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Supporting Information

Triple conjugated Carbon dots as a nano-drug delivery model for glioblastoma brain tumors

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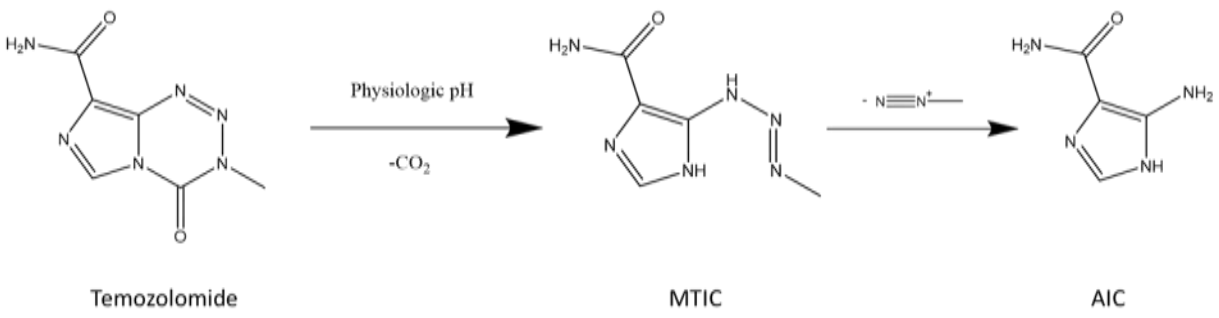


Figure S1: The activation process of temozolomide with the addition of water in to the carbonyl moiety of temozolomide at physiological pH. It changes to AIC structure via MTIC after the breakage of the tetrazinone ring as shown above.

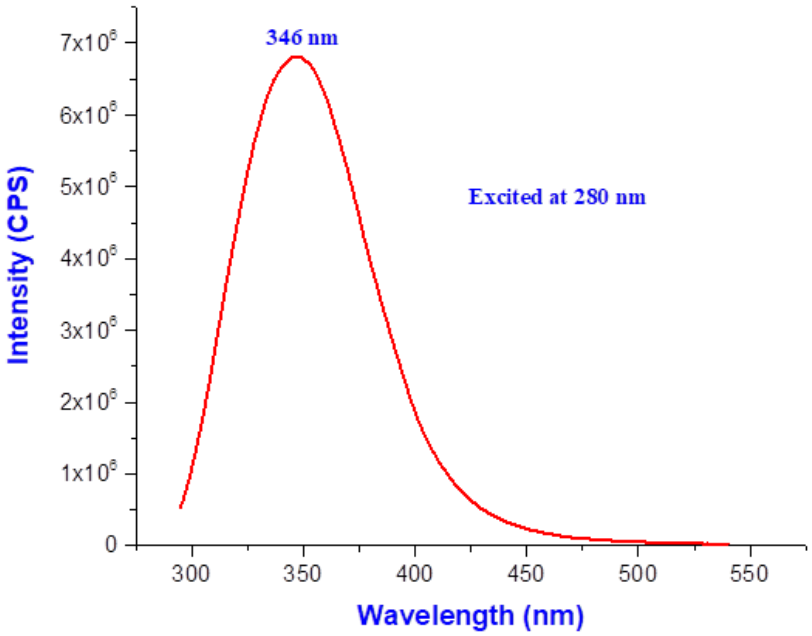


Figure S2: The fluorescence emission spectrum of free transferrin at the excitation wavelength of 280 nm. The sample was tested in 1 cm (optical path length) quartz cell at the concentration of 20 µg mL^{-1}.

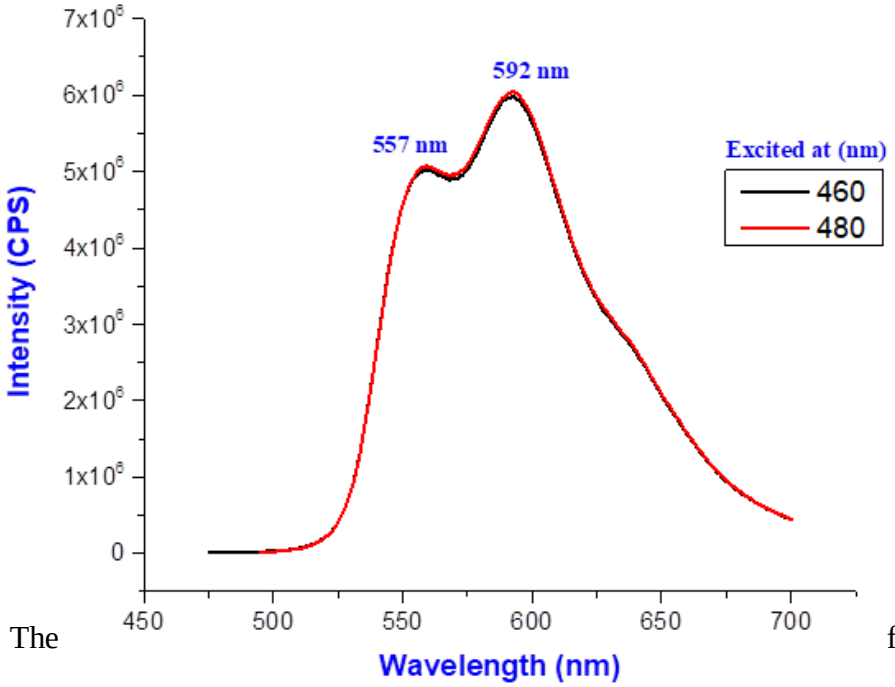


Figure S3: The fluorescence emission spectra of free epirubicin at the excitation wavelengths of 460 and 480 nm. The sample was tested at 10 µg mL^{-1} concentration, in 1cm quartz cell.

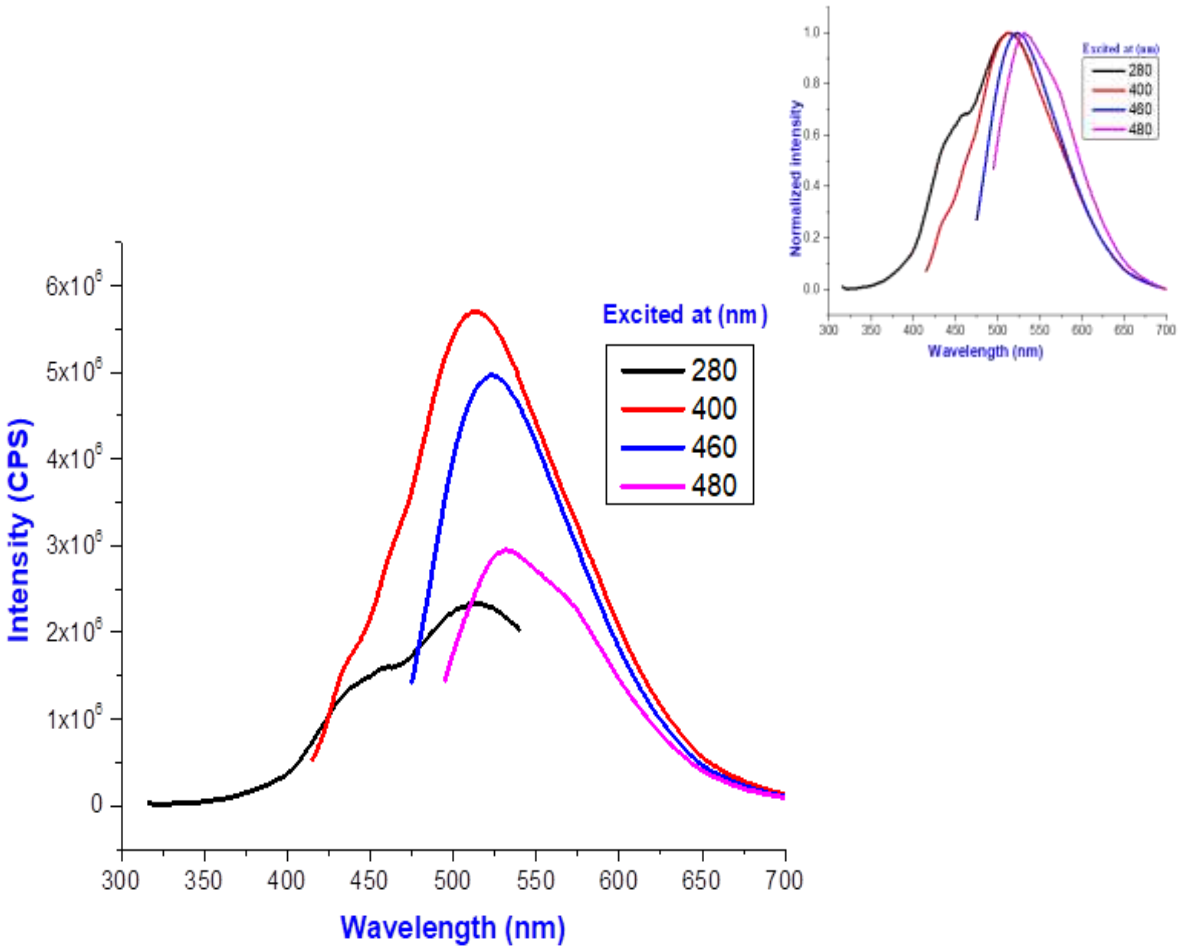


Figure S4: The fluorescence emission spectra of free C-dots at various excitation wavelengths of 280-480 nm. Inset, normalized fluorescence emission spectra. The tested C-dot sample was at 10 µg mL^{-1} concentration and used the 1cm (optical path length) quartz cell.

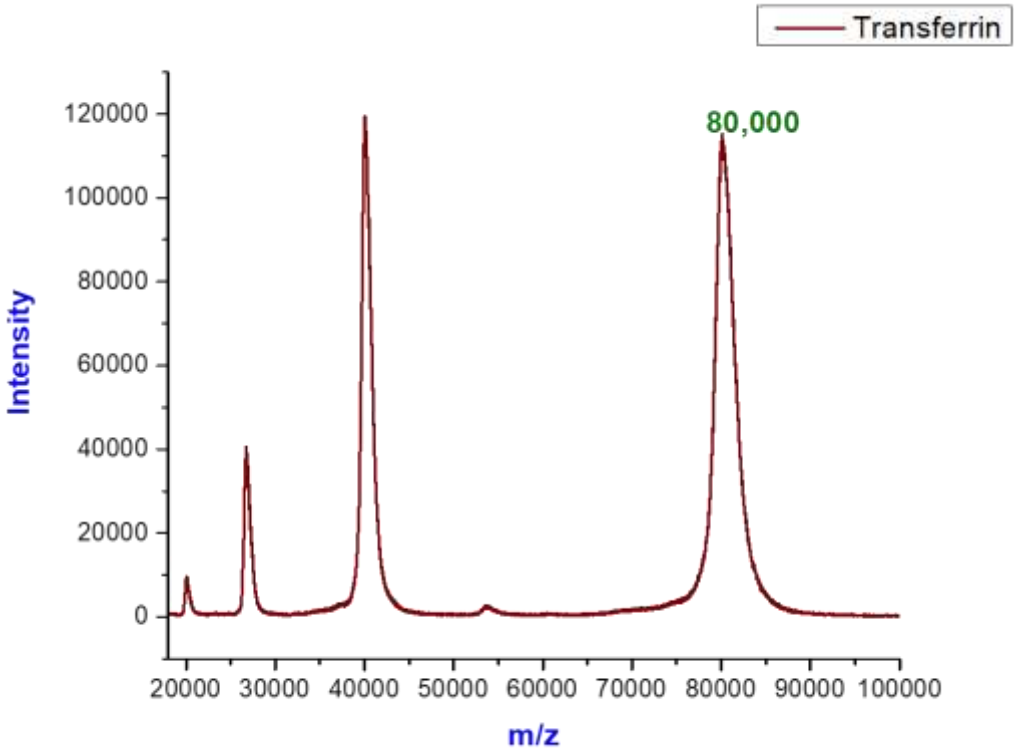


Figure S5: MALDI-TOF spectrum of free transferrin at the concentration of 1 mg mL^{-1}.

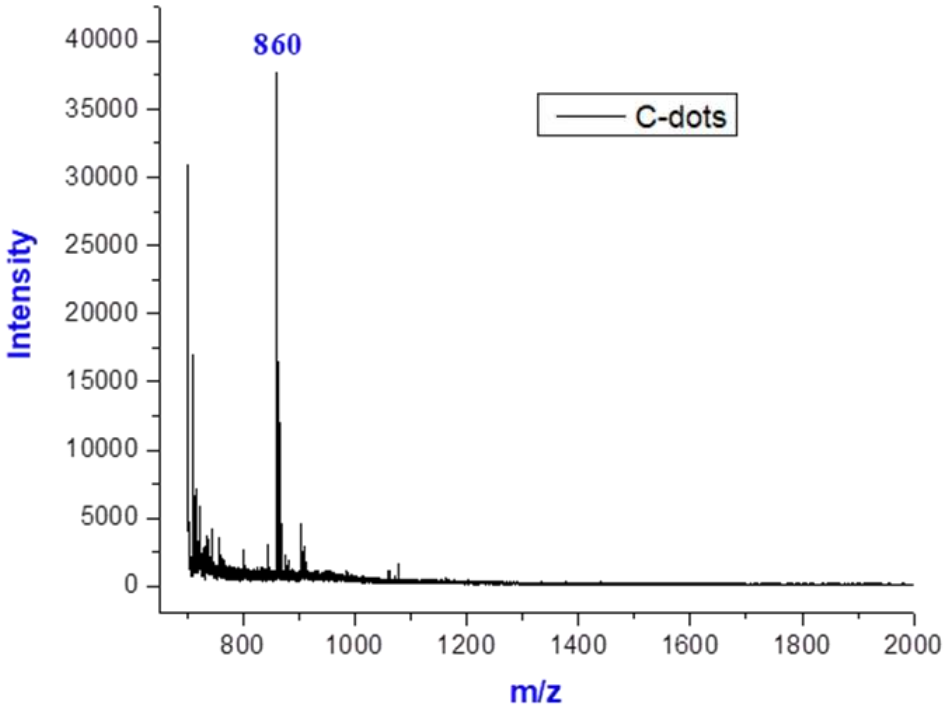


Figure S6: ESI-Mass spectrum of free C-dots at the concentration of 1 mg mL^{-1}.

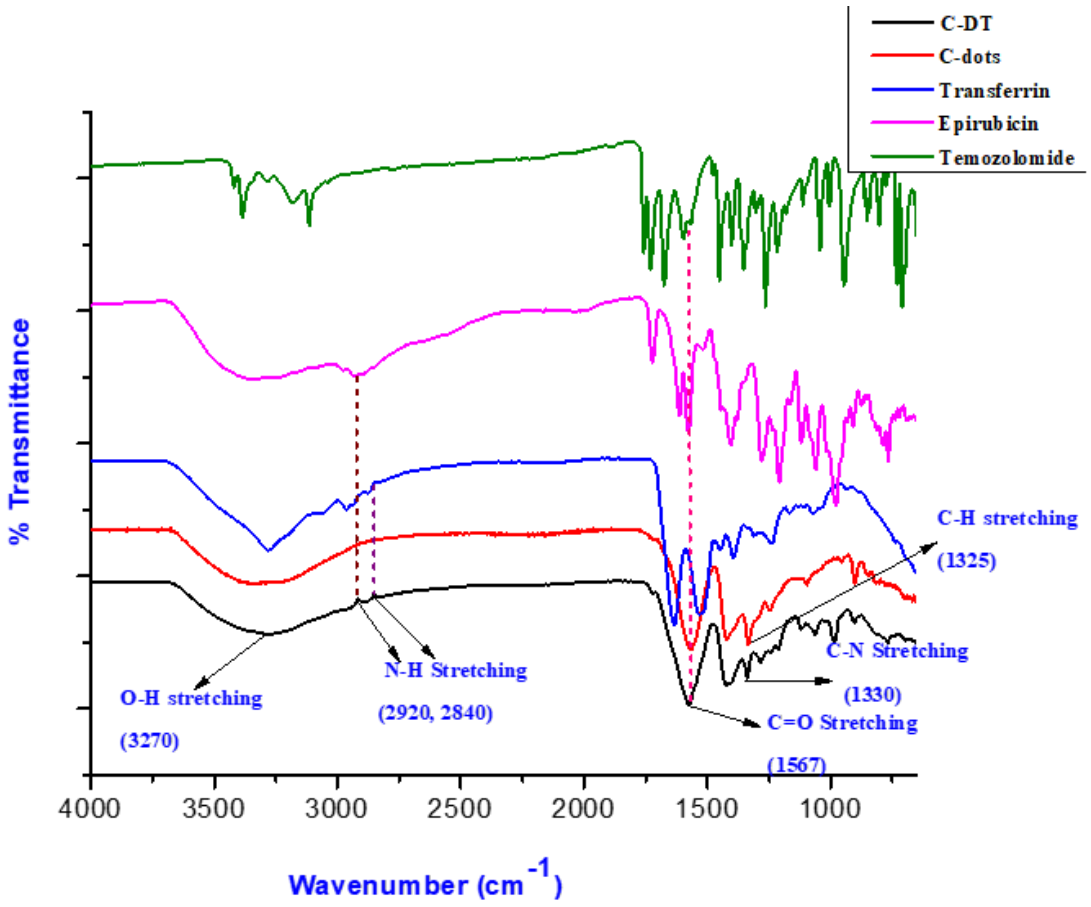


Figure S7: FTIR-ATR Spectra comparison of C-dots-transferrin-epirubicin-temozolomide (C- DT)

Calculation of the loading capacity/concentration of each drug on C-dots

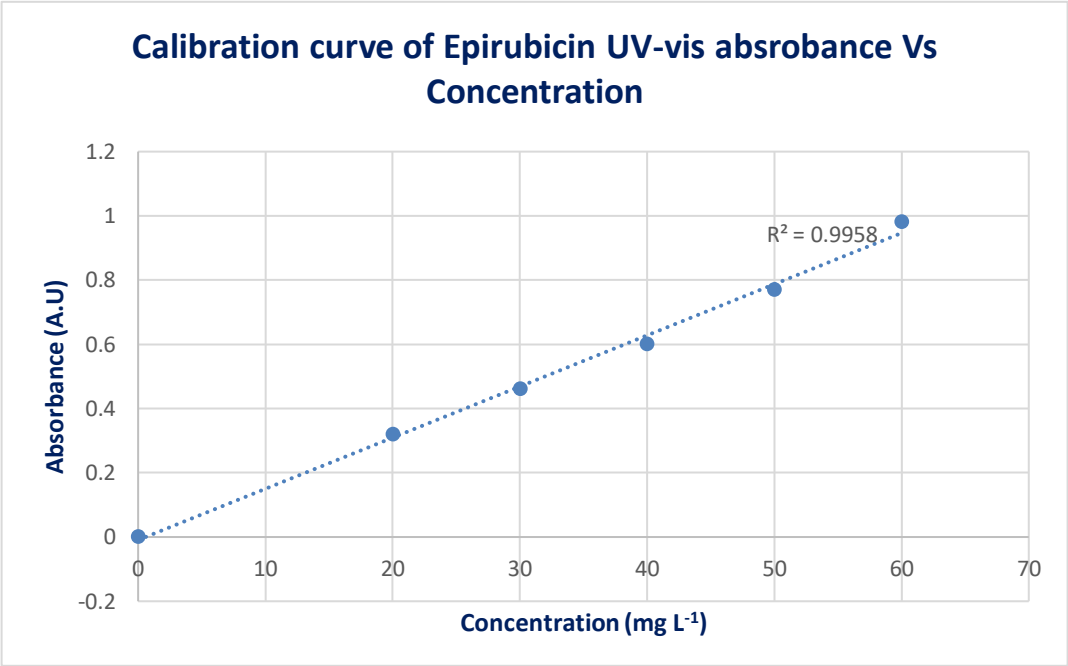


Figure S8: Calibration curve of epirubicin UV-vis absorbance Vs concentration

Sample	Concentration of the sample (mg L^{-1})	UV-Vis absorbance (A.U)	Loaded epirubicin concentration in each sample (mg L^{-1})
C-DT	**60**	**0.23**	**15**
C-ET	**60**	**0.57**	**37**
C-dots-epi-temo	**60**	**0.69**	**45**
C-dots-epi	**60**	**0.88**	**56**

Table S1: Loaded epirubicin concentration analysis of the each epirubicin loaded samples. Each sample concentration is 60 mg L^{-1}.

As shown in above Table S1, the loaded epirubicin concentration was measured in each 60 mg L^{-1} sample by using an epirubicin calibration curve analysis. According to the table, loaded epirubicin concentration was low in C-DT than all the other samples. Also, it indicated the transferrin conjugated samples (C-DT and C-ET) has lower concentration of epirubicin than non-transferrin conjugated samples (C-dots-epi-temo and C-dots-epi). Therefore, this loading concentration analysis further confirms that the triple conjugated C-DT carries less concentration of epirubicin than all the other samples. Temozolomide is not UV-vis and fluorescence active to measure the loading concentration.

Transferrin Receptor 1 (TFR1) in SJGBM2, CHLA266, U87 and CHLA200 cells

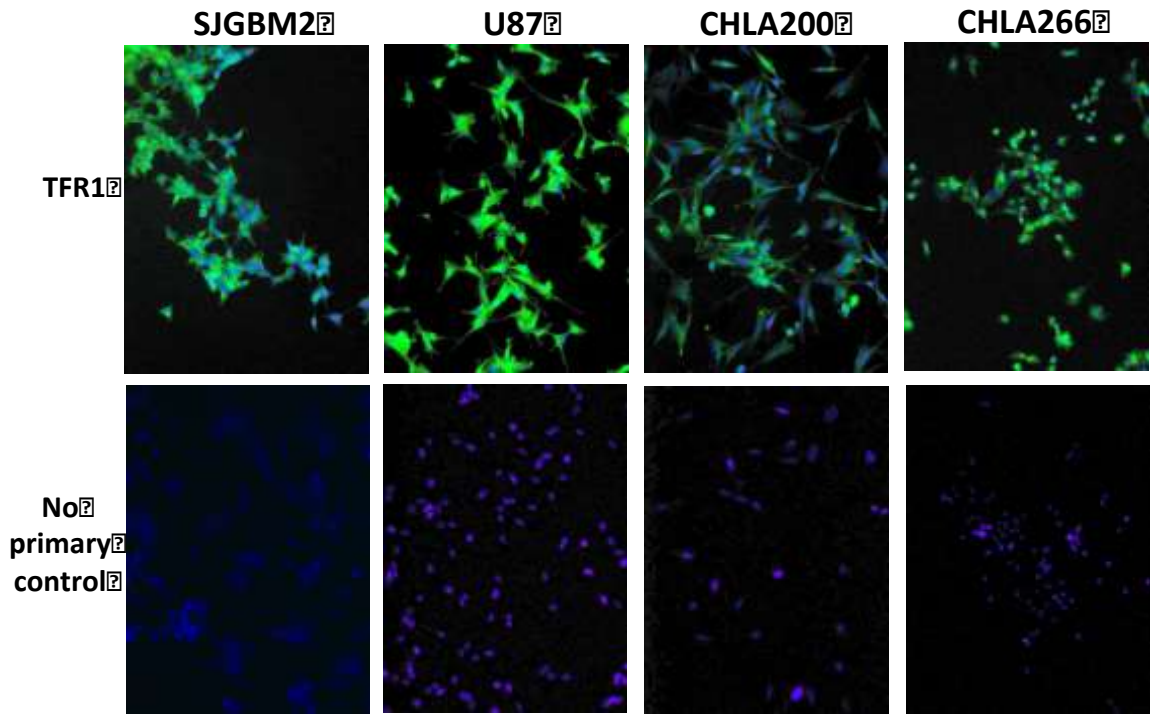


Figure S9. Transferrin Receptor 1 (TFR1) expression was determined using immunocytochemistry. TFR1 is shown in green. Nuclei are shown in blue (DAPI staining) TFR1 was observed in all cells lines, however CHLA200 cell demonstrated reduced expression compared to the other cell lines. A no primary control was included and serves as a negative control.

Nuclear localization for C-dot drug conjugates in SJGBM2 cells

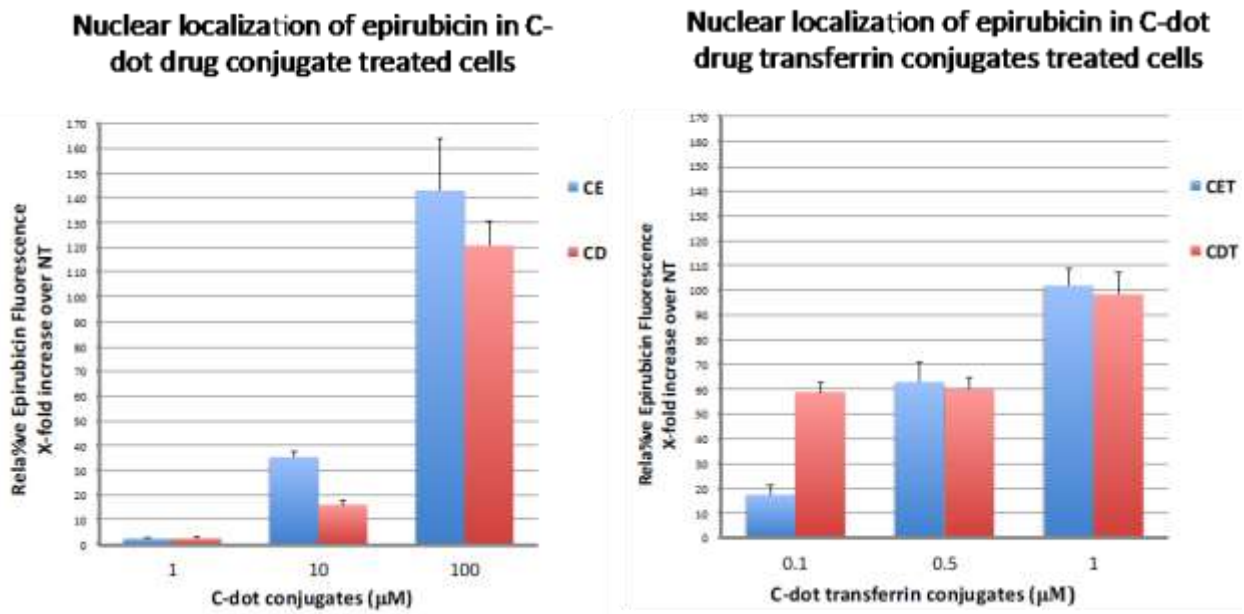


Figure S10. Relative levels of nuclear epirubicin in SJGBM2 cells. Cells were treated with increasing concentrations of either C-dot drug conjugates, C-dot-epi (CE) and C-dot-dual drug (CD) (left panel) or C-dot drug transferrin conjugates, C-dot-epi-transferrin (CET) and C-dot dual drug-transferrin (CDT) (right panel) for 18 hours and the epirubicin fluorescence was quantitated using Thermo Fisher Array Scan VTI with Target Activation algorithm run at 10X objective.

Relative levels of nuclear epirubicin in all cell lines following 6 hours of treatment

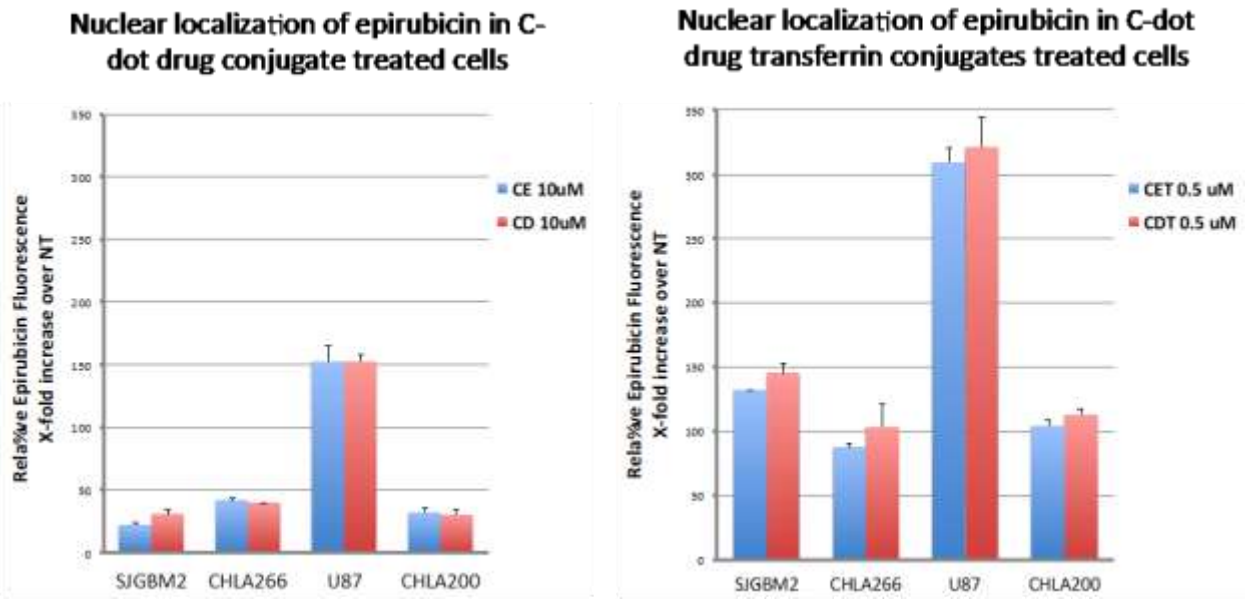
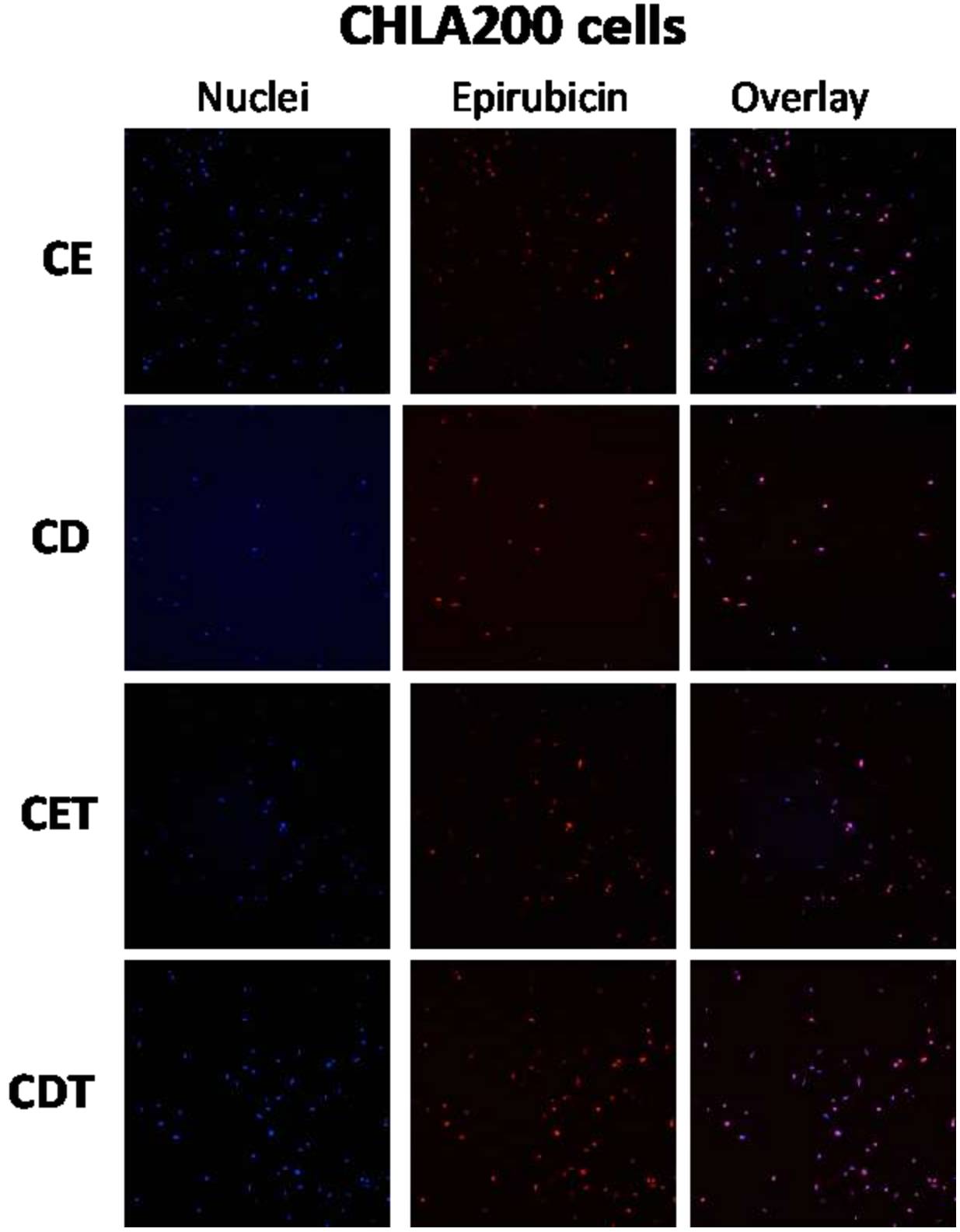
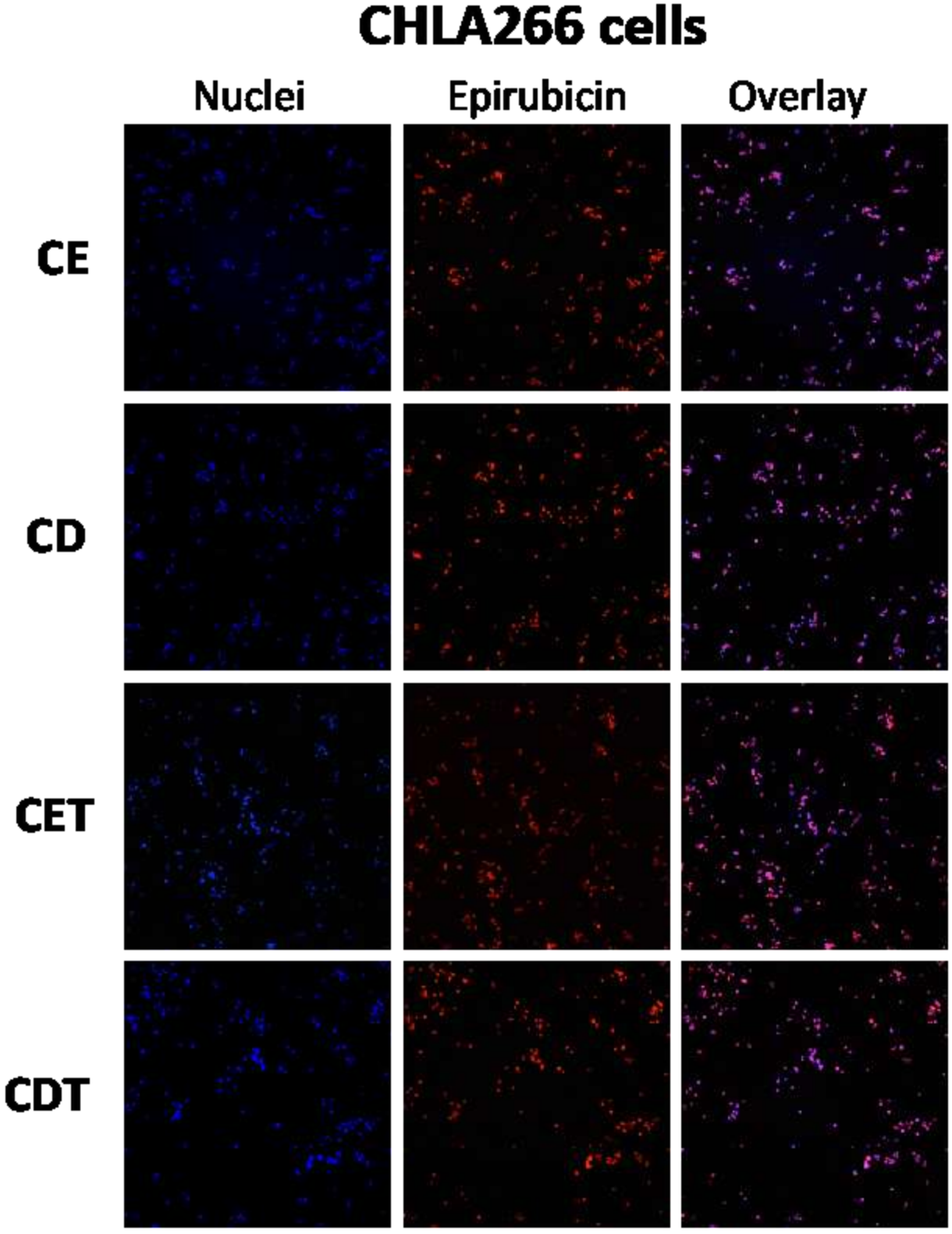
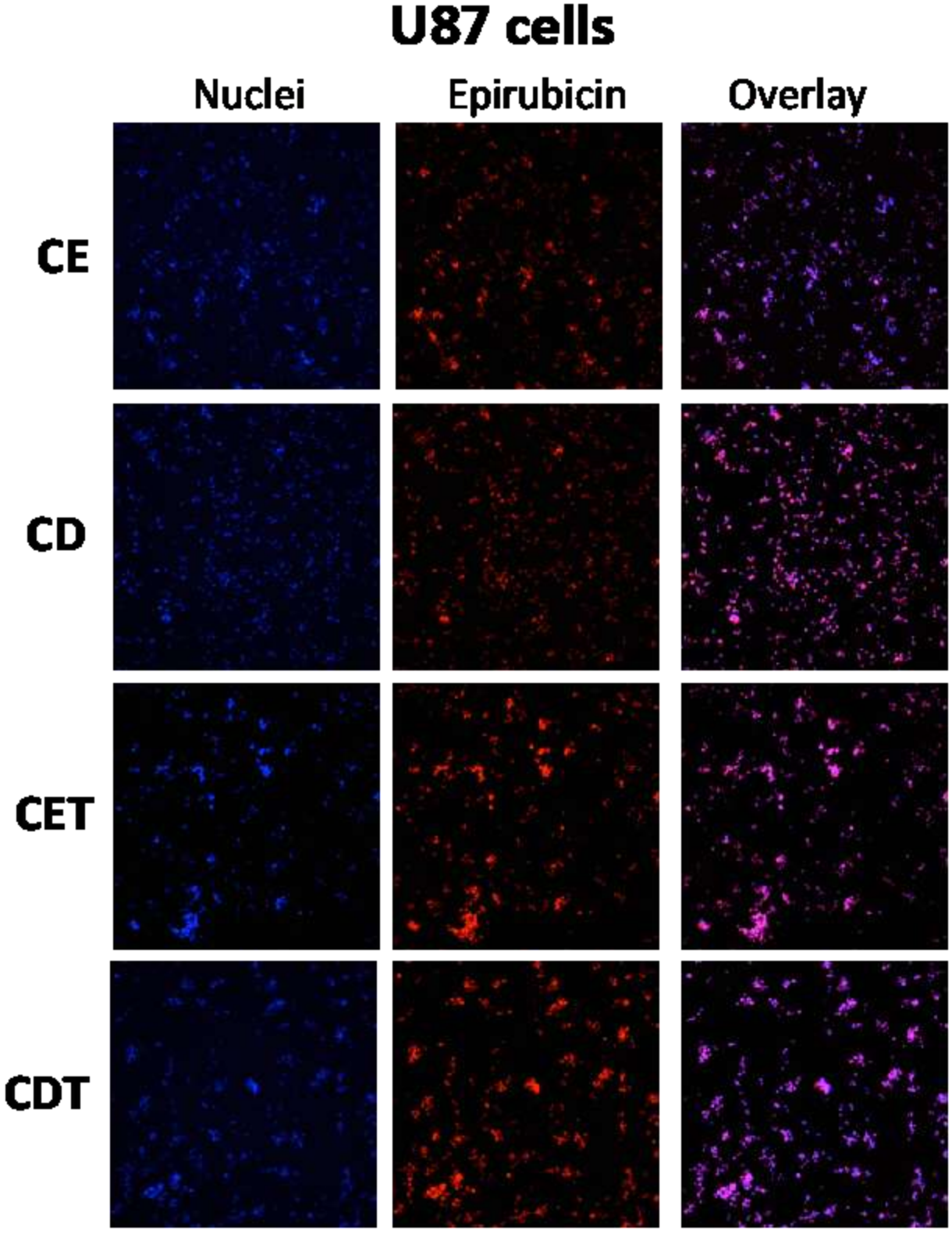
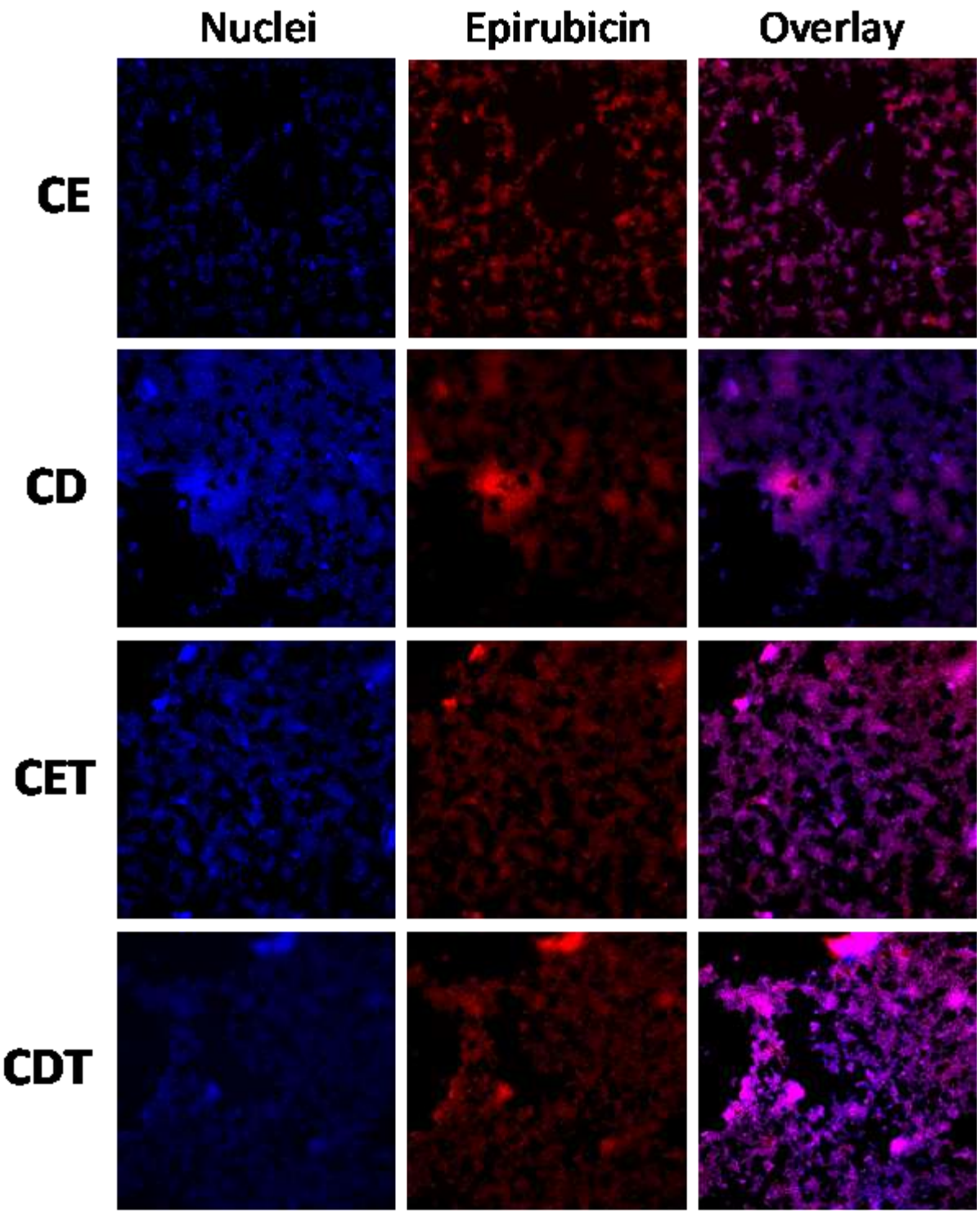


Figure S11. **A**. Relative levels of nuclear epirubicin in SJGBM2, CHLA266, U87 and CHLA200 cells. Cells were treated with either 10 μM C-dot drug conjugates, C-dot-epi (CE) and C-dot-dual drug (CD) (left panel) or 0.5 μM C-dot drug transferrin conjugates, C-dot-epi-transferrin (CET) and C-dot dual drug-transferrin (CDT) (right panel) for 6 hours and the epirubicin fluorescence was quantitated using Thermo Fisher Array Scan VTI with Target Activation algorithm run at 10X objective. **B**. Images (shown blow) were obtained and pseudocolored with imageJ. First panel is the hoechst stain (nuclei) in blue, second panel is the epirubicin fluorescence in red and the third panel is the overlay of the first two images. *Purple* color indicates overlap of nuclear and epirubicin fluorescence.


CHLA200 cells
Nuclei
Epirubicin
Overlay
CE
CD
CET
CDT


CHLA266 cells
Nuclei
Epirubicin
Overlay
CE
CD
CET
CDT


U87 cells
Nuclei
Epirubicin
Overlay
CE
CD
CET
CDT


SJGBM2 cells
Nuclei
Epirubicin
Overlay
CE
CD
CET
CDT

Supplemental methods

Immunocytochemistry

Our protocol for immunocytochemistry has bee previously described (BMC Cancer. 2017 Feb 4;17(1):99). Briefly, Cells were fixed in 4% paraformaldehyde, blocked and permeabilized with methanol and then incubated overnight with the primary antibody (TFR1, Cell Signaling Technology). A "no primary control" was included for all cell lines in which the cells were incubated with only the antibody diluent (2.5% BSA, balance PBS). Cells were then treated with a fluorochrome-conjugated secondary antibody followed by Prolong Gold Antifade Reagent with DAPI (Thermo Fisher Scientific, Waltham, MA). Samples were examined under an EVOS FLoid Cell Imaging Station fluorescent microscope (Thermo Fisher Scientific, Waltham, MA).

Quantitation of nuclear Epirubicin levels

Our method for quantitating nuclear uptake of drug containing nanoparticles has previously been described (ref # 30). Briefly, cells were treated with C-dot conjugates for either 18 hours or 6 hours, incubated with Hoechst stain to identify the nucleus, washed and media replaced. The nuclear level of epirubicin containing C-dot conjugates was quantitated using Thermo Fisher Array Scan VTI with Target Activation algorithm run at 10X objective. The Hoechst stain (channel 1) was used to define the cell mask (the nuclei) for the epirubicin fluorescence (channel 2). Approximately 1000 cells per well were analyzed. Data is presented as relative fold-increase in nuclear epirubicin fluorescence compared to non-treated control cells. Images were obtained and pseudocolored using ImageJ. Hoechst stain (nuclei) is blue, epirubicin shown in red. An overlay of the two images was generated using ImageJ to examine co-localization of nuclear and epirubicin fluorescence.