Article

Low-Dose Near-Infrared Light-Activated Mitochondria-Targeting Photosensitizers for PDT Cancer Therapy

Wenyu Wu Klingler 1,2,*, Nadine Giger 1, Lukas Schneider 1,*, Vipin Babu 1,*, Christiane König 3, Patrick Spielmann 4,*, Roland H. Wenger 1,*, Stefano Ferrari 3,* and Bernhard Spingler 1,*

1 Department of Chemistry, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
2 Laboratory for Advanced Fibers, Empa Swiss Federal Laboratories for Materials Science and Technology, Lerchenfeldstrasse 5, 9014 St. Gallen, Switzerland
3 Institute of Molecular Cancer Research, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
4 Institute of Physiology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
* Correspondence: sferrari@imcr.uzh.ch (S.F.); spingler@chem.uzh.ch (B.S.); Tel.: +41-44-635-46-56 (B.S.)

Abstract: Phthalocyanines (Pcs) are promising candidates for photodynamic therapy (PDT) due to their absorption in the phototherapeutic window. However, the highly aromatic Pc core leads to undesired aggregation and decreased reactive oxygen species (ROS) production. Therefore, short PEG chain functionalized A3B type asymmetric Pc photosensitizers (PSs) were designed in order to decrease aggregation and increase the aqueous solubility. Here we report the synthesis, characterization, optical properties, cellular localization, and cytotoxicity of three novel Pc-based agents (LC31, MLC31, and DMLC31Pt). The stepwise functionalization of the peripheral moieties has a strong effect on the distribution coefficient (log P), cellular uptake, and localization, as well as photocytotoxicity. Additional experiments have revealed that the presence of the malonic ester moiety in the reported agent series is indispensable in order to induce photocytotoxicity. The best-performing agent, MLC31, showed mitochondrial targeting and an impressive phototoxic index (p.i.) of 748 in the cisplatin-resistant A2780/CP70 cell line, after a low-dose irradiation of 6.95 J/cm². This is the result of a high photocytotoxicity (IC50 = 157 nM) upon irradiation with near-infrared (NIR) light, and virtually no toxicity in the dark (IC50 = 117 µM). Photocytotoxicity was subsequently determined under hypoxic conditions. Additionally, a preliminary pathway investigation of the mitochondrial membrane potential (MMP) disruption and induction of apoptosis by MLC31 was carried out. Our results underline how agent design involving both hydrophilic and lipophilic peripheral groups may serve as an effective way to improve the PDT efficiency of highly aromatic PSs for NIR light-mediated cancer therapy.

Keywords: phthalocyanine; cancer treatment; photodynamic therapy (PDT); phototoxic index (p.i.); cisplatin; cytotoxicity; photocytotoxicity; crystal structure

1. Introduction

Photodynamic therapy (PDT) serves as a clinically approved non-invasive treatment for a broad range of cancers with selective and modulable cytotoxic activity [1–3]. PDT treatment requires the presence of three essential components (photosensitizer (PS), light, and oxygen). After application, a local irradiation of the tissue using light of an appropriate wavelength at the target tumor site activates the PS. Upon light irradiation, the PS can either transfer the energy from its excited state to molecular oxygen or directly react with biomolecules. Both pathways lead to the generation of cytotoxic reactive oxygen species (ROS) [4,5]. These locally generated ROS then react with the cellular microenvironment or organelles, leading to apoptosis, necrosis, or autophagy of the tumor cells. This can be
accompanied by an acute local inflammatory reaction that leads to the removal of dead cells, restoration of normal tissue homeostasis and occasionally systemic immunity [2,6]. Since PSs are ideally only activated upon intentional irradiation with light, they induce minimal toxic side effects and the PDT treatment can be repeated without the appearance of an accumulative toxicity. Therefore, PDT has been an attractive alternative treatment option for different severe cancers, while improving the survival rate without compromising the life quality of the affected patients [2,7].

Despite the many merits, PDT has not yet been widely accepted in clinics due to certain limitations of the reported PSs. Phthalocyanines (Pcs) are considered promising candidates for PDT because of their intense optical absorption in the red region of the visible light spectrum [8–13]. Their absorption spectra perfectly match the desired PDT phototherapeutic window of 650–850 nm [14–16], which has an excellent penetration depth in human tissue [17]. Normally, most of the PSs have an extended aromatic ring system, which makes them highly hydrophobic and susceptible to $\pi-\pi$ stacking. This then results in poor solubility in aqueous environments, a rapid clearance from the blood circulation, and therefore a low bioavailability. Additionally, the high aromaticity results in undesired aggregation, leading to a quenching of the singlet oxygen generation and thereby to a reduced therapeutic effect [18]. An important parameter to evaluate the effect of PSs is the phototoxic index (p.i.), i.e., the ratio of dark to light toxicity [19–27]. Reducing the lipophilicity and minimizing aggregation of the planar PS, for example by PEGylation, plays a critical role for improving PDT therapeutic efficiency and improving the p.i. [28–39]. Hence, exploring suitable designs to modify PSs for improving their amphipathicity is of high importance.

Herein, we have designed amphiphilic peripheral units to reduce the aggregation and self-quenching of the A$_3$B asymmetric type Pc PSs. Through stepwise modification, three PSs were obtained, each with very different distribution coefficients (log P) and, therefore, differential cellular uptake behaviors. In this work, we report novel, amphipathic Pc PSs LC31, MLC31, and DMLC31Pt and their photocytotoxicities under normoxic and hypoxic conditions.

2. Results

A series of three novel A$_3$B type Pcs with three glyoxylic substitution groups and one functional moiety was synthesized (Figure 1 and Scheme S1, Supporting Information). The phthalonitriles 3 and 6 (in a ratio of 3:1) were reacted with zinc(II) acetate and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as a base in a macrocyclization reaction in n-pentanol to give the novel A$_3$B disubstituted non-symmetrical zinc(II) Pc LC31 (Scheme S1) according to reported procedures [9,40]. MLC31 was obtained through a coupling of LC31 with amino diethylmalonate, which has excellent and specific mitochondria-targeting properties in cancer cells, leading to an efficient NIR light-induced cytotoxicity (Scheme 1). After deprotection of MLC31, the acid moiety was coordinated further with activated cisplatin in order to obtain DMLC31Pt. Analysis by $^{195}$Pt-NMR showed that the obtained platinated PS DMLC31Pt is a mixture of N,O—Pt and O,O′—Pt chelates in a ratio of about 8.5:1 (Figure S2 and Table S1, Supporting Information). In the N,O—Pt chelate (−2102.0 ppm) the platinum is coordinated to the nitrogen of the amide group and to the carboxylate oxygen atom of the decomposed malonate moiety (DMLC31Pt), while in the O,O′—Pt chelate (−1760.4 ppm) the platinum is coordinated to the two carboxylate oxygen atoms of the malonate moiety. The photophysical properties, ability to generate ROS, photocytotoxicity, and cellular uptake properties of these PSs, in particular MLC31, have been studied and compared in order to evaluate the different modifications.
Figure 1. Studied structures: LC31, MLC31, and DMLC31Pt.

Scheme 1. The light-activated function of PS MLC31. Top panel: MLC31 uptake into mitochondria of HeLa cells (200 nM, 4 h incubation). Bottom panel: the proposed cellular response of MLC31 (marked in green) within a mitochondrion of a cancer cell after activation by NIR light (left), and chemical structure of photosensitizer MLC31 (right).
Pcs LC31, MLC31, and DMLC31Pt all display a B-band at approximately 340 nm and a Q-band at approximately 695 nm. Of all the tested PSs, MLC31 has the highest Q-band extinction coefficient (ε) in all tested solvent systems (Table 1), especially in aqueous environments such as phosphate-buffered saline PBS (57,900 M⁻¹ cm⁻¹, compared to 37,400 M⁻¹ cm⁻¹ in the case of LC31 and 19,500 M⁻¹ cm⁻¹ in the case of DMLC31Pt). Yet for all PSs, an aggregation tendency in PBS was observed, leading to split, broadened and blueshifted Q-bands when compared to solvent systems like dimethyl sulfoxide (DMSO) or MeOH (Figures 2a–c and S3). In terms of fluorescence, MLC31 displayed emission in DMSO and MeOH but not in PBS. In DMSO, the peak is at 704 nm, which corresponds to a 7 nm Stokes shift. The property of strong fluorescence emission in the NIR range allows for the tracking of these PSs in cell localization studies via fluorescence microscopy and in vivo during the treatment.

**Figure 2.** UV-Vis absorption spectra of (a) LC31, (b) MLC31, and (c) DMLC31Pt in DMSO or PBS, and the corresponding normalized emissions in DMSO. Emission in PBS is not shown because none could be observed. (d) Plots of optical density change (ΔOD) of DPBF in methanol monitored at 410 nm versus the irradiation time. Light of wavelengths in the range of 600–720 nm with a power intensity of 20 mW cm⁻² was applied with a halogen light source.

ROS, especially singlet oxygen (¹O₂), are considered to be the main cytotoxic agents in PDT, therefore the ¹O₂ quantum yields (Φ₅) of the PSs have been investigated by comparison with a standard [41,42]. The Φ₅ upon irradiation with light was evaluated with a steady-state method while monitoring the PS-mediated photo-oxidation of 1,3-diphenylisobenzofuran (DPBF) using methylene blue (MB) as a standard (Φ₅ of 0.52 in MeOH [43]). Therefore, a solution of the corresponding Pc-based PS containing DPBF was irradiated using a halogen lamp with a 600 nm long-pass filter over a time period of 0 to
150 s while the decrease of the absorption band of DPBF at 410 nm was monitored. The $\Phi_{\Delta}$ was then calculated by comparing the decrease to that of MB (Figure S4, Supporting Information) through plotting the optical density change ($\Delta$OD) of DPBF against the irradiation time (Figure 2d). As illustrated in Figure S4, the absorbance of the mixtures of the corresponding PS and DPBF decreased quickly at 410 nm upon irradiation with light, indicating an efficient generation of $^{1}$O$_{2}$. In the control experiment without PSs, no significant decrease could be observed at 410 nm during an irradiation time of 1 min. The $\Phi_{\Delta}$s for LC31, MLC31, and DMLC31Pt in methanol were determined to be 0.49, 0.84, and 0.39, respectively. Surprisingly, the $\Phi_{\Delta}$ of MLC31 is clearly higher compared to that of LC31 and DMLC31Pt. Possible reasons for this observation are the higher $\epsilon$ of MLC31 at 700 nm and the slightly different aggregation form, described below. Additionally, the ability of all three Pc-based PSs to generate hydroxyl radicals ($\cdot$OH) upon irradiation with NIR light was determined with help of the APF sensor (Supporting Information). Interestingly, LC31 generated the most $\cdot$OH, followed by DMLC31Pt and MLC31 (Figure S5).

### Table 1. Photophysical properties of LC31 and MLC31 in PBS, DMSO and MeOH.

|         | B-Band (nm) | Q-Band (nm) | $\epsilon$ of the Q-Band (M$^{-1}$ cm$^{-1}$) | $\lambda_{em}$ (nm) | $\tau_{F}$ (ns) | Stokes shift (nm) | $\Phi_{\Delta}$ b |
|---------|-------------|-------------|---------------------------------------------|---------------------|-----------------|-------------------|------------------|
|         | DMSO        | DMSO        | PBS                                         | DMSO                | MeOH            | DMSO              |                  |
| LC31    | 338         | 696         | 37,400                                      | 114,800             | 105,000         | 704               | 2                | 8                | 0.49             |
| MLC31   | 345         | 694         | 57,900                                      | 206,900             | 145,800         | 707               | 3                | 13               | 0.84             |
| DMLC31Pt| 350         | 693         | 19,500                                      | 79,000              | 31,000          | 697               | -                | 4                | 0.39             |

*a* in MeOH. *b* $\Phi_{MB} = 0.52$ in MeOH [44]. Light with a wavelength cutoff below 600 nm with a power intensity of 5.8 mW cm$^{-2}$ was applied with a white light projector. The total light dose equals to 6.96 J cm$^{-2}$.

The aggregation of highly aromatic dye molecules plays an important role in energy and electron transfer and light harvesting systems [45]. It is well known that Pcs tend to form aggregates caused by the strong $\pi-\pi$ interactions between the planar macrocycles in solution. The relative geometry of the macrocycles reflects on the spectroscopic behavior of the aggregates. Previous reports have shown that the Q-band of aggregates is split compared to well-dispersed single molecules (Figure 2) when the angle between the polarization axes of monomers differs or the monomer center of the aggregate shifts. If the Pc centers are overlapping, with the ring systems being stacked in a parallel way, the aggregates are called H-type aggregates, which lead to a blueshifted band. On the other hand, when the molecules are aggregated in a head-to-tail form and the centers are further apart, the aggregates are called J-type aggregates and the band is redshifted [46]. The co-facial arrangement, which is common in most Pc aggregates, generally yields a blueshifted H-aggregates, whereas an edge-to-edge arrangement of the J-aggregates is rare. Compound MLC31 containing triethylene glycol substituents resulted in an unusually mixed Q-band with a redshifted peak (Q$^{-}$ = 698 nm, compared to Q = 694 nm) and a blueshifted peak (Q$^{+}$ = 646 nm) upon aggregation in aqueous media (Figure 2b). This suggests a co-existence of H-type and J-type aggregates. In contrast to MLC31, LC31 showed no redshift (Q$^{-}$ = 696 nm, compared to Q = 696 nm) but only a blueshifted peak (Q$^{+}$ = 653 nm) upon aggregation in aqueous media (Figure 2a), suggesting the existence of the H-type aggregates only. A similar behavior was displayed by DMLC31Pt, where Q$^{-}$ = 693 nm (compared to Q = 693 nm) was observed, and a blueshifted peak at 644 nm.

In sharp contrast to the protonation of the porphyrin rings at the pyrrole nitrogen atoms, Pcs can undergo protonation at two different sites on the isoindole nitrogen atoms, as well as the meso-nitrogen atoms [47]. To elucidate the protonation site of LC31 in solution, the absorption spectral changes upon addition of TFA to the solution of LC31 in CHCl$_{3}$ were measured. The titration of LC31 with TFA in CHCl$_{3}$ allowed observation of the one-step spectral change, with one isosbestic point at 705 nm in the case of LC31 (Figure S6a). The split and shifted Q-bands indicate spectral changes accompanying the
mono-protonation of LC31. These spectral changes could be attributed to the mono-protonation of the azomethine nitrogen bridges in the ZnPc ring, as according to previous reports [48,49], resulting in progressive redshifts and loss of symmetry. This apparent one-step mono-protonation is ascribed to the high basicity of the Pc meso-nitrogen bridge in the structural backbone, similar to mono-protonated porphyrins [50]. The equilibrium constant $K$ of the Pc backbone mono-protonation was determined to be $7.1 \pm 0.1 \times 10^4 \text{M}^{-1}$ for LC31 in CHCl$_3$ (Figure S6b) according to Equation (1):

$$\text{LC31} + \text{HA} \rightleftharpoons [\text{LC31H}^+ \text{A}^-]$$

(1)

The redshift is a result of the reduction of the HOMO–LUMO gap in the electronic structure. The a$_{1u}$ orbital has a nodal plane through the meso-nitrogen atoms [49,51]. The LUMO, unlike the HOMO, has a significant electron density on the azomethine nitrogen atoms. Engaging the nitrogen lone pairs withdraws electrons from the LUMO, thereby stabilizing it relative to the HOMO, whose energy is conserved. While mono-protonation of ZnPc was successful, TFA did not lead to the di-protonation of the ZnPc, compared to similar reports. For a di- or even tri-protonation, sulfuric acid might be used.

2.1. Photocytotoxicity

A successful PS for PDT exhibits an efficient off-on cytotoxicity in the absence and presence of the light used for the activation of the PS. Therefore, the cytotoxicity of the Pc-based compounds was evaluated against different cell lines both under irradiation and in the dark. Compounds LC31, MLC31, and DMLC31Pt, as well as cisplatin were screened against several cancer cell lines representative of human cervical cancer (HeLa), cisplatin-sensitive human ovarian carcinoma (A2780), cisplatin-resistant ovarian endometrioid adenocarcinoma (A2780/C70), and non-cancerous fibroblast (MRC-5) cell lines. The cytotoxicity parameters are reported in Table 2 in terms of the determined IC$_{50}$ value (the median half-maximal growth inhibitory concentration calculated from dose–survival curves (Figure S7), obtained after 4 h incubation with respective compounds, 20 min of irradiation with a white light projector (filter cutoff below 600 nm, light intensity 5.8 mW cm$^{-2}$, light dose: 6.96 J cm$^{-2}$) and further 72 h of incubation). Overall, the tested agents were found to be much more active towards the cancerous cell lines after light activation compared to cisplatin and to the condition without activation by light.

Table 2. Cytotoxic activity data (IC$_{50}$) for Pc-based agents LC31, MLC31, DMLC31Pt, and cisplatin against human cervical cancer (HeLa), human ovarian carcinoma (A2780), its cisplatin resistant variant (A2780/C70), and non-cancerous fibroblast (MRC-5) cell lines.

| Comp. | HeLa (µM) | A2780 (µM) | A2780/C70 (µM) | MRC-5 (µM) |
|-------|-----------|-----------|----------------|------------|
|       | Dark $^a$ | Light $^b$ | p.i. $^c$      | Dark $^a$  | Light $^b$ | p.i. $^c$      | Dark $^a$  | Light $^b$ | p.i. $^c$      |
| LC31  | 14.7      | 0.426     | 35             | 31.0       | 0.211     | 147           | 69.5       | 1.2        | 58             | 48.1       | 1.1        | 44             |
| MLC31 | 5.2       | 0.009     | 578            | 44.0       | 0.018     | 2444          | 117.5      | 0.157      | 748            | 12.8       | 0.019      | 674            |
| DMLC31Pt | 8.0     | 0.775     | 10             | 25.0       | 0.199     | 126           | 88.7       | 0.773      | 115            | 61.5       | 0.845      | 73             |
| cisplatin | 1.3 | 1.7       | 0.76           | 2.8        | 4.0       | 0.7           | 102.3      | 128.8      | 0.79           | 5.4        | 4.5        | 1.2            |

$^a$ Cells were incubated with the indicated compounds in the dark for 4 h. $^b$ Cells were incubated with the indicated compounds for 4 h in the dark and then irradiated with a white light projector (filter cutoff below 600 nm, light intensity 5.8 mW cm$^{-2}$, light dose 6.96 J cm$^{-2}$). $^c$ p.i. = phototoxicity index, the ratio of the IC$_{50}$ values in the dark to those obtained after light irradiation.

MLC31 was obtained by coupling LC31 with amino-diethyl-malic ester, thereby not only improving the lipophilicity, but also attaching a functional group. Surprisingly, MLC31 showed the lowest IC$_{50}$ value of the three tested Pc-based agents in HeLa cells after irradiation with NIR light (9 nM, Table 2), in comparison to 5.2 µM in the dark. The p.i. of MLC31 is around 578. The IC$_{50}$ value for LC31 is 426 nM under light irradiation, lower than the IC$_{50}$ value of cisplatin (1.7 µM). The p.i. of LC31, however, is over 30. In
this context, it is interesting to note that Xue et al. found that their mono-PEGylated Pcs were much more toxic against HepG2 cells (IC\(_{50}\) values in the range of 12–17 nM) than the analogue Pcs, which were four-fold substituted with the same PEG substituent (IC\(_{50}\) values between 8 and >40 µM) [32]. After a coupling with cisplatin, DMLC31Pt might offer a double-functional chemotherapy/PDT mode of action. Yet, the observed cytotoxicity did not give solid evidence of an improved therapeutic efficiency of such a design. The IC\(_{50}\) values of DMLC31Pt upon light irradiation and in the dark tested in the HeLa cell line are 775 nM and 8.0 µM, respectively, with the p.i. being around 10. In conclusion, the observed phototoxicities of the three studied PSs could be positively correlated with their Φ\(_{Δ}\)s, but not with their abilities to generate •OH. This indicates that 1\(^{\text{O}}\)\(_{2}\) is the dominant agent responsible for the phototoxicity of LC31, MLC31, and DMLC31Pt.

2.2. DNA Damage

To gain mechanistic insights into the cellular effect of DMLC31Pt, we examined the phosphorylation of Ser139 of histone variant H2AX to give γH2AX, an established marker of the DNA damage response (DDR) [52]. An immunofluorescence-based method was used to detect γH2AX according to an established protocol [53]. HeLa cells incubated with DMLC31Pt for 16 h but not activated by NIR light showed only minor formation of γH2AX, while cells treated with DMLC31Pt and activated by NIR light showed a more intense γH2AX signal (Figure S8). Considering that DMLC31Pt is predominantly localized in the cytoplasm, generation of γH2AX in response to light irradiation of DMLC31Pt could be either triggered by low amounts of compound entering the nuclei or be a consequence of the activation of cell death pathways by irradiated DMLC31Pt that is present outside the nucleus.

A comparison of the relative cytotoxicities of LC31, MLC31, DMLC31Pt, and cisplatin was also made between the A2780 cell line and cisplatin-resistant A2780/CP70 cell lines (Figure S7). As expected, cisplatin resulted in no obvious cytotoxicity difference upon irradiation with light. Notably, after light irradiation at a very low intensity (2.0 W cm\(^{-2}\)) for 20 min, the tested agents were found to be much more active in the cisplatin-resistant cell line (MLC31 with IC\(_{50}\) around 157 nM) than cisplatin itself (129 µM), which indicates its therapeutic potential, particularly in light of the worrying emergence of cisplatin resistance in tumors [54]. Moreover, the finding that MLC31 was found to be less cytotoxic than cisplatin under dark conditions in the non-cancerous MRC-5 cell line also implies that MLC31 has a better therapeutic profile when compared to cisplatin.

The IC\(_{50}\) phototoxicity values after activation by NIR light irradiation follow the general trend of LC31 ≈ DMLC31Pt >> MLC31 (Table 2). It is surprising to note that MLC31, which is structurally very similar to LC31 and DMLC31Pt, displayed quite different IC\(_{50}\) values than the latter two PSs (>5-fold for the A2780/CP70 cell line, >10-fold for the A2780, and >80-fold for the HeLa cell line) upon light irradiation. Interestingly, the photo-induced cytotoxicity of these PSs correlates rather well with the production of 1\(^{\text{O}}\)\(_{2}\) (Table 1). In order to evaluate the action of the best PS, MLC31, against HeLa cells further, a comparison between its phototoxicity and those of the best molecular Pc-based PSs reported in literature was made (Table 3) [55]. This shows a clear trend that an increasing positive charge leads to a more phototoxic PS. However, the neutrally charged MLC31 is clearly the most toxic Pc in the comparison series.
Table 3. Comparison of toxicities in the dark and upon light irradiation of Pcs with a neutrally charged central unit against the human cervical cancer (HeLa) cell line. Only the best compound from each publication is taken.

| Comp. | Dark Toxicity [µM] | Light Toxicity [µM] | p.i. | Wavelength [nm] | Fluence [J cm\(^{-2}\)] | Ref. |
|-------|-------------------|---------------------|------|-----------------|-----------------------|------|
| MLC31 | 5.2               | 0.009               | 578  | \(\lambda > 600\)\(^{a}\) | 6.96                  | This work |
| (SiPc)((OC\(_2\)H\(_4\))\(_2\)OH)\(_2\) | >3                | 0.3                 | >10  | \(690\)\(^{b}\) | 2                    | [56] |
| SiPc(R-Biotin)\(_2\) | >10               | 0.4                 | >25  | \(690\)\(^{b}\) | 2                    | [57] |
| ZnPc(branchedPEG)\(_2\) biotin | >5                | 1.5                 | >3.3 | \(690\)\(^{b}\) | 2                    | [58] |
| ZnPc(1\(_3\))R-Biotin | >10               | 2.2                 | >4.6 | \(690\)\(^{b}\) | 2                    | [59] |
| [(SiPc)(RNEt\(_2\)Me)\(_2\)]\(^{2+}\) | >10               | 1.5                 | >6.7 | \(690\)\(^{b}\) | 2                    | [60] |
| [ZnPc]\(^{8+}\) | >100              | 0.04                | 2500 | \(740\)\(^{c}\) | 15                   | [61] |
| [ZnPc-(R(pyMe)\(_4\))\(_4\)]\(^{16+}\) | 675               | 0.48                | 1409 | \(\lambda > 570\)\(^{d}\) | 11.2 | [62] |
| [ZnPc-(R’imidazolium\(_4\))\(_4\)]\(^{16+}\) | 395               | 0.037               | 10675 | \(\lambda > 570\)\(^{d}\) | 11.2 | [63] |

\(^{a}\) Halogen lamp, \(^{b}\) Lumacare (LC-122 fiber-optic probes), \(^{c}\) LED, \(^{d}\) Xe lamp.

2.3. Cellular Uptake of LC31, MLC31, and DMLC31Pt

HeLa cells were seeded in 6 cm dishes and three different concentrations (500 nM, 2 µM and 8 µM) of LC31, MLC31, and DMLC31Pt were added in triplicates, respectively. After 4 h of incubation with the PSs, the cellular uptake was investigated by flow cytometry. As shown in Figure 3, an intracellular accumulation of PSs was clearly observed, and the fluorescence intensity exhibited an increasing tendency with the increase of the PS concentrations (Figure 3a–c), consistent with the quantitative analysis of the mean fluorescence intensity (Figure 3d). At all of these tested concentrations, MLC31 showed the highest cellular fluorescence intensity, which indicates the highest cellular uptake of these three PSs. The cellular uptake of the PSs was evaluated at two different temperatures (4 °C and 37 °C) to determine whether the internationalization occurred in an energy-dependent manner [64]. Compared to 37 °C, the cellular uptake of the PSs was significantly inhibited at 4 °C, according to the fluorescence intensity analyzed by flow cytometry (Figure S9). This indicates that MLC31 was internalized into the cells via an energy-dependent active pathway.

2.4. Distribution Coefficient (logP)

The lipophilicity of a compound has a strong influence on its cellular uptake, localization, and further biodistribution. Chemical drugs have to cross a series of barriers in the body by either passive diffusion or a carrier-mediated transport [65]. The distribution coefficient (logP), the logarithm of its partition coefficient between n-octanol and water (log(c\(_{\text{octanol}}\)/c\(_{\text{water}}\))), is used as one of the principal parameters to evaluate the lipophilicity of chemical compounds, therefore influencing the pharmacokinetic properties of drugs. Low hydrophilicities and thus high logP values normally indicate poor absorption or permeation. The lipophilicity of the Pc agents reported here was determined by measuring the distribution coefficients in PBS at a pH = 7.4, an approximation of physiological conditions, using the shake-flask method [66,67]. As shown in Figure 3e, MLC31 has the highest lipophilicity of the series due to the presence of the ethyl malonic ester moiety, with a logP value around 1.316. LC31 has a logP value of 0.908, and DMLC31Pt has the lowest logP value of 0.735. These distribution coefficient values are in line with the cellular uptake measured using flow cytometry, as a certain lipophilicity of MLC31 leads to higher cellular uptake. These results also correlate with the NIR light-induced intracellular ROS intensities, showing that MLC31 has the highest ability to generate ROS in cells (Figure S10).
2.5. Cell Localization via In Vitro Fluorescence Evaluation

The mechanism of PDT-induced cell death via apoptosis or necrosis is highly dependent upon the localization of the PS within the cell and the amount of cytotoxic $^{1}$O$_{2}$ generated [68]. Some evidence suggests that a PS, which is localized in the mitochondria or the endoplasmic reticulum (ER), is a better inducer of apoptosis, whereas PS localized in the plasma membrane or lysosomes is more conducive to necrosis [69]. The diverse photophysical properties of metal–Pc complexes, especially the fluorescent properties, can be utilized to study their cellular accumulation, biodistribution, and metabolic pathways. This, in turn, allows for decision-making on whether they are ideal model compounds to construct novel theragnostic platforms combining diagnostic and therapeutic purposes.

As a first step towards elucidating the cellular uptake action of MLC31, favorable NIR emission has been used to evaluate the localization of the Pc-based agents in HeLa cells. Cellular localization of LC31, MLC31, and DMLC31Pt was assessed by fluorescence confocal microscopy. All investigated PSs were effectively taken up by HeLa cells, and the emerging fluorescence emission showed that LC31 and MLC31 co-localized well with the MitoTracker dye (Figures 4 and S11). This shows that LC31 and MLC31 tend to specifically accumulate in mitochondria, more precisely in the mitochondrial outer membrane, while DMLC31Pt accumulated in the cytoplasm and nucleoli (Figure S8). Unlike DMLC31Pt, MLC31 did not accumulate in the cell nucleus, therefore limiting potential DNA damage that could be carcinogenic or lead to the development of resistant clones. Within PDT, the primary pathway for the execution of mitochondria-targeting PS-induced cell death has been attributed to apoptosis [69,70]. Induction of apoptosis by MLC31 was observed by detecting changes of the cell morphology from image acquisitions in HeLa cells. As shown
in Figure S12, a longer light irradiation (633 nm) of cells treated with MLC31 resulted in increasing abnormal cell morphology within few minutes. More specifically, plasma membrane blebbing (zeiosis) occurred, one of the defining characteristics of apoptosis [71]. The fluorescent signal of MLC31 remained in the mitochondria during the procedure of the image acquisition.

Figure 4. Subcellular localization of (a) LC31 and (b) MLC31 by MitoTracker Deep Red FM and DAPI staining. HeLa cells were incubated with 1 µM LC31 or 500 nM MLC31 for 2 h. The PSs were visualized upon excitation at 633 nm. Images merging and multi-channel intensity line profile plots were obtained using the software Fiji [72].
2.6. Detection of the Loss of the Mitochondrial Membrane Permeabilization

Mitochondria play a central role in cellular homeostasis, not only providing energy from ATP by the process of oxidative phosphorylation, but also by decisively regulating the intrinsic pathway of apoptosis [73]. The integrity of the mitochondrial membrane, and especially an intact electrochemical gradient, are responsible for ATP production, while dysfunction of the latter is linked to the release of pro-apoptotic factors [74,75]. The hallmark of mitochondrial dysfunction is the loss of the mitochondrial membrane potential (MMP, $\Delta \Psi_m$). The collapse of the $\Delta \Psi_m$ coincides with the opening of the mitochondrial permeability transition pore, leading to the release of soluble proteins such as cytochrome c (cyt c) into the cytosol. The study of the $\Delta \Psi_m$ is essential for an integrated appraisal of mitochondrial function, since it reflects differences in the electrical potential and represents the main component of the proton electrochemical gradient—accounting for more than 90% of the total available respiratory energy. The collapse of $\Delta \Psi_m$ in tumor cells constitutes one of the goals of anticancer chemotherapy. As the aforementioned results confirmed that MLC31 localizes mainly in mitochondria, an attempt to better understand the kinetics and consequences of apoptotic damage was performed though the detection of the cellular MMP with or without irradiation with NIR light.

As shown in Figure 5, no detectable loss in the $\Delta \Psi_m$ was found without irradiation by light for cells treated with MLC31, or in the control condition without administration of the compound and activation by NIR light. However, MLC31 in the presence of NIR light led to an extensive decrease in the $\Delta \Psi_m$ together with a decrease in the fluorescence signal, which occurred within few minutes (Figures 5b, S12 and S13). The $\Delta \Psi_m$ collapse leads to release of cyt c into the cytosol, starting the apoptotic pathway.

2.7. Effects under Hypoxia

Solid tumors are known to contain regions with oxygen deficiency (hypoxia), which triggers reprogramming of tumor metabolism and angiogenesis, leading to metastasis and decreased patient survival [76]. Tumor hypoxia not only increases the resistance to radiotherapy and chemotherapy, but also plays a critical role in PDT, because the ROS generation is oxygen dependent. In order to evaluate the effect of oxygen availability, the light irradiation-induced activity of MLC31 in HeLa cells was investigated under normoxic (18.6% $O_2$) and hypoxic (0.2% $O_2$) conditions.

As shown in Figure 6a, HeLa cells were incubated with PS and subjected to photoirradiation under hypoxic conditions before being returned to normoxia. While for all tested compounds the inhibition efficiency decreased only slightly under hypoxic conditions (Figure 6b–d and Table 4), a high level of cytotoxicity was still observed, even at an oxygen concentration as low as 0.2%, with IC$_{50}$ values of 56 nM and a p.i. of 74 (Figure 6). The different IC$_{50}$ values obtained in this series of experiments compared with the ones reported in Table 1 can be explained by the use of different light sources (a projector with 600 nm cutoff long-pass filter was used to obtain the results reported in Table 1, while a 660 nm LED light source was used for the results reported in Table 4). These are promising results since tumor hypoxic microenvironments exhibit a wide range of different oxygen partial pressures, corresponding to gas phase concentrations in the range of 0 to 5%, while MLC31 is effective even at an oxygen concentration of 0.2% [77]. This suggests that MLC31 is able to ablate cells even in harsh hypoxic environments. Based on these results, we suggest that MLC31 could potentially also be highly effective in solid tumors.
Figure 5. (a) NIR light-induced $\Delta \Psi_m$ change measured by the MMP assay. (b) Timelapse of intensity variation analysis of MLC31 and $\Delta \Psi_m$ channels obtained through the ROI Manager for intensity analysis using Fiji [72].
Figure 6. (a) Schematic protocol of the cytotoxicity determination in HeLa cells under hypoxic conditions. Cells were treated with (b) LC31, (c) MLC31, and (d) DMLC31Pt, respectively, for 4 h under hypoxic or normoxic conditions (inhibition of viability shown, LED light source of 660 nm, light intensity = 9 mW cm$^{-2}$, light dose = 10.8 J cm$^{-2}$).

Table 4. Comparison of the IC$_{50}$ values (µM) of LC31, MLC31, and DMLC31Pt under hypoxic or normoxic conditions.

|          | 18.6% O$_2$ | 0.2% O$_2$ |          |
|----------|-------------|------------|----------|
|          | Dark  | Light * | p.i.  | Dark  | Light * | p.i.  |
| LC31     | >20   | 0.169    | >118   | >20   | 0.177    | >113  |
| MLC31    | 14.8  | 0.051    | 290    | >20   | 0.061    | 328   |
| DMLC31Pt | 16.7  | 7.0      | 2.4    | >20   | 8.1      | >2.5  |

*a Irradiation experiment with an LED 660 nm light source, light intensity = 9 mW cm$^{-2}$, light dose = 10.8 J cm$^{-2}$.

3. Materials and Methods
3.1. General Materials and Instrumentation

Unless otherwise stated, all chemicals were of reagent grade and purchased from Sigma-Aldrich (Darmstadt, Germany), Merck (Darmstadt, Germany), Alfa Aesar (Kandel, Germany), or Fluorochem (Hadfield, United Kingdom). Reactions were carried out under N$_2$ and monitored for completion by analysing a small sample by TLC or UPLC. Solvents for reactions were of p.a. grade. Evaporation of the solvents in vacuo was done with a rotary evaporator at the given bath temperature and pressure. A vacuum line and Schlenk glassware were employed when reactions had to be carried out under a dry atmosphere. Assemblies were protected from light, if necessary, by wrapping them with aluminium foil. Antibodies: rabbit polyclonal antibodies to phosphorylated histone H2AX (S$_{139}$) from Cell Signalling Technology (Beverly, MA, USA) were used. HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Amersham-Biosciences/GE-Healthcare (Otelfingen, Switzerland). Alexa Fluor® 488 anti-rabbit secondary antibody was obtained from Cell Signalling Technology (Beverly, MA, USA). pH: Merck indicator paper pH 1–14 (universal indicator). Chromatography: Grace silica gel (10–14 µm) with the indicated solvent system. Eluent mixtures are expressed as volume to volume ($v/v$) ratios. Thin-layer chromatography (TLC): Merck TLC plates silica gel 60 on Alox with the indicated solvent system and the spots were visualized with UV light (254 and 366 nm). UV-Vis spectra: Specord 250 Plus spectrophotometer (Analytik Jena, Jena, Germany); $\lambda_{\text{max}}$ (log$e$) and $\lambda_{\text{min}}$
(log ε) in nm. Emission spectra were obtained on a Perkin-Elmer Luminescence spectrometer LS 50 B and were corrected for instrumental response using the provided correction factors. The excitation and emission slit width were set to 5.0 and 5.0 nm, respectively. IR spectra: SpectrumTwo FT-IR Spectrometer (Perkin–Elmer, Schwerzenbach, Switzerland) equipped with a Specac Golden Gate™ ATR (attenuated total reflection) accessory, applied as neat samples, 1/λ in cm⁻¹. ¹H-NMR spectra in CDCl₃ or DMSO-d₆; Bruker AV-400 (400 MHz) or Bruker AV-500 (500 MHz). δ in ppm refer to the deuterated solvent CDCl₃ (δ 7.26) or DMSO-d₆ (δ 2.5) in Hz. ¹³C-NMR spectra in CDCl₃ or DMSO-d₆; multiplicities from DEPT-135 and DEPT-90 experiments. The abbreviations for the peak multiplicities are as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet), and br (broad).

The Acquity Waters UPLC (BEH C18 analytical column, 1.7 μm, 50 × 2.1 mm), equipped with a DAD detector and an auto sampler, was performed with a linear gradient of solvent A (distilled H₂O containing 0.1% v/v TFA) and solvent B (CH₃CN (Sigma-Aldrich HPLC-grade)); t = 0–0.5 min, 5% B; t = 0.51–4 min, from 95% A (5% B) to 0% A (100% B); t = 4–5 min, 0% A, 100% B. Detection was performed at 250 nm, 313 nm, and 640 nm. HR-EI-MS was performed on a Thermo DRS (ThermoFisher Scientific, Bremen, Germany) double-focus magnetic sector mass spectrometer (geometry BE). Mass spectra were measured in electron impact (EI) mode at 70 eV, with a solid probe inlet, a source temperature of 200 °C, acceleration voltage of 5 kV, and resolution of 2500. The instrument was scanned between m/z 30 and 900 at scan rate of 2 s/decade in the magnetic scan mode. Perfluorokerosene (PFK, Fluorochem, Derbyshire, UK) was used for calibration. The UPLC–ESI–MS spectra of PSs were measured on an Acquity Waters UPLC system coupled to Bruker HCT™ (Bremen, Germany) for the MS measurements, equipped with a DAD detector and an auto sampler using an Acquity UPLC BEH C18 analytical column (1.7 μm, 50 × 2.1 mm). The LC run (flow rate: 0.6 mL/min) was performed with a linear gradient of solvent A (distilled H₂O containing 0.1% v/v formic acid) and solvent B (CH₃CN (Sigma-Aldrich HPLC-grade)); t = 0–0.5 min, 5% B; t = 0.51–4 min, from 95% A (5% B) to 0% A (100% B); t = 4–5 min, 0% A, 100% B. UV-Vis detection was collected from 200 to 480 nm.

3.2. Syntheses

The phthalocyanines were isolated as green solids after column chromatography.

**LC31** (analogous to [9,40]):

\[
\text{Zn(OAc)}_2 (1.19 \text{ mmol}, 0.22 \text{ g}) \text{ and } 4-(4\text{-carboxyphenoxy})\text{-phthalonitrile (6,1.17 mmol, 0.31 g) were dissolved in 1-pentanol (17 mL) in a one-necked 100 mL round-bottom flask equipped with a reflux condenser. The mixture was stirred at 110 °C for 20 min. Then, the glycol-substituted nitrophthalonitrile (3,3.69 mmol, 1.07 g) was added, followed by DBU (769 \mu L), and the colour of the mixture changed from orange to brown. The reaction mixture was then stirred at 160 °C under N₂ atmosphere. After 48 h, the green reaction mixture was cooled down to room temperature and hexane (185 mL) was added. A green suspension/precipitate was formed, which was filtered off to obtain the crude product (7, 2.02 g). The crude product was purified by column chromatography on silica gel with chloroform and increasing amounts of MeOH. Analysis by UPLC showed a purity of 95%. NMR data were not obtained as LC31 consists of a mixture of two isomers. UV-Vis (CHCl₃; \lambda_{\text{max}} \text{ nm, log } ε): 339 (6.01), 624 (5.82), 694 (6.55), 740 (5.69). (+)-UPLC-MS: 1199.7 (100, [M + H]^+); calculated 1199.4, (100, [M + H]^+). UPLC: Rₘ = 3.1.
\]

**MLC31**:

To a suspension of 44.0 mg (0.037 mmol) of compound LC31 in 3 mL of DMF, 11.6 mg (0.18 mmol) of N-methylmorpholine was added, followed by the addition of 9.0 mg (0.051 mmol) of 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) at 0 °C. The resulting mixture was stirred for 1.5 h until analysis by HPLC showed completeness of the activation. Dimethyl aminomalonate hydrochloride (10.8 mg, 0.051 mmol) was added, and the mixture was stirred at 25 °C until a complete conversion of MLC31 was observed by HPLC. A total of 10 mL of methylene chloride was then added to the reaction mixture together with 10 mL of deionized water, and the mixture was stirred further for 15 min. The layers were then...
separated, and the organic dark green layer was mixed with Celite before the solvent was evaporated to obtain a dry residue. Flash column chromatography (EtOAc/MeOH = 10:1 and CH₂Cl₂/CH₃OH = 10:1) yielded MLC31 (19 mg, 37% yield) as a dark green solid. The chemical purity assessed by NMR is 97%. M.p. > 300 °C, Rf (CH₂Cl₂/CH₃OH = 10:1) = 0.14.

UV–Vis (CDCl₃, λ_{max} nm, log ε): 694 (7.05), 626 (6.27), 336 (6.51). ¹H-NMR (400 MHz; DMSO-d₆): 11.99 (s, 1 H), 9.46–9.34 (m, 2 H), 9.13–8.90 (m, 4 H), 8.18–8.07 (m, 4 H), 7.94 (d, J = 8.2 Hz, 1 H), 7.84 (d, J = 8.2 Hz, 2 H), 7.74 (d, J = 7.6 Hz, 1 H), 7.49 (d, J = 8.3 Hz, 2 H), 5.38 (d, J = 7.6 Hz, 1 H), 5.19 (s, 3 H), 4.90 (s, 3 H), 4.40 (s, 3 H), 4.42 (q, J = 6.8 Hz, 2 H), 4.19 (s, 4 H), 4.07 (s, 3 H), 3.75 (s, 6 H), 3.60–3.52 (m, 4 H), 3.45 (s, 4 H), 3.42–3.37 (m, 3 H), 3.17 (s, 3 H), 3.11 (s, 6 H), 1.25 (t, J = 7.1 Hz, 6 H).

Conclusions

A preliminary investigation of its behavior in cells and its mechanism of action showed how the functional groups affect the distribution coefficient (log P) and thereby the cellular uptake. Additionally, how MLC31 disrupts the ΔΨₐ and induces apoptosis upon activation by light was investigated. Surprisingly, MLC31 showed high cytotoxicity under hypoxic conditions, which is due to its mitochondria-targeting property. In conclusion, the amphiprotic property of MLC31 dramatically enhances the intercalation with the mitochondria in hypoxic conditions, which is due to its mitochondria-targeting property. In conclusion, the amphiprotic property of MLC31 dramatically enhances the intercalation with the mitochondria in hypoxic conditions, which is due to its mitochondria-targeting property.
mitochondrial membrane, thus improving the mitochondria-targeting uptake. Such an asymmetric design with both hydrophilic and lipophilic peripheral groups may serve as an effective way to improve the PDT efficiency of highly aromatic PSs for NIR light-mediated cancer therapy.

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23179525/s1. References [9,26,33,40,43,66,67,79–89] are cited in the supplementary materials.

Author Contributions: Conceptualization, W.W.K. and B.S.; investigation, W.W.K., N.G., L.S., V.B., C.K., P.S. and B.S.; writing—original draft preparation, W.W.K.; writing—review and editing, L.S., R.H.W., S.F. and B.S.; visualization, W.W.K.; supervision, R.H.W., S.F. and B.S.; funding acquisition, W.W.K. and B.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the University of Zurich, the Swiss National Science Foundation (project number 205321_159976), and its R’Equip programme (project number 206021_164018). W.W.K. was supported by a Swiss Government Excellence Scholarship and the Novartis Foundation for Medical–Biological Research (project number #18B085).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Additional data are available as the Supplementary Information.

Acknowledgments: Imaging was performed with support and equipment maintained by the Center for Microscopy and Image Analysis, University of Zurich. We thank Thomas Fox for recording the 195Pt-NMR spectra and Frank Schumer for synthesizing the APF.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Dolmans, D.E.J.G.J.; Fukumura, D.; Jain, R.K. Photodynamic therapy for cancer. Nat. Rev. Cancer 2003, 3, 380–387. [CrossRef] [PubMed]
2. Agostinis, P.; Berg, K.; Cengel, K.A.; Foster, T.H.; Girotti, A.W.; Gollnick, S.O.; Hahn, S.M.; Hamblin, M.R.; Juzeniene, A.; Kessel, D.; et al. Photodynamic Therapy for Anticancer: An Update. CA Cancer J. Clin. 2011, 61, 250–281. [CrossRef] [PubMed]
3. Zhang, J.; Jiang, C.; Figueiró Longo, J.P.; Azzevedo, R.B.; Zhang, H.; Muehlmann, L.A. An updated overview on the development of new photosensitizers for anticancer photodynamic therapy. Acta Pharm. Sin. B 2018, 8, 137–146. [CrossRef] [PubMed]
4. Antoni, P.M.; Naik, A.; Albert, I.; Rubbiani, R.; Gupta, S.; Ruiz-Sanchez, P.; Munikorn, P.; Mateos, J.M.; Luginbuehl, V.; Thamyonkit, P.; et al. (Metallo)porphyrins as Potent Phototoxic Anti-Cancer Agents after Irradiation with Red Light. Chem. Eur. J. 2015, 21, 1179–1183. [CrossRef]
5. Pham, T.C.; Nguyen, V.-N.; Chou, Y.; Lee, S.; Yoon, J. Recent Strategies to Develop Innovative Photosensitizers for Enhanced Photodynamic Therapy. Chem. Rev. 2021, 121, 13454–13619. [CrossRef]
6. Nath, S.; Obaid, G.; Hasan, T. The Course of Immune Stimulation by Photodynamic Therapy: Bridging Fundamentals of Photochemically Induced Immunogenic Cell Death to the Enrichment of T-Cell Repertoire. Photochem. Photobiol. 2019, 95, 1288–1305. [CrossRef]
7. Wagner, A.; Denzer, U.W.; Neureiter, D.; Kiesslich, T.; Puespoeck, A.; Rauws, E.A.J.; Emmanuel, K.; Degenhardt, N.; Frick, U.; Beuers, U.; et al. Temoporfin improves efficacy of photodynamic therapy in advanced biliary tract carcinoma: A multicenter prospective phase II study. Hepatology 2015, 62, 1456–1465. [CrossRef]
8. Singh, S.; Aggarwal, A.; Bhupathiraju, N.V.S.D.K.; Arianna, G.; Tiwari, K.; Drain, C.M. Glycosylated Porphyrins, Phthalocyanines, and Other Porphyrinoids for Diagnostics and Therapeutics. Chem. Rev. 2015, 115, 10261–10306. [CrossRef]
9. Liu, J.-Y.; Jiang, X.-J.; Fong, W.-P.; Ng, D.K.P. Highly photocytotoxic 1,4-dipegylated zinc(II) phthalocyanines. Effects of the chain length on the in vitro photodynamic activities. Org. Biomol. Chem. 2008, 6, 4560–4566. [CrossRef]
10. Ogunsipe, A.; Durmus, M.; Atilla, D.; Gürek, A.G.; Ahsen, V.; Nyokong, T. Synthesis, photophysical and photochemical studies on long chain zinc phthalocyanine derivatives. Synth. Met. 2008, 158, 839–847. [CrossRef]
11. Moreira, L.M.; dos Santos, F.V.; Lyon, J.P.; Maftoum-Costa, M.; Pacheco-Soares, C.; da Silva, N.S. Photodynamic therapy: Porphyrins and Phthalocyanines as Photosensitizers. Aust. J. Chem. 2008, 61, 741–754. [CrossRef]
12. McRae, E.K.S.; Nevonen, D.E.; McKenna, S.A.; Nemykin, V.N. Binding and photodynamic action of the cationic zinc phthalocyanines with different types of DNA toward understanding of their cancer therapy activity. J. Inorg. Biochem. 2019, 199, 110793. [CrossRef] [PubMed]
13. Furuyama, T.; Miyaj, Y.; Maeda, K.; Maeda, H.; Segi, M. Extremely Photostable Electron-Deficient Phthalocyanines that Generate High Levels of Singlet Oxygen. Chem. Eur. J. 2019, 25, 1678–1682. [CrossRef] [PubMed]
14. Hamblin, M.R.; Huang, Y. Imaging in Photodynamic Therapy, 1st ed.; CRC Press: Boca Raton, FL, USA, 2017; p. 479.

15. Sen, P.; Managa, M.; Nyokong, T. New type of metal-free and Zinc(II), In(III), Ga(III) phthalocyanines carrying biologically active substituents: Synthesis and photophysics and photophysical properties and photodynamic therapy activity. Inorg. Chim. Acta 2019, 491, 1–8. [CrossRef]

16. Lo, P.C.; Rodriguez-Morgade, M.S.; Pandey, R.K.; Ng, D.K.P.; Torres, T.; Dumoulin, F. The unique features and promises of phthalocyanines as advanced photosensitisers for photodynamic therapy of cancer. Chem. Soc. Rev. 2020, 49, 1041–1056. [CrossRef] [PubMed]

17. Plaetzer, K.; Krammer, B.; Berlanda, J.; Berr, F.; Kiesslich, T. Photophysics and photochemistry of photodynamic therapy: Fundamental aspects. Lasers Med. Sci. 2009, 24, 259–268. [CrossRef] [PubMed]

18. Safar Sajadi, S.M.; Khoee, S. The simultaneous role of porphyrins’ H- and J-aggregates and host–guest chemistry on the fabrication of reversible Dextran-PMMA polymersome. Sci. Rep. 2021, 11, 2832. [CrossRef]

19. Naik, A.; Rubbiani, R.; Gasser, G.; Spingler, B. Visible-Light-Induced Annihilation of Tumor Cells with Platinum–Porphyrin Conjugates. Angew. Chem. Int. Ed. 2014, 53, 6938–6941. [CrossRef]

20. Schneider, L.; Larocca, M.; Wu, W.; Babu, V.; Padrutt, R.; Slyshkina, E.; König, C.; Ferrari, S.; Spingler, B. Exocyclically metallated tetrapyridinoporphyrazine as a potential photosensitizer for photodynamic therapy. Photochem. Photobiol. Sci. 2019, 18, 2792–2803. [CrossRef]

21. Rubbiani, R.; Wu, W.; Naik, A.; Larocca, M.; Schneider, L.; Padrutt, R.; Babu, V.; König, C.; Hinger, D.; Maake, C.; et al. Studying the cellular distribution of highly photosensitive platination metalloporphyrins by isotope labelling. Chem. Commun. 2020, 56, 14373–14376. [CrossRef]

22. Dingiswayo, S.; Babu, B.; Prinsloo, E.; Mack, J.; Nyokong, T. A comparative study of the photophysicochemical and photodynamic activity properties of meso-4-methylthiophenyl functionalized Sn(IV) tetraarylporphyrins and triarylcorroles. J. Porphyr. Phthalocyanines 2020, 24, 1138–1145. [CrossRef]

23. Deng, J.; Li, H.; Yang, M.; Wu, F. Palladium porphyrin complexes for photodynamic cancer therapy: Effect of porphyrin units and metal. Photoch. Photobiol. Sci. 2020, 19, 905–912. [CrossRef] [PubMed]

24. Padrutt, R.; Babu, V.; Klingler, S.; Kalt, M.; Schumers, F.; Anania, M.; Schneider, L.; Spingler, B. Distyryl-BODIPY-Transplatin Conjugates as Highly Phototoxic Photosensitizers for Photodynamic Therapy. ChemMedChem 2021, 16, 694–701. [CrossRef] [PubMed]

25. Gandosio, A.; Purkait, K.; Gasser, G. Recent Approaches towards the Development of Ru(II) Polyphenyl Complexes for Anticancer Photodynamic Therapy. Chimia 2021, 75, 845–855. [CrossRef]

26. Le, N.A.; Babu, V.; Kalt, M.; Schneider, L.; Schumers, F.; Spingler, B. Photo-stable platinated bacteriochlorins as potent photodynamic agents. J. Med. Chem. 2021, 64, 6792–6801. [CrossRef]

27. Schneider, L.; Kalt, M.; Larocca, M.; Babu, V.; Spingler, B. Potent PBS-Soluble Transplatin Derived Porphyrin-Based Photosensitizer for Photodynamic Therapy. Inorg. Chem. 2021, 60, 9416–9426. [CrossRef] [PubMed]

28. Li, H.; Froncek, F.R.; Vicente, M.G.H. Pegylated phthalocyanines: Synthesis and spectroscopic properties. Tetrahedron Lett. 2011, 52, 6675–6678. [CrossRef]

29. Tuncel, S.; Dumoulin, F.; Gailer, J.; Sooriyaarachchi, M.; Atilla, D.; Durmuş, M.; Bouchou, D.; Savoie, H.; Boyle, R.W.; Ahsen, V. A set of highly water-soluble tetrachlorin-phytoporphyrin-substituted Zn(ii) phthalocyanines: Synthesis, photophysical and photochemical properties, interaction with plasma proteins and in vitro photocytotoxicity. Dalton Trans. 2011, 40, 4067–4079. [CrossRef]

30. Akkus, F.; Kabay, N.; Gök, Y. The first synthesis and characterization of new metal-free and metallophthalocyanine containing 33-membered crown ether phthalocyanines. J. Porphyr Phthalocyanines 2013, 17, 473–479. [CrossRef]

31. Wierczowski, M.; Sobotta, L.; Skupin-Mrugalska, P.; Kruk, J.; Jusiak, W.; Ye, M.; Konopka, K.; Düzgünş, N.; Tykarska, E.; Gdaniec, M.; et al. Phthalocyanines functionalized with 2-methyl-5-nitro-1H-imidazolylthio and 1,4,7-trioxanonyl moieties and the effect of metronidazole substitution on photocytotoxicity. J. Inorg. Biochem. 2013, 127, 62–72. [CrossRef]

32. Jia, X.; Yang, F.-F.; Li, J.; Liu, J.-Y.; Xue, J.-P. Synthesis and in Vitro Photodynamic Activity of Oligomeric Ethylene Glycol-Quinoline Substituted Zinc(II) Phthalocyanine Derivatives. J. Med. Chem. 2013, 56, 5797–5805. [CrossRef] [PubMed]

33. Tuncel, S.; Trivella, A.; Atilla, D.; Bennis, K.; Savoie, H.; Albriex, F.; Delort, L.; Billard, H.; Dubois, V.; Ahsen, V.; et al. Assessing the Dual Activity of a Chalcone-Phthalocyanine Conjugate: Design, Synthesis, and Antivascular and Photodynamic Properties. Mol. Pharm. 2013, 10, 3706–3716. [CrossRef] [PubMed]

34. Sobotta, L.; Wierczowski, M.; Mierzwicki, M.; Gdaniec, Z.; Mielenk, J.; Persoons, L.; Goslinski, T.; Balzarini, J. Photochemical studies and nanomolar photodynamic activities of phthalocyanines functionalized with 1,4,7-trioxanonyl moieties at their non-parallel positions. J. Inorg. Biochem. 2016, 155, 76–81. [CrossRef] [PubMed]

35. Topkaya, D.; Lafont, D.; Poyet, F.; Garcia, G.; Albriex, F.; Maillard, P.; Bretonniere, Y.; Dumoulin, F. Design of an amphiphilic porphyrin exhibiting high in vitro photocytotoxicity. New J. Chem. 2016, 40, 2044–2050. [CrossRef]

36. Aribi, F.; Vey, C.; Topkaya, D.; Kostakoglou, S.T.; Fournier-dit-Chabert, J.; Buyukdeki, S.; Taskin, G.C.; Alpugan, S.; Albriex, F.; Gurek, A.G.; et al. Phthalocyanine-chalcone conjugates. J. Porphyr. Phthalocyanines 2016, 20, 497–504. [CrossRef] [PubMed]

37. Kasprzycki, P.; Sobotta, L.; Lijewski, S.; Wierczowski, M.; Goslinski, T.; Mielenk, J.; Radzewicz, C.; Fita, P. Unusual cisdiprotonated forms and fluorescent aggregates of non-peripherally alkoxy-substituted metallophthalocyanines. Phys. Chem. Chem. Phys. 2017, 19, 21390–21400. [CrossRef]
