ChemComm

RSCPublishing

COMMUNICATION

Iodinated Cyanine Dyes: A New Class of Sensitisers for use in NIR Activated Photodynamic Therapy (PDT)†

Cite this: DOI: 10.1039/x0xx00000x

Jordan Atchison[1], Sukanta Kamila[1], Heather Nesbitt[1], Kieran A. Logan[1], Dean M Nicholas[1], Colin Fowley[1], James Davis[2], Bridgeen Callan[1], Anthony P McHale[1] and John F Callan[1]*

Received 00th January 2016,
Accepted 00th January 2016

DOI: 10.1039/x0xx00000x

www.rsc.org/

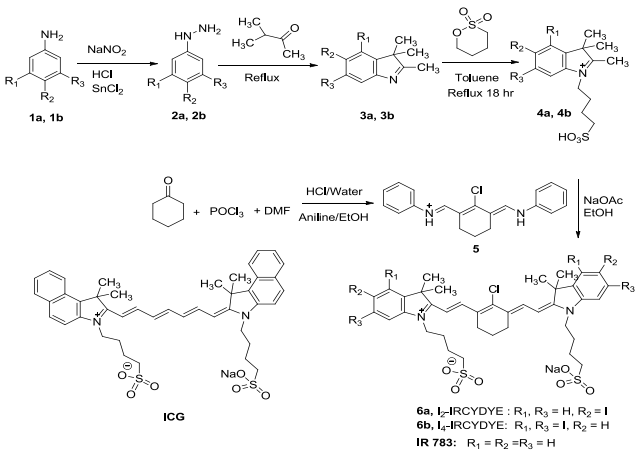
A new class of iodinated cyanine dyes have been prepared for use in NIR excited photodynamic therapy (PDT) and demonstrated improved efficacy in three pancreatic cell lines as well as excellent tumour control in a murine model of the disease.

Photodynamic therapy (PDT) involves the pre-treatment of a cancerous lesion(s) with an otherwise inactive photosensitising drug that sensitises the tissue to light. Upon light activation, the drug undergoes a series of photochemical reactions that generate toxic quantities of reactive oxygen species (ROS) resulting in cell death.[1] The main attraction of using PDT as an anti-cancer treatment is that ROS generation can be controlled by exposing only target tissues to light, thereby limiting undesirable off-target toxic effects to normal tissue.[2] The life-time and diffusion distance of ROS are also extremely short, meaning cellular damage is largely confined to the site of ROS generation.[2] While PDT has proven effective for the treatment of superficial lesions, its ability to treat larger or more deeply-seated tumours is hampered by the inability of visible light to penetrate deeply through human tissue. As tissue attenuation is inversely related to the wavelength of light, longer wavelength light penetrates much deeper through human tissue than shorter wavelength light.[3] Indeed, the phototherapeutic window, where tissue has maximum transparency to light, is in the near infrared (NIR) region between 750-900 nm.[3-5] At these wavelengths light is less prone to being filtered by other endogenous chromophores and as a result penetrates much deeper through living tissue. It is no surprise, therefore, that a significant body of work has focussed on developing new photosensitisers with absorption maxima in the phototherapeutic window, in an attempt to widen the applicability of PDT.[4,6,7]

Indocyanine green (ICG), also referred to as Cardio Green, is a widely used NIR fluorophore that gained clinical acceptability in the early 1950's.[8] ICG has also been explored as a potential photosensitiser but an extremely low singlet oxygen quantum yield (Φ = 0.077) has limited its practical utility.[9] Singlet oxygen generation in PDT involves energy transfer from the sensitisers excited triplet state to the ground state of molecular oxygen. Excited triplet states are formed from excited singlet states via a process known as intersystem crossing (ISC).[1,10] As fluorescence occurs as a result of radiative deactivation from a fluorophores excited singlet state, the fluorescence and singlet oxygen quantum yields are invariably linked, with good fluorophores typically being poor sensitisers and vice versa.[11,12] Addition of heavy atoms to the structural skeleton of conjugated aromatics is known to increase the ISC process and improve singlet oxygen generation.[7,13] Naturally, this usually comes at a price of reduced fluorescence emission. In this manuscript, we modify the structure of a basic cyanine dye by substituting hydrogen atoms with iodine atoms. The effect of these structural modifications on the compound's absorption and fluorescence properties as well as their singlet oxygen generating potential was determined. Furthermore, the NIR activated PDT efficacy of the iodinated analogues is also established in two pancreatic cancer cell lines (BxPC-3 and MIA-PaCa-2) and in an *in vivo* murine model of the disease.

IR-783 is a commercially available NIR cyanine dye with similar absorption and emission properties to ICG but has a modified chemical structure.[14] We prepared two iodinated derivatives of IR-783, where each indole ring was either mono-iodinated (**6a**) or di-iodinated (**6b**) (Scheme 1). In the case of **6a**, this involved first forming the diazonium salt of 4-iodoaniline (**1a**) followed by reduction using $SnCl_2$ / $NaBH_4$ to afford the hydrazine (**2a**). This was then refluxed with 3-methyl-2-butanone to produce the indole **3a**, which after N-alkylation with 1,4-butane-sultone resulted in the formation of sulfonic acid derivative **4a**. In parallel, compound **5** was prepared by a Vilsmeier-Haack reaction involving cyclohexanone, phosphoryl chloride and dimethylformamide. Compounds **4a** and **5** were then coupled together in a 1:2 molar ratio to produce target compound **6a**. In the case of **6b**, the precursor compound 3, 5-diiodoaniline (**1b**) was prepared according to a literature procedure.[15]

[1] *Biomedical Sciences Research Institute, University of Ulster, Coleraine, Northern Ireland, BT521SA. E-mail: j.callan@ulster.ac.uk*
[2] *Engineering Research Institute, University of Ulster, Coleraine, Northern Ireland, BT521SA.*
†Electronic Supplementary Information (ESI) available: Synthetic and experimental procedures. See DOI: 10.1039/b000000x/



Scheme 1. Scheme 1 Synthetic scheme for preparation of **6a** and 6**b.**

The same series of reactions used to furnish **6a** from **1a** were also used to generate **6b** from **1b**.

The effect of iodination on the photophysical properties of **6a** and **6b** was determined by studying their UV-Vis and fluorescence spectra in aqueous solution. For comparative purposes, the UV-Vis and fluorescence spectra of ICG were also recorded.[16] The results are shown in fig S7a and reveal that ICG and **6a** have similar absorption λ_{MAX} values (780 and 790 nm respectively). In contrast, the UV-Vis spectrum of **6b** was much broader with a significantly blue shifted λ_{MAX} value (687 nm). The fluorescence spectra, recorded after excitation at 775 nm for ICG and **6a**, and 687 nm for **6b**, revealed a significantly quenched emission for **6b** ($\Phi_f = 0.009$) when compared to either ICG ($\Phi_f = 0.27$) or **6a** ($\Phi_f = 0.22$) (fig S7b). Therefore, these results indicate that the number and/or position of iodine atom substitution on the indole ring of indocyanine dyes dramatically affects the photophysical properties of the resulting compounds with increasing iodination producing more dramatic effects on the UV-Vis and fluorescence spectra.

To determine the ability of **6a** and **6b** to generate singlet oxygen upon NIR excitation, the fluorescent probe Singlet Oxygen Sensor Green (SOSG) was utilised.[17] SOSG is non-fluorescent in its reduced form but reacts specifically with singlet oxygen to generate a fluorescent product. The intensity of the fluorescence signal at 525 nm is indicative of the amount of singlet oxygen generated. PBS solutions containing SOSG and matched concentrations of either ICG, **6a** or **6b** were prepared and irradiated with 780 nm light (100 mW). The fluorescence intensity of the solutions at 525 nm was recorded before and 10 min after irradiation. A control experiment involving light irradiation of SOSG alone (i.e. no sensitiser) was also undertaken for comparative purposes. The results are shown in fig S8 and reveal significantly enhanced singlet oxygen generation for both **6a** (7.9 fold, $\Phi^1O_2 = 0.66$) and **6b** (4.4 fold, $\Phi^1O_2 = 0.44$) relative to ICG. The increased singlet oxygen generation for **6a** compared to **6b** was surprising given the significantly reduced emission observed for the latter.

To investigate if the improved $^1O_2$ generation of **6a** and **6b** with respect to ICG would manifest itself in enhanced cellular toxicity, two pancreatic cancer cell lines (BxPC-3 and MIA PaCa-2) were seeded in 384 well plates and incubated with increasing concentrations (0-50 µM) of the three compounds for 3 hours. Selected wells were then exposed to 780 nm light (100 mW) for 1 min and incubated in fresh

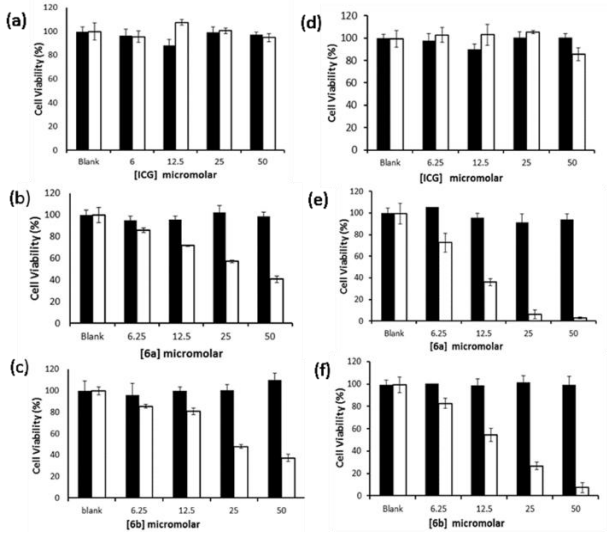


Fig. 1 Plot of cell viability for BxPC-3 cells (left panel) and MiaPaCa-2 cells (right panel) treated with ICG (a+d), **6a** (b and e) and **6b** (c and f) with (white bars) and without (black bars) 780 nm (100 mW) light treatment for 1 min.

media for a further 24 hr before cell viability was determined using a MTT assay. Cells incubated with either ICG, **6a** or **6b** alone (i.e. no light treatment) and cells exposed to light treatment alone (i.e. no drug) were also undertaken as controls. The results are shown in fig 1 and reveal no noticeable PDT mediated reduction in cell viability for ICG in either cell line over the concentration range tested. In contrast, a dose-dependent PDT mediated reduction in cell viability was observed for **6a** and **6b** in both cell lines. Interestingly, the effect was more dramatic in the MIA PaCa-2 cell line with less than 10% viable cells remaining at 50 µM drug concentration while the value was approximately 40% for BxPc-3 at the same concentration. While there was no significant difference between the efficacy of **6a** or **6b** in the BxPC-3 cell line, **6a** proved more potent in the MIA PaCa-2 cell line with 10.2, 18.3 and 20.3 % lower cell viability at 6.25, 12.50 and 25.0 µM respectively. To put these *in vitro* results into perspective, cell viability experiments were also performed using the commonly used pancreatic cancer antimetabolite drug 5-fluoruracil (5-FU). Both cell lines were treated with 5-FU at concentrations of 50, 100 and 250 µM and cell viability recorded 24 h later using the MTT assay. As shown in fig S9, the maximum reduction in cell viability observed was similar at 23 % and 26 % for MIA PaCa-2 and BxPC-3 respectively at 250 µM 5-FU. Therefore, these results confirm that PDT using **6a** or **6b** is significantly more effective than 5-FU at killing these pancreatic cancer cell lines.

Based on its singlet oxygen generation and *in vitro* toxicity when compared to **6b**, in addition to its inherent NIR fluorescence, **6a** was selected as a candidate for *in vivo* studies. Ectopic BxPC-3 Luc pancreatic tumours were established in SCID mice and once the tumours had reached an average volume of 205 mm^3, the mice were randomly separated into two groups. One group received no treatment (control) and one group received an intra-tumoural injection (2.5 mg/kg) of **6a** followed by 9 min exposure to 780 nm light (100 mW) with a 1 min lag after 3 and 6 min. The latter group also received a second treatment at day 8. The tumour volumes were monitored in each group and the results shown in Fig 2a reveal that by day 11, tumours in the untreated group had grown by almost 500 % from their pre-treatment value, while PDT treated tumours had increased in

volume by only 39 % over the same time period ($p < 0.001$). Indeed, 4 days after PDT treatment, the tumours reduced from their pre-treatment size by 10 %. Between days 4-7 the tumours gradually increased back to their pre-treatment values but following a second PDT treatment on day 8 they again reduced in volume until day 11, when the experiment was terminated due to the control tumours reaching their maximum permissible size.

In the above *in vivo* study, an intra-tumoral injection was used to preclude variables associated with systemic delivery and guarantee that a consistent dose of sensitiser was administered to each subject. In the clinic however, PDT treatment of pancreatic cancer requires intravenous (IV) administration of the sensitiser followed by ultrasound or MRI guided interstitial light treatment. We were therefore interested in examining the specificity of uptake of **6a** by pancreatic tumours following IV administration.[18] In addition, this experiment would also allow us to assess the potential of **6a** as a real-time NIR fluorescence imaging agent. A solution of **6a** was

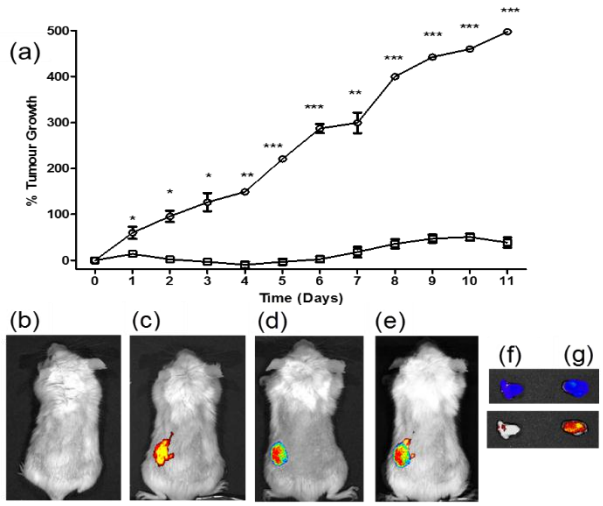


Fig. 2 (a) Plot of tumour volume against time for SCID mice bearing ectopic BxPC3-Luc tumours following treatment with (squares) or without (circles) **6a** and 780 nm light (3 x 3 min). Arrow indicates 2nd treatment. *$p \leq 0.05$; **$p \leq 0.01$ and ***$p \leq 0.001$. Whole body fluorescence (b and c), bioluminescence (d) and combined fluorescence / bioluminescence overlay image (e) of SCID mice bearing ectopic BxPC3-Luc tumours recorded before (b) and 18 h after (c-e) tail vein administration of **6a**. Images f-g show bioluminescence (top) and fluorescence (bottom) of BxPC-3Luc tumours excised 18h after tail vein administration of vehicle only (f) or **6a** (g).

administered by tail vein injection and imaged 18 h later using an IVIS whole body imaging system with images collected in the bioluminescence, fluorescence and overlay modes. The results are shown in fig 2b-e and reveal an intense NIR fluorescence signal from the tumour region with an almost identical overlap between the fluorescence of **6a** and tumour bioluminescence signal. Indeed, there was no evidence of fluorescence in any other region 18h post administration illustrating effective uptake of **6a** by the tumour and clearance from non-target tissue. While PDT is a targeted cancer treatment, it is still beneficial for the sensitiser to accumulate in the tumour to avoid collateral damage to surrounding healthy tissue in the event of misguided light treatment. This is particularly relevant in the treatment of pancreatic cancer where the tumour is often located close to critical anatomical structures such as veins or arteries and where inadvertent tissue damage could have serious consequences for the patient.

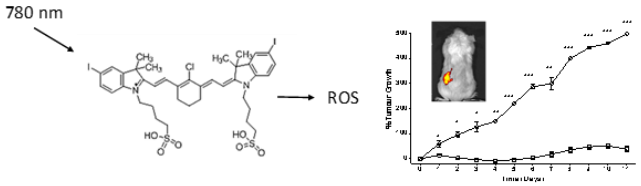
In conclusion, we have demonstrated that the addition of iodine atoms to the basic structural skeleton of an NIR absorbing indocyanine dye, improves singlet oxygen production and enhances PDT mediated cytotoxicity when compared to the non-iodinated and clinically-approved analogue ICG. PDT treatment of mice bearing ectopic human xenograft BxPC-3 pancreatic tumours using 6a significantly reduced tumour burden and was sufficiently fluorescent to enable real-time NIR fluorescence imaging in vivo. Pancreatic cancer remains one of the most recalcitrant forms of the disease in which survival statistics haven't changed in over 40 years, illustrating a clear unmet need for alternative approaches to treat the disease. NIR activated PDT using **6a** may provide an alternative approach to conventional chemotherapy as a neo-adjuvant or palliative treatment for patients who present with locally advanced or metastatic disease. Since the latter represent over 70% of patients, such a treatment could find widespread appeal as a minimally invasive treatment with limited off-target side-effects.

Acknowledgements: JFC thanks Norbrook Laboratories Ltd for an endowed chair.

References

1. Triesscheijn, M.; Baas, P.; Schellens, J. H.; Stewart, F. A.; Photodynamic therapy in oncology. *Oncologist*. 2006, **9**, 1034-1044.
2. Luo, Y.; Kessel, D.; *Photochem. Photobiol*. 1997, **66**, 479-483.
3. Sternberg, E. D.; Dolphin, D.; Bruckner, C.; *Tetrahedron*. 1998, **54**, 4151-4202.
4. Luan L.; Ding L.; Zhang W.; Shi J.; Yu, X.; Liuet, W. *Bioorg. Med. Chem. Lett*. 2013, **2**3, 3775-3779.
5. Fowley C.; Nomikou N.; McHale A. P.; McCaughan, B.; Callan, J. F. *Chem. Commun*. 2013, **49**, 8934-8936.
6. Lu, T.; Saho, P.; Matthew, S.; Sand, A.; Sun, F. *J. Am. Chem. Soc*. 2008, **130**, 15782-15783.
7. He, J.; Wang, Y.; Missinato, M. A.; Onuoha, E.; Perkins, L. A.; Watkins, S. C.; St Croix, C. M.; Tsang, M.; Bruchez, M. P.; *Nat. Methods*. 2016, **13**, 263-268.
8. Alander, J.T.; Kaartinen, I.; Laakso, A.; Patila, T.; Spillmann, T.; Tuchin V. V.; Venermo, M.; Välisuo, P.; *Int. J. Biomed. Imaging*. 2012. Article ID 940585
9. Cardillo J. A.; Jorge, R.; Costa R. A.; Nunes S. M. T.; Lavinsky, D.; Kuppermann, B. D.; Tedesco, A. C.; Farah, M. E.; *Br. J. Ophthalmol*. 2008, **92**, 276-280.
10. Atchison, J.; Kamila, S.; McEwan, C.; Nesbitt, H.; Davis, J.; Fowley, Colin.; Callan, B.; McHale, A. P.; Callan, J. F.; *Chem. Commun*. 2015, **51**, 16832-16835.
11. Wu, W.; Guo, H.; Wu, W.; Ji, S.; Zhao, J.; *J. Org. Chem.*, 2011, **76**, 7056-7064.
12. Swaminathan, S.; Fowley C.; Thapaliya, E. R.; McCaughan, B.; Tang, S.; Fraix, A.; Captain, B.; Sortino, S.; Callan, J.; Raymo, F.M.; *Nanoscale*. 2015, **7**, 14071-14079.
13. Kamkaew, A.; Lim S. H.; Lee, H. B.; Kiew, L.V.; Chung, L.Y.; Burgess, K.; *Chem. Soc. Rev*., 2013, **42**, 77-88.
14. Yang, X.; Shi, C.; Tong, R.; Qian, W.; Zhau, H. E.; Wang, R.; Zhu, G.; Cheng. J.; Yang, V. W.; Cheng, T.; Henary, M.; Strekowski, L.; Chung, L. W.; *Clin. Cancer Res*. 2010, **16**, 2833-2844.
15. Berube, M.; Poirier, D.; *Org. Lett.*, 2004, **6**, 3127-3130.
16. ICG was used instead of IR 783 to compare the properties of **6a** and **6b** given its widespread use as an imaging agent and sensitiser. Both ICG and IR 783 have similar absorption profiles, extinction co-efficient and singlet oxygen generating potential (Fig S10).
17. See: http://www.lifetechnologies.com.
18. Bown S.G.; Rogowska A.Z.; Whitelaw D.E.; Lees W.R.; et al., *Gut*, 2002, **50**, 549-557

Table of Contents



Iodinated cyanine dye **6a** has been developed for use as a NIR excited photosensitiser in Photodynamic Therapy.