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Novel pyrazolo[1,5-*a*]pyrimidines as c-Src kinase inhibitors that reduce I_{Kr} channel blockade

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Abstract—To improve the in vitro potency of the c-Src inhibitor **1a** and to address its hERG liability, a structure–activity study was carried out, focusing on two regions of the lead compound. The blockade of the delayed cardiac current rectifier K^+ (I_{Kr}) channel was overcome by replacing the ethylenediamino group with an amino alcohol group at the 7-position. In addition, modifying the substituents at the 5-position and the side chain groups on the amino alcohols at the 7-position enhanced the intracellular c-Src inhibitory activity and increased central nervous system (CNS) penetration. In the present study, **6l** exhibited significant in vivo efficacy in a middle cerebral artery (MCA) occlusion model in rats.

1. Introduction

In cerebral ischemia, c-Src[1] is intimately involved in a multitude of pathways that may be important in ischemic brain pathology. For instance, c-Src interacts with the *N*-methyl-D-aspartate (NMDA) receptor, suggesting a role in excitotoxic damage.[2,3] In addition, c-Src participates in pathways that are activated by reactive oxygen species, and neutrophil degranulation appears to depend on signaling that involves c-Src, which may have relevance for inflammatory damage.[4,5] Furthermore, c-Src has been reported to be necessary for stimulation of vascular endothelial growth factor (VEGF), which induces vasogenic edema.[6–8] These studies suggest that c-Src is a key intermediate and novel therapeutic target in the pathophysiology of cerebral ischemia.[7]

Previously, we reported the initial structure–activity relationships (SAR) of pyrazolo[1,5-*a*]pyrimidine derivatives as novel c-Src kinase inhibitors.[9] Of these compounds, compound **1a** was the best c-Src kinase inhibitor (Table 1) and demonstrated remarkable neuroprotective efficacy in a rat model. Unfortunately, this compound inhibited I_{Kr} channels, which would markedly complicate its clinical use due to the cardiovascular toxicity associated with QT interval prolongation.[10,11] Several drugs, including terfenadine and astemizole, have been withdrawn because they bind the product of the human ether-a-go-go-related gene (hERG) and prolong the QT interval.[12] Furthermore, Cayabyab and Schlichter have reported that Src tyrosine kinase exists in a signaling complex with the rat ether-a-go-go-related gene (r-ERG) product, which underlies a component of the I_{Kr} channel, and regulates the endogenous r-ERG current.[13] However, no quantitative activity relationship between I_{Kr} channel inhibition and c-Src inhibition was described in their paper. Thus, we sought to vary the substituents on the pyrazolo[1,5-*a*]pyrimidine core to investigate whether it would be possible to attenuate blockade of the I_{Kr} channels.

In recent years, there has been significant progress in the structural understanding of drugs that bind to hERG channels[14] and the relationship between hERG binding potency and preclinical QT prolongation in vivo.[15] Pharmacophore modeling and quantitative structure–activity relationship (QSAR) analysis have also provided insight into the structural requirements.

To minimize blockade of the I_{Kr} channel, we conducted an in silico study that involved compound **1a**

Keywords: c-Src; Delayed cardiac current rectifier K^+ (I_{Kr}) channel; Central nervous system (CNS) penetration; Middle cerebral artery (MCA) occlusion.

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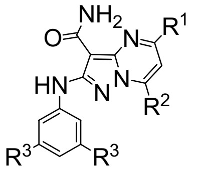
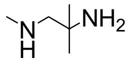
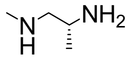
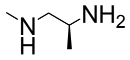
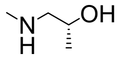
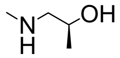
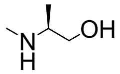
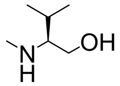
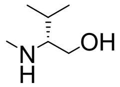
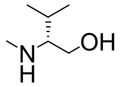
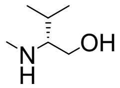
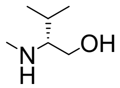
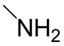
Table 1. In vitro data for the pyrazolo[1,5-*a*]pyrimidine derivatives



Compound	R^1	R^2	R^3	c-Src	ELISA		I_{Kr}
				IC_{50}[a] (μM)	IC_{50}[b] (μM)	% Inhibition[c] at 1 μM	% Inhibition
1a[g]	Me		MeO	0.175	0.9	51.5	90.7[d]
1b[g]	Me		MeO	0.408	NT[f]	0	47.9[d]
1c[g]	Me		MeO	2.99	NT[f]	0	99.1[d]
6a	Me		MeO	6.69	NT[f]	NT	NT[f]
6b	Me		MeO	9.2	NT[f]	NT[f]	NT[f]
6c	Me		MeO	3.48	NT[f]	NT[f]	NT[f]
6d	Me		MeO	1.21	NT[f]	NT[f]	NT[f]
6e	Me		MeO	0.432	NT[f]	13.3	NT[f]
6f	Ph		MeO	0.068	NT[f]	NT[f]	12.4[e]
6g	*i*-Pr		MeO	0.041	NT[f]	60.8	14.6[e]
6h	*c*-Pr		MeO	0.008	0.1	100	0.1[e]
6i	*c*-Pr		H	0.093	NT[f]	69.0	66.6[e]

[a] The IC_{50} for the inhibition of c-Src (determined in duplicate).

[b] The IC_{50} for the inhibition of intracellular c-Src (determined in duplicate).

[c] The inhibition (%) of intracellular c-Src kinase with 1 μM of each compound (determined in duplicate).

[d] The percentage (%) blockade of I_{Kr} channels with 10 μM of each compound (determined in duplicate).

[e] The percentage (%) blockade of I_{Kr} channels with 30 μM of each compound (determined in duplicate).

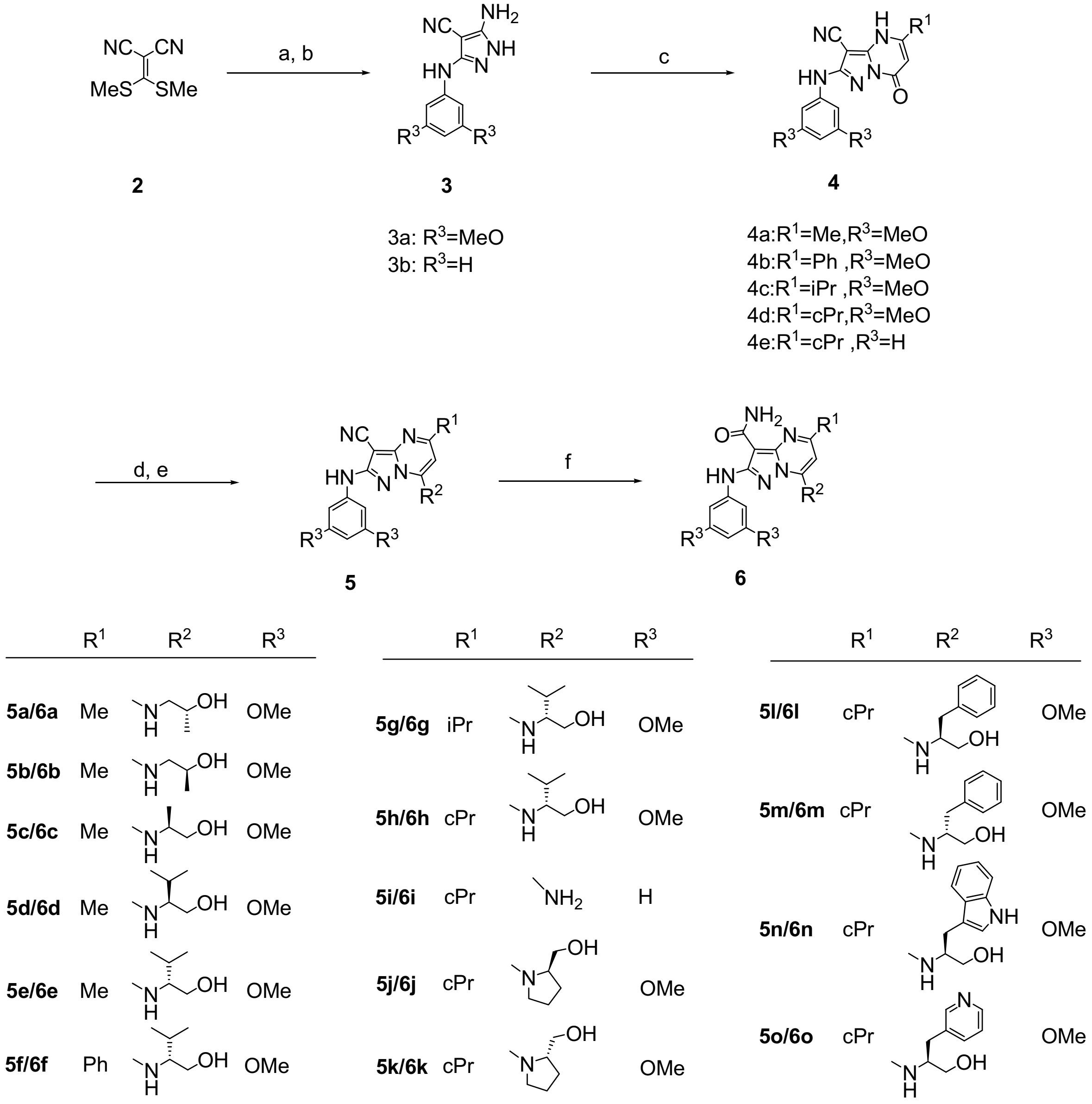
[f] NT, not tested.

[g] See Ref. 9.

and the hERG pharmacophore model using Catalyst.[16] In addition, we attempted to decrease I_{Kr} channel blockade and obtained pyrazolo[1,5-*a*]pyrimidine derivatives with excellent in vitro inhibitory activities and CNS penetration. Here, we report the pharmacological profile and in vivo efficacy of the resulting compound **6l**.

2. Chemistry

Scheme 1 outlines the general method for the synthesis of 5,7-disubstituted 2-anilinopyrazolo[1,5-*a*]pyrimidine derivatives **6**. The synthetic intermediates were prepared by replacing the corresponding anilines with [bis(methylthio)methylene]propanedinitrile **2**, and then cyclizing



Scheme 1. Reagents and conditions: (a) 3,5-dimethoxyaniline or aniline, MeOH, reflux; (b) hydrazine monohydrate, DMF, 100°C; (c) $R^1(CO)CH_2CO_2Et$, AcOH, reflux; (d) $POCl_3$, *N*,*N*-dimethylaniline, 60 °C; (e) R^2 (various amino alcohols), NEt_3, 1-methyl-2-pyrrolidinone (NMP), rt; (f) NaOH, H_2O_2, DMSO, 60 °C.

each compound with hydrazine monohydrate to yield the pyrazolo derivatives **3**. Cyclization of **3** using β-keto ester derivatives generated 2-anilinopyrazolo[1,5-*a*]pyrimidine-7-one analogs **4**.[17] Treatment of **4** with phosphorus oxychloride in *N*,*N*-dimethylaniline provided the corresponding chloro derivatives, which were converted to 3-cyanopyrazole [1,5-*a*]pyrimidines **5**, by substitution of the 7-chlorine with the substituted amino alcohols. Finally, the target compounds **6** were prepared by hydroxylation of the corresponding nitriles **5** with basic hydrogen peroxide in dimethylsulfoxide (DMSO).[18]

3. Results and discussion

As reported previously, rabbit I_{Kr} channel blockade, caused by the pyrazolo[1,5-*a*]pyrimidine derivatives, was measured using a single rabbit ventricular myocyte.[19] The c-Src inhibitory activities of these products were also evaluated using a coupled spectrophotometric enzyme assay (Table 1). The lead compound **1a**, which has a terminal primary amine, strongly inhibited tyrosine kinase c-Src ($IC_{50} = 0.175$ μM) and inhibited intracellular c-Src ($IC_{50} = 0.9$ μM) in an enzyme-linked immunosorbent assay (ELISA) utilizing COS7 cells. Unfortunately, **1a** blocked the I_{Kr} channel by 90.7% at a concentration of 10 μM. The (*S*)-2′-aminopropyl derivative **1c** also blocked the I_{Kr} channel, although it exhibited less c-Src inhibition than **1a**. Despite its terminal primary amine, **1b** interacted somewhat with the I_{Kr} channel and was a potent c-Src inhibitor.

In a previous study, X-ray analysis showed that the terminal primary amino group of **1a** was hydrogen bonded to the water bridging Asn-319.[9] This result suggested that (*R*)-**1b** may have advantages over (*S*)-**1c** in hydrogen bonding to them. Furthermore, we considered the hERG inhibitory activity of **1a**–**c**, based on a docking model between the hERG homology model and these compounds. The homology model was created with FAMS[20] using the KcsA (PDB code:1BL8)[21] structure as the template. In the binding model, the terminal amino groups of **1a**–**c** commonly interact with the phenyl ring of F656 of the hERG channel through a cation–π interaction. The methyl group at the α-position of the terminal amino group (**1c**) interacts with the phenyl ring of Y652, through a CH–π interaction. In contrast, the methyl group of **1b** does not interact with the phenyl ring of Y652 (data not shown). Hence, the stereochemistry of the α-methyl group seemed to play a significant role in the potency of hERG blockade.

Compound **1b** was assumed to be the key compound in this phenomenon, and these results indicate that varying the substituents at the 7-position may alter the blockade of the I_{Kr} channel independent of the c-Src kinase inhibitory activity.

3.1. In silico study of 1a with hERG pharmacophore model

Mutation studies of the hERG channel by Mitcheson et al.[22] demonstrated that basic nitrogen-containing substituents produce a good cation–π interaction with the aromatic residues (F656 and Y652) of the hERG ion channel, and compounds with such substituents are often potent I_{Kr} channel blockers. To identify the structural features of these compounds that are important for I_{Kr} channel blockade, Ekin's hERG pharmacophore model was applied to compound **1a** (Fig. 1). The model possessed four hydrophobic features and one positive ionizable feature. When superimposed on the pharmacophore model, compound **1a** showed a good fit with all five features. The central core, the methyl moiety of the methoxy group, and phenyl part matched the four hydrophobic features. In addition, the terminal primary amine fitted the positive ionizable feature. Thus, we attempted to remove the terminal primary amine on the 7-substituent and replace it with a neutral hydroxyl group, to diminish interactions with the I_{Kr} channel.

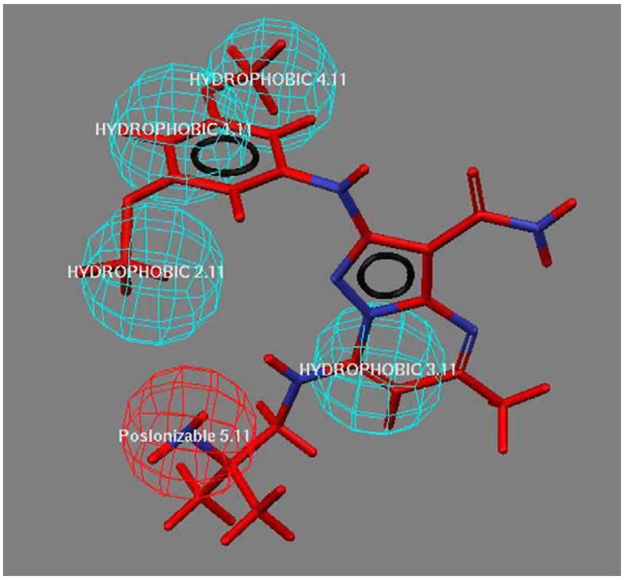


Figure 1. Structure of compound **1a** fitted to the catalyst general hERG pharmacophore. The pharmacophore features are indicated with spheres (hydrophobic feature, cyan; positive ionizable feature, red).

3.2. c-Src kinase and cellular inhibitory activity

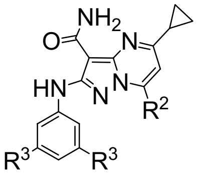
Replacing the terminal primary amine (**1b** and **1c**) with a hydroxyl group (**6a** and **6b**) decreased c-Src inhibitory activity (Table 1). Shifting the methyl group from the α-position of the hydroxyl group to the β-position had a positive effect on c-Src inhibitory activity (**6b** vs **6c**). Replacing the methyl groups with a sterically bulky alkyl group, for example, isopropyl (**6d**), enhanced c-Src inhibitory activity as compared to **6c**. In addition, the (*R*)-valinol analog **6e** had superior activity to the (*S*)-valinol analog **6d**. Nonetheless, compound **6e** with a 7-valinol substituent showed weak cellular c-Src inhibition in the ELISA, as compared to **1a**. A previous study in our laboratory showed that substituent variation at the 5-position on the pyrazolo[1,5-*a*]pyrimidine core affected the inhibitory activity in both a c-Src enzyme assay and a cellular assay.[9] Replacing the methyl group (**6e**) at the 5-position with a phenyl group (**6f**) increased the inhibitory activity. Introducing various alkyl groups at the 5-position, such as 5-isopropyl (**6g**) and 5-cyclopropyl (**6h**), enhanced the activity to a greater extent than that of **6f**. The 5-cyclopropyl derivative **6h** had the most potent activity in this series, with an IC_{50} of 0.008 μM in the kinase assay and 0.1 μM in the cellular kinase assay.

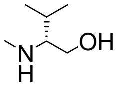
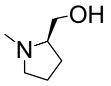
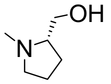
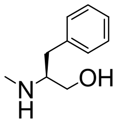
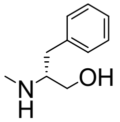
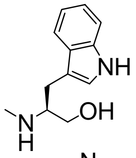
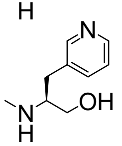
Adding aromatic-substituted amino alcohol derivatives, such as the phenylalaninol analog **6l**, tryptophanol analog **6n**, and 3-pyridinyl amino alcohol analog **6o**, did not adversely affect either the c-Src kinase or the intracellular inhibitory activity, as compared with **6h** (Table 2). Interestingly, the (*S*)-phenylalaninol analog **6l** was a more effective inhibitor than the (*R*)-phenylalaninol analog **6m** in both the c-Src kinase and cellular assays. Conversely, the (*S*)-prolinol derivative **6k** had less c-Src inhibitory activity than the (*R*)-prolinol derivative **6j**. The terminal hydroxyl groups of the amino alcohol derivatives (**6h** and **6j**–**o**) seemed to be similar to the terminal primary amino group of **1a**, hydrogen bonded to the amino acids on the solvent side.[9] These results suggest that the stereochemistry of the amino alcohols played an important role in hydrogen bonding between the terminal alcohols and the amino acids on the solvent side.

3.3. Reducing I_{Kr} channel blockade

The introduction of an amino alcohol (**6f**–**h**) at the 7-position on the pyrazolo[1,5-*a*]pyrimidine core attenuated the blockade of the I_{Kr} channel compared to **1a** (Table 1). In particular, the interaction of compound **6h** with the I_{Kr} channel was markedly reduced, by 0.1%, at a concentration of 30 μM. Conversion of the terminal

Table 2. In vitro data for the 5-cyclopropylpyrazolo[1,5-*a*]pyrimidine derivatives



Compound	R_2	R_3	c-Src	ELISA		I_{Kr}
			IC_{50}[a] (μM)	IC_{50}[b] (μM)	% Inhibition[c] at 1 μM	% Inhibition[d] at 30 μM
6h		MeO	0.008	0.1	100	0.1
6j		MeO	0.009	NT[e]	41.6	14.2
6k		MeO	0.025	NT[e]	14.8	NT[e]
6l		MeO	0.003	0.1	100	23.4
6m		MeO	0.012	NT[e]	36.1	NT[e]
6n		MeO	0.005	0.5	71.2	5.4
6o		MeO	0.005	0.1	100	NT[e]

[a] The IC_{50} for the inhibition of c-Src (determined in duplicate).

[b] The IC_{50} for the inhibition of intracellular c-Src (determined in duplicate).

[c] The inhibition (%) of intracellular c-Src kinase with 1 μM of each compound (determined in duplicate).

[d] The percentage (%) blockade of I_{Kr} channels with 30 μM of each compound (determined in duplicate).

[e] NT, not tested.

primary amine into a hydroxyl group at the 7-substituent also attenuated blockade of the I_{Kr} channel. Conversely, removal of the terminal hydroxyl alkyl group from the 7-substituent (**6i**) had moderate inhibitory activity on I_{Kr} channel current. Several groups have used various approaches to decrease hERG channel blockade with considerable success. Zolotoy et al.[23] reported that shielding the cationic amines by bulky substituents prevented deprotonation of the cationic amines by external water and thereby enhanced I_{Kr} channel blockade. On the other hand, they reported that the introduction of a hydroxyl group at the β-position relative to the cationic amine reduced I_{Kr} channel blockade. These results suggested that introduction of the substituted β-amino alcohol would provide easy access for the hydroxyl group to the proton, leading to fast deprotonation of the cationic amine.

3.4. Central nervous system penetration

For effective treatment of stroke, it is important that these compounds penetrate the brain to pharmacologically active levels. The concentrations of **6h** in the brain and plasma were measured 1 h after intravenous injection (Table 3). Unfortunately, **6h** showed poor CNS penetration in the rat, with a brain:plasma ratio of 0.4 at an intravenous dose of 3 mg/kg. Next, we examined the CNS penetration of some of the derivatives with potent inhibitory activities in the ELISA. The hydrophobic aromatic amino alcohol **6l** showed enhanced CNS penetration as compared with **6h**, while the hydrophilic aromatic amino alcohol **6o** did not penetrate the CNS despite its potent c-Src cellular inhibitory activity. Of these compounds, **6l** reached sufficient levels in the brain. In a pharmacokinetic

Table 3. The CNS penetration of selected c-Src kinase inhibitors in rats[a]

Compound[b]	Brain[c] C_{1h} (ng/g)	Plasma[c] C_{1h} (ng/mL)	Ratio (brain/plasma)
6h	126	346	0.4
6l	256	273	0.9
6n	89	80	1.1
6o	ND[d]	81	

[a] The results are shown as mean values.

[b] Each compound was administered by intravenous injection (3 mg/kg) in a solution of EtOH/dimethyl isosorbide/propylene glycol 400/5% glucose (15:10:35:40).

[c] The concentrations were measured after 1 h.

[d] ND, not detected.

study, compound **6l** was characterized as having moderate pharmacokinetic parameters in rats (Table 4). We concluded that **6l** was a suitable candidate for an in vivo study.

3.5. In vivo efficacy study

The in vivo efficacy of **6l** was evaluated using the rat middle cerebral artery (MCA) occlusion model reported by Umemura et al.[24] The compound was administered intravenously at doses of 0.3, 1, and 3 mg/kg in Cremophor/EtOH/5% glucose (13:7:80), starting immediately after MCA occlusion. The neuroprotective effect was assessed using the infarct volume (Fig. 2). Compound **6l** reduced the infarct volume in a dose-dependent manner, reducing the infarct volume by 20.5% at the highest dose of 3 mg/kg.

Table 4. The pharmacokinetic parameters of c-Src inhibitor **6l** in rats[a]

Pharmacokinetic parameters (iv (3 mg/kg))	AUC[b] (μg h/mL)	$t_{1/2}$ (min)	Cl (mL/min/kg)	V_{ss} (L/kg)
6l	1.99	22	25	0.53

[a] The results are shown as mean values.

[b] The area under the curve represents 0 h to infinity.

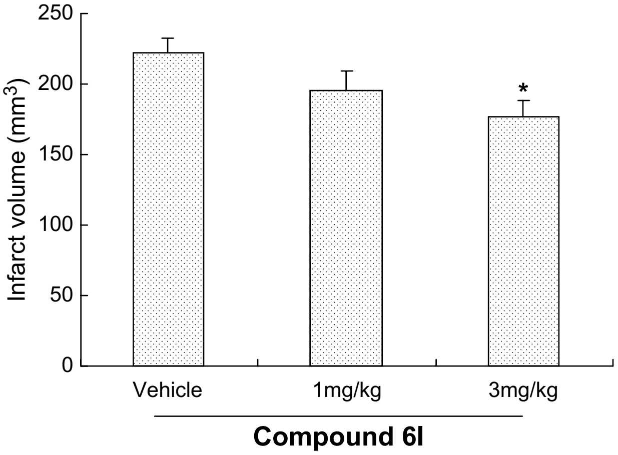


Figure 2. Neuroprotective effects in middle cerebral artery (MCA) thrombosis. Compound **6l** or vehicle (Cremophor/EtOH/5% glucose at 13:7:80) was administered intravenously. $^{*}P < 0.05$ versus vehicle (Dunnett's test; $n = 8$–10).

3.6. Selectivity of compound 6l for other kinase family members

Selectivity for other kinases is important for developing a therapeutic agent. The effect of compound **6l** on kinases other than c-Src kinase was examined (Table 5). Compound **6l** had significant activity against other c-Src kinase family members, such as Lck. However, **6l** showed excellent selectivity for inhibiting Src-family kinases, such as Lck, over other kinases.

4. Conclusions

A series of 2-anilinopyrazolo[1,5-*a*]pyrimidine derivatives was optimized to obtain c-Src kinase inhibitors with optimal properties. The initial problem encountered with compound **1a** was its potent blockade of I_{Kr} channels. The problem associated with I_{Kr} blockade was resolved by replacing the terminal amino group with a hydroxyl group on the 7-substituents. The introduction of a cyclopropyl group at the 5-position increased the cellular inhibitory activity. Furthermore, the introduction of various substituted amino alcohols influenced CNS penetration. The optimized compound **6l** showed low I_{Kr} channel blockade, good CNS penetration, and potent neuroprotective efficacy in the rat MCA occlusion model. Therefore, this novel compound may be useful for treating acute ischemic stroke without cardiac side effects.

5. Experimental

5.1. Chemistry

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

Table 5. The selectivity profile of compound **6l** for various kinases

Compound	IC_{50}[a] (μM)						
	c-Src	Lck	ZAP	Syk	PKC-β	EGFR	KDR
6l	0.003	0.007	>10.0	>10.0	>10.0	2.40	>10.0

[a] The IC_{50} for the inhibition of each kinase (determined in duplicate).

q, quartet. The mass spectra (MS) were carried out with a Thermo Quest FINNIGAN AQA electrospray ionization mass spectrometer. Elemental analyses were performed on an Elementar Vario EL analyzer (C, H, and N). The analytical results obtained were within ±0.4% of the theoretical values unless otherwise stated. Silica gel $60F_{254}$ 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 column chromatography (MPLC) was performed on silica gel 60N (particle size 40–50 μm) from Kanto Chemical Co., Inc. or APS Daisogel IR-60 (particle size 25–40 μm) from Daiso Co., Ltd. All reagents and solvents were commercially available unless otherwise indicated.

The following compounds **1a–c** and intermediates **4a–e** were prepared according to the procedure described previously.[9]

5.1.1. 2-(3,5-Dimethoxyphenylamino)-7-((*R*)-2-hydroxypropylamino)-5-methylpyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (5a). A mixture of 2-(3,5-dimethoxyphenylamino)-5-methyl-7-oxo-4,7-dihydropyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (**4a**, 2.59 g, 7.96 mmol) and phosphorus oxychloride (15 mL, 0.16 mol) in *N*,*N*-dimethylaniline (15 mL, 0.12 mol) was stirred for 3 h at 60 °C. After cooling at room temperature, the mixture was poured into ice-water. The resulting precipitates were collected by filtration and washed with water (50 mL). After drying under vacuum, 1.72 g (63%) of 7-chloro-2-(3,5-dimethoxyphenylamino)-5-methylpyrazolo[1,5-*a*]pyrimidine-3-carbonitrile was obtained as a crude mixture. To a suspension of 7-chloro-2-(3,5-dimethoxyphenylamino)-5-methylpyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (517 mg, 1.50 mmol) and (*R*)-(−)-amino-2-propanol (191 mg, 2.54 mmol) in 1-methyl-2-pyrrolidinone (3.0 mL) was added triethylamine (0.42 mL, 3.10 mmol), and the mixture was stirred for 24 h at room temperature. The resulting mixture was diluted with water (3.0 mL), and the obtained precipitates were collected by filtration and washed with water (3.0 mL) to give **5a** (498 mg, 87%) as an off-white solid: mp 282–283 °C (MeOH); $[\alpha]_D^{30}$ −6.28° (*c* 1.00, DMSO); IR (KBr) 3322, 2210, 1596, 1154 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.13 (3H, d, $J = 6.3$ Hz), 2.40 (3H, s), 3.20–3.30 (1H, m), 3.35–3.45 (1H, m), 3.73 (6H, s), 3.85–3.95 (1H, m), 4.99 (1H, d, $J = 4.7$ Hz), 6.08 (1H, t, $J = 2.2$ Hz), 6.36 (1H, s), 6.97 (2H, d, $J = 2.2$ Hz), 7.33 (1H, t, $J = 6.0$ Hz), 9.21 (1H, s). Anal. Calcd for $C_{19}H_{22}N_6O_3 \cdot 0.5H_2O$: C, 58.30; H, 5.92; N, 21.47. Found: C, 58.54; H, 5.89; N, 21.79.

5.1.2. 2-(3,5-Dimethoxyphenylamino)-7-((*S*)-2-hydroxypropylamino)-5-methylpyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (5b). The title compound was prepared from **4a** and (*S*)-(+)-amino-2-propanol as described for the synthesis of **5a**, and obtained as an off-white solid (84%): mp 277–278 °C (MeOH); $[\alpha]_D^{30}$ +6.40° (*c* 1.00, DMSO); IR (KBr) 3322, 2210, 1596, 1154 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.13 (3H, d, $J = 6.3$ Hz), 2.40 (3H, s), 3.20–3.30 (1H, m), 3.35–3.45 (1H, m), 3.73 (6H, s), 3.85–3.95 (1H, m), 4.99 (1H, d, $J = 4.7$ Hz), 6.08 (1H, t, $J = 2.2$ Hz), 6.36 (1H, s), 6.97 (2H, d, $J = 2.2$ Hz), 7.33 (1H, t, $J = 6.0$ Hz), 9.21 (1H, s). Anal. Calcd for $C_{19}H_{22}N_6O_3 \cdot 0.5H_2O$: C, 58.30; H, 5.92; N, 21.47. Found: C, 58.50; H, 5.87; N, 21.67.

5.1.3. 2-(3,5-Dimethoxyphenylamino)-7-((*S*)-2-hydroxy-1-methylethylamino)-5-methylpyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (5c). The title compound was prepared from **4a** and L-alaninol as described for the synthesis of **5a**, and obtained as an off-white solid (80%): mp 269–270 °C (MeOH); $[\alpha]_D^{30}$ +3.43° (*c* 1.11, DMSO); IR (KBr) 3310, 2214, 1618, 1593, 1487, 1151 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.24 (3H, d, $J = 6.3$ Hz), 2.40 (3H, s), 3.50–3.60 (2H, m), 3.74 (6H, s), 3.80–3.95 (1H, m), 5.04 (1H, t, $J = 5.4$ Hz), 6.07 (1H, t, $J = 2.2$ Hz), 6.39 (1H, s), 6.98 (2H, d, $J = 2.2$ Hz), 7.01 (1H, d, $J = 8.8$ Hz), 9.24 (1H, s). Anal. Calcd for $C_{19}H_{22}N_6O_3 \cdot 0.1H_2O$: C, 59.39; H, 5.82; N, 21.87. Found: C, 59.59; H, 5.78; N, 22.23.

5.1.4. 2-(3,5-Dimethoxyphenylamino)-7-((*S*)-1-hydroxymethyl-2-methylpropylamino)-5-methylpyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (5d). The title compound was prepared from **4a** and L-valinol as described for the synthesis of **5a**, and obtained as an off-white solid (83%): mp 204–205 °C (MeOH); $[\alpha]_D^{30}$ +11.03° (*c* 1.09, DMSO); IR (KBr) 3367, 2213, 1613, 1570, 1488, 1154 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95 (3H, d, $J = 6.9$ Hz), 0.97 (3H, d, $J = 6.9$ Hz), 1.95–2.05 (1H, m), 2.40 (3H, s), 3.50–3.70 (3H, m), 3.73 (6H, s), 4.94 (1H, t, $J = 5.0$ Hz), 6.07 (1H, t, $J = 2.2$ Hz), 6.44 (1H, s), 6.86 (1H, d, $J = 9.8$ Hz), 7.00 (2H, d, $J = 2.2$ Hz), 9.28 (1H, s). Anal. Calcd for $C_{21}H_{26}N_6O_3 \cdot 0.5H_2O$: C, 60.13; H, 6.49; N, 20.03. Found: C, 60.36; H, 6.44; N, 20.42.

5.1.5. 2-(3,5-Dimethoxyphenylamino)-7-((*R*)-1-hydroxymethyl-2-methylpropylamino)-5-methylpyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (5e). The title compound was prepared from **4a** and D-valinol as described for the synthesis of **5a**, and obtained as an off-white solid (76%): mp 204–205 °C (MeOH); $[\alpha]_D^{28}$ −8.45° (*c* 1.04, DMSO); IR (KBr) 3319, 2211, 1618, 1593, 1486, 1153 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95 (3H, d, $J = 6.9$ Hz), 0.97 (3H, d, $J = 6.9$ Hz), 1.95–2.05 (1H, m), 2.40 (3H, s), 3.50–3.70 (3H, m), 3.73 (6H, s), 4.94 (1H, t, $J = 5.0$ Hz), 6.07 (1H, t, $J = 2.2$ Hz), 6.44 (1H, s), 6.86 (1H, d, $J = 9.8$ Hz), 7.00 (2H, d, $J = 2.2$ Hz), 9.28 (1H, s). Anal. Calcd for $C_{21}H_{26}N_6O_3$: C, 61.45; H, 6.38; N, 20.47. Found: C, 61.42; H, 6.39; N, 20.51.

5.1.6. 2-(3,5-Dimethoxyphenylamino)-7-((*R*)-1-hydroxymethyl-2-methylpropylamino)-5-phenylpyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (5f). The title compound was prepared from **4b** and D-valinol as described for the synthesis of **5a**, and obtained as an off-white solid (39%): mp 225–226 °C (MeOH); $[\alpha]_D^{28}$ −12.2° (*c* 1.12, DMSO); IR (KBr) 2478, 2223, 1614, 1204 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.97 (3H, d, $J = 6.6$ Hz), 1.02 (3H, d, $J = 6.6$ Hz), 2.00–2.10 (1H, m), 3.60–3.75 (2H, m), 3.75 (6H, s), 3.85–3.90 (1H, m), 4.96 (1H, t, $J = 5.4$ Hz), 6.09 (1H, d, $J = 2.2$Hz), 7.01 (1H, d, $J = 8.8$ Hz), 7.03 (2H, d, $J = 2.2$ Hz), 7.06 (1H, s), 7.50–7.60 (3H, m), 8.15–8.25 (2H, m), 9.38 (1H, s). Anal. Calcd for $C_{26}H_{28}N_6O_3$: C, 66.09; H, 5.97; N, 17.78. Found: C, 66.11; H, 5.91; N, 17.75.

5.1.7. 2-(3,5-Dimethoxyphenylamino)-7-((*R*)-1-hydroxymethyl-2-methylpropylamino)-5-isopropylpyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (5g). The title compound was prepared from **4c** and D-valinol as described for the synthesis of **5a**, and obtained as an off-white solid (80%): mp 173–174 °C (MeOH); $[\alpha]_D^{28}$ −4.8° (*c* 1.00, DMSO); IR (KBr) 3364, 2963, 2214, 1614, 1591 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.93 (3H, d, *J* = 6.9 Hz), 0.98 (3H, d, *J* = 6.9 Hz), 1.25 (6H, d, *J* = 6.9 Hz), 1.95–2.05 (1H, m), 2.85–3.00 (1H, m), 3.55–3.70 (3H, m), 3.73 (6H, s), 4.93 (1H, t, *J* = 5.4 Hz), 6.07 (1H, d, *J* = 2.2Hz), 6.42 (1H, s), 6.86 (1H, d, *J* = 9.5 Hz), 6.99 (2H, d, *J* = 2.2 Hz), 9.28 (1H, s). Anal. Calcd for $C_{23}H_{30}N_6O_3$: C, 62.99; H, 6.90; N, 19.16. Found: C, 62.88; H, 6.87; N, 19.14.

5.1.8. 5-Cyclopropyl-2-(3,5-dimethoxyphenylamino)-7-((*R*)-1-hydroxymethyl-2-methylpropylamino)pyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (5h). The title compound was prepared from **4d** and D-valinol as described for the synthesis of **5a**, and obtained as an off-white solid (71%): mp 205–206 °C (MeOH); $[\alpha]_D^{28}$ −0.20° (*c* 0.98, DMSO); IR (KBr) 3354, 2958, 2211, 1616, 1590, 1489 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.93 (3H, d, *J* = 6.9 Hz), 0.95–1.10 (4H, m), 0.97 (3H, d, *J* = 6.9 Hz), 1.95–2.10 (2H, m), 3.55–3.70 (3H, m), 3.73 (6H, s), 4.95 (1H, t, *J* = 2.2 Hz), 6.06 (1H, t, *J* = 2.2 Hz), 6.50 (1H, s), 6.82 (1H, d, *J* = 9.8 Hz), 6.99 (2H, d, *J* = 2.2 Hz), 9.26 (1H, s). Anal. Calcd for $C_{23}H_{28}N_6O_3$: C, 63.29; H, 6.47; N, 19.25. Found: C, 63.00; H, 6.44; N, 19.21.

5.1.9. 7-Amino-5-cyclopropyl-2-phenylaminopyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (5i). The title compound was prepared from **4e** and 28% aqueous ammonium solution as described for the synthesis of **5a**, and obtained as an off-white solid (59%): mp 257–258 °C (MeOH); IR (KBr) 3335, 2201, 1642, 1610, 1565 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.90–1.00 (4H, m), 1.95–2.05 (1H, m), 6.15 (1H, s), 6.91 (1H, t, *J* = 7.6 Hz), 7.27 (2H, t, *J* = 7.6 Hz), 7.79 (2H, br s), 7.80–7.85 (2H, m), 9.14 (1H, s). Anal. Calcd for $C_{16}H_{14}N_6{\cdot}0.1H_2O$: C, 65.78; H, 4.90; N, 28.77. Found: C, 65.73; H, 4.75; N, 28.85.

5.1.10. 5-Cyclopropyl-2-(3,5-dimethoxyphenylamino)-7-((*R*)-2-hydroxymethylpyrrolidin-1-yl)pyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (5j). The title compound was prepared from **4d** and D-prolinol as described for the synthesis of **5a**, and obtained as an off-white solid (74%): mp 220–221 °C (MeOH); $[\alpha]_D^{28}$ +125.0° (*c* 0.98, DMSO); IR (KBr) 3319, 2202, 1611, 1577, 1489 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95–1.10 (4H, m), 1.80–1.95 (1H, m), 1.95–2.10 (4H, m), 3.46 (2H, t, *J* = 5.4 Hz), 3.71 (6H, s), 3.80–3.95 (1H, m), 4.00–4.10 (1H, m), 4.80–4.90 (1H, m), 4.81 (1H, t, *J* = 5.7 Hz), 6.06 (1H, t, *J* = 2.2 Hz), 6.28 (1H, s), 6.89 (2H, d, *J* = 2.2 Hz), 9.13 (1H, s). Anal. Calcd for $C_{23}H_{26}N_6O_3{\cdot}H_2O$: C, 61.05; H, 6.24; N, 18.57. Found: C, 61.10; H, 6.44; N, 18.53.

5.1.11. 5-Cyclopropyl-2-(3,5-dimethoxyphenylamino)-7-((*S*)-2-hydroxymethylpyrrolidin-1-yl)pyrazolo[1,5-*a*]-pyrimidine-3-carbonitrile (5k). The title compound was prepared from **4d** and L-prolinol as described for the synthesis of **5a**, and obtained as an off-white solid (74%): mp 224–225 °C (MeOH); $[\alpha]_D^{28}$ −128.4° (*c* 1.05, DMSO); IR (KBr) 3321, 2201, 1610, 1577, 1488 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95–1.10 (4H, m), 1.80–1.95 (1H, m), 1.95–2.10 (4H, m), 3.46 (2H, t, *J* = 5.4 Hz), 3.71 (6H, s), 3.80–3.95 (1H, m), 4.00–4.10 (1H, m), 4.80–4.90 (1H, m), 4.81 (1H, t, *J* = 5.7 Hz), 6.06 (1H, t, *J* = 2.2 Hz), 6.28 (1H, s), 6.89 (2H, d, *J* = 2.2 Hz), 9.13 (1H, s). Anal. Calcd for $C_{23}H_{26}N_6O_3{\cdot}H_2O$: C, 61.05; H, 6.24; N, 18.57. Found: C, 60.97; H, 6.38; N, 18.38.

5.1.12. 7-((*S*)-1-Benzyl-2-hydroxyethylamino)-5-cyclopropyl-2-(3,5-dimethoxyphenylamino)pyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (5l). The title compound was prepared from **4d** and L-phenylalaninol as described for the synthesis of **5a**, and obtained as an off-white solid (55%): mp 183–184 °C (MeOH); $[\alpha]_D^{28}$ −180.8° (*c* 1.07, DMSO); IR (KBr) 3349, 2213, 1614, 1590 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.90–1.00 (4H, m), 0.95–2.05 (1H, m), 2.93 (1H, dd, *J* = 13.6, 8.2 Hz), 3.00 (1H, dd, *J* = 13.6, 6.0 Hz), 3.50–3.65 (2H, m), 3.74 (6H, s), 4.00–4.10 (1H, m), 5.11 (1H, t, *J* = 5.4 Hz), 6.09 (1H, t, *J* = 2.2 Hz), 6.26 (1H, s), 6.95 (2H, d, *J* = 2.2 Hz), 7.04 (1H, d, *J* = 9.5 Hz), 7.17 (1H, t, *J* = 7.6 Hz), 7.25 (2H, t, *J* = 7.6 Hz), 7.31 (2H, d, *J* = 7.6 Hz), 9.19 (1H, s). Anal. Calcd for $C_{27}H_{28}N_6O_3$: C, 66.93; H, 5.82; N, 17.34. Found: C, 67.05; H, 5.78; N, 17.43.

5.1.13. 7-((*R*)-1-Benzyl-2-hydroxyethylamino)-5-cyclopropyl-2-(3,5-dimethoxyphenylamino)pyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (5m). The title compound was prepared from **4d** and D-phenylalaninol as described for the synthesis of **5a**, and obtained as an off-white solid (37%): mp 183–184 °C (MeOH); $[\alpha]_D^{28}$ +182.6° (*c* 1.11, DMSO); IR (KBr) 3347, 2213, 1616, 1590 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.90–1.00 (4H, m), 0.95–2.05 (1H, m), 2.93 (1H, dd, *J* = 13.6, 8.2 Hz), 3.00 (1H, dd, *J* = 13.6, 6.0 Hz), 3.50–3.65 (2H, m), 3.74 (6H, s), 4.00–4.10 (1H, m), 5.11 (1H, t, *J* = 5.4 Hz), 6.09 (1H, t, *J* = 2.2 Hz), 6.26 (1H, s), 6.95 (2H, d, *J* = 2.2 Hz), 7.04 (1H, d, *J* = 9.5 Hz), 7.17 (1H, t, *J* = 7.6 Hz), 7.25 (2H, t, *J* = 7.6 Hz), 7.31 (2H, d, *J* = 7.6 Hz), 9.19 (1H, s). Anal. Calcd for $C_{27}H_{28}N_6O_3$: C, 66.93; H, 5.82; N, 17.34. Found: C, 67.02; H, 5.75; N, 17.34.

5.1.14. 5-Cyclopropyl-2-(3,5-dimethoxyphenylamino)-7-[(*S*)-2-hydroxy-1-(1*H*-indol-3-ylmethyl)ethylamino]pyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (5n). The title compound was prepared from **4d** and L-tryptophanol as described for the synthesis of **5a**, and obtained as an off-white solid (43%): mp 220–221 °C (MeOH); $[\alpha]_D^{28}$ −228.9° (*c* 1.00, DMSO); IR (KBr) 3339, 2213, 1616, 1589, 1488 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.80–0.95 (4H, m), 1.80–1.90 (1H, m), 3.03 (1H, dd, *J* = 14.8, 7.9 Hz), 3.12 (1H, dd, *J* = 14.8, 5.7 Hz), 3.65 (2H, t, *J* = 4.7 Hz), 3.72 (6H, s), 4.00–4.10 (1H, m), 5.10 (1H, t, *J* = 5.4 Hz), 6.08 (1H, t, *J* = 2.2 Hz), 6.10 (1H, s), 6.95–7.10 (3H, m), 6.97 (2H, d, *J* = 2.2 Hz), 7.16 (1H, d, J = 2.2 Hz), 7.31 (1H, d, J = 7.6 Hz), 7.62 (1H, d, J = 7.6 Hz), 9.20 (1H, s), 10.79 (1H, br s). Anal. Calcd for $C_{29}H_{29}N_7O_3 \cdot 0.5H_2O$: C, 65.40; H, 5.68; N, 18.41. Found: C, 65.57; H, 5.55; N, 18.38.

5.1.15. 5-Cyclopropyl-2-(3,5-dimethoxyphenylamino)-7-((*S*)-1-hydroxymethyl-2-pyridin-3-ylethylamino)pyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (5o). The title compound was prepared from **4d** and (*S*)-2-amino-3-pyridin-3-ylpropanol as described for the synthesis of **5a**, and obtained as an off-white solid (37%): mp 253–254 °C (MeOH); $[\alpha]_D^{28}$ −168.7° (c 1.09, DMSO); IR (KBr) 3322, 2210, 1593, 1486, 1152 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.90–1.00 (4H, m), 1.90–2.00 (1H, m), 2.96 (1H, dd, J = 13.9, 8.5 Hz), 3.02 (1H, dd, J = 13.9, 5.7 Hz), 3.55–3.65 (2H, m), 3.74 (6H, s), 4.05–4.15 (1H, m), 5.16 (1H, t, J = 5.0Hz), 6.08 (1H, t, J = 2.2 Hz), 6.26 (1H, s), 6.95 (2H, d, J = 2.2 Hz), 7.15 (1H, d, J = 9.8 Hz), 7.26 (1H, ddd, J = 7.9, 4.7, 0.6 Hz), 7.73 (1H, dt, J = 7.9, 1.9 Hz), 8.37 (1H, dd, J = 4.7, 1.6 Hz), 8.50 (1H, d, J = 1.9 Hz), 9.18 (1H, s). Anal. Calcd for $C_{26}H_{27}N_7O_3$: C, 64.32; H, 5.60; N, 20.19. Found: C, 64.03; H, 5.61; N, 20.00.

5.1.16. 2-(3,5-Dimethoxyphenylamino)-7-((*R*)-2-hydroxypropylamino)-5-methylpyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6a). To a solution of **5a** (308 mg, 0.81 mmol) and 5 M aqueous NaOH (1.12 mL, 5.64 mmol) in DMSO (2.0 mL) and EtOH (2.0 mL) was added dropwise 30% aqueous hydrogen peroxide (0.64 mL, 5.64 mmol) at 60 °C, and the mixture was stirred for 3 h at same temperature. After cooling at room temperature, water (2.0 mL) was added to the mixture and the resulting precipitate was collected by filtration. The obtained solid was crystallized from MeOH and DMSO to give **6a** (286 mg, 89%) as an off-white solid: mp 259–260 °C (MeOH); $[\alpha]_D^{28}$ −4.6° (c 1.04, DMSO); IR (KBr) 3351, 1590, 1507, 1151 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.15 (3H, d, J = 6.3 Hz), 2.43 (3H, s), 3.20–3.30 (1H, m), 3.40–3.50 (1H, m), 3.77 (6H, s), 3.85–4.00 (1H, m), 5.03 (1H, d, J = 5.0 Hz), 6.09 (1H, t, J = 2.2 Hz), 6.32 (1H, s), 6.89 (2H, d, J = 2.2 Hz), 7.33 (1H, t, J = 6.0 Hz), 7.37 (1H, d, J = 2.5 Hz), 7.60 (1H, d, J = 2.5 Hz), 9.58 (1H, s). Anal. Calcd for $C_{19}H_{24}N_6O_4$: C, 56.99; H, 6.04; N, 20.99. Found: C, 56.87; H, 6.00; N, 21.13.

5.1.17. 2-(3,5-Dimethoxyphenylamino)-7-((*S*)-2-hydroxypropylamino)-5-methylpyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6b). The title compound was prepared from **5b** as described for the synthesis of **6a** and obtained as an off-white solid (88%): mp 257–258 °C (MeOH); $[\alpha]_D^{28}$ +5.0° (c 1.00, DMSO); IR (KBr) 3351, 1590, 1507, 1151 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.15 (3H, d, J = 6.3 Hz), 2.43 (3H, s), 3.20–3.30 (1H, m), 3.40–3.50 (1H, m), 3.77 (6H, s), 3.85–4.00 (1H, m), 5.03 (1H, d, J = 5.0 Hz), 6.09 (1H, t, J = 2.2 Hz), 6.32 (1H, s), 6.89 (2H, d, J = 2.2 Hz), 7.33 (1H, t, J = 6.0 Hz), 7.37 (1H, d, J = 2.5 Hz), 7.60 (1H, d, J = 2.5 Hz), 9.58 (1H, s). Anal. Calcd for $C_{19}H_{24}N_6O_4$: C, 56.99; H, 6.04; N, 20.99. Found: C, 56.79; H, 6.01; N, 21.21.

5.1.18. 2-(3,5-Dimethoxyphenylamino)-7-((*S*)-2-hydroxy-1-methylethylamino)-5-methylpyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6c). The title compound was prepared from **5c** as described for the synthesis of **6a** and obtained as an off-white solid (91%): mp 236–237 °C (MeOH); $[\alpha]_D^{28}$ +31.5° (c 1.04, DMSO); IR (KBr) 3397, 1589, 1567, 1160 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.27 (3H, d, J = 6.6 Hz), 2.44 (3H, s), 3.50–3.65 (2H, m), 3.77 (6H, s), 3.85–3.95 (1H, m), 5.09 (1H, t, J = 5.4 Hz), 6.08 (1H, t, J = 2.2 Hz), 6.34 (1H, s), 6.91 (2H, d, J = 2.2 Hz), 7.00 (1H, d, J = 8.8 Hz), 7.38 (1H, d, J = 2.5 Hz), 7.59 (1H, d, J = 2.5 Hz), 9.58 (1H, s). Anal. Calcd for $C_{19}H_{24}N_6O_4$: C, 56.91; H, 6.04; N, 20.99. Found: C, 56.79; H, 6.01; N, 21.17.

5.1.19. 2-(3,5-Dimethoxyphenylamino)-7-((*S*)-1-hydroxymethyl-2-methylpropylamino)-5-methylpyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6d). The title compound was prepared from **5d** as described for the synthesis of **6a** and obtained as an off-white solid (87%): mp 217–218 °C (MeOH); $[\alpha]_D^{28}$ +14.0° (c 1.13, DMSO); IR (KBr) 3374, 1585, 1567, 1153 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95 (3H, d, J = 6.9 Hz), 0.98 (3H, d, J = 6.9 Hz), 1.95–2.10 (1H, m), 2.43 (3H, s), 3.55–3.75 (3H, m), 3.76 (6H, s), 5.00 (1H, t, J = 4.7 Hz), 6.08 (1H, t, J = 2.2 Hz), 6.40 (1H, s), 6.86 (1H, d, J = 9.8 Hz), 6.92 (2H, d, J = 2.2 Hz), 7.40 (1H, d, J = 2.5 Hz), 7.59 (1H, d, J = 2.5 Hz), 9.59 (1H, s). Anal. Calcd for $C_{21}H_{28}N_6O_4 \cdot 0.25H_2O$: C, 58.25; H, 6.63; N, 19.41. Found: C, 58.11; H, 6.52; N, 19.56.

5.1.20. 2-(3,5-Dimethoxyphenylamino)-7-((*R*)-1-hydroxymethyl-2-methylpropylamino)-5-methylpyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6e). The title compound was prepared from **5e** as described for the synthesis of **6a** and obtained as an off-white solid (84%): mp 213–214 °C (MeOH); $[\alpha]_D^{28}$ −8.9° (c 1.05, DMSO); IR (KBr) 3449, 1643, 1609, 1572, 1153 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95 (3H, d, J = 6.9 Hz), 0.98 (3H, d, J = 6.9 Hz), 1.95–2.10 (1H, m), 2.43 (3H, s), 3.55–3.75 (3H, m), 3.76 (6H, s), 5.00 (1H, t, J = 4.7 Hz), 6.08 (1H, t, J = 2.2 Hz), 6.40 (1H, s), 6.86 (1H, d, J = 9.8 Hz), 6.92 (2H, d, J = 2.2 Hz), 7.40 (1H, d, J = 2.5 Hz), 7.59 (1H, d, J = 2.5 Hz), 9.59 (1H, s). Anal. Calcd for $C_{21}H_{28}N_6O_4$: C, 58.86; H, 6.59; N, 19.61. Found: C, 58.53; H, 6.59; N, 19.57.

5.1.21. 2-(3,5-Dimethoxyphenylamino)-7-((*R*)-1-hydroxymethyl-2-methylpropylamino)-5-phenylpyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6f). The title compound was prepared from **5f** as described for the synthesis of **6a** and obtained as an off-white solid (quant.): mp 245–246 °C (MeOH); $[\alpha]_D^{28}$ −7.8° (c 1.07, DMSO); IR (KBr) 3404, 1643, 1161, 1572, 1152 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.99 (3H, d, J = 6.9 Hz), 1.03 (3H, d, J = 6.9 Hz), 2.00–2.15 (1H, m), 3.60–3.75 (2H, m), 3.78 (6H, s), 3.85–4.00 (1H, m), 5.01 (1H, t, J = 4.4 Hz), 6.10 (1H, d, J = 2.2 Hz), 6.96 (2H, d, J = 2.2 Hz), 7.00–7.05 (2H, m), 7.50 (1H, d, J = 2.2 Hz), 7.52–7.60 (3H, m), 7.69 (1H, d, J = 2.2 Hz), 8.21 (2H, dd, J = 8.1, 1.6 Hz), 9.68 (1H, s). Anal. Calcd for $C_{26}H_{30}N_6O_4 \cdot H_2O$: C, 61.40; H, 6.34; N, 16.52. Found: C, 61.49; H, 6.23; N, 16.64.

5.1.22. 2-(3,5-Dimethoxyphenylamino)-7-((*R*)-1-hydroxymethyl-2-methylpropylamino)-5-isopropylpyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6g). The title compound was prepared from **5g** as described for the synthesis of **6a** and obtained as an off-white solid (84%): mp 177–178 °C (MeOH); $[\alpha]_D^{28}$ −8.3° (*c* 1.09, DMSO); IR (KBr) 3381, 2964, 1649, 1585, 1154 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95 (3H, d, *J* = 6.9 Hz), 0.99 (3H, d, *J* = 6.9 Hz), 1.26 (6H, d, *J* = 6.9 Hz), 1.95–2.10 (1H, m), 2.90–3.00 (1H, m), 3.60–3.75 (3H, m), 3.77 (6H, s), 4.99 (1H, t, *J* = 5.0 Hz), 6.08 (1H, d, *J* = 2.2 Hz), 6.38 (1H, s), 6.87 (1H, d, *J* = 9.5 Hz), 6.93 (2H, d, *J* = 2.2 Hz), 7.40 (1H, d, *J* = 2.8 Hz), 7.63 (1H, d, *J* = 2.8 Hz), 9.58 (1H, s). Anal. Calcd for $C_{23}H_{32}N_6O_4$: C, 60.51; H, 7.06; N, 18.41. Found: C, 60.36; H, 7.04; N, 18.38.

5.1.23. 5-Cyclopropyl-2-(3,5-dimethoxyphenylamino)-7-((*R*)-1-hydroxymethyl-2-methylpropylamino)pyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6h). The title compound was prepared from **5h** as described for the synthesis of **6a** and obtained as an off-white solid (89%): mp 204–205 °C (MeOH); $[\alpha]_D^{28}$ −2.4° (*c* 0.99, DMSO); IR (KBr) 3387, 1585, 1463, 1207 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95 (3H, d, *J* = 6.9 Hz), 0.99 (3H, d, *J* = 6.9 Hz), 1.00–1.10 (4H, m), 1.95–2.15 (2H, m), 3.60–3.75 (3H, m), 3.76 (6H, s), 4.99 (1H, br s), 6.07 (1H, t, *J* = 2.2 Hz), 6.47 (1H, s), 6.81 (1H, d, *J* = 9.5 Hz), 6.91 (2H, d, *J* = 2.2 Hz), 7.33 (1H, d, *J* = 2.2 Hz), 7.42 (1H, d, *J* = 2.2 Hz), 9.58 (1H, s). Anal. Calcd for $C_{23}H_{30}N_6O_4$·H_2O: C, 58.46; H, 6.83; N, 17.78. Found: C, 58.53; H, 6.69; N, 17.87.

5.1.24. 7-Amino-5-cyclopropyl-2-phenylaminopyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6i). The title compound was prepared from **5i** as described for the synthesis of **6a** and obtained as an off-white solid (61%): mp 269–270 °C (MeOH; IR (KBr) 3304, 1649, 1597, 1561 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95–1.05 (4H, m), 1.95–2.10 (1H, m), 6.13 (1H, s), 6.92 (1H, t, *J* = 6.9 Hz), 7.26 (1H, br s), 7.30 (2H, t, *J* = 7.6 Hz), 7.43 (1H, br s), 7.74 (2H, d, *J* = 7.9 Hz), 7.82 (2H, br s), 9.57 (1H, s). Anal. Calcd for $C_{16}H_{16}N_6O$: C, 62.32; H, 5.23; N, 27.26. Found: C, 62.01; H, 5.10; N, 27.08.

5.1.25. 5-Cyclopropyl-2-(3,5-dimethoxyphenylamino)-7-((*R*)-2-hydroxymethylpyrrolidin-1-yl)pyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6j). The title compound was prepared from **5j** as described for the synthesis of **6a** and obtained as an off-white solid (88%): mp 197–198 °C (MeOH); $[\alpha]_D^{28}$ +136.8° (*c* 1.03, DMSO); IR (KBr) 3462, 1604, 1574, 1155 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95–1.05 (4H, m), 1.85–1.95 (1H, m), 2.00–2.10 (4H, m), 3.49 (2H, t, *J* = 5.0 Hz), 3.74 (6H, s), 3.85–4.00 (1H, m), 4.05–4.15 (1H, m), 4.84 (1H, t, *J* = 5.4 Hz), 4.90–5.05 (1H, m), 6.06 (1H, t, *J* = 2.2 Hz), 6.26 (1H, s), 6.77 (2H, d, *J* = 2.2 Hz), 7.31 (1H, d, *J* = 2.8 Hz), 7.60 (1H, d, *J* = 2.8 Hz), 9.59 (1H, s). Anal. Calcd for $C_{23}H_{28}N_6O_4$·0.25H_2O: C, 60.45; H, 6.29; N, 18.39. Found: C, 60.49; H, 6.23; N, 18.45.

5.1.26. 5-Cyclopropyl-2-(3,5-dimethoxyphenylamino)-7-((*S*)-2-hydroxymethylpyrrolidin-1-yl)pyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6k). The title compound was prepared from **5k** as described for the synthesis of **6a** and obtained as an off-white solid (77%): mp 196–197 °C (MeOH); $[\alpha]_D^{28}$ −137.3° (*c* 1.05, DMSO); IR (KBr) 3466, 1605, 1574, 1153 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95–1.05 (4H, m), 1.85–1.95 (1H, m), 2.00–2.10 (4H, m), 3.49 (2H, t, *J* = 5.0 Hz), 3.74 (6H, s), 3.85–4.00 (1H, m), 4.05–4.15 (1H, m), 4.84 (1H, t, *J* = 5.4 Hz), 4.90–5.05 (1H, m), 6.06 (1H, t, *J* = 2.2 Hz), 6.26 (1H, s), 6.77 (2H, d, *J* = 2.2 Hz), 7.31 (1H, d, *J* = 2.8 Hz), 7.60 (1H, d, *J* = 2.8 Hz), 9.59 (1H, s). Anal. Calcd for $C_{23}H_{28}N_6O_4$·0.8H_2O: C, 59.16; H, 6.39; N, 18.00. Found: C, 59.20; H, 6.28; N, 18.13.

5.1.27. 7-((*S*)-1-Benzyl-2-hydroxyethylamino)-5-cyclopropyl-2-(3,5-dimethoxyphenylaminopyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6l). The title compound was prepared from **5l** as described for the synthesis of **6a** and obtained as an off-white solid (quant.): mp 196–197 °C (MeOH); $[\alpha]_D^{28}$ −186.2° (*c* 1.06, DMSO); IR (KBr) 3368, 1634, 1592, 1557 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.90–1.05 (4H, m), 1.95–2.05 (1H, m), 2.95 (1H, dd, *J* = 13.6, 8.5 Hz), 3.03 (1H, dd, *J* = 13.6, 6.3 Hz), 3.55–3.65 (2H, m), 3.78 (6H, s), 4.00–4.10 (1H, m), 5.17 (1H, br s), 6.10 (1H, t, *J* = 2.2 Hz), 6.25 (1H, s), 6.90 (2H, d, *J* = 2.2 Hz), 7.04 (1H, d, *J* = 9.1 Hz), 7.17 (1H, t, *J* = 7.6 Hz), 7.26 (2H, t, *J* = 7.6 Hz), 7.30–7.35 (3H, m), 7.39 (1H, d, *J* = 2.5 Hz), 9.54 (1H, s). Anal. Calcd for $C_{27}H_{30}N_6O_4$·0.5H_2O: C, 63.39; H, 6.11; N, 16.43. Found: C, 63.41; H, 5.81; N, 16.35.

5.1.28. 7-((*R*)-1-Benzyl-2-hydroxyethylamino)-5-cyclopropyl-2-(3,5-dimethoxyphenylamino)pyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6m). The title compound was prepared from **5m** as described for the synthesis of **6a** and obtained as an off-white solid (52%): mp 194–195 °C (MeOH); $[\alpha]_D^{28}$ +191.4° (*c* 1.05, DMSO); IR (KBr) 3463, 1636, 1592, 1556 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.90–1.05 (4H, m), 1.95–2.05 (1H, m), 2.95 (1H, dd, *J* = 13.6, 8.5 Hz), 3.03 (1H, dd, *J* = 13.6, 6.3 Hz), 3.55–3.65 (2H, m), 3.78 (6H, s), 4.00–4.10 (1H, m), 5.17 (1H, br s), 6.10 (1H, t, *J* = 2.2 Hz), 6.25 (1H, s), 6.90 (2H, d, *J* = 2.2 Hz), 7.04 (1H, d, *J* = 9.1 Hz), 7.17 (1H, t, *J* = 7.6 Hz), 7.26 (2H, t, *J* = 7.6 Hz), 7.30–7.35 (3H, m), 7.39 (1H, d, *J* = 2.5 Hz), 9.54 (1H, s). Anal. Calcd for $C_{27}H_{30}N_6O_4$: C, 64.53; H, 6.02; N, 16.72. Found: C, 64.22; H, 6.01; N, 16.70.

5.1.29. 5-Cyclopropyl-2-(3,5-dimethoxyphenylamino)-7-[(*S*)-2-hydroxy-1-(1*H*-indol-3-ylmethyl)ethylamino]pyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6n). The title compound was prepared from **5n** as described for the synthesis of **6a** and obtained as an off-white solid (98%): mp 250–251 °C (MeOH); $[\alpha]_D^{28}$ −226.1° (*c* 1.03, DMSO); IR (KBr) 3419, 1643, 1587, 1157 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.80–1.00 (4H, m), 1.85–1.95 (1H, m), 3.06 (1H, dd, *J* = 14.8, 7.9 Hz), 3.15 (1H, dd, *J* = 14.8, 6.0 Hz), 3.65–3.70 (2H, m), 3.76 (6H, s), 4.00–4.15 (1H, m), 5.15(1H, t, *J* = 5.0 Hz), 6.05–6.15 (2H, m), 6.90 (2H, d, *J* = 6.9 Hz), 6.95–7.10 (3H, m), 7.19 (1H, d, *J* = 2.2 Hz), 7.25–7.35 (2H, m), 7.39 (1H, d, *J* = 2.2 Hz), 7.66 (1H, d, *J* = 8.2 Hz), 9.55 (1H, s), 10.80 (1H, br s). Anal. Calcd for $C_{29}H_{31}N_7O_4$·H_2O: C, 62.24; H, 5.94; N, 17.52. Found: C, 62.47; H, 5.76; N, 17.60.

5.1.30. 5-Cyclopropyl-2-(3,5-dimethoxyphenylamino)-7-((*S*)-1-hydroxymethyl-2-pyridin-3-ylethylamino)pyrazolo[1,5-*a*]pyrimidine-3-carboxamide (6o). The title compound was prepared from **5o** as described for the synthesis of **6a** and obtained as an off-white solid (96%): mp 173–174 °C (MeOH); $[\alpha]_D^{28}$ −186.3° (*c* 0.85, DMSO); IR (KBr) 3388, 1605, 1586, 1153 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.90–1.05 (4H, m), 1.95–2.05 (1H, m), 2.99 (1H, dd, *J* = 13.9, 8.5 Hz), 3.05 (1H, dd, *J* = 13.9, 6.0 Hz), 3.55–3.70 (2H, m), 3.78 (6H, s), 4.05–4.15 (1H, m), 5.21 (1H, t, *J* = 5.0Hz), 6.09 (1H, t, *J* = 2.2 Hz), 6.24 (1H, s), 6.90 (2H, d, *J* = 2.2 Hz), 7.13 (1H, d, *J* = 9.5 Hz), 7.27 (1H, dd, *J* = 7.9, 4.7 Hz), 7.30 (1H, d, *J* = 2.5 Hz), 7.39 (1H, d, *J* = 2.5 Hz), 7.75 (1H, dt, *J* = 7.9, 1.9 Hz), 8.37 (1H, dd, *J* = 4.7, 1.9 Hz), 8.52 (1H, d, *J* = 1.9 Hz), 9.55 (1H, s). Anal. Calcd for $C_{26}H_{29}N_7O_4$: C, 62.01; H, 5.80; N, 19.47. Found: C, 61.91; H, 5.87; N, 19.46.

5.2. Biology

5.2.1. Kinase assays. A coupled spectrophotometric assay was used wherein ADP generated by Src 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, initiated by adding ATP. PK (100 μg/mL), LDH (50 μg/mL), PEP (2 mM), and NADH (140 μM) were also added. Kinase activity was measured by adding 100 μM Src optimal peptide substrate (peptide sequence: AEEEIYGEFEAKKKK, Sawady, Tokyo). The methods for the other kinase assays except c-Src were constructed on the basis of the previous report.[25]

5.2.2. Intracellular kinase inhibition assay. The kinase domain of human Src kinase (NM_005417, base# 790-1650) was cloned by PCR amplification, using the linker-containing primers 5′-AAACTTAAGCTTCATAT GTCCAAGCCGCAGAC-3′ and 5′-CTGCAGATATC CCTAGAAGTAGTCCTCCAGGAA-3′. The gene for the Src kinase domain was integrated between the HindIII and EcoRV restriction sites within the multiple cloning site of the pcDNA3.1(+) expression vector. The vector was transfected into COS7 cells according to the calcium phosphate method.[26] Subsequently, 8.8 μg DNA was mixed with 220 μL of 1 mM Tris–HCl, 0.1 mM EDTA buffer (pH 8.0), 250 μL 2× Hepes buffered saline, and 31 μL of 2 M $CaCl_2$. The solution was incubated at room temperature for 15 min and then used to resuspend a pellet of 10^6 COS7 cells. The cell suspension was incubated at room temperature for 15 min; then, 4.5 mL Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics was added, and the solution was pre-warmed to 37 °C. Cells were seeded into 96-well multi-well plates with 2×10^4 cells/well/100 μL. A day after transfection, the culture media were replaced with media containing fixed concentrations of test compounds. On the third day, the cellular phosphotyrosine contents were determined using a commercially available phosphotyrosine ELISA kit (Upstate Biotechnology, NY, USA. Cellular Phosphotyrosine ELISA Kit™, Catalog #17-182). Any basal phosphotyrosine content unrelated to c-Src activity was screened for using vector-transfected cells. Intracellular c-Src inhibition was expressed as the percentage of specific phosphotyrosine levels in drug-treated cells compared to the levels in control cells without drug. The toxicity of each chemical was determined with another replica plate using a colorimetric MTT assay kit (Chemicon International, CA, USA. Cat. #CT02).

5.2.3. Measuring the delayed rectifier K$^+$ currents in single rabbit ventricular myocytes

5.2.3.1. Isolation of single rabbit ventricular myocytes. Japanese White rabbits (1.5–2.0 kg) were anesthetized with thiamylal sodium after being heparinized. Single myocytes were isolated enzymatically from the middle left ventricular free wall using the Langendorff technique.[27]

5.2.3.2. Electrophysiological recordings. The single pipette, whole-cell clamp method was used to record the currents. The resistance of the grass pipette was 2–3 MΩ after it was filled with an internal pipette solution. The cell capacitance was determined by varying the voltage between −50 and +20 mV. An aliquot of the cell suspension was placed in the recording chamber on the stage of an inverted microscope, and the cells were perfused with solution. The voltage and current signals were stored on a personal computer using the PCLAMP version 8.2 software (Axon Instruments) for analysis.

5.2.3.3. Solution and drugs. Tyrode solution, which was used for cell isolation, consisted of (in mmol/L) NaCl 143, KCl 5.4, $MgCl_2$ 0.5, NaH_2PO_4 0.25, Hepes 5.0, $CaCl_2$ 1.8, and glucose 5.6 (pH adjusted to 7.35 with NaOH). The internal pipette solution consisted of (in mmol/L) KOH 60, KCl 80, aspartate 40, Hepes 5.0, EGTA 10, MgATP 5.0, sodium creatinine phosphate 5.0, and $CaCl_2$ 0.65 (pH adjusted to 7.2 with NaOH; pCa 8.0). When the I_{Kr} was measured, the cells were superfused with a Na$^+$- and K$^+$-free NMG solution that consisted of (in mmol/L) *N*-methyl-D-glucamine 149, $MgCl_2$ 5, $CaCl_2$ 0.9, Hepes 5, and nisoldipine 0.003. The bath temperature in all of the experiments was 34 °C. I_{Kr} was measured during blockade of the slow component of the delayed rectifier potassium current (I_{Ks}) with 30 μmol/L chromanol 293B. Chromanol 293B and the test substances were dissolved in dimethylsulfoxide (DMSO) as 100 and 30 mmol/L stock solutions, respectively, and diluted in the superfusates to the final concentration immediately before each application.

5.2.3.4. Data analysis. The effects of the test compounds on the currents were expressed as changes relative to the currents recorded before the test compounds were added.

5.2.4. Analysis of c-Src inhibitors in the brain and plasma. To inhibit c-Src kinase in the brain, we first determined the brain and plasma levels of the test compounds. Sprague–Dawley rats (300–350 g: $n = 3$/study) were anesthetized with 25% urethane (1.1 g/kg, sc), and the femoral vein was cannulated to deliver the test compounds. Tracheal intubation was performed for artificial respiration. The animal's body temperature was maintained at 37.5 °C with a heating-pad. Test compounds were administered by an intravenous injection at 3 mg/kg. One hour later, blood samples were taken from the aorta and centrifuged to separate the plasma. Brain tissue was homogenized with a threefold volume of saline.

Plasma and brain tissue homogenate (50 μL) was mixed with 200 μL of 100% acetonitrile, 100 μL of 0.1% heptafluorobutyric acid, and 200 μL internal standard solution. After centrifugation at 3300 rpm for 20 min, the supernatant was removed and transferred to the column. Samples were quantified using liquid chromatography–tandem mass spectrometry (LC–MS/MS). The LC–MS/MS system consisted of an Alliance HPLC (Waters 2690) with a SYNERGI MAX-RP 80 A column (4 μm, 50 × 4.6 mm, Phenomenex, Torrance, CA) and a Sciex API 365 mass spectrometer (Perkin-Elmer Sciex, Toronto, Canada) equipped with a turbo ion spray source. The mobile phase consisted of 100% acetonitrile and 0.1% acetic acid in water (50:50 v/v). The column was maintained at 50 °C with a constant flow rate of 0.4 mL/min. The data were processed using Mass Chrom 1.1 software. The standard curves plotted for each test compound demonstrated good linearity (coefficient of determination >0.99). The limits of quantification of the compounds in plasma and brain were 50 ng/mL.

5.2.5. Photochemically induced middle cerebral artery occlusion in rats. Male Sprague–Dawley rats weighing 260–320 g were anesthetized with enflurane. The animal's body temperature was maintained at 37.5 °C with a heating pad. We performed middle cerebral artery (MCA) thrombosis as previously described.[22] A catheter was inserted into the femoral vein to administer the drug and rose bengal dye. The scalp and temporalis muscle were folded over. A subtemporal craniotomy was performed using a dental drill under an operating microscope to open a 3-mm-diameter oval bony window. The window was irradiated with green light (540 nm wavelength) using a xenon lamp (L4887, Hamamatsu Photonics, Hamamatsu, Japan), with both heat-absorbing and green filters. The irradiation was directed by a 3-mm-diameter optic fiber mounted on a micromanipulator. The head of the optic fiber was placed on the window in the skull base at a distance of 2 mm above the vessel, providing an irradiation dose of 0.62 W/cm^2. Rose bengal (20 mg/kg) was injected intravenously. Photo-irradiation was continued for another 10 min. Compound **6l** was administered at doses of 1 and 3 mg/kg, starting immediately after the MCA occlusion. Twenty-four hours after surgery, the rats were sacrificed by administering an overdose of pentobarbital, and their brains were quickly removed. The cerebrum was separated from the other parts of the brain, and cut into six 2-mm-thick slices using a Brain Matrix (Muromachi Kikai, Japan). Each slice was incubated in 1% tetratrihydrochloride (TTC) solution at room temperature for 30 min and then photographed. The area of infarction was measured for each slice using a computerized image analysis system (Mac scope, Japan), and the ratio of infarction size was calculated by dividing the whole area by the region of cerebral ischemic damage. All animal studies were approved by the Kissei Pharmaceutical Co., Ltd Committee on Ethics of Animal Experimentation, and special care was taken to prevent animal suffering.

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