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Synthesis of pheophorbide-*a* conjugates with anticancer drugs as potential cancer diagnostic and therapeutic agents

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ABSTRACT

Pheophorbide-*a*, a chlorine based photosensitizer known to be selectively accumulated in cancer cells, was conjugated with anticancer drugs, doxorubicin and paclitaxel in the purpose of selective cancer diagnosis and therapy. Pheophorbide-*a* was conjugated with anticancer drugs via directly and by the use of selective cleavage linkers in cancer cell. The fluorescence of pheophorbide-*a* and doxorubicin conjugate by excitation at 420 or 440 nm was greatly diminished possibly by the energy transfer mechanism between two fluorescent groups. However, upon treatment in cancer cells, the conjugate showed to be cleaved to restore each fluorescence of pheophorbide-*a* and doxorubicin after 48 h of incubation. Also, pheophorbide-*a* conjugates either with doxorubicin and paclitaxel inhibited the growth of various cancer cells more potently than pheophorbide-*a*, which displayed very weak inhibitory activity. The results indicated that the pheophorbide-*a* conjugates with anticancer drugs could be utilized for selective cancer therapy as well as for the fluorescence detection of cancer.

1. Introduction

Many drugs are designed based on the prodrug approach as the reason for improvement of physicochemical, biopharmaceutical or pharmacokinetic properties.[1] Chemical linker, targeting-ligand, membrane transporter, and polymer have been used as the prodrug conjugation motives.[2] As one of the targeting-ligand, photosensitizers have been utilized in the development of cancer diagnostic agents as well as in photodynamic therapy (PDT) which could treat cancer and non malignant tumor by reactive oxygen species generated by light, oxygen and photosensitizer.[3]

Several photosensitizer conjugates have been developed. The carbohydrate moieties on conjugation with 3-(10-hexyloxyethyl)-3-devinyl pyropheophorbide-*a* (HPPH),[4] porphyrin conjugates with monocolonal antibodies[5] and with spermine,[6] pyropheophorbide conjugate with tamoxifen,[7] tetra(pentafluorophenyl)porphyrin conjugate with thiosaccharide,[8] and Chlorin p6 and histamine conjugate[9] were reported to increase cellular accumulation of photosensitizer in tumor cells. In addition, anticancer drugs such as doxorubicin have been reported that their conjugated compounds enhanced selective cellular uptake and reduced the side effects.[10–13] Pheophorbide-*a* (Pa, **1**) is a chlorine based photosensitizer derived from chlorophyll-*a* with photo-dependent or -independent cytotoxic activity.[14,15] Herein we report our investigations on the effects of pheophorbide-*a* conjugates with anticancer drugs such as doxorubicin (DOX, **2**) and paclitaxel (PTX, **3**) (Fig. 1) either by direct coupling or via linkers which are known for the characteristics of selective cleavage in cancer cell. The analysis of fluorescence spectrum, cellular uptake using confocal microscopy and in vitro anti-cancer activities in various cancer cell lines of the new pheophorbide-*a* conjugates are reported.

2. Results and discussion

2.1. Chemistry

The general synthetic procedure for pheophorbide-*a* (Pa, **1**) is described in Scheme 1. Treatment of the ethanol solution of chlorophyll *a* (**4**)[16] in an acidic condition (1 N HCl, pH 2.5) enabled to remove the Mg^{2+} ion easily to afford a crude pheophytin (**5**) in the form of precipitate. The pheophytin (**5**) was subsequently hydrolyzed by reacting with 80% TFA in water to afford pheophorbide-*a* as a fine powder. The synthesized Pa was conjugated directly or indirectly using self-immolative linkers with doxorubicin (DOX, **2**) or paclitaxel (PTX, **3**). The directly conjugated product, Pa–DOX (**6**) and Pa–PTX (**7**) were synthesized by the reaction using EDCI as a coupling reagent[17,18] (Scheme 2).

For the synthesis of Pa–DOX conjugates (**10** and **13**), hydroxycinnamoyl moiety and aminobenzyloxycarbonyl moiety as self-immolative linkers were employed. Briefly, 2-hydroxycinnamic acid (**8**) was first reacted with doxorubicin hydrochloride (**2**) and

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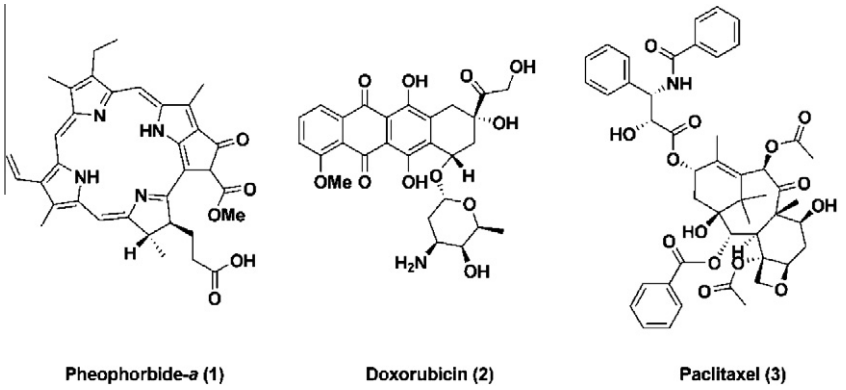
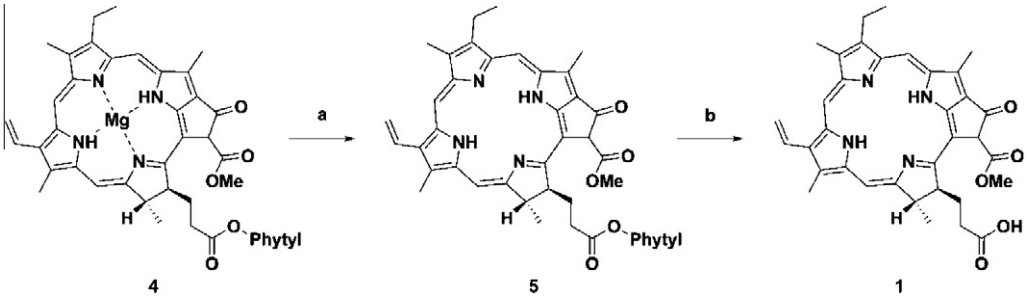
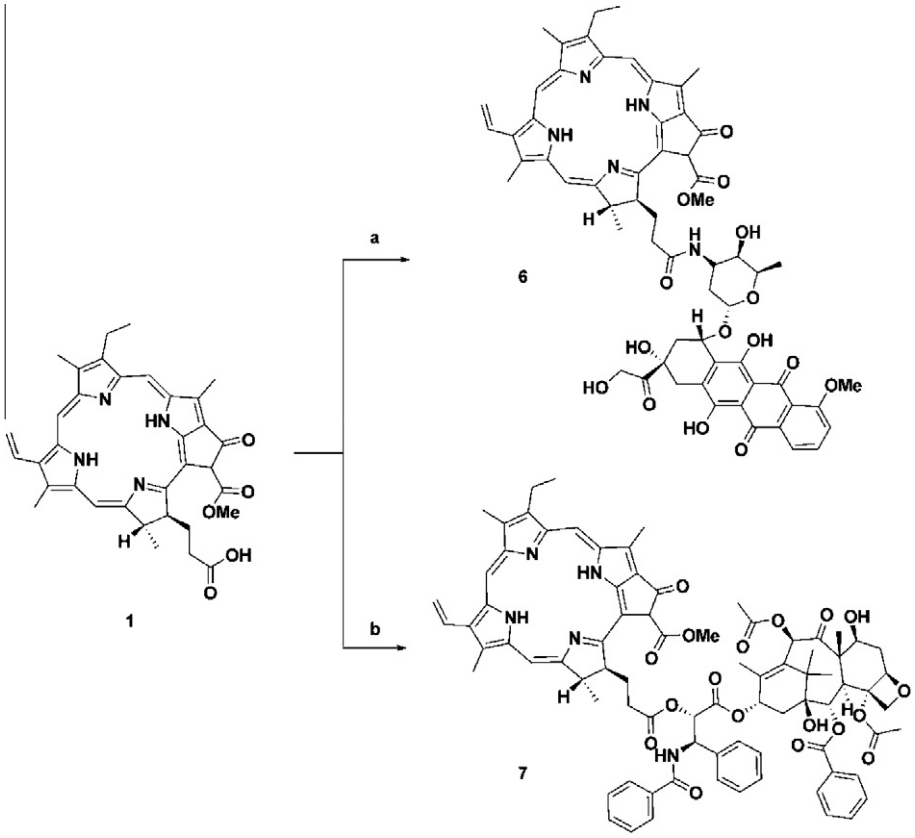


Figure 1. Structure of pheophorbide-*a* (**1**) as photosensitizer, and doxorubicin (**2**), paclitaxel (**3**) as anticancer agents.



Scheme 1. Synthesis of pheophorbide-*a*. Reagents and conditions: (a) 1 N HCl, acetone, MeOH, rt, 4 h, 88%; (b) 80% aqueous TFA, 0 °C, 1 h, 58%.



Scheme 2. Synthesis of Pa–DOX and Pa–PTX. Reagents and conditions: (a) DOX (**2**), EDC, HOBt, TEA, DCM, DMF, rt, 4 h, 53%; (b) PTX (**3**), EDC, DMAP, DCM, rt, 4 h, 54%.

subsequently reacted with Pa (**1**) using EDCI as a coupling reagent to produce the Pa–(hydroxycinnamoyl)linker–DOX compound (**10**) (Scheme 3). For the aminobenzyoxycarbonyl linker, Pa (**1**) was coupled with 4-aminobenzyl alcohol to synthesize compound (**11**), which was further reacted with 4-nitrophenyl chloroformate to afford carbonate ester compound (**12**). Finally, doxorubicin hydrochloride (**2**) and compound (**12**) were conjugated to synthesize the Pa–(aminobenzyloxycarbonyl)linker–DOX compound (**13**) bearing the carbamate ester (Scheme 4).

2.2. Cell viability study

A cell viability assay was conducted to test the synthesized compounds on various cancer cells including MCF7 (breast adenocarcinoma), KB (mouth carcinoma), HeLa (cervical cancer), U-87MG (glioblastoma), A549 (lung adenocarcinoma), AT-84 (oral cancer), and YD-10B (oral cancer) cells. Briefly, the cells were incubated for three days with the synthesized compounds (**6**, **7**, **10**, and **13**) and the viability was measured by the protocol of SRB assay. The activity of the compounds is shown in Figure 2. The results showed that 10 μM of Pa–DOX direct conjugate (**6**) moderately inhibited the growth of cancer cells including MCF7, HeLa, U-87MG, and AT-84 cells, but lower activity than DOX itself. Although the tumor specific self-immolative linkers in the conjugates (**10** and **13**) would be theoretically cleaved to generate Pa and DOX, the Pa–linker–DOX compounds showed generally less inhibitory activity of the cell viability compared to the same concentration of Pa–DOX direct conjugate. The results may be interpreted with possibilities that the conjugates may suffer from entering the cells or being cleaved in the cells. In fact, the cellular uptake of the conjugates was occurred slower than Pa itself as shown in the study of Section 2.4.

On the other hand, Pa–PTX conjugate (**7**) showed potent inhibitory activity of the viability of MCF7, HeLa, KB, and YD-10B cells at 10 μM. Therefore, the ester bond between Pa and PTX in the conjugate (**7**) may be more easily cleaved than the amide bond of Pa–DOX conjugates in those cancer cells.

2.3. Analysis of fluorescence spectrum

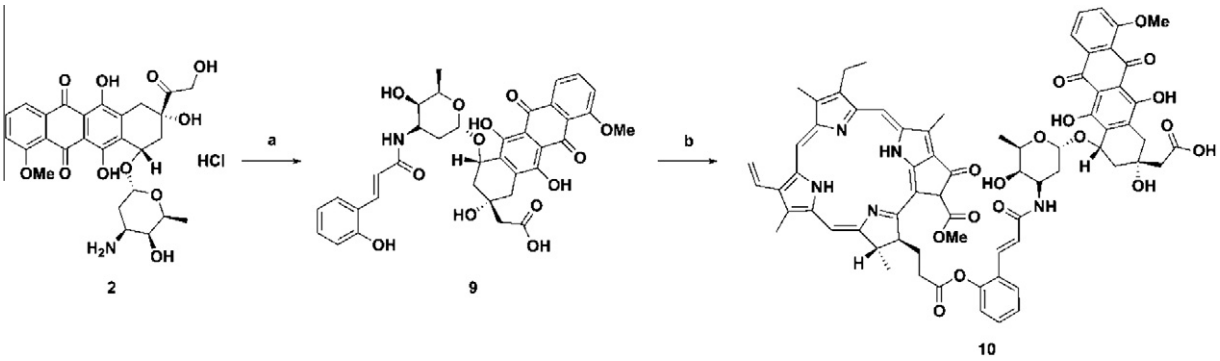
To determine fluorescence quenching effect of the conjugates, the intensity of fluorescence of each compound with same concentration was analyzed using fluorescence spectrophotometer. The fluorescence emission spectrum of compounds at 1 μM (**1**, **2**, **6**, **10**, and **13**) and 0.5 μM (**7**) was obtained by excitation wavelengths (420 and 440 nm). The fluorescence emission of Pa (**1**) showed a peak at 665 nm, the highest fluorescence being generated by 420 nm excitation. The fluorescence emission of DOX (**2**) showed a peak at 585 nm either by 420 and 440 nm excitation. The fluorescence emission of Pa–DOX conjugate (**6**, **10**, and **13**) showed a peak at 670 nm, the highest fluorescence being generated by 420 nm excitation. The fluorescence intensity of the emission at 670 nm of all conjugates of Pa–DOX was significantly less than that of Pa when excited at 440 nm. This data suggested that photo-activity of the conjugates may be suppressed by the self-quenching effect between Pa and DOX. Therefore, it could be speculated that the background fluorescence of the conjugates as they are administered in-vivo, may not be detected until internalization and cleavage of the conjugates by enzymatic attack in cancer cells are occurred so that the photoactivity of Pa and DOX may be recovered. In the case of Pa–PTX conjugate (**7**), fluorescence quenching effect was not observed as PTX is not a fluorophore (Fig. 3).

To verify fluorescence quenching due to a close contact effect, spectral comparison of fluorescence emission of compounds **6** (1 μM) and **7** (0.5 μM) in aqueous and organic solvent was investigated by 420 nm excitation wavelength using fluorescence spectrophotometer. In contact quenching, two molecules interact by proton-coupled electron transfer through the formation of hydrogen bonds. In aqueous solutions, electrostatic, steric and hydrophobic forces control the formation of hydrogen bonds. Therefore, the quenching efficiency of conjugates could depend on the employed solvents. In fact, the intensity of fluorescence of conjugates (**6** and **7**) in 50% aqueous MeOH was significantly reduced compared with that in 100% MeOH. This data suggested that two molecules of the conjugates stack together by proton-coupled electron transfer through the formation of hydrogen bonds to form ground state complex, nonfluorescent species as a contact quenching mechanism because energy transfer was not observed with the conjugates (Fig. 4).

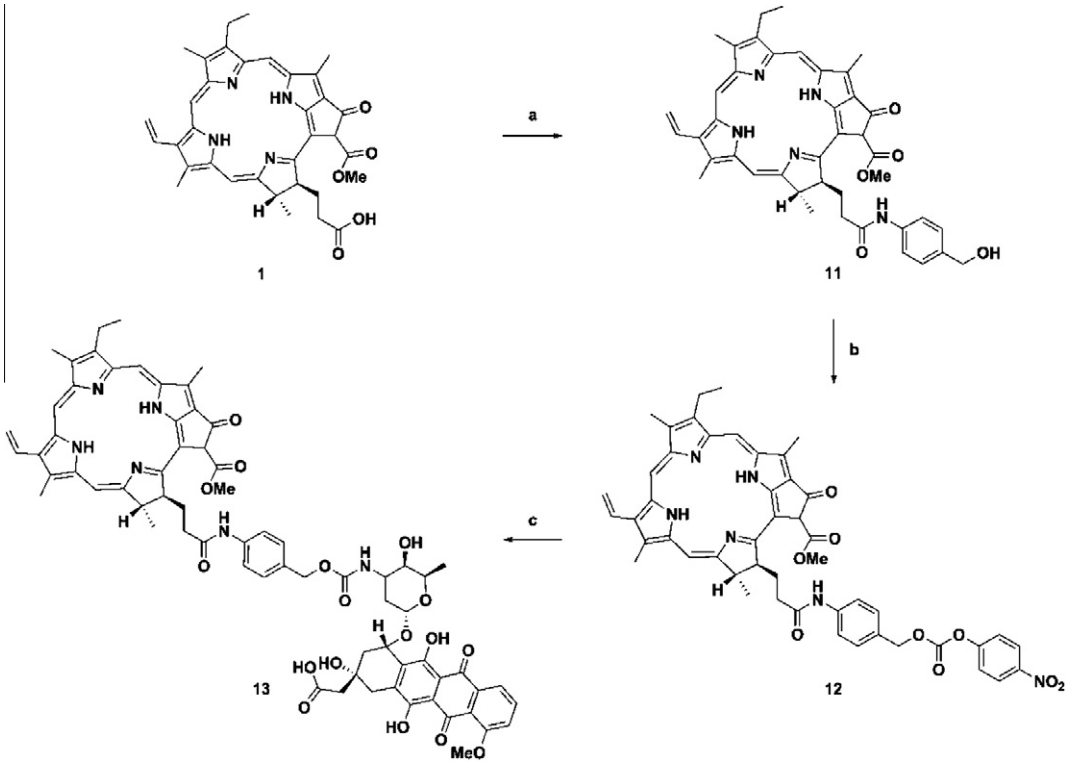
Since the environment of cancers generally exhibits lower pH, fluorescence profiles of conjugates, **6** and **7** either in acidic and basic conditions were explored by measuring the emission spectrum and intensity of fluorescence of compounds **6** (1 μM) and **7** (0.3 μM) at pH 4 and 10 using 420 nm of excitation wavelength. The emission spectra of fluorescence of **6** and **7** at pH 4 were blue-shifted about 10 nm from those at pH 10 and the intensity of fluorescence was increased at pH 4 but decreased at pH 10. These results suggested that higher fluorescence of the conjugates at lower pH as in the case of cancer environment may be advantageous in utilizing them for the fluorescence detection of cancers (Fig. 5).

2.4. Cellular uptake study by confocal fluorescence microscopy

The cellular uptake of compounds (**2**, **6**, **10**, and **13**) with different structural features was examined using confocal microscopy. For the measurement of cellular localization of the conjugates



Scheme 3. Synthesis of Pa–(hydroxycinnamoyl)linker–DOX. Reagents and conditions: (a) EDC, HOBt, TEA, DCM, DMF, rt, 4 h, 11%; (b) Pa (**1**), EDC, DMAP, DCM, DMF, rt, overnight, 24%.



Scheme 4. Synthesis of Pa–(aminobenzyloxycarbonyl)linker–DOX. Reagents and conditions: (a) 4-aminobenzyl alcohol, EDC, HOBt, TEA, DCM, DMF, rt, 4 h, 38%; (b) 4-nitrophenyl chloroformate, DIPEA, THF, rt, overnight, 41%; (c) DOX (**2**), TEA, DMF, rt, 6 h, 19%.

when they are cleaved, confocal microscopy was performed in HeLa cells based on the cell viability assay result. In a preliminary study, HeLa cells pretreated with compounds at 10 μM exhibited fluorescence at 570–600 nm and 655–755 nm emission when excited at 440 nm, which are corresponding to those of DOX and Pa, respectively, according to the fluorescence microscopy data. The fluorescence confocal images of HeLa cells after incubation with compounds for 48 h are shown in Figure 6, where the fluorescence of the Pa is shown in red and the fluorescence of the DOX is shown in green, respectively. Although Pa–DOX conjugates incubated with HeLa cells emitted lower fluorescence intensity compared with free Pa and DOX, the florescence corresponding to DOX and Pa, the components of conjugates was gradually increased up to 48 h, which could be detected only when the cleavage occur. Therefore, the conjugates of Pa–DOX system developed in this study may possess the potential properties to be used in cancer diagnosis as well as therapeutic treatment such as more efficient photodynamic therapies. Since Pa–linker–DOX conjugates showed similar behavior of uptake and cleavage to that of Pa–DOX, the reported cancer specific linkers seems not to be effective in the system for the Pa–DOX conjugates.

3. Conclusion

New conjugates of photosensitizer and anticancer drug using pheophorbide-*a* and doxorubicin showed potential characteristics of cancer diagnosis as well as cancer therapeutics by the study of inhibitory activities of cancer cell viability and background fluorescent quenching effect of the conjugates, and cellular uptake and cleavage monitored by confocal fluorescence microscopy. Also, dual anticancer effect by the photosensitizer, Pa in photodynamic therapy and anticancer drug, DOX after cell uptake and cleavage could be expected and further study on this field is in progress.

4. Experimental protocols

4.1. Chemistry

Starting materials, reagents, and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI) and TCI (Tokyo) and used as supplied without further purification. Proton nuclear magnetic resonance spectroscopy was performed on a JEOL JNM-LA 300WB and 400WB spectrometer, and spectra were taken in $CDCl_3$ or DMSO-d_6. Unless otherwise noted, chemical shifts are expressed as ppm downfield from tetramethylsilane as the internal standard, and *J* values are given in Hz. Data are reported as follows: chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet; b, broad; app., apparent), coupling constants, and integration. Mass spectroscopy was carried out on FAB (fast atom bombardment) instruments. [FAB source: JEOL FAB source and ion gun (Cs ion beam, 30 kV acceleration)]. High-resolution mass spectra (*m*/*z*) were recorded on a FAB (JEOL: mass range 2600 amu, 10 kV acceleration) at Korea Basic Science Institute (Daegu).

4.1.1. Procedure for the synthesis of pheophorbide-*a* (1)

50% ethanol in water (9 L) was treated to chlorella 6 L for removing polar materials and the residue was extracted twice with 100% ethanol (9 L) to obtain chlorophyll-*a* (**4**, 4.5 g). Chlorophyll-*a* (**4**, 4.0 g, 4.38 mmol) was dissolved in acetone (40 mL) and diluted with MeOH (100 mL). 1 N HCl (aq) (25 mL) was added to adjust pH 2.5 and reaction mixture was stirred for 4 h at room temperature. After 4 h, water was added in reaction flask and the mixture was stored in refrigerator for 1 day. The reaction mixture was filtered and the precipitate was obtained as a black solid, pheophytin (**5**, 3.38 g). Yield = 88%.

Pheophytin (**5**, 3.3 g, 3.78 mmol) was dissolved in 80% aqueous TFA (70 mL) at 0 °C, which had been bubbled with nitrogen for

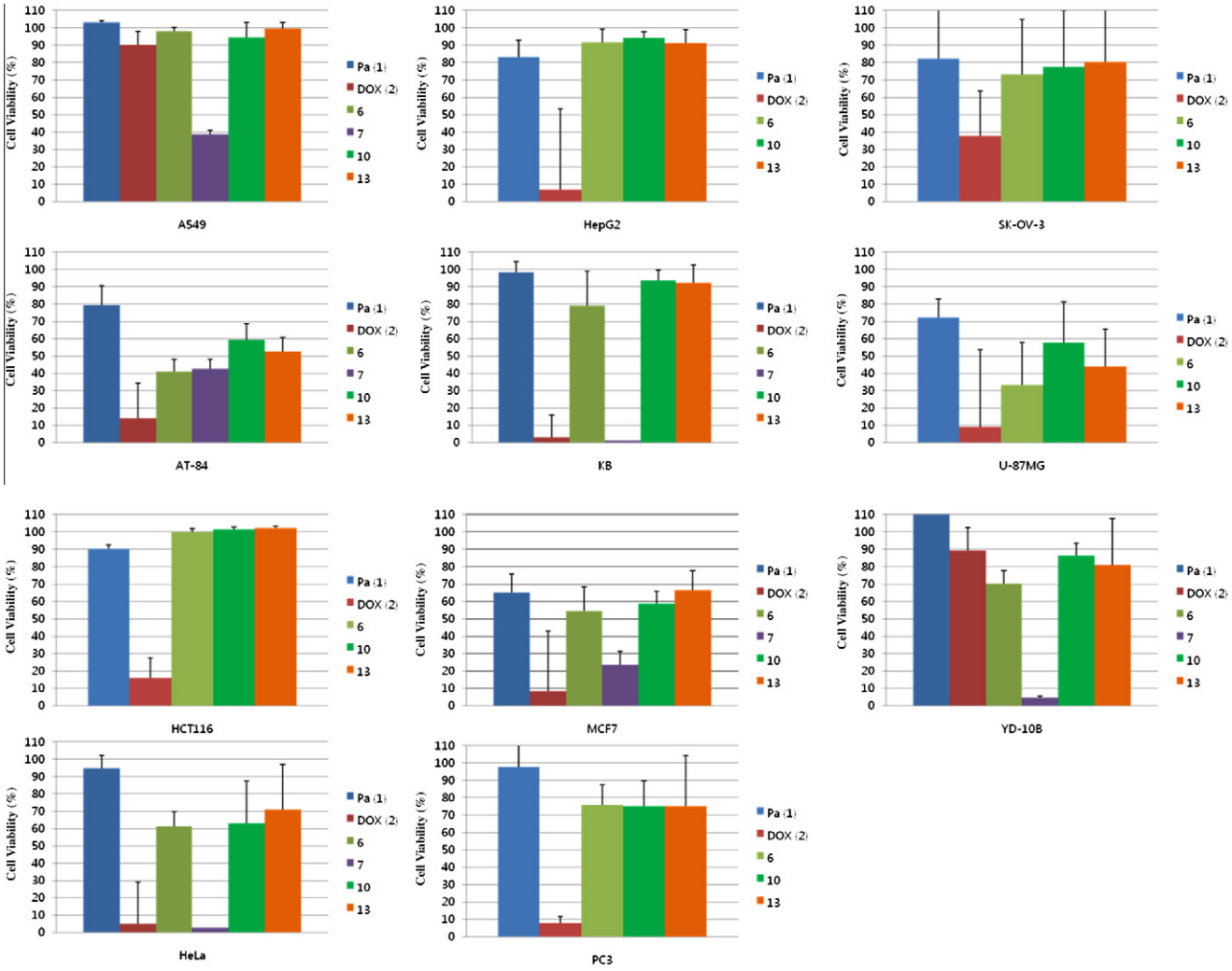


Figure 2. Cancer cell viability after 3 days in the presence of 10 μM concentrations of Pa (**1**), DOX (**2**) and conjugates (**6**, **7**, **10**, and **13**).

10 min. The resulting solution was protected from light and stirred under a nitrogen blanket at 0 °C for 1 h. The reaction mixture was concentrated to remove TFA, added to 4 N HCl (aq) (400 mL) and extracted with ethyl acetate (3 × 400 mL) three times. The combined organic layers were dried with Na_2SO_4 and concentrated. The residue was purified by silica column chromatography (chloroform:methanol = 30:1) to give Pa as a black solid (**1**, 1.3 g). Yield = 58%; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 9.51 (s, 1H), 9.40 (s, 1H), 8.62 (s, 1H), 8.00 (dd, J = 11.6, 6.4 Hz, 1H), 6.34–6.16 (m, 3H), 4.48 (m, 1H), 4.42 (m, 1H), 3.83 (s, 3H), 3.69 (m, 2H), 3.61 (s, 3H), 3.38 (s, 3H), 3.21 (s, 3H), 2.66–2.52 (m, 2H), 2.32–2.21 (m, 2H), 1.81 (d, J = 6.8 Hz, 3H), 1.67 (t, J = 7.6 Hz, 3H); ESI [M+H] = 593.5; HRMS (FAB) ($C_{35}H_{36}N_4O_5$): calcd 593.2764, found 592.2690.

4.1.2. Procedure for the synthesis of pheophorbide-*a*–doxorubicin (6)

EDC (50.0 mg, 0.25 mmol), HOBt (23.0 mg, 0.17 mmol), and TEA (25.0 μL, 0.17 mmol) were add to Pa (**1**, 50.0 mg, 0.08 mmol) in DCM (3 mL). Then, doxorubicin hydrochloride (**2**, 60.0 mg, 0.10 mmol) in DMF was added to the resulting solution and the reaction mixture was stirred for 4 h at room temperature. The reaction mixture was added to $NaHCO_3$ (aq) (30 mL) and extracted with chloroform (2 × 30 mL). The combined organic layers were dried over $MgSO_4$, concentrated and purified by silica gel column chromatography (chloroform:methanol = 50:1) to give **6** as a brown sticky solid (50.0 mg). Yield: 53%; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 9.22 (s, 1H), 9.17 (s, 1H), 8.43 (s, 1H), 7.91 (m, 1H), 7.54 (m, 1H), 7.39 (m, 1H), 6.94 (d, J = 8.0 Hz, 1H), 6.14 (m, 3H), 5.90 (s, 1H, –OH), 4.90 (s, 2H), 4.62 (s, 2H), 4.41 (m, 1H), 4.21 (m, 2H), 4.12 (m, 1H), 4.04 (m, 1H), 3.85 (m, 3H), 3.72 (s, 3H), 3.52 (m, 2H), 3.38 (s, 1H, –OH), 3.23 (s, 6H), 3.14 (s, 3H), 3.02 (m, 1H), 2.86 (d, J = 18.0 Hz, 1H), 2.60 (m, 2H), 2.36 (m, 2H), 2.04 (m, 2H), 2.01 (m, 1H), 1.85 (d, J = 14.0 Hz, 1H), 1.69 (d, J = 7.2 Hz, 3H), 1.62 (t, J = 7.6 Hz, 3H), 1.09 (d, J = 6.0 Hz, 3H); ESI [M+H] = 1118.4; HRMS (FAB) ($C_{62}H_{63}N_5O_{15}$): calcd 1118.4399, found 1118.4403.

4.1.3. Procedure for the synthesis of pheophorbide-*a*–paclitaxel (7)

EDC (10.0 mg, 0.06 mmol) and DMAP (0.2 mg, 0.004 mmol) were added to Pa (**1**, 10.0 mg, 0.02 mmol) in DCM (2 mL). Then, paclitaxel (**3**, 60.0 mg, 0.70 mmol) was added to the resulting solution and the reaction mixture was stirred for 4 h at room temperature. The reaction mixture was added to $NaHCO_3$ (aq) (20 mL) and extracted with chloroform (2 × 20 mL). The combined organic layers were dried over $MgSO_4$, concentrated and purified by silica gel column chromatography (chloroform:methanol = 70:1) to give **7** as a black sticky solid (11.5 mg). Yield: 54%; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 9.49 (s, 1H), 9.36 (s, 1H), 8.52 (s, 1H), 8.13 (m, 2H), 8.00 (m, 1H), 7.77 (m, 2H), 7.53 (m, 1H), 7.41–7.27 (m,

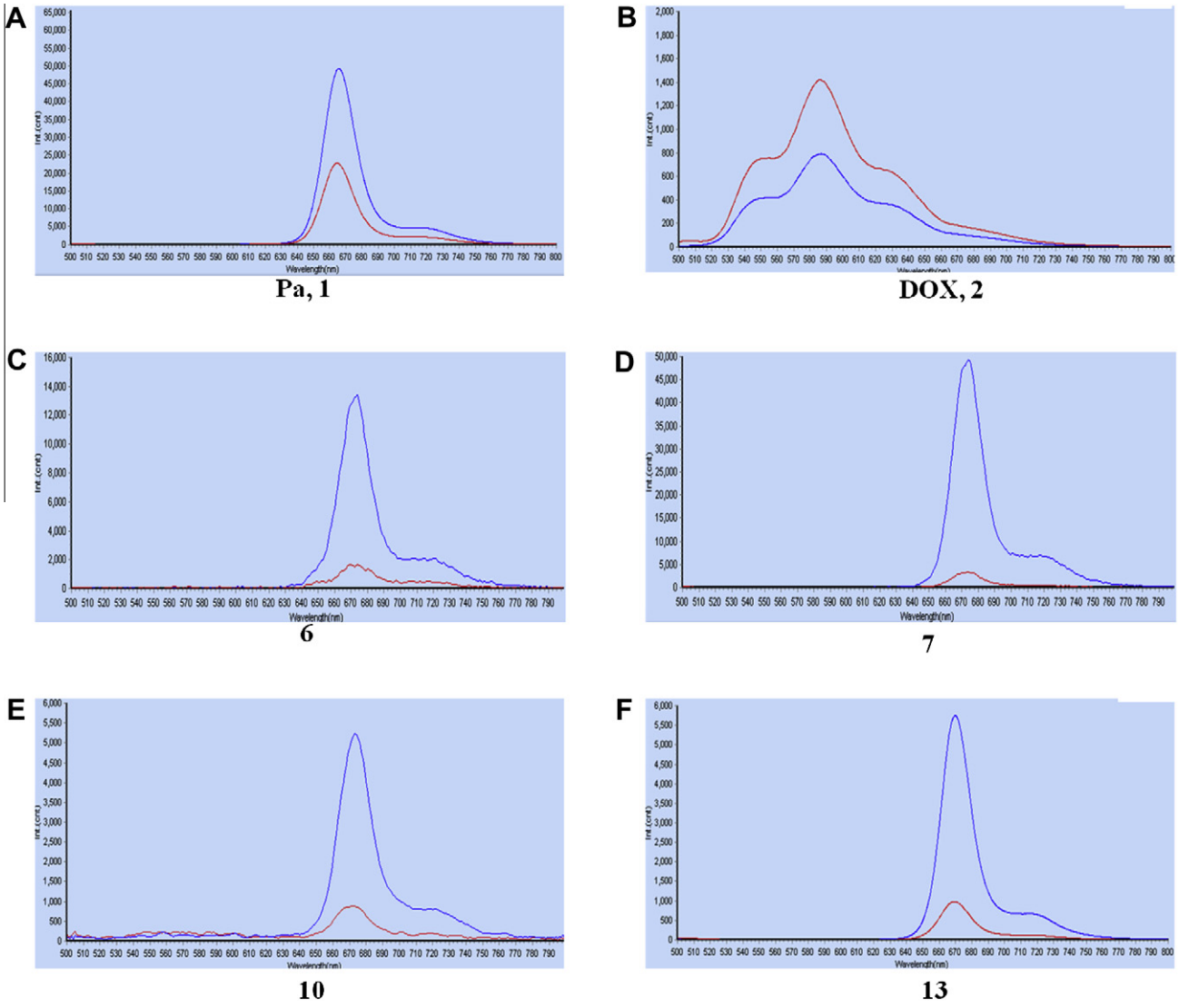


Figure 3. Fluorescence spectra of Pa (A), DOX (B) and conjugates (C–F). The emission wavelength with 420 and 440 nm excitation wavelength was shown in blue and red, respectively.

10H), 7.21 (d, J = 7.2 Hz, 1H, –NH), 6.30–6.16 (m, 3H), 6.08 (s, 1H), 6.05 (s, 1H), 5.69 (d, J = 7.2 Hz, 1H), 5.62 (d, J = 3.6 Hz, 1H), 4.97 (d, J = 9.6 Hz, 1H), 4.45 (m, 1H), 4.30 (d, J = 8.4 Hz, 1H), 4.22 (m, 2H), 3.81 (d, J = 7.2 Hz, 1H), 3.75 (s, 3H), 3.69 (m, 5H), 3.61 (d, J = 4.8 Hz, 1H), 3.38 (s, 3H), 3.21 (s, 3H), 2.67–2.51 (m, 5H, –OH), 2.45 (s, 3H), 2.42–2.29 (m, 3H), 2.21 (s, 3H), 2.18 (m, 1H), 1.94 (m, 5H), 1.87 (s, 1H, –OH), 1.79 (d, J = 6.8 Hz, 3H), 1.70–1.65 (m, 6H), 1.23 (s, 3H), 1.13 (s, 3H); ESI [M+H] = 1429.4; HRMS (FAB) ($C_{82}H_{85}N_{18}O_5$): calcd 1428.5968, found 1428.5973.

4.1.4. Procedure for the synthesis of pheophorbide-*a*–linker–doxorubicin

4.1.4.1. Synthesis of pheophorbide-*a*–(hydroxycinnamoyl)-linker–doxorubicin. *4.1.4.1.1. Pheophorbide-**a** –2-hydroxycinnamic acid (**9**).* EDC (661.4 mg, 3.45 mmol), HOBt (349.7 mg, 2.59 mmol), and TEA (360.7 μL, 2.59 mmol) were added to 2-hydroxycinnamic acid (**8**, 283.2 mg, 1.73 mmol) in DCM (10 mL). Then, doxorubicin hydrochloride (**2**, 1.0 g, 1.73 mmol) in DMF was added to the resulting solution and the reaction mixture was stirred for 4 h at room temperature. The reaction mixture was added to $NaHCO_3$ (aq) (100 mL) and extracted with chloroform (2 × 100 mL). The combined organic layers were dried over $MgSO_4$, concentrated and purified by silica gel column chromatography (chloroform:methanol = 60:1) to give **9** as a brown sticky solid (137.0 mg). Yield: 11%; ^{1}H NMR (400 MHz, DMSO) δ (ppm) 9.92 (s, 1H, –OH), 7.88 (m, 1H), 7.76 (d, J = 8.8 Hz, 1H), 7.62 (m, 1H), 7.56 (d, J = 16.0 Hz, 1H), 7.36 (d, J = 8.0 Hz, 1H), 7.13 (t, J = 8.0 Hz, 1H), 6.83 (d, J = 8.4 Hz, 1H), 6.78 (t, J = 7.6 Hz, 1H), 6.71 (d, J = 16.0 Hz, 1H), 5.47 (s, 1H), 5.22 (s, 1H), 4.93 (s, 1H, –OH), 4.84 (m, 2H), 4.56 (d, J = 5.6 Hz, 2H), 4.18 (m, 1H), 3.94 (s, 3H), 3.41 (s, 1H, –OH), 3.00 (m, 1H), 2.94 (m, 1H), 2.20 (m, 2H), 1.89 (m, 1H), 1.46 (m, 1H), 1.11 (d, J = 6.4 Hz, 3H); ESI [M−H] = 687.8.

*4.1.4.1.2. Pheophorbide-a –2-hydroxycinnamic acid–doxorubicin (**10**).* EDC (64.4 mg, 0.336 mmol) and DMAP (2.5 mg, 0.02 mmol) were added to Pa (**1**, 100.0 mg, 0.17 mmol) in DCM (5 mL). Then, the solution of **9** (115.9 mg, 0.86 mmol) in DMF (2 mL) was added to the resulting solution and the reaction mixture was stirred for overnight at room temperature. The reaction mixture was added to NH_4Cl (aq) (30 mL) and extracted with chloroform (2 × 30 mL). The combined organic layers were dried over $MgSO_4$, concentrated and purified by silica gel column chromatography (chloroform:methanol = 70:1) to give **10** as a brown sticky solid (51.5 mg). Yield: 24%; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 9.42 (s, 1H), 8.91 (s, 1H), 8.38 (s, 1H), 7.99 (d, J = 15.2 Hz, 1H), 7.69 (d,

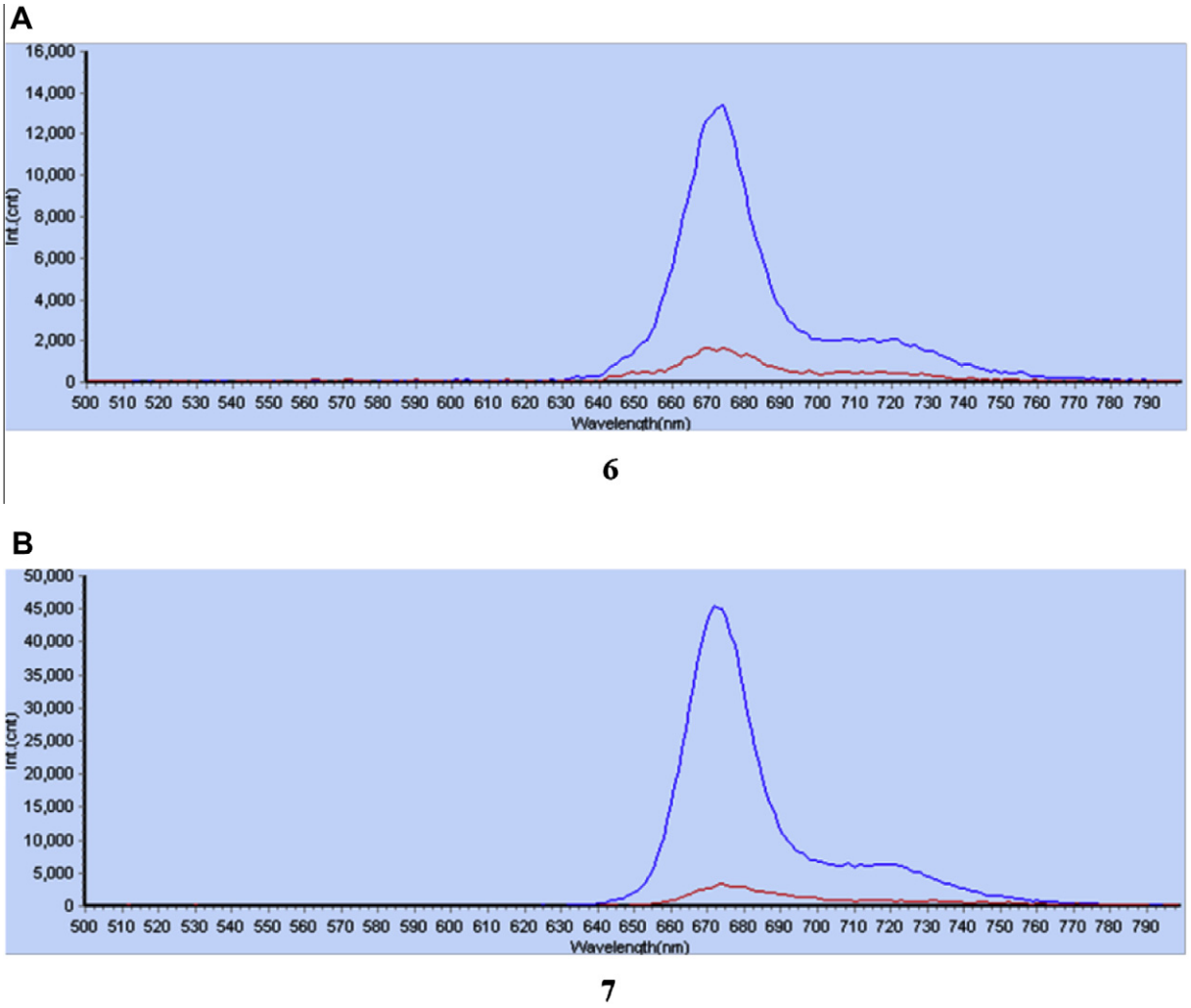


Figure 4. Fluorescence spectra of compounds **6** (A) and **7** (B) in organic and aqueous solvent with 420 nm excitation wavelength. The emission wavelength in MeOH and aqueous solvent was shown in blue and red, respectively.

J = 16.8 Hz, 1H), 7.64 (d, *J* = 6.7 Hz, 1H), 7.51 (m, 1H), 7.38 (m, 3H), 7.20 (t, *J* = 7.6 Hz, 1H), 7.06 (d, *J* = 8.0 Hz, 1H), 6.52 (d, *J* = 15.6 Hz, 1H), 6.23 (s, 1H), 6.05 (d, *J* = 8.0 Hz, 1H), 5.78 (m, 2H), 4.82 (s, 1H), 4.67 (m, 1H), 4.54 (m, 1H), 4.46 (m, 1H), 4.33 (m, 2H), 3.97–3.83 (m, 6H), 3.71–3.59 (m, 6H, –OH(1)), 3.35 (s, 1H), 3.14 (s, 3H), 3.02 (s, 1H), 2.88 (m, 2H), 2.82 (m, 2H), 2.16 (m, 2H), 1.85–1.75 (m, 6H), 1.63 (s, 4H), 1.27 (d, *J* = 6.4 Hz, 3H); ESI [M+H] = 1264.9; HRMS (FAB) ($C_{71}H_{69}N_5O_{17}$): calcd 1264.4767, found 1264.4763.

4.1.4.2. Synthesis of pheophorbide-*a*–(aminobenzyloxycarbonyl)linker–doxorubicin. *4.1.4.2.1. Pheophorbide-**a**–4-aminobenzyl alcohol (**11**).* EDC (485.4 mg, 2.53 mmol), HOBt (228.1 mg, 1.69 mmol), and TEA (235.3 μL, 2.59 mmol) were added to Pa (**1**, 500.0 mg, 0.84 mmol) in DCM (7 mL). Then, 4-aminobenzyl alcohol (104.3 mg, 0.84 mmol) in DMF was added to the resulting solution and the reaction mixture was stirred for 4 h at room temperature. The reaction mixture was added to $NaHCO_3$ (aq) (100 mL) and extracted with chloroform (2 × 100 mL). The combined organic layers were dried over $MgSO_4$, concentrated and purified by silica gel column chromatography (chloroform:methanol = 80:1) to give **11** as a brown sticky solid (226.5 mg). Yield: 38%; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 9.34 (s, 1H), 9.01 (s, 1H), 8.51 (s, 1H), 7.98 (m, 1H), 7.15 (d, *J* = 8.4 Hz, 2H), 6.66 (d, *J* = 8.8 Hz, 2H), 6.29 (m, 3H), 4.52 (s, 2H), 4.42 (s, 1H), 3.79 (s, 3H), 3.35 (m, 2H), 3.31 (s, 3H), 3.22 (s, 3H), 3.07 (s, 3H), 2.68 (m, 2H), 2.25 (m, 2H), 1.78 (d, *J* = 7.2 Hz, 3H), 1.59 (t, *J* = 7.6 Hz, 3H); ESI [M−H] = 695.6.

*4.1.4.2.2. Pheophorbide-a –4-aminobenzyl alcohol–4-nitrophenyl formate (**12**).* DIPEA (111.5 μL, 0.64 mmol) were added to **11** (226.5 mg, 0.32 mmol) in THF (5 mL). Then, 4-nitrophenyl chloroformate (96.7 mg, 0.45 mmol) in THF was dropwised to the resulting solution and the reaction mixture was stirred for overnight at room temperature. The reaction mixture was added to $NaHCO_3$ (aq) (70 mL) and extracted with chloroform (2 × 70 mL). The combined organic layers were dried over $MgSO_4$, concentrated and purified by silica gel column chromatography (chloroform:methanol = 130:1) to give **12** as a brown sticky solid (85.5 mg). Yield: 41%; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 9.43 (s, 1H), 9.42 (s, 1H), 8.54 (s, 1H), 8.26 (d, *J* = 9.2 Hz, 2H), 8.04 (m, 1H), 7.36 (d, *J* = 9.2 Hz, 2H), 6.88 (d, *J* = 8.8 Hz, 2H), 6.33–6.18 (m, 3H), 5.04 (s, 2H), 4.53 (m, 1H), 4.30 (m, 1H), 3.84 (s, 3H), 3.70 (m, 2H), 3.55 (s, 3H), 3.35 (s, 3H), 3.25 (s, 3H), 2.73 (m, 2H), 2.03 (m, 2H), 1.81 (d, *J* = 7.2 Hz, 3H), 1.70 (t, *J* = 8.0 Hz, 3H); ESI [M+H] = 862.8.

*4.1.4.2.3. Pheophorbide-a –4-aminobenzyl alcohol–4-nitrophenyl formate–doxorubicin (**13**).* TEA (25.8 μL, 0.19 mmol) were added to **12** (80.0 mg, 0.09 mmol) in DMF (4 mL). Then, doxorubicin hydrochloride (**2**, 53.7 mg, 0.09 mmol) in DMF (1 mL) was dropwised to the resulting solution and the reaction mixture was stirred

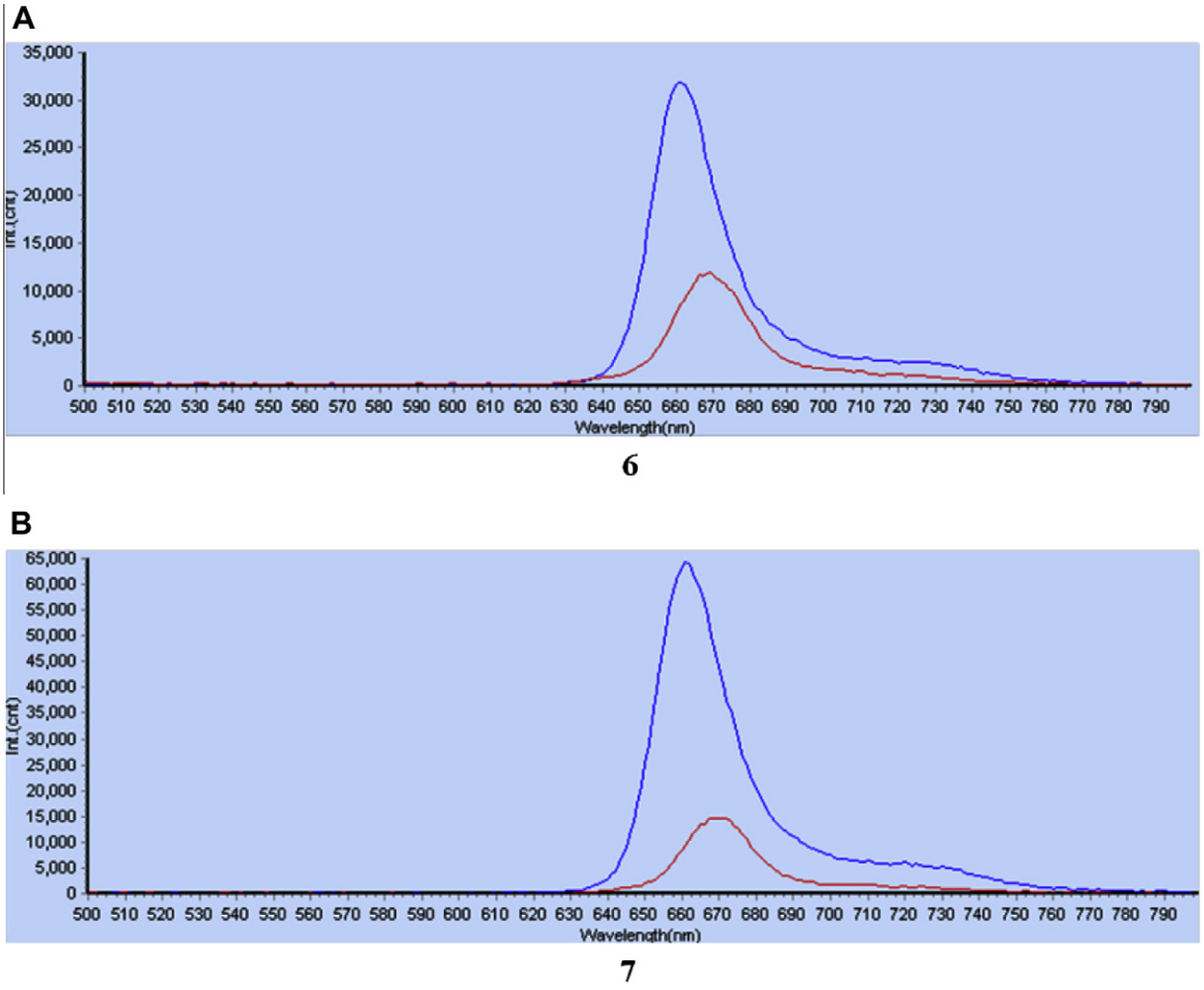


Figure 5. pH profile of fluorescence spectra of **6** (A) and **7** (B) at pH 4 and 10 with 420 nm excitation wavelength. The emission wavelength at pH 4 and 10 was shown in blue and red, respectively.

for 6 h at room temperature. The reaction mixture was added to $NaHCO_3$ (aq) (40 mL) and extracted with chloroform (2 × 40 mL). The combined organic layers were dried over $MgSO_4$, concentrated and purified by silica gel column chromatography (chloroform:methanol = 80:1) to give **13** as a brown sticky solid (21.4 mg). Yield: 19%; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 9.19 (s, 1H), 9.14 (s, 1H), 8.39 (s, 1H), 7.90 (m, 1H), 7.42 (d, J = 7.2 Hz, 1H), 7.17 (t, J = 8.0 Hz, 1H), 6.87 (m, 4H), 6.64 (d, J = 6.8 Hz, 1H), 6.25 (m, 3H), 6.15 (d, J = 6.8 Hz, 1H), 4.90 (s, 1H), 4.72 (m, 4H), 4.43 (m, 1H), 4.18 (m, 3H), 3.90 (m, 1H), 3.79 (s, 3H), 3.70 (m, 2H), 3.59 (m, 5H), 3.47 (s, 1H), 3.30 (s, 1H), 3.21 (s, 3H), 3.19 (s, 3H), 3.05 (m, 1H), 2.98 (m, 1H), 2.67 (m, 2H), 2.24 (m, 2H), 2.06 (m, 2H), 1.77 (m, 1H), 1.72 (m, 4H), 1.60 (s, 3H), 1.18 (d, J = 6.0 Hz, 3H); ESI [M+H] = 1268.1; HRMS (FAB) ($C_{70}H_{71}N_6O_{17}$): calcd 1267.4876, found 1267.4871.

4.2. HPLC analysis

HPLC was used for purification of all final products. The purification was performed on a Shimadzu SCL-10A VP HPLC system using a Shimadzu Shim-pack C18 analytical column (250 mm × 4.6 mm, 5 μm, 100 Å) in isocratic solvent systems. Solvent system was 0.1% TFA in H_2O:CH_3CN = 5:95 over 30 min at a flow rate = 1 mL/min. Peaks were detected by UV absorption using a diode array detector.

4.3. Cell viability assay

4.3.1. Cell culture

The AT-84 is a squamous cell carcinoma, spontaneously arising from oral mucosa and syngenic to C3H/HeJ mice (which were kindly provided by Dr. E.J. Shillitoe, State University of New York, Upstate Medical University). MCF7, KB, HCT116, PC3, U-87MG, SK-OV-3, AT-84 cells were grown in RPMI 1640. The adherent cell line HepG2, HeLa, A549, YD-10B cells were cultured in DMEM medium. Both media were supplemented with 10% FBS (Invitrogen Co.), and antibiotic–antimycotic (Invitrogen Co.). All cell lines were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. The adherent cells were detached from the culture flasks by removal of the growth medium and addition of 1 mL trypsin/EDTA solution (0.05% w/v trypsin, 0.016% w/v EDTA). After 1–2 min incubation at 37 °C, when the cells had detached from the surface, trypsinization was stopped by the addition of 4 mL of DMEM medium containing 10% FBS.

4.3.2. SRB assay

The cells were plated at a density of 50,000 cells/mL and well in 96-well plates and incubated with inhibitors for the time indicated. The SRB assay was carried out as described by Skehan et al.[19] The drug incubation period of the cells was stopped by the addition of 50 μL of ice cold TCA solution into the growth

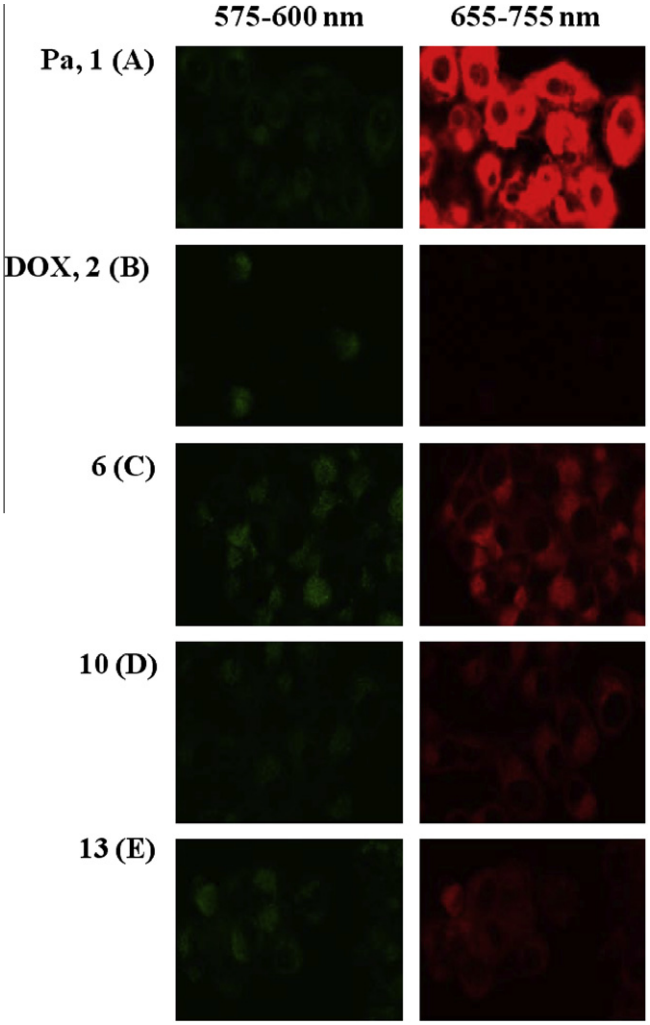


Figure 6. Confocal fluorescence microscope images on HeLa cell after incubation with 10 μM Pa (A), DOX (B) and conjugates (C–E) for 48 h. 575–600 nm emission wavelength with 440 nm excitation wavelength was shown in green, and the 655–755 nm emission wavelength with 440 nm excitation wavelength was shown in red.

medium. After 1 h incubation in the refrigerator, the supernatant was discarded and the dishes were rinsed with deionized water five times, and dried at room temperature. One hundred microliters of SRB solution was pipetted into each well and incubated at room temperature for 1 h. Then, the staining solution was decanted, the dishes were washed four times with 1% (v/v) acetic acid and dried again at room temperature. SRB stain unspecifically bound to protein was released by adding 1 mL of 10 mM. Tris buffer per well shakes gently for 20 min. Finally the light extinction at 515 nm wavelength was read in an ELISA-reader. The mean values of four duplicated samples were calculated. These results were expressed as a percentage, relative to solvent treated control incubations.

4.4. Fluorescence spectrometry

Fluorescence measurements were done using a FluoroMate FS-2. Steady-state fluorescence spectra were generally taken at 1 μM of compounds (**1**, **2**, **6**, **10**, and **13**) and 0.5 μM of compound **7** in MeOH. The samples were illuminated with 420 and 440 nm of light and fluorescence emission was scanned from 500 to 800 nm. Fluorescence spectra in both aqueous and organic solvent were generally taken at 1 μM of compound **6** and 0.5 μM of compound **7** in 100% MeOH and 50% aqueous MeOH by illumination with 420 nm of light. The pH profiles of fluorescence spectra were generally taken at 1 μM of compound **6** and 0.3 μM of compound **7** at pH 4 and 10 in MeOH by illumination with 420 nm of light.

4.5. Confocal fluorescence microscopy

HeLa Cells were plated in the slide dishes and incubated in RPMI 1640 medium for overnight. The next day, compound of 10 μM in complete medium was added and incubated for 48 h in a humidified atmosphere of air/CO_2 (95%:5%). After washing three times with PBS, the cells were viewed under a FluoView™ FV1000 Confocal Microscope. Compounds' fluorescence was observed at 440 nm for an excitation wavelength and at 575–600 nm and 655–755 nm for an emission wavelength. The images of Pa (**1**) and DOX (**2**) were assigned as red and green color.

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