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Research Article

Anticancer Activity of Mixed Doxorubicin and Pravastatin in Nanoemulsions Against HCT 116 Colon Cancer Cells

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ABSTRACT

Combining drugs with different mechanism of action in nanocarriers is becoming a promising strategy in cancer therapy. In the present study, the anticancer activity of the combination of doxorubicin (DOX) and pravastatin (PRV) loaded in nanoemulsions (NEs) was evaluated in HCT 116 colon cancer cells. The NE formulas (NEa and NEb) consisted of different weight fractions of the surfactant mixture of Eumulgin HRE 40/ Soya phosphatidylcholine/ sodium oleate at a fixed weight ratio of 3.5:3.0:3.5, cholesterol (CHO), Tris- HCl buffer (pH 7.22), and 1-octanol. The cytotoxicity of the drug formulas, loaded in either water or NEs, was assessed through 3-(4, 5 Dimethylthiazole- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay, while the mechanism of cell death was determined by observing the morphological changes of treated cells under light microscope and identifying apoptosis by using the ApopNexin FITC kit and DAPI nuclear staining. It has been found that reducing the concentration of DOX from 15 to 7.5µM by formulating it with 7.5µM of PRV in NEa (NEa (1 DOX:1 PRV)) has preserved its cytotoxicity against HCT-116 cancer cells. The present study proved that the combination of the PRV and DOX loaded in NEa formulations improved the therapeutic potential of both of PRV and DOX as anticancer drugs.

Keywords: Combination chemotherapy, Nanoemulsions, Cytotoxicity, Apoptosis, antitumor activity.

INTRODUCTION

Cancer, the second most common causes of death worldwide, is one of the most debilitating disease that continue to mystify researchers and clinicians[1]. In spite of the extensive researches on cancer therapy, the severe side effects of the chemotherapeutic agents is restraining its antitumor effect[2]. Mixing drugs in nanocarriers is grabbing the attention of the pharmaceutical industries due to their synergestic antiproliferative effects, decreased toxicity attributed by the lone drug and the suppression of the cancer cell resistance[3]. Pravastatin (PRV), a hydrophilic statin, is an HMG-CoA reductase inhibitor used to lower the cholesterol in the blood. Many research studies have reported that statins could have antitumor effect by impeding the cancer cell growth[4-6]. Doxorubicin (DOX), an anthracycline chemotherapeutic agent, is extensively used as anticancer drug. However, its cardiotoxicity is adversing its therapeutic effect[7]. Mixing the drugs in nanoparticles would facilitate the permeation and accumulation of drugs in the cancer cells without affecting the normal cells. Nanoemulsions (NEs) are heterogeneous colloidal systems that consist of oil, water, 5 -10 % of surfactant and most frequently cosurfactant. They require large input of energy to produce single transparent phase containing nano droplets that has diameter of 20 -200 nm[8]. The microstructure of the nanodroplets can be micelles (o/w), reverse micells (w/o) or bicontinuous. NEs holds great potential to act as nanocarriers for many pharmaceutics and nutraceutics agents[9]. The objective of the present study was to *in vitro* evaluate the NE formulations designed by Alkhatib and Albishi[10] with some modifications as a drug carrier for the mixed PRV and DOX applied into HCT-116 cells.

MATERIALS AND METHODS

Chemicals and subjects

Doxorubicin (DOX) hydrochloride and pravastatin (PRV) sodium were purchased from U.S. Pharmacopeia (Rockville, US). Soya phosphatidylcholine (SPC), cholesterol (CHO), sodium oleate (SO), Tris (hydroxymethyl) aminomethane, 1-octanol, Polyoxyethylenglycerol trihydroxystearate 40 (Eumulgin® HRE 40, EU), dimethyl sulfoxide (DMSO), Penicillin-Streptomycin antibiotic solution were purchased from Sigma Aldrich (Missouri, US). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), 10 mM phosphate buffer saline (PBS) (pH 7.4), Coomassie blue and 4', 6-Diamidino-2-Phenylindole, dihydrochloride (DAPI) were purchased from Invitrogen life technologies (New York, US). The 3-(4, 5 Dimethylthiazole- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) cell proliferation assay kit was obtained from the Cayman's Chemical Company (Michigan, US). ApopNexin Annexin V FITC Apoptosis Kit was purchased from Millipore (MA, US). Trypsin was obtained from HyClone (Utah, US). The HCT-116 colon cancer cell line was procured from from America Type Tissue Culture Collection (Manassas, VA, USA).

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Preparation of drug formulas

NE formulas were produced as described elsewhere[10] with some modifications. The different blank o/w NE formulations (NEa, NEb) were prepared by mixing different weight fractions of the surfactant mixture of EU/SPC/SO at a fixed weight ratio of 3.5:3.0:3.5, the oil phase of CHO, the aqueous phase of 0.1 M Tris-HCl buffer (PH 7.22), and the cosurfactant of 1-octanol, as illustrated in Table 1. The components of the NE formulas were blended in subsequent steps. First, the mixture, EU/SPC/SO, was mixed well and homogenized. Then, 1-octanol was added dropwise followed by mixing CHO slowly. Finally, the desired fraction of the aqueous phase was added at a slow rate with gradual stirring. For the mixture to be clear and transparent, it was vortexed and kept in the water bath at 90 ºC for 3 h. The resulting blank NE formulas were stored at room temperature. The desired NE drug formulas, prepared by directly dissolving 1mg/ml of either DOX, PRV or their combination to the selected blank NE formula, were DOX-loaded-NEa (NEa (DOX)), PRV-loaded-NEa (NEa (PRV)), a 1:1 ratio of DOX: PRV – loaded-NEa (NEa (1 DOX: 1 PRV)), a 1:2 ratio of DOX: PRV –loaded NEa (NEa (1 DOX: 2 PRV)), DOX-loaded-NEb (NEb (DOX)), PRV-loaded-NEb (NEb (PRV)), a 1:1 ratio of DOX: PRV – loaded-NEb (NEb (1 DOX: 1 PRV)), and a 1:2 ratio of DOX:PRV –loaded NEb (NEb (1 DOX: 2 PRV)). Further drug formulas were formed by replacing the blank NE by water and were designated as W (DOX), W (PRV), W (1 DOX: 1 PRV) and W (1 DOX: 2 PRV). The micromolar composition of the drug fromulas in illustrated in Table 2.

In Vitro evaluation of antitumor activity

Cell culture

HCT-116 colon cancer cells were grown in a tissue culture flask (25 cm^2) containing 6 ml of DMEM supplemented with 10 % (v/v) heat inactivated FBS and 1% (v/v) Penicillin-Streptomycin antibiotics at 37 °C in a 95 % air and 5 % humidified CO_2 incubator. Spent medium was discarded from the tissue culture flask and changed at 48 h intervals. Cells were fed until confluence and the confluent cells were split by washing with 3 ml of 10 mM PBS (pH 7.4). After that, PBS was replaced with 2 ml of trypsin (0.15 %) in order to dissociate the cells. After 2 min of incubation, a 4 ml of DMEM media was added. The experimental cells were incubated in 10 % of DMEM culture medium for 24 h in a 95 % air and 5 % humidified CO_2 incubator at 37 °C.

MTT assay for cell proliferation

The effect of DOX, PRV and their combination on the proliferation of HCT-116 cells was measured by the MTT assay. Cells were seeded in 96-well plate at a density of 5 $x10^3$ cells per well in 100 µl culture media and incubated for 24 h at 37 °C in a humidified 5 % CO_2 (70–80% confluent). Cells were treated with 100 µl of 1, 10 and 15 µM of the examined drug formula. Triplicate wells were prepared for each individual formulation and re-incubated for 48 h at 37 °C in a humidified 5% CO_2. Untreated cells were used as control. At the end of the incubation, a 5 µl of MTT reagent was added to each well followed by incubating the cells for 4 h at 37°C in CO_2 incubator. After the incubation time, the culture medium was discarded from each well carefully to prevent disruption of the cell monolayer followed by the addition of 100 µl of the crystal dissolving solution to each well to dissolve the formazan crystals and produce a purple color. Finally, cells were shaken for 10 min and the absorbance of each sample was measured at a wavelength of 562 nm by ELISA plate reader (BioTek, US). The cytotoxicity of the drugs on the cells was determined according to the percentages of cell viabilities calculated by dividing the optical density (OD) of the treated cells by the OD of the untreated cells.

Light microscopy for cell morphology characterization

Cultured cells of HCT-116 were counted and cultured at a density of 1 $x10^5$ cells per well into 24-well, flat-bottomed tissue culture plates containing 500 µl of growth medium per well. Cells were incubated with 500 µl of 15µM of desired drug formula for 48 h. After that, they were washed twice with PBS for 5 min and fixed by the addition of 4 % formaldehyde. After 5 min, the fixation solution was discarded and the cells were stained with 10 % Coomassie blue dye for 10 min. Finally, the stain was washed with distilled water twice, and left to dry one hour at room temperature. The morphological changes were observed by phase contrast inverted microscope (1X2-SP Olympus, Japan).

ApopNexin FITC assay for apoptosis detection

The HCT 116 cells were cultured in a 24-well plates (5×10^4 cells per well) and incubated for 24 h. Cells were treated with 500µl of 15 µM of the desired drug formula and incubated for 48 h. Then, cells were rinsed with 300 µl 10 mM PBS (pH 7.4). After that, PBS was discarded and cells were dissociated with 200 µl of trypsin (0.15 %). After 2 min incubation, a 500 µl DMEM media was added to each well. Then, cells were transferred to a flow cytometry tube, precipitated by centrifugation at 1000 rpm for 5 min, and washed twice with cold 10mM PBS (pH 7.4). After removing the supernatant, cells were re-suspended in a 200 µl of ice cold 1X binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 10^6 cells/ml. Finally, the cell suspension was mixed with a 3 µl of FITC and 2 µl of PI, incubated for 15 min at room temperature in the dark and placed on ice to be analyzed by flow cytometer (BD Biosciences, US). The positive of Annexin FITC indicate the release of PS, which happens in the early stage of apoptosis. Therefore, the apoptotic cells were identified as Annexin FITC+ and PI−. The nonviable cells were identified as Annexin FITC+ and PI+, viable cells as Annexin FITC− and PI− and the inflammation cells as Annexin FITC− and PI+.

DAPI assay for nuclear staining

DAPI is a blue fluorescent stain that binds strongly to A-T rich regions in the dsDNA. When DAPI is added to the cells, undergoing apoptosis, it is rapidly taken into the cellular DNA yielding highly fluorescent nuclei and no detectable cytoplasmic fluorescence. DAPI staining revealed that nuclei with chromatin condensation and apoptotic bodies can be assessed by the intensity of DAPI labeling and the shape of the chromatin. The HCT 116 cells were counted and cultured in a 24-well plates (5×10^4 cells

Table 1: The blank NE formulations prepared at different weights of the surfactant mixture EU/SPC/SO at a fixed weight ratio of 3.5:3.0:3.5, respectively, and CHO. The weight fraction of the aqueous phase of 0.1 M Tris-HCl buffer (pH 7.22) and the cosurfactant of 1-octanol were fixed at 99.9 and 0.1, respectively.

Component	Weight (g)	
	NEa	NEb
SPC	0.0375	0.0600
SO	0.0437	0.0700
EU	0.0437	0.0700
CHO	0.0125	0.0050

SPC: Soya phosphatidylcholine; SO: Sodium Oleate; EU: Eumulgin® HRE 40; CHO: Cholesterol.

per well) and incubated for 24 h. Cells, treated with 500 µl of 15 µM of the examined drug formula, were incubated for 48 h and rinsed with 500 µl of 10 mM PBS (pH 7.4). After that, a 300 µl of DAPI reagent was added to each well followed by incubation for 1-5 min in a humidified 5 % CO_2 incubator. Finally, the cells were rinsed several times with PBS and the excess buffer was drained from plate. Cells were viewed under a fluorescence microscope (Leica DMI6000 B, Germany) with appropriate filters at 461 nm.

Statistical analysis

The discrepancies between the samples were identified by using the MegaStat Excel (version 10.3, Butler University) for the hypothesis testing, one- way analysis of variance (ANOVA) and two- way ANOVA. The considerable differences were determined when the p-value was less than 0.05.

RESULTS

MTT assay for cell proliferation

The effect of the different concentrations of the solution and NE formulations on the percentages of cell viability of HCT-116 cells is displayed in Table 3. Regarding the DOX formulations, NEb (DOX) was the most cytotoxic formula at 1 µM while NEa (DOX) was the most cytotoxic formula at 10 µM. However, all of the DOX formulations were having similar cytotoxicity at 15 µM. In contrast, PRV, loaded in either water or NE's, did not show any cytotoxic effect at all of the selected concentrations. Formulating different ratios of 1:1 or 1:2 of DOX to PRV in NEa at different concentrations did not result in a change in the cytotoxicity against HCT-116. Nevertheless, NEa formulations were having more cytotoxicity than the other formulas. On the other hand, NEb combination formulas have shown cytotoxicity at only 1 and 15 µM when DOX and PRV were mixed at 1:1 ratio, but they showed slight toxicity when DOX and PRV were combined in 1:2 ratio, respectively. In contrast, the combination formulas in water were cytotoxic at only 15 µM. Interestingly, the blank NEa and NEb were having increased and similar cytotoxicity at 15 µM but very slight toxicity at 1 and 10 µM. Based on the results of the anti-proliferative MTT assay, it has been found that the best inhibitory effect of the combination formulas in either water or NE was observed at 15 µM. Additionally, NEa have more inhibition effect than NEb on the HCT 116 cells. NEa (1 DOX: 1PRV) was considered for more in-depth studies on its antitumor activity as it has the best inhibitory effect on the HCT 116 cells.

Light microscopy for cell morphology characterization

HCT-116 cells were slightly affected by NEa, W (PRV) and NEa (PRV) as demonstrated in Figure 1. In contrast, they were more affected by W (DOX), NEa (DOX), NEa (1 DOX: 1 PRV) and W (1 DOX: 1 PRV) as more intracellular spaces between the cells, membrane blebbing, chromatid condensation and fragmentation were observed.

ApopNexin FITC assay for apoptosis detection

ApopNexin FITC apoptosis detection kit contain double staining with two fluorescents, FITC/PI, can distinguish between necrotic (Q1), viable (Q3), early apoptotic (Q4) and late apoptotic (Q2) cells. As displayed in Figure 2 and illustrated in Table 4, among all of the formulations, NEa (DOX) was the only formula that induced apoptosis as the sum of the percentages of the early and late apoptotic cells was more than 30%. PRV, formulated in either water or NE, was having a necrotic effect on the HCT-116 cells as the percentages of the necrotic were more than 30%. Combining PRV with DOX in either water or NEa have reduced the apoptotic effect of DOX on HCT-116.

DAPI assay for nuclear staining

DAPI assay was employed in order to detect the effect of the drug formulations on the DNA of the cells. The nuclei of HCT-116 cells were slightly stained when subjected into NEa, W (PRV), NEa (PRV) as viewed in Figure 3. In contrast, among all of the formulas, the greatest numbers of nuclei, that were condensed, got enlarged and have strongly fluoresced, were detected in NEa (DOX) and W (DOX) treated cells. Interestingly, incorporating PRV into the DOX formulas (W (1 DOX: 1 PRV) and NEa (1 DOX: 1 PRV)) has resulted in the formation of the digestive vesicles in the treated cells as the enlarged and condensed nuclei have contained black spots indicating that the induced apoptosis was integrated with autophagocytosis.

DISCUSSION

In this study, it has been found that the best inhibitory effect of the combination formulas in either water or NE was observed at 15 µM according to the results of the cytotoxicity screening using the MTT assay. Additionally, NEa, composed of less surfactant mixture and more oil fraction relative to NEb, have a more inhibitory effect than

Table 2: The micromolar composition of the prepared drug formula, loaded in either water or NE, when subjected into the cells.

Concentration (µM)	Micormolar composition of the drug formulas			
	DOX	PRV	1 DOX : 1 PRV	1 DOX : 2 PRV
1	1	1	0.5 DOX: 0.5 PRV	0.33 DOX: 0.66 PRV
10	10	10	5 DOX: 5 PRV	3.33 DOX: 6.66 PRV
15	15	15	7.5 DOX: 7.5 PRV	5 DOX: 10 PRV

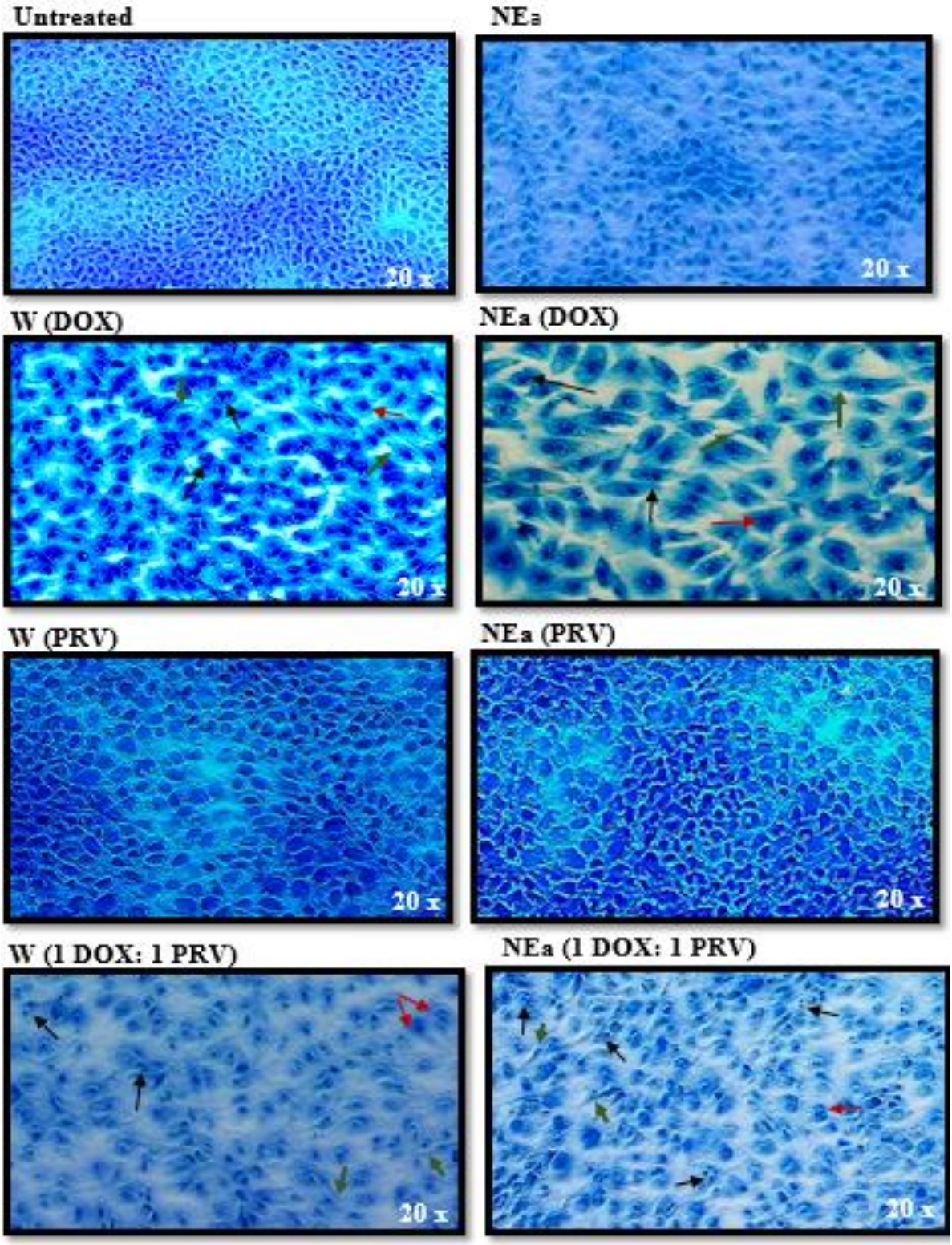

Figure 1: Light microscopy images of HCT-116 colon cancer cells treated with 15 µM of water and NE formulations. Black arrows represent chromatin fragmentation; green bold arrows represent membrane blebbing; and red arrows represent chromatin condensation.

NEb which could help the nanocarriers to permeate the cells more efficiently[11]. Based on the MTT assay, morphological changes seen under light microscopy and DAPI labeling, and according to the results of the ApopNexin FITC kit, among all of the treated cells, NEa (DOX), NEa (1 DOX: 1 PRV), W (DOX) and W (1 DOX: 1 PRV) have similar and highly cytotoxic effect as the morphology changes and signs of apoptosis were seen. It is interesting to note here that although NEa (1 DOX: 1 PRV) contained less amount of DOX by half than the NEa (DOX) and W (DOX), it was having a similar toxic effect on HCT-116 cells, indicating that incorporating PRV into

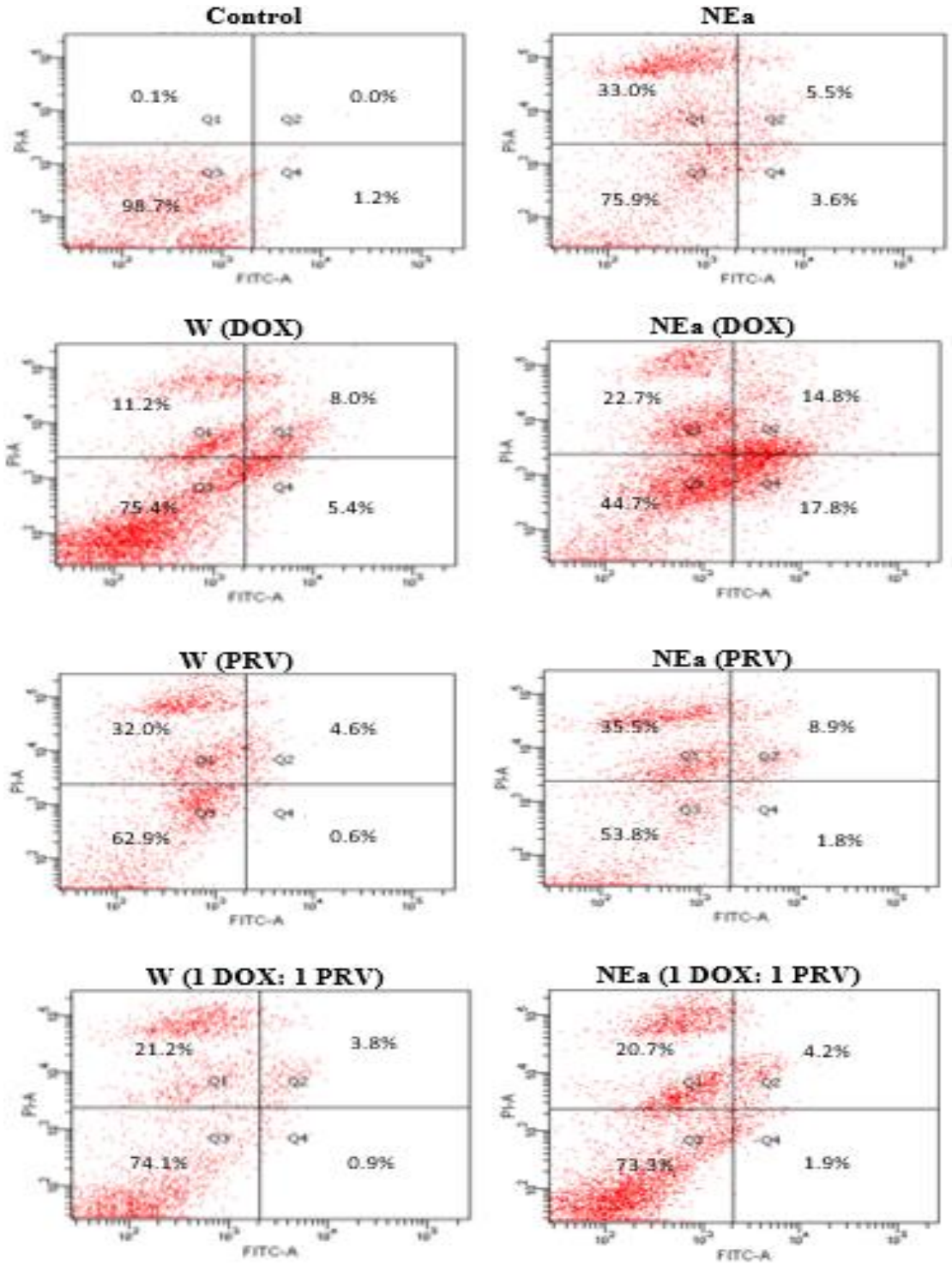

Figure 2: FITC/PI flow cytometry plot of drug formulations subjected into HCT-116 cells.

the combination formula augmented the cytotoxicity and apoptotic effect of DOX. In agreement with our study, Werner *et al.*[12] found out that incorporating simvastatin with DOX caused a considerable enhancement in impeding the activity of topoisomerase II and hence resulted in the cleavage of DNA double strand of the cancer cells. Furthermore, previous studies demonstrated that the lovastatin ameliorated the anti-proliferative effect of DOX and diminished its cardiotoxicity in the mice[13,14]. It is worth mentioning that PRV administered into mice treated with cisplatin has reduced its nephrotoxicity by suppressing the stress of the reactive oxygen and nitrogen species[15]. Another study performed by Cheng *et al.*[16] found out that PRV has protected the cardiac tissue *in vivo* and in cultured cells from the toxicity caused by carboplatin. The single treatments of PRV, loaded in either NEa or water, have shown a slight cytotoxic effect and caused little morphological changes in the HCT-116 cells. In agreement with this study, Menter *et al.*[17] demonstrated that PRV was less effective than simvastatin in impeding the growth of

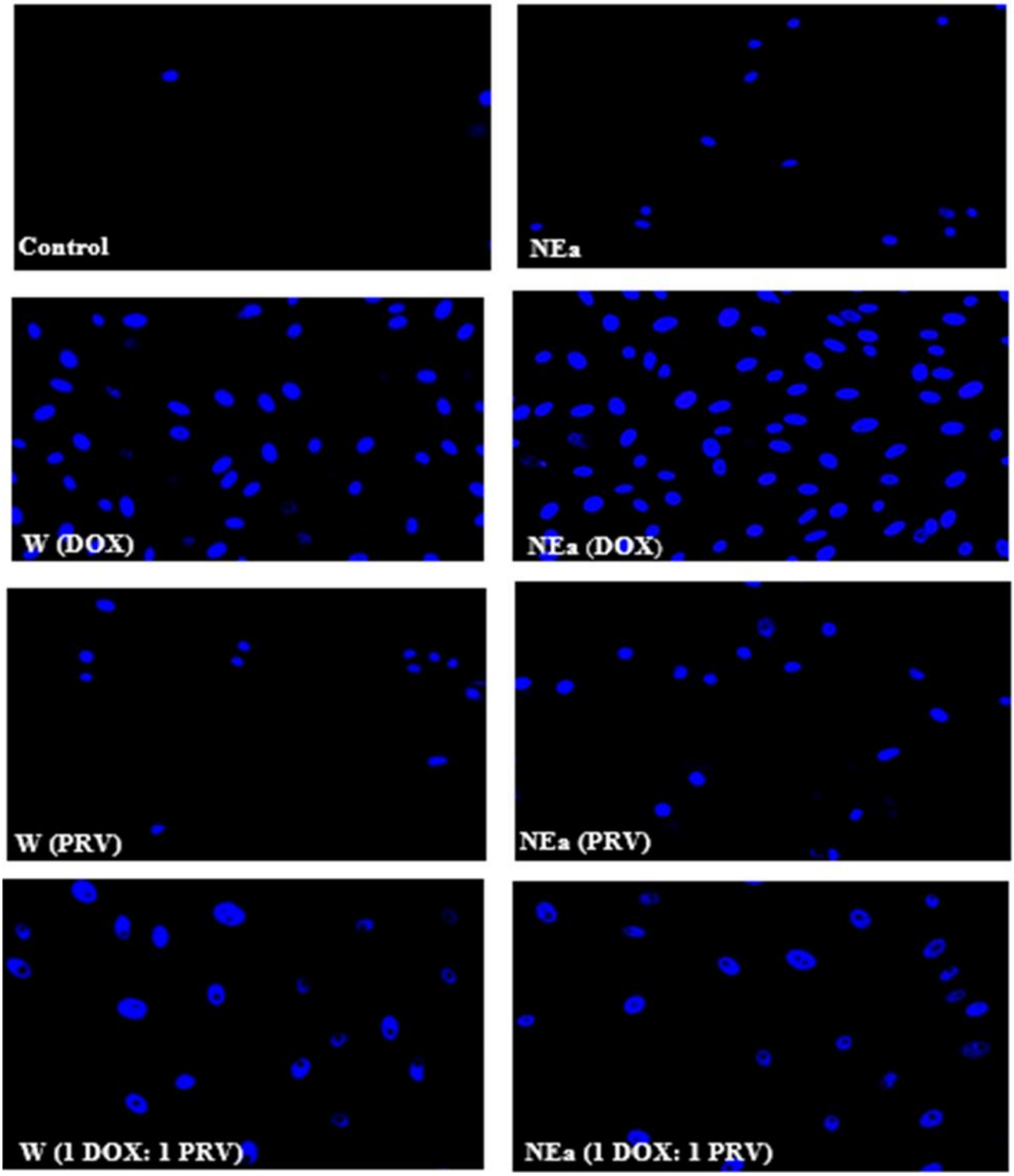

Figure 3: Fluorescence microscopic images of HCT-116 colon cancer cells treated with different drugs formulations, labeled with DAPI and magnified at 20x.

most cancer cells. In fact, the hydrophilicity of PRV minimizes its penetration into the non-hepatic tissues[18].

CONCLUSION

In this study, two NE formulations, differ in oil fractions and surfactant contents, were evaluated *in vitro* as a drug carrier for the mixed PRV and DOX applied into HCT-116 cells. It has been found that mixing 7.5µM of DOX and 7.5µM of PRV in NEa which has more oil fraction and lesser surfactant amounts has similar antiproliferative effect as the single treatment of 15µM of DOX solubilized in NEa. Based on this study, it is recommended to perform further *in vivo* researches in order to give a complementary study of the NEa combination formulas effect on the body tissues.

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Table 3: Cytotoxic screening of 1, 10 and 15 µM of the water and NE formulations subjected into HCT-116 cells. The percentages of cell viabilities were expressed as $\bar{X}\pm$ SD.

	Cell Viability (%)		
Formula	1 µM	10 µM	15 µM
W (DOX)	93.89 ± 3.02	63.31 ± 3.38	53.46 ± 5.9
NEa (DOX)	80.69 ± 11.99	46.93 ± 8.73	52.85 ± 8.23
NEb (DOX)	43.10 ± 6.89	87.57 ± 8.14	41.99 ± 16.23
W (PRV)	90.54 ± 3.20	99.82 ± 11.68	87.98 ± 16.21
NEa (PRV)	85.68 ± 17.22	79.64 ± 11.00	92.42 ± 16.79
NEb (PRV)	83.52 ± 8.42	97.39 ± 4.23	78.76 ± 7.06
W (1 DOX: 1 PRV)	82.49 ± 18.70	108.85 ± 14.15	62.29 ± 6.99
NEa (1 DOX: 1 PRV)	66.49 ± 12.96	68.51 ± 6.94	52.96 ± 0.72
NEb (1 DOX: 1 PRV)	53.04 ± 9.84	90.81 ± 6.12	60.48 ± 11.83
W (1 DOX: 2 PRV)	95.25 ± 3.62	85.93 ± 8.84	76.62 ± 21.10
NEa (1 DOX: 2 PRV)	51.40 ± 1.08	58.39 ± 9.12	60.92 ± 4.69
NEb (1 DOX: 2 PRV)	84.61 ± 10.38	82.03 ± 7.18	84.96 ± 14.29
NEa	98.95 ± 10.14	92.95 ± 4.68	67.73 ± 4.76
NEb	87.84 ± 1.95	101.35 ± 3.92	61.80 ± 6.04

Table 4: The percentages of the HCT-116 cells distinguished between necrotic (Q1), viable (Q3), early apoptotic (Q4) and late apoptotic (Q2) when subjected into different formulations.

Formula	Q1	Q2	Q3	Q4
Control	0.1	0	89.7	1.2
NEa	33	5.5	57.9	3.6
W(DOX)	11.2	8	75.4	5.4
NEa (DOX)	22.7	14.8	44.7	17.8
W(PRV)	32	4.6	62.9	0.6
NEa (PRV)	35.5	8.9	53.8	1.8
W(1 DOX: 1 PRV)	21.2	3.8	74.1	0.9
NEa (1 DOX: 1 PRV)	20.7	4.2	73.3	1.9

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