Self-assembling Releasable Thiocolchicine–Diphenylbutenylaniline Conjugates

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S Supporting Information

ABSTRACT: The design and the synthesis of new self-assembling conjugates is reported. The target compounds are characterized by the presence of a self-immolative linker that secures a controlled release induced by lipase cleavage. 4-(1,2-Diphenylbut-1-en-1-yl)aniline is used as a self-assembling inducer and amino-thiocolchicine as prototype of drug. The release of thiocolchicine derivative has been demonstrated *in vitro* in the presence of porcine pancreatic lipase and Celite-supported lipase. The formation of nanoparticles is confirmed by dynamic light scattering, atomic force microscopy, and fluorescence microscopy. The antiproliferative activity has been proved on two human cancer cell lines.

KEYWORDS: Self-assembled nanoparticles, self-immolative linker, thiocolchicine derivative, drug release, anticancer drug

In recent years we have been attracted by modification of anticancer drugs to obtain self-assembling drug conjugates that spontaneously form nanoparticles (NPs) in aqueous media.^{1–6} We addressed different constructs: NPs composed by conjugate releasable compounds;² single and dual drug fluorescent hetero-NPs;^{3,4} dual drug hetero-NPs (cyclopamine/taxol,⁴ cyclopamine/doxorubicin,⁵ ecdysteroid/doxorubicin⁶); and NPs by self-assembling conjugate dual drugs.⁷ We focused in general on the preparation of compounds composed by a squalene tail^{2–6} or 4-(1,2-diphenylbut-1-en-1-yl)aniline⁷ that makes them able to self-assemble in water. The release inside the cell was favored by the introduction of a disulfide-containing linker. In this scenario, we turned our attention to the replacement of the previously described disulfide linker² with a self-immolative one,⁸ in order to improve the release of the active drugs.

We took inspiration from known self-immolative linkers,⁹ and we focused on the controlled release induced by the self-immolative ability of a *p*-hydroxylbenzyl alcohol based linker.¹⁰ The enzymatic cleavage of a scissile ester bond (acetate) affords a strongly electron-donating phenoxide that facilitates the formation of quinone methide intermediate (Figure 1).

We planned the use of a benzyl alcohol function for the conjugation of the drug. We then designed a *p*-hydroxylbenzyl bearing a proper chain for the introduction of the self-assembling inducer. We selected thiocolchicine as drug and 4-(1,2-diphenylbut-1-en-1-yl)aniline as self-assembling inducer that prompted us to design and synthesize compounds **1** and **2** (Figure 2). They differ in the presence of a disulfide bond in the spacer (spacer A in Figure 1). The synthesis of the self-immolative linker started from compound **5**, obtained in good yield from **3** following a reported procedure.¹¹

The acylation with acetic anhydride and subsequent NaBH₄ reduction led to compound **7**, which was reacted with 4-nitrophenylchloroformate to yield carbonate **8**. Hydroboration secured the production of the activated compound **9** (Scheme 1).

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Figure 1. Structures of building blocks, general structure of the designed conjugates, and mechanism of the release process.

Figure 2. Structures of the obtained conjugates 1 and 2.

Scheme 1. Synthesis of Intermediate 9^a

^aReagents and conditions: (a) allyl bromide, K₂CO₃, KI; (b) MW, 100°C; (c) Ac₂O, Py; (d) NaBH₄; (e) 4-nitrophenylchloroformate, Py; (f) BH₃·THF, H₂O₂.

Compound 9 was then reacted with thiocolchicine derivative 11¹² obtained by deacetylation of thiocolchicine 10, condensation with *N*-Boc-glycine, and *N*-Boc deprotection (Scheme 2), to give compound 12. The final condensation between the primary alcohol 12 and the acid 13a or 13b⁷ in the presence of EDC and DMAP led to the final target compounds 1 and 2.

The cleavage of the designed linker to promote the release of the two bioactive moieties was studied *in vitro* via esterase-catalyzed hydrolysis using porcine pancreas lipase type II (PPL)¹³ or a Celite-supported formulation of lipase PS (1% w/

Scheme 2. Synthesis of Conjugates 1 and 2^a

^aReagents and conditions: (a) HCl, MeOH; (b) *N*-Boc-glycine, DCC, DMAP; (c) TFA; (d) 9, DMAP; (e) 13a or 13b, EDC-HCl, DMAP.

w). The reactions were run on analytical scale and analyzed by means of reversed-phase HPLC (see Supporting Information). Overnight incubation of 1 in a 15:85 mixture of DMSO and phosphate buffer (20 mM, pH 7) in the presence of 1% of *n*-BuOH and PPL, resulted in the hydrolysis of 85% of starting compound, leading to the formation of 11 (amino-thiocolchicine), 13a with traces of desacetylthiocolchicine 14, and aniline derivative 15 (Figure 3). In the same conditions,

Figure 3. Structures of desacetylthiocolchicine and 4-(1,2-diphenylbut-1-en-1-yl)aniline.

but in the presence of lipase PS on Celite, 88% of 2 was successfully hydrolyzed, leading to the formation of 11, 13b, and traces of desacetylthiocolchicine 14 and aniline derivative 15. These simple preliminary experiments confirmed the expected self-immolative character of the proposed linker.

We evaluated the successful formation of nanoparticles by means of dynamic light scattering (DLS) measurements (Table 1). Compounds 1 and 2 were able to give a stable

Table 1. Hydrodynamic Diameter, ζ -Potential, and Polydispersity Index of Nanoformulated Compounds 1 and 2

	hydrodynamic diameter (nm)	ζ -potential (mV)	polydispersity index
1	412.9 $\pm$ 5.9	−33.51 $\pm$ 0.32	0.170 $\pm$ 0.022
2	344.0 $\pm$ 8.2	−46.47 $\pm$ 0.48	0.147 $\pm$ 0.031

suspension of NPs, characterized by hydrodynamic diameters (HD) in the range of 340–420 nm and a negative ζ -potential (less than −30.0 mV). The nanoparticles resulted to be stable in aqueous solution over the time, and even after 1 week, the hydrodynamic diameters were only slightly affected.

To gain more information about the colloidal stability of 1 and 2, we measured their ζ -potential as a function of pH and ionic strength of the media.¹⁴ At a fixed ionic strength ([KCl]

= 1 mM) and at compound concentration of 0.5 mg/mL, the pH has been modulated between 2 and 12 by adding NaOH or HCl 0.1 M. As depicted in Figure 4a, the trend was similar for

Figure 4. Colloidal stability as a function of (a) pH and (b) ionic strength (trend of ζ -potential).

both NPs, registering the lowest value of ζ -potential at pH 8. In acidic condition, the ζ -potential increased to positive value, which did not ensure a prolonged colloidal stability. The influence of the ionic strength on ζ -potential was evaluated by increasing KCl concentration (0, 10, 25, 50, and 100 mM), keeping fixed the NP concentration at 0.5 mg/mL and the pH of the media, buffered at 7.4 by using PBS. The colloidal solutions proved to be very stable, even at high saline concentration (Figure 4b). In the same range of pH and ionic strength, the variation of the hydrodynamic diameter was also evaluated. At low pH, the population of the two solutions is not monodispersed and aggregations occurred, confirming the loss in stability observed by ζ -potential measures. The presence of more than one population was registered even under very basic conditions, stating that the best operational pH range is between 7 and 8 (Figures 1a and S1b). By dispersing the compounds in phosphate buffered solution and increasing the KCl concentration, we observed a similar trend. The colloids were stable, but the formation of new aggregates was registered when the ionic strength increased (Figures 1c and S1d).

In order to get more information on the newly formed nanoconjugates, nanoparticle tracking analysis was performed by atomic force microscopy (AFM) and by fluorescence microscopy (Figure 5). The AFM analysis of the nanoparticles of compounds 1 and 2 deposited on a glass slide showed the

Figure 5. Three-dimensional AFM images recorded in tapping mode (a,d), diameter distribution extrapolated from $50 \times 50 \mu\text{m}^2$ AFM scans (b,e), and fluorescence microscopy images taken with 360–370 nm excitation filter (c,f) of nanoparticles obtained with compounds 1 (a–c) and 2 (d–f)

copresence of some nearly micrometric particles, evidenced by the 3d reconstruction image, together with submicrometric particles, representing the majority of the population. The same scenario was confirmed by fluorescence microscopy (shown in frames c and f), where the blue emission ascribable to the *trans*-stilbene structure, comprised in the self-assembly inducer, is particularly evident for the bigger particles.

The antiproliferative activity of the NPs obtained from compounds 1 and 2 was evaluated on three human tumor cell lines: HeLa (human cervix adenocarcinoma), HepG2 (human hepatocellular carcinoma), and MCF-7 (human breast adenocarcinoma) (Table 2). The building blocks 13a, 13b, 14, and the drug 11a were also taken into consideration as reference.

Table 2. Cell Growth Inhibition (GI_{50}) of the NPs from 1 and 2, Building Blocks 13a, 13b, 14, and the Drug 11a on HeLa, HepG2, and MCF-7 Cell Lines

	HeLa (GI_{50}) μM^a	HepG2 (GI_{50}) μM^a	MCF-7 (GI_{50}) μM^a
13a	>30 ^b	25.6 ± 1.1^b	>30
13b	>30	>30	>30
11a	0.014 ± 0.004	0.018 ± 0.002	0.018 ± 0.004
14	>30 ^b	28.2 ± 1.0^b	>30 ^c
1-NP	4.2 ± 0.2	>30	0.87 ± 0.08
2-NP	2.8 ± 0.3	>30	0.71 ± 0.15

^a GI_{50} values are the concentration of tested agent inducing 50% reduction in cell number compared to control cultures after 72 h of incubation. ^bFrom ref 7. ^cFrom ref 15.

In detail, the GI_{50} values indicated for both NPs 1 and 2 the ability to exert a significant antiproliferative effect on HeLa and MCF-7 cell lines, even if lower with respect to that of the reference drug 11a, while no cytotoxicity was observed on HepG2 cells. Otherwise, the building blocks 13a, 13b, and 14 are unable to induce any cytotoxic effect on cells. In particular, 1 and 2 show GI_{50} values in the low micromolar range on both HeLa and MCF-7 cell lines. Moreover, a comparison between the cytotoxicity of these two NPs does not highlight any appreciable difference, suggesting a negligible role for the disulfide bond in spacer A.

In summary, we report the design and the synthesis of new self-assembling conjugates. The obtained compounds are characterized by the presence of a self-immolative linker that secures a controlled release induced by lipase cleavage and a proper appendage for anchoring the self-assembling inducer. The release of thiocolchicine derivative, which we used as drug prototype, has been demonstrated in vitro in the presence of porcine pancrease lipase (PPL) and Celite-supported lipase (PS). The formation of nanoparticles is confirmed by DLS, AFM, and fluorescence microscopy. The antiproliferative activity of the NPs obtained with 1 and 2 has been proved on two human cancer cell lines (HeLa and MCF-7). All together the described results are a further demonstration of the possibility to modulate the characteristics of the self-assembled nanoparticles that depend on the self-assembling inducer, self-immolative linker, and drug.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00605.

Experimental details regarding preparation of compounds 1–12 and 13a,b, nanoparticle preparation and characterization, biological evaluation (cell cultures, inhibition growth assay, cytofluorometric analysis, confocal microscopy analysis) (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

DMAP, 4-dimethylaminopyridine; DCC, *N,N'*-dicyclohexylcarbodiimide; NP, nanoparticles; AFM, atomic force microscopy; DLS, dynamic light scattering

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