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Figure 1:

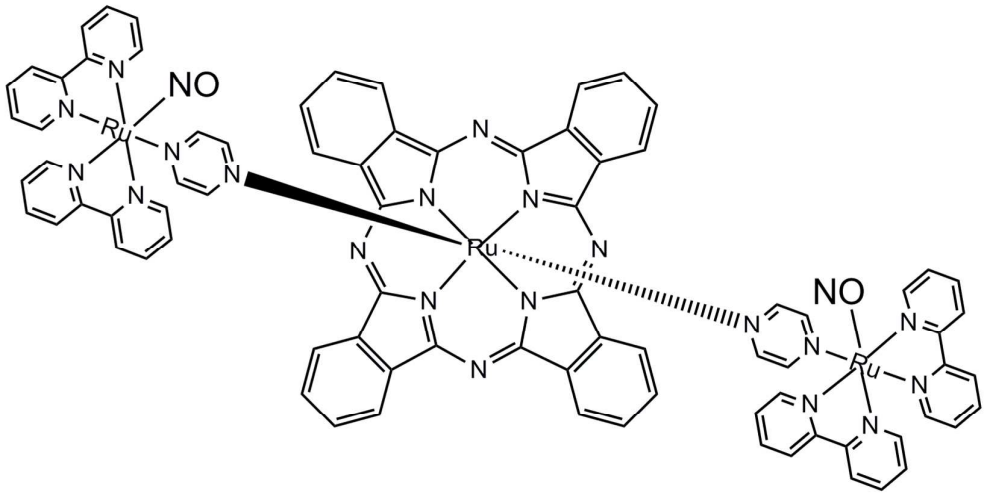


Figure 1: Molecular structure of $[Ru(phthalocyanine)(pz)_2\{Ru(bpy)_2NO\}_2]^{6+}$.

Figure 2:

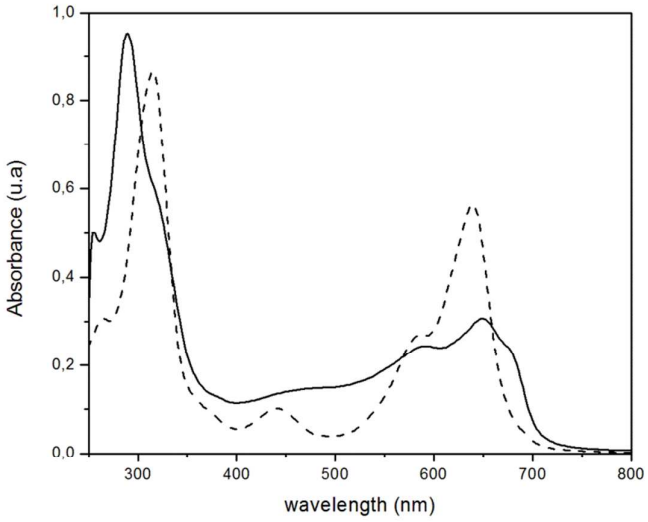


Figure 2. Absorption spectra of **(I)** ($4.0x10^{-5}$ M) in DMSO (solid line) and $[Ru(pc)(pz)_2]$ (1.84×10^{-5} M) in chloroform (dashed line).

Figure 3:

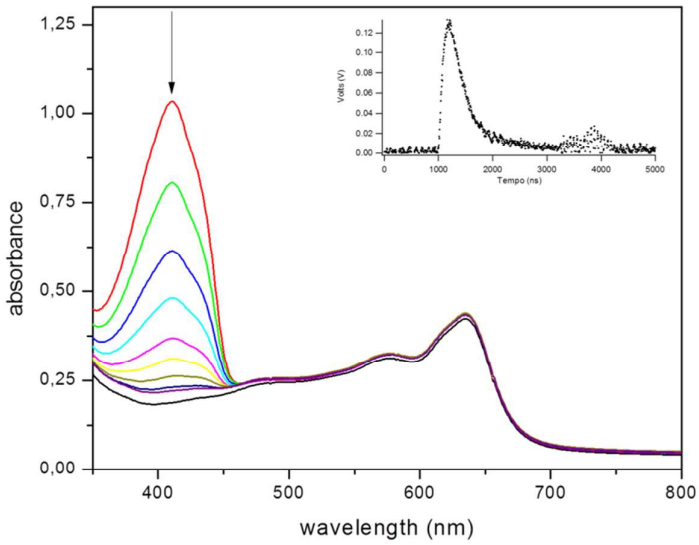


Figure 3. Absorption spectra of **(I)** in the presence of DPBF after different times of irradiation (30s) with a 660 nm laser. **Inset:** Exponential decay of singlet oxygen emission after excitation at 355nm with Nd-YAG laser.

Figure 4:

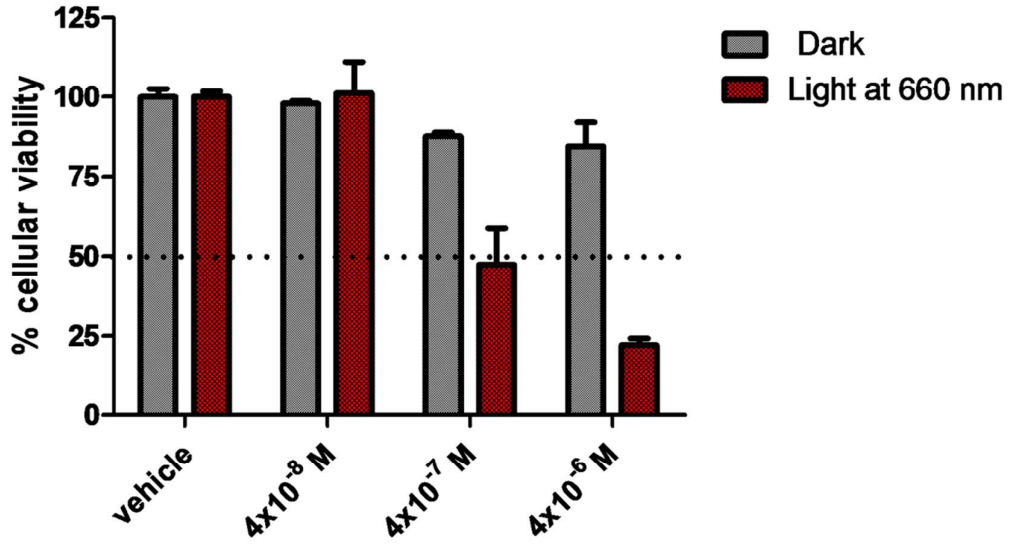


Figure 4: Cell viability *vs*. concentration for **(I)** against the B16F10 cell line. The cells were treated with **(I)** under the indicated concentration. The number of viable cells was assessed by MTT 24 hours after light exposure. Results are presented as a percentage compared with the control and the values are the mean ± SEM of three independent experiments carried out in duplicate.

Figure 5

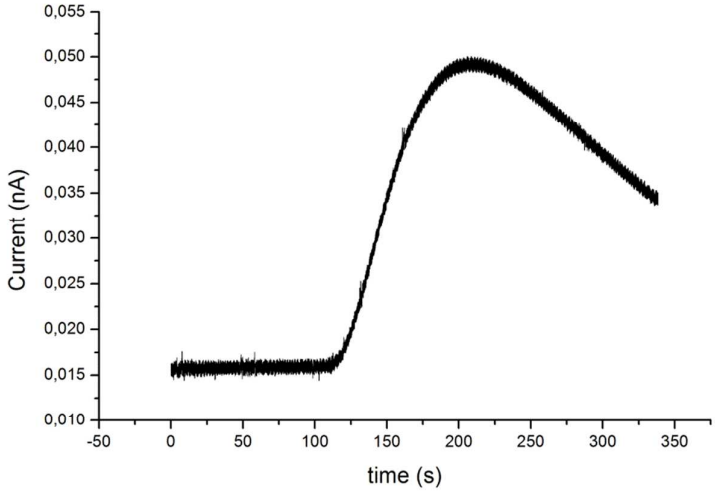


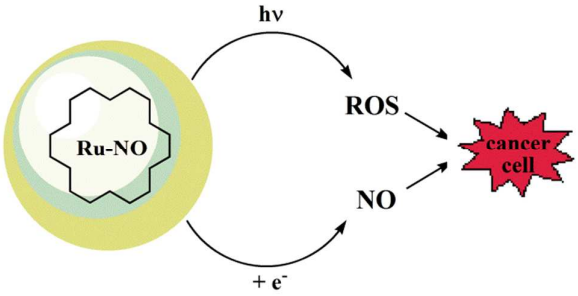
Figure 5: Time course of NO release from **(I)** (2.0 x 10^{-6} M) in aqueous/DMSO solution under reduction with ascorbic acid

Table 1. Concentration of nitric oxide released ($mol.L^{-1}$) from **(I)** upon photolysis. Correlations between pH and light irradiation wavelength.

pH / λ_{irr}	377 nm	447 nm	660 nm
pH 3.0	2.08×10^{-5}	1.43×10^{-5}	1.02×10^{-5}
pH 7.4	8.14×10^{-6}	3.63×10^{-7}	$< 10^{-7}$

Graphical Abstract text

Nitric oxide and reactive oxygen species originated from $[Ru(pc)(pz)_2\{Ru(bpy)_2NO\}_2](PF_6)_6$ (**I**) have been postulated to act synergistically against cancer cell. we have evaluated this in B16F10 cell line. Nitric oxide is released from **(I)** by reduction process followed singlet oxygen formation by light irradiation at 660 nm.



Production of reactive oxygen and nitrogen species by light irradiation of a nitrosyl phthalocyanine ruthenium complex as a strategy for cancer treatment

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Abstract

Production of reactive oxygen species have been used in clinical therapy for cancer treatment in a technique known as Photodynamic Therapy (PDT. The success of this therapy depends on oxygen concentration since hypoxia limits the formation of reactive oxygen species with consequent clinical failure of PDT. Herein, a possible synergistic effect between singlet oxygen and nitric oxide (NO) is examined since this scenario may display increased tumoricidal activity. To this end, the trinuclear species $[Ru(pc)(pz)_2\{Ru(bpy)_2(NO)\}_2](PF_6)_6$ (**I**) (pc = phthalocyanine; pz = pyrazine; bpy = bipyridine) was synthesized to be a combined NO and singlet oxygen photogenerator.

Photobiological assays using **(I)** at $4x10^{-6}$ M in the B16F10 cell line results in cell viability decreased to 21.78 $\pm$ 0.29 % of normal under light irradiation at 660 nm. However, in the dark and at the same concentration of compound **(I)** viability was 91.82 $\pm$ 0.37 % of normal. The potential application of a system like **(I)** in clinical therapy against cancer may be an upgrade to normal photodynamic therapy.

Introduction

The importance of oxygen to living organisms and its essentiality to life is clear. Significantly, variation in the concentration of this element is generally associated with the etiology of some diseases [1, 2, 3, 4]. For example, tumors have been described as often being relatively low in oxygen concentration. The main reason for this hypoxia lies in the imbalance between oxygen supply and consumption. Insufficient supply of this vital element is normally attributed to abnormal structure and impaired function of the microvessels responsible for blood vessel nutrition [5, 6, 7]. The occurrence of hypoxia could negatively affect oxygen-based therapies against cancer such as is the case of Photodynamic Therapy (PDT), which requires the combination of light irradiation and the presence of dioxygen [8, 9, 10, 11]. Indeed, when it comes to cancer treatment, the success of this therapy depends on oxygen concentration, and a hypoxic event usually results in diminished formation of reactive oxygen species and consequent clinical failure of PDT [12, 13]. The use of other classes of reactive species has been proposed as an attempt to improve PDT, but the outcome remains uncertain. Nitric oxide (NO) is one such proposed radical molecule, since it displays tumoricidal character depending on the NO concentration [14, 15, 16, 17] and also could give characteristic of vasodilation improving blood flow with consequent increase of oxygen

concentration. The key to the successful application of NO in anticancer therapy is to achieve control of the NO concentration while ensuring that the target tissue is reached. Several NO donor agents have been studied for this purpose [18, 19, 20, 21, 22], including transition metal nitrosyl species. Owing to the specific features of the nitrosyl ligand, ruthenium complexes are one of the most extensively investigated systems [23, 24, 25, 26]. Among them $[RuL_5NO]^{n+}$ complexes, where L is a nitrogen-type ligand have been shown to be remarkable as NO deliver agents by pharmacological viewpoint [27, 28, 29, 30, 31, 32]. The vasorelaxation properties of these complexes [33, 34, 35], and some cytotoxicity properties also imply that these species can be potentially useful in clinical therapy [36, 37, 38, 39]. Therefore, the aim of this work was to evaluate the synergistic effect elicited by nitric oxide and singlet oxygen produced by external stimulation of the same molecule as a function of time. To this end, the trinuclear species $[Ru(pc)(pz)_2\{Ru(bpy)_2(NO)\}_2](PF_6)_6$ **(I)** (Fig 1) was synthesized and photobiological properties are described in B16F10 cells.

INSERT FIGURE 1

Experimental Section

Apparatus

The ultraviolet–visible (UV–Vis) spectra were recorded on a on an Agilent 8753 Diode Array UV/Vis Spectrophotometer. Infrared spectra were obtained with a Prestige-21 Schimazur 02190 FTIR spectrophotometer, at a resolution of 4 cm^{-1}, using solid samples pressed in KBr pellets. The pH measurements were accomplished by means of a DM-20 pH meter from Digimed. Singlet oxygen was indirectly measured using 1,3-

diphenylisobenzofuran (DPBF) as a probe as previously described [36,40]. DPBF reacts with $^1O_2$ irreversibly, leading to a decrease in the band at 415 nm. The solutions containing DPBF in ethanol (8.0×10^{-3} M) and the sensitizers (OD = 0.2 at the irradiation wavelength) in PBS were prepared on the day of the analysis and stored in the dark. A volume of 40 μL of the DPBF solution was added to 2.0 mL of a solution of the phthalocyanine ruthenium complex, which was then irradiated at 660 nm with a diode laser (Colibri Quantum Tec). The irradiation period, controlled by a shutter, was selected in such a way that around 5% of the DPBF would be bleached in the first cycle (5–30 s depending on the employed sensitizer). This procedure was repeated at least 15 times. The DPBF absorption decay at 415 nm was recorded after each cycle. The measured absorbance was corrected by the absorbance of the sensitizer at the respective detection wavelength. The experiment was carried out at least three times for each sensitizer. The DPBF quantum yields for each irradiation cycle were calculated as previously described [40]. The luminescence measurement of singlet oxygen were determined in an apparatus consisting of a Nd-YAG laser (Continuum, Santa Clara, CA) with excitation (8 nm half life) provided by the third harmonic at 355 nm. The luminescent decay kinetics was detected by employing a germanium detector operating at liquid nitrogen temperature. A silicon filter was used to avoid any fluorescent signal interfering with the singlet oxygen measurements. NO release was measured with an ISO–NOP NO meter from Word Precision Instruments.

Synthesis of ruthenium complexes

[Ru(pc)(pz)$_2$]. The synthesis of [Ru(pc)] (pc = phthalocyanine) was prepared following published procedure [41]. [Ru(pc)(pz)$_2$] (pz = pyrazine) complex was synthesized with slight modifications of the well established procedure [40]. Briefly, 0.600 g (1.60×10^{-3} mol) of [Ru(pc)] complex was melted with pyrazine 0,016 g (2.00×10^{-4} mol) at 90.0 ◦C. The reaction flask was kept for 48 h and [Ru(pc)(pz)$_2$] was obtained by addition of methanol. The solid was separated by vacuum filtration; washed three times with concentrated acetic acid, water, ethanol, and diethyl ether; and finally dried under vacuum. Yield: 40.0%. The synthesized [Ru(pc)(pz)$_2$] complex was characterized with respect to UV-visible spectrum and compared to published data [40].

[{Ru(NO)(bpy$_2$)}$_2$RuPc(pz)$_2$](PF$_6$)$_6$. The *cis*-[Ru(NO$_2$)(bpy)$_2$(NO)](PF$_6$)$_2$ complex was prepared according to method previously described in the literature [42]. The *cis*-[Ru(NO$_2$)(bpy)$_2$(NO)](PF$_6$)$_2$ salt (0.100 g, 1.92 10^{-4} mol) was dissolved in acetone (20 mL). An equimolar amount of NaN_3 (0.013 g) was then dissolved in methanol (5 mL) and added dropwise to the above solution. After 20 min, 1.00×10^{-4} of [Ru(pc)(pz)$_2$] previously dissolved in acetone (5 mL) was added to the stirred solution and the reaction was allowed to proceed for 17 h. After that 0.5 mL of concentrated HPF_6 was added to the stirred solution to produce a blue-green precipitate. The solution was filtered off, and the solid washed three times with concentrated acetic acid, water, ethanol, and diethyl ether; and finally dried under vacuum. Yield 45.0 %. (Anal. Calc. for $Ru_3C_{80}H_{58}N_{22}O_2P_6F_{36}$: C, 37.94; H, 2.31; N, 12.17. Found: C, 41.22; H, 2.40; N, 12.14).

B16F10 cell culture

B16F10 cells were cultured in 75 cm^2 flasks with RPMI medium (Gibco) containing 10% defined supplement fetal bovine fetal serum plus 100 IU/mL penicillin G, 100 $mg.mL^{-1}$ streptomycin, and 1 $\mu g.mL^{-1}$ amphotericin. Cells were seeded until 75–90 % confluence in 6, 12, and 96-well plates, depending on the applied assay, and cultured in a humidified incubator at 37.0 °C with 5.0 % CO_2 for 2 h.

Cell viability assay

Cells proliferation was assessed using the classical [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) colorimetric assay the MTT colorimetric assay, as described previously [43]. To this end, 106 cells were incubated for 3 h in 96-well cell culture plates containing the trinuclear specie **(I)** dissolved in DMSO solution and cells were incubated for 3 h at 37.0 °C in culture medium containing 10 μM MTT diluted in phosphate-buffered saline (PBS). The blue MTT formazan precipitate was then dissolved in 200 μL DMSO, and the absorbance was measured at 570 nm on ELISA reader (Power Wave X equipped with KC4 Kineticalc for Windows. Cell viability is expressed as the percentage of these values in treated cells compared with the non-treated (control) cells. Data are presented as the mean ± standard error of triplicate cultures.

Phototoxicity assays

B16F10 cells were incubated for 4 h under the same conditions as those used during the cellular viability assay. After incubation with **(I)** in solution ($4.0 - 0.04 \times 10^{-6}$ M) the cells were irradiated at 660 nm (5 $J.cm^{-2}$) using a diode laser (Quantum Tech, São Carlos, Brazil). Immediately after light exposure, the cells were rinsed with PBS and incubated again with RPMI for 4 h at 37.0 °C, for accomplishment of the MTT cell

viability assay. Control cells were incubated without **(I)** and irradiated under the same conditions.

Student's t-test and GraphPad Prism software were employed for statistical analysis of the cell assays.

Results and Discussion

The synthesis of the trinuclear specie **(I)**, as far as we know is the first example of two bonded nitrosyl ruthenium complexes in photosensitized phthalocyanine moiety. The complex **(I)** is stable in aqueous solution in the dark, but upon irradiation with visible light, nitric oxide were selectively photodissociated from the nitrosyl ruthenium complex, as inferred by UV–vis spectroscopy and NO-sensor measurement.

The FTIR spectrum of **(I)** displayed an usual NO frequency at 1940 cm^{-1}, characteristic of nitrosyl stretching [41] (Figure 1: Supplementary Material). The UV-visible spectrum in dimethylsulfoxide (DMSO) (Fig 2) consisted of three distinct regions, for which the assignments were made on the basis of similar mononuclear species [39, 41]. The bands at 634 nm (log ε = 4.52 $M^{-1}cm^{-1}$) and 576 nm (log ε = 4.39 $M^{-1}cm^{-1}$) were assigned as being due to the Q-band described for phthalocyanines. The shoulder at 318 nm (log ε = 4.89 $M^{-1}cm^{-1}$) was attributed to metal ligand charge transfer (MLCT) from ruthenium(II) to the bipyridine $d\pi(Ru^{II}) \rightarrow \pi^{*}$(bpy) and nitrosyl $d\pi(Ru^{II}) \rightarrow \pi^{*}$(NO) ligands, respectively. A band ascribed to the intraligand transition was detected at 290 nm (log ε = 5.17 $M^{-1}cm^{-1}$). Compared with $[Ru(pc)(pz)_2]$ [40], the Q-band was shifted to low energy by 20 nm, probably as a result of the structural change undergone by the phthalocyanine ring in the trinuclear complex (Fig 2). The luminescence spectrum of **(I)** in acetonitrile upon excitation at the Q-band presented an emission band at 690 nm.

INSERT FIGURE 2

As for the NO measurement in complex **(I)**, there was nitric oxide (NO) release upon light irradiation at 377 nm (Table 1). The photochemical pathway for the photolysis at high energy was attributed to the electron transfer mechanism centered on the MLCT band originated from the $d\pi(Ru^{II})$-$\pi^*(NO^+)$ transition, by analogy with the similar photochemical behavior observed for ruthenium bipyridine species [42], as depicted in the equation below:

$$[Ru^{II}(bpy)_2NO^+pzRu(Pc)pz\ Ru^{II}(bpy)_2NO^+]^{6+} \xrightarrow[H_2O]{h\nu = 377\ nm} [Ru^{III}(bpy)_2NO^0pzRu(Pc)pz\ Ru^{III}(bpy)_2NO^0]^{6+}$$

$$\downarrow$$

$$[Ru^{III}(bpy)_2(H_2O)pzRu(Pc)pzRu^{III}(bpy)_2(H_2O)]^{6+} + 2NO$$

The produced NO amount decreased with increasing pH (Table 1), which was attributed to the interconversion nitrosyl-nitro species due to the attack of the hydroxide ion, which should diminish the nitrosyl character of the nitrogen oxide ligand. A very low NO quantity was also observed for light irradiation at 660 nm, probably because the photochemical mechanism for this process involves a photoinduced electron transfer from the phthalocyanine site to the nitrosyl moiety.

INSERT TABLE 1

Direct singlet oxygen production was measured in ethanolic solution and determined by time-resolved phosphorescence studies at 1270 nm by using an Nd-YAG laser λ_{ex} operating at 355 nm (inset Fig 3) [44]. The assays were also conducted by photooxidation using 1,3-diphenylisobenzofuran (DPBF) as substrate. The singlet

oxygen measurement was performed via an indirect method monitoring DPBF consumption by UV-Vis spectroscopy [45]. The sample with **(I)** and DPBF was irradiated with a laser (Colibri, Quantum Tech^{R}), λ_{irr} 660 nm. The decrease in DPBF concentration was followed at 415 nm (Fig 3). The measured singlet oxygen quantum yield was 0.35 when the sample was excited 377 nm, which is in the range of some phthalocyanine complexes [46, 47], and 0.80 when the excitation was carried out at 660 nm. There was no reduction in DBPF absorbance when the sample was irradiated at 377 nm.

INSERT FIGURE 3

Photocytotoxicity assays using **(I)** were accomplished against B16F10 cells. For this purpose, a stock solution of the compound was stored in DMSO. The cells were seeded in a 96-well plate for 4 hours, prior to the treatment. Then, the cellular medium was removed, and different concentrations of **(I)** were added. Cells were exposed to the compounds for four hours, which was followed by exposure to light irradiation dose (λ_{irr} 660 nm; D = 5 J.cm^{-2}). The response to the light treatment was measured 24 hours after irradiation. The same experimental conditions were employed in a control assay carried out without light stimulus. Cell viability was measured by means of the MTT assay (Fig 4) [43].

INSERT FIGURE 4

These preliminary results showed that in the presence of **(I)** at 4.0x10^{-6} M, the cell viability decreased to 21.78 $\pm$ 0.29 % under light irradiation compared to controls (no compound) while in the dark and at the same concentration of compound **(I)** there was little cytotoxicity (91.82 $\pm$ 0.97 % viability compared to control). A similar experiment performed with [Ru(pc)] [36] demonstrated that the cell viability was

reduced to 80.00 % ± 0.69 of control (no compound) under 5 J.cm^{-2} light irradiation at 660 nm, while no effect was observed in the dark.

The reduction of complex **(I)** with concomitant generation of NO can be easily assessed in biological medium, as verified by carrying out reduction with ascorbic acid (Fig 5). Because it is known that NO donors can be used as cancer chemotherapeutic agents [23, 38, 43, 48, 49] depending on their concentration, we pursued the hypothesis that the NO released by photolysis of **(I)** could act as an anticancer agent, which could explain the cytotoxicity observed in Fig 4.

INSERT FIGURE 5

Conclusions

The effect of **(I)** on cell viability seems to be due to a synergism between NO and singlet oxygen. The potential application of a system like **(I)** in clinical therapy against cancer should be considered an upgrade to normal photodynamic therapy. The use of low intensity laser and the consequent marked cell viability decrease in the B16F10 cell line described in this work is novelty and has never been observed by photobiological assays using a photosensitizer. A possible biological mechanism related to this cytotoxicity could be due inhibition of mitochondrial respiration by nitric oxide increasing the bioavailability of oxygen to produce ROS [50]. The details of NO and singlet oxygen generation and the relationship to the biological applications of this complex are currently under investigation.

Acknowledgments

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