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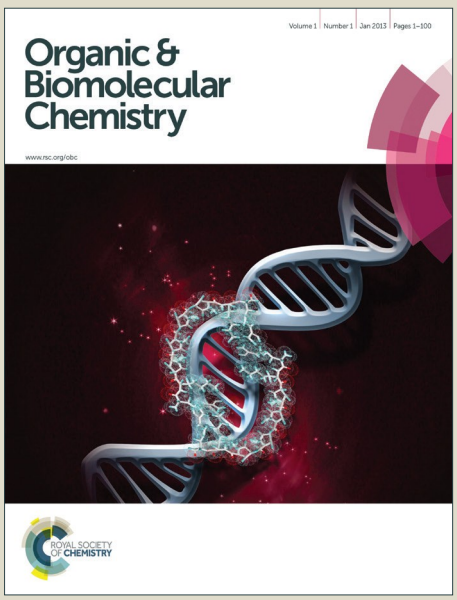

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ARTICLE

Reversibility of thia-Michael reaction of the cytotoxic C_5-curcuminoid and structure-activity relationship of the bis-thiol-adducts thereof

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C_5-curcuminoids [a.k.a. bis(arylmethylidene)acetones] are curcumin analogues bearing a reactive cross-conjugated dienone structure essential for eliciting cytotoxicity. To gain insight into the mode of action of C_5-curcuminoids, we investigated the reversibility of the thia-Michael reaction of 1,5-bis(3,5-bis(methoxymethoxy)phenyl)-1,4-pentadiene-3-one, named GO-Y030 which is the most potent cytotoxic C_5-curcuminoid, using spectroscopic methods. A panel of GO-Y030-bis-thiol-adducts were synthesized and the structure-reactivity relationship regarding retro thia-Michael reaction as well as the cell growth inhibitory activity against human colon cancer HCT116 were evaluated. Some C_5-curcuminoid thiol-adducts exhibited comparable cytotoxicity with GO-Y030, demonstrating their potential use as prodrugs. These results imply that C_5-curcuminoids elicit cytotoxicity by covalently interacting with various biothiols via a reversible thia-Michael reaction.

Introduction

Owing to their inherent reactivity as Michael acceptors that would form a covalent bond with multiple nucleophilic biomolecules, α,β-unsaturated carbonyl compounds have traditionally been research subjects in toxicology and thus have been avoided in modern medicinal chemistry.[1] However, recently, the druggability of Michael acceptors has substantially been reviewed in light of accumulating knowledge that the modulation of the Michael reaction could lead to significant improvement of pharmacological properties, and a novel design concept of covalent drugs has been formulated.[2]

Previously, we disclosed that some bis(arylmethylidene)-acetones, which we named C_5-curcuminoids in connection with naturally occurring C_5-curcumin, exhibit antitumor activities without any remarkable toxicity in common with curcumin (Figure 1).[3] Thus, we identified 1,5-bis(3,5-bis(methoxy-methoxy)-phenyl)-1,4-pentadiene-3-one (GO-Y030) out of more than 100 synthetic analogues as the most potent and promising cytotoxic agent that successfully prevents colorectal carcinogenesis in familial adenomatous polyposis (FAP) mice *in vivo*.[3b] We then explored molecular targets of C_5-curcuminoids using GO-Y086, a biotinylated analogue of a C_5-curcuminoid, to identify that GO-Y086 covalently binds to FUBP2 (far upstream element binding protein 2) at C500, which ultimately resulted in a marked inhibition of the expression of the c-Myc protein.[4]

Considering the high reactivity of C_5-curcuminoids as Michael acceptors, GO-Y086 should experience instantaneous Michael reaction with biothiols upon administration, particularly with glutathione, which is the most abundant (2-3 mM) nonprotein thiol in eukaryotic cells, to give the corresponding thia-Michael adducts. Therefore, it was presumed that GO-Y086 survives via a retro thia-Michael reaction before it participates in the crucial irreversible Michael reaction with binding protein(s). Such a hypothesis prompted us to obtain evidence that C_5-curcuminoids undergo a reversible thia-Michael reaction.

Aside from its promising antitumor activity, GO-Y030 has a low water solubility[6], which led us to envisage the development of a prodrug employing the reversibility of the thia-Michael

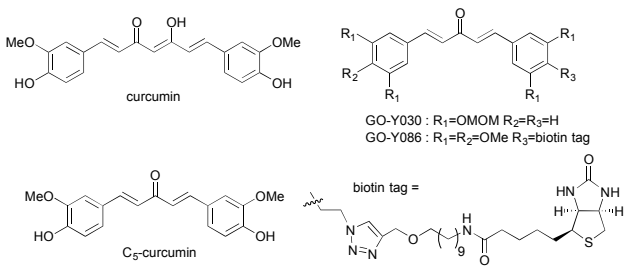

Figure 1 Structure of curcumin and C_5-curcuminoid

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†Electronic supplementary information (ESI) available: General synthetic methods, experimental data, and spectral data are presented in pdf format. See DOI: 10.1039/x0xx00000x

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reaction.[7] In this context, we intended to gain insight into the structure-reactivity relationship focusing on the thiol moiety.

In this paper, we describe our investigation into the reversibility of the thia-Michael reaction between the cytotoxic C_5-curcuminoid GO-Y030 and thiols using spectroscopic methods. A panel of GO-Y030-thiol-adducts were synthesized to examine the structure-reactivity relationship in the retro thia-Michael reaction in aqueous media as well as to probe its potential use in prodrugs.

Results and discussion

Observation of reversible Michael reaction between C_5-curcuminoid and cysteamine

To observe a reversible thia-Michael reaction of GO-Y030 (Figure 2a), we conducted a cysteamine assay using a ^{1}H-NMR spectroscopic method,[8] devised by Appendino and coworkers. Upon the addition of 4 equiv. of cysteamine (Figure 2b), the olefinic protons of GO-Y030 (δ 7.7 and 7.3 ppm in DMSO-d_6) disappeared instantly, giving a spectrum consisting of mono-adduct **B** and bis-adduct **C** (5 min), thereby confirming the thia-Michael reaction. Then, according to Appendino's protocol, an aliquot of the DMSO-d_6 solution of the in-situ-generated Michael adducts was diluted 1:20 with $CDCl_3$ to change the position of equilibrium of the reversible thia-Michael reaction, however, the spectrum showed only tiny peaks corresponding to the dienone (See SI pp 96-98). During our attempts to monitor a clear retro thia-Michael reaction, we eventually found that a simple exposure of the DMSO-d_6 solution of the in-situ-generated Michael adducts (prepared by mixing cysteamine with GO-Y030) tends to shift to the retro-Michael reaction. As shown in Figure 2b, a time dependent restoration of the olefin resonance was clearly observed, (Figure 2b, from 5 min to 6 hr), confirming that the retro-Michael reaction of GO-Y030-cysteamine-adducts (**B** or **C**) occurred after the rapid Michael reaction between GO-Y030 and cysteamine. It is important to point out that the thia-Michael addition proceeded in a stereo-indiscriminating manner to give the bis-adduct **C** as an ca. 1:1 diastereomeric mixture, and both diastereomers exhibited almost the same reactivity in the retro thia-Michael reaction. Also no retro-Michael reaction was observed under anaerobic conditions, indicating that autoxidation of cysteamine to give cystamine operates to shift the equilibrium. A negative control experiment was conducted using maleimide, which is most widely used Michael acceptor for labelling proteins. [9] As a result, no reappearance of the olefin resonance was observed, even after prolonged exposure of the thia-Michael adduct to air, although a rapid thia-Michael reaction occurred. This result supports the validation of this modified cysteamine assay to assess reversibility of the thia-Michael reaction (See SI pp 96-102).

Having confirmed the usefulness of the modified cysteamine assay, we examined its adoptability to a UV spectroscopic method that should enable the high-throughput screening of the retro thia-Michael reactions of GO-Y030-thiol-adducts in aqueous systems. It was found that monitoring the change in the absorption at 340 nm, the maximum absorption wavelength attributed to the cross-conjugated dienone moiety of GO-Y030, provided a comparable result with the NMR adducts in aqueous systems. It was found that monitoring the change in the absorption at 340 nm, the maximum absorption wavelength attributed to the cross-conjugated dienone moiety of GO-Y030, provided a comparable result with the NMR method: upon addition of 24 equiv. of cysteamine, the absorption at 340 nm derived from GO-Y030 in DMSO-d_6 was instantaneously depleted, and it gradually recovered up to its original level (Figure 2c). Note that the UV assay could be conducted in a 96-well plate, for which less than one-fifth of the reagents used in the NMR assay was needed.

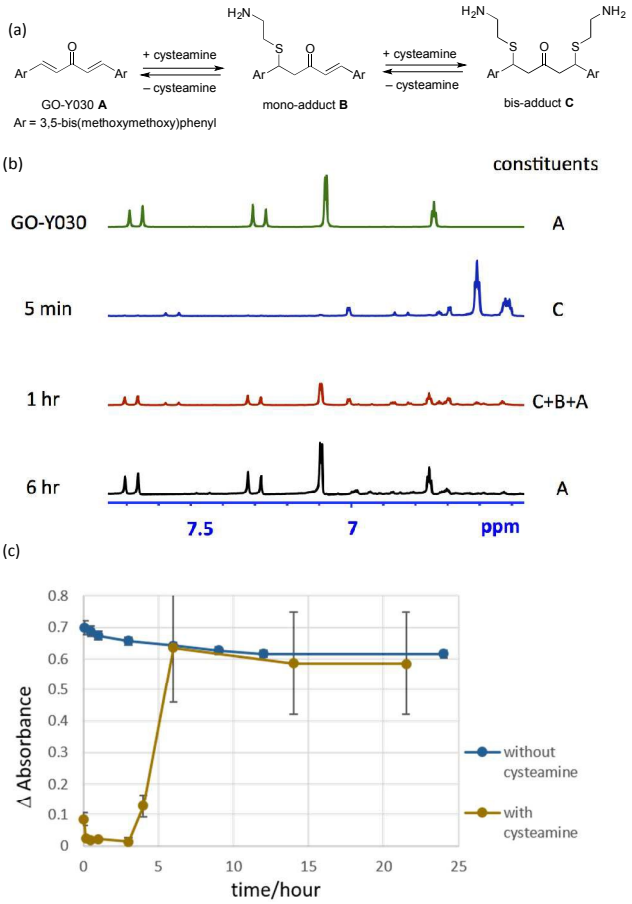

Figure 2 (a) Possible thia-Michael/retro thia-Michael reactions between GO-Y030 and cysteamine. (b) H^1-NMR spectra of GO-Y030 after addition of cysteamine (4 eq.) in DMSO-d_6 (c) Time-dependent change of Δ Absorbance at 340 nm of GO-Y030 without and with cysteamine (4 equiv.) in DMSO-d_6

Synthesis of GO-Y030-bis-thiol-adducts

With the recognition of the potential of GO-Y030 to undergo a reversible thia-Michael reaction, we planned to develop a thia-Michael adduct-type prodrug of GO-Y030. To gain a basic understanding of the structure-reactivity relationship of GO-Y030-thiol-adducts in the retro thia-Michael reaction, a panel of GO-Y030-thiol-adducts were designed using 14 thiols with different physical and stereoelectronic properties. At this

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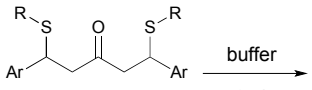
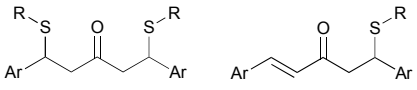
Table 1 Structure-reactivity relationships of retro-Michael reaction profiles and antiproliferative activity of of GO-Y030 thiol-adducts

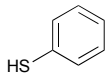
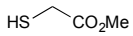
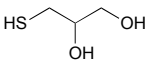
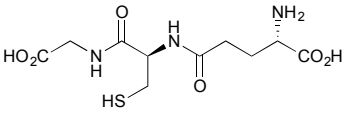
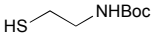
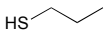
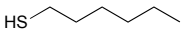
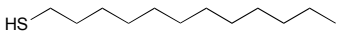
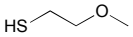
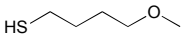
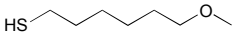
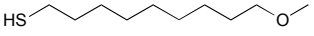
(a) Reactivity of GO-Y030-bis-thiol-adducts towrard reteo thia-Michael reaction



Ar = 3,5-bis(methoxymethoxy)phenyl

(b) Antiproliferative activity against HCT-116[d]



R-SH	name	Yield of GO-Y030 (%)[a]			GI_{50} (μM)	name	GI_{50} (μM)
		pH 3	pH 7.3	pH 8.5			
	GO-Y135	< 5	6	92	0.92	GO-Y181	0.84
	GO-Y137	< 5	5	78	0.97	GO-Y136	0.89
	GO-Y139	< 5	73	79	0.56	GO-Y138	0.72
	GO-Y140[b]	< 5	95	100	0.98	—	—
	GO-Y142	< 5	5	60	0.84	GO-Y141	0.91
	GO-Y146	< 5	12	98	1.00	GO-Y145	0.99
	GO-Y174	< 5	< 5	27	0.72	GO-Y173	1.00
	GO-Y075[c]	< 5	< 5	49	2.00	GO-Y077[c]	0.82
	GO-Y178	< 5	9	9	> 40	GO-Y177	0.92
	GO-Y144	< 5	5	< 5	> 40	GO-Y143	> 40
	GO-Y180	7	6	61	0.72	GO-Y179	0.34
	GO-Y185	< 5	< 5	70	0.76	GO-Y184	0.34
	GO-Y187	10	10	17	0.78	GO-Y186	0.42
	GO-Y189	9	7	11	> 40	GO-Y188	0.67

[a] Yield of GO-Y030 = [$Abs_{\text{thiol-adduct 24 hr}} - Abs_{\text{thiol-adduct 5 min}}$] / $Abs_{\text{GO-Y030}}$ (eq. 1). [b] GO-Y140 was obtained as salts of Et_3N. [c] GO-Y075 and GO-Y077 were reported in reference 3(d). *d* GI_{50} value of GO-Y030 is 0.3 μM.

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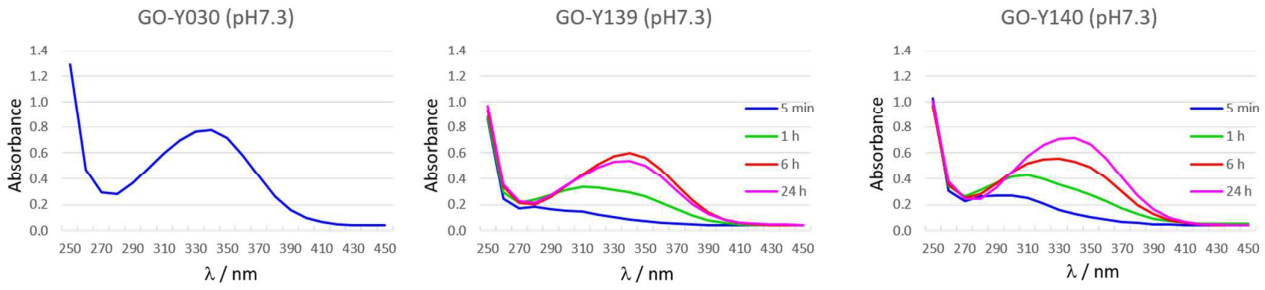

Figure 3 UV spectra of GO-Y030, GO-Y139, and GO-Y140 after dilution with pH 7.3 phosphate buffer

juncture, we focused on the bis-thiol-adducts for their synthetic accessibility, and we particularly envisioned GO-Y139 (GO-Y030-bis(2,3-dihydroxy-propanethiol-adduct) and GO-Y140 (GO-Y030-bis-glutathione-adduct) to be possible water soluble prodrugs of GO-Y030. On treatment with 4 equiv. of thiol in the presence of Et_3N in an appropriate solvent at rt, GO-Y030 quantitatively gave GO-Y030-bis-thiol-adducts as an ca. 1:1 nseparable mixture of diastereomers. As references, the corresponding GO-Y030-mono-thiol-adducts were prepared in a similar manner by decreasing the amount of thiol (1–2 equiv.) (See SI).

Retro thia-Michael reaction of GO-Y030-bis-thiol-adduct

The retro thia-Michael reactions of each GO-Y030-bis-thiol-adducts at three different pH (*vide infra*) were monitored by using a 96-well-plate-based assay system.[10] The amount of GO-Y030, generated from GO-Y030-bis-thiol-adduct via two retro thia-Michael reactions, was assessed by measuring the absorbance at 340 nm, where GO-Y030-mono-thiol-adducts (λ_{max} ~302 nm) interfere only slightly (Figure S7). GO-Y030-bis-thiol-adducts (in 100 μL DMSO) were mixed with 100 μL of buffer (pH 3 glycine·HCl or pH 7.3 phosphate or pH 8.5 Tris·HCl) in each well, and the yield of GO-Y030 after 24 hr was estimated from the changed in absorbance. In acidic medium (50% DMSO in pH 3 glycine-HCl buffer), the yield of GO-Y030 for all analogues was ≤10%, indicating that GO-Y030-bis-thiol-adducts hardly undergo retro thia-Michael reaction in acidic medium (Figures S1, S4). In nearly neutral medium (50% DMSO in pH 7.3 phosphate buffer), two adducts bearing hydrophilic functionalities, namely, GO-Y139 and GO-Y140, exhibited marked reactivity in the retro thia-Michael reaction to give GO-Y030 in 73% and 95% yield after 24 hr (Figure 3), whereas others exhibited ≤10% yield of GO-Y030, demonstrating that GO-Y139 and GO-Y140 have an exceptionally high reactivity for the retro thia-Michael reaction (Figures S2, S5). In basic medium (50% DMSO in pH 8.5 Tris-HCl buffer), most of the bis-thiol-adducts showed a moderate to high reactivity to yield GO-Y030 in 27% to 100% yield after 24 hr, except for GO-Y178, GO-Y144, GO-Y187, and GO-Y189, which bear a lipophilic *S*-alkyl chain (Figures S3, S6). The quite similar structure-reactivity profiles were observed in the retro thia-Michael reaction of a panel of GO-Y030-mono-thiol-adducts (Figures S8-S10). The results suggest that lipophilic substituents hinder the retro thia-Michael reaction.

Biological evaluation of GO-Y030-thiol-adduct

The cytotoxic activity of GO-Y030-thiol-adducts was assessed by cell growth inhibitory testing against HCT116 (Table 1b). In general, irrespective of the mono- or bis-adducts, GO-Y030-thiol-adducts that regenerate GO-Y030 in a buffered solution via retro thia-Michael reaction(s) exhibit a comparable antiproliferative activity with GO-Y030. Specifically, GO-Y143, GO-Y144, GO-Y178, and GO-Y189, which were inactive toward retro thia-Michael reaction did not exhibit apparent cytotoxicity under the assay conditions. These results suggested that the cytotoxic thiol-adducts release GO-Y030 through the retro-Michael reaction after the treatment of cancer cells with each analogue. Importantly the glutathione adduct GO-Y140 had high water solubility compared with GO-Y030 and exhibited moderate cytotoxicity.

Discussion

Focusing on the reversible thia-Michael reaction, fourteen kinds of GO-Y030-bis-thiol-adducts and thirteen kind of GO-Y030-mono-thiol-adducts were synthesized to investigate the structure-activity relationship towards the retro-Michael reaction in aqueous medium as well as to evaluate their potential as prodrugs.

Our results showed that GO-Y030-bis-thiol-adducts exhibited a pH-dependent reactivity toward the retro thia-Michael reaction, in which the greater the pH of the medium, the faster the retro thia-Michael reaction. At this juncture, it was shown that the *S*-substituent exerts a marked impact on the retro thia-Michael reaction to regenerate the parent drug GO-Y030: the more hydrophilic or polar functionality the *S*-substituent equipped with, the more efficient the retro thia-Michael reaction. Note that GO-Y139 and GO-Y140, bearing a highly hydrophilic *S*-substituent, exhibited a pronounced reactivity in the retro-thia-Michael reaction even in nearly neutral medium (50% DMSO-pH7.3 buffer). In contrast, GO-Y178, GO-Y144, GO-Y187, and GO-Y189, bearing a highly hydrophobic *S*-substituent, underwent the retro thia-Michael reaction only sluggishly to give GO-Y030 in less than 17%

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yield. It was unexpected to find that GO-Y178 and GO-Y185 showed the marked difference in retro thia-Michael reaction, suggesting that even a change (-CH_2- to -O-) at the ε-position from sulfur exerted impact on the reactivity, probably by facilitating solvation of the reactive site. Further implication on the solvation issue would be provided from ClogP values: GO-Y030-thiol-adducts with Clog P > 10 did not undergo retro-Michael reaction (see SI Table S1).

Our results also indicated that there is a correlation between the tendency towards the retro thia-Michael reaction and the cytotoxicity of GO-Y030-thiol-adducts: GO-Y030-thiol-adducts that are prone to regenerate GO-Y030 through the retro-Michael reaction exhibit comparable cytotoxicity to GO-Y030. In this context, GO-Y187 represents a surprising exception: comparison with GO-Y144 illuminates impact of etheric O atom at η-position from sulfur atom, which might affect on the solvation or aggregation. Relating to this issue, the salient differences observed between particular mono-thiol-adducts and bis-thiol adducts, namely, (i) GO-Y188 and GO-Y189, and (ii) GO-Y177 and GO-Y178, in terms of reactivity toward retro thia-Michael reaction as well as the antiproliferative activity against HCT-116 would indicate that mono-thiol-adducts undergo a more facile retro thia-Michael reaction.

That GO-Y030-thiol-adducts bearing a hydrophilic group - in particular, GO-Y140 (bis-glutathione-adducts of GO-Y030)- are freely miscible in water (Figure S14), will encourage further studies for the clinical use. In this context, Snyder and co-workers should be credited for their pioneering work on the development of EF24-$(GSH)_2$ which is, to the best of our knowledge, the first water-soluble prodrug of C_5-curcuminoid that employs the reversible thia-Michael reaction. The structure-activity-relationship (SAR) information on the reactivity of GO-Y030-thiol-adducts toward retro-thia-Michael reaction gained in this study will be useful for designing a prodrug with advanced pharmacological properties based on retro thia-Michael reactions.

The SAR information of GO-Y030-thiol-adducts also provides a consistent rationale why C_5-curcuminoids induce multiple biological activities and interact with various molecules[3a], such as FUBP2[4], Trx-1, GSH,[11] and Keap1,[12] in either an irreversible or reversible manner. C_5-curcuminoids experience a random thia-Michael reaction with biothiols and a retro thia-Michael reaction depending on the nature of their thiol, in which equilibrium dominates its temporal binding until C_5-curcuminoids lose reactivity. A similar reversible Michael system was proposed by Suzuki and coworkers to explain influx-efflux phenomena observed for cytotoxic prostaglandin A (PGA) methyl ester in glutathione in cells.[13] The design and synthesis of thia-Michael-adduct-type prodrugs may promote the clinical use of C_5-curcuminoids for cancer chemotherapy.

Conclusions

In summary, we have demonstrated that the cytotoxic C_5-curcuminoid GO-Y030 is potentially a reversible thia-Michael acceptor and that GO-Y030-thiol-adducts elicited cytotoxicity depending on the structure of *S*-substituents and the pH of the reaction medium. Our study suggests that a reversible thia-Michael/retro thia-Michael system between C_5-curcuminoid and biothiols is operative in cells. Some GO-Y030-thiol-adducts exhibited identical cytotoxicity to GO-Y030, indicating their potential as prodrugs. The results on the retro thia-Michael reaction of GO-Y030-thiol-adducts coupled with the cytotoxicity should inspire new avenues for the design of prodrugs derived from Michael acceptors.

Experimental

Supplementary data associated with this article can be found, in the online version, at xxx.

Acknowledgements

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