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Discovery of 7-*N*-Piperazinylthiazolo[5,4-*d*]pyrimidine Analogues as a Novel Class of Immunosuppressive Agents with in Vivo Biological Activity

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Herein we describe the synthesis and in vitro and in vivo activity of thiazolo[5,4-*d*]pyrimidines as a novel class of immunosuppressive agents, useful for preventing graft rejection after organ transplantation. This research resulted in the discovery of a series of compounds with potent activity in the mixed lymphocyte reaction (MLR) assay, which is well-known as the in vitro model for in vivo rejection after organ transplantation. The most potent congeners displayed IC_{50} values of less than 50 nM in this MLR assay and hence are equipotent to cyclosporin A, a clinically used immunosuppressive drug. One representative of this series was further evaluated in a preclinical animal model of organ transplantation and showed excellent in vivo efficacy. It validates these compounds as new promising immunosuppressive drugs.

Introduction

Solid organ transplantation has become a common medical procedure with considerable impact on extending and improving the quality of life of patients with end stage renal, cardiac, hepatic, or pulmonary failure.[1] Several immunosuppressive agents to prevent graft rejection after organ transplantation are available such as calcineurin inhibitors (cyclosporin A, tacrolimus), antimetabolites (mycophenolate mofetil, azathioprine), mammalian target of rapamycin (mTOR[a]) inhibitors (sirolimus, everolimus), and corticosteroids. However, continuous administration of these agents for the entire life is difficult for the patient because immunosuppressive drugs generally suppress the host's immunocompetence and the treatment is accompanied by serious side effects such as opportunistic infections, nephrotoxicity, neurotoxicity, hyperlipidaemia, new-onset post-transplant diabetes mellitus, and hypertension.[2] Thus, a high medical need exists for safe and specific inhibitors of early T-cell activation with a novel mechanism of action.

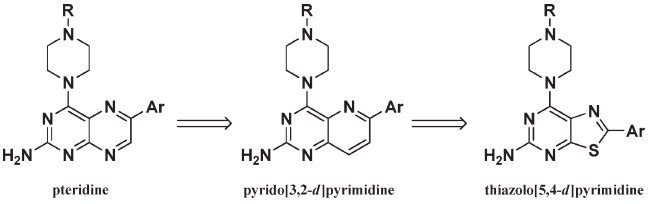

Figure 1. Scaffold evolution.

Over the past few years, our group has been actively involved in the search for new immunosuppressive compounds, based on a bicyclic heteroaromatic scaffold (Figure 1). This research started in the late 1990s, with the discovery of 4-*N*-piperazinylpteridines as immunosuppressive agents.[3] Further scaffold modification led to the synthesis of pyrido[3,2-*d*]pyrimidines as potent immunosuppressants.[4]

The concept of bioisosterism is a useful way to come up with novel scaffolds, starting from a known, biologically active compound. In drug design, the purpose of exchanging one bioisostere for another is to enhance the desired biological or physicochemical properties of a compound without changing the target. Bioisosteric replacements often provide the foundation for the development of new structure−activity relationships, and ring equivalent bioisosteres have therefore been used frequently in drug discovery programs.[5] As a 1,3-thiazole is a well-known isostere of the pyridine ring, we envisaged synthesis of thiazolo[5,4-*d*]pyrimidines as potential surrogates of the pyrido[3,2-*d*]pyrimidine scaffold (Figure 1).

The thiazolo[5,4-*d*]pyrimidine scaffold is not very frequently used in drug discovery programs, as the number of medicinal chemistry projects that describes the synthesis and biological evaluation of thiazolopyrimidines is limited. However, certain thiazolo[5,4-*d*]pyrimidines have been reported as templates

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[a] Abbreviations: AcOH, acetic acid; APBS, adaptive Poisson−Boltzmann solver; Boc, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; CsA, cyclosporin A; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC, dicyclohexylcarbodiimide; DIPEA, diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; HOBt, hydroxybenzotriazole; HSP, heat shock protein; IC_{50}, half maximal (50%) inhibitory concentration; *m*CPBA, *m*-chloroperoxybenzoic acid; MLR, mixed lymphocyte reaction; mTOR, mammalian target of rapamycin; NEt_3, triethylamine; PCC, pyridinium chlorochromate; PNP, purine nucleoside phosphorylase; ROCS, rapid overlay of chemical structures; rt, room temperature; SAR, structure−activity relationship; TBTU, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate; TFA, trifluoroacetic acid.

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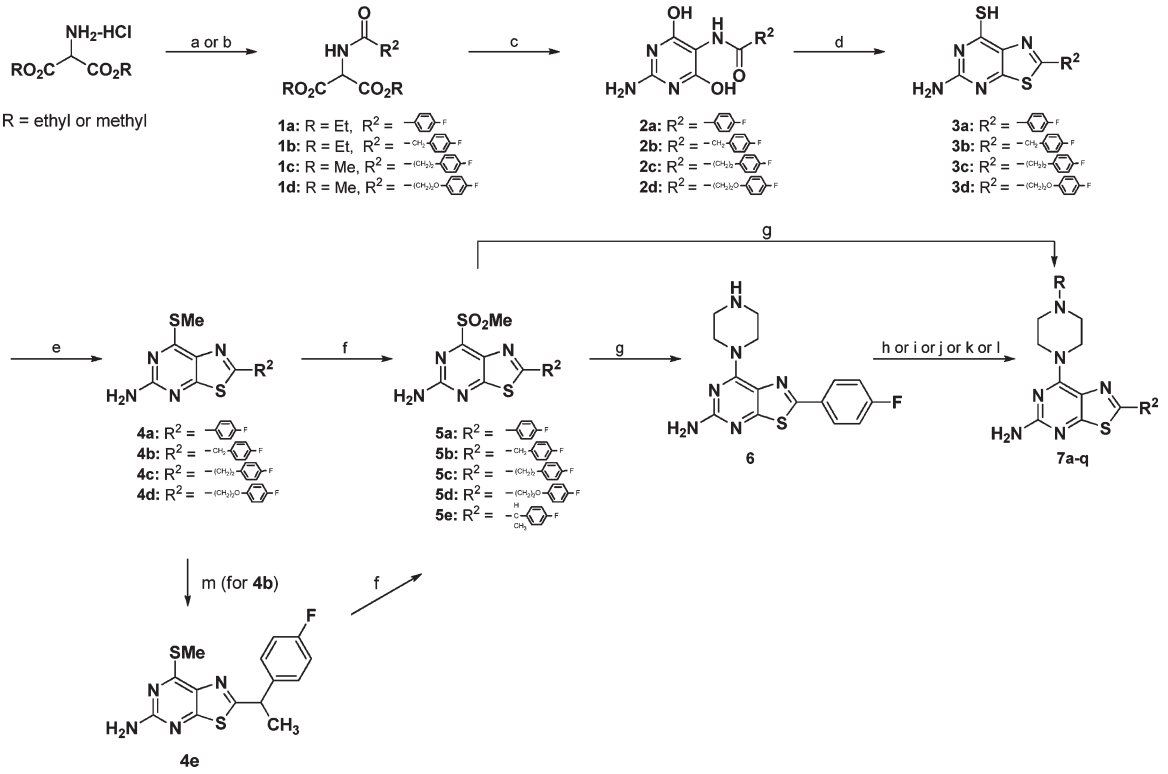
Scheme 1[a]



[a] Reagents and conditions: (a) 4-fluorobenzoyl chloride, pyridine, DMF, rt; (b) carboxylic acid, DCC, DMF/CH_2Cl_2 (1/10), rt; (c) guanidine hydrochloride, Na, EtOH, reflux; (d) P_2S_5, pyridine, reflux; (e) iodomethane, NEt_3, DMSO, rt; (f) *m*CPBA, CH_2Cl_2, 0 °C to rt; (g) piperazine or N-substituted piperazine, dioxane, 60 °C; (h) isocyanate, DMF, rt; (i) acyl chloride, pyridine, DMF, rt; (j) benzenesulfonyl chloride, pyridine, DMF, rt; (k) benzyl chloroformate, pyridine, DMF, rt; (l) carboxylic acid, TBTU, DIPEA, DMF, rt; (m) iodomethane, 2 N NaOH, DMSO, rt.

that have been functionalized to achieve biological activities such as inhibitors of purine nucleoside phosphorylase (PNP),[6] as vanilloid receptor antagonists,[7] as activators of caspases and inducers of apoptosis,[8] as antiangiogenic agents,[9] as growth factor receptor inhibitors,[10] and as heat shock protein 90 (HSP-90) inhibitors.[11]

In this paper, we describe the synthesis of a new class of thiazolo[5,4-*d*]pyrimidine analogues with in vitro as well as in vivo immunosuppressive activity. The structure−activity relationship (SAR) studies are described in which both the substitution pattern and the scaffold are modified.

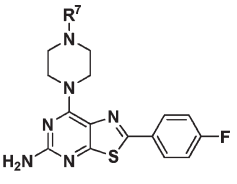
Chemistry

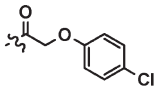
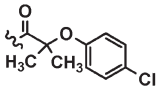
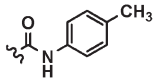
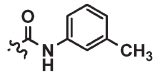
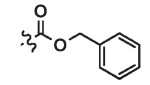
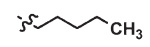
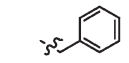
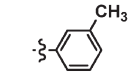
Synthesis of Thiazolo[5,4-*d*]pyrimidine Analogues. The synthesis of thiazolo[5,4-*d*]pyrimidine derivatives starts from commercially available diethyl aminomalonate hydrochloride or dimethyl aminomalonate hydrochloride by a known procedure (Scheme 1).[12] Dialkyl aminomalonate hydrochloride was converted to amides by direct acylation with acyl chlorides or by standard peptide coupling procedures, using carboxylic acids, hydroxybenzotriazole (HOBt), and dicyclohexylcarbodiimide (DCC).[13] In order to construct the pyrimidine scaffold, dialkyl acylaminomalonates **1a**−**d** were treated with guanidine in an ethanolic sodium ethoxide solution yielding pyrimidines **2a**−**d**. Conversion of the lactam functionalities of 5-acylaminopyrimidine-4,6-diols **2a**−**d** into thiolactam groups was achieved with phosphorus pentasulfide in pyridine, giving access to 2,5,7-substituted-thiazolo[5,4-*d*]pyrimidines **3a**−**d**. In order to introduce a high degree of molecular diversity at position 7, alkylation of the thio group to the corresponding thiomethylethers **4a**−**d** was effected by treatment with iodomethane in the presence of triethylamine in DMSO.

Alkylation reaction on the thiazolo[5,4-*d*]pyrimidine **4b** was also performed, using iodomethane and a base (2 N NaOH) in DMSO. Careful analysis of the ^{1}H NMR spectrum of the isolated compound confirmed that in these reaction conditions the benzylic methylene group is methylated, yielding compound **4e**. The signal of the methyl ($C\underline{H}_3$) protons is observed at 1.8 ppm (doublet, 3H). *N*-Alkyl signals are usually observed around 3 ppm. Moreover, the deuterium exchange spectrum of compound **4e** indicates that there are two amino protons, further supporting the fact that alkylation takes place at the benzylic position and not at the exocyclic amino group or on the pyrimidine ring nitrogens.

The direct introduction of a piperazinyl group from the thiomethyl group did not work, as only starting material could be recovered. Therefore, the thiomethyl group was first oxidized with *m*-chloroperoxybenzoic acid (*m*CPBA) in dichloromethane, yielding the corresponding sulfone derivatives **5a**−**e**. The reaction of the sulfone **5a** with piperazine at 60 °C afforded the desired 2-(4-fluorophenyl)-7-(piperazin-1-yl)thiazolo[5,4-*d*]pyrimidin-5-amine **6** which could be further reacted with an isocyanate, an acyl chloride, a carboxylic acid,[14] a sulfonyl chloride, or a chloroformate, yielding urea (compounds **7c** and **7d**), amides (compound **7a** using an acyl chloride and compounds **7b**, **7n**−**q** from carboxylic acids), sulfonamide (compound **7e**), and carbamate (compound **7f**), respectively (Tables 1 and 4). Alternatively, it is also possible to introduce commercially available N-substituted piperazine derivatives directly onto the thiazolo-[5,4-*d*]pyrimidine skeleton, yielding derivatives **7g**−**i** (Table 1).

Table 1. SAR of the Piperazinyl Substituent of 5-Amino-2-(4-fluorophenyl)-thiazolo[5,4-*d*]pyrimidines



Compd	R^7	IC_{50} (μM)[a]
6	H	4.3
7a		0.49
7b		>10
7c		0.7
7d		0.3
7e	-SO_2Ph	>10
7f		4.9
7g		>10
7h		>10
7i		>10
CsA[b]		0.054

[a] Values are the mean of two independent experiments. [b] Cyclosporin A.

As amide derivatized compound **7a** exhibited potent immunosuppressive activity, 2-(4-chlorophenoxy)-1-(piperazin-1-yl)ethanone **9** was synthesized according to the procedure outlined in Scheme 2. Reaction of *tert*-butyl piperazine-1-carboxylate with *p*-chlorophenoxyacetyl chloride followed by acidic deprotection of the Boc group furnished piperazine analogue **9**. The availability of compound **9** allows a one-step displacement of the sulfone group of compounds **5b**−**e**, yielding derivatives **7j**−**m** (Table 3).

The synthetic route described in Scheme 1 allows introduction of structural variety easily at position 7. However, as we would like to make variations at position 2, we preferred another route that makes it possible to introduce the substituent at position 2 at a later stage of the synthesis (Scheme 3). Commercially available 2,5-diamino-4,6-dihydroxypyrimidine hydrochloride was converted to 2,5-diamino-4,6-dichloropyrimidine **10** by the reaction with phosphorus oxychloride in the presence of tetraethylammonium chloride.[15] Displacement of one of the chlorines with 1 equiv of *tert*-butyl piperazine-1-carboxylate furnished *tert*-butyl 4-(2,5-diamino-6-chloropyrimidin-4-yl)piperazine-1-carboxylate **11**. By heating with sodium sulfide nonahydrate in DMSO, the remaining chlorine was converted into a thio group, yielding the valuable key intermediate *tert*-butyl 4-(2,5-diamino-6-mercaptopyrimidin-4-yl)piperazine-1-carboxylate **12**.

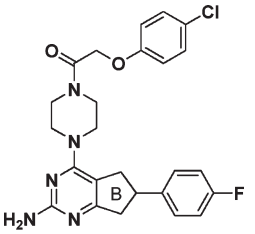
5-Amino-7-piperazinyl-2-substituted-thiazolo[5,4-*d*]-pyrimidines **14a**−**h** were prepared from pyrimidine **12** in two ways. It is well-known that a thiazole ring can be mounted onto a 2-aminobenzenethiol by the reaction with an acyl chloride[16] or an aldehyde.[17] Therefore, we envisaged construction of a thiazole ring onto pyrimidine **12** by condensation with an appropriate acyl chloride or aldehyde. First, pyrimidine **12** was reacted with an acyl chloride in DMF. However, in the absence of base, the Boc group of piperazinyl moiety was cleaved off and the free amine was reacted with an acyl chloride, yielding 7-*N*-acylpiperazinyl-thiazolo[5,4-*d*]pyrimidine. In the presence of base, 5- or 6-acylated pyrimidines were isolated and no ring closure took place. Fortunately, heating of 5- or 6-acylated pyrimidines in 3 M HCl in dioxane afforded thiazolo[5,4-*d*]pyrimidines **14d**−**h**. Alternatively, thiazolo[5,4-*d*]pyrimidines **14a**−**c** were prepared by heating the pyrimidine **12** with an appropriate aldehyde in DMSO. The Boc group of **13a**−**c** was cleaved off under acidic conditions, yielding compounds **14a**−**c**. The piperazine moiety of compounds **14a**−**h** was further derivatized to amides **15** by the reaction with *p*-chlorophenoxyacetyl chloride (yielding compounds **15a**−**h**, Table 3) or appropriate carboxylic acids (yielding compounds **15i**,**j**, Table 4).

Synthesis of Oxazolo[5,4-*d*]pyrimidine 17. Oxazolo[5,4-*d*]pyrimidine **17** was synthesized from 5-acylaminopyrimidine-4,6-diol **2a** which is described in Scheme 1 as an intermediate in the synthesis of the thiazolo[5,4-*d*]pyrimidine analogues (Scheme 4). By heating with phosphorus oxychloride, compound **2a** underwent a cyclodehydration reaction yielding 7-chlorooxazolo[5,4-*d*]pyrimidine **16**.[18] Displacement of the 7-chloro substituent was easily accomplished by heating with piperazine analogue **9**, affording compound **17**.

Synthesis of Thiazolo[4,5-*d*]pyrimidine 23. The synthesis of thiazolo[4,5-*d*]pyrimidine **23** starts from commercially available 2,6-diamino-4-chloropyrimidine. Nucleophilic displacement of the chlorine by heating with *N*-acetylpiperazine in water yields the pyrimidine analogue **18** (Scheme 5).[3b] Thiocyanation of compound **18** by treatment with potassium thiocyanate, bromine, and pyridine in DMF led to the construction of the thiazolo[4,5-*d*]pyrimidine scaffold (compound **19**).[19] The 2-amino group was converted to chlorine via diazotation of compound **19** by treatment with sodium nitrite and CuCl (Sandmeyer reaction).[19,20] Suzuki coupling of **20** with 4-fluorophenylboronic acid furnished compound **21**. The acetyl group of **21** was cleaved off under acidic conditions and the piperazine moiety was further derivatized with *p*-chlorophenoxyacetyl chloride, affording compound **23**.

Synthesis of Purine Analogue 26. Purine based compound **26** was synthesized from commercially available 2-amino-6-chloropurine in three steps (Scheme 6). Bromination of 2-amino-6-chloropurine by treatment with bromine in aqueous acetic acid afforded 2-amino-8-bromo-6-chloropurine **24**.[21] Suzuki reaction with 1 equiv of 4-fluorophenylboronic

Table 2. SAR of Different Scaffolds



Compd	scaffold	B	IC_{50} (μM)[a]	EON	ROCS	Combination
7a	thiazolo[5,4-*d*]pyrimidine		0.49	1.000	1.000	2.000
17	oxazolo[5,4-*d*]pyrimidine		3	0.882	0.963	1.845
23	thiazolo[4,5-*d*]pyrimidine		>10	0.494	0.859	1.353
26	purine		0.8	0.238	0.971	1.209
30	thieno[2,3-*d*]pyrimidine		0.7	0.562	1.000	1.562

[a] Values are the mean of two independent experiments.

acid provided compound **25** as a major product. Displacement of the 6-chloro substituent was easily accomplished by heating with piperazine analogue **9**, affording compound **26**.

Synthesis of Thieno[2,3-*d*]pyrimidine 30. The synthesis of thieno[2,3-*d*]pyrimidine derivative **30** has been described before,[22] however without experimental data. Therefore, we repeated this chemistry (Scheme 7), generating spectral data of the intermediates and the final compound. 2-(4-Fluorophenyl)acetaldehyde **27** was prepared by oxidation of 2-(4-fluorophenyl)ethanol with pyridinium chlorochromate (PCC) in moderate yield. The condensation of aldehyde **27** with ethyl cyanoacetate in the presence of a base and elemental sulfur (Gewald reaction) furnished ethyl 2-amino-5-(4-fluorophenyl)thiophene-3-carboxylate **28**.[23] Reaction of compound **28** with chloroformamidine hydrochloride provided the thieno[2,3-*d*]pyrimidine analogue **29** in good yield. By a benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP) promoted direct amination reaction,[24] piperazine analogue **9** was introduced in a single step, yielding final compound **30**.

Biological Evaluation and Structure−Activity Relationship (SAR) Studies

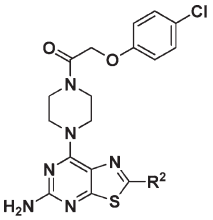
Biological Evaluation. For evaluation of the immunosuppressive activity of the compounds, an allogeneic mixed lymphocyte reaction (MLR) assay was used. The MLR assay is a fundamental benchmark test for immunosuppressants activity. Most of the current immunosuppressive drugs are very potent in MLR assay, and several of them were discovered by this assay. Therefore, it is appropriate to evaluate the immunosuppressive activity of new compounds with a MLR assay. This screening is used as a predictive in vitro test of in vivo transplant rejection. Peripheral blood lymphocytes from two individuals are mixed together in tissue culture for several days. Lymphocytes from incompatible individuals will stimulate each other to proliferate significantly (measured by tritiated thymidine uptake), whereas those from compatible individuals will not. In the one-way MLR test, the lymphocytes from one of the individuals are inactivated (usually by treatment with mitomycin or radiation), thereby allowing only the untreated remaining population of cells to proliferate in response to foreign histocompatibility antigens.

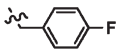
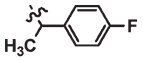
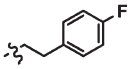
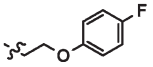
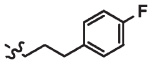
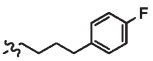
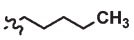
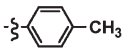
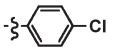
Comparison of Different Scaffolds. The first analogue synthesized (compound **7a**) was based on a thiazolo[5,4-*d*]-pyrimidine scaffold, as this is a direct isostere of a pyrido[3,2-*d*]-pyrimidine structure. Furthermore, the thiazolo moiety was replaced by an isomeric thiazole (affording thiazolo-[4,5-*d*]pyrimidine **23**), by an imidazole (yielding purine **26**), by an oxazole (furnishing oxazolo[5,4-*d*]pyrimidine **17**), and by a thiophene ring (yielding thieno[2,3-*d*]pyrimidine **30**).

Previous SAR studies on pyrido[3,2-*d*]pyrimidines[3] and pteridines[4] have determined the optimal substituents attached to the central heterocyclic ring system. The amino, the *N*-*p*-chlorophenoxyacetylpiperazinyl, and the *p*-fluorophenyl groups are known to contribute to the immunosuppressive activity. The SAR study started by comparing the five different scaffolds, bearing an identical substitution pattern that allows us to study the effect of scaffold variation on immunosuppressive activity (Table 2).

From the data in Table 2, it can be deduced that the thiazolo[4,5-*d*]pyrimidine derivative is the least active with a MLR $IC_{50} > 10$ μM. The oxazolo[5,4-*d*]pyrimidine scaffold is 6-fold less potent than the reference thiazolo-[5,4-*d*]pyrimidine scaffold. The thiazolo[5,4-*d*]pyrimidine,

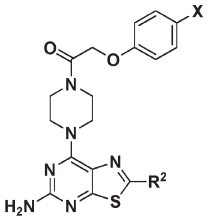
Table 3. SAR of 2-Substituted 5-Amino-7-*N*-piperazinylthiazolo[5,4-*d*]-pyrimidines



Compd	R^2	IC_{50} (μM)[a]
7a		0.49
7j		0.7
7k		4.38
7l		4.4
7m		3.54
15a		0.96
15b		0.92
15c		0.69
15d		0.35
15e		0.27
15f		0.04
15g		0.05
15h		0.23

[a] Values are the mean of two independent experiments.

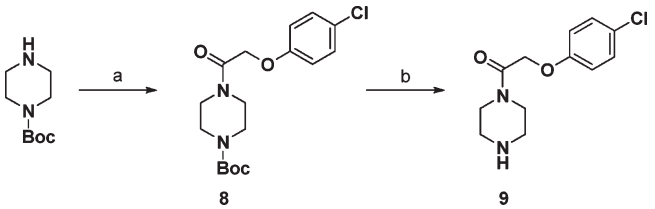
Table 4. SAR of the Phenoxy Moiety of 2-Substituted 5-Amino-7-*N*-piperazinylthiazolo[5,4-*d*]pyrimidines



Compd	R^2	X	IC_{50} (μM)[a]
7a		Cl	0.49
7n	"	H	0.09
7o	"	F	0.07
7p	"	Br	0.38
7q	"	OCH_3	0.04
15i		F	0.012
15j	"	OCH_3	0.012

[a] Values are the mean of two independent experiments.

Scheme 2[a]



[a] Reagents and conditions: (a) *p*-chlorophenoxyacetyl chloride, NEt_3, CH_2Cl_2, rt; (b) TFA, CH_2Cl_2, rt.

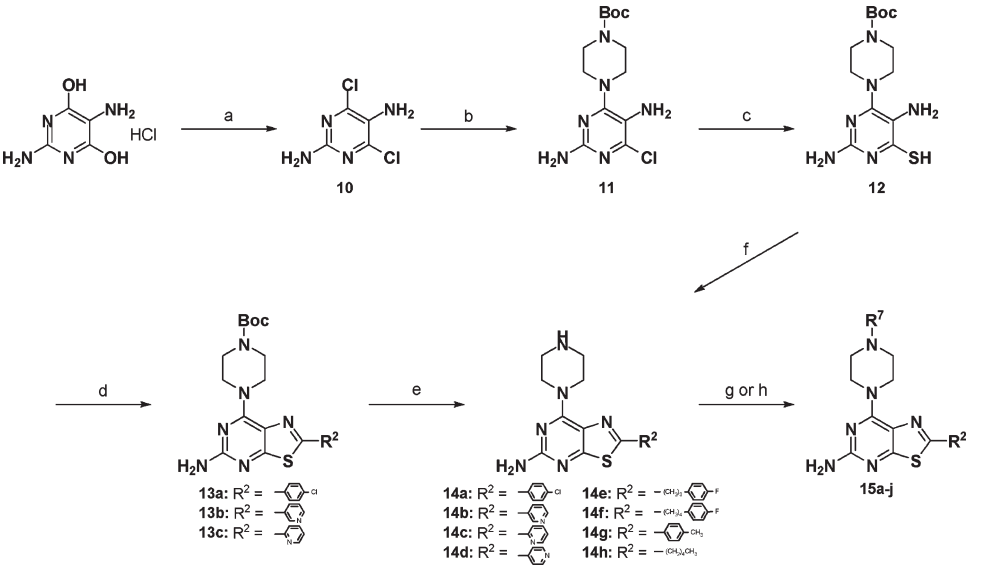
thieno[2,3-*d*]pyrimidine, and purine scaffold are equally active with IC_{50} values ranging from 0.49 to 0.8 μM.

The MLR test is a cell-based assay and does not give any information about the possible molecular targets of the immunosuppressive compounds. So 3D experimental structural information of the target protein, which could help us to understand the differences in biological activity between the different scaffolds, cannot be used for modeling and docking experiments.

However, both shape and electrostatic components of the molecules are known to play a critical role in recognition of a ligand by a target protein. In order to evaluate the similarity (or dissimilarity) between the different scaffolds, new scaffolds have been overlaid with a reference scaffold. The thiazolo[5,4-*d*]pyrimidine scaffold was taken as a reference. Then a Tanimoto index based on shape and electrostatic similarity has been calculated in order to quantify the similarity between the scaffolds.[25] A score of 1.000 means similar molecules. The OpenEye software packages ROCS and EON are well suited for this purpose.[26] Only the parts of the molecules that contain the differences were used for ROCS and EON calculations.

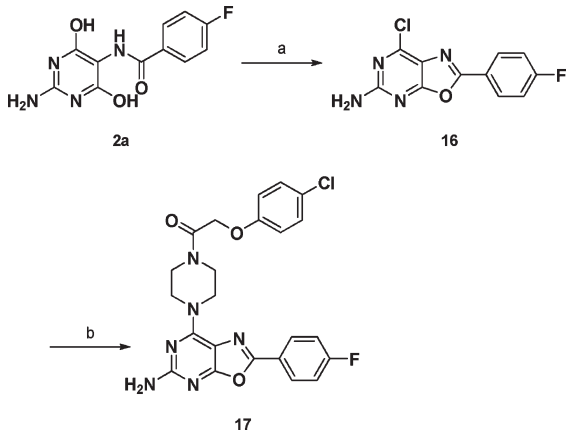
ROCS (rapid overlay of chemical structures) is a fast shape comparison application, providing a measure of molecular shape complementarity through maximizing of shape overlap between molecules. The Tanimoto shape scores were obtained from the ROCS software after superposition of the

Scheme 3[a]



[a] Reagents and conditions: (a) tetraethylammonium chloride, $POCl_3$, reflux; (b) *tert*-butyl piperazine-1-carboxylate, DIPEA, dioxane, reflux; (c) sodium sulfide nonahydrate, DMSO, 50 °C; (d) aldehyde, DMSO, 150 °C; (e) TFA, CH_2Cl_2, rt; (f) acyl chloride, pyridine, DMF, rt, then 3 M HCl in dioxane, dioxane, 60 °C; (g) acyl chloride, pyridine, DMF, rt; (h) carboxylic acid, TBTU, DIPEA, DMF, rt.

Scheme 4[a]



[a] Reagents and conditions: (a) $POCl_3$, DIPEA, 90 °C; (b) 2-(4-chlorophenoxy)-1-(piperazin-1-yl)ethanone **9**, dioxane, 80 °C.

different scaffolds on the thiazolo[5,4-*d*]pyrimidine scaffold, which was used as a reference (Figure 2 and Table 2).

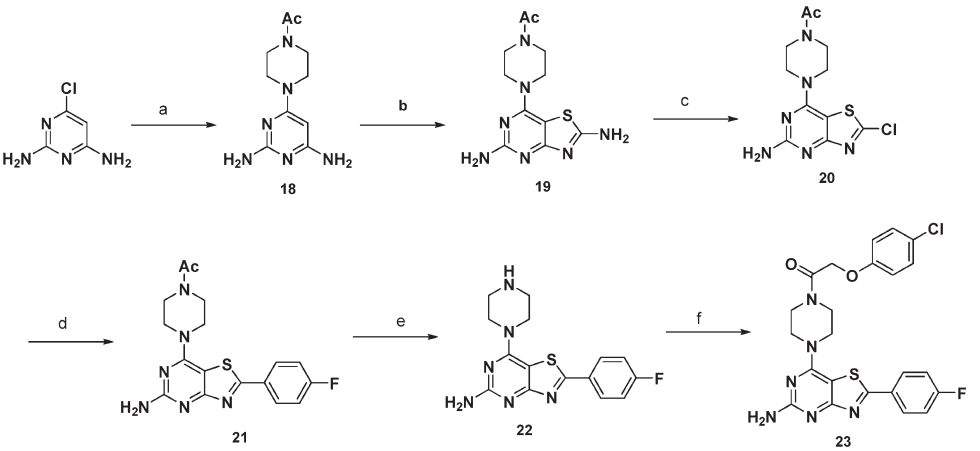
EON compares electrostatic potential distribution overlap between molecules.[27] The Adaptive Poisson-Boltzmann Solver software package (APBS) was used to calculate the electrostatic potential around the different scaffolds.[28] The resulting potential maps were mapped onto the solvent accessible surface (calculated by MSMS)[29] of the molecules and visualized by Chimera.[30] Pictures showing the electrostatic potentials mapped on the surface of the five molecular scaffolds are given in Figure 3. The electrostatic Poisson−Boltzmann scores (electrostatic Tanimoto score) were calculated by OpenEye's EON software and are also mentioned in Table 2. On the basis of the calculations, it can be concluded that the shape of the molecules seems to be more important for the activity than the electrostatic potential map in the neighborhood of the five-membered ring.

Having the biological data in hand, a selection of scaffolds for further optimization has to be done. Priority was given to the thieno[2,3-*d*]pyrimidine and thiazolo[5,4-*d*]pyrimidine scaffolds. The SAR of thieno[2,3-*d*]pyrimidines has been reported by our group in a separate paper.[22] Because of the innovative character of thiazolo[5,4-*d*]pyrimidine chemistry, we further explored the immunosuppressive properties of this scaffold.

SAR of the Thiazolo[5,4-*d*]pyrimidine Analogues. The chemistry of the thiazolopyrimidine scaffold is centered around the 5-amino-7-*N*-piperazinylthiazolo[5,4-*d*]pyrimidine pattern. The SAR started with the biological evaluation of the unsubstituted piperazinyl group (compound **6**), which shows already reasonable MLR activity (IC_{50} = 4.3 μM) and functions as an excellent starting point for further optimization. In order to improve the in vitro potency, a series of substituents was introduced (Table 1). The introduction of aryl groups (compounds **7h** and **7i**) or alkyl group (compound **7g**) led to analogues that did not show any immunosuppressive activity at 10 μM, the highest tested concentration. The piperazinyl group also offers the opportunity to be derivatized as amides, urea, carbamates, and sulfonamides. The sulfonamide derivative (compound **7e**) did not show any activity at 10 μM, and the carbamate derivative (compound **7f**) showed similar activity as unsubstituted compound **6**. On the other hand, potent activity was associated with urea and amide. The choice for the *m*- and *p*-tolylamide (compounds **7d** and **7c**) and the *p*-chlorophenoxyacetyl (compound **7a**) side chain is based on the fact that these substituents are known from previous SAR studies on other heterocycles to impart potent immunosuppressive activity. The introduction of a geminal dimethyl substituent in α-postion of the carbonyl moiety of compound **7a** led to compound **7b**, which does not show any immunosuppressive activity at 10 μM.

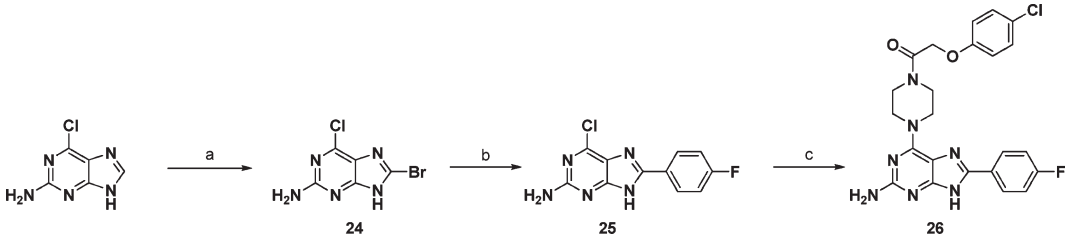
The SAR at position 2 has been investigated intensively (Table 3). Different linkers were introduced between the aromatic *p*-fluorophenyl ring and the heterocyclic scaffold. No linker (compound **7a**) and a methylene (compound **7j**), a propyl (compound **15a**), and a butyl (compound **15b**) linker all show very comparable MLR activity with IC_{50} ranging

Scheme 5[a]



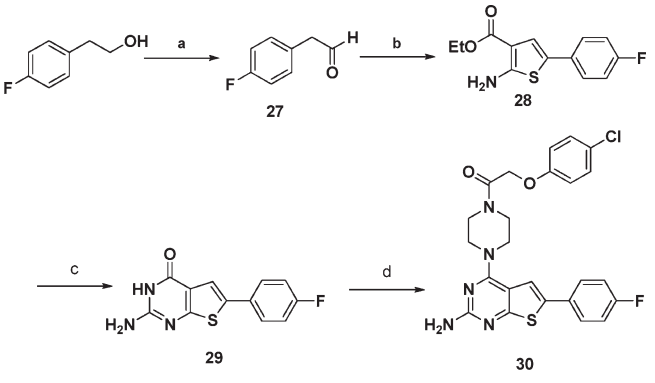
[a] Reagents and conditions: (a) 1-acetylpiperazine, water, reflux; (b) KSCN, pyridine, Br_2, DMF, 65−5 °C; (c) $NaNO_2$, conc H_2SO_4, 80 °C, then conc HCl, CuCl, 40 °C; (d) 4-fluorophenylboronic acid, K_2CO_3, $Pd(PPh_3)_4$, dioxane/water (3/1), 100 °C; (e) 5% HCl, 100 °C; (f) 4-chlorophenoxyacetyl chloride, pyridine, DMF, rt.

Scheme 6[a]



[a] Reagents and conditions: (a) Br_2, $AcOH/H_2O$, 60 °C; (b) 4-fluorophenylboronic acid, K_2CO_3, $Pd(PPh_3)_4$, dioxane/water (3/1), 100 °C; (c) 2-(4-chlorophenoxy)-1-(piperazin-1-yl)ethanone **9**, dioxane, 80 °C.

Scheme 7[a]



[a] Reagents and conditions: (a) PCC, CH_2Cl_2, rt; (b) ethyl cyanoacetate, S, NEt_3, DMF, 50 °C to rt; (c) chloroformamidine hydrochloride, dimethylsulfone, 130 °C; (d) BOP, DBU, 2-(4-chlorophenoxy)-1-(piperazin-1-yl)ethanone **9**, CH_3CN, rt to 60 °C.

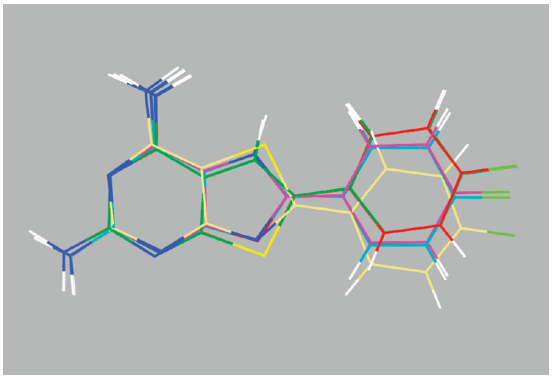


Figure 2. Predicted three-dimensional shape of compounds **7a**, **17**, **23**, **26**, and **30**. Color code of carbon atoms is as follows: for compound **7a**, red; for compound **17**, green; for compound **23**, khaki; for compound **26**, magenta; for compound **30**, cyan.

from 0.49 to 0.92 μM. On the other hand, an ethyl (compound **7l**) or an ethoxy linker (compound **7m**) led to a 10-fold decrease in MLR activity. Branching of the benzylic carbon of compound **7j** led to the preparation of compound **7k**. This compound (which was tested as a racemate) shows a 6-fold drop in immunosuppressive activity when compared with its unsubstituted analogue **7j**.

In order to further establish the SAR at position 2, a number of additional analogues was made in which the *p*-fluorophenyl group was replaced by a *p*-methylphenyl (compound **15d**) and a *p*-chlorophenyl (compound **15e**). These two compounds all exhibited IC_{50} values around 300 nM. Even an aliphatic alkyl chain (compound **15c**) retained immunosuppressive activity with a MLR IC_{50} of 0.69 μM. It indicated that quite some structural variation is tolerated at that position. Furthermore, a number of heterocyclic aromatics were introduced, as exemplified by the synthesis of the three pyridyl analogues **15f**−**h**. The 2-pyridyl analogue **15h** is equipotent with the original lead compound **7a**, whereas the 3- and 4-pyridyl analogues show a 10-fold increase in MLR activity, yielding IC_{50} values of 50 and 40 nM, respectively. This is as potent as cyclosporin A (MLR IC_{50} = 54 nM), a

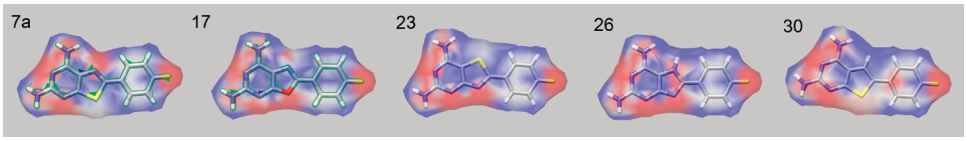


Figure 3. Electrostatic potentials mapped on the surface of the compounds **7a**, **17**, **23**, **26**, and **30**: −0.5, red; 0, white; 0.5, blue.

Table 5. Effect of Compound **15g** in Suppression of Rejection of C57-to-Balb/c Mouse Cardiac Allografts

compd	dose[a] ((mg/kg)/day)	surviving days	p[b]
vehicle		6, 7, 8, 8, 9, 9	
CsA	40	>30, >30, >30, >30	<0.001
15g	40	17, >30, >30, >30	<0.03

[a] By daily gavage. [b] Student t test; vs vehicle group.

clinically used immunosuppressive drug, which was included as positive control.

In order to probe the optimal substitution pattern on the phenoxy ring, a number of additional analogues were made (Table 4). The *p*-chloro and *p*-bromo analogues display comparable immunosuppressive activity, whereas the unsubstituted analogue (compound **7n**) and especially the *p*-fluoro (compound **7o**) and the *p*-methoxy (compound **7q**) congeners demonstrate profound immunosuppressive activity with MLR IC_{50} values of 70 and 40 nM, respectively.

The combination of both optimized structural features (the 3-pyridinyl ring at position 2 and a 4-fluoro/methoxy phenoxy acetyl side chain on the piperazinyl moiety) led to the synthesis of compounds **15i** and **15j**. Both analogues demonstrate very potent immunosuppressive activity in the MLR assay (IC_{50} for both compounds is 12 nM).

In Vivo Evaluation of Compound 15g. The procedures described above led to the identification of compounds with strong immunosuppressive activity in the MLR assay. This, however, does not guarantee in vivo efficacy of the compounds. Therefore, we selected compound **15g**, as representative compound, for in vivo evaluation of its immunosuppressive activity.

Graft Survival. The in vivo efficacy of compound **15g** was studied in a mouse model of cardiac allograft transplantation. Drug vehicle (n = 6) or compound **15g** (n = 4) was given by oral gavage daily, from the day of transplantation, until day 30 after transplantation. Cyclosporin A, the major immunosuppressive drug used in organ transplantation, was used as a reference and was also administered by daily gavage (n = 4). The results of this experiment are shown in Table 5.

Animals treated with vehicle alone rejected their allograft within 6−9 days after transplantation. CsA at the given dose achieved survival of all four grafts as long as the treatment was continued. Oral administration of compound **15g** (at a dose of 40 mg/kg) resulted in continuous graft survival in 3 out of 4 grafts. No signs of toxicity were observed by treatment of mice with compound **15g** at a dose of 40 (mg/kg)/day for 30 days.

These data indicate that compound **15g** can suppress a robust in vivo allogeneic response.

Conclusion

On the basis of previously published research and using the concept of isosterism, a novel series of immunosuppressive compounds have been designed and synthesized. The SAR studies have been guided by a cell-based MLR assay and led to the synthesis of immunosuppressive compounds based on a thiazolo[5,4-*d*]pyrimidine scaffold. By selection of the right decoration pattern of the scaffold, potent in vitro immunosuppressive activity could be obtained. One representative of this series (compound **15g**) was selected to demonstrate in vivo biological activity in a mouse model of cardiac allograft transplantation. From these in vitro and in vivo studies, we can conclude that novel immunosuppressive agents have been discovered that are equally active to cyclosporin A, a marketed immunosuppressive drug that is routinely used in transplant patients. Therefore, these findings are an excellent starting point for the development of a new generation of immunosuppressive drugs. Further pharmacokinetic and pharmacodynamic studies are underway to evaluate the importance of those findings.

Experimental Section

Chemistry. For all reactions, analytical grade solvents were used. All moisture-sensitive reactions were carried out in oven-dried glassware (135 °C). ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Advance 300 instrument (^{1}H NMR, 300 MHz; ^{13}C NMR, 75 MHz), using tetramethylsilane as internal standard for ^{1}H NMR spectra and DMSO-d_6 (39.5 ppm) or $CDCl_3$ (77.2 ppm) for ^{13}C NMR spectra. Abbreviations used are the following: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br s = broad signal. Coupling constants are expressed in hertz. Mass spectra are obtained with a Finnigan LCQ advantage Max (ion trap) mass spectrophotometer from Thermo Finnigan, San Jose, CA, U.S. Exact mass measurements are performed on a quadrupole time-of-flight mass spectrometer (Q-tof-2, Micromass, Manchester, U.K.) equipped with a standard electrospray-ionization (ESI) interface. Samples were infused in *i*-PrOH/H_2O (1:1) at 3 μL/min. Melting points are determined on a Barnstead IA 9200 and are uncorrected. Precoated aluminum sheets (Fluka Silica gel/TLC-cards, 254 nm) were used for TLC. Column chromatography was performed on ICN silica gel 63-200, 60 Å. All final compounds possess a purity of at least 95%, as determined by a RP-HPLC analysis.

Diethyl 2-(4-Fluorobenzamido)malonate (1a). To a solution of diethyl aminomalonate hydrochloride (5.0 g, 23.6 mmol) and pyridine (7.64 mL, 94.5 mmol) in DMF (60 mL) was added *p*-fluorobenzoyl chloride (4.19 mL, 35.4 mmol). The reaction mixture was stirred at room temperature for 15 h. After removal of the solvents, the residue was redissolved in dichloromethane, washed with water, brine, and dried over Na_2SO_4. After removal of the solvent, the crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 30:1) to yield the title compound as a white solid (4.9 g, 70%). Mp 108−109 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 9.37 (d, J = 7.5 Hz, 1H, NH), 7.96−8.01 (m, 2H, ArH), 7.33 (t, J = 8.8 Hz, 2H, ArH), 5.30 (d, J = 7.5 Hz, 1H, CH), 4.12−4.28 (m, 4H, CH_2), 1.22 (t, J = 7.1 Hz, 6H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 166.5, 165.4, 164.2 (d, J = 247.9 Hz), 130.4 (d, J = 9.2 Hz), 129.4 (d, J = 2.8 Hz), 115.4 (d, J = 21.8 Hz), 61.7, 56.5, 13.9 ppm. HRMS: calcd for $C_{14}H_{17}FNO_5$ 298.1091, found 298.1092.

General Method for Preparation of Dialkyl 2-Acylaminomalonate (1b−d). To a solution of carboxylic acid (9.3 mmol) and 1-hydroxybenzotriazole (32.2 mmol) in dichloromethane (140 mL) was added DCC (32.2 mmol). The reaction mixture was stirred at room temperature for 2 h. The resulting solution was cooled to 0 °C. Then a solution of diethyl aminomalonate hydrochloride

(29.3 mmol) and pyridine (29.3 mmol) in DMF (10 mL) was added. The temperature was allowed to rise to ambient temperature. After 1 h, the reaction mixture was concentrated under reduced pressure, and the residue was partitioned between ethyl acetate and a 5% $NaHCO_3$ solution. The organic layer was washed with water, brine and was dried over Na_2SO_4. After removal of the solvent, the crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 50:1) to yield the title compound.

Diethyl 2-(2-(4-Fluorophenyl)acetamido)malonate (1b). White solid (yield 93%). Mp 91−92 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ 7.26−7.29 (m, 2H, ArH), 7.05 (t, J = 8.7 Hz, 2H, ArH), 6.44 (d, J = 6.9 Hz, 1H, NH), 5.12 (d, J = 6.9 Hz, 1H, CH), 4.16−4.32 (m, 4H, CH_2), 3.61 (s, 2H, $C\underline{H}_2$Ph), 1.27 (t, J = 7.1 Hz, 6H, CH_3) ppm. ^{13}C NMR (75 MHz, $\underline{C}DCl_3$, 25 °C): δ 170.6, 166.3, 162.2 (d, J = 244.2 Hz), 131.0 (d, J = 7.7 Hz), 130.1 (d, J = 3.4 Hz), 115.8 (d, J = 21.3 Hz), 62.7, 56.6, 42.1, 14.0 ppm. HRMS: calcd for $C_{15}H_{19}FNO_5$ $[M + H]^+$ 312.1247, found 312.1247.

General Method for Preparation of 5-Acylamino-2-amino-4,6-dihydroxypyrimidine (2a−d). Guanidine hydrochloride (14.1 mmol) and dialkyl 2-aminoacylmalonate (10.1 mmol) were added to a solution of sodium (20.2 mmol) in ethanol (50 mL). The reaction mixture was refluxed for 3 h. The mixture was cooled to room temperature. The solid product was filtered off and washed with ethanol. The product was dissolved in the minimum amount of water and acidified to pH 4−5 with 5 M HCl. The precipitate was collected, washed with water, and dried to give the title compound.

***N*-(2-Amino-4,6-dihydroxypyrimidin-5-yl)-4-fluorobenzamide (2a).** White solid (yield 53%). Mp 298 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 10.62 (s, 2H, OH), 8.81 (s, 1H, NH), 7.96−8.01 (m, 2H, ArH), 7.28 (t, J = 8.8 Hz, 2H, ArH), 6.63 (s, 2H, NH_2) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 164.6, 163.7 (d, J = 245.8 Hz), 162.3, 152.4, 131.3, 130.2 (d, J = 9.0 Hz), 114.9 (d, J = 21.5 Hz), 91.1 ppm. HRMS: calcd for $C_{11}H_{10}FN_4O_3$ $[M + H]^+$ 265.073 69, found 265.071 48

General Method for Preparation of 5-Amino-7-thio-2-substituted-thiazolo[5,4-*d*]pyrimidine (3a−c). A solution of 5-acylamino-2-amino-4,6-dihydroxypyrimidine (4.92 mmol) and phosphorus pentasulfide (9.84 mmol) in dry pyridine (25 mL) was refluxed for 6 h. The solvents were evaporated in vacuo. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 30:1), yielding the title compound.

5-Amino-2-(4-fluorophenyl)thiazolo[5,4-*d*]pyrimidine-7-thiol (3a). Yellow solid (yield 93%). Mp 314 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 12.51 (s, 1H, SH), 7.96−8.01 (m, 2H, ArH), 7.38 (t, J = 8.8 Hz, 2H, ArH), 7.10 (s, 2H, NH_2) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 176.3 163.5, 163.4 (d, J = 247.5 Hz), 157.2, 140.7, 129.4 (d, J = 2.9 Hz), 128.7 (d, J = 8.9 Hz), 116.4 (d, J = 22.1 Hz) ppm. HRMS: calcd for $C_{11}H_8FN_4S_2$ $[M + H]^+$ 279.0174, found 279.0165

General Method for Preparation of 5-Amino-7-methylthio-2-substituted-thiazolo[5,4-*d*]pyrimidine (4a−c). To a solution of 5-amino-7-thio-2-substituted-thiazolo[5,4-*d*]pyrimidine (4.31 mmol) and triethylamine (10.8 mmol) in DMSO (25 mL) was added iodomethane (8.62 mmol). The reaction mixture was stirred for 12 h under N_2 at 25 °C. The mixture was poured onto water and extracted with EtOAc. The organic extracts were dried over Na_2SO_4, and the solvent was removed under reduced pressure. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 80:1), yielding the title compound.

2-(4-Fluorophenyl)-7-(methylthio)thiazolo[5,4-*d*]pyrimidin-5-amine (4a). Light yellow solid (yield 60%). Mp 217−218 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 7.98−8.03 (m, 2H, ArH), 7.39 (t, J = 8.8 Hz, 2H, ArH), 7.09 (s, 2H, NH_2), 2.59 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 164.5, 163.6 (d, J = 247.7 Hz), 163.3, 159.8, 157.5, 134.7, 129.3 (d, J = 2.8 Hz), 128.9 (d, J = 8.9 Hz), 116.4 (d, J = 22.2 Hz), 11.3 ppm. HRMS: calcd for $C_{12}H_{10}FN_4S_2$ $[M + H]^+$ 293.0331, found 293.0328

2-(2-(4-Fluorophenoxy)ethyl)-7-(methylthio)thiazolo[5,4-*d*]pyrimidin-5-amine (4d). A solution of *N*-(2-amino-4,6-dihydroxypyrimidin-5-yl)-3-(4-fluorophenoxy)propanamide **2d** (2.5 g, 8.11 mmol) and phosphorus pentasulfide (3.60 g, 16.2 mmol) in dry pyridine (40 mL) was refluxed for 6 h. After the mixture was cooled to room temperature, the precipitate was filtered off, washed with EtOAc, and dried. The crude 5-amino-2-(2-(4-fluorophenoxy)ethyl)thiazolo[5,4-*d*]pyrimidine-7-thiol (2.0 g, 6.20 mmol) was redissolved in DMSO (30 mL). Triethylamine (0.85 mL, 6.12 mmol) and iodomethane (0.31 mL, 4.90 mmol) were added. The reaction mixture was stirred for 12 h at room temperature under a nitrogen atmosphere. The mixture was poured into water and extracted with EtOAc. The combined organic layers were dried over Na_2SO_4, and the solvents were removed under reduced pressure. The crude residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 100:1), yielding the title compound as a light yellow solid (0.45 g, 15% over two steps). Mp 115 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ 6.96 (t, J = 9.1 Hz, 2H, ArH), 6.34−6.88 (m, 2H, ArH), 5.19 (s, 2H, NH_2), 4.31 (t, J = 6.2, 2H, CH_2), 3.48 (t, J = 6.2, 2H, CH_2) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ 164.7, 164.3, 161.9, 159.2, 157.6 (d, J = 237.1 Hz), 135.4, 116.0 (d, J = 23.0 Hz), 115.9 (d, J = 7.8 Hz), 66.8, 34.7, 12.1 ppm. HRMS: calcd for $C_{14}H_{14}FN_4OS_2$ $[M + H]^+$ 337.0593, found 337.058 27.

2-(1-(4-Fluorophenyl)ethyl)-7-(methylthio)thiazolo[5,4-*d*]pyrimidin-5-amine (4e). To a solution of 2-(4-fluorobenzyl)-7-(methylthio)thiazolo[5,4-*d*]pyrimidin-5-amine **4b** (0.4 g, 1.31 mmol) and 2 N NaOH (0.65 mL, 1.31 mmol) in DMSO (7 mL) was added iodomethane (81 μL, 1.31 mmol). The reaction mixture was stirred at room temperature for 2 h. The mixture was poured into water and extracted with ethyl acetate, brine and dried over Na_2SO_4. After removal of the solvents under reduced pressure, the residue was purified by chromatography on silica gel (hexane/EtOAc 5:1), yielding the title compound as a white solid (0.29 g, 69%). Mp 134−135 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ 7.31(br s, 2H, ArH), 7.02 (br s, 2H, ArH), 5.16 (s, 2H, NH_2), 4.47 (br s, 1H, CH), 2.58 (s, 3H, CH_3), 1.76 (d, J = 6.6 Hz, 3H, $CHC\underline{H}_3$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ 169.9, 164.8, $\underline{1}64.4$, 162.2 (d, J = 244.5 Hz), 159.1, 138.5 (d, J = 3.1 Hz), 135.6, 129.3 (d, J = 7.9 Hz), 115.8 (d, J = 21.3 Hz), 44.4, 21.3, 12.2 ppm. HRMS: calcd for $C_{14}H_{14}FN_4S_2$ $[M + H]^+$ 321.064 39, found 321.063 77.

General Method for Preparation of 5-Amino-7-methylsulfonyl-2-substituted-thiazolo[5,4-*d*]pyrimidine (5a−e). To a solution of 5-amino-7-thio-2-substituted-thiazolo[5,4-*d*]pyrimidine (1.03 mmol) in dichloromethane (5 mL) was added *m*CPBA (70%, 2.57 mmol) at 0 °C. The reaction mixture was stirred for 3 h, whereby the reaction temperature was gradually increased from 0 °C to room temperature. The reaction mixture was diluted with $CHCl_3$ and was washed with a saturated $NaHCO_3$ solution, brine and was dried over Na_2SO_4. After removal of the solvent under reduced pressure, the residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 50:1), affording the title compound.

2-(4-Fluorophenyl)-7-(methylsulfonyl)thiazolo[5,4-*d*]pyrimidin-5-amine (5a). White solid (yield 93%). Mp 231 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 8.11−8.16 (m, 2H, ArH), 7.12 (s, 2H, NH_2), 7.44 (t, J = 8.8 Hz, 2H, ArH), 3.58 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 171.5, 164.2 (d, J = 249.2 Hz), 160.9, 159.7, 156.9, 131.5, 129.9 (d, J = 9.1 Hz), 128.8 (d, J = 2.9 Hz), 116.6 (d, J = 22.3 Hz), 41.4 ppm. HRMS: calcd for $C_{12}H_{10}FN_4O_2S_2$ $[M + H]^+$ 325.0229, found 325.0222

General Method for Introduction of Amine at Position 7 (6, 7g−m). To a solution of 5-amino-7-methylsulfanyl-2-substituted-thiazolo[5,4-*d*]pyrimidine (1.23 mmol) and triethylamine (1.85 mmol) in dioxane (6 mL) was added amine (1.85 mmol). The reaction mixture was heated at 60 °C for 5 h. After the mixture was cooled, the volatiles were removed under reduced pressure. The crude residue was purified by flash chromatography

on silica (CH_2Cl_2/MeOH 15:1 for comound **6** and CH_2Cl_2/MeOH 50:1 for the others), furnishing the title compound.

2-(4-Fluorophenyl)-7-(piperazin-1-yl)thiazolo[5,4-*d*]pyrimidin-5-amine (6). Light yellow solid (yield 67%). Mp 205 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 9.09 (t, J = 9.2 Hz, 1H, NH), 7.98−8.03 (m, 2H, ArH), 7.37 (t, J = 8.8 Hz, 2H, ArH), 6.58 (s, 2H, NH_2), 4.45 (br s, 4H, NCH_2), 3.26 (br s, 4H, $NHC\underline{H}_2$) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 167.9, 163.1 (d, J = 246.5 Hz), 159.9, 154.5, 151.5, 129.8 (d, J = 3.0 Hz), 128.3 (d, J = 8.6 Hz), 116.3 (d, J = 22.0 Hz), 46.5, 45.7 ppm. HRMS: calcd for $C_{15}H_{16}FN_6S$ $[M + H]^+$ 331.1141, found 331.1129

General Method of Coupling of 5-Amino-7-(piperazin-1yl)-2-substituted-thiazolo[5,4-*d*]pyrimidine with Acyl Chloride (7a, 15a−h). To a solution of 5-amino-7-(piperazin-1-yl)-2-substituted-thiazolo-[5,4-*d*]pyrimidine (0.12 mmol) and pyridine (0.18 mmol) in DMF (1 mL) was added 4-chlorophenoxyacetyl chloride (0.13 mmol). The reaction mixture was stirred for 2 h at room temperature. The reaction mixture was quenched with water, extracted with EtOAc, brine, and dried over Na_2SO_4. After removal of the solvents, the crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 50:1) to yield the title compound.

1-(4-(5-Amino-2-(4-fluorophenyl)thiazolo[5,4-*d*]pyrimidin-7-yl)-piperazin-1-yl)-2-(4-chlorophenoxy)ethanone (7a). White solid (yield 53%). Mp 240 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ 7.86−7.91 (m, 2H, ArH), 7.26 (d, J = 9.0 Hz, 2H, ArH), 7.15 (t, J = 8.4 Hz, 2H, ArH), 6.93 (d, J = 9.0 Hz, 2H, ArH), 4.81 (s, 2H, NH_2), 4.75 (s, 2H, CH_2), 4.32 (br s, 4H, $N(CH_2)_2$), 3.75 (quint, J = 5.0 Hz, 4H, $CON(C\underline{H}_2)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ 168.3, 166.6, 164.2 (d, J = 249.9 Hz), 159.4, 156.6, 155.4, 155.2, 130.1 (d, J = 3.1 Hz), 129.8, 129.0, 128.7 (d, J = 8.6 Hz), 127.0, 116.3 (d, J = 22.0 Hz), 116.1, 68.2, 45.7, 42.5 ppm. HRMS: calcd for $C_{23}H_{21}ClFN_6O_2S$ $[M + H]^+$ 499.1119, found 499.1130.

General Method For Coupling of 5-Amino-7-(piperazin-1-yl)-2-substituted-thiazolo[5,4-*d*]pyrimidine with Carboxylic Acids (7b,n−q and 15i,j). To a solution of 5-amino-7-(piperazin-1-yl)-2-substituted-thiazolo[5,4-*d*]pyrimidine (0.09 mmol) and carboxylic acid (0.14 mmol) in DMF (2 mL) was added TBTU (*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate) (0.14 mmol), followed by DIPEA (0.14 mmol). The reaction mixture was stirred at room temperature for 3 h. The mixture was diluted with water and extracted with dichloromethane. The combined organic layers were washed with brine and dried over Na_2SO_4. After removal of the solvents under reduced pressure, the crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 50:1), yielding the title compound.

1-(4-(5-Amino-2-(4-fluorophenyl)thiazolo[5,4-*d*]pyrimidin-7-yl)-piperazin-1-yl)-2-(4-chlorophenoxy)-2-methylpropan-1-one (7b). White solid (yield 59%). ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 7.94−7.98 (m, 2H, ArH), 7.32−7.38 (m, 4H, ArH), 6.87 (d, 2H, ArH), 6.46 (s, 2H, NH_2), 4.14 (br s, 2H, NCH_2), 3.93 (br s, 4H, $N(CH_2)_2$), 3.68 (br s, 2H, NCH_2), 1.56 (s, 6H, CH_3, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 170.2, 167.8, 163.1 (d, J = 246.6 Hz), 159.8, 154.4, 153.8, 152.0, 129.7 (d, J = 3.2 Hz), 129.3, 128.5 (d, J = 8.6 Hz), 125.3, 124.7, 118.8, 116.2 (d, J = 21.9 Hz), 80.8, 45.2, 42.6, 25.6 ppm. HRMS: calcd for $C_{25}H_{25}ClFN_6O_2S$ $[M + H]^+$ 527.1432, found 527.1445.

4-(5-Amino-2-(4-fluorophenyl)thiazolo[5,4-*d*]pyrimidin-7-yl)-*N*-*p*-tolylpiperazine-1-carboxamide (7c). To a solution of 2-(4-fluorophenyl)-7-(piperazin-1-yl)thiazolo[5,4-*d*]pyrimidin-5-amine **6** (50 mg, 0.15 mmol) in DMF (1 mL) was added *p*-tolyl isocyanate (21 μL, 0.17 mmol) in DMF (0.3 mL). The reaction mixture was stirred for 2 h at room temperature. The reaction mixture was quenched with water, extracted with EtOAc, brine, and dried over Na_2SO_4. After removal of the solvent, the crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 100:1) to yield the title compound as a white solid (31 mg, 44%). Mp 233−235 °C.^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ 7.86−7.91 (m, 2H, ArH), 7.26 (d, J = 1.6 Hz, 2H, tolyl H), 7.10−7.23 (m, 4H, ArH, tolyl H), 6.34 (s, 1H, NH), 4.81 (s, 2H, NH_2), 4.42 (br s, 4H, NCH_2), 3.28 (t, J = 5.3 Hz, 4H, $CONC\underline{H}_2$), 2.31 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ 168.3, 164.1 (d, J = 249.8 Hz), 159.5, 155.4, 155.3, 155.2, 136.3, 133.3, 130.2 (d, J = 3.3 Hz), 129.7, 128.6 (d, J = 8.6 Hz), 126.8, 120.5, 116.3 (d, J = 21.9 Hz), 45.6, 44.1, 20.9 ppm. HRMS: calcd for $C_{23}H_{23}FN_7OS$ $[M + H]^+$ 464.1669, found 464.1671.

2-(4-Fluorophenyl)-7-(4-(phenylsulfonyl)piperazin-1-yl)thiazolo-[5,4-*d*]pyrimidin-5-amine (7e). To a solution of 2-(4-fluorophenyl)-7-(piperazin-1-yl)thiazolo[5,4-*d*]pyrimidin-5-amine **6** (50 mg, 0.15 mmol) and pyridine (18 μL, 0.23 mmol) in DMF (1 mL) was added benzenesulfonyl chloride (21 μL, 0.17 mmol). The reaction mixture was stirred for 3 h at room temperature. The reaction mixture was quenched with water, extracted with EtOAc, brine, and dried over Na_2SO_4. After removal of the solvent, the crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 80:1) to yield the title compound as a white solid (32 mg, 45%). Mp 280 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 7.94−7.98 (m, 2H, ArH), 7.64−7.79 (m, 5H, ArH), 7.35 (t, J = 8.6 Hz, 2H, ArH), 6.48 (s, 2H, NH_2), 4.35 (br s, 4H, NCH_2), 3.06 (br s, 4H, $CONC\underline{H}_2$) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 167.9, 163.2 (d, J = 246.8 Hz), 159.9, 154.3, 152.2, 134.7, 133.4, 129.5 (d, J = 3.0 Hz), 129.5, 128.5 (d, J = 8.6 Hz), 127.6, 124.7, 116.2 (d, J = 21.9 Hz), 45.9, 44.3 ppm. HRMS: calcd for $C_{21}H_{20}FN_6O_2S_2$ $[M + H]^+$ 471.107 32, found 471.106 94.

Benzyl 4-(5-Amino-2-(4-fluorophenyl)thiazolo[5,4-*d*]pyrimidin-7-yl)piperazine-1-carboxylate (7f). To a solution of 2-(4-fluorophenyl)-7-(piperazin-1-yl)thiazolo[5,4-*d*]pyrimidin-5-amine **6** (50 mg, 0.15 mmol) and pyridine (18 μL, 0.23 mmol) in DMF (1 mL) was added benzyl chloroformate (24 μL, 0.17 mmol). The reaction mixture was stirred for 3 h at room temperature. The reaction mixture was quenched with water, extracted with EtOAc, brine, and dried over Na_2SO_4. After removal of the solvent, the crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 100:1) to yield the title compound as a white solid (54 mg, 77%). Mp 196 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ 7.85−7.89 (m, 2H, ArH), 7.40−7.35 (m, 5H, ArH), 7.13 (t, J = 8.6 Hz, 2H, ArH), 5.19 (s, 2H, CH_2), 4.82 (s, 2H, NH_2), 4.34 (br s, 4H, NCH_2), 3.66 (br s, 4H, $CONC\underline{H}_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ 168.3, 164.1 (d, J = 249.8 Hz), 159.5, 155.5, 155.3, 155.02, 155.00, 136.7, 130.1 (d, J = 3.3 Hz), 128.7, 128.6 (d, J = 8.3 Hz), 128.4, 128.3, 128.2, 126.7, 116.2 (d, J = 22.0 Hz), 45.8, 44.1 ppm. HRMS: calcd for $C_{23}H_{22}FN_6O_2S$ $[M + H]^+$ 465.150 90, found 465.150 05.

***tert*-Butyl 4-(2-(4-Chlorophenoxy)acetyl)piperazine-1-carboxylate (8).** To a solution of *tert*-butyl piperazine-1-carboxylate (0.30 g, 0.16 mmol) and triethylamine (0.34 mL, 2.42 mmol) in dichloromethane (8 mL) was added *p*-chlorophenoxyacetyl chloride (0.36 mg, 1.77 mmol). The reaction mixture was stirred at room temperature overnight. The mixture was diluted with dichloromethane and washed with water, brine and dried over Na_2SO_4. After removal of the solvents, the crude residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 80:1) to yield the title compound as a white solid (0.57 g, 100%). Mp 89 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ 7.25 (d, J = 8.9 Hz, 2H, ArH), 6.88 (d, J = 8.9 Hz, 2H, ArH), 4.68 (s, 2H, CH_2), 3.57 (br s, 4H, $CON(C\underline{H}_2)_2$), 3.41 (br s, 4H, $CON(C\underline{H}_2)_2$), 1.46 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ 166.5, 156.5, 154.6, 129.7, 126.9, 116.0, 80.6, 68.1, 45.4, 43.8, 42.1, 28.5 ppm. HRMS: calcd for $C_{17}H_{24}ClN_2O_4$ $[M + H]^+$ 355.142 46, found 355.141 67.

2-(4-Chlorophenoxy)-1-(piperazin-1-yl)ethanone (9). A suspension of *tert*-butyl 4-(2-(4-chlorophenoxy)acetyl)piperazine-1-carboxylate **8** (0.58 g, 0.16 mmol) in dichloromethane (8 mL) was treated dropwise at room temperature with TFA until the solid completely dissolved. The reaction mixture was stirred under nitrogen at room temperature overnight. The volatiles were

evaporated to dryness, diluted with water, and the solid was collected by filtration. The solid was washed with water and dried to yield the title compound as a white solid (0.30 g, 72%). Mp 239−240 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 9.02 (s, 1H, NH), 7.32 (d, J = 8.8 Hz, 2H, ArH), 6.96 (d, J = 8.8 Hz, 2H, ArH), 4.90 (s, 2H, CH_2), 3.65 (br s, 4H, CON(CH_2)), 3.18 (br s, 2H, NCH_2), 3.10 (br s, 2H, NCH_2) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 165.9, 156.9, 129.1, 124.6, 116.5, 65.7, 42.6, 41.2, 38.1 ppm. HRMS: calcd for $C_{12}H_{16}ClN_2O_2$ $[M + H]^+$ 255.090 03, found 255.089 13.

4,6-Dichloropyrimidine-2,5-diamine (10). A suspension of 2,5-diamino-4,6-dihydroxypyrimidine hydrochloride (5.0 g, 28 mmol) and tetraethylammonium chloride (27.8 g, 0.167 mmol) in phosphorus oxychloride (80 mL) was heated at 105 °C for 20 h. After the mixture was cooled, excess phosphorus oxychloride was distilled off under vacuum. The reaction mixture was poured into ice−water and the pH adjusted to 4, and the mixture was stirred for 1 h at 50 °C. The pH was adjusted to 7, cooled to 35 °C, and the product was extracted with ethyl acetate, washed with a saturated $NaHCO_3$ solution, brine, and dried over Na_2SO_4. After removal of the solvents under reduced pressure, the residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 50:1), affording the title compound (3.21 g, 64%). ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 6.49 (s, 2H, NH2), 4.71 (s, 2H, NH_2) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 154.6, 145.7, 126.3 ppm. HRMS: calcd for $C_4H_5Cl_2N_4$ $[M + H]^+$ 178.9891/ 1780.9862, found 178.9910/180.9884.

***tert*-Butyl 4-(2,5-Diamino-2-substituted-6-chloropyrimidin-4-yl)piperazine-1-carboxylate (11).** To a solution of 2,5-diamino-4,6-dichloropyrimidine (2.0 g g, 11.2 mmol) and DIPEA (2.9 mL, 16.8 mmol) in dioxane (40 mL) was added *tert*-butyl piperazine-1-carboxylate (3.12 g, 16.8 mmol). The reaction mixture was heated at 100 °C for overnight. After the mixture was cooled, the volatile was removed under reduced pressure. The crude residue was diluted with $CHCl_3$ and was washed with a saturated $NaHCO_3$ solution, brine and dried over Na_2SO_4. After removal of the solvents under reduced pressure, the residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 50:1), affording the title compound. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 5.81 (s, 2H, NH2), 3.85 (s, 2H, NH_2), 3.43 (br s, 4H, N(CH_2)$_2$), 3.25 (br s, 4H, N(CH_2)$_2$), 1.41 (s, 9H, *t*-Bu) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ 158.4, 155.3, 154.9, 146.0, 119.6, 80.2, 47.2, 43.5, 28.6 ppm. HRMS: calcd for $C_{13}H_{22}ClN_6O_2$ $[M + H]^+$ 329.1493, found 329.1516.

***tert*-Butyl 4-(2,5-Diamino-6-mercaptopyrimidin-4-yl)piperazine-1-carboxylate (12).** To a solution of *tert*-butyl 4-(2,5-diamino-6-chloropyrimidin-4-yl)piperazine-1-carboxylate (2.0 g, 6.1 mmol) in DMSO (15 mL) was added sodium sulfide nonahydrate (2.9 g, 12.1 mmol). The reaction mixture was heated at 50 °C for overnight., After the mixture was cooled, water (15 mL) was added and the solution was evaporated under reduced pressure. The crude residue was diluted with water (20 mL) and neutralized with HCl. The precipitates were collected by filtration, washed with water, and dried over P_2O_5, yielding the title compound. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 11.60 (s, 1H, SH), 6.23 (s, 2H, NH_2), 3.99 (s, 2H, NH_2), 3.39 (br s, 8H, N(CH_2)$_2$, N(CH_2)$_2$), 1.41 (s, 9H, *t*-Bu) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 164.5, 153.9, 151.1, 148.6, 119.6, 78.9, 45.7, 43.1, 28.0 ppm. MS: 326.65 $[M + H]^+$

General Method for Condensation of Pyrimidine with Aldehydes (13a,c,d). A solution of *tert*-butyl 4-(2,5-diamino-6-mercaptopyrimidin-4-yl)piperazine-1-carboxylate **12** (6.1 mmol) in DMSO (10 mL) was added aldehyde (2.36 mmol). The reaction mixture was heated at 150 °C for 1 h. After the mixture was cooled, the mixture was diluted with ethyl acetate and washed with water, brine and dried over Na_2SO_4. After removal of the solvent, the crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 100:1) to yield the title compound.

***tert*-Butyl 4-(5-Amino-2-(4-chlorophenyl)thiazolo[5,4-*d*]pyrimidin-7-yl)piperazine-1-carboxylate (13a).** White solid (yield 55%). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ 7.83 (d, J = 7.7 Hz, 2H, ArH), 7.42 (d, J = 7.7 Hz, 2H, ArH), 4.85 (s, 2H, NH_2), 4.32 (br s, 4H, N(CH_2)$_2$), 3.59 (br s, 4H, N(CH_2)$_2$), 1.52 (s, 9H, *t*-Bu) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ 168.2, 159.5, 155.3, 154.9, 154.6, 136.2, 132.3, 129.3, 127.8, 126.8, 80.2, 45.8, 43.9, 28.6 ppm. HRMS: calcd for $C_{20}H_{24}ClN_6O_2S$ $[M + H]^+$ 447.1370, found 447.1369.

General Method of Deprotection of Boc Group (14a,c,d). To a solution of *tert*-butyl 4-(5-amino-2-substituted-thiazolo[5,4-*d*]-pyrimidin-7-yl)piperazine-1-carboxylate (0.51 mmol) in dichloromethane (5 mL) was added TFA (0.38 mL). The reaction mixture was stirred at room temperature overnight. The volatile was evaporated under reduced pressure, and the residue was diluted with water and neutralized with 1 N NaOH. The precipitates were collected by filtration, washed with water, and dried over P_2O_5, yielding the title compound.

2-(4-Chlorophenyl)-7-(piperazin-1-yl)thiazolo[5,4-*d*]pyrimidin-5-amine (14a). White solid (yield 88%). ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 7.47 (d, J = 7.9 Hz, 2H, ArH), 7.57 (d, J = 7.9 Hz, 2H, ArH), 6.62 (s, 2H, NH_2), 4.45 (br s, 4H, N(CH_2)$_2$), 3.29 (br s, 4H, N(CH_2)$_2$) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 168.1, 159.9, 154.4, 152.2, 134.8, 131.8, 129.2, 127.8, 124.8, 42.6, 42.1 ppm. MS: 346.94 $[M + H]^+$

General Method for Condensation of Pyrimidine with Acyl Chloride (14b,e−h). To a solution of *tert*-butyl 4-(2,5-diamino-6-mercaptopyrimidin-4-yl)piperazine-1-carboxylate **12** (0.31 mmol) and pyridine (0.46 mmol) in DMF (3 mL) was added acyl chloride (0.34 mmol). The reaction mixture was stirred for 2 h. The mixture was extracted with ethyl acetate, washed with water, brine, and dried over sodium sulfate. After removal of the solvent under reduced pressure, the crude mixture was diluted with dioxane (5 mL) and 3 M HCl in dioxane (1 mL) was added and the mixture was heated at 60 °C for 5 h. After cooling, the mixture was concentrated under reduced pressure and the residue was diluted with water and neutralized with 1 N NaOH. The precipitates were collected by filtration, washed with water, and dried over P_2O_5, yielding the title compound.

7-(Piperazin-1-yl)-2-(pyridin-4-yl)thiazolo[5,4-*d*]pyrimidin-5-amine (14b). Yellow solid (yield 48%). ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 8.69 (d, J = 4.6 Hz, 2H, pyridyl-H), 7.87 (d, J = 4.6 Hz, 2H, pyridyl-H), 6.70 (s, 2H, NH_2), 4.46 (br s, 4H, N(CH_2)$_2$), 3.19 (br s, 4H, N(CH_2)$_2$) ppm.

7-Chloro-2-(4-fluorophenyl)oxazolo[5,4-*d*]pyrimidin-5-amine (16). To a solution of *N*-(2-amino-4,6-dihydroxypyrimidin-5-yl)-4-fluorobenzamide **2** (0.30 g, 1.14 mmol) in $POCl_3$ (6 mL) was added diisopropylethylamine (0.39 mL, 2.27 mmol). The reaction mixture was stirred under N_2 at 90 °C for 3.5 h. The reaction mixture was allowed to cool to room temperature, and the volatiles were evaporated to dryness. The residue was diluted with water, and the aqueous phase was extracted with diethyl ether. The combined organic layers were washed with a saturated $NaHCO_3$ solution and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 100:1) to yield the title compound as a white solid (0.085 g, 28%). ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 8.13−8.18 (m, 2H, ArH), 7.48 (s, 2H, NH_2), 7.45 (t, J = 8.9 Hz, 2H, ArH) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 167.1, 164.3 (d, J = 249.4 Hz), 160.7, 157.5, 149.2, 129.7 (d, J = 9.7 Hz), 122.3 (d, J = 2.6 Hz), 120.7, 116.6 (d, J = 22.3 Hz) ppm. HRMS: calcd for $C_{11}H_7ClFN_4O$ $[M + H]^+$ 265.029 24, found 265.028 51.

1-(4-(5-Amino-2-(4-fluorophenyl)oxazolo[5,4-*d*]pyrimidin-7-yl)-piperazin-1-yl)-2-(4-chlorophenoxy)ethanone (17). To a solution of 7-chloro-2-(4-fluorophenyl)oxazolo[5,4-*d*]pyrimidin-5-amine **16** (30 mg, 0.11 mmol) and triethylamine (24 μL, 0.17 mmol) in dioxane (1 mL) was added 2-(4-chlorophenoxy)-1-(piperazin-1-yl)-ethanone **9** (43 mg, 0.17 mmol). The reaction mixture was heated at 70 °C for 3 h. After the mixture was cooled, the volatiles were removed under reduced pressure. The crude residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 70:1) to yield the title compound as a white solid (40 mg, 73%). Mp 244 °C.

^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ 8.03−8.08 (m, 2H, ArH), 7.40 (t, J = 8.8 Hz, 2H, ArH), 7.33 (d, J = 8.8 Hz, 2H, ArH), 6.98 (d, J = 8.8 Hz, 2H, ArH), 6.54 (s, 2H, NH_2), 4.93 (s, 2H, CH_2), 4.19 (br s, 2H, NCH_2), 4.09 (br s, 2H, $N(CH_2)_2$), 3.62 (br s, 4H, $CON(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ 167.4, 165.9, 163.5 (d, J = 247.4 Hz), 160.5, 156.9, 153.7, 152.5, 129.1, 128.5 (d, J = 8.9 Hz), 124.5, 123.1 (d, J = 2.9 Hz), 116.4, 116.3 (d, J = 22.1 Hz), 108.6, 65.9, 43.9, 41.1 ppm. HRMS: calcd for $C_{23}H_{21}ClFN_6O_3$ $[M + H]^+$ 483.134 77, found 483.133 67.

1-(4-(2,6-Diaminopyrimidin-4-yl)piperazin-1-yl)ethanone (18). To a solution of 2,6-diamino-4-chloropyrimidine (3.0 g, 20.8 mmol) in water (100 mL) was added *N*-acetylpiperazine (10.64 g, 83.0 mmol). The mixture was refluxed for 21 h. The orange solution was cooled and made alkaline with 10 M NaOH to give a white precipitate. The solution was filtered, washed with cold water, and dried over P_2O_5 in a vacuum desiccator to yield the title compound as a white solid (3.3 g, 67%). Mp 274 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 5.74 (s, 2H, NH_2), 5.51 (s, 2H, NH_2), 5.04 (s, 1H, CH), 3.33−3.46 (m, 8H, CH_2), 2.02 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 168.3, 165.2, 163.5, 162.7, 74.1, 45.2, 43.7, 43.3, 40.5, 21.2 ppm. HRMS: calcd for $C_6H_{11}N_4O$ $[M + H]^+$ 155.093 29, found 155.092 60.

1-(4-(2,5-Diaminothiazolo[4,5-*d*]pyrimidin-7-yl)piperazin-1-yl)-ethanone (19). A solution of 1-(4-(2,6-diaminopyrimidin-4-yl)-piperazin-1-yl)ethanone **18** (3.3 g, 26.2 mmol) and potassium thiocyanate (15.2 g, 0.16 mol) in DMF (120 mL) was heated at 65 °C. Pyridine (4.2 mL, 52.3 mmol) was added and the solution cooled to 5 °C. Bromine (1.34 mL, 26.2 mmol) was added slowly and the reaction mixture stirred for 2 h at 5−10 °C. The reaction mixture was poured into ice−water (100 mL) and stirred for 1 h. The volatiles were removed under reduced pressure and the resulting solid was diluted with water, filtered, and dried over P_2O_5, yielding the title compound as a pale yellow solid (2.5 g, 52%). Mp 263 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 7.85 (s, 2H, NH_2), 5.90 (s, 2H, NH_2), 3.53−3.64 (m, 8H, CH_2), 2.03 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 172.2, 170.2, 168.5, 161.6, 157.5, 90.9, 45.3, 45.2, 44.9, 40.6, 21.2 ppm. HRMS: calcd for $C_6H_{11}N_4O$ $[M + H]^+$ 155.093 29, found 155.092 60.

1-(4-(5-Amino-2-chlorothiazolo[4,5-*d*]pyrimidin-7-yl)piperazin-1-yl)ethanone (20). A solution of $NaNO_2$ (94 mg, 1.36 mmol) in H_2SO_4 (1.7 mL) was heated at 80 °C until the solid dissolved. The solution was cooled to room temperature, and a solution of 1-(4-(2,5-diaminothiazolo[4,5-*d*]pyrimidin-7-yl)piperazin-1-yl)ethanone **19** (0.1 g, 0.34 mmol) in acetic acid (0.5 mL) was added. After the addition was completed, the solution was stirred at 40 °C for 30 min. A solution of CuCl (0.1 g, 1.02 mmol) in HCl (0.5 mL) was added, and the mixture was heated at 80 °C for 30 min. After the mixture was cooled, water (3 mL) was added. The mixture was neutralized with a 10 N NaOH solution and extracted with a $CHCl_3$/EtOH (4/1) solution, brine and dried over Na_2SO_4. After removal of the solvents under reduced pressure, the crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 20:1), affording the title compound as a white solid (65 mg, 61%). Mp 230 °C (dec). ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 6.46 (s, 2H, NH_2), 3.71−3.80 (m, 4H, $N(CH_2)_2$), 3.56−3.61 (m, 4H, $CON(CH_2)_2$), 2.04 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 169.3, 168.6, 162.1, 158.1, 157.8, 86.1, 44.9, 44.8, 44.6, 40.4, 21.2 ppm. HRMS: calcd for $C_{11}H_{14}ClN_6OS$ $[M + H]^+$ 313.0638, found 313.0622.

1-(4-(5-Amino-2-(4-fluorophenyl)thiazolo[4,5-*d*]pyrimidin-7-yl)-piperazin-1-yl)ethanone (21). To a solution of 1-(4-(5-amino-2-chlorothiazolo[4,5-*d*]pyrimidin-7-yl)piperazin-1-yl)ethanone **20** (0.10 g, 0.32 mmol) in dioxane/water (3:1, 4 mL) were added 4-fluorophenylboronic acid (45 mg, 0.32 mmol), K_2CO_3 (0.18 g, 1.28 mmol), and $Pd(PPh_3)_4$ (37 mg, 0.03 mmol). The reaction mixture was refluxed under N_2 overnight. After the mixture was cooled to room temperature, 1 N HCl was added slowly to neutralize the mixture to pH 7−8. The mixture was extracted with dichloromethane, brine and dried over Na_2SO_4. After removal of the solvent, the residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 30:1) to yield the title compound as a pale yellow solid (0.13 g, 73%). Mp 280 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 8.09−8.14 (m, 2H, ArH), 7.42 (t, J = 8.8 Hz, 2H, ArH), 6.33 (s, 2H, NH_2), 3.88 (br s, 2H, CH_2), 3.81 (br s, 2H, CH_2), 3.61 (br s, 4H, CH_2), 2.06 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 172.4, 169.4, 168.6, 164.2 (d, J = 248.7 Hz), 162.2, 158.5, 129.2 (d, J = 9.0 Hz), 128.9 (d, J = 2.9 Hz), 116.6 (d, J = 22.1 Hz), 97.5, 45.1, 44.8, 40.5, 40.3, 21.2 ppm. HRMS: calcd for $C_{20}H_{24}FN_6O_2S$ $[M + H]^+$ 431.166 55, found 431.165 58.

2-(4-Fluorophenyl)-7-(piperazin-1-yl)thiazolo[4,5-*d*]pyrimidin-5-amine (22). The mixture of 1-(4-(5-amino-2-(4-fluorophenyl)-thiazolo[4,5-*d*]pyrimidin-7-yl)piperazin-1-yl)ethanone **21** (0.1 g, 0.27 mmol) in 5% HCl (2 mL) was heated at 100 °C for 4 h. After cooling, the mixture was neutralized with 2 N NaOH to pH 7. The precipitate was filtered and washed with water and dried. The crude compound was purified by flash chromatography on silica (CH_2Cl_2/MeOH 5:1) to yield the title compound as a yellow solid (81 mg, 91%). Mp 260 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 8.10−8.15 (m, 2H, ArH), 7.41 (t, J = 8.8 Hz, 2H, ArH), 6.26 (s, 2H, NH_2), 3.76 (br s, 4H, CH_2), 2.82 (br s, 4H, CH_2) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 172.4, 169.1, 164.2 (d, J = 248.6 Hz), 162.2, 158.6, 129.2 (d, J = 9.0 Hz), 128.9 (d, J = 3.1 Hz), 116.5 (d, J = 22.1 Hz), 97.3, 46.5, 45.4 ppm. HRMS: calcd for $C_{20}H_{24}FN_6O_2S$ $[M + H]^+$ 431.166 55, found 431.165 58.

1-(4-(5-Amino-2-(4-fluorophenyl)thiazolo[4,5-*d*]pyrimidin-7-yl)-piperazin-1-yl)-2-(4-chlorophenoxy)ethanone (23). This compound was synthesized from compound **22** according to a procedure for the preparation of compound **7a**. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 40:1) to yield the title compound as a yellow solid (45 mg, 99%). Mp 267 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 8.10−8.15 (m, 2H, ArH), 7.43 (t, J = 8.6 Hz, 2H, ArH), 7.33 (d, J = 8.6 Hz, 2H, ArH), 6.97 (d, J = 8.6 Hz, 2H, ArH), 6.34 (s, 2H, NH_2), 4.92 (s, 2H, OCH_2), 3.92 (br s, 2H, CH_2), 3.87 (br s, 2H, CH_2), 3.65 (br s, 4H, CH_2) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 172.4, 169.4, 165.9, 164.2 (d, J = 248.7 Hz), 162.2, 158.5, 156.9, 129.2 (d, J = 9.0 Hz), 129.1, 128.9 (d, J = 3.0 Hz), 116.5 (d, J = 21.6 Hz), 116.4, 97.6, 65.9, 44.9, 44.7, 43.5, 40.9 ppm. HRMS: calcd for $C_{23}H_{21}ClFN_6O_2S$ $[M + H]^+$ 499.111 93, found 499.110 23.

8-Bromo-6-chloro-9*H*-purin-2-amine (24). To a solution of 2-amino-6-chloropurine (1.00 g, 5.90 mmol) in acetic acid (25 mL) was added bromine (1.21 mL, 23.6 mmol) slowly. And water (5 mL) was added to the mixture. The temperature was maintained between 50 and 60 °C for 36 h, and the volatiles were evaporated under reduced pressure. The crude solid was redissolved in MeOH, preadsorbed on silica gel, and purified by chromatography on silica gel (CH_2Cl_2/MeOH 30:1) to yield 8-bromo-6-chloro-9*H*-purin-2-amine as a yellow solid (0.69 g, 47%). ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 13.67 (s, 1H, NH), 6.88 (s, 2H, NH_2) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 159.8, 156.5, 147.2, 126.8, 124.0 ppm. HRMS: calcd for $C_5H_4BrClN_5$ $[M + H]^+$ 247.933 86, found 247.933 26.

6-Chloro-8-(4-fluorophenyl)-9*H*-purin-2-amine (25). To a solution of 8-bromo-6-chloro-9*H*-purin-2-amine **24** (0.30 g, 1.21 mmol) in dioxane/water (3:1, 6 mL) was added 4-fluorophenylboronic acid (0.17 g, 1.21 mmol), K_2CO_3 (0.67 g, 4.83 mmol), and $Pd(PPh_3)_4$ (0.14 g, 0.12 mmol). The reaction mixture was refluxed under N_2 for 2 h. After the mixture was cooled to room temperature, 1 N HCl was added slowly to neutralize the mixture to pH 7−8. The solvents were removed under reduced pressure. The residue was diluted with water (10 mL) and then filtered. The crude solid was redissolved in MeOH, preadsorbed on silica gel, and purified by chromatography on silica gel (CH_2Cl_2/MeOH 70:1), yielding 6-chloro-8-(4-fluorophenyl)-9*H*-purin-2-amine as a pale yellow solid (0.10 g, 31%). ^{1}H NMR (300 MHz, DMSO, 25 °C): δ

13.31 (s, 1H, NH), 8.14−8.19 (m, 2H, ArH), 7.39 (t, J = 8.6 Hz, 2H, ArH), 6.84 (s, 2H, NH_2) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 163.3 (d, J = 246.7 Hz), 159.7, 156.4, 149.2, 148.4, 8.8 (d, J = 8.6 Hz), 125.8 (d, J = 2.7 Hz), 116.1 (d, J = 21.9 Hz) ppm. HRMS: calcd for $C_{11}H_8ClFN_5$ $[M + H]^+$ 264.045 23, found 264.044 61.

1-(4-(2-Amino-8-(4-fluorophenyl)-9*H*-purin-6-yl)piperazin-1-yl)-2-(4-chlorophenoxy)ethanone (26). To a solution of 6-chloro-8-(4-fluorophenyl)-9*H*-purin-2-amine **25** (35 mg, 0.13 mmol) and triethylamine (37 μL, 0.27 mmol) in dioxane (1 mL) was added 2-(4-chlorophenoxy)-1-(piperazin-1-yl)ethanone **9** (51 mg, 0.20 mmol). The reaction mixture was heated at 70 °C for 3 h. After the mixture was cooled, the volatiles were removed under reduced pressure. The crude residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 20: 1) to yield 1-(4-(2-amino-8-(4-fluorophenyl)-9*H*-purin-6-yl)piperazin-1-yl)-2-(4-chlorophenoxy)ethanone as a white solid (50 mg, 78%). ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 12.76 (s, 1H, NH), 8.05−8.09 (m, 2H, ArH), 7.31−7.36 (m, 4H, ArH), 6.98 (d, J = 7.9 Hz, 2H, ArH), 4.32 (s, 2H, NH_2), 4.92 (s, 2H, CH_2), 4.32 (br s, 2H, NCH_2), 4.14 (br s, 2H, NCH_2), 3.60 (s, 4H, $CONCH_2$) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 172.6, 165.8, 162.5 (d, J = 246.0 Hz), 159.5, 156.9, 155.6, 153.2, 129.1, 127.6 (d, J = 8.6 Hz), 126.7 (d, J = 3.0 Hz), 116.4, 115.8 (d, J = 21.8 Hz), 114.4, 65.9, 44.2, 41.2 ppm. HRMS: calcd for $C_{23}H_{22}ClFN_7O_2$ $[M + H]^+$ 482.150 75, found 482.149 55.

2-(4-Fluorophenyl)acetaldehyde (27). To a stirred suspension of pyridinium chlorochromate (6.9 g, 21.4 mmol) in CH_2Cl_2 (100 mL) was added a solution of 2-(4-fluorophenyl)ethanol (3.0 g, 21.4 mmol) in CH_2Cl_2 (10 mL). The resulting suspension was stirred for 2 h at room temperature and was then diluted with ether. The resulting suspension was filtered through a pad of Celite and washed with ether. The solvents were removed under reduced pressure to yield the crude title compound as a green oil (2.6 g, 86%), which was used as such for further reaction. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ 9.75 (s, 1H, CH), 7.19−7.22 (m, 2H, ArH), 7.06 (t, J = 8.5 Hz, ArH), 3.68 (s, 2H, CH_2) ppm.

Ethyl 2-Amino-5-(4-fluorophenyl)thiophene-3-carboxylate (28). Triethylamine (0.98 mL, 7.01 mmol) was added to a stirred suspension of ethyl cyanoacetate (2.79 mL, 13.7 mmol) and sulfur (0.44 g, 13.7 mmol) in DMF (70 mL). A solution of 2-(4-fluorophenyl)acetaldehyde **27** (1.9 g, 13.7 mmol) in DMF (5 mL) was added dropwise over a period of 50 min, while the temperature was maintained at 50 °C. The solution was cooled to room temperature and stirred overnight. The mixture was poured into water, and the aqueous phase was extracted with diethyl ether. The organic layer was separated and washed with water, brine and dried over Na_2SO_4. The solvents were evaporated and the crude residue was purified by flash chromatography on silica gel (EtOAc/hexane 1:15) to yield the title compound as a white solid (1.3 g, 38%). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ 7.33−7.39 (m, 2H, ArH), 7.14 (s, 1H, CH), 6.99 (t, J = 8.6 Hz, ArH), 6.06 (s, 2H, NH_2), 4.29 (q, J = 7.1, 2H, CH_2), 1.36 (t, J = 7.1, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ 165.5, 162.3, 161.8 (d, J = 244.6 Hz), 130.4 (d, J = 3.3 Hz), 126.4 (d, J = 7.4 Hz), 123.8, 121.3, 115.8 (d, J = 21.7 Hz), 107.9, 60.0, 14.6 ppm. HRMS: calcd for $C_{13}H_{13}FNO_2S$ $[M + H]^+$ 266.065 10, found 266.064 25.

2-Amino-6-(4-fluorophenyl)thieno[2,3-*d*]pyrimidin-4(3*H*)-one (29). A mixture of ethyl 2-amino-5-(4-fluorophenyl)thiophene-3-carboxylate **28** (0.3 g, 1.13 mmol), chloroformamidine hydrochloride (0.33 g, 2.83 mmol), and dimethylsulfone (0.53 g, 5.65 mmol) was heated at 120−130 °C for 30 min. After the mixture was cooled to room temperature, water (10 mL) was added and ammonium hydroxide was used to neutralize the suspension. The solid was filtered off, washed with water, and dried. The crude residue was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 10:1) to yield the title compound as a white solid (0.28 g, 95%). Mp 376 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 11.05 (s, 1H, NH), 7.66−7.71 (m, 2H, ArH), 7.51 (s, 1H, CH), 7.23 (t, J = 8.8 Hz, ArH), 6.73 (s, 2H, NH_2) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 167.8, 161.4 (d, J = 243.4 Hz), 158.0, 153.5, 130.9, 130.2 (d, J = 3.0 Hz), 127.0 (d, J = 8.1 Hz), 117.3, 116.9, 115.9 (d, J = 21.6 Hz) ppm. HRMS: calcd for $C_{12}H_9FN_3OS$ $[M + H]^+$ 262.045 04, found 262.044 13.

1-(4-(2-Amino-6-(4-fluorophenyl)thieno[2,3-*d*]pyrimidin-4-yl)-piperazin-1-yl)-2-(4-chlorophenoxy)ethanone (30). To a solution of 2-amino-6-(4-fluorophenyl)thieno[2,3-*d*]pyrimidin-4(3*H*)-one **29** (40 mg, 0.15 mmol) and BOP (88 mg, 0.20 mmol) in CH_3CN (1 mL) was added DBU (34 μL, 0.23 mmol). After the mixture was stirred for 10 min at room temperature, 2-(4-chlorophenoxy)-1-(piperazin-1-yl)ethanone (59 mg, 0.23 mmol) was added. The mixture was stirred at room temperature overnight and then heated at 60 °C for 4 h. The solvents were removed under reduced pressure, and the crude residue was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 60:1) to yield the title compound as a white solid (42 mg, 55%). Mp 128 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ 7.52−7.57 (m, 2H, ArH), 7.27 (s, 1H, CH), 7.22 (d, J = 8.9 Hz, 2H, ArH), 7.10 (t, J = 8.7 Hz, ArH), 6.91 (d, J = 8.9 Hz, 2H, ArH), 4.78 (s, 2H, NH_2), 4.74 (s, 2H, CH_2), 3.86 (br s, 4H, NCH_2), 3.78 (br s, 4H, NCH_2) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ 171.1, 165.9, 161.5 (d, J = 243.5 Hz), 159.7, 158.6, 156.9, 130.4 (d, J = 3.3 Hz), 129.0, 128.6, 127.3 (d, J = 7.9 Hz), 124.5, 117.4, 116.4, 115.8 (d, J = 21.5 Hz), 109.9, 65.9, 45.8, 45.6, 43.5, 40.9 ppm. HRMS: calcd for $C_{24}H_{22}ClFN_5O_2S$ $[M + H]^+$ 498.116 68, found 498.115 11.

MLR Assay. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of healthy donors by density-gradient centrifugation over Lymphoprep. PBMCs were resuspended at a concentration of 1.6×10^6 cells/mL in complete medium (RPMI-1640 containing 10% heat-inactivated fetal calf serum (FCS) and antibiotics). RPMI1788 cells were treated with 30 μg/mL mitomycin C for 20 min at 37 °C, washed four times with medium, and finally suspended in complete RPMI medium to a density of 0.6×10^6 cells/mL. An amount of 75 μL of each cell suspension was mixed with 50 μL of diluted compound. The mixed cells were cultured at 37 °C for 5 days in 5% CO_2. DNA synthesis was assayed by the addition of 1 μCi (methyl-^{3}H)thymidine per well during the last 18 h in culture. Thereafter, the cells were harvested on glass filter paper and the counts per minute (cpm) determined in a liquid scintillation counter.

In Vivo Evaluation. Animals and Surgery. Inbred C57BL/6 H-2^b and Balb/c H-2^d female mice, 8−10 weeks old, 20−25 g, were used as donor and recipient, respectively. Heterotopic heart transplantation was performed by implanting the donor heart on the neck of recipients using conventional microsurgery techniques as described previously.[31] Grafts were implanted in the recipient neck, and graft beating was checked daily by inspection and palpation. Cessation of beating indicated graft rejection, which was confirmed by histological examination. Housing and all experimental animal procedures were approved by the Institutional Animal Care and Research Advisory Committee of the KU Leuven, Belgium.

Animals were randomly divided into three groups: (i) vehicle group, with vehicle (30% 2-hydroxypropyl-β-cyclodetrin) only by daily gavage, n = 6; (ii) reference drug group, with CsA of 40 mg/kg by daily gavage, n = 4; (iii) compound **15g** group, with 40 mg/kg by daily gavage, n = 4.

Treatment started from the day of transplantation until day 30 after transplantation. Long-term surviving grafts after withdrawing treatment remained under monitoring until day 60 after transplantation.

Supporting Information Available: Experimental and spectroscopic data of all compounds as well as HPLC purity determination of all tested compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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