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Inclusion complexation with the derivatives of β -cyclodextrin alters biodistribution and photodynamic efficacy of mTHPC

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Abstract

Low aqueous solubility of the clinical approved photosensitizer, meta-tetra(hydroxyphenyl)chlorin (mTHPC), complicates its intravenous administration and impairs pharmacokinetics and bioavailability. Two modified cyclodextrins (CDs), methyl- β -cyclodextrin (M- β -CD) and 2-hydroxypropyl- β -cyclodextrin (Hp- β -CD), have been studied in order to improve mTHPC physicochemical and pharmacokinetic properties via the formation of a complex. The physicochemical characterization showed that the formation of an inclusion complexes with mentioned CDs provides significant enhancement of mTHPC solubility and of its photophysical properties. Based on fluorescence polarization measurements and chromatographic techniques, the influence of CDs on mTHPC distribution in serum solution has been established. Furthermore, the effect of M- β -CD and Hp- β -CD on mTHPC cellular uptake and distribution, as well as the efficiency of photosensitized damage of HT29 human adenocarcinoma cells were obtained. Cellular uptake of mTHPC critically depends on CD concentrations and can be observed both acceleration and inhibition of mTHPC accumulation. Photocytotoxicity data clearly demonstrated a much enhanced photokilling ability of mTHPC complexed with CDs compared to mTHPC alone. mTHPC accumulation after intravenous injection in different organs of tumor bearing mice assessed by fluorescence imaging displayed significant alteration of mTHPC biodistribution via complexation with CDs. In case of M- β -CD, a reduction is observed in the accumulation of mTHPC in the skin and muscles, as well as an increase in the drug level in the tumor compared to mTHPC alone.

Keywords: mTHPC, Cyclodextrin, Inclusion complex, Photodynamic Therapy, Biodistribution

Introduction

Photodynamic therapy (PDT) is a minimally invasive photochemical-based treatment with a promising clinical track record for oncological diseases (Agostinis et al. 2012; Allison 2014). PDT uses light-activated drugs (photosensitizers, PSs) to induce cell death mediated by the production of singlet oxygen and oxygen radicals. These reactive oxygen species induce damage in malignant and nonmalignant cells alike, depending on the PS distribution (Castano et al. 2004). Clinical application of PDT encounters several difficulties due to high hydrophobicity of most PSs, thus leading to poor water solubility and aggregation within the vasculature after intravenous injection (Josefsen and Boyle 2012). *In fine*, a decrease in photosensitizing efficiency and extended skin photosensitivity caused by unfavorable biodistribution are considered as major drawbacks of PDT.

The use of biocompatible nanoscale systems for PS delivery demonstrated a significant improvement in PS bioavailability, biodistribution and in treatment efficacy (Marchal et al. 2015). Special delivery forms such as liposomes (Reshetov et al. 2013), polymeric nanoparticle (Ogawara et al. 2016) and dendrimers (Bastien et al. 2015) were extensively studied and showed important progress in drug delivery and PDT. Among the investigated drug delivery systems, cyclodextrins (CDs) are promising PS carriers (Kryjewski et al. 2015; Mazzaglia 2011). CDs are a family of cyclic oligosaccharides with a hydrophobic internal cavity and a hydrophilic outer space. These structural features allow CDs to form inclusion complexes with various organic and inorganic compounds, providing a unique opportunity to assemble host-guest complexes. Due to its appropriate cavity size, β -CD and its derivatives are most commonly used for inclusion complexation with PSs, especially aryl substituted porphyrins. PS/CD complexes were tested mostly in solution and *in vitro*, in cultured cells. Several reports documented that PSs binding to CDs increased the solubility of highly hydrophobic drugs, their physical and chemical stability, enhanced PSs delivery into cells and their photokilling ability (Kolarova et al. 2003, 2004; Lang et al. 2004; Kitagishi et al. 2015). It should be noted that currently available experimental data on the influence of CDs on PS photosensitized processes in cells are highly contradictory.

In the present study, we analyzed the influence of methyl- β -cyclodextrin (M- β -CD) and 2-hydroxypropyl- β -cyclodextrin (Hp- β -CD) at wide range concentrations on the processes of meta-tetra(hydroxyphenyl)chlorin (mTHPC) biodistribution and its photosensitized activity. mTHPC is one of the most potent PS, clinically approved in the European Union, Norway & Iceland as a solvent-based formulation Foscan[®] (Biolitec Research GmbH, Jena, Germany) for the PDT treatment of head and neck cancers. Formation of inclusion complexes between mTHPC and CDs and their physico-chemical properties were studied by the P. Prognon and A. Kasselouri group. Introduction of β -CDs derivatives into aqueous solutions of mTHPC causes inclusion complexation providing PS disaggregation with the restoration of the absorption and fluorescence spectra of the monomer form

(Demore et al. 1999; Desroches et al. 2001; Bautista-Sanchez et al. 2005). However, to date there is no available data on the interaction between CDs and mTHPC on *in vitro* and *in vivo* models.

The aim of this work was to evaluate the effect of CDs on mTHPC behavior at various stages of its distribution *in vitro* and *in vivo*. Binding to serum proteins, the intracellular accumulation and distribution, the efficiency of mTHPC photosensitized damage were investigated, as well as the pharmacokinetics and biodistribution after intravenous injection in tumor bearing mice.

1. Material and methods

2.1. Chemical and reagent preparation

The photosensitizer mTHPC (temoporfin) was kindly provided by Biolitec Research GmbH (Germany). Phosphate buffered saline (PBS), phenol-red-free RPMI-1640 medium, penicillin-streptomycin and L-glutamine were supplied by GibcoBRL (Belgium). Fetal bovine serum (FBS) was purchased from PANTM BIOTECH GmbH (South America). M- β -CD and Hp- β -CD were purchased from AraChem (Netherlands).

mTHPC stock solution (1 mM) was prepared in absolute ethanol ($\geq 99.6\%$) and was kept at 4 °C in the dark. The concentration of mTHPC in the solution was estimated by a spectrophotometric method using an mTHPC molar extinction coefficient of 30 000 M⁻¹·cm⁻¹ at 650 nm in ethanol.

CDs and mTHPC complexes were formed using co-precipitation method (Martin Del Valle 2004). Briefly, CDs were dissolved in PBS at the required concentrations with the subsequent addition of mTHPC stock solution. The final content of ethanol in the mTHPC/CD solutions did not exceed 1%. Solution was thoroughly mixed over 15 min under magnetic stirring at 25°C.

For *in vitro* cell culture experiments mTHPC stock solution was diluted in RPMI medium, supplemented with 2% FBS to reach unless otherwise indicated the final mTHPC concentration of 1.47 μ M.

All other chemicals and solvents used in our experiments were commercial products of the highest grade of purity and its manufacturers will be specified below.

2.2. Spectroscopic measurements

The absorption spectra were measured on PerkinElmer Lambda 35 UV/Vis (PerkinElmer, Waltham, USA) and Solar PV1251 (Solar, Belarus) spectrophotometers using 1 cm optical path quartz cuvettes. Fluorescent measurements were performed on PerkinElmer LS55B (Perkin-Elmer, USA) and Solar SFL 1211A (Solar, Belarus) fluorescence spectrometers equipped with thermostat cuvette compartments (PTP-1 Peltier temperature controller in the case of LS55B) and magnetic stirring. Only slight spectral changes in the absorption and fluorescence spectra (1-2 nm shifts of the spectra

maxima) of mTHPC complexed with CDs are observed as compared to the PS in the ethanol and serum solution. The factor of change of mTHPC fluorescence intensity at the maximum compared to PS in ethanol solution (100%) is 140 % for PBS solution of M- β -CD, 115 % for PBS solution of Hp- β -CD, 70 % for serum solution (2% FBS) and less than 0.5% for PBS solution only. mTHPC fluorescence lifetime was measured in a pulsed fluorometer PRA-3000 (Photochemical Research Associates, Canada), working in photon counting mode. The values of mTHPC fluorescence lifetimes in 2% FBS solution and in solution of 50 μ M M- β -CD (Hp- β -CD) are 10.2 \pm 0.1 and 10.8 \pm 0.1 ns, respectively. For mTHPC fluorescence polarization measurements, samples were excited at 435 nm and fluorescence was registered at 652 nm. The degree of fluorescence polarization (**P**) of mTHPC complexed with CDs is 4.5% and 25% for PS in FBS solution. The obtained data are consistent with those previously published (Demore et al. 1999; Desroches et al. 2001; Bautista-Sanchez et al. 2005).

2.3. Application of fluorescence polarization for estimation of mTHPC distribution between CDs and serum

To analyze the kinetic and equilibrium characteristics of mTHPC distribution, the method of fluorescence polarization was used. The measurements were carried out at 25 °C at 0.2 μ M of mTHPC. A small aliquot of serum (or CDs) was added to mTHPC solution associated with CDs (or serum proteins). mTHPC ethanol solution was added to PBS containing 2% of FBS and was incubated 1h at 37 °C. The photosensitizer complexes with CDs prepared according to the method described above. The equilibrium distribution of the photosensitizer was studied after 24 hours of incubation and is not depended on the order you added the whey and CDs. Binding of mTHPC to various biological structures alters the mobility of PS molecules with the corresponding changes in the value of fluorescence polarization degree of mTHPC (Reshetov et al. 2011). It is well known that when binding a fluorescent dye with macromolecules the **P** value is determined by spectroscopic property of the fluorophore (its lifetime) and on its rotational motion (volume and shape of the complex, temperature etc.). Since the fluorescence lifetimes of mTHPC change slightly between PS associated with serum proteins and CDs. Thus, the **P** value of mTHPC fluorescence is mainly determined by the size of the structures with which it is associated. Indeed, the **P** value of mTHPC complexed with small CD molecules 4.5% while fluorescence of PS associated with large serum proteins (mostly lipoproteins) is characterized by high polarization degree (**P** = 25%). Thus, the measurement of mTHPC fluorescence **P** value allows estimating the relative binding of the PS in the mixed solutions of CDs and serum, if differences of the values of the quantum yield of fluorescence will be taken into account.

2.4. Size-exclusion chromatography

mTHPC (1.47 μ M) was incubated during 2h with different M- β -CD concentrations in PBS supplemented with 2% FBS at 37°C under light protection.

Chromatography experiments were performed as described earlier (Reshetov et al. 2012) except that we used Sigma 1.5 \times 35 cm column filled with Sephadex G-200 gel (GE Healthcare, USA), pre-equilibrated with PBS and a total bed volume of 62 mL. mTHPC content in the chromatographic fractions was estimated by fluorescence intensity measured after the addition of 0.2 % neutral detergent Triton®X-100 (Sigma–Aldrich, USA) to the samples. Intensities were corrected for fraction volume. No background fluorescence of serum was registered in the spectral region of 630–670 nm.

2.5 Cell line and culture conditions

HT29 human adenocarcinoma cells were obtained from the ATCC® (LGC Standards, France) and regularly controlled for mycoplasma contamination. HT29 cells were maintained in RPMI medium supplemented with 9% (vol/vol) heat-inactivated FBS, penicillin (10.000 IU), streptomycin (10.000 μ g/mL), and 1% (vol/vol) 0.1 M glutamine. Cells were kept as a monolayer culture in a humidified incubator (5% CO₂) at 37°C. Cell culture was reseeded every week to ensure exponential growth.

2.6. mTHPC cellular uptake

HT29 cells (5 \times 10⁴ cells per ml) were seeded in 6-wells plate, 48 h later the cells were washed twice with PBS and incubated in RPMI supplemented with 2% FBS, containing 1.47 mTHPC alone or complexed with CD at various CD concentrations. After three consecutive washings with PBS, cells were trypsinized, centrifuged, the pellet washed with PBS, centrifuged once more at 300 g., then the supernatant was discarded and the methanol was added to the pellet. Afterwards, the suspension was sonicated during 15 min and centrifuged. The mTHPC fluorescence contained in the supernatant was measured with Perkin-Elmer LS 55B spectrometer and mTHPC concentration was evaluated by fluorescence calibration curves constructed by plotting the peak height of PS standard solutions in methanol. The obtained mTHPC concentration was normalized to the number of viable cells.

2.7. Fluorescence microscopy

HT29 cells (1.5 \times 10⁴ cells per ml) were plated into Lab-Tek II chamber Slide (Roskilde, Denmark), incubated in the dark at 37°C with 1.47 μ M of mTHPC alone or complexed with M- β -CD (10 μ M) during 1 h, then rinsed with PBS. Fluorescence of mTHPC was observed under an upright epifluorescence microscope (AX-70 Provis, Olympus, France) equipped with a 100 W mercury vapor lamp and a Peltier cooled CCD camera (DP70, Olympus). The filter was set at 400–440 nm band pass

excitation associated with a 570 nm dichroic mirror and a 590 nm long pass emission filter for mTHPC fluorescence measurements. Fluorescence images were recorded using an oil immersion $\times 40$ objective.

Fluorescence microscopy technique based on Filipin III cholesterol assay kit (ab133116 Abcam, UK) was used to estimate of CD effect on plasma membrane cholesterol content (Wolfe et al. 2015). The filter was set at 330–385 nm band pass excitation associated with a 400 nm dichroic mirror and a 420–460 nm long pass emission filter for Filipin III fluorescence measurements. A cholesterol inhibitor, U-18666A, was used as a positive control.

To prevent the photobleaching of dyes, the samples were analyzed immediately after staining. Fluorescence microscopy data were analyzed using ImageJ software.

2.8. Assessment of cell viability

Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma] colorimetric assay, which measures the capacity of mitochondrial dehydrogenase in viable cells to reduce MTT to purple formazan crystals. HT29 cells (5×10^4 cells per ml) were seeded in 96-wells plates, 48 h later the cells were washed twice with PBS and incubated during 3 h or 24 h in RPMI supplemented with 2% FBS containing 1.47 μM of mTHPC only or in presence of 10 μM of M- β -CD or 200 μM of Hp- β -CD. Irradiation was performed at room temperature with a laser diode (Biolitec AG, Germany) at 652 ± 4 nm at different exposition times varying from 4 s to 500 s, power output was adjusted to 2 mW/cm^2 . Following irradiation, cells were washed twice with cold PBS, the fresh medium was added and cells were maintained in the humidified incubator. 24 h later, the medium was removed, the MTT solution was added to each dish and cells were kept in the humidified incubator for 3 h. The formazan crystals were solubilized by adding dimethyl sulfoxide and absorbance was measured at 540 nm with a Multiskan Ascent spectrometer (Labsystems, USA). From these values, cell viability percentages of treated and non-irradiated control samples were calculated. All experiments were carried out in triplicate. The efficacy of mTHPC phototoxicity was based on LD₅₀ (Lethal Dose inducing 50% cell death).

2.9. Fluorescence analysis of in vivo distribution of mTHPC

All experiments were performed in accordance with animal care guidelines (Directive 2010/63/EU) and carried out by authorized persons (personal authorization number 54-116) issued by the Department of Veterinary Services in a registered establishment (establishment number A54-547-21) issued by the Department of Veterinary Services.

All procedures involving animals were performed under general anesthesia with inhaled isoflurane (Forene; Abbott Laboratories, Abbott Park, IL, USA) using a Univentor 400 anesthesia unit

(Genestil, Royaucourt, France), with every effort made to minimize suffering. Female NMRI^{nu/nu} mice (Janvier, St Berthevin, France) aged 9–10 weeks were used, with a mean body weight of 29±2 g. Mice were inoculated subcutaneously in the right flank with 8×10⁶ exponentially growing HT29 cells. When the tumor volume reached ~ 100 mm³, the mice were randomized into three groups (n = 3-4 mice/group) according to the injected formulation: mTHPC alone, mTHPC/M-β-CD and mTHPC/Hp-β-CD. All drugs were administered intravenously by a tail vein injection at a dose of 0.5 mg/kg of mTHPC. Following injection, mice were kept in the dark and experiments were undertaken with minimal ambient light.

PDT fluorometer (JETI Technische Instrumente GmbH, Germany) was used to measure the *in vivo* mTHPC fluorescence after injection. Excitation wavelength was 405 nm, emission wavelength 600-700 nm. At each time interval the mTHPC fluorescence was measured in three points on the surface of both tumor and healthy tissues.

To compare tissue and tumors distribution of mTHPC alone and complexed to CD 24 h after i.v. injection, the excised tissues including liver, spleen, kidney, muscle, lungs, heart and skin as well as the tumor were analyzed by fluorescence imaging. The FluorVivoTM 300 image system (Indec Systems, Santa Clara, CA, USA) equipped with a LED at 510-550 nm and a long wave emission filter (630-690 nm). Imaging data were analyzed using ImageJ software.

2.10. Statistical analysis

Each experiment was repeated at least three times. Data are presented as mean ± SD. The data were evaluated using Student's t-test. The difference was considered significant at $p < 0.05$. Data analysis was carried out with the STATISTICA 10 (StatSoft, Inc., USA).

2. Results

3.1. Kinetic and equilibrium distribution of mTHPC between CDs and serum

Introduction of M-β-CD and HP-β-CD into aqueous solution of mTHPC results in an inclusion complexation providing complete PS monomerization. This binding is a reversible process, i.e. each molecule can dissociate from the inclusion complex with the subsequent association with other molecules (like plasma proteins). Therefore, the introduction of mTHPC/CD complexes into the serum is accompanied by the processes of PS redistribution to plasma proteins. We have estimated the rates of mTHPC transfer from its inclusion complexes with CD to serum proteins and in opposite direction from serum to CDs. A convenient tool for this purpose is the measurements of mTHPC fluorescence polarization changes (see 2.4). Fig. 1 shows typical kinetics of *P* changes when mTHPC/M-β-CD complexes are mixed with 2% serum solution. A competition between M-β-CD and serum proteins

leads to redistribution of mTHPC molecules from inclusion complexes to proteins and as a result to the growth of fluorescence polarization values. When CD concentration is low (Fig. 1, curve 1) P reaches 24-25% in 5-7 minutes of incubation indicating that almost all mTHPC molecules have changed their location. A transfer of mTHPC molecules in opposite direction (from lipoproteins to CDs) also occurs very quickly. According to Fig.1 (curve 4), after addition of 100 μ M M- β -CD to sample of PS preloaded serum P value drops from 25% to 4-5% in several minutes. In the presence of moderate CD concentration mTHPC transfer in both directions rather quickly leads to equilibrium with transitional P values (Fig. 1, curves 2 and 3).

Fig. 2 shows the dependence of mTHPC fluorescence polarization degree in 2% FBS as function of M- β -CD and HP- β -CD concentration. According to the data obtained the detectable amount of CD-bound mTHPC in serum appears at 2 μ M M- β -CD and 50 μ M HP- β -CD. Minimal values of P corresponding to complete mTHPC transfer from serum proteins to CDs are observed at 200 μ M M- β -CD and 2 mM HP- β -CD.

To further characterize the influence of CDs on the processes of mTHPC distribution in serum, we used size exclusive gel chromatography technique. Fig 3 shows profiles of elution for samples of 2% serum loaded with 1.47 μ M mTHPC and different bulk of M- β -CD. In control samples (mTHPC in FBS) mTHPC is tightly bound to the main transport proteins with the elution peaks in the range of 20-50 mL (Fig. 3 curve 1). Bioluminescence analysis of protein eluting curves shows that these elution peaks of mTHPC correspond to low density lipoproteins (LDL, band with maxima at 23 mL) and high density lipoproteins (HDL, band with maxima at 31 mL) fraction (data not shown). Relative weight of mTHPC bound to serum albumin (band with maxima at 40 mL) does not exceed 5%. Introduction of M- β -CD at the concentrations higher 1 μ M leads to decrease in the main elution protein-bound peaks with a simultaneous appearance of a new peak of mTHPC bound to M- β -CD (50-70 mL). The relative weight of this fraction increases with increasing M- β -CD concentrations and at 200 μ M all bulk of mTHPC is associated with M- β -CD (Fig. 3, curve 5). It should be noted that CDs do not affect the distribution profile of the drug between the major serum transport proteins (excepting free inclusion complexes mTHPC/CD). Data of mTHPC distribution between serum proteins and CDs obtained by chromatography are in full agreement with those obtained by fluorescence polarization.

3.2. Influence of the CDs on the rate of mTHPC cellular uptake

Cells were incubated with mTHPC alone (control) or mTHPC/CD complexes. mTHPC concentration was assessed by fluorescence after extraction and was either normalized to that of controls (Fig. 4) or presented in its absolute values (Fig.5).

As shown in Fig. 4, cellular uptake of mTHPC critically depends on M- β -CD and Hp- β -CD concentrations. Low concentrations of CDs result in considerable increase of mTHPC cellular uptake.

A maximal cellular uptake of mTHPC was observed at 10 μ M and 200 μ M of M- β -CD and Hp- β -CD, respectively. Further increase in CD concentrations is accompanied by a gradual decrease of mTHPC cellular uptake, which at the higher concentrations ranging from 60 μ M for M- β -CD and 2 mM for Hp- β -CD becomes lower than that in control. The listed above patterns were similar at short (3 h) and long (24 h) incubation times for both CDs (Fig. 4). It should be noted that the observed changes in the mTHPC accumulation by cells in the presence of CDs is not associated with a decrease in cell viability, because its level was not less than 95% compared to a control sample.

Fig. 5 shows the kinetics of mTHPC accumulation alone or in the presence of optimal CD concentrations that provide maximum rate of mTHPC cellular uptake. mTHPC alone shows gradual increase of PS cellular uptake without reaching a plateau during 24h. Addition of CD substantially increases the rate of mTHPC intracellular accumulation. The highest rate of mTHPC intracellular accumulation is observed immediately after the addition of CD with a gradual decrease in the rate of PS uptake with time. For example, within 0-3h of incubation the rates of mTHPC cellular uptake are 0.21, 0.60 and 1.63 $\text{pg}\cdot\text{cell}^{-1}\cdot\text{h}^{-1}$ while within 10-24 h of incubation the rates of PS uptake are 0.15, 0.16 and 0.15 $\text{pg}\cdot\text{cell}^{-1}\cdot\text{h}^{-1}$ for mTHPC alone, mTHPC/M- β -CD and mTHPC/Hp- β -CD, respectively.

3.3. Photocytotoxicity of mTHPC/CD inclusion complexes

To evaluate photo-induced cytotoxicity of mTHPC alone and its complexes with CDs at concentrations providing optimal PS accumulation (10 μ M for M- β -CD and 200 μ M for Hp- β -CD) we have determined the parameter LD₅₀, which was derived from dose-response curves. No dark cytotoxicity of mTHPC, CDs and their mixtures was observed at investigated conditions (data not show). Under photoirradiation mTHPC demonstrated a high cytotoxicity, which was significantly enlarged by the presence of CDs (Fig. 6). For short term incubation this CD-dependent effect was more evident. According to our data, for 3h incubation LD₅₀ decreased from 330 $\text{mJ}\cdot\text{cm}^{-2}$ for mTHPC alone to 70 $\text{mJ}\cdot\text{cm}^{-2}$ for mTHPC/M- β -CD and 27 $\text{mJ}\cdot\text{cm}^{-2}$ for mTHPC/Hp- β -CD. For longer duration of incubation these differences in the LD₅₀ values decrease. LD₅₀ values were 39 $\text{mJ}\cdot\text{cm}^{-2}$ for mTHPC alone, 14 $\text{mJ}\cdot\text{cm}^{-2}$ for mTHPC/M- β -CD and 8 $\text{mJ}\cdot\text{cm}^{-2}$ for mTHPC/Hp- β -CD after 24 h of incubation.

3.4. Fluorescent microscopy studies of mTHPC accumulation by HT29 cells

The intracellular distribution of free mTHPC and mTHPC/CD complexes was assessed using epifluorescence microscopy (Fig. 7). mTHPC fluorescence images revealed diffuse pattern of dye localization throughout the cytoplasm with fluorescence intensification in the perinuclear region sparing the nucleus. After incubation with M- β -CD, the overall level of mTHPC intracellular fluorescence increased. Simultaneously, we observed the appearance of bright spots in the periphery of cell fluorescence image indicating about heterogeneous distribution pattern of PS in the plasma

membrane (Fig. 7). Using Filipin III fluorescent we assumed the involvement of CD-mediated changes of plasma membrane cholesterol content into regulation of mTHPC binding to cells. Treatment of cells by 10 μ M of M- β -CDs and 200 μ M of Hp- β -CDs causes 15-20% decrease of mean fluorescence intensity of Filipin III in cells indicating reduction of cholesterol content in plasma membrane (data not show).

3.5. Biodistribution of mTHPC in tumor-bearing mice

Noninvasive fluorescence measurements with PDT fluorometer allow to obtain objective information about the kinetics of PS biodistribution in tumor bearing mice. *In vivo* kinetics of mTHPC fluorescence from the surface of grafted HT29 tumor and symmetrically located area of muscles tissue after i.v. injection of PS alone (curve 3) or its mixture with M- β -CDs (curves 1) are presented in Fig. 8. Injection of mTHPC alone demonstrates a gradual slow increase in the fluorescence intensity during 24 hours, while mTHPC/CD introduction causes a rapid rise of fluorescence signal from both tumor and muscles surfaces with the achievement of a plateau after 30 minutes. According to PDT fluorometry data, there were no significant difference between tumor and muscles fluorescence levels for both formulations of mTHPC.

Several organs and tissues of the injected mice were harvested for *ex vivo* fluorescence imaging to visualize mTHPC distribution 24h following injection (Fig. 9). Fluorescent imaging technique was to quantify mTHPC accumulation in these samples (Table 1). According to the data obtained, CDs strongly influence mTHPC distribution between different organs and tissues. Thus, introduction of mTHPC with CDs results in 1.5-3 times decreasing of PS accumulation in skin and muscles tissue. Another interesting observation deals with opposite changes in mTHPC accumulation in the kidneys and liver. Injection of mTHPC/M- β -CD based formulation decreased 1.5 fold the level of mTHPC in the liver and increased 1.8 fold mTHPC accumulation by kidneys compared to introduction of mTHPC alone. Similar changes were also observed in the case of Hp- β -CD based formulation. Changes in the level of PS accumulation associated with the use of CDs were expressed weaker in lungs and spleen, and no significant changes were observed in the heart. The PS distribution between the tumor (T) and normal muscle (M) tissue is of special interest. The highest mTHPC fluorescent signal in the tumor was observed when mTHPC introduced with M- β -CD. Calculated from obtained data T/M ratio for mTHPC/M- β -CD, mTHPC/Hp- β -CD and mTHPC alone decreased in a range $12 > 7 > 5$.

3. Discussion

Despite the high efficacy of mTHPC as a photosensitizer for PDT, its low water solubility causes non-optimal pharmacokinetics, biodistribution and relatively low bioavailability which induce adverse effects of mTHPC application. The use of CDs as carriers of porphyrins PS can be useful to avoid

aggregation and, hence, provide favorable characteristics of pharmacokinetics and biodistribution of drug (Kryjewski et al. 2015). It is well documented that mTHPC similarly to other arylporphyrins is able to bind 1 or 2 molecules of β -CD derivatives and the side phenyl groups penetrate into CD cavity (Demore et al. 1999). According to Bautista-Sanchez et al. (2005) binding constants for mTHPC with several β -CD derivatives are very high especially in the case of Hp- β -CD ($4.8 \times 10^4 \text{ M}^{-1}$) and M- β -CD ($1.2 \times 10^5 \text{ M}^{-1}$). So the effective formation of CD/mTHPC inclusion complex can be obtained in the whole plasma. Such an effect allows exploiting fluorescence measurements for direct mTHPC pharmacokinetic studies (Desroches et al. 2001).

Inclusion in CDs exerts a profound effect on physicochemical properties of guest molecules as they are temporarily locked or partially caged within the host cavity giving rise to beneficial modification of guest molecule properties. Indeed, in the case of hydrophobic drugs, CDs increase drug solubility, dissolution, circulation time thus enhancing its bioavailability (Loftsson and Brewster 2013; Stella and He 2008). Following this approach, it is reasonable to assume that β -CD derivatives can be used to improve the pharmacokinetics of mTHPC.

It is well recognized that the distribution of PS molecules during PDT of solid tumors may be defined by three main stages: PS interactions with plasma proteins after injection, Ps transfer from the bloodstream to the extravascular tissue and its interaction with intracellular targets within the tumor environment. Data obtained in this work allow assessing the possible influence of CDs on the mTHPC behavior at all these stages.

Plasma protein binding pattern is an important factor governing PS pharmacokinetics, biodistribution and efficiency during PDT. Previous studies have revealed 2 features of the interaction of mTHPC with plasma proteins. First, about 95 % of the sensitizer in plasma is associated with HDL and LDL, and the binding with HDL is twice higher than with LDL (Reshetov et al. 2012, Triesscheijn et al. 2007). Secondly, as compared with other non-polar drugs, mTHPC releases from lipoprotein carrier very slowly and proceeds on a timescale of hours (Sasnouski et al. 2006). According to the fluorescence polarization measurements mTHPC molecules in the presence of M- β -CD quickly dissociate from complexes with proteins, resulting in an equilibrium distribution attained in 10-15 minutes (Fig.1 curve 4). Since mTHPC dissociation from the inclusion complexes and subsequent binding to serum proteins is achieved even faster, it can be concluded that CD causes an acceleration of the diffusion displacements of sensitizer molecules in plasma.

The equilibrium distribution of PS in serum is dependent on CD concentration. According to our chromatography data (Fig. 3), at low concentrations of M- β -CD almost all mTHPC molecules are associated with serum proteins. This conclusion is consistent with the results of fluorescence polarization measurements (Fig. 2). At low CD concentrations (less than 5 μM for M- β -CD and 50 μM for HP- β -CD) in 2% FBS solution, the degree of mTHPC fluorescence polarization takes the

maximum value 25% and, hence, the relative weight of mTHPC inclusion complexes, is minor. Increasing the concentration of CDs shifts the equilibrium in the distribution of PS molecules towards the formation of inclusion complexes. As a result, at high concentrations of CDs, most of the sensitizer molecules are not bound to plasma proteins. It is obvious that the value of CD concentration required to change the mechanism mTHPC transport in the blood is determined by the constant of inclusion complex formation and may differ significantly in the case of use of various β -CD derivatives.

The formation of inclusion complexes in the plasma can have a significant impact on the distribution of mTHPC from the bloodstream and localization in extravascular tissues including tumor transformed one. Indeed, the interstitial transport of molecules and particles is characteristically described by their effective interstitial diffusion coefficient, which decreases as their size is increased (Pluen et al. 2001). The smaller CD/mTHPC inclusion complexes, which size is 1,5-2,0 nm, must penetrate into extravascular tissues much faster as compare to mTHPC/HDL (8-12 nm) or mTHPC/LDL (20-25 nm) complexes. In addition, significantly lower concentration of cyclodextrin in the tissue compared to the bloodstream stimulates the mTHPC dissociation from the complexes with CDs and its subsequent binding by tissue structures (cells, interstitial proteins).

The influence of CDs on the accumulation of arylporphyrins and some other porphyrin-like sensitizers in cellular systems have been studied by several research groups (Kitagishi et al. 2015; Kolarova et al. 2003, 2004). It should be noted that the results on this subject are extremely contradictory. In the study by Kolarova et al. (2004) the one hundred excess of Hp- β -CD in relation to the PSs 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin (TTPS₄) and Pd-TTPS₄ leads to a 2-fold increase of TTPS₄ and Pd-TTPS₄ uptake in melanoma G361 cells. At the same time, Kitagishi et al. (2015) have observed that the addition of TTPS₄ complexed with 2.4 times excess of trimethyl- β -CD leads to approximately 30% reduction of PS cellular uptake and consequently cell-photokilling ability in HeLa cells. Similarly, Dentuto et al. (2007) showed that the interaction of *chlorophyll a* with TM- β CD at a ratio 1:10, Hp- β CD (1:100) or Hp- γ -CD (1:100) results in a 2-4 times decrease of phototoxicity in Jurkat cell line.

Results presented in this paper show that accumulation of mTHPC by HT29 cells critically depends on Hp- β -CD and M- β -CD concentration and CDs may cause both acceleration and inhibition of mTHPC cellular uptake (Fig. 4). It is well documented that CDs are not able to penetrate into cells. So, their effect on accumulation of hydrophobic drugs should be attributed to increase the permeability by enhancing drug solubility, dissolution and thus making the drug available at the surface of the biological barrier – cellular membrane, from where it partitions into intracellular compartments (Loftsson and Brewster 2013). In such cases it is important to use proper CD concentration since excess may decrease the drug availability. Actually, at high concentrations of CD, the rate of mTHPC

accumulation in the cells is reduced due to the high probability of formation of inclusion complexes in the extracellular environment.

Another possible mechanism affecting mTHPC absorption may be mediated by CD-dependent perturbations in cellular membrane. CDs are able to bind several components of the cell plasma membrane that causes changes in membrane composition and structure, modifying its binding and barrier functions (Zidovetzki and Levitan 2007; Lopez et al. 2011). In this connection, it is interesting to consider the involvement of cellular cholesterol, the content of which significantly affects the relative affinity of porphyrin PSs to cellular and model biological membranes (Cohen et al. 1985; Zorin et al. 1997). Numerous studies have shown that exposing cells or model membranes to CDs results in removal of cellular cholesterol (Fenyvesi et al. 2008; Kiss et al 2010; Lopez et al. 2011). The degree of cholesterol depletion is a function of the CD derivative used, its concentration, incubation time, temperature and cell type. In particular b-CDs have been shown to be the most efficient sterol-acceptor molecule, apparently due to the diameter of its internal cavity that matches the size of these molecules (Zidovetzki and Levitan 2007). As observed in this work, changes in cholesterol-specific probe fluorescence Filipin III are fully consistent with the assumption of possible cholesterol depletion in the plasma membrane of cells HT29 under the action of CD. It is important to note that CDs can modify not only the content of cholesterol, but also to influence its distribution into the cellular membranes. The uneven distribution of cholesterol may lead to increased accumulation of mTHPC in the cholesterol depleted domains in the cell membrane. Indeed, Decker et al. (2013) concluded that mTHPC prefers cholesterol poor membrane compartments.

Our data demonstrate that the administration of mTHPC together with CDs significantly increase its photosensitized damage of HT29 tumor cells resulting in a decrease in the LD₅₀ value (Fig. 6). It is well known that the efficiency photosensitized damage of the PS correlated well with the level of its accumulation in cells. At the same time reducing of the LD₅₀ values for mTHPC administered together with CDs more significant than the increase in the accumulation level compared with PS alone. The photokilling of mTHPC/CD complexes in terms of equivalent concentration of PS appears 1.5-2 times more efficient for both CDs compared to mTHPC alone. This increase is unlikely due to the increase in the quantum yield of singlet oxygen $\Phi_{\Delta} (^1O_2)$. According to Bautista-Sanchez et al. (2005), depending on used techniques of singlet oxygen measurements, the values $\Phi_{\Delta} (^1O_2)$ were either not affected or were even 1.5 lower by the mTHPC/CDs associations. It can be assumed that the most likely reasons for the increase in efficiency of photosensitized damage are changing intracellular distribution of mTHPC, and the interaction of CDs with plasma membrane.

Changes of physico-chemical properties of sensitizer via inclusion complexation with CDs should obviously influence its distribution behavior in the body. Our results of *in vivo* fluorescence (Fig. 8) and fluorescent imaging of mTHPC distribution in various tissues (Fig. 9) confirm this

assumption. It was shown that administration of mTHPC alone into the blood leads to formation of large non-fluorescent aggregates distorting the pharmacokinetics and reducing drug bioavailability []. The gradual destruction of these aggregates is accompanied by an increase of monomeric mTHPC concentration in bloodstream and consequently its fluorescence intensity (Fig.8, curves 3,4). Co-administration of mTHPC with CDs provide PS monomerization that leads to a rapid attainment of plateau of *in vivo* mTHPC fluorescence (Fig.8, curve 1,2). This pharmacokinetic pattern should yield an increase in PS bioavailability after injection.

The fluorescence analysis of the mTHPC distribution in the various organs and tissues of HT29 tumor-bearing mice indicates considerable influence of CDs on these processes. It should be noted that the fluorescence intensity of the sensitizer studied by fluorescence imaging depends on the optical properties of studied tissues. Therefore, for comparison of PS accumulation in different organs should be taken into account their optical properties. At the same time the change in fluorescent signal in the same type of tissue makes it possible to objectively judge the influence of different formulation for the accumulation of PS. The tumor and muscles uptake indicates that complexation of mTHPC with CDs especially with M- β -CD results in higher tumor-to-muscles ratio than mTHPC alone that may induce a better selectivity of PDT treatment (Roby et al. 2007). Moreover, formulation of mTHPC with M- β -CD and Hp- β -CD showed lower drug levels in the skin compared to mTHPC alone (Fig. 9), thus indicating that the side effects with both CDs may prove less severe. It should be noted, that CDs are rapidly eliminated in the urine and can increase renal clearance of lipophilic water-insoluble drugs (Stella et al. 1999). This is in accordance with a significant increase in the fluorescence level of mTHPC in the kidney as well as a reduction in the PS fluorescence level in the liver when applying a formulation with M- β -CD compared to mTHPC alone. This indirectly confirms that even after administration, mTHPC remains associated with M- β -CD.

Clearly, the pharmacokinetics and biodistribution of mTHPC complexed with CDs are a superposition of different parameters, including volume of distribution and half-lives of CDs elimination, association constant and concentrations of PS and CD, binding constant and concentration of protein and competing agent, features of the interaction of CD and PS with tissues etc. Thus, the determination of complete mechanisms of CDs influence on the pharmacokinetics and biodistribution of tetrapyrrole compounds requires more detailed studies.

4. Conclusion

The use of cyclodextrins makes it possible to overcome most of the problems associated with the introduction of mTHPC into the body. CDs significantly increase the solubility and dissolution rate of mTHPC, preventing its aggregation. Unlike other delivery vehicles cyclodextrins at moderate concentrations provide a rapid serum protein binding and also accelerate its distribution in the body.

CDs cause a significant acceleration of mTHPC molecular mobility increasing the bioavailability of the photosensitizer. In addition, based on general considerations, it is possible to regulate the pharmacokinetics and biodistribution of mTHPC in the body through the use of CDs with different affinity to the drug, different concentrations and protocols of introduction (the sequence of drug administration).

Abbreviations

mTHPC - meta-tetra(hydroxyphenyl)chlorin

CD - Cyclodextrin

M- β -CD - Methyl- β -cyclodextrin

Hp- β -CD - 2-Hydroxypropyl- β -cyclodextrin

PDT - Photodynamic therapy

PS - Photosensitizer

PBS - Phosphate buffered saline

RPMI - Roswell Park Memorial Institute

FBS - Fetal bovine serum

P - The degree of fluorescence polarization

LD₅₀ - Lethal Dose, inducing 50% cell death

LDL - low density lipoproteins

HDL - high density lipoproteins

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Figure Legends

Fig. 1. Time-dependent changes of mTHPC fluorescence polarization degree in the process of mTHPC (0.2 μM) redistribution between M- β -CD and FBS (2 %). The kinetic curves 1, 2 refer to the transfer of mTHPC from M- β -CD to serum. The kinetic curves 3, 4 refer to the transfer of mTHPC from serum to M- β -CD. Concentrations of M- β -CD are (1) 1 μM , (2, 3) 10 μM and (4) 100 μM . Temperature T=25 $^{\circ}\text{C}$.

Fig. 2. Dependence of fluorescence polarization degree of mTHPC (0.2 μM) on concentration of M- β -CD (■) and Hp- β -CD (●) in 2% FBS solution. Temperature T=25 $^{\circ}\text{C}$.

Fig. 3. Sephadex G-200 chromatography of mTHPC (1.47 μM) incubated with 2% FBS for 2 h in the presence of various concentrations of M- β -CD. Elution curves of mTHPC (1) alone and in the presence of (2) 5 μM , (3) 10 μM , (4) 20 μM and (5) 200 μM of M- β -CD. Samples were incubated at 37 $^{\circ}\text{C}$. mTHPC profiles are measured by fluorescence in every eluted fraction of 2 mL.

Fig. 4. CD-dependent photosensitizer uptake. HT29 were incubated with 1.47 μM of mTHPC alone or mTHPC mixed with various M- β -CD (a) and Hp- β -CD (b) concentrations in medium supplemented with 2% of FBS for 3h and 24h. mTHPC uptake with CDs is normalized to mTHPC alone (controls, taken as 100%). Results are expressed as the mean with the vertical bar showing S.D. (n = 3). *p < 0.05 and **p < 0.01, compared with cellular uptake of mTHPC alone for a given incubation time.

Fig. 5. Accumulation profile of mTHPC alone (■), mTHPC complexed with 10 μM of M- β -CD (●) or 200 μM of Hp- β -CD (▲). Concentration of mTHPC is 1.47 μM . Results are expressed as the mean with the vertical bar showing S.D. (n = 3). *p < 0.05 and **p < 0.01, compared with cellular uptake of mTHPC alone for a given incubation time.

Fig. 6. Influence of CDs on mTHPC photosensitized damage of HT29 cells based on the LD₅₀ values. Keys; filled column, mTHPC alone (control); hatched column, mTHPC complexed with 10 μM of M- β -CD; empty column, mTHPC complexed with 200 μM of Hp- β -CD. Concentration of mTHPC is 1.47 μM . Results are expressed as the mean with the vertical bar showing S.D. (n = 3). *p < 0.05 and **p < 0.01, compared with cellular uptake of mTHPC alone for a given incubation time.

Fig. 7. Cellular uptake and intracellular distribution of mTHPC administrated alone or complexed with M- β -CD (10 μM) studied with fluorescence microscopy. HT29 cells were cultured on

glass slides and incubated with 1.47 μ M mTHPC alone or mTHPC/M- β -CD complexes for 1 h at 37 °C. Scale bar = 10 μ m.

Fig. 8. Comparison of in vivo mTHPC fluorescence kinetic profiles of the two different formulations from the surface of healthy tissue (muscles, curves 2, 4) or tumor (curves 1, 3) through the skin of HT29 tumor bearing mice. 0.5 mg/kg of mTHPC was injected alone (curves 3, 4) or in the presence of 2.5 mg/kg M- β -CD (curves 1, 2). Results are expressed as the mean with the vertical bar showing S.D. (n = 3-4).

Fig. 9. Estimation of CDs effect on mTHPC distribution in different organs and tissues of HT29 tumor-bearing mice assessed by fluorescence imaging. HT29 tumor-bearing mice were i.v. injected with mTHPC (0.5 mg/kg) either alone, in the presence of 2.5 mg/kg M- β -CD or 25 mg/kg Hp- β -CD. Twenty-four h after injection the mice were sacrificed, the selected organs were excised and subjected to small animal fluorescence imaging.

Table 1. Fluorescence intensity of mTHPC in various organs of tumor-bearing mice 24 h after injection

Formulations of mTHPC	Fluorescence intensity (a.u.)							
	Tumor	Muscles	Skin	Liver	Kidney	Spleen	Heart	Lung
Alone	196±26	38.7±5.6	323±45	198±41	135±32	60±6	85±12	248±6
M-β-CD	247±30*	20.6±8.9*	203±87*	130±36*	241±39**	54.3±4.2	85±11	150±5**
Hp-β-CD	143±28*	20.3±9.6*	119±23**	157±26	183±72	39±14*	77±5	204±33

The experimental conditions as indicated in the legend to Fig. 9.

Results are presented as mean ± SD (n= 3-4).

Significant difference compared to mTHPC alone: * p< 0.05; ** p<0.01.

Fig. 1 I. Yankovsky et al.

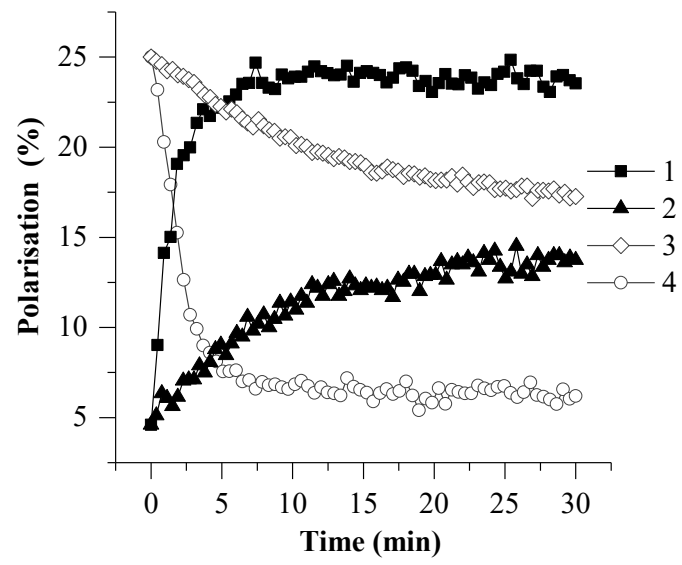


Fig. 2 I. Yankovsky et al.

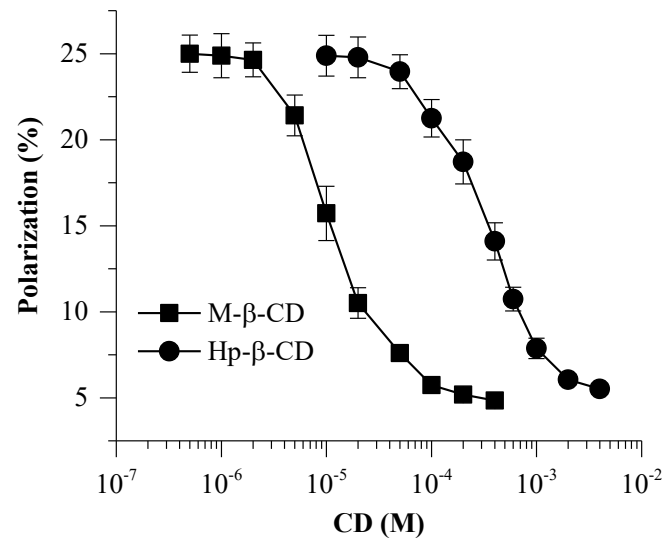


Fig. 3 I. Yankovsky et al.

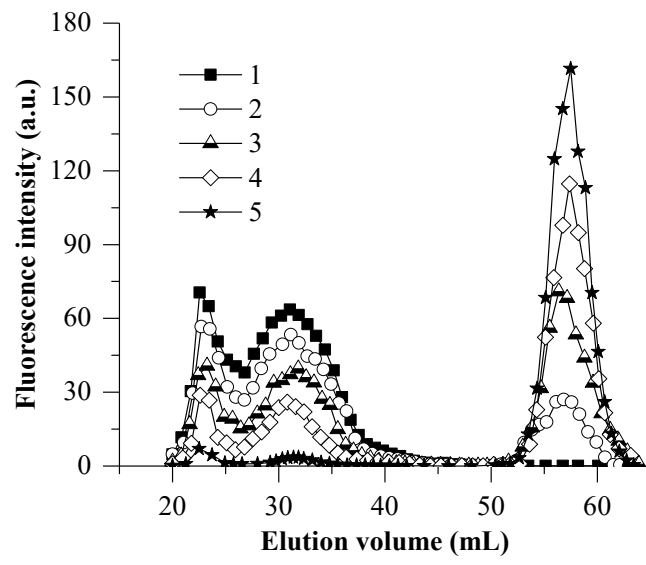


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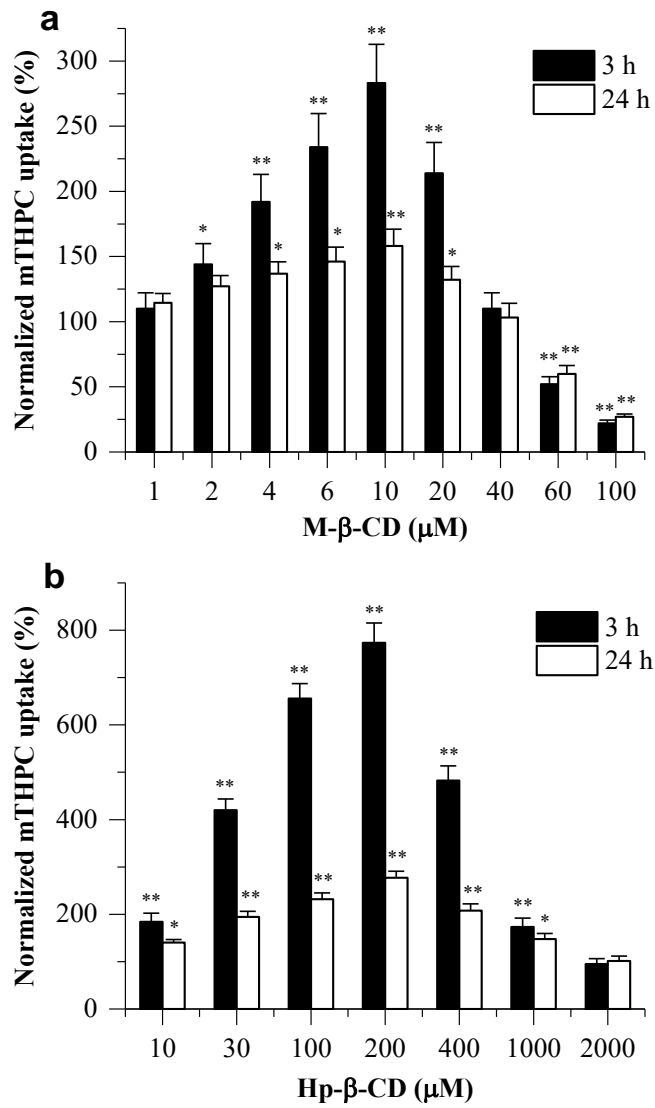


Fig. 5 I. Yankovsky et al.

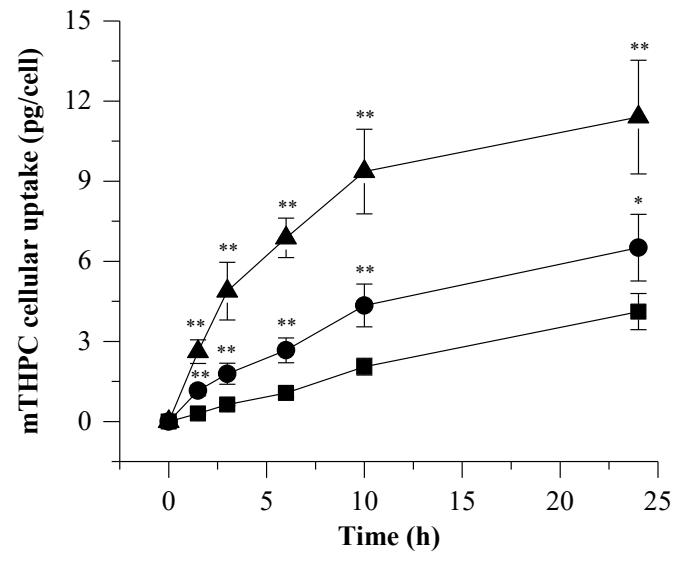


Fig. 6 I. Yankovsky et al.

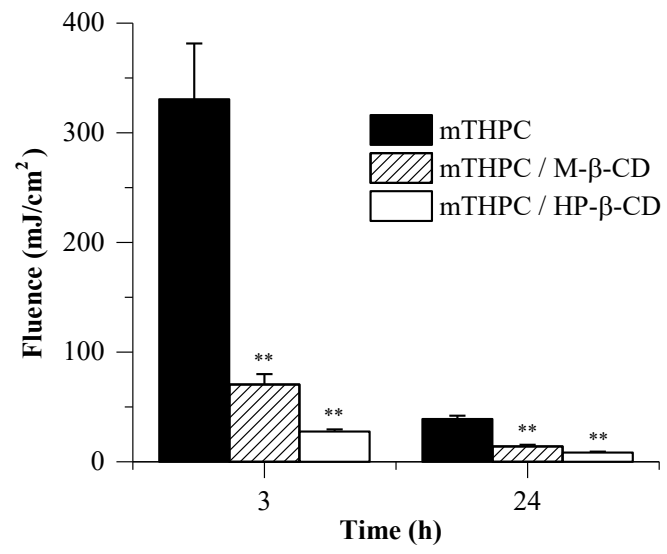


Fig. 7 I. Yankovsky et al.

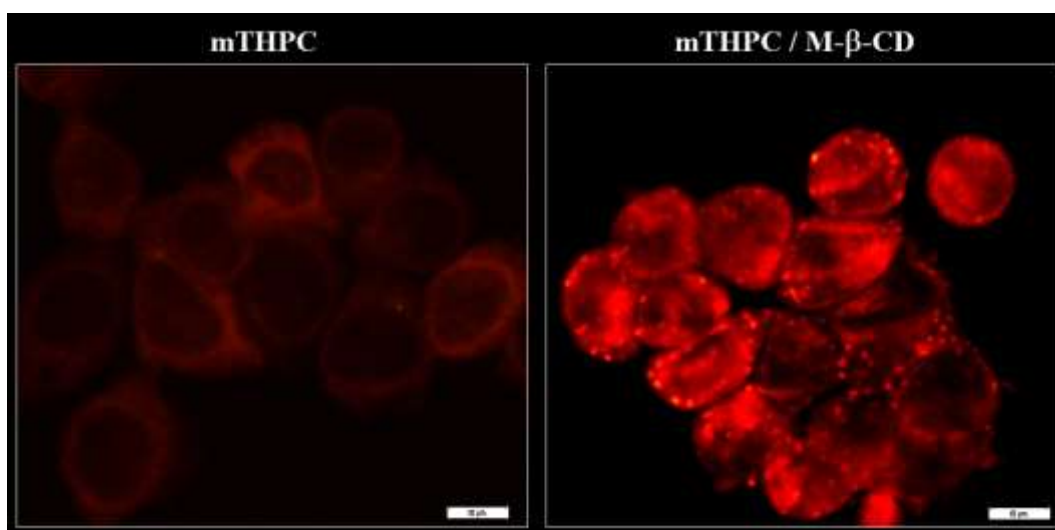


Fig. 8 I. Yankovsky et al.

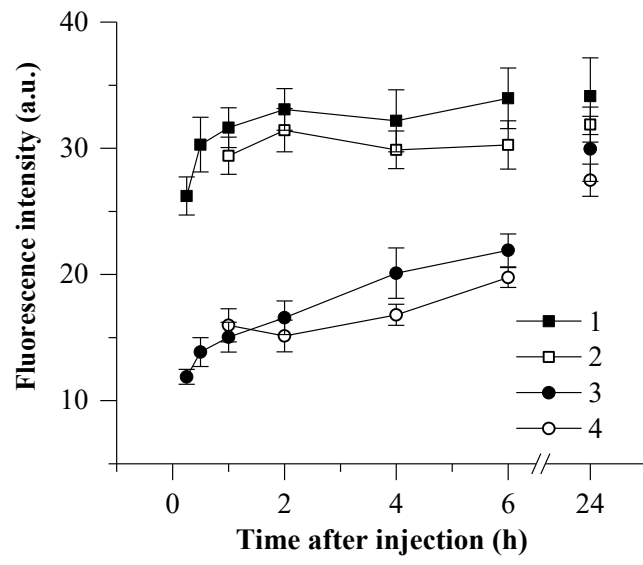
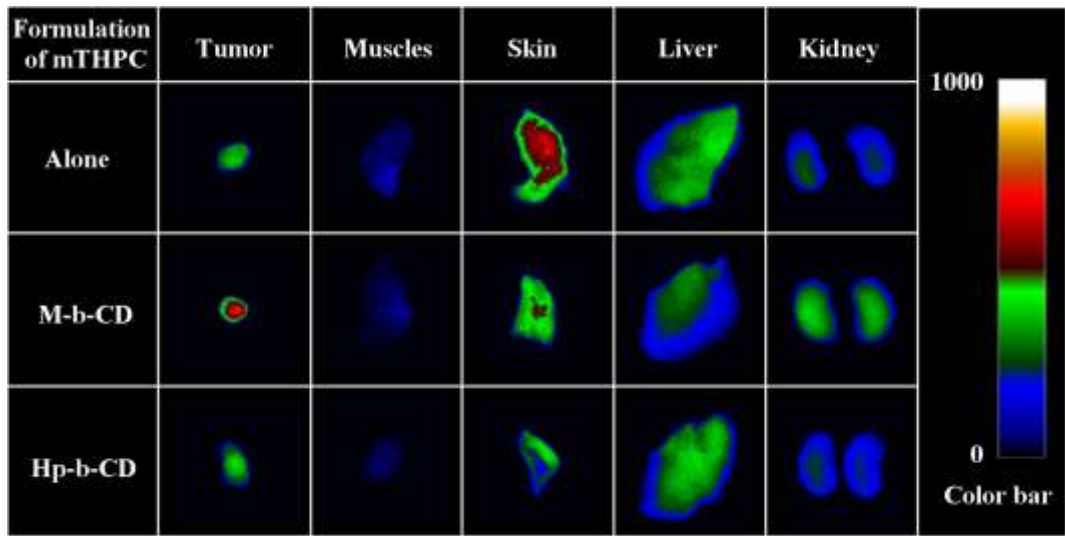


Fig. 9 I. Yankovsky et al.



Graphical abstract I. Yankovsky et al.

