A New Inhibitor of ADAM17 Composed of a Zinc-Binding Dithiol Moiety and a Specificity Pocket-Binding Appendage

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A disintegrin and metalloproteinase 17 (ADAM17) is a zinc-dependent enzyme that catalyzes the cleavage of the extracellular domains of various transmembrane proteins. ADAM17 is regarded as a promising drug target for the suppression of various diseases, including cancer metastasis. We synthesized a new ADAM17 inhibitor, SN-4, composed of a zinc-binding dithiol moiety and an appendage that specifically binds to a pocket of ADAM17. We show that SN-4 inhibits the ability of ADAM17 to cleave tumor necrosis factor α (TNF-α) in vitro. This activity was reduced by the addition of zinc, indicating the importance of the zinc chelating dithiol moiety. Inhibition of TNF-α cleavage by SN-4 in cells was also observed, and with an IC 50 of 3.22 µM, SN-4 showed slightly higher activity than the well-studied ADAM17 inhibitor marimastat. Furthermore, SN-4 was shown to inhibit cleavage of CD44 by ADAM17, but not by ADAM10, and to suppress cell invasion. Molecular docking showed good fitting of the specificity pocket-binding group and one SH of SN-4 and hinted at possible means of structural optimization. This study provides clues for the development of potent and selective ADAM17 inhibitors.

Key words A disintegrin and metalloproteinase 17 (ADAM17) inhibitor; dithiol; zinc-binding

Introduction

Despite recent progress in the development of new therapies, cancer remains one of the leading causes of death worldwide.1 Cancer is notoriously difficult to treat once it has metastasized.2–4 Therefore, the molecular mechanisms behind metastasis have been extensively studied, and the development of anti-metastasis drugs2–4 has been attempted, albeit with limited success.

A well-studied protein related to metastasis is a disintegrin and metalloproteinase 17 (ADAM17), a member of the ADAM family of metallopeptinases, which has been identified as a tumor necrosis factor-α (TNF-α)-converting enzyme.5,6 ADAM17 is a membrane protein and contains a catalytic center with zinc7 in its extracellular domain. It functions by catalyzing the cleavage of the extracellular domains of other transmembrane proteins and is triggered by activation of signaling proteins such as protein kinase C, Rac, and mitogen-activated protein kinase 8, 9. Some of its substrates are important for cell adhesion; for example, CD44, which interacts with hyaluronic acid, is cleaved by ADAM17 to induce metastatic spread and invasion of cancer cells.10 Thus, ADAM17 is regarded as a promising drug target for the suppression of metastasis. Many inhibitors of ADAM17 have been synthesized,11–14 most of which comprise hydroxamic acid, which is a general zinc-binding group, and a moiety that interacts with a pocket in the vicinity of the zinc ion. However, the hydroxamic acid moiety has been shown to cause side effects in vivo,15 and some ADAM17 inhibitors with hydroxamic acid have failed clinical testing because of this toxicity.11,14 We herein synthesized a new inhibitor of ADAM17, and its activity was evaluated.

Results and Discussion

Design and Synthesis

We previously reported a zinc-binding molecule SN-1 (Fig. 1) containing two SH groups16 and demonstrated that SN-1 and its derivatives bind and regulate the function of various zinc proteins, including human immunodeficiency virus-1 (HIV-1) enhancer binding protein,17 apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G (APOBEC3G),17,18 farnesyltransferase,19,20 and TNF receptor-associated factor 6 (TRAF6).21-22 Molecular docking indicated that one SH binds to the zinc ion and the other interacts with neighboring amino acids.18,20–22 We reasoned that SN-1 would attain ADAM17 selectivity by the introduction of a 4-(but-2-yn-1-yloxy)benzenesulfonyl amide group, which selectively binds to the S1′ pocket of ADAM17,23-25 and designed such a derivative, SN-4 (Fig. 1).

Dithiol is easily air-oxidized to disulfide. We previously reported that an oxidized form of SN-1, produced by the introduction of two Nps groups, works as a prodrug and releases SN-1 under reducing conditions inside cells more efficiently than intra- or inter-molecular oxidized forms of SN-1.22 Thus, SN-4(Nps)2 (Fig. 1) was synthesized as a prodrug of SN-4.

The synthesis was carried out as shown in Chart 1. Reductive amination of aldehyde 1 using 2-(tert-butyllthio)-
ethan-1-amine afforded 2 (84%). Secondary amine 2 was treated with 4-(2-butyn-1-yloxy)benzenesulfonyl chloride and Et₃N to give acetal 3 (65%), which was converted to aldehyde 4 (80%) under acidic conditions. 2-(tert-Butylthio)ethan-1-amine was condensed with aldehyde 4 followed by reduction with NaCNBH₃ to yield 5 (64%). Treatment of 5 with 2-nitrobenzenesulfonyl chloride (Nps-Cl) provided 6, and the Nps group on the amino nitrogen of 6 was selectively removed by 3-methylindole to afford the final SN-4(Nps)₂ compound (54%).

**Biological Evaluation** The ADAM17 inhibitory activity of SN-4 was examined. TNF-α is a well-known substrate of ADAM17, and soluble TNF-α (sTNF-α) is released by ADAM17-mediated extracellular domain cleavage. To examine ADAM17 inhibition by SN-4 in vitro, ADAM17, full-length TNF-α (pro-TNF-α), and SN-4 generated from SN-4(Nps)₂ and three equivalents of dithiothreitol (DTT) were used. Immunoblot analysis showed that pro-TNF-α was cleaved by ADAM17, and this activity was suppressed by the addition of SN-4 (Fig. 2A, lane 1–3). The same experiment in the presence of zinc ions resulted in a decrease in SN-4 activity (Fig. 2A, lane 4), demonstrating the importance of the zinc chelating function of the dithiol moiety. Next, cellular experiments were conducted. Stimulation of differentiated THP-1 cells by lipopolysaccharide (LPS) activates ADAM17 to release sTNF-α. Here, THP-1 cells were incubated with SN-4(Nps)₂ or marimastat (control, Fig. 2B) before the addition of LPS. After incubation, the amount of sTNF-α in the supernatant was evaluated by immunoblotting. As shown in Figs. 2C and D, the increase of sTNF-α by LPS stimulation was inhibited by SN-4 and marimastat in a dose-dependent manner. Using enzyme-linked immunosorbent assay (ELISA), we then determined the IC₅₀ values of SN-4 and marimastat, which were 3.22 and 4.75 µM, respectively (Figs. 2E, F). The IC₅₀ value of...
marimastat was almost identical to that reported, and SN-4 showed slightly higher activity than marimastat. Furthermore, like marimastat, SN-4 showed no toxicity at 0.1–10 µM (Fig. 2G). Together, these results demonstrate the ability of SN-4 to inhibit ADAM17.

The selectivity of SN-4 was then examined using two highly similar metalloproteinases, ADAM17 and ADAM10. Cleavage of the extracellular domain of CD44 is known to be regulated by ADAM10 and ADAM17 in response to distinct stimulation; ionomycin and PMA activate ADAM10 and ADAM17, respectively. U251 MG cells were incubated with SN-4(Nps)2 or marimastat followed by addition of ionomycin or PMA. After incubation, CD44EXT, generated by cleavage together with soluble CD44, was evaluated by immunoblotting. In the case of ionomycin treatment, CD44 cleavage was inhibited by marimastat but not by SN-4 (Figs. 3A–C). On the other hand, both compounds inhibited CD44 cleavage by PMA stimulation (Figs. 3D–F). This is consistent with a previous report that showed that marimastat inhibits both ADAM10 and ADAM17. In contrast, SN-4 selectively inhibited ADAM17. Furthermore, fluorescent immunostaining confirmed that CD44 was maintained on the membranes of PMA-treated cells after addition of SN-4 or marimastat (Fig. 3G).

Since CD44 is important for the adhesion of cells, we assessed whether SN-4 inhibits cellular invasion. U252 MG cells were cultured on a Transwell plate in the presence of SN-4 for 24 h, and invaded cells were stained with crystal violet (Fig. 3H) or evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 3I). The results showed that SN-4 clearly suppresses the invasion of cells to the same magnitude as that of marimastat.

**Molecular Docking Study**

Selective ADAM17 inhibition of SN-4 could be attributed to binding of its 4-(but-2-yn-1-yloxy) benzenesulfonyl amide group to the S1 pocket of ADAM17. To test this, a molecular docking study of ADAM17-SN-4 was performed. As the template, we employed the X-ray crystal structure (PDB code: 2OI0) of ADAM17 co-crystalized with an aryl sulfonamide 4b, which has the same ADAM17-binding 4-(but-2-yn-1-yloxy)-benzenesulfonyl amide group. In this model, shown in Fig. 4A, the ADAM17 binding moiety of SN-4 can almost be overlaid on the co-crystalized compound 4b, occupying the S1 pocket, extending through a narrow channel, and reaching the S3 subsite. This imparts a significant selectivity towards ADAM-17 over other metalloproteinases in which this channel is blocked by a conserved tyrosine. As shown in Fig. 4B, the sulfone linker contributes to overall complex stability via hydrogen bonding with Leu348 and Gly349. Furthermore, the SH group of SN-4 strongly coordinates with the active site zinc ion, affording a stable tetrahedron with amino...
Concomitantly, the pyridinium nitrogen is implicated in hydrogen bonding with water molecule HOH578. Of note, the other SH group lies in proximity to hydrophobic amino acid residues (Val314, Lys315, Leu350, and Ala351) in the neighboring subsite, but its interaction with the amino acids could not been observed.

**Conclusion**

In this study, an ADAM17 specificity pocket-binding moiety was introduced into zinc-binding dithiol molecule SN-1 to construct a new compound, SN-4. SN-4 was shown to selectively inhibit ADAM17, cleave the extracellular domains of TNF-α and CD44, and suppress cell invasion. The molecular docking study showed accommodation of the specificity pocket-binding group leading to specific ADAM17 inhibitory activity and interaction of one SH with the zinc ion, while the other SH group was shown to be free. Modification of the dithiol group to optimize fitting to neighboring amino acids could increase the inhibitory activity of SN-4. Recently, we reported compounds that suppress the expression of CD44 and migration of cells. Together with these compounds, SN-4 is considered to be a lead compound for the development of anti-metastasis drugs in the near future. Furthermore, since ADAM17 was reported to play an important role in symptoms of coronavirus disease 2019 (COVID-19), this study may also be useful in the fight against the ongoing pandemic.

**Experimental**

**General Procedure Pertaining to Synthesis** All reagents were commercially available with high purity grade. TLC was performed on precoated plates, TLC sheets silica 60 F254 (Merck, Darmstadt, Germany) or TLC sheets Chromatorex NH silica (Fuji Silysia Chemical, Kasugai, Japan). Chromatography was carried out on Silica Gel 60N (40–100 mesh)
(Kanto Chemical, Tokyo, Japan) and NH silica gel Chromatorex (NH, 100–200 mesh) (Fuji Silysia Chemical). NMR spectra were recorded on a JEOL (Tokyo, Japan) JNM-AL300 (300MHz) and Bruker (Billerica, MA, U.S.A.) Avance 600 (600MHz). Chemical shifts were referenced to tetramethylsilane (TMS). Mass spectrum (FAB) and high-resolution mass spectra (HRMS) were recorded by a JEOL JMS-DX303. HRMS were recorded by using positive fast atom bombardment (FAB) with 3-nitrobenzyl alcohol (NBA) as the matrix. IR-red spectra were recorded on a JASCO (Tokyo, Japan) FT/IR-410. The samples were prepared as KBr discs or thin films between sodium chloride discs. Melting points were determined on a Yanaco (Kyoto, Japan) melting point apparatus and were uncorrected.

**Synthesis of SN-4(Nps)₂**

2-({[2-(tert-Butylthio)ethyl]amino}methyl)-6-(diethoxymethyl)-N,N-dimethylpyridin-4-amine (2).

Aldehyde 1⁵⁶ (1.6 g, 6.4mmol) was dissolved in 45mL of 1:1 MeOH of dist. MeOH, molecular sieves 4A and (tert-butylthio)ethan-1-amine (2.7 g, 16mmol) were added, and the mixture was stirred under an argon atmosphere for 25h. Then, NaCNBH₃ (1.2 g, 19mmol) was added, and the mixture was stirred for 20h. The mixture was filtered to remove molecular sieves, 10 mL of purified water was added, and the mixture was stirred for 30min, and then the solvent was evaporated under reduced pressure. After adding 10 mL of purified water to the residue and extracting with CH₂Cl₂ (30 mL × 3), the organic layer was dried with MgSO₄. The organic layer was concentrated under reduced pressure and purified by column chromatography (CH₂Cl₂:MeOH = 20:1). 2 (2.0 g, 99%) was obtained as a brown oil. ¹H-NMR (CDCl₃) δ: 1.24 (6H,

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Fig. 4. Molecular Docking Analysis of the Binding Mode of SN-4 to the Catalytic Site of ADAM17

(A) Superimposed SN-4 (green) and the co-crystalized native ligand 4b shown on the right (magenta) placed into the ADAM17 active site (PDB code: 2OI0). Molecular Operating Environment was used for the calculations as previously described.⁶⁵ (B) 3D binding mode of SN-4 in the ADAM17 active site and neighboring subsite for the analysis conducted in (A). Amino acid residues and water molecule are shown in off-white, and the zinc ion is the cyan sphere. Hetero-atoms are colored by element and intermolecular bonds are represented by the black dashed lines. Amino acids, water molecule, and zinc ion, which directly interact with SN-4 are black-colored, and the others are red-colored. (Color figure can be accessed in the online version.)
t, \( J = 7.03 \text{ Hz}, \text{CH}_2 \times 2 \), 1.32 (9H, s, \text{CH}_3 \times 3), 2.73 (2H, t, \( J = 6.57 \text{ Hz} \)), 2.84 (2H, \( J = 6.03 \text{ Hz} \)), 3.01 (6H, s, \text{CH}_2 \times 2), 3.55–3.75 (4H, m, \text{CH}_2 \times 2), 3.87 (2H, s, \text{CH}_2), 5.32 (1H, s, \text{CH}) , 6.35 (1H, s, \text{CH}_3 \text{N(CH)}_2 \), 6.71 (1H, s, \text{CH}_3 \text{N(CH)}_2 ). \( ^{13}\text{C}-\text{NMR} \) (CDCl\(_3\)) \( \delta \): 151.7, 27.6, 30.9, 40.1, 42.6, 48.7, 63.1, 98.9, 103.7, 104.6, 151.4, 154.4, 157.4. FAB-MS (m/z): 370 (M + H)\(^+\).

4-(But-2-yn-1-yl)-N-[2-( tert-butylthio)ethyl] N-[(6- (diethylamino)-4-(dimethylamino)pyridin-2-yl)methyl] benzenesulfonamide (3):

Amine 2 (2.0 g, 5.4 mmol) was dissolved in 18 mL of tetrahydrofuran (THF) and 0.98 mL of Et\(_3\)N, 4-(but-2-yn-1-yl)benzenesulfonfonyl chloride \( ^{27} \) (2.0 g, 8.1 mmol) dissolved in 7 mL of THF was added at 0°C, and the mixture was stirred at room temperature for 11 h. Then, water was added to make the mixture at 10 h. The mixture was stirred for 10 min. The solution was concentrated under reduced pressure and extracted with CH\(_2\)Cl\(_2\). The extract was dried over MgSO\(_4\) and concentrated under reduced pressure. The residue was purified by column chromatography (CH\(_2\)Cl\(_2\) : MeOH = 20 : 1) to afford 5 (1.1 g, 64%) as a brown liquid. \( ^{1}\text{H}-\text{NMR} \) (CDCl\(_3\)) \( \delta \): 1.21 (9H, s, \text{CH}_3 \times 3), 1.32 (9H, s, \text{CH}_3 \times 3), 1.88 (3H, s, \text{CH}_3), 2.13 (1H, s, NH), 2.57 (2H, \( J = 8.11 \text{ Hz} \)), 2.72 (2H, \( J = 6.61 \text{ Hz} \)), 2.83 (2H, \( J = 6.66 \text{ Hz} \)), 2.99 (6H, s, \text{CH}_2 \times 2), 3.28 (2H, \( J = 8.11 \text{ Hz} \)), 3.72 (2H, s, CH), 4.35 (2H, s, CH), 4.71 (2H, s, CH), 6.46 (1H, s, \text{CH}_3 \text{N(CH)}_2 \), 6.54 (1H, s, \text{CH}_3 \text{N(CH)}_2 \), 7.95 (2H, \( J = 8.81 \text{ Hz} \)), \text{CH}_3 \text{N(CH)}_2 \times 2), 7.79 (2H, \( J = 8.81 \text{ Hz} \)), \text{CH}_3 \text{N(CH)}_2 \times 2). \( ^{13}\text{C}-\text{NMR} \) (CDCl\(_3\)) \( \delta \): 37.2, 27.5, 28.7, 31.0, 39.3, 42.1, 42.5, 49.2, 54.8, 54.9, 56.7, 73.4, 84.8, 103.7, 103.9, 115.1, 129.2, 131.6, 155.7, 156.2, 161.0. IR (neat) 2973, 2314, 1604, 1500, 1442, 1330, 1153, 730, 1002, 917, 836 cm\(^{-1}\). High resolution (HRMS) (FAB) \( m/z \) Caled for C\(_{34}\)H\(_{33}\)N\(_3\)O\(_4\)S\(_2\)Na (M + H\(^+\)) 621.2967. Found: 621.2963.

4-(But-2-yn-1-yl)-N-(4-(dimethylamino)-6-[{(2-( tert-butylthio)ethan-1-amine}methyl]-4-(dimethylamino)pyridin-2-yl)(methyl)-N-[(2-(nitrphenyl)dithio]ethyl]benzenesulfonamide (6):

Compound 5 (41 mg, 0.066 mmol) was dissolved in 0.7 mL of DMF and 1.5 mL of AcOH, 2-nitrobenzensulfonamide (Nps-Cl) (63 mg, 0.33 mmol) was added at 0°C, and the mixture was stirred for 5 h. The resulting mixture was neutralized with sat. NaHCO\(_3\), and extracted with CH\(_2\)Cl\(_2\). The extract was dried over MgSO\(_4\) and concentrated under reduced pressure. The residue was purified by column chromatography (Hexane:AcOEt = 1 : 1) to afford 6 (45 mg, 70%) as a yellow solid. \( ^{1}\text{H}-\text{NMR} \) (CDCl\(_3\)) \( \delta \): 1.88 (3H, s, CH\(_3\)), 2.81 (2H, \( J = 7.22 \text{ Hz} \)), 2.74–2.97 (8H, m, CH\(_2\) \times 2), 3.33–3.46 (4H, m, CH\(_2\) \times 2), 4.19 (2H, s, CH\(_3\)), 4.28 (2H, s, CH\(_3\)), 4.71 (2H, s, CH\(_3\)), 6.31 (1H, s, CH\(_3\text{N(CH)}_2 \)), 6.64 (1H, s, CH\(_3\text{N(CH)}_2 \)), 7.03 (2H, \( J = 8.98 \text{ Hz} \)), 7.17 (1H, m, CH\(_3\)), 7.30–7.33 (2H, m, CH\(_3\)), 7.65–7.69 (2H, m, CH\(_3\)), 7.71 (2H, \( J = 8.86 \text{ Hz} \)), 8.07 (1H, d, \( J = 12.00 \text{ Hz} \)), 8.14 (1H, d, \( J = 8.23 \text{ Hz} \)), 8.17 (1H, d, \( J = 8.29 \text{ Hz} \)), 8.20–8.23 (2H, m, CH\(_3\), \text{CH}_3 \times 2), 8.28 (1H, d, \( J = 8.23 \text{ Hz} \)), (CH\(_3\)). \( ^{13}\text{C}-\text{NMR} \) (CDCl\(_3\)) \( \delta \): 37.3, 36.4, 36.8, 39.2, 47.9, 55.6, 56.8, 60.4, 64.4, 73.1, 76.8, 78.0, 77.0, 104.6, 104.7, 114.9, 115.2, 124.9, 125.3, 125.9, 126.0, 126.1, 126.2, 127.3, 129.4, 134.1, 137.4, 161.2. IR (KBr) 2341, 1601, 1511, 1446, 1338, 1135, 925, 848, 736 cm\(^{-1}\). HRMS (FAB) \( m/z \) Caled for C\(_{27}\)H\(_{32}\)N\(_4\)O\(_4\)S\(_2\) (M + H\(^+\)) 968.1368. Found: 968.1352.

4-(But-2-yn-1-yl)-N-((4-(dimethylamino)-6-[{(2-(nitrphenyl)dithio]ethyl]amino)methyl]pyridin-2-yl)methyl)-N-[(2-(nitrphenyl)dithio]ethyl]benzenesulfonamide (SN-4(Nps)):

Compound 6 (40 mg, 0.041 mmol) was dissolve in a mixture of CH\(_2\)Cl\(_2\) and MeOH (4.5 mL, 2:3), 3-methylindole (22 mg, 0.166 mmol) and 0.5 M HCl (0.2 mL) were added at 0°C, and the mixture was stirred at room temperature for 6 h. The solution was concentrated under reduced pressure, neutralized with sat. NaHCO\(_3\), and extracted with CH\(_2\)Cl\(_2\). The extract was concentrated under reduced pressure. The residue was purified by amino silica gel column chromatography (CH\(_2\)Cl\(_2\); AcOEt = 15 : 1) to afford a yellow solid SN-4(Nps),
(18 mg, 54%)

1H-NMR (CDCl 3) δ: 1.88 (3H, s, CH₃), 2.80 (2H, t, CH₂), 2.86–2.93 (10H, m, CH₂ × 2, CH₂ × 2), 3.40 (2H, t, J = 7.7 Hz, CH₂), 3.64 (2H, s, CH₂), 4.29 (2H, s, CH₂), 4.71 (2H, s, CH₂), 6.34 (1H, s, C₆H₄N (CH)), 6.47 (1H, s, C₆H₄N (CH)), 7.02 (2H, d, J = 8.9 Hz, C₆H₄ (CH × 2)), 7.33–7.35 (2H, m, C₆H₄ (CH)), 7.64–7.66 (2H, m, C₆H₄ (CH × 2)), 7.74 (2H, d, J = 8.9 Hz, C₆H₄ (CH × 2)), 8.14 (1H, d, J = 9.4 Hz, C₆H₄ (CH)), 8.25 (2H, d, J = 9.6 Hz, C₆H₄ (CH × 2)), 8.30 (1H, d, J = 9.4 Hz, C₆H₄ (CH)). 13C-NMR(CDCl 3) δ: 37.2, 39.2, 47.7, 48.0, 54.7, 55.0, 56.8, 73.0, 84.9, 104.0, 115.2, 126.1, 126.2, 127.2, 127.4, 129.4, 130.8, 134.1, 137.3, 137.7, 145.5, 145.7, 155.6, 155.6, 161.2. IR (KBr) 3085, 2337, 1600, 1511, 1446, 1334, 1160, 917, 848, 744 cm⁻¹. HRMS(FAB) m/z Calcd for C₂₆H₂₆N₆O₇S₅ (M + H⁺) 815.1484. Found: 815.1501. mp: 105°C. Purity: 97.1% (See supplementary materials).

Cell Culture, Differentiation, and Stimulation The human monocytic cell line THP-1 was cultured in RPMI-1640 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, U.S.A.) and 55 μM 2-mercaptoethanol. The human astrocytoma cell line U251 MG was cultured in Dulbecco’s modified Eagle’s medium (DMEM) (FUJIFILM Wako Pure Chemical Corporation) (1 : 500), or anti-CD44 monoclonal antibody BU52 (Ancell, Bayport, MN, U.S.A.) (1 : 400) in PBS containing 1% BSA (Nacalai Tesque) and 0.3% Triton X-100 overnight at 4°C. The cells were then washed three times with PBS, reacted with goat anti-mouse immunoglobulin G (IgG) (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Thermo Fisher Scientific, Waltham, MA, U.S.A.) at room temperature in the dark for 1 h. After washing three times with PBS, the cells were incubated with Hoechst 33342 (Dojin Molecular Technologies) solution at RT for 15 min, followed by washing three times with PBS. Microscopic observation was performed using TCS SP confocal laser-scanning microscope (Leica, wetzlar, Germany).

Immunoblot Analysis and ELISA Immunoblot analysis was performed using cells lysed in PBS/Laemmli sample buffer (1 : 1) or cell culture supernatant as described previously.39 As an antibody, Human TNF-alpha Antibody (R&D Systems, Minneapolis, MN, U.S.A.) (1 : 500), anti-CDD4 ICD (intracellular domain) Polyclonal antibody (TransGenie, Kobe, Japan) (1 : 500), or anti-β-actin clone AC-15 (Sigma-Aldrich) (1 : 1000) was used. Immunoreactivity was detected by using ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation). Intensity of bands was quantified by ImageJ (National Institute of health, Bethesda, MD, U.S.A.). ELISA was conducted by using Human TNF-α ELISA kit (Thermo Fisher Scientific).

In Vitro TNF-α Cytotoxic Assay Mixture of Recombinant Human TACE/ADAM17 Protein (R&D Systems), SN-4(Nps)₂, and 1,4-dithiotheritol (DTT) (FUJIFILM Wako Pure Chemical Corporation) with or without zinc chloride (FUJIFILM Wako Pure Chemical Corporation) in Tris–HCl buffer (50 mM, pH 8.0) was incubated at 37°C for 30 min. Then Recombinant Pro-TNF-alpha Fusion Protein (R&D Systems) was added. The solution was incubated at 37°C overnight followed by immunoblot analysis. The final concentrations are as follows. ADAM17, 100 μg/mL; SN-4(Nps)₂, 10 μM; DTT, 30 μM; zinc chloride; 20 μM, Pro-TNF-α, 100 μg/mL.

Molecular Docking Analysis ADAM-17 X-ray crystal structure co-crystalized with an Aryl-sulfonamide ligand (PDB code 2O10)24 was retrieved from Protein Data Bank to be utilized as a model in the present study. The protein structure was prepared using QuickPrep module of MOE (Version 2019.0) (Chemical Computing Group, Montreal, Canada). Pocket water molecules and zinc ion were conserved. The docking study was conducted using the rigid-receptor method.36,40 The co-crystallized ligand was defined as the center of the binding site. All other options were left at their default values. One hundred docking positions were generated for each ligand. The generated docking positions were visualized using MOE.41

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Supplementary Materials The online version of this ar-

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