Photobinding of Flutamide to Phospholipid Vesicles: Additional Evidence for Photoprocesses Unexpectedly Triggered by Conformational Changes in the Bilayer

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It is demonstrated that irradiation of the anticancer drug flutamide (FM) in the presence of unilamellar phospholipid bilayer vesicles, chosen as a model for cell membranes, leads to an efficient photobinding process. This pathway is triggered by an unexpected hydrogen-abstraction reaction of the excited drug by the lipid chain and provides additional evidence that the overall photochemical scenario observed in the vesicles is unusually triggered by conformational change of the drug's molecular structure upon incarceration in the 'close-packed' bilayer. The present investigation, beyond contributing to the general picture concerning fundamental aspects of the light-induced processes in self-organized assemblies, may also provide important insights for a more satisfactory understanding of the recently reported adverse photoallergic and phototoxic phenomena displayed by this anticancer drug.

1. Introduction. – Exploration of light-induced processes in biological mimick systems is an extremely active field of research that continues to capture interest in interdisciplinary areas of the scientific community [1–6]. Photochemical and photophysical studies carried out in a large variety of organized assemblies such as micelles, vesicles, microemulsions, monolayers, and cyclodextrins have commonly been addressed to two main goals: *i*) to make use of the photochemical and photophysical properties of guest molecules to obtain a better and more detailed understanding of the organized media; *ii*) to gain insight into the influence of the organized media on the photobehavior of guest molecules [7]. In particular, in the last few years, a general aspect embraced by this latter objective concerns phototoxic drugs [8–10]. In this regard, it is important to highlight that, although the knowledge of the drug's photochemical behavior in homogeneous solution represents the first step toward understanding the molecular basis of drug-photoinduced disorders, photoreactivity and phototoxicity are often not directly correlated with each other. The main reason for these inconsistencies is that real-life photoprocesses occur at surfaces, interfaces, and in multiphase heterogeneous systems. Therefore, the compartmentalization of a certain drug molecule in specific sites of a biological microenvironment characterized by confined space, particular polarity features, specific interactions, and/or steric constraints, can, in fact, lead to profound modifications of both nature and efficiency of the photodeactivation pathways with respect to those observed in homogeneous solution. From this picture, it emerges clearly that a stepwise approach consisting of the investigation of the drug photobehavior in biomimicking systems of increasing complexity represents an adequate strategy for more appropriate correlation between photoreactivity and phototoxicity.

Flutamide (FM), 2-methyl-*N*-[4-nitro-3(trifluoromethyl)phenyl]propanamide, is a nonsteroidal antiandrogen drug that blocks androgen receptor sites and is widely used in advanced prostate cancer [11–13]. Recently, it was pointed out that FM is able to induce *in vivo* phototoxic and photoallergic effects upon UVA light excitation in patients after drug treatment [14–19].

In our previous study, we have reported on the FM photochemistry in homogeneous solvents and in the β-cyclodextrin cavity [20]. This latter medium was chosen to model the influence of nonaqueous biological environments on the drug photoreactivity.

To gain deeper knowledge about the light-induced processes of this phototoxic anticancer compound in a biomimetic medium of higher complexity, we decided to explore the FM photobehavior in the presence of unilamellar phospholipid bilayer vesicles (liposomes) of α-L-phosphatidylcholine (PC). Such self-assembled structures are smectic mesophases of phospholipids with water interspaced among them, characterized by both high aggregation and high occupancy number. They are commonly accepted to be a suitable functional membrane model [21][22]. In our brief preliminary communication, we have shown that FM self-incorporates in the phospholipid bilayer, and that such a microenvironment exerts a decisive role in modifying the drug photoreactivity [23]. In this paper, we report new results on the photoreactivity of FM in PC vesicles that *a*) further corroborate what was proposed in our preliminary study, and *b*) contribute to the depiction of a more complete photochemical scenario of this phototoxic anticancer drug that can be both fundamentally and practically useful.

It is finally noteworthy that, due to the poor water solubility of FM, efforts have been made to improve the bioavailability of the drug with suitable carrier systems based on the formation of supramolecular complexes [24]. In this respect, the widespread application of liposomes as drug carrier systems to enhance drug solubilization [22] further reinforces our interest in the present topic.

2. Results and Discussion. – Before discussing the liposome vesicles' effects on the photoprocesses of FM, for sake of clarity, we recall briefly the main features regulating the FM photoreactivity in homogeneous solvents. In these media, the drug photochemistry is almost exclusively characterized by a nitro to nitrite photorearrangement leading to the phenol derivative **1** as the main stable photoproduct (*Scheme 1*)[1]). Interesting structural factors are responsible for the formation of this compound. Indeed, it has been pointed out that, due to the presence of the CF_3 substituent, the NO_2 group is placed almost perpendicularly to the aromatic plane and, as a consequence, is not conjugated with the latter. This twisted conformation plays a dominant role in triggering the photorearrangement. In fact, the 'out of plane' geometry makes the p-orbital of the O-atom constructively overlap with the adjacent p-orbital of the aromatic ring in the ground state (see *Scheme 1*). As a result of this molecular structure, the lowest excited triplet state is characterized by low biradical character and, as a consequence, by considerable inefficiency towards H-abstraction even in H-donating solvents [20] contrary to what was commonly observed for nitro

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[1]) Our results presented in [20] are partially in contrast with those reported by *Vargas et al.* [25], who found in addition to the formation of **1**, that other photolysis products were also found. This apparent incongruity is probably due to the different experimental conditions used in the two works (*i.e.*, light excitation wavelength, ground-state concentrations of FM).

aromatic compounds in which the NO_2 group is conjugated with the aromatic plane [26][27]. The optimized structures (MM+) of the two relevant conformations are displayed in *Fig. 1*.

Scheme 1

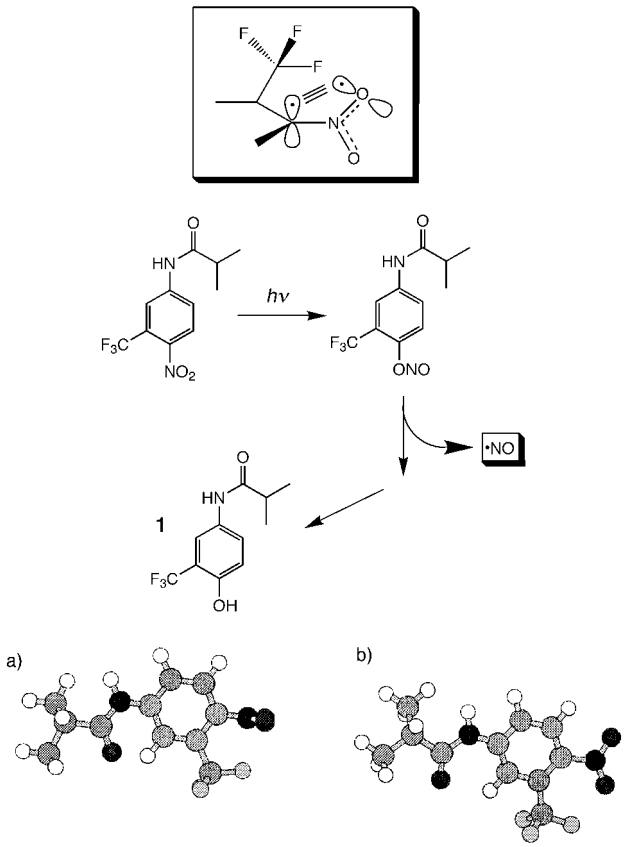


Fig. 1. *Optimized structure* (MM+) *of the two relevant conformations of FM with the* NO_2 *group* a) *perpendicular and* b) *almost coplanar to the aromatic ring*

Self-incorporation of FM into the vesicles leads to profound effects on the drug photoreactivity. *Fig. 2* shows the spectral changes observed upon 325-nm-light irradiation of FM in the presence and, for sake of comparison, in the absence of PC vesicles. Such markedly different spectral behavior is reflected in both the efficiency of FM photodegradation and the nature of the photoproducts. The analysis of the irradiated mixture showed, in fact, a 30-fold increase of the drug-disappearance quantum yield, the total suppression of product **1**, and the formation of the new photoproduct **2** (see *Scheme 2*)[2]. These results account for a highly selective shutdown

[2] Full spectroscopic data of **2** are available in [20]. Such a compound was indeed one of the components of the products mixture observed in the case of the FM-β-CD inclusion complex.

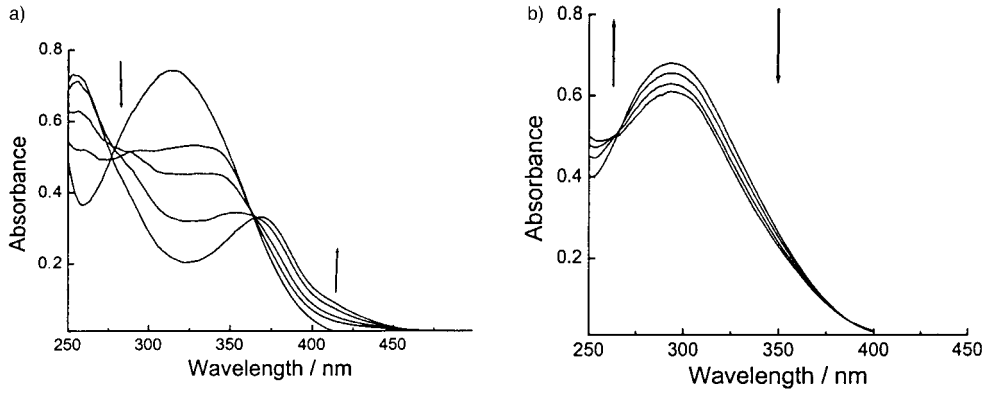
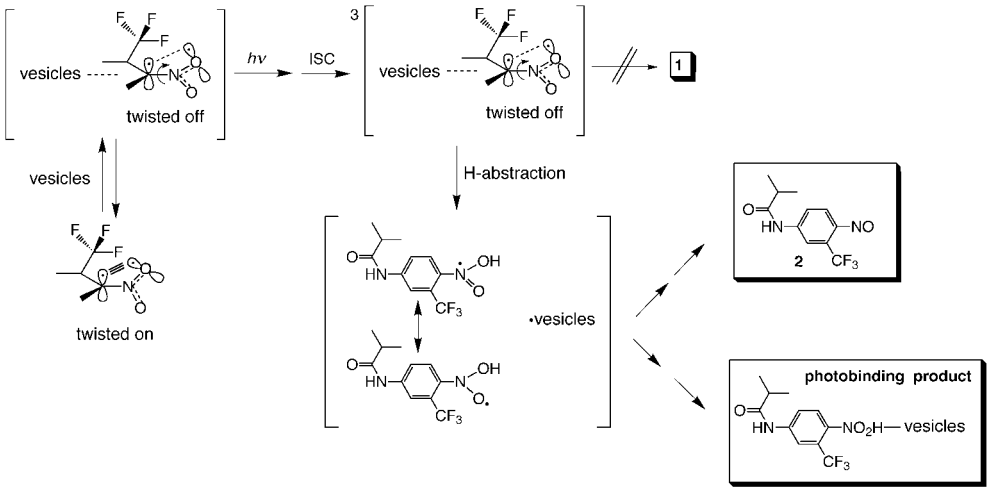


Fig. 2. *Spectral changes observed in N_2-flushed 10^{-4} M FM solution at pH 7.4* (a) *in the presence of PC vesicles upon regular irradiation time intervals of 1 min and* (b) *in the absence of vesicles upon time intervals of 10 min.* [PC] = 10^{-3} M. Cell path 1 cm. The absorption spectra in the presence of PC vesicles were taken *vs.* a reference solution containing the same concentration of PC vesicles.

Scheme 2



of the NO_2 to nitrite photorearrangement and for an unexpected activation of an efficient photonitroreductive pathway responsible for the formation of **2**.

These dramatic changes of the photochemical outcome observed in the phospholipid vesicles cannot be roughly rationalized on the basis of the presence of the abstractable H-atoms of the bilayer or its low polarity. Actually, as observed in our previous work, the irradiation of FM performed in solvents characterized by good H-donating properties and polarity similar to the vesicles interior did not activate the photonitroreductive pathway [20]. On the contrary, it was recently proposed that a plausible explanation to account for the inhibition of **1** and the photogeneration of **2** might be consistent with structural changes of FM occurring upon its self-compartmentalization in the bilayer structure [23]. In this regard, a less perpendicular geometry

of the NO_2 group with respect to the aromatic ring, more likely caused by steric constrains and specific weak interactions (*i.e.*, H-bond involving the CF_3 and/or NO_2) with the 'close-packed' lipid chains, was proposed to be responsible for the behavior observed.

The above picture is fully supported by the results obtained during simultaneous monitoring of the FM photodecomposition and the corresponding formation of photoproduct **2** as a function of the irradiation time. From the trend shown in *Fig. 3*, it can readily be seen that the disappearance of FM is not accompanied by a corresponding increase of **2**. In fact, the mass balance obtained from the difference of the slopes related to the disappearance of FM and formation of **2** is indicative for the lack of *ca*. 40% of products. We assume that this apparent incongruity might be due to the formation of photoproducts that are covalently bound to the vesicles and, thus, not detectable by usual HPLC procedures. To confirm this hypothesis, we carried out an experiment in which the UV/VIS absorption changes were monitored upon irradiation of FM in the presence of vesicles and subsequent separation of the vesicles by gel-filtration chromatography prior their destruction with some drops of *Triton X-100*. This procedure allows the complete release of noncovalently bound substances. *Fig. 4* shows the UV absorption obtained in the case of nonirradiated and irradiated samples, respectively. In the former case, no relevant absorption was observed beyond 300 nm, indicating that all FM was retained by column. On the contrary, in the case of the irradiated sample, a significant absorption extending beyond 400 nm was present. This finding provides unequivocal evidence for the formation of liposome covalently bound product originated in a photobinding process. Observation of this new reaction pathway rationalizes well the apparent incongruity in the mass balance and provides strong support to our previous proposal of changes in the perpendicularity of the NO_2 group upon drug self-incarceration in the vesicles microenvironment. In light of this new result, the overall picture of FM photoreactivity in the vesicles is depicted in the *Scheme 2* and discussed below.

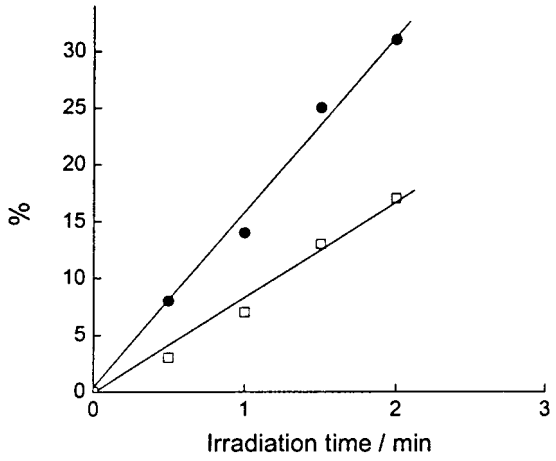


Fig. 3. *Percent of disappearance of FM* (●) *and formation of* **2** (□) *in the presence of PC vesicles as a function of the irradiation time.* [PC] = 10^{-3} M.

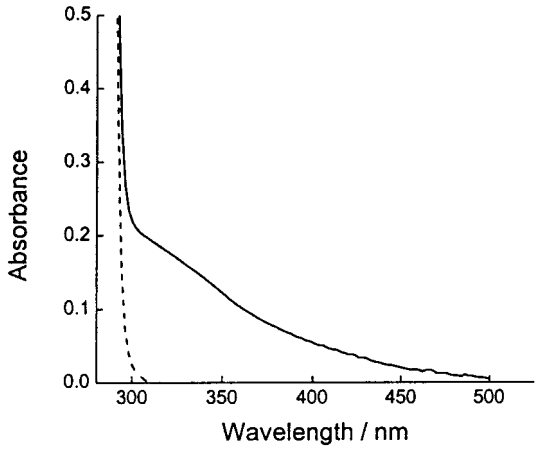


Fig. 4. *Absorption spectra recorded after* Sephadex *filtration of FM/vesicles irradiated* (—) *and non-irradiated* (···)

Planarization of the NO_2 group with consequent loss of the twisted conformation would lead in fact to a less extended overlap of the p-atomic-orbital of the O-atom with the adjacent orbital of the aromatic ring. As well-documented in the literature [28][29], the twisted conformation is an essential prerequisite for the NO_2 to nitrite photorearrangement responsible for the formation of **1**. The consequence of the planarization is, in turn, reflected in the logical increase of biradical character of the n,π^* triplet and in the consequent high ability of this latter to abstract H-atoms in the presence of a suitable H-donor. Under these conditions, an intravesicular H-abstraction photoprocess involving the NO_2 group and the H-atoms of the lipid chains might be activated. As shown in *Scheme 2*, the 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 lipid chain and eventually to give rise to **2** according to the well-known mechanistic pathways [26][27]; *ii*) to remain close to the former reaction site and recombine after intersystem crossing (ISC) with the vesicle-centered radical, giving rise to the vesicle covalently linked product. The considerable amount of photobinding product observed is consistent with a high efficiency of pathway *ii*. According to this view, one has to consider that the dynamics of solutes incorporated in the phospholipid vesicles is known to be very slow in comparison to other organized systems (*i.e.*, micelles, cyclodextrins) [2][3][6][21][22]. Thus, it is reasonable to assume that the radical in question may 'wait' close to the lipid-centered radical for a time long enough to make ISC and subsequent radical-radical recombination processes highly competitive with the radical diffusion.

The absorption shown in *Fig. 4* is in good agreement with the structure tentatively proposed in *Scheme 2*. A quantum-mechanical semiempirical calculation (AM1) of the absorption spectrum, carried out on a probable structure of the product with an O-atom covalently bound to the PC (see *Fig. 5*) compared with that of the PC itself, shows the appearance of three bands in the region of 336–309 nm, absent or very weak in vesicle. Indeed, the band at 314 nm, very weak in PC ($f = 0.05$), acquires a more permitted

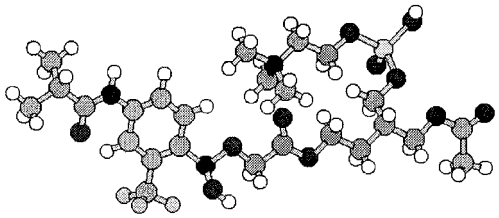


Fig. 5. *Possible structure of the product covalently bound with a model PC* (for simplicity $R^1 = R^2$ has been replaced by CH_3)

character for the bound product ($f = 0.143$), and a completely new band appears at 336 nm with $f = 0.05$. These new bands can be fairly well related to the long wavelength features ($\lambda > 300$ nm) of the spectrum.

In conclusion, we believe that the general picture presented herein may be of relevance for interdisciplinary areas of the scientific community.

1) From a strictly photochemical point of view, the reactivity of FM in PC vesicles represents a significant case of a remarkably selective and efficient photochemical modification fairly unusually piloted by conformational changes of a guest molecule upon its compartmentalization in a self-organized medium. In this connection, the multifaceted role of the vesicle bilayer in modifying the FM photoreactivity should be stressed. In fact, in this case, the phospholipid bilayer vesicles behave as unique reaction vessel that *i*) makes the drug molecular geometry suitable for a potential H-abstraction process, *ii*) provides a source of abstractable H-atoms, and *iii*) slows down the molecular motions of the photogenerated radical species, allowing the formation of a photobinding product. However, although the two latter factors are commonly accepted to play a role in numerous photochemical reactions, the former effect is quite uncommon, and remains the only indispensable prerequisite for observation of the present photochemical scenario.

2) From a photobiological point of view, given the potential of the bilayer to provide a suitable model mimicking the biological membrane, and, by taking into account that the photoprocesses initiated in the vesicles are mediated by radical pathways, the photochemical response of FM observed in this medium represents a good step forward toward gaining insight into the origin of the phototoxic and photoallergic effects displayed by FM. Actually, compartmentalization of the drug in particular biological sites in the presence of steric constraints and specific interactions could lead not only to a relevant increase in the photoproduction of reactive radical species but also to membrane-photobinding processes that, as generally accepted, are the first step in the onset of the photoallergy [30–32].

3) Finally, from an industrial point of view, by taking into account the efforts of the scientific community in the development of suitable carrier systems able to increase the FM solubility, our study suggests that, in light of the high photolability of the FM-liposome adduct in comparison with the 'free molecule', the use of liposome-based drug/carrier systems might not be the best approach toward achieving the aforementioned goal.

Experimental Part

General. Flutamide (M_r 250.2) and phosphatidylcholine were purchased from *Sigma Chemical Company* (St. Louis MO, USA) and used as received. Phosphate buffer (10^{-2} M, pH 7.4) was prepared from reagent-grade products. The pH of solns. was measured with a glass electrode. MeCN from *Carlo Erba* (Milan, Italy) was HPLC-grade.

Unilamellar liposomes were prepared by the solvent injection method [33]. Aliquots (0.4 ml) of egg PC standard soln. (0.04M) in abs. EtOH were introduced by means of a micrometric syringe *Chemetron* into 5.6 ml of phosphate buffer and were thermostatted at 60°. The injection rate was regulated at *ca*. 40 ml min^{-1}. This method yielded vesicles of homogeneous size (*ca*. 25-nm radius) [34].

Steady-state absorptions were recorded with a *Beckman 650 DU* spectrophotometer. The reaction mixture was analyzed with a *Hewlett-Packard* LC/MS system equipped with on-line photodiode-array detector (DAD) and a *LiChroCart 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, resp. Both retention time and integrated area for the nonirradiated FM were the same either in the absence or in the presence of vesicles, suggesting that no complex existed during the elution.

The separation of the photobinding product was achieved by gel filtration with a *Sephadex G-10* column equilibrated with phosphate buffer. To both irradiated and nonirradiated samples were added some drops of *Triton X-100* to destroy the vesicles and allow the complete release of noncovalently bound substance remaining trapped in the column.

Irradiation Conditions. Irradiations of FM were performed by means of 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 [35].

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