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Convenient screening methods for the photosensitivity

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Abstract

Two kinds of convenient photosensitivity screening methods (Methods A and B) were established to find new photosensitizers for photodynamic therapy (PDT). Method A is based on oxidation mechanism of cholesterol and types I and II can be specified. Method B is performed using dansyl-L-methionine and offers the benefits of fast response time. Various porphyrin, chlorin and phthalocyanine derivatives were synthesized and subjected to the screening methods. The assays have yielded similar results. The results were also identical to the photosensitivity (Ga > Zn > Mn-complex) of water-soluble metalloporphyrins bearing the same side chains. Therefore, the Methods A and B were thought to be appropriate as screening methods to find lead compounds of photosensitizers for PDT.

Keywords: Photodynamic therapy; Photosensitizer; Photosensitivity; Screening method

1. Introduction

Photodynamic therapy as applied to the therapy of the superficial cancers or age-related macular degenerations generally consists of two crucial steps, an administration of a photosensitizer and subsequent laser irradiation [1,2]. In 1900, Raab [3] reported that photo irradiation with acridine killed *Paramecium caudatum*. Hematoporphyrin was revealed to be accumulated in cancerous tissues. Based on this observation, photodynamic therapy was proposed in 1940s [4,5]. Porphyrins and chlorins play key roles in respiration or photosynthesis. Some of them are also selectively accumulated in cancers and generate singlet oxygen upon irradiation with, e.g., laser light, consequently damaging the cancerous tissues. A number of porphyrin-related compounds have been investigated as photosensitizers for photodynamic therapy [6].

Photo-oxidations are classified into types I and II by reaction mechanism [7]. The type I mechanism is that the excited triplet ($^3M^*$) of a photosensitizer (M) reacts directly with the substrate to generate a radical ion then the radical of the substrate reacts with dissolved oxygen. In the type II mechanism, the energy is transferred from the excited triplet $^3M^*$ of the photosensitizer to $^3O_2$ to generate singlet oxygen $^1O_2$. Subsequently, the singlet oxygen reacts with the substrate. In photodynamic therapy, type II photo-oxidation is thought to damage cancerous cells [8].

Photodynamic therapy is a safe and non-invasive treatment and is widely applicable to early lung cancers, cervical cancers and so on. However, the therapy is not effective on deep-seated or advanced cancers due to the lack of an appropriate photosensitizer. We have started the establishment of convenient photosensitivity and tumor-localizing property screening methods to find new excellent photo-

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sensitizers. Here, we present the photosensitivity screening methods.

2. Experimental

2.1. Materials

5,10,15,20-Tetraphenyl-21H,23H-porphine (TPP, **1**), protoporphyrin IX dimethyl ester (PP-Me, **2**) and 29H,31H-phthalocyanine (PC, **3**) were purchased from Aldrich (Tokyo). Pheophorbide a (PPB-A, **4**) and chlorin e6 trimethyl ester (Ce6-Me, **5**) were purchased from Wako Pure Chemical (Osaka). A series of metal complexes (**7**–**18**) were synthesized according to the literature (Fig. 1) [9–13].

Photoprotoporphyrin dimethylester (P-Me, **6**) was obtained by the following procedure: halogen light (150 W, 100 000 lux, 1 week) was irradiated to protoporphyrin IX dimethyl ester (20 g) in chloroform (4 L). The solution was concentrated and purified by silica gel column chromatography (chloroform/MeOH). The eluate with 1% MeOH/chloroform was evaporated to dryness and re-

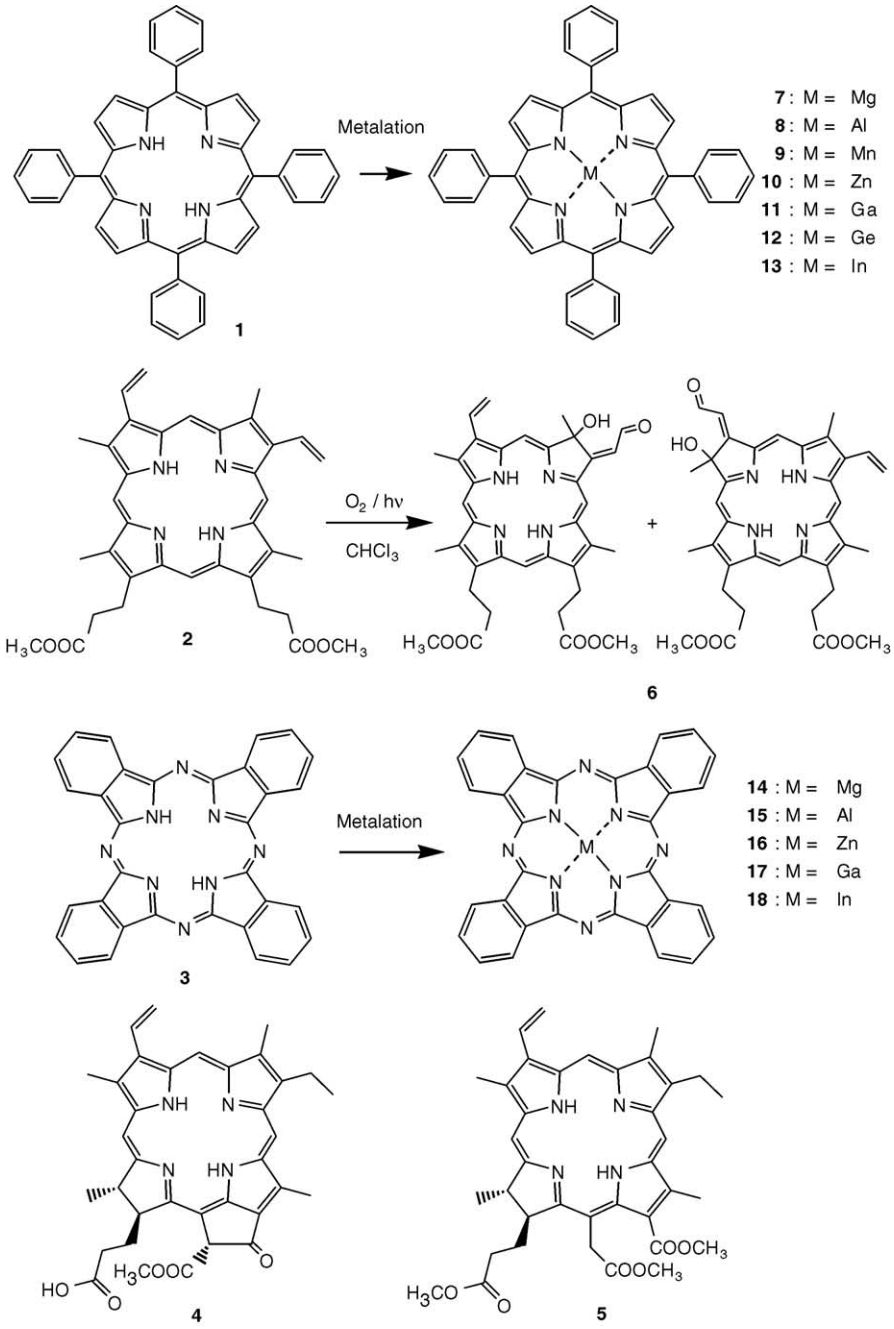

Fig. 1. Chemical structures of test compounds.

crystallized from chloroform to obtain P-Me (3 g, 15%). HRMS (FAB$^+$) *m/z*: calcd for $C_{36}H_{38}N_4O_6$622.2791, found 622.2783. IR (KBr) *ν*: 3342, 1730, 1717, 1654, 1437, 1343, 1266, 1190, 1153, 1135, 1092, 1068, 720, 672 cm^{-1}. UV (pyridine) λ_{max} (ε): 393 (63 000), 439 (94 000), 568 (14 000), 613 (6900), 672 (46 000) nm. Anal. calcd for $C_{36}H_{38}N_4O_6$: C, 69.44; H, 6.15; N, 9.00. Found: C, 69.48; H, 6.08; N, 8.94.

Cholesteryl 3,5-dinitrobenzoate (DNB-cholesterol, **19**) was obtained by the following procedure: cholesterol (5 g, 12.9 mmol) and 3,5-dinitrobenzoyl chloride (25 g, 108.4 mmol, 8.4 equiv.) were dissolved in THF (250 mL) and stirred at 60 °C for 6 h. The solution was evaporated to dryness and re-dissolved in MeOH. The crude product was purified by silica gel column chromatography (*n*-hexane/EtOAc). The eluate with *n*-hexane/EtOAc = 10:1 was re-crystallized from EtOAc to obtain cholesteryl 3,5-dinitrobenzoate (2.1 g, 28%). HRMS (FAB$^+$) *m/z*: calcd for $C_{34}H_{48}N_2O_6$580.3512, found 580.3521. IR (KBr) *ν*: 3109, 2937, 2868, 1728, 1629, 1547, 1464, 1344, 1286, 1173, 1074, 995, 980, 920, 729, 719 cm^{-1}. UV ($CHCl_3$) λ_{max} (ε): 251 (10 400) nm. Anal. calcd for $C_{34}H_{48}N_2O_6$: C, 70.32; H, 8.33; N, 4.82. Found: C, 70.41; H, 8.29; N, 4.78.

Dansyl-L-methionine sulfoxide (d-Met-SO, 20) was obtained by the following procedure: halogen light (150 W, 100 000 lux, 10 min) was irradiated to dansyl-L-methionine cyclohexylammonium salt (100 mg, 0.18 mmol) and 30% hydrogen peroxide in water. The solution was concentrated and purified by silica gel column chromatography (chloroform/MeOH). The eluate with 10% MeOH/chloroform was evaporated to dryness to obtain dansyl-L-methionine sulfoxide (24 mg, 0.06 mmol, 23%). HRMS (FAB$^+$) *m/z*: calcd for $C_{17}H_{22}N_2O_5S_2$398.0970, found 399.0962. IR (KBr) *ν*: 2943, 2789, 1612, 1589, 1576, 1506, 1456, 1321, 1232, 1202, 1144, 1094, 1032, 943, 878, 793, 752, 685, 627, 579 cm^{-1}. UV ($CHCl_3$) λ_{max} (ε): 256 (12200), 341 (3700) nm. Anal. calcd for $C_{17}H_{22}N_2O_5S_2$: C, 51.24; H, 5.56; N, 7.03. Found: C, 51.18; H, 5.52; N, 7.11.

2.2. Instrumentation

UV–vis spectra were obtained on a spectrometer UV-2400PC (Shimadzu) and infrared spectra on a FTIR-8200 (Shimadzu) using KBr pallete method. FAB mass spectra were obtained on a mass spectrometer M-80B (Hitachi) using 3-nitrobenzyl alcohols as matrix. Thin-layer chromatography was carried out with silica gel 60 F_{254} (Merck). Column chromatography was carried out with Wakogel C-200 (Wako). Elemental analysis were carried by a vario EL (Elementar).

2.3. The screening method with DNB-cholesterol as a probe substrate (Method A)

DNB-cholesterol (20 mg, 0.03 mmol) and test compound (5 mg) were dissolved in pyridine (12 mL) and halogen light (150 W, 100 000 lux, 3 h, Nippon P.I. Co. Ltd.) was irradiated under the stirring. The solution was applied to thin-layer chromatography (TLC) and developed with *n*-hexane/EtOAc = 5:1. After developing, UV absorption intensity at 254 nm of each spots was measured by densitometry (CS-9300PC, Shimadzu).

2.4. The screening method with dansyl-L-methionine as a probe substrate (Method B)

Dansyl-L-methionine (0.01 mmol) and test compound (100 mmol) were dissolved in chloroform (1 mL). Halogen light (150 W, 100 000 lux, 10 min, Nippon P.I. Co. Ltd.) was irradiated under the stirring. The solution was applied to thin-layer chromatography (TLC) at the every 1 min from the start and developed with chloroform/methanol = 3:2.

3. Results

3.1. Screening method with DNB-cholesterol as a probe substrate (Method A)

Cholesterol is known to be specifically converted to the corresponding 5-hydroperoxide by a singlet oxygen-driven type II reaction. Otherwise, it would be converted to the corresponding 7-α- or 7-β-hydroperoxide by a radical driven auto-oxidation, i.e., a type I reaction (Fig. 2) [14,15]. Indeed, light-activated pheophorbide-a (PPB-A, **4**) oxidizes cholesterol to the corresponding 5-hydroperoxide according to a type II reaction.

We employed UV-detectable probe substrates for quantitative evaluation. Pheophorbide-a (PPB-A, **4**) was tested by a screening method with a UV active-labeled cholesterol (Method A) to observe a non-DNB-cholesterol (**19**) spot at R_f 0.5. This spot was purified by silica gel column chromatography and subjected to FAB-MS analysis and identified as DNB-cholesterol 5-hydroperoxide. Some of the chlorin derivatives were investigated and also showed a spot at R_f 0.4. MS spectrum of this spot revealed the corresponding DNB-cholesterol 7-hydroperoxides (Fig. 3). Photosensitivity of each derivative was evaluated from the UV ab-

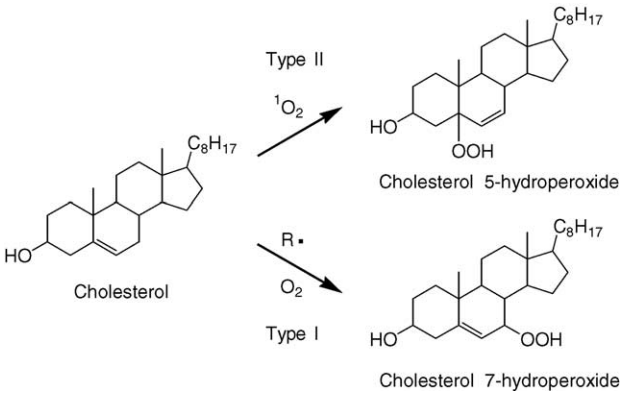

Fig. 2. Photo-oxidation mechanisms of cholesterol.

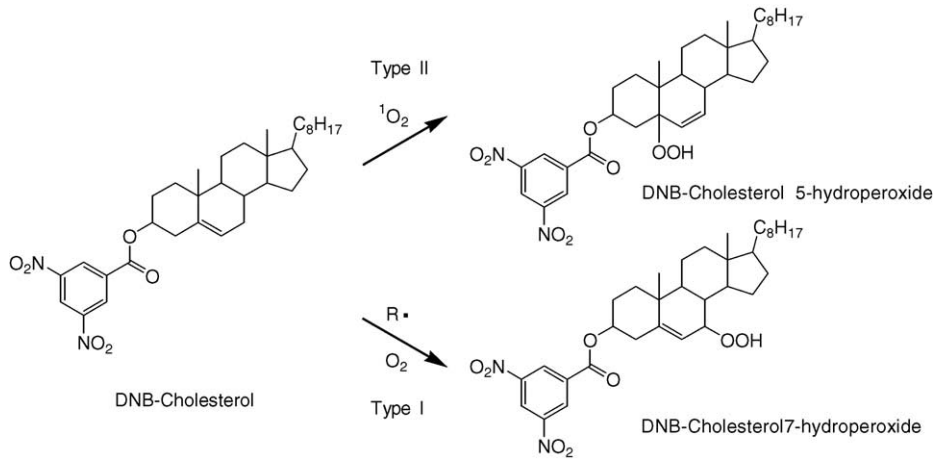

Fig. 3. Photo-oxidation mechanisms of DNB-cholesterol.

Table 1
Photo-oxidation potency of porphyrins, chlorins and phthalocyanines

Code	Name	Method A (peak area)			Method B (min)
		I[a]	II[b]	I + II[c]	
1	TPP	0	4800	4800	4
2	PP-Me	0	5100	5100	3
3	PC	0	0	0	10
4	PPB-A	0	5000	5000	3
5	Ce6	1500	5200	6700	2
6	P-Me	1600	5300	6900	2
7	Mg-TPP	0	2100	2100	7
8	Al-TPP	0	2200	2200	8
9	Mn-TPP	0	0	0	10
10	Zn-TPP	0	2300	2300	9
11	Ga-TPP	0	5500	5500	3
12	Ge-TPP	0	2100	2100	9
13	In-TPP	0	5300	5300	3
14	Mg-PC	0	900	900	9
15	Al-PC	0	200	200	10
16	Zn-PC	900	3900	4800	3
17	Ga-PC	0	1200	1200	6
18	In-PC	0	700	700	9

[a] Potency by type I.
[b] Potency by type II.
[c] The total amount of types I and II.

sorbance measured by densitometry (Table 1). The highest photosensitivities among the non-metalated derivatives were Ce6-Me (**5**) and P-Me (**6**). Ga-porphyrin complex (**11**) and In-porphyrin complex (**13**) showed the highest photosensitivities among the metal complexes. Oxidation with porphyrins indicated only type II reactions, while chlorins and the metal-phthalocyanine complexes yielded a mixture of 5- and 7-hydroperoxides.

3.2. Screening method with dansyl-L-methionine (Method B)

Subsequently, we established a more convenient method based on the principle that methionine is oxidized in vivo to generate methionine sulfoxide [16]. Dansyl-L-methionine was employed as a probe substrate. Dansyl-L-methionine was irradiated with UV at 254 nm in hydrogen peroxide-water. The reaction mixture was subjected to TLC analysis and revealed a new spot (R_f 0.5). The new spot was identified as dansyl-L-methionine sulfoxide (**20**) (Fig. 4). The samples previously tested were subjected to Method B; the disappearance of dansyl-L-methionine after 10 min from the start of reaction was observed in almost every case. A time course with sampling at 1-min time intervals was performed for quantitative evaluation. The time periods required for the total disappearance of dansyl-L-methionine are summarized in Table 1. These should correspond to photosensitivity. As a result, the chlorins (**5**, **6**), Ga-porphyrin complex (**11**), In-porphyrin complex (**13**) and Zn-phthalocyanine (**16**) showed the highest photosensitivities, and this tendency was almost identical to the previous method. Although, Method B cannot identify the oxidation mechanism, it can allow rapid evaluation.

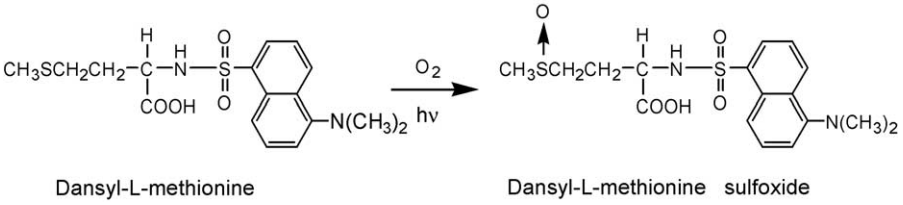

Fig. 4. Photo-oxidation mechanism of dansyl-L-methionine.

4. Discussion

The results derived from the Methods A and B were closely related. Therefore, the Method B is considered to be appropriate for initial screening. If the method with DNB-cholesterol (**19**) is used as a second screening, the oxidation mechanism can be specified.

The photosensitivity of the metalloporphyrins bearing same side chains was Ga > Zn > Mn complexes. The results were similar to literature reports [17,18] describing the photosensitivity of water-soluble metalloporphyrins bearing same side chains. Photosensitivity depends essentially on the lifetimes of photosensitizers in these references. The measurement of the triplet lifetime is thought to be the effective screening method for the photosensitivity. However, it is not convenient because special devices should be necessary. The different side chains and the same skeletons bearing compounds (**1**, **2**) showed different photosensitivity. Therefore, Methods A and B are thought to be widely applicable to the evaluations for the skeleton-selection and the precursors of final water-soluble photosensitizers.

Oxidation with porphyrins indicated only type II reactions, while chlorins and the metal-phthalocyanine complexes yielded a mixture of 5- and 7-hydroperoxides. The general PDT is known to consume oxygen molecules by the photo-oxidation and induce locally the state of the lower oxygen concentration. PDT based on the type II oxidation is not effective under this state. Molecular oxygen is required in type II oxidations, but not in type I. Chlorins and metal-phthalocyanines are also expected to be effective for the destruction of hypoxic cells.

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