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Biochemical analysis of cellular target of *S*-trityl-L-cysteine derivatives using affinity matrix

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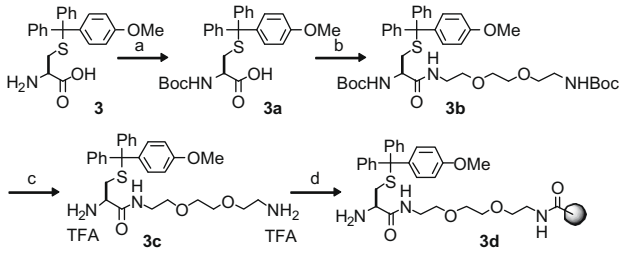
ABSTRACT

Biochemical analysis of the cellular target of *S*-trityl-L-cysteine (STLC) derivatives was performed by using the newly synthesized STLC derivative-immobilized affinity beads (**3d**). The affinity beads efficiently captured KSP in HCT116 cytoplasmic cell lysate. The results obtained from pull-down and competition experiments using **3d** with STLC derivatives provided the first evidence for direct interaction of these derivatives with KSP in cancer cells. Design, synthesis and application of **3d** were reported.

KSP is a member of the kinesin superfamily, and a microtubule motor protein, that is, essential for the formation of bipolar spindles and the proper segregation of sister chromatides during mitosis.[1–4] It belongs to the bimC family of kinesin-related microtubule-associated motor proteins, which hydrolyze ATP such that it moves toward the plus ends of microtubules.[5,6] Inhibition of KSP causes the formation of monopolar mitotic spindles, so-called monoasters, activates spindle assembly checkpoint, and arrests cells at mitosis, which leads to subsequent cell death.[1–3,7] The first small-molecular inhibitor of KSP, monastrol, was discovered in phenotype-based screening.[8] Monastrol specifically inhibits KSP ATPase activity without affecting interphase microtubules.[9] A number of KSP inhibitors have been found, some of which exhibited therapeutic potential as anticancer drugs.[10] Recently, we and another group rediscovered *S*-trityl-L-cysteine (STLC) derivatives as KSP ATPase inhibitors through the study of structure–activity relationships.[11,12] Several STLC derivatives such as compounds **1** and **3** were 7–10-fold more potent than original STLC, both in terms of KSP ATPase inhibition and HeLa cell cytotoxicity, and demonstrated M-phase accumulation in HeLa cells with 'monoasters', which is the typical phenotype of KSP inhibition.[11]

In order to analyze the interaction between STLC derivatives and cellular KSP, we prepared compound **3** immobilized on affinity beads (**3d**). As we previously reported, acylation of the amino terminal in **3** resulted in loss of potency in both of KSP ATPase inhibition and cytotoxicity, whereas modification of the carboxylic acid terminal in **3** (KSP ATPase IC_{50} = 0.15 μmol/L) to the methyl ester (KSP ATPase IC_{50} = 0.68 μmol/L) yielded little loss of KSP ATPase activity.[11] For that reason, we selected the carboxylic acid terminal of compound **3** as the immobilized position on Sepharose beads, where is believed to be tolerated affinity to KSP by the chemical modifications (Scheme 1). Specifically, we designed affinity beads **3d** with a widely used water soluble polyethylene glycol (PEG) linker, the length of which is expected maintain a sufficient distance from the KSP molecule. The synthetic procedure for the target affinity matrix is shown in Scheme 1. *N*-Boc-protected carboxylic acid **3a** (prepared from compound **3**) was condensed with *tert*-butyl 2-[2-(2-aminoethoxy)ethoxy)]ethylcarbamate to yield compound **3b**.[13] Deprotection of **3b** with 1 N HCl/AcOEt (purified by reversed phase HPLC), and subsequent condensation with NHS-activated Sepharose provided the desired **3**-immobilized affinity beads **3d**.[14] Incorporation of PEG linker to the carboxylic acid terminal in **3** preserved the KSP inhibitory activity (**3c**; KSP ATPase IC_{50} = 0.29 μmol/L).



Scheme 1. Reagents and conditions: (a) Boc_2O, 1 N NaOH aq, 1,4-dioxane, rt; (b) CDI, CH_2Cl_2, *tert*-butyl 2-[2-(2-aminoethoxy)ethoxy]ethylcarbamate, rt; (c) 1 N HCl/AcOEt, rt; (d) NHS-activated Sepharose.

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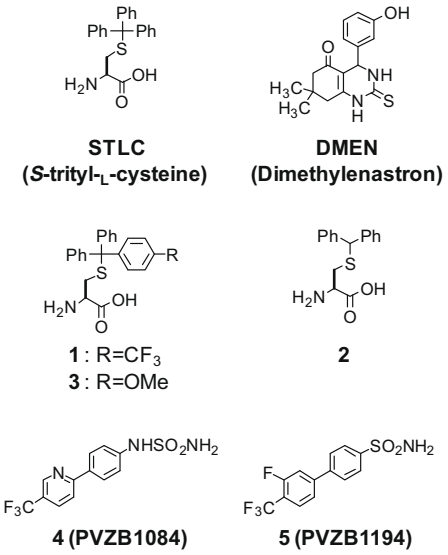

Figure 1. Chemical structure of STLC, DMEN and compounds **1**–**5**.

We examined the binding of drug-immobilized affinity beads **3d** to intrinsic KSP protein using cytoplasmic protein extract from human colon carcinoma cell-line HCT116.[15] Affinity beads **3d** were mixed with HCT116 cell extract and incubated at 4 °C for 2 h. The beads and the supernatant were collected separately by centrifuge, and the amount of KSP in these fractions was analyzed by Western blotting with anti-KSP antibody. As shown in Figure 2a, no band identical to KSP was observed when the control ethanolamine-fixed beads were employed, while the KSP band was clearly detected on the drug-fixed beads **3d** after incubation with the cell extract. On the other hand, KSP was detected in the supernatant after reaction with control beads, and KSP was hardly detected in the supernatant after reaction with **3d** (Fig. 2b). Thus, affinity beads **3d** have the ability to capture KSP efficiently from the mixture of proteins in cell extract.

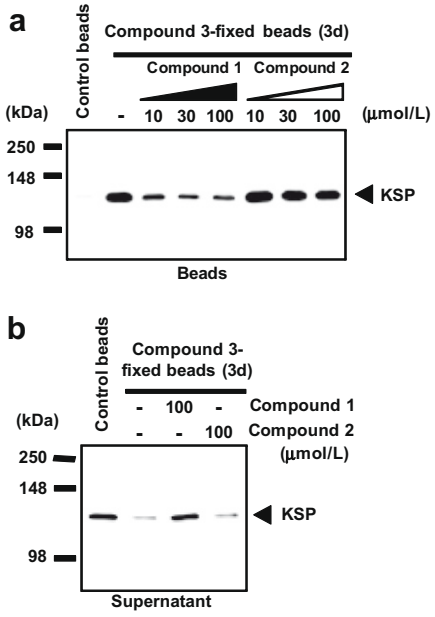

Figure 2. Binding of affinity beads **3d** and KSP extracted from HCT116 cells. Competitive inhibition of the binding between KSP and **3d** by STLC derivatives was evaluated by Western blot analysis of KSP bound to **3d** (a) and KSP in supernatant after reaction with **3d** (b).

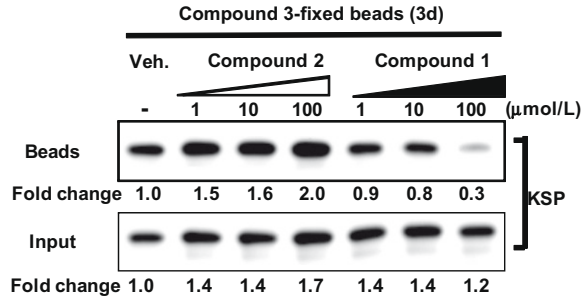

Figure 3. Binding of the affinity beads **3d** with KSP in cell extract prepared from drug treated HCT116 cells. Fold changes were determined from relative intensity compared to the vehicle control.

Next, we examined whether the binding of the beads **3d** and KSP would be inhibited by free STLC derivatives. Two representative STLC derivatives (Fig. 1) were used: compound **1** (KSP ATPase IC_{50} = 0.22 μmol/L, cytotoxicity IC_{50} = 0.53 μmol/L), which has strong ATPase inhibitory and cell-proliferation inhibitory activities, and compound **2** (KSP ATPase IC_{50} >63 μmol/L, cytotoxicity IC_{50} >50 μmol/L), which has weak activity.[11] Affinity beads **3d** were suspended with binding buffer and compound **1** or **2** with cell extract.[16] The amount of KSP bound with affinity beads **3d** decreased in a concentration-dependent manner when active STLC derivative **1** was added; however, the same reduction in KSP was not seen when inactive STLC derivative **2** was used (Fig. 2a). On the other hand, compound **1** restored the amount of KSP in the supernatant after treatment with beads **3d** but compound **2** did not (Fig. 2b). These results suggest that active STLC derivative **1** possesses binding activity to KSP and competitively inhibits the binding of KSP to beads **3d**, while inactive STLC derivative **2** does not. Although a higher amount of compound **1** was required, similar results were obtained when HCT116 cells were pre-treated with compound **1** for 3 h (Fig. 3). On the other hand, exposure of cells to compound **2** did not result in a decrease in the KSP band. Furthermore, KSP retained on affinity beads **3d** could be easily eluted with the addition of excess compound **1**, as shown in Figure 4. This result indicates the reversible nature of the interaction between KSP and STLC derivatives. This reversible binding of STLC derivatives to KSP was consistent with the results previously reported by Skoufias et al.[17]

We previously reported that compound **1** induced M-phase arrest with the monoastral phenotype, that is, typical to KSP inhibition in cells. In addition, KSP inhibitory activities in vitro correlated with cytotoxic activities in STLC derivatives.[11] Consequently, KSP was anticipated to be a cellular target of STLC and its derivatives. However, direct evidence that shows the interaction of STLC derivatives with cellular KSP has not been obtained to date. Taken together, our experiments using drug-immobilized affinity beads provide the first direct evidence of the interaction of STLC derivatives with KSP in cells and demonstrated that affinity beads **3d** are a useful tool for analyzing the mode of action of KSP inhibitors.

Using this system, we next investigated the interaction of KSP with different chemotypes of KSP ATPase inhibitors, such as dime-

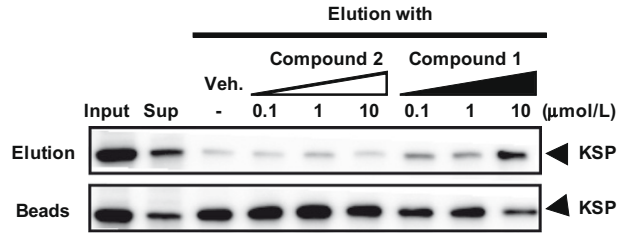

Figure 4. Reversibility of the binding between affinity beads **3d** and KSP.

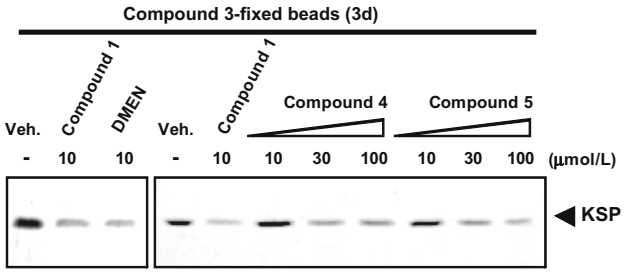

Figure 5. Competitive inhibition of the binding between **3d** and KSP by various KSP inhibitors.

thylenastron (DMEN; KSP ATPase IC_{50} = 1.1 μmol/L), and biaryl derivatives **4** (KSP ATPase IC_{50} = 0.70 μmol/L) and **5** (KSP ATPase IC_{50} = 0.12 μmol/L). These biaryl derivatives showed similar ATPase activity to STLC derivative **1** in vitro.[18] However, as shown in Figure 5, the KSP band was attenuated by 10 μmol/L STLC derivative **1** or DMEN, while no decrease in the band was observed when 10 μmol/L biaryl derivative **4** or **5** was added. These results demonstrated that the binding of KSP and affinity matrix **3d** was effectively inhibited by compound **1** or DMEN, but not by compound **4** or **5**. The addition of a higher amount of **4** or **5** (>30 μmol/L) resulted in a decrease in the KSP band. These results imply that biaryl derivatives have a different mode of binding from STLC derivatives for abrogation of ATPase activity of KSP. We have already reported that the biaryl derivatives inhibit KSP ATPase in a microtubule dependently, and that STLC derivatives inhibited KSP ATPase in a microtubule independently.[18] These observations might be due to the difference in the KSP binding sites between both chemotypes of inhibitors. Furthermore, it was suggested that the binding site of other biaryl derivatives was not the loop 5 allosteric site,[19] as distinct from most other KSP inhibitors, including STLC.[12,20] Further investigations on the detailed mode of action by biaryl derivatives are currently underway.

In conclusion, we synthesized an STLC derivative immobilized on affinity beads **3d** and firstly demonstrated the direct binding of STLC derivatives to KSP in cancer cells by using **3d**. Application of **3d** including, pull-down and competition experiments with a variety of KSP inhibitors could be successfully performed. Detailed investigations with **3d** will provide further insight into the mode of action of STLC derivatives and other chemotypes of KSP inhibitors.

Acknowledgment

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13. *Synthesis of* **3b**: To a solution of **3a** (493 mg, 1.00 mmol) in 10 mL of CH_2Cl_2 was added carbodiimidazole (211 mg, 1.30 mmol). After the mixture was stirred for 5 min at room temperature, *tert*-butyl 2-[2-(2-aminoethoxy)ethoxy]ethylcarbamate (496 mg, 2.00 mmol) was added. After the mixture was stirred overnight at room temperature, the mixture was partitioned between CH_2Cl_2 and H_2O, and the organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by silica gel chromatography (AcOEt/*n*-hexane = 2/3, v/v) to afford **3b** (461 mg, 64%) as amorphous material. ^1H NMR ($CDCl_3$) δ: 1.41–1.44 (18H, m), 2.50–2.54 (1H, m), 2.69–2.73 (1H, m), 3.28–3.31 (2H, m), 3.40–3.55 (10H, m), 3.79 (3H, s), 3.88–3.91 (1H, m), 4.90 (1H, br s), 5.09 (1H, br s), 6.48 (1H, br, s), 6.81 (2H, d, *J* = 8.8 Hz), 7.19–7.41 (12H, m); MS (ESI, $^+$) *m*/*z* 746 $[M+Na]^+$. *Synthesis of* **3c** (KSP ATPase IC_{50} = 0.29 μM): To a solution of **3b** (183 mg, 0.252 mmol) in 1.0 mL of AcOEt was added a solution of 1 N HCl in AcOEt (1.3 mL, 1.30 mmol). After the mixture was stirred for 7 h at room temperature, the mixture was concentrated. The residue was purified by a reversed phase HPLC to afford **3c** (73 mg, 56%) as amorphous material. ^1H NMR (DMSO-d_6) δ: 2.38–2.42 (2H, m), 2.92–2.96 (2H, m), 3.24–3.28 (2H, m), 3.46–3.57 (8H, m), 3.75 (3H, s), 3.75–3.77 (1H, m), 6.91 (2H, d, *J* = 8.8 Hz), 7.17 (2H, d, *J* = 8.8 Hz), 7.27–7.38 (10H, m), 7.83 (2H, br s), 8.28 (2H, br s), 8.58 (1H, br s); MS (ESI, $^+$) *m*/*z* 546 $[M+Na]^+$.
14. *Preparation of drug-immobilized beads* (**3d**)*:* NHS-activated Sepharose 4 Fast Flow (16–23 μmol/mL, 600 μL) was washed with 1 mmol/L HCl (7.50 mL) and H_2O (5.0 mL), and suspended in H_2O (500 μL). A solution of **3c** in DMSO (100 mmol/L, 140 μL) was added, and the suspension was brought to pH 8 with Et_3N and gently shaken 24 h at room temperature. The resin was filtered, washed with H_2O (1 mL, three times), 1 mmol/L ethanolamine (2 mL), 50 mmol/L Tris buffer (1 mL, pH 8), and 70 mmol/L AcOH (1 mL, pH 4). Tris buffer and AcOH washings were repeated three times and the resin was washed with ultrapure water (2 mL, three times), and stored in ultrapure water/ethanol (4/1, v/v) until use. For the control beads, a solution of ethanolamine in DMSO (100 mmol/L, 140 μL) was used instead of the solution of **3c** in DMSO.
15. HCT116 cells were trypsinized and collected by centrifugation and washed with ice-cold PBS. After removed supernatant, the pellet was resuspended in binding buffer (25 mmol/L Tris-OAc, pH 7.5, 5% glycerol, 20 mmol/L KCl, 5 mmol/L β-mercaptoethanol, 0.02% NP-40, 2 mmol/L $MgCl_2$) and lysed by three cycles of freezing and thawing.
16. Drug-immobilized or control ethanolamine-immobilized beads were washed with binding buffer [25 mmol/L Tris-OAc (pH 7.5), 5% glycerol, 20 mmol/L KCl, 5 mmol/L β-mercaptoethanol, 0.02% NP-40, 2 mmol/L $MgCl_2$]. Resuspended in binding buffer including DMSO or DMSO solution of STLC derivatives and incubated with 50 mg/mL of BSA and the 8 mg/mL cytoplasmic cell extract with continuous rotation for two hours at 4 °C. After centrifugation, supernatant were removed and the beads were washed three times with binding buffer. KSP bound on the beads or supernatant was analyzed by Western blotting with anti-KSP antibodies.
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