Volume 52 | Number 89 | 18 November 2016 | Pages 13049–13182

ChemComm

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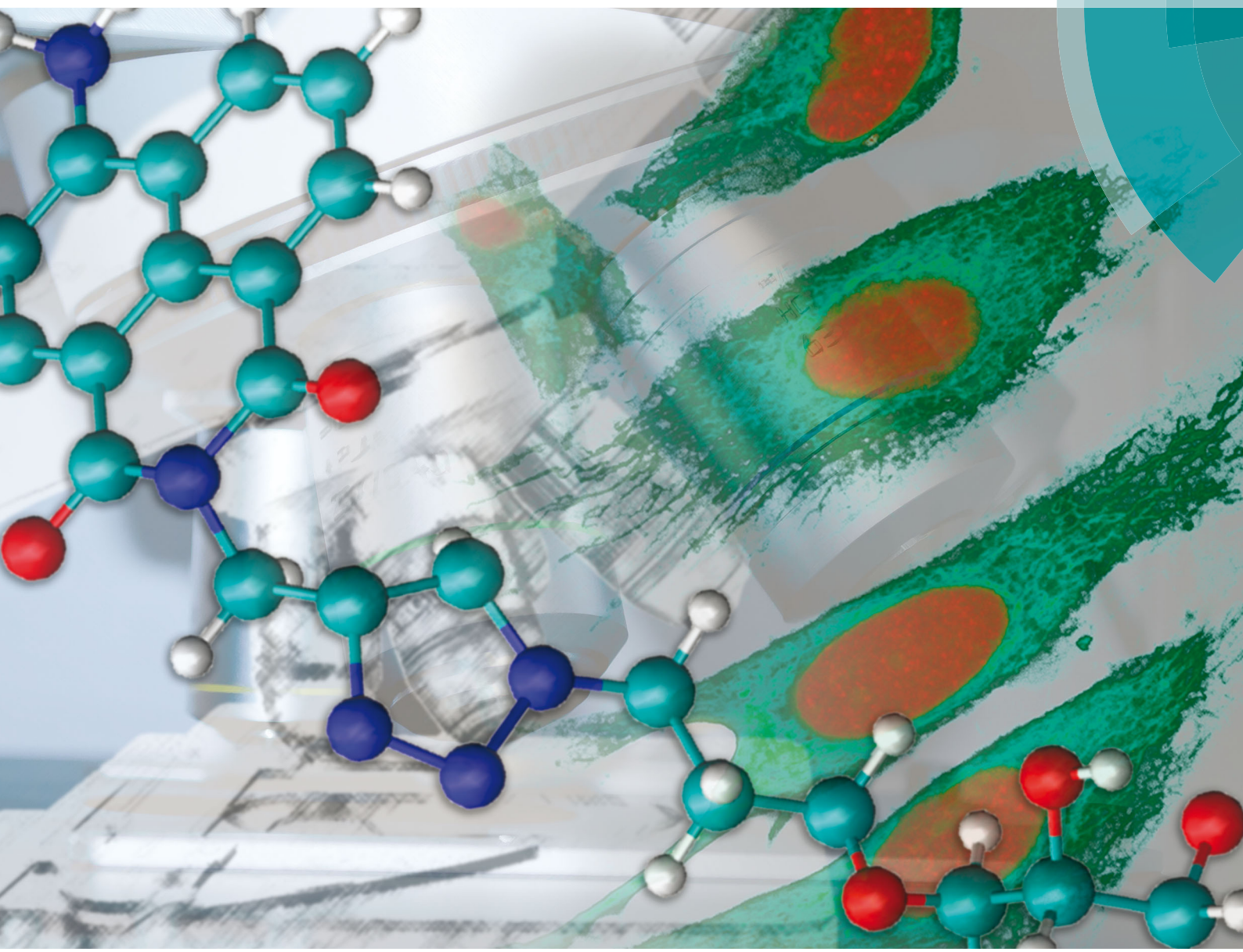

ISSN 1359-7345

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Cite this: *Chem. Commun.*, 2016, 52, 13086

Received 4th August 2016,
Accepted 22nd September 2016

DOI: 10.1039/c6cc06451e

www.rsc.org/chemcomm

Glycosidase activated release of fluorescent 1,8-naphthalimide probes for tumor cell imaging from glycosylated 'pro-probes'†

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Glycosylated 4-amino-1,8-naphthalimide derivatives possess a native glycosidic linkage that can be selectively hydrolysed *in situ* by glycosidase enzymes to release the naphthalimide as a fluorescent imaging or therapeutic agent. *In vitro* studies using a variety of cancer cell lines demonstrated that the naphthalimides only get taken up into cells upon enzymatic cleavage from the glycan unit; a mechanism that offers a novel approach for the targeted delivery of probes/drugs.

The 1,8-naphthalimide (Nap) is a versatile fluorescent building block that has been extensively used in both supramolecular and medicinal chemistry.[1] Being synthetically readily accessible, Nap compounds have been developed as luminescent sensors, cellular probes and as DNA targeting and binding agents. They have also been shown to demonstrate potent anti-cancer activity, particularly active against a variety of murine and human cancer cell lines.[2] An example of this is Amonafide, a 3-amino-Nap structure developed as a topoisomerase-II inhibitor; which displays potent activity against acute myeloid leukemia (AML).[3] The Naps can display tunable photophysical properties that can be carefully controlled through synthesis at the aromatic core or the imide site.[4] In particular, 3- and 4-amino-Naps possess internal charge transfer (ICT) excited state properties that are highly solvent/polarity dependent, making them particularly attractive as fluorescent probes for observing the microenvironment of biological systems.[5] Small-molecule glycoconjugates are now widely employed as both biological probes and anticancer therapeutics.[6] In these systems, the presence of the glycan unit modulates hydrophilicity and lectin binding properties. This endows a number of beneficial properties, such as enhanced aqueous solubility, tumor cell targeting and cellular uptake, all of which can result in improved pharmacokinetics for such derivatives.[7] Importantly, glycosidase enzymes, either extra- or intracellular, can activate glycoconjugates; a mechanism that can be employed to enable targeted delivery of a therapeutic payload or an imaging agent.[7b,8] With this in mind, we set out to prepare and investigate the biological applications of various glycosylated-Nap derivatives (**1–4** and **12**, Schemes 1 and 2) that could be 'activated' using enzymatic hydrolysis of the glycosidic bond. The concept of a 'prodrug' is well established in medicinal and pharmaceutical chemistry.[9] The rationale behind our design is depicted schematically in Fig. 1, and draws a parallel to the concept of a 'prodrug' where the glycosylated-Naps can be considered as a 'pro-probe' (*i.e.* a 'prodrug' equivalent), a structure that does not undergo cellular uptake in its glycoconjugated form (*i.e.* it can be considered as 'inactive'). However, upon enzymatic hydrolysis *in situ*, using specific glycosidase enzymes, the Nap 'probe' is released or 'activated', which facilitated cellular uptake allowing for imaging of cellular function. The use of luminescent probes to monitor enzymatic reactions is of current interest, and we have recently developed a Tb(III) based luminescent 'Switch On Assay' for monitoring

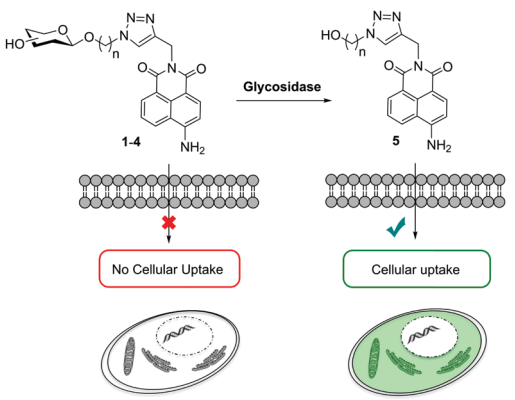

Fig. 1 Glycosidase activation of the glycosylated naphthalimide 'pro-probes' (**1–4**), which results in the release of a fluorescent probe (**5**) that can undergo endocytosis into cancer cells.

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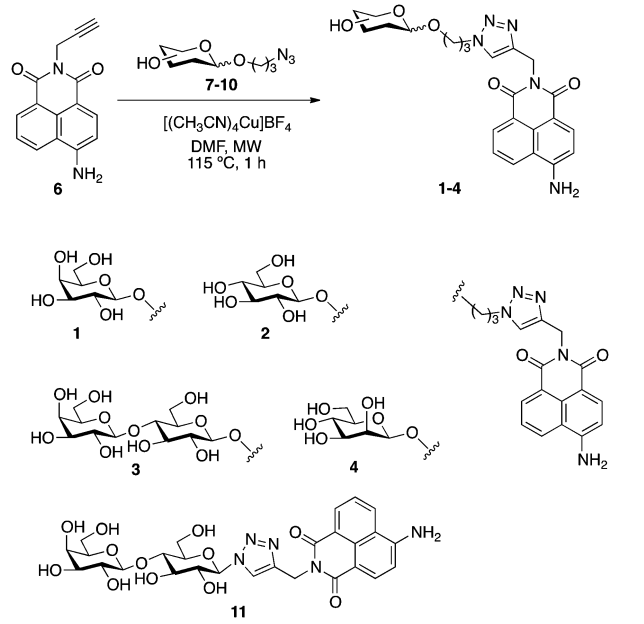
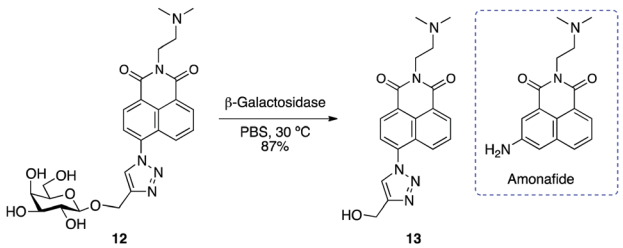
† Electronic supplementary information (ESI) available: Experimental procedures and characterization of new compounds. See DOI: 10.1039/c6cc06451e



Scheme 1 Synthesis of glycosylated naphthalimides **1–4** from **6**.



Scheme 2 Glyconaphthalimide **12** displaying structural features of Amonafide and concomitant release of **13** by galactosidase.

glutathione reductase activity *in situ*.[10] To date, only a limited number of examples have been reported where the Nap moiety has been included in such agents. Recent examples include that of Wong *et al.* who used a 'click activated' fluorescent Nap probe for azido labeling of fucosylated glycoproteins;[11] Shi *et al.* who developed a galactosyl azido-Nap probe for the imaging of hepatocellular H_2S[12] and Dong *et al.* who reported the effect of glycosylation on a Nap Zn(II) probe.[13] While these are excellent examples of the application of Nap in biotechnology; none of these examples contain a native glycosidic bond between the carbohydrate and the Nap moiety. While this may be considered advantageous for metabolic stability, it greatly limits the application of such compounds for use in glycosidase-triggered release. Herein we demonstrate that **1–4** and **12** are viable candidates for use in cellular targeted delivery, and that upon enzymatic activation *in vitro* a targeted delivery of a therapeutic or imaging payload occurs.

The synthesis of **1–4** was achieved using a facile and high-yielding synthetic route (see ESI†). These structures or so called 'pro-probes' all contain a native glycosidic bond that covalently links the carbohydrate unit and the Nap moiety. This feature enables enzyme-mediated cleavage of the carbohydrate unit from the Nap core, resulting in release of the naphthalimide fluorophore. The galactose, glucose, lactose and mannose Nap derivatives **1–4** (Scheme 1) were prepared in high yield *via* copper catalyzed azide–alkyne 'click' ligation[14] between an unprotected carbohydrate derivative (displaying an alkyl azide at the anomeric position) and *N*-propargyl-4-amino-1,8-naphthalimide **6**; itself being prepared in two steps from commercially available 4-nitro-1,8-naphthalic anhydride. An example of a disaccharide conjugated Nap, compound **11**, that lacks the native glycosidic bond, was also prepared, from a lactose moiety displaying an anomeric azido group. Although this example does not possess a native glycosidic bond at the reducing terminus, the disaccharide portion is itself susceptible to hydrolysis by a lactase enzyme. Since this will release the glucosylated Nap, we postulated that the lactose derivative may also function as a fluorescent probe for use within cancer cells.

As outlined above, Amonafide, a topoisomerase-II inhibitor, has been shown to have potent anti-cancer activity. While demonstrating excellent activity in clinical phase II breast cancer trials it failed in clinical phase III trials due to acute side effects and dose-limiting bone marrow toxicity. Developing alternative derivatives of Amonafide that are cytotoxic, but without the associated severe side effects is of major clinical interest.[15] Hence, we also prepared the galactosylated Amonafide analogue **12**, Scheme 2, using a click reaction between galactose-β-*O*-propargyl and an azido Nap (see ESI†) in 92% yield.

The photophysical properties of all of the glycosylated-Naps pro-probes were investigated; the 4-amino-Naps display classical Nap ICT excited state properties as determined by recording the UV-Vis absorption, the fluorescence excitation and the emission spectra in a variety of solvents (Fig. S1, ESI†), Fig. 2. This solvatochromic effect was also studied using **1** and **11**. While only minor changes were seen in the absorption spectra, a significant quenching was observed in the emission spectra when recorded in both H_2O or phosphate buffered saline (PBS) solution compared to that seen in CH_3CN; the latter possibly being due to the presence of a twisted internal charge transfer excited state (TICT) in **11**. The Amonafide derivative, **12**, possessing the triazole at the Nap moiety was, in comparison, blue shifted (Fig. 2), with a λ_{abs} centered at 347 nm (*vs.* 430 nm for **1–4**) and λ_{flu} at 425 nm (*vs.* 550 nm for **1–4**). Quantum yield and extinction coefficient values were determined (Tables S1 and S3, ESI†). All of the probes were found to be photo-stable, with no noticeable

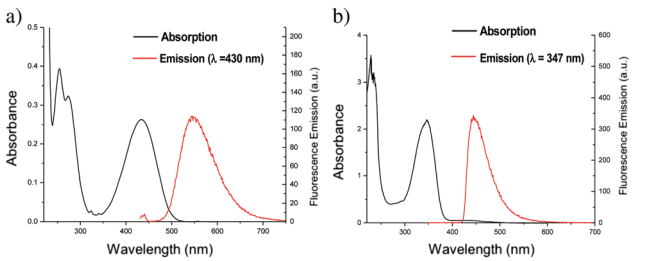


Fig. 2 UV/Vis absorbance and fluorescence spectra of (a) **1** (0.1 mM) and (b) **12** (0.1 mM), respectively.

bleaching being observed upon prolonged excitation of the Nap fluorophores in aqueous solution.

As the activity of these probes should be within the physiological pH window, they were subjected to a spectroscopic pH titration where both the ground and the excited state were monitored. As an example, for compound **12**, from pH 2.5–10 the Nap emission remained constant and only 'switched off' in highly alkaline solution, above pH 10 (Fig. S3, ESI†). The analysis of the concomitant changes in the absorption confirmed this; while an isosbestic point was seen at 314 nm, with λ_{abs} being shifted to 298 nm as a function of pH.

To test our hypothesis that the glycosylated Nap derivatives can be used as probes for cancer, compounds **2–4** and **11** were incubated with HeLa cell lines (cervical cancer) at 0.1 mM concentration for 3 h, after which time they were analysed (as live cells) by using confocal fluorescence microscopy, upon excitation at 405 nm. Glycosylation typically enhances tumor cell endocytosis, either through ASGPr-mediated endocytosis or *via* the insulin-independent glucose transporter GLUT-1.[7a] However, as can be seen in Fig. 3, no cellular localisation occurred for any of the compounds; the recorded green-channel fluorescence only arising from compound within the extracellular media excited with a high laser power (the cells appearing black with the nucleus stained red using DRAQ5). Longer incubation times up to 24 h did not show any change in the cellular uptake processes, whilst viability studies demonstrated that these compounds did not display significant cytotoxicity against HeLa cells (Table S5, ESI†).

Prior to *in vitro* evaluation, the affinity of the glycosylated naphthalimides as substrates for glycosidase enzymes was evaluated using commercially available β-galactosidase and β-glucosidase enzymes. Compounds **1** and **2** were treated with β-galactosidase and β-glucosidase, respectively. Lactose-containing naphthalimides (**3** and **11**) were treated first with β-galactosidase followed by β-glucosidase. Glycosidase enzymes exerted their activity on compounds **1–3**, rapidly hydrolysing the glycosidic bonds and releasing **5**. The completion of the reaction within 30 min was confirmed by mass spectrometry. Compound **11** was designed to be a negative control for the β-glucosidase, as there is no glycosidic linkage between the glucose and the triazole, therefore the enzyme would be unable to exert its activity. As predicted, compound **11**, only showed activity for the β-galactosidase enzyme and therefore compound **5** was not released. The activity of the Amonafide analogue **12** in the presence of β-galactosidase was also investigated and found to undergo complete and rapid hydrolysis of the glycosidic bond in the presence of the enzyme, demonstrating that our design is applicable for use in enzymatic triggered delivery of a therapeutic payload.

Compounds **5** and **13** were used as positive controls for the *in vitro* studies, and were synthesised from **1** and **12**, respectively, using β-galactosidase (0.01 U) in buffered solutions with high isolated yields of 97 and 87%, respectively. The absorption, excitation and fluorescence emission spectra of compounds **5** and **13** were recorded (see ESI†). Compound **5** exhibits the same characteristic bands as the precursors **1–3**, demonstrating that the conjugation to a glycan does not affect the ICT band of the naphthalimides. However, a significant decrease in the fluorescence intensity of compound **13** *vs.* compound **12** was observed. Importantly, no significant changes were seen in the absorption spectrum of **1–3** and **12** upon addition of enzymes, which rules out any non-specific or strong association between the enzyme and the Nap substrate.

Having demonstrated that **1–3** and **12** can undergo rapid and controlled enzymatic hydrolysis, we subsequently tested the effect on cellular uptake of the released naphthalimide core upon enzymatic activation *in vitro*. A 3 h incubation of HeLa cells with the compound **1** (0.1 mM) was followed by the subsequent addition of the β-galactosidase enzyme (1.0 U) 1.5 h later. Confocal microscopy visualisation showed that the naphthalimide moiety had entered the cells upon cleavage of the glycosidic linkage by the glycosidase enzyme (Fig. 4). At a lower concentration of compound **1** (0.05 mM), cellular uptake of the naphthalimide was once again only observed in the presence of the glycosidase enzyme. At a further reduced concentration of **1** (0.01 mM), only trace fluorescence was observed inside the cells in the presence of the enzyme (Fig. S6, ESI†). *In vitro* studies of **2** with the β-glucosidase enzyme were not successful, despite the growth of the cells in a glucose-free medium, to prevent saturation of the enzyme. Different concentrations of the enzyme were also investigated (0.01, 0.1 and 1 U), however, no improvement was seen and, unfortunately, β-glucosidase mediated release appeared inactive *in vitro*.

In order to further investigate the generality of the enzyme release approach, two additional cancer cell lines, a colon

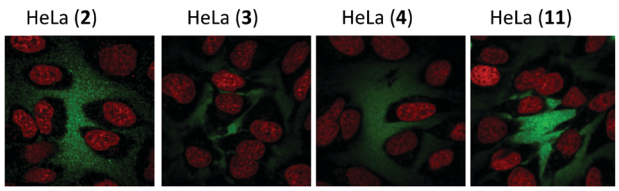


Fig. 3 Incubation of compounds **2–4** and **11** (0.1 mM) for 3 h in HeLa cells. Compounds were excited with a high power laser.

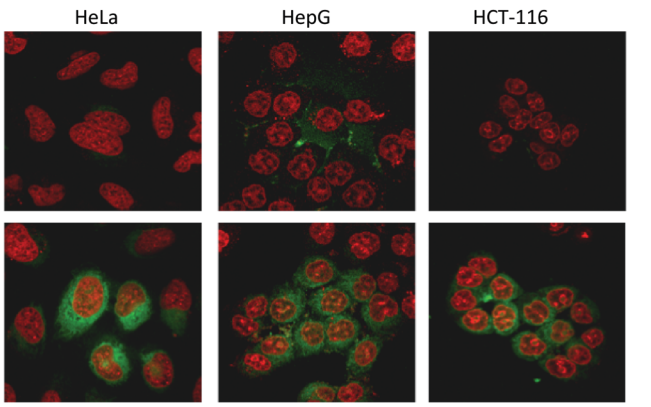


Fig. 4 Incubation of compound **1** (0.1 mM) for 3 h in cancer cell lines, first row, and incubation of compound **1** (0.1 mM) for 3 h and β-galactosidase (1.0 U) for 1.5 h, second row.

carcinoma (HCT-116) and hepatocellular carcinoma (HepG2), the latter known to express ASGP-R, were investigated with compound **1** and β-galactosidase. As shown in Fig. 4, no uptake of **1** was observed in the absence of the enzyme, suggesting that the enzyme-mediated process is independent of the specific-receptor interaction. Crucially, this finding demonstrates that the presence of the galactose binding receptor protein does not inhibit activity of the β-galactosidase enzyme and that the galactose moiety is available for hydrolysis. It is possible however, that while the expression of ASGP-R did not enhance cellular uptake, expression of ASGP-R in certain cancers *in vivo* may promote increased localisation of compounds to cancers which in turn would increase cell uptake upon cleavage. Compound **5** was investigated as a positive control for the three cell lines. When **5** (0.1 mM) was incubated in cells, strong fluorescence inside the cells was observed, proving that the non-glycosylated naphthalimide undergoes rapid internalisation by the cells (Fig. S6, ESI†).

Finally, *in vitro* studies with Amonifide analogue **12** showed a similar behavior to compound **1**. Release of the Amonafide derivative required the presence of β-galactosidase, the released **13** underwent uptake into all three cell lines that could be monitored by fluorescence enhancement within the cytosol (Fig. S5, ESI†). As is evident from Fig. 4, the proof of principle that cytotoxic agents could also be released by this enzymatic approach has been clearly demonstrated.

In conclusion, we have designed and developed the first examples of glycosylated Naps as fluorescent pro-probes for use in enzymatic triggered release *in vitro*, which results in uptake across a range of cancer cell lines. The strategy offers significant potential for the release of therapeutic or imaging agents through activation by expressed glycosidases *in vivo*. For example, it is known that high concentrations of β-glucuronidase are expressed in a wide range of malignancies including lung, breast, ovarian and gastrointestinal tract carcinomas as well as in certain melanomas.[16] In the tumour environment, β-glucuronidase is secreted extracellularly in necrotic areas by inflammatory cells (monocytes/granulocytes) while in healthy tissue activity is confined to lysosomes.[16c] Glycosidases are also expressed in high concentrations by colonic microflora, which can then release the aglycon probe/therapeutic in the colon in a site-specific manner.[17] The process appears to be independent of the glycosyl receptor. Our results demonstrate that the combination of carbohydrate binding receptor and enzyme-mediated release represents a potentially powerful approach for cancer therapy. We are currently pursuing this line of investigation in our laboratories.

We thank School of Chemistry TCD and Science Foundation Ireland (SFI) (for PI 2013 13/IA/1865 grant), for financially supporting this research.

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