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The NSAIDs Indomethacin and Diflunisal as Scavengers of Photogenerated Reactive Oxygen Species†

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ABSTRACT

Diflunisal (DFNS) and Indomethacin (IMTC) are two profusely employed NSAIDs that provide anti-inflammatory and analgesic effects in humans. The scavenging of reactive oxygen species (ROS) by both NSAIDs was systematically studied in pH 7 aqueous solution. The ROS $O_2(^1\Delta_g)$, $O_2^{\bullet-}$ and H_2O_2, generated by visible light irradiation of Riboflavin (Rf) in the presence of DFNS and IMTC, are deactivated by the NSAIDs. The ROS scavenging action by both NSAIDs constitutes an interesting result and adds one more positive aspect to the beneficial actions attributed to these drugs. Nevertheless it should be taken into account that several NSAIDs, in particular IMTC, have been connected to the pathogenesis of gastric mucosal lesions, which in some cases includes ROS generating-ability. DFNS quenches ROS in a dominant physical fashion. It constitutes an excellent protective-antioxidant provided that is practically not destroyed/oxidized after the ROS scavenging action. IMTC, being also an efficient interceptor of ROS, belong to the so-called group of *sacrificial*-ROS quenchers: It is easily degraded by the oxidative species in the scavenging action. Although this property is negative in the context of prolonged ROS elimination, exhibits a promissory aspect for the degradation of pharmaceutical contaminants, such as NSAIDs, in waste waters.

Abbreviations: CAT, Catalase; DFNS, Diflunisal; D_2O, Deuterium oxide; FFA, Furfuryl alcohol; H_2O_2, Hydrogen peroxide; IMTC, Indomethacin; KH_2PO_4, Potassium phosphate monobasic; K_2HPO_4, Potassium phosphate dibasic; NaN_3, Sodium azide; ND, Neurodegenerative disorders; NSAIDs, Nonsteroidal anti-inflammatory drugs; $O_2(^1\Delta_g)$, Singlet molecular oxygen; $O_2^{\bullet-}$, Superoxide radical anion; RB, Rose Bengal; Rf, Riboflavin (vitamin B2); ROS, Reactive oxygen species; SOD, Superoxide dismutase; SPC, Time-correlated single photon counting; TRPD, Time resolved phosphorescence detection.

INTRODUCTION

Many neuroinflammatory mediators, including oxidative agents such as reactive oxygen species (ROS) were found to be upregulated in human brain areas affected by neurodegenerative disorders (ND) (1,2). In fact, oxidative stress is considered an identifier mark as evaluated by DNA, RNA, lipids and proteins oxidation level when NDs are detected (3–5). Several studies have established an inverse correspondence between ND manifestation and prolonged administration of anti-inflammatory medicaments, especially non-steroidal anti-inflammatory drugs (NSAIDs) [for a review see reference (6)]. These findings suggest that potential antioxidant properties of the NSAIDs could be related, in some degree, with the retardation of ND. In this context, we decided to investigate two profusely employed NSAIDs named Indomethacin (IMTC) and Diflunisal (DFNS) (For structural formulae of the NSAIDs see Table 1), in connection with their possible scavenging action towards photogenerated ROS. Both IMTC, an indolic acid derivative, and DFNS, a salicylic acid derivative, are NSAIDs widely used due to their anti-inflammatory and analgesic properties (7). Nevertheless several NSAIDs, especially IMTC, have been reported due to their adverse effects in the human body, particularly related to gastro-intestinal disorders. In most of the cases the administration of IMTC has been connected to the pathogenesis of gastric mucosal lesions (8,9) that includes ROS generating-ability (10). Besides, the administration of IMTC caused a significant decrease in the levels of native protecting antioxidants and a concomitant increase in the lipid peroxidation (11). The case of DFNS is less dramatic in this sense, since the limited literature on the topic only mentions *rare but possible* gastrointestinal adverse effects (12).

We focused in the present paper the study of kinetic and mechanistic aspects of the eventual ROS-quenching ability of two selected NSAIDs. The oxidative species were produced by visible-light irradiation of vitamin B2 (Riboflavin, Rf). We think This arrangement roughly models a scenery with ROS generated in the presence of potential antioxidants, in a given biological environment.

Riboflavin has been reported as a frequent sensitizer for the oxidative degradation of numerous relevant natural substrates (13). The pigment produced the highly reactive species $O_2(^1\Delta_g)$. Besides numerous cases of other aggressive ROS such as superoxide radical anion ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2) produced through Rf- photosensitization by Rf, have been reported (14).

Although the main objective of the work was the evaluation of the eventual overall protective effect exerted by IMTC and DFNS against photogenerated ROS, kinetic and mechanistic details of the scavenging process will help to predict the fate of

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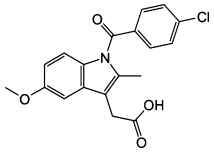
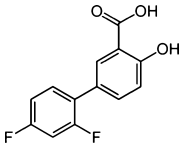
Table 1. Rate constant values for the quenching of electronically excited singlet ($k_{q1,}$ $M^{-1}s^{-1}$), and triplet Riboflavin ($k_{q3,}$ $M^{-1}s^{-1}$) by Indomethacin and Diflunisal. Overall ($k_{t,}$ $M^{-1}s^{-1}$) and reactive ($k_{r,}$ $M^{-1}s^{-1}$) rate constants for the interaction of $O_2(^1\Delta_g)$ with Indomethacin and Diflunisal, $\boldsymbol{k_r}$ **/**$\boldsymbol{k_t}$ ratio and relative rates of oxygen uptake by Indomethacin and Diflunisal upon Rf- (**V_{ox}Rf**) and RB-photosensitization (**V_{ox}RB**).

Compound	$k_{q1} \times 10^{-9}$(a)	$k_{q3} \times 10^{-9}$	$k_t \times 10^{-7}$	$k_r \times 10^{-7}$	k_r/k_t	V_{ox}Rf	V_{ox}RB
Indomethacin (IMTC)	8.9 ± 0.06	1.8 ± 0.3	2.6 ± 0.2	2.7 ± 0.2	~1	1	1
							
Diflunisal (DFNS)	9.0 ± 0.05	2.1 ± 0.5	1.7 ± 0.3	0.19 ± 0.1	0.11	0.26	0.07
							

In MeOH

both reaction partners. This is an essential point not only in the context of eventual ROS degradation but also enables the estimation of the chemical resistance of the NSAIDs under natural oxidative conditions. In this line a new important aspect arises as a secondary objective of the present research work. It is related to the elimination of contaminant residues from the aquatic environment: The NSAIDs, profusely employed as medicaments in many therapeutic options, belong to one of the most significant groups of pharmaceuticals incorporated to waste waters through different sources (15). This fact constitutes a serious potential risk for the natural environment. Thus, the persistence/oxidability of these drugs under natural-light- irradiation in the presence of a native dye-sensitizer constitutes a topic of environmental relevance.

MATERIALS AND METHODS

Materials. Riboflavin, Indomethacin (2-{1-[(4-chlorophenyl)carbonyl]-5-methoxy-2-methyl-1*H*-indol-3-yl}acetic acid), Diflunisal (2′,4′-difluoro-4-hydroxybiphenyl-3-carboxylic acid), superoxide dismutase (SOD) from boverythrocytes, catalase from bovine liver (CAT) and deuterium oxide 99.9% (D_2O) were purchased from Sigma Chem. Co. Rose Bengal (RB), were from Aldrich. Sodium azide (NaN_3) was from Merck. Furfuryl alcohol (FFA) was from Riedel de Haën. All these compounds were used as received. Methanol (MeOH) HPLC quality, was from Sintorgan. All measurements were carried out at room temperature and with freshly prepared solutions. D_2O was employed in the time-resolved determinations of $O_2(^1\Delta_g)$ phosphorescence in order to enlarge the lifetime of this species. Water was triply distilled. The pH-pD of the final aqueous solutions for all photochemical experiments, unless specified, was in the range 7.0 ± 0.2, employing buffered aqueous solutions prepared, with 0.025 M KH_2PO_4/0.025 M Na_2HPO_4 (16). The presence of the salts in the mentioned concentrations did not affect neither the lifetimes nor the profiles of the optical spectra of Rf electronically excited states, as compared to those obtained in pure water.

Due to the reported pK_a values of 4.5 and 3.3 for IMTC and DFNS respectively, the species present in pH 7 aqueous solution were the corresponding carboxylate ones (17,18).

Methods. Stationary photolysis. Continuous aerobic photolysis of aqueous solutions of NSAIDs with RB or Rf as sensitizers was carried out in a home-made photolyser with the filtered light from a 150-W quartz-halogen lamp. In the Rf- or RB-sensitized photoirradiations, cut-off at 400 nm was employed. Hence none of the substrates employed, IMTC, DFNS and the respective indolic and salicylic acid model compounds absorbed any incident light.

Ground state absorption spectra were registered in a Hewlett Packard 8452A diode array spectrophotometer.

The anaerobic photodegradation rates of Rf were deduced from the decrease of the 445-nm absorption band under Argon saturation.

The rate constant k_r for the reaction $O_2(^1\Delta_g)$ + NSAIDs [process (16)], see Scheme 1) was determined using a described method (19), for which the knowledge of the reactive rate constant k_{rR} for the photooxidation of a reference compound R is required, using the expression slope/slope$_R$ = k_r [NSAID]/k_{rR} [R], where slope and slope$_R$ are the respective slopes of the first-order plots of NSAID and R consumption. The reference compound employed was FFA, with a reported value for k_{rR} of 1.2×10^8 $M^{-1}s^{-1}$ (15).

The rates of oxygen consumption were determined with a specific oxygen electrode Orion 97-08 in aqueous solution. The electrode was immersed in a 150 mL hermetically sealed quartz cell containing the mixture sensitizer + NSAID. The system was irradiated with the above described photolysis device.

Quenching of Rf electronically excited states. Fluorescence quenching of Rf was determined employing a JASCO FP6200 apparatus. Excitation and emission wavelengths for Rf were 445 and 525 nm, respectively. The fluorescence quenching rate constant ^1k_q for the interaction of ^{1}Rf* with NSAIDs [process (2)] was determined by a classical Stern-Volmer treatment with the expression $I_0/I = 1 + {}^1k_q\ {}^1\tau_0$ [NSAIDs], where I_0 and I are the respective stationary fluorescence intensities in the absence and in the presence of NSAIDs and $^1\tau_0$ is the Rf fluorescence lifetime. Only these fluorescence quenching experiments, due to limitations in the solubility of the NSAIDs, were done in MeOH.

Argon-saturated 0.04 mM Rf aqueous solutions were photolyzed using a previously reported flash photolysis apparatus (20): A ns Nd:YAG laser system (Spectron) at 355 nm was the excitation source, employing a 150 W Xenon lamp as analyzing light. The detection system comprised a PTI monochromator and a red-extended photomultiplier (Hamamatsu R666). The signal, acquired and averaged by a digital oscilloscope (Hewlett-Packard 54504A), was transferred to a PC via a HPIB parallel interface, where it was analyzed and stored. ^{3}Rf* disappearance was monitored from the first-order decay of the absorbance at 670 nm, a zone where the interference from other possible species is negligible. To avoid self-quenching and triplet-triplet annihilation, the triplet decay was measured at low Rf concentration (typically 0.05 mM) and at low enough

laser energy. The rate constant ^3k_q of the interaction ^{3}Rf* + NSAIDs [process (8)] was deduced by the Stern-Volmer expression $1/^3\tau = (1/^3\tau_0) + {}^3k_q$ [NSAID], where $^3\tau_0$ and $^3\tau$ are the experimentally determined lifetimes of ^{3}Rf* in the absence and in the presence of NSAIDs, respectively.

$O_2(^1\Delta_g)$ phosphorescence detection. The overall quenching rate constant of $O_2(^1\Delta_g)$ by a quencher Q, k_t, was determined using time resolved phosphorescence detection (TRPD) in a system previously described (21). Briefly, the $O_2(^1\Delta_g)$ phosphorescence at 1270 nm, generated by excitation with the 532-nm emission from a Nd:YAG laser (Spectron), was detected at right angles using an amplified Judson J16/8Sp Germanium detector, after filtering through a 1270-nm interference and a wratten filter. The output of the detector was coupled to a digital oscilloscope and to a personal computer for the signal processing. Usually, ten shots were needed for averaging so as to achieve a good signal-to-noise ratio, from which the decay curve was obtained. Air equilibrated solutions were employed in all cases. In the dynamic determinations, solutions of RB in D_2O ($Abs_{560} = 0.3$) were used. $O_2(^1\Delta_g)$ lifetimes were evaluated in the presence (τ) and in the absence (τ_0) of the quencher (NSAIDs), and the data were plotted as a function of IMTC or DFNS concentration, according to a simple Stern-Volmer treatment: $\tau_0/\tau = 1 + k_t\,\tau_0$ [NSAID].

RESULTS

Proposed kinetic scheme

Scheme 1 depicts a generic Rf-photosensitized process, employed in this work for interpretation and discussion of the results.

Scheme 1 indicates that the sensitizer (Rf, in ground state) is promoted to electronically excited singlet and triplet states [^{1}Rf* and ^{3}Rf* respectively, process (1)] upon absorption of the incident light. Both states can either decay to ground state Rf (reactions not shown) or can be quenched through processes (2)–(4) and/or (11). The species superoxide radical anion ($O_2^{\bullet-}$) can be formed through reaction of ^{3}Rf* with ground state oxygen [$O_2(^3\Sigma_g^-)$]. A quantum yield of 0.009 was reported for process (3) (13,22). In the presence of electron donors as the NSAIDs, an electron transfer process to ^{3}Rf* would give rise to the respective semioxidized ($NSAIDs^{\bullet+}$) and semireduced ($Rf^{\bullet-}$) [process (4)]. The interaction $Rf^{\bullet-}$–$O_2(^3\Sigma_g^-)$ generates the reactive species $O_2^{\bullet-}$ process (7), with a reported value for $k_7 = 1.4 \times 10^8\ M^{-1}s^{-1}$) (23,24). At neutral pH, the species $RfH^{\bullet}$ (pKa = 8.3) is formed through protonation of the species $Rf^{\bullet-}$ [process (5)] (25,26). The bimolecular decay of $RfH^{\bullet}$ is known to proceed through disproportion reaction to yield equimolar Rf and fully reduced Rf (RfH_2) [process (6)], which in the presence of $O_2(^3\Sigma_g^-)$ is reoxidized to give initially Rf radical and $O_2^{\bullet-}$ [process (7)] and finally Rf and H_2O_2 [process (8)] (27). The species $O_2(^1\Delta_g)$ is formed by means of an energy transfer process from ^{3}Rf* to $O_2(^3\Sigma_g^-)$, with a quantum yield of 0.49 in MeOH (28) [step (11)]. The excited oxygenated species decays by interaction with NSAIDs through physical and/or chemical/reactive processes or by collision with surrounding solvent molecules [processes (13) and (14) or step (12) respectively].

Riboflavin-photosensitization

The visible-photoirradiation of the mixture of 0.04 mM DFNS + 0.04 mM Rf or 0.05 mM IMTC + 0.04 mM Rf, both in aerated neutral aqueous solution, induces changes in the absorption spectra that are attributable to transformations in the NSAIDs (Fig. 1).

$Rf + h\nu \rightarrow {}^1Rf^* \longrightarrow {}^3Rf^*$ [1]

$^1Rf^* + NSAIDs \longrightarrow Rf + NSAIDs$ or P(2) [2], rate constant ^1k_q

$^3Rf^* + O_2(^3\Sigma_g^-) \longrightarrow Rf^{\bullet+} + O_2^{\bullet-}$ [3]

$^3Rf^* + NSAIDs \longrightarrow Rf^{\bullet-} + NSAIDs^{\bullet+}$ [4] rate constant ^3k_q

$Rf^{\bullet-} + H^+ \rightleftharpoons RfH^{\bullet}$ [5]

$2\ RfH^{\bullet} \longrightarrow Rf + RfH_2$ [6]

$RfH_2 + O_2(^3\Sigma_g^-) \longrightarrow RfH_2^{\bullet+} + O_2^{\bullet-}$ [7] rate constant k_7

$RfH_2^{\bullet+} + O_2^{\bullet-} \longrightarrow Rf + H_2O_2$ [8]

$O_2^{\bullet-} + NSAIDs \longrightarrow P(9)$ [9] rate constant k_9

$H_2O_2 + NSAIDs \longrightarrow P(10)$ [10]

$^3Rf^* + O_2(^3\Sigma_g^-) \longrightarrow Rf + O_2(^1\Delta_g)$ [11] rate constant k_{ET}

$O_2(^1\Delta_g) \longrightarrow O_2(^3\Sigma_g^-)$ [12]

$O_2(^1\Delta_g) + NSAIDs \longrightarrow O_2(^3\Sigma_g^-) + NSAIDs$ [13] rate constant k_q

$O_2(^1\Delta_g) + NSAIDs \longrightarrow P(16)$ [14] rate constant k_r

Being $k_t = k_r + k_q$

Scheme 1. Possible pathways in a Riboflavin-photosensitized process in the presence of an electron donor transparent to the incident light (NSAIDs).

The irradiation of aerated aqueous solutions of the individual 0.5 mM NSAIDs + 0.04 mM Rf gave rise to oxygen uptake, as shown in Fig. 2. The rate of oxygen consumption ($V_{ox}Rf$) for IMTC is higher than the corresponding one for DFNS. The relative rate values are shown in Table 1). The photoirradiation of a similar solution of 0.04 mM of the isolated sensitizer does not consume any oxygen within irradiation times even superior to those employed for the above described mixtures.

The anaerobic photodegradation of Rf, upon stationary irradiation, proceeds in a predominant fashion through ^{3}Rf* (13). The rate of the process can be deduced from the absorbance decrease of the 445-nm band. Comparative irradiations of N_2-bubbled solutions *ca.* 0.02 mM of the sensitizer show that this rate decreases when the experiment is performed in the presence of 0.2 mM DFNS or IMTC. This result strongly suggests the occurrence of a quenching process of the excited state ^{3}Rf* by the NSAIDs (data not shown).

A coarse analysis of the preceding results indicates that: (a) DFNS and IMTC are photodegraded in a Rf-sensitized process; (b) ROS seems to participate in the photodegradation of the NSAIDs and (c) both NSAIDs apparently interact with ^{1}Rf* and/or ^{3}Rf*. On these grounds, we carried out a systematic study, by evaluating the nature and extent of kinetic and mechanistic aspects involved in the processes started with the visible-light irradiation of the aqueous mixtures Rf + NSAIDs.

Quenching of electronically excited singlet and triplet sates of Riboflavin

Rf shows a fluorescence emission band centered at 525 nm with a quantum yield $\Phi_f = 0.25$ (8), in air-equilibrated aqueous

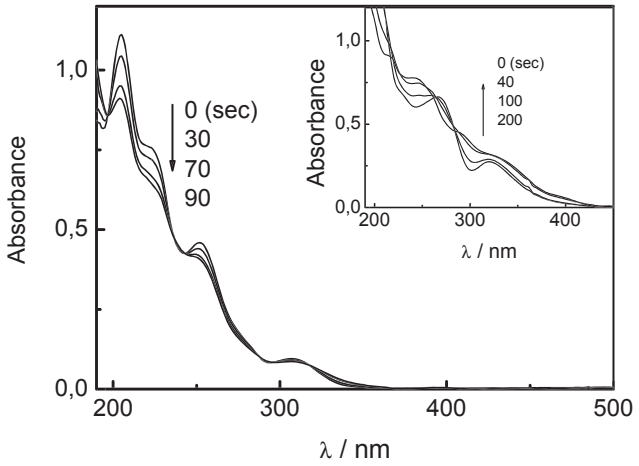


Figure 1. Changes in the UV–vis absorption spectrum of a pH 7 aqueous solution of 0.04 mM Rf plus 0.03 mM DFNS upon photoirradiation, taken *vs.* of 0.04 mM Rf in the same solvent. Inset: Changes in the UV–vis absorption spectrum of a pH 7 aqueous solution of 0.04 mM Rf plus 0.04 mM IMTC upon photoirradiation, taken *vs.* of 0.04 mM Rf in the same solvent. Cut-off 400 nm, under air-saturated conditions. Numbers on the spectra represent photoirradiation time.

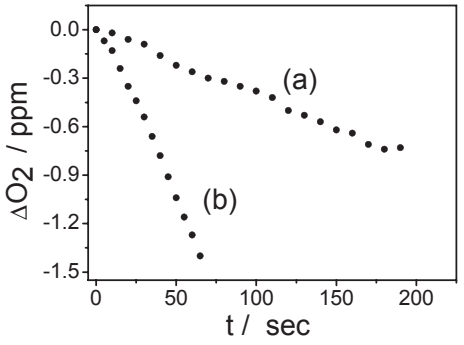


Figure 2. Oxygen consumption *vs.* photoirradiation time in pH 7 aerated aqueous solutions for the systems: (a) Rf (A_{446} = 0.46) plus DFNS (0.4 mM); (b) Rf (A_{446} = 0.46) plus IMTC (0.4 mM).

solution. In the presence of ≥10 mM IMTC or DFNS, a fluorescence quenching is detectable. The stationary emission intensity decreases while the spectral shape does not change. The interaction was quantified in MeOH solutions, by means of the respective Stern-Volmer constants ${}^1K_{SV} = {}^1k_q \times {}^1\tau_o$. The reported value of 5 ns for ${}^1\tau_o$ was employed in the calculations (29). The respective ${}^1k_q$ values [process (2)], both in the range of the diffusion-controlled ones, are shown in Table 1.

The interaction ^{3}Rf*-NSAIDs [process (4)] was studied through laser flash photolysis experiments in Argon-saturated solutions. The ^{3}Rf* lifetime (17 μs, as experimentally determined) decreases in the presence of the individual NSAIDs in the mM-concentration range. Again through a classical Stern-Volmer treatment the value for the bimolecular rate constant ${}^3k_q$ [process (4)] was determined (Fig. 3, inset and Table 1). The shape of the long-life absorption, obtained in the absence of NSAIDs corresponds to the well known ^{3}Rf* (30). The shape of the transient signal obtained in the presence of 1 mM NSAIDs (more than 97% ^{3}Rf* quenched) is in perfect agreement with that of the species $RfH^{\bullet}$ (neutral semiquinone radical), already reported (27). This radical is formed [process (5)] after

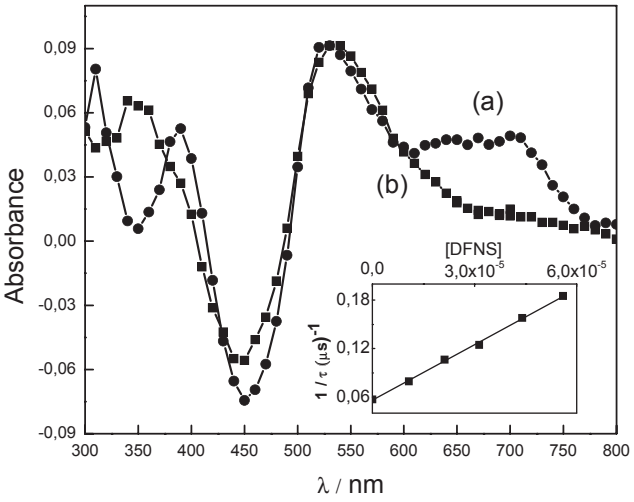


Figure 3. Transient absorption spectra of Rf (0.05 mM) in argon-saturated aqueous buffer pH 7 in the absence (a) 2 μs after the laser pulse and in the presence (b) of DFNS 4 μs after the laser pulse. Inset: Stern-Volmer plot for the quenching of ^{3}Rf* by DFNS. $1/\tau$ refer to the reciprocal of ^{3}Rf* lifetime in the in the presence of different DFNS concentrations.

protonation of $Rf^{\bullet -}$, generated in process (4). The transient spectrum corresponding to the mixture Rf + IMTC did not show any significant absorption in the range 350–800 nm due to IMTC radical cation species. In the system Rf + DFNS a new absorption was observed in the region of 350 that could be attributable to the generation of the $DFNS^{\bullet +}$ species (Fig. 3, main).

Oxygen consumption upon Rf-sensitization. Participation of ROS

The molecular moiety responsible for the Rf-sensitized photooxigenation of the NSAIDs, was identified through comparative experiments of oxygen uptake. For IMTC 2-methylindole and 1-acetylindole were employed as possible molecular models and salicylic acid was used in the case of DFNS, all in a concentration 0.5 mM and in the presence of 0.05 mM Rf. No oxygen consumption was detected for 1-acetylindole within typical irradiation times employed for IMTC, whereas the rate of oxygen uptake for 2-methylindole was practically twice to that of IMTC. The rates of oxygen uptake by salicylic acid and DFNS were closely the same.

The evaluation the potential participation of $O_2({}^1\Delta_g)$, $O_2^{\bullet -}$ and H_2O_2 in the degradation of the NSAIDs was done through oxygen consumption experiments upon Rf-photosensitization, employing specific ROS interceptors. The rates of oxygen consumption by the NSAIDs was decreased in different extents in the presence of individual 1 mM NaN_3, 1 μg/mL SOD and 1 μg/mL CAT. The rate for DFNS was reduced in 87% in the presence of NaN_3; 21% in the presence of SOD and 20% and in the presence of CAT. Similar qualitative results are shown for IMTC in the bars diagram of Fig. 4. These experiments, using specific ROS-interceptors have been formerly employed to discard/confirm the participation of $O_2({}^1\Delta_g)$, $O_2^{\bullet -}$ and H_2O_2 respectively in a given oxidative event (31–33). The salt NaN_3 deactivates physically the species $O_2({}^1\Delta_g)$ [reaction (13) with NaN_3 instead of NSAIDs] whereas SOD dismutates the species $O_2^{\bullet -}$ [reaction (15)] and CAT decomposes H_2O_2 [reaction (16)]).

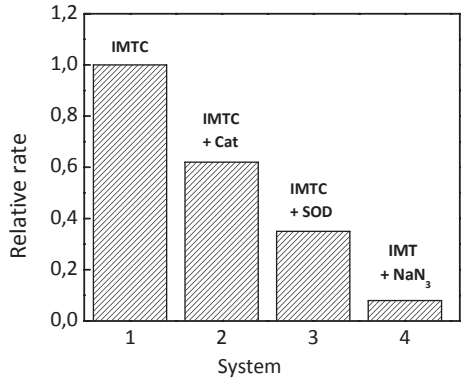


Figure 4. Bars diagram for the relative rates of oxygen uptake for 0.5 mM IMTC + Riboflavin (A_{445}) = 0.5 as a function of photoirradiation time (cut-off 400 nm), in pH 7 aqueous solution, in the absence (1) and in the presence of 1 μg/mL CAT (2); 1 μg/mL SOD (3) and 1 mM NaN_3 (4).

$$2O_2^{\bullet -} + 2H^+ + SOD \rightarrow O_2(^3\textstyle\sum_g^-) + H_2O_2 \qquad 15$$

$$2H_2O_2 + CAT \rightarrow 2H_2O + O_2(^3\textstyle\sum_g^-) \qquad 16$$

The observed decrease in the oxygen consumption rates in the presence of the selective ROS scavengers indicates the participation of $O_2(^1\Delta_g)$, $O_2^{\bullet -}$ and H_2O_2 in the overall NSAIDs oxidative event.

Rose Bengal-photosensitization

In the $O_2(^1\Delta_g)$-mediated experiments the xantenic dye RB was employed as a photosensitizer instead of vitamin B2. The change eliminates possible interferences due to radical-mediated reactions promoted by Rf. RB is one of the most commonly employed photosensitizer in $O_2(^1\Delta_g)$ reactions. It generates this oxidative species in a predominant fashion, with a quantum yield of *ca.* 0.7 in aqueous media (34,35).

The overall quenching constant values k_t for IMTC and DFNS were determined employing time-resolved detection of $O_2(^1\Delta_g)$ phosphorescence (Table 1). In Fig. 5 the case of IMTC is shown.

The k_r values for both NSAIDs (see Table 1) and for the model compounds 2-methylindole ($k_r = 2 \times 10^7\ M^{-1}s^{-1}$) and salicylic acid ($k_r = 5.2 \times 10^7\ M^{-1}s^{-1}$) were determined employing independent experiments, by means of oxygen uptake measurements (see experimental) in the presence of 0.05 mM RB as a dye-sensitizer (Fig. 5).

The combination of stationary and time resolved experiments unambiguously demonstrates the interaction of $O_2(^1\Delta_g)$ with IMTC and DFNS.

Regarding the $O_2(^1\Delta_g)$-mediated oxidation quantum efficiency, it can be evaluated by means of the expression $\Phi_r = k_r\ [Q]/(k_d + k_t\ [Q])$, being k_d is the rate constant of the $O_2(^1\Delta_g)$-deactivation by interaction of the oxidative species with solvent molecules [process (12)]. This expression can be hardly applied when the substrate concentration is uncertain, as in the cases of complex biological or natural environments. In these condition a useful and simpler approach is the evaluation of the k_r/k_t ratio (Table 1), *i.e.* the effective fraction of overall quenching that leads to chemical reaction.

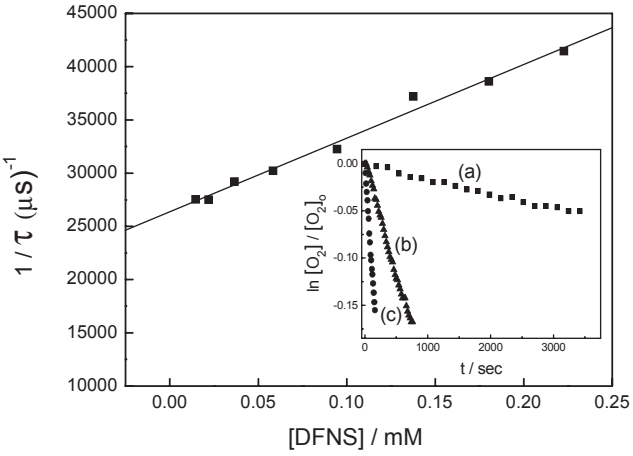


Figure 5. Stern-Volmer plot for the quenching of $O_2(^1\Delta_g)$ phosphorescence by DFNS in pD 7 D_2O solution. Inset: first order plots for oxygen uptake in buffer pH 7 aqueous solutions by 0.5 mM DFNS (a), 0.5 mM IMTC (b) and 0.5 mM FFA (c), all in the presence of RB (A_{548} = 0.5) as a sensitizer.

Photoprotective effect of NSAIDs towards peptide oxidation

As mentioned in the introductory section the damage of several biological targets, including proteins, produced by exacerbated oxidative stress, is considered an important mechanism operating in ND (3–5). In order to evaluate the eventual protective effect of the NSAIDs IMTC and DFNS on the ROS-mediated oxidation of proteinaceous materials, the Rf- and RB-sensitized photooxidation of the peptide Trp-Tyr was employed.

The oxidizable dipeptide was selected in this work as a model for protein-sensitive sites. Trp-Tyr has been also employed to evaluate the effect of the peptide bond on the ROS-mediated oxidation of the biopolymers (36). Tyr and Trp participate in practically all oxidative damage of proteins through photodynamic oxidation (37).

The rates of oxygen consumption by 0.5 mM Trp-Tyr in neutral water were determined in the presence and in the absence of 0.5 mM of the individual NSAIDs. These rates were taken as a measure of the global photooxidative progress by monitoring up to 10% conversion of the starting material. The RB-sensitized degradation of Trp-Tyr in aqueous solution operates through a $O_2(^1\Delta_g)$-mediated mechanism with a reported $k_r = 5.9 \times 10^7 M^{-1}s^{-1}$ (36). Besides, it is well known that Trp and Tyr are photooxidizable upon Rf-sensitization (38). Results, shown in Fig. 6A indicate that the photooxidation rate of the peptide + DFNS, as estimated by the rate of oxygen consumption upon RB-photosensitization, suffers a decrease of *ca.* 54%, as compared to the oxygen uptake rate for the isolated NSAID. In parallel, the rate value for the mixture Trp-Tyr + IMTC is close to the simple addition of the respective individual rates for the peptide and the NSAID. The rates of oxygen uptake behave in similar fashion when RB was replaced by Rf (Fig. 6B).

DISCUSSION

Experimental evidence strongly support the occurrence of effective quenching of ROS by IMTC and DFNS. Apparently the molecular moieties 2-methylindole and salicylic acid respectively are responsible for the oxidative process in the NSAIDs.

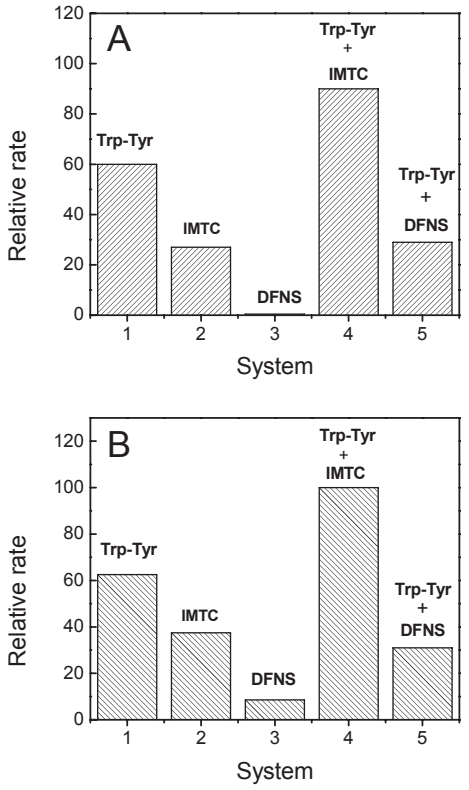


Figure 6. A. Bars diagrams for the relative rates of oxygen uptake upon RB (Abs_{560} = 0.4) photosensitization in pH 7 buffered aqueous solution by Trp-Tyr (1); IMTS (2); DFNS (3); Trp-Tyr + IMTC (4); Trp-Tyr + and DFNS (5), all in individual concentration 0.5 mM. **B.** The same as in Figure (A), upon 0.04 mM Rf photosensitization instead of RB sensitization.

Results will be discussed on the basis of the mechanistic pathways of Scheme 1.

Quenching of Rf electronically excited states and ROS photogeneration and quenching

The effects derived from the interaction of the NAIDs with electronically excited Rf under work conditions, *i.e.* NAIDs 0.5 mM –similar to those employed in the experiments of Rf-photosensitization– should be ascribed to an interaction with ^{3}Rf*. According to a simple Stern-Volmer calculation, the lifetime of ^{1}Rf* would be decreased in a proportion lower than 2%.

The electron transfer reaction represented in reaction (4) is thermodynamically feasible in the cases of DFNS and IMTC. According to the Rehm-Weller treatment (39), a value of $\Delta G^0 = -0.78$ eV for DFNS and -1.04 eV for IMTC can be deduced from Eq. (17):

$$\Delta_{ET}G_0 = E^0(\text{NSAID/NSAID}^{\bullet+}) - E^0(\text{Rf/Rf}^{-\bullet})] - \Delta E_{0,0} + C \quad (17)$$

where E^0(NSAID/NSAID$^{\bullet+}$) is the standard electrode potential of the donor, E^0(Rf/ Rf$^{-\bullet}$) is the standard electrode potential for the acceptor (-0.80 V), $\Delta E_{0,0}$ is the vibrational zero energy of the triplet excited partner (2.17 eV) and C (-0.06 V) is a coulombic energy term (40).

In the present case the values of 0.65 and 0.39 V were employed as the E^0(NSAID/NSAID$^{\bullet+}$) values of DFNS and IMTC respectively, after conversion to a standard hydrogen electrode (41). The original literature values were 0.9 V for DFNS and 0.64 V for IMTC, measured vs. a Ag/AgCl$^-$ electrode (42,43).

The operation of reaction (7), under aerobic conditions, depends on whether it is kinetically competitive with reaction (11). The k_{ET} value of reaction (11) in H_2O is *ca.* 1/9 of the diffusional value (44) i.*e.* 7×10^8 M^{-1}s^{-1}, while the values obtained for the rate constant ^3k_q in the case of both NSAIDS have a mean value of *ca.* 2×10^9 M^{-1}s^{-1} (Table 1). Hence, for the same concentrations of NSAIDs and dissolved $O_2(^3\Sigma_g^-)$, the rate of generation of the initial $O_2^{\bullet-}$ precursor *via* Rf$^{\bullet-}$ [reaction (7)], is *ca.* 3-fold higher than the corresponding one for the oxidative species $O_2(^1\Delta_g)$. This means that both rate values are relatively close and could compete. In other words, the photoirradiation of Rf in the presence of NSAIDs generates the precursory species for the further production of $O_2^{\bullet-}$ and H_2O_2 and also produces $O_2(^1\Delta_g)$ as indicated in the kinetic scheme [reactions (7), (8) and (11)].)

Quenching of $O_2(^1\Delta_g)$

Employing RB and Rf as photosensitizers, the rates of oxygen consumption by DFNS and IMTC exhibit a close parallelism (Figs. 6A and B). This fact, on the basis that RB is a $O_2(^1\Delta_g)$ generator, strongly suggests that this oxidative species plays a dominant role in the global oxygen uptake mechanism. The overall rate constants for the interaction of NSAIDs with $O_2(^1\Delta_g)$, in the range of 10^7 M^{-1}s^{-1} (Table 1), indicate that IMTC and DFNS can be considered good quenchers of this ROS.

The considerably low values for the quotient k_r/k_t exhibited by DFNS (Table 1) is, in the present case, a desirable quality. These values indicate the elimination of $O_2(^1\Delta_g)$ without significant loss of the scavenger. This action, in a biological medium, constitutes a protective action for, DNA, proteins and other cell matrix components, which due to their high local concentration and known reactivity, constitute primary targets for the attack of oxidative species. This photoprotective effect exerted by DFNS was evident as observed by the clear decrease in the rate of oxygen uptake of the photosensitized system Trp-Tyr – DFNS as compared to the rate in the absence of DFNS (Fig. 6A). In the case of IMTC, although the overall $O_2(^1\Delta_g)$ quenching rate constant k_t is higher than the corresponding one for DFNS, the actual protective efficiency is diminished by the fact that every quenching event produces the degradation of the scavenger ($k_r/k_t \sim 1$, Table 1). This fact is clearly shown by the high rate of oxygen uptake, reflecting the photooxidation of both, the peptide and IMTC in the RB-sensitized runs (Fig. 6A).

Evaluation of the quenching of ROS by IMTC and DFNS

A recent paper from Choi *et al.* clearly describe and discuss the beneficial effects of natural polyphenols on AD, operated through free radicals scavenging and increase of the antioxidative activity (5). In the same context, we demonstrate in this work that both DFNS and IMTC possess an acceptable quenching efficiency against oxidative species. Even though many pathways and mechanisms considered for mediating these effects are rather general than specific, in most cases the overall antioxidant

effectiveness is warranted due to the high doses of NSAIDs incorporated by the human body in cases of chronic pain or serious inflammatory deleterious that require daily intake of the drug for prolonged periods. By this reason the undesired effects of NSAIDs must be especially considered. IMTC constitutes an archetypical example: It is a good quencher of ROS, as demonstrated in this paper, but at the same time it has been reported as a promotor of ROS-mediated gastric damage (9). As said, DFNS appears as a less aggressive medicament as referred to secondary undesired effects, especially those related to the promotion of oxidative-mediated injuries (11).

Although known commercial NSAIDs possess demonstrated therapeutic effects, particularly as anti-inflammatory, analgesic and anti-fever agents, the antioxidative activity is not an immediate consequence of such a properties. This capacity must be tested for each drug, a work that is presently in progress in our laboratory for several NSAIDs.

Regarding the possible environmental degradation of pharmaceutical residues by sustainable methods, evaluation of the kinetic data offers a promissory panorama, especially in the case of IMTC. Its high photodegradation efficiency strongly suggests that photosensitization appear as a plausible way for environmental degradation of the NSAID in waste waters, induced by daylight. Rf, a naturally-occurring pigment present as traces in practically all kind of water courses, can play the photosensitizing role (31,45).

CONCLUSIONS

The ROS $O_2(^1\Delta_g)$, $O_2^{\bullet-}$ and H_2O_2, generated through Rf-photosensitization in the presence of DFNS and IMTC, are effectively intercepted by the NSAIDs. These results add another positive indicator to the already recognized properties of different NSAIDs.

Apparently the $O_2(^1\Delta_g)$-mediated process is the prevailing quenching mechanism in the Rf-photosensitized process. DFNS qualifies as an ideal scavenger of $O_2(^1\Delta_g)$ provided that eliminates physically the oxidative species, it means without a substantial self-degradation. To the contrary, IMTC, also a kinetically efficient interceptor of the ROS, is easily degradable and constitutes an excellent candidate for environmental degradation in waste waters, in the presence of natural day-light absorbing sensitizers.

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