



Cite this: *Chem. Commun.*, 2022, 58, 5518

Received 25th February 2022,
Accepted 8th April 2022

DOI: 10.1039/d2cc01170k

rsc.li/chemcomm

Dynamic nucleophilic aromatic substitution of tetrazines (S_NTz) 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.¹ 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,² 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.³ 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.^{4–7} 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

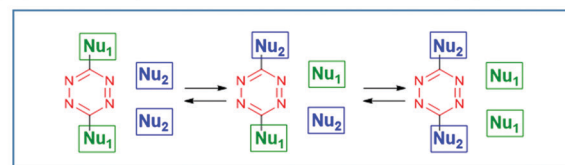
Hydrogen sulphide-triggered theranostic prodrugs based on the dynamic chemistry of tetrazines†

Marcelle D. Perretti,^{‡,ab} Yaiza Pérez-Pérez,^{‡,a} Kevin Soler-Carracedo,^c Endika Martín-Encinas,^d Concepción Alonso,^{id} *^d Jimena Scoccia^{id} *^b and Romen Carrillo^{id} *^{ab}

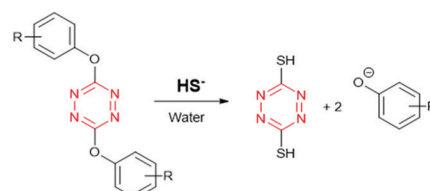
tetrazines (S_NTz , Fig. 1a).^{8–10} This reaction quickly exchanges phenols and thiols in positions 3 and 6 of the tetrazine ring, at

Previous Works:

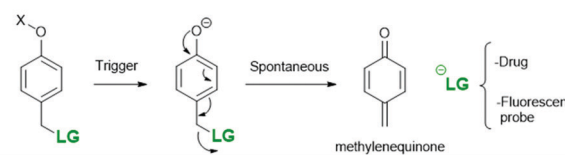
a) Dynamic Nucleophilic Aromatic Substitution of Tetrazines (S_NTz)



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:

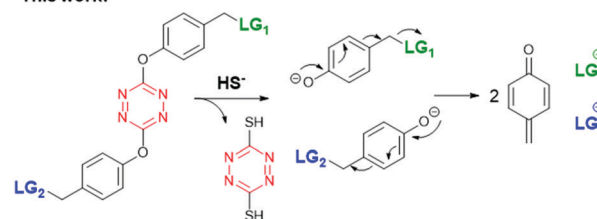


Fig. 1 (a) In previous works, the dynamic nature of the aromatic substitution of tetrazines has been reported. (b) Due to such dynamic behaviour, hydrogen sulphide in water is able to irreversibly displace any phenol from the tetrazine ring. (c) It is also well known that some phenols can act as self-immolative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

^a Instituto de Productos Naturales y Agrobiología (IPNA-CSIC), Avenida Astrofísico Francisco Sánchez 3, 38206, La Laguna, Spain. E-mail: rcarrillo@ipna.csic.es

^b Instituto Universitario de Bio-Organica "Antonio González", Universidad de La Laguna, Avenida Astrofísico Francisco Sánchez 2, P.O. Box 456, 38200, La Laguna, Spain. E-mail: jscoccia@ull.es

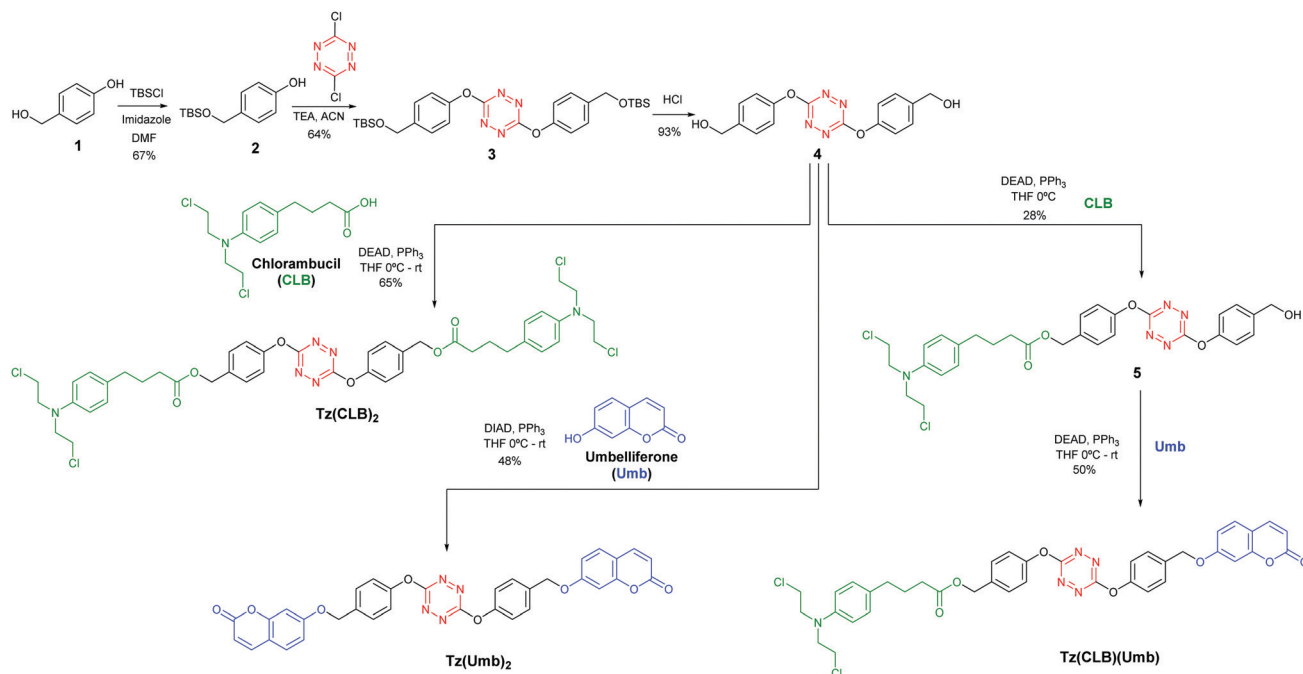
^c Departamento de Física, Universidad de La Laguna, Apdo. 456, E-38200, San Cristóbal de La Laguna, Santa Cruz de Tenerife, Spain

^d Department of Organic Chemistry I, Faculty of Pharmacy and Lascaray Research Center, University of Basque Country (UPV/EHU), 01006 Vitoria-Gasteiz, Spain. E-mail: concepcion.alonso@ehu.es

† Electronic supplementary information (ESI) available: Synthetic procedures, characterization of the compounds, cell cultures and *in vitro* screenings. See DOI: <https://doi.org/10.1039/d2cc01170k>

‡ These authors contributed equally.





Scheme 1 Synthetic pathway to obtain the tetrazine responsive systems.

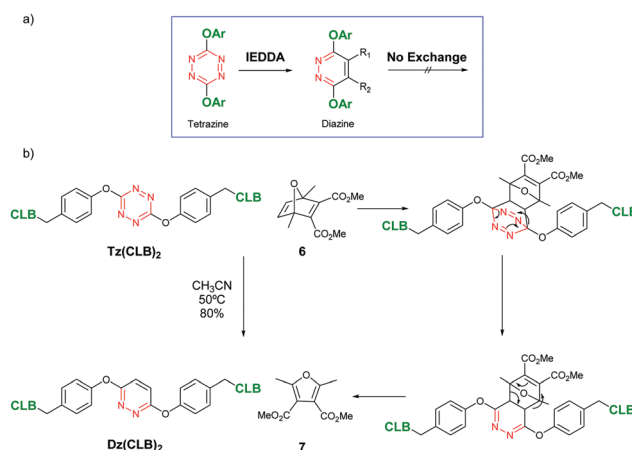
room temperature and it is compatible with aqueous environments.

We discovered that thiols efficiently release any phenol quantitatively,⁸ 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).⁹ 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.^{11–17} 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 cystathionine beta synthase (CBS).¹⁸ 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.^{19–21} Indeed, self-immolation has been extensively used as an efficient drug-releasing method.²² 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;²³ 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.²⁴

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.



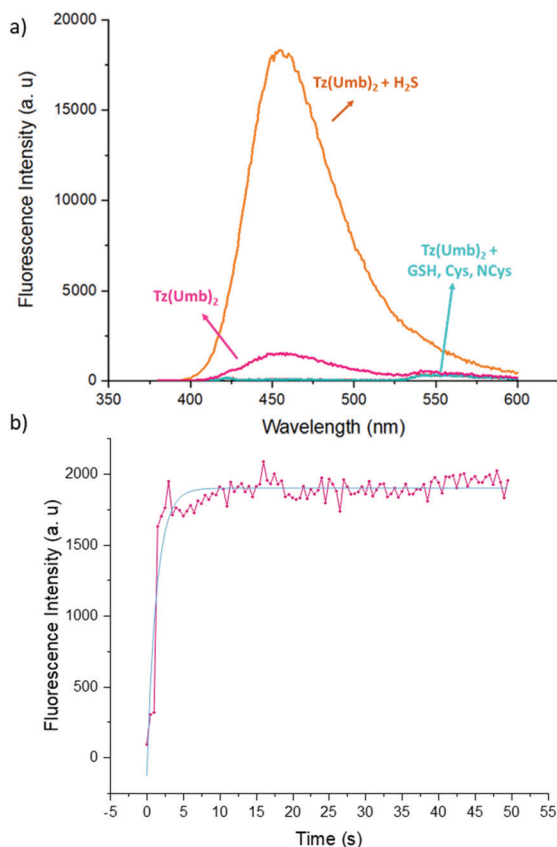


Fig. 2 (a) Relative fluorescence intensity at $\lambda_{\text{ex}} = 360$ nm of probe **Tz-(Umb)₂** (10 μM) with various species (200 μM) at 37 $^{\circ}\text{C}$ in $\text{H}_2\text{O}/\text{DMSO}$ (92:8). Glutathione (GSH, 200 μM), cysteine (Cys, 200 μM), *N*-acetylcysteine (NCys, 200 μM) and H_2S (Na_2S , 200 μM). (b) Time-course fluorescence intensity at $\lambda_{\text{em}} = 451$ nm of 13 μM probe **Tz-(Umb)₂** towards 20 μM H_2S in $\text{H}_2\text{O}/\text{DMSO}$ (92:8).

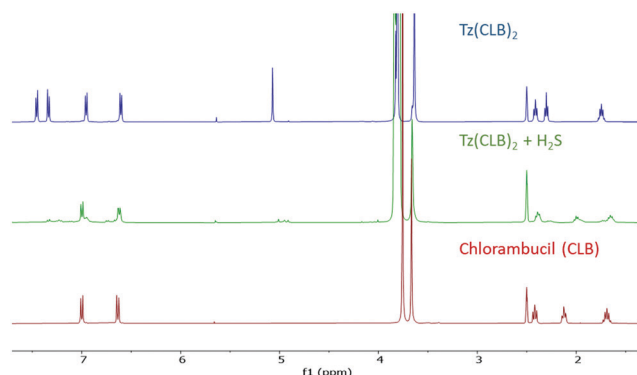


Fig. 3 Stacked ^1H NMR spectra of **Tz(CLB)₂** before (top spectrum in blue) and after addition of H_2S in $\text{DMSO}-d_6:\text{D}_2\text{O}$ (9:1) (middle spectrum in green), compared to the chlorambucil spectrum (bottom in red).

umbelliferone molecules were linked through Mitsunobu reactions, giving **Tz(CLB)₂** and **Tz(Umb)₂**, 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)₂** (Scheme 2). Indeed, it has been proved that IEDDA reaction cancels the exchange, as the resulting diazine ring is electron richer.^{8,9} 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)₂** where the only difference with respect to **Tz(CLB)₂** is a substitution of two nitrogen atoms for two C–H groups.

Then, we tested the H_2S induced release with **Tz(Umb)₂**, as it could be followed by the increasing fluorescence of the reaction mixture due to the released umbelliferone. We employed a solution of Na_2S in water, as it is a commonly used surrogate of H_2S . As expected, while the original compound shows very little fluorescence, an aqueous solution of Na_2S 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)₂** (Fig. 2a).¹⁵ The release could also be followed by NMR with compound **Tz(CLB)₂**: upon addition of a solution of Na_2S in D_2O , 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 H_2S ;¹⁸ 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).^{25–27} As expected, (Table 1, entry 1), compound **Tz(CLB)₂** 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 ($\text{IC}_{50} > 50 \mu\text{M}$).²⁸ This selective performance is also evident under microscope observation (see Fig. S19 in the ESI†). Furthermore, compound **Dz(CLB)₂**, 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)₂**, **Dz(CLB)₂**, **Tz(Umb)₂** and **Tz(CLB)(Umb)**.^a

Entry	Compound	Cell line, IC_{50} (μM)		
		SK-OV3	HCT-116	MRC-5
1	Tz(CLB)₂	> 50	3.72 ± 1.90	> 50
2	Dz(CLB)₂	> 50	> 50	> 50
3	Tz(Umb)₂	> 50	19.45 ± 2.18	> 50
4	Tz(CLB)(Umb)	> 50	36.50 ± 2.61	> 50

^a 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)**₂ (Table 1, entries 3 and 4). Curiously, **Tz(Umb)**₂ 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.²⁹ 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,³⁰ and therefore it probably contributes to the cytotoxicity of the tetrazine prodrugs.³¹ 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 H₂S and consequently very relevant for colon cancer and any other disease where there is a high concentration of H₂S. Fluorescent compounds can also be released and consequently this method shows great potential for the development of H₂S sensors, chemiluminescent detection,^{32,33} 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.

This work was financially supported by Ministerio de Ciencia e Innovación (PGC2018-094503-B-C21). J.S. thanks Cabildo de Tenerife for the Agustín de Betancourt programme. M.D.P. thanks the ACIISI and the European Social Fund (ESF) for a predoctoral grant.

Conflicts of interest

There are no conflicts to declare.

Notes and references

- 1 P. T. Wong and S. K. Choi, *Chem. Rev.*, 2015, **115**, 3388; M. Srinivasarao and P. S. Low, *Chem. Rev.*, 2017, **117**, 12133; E. A. Ashley, *Nat. Rev. Genet.*, 2016, **17**, 507.
- 2 J. Shi, P. W. Kantoff, R. Wooster and O. C. Farokhzad, *Nat. Rev. Cancer*, 2017, **17**, 20.
- 3 G. Kroemer and J. Pouyssegur, *Cancer Cell*, 2008, **13**, 472; W. C. Hahn and R. A. Weinberg, *Nat. Rev. Cancer*, 2002, **2**, 331.
- 4 S. J. Rowan, S. J. Cantrill, G. R. L. Cousins, J. K. M. Sanders and J. F. Stoddart, *Angew. Chem., Int. Ed.*, 2002, **41**, 898.
- 5 *Dynamic Covalent Chemistry: Principles, Reactions, and Applications*, ed. W. Zhang and Y. Jin, Wiley, Hoboken, 2018.
- 6 Y. Jin, C. Yu, R. J. Denman and W. Zhang, *Chem. Soc. Rev.*, 2013, **42**, 6634.
- 7 P. T. Corbett, J. Leclaire, L. Vial, K. R. West, J. L. Wietor, J. K. M. Sanders and S. Otto, *Chem. Rev.*, 2006, **106**, 3652.
- 8 T. Santos, D. S. Rivero, Y. Pérez-Pérez, E. Martín-Encinas, J. Pasán, A. H. Daranas and R. Carrillo, *Angew. Chem., Int. Ed.*, 2021, **60**, 18783.
- 9 D. S. Rivero, R. E. Paiva-Feener, T. Santos, E. Martín-Encinas and R. Carrillo, *Macromolecules*, 2021, **54**, 10428.
- 10 It has not been proved yet, although it is very likely that mono-substituted tetrazines also display a dynamic behaviour. See: S. D. Schnell, L. V. Hoff, A. Panchagnula, M. H. H. Wurzenberger, T. M. Klapötke, S. Sieber, A. Linden and K. Gademann, *Chem. Sci.*, 2020, **11**, 3042; W. Mao, W. Chi, X. He, C. Wang, X. Wang, H. Yang, X. Liu and H. Wu, *Angew. Chem., Int. Ed.*, 2022, **61**, e202117386.
- 11 M. D. Hartle and M. D. Pluth, *Chem. Soc. Rev.*, 2016, **45**, 6108.
- 12 A. K. Steiger, S. Pardue, C. G. Kevil and M. D. Pluth, *J. Am. Chem. Soc.*, 2016, **138**, 7256.
- 13 E. J. Mitchell, A. J. Beecroft, J. Martin, S. Thompson, I. Marques, V. Félix and P. D. Beer, *Angew. Chem., Int. Ed.*, 2021, **60**, 24048.
- 14 X. Wang, L. An, Q. Tian and K. Cui, *RSC Adv.*, 2019, **9**, 33578.
- 15 I. Ismail, Z. Chen, L. Sun, X. Ji, H. Ye, X. Kang, H. Huang, H. Song, S. G. Bolton, Z. Xi, M. D. Pluth and L. Yi, *Chem. Sci.*, 2020, **11**, 7823; K. Zhang, J. Zhang, Z. Xi, L. Y. Li, X. Gu, Q. Z. Zhang and L. Yi, *Chem. Sci.*, 2017, **8**, 2776.
- 16 N. Singh, S. Sharma, R. Singh, S. Rajput, N. Chattopadhyay, D. Tewari, K. B. Joshi and S. Verma, *Chem. Sci.*, 2021, **12**, 16085.
- 17 N. Singh, R. Singh, M. Shukla, G. Kaul, S. Chopra, K. B. Joshi and S. Verma, *ACS Infect. Dis.*, 2020, **6**, 2441.
- 18 C. Szabo, C. Coletta, C. Chao, K. Módis, B. Szczesny, A. Papapetropoulos and M. R. Hellmich, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 12474; M. R. Hellmich, C. Coletta, C. Chao and C. Szabo, *Antioxid. Redox Signaling*, 2015, **22**, 424.
- 19 M. Fernández, A. Shamsabadi and V. Chudasama, *Chem. Commun.*, 2020, **56**, 1125.
- 20 Y. Yang, T. Zhou, M. Jin, K. Zhou, D. Liu, X. Li, F. Huo, W. Li and C. Yin, *J. Am. Chem. Soc.*, 2020, **142**, 1614.
- 21 A. Dal Corso, V. Borlandelli, C. Corno, P. Perego, L. Belvisi, L. Pignataro and C. Gennari, *Angew. Chem., Int. Ed.*, 2020, **59**, 4176.
- 22 M. E. Roth, O. Green, S. Gnaim and D. Shabat, *Chem. Rev.*, 2016, **116**, 1309.
- 23 R. T. Dorr and W. L. Fritz, *Cancer Chemotherapy Handbook*, Elsevier Science, New York, 1982.
- 24 R. Weinstein, E. Segal, R. Satchi-Fainaro and D. Shabat, *Chem. Commun.*, 2010, **46**, 553.
- 25 R. C. Bast Jr., B. Hennessy and G. B. Mills, *Nat. Rev. Cancer*, 2009, **9**, 415.
- 26 X. Yang and M. Pagé, *Oncol. Res.*, 1995, **7**, 619.
- 27 S. Bhattacharyya, S. Saha, K. Giri, I. R. Lanza, K. S. Nair, N. B. Jennings, C. Rodriguez-Aguayo, G. López-Berestein, E. Basal, A. L. Weaver, D. W. Visscher, W. Cliby, A. K. Sood, R. Bhattacharya and P. Mukherjee, *PLoS One*, 2013, **8**, e79167.
- 28 CLB shows no cytotoxicity with any of the cell lines tested, as it is seen in Table S1 (ESI[†]). Indeed, CLB is usually only active when an improved internalization takes place. See: P. Huang, D. Wang, Y. Su, W. Huang, Y. Zhou, D. Cui, X. Zhu and D. Yan, *J. Am. Chem. Soc.*, 2014, **136**, 11748.
- 29 N. E. B. Saidu, S. Valente, E. Bana, G. Kirsch, D. Bagrel and M. Montenarh, *Bioorg. Med. Chem.*, 2012, **20**, 1584.
- 30 H. Hagen, P. Marzenell, E. Jentzsch, F. Wenz, M. R. Veldwijk and A. Mokhir, *J. Med. Chem.*, 2012, **55**, 924; J. Noh, B. Kwon, E. Han, M. Park, W. Yang, W. Cho, W. Yoo, G. Khang and D. Lee, *Nat. Commun.*, 2015, **6**, 6907.
- 31 A dimercaptotetrazine is also released, that might enhance the global effect of the prodrug, only when activated.
- 32 S. P. Gholap, C. Yao, O. Green, M. Babjak, P. Jakubec, T. Malatinský, J. Ihssen, L. Wick, U. Spitz and D. Shabat, *Bioconjugate Chem.*, 2021, **32**, 991; T. S. Bailey and M. D. Pluth, *J. Am. Chem. Soc.*, 2013, **135**, 16697.
- 33 Y. Chen, R. Zhao, C. Tang, C. Zhang, W. Xu, L. Wu, Y. Wang, D. Ye and Y. Liang, *Angew. Chem., Int. Ed.*, 2022, **61**, e202112734.

