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Photo-induced anticancer activity and singlet oxygen production of prodigiosenes

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Abstract

The photo-induced cytotoxicity of prodigiosenes is reported. One prodigiosene represents a synthetic analogue of the natural product prodigiosin, and two are conjugated to molecules that target the estrogen receptor (ER). A comparison of incubation and irradiation frameworks for the three prodigiosenes is reported, with activity against ER- and ER+ lines explored. Furthermore, the ability of the three prodigiosenes to photosensitise the production of singlet oxygen is demonstrated, shedding mechanistic light onto possible photodynamic therapeutic effects of this class of tripyrroles.

Introduction

Photodynamic therapy (PDT) is a light-induced, non-invasive cancer treatment, which is effective for both tumour cell killing and ablation.[1-7] PDT usually involves the use of a photosensitizer (PS), which, upon irradiation of the tumour site with light, triggers a series of photochemical and photobiological reactions generating reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), that are directly toxic to cells.[8] PDT also induces vascular damage and blood flow stasis that deprive the tumour cells of their nutrients, thus indirectly leading to both apoptosis and inflammation around the tumour site and stimulating an immune response (both local and systemic).[9-14] Advantages of localizing light irradiation to the cancerous area include limited damage to healthy tissues and minimized long-term systemic toxicity. However, PDT is not without its drawbacks. The most common side-effect, sunlight sensitivity, can last from a few days to 4-6 weeks due to the accumulation of PS in the skin. PDT can be employed to treat a wide range of solid tumours (*e.g.* brain, breast, lung, pancreas, prostate) and more advanced cancers when combined with other therapies (chemotherapy, radiotherapy, surgery). In order to ensure an efficient PDT regime, the PS should absorb light in the range of 650-800 nm (near-infrared region, NIR), where light penetration through the human tissues is optimal,[6] and effectively produce $^1O_2$.[15, 16] Therefore, the PS should have high quantum yields of triplet formation (>50%), long triplet lifetimes (microseconds) and low quantum yields of photobleaching.[16, 17] Furthermore, the PS should ideally be chemically pure, accumulate selectively in the tumour area, have no dark toxicity and be cleared from the body within days in order to minimize skin phototoxicity.[15]

Tetrapyrrole-based photosensitizers, including porphyrins, chlorins and phthalocyanines with their highly conjugated structures absorbing in the red/NIR, have been studied as PS for PDT for many years. Several have been used clinically[5, 17] (*e.g.* porfimer sodium, verteporfin, temoporfin).[17-21] The addition of heavy atoms, usually transition metals,[22] the encapsulation of the PS into nanoparticles[23] and the use of supramolecular structures[24] are among the strategies employed to design new tetrapyrrolic PS for PDT. Furthermore, conjugation to bioactive molecules, such as antibodies,[25] significantly increases the selectivity of the PDT agent towards the targeted cancerous cells.[18, 26, 27] However, cyclic tetrapyrrolic compounds are often

synthetically challenging and structure modifications can be rather difficult. Consequently, recent studies have been focused on other pyrrolic structures such as 4,4-difluoro-4-bora-3a,4a-diaza-s-indacenes (BODIPYs) and aza-BODIPYs.[17, 28]

Prodigiosin is a tripyrrolic, red pigmented natural product produced by certain strains of *Serratia* and *Streptomyces* bacteria (**1**, Figure 1).[29, 30] It exhibits a multitude of biological responses including immunosuppressive,[31] antimicrobial[32-35] and anticancer properties[36, 37] through several modes of action, e.g. H^+/Cl^- exchange,[38-41] Cu-mediated DNA cleavage[42-44] and signal-transduction interference.[45-47] However, clinical applications have been limited due to poor selectivity.[29] Derivatives of prodigiosin, namely prodigiosenes,[48] featuring various modifications on the pyrrolyldipyrrin core of the natural product, have been shown to maintain the anticancer activity of the parent compound, as well as result in a reduced toxicity profile.[49-56]

Despite the similarity of the tripyrrolic core with both porphyrins and BODIPYs, only a few studies have reported the use of prodigiosin and its synthetic derivatives as potential PS for PDT treatments. In 1967 exposure of colourless mutant *Sarcina lutea* cells to prodigiosin and irradiation with visible light was shown to cause cell death.[57] Forty years later the anticancer activity of prodigiosin **1** and the synthetic analogues **2-4** against the HL-60 cancer cells after 30 minutes of irradiation with visible light (Figure 1) was reported.[58, 59] The dark cytotoxicity, featured by the natural product,[29] was shown to be prevented using either an *N*-methyl group on the C-ring (**2**) or a phenyl group as the A-ring (**3** and **4**), which both block the coordination of metal cations, usually copper, and subsequent DNA damage.[42-44] Although synthetic analogues **2-4** were demonstrated to exhibit a significant photo-induced cytotoxicity (in the case of analogue **4**, the IC_{50} value is almost equal to that of the natural product **1**, 3.6 and 2.5 μM respectively),[58] the experimental details for assessing the anticancer activity do not include critical details such as fluence. Furthermore, suggestions as to underlying mechanistic considerations are sparse.[58, 59]
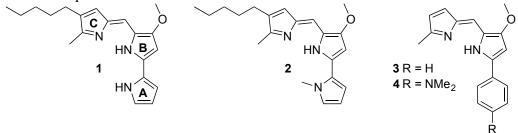

Figure 1. Prodigiosin **1** and synthetic prodigiosenes **2-4**

Conjugation of potential drugs to bioactive molecules[60-66] represents a very effective strategy[67-69] to target specific cancerous tissues,[70] and accordingly minimize toxicity towards healthy cells. Previously, we reported the synthesis of the first series of prodigiosenes conjugated to estradiol and 4-hydroxytamoxifen derivatives.[51] Herein, we report the investigation of the anticancer activity of two representatives of this class, conjugates **5** and **6**, against ER+ (T-47D) and ER- (MDA-MB-231) breast cancer cell lines, in the dark and under visible light irradiation (Figure 2). The tripyrrolic core of prodigiosene serves as chromophore, while the estradiol- and tamoxifen-moieties are intended to function as bio-vectors to target cancer tissues overexpressing estrogen receptors.[70-74] Ready available synthetic prodigiosene **7**,[52] featuring only an extra methyl group on the C-ring compared to the natural product, is used as a control through which to assess the selectivity of **5** and **6** towards the targeted ER+ breast cancer cells (Figure 2).
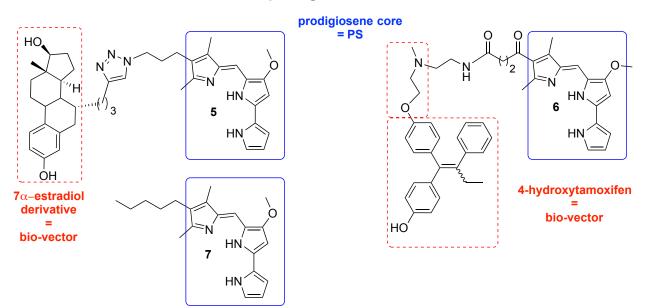
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Figure 2. Synthetic prodigiosenes discussed herein

Results and discussion

In order to study the effects of conjugating prodigiosenes to the bio-vectors estradiol and 4-hydroxytamoxifen, conjugates **5** and **6**, along with control compound **7**, were incubated with ER+ (T-47D) and ER- (MDA-MB-231) breast cancer cell lines at a range of concentrations for 24, 48 and 72 hours. In order to solubilise them **5**, **6**, and **7** were dissolved in DMSO and diluted with media to the appropriate concentrations. Cells were then either irradiated with visible light (400 – 700 nm; 10 J cm^{-2}) or protected from light, and cell viability was assessed 24 hours later by MTT assay.[75] Cytotoxicity results are shown, graphically, in Figure 3. The native toxicity of non-conjugated prodigiosene **7** (Figure 3, bottom) results in 90% or greater cell killing for both ER+ and ER- cells at the highest concentration (10 μM), and shows little difference between irradiated and non-irradiated cells. Some differentiation between ER+ and ER- cells can be seen at lower concentrations, but as **7** has no targeting component this may simply be due to differences in metabolism between the two cell lines.

A more complicated set of results was observed for the prodigiosin conjugates **5** and **6**. Estradiol conjugate **5** demonstrated negligible toxicity (Figure 3, top), either with or without light, against the ER- cell line; this contrasts with the same conjugate for ER+ cells, where at the shortest incubation time (24 hr), and highest doses (10 and 5 μM), very little or no toxicity is seen in the absence of light. However, at the same time point cell killing is increased by approximately 30% upon irradiation. Longer incubation times result in a steady increase in toxicity for non-irradiated cells and a decrease in the cell killing enhancement gained from irradiation. The increase in "dark" toxicity with time may be associated with a requirement for internalisation, whereas light-mediated toxicity resulting from ROS mediated damage would be expected to be more immediate. However, this could be masked at later time points by the inherent cytotoxicity of the prodigiosin, as shown for the non-conjugated analogue. Thus, the conjugation of estradiol "protects" both cell lines from "dark" toxicity at early time points, but this is re-established with longer incubations.

4-Hydroxytamoxifen conjugate **6** shows a similar patter of activity to **5** in terms of "protection" of the cells c.f. the toxicity of free prodigiosin **7**. However, the enhancement of

cytotoxicity mediated by light is more extreme at the two higher concentrations (10 and 5 µM) – approximately 70% and 80%, respectively. Disappointingly, this photo-enhancement of cell killing is seen for both ER+ and ER- cells, suggesting the effect is general, and not associated with active targeting by the 4-hydroxytamoxifen.

Taking all the results together, the conjugation process significantly reduces the inherent cytotoxicity of prodigiosin, especially at shorter incubation times and higher concentrations and, while not selective for ER+, a significant phototoxicity can be induced at higher concentrations.
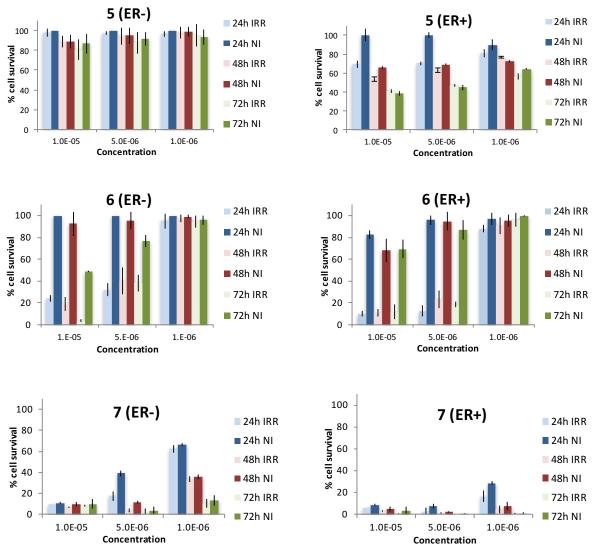

Figure 3. Cytotoxicity results for prodigiosenes **5-7 against ER- (MDA-MB-231) and ER+ (T-47D) cell lines**, either irradiated (IRR) or non-irradiated (NI), as a function of incubation time and concentration (M).

In an effort to gain a more thorough understanding of the mechanism (type I or II)[6, 7] of the phototoxicity of conjugated **5** and **6,** and the unconjugated prodigiosin **7**, a UV-vis analysis was carried out using 9,10-dimethylantracene (DMA), as a diagnostic tool.[76] DMA absorbs in a region that is transparent to molecules **5**, **6** and **7** and has been found to rapidly scavenge $^1O_2$

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(type II mechanism) to generate an endoperoxide (Figure 4). This endoperoxide remains undetectable in the regions of both the photosensitizers of interest and, most importantly, the DMA probe. By monitoring the disappearance of DMA, using absorption spectroscopy as a function of time, endoperoxide formation and, thus, $^1O_2$ generation can be estimated.[76, 77]
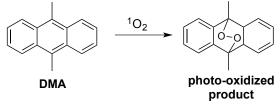

Figure 4. DMA photo-oxidation reaction.

Prior to introduction of DMA into the reaction mixture, the photostability of **5**, **6** and **7** was assessed *via* 480 nm LED excitation. This LED light source was chosen as it is close to the maximum spectral absorption the photosensitizers of interest. Figure 5 illustrates absorption decreases of **5** (λ_{max} = 473 nm), **6** (λ_{max} = 453 nm) and **7** (λ_{max} = 473 nm) by 22 %, 51 % and 63%, respectively, following 120 min of irradiation. Previous work[59] has related photoactivity and, thus, photocytotoxicity, of prodigiosin analogues to absorption loss with exposure to visible light. However, it has also been shown that methylene blue photosensitizers exhibit little change in absorption with extended visible light irradiation.[78] Given the seemingly contradictory nature of these contributions, it is possible that observed variations in dye absorption, as a function of extended irradiation, may be a hybrid of the two and attributed to both photoactivity and photostability of the molecule. The λ_{max} for the unconjugated control (**7**) is nearly at the λ_{max} of the LED excitation source, resulting in larger absorption of the light energy. Conversely, prodigiosene **6** is observed to have a λ_{max} ~30 nm blue shifted from the 480 nm light source, resulting in decreased light absorption by the molecule and may be responsible for the marginally smaller decrease in absorption observed in Figure 5b. Furthermore, **5** clearly shows lower absorption at 480 nm (Figure 5a) as compared to solutions of **6** and **7** at the same concentration. The diminished molecular absorptivity at the excitation wavelength likely contributes to the smaller % loss of **5** over 120 min irradiation. Thus, the observed decrease in the absorption intensity at λ_{max} for **5**, **6** and **7** is proposed to be due to, in part, photobleaching of the molecule as a result of extended LED light exposure, as well as the photoresponse of the molecule at λ_{max} of LED light source.
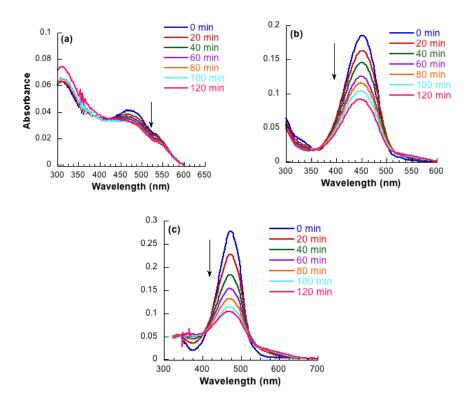
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Figure 5. Time monitored absorption of 10 μM(a) **5**, (b) **6** and (c) **7** in CH$_3$CN following 480 nm LED irradiation.

In order to discern the mechanism responsible for the observed photoactivity of syntheized prodigiosenes presented in Figure 2, DMA was added to allow for both $^1O_2$ detection and $^1O_2$ quantification. Importantly, in the absence of either **6** or **7**, no decrease in DMA absorption was observed, indicating that the probe molecule is photo-inactive at the chosen excitation wavelength of 480 nm (see Figure S1). Figure 6 illustrates the time-monitored UV-visible absorption spectra obtained for conjugated molecules **5** and **6** following exposure to 480 nm LED light in the presence of DMA. The DMA absorption (398 nm) decreases 32 and 15% after 20 min of irradiation of **5** and **6**, respectively. DMA absorption continued to diminish over 120 nm, after which time absorption due to **5** and **6** was negligible. The diminished DMA absorption following excitation of the synthetic prodigiosenes strongly suggests that $^1O_2$ generation from the these molecules is responsible for the observed decrease due to the known capability of the probe anthracene to rapidly trap $^1O_2$ and strongly suggests that a type II mechanism is responsible for the observed photocytotoxicity illustrated in Figure 3.

The photoactivity of **7** was also examined in the presence of the DMA (Figure S2) to allow for direct comparison of $^1O_2$ yields generated from both the unconjugated control and the corresponding estradiol and hydroxytamoxifen derivatives (**5** and **6**). The DMA absorption at 398 nm decreases a minimal 5 % following exposure of **7** to 20 min of 480 nm LED light. This result suggests that prodigiosene conjugation is highly influential on the light-induced toxicity of the photosensitizer.
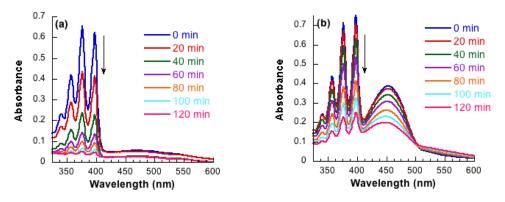
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Figure 6. UV-visible spectra of 10 µM (a) **5** and (b) **6** and 70 µM DMA collected in CH₃CN over 120 min of 480 nm LED light exposure

The photoefficiency, or quantum yield, of $^1O_2$ (Φ_Δ) is commonly used to assess the overall phototoxicity of a given photosensitizer and provides a quantitative means of assessing the capacity of a molecule to facilitate cell death. Φ_Δ of **5** and **6** and **7** was calculated using Rose Bengal as a reference actinometer (Φ_Δ in CH₃CN = 0.54).[79] to evaluate the overall efficacy of visible light-induced phototoxicity of the discussed synthetic prodigiosenes. The time-resolved absorption spectra of DMA following 480 nm irradiation of Rose Bengal is shown in Figure S3 and the method used for Φ_Δ calculation is discussed in the Supporting Information. Φ_Δ for **5**, **6** and **7** were calculated as 0.32, 0.69 and 0.38, respectively. These values closely mirror the results observed in Figure 3, with **6** exhibiting the most pronounced light-induced cytotoxicity and **5** the least. These behaviours align well with the calculated efficiencies of the synthetic prodigiosenes to generate cytotoxic $^1O_2$. The smaller Φ_Δ for **5** and **7** may be a consequence of lower photoresponse or bio-vector conjugation, respectively. Table 1 summarizes the photophysical properties determined for **5**, **6** and **7** and, again, strongly suggests a type II phototoxicity mechanism.

Table 1. Photophysical properties calculated for **5**, **6** and **7** in CH₃CN

Prodigiosene	λ_{max} (nm)	Φ_Δ
5	473	0.32
6	453	0.69
7	473	0.38

Conclusions

The results of our photocytotoxicity studies for prodigiosenes with, and without, conjugated ER receptor targeting molecules, confirms the previously reported photodynamic activity of this class of chromophore. However, while prodigiosenes undoubtedly exhibit both direct anti-cancer and photodynamic properties, their practical applications in cancer therapy have been hindered by a lack of selectivity for specific cancers. Comparison of the behaviour of prodigiosenes conjugated to ER-targeting molecules estradiol and hydroxytamoxifen (**5** and **6**) with the same tripyrrolic chromophore without active targeting (**7**) shows that, while the general cytotoxicity is reduced for **5** and **6**, relative to the core tripyrrolic unit of **7**. Interestingly, enhancement of anticancer activity upon irradiation is also observed, especially for conjugates **5** and **6** at shorter

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incubation times and higher concentrations. By using 9,10-dimethylanthracene as a $^1O_2$ scavenger, photophysical properties of both the unconjugated (**7**) and conjugated (**5** and **6**) photosensitizers clearly suggest that $^1O_2$ is produced following visible light irradiation. This suggests that a type II mechanism is responsible for the observed phototoxicity of prodigiosenes. The photophysical studies further corroborate that conjugation of prodigiosenes to hydroxytamoxifen (**6**) exhibits enhanced, light-dependent, anticancer activity through increased photoefficiency of $^1O_2$ formation. These results suggest that suitable prodigiosin conjugates may be capable of exhibiting dual photodynamic and inherent anticancer activity.

Experimental Procedures
Synthesis
Compounds **5**,[51] **6**[51] and **7**[54] were prepared according to literature procedures.
Photocytotoxicity Assay
Cells (5 x 10^3; 100 µl /well) were plated into 96-well plates in duplicate and left overnight to attach in an incubator set at 37 ºC and 5% CO_2. The media was carefully removed the next day and medium only (controls), or medium plus dilutions of prodigiosin compounds, were added to each seeded well. The plates were then incubated for 24, 48 or 72 hrs. At the appropriate time point, the two duplicate plates were taken out. One was kept in the dark while the other one was irradiated with broad band visible light (400 – 700 nm, Oriel light system: 100 Quartz Tungsten-halogen lamp housing powered by a 1100 W radiometric power supply) at a dose of 10 J cm⁻2, after which both plates were returned to the incubator. 24 hrs later an MTT assay[75] was performed and the colorimetric changes read (Biotek plate reader). Results were expressed as a % of the cells only readings taken as 100% cell survival.
Photophysical Studies
9,10-Dimethylantracene (DMA) was purchased from Sigma-Aldrich and used as received. HPLC-grade CH_3CN used in sample preparation and analysis was purposed from Fisher Chemicals Canada and used as received. Samples were prepared in 1 cm x 1 cm quartz cuvettes. For each trial 70 µM DMA, 10 µM photosensitizer, or a combination of the two, were prepared in a 3 mL solution of CH_3CN and exposed to 480 nm LED light (Mouser, Inc.) set at a power of 1.1 W. UV-visible spectra of the sample were taken every 20 min using a Cary 100 UV-visible spectrophotometer in dual beam mode. Concentration of the photosensitizers was chosen based on the largest concentration used in the photocytotoxicity studies.

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Conflicts of interest
There are no conflicts of interest to declare.

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Supplementary information

Electronic supplementary information (ESI) available: Description of Φ_Δ calculations, time-resolved UV-visible spectra of 70 μM DMA, 10 μM control **7** and 70 μM DMA + 10 μM **7** following 480 nm LED irradiation, time-resolved UV-visible spectra of 70 μM DMA + 1.6 μM Rose Bengal following 480 nm LED irradiation, ΔA of DMA at 398 nm *vs.* irradiation time, 480 nm LED apparatus.
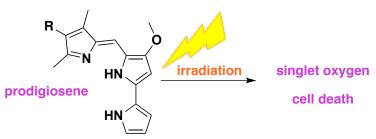
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Photo-induced anticancer activity and singlet oxygen production of prodigiosenes

Table of contents entry

The photo-induced bioactivity of prodigiosenes is demonstrated. A study investigating the cause of such activity identifies that irradiation of prodigiosenes causes the generation of singlet oxygen.