Synthesis of a 2,4,6-trisubstituted 5-cyano-pyrimidine library and evaluation of its immunosuppressive activity in a Mixed Lymphocyte Reaction assay

Alessandro Stella,[a,b] Kristien Van Belle,[a,c] Steven De Jonghe,[a,b] Thierry Louat,[a,c] Jean Herman,[a,d] Jef Rozenski,[b] Mark Waer,[a,c] Piet Herdewijn*[a,b]

a) Katholieke Universiteit Leuven, Interface Valorisation Platform, Kapucijnenvoer 33, 3000 Leuven, Belgium.
b) Katholieke Universiteit Leuven, Rega Institute for Medical Research, Laboratory of Medicinal Chemistry, Minderbroedersstraat 10, 3000 Leuven, Belgium.
c) Katholieke Universiteit Leuven, Laboratory of Experimental Transplantation, Campus Gasthuisberg O&N I box 811, Herestraat 49, B-3000 Leuven, Belgium.
d) University Hospitals Leuven, Department of Pediatric Transplantation, Herestraat 49, B-3000 Leuven, Belgium.

* Corresponding author. Tel. +32 (0)16/33.73.87 ; Fax : +32 (0)16/33.73.40 ; E-mail : piet.herdewijn@rega.kuleuven.be

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Abstract

A series of novel pyrimidine analogues were synthesized and evaluated for immunosuppressive activity in the Mixed Lymphocyte Reaction assay, which is well-known as the *in vitro* model for *in vivo* rejection after organ transplantation. Systematic variation of the substituents at positions 2, 4 and 6 of the pyrimidine scaffold led to the discovery of 2-benzylthio-5-cyano-6-(4-methoxyphenyl)-4-morpholinopyrimidine with an IC_{50} value of 1.6 μM in the MLR assay.

Introduction

The central issue in organ transplantation remains suppression of allograft rejection. In recent years, the patient and graft survival have dramatically increased, mainly due to improvements in immunosuppressive therapy [1].

Current immunosuppressive regimens are mostly based on a combination of a calcineurin inhibitor (cyclosporine or tacrolimus), a glucocorticoid (prednisone) and an antiproliferative agent (azathioprine or mycophenolate). In recent years, two new drugs (sirolimus and

everolimus) were made available. They have a distinct molecular target, as they inhibit a kinase, called the mammalian-target of rapamaycin (mTOR). This current armamentarium of immunosuppressive drugs is efficient for the inhibition of T-lymphocyte dependent rejection, but is quite ineffective in preventing or treating chronic rejection. In addition, most of these drugs show toxicities that impair patient and graft survival. So, there is a continuous need for novel immunosuppressive agents. Besides their use for the prevention of graft rejection, immunosuppressive agents are also often used for the treatment of auto-immune diseases [2]. More than 70 auto-immune diseases have been described. The most prevalent conditions are rheumatoid arthritis, psoriasis, multiple sclerosis, Crohn's disease and systemic lupus erythematosus. Because of the high prevalence of these diseases, the pharmaceutical sector is heavily interested in pursuing new therapies for these diseases.

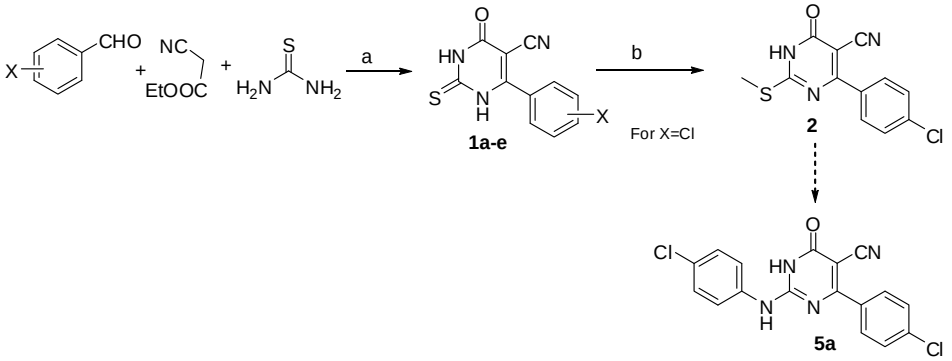
Pyrimidines have been heavily explored in medicinal chemistry and they have been shown to be associated with a wide range of biological activities. Dihydro-alkoxy-benzyl-oxopyrimidines (DABOs) are potent HIV-1 non-nucleoside reverse transcriptase inhibitors (NNRTIs) [3]. Pyrimidine-based compounds have been prepared as dual reverse transcriptase and integrase inhibitors of HIV [4]. Pyrimidines have been often used as central core structure for the design and synthesis of inhibitors of a wide range of kinases, such as for example Axl kinase [5], phosphoinositide-3-kinase [6], Fibroblast Growth Factor Receptor [7] , Cyclin-Dependent Kinase 4/6 [8], Tie kinase [9] and Janus Tyrosine kinase 3[10].

Although the biological activity of a wide variety of pyrimidines has been described, their immunosuppressive activity has, to our knowledge, not been reported in literature. As part of our ongoing interest in the synthesis of small-molecule based heterocyclic structures as potential novel immunosuppressive agents, we report the discovery of a series of pyrimidines with immunosuppressive activity in a Mixed Lymphocyte Reaction (MLR) assay.

Chemistry

The generation of a pyrimidine library started with the synthesis of 2-thiouracil derivatives **1a-e** as key intermediates (Scheme 1), which are versatile building block as the thio-urea moiety, as well as the lactam group, can be further derivatised. The construction of the pyrimidine core has been achieved *via* a Biginelli–type multicomponent reaction between a substituted benzaldehyde, ethylcyanoacetate, thiourea, and piperidine as a base, yielding the 2-thiouracil congeners **1a-e**. Treatment of compound **1a** (X=Cl) with iodomethane in the presence of potassium carbonate yielded the thiomethyl derivative **2.** It was anticipated that this compound would be an ideal key intermediate for structural variation at position 2 by

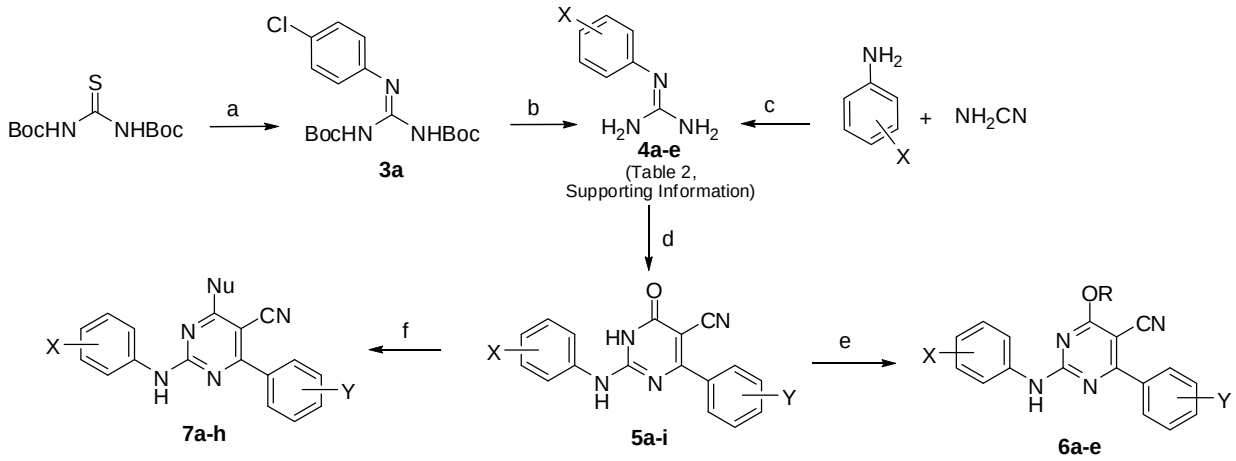
nucleophilic aromatic substitutions. In order to introduce an aniline substituent at position 2, a wide range of different conditions (solvents, temperature, reaction time) have been explored, but the desired compounds could never been isolated in acceptable yields. The best result was obtained performing the reaction with *p*-chloroaniline, under microwave irradiation at 160°C, using DMF as solvent. Under these reaction circumstances, the desired 2-(4-chloroanilino)pyrimidine derivative **5a** was isolated in only 24% yield, after complex and tedious chromatographic separations. Attempts to oxidize the thiomethyl group of compound **2** to the corresponding sulfone or sulfoxide with *m*-chloroperoxybenzoic (*m*-CPBA) prior to nucleophilic displacement was unsuccessful, as only decomposition of the starting material was observed.



Scheme 1. Reagents and conditions: a) piperidine, EtOH, reflux, overnight; b) MeI, K_2CO_3, CH_3CN.

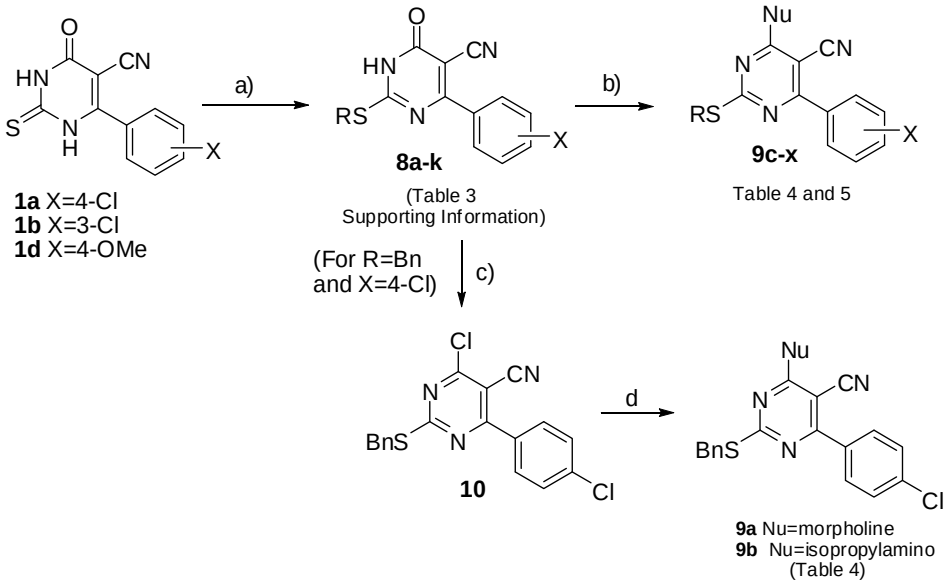
Therefore, alternative strategies for the introduction of an anilino moiety at C-2 have been explored. The availability of a substituted phenylguanidine moiety such as **4a-e** in a Biginelli-type multicomponent reaction would allow the direct synthesis of a 2-anilino-pyrimidine library. Two different methods have been explored for the synthesis of this phenylguanidine counterparts **4a-e** (Scheme 2). A first methodology is based on the *N*-iodosuccinimide (NIS)-promoted guanylation of thiourea [11]. As this reaction only works with electron-withdrawing groups on both nitrogens, commercially available di-Boc-thiourea was selected as starting material. Reaction with *p*-chloroaniline, in the presence of NIS and triethylamine afforded the protected guanidine **3a** in good yields. Acidic deprotection furnished the desired phenylguanidines **4a**. Although this method works well, the protection/deprotection sequence makes the synthetic route long and not suitable for parallel chemistry. A straightforward and shorter approach is based on the reaction between a substituted aniline and cyanamide (in a mixture of ethanol/water, under acidic conditions), affording directly the desired substituted phenylguanidine **4b-e** in good yield. Guanidines **4a-e** were used in the multicomponent reaction with substituted benzaldehydes and ethylcyanoacetate [12], with piperidine as base

and ethanol as solvent, permitting the direct construction of a 2-anilino-pyrimidine skeleton. By the selection of different substituents on the benzaldehyde and phenylguanidine counterpart, a small library of 2-anilino-5-cyano-pyrimidin-4-(3H)-one analogues **5a-i** was prepared. A summary of the synthesized compounds is reported in Table **2**. For further derivatisation of the scaffold, a series of nitrogen-containing nucleophiles has been introduced at C-4 of the pyrimidine ring by a one-step amination reaction, using benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP) as coupling partner [13], yielding compounds **7a-h**. Moreover, alkylation of lactam moiety of the pyrimidine scaffold has been carried out with different alkyl halides (methyl iodide, ethyl iodide and benzyl bromide), using potassium carbonate as base in DMF as solvent. These reaction conditions lead selectively to *O*-alkylated derivatives **6a-e** (Scheme 2). The presence of an *O*-alkyl group was easily derived from the ^{13}C-NMR spectra. The signals of the C-atom linked to oxygen are observed between 50 - 60 ppm. On the contrary, if the C-atom would be directly linked to the nitrogen, the ^{13}C signals should be visible between 35 and 40 ppm.



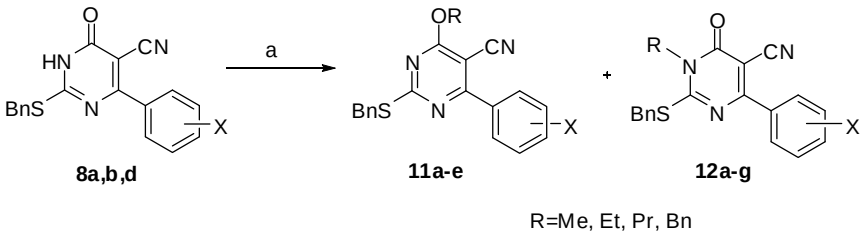
Scheme 2. Reagents and conditions: a) *p*-chloroaniline, NIS, Et_3N, CH_2Cl_2, 24h, rt. b) TFA, CH_2Cl_2, rt. c) HNO_3, $EtOH/H_2O$; d) Substituted benzaldehydes, ethylcyanoacetate, piperidine, EtOH, reflux; e) alkyliodides, K_2CO_3, DMF, RT; f) NuH, DBU, BOP, MeCN, 12h, rt.

In order to expand the structural variety, thiouracil derivatives **1a, 1b** and **1d** have been alkylated with different alkyl and benzyl halides, affording compounds **8a-k** (Scheme 3 and Table 3 of the Supporting Information). These 4-oxo-derivatives were used for further derivatisation by introducing nitrogen containing nucleophiles using the BOP-mediated nucleophilic arylic substitution reaction (compounds **9c-x**, Tables 4 and 5). Alternatively, compound **8a** was treated with $POCl_3$, furnishing a 4-chloro-pyrimidine analogue **10**. The latter has been used as starting point for direct nucleophilic aromatic substitution reaction with morpholine and isopropylamine, yielding compounds **9a** and **9b**, respectively.



Scheme 3. Reagents and conditions: a) RBr or RI, K_2CO_3, CH_3CN, rt; b) NuH, BOP, DBU, CH_3CN, rt; c) $POCl_3$, dioxane, reflux; d) NuH, dioxane, rt.

The 2-thiobenzyl derivatives **8a, 8b** and **8d** were alkylated with alkyl iodides or benzylbromide using K_2CO_3 as base and DMF as solvent (Scheme 4). These conditions were identical to the procedure used for the alkylation of 2-anilino-pyrimidin-4-(3H)-one derivatives **5a-k**. However, for the 2-thiobenzyl congeners the reaction is less selective affording a mixture of *O*- and *N*- alkylated products (Table 1). In most cases, the two isomers could be separated by flash chromatography and the ratio between *O*- versus *N*-alkylated derivatives was determined by mass balance after chromatographic separation. In some cases, only one of the regioisomers could be isolated in pure form (entries 6 and 7).



Scheme 4: Reagents and conditions: a) RI or RBr, K_2CO_3, DMF, rt.

Entry	Substrate	R	Product	Ratio *O*-alkyl/*N*-alkyl
1	**8a** (X=4-Cl)	Me	**11a/12a**	(**11a**) 2:1 (**12a**)
2	**8a** (X=4-Cl)	Et	**11b/12b**	(**11b**) 3:1 (**12b**)
3	**8a** (X=4-Cl)	Bn	**11c/12c**	(**11c**) 1:2 (**12c**)
4	**8b** (X=3-Cl)	Me	**11d/12d**	(**11d**) 3:1 (**12d**)
5	**8d** (X=4-OMe)	Et	**11e/12e**	(**11e**) 2:1 (**12e**)
6	**8b** (X=3-Cl)	Pr	**12f**	Only *N*-Pr derivative was isolated

				in pure form (**12f**)
7	**8d** (X=4-OMe)	Me	**12g**	Only N-Me derivative was isolated in pure form (**12g**)

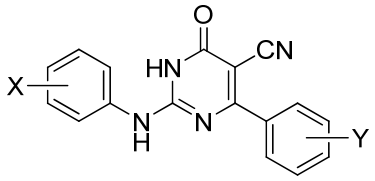
Table 1: *O*/*N* Alkylation of 2-thiobenzylpyrimidine derivatives.

Biological evaluation

To screen for compounds that have immunosuppressive properties, the MLR assay has been used, which is considered to be a model assay for T-cell responses against allogenic antigens. It is a clinically relevant test for histocompatibility between donor and recipient where lymphocytes from one individual (effector) are incubated with the lymphocytes of another individual (stimulator), which have been previously rendered incapable of blast transformation by treatment with mitomycin. The degree of incompatibility is reflected in the amount of cell division as measured by the uptake of tritium-labeled thymidine, whereby incompatibility increases its uptake.

In a first round of screening, compounds were tested at a concentration of 10 μM. Compounds displaying more than 50% inhibition were subjected to dose-response curves in order to determine IC_{50} values.

The first series of compounds screened for immunosuppressive activity were the 2-anilino-5-cyano-pyrimidin-4-(3H)-one analogues **5a-i**, bearing structural variety on both phenyl rings. These compounds lack immunosuppressive activity, all displaying IC_{50} values greater than 10 μM (Table 2).

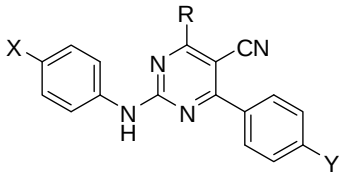


5a-i

Comp.	X	Y	MLR IC_{50} (μM)
5a	4-Cl	4-Cl	>10
5b	4-Cl	3-Cl	>10
5c	3-F	4-Cl	>10
5d	3-Et	4-Cl	>10
5e	H	4-Cl	>10
5f	4-Cl	H	>10
5g	4-Cl	4-OMe	>10
5h	4-Me	4-Cl	>10
5i	4-Me	H	>10

Table 2: SAR of 2-anilino-5-cyano-pyrimidin-4-(3H)-one analogues.

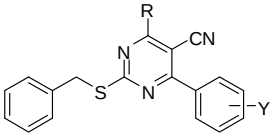
In an attempt to increase the biological activity, a novel series of pyrimidine analogues was prepared with structural modifications at position 4 of the pyrimidine scaffold (Table 3). The presence of an alkoxy substituent (compounds **6a-e**) never imparted immunosuppressive activity to these compounds. On the other hand, the presence of a piperidino moiety, combined with an appropriate substitution pattern at positions 2 and 6, yielded compounds **7g** and **7h**, with immunosuppressive activity, displaying IC_{50} values of 4.3 and 6.2 μM, respectively.



Comp	X	Y	R	MLR IC_{50} (μM)
6a	Cl	Cl	Benzyloxy	>10
6b	Cl	Cl	Methoxy	>10
6c	Cl	Cl	Ethoxy	>10
6d	Cl	H	Ethoxy	>10
6e	Cl	H	Propoxy	>10
7a	Cl	Cl	Morpholine	>10
7b	Cl	Cl	Isopropylamino	>10
7c	Cl	Cl	Piperidino	>10
7d	Cl	Cl	Cyclohexylamino	>10
7e	Cl	H	Isopropylamino	>10
7f	CH_3	Cl	Piperidino	>10
7g	CH_3	H	Piperidino	4.3
7h	Cl	H	Piperidino	6.2

Table 3: SAR of 2-anilino-4-substituted-5-cyano-6-aryl pyrimidine analogues

Driven by the fact that simultaneous variation of substituents at positions 2, 4 and 6 of the pyrimidine scaffold can lead to immosuppressive compounds, a focused library of 5-cyano-pyrimidines was prepared. For the ease of synthesis, a thiobenzyl group was selected at position 2. The installation of alkoxy substituents at position 4 (compounds **11a-e**) did not afford any immunosuppressive agents. This is in complete agreement with the MLR data of the 4-alkoxy-2-anilino pyrimidine analogues **6a-e**, which were also completely inactive.



Comp	Y	R	MLR IC_{50} (μM)
11a	4-Cl	Methoxy	>10
11b	4-Cl	Ethoxy	>10
11c	4-Cl	Benzyloxy	>10
11d	3-Cl	Ethoxy	>10
11e	4-OMe	Ethoxy	>10
9a	4-Cl	Morpholine	8.3
9b	4-Cl	Isopropylamine	>10
9c	4-Cl	Cyclohexylamine	6.4
9d	4-Cl	Isonipecotinamide	6.4
9e	4-Cl	Nipecotinamide	>10
9f	4-Cl	Benzylpiperidine	>10
9g	4-Cl	Benzoylpiperidine	9.8
9h	4-OCH_3	2-Thienylmethylamine	5.0
9i	4-OCH_3	Benzylamine	2.5
9j	4-OCH_3	Phenethylamine	>10
9k	4-OCH_3	Morpholine	1.6
9l	4-OCH_3	Thiomorpholine	>10
9m	4-OCH_3	4-Me-piperidine	>10
9n	4-OCH_3	4-Benzylpiperidine	>10
9o	4-OCH_3	Isonipecotinamide	4.9
9p	4-OCH_3	4-Benzoylpiperidine	2.1
9q	4-OCH_3	4-Phenylpiperidine	>10
9r	3-Cl	Morpholine	5.5

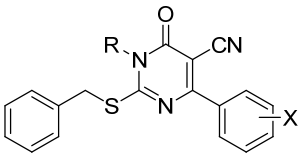
Table 4: SAR of 2-thiobenzyl-4-substituted-5-cyano-6-aryl pyrimidines

Primary, secondary, aliphatic, as well as aromatic amines were incorporated to maximize structural diversity. In a first series of analogues, a 3- or 4-chlorophenyl moiety was fixed at position 6 of the pyrimidine scaffold. A small, aliphatic substituent at position 4 such as isopropylamine (compound **9b**) did not impart any immunosuppressive activity to the pyrimidine scaffold. On the other hand, bulkier amines displayed immunosuppressive activity. The 4-*N*-cyclohexylamino derivative (compound **9c**) displayed an IC_{50} value of 6.4 μM. The presence of a morpholine moiety in combination with a 3-Cl-phenyl (compound **9r**) or 4-Cl-phenyl moiety (compound **9a**) furnished pyrimidine analogues with immunosuppressive activity (MLR IC_{50} values of 5.5 μM and 8.3 μM, respectively). A number of larger substituents was also introduced. The insertion of a carboxamide group on the piperidine moiey at the right position (i.e. an isonipecotinamide) yielded derivative **9d**, which displayed an MLR IC_{50} value of 6.4 μM. The insertion of the corresponding isomeric nipecotinamide led to compound **9e**, which was devoid of immunosuppressive activity. The addition of a

benzyl group on the piperidine moiety led to an inactive compound **9f** (MLR IC_{50} > 10 μM), whereas the benzoylpiperidine analogue **9g** showed weak immunosuppressive activity (MLR IC_{50} = 9.8 μM).

In order to probe the influence of the substituents on the 6-phenyl ring, a number of analogues was made carrying a 4-methoxy-phenyl moiety at position 6 instead of the Cl-phenyl group. Among these congeners, aromatic amines, such as 2-thienylmethylamine (compound **9h**) and benzylamine (compound **9i**) displayed strong immunosuppressive activity with IC_{50} values of 5.0 μM and 2.5 μM, respectively. However, insertion of an additional methylene linker between the amino group and the phenyl ring (phenethylamine at position 4; compound **9j**) led to a loss of activity. Within the 6-(4-OCH_3-phenyl) series, a morpholine at position 4 seems to be optimal for immunosuppressive activity with an IC_{50} value of 1.6 μM (compounds **9k**). Remarkably, changing the morpholine substituent for a thiomorpholine moiety (compound **9l**) resulted in complete loss of immunosuppressive activity. Analogously to the Cl-phenyl series, a number of substituted piperidine substituents were also incorporated. The insertion of a methyl group (compound **9m**) or a benzyl group (compound **9n**) led to pyrimidine analogues lacking immunosuppressive activity, whereas a nipecotinamide (compound **9o**) or a benzoylpiperidine (compound **9p**) gave potent immunosuppressive activity (MLR IC_{50} values of 4.9 μM and 2.1 μM, respectively).

Besides the insertion of oxygen- and nitrogen-containing nucleophiles at position 4 of the pyrimidine scaffold, the nitrogen of the lactam moiety was also alkylated with a number of alkylhalides (Table 5). None of these N-substituted derivatives showed any immunosuppressive activity at 10 μM.



Cmpd	R	X	MLR IC_{50} (μM)
12a	Me	4-Cl	>10
12b	Et	4-Cl	>10
12c	Bn	4-Cl	>10
12d	Me	3-Cl	>10
12e	Et	4-OMe	>10
12f	Pr	3-Cl	>10
12g	Me	4-OMe	>10

Table 5 : SAR of N(3)-alkyl-2-thiobenzyl-4-oxo-5-cyano-6-aryl pyrimidines

To examine the optimal substitution pattern at position 2, different thio-alkyl groups were introduced. This was combined with a 4-methoxyphenyl moiety at C-(6) and a morpholino at

C-(4) as this was the optimal substitution pattern for immunosuppressive activity in compound **9c**.

Comp	R	MLR IC_{50} (µM)
9s	CH_2Bn	3.9
9t	Me	4.9
9u	4-Br-Bn	>10
9v	*n*-butyl	2.3
9w	*n*-hexyl	5.3
9x	allyl	3.4

Table 6: SAR of 2-thioalkyl-4-morpholino-5-cyano-6-phenyl-pyrimidines

From the data in Table 6, it is clear that structural variety is tolerated at position 6. Replacement of the bulky benzyl group by smaller alkyl groups furnished analogues (compounds **9t, 9v, 9w, 9x**) that showed noteworthy inhibitory activity in the MLR assay with IC_{50} values ranging from 2.3 µM to 5.3 µM. Elongation of the linker between the sulfur atom and the phenyl group from a methylene group in compound **9k** to an ethylene spacer in derivative **9s** led to a modest decrease of activity in the MLR assay. Surprisingly, a 4-bromothiobenzyl group is not tolerated, as compound **9u** shows a IC_{50} higher than 10 µM.

In order to exclude that the immunosuppressive activity is due to a general cytotoxic effect, the cellular toxicity of the most potent immunosuppressive compounds was evaluated by a WST assay using Jurkat cells. From the data in Table 7, it can be deduced that most compounds lack cytotoxic activity, with compound **9t** being the exception. Its observed immunosuppressive activity is due to a cytotoxic effect and therefore, this compound is not useful for further optimization as immunosuppressive drug.

Comp	Cellular Toxicity CC_{50} (µM)
7h	>10
7g	>10
9a	>10
9c	>10
9d	>10
9g	>10
9h	>10
9i	>10
9k	>10
9o	>10
9p	>10
9r	>10
9s	>10
9t	0.1
9v	>10
9w	>10
9x	>10

Table 7: Toxicity data on Jurkat T cell line

Conclusion

A library of novel 2,4,6-trisubstituted-5-cyano-pyrimidine analogues has been synthesized and evaluated for its immunosuppressive activity in the MLR assay. The SAR study revealed that a hydroxyl group or an alkoxy group at position 4 is not tolerated for immunosuppressive activity, whereas the presence of larger amines (in particular morpholine) yielded compounds that showed a better activity in the MLR assay. Position 2 of the pyrimidine scaffold tolerates structural variety with respect to immunosuppressive activity, as the presence of aniline groups, as well as different thioalkyl moieties, furnished compounds with IC_{50} values of less than 5 μM in the MLR assay. At position 6, only phenyl groups were investigated as substituent, whereby the 4-OCH_3-phenyl moiety seem to be optimal for immunosuppressive activity, yielding compound **9k** with a IC_{50} of 1.6 μM in the MLR assay. Counterscreening revealed that pyrimidine derivative **9k** was devoid of cellular toxicity (CC_{50} > 10 μM) and is therefore an interesting starting point for further optimization towards more potent immunsuppressive drugs.

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Supplementary data

Supplementary data (Characterization data for the compounds **1a-b**, **4c-e**, **5c-i**, **6c-e**, **11c-e**, **12c-g**, **8d-k**, **7d-h**, **9f-x**) associated with this article can be found in the online version.

Experimental section

General

For all reactions, analytical grade solvents were used. All moisture-sensitive reactions were carried out in oven-dried glass-ware (135°C). ^1H and ^{13}C NMR spectra: *Bruker Advance 300* (^1H-NMR: 300 MHz, ^{13}C-NMR: 75 MHz), using tetramethylsilane as internal standard for ^1H-NMR spectra and (D_6)-DMSO (39.5 ppm) or $CDCl_3$ (77.2 ppm) for ^{13}C-NMR spectra. Abbreviations used are: *s* = singlet, *d* = doublet, *t* = triplet, *q* = quartet, *m* = multiplet, br. *s* =

broad singlet. Coupling constants are expressed in Hz. Mass spectra are obtained with a Finnigan LCQ Advantage Max (ion trap) mass spectrophotometer from Thermo Finnigan, San Jose, CA, USA. High resolution mass spectrometry: spectra were acquired on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Milford, MA). Samples were infused at 3 μL/min and spectra were obtained in positive (or negative) ionization mode with a resolution of 15000 (FWHM) using leucine enkephalin as lock mass.

Precoated aluminum sheets (*Fluka* Silica gel/TLC-cards, 254 nm) were used for TLC. Column chromatography (CC) was performed on *ICN* silica gel 63-200, 60 Å.

General procedure A for the synthesis of 5-cyano-6-phenyl-2-thiouracils (1a-e): To a solution of an appropriate benzaldehyde (6.0 mmol) in EtOH (20 mL) were added ethylcyanoacetate (6.3 mmol), thiourea (6.3 mmol) and piperidine (12 mmol). The resulting reaction mixture was refluxed overnight. The solution was cooled down at 0°C, the precipitate was filtered off and washed several times with cold EtOH. The crude products were used in the next step without any further purification.

5-Cyano-6-(4-toluil)-2-thiouracil (1c)

This compound was prepared according to General Procedure A using *p*-tolualdehyde in a yield of 61%. ^{1}H-NMR (300 MHz, (D6)-DMSO): 7.59 (d, *J*=7.95 Hz, 2H), 7.40 (d, *J*=7.95 Hz, 2H), 2.40 (s, 3H). ^{13}C-NMR (75 MHz, (D6)-DMSO): 168.2, 153.2, 135.3, 121.2, 120.1, 118.4, 105.9, 81.7, 12.2. ESI-MS (pos): 244.0 ($[M+H]^+$).

5-Cyano-6-(4-methoxyphenyl)-2-thiouracil (1d)

This compound was prepared according to General Procedure A using 4-anisaldehyde in a yield of 47%. ^{1}H-NMR (300 MHz, (D6)-DMSO): 13.1 (br, s, 1H), 7.65 (d, *J*=8.55 Hz, 2H), 7.11 (d, *J*=8.55 Hz, 2H), 3.86 (s, 3H). ^{13}C-NMR (75 MHz, (D6)-DMSO) 176.3, 162.5, 160.6, 158.8, 131.0, 121.2, 115.2, 114.0, 89.9, 55.7. ESI-MS (pos): 260.1 ($[M+H]^+$).

5-Cyano-6-phenyl-2-thiouracil (1e)

This compound was prepared according to General Procedure A using benzaldehyde in a yield of 41%. ^{1}H-NMR (300 MHz, (D6)-DMSO): 12.86 (br s, 1H), 8.5 (br s, 1H), 7.68 (d, *J*=7.56 Hz, 2H), 7.61 m, 1H), 7.56 (d, *J*=7.56 Hz, 2H). ^{13}C-NMR (75 MHz, (D6)-DMSO): 176.0, 160.2, 158.2, 132.0, 128.6, 128.2, 113.8, 90.2. ESI-MS (pos): 230.1 ($[M+Na]^+$).

Synthesis of *N,N'*-Bis(*tert*-butoxycarbonyl)-*N"*-*p*-chlorophenylguanidine (3a)

To a solution of *p*-chloroaniline (0.3 mmol), di-Boc-thiourea (0.25 mmol) and Et_3N (0.50 mmol) in CH_2Cl_2, was added *N*-iodosuccinimide (0.25 mmol) in one portion and the reaction was stirred at room temperature for 12 h. The reaction was quenched by adding 3 mL of a $Na_2S_2O_3$ solution. The organic phase was separated and washed twice with brine. The crude product was purified by silica gel chromatography (Heptane/AcOEt 5:1). ^{1}H-NMR (300 MHz, (D6)-DMSO): 11.6 (s, 1H), 10.4 (s, 1H), 7.58 (d, *J*=8.79 Hz, 2H), 7.29 (d, *J*=8.79 Hz, 2H), 1.55 (s, 9H), 1.53 5s, 9H). ESI-MS (pos): 370.2 ($[M+H]^+$).

Synthesis of *p*-Chlorophenylguanidine (4a)

A solution of **3a** in a mixture of CH_2Cl_2/TFA (1:1; 2 mL) was stirred at room temperature for 12 h. The mixture was basified by slowly adding a saturated $NaHCO_3$ solution till pH = 8. The crude product was used in the next step without any further purification. ^{1}H-NMR (300 MHz, (D6)-DMSO): 7.50-7.25 (m, 4H), 7.05 (br s, 4H). ESI-MS (pos): 170.0 ($[M+H]^+$).

General procedure B for the synthesis of substituted arylguanidines (4b-e)

To a solution of the appropriate aniline (7 mmol) in a mixture of EtOH/H_2O (1:1 ; 20 mL) were added cyanoamide (10 mmol) and a concentrated nitric acid solution (7 mmol). The resulting reaction mixture was refluxed overnight. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography, using the mobile phase as mentioned below for each derivative.

3-Fluorophenylguanidine (4b)

This compound was prepared using 3-fluoro-aniline, following the general procedure **B**. The crude residue was purified by silica gel chromatography (CH_2Cl_2/MeOH 99:1→95:5), affording the title compound in 39% yield. ^{1}H-NMR (300 MHz, (D6)-DMSO): 9.84 (br s, 1H), 7.50 (br m, 4H), 7.65 (d, *J*=7.89 Hz, 1H) 7.16-7.06 (m, 4H). ESI-MS (pos): 154.2 ($[M+H]^+$).

General procedure C for the synthesis of 2-anilino-4-oxo-5-cyano-6-phenylpyrimidines (5a-j) To a solution of the substituted arylguanidines (3 mmol) in EtOH (10 mL), were added the appropriate substituted benzaldehyde (3.15 mmol), ethylcyanoacetate (3.15 mmol) and piperidine (6 mmol) and the mixture was refluxed overnight. The solution was cooled down at

0°C. The reaction was then quenched with brine and the organic phase was extracted with AcOEt. The crude product was purified by silica gel chromatography, using a mobile a phase as mentioned below for each derivative.

2-(4-Chlorophenylamino)-4-oxo-5-cyano-6-(4-chlorophenyl)-3,4-dihydropyrimidine (5a)
This compound was prepared according to General Procedure **C,** using *p*-chlorophenylguanidine and *p*-chlorobenzaldehyde. The crude product was purified by silica gel chromatography, using a mixture of CH_2Cl_2/MeOH (in a ratio of 99:1) as mobile phase, affording the title compound in a yield of 49%. ^{1}H-NMR (300 MHz, (D6)-DMSO): 10.19 (s, 1H); 7.88 (d, *J*=8.52 Hz, 1H), 7.65 (dd, *J*=4.38, 8.79 Hz, 4H), 7.62 (d, *J*=8.52 Hz, 1H). ^{13}C-NMR (75 MHz, (D6)-DMSO): 169.2, 136.4, 136.2, 135.1, 130.4, 128.9, 128.8, 128.3, 123.2, 116.6, 88.5. HRMS: ESI$^+$ calc. for $(C_{17}H_{11}Cl_2N_4O)^+$ 357.0310, found: 357.0321.

2-(4-Chlorophenylamino)-4-oxo-5-cyano-6-(3-chlorophenyl)-3,4-dihydropyrimidine (5b)
This compound was prepared according to General Procedure **C**, using a mixture of *p*-chlorophenylguanidine and *m*-chlorobenzaldehyde. The crude product was purified by silica gel chromatography, using a mixture of CH_2Cl_2/MeOH (in a ratio of 99:1) as mobile phase, affording the title compound in 39% yield. ^{1}H-NMR (300 MHz, (D6)-DMSO): 7.83 (m, 4H), 7.57 (d, *J*=8.41 Hz, 2H), 7.33 (d, *J*=8.58 Hz, 2H). ^{13}C-NMR (75 MHz, (D6)-DMSO): 172.2, 168.0, 139.6, 138.8, 133.1, 130.4, 130.2, 128.5, 128.1, 127.1, 125.9, 121.5, 118.7, 86.4. HRMS: ESI$^+$ calc. for $(C_{17}H_{11}Cl_2N_4O)^+$ 357.0310, found: 357.0320.

General procedure D for the synthesis of compounds 6a-e, 11a-e, 12a-g.
To a solution of a pyrimidine derivative (0.3 mmol) in DMF (7 mL) were added an appropriate alkylating agent (1.05 eq) and K_2CO_3 (1.5 eq). The solution was stirred at room temperature for 12 hours. The reaction was quenched with brine and the organic phase was extracted twice with AcOEt, dried over $MgSO_4$, filtered and evaporated ander reduced pressure. The crude residue was purified by silica gel chromatography, using the mobile phase as specified below for each derivative.

2-(4-Chlorophenylamino)-4-benzyloxy-5-cyano-6-(4-chlorophenyl)pyrimidine (6a)
This compound was prerared according to the General Procedure **D**, starting from compound **5a** and benzylbromide. The crude residue was purified by silica gel chromatography (Heptane/AcOEt 6:1), affording the title compound in 79% yield. ^{1}H-NMR (300 MHz,

$CDCl_3$): 10.1 (br s, 1H), 7.93 (d, *J*=8.40 Hz, 2H), 7.74 (br s, 2H), 7.64 (d, *J*=8.40 Hz, 2H), 7.47 (br d, 3H), 7.43 (d, *J*=8.52 Hz, 2H), 7.38 (d, *J*=8.52 Hz, 2H), 5.57 (s, 2H). ^{13}C-NMR (75 MHz, (D6)-DMSO): 169.0, 159.1, 137.8, 136.3, 135.7, 134.6, 130.5, 128.8, 128.7, 128.4, 127.2, 122.1, 115.6, 69.1. HRMS: ESI$^+$ calc. for $(C_{24}H_{17}Cl_2N_4O)^+$ 447.0779, found: 447.0782.

2-(4-Chlorophenylamino)-4-methoxy-5-cyano-6-(4-chlorophenyl)pyrimidine (6b)

This compound was prepared according to the General Procedure **D**, starting from compound **5a** and methyliodide. The crude residue was purified by silica gel chromatography (Heptane/AcOEt 8:1), affording the title compound in 61% yield. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.97 (d, *J*=7.89 Hz, 2H), 7.60 (d, *J*=7.89 Hz, 2H), 7.52 (d, *J*=8.55 Hz, 2H), 7.37 (d, *J*=8.55 Hz, 2H), 4.13 (s, 3H).^{13}C-NMR (75 MHz, $CDCl_3$): 168.8, 158.6, 137.6, 136.1, 129.8, 129.0, 128.8, 128.7, 121.2, 114.9, 93.4, 55.0. HRMS: ESI$^+$ calc. for $(C_{18}H_{13}Cl_2N_4O)^+$ 371.0466, found: 371.0450.

2-Benzylthio-4-methoxy-5-cyano-6-(4-chlorophenyl)pyrimidine (11a) and 2-Benzylthio-N(3)-methyl-5-cyano-6-(4-chlorophenyl)pyrimidine (12a)

These compounds were prepared according to the General Procedure **D**, starting from compound **8a** and methyliodide. The mixture of isomers was purified by silica gel chromatography (Heptane/AcOEt 5:1), after which the ratio O-alkyl/N-alkyl was determined to be as 2:1, and the overall yield was 71%. Compound **11a**: ^{1}H-NMR (300 MHz, $CDCl_3$): 8.0 (d, *J*=8.61 Hz, 2H), 7.97 (d, *J*=8.61 Hz, 2H), 7.49 (d, *J*=6.66 Hz, 2H), 7.35-7.28 (m, 3H), 4.48 (s, 2H), 4.14 (s, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$): 174.6, 169.6, 166.9, 137.9, 136.3, 133.1, 130.0, 128.7, 128.5, 128.3, 127.2, 114.2, 87.6, 55.2, 35.5. HRMS: ESI$^+$ calc. for $(C_{19}H_{15}ClN_3OS)^+$ 368.0624, found: 368.0625. Compound **12a**: ^{1}H-NMR (300 MHz, $CDCl_3$): 7.99 (d, *J*=8.28 Hz, 2H), 7.51 (d, *J*=8.28 Hz, 2H), 7.38-7.28 (m, 5H), 4.57 (s, 2H), 3.60 (s, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$): 165.9, 164.0, 159.7, 138.0, 134.1, 133.0, 130.0, 128.8, 128.7, 128.6, 127.9, 114.8, 92.7, 37.0, 30.6. HRMS: ESI$^+$ calc. for $(C_{19}H_{15}ClN_3OS)^+$ 368.0624, found: 368.0622.

2-Benzylthio-4-ethoxy-5-cyano-6-(4-chlorophenyl)pyrimidine (11b) and 2-Benzylthio-N(3)-ethyl-5-cyano-6-(4-chlorophenyl)pyrimidine (12b)

These compounds were prepared according to the General Procedure **D**, starting from compound **8a** and ethyliodide. The mixture of isomers was purified by silica gel

chromatography (Heptane/AcOEt 5:1), after which the ratio O-alkyl/N-alkyl was determined to be 3:1 and the overall yield was 63%. Compound **11b**: ^{1}H-NMR (300 MHz, $CDCl_3$): 8.00 (d, *J*=8.58 Hz, 2H), 7.52 (d, *J*=8.58 Hz, 2H), 7.44 (br d, 2H), 7.34-7.28 (m, 3H), 4.63 (q, *J*=7.08 Hz, 2H), 4.47 (s, 2H), 1.48 (t, *J*=7.08 Hz, 3H). ^{13}C-NMR (75 MHz, (D6)-DMSO): 174.5, 169.2, 167.0, 137.8, 136.3, 133.2, 130.0, 128.7, 128.4, 128.3, 127.2, 114.3, 87.7, 64.5, 35.5, 13.9. HRMS: ESI$^+$ calc. for $(C_{20}H_{17}ClN_3OS)^+$ 382,0781, found: 382,0796. Compound **12b**: ^{1}H-NMR (300 MHz, $CDCl_3$): 7.99 (d, *J*=8.67 Hz, 2H), 7.51 (d, *J*=8.67 Hz, 2H), 7.40-7.35 (m, 5H), 4.56 (s, 2H), 4.20 (q, *J*=7.14 Hz, 2H), 1.40 (t, *J*=7.14 Hz, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$): 165.1, 164.0, 159.4, 138.0, 134.1, 133.1, 130.0, 128.8, 128.7, 128.6, 127.9, 114.4, 93.1, 40.5, 36.9, 11.9. HRMS: ESI$^+$ calc. for $(C_{20}H_{17}ClN_3OS)^+$ 382.0781, found: 382.0794.

General procedure E for the synthesis of compounds 2 and 8a-k.

To a solution of a 2-thiouracil analogue (0.61 mmol) in CH_3CN (8 mL), were added K_2CO_3 (0.64 mmol) and an alkyl or benzyl halide (0.64 mmol). The mixture was stirred overnight at room temperature. The reaction was quenched with brine and diluted with AcOEt. The organic phase was extracted, dried over $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by silica gel chromatography using the mobile phase as mentioned below for each derivative.

2-Methylthio-5-cyano-6-(4-chlorophenyl)uracil (2)

This compound was prepared according to General Procedure **E**, starting from **1a** and methyliodide. The crude residue was purified by silica gel chromatography (Heptane/AcOEt1:1), affording the title compound in 88% yield. ^{1}H-NMR (300 MHz, (D6)-DMSO): 7.91 (d, *J*=8.07 Hz, 2H), 7.79 (d, *J*=8.07 Hz, 2H), 2.60 (s, 3H). ESI-MS (pos): 278.0 ($[M+H]^+$).

2-Benzylthio-5-cyano-6-(4-chlorophenyl)uracil (8a)

This compound was prepared according to General Procedure **E**, starting from **1a** and benzylbromide. The crude residue was purified by silica gel chromatography (Heptane/AcOEt 5:1→1:1), affording the title compound in 61% yield. ^{1}H-NMR (300 MHz, (D6)-DMSO): 7.90 (d, *J*=8.55 Hz, 2H), 7.61 (d, *J*=8.55 Hz, 2H), 7.40 (d, *J*=6.75 Hz, 2H), 7.33-7.24 (m, 3H), 4.45 (s, 2H). HRMS: ESI$^+$ calc. for $(C_{18}H_{13}ClN_3OS)^+$ 354.0468, found: 354.0486.

2-Benzylthio-5-cyano-6-(3-chlorophenyl)uracil (8b)

This compound was prepared according to General Procedure **E**, starting from **1b** and benzylbromide. The crude residue was purified by silica gel chromatography (CH_2Cl_2/MeOH 100:0→97:3), yielding the title compound in a yield of 57%. ^{1}H-NMR (300 MHz, (D6)-DMSO): 7.88 (d, *J*=7.94 Hz, 2H), 7.68 (d, *J*=8.07 Hz, 1H), 7.62 (d, *J*=8.12 Hz, 1H), 7.35 (d, *J*=7.45 Hz, 2H), 7.33-7.27 (m, 3H), 4.52 (s, 2H). ^{13}C-NMR (75 MHz, (D6)-DMSO): 166.3, 165.9, 161.1, 137.4, 136.6, 133.4, 131.9, 131.6, 130.7, 129.1, 128.7, 128.5, 127.6, 127.5, 93.9, 34.4. HRMS: ESI$^+$ calc. for $(C_{18}H_{13}ClN_3OS)^+$ 354.0468, found: 354.0456.

2-Benzylthio-5-cyano-6-(4-methylphenyl)uracil (8c)

This compound was prepared according to General Procedure **E**, starting from **1c** and benzylbromide. The crude residue was purified by silica gel chromatography (CH_2Cl_2/MeOH 100:0→95:5), furnishing the title compound in a yield of 61%. ^{1}H-NMR (300 MHz, (D6)-DMSO): 7.87 (d, *J*=7.89 Hz, 2H), 7.43-7.37 (m, 4H), 7.35-7.27 (m, 3H), 4.45 (s, 2H), 2.40 (s, 3H). ^{13}C-NMR (75 MHz, (D6)-DMSO): 167.2, 165.5, 161.2, 142.6, 142.3, 136.6, 132.5, 129.3, 129.1, 128.9, 128.7, 127.6, 116.1, 92.7, 34.3, 21.22. HRMS: ESI$^+$ calc. for $(C_{19}H_{16}N_3OS)^+$ 334.1014, found: 334.1031.

General procedure F for the BOP mediated nucleophilic arylic substitution.

To a solution of a 4-oxopyrimidine derivative (0.280 mmol) in MeCN (5 mL), were added BOP (0.363 mmol), the appropriate amine (0.7 mmol) and DBU (0.42 mmol). The reaction mixture was stirred at room temperature for 12 hours. The solution was diluted with AcOEt and brine, the organic phase was separated and washed twice with brine. The combined organic phases were dried over $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by silica gel chromatography using the mobile phase as mentioned below for each derivative.

2-(4-Chlorophenylamino)-4-morpholino-5-cyano-6-(4-chlorophenyl)pyrimidine (7a)

This compound was prepared according to General Procedure **F** starting from compound **5a** and morpholine. The crude residue was purified by silica gel chromatography (Heptane/AcOEt 4:1), furnishing the title compound in 71% yield. ^{1}H-NMR (300 MHz, (D6)-DMSO): 10.2 (br s, 1H), 7.88 (d, *J*=8.19 Hz, 2H), 7.71 (d, *J*=8.64 Hz, 2H), 7.62 (d, *J*=8.64 Hz, 2H), 7.37 (d, *J*=8.19 Hz, 2H), 3.88 (br s, 4H), 3.76 (br s, 4H). ^{13}C-NMR (75 MHz, (D6)-

DMSO) 170.6, 162.4, 158.3, 138.3, 135.6, 130.8, 128.4, 126.3, 124.1, 121.6, 118.6, 65.8, 47.3. HRMS: ESI$^+$ calc. for $(C_{21}H_{18}Cl_2N_5O)^+$ 426.0888, found: 426.0885.

2-(4-Chlorophenylamino)-4-isopropylamino-5-cyano-6-(4-chlorophenyl)pyrimidine (7b)
This compound was prepared according to General Procedure **F** starting from compound **5a** and isopropylamine. The crude residue was purified by silica gel chromatography (Heptane/AcOEt 2:1), affording the title compound in 62% yield. ^{1}H-NMR (300 MHz, (D6)-DMSO): 10.05 (br s, 1H), 7.85 (d, *J*=8.43 Hz, 2H), 7.81 (d, *J*=8.88 Hz, 2H), 7.614 (d, *J*=8.43 Hz, 2H), 7.37 (d, *J*=8.88 Hz, 2H), 4.41 (br m, 1H), 1.27 (d, *J*=6.42 Hz, 6H). ^{13}C-NMR (75 MHz, (D6)-DMSO): 168.1, 162.0, 159.2, 138.9, 135.8, 135.4, 130.3, 128.6, 128.5, 126.1, 12 1.4, 117.2, 43.0, 21.9. HRMS: ESI$^+$ calc. for $(C_{20}H_{18}Cl_2N_5)^+$ 398.0939, found: 398.0951.

2-(4-Chlorophenylamino)-4-piperidino-5-cyano-6-(4-chlorophenyl)pyrimidine (7c)
This compound was prepared according to General Procedure **F** starting from compound **5a** and piperidine. The crude residue was purified by silica gel chromatography (Heptane/AcOEt 2:1), yielding the title compound in 71% yield. ^{1}H-NMR (300 MHz, (D6)-DMSO): 10.11 (br s, 1H), 7.87 (d, *J*=8.46 Hz, 2H), 7.76 (d, *J*=8.82 Hz, 2H), 7.63 (d, *J*=8.46 Hz, 2H), 8.76 (d, *J*=8.82 Hz, 2H), 3.84 (br s, 4H), 1.67 (br s, 6H). HRMS: ESI$^+$ calc. for $(C_{22}H_{20}Cl_2N_5)^+$ 424.1096, found: 424.1085.

2-Benzylthio-5-cyano-6-(4-chlorophenyl)-4-cyclohexylaminopyrimidine (9c)
This compound was prepared according to General Procedure **F** starting from compound **8a** and cyclohexylamine. The crude product was purified by silica gel chomarography (Heptane/AcOEt 5:1) affording the title compound in 58% yield . ^{1}H-NMR (300 MHz, (D6)-DMSO): 7.83 (d, *J*=8.55 Hz, 2H), 7.60 (d, *J*=8.55 Hz, 2H), 7.41 (d, *J*=6.87 Hz, 2H), 7.33-7.24 (m, 3H), 4.42 (d, 2H), 4.05 (br m, 1H), 1.75 (t, *J*=12.18 Hz, 3H), 1.60 (d, *J*=11.52 Hz, 1H), 1.51-1.40 (m, 2H), 1.29-1.22 (m, 2H). ^{13}C-NMR (75 MHz, (D6)-DMSO): 174.2, 165.4, 160.6, 137.2, 137.0, 034.1, 029.7, 128.6, 128.4, 128.2, 126.9, 83.0, 50.0, 35.1, 32.5, 25.1, 24.5. HRMS: ESI$^+$ calc. for $(C_{24}H_{24}ClN_4S)^+$ 435.1110, found: 435.1122.

2-Benzylthio-4-(4-carboxyamidepiperidine)-5-cyano-6-(4-chlorophenyl)pyrimidine (9d)
This compound was prepared according to General Procedure **F**, starting from compound **8a** and 4-carboxyamide-piperidine. The crude residue was purified by silica gel chromatography (Heptane/AcOEt 8:1), affording the title compound in 55% yield. ^{1}H-NMR (300 MHz, (D6)-

DMSO): 7.88 (d, *J*=8.58 Hz, 2H), 7.63 (d, *J*=8.58 Hz, 2H), 7.44 (br d, *J*=7.08 Hz, 2H), 7.34-7.25 (m, 3H), 6.85 (br s, 1H), 4.60 (d, *J*=13.38 Hz, 2H), 3.28 (m, 3H), 1.91-1.85 (m, 2H), 1.69-1.55 (m, 2H). ^{13}C-NMR (75 MHz, (D6)-DMSO): 175.8, 172.2, 169.0, 161.3, 137.7, 136.2, 135.1, 131.2, 128.9, 128.7, 128.6, 128.4, 127.3, 118.0, 83.9, 46.7, 41.0, 34.7, 28.4. HRMS: ESI$^+$ calc. for $(C_{24}H_{23}ClN_5OS)^+$ 464.1312, found: 464.1315.

2-Benzylthio-4-(3-carboxyamidepiperidine)-5-cyano-6-(4-chlorophenyl)pyrimidine (9e)
This compound was prepared according to General Procedure **F**, starting from compound **8a** and 3-carboxyamide piperidine. The crude residue was purified by silica gel chromatography (CH_2Cl_2/MeOH 99:1), affording the title compound in 71% yield. ^{1}H-NMR (300 MHz, (D6)-DMSO) 7.87 (d, *J*=8.58 Hz, 2H), 7.61 (d, *J*=8.58 Hz, 2H), 7.42 (d, *J*=6.96 Hz, 2H), 7.35-7.25 (m, 3H), 6.85 (br s, 1H), 4.58 (d, *J*=13.44 Hz, 2H), 4.43 (s, 2H), 3.27 (t, *J*=11.52 Hz, 2H), 2.54 (s, 1H), 1.91-1.88 (br m, 2H), 1.68-1.61 (br m, 2H). ^{13}C-NMR (75 MHz, (D6)-DMSO): 175.8, 172.2, 169.0, 161.4, 137.7, 136.2, 135.1, 131.3, 128.9, 128.7, 128.6, 127.3, 118.0, 83.9, 46.7, 41.0, 36.6, 34.7, 28.4. HRMS: ESI$^+$ calc. for $(C_{24}H_{23}ClN_5OS)^+$ 464.1312, found: 464.1328.

2-Benzylthio-4-chloro-5-cyano-6-(4-chlorophenyl)pyrimidine (10)
To a solution of **8a** (100 mg, 0.282 mmol) in dioxane (5 mL) was added $POCl_3$ (155 µL, 1.69 mmol) and the reaction was stirred at 80°C for 2 hours. The solvent was then evaporated and the crude product was purified by silica gel chromatography using a mixture of heptane and AcOEt (in a ratio of 4:1) as mobile phase, furnishing the title compound in a yield of 85%. ^{1}H-NMR (300 MHz, (D6)-DMSO): 8.00 (d, *J*=8.43 Hz, 2H), 7.73 (d, *J*=8.43 Hz, 2H), 7.47 (d, *J*=6.84 Hz, 2H), 7.36 (m, 3H), 4.53 (s, 2H).

General Procedure F for the synthesis of compounds 9a-b.
To a solution of **10** (0.25 mmol) in dioxane (5 mL) was added the appropriate amine (0.5 mmol) and the mixture was stirred at room temperature for 2 hours. The solution was diluted with brine and AcOEt. The organic phase was extracted, dried over $MgSO_4$ and evaporated under reduced pressure. The crude mixture was purified by silica gel cromatography using the mobile phase as mentioned below for each derivative.

2-Benzylthio-4-morpholino-5-cyano-6-(4-chlorophenyl)pyrimidine (9a)

This compound was prepared according to General Procedure **F** using morpholine,. The crude residue was purified by silica gel chromatography (Heptane/AcOEt 6:1) yielding the title compound in a yield of 55%. ^{1}H-NMR (300 MHz, (D6)-DMSO): 7.81 (d, *J*=8.61 Hz, 2H), 7.46 (m, 4H), 7.33 (m, 3H), 4.41 (s, 2H), 3.97 (s, 4H), 3.80 (s, 4H). ^{13}C-NMR (75 MHz, (D6)-DMSO): 173.2, 169.4, 162.1, 137.3, 136.6, 134.2, 130.4, 128.5, 128.4, 128.3, 127.0, 117.7, 83.4, 77.2, 76.7, 76.3, 69.3, 47.2, 35.3. HRMS: ESI^{+} calc. for $(C_{22}H_{20}ClN_4OS)^{+}$ 423.1046, found: 423.1050.

2-Benzylthio-4-isopropylamino-5-cyano-6-(4-chlorophenyl)pyrimidine (9b)

This compound was prepared according to General Procedure **F**, using isopropylamine. The crude residue was purified by silica gel chromatography (Heptane/AcOEt 4:1), affording the title compound in a yield of 72%. ^{1}H-NMR (300 MHz, (D6)-DMSO):7.84 (d, *J*=8.61 Hz, 2H), 7.63 (d, *J*=8.61 Hz, 2H), 7.43 (d, *J*=8.61 Hz, 2H), 7.34 (m, 3H), 4.41 (s, 3H), 1.21 (d, *J*=6.54 Hz, 6H). ^{13}C-NMR (75 MHz, (D6)-DMSO) 173.2, 166.3, 160.2, 137.8, 136.0, 134.9, 130.5, 129.0, 128.8, 128.7, 128.6, 127.2, 116.2, 83.5, 43.0, 34.5, 21.8. HRMS: ESI^{+} calc. for $(C_{22}H_{19}ClN_4S)^{+}$ 395.1017, found: 395.1020.

MLR

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 1.2 x 10^6 cells/mL in complete medium (RPMI-1640 containing 10% heat-inactivated fetal calf serum and antibiotics). RPMI 1788 cells (human B lymphocyte cell line) were treated with 30 µg/mL of mitomycin C for 20 min at 37°C, washed four times with medium and finally suspended in complete medium to a density of 0.45 10^6 cells/mL. One hundred µL of each cell suspension were mixed with 20 µL of diluted compound in 96-well microtiter plates. The mixed cells were cultured for 6 days at 37°C in a humidified atmosphere of 5% CO_2 95% air. DNA synthesis was assayed by the addition of 1 µCi (methyl-^{3}H) thymidine per well during the last 18 hours of culture. Thereafter, the cells were harvested on glass filter paper and the counts per minute determined in a liquid scintillation counter.

Cytotoxicity assay

Cytotoxicity of compounds was measured using a colorimetric cell viability assay, the WST-1 assay, on Jurkat T-cell line. Cells were seeded in 96-well plates (5.10^4 cells per well) and

exposed to serial dilutions of compounds for 48 hours at 37°C and 5% CO_2. After removal of 100 µL of medium, cell viability was checked by adding 10µL of a solution of tetrazolium salt, 4-[3-[4-Iodophenyl]-2-4-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate (WST-1, Roche Diagnostics, Switzerland). After incubation at 37°C and 5% CO_2, absorbance at 450nm was measured with a spectrophotometer (2103 Envision Multilabel Reader, Perkin Elmer Waltham, MA). Results were expressed as dilution as percentage of cytotoxicity at each dilution and concentration at which 50% of the cells had died (CC_{50}) was calculated.

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