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Synthesis, immunosuppressive activity and structure–activity relationship study of a new series of 4-*N*-piperazinyl-thieno[2,3-*d*]pyrimidine analogues

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ABSTRACT

The synthesis of a new series of 4-*N*-piperazinyl-thieno[2,3-*d*]pyrimidines is described. The synthetic route allows introducing structural variety at positions 2, 4 and 6 of the scaffold. Evaluation of their immunosuppressive activity in a Mixed Lymphocyte Reaction (MLR) assay revealed that the most potent compound has an IC_{50}-value of 66 nM and therefore deserves attention for further medicinal chemistry optimization.

In the last 50 years, solid organ transplantation has become the therapy of choice for end stage liver, lung and heart diseases. This is mainly due to the fact that more effective immunosuppressive medication became available.[1] Cyclosporine and tacrolimus belong to the most frequently used drugs for transplant patients.[2] Their immunosuppressive activity is linked to inhibition of calcineurin, a serine-threonine phosphatase that activates intracellular gene-promoting transcription factors involved in IL-2 activation. In order to achieve calcineurin inhibition, cyclosporine binds to an intracellular cyclophilin, whereas tacrolimus interacts with another cytoplasmic protein, called FK-binding protein. The ultimate biological effect of calcineurin inhibition is a decreased IL-2 production and a resultant decreased IL-2–mediated proliferation of T-cells. Sirolimus (rapamycin)[3] also binds to a cytosolic immunophilin, called FKBP12. However, the sirolimus–FKBP12 complex does not inhibit calcineurin, nor does it inhibit pathways of IL-2 production. The sirolimus–FKBP12 complex binds proteins downstream of IL-2 in T-cell activation pathways, known as the mammalian target of rapamycin (mTOR) that prevent DNA and protein synthesis in T-cells. Antiproliferative agents, such as azathioprine and mycophenolate mofetil (MMF) are commonly used in transplant centers.[4] Azathioprine is an inhibitor of purine biosynthesis. This leads to inhibition of proliferation of many cell types and azathioprine is therefore a quite toxic drug. Inhibition of inosine monophosphate dehydrogenase by MMF leads to a depletion of guanine nucleotides and an inhibition of proliferating lymphocytes. In addition, corticosteroids have played a central role in the maintenance of immunosuppression as well as the treatment of acute rejection.

Despite intensive efforts to overcome rejection following organ transplantation, the strong demand for effective and safe immunosuppressants still remains.[5] Most of the currently available drugs for transplantation are known to be accompanied with serious side effects[5,3] such as nephrotoxicity, neurotoxicity, hyperlipidaemia, new-onset post-transplant diabetes mellitus and hypertension.[6] Thus, development of potent and safe immunosuppressants has been desired.

However, in the first decade of the new millennium, no new medication specifically indicated for organ transplantation has been approved. There are currently three small compounds in various stages of clinical development in renal transplantation.[7] ISA247 is a semisynthetic cyclosporine analogue. It is more potent than cyclosporine in the calcineurin enzymatic assay and in preclinical animal models of organ transplantation. Furthermore, studies in monkeys suggest that this compound has no nephrotoxicity. Pfizer is currently developing CP-690550, a selective Janus kinase 3 (JAK3) inhibitor. AEB071 is currently in clinical trials by Novartis. This compound blocks early T-cell activation by selective inhibition of protein kinase C.

Over the last few years, our group has been active in the search for novel immunosuppressive agents based on a bicyclic, heteroaromatic scaffold. Immunosuppressive activity has been associated

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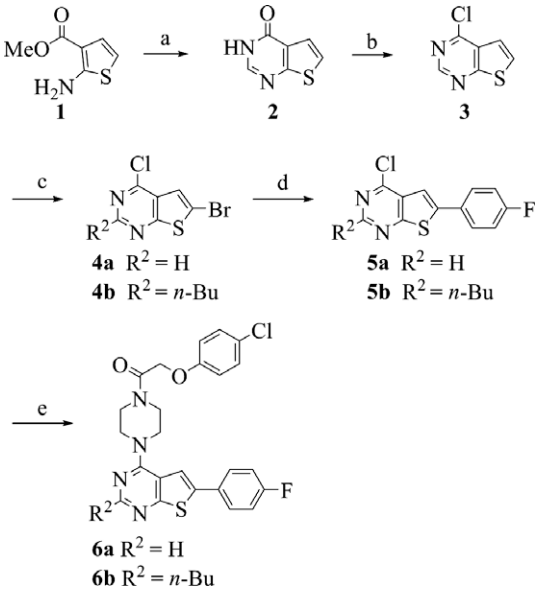
with pteridines,[8] 5-deazapteridines[9] (pyrido[2,3-*d*]pyrimidines) and 8-deaza-pteridines[10] (pyrido[3,2-*d*]pyrimidines). A common element is that these are all bicyclic hetero-aromatic flat structures. One of the main conclusions of our structure–activity relationship (SAR) study was that a phenoxyacetyl-piperazinyl group at position 4 is necessary to obtain potent immunosuppressive activity. As part of further exploration of the SAR of these compounds, we envisaged to use the thieno[2,3-*d*]pyrimidine scaffold as core scaffold, as described in this Letter.

For evaluation of the immunosuppressive activity of the compounds, an allogeneic Mixed Lymphocyte Reaction (MLR) assay was used.[11] The MLR assay is a fundamental benchmark test for immunosuppressants activity. Most of the current immunosuppressive compounds are very potent in MLR assay and several of them were discovered by this assay.[12] 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.

Within the isomeric thieno[3,2-*d*]pyrimidine series, a versatile starting material has been described in literature.[13] 6-Bromo-4-chloro-thieno[3,2-*d*]pyrimidine can be regioselectively functionalized at positions 4 and 6. Palladium-catalyzed cross-coupling reactions, occur exclusively at position 6, whereas the reaction with amines results in displacement of the chlorine at position 4. Therefore, it was envisaged that the corresponding 6-bromo-4-chloro-2-substituted-thieno[2,3-*d*]pyrimidine could similarly act as a versatile building block for the introduction of various substituents at position 4 and 6 to build up thieno[2,3-*d*]pyrimidine libraries. In order to prepare this building block, methyl 2-amino-thiophene-3-carboxylate **1** was heated in formamide yielding thieno[2,3-*d*]pyrimidin-4(3*H*)one **2** (Scheme 1).[14] Conversion of the 4-oxo group into chlorine was achieved in good yield with oxalyl chloride and DMF (Vilsmeier reagent). Regioselective introduction of a bromine at position 6 was performed with *n*-BuLi and carbon tetrabromide as bromine source at low temperature (−78 °C). Two major products (**4a** and **4b**) were isolated in a ratio of 1:1, after purification by flash chromatography. The mass spectral and ^{1}H NMR data of **4b**, indicate that a butyl group is present on the thienopyrimidine scaffold. Heteronuclear Multiple Bond Correlation (HMBC) spectroscopy was used to determine the exact regiochemistry (Fig. 1). A direct coupling (^1J) of the aromatic proton (H_a: δ = 7.4 ppm) and a ^{13}C signal at 122 ppm was detected. The carbon signal of C(2) is usually seen above 150 ppm, whereas the carbon at position 5 is observed around 120 ppm, indicating that the aromatic proton is present at position 5. Only one HMBC correlation (^2J) between the methylene protons (H_b) of the *n*-butyl substituent and a carbon (at δ = 150 ppm) is observed, which supports the fact that the *n*-butyl group is attached to C(2). These NMR data also confirm that bromination takes place at position 6 of the thieno[2,3-*d*]pyrimidine scaffold. Standard reaction conditions for Suzuki coupling of **4a** and **4b** with 4-fluorophenylboronic acid afforded compounds **5a** and **5b**, respectively. The remaining chlorine atom was displaced by 2-(4-chlorophenoxy)-1-(piperazin-1-yl)ethanone[15] under mild conditions yielding compounds **6a** and **6b**.



Scheme 1. Reagents and conditions: (a) formamide, reflux, 3 h; (b) oxalyl chloride, DMF, CH_2Cl_2, 0 °C to reflux, 2.5 h; (c) *n*-BuLi, CBr_4, THF, −78 °C, 20 min, then rt, 2 h; (d) 4-fluorophenylboronic acid, $Pd(PPh_3)_4$, K_2CO_3, dioxane/water (3:1 v/v), reflux, 3 h; (e) 2-(4-chlorophenoxy)-1-(piperazin-1-yl)ethanone, dioxane, NEt_3, 70 °C, 24 h.

Although 6-bromo-4-chloro-thieno[2,3-*d*]pyrimidine **4a** is a versatile building block, due to synthetic difficulties (low yields and the formation of side products), an alternative route was explored (Scheme 2), starting from an appropriate phenylacetaldehyde (either commercially available when R = H; or synthesized by oxidation of 2-(4-fluorophenyl)ethanol with pyridinium chlorochromate if R = F). The condensation of aldehyde **7** with ethyl cyanoacetate in the presence of a base and elemental sulfur (Gewald reaction) furnished ethyl 2-amino-5-aryl-thiophene-3-carboxylate **8**.[16] Reaction of compound **8** with several nitriles under acidic conditions or with chloroformamidine hydrochloride provided the 4-oxo-thieno[2,3-*d*]pyrimidine analogues **9a–e** in good yields. For the introduction of the piperazine moiety at position 4, a convenient phosphonium-mediated S_NAr reaction for the derivatisation of the lactam functionality is used.[17] Treatment of **9a** and **9b** with benzotriazol-1-yloxytris(dimethylamino)-phosphonium-hexafluorophosphate (BOP), DBU and piperazine leads to the formation of the 7-*N*-piperazinyl-thieno[2,3-*d*]pyrimidine analogues **10a,b**. The piperazine moiety can be further derivatised to amides (compound **11g–o**) by reaction with appropriate acid chlorides, or to urea (compound **11p–s**) by coupling with isocyanates. Alternatively, it is also possible to introduce substituted piperazine derivatives directly in one step by the BOP-mediated reaction (compound **11a–f**, **t**, **u**). An overview of the compounds **11a–u** synthesized according to this general synthetic scheme is shown in Table 1, together with their MLR data. The IC_{50} value represents

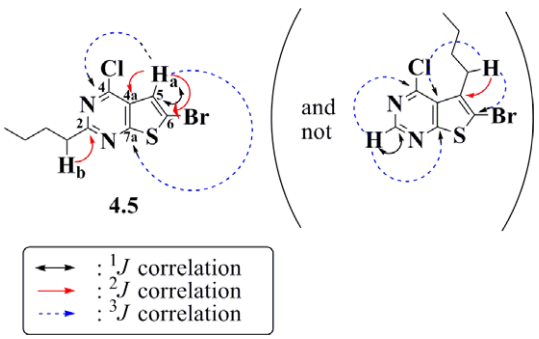
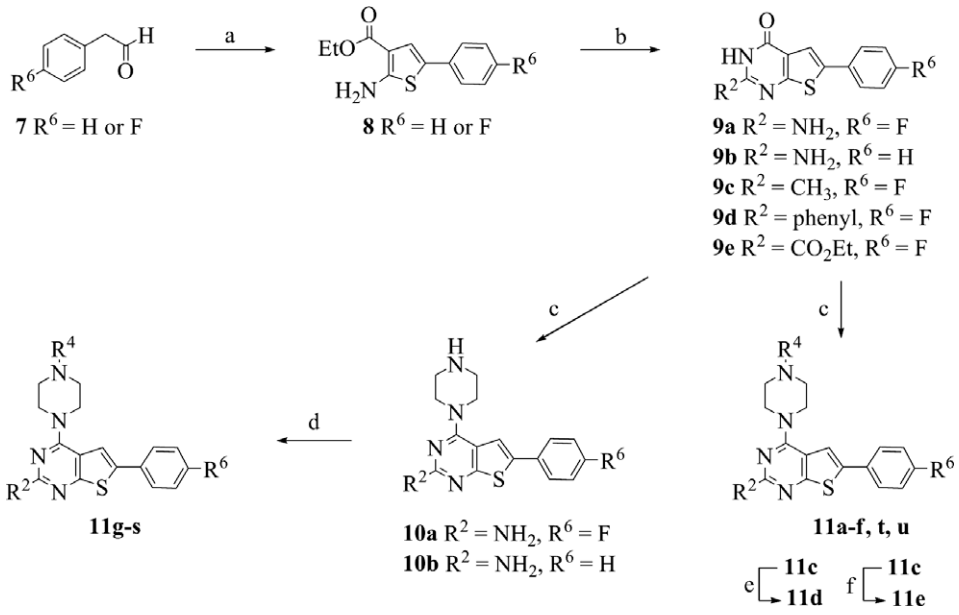
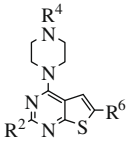


Figure 1.



Scheme 2. Reagents and conditions: (a) ethyl cyanoacetate, S, NEt_3, DMF, 50 °C to rt; (b) RCN, 4 M HCl in dioxane, rt then DMF, 100 °C or chloroformamidine hydrochloride, dimethylsulfone, 130 °C; (c) BOP, DBU, amine, CH_3CN, rt to 60 °C; (d) acid chloride or isocyanate, NEt_3, DMF, rt; (e) 2 M NaOH, MeOH, rt; (f) 7N NH_3 in MeOH, rt.

Table 1
Overview of synthesized compounds and their MLR inhibition data[a]



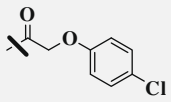
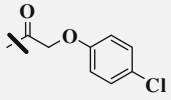
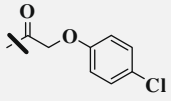
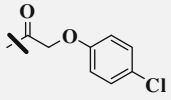
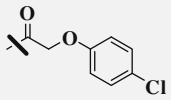
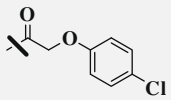
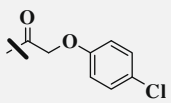
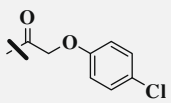
Compd	R^2	R^4	R^6	MLR IC_{50}[a] (μM)
6a	H		F	6.35
6b	*n*-Bu		F	1.33
11a	CH_3		F	3.39
11b	Ph		F	>10
11c	CO_2Et		F	>10
11d	CO_2H		F	>10
11e	$CONH_2$		F	>10
11f	NH_2		F	0.7

Table 1 (*continued*)

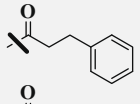
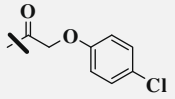
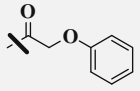
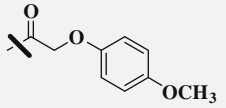
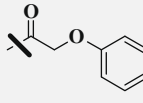
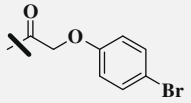
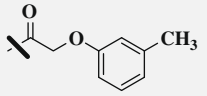
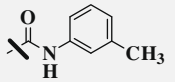
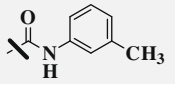
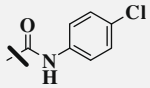
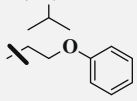
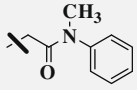
Compd	R^2	R^4	R^6	MLR IC_{50}[a] (μM)
11g	NH_2		F	6.99
11h	NH_2		F	>10
11i	NH_2		F	>10
11j	NH_2		H	0.33
11k	NH_2		H	0.78
11l	NH_2		H	0.072
11m	NH_2		H	0.066
11n	NH_2		H	0.32
11o	NH_2		H	0.21
11p	NH_2		F	2.8
11q	NH_2		H	0.93

Table 1 (*continued*)

Compd	R^2	R^4	R^6	MLR IC_{50}[a] (μM)
11r	NH_2		H	6.7
11s	NH_2		F	8.2
11t	NH_2		H	9.64
11u	NH_2		H	8.12
CsA[b]				0.054

[a] Values are means of two independent experiments.
[b] Cyclosporine A.

the lowest concentration of the compound (expressed in μM) that resulted in a 50% inhibition of the MLR.

Inspection of the SAR at position 2 (substituent R^2 in Table 1) reveals that an amino group is optimal for biological activity (MLR IC_{50} = 0.7 μM). An *n*-butyl group has very similar activity (only twofold less active than the amino congener), whereas a methyl is not far off (fivefold less active than the amino congener). Other substituents (hydrogen, phenyl, carboxylic acid and its amide and ester analogue) are less active, displaying MLR IC_{50} values of 6 μM or more.

By direct comparison of the fluorophenyl (R^6 = F) and phenyl (R^6 = H) congeners, it seems that fluorine substitution has a slightly negative impact on activity. For example, compare the urea derived piperazines **11q** and **11p** with IC_{50} values of 0.93 μM and 2.8 μM, respectively, and the amide derived piperazines **11j** and **11f** with MLR IC_{50} values of 0.33 μM and 0.7 μM, respectively.

The SAR of the piperazine moiety has been extensively investigated. It seems that the second nitrogen atom of the piperazine moiety needs to be nonbasic as compounds in which the second nitrogen atom was basic show much less immunosuppressive activity. Compound **11t** is 12-fold less active than its direct analogue with a carbonyl functionality (compound **11k**). Insertion of a methylene group between the carbonyl moiety and the nitrogen atom of the piperazinyl group affords compound **11u** with a MLR IC_{50} of 8.12 μM. For the urea substituted piperazine derivatives (**11p**–**s**), most potent activity is associated with a *m*-tolylacetamide substituent (compounds **11p** and **11q**). The choice of substituents on the phenyl group is important as the activity of the *p*-chloro-analogue **11r** drops by a factor 7, as compared to the *m*-methyl substituted analogue **11q**. The aliphatic urea (compound **11s**) lacks considerable MLR activity.

In agreement with previous research,[8–10] phenoxyacetyl-piperazine derivatives show potent activities, while a nicotinoyl amide (compound **11i**) and an aliphatic amide (compound **11h**) do not show any activity at 10 μM. The closely related hydrocinnamoyl side chain (compound **11g**) is also less active. To probe the optimal substitution pattern on the phenyl ring of the phenoxyacetyl side chain, a number of analogues were prepared (compounds **11k**–**o**). The unsubstituted congener **11k**, the *p*-bromo analogue **11n**, and the *m*-methyl congener **11o** show comparable activity as *p*-chloro compound **11j**, with MLR IC_{50} between 0.2 and 0.7 μM. The *p*-methoxy (compound **11l**) and the *p*-fluoro (compound **11m**) analogue do show excellent in vitro MLR activity with IC_{50} values of 72 nM and 66 nM, respectively, This is almost as potent as Cyclosporine (IC_{50} = 54 nM), a clinically used immunosuppressive agent.

In summary, the discovery of a new series of immunosuppressive agents based on a thieno[2,3-*d*]pyrimidine scaffold is described. The preliminary SAR studies led to the identification of compound **11m** with an IC_{50} value of 66 nM in the cellular MLR assay, which is equipotent to Cyclosporine. These compounds represent a valid starting point for a new generation of immunosuppressive drugs.

References and notes

1. (a) Golshayan, D.; Buhler, L.; Lechler, R. I.; Pascual, M. *Transplant. Int.* **2007**, *20*, 12; (b) Lechler, R.; Sykes, M.; Thomson, A. W.; Turka, L. A. *Nat. Med.* **2005**, *6*, 605.
2. Pratschke, J.; Tullius, S. G.; Neuhaus, P. *Graft* **2002**, *5*, 338.
3. Gummert, J. F.; Ikonen, T.; Morris, R. E. *J. Am. Soc. Nephrol.* **1999**, *10*, 1366.
4. Allison, A. C. *Immunopharmacology* **2000**, *47*, 63.
5. Smith, J. M.; Nemeth, T. L.; McDonald, R. A. *Pediatr. Clin. North. Am.* **2003**, *50*, 1283.
6. (a) O'Shea, J. J.; Pesu, M.; Borie, D. C.; Changelian, P. S. *Nat. Rev. Drug Disc.* **2004**, *3*, 555; (b) Hong, J. C.; Kahan, B. D. *J. Semin. Nephrol.* **2000**, *20*, 108.
7. Vincenti, F.; Kirk, A. D. *Am. J. Transplant.* **2008**, *8*, 1972.
8. (a) Shen, C.; Dillissen, E.; Kasran, A.; Lin, Y.; Herman, J.; Sienaert, I.; De Jonghe, S.; Kerremans, L.; Geboes, K.; Boon, L.; Rutgeerts, P.; Ceuppens, J. *Clin. Immunol.* **2007**, *122*, 53; (b) Waer, M.; Herdewijn, P.; De Jonghe, S.; Marchand, A.; Gao, L. J. WO2005/039587.
9. De Jonghe, S.; Herdewijn, P.; Gao, L. J. WO2006/087229.
10. (a) De Jonghe, S.; Dolusic, E.; Gao, L. J.; Herdewijn, P.; Pfleiderer, W. WO2006/069805.; (b) De Jonghe, S.; Dolusic, E.; Gao, L. J.; Herdewijn, P.; Pfleiderer, W. WO2009/003669.
11. Waer, M.; Vanrenterghem, Y.; Van der Schueren, E.; Michielsen, P.; Vandeputte, M. *Transplant. Proc.* **1987**, *19*, 1570.
12. Kino, T.; Hatanaka, H.; Hashimoto, M.; Nishiyama, M.; Goto, T.; Okuhara, M.; Kohsaka, M.; Aoki, H.; Imanaka, H. *J. Antibiot.* **1987**, *40*, 1249.
13. Munchhof, M. J.; Beebe, J. S.; Casavant, J. M. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 21.
14. El-Bahaie, S.; Assy, M. G.; Ibrahim, Y. A. *Sulfur Lett.* **1988**, *8*, 199.
15. 2-(4-Chlorophenoxy)-1-(piperazin-1-yl)ethanone was prepared from *tert*-butyl piperazine-1-carboxylate by the reaction with 4-chlorophenoxyacetyl chloride and triethylamine in dichloromethane followed by acidic cleavage of Boc group by treatment of trifluoroacetic acid in dichloromethane.
16. Donkor, I. O.; Li, H.; Queener, S. F. *Eur. J. Med. Chem.* **2003**, *38*, 605.
17. (a) Wan, Z. K.; Wacharasindhu, S.; Levins, C. G.; Lin, M.; Tabei, K.; Mansour, T. S. *J. Org. Chem.* **2007**, *72*, 10194; (b) Wan, Z. K.; Wacharasindhu, S.; Binnun, E.; Mansour, T. S. *Org. Lett.* **2006**, *8*, 2425.