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A Stable and Cleavable *O*-Linked Spacer for Drug Delivery Systems

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Summary

Anti-cancer chemotherapy with good efficacy and fewer side effects is highly desirable. A drug delivery system comprising a cancer-targeting module and a cytotoxic agent connected with a cleavable linker is promising for reducing side effects. The development of a cleavable linker satisfying the requirements of both stability and cleavability, however, is difficult, especially when a carbonate moiety is used for conjugating the linker to a hydroxy group in a drug of interest. We herein report a new stable linker comprising carbamate and ester spacers, which can be introduced on a hydroxy group of a drug. This linker is more stable in aqueous neutral buffer than a corresponding carbonate-type linker, and releases a payload anti-cancer drug, SN-38, through a two-step sequence upon cathepsin B treatment. This linker may have potential use in other drug delivery systems to lower side effects by selectively transporting cytotoxic drugs to tumor cells.

Keywords

cleavable linker, drug delivery, cathepsin, SN-38, antibody

Main Text

Cancer is one of the leading causes of death globally.[1)] Among possible anti-cancer strategies, chemotherapy with cytotoxic agents is widely used for many patients. The strategy is effective in killing cancer cells, but it often causes serious side effects, such as diarrhea and nausea, because most of the cytotoxic agents also damage fast-growing healthy cells.[2)] An anti-cancer therapy with high cancer-selectivity is thus highly desired. A drug delivery system to selectively transport cytotoxic compounds to cancer cells is a promising strategy for this purpose,[3)] and many modes of delivery have been developed, including encapsulation strategies such as liposomes[4)] and nanoparticles,[5)] and bivalent strategies comprising cytotoxic drugs and cancer cell-targeting carrier modules such as peptides,[6)] oligosaccharides,[7)] and antibodies.[8)] In the latter approach, a cleavable linker connecting the two modules is often used, and the linker plays a crucial role in the stability and drug-releasing ability of the conjugates, thus significantly affecting their therapeutic efficacy and side effects. Based on this background, many linkers cleaved under acidic conditions,[9)] under reducing conditions,[10)] or by enzymes[11)] have been developed.

Among the cleavable linkers, valine-citrulline (Val-Cit) or phenylalanine-lysine (Phe-Lys) dipeptide linker with a *p*-aminobenzyloxy (PABO) spacer have been utilized in many drug delivery systems due to their good balance between plasma stability and cleavability by an intracellular cathepsin B protease.[12)] Although these have good stability when conjugated with an amino group in drugs through a carbamate linkage, it is indicated that they have low stability when conjugated with a hydroxy group through a carbonate

linkage[13, 14] because a carbonate moiety is generally unstable in serum,[15] plasma,[16] and even aqueous neutral buffer.[17] Since it is not always possible to conjugate the linker to an amino group of a drug of interest, identification of an alternative method of enabling stable *O*-linkage is necessary. Here, we report a new stable linker comprising a carbamate and an ester moiety, which can be introduced on a hydroxy group of a drug (Fig. 1). The linker is stable in aqueous neutral buffer and releases a payload through a two-step sequence upon cathepsin B treatment.

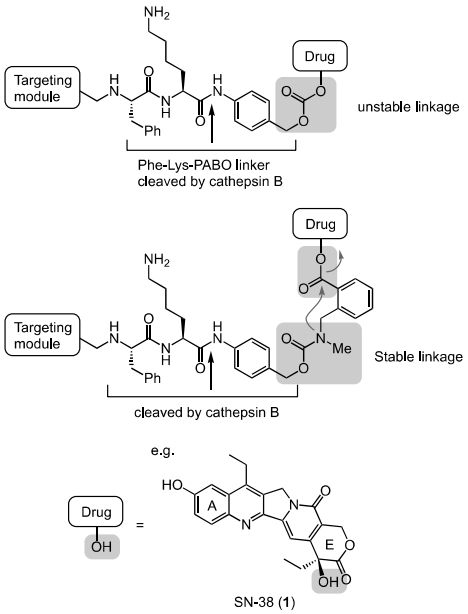

Fig. 1. Cathepsin B-reactive cleavable linkers conjugated with SN-38. A carbonate linker used previously (up) and a carbamate-ester linker developed in this study (down) are shown.

As a model system for the development of stable linkers, we selected a

topoisomerase inhibitor SN-38 (**1**)[18] as a payload anti-cancer drug, since it does not have an amino group for conjugation, and it is frequently used in studies of drug delivery systems (Fig. 1).[19] There are two hydroxy groups for conjugation in SN-38: a phenolic hydroxy group in ring A and a tertiary hydroxy group in ring E. It has been reported that the lactone ring E is critical for the drug activity,[20] and acylation of the hydroxy group in ring E increases its stability for hydrolysis.[21] We thus decided to conjugate linker derivatives at the tertiary hydroxy group.

Previously, the Phe-Lys-PABO linker was conjugated to the tertiary hydroxy group in SN-38 through a carbonate linkage, but its half-life in aqueous neutral buffer (pH 7, 37 °C) was less than 6 hours.[14] As an alternative linkage to the unstable carbonate moiety, we selected an ester moiety, which is often used in a prodrug approach due to its stability under physiological conditions.[16, 22] To release SN-38 in cancer cells by the action of cathepsin B, we conceived a two-step drug releasing sequence: (1) cathepsin B-mediated cleavage of the dipeptide linker, liberating the amino group through the PABO-carbamate spacer, and (2) an intramolecular ester-amide exchange to release the drug payload (Fig. 1). Since a drug-releasing rate is dependent upon the structure and electronic property of the amino-ester moiety between the carbamate and the ester moieties, we first investigated their influence by synthesizing several model compounds (Fig. 2A).

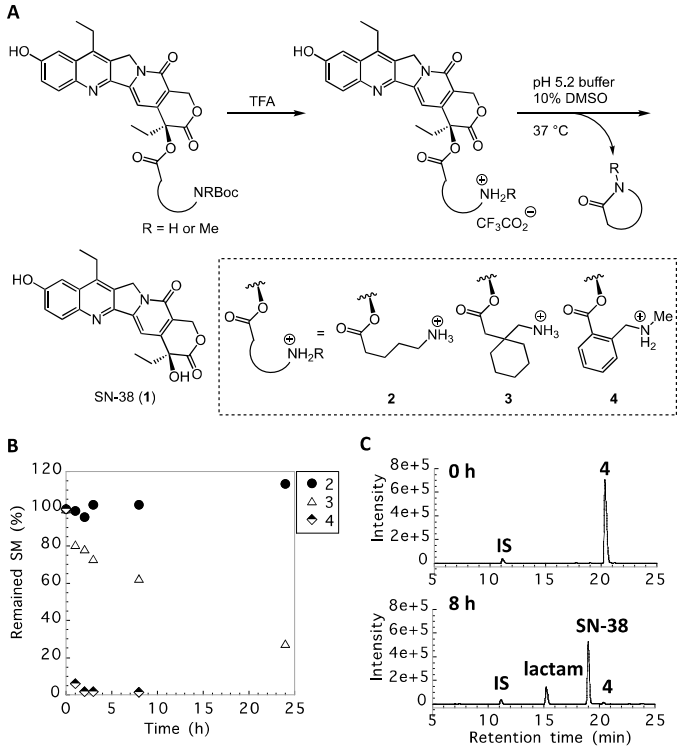

Fig. 2. Investigation of cleavage rates of model compounds. **A**) Structures of model compounds and the reaction scheme for the cleavage analysis, **B**) Time course profile of the linker cleavage, **C**) Representative HPLC charts (254 nm) of compound **4** analysis (up: 0 h, down: 8 h). IS: internal standard (tyrosine methyl ester).

Three model compounds (**2**, **3**, **4**) with varying structural flexibility and electronic properties were designed and synthesized as their Boc-carbamates (for the syntheses, see Supplementary Materials). The cyclization rate was evaluated after cleaving the Boc groups with trifluoroacetic acid treatment, followed by dissolving the deprotected amine salts in acidic buffer (citric acid/Na_2HPO_4, pH 5.2), since cathepsin B works in lysosomes.[23] The most flexible compound, **2**, was stable under these conditions, and only a tiny amount of SN-38 was released, even after 24 hours (Fig. 2B and Fig. S1A). Compound **3**, containing a

cyclohexyl ring, was much more reactive than **2** due to the Thorp-Ingold effect,[24] and 70% of **3** was consumed to release SN-38 after 24 hours of incubation (Fig. 2B and Fig. S1B). The most promising compound was **4**, possessing a conformationally rigid *o*-aminomethylbenzoic acid structure whose ester moiety is electronically more reactive than that of **2** and **3**. Cyclization was rapidly completed within 1 hour, and SN-38 was released up to the saturation limit of the buffer (Fig. 2B, C, and Fig. S1C). Interestingly, a derivative lacking the *N*-methyl group of **4** was unstable, and released SN-38 at the stage of the precursor Boc carbamate through auto-cyclization to the ester moiety. These results clearly showed that the *o*-*N*-methylaminomethylbenzoic acid ester is the most effective spacer for further investigations.

Next, we synthesized a Phe-Lys-PABO linker-conjugated derivative of the most promising compound, **4**, and assessed its stability and drug-releasing ability. For future conjugation to a tumor-targeting module, an azido group was introduced at its terminus (Chart 1). An activated carbonate ester **5** was coupled with phenylalanine methyl ester hydrochloride **6** to produce **7**. Hydrolysis of the methyl ester, followed by coupling with protected lysine derivative **8** with a PABO moiety, afforded **9**. The hydroxy group at the PABO moiety was activated as its nitrophenyl carbonate, and reacted with aminoalcohol **11** to produce **12**. The hydroxy group at the benzylic position was oxidized in a stepwise manner to afford carboxylic acid **13**. Finally, the ester condensation between **13** and triisopropylsilyl (TIPS)-protected SN-38 (**14**) was performed with water soluble carbodiimide (WSCI) and 4-(dimethylamino)pyridine (DMAP), and the TIPS group and

the (4-methoxyphenyl)diphenylmethyl (Mmt) group were removed by treatment with triethylamine trihydrofluoride and formic acid, respectively, to produce Phe-Lys-PABO-containing masked SN-38 (**15**). A corresponding carbonate derivative **16** was also synthesized for comparison (Supplementary Materials).

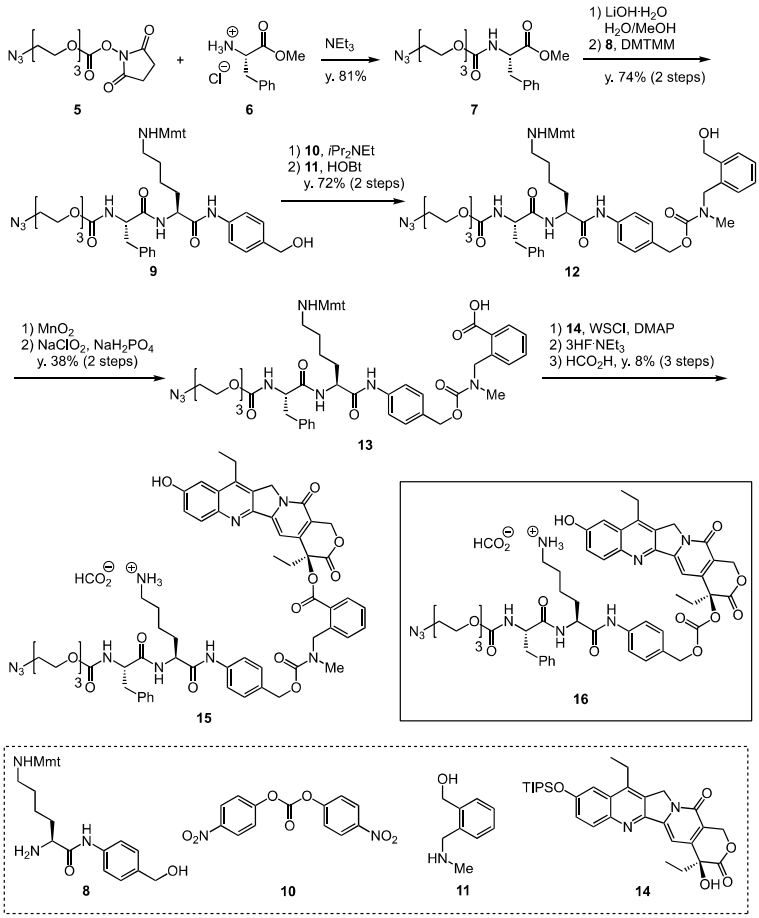

Chart 1. A synthetic scheme for SN-38 conjugated with the newly developed cathepsin B-reactive carbamate-ester linker. A corresponding carbonate derivative **16** is shown in the square.

Stability of the newly synthesized SN-38 conjugate **15** and corresponding carbonate conjugate **16** in aqueous neutral buffer (PBS: phosphate buffered saline, pH 7.4) was evaluated (Fig. 3A). Each compound (50 μM) was dissolved in PBS containing 1.5% DMSO and incubated at 37°C, and the release of SN-38 was monitored with high performance liquid chromatography (HPLC). SN-38 conjugate **16**, with the carbonate linkage, released free SN-38 gradually, reaching 30% release of SN-38 after 24 hours of incubation. In striking contrast, the newly synthesized SN-38 conjugate **15** was stable under the same conditions, and the amount of free SN-38 in the sample (originally containing 5%) did not increase, even after 24 hours. These results clearly showed that the carbamate-ester linkage has an advantage in providing stability under aqueous neutral conditions.

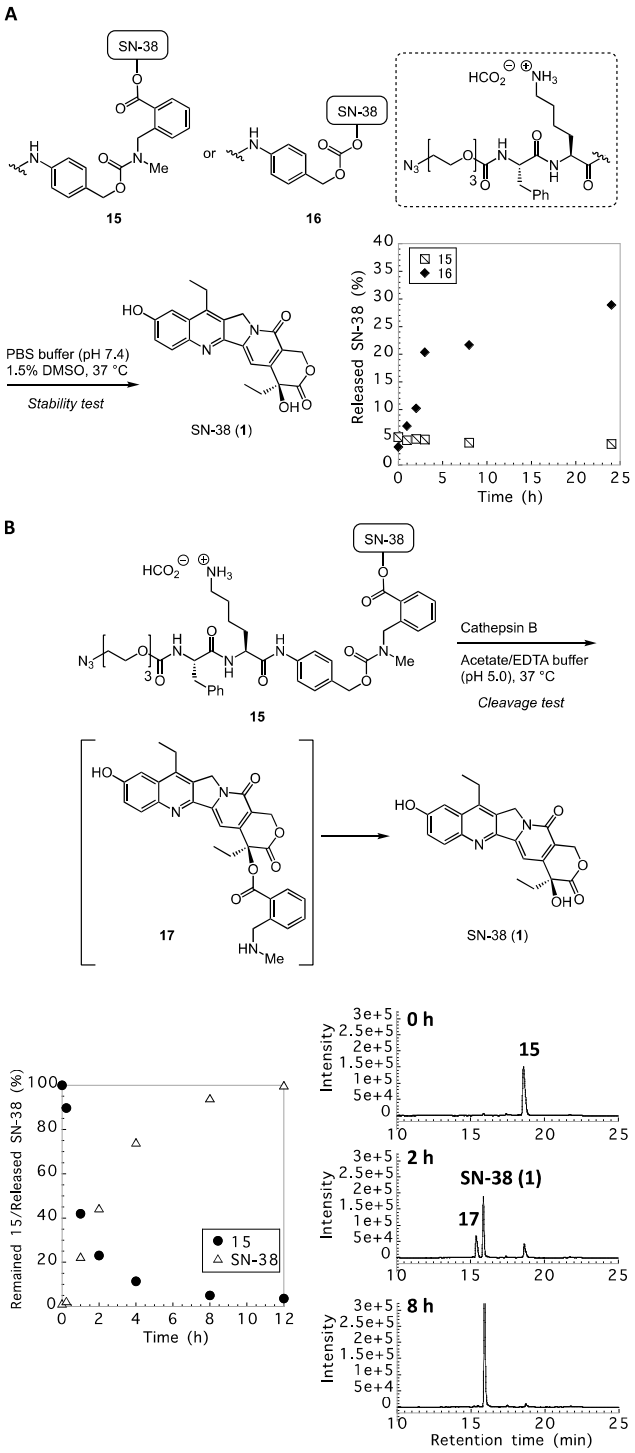

Fig. 3. Stability and cleavability analyses of cathepsin B-reactive SN-38 conjugates. **A**) Time course profile of SN-38 release from the conjugates with a carbamate-ester linker (**15**) and a carbonate linker (**16**) under aqueous neutral buffer conditions, **B**) Time course profile of SN-38 release from **15** upon cathepsin B treatment. Representative HPLC charts

(360 nm, up: 0 h, middle: 2 h, down: 8 h) are shown.

Next, we evaluated cathepsin B-induced SN-38 release from conjugate **15**. When **15** was treated with cathepsin B from bovine spleen at pH 5 at 37°C, the time-dependent release of SN-38 (**1**) was observed (Fig. 3B, and Fig. S3). During the course of the reaction, intermediate **17** with an amino ester spacer was also observed, and all the starting material **15** and intermediate **17** were finally consumed after 12 hours, releasing SN-38 in a very clean manner. Taken together, the newly developed carbamate-ester spacer has sufficient stability under aqueous neutral conditions, and releases its payload drug upon treatment with cathepsin B.

In summary, we developed a new cathepsin B-reactive cleavable linker, which can be introduced at a hydroxy group of a drug of interest. This linker was more stable under aqueous neutral conditions than the previously used carbonate linker, and released its payload anti-cancer drug SN-38 through a two-step reaction sequence upon treatment with cathepsin B protease, which is overexpressed in cancer cells. Since drug delivery systems in which a linker between a targeting module and a payload drug plays an important role are increasingly becoming important, our newly developed linker would have potential use in these systems to lower side effects by selectively transporting cytotoxic drugs to pathogenic cells, such as cancers.

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Conflict of Interest (COI)

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials.

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