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**Dynamic nucleophilic aromatic substitution of tetrazines ( $S_N Tz$ )** has been employed to build theranostic prodrugs that are activated by hydrogen sulfide.  $H_2S$  is typically found in high concentrations in some kinds of cancer cells and it is able to trigger the disassembly of tetrazine prodrugs. In such a way, a dual release of drugs and/or fluorescent compounds can be selectively triggered.

A targeted delivery approach is a long sought aim of drug delivery.<sup>1</sup> Its classic goals are to avoid unfavourable side effects and also to increase the efficiency of the drug. Therefore, it is particularly interesting in cancer treatments,<sup>2</sup> in which traditional chemotherapy has remained basically the same for decades and it lacks any intrinsic selectivity inducing systemic toxicity to the patient. In order to achieve efficient targeted drug delivery, two main features are required: a distinctive hallmark in the tumoral cell, and a chemical mechanism designed to release the active drug selectively in the presence of such hallmark.<sup>3</sup> Regarding the latter, dynamic covalent chemistry (DCvC) might be considerably helpful. DCvC deals with reversible reactions that allow the system to reach thermodynamic equilibrium and also to respond to changes in the environment.<sup>4–7</sup> Thus, dynamic chemical systems are intrinsically responsive to a specific stimulus. Obviously, any dynamic system aimed at releasing drugs should be inherently stable in aqueous conditions.

In this regard, our group has recently developed a novel dynamic reaction: the nucleophilic aromatic substitution of

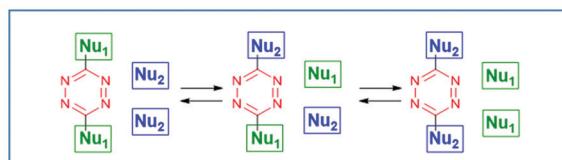
## Hydrogen sulphide-triggered theranostic prodrugs based on the dynamic chemistry of tetrazines<sup>†</sup>

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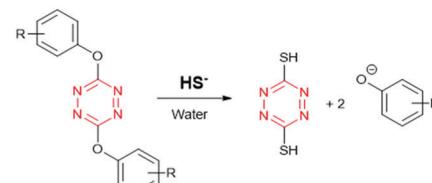
tetrazines ( $S_N Tz$ , Fig. 1a).<sup>8–10</sup> This reaction quickly exchanges phenols and thiols in positions 3 and 6 of the tetrazine ring, at

### Previous Works:

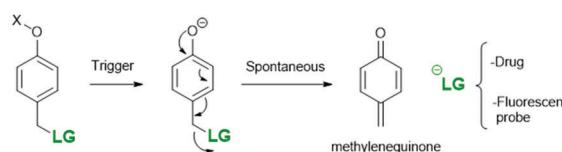
#### a) Dynamic Nucleophilic Aromatic Substitution of Tetrazines ( $S_N Tz$ )



#### b) Release of phenols from the tetrazine ring induced by hydrogen sulfide in water



#### c) Self-immolative release of drugs or fluorescent probes



#### This work:

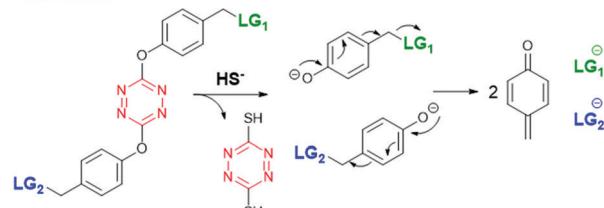


Fig. 1 (a) In previous works, the dynamic nature of the aromatic substitution of tetrazines has been reported. (b) Due to such dynamic behaviour, hydrogen sulphide in water is able to irreversibly displace any phenol from the tetrazine ring. (c) It is also well known that some phenols can act as self-immolative moieties. Herein we combine both properties to create molecular systems that respond to the presence of hydrogen sulphide by releasing drugs or fluorescent compounds.

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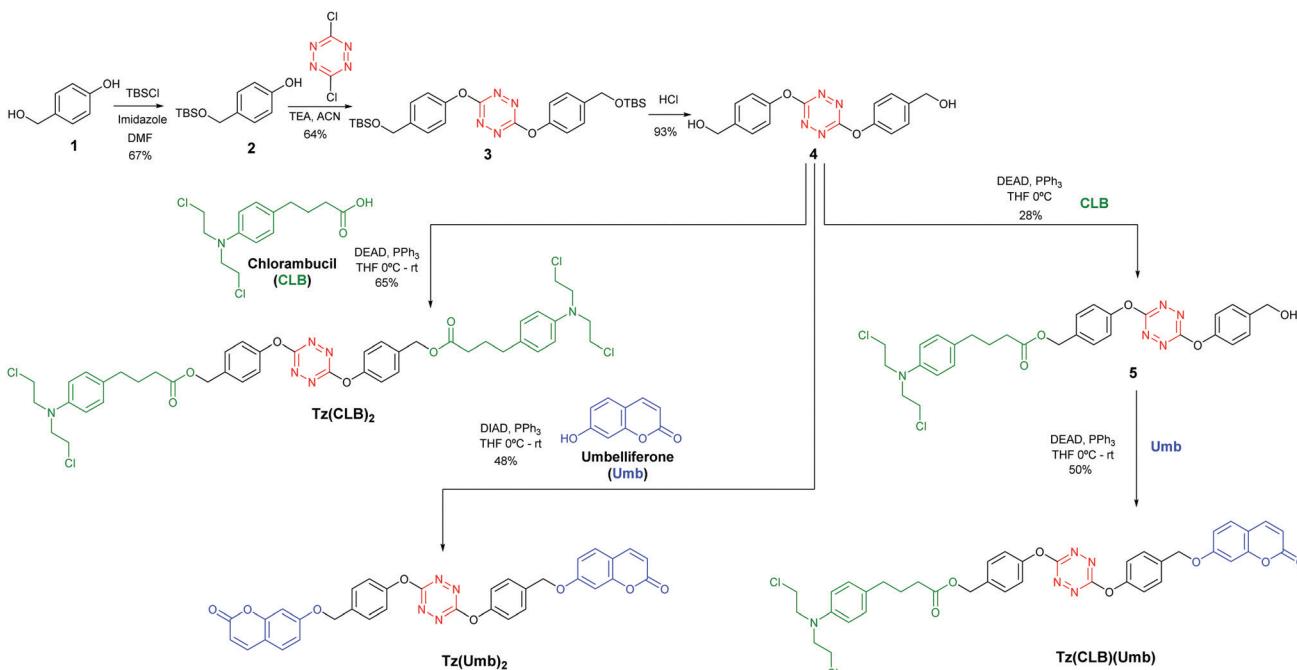
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Scheme 1 Synthetic pathway to obtain the tetrazine responsive systems.

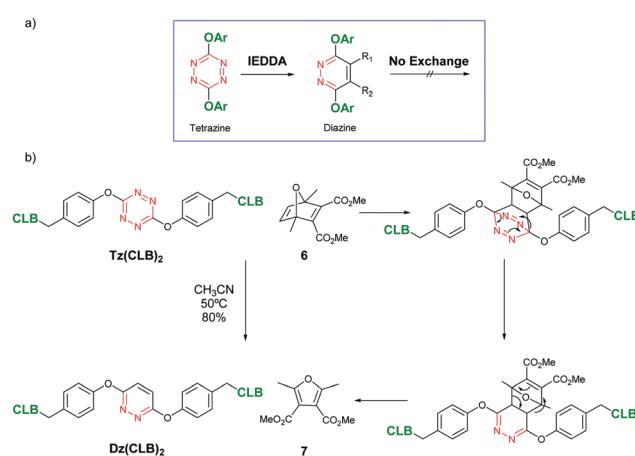
room temperature and it is compatible with aqueous environments.

We discovered that thiols efficiently release any phenol quantitatively,<sup>8</sup> although usually a base is required to initiate the process, unless the thiol is acidic enough. Indeed, hydrogen sulfide ( $pK_{a1}$  6.9) is able to displace phenols from the tetrazine ring in water with no base required (Fig. 1b).<sup>9</sup> This fact allowed a hydrogen sulfide triggered release event to be envisioned. Although  $H_2S$  has been considered traditionally as an irritating gas with a smell of rotten eggs, recently its relevance in several physiological events has turned it into an interesting biomarker to detect or into a trigger to induce the activation of therapeutic systems.<sup>11–17</sup> It is worth mentioning that some kinds of cancer cells, such as colon cancer cells, display a characteristic high intracellular concentration of  $H_2S$  due to the overexpression of the enzyme cystathione beta synthase (CBS).<sup>18</sup> Therefore, tetrazine prodrugs could be useful to selectively release molecules in colon cancer cells.

The released compounds do not have to display a phenol necessarily, but the tetrazine release process can be combined with a self-immolative spacer.<sup>19–21</sup> Indeed, self-immolation has been extensively used as an efficient drug-releasing method.<sup>22</sup> One of the most common self-immolative linkers is a phenol bearing a reasonably good leaving group (LG) on a methylene in the ortho or para position (Fig. 1c). Therefore, a self-immolative phenol, bearing a drug or a fluorophore, could be attached to a tetrazine (Fig. 1, bottom). Such a system would be selectively disassembled in physiological conditions by  $H_2S$ . Interestingly, as the tetrazine has two reactive points, a dual release is possible, and therefore a theranostic compound can be designed by incorporating an active drug and a fluorescent probe.

As a proof of concept, we have chosen chlorambucil (CLB) as the active drug to be released, a well-known FDA-approved anticancer drug limited by adverse effects due to its lack of selectivity;<sup>23</sup> on the other hand, umbelliferone was chosen as a fluorescent probe, as it is known to be virtually non-fluorescent as long as it is attached by the hydroxyl group, but it turns highly luminescent once it is released.<sup>24</sup>

The synthesis of the prodrugs starts by selectively protecting the primary alcohol in **1** and then attaching phenol **2** to the dichlorotetrazine (Scheme 1). The resulting product (**3**) yields tetrazine-diol **4** by deprotection by acid hydrolysis. **4** is the starting compound for any other product: two chlorambucil or

Scheme 2 (a) IEDDA reaction post-functionalizes the tetrazine ring and cancels the exchange; (b) pro-drug **Tz(CLb)2** can be easily modified by IEDDA reaction into **Dz(CLb)2** which should not be able to be activated.

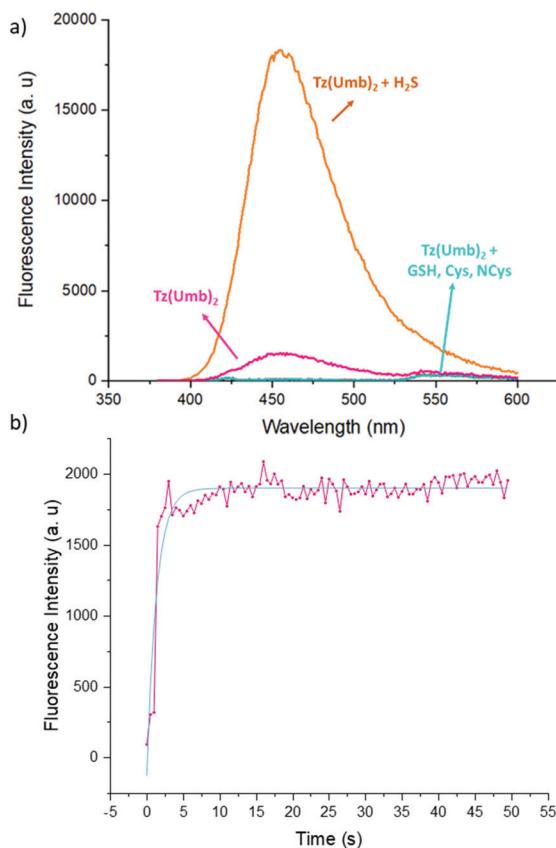


Fig. 2 (a) Relative fluorescence intensity at  $\lambda_{\text{ex}} = 360$  nm of probe **Tz-(Umb)<sub>2</sub>** (10  $\mu\text{M}$ ) with various species (200  $\mu\text{M}$ ) at 37 °C in  $\text{H}_2\text{O}/\text{DMSO}$  (92:8). Glutathione (GSH, 200  $\mu\text{M}$ ), cysteine (Cys, 200  $\mu\text{M}$ ), *N*-acetylcysteine (NCys, 200  $\mu\text{M}$ ) and  $\text{H}_2\text{S}$  ( $\text{Na}_2\text{S}$ , 200  $\mu\text{M}$ ). (b) Time-course fluorescence intensity at  $\lambda_{\text{em}} = 451$  nm of 13  $\mu\text{M}$  probe **Tz-(Umb)<sub>2</sub>** towards 20  $\mu\text{M}$   $\text{H}_2\text{S}$  in  $\text{H}_2\text{O}/\text{DMSO}$  (92:8).

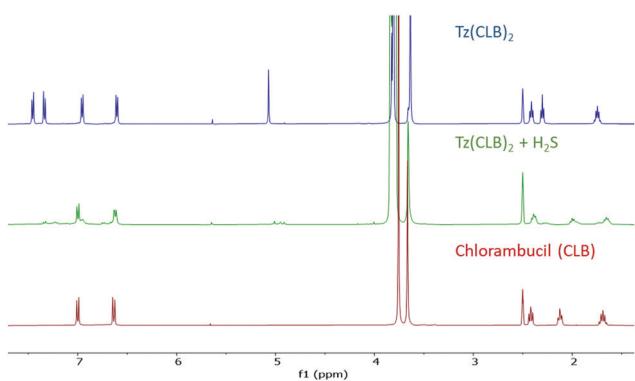


Fig. 3 Stacked <sup>1</sup>H NMR spectra of **Tz(CLB)<sub>2</sub>** before (top spectrum in blue) and after addition of  $\text{H}_2\text{S}$  in  $\text{DMSO-d}_6/\text{D}_2\text{O}$  (9:1) (middle spectrum in green), compared to the chlorambucil spectrum (bottom in red).

umbelliferone molecules were linked through Mitsunobu reactions, giving **Tz(CLB)<sub>2</sub>** and **Tz(Umb)<sub>2</sub>**, respectively. The theranostic compound **Tz(CLB)(Umb)** was synthesized by performing a sequential Mitsunobu reaction, attaching

chlorambucil first (5), followed by the umbelliferone. Finally, as a control compound, an Inverse Electron Demand Diels Alder (IEDDA) reaction was carried out on compound **Tz(CLB)<sub>2</sub>** (Scheme 2). Indeed, it has been proved that IEDDA reaction cancels the exchange, as the resulting diazine ring is electron richer.<sup>8,9</sup> Therefore, the prodrug should be deactivated by such Diels Alder reaction, and no release is expected in the analogous diazine compound. In order to keep the size and geometry as similar as possible, IEDDA reaction was performed with strained alkene 6, which after elimination of nitrogen, followed by a retro-Diels Alder reaction, yields diazine **Dz(CLB)<sub>2</sub>** where the only difference with respect to **Tz(CLB)<sub>2</sub>** is a substitution of two nitrogen atoms for two C–H groups.

Then, we tested the  $\text{H}_2\text{S}$  induced release with **Tz(Umb)<sub>2</sub>**, as it could be followed by the increasing fluorescence of the reaction mixture due to the released umbelliferone. We employed a solution of  $\text{Na}_2\text{S}$  in water, as it is a commonly used surrogate of  $\text{H}_2\text{S}$ . As expected, while the original compound shows very little fluorescence, an aqueous solution of  $\text{Na}_2\text{S}$  induces a ten-fold increase in the fluorescence (Fig. 2a), in a very fast way (Fig. 2b).

Fortunately, any other biothiol tested in the same conditions (cysteine, *N*-acetylcysteine and glutathione) causes no remarkable increase in the fluorescence of **Tz(Umb)<sub>2</sub>** (Fig. 2a).<sup>15</sup> The release could also be followed by NMR with compound **Tz(CLB)<sub>2</sub>**: upon addition of a solution of  $\text{Na}_2\text{S}$  in  $\text{D}_2\text{O}$ , free chlorambucil is clearly seen in the spectrum (Fig. 3).

Finally, the cytotoxicity of all the compounds was investigated *in vitro* by testing the antiproliferative activities against three human cell lines: on one hand HCT-116, a human colorectal carcinoma cell line in which enzyme CBS is overexpressed and therefore there is a high intracellular concentration of  $\text{H}_2\text{S}$ ,<sup>18</sup> on the other hand, MRC-5 (non-malignant lung fibroblasts-like) that displays normal levels of hydrogen sulfide, and can also be considered as a test for selective toxicity between cancer and normal cells; and finally SK-OV3 (human ovarian carcinoma).<sup>25–27</sup> As expected, (Table 1, entry 1), compound **Tz(CLB)<sub>2</sub>** is only active against HCT-116 ( $\text{IC}_{50} = 3.72 \mu\text{M}$ ) while it shows no cytotoxicity with the other two cell lines ( $\text{IC}_{50} > 50 \mu\text{M}$ ).<sup>28</sup> This selective performance is also evident under microscope observation (see Fig. S19 in the ESI†). Furthermore, compound **Dz(CLB)<sub>2</sub>**, bearing a diazine instead of a tetrazine, was not active against any of the cell lines, not even HCT-116 (Table 1, entry 2). Therefore, it has been also proved that the dynamic behaviour of the tetrazine is essential

Table 1 Antiproliferative activity of **Tz(CLB)<sub>2</sub>**, **Dz(CLB)<sub>2</sub>**, **Tz(Umb)<sub>2</sub>** and **Tz(CLB)(Umb)**.<sup>a</sup>

Entry	Compound	Cell line, $\text{IC}_{50}$ ( $\mu\text{M}$ )		
		SK-OV3	HCT-116	MRC-5
1	<b>Tz(CLB)<sub>2</sub></b>	>50	$3.72 \pm 1.90$	>50
2	<b>Dz(CLB)<sub>2</sub></b>	>50	>50	>50
3	<b>Tz(Umb)<sub>2</sub></b>	>50	$19.45 \pm 2.18$	>50
4	<b>Tz(CLB)(Umb)</b>	>50	$36.50 \pm 2.61$	>50

<sup>a</sup> For a detailed experimental procedure of the antiproliferative assays, please see the ESI.

for a proper bioactivity and, as a consequence, IEDDA reaction prevents drug-release. Compounds bearing coumarins were again only active against HCT-116 although to a lesser extent than **Tz(CLB)<sub>2</sub>** (Table 1, entries 3 and 4). Curiously, **Tz(Umb)<sub>2</sub>** is more cytotoxic than **Tz(CLB)(Umb)**, even when the latter contains one molecule of chlorambucil. In this regard, it is worth mentioning that some coumarins have been reported to show cytotoxicity against HCT-116 cells.<sup>29</sup> It also has to be considered that the release of the attached compounds concomitantly delivers a methylenquinone (Fig. 1c), that has been reported to deplete glutathione levels in the cell,<sup>30</sup> and therefore it probably contributes to the cytotoxicity of the tetrazine prodrugs.<sup>31</sup> Further studies will be required to better understand the reasons underlying cytotoxicity.

In conclusion, a novel method for a controlled drug release based on the dynamic chemistry of tetrazines has been described herein. It is selectively triggered by H<sub>2</sub>S and consequently very relevant for colon cancer and any other disease where there is a high concentration of H<sub>2</sub>S. Fluorescent compounds can also be released and consequently this method shows great potential for the development of H<sub>2</sub>S sensors, chemiluminescent detection,<sup>32,33</sup> and theranostic compounds. Obviously, this strategy is modular, and therefore other cytotoxic compounds and luminescent probes could be incorporated. Tetrazine responsive systems can also easily grow in complexity and in sophistication and therefore they could probably be very helpful in the development of innovative sensing and therapeutic solutions.

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## Conflicts of interest

There are no conflicts to declare.

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