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. 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. 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. Indeed, the phototherapeutic window, where tissue has maximum transparency to light, is in the near infrared (NIR) region between 750-900 nm. 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. 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. 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-idoaniline (1a) followed by reduction using SnCl2 / NaBH4 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 Wilsmeier-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-diidoaniline (1b) was prepared according to a literature procedure. 

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

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*Electronic Supplementary Information (ESI) available: Synthetic and experimental procedures. See DOI: 10.1039/b000000x/
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. The results are shown in fig S7a and reveal that ICG and 6a have similar absorption $\lambda_{\text{MAX}}$ values (780 and 790 nm respectively). In contrast, the UV-Vis spectrum of 6b was much broader with a significantly blue shifted $\lambda_{\text{MAX}}$ 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. 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 sensitisers) 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, $\Phi_f \text{O}_2 = 0.66$) and 6b (4.4 fold, $\Phi_f \text{O}_2 = 0.44$) 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 $^{1} \text{O}_2$ 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 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 efficacies 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-fluorouracil (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 as a real-time NIR fluorescence imaging agent. A solution of 6a was administered by tail vein injection and imaged 18 hours 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 shown in fig 2f-g.

In addition, this experiment would also allow us to assess the potential of 6a as a real-time NIR fluorescence imaging agent. Fig 2a shows the % tumour growth against time for SCID mice bearing ectopic BxPC3-Luc tumours following IV administration of the sensitiser. While PDT i

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

References

16. ICG was used instead of IR 783 to compare the properties of 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.
Iodinated cyanine dye 6a has been developed for use as a NIR excited photosensitiser in Photodynamic Therapy.