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ORIGINAL ARTICLE

Comparative study of photodynamic activity of methylene blue in the presence of salicylic acid and curcumin phenolic compounds on human breast cancer

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Abstract
Curcumin and salicylic acid are both phenolic compounds and they can both affect cancer treatment efficacy. In this study, the effects of methylene blue-curcumin (CU-MB) and methylene blue-salicylic acid (SA-MB) ion pair complexes on MDA-MB-231 human breast cancer cells are studied. According to the thermodynamic parameters, the stability of curcumin and salicylic acid complexes ion pair complexes was compared. The free energy of ion pair interactions was calculated based on binding constants. A comparison of the free energies of the complexes (CU-MB: $\Delta G^{\circ}_{b1} = -21.11$ kJ/mol and $\Delta G^{\circ}_{b2} = -8.37$ kJ/mol, SA-MB: $\Delta G^{\circ}_{b1} = -12.92$ kJ/mol and $\Delta G^{\circ}_{b2} = -9.02$ kJ/mol) indicates that the interaction of methylene blue in first binding interaction with curcumin is greater than that of methylene blue with salicylic acid. Electrostatic interactions are the main forces in the binding of both compounds to methylene blue. All forces are inter-molecular physical interactions. The results of cellular experiments show that ion pairing has enhanced the reduction of cell viability. By increasing molecular stability and prevention of dimerization of methylene blue, the cell killing potential of methylene blue increases and it subsequently causes enhancement of photodynamic efficacy.

Keywords Photodynamic therapy · Molecular stability · Polyphenolics · Biothermodynamics · Photosensitizer

Introduction

A wide range of drugs are amphiphilic, ionizable, weak acids or bases and are dissociated to release cations and anions under body's physiological conditions. Penetration of hydrophilic ionizable drugs across cell membranes, as a physiological barrier, is limited by structural properties. Hydrophilic ionic compounds are known to be poor penetrants across biological membranes, non-porous polymers, and artificial membranes. Swarbrick and coworkers reported that the permeation coefficient of these drugs has been estimated to be about 10^4 times smaller than that of uncharged compounds [1, 2]. Various techniques have been developed for increasing the penetration of hydrophilic drugs by using penetration enhancers [3]. These enhancing materials may have the potential to cause skin irritation. Therefore, more effective and safer penetration enhancement techniques must be developed. Ion pairing of ionizable drugs has recently been reported for enhancement of membrane permeability [4–11]. The complex is composed of oppositely charged ions which are bound together by coulomb forces and electrostatic interactions without covalent or chemical bonds.

Ionic compounds have high water solubility that leads to poor membrane penetration and consequently, it decreases cellular uptake [12]. Ion pairing reduces or neutralizes the total electrostatic charge of the complex and increases the total hydrophobicity of ion pairs. Drug penetration increases by enhancing hydrophobicity due to hydrophobic structural properties of cellular or artificial membranes [4, 5, 13, 14]. However, it must be noted that the hydrophobicity/hydrophilicity balance

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is very important in functioning of drugs and their distribution in cells and body fluids [15]. Some reports suggest application of salicylic acid and its derivatives in cellular uptake of hydrophilic drugs (S. A. Megwa et al. 2000; S. A. Megwa, Cross, Benson, and Roberts 2000). Bioavailability of hydrophilic drugs, i.e., pholedrine and bretylium, is enhanced by pairing with hexyl-salicylate after oral and rectal application [3, 13]. Ion pairing of salicylate using alkyl amines increases the penetration of salicylate through shed snakeskin. The effects of alkyl chain type and length were investigated by Megwa and coworkers. According to in vitro studies, secondary, tertiary, and quaternary amines increase salicylate permeability through human epidermal membranes. The permeability enhancement effect of tertiary amines is greater than that of other types of amines and it was found to increase by increasing alkyl chain length of amines [13, 16]. Bioavailability of various drugs and therapeutic compounds depends on their molecular structure. In other words, it may be suggested that ion pairing can enhance permeability of both hydrophilic and hydrophobic drugs by balancing the hydrophobic and hydrophilic interactions [12].

In this study, the effect of salicylate and curcumin on cellular bioavailability of methylene blue is investigated by considering spectrophotometric molecular interactions and biothermodynamic aspects. Methylene blue is a water soluble photosensitizer which is used in photodynamic therapy. The reduction of methylene blue to leuco-methylene blue in the body and its high water solubility lead to low photodynamic performance [17]. Salicylic acid was used as a cancer chemotherapeutic agent [18]. Also, many studies have reported curcumin as antioxidant photoactive biomaterials that can be used in cancer treatment [19]. Nowadays, application of natural plant metabolites such as curcumin and salicylate phenolic compounds in treatment of diseases has attracted a lot of interest from the scientists due to their inexpensive availability, biocompatibility, and lower side effects. The bioavailability enhancement of methylene blue and its counter ion (salicylic acid and curcumin) can increase the cellular uptake of both ion pairing complexes which would consequently increase their therapeutic effects.

Materials and methods

Dimethyl sulfoxide (DMSO), salicylic acid, methylene blue, and tryphan blue were supplied by Merck (Germany). Curcumin, tetrazolium dye, MTT, and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich. Antibiotics and fetal bovine serum (FBS) were purchased from Gibco (Gibco BRL). DMEM medium (Dulbecco's Modified Eagle Medium) was purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA). Buffer salts and other chemicals were supplied by Merck Co. Fresh double distilled deionized water was used for preparation of solutions. Human breast cancer cell line, MDA-MB-231, was provided by the Institute of Pasture, Tehran, Iran.

The UV–Vis absorption spectra were recorded by using a Cary 60 UV–Vis spectrophotometer, equipped with quartz cells. Red light emitting LED (660 nm; power density 30 mW cm^{-2}) was used as a light source. Spectrophotometric ELISA reader (Hyperion, Inc., FL, USA) was used for cell viability measurements.

Spectrophotometric study of methylene interactions with curcumin and salicylic acid

Photosensitizer (methylene blue) (5×10^{-6} mol L^{-1}) and analytes (curcumin and salicylic acid) stock solution was prepared by dissolving a specific amount of each in double distilled deionized water. Variations of absorbance spectrum of methylene blue with increased analytes concentrations were recorded at 200–800 nm wavelength using water as a blank. The obtained data were analyzed and molecular binding constants were determined using a suitable theoretical method.

MDA-MB-231 human breast cancer cell culture

The cells were grown using DMEM medium containing fetal bovine serum (FBS 10%), penicillin (100 IU/mL) and 100 μg/mL of streptomycin. Then, they were incubated in a humidified incubator containing 5% CO_2 at 37 °C. For the experiments, the cells were removed by trypsinizing (trypsin 0.025%, EDTA 0.02%) and washed with phosphate-buffered saline (PBS), and re-cultured and treated using the mentioned compounds.

Cell viability determination by MTT assay

Cellular viability was determined using colorimetric MTT assay. Cell viability can be measured as a function of cells redox potential. Living cells convert the MTT to an insoluble formazan form. The resulting formazan is soluble in dimethyl sulfoxide (DMSO) by inducing color change and its concentration can be determined using spectrophotometric methods. Briefly, culture medium was removed after treatment and the cells were incubated in a medium containing 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for 4 h at 37 °C. The resulting purple formazan crystals were dissolved in 100 μL DMSO and shaken for 15 min.

The absorbance of solutions was measured at 570 nm by an ELISA reader (Hyperion, Inc., FL, USA). All experiments were repeated three times.

In vitro photodynamic experiment

The MDA-MB-231 breast cancer cell line was grown in medium culture cell and the cells were washed with PBS after

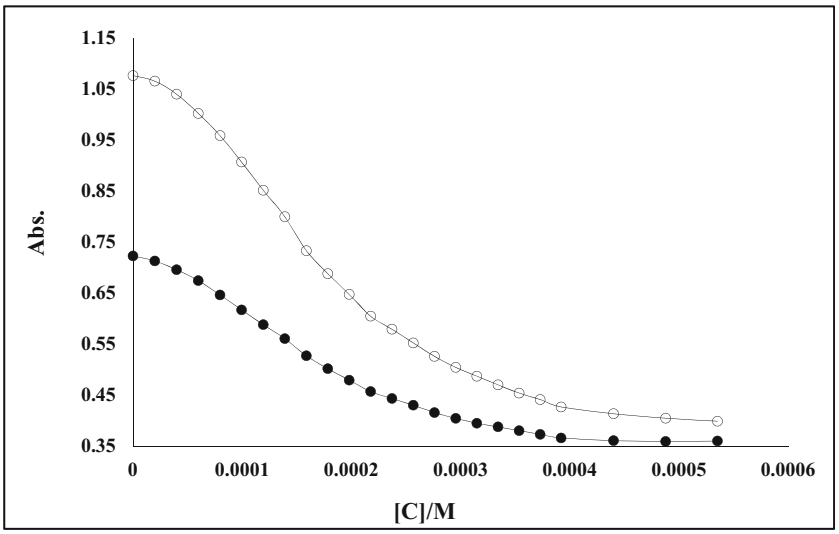

Fig. 1 Alternation in absorbance at 620 nm (dimeric form of MB) (●) and 668 nm (monomeric form of MB) (○) by increasing of curcumin concentration

reaching 80–90% confluence. Then, they were detached from the flask by addition of 1.0 mL of 0.25% trypsin for 1–3 min at 37 °C. The cells (1×10^4 cells/well) were seeded into 96-well plates. The cells were treated with analytes at different concentrations (4-h incubation at 0, 10, 25, 50, and 100 μg/mL) and various experimental conditions (dark and irradiation). After a certain incubation time, one plate was considered as dark (control plate) and the other plates were illuminated as PDT experiment. The treated cells were used for cell viability study using MTT assay. All experiments were repeated three times.

Statistical analysis

Statistical analysis was performed with Student's *t* test (two-tailed). All values are expressed as means ± SD. The results are expressed with *n* denoting the number of experiments.

Results and discussions

Spectroscopic and thermodynamic studies

Titration of methylene blue solution using salicylic acid and curcumin stock solutions makes bathochromic or red shift in the absorption spectra of methylene blue (MB). This effect is due to increased hydrophobicity of compounds by ion pair interactions. Electrostatic interactions have occurred between the positive charges of methylene blue molecules with the negative charge counter ions by addition of opposite charge counter ions to the methylene blue solution. According to the physicochemical properties of ionic compounds, it is clear that the net electrostatic charge on molecules increases the solubility of ionic compounds in polar solvents such as water. The water solubility of these materials decreases by a reduction of the net charges of ionic molecules. Hydrophobic forces

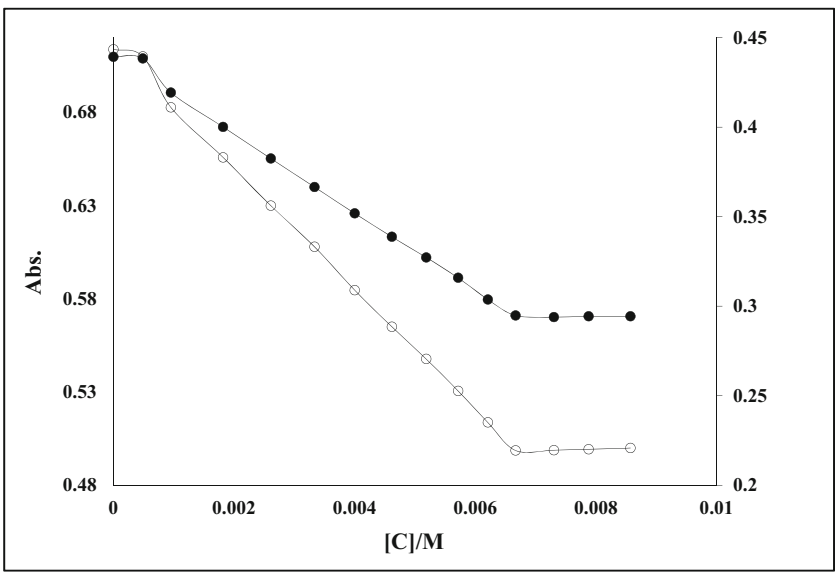

Fig. 2 Alternations in absorbance at 620 nm (dimeric form of MB) (●) and 668 nm (monomeric form of MB) (○) by increasing salicylic acid concentration

Table 1 Thermodynamic parameters related to the first and second binding sets in MB interaction with curcumin and salicylic acid, obtained based on the Benesi-Hildebrand equation

	K_{b1}	K_{b2}	ΔG_{b1} (kJ/mol)	ΔG_{b2} (kJ/mol)
MB-CU	5016.25	29.32	− 21.11	− 8.37
MB-SA	183.97	38.12	− 12.92	− 9.02

increase with the reduction of electrostatic interactions between ionic compounds with polar bulk solution. Electron transfer between electron layers of molecules can be changed by alternation in molecular forces around the target molecule. Therefore, the microenvironment of molecules has a great effect on electron transfer between electron layers that could be detect by molecular absorption spectrophotometry.

The methylene blue UV–Vis spectrum shows the strong absorption band in the 500–700 nm. The maximum absorbance can be seen at 664–668 nm with molar absorptivity coefficient of 85,000 M^{-1} cm^{-1} at 664 nm [17]. Dimerization of methylene blue is depending on concentration and its equilibrium constant is 3.8×10^3 M^{-1} in water. Figure 1 represents reduction of absorbance related to monomeric form (668 nm) and dimeric form (620 nm) of methylene blue with increased curcumin concentration. Absorbance changes prove the interaction of methylene blue with curcumin. The reduction of absorbance at 668 nm has a sharper slope when compared with the reduction in absorbance at 620 nm. Moreover, Fig. 2 shows the variation of absorbance related to the dimeric form (620 nm) and the monomeric form (668 nm) of methylene blue with increased concentration of salicylic acid. According to Figs. 1 and 2, it is clear that the interaction between the monomer form of methylene blue is greater than the interaction of the dimmer form with curcumin and salicylic acid.

According to the principals of equilibrium of reactions, dimerization of methylene blue decreases upon decreasing monomer concentration. In the presence of both counter ions, the ion pairing reaction decreases the dimerization of methylene blue. It should be noted that the active form of methylene blue in PDT is monomeric and dimerization decreases PDT efficacy. Thermodynamic parameters of methylene blue interaction with curcumin and salicylic acid have been obtained (as shown in Table 1) [20, 21]. The binding constants of methylene blue interactions are obtained based on the Benesi-Hildebrand method. In both interactions, 1:2 binding complex between methylene blue and analytes is induced. The first binding set is related to electrostatic and hydrophobic interactions and the second binding set is related to hydrophobic interactions. Gibbs free energy of binding sets is calculated based on the obtained binding constant.

According to the obtained binding constants and the associated Gibbs free energies, it is clear that the interactions are inter-molecular and weak. The first sets are related to inter-ion electrostatic interactions and binding is a spontaneous process. The second binding sets are related to inter-molecular Van Der Waals interactions and hydrophobic bindings. The results show that the interaction of methylene blue with curcumin in the first binding set is greater than salicylic acid. The strength of the second binding set is similar in both curcumin and salicylic acid. This effect shows that curcumin-methylene blue ion-pair formation is more favorable than salicylate-methylene blue. Electrostatic interactions are the main forces in binding of both compounds to methylene blue. By addition of curcumin and salicylic acid, the hydrophobic forces increase due to ionic neutralization. After complete ionic neutralization of free methylene blue molecules, hydrophobic interactions are the main forces.

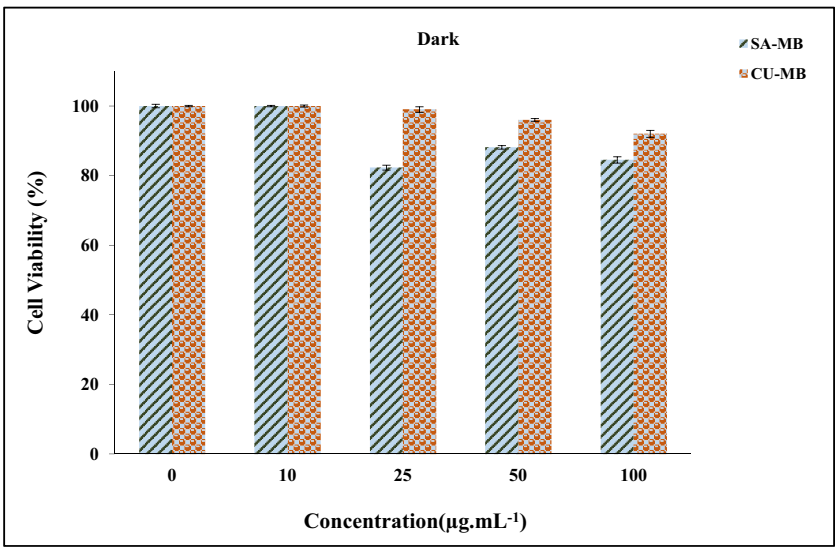

Fig. 3 Dark cytotoxicity of ionic complex of methylene blue, SA-MB (salicylate-methylene blue) and CU-MB (curcumin-methylene blue) complexes. The results are expressed as mean ± SD ($n = 3$)

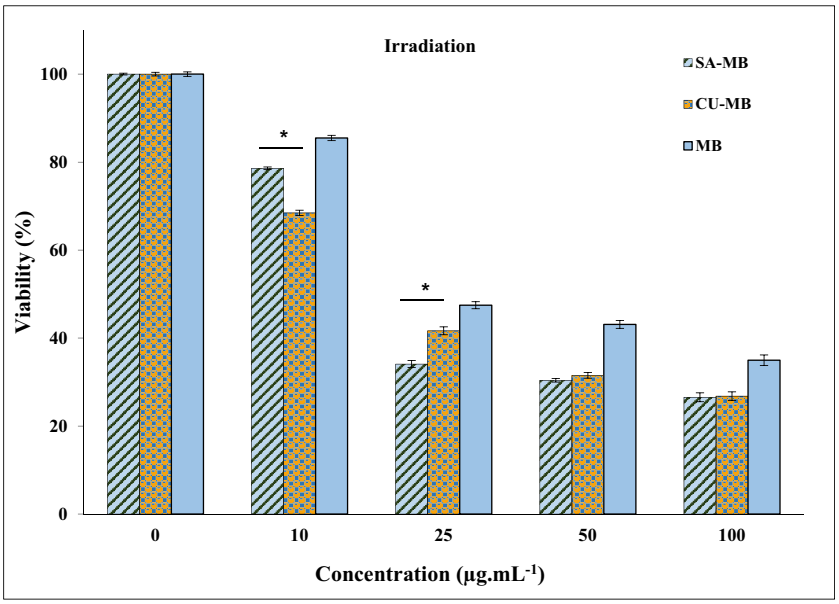

Fig. 4 Cell viability of MDA-MB-231 breast cancer cell line treated with MB, SA-MB (salicylate-methylene blue), and CU-MB (curcumin-methylene blue) complexes under red light irradiation. The results are expressed as the mean ± SD ($n = 3$, $^*P < 0.05$ the SA-MB group compared with CU-MB group)

Comparing dark cytotoxicity of ion-pairing complexes

The dark toxicity of the complex was studied. In order to consider the effectiveness of ionic complex in reducing cancer cell viability, it is important to compare the toxicity of the complex with the toxicity of free SA, CU, and MB alone. According to previously reported results, salicylic acid shows dark toxicity by decreasing MDA-MB-231 cancer cells viability by about 30%. Decreasing of cytotoxicity of salicylic acid in the complex results in selectivity in photo activation of toxicity of the complex that is favorable in treatments and reduces undesirable side effects. Curcumin does not show any toxicity when used alone. Photodynamic activity is one of the main characteristics of photosensitizers for light selective activation. Photo activation yield is related to light-induced cytotoxicity. In order to consider the dark toxicity of the complexes, dark experiments were designed for methylene blue-curcumin and methylene blue-salicylate complexes. The results are shown in Fig. 3.

Curcumin-based methylene blue ionic complex has low cytotoxicity while salicylate-methylene blue complex shows dark cytotoxicity in concentrations higher than 10 µg/mL. Phenolic compounds have antioxidant properties that can scavenge free radicals in the biological system and prevent the system from oxidative damages. The structural and electron-resonance stability of compounds is one of the effective parameters in radical scavenging or

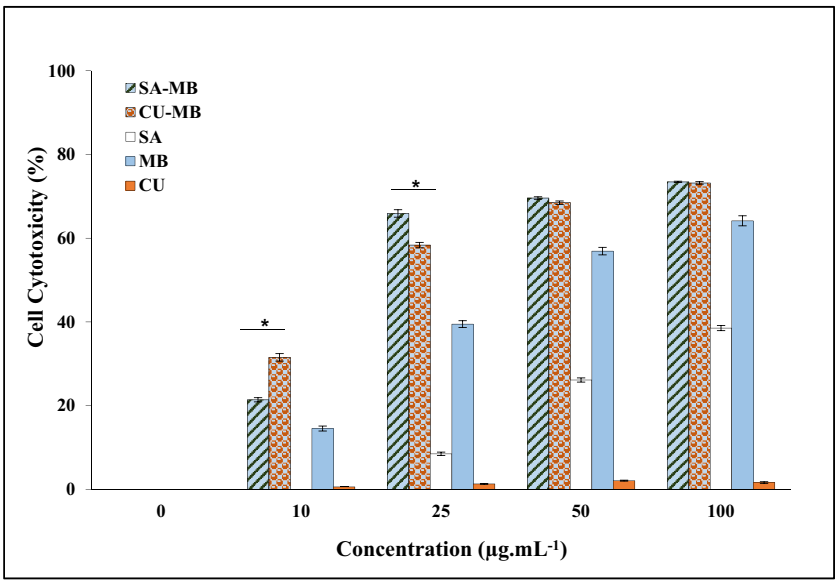

Fig. 5 Cell cytotoxicity potential of SA, MB, CU, SA-MB (salicylate-methylene blue), and CU-MB (curcumin-methylene blue) ion pairs under red light irradiation. The results are expressed as the mean ± SD ($n = 3$, $^*P < 0.05$ the SA-MB group compared with the CU-MB group)

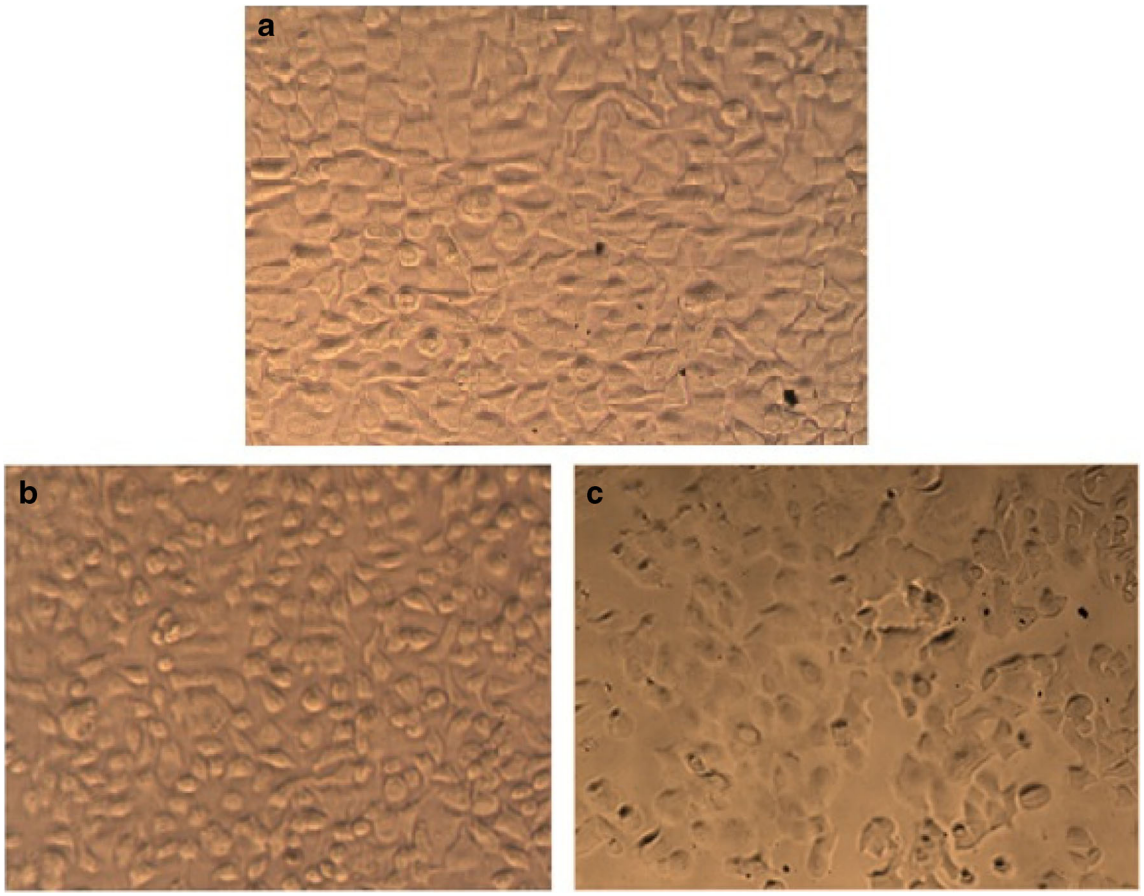

Fig. 6 Morphological microscopic images of MDA-MB-231 breast cancer cell line treated with ion-pair complexes (25 μg/mL) at dark. **a** Blank. **b** CU-MB. **c** SA-MB

radical chain inducing potential of organic compounds. Curcumin has internal molecular resonance stability and two enol-ketone isoforms that provide further molecular stability. Previously, it has been reported that methylene blue has dark toxicity, but its cell toxicity decreases in the presence of phenolic counter ions. This may be associated with increased molecular stability of methylene blue in ion-pairing form [22]. The results obtained indicate that the dark toxicity of salicylate-methylene blue is higher than that of curcumin-methylene blue (for which dark toxicity is negligible). The dark toxicity of salicylic acid, curcumin, and methylene blue is higher than that of the ionic complex forms [20, 21].

Photo-induced cytotoxicity of ion-pairing complexes

Red light emitting LED (660 nm; power density 30 mW cm^{-2}) was used as the light source [20, 21]. The results showed that the photodynamic efficacy of combination methylene blue is higher than methylene blue alone.

Curcumin-methylene blue ion pair shows a higher photodynamic efficacy than salicylate-methylene blue complex as shown in Fig. 4. Cytotoxicity potentials are presented in Fig. 5. The results show that IC_{50} for curcumin-methylene blue ion-pair complex is lower than 25 μg/mL while it is higher than 25 μg/mL for salicylate-methylene blue. Concentrations higher than 50 μg/mL has no significant change in cytotoxicity for both complexes. With a fivefold increase in concentration of complexes, the cell cytotoxicity 2.7× for salicylate-methylene blue while it is 2.5× for curcumin-methylene blue. It can be concluded that curcumin-methylene blue induces effective cytotoxicity at low concentrations when compared with salicylate-methylene blue ion pair.

Cellular morphology study

The cellular morphology changes after treatment were investigated using inverted light microscope images (× 40). Figure 6 shows morphological changes in the cells that were incubated in the dark at the presence of SA-MB and CU-MB ion-pairing complexes (25 μg/mL). The figure shows that with increase in the cells, they became rounder in appearance. Figure 7 shows the morphology changes in cells treated with ion-pair complexes under red light LED irradiation. As seen in

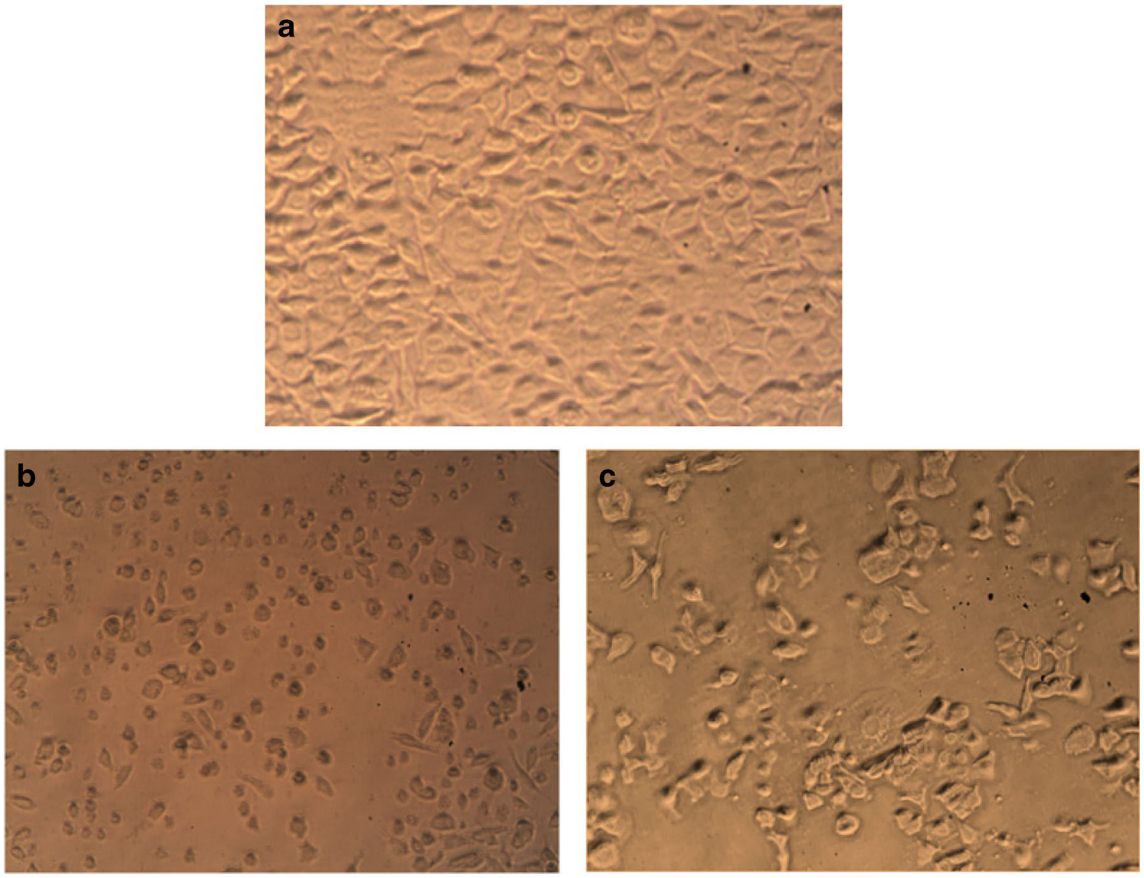

Fig. 7 Morphological microscopic images of MDA-MB-231 breast cancer cell line treated with ion-pair complexes (25 μg/mL) under red light irradiation. **a** Blank. **b** CU-MB. **c** SA-MB

Figs. 6 and 7, the morphological difference between dark and irradiated samples can be detected. Under irradiation, the round shape cells increased. Moreover, cells treated with ion-pair complexes exhibit feature characteristic of cell death, including cell shrinkage. These results are in agreement with spectroscopic and cell viability studies.

Conclusion

Ion pairing is a simple and valuable method of enhancing solubility, stability, and delivery of therapeutic compounds. Moreover, this approach can also improve permeability across biological membranes, enhance availability, and decrease effective therapeutic dosage. Spectroscopic and thermodynamic results indicate that electrostatic and hydrophobic interactions are the main forces in complex formation. Ion pair complexes are formed by inter-molecular physical interactions which do not entail alterations in structure and function of therapeutic compounds. Cellular experiments showed that ion pairing enhances cell cytotoxicity of ion-paired methylene blue due to better cell permeability. Thermodynamic data shows that the free energy of interactions is negative. Therefore, molecular stability in the complex is higher than the single molecule forms of both curcumin and salicylic acid. By increasing molecular stability and preventing dimerization of methylene blue, the cytotoxicity potential of methylene blue increases and this subsequently leads to improvement of its photodynamic efficacy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All of ethics regrading this work were provided and considered and authors approve this matter.

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