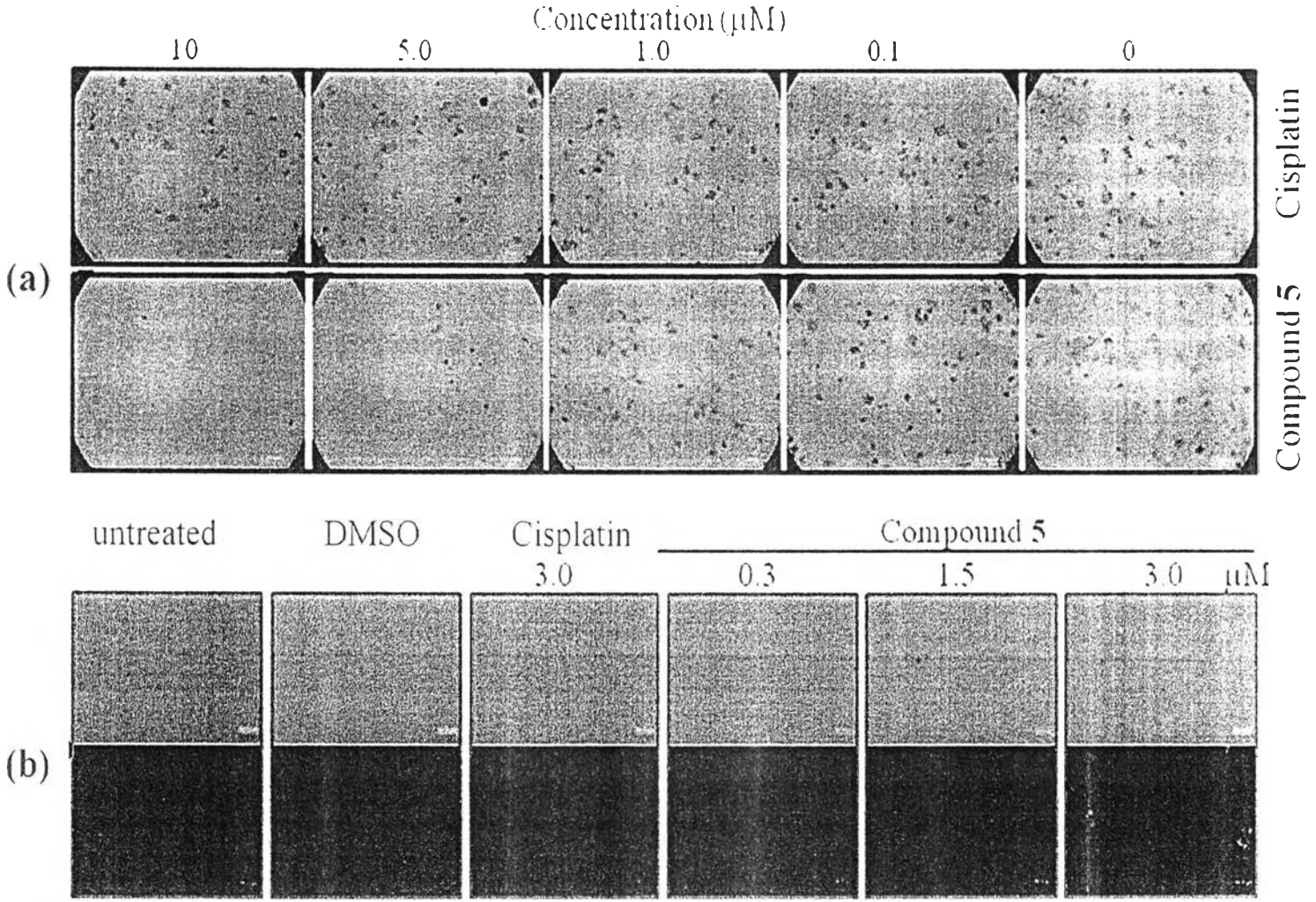


Figure 10. (a) Colony formation in CaSki treated with cisplatin or compound 5 at 10, 5, 1, 0.1, 0.01 μM (b) treatment with compound 5 induces apoptosis in a dose-dependent manner, compared with an untreated cells, DMSO and cisplatin (3.0 μM). Profiles shown are representative from two independent trials.

Next experiment, we studied inducing apoptosis by compound 5 using DAPI staining. DAPI was performed to identify the chromatin condensation as a marker for apoptosis using fluorescence microscope as described in materials and methods. After treatment of Ca Ski cells with compound 5 at 0.3, 1.5 and 3.0 μM, chromosome condensations were increased at a dose-dependent manner (Figure 10b); while a little chromatin condensation was found after treatment by cisplatin at 3 μM. Especially at the highest concentration (3 μM), compound 5 completely induced chromosome condensations compared to cisplatin, suggesting that compound 5 induces apoptosis in Ca Ski cells.

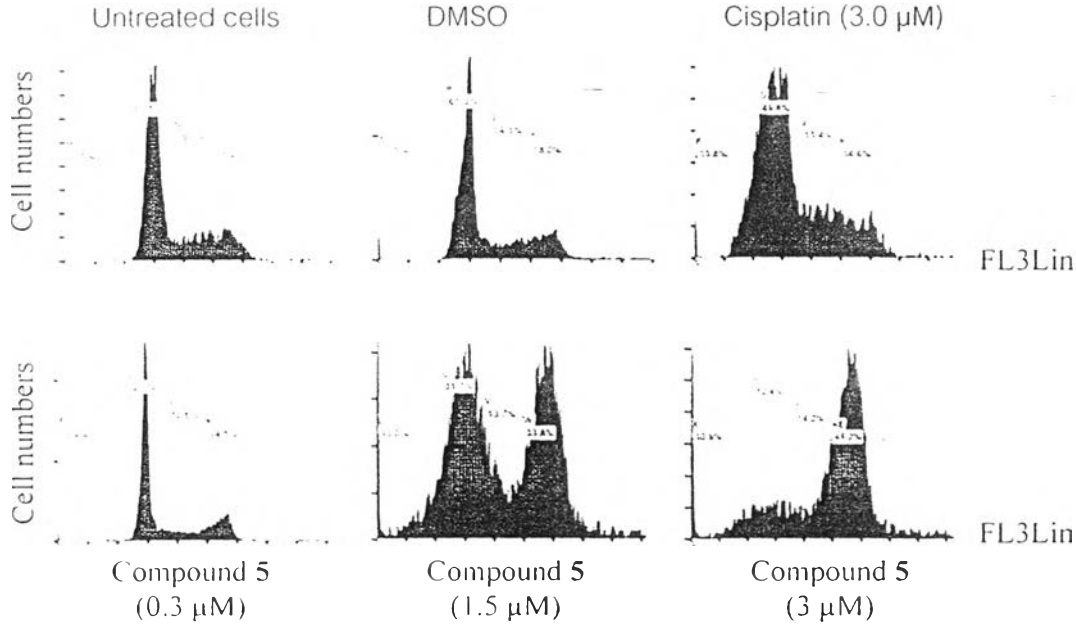


Figure 11. Compound 5 arrest cell in G_2/M. Cell cycle profiles of Ca Ski cells at 24 h after treatment with compound 5 at 1.5 and 3.0 μM compared to cisplatin at 3 μM. Control cells (Un-treatment and DMSO) shown were measured at 24 h and no significant changes were observed. In all profiles at least two events (cells) were preformed.

Flow cytometry was used to examine the effects of compound 5 on cell cycle progression in Ca Ski cells. Basically, the profiles represent each phase of cell cycle

(Untreated cells and DMSO, Figure 11); the first peak (left) represents G_0/G_1 phase, the small last peak (right) represents G_2/M phase and between G_0/G_1 and G_2/M represents S phase. After treatment with compound 5 radically changed when compared with those of untreated and DMSO treated cells as shown in Figure 11. At the lowest concentration of compound 5 (0.3 μM), the percentages of cells did not change in all cell cycle phases while at the higher concentration (1.5 μM), the percentage of cells in the G_2/M phase significantly increased from 18.0 to 33.8% (Δ15.8%). Especially at 3.0 μM, the cells in the G_2/M phase significantly increased while those in the G0/G1 phase inversely decreased as compared to cisplatin at the same concentration (3.0 μM) which did not affect the cell cycle profile. Compound 5 disrupted cell cycle progression by pushing cells to accumulate in the G_2/M phase in a dose-dependent manner at 24 h. Our data is consistent with previous report that anthraquinone derivatives including doxorubicin also induce cell cycle arrest in cancer cells in the G_2/M phase. This result indicated that compound 5 disrupted cell cycle progression which is different from cisplatin.

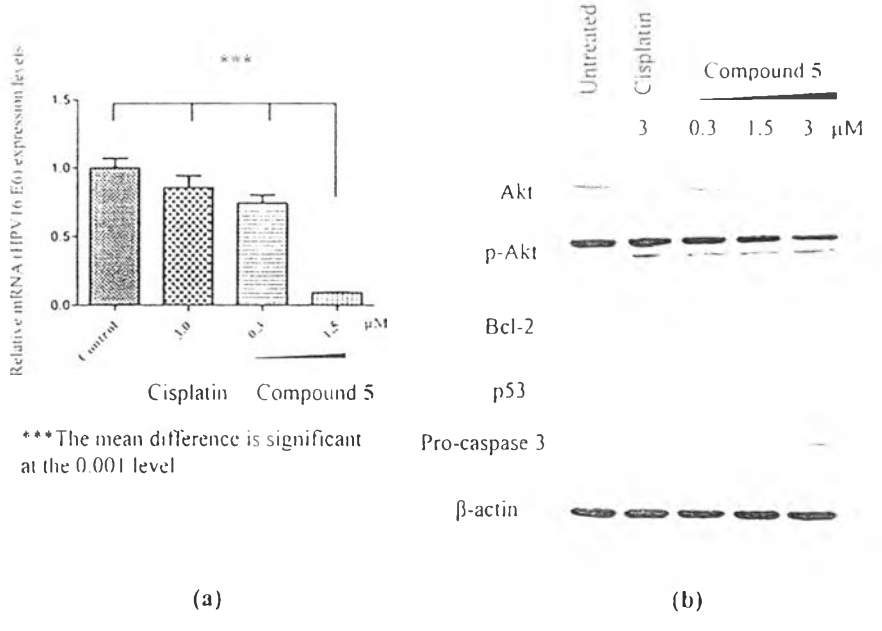


Figure 12. (a) Total RNA from HPV positive Ca Ski cells was analyzed for mRNA expression by real time RT-PCR, and (b) effect of compound 5 treatment in a does-

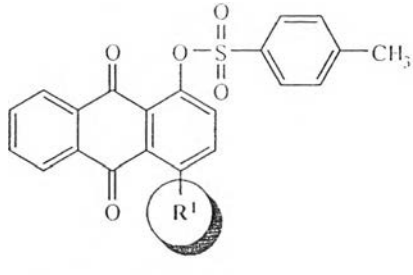
dependent manner (0.3 and 1.5 and 3.0 μM) on protein expression in Ca Ski cells for 24 h, comparing with cisplatin at 3.0 μM as a positive control. Forty microgram of total protein from each sample was analyzed by Western blot, and profiles are shown representative of those seen from two independent trials.

Since degradation of the tumor suppressor p53 by HPV E6 in HPV positive cervical cancer cells can suppress cell cycle and apoptosis. Thus, we also studied whether compound 5 could inhibit the cancer cells carrying HPV by real-time RT-PCR. The mRNA levels of E6 of HPV type 16 were significantly reduced by compound 5 treatment at 0.3 μM, compared with treatment with cisplatin at a 10-fold higher concentration (3 μM) (p value < 0.001) as shown in Figure 12a. This data suggests that compound 5 suppresses E6 expression at the transcription level.

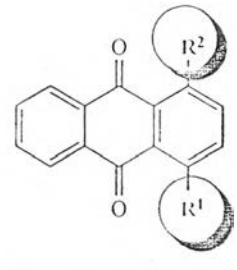
Because various apoptosis-inducing agents play an important role for anti-cancer development, we investigated the effect of compound 5 on the level of bcl-2 as anti-apoptosis and p53. Compound 5 completely inhibited bcl-2 expressions even at a low concentration (0.3 μM) compared with untreated and cisplatin at 3 μM. Moreover, compound 5 treatment increased p53 and pro caspase-3, while phospho-Akt was down-regulated at a dose-dependent manner (Figure 12b). In contrast, treatment with cisplatin at 3 μM as positive control, increased bcl-2 level and decreased pro-caspase-3 level. The opposite result between compound 5 and cisplatin suggest that compound 5 may induce apoptosis indicated by decreasing E6 expression and bcl-2 and increase p53 level.

Part II. Anthracene, 9-10-dione derivatives with overcoming drug resistance

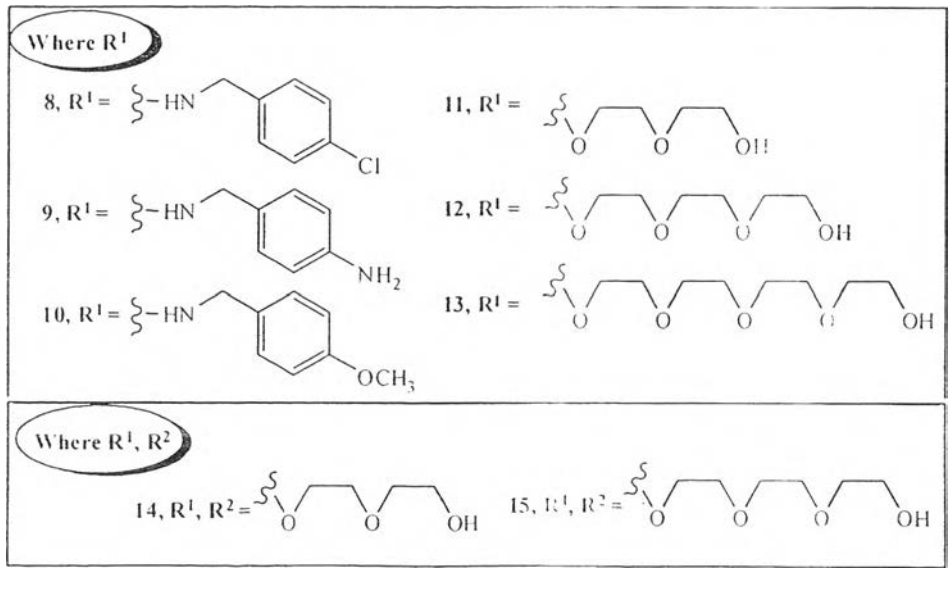
To investigate the effects of both asymmetric and symmetric substituents at the 1- and/or 4-position on cytotoxicity and anti-cancer properties, 1,4-dihydroxy anthraquinone (compound 1) was modified according to the procedures shown in Scheme 1. Two hydroxyl groups of 1,4-dihydroxy anthraquinone were substituted with tosyl chloride (TsCl) to obtain compoun 2 as an intermediate, the 4-position of compound 2 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 8-10 and (ii) a group of aliphatic chain of di, tri, tetra-ethylene glycol substituants to provide 11-13, respectively. Finally, Position 4 of compound 11 & 12 were reacted with di-ethylene (14) and tri-ethylene glycol (15) in dry acetonitrile followed by treatment of K_2CO_3. The purity and structural assignments of synthesized compounds were determined by ^{1}NMR, ^{13}NMR, and MS spectral analysis.



Compounds 8-13



Compounds 14-15



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 8-15 and doxorubicin as a reference. Cell proliferation was measured using MTT as reported. 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 4 %Cell Proliferation of anthraquinone derivatives (8-15) compared to doxorubicin (DOX)

Compd (20 μM)	Cell Proliferation (% Inhibition)								
	BXPC3	MIAPaCa-2	NCI/ADR-RES	CAOV3	OVCAR-8	PC-3	DU145	LNCaP	H1229
8	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
9	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
10	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
11	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
12	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
13	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
14	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
15	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
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

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.

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 cell line that over expresses multi-drug resistance transporter, MDR1, conferring resistance to various chemotherapies including doxorubicin. Among the eight new anthraquinone derivatives, three compounds bearing the benzylamine (**8** (-Cl), **9** ($-NH_2$), and **10** ($-OCH_3$)) inhibited cell proliferation more than 50% at 20 μM in all cancer cell lines tested, especially in the NCI/ADR-RES resistant cell lines which were inhibited more than 78% (Table 4). However, compounds **11-15** 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 tested cell lines. This data demonstrates that the benzylamine substituents at the 4-position of the quinone core are important for activity, while compounds **11-15** with flexible long glycol chain were inactive perhaps by steric effect.

In comparison, asymmetric anthraquinones (**8-10**) with containing substitution (-Cl, -NH2, $-OCH_3$) on the 4-position of benzylamine showed similar activity; compound **9** inhibited cell proliferations with IC_{50} values within 0.4-6.5 μM and compounds **8** and **10** also inhibited cell proliferation with IC_{50} values within 0.6-8.0 μM against almost all cell lines except PC-3 for compound **8** and H1299 for compound **10** (Table 3, Figure 2B). This data suggests that only the benzylamine at 4-position of anthraquinone plays an important role in cytotoxic activity, but the electrophile substitutions (-Cl, $-NH_2$, $-OCH_3$) at 4-position of the bezylamine do not significantly affect the compound's 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 described (Lutzker and Levine, 1996). The colony numbers were suppressed after treatment of compounds **8-10** at 24 h as dose dependence (Figure 13A). Surprisingly, compound **8** at concentration of 1, 2.5 and 5 μM significantly inhibited the colony-formating abilities (Figure 13C) compared to its level of inhibition of untreated control

culture correlated with MTT results (IC_{50} = 0.8 μM). Our result indicates that compound 9 affects the cell's reproduction ability to form progenies in NCI/ADR-RES cell leading to cell death. The advancements of inhibiting colony formation assay have been made for benefit of cancer therapy in recent years with combination of previous individual modalities such as radiation and chemotherapy. Thus compound 9 as the most active compound was chosen for the future experiments.

Table 5. IC_{50} of anthraquinone derivatives (8-10) compared to doxorubicin (Dox)

Com	Cell Proliferation Inhibition, IC_{50} (μM)[a]									
	BXP3	MIAPaCa-2	NCI/ADR-RES	CAOV3	OVCAR-8	PC-3	DU145	LNCaP	H1229	U20S
8	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
9	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
10	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

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.

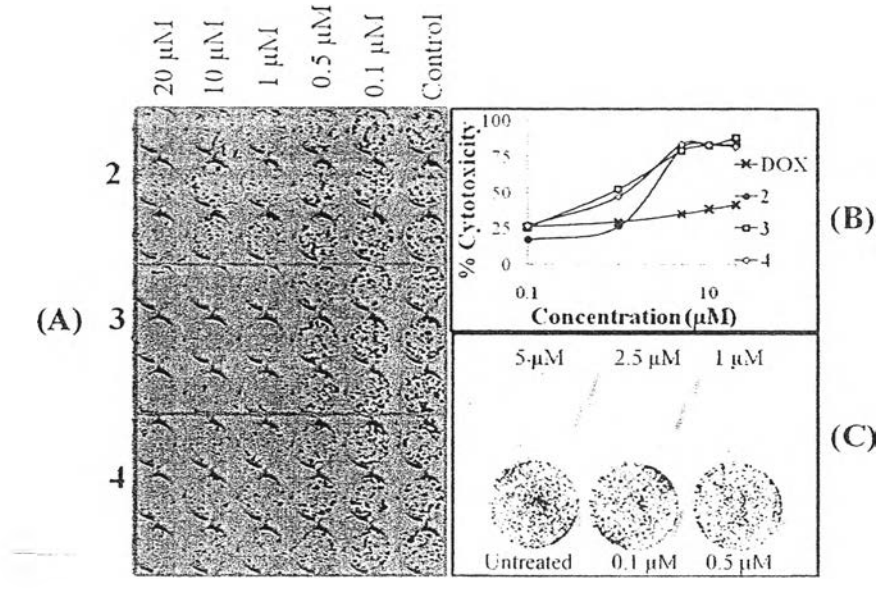


Figure13. Compound 8-10 inhibit cell proliferation and colony formation (A) representative (of three independent trials) colony formation assays, (B) cytotoxicity, as % killed cells, of 8-10 against the NCI/ADR-RES cell line assayed after 72 h exposure and shown as the mean ± 1 SD and are derived from triplicate trials, and (C) complete inhibition of NCI/ADR-RES cells by 9 at 1 μM (result shown is representative of three independent repeats).

To test the hypothesis that compound 9-induced inhibition of colony formation is mediated by modulation of the expression of cell cycle (incomplete sentence). Flow cytometry was used to examine the effects of compound 9 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) represents cell in G0/G1, the intervening area represents cells in S phase and the final tail/small peak (right) represents cell in G_2/M (Figure 14). The percentage of cells in G0/G1 phase decreased from 51% to 30%, while in G_2/M increased from 35% to 55% in NCI/ADR-RES after treatment of compound 9 at 10 μM. In our observation, Compound 9 inhibited cell proliferation by arresting G_2/M phase. This data is consistent with

previously reported anthraquinone derivatives such as an emodin that also arrest cancer cells in the G_2/M phase (Cui *at al.*, 2002).

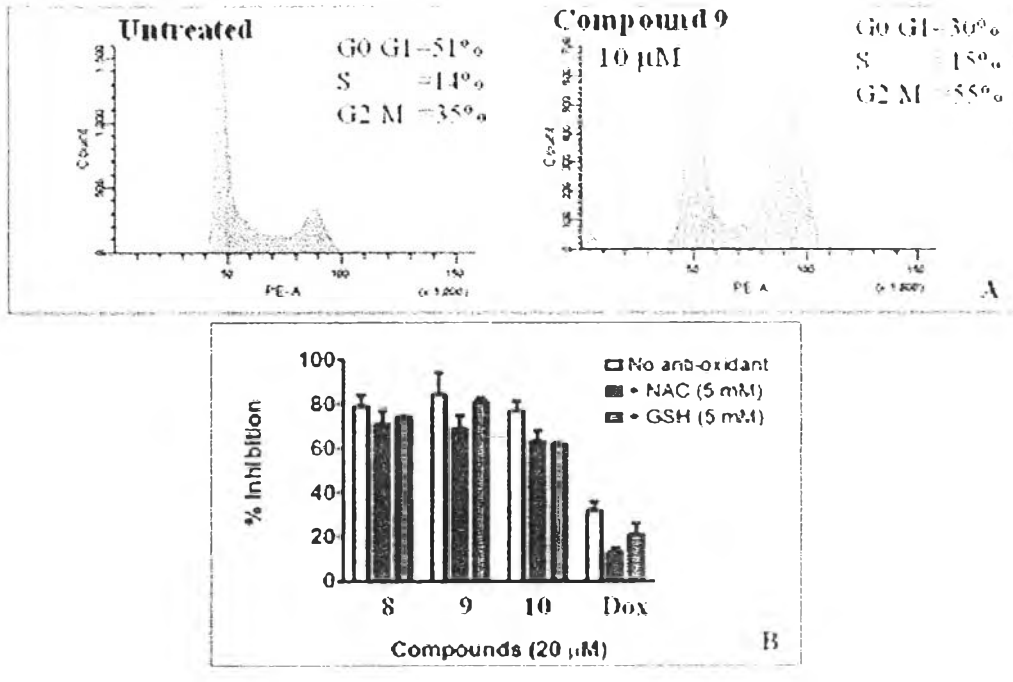


Figure 14. (A) Compound 9 arrest cells in G_2/M of NCI/ADR-RES cells treated with compound 9 at 10 μM for 24 h. Control cells were untreated (B) antioxidants have no effect on compound 3-mediated cytotoxicity. NCI/ADR-RES cells were pretreated with NAC or GSH prior to 72 h treatment with compound 9.

Anthraquinone including emodin and doxorubicin has been reported to induce ROS-mediated apoptosis (Lu, *at al.*, 1993, Kuo, 2009 and Siddik, 2003). Therefore, we sought to determine the role of ROS on our compound's 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 8-10 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 14) while compounds 8-10 still showed cytotoxicity more than 60% inhibition. Our observation showed that pre-

treatment with antioxidants did not decrease the activity of compounds 8-10, suggesting the activities of these compounds may not be ROS-mediated as observed with doxorubicin.

Since doxorubicin-induced apoptosis in response to DNA damage is p53-mediated, we also assessed whether the cytotoxic effects of compound **9** are p53-mediated (Cai, 2008). Because OVACAR-8 and NCI/ADR-RES are carrying a p53 mutant (Liu, *at al.*, 2011 and Ogretmen, *at. al.*, 1997), we assessed 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, we assessed the protein levels of p53 and caspase 9 upon treatment of doxorubicin and compound **9** 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 **9** at the same concentration did not alter p53 but slightly activated caspase-9, suggesting the cytotoxic effects of compound **9** is not p53-mediated (Figure 15). This is further supported by our MTT results that no comparable difference in activity in p53 wild type (U2OS) and p53 null (PC3 and H1299) cell lines (Table 5) were shown.

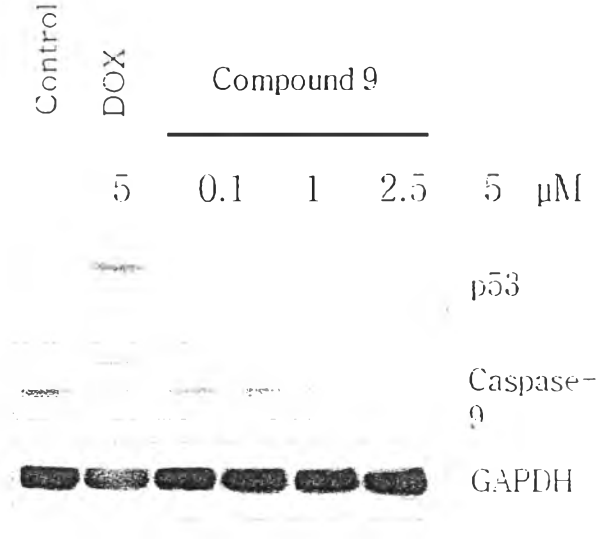


Figure 15. Compound 9 does not upregulate p53 in LNCaP cells for 24 h. Thirty microgram of protein was loaded per track and analyzed by western blot.

We have discovered a new class of anthraquinone derivatives that inhibit cancer cell proliferation in the low micromolar range. We have shown compound 9 can overcome drug resistance in NCI/ADR-RES, with IC_{50}'s 25 times greater than doxorubicin. Interestingly, compound 9's cytotoxic effect does not appear to be ROS or p53-mediated suggesting its mechanism of action may be different from previously reported anthraquinones. Further studies are required to further assess the precise mechanism of action, perhaps targeting potential new anti-cancer targets. Additionally, these new compounds may be beneficial for the treatment of resistant cancer or used in adjuvant therapy with other anti-cancer agents.