Dual-Locked Macrocyclic "Turn-On" Drug for Selective and Traceless Release in Cancer Cells

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Abstract

Drug safety and efficacy due to premature drug release in the bloodstream and poor biodistribution remain challenging issues despite seminal advances in the field. To circumvent these limitations, we report a directed-macrocylization as a dual lock for camptothecin (CPT), a small molecule anticancer drug. In this way, the activity is "locked" within the cyclic structure by the redox responsive disulfide and pH-responsive boronic acid-salicylhydroxamate and turned on only in the presence of acidic pH and glutathione through traceless release. Notably the dual-responsive CPT is more active (100-fold) compared to the non-cleavable (closed) analogue. We further include bioorthogonal handle in the cyclic backbone for subsequent functionalization to generate cell-targeting peptide-macrocyclic and protein-macrocyclic CPTs for targeted, traceless drug release in triple negative metastatic breast cancer cells to inhibit cell growth in the low nanomolar concentration.

Introduction

Cancer remains a major global healthcare challenge with significant mortality rate despite medical advances.[1] Chemotherapy is one of the mainstream treatments to combat cancer but safety and efficacy limitations of anti-cancer drugs persist due to their indiscriminate biodistribution in healthy and tumor tissue.[2] To improve the efficacy of existing small molecule anti-cancer drugs, targeted therapeutics have been developed that combine a small molecule drug with a recognition moiety which specifically binds to and penetrate cancer cells.[3] Examples include peptide- or antibody-drug conjugates and targeted nanoparticle delivery systems.[4] Peptide- and antibody-drug conjugates usually offer advantages over targeted nanoparticle systems, as the nanoparticle can bind to plasma proteins that shield the targeting groups and thus compromise the targeting effect *in vivo*.[5] Besides targeting,

stability in blood plasma without burst or premature release of cargo in low glutathione (GSH) concentrations (μM) through disulfide exchange reactions or in acidic extracellular tumor microenvironments (TME) of glycolytic tumors (extracellular pH<7) are required to reduce off-target toxicity.[6] Additionally, controlled drug release from the targeting group within tumor cells or a nanoparticle formulation should, ideally, be quantitative for high therapeutic efficacy. However, to date, there are limited linker chemistries that fulfill both criteria to prevent premature release. Taking all the criteria above into consideration, new strategies are urgently needed to furnish an anti-cancer drug that possesses: (1) the availability of a targeting group to bind to cell surface receptors with high affinity; (2) quantitative traceless drug release to convert the inactive drug into its active form; (3) controlled drug release in the cytoplasm of cancer cells to prevent premature release in the blood plasma and in the acidic TME.

Nature uses cyclization to improve the bioavailability and stability of peptides, e.g. in peptide hormones[7] or snake venom[8], which has inspired the design of excellent drug candidates.[9] Macrocyclic natural products have been developed as therapeutics for cancer therapy due to their high target binding affinity and favorable stability *in vivo*.[10] Bioactivity can be influenced by a conformational switch from cyclic (closed) to linear (open), suggesting that embedding small-molecule drugs in a macrocyclic scaffold would be a viable approach to improve their efficacy and specificity by intracellular drug activation.[11] However, it is synthetically challenging to combine a small molecule drug, targeting groups and stimuli-responsiveness within a cyclic structure, with limited examples in literature.[12] We have recently reported the combination of redox responsive disulfides and pH responsive catechol – boronic acids (BAs) containing interlocked, peptide linkers that display dual-stimuli-responsiveness, with improved stability and controlled disassembly in tumor cells.[13] Therefore, this could offer a broadly applicable strategy over the classical head-to-tail approach, by enabling directionality for efficient cyclization to form stimuli-responsive macrocyclic drugs with cell targeting features (Figure 1).

Boronic acids (BAs) can undergo pH-reversible coordination to diols and salicylhydroxamates (SHA), as well as provide traceless release in the presence of a high concentration of H_2O_2 in cancer cells to restore the alcohol groups in cargo drugs.[14] We selected SHA, which offers several advantages over catechol, with better binding affinity to BAs (μM versus mM) to afford stable conjugates at lower concentrations, while being rapidly and quantitatively

cleavable at acidic pH (Figure 1).[15] Under physiological pH in healthy tissue, the macrocyclic structure remains in its "closed" form and only opens in the acidic environment of subcellular compartments.[16] Additionally, BAs offer more chemical innovation space for pharmaceuticals as they are non-toxic, as exemplified by several marketed drugs with boronic acid groups such as Eucrisa™ or Vabomere™, among others, which are used in clinical trials.[17] A disulfide linkage is introduced as a second chemical lock to control the release within cytosolic compartments and prevent the release in the acidic extracellular space within the TME.[18] Disulfides remain stable over several pH units and can undergo self-immolative intracellular reduction for traceless release in tumor cells with high intracellular GSH concentration.[19] This restores the activity of the drug cargo, which may be an advantage over conventional disulfide linkers that result in loss of drug activity due to the formation of less active glutathione-drug conjugates.[20] However, premature burst release of disulfide containing pro-drugs in the blood circulation still represents a concern that could potentially be prevented in combination with an intramolecular SHA-BA lock.[21]

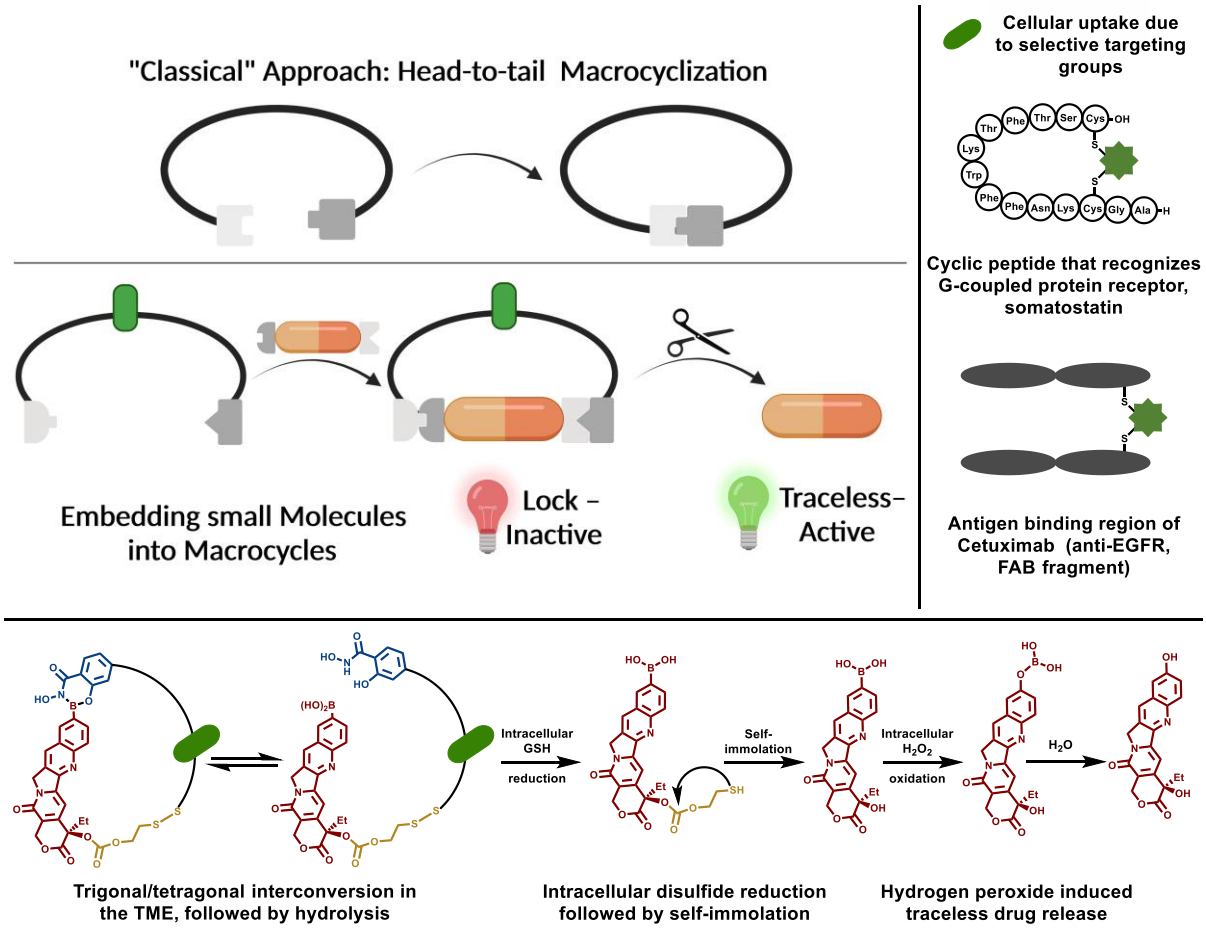
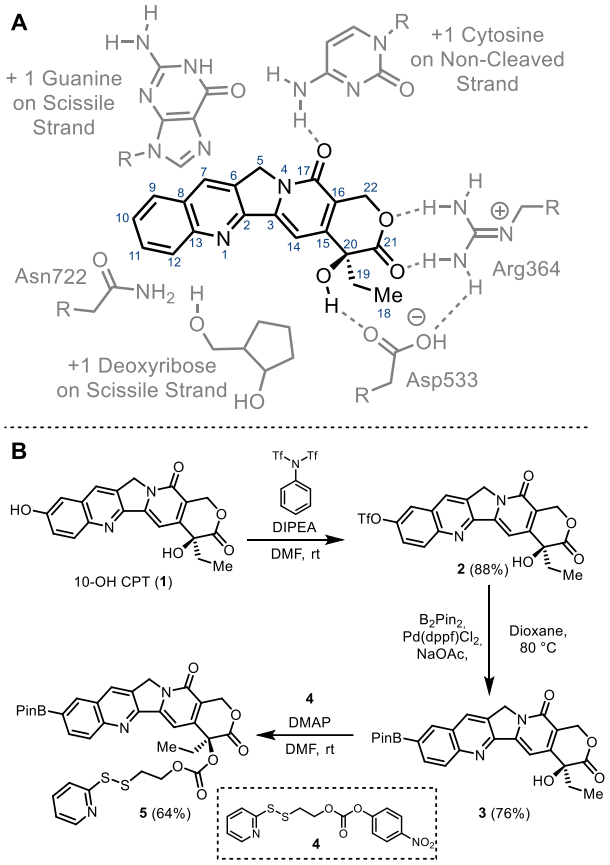


Figure 1: Conceptual overview of "classical approach" for macrocyclization and dual-locked CPT-macrocycle that potentially enables selective and traceless release in cancer cells. Image shows a possible pathway of sequential cleavage and traceless release.

We choose the pentacyclic natural product, camptothecin (CPT), as the drug lead structure (Scheme 1A).[22] CPT binds to nuclear enzyme DNA topoisomerase I (TOP1) and DNA complex, which ultimately leads to apoptosis.[23] Different analogues of the topoisomerase I inhibitor are FDA approved, highly potent (IC_{50} low nM), and have been widely applied for treatment of lung cancer, breast cancer, among others.[24] In addition, we introduced a bioorthogonal reactive handle for post-functionalization of the macrocyclic drug, where the targeting moiety is linked *via* a stable covalent linkage. In this way, we could generate cell-targeting peptide-macrocyclic and protein-macrocyclic CPTs for traceless drug release in triple negative metastatic breast cancer cells to inhibit cell growth. We envision that dual-stimuli-responsive macrocyclic chemotherapeutics can be positioned as an efficient strategy to generate drugs without premature release into the bloodstream and in the acidic TME with selective and traceless drug release into the cytosol of cancer cells.

Results and discussion

To prepare a dual-responsive cyclic CPT analogue, we introduced a boronic acid moiety on the quinoline ring (Scheme 1B), as previously shown for the prodrug of SN-38.[25] The alcohol of the α-hydroxy lactone was modified with a self-immolative disulfide linker.[26] In this way, traceless CPT release could be achieved, which is essential for the inhibitory effects. We started the synthesis with the trifluoromethanesulfonation of commercially available 10-OH CPT (**1**) using *N*-phenyl-bis-(trifluoromethanesulfonimide) followed by palladium catalyzed Miyaura-borylation resulting in 10-BPin CPT (**3**, see Scheme 1B). The alcohol functionality on the stereocenter (C20) of **3** was transformed to the corresponding carbonate, using the activated disulfide **4** in the presence of DMAP, providing compound **5** in an overall yield of 42% (over three steps).



Scheme 1: A: Proposed CPT mechanism of action. Schematic representation of the key hydrogen bonds and ring-stacking interactions between the human topoisomerase I–DNA covalent complex and CPT in the proposed CPT binding mode. The atomic nomenclature for CPT is also indicated.[20] B: Synthesis of 10-BPin CPT pyridyl disulfide (**5**).

With 10-BPin-CPT pyridyl disulfide (**5**) in hand, we performed thiol exchange with *N*-acetyl cysteine amide (**7**). The pinacolborane conveniently hydrolysed during HPLC purification, yielding the unprotected boronic acid **7** (Figure 2A). A time course for the cleavage of the disulfide bond, followed by self-immolative release of CPT-boronic acid (10-BA CPT, **6**) was conducted in "cell-like" reducing conditions (SI-Figure 44 and 45). Therefore, **7** (50 µM) was dissolved in ammonium bicarbonate buffer (50 mM, pH 7.4 or 6.5) containing 10 mM GSH, 1 mM GSSG and the reaction was monitored by LC-MS (SI-Figures 44 and 45). Clean conversion of the disulfide to 10-BA CPT was observed at both pH values. As a non-cleavable analogue, a cysteine thioether was synthesized (**8**, Figure 2A). The prepared CPT derivatives were subjected to cellular growth assays using MDA-MB-231 triple-negative breast cancer cells. Cellular growth was monitored over 110 h using an Incucyte® (Figure 2B).

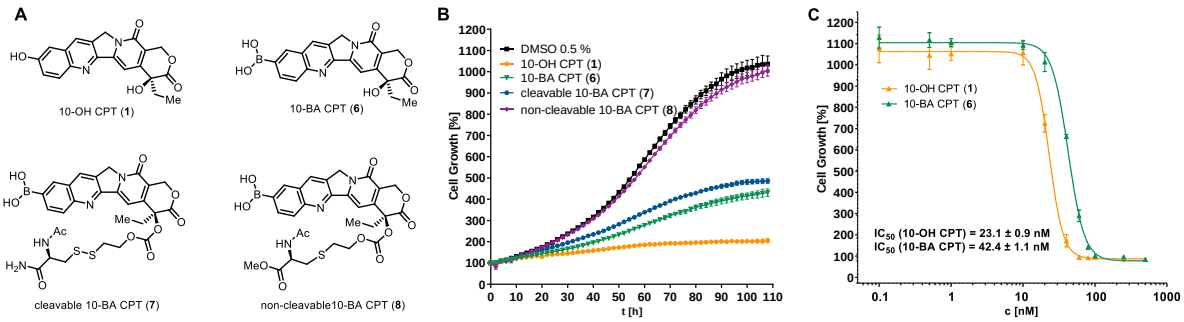


Figure 2: A: Structures of CPT derivatives subjected to proliferation assay. B: Cellular growth of MDA-MB-231 cells by treatment with different CPT derivatives at 50 nM concentration. C: Determination of IC_{50} values for 10-OH CPT (**1**) and 10-BA-CPT (**6**) for MDA-MB-231 cells.

As expected, 10-OH CPT (**1**) displayed high inhibition in the proliferation assay. At a concentration of 50 nM, no significant cell growth was observed. The IC_{50} value was determined to be 23.1 ± 0.9 nM. Boronic acid modified CPT (**6**), showed similar activity with an IC_{50} value of 42.4 ± 1.1 nM. In contrast, the non-cleavable analogue (**8**), resulted in almost no difference in cell growth inhibition when compared to the DMSO control. The disulfide containing, self-immolative analogue (**7**) was as potent as 10-BA CPT at a concentration of 50 nM, suggesting that efficient disulfide reduction took place under these conditions. Based on these results, our next step was to embed the drug in a cyclic structure to achieve selective, dual-responsive and traceless release in the unique intracellular cancer environment.

Design and synthesis of cyclic CPT derivatives

To enable dual-stimuli responsive delivery of CPTs, a tri-functional linker was required. The scaffold, containing SHA, a thiol, an appropriate number of PEG linkers in between the SHA and thiol to form the cyclic structure, and a bioorthogonal chemical handle for further conjugation (azide or DBCO), were conveniently assembled on solid support, with only one step-purification required by HPLC (Figure 3A).

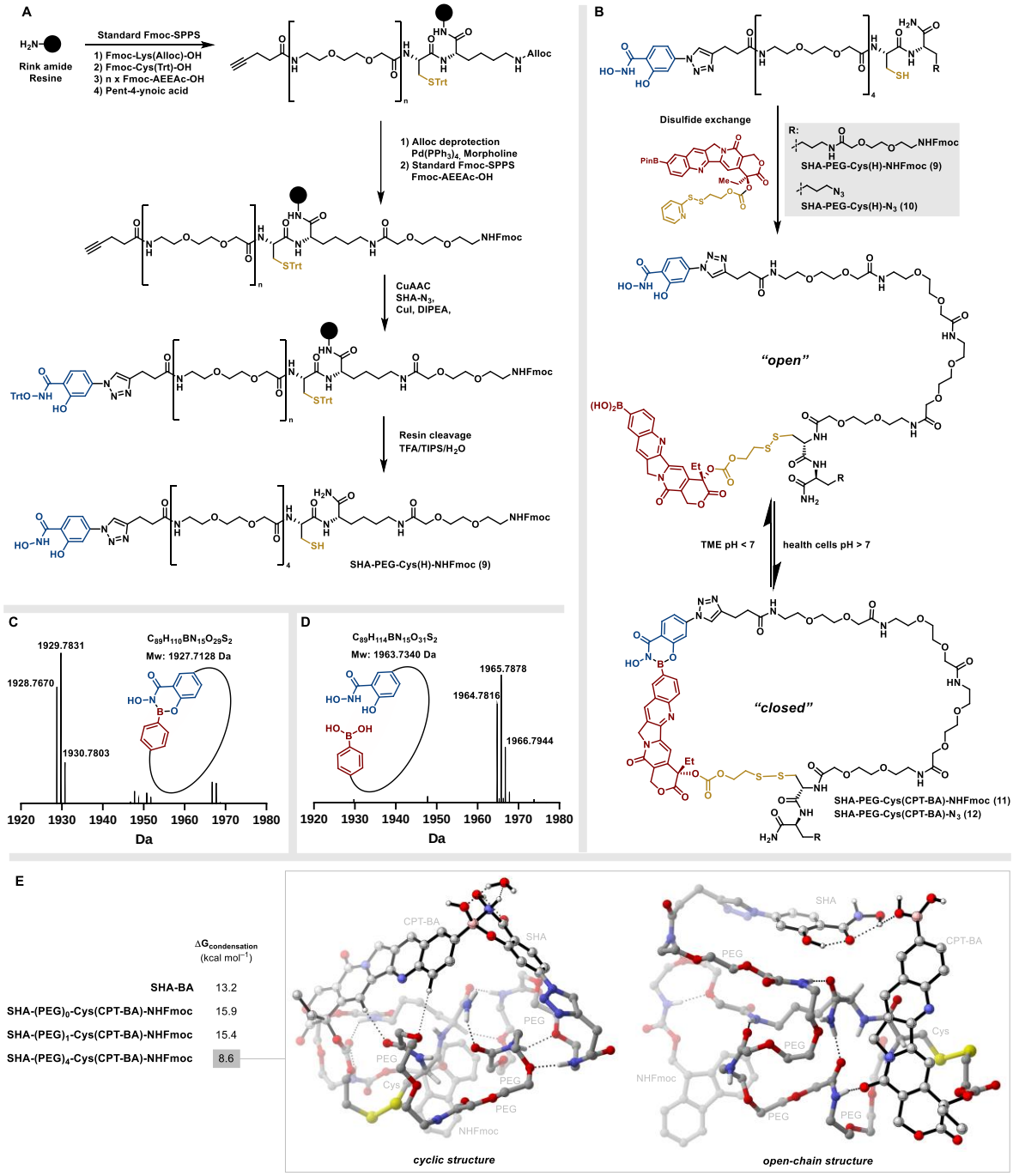


Figure 3: Synthesis of dual-stimuli responsive CPT analogue. A: Assembly of the tri-functional linker by Fmoc-SPPS ("standard Fmoc-SPPS" refers to: Fmoc-deprotection using 20 vol% piperidine in DMF, pre-activation of the AA with HATU/DIPEA, DIC/Oxyma (for Cys) and capping using 20 vol% of acetic anhydride in DMF, respectively. B: "Loading" of CPT analogue (**5**) by disulfide exchange, structure of the "opened" and the "closed" construct. (Isolated yield of **11**, 30% from initial SPPS loading). Intramolecular cyclization of CPT. C: HR-ESI-MS analysis of dual-stimuli responsive CPT (**11**) in ammonium acetate buffer at pH 7.4, "cyclic" structure (calc. for $C_{89}H_{110}BN_{15}O_{29}S_2$: 1927.7128 Da); D: HR-ESI-MS analysis of dual-stimuli responsive CPT (**11**) in ammonium acetate buffer at pH 6.5, "open" structure (calc. for $C_{89}H_{114}BN_{15}O_{31}S_2$: 1963.7340 Da). E: Molecular modeling (some hydrogens are omitted for clarity) and theoretical evaluation of the macrocyclization process. DFT calculations *at M06 2X/Def2-TZVPP/SMD(water)//B3LYP/6-31G(d) level of theory.*

Besides "standard" Fmoc-SPPS, we utilized copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC), palladium(0)-catalyzed Alloc deprotection, acid mediated monomethoxytrityl

(MMT) deprotection and thio-ether bond formation, directly on the resin (Figure 3A). After global deprotection, disulfide exchange of **5** to the Cys-side chain was performed (Figure 3B). The length of the linker was predicted by molecular modeling to determine the number of Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-AEEAc-OH) spacers necessary to enable intramolecular cyclization of the SHA to the BA. (Figure 3E). The theoretical feasibility of the intramolecular cyclization was assessed by analyzing the macrocyclization equilibria, assuming a thermodynamically controlled process. To determine the relative Gibbs free energies of cyclic and open-chain structures for SHA-$(PEG)_n$-Cys(CPT-BA)-NHFmoc (n=0, 1, 4), density functional theory (DFT) calculations were performed. The results indicated that a longer linker (e.g., 4 PEG linkers) would enhance the efficiency of macrocyclization, most likely due to the planarity of the SHA and CPT-BA, which hinders intramolecular cyclization. In fact, the energy difference between the open and closed structures for SHA-$(PEG)_4$-Cys(CPT-BA)-NHFmoc is 8.6 kcal mol^{-1}, whereas for SHA-$(PEG)_1$-Cys(CPT-BA)-NHFmoc is 15.4 kcal mol^{-1}.

The successful switching between intramolecular cyclization ("closed") and linear ("opened") structure was characterized and confirmed by high resolution electrospray ionization (ESI) (Figure 3C and 3D), which shows a change in a *m/z* of 36 Da (two molecules of water) and corresponds to the calculated exact mass of the respective structures.

To explore dual-stimuli-responsive drug release, the intrinsic fluorescence of CPT was utilized for Förster Resonance Energy Transfer (FRET) studies. FRET occurs when two appropriate fluorescent dyes (a donor-acceptor pair) are in close proximity and thus could be exploited to provide information on if linker cleavage had proceeded.[27] To demonstrate the release of CPT, *N*-ethylamide-4-dimethyl-1,8-naphthalimide was installed on a Lys-side chain of the linker as an acceptor dye in the dual-responsive CPT (**13**) and mono-responsive CPTs (**14** and **15**) (Figure 4A-D), based on the measurements by Hu and Zeng.[28] As a control, the fluorescence of 10-BA CPT (**6**) was first evaluated at pH 7.4 and 6.5, as well as in the presence and absence of GSH (25 µM in PB buffer containing 10% DMSO at rt). A strong fluorescence band with a maximum at 428 nm under excitation at 385 nm was found (Figure 4E). Fluorescence intensity was two times higher at pH 7.4 than at pH 6.5. Addition of GSH (1.0 mM, 40 equiv.) led to a decrease in the fluorescence intensity. Mono-responsive CPT with a disulfide bond (**14**, Figure 4B), showed strong, pH independent fluorescence quenching, as expected. After addition of GSH, a significant increase in fluorescence intensity (50-fold) was observed, suggesting cleavage of the disulfide and release of the CPT.

Quenching of the pH responsive BA-SHA CPT conjugate showed release at pH < 7.0 (**15**, Figure 4C). However, the degree of quenching was lower at pH 7.4 than that observed for **14**. This may be due to the greater distance between the two fluorophores, the enlarged chromophore after BA-SHA condensation or to the fact there is no pre-coordination. Nevertheless, a clear difference in fluorescence intensity (a 10-fold increase) for the two pH values was observed. At pH 6.5, similar fluorescence intensity of **14** was detected as for **6** under these conditions. The results show that the two different stimuli can act independently for mono-responsive CPT derivatives (Figure 4E). Next, we focused on FRET studies of the dual-stimuli-responsive CPT (**13**, Figure 4A). Fluorescence measurements at pH 7.4 and 6.5 (releasing the SHA-BA conjugate) showed no difference in fluorescence quenching and addition of GSH at pH 7.4 did not increase the fluorescence either. Furthermore, pre-coordination of SHA and BA by the disulfide bond has a clear effect on fluorescence quenching. A strong fluorescence signal can be detected only when both stimuli are present (pH 6.5 and GSH) (Figure 4F). Taken together, these results demonstrate how both chemical triggers (GSH and pH) are required to unlock the double hinges and achieve controlled release of the CPT, which is critical for selective intracellular release.

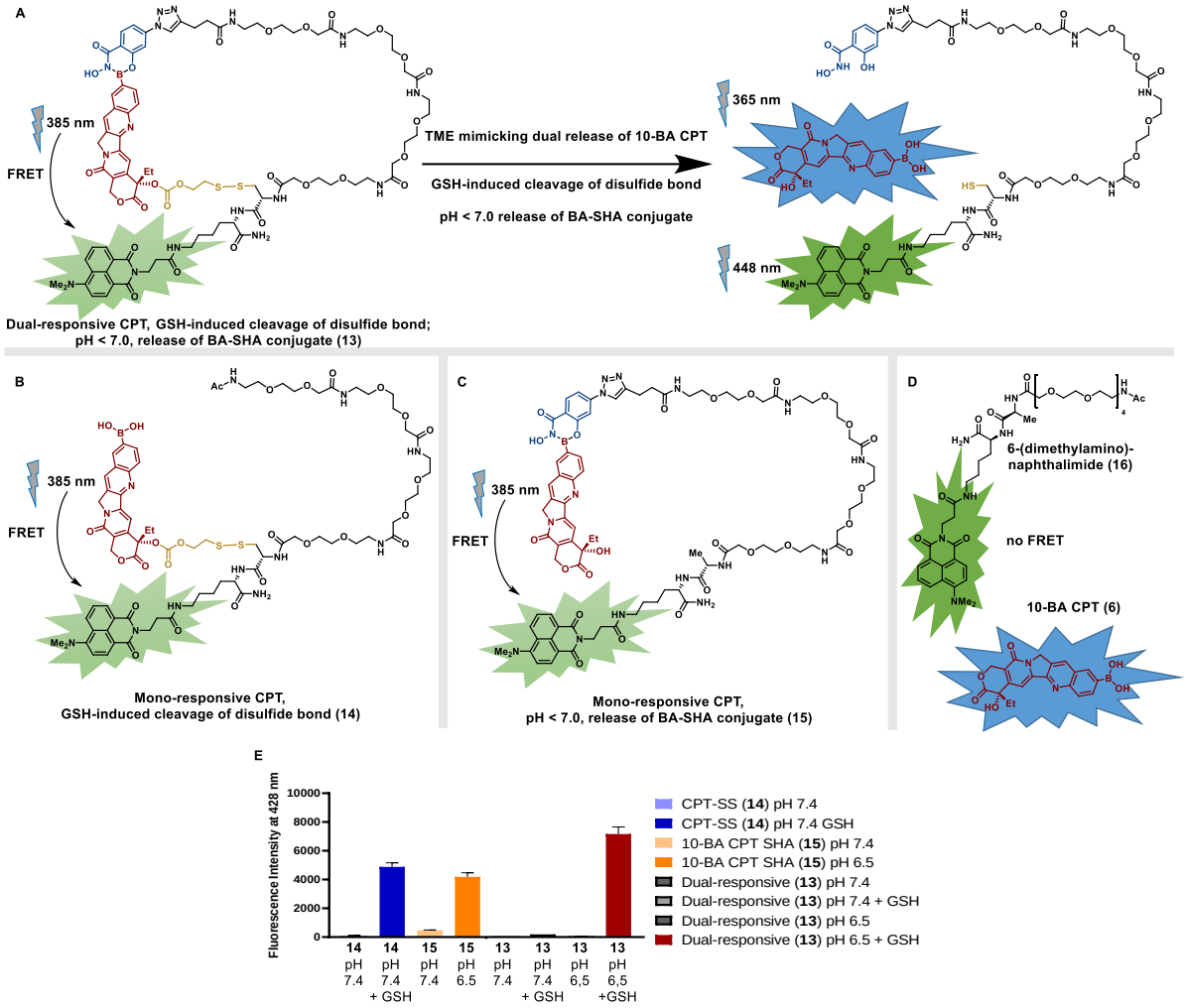


Figure 4: Förster Resonance Energy Transfer (FRET) of CPT derivatives. A: Dual-responsive CPT, GSH-induced cleavage of disulfide bond; pH<7.0, release of BA-SHA conjugate (**13**); B: Mono-responsive CPT, GSH-induced cleavage of disulfide bond (**14**); C: Mono-responsive CPT, pH<7.0, release of BA-SHA conjugate (**15**); D: 4-dimethylamino-1,8-naphthalimide derivative (**16**) and 10-BA CPT (**6**). E: Comparison of fluorescence intensity (excitation at 385 nm, emission at 428 nm, 25 µM in PB buffer containing 10% DMSO at rt) for CPT derivatives and stimuli responsive release.

Synthesis of macrocyclic CPT bioconjugates and *in vitro* activity

We further investigate if the dual-stimuli-responsive CPT variant could be activated in the presence of chemical stimuli in cancer cells, as compared to non-cleavable CPTs that retain their cyclic structure in the TME (extra- and intracellular). Therefore, a benzyl thio-ether, similar in size to the salicylhydroxamate (SHA) was introduced at the 10-position, as a "non-cleavable — open" analogue (compound **17**, Figure 5A). A "non-cleavable — cyclic" CPT derivative (**18**) was prepared by cyclization through CuAAc to propargyl glycine side chain.

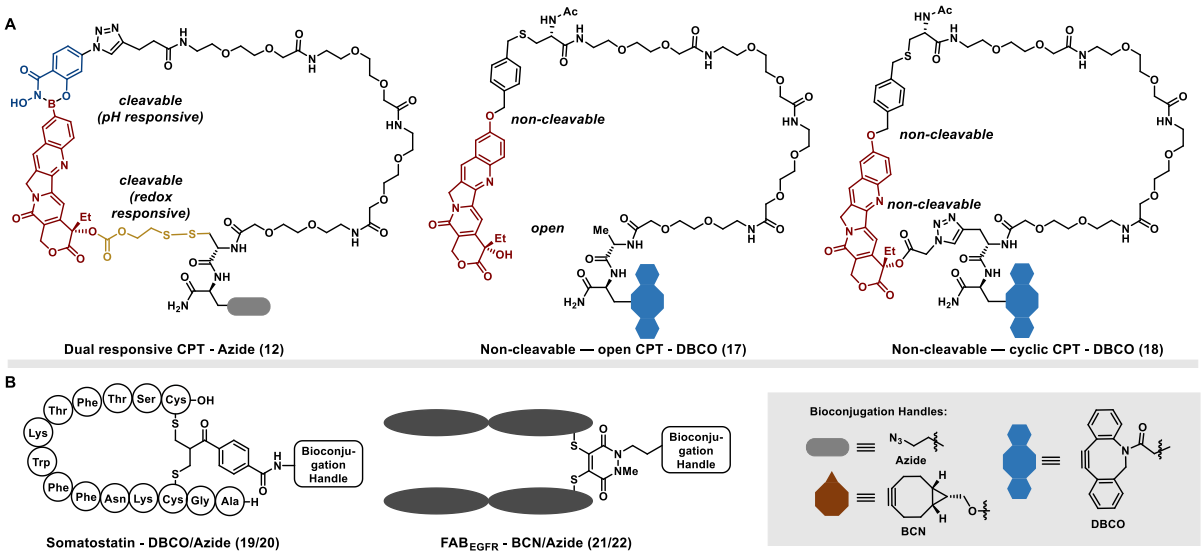


Figure 5: Prepared CPT derivatives and tumor selective targeting moieties.

To implement targeting through binding to overexpressed surface receptors on cancer cells, we further demonstrated the feasibility of post-functionalization of the newly designed macrocycle. Two different cancer cell-targeting entities were attached by strain-promoted copper-free azide-alkyne cycloaddition (SPAAC). First, the cyclic growth hormone peptide somatostatin, which binds to G-coupled protein receptors (SSTR), known to be overexpressed in triple negative MDA-MB-231 cancer cells was chosen (Figure 6A).[29] Radioligand binding assays of dual-responsive CPT-SST (**23**) showed binding to the somatostatin receptors of human recombinant CHO-K1 cells with low nanomolar IC_{50} value (3.5 nM, SI-Figure 55), suggesting that modified SST retains its receptor binding affinity expressed on the CHO-K1 cells. Thus, we further treated the prepared CPT-SST derivatives on MDA-MB-231 cells and determined their effects on cellular growth using Incucyte®. Dual-stimuli-responsive CPT-somatostatin construct **23**, which theoretically should enable traceless drug release, showed high toxicity in the cell proliferation assays (Figure 6D). Remarkably, the IC_{50} value was determined to be 119 ± 9 nM, which is in a similar range as 10-BA CPT (**6**, Figure 2C, 42.4 ± 1.1 nm). In clear contrast to this, cyclic CPT-somatostatin construct (**25**), retaining its "closed" ring structure in the TME showed no cell growth inhibition at this concentration range. Inhibitory effects were only observed starting at concentration of 5 µM. The IC_{50} value was estimated to be in the order of 10 µM, being ~100-times higher than the dual-responsive analog. The non-cleavable open CPT (**24**), which can be seen as an intermediate where one bond has already been cleaved, was more active than **25** (IC_{50} value 2.7 ± 0.1 µM), but still ~20-fold less potent

than the dual-responsive derivative **23**, corroborating that both traceless stimuli are required to regenerate the efficient chemotherapeutic.

Besides using small peptide somatostatin, we conjugated the anti-EGFR Cetuximab antibody fragment (FAB, MW ~50 kDa) with a single CPT derivative (Figure 6B). The epidermal growth factor receptor (EGFR) is highly expressed in different cancer types including MDA-MB-231, as shown by western blot analysis (SI-Figure 67). Thus, we conjugated the cyclic CPT to a FAB targeting EGFR as a proof-of-concept.[30] Cellular uptake of the Fab-drug conjugate in MDA-MB-231 cells was monitored using Alexa Fluor™ 594 labeled dual-responsive CPT-FAB (Alexa-**26**, Figure 6C), suggesting that the CPT-FAB conjugate penetrated into the EGFR-expressing MDA-MB-231 cells. A proliferation assay of dual-responsive CPT-FAB (**26**) displayed high drug toxicity with an IC_{50} value of 48.4 ± 1.6 nM (Figure 6E), which was comparable to 10-BA CPT (**6**). On the other hand, non-cleavable-cyclic CPT-FAB (**27**) displayed no noticeable toxicity up to the tested maximum concentration of 5 µM. These results clearly demonstrate that only the dual-stimuli-responsive CPT conjugates **23** and **26** showed high cell toxicity comparable to the unmodified drug 10-BA CPT (**6**). This further confirms the observation that traceless release is required for recovery of CPT activity. Notably, we also demonstrated that conjugation of the macrocyclic CPT to macromolecules such as peptides and proteins had no detrimental effects on the toxicity of the released CPT (Figure 6). Our results clearly indicate that the dual-responsive macrocycles designed herein represent novel “turn-on” drugs for selective and traceless release, a versatile approach for improving the delivery of existing small molecule therapeutics.

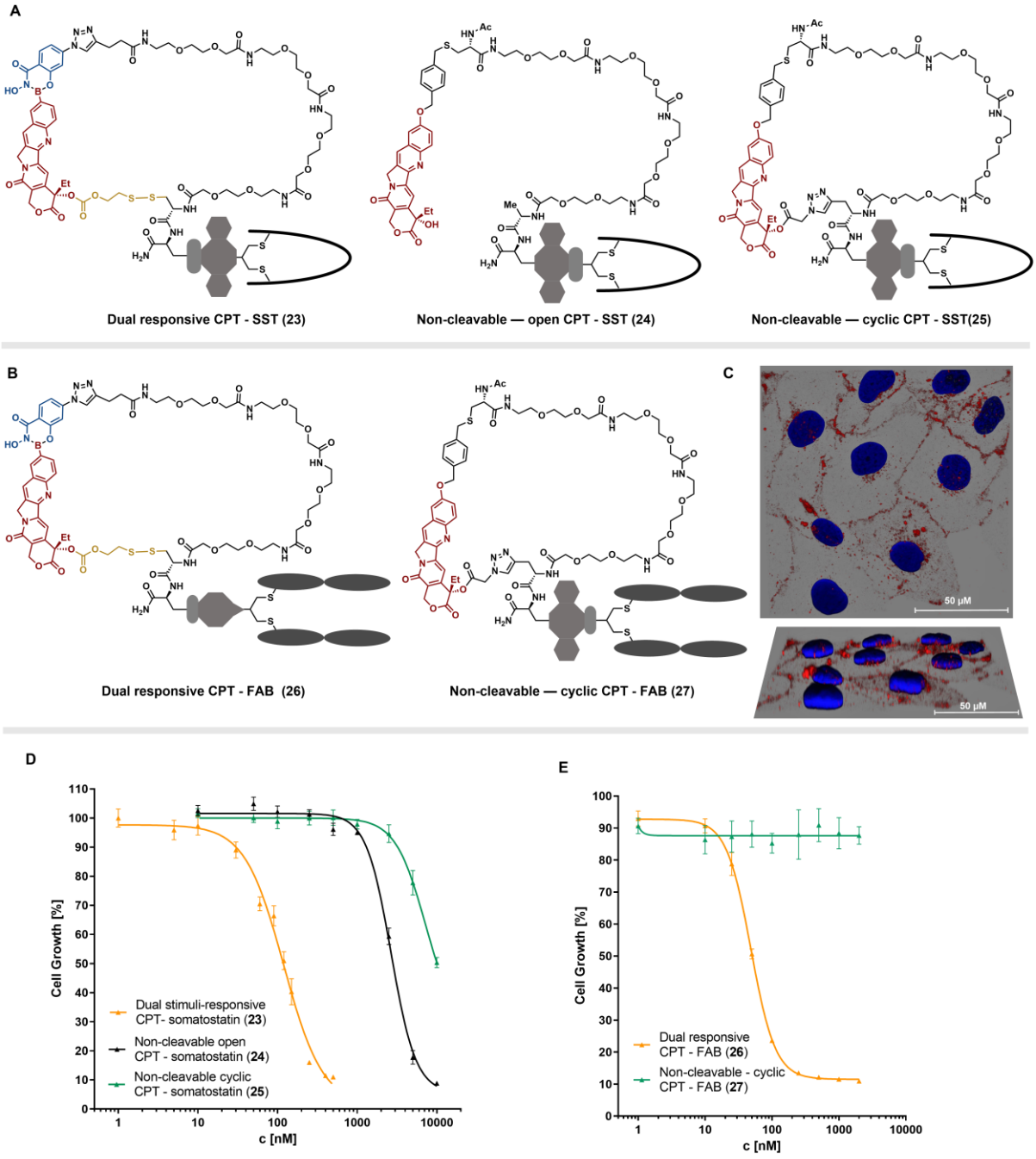


Figure 6: Structures of CPT derivatives conjugated to A: somatostatin and B: anti-EGFR, FAB fragment, which were subjected to proliferation essay of MDA-MB-231 cells. C: 3D visualization of z-stacked confocal laser scanning micrographs of MDA-MB-231 cells treated with 200 nM Alexa Fluor™ 594 labeled dual-responsive CPT - FAB (Alexa-**26**). Nucleus stained with Hoechst 33342 dye. D: Determination of IC_{50} values for somatostatin conjugates using **23**, **24**, and **25**. E: Determination of IC_{50} values for FAB conjugates using **26** and **27**.

Conclusion

In this work we demonstrate the potential of cooperative, dynamic covalent chemistry to direct the macrocyclization of a small molecule anti-cancer drug, CPT. Our strategy enables: (1) the incorporation of a targeting group that binds with high affinity to cell surface

receptors; (2) the combination of two cross-orthogonal chemistries to direct cyclization in a stimuli-responsive manner; and (3) traceless release of free drug to its active form. At the same time, the dual-latch mechanism holds great promise to overcome the current limitations of existing systems, i.e., to prevent premature release of the active drug in the extracellular and often acidic TME or disulfide burst release during circulation in the bloodstream. Furthermore, we were able to achieve proof-of-concept of the dual-locked cyclic CPT with two therapeutically relevant targeting entities: a cyclic peptide somatostatin that recognizes G-coupled protein receptor; and an antibody protein fragment of Cetuximab (anti-EGFR, FAB fragment). Notably, we demonstrated that release of the CPT from the cyclic drug conjugates took place in the intracellular space of cancer cells, with high recovery of CPT potency due to traceless release induced by stimuli present in the cytosol. Overall, we have thus devised a new strategy for the convenient synthesis of stimuli responsive macrocyclic drugs, as well as enhanced the features of current repertoire of small molecule therapeutics with the possibility for targeted delivery with controlled intracellular release. Furthermore, by introducing a boronic acid into the design, we provide new chemical space for the innovation of medicinal drugs, which holds immense promise for the pharmaceutical development of safer and more efficient biotherapeutics.

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