Direct Spectroscopic Evidence that the Photochemical Outcome of Flutamide in a Protein Environment is Tuned by Modification of the Molecular Geometry: A Comparison with the Photobehavior in Cyclodextrin and Vesicles

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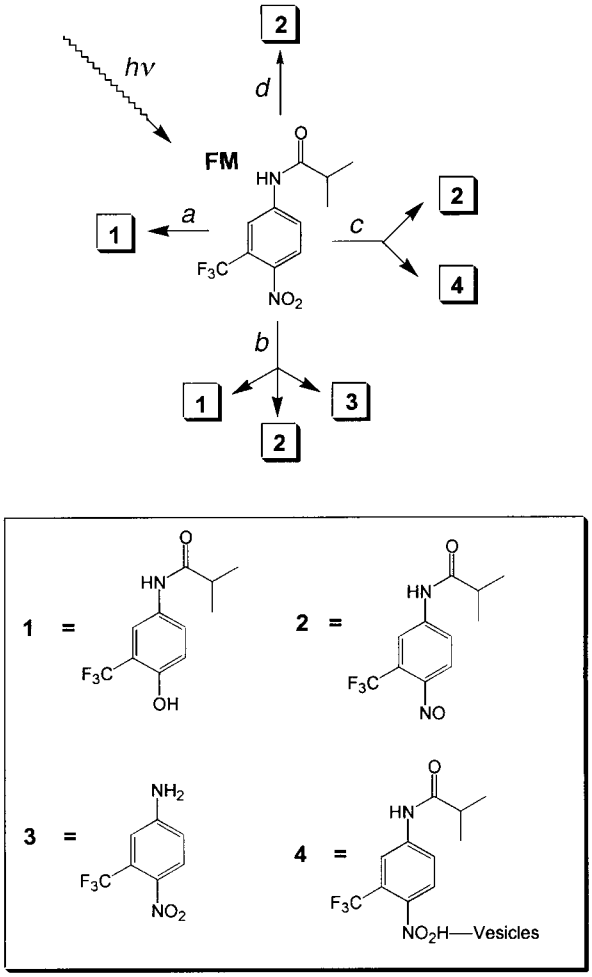
The photoreactivity of the phototoxic anticancer drug flutamide (FM) in the presence of bovine serum albumin (BSA) has been investigated. The presence of BSA induces a remarkable modification of the photochemical outcome of the drug with respect to that observed in aqueous solution. Induced circular dichroism (ICD) measurements combined with theoretical calculations provide strong evidence that the new photochemical scenario is tuned by changes of the molecular geometry of FM when incorporated in the protein microenvironment. This behavior presents close analogies to that found in the presence of either cyclodextrin or phospholipid vesicles, chosen as models for biological systems, and delineates a quite general photochemical picture that can be useful for a more appropriate understanding of the adverse phototoxic effects induced by this drug.

Introduction. – Flutamide (FM; *2-methyl*-N-*[4-nitro-3-(trifluoromethyl)phenyl]-propanamide*), is a nonsteroidal anticancer drug [1–3] known to induce *in vivo* phototoxic and photoallergic effects [4–9]. In general, since real-life photoprocesses occur at surfaces, interfaces, and in multiphase heterogeneous systems [10–12], a suitable strategy for an appropriate understanding of the molecular basis at the origin of the drug-photoinduced disorders consists of extending the drug's photobehavior in homogeneous solution to biological model systems of increasing complexity. Cyclodextrins, micelles, and vesicles represent some of the most exploited biological mimicking systems [10–14]. The photoreactivity of molecules incorporated in such systems is an extremely active field of research that continues to capture the interest of interdisciplinary areas in the scientific community [13–18]. The drug compartmentalization in specific sites of a biological microenvironment characterized by confined space, peculiar polarity features, specific interactions, and/or steric constraints may actually lead to dramatic modifications to the nature of the photodeactivation pathways, to their efficiency, or to both, with respect to those observed in homogeneous solution. Our recent studies, addressed to elucidate the photoreactivity of FM in different media, allowed us to gain interesting mechanistic insights useful from both fundamental and practical standpoints [19–22]. We pointed out that the photochemical response of FM to UVA excitation is particularly sensitive to the molecular microenvironment. The main results are briefly summarized below for the sake of clarity. UVA Irradiation of FM in homogeneous solvents such as H_2O, MeOH or

i-PrOH gave rise almost exclusively to the phenol derivative **1** as the main stable photoproduct (*Scheme 1*, path *a*) [19]. It was demonstrated that the twisted geometry of the NO_2 group with respect to the aromatic plane (see *Fig. 1*) plays a key role in the formation of **1** through the well-known nitro-to-nitrite photorearrangement followed by cleavage of the O–N bond [19][20]. In the presence of β-cyclodextrin (β-CD), chosen as suitable model to mimic the hydrophobic pockets of cell components, the formation of **1** was accompanied by two new photoproducts originating from the photoreduction of the NO_2 group, **2**, and the photocleavage of the amide bond, **3** (*Scheme 1,* path *b*) [19]. In the presence of phospholipid vesicles (PV), chosen as a model compound to mimic biological membranes, generation of **1** was totally suppressed, and the formation of **2** accompanied by a photobinding product **4** was instead observed (*Scheme 1,* path *c*) [21][22].

Scheme 1. *Photochemical Scenario of FM in Different Environments*



The photobehavior of these supramolecular complexes was rationalized on the basis of conformational changes in the structure of FM, occurring upon its compartmentalization in the new microenvironments. In this regard, the loss of the twisted conformation in favor of the almost coplanar geometry of the NO_2 group (see *Fig. 1*) was proposed to take place likely due to steric constraints and specific weak interactions (*i.e.*, H-bond involving the CF_3 and/or NO_2) within either the restricted β-CD cage or the close-packed vesicles bilayer [19][21][22].

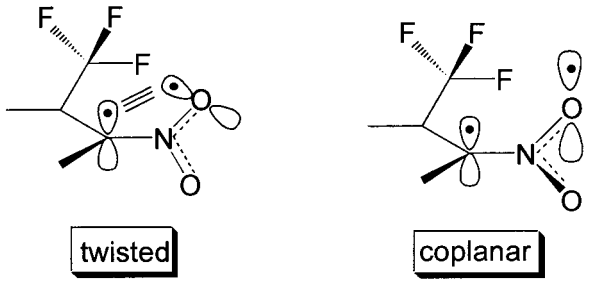


Fig. 1. *Schematic view of the two relevant conformations of FM*

This paper aims to investigate the photobehavior of FM in the presence of bovine serum albumin (BSA) to gain further insights into the photochemical reactivity of the drug in biologically mimicking systems of increasing complexity.

Results and Discussion. – Irradiation of FM (10^{-4} M) in the presence of BSA at pH 7.4 led to remarkable changes of both efficiency and nature of the photochemical deactivation pathways with respect to those found in aqueous medium. *Fig. 2* shows the transformation percentage in the presence and, for comparison, in the absence of 1 mg/ml BSA[1]).

Since the amount of absorbed photons was basically the same in both cases, from the ratio of the slopes obtained in the linear part of the plots, one can readily notice that FM photodegradation in the presence of BSA occurs *ca.* ten times more efficiently than in its absence. Interestingly, we observed the total suppression of photoproduct **1** and the formation of the nitroso derivative **2** (path *d* in *Scheme 1*). As displayed in *Fig. 2*, the percentage of disappearance of FM was accompanied by a corresponding increase in **2** in the early stage of the photoreaction (*ca.* within 10% of conversion of the starting compound). Such mass balance ruled out any formation of potential photobinding products of FM with BSA and confirmed **2** as the sole photoreaction product. These findings account for a higher selectivity in the product formation than that previously observed in the presence of either β-CD or PV [19][21][22]. Similarly to what was proposed in these two biological mimicking systems, also in the present case the behavior observed seems to be tuned by changes in the molecular geometry of FM upon its likely compartmentalization in the restricted and hydrophobic pockets of the protein. More specifically, the loss of the twisted geometry indispensable to initiate the nitro-to-nitrite photorearrangement at the basis of the formation of **1** would lead to a

[1]) Under these experimental conditions, FM is almost totally complexed with BSA (see ICD measurements).

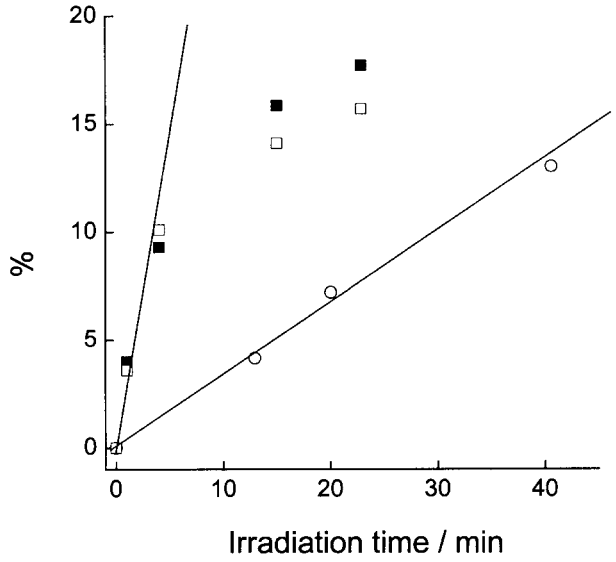


Fig. 2. *Percentage of FM disappearance in the absence* (○) *and in the presence* (■) *of BSA, and % of photoproduct* **2** *formation* (□) *in the presence of BSA as a function of the irradiation time.* pH 7.4; [BSA] = 1 mg/ml.

less-extended overlap of the p orbital of the O-atom with the adjacent orbital of the aromatic ring with consequent increase of the biradical character of the n,π^* triplet state (see *Fig. 1*) [19][21][22]. Inhibition of **1** and activation of an intermolecular H-abstraction process of the NO_2 group from the protein sites to give product **2** may, thus, reasonably come into play as a result of these conformational changes (see *Scheme 2*).

It should now be highlighted that the resonance-stabilized radical photogenerated after H-abstraction may follow two different pathways: *i*) to diffuse away from the former reaction site to abstract a further H-atom from a different part of the protein and eventually to originate **2** according to well-known mechanistic pathways [23][24]; *ii*) to remain close to the former reaction site and recombine after intersystem crossing (ISC) with the protein-centered radical, giving rise to a protein covalently linked product. The lack of photobinding product in the present case accounts for a large predominance of pathway *i*. This may be attributable to a fast dynamic of the radical species in the BSA environment when compared with the slow motion in phospholipid vesicles [25][26]. According to this view, a considerable amount of photobinding product was, in fact, noted in this medium [22].

Spectroscopic evidences combined with theoretical calculations support well the hypothesis that the FM photoprocesses in the presence of BSA are tuned by conformational changes of the drug molecular structure.

Fig. 3 shows the induced circular dichrosim (ICD) spectra of FM obtained in the presence of increasing amounts of BSA. ICD Signals arise as a consequence of the complexation of the nonchiral drug with the optically active protein. As reported in the inset of *Fig. 3*, the intensity of the ICD signal reached a plateau value at BSA

Scheme 2

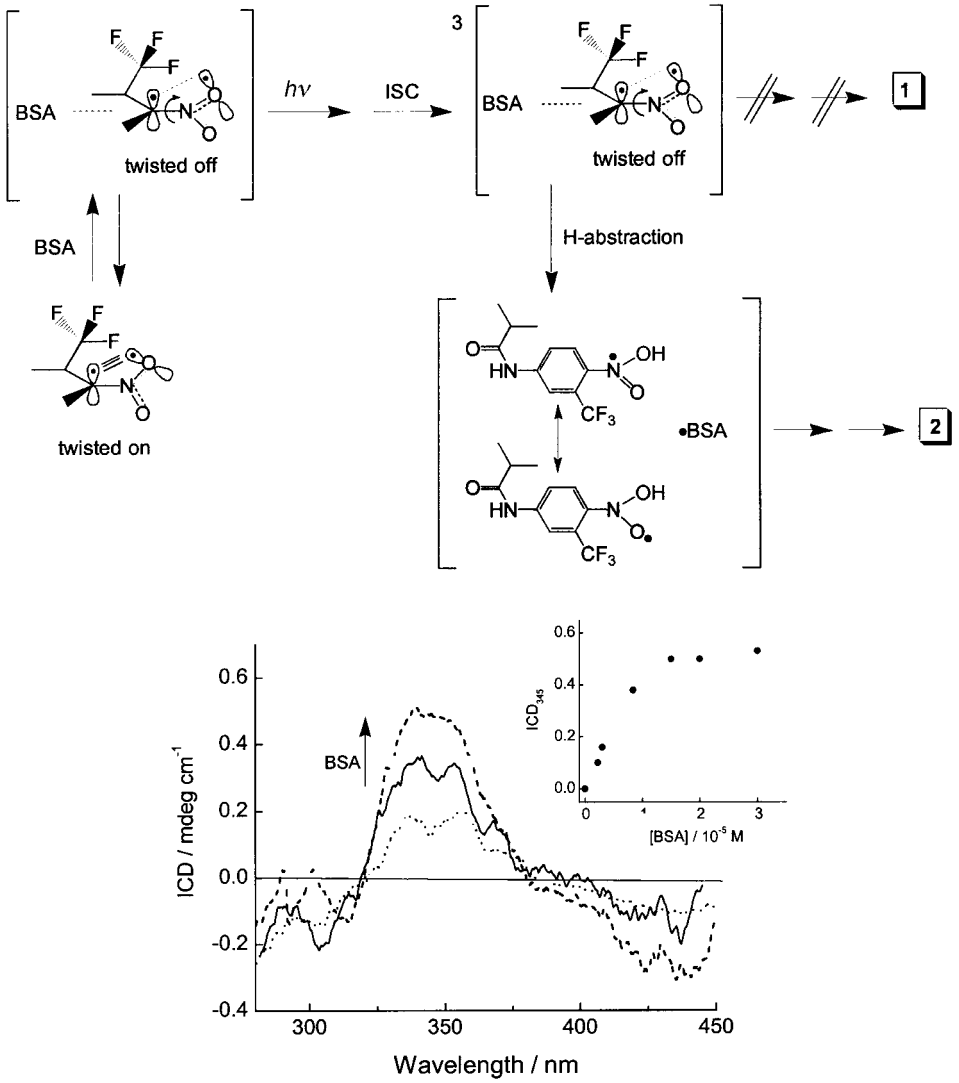


Fig. 3. *ICD Spectrum of 10^{-4}* M *FM in the presence of increasing amount of BSA at pH 7.4* (some spectra are omitted for sake of clarity). The inset shows the dependence of the ICD signal at 345 nm on BSA concentration. Cell path: 10 mm. All spectra were recorded against reference solutions containing the same concentrations of BSA.

concentration above 1.5×10^{-5} M (*ca.* 1 mg ml^{-1}). From a *Scatchard* plot [27] of the data obtained, a binding constant K_{ass} of *ca.* 2×10^5 M^{-1} could be estimated. This value is higher than that obtained for FM complexation with β-CD. By taking into account that $K_{ass} = k_+/k_-$ (where k_+ and k_- are the kinetic rate constants of entry and exit of FM 'in' and 'from' BSA) and that k_+ is, in general, diffusion-controlled [16], the difference in

K_{ass} obtained in the two cases reflects a slower exit dynamic of the drug from the protein microenvironment. FM compartmentalization in regions of BSA characterized by lower micropolarity and higher number of bindung sites than β-CD might be tentatively proposed to explain such difference in K_{ass}.

The ICD spectra clearly show a negative signal centered at *ca.* 430 nm and a larger, positive signal at *ca.* 340 nm. On the basis of the calculated energies for the excited states of FM [19], one can attribute the negative signal to the lowest n,π* states, centered mainly on the NO_2 group, whereas the positive signal can be assigned to the first allowed π,π* transition. Inspection of the sign of the ICD bands provides unambiguous confirmation of the molecular structure of the FM when complexed with BSA. The effect of the geometry of the NO_2 group (planar or perpendicular to the ring) on the sign of the ICD of the first band was investigated by a calculation of the m-μ term [28][29][2]), which appears relevant for an isolated n,π* transition centered on this group. It was found that the different symmetry of the magnetic moment in the two different situations plays a decisive role, giving rise to a fairly large negative rotatory power for the planar geometry and to a small but positive term for the perpendicular one. On the other hand, the ICD band of the π,π* state, centered on the phenyl ring, is calculated to be positive through the μ-μ electric dipole-dipole term. These results represent a strong support for the model invoked to explain the photoreactivity of FM in the presence of BSA.

In conclusion, we have demonstrated that the photochemical reactivity of FM in a protein environment is tuned by changes of the molecular geometry. Owing to this conformational modification, a relevant increase of the drug photodegradation efficiency accompanied by drastic changes of the primary photochemical processes has been observed.

It should be stressed that, in the light of our previous studies carried out with cyclodextrin cavities and phospholipid bilayer vesicles, the findings presented herein allow to draw a quite common photochemical scenario for FM in biological mimicking systems that may help in the understanding of the phototoxic effects induced by this drug. Actually, it seems quite general that accommodation of FM in such micro-environments switches the molecular geometry from twisted to almost coplanar. The photochemical reactivity observed in all the biomimicking systems we have explored to date seems to be governed by the harmonic synergism of effects induced by each specific microenvironment, such as *i*) its capability to induce the above structural modification, *ii*) its participation as a reactant providing a source of abstractable H-atoms, *iii*) its role in influencing the mobility of the radical species photogenerated, *iv*) its structure and hydrophobicity. Although the three latter factors are commonly accepted to play a role in numerous photochemical reactions and, in our case, influence both photodegradation efficiency and photoproducts distribution, the former effect is quite uncommon and remains the only indispensable prerequisite to observe these new photochemical scenarios.

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[2]) The magnetic moments were computed by the complete angular-momentum operator method (for details, see [28]).

Studies addressed to verify the validity of the model proposed in more complex biological systems represent the future development of this work and are now being conducted.

Experimental. – Flutamide (M_r 250.2) and BSA were purchased from *Sigma Chemical Company* (Milano, Italy) and used as received. Phosphate buffer (10^{-2} M, pH 7.4) was prepared from reagent-grade products. pH Values of solns. were measured with a glass electrode. MeCN from *Carlo Erba* (Milan, Italy) was HPLC-grade.

Steady-state absorption spectra were recorded with a *Beckman 650 DU* spectrophotometer. ICD Spectra were recorded with a *Jasco J-615* dichrograph.

Solns. of FM and BSA in phosphate buffer was allowed to incubate in the dark for 30 min. The samples were then irradiated in 3-ml quartz cuvettes for different lengths of time. The irradiated mixtures were filtered through a *Millipore* cut-off filter *Ultrafree-MC* to separate the protein from FM and its photoproducts and analyzed through a *Hewlett-Packard* LC-ESI-MS system equipped with on-line photodiode-array detector (DAD) and a *LiChro Cart RP-18* column (5 μm packing, 4 × 250 mm *Hewlett Packard*). The gradient used for elution was MeCN in 0.01M phosphate buffer (pH 7) from 0 to 75% in 25 min, at a flow rate of 1 ml min^{-1}. The retention times of FM and photoproduct **2** were 16.7 and 17.3 min, respectively. Both retention time and integrated area for the nonirradiated FM were the same either in the absence or in the presence of BSA after filtration, suggesting that no complex existed during the elution. The mass balance was checked by performing the chromatographic analysis with DAD at 350 nm and by correcting the integrated areas related to FM and photoproduct **2** for the different extinction coefficients at this wavelength.

Irradiations of FM were performed with monochromatic irradiation obtained from a series *200 He-Cd 325* nm laser (*Liconix*, St. Clara CA, USA). The incident photon flux on 3-ml quartz cuvettes was *ca.* 5×10^{15} quanta s^{-1}. The experimental procedures of irradiation and the light intensity measurements have been described in [30].

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