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Photosensitizers Related to Purpurin-18-*N*-alkylimides: A Comparative in vivo Tumoricidal Ability of Ester versus Amide Functionalities

Gang Zheng,[a] William R. Potter,[a] Adam Sumlin,[a] Thomas J. Dougherty[a] and Ravindra K. Pandey[a,b,*]

[a]*Photodynamic Therapy Center, Roswell Park Cancer Institute, Buffalo, NY 14263, USA*
[b]*Department of Nuclear Medicine, Roswell Park Cancer Institute, Buffalo, NY 14263, USA*

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Abstract—For a comparative study, 3-(alkyloxyethyl)-3-devinylpurpurin-18-*N*-hexylimides with ester and amide functionalities were investigated for tumor selectivity and in vivo photosensitizing efficacy. Compared to amide analogues, the related photosensitizers with ester functionalities were found to be more effective. Among these compounds the 3-devinyl-(3-hexyloxyethyl)-purpurin-18-*N*-hexylimide as methyl ester **12** showed excellent tumor uptake (tumor versus muscle ratio: 8:1), and produced 100% tumor cure on day 30 at a dose of 1.0 μmol/kg. The mice were treated with light (135 J/cm^2, 705 nm) at 24 h post injection of the drug.

Photodynamic therapy (PDT), now a well recognized treatment for the destruction of tumors, utilizes the ability of a selectively retained photosensitizer to elicit an efficient photodynamic reaction upon activation with tissue penetrating light.[1] Photofrin,® a mixture of porphyrin oligomers, is currently the only PDT drug approved by the Health Agencies in the United States and elsewhere. Despite its excellent PDT efficacy, Photofrin® suffers with several drawbacks: (a) it is a chemically complex mixture, (b) its long wavelength absorption at 630 nm lies well below the wavelength necessary for the maximum tissue penetration, and (c) it induces cutaneous prolonged photosensitivity. Therefore, during the past decade, numerous effort has been devoted in developing long wavelength absorption photosensitizers, the so called 'second and third generation PDT drugs' with well defined structures and low phototoxicity.[2]

The earlier contributions from our laboratories have shown that variation of substituents in parent molecules of a variety of systems makes a significant difference in PDT biological activity. For example, in a congeneric series of alkyl ether analogues of pyropheophorbide *a* the in vivo photodynamic efficacy demonstrated a parabolic relationship being maximum in compounds with *n*-hexyl and *n*-heptyl chains at position-3 (ring A).[3] The structural elements evaluated in this in vivo quantitative structure/activity relationship (QSAR) study include the length and shape (alkyl, alkenyl, cyclic, and secondary analogues) of the ether side chain. Thus, three end points including tumor growth delay, tumor cell lethality, and vascular perfusion after treatment revealed highly similar QSAR patterns that constituted a function of the alkyl ether chain length and drug lipophilicity.

Recently, Dagan and co-workers[4] reported in vitro photodynamic efficacy of a series of pheophorbide *a* derivatives in which the carboxylic acid group of the 17-propionic acid functionality was replaced by alkyl amide substituents with variable carbon numbers and terminal groups. Optimal photosensitizer uptake in cells and photosensitizing activity were observed with compounds containing side-chain lengths of 4–6 carbon units with -OH and -CH_3 terminal ends. The most effective compound, the *N*-(4-hydroxybutyl)amide derivative, was found to be more effective than Photofrin® in vitro. Unfortunately, there is no report regarding their in vivo studies.

*Corresponding author. Tel.: +1-716-845-3203; fax: +1-716-845-8920; e-mail: rpandey@sc3103.med.buffalo.edu

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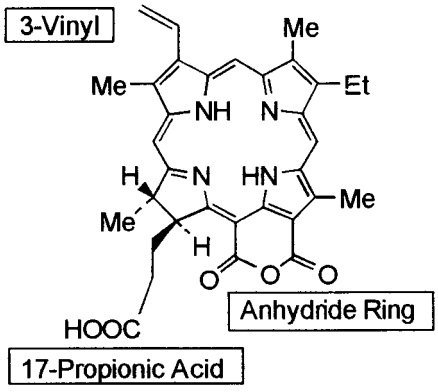


Having developed a QSAR for the alkyl ether analogues of pyropheophorbide series, we decided to expand this approach to photosensitizers with longer wavelength absorption and try to establish a generic requirement for effective photosensitizers. For our study, purpurin-18 **1** was selected as a starting material due to: (a) its ready availability from chlorophyll *a*, (b) its strong absorption near 700 nm and thus it has advantage over other porphyrin and chlorin-based photosensitizers in treating tumors that are deeply seated and (c) its high singlet oxygen yield as well as inherent photosensitizing ability demonstrated by in vitro studies. Purpurin-18 also provides us an opportunity for modifying the functional groups substituted at the peripheral positions such as the vinyl, fused anhydride ring and propionic acid side chains. These modifications could generate a series of photosensitizers with variable lipophilicity thus avoiding multistep total syntheses.

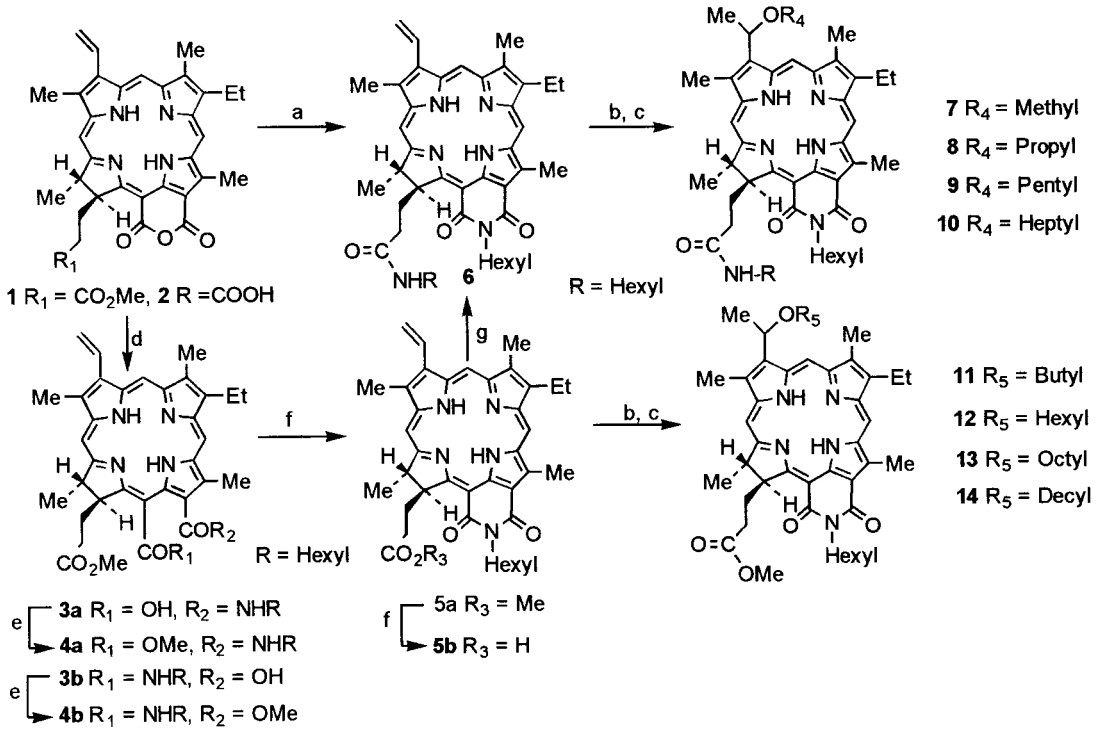
For our studies, we initially prepared a series of 3-devinyl-3-alkyl ether analogues of purpurin-18 methyl ester. Unfortunately, these compounds, containing a fused anhydride ring, were found to be unstable in vivo. Therefore, in our attempt to investigate the effect of a series of alkyl ethers versus ester amide substituents the anhydride ring **1** was first converted into a stable six membered imide ring system **5a** by following the methodology developed in our laboratory.[5] Starting from chlorin **5a**, two series of compounds were synthesized. In one series, the 17-propionic ester and the *N*-hexyl functionality of the fused imide ring system were kept constant. The vinyl group present at position-3 of the macrocycle was converted into a series of 3-devinyl-3-*O*-alkyl ether analogues [e.g. *O*-butyl **11** (log *P* 9.30), *O*-hexyl **12** (log *P* 10.30), *O*-Octyl **13** (log *P* 11.30), and *O*-Decyl **14** (log *P* 12.30), with variable lipophilicity (the log *P* values of these compounds are listed in Table 1). The second series of compounds was designed to investigate the importance of lipophilicity as well as the effect of amide versus ester functionalities in PDT efficacy. For the synthesis of such analogues, *N*-hexylimide (**5a**) was first converted into the corresponding hexylamide **6** by following the methodology shown in Scheme 1. Reaction of **6** with HBr/Acetic acid gave the corresponding 1-bromoethyl intermediate. The bromo-intermediate was not characterized and was immediately reacted with various alkyl alcohols. On the basis of the alkyl alcohol used, the *O*-methyl **7** (log *P* 9.3); *O*-propyl **8** (log *P* 10.3), *O*-pentyl **9** (log *P* 11.3) and *O*-heptyl **10** (log *P* 12.3) derivatives with log *P* values similar to those chlorins bearing ester functionalities **11**–**14** were synthesized (see Table 1). In our approach, among photosensitizers containing either propionic ester or amide functionality, out of the three possible variables only one was modified (the vinyl functionality) while the other two were kept constant.

Recently, Reddy et al.[6] have reported a facile method for the preparation of various *N*-alkyl and *N*-arylimides by reacting phthalic anhydride with various alkyl- or aryl amines in presence of Lewis acid/hexamethyldisilazane (HMDS). We extended this approach to the purpurin-18 series, and to our surprise the expected hexylimide **5a** was obtained only in small quantity, whereas the related hexyl amide analogue **6** [in which the methoxycarbonyl functionality (CO_2Me) of 17-propionic ester ($CH_2CH_2CO_2Me$) was replaced with hexyl amide (-CONH-hexyl)] was obtained as a major product. Thus, this method appeared to be an efficient approach for preparing photosensitizers with both *N*-alkyl-imide and alkylamide substituents that can be prepared in excellent yield directly from purpurin-18 methyl ester via a one-pot synthesis (Scheme 1). The presence of the amide functionality in chlorin **6** was confirmed by NMR and mass spectrometry analyses. Compared to purpurin-imide **5a** the NMR spectrum of

Table 1. Comparative in vivo studies of purpurin-imides with amide versus ester functionalities

Compound number	Log *P* value	λ_{max} nm (CH_2Cl_2)	λ_{max} nm (in vivo)	% Tumor response[a] (at day 7)	Tumor versus muscle ratio[b]	Tumor uptake (μmol/kg)
7	9.3	699	705	0	4.35	6.40
11	9.3	699	705	100	2.22	12.90
8	10.3	699	705	67	2.69	7.00
12	10.3	699	705	100	8.00	7.27
9	11.3	699	705	20	3.32	7.33
13	11.3	699	705	100	2.90	15.70
10	12.3	699	705	50	3.59	6.58
14	12.3	699	705	100	3.23	4.20

[a]Groups of six mice implanted with RIF tumor were treated with light (705 nm at 135 J/cm^2 of light) at 24 h post injection of the drug (dose: 1.0 μmol/kg).
[b]Tumor versus muscle uptake in each mouse implanted with RIF tumor was determined by in vivo reflectance spectroscopy at 5 min, 24 h and 48 h post injection of the drug (dose: 5.0 μmol/kg).



Scheme 1. (a) $Zn(Br)_2$/HMDS, hexylamine, toluene, reflux; (b) HBr/AcOH; (c) alkyl alcohol; (d) alkyl amine; (e) CH_2N_2; (g) MeOH/KOH; (f) aq LiOH/MeOH/THF; (g) DCC/H_2N–$(CH_2)_5CH_3$.

compound **6** showed the disappearance of a singlet at δ 3.6 ppm (-CO_2CH_3 protons), and appearance of a broad peak at δ 6.9 ppm assigned to -CO-NH-proton (Fig. 1).

Preliminary in vivo biological studies

To relate the molecular structure of photosensitizers to their in vivo therapeutic response, the anti-tumor activity of all new compounds was evaluated. Briefly, groups of C3H mice (6 mice/group) implanted subcutaneously with RIF tumors were injected at a dose of 1 μmol/kg of the photosensitizers and treated with laser light (135 J/cm^2) at λ_{max} 705 nm (determined by in vivo reflectance spectroscopy)[7] at 24 h post injection of the drug. The data are summarized in Table 1. For preliminary evaluation, day 7 response (no tumor regrowth after PDT treatment) was selected as the tumor response endpoint.[8]

As shown in Table 1, all compounds with ester functionality, regardless of lipophilicity, produced no tumor regrowth at a dose of 1 μmol/kg, while compounds with amide functionality, showed a significant decrease in in vivo PDT efficacy. Among compounds with low lipophilicity, amide analogue **7** (log $P=9.3$) produced no response at a dose of 1 μmol/kg, while its ester counterpart with the same lipophilicity, **11** (log $P=9.3$) under similar treatment conditions gave excellent results. In the case of compounds with medium to high lipophilicity, all three analogues **8** (log $P=10.3$), **9** (log $P=11.3$) and **10** (log $P=12.3$), produced a partial response.

Compared to their amide counterparts, photosensitizer **12** (log $P=10.3$), **13** (log $P=11.3$), and **14** (log $P=12.3$) produced 100% tumor response. These results were in contrast to those reported by Dagan and co-workers[3] in their in vitro studies with certain pyropheophorbide analogues containing alkyl amide functionalities.

To further correlate the molecular structure of the photosensitizers to their in vivo biological behavior, the in vivo tumor uptake and the tumor versus muscle ratio of the newly synthesized photosensitizers were determined by the in vivo reflectance spectroscopy. As shown in Table 1, purpurin-imides with ester or amide

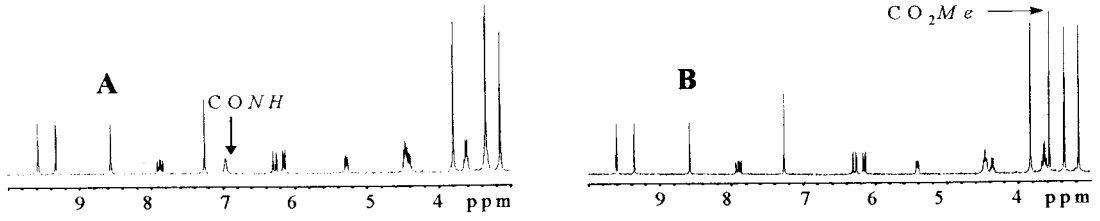


Figure 1. Partial NMR spectra ($CDCl_3$, δ ppm) of purpurin-imide with amide **6** (A) and ester **5a** (B) linkages.

functionalities showed good tumor uptake (4.20–15.70 μmol/kg) and selectivity (tumor versus muscle ratio was observed in the range of 2.22–8.36). However, it was interesting to observe that high tumor uptake of the drug was not the only factor for improved PDT efficacy. For example compound **7** has higher tumor uptake (6.40 μmol/kg) than **14** (4.20 μmol/kg), but did not show any PDT efficacy, whereas compound **14** at similar treatment conditions gave 100% tumor response (no tumor regrowth) on day 7.

The other important factor which is crucial and should be considered for selecting a photosensitizer is its high selectivity in tumor versus surrounding muscle (i.e. tumor versus muscle ratio). This may minimize the skin phototoxicity, a drawback associated with the drug Photofrin® and some other second generation photosensitizers such as; tetra(*m*-hydroxyphenyl)chlorin (THMPC) and Sn(II)purpurin, currently at various stages of human clinical trials. As can be seen from Table 1, among the drugs tested so far, compound **12** (R_1 = hexyl, log P = 10.32) produced the best tumor versus muscle ratio (8:1) (see Fig. 2) with excellent PDT efficacy at a dose of 1.0 μmol/kg (in vivo uptake and absorption was determined by in vivo reflectance spectroscopy) at 5 min, 1 h, 3 h and 24 h post injection. On the basis of our preliminary results, a lower dose of photosensitizer **12** was studied. Thus two groups of C3H mice (six mice each group) bearing RIF tumors were treated at two different drug doses (1.0 and 0.4 μmol/kg), and the same light dose (135 J/cm^2). The day 30 tumor response (no tumor regrowth) was selected as the end point. As can be seen from Figure 3, compound **12** at a dose of 1.0 μmol/kg was extremely effective and produced 100% tumor cure on day 30. Lowering the dose to 0.4 μmol/kg resulted in a decreased PDT efficacy (complete response on day 10, 80% response on day 15, 60% on day 20 and tumor regrowth on day 25). See Figure 3.

It has been observed by us that among certain photosensitizers the methyl ester functionalities can be hydrolyzed in vivo by esterases into the corresponding carboxylic acids.[9] Therefore, in order to investigate the effect of such functionalities in PDT efficacy, the most effective photosensitizer **12** was also evaluated as the corresponding propionic acid analogue and produced

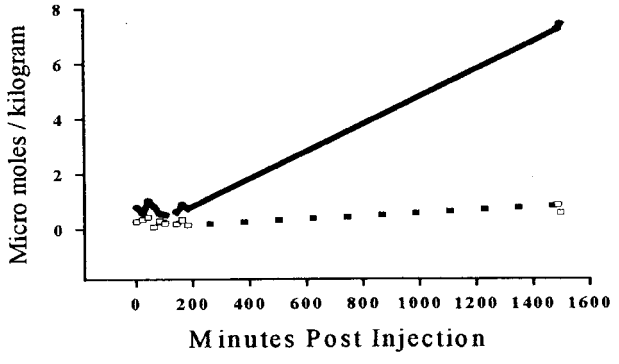


Figure 2. Tumor uptake of photosensitizer **12** in tumor (——) versus muscle (- - - - -) (drug dose 5.0 μmol/kg).

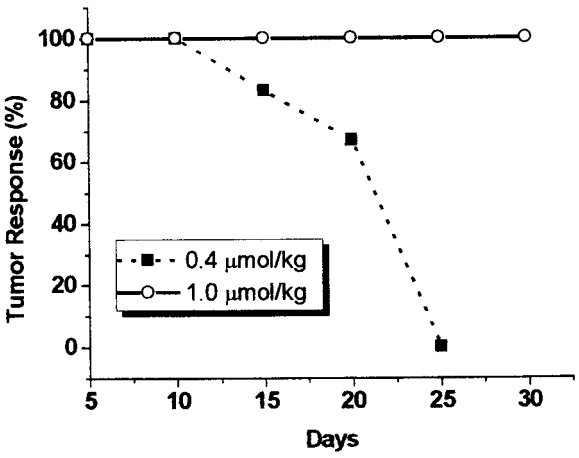


Figure 3. PDT efficacy of photosensitizer **12** at variable doses.

similar in vivo PDT efficacy and tumor uptake as observed for the related ester derivative.

In summary, we have demonstrated that the alkyl side chains and a variety of other functional groups with variable lipophilicity can be introduced at several position(s) of the purpurin-18 macrocycle by following three different approaches. Unlike purpurin-18, purpurinimides containing a fused six membered *N*-substituted imide ring system are stable in vivo and seem to be ideal candidates to study the structure activity relationship in a particular series of compounds. The preliminary in vivo data indicate that among the purpurin-imides, the replacement of the ester with an amide group (with similar lipophilicity), diminishes the in vivo activity, the reasons of which are unclear. Further studies to determine differences between their cellular/subcellular localization are underway and will be presented in our full paper.

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