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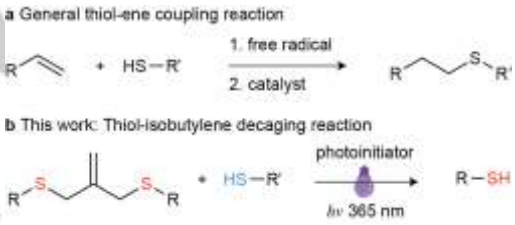
Radical-mediated thiol-ene strategy for photoactivation of thiol-containing drugs in cancer cells

Shuang Sun,[a] Bruno L. Oliveira,[a] Gonzalo Jiménez-Osés,[b] and Gonçalo J. L. Bernardes*[a,c]

Abstract: Photo-activated drugs provide an opportunity to improve efficacy alongside reducing side-effects in the treatment of severe diseases, such as cancer. Herein, we describe a photoactivation decaging method of isobutylene-caged thiols through a UV-initiated thiol-ene reaction. The method was demonstrated with an isobutylene-caged cysteine, cyclic disulfide-peptide and thiol-containing drug, all of which were rapidly and efficiently released under mild UV irradiation in the presence of thiol sources and a photoinitiator. Importantly, we show that the activity of histone deacetylase inhibitor Largazole can be switched-off when stapled, but selectively switched-on when irradiated with non-phototoxic light in cancer cells.

In recent decades, precision medicine has drawn a lot of attention for the effective treatment of various diseases, especially cancer. Currently, as a result of a lack of selectivity in the pathological sites, the development of new, effective and safe therapies remains challenging. Among various new methods, the recently developed light-mediated treatment is recognized as a promising approach to achieve controlled activation of medicine at pathological sites[1], which could significantly reduce the side effects of chemotherapy. So far, in the battle against cancer, several types of light-activated anti-cancer reagents, which can be switched on conditionally with irradiation, have been investigated[2]. The structures of these photocaged drugs include various ultraviolet, near infra-red or visible responsive moieties[3], such as *o*-nitrobenzyl[2a, 4], coumarinyl ester[5], metal complexes[2e, 6]. These photo-responsive structures offer an extensive toolbox for use in cancer therapies and other biological applications. However, issues and challenges remain in this field, such as using non-toxic wavelength, achieving rapid and efficient conversion and improving the bioavailability of the prodrug. Therefore, there is still demand for new designs and new developments for photo-mediated therapy.

Thiol-ene reactions (Scheme 1), a conjugation between a thiol and an alkene, have been known since the early 1900s[7]. The coupling reaction proceeds through two mechanisms, photo-initiated free-radical addition and catalyzed Michael addition reactions. There are several desirable features of a click reaction, rapid reaction rates, ease of implementation, high yields and rates of conversion[8], so thiol-ene 'click' reactions have been increasingly used for various applications, such as biofunctionalization[9], surface and polymer modification, polymerization[10], and so on[8, 11]. Recently, isobutylene-bridged polymer networks have been extensively studied to synthesize polymer networks through radical-initiated thiol-ene chemistry[12]. This covalently cross-linked network is able to undergo photo-mediated, reversible cleavage of its isobutylene backbone to allow chain rearrangement and relieve of structural strain. This method has also been used to provide a reactive handle for reversible addition and exchange of biochemical moieties under cytocompatible conditions[12d]. Key to this reaction is the isobutylene structure capable of addition-fragmentation chain transfer (AFCT), in which the structure is attacked by the photo-initiated thiol radical in the presence of a photoinitiator (PI) to release the caged thiol part.



Scheme 1. a. General thiol-ene coupling reaction; **b.** the thiol-isobutylene decaging reaction.

Inspired by this AFCT reaction, we hypothesized that the isobutylene structure could be used as a bridging graft to cage thiol-drugs and allow further controlled activation of anti-cancer drugs by means of a radical-mediated thiol-ene mechanism (Scheme 1). Previously, we reported a one-pot macrocyclization strategy [with tris(2-carboxyethyl)phosphine (TCEP)] for thiol-containing peptides by using an isobutylene graft, which can significantly enhance both membrane permeability and binding activity of the corresponding macrocycles[13]. The isobutylene graft can be rapidly and efficiently installed onto reduced thiol groups in a biocompatible manner because of the high reactivity of the bis-bromo isobutylene. Our research began with *N-tert*-butyloxycarbonyl(Boc)-L-cysteine **1**, which was protected with bis-bromo isobutylene (Table 1 and Supporting Information). Stapled cysteine **2** was then screened under a series of conditions. Firstly, different PIs were tested with the same amounts of thiol source, 1-thio-*β*-D-glucose tetraacetate, and stapled cysteine under irradiation at 365 nm. The reaction with water-soluble PI 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (Vazo 44) did not progress after 2 hours. However, the other PI, 2,2-dimethoxy-2-phenylacetophenone (DPAP) successfully promoted the reaction and released *N*-Boc-cysteine **1**. *β*-

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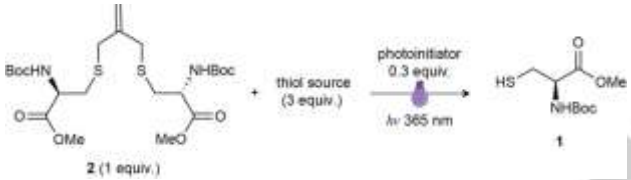
Mercaptoethanol (BME) was also tested as the thiol compound under the same conditions. This reaction released the cysteine in a slightly lower yield, which suggests that different thiol sources could be used to promote the reaction. However, when *N,N*-dimethylformamide (DMF) was used as solvent, two disulfides between the thiol-glucose and *N*-Boc-cysteine **1**, and *N*-Boc-cystine were observed. To evaluate the reaction and calculate the isolated yield, TCEP was added to reduce the disulfides after the reaction. Under these conditions, the reaction was complete within 15 mins and gave a relatively high yield (65%). Moreover, the reaction was also rapid and efficient when glutathione was used as a thiol source in a mixed solvent of DMF and water, which shows that the reaction is practical for an aqueous environment. Then, the feasibility of our strategy was investigated with an isobutylene cyclized 5-mer peptide that bears two terminal cysteines and synthesized by solid-phase peptide synthesis[13]. The short peptide was completely converted into the disulfide derivative within 15 mins under the same conditions described previously (Figure 1).

Table 1. Optimization of the thiol-ene decaging reaction.



Entry	PI	Thiol	Solvent	TCEP	Time (min)	Yield (%)
1	Vazo44	4AcGlcSH	MeOH	-	120	0
2	DPAP	4AcGlcSH	CH_2Cl_2	-	120	54
3	DPAP	BME	CH_2Cl_2	-	120	37
4	DPAP	4AcGlcSH	DMF	-	120	[a]
5	DPAP	4AcGlcSH	DMF	-	15	[a]
6	DPAP	4AcGlcSH	DMF	+	15	65
7	DPAP	GSH	DMF/H_2O 1/1	+	15	67

[a]A mixture of disulfides between cysteine and thiol-glucose and cystine. PI, Photoinitiator; 4AcGlcSH, 1-thio-*β*-D-glucose tetraacetate; BME, *β*-mercaptoethanol; GSH, glutathione.

To demonstrate that the release of the thiol compound is by means of a radical-mediated thiol-ene mechanism, a series of control experiments were conducted (Table 2 and Supporting Information). As shown in the table, a thiol source, UV irradiation and a PI are essential for the decaging reaction. When radical scavenger (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) was added to the reaction, it terminated the reaction by forming an intermediate with the DPAP fragmentation, which suggests that the reaction is mediated by radicals.

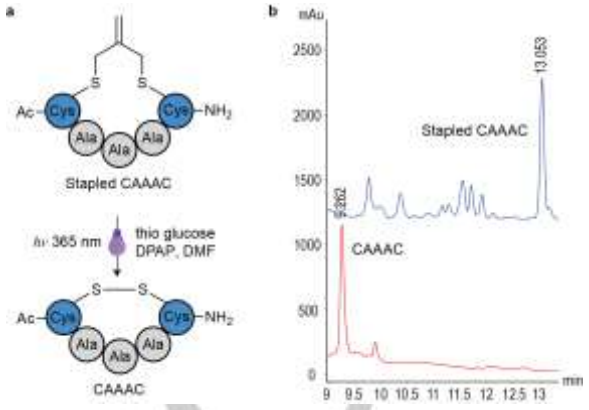


Figure 1. Thiol-ene decaging reaction of isobutylene-cyclized peptide. **a.** The decaging reaction of stapled CAAAC peptide with 1-thio-*β*-D-glucose tetraacetate. **b.** HPLC traces of the starting material and reaction mixture. Blue, stapled CAAAC peptide; Red, reaction mixture after 15 min.

The proposed mechanism for the radical-mediated thiol-ene decaging reaction was studied by Quantum Mechanical calculations using abbreviated thiol models (see Supporting Information) and is shown as Scheme 2. After generation of the thiol radical by the PI under UV irradiation, the isobutylene grafted structure undergoes a fast thiol-ene anti-Markovnikov addition with a calculated activation energy of $\Delta G^{\ddagger} \sim 14$ kcal mol^{-1} at the PCM(H_2O)/M06-2X/6-31++G(2,p) theory level, to generate a symmetric tertiary carbon-centered radical intermediate. Then, the unstable radical intermediate undergoes a *β*-scission at a very similar reaction rate regenerating the isobutylene linkage and resulting in a mixed caged compound, which can be again attacked by another thiol radical following the same mechanism to release the other unit of the caged thiol compound. The process is nearly thermoneutral and reversible until two decaged radical thiols collapse forming a stable disulfide bond (Scheme 2).

Table 2. Control studies of thiol-ene decaging reactions between isobutylene-grafted cysteine and thiol-glucose.

Entry	Thiol	Stapled Cys **2**	UV	DPAP	TEMPO	Conversion (%)
1	+	+	+	+	-	100
2	+	+	-	+	-	no reaction
3	+	-	+	+	-	no reaction
4	-	+	+	+	-	no reaction
5[a]	+	+	+	+	+	no reaction

[a]A mixture of disulfides between cysteine and thiol-glucose and cystine. PI, Photoinitiator; 4AcGlcSH, 1-thio-*β*-D-glucose tetraacetate; BME, *β*-mercaptoethanol; GSH, glutathione.

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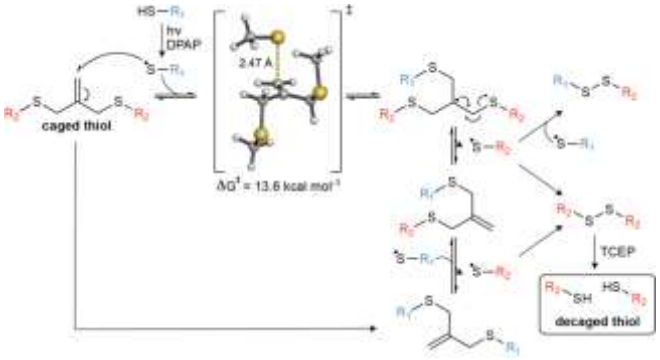

Scheme 2. Proposed mechanism of the radical-mediated thiol-isobutylene decaging reaction.

With all this knowledge in hand and to demonstrate that this strategy is practical to activate drugs *in vitro*, we applied our method to potent histone deacetylase inhibitor (HDAC) Largazole. Cyclic depsipeptide Largazole is a marine natural product, and its derivatives are recognized as promising potential anticancer therapeutics. Cyclic depsipeptide Largazole is a marine natural product, and its derivatives are recognized as promising potential anticancer therapeutics. Largazole possesses remarkable and preferential growth-inhibitory activity against cancer cell lines relative to corresponding non-transformed cells[14]. The octanoyl tail in Largazole has better cell-permeability than the active free thiol species, Largazole thiol, the latter of which is formed inside cells by esterase or lipase-based cleavage of the octanoyl residue[15]. The free thiol group binds to the active site Zn^{2+}-domain within the HDAC enzyme, which results in a potent inhibitory effect. Thus, the thiol-ene decaging strategy can be used to protect the thiol group, improve the cell-permeability and allow controlled activation with UV. Therefore, we synthesized three Largazole derivatives; Largazole, Largazole thiol and stapled Largazole (see Supporting Information). The result of the parallel artificial membrane permeability assay indicated that stapled Largazole is a highly passively permeable compound ($logP_e$ –5.29 and P_e $5.3x10^{-6}$ cm/sec; Supporting Information Table S4). Then, the stapled Largazole was reacted with 1-thio-*β*-D-glucose tetraacetate and DPAP under UV irradiation for 15 min (Figure 2a). Full conversion of the stapled Largazole was observed in the HPLC trace along with the appearance of Largazole thiol signal (Figure 2b). Next, the growth-inhibitory activity was evaluated with human colon carcinoma cell lines, HCT-116 (Figure 2c). As expected[16], Largazole (GI_{50} 1.433 nM) is more potent than the corresponding free thiol species (GI_{50} 185.1 nM) owing to its octanoyl side-chain which improves cell permeability and allows facile presentation of the free thiol within the cell[15]. The stapled Largazole (GI_{50} 407.7 nM) is a much less potent compound because the thiol group is protected by the isobutylene structure, which prevents binding with the Zn^{2+} domain in cells. Before testing the decaging condition in cells, we investigated the toxicity of DPAP and phototoxicity of the light in terms of the power and the irradiation time (see Supporting Information, Figure S7). A set of cytocompatible conditions, 15 min irradiation at 80 W, were chosen to conduct further investigations.

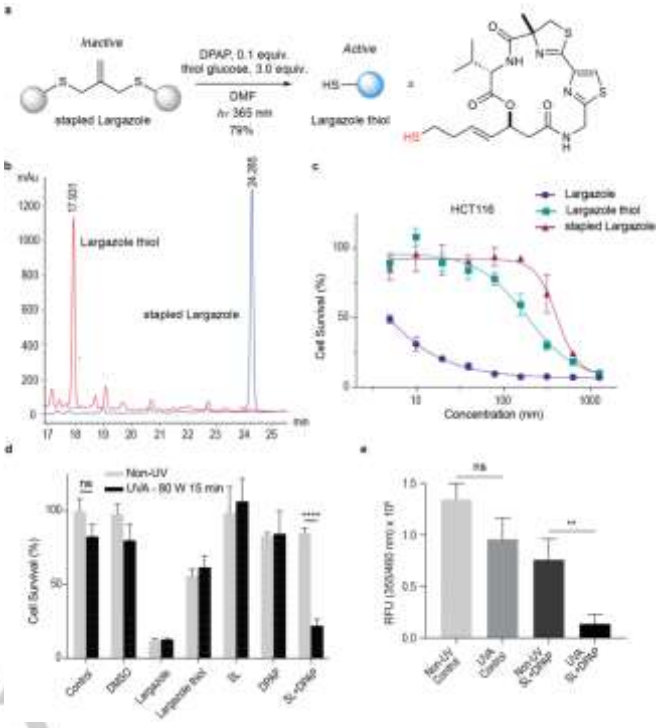

Figure 2. The photoactivation of isobutylene-grafted Largazole thiol. **a.** Thiol-ene decaging reaction of stapled Largazole with 1-thio-*β*-D-glucose tetraacetate. HPLC traces of the reaction mixture. Blue, stapled Largazole; Red, reaction mixture after 15 min. **b.** Growth inhibitory effects of Largazole, Largazole thiol and stapled Largazole on HCT-116 colon carcinoma cells, Largazole GI_{50} 1.433 nm, Largazole thiol GI_{50} 185.1 nm, stapled Largazole GI_{50} 407.7 nm. **c.** Cell survival under different conditions; SL, stapled Largazole, UV condition, 365 nm 80 W, 15 min. **d.** HDAC activity assay of control group and UV group. Data are representative of three independent tests and analyzed by the two-tailed unpaired Student's t-test. **, P < 0.01; ****, P < 0.0001. Error bars reflect one standard deviation from the mean.

The decaging reaction of stapled was tested with HCT-116 cells at 150 nM. McCoy's 5A culturing medium contains cysteine and glutathione, so no other thiol source was added to the medium. The cell viabilities of the three drug groups with/without UV irradiation were consistent with the growth-inhibitory assay (Figure 2d). The UV-irradiated premixed group of stapled Largazole and DPAP showed significantly lower cell viability than the corresponding non-irradiation group, the stapled Largazole group and the Largazole thiol group. To confirm the results, a fluorometric HDAC activity assay was conducted with HCT-116 cell lysates (Figure 2e). A significant decrease of fluorescence was observed in the UV-irradiated premixed group relative to the control groups and the non-irradiation group. Both the cell viability and the enzyme activity results indicated that the stapled Largazole was successfully activated by UV light.

In summary, we have developed a rapid and efficient thiol-ene based photoactivation strategy for thiol-containing drugs, caged with isobutylene. The radical-mediated reaction, that is triggered by UV light, undergoes a thiol-ene addition step to form an unstable radical intermediate, which is further cleaved by *β*-scission to release the caged thiols. We applied this method to various substrates, such as *N*-Boc-cysteine, a cysteine-containing peptide and the HDAC inhibitor Largazole, and

showed that the caged thiol-molecules, unlike their free counterparts, display high membrane permeability. The successful activation of Largazole in HCT-116 cells demonstrates its potential as a drug delivery and activation method for cancer therapy. Further investigation of the use of this strategy on proteins could also be extremely useful for photo-controlled protein activation and drug-release from antibody-drug conjugates.

Acknowledgements

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Keywords: photoactivation • caged drugs • radical reaction • thiol-ene • isobutylene

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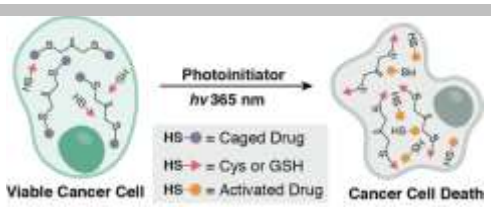

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