Supplementary Information

Selective decoy-doxorubicin complex for targeted co-delivery, STAT3 probing and synergistic anti-cancer effect

Shao-Jen Wang, Yung-Te Hou, and Lin-Chi Chen

Department of Bio-Industrial Mechatronics Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 10617, Taiwan (R.O.C.).

Materials and methods

Assembly of doxorubicin intercalated hpdODN

The doxorubicin hydrochloride was obtained from the Sigma-Aldrich Taiwan. The sequences of the hpdODN in this study was: 5'-CGTTTCTCATAAAGCGAAGCTTTATGAGAAACG-3'. It is in a structure of hairpin helix. With a simple mixture of doxorubicin and hpdODN (1 μg of each) and the remove of un-bonding reagent by Amicon Ultra centrifugal filter (10KDa), the doxorubicin-intercalated hpdODN, DOXON, was obtained. The doxorubicin used in this study was suitable for fluorescence related experiments.

Circular dichroism and UV-Vis characterization

The circular dichroism (CD) spectropolarimeter (Aviv Biomedical Inc., Model 202) was used to characterize the secondary structures of the DOXON. A quartz cuvette with the 1 mm optical path was filled with 5 μM of sample solution in DPBS, and the CD spectra were obtained by taking the average of triplicates from 300 nm to 220 nm at 37℃. The UV-Vis absorption spectra were measured on an Agilent UV-Vis spectrophotometer, Perkin Elmer (Lambda 25), with the use of 1.0 cm quartz cells. To determine the release profile, biotinylated DOXON was immobilized on the 24-well streptavidin coated plate. With the addition of STAT3 or BSA, the doxorubicin in the supernatant solution was measured with the UV-Vis.

In vitro STAT3-triggered DOX release

Biotinylated DOXON is immobilized onto a streptavidin-coated 24-well plate and followed by adding the DPBS containing 0.5 μM of STAT3 or BSA protein. The fluorescence intensity of released DOX was measured at 596 nm under an excitation wavelength of 480 nm through a micro-plate reader (Infinite M200 PRO, Tecan) every 30 minutes after incubation. The saturated fluorescence intensity is determined by measuring the signal with 50 μM STAT3 protein (30-min incubation) and considered as the maximum intensity (set as 100%). The signal obtained from the just-prepared complex is considered as the background (set as 0%).

ELONA assays for K_D estimation

ELONA assays were used for the estimation of the binding affinity (K_D) of DOXON toward phosphorylated STAT3 and the BSA protein was used as the negative control (obtained from Cell Signaling Technology). For protein coating, 100 μL of 10 nM protein in DPBS was added to each well of 96-well polystyrene plate and incubated at 4 ℃ overnight. The wells were then washed four times with 0.05% Tween 20 in DPBS before being blocked with 1% BSA. A 100 □L of biotinylated DOXON in the DPBS was then added and incubated at room temperature for 2 hours. The wells were than washed four times with 0.05% Tween 20 in DPBS. The 100 □L of streptavidin–HRP solution was added and incubated at room temperature for 30 minutes. Afterward, the wells were washed with DPBS supplemented with 0.05% (v/v) Tween 20 and 0.1% (w/v) BSA. Then the TMB substrate solution was used to quantify the binding complex. The enzymatic reaction was stopped by adding 100 □L of 2 M H_2SO_4 and followed by absorbance measurement at 450 nm.

MTT cell proliferation assays

The cell viability of DOXON-treated cells were assessed with Vybrant® MTT cell proliferation assay. 50 □L of 12 mM MTT solution was added to each well with 1 mL medium. After 2 hours of incubation, 750 □L of the medium was removed and 500 □L of DMSO was added into each well. After another 10 minutes of incubation, the samples were well mixed and measured for the absorbance at 540 nm.

Gene expression determination

The down-stream mRNA expressions of STAT3 and *p53* in response to the ligand transfection were measured using reverse transcription-polymerase chain reaction (RT-PCR) with Qiagen Rotor-Gene Q®. The mRNA extraction and cDNA amplification were carried out with the Cells-to-CT™ Power SYBR® Green Kit according to the protocol from the manufacturer. The primer sequences are *Bcl-xL*: 5'- GGAAAGCGTAGACAAGGAGATGC-3' and 5'- GGTGGGAGGGTAGA - GTGGATGGT-3'; *Bax*: 5'-CATGTTTTCTGACGGCAACTTC-3' and 5'-AGGGC - CTTGAGCACCAGTTT-3', and *GAPDH*: 5'- GACCACAGTCCATGCCATC - ACTGC-3' and 5'- ATGACCTTGCCCACAGCCTTGG-3'.

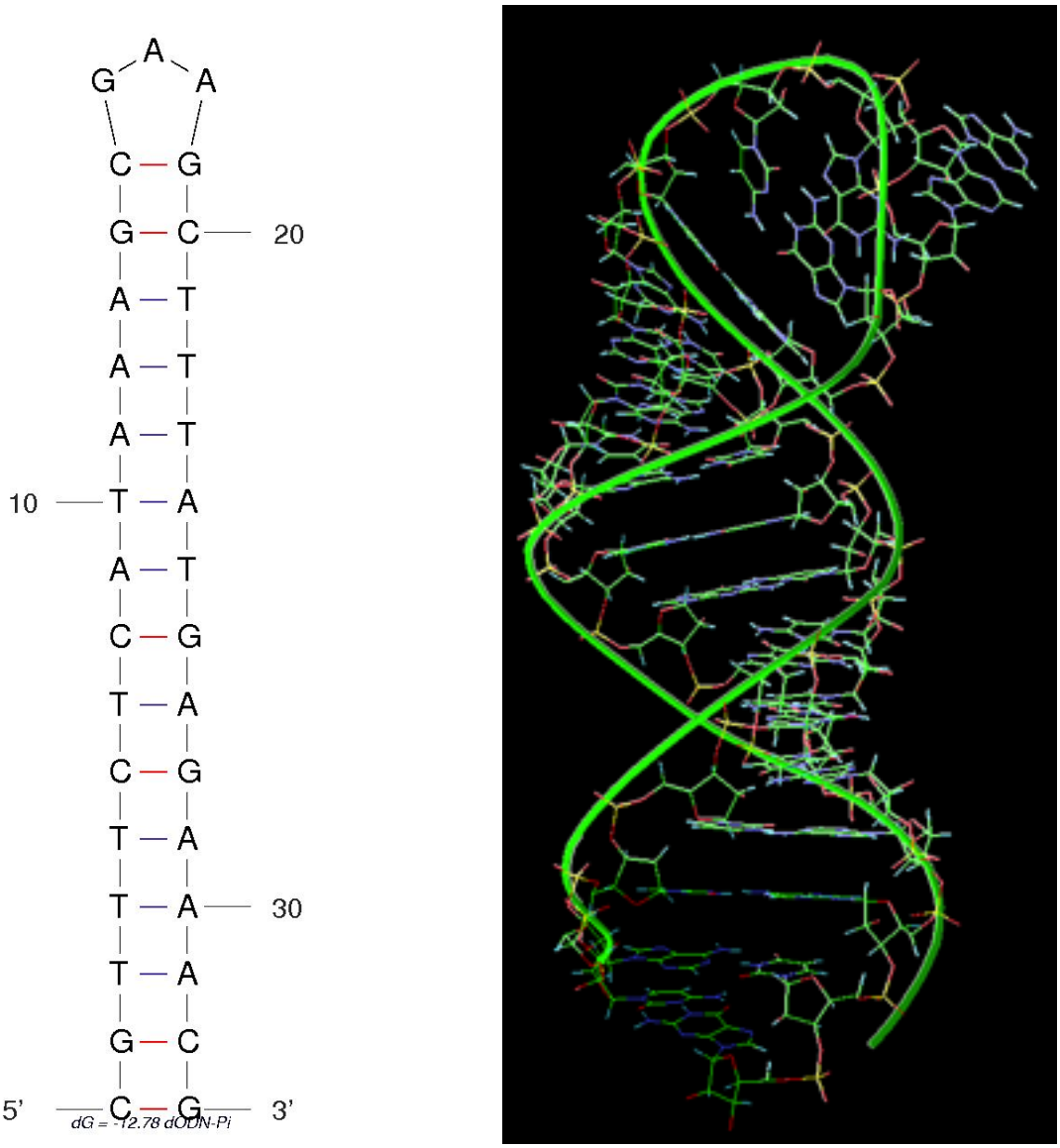

Figure S1. The 2D and 3D structure prediction of the hpdODN generated by the mfold web server [i] and RNA composer web server [ii].

([i] http://mfold.rna.albany.edu/ and [ii] http://rnacomposer.cs.put.poznan.pl/)

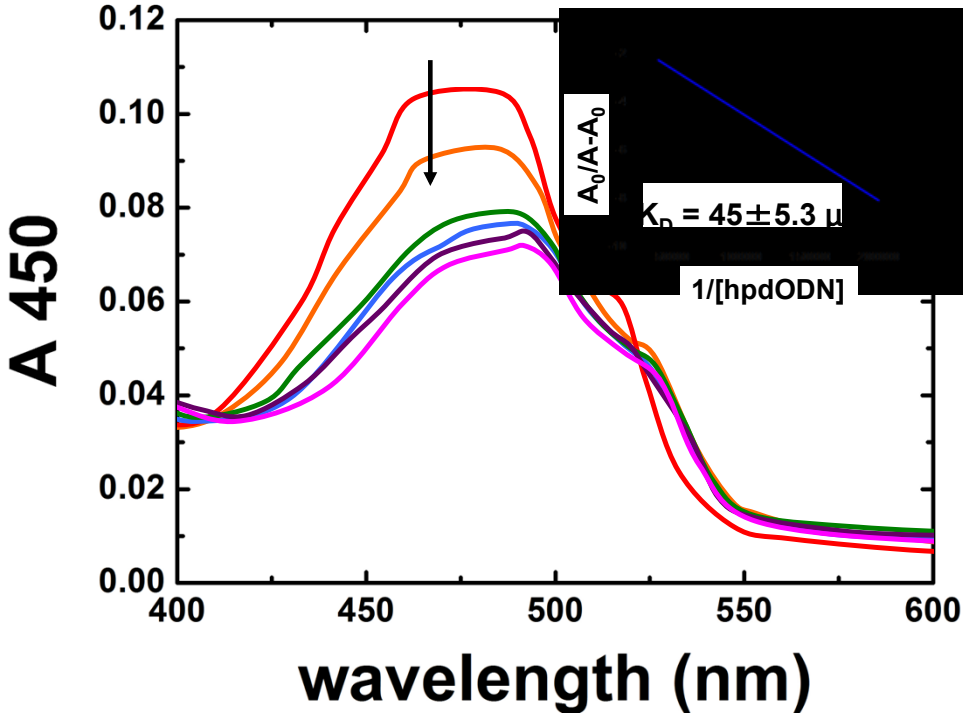

Figure S2. UV-Vis absorption spectra of doxorubicin in the presence of hpdODN at the concentrations = 0, 5.0, 10.0, 15.0, 20.0 and 25.0 μM. The doxorubicin concentration is 10 μM in DPBS.

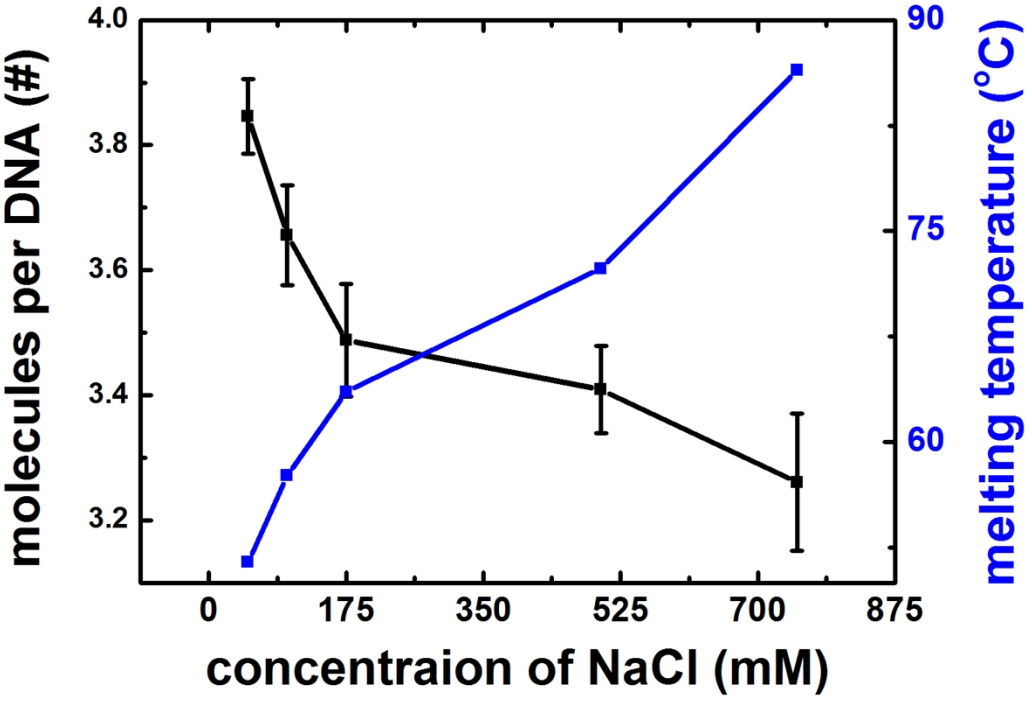

Figure S3. Drug capacity of the DOXON (black line) and melting temperature of the hpdODN (blue line) under different ion strength condition.

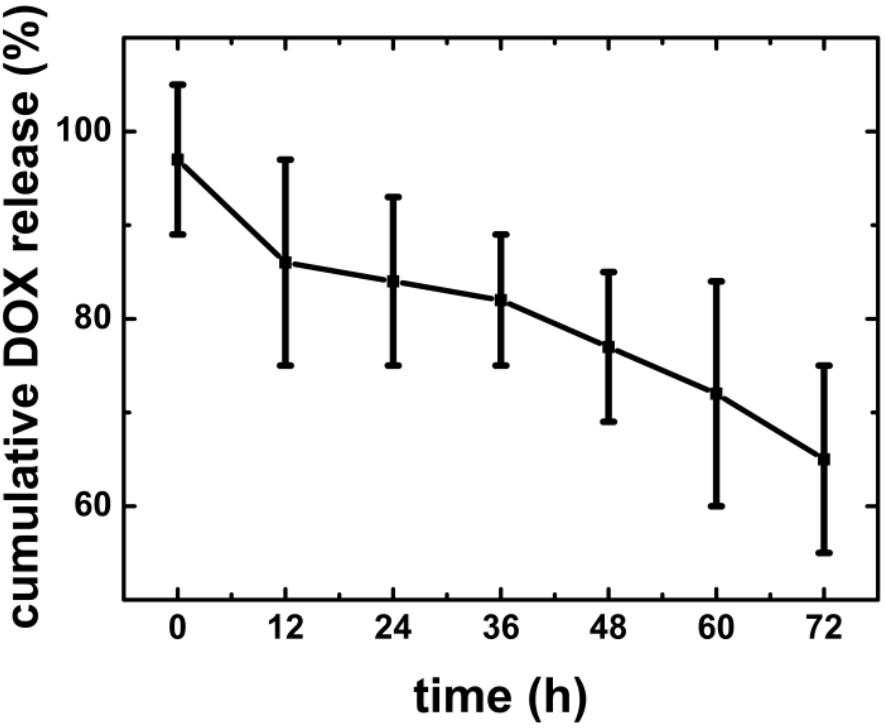

Figure S4. The stability of DOXON in the 10% FBS-containing cell growth medium (MEMα) evaluated by measuring the leaked DOX during incubation.

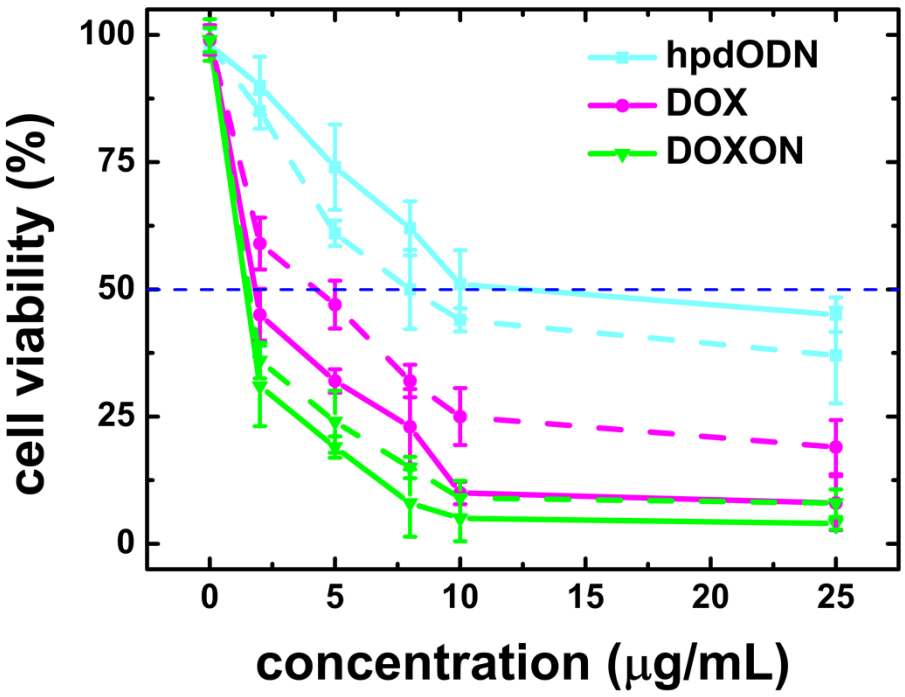

Figure S5. Dose dependent cell death caused by the hpdODN, doxorubicin, and DOXON in the MCF-7 cell model. The solid and dash line are representing the condition without (—) and with (---) the Lipofectamine®. We follow the instruction from manufacture to use the Lipofectamine® 2000 (which is available at: http://tools.lifetechnologies.com/content/sfs/manuals/Lipofectamine_2000_Reag_protocol.pdf). The cell viability is measured after 48 hours of incubation.

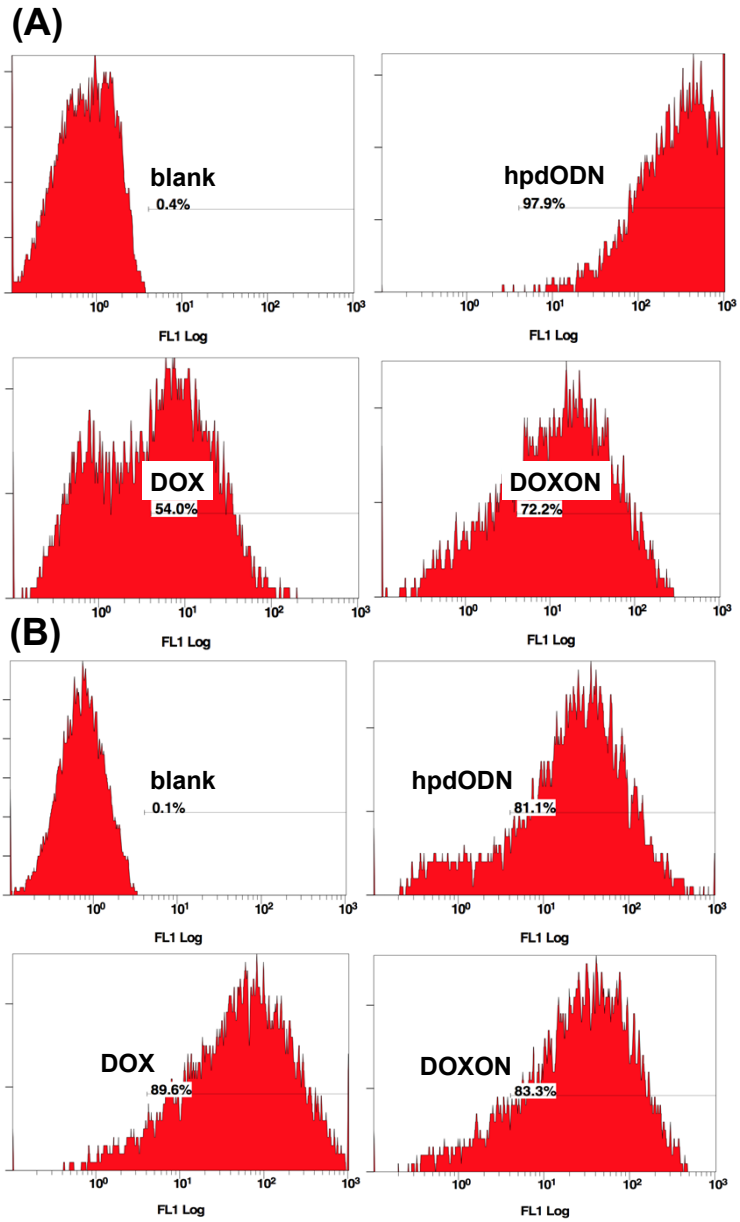

Figure S6. Using flow cytometry to determine the transfection rate. With the Lipofectamine®, (A) blank (untreated cell): 0.4%; hpdODN-treated cell: 97.9%; DOX-treated cell: 54.0%; DOXON-treated cell: 72.2%. Without the Lipofectamine, (B) blank (untreated cell): 0.1%; hpdODN-treated cell: 81.1%; DOX-treated cell: 89.6%; DOXON-treated cell: 83.3%. The sequence was tracked with Alexa Fluor® 546 dye (for both hpdODN pane and DOXON pane) and the DOX was exited with 488 nm light (for DOX pane). The blank was measured from both the 546 nm and 488 nm light excitation, which showed exactly the same result. The obtained result suggests that the ODN is able to enter the cell without the carrier[1]. The endocytosis and macro pinocytosis can be involved to achieve these obtained transfection results [2].

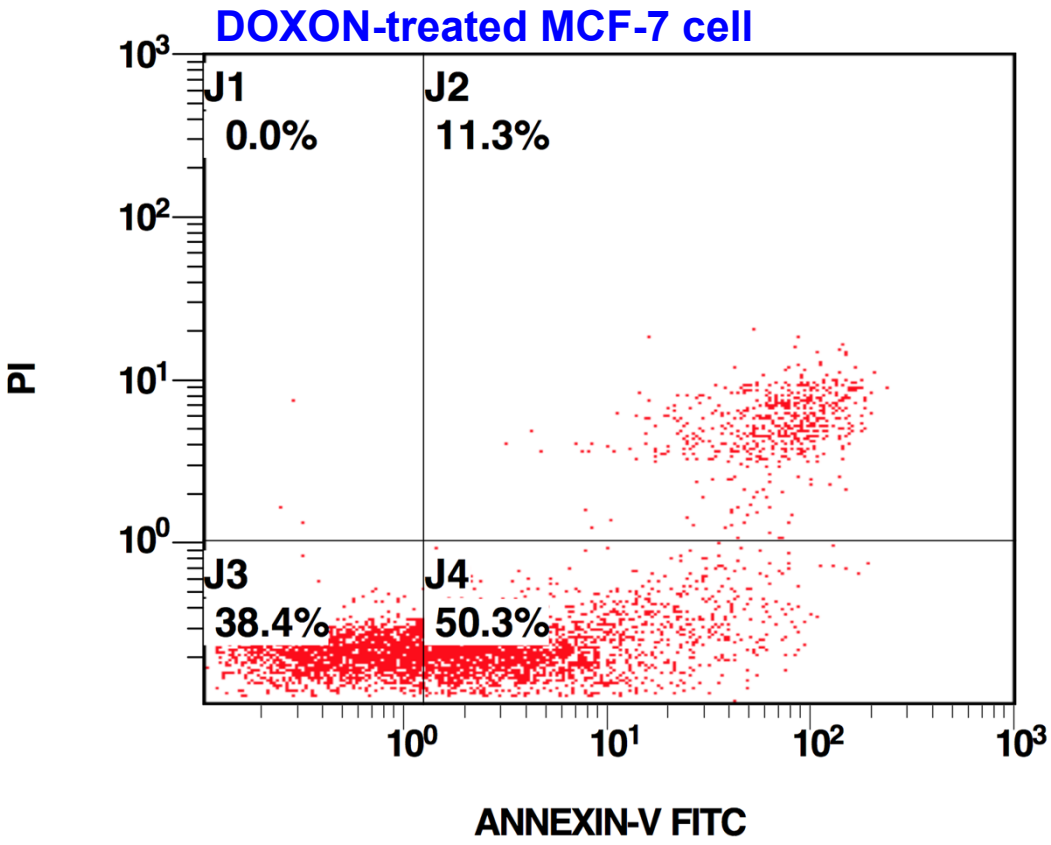

Figure S7. The scatter plot of flow cytometry of DOXON-treated MCF-7 cell shows the high quantity of apoptosis (50.3%, J4 region) with 5 μg/mL, 6 hours incubation. The apoptosis behaviour was characterized by an Annexin V-PI assay using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit and measured by the Beckman Coulter FC500 flow cytometry according to the manufacture instruction.

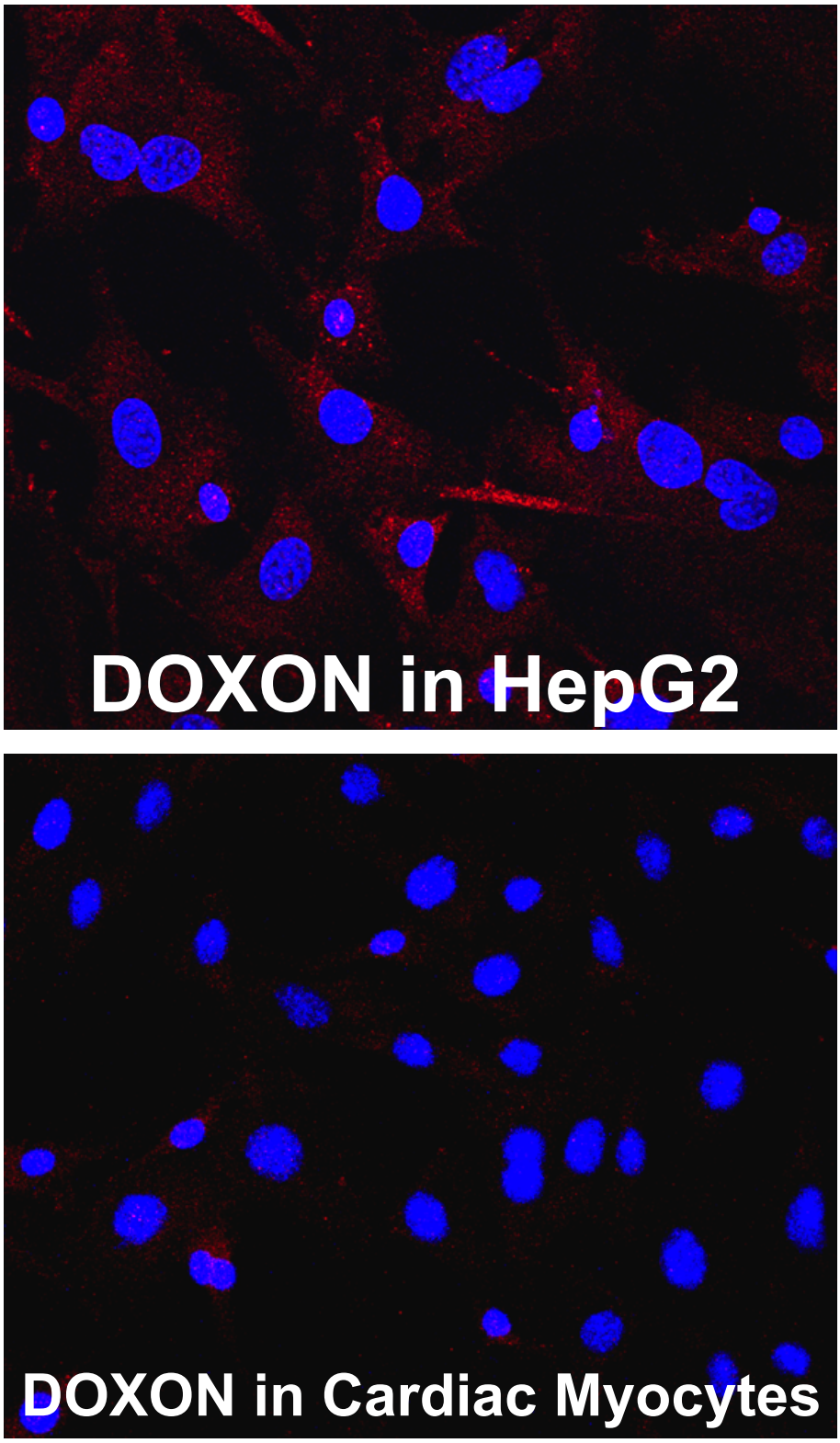

Figure S8. The images obtained from the confocal fluorescence microscopy of DOXON-treated HepG2 (with STAT3 over-activation) and human cardiac myocyte (without STAT3 over-activation). The nucleus is stained with DAPI (blue), and the doxorubicin is exited with 488 nm light (red).

Supporting references:

1. M. Sen, S. M. Thomas, S. Kim, J. I. Yeh, R. L. Ferris, J. T. Johnson, U. Duvvuri, J. Lee, N. Sahu, S. Joyce, M. L. Freilino, H. Shi, C. Li, D. Ly, S. Rapireddy, J. P. Etter, P.-K. Li, L. Wang, S. Chiosea, R. R. Seethala, W. E. Gooding, X. Chen, N. Kaminski, K. Pandit, D. E. Johnson and J. R. Grandis, *Cancer Discov*, 2012, **2**, 694-705.

2. E. Merit Reyes-Reyes, Yun Teng, and Paula J. Bates, *Cancer Res*, 2010, **70**, 8617–8629.