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Design and synthesis of releasable folate–drug conjugates using a novel heterobifunctional disulfide-containing linker

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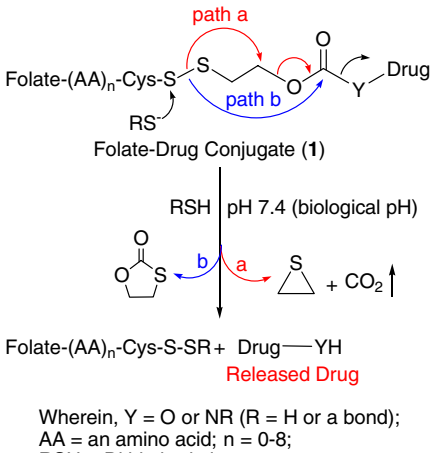
Abstract—Cellular uptake of vitamin folic acid occurs via folate-receptor mediated endocytosis. Many types of cancer cells express high levels of folate receptors as they need continuous supply of this vitamin for their proliferation. With an objective to use folic acid as a 'Trojan Horse' to transport anticancer drugs into cancer cells, a novel heterobifunctional disulfide-containing linker was synthesized and utilized to covalently link an amino- and hydroxyl-containing anticancer drug, and an appropriately functionalized folic acid to create novel targetable folate–drug conjugates that are shown to release free drugs under biologically relevant pH via sulfhydryl-assisted cleavage of the self-immolative disulfide-containing linker.

Tumor cells use unique defensive pathways for their survival and proliferation. This very intrinsic property of cancer cells offers great opportunity for identification of molecular targets or pathways that can be utilized to achieve targeted delivery of anticancer drugs selectively into cancer cells. Some of the unique characteristics of cancer cells that have been exploited include: (1) Highly proliferating cancer cells need continuous supply of vitamins and tend to express high levels of vitamin receptors on their cell surfaces. For instance, vitamin folic acid is transported into cells via receptor-mediated endocytosis.[1,2] Because of that reason, folic acid has been successfully exploited as a 'Trojan horse' to transport anticancer drugs selectively into cancer cells;[3] (2) Cancer cells express high levels of detoxifying enzymes such as glutathione-*S*-transferases (GSTs)[4] and glutathione (GSH) to protect themselves from toxic xenobiotics including therapeutic agents. In our earlier work, we have successfully exploited this intrinsic property of cancer cells to our advantage by designing isozyme selective GST inhibitors[5] and also GST-activated latent alkylating agents[6,7] that are currently in clinical trials; and (3) Higher levels of glutathione present in cancer cells make them resistant to chemotherapeutic agents and this particular condition of cancer cells has been exploited by designing novel molecules that are activated selectively by glutathione to release lethal amounts of nitric oxide (NO) intracellularly thereby selectively killing those cancer cells.[8]

To exploit some of the above-mentioned inherent properties of cancer cells to our advantage and also to achieve targeted delivery of effective amounts of chemotherapeutic agents selectively into cancer cells, we have designed and synthesized novel folate–drug conjugates of the general structure **1** (Scheme 1).

The folic acid moiety in the conjugates **1** is expected to not only act as a necessary vitamin for the proliferating cells



Scheme 1. Proposed mechanism of drug release.

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but also serve as a targeting ligand to transport the appended drug selectively in to cancer cells via receptor-mediated endocytosis. The spacer portion consists of a bond or a peptide made of 0–8 suitable amino acid residues and a free sulfhydryl-containing terminal residue such as cysteine and the spacer is expected to impart desirable physicochemical properties such as affinity and water-solubility, to the resulting conjugate **1**. The folate–drug conjugates **1** also contain a novel self-immolative and drug releasable disulfide-containing linker[9,10] as an integral part of their structure. The drug portion of the conjugate **1** clearly illustrates the broad scope of this linker technology where any (primary or secondary) amino- or (primary, secondary, tertiary, or phenolic) hydroxyl-containing drug can be covalently coupled to the linker to generate the corresponding releasable folate–drug conjugates of the general structure **1**. Thus, these folate–drug conjugates **1** are expected to be selectively internalized by the cancer cells having high levels of folate receptors and release lethal amounts of free drug intracellularly by the action of sulfhydryl-containing species such as GSH on the disulfide-containing folate–drug conjugate via a probable mechanism as proposed in Scheme 1.

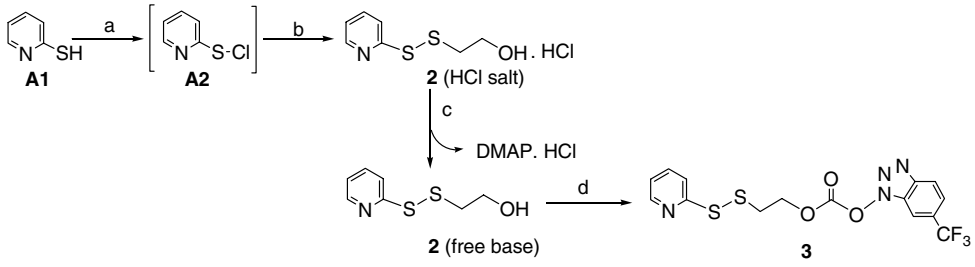
It was a challenging objective for us to develop linkers that would release free drugs intracellularly once they get internalized by the cancer cells. After considerable experimentation, we have successfully met this objective by rationally designing, synthesizing, and utilizing a novel heterobifunctional linker **3** and those preliminary results are reported here. As shown in the Scheme 2, we have synthesized the linker **3** by reacting 2-(2-hydroxyethyldithio)-pyridine (**2**)[11] with 1,1-bis [6-(trifluoromethyl)benzotriazolyl]-carbonate (BTBC).[12] Obviously, utilization of BTBC in the synthesis of **3** eliminates the use of highly toxic phosgene.

Thus, we have successfully utilized the novel heterobifunctional linker **3** to covalently link an (both primary and secondary) amino-, a (primary, secondary, or phenolic) hydroxyl-containing drug to one side of the linker to generate drug-linker intermediate **4** (Scheme 3).

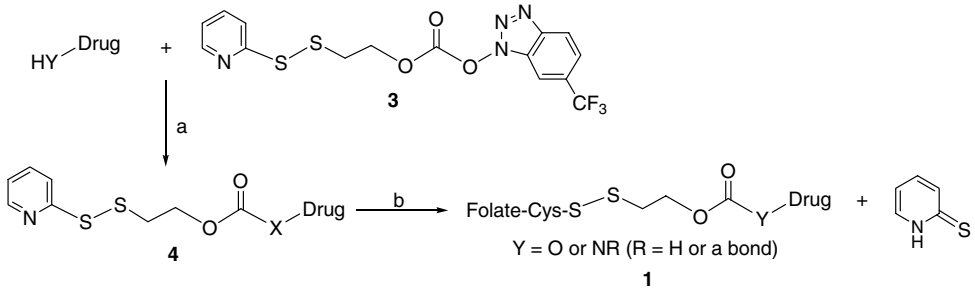
Because of its facile reaction even with secondary alcohols and phenols,[9] we often called this heterobifunctional linker **3** as 'Magic Carbonate'! Thus, as shown in Scheme 3 and Figure 1, the drug-linker intermediates **4** were made by reacting the heterobifunctional linker **3** with an amino-containing or hydroxyl-containing drugs. Further reaction of the intermediates **4** with an appropriately functionalized Folate-Cys-OH (**5**) containing a terminal sulfhydryl group (most often a cysteine residue) gave the folate–drug conjugates **1**.

This novel linker technology was demonstrated by making a few representative examples. Thus, the reaction of an amino-containing drug such as daunomycin gave the corresponding drug-linker intermediate **4a** (Fig. 1), which upon reaction with Folate-Cys-OH (**5**) yielded the desired folate–daunomycin conjugate **1a** (Fig. 2).

As shown in Scheme 4, synthesis of the paclitaxel-linker intermediate **4b** was performed via transient protection and deprotection of 2′-hydroxyl group of paclitaxel. Thus, the more reactive 2′-hydroxyl group of paclitaxel was selectively protected first with the allyloxycarbonyl (Alloc) group to give (2-*O*-Alloc)paclitaxel **6**, which was then treated with the linker **3** to yield the fully protected paclitaxel-linker derivative **7**. Finally, palladium-catalyzed removal of Alloc protecting group from the intermediate **7** yielded the desired paclitaxel-linker intermediate **4b**.



Scheme 2. Reagents and conditions: (a) SO_2Cl_2, DCM, 0–5 °C to rt, quant.; (b) 2-mercaptoethanol (**A3**), DCM, 0–5 °C to rt, 79.4%; (c) dimethylaminopyridine (DMAP), DCM, chromatography on silica gel, 90–95%; (d) 1,1-bis [6-(trifluoromethyl)benzotriazolyl]-carbonate (BTBC), acetonitrile, rt, 81%.



Scheme 3. Reagents and conditions: (a) triethylamine/DMAP, acetonitrile, 0 °C to rt, 51–81%; (b) Folate-Cys-OH (5), acetonitrile, 0.5–1.0 N sodium bicarbonate, water, rt, 30–50%.

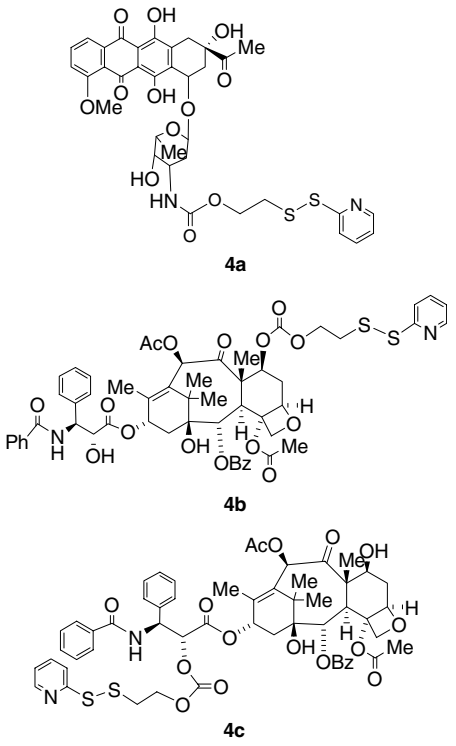


Figure 1. Structures of intermediates **4a–c**.

Similarly, direct reaction of the linker **3** with paclitaxel selectively gave the drug-linker intermediate **4c** (Fig. 1) as the major product. The minor product from the reaction was identified as the disubstituted paclitaxel derivative where both the 2′-hydroxyl and 7-hydroxyl groups of paclitaxel were derivatized with the linkers.

Further reaction of the intermediate **4b** with Folate-Cys-OH (**5**) gave the expected folate–paclitaxel conjugates **1b** (Fig. 2). The Folate-Cys-OH (**5**) was made by using the standard manual fluorenylmethyloxycarbonyl-based solid-phase peptide synthesis (FMOC-based SPPS) on Wang resin. We have synthesized and evaluated the folate–Cys(CO_2Me)–paclitaxel conjugate **1c** by an alternative method starting from the paclitaxel-linker intermediate **4c** and Folic acid-OSu (**9**)[13,14] as shown in Scheme 5.

As a 'Proof of the Principle', all the three folate–drug conjugates **1a–c** released their respective free drugs upon treatment with sulfhydryl-containing compounds such as dithiothreitol (DTT) or dithioerythritol (DTE) or glutathione (GSH) at biologically relevant pH of ~7.4. This drug release study was monitored by reverse-phase HPLC.[15]

Interestingly, a few promising preclinical[16,17] and clinical[18,19] candidates have already been identified by the

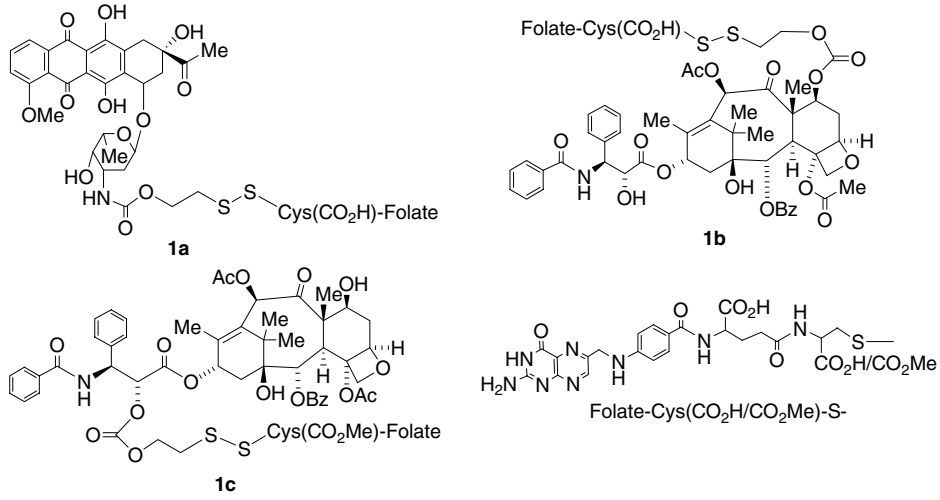
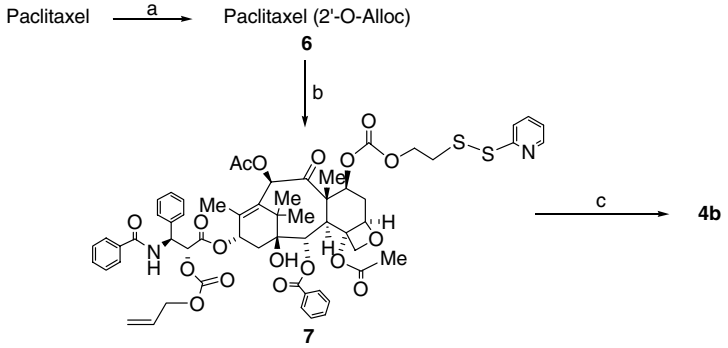
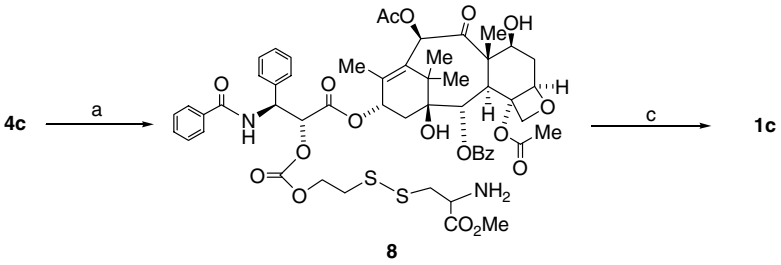


Figure 2. Structures of folate–drug conjugates **1a–c**.



Scheme 4. Reagents and conditions: (a) allyloxycarbonyl chloride, diisopropylethyl amine, DCM, 0–5 °C, 98%; (b) 3, DMAP, acetonitrile, reflux, 58%; (c) diethylamine, Pd (PPh_3)$_4$, THF, rt, 64%.



Scheme 5. Reagents and conditions: (a) H-Cys-OMe.HCl, 1 N $NaHCO_3$, acetonitrile, water, pH 7.5–8.0, rt, 59%; (b) folic acid-OSu (9),[13,14] *N*-methylmorpholine, DMSO, rt, 14.8%.

application of this novel disulfide-carbonate linker technology, which can be fine tuned[10] further for improving stability of the conjugates as well as achieving controlled/sustained release of free drugs from the conjugates. We also made a Folate-*seo*-CBI analog (a phenolic hydroxyl-containing *seo*-CBI[20] as an example), a folate–Normustard conjugate, and a folate–NBD conjugate (as a model compound), and the data associated with these folate–drug conjugates[9] will be reported in due course.

In summary, we have designed and synthesized a novel heterobifunctional disulfide-containing linker **3** which could be conveniently reacted with representative amino-containing and hydroxyl (including phenolic hydroxyl)-containing therapeutic agents under mild conditions to generate the corresponding drug-linker intermediates **4**, which in turn could be successfully conjugated to sulfhydryl-containing folate derivatives **5** to give the corresponding folate–drug conjugates **1**. Thus, the heterobifunctional disulfide linker **3** has been proved to be a versatile reagent with a broad scope of applicability to generate targetable as well as releasable folate–drug conjugates that release free drugs at biologically relevant pH via sulfhydryl-assisted cleavage of self-immolative disulfide-containing linker.

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Supplementary data

Experimental procedures, analytical, and spectral data can be found, in the online version, at doi:10.1016/j.bmcl.2008.04.063.

References and notes

1. Kamen, B. A.; Capdevila, A. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 5983.
2. Rothberg, K. G.; Ying, Y.; Kolhouse, J. F.; Kamen, B. A.; Anderson, R. G. *J. Cell. Biol.* **1990**, *110*, 637.
3. For a recent review see: Leamon, C. P.; Reddy, J. A. *Adv. Drug Delivery Rev.* **2004**, *56*, 1127.
4. Howie, A. F.; Farrester, L. M.; Glancey, M. J.; Schlager, J. J.; Powis, G. G.; Beckett, G. J.; Hayes, J. D.; Wolf, C. R. *Carcinogenesis* **1990**, *11*, 451.
5. Lyttle, M. H.; Hocker, M. D.; Hui, H. C.; Caldwell, C. G.; Aaron, D. T.; Goldstein, A. E.; Flatgard, J. E.; Bauer, K. E. *J. Med. Chem.* **1994**, *37*, 189.
6. Satyam, A.; Hocker, M. D.; Kane-Maguire, K. A.; Morgan, A. S.; Villar, H. O.; Lyttle, M. H. *J. Med. Chem.* **1996**, *39*, 1736.
7. Lyttle, M. H.; Satyam, A.; Hocker, M. D.; Bauer, K. E.; Caldwell, C. G.; Hui, H. C.; Morgan, A. S.; Mergia, A.; Kauvar, L. M. *J. Med. Chem.* **1994**, *37*, 1501.
8. Saavedra, J. E.; Srinivasan, A.; Buzard, G. S.; Davies, K. M.; Waterhouse, D. J.; Inami, K.; Wilde, T. C.; Citro, M. L.; Cuellar, M.; Deschamps, J. R.; Parrish, D.; Shami, P. J.; Findlay, V. J.; Townsend, D. M.; Tew, K. D.; Singh, S.; Jia, L.; Ji, X.; Keefer, L. K. *J. Med. Chem.* **2006**, *49*, 1157.
9. Vlahov, I. R.; Leamon, C. P.; Parker, M. A.; Howard, S. J.; Santhapuram, H. K.; Satyam, A.; Reddy, J. A. US Patent Application No. 2005/0002942 A1 (10/765,336), 2005.
10. Jones, L. R.; Goun, E. A.; Shinde, R.; Rothbard, J. B.; Contag, C. H.; Wender, P. A. *J. Am. Chem. Soc.* **2006**, *128*, 6526.
11. Kaneko, T.; Willner, D.; Monkovic, I.; Knipe, J. O.; Braslawsky, G. R.; Greenfield, R. S.; Vyas, D. M. *Bioconjugate Chem.* **1991**, *2*, 133, Also, see supplementary material for alternative methods of synthesis of this compound as shown in Scheme 2.
12. Takeda, K.; Tsuboyama, K.; Hoshino, M.; Kishino, M.; Ogura, H. *Synthesis* **1987**, 557.
13. Leamon, C. P.; Low, P. S. S. *J. Biol. Chem.* **1993**, *268*, 24847.
14. Santos, M. A.; Enyedy, E. A.; Nuti, E.; Rossello, A.; Krupenko, N. I.; Krupenko, S. A. *Bioorg. Med. Chem.* **2007**, *15*, 1266.
15. Refer to supporting data for details on drug release studies.
16. Henne, W. A.; Doorneweerd, D. D.; Hilgenbrink, A. R.; Kularatne, S. A.; Low, P. S. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5350.
17. Vlahov, I. R.; Santhapuram, H. K.; Kleindl Wang, Y.; Kleindl, P. J.; You, F.; Howard, S. J.; Westrick, J. A.; Reddy, J. A.; Leamon, C. P. *J. Org. Chem.* **2007**, *72*, 5968.
18. Vlahov, I. R.; Santhapuram, H. K.; Kleindl, P. J.; Howard, S. J.; Stanford, K. M.; Leamon, C. P. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5093.
19. Leamon, C. P.; Reddy, J. A.; Vlahov, I. R.; Westrick, E.; Dawson, A.; Dorton, R.; Vetzel, M.; Santhapuram, H. K.; Wang, Y. *Mol. Pharmaceutics* **2007**, *4*, 659.
20. Boger, D. L.; Ishizaki, T.; Kitos, P. A.; Suntornwat, O. *J. Org. Chem.* **1990**, *55*, 5823.