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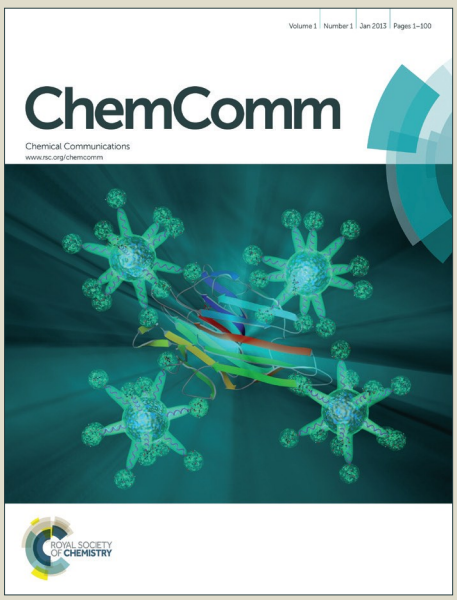

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Modulation of ROS production in Photodynamic Therapy using a pH controlled Photoinduced Electron Transfer (PET) based sensitiser. †

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Jordan Atchison[1], Sukanta Kamila[1], Conor McEwan[1], Heather Nesbitt[1], James Davis[2], Colin Fowley[1], Bridgeen Callan[1], Anthony P. McHale[1]* and John F. Callan[1*]

A new sensitiser (4) for use in photodynamic therapy (PDT) has been developed to enable control ROS production as a function of pH. This pH dependent PDT behaviour was tested in HeLa cells and in SCID mice bearing human xenograft pancreatic cancer (BxPC-3) tumours.

Photodynamic Therapy (PDT) has emerged as alternative to conventional chemotherapy in the treatment of solid cancerous tumours due to its high specificity and minimally invasive nature.[1] PDT involves the interaction of a non-toxic sensitising drug with light of an appropriate wavelength that, in the presence of molecular oxygen, generates cytotoxic reactive oxygen species (ROS).[1] Such ROS are highly reactive and oxidise cellular substrates leading to cell death via apoptotic or necrotic pathways.[2] PDT is approved in the UK for the treatment of non-melanoma skin cancer and has been trialled in the treatment of other cancers such as head and neck, oesophageal, bladder and prostate.[3] The attraction of PDT as a clinical treatment stems from the fact that ROS can be selectively generated at a target site by careful positioning of the light source, reducing collateral damage to surrounding healthy tissue. However, this therapeutic approach is limited not only by the poor penetration capability of visible light through human tissue but also a lack of sensitiser specificity for tumour tissue.[4,5] The latter has meant that patients receiving PDT are hypersensitive to light and are advised to remain in low level lighting for several days post treatment.[4] The emergence of so-called "third generation sensitisers" in the past decade, where conventional sensitisers have been adapted to include targeting motif's such as antibodies, peptides and small molecules such as folic acid, promises to improve sensitiser specificity and reduce off-target effects.[4,5] These approaches, however, can involve cumbersome synthetic and purification procedures that in certain instances may be cost prohibitive. Another strategy, first adopted by O'Shea and co-workers, exploited targeting the lower pH of tumour tissue interstitial fluid (~ pH 6.0) in an attempt to selectively control singlet oxygen generation.[6] This approach involved arming halogenated BODIPY sensitisers with pH sensitive receptors in a photoinduced electron transfer (PET) format.[6] The PET mechanism has proven popular in the design of optical sensors and involves attachment of a receptor with specificity for a target analyte to a fluorophore (or sensitiser) via a short spacer unit (usually a methylene or ethylene unit).[7,8] In the absence of a target analyte, the excited state energy of the fluorophore is used to oxidise the receptor resulting in non-radiative excited state decay. Upon binding a target analyte, the oxidation potential of the receptor is raised making receptor oxidation energetically unfavourable and the excited state energy is deposited as fluorescence.[8] When a sensitiser is used in place of a fluorophore, the excited state deactivation that results from PET prohibits population of the sensitisers triplet excited state, resulting in negligible ROS production. Such a strategy has proven capable of modulating the generation of ROS in acidic (ROS on) or basic (ROS off) media through the use of amine functionalised receptors.[9] However, such sensitisers need to be carefully designed so that the amine pKa is suitable to enable protonation when the pH is lowered from 7.4 to 6.0. While previous studies have successfully proven the feasibility of such an approach, their investigations were limited to cell free experiments and failed to explore the potential of such sensitisers in a cellular or *in vivo* based environment.[9,10]

In this manuscript, we describe the preparation of an amine functionalsied emodin based photosensitiser that is capable of modulating its singlet oxygen production with changes in pH. Emodin (**1**) is a naturally occurring anthraquinone derivative that is a key precursor in the synthesis of hypericin.[11] Hypericin has been widely studied as a sensitiser in PDT but has suffered from poor aqueous solubility and a relatively short absorption maximum limiting its ability to be activated at depth in human tissue.[12] In a quest to overcome these shortcomings, Lackner *et al.* developed a range of amine functionalised hypericin derivatives that boasted improved water solubility and longer absorption maxima than hypericin itself.[12] In contrast to hypericin, emodin's use as a sensitiser in PDT has not been widely investigated, most likely due to its even shorter absorption maximum than hypericin.[13] As hypericin is essentially a dimer of emodin anthrone, we have reasoned that amine functionalization of emodin may also result in a favourable bathocromic shift in its UV-Vis spectrum. Therefore, we have prepared emodin derivative **4**, with two PET active tertiary amine receptors. We investigate the effect of introducing this

[1]*Biomedical Sciences Research Institute, University of Ulster, Coleraine, Northen Ireland, BT521SA. E-mail: j.callan@ulster.ac.uk* [2] *Engineering Research Institute, University of Ulster, Jordanstown, Northern Ireland, U.K. BT37 0QB*

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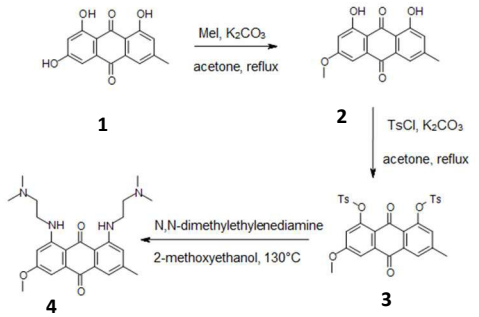
functionality on its photophysical properties and examine its PDT efficacy in HeLa cells when the cellular medium was adjusted from pH 7.4 to pH 6.0. Finally, the ability of the conjugate to reduce the size of ectopic human xenograft BxPc-3 tumours in mice upon light irradiation was also determined.



Scheme 1 Synthesis of emodin derivative **4.**

Diamine functionalised emodin derivative **4** was prepared by first selectively alkylating the C-3 hydroxyl of emodin (**1**) to form intermediate **2** (Scheme 1). The remaining two phenolic hydroxyls of **2** were then tosylated to form compound **3** which was reacted with *N,N*-dimethylethylenediamine to form diamine functionalised target compound **4**. The effect of adding this amine functionality on the photophysical properties of **4** was first examined by UV-Vis spectroscopy. The absorption profile of **1** and **4**, recorded in MeOH are shown in fig. S4a and reveal a significant red-shift in the absorption maximum of **4** (λ_{max} 550 nm) compared to **1** (λ_{max} = 425 nm). Such an increase in the absorption maximum is hugely beneficial for PDT applications and allows activation of the sensitiser by light at a greater depth in mammalian tissue.[5] Interestingly, the absorbance spectrum of **4** shows only minor changes when recorded at various pH values with a slight bathochromic shift observed in alkaline pH, most likely due to deprotonation of the anilino proton (Fig S4b).[14] This is in contrast to **1** which shows a significant bathochromic shift across a broad pH range due to the presence of three phenolic groups (Fig S5).[15] To determine the effect of pH on the emission properties of **4**, a pH-fluorescence titration was performed in a H_2O:MeOH (1:1) solvent system. As shown in figure S6a, a broad emission with a maximum centred at 646 nm was observed upon excitation at 535 nm which was strongly dependent on solution pH. Indeed, a 7-fold increase in the emission intensity was observed when the pH was decreased from 10.6 to 2.0 with no change in the position of the emission maximum. Such features, where the UV-Vis spectrum remains relatively unchanged and the fluorescence emission is enhanced upon analyte recognition are characteristic of PET type systems.[14] In addition, the fluorescence intensity of **4** was observed to decrease with increasing solvent dipolarity, further supporting the existence of the PET mechanism (Fig S7).[7] Indeed, the fluorescence enhancement observed at low pH for **4** can be attributed to protonation of the tertiary amine groups that cancels the PET process and switches fluorescence "On".[8] When the fluorescence intensity of **4** was plotted as a function of pH a sigmoidal type curve was observed with a significant increase in fluorescence between pH 6-8 (Fig S6b). From this fluorescence data the pKa was determined as 6.7 using a modification of the Henderson-Hasslebach equation.[17]

To determine the ability of **4** to generate singlet oxygen at different pH values, the fluorescent probe "singlet oxygen sensor green" (SOSG) was used. This probe is non-fluorescent in its reduced form but is highly fluorescent upon reaction with singlet oxygen.[17] Solutions of SOSG containing **4** in either acidic (pH 3.3) or basic (pH 10.3) solution were prepared and exposed to white light or kept in the dark for 5 minutes. The intensity of SOSG fluorescence at 525 nm upon excitation at 505 nm was recorded at the beginning and at the end of each experiment. The results are shown in figure 3 and reveal a statistically significant ($p \leq 0.05$) increase in SOSG fluorescence for light treated **4** at pH 3.3 compared to the analogous solution kept in the dark. In contrast, there was no noticeable difference in SOSG fluorescence between light or dark treated solutions of **4** at pH 10.3. These results are consistent with the pH-fluorescent titrations described above (Fig 1a) and suggest that in basic solution, the excited singlet state of **4** is quenched by PET from the tertiary amine that restricts intersystem crossing to the excited triplet state, and subsequent energy transfer to molecular oxygen to generate singlet oxygen. In acidic solution, the amines become protonated increasing their oxidation potential and making PET no longer energetically feasible.[7,8] Thus, the excited singlet state is free to engage in intersystem crossing enabling population of the excited triplet state and subsequent singlet oxygen generation with a singlet oxygen quantum yield of 0.19.

The results outlined above indicate that the fluorescence and singlet oxygen produced by **4** can be controlled by changing solution pH. However, the pH difference between healthy and tumour tissue interstitial fluid is approximately 1.5 units, reflecting a narrow pH window over which to control singlet oxygen production.[18] Using the pKa value determined above (6.7), the % ionisation of **4** at pH 7.4 (17%) and pH 6.0 (84%) was calculated using the Henderson-Hasslebach equation. To investigate if such a change in ionisation over this pH range would be sufficient to control the PDT mediated cytotoxicity in a cancer cell line, HeLa cells were seeded in a 96 well plate and incubated for 24 h at 37°C in a humidified CO_2 (5%) atmosphere. Solutions of **4** were prepared at three different concentrations (3, 5 and 10 μM) with the solution pH buffered at either pH 6.0 or pH 7.4. Cells were then incubated with solutions of **4** at the appropriate concentration and pH for 3 h after which the solution of **4** was removed and the cells washed twice with buffer at the appropriate pH. Selected wells were then treated with white light for 30 seconds. Control experiments were also performed in the absence of light treatment. The buffer was then replaced with fresh medium and the cells incubated for a further 24h before determining cell viability using the MTT assay. As shown in Fig 1b-c, the difference between light and dark toxicity at each concentration was greater for cells treated at pH 6.0 compared to pH 7.4. Indeed, a statistically significant reduction in cell viability of 25.3 % ($p \leq 0.01$) was observed for cells treated at pH 6.0 when the concentration of **4** was 3 μM, while cells treated with the same concentration of **4** at pH 7.4 reduced by only 4.8 %, which was not statistically significant (Fig 1d). When the concentration of **4** was increased further to 5 μM or 10 μM, the difference in cytotoxicity between the dark and light treated cells at pH 6.0 (41.0 % at 5 μM and 46.4% at 10 μM) and pH 7.4 (32.8 % at 5 μM and 35.4% at 10 μM) was less evident. One possible explanation for these results is that at a concentration of 3 μM there was insufficient ionised **4** available at pH 7.4 to generate singlet oxygen upon light treatment

while at pH 6.0, a sufficient amount was available to produce the observed cytotoxic effect. However, when the concentration of **4** was increased to 5 μM or 10 μM, then even at pH 7.4, the amount of ionised **4** present was sufficient to exert a substantial cytotoxic effect. Therefore, by carefully choosing the appropriate concentration of **4**, it should be possible to selectively activate its PDT activity in tumour tissue while reducing it in healthy tissue.

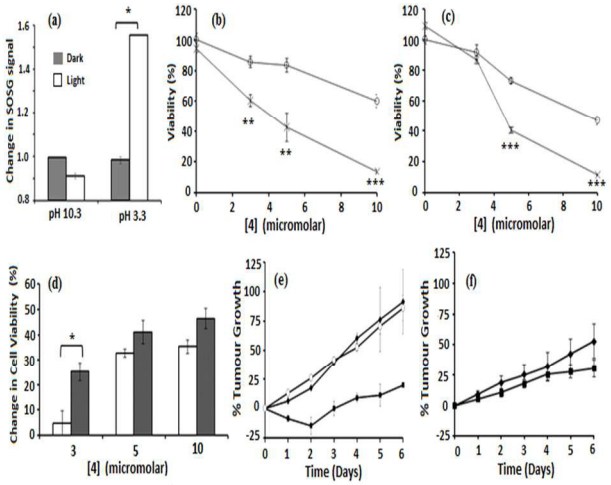


Figure 1 (a) Relative intensity (I/Io) of SOSG fluorescence at 525 nm for solutions at pH 10.3 and pH 3.3 containing **4** (5 μM) and SOSG (2.5 μM) after 5 minutes exposure to white light. (b) Plot of cell viability against concentration for HeLa cells treated with **4** and white light (X) or without white light (o) in (b) pH 6.0 buffer or (c) pH 7.4 buffer. (d) Plot of the difference in cell viability between cells treated with **4** in the absence and presence of white light at pH 6.0 (grey bars) and pH 7.4 (white bars). Error bars represent ± the standard error where n=6. *p < 0.05, **p < 0.01 and ***p <0.001.(e) Plot of % change in tumour volume for mice bearing ectopic Bx-PC-3 tumours treated with (i) no treatment (open circles) (ii) **4** only (filled diamonds) and (iii) **4** + white light (filled squares). (f) Plot of % change in tumour volume for mice bearing ectopic Bx-PC-3 tumours treated with (i) no treatment (filled diamonds) and (ii) IP gemcitabine (filled squares) (120 mg/kg) administered on days 0 and 3.

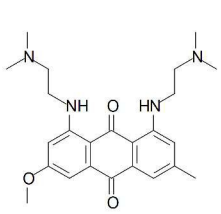
To determine the effectiveness of **4** in vivo we chose the human pancreatic cancer cell line BxPc-3 to establish ectopic xenograft tumours in SCID mice. Pancreatic ductal adenocarcinoma (PDA) remains one of the most challenging diseases to treat with less than 5% of patients diagnosed surviving 5 years or more.[19] Indeed, this survival statistic has not changed over the past 40 years.[20] Therefore, it is clear that current treatment approaches have proven ineffective and that the development of new strategies for treatment of this disease are required. One characteristic feature of PDA is a pronounced hypoxic tumour microenvironment, with high levels of expression of the hypoxia biomarker HIF-1α (hypoxia inducible transcription factor) serving as a predictor of poor clinical outcome.[21] Hypoxia is known to generate an overproduction of acid as it induces the production of energy from glycolysis via the Pasteur effect.[22] Therefore, BxPC-3 tumours provide an ideal target to determine the PDT activity of **4**, as the low pH should ensure almost complete protonation. Ectopic tumours were established and the mice separated into three groups. One group received vehicle only, the second group received **4** only (1.25 mg/kg) while the third group received both **4** (1.25 mg/kg) and exposure to white light. Following treatment, the tumour volume was measured each day for six days and the percentage change in tumour volume plotted as a function of time (Fig1e). A second treatment was also administered on day 3 using 4mg/kg of **4** under otherwise identical conditions. The results reveal a significant reduction in tumour volume for those animals treated with both light and **4** compared to either of the control groups. Indeed, six days following the initial treatment, tumours in the treatment group were only 20% above pre-treatment size while the control groups increased by 91% (**4** only) and 86% (vehicle only). We were interested to know how significant this PDT tumour reduction was when compared to the benchmark pancreatic cancer chemotherapy gemcitabine.[23] Therefore, we treated the same tumour model with gemcitabine (intraperitoneal injection, 2 x 120 mg/kg at day 0 and day 3) and measured the tumour volume with time. The results are shown in Fig 1f and reveal a significantly lower reduction in tumour volume (22.5%) between the vehicle only and gemcitabine treated tumours 6 days after treatment compared to a 71% difference between the vehicle only and PDT treated groups. This is a significant improvement in efficacy and suggests PDT treatment of pancreatic cancer tumours using **4** may be a promising alternative to conventional chemotherapy. We are also exploring the effect of combining PDT and gemicitabine therapy for the treatment of pancreatic cancer and hope to report on this soon.

In conclusion, we have developed a new emodin derivative **4** for use as a sensitiser in PDT. Functionalising emodin with tertiary amine groups not only results in a significant bathochromic shift in the absorbance spectrum but also introduces PET active receptors that enable control over singlet oxygen generation using simple protonation / deprotonation equilibria. The ability to control the cytotoxic behaviour of **4** in cancer cells where the extracellular pH was adjusted to either pH 6.0 or pH 7.4, was found to be dependent on the concentration of **4** with an optimum concentration of 3 μM identified. PDT treatment of ectopic BxPC-3 tumours using **4** proved effective at reducing tumour volume by 66% compared to untreated mice. Therefore, PDT treatment using **4** may be a promising alternative for the treatment of PDA with the added benefit of reduced light sensitivity in healthy tissue.

Notes and references

1. Triesscheijn M.; Baas P.; Schellens J.H.; Stewart F.A., *Oncologist,* 2006, **9**, 1034–1044
2. Luo Y.; Kessel D., Photochem. Photobiol., 1997, **66**, 479-483
3. *See http://www.cancerresearchuk.org/cancer-help/aboutcancer/treatment/other/photodynamic-therapy-pdt*
4. Reddi E., *J. Photochem. Photobiol. B,* 1997, **37**, 189-195
5. Cui S. *et al., ACS Nano,* 2013, **7**, 676-688
6. McDonnell S.O. *et al., J. Am. Chem. Soc.,* 2005, **127**, 16360-16361
7. de Silva A.P. et al., *Chem. Rev*. 1997, **97**, 1515-1566
8. de Silva A.P *et al., Analyst,* 2009, **134**, 2385-2393
9. Erbas-Cakmak S. *et al. Chem.Sci.,* 2013, **4**, 858-862
10. Ozlem S.; Akkaya E.U., *J. Am. Chem. Soc.,* 2009, **13**1, 48-49
11. Mazur Y. *et al., Can Pat Appl,* 1991, CA 2, 029, 993.
12. Lackner B. *et al. Monatsh. Chem.,* 2005, **136**, 2067-2082
13. Buchalter L., *J. Pharm. Sci.,* 1969, **58**, 904
14. de Silva A.P. et al., *Arkivoc,* 2003, 229-243
15. Lee M. *et al., J. Am. Chem. Soc.,* 2010, **132**, 8828-8830
16. de Silva A.P. *et al., J. Chem. Soc. Chem. Commun.,* 1989, 1054-1056
17. See: http://www.lifetechnologies.com
18. Tannock I.F.; Rotin D., *Cancer Res.,* 1989, **49**, 4373-4384
19. See CRUK website*: http://www.cancerresearchuk.org/*
20. Li D. *et al. Lancet,* 2004, **363**, 1049-1057
21. Brown J.M.; Giaccia A.J.; *Cancer Res.,*1998, **58**, 1408–1416
22. Harris A.L., *Nat. Rev. Cancer,* 2002, 2, 38-47
23. Burris H.A. *et al., J. Clin. Oncol.,* 1997, **15**, 2403-2413.

Graphical Abstract



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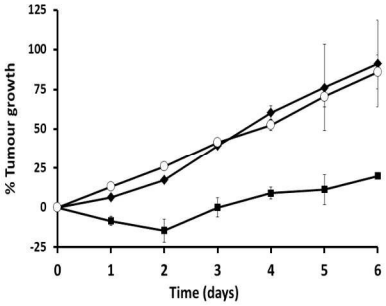

Compound **4** has been developed as a pH dependent sensitiser for use in Photodynamic Therapy.