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A novel Syk family kinase inhibitor: Design, synthesis, and structure–activity relationship of 1,2,4-triazolo[4,3-*c*]pyrimidine and 1,2,4-triazolo[1,5-*c*]pyrimidine derivatives

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ABSTRACT

Splenic tyrosine kinase (Syk) family kinases, which are members of the protein tyrosine kinase family, play crucial roles in immune responses, with Syk participating in B-cell activation and the zeta-associated protein 70 kDa (ZAP-70) kinase being involved in T-cell activation. Therefore, Syk family kinase inhibitors are candidate therapeutic agents for the treatment of various allergic disorders and autoimmune diseases. We designed 1,2,4-triazolo[4,3-*c*]pyrimidine and 1,2,4-triazolo[1,5-*c*]pyrimidine derivatives as Syk family kinase inhibitors, based on literature reports and structure-based drug design. These derivatives showed significant Syk inhibitory activities, with ZAP-70 inhibition. Representative compounds **10d** and **11** not only exhibited strong inhibition of both Syk and ZAP-70 kinase but also suppressed IL-2 production by peripheral blood mononuclear cells and whole blood.

1. Introduction

The splenic tyrosine kinase (Syk) family kinases are cytosolic non-receptor tyrosine kinases, represented by Syk and zeta-associated protein 70 kDa (ZAP-70).[1,2] Syk is present in platelets, B lymphocytes, mast cell, basophils, neutrophils, dendritic cells, macrophages, and monocytes. Syk kinase has functional roles in the signal transduction mediated by immunoreceptor tyrosine-based activation motifs expressed in various hematopoietic cells. ZAP-70 is found in T lymphocytes and natural killer cells. Syk family kinases play important roles in immune responses, and are involved in inflammatory and autoimmune diseases.[3–5] Recently, it has been reported that several Syk inhibitors are effective in the treatment of inflammation and autoimmune diseases.[6–10] Thus, Syk family tyrosine kinase inhibitors are candidate therapeutic agents for the treatment of various allergic and autoimmune disorders.

In our search for protein tyrosine kinase inhibitors, we discovered the following compounds, based on literature reports and structure-based drug design (SBDD): 1,2,4-triazolo[4,3-*c*]pyrimidine and 1,2,4-triazolo[1,5-*c*]pyrimidine.[11] These compounds showed highly potent inhibitory activities for Syk and/or ZAP-70 kinase. In the present study, we describe the discovery process and investigate the structure–activity relationships (SARs) of 1,2,4-triazolo[4,3-*c*]pyrimidine and 1,2,4-triazolo[1,5-*c*]pyrimidine derivatives as Syk family kinase inhibitors.

2. Chemistry

To prepare the 1,2,4-triazolo[4,3-*c*]pyrimidine and 1,2,4-triazolo[1,5-*c*]pyrimidine derivatives, we selected 5-cyano-2,4,6-trichloropyrimidine **4** as the starting material and used the synthetic route shown in Scheme 1. Substitution of compound **4** with 3,5-dimethoxyaniline in THF at −78 °C gave the 6-anilinopyrimidine derivative **5**, along with the undesired 4-anilinopyridine derivative. These compounds were easily separated by crystallization using appropriate solvents. Compound **5** was reacted with various amines to afford **6a**–**g**. Protection of the hydroxyl group of **6b** with *tert*-butyldimethylsilyl (TBS) group gave **6c**. Each compound was treated with hydrazine in THF, and the corresponding intermediate was heated with trimethylorthoformate to afford 1,2,4-triazolo-[4,3-*c*]pyrimidine derivatives **8a**–**f**.[12] Hydrolysis of the nitrile group using 30% H_2O_2 and 5 M NaOH generated the carbamoyl derivatives **9a**–**f**. Furthermore, removal of the Boc groups from compounds **9c**–**f** was achieved by treatment with 4 M HCl–AcOEt, to give compounds **10a**–**d**.

Synthesis of 1,2,4-triazolo[1,5-*c*]pyrimidine derivative **11** was readily achieved by the Dimroth rearrangement under the heating conditions illustrated in Scheme 2.[13] The 2-phenyl-1,2,4-triazolo [4,3-*c*]pyrimidine derivative **13a** and 2-phenyl-1,2,4-triazolo[1,5-*c*] pyrimidine derivatives **14a**–**c** were synthesized according to the

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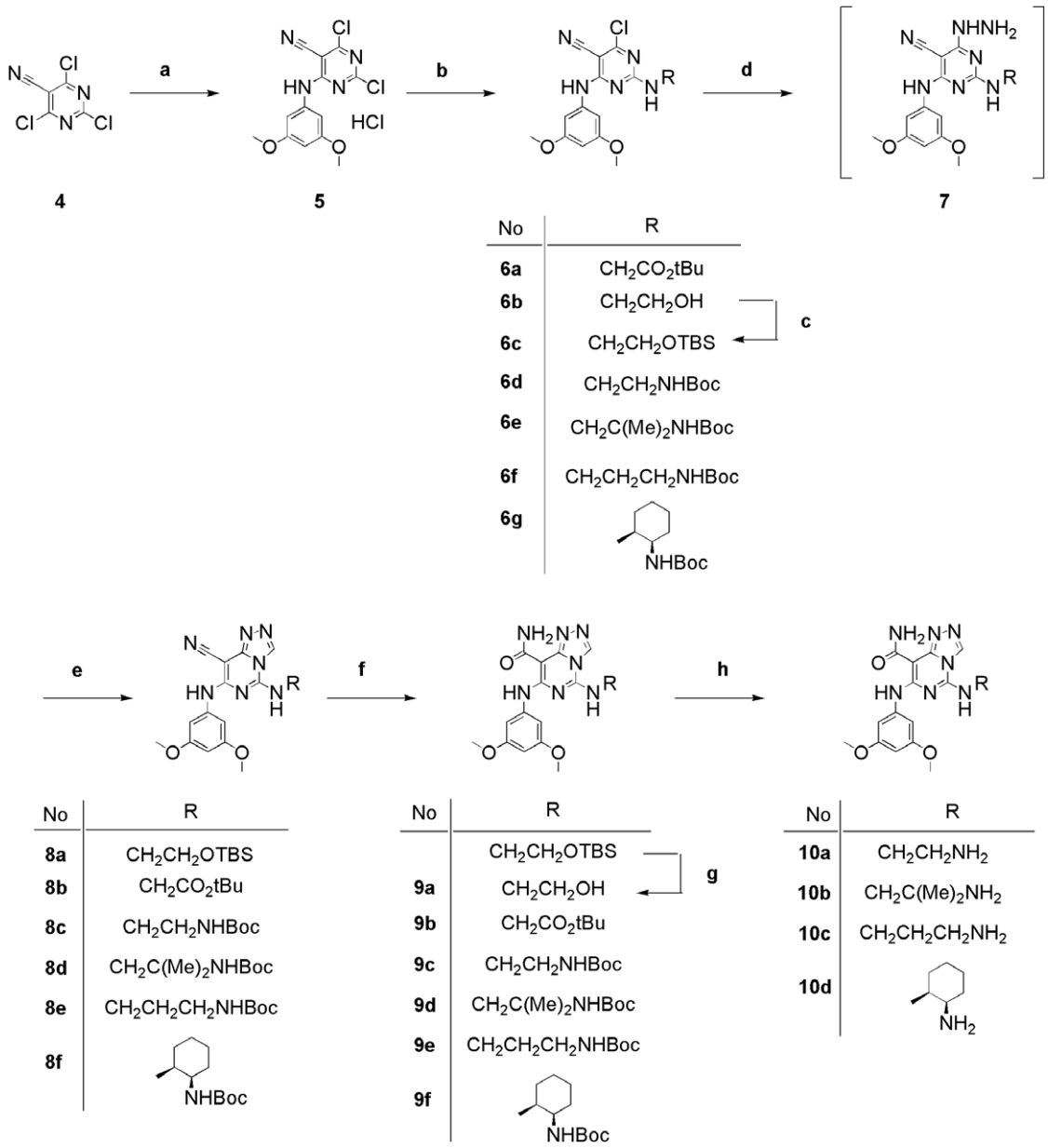
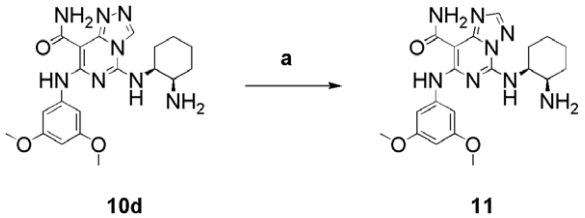


Scheme 1. Reagents: (a) 3,5-dimethoxyaniline, *i*-Pr_2NEt, THF; (b) RNH_2, *i*-Pr_2NEt, THF; (c) TBSCl, imidazole, DMF; (d) $NH_2NH_2H_2O$, THF; (e) $HC(OMe)_3$; (f) 30% H_2O_2, 5 M NaOH, DMSO, EtOH; (g) 1 M TBAF, THF; (h) 4 M HCl–AcOEt.



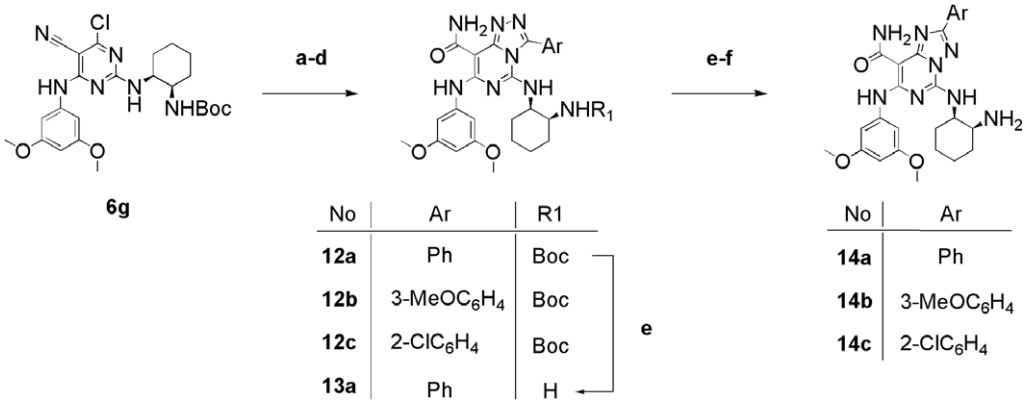
Scheme 2. Reagent and condition: (a) DMF, 100 °C.

method shown in Scheme 3. Compound **6g** was treated with hydrazine monohydrate in THF and was mixed with the corresponding benzaldehyde derivatives in MeCN. These mixtures were hydrolyzed with 30% H_2O_2 and 5 M NaOH, and the resulting carbamoyl products were treated with diisopropyl azodicarboxylate (DIAD) in MeCN, to provide the 3-phenyl-1,2,4-triazolo[4,3-*c*]pyrimidine derivatives **12a**–**c**. Compound **13a** was prepared by deprotection of the Boc group of **12a** under acidic conditions. Dimroth rearrangement products were synthesized in accordance with the following method. Removal of the Boc group from **12a**–**c** was achieved by treating under acidic conditions, and the crude compounds were heated in DMF, to give 2-phenyl-1,2,4-triazolo[1,5-*c*]pyrimidine derivatives **14a**–**c**. The chemical structures of the synthesized compounds were determined by spectroscopy (^1H NMR, mass spectrometry) and elemental analysis.

3. Results and discussion

3.1. Design of novel Syk family tyrosine kinase inhibitors

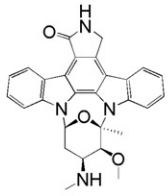
Initially, we sought lead compounds based on a combination of literature reports and SBDD. At that time, there was little structural information available on human ZAP-70[14] and Syk.[15] A comparison of the primary amino acid sequence of the ZAP-70 kinase domain with Lck revealed approximately 34% homology, and a comparison

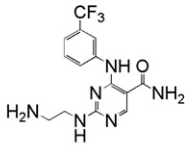


Scheme 3. Reagents and condition: (a) $NH_2NH_2H_2O$, THF; (b) ArCHO, MeCN; (c) DIAD, Ph_3P, MeCN; (d) 30% H_2O_2, 5 M NaOH, DMSO, EtOH; (e) 4 M HCl–AcOEt; (f) DMF, 100 °C.

of the ATP-binding site between ZAP-70 and Lck revealed approximately 57% homology.[16] Given the relatively high degree of homology at the ATP-binding site between Lck and ZAP-70 (or Syk), we constructed a ZAP-70 homology model, based on the published crystal structure of the activated Lck kinase domain,[16,17] using FAMS.[18] The binding properties of several compounds derived from the model were examined using the ADAM software.[19] Initially, we adopted the well-known tyrosine kinase inhibitor staurosporine **1**,[20] which showed strong inhibitory activities for both Syk and ZAP-70 kinase in our evaluation (Table 1). Staurosporine **1** also demonstrated strong suppression of IL-2 production by peripheral blood mononuclear cells (PBMCs) and by whole blood (Table 5), as well as in an in vivo murine model (data not shown). Several research groups have reported Syk kinase inhibitors.[21] Researchers at Astellas Pharmaceutical have reported that the potent and selective low-molecular-weight Syk kinase inhibitor **2** is highly potent in vitro and efficacious against the anaphylaxis reaction in vivo.[9] We compared staurosporine **1** with the 4-anilinopyrimidine-5-carboxamide derivative **2** in the ZAP-70 receptor model, to identify structural differences in their inhibition profiles. The binding modes of the ATP-binding site on staurosporine **1** and compound **2** are shown in Figure 1.[22,9] We examined the regions surrounding the adenosine pockets, namely sites **A**, **B**, and **C**.[14] After the binding of staurosporine **1**, sites **A** and **B** formed a CH-π interaction[23] between the aromatic ring and the methylene or methyl groups. Thus, the indole skeleton at site **A** formed a CH-π interaction with the methyl group of Val352, and the phenyl group of site **B** formed a CH-π interaction with the methylene groups of Leu344 and Gly420 (Fig. 1B). Site **C** corresponded to a sugar pocket based on the crystal structures of ATP-containing analogues. In the binding mode, staurosporine **1** effectively occupied all the **A**, **B**, and **C** sites, whereas compound **2** was shared at sites **B** and **C** and did not occupy site **A**. The inhibitory activity of compound **2** for ZAP-70 was extremely low compared with that for Syk kinase. We hypothesized that the occupation of all the **A**, **B**, and **C** sites was required to inhibit both the Syk and ZAP-70 kinases via hydrogen-bonding interactions and CH-π interactions. Based on this hypothesis, we designed the virtual 1,2,4-triazolo[4,3-*c*]pyrimidine derivative **3** (Fig. 1). Examination of the binding mode between ZAP-70 and compound **3** revealed that (1) the N–H group and carbonyl group of the 8-carbamoyl group interacted with the carbonyl group of Glu415 and the N–H group of Ala417, respectively; (2) the N–H group of the aniline formed a hydrogen bond with the carbonyl group of Ala417, similar to compound **2**; (3) the triazole core of compound **3** occupied site **A** to form a CH-π interaction with the methyl group of Val352; and (4) the 7-anilino group of compound **3** on site **B** formed a CH-π interaction with the methylene groups of Leu344 and Gly420. Regarding substituents on the 7-anilino group, several literature reports have pointed out that *meta* substituents were favorable for Syk inhibition.[9,24,25] Thus, we investigated primarily *meta*-substituted benzene derivatives. Moreover, it was conceivable that the cycloalkyldiamino group at the 5-position of 1,2,4-triazolo[4,3-*c*]pyrimidine core contributed to effective occupation of site **C**, similar to the cyclohexane core of staurosporine **1**. In fact, the cyclohexyldiamino derivative showed excellent Syk inhibitory activity.[26] Based on these considerations, we synthesized 1,2,4-triazolo[4,3-*c*]pyrimidine derivatives such as virtual compound **3** and 1,2,4-triazolo[1,5-*c*]pyrimidine derivatives, and studied their SARs.

Table 1
Inhibitory effects of staurosporine **1** and compound **2** on Syk family kinases


Staurosporine **1**


Compound **2**

Compound	IC_{50} (μM)	
	Syk	ZAP-70
1	0.009[a]	0.053[a]
2	0.041[b]	11.2[b]

[a] Results from in-house evaluations. The IC_{50} values for inhibition of Syk and ZAP-70 kinases were determined in duplicate.
[b] The results are presented in the following Ref. 9.

3.2. SARs for Syk and ZAP-70 kinase inhibition

The Syk and ZAP-70 inhibitory activities of our synthesized compounds were evaluated using a coupled spectrophotometric enzyme assay (Tables 1–4). Potent compounds were further investigated for enzyme selectivity compared with other tyrosine kinases (Lck and PKCβII) (Table 5).

We investigated the inhibitory effects of various substituents at the 5-position of the 1,2,4-triazolo[4,3-*c*]pyrimidine core on Syk family kinases (Table 2). Compound **9a**, which possesses a hydroxyethylamino group at the 5-position, showed weak inhibitory activities for Syk and ZAP-70. Similarly, low inhibitory activities for both Syk and ZAP-70 were observed with compound **9b**, in which *tert*-butyl glycine is substituted at the 5-position. The 5-aminoethylenediamino derivative **10a** exhibited highly potent

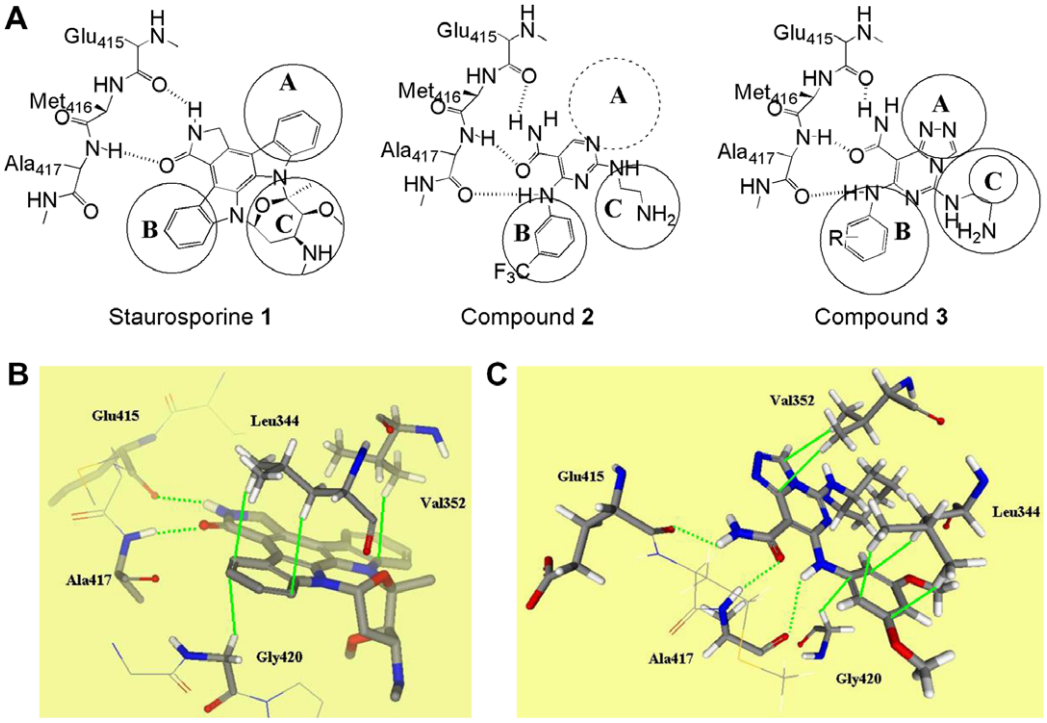
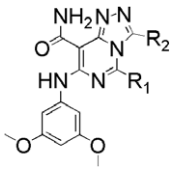
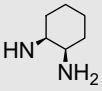
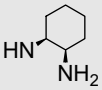


Figure 1. (A) Proposed docking modes of staurosporine **1**, compound **2**, and compound **3** to the ATP-binding site of the ZAP-70 kinase. (B) Proposed docking mode of staurosporine **1** to the ZAP-70 kinase complex. (C) Proposed docking mode of compound **10d** to the ZAP-70 kinase complex.

inhibition of Syk (IC_{50} = 0.009 μM) but was less potent at inhibiting ZAP-70 (IC_{50} = 8.3 μM). This result was similar to the outcome observed previously for compound **2**.[9] Compound **10b**, which is substituted with a dimethyl group at the approximate terminal primary amine at the C5 position in **10a**, showed lower inhibition of Syk kinase (IC_{50} = 0.15 μM), as compared with **10a**. Compound **10c**, which bears a 3-propylenediamino group, had significantly lower inhibitory activities for Syk and ZAP-70 (IC_{50} = 7.7 μM for Syk, IC_{50} = 50 μM for ZAP-70). Compound **10d**, in which a *cis*-cyclohexyldiamino group is introduced at the 5-position of the 1,2,4-triazolo[4,3-*c*]pyrimidine core, indicated excellent Syk kinase inhibition (IC_{50} = 0.009 μM) and improved ZAP-70 inhibitory activity (IC_{50} = 0.12 μM), as compared with compound **10a**. These findings indicate that an amino group introduced at the 5-position of the 1,2,4-triazolo[4,3-*c*]pyrimidine skeleton has an important impact on Syk family kinase inhibition. A specific length of the connecting diamino group is also crucial for kinase inhibition.

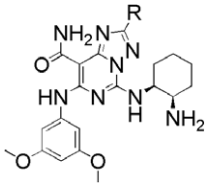
Table 2
Inhibition of Syk and ZAP-70 activities by 1,2,4-triazolo[4,3-*c*]pyrimidine derivatives



Compound	R_1	R_2	IC_{50} (μM)[a]	
			Syk	ZAP-70
9a	$NHCH_2CH_2OH$	H	6.1	6.4
9b	$NHCH_2CO_2tBu$	H	79.1	9.0
10a	$NHCH_2CH_2NH_2$	H	0.009	8.3
10b	$NHCH_2C(Me)_2NH_2$	H	0.15	1.1
10c	$NHCH_2CH_2CH_2NH_2$	H	7.7	50
10d		H	0.009	0.12
13a		Ph	15.2	26

[a] The IC_{50} values were determined in duplicate.

Table 3
Inhibition of Syk and ZAP-70 activities by 1,2,4-triazolo[1,5-*c*]pyrimidine derivatives



Compound	R	IC_{50} (μM)[a]	
		Syk	ZAP-70
11	H	0.004	0.33
14a	Ph	0.083	0.11
14b	3-$MeOC_6H_4$	0.12	0.12
14c	2-ClC_6H_4	0.050	0.097

[a] The IC_{50} values were determined in duplicate.

Table 4
Suppression of IL-2 production in PBMCs and WB, by Syk family kinase inhibitors

Compound	X	Y	R	IC_{50} (μM)[a]			
				Syk	ZAP-70	PBMCs	WB
10d	N	CH	–	0.009	0.12	0.046	0.44
11	CH	N	H	0.004	0.33	0.076	0.48
14a	CH	N	Ph	0.083	0.11	0.21	2.0
14c	CH	N	2-ClC_6H_4	0.050	0.097	1.76	NT[b]

[a] The IC_{50} values were determined in duplicate.
[b] NT, not tested.

Table 5
Summary of the effects of the Syk family kinase inhibitors

Compound	IC_{50} (μM)[a]					
	Syk	ZAP-70	Lck	PKCβII	PBMC	WB
1	0.003	0.053	0.018	0.004	0.016	0.10
10d	0.009	0.12	0.18	0.68	0.046	0.44
11	0.004	0.33	0.59	2.66	0.076	0.48

[a] The IC_{50} values were determined in duplicate.

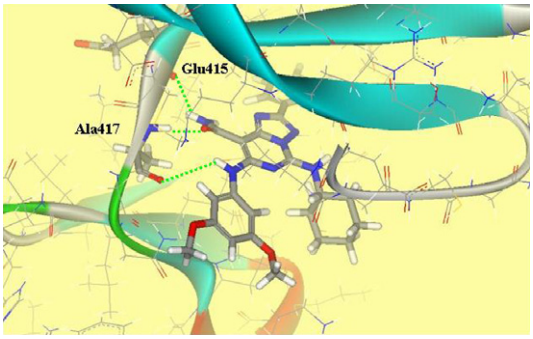


Figure 2. Proposed docking mode of compound **14a** to the ZAP-70 kinase complex.

Furthermore, incorporation of the cyclohexyldiamino group was very effective at inhibiting Syk family kinases. In particular, ZAP-70 inhibition was strongly enhanced by this modification. Dimroth rearrangement derivative 1,2,4-triazolo[1,5-*c*]pyrimidine (derivative **11**) showed strong inhibition of Syk family kinases (IC_{50} = 0.004 μM for Syk; IC_{50} = 0.33 μM for ZAP-70; Table 3), similar to compound **10d**. As they showed almost identical inhibitory profiles, compounds **10d** and **11** were considered to have similar pharmacophores. We consider that these results are in accordance with our binding mode hypothesis.

We also studied the effect of substitutions at the 3-position in the 1,2,4-triazolo[4,3-*c*]pyrimidine core and the 2-position in the 1,2,4-triazolo[1,5-*c*]pyrimidine core. Compound **13a**, with a phenyl group at the 3-position in the 1,2,4-triazolo[4,3-*c*]pyrimidine skeleton, showed complete loss of ability to inhibit Syk and ZAP-70 kinases (Table 2). Compounds that incorporated the phenyl group at the 2-position in the 1,2,4-triazolo[1,5-*c*]pyrimidine core were also evaluated (Table 3). The inhibitory activity of compound **14a** for ZAP-70 kinase was slightly improved (IC_{50} = 0.11 μM). Contrary to our expectations, substitution of the phenyl group decreased Syk kinase inhibition (IC_{50} = 0.083 μM). Compounds **14b** and **14c** exhibited Syk family kinase inhibitory activities similar to that of compound **14a** (see Fig. 1C).

An examination of the binding profile of compound **13a**, as predicted by the ADAM[19] software, revealed a narrow space around the 3-position in the 1,2,4-triazolo[4,3-*c*]pyrimidine core and a phenyl group at the 3-position that might interact with Lys369 and/or Asp479. This repulsion would abolish the Syk family kinase inhibitory activity of compound **13a**. Regarding the binding of **14a** (Fig. 2) to ZAP-70, there is a comparatively large space at the 2-position in the 1,2,4-triazolo[1,5-*c*]pyrimidine skeleton. The phenyl group of compounds **14a–c** fitted well into this space. As these derivatives effectively occupied sites **A**, **B**, and **C** (Fig. 1), we anticipated that the Syk kinase inhibitory profile of **14a–c** would resemble that of staurosporine **1**.

3.3. IL-2 production

IL-2 production levels of PBMCs and WB were assayed by enzyme-linked immunosorbent assay (ELISA). Compounds with high inhibitory activities for Syk and/or ZAP-70 kinase (i.e., compounds **10d**, **11**, **14a**, and **14c**) were tested for suppressive effects on IL-2 production (Table 4). In PBMCs, compounds **10d** and **11** demonstrated strong suppression of IL-2 production, whereas the 2-phenyl derivatives **14a** and **14c** were less potent in this regard. In WB, compound **14a** showed decreased suppressive potency for IL-2 production. We considered that the higher molecular weights of **14a** and **14c** as compared with theose of compounds **10d** and **11** or the weaker solubility in water of **14a** (1.2 μg/mL) as compared with that of compounds **10d** (351 μg/mL) and **11** (42 μg/mL) influenced the cellular activities. Compounds **10d** and **11** showed excellent suppression of IL-2 production in WB. Compounds **10d** and **11** demonstrated almost equal potencies for IL-2 suppression to staurosporine **1** in the cellular assay system.

3.4. In vitro selectivity of Syk family kinase inhibition over Lck and PKCβII

We assessed the selectivity of the actions of compounds **10d**, **11**, and staurosporine **1** (Table 5). Staurosporine **1** strongly inhibited not only Syk family kinases but also Lck and PKCβII. On the other hand, compounds **10d** and **11** showed potent inhibitory activities against Syk family kinases, but less potent inhibitory activities against Lck and PKCβII than staurosporine **1**. Whereas staurosporine **1** formed a hydrogen bond with Ser323 in Lck, no interaction occurred between compound **10d** and Ser323 in Lck (Fig. 3A). This interaction may be responsible for the Syk family selectivity of compound **3**, as compared with that of staurosporine **1**. Regarding the selectivity of compound **10d** for PKCβII, we assumed that the hydrogen bonding between the N–H group and Asp470 was important. The N–H in the methylamino group on the tetrahydropyran core of staurosporine **1** interacted with the carboxyl group in Asp 470, whereas there was no interaction between the NH_2 group in compound **10d** and the carboxyl group in Asp470 (Fig. 3B).

4. Conclusions

We have discovered novel Syk family kinase inhibitors based on literature reports and SBDD and ZAP-70 homology models constructed from the published Lck crystal structure. The 1,2,4-triazolo[4,3-*c*]pyrimidine derivative **10d** and its regioisomer 1,2,4-triazolo[1,5-*c*]pyrimidine derivative **11** exhibited strong kinase inhibitory activities. Moreover, the potencies of the IL-2-suppressive effects of **10d** and **11** on PBMCs and WB were almost equivalent to that of staurosporine **1**. Concerning Syk family kinase inhibition, similar selectivity profiles were observed in staurosporine **1** and compounds **10d** and **11**. However, compounds **10d** and **11** had higher selectivity than staurosporine **1** for Lck and PKCβII. As these derivatives possess promising in vitro potencies, we intend to investigate their in vivo efficacies, the results of which will be reported in due course.

5. Experimental procedures

5.1. Chemistry

Melting points were taken on a Yanako MP-3S Micro melting point apparatus and were uncorrected. Infrared spectra were measured on a Nicolet 510 FT-IR spectrophotometer and were reported in reciprocal centimeters. Proton NMR spectra were recorded at 400 or 500 MHz with a Brucker AMX 400 or DRX 500 instrument, and chemical shifts were reported in parts per million (δ) downfield from tetramethylsilane as internal standard. The peak patterns were shown as the following abbreviations: br, broad; d,

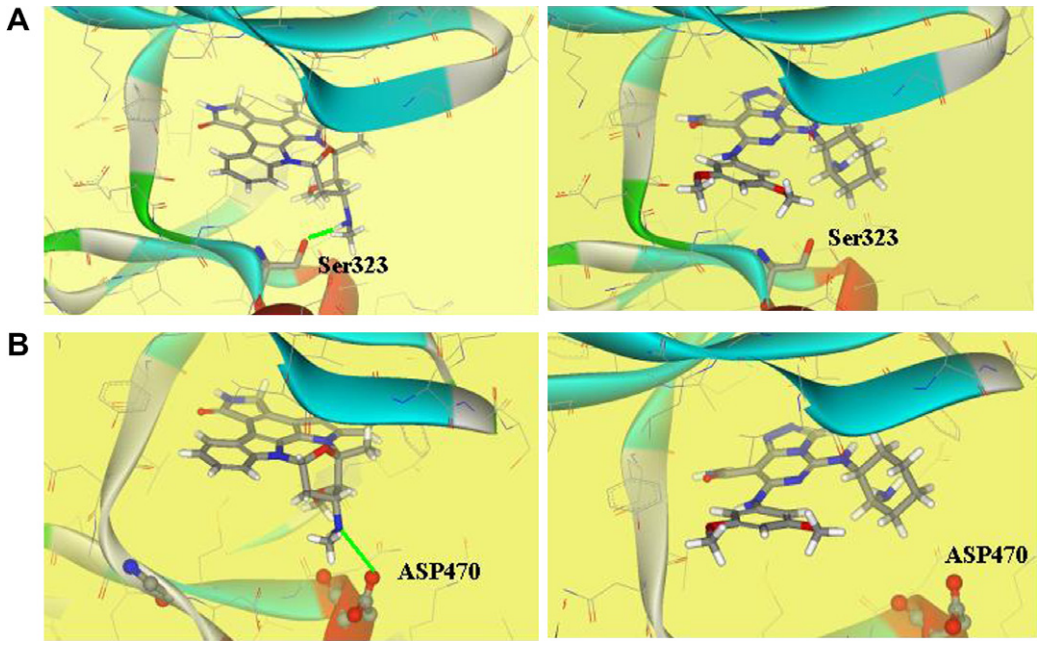


Figure 3. (A) Proposed docking mode of staurosporine **1**, compound **10d**, and Lck kinase complex. (B) Proposed docking modes of staurosporine **1**, compound **10d**, and PKCβII kinase complex.

doublet; m, multiplet; s, singlet; t, triplet; q, quartet. The mass spectra (MS) were carried out with Thermo Quest FINNIGAN AQA electrospray ionization mass spectrometer. Elemental analyses were performed on an Elementar Vario EL analyzer (C, H, and N). Silica gel 60F254 precoated plates on glass from Merck KgaA or aminopropyl silica gel (APS) precoated NH plates from Fuji Silysia Chemical Ltd were used for thin layer chromatography (TLC). Flash or medium-pressure liquid chromatography (MPLC) was performed on silica gel BW-350 from Fuji Silysia Chemical Ltd or APS Daisogel IR-60 (particle size 25–40 μM) from Daiso Co., Ltd. All reagents and solvents were commercially available unless otherwise indicated.

5.1.1. 2,4-Dichloro-6-(3,5dimethoxyphenylamino)pyrimidine-5-carbonitrile hydrochloride (5)

To a stirred suspension of 2,4,6-trichloropyrimidine-5-carbonitrile **4** (2.00 g, 9.60 mmol) in THF (50 mL) were added 3,5-dimethoxyaniline (1.47 g, 9.60 mmol) in THF (30 mL) and diisopropylethylamine (1.84 mL, 10.6 mmol) at −70 °C under argon atmosphere for 1 h and stirred for 2 h at 0 °C. Saturated ammonium chloride solution was added to the mixture and extracted with AcOEt. Organic phase was washed with brine, dried over anhydrous $MgSO_4$, and evaporated in vacuo. Tetrahydrofuran was added to the residue and resulting precipitate was collected by suction. The filtrate was concentrated in vacuo and crystallized from AcOEt to give **5** (0.800 g, 26%) as pale yellowish solid: ^{1}H NMR ($CDCl_3$) δ: 3.82 (6H, s), 6.38 (1H, t, J = 2.0 Hz), 6.76 (2H, d, J = 2.0 Hz), 7.32 (1H, br s); Anal. Calcd for $C_{13}H_{10}Cl_2N_4O_2$·1.0 HCl: C 43.18; H 3.07; N 15.49. Found: C, 43.03; H, 2.80; N, 15.22.

5.1.2. *tert*-Butyl [4-chloro-5-cyano-6-(3,5-dimethoxyphenylamino)pyrimidin-2-ylamino]acetate (6a)

To a stirred suspension of compound **5** (0.430 g, 1.32 mmol) and *tert*-butyl aminoacetate hydrochloride (0.244 g, 1.45 mmol) in THF (10 mL) was added diisopropylethylamine in THF (5 mL) at −70 °C under argon atmosphere and the mixture was gradually warmed to room temperature, then stirred overnight at ambient temperature. Saturated ammonium chloride solution was added to the mixture and extracted with AcOEt. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and evaporated in vacuo to give **6a** (0.546 g, 98%) as white solid: mp 213–214 °C (THF); ^{1}H NMR ($CDCl_3$) δ: 1.40–1.50 (9H, m), 3.78–3.83 (6H, m), 4.07–4.15 (2H, m), 5.82–6.02 (1H, m), 6.28–6.32 (1H, m), 6.73–6.78 (2H, m), 6.92–7.08 (1H, m); Anal. Calcd for $C_{19}H_{22}ClN_5O_4$·0.2H_2O: C, 53.89; H, 5.33; N, 16.53. Found: C, 54.10; H, 5.41; N, 16.19.

5.1.3. 4-Chloro-6-(3,5-dimethoxyphenylamino)-2-(2-hydroxyethylamino)pyrimidine-5-carbonitrile (6b)

To a stirred suspension of compound **5** (0.138 g, 0.424 mmol) in THF (10 mL) was added 2-aminoethanol (0.128 mL, 2.12 mmol) at −70 °C under argon atmosphere and the mixture was stirred for 2 h at −20 °C. Water was added to the mixture and extracted with AcOEt. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and evaporated in vacuo to give **6b** (0.147 g, 99%) as white solid: mp 223–224 °C (THF); ^{1}H NMR ($CDCl_3$) δ: 3.30–3.38 (3H, m), 3.46–3.52 (2H, m), 3.71–3.76 (6H, m), 4.66–4.73 (1H, m), 6.24–6.28 (1H, m), 6.89–6.99 (2H, m), 8.02–8.28 (1H, m); Anal. Calcd for $C_{15}H_{16}ClN_5O_3$: C, 51.51; H, 4.61; N, 20.02. Found: C, 51.31; H, 4.65; N, 19.94.

5.1.4. *tert*-Butyl *N*-{2-[4-chloro-5-cyano-6-(3,5-dimethoxyphenylamino)pyrimidin-2-ylamino]ethyl}carbamate (6d)

To a stirred suspension of compound **5** (0.358 g, 1.10 mmol) in THF (15 mL) was added *tert*-butyl *N*-(2-aminoethyl)carbamate (0.873 mL, 8.81 mmol) at −70 °C under argon atmosphere and the mixture was stirred for 2 h at −20 °C. Water was added to the mixture and extracted with AcOEt. Organic layer was washed with brine, dried over anhydrous $MgSO_4$, and evaporated in vacuo to give **6d** (0.400 g, 81%) as white solid: mp 223–224 °C (THF); ^{1}H NMR ($CDCl_3$) δ: 1.36 (9H, s), 3.06–3.13 (2H, m), 3.25–3.31 (2H, m), 3.71–3.73 (6H, m), 6.25–6.27 (1H, m), 6.70–6.87 (2H, m), 6.88-6.97 (1H, m), 8.05–8.25 (1H, m), 9.18–9.37 (1H, m); Anal. Calcd for $C_{20}H_{25}ClN_6O_4$: C, 53.51; H, 5.61, N, 18.72. Found: C, 53.27; H, 5.62; N, 18.50.

5.1.5. *tert*-Butyl {2-[4-chloro-5-cyano-6-(3,5-dimethoxyphenylamino)pyrimidin-2-ylamino]-1,1-dimethylethyl}carbamate (6e)

The title compound was prepared from **5** and *tert*-butyl (2-amino-1,1-dimethylethyl)carbamate in the same manner as described for **6d**, and was obtained as pale yellowish solid (69%): mp 174–176 °C (AcOEt); ^{1}H NMR ($CDCl_3$) δ: 1.23–1.38 (6H, m), 1.40–1.49 (9H, m), 3.63–3.74 (2H, m), 3.78–3.82 (6H, m), 4.52–4.76 (1H, m), 6.23–6.31 (2H, m), 6.75–7.04 (3H, m); Anal. Calcd for $C_{22}H_{29}ClN_6O_4$: C, 55.40; H, 6.13; N, 17.62. Found: C, 55.12; H, 6.13; N, 17.61.

5.1.6. *tert*-Butyl {3-[4-chloro-5-cyano-6-(3,5-dimethoxyphenylamino)pyrimidin-2-ylamino]propyl}carbamate (6f)

The title compound was prepared from **5** and *tert*-butyl (3-aminopropyl)carbamate in the same manner as described for **6d**, and was obtained as pale yellowish solid (40%): mp 183–184 °C (AcOEt); ^{1}H NMR ($CDCl_3$) δ: 1.40–1.47 (9H, m), 1.67–1.80 (2H, m), 3.17–3.25 (2H, m), 3.45–3.55 (2H, m), 3.77–3.84 (6H, m), 4.70–4.88 (1H, m), 6.16–6.30 (2H, m), 6.78–6.83 (2H, m), 6.90–7.05 (1H, m); Anal. Calcd for $C_{21}H_{27}ClN_6O_4$: C, 54.48; H, 5.88; N, 18.15. Found: C, 54.39; H, 5.87; N, 18.13.

5.1.7. *tert*-Butyl *cis*-{2-[4-chloro-5-cyano-6-(3,5-dimethoxyphenylamino)pyrimidin-2-ylamino]cyclohexyl}carbamate (6g)

The title compound was prepared from **5** and *tert*-butyl *cis*-(2-aminocyclohexyl)carbamate in the same manner as described for **6d**, and was obtained as pale yellowish solid (40%): mp 207–208 °C (MeOH–AcOEt); ^{1}H NMR ($CDCl_3$) $_{\delta:}$ 1.16–1.25 (2H, m), 1.33 (9H, s), 1.35-1.76 (6H, m), 3.73 (6H, s), 3.74–4.00 (2H, m), 6.26–6.30 (1H, m), 6.50–6.60 (1H, m), 6.82–6.90 (2H, m), 7.48–8.20 (1H, m), 9.20–9.45 (1H, m); Anal. Calcd for $C_{24}H_{31}ClN_6O_4$: C, 57.31; H, 6.21; N, 16.71. Found: C, 57.26; H, 6.25; N, 16.57.

5.1.8. 5-[2-(*tert*-Butyldimethylsilanyloxy)ethylamino]-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidine-8-carbonitrile (8a)

To a mixture of compound **6b** (0.323 g, 0.923 mmol) in DMF (5 mL) were added imidazole (0.069 g, 1.02 mmol) and *tert*-butyl dimethylchlorosilane (0.153 g, 1.02 mmol) and the mixture was stirred for 20 min at room temperature. Water was added and the mixture was extracted with AcOEt. Organic phase was washed with water and brine, successively, dried over anhydrous $MgSO_4$, and evaporated in vacuo to give **6c**. Crude **6c** was dissolved in THF (10 mL) and hydrazine monohydrate (0.448 mL, 9.23 mmol) was added at room temperature. After being stirred overnight, water was added to the mixture and resulting precipitate was collected by suction filtration. Without purification a mixture of this precipitate in trimethylorthoformate was heated overnight at 100 °C. After being cooled to room temperature, resulting precipitate was collected by suction filtration to give **8a** (0.048 mg, 11%) as white solid: mp 243–245 °C (THF); ^{1}H NMR (DMSO-d_6) δ: −0.06 (6H, s), 0.77 (9H, s), 3.56–3.61 (2H, m), 3.72 (6H, s), 3.75 (2H, d, J = 6.0 Hz), 6.23 (1H, t, J = 2.0 Hz), 6.80 (2H, d, J = 2.0 Hz), 9.03–9.08 (1H, m), 9.18 (1H, s), 9.36 (1H, s); MS m/z: 470 $(M+H)^+$. Anal. Calcd for $C_{22}H_{31}N_7O_3Si{\cdot}0.4H_2O$: C, 55.41; H, 6.72; N, 20.56. Found: C, 55.51; H, 6.60; N, 20.90.

5.1.9. *tert*-Butyl [8-cyano-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidin-5-ylamino]acetate (8b)

To a stirred solution of compound **6a** (1.10 g, 2.62 mmol) in THF (10 mL) was added hydrazine monohydrate (1.27 mL, 26.2 mmol) at room temperature and the mixture was stirred overnight at ambient temperature. Water was added to the mixture and the resulting precipitates were collected by suction filtration and dried in vacuo. The precipitate and trimethylorthoformate (8.53 mL, 80.0 mmol) were heated for 2 days at 90 °C. After being cooled to room temperature, the resulting residue was collected and washed with Et_2O to give **8b** (0.278 g, 25%) as white solid: mp 230–231 °C (THF); ^{1}H NMR (DMSO-d_6) δ: 1.24 (9H, s), 3.73 (6H, s), 4.14 (2H, d, J = 5.5 Hz), 6.25 (1H, t, J = 2.5 Hz), 6.76 (2H, d, J = 2.5 Hz), 9.20 (1H, s), 9.40 (1H, s), 9.52 (1H, t, J = 5.5 Hz); MS m/z: 426 $(M+H)^+$. Anal. Calcd for $C_{20}H_{23}N_7O_4{\cdot}0.7H_2O$: C, 54.84; H, 5.61; N, 22.38. Found: C, 55.01; H, 5.33; N, 22.17.

5.1.10. *tert*-Butyl {2-[8-cyano-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidin-5-ylamino]ethyl}carbamate (8c)

The title compound was prepared from **6d** in the same manner as described for **8b**, and was obtained as pale white solid (32%): ^{1}H NMR (DMSO-d_6) δ: 1.35 (9H, s), 3.11–3.12 (2H, m), 3.70–3.75 (6H, m), 6.26 (1H, s), 6.68–6.86 (1H, m), 6.87–6.98 (2H, m), 8.00–8.25 (1H, m), 9.16–9.36 (1H, m); MS m/z: 455 $(M+H)^+$.

5.1.11. *tert*-Butyl {2-[8-cyano-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidin-5-ylamino]-1,1-dimethylethyl}carbamate (8d)

The title compound was prepared from **6e** in the same manner as described for **8b**, and was obtained as pale yellowish solid (40%): mp 216–217 °C (THF–MeCN); ^{1}H NMR (DMSO-d_6) δ: 1.20 (6H, s), 1.32 (9H, s), 3.66 (2H, d, J = 5.0 Hz), 3.73 (6H, s), 6.25–6.30 (1H, m), 6.35–6.46 (1H, m), 6.80 (2H, d, J = 2.0 Hz), 8.75–8.83 (1H, m), 9.25 (1H, s), 9.35 (1H, s); Anal. Calcd for $C_{23}H_{30}N_8O_4$: C, 57.25; H, 6.27; N, 23.22. Found: C, 56.98; H, 6.18; N, 23.17.

5.1.12. *tert*-Butyl {3-[8-cyano-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidin-5-ylamino]propyl}carbamate (8e)

The title compound was prepared from **6f** in the same manner as described for **8b**, and was obtained as pale yellowish solid (60%): mp 202–203 °C (THF–MeCN); ^{1}H NMR (DMSO-d_6) δ: 1.34 (9H, s), 1.70–1.78 (2H, m), 2.94–3.02 (2H, m), 3.44 (2H, t, J = 5.5 Hz), 3.72 (6H, s), 6.23 (1H, t, J = 2.0 Hz), 6.73–6.80 (1H, m), 6.83 (2H, d, J = 2.0 Hz), 8.88–8.94 (1H, m), 9.15 (1H, s), 9.32 (1H, s); Anal. Calcd for $C_{22}H_{28}N_8O_4{\cdot}0.3H_2O$: C, 55.75; H, 6.08; N, 23.64. Found: C, 56.02; H, 6.09; N, 23.24.

5.1.13. *tert*-Butyl *cis*-2-[8-cyano-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidin-5-ylamino]cyclohexyl}-carbamate (8f)

The title compound was prepared from **6g** in the same manner as described for **8b**, and was obtained as pale yellowish solid (77%): mp 232–234 °C (THF–MeCN); ^{1}H NMR (DMSO-d_6) δ: 0.90–0.98 (2H, m), 1.24 (9H, s), 1.30–1.80 (6H, m), 3.73 (6H, s), 3.90–4.05 (1H, m), 4.06–4.14 (1H, m), 6.25–6.29 (1H, m), 6.76–6.81 (2H, m), 6.87 (1H, d, J = 9.0 Hz), 8.37–8.44 (1H, m), 9.33 (1H, s), 9.39 (1H, s); MS m/z: 509 $(M+H)^+$. Anal. Calcd for $C_{25}H_{32}N_8O_4$: C, 59.04; H, 6.34; N, 22.03. Found: C, 58.93; H, 6.36; N, 21.96.

5.1.14. 7-(3,5-Dimethoxyphenylamino)-5-(2-hydroxyethylamino)-1,2,4-triazolo[4,3-*c*]pyrimidine-8-carboxamide (9a)

To a solution of **8a** (0.040 g, 0.085 mmol) in DMSO (0.4 mL) and EtOH (0.4 mL) were added 5 M NaOH solution (0.170 mL, 0.852 mmol) and 30% H_2O_2 solution (0.097 mL, 0.852 mmol), then the mixture was stirred overnight at room temperature. Water (1 mL) was added and the mixture was neutralized with 1 M HCl solution. The obtained pale yellow precipitates were collected by filtration and washed with H_2O. The precipitates were dissolved in THF and to the mixture was added 1 M tetrabutylammonium fluoride in THF solution (0.086 mL, 0.085 mmol). After being stirred for 2 h, the mixture was purified by column chromatography on APS with MeOH/AcOEt = 1:20 to give **9a** (0.005 g, 16%) as white solid: mp 280–281 °C (THF); ^{1}H NMR (DMSO-d_6) δ: 3.11–3.20 (3H, m), 3.51–3.62 (4H, m), 3.73 (6H, s), 6.02–6.04 (1H, br s), 7.03–7.10

(2H, br s), 8.61 (2H, br), 12.52 (1H, s); MS *m*/*z*: 374 $(M+H)^+$. Anal. Calcd for $C_{16}H_{19}N_7O_4{\cdot}0.3H_2O$: C, 50.74; H, 5.22; N, 25.88. Found: C, 50.82; H, 5.26; N, 25.58.

5.1.15. *tert*-Butyl [8-carbamoyl-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidin-5-ylamino]acetate (9b)

To a solution of **8b** (0.114 g, 0.268 mmol) in DMSO (1 mL) and EtOH (1 mL) were added 5 M NaOH solution (0.535 mL, 2.68 mmol), and 30% H_2O_2 solution (0.305 mL, 2.68 mmol) then the mixture was stirred overnight at room temperature. Water (1 mL) was added and the mixture was neutralized with 1M HCl solution. The obtained pale yellow precipitates were collected by filtration and washed with H_2O. The precipitates were purified by column chromatography on silica gel with MeOH/AcOEt = 1:20 → 1:8 to give **9b** as yellowish solid: mp 285 °C (dec); ^{1}H NMR (DMSO-d_6) δ: 1.29 (9H, s), 3.77 (6H, s), 4.24 (2H, s), 6.24 (1H, t, J = 2.5 Hz), 6.75 (2H, d, J = 2.5 Hz), 7.61 (1H, s), 8.76–8.79 (1H, m), 9.21 (1H, s), 9.43 (1H, s), 12.52 (1H, s); MS *m*/*z*: 444 $(M+H)^+$. Anal. Calcd for $C_{20}H_{25}N_7O_5{\cdot}\ 0.3H_2O$: C, 53.52; H, 5.75; N, 21.84. Found: C, 53.52; H, 5.66; N, 21.50.

5.1.16. *tert*-Butyl {2-[8-carbamoyl-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidin-5-ylamino]ethyl}carbamate (9c)

To a solution of **8c** (0.097 g, 0.213 mmol) in DMSO (1 mL) and EtOH (1 mL) were added 5 M NaOH solution (0.480 mL, 4.25 mmol) and 30% H_2O_2 solution (2.13 mL, 4.25 mmol), then the mixture was stirred overnight at room temperature. Water (mL) was added and the mixture was neutralized with 1M HCl solution. The obtained pale yellow precipitates were collected by filtration and washed with H_2O to give **9c** (0.072 g, 72%) as brown solid: ^{1}H NMR (DMSO-*d6*) δ: 1.37 (9H, s), 3.10–3.17 (2H, m), 3.35–3.34 (2H, m), 3.73 (6H, s), 6.20 (1H, t, J = 2.0 Hz), 6.81 (2H, d, J = 2.0 Hz), 6.83–6.89 (2H, m), 7.06–7.09 (1H, br s), 9.15 (1H, br s), 10.62 (1H, br s), 13.05 (1H, br s); MS *m*/*z*: 473 $(M+H)^+$.

5.1.17. *tert*-Butyl {2-[8-carbamoyl-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidin-5-ylamino]-1,1-dimethylethyl}carbamate (9d)

The title compound was prepared from **8d** in the same manner as described for **9b**, and was obtained as pale yellowish solid (21%): mp 207–210 °C (THF); ^{1}H NMR (DMSO-d_6) δ: 1.27 (6H, s), 1.36 (9H, s), 1.70–1.78 (2H, m), 3.64–3.70 (2H, m), 3.76 (6H, s), 6.26 (1H, br s), 6.88 (2H, d, J = 2.0 Hz), 7.64 (1H, br s), 8.65 (1H, br s), 8.72 (1H, br s), 12.43 (1H, s); MS *m*/*z*: 501 $(M+H)^+$. Anal. Calcd for $C_{23}H_{32}N_8O_5{\cdot}0.2H_2O$: C, 54.79; H, 6.48; N, 22.22. Found: C, 54.69; H, 6.39; N, 21.99.

5.1.18. *tert*-Butyl {3-[8-carbamoyl-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidin-5-ylamino]propyl}carbamate (9e)

The title compound was prepared from **8e** in the same manner as described for **9b**, and was obtained as pale yellowish solid (21%): ^{1}H NMR (DMSO-d_6) δ: 1.45 (9H, s), 1.87–1.95 (2H, m), 3.25–3.33 (2H, m), 3.68–3.75 (2H, m), 3.80 (6H, s), 5.50 (1H, br s), 6.23 (1H, t, J = 2.0 Hz), 6.93 (2H, d, J = 2.0 Hz), 7.64 (1H, br s), 8.03 (1H, s), 8.65 (1H, br s), 9.08 (1H, br s), 12.20 (1H, s); MS *m*/*z*: 487 $(M+H)^+$.

5.1.19. *tert*-Butyl {*cis*-2-[8-carbamoyl-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidin-5-ylamino]cyclohexyl}carbamate (9f)

The title compound was prepared from **8f** in the same manner as described for **9b**, and was obtained as pale yellowish solid (33%): mp 222–224 °C (THF); ^{1}H NMR (DMSO-d_6) δ: 0.86–0.96 (2H, m), 1.23 (9H, s), 1.26–1.84 (6H, m), 3.76 (6H, s), 4.11–4.20 (2H, m), 6.25 (1H, br s), 6.79 (2H, d, br s), 6.82–6.89 (1H, m), 7.52 (1H, d, J = 3.0 Hz), 8.19–8.27 (1H, m), 8.77 (1H, s), 9.36 (1H, s), 12.44 (1H, s); MS *m*/*z*: 527 $(M+H)^+$. Anal. Calcd for $C_{25}H_{34}N_8O_5{\cdot}0.4H_2O$: C, 56.25; H, 6.57; N, 20.99. Found: C, 56.40; H, 6.46; N, 20.74.

5.1.20. 5-(2-Aminoethylamino)-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidine-8-carboxamide (10a)

A mixture of compound **9c** in HCl–MeOH was stirred for 1 h at room temperature. Resulting precipitates were collected by suction filtration and dried under reduced pressure to give **10a** as pale yellowish solid (40%): mp 234–236 °C; ^{1}H NMR (DMSO-d_6) δ: 3.19–3.26 (2H, m), 3.64–3.71 (2H, m), 3.76 (6H, s), 6.22 (1H, t, J = 2.0 Hz), 6.79 (2H, d, J = 2.0 Hz), 8.30 (3H, br s), 8.80 (1H, br s), 9.65 (1H, s), 9.70 (1H, t, J = 5.0 Hz), 12.52 (1H, s); MS *m*/*z*: 371 $(M-H)^-$. Anal. Calcd for $C_{16}H_{20}N_8O_3{\cdot}3.0HCl{\cdot}2.0H_2O$: C, 37.11; H, 5.26; N, 21.64. Found: C, 37.35; H, 5.25; N, 21.79.

5.1.21. 5-(2-Amino-2-methylpropylamino)-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidine-8-carboxamide (10b)

The title compound was prepared from **9d** in the same manner as described for **10a**, and was obtained as pale yellowish solid (21%): mp 274–275 °C; ^{1}H NMR (DMSO-d_6) δ: 1.29 (6H, s), 3.72–3.75 (2H, m), 3.76 (6H, s), 6.29 (1H, t, J = 2.0 Hz), 6.84 (2H, d, J = 2.0 Hz), 7.67 (1H, br s), 7.87 (3H, br s), 8.44 (1H, s), 8.73 (1H, br s), 8.81 (1H, t, J = 6.0 Hz), 12.38 (1H, s); MS *m*/*z*: 401 $(M+H)^+$. Anal. Calcd for $C_{18}H_{24}N_8O_3{\cdot}1.6HCl$: C, 47.12; H, 5.62; N, 24.42. Found: C, 47.34; H, 5.78; N, 24.20.

5.1.22. 5-(3-Aminopropylamino)-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidine-8-carboxamide (10c)

The title compound was prepared from **9e** in the same manner as described for **10a**, and was obtained as pale yellowish solid (33%): mp 208–210 °C; ^{1}H NMR (DMSO-d_6) δ: 1.93–2.01 (2H, m), 2.92–3.00 (2H, m), 3.77 (6H, s), 4.16 (2H, t, J = 7.0 Hz), 4.40–4.65 (2H, br s), 6.50 (1H, t, J = 2.0 Hz), 6.63 (2H, d, J = 2.0 Hz), 7.84–7.98 (1H, m), 8.08 (3H, br s), 9.00 (1H, s), 11.82 (1H, br s), 15.04 (1H, br s); MS *m*/*z*: 385 $(M-H)^-$. Anal. Calcd for $C_{17}H_{22}N_8O_3{\cdot}2.0HCl{\cdot}1.8H_2O$: C, 41.52; H, 5.66; N, 22.79. Found: C, 41.75; H, 5.61; N, 22.76.

5.1.23. *cis*-5-(2-Aminocyclohexylamino)-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidine-8-carboxyamide (10d)

The title compound was prepared from **9f** in the same manner as described for **10a**, and was obtained as pale yellowish solid (33%): mp 208–210 °C; ^{1}H NMR (CD_3OD) δ: 1.50–2.00 (8H, m), 3.81 (6H, s), 3.94–3.99 (1H, m), 4.30–4.37 (1H, m), 6.39 (1H, t, J = 2.0 Hz), 6.73 (2H, d, J = 2.0 Hz), 9.47(1H, s); MS *m*/*z*: 427 $(M+H)^+$. Anal. Calcd for $C_{20}H_{26}N_8O_3$ 2.0HCl 2.0H_2O: C, 44.86; H, 6.02; N, 20.93. Found: C, 44.63; H, 6.02; N, 21.13.

5.1.24. *cis*-5-(2-Aminocyclohexylamino)-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[1,5-*c*]pyrimidine-8-carboxamide (11)

A mixture of **10d** (0.050 g, 0.108 mmol) in DMF (1 mL) was stirred for 2 days at 100 °C. After being cooled to room temperature, resulting residue was collected by suction filtration and washed with AcOEt to give **11** (0.025 g, 50%) as white solid. mp 293 °C (dec); ^{1}H NMR (DMSO-d_6) δ: 1.30–2.10 (8H, m), 3.77 (6H, s), 4.20–4.35 (1H, m), 6.29 (1H, t, J = 2.0 Hz), 6.74 (2H, d, J = 2.0 Hz), 7.62 (1H, br s), 7.80–8.16 (4H, m), 8.43 (1H, s), 8.80 (1H, br s), 9.58 (1H, s), 12.36 (1H, s); MS *m*/*z*: 427 $(M+H)^+$. Anal. Calcd for $C_{20}H_{26}N_8O_3{\cdot}1.0HCl{\cdot}0.2H_2O$: C, 51.49; H, 5.92; N, 24.02. Found: C, 51.16; H, 5.81; N, 23.65.

5.1.25. *tert*-Butyl *cis*-{2-[8-carbamoyl-7-(3,5-dimethoxyphenylamino)-3-phenyl-1,2,4-triazolo[4,3-*c*]pyrimidin-5-ylamino]cyclohexyl}carbamate (12a)

To a stirred solution of compound **6g** (1.00 g, 1.99 mmol) in THF (8 mL) and EtOH (8 mL) was added hydrazine monohydrate (0.964 mL, 19.9 mmol) at room temperature and the mixture was stirred for 3 h at ambient temperature. Water was added to the mixture and resulting precipitates were collected by suction filtration and dried in vacuo. A mixture of the precipitates and benzaldehyde (0.305 mL, 2.99 mmol) in CH_2Cl_2 (5 mL) was stirred for 2 h at room temperature. The mixture was purified by column chromatography on APS with AcOEt to give crude product. To this product (0.472 g) in DMSO (2.5 mL) and EtOH (2.5 mL) were added 5 M NaOH solution (1.13 mL, 5.63 mmol) and 30% H_2O_2 solution (0.640 mL, 5.63 mmol), then the mixture was stirred overnight at room temperature. To the mixture was added 2 M HCl solution (2.85 mL) at 0 °C and resulting residue was collected by suction filtration. This precipitate was washed with water and dried under reduced pressure. This precipitate (0.100 g) was dissolved in MeCN (3 mL) and 40% diisopropyl azodicarboxylate in toluene (0.500 mL, 0.993 mmol) was added. After being stirred for 24 h at 110 °C, the mixture was purified by column chromatography on silica gel with hexane/AcOEt = 2:3 → 1:4 and recrystallized from MeOH/AcOEt to give P1 as white solid.give **12a** (0.020 g, 17%) as white solid: mp 214–216 °C (MeOH–AcOEt); ^{1}H NMR (DMSO-d_6) δ: 0.55–0.74 (2H, m), 1.22 (9H, s), 1.20–1.62 (6H, m), 3.56–3.64 (1H, m), 3.73 (6H, s), 4.40–4.49 (1H, m), 5.50–5.56 (1H, m), 6.19 (1H, s), 6.74–6.80 (1H, m), 6.79 (2H, d, J = 2.0 Hz), 7.62 (1H, d, J = 3.0 Hz), 7.65–7.71 (3H, m), 7.81–7.87 (2H, m), 8.95 (1H, s), 12.60 (1H, s); MS m/z: 603 $(M+H)^+$. Anal. Calcd for $C_{31}H_{38}N_8O_5$: C, 61.78; H, 6.36; N, 18.59. Found: C, 61.38; H, 6.31; N, 18.41.

5.1.26. *tert*-Butyl *cis*-2-[8-carbamoyl-7-(3,5-dimethoxyphenylamino)-3-(3-methoxyphenyl)-1,2,4-triazolo[4,3-*c*]pyrimidin-5-ylamino]cyclohexylcarbamate (12b)

The title compound was prepared from **6f** in the same manner as described for **12a**, and was obtained as pale yellowish solid (36%): mp 169–171 °C (AcOEt); ^{1}H NMR ($CDCl_3$) δ: 1.35 (9H, br s), 1.36–1.70 (8H, m), 3.78 (6H, s), 3.84–3.91 (1H, m), 3.89 (3H, s), 4.26–4.50 (2H, m), 5.60–5.64 (2H, m), 6.22 (1H, t, J = 2.0 Hz), 6.79 (2H, d, J = 2.0 Hz), 7.15–7.23 (3H, m), 7.53 (1H, t, J = 8.0 Hz), 9.33 (1H, br s), 12.25 (1H, br s); MS m/z: 633 $(M+H)^+$. Anal. Calcd for $C_{32}H_{40}N_8O_6{\cdot}0.4H_2O$: C, 60.06; H, 6.43; N, 17.51. Found: C, 59.95; H, 6.29; N, 17.31.

5.1.27. *tert*-Butyl *cis*-2-[8-carbamoyl-3-(2-chlorophenyl)-7-(3,5-dimethoxyphenylamino)-1,2,4-triazolo[4,3-*c*]pyrimidin-5-ylamino]cyclohexylcarbamate (12c)

The title compound was prepared from **6f** in the same manner as described for **12a**, and was obtained as pale yellowish solid (12%): mp 192–194 °C (AcOEt); ^{1}H NMR ($CDCl_3$) δ: 1.15–1.35 (6H, m), 1.39 (9H, s), 1.40–1.70 (2H, m), 3.60–3.77 (1H, m), 3.78 (6H, s), 4.20–4.50 (2H, m), 5.15–5.26 (1H, m), 5.63 (1H, br s), 6.22 (1H, t, J = 2.0 Hz), 6.77–6.80 (2H, m), 7.52–7.75 (4H, m), 9.29 (1H, br s), 12.25–12.35 (1H, m); MS m/z: 637 $(M+H)^+$. Anal. Calcd for $C_{31}H_{37}ClN_8O_5$: C, 58.44; H, 5.85; N, 17.59. Found: C, 58.11; H, 5.82; N, 17.45.

5.1.28. *cis*-5-(2-Aminocyclohexylamino)-7-(3,5-dimethoxyphenylamino)-3-phenyl-1,2,4-triazolo[4,3-*c*]pyrimidine-8-carboxamide (13a)

A mixture of **12a** (0.092 g, 0.153 mmol) in 4 M HCl–AcOEt (1.14 mL, 4.58 mmol) was stirred overnight at room temperature. Resulting precipitates were collected by suction filtration and dried under reduced pressure to give **13a** (0.060 g, 72%) as white solid: mp 210–212 °C (AcOEt); ^{1}H NMR (DMSO-d_6) δ: 1.14–1.66 (8H, m), 3.73–3.79 (1H, m), 3.76 (6H, s), 3.90 (1H, br s), 4.36 (1H, br s), 5.48–5.53 (1H, m), 6.27 (1H, t, J = 2.0 Hz), 6.76 (2H, d, J = 2.0 Hz), 7.64–7.76 (6H, m), 7.85–7.90 (2H, m), 8.94 (1H, br s), 12.53 (1H, s); MS m/z: 503 $(M+H)^+$. Anal. Calcd for $C_{26}H_{30}N_8O_3{\cdot}2.0HCl{\cdot}0.8H_2O$: C, 52.94; H, 5.74; N, 19.00. Found: C, 53.10; H, 5.88; N, 18.65.

5.1.29. *cis*-5-(2-Aminocyclohexylamino)-7-(3,5-dimethoxyphenylamino)-2-phenyl-1,2,4-triazolo[1,5-*c*]pyrimidine-8-carboxamide (14a)

A mixture of **13a** (0.030 g, 0.056 mmol) DMF (0.5 mL) and stirred overnight at 100 °C. Resulting precipitates were collected by suction filtration to give **14a** (0.020 g, 67%) as white solid: mp 260–261 °C (AcOEt); ^{1}H NMR (DMSO-d_6) δ: 1.38–2.08 (8H, m), 3.78 (6H, s), 3.80–3.86 (1H, m), 4.27–4.33 (1H, m), 6.30 (1H, t, J = 2.0 Hz), 6.81 (2H, d, J = 2.0 Hz), 7.56–7.60 (3H, m), 7.75 (1H, d, J = 2.5 Hz), 7.86–8.20 (2H, br), 8.20–8.29 (3H, m), 8.89 (1H, d, J = 2.5 Hz), 12.41 (1H, s); MS m/z: 503 $(M+H)^+$. Anal. Calcd for $C_{26}H_{30}N_8O_3{\cdot}1.5HCl$: C, 56.04; H, 5.70; N, 20.10. Found: C, 56.30; H, 5.81; N, 19.78.

5.1.30. *cis*-5-(2-Aminocyclohexylamino)-7-(3,5-dimethoxyphenylamino)-2-(3-methoxyphenyl)-1,2,4-triazolo[1,5-*c*]-pyrimidine-8-carboxamide (14b)

The title compound was prepared from **12b** in the same manner as described for **13a** and **14a**, and was obtained as pale yellowish solid (33%): mp 283–285 °C (AcOEt); ^{1}H NMR (DMSO-d_6) δ: 1.30–2.20 (8H, m), 3.77 (6H, s), 3.81–3.90 (2H, m), 3.86 (3H, s), 4.20–4.35 (1H, m), 6.30 (1H, t, J = 2.2 Hz), 6.81 (2H, d, J = 2.2 Hz), 7.10–7.20 (1H, m), 7.49 (1H, t, J = 8.0 Hz), 7.70–8.10 (5H, m), 8.85–8.90 (1H, m), 12.41 (1H, s); MS m/z: 533 $(M+H)^+$. Anal. Calcd for $C_{27}H_{32}N_8O_4{\cdot}6.0HCl{\cdot}4.\ 0C_2H_7N{\cdot}2.0H_2O$: C, 43.44; H, 7.29; N, 17.37. Found: C, 43.31; H, 7.44; N, 17.79.[27]

5.1.31. *cis*-5-(2-Aminocyclohexylamino)-7-(3,5-dimethoxyphenylamino)-2-(2-chlorophenyl)-1,2,4-triazolo[1,5-*c*]pyrimidine-8-carboxamide (14c)

The title compound was prepared from **12c** in the same manner as described for **13a** and **14a**, and was obtained as pale yellowish solid (8.8%): mp 249–251 °C (AcOEt); ^{1}H NMR (DMSO-d_6) δ: 1.36–2.05 (8H, m), 3.78 (6H, s), 3.78–3.87 (2H, m), 4.25–4.33 (1H, m), 6.31 (1H, t, J = 2.0 Hz), 6.81 (2H, d, J = 2.0 Hz), 7.53 (1H, dt, J = 8.0 Hz, 1.0 Hz), 7.59 (1H, dt, J = 8.0 Hz, 2.0 Hz), 7.67 (1H, dd, J = 8.0 Hz, 1.0 Hz), 7.72 (1H, d, J = 2.5 Hz), 7.95 (1H, dd, J = 8.0 Hz, 2.0 Hz), 8.28–8.48 (2H, m), 8.78 (1H, d, J = 2.5 Hz), 12.37 (1H, s); MS m/z: 537 $(M+H)^+$. Anal. Calcd for $C_{26}H_{29}ClN_8O_3{\cdot}4.0HCl{\cdot}2.\ 0C_2H_7N{\cdot}1.0H_2O$: C, 45.55; H, 6.24; N, 17.71. Found: C, 45.38; H, 6.42; N, 17.38.[27]

5.2. Molecular modeling

The 3D coordinates of ZAP-70 receptor model were constructed based on the published crystal structure of an activated Lck kinase domain[16,17] (PDB code. 1QPD) using program FAMS.[18] The structure of staurosporine **1** was extracted from X-ray structure of LCK (PDB code. 1QPD) and manually docked into the ATP-binding site of ZAP-70 receptor model using QUANTA2000[28] (Accelrys Inc.: San Diego, CA, U.S.A.). All docking calculations were performed by using program ADAM.[19]

5.3. Biology

5.3.1. Intracellular ZAP-70 kinase inhibition assay

The kinase domain of human ZAP-70 kinase (Leu325-Ala619) was cloned to NdeI and XhoI sites of pET-19b expression vector (Novagen Inc.) by PCR amplification from human thymus Marathon-Ready™ cDNA (CLONTECH Inc.). The ZAP-70 kinase domain binding with pET-19b His-tag gene at 5′ region was integrated into

pFASTBAC1 vector of the BAC-TO-BAC™ (GIBCO BRL Inc.) baculovirus expression system. The transfected virus was obtained by transfecting Sf-9 cell (Invitrogen Inc.) with pFASTBAC1 containing His-tag-fused ZAP kinase domain as described above. High Five™ baculocells, which were infected with this transfected virus, were recovered and these cells were dissolved by ultrasonication. After soluble fraction being separated by centrifuge, the supernatant was added to TALON™ metal affinity resin (CLONTECH Inc) and to this resin was adsorbed His-tag-fused protein of ZAP-70 kinase domain. The resin was washed several times and the His-tag-fused protein of ZAP-70 kinase domain was extracted by a buffer containing imidazole. A coupled spectrophotometric assay was used wherein ADP generated by ZAP-70 kinase was converted to ATP by pyruvate kinase (PK), with concomitant production of pyruvate from phosphoenolpyruvate (PEP). LDH reduces pyruvate to lactate by oxidizing NADH. NADH depletion was monitored at 340 nm using a microplate reader (Spectra Max 250, Molecular Device) at 30 °C for 20 min. Reactions were performed at 30 °C in 100 mM HEPES buffer (pH 7.6) containing 20 mM $MgCl_2$ and 10% glycerol, and started by adding ATP. PK (150 μg/ml), LDH (500 μg/mL), PEP (2.5 mM), and NADH (150 μM) were added in large excess. Addition of 100 μM ZAP-70 optimal peptide substrate (peptide sequence: AEEEIYGEFEAKKKK, Sawady, Tokyo) allowed measurement of kinase activity.

5.3.2. Intracellular Syk kinase inhibition assay

The kinase domain of human Syk kinase (Met343–Asn635) was cloned to Ndel and Xhol sites of pET-19b expression vector (Novagen Inc) by PCR amplification from human thymus Marathon-Ready™ cDNA (CLONTECH Inc). The Syk kinase domain binding with pET-19b His-tag gene at 5′ region was integrated to pFASTBAC1 vector of the BAC-TO-BAC™ baculovirus expression system (GIBCO BRL Inc.). The transfected virus was obtained by transfecting Sf-9 cell (Invitrogen Inc.) with pFASTBAC vector containing His-tag-fused Syk kinase domain as described above. High Five™ baculocells, which were infected with this transfected virus, were recovered and dissolved by ultrasonication. After soluble fraction being separated by centrifuge, the supernatant was mixed with TALON™ metal affinity resin (CLONTECH Inc) and to this resin was adsorbed His-tag fused protein of Syk kinase domain. The resin was washed several times and the His-tag-fused protein of Syk kinase domain was extracted by a buffer containing imidazole. A coupled spectrophotometric assay was used wherein ADP generated by Syk kinase was converted to ATP by pyruvate kinase (PK), with concomitant production of pyruvate from phosphoenolpyruvate (PEP). LDH reduces pyruvate to lactate by oxidizing NADH. NADH depletion was monitored at 340 nm using a microplate reader (Spectra Max 250, Molecular Device) at 30 °C for 20 min. Reactions were performed at 37 °C in 100 mM HEPES buffer (pH 7.6) containing 40 mM $MgCl_2$ and 10% glycerol, and started by adding ATP. PK (150 μg/mL), LDH (50 μg/mL), PEP (2.5 mM), and NADH (200 μM) were added in large excess. Addition of 100 μM Syk optimal peptide (peptide sequence: AEEEIYGEFEAKKKK, Sawady, Tokyo) allowed measurement of kinase activity.

5.3.3. Peripheral blood mononuclear cells (PBMCs) and whole blood (WB) assay

5.3.3.1. Preparation of PBMCs. Heparinized human peripheral blood was obtained from healthy donors. PBMCs were isolated by the Ficoll-Hypaque gradient density method as described previously. Blood cells were diluted with PBS buffer, and then centrifuged in a Ficoll-Hypaque discontinuous gradient at 1500 rpm for 30 min. The PBMC layers were collected and washed with cold distilled water and 10× Hanks' buffer saline solution (HBSS) to remove red blood cells. The cells were resuspended to a concentration of 2×10^6 cells/mL inAIM-V medium (Invitrogen) containing100 U/mL penicillin and 100 μg/mL streptomycin.

5.3.3.2. Determination of IL-2 Production by PBMCs and WB. PBMCs or WB were cultured with 10 μg/mL PHA alone or in combination with varying concentrations of compounds in 5% CO_2-air humidified atmosphere at 37 °C for 24 h. The cell supernatants were then collected and assayed for IL-2 concentrations by the enzyme-linked immunosorbent assay (ELISA). The ELISA used here is not reported to exhibit detectable cross-reactivity with the other cytokines.

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