Photo-Induced Cytotoxicity of Prodigiosin Analogues

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Prodigiosin (**1**) is the parent member of a class of polypyrrole natural products that exhibit promising anticancer activities. They can facilitate copper-promoted oxidative DNA damage by binding to copper ions, and this activity is thought to represent their mechanism of cytotoxicity in the dark. They also possess photoinduced cytotoxicity, although **1** is too toxic in the dark to be used effectively for the treatment of cancer by photodynamic therapies. To circumvent dark toxicity by prodigiosins, the semi-synthetic analogue **2**, in which the N-pyrrolic atoms of **1** are methylated to block copper coordination, and the synthetic phenyl analogues **3** and **4**, which lack the copper-coordinating **A**-pyrrole ring of **1**, were tested for their ability to inhibit colony formation of HL-60 cancer cells in the absence and presence of visible light (λ > 495 nm). Our results show that **2-4** lack cytotoxicity in the dark, but are able to inhibit colony formation of HL-60 cells following irradiation for 30 min. The synthetic derivative **4** exhibits photo-induced cytotoxicity similar to that of the natural product **1**, demonstrating the potential use of prodigiosin-based compounds for treatment of cancers following irradiation with visible light.

Key Words : Prodigiosin, Cytotoxicity, Photophysical property, Cancer treatment

Introduction

Prodigiosin (**1**, Fig. 1) is the parent member of a family of red pigments produced by some strains of *Serratia marcesens* that contain a characteristic pyrrolylpyrromethene skeleton with a **B**-ring methoxy group.[1-3] They are noted for their promising immunosuppressive[4,5] and cytotoxic activities[6] and have been identified as mediators of apoptosis in dozens of human cancer cells with little effect on non-malignant cells.[7-9]

The therapeutic potential of prodigiosins has stimulated research into their mechanism of action. Here a possible relationship between the cytotoxicity of the prodigiosins and their DNA-damaging capacity has been demonstrated.[10-15] In the presence of redox-active metal cations, preferably copper^{2+} (Cu(II)), prodigiosins facilitate single- (ss) and double-strand (ds) DNA cleavage.[11-14] These events are thought to derive from formation of the π-radical cation at the electron-rich pyrrolylpyrromethene chromophore through interaction with Cu(II) to yield Cu(I), which fosters reductive-activation of molecular oxygen (O_2) to form the superoxide radical anion ($O_2^{\bullet-}$) and hence hydrogen peroxide (H_2O_2).[11] The interaction of H_2O_2 with a Cu-bound prodigiosin species[16] is thought to initiate dsDNA cleavage. Structure-activity relationships demonstrate that replacement of the individual metal-coordinating pyrrole rings by other weaker Cu(II)-ligating arenes results in marked loss of nuclease activity and cytotoxicity.[11,13,14,17]

While coordination of **1** to a redox-active metal cation represents one way of triggering oxidation of the natural alkaloid to furnish an oxidative stress, Roth noted in 1967 that prodigiosin also possesses photosensitizing activity.[18] Here exposure of colourless mutant *Sarcina lutea* cells to prodigiosin and visible light led to cell death in an O_2-dependent process. More recent studies have shown that prodigiosin undergoes degradation upon exposure to blue (470 nm) light.[19] In view of our findings regarding the interaction of **1** with Cu(II)[11,12,14,16] it was speculated that **1** would undergo a photo-oxidative process to reductively activate O_2 to yield H_2O_2 and prodigiosin-derived π-radical cations that may act to facilitate tumour destruction. While prodigiosin (**1**) itself is too cytotoxic in the dark to act as a useful phototherapeutic agent that should deliver chemical reactivity on demand through interaction of the pigment with visible light,[20] the ability to chemically synthesize new prodigiosin analogues with diminished cytotoxicity in the dark[13,14,17] suggested the possible development of prodigiosin-like pigments with photochemical reactivity suitable for therapeutic applications. Presently, we report on our initial

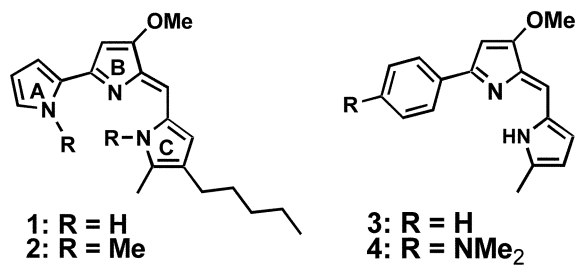


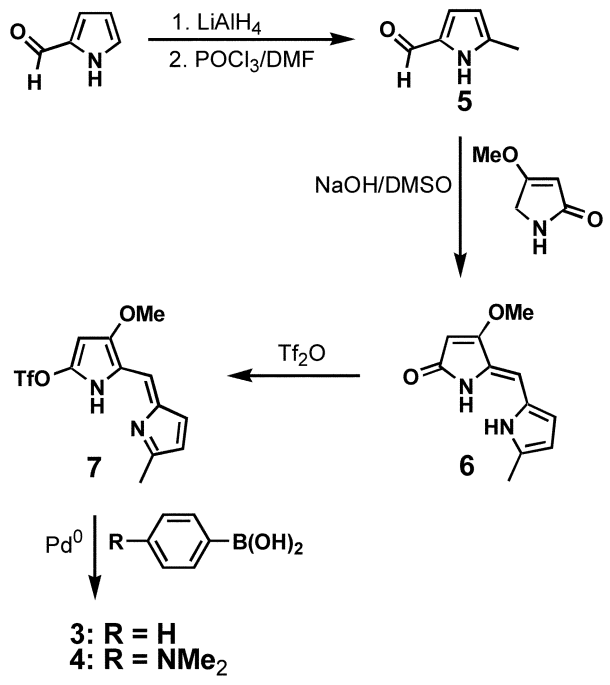
Figure 1. Structures of prodigiosin (**1**) and analogues **2-4**.

findings regarding the photoinduced cytotoxicity of prodigiosin analogues **2-4** shown in Figure 1. Within this subset the *N,N*-dimethyl-aniline analogue **4** has emerged as a lead compound for development of prodigiosin-based phototherapeutic agents.

Results and Discussion

Synthesis. It has been established that an N-containing heterocyclic **A**-ring along with the lone-pair N-electrons in conjugation with the tricyclic frame[4] and the **B**-ring methoxy group[6] are critical for the cytotoxic potency of prodigiosins in the dark. It has also been speculated that the biological activity of the prodigiosins stem from their ability to form the complexes with Cu(II).[13] Our laboratory has demonstrated that all N-atoms of prodigiosin (**1**) bind Cu(II) to form a distorted square-planar 1 : 1 complex.[16] The participation of all N-atoms in Cu(II)-binding appears to be important for nuclease activity by **1**, as replacement of the **A**-pyrrole ring with alternative, non-coordinating, arenes inhibits DNA strand scission.[13,14] Thus, synthetic strategies to inhibit the cytotoxic potency of **1** in the dark would include methylation of the pyrrolic N-atoms to block Cu(II)-coordination, or to replace the **A**-pyrrole ring system of **1** with an alternative arene.

Treatment of **1** with NaH/MeI afforded a sample of **2** (Fig. 1) in which the **A**- and **C**-N atoms are methylated. Unambiguous structural assignment of **2** was afforded by two-dimensional (2D) NMR spectroscopy. The synthetic analogues **3** and **4** (Fig. 1) that contain a phenyl **A**-ring were also prepared using the strategies outlined in Scheme 1. For the synthetic derivative **4** the **A**-ring also contains lone-pair N-electrons (NMe_2 group) in conjugation with the pyrromethene moiety and thus the electron distribution of **4** and **1** should be similar. However, the lone-pair N-electrons in **4** cannot participate in coordination to Cu(II) by the metal-coordinating pyrromethene entity,[16] and it was expected that **4** and **3** would show diminished cytotoxic potency in the dark.[17]



Scheme 1

Table 1. Inhibition of HL-60 leukemia cancer cell growth by prodigiosin analogues[a]

Compound	IC_{50} (dark)	IC_{50} (light)[d]
1	6.6[b]	2.53
2	>25[c]	10.62
3	>25[c]	16.18
4	>25[c]	3.63

[a]Inhibition of colony formation was assessed using the soft agar clonogenic survival assay as described in the Experimental Section. Values in μM are expressed as the Mean of at least three determinations. [b]From ref 26. [c]No inhibition at 25 μM drug. [d]Inhibition following 4 h exposure to drug in the presence of 30 min exposure to visible light (> 495 nm).

Photoinduced Cytotoxicity. Preliminary studies conducted with human promyelocytic leukemia (HL-60) cells demonstrated that prodigiosin (**1**) inhibits colony formation and possesses an IC_{50} value = 6.6 μM (4 h drug exposure).[11] Under analogous conditions the N-methyl analogue **2** and the synthetic derivatives **3** and **4** failed to inhibit colony formation at 25 μM, which was the maximum drug concentration examined (IC_{50} (dark), see Table 1). The photo-induced cytotoxicity of **1-4** was then determined by exposing HL-60 cells to drug and visible light (λ > 495 nm) for 30 min followed by incubation for an additional 3.5 h in the absence of light. Cells exposed to 30 min light in the absence of drug showed no inhibition of colony formation. As shown in Table 1, prodigiosin **1** was ~3-fold more active with irradiation (IC_{50} (dark) *vs*. IC_{50} (light)). In fact, all derivatives inhibited colony formation with the analogue **4** (IC_{50} (light) = 3.63 μM) showing potency almost equal to that of the natural product **1**.

In summary, our findings demonstrate the photo-induced cytotoxicity of the natural product prodigiosin 1 along with three structure analogues 2-4. The natural prodigiosin 1 is too cytotoxic in the dark to be considered an effective photo-therapeutic agent even though it shows ~3-fold enhancement in cytotoxic potency upon irradiation with visible light. Of the structural analogues tested, the derivative 4 shows similar potency to 1 in the presence of visible light, but unlike 1, it fails to inhibit colony formation of leukemia (HL-60) cells in the dark. These results demonstrate the potential of prodigiosin-based compounds, such as 4, for cancer treatment by photodynamic therapy. Current efforts are focused on gaining an understanding of the photophysical properties of prodigiosin-based pigments and on determining whether these compounds trigger photochemical DNA damage, which may provide a rationale for their photo-induced cytotoxicity.

Experimental Section

Materials and Methods. Prodigiosin (**1**) was a gift from

the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (NCI). It was received as the free base and confirmation of structure was obtained by ^{1}H NMR spectroscopy and positive ionization electrospray mass spectrometry (ES^+): *m/z* 324.2 $[M + H]^+$. Starting materials and solvents were purchased from Aldrich (Milwaukee, WI), Sigma (St. Louis, MO), Fisher Scientific (Itasca, IL), Frontier Scientific (Logan, UT), or Alfa Aesar (Ward Hill, MA), and were used without further purification. Column chromatography was performed using ICN 60, 230-400 mesh silica gel or 60-325 mesh alumina (Fisher Scientific, Brockman activity II-III). TLC was carried out on Analtech 250 mM layer, UV254 silica gel plates with glass backing. Distilled, deionized water from a Milli-Q system was used for all aqueous solutions and manipulations. Other solvents were purified and dried according to standard procedures.

Elemental analyses were carried out by Atlantic Microlab Inc (Atlanta, GA). High-resolution mass spectra (HRMS) were carried out by the Duke University Mass Spectrometry Facility (Durham, NC). In-house low-resolution ES^+ spectra were acquired using an Agilent 1100 series LC/MSD_SL_00045 Trap. Semipreparative purification of prodigiosin analogues were performed on a Hitachi 7400 series HPLC system with a L7455 diode array detector. Separations were carries out on a 5 mm Phenomenex C-8 column (250×10 mm) at ambient temperature using a 5.90 mL/min flow rate. NMR spectra of synthetic prodigiosins were recorded on a Bruker AVANCE 300DMX (300 MHz) spectrometer in $CDCl_3$ or DMSO-d_6 and peaks were referenced to the residual $CHCl_3$ and DMSO-d_5H peaks. All 2D spectra were collected with 2K points in F2 (direct dimension) and 512 points in F1 (indirect dimension). The number of scans in the 2D experiments varied as follows; gs-COSY (8 scans), gs-HMQC (32 scans), gs-HMBC (128 scans), 600 ms NOESY (64 scans) and gs-DQFCOSY (64 scans). All 2D spectra were processed to 1K in the F2 and F1 dimensions. Chemical shifts are given in ppm relative to TMS, and coupling constants (*J*) are reported in hertz (Hz). Absorption measurements were made on a Hewlett Packard (HP-8453) spectrophotometer equipped with a thermostated cell compartment and samples were stirred using a magnetic stir bar.

Methylation of Prodgiosin. To a DMF solution of prodigiosin (**1**, 0.062 mmol) was added 3 mg NaH (0.124 mmol) and the solution was allowed to stir at room temperature for 30 min. To the reaction mixture 0.176 g (0.124 mmol) of MeI was added and the mixture was stirred for an additional 30 min. Following aqueous workup, the crude product was purified by silica gel flash chromatography (100% EtOAc) and semipreparative HPLC using a mobile phase of 65/35 solvent A/B with solvent B increased to 80% over 20 min (solvent A: 0.1% formic acid in H_2O; solvent B: 0.1% formic acid in MeCN). The combined fractions were collected to afford N-methyl-prodigiosin **2** as a dark red solid. Confirmation of structure was obtained by gs-COSY, gs-HMQC, gs-HMBC and NOESY spectra (**2**): ^{1}H NMR ($CDCl_3$): δ 1.32 (m, 6H), 1.55 (t, 3H), 2.19 (s, 3H), 2.39 (t, 2H), 3.57 (s, 3H), 3.86 (s, 3H), 4.20 (s, 3H), 5.90 (s, 1H), 6.16 (m, 1H), 6.43 (m, 1H), 6.76 (s, 2H), 7.47 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 10.23, 13.90, 23.36, 26.09, 30.05, 30.17, 31.30, 37.54, 57.91, 96.29, 107.98, 111.87, 113.70, 118.00, 124.66, 127.78, 129.06, 131.98, 141.99, 160.20, 167.99; ES^+: MH^+ = 352; UV-vis (MeOH): 544 nm.

Synthesis of Prodigiosins. The strategy used for the preparation of prodigiosin analogues **3** and **4** was derived from the procedure by D'Alessio and coworkers.[4,21] As outlined in Scheme 1, the syntheses started from pyrrole-2-carboxaldehyde (Aldrich), which was treated with $LiAlH_4$ in THF to afford 2-methyl-pyrrole (~75%), which was subsequently treated with $POCl_3$/DMF to yield 2-formyl-5-methylpyrrole **5**, as outlined previously by Liddell and coworkers.[22]

*4-Methoxy-5-(5-methyl-1H-pyrrol-2-ylmethylidene)-1,5-dihydropyrrol-2-one (**6**)*: A DMSO solution (20 mL) of 2-formyl-5-methylpyrrole **5** (1.12 g, 1.03×10^{-3} mol) and 4-methoxy-3-pyrrolin-2-one (2.32 g, 2.05×10^{-3} mol, Aldrich) was treated with 2 N NaOH and stirred at 60 °C for 20 h. The mixture was then poured into H_2O (100 mL) and extracted with $CHCl_3$ (3×20 mL). The combined organic fractions were dried ($MgSO_4$), filtered, and concentrated under reduced pressure to afford **6** (2.03 g, 97%) as a yellow solid. (**6**): ^{1}H NMR (DMSO-d_6): δ 2.21 (s, 3H), 3.83 (s, 3H), 5.19 (s, 1H), 5.85 (d, *J* = 2.4, 1H), 6.04 (s, 1H), 6.52 (d, *J* = 2.4, 1H), 9.36 (bs, 1H), 10.78 (bs, 1H); ^{13}C NMR (DMSO-d_6): δ 13.2, 58.6, 91.4, 98.6, 109.1, 113.5, 124.7, 125.5, 131.7, 167.2, 171.0. Anal. Calcd. for $C_{11}H_{12}N_2O_2$: C, 64.69; H, 5.92; N, 13.72. Found: C, 64.47; H, 6.07; N, 13.59.

*2-Trifluoromethansulfonyl-4-methoxy-5-[5-methyl-2H-pyrrol-2-ylidene]methyl]-1H-pyrrole (**7**)*: Trifluoromethansulfonic anhydride (Aldrich) (2 equiv., 0.50 mL, 2.94×10^{-3} mol) was added dropwise to a solution of **6** (0.30 g, 1.47×10^{-3} mol) in dichloromethane (10 mL, 0-5 °C, argon atmosphere, 3 h reaction time). The triflate **7** (yellow solid, 65%) was isolated and purified by standard work-up and silica gel flash chromatography (80 : 20 hexane : ethyl acetate). (**7**): ^{1}H NMR ($CDCl_3$): δ 2.37 (s, 3H), 3.86 (s, 3H), 5.41 (s, 1H), 6.02 (d, *J* = 3.6, 1H), 6.62 (d, *J* = 3.6, 1H), 7.01 (s, 1H), 10.75 (bs, 1H); ^{13}C NMR ($CDCl_3$): δ 14.1, 59.1.9, 87.5, 111.6, 122.4, 123.7, 125.5, 129.0, 132.7, 140.7, 161.5, 168.4.

*General Procedure for Synthesis of Prodigiosin Analogues **3** and **4***: A solution of the triflate (**7**, 1-0.5 mmol) in 1,4-dioxane (5 mL) was treated in sequence with 4 equiv of the desired boronic acid (R-$B(OH)_2$) and 8 equiv potassium carbonate. The boronic acids were purchased from Frontier Scientific. For the syntheses, 1,4-dioxane solutions were purged with argon for 25 min, treated with 0.1 equiv tetrakis(triphenylphosphine) palladium(0), and heated to 70 °C with stirring for 6 h. Following standard work-up, the crude material was purified over an alumina column with 100% hexane, followed by 8 : 2 hexane : ethyl acetate (EtOAc). The collected fractions were concentrated to yield the desired prodigiosin analogues as free bases.

2-Phenyl-4-methoxy-5-[(5-methyl-2H-pyrrol-2-ylidene)-

*methyl]-1H-pyrrole (**3**)*: 132 mg, 41%; ^{1}H NMR ($CDCl_3$): δ 2.44 (s, 3H), 3.91 (s, 3H), 6.01 (d, J = 3.7, 1H), 6.08 (s, 1H), 6.58 (d, J = 3.7, 1H), 6.90 (s, 1H), 7.44 (m, 3H), 8.00 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 15.0, 59.2, 95.6, 111.4, 119.2, 121.7, 127.7, 129.5, 130.5, 131.1, 136.0, 139.3, 141.8, 166.6, 169.4; UV-vis (MeOH): 495 nm (ε = 57,238); HRMS (FAB) $[M + H]^+$ calcd for $C_{17}H_{17}N_2O$ 265.1262, found 265.1261. Anal. Calcd. for $C_{17}H_{16}N_2O(H_2O)_{0.05}(EtOAc)_{0.25}$: C, 75.26; H, 6.35; N, 9.75. Found: C, 75.51; H, 6.34; N, 9.37.

*2-(4-N,N-Dimethylamino-phenyl)-4-methoxy-5-[(5-methyl-2H-pyrrol-2-ylidene)-methyl]-1H-pyrrole (**4**)*: 103 mg, 72%; ^{1}H NMR ($CDCl_3$): δ 2.41 (s, 3H), 3.02 (s, 6H), 3.87 (s, 3H), 5.97 (d, J = 3.5, 1H), 6.03 (s, 1H), 6.49 (d, J = 3.5, 1H), 6.74 (d, J = 8.8, 2H), 6.76 (s, 1H), 7.89 (d, J = 8.8, 2H). ^{13}C NMR ($CDCl_3$): δ 14.4, 40.6, 58.6, 95.0, 110.1, 112.2, 115.6, 119.1, 123.4, 128.7, 130.8, 136.9, 142.2, 151.9, 166.6, 168.7; HRMS (FAB) calcd for $C_{19}H_{22}N_3O$ $[M + H]^+$ 308.1762, found 308.1769. Anal. Calcd. for $C_{19}H_{21}N_3O(H_2O)_{0.25}$: C, 73.17; H, 6.95; N, 13.47. Found: C, 73.05; H, 6.81; N, 13.14.

Photo-induced Cytotoxicity. HL-60 cells, a model of human promyelocytic leukemia, were maintained in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). The cells were kept in a humidified atmosphere of 5% CO_2-95% air at 37 °C. Cell doubling time was ca. 24 h under these culture conditions and the cells tested negative for mycoplasma contamination, as evidenced by a Mycoplasma Detection Kit from Stratagene (La Jolla, CA). Cell numbers were measured with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). The photo-cytotoxicity of prodigiosin ananlogues was assessed using a soft agar clonogenicity assay. HL-60 leukemia cells growing in log phase were treated with various concentrations of drug, exposed to light (250 W Xe arc, λ > 495 nm) for 30 min, and incubated for another 3.5 h at 37 °C in a CO_2 incubator. Control reactions consisted of cells incubated with drug for 4 h in the dark and of cells exposed to light for 30 min in the absence of drug. Following drug treatment, cells were washed with culture media and embedded in soft agar (4,500-45,000 in 1.5 mL of RPMI-1640 media supplemented with 10% FBS). Colony formation was assessed 7-10 days later, at which time colonies (> 50 cells) were counted using an inverted light microscope. For prodigiosins showing cytotoxicity at 25 μM, the percent inhibition of colony formation was determined using the formula: % Inhibition = [1 – (Number of colonies in compound-treated test cultures/Number of colonies in control cultures)] × 100. The IC_{50} values were calculated by non-linear regression analysis using the Graphpad Prism Software version 2.0 (Graphpad Software, Inc., San Diego, CA). Results were based on three independent experiments assayed in quadruplicate.

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