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Photoinduced processes in flurbiprofen–carprofen dyads

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Dedicated to Professor Haruo Inoue on the occasion of his 60th birthday.

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ABSTRACT

A common tool for investigating the specific distribution of a ligand between different protein microenvironments is the use of displacement probes, which force the ligand to move from one to another site or from the protein to the bulk aqueous solution. Open problems associated with this methodology are the possibility of two different molecules sharing the same site and the potential appearance of allosteric effects. A possible approach to address these issues could be based on the use of two different singlet excited states, together with the corresponding triplets, acting as reporters.

As a first step towards the development of this concept, we have synthesized two model diastereomeric dyads containing covalently linked flurbiprofen (FBP) and carprofen (CPF) moieties and studied their photophysical and photochemical behaviour, looking for spectroscopically detectable excited state interactions between the two chromophores.

The main deactivation processes that take place upon excitation of dyads (*R*,*R*)-FBP–CPF and (*S*,*R*)-FBP–CPF are the following: initial excitation at 266 nm leads to the first singlet excited states of both subunits. Singlet–singlet energy transfer (SSET) from ^{1}FBP* to CPF is thermodynamically allowed and indeed it appears to take place very efficiently. Radiative deactivation of ^{1}CPF* is followed by intersystem crossing (ISC) and triplet–triplet energy transfer (TTET) from ^{3}CPF* to FBP, which is also downhill in energy. The final step corresponds to deactivation of ^{3}FBP* to the ground state.

In connection with the possibility of making use of the photophysical properties of FBP and CPF to investigate drug–drug interactions in protein binding studies, the most clear-cut conclusions are the following: (a) if the two drugs are within the same protein molecule (irrespective of the site) the only detectable emission will likely correspond to CPF, as its excited singlet is lower in energy, and SSET *via* the Förster mechanism is feasible, and (b) the transient triplet–triplet absorptions corresponding to the two chromophores are in principle detectable by laser flash photolysis; however, if the two drugs share the same binding site of a protein only the FBP triplet will be observed, as TTET occurs *via* the Dexter mechanism, which can only operate at very short distances.

1. Introduction

Dyads containing two covalently attached active chromophores have been widely used as model systems to gain mechanistic insight into some fundamental aspects of photochemical and photobiological processes. Thus, key events such as exciplex formation, energy, electron or proton transfer can be thoroughly investigated in bichromophoric dyads [1–22].

Flurbiprofen [(+)-(*S*)- or (−)-(*R*)-2-(2-fluorobiphenyl-4-yl)propanoic acid], from now on (*S*)- or (*R*)-FBP, is a disubstituted biphenyl derivative (Scheme 1). It is a nonsteroidal anti-inflammatory drug, prescribed for the treatment of inflammation, pain and fever [23–25]. Recently, FBP has also shown potential for prevention of the Alzeimer's disease [26]. It does not absorb light above 300 nm and consequently it does not lead to phototoxic effects upon exposure to the UVB–UVA–vis fractions of solar radiation [27]; however, its photophysical and photochemical behaviour have been established in detail, in order to obtain relevant information concerning the microenvironments experienced by drugs within the binding sites of serum albumins [28]. In this context, the main features of FBP in MeCN solution under anaerobic conditions are a singlet-excited state energy of *ca.* 99 kcal mol^{-1}, with a fluorescence maximum at 310 nm and a quantum yield of 0.21, together with a triplet–triplet (T–T) transient absorption spectrum detectable by laser flash photolysis (LFP), with a maximum centered at 360 nm and a relatively high value of intersystem crossing ($\phi_{ISC} = 0.71$); the triplet energy has been estimated between 65 and 69 kcal mol^{-1}. The FBP photoreactivity is very low: long irradiation times are needed to obtain detectable amounts of photoproducts ($\phi_{reaction} < 0.01$), essentially formed through decarboxylation or (in aqueous media) nucleophilic substitution of the fluoride ion [27].

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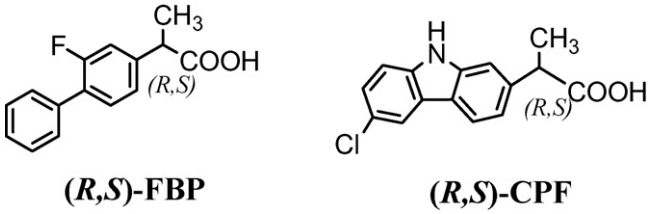


Scheme 1. Chemical structures of flurbiprofen (FBP) and carprofen (CPF).

Carprofen [(+)-(*S*)- or (−)-(*R*)-2-(6-chloro-9*H*-carbazol-2-yl)propanoic acid], abbreviated as (*S*)- or (*R*)-CPF, is a chlorocarbazole derivative (Scheme 1) with anti-inflammatory effects, currently used for veterinary purposes [29–31]. It is able to induce photosensitivity disorders, since its absorption spectrum reaches wavelengths in the UVA range (until *ca.* 360 nm) [32]. Likewise, CPF has been shown to undergo photobinding to cell constituents, which is the primary event involved in the development of photoallergy. Upon excitation at 300 nm, the fluorescence spectrum of CPF in acetonitrile shows two bands around 367 and 352 nm, with an emission quantum yield of 0.068 and a singlet energy of 81 kcal mol^{-1}. The T–T transition (λ_{max} = 430 nm) is observed after LFP in methanolic solution, with a ϕ_{ISC} = 0.37 and an energy of *ca.* 69 kcal mol^{-1}. At a longer timescale, a second transient absorbing at *ca*. 640 nm has been detected and assigned to the corresponding *N*-centered carbazolyl radical. Irradiation of CPF leads to different photoproducts, but the predominating one is by far its dehalogenated derivative, which is efficiently formed from the excited triplet state [32].

Being 2-arylpropionic acids, FBP and CPF are chiral compounds, due to the asymmetric carbon atom of the side chain. In fact, the pharmacological activity of these drugs is mainly attributed to the (*S*)-enantiomer [33]. Interestingly, stereoselective interactions between FBP or CPF and human serum albumin (HSA) have been previously observed by our group using photophysical techniques [4,28,34]. In the presence of protein, the same T–T transition has been detected for both enantiomers of each drug, but a remarkable stereodifferentiation in the drug/HSA interaction has been revealed by means of time-resolved measurements. Thus, for (*S*)-FBP/HSA and (*R*)-FBP/HSA systems the triplet decay evidences the presence of two components with different lifetimes that can be correlated with complexation of the drug to site I and site II of the albumin [28]. An analogous situation is observed for (*S*)- and (*R*)-CPF in the presence of HSA [4,34].

The LFP methodology has proven to be very useful for determination of the binding degree of a given drug to HSA, as well as the site selectivity, under non-saturating conditions [28,34]. In this connection, a common tool for investigating the specific distribution of a ligand between the different protein microenvironments is the use of displacement probes, which force the ligand to move from one to another site or from the protein to the bulk aqueous solution. Open problems associated with this approach are the possibility of two different drug molecules sharing the same site and the potential appearance of allosteric effects; these are very interesting issues, which are difficult to address by the existing techniques. For this purpose, an approach based on the use of two different singlet excited states, together with the corresponding triplets, acting as reporters could be very powerful. As a first step towards the development of this concept, we have synthesized two model diastereomeric dyads containing covalently linked FBP and CPF moieties (Scheme 2) and studied their photophysical and photochemical behaviour, looking for spectroscopically detectable excited state interactions between the two chromophores. Such interactions would provide relevant information on the photoinduced processes occurring when both drugs are placed at a very short distance, as it would be expected when sharing the same binding site. Moreover, in the diastereomeric dyads, a stereodifferentiation in the photophysical properties would indicate the involvement of a specific process requiring a close approach between the two active units. It will be shown that the selected FBP–CPF systems actually exhibit strong excited state interchromophoric interactions, basically of the energy transfer type, which can be exploited for a better understanding of the use of displacement probes in drug–protein binding studies.

2. Experimental

2.1. Materials and solvents

(*S*)- and (*R*)-FBP, racemic CPF, LiH_4Al, *N*,*N*′-DCC (*N*,*N*-dicyclohexylcarbodiimide) and 4-DMAP (4-dimethylaminopyridine) were commercially available. Their purity was checked by ^{1}H NMR and HPLC analysis. The reagent grade solvents (methanol, dioxane, ethyl acetate, and acetonitrile) were used without further purification.

2.2. General

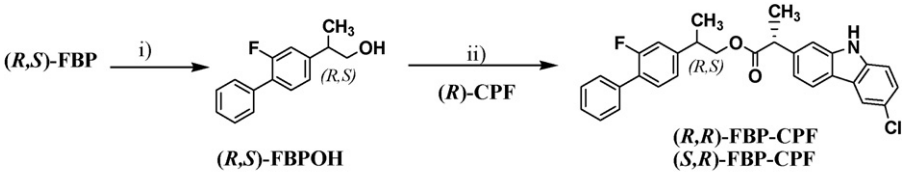
UV spectra were recorded in acetonitrile with a Shimadzu UV-160A; λ_{max} (nm) and log ε values (in brackets) are given for each significant absorption band. IR spectra were obtained with a Jasco FT/IR-460 Plus; ν_{max} (cm^{-1}) is given for the main absorption bands. The ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ as solvent at 300 and 75 MHz, respectively; chemical shifts are reported in ppm downfield from TMS. Exact mass values were obtained by electron impact in a VG Autospec high-resolution mass spectrometer (HRMS). As regards the standard MS data, the *m*/*z* ratios and the relative intensities (%) are indicated for the main peaks. Fluorescence spectra were recorded with a PerkinElmer LS50 instrument. All reactions were monitored by analytical TLC with silica gel 60 F_{254} (Merck). The residues were purified through silica gel 60 (0.063–0.2 mm).

2.3. Synthesis of the substrates

The two enantiomers of carprofen were obtained by chiral HPLC separation of the racemic mixture, using hexane/methyl *tert*-butyl ether/acetic acid (45:55:0.1, v/v/v) as the mobile phase; flow-rate 2.2 mL/min. Samples were injected onto a semipreparative column (Kromasil CHI-TBB). Chromatographic HPLC separation was performed coupled with a chiral detector.

(*R*)- and (*S*)-FBPOH [(−)-(*S*)- or (+)-(*R*)-2-(2-fluorobiphenyl-4-yl)propanol] [35], were prepared by reduction of (*S*)- or (*R*)-FBP, respectively. Thus, FBP (300 mg, 1.23 mmol) was dissolved in 12 mL of dry ether at 0 °C; then 5 mL (4.9 mmol) of 1 M $LiAlH_4$ in ether was added dropwise, and the mixture was heated under reflux for 1 h. The reaction mixture was cooled to room temperature and then it was washed with water (3× 10 mL). The organic phase was dried over Mg_2SO_4 and evaporated to afford quantitatively (*R*)- and (*S*)-FBPOH. The residue was used directly in the following reaction without further purification.

(*R*,*R*)-FBP–CPF and (*S*,*R*)-FBP–CPF dyads were synthesized as follows: to a cold of solution of (*R*)-FBPOH or (*S*)-FBPOH (200 mg, 0.87 mmol) in ether (25 mL), dicyclohexylcarbodiimide (DCC, 268 mg, 1.30 mmol) was added portionwise. Then, a solution of (*R*)-CPF (238 mg, 0.87 mmol) and 4-dimethylaminopyridine (DMAP, catalytic amounts) in ether (25 mL) was added, and the mixture was stirred for 24 h at room temperature. After this time, brine was added, and the mixture was extracted with ether (3× 5 mL). The combined organic layers were washed with water, the organic phase was dried over Na_2SO_4, evaporated and further purified by



Scheme 2. Synthesis of (*R*,*R*)-FBP–CPF and (*S*,*R*)-FBP–CPF.

column chromatography (eluent: hexane/dichloromethane/ethyl acetate 70:20:10, v/v/v) to yield the corresponding esters (*R*,*R*)-FBP–CPF (64%) and (*S*,*R*)-FBP–CPF (50%) as white solids.

2.4. Fluorescence measurements

Emission spectra were recorded on a spectrofluorometer system, provided with a monochromator in the wavelength range 200–900 nm. The solutions were placed into 10 mm × 10 mm quartz cells with a septum cap and were purged with nitrogen for at least 15 min before the measurements. The absorbance of the samples at the excitation wavelength was kept lower than 0.2. Fluorescence quantum yields were determined using FBP as standard (ϕ_F = 0.21 at λ_{exc} = 266 nm, MeCN, N_2). For each compound, the singlet energy was obtained after normalization of the excitation and emission spectra. For time-resolved fluorescence decay measurements, the conventional single photon counting technique was used. All the experiments were carried out at room temperature (22 °C).

2.5. Laser flash photolysis experiments

LFP was performed by using a Q-switched Nd:YAG laser (266 nm, 4 mJ per pulse, ~5 ns fwhm) coupled to a turn-key mLFP-miniaturized equipment from Luzchem. For the excitation at 308 nm, a pulsed excimer laser system with Xe/HCl/Ne mixture was used. The single pulses were ~17 ns duration and the energy was ≤100 mJ/pulse. A pulsed Lo255 Oriel xenon lamp was employed as the detecting light source. The laser flash photolysis apparatus consisted of the pulsed laser, the Xe lamp, a 77200 Oriel monochromator, and an Oriel photomultiplier tube (PMT) system made up of a 77348 side-on PMT tube, 70680 PMT housing, and a 70705 PMT power supply. The oscilloscope was a TDS-640A Tektronix. The output signal from the oscilloscope was transferred to a personal computer. All transient spectra were recorded employing 10 mm × 10 mm quartz cells with 4 mL capacity and were bubbled during 30 min with N_2 before acquisition. The absorbance of the samples was 0.2 at the laser wavelength. All the experiments were carried out at room temperature.

2.6. Spectral data of the new compounds

(+)-(*R*)-2-(2-Fluorobiphenyl-4-yl)propanol, (*R*)-FBPOH. UV: 246 (4.2); FTIR: 3391 (OH), 2927, 1626, 1483, 1410, 1027; ^{1}H NMR: 1.33 (d, *J* = 7 Hz, 3H, CH_3), 3.03 (m, 1H, C<u>H</u>–CH_3), 3.77 (d, *J* = 7 Hz, 2H, C<u>H</u>$_2$OH), 7.05 (d, *J* = 1.8 Hz, 1H, ArH), 7.05–7.15 (m, 4H, ArH), 7.30–7.60 (m, 4H, ArH); MS: 230, (M^+, 35), 212 (18), 199 (100), 178 (16); HRMS Calcd. for $C_{15}H_{15}FO$: 230.11069. Found: 230.11179.

(*S*)-2-(2-Fluorobiphenyl-4-yl)propyl (*R*)-2-(6-chloro-9*H*-carbazol-2-yl)propanoate, (*S*,*R*)-FBP–CPF. UV: 239 (4.8), 300 (4.3); FTIR: 3417 (NH), 2962, 1720 (CO), 1620, 1466, 1265, 1072, 802; ^{1}H NMR: 1.14 (d, *J* = 7.2 Hz, 3H, CH_3), 1.47 (d, *J* = 7.2 Hz, 3H, CH_3), 3.01 (m, 1H, CH_2–C<u>H</u>–CH_3), 3.76 (q, *J* = 7.2 Hz, 1H, C<u>H</u>–CH_3), 4.05 (m, 1H, CH_2O–CO–), 4.20 (m, 1H, CH_2O–CO–), 6.79–7.36 (m, 12H, ArH), 7.78 (d, *J* = 8.1 Hz, 1H, ArH), 7.85 (d, *J* = 2.1 Hz, 1H, ArH), 7.86 (broad s, 1H); MS: 485, (M^+, 67), 273 (54), 228 (100), 212 (36), 193 (44); HRMS Calcd. for $C_{30}H_{25}FClNO_2$: 485.15578. Found: 485.15504.

(*R*)-2-(2-Fluorobiphenyl-4-yl)propyl (*R*)-2-(6-chloro-9*H*-carbazol-2-yl)propanoate, (*R*,*R*)-FBP–CPF. UV: 239 (4.8), 300 (4.3); FTIR: 3417 (NH), 2962, 1720 (CO), 1627, 1458, 1265, 1072, 925, 802; ^{1}H NMR: 1.14 (d, *J* = 7.2 Hz, 3H, CH_3), 1.46 (d, *J* = 7.2 Hz, 3H, CH_3) 2.99 (m, 1H, CH_2–C<u>H</u>–CH_3), 3.74 (q, *J* = 7.2 Hz, 1H, C<u>H</u>–CH_3), 4.08 (m, 1H, CH_2O–CO–), 4.16 (m, 1H, CH_2O–CO–), 6.74–7.36 (m, 12H, ArH), 7.80 (d, *J* = 7.8 Hz, 1H), 7.86 (d, *J* = 2.1 Hz, 1H, ArH), 7.93 (broad s, 1H); MS: 485 (M^+, 59), 273 (11), 228 (70), 212 (100), 193 (29); HRMS Calcd. for $C_{30}H_{25}FClNO_2$: 485.15578. Found: 485.15504.

3. Results and discussion

The title dyads (*R*,*R*)-FBP–CPF and (*S*,*R*)-FBP–CPF were obtained by reaction of (*R*)- or (*S*)-FBPOH with (*R*)-CPF (Scheme 2); they were submitted to fluorescence (steady-state and time resolved) as well as laser flash photolysis studies, in order to obtain relevant information on the excited state interactions between both chromophoric units.

The photophysical experiments were performed in acetonitrile. The UV absorption spectra of (*R*,*R*)-FBP–CPF and (*S*,*R*)-FBP–CPF are shown in Fig. 1; they were identical to the added spectra of the corresponding isolated CPF and FBPOH reference compounds, at the same concentrations. This reveals the absence of any significant ground-state intramolecular interaction between the two units. The fraction of light absorbed by each chromophore was determined by comparing the absorbance of solutions of CPF and FBPOH at the same concentration; it was found that, at 266 nm, 32% of the incident light reaches the biphenyl and 68% the carbazole chromophore.

3.1. Fluorescence studies on FBP–CPF dyads

Upon excitation at 266 nm, the main features of FBPOH (MeCN, N_2) were a maximum at 310 nm, a fluorescence quantum yield

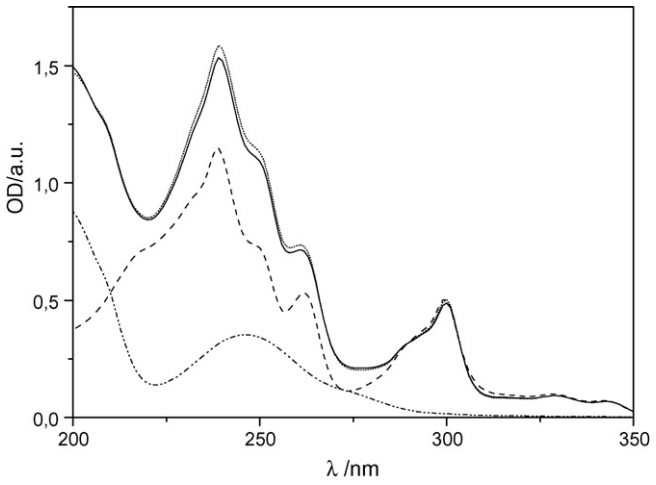


Fig. 1. UV–vis absorption spectra of FBPOH (- · - · -), CPF (- - - -), (*R*,*R*)-FBP–CPF (—) and (*S*,*R*)-FBP–CPF (· · · · ·) at 2.5 × 10^{-5} M.

of 0.21 and a singlet energy $E_s = 99\,\text{kcal mol}^{-1}$. Under the same conditions, CPF exhibited the known maxima at 367/353 nm, with a fluorescence quantum yield $\Phi_F = 0.05$. Similar experiments were conducted with (*R*,*R*)-FBP–CPF and (*S*,*R*)-FBP–CPF; the most remarkable aspect observed in both dyads was a very efficient singlet–singlet energy transfer (SSET) (see Fig. 2A). Thus, no emission was observed to take place from FBP, although under these conditions it was absorbing a non-negligible fraction (32%) of the incident light. Accordingly, the excitation spectra for the dyads showed a clear contribution of the FBP absorption band, providing further experimental support for the abovementioned intramolecular SSET (Fig. 2B). The spectrum recorded for the dyads matched in shape and position with that of CPF, whereas the fluorescence quantum yields were slightly (but significantly) higher, with a value of 0.06 (Fig. 2). This can be explained as a result of a more efficient population of the lowest-lying ^{1}CPF* level when the energy transfer process takes place from ^{1}FBP* than when it is reached after internal conversion, upon direct 266 nm excitation of CPF to higher singlet states.

In addition to the observation of energy transfer, elucidation of the operating mechanism is essential for a correct analysis of drug–drug interactions inside the protein pockets. In this context, calculation of the spectral overlap integrals for singlet–singlet

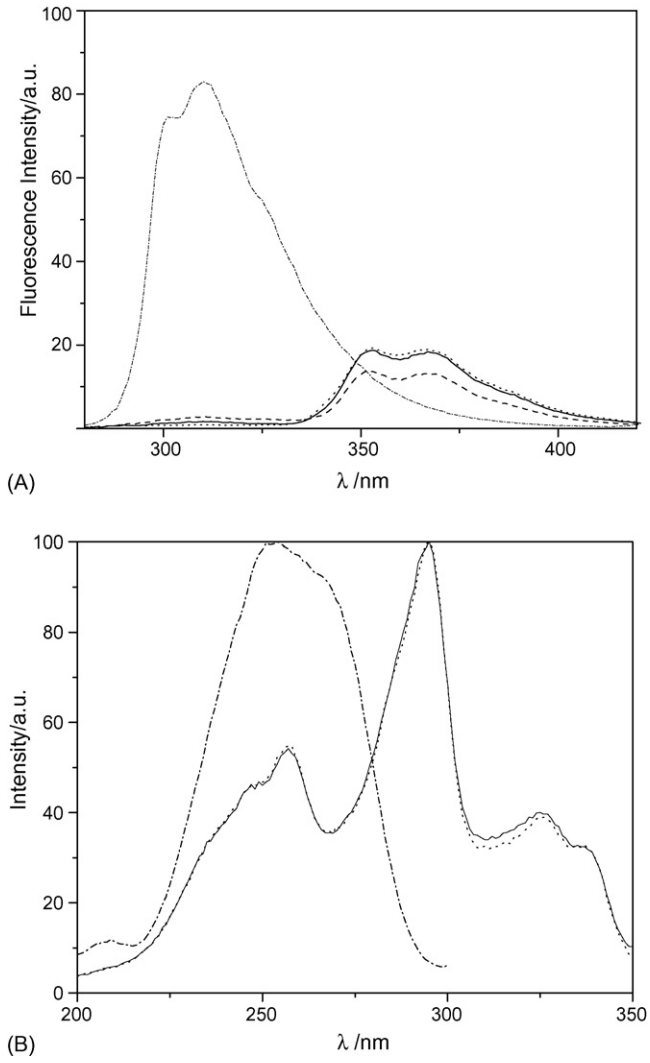


Fig. 2. (A) Fluorescence spectra (MeCN, N_2) of isoabsorptive solutions ($A = 0.2$ at $\lambda_{exc} = 266$ nm) of FBPOH (- · - · -), CPF (- - - -), (*R*,*R*)-FBP–CPF (—) and (*S*,*R*)-FBP–CPF (· · · · ·). (B) Excitation spectra at the emission maxima.

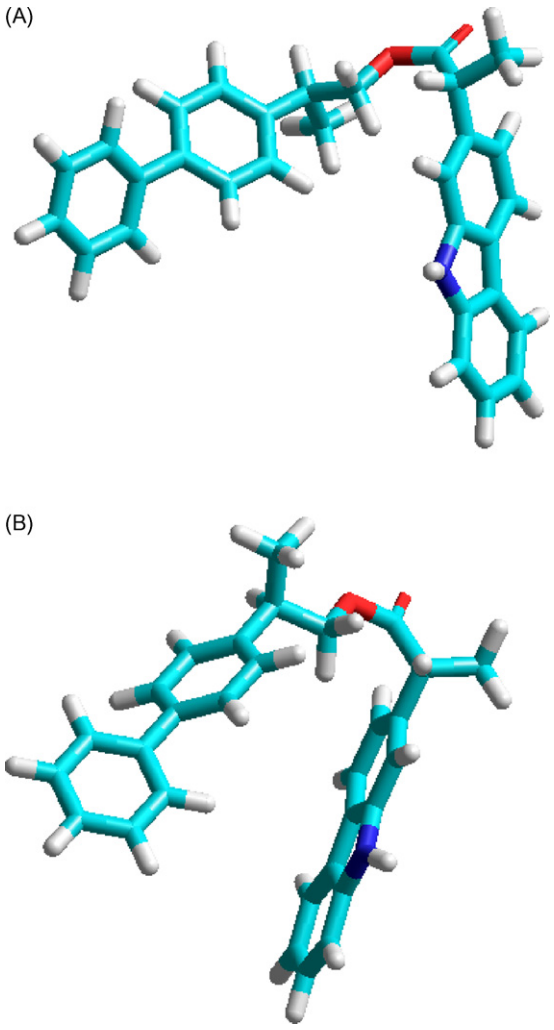


Fig. 3. Model structures of (A) (*S*,*R*)-FBP–CPF and (B) (*R*,*R*)-FBP–CPF obtained using HyperChem Release 8.0.3 for Windows Molecular Model System.

energy transfer in the dyads for the Förster ($J_{\text{dipole–dipole}}$) mechanism [36,37] has been done using the conventional equation (Eq. (1)):

$$J_{\text{dipole–dipole}} = \int_0^\infty \frac{\overline{F_D}(\bar{\nu}) \in_A(\bar{\nu})\, d\bar{\nu}}{\bar{\nu}^4} \quad (1)$$

where $\bar{F}_D(\bar{\nu})$ represents the normalized donor emission spectrum, $\in_A(\bar{\nu})$ the absorption spectrum of the acceptor expressed by its molar absorption coefficient, and $\bar{\nu}$ the average transition frequency.

The value obtained was $J_{\text{dipole–dipole}} = 7.3\ 10^{-14}\,\text{M}^{-1}\,\text{cm}^3$. The Förster theory defines R_0 as the critical distance where the probability of energy transfer is 50% (Eq. (2)):

$$R_0\,(\text{Å}) = 9.78 \times 10^3 \left(\frac{\kappa^2 \phi_D J_{\text{dipole–dipole}}}{n^4} \right)^{1/6} \quad (2)$$

For random orientations $\kappa^2 = 2/3$; $\phi_D = \phi_{\text{FBPOH}} = 0.21$ in MeCN and n, the refractive index of acetonitrile, is equal to 1.34. For the investigated dyads, R_0 was estimated to be higher than 20 Å.

On the other hand, simple molecular modelling (AM1) resulted in a center-to-center distance of *ca.* 8 Å between both moieties (Fig. 3).

Based on these observations, it can be safely concluded that participation of both the Förster and Dexter mechanisms is actu-

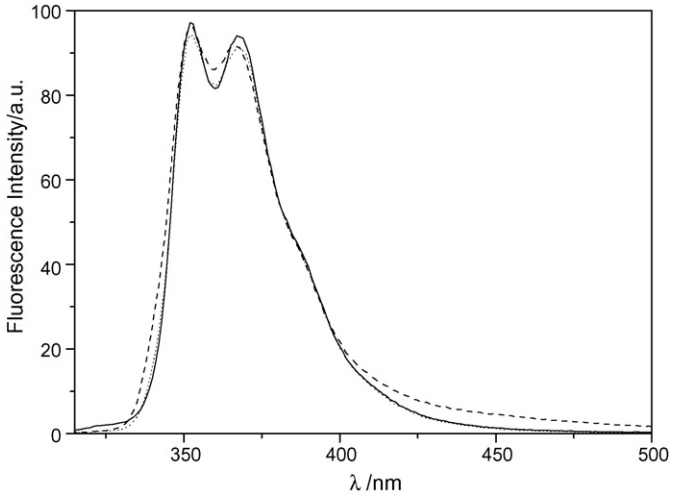
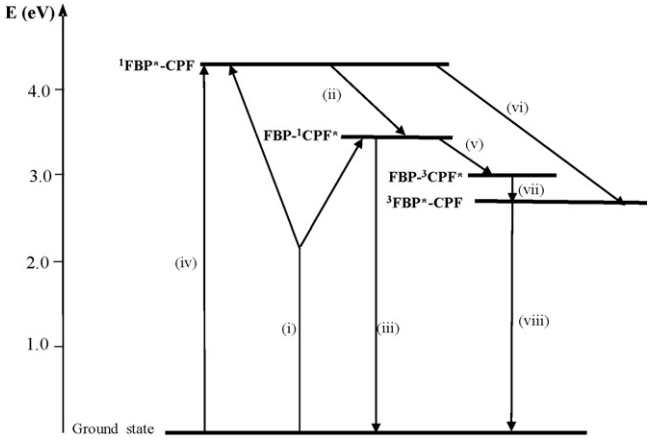


Fig. 4. Fluorescence spectra (MeCN, N_2) of isoabsorptive solutions ($A = 0.2$ at $\lambda_{exc} = 308$ nm) of CPF (- - - -), (*R*,*R*)-FBP–CPF (—) and (*S*,*R*)-FBP–CPF (· · · · ·).



Scheme 3. Qualitative energetic diagram for the different excited states and reactive intermediates generated upon excitation of FBP–CPF dyads.

ally feasible. In the more stable folded conformation, a substantial contribution of the Dexter mechanism would be expected. However, due to the high flexibility of the spacer in the dyads, extended conformations with larger donor–acceptor distances can also be found as local minima; for these situations, Förster energy transfer should be dominating, due to the missing orbital overlap between donor and acceptor, as it is required for operation of the Dexter process.

Irrespective of the specific pathways leading to population of the lowest singlet excited state of the chlorocarbazole chromophore as the only emitting species, time-resolved measurements revealed that the fluorescence lifetimes were coincident within the experimental errors for CPF, (*R*,*R*)-FBP–CPF, and (*S*,*R*)-FBP–CPF (1.3 ns). This indicates that intramolecular interactions involving such excited state, in particular electron transfer, are not significant.

Concerning a possible stereodifferentiation in the SSET process, no clear-cut differences were observed between both dyads. This could be attributed to a similar spatial arrangement of both chromophores in the diastereomers, leading to average FBP–CPF distances of the same order.

Parallel experiments were also carried out with 308 nm as the excitation wavelength, to prevent direct absorption of the incident light by the flurbiprofen moiety and thus bypass the singlet–singlet energy transfer step. As expected, all the investigated systems (*R*,*R*)-FBP–CPF, (*S*,*R*)-FBP–CPF, and CPF exhibited the same type of band, which matched in shape, position and intensity (Fig. 4); the lifetimes were also very similar, basically the same as those observed upon 266 nm excitation.

From the fluorescence results, it can be anticipated that SSET between the two drugs is likely to occur when they are occupying the same binding site, or even when they are located in different microenvironments of the same protein molecule, where the typical distances between the two sites are compatible with a Förster mechanism.

3.2. Laser flash photolysis of FBP–CPF dyads

The typical FBP T–T transient absorption spectrum was obtained upon LFP of (*R*,*R*)-FBP–CPF and (*S*,*R*)-FBP–CPF at 266 nm (Fig. 5A). This is consistent with initial formation of ^{3}CPF*, which can undergo triplet–triplet energy transfer (TTET) to afford ^{3}FBP*. The triplet quantum yields for FBPOH, (*R*,*R*)-FBP–CPF and (*S*,*R*)-FBP–CPF were obtained by comparison of the respective ΔOD at 370 nm (optically matched at the laser excitation wavelength) using Eq. (3) and were found to be 0.25 in both dyads:

$$\phi_T^i = \frac{\Delta OD_{max}^i}{\Delta OD_{max}^{FBP}} \phi_T^{FBP} \quad (3)$$

The top OD at 370 nm was less intense in the dyads than when the model FBPOH was directly excited under the same conditions

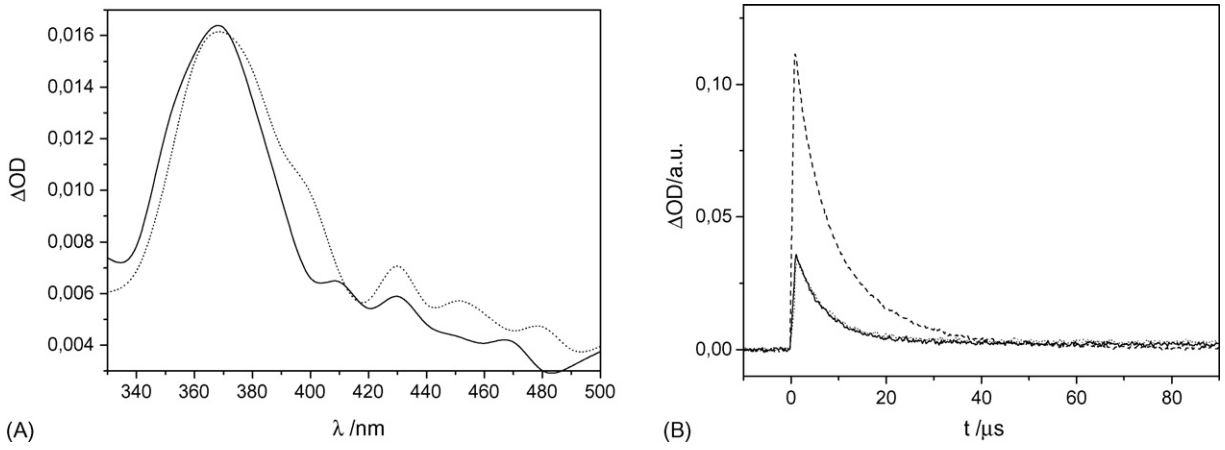


Fig. 5. Laser flash photolysis (266 nm excitation) of FBPOH (- · - · -), (*R*,*R*)-FBP–CPF (—) and (*S*,*R*)-FBP–CPF (· · · · ·) 2.5×10^{-5} M in PBS, N_2. (A) Spectra recorded under the same conditions, 2 μs after the laser pulse. (B) Transient decay monitored at 370 nm.

(i) FBP-CPF $\xrightarrow{h\nu}$ ^{1}FBP*-CPF + FBP-^{1}CPF*

(ii) ^{1}FBP*-CPF $\xrightarrow{SSET}$ FBP-^{1}CPF*

(iii) ^{1}FBP*-CPF $\xrightarrow{-h\nu'}$ FBP-CPF

(iv) FBP-^{1}CPF* $\xrightarrow{-h\nu''}$ FBP-CPF

(v) ^{1}FBP*-CPF $\xrightarrow{ISC_{FBP}}$ ^{3}FBP*-CPF

(vi) FBP-^{1}CPF* $\xrightarrow{ISC_{CPF}}$ FBP-^{3}CPF*

(vii) FBP-^{3}CPF* $\xrightarrow{TTET}$ ^{3}FBP*-CPF

(viii) ^{3}FBP*-CPF $\longrightarrow$ FBP-CPF

Scheme 4. Photophysical processes in (*R*,*R*)-FBP–CPF and (*S*,*R*)-FBP–CPF.

(Fig. 5B). This is due to the lower ϕ_{ISC} of CPF compared with that of FBP.

It is known that triplet–triplet energy transfer can only take place through electron exchange mechanism, which requires an overlap of the wave functions of donor and acceptor [38]. This would be a critical information for CPF and FBP in the presence or serum albumin, as TTET between both drugs would indicate close proximity between them, and hence would constitute an unambiguous proof for both compounds sharing the same binding site.

4. Conclusions

The main deactivation processes that take place upon excitation of dyads (*R*,*R*)-FBP–CPF and (*S*,*R*)-FBP–CPF are detailed in Schemes 3 and 4. Thus, initial excitation at 266 nm: (i) leads to the first singlet excited states of both the FBP and the CPF subunits. Singlet–singlet energy transfer from ^{1}FBP* (E_S = 99 kcal mol^{-1}) [27] to CPF (E_S = 81 kcal mol^{-1}) [32] would be thermodynamically allowed (ii) and indeed it appears to take place very efficiently. Radiative deactivation from ^{1}FBP* or ^{1}CPF* is represented in steps (iii) and (iv), respectively, whereas routes (v) and (vi) correspond to intersystem crossing (ISC) processes. In practice, (iii) and (v) are negligible, as they cannot compete with the ultrafast SSET occurring from the same excited state ^{1}FBP* (process ii). Triplet–triplet energy transfer from ^{3}CPF* (E_T = 69 kcal mol^{-1}) [30] to FBP (E_T = 65 kcal mol^{-1}) [27] would also be downhill in energy and actually it clearly occurs in the dyads (step vii). The final step corresponds to deactivation *via* ISC to the ground state.

In connection with the possibility of making use of the photophysical properties of the singlet and triplet excited states of FBP and CPF to investigate drug–drug interactions in protein binding studies, the most clear-cut conclusions are the following: (a) if the two drugs are within the same protein molecule (irrespective of the site) the only detectable emission will likely correspond to CPF, as its excited singlet is lower in energy, and SSET *via* the Förster mechanism is feasible, and (b) the transient triplet–triplet absorptions corresponding to the two chromophores are in principle detectable by laser flash photolysis; however, if the two drugs share the same binding site of a protein only the FBP triplet will be observed, as TTET occurs *via* the Dexter mechanism, which can only operate at very short distances.

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