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Supplementary Information

Antineoplastic Tropolone Derivatives as Useful Biomarkers: Fluorescence Enhancement Upon Binding to Biological Targets

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General experimental:

Materials. Colchicine (COL), Deacetylcolchicine (DCOL), Human serum albumin fatty free (HSA), ibuprofen (IBP), sodium bis(2-ethylhexyl) sulfosuccinate (AOT) and warfarin (WAR) were commercial products obtained from Sigma-Aldrich Chemical Company. Sodium phosphate buffer (PB) was prepared from reagent-grade products using milli-Q water; the pH of the solutions was measured through a glass electrode and adjusted with NaOH to pH 7.4. Other chemicals were reagent grade and used as received.

Synthesis of Colchiceine (CEI) and deacetylcolchiceine DCEI). They were obtained as previously have been described (Dumont, R.; Brossi, A.; Silverton, J. V. *J. Org. Chem.* **1986**, *51*, 2515). The synthesis of CEI (300 mg) was performed by hydrolysis of colchicine (COL, 500 mg, 1,25 mmol) in 0.5 ml of acetic acid with 0.1 N HCl (30ml) heating at 100 ^{0}C for 2 h. The hot solution was cooled and Na_2CO_3 was added until the pH reached 6-7. The organic extract obtained with dichloromethane was purified by column chromatography using dichloromethane/ethanol (10/2). To obtain DCEI: COL (500 mg, 1,25 mmol) was dissolved in 5ml of acetic acid and 10 ml of H_2SO_4 (20%) and the mixture was heated at 90 ^{0}C for 5 h. The hot solution was neutralized with solid Na_2CO_3 and allowed to cool down to to room temperature. Afterward, extracted with CH_2Cl_2, concentrated to dryness and crystallized with methanol/chloroform to give 200 mg of DCEI.

Samples preparation. The tropolone derivative samples were prepared at 2 mM PB concentrations starting from a mother solution of 200 mM PB adjusted at pH 7.4. The pH

changes were induced adding different amouns of HCl or NaOH (12 M) to the neutral samples. These measurements were performed with a Crison pH-meter.

Each mixture was kept in the dark during 15 min at room temperature before performing measurements (it was ensurated that samples do not need more time to equilibrate).

Absorption and emission measurements. Ultraviolet spectra were recorded on a UV/Vis scanning spectrophotometer with a slit width of 5 nm. Fluorescence emission spectra were recorded on a Photon Technology International (PTI) LPS-220B fluorimeter. Measurements were done under aerated conditions at room temperature (25 ºC) in cuvettes of 1 cm path length.

Fluorescence measurements of tropolone derivatives with and without the presence of HSA. The analysis of the fluorescence enhancements were performed at 370 nm excitation wavelength using COL and DCOL at 10^{-4} M and CEI and DCEI at 5 x 10^{-5} M in buffered aqueous solutions (10^{-4} M PB, pH ca. 7.4). The HSA concentrations ranged from 10^{-5} up to 2 x10^{-4} M to avoid viscosity changes.

The fluorescence quantum yields of tropolone derivatives aqueous solutions (2 mM PB) of with and without the presence of albumin were performed at room temperature under aerobic conditions. The samples were excited at 370 nm (absorbance = 0.15) and the emission measurements were performed in the region 380-630 nm.

In this context, we determine K_a using equation 1 from the Clark's theory (Kenakin, T. *Trends in Pharmacological Sciences* **2004**, *25*, 186):

$$F/F_T = f = [HSA]/(K_d + [HSA]) \qquad (1)$$

where F is the fluorescence at different HSA-drug concentrations, F_T is the total emission when the HSA-drug concentration corresponds to 100 % saturation of drug bound. The ratio F/F_T is also referred to as f, the fractional bound, [HSA] is the free albumin concentration and K_d is the dissociation constant ($1/K_a$). To calculate K_d from equation 1, the initial HSA concentrations ($[HSA]_0$) up to 2 x 10^{-5} M were used as a good approximation of [HSA] in the fluorescence assays performed with CEI because, with these settings, the amount of drug-albumin complex is a very small percentage (less than 15% of $[HSA]_0$). In the case of DCEI, $[HSA]_0$ was used as [HSA] up to 8 x 10^{-5} M.

Fluorescence quantum yields (ϕ_F) were determined at the excitation wavelength of 370 nm using integrated peak areas by comparison with the areas under the emission curves with that obtained with quinine bisulfate in 1 N H_2SO_4, a widely used fluoresence standard (ϕ_{Fl} = 0.546). When the same experiments were performed at 400 nm, fluorescence quantum yield of CEI and DCEI decreased from ca. 0.0002 and 0.0004, respectively, to ca. 0.0001 for both.

The study of fluorescence measurements of colchiceine (CEI) at different pHs, including determination of ϕ_F, was performed using the isosbestic point appeared at 338 nm as the excitation wavelength to easily prepare isoabsorptive samples ($A_{338\ nm}$ = 0.15).

Competitive reactions between tropolone derivatives and ibuprofen (IBP) or warfarin (WAR) to bind HSA were carried out with 8 x 10^{-5} M HSA aqueous solutions (2 mM PB) containing 10^{-4} M COL or 2 x 10^{-4} DCOL and 10^{-4} M because at this concentrations ca. 95 % of HSA is complexed with the drugs. The experiment consisted of registering the fluorescence emission of CEI and DCEI before and after addition of increasing amounts of

IBP (1 x 10^{-5} M to 4 x 10^{-4} M) or WAR, which were added only up to 2 x 10^{-4} M to avoid absorption at the excitation wavelength (370 nm).

Albumin fluorescence quenching by tropolone derivatives. Phosphate buffered (2 m M, pH ca 7.4) aqueous solutions containing 10^{-5} M HSA display a fluorescence band centered at 344 nm, after excitation at 295 nm. Then, fluorescence quenching of this band after addition of increasing amounts of the quenchers (COL, DCOL, CEI and DCEI from 10^{-6} to 1.2 x 10^{-5} M) was monitored. Before analyzing the data, an inner filter effect correction (IFE) was applied because the three compounds show absorptions at the excitation and emission wavelengths. The IFE correction was done using equation (2):

$$F_{corr} = F_{obs} \times 10^{(Aex+Aem)/2} \quad (2)$$

where F_{corr} and F_{obs} are the corrected and observed fluorescence respectively, and A_{ex} and A_{em} the absorbance values at the excitation and emission wavelength respectively.

To determine the drugs-HSA binding constants from quenching data equation 3 was selected (Bosca, F. *J Phys Chem B* **2012**, *116*, 3504):

$$F_0/F = 1 + K_{SV}\,[Drug] \quad (3)$$

where F_0 and F are the fluorescence intensity in the absence and presence of the quencher ([Drug]) respectively, and K_{SV} is the Stern-Volmer quenching constant, which can be a good approximation for K_a in this type of static quenching process (Ahmad, B.; Parveen, S.; Khan, R. H. *Biomacromolecules* **2006**, *7*, 1350.).

Wide-field fluorescence microscopy. Images of reverse microemulsions were obtained by using conventional epifluorescence microscopy: Nikon Elipse E-800 fluorescence microscope. Imaging was accomplished with Nikon DMX1200 Digital color camera driven by ACT-1 VS.2.63 software. Appropriate dichroic and emission filters were employed to avoid crossover of fluorescence emission.

Reverse microemulsions were prepared using similar methodology to that used in reverse micelle environments with AOT/hexane/aqueous media with and without albumins (Itri, R.; Amaral, C. L. C.; Politi, M. J. *J. Chem. Phys.* **1999**, *111*, 7668. Itri, R.; Caetano, W.; Barbosa, L. R. S.; Baptista, M. S. *Braz. J. Phys.* **2004**, *34*, 58). Thus, the samples were made by adding a desirable volume of 10^{-4} M CEI aqueous solutions (5 mM phosphate buffer) with and without the presence of 10^{-4} M HSA into a 0.1 M AOT/hexane solution.

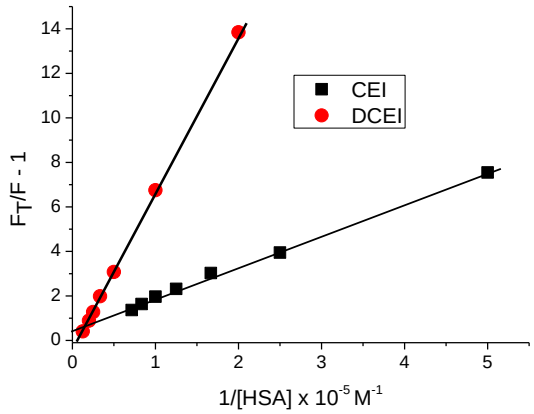
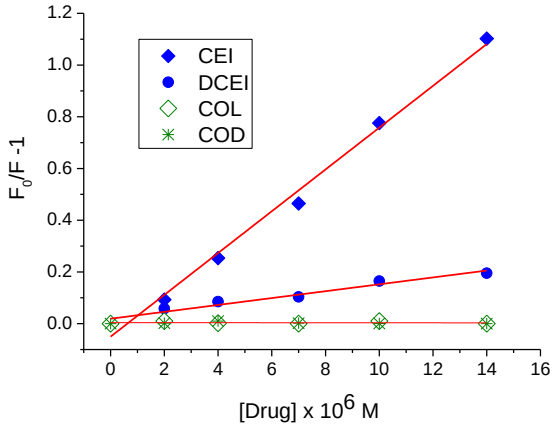


Fig. 1S Plots of F_T/F values obtained from 8 x 10^{-5} CEI and DCEI aqueous solutions *versus* reciprocal of [HSA].



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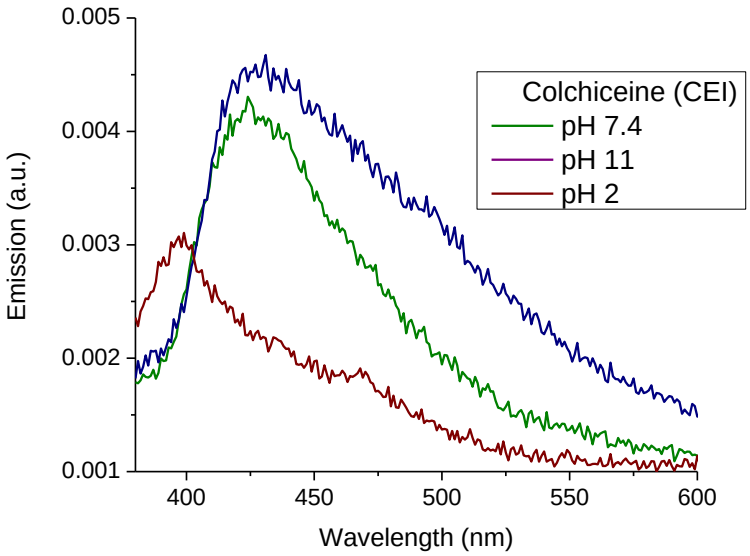

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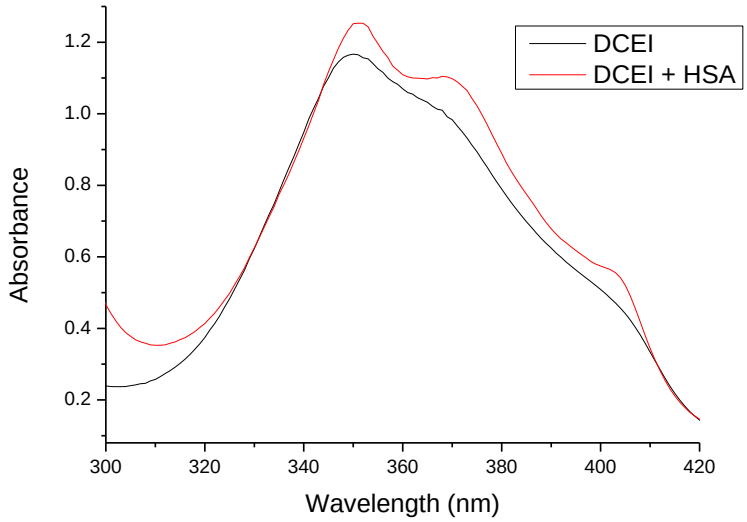

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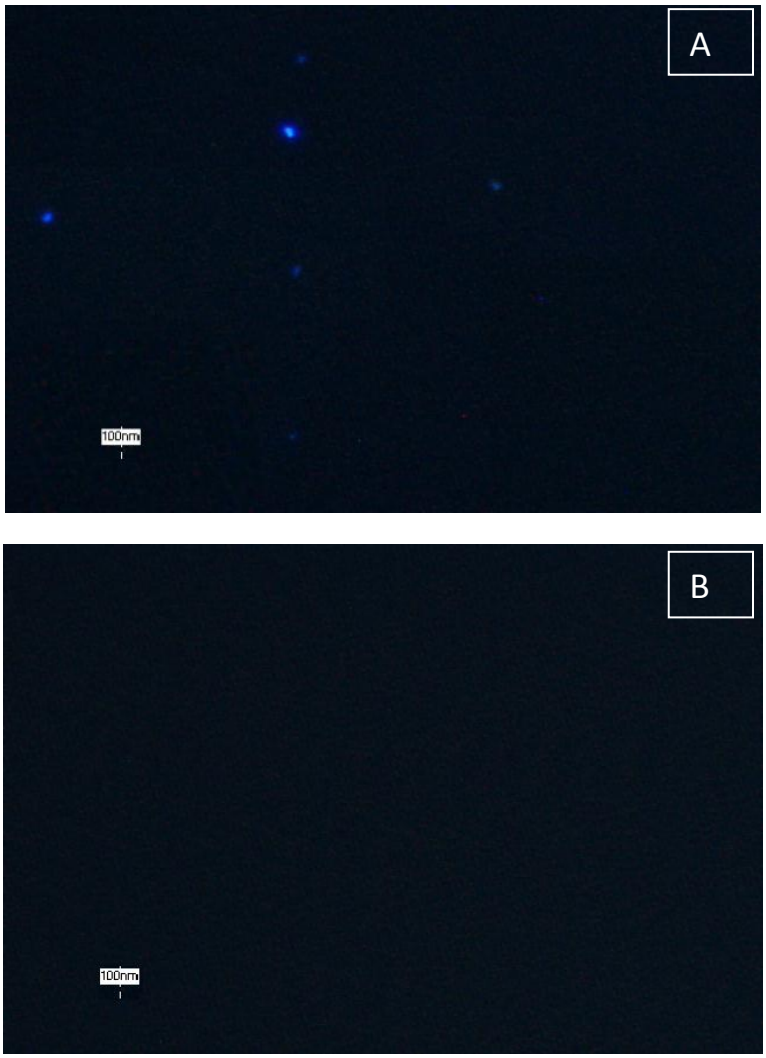

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