Photodynamic activity of thiophene-derived lysosome-specific dyes

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ABSTRACT

The photodynamic activity occurring through the lysosomes photo-damage is effective in terms of triggered synergic effects which can avoid chemo-resistance pathways. The potential photodynamic activity of two fluorescent lysosomes-specific probes was studied providing their interaction with human serum albumin, demonstrating their *in vitro* generation of singlet oxygen and investigating the resulted photo-toxic effect in human cancer cells.

Highlights

- TC dyes interact with HSA as indicated by UV-Vis and Fluorescence measurements
- TC1 and TC2 result efficient $^1O_2$ generators
- TC1 and TC2 cause a rapid intracellular ROS release following Light Beam exposure
- TC1 compound has a considerable photo-toxic effect in human fibrosarcoma cells

Keywords:

Thiophene dyes
Lysosomes
Photosensitizers
Singlet oxygen
Reactive Oxygen Species
Human Serum Albumin

Abbreviations [i]

[i] *Abbreviations*: DPBF, 1,3-diphenylisobenzofuran; HSA, human serum albumin; lyso-PDT, photo-damage of lysosomes; mito-PDT, photo-damage of mitochondria; PDT, photodynamic therapy; PS, photosensitizer; ROS, reactive oxygen species.

1. Introduction

Photodynamic activity is a photochemistry-based mechanism in which a light-activatable dye, the photosensitizer (PS), is capable of generating reactive oxygen species (ROS) upon light irradiation [1,2].

This combination of light and non-toxic PS is the basis of the emerging photodynamic therapy (PDT), a minimally invasive treatment of various pathologies including cancer [3-5]. PDT has several advantages compared to conventional protocols (such as chemotherapy and radiotherapy) due to its selectivity, non-invasiveness, loss of systemic side effects and activation of pathways which overcome cancer drug resistance [6]. The produced ROS can trigger cascading biochemical and molecular events which finally lead to cell death and cancer disruption due to the oxidation of biological molecules such as proteins, DNA and phospholipids [7-10]. So the photo-damage acts to various levels inducing apoptosis or necrosis and bypassing the cancer cell chemo and radio-resistance pathways. PDT can directly induce damage to proteins involved in cancer resistance mechanisms such as the antiapoptotic BCL-2 family proteins that is one of the principle drug resistance pathway [6,11]. A number of PSs are already in clinical use or in clinical trials for the treatment of different solid tumors in skin, brain, lung, bone, cervix, prostate and ovary [12]. PSs are commonly divided into three main classes: the first generation of PSs includes porphyrin-based dyes with a good photodynamic activity, widely used for the treatment of lung and esophageal cancers [13]; the second generation includes PSs with improved pharmacokinetic and reduced tissues photosensitivity because of their near-infrared (NIR) wavelength absorption such as benzoporphyrin derivatives, Bodipy dyes, phthalocyanines and many others which were projected with selected properties [14]. Currently photochemistry research goes toward two-photon bio-imaging [15] that offers new PSs, the third generation, which can be excited with a twice wavelength providing a better tissue penetration and a more sensitive therapeutic effect [16,17]. Many factors can influence the PS activity: absorption band, fluorescence quantum yield, photo-stability, dark toxicity and hydrophobicity. A PS should have relatively high absorption bands (> 20,000–30,000 M^{-1} cm^{-1}) in order to minimize the dose of PS to achieve the desired effect [12]. The fluorescence of the PS led to the development of protocols for dyes quantification in cells and tissues and allows bio-imaging techniques to locate and monitor the disease development [2,18]. For clinical applications an important issue is the solubility of PS in water or in a harmless aqueous solvent mixture without phenomena of aggregation which could determine the loss of photodynamic activity [19]. Another important characteristic of the PS is its hydrophobic nature that may interfere with the solubility but may facilitate the crossing of biological membranes optimizing PS cellular uptake. The PDT treatment implies the PS accumulation in cancer tissue through two mechanisms: using a selective drug delivery systems (active targeting) or exploiting the enhanced vascular permeability and retention (EPR) effect (passive targeting) [20,21]. Therefore it is possible to perceive that the PSs biodistribution and the achievement of the target site are linked to various factors such as their hydrophobic nature, the undesirable uptake by phagocytic cells and the binding of plasma molecules [22]. In this context the human serum albumin (HSA) plays a crucial role. HSA is the main plasma protein and it is responsible of the binding and transport of many endogenous and exogenous molecules including drugs [23,24]. The nature and the efficiency of the binding between the PS and serum albumin plays an important role for PDT efficiency. Recently, it has been shown that if a molecule possesses affinity for serum albumin, it would probably exhibit efficient PDT applications [25,26]. The effective cancer cellular uptake is essential but the triggered cell mechanisms and the degree of photo-damage is closely linked to the subcellular localization of the PS [27-29]. ROS have high reactivity, short half-life and their radius of action is of the order of few nm, only the biomolecules proximal to the area of its production are directly affected by PDT [30]. Therefore the subcellular localization of PS is a crucial factor which depends on the target of the photo-damage and on the induced cell death mechanism [31]. Most PSs preferentially localize in plasma membrane, Golgi apparatus, endoplasmic reticulum, nucleus, mitochondria and lysosomes [2]. The two most projected and exploited PDT methods are the photo-damage of mitochondria (mito-PDT) and the photo-damage of lysosomes (lyso-PDT). The mito and lyso-PDT promote the pro-apoptosis pathways bypassing the cancer drug resistance [6].

In particular lysosomes had shown a relevant role in cell death, representing a promising target for effective PDT [32]. In fact the lyso-photodamage in cancer cell models causes the lysosomes permeabilization with release of proteolytic enzymes that trigger the apoptosis through different pathways such as the activation of pro-apoptotic factors [33-37]. Thus the advantage of lyso-PDT is the multiple response bypassing the autophagic mechanism [38]. The lyso-PDT response implies not only the destruction of these organelles, but also the permeabilization of mitochondria membranes with the loss of membrane potential, release of cytochrome c and activation of caspases [39-41]. The PDT mechanism is influenced also by the photodynamic dose [42]. Therefore recent works have demonstrated a synergic enhancement of photo-damage by using PS with different compartments targeting [43-45]. In particular an enhanced photo-toxic effect is obtained with a sequential low dose lyso and mito-PDT [46]. The lysosomal PSs are also good candidates for the photochemical internalization process (PDI) that is a drug delivery mechanism allowing the internalization of therapeutic macromolecules which do not reach their target, remaining entrapped in lyso and endocytic vesicles. In this case the photodynamic activity of PS is exploited to induce the destabilization of lysosomes membranes allowing the drugs release in the cytosol [47]. In our recent paper, we have synthetized and characterized two Thiophene-derived Lysosomes-specific dyes (TC1 and TC2), whose structures are shown in Figure 1 [48]. These dyes showed high selectivity for fluorescent staining of lysosomes (co-localization coefficient of > 95%), high photostability, low toxicity, moderate fluorescence quantum yield and extinction coefficient. These features make TC1 and TC2 perfect candidates as PSs.

In this present study we investigate the photodynamic activity of TC1 and TC2 studying the interaction of these dyes with HSA, ensuring the *in vitro* singlet oxygen production and their phototoxic effect in human fibrosarcoma cells.

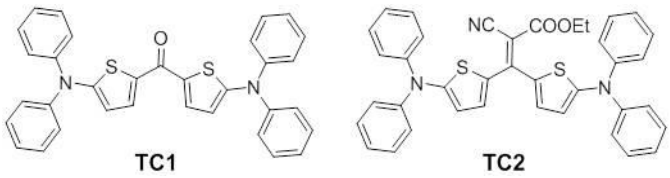

Fig. 1. Structures of TC1 and TC2 dyes. **1 column fitting image**

2 Materials and Methods

2.1 Materials

All reagents were of analytical reagent grade and double distilled water was used. The singlet oxygen probe 1,3- diphenylisobenzofuran (DPBF), Human Serum Albumin (HSA), the intracellular probe for oxidative stress 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) and the phosphate buffered saline (PBS) were purchased from Sigma-Aldrich. The WST-1 cell proliferation assay was purchased from Roche Applied Science (Penzberg, Germany). The thiophene-based fluorescent probes, TC1 and TC2, were synthesized as already described in our previous work [48].

2.2 Spectroscopic measurements

UV-Vis measurements were recorded using a Cary 300 spectrophotometer. Fluorescence measurements were recorded using a Fluorolog 2 from Jobyn Ivon. Excitation and emission slits (10 nm) were arranged in order that all fluorescence spectra could be recorded under the same conditions. Fluorescence lifetimes were obtained on a Fluorolog2 spectrofluorimeter, equipped with proper NanoLED source (emitting at 530nm)

and using a photon counting detector (TBX04)The interaction between HSA and TC dyes was studied keeping the dye concentration constant (6 μM) and increasing HSA concentration (from 1 μM to 20 μM). The TC mother solution was prepared in DMSO (10 mM) instead the HSA mother solution was prepared in PBS (50 μM). Dilutions for the experiments were performed in PBS buffer (2 mM, pH 7.4). The UV spectra were recorded in the range of 200-600 nm while the fluorescence spectra were recorded in the range of 500–700 nm. Samples were excited at 485 nm and monitored at 597 nm to check TC1 fluorescence and at 495 nm and monitored at 599 nm to check TC2 fluorescence. Fluorescence lifetime measurements of TC in presence of HSA were performed keeping a constant concentration of dye (6μM) and an increasing HSA concentration (1-12 μM).

The *in vitro* generation of singlet oxygen was detected using the well-known singlet oxygen probe DPBF [49]. The experiments were performed in PBS at an initial concentrations of 12 μM of TC and 20 μM of DPBF. The solutions were exposed to a broad band light at 250 mW/m^3 with 455 nm and 495 nm cut-off filters respectively for TC1 and TC2. The reaction of DPBF oxidation was monitored spectrophotometrically recording the decrease of the adsorption at 414 nm.

2.3 Cell culture

The HT-1080 human fibrosarcoma cell line (ATCC, Rockville, MD, USA) was cultured in Minimum Essential Medium Eagle (EMEM) supplemented with 2 mM L-glutamine, 100 UI/mL penicillin, 100 μg/mL streptomycin and (Sigma-Aldrich, Milano, Italy) in a humidified atmosphere of 5% CO_2 air at +37°C. The HT-1080 was detached using 0.05% trypsin-0.02% EDTA solution (Sigma-Aldrich), resuspended in culture medium and seeded at the appropriate cell concentration for cell culture experiments.

2.4 *In vitro* photodynamic treatment

HT-1080 cells in the exponential growth phase was pre-incubated in the dark for 24 hours with culture medium containing TC dyes at concentration of 1 μg/ml. The cells were then removed from the flask with 0.05% trypsin-0.02% EDTA solution and normalized to 5×10^5 cells in 1 mL of PBS into polystyrene tubes. HT-1080 cells, pre-incubated with TC1 or TC2, were treated with a custom compact system for PDT with fixed source-cuvette positioning and light diffuser all-around cell culture. The light-emitting source of the system is based on InGaN light-emitting diodes (Cree Inc, Durham, NC, USA) with 20 mW max radiant power (emitted flux) and a central wavelength of 405 nm for TC1 and 470 nm for TC2 respectively. The system allows continuous radiant flux regulation from 0 to 20 mW with a programmable non-switching diode current source. The energy fluency rates of the UV radiation were adjusted to 15 mW/cm^2 for 40 minutes, measured using Actinic UV-meter (Jelosil, Le Landeron, Switzerland).

2.5 Cell proliferation assay

The WST-1 cell proliferation assay was used to evaluate the cytotoxic effect of *in vitro* PDT treatment on the HT-1080 cells. After the various treatments, 1.5×10^3 cells were seeded in 100 μL of culture medium in replicates (n=8) in 96-well culture plates. WST-1 reagent (10 μL) was added at 24, 48, and 72 hours and the plates were incubated at +37°C in 5% CO_2 for 1.5 hours. The well absorbance was measured at 450 and 620 nm (reference wavelength) in a microplate reader (Asys UV340; Biochrom, Cambridge, UK).

2.6 Flow cytometric analysis

The ROS generation was measured with the DCFH-DA as intracellular probe for oxidative stress. DCFH-DA is a stable, non-fluorescent molecule that readily crosses the cell membrane and is hydrolyzed by intracellular esterases to the non-fluorescent DCFH, which is rapidly oxidized in the presence of peroxides to highly fluorescent dichlorofluorescein (DCF) upon oxidation by ROS. After the PDT treatment, cells were incubated with 10 μM DCFH-DA for 30 minutes, washed with PBS, trypsinized, collected in 1 mL of PBS,

and analyzed by a C6 flow cytometer (Accuri Cytometers, Milano, Italy) evaluating 10,000 events. The ROS production was expressed as the integrated mean fluorescence intensity (iMFI), which was the product of the frequency of ROS-producing cells and the median fluorescence intensity of the cells. The iMFI was determined at different time points ,i.e., 1, 3, 5, 10, 15, 30 minutes.

3. Results and Discussion

3.1 Interaction between TC1-TC2 and HSA

The nature of the binding between the PSs and HSA could influence PDT efficacy [50]; for example a tight binding can imply a slow release of the drug compromising its efficacy. Studies of PS interaction with bio-macromolecules and isolated biological structures are relevant in view of the PS biodistribution and pharmacokinetic. The nature of PS-HSA interaction can result with different kinetic such as it was demonstrated for porphyrin and squaraine dyes [51]. Moreover, it was established that also the quantum yield of singlet oxygen is influenced by HSA [52]. Thus preliminary steady-state absorption and fluorescence measurements were conducted to study the interaction of TC dyes with HSA.

UV-Vis absorption measurement is a simple and applicable method to investigate the formation of a complex [53,54]. Figure 2 shows that the absorbance of TC-HSA complex differ from the spectra of TC dyes alone and HSA alone suggesting the formation of a complex between the PSs and the protein. In fact, as an example, the absorption peaks at 278 nm and 458 nm are higher for the TC1-HSA complex than for the dye and protein spectra alone at the same concentration. A titration experiment was then conducted for both dyes and the absorption spectra of TC1 and TC2 in absence and in presence of different concentrations of HSA are presented in Fig.S1. Since a quantitative study was not appropriate due to the low sensitivity of the technique, we decided to perform a titration using fluorescence spectroscopy.

Figure 3 shows an increase of fluorescence emission of TC dyes with addition of an increasing concentration of HSA. If we plot the emission intensity with the HSA concentration we can fit these data by an hyperbola equation for the evaluation of the binding constants [55]. There is nearly no difference in the binding to HSA since the constants are very closed for TC1 (24.7 μM) and TC2 (22.0 μM) suggesting that the degree of interaction with the protein is not influenced by the different substituents on the dye.

Using time-correlated single-photon counting (TCSPC) to measure fluorescence decay of TC-HSA, we found that, at zero HSA concentration, the decay could be best fitted by a biexponential model with decay constants of 0.20 and 0.80 ns for TC1 with a mean lifetime of 0.5 ns and 0.26 and 2.25 ns for TC2 with a mean lifetime of 1.25 ns. On addition of the protein, the mean lifetime of the complex increased by a maximum of 1.80 ns for TC1 and 1.55 ns for TC2 (see Table1 and Table2 in S2). This clear increase in the average decay time is indicative of the TC dyes interacting with HSA, confirming the formation of the complex.

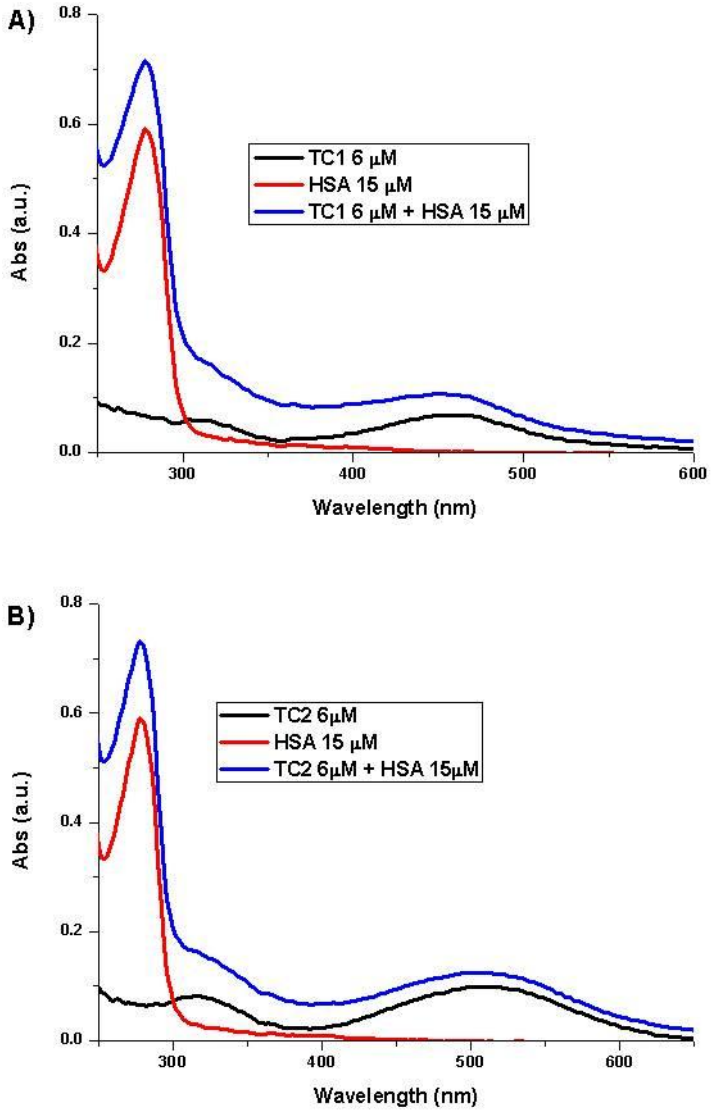

Fig. 2. UV-Vis spectra of A) TC1-HSA interaction and B) TC2-HSA interaction.

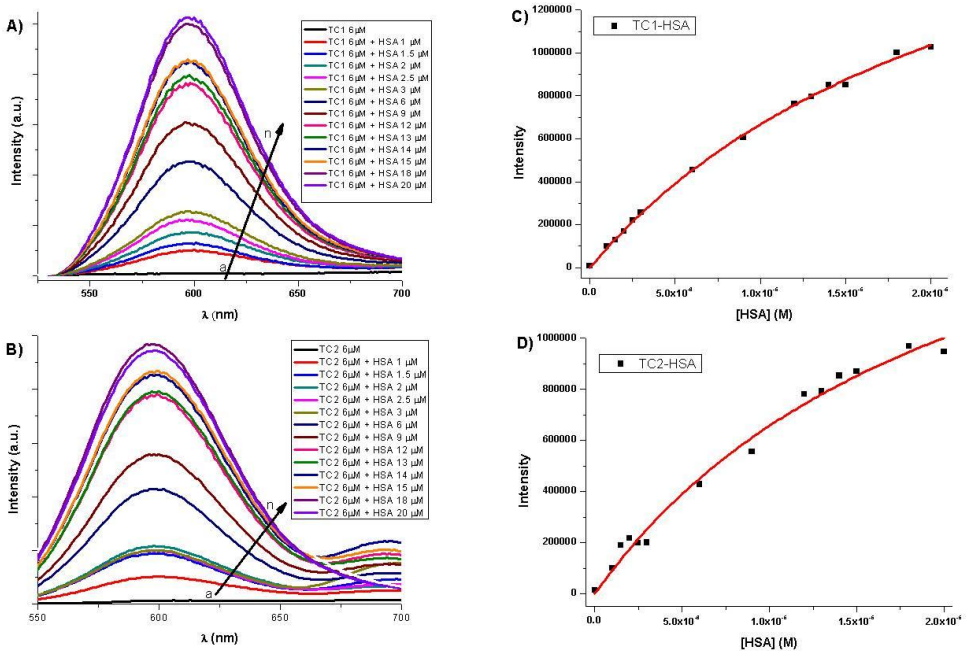

Fig. 3. Change in fluorescence emission of A) TC1 (6 μM) and B) TC2 (6 μM) with the addition of HSA. [HSA] (a) 0, (b) 1 μM, (c) 1.5 μM, (d) 2 μM, (e) 2.5 μM, (f) 3 μM, (g) 6 μM, (h) 9 μM, (i) 12 μM, (j) 13 μM, (k) 14 μM (l) 15 μM, (m) 18 μM, (n) 20 μM. The [HSA]-dependence of fluorescence signal intensity at 598 nm. The solid line is the best fit to a hyperbola equation yielding a Kd of 24.7 μM for TC1-HSA complex (C) and 22.0 μM for the TC2-HSA complex (D).

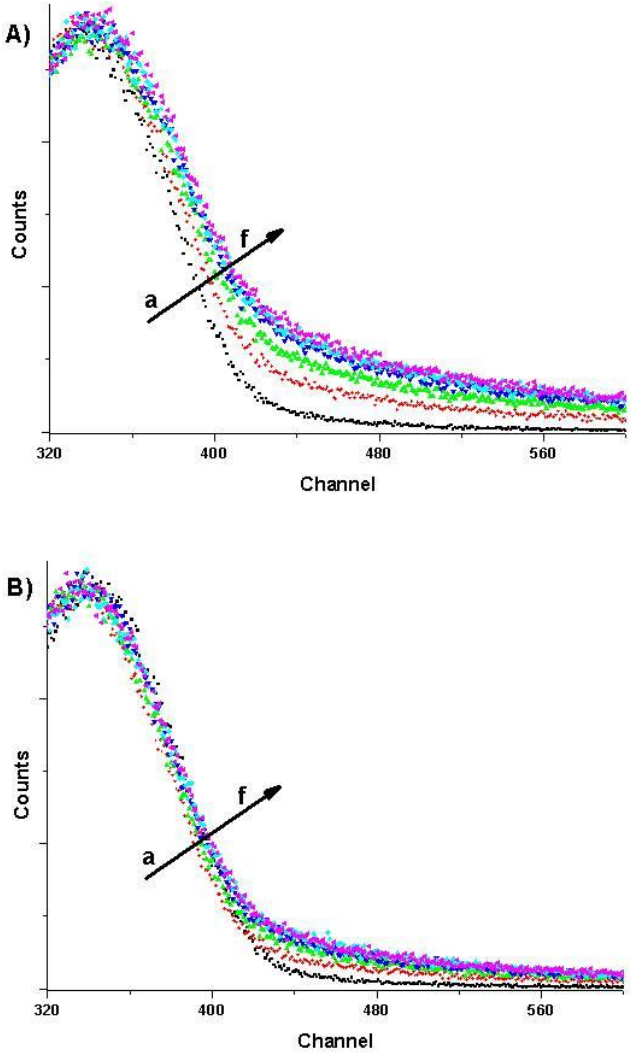

Fig. 4. Time-resolved fluorescence decay of TC1 (A) and TC2 (B) (6 μM) with the increase in addition of HSA. [HSA]: a) 0, b) 1, c) 3, d) 6, e) 9 and f) 12 μM.

3.2 Generation of singlet oxygen ability of TC1 and TC2

A qualitative comparative study of the abilities of TC1 and TC2 to generate singlet oxygen was detected by trapping $^1O_2$ with 1,3-diphenylisobenzofuran (DPBF). In fact DPBF reacts rapidly with $^1O_2$ by forming the colorless o-dibenzoylbenzene derivative resulting in the disappearance of DPBF characteristic absorption band at 415 nm. Thus, a solution of DPBF and the dyes in PBS pH 7.4 and DMSO (1%) was irradiated in a solar box filtered to remove both wavelengths below 515 nm; the DPBF consumption was monitored over time. The decrease of the quencher absorption at 415nm as a function of the irradiation time of each dye compared to that obtained by methylene blue (MB), an efficient and well-known singlet oxygen generator, shows that both TC1 and TC2 posses $^1O_2$ generation ability (Figure 5).

The dyes promoted the nearly complete decay of DPBF absorption within 4 minutes.

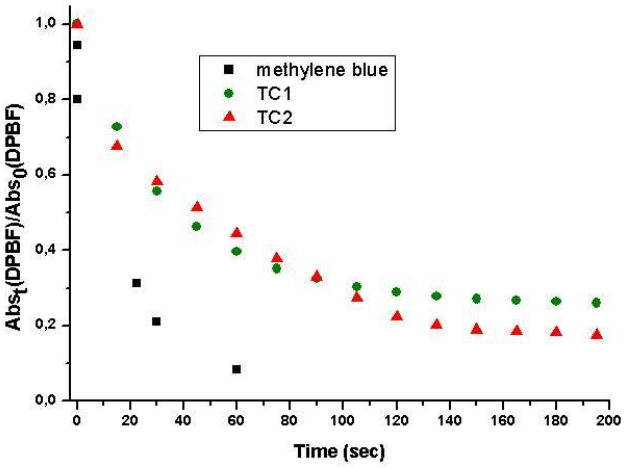

Fig.5. Time-dependent decrease of the absorbance at 414 nm by oxidation of DPBF (20 µM) with dyes (12 µM) under broad light (using appropriate cut-off filters). Comparative singlet oxygen generation ability of TC dyes and methylene blue. Abs_0 (DPBF): absorbance of DPBF before irradiation; Abs_t (DPBF): absorbance of DPBF after irradiation time t.

3.3 Photodynamic activity of TC1 and TC2

The *in vitro* photo-toxic effect of TC probes was studied in human fibrosarcoma cells, HT-1080. Briefly, cells were incubated with the dyes at the low concentration of 1 µg/ml for 24 hours to ensure the efficient cellular uptake and localization. The effect of the PDT treatment was investigated exposing the cells to the appropriate light beam (LB) for 40 minutes. The WST-1 assay allowed the evaluation of cell proliferation over time. As shown in the figure 6A-B, only when the cells were pre-incubated with TC1 probe the LB was able to significantly affect the HT-1080 cell proliferation. The TC2 probe does not shown the same photo-toxic effect with the identical PDT protocol conditions. Control treatments, i.e, exposure to fluorescence probes alone and exposure to LB alone, did not significantly affect cell proliferation comparing with the untreated cells. DCFH-DA was used as ROS probe to evaluate the intracellular ROS production following the PDT treatment. The fluorescence of the formed DCF was measured by flow cytometry and so the intracellular ROS levels were determined after 1, 3, 5, 10, 15, 30 minutes from the PDT treatment. The control treatments were made in the same way of the previous experiment. As shown in the figure 6C-D the PDT protocol led to a net ROS production only one minute after treatment. Then a reduction of released ROS in cells has been observed up to a reset after 30 minutes. With respect to what observed for the WST-1 assay, TC2 also showed a rapid increase in ROS production following LB exposure. Moreover, the LB exposure alone led to a small ROS production in HT-1080 cells as the exposure to TC1 probe alone, either without significant effect on cell proliferation. However, the ROS intracellular generation following TC2 photo-activity was not sufficient to induce a strong cell death for the tested concentration and observed times. We know that TC1 and TC2 have the same internalization kinetics (measured by flow cytometry, A.L. Capodilupo et al.) so this different photo-toxic effect could only be due to a different dose-dependent response. Noteworthy is the drastic photodynamic effect of the TC1 at the lower concentration (1µg/ml) than other lysosomal probes which were presented as effective PSs [37, 41].

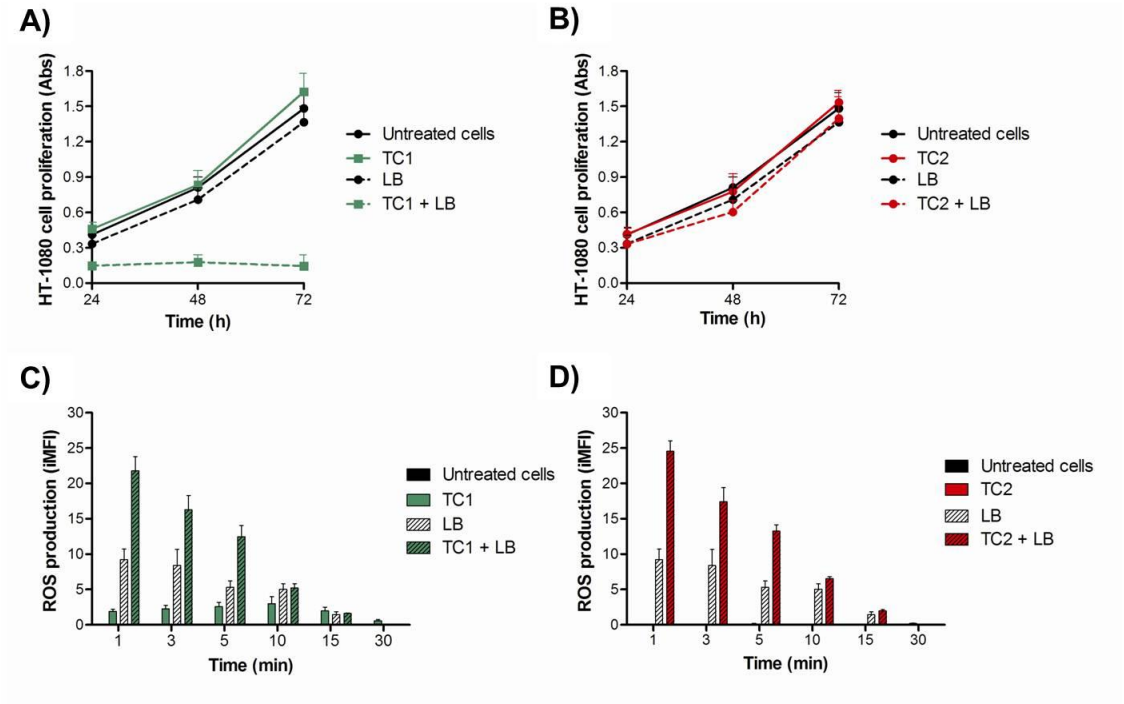

Fig.6. Effect of different treatments on HT-1080 cells proliferation and ROS production as a function of time. Cells were incubated for 24 hours with TC1 (A, C) or TC2 (B, D) dyes (1 μg/ml). The PDT protocol was performed with LB (TC1 - 405 nm and TC2 - 470 nm) at 15 mW/cm^2 for 40 minutes. Cell proliferation was evaluated after 24, 48 and 72 hours by WST-1 assay and ROS production was determined according to the DCF-DA assay by flow cytometry.

4. Conclusions

The subcellular localization of the PSs is determinant for the targeting of the photo-damage and the induction of cell death mechanism. In particular lysosomes had shown a specific role in cell death for PDT. TC1 and TC2 are known as no toxic, specific lysosomes-probes with high photostability and pH-insensitivity. In this work we showed that these dyes are potential effective lysosomes PSs. The interaction between TC dyes and HSA results in their fluorescence intensity increase. Both probes have a very fast production of singlet oxygen upon light irradiation. Moreover, the phototoxic effect was studied in human fibrosarcoma cells under specific PDT protocols. The light irradiation caused the rapid ROS intracellular release for both the PSs candidates. However cells proliferation is drastically inhibited only by the photo-activity of TC1 dye. Despite the moderate fluorescence quantum yield and extinction coefficient, TC1 and TC2 have shown a good *in vitro* photo-activity. Finally TC1 compound has a considerable photo-toxic effect in cancer cells at lower dose compared to other lysosomes PSs so it represents a new potential candidate for lyso-PDT.

Acknowledgements

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