THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

pubs.acs.org/JPCB

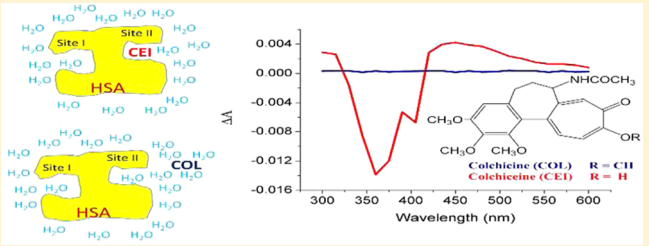
Behavior of Drug Excited States within Macromolecules: Binding of Colchicine and Derivatives to Albumin

Francisco Bosca*,† and Rosa Tormos*,‡

†Instituto Universitario Mixto de Tecnologia Quimica (UPV-CSIC), Universitat Politecnica de Valencia, Avenida de los Naranjos s/n, 46022 Valencia, Spain

‡Departamento de Química UPV, Universitat Politecnica de Valencia, Camino de Vera sn, 46022-Valencia, Spain

ABSTRACT: The aqueous solutions of colchicine (COL) and analogues such as colchiceine (CEI), and *N*-deacetylcolchiceine (DCEI) have been studied by laser flash photolysis (LFP) with and without the presence of human serum albumin (HSA) to determine the possible interactions between the drugs and the albumin. When irradiation of these drugs was performed in aqueous media, transient absorption species were not detected. However, triplet excited states of CEI and DCEI were detected when the experiments were carried out in the presence of albumin. Contrary to expectations, in the case of COL, no transient absorption species was observed. A deep study of COL triplet excited-state properties has revealed that intersystem crossing quantum yield (ϕ_{ISC}) decreases from organic media such as dichloromethane to water with ϕ_{ISC} values ca. 0.035 and <0.001 respectively. This fact together the inappreciable reactivity of ^{3}COL with tyrosine and tryptophan agree with the unbinding of COL to HSA. This study will show for the first time a way to determine the association constant (K_a) using the LFP technique. Thus, for CEI and DCEI binding to HSA, K_a values of $8 \pm 3 \times 10^4$ M^{-1} and $2 \pm 1 \times 10^4$ M^{-1} were obtained, respectively. Moreover, it was also established that these drugs are mainly placed into site II of the albumin using this technique. Therefore, this study validates the LFP as a useful methodology to study the interactions of COL and its analogues with serum albumins, and consequently it could be applied to others proteins such as tubulin.



INTRODUCTION

The interest in supramolecular systems has been growing during the last several years. In this context, the supramolecular photochemistry is a tool to control the regio- and stereoselectivity of a number of reactions.[1] A variety of hosts have been employed for this purpose, including zeolites,[2] cyclodextrins,[3] or biomolecules[4−6] such as serum albumins, which are the proteins most extensively studied because of their abundance, low cost, and stability.[7,8] They are attractive entities with wide application possibilities in photochemistry, from the investigation of fundamental mechanisms[9] to the development of tools for molecular biology[10] or photocatalytic antibodies.[11] Moreover, the albumins are very important from the biopharmacological point of view because of their capability to carry and to deliver endogenous and exogenous substances such as fatty acids, cholesterol, or drugs to the tissues. Thus, these biomolecules allow the solubilization of hydrophobic compounds and produce their homogeneous distribution in the body.[12,13]

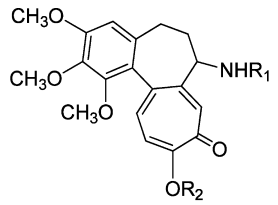
Human serum albumin (HSA) displays basically two high affinity binding sites for drugs, one located in subdomain IIA (site I) and one located in IIIA (site II).[13] The drug−protein binding constant is a fundamental physicochemical parameter for understanding the absorption, transport, and the target molecules of the drugs at the cellular level.[14] The techniques to determine association constant (K_a) are based on the separation of the free and the bound fraction of the drug and in the detection of physicochemical changes of the properties of the complexed drugs or in the behavior of the bound protein. Conventional techniques such as dialysis, ultrafiltration or calorimetry, separation methods such as liquid chromatography, microdialysis, or capillary electrophoresis as well as spectroscopic measurements of absorption, fluorescence, room-temperature phosphorescence have been used to determine binding parameters, that is, the number of the binding sites and affinity constants.[15] During the past few years, new and more specific assays for ligand−protein interactions have been achieved in the analytical methodology. In fact, recently, researchers have shown that the laser flash photolysis (LFP) is a fast and sensitive technique to analyze some properties of drug−protein interactions.[5,6]

Colchicine (COL), a bioactive plant alkaloid (Chart 1) extracted from *Colchicum autumnale* and *Gloriosa superba*, is a drug that has long been used for treatment of arthritic gout that in the last decades has received considerable attention in cancer research because of its binding to tubulin disrupts mitosis, ending this process at the methaphase and hence stopping cell division.[16] Nevertheless, because the clinical usefulness of COL

Received: March 12, 2013
Revised: May 9, 2013

   dx.doi.org/10.1021/jp402489j | *J. Phys. Chem. B* XXXX, XXX, XXX−XXX

Chart 1. Structure of Colchicine (COL) and Analogues Such As Colchiceine (CEI) and Deacetylcolchiceine (DCEI)



	COL	CEI	DCEI
R_1 =	$COCH_3$	$COCH_3$	H
R_2 =	CH_3	H	H

is limited by severe toxicity,[17] important efforts have been devoted to the design of less toxic derivatives. Thereby, new compounds such as colchiceine (CEI) and desacetylcolchiceine (DCEI, see Chart 1) were synthesized with this aim.[18] The therapeutic activity of COL and its derivatives and their ability to bind biomolecules such as tubulin have been well-established.[18−21] However, contradictory reports about the binding of COL and derivatives to HSA have been found in the literature.[22,23] A former study of the issue, using equilibrium dialysis, stated that the COL is not bound to proteins, while, under the same conditions, the binding of the analogous DCEI to HSA was 80% and an association constant (K_a) for a single binding site ca. 8×10^3 M^{-1} has been reported.[22] Conversely, when the interaction of COL with albumins such as HSA and bovine serum albumin (BSA) was investigated by means of protein fluorescence quenching, K_a values of 4.3 and 4.9×10^4 M^{-1} (298 K) were reported for COL binding to HSA and BSA, respectively.[23,24] With this background, we decide to study the COL−albumin binding by LFP because when a complexation process between drugs and albumins is produced, changes in the triplet excited-state lifetimes of the drugs or generation of new intermediates can be detected.[5,6,25] In this context, to shed some light on the issue, COL derivatives as CEI and DCEI were also included in this study.

LFP results will provide strong evidence that under physiological conditions CEI and DCEI are bound to HSA but not COL. A clear characterization of COL triplet excited state has been done to confirm its negligible binding to albumin.

Moreover, K_a values for drug−biomolecule binding will be determined for the first time using the LFP technique.

EXPERIMENTAL SECTION

General. Materials. COL, HSA fatty free, ibuprofen (IBP), *N*-acetyl-L-tryptophan (Trp), L-tyrosine methyl ester (Tyr), and warfarin (WAR) were commercial products obtained from Sigma-Aldrich Chemical. 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.

The samples were prepared with a mother solution of 300 mM PB adjusted at pH 7.4 with a Crison pH-meter.

CEI and DCEI have been obtained as previously described.[26] The synthesis of CEI (300 mg) was performed by hydrolysis of COL (500 mg, 1.25 mmol) in 0.5 mL of acetic acid with 0.1 N HCL (30 mL) heating at 100 °C for 2 h. The hot solution was cooled, and Na_2CO_3 was added until the pH reached 6 to 7. The organic extract obtained with dichloromethane was purified by column chromatography using dichloromethane/ethanol (10/2). In the case of DCEI, COL (500 mg, 1,25 mmol) was dissolved in a mixture of 5 mL of acetic acid and 10 mL of H_2SO_4 (20%), which was heated to 90 °C for 5 h. The hot solution was neutralized with solid Na_2CO_3 and cooled to room temperature. Afterward, it was extracted with CH_2Cl_2, concentrated to dryness, and crystallized with methanol/chloroform to obtain 200 mg of DCEI.

Laser Flash Photolysis Experiments. A pulsed Nd:YAG laser was used at 355 nm. The single pulses were ∼10 ns duration, and the energy was from 10 to 1 mJ/pulse. A pulsed xenon lamp was employed as detecting light source. The LFP apparatus consisted of the pulsed laser, the Xe lamp, a monochromator, and a photomultiplier made up of a tube, housing, and power supply. The output signal from the oscilloscope was transferred to a personal computer.

All solutions of COL, CEI, and DCEI were prepared at 5×10^{-5} M and excited using a 355 nm laser pulse.

Colchicine Triplet Excited State Measurements. The molar absorption coefficient of COL triplet state (^{3}COL) in acetonitrile was estimated by monitoring the energy transfer reaction between ^{3}COL and β-carotene ground state (β-Car). Because the intersystem crossing quantum yield (Φ_{ISC}) of β-Car is exceedingly small, ^{3}β-Car can only be populated via energy transfer from triplet excited-state molecules with energies higher than that of ^{3}β-Car (eq 1). Then, ^{3}COL can act as an energy donors to β-Car, whose low-lying triplet excited-state energy is ca. 19 kcal mol^{-1}.[27]

$$^3\text{COL} + \beta\text{-Car} \rightarrow \text{COL} + {}^3\beta\text{-Car} \quad (1)$$

The study was performed with deaerated acetonitrile solutions of COL with and without the presence of β-Car (1 to 5×10^{-5} M) using a 355 nm laser pulse. By this means, the molar absorption coefficient (ε) of ^{3}COL was calculated using the eq 2:

$$k_2/(k_2 - k_1) \times \Delta A({}^3\beta\text{-Car}(520\ \text{nm})) \times \varepsilon({}^3\text{COL}(420\ \text{nm})) = \Delta A({}^3\text{COL}(420\ \text{nm})) \times \varepsilon({}^3\beta\text{-Car}(520\ \text{nm})) \quad (2)$$

where the ΔA values refer to the absorbance at 420 nm of ^{3}COL triplet state at the beginning of the reaction and ^{3}β-Car (at 520 nm) at the end of the reaction, k_1 is the ^{3}COL decay rate constant without β-Car, and k_2 is the different ^{3}COL decay rate constant obtained at the different concentrations of β-Car. The molar absorption coefficient for ^{3}β-Car in acetonitrile at 520 nm was taken to be 100 000 M^{-1} cm^{-1}, as described in toluene.[27]

The intersystem crossing quantum yield (Φ_{ISC}) of ^{3}COL was obtained by the comparative method,[28] assuming that $\varepsilon(^3\text{COL})$ is similar in all solvents. Hence, excitation of benzophenone (BZPH) and COL were carried out separately using solutions with identical absorbance at the excitation wavelength (0.3 at 355 nm). Then, eq 3 was applied:

$$\Phi_{ISC}(\text{COL}) = \Phi_{ISC}(\text{BPZH}) \times \Delta A({}^3\text{COL}(420\ \text{nm})) \times \varepsilon({}^3\text{BZPH}(525\ \text{nm})) / (\Delta A({}^3\text{BZPH}^*(525\ \text{nm})) \times \varepsilon({}^3\text{COL}(420\ \text{nm}))) \quad (3)$$

dx.doi.org/10.1021/jp402489j | J. Phys. Chem. B XXXX, XXX, XXX−XXX

where the ΔA values refer to the absorbance for ^{3}COL at 420 nm and ^{3}BZPH at 525 nm. The benzophenone molar absorption coefficient ($\varepsilon(^3$BZPH(525 nm))) and its triplet-state quantum yield (Φ_{ISC} (BPZH)) in acetonitrile were taken to be 6500 M^{-1} cm^{-1} and 1, respectively.[29]

The ^{3}COL quenching rate constants by oxygen, β-Car, and other quenchers were determined using the Stern–Volmer eq 4:

$$1/\tau = 1/\tau_0 + k[\text{quencher}] \quad (4)$$

The reactivity of ^{3}COL with Trp and Tyr was analyzed in acetonitrile using increasing amounts of the quenchers (from 10^{-3} to 10^{-2} M).

Study of COL, CEI, and DCEI in Aqueous Media with and without the Presence of HSA. Aqueous solutions of COL, CEI, and DCEI were prepared in 2×10^{-3} M PB with and without the presence of HSA under aerobic and anaerobic conditions (bubbling N_2O and N_2). The samples containing albumins needed special manipulation due to the impossibility of bubbling the solutions to remove oxygen. Thus, N_2O and N_2 were introduced inside of the sample quartz cells, flowing the gas during 20 min without generating bubbles and stirring the solution. Transient absorption spectra at different times after the laser pulse were obtained for each sample in the presence and the absence of HSA (10^{-4} M).

LFP assays with 5×10^{-5} M COL, CEI, and DCEI in buffered aqueous solutions (2×10^{-3} M PB, pH ca. 7.4) using HSA concentrations ranging from 10^{-5} up to 4×10^{-4} M were performed to determine their associations constant (K_a) to albumin and their Φ_{ISC} when they are bound to HSA. The experiment consisted of registering the absorption of the intermediate before and after the addition of increasing amounts of the albumin. The absorptions were registered three times (with three different samples), and the results include the average.

The study of competitive reactions between CEI and its derivative DCEI with IBP or WAR to bind HSA were carried out using 2×10^{-3} M PB aqueous solutions containing 10^{-4} M of HSA, CEI, and DCEI. As described above, the experiment consisted of registering the absorption of the intermediate before and after the addition of increasing amounts of IBP (5×10^{-5} to 4×10^{-4} M) or WAR. To avoid absorption at 355 nm, WAR was added only up to 2×10^{-4} M.

RESULTS AND DISCUSSION

LFP of COL (5×10^{-5} M) was carried out in phosphate-buffered solutions (2×10^{-3} M PB, at pH ca. 7.4) under N_2O, N_2, and aerated atmospheres with and without the presence of HSA (10^{-4} M) using a 355-nm Nd:YAG laser, but no transient species was detected. These results agree with a recent LFP study of COL with and without the presence of albumins,[30] but they are in disagreement with the results of another study where it was claimed that triplet excited state of COL can be detected in different solvents including water.[31] Thus, before analyzing in depth the COL results obtained in water, we decide to perform a LFP study of COL in different solvents.

Properties of Colchicine Triplet Excited State. LFP experiments with 5×10^{-5} M COL in H_2O, methanol, ethanol, acetonitrile, and dichloromethane under aerated and anaerated conditions were performed. Thus, although no intermediate was detected in aqueous medium, an almost identical transient absorption species with a λ_{max} ca. 420 nm was observed in the other solvents (see Figure 1). Our results were not in agreement with the data described in the literature.[31] The absorption of the detected intermediate decreased when the protic character of solvent increased to the extent that no signal was observed in water. Besides, the lifetimes of the intermediate in the different solvents result to be longer (between 2.2 and 3.6 μs, Table 1) than those described in the literature (close to 1 μs in all solvents including water).[31] With these results and considering that the intermediate has been assigned to COL triplet excited estate (^{3}COL) only by its efficient quenching by molecular oxygen, we decided to perform new experiments to prove the triplet character of this transient species. Thereby, first, its reactivity was checked with molecular oxygen in all solvents, and quenching rate constant values of 2 to 3×10^9 M^{-1} s^{-1} were determined. The next step was to perform an energy-transfer study of COL with β-carotene (β-Car) in acetonitrile because if an energy-transfer reaction between transient species of COL and β-Car will occur (eq 1 in the Experimental Section), the COL intermediate would be unequivocally ^{3}COL. As it was observed that the decay of the intermediate absorbing at λ_{max} ca. 420 nm was concomitant with the growth of $^3\beta$-Car (Figure 2), the assignment ^{3}COL was confirmed. Moreover, this reaction was diffusion-controlled (k_q ca. 10^{10} M^{-1} s^{-1}), which was in agreement with the energy of ^{3}COL described in the literature (45 kcal mol^{-1})[31] because the $^3\beta$-Car energy is only 19 kcal mol^{-1}.

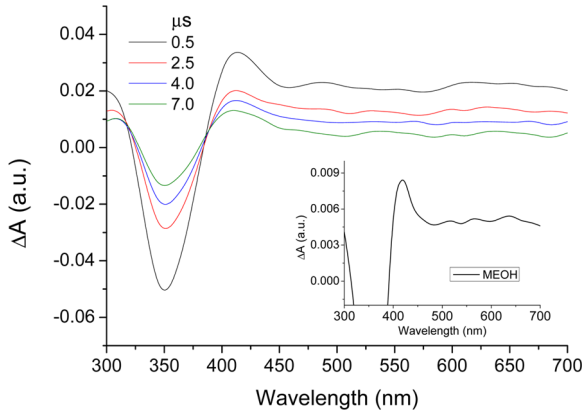


Figure 1. Absorption spectra of 5×10^{-5} M COL in dichloromethane at different times (microseconds) after laser excitation Inset: Absorption spectra of 5×10^{-5} M COL in methanol 30 ns after the laser pulse.

In this context, applying eq 2, as indicated in the Experimental Section, a triplet molar absorption coefficient (ε) at 420 nm was found to be 20 100 ± 1100 M^{-1} cm^{-1} for ^{3}COL in acetonitrile. When this parameter was applied in eq 3 (see eq 3 in the Experimental Section) to determine the intersystem crossing quantum yield (ϕ_T) of COL in each solvent, important differences were obtained (Table 1). The proticity of the solvents can be correlated with the ϕ_T changes. The polarity of the solvents and other properties such as the dielectric constant or the dipole moment cannot be interrelated with all photophysical and photochemical properties of COL, as it has been suggested (see Table 1).[32] Analysis of the data shown in Table 1 also confirm that COL photodegradation is not produced from ^{3}COL[31,33] because there is no relationship between the photodegradation quantum yields (ϕ_D) of COL and its intersystem quantum yields (ϕ_T).

Study of the Interactions Colchicine Triplet Excited State and Albumin. As commented above, LFP experiments

dx.doi.org/10.1021/jp402489j | J. Phys. Chem. B XXXX, XXX, XXX–XXX

Table 1. Photophysical and Photochemical Properties of Colchicine in Different Solvents

solvent	absorption (λ_{max}, nm)	τ_T (μs)	ϕ_T	ϕ_D	solvent dielectric constant
H_2O	353		<0.001	0.003[a]-0.006[b]	78.3
CH_3OH	350	2.4	0.009	0.02[a]-0.037[b]	32.6
CH_3CH_2OH	348	3.6	0.012	0.051[b]	24.5
CH_3CN	342	2.2	0.037	0.026[b]	37.5
CH_2Cl_2	345	2.5	0.035	0.03[a]	8.9
$CHCl_3$	350[a]			0.05[a]-0.06[b]	4.8

[a]Ref 32. [b]Ref 31.

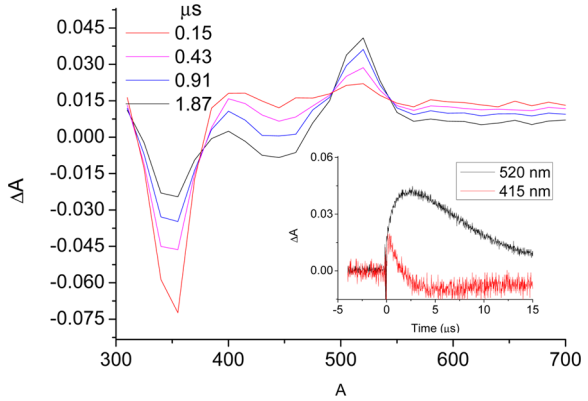


Figure 2. Laser flash photolysis spectra of 5×10^{-5} M COL in acetonitrile in the presence of β-Car 5×10^{-5} M, 0.1 μs, 0.43 μs, 0.91 and 1.87 μs after the laser pulse. Inset: Decay and growth traces at 415 (red) and 520 nm (black), corresponding to ^{3}COL and $^3\beta$-Car, respectively.

with COL (5×10^{-5} M) with and without the presence of HSA (10^{-4} M) did not display transient absorption species. Thus, taking into account the properties of ^{3}COL, this fact could be attributed to: (i) COL binding to albumin is located in subdomain IIA (site I of HSA) or in the subdomain IIIA (site II) and fast electron-transfer reactions of ^{1}COL or ^{3}COL with Tyr or Trp generate undetectable intermediates by nanosecond LFP, (ii) COL binding to proteins is produced but ^{3}COL is quickly expelled from the cavities of the proteins, and (iii) COL is not into the HSA cavities.

Interestingly, taking into account the albumin−drug equilibrium:

$$[\text{COL}] + [\text{albumin}] \underset{K_d}{\overset{K_a}{\rightleftarrows}} [\text{COL-albumin}] \quad (5)$$

the eq 6:

$$K_a = [\text{COL-albumin}]/([\text{albumin}] \times [\text{COL}]) \quad (6)$$

and the LFP initial concentrations of drug and albumin (10^{-4} M), the percentage of complexed drug should be ca. 70% for a COL−albumin association constants (K_a) of 4.3×10^4 M^{-1}, the value determined for COL by fluorescence spectroscopy.[23] However, the complexation should be <8% if the K_a value is below 10^3 M^{-1}, as it can be estimated from the COL equilibrium dialysis study.[22]

The reactivity of ^{3}COL with the most reactive amino acid residues (Trp and Tyr) of the major selective binding sites of HSA (site I and II) was checked to analyze the first hypothesis. It is important to note that site I is dominated by the presence of hydrophobic residues including Trp214 in HSA, and the probable candidate for a complex formation at site II is Tyr411 in HSA.[34] Therefore, LFP experiments with COL acetonitrile solutions in the presence of increasing amounts of Trp, L-tyrosine methyl ester (Tyr) were conducted, but no quenching was observed, which discards the first hypothesis.

The possibility that ^{3}COL is quickly expelled from the albumin cavity was also analyzed using the receptor−ligand complex eq 7:[35]

$$K_a = k_{on}/k_{off} \quad (7)$$

assuming that the association constant (K_a) for the ground state is similar to that expected for ^{3}COL and that the association rate constant (k_{on}) is a diffusion limit control process in water (5×10^9 M^{-1} s^{-1}). Therefore, for a COL−albumin K_a ca. 4.3×10^4 M^{-1}, the ^{3}COL−albumin complexation should be >70% under our experimental conditions and the dissociation rate constants (k_{off}) of ^{3}COL-albumin would be ca. 1.6×10^5 s^{-1}, which would produce the detection of the ^{3}COL placed into HSA. The fact that this was not observed discards the second hypothesis. Hence, all results are in favor that COL is predominantly outside of the protein cavities, and, accordingly, K_a ought to be below 10^3 M^{-1}.

Study of the Interactions of Colchiceine and *N*-Deacetylcolchiceine with HSA. Laser excitation of 5×10^{-5} M DCEI and CEI in buffered aqueous solutions under aerobic and anaerobic conditions does not show transient absorption spectra, which is concordant with the results obtained for COL. However, when both compounds were studied in the presence of albumin (HSA, 10^{-4} M), transient absorption spectra very similar between them and to those registered for ^{3}COL in acetonitrile were detected (see Figure 2−4).

These intermediates, with lifetimes (τ) longer than 250 μs under anaerobic conditions, are quenched in the presence of

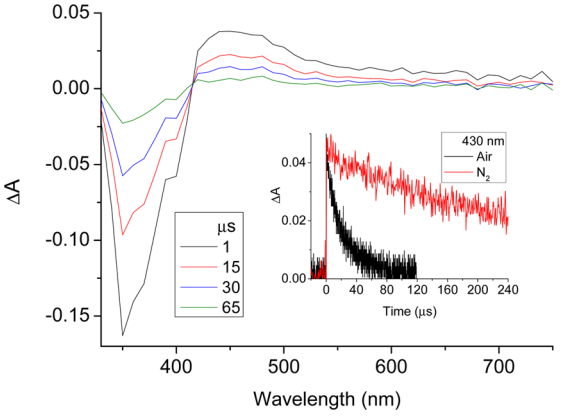


Figure 3. Absorption spectra of 5×10^{-5} M DCEI in aqueous 2×10^{-3} M PB in the presence of 10^{-4} M HSA and air at different times after laser excitation. Inset: Decay traces at 430 nm of DCEI in air (black) and under anaerobic conditions (red).

dx.doi.org/10.1021/jp402489j | J. Phys. Chem. B XXXX, XXX, XXX−XXX

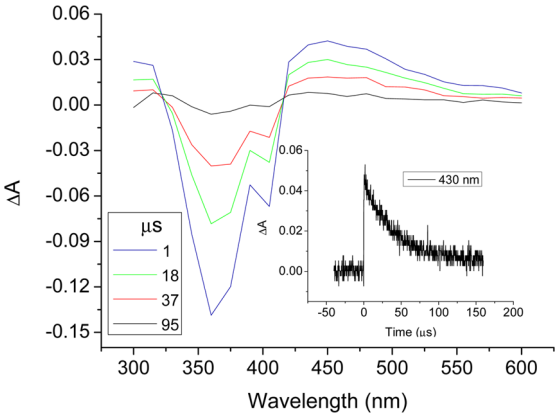


Figure 4. Absorption spectra of 5×10^{-5} M CEI in aqueous 2×10^{-3} M PB in the presence of 10^{-4} M HSA and air at different times after laser excitation. Inset: Decay traces at 430 nm of DCEI under aerobic conditions.

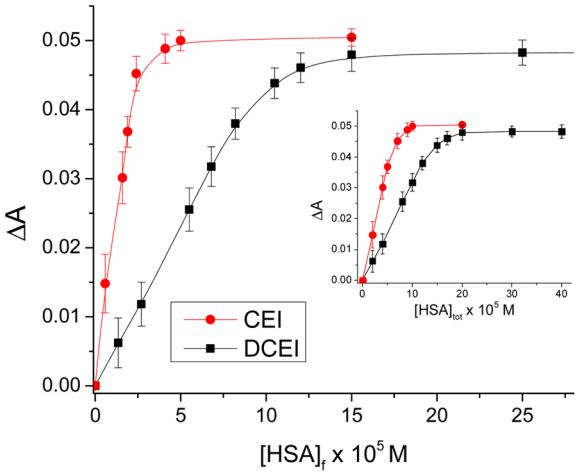


Figure 5. Absorbance increases at 430 nm of 5×10^{-5} M CEI (red circles) and DCEI (black square) in 2 mM PB aqueous solutions versus free albumin $[HSA]_f$ and inset versus total albumin $[HSA]_{tot}$.

oxygen (τ ca. 18 and 38 μs for DCEI and CEI). These facts suggest that the transient absorption species of DCEI and CEI detected are assignable to triplet excited states. Further support was found when performing LFP of CEI in acetonitrile and ethanol; an intermediate was detected in both solvents that was efficiently quenched by oxygen (k_q ca. 4×10^{-9} M^{-1} s^{-1}) and displayed a transient absorption spectrum that matched that registered for CEI in aqueous solutions with albumin. Lifetime of ^{3}CEI under anaerobic conditions was found to be ca. 8.1 μs in acetonitrile and 5.1 μs in ethanol. It is noteworthy that the lifetime increase in intermediates by the presence of albumin is usually observed in triplet excited states linked to albumins.[5,6,25,36] Therefore, the affinity of COL analogues for binding to albumin is higher when the OH group of their tropolone ring is free.

Assuming an molar absorption coefficient for ^{3}CEI and ^{3}DCEI at 430 nm similar to that determined for ^{3}COL, an intersystem crossing quantum yield (Φ_{ISC}) of 0.035 was estimated for CEI and DCEI in acetonitrile.

The addition of increasing amounts of HSA ($[HSA]_{tot}$ from 10^{-5} to 4×10^{-4} M) to CEI and DCEI aqueous solution resulted in important CEI and DCEI triplet absorption enhancements (inset Figure 5). Thus, as CEI and DCEI at 2 and 4×10^{-4} M, respectively, are mostly bound to HSA, a Φ_{ISC} of ca. 0.035 was also obtained for CEI and DCEI using the comparative method and the maxima absorbance obtained for both drugs using the albumin concentrations described above. Moreover, the triplet excited-state absorption enhancements of CEI and DCEI were also used to determine their K_a ($1/K_d$), applying eq 8 from the Clark's theory:[37]

$$\Delta A/\Delta A_T = f = [HSA]_f/(K_d + [HSA]_f) \quad (8)$$

where ΔA is the relative absorbance at the different HSA−drug concentrations and ΔA_T is the total absorbance when the HSA−drug concentration corresponds to 100% saturation of drug bound (at this point [HSA − drug] = [drug]). The ratio $\Delta A/\Delta A_T$ is also referred to as f, the fractional bound, $[HSA]_f$ is the free albumin concentration ($[HSA]_{tot} - [HSA-drug]$), and K_d is the dissociation constant ($1/K_a$). Consequently, when K_d is defined as the concentration of free HSA at which 50% of the drug is bound (50% of relative absorbance with a fractional binding of 0.5, see eq 9 and Figure 5), a K_d of ca. $1.3 \pm 0.5 \times 10^{-5}$ M for CEI (K_a $8 \pm 3 \times 10^4$ M^{-1}) and $5 \pm 2.5 \times 10^{-5}$ M for DCEI (K_a $2 \pm 1 \times 10^4$ M^{-1}) were obtained.

$$F/F_T = f = 0.5 = [HSA]_f/(K_d + [HSA]_f) \rightarrow K_d = [HSA]_f \quad (9)$$

This methodology could be valid not only when the free drugs do not contribute to the triplet signal and the triplet complex disappears with monoexponential time profile but also when the triplet drug is populated outside and bound to one or both albumin sites. In these cases, as the overall T−T signal would correspond to the amount of the signal of two or three T−T signal fractions with different monoexponential time profiles, then, the absorption of the free triplet drug would be subtracted before to apply eq 8. However, this methodology may not be applied when the triplet drug reacts with the protein or when the triplet lifetimes are similar outside and complexed in some of the cavities.

The use of specific probes to move a protein ligand is a well-established method for binding site assignment. IBP and WAR were chosen as stereotypical ligands for sites II and I, respectively.[38,39] The experiments were performed on 1:1 CEI/HSA and DCEI/HSA aqueous PB (2 mM) solutions, and titration was conducted by the addition of increasing amounts of IBP (up 6×10^{-4} M) or WAR (up to 2×10^{-4} M) after excitation at 355 nm. The analysis of the results showed a decrease in the absorbance of the ^{3}CEI and ^{3}DCEI by the presence of IBP, while nothing changed when WAR was added. In Figure 6 are shown the decay traces registered at 430 nm for DCEI. It is noteworthy from these results that CEI and DCEI are mainly associated with site II of HSA. However, although a similar behavior was observed for CEI than for DCEI, the decrease in the absorption at 430 nm was lower for CEI. This fact can be attributable to the higher association constant for CEI than for DCEI, which reveals that N-deacetylation of CEI produces an affinity decrease for albumin site II.

CONCLUSIONS

The most relevant properties of colchicine triplet excited state (^{3}COL) have been determined, highlighting the decrease in ^{3}COL quantum yield in protic media. The reactivity of ^{3}COL with the most reactive amino acids of the HSA binding sites

dx.doi.org/10.1021/jp402489j | J. Phys. Chem. B XXXX, XXX, XXX−XXX

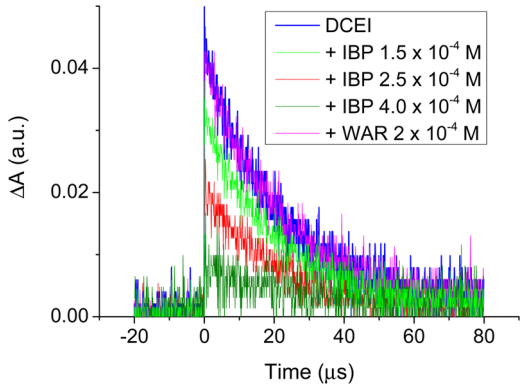


Figure 6. Decay traces at 430 nm of 2 m M PB aqueous solutions upon 355 nm laser excitation of 5×10^{-5} M DCEI (black) in the presence 10^{-4} M HSA and increasing amounts of ibuprofen ((1.5 and 4) $\times 10^{-4}$ M) or warfarin (2×10^{-4} M).

(tryptophan and tyrosine) has also been discarded. LFP experiments in aqueous media have confirmed the very low binding affinity of COL to HSA. (Its association constant must be below 10^3 M^{-1}.) By contrast, an important binding of CEI and DCEI to site II of HSA has been detected. In this context, this study has shown a procedure to determine association constants using the LFP technique. Thus, for CEI and DCEI binding to HSA K_a values of $8 \pm 3 \times 10^4$ M^{-1} and $2 \pm 1 \times 10^4$ M^{-1} were obtained, respectively. Moreover, it was also established that these drugs are mainly placed into site II of the albumin using this technique. Therefore, these findings, besides to validate the LFP as a useful methodology to study the interactions of COL derivatives with albumins, open a new way to evaluate their interactions with other proteins such as tubulin, which can be highly relevant to understanding the primary event in the cancer treatment by these family of drugs. Moreover, the knowledge of the binding affinities of COL and its derivatives CEI and DCEI to HSA contributes to the understanding of the higher toxicity of COL than those described for its derivatives[40] as well as to design new compounds taking into account this property.

AUTHOR INFORMATION

Corresponding Author

*E-mail: fbosca@itq.upv.es (F.B.); rtormos@qim.upv.es (R.T.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Financial support from Spanish government (CTQ2010-19909) is gratefully acknowledged.

REFERENCES

(1) Nishijima, M.; Wada, T.; Mori, T.; Pace, T. C. S.; Bohne, C.; Inoue, Y. Highly Enantiomeric Supramolecular 4 + 4 Photocyclodimerization of 2-Anthracenecarboxylate Mediated by Human Serum Albumin. *J. Am. Chem. Soc.* **2007**, *129*, 3478−3484.

(2) Sivaguru, J.; Poon, T.; Franz, R.; Jockusch, S.; Adam, W.; Turro, N. J. Stereocontrol within Confined Spaces: Enantioselective Photooxidation of Enecarbamates Inside Zeolite Supercages. *J. Am. Chem. Soc.* **2004**, *126*, 10816−10817.

(3) Fukuhara, G.; Mori, T.; Wada, T.; Inoue, Y. Entropy-Controlled Supramolecular Photochirogenesis: Enantiodifferentiating Z-E Photoisomerization of Cyclooctene Included and Sensitized by Permethylated 6-O-Modified Beta-Cyclodextrins. *J. Org. Chem.* **2006**, *71*, 8233−8243.

(4) Wada, T.; Nishijima, M.; Fujisawa, T.; Sugahara, N.; Mori, T.; Nakamura, A.; Inoue, Y. Bovine Serum Albumin-Mediated Enantiodifferentiating Photocyclodimerization of 2-Anthracenecarboxylate. *J. Am. Chem. Soc.* **2003**, *125*, 7492−7493.

(5) Lhiaubet-Vallet, V.; Sarabia, Z.; Bosca, F.; Miranda, M. A. Human Serum Albumin-Mediated Stereodifferentiation in the Triplet State Behavior of (S)- and (R)-Carprofen. *J. Am. Chem. Soc.* **2004**, *126*, 9538−9539.

(6) Lhiaubet-Vallet, V.; Bosca, F.; Miranda, M. A. Stereodifferentiating Drug-Biomolecule Interactions in the Triplet Excited State: Studies on Supramolecular Carprofen/Protein Systems and on Carprofen-Tryptophan Model Dyads. *J. Phys. Chem. B* **2007**, *111*, 423−431.

(7) Carter, D. C.; He, X. M. Structure of Human Serum-Albumin. *Science* **1990**, *249*, 302−303.

(8) Carter, D. C.; Ho, J.; Wang, Z.; Ho, J. X. Evaluating Drug Compounds Based on Their Albumin Binding Properties Comprises Obtaining Information About the Three-Dimensional Structures of Drugs That Bind to Human Serum Albumin Binding Sites. WO2005041895-A2; EP1684705-A2; US2007043509-A1, 2005.

(9) Alvarez, E. J.; Vartanian, V. H.; Brodbelt, J. S. Metal Complexation Reactions of Quinolone Antibiotics in a Quadrupole Ion Trap. *Anal. Chem.* **1997**, *69*, 1147−1155.

(10) Bergogne-Berezin, E. Clinical Role of Protein Binding of Quinolones. *Clin. Pharmacokinet.* **2002**, *41*, 741−750.

(11) Balfour, J. A. B.; Lamb, H. M. Moxifloxacin - A Review of Its Clinical Potential in The Management of Community-Acquired Respiratory Tract Infections. *Drugs* **2000**, *59*, 115−139.

(12) He, X. M.; Carter, D. C. Atomic-Structure and Chemistry of Human Serum-Albumin. *Nature* **1992**, *358*, 209−215.

(13) Carter, D. C.; Ho, J. X. Structure of Serum-Albumin. *Adv. Protein. Chem.* **1994**, *45*, 153−203.

(14) Herve, F.; Urien, S.; Albengres, E.; Duche, J. C.; Tillement, J. P. Drug-Binding in Plasma - A Summary of Recent Trends in the Study of Drug and Hormone-Binding. *Clin. Pharmacokinet.* **1994**, *26*, 44−58.

(15) Bosca, F. Seeking to Shed Some Light on the Binding of Fluoroquinolones to Albumins. *J. Phys. Chem. B* **2012**, *116*, 3504−3511.

(16) Bhattacharyya, B.; Panda, D.; Gupta, S.; Banerjee, M. Anti-Mitotic Activity of Colchicine and the Structural Basis for Its Interaction with Tubulin. *Med. Res. Rev.* **2008**, *28*, 155−183.

(17) Levy, M.; Spino, M.; Read, S. E. Colchicine - A State of the Art Review. *Pharmacotherapy* **1991**, *11*, 196−211.

(18) Bhattacharyya, B.; Wolff, J. Promotion of Fluorescence Upon Binding of Colchicine to Tubulin. *Proc. Natl. Acad. Sci.* **1974**, *71*, 2627−2631.

(19) Bhattacharyya, B.; Howard, R.; Maity, S. N.; Brossi, A.; Sharma, P. N.; Wolff, J. B Ring Regulation of Colchicine Binding - Kinetics and Fluorescence. *Proc. Natl. Acad. Sci.* **1986**, *83*, 2052−2055.

(20) Bhattacharyya, B.; Wolff, J. Immobilization-Dependent Fluorescence of Colchicine. *J. Biol. Chem.* **1984**, *259*, 1836−1843.

(21) Hastie, S. B.; Macdonald, T. L. Binding of Colchiceine To Tubulin - Mechanisms of Ligand Association with Tubulin. *Biochem. Pharmacol.* **1990**, *39*, 1271−1276.

(22) Trnavska, Z.; Kuchar, M.; Rejholec, V.; Trnavsky, K. Binding of Colchicine and Its Derivatives to Bovine and Human-Serum Albumin and Human-Plasma. *Pharmacology* **1979**, *18*, 123−127.

(23) Hu, Y. J.; Liu, Y.; Zhao, R. M.; Qu, S. S. Interaction of Colchicine with Human Serum Albumin Investigated by Spectroscopic Methods. *Int. J. Biol. Macromol.* **2005**, *37*, 122−126.

(24) Hu, Y. J.; Liu, Y.; Zhang, L. X.; Zhao, R. M.; Qu, S. S. Studies of Interaction Between Colchicine and Bovine Serum Albumin by Fluorescence Quenching Method. *J. Mol. Struct.* **2005**, *750*, 174−178.

(25) Monti, S.; Manet, I.; Manoli, F.; Capobianco, M. L.; Marconi, G. Gaining an Insight into the Photoreactivity of a Drug in a Protein Environment: A Case Study on Nalidixic Acid and Serum Albumin. *J. Phys. Chem. B* **2008**, *112*, 5742−5754.

(26) Dumont, R.; Brossi, A.; Silverton, J. V. Facile Conversion of Natural Colchicine into (±)-Congeners and (+)-Enantiomers Including 2-Demethyl Analogs. *J. Org. Chem.* **1986**, *51*, 2515−2521.
(27) Silva, J. N.; Bosca, F.; Tome, J. P. C.; Silva, E. M. P.; Neves, M. G. P. M. S.; Cavaleiro, J. A. S.; Patterson, L. K.; Filipe, P.; Maziere, J.-C.; Santus, R.; Morliere, P. Tricationic Porphyrin Conjugates: Evidence for Chain-Structure-Dependent Relaxation of Excited Singlet and Triplet States. *J. Phys. Chem. B* **2009**, *113*, 16695−16704.
(28) Land, E. J. Pulse Radiolysis and Flash Photolysis - Some Applications in Biology and Medicine. *Biochimie* **1980**, *62*, 207−221.
(29) Bensasson, R. V.; Gramain, J. C. Benzophenone Triplet Properties in Acetonitrile and Water - Reduction By Lactams. *J. Chem. Soc., Faraday Trans. 1* **1980**, *76*, 1801−1810.
(30) Bartovsky, P.; Tormos, R.; Miranda, M. A. Colchicine-Protein Interactions Revealed by Transient Absorption Spectroscopy after in Situ Photoisomerization to Lumicolchicines. *Chem. Phys. Lett.* **2009**, *480*, 305−308.
(31) Nery, A. L. P.; Quina, F. H.; Moreira, P. F.; Medeiros, C. E. R.; Baader, W. J.; Shimizu, K.; Catalani, L. H.; Bechara, E. J. H. Does the Photochemical Conversion of Colchicine into Lumicolchicines Involve Triplet Transients? A Solvent Dependence Study. *Photochem. Photobiol.* **2001**, *73*, 213−218.
(32) Roigt, H.; Leblanc, R. M. Photophysical Processes in Colchicine Molecule. *Can. J. Chem.* **1973**, *51*, 2821−2827.
(33) Bussotti, L.; Cacelli, I.; D'Auria, M.; Foggi, P.; Lesma, G.; Silvani, A.; Villani, V. Photochemical Isomerization of Colchicine and Thiocolchicine. *J. Phys. Chem. A* **2003**, *107*, 9079−9085.
(34) Jisha, V. S.; Arun, K. T.; Hariharan, M.; Ramaiah, D. Site-Selective Interactions: Squaraine Dye-Serum Albumin Complexes with Enhanced Fluorescence and Triplet Yields. *J. Phys. Chem. B* **2010**, *114*, 5912−5919.
(35) Hulme, E. C.; Trevethick, M. A. Ligand Binding Assays at Equilibrium: Validation and Interpretation. *Br. J. Pharmacol.* **2010**, *161*, 1219−1237.
(36) Vaya, I.; Bueno, C. J.; Jimenez, M. C.; Miranda, M. A. Determination of Enantiomeric Compositions by Transient Absorption Spectroscopy using Proteins as Chiral Selectors. *Chem.—Eur. J.* **2008**, *14*, 11284−11287.
(37) Kenakin, T. Principles: Receptor Theory in Pharmacology. *Trends Pharmacol. Sci.* **2004**, *25*, 186−192.
(38) Ascoli, G. A.; Domenici, E.; Bertucci, C. Drug Binding to Human Serum Albumin: A Bridged Review of Results Obtained with High-Performance Liquid Chromatography and Circular Dichroism. *Chirality* **2006**, *18*, 667−679.
(39) Ghuman, J.; Zunszain, P. A.; Petitpas, I.; Bhattacharya, A. A.; Otagiri, M.; Curry, S. Structural Basis of the Drug-Binding Specificity of Human Serum Albumin. *J. Mol. Biol.* **2005**, *353*, 38−52.
(40) Hu, E.; Ko, R.; Koda, R.; Rosen, P.; Jeffers, S.; Scholtz, M.; Muggia, F. Phase-I Toxicity and Pharmacology Study of Trimethylcolchicinic Acid in Patients with Advanced Malignancies. *Cancer Chemother. Pharmacol.* **1990**, *26*, 359−364.