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RESEARCH ARTICLES

Fluorescence-Based Assays for Measuring Doxorubicin in Biological Systems

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Kauffman MK et al. Reactive Oxygen Species 2(6):432–439, 2016; ©2016 Cell Med Press
http://dx.doi.org/10.20455/ros.2016.873
(Received: October 3, 2016; Revised: October 18, 2016; Accepted: October 19, 2016)

ABSTRACT | Detection and measurement of doxorubicin in biological systems, including body fluids, cells, and tissues, are instrumental in understanding the mechanisms of action of this widely used drug in treating cancer as well as in causing adverse effects. In this article, we, for the first time, characterized the use of fluorescence-based techniques, including fluorescence spectrometry, microscopy, and flow cytometry in measuring and/or detecting doxorubicin in biological systems, including cell lysates and cultured intact cells. We showed that doxorubicin has a maximum excitation and emission wavelength of 470 and 560 nm, respectively. The detection sensitivity by fluorescence spectrometry is less than 0.1 μM in buffers and cell lysates. Fluorescence microscopy demonstrated the readily detection of concentration-dependent accumulation of doxorubicin in cultured cells via either green or red fluorescence, but with green fluorescence showing a higher sensitivity of detection. Flow cytometry also revealed sensitive detection of doxorubicin accumulation in cell suspensions in a concentration-dependent manner. The readily and sensitive measurement and detection of doxorubicin by the above three fluorescence-based techniques has important implications in studying the cellular dynamics of doxorubicin in both cancer and normal cells under various experimental conditions.

KEYWORDS | Fluorescence; Flow cytometry; Doxorubicin; Microscopy; Spectrometry

ABBREVIATIONS | CPBS, complete phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; ROS, reactive oxygen species

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1. INTRODUCTION

Doxorubicin is one of the most widely used and effective drugs for treating a wide variety of human cancers. These include breast cancer, acute lymphoblastic leukemia, acute myeloblastic leukemia, Hodgkin lymphoma, non-Hodgkin lymphoma, Wilms' tumor, neuroblastoma, soft tissue sarcoma, bone sarcoma, ovarian carcinoma, bladder carcinoma, thyroid carcinoma, gastric carcinoma, and bronchogenic carcinoma. Recently, doxorubicin plus sorafenib was shown to be effective in treating advanced hepatocellular carcinoma [1]. Doxorubicin causes adverse effects, especially cardiotoxicity and heart failure, which limit its clinical use [2–4]. The mechanisms of action of doxorubicin regarding both tumor killing and adverse effects remain partially understood. It is partly for this reason that doxorubicin is currently among the most extensively studied anticancer drugs in the fields of both basic biomedical research and clinical medicine [5]. In this context, determination of the cellular dynamics of doxorubicin is of crucial importance in understanding its cellular effects in both cancer and normal cells. To this end, in this paper, we have characterized three fluorescence-based techniques (i.e., spectrometry, microscopy, and flow cytometry) to measure and/or detect doxorubicin in cultured cells. Our results demonstrated the value of these 3 techniques in determining the dynamics of doxorubicin in cancer cells as well as other biological systems.

2. MATERIALS AND METHODS

2.1. Materials

B16-F10 melanoma cell line (CRL-6475) was from American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fungizone, fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were from Thermo Fisher Scientific (Grand Island, NY, USA). Cell culture flasks and other plasticwares were from Corning (Corning, NY, USA). Doxorubicin and other chemicals and reagents of analytical grade were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell Culture

The B16-F10 cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of fungizone in a 150 cm^2 tissue culture flask at 37°C in a humidified atmosphere of 5% CO_2, as described before [6]. The cells were subcultured once they reached 90% confluence.

2.3. Fluorescence Spectrometric Measurement

To characterize the fluorescence spectrometric properties of doxorubicin, the emission and excitation spectra of the drug were obtained with a Perkin Elmer LS-50B fluorescence spectrometer (Waltham, MA, USA). The maximum wavelengths for excitation and emission were then used for measuring the fluorescence intensity of samples containing doxorubicin. For measuring cellular doxorubicin, cells were incubated with various concentrations of doxorubicin in complete PBS (CPBS) (PBS containing 0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$, and 0.1% glucose). Following the incubation, cells were washed once with PBS and then lysed in the lysis buffer followed by the measurement of the fluorescence intensity (λex = 480 nm; λem = 560 nm).

2.4. Fluorescence Microscopic Imaging

The cells were seeded into a 6-well tissue culture plate and cultured overnight. The media were then replaced with CPBS containing various concentra-

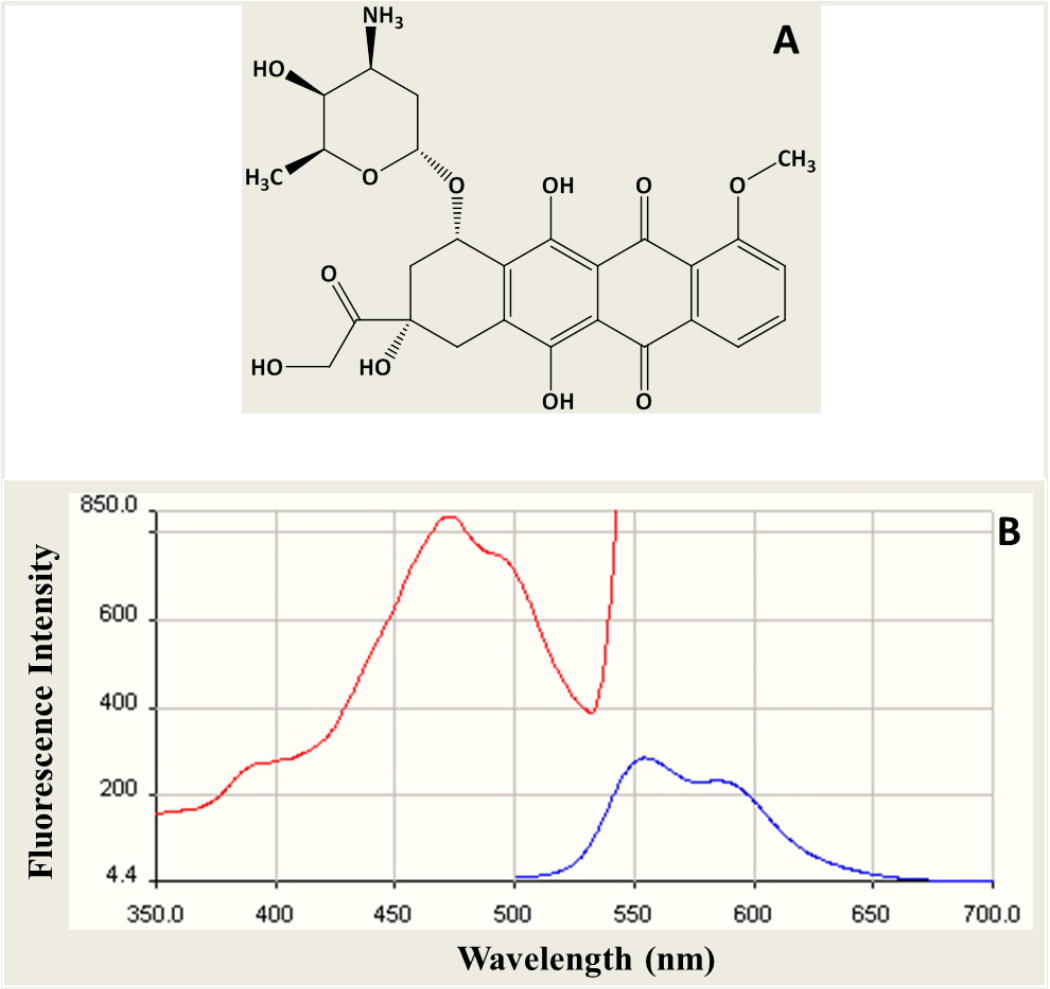

FIGURE 1. Doxorubicin chemical structure (A) and fluorescence spectra (B). In panel B, red line indicates the excitation spectrum, and blue line indicates the emission spectrum. The fluorescence spectra were recorded with a Perkin Elmer LS-50B fluorescence spectrometer.

tions of doxorubicin. The plate was incubated at 37°C for 30 min followed by examination with an EVOS Floid cell imaging system (Thermo Fisher, Waltham, MA, USA).

2.5. Flow Cytometric Detection

The cells were incubated in tissue culture tubes in CPBS containing various concentrations of doxorubicin at 37°C for 30 min. Immediately after this incubation, the cells were spun down and washed once and then resuspended in CPBS for flow cytometric measurement with a Muse cell analyser (EMD Millipore, Temecula, CA, USA).

3. RESULTS AND DISCUSSION

3.1. Fluorescence Spectrometry

During our studies on MitoSOX-based fluorescence assays, we observed that doxorubicin has similar fluorescence spectrometric properties to MitoSOX, a probe for detecting reactive oxygen species (ROS),

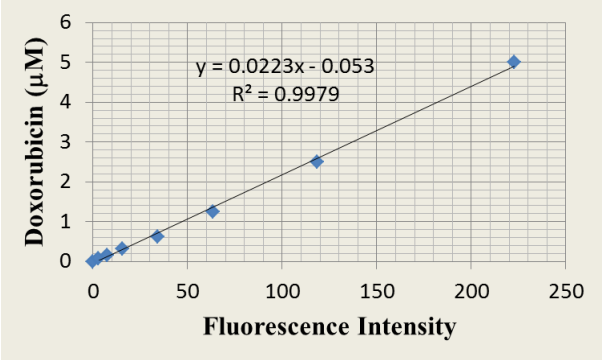

FIGURE 2. Standard curve of doxorubicin. Fluorescence intensity was measured at the emission wavelength of 560 nm and excitation wavelength of 470 nm with a Perkin Elmer LS-50B fluorescence spectrometer. The lowest concentration of doxorubicin used was 0.078 μM, which was readily measurable under the experimental conditions described.

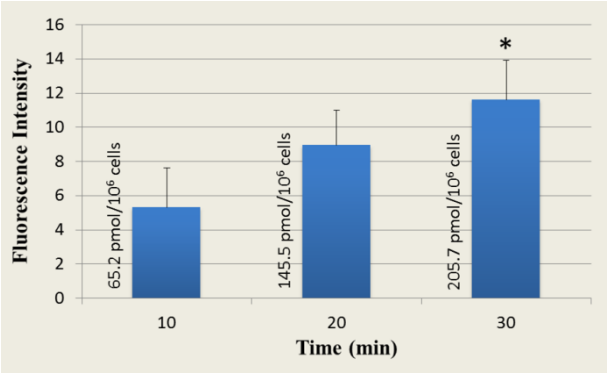

FIGURE 3. Measurement of time-dependent accumulation of doxorubicin in B16-F10 melanoma cells. The cells (2×10^6 cells) were incubated with 5 μM doxorubicin in 2 ml CPBS at 37°C for the indicated time points. The cells were then washed once in PBS and lysed in 2 ml lysis buffer followed by measuring the florescence intensity at the emission wavelength of 560 nm and excitation wavelength of 470 nm with a Perkin Elmer LS-50B fluorescence spectrometer. Bar graph data represent mean ± SD (n = 3), and inserted data show the mean cellular doxorubicin amounts (pmol per 10^6 cells) calculated based on the standard curve shown in Figure 2. *, $p < 0.05$ compared with the 10 min group.

especially superoxide [7]. We thus further characterized the fluorescence properties of doxorubicin in a hope to develop fluorescence-based assays for detecting this commonly used anticancer drug in biological systems. As shown in **Figure 1**, doxorubicin exhibits characteristic fluorescence spectra of excitation and emission with maximum excitation (λex) and emission (λem) wavelengths being 470 and 560 nm, respectively. Notably, the λex and λem are very similar to those of ROS-oxidized MitoSOX [7]. This observation has important implications in using MitoSOX assays to detect the ROS formation induced by doxorubicin. In this context, doxorubicin is widely viewed as a redox cycling quinone to produce ROS in biological systems though recent evidence does not support this notion [5].

With the above λex and λem, the amount of doxorubicin could be sensitively measured. As shown in **Figure 2**, there is a linear relationship between the concentrations of doxorubicin and the fluorescence intensity. The minimal detection limit for doxorubicin was less than 0.1 μM.

To determine if the fluorescence spectrometry could be used to measure doxorubicin levels in cells, the B16-F10 cells were incubated with 5 μM doxorubicin for various times. Following the incubation, the cells were pelleted and washed once in PBS and then lysed in a lysis buffer containing 0.1% Triton X-100. As shown in **Figure 3**, incubation of cells with doxorubicin led to a time-dependent accumulation of doxorubicin in the cells.

Collectively, the above results demonstrate that fluorescence spectrometry can be used to sensitively measure the amounts of doxorubicin in aqueous solutions and cells. This fluorescence spectrometric procedure can also be adopted to measure doxorubicin using a fluorescence microplate reader.

3.2. Fluorescence Microscopy

Fluorescence microscopic imaging of molecules in cells has become instrumental in studying modern cell biology. In this context, we investigated the possibility of using fluorescence microscopy to visualize intracellular doxorubicin. As shown in **Figure 4B**, the emission wavelength of doxorubicin covers both green and red light, with green light being predominant. In line with this, green fluorescence imaging of

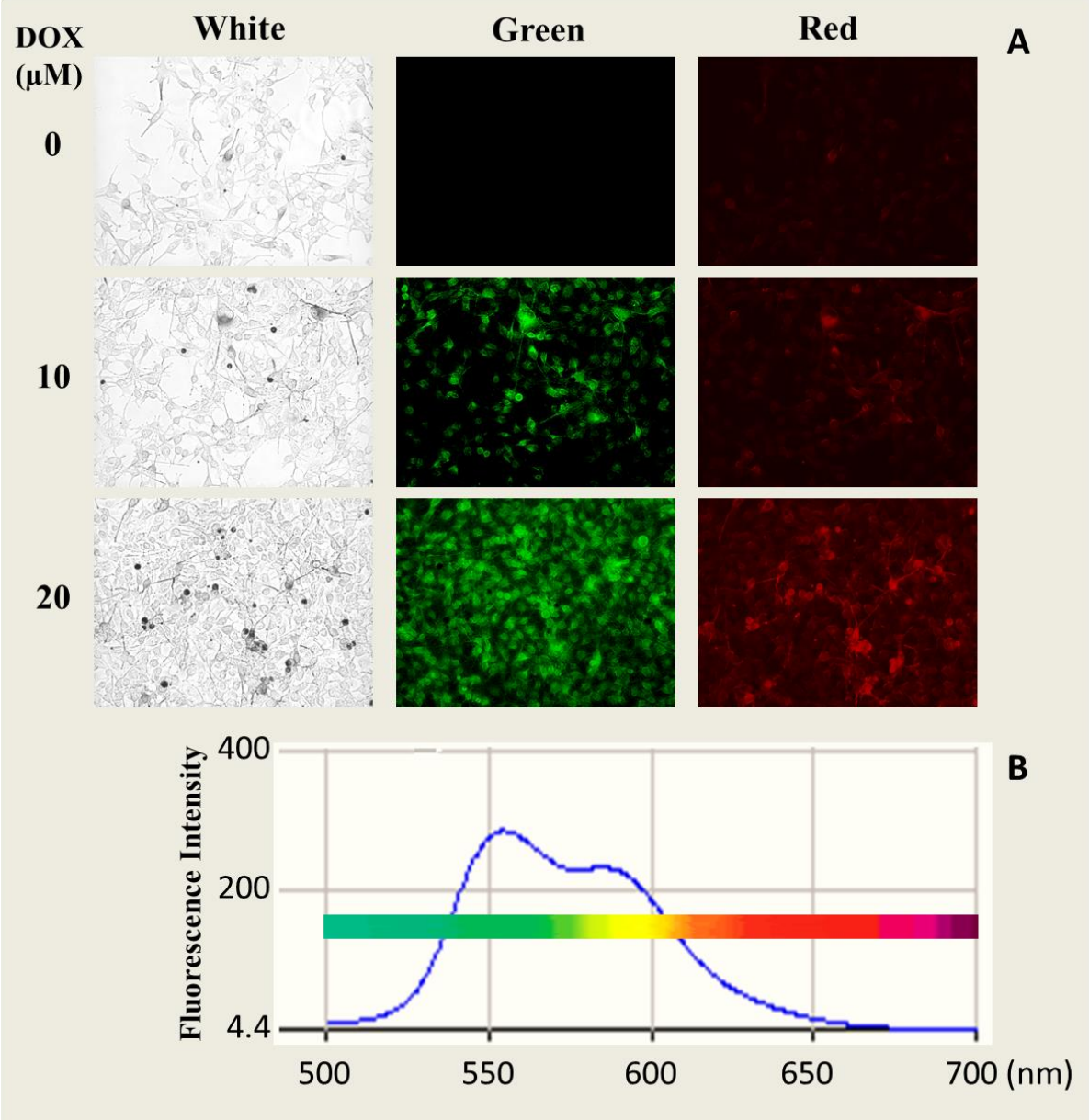

FIGURE 4. Fluorescence microscopic detection of accumulation of doxorubicin in B16-F10 melanoma cells. The cells (2 ×10^6 cells/2 ml per well) were first seeded into a 6-well culture plate (overnight) and then incubated with the indicated concentrations of doxorubicin in 2 ml CPBS at 37°C for 30 min. The cells were then washed once with CPBS followed by imaging under an EVOS Floid cell imaging system with white, green, and red light (panel A). Panel B shows the overlap of the doxorubicin fluorescence emission spectrum with the visible spectrum of wavelengths between 500 and 700 nm.

cellular doxorubicin showed a higher sensitivity than red fluorescence (**Figure 4A**). Notably, the imaging intensity of cellular doxorubicin showed a concentration-dependent manner with both green and red fluo-

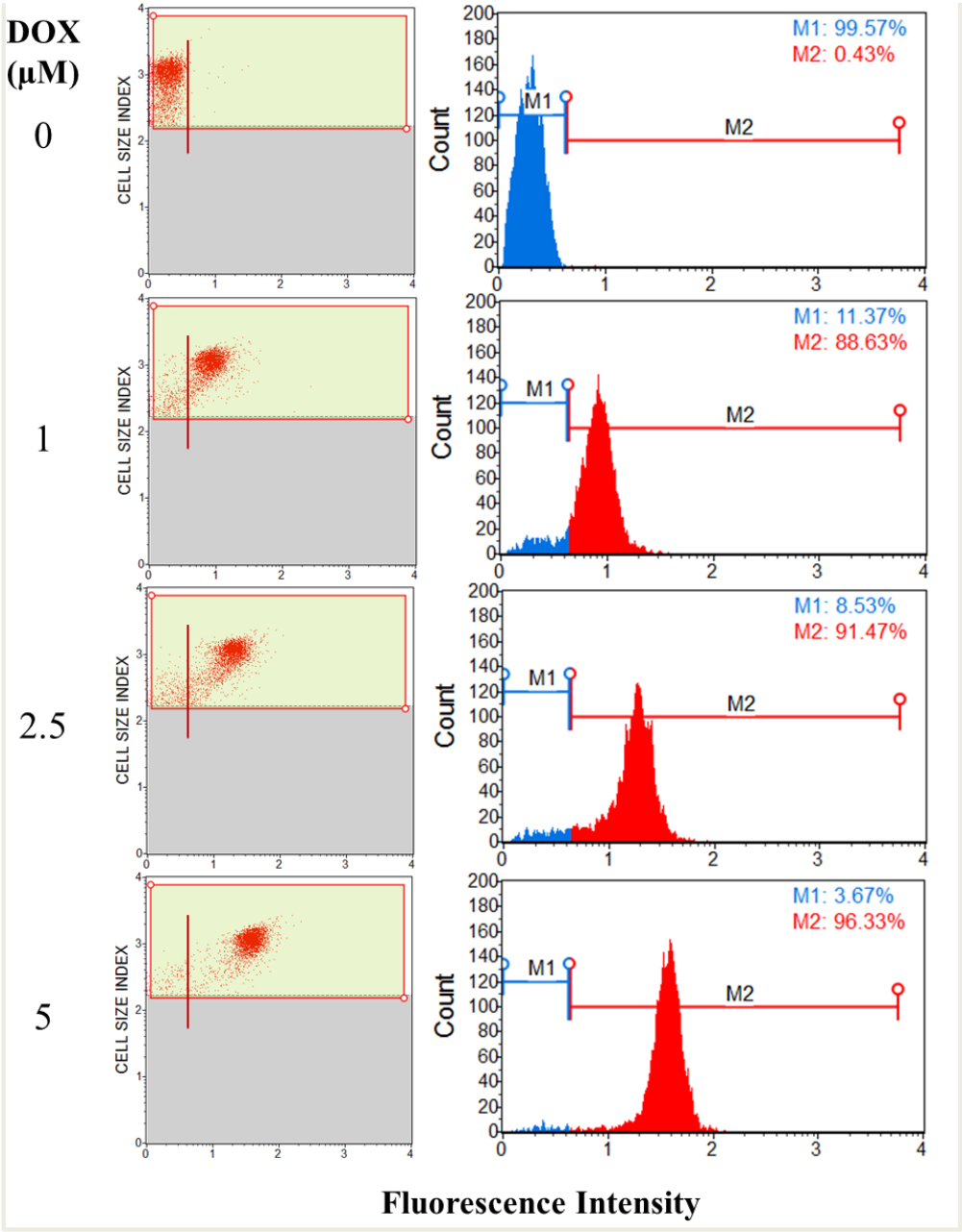

FIGURE 5. Detection of doxorubicin levels in B16-F10 melanoma cells by flow cytometry. The cells (2×10^6 cells) were incubated with the indicated concentrations of doxorubicin in 2 ml CPBS at 37°C for 30 min. The cells were washed once in CPBS followed by measurement with a Muse cell analyser. Left panels show dot plots and right panels show histograms.

rescence. Although confocal fluorescence microscopy was not used in this study, it could certainly be used to image the subcellular distribution of doxorubicin. Studies are currently underway in our laboratories to apply confocal fluorescence microscopy to visualize the subcellular distribution of doxorubicin

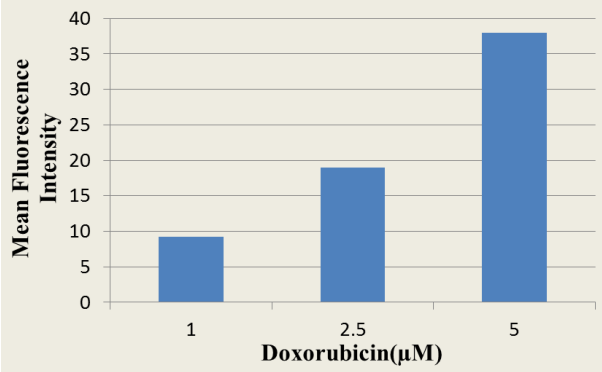

FIGURE 6. Mean fluorescence intensity of doxorubicin in B16-F10 melanoma cells detected by flow cytometry. The experimental condition was described in Figure 5 legend and the data represent the mean fluorescence intensity of the cell populations following incubation with 1, 2.5, and 5 μM doxorubicin at 37°C for 30 min.

under various conditions. This may help elucidate the intracellular targets (e.g., mitochondria) of doxorubicin-mediated cancer cell killing as well as cardiomyocyte cytotoxicity.

3.3. Flow Cytometry

The success in measuring and detecting doxorubicin with both fluorescence spectrometry and microscopy prompted us to determine if flow cytometry could also be used to sensitively detect doxorubicin in cells. As shown in **Figures 5** and **6**, flow cytometry was able to sensitively detect doxorubicin amounts in cells in a concentration-dependent manner. Both the dot plots and histograms (**Figure 5**) clearly showed a doxorubicin concentration-dependent increase in both the proportion of doxorubicin-containing cells and the fluorescence intensity. The mean fluorescence intensity of the cells also exhibited a doxorubicin concentration-dependent relationship (**Figure 6**). Although 1 μM was the lowest concentration of doxorubicin used, it was apparent that the flow cytometry could easily be applied to study the cellular accumulation of doxorubicin at concentrations lower than 1 μM.

Because flow cytometry provides quantitative data at the level of cell populations, the procedure characterized in this study can have significant applications in studying the cellular dynamics of doxorubicin in different cell types including both cancer and normal cells. One application would be the determination of the accumulation and efflux of doxorubicin in cancer cells. Another application would be the assessment of cardioprotective agents on cellular doxorubicin accumulation in cardiomyocytes so as to better understand the mechanisms of action of such cardioprotective modalities.

3.4. Conclusions

In conclusion, this study demonstrated that the characteristic fluorescence properties of doxorubicin made it possible to detect and/or measure this anticancer drug in biological systems via the use of fluorescence-based techniques, including spectrometry, microscopy, and flow cytometry. Simultaneous use of these techniques would produce a comprehensive picture on cellular dynamics of doxorubicin in both cancer and normal cells. Such studies would help understand the detailed mechanisms of action of this widely used anticancer drug as well as the development of novel modalities to enhance its therapeutic index.

ACKNOWLEDGMENTS

The work was supported in part by a grant from the United States National Institutes of Health/National Cancer Institute (CA192936) and an investigator-initiated grant (IIG) (09A084) from the American Institute for Cancer Research (AICR). Melinda K. Kauffman is currently an undergraduate student. Megan E. Kauffman is currently a second year medical student.

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