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Analysis of mebendazole binding to its target biomolecule by laser flash photolysis

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ABSTRACT

Mebendazole (MBZ) and related anticancer benzimidazoles act binding the β-subunit of Tubulin (TU) before dimerization with α-TU with subsequent blocking microtubule formation. Laser flash photolysis (LFP) is a new tool to investigate drug–albumin interactions and to determine binding parameters such as affinity constant or population of binding sites. The aim of this study was to evaluate the interactions between the nonfluorescent mebendazole (MBZ) and its target biomolecule TU using this technique. Before analyzing the MBZ@TU complex it was needed to determine the photophysical properties of MBZ triplet excited state (^{3}MBZ*) in different media. Hence, ^{3}MBZ* showed a transient absorption spectrum with maxima at 520 and 375 nm and a lifetime much longer in acetonitrile (12.5 μs) than in water (260 ns). The binding of MBZ to TU produces a greater increase of the lifetime of ^{3}MBZ* (25 μs). This fact and the strong electron acceptor capability observed for ^{3}MBZ* evidence that MBZ must not be located close to any electron donor amino acid of TU such as its tryptophan or cysteine residues. Adding increasing amounts of MBZ to aqueous TU was determined the MBZ-TU binding constant ($2.0 \pm 0.5 \times 10^5$ M^{-1} at 298 K) which decreased with increasing temperature. The LFP technique has proven to be a powerful tool to analyze the binding of drug–TU systems when the drug has a detectable triplet excited state. Results indicate that LFP could be the technique of choice to study the interactions of non-fluorescent drugs with their target biomolecules.

1. Introduction

Microtubules, major structural components in cells, represent the single best cancer target identified to date [1]. Thzey are highly dynamic assemblies of the protein Tubulin (TU) which is a heterodimeric globular protein formed by α and β subunits that plays a key role in the mitosis, the process of chromosomal division to form new cells [2–5]. Hence, the antitumor drugs act by binding to diverse TU sites and at different positions within the microtubule and their common mechanism is the alteration of the polymerization dynamics of microtubules, blocking mitosis, and subsequently, inducing cell death by apoptosis [1,6]. Thus, the study of drug–TU interactions is of paramount biological importance. Interestingly, there are TU high affinity binding sites for a variety of drugs including colchicine [7], maytansine [8], epothilone [9], peloruside [10] and the vinca alkaloids [11]. In this context, benzimidazoles are drugs used in both human and veterinary medicine that act binding the β-TU subunit before dimerization with α-TU with subsequent blockade of microtubule formation [12]. Specifically, mebendazole (MBZ, see Chart 1), a human anthelmintic commercially known as Vermox used to treat infestations by worms including pinworms, roundworms, tapeworms, hook worms, and whipworms, induces mitotic arrest and apoptosis in human lung cancer cells [13–14]. More interestingly MBZ displays cytotoxicity on glioblastoma multiforme (GBM) cell lines, the most common brain cancer, and has been identified as a promising drug for GBM therapy [15]. Literature reports have described that MBZ works by inhibiting the synthesis of microtubules upon binding to the colchicine site of TU and the affinity constant of MBZ to brain bovine TU has been determined to be 2.8×10^5 M^{-1} [16]. The TU binding behavior of MBZ and related benzimidazoles have only been analyzed by conventional techniques such as inmunofluorescence, ultramicroscopic or turbidimetric assays [16–18]. However, during the past few years, new and more specific assays for ligand–protein interactions have been achieved in the analytical methodology [19]. In this context, the interactions between drugs and transport proteins (serum albumins and α1-acid glycoproteins) have been studied using the laser flash photolysis (LFP) technique to detect minute amounts of ligands and to determine binding parameters such as affinity constant or population of binding sites [19]. Nevertheless, although this technique does not require separation of free and complexed species as it also occurs in fluorescence spectroscopy [19], the binding of non-fluorescent anticancer drugs to their target biomolecules has not been studied using the LFP technique.

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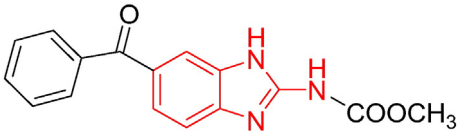

Chart 1. Mebendazole structure and its benzimidazole subunit (in red).

With this background, the aim of the present work was to evidence the potentials of this technique in this type of interactions. Thus, the selected target biomolecule was TU and the drug was MBZ because it is a non-fluorescent drug that shows phosphorescence in low-temperature matrices [20] which suggests that MBZ–TU complex could be analyzed at room temperature using the sensitivity and/or reactivity of MBZ triplet excited state. Hence, the results of the full spectroscopic characterization of MBZ triplet excited state in different media as well as in the presence of TU have allowed to determine the equilibrium binding constant of tubulin with MBZ, to evidence the influence of temperature in the MBZ-TU binding and to prove the absence of electron donor amino acids interacting with MBZ in the colchicine site of TU.

2. Materials and Methods

2.1. Chemicals

Benzophenone (BZP), 1,4-cyclohexadiene, dibenzofuran, fluorene, mebendazole (MBZ), naphthalene (NP), phenanthrene and tryptophan methyl ester were purchased from Aldrich. Their purity was checked by ^{1}H NMR spectroscopy and high performance liquid chromatography (HPLC) analysis. Reagent grade solvent acetonitrile and methanol were purchased from Scharlau and without further purification. Tubulin was purified from bovine brain and stored as described [21]. Before use, concentrated TU was diluted more than 50-fold into GTP buffer to give the desired final concentration and employed within the next 4 h. GTP buffer: 10 mM sodium phosphate and 0.1 mM GTP at pH 7.

2.2. Absorption and Emission Measurements

Optical spectra in different media were recorded on a Perkin-Elmer Lambda 35 UV/vis spectrophotometer. Phosphorescence measurements were registered with a time-resolved spectrometer (TimeMaster fluorescence lifetime spectrometer TM-2/2003) from Photon Technology International. The spectra of the samples were performed at 77 K in methanol solution using a single-cell Peltier cooler. The emission spectral bandwidths were set to 10 nm at 350 nm excitation wavelength. The phosphorescence emission spectra were taken with a delay time of 0.5 ms and a total gate time of 10 ms.

2.3. Laser Flash Photolysis Experiments

The LFP apparatus consisted of two pulsed laser (355 nm INDI Nd:YAG, Spectra-Physics with single pulse of ca. 10 ns duration and an energy between 10 to 1 mJ pulse^{-1} and 308 nm (Excimer LEXTRA-MC Lambda Physic with a Xe/HCl/Ne mixture for the selected excitation wavelength, a single pulse of ca. 17 ns duration and an energy of ca. 10 mJ/pulse), a pulsed Xe lamp (Lo255 Oriel), 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 computer.

The transient spectra were recorded using 10×10 mm^2 quartz cells with 3 mL capacity, and were bubbled during 20 min with N_2O, N_2 or O_2. Absorbance of the samples was kept between 0.1 and 0.3 at the excitation laser wavelength. The experiments were carried out at room temperature and each measurement was registered three times (with different samples) and the results are an average of them.

2.3.1. Mebendazole Triplet Excited State Measurements

The molar absorption coefficient of MBZ triplet state (^{3}MBZ*) in acetonitrile was determined by monitoring the energy transfer reaction between ^{3}MBZ* and NP ground state. As NP does not absorb 355 nm light, ^{3}NP* can only be populated via energy transfer from ^{3}MBZ* (Eq. (1)) [22].

$$^3\mathrm{MBZ}^* + \mathrm{NP} \rightarrow \quad \mathrm{MBZ} + {}^3\mathrm{NP}^* \tag{1}$$

The measurements were performed with deaerated acetonitrile solutions of MBZ alone and in the presence of NP ($1–5 \times 10^{-5}$ M) using a 355 nm laser pulse, and the molar absorption coefficient (ε) of ^{3}MBZ* was calculated using the Eq. (2):

$$k_2/(k_2 - k_1) \times \Delta A({}^3\mathrm{NP}^*(415\,\mathrm{nm})) \times \varepsilon({}^3\mathrm{MBZ}^*(520\,\mathrm{nm})) = \Delta A({}^3\mathrm{MBZ}^*(520\,\mathrm{nm})) \times \varepsilon({}^3\mathrm{NP}^*(415\,\mathrm{nm})) \tag{2}$$

where the ΔA values refer to the absorbance at 520 nm of ^{3}MBZ* at the beginning of the reaction and ^{3}NP* (at 415 nm) at the end, k_1 is the ^{3}MBZ* decay rate constant in the absence of NP, and k_2 is the different ^{3}MBZ* decay rate constant determined at the different concentrations of NP. The molar absorption coefficient for ^{3}NP* in acetonitrile at 415 nm was taken 24,000 $\pm$ 3000 M^{-1} cm^{-1} as described in cyclohexane [23].

The intersystem crossing quantum yield (ϕ_{ISC}) of MBZ was obtained by the comparative method [24] assuming that ε (^{3}MBZ*) is very similar in all solvents. Hence, excitation of BZP and MBZ was carried out separately using solutions with identical absorbance (0.3) at the excitation wavelength of 355 nm. Then Eq. (3) was applied:

$$\phi_{ISC}(\mathrm{MBZ}) = \phi_{ISC}(\mathrm{BPZ}) \times \Delta A({}^3\mathrm{MBZ}^*(520\,\mathrm{nm})) \times \varepsilon({}^3\mathrm{BZP}^*(525\,\mathrm{nm}))/\Delta A({}^3\mathrm{BZP}^*(525\,\mathrm{nm})) \times \varepsilon({}^3\mathrm{MBZ}^*(520\,\mathrm{nm})) \tag{3}$$

where the ΔA values refer to the absorbance for ^{3}MBZ* at 520 nm and ^{3}BZP* at 525 nm. The BZP molar absorption coefficient (ε (^{3}BZP*(525 nm))) and its triplet state quantum yield (ϕ_{ISC} (BPZ)) in acetonitrile were taken to be 6500 M^{-1} cm^{-1} and 1, respectively [25].

The ^{3}MBZ* quenching rate constants by oxygen, NP and other quenchers were determined using the Stern–Volmer Eq. (4):

$$1/\tau = 1/\tau_0 + k[\mathrm{Quencher}]. \tag{4}$$

2.3.2. Transient Ppectrum of MBZ in Buffered Aqueous Media in Absence and Presence of Tubulin

Aqueous solutions of 1×10^{-5} M MBZ were prepared in GP buffer at pH 7 with and without the presence of TU under aerobic and anaerobic conditions. The samples containing protein needed special manipulation to remove oxygen. Thus, deaerated media (N_2O and N_2) were introduced inside the sample quartz cells flowing the gas during 20 min without generating foams and stirring the solution. Transient absorption spectra at different times after the laser pulse were obtained for each sample with and without TU (1×10^{-5} M).

3. Results and Discussion

3.1.1. Detection and Characterization of MBZ Triplet Excited State by LFP

Laser flash photolysis ($\lambda_{exc} = 308$ nm) of deaerated acetonitrile solutions of MBZ (1×10^{-5} M) led to a transient absorption spectrum showing the main maximum at 520 nm (Fig. 1). It was quenched by oxygen with a k_{qO2} of 5.3×10^9 M^{-1} s^{-1}, suggesting that this transient species can be ascribed to the triplet excited state (^{3}MBZ*).