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Dyes and Pigments 49 (2001) 75–82

DYES and PIGMENTS

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Characterization of photodynamic effects of meso-tetrakis-(4-methoxyphenyl) porphyrin: biological consequences in a human carcinoma cell line

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Received 15 June 2000; received in revised form 31 July 2000; accepted 10 January 2001

Abstract

The photodynamic behavior of 5,10,15,20-tetrakis(4-methoxyphenyl)porphyrin (TMP) has been investigated in solutions containing photo-oxidizable substrates and in the presence of Hep-2 human larynx-carcinoma cell line. It has been found that Hep-2 cells rapidly incorporate TMP until an intracellular concentration of ~0.018 μmol of TMP/10^6 cells is reached. The survival of the irradiated cells treated with TMP was dependent upon both light exposure level and intracellular sensitizer concentration. In addition, a linear dependence of the photoinactivation rate ($1/D_{50}$, where D_{50} represents the light exposure level required to inactivate 50% of the cell population) and TMP intracellular concentration was found, indicating that TMP localizes at unsaturated sites within the cells. TMP photosensitized singlet molecular oxygen ($^1\Delta_2$) with a quantum yield of 0.65. When the amino acid L-tryptophan was used to assess $^1\Delta_2$-mediated photooxidation in a homogeneous medium, singlet molecular oxygen ($^1\Delta_g$) mediation appeared to be mainly responsible for the cell inactivation. These in vitro results enhance our understanding of the phototoxicity of porphyrins in cellular media and the sensitivity of Hep-2 cells to photodamage.

Keywords: Porphyrin; Photosensitizer; Singlet oxygen; Photodynamic therapy

1. Introduction

Tetrapyrrolic compounds have attracted considerable attention as phototherapeutic agents [1,2] and recently several porphyrin derivatives have been synthesized for potential use in the treatment of tumors [3–7]. It is well known that photodynamic therapy (PDT) is based on the administration of a photosensitizer that concentrates in tumor cells and, upon subsequent irradiation with visible light in the presence of oxygen, selectively destroys the cancerous cells [8,9]. Although the photodynamic process that characterises the action of sensitizers on neoplastic tissues is still not well understood [10], two types of reactions generally occur after activation of the photosensitizer. One reaction involves the generation of free radicals

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0143-7208/01/$ - see front matter
PII: S0143-7208(01)00010-9

(type I photochemical reaction) and in the other, singlet molecular oxygen ($^1\Delta_g$) produced after exposure of the sensitizer to light (type II) is the species mainly responsible for cell inactivation [9].

Spectroscopic studies involving the triplet state were carried out with a series of 5,10,15,20-tetrakis(methoxyphenyl)porphyrins and the results showed that such porphyrins were effective photosensitizers and that they could be used as model compounds for investigating the theoretical and experimental aspects of PDT [11]. With this in mind, the aim of the present study was to characterize the photodynamic effects of 5,10,15,20-tetrakis(4-methoxyphenyl) porphyrin (TMP) in a biological system, using a human carcinoma cell line.

The spectroscopic properties, fluorescence, and singlet molecular oxygen quantum yield for TMP were evaluated in tetrahydrofuran solution. The role of singlet molecular oxygen in the photo-oxidation process was analyzed in a homogeneous medium containing the photo-oxidizable substrate L-tryptophan. The response of Hep-2 human larynx-carcinoma cell line to cytotoxicity photoinduced by TMP was evaluated in a biological medium.

2. Experimental

2.1. General

Flash column chromatography was conducted using silica gel 200–400 mesh from Aldrich and TLC analysis was conducted using Uniplate Silica gel GHLF 250 μm thin layer chromatography plates from Analtech. HPLC was conducted with a Varian 5000 LC instrument equipped with a Varian 2550 UV-visible variable-wavelength detector, and a Varian MicroPak SI-5 column. The solvent used was 3% 2-propanol/*n*-hexane and the flow rate was 0.5 ml/min. NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer using TMS as the internal standard. Mass spectra were recored on a Varian Matt 312 instrument operating in the EI mode at 70 eV. Light intensity measurements were determined using a Radiometer Laser Mate-Q (Coherent).

9,10-Dimethylanthracene (DMA) and L-tryptophan (Trp) from Aldrich were used without further purification. Sodium dodecyl sulphate (SDS) from Merck was used as received. D,L-α-Dipalmitoyl phosphatidylethanolamine from Sigma was used in liposome preparation. Solvents from Merck (GR grade) were distilled and stored over 4 Å molecular sieves. Tetrahydrofuran (THF) was distilled from lithium aluminium hydride under an argon atmosphere.

2.2. TMP

5,10,15,20-Tetrakis(4-methoxyphenyl) porphyrin (TMP) was synthesized by the condensation of 4-methoxybenzaldehyde and pyrrole [12]. Flash column chromatography using dichloromethane afforded pure TMP. TLC: $R_f = 0.45$. HPLC: $t_R =$ 7.45 min. ^{1}H NMR ($CDCl_3$) δ: 2.76 (brs, 2H, pyrrole N-H), 4.08 (s, 12H, Ar-OCH_3), 7.26 (d, 8H, $J = 8.5$ Hz, 5,10,15,20-Ar 3,5-H), 8.10 (d, 8H, $J = 8.5$ Hz, 10,15,20-Ar 2,6-H), 8.8–8.90 (s, 8H, pyrrole). EI–MS [m/z]: 734.3 (M^+), (734.29 calculated for $C_{48}H_{38}N_4O_4$).

2.3. Light exposures

The light source used was that of a Kodak slide projector equipped with a 150-W lamp. The light was filtered through a 3-cm water layer to absorb heat. A wavelength range of 350–800 nm was selected with the aid of optical filters [13], and the light intensity at the treatment site was 18 mW/cm^2.

2.4. Spectroscopic studies

Absorption spectra were recorded on a Shimadzu UV-2401PC spectrophotometer at 25.0 ± 0.5°C using 1-cm path length cells. Wavelength maxima (λ_{max}) were determined by taking the midpoint between the two positions of the spectrum where the absorbance of the band was equaled to 0.9 A_{max} [14]. Fluorescence measurements were performed on a Spex FluoroMax spectrofluorimeter. The fluorescence quantum yield (ϕ_F) for TMP was calculated by comparing the area below the corrected emission spectrum in tetrahydrofuran with that of tetraphenylporphyrin (TPP) as a fluorescence standard, exciting at $\lambda_{ex} = 550$ nm [15]. A value of $\phi_F = 0.10$ for TPP in tetrahydrofuran was calculated

by comparison with the fluorescence spectrum in toluene using $\phi_F = 0.11$ [16]. Singlet molecular oxygen sensitization was measured using a 1270 nm time-resolved phosphorescence detection system. A Q-switched Nd:YAG laser (Spectron) was used as the excitation source. The frequency-doubled output at 532 nm was employed to excite the porphyrins. TPP in tetrahydrofuran was used as the standard ($\Phi_\Delta = 0.62$) [17]. Measurements of sample and reference under similar conditions afforded Φ_Δ for TMP by direct comparison of the slopes in the linear region of the plots of the amplitude of phosphorescence extrapolated to zero versus the laser-pulse fluency [18]. The photooxidation of Trp was evaluated using DMA as actinometer under the same experimental conditions [2]. DMA was employed because the singlet oxygen quenching process for this compound occurs exclusively through a chemical reaction. A value of 5×10^7 s^{-1} M^{-1} was estimated for the bimolecular rate constant of DMA [20]. The kinetics of DMA and Trp photooxidation in tetrahydrofuran were studied by following the decrease in the absorbance at $\lambda_{max} = 378$ nm and the fluorescence intensity at $\lambda = 330$ nm, respectively. The Trp fluorescence was induced by 290 nm light [21]. Control experiments showed that under these experimental conditions the fluorescence intensity correlated linearly with Trp concentration.

2.5. Liposome preparation

The incorporation of TMP into the phospholipid bilayer of the D,L-α-dipalmitoyl phosphatidylethanolamine was achieved by a modification of the ethanol injection method [22]. Typically, a solution containing phospholipid (9.60 mM), cholesterol (1.91 mM), and TMP (0.27 mM) in ethanol–tetrahydrofuran (2 ml, 1:1 v/v) was injected into phosphate-buffered saline (PBS, 10 ml) at 80°C. The injection was performed at a speed of 50 μl/min with magnetic stirring [23].

2.6. Cell culture

The Hep-2 human larynx-carcinoma cell line (Asociación Banco Argentino de Células, ABAC, Instituto Nacinal de Enfermedades Virales Humanas, Pergamino, Argentina) was stored frozen in liquid nitrogen. The cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 50 μg gentamycin as an antibiotic. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere and the medium was changed daily. The cell line was routinely checked for the absence of mycoplasma contamination.

2.7. TMP uptake and quantification

TMP uptake by Hep-2 cells was determined by fluorescence spectroscopy. Cells were inoculated in 25 cm^2 culture flasks and incubated to obtain nearly confluent cell layers ($\sim1\times10^6$ cells). Then, an appropriate volume of the TMP incorporated into liposomes was added to the culture flask containing 5 ml of medium. Cells were treated with various TMP concentrations at several incubation times. Afterwards, the medium containing TMP was removed, and the cells were washed three times with phosphate-buffered solution (PBS) and suspended in 1 ml of PBS. The number of cells in each suspension was estimated via the trypan blue (TB) exclusion test [24] using a Neubauer chamber counter. Sodium dodecyl sulphate (SDS, 1 ml, 4%) was added to the cellular suspension. The suspension was incubated for 15 min in the absence of light at room temperature and centrifuged at 9000 rpm for 30 min. The TMP concentration in the supernatant was measured by spectrofluorimetry ($\lambda_{exc} = 420$ nm, $\lambda_{em} = 658$ nm, and the fluorescence values obtained from each sample were designated as the total number of cells contained in the suspension. The concentration of TMP in the supernatant was estimated by comparison with a calibration curve obtained with standard solutions of TMP in 2% SDS where the TMP concentration was $\sim$0.5–10 μM. Each experiment was repeated three times and four culture flasks were used for each incubation time.

2.8. Cell photosensitization

Cells were seeded, incubated and treated with TMP in 25 cm^2 culture flasks for the predetermined time in the absence of light, as described in Section

2.7. Afterwards, the medium containing TMP was discharged. Cells were washed three times with medium (DMEM) and kept in 5 ml of this medium. The cells were exposed to visible light for different time periods and after each light exposure, cell viability was assessed microscopically using TB [24]. The procedure was repeated without light exposures. Four culture flasks were used for each incubation time and sets of experiments were compared with a culture control without TMP.

3. Results and discussion

3.1. Spectroscopic studies

The absorption spectrum for TMP in dichloromethane shows the typical Soret band at 422 nm ($\varepsilon_{422} = 463{,}270$) and less intense Q-bands at 518 ($\varepsilon_{518} = 14{,}130$), 556 ($\varepsilon_{556} = 9750$), 593 ($\varepsilon_{518} = 4347$), and 650 nm ($\varepsilon_{518} = 4827$). The band at 422 nm was used to assess the effects of solvents and biological environments, and the results are summarized in Table 1. It can be seen that solvents cause a slight red shift in a medium of low-polarity. A sharp absorption band was obtained when there was no aggregation of the TMP [25–27].

Although TMP was not soluble in phosphate-buffered solution (PBS) water solution when fetal calf serum (FCS) was added, a Soret band appeared at 409 nm. Titration of 0.5 μM TMP with FCS in PBS (0–16% v/v) led to a narrowing and enhancement in Soret band intensity until ~10%. An increase in FCS produced monomeric TMP, which was preferentially bound to the hydrophobic lipoproteins in the serum [28]. The observation of selective TMP uptake by lipoproteins enhances the potential for selective photosensitizer absorption by the target tumor, since mainly the low-density lipoprotein (LDL) interacts with neoplastic cells [23,29]. In a suspension of Hep-2 cells, the Soret band absorption maximum was close to the maximum in liposomes, suggesting that TMP binding in the cells may involve a micro environment which is similar to that in liposomes [25,27].

The steady-state fluorescence emission spectra of TMP in different media are characterised by two maxima, as showed in Table 2. The same values for λ_{em} were obtained when exciting the sample at the Soret and Q-band maxima. These bands are characteristic of free base porphyrins and have been assigned to $Q(0–0)$ and $Q(0–1)$ transitions [30]. A Stokes shift of ~7 nm was found by comparing the absorption and emission spectra for TMP in dichloromethane. A very small Stokes shift is expected for tetraphenylporphyrin derivatives [30], indicating that the excited state (S_1) energy is nearly identical to the energy of the ground state (S_0). The data in Table 2 show that the λ_{em} values were not very different in organic solvents versus the biological environments. Moreover, no changes in the fluorescence profile were observed, showing that the same species (monomer) was responsible for fluorescence emission in each medium. The fluorescence quantum yield (ϕ_F) for TMP was calculated by the steady state comparative method using TPP as a reference [15,16]. A value of $\phi_F = 0.14$ was obtained in tetrahydrofuran.

Table 1
The Soret band absorption peak for TMP in solvent and biological media

Medium	λ_{max} (nm)	Medium	λ_{max} (nm)
n-Heptane	417.8	Liposomes	423.8
Tetrahydrofuran	419.9	FCS[a]	409.0
Dichloromethane	421.1	SDS[b]	428.8
N,*N*-dimethylformamide	421.9	Hep-2 cells[c]	424.0

[a] 10% (v/v) in PBS.
[b] 2% in PBS.
[c] Cell suspension in PBS.

Table 2
Fluorescence emission maxima for TMP in solvent and biological media

Medium	λ_{em} (nm)	
Dichloromethane	657	722
Tetrahydrofuran	658	722
Liposome	665	727
SDS[a]	660	725
FCS[b]	655	720
Hep-2 cells[c]	660	726

[a] 10% (v/v) in PBS.
[b] 2% in PBS.
[c] Cell suspension in PBS.

3.2. Singlet oxygen production and photo-oxidation in a homogeneous medium

In the presence of air, the emission signal of the singlet molecular oxygen formed after laser excitation of a TMP solution in tetrahydrofuran showed first-order exponential decay. The initial singlet molecular oxygen emission intensities were calculated from extrapolation to $t=0$. These initial signal intensities were plotted against laser intensities. The quantum yield of singlet molecular oxygen production (Φ_Δ) was calculated from the slopes of the plots for TMP and the corresponding slopes obtained for the reference compound (TPP). A value of $\Phi_\Delta=0.65$ was found for TMP in tetrahydrofuran, which is consistent with data reported for similar porphyrins [11].

A solution of TMP in tetrahydrofuran was irradiated with visible light in the presence of photo-oxidizable substrates, under aerobic conditions. The stability of TMP was monitored by absorption spectroscopy and no consumption of the photosensitizer was observed during 1 h of irradiation. When the system was irradiated in the presence of L-tryptophan (Trp) [21,31], a time-dependent decrease in Trp concentration was observed by following a decrease in its fluorescence emission at 350 nm. The decrease in Trp concentration followed first-order kinetics, as indicated in Fig. 1. A value of $(1.20\pm0.07)\times10^{-4}$ s^{-1} was obtained for the Trp experimental rate constant (k^{Trp}) and this rate constant did not change when Trp concentration was varied within the 2–20 μM range.

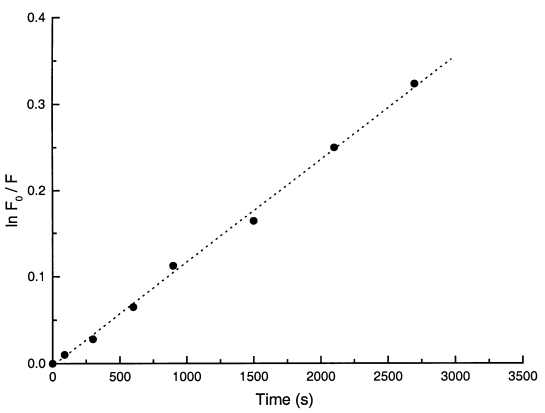

Fig. 1. Effects of irradiation time and TMP sensitizer on the decrease in Trp concentration, where [Trp] = 5 μM and [TMP] = 3 μM.

To evaluate the second-order process for Trp photo-oxidation (k_r^{Trp}), DMA was used as the actinometer, because of its ability to quench singlet molecular oxygen exclusively by chemical reaction [19]. When the excitation of TMP in tetrahydrofuran was performed in the presence of DMA, a plot similar to that in Fig. 1 was obtained, by following the decrease in TMP absorbance at 378 nm. A value of $k^{DMA}=(2.90\pm0.05)\times10^{-3}$ s^{-1} was obtained and from the ratio of the first-order slopes (k^{Trp}/k^{DMA}), the bimolecular rate constant for Trp ($k_r^{Trp}=2.1\times10^6$ s^{-1} M^{-1}) was estimated [19]. As expected, k_r^{Trp} was about two orders of magnitude lower in THF than in water [20].

Trp is sensitive to singlet molecular oxygen damage and this behavior has been used to analyze TMP activity towards biological substrates in different media [21,31–33]. The independence of the rate constant for Trp (k_{obs}^{Trp}) photooxidation over a wide range of Trp concentrations shows that TMP appears to exhibit photosensitization via the intermediacy of singlet molecular oxygen. Alternative reaction pathways involving a direct interaction between the excited photosensitizer and the substrate are concentration dependent [21]

3.3. Hep-2 cells studies

TMP was added to a liposomal solution of L,D-α-dipalmitoylphosphatidyl-ethanolamine containing cholesterol. The use of phospholipid liposomes as vehicles to transport hydrophobic photo sensitizer would constitute an advantage for its application in PDT, since liposomes optimize the release of lipophilic sensitizer to LDL [23]. Hep-2 cells were treated with TMP (1, 5 and 10 μM) and intracellular TMP incorporation into Hep-2 cells as a function of incubation time was assessed. The results are summarized in Fig. 2. It is clear that cellular uptake increased with increasing TMP concentration. In all cases, TMP underwent rapid incorporation at low incubation times (<5 h) and tended to saturate the substrate at incubation times $\geqslant$ 24 h. It is interesting to note that a TMP uptake value of $\sim$0.018 μmol/10^6 cells was calculated after 24 h incubation, by comparison with a

calibration curve for TMP (~0.5–10 μM) in 2% SDS (see Section 2.7). It was reported previously that the cellular uptake of porphyrins increases with lipophilicity and, consequently, the high uptake value could be due to an increase in membrane permeability towards lipophilic compounds [34].

TMP-induced cell toxicity was first analyzed in the absence of light at different photosensitizer concentrations and incubation times. When the Hep-2 cells were treated with 1 μM TMP for 45 min and then incubated without light exposure at 37°C, no toxicity was detected after 0, 4, 8, 12 and 24 h. The same results were obtained by treating the cells with 1 μM TMP for 15 and 24 h. In addition, no toxicity was found when cell cultures not treated with TMP were irradiated under the same experimental conditions used to obtain the survival curves. Therefore, the cell mortality observed after visible light exposures is due to the sensitization effects of TMP produced by irradiation.

The survival data obtained after the treatment of cells at different incubation times with 1 μM TMP and visible light are shown in Fig. 3. As expected, cell inactivation depends on the light exposure time and the time that the cells were incubated with TMP before irradiation. The cell incubation time was characterised by considering the intracellular fraction (f_i) of TMP incorporated into the cells, taking into account the point at which the cells were saturated with TMP (0.018 μmol/10^6 cells). Survival curves were also obtained using cells treated with 5 and 10 μM of TMP for different incubation periods (Fig. 4). In all cases, the cell inactivation curves followed approximately exponential decay (Figs. 3 and 4) and the light exposure level required to inactivate 50% of cell population (D_{50}) was calculated for different f_i of TMP, considering light intensity at the treatment site. The results are shown in Table 3. It is evident that an

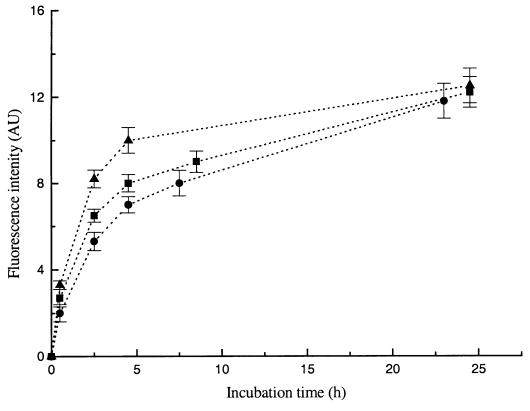

Fig. 2. Uptake of TMP into Hep-2 cells as a function of incubation time for different TMP concentrations: (●) 1 μM; (■) 5 μM; (▲) 10 μM .

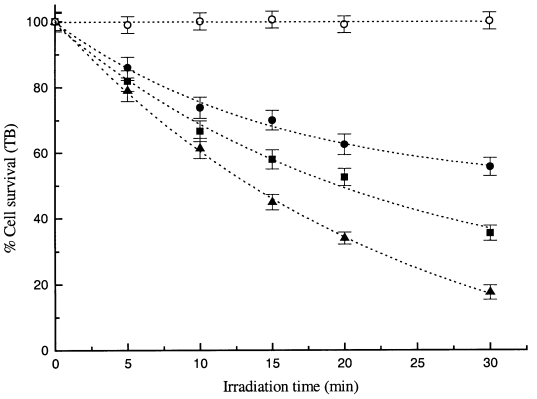

Fig. 3. Inactivation of the cells incubated with 1 μM TMP for various times and then exposed to visible light. Incubation times: (●) 45 min; (■) 9 h; (▲) 24 h; (○) the corresponding control in the absence of light. Values represent mean ± standard deviation of three experiments.

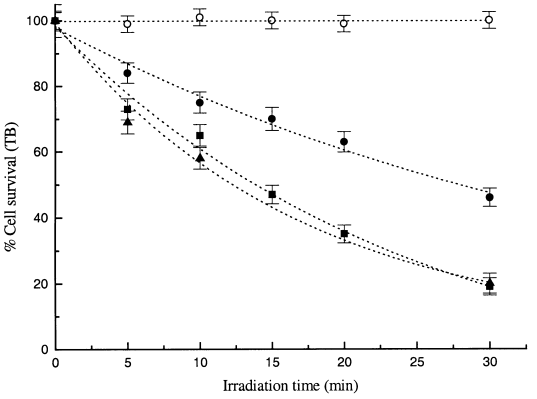

Fig. 4. Inactivation of the cells incubated with different TMP concentrations and then exposed to visible light. [TMP] and incubation times: (●) 5 μM, 45 min; (■) 5 μM, 23 h and (▲) 10 μM, 24 h; (○) the corresponding control in the absence of light. Values represent mean ± standard deviation of three experiments.

Table 3
Effects of TMP concentration and incubation time on cell inactivation

[TMP] (μM)	Incubation time	f_i[a]	$\tau_{1/2}$ (min)[b]	D_{50} (J/cm^2)[c]
1	45 min	0.22	44.2±0.7	47.7±0.7
1	9 h	0.70	19.3±0.6	20.8±0.6
1	24 h	1.00	13.2±0.5	14.2±0.5
5	45 min	0.28	35.5±0.6	38.3±0.6
5	23 h	1.00	13.4±0.5	14.5±0.5
10	24 h	1.00	12.8±0.5	13.8±0.5

[a] Fraction of molecules incorporated into the cells with respect to the value obtained at 24 h.
[b] Irradiation time at which 50% of the cells were inactivated under the conditions shown.
[c] Light exposure level required to inactivate 50% of the cell population.

increase in TMP intracellular concentration produced a decrease in D_{50} values, until the TMP intracellular saturation value was reached. At this point ($f_i = 1$), a D_{50} of ~14 J/cm^2 was calculated from cells saturated with TMP. The same D_{50} values were obtained from independent experiments, by saturating the Hep-2 cells at different TMP concentrations (1–10 μM), Table 3. When the relative photosensitizing effectiveness of TMP was expressed as a function of photoinactivation rate ($1/D_{50}$), a linear relationship between $1/D_{50}$ and f_i was found (Fig. 5). These results suggest that TMP localized at nonsaturated sites within cells, at the concentration range employed [35].

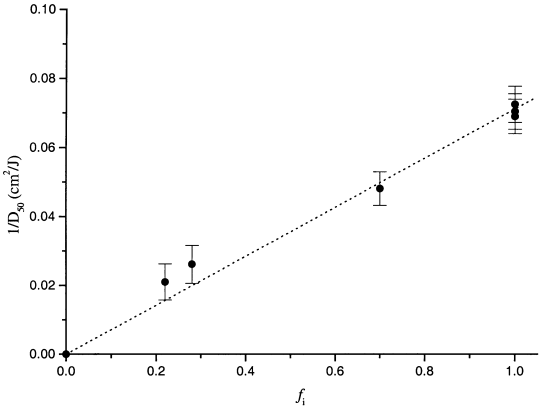

Fig. 5. Photoinactivation rates ($1/D_{50}$) for Hep-2 cells treated with TMP as a function of TMP intracellular fraction (f_i). Values represent mean ± standard deviation of three or more experiments.

It can be concluded that TMP efficiently photosensitizes singlet molecular oxygen and is the principal mediator in the photooxidation of L-tryptophan. It is also clear that Hep-2 cells incorporate TMP rapidly until the intracellular concentration of ~0.018 μmol/10^6 cells is reached. Upon irradiation with visible light, Hep-2 cells treated with TMP undergo considerable damage. A high intracellular TMP concentration (~40 J/cm^2) produced 90% cell mortality. A similar response has been reported for other human cells using *meso*-tetrahydroxy phenylchlorin as the photosensitizer [36]. It has also been shown that TMP appears to function as a photosensitizer through the intermediacy of singlet molecular oxygen, based on the photooxidation of biological substrates.

Acknowledgements

The authors are grateful to Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) of Argentina, Agencia Nacional de Promoción Científica y Técnica and SECYT of Universidad Nacional de Río Cuarto for financial support. V.R. and E.N.D. hold a position of Researchers at CONICET. M.G.A. thanks CONICET for a research fellowship.

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