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Minimum structural requirements for inhibitors of the zinc finger protein TRAF6

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ABSTRACT

Zinc fingers have rarely been regarded as drug targets. On the contrary, the zinc-binding site of enzymes has often been considered a target of inhibitors. We previously developed a dithiol compound called SN-1 that binds to the zinc finger protein tumor necrosis factor receptor-associated factor 6 (TRAF6) and suppresses downstream nuclear factor-κB (NF-κB) signaling. To determine the minimal structure requirements of TRAF6 inhibitors based on SN-1, NF-κB inhibitory activity and cytotoxicity of its derivatives including new compounds were examined. SN-2, an oxidative type of prodrug of SN-1 with 2-nitrophenylthio groups via disulfide, has the minimum structure for an inhibitor of TRAF6, as seen with cellular experiments. The importance of two side chains with a thiol group was shown with molecular modelling. This study may lead to development of selective TRAF6 inhibitors in the near future.

Introduction

Zinc is often present in the catalytic center of enzymes that catalyze various biological reactions.[1] In other cases, zinc contributes to protein folding to maintain the stability of the protein,[2–5] and to the functions of the protein via formation of a zinc finger moiety.[1] Initially, zinc fingers were investigated as DNA binding motifs of transcription factors. Later, some cytoplasmic proteins were found to have zinc fingers that exert these functions. One explicit example is tumor necrosis factor receptor-associated factor 6 (TRAF6), a signaling transducer implicated in cancer and inflammation.[6,7] TRAF6 has five zinc fingers C-terminal to the N-terminal RING finger domain, which has E3 ubiquitin ligase activity against TRAF6 itself and other signaling proteins and leads to downstream signaling.[8–10] The first zinc finger regulates poly-ubiquitination of TRAF6.[11]

The zinc-binding site of enzymes has a pocket to accommodate these substrates,[12] and thus, this site has been considered a target of inhibitors. Among the inhibitors, captopril, an inhibitor of zinc-containing angiotensin-converting enzyme,[13] entered the market and is used widely. On the other hand, zinc fingers have been rarely regarded as drug targets. Previously, we synthesized a zinc protein-binding compound called SN-1[14–17] (Fig. 1). SN-1 binds to the zinc finger protein, TRAF6, and suppresses its ubiquitination and downstream nuclear factor-κB (NF-κB) signaling.[18] Introduction of an SN-1 recognition moiety that can specifically bind TRAF6 zinc fingers may eventually lead to a selective TRAF6 inhibitor drug. In our endeavors to achieve this goal, here we determined the minimal structure requirements of TRAF6 inhibitors based on SN-1.

SN-1 has two thiol groups, and thus, the compound is easily oxidized to cyclic disulfide oligomeric mixtures (SN-3) (Fig. 1). To surmount this obstacle, we have used a compound with 2-nitrophenylthio groups via disulfide (SN-2) as an oxidative type of prodrug of SN-1 in

Abbreviations: DTT, DL-dithiothreitol; IL-1α, interleukin-1α; MTT, 3-(4,5-dimethylthial-2-yl)-2,5-diphenyltetrazalium bromide; NF-κB, nuclear factor-κB; NpSH, *o*-nitrobenzenethiol; TRAF6, tumor necrosis factor receptor-associated factor 6

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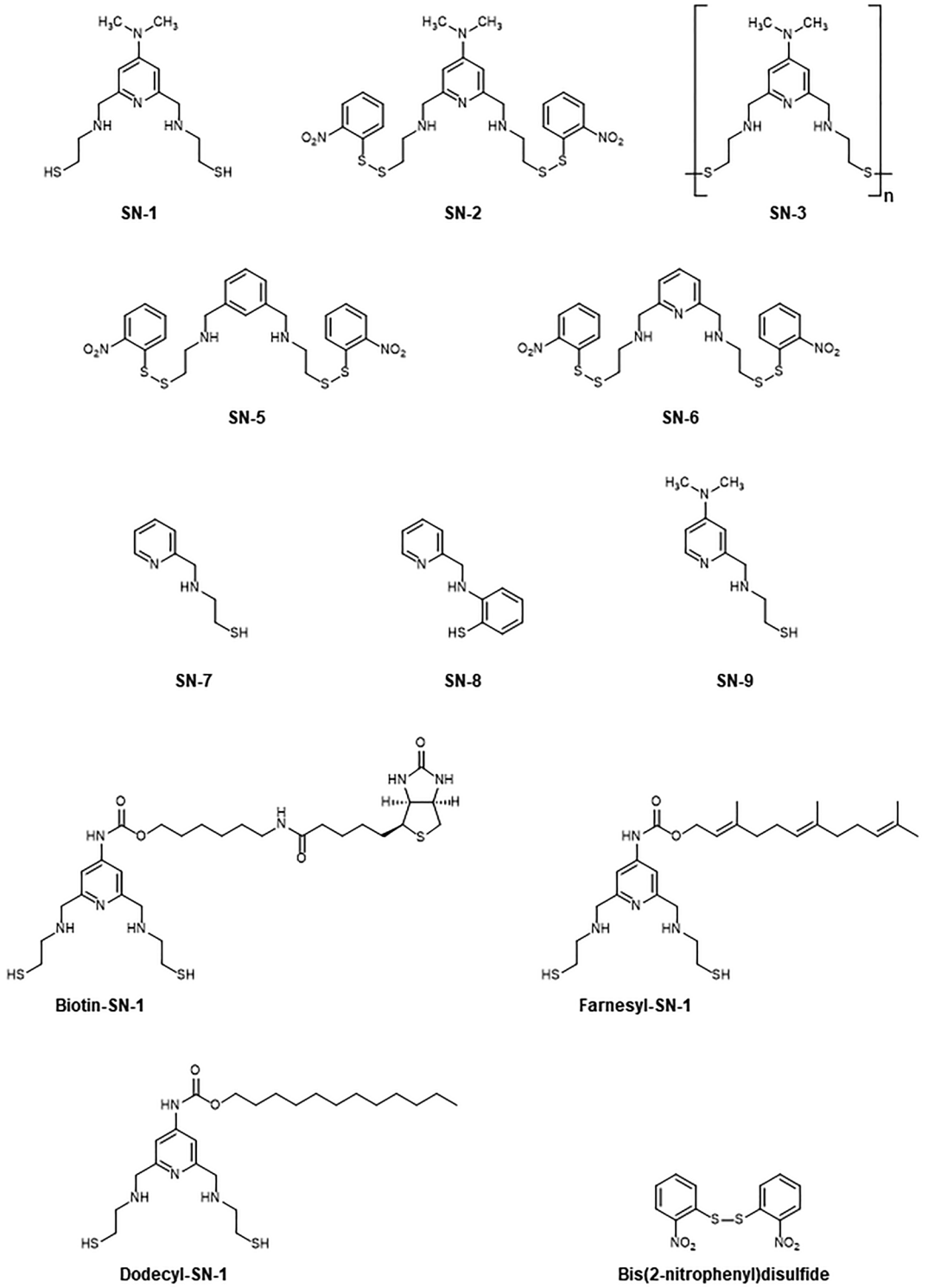
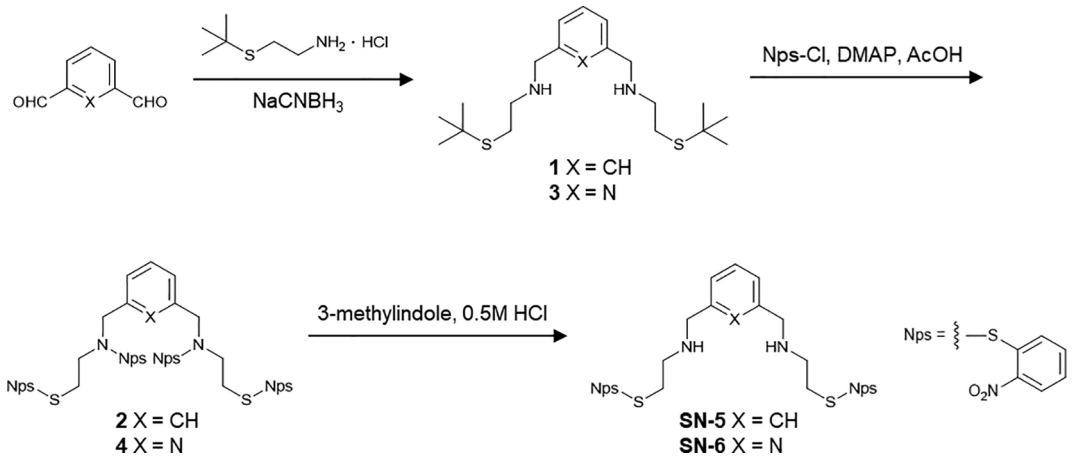


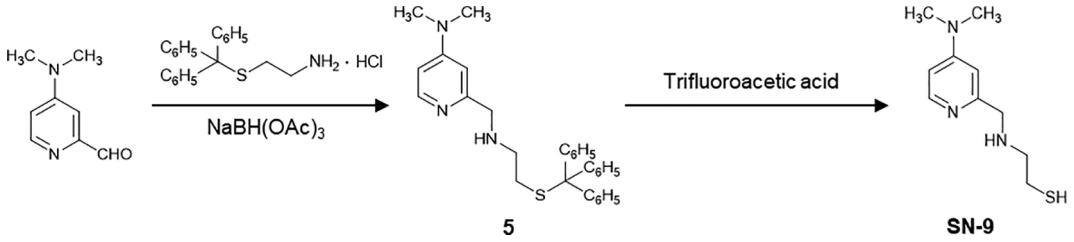
Fig. 1. Structures of SN-1 and its derivatives used in this study. SN-1[14], SN-2[14], SN-3[16], Biotin-SN-1[17], Farnesyl-SN-1[21], and Dodecyl-SN-1[21] were previously reported. SN-5, SN-6, SN-7, SN-8, and SN-9 were synthesized in this study. Bis(2-nitrophenyl)disulfide was commercially available.

cellular experiments.[16,18] To explore the structure-activity relationship in more detail, compounds SN-5 and SN-6 with phenyl and pyridinyl groups, respectively, instead of the 4-dimethylaminopyridinyl group of SN-2 were synthesized. Compound SN-9 that lacks one side chain of SN-1, and SN-7 that does not have the dimethylamino group present in SN-9 were also designed. Furthermore, compound SN-8 in which the mercaptoethylamino group is fixed by introduction of a phenyl ring was designed. In these compounds with one thiol group, complex oxidative products are expected to not be produced. Thus, we did not need to change the thiol group to a disulfide.

SN-5 and SN-6 were synthesized with the same method as SN-2[14] (Scheme 1). The corresponding aryl dialdehyde was reductively aminated with *S-tert*-butylcysteamine hydrochloride in the presence of sodium cyanoborohydride to give compounds **1** and **3**. Reacting **1**/**3** with 2-nitrophenylsulfenyl chloride followed by selective release of the nitrophenylsulfenyl moiety from the amino groups afforded SN-5/SN-6



Scheme 1. Synthetic route of SN-5 and SN-6.



Scheme 2. Synthetic route of SN-9.

via **2**/**4** (Scheme 1). The overall yields of SN-5 and SN-6 were 3.7% and 13%, respectively. SN-7 was obtained by a straightforward reaction between 2-(bromomethyl)pyridine hydrobromide and 2-aminoethanethiol hydrochloride in the presence of sodium bicarbonate (94% yield) as reported previously.[19] To obtain SN-8, 2-(pyridin-2-yl)-2,3-dihydrobenzo[*d*]thiazole was synthesized[20] (87% yield), and then its thiazoline ring was reductively opened with sodium borohydride[21] (70% yield), as previously described. SN-9 was synthesized by reductive amination of 4-(dimethylamino)picolinaldehyde with *S*-tritylcysteamine hydrochloride in the presence of triacetoxyborohydride (18% yield), followed by removal of the trityl group (22% yield) (Scheme 2). Whereas the yields of reductive amination products were generally low, the final compounds were obtained in good purity. Additionally, we included the previously reported compounds Biotin-SN-1,[17] Farnesyl-SN-1, and Dodecyl-SN-1[22] in our study to obtain more thorough structure-activity relationship information (Fig. 1).

To assess the inhibitory activity of TRAF6, its downstream NF-κB signaling was examined with a cellular reporter assay.[18,23] HeLa S3 cells co-transfected with 3κB-tk-luc (with a triple κB sequence that binds to NF-κB joined to a downstream firefly luciferase gene) and control vector pRL-Luc (with a β-actin promoter joined to a downstream renilla luciferase gene) were incubated for 1 day, and SN-1 or its derivative (10 μM) was added. After 1 h of incubation, cells were stimulated with interleukin-1α (IL-1 α) and further incubated for 3 h. Cells were lysed, and a dual-luciferase reporter assay was conducted. The value of luminescence after addition of a substrate of firefly luciferase was normalized to that using a substrate of renilla luciferase to determine NF-κB activity.

The result is shown in Fig. 2A. As previously reported, SN-2 showed a pronounced inhibitory effect (80%) against NF-κB activation.[18] SN-6 showed a similar effect (76%), whereas no activity of SN-3 was observed. SN-2 and SN-3 are expected to generate the same SN-1 by reductive agents inside cells, and thus, we deduced that SN-3 is a mixture of large molecules formed by oxidation of thiol groups. To correctly evaluate the activity of the reductive type of these compounds, DL-dithiothreitol (DTT) (30 μM) was added to the compounds first, and the experiment was repeated using the same conditions.

As shown in Fig. 2B, SN-3 showed similar activity (85%) to that of SN-2 (89%), demonstrating that this assay system is valid. Compared with SN-2, SN-6 had similar (84%) but slightly weaker activity, showing the small effect of a dimethylamino group on the pyridine ring. SN-5 did not show clear inhibitory activity. This demonstrates the importance of the nitrogen atom in the pyridine ring. Notably, 2-nitrobenzenethiol (NpSH), which is generated from Bis(2-nitrophenyl) disulfide and is also formed by reductive cleavage of SN-2, SN-5, and SN-6, did not show activity. SN-7, SN-8, and SN-9 lacked any detectable activity, implying the importance of the two side chains of the pyridine ring. Among Biotin-SN-1, Farnesyl-SN-1, and Dodecyl-SN-1, only Biotin-SN-1 showed activity (78%), although it was weaker than that of SN-3. This suggests that a long substituent at position 4 of the pyridine group is not a major obstacle to activity. The reason that Farnesyl-SN-1 and Dodecyl-SN-1 had no activity may be because these compounds localize to the membrane or to other proteins with a hydrophobic pocket. In these experiments, SN-2 showed the best activity to suppress downstream signaling of TRAF6.

The experimental results can be explained by our previous molecular docking study of SN-1 into the first zinc finger of TRAF6 using the Molecular Operating Environment.[18] Both the pyridyl nitrogen and two sulfurs bind to an amino acid or zinc of TRAF6, showing their importance (binding score −8.5 kcal/mol). Herein, we predicted that SN-9 interacts in the same shallow pocket. The ionized sulfur interacts with zinc and Cys[155] by ionic and H-bonds, respectively. One more

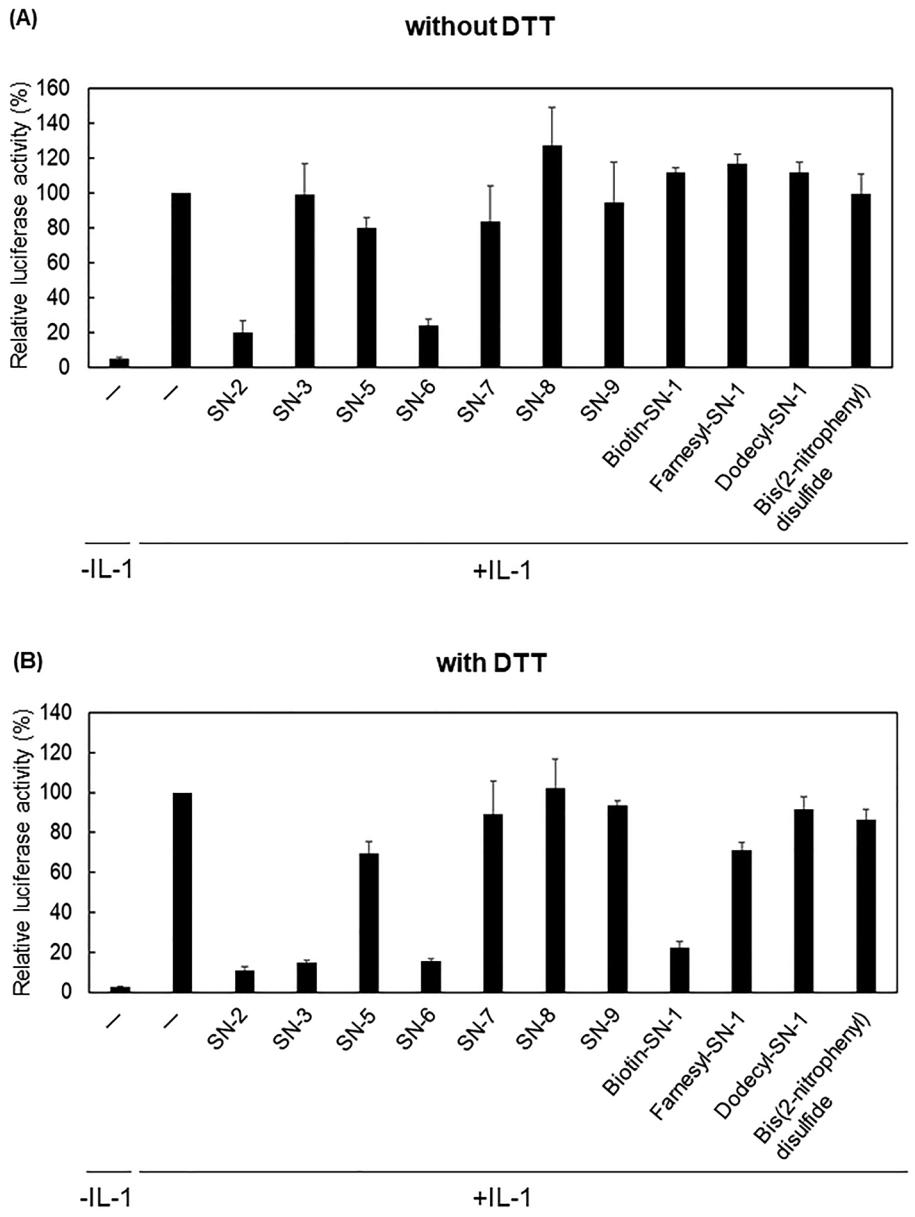


Fig. 2. Inhibitory activity against NF-κB activation determined by the reporter assay. HeLa S3 cells were transfected with 3κB-tk-luc and pRL-Luc and incubated for 1 day, followed by treatment with compounds (10 μM) in the absence (A) or presence (B) of DTT (30 μM) and incubated for 1 h. The cells were further treated with IL-1α (10 ng/mL), and the dual-luciferase reporter assay was performed 3 h post-transfection. The transcription activity from the κB site (in 3κB-tk-luc) was normalized to the activity from the β-actin promoter (in pRL-Luc). The relative NF-κB activity is shown.

intermolecular bond is formed between the pyridyl moiety and the His^{151} residue (binding score −6.2 kcal/mol) (Fig. 3). Visual analysis and binding score values can provide information about the key role of the second mercaptoethyl amino chain that conferred superior TRAF6 inhibitory activity on SN-1 by forming a more favorable complex with the first zinc finger of TRAF6.

Finally, the cytotoxicity of select compounds was examined. HeLa S3 cells were incubated with SN-2, SN-3, SN-6, Biotin-SN-1, or bis(2-nitrophenyl)disulfide with anti-NF-κB activity that were pretreated with DTT. An MTT [3-(4,5-Dimethylthial-2-yl)-2,5-Diphenyltetrazalium Bromide] assay was performed after 8 h of incubation, which was a longer time than that in the experiment to determine NF-κB activity. As shown in Fig. 4, SN-3 induced death in around half of the cells. In contrast, SN-2 did not show cytotoxicity. Both SN-2 and SN-3 are expected to generate SN-1 due to reductive agents in the cells. The difference is that only SN-2 will generate NpSH. One plausible mechanism for the absence of toxicity of SN-2 is that NpSH inhibits the interaction of SN-2 with various proteins to exert toxicity. However, NpSH itself shows weak toxicity (24%) (Fig. 4), and thus, the mechanism is not simple. Importantly, SN-6 showed cytotoxicity unlike SN-2, showing that the dimethylamino group in the pyridine ring decreases toxicity. Biotin-SN-1 with a long substituent had similar toxicity to that of SN-3 with a dimethylamino group.

Taken together, our results suggest that SN-2 has the structure with the best activity to suppress downstream signaling by binding to TRAF6. Especially in SN-2, the nitrogen atom of the pyridine ring and

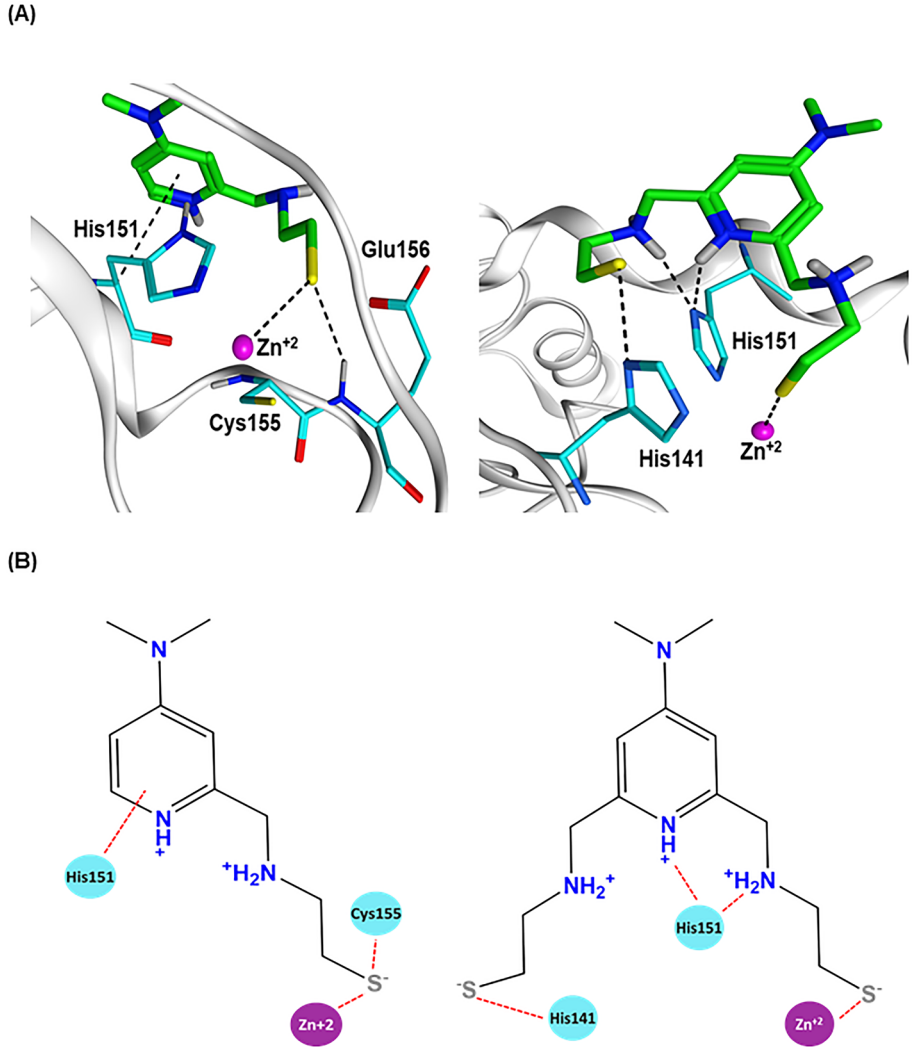


Fig. 3. Binding mode of SN-1 or SN-9 to the first zinc finger of TRAF6 (PDB ID: 3HCS) predicted by Molecular Operating Environment. The modeling of SN-1 was described previously.[18] (A) 3D ribbon diagram of SN-9 (left) and SN-1 (right). Hydrogen bonds between compounds and TRAF6 are shown as black dashed lines. Amino acids interacting with the compounds are shown in pale blue, and the compounds are in green. Nitrogen, oxygen, and sulfur are colored in blue, red, and yellow, respectively. (B) 2D depiction of SN-9 (left) and SN-1 (right). Hydrogen bonds between compounds and TRAF6 are shown as red dashed lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

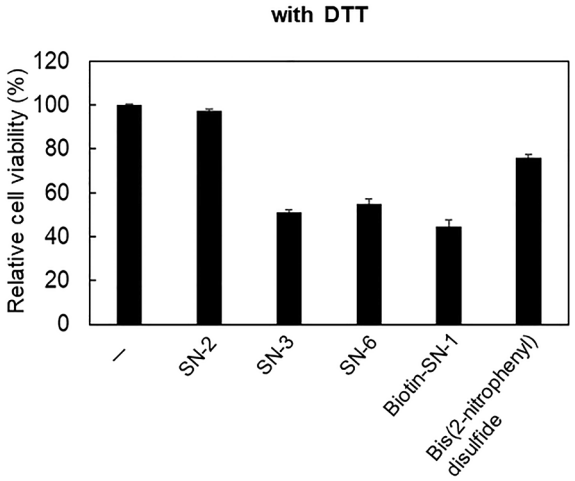


Fig. 4. Cytotoxic activity of compounds. HeLa S3 cells were treated with compounds (10 μM) in the presence of DTT (30 μM). After 8 h of incubation, the MTT assay was performed. The relative activity is shown.

its two side chains are required for the activity. The dimethylamino group of the pyridine ring and the nitrophenylsulfenyl group are important for decreasing cytotoxicity. We conclude that SN-2 has the minimum structure for an inhibitor of TRAF6 as shown with the cellular experiments. This study may become the basis for a new category of drugs targeting zinc fingers. We have already succeeded in adding target specificity to SN-1 by changing its structure[15] or introducing a moiety to selectively bind to target proteins[22,24]. Development of TRAF6-specific inhibitors based on SN-2 is in progress.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.06.050.

References

1. Coleman JE. Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins. *Annu Rev Biochem.* 1992;61:897–946.
2. Knaus T, Uhl MK, Monschein S, Moratti S, Gruber K, Macheroux P. Structure and stability of an unusual zinc-binding protein from Bacteroides thetaiotaomicron. *BBA.* 2014;1844(12):2298–2305.
3. Iannuzzi C, Adrover M, Puglisi R, Yan R, Temussi PA, Pastore A. The role of zinc in the stability of the marginally stable IscU scaffold protein. *Protein Sci.* 2014;23(9):1208–1219.

4. Yamamoto M, Koga R, Fujino H, et al. Zinc-binding site of human immunodeficiency virus 2 Vpx prevents instability and dysfunction of the protein. *J Gen Virol.* 2017;98(2):275–283.
5. Koga R, Yamamoto M, Ciftci HI, Otsuka M, Fujita M. Introduction of H2C2-type zinc-binding residues into HIV-2 Vpr increases its expression level. *FEBS Open Bio.* 2018;8(1):146–153.
6. Inoue J, Gohda J, Akiyama T. Characteristics and biological functions of TRAF6. *Adv Exp Med Biol.* 2007;597:72–79.
7. Walsh MC, Lee J, Choi Y. Tumor necrosis factor receptor- associated factor 6 (TRAF6) regulation of development, function, and homeostasis of the immune system. *Immunol Rev.* 2015;266(1):72–92.
8. Deng L, Wang C, Spencer E, et al. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell.* 2000;103(2):351–361.
9. Sun L, Deng L, Ea CK, Xia ZP, Chen ZJ. The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. *Mol Cell.* 2004;14(3):289–301.
10. Yamazaki K, Gohda J, Kanayama A, et al. Two mechanistically and temporally distinct NF-kappaB activation pathways in IL-1 signaling. *Sci Signal.* 2009;2(93):ra66.
11. Lamothe B, Campos AD, Webster WK, Gopinathan A, Hur L, Darnay BG. The RING domain and first zinc finger of TRAF6 coordinate signaling by interleukin-1, lipopolysaccharide, and RANKL. *J. Biol. Chem.* 2008;283(36):24871–24880.
12. Supuran CT, Winum JY, eds. *Drug design of zinc-enzyme inhibitors: functional, structural, and disease applications.* Wiley; 2009.
13. Cushman DW, Ondetti MA. Design of angiotensin converting enzyme inhibitors. *Nat Med.* 1999;5(10):1110–1113.
14. Fujita M, Otsuka M, Sugiura Y. Metal-chelating inhibitors of a zinc finger protein HIV-EP1. Remarkable potentiation of inhibitory activity by introduction of SH groups. *J Med Chem.* 1996;39(2):503–507.
15. Otsuka M, Fujita M, Sugiura Y, et al. Synthetic inhibitors of regulatory proteins involved in the signaling pathway of the replication of human immunodeficiency virus 1. *Bioorg Med Chem.* 1997;5(1):205–215.
16. Ejima T, Hirota M, Mizukami T, Otsuka M, Fujita M. An anti-HIV-1 compound that increases steady-state expression of apoplipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G. *Int J Mol Med.* 2011;28(4):613–616.
17. Radwan MO, Sonoda S, Ejima T, et al. Zinc-mediated binding of a low-molecular-weight stabilizer of the host anti-viral factor apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G. *Bioorg Med Chem.* 2016;24(18):4398–4405.
18. Koga R, Radwan MO, Ejima T, et al. A dithiol compound binds to the zinc finger protein TRAF6 and suppresses its ubiquitination. *ChemMedChem.* 2017;12(23):1935–1941.
19. De Almeida MV, Chaves JDS, Fontes APS, César ET, Gielen M. Synthesis and characterization of platinum(II) complexes from trifluoromethyl phenylenediamine, picoline and N-benzyl ethylenediamine derivatives. *J Braz Chem Soc.* 2006;17(7):1266–1273.
20. Carlson LJ, Welby J, Zebrowski KA, et al. Spectroscopic differences between heterocyclic benzothiazoline, -thiazole and imine containing ligands and comparison of the Co and Cu pyridine benzothiazole and imine complexes. *Inorg Chim Acta.* 2011;365(1):159–166.
21. Brand U, Vahrenkamp H. Zinc complexes of the N, N, S ligand (mercaptophenyl) (picolyl)amine. *Chem Ber.* 1996;129(4):435–440.
22. Tanaka A, Radwan MO, Hamasaki A, et al. A novel inhibitor of farnesyltransferase with a zinc site recognition moiety and a farnesyl group. *Bioorg Med Chem Lett.* 2017;27(16):3862–3866.
23. Kanemaru Y, Momiki Y, Matsuura S, et al. An artificial copper complex incorporating a cell-penetrating peptide inhibits nuclear factor-kappaB (NF-kappaB) activation. *Chem Pharm Bull.* 2011;59(12):1555–1558.
24. Hamasaki A, Naka H, Tamanoi F, Umezawa K, Otsuka M. A novel metal-chelating inhibitor of protein farnesyltransferase. *Bioorg Med Chem Lett.* 2003;13(9):1523–1526.