pH effect on cellular uptake of Sn(IV) chlorine e6 dichloride trisodium salt by cancer cells in vitro

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Abstract The effects of pH value and presence of serum in an incubation medium on photosensitizer drug cellular uptake in MCF7 cancer cells have been investigated. The results showed that the presence of serum in an incubation medium reduced the drug cellular uptake at all pH values. It has been found that decreasing pH values of the incubation medium increased the cellular uptake of the drug, demonstrating selective uptake of the sensitizer. The HepG2 liver cancer cells exhibited more drug cellular uptake than CCD-18CO normal colon cells, which assessed the selectivity uptake of photosensitizer on cancerous cells. The concentration of photosensitizer measured in 10^6 cells showed a good correlation to the incubation time. Fluorescence and absorption spectroscopy been have used to examine the cells.

Keywords Sn(IV) chlorin e6 · Photodynamic therapy · Cellular uptake

1 Introduction

Photodynamic therapy (PDT) is considered as a preferable treatment for a variety of malignant and nonmalignant diseases. PDT is based on a combination of suitable wavelength of light and photosensitizer. To avoid damage of normal cells, it is important to reduce the PDT treatment area. The selectivity of tumor uptake for PDT allows a reduction of the illumination to the tumor tissue. Chlorine has been widely used as photosensitizing agent of PDT due to its selective tumor uptake in tissues and efficient phototoxicity [1]. There is still ongoing investigation on certain properties of these substances regarding to tumor selective uptake and phototoxicity [2]. Researchers have found tumor-selective uptake in hematoporphyrin derivatives (HpD) [3].

Various photosensitizers have been applied in PDT. Previous studies reported that photosensitizers are selectively distributed in tumor tissues [4–7]. In comparison, the pH

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in the tumor tissues is lower than in normal tissues, which could play a role in selective distribution of photosensitizer [8]. The change in the lipophilicity is related to pH dependant drug uptake [9, 10]. It is found that the cancer cells and proliferating cells were depolarized in comparison with normal nonproliferating cells [11, 12]. The points of interest are the effect of pH value on cells and the addition of serum to the medium, which are considered as a function of incubation time of the photosensitizer in the medium which contains the cells. This work has studied whether pH dependence affects the cellular uptake of photosensitizer (Sn (IV) chlorine e6 dichloride trisodium salt) or not. The uptake of photosensitizer in MCF7 breast cancer cells at three pH values corresponding to pH in tumor with and without serum in an incubation medium were investigated. The cellular uptake of the photosensitizer in HepG2 liver cancer cells and CCD-18CO colon normal cells is studied as well.

2 Methodology

2.1 Cell culturing

Photosensitizer (Sn (IV) chlorine e6 dichloride trisodium salt) was purchased from Frontier Scientific Inc. (Logan, USA); its chemical structure is shown in Fig. 1. A stock solution of 1 mg/ml was prepared by PBS in adding 1 mg of Sn (IV) chlorine e6 into 1 ml of PBS and kept in the dark at 4°C until use. Cultured MCF7 breast adenocarcinoma, and HepG2 liver carcinoma, and normal cultured CCD-18CO colon cell lines were purchased from American Type Culture Collection (ATCC), USA. All chemical products were purchased from Sigma (Malaysia). All cells were incubated in Eagle's minimal essential medium supplemented, under an environment of 5% CO₂ and 37°C, with 10% fetal bovine serum (FBS), 1% penstrep (mixture of penicillin and streptomycin used in cell cultures as an antibiotic), 1% nonessential amino acid, 1% sodium pyruvate, 1% L-glutamine, and 0.1% bovine insulin for MCF7 cells, with 10% FBS, 1% penstrep, 1% nonessential amino acid, 1% sodium pyruvate, and 1% L-glutamine for HepG2 cells, and with 10% fetal bovine serum, 1% L-glutamine, 1% nonessential amino acid, and 1% penicillin/streptomycin for CCD-18CO normal cells.

2.2 Cellular uptake

A carbon dioxide sodium-bicarbonate system has been used to prepare the pH values of medium [13–15]; 10⁶ cells/well were incubated for 24 h at 5% CO₂ and 37°C in the culture

Fig. 1 The chemical structure of Sn(IV) chlorine e6 dichloride trisodium salt

Fig. 2 Absorption of Sn(IV) chlorine e6 dichloride trisodium salt

medium. The culture medium was used with NaHCO_3 (2.0 g/l for pH 7.3, 7.6 and 0.5 g/l for pH 6.7) to retain a constant pH value, and both with and without supplemented serum. A Hach Sens Ion pH meter was used to measure pH values of the media. The partial pressures of CO_2 in the incubator were 3.5%, 5%, and 17% for pH 7.6, 7.3, and 6.7, respectively. The media were pre-incubated overnight without cells under mentioned CO_2 partial pressures. The cells were washed twice with pre-incubated medium. The cells were then incubated in pre-incubated medium, which contained 10 $\mu\text{g/ml}$ of photosensitizer from stock solution prepared in PBS (1 mg/ml). The medium was removed and the cells washed in PBS, then incubated in 3 ml of 100% methanol for 1 h at 37°C to extract photosensitizer out of cells after 2–12 h incubation times [16]. All phases of this work were carried out in darkness at room temperature without photosensitizer as a control and with photosensitizer, to measure the fluorescence and absorption of the medium. Fluorescence spectra were recorded using a Jasco FB-750 Spectrofluorometer (Jasco, UK) with a 1×1 cm crystal cuvette. The emission and absorption wavelength was 635 nm, and the excitation wavelength was 410 nm. The fluorescence data are mainly presented in relative units. Absorbance spectra of the extracted photosensitizer were recorded using a U-2000 Hitachi spectrophotometer. The extracted methanol control sample was used as a reference. The concentrations and absorption of photosensitizer in the cells were calculated. The absorption and fluorescence spectra of the photosensitizer are shown in Figs. 2 and 3, respectively.

Fig. 3 The fluorescence excitation spectra of Sn(IV) chlorine e6 dichloride trisodium salt

3 Results and discussion

Drug uptake of photosensitizer of MCF7 cancer cells at different pH values was investigated both with and without presence of fetal bovine serum, as shown in Fig. 4a–c. In all figures, error bars show standard deviation over three measurements. The presence of 10% FBS in the incubation medium caused a 60–75% reduction of the cellular uptake of the photosensitizer. Figure 4 shows the absorption of the extracted photosensitizer at the Soret band, with and without presence of serum at pH values of 6.7, 7.3, and 7.6, respectively. The presence of serum could reduce the amount of the drug taken up by the cells; as a result, the absorbance is reduced, but the serum would probably not reduce the absorption of the photosensitizer.

Fig. 4 The effect of serum on cellular uptake of photosensitizer in MCF7 cancer cells showed by Soret band absorption for different pH values

Fig. 5 The fluorescence of cellular uptake of photosensitizer in MCF7 with 10% FBS, at different pH values, and different incubation times: pH 6.7 (solid line), pH 7.3 (dashed line), and pH 7.6 (dotted line)

Fig. 6 The fluorescence of cellular uptake of photosensitizer in MCF7 without 10% FBS, at different pH values, and different incubation times: pH 6.7 (solid line), pH 7.3 (dashed line), and pH 7.6 (dotted line)

Fig. 7 The absorption at Soret band of cellular uptake of photosensitizer in MCF7, with 10% FBS at different pH values: pH 6.7 (solid line), pH 7.3 (dashed line), and pH 7.6 (dotted line)

Fig. 8 The absorption at Soret band of cellular uptake of photosensitizer in MCF7, without 10% FBS at different pH values: pH 6.7 (*solid line*), pH 7.3 (*dashed line*), and pH 7.6 (*dotted line*)

Fig. 9 Fluorescence emission of extracted photosensitizer in HepG2 cancer cells with and without 10% FBS at different incubation times: without 10% FBS (*solid line*), with 10% FBS (*dashed line*) at various incubation times and pH value of 7.3

Fig. 10 Maximum absorbance of extracted photosensitizer in HepG2 cells at Soret band with and without serum at different incubation times and pH value of 7.3

Table 1 Concentration of Sn(IV) chlorin e6 in HepG2 cancer cells with, and without the presence of 10% FBS at pH value of 7.3

Incubation time (h)	Concentration of Sn(IV) chlorin e6 in HepG2 cells without 10% FBS $\mu\text{g}/10^6\text{cells}$	Concentration of Sn(IV) chlorin e6 in HepG2 cells with 10% FBS $\mu\text{g}/10^6\text{cells}$
2	0.48 ± 0.02	0.3 ± 0.02
4	0.78 ± 0.02	0.48 ± 0.02
6	1.54 ± 0.02	0.92 ± 0.02
8	1.68 ± 0.02	1.08 ± 0.02
10	1.92 ± 0.02	1.18 ± 0.02
12	2.02 ± 0.02	1.24 ± 0.02

The cellular uptake of photosensitizer was strongly affected the pH values of the incubation medium, both with and without presence of serum. The drug uptake in MCF7 cancer cells increased with decreasing pH values as shown in Figs. 5 and 6. The cellular uptake of photosensitizer without presence of serum increased rapidly and reached a steady state after 6 h of incubation as shown in Fig. 6. Figures 7 and 8 show the absorption of the extracted photosensitizer at the Soret band with, and without serum. Initially, there is a rapid increase in absorption, which reaches a steady state after 8 h as shown in Fig. 8. The absorption of the extracted photosensitizer with serum resulted in a less steep curve as shown in Fig. 7. A standard absorption curve of known concentration was obtained, and used to determine the photosensitizer concentration.

Drug uptake of photosensitizer in HepG2 cells with and without presence of serum was studied using fluorescence and absorbance spectroscopy as shown in Figs. 9 and 10, respectively. The presence of serum reduced the photosensitizer uptake in the cells for both fluorescence and absorption. The concentrations of photosensitizer were calculated, as shown in Table 1. The presence of serum reduced the cell uptake by 25–43%. Figs. 11 and 12 showed the uptake of photosensitizer in HepG2 cancer cells and CCD-18CO normal cells, with and without 10% FBS. In comparison, the uptake of photosensitizer is higher in cancer cells than in normal cells with and without presence of serum at all incubation times with pH value of 7.3. Finally, the absorption data supported the fluorescence emission results. The chemical structure and lipophilicity of a photosensitizing drug are responsible for the drug's uptake mechanisms. The uptake mechanisms are probably influenced by the change on drug lipophilicity as a result of the pH value by the incubation medium [17].

Passive and active transport are the main responsible mechanisms for drug cellular uptake [18]. Passive transport depends on the electrochemical gradient over the cellular membrane. The electrical potential difference across the plasma membrane favors the entry

Fig. 11 Cellular uptake in HepG2 liver cancer (filled) and CCD-18CO colon normal cells (not filled) with 10% FBS in incubation medium at different incubation times and pH value of 7.3

Fig. 12 Cellular uptake in HepG2 liver cancer (*filled*) and CCD-18CO colon normal cells (*not filled*) without 10% FBS in incubation medium at different incubation times and pH value of 7.3

of positively charged ions into the cells and opposes the entry of negatively charged ions. The active transport occurs mainly during receptor-mediated endocytosis when the cells internalize drugs with inward budding of plasma membrane vesicles and transports them to the lysosomes. Due to this process, passive transport across the cellular membrane is a much more rapid process than active transport. The rapid increment of photosensitizer uptake with and without presence of serum showed that the uptake mechanism is passive transport across the plasma membrane.

pH values clearly affect photosensitizer uptake. The cellular uptake of photosensitizer is significantly lower with serum than without serum due to the binding of the drug to serum components. Therefore, these results are found to be consistent with Barel et al. and Jori et al., when they mentioned that 80–90% of photosensitizing drugs bound to different serum proteins [19–21].

A modification of the binding behavior of the photosensitizer to different serum components resulted from decreasing in pH value. The acidification of the medium resulted in higher lipophilicity of photosensitizer, which is considered to increase the cellular uptake with low pH values.

In comparison, HepG2 cancer cells and CCD-18CO normal cells revealed that the drug cellular uptake in cancer cells is higher than in normal cells, by a factor of 1.55.

4 Conclusion

Protonation of hydrophilic drugs is considered as a photosensitizing mechanism and used to increase lipophilicity, which enhances cellular uptake. The result was assessed using HepG2 cancer cells and CCD-18CO normal cells; it is found that the uptake of the drug in the cancer cells is more than in the normal cells. The effect of serum on the cellular uptake decreased with increasing pH value. The cellular uptake of the photosensitizer is significantly higher without presence of serum than with presence of serum, which is due to the binding of the drug to serum components. The concentration of free photosensitizer molecules in the incubation medium without serum is expected to be higher than in medium with serum. These increments tend to increase aggregation, which finally increased the cellular drug uptake. Photosensitizer showed a pH dependent cellular drug uptake. The acidification of the environment effected a protonation of carboxylic groups, which made the photosensitizer more lipophilic and, therefore, enhanced the cellular drug uptake by passive diffusion. Thus, we observed pH dependent increasing in cellular uptake of porphyrin-type photosensitizer in vitro due to the modification of the drug lipophilicity. The reduction of pH value of the incubation medium caused a modification of the binding

behavior of the photosensitizer for different serum components. The acidification of the medium resulted in higher lipophilicity of the photosensitizer, which could increase the cellular uptake at low pH values. The presence of Sn(IV) in the centre of pyrole ring makes the photophysical properties of the photosensitizer more stable with change on pH values in media.

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