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Singlet oxygen production and *in vitro* phototoxicity studies on fenofibrate, mycophenolate mofetil, trifusal and their active metabolites

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Dedicated to Prof. Waldemar Adam on the occasion of his 80th birthday.

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Abstract

Singlet oxygen photosensitization (using time-resolved near infrared emission) and i*n vitro* phototoxicity (by means of the 3T3 Neutral red uptake assay) have been investigated for the prodrugs fenofibrate (**FFB**), mycophenolate mofetil (**MMP**) and trifusal (**TFS**) as well as for their active metabolites fenofibric acid (**FFA**), mycophenolic acid (**MPA**) and 2-hydroxy-4-(trifluoromethyl)benzoic acid (**HTB**). The results show that **FFB** and its active metabolite **FFA** generate $^1O_2$ with a quantum yield in the range 0.30-0.40 and show a photo-irritation factor (PIF) higher than 40. By contrast, **MMP**/**MPA** and **TFS**/**HTB** are not photoactive in the employed assays. These results correlate well with the previously reported *in vivo* phototoxicity in treated patients.

Introduction

The metabolic processes, achieved by the enzymatic machinery of living organisms, produce chemical modifications on drugs, which may lead to activation/deactivation and transformation into more hydrophilic derivatives that facilitate elimination from the body.[1] These changes are the result of phase I reactions (such as oxidations, reductions or hydrolysis) and/or phase II reactions (*i. e*. glucuronidation, sulfation, acetylation or methylation).[2]

The interaction of UV-radiation with drugs may result in photosensitization *via* a Type I or a Type II mechanism. The former is related to hydrogen abstraction or electron transfer and leads to production of radical species that can oxidize biomolecules, whereas the latter involves singlet oxygen ($^1O_2$) generation and its attack to biomolecules such as proteins, nucleic acids and lipids. [3] Although determination of the photosensitizing potential of drugs is an active field of research, much less has been done with metabolites, even if they might act as suitable photoactive compounds. Actually, most metabolites retain the main chromophore of the drug and hence their photosensitizing ability, which can even be enhanced after the chemical modifications occurring during the metabolic processes. Hence, identification of the phototoxic potential of metabolites is important, specially when they are the pharmacologically active compounds.

In this context, we have undertaken a systematic study on the prodrugs fenofibrate (**FFB**),[4] mycophenolate mofetil (**MMP**)[5] and trifusal (**TFS**)[6] as well as on their active metabolites fenofibric acid (**FFA**),[7] mycophenolic acid (**MPA**)[8] and 2-hydroxy-4-(trifluoromethyl)benzoic acid (**HTB**)[9]. Their chemical structures are shown in Chart 1. The investigated drugs possess different pharmacological effects. Thus, while **FFB** is prescribed for the treatment of hyperlipidemia, **MMP** is employed for the prevention of organ transplant rejection and **TFS** is an antiplatelet agent. In all cases, we have investigated the formation of $^1O_2$ by time-resolved near infrared emission and the i*n vitro* phototoxicity using the 3T3 neutral red uptake (NRU) assay.

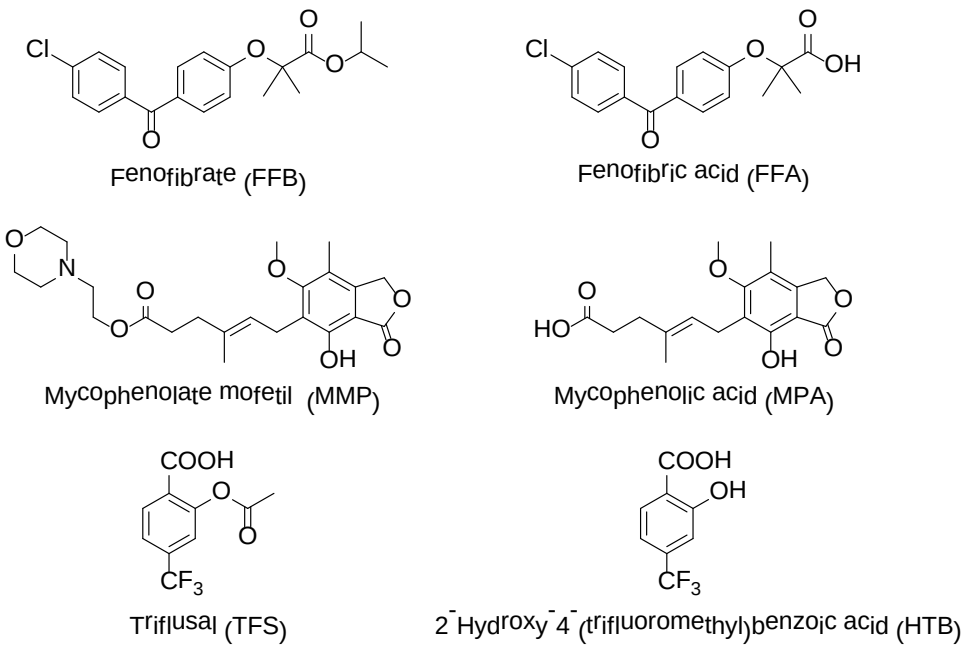

Chart 1. Chemical structure of drugs and metabolites investigated in this work.

Results

First, $^1O_2$ production was studied in the polar non-protic solvent acetonitrile, taking advantage of the characteristic $^1O_2$ near-infrared phosphorescence at 1270 nm and using phenalenone (PN) as standard for comparative purposes. Except **TFS**, which does not absorb at the laser excitation wavelength (355 nm), the other drugs and metabolites investigated were able to generate $^1O_2$ upon exposure to UV radiation, although to different extent (Figure 1). The $^1O_2$ quantum yield (Φ_Δ) and the rate constant for $^1O_2$ quenching by the drug or its metabolites (k_q) are collected in Table 1. For **FFA** and **FFB**, the Φ_Δ values were in the range from 0.30 to 0.40 (Figure 1A), similar to that of model benzophenone (Φ_Δ = 0.35). [10] For the other compounds, Φ_Δ were in the range 0.05-0.10 (Figures 1B, C). The kinetics of $^1O_2$ showed the characteristic rise and decay profile of reactive intermediates. Except for **HTB**, the $^1O_2$ decay lifetime (τ_Δ) was noticeably shorter than the accepted value for neat acetonitrile (81 μs),[11] indicating that the compounds are good $^1O_2$ quenchers (Figures 1D-F). Analysis of τ_Δ dependence with drug/metabolite concentration allowed us to calculate k_q; the quenching was remarkable for **MPA** and **MMP** (k_q in the range of 10^7 $M^{-1}s^{-1}$), which is in agreement with the presence of phenolic rings in their chemical structure [12] and, to a lesser extent, for **FFA** and **FFB**. Generation of $^1O_2$ by the more hydrophilic active metabolites was studied also

in d-PBS, using sodium 1*H*-phenalen-1-one-2-sulphonate (PNS) as standard. A clear decrease of Φ_Δ was observed for all compounds (Figures 1G-I), but particularly for **FFA**; this is likely the result of the competition between energy transfer to oxygen and decarboxylation. [13]

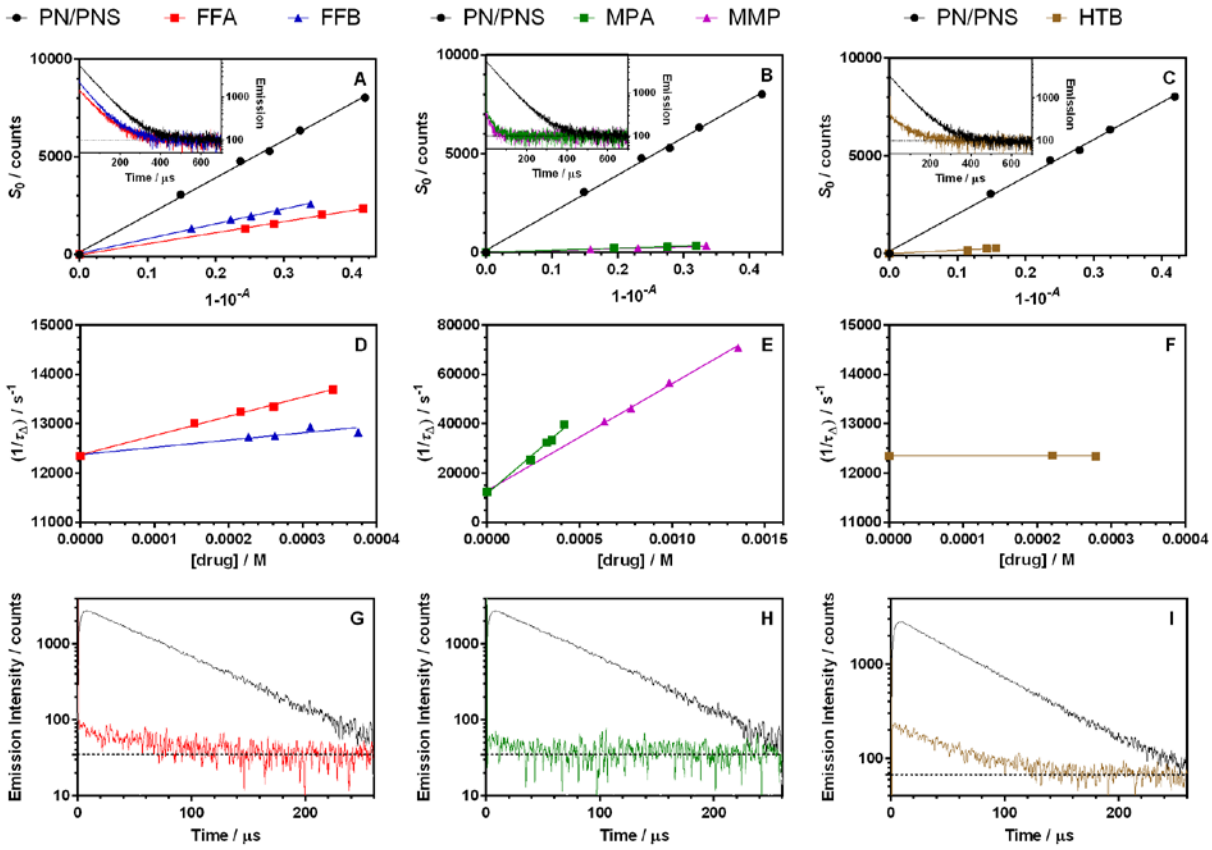

Figure 1: A-C: Determination of $^1O_2$ quantum yields (Φ_Δ). Absorbance dependence of the $^1O_2$ phosphorescence intensity (S_0) for the drugs and PN in acetonitrile; λ_{exc} = 355 nm. Inset: kinetic traces for $^1O_2$ phosphorescence signals. D-F: Drug concentration effect on the $^1O_2$ lifetime (τ_Δ). G-I: $^1O_2$ phosphorescence signals in d-PBS for optically matched solutions of the drugs and PNS. Colour codes: PN/PNS: black; FFA: red; FFB: blue; MPA: green; MMP: magenta, and HTB: brown.

Table 1: Quantum yield of $^1O_2$ production (Φ_Δ) and rate constant for $^1O_2$ quenching (k_q) for drugs and metabolites.

Compound	Φ_Δ[a]	k_q ($M^{-1}s^{-1}$)[a]	Φ_Δ[b]
FFB	0.40 ± 0.04	$(1.5 \pm 0.3) \times 10^6$	-
FFA	0.30 ± 0.03	$(3.9 \pm 0.2) \times 10^6$	0.016 ± 0.002
MMP	0.050 ± 0.005	$(4.3 \pm 0.1) \times 10^7$	-
MPA	0.055 ± 0.005	$(6.4 \pm 0.4) \times 10^7$	0.009 ± 0.001
HTB[c]	0.09 ± 0.01	$< 1 \times 10^5$	0.052 ± 0.005

[a]In CH_3CN; [b]in d-PBS, only for the metabolites, due to the scarce solubility of the drugs in aqueous media; [c] **TFS** was not measured because it does not absorb at the excitation wavelength (355 nm).

In another series of experiments, cell viability upon exposure to all compounds, in combination with UVA irradiation was evaluated by the in vitro 3T3 neutral red uptake (NRU) phototoxicity assay. [14] Hence, cytotoxicity profiles of BALB/c 3T3 fibroblasts treated with **FFB**, **FFA**, **MMP**, **MPA**, **TFS** and **HTB** were measured, using neutral red as vital dye, both in the dark and under UVA irradiation (Figure 2). The IC50 values were determined from dose-response curves for cell viability of cell treated under conditions described above (more details are provided in Experimental Section).

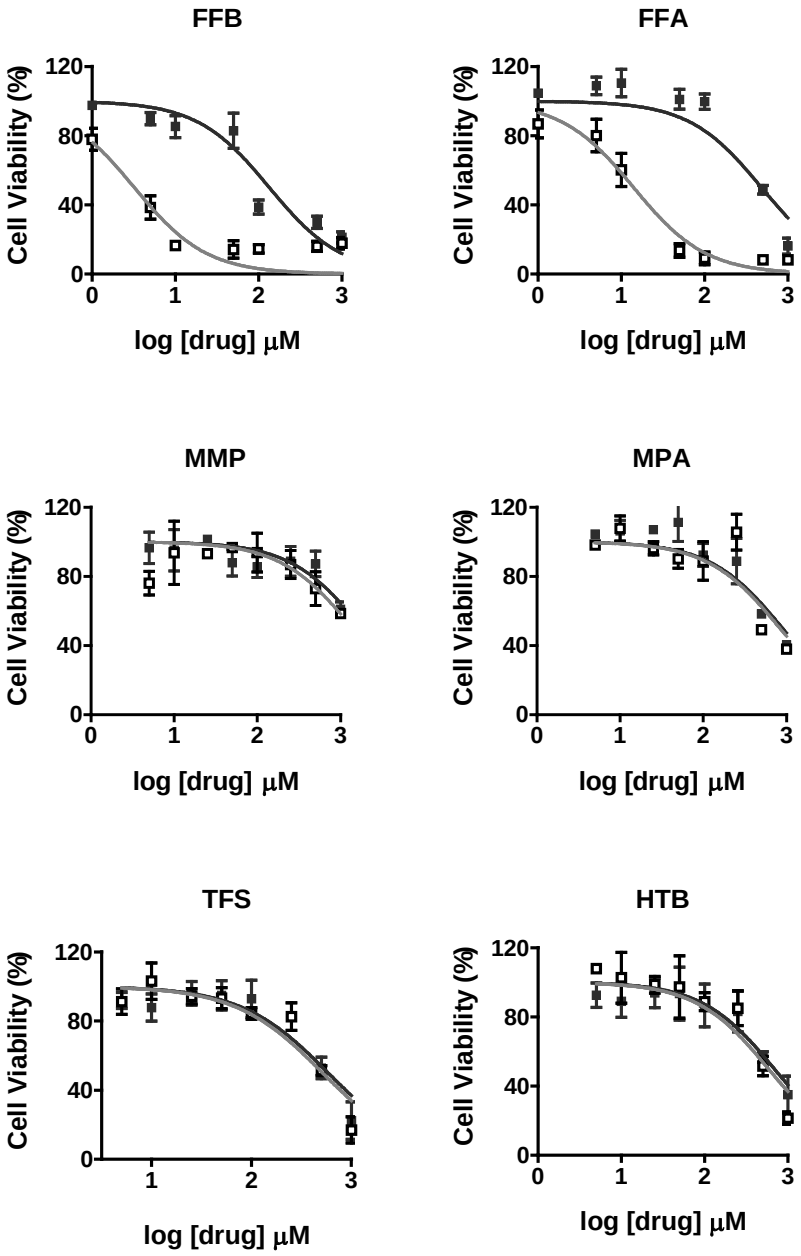

Figure 2. Dose-Response curves for cell viability of 3T3 cells treated with compounds drugs and metabolites by using NRU assay in the presence (□) or absence (■) of 5 J/cm^2 UVA radiation. Data represent Mean ± SD from four independent experiments.

The main goal of NRU test is to calculate the photo-irritation factor (PIF), which corresponds to the ratio of the IC50 under dark and light conditions for each compound. As inferred from the data in Figure 3, **FFB** and **FFA** resulted to be potentially phototoxic with a PIF value >40, whereas all other compounds were basically non-phototoxic (PIF ~ 1).

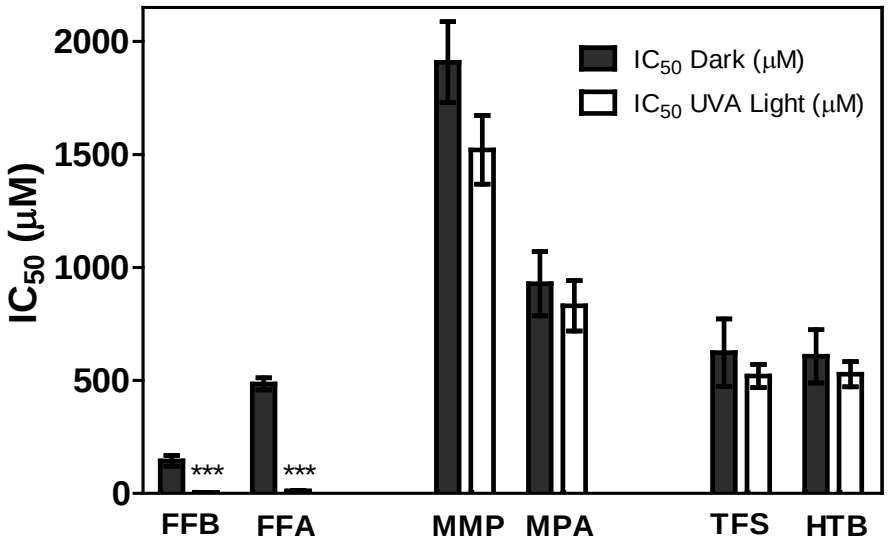

Figure 3. Phototoxicity of drugs and metabolites in the 3T3 NRU assay. Data represent mean ± SD from four independent experiments and asterisks denote significant differences relative to the IC50 Dark by the TStudent test (***$p \geq 0.001$).

Discussion

Overall, the results obtained in the two types of experiments indicate that the benzophenone chromophore present in **FFB** and **FFA** is the only significant active moiety as regards $^1O_2$ production and phototoxicity within the investigated series of compounds. This is in agreement with the case reports on the appearance of phototoxic reactions experienced after administration of the drugs to a number of patients.[15]

Upon 355 nm excitation of both **FFB** and **FFA** in acetonitrile, $^1O_2$ is generated with Φ_Δ in the range 0.30-0.40, whereas in neutral aqueous medium the Φ_Δ value of **FFA** is *ca.* 20 times lower. This can be attributed to the different photochemical behavior previously reported for the free carboxylic acid and the carboxylate salt. Thus, the latter is much more reactive from its triplet excited state, giving rise to decarboxylation products. [16] Interestingly, the phototoxicity (as quantified by the PIF value) does not differ significantly from **FFB** to **FFA**. This suggests that in the cellular medium, both the drug and its metabolite are mainly located in a hydrophobic environment, probably the cell membrane. Moreover, under the steady-state irradiation conditions employed in the

in vitro assay, the **FFA** photoproducts that can be formed retain the benzophenone chromophore and are also expected to be phototoxic.[17]

In the case of the **MMP**/**MPA** pair, both $^1O_2$ production and phototoxicity are very low, consistently with the lack of clinical data on *in vivo* phototoxicity found in treated patients. Interestingly, **MMP** and **MPA** may even behave as scavengers of $^1O_2$, owing to the high rate constants determined for the quenching of this reactive oxygen species.

Finally, **TFS** and its active metabolite **HTB** are also inefficient in the $^1O_2$ as well as in the phototoxicity measurements. It is noteworthy that this drug has been reported to elicit photosensitivity disorders; however, these side effects appear to be photoallergic rather than phototoxic in nature. Photoallergy is accepted to involve the immunological system and is triggered by photohaptenation of a protein through irreversible covalent photobinding to specific amino acid residues. Actually, **HTB** photoreacts with the ε-amino group of Lys residues, not only in the free amino acid, but also in poly-Lys models and in the whole proteins. [18]

Conclusions

Within the series of compounds investigated in the present work, $^1O_2$ production correlates well with the *in vitro* phototoxicity assessed by the 3T3 NRU phototoxicity test. Thus, fenofibrate and its active metabolite fenofibric acid generate $^1O_2$ with a quantum yield ranging from 0.30 to 0.40 and show a photo-irritation factor (PIF) higher than 40. By contrast, mycophenolate mofetil /mycophenolic acid and triflusal/2-hydroxy-4-(trifluoromethyl)benzoic acid are not photoactive in the employed assays. This correlates well with the previously reported *in vivo* phototoxicity. A special case is trifusal that gives rise to photosensitivity disorders of photoallergic nature, whose detection should be anticipated following a different experimental approach.

Experimental Section

Chemicals. Acetonitrile, deuterium oxide, phenalenone, all drugs and metabolites (except **HTB**, which was from Wako) as well as neutral red-based *in vitro* toxicology test kit, were purchased from Sigma-Aldrich and used as received. Phosphate buffered saline solution was

prepared dissolving the required amount of a PBS tablet (Sigma) in deuterium oxide or deionized water. Sodium 1H-phenalen-1-one-2-sulphonate was synthesized as described previously.[19]

Singlet Oxygen Experiments. Production of $^1O_2$ was studied by time-resolved near-infrared phosphorescence using a setup described in details elsewhere.[20] Briefly, a pulsed Nd:YAG laser (FTSS355-Q, Crystal Laser,) working at 1 kHz repetition rate at 355 nm (third harmonic; 0.5 μJ per pulse) was used for sample excitation. A 1064 nm rugate notch filter (Edmund Optics) and an uncoated SKG-5 filter (CVI Laser Corporation) were placed at the exit port of the laser to remove any residual component of its fundamental emission in the near-infrared region. The luminescence exiting from the sample was filtered by a 1100 nm long-pass filter (Edmund Optics) and a narrow bandpass filter at 1275 nm (BK-1270-70-B, bk Interferenzoptik). A thermoelectric-cooled near-infrared sensitive photomultiplier tube assembly (H9170-45, Hamamatsu Photonics) was used as detector. Photon counting was achieved with a multichannel scaler (NanoHarp 250, PicoQuant). The time dependence of the $^1O_2$ phosphorescence with the signal intensity $S(t)$ is described by *Eq. 1*, where τ_T and τ_Δ are the lifetimes of the photosensitizer triplet state and of $^1O_2$, respectively, and $S(0)$ is a quantity proportional to Φ_Δ as shown in *Eq. 2*; κ is a proportionality constant, which includes electronic and geometric factors, k_R is the $^1O_2$ radiative rate constant, E is the incident laser energy and A is the sample absorbance at 355 nm.

$$S(t) = S(0)\frac{\tau_\Delta}{\tau_\Delta - \tau_T}\left(e^{\frac{-t}{\tau_\Delta}} - e^{\frac{-t}{\tau_T}}\right) \qquad Eq.1$$

$$S(0) = \kappa k_r \phi_\Delta E(1 - 10^{-A}) \qquad Eq.2$$

In acetonitrile, the procedure for determining Φ_Δ involved measuring $S(0)$ value for a series of solutions of increasing absorbance and then plotting $S(0)$ versus the sample absorption factor ($1\text{-}10^{-A}$), which yielded linear plots. Then Φ_Δ was obtained by comparison of the slopes of such plots for a suitable reference (PN, $\Phi_\Delta = 1.00 \pm 0.03$) [21] and the drug, using Eq. 3.

$$\phi_{\Delta,drug} = \frac{Slope_{drug}}{Slope_{reference}}\phi_{\Delta,reference} \qquad Eq.3$$

In deuterated-PBS, Φ_Δ was determined by comparing the $S(0)$ values of optically-matched solutions of the drug and the reference (PNS, $\Phi_\Delta = 0.97 \pm 0.06$) [22] at 355 nm as described by Eq.4.

$$\phi_{\Delta,drug} = \frac{S(0)_{drug}}{S(0)_{reference}}\phi_{\Delta,reference} \qquad Eq.4$$

***In Vitro* 3T3 Neutral Red Uptake (NRU) Phototoxicity Test.** The fibroblast cell line BALB/c 3T3 was grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS), 4 mM glutamine and 1% penicillin/streptomycin and maintained twice a week in exponential growth in 75 cm^2 plastic flasks in a humidified incubator at 37 ºC under 5 % CO_2 atmosphere. The NRU was performed as previously described by the OECD guideline 432 [14] with minor modifications. In brief, for each compound two 96-wells plates were seeded (2.5×10^4 cells/well). Cells were incubated with test compounds at eight concentrations ranging from 0.5 µM to 1000 µM for 1 h. After that, one plate was irradiated on ice for 11 min to reach a dose of UVA equivalent to 5 J/cm^2, whereas the other plate was kept in a dark box. For this purpose, a photoreactor model LZC-4 (Luzchem, Canada) equipped with 14 UVA lamps for top and side irradiation (λ_{max} = 350 nm, Gaussian distribution) was employed as the light source. All irradiations were carried out through the lid of the plates and the temperature was maintained by ventilation during the irradiation step. The viability of UVA-treated control cells in the absence of test compounds was higher than 90% of those kept in the dark, demonstrating the suitability of the UVA dose. Next, the compound solutions were replaced with DMEM medium, and plates were incubated overnight. After this time, neutral red solution (50 µg/mL) was added into each well and incubated for 2 h. Cells were washed with PBS and neutral red was extracted in 100 µL with the desorbs solution (water 49 % (v / v), ethanol 50 % (v / v) and acetic acid 1 % (v / v). With a Multiskan Ex microplate reader, the absorbance was measured at 550 nm. Dose-response curves were developed for each compound in order to determine the concentration of compound that causes a 50% decrease in neutral red uptake (IC_{50}) in the absence and in the presence of irradiation. As a final point, the Photo Irritation Factor (PIF) was calculated by means of Eq. 5

$$PIF = \frac{\text{IC50 DARK}}{\text{IC50 UVA LIGHT}} \qquad Eq.5$$

A compound is labelled as phototoxic if PIF > 5, probably phototoxic when 5 > PIF > 2, and non phototoxic when PIF < 2, according to the OECD Test Guideline.[14] Chlorpromazine and sodium dodecyl sulphate were used as positive and negative controls, respectively.

Acknowledgements

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