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Different Doxorubicin Sensitivity Across Various Human Cancer Cell Lines

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Abstract

Doxorubicin (Dox) is a highly potent chemotherapeutic agent, approved by FDA since 1974, for treating a broad spectrum of cancers. However, development of severe side effects and drug resistance are main issues that limit the clinical use of Dox. Herein, we aimed to investigate the sensitivity of Dox in a variety of human cancer cell lines. Eleven cell lines were tested in this study including 2 hepatocellular carcinoma (HCC) cell lines (HepG2 and Huh7), 4 bladder cancer (BlCa) cell lines (UMUC-3, VMCUB-1, TCCSUP and BFTC-905), a lung cancer cell line (A549), a cervical carcinoma cell line (HeLa), a breast cancer cell line (MCF-7), a skin melanoma cell line (M21) and a noncancer human kidney cell line (HK-2). The MTT assay was employed for the determination of cytotoxicity. Cells were treated with various concentrations of Dox for 24 h. The half-maximal inhibitory concentration (IC50) of Dox was calculated to compare the Dox sensitivity among tested cell lines. The result showed that IC50 of Dox in HepG2, Huh7, UMUC-3, VMCUB-1, TCCSUP, BFTC-905, A549, HeLa, MCF-7, M21 and HK-2 cells were 12.2, > 20, 5.1, > 20, 12.6, 2.3, > 20, 2.9, 2.5, 2.8 and > 20 μM, respectively. BFTC-905 had the lowest IC50 value, therefore, it was the most sensitive to Dox. In contrast, Huh7, VMCUB-1 and A549 cells were resistant to Dox with IC50 values > 20 μM. Conclusions, we demonstrated that different human malignant cell lines had different sensitivity to Dox. BFTC-905 BlCa cell line had the highest sensitivity to Dox. Huh7, VMCUB-1 and A549 cell lines were resistant to Dox. Perhaps, the different Dox sensitivity in different cell lines was due to the different acquisition of Dox resistance mechanisms. Huh7, VMCUB-1 and A549 cell lines could be suitable cell models to investigate the molecular mechanism of Dox resistance in different cancers.

Keywords: Doxorubicin, Cancer cell lines, Drug sensitivity, Anticancer drug resistance, Chemotherapy

Introduction

Historically, doxorubicin (Dox) is an anthracycline antibiotic produced by *Streptomyces peucetiu* isolated from the soil sample, collected from the area near the Castel del Monte in Italy in 1957, and it was first purely produced by Cassinelli [1]. Biologically, Dox has highly potent antimicrobial and antitumor activities. It was approved by FDA in 1974 as chemotherapeutic agent for the treatment of a variety of cancers including, but not limited to breast, ovary, bladder, thyroid and lung cancers as well as liquid tumors such as leukemia and lymphoma [2]. Although Dox is highly effective for killing cancer cells, it commonly causes several side effects that limits its long-term use. The serious life-threatening side effect of Dox is the Dox-induced cardiotoxicity that has been reported up to 9 - 11 % [3,4].

The mechanism of action of Dox is not fully understood, but the primary mechanisms involve intercalating into DNA and RNA, introducing DNA strand breaks, blocking DNA and RNA synthesis and inhibiting topoisomerase-II-mediated DNA repair [5-8]. Furthermore, Dox induces oxidative stress through an increased generation of reactive oxygen species (ROS) in mitochondria leading to cell injury and death, and this mechanism of action is primary mechanism responsible for the Dox-induced cardiac toxicity [9,10]. Resistance to Dox can be developed after the treatment. Basically, development of anticancer drug resistance is complex and depends on several factors such as types of cancers, duration of drug exposure, tumor microenvironment as well as genetic and epigenetic components [11]. Dox is currently the most effective chemotherapeutic drug in breast cancer, and the data suggest that activation of MAPK/ERK signaling pathway is an adaptive mechanism to help protect breast cancer cells and eventually make them become Dox-resistant [12]. In hepatocellular carcinoma (HCC), resistance to Dox is caused by the reduction of accumulated Dox in cells that is primarily due to an upregulation of ABC family efflux pumps [13]. Cancer cell lines are the simplest and cheapest, but still effective, *in vitro* models for investigating the sensitivity and resistance to chemotherapeutic drugs. To our knowledge, the comparative evidence for Dox sensitivity across several human cancer cell lines remains limited. We found only 1 study by Kibria *et al.* [14] that reported the sensitivities of various cancer cell lines to Dox, and they classified cell lines according to the Dox sensitivity into 3 groups, i.e., Dox-sensitive, Dox-moderately sensitive and Dox-resistant cells.

In this study, we aimed to investigate the sensitivity of commercially available human cancer cell lines to Dox. Cytotoxicity of Dox was evaluated using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in 10 cancer cell lines including 2 hepatocellular carcinoma (HCC) cell lines (HepG2 and Huh7), 4 bladder cancer (BlCa) cell lines (UMUC-3, VMCUB-1, TCCSUP and BFTC-905), a lung cancer cell line (A549), a cervical carcinoma cell line (HeLa), a breast cancer cell line (MCF-7) and a melanoma cell line (M21). Normal human kidney cell line (HK-2) was also tested as a representative of noncancer cell line. The half-maximal inhibitory concentration (IC50) of Dox was calculated and compared among the tested cell lines.

Materials and methods

Human cell lines

HepG2, Huh7, UMUC-3, VMCUB-1, TCCSUP, BFTC-905 and HK-2 cell lines were purchased from ATCC. M21 was obtained from the lab of Prof. Tambet Teesalue at University of Tartu, Estonia. A549,

HeLa and MCF-7 were obtained from the lab of Dr. Sergio Moya at CICbiomaGUNE, Spain. HepG2 and Huh7 cell lines were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 1× penicillin /streptomycin and 2 % non-essential amino acid. Others cell lines were cultured and maintained in the same medium, but without 2 % non-essential amino acids. Dox was purchased from Toronto Research Chemicals Inc. MTT was purchased from Invitrogen.

MTT assay

The cytotoxicity of Dox in cell lines was evaluated using the MTT assay in a dose-dependent manner. HepG2, Huh7, UMUC-3, VMCUB-1, TCCSUP, BFTC-905, A549, HeLa, MCF-7, M21 and HK-2 cells were seeded into a 96-well plate at 1×10^4 cells/well and cultured for 24 h. Before the Dox treatment, cell density of about 80 % confluency was ensured by light microscope. All cell lines were treated with 6 different concentrations of Dox including 0 (control), 1.25, 2.5, 5, 10 and 20 µM for 24 h. After the 24-hour treatment, the treated medium was removed, and MTT solution was added and incubated for 4 h. In viable cells, formazan crystals were formed due to the viable activity of mitochondrial reductase enzymes. After 4-hour incubation, the MTT solution was carefully removed from wells. Cells were lysed and formazan crystals were dissolved by dimethyl sulfoxide. Absorbance at 560 nm was measured using a microplate reader. The experiments were done in triplicate separately. Cell viability (%) was calculated from the following formula.

$$\%\ \text{Cell viability} = \frac{\text{OD}_{\text{sample}} \times 100}{\text{OD}_{\text{control}}}$$

The cell imaging was observed and captured by the EVOS cell imaging system (Thermo Fisher Scientific Inc.). All cell lines were imaged before and after the treatment with doxorubicin. Formazan crystals formed after incubation with MTT solution for 4 h were also observed under the EVOS microscope.

Statistical analysis

All data were presented with mean ± standard deviation (SD) or standard error of mean (SEM) as appropriate. One-way ANOVA was used for a comparison among treatment conditions, followed by Dunnett's multiple comparisons test. The absolute IC50 value of Dox for each cell line was calculated using the GraphPad Prism Software version 10. Graphs were created by the GraphPad Prism Software version 10. Statistically significant level was set at p value < 0.05.

Results and discussion

Cells were seeded and grown overnight before the Dox treatment (to obtain about 80 % confluent). The morphology of each cell line is shown in **Error! Reference source not found.**. UMUC-3, VMCUB-1 and M21 cells exhibited fibroblast-like shapes. All cell lines were ensured to be in good condition and free from contamination before performing Dox treatment.

Media containing Dox at concentration of 0, 1.25, 2.5, 5, 10 and 20 μM were freshly prepared. Cell survival after Dox treatment was measured by MTT assay. A549 and HeLa cells were selected to be representatives of Dox-resistant (high IC50 value) and Dox-sensitive (low IC50 value) cells, respectively, and their representative micrographs after the 24-hour Dox treatment are shown in **Error! Reference source not found.**. In HeLa cells, cells containing purple formazan crystals (reflective of viable cells) were gradually decreasing when Dox concentrations were increasing (**Error! Reference source not found.(D)**). In contrast, the number of formazan-containing cells remained unchanged (only slightly altered) in A549 cells treated with increasing Dox concentrations (**Error! Reference source not found.(B)**). These results suggested that A549 cells were more resistant to Dox than HeLa cells.

IC50 values of Dox in all tested cell lines were calculated to assess how sensitive or resistant each cell line to Dox. In this study, we calculated the absolute IC50 from the MTT data because we aimed to obtain the concentration of Dox that killed 50 % of cells. In other word, the Dox concentration that provoked a response (cell survival) halfway (50 % survival) between blank (without Dox, 100 % survival) and positive control (0 % survival) [15]. Our result showed that absolute IC50 values of Dox in HepG2, Huh7, UMUC-3, VMCUB-1, TCCSUP, BFTC-905, A549, HeLa, MCF-7, M21 and HK-2 cells were 12.18 ± 1.89, > 20, 5.15 ± 1.17, > 20, 12.55 ± 1.47, 2.26 ± 0.29, > 20, 2.92 ± 0.57, 2.50 ± 1.76, 2.77 ± 0.20 and > 20 μM, respectively (**Table 1**).

We calculated the percentile rank of all 11 calculated IC50 values. The result revealed that the 50 % percentile (median) was 12.18 μM, 25 % percentile was 2.77 μM and 75 % percentile was 20 μM. We further categorized cell lines according to the percentile rank of IC50 values into 3 groups, 1) cell lines with IC50 < 25 % percentile, 2) cell lines with IC50 between 25 and 75 % percentiles and 3) cell lines with IC50 > 75 % percentile. Finally, we arbitrarily defined those cell lines with IC50 < 25 % percentile as Dox-sensitive cells (IC50 < 2.77 μM), those cell lines with IC50 between 25 and 75 % percentiles as Dox-moderately sensitive cells (IC50 between 2.77 and 20 μM), and those cell lines with IC50 > 75 % percentile as Dox-resistant cells (IC50 > 20 μM).

According to our arbitrary classification criteria, BFTC-905, MCF-7 and M21 cell lines were classified as Dox-sensitive. HepG2, UMUC-3, TCCSUP and HeLa cells were moderately sensitive to Dox. Huh7, VMCUB-1, A549 and HK-2 cells were Dox-resistant cells.

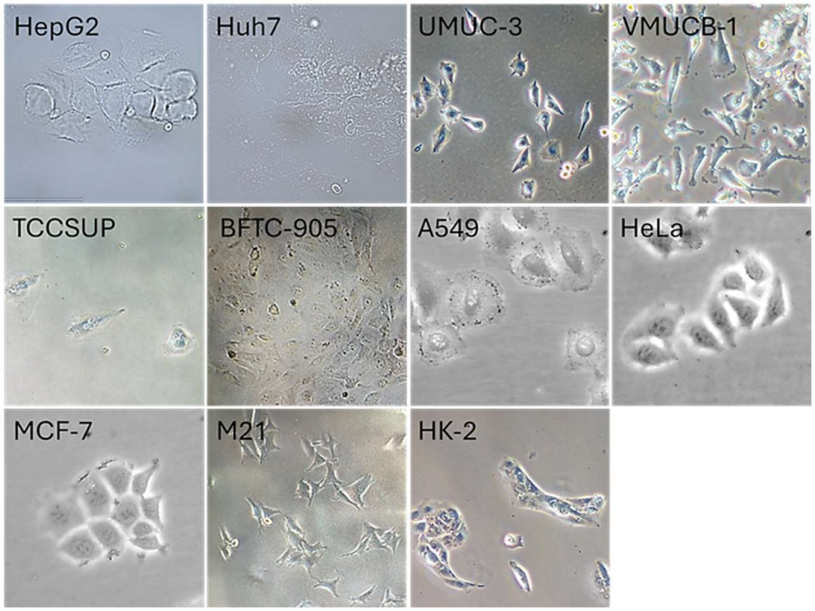

Figure 1 Representative micrographs of each cell line (before Dox treatment) imaged under the light microscope. Magnification: 400×.

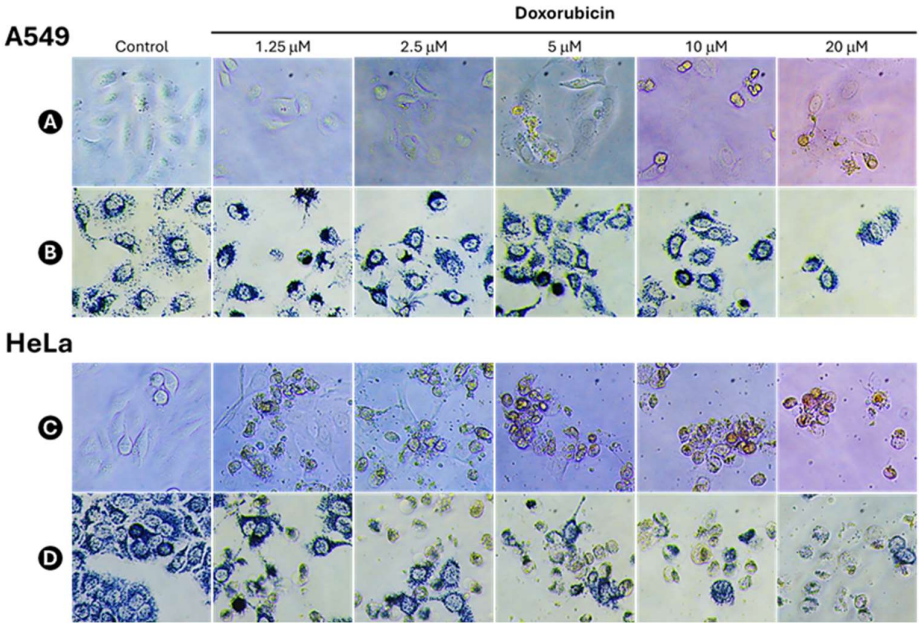

Figure 2 Representative micrographs of cells treated with Dox (24 h). A549 and HeLa cells were selected as representatives of all cell lines. (A) and (C): Micrographs of cells after 24-hour Dox treatment. (B) and (D): Micrographs of cells after 4-hour incubation with MTT, cells containing purple formazan crystals indicate viable cells. Magnification: 400×.

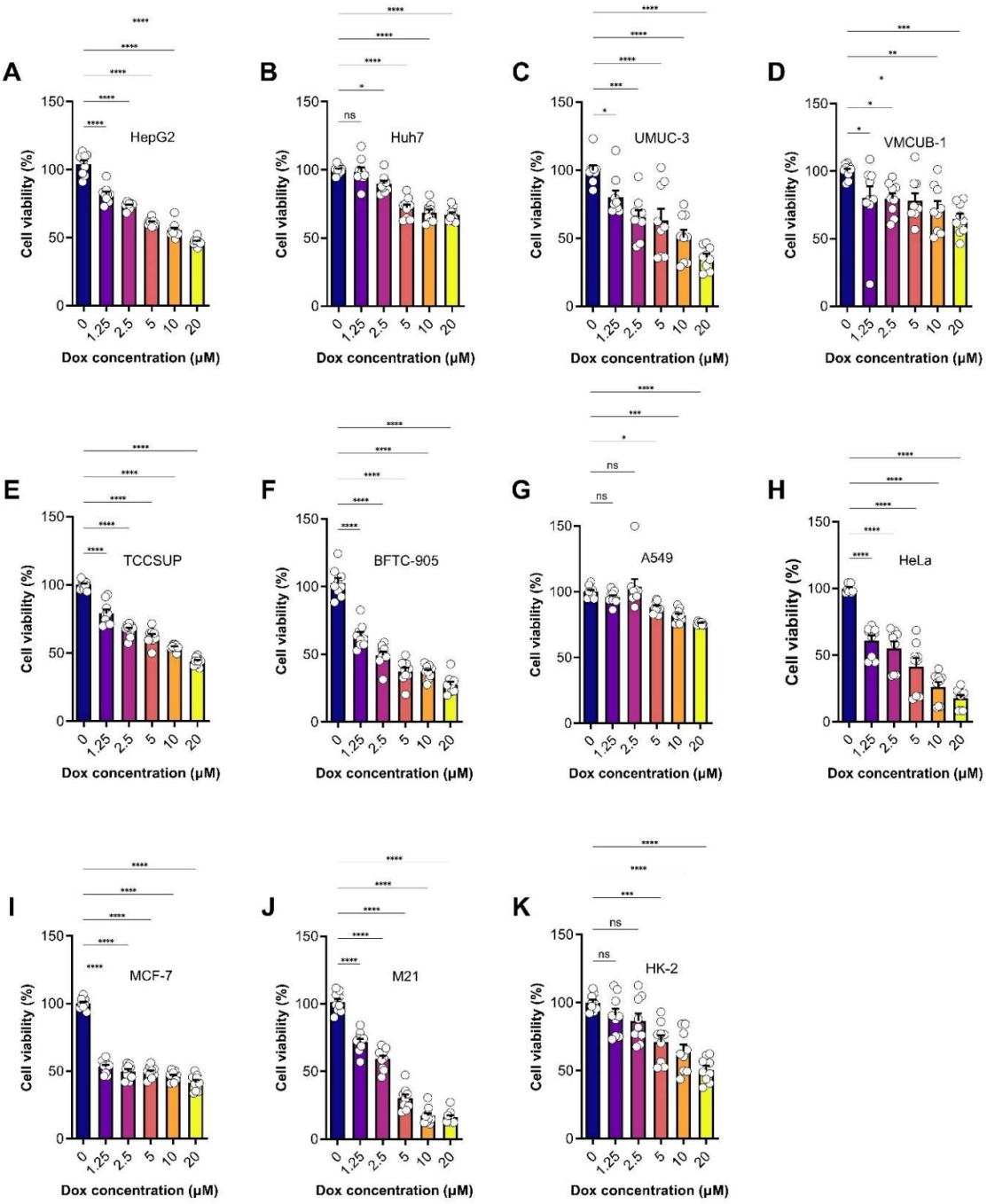

Figure 1 MTT result showing cytotoxicity of Dox in each cell line. (A) HepG2, (B) Huh7, (C) UMUC-3, (D) VMCUB-1, (E) TCCSUP, (F) BFTC-905, (G) A549, (H) HeLa, (I) MCF-7, (J) M21 and (K) HK-2. Bars and error bars indicate mean ± SEM. *, **, *** and **** indicate statistically significant. ns: Not statistically significant.

Table 1 IC50 and area under curve values of Dox in each tested cell lines.

Tissues sources	Cell lines	IC50 (μM)	Area under curve
Hepatocellular carcinoma	HepG2	12.18 ± 1.89	1,142 ± 44.6
	Huh7	> 20	1,475 ± 53.4
Bladder cancer	UMUC-3	5.15 ± 1.17	942 ± 85.1
	VMCUB-1	> 20	1,530 ± 114.5
	TCCSUP	12.55 ± 1.47	1,145 ± 27.4
	BFTC-905	2.26 ± 0.29	792.1 ± 52.7
Lung cancer	A549	> 20	1,700 ± 40.8
Cervical cancer	HeLa	2.92 ± 0.57	682 ± 93.7
Breast cancer	MCF-7	2.50 ± 1.76	960 ± 39.9
Melanoma	M21	2.77 ± 0.20	586 ± 50.9
Human kidney (noncancer)	HK-2	> 20	1,339 ± 107.5

In discussion, IC50 is the most widely used parameter for initially screening the efficacy of the drug of interest, because it indicates how much drug is required to inhibit the biological process of interest by 50 % [16]. In case of anticancer drug, IC50 indicates the dose of drug needed to kill cancer cells by half. The method that is mostly employed for measuring the IC50 value is MTT assay [17]. The purpose of the present study was to determine the sensitivity to Dox using MTT assay in the human cancer cell lines that are commonly used in several laboratories. We found that the IC50 values of Dox in tested cell lines varied from 2.26 to > 20 μM. The exact IC50 values of Dox in Huh7, VMCUB-1, A549 and HK-2 cells could not be calculated, because even at the highest concentration of Dox (20 μM) these cells could not be killed by half. Therefore, the IC50 for Dox in these 4 cell lines were certainly > 20 μM. Based on the percentile rank of IC50 values, we classified BFTC-905, MCF-7 and M21 cell lines as Dox-sensitive cells (IC50 < 2.77 μM), HepG2, UMUC-3, TCCSUP and HeLa cells as Dox-moderately sensitive cells (IC50 between 2.77 and 20 μM), and Huh7, VMCUB-1, A549 and HK-2 as Dox-resistant cells (IC50 > 20 μM). Furthermore, A549 had the highest area under curve compared with others (**Table 1**), suggesting that it was the most Dox-resistant cell line.

The Genomics of Drug Sensitivity in Cancer Project (GDSC project, a collaboration between the Cancer Genome Project at the Wellcome Sanger Institute (UK) and the Center for Molecular Therapeutics, Massachusetts General Hospital Cancer Center (MGH, USA) is known as the large drug sensitivity screening study for anticancer therapeutics in more than 1,000 human cancer cell lines. In this project, IC50 values for therapeutic agents were measured by fluorescent Syto60 staining and resazurin assay, and they are publicly available (https://www.cancerrxgene.org/) [18]. The IC50 values for Dox were screened in 870 cell lines at MGH, USA. They reported the IC50 values for Dox in TCCSUP, UMUC-3, BFTC-905, HeLa, MCF-7, Huh7 and A549 at 0.60, 0.09, 0.02, 0.14, 0.01, 0.27 and 0.24 μM, respectively [19]. It should be noted that although the IC50 is very useful for screening the anticancer efficacy of the drug, the IC50 values obtained from different laboratories are hardly comparable, mainly because those measurements are carried

out under different conditions, for instance, different cell passage numbers [20]. This could be the reason why our reported IC50 values were different from those reported in the GDSC project. However, our data and GDSC data provided the same conclusion that BFTC-905 and MCF-7 were markedly more sensitive to Dox than Huh7 and A549.

Our data also corresponded well with the results from a study by Dubbelboer *et al.* [21]. They reported that the IC50 values of Dox for HepG2 and Huh7, measured by resazurin reduction assay (24-hour treatment), were 1.3 ± 0.18 and 5.2 ± 0.49 μM, respectively. We also demonstrated that Huh7 was remarkably more resistant to Dox than HepG2. Study by Louisa *et al.* [22] showed that HepG2 cells expressed the drug transporter genes (i.e., P-glycoprotein, OATP1B1 and OCT1) lower than Huh7 cells. Our previous study showed that Huh7 was more aggressive and more resistant to H_2O_2 than HepG2 [23]. These might be the explanations why HepG2 is more sensitive to Dox than Huh7.

For BlCa cell lines, we found that VMCUB-1 was more resistant to Dox than TCCSUP, UMUC-3 and BFTC-905, respectively. To our knowledge, there is no study that compared the IC50 values of Dox among these 4 BlCa cell lines. However, our previous study showed that VMCUB-1 had a more aggressive phenotype than TCCSUP and UMUC-3, and it might be an indirect explanation for increased Dox resistance in VMCUB-1 [24,25]. Further study should be performed to explore the Dox resistance mechanism in VMCUB-1 cell line.

Study by Kibria *et al.* [14] showed that HeLa, A549 and HepG2 cell lines were Dox-sensitive compared with very low EC50 values (determined by WST-8 cytotoxicity assay) of 0.012, 0.001 and 0.0003 μM, respectively. Our finding was discrepant with this result. We found that A549 was Dox-resistant, but HeLa and HepG2 were moderately sensitive to Dox. Differences in assay and cell passage number might be responsible for this discrepancy. In the previous study, NRF2 is one of the regulators of anti-oxidant protein expression [26]. Increasing ROS and oxidative stress increased NRF2 expression and led to increasing anti-oxidant protein [27]. However, one of the DOX mechanisms is inducing ROS in cell [28]. Moreover, A549 cells have NRF2 expression higher than basal expression of NRF2 [29]. Therefore, increasing ROS from DOX could be reduced by anti-oxidant protein via increasing NRF2 expression. In the other studies, however, the IC50 values of Dox in HeLa cells were reported at 1.39 μM (measured by neutral red assay) and 0.34 μM (by MTT assay) [30] that was comparable to the IC50 value (2.9 μM) observed in our study.

In breast cancer MCF-7 cell line, the IC50 value for Dox was observed at 2.50 μM, and we classified it as Dox-sensitive cells. Other studies reported that the IC50 values for Dox in MCF-7 were 0.1 μM (by MTT assay for 3 - 5 days) [31] and 0.68 μg/mL (1.25 μM, by MTT assay for 48 h) [32]. By contrast, the IC50 value of Dox tested in Dox-resistant MCF-7 cells was reported at 1.9 μM [33]. Again as mentioned above, the IC50 values between laboratories cannot be directly compared due to several uncontrolled factors such as assay, treatment condition and genetic and epigenetic heterogeneities of cell lines [20]. For human skin melanoma M21 cell line, we did not find a report of IC50 for Dox in the literature. Based on findings in this study, we concluded that our M21 cell line was sensitive to Dox.

We found that our HK-2 cells were resistant to Dox, even though HK-2 cells were derived from normal human kidney cells. According to the literature, functional p53 is required for cytotoxicity of doxorubicin by promoting apoptosis in cancer cells [34]. The presence of mutant p53 causes reduced apoptosis leading to Dox resistance. Detection of p53 mutation in the HK-2 cells should be further investigated to explain the Dox-resistant phenotype of HK-2 seen in this study.

To study the mechanism of how cells overcome the cytotoxicity of Dox, both *in vitro* (both 2D and 3D) and *in vivo* studies are needed. Protein and mRNA expression of genes related to Dox (both signaling pathway and efflux pumps) are measured to compare between Dox-resistant cells and Dox-sensitive cells. The resistant mechanism proposed from *in vitro* findings should be further verified by animal model.

Limitations of the present study should be mentioned. The number of cell lines tested in this study (n = 11) was small. Expansion to other human cancer cell lines should be carried out in further study. *In vivo* animal models should be performed to warrant the Dox sensitivity observed in this *in vitro* study. Insight into mechanism of Dox resistance in Huh7, VMCUB-1 and A549 cancer cell lines was not explored in the present study.

Conclusions

In conclusion, we reported the IC50 values of Dox in 10 human cancer cell lines (HepG2, Huh7, UMUC-3, VMCUB-1, TCCSUP, BFTC-905, A549, HeLa, MCF-7 and M21) and a noncancer cell line (HK-2). The findings showed that different cancer cell lines had different sensitivity to Dox. BFTC-905 BlCa cell line had the lowest IC50 value, indicating the highest sensitivity to Dox. By contrast, Huh7 (HCC), VMCUB-1 (BlCa) and A549 (lung cancer) cancer cell lines were resistant to Dox. Perhaps, the different sensitivity of Dox in different cell lines was due to the different acquisition of Dox resistance mechanisms. Huh7, VMCUB-1 and A549 cell lines could be suitable cell models for investigating the mechanistic pathways of Dox resistance in different types of cancers.

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