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Drug–Drug Interactions within Protein Cavities Probed by Triplet–Triplet Energy Transfer

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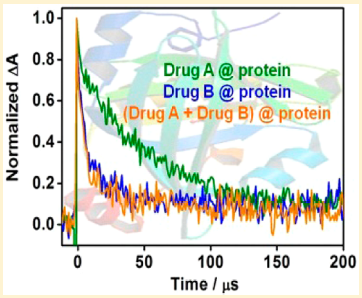
Supporting Information

ABSTRACT: A new direct and noninvasive methodology based on transient absorption spectroscopy has been developed to probe the feasibility of drug–drug interactions within a common protein binding site. The simultaneous presence of (*R*)-cinacalcet (CIN) and (*S*)-propranolol (PPN) within human or bovine α_1-acid glycoproteins (AAGs) is revealed by detection of ^{3}CIN* as the only transient species after laser flash photolysis of CIN/PPN/AAG mixtures at 308 nm. This is the result of triplet–triplet energy transfer from ^{3}PPN* to CIN, which requires close contact between the two drugs within the same biological compartment. Similar results are obtained with nabumetone and CIN as donor/acceptor partners. This new methodology can, in principle, be extended to a variety of drug/drug/biomolecule combinations.



SECTION: Biophysical Chemistry and Biomolecules

The phenomenon of drug–drug interactions (DDI) is becoming a common issue in modern medicine and drug development. It is defined as a set of measurable modifications of the action of one drug by prior or concomitant coadministration of another drug.[1−4] The outcome of such interactions includes beneficial effects (i.e., additive and potentiating) but also undesired side effects, such as loss of therapeutic properties or toxicity. For instance, DDI are the origin of many hospital admissions, as they may be associated with severe or even fatal medical episodes.[5] As a result, a number of established drugs have been withdrawn from the market after discovering their involvement in medically important, unwanted DDI. In general, DDI may become of special relevance in high-risk patients receiving multiple drug therapy (polypharmacy), a common situation in the elderly.[6] It is clear that a scientifically sound understanding of the potential for DDI should be required before introducing new chemical entities.[7−9]

The mechanism of DDI is currently a matter of intensive research activity. It may involve pharmacokinetic interactions, which affect absorption, distribution, biotransformation, and excretion of the drugs.[10−12] A key factor in pharmacokinetics is plasma protein binding, where DDI may play a clinically significant role by affecting the blood drug concentration. Interactions at the transport protein level may lead to altered drug distribution to the target organs via displacement from the protein binding sites.[13,14] Hence, the possibility of two drugs sharing the same protein binding site should be considered in the case of tightly bound drugs having a narrow therapeutic window.

Detection of DDI is not straightforward, especially if looking at direct, noninvasive in situ determinations. The employed in vitro methods include assays with cells or artificial membranes as well as physical measurements based on nuclear magnetic resonance or fluorescence spectroscopy, among others.[3,4] Specifically, the well-known Förster resonance energy transfer (FRET) involves singlet–singlet energy transfer, which occurs by dipole–dipole interaction and can operate at long distances between chromophores located in different biological compartments.[15,16] Hence, the development of complementary strategies for assessment of DDI within the same protein binding sites is an important field of interest that requires further research effort.[17]

In this context, the behavior of triplet excited states is highly sensitive to the microenvironment, a property that has been exploited to address a number of biologically relevant problems. In particular, laser flash photolysis (LFP) of drug@protein systems has allowed us to obtain a wealth of information on the binding of flurbiprofen, its methyl ester, carprofen, naproxen, propranolol, or cinacalcet to a transport protein, that is, human or bovine serum albumins (HSA, BSA) or α_1-acid glycoproteins (HAAG, BAAG).[18−27] With this background, it appeared interesting to explore the potential of triplet excited states as reporters for intraprotein DDI detection. In the case of two different drugs sharing a common binding site, triplet–triplet energy transfer (TTET) could be revealed by transient absorption spectroscopy, as this phenomenon occurs exclusively through a Dexter mechanism, which requires electron

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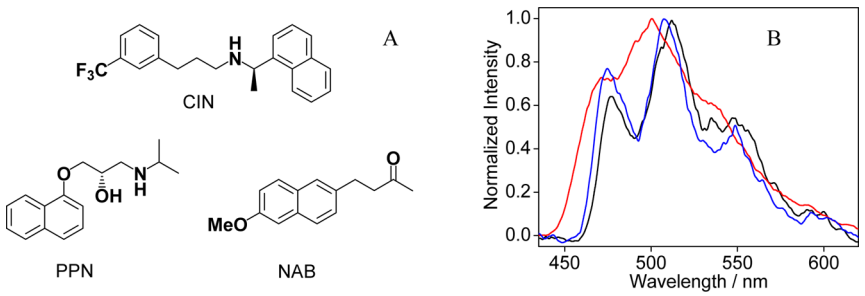

Figure 1. (A) Chemical structures of CIN, PPN, and NAB. (B) Normalized phosphorescence spectra of CIN (black), PPN (blue), and NAB (red) in ethanol matrix at 77 K. The absorbance of the samples was 0.8 at the excitation wavelength (308 nm).

exchange and therefore a close contact between the donor and acceptor partners.

To prove the concept, we have chosen HAAG and BAAG as biological hosts due to their ability to bind and transport endogenous or exogenous ligands, mainly with basic or neutral character. These proteins consist of one polypeptide chain and are highly glycosylated. Although several binding sites have been described for AAGs, most drugs and small organic molecules bind almost exclusively to one of them, which is large and flexible. The other binding sites have much lower importance; therefore, their role in the transport of substrates is marginal.[28−35] The hydrophobic nature of the binding pockets of HAAG and BAAG is similar, although their microviscosities are markedly different.[36]

The drugs selected for interrogation of DDI were (*R*)-cinacalcet (CIN), (*S*)-propranolol (PPN), and nabumetone (NAB); their chemical structures are shown in Figure 1A. They possess a naphthalene (NP) chromophore, appropriate for generation and detection of the triplet excited state. The different NP substitution was expected to modulate the position of the transient absorption maxima and the triplet energy values, thus making the TTET process possible and facilitating its monitorization by LFP.

CIN is a calcimimetic, prescribed for persistent hyperparathyroidism in renal transplanted patients; in blood, at therapeutic doses, it is found quantitatively bound to transport proteins.[37] PPN is a nonselective *β*-blocker used for the treatment of hypertension that binds strongly to HAAG (K_B = 2−4 × 10^6 M^{-1}).[38] Co-administration of CIN and PPN constitutes a real case of DDI,[39] whose most common side effects experienced by patients include anxiety, diarrhea, and asthenia. NAB is a nonsteroidal anti-inflammatory drug commonly used to treat pain and inflammation associated with arthritis.[40]

To check the feasibility of TTET between the selected drugs, their phosphorescence spectra were recorded in ethanol matrix at 77 K. The triplet energy values determined therefrom were found to be 60.9, 61.4, and 62.9 kcal mol^{-1} for CIN, PPN, and NAB, respectively. Thus, it should, in principle, be possible to observe TTET from PPN to CIN and from NAB to CIN.

Before studying the complex, three-component systems, we investigated the behavior of the separate drugs in the absence of protein under our experimental conditions, both in organic solution and in aqueous medium. LFP of deareated MeCN solutions of CIN, PPN, and NAB (5.8 × 10^{-4} M, λ_{exc} = 308 nm) allowed us to obtain their transient absorption spectra and triplet lifetimes (τ_T); the results were in agreement with literature data, when available.[26,27,41] For CIN, the maximum was located at 420 nm (τ_T = 4.0 *μ*s), while for PPN and NAB it was centered at 450 nm (τ_T = 6.1 and 10.4 *μ*s, respectively). Under air, the triplet lifetimes were markedly shorter; in addition, the triplet decays under aerated conditions were much longer in the protein microenvironment (Table 1). Interestingly, both the position of the transient absorption maximum and the triplet lifetime of AAG-bound CIN were sufficiently different from those of PPN and NAB to monitor a possible TTET process.

Table 1. Triplet Lifetimes of CIN, PPN, and NAB in Different Media

	τ_T (*μ*s)[a]			
drug	MeCN/N_2	MeCN/air	HAAG/air[b]	BAAG/air[b]
CIN	4.0	0.2	5.7	10.5
PPN	6.1	0.1	25.2	51.8
NAB	10.4	0.1	16.6	25.8

[a]Obtained upon LFP at λ_{exc} = 308 nm. [b]Drug@protein systems at 1:1 molar ratio, in 0.01 M PBS.

After examining the individual AAG-bound drugs, we investigated the behavior of encapsulated donor/acceptor pairs to detect potential DDI. Attempts to dissolve CIN/PPN and CIN/NAB mixtures in PBS at the required concentrations failed, giving rise to turbid suspensions (Figure 2a,b). Complexation with AAGs was easily followed by the naked eye through a complete solubilization resulting in clear preparations (Figure 2c,d).

Remarkably, when a solution of CIN/PPN/HAAG at 1:1:1 molar ratio was submitted to LFP, the decay trace monitored at 450 nm matched that of the CIN/HAAG system (Figure 3A); a

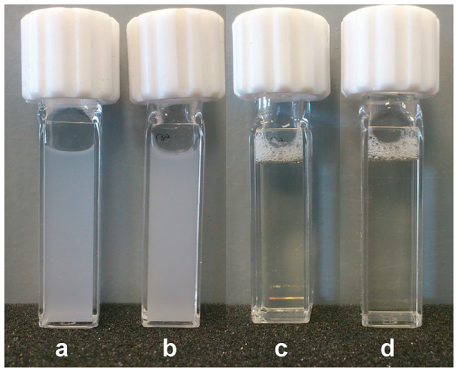

Figure 2. Photograph of drug mixtures in PBS in the absence and presence of protein: (a) CIN/PPN, (b) CIN/NAB, (c) CIN/PPN/HAAG, and (d) CIN/NAB/HAAG. Concentration of each component was 5.8 × 10^{-4} M.

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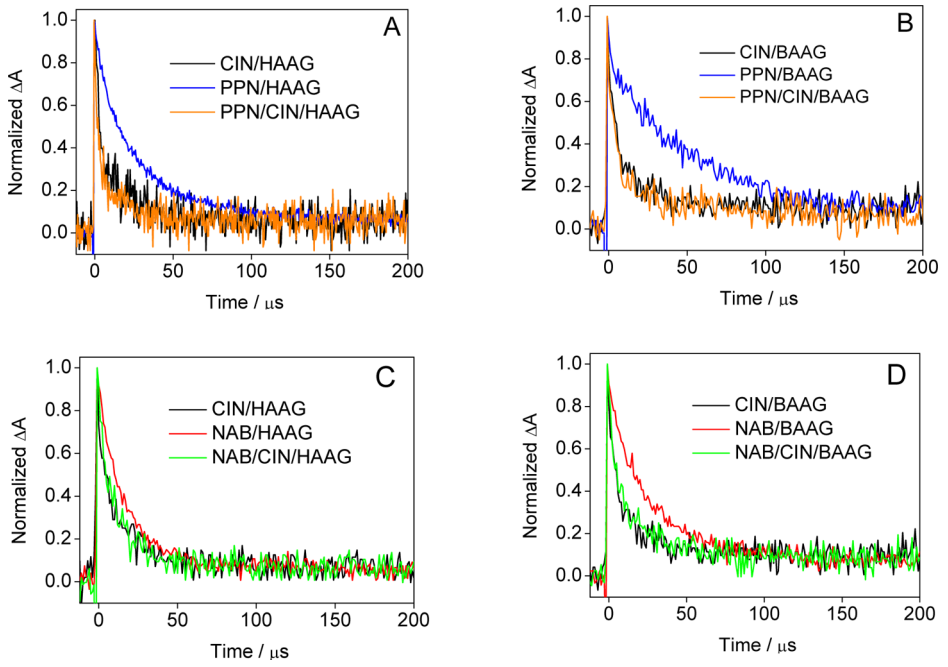
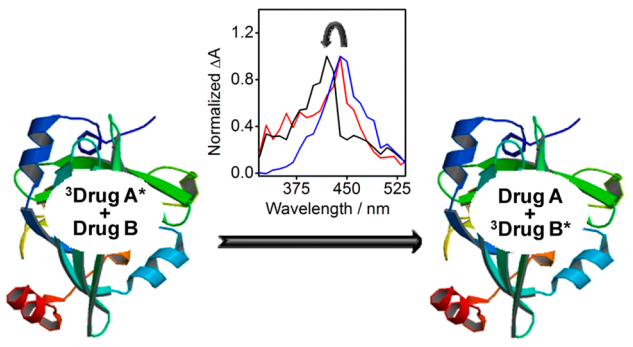


Figure 3. Laser flash photolysis (λ_{exc} = 308 nm, PBS/air) of CIN/PPN or NAB/AAG systems. Normalized decay traces monitored at λ_{max} = 450 nm for CIN/AAG (black), PPN/AAG (blue), NAB/AAG (red), CIN/PPN/AAG (orange), and CIN/NAB/AAG (green). (A,C) HAAG. (B,D) BAAG. The concentration of each component was 5.8×10^{-4} M.

similar situation was observed in the presence of BAAG (Figure 3B). No contribution from ^{3}PPN* was observed in the kinetic decays. Likewise, for CIN/NAB/AAG solutions, the decay traces at 450 nm matched those of the CIN/AAG mixtures (Figures 3C,D). Accordingly, the transient absorption spectra obtained for the drug mixtures inside the proteins were identical to those of encapsulated CIN (Scheme 1). These results clearly indicated an efficient energy transfer from ^{3}PPN* or ^{3}NAP* to CIN and revealed the DDI between both pairs of drugs, which share a common AAG binding site.

Because the TTET process was completed within the duration of the laser pulse (<20 ns), deactivation of the excited triplet states has to be extremely fast, with a rate constant higher than 5×10^7 s^{-1}. Such a high value is not compatible with the expectedly slow diffusion inside the AAG binding sites and points to the formation of preassociated complexes between donor (PPN or NAB) and acceptor (CIN). This was confirmed by theoretical calculations, which were performed to find the binding site of CIN and PPN at HAAG by means of a combined atomistic and quantum-semiempirical methodology, taking the HAAG structure (2868 atoms) from a recent determination.[42] Attractive energetic interactions were found between CIN and PPN (−14 kJ/mol) and between HAAG and the associated drugs CIN+PPN (−46 kJ/mol), indicating that this protein can successfully host DDI, owing to the large size of the binding site and its intrinsic flexibility. The extra stabilization associated with pairing of PPN and CIN inside the protein would explain the lack of ^{3}PPN* detection that could arise from two PPN molecules sharing the same binding site. Within the found equilibrium conformation (Figure 4), a more sophisticated DFT method using the accurate functional M06-2X and the large basis set Def2-TZVP revealed that TTET from PPN to CIN is indeed possible due to the large overlap between the relevant molecular orbitals and the small energy gap between them. Further details can be found in the Supporting Information.

Scheme 1. Representation of the Use of TTET to Detect DDI between Two Drugs Sharing a Common Protein Binding Site



Drug A = (*S*)-PPN or NAB.
Drug B = (*R*)-CIN.

In summary, the feasibility of DDIs within the binding sites of transport proteins can be probed by transient absorption spectroscopy. The concept involves detection of TTET, which requires a close proximity between the donor and acceptor partners and can only occur within a common biological compartment (Scheme 1). This constitutes a new methodology, which can, in principle, be extended to a variety of drug/drug/biomolecule combinations.

METHODS

Materials and Solvents. HAAG (99%), BAAG (99%), NAB, and PPN were purchased from Sigma-Aldrich (Steinheim, Germany), whereas CIN hydrochloride was extracted from commercial Mimpara 60 mg (Amgen, Spain). The pills were powdered in a mortar, suspended in 1 M NH_4OH, and extracted with CH_2Cl_2. The combined organic layers were

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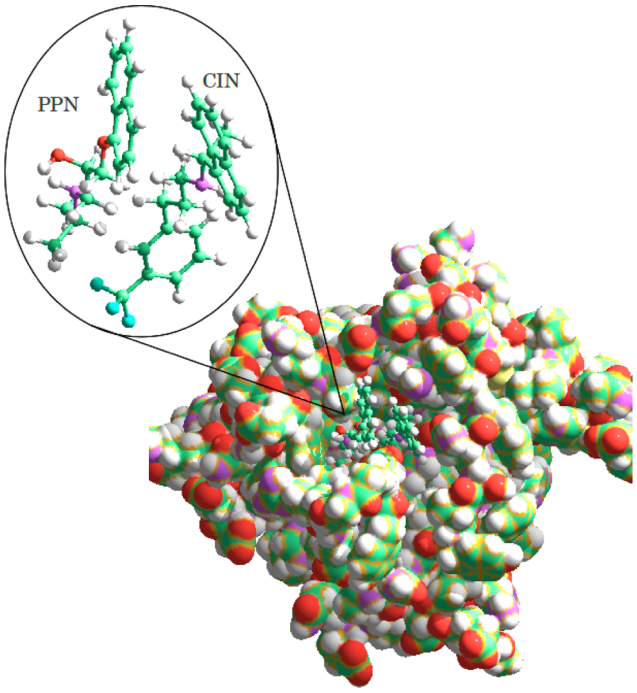

Figure 4. Optimized geometry of PPN and CIN within the binding site of HAAG.

washed with brine and water, dried over $MgSO_4$, and evaporated under reduced pressure. The residue was purified by column chromatography through silica gel 60 (CH_2Cl_2/CH_3OH/15 M NH_4OH 98:2:0.1 v/v/v) to give the corresponding CIN free base as a colorless oil. Phosphate-buffered saline solution (PBS, pH 7.4, 0.01 M) was prepared by dissolving Sigma tablets in the appropriate amount of deionized water.

Phosphorescence Measurements. Phosphorescence spectra were obtained from a Photon Technology International (PTI, TimeMaster TM-2/2003) spectrofluorometer equipped with a pulsed Xe lamp. The apparatus was operated in time-resolved mode, with a delay time of 0.5 ms. Compounds were dissolved in ethanol, placed in a quartz tube (5 mm of diameter), and cooled to 77 K. The absorbance of the samples was 0.8 at the excitation wavelength (308 nm).

Laser Flash Photolysis Experiments. LFP experiments were carried out with a pulsed XeCl excimer laser (λ_{exc} = 308 nm, ca. 17 ns pulse width, <100 mJ per pulse). In general, samples received between one and three pulses for all of the kinetic experiments. A pulsed Lo255 Oriel Xenon lamp was used as detecting light source. The observation wavelength was selected with a 77200 Oriel monochromator, and the signal was amplified by an Oriel photomultiplier tube (PMT) system made up of a 77348 side-on tube, 70680 housing, and a 70705 power supply. The signal was registered with a TDS-640A Tektronix oscilloscope and subsequently transferred to a personal computer. Experiments conducted in the presence of different proteins were performed in air-saturated PBS; solutions were incubated 24 h in the dark before acquisition, and the decay traces (at 450 nm) were fitted to mono-exponential functions to determine the triplet lifetimes. The concentrations of the samples were fixed at the arbitrary value of 5.8×10^{-4} M. All measurements were recorded at room temperature using 10 × 10 mm^2 quartz cells with 4 mL capacity.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Theoretical calculations on the binding of PPN and CIN to HAAG. This material is available free of charge via the Internet http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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