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Original article

Cyclic nucleotide phosphodiesterase type 4 inhibitors: Evaluation of pyrazolo[1,5-*a*]-1,3,5-triazine ring system as an adenine bioisostere

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Abstract

A series of 8-substituted pyrazolo[1,5-*a*]-1,3,5-triazines were considered as a bioisosteric replacement for the 9-substituted adenine derivatives resulting in the discovery of 8-(2-methoxybenzyl)-4-(*N*-methylamino)-2-*n*-propylpyrazolo[1,5-*a*]-1,3,5-triazine (**14d**) and 2-trifluoromethyl-8-(2-methoxybenzyl)-4-(*N*-methylamino)pyrazolo[1,5-*a*]-1,3,5-triazine (**14e**) as a new structural class of potent phosphodiesterase type 4 inhibitors ($IC_{50} = 13$ nM and 11 nM, respectively) with high isoenzyme selectivity. An original tandem of reactions involving a palladium-mediated cross-coupling reaction (PMCCR) of the readily available 8-iodo-2-methyl-4-(*N*-methyl-*N*-phenylamino)pyrazolo[1,5-*a*]-1,3,5-triazine (**11a**) and arylboronic acids or alkynes followed by the displacement of the *N*-methyl-*N*-phenylamino group constitute the key steps in a novel synthetic approach developed herein. The treatment of **11a–c** with *n*-BuLi and selected aldehydes represents an interesting alternative to the PMCCR for the synthesis of benzylic derivatives **14a–i**. Preliminary biological testing has shown that compounds **14d** and **14e** strongly inhibit LPS-induced TNFα release from human mononuclear cells from healthy subjects. These two compounds were selected for further biological evaluation.

Keywords: Bioisostere; Pyrazolo[1,5-*a*]-1,3,5-triazines; Phosphodiesterase; Type 4; TNFα; Inflammation

1. Introduction

Cyclic AMP phosphodiesterases (PDE4), responsible for the hydrolysis of key second messenger cyclic AMP (cAMP), mediate a wide array of activities and are particularly abundant in airway smooth muscle, and in inflammatory and immune cells, where PDE4s regulate the production of inflammatory mediators, cytokines and reactive oxygen species [1,2]. These findings have led to intense efforts in the last few years to identify highly potent PDE4 inhibitors as promising treatment for autoimmune and inflammatory diseases [1,3,4]. Moreover, several PDE4 inhibitors have demonstrated clinical efficacy [4]. Unfortunately, the development of pioneer PDE4 inhibitors, such as the archetypal rolipram (**1**, Fig. 1) and structurally-related compounds (e.g. Ariflo, **2**) has been hampered by their propensity to induce various side effects, such as nausea, emesis, gastric acid secretion, or central nervous system activation [2,4–7]. As a result, these compounds suffered from a limited therapeutic index [8]. Thus, the design of novel, potent and selective second-generation PDE4

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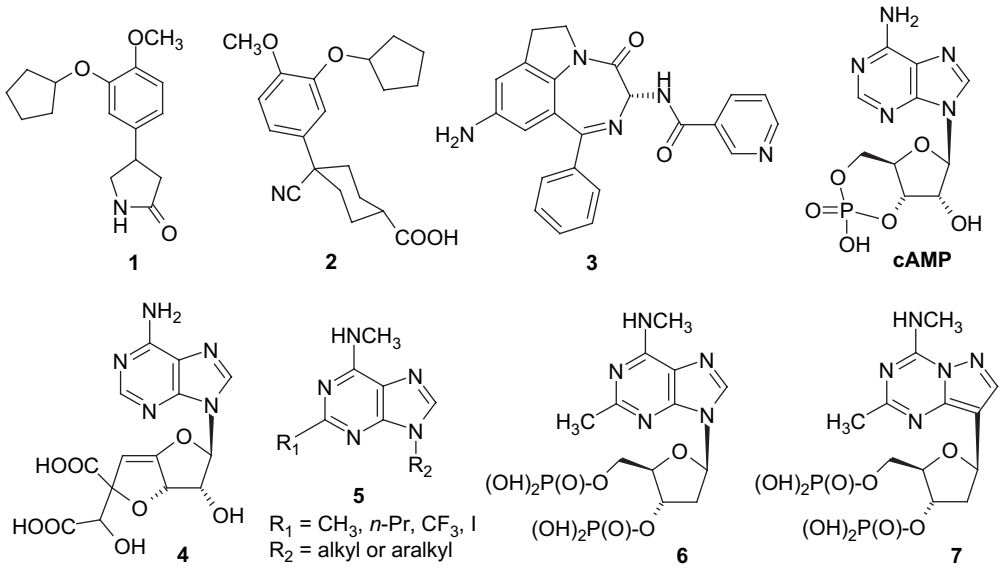

Fig. 1.

inhibitors with reduced emetogenic properties represents a critical need and is still a challenge in the pharmaceutical industry [4].

It was initially proposed that rolipram produced side effects via the binding to a high affinity binding site (HARBS) distinct from the catalytic site of PDE4 [9]. It has now been clarified that the HARBS corresponds to the holoenzyme conformer of the PDE4 [10], and that the emetic response is a consequence of inhibition of PDE4, especially the PDE4D, in nontarget tissues [11]. Therefore, two ways to obtain potent selective PDE4 inhibitors with an improvement in the therapeutic index are the following: (i) to develop new compounds selective toward one subtype, especially the PDE4A and C [12] and/or (ii) with original chemical structures, which are completely unrelated to catechol ether derivatives (e.g. rolipram) [9]. The utility of the second approach has been successfully demonstrated by the recently reported benzodiazepine **3** (CI-1044) [13], which is well tolerated by ferrets receiving **3** at a dose of 40 mg kg^{-1} i.v., whereas **2** was emetic at 10 mg kg^{-1} i.v. in the same model [4]. Another method to obtain a potent and specific inhibitor that binds the PDE4 in the catalytic site with improved margin of safety is to start from the structure of the natural substrate (cAMP). The natural product griseolic acid (**4**) was one of the first reported substrate-related PDE4 inhibitors [14]. Moreover, several years ago, we reported that adenine derivatives substituted at position 9 (**5**, Fig. 1) strongly and selectively inhibited PDE4 and elicited anti-inflammatory properties [15,16]. The fact that this series of PDE4 inhibitors did not stimulate the in vivo gastric acid secretion in rats suggests that they may produce fewer gastrointestinal side effects [16].

However, since 9-substituted adenine derivatives have been extensively studied, it is very difficult to patent such compounds. One way to overcome this difficulty is to transfer the structure–activity relationships from the purine ring to a novel bioisosteric heterocycle while maintaining or improving the therapeutic interest over the parent adenines [17,18].

We have recently reported that the pyrazolo[1,5-*a*]-1,3,5-triazine C-nucleoside (**7**) as a potent purinergic $P2Y_1$ receptor antagonist with enhanced metabolic stability compared to the parent purine derivative (**6**) [19]. In view of this promising result, we sought to apply the same strategy to the synthesis of C-analogues of 9-substituted N^6-methyladenines **5** as PDE4 inhibitors. In this article, we report the details of the synthetic studies which have successfully led to the preparation of novel pyrazolo[1,5-*a*]-1,3,5-triazines (**19**, **14a–i**, **16a**,**b**, **18a–k**) as potent and selective PDE4 inhibitors. Subsequently, the inhibition of TNFα release from mononuclear cells stimulated with lipopolysaccharide (LPS) was also evaluated.

2. Semi-empirical calculations

In order to assess the similarity of the two scaffolds, compounds **8** and **9** were first sketched in the Sybyl 6.8 package [20] and minimized with the Tripos force field (Fig. 2) [21]. Then, a subsequent geometry optimization with the AM1 parameterization was performed [22] in Mopac [23] module of Sybyl, with default parameters. Connolly surfaces were calculated with Molcad, and electrostatic potential properties were mapped onto them. Fig. 2 shows the surface of each scaffold colored by electrostatic potential values as determined from the final minimized structures. The electrostatic potential surfaces of the two scaffolds (pyrazolotriazine **9** and adenine **8**) were found to be strikingly similar. Moreover, the amount of surface area corresponding to the negative value is very close (136 $Å^2$ for **8** and 137 $Å^2$ for **9**).

In view of these promising preliminary results obtained by comparing the two bicyclic compounds **8** and **9**, we decided to compare the charges (Fig. 3) and isopotential curve (Fig. 4) properties [24–28] of the adenine **20d** and its pyrazolo[1,5-*a*]-1,3,5-triazine analogue **14d**. Details of the calculations

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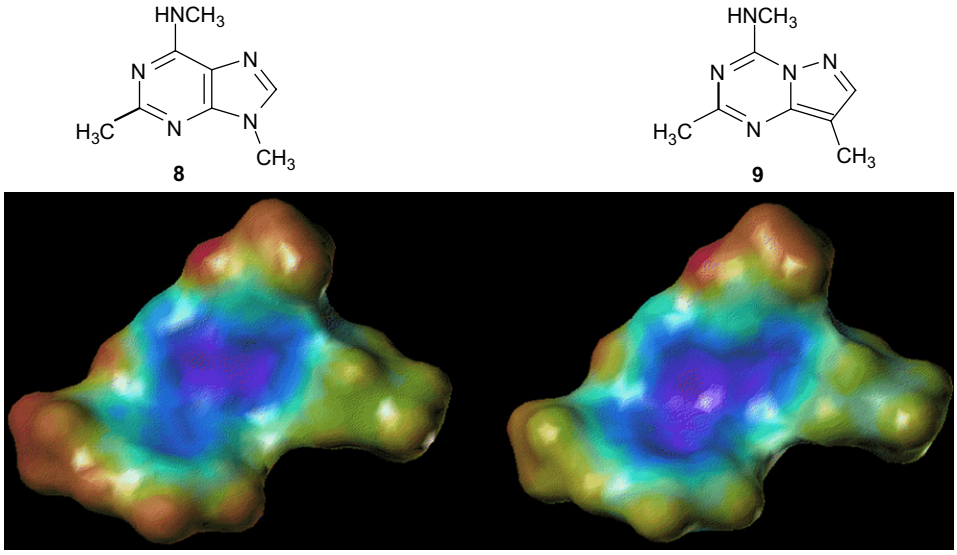

Fig. 2. Electrostatic potential: blue colors represent negative values while red colors positive values. Values close to zero are colored in green (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

are presented in the Section 6. Despite the existence of small differences, these two heterocycles possess common features and are structurally very similar. These computational studies prompted the synthesis of novel pyrazolo[1,5-*a*]-1,3,5-triazines substituted in position 8 (**19**, **14a–i**, **16a,b**, **18a–k**), which were tested as PDE4 inhibitors that were expected to act at the catalytic site of the PDE4 with the same pharmacophoric contact points as adenines **5**.

3. Chemistry

The synthetic strategies used for the preparation of the target pyrazolo[1,5-*a*]-1,3,5-triazines are summarized in Figs. 5 and 6. The starting materials, *N*-methyl-*N*-phenylamino derivatives **11a–c**, were prepared as previously reported [19,30] by treatment of the corresponding pyrazolo[1,5-*a*]-1,3,5-triazin-4-ones (**10a–c**) with phosphorus oxychloride and dimethylaniline under high pressure, followed by the regioselective iodination at the 8-position by *N*-iodosuccinimide. Introduction of substituents and functions at the 8-position was then carried out by two novel and versatile methods, both employing the suitable *N*-methyl-*N*-phenylamino (NMNPA) activating group, which possesses a number of favorable characteristics including stability, ease of handling, and reactivity [19,30]. The first process (Fig. 5) utilizes the lithiation of key 8-iodo intermediates **11a–c** with *n*-BuLi and subsequent treatment with selected aldehydes, followed by reduction of the hydroxy group of intermediates **12b–j** leading to desired 8-substituted derivatives **13a–i** in reasonably good yields (40–93%). Subsequent displacement of the NMNPA group with methylamine provided the target pyrazolotriazines **19**, **14a–i** (Table 1, 53–87% yield). The alternative route to 8-substituted pyrazolotriazines **16a–j** utilizes an original tandem of reactions involving a palladium-mediated cross-coupling

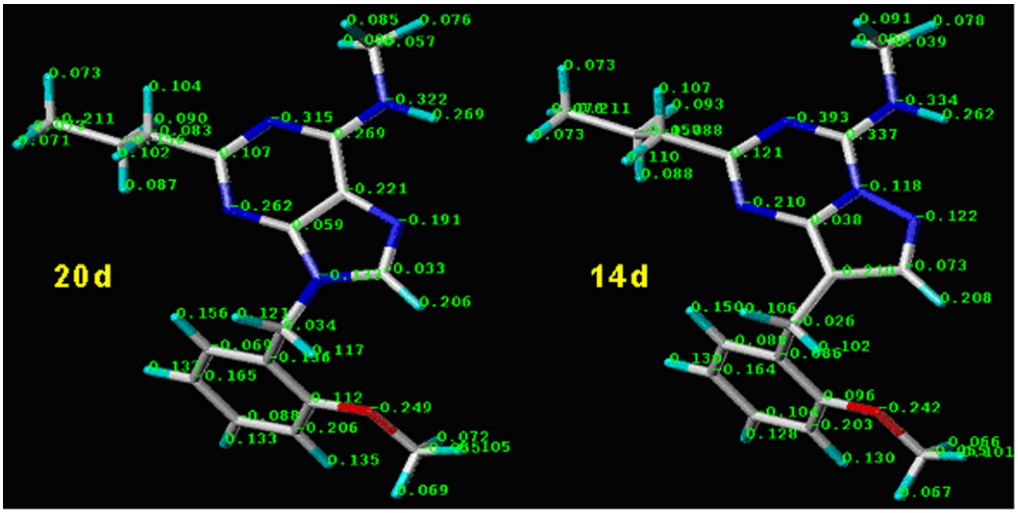

Fig. 3. Mopac charges as calculated with AM1 and labelled on heavy atoms for **20d** (right) and **14d** (left).

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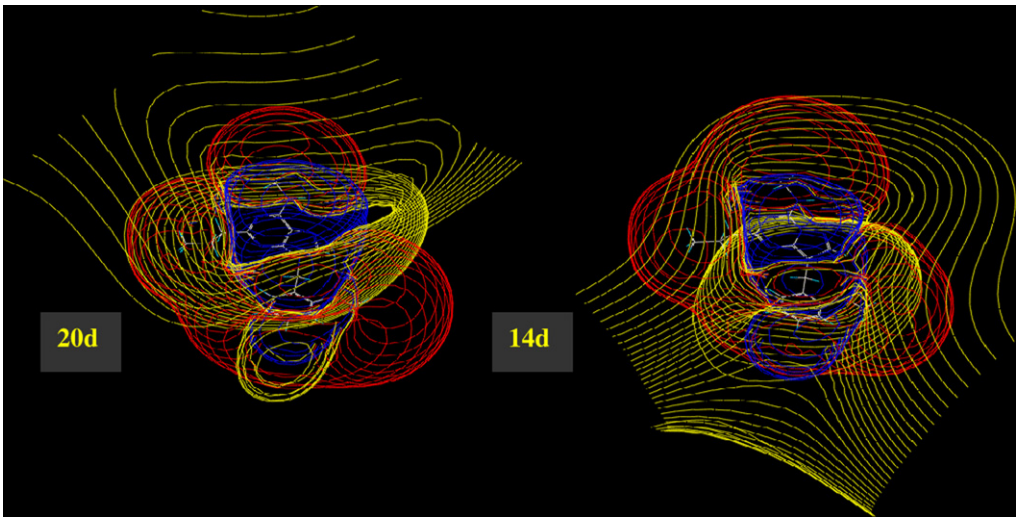

Fig. 4. Isopotential curves for **20d** (right) and **14d** (left): red corresponds to positive values, blue to negative values, and yellow is neutral (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

reaction of the readily available 8-iodo-2-methyl-4-(*N*-methyl-*N*-phenylamino)pyrazolo[1,5-*a*]-1,3,5-triazine (**11a**) and alkynes or boronic acids leading to intermediates **15a**,**b** and **17a**–**j** in 26–87% yield (Fig. 6). Subsequent displacement of the NMNPA moiety with methylamine [19,30] afforded final compounds **16a**,**b** and **18a**–**k** (Table 1, 21–95% yield).

4. Pharmacology, results and discussion

The 8-substituted pyrazolo[1,5-*a*]-1,3,5-triazines were screened for their activity against PDE4 as described previously [15]. Results are summarized in Table 1. As reported previously, a single substitution with a methyl group on the exocyclic nitrogen atom at position 6 of the adenine ring (corresponding to position 4 of the pyrazolotriazine ring) was optimal [29]. Thus, the study was performed with *N*-methyl derivatives. Comparison of adenines and their corresponding pyrazolo[1,5-*a*]-1,3,5-triazines revealed that the structure–activity relationships are very similar in both series. Recently, we reported that the introduction of a *n*-propyl or a trifluoromethyl group at position 2 of the adenine ring strongly increased the potency of the resulting compounds (compare **20a** with **20b**, **20c** with **20d** and **20e**) [29]. In a similar manner, when compared to **14a** ($IC_{50} = 98$ nM), the *n*-propyl and trifluoromethyl derivatives (**14b** and **14c**, respectively) were found to be almost two fold more potent ($IC_{50} = 48$ nM and 77 nM, respectively). Moreover, the 2-methoxybenzyl group at position 9 of the adenine (corresponding to the position 8 of the pyrazolotriazine) appeared to be especially beneficial in both series, and increased by —four to eight fold the potency of the resulting compound, when compared to the benzyl derivatives (compare **20a** with **20c**, **20b** with **20d**, **14b** with **14d**, and **14c** with **14e**). Although the 2-furylmethyl (**14g**, $IC_{50} = 78$ nM) and 2-thienylmethyl (**14h**, $IC_{50} = 70$ nM) compounds were found to be slightly more potent than the benzyl derivative (**14a**, $IC_{50} = 98$ nM), all other substitutions at position 8 of the 2-methylpyrazolotriazine

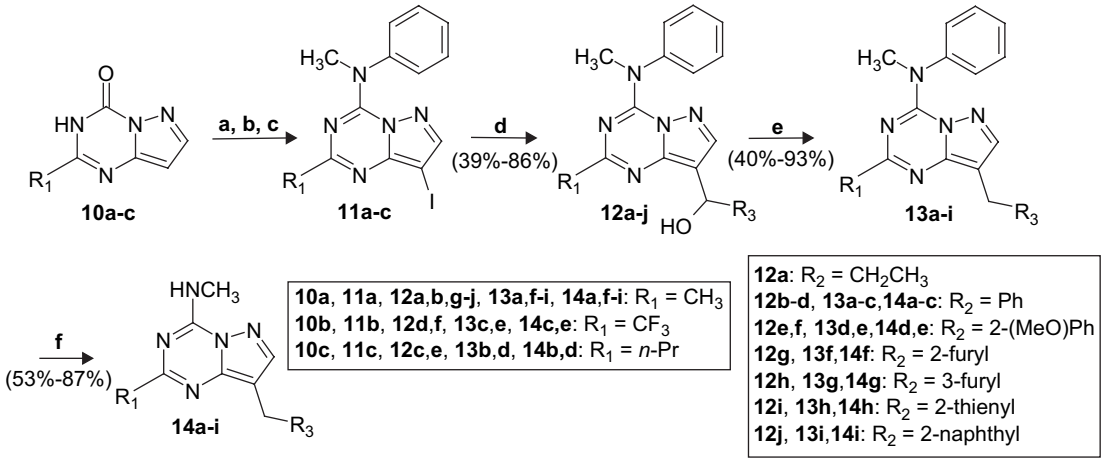

Fig. 5. (a) $POCl_3$, DMAP, $CHCl_3$, 120 °C, sealed tube, (b) *N*-methylaniline, CH_2Cl_2, 25 °C, (c) NIS, $CHCl_3$, reflux, (d) *n*-BuLi, R_3CHO, THF, −78 °C, (e) TMSCl, NaI, CH_3CN, (f) NH_2CH_3, EtOH, 100 °C, sealed tube.

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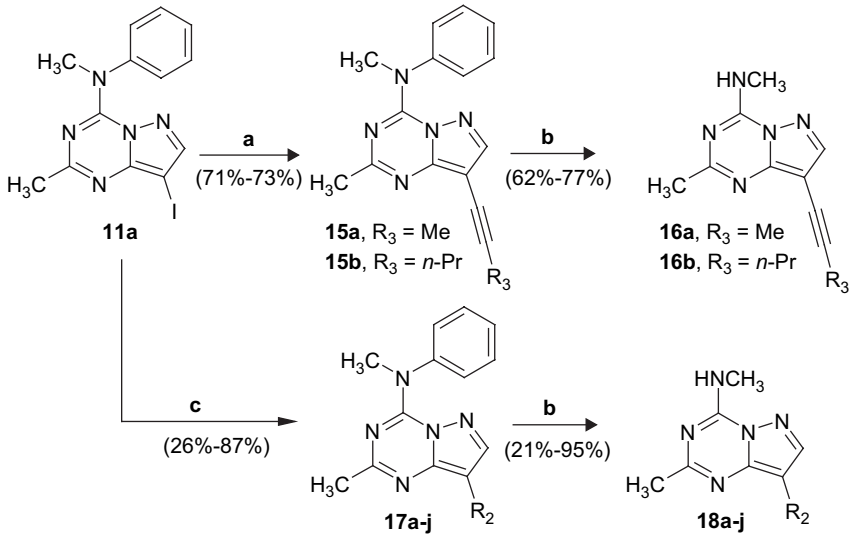

	R2		R2
17a, 18a	Ph	**17f, 18f**	2-furyl
17b, 18b	2-ClPh	**17g, 18g**	2-thienyl
17c, 18c	3-acetylPh	**17h, 18h**	1-naphthyl
17d, 18d	3-NO2Ph	**17i, 18i**	2-benzo[b]furyl
17e, 18e	3-CF3Ph	**17j, 18j**	2-benzo[b]thienyl

Fig. 6. (a) R_3–C≡CH, CuI, $PdCl_2$, TEA, CH_3CN, 25 °C, (b) NH_2CH_3, EtOH, 100 °C, sealed tube, (c) $R_2B(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, toluene, EtOH, H_2O, 90 °C.

were found to be detrimental to the PDE4 inhibitory activity. Finally, combining beneficial substituent effects in both positions R_1 and R_2 led to new compounds with increased potencies. Thus, synergistic effects were observed when the 9-(2-methoxybenzyl) moiety was introduced in derivatives with the most appropriate substituent in position 2. In particular, the 2-*n*-propylpyrazolotriazine (**14d**) exhibited almost the same activity as that of the corresponding adenine **20d** ($IC_{50} = 13$ nM and 7 nM, respectively). However, the beneficial effect of the 2-trifluoromethyl substitution was only partially recovered for the pyrazolotriazine **14e** ($IC_{50} = 11$ nM) when compared to the parent adenine **20e** ($IC_{50} = 1.4$ nM). Interestingly, this novel series of pyrazolotriazine derivatives possess improved PDE4 selectivity profiles vs PDE1, -2, -3, and -5 as demonstrated with compounds **14a**, **14b**, **14d**, **14e**, **18a**, and **18k** (Table 2).

With the above promising data, we selected compounds **14c–e** for further evaluation of their in vitro anti-inflammatory activities by examining the suppression of TNFα release by lipopolysaccharide (LPS)-activated peripheral blood mononuclear cells from healthy donors (Fig. 7). These compounds elicited a strong concentration-dependent inhibition of the TNFα release. However, we found no direct correlation between the activity of the compounds **14c–e** in PDE4 inhibition and TNFα inhibition. These findings might be ascribed to the difference of physicochemical properties (cell permeability, water solubility) between these compounds or may suggest that accumulation of cAMP is a major way to decrease TNFα secretion but certainly not the only one.

5. Conclusion

The main objective of the present study was the bioisosteric replacement of the adenine ring by the pyrazolo[1,5-*a*]-1,3,5-triazine in the field of PDE4 inhibitors. Thus, a series of 8-substituted derivatives were synthesized using very efficient and particularly attractive routes involving a palladium-mediated cross-coupling reaction of the readily available 8-iodo-2-methyl-4-(*N*-methyl-*N*-phenylamino)pyrazolo[1,5-*a*]-1,3,5-triazines (**11a**) and arylboronic acids or alkynes, or by the treatment of **11a–c** with *n*-BuLi and selected aldehydes. The *N*-methyl-*N*-phenylamino group was found to be particularly stable under these conditions and was readily displaced at the end of the reaction sequence with methylamine to give the target compounds **14a–i**, **16a**,**b**, **18a–k**, and **19**. As anticipated from computational analysis, we have demonstrated that this series of pyrazolotriazine analogues **14a–i**, **16a**,**b**, **18a–k**, and **19** are pharmacologically bioequivalent to the parent 9-substituted adenines **20a–e** and exhibit similar structure–activity relationships. Among these novel derivatives, 8-(2-methoxybenzyl)-4-methylamino-2-*n*-propylpyrazolo[1,5-*a*]-1,3,5-triazine (**14d**) and 2-trifluoromethyl-8-(2-methoxybenzyl)-4-methylaminopyrazolo [1,5-*a*]-1,3,5-triazine (**14e**) emerged as the most potent PDE4 inhibitors with IC_{50} values of 11 nM and 13 nM, respectively, which was 100 fold more potent than rolipram (1.2 μM) and 2.5 fold when compared to Ariflo (30 nM). Additionally, preliminary testing has shown that both **14d** and **14e** strongly inhibit cytokine production in LPS-induced human blood

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Table 1

R_1	R_2	Compd	PDE4 IC_{50}[a] (μM)	Compd	PDE4 IC_{50}[a] (μM)
CH_3	$CH(OH)C_2H_5$	–	–	**19**	4.8
CH_3	Bn	**20a**, [29]	0.28	**14a**	0.098
n-C_3H_7	Bn	**20b**, [29]	0.088	**14b**	0.048
CF_3	Bn	–	–	**14c**	0.077
CH_3	2-(MeO)Bn	**20c**, [29]	0.061	–	–
n-C_3H_7	2-(MeO)Bn	**20d**, [29]	0.007	**14d**	0.013
CF_3	2-(MeO)Bn	**20e**, [29]	0.0014	**14e**	0.011
CH_3	Furfuryl	–	–	**14f**	0.10
CH_3	3-Furylmethyl	–	–	**14g**	0.078
CH_3	2-Thienylmethyl	–	–	**14h**	0.070
CH_3	2-Naphthylmethyl	–	–	**14i**	0.27
CH_3	1-Propynyl	–	–	**16a**	6.4
CH_3	1-Pentynyl	–	–	**16b**	0.41
CH_3	Ph	–	–	**18a**	0.27
CH_3	2-ClPh	–	–	**18b**	2.9
CH_3	3-Acetylph	–	–	**18c**	0.54
CH_3	3-NO_2Ph	–	–	**18d**	1.2
CH_3	3-CF_3Ph	–	–	**18e**	0.13
CH_3	2-Furyl	–	–	**18f**	3.1
CH_3	2-Thienyl	–	–	**18g**	2.4
CH_3	1-Naphthyl	–	–	**18h**	0.21
CH_3	2-Benzo[*b*]furyl	–	–	**18i**	6.2
CH_3	2-Benzo[*b*]thienyl	–	–	**18j**	3.5
CH_3	Bz	–	–	**18k**, [30]	0.12

[a] The IC_{50} was calculated by linear regression (correlation coefficient $r = 0.95$) and represents the mean value of three determinations; the experimental error is about 15%.

mononuclear cell preparations. Therefore, compounds **14d** and **14e** were selected for further biological evaluation.

As demonstrated in our previous work on $P2Y_1$ receptor antagonists, the results presented in this study indicate that the nitrogen in position 9 of adenine is unnecessary for activity in both systems and appeared as a point of anchorage for the N9 substituent. Moreover, the replacement of the purine ring with the pyrazolotriazine would provide an increased in

Table 2
IC_{50}[a] or % of inhibition at 10 μM

Compd	PDE1		PDE2		PDE3 (%)	PDE5
	−CaM (%)	+CaM	−GMPc (%)	+GMPc		
14a	37	14 μM	19	56 μM	8.6	33
14b	24	25	20	20	10	16
14d	27	35	19	31	16	38
14e	20	–	16	–	24	74
18a	21	20	3.6	11	2.7	11
18k	16	29	15	17	11	8

[a] The IC_{50} was calculated by linear regression (correlation coefficient $r = 0.95$) and represents the mean value of three determinations; the experimental error is about 15%.

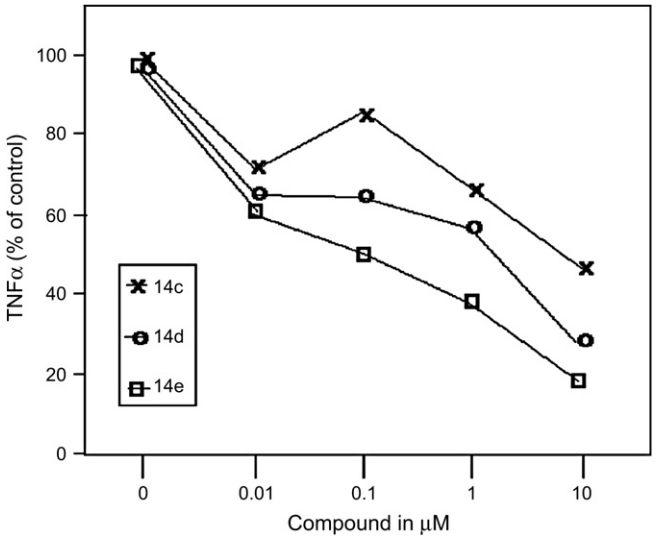

Fig. 7. Suppression of TNFα release by LPS-activated peripheral blood mononuclear cells from healthy donors.

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vivo stability when compared to the parent adenines [19]. Thus, pyrazolo[1,5-*a*]-1,3,5-triazine does appear to serve as a valuable potent ''carbabioisostere'' for the purine nucleus present in various biologically active molecules.

6. Experimental section

6.1. Chemical synthesis

6.1.1. General

Reagents used for the synthesis were purchased from Sigma-Aldrich (Isle d'Abeau Chesnes, France) and Lancaster (Bischheim-Strasbourg, France). With the exception of THF and Et_2O, all solvents were obtained from commercial suppliers and used without further purification. These two solvents were freshly distilled from sodium benzophenone ketyl. Flash chromatography was performed on Geduran® Silica gel Si 60 (40–63 μm, Merck). Thin-layer chromatography was carried out using Silica gel 60 F_{254} plates (Merck). The spots were visualized either under UV light ($\lambda = 254$ nm) or by spraying with molybdate reagent (H_2O/concentrated H_2SO_4/$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$/$(NH_4)_2$-$Ce(SO_4)_4\cdot 2H_2O$, 90:10:25:1, v/v/w/w) and charring at 140 °C for a few minutes. All chemical yields are unoptimized and generally represent the result of a single experiment.

^{1}H NMR spectra were recorded on a Bruker AC 200 (200 MHz) or a Bruker DPX 300 (300 MHz) spectrophotometer at room temperature. Chemical shifts are given in ppm (δ), coupling constants (J) are in Hertz (Hz) and signals are designated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; quint., quintuplet; m, multiplet; br s, broad singlet; etc.

The mass spectra were obtained on a Mariner API-TOF.

Melting points were determined with a Mettler FP62 apparatus and are uncorrected. Elemental analyses were performed by the CNRS department of microanalysis (CNRS, Vernaison, France) and are indicated only by the elemental symbols within ±0.4% of the theoretical values unless otherwise noted.

6.1.2. 2-Trifluoromethylpyrazolo[1,5-a]-1,3,5-triazin-4-one (**10b**)

To a stirred solution of 3-aminopyrazole (1.18 g, 14.2 mmol) in acetonitrile (15 mL) at room temperature was added *S*-*p*-chlorophenyltrifluorothioacetimidate (3.4 g, 14.2 mmol). After 5 min of stirring at 20 °C, acetic acid (812 μL, 14.2 mmol) was added dropwise under argon. After 8 h, the solvent was evaporated to dryness and the residue was triturated with ether (5 mL) and hexane (30 mL), filtered and washed successively with hexane and water to yield *N*-(pyrazol-3-yl)trifluoroacetamidine (2.35 g, 93%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.38 (d, $J = 2.4$, 1H), 7.51 (d, $J = 2.4$, 1H); *m/z* 201 $(M + Na)^+$.

Sodium ethoxide (1.0 M in EtOH, 5.45 mL) was slowly added, at room temperature, to a stirred solution of *N*-(pyrazol-3-yl)trifluoroacetamidine (178 mg, 1.0 mmol) and diethyl carbonate (605 μL, 5.45 mmol) in anhydrous ethanol (5 mL). The resulting mixture was heated at reflux for 5 h under argon. After the mixture was cooled to room temperature, the solvent was evaporated in vacuo. The residue was diluted with ice-cold water (7 mL) and the pH was adjusted to 7 with HCl 1 N. After 2 h at 0 °C, the precipitate was collected by filtration and washed with water, ethanol and ether. Recrystallization from ethanol/ether gave compound **10b** (182 mg, 89%) as a colorless solid: mp > 300 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ 6.20 (d, $J = 1.8$, 1H), 7.79 (d, $J = 1.8$, 1H); *m/z* 205 $(M + H)^+$.

6.1.3. 2-Trifluoromethyl-8-iodo-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**11b**)

A solution of **10b** (1.0 g, 4.87 mmol), 4-(*N*,*N*-dimethylamino)pyridine (2.90 g, 23.7 mmol), and phosphorus oxychloride (8 mL, 107 mmol) was refluxed for 2 h. After the solution was cooled to room temperature, the excess of phosphorus oxychloride was removed under reduced pressure. Then, the residue was redissolved in CH_2Cl_2 (40 mL), and *N*-methylaniline (1.5 mL, 11.8 mmol) was added dropwise with stirring at 0 °C under argon. After 10 min, the reaction mixture was allowed to warm to room temperature. After evaporation of the solvent, the residue was chromatographed on silica (EtOAc/hexanes, 1:1) to give 2-trifluoromethyl-4-(*N*-methyl-*N*-phenylamino)-pyrazolo[1,5-*a*]-1,3,5-triazine (1.27 g, 88%), as a white powder: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.82 (s, 3H, CH_3), 6.54 (d, $J = 2.2$, 1H, 8-H), 7.21–7.24 (m, 2H, ArH), 7.42–7.44 (m, 3H, ArH), 7.81 (d, $J = 2.2$, 1H, 7-H); *m/z* 296 $(M + H)^+$.

To a solution of 2-trifluoromethyl-4-(*N*-methyl-*N*-phenylamino)pyrazolo[1,5-*a*]-1,3,5-triazine (1.23 g, 4.18 mmol) in dry chloroform (100 mL) was added *N*-iodosuccinimide (1.33 g, 5.91 mmol), and the mixture was refluxed for 0.5 h. After the mixture was cooled at room temperature, the solvent was evaporated in vacuo. Then, the residue was partitioned between dichloromethane (100 mL) and water (70 mL). The aqueous layer was extracted twice with additional dichloromethane (2 × 50 mL). The combined organic phases was washed with 10% sodium bisulfite (70 mL). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The crude material was purified by column chromatography on silica (EtOAc/CH_2Cl_2/hexanes, 2:3:5). Recrystallization from ethanol yielded compound **11b** (1.42 g, 81%) as colorless crystals: mp 155 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.81 (s, 3H, CH_3), 7.19–7.24 (m, 2H, ArH), 7.40–7.45 (m, 3H, ArH), 7.79 (s, 1H, 7-H); *m/z* 420 $(M + H)^+$.

6.1.4. 8-Iodo-4-(N-methyl-N-phenylamino)-2-n-propylpyrazolo[1,5-a]-1,3,5-triazine (**11c**)

Prepared from 4-(*N*-methyl-*N*-phenylamino)-2-*n*-propyl-pyrazolo[1,5-*a*]-1,3,5-triazine (**10c**) [30] using the procedure described for **11b**. Compound **11c** was obtained (76%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.03 (t, $J = 7.3$, 3H, CH_3), 1.85–1.89 (m, 2H, CH_2), 2.80 (t, $J = 7.3$, 2H, CH_2), 3.73 (s, 3H, CH_3), 7.13–7.44 (m, 5H, ArH), 7.68 (s, 1H, 7-H); *m/z* 394 $(M + H)^+$.

*6.1.5. 8-(1-Hydroxypropyl)-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**12a**)*

A solution of *n*-BuLi (15% in hexane, 220 μL, 0.52 mmol) was slowly added under nitrogen at −78 °C, to a stirred solution of **11a** [19] (157 mg, 0.43 mmol) in anhydrous THF (25 mL). After 5 min, propionaldehyde (93 μL, 1.29 mmol) was added and the reaction mixture was allowed to warm up to 0 °C, then quenched with acetic acid (50 μL). The mixture was diluted with ethyl acetate, washed with water, dried (Na_2SO_4), and concentrated to dryness under reduced pressure. Chromatography on silica (AcOEt/hexane, 1:1) afforded the desired product **12a** (50 mg, 39%) as a white powder. ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.96 (t, $J = 7.5$, 3H, CH_3), 1.82−1.94 (m, 2H, CH_2), 2.53 (s, 3H, CH_3), 3.08 (d, $J = 3.3$, 1H, OH), 3.72 (s, 3H, CH_3), 4.89−4.95 (m, 1H, CH), 7.16−7.20 (m, 2H, ArH), 7.27−7.42 (m, 3H, ArH), 7.65 (s, 1H, 7-H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 10.41, 26.01, 31.11, 42.42, 67.82, 111.06, 126.49, 127.43, 129.36, 143.10, 145.11, 148.74, 149.40, 162.37; *m/z* 298 $(M + H)^+$.

*6.1.6. 8-[(Hydroxy)(phenyl)methyl]-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**12b**)*

Prepared from **11a** [19] and benzaldehyde using the procedure described for **12a**. Compound **12b** was obtained (82%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.57 (s, 3H, CH_3), 3.72 (s, 3H, CH_3), 3.74 (br s, 1H, OH), 6.14 (d, $J = 1.7$, 1H, CH), 7.13−7.45 (m, 11H, ArH + 7-H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 25.95, 42.44, 68.30, 111.28, 126.61, 126.67, 127.52, 127.81, 128.73, 129.33, 143.79, 143.98, 144.99, 148.70, 149.33, 162.87; *m/z* 346 $(M + H)^+$.

*6.1.7. 8-[(Hydroxy)(phenyl)methyl]-4-(N-methyl-N-phenylamino)-2-n-propylpyrazolo[1,5-a]-1,3,5-triazine (**12c**)*

Prepared from **11c** and benzaldehyde using the procedure described for **12a**. Compound **12b** was obtained (83%) as a white powder: ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.03 (t, $J = 7.5$, 3H, CH_3), 1.88−1.90 (m, 2H, CH_2), 2.81 (t, $J = 7.5$, 2H, CH_2), 3.73 (s, 3H, CH_3), 3.74 (br s, 1H, OH), 6.12 (d, $J = 1.7$, 1H, CH), 7.13−7.45 (m, 11H, ArH + 7-H).

*6.1.8. 2-Trifluoromethyl-8-[(hydroxy)(phenyl)methyl]-4-(N-methyl-N-phenylamino)pyrazolo [1,5-a]-1,3,5-triazine (**12d**)*

Prepared from **11b** and benzaldehyde using the procedure described for **12a**. Compound **12d** was obtained (86%) as a white powder: ^{1}H NMR (200 MHz, DMSO-d_6) δ 3.76 (s, 3H, CH_3), 5.91 (d, $J = 4.4$, 1H, CH), 5.99 (d, $J = 4.4$, 1H, OH), 7.21−7.45 (m, 10H, ArH), 7.94 (s, 1H, 7-H); *m/z* 400 $(M + H)^+$.

*6.1.9. 8-[(Hydroxy)(2-methoxyphenyl)methyl]-4-(N-methyl-N-phenylamino)-2-n-propyl pyrazolo[1,5-a]-1,3,5-triazine (**12e**)*

Prepared from **11c** and 2-methoxybenzaldehyde using the procedure described for **12a**. Compound **12e** was obtained (80%) as a white powder: ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.02 (t, $J = 7.5$, 3H, CH_3), 1.88−1.91 (m, 2H, CH_2), 2.83 (t, $J = 7.5$, 2H, CH_2), 3.73 (s, 3H, CH_3), 3.74 (br s, 1H, OH), 3.84 (s, 3H, CH_3), 6.12 (d, $J = 1.7$, 1H, CH), 7.11−7.46 (m, 10H, ArH + 7-H).

*6.1.10. 2-Trifluoromethyl-8-[(hydroxy)(2-methoxyphenyl) methyl]-4-(N-methyl-N-phenyl amino)pyrazolo[1,5-a]-1,3,5-triazine (**12f**)*

Prepared from **11b** and 2-methoxybenzaldehyde using the procedure described for **12a**. Compound **12f** was obtained (80%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.78 (s, 3H, CH_3), 3.82 (s, 3H, CH_3), 4.70 (d, $J = 4.2$, 1H, CH), 6.38 (d, $J = 4.2$, 1H, OH), 6.86−6.98 (m, 3H, ArH), 7.18−7.45 (m, 6H, ArH), 7.66 (s, 1H, 7-H); *m/z* 430 $(M + H)^+$.

*6.1.11. 8-[(2-Furyl)(hydroxy)methyl]-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**12g**)*

Prepared from **11a** [19] and 2-furaldehyde using the procedure described for **12a**. Compound **12g** was obtained (81%) as a white powder: ^{1}H NMR (300 MHz, $CDCl_3$/DMSO-d_6) δ 2.35 (s, 3H, CH_3), 3.54 (s, 3H, CH_3), 4.66 (d, $J = 5.1$, 1H, OH), 5.92 (d, $J = 5.1$, 1H, CH), 5.99−6.12 (m, 2H, ArH), 6.97−7.28 (m, 6H, ArH), 7.59 (s, 1H, 7-H); *m/z* 336 $(M + H)^+$.

*6.1.12. 8-[(3-Furyl)(hydroxy)methyl]-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**12h**)*

Prepared from **11a** [19] and 3-furaldehyde using the procedure described for **12a**. Compound **12h** was obtained (82%) as a white powder: ^{1}H NMR (200 MHz, DMSO-d_6) δ 2.46 (s, 3H, CH_3), 3.68 (s, 3H, CH_3), 5.54 (d, $J = 4.2$, 1H, OH), 5.89 (d, $J = 4.2$, 1H, CH), 6.49 (s, 1H, ArH), 7.26−7.56 (m, 7H, ArH), 7.82 (s, 1H, 7-H); *m/z* 336 $(M + H)^+$.

*6.1.13. 8-[(Hydroxy)(2-thienyl)methyl]-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**12i**)*

Prepared from **11a** [19] and thiophene-2-carboxaldehyde using the procedure described for **12a**. Compound **12i** was obtained (85%) as a white powder: ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.53 (s, 3H, CH_3), 3.74 (s, 3H, CH_3), 4.15 (d, $J = 4.7$, 1H, OH), 6.38 (d, 1H, $J = 4.7$, CH), 6.92−6.97 (m, 2H, ArH), 7.16−7.40 (m, 6H, ArH), 7.59 (s, 1H, 7-H); *m/z* 352 $(M + H)^+$.

*6.1.14. 8-[(Hydroxy)(2-naphthyl)methyl]-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**12j**)*

Prepared from **11a** [19] and 2-naphthaldehyde using the procedure described for **12a**. Compound **12j** was obtained (80%) as a white powder: ^{1}H NMR (200 MHz, DMSO-d_6) δ 2.49 (s, 3H, CH_3), 3.68 (s, 3H, CH_3), 5.87 (d, $J = 4.6$, 1H, CH), 6.13 (d, $J = 4.6$, 1H, OH), 7.24−7.57 (m, 8H, ArH), 7.75 (s, 1H, 7-H), 7.81−7.91 (m, 4H, ArH); *m/z* 396 $(M + H)^+$.

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*6.1.15. 8-Benzyl-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**13a**)*

To a solution of NaI (260 mg, 1.74 mmol) in acetonitrile (5 mL) was added chlorotrimethylsilane (220 μL, 1.74 mmol) under argon atmosphere. After 10 min of stirring at 20 °C, a solution of **12b** (100 mg, 0.29 mmol) in acetonitrile (5 mL) was added. The mixture was stirred at room temperature under argon for 15 min, then diluted with ether (60 mL) and washed with water (30 mL). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography on silica (AcOEt/hexane, 1:1) afforded the desired product **13a** (79 mg, 83%) as colorless oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.60 (s, 3H, CH_3), 3.74 (s, 3H, CH_3), 4.02 (s, 2H, CH_2), 7.16–7.43 (m, 10H, ArH), 7.50 (s, 1H, 7-H); *m/z* 330 $(M + H)^+$.

*6.1.16. 8-Benzyl-4-(N-methyl-N-phenylamino)-2-n-propylpyrazolo[1,5-a]-1,3,5-triazine (**13b**)*

Prepared from **12c** using the procedures described for **13a**. Compound **13b** was obtained (54%) as colorless oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.05 (t, $J = 7.5$, 3H, CH_3), 1.88–1.90 (m, 2H, CH_2), 2.79 (t, $J = 7.5$, 2H, CH_2), 3.73 (s, 3H, CH_3), 4.02 (s, 2H, CH_2), 7.06–7.43 (m, 10H, ArH), 7.50 (s, 1H, 7-H); *m/z* 358 $(M + H)^+$.

*6.1.17. 8-(Benzyl)-2-trifluoromethyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**13c**)*

Prepared from **12d** using the procedure described for **13a**. Compound **13c** was obtained (84%) as a white powder: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.80 (s, 3H, CH_3), 4.06 (s, 2H, CH_2), 7.22–7.40 (m, 10H, ArH), 7.59 (s, 1H, 7-H); *m/z* 384 $(M + H)^+$.

*6.1.18. 8-(2-Methoxybenzyl)-4-(N-methyl-N-phenylamino)-2-n-propylpyrazolo[1,5-a]-1,3,5-triazine (**13d**)*

Prepared from **12e** using the procedures described for **13a**. Compound **13d** was obtained (40%) as colorless oil: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.07 (t, 3H, $J = 7.4$, CH_3), 1.89–1.93 (m, 2H, CH_2), 2.81 (t, $J = 7.4$, 2H, CH_2), 3.74 (s, 3H, CH_3), 3.85 (s, 3H, CH_3), 4.03 (s, 2H, CH_2), 6.80–7.45 (m, 9H, ArH), 7.58 (s, 1H, 7-H); *m/z* 388 $(M + H)^+$.

*6.1.19. 2-Trifluoromethyl-8-(2-methoxybenzyl)-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**13e**)*

Prepared from **12f** using the procedure described for **13a**. Compound **13e** was obtained (88%) as a white powder: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.78 (s, 3H, CH_3), 3.82 (s, 3H, CH_3), 4.04 (s, 2H, CH_2), 6.83–6.88 (m, 2H, ArH), 7.16–7.22 (m, 4H, ArH), 7.36–7.42 (m, 3H, ArH), 7.65 (s, 1H, 7-H); *m/z* 414 $(M + H)^+$.

*6.1.20. 8-(Furfuryl)-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**13f**)*

Prepared from **12g** using the procedure described for **13a**. Compound **13f** was obtained (53%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.46 (s, 3H, CH_3), 3.67 (s, 3H, CH_3), 3.96 (s, 2H, CH_2), 6.02–6.04 (m, 1H, ArH), 6.31–6.34 (m, 1H, ArH), 7.26–7.50 (m, 6H, ArH), 7.75 (s, 1H, 7-H); *m/z* 320 $(M + H)^+$.

*6.1.21. 8-[(3-Furfuryl)methyl]-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**13g**)*

Prepared from **12h** using the procedure described for **13a**. Compound **13g** was obtained (93%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.61 (s, 3H, CH_3), 3.76 (s, 3H, CH_3), 3.84 (s, 2H, CH_2), 6.34 (s, 1H, ArH), 7.18–7.43 (m, 7H, ArH), 7.58 (s, 1H, 7-H); *m/z* 320 $(M + H)^+$.

*6.1.22. 2-Methyl-4-(N-methyl-N-phenylamino)-8-(2-thienylmethyl)pyrazolo[1,5-a]-1,3,5-triazine (**13h**)*

Prepared from **12i** using the procedure described for **13a**. Compound **13h** was obtained (86%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.59 (s, 3H, CH_3), 3.72 (s, 3H, CH_3), 4.01 (s, 2H, CH_2), 7.14–7.42 (m, 8H, ArH), 7.47 (s, 1H, 7-H); *m/z* 336 $(M + H)^+$.

*6.1.23. 2-Methyl-4-(N-methyl-N-phenylamino)-8-(2-naphthylmethyl)pyrazolo[1,5-a]-1,3,5-triazine (**13i**)*

Prepared from **12j** using the procedure described for **13a**. Compound **13i** was obtained (81%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.64 (s, 3H, CH_3), 3.77 (s, 3H, CH_3), 4.22 (s, 2H, CH_2), 7.19–7.90 (m, 13H, ArH + 7-H); *m/z* 380 $(M + H)^+$.

*6.1.24. 8-Benzyl-2-methyl-4-(N-methylamino)pyrazolo[1,5-a]-1,3,5-triazine (**14a**)*

A solution of **13a** (1.01 mmol) and methylamine (2 M, 2.0 mL, 4.0 mmol) in ethanol (10 mL) was stirred at 100 °C in a sealed tube for 12 h. After the mixture was cooled the solvent was evaporated under reduced pressure. The crude reaction product was purified by column chromatography on silica gel (EtOAc/CH_2Cl_2/EtOH, 4:5:1). Recrystallization from ethanol and diethyl ether yielded compound **14a** (53%) as colorless prisms: ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.98 (s, 3H, CH_3), 3.41 (d, $J = 5.1$, 3H, CH_3), 4.42 (s, 2H, CH_2), 7.18–7.39 (m, 5H, ArH), 7.79 (s, 1H, 7-H), 7.90 (q, $J = 5.1$, 1H, NH); *m/z* 254 $(M + H)^+$. Anal. Found: $C_{14}H_{15}N_5$ (C, H, N).

*6.1.25. 8-Benzyl-4-(N-methylamino)-2-n-propylpyrazolo [1,5-a]-1,3,5-triazine hydrochloride (**14b**)*

Prepared from **13b** using the procedure described for **14a**. Compound **14b** was obtained (68%) as colorless prisms: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.11 (t, $J = 7.3$, 3H, CH_3), 1.95–1.98 (m, 2H, CH_2), 3.26 (t, $J = 7.6$, 2H, CH_2), 3.42 (d, $J = 5.0$, 3H, CH_3), 4.42 (s, 2H, CH_2), 7.19–7.36 (m, 5H, ArH), 7.74 (s, 1H, 7-H), 8.08 (q, $J = 5.0$, 1H, NH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.32, 21.35, 28.31, 28.86, 37.32, 107.32, 126.93, 129.22, 129.33, 138.46, 141.27, 147.45, 149.67, 165.44; *m/z* 282 $(M + H)^+$. Anal. Found: $C_{16}H_{19}N_5$.HCl (C, H, N).

*6.1.26. 8-Benzyl-2-trifluoromethyl-4-(N-methylamino) pyrazolo[1,5-a]-1,3,5-triazine (**14c**)*

Prepared from **13c** using the procedure described for **14a**. Compound **12c** was obtained (79%) as colorless prisms: ^{1}H

NMR (200 MHz, $CDCl_3$) δ 3.28 (d, $J = 5.1$, 3H, CH_3), 4.11 (s, 2H, CH_2), 6.60 (br s, 1H, NH), 7.13–7.42 (m, 5H, ArH), 7.83 (s, 1H, 7-H); *m/z* 308 $(M + H)^+$. Anal. Found: $C_{14}H_{12}F_3N_5$ (C, H, N).

6.1.27. 8-(2-Methoxybenzyl)-4-(N-methylamino)-2-n-propylpyrazolo[1,5-a]-1,3,5-triazine (**14d**)

Prepared from **13d** using the procedure described for **14a**. Compound **14d** was obtained (76%) as colorless prisms: ^1H NMR (300 MHz, $CDCl_3$) δ 1.04 (t, $J = 7.4$, 3H, CH_3), 1.86–1.89 (m, 2H, CH_2), 2.77 (t, $J = 7.7$, 2H, CH_2), 3.21 (d, $J = 5.3$, 3H, CH_3), 3.87 (s, 3H, CH_3), 4.04 (s, 2H, CH_2), 6.41 (br s, 1H, NH), 6.85–6.90 (m, 2H, ArH), 7.18–7.22 (m, 2H, ArH), 7.58 (s, 1H, 7-H); *m/z* 312 $(M + H)^+$. Anal. Found: $C_{17}H_{21}N_5O \cdot 0.1H_2O$ (C, H, N).

6.1.28. 2-Trifluoromethyl-8-(2-methoxybenzyl)-4-(N-methylamino)pyrazolo[1,5-a]-1,3,5-triazine (**14e**)

Prepared from **13e** using the procedure described for **14a**. Compound **14e** was obtained (76%) as colorless prisms: ^1H NMR (300 MHz, $CDCl_3$) δ 3.30 (d, $J = 4.9$, 3H, CH_3), 3.87 (s, 3H, CH_3), 4.09 (s, 2H, CH_2), 6.72 (br s, 1H, NH), 6.87–6.92 (m, 2H, ArH), 7.19–7.28 (m, 2H, ArH), 7.89 (s, 1H, 7-H); *m/z* 338 $(M + H)^+$. Anal. Found: $C_{15}H_{14}F_3N_5O$ (C, H, N).

6.1.29. 8-(Furfuryl)-2-methyl-4-(N-methylamino)pyrazolo[1,5-a]-1,3,5-triazine (**14f**)

Prepared from **13f** using the procedure described for **14a**. Compound **14f** was obtained (62%) as colorless prisms: ^1H NMR (200 MHz, $CDCl_3$) δ 2.57 (s, 3H, CH_3), 3.21 (d, $J = 4.9$, 3H, CH_3), 4.06 (s, 2H, CH_2), 6.01–6.04 (m, 1H, ArH), 6.27–6.30 (m, 1H, ArH), 6.45 (br s, 1H, NH), 7.25–7.34 (m, 1H, ArH), 7.84 (s, 1H, 7-H); *m/z* 244 $(M + H)^+$. Anal. Found: $C_{12}H_{13}N_5O$ (C, H, N).

6.1.30. 8-[(3-Furyl)methyl]-2-methyl-4-(N-methylamino)pyrazolo[1,5-a]-1,3,5-triazine (**14g**)

Prepared from **13g** using the procedure described for **14a**. Compound **14g** was obtained (64%) as colorless prisms: ^1H NMR (200 MHz, $CDCl_3$) δ 2.57 (s, 3H, CH_3), 3.22 (d, $J = 4.9$, 3H, CH_3), 3.84 (s, 2H, CH_2), 6.30–6.34 (m, 1H, ArH), 6.48 (br s, 1H, NH), 7.25–7.37 (m, 2H, ArH), 7.56 (s, 1H, 7-H); *m/z* 244 $(M + H)^+$. Anal. Found: $C_{12}H_{13}N_5O$ (C, H, N).

6.1.31. 2-Methyl-4-(N-methylamino)-8-[(2-thienyl)methyl]pyrazolo[1,5-a]-1,3,5-triazine (**14h**)

Prepared from **13h** using the procedure described for **14a**. Compound **14h** was obtained (87%) as colorless prisms: ^1H NMR (200 MHz, $CDCl_3$) δ 2.57 (s, 3H, CH_3), 3.22 (d, $J = 4.9$, 3H, CH_3), 4.25 (s, 2H, CH_2), 6.45 (br s, 1H, NH), 7.86–7.94 (m, 2H, ArH), 7.11–7.25 (m, 1H, ArH), 7.81 (s, 1H, 7-H); *m/z* 260 $(M + H)^+$. Anal. Found: $C_{12}H_{13}N_5S$ (C, H, N).

6.1.32. 2-Methyl-4-(N-methylamino)-8-[(2-naphthyl)methyl]pyrazolo[1,5-a]-1,3,5-triazine (**14i**)

Prepared from **13i** using the procedure described for **14a**. Compound **14i** was obtained (86%) as colorless prisms: ^1H NMR (200 MHz, $CDCl_3$) δ 2.63 (s, 3H, CH_3), 3.26 (d, $J = 5.1$, 3H, CH_3), 4.25 (s, 2H, CH_2), 6.47 (br s, 1H, NH), 7.43–7.82 (m, 7H, ArH), 7.86 (s, 1 H, 7-H); *m/z* 304 $(M + H)^+$. Anal. Found: $C_{18}H_{17}N_5 \cdot 0.3H_2O$ (C, H, N).

6.1.33. 2-Methyl-4-(N-methyl-N-phenylamino)-8-(prop-1-ynyl)pyrazolo[1,5-a]-1,3,5-triazine (**15a**)

Methylacetylene (2 mL) was condensed in a sealed tube at −78 °C. Then, **11a** [19] (157 mg, 0.43 mmol), CuI (12 mg, 0.063 mg), $PdCl_2$ (7 mg, 0.039 mmol), triphenylphosphine (23 mg, 0.088 mmol), triethylamine (2 mL, 14 mmol) and acetonitrile (3 mL) were added. The reaction mixture was stirred at room temperature for 24 h. After the solution was cooled to −78 °C, the sealed tube was opened and slowly heated at room temperature. The solvent was evaporated under reduced pressure and the crude reaction product was purified by column chromatography on silica gel (EtOAc/hexane, 1:1) as a yellow solid (73%): ^1H NMR (200 MHz, $CDCl_3$) δ 2.07 (s, 3H, CH_3), 2.64 (s, 3H, CH_3), 3.78 (s, 3H, CH_3), 7.14–7.20 (m, 2H, ArH), 7.36–7.48 (m, 3H, ArH), 7.74 (s, 1H, 7-H).

6.1.34. 2-Methyl-4-(N-methyl-N-phenylamino)-8-(pent-1-ynyl)pyrazolo[1,5-a]-1,3,5-triazine (**15b**)

A solution of **11a** [19] (200 mg, 0.55 mmol), 1-pentyne (980 μL, 10 mmol), CuI (12 mg, 0.063 mmol), $PdCl_2$ (7 mg, 0.039 mmol), triphenylphosphine (23 mg, 0.088 mmol), triethylamine (2 mL, 14 mmol) and acetonitrile (3 mL) was stirred under nitrogen at 25 °C for 24 h. The solvent was evaporated under reduced pressure and the crude reaction product was purified by column chromatography on silica gel (EtOAc/hexane, 1:1). Recrystallization from dichloromethane and hexanes yielded compound **15b** (119 mg, 71%) as colorless solid: ^1H NMR (200 MHz, $CDCl_3$) δ 1.07 (t, $J = 7.3$, 3H, CH_3), 1.65–1.69 (m, 2H, CH_2), 2.47 (t, $J = 7.3$, 2H, CH_2), 2.63 (s, 3H, CH_3), 3.76 (s, 3H, CH_3), 7.15–7.20 (m, 2H, ArH), 7.35–7.45 (m, 3H, ArH), 7.76 (s, 1H, 7-H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.07, 22.32, 22.68, 26.21, 42.54, 70.03, 92.42, 94.24, 126.35, 127.49, 129.40, 144.96, 147.19, 149.54, 152.02, 164.12; *m/z* 306 $(M + H)^+$.

6.1.35. 2-Methyl-4-(N-methylamino)-8-(prop-1-ynyl)pyrazolo[1,5-a]-1,3,5-triazine hydrochloride (**16a**)

Prepared from **15a** using the procedure described for **14a**. Compound **16a** was obtained (62%) as a white solid: ^1H NMR (200 MHz, $CDCl_3$) δ 2.05 (s, 3H, CH_3), 2.75 (s, 3H, CH_3), 3.20 (d, $J = 4.2$, 3H, CH_3), 7.91 (s, 1H, 7-H), 9.50 (br s, 1H, NH); *m/z* 202 $(M + H)^+$. Anal. Found: $C_{10}H_{11}N_5 \cdot HCl \cdot 0.2H_2O$ (C, H, N).

6.1.36. 2-Methyl-4-(N-methylamino)-8-(pent-1-ynyl)pyrazolo[1,5-a]-1,3,5-triazine (**16b**)

Prepared from **15b** using the procedure described for **14a**. Compound **16b** was obtained (77%) as colorless prisms: ^1H NMR (300 MHz, $CDCl_3$) δ 1.06 (t, $J = 7.3$, 3H, CH_3), 1.62–1.72 (m, 2H, CH_2), 2.46 (t, $J = 7.2$, 2H, CH_2), 2.60 (s, 3H, CH_3), 3.23 (d, $J = 4.9$, 3H, CH_3), 6.49 (br s, 1H, NH), 7.9 (s, 1H, 7-H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.07, 22.28, 22.65, 26.47, 27.67, 69.84, 85.81, 93.56, 94.18,

147.67, 149.53, 165.27; *m/z* 230 $(M+H)^+$. Anal. Found: $C_{12}H_{15}N_5$ (C, H, N).

*6.1.37. 2-Methyl-4-(N-methyl-N-phenylamino)-8-phenylpyrazolo[1,5-a]-1,3,5-triazine (**17a**)*

A solution of **11a** [19] (157 mg, 0.43 mmol), tetrakis(triphenylphosphine)palladium(0) (50 mg, 0.043 mmol), sodium carbonate (2 M in H_2O, 430 μL, 0.86 mmol), benzeneboronic acid (58 mg, 0.47 mmol), and ethanol (500 μL) in toluene (10 mL) was stirred under nitrogen at 90 °C for 16 h. After the solution was cooled to room temperature, the solvent was evaporated under reduced pressure. The crude reaction product was purified by column chromatography on silica gel (EtOAc/hexanes, 1:1) to give **17a** (94 mg, 69%) as a colorless solid: mp 195 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.67 (s, 3H, CH_3), 3.78 (s, 3H, CH_3), 7.20–7.50 (m, 8H, ArH), 7.95–8.00 (m, 2H, ArH), 8.05 (s, 1H, 7-H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 26.34, 31.33, 108.39, 126.38, 126.44, 126.60, 127.33, 129.09, 129.39, 132.31, 143.34, 145.29, 147.99, 149.71, 163.35; *m/z* 316 $(M+H)^+$.

*6.1.38. 8-(2-Chlorophenyl)-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**17b**)*

Prepared from **11a** [19] and 2-chlorophenylboronic acid using the procedure described for **17a**. Compound **17b** was obtained (79%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.62 (s, 3H, CH_3), 3.80 (s, 3H, CH_3), 7.19–7.51 (m, 8H, ArH), 7.86–7.91 (m, 1H, ArH), 8.19 (s, 1H, 7-H); *m/z* 350 $(M+H)^+$.

*6.1.39. 8-(3-Acetylphenyl)-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**17c**)*

Prepared from **11a** [19] and 3-acetylphenylboronic acid using the procedure described for **17a**. Compound **17c** was obtained (58%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.67 (s, 3H, CH_3), 2.68 (s, 3H, CH_3), 3.78 (s, 3H, CH_3), 7.24–7.86 (m, 7H, ArH), 8.08 (s, 1H, 7-H), 8.18–8.24 (m, 1H, ArH), 8.56–8.58 (m, 1H, ArH); *m/z* 358 $(M+H)^+$.

*6.1.40. 2-Methyl-4-(N-methyl-N-phenylamino)-8-(3-nitrophenyl)pyrazolo[1,5-a]-1,3,5-triazine (**17d**)*

Prepared from **11a** [19] and 3-nitrophenylboronic acid using the procedure described for **17a**. Compound **17d** was obtained (77%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.66 (s, 3H, CH_3), 3.77 (s, 3H, CH_3), 7.18–7.59 (m, 6H, ArH), 8.02–8.04 (m, 1H, ArH), 8.06 (s, 1H, 7-H), 8.30–8.35 (m, 1H, ArH), 8.80–8.82 (m, 1H, ArH); *m/z* 361 $(M+H)^+$.

*6.1.41. 8-[3-(Trifluoromethylphenyl)]-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**17e**)*

Prepared from **11a** [19] and 3-trifluoromethylphenylboronic acid using the procedure described for **17a**. Compound **17e** was obtained (87%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.64 (s, 3H, CH_3), 3.76 (s, 3H, CH_3), 7.18–7.52 (m, 7H, ArH), 8.03 (s, 1H, 7-H), 8.14–8.21 (m, 2H, ArH); *m/z* 384 $(M+H)^+$.

*6.1.42. 8-(2-Furyl)-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**17f**)*

Prepared from **11a** [19] and furan-2-boronic acid using the procedure described for **17a**. Compound **17f** was obtained (51%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.63 (s, 3H, CH_3), 3.74 (s, 3H, CH_3), 6.47–6.50 (m, 1H, ArH), 6.77–6.80 (m, 1H, ArH), 7.15–7.20 (m, 2H, ArH), 7.33–7.44 (m, 4H, ArH), 7.99 (s, 1H, 7-H); *m/z* 306 $(M+H)^+$.

*6.1.43. 2-Methyl-4-(N-methyl-N-phenylamino)-8-(2-thienyl)pyrazolo[1,5-a]-1,3,5-triazine (**17g**)*

Prepared from **11a** [19] and thiphene-2-boronic acid using the procedure described for **17a**. Compound **17g** was obtained (54%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.67 (s, 3H, CH_3), 3.78 (s, 3H, CH_3), 7.08–7.48 (m, 8H, ArH), 7.93 (s, 1H, 7-H); *m/z* 322 $(M+H)^+$.

*6.1.44. 2-Methyl-4-(N-methyl-N-phenylamino)-8-(1-naphthyl)pyrazolo[1,5-a]-1,3,5-triazine (**17h**)*

Prepared from **11a** [19] and 1-naphthaleneboronic acid using the procedure described for **17a**. Compound **17h** was obtained (57%) as a white powder: ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.57 (s, 3H, CH_3), 3.80 (s, 3H, CH_3), 7.26–7.98 (m, 12H, ArH), 7.95 (s, 1H, 7-H); *m/z* 366 $(M+H)^+$.

*6.1.45. 8-(2-Benzo[b]furyl)-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**17i**)*

Prepared from **11a** [19] and benzo[*b*]furan-2-boronic acid using the procedure described for **17a**. Compound **17i** was obtained (26%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.69 (s, 3H, CH_3), 3.79 (s, 3H, CH_3), 7.22–7.63 (m, 10H, ArH), 8.17 (s, 1H, 7-H); *m/z* 356 $(M+H)^+$.

*6.1.46. 8-(2-Benzo[b]thienyl)-2-methyl-4-(N-methyl-N-phenylamino)pyrazolo[1,5-a]-1,3,5-triazine (**17j**)*

Prepared from **11a** [19] and benzo[*b*]thiophene-2-boronic acid using the procedure described for **17a**. Compound **17j** was obtained (59%) as a white powder: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.69 (s, 3H, CH_3), 3.76 (s, 3H, CH_3), 7.18–7.43 (m, 7H, ArH), 7.73–7.80 (m, 3H, ArH), 8.06 (s, 1H, 7-H); *m/z* 372 $(M+H)^+$.

*6.1.47. 2-Methyl-4-(N-methylamino)-8-phenylpyrazolo[1,5-a]-1,3,5-triazine (**18a**)*

Prepared from **17a** using the procedure described for **14a**. Compound **18a** was obtained (83%) as colorless prisms: ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.63 (s, 3H, CH_3), 3.25 (d, $J = 5.0$, 3H, CH_3), 6.51 (br s, 1H, NH), 7.23–7.28 (m, 1H, ArH), 7.41–7.46 (m, 2H, ArH), 7.98–8.01 (m, 2H, ArH), 8.24 (s, 1H, 7-H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 26.64, 27.64, 109.83, 126.50, 129.16, 132.27, 143.36, 145.72, 149.59, 164.47; *m/z* 240 $(M+H)^+$. Anal. Found: $C_{13}H_{13}N_5$ (C, H, N).

*6.1.48. 8-(2-Chlorophenyl)-2-methyl-4-(N-methylamino)pyrazolo[1,5-a]-1,3,5-triazine (**18b**)*

Prepared from **17b** using the procedure described for **14a**. Compound **18b** was obtained (64%) as colorless prisms: ^{1}H NMR (200 MHz, DMSO-d_6) δ 2.44 (s, 3H, CH_3), 3.06 (d, $J = 4.6$, 3H, CH_3), 7.31–7.86 (m, 4H, ArH), 8.46 (s, 1H, 7-H), 8.82 (q, $J = 4.6$, 1H, NH); *m/z* 274 $(M + H)^+$. Anal. Found: $C_{13}H_{12}ClN_5 \cdot 0.5H_2O$ (C, H, N).

*6.1.49. 8-(3-Acetylphenyl)-2-methyl-4-(N-methylamino)pyrazolo[1,5-a]-1,3,5-triazine (**18c**)*

Prepared from **17c** using the procedure described for **14a**. Compound **18c** was obtained (43%) as colorless prisms: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.55 (s, 3H, CH_3), 2.68 (s, 3H, CH_3), 3.26 (d, $J = 5.0$, 3H, CH_3), 6.56 (br s, 1H, NH), 7.51–7.56 (m, 1H, ArH), 7.81–7.84 (m, 1H, ArH), 8.26–8.30 (m, 2H, ArH), 8.56 (s, 1H, 7-H); *m/z* 282 $(M + H)^+$. Anal. Found: $C_{15}H_{15}N_5O$ (C, H, N).

*6.1.50. 2-Methyl-4-(N-methylamino)-8-(3-nitrophenyl)pyrazolo[1,5-a]-1,3,5-triazine hydrochloride (**18d**)*

Prepared from **17d** using the procedure described for **14a**. Compound **18d** was obtained (32%) as colorless prisms: ^{1}H NMR (200 MHz, DMSO-d_6) δ 2.52 (s, 3H, CH_3), 3.07 (d, $J = 2.3$, 3H, CH_3), 7.66–7.71 (m, 1H, ArH), 8.01–8.04 (m, 1H, ArH), 8.46–8.49 (m, 1H, ArH), 8.63 (br s, 1H, NH), 8.73 (s, 1H, 7-H), 8.96 (s, 1H, ArH); *m/z* 285 $(M + H)^+$. Anal. Found: $C_{13}H_{12}N_6O_2 \cdot 1H_2O$ (C, H, N).

*6.1.51. 8-(3-Trifluoromethylphenyl)-2-methyl-4-(N-methylamino)pyrazolo[1,5-a]-1,3,5-triazine (**18e**)*

Prepared from **17e** using the procedure described for **14a**. Compound **18e** was obtained (29%) as colorless prisms: ^{1}H NMR (200 MHz, DMSO-d_6) δ 2.56 (s, 3H, CH_3), 3.12 (d, $J = 4.2$, 3H, CH_3), 7.32–7.45 (m, 2H, ArH), 7.97 (s, 1H, 7-H), 8.04–8.14 (m, 2H, ArH), 8.29 (br s, 1H, NH); *m/z* 308 $(M + H)^+$. Anal. Found: $C_{14}H_{12}F_3N_5$ (C, H, N).

*6.1.52. 8-(2-Furyl)-2-methyl-4-(N-methylamino)pyrazolo[1,5-a]-1,3,5-triazine (**18f**)*

Prepared from **17f** using the procedure described for **14a**. Compound **18f** was obtained (21%) as colorless prisms: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.62 (s, 3H, CH_3), 3.25 (d, $J = 5.0$, 3H, CH_3), 6.49–6.51 (m, 2H, ArH + NH), 6.79 (d, $J = 3.1$, 1H, ArH), 7.45 (d, $J = 1.9$, 1H, ArH), 8.20 (s, 1H, 7-H); *m/z* 230 $(M + H)^+$. Anal. Found: $C_{11}H_{11}N_5O$ (C, H, N).

*6.1.53. 2-Methyl-4-(N-methylamino)-8-(2-thienyl)pyrazolo[1,5-a]-1,3,5-triazine hydrochloride (**18g**)*

Prepared from **17g** using the procedure described for **14a**, then converted to the HCl salt. Compound **18g** was obtained (42%) as colorless prisms: ^{1}H NMR (200 MHz, DMSO-d_6) δ 2.50 (s, 3H, CH_3), 3.05 (d, $J = 4.6$, 3H, CH_3), 7.09–7.14 (m, 1H, ArH), 7.43–7.55 (m, 2H, ArH), 8.51 (s, 1H, 7-H), 8.91 (q, $J = 4.6$, 1H, NH); *m/z* 246 $(M + H)^+$. Anal. Found: $C_{11}H_{11}N_5S \cdot 0.5H_2O$ (C, H, N).

*6.1.54. 2-Methyl-4-(N-methylamino)-8-(1-naphthyl)pyrazolo[1,5-a]-1,3,5-triazine hydrochloride (**18h**)*

Prepared from **17h** using the procedure described for **14a**, then converted to the HCl salt. Compound **18h** was obtained (40%) as colorless prisms: ^{1}H NMR (200 MHz, DMSO-d_6) δ 2.43 (s, 3H, CH_3), 3.15 (d, $J = 4.8$, 3H, CH_3), 7.48–7.65 (m, 4H, ArH), 7.86–8.30 (m, 3H, ArH), 8.46 (s, 1H, 7-H), 9.48 (q, $J = 4.8$, 1H, CH_3); *m/z* 290 $(M + H)^+$. Anal. Found: $C_{17}H_{15}N_5 \cdot HCl \cdot 0.7H_2O$ (C, H, N).

*6.1.55. 8-(2-Benzo[b]furyl)-2-methyl-4-(N-methylamino)pyrazolo[1,5-a]-1,3,5-triazine (**18i**)*

Prepared from **17i** using the procedure described for **14a**. Compound **18i** was obtained (95%) as colorless prisms: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.69 (s, 3H, CH_3), 3.30 (d, $J = 4.9$, 3H, CH_3), 6.57 (q, $J = 4.9$, 1H, NH), 7.24–7.33 (m, 3H, ArH), 7.52–7.63 (m, 2H, ArH), 8.40 (s, 1H, 7-H); *m/z* 280 $(M + H)^+$. Anal. Found: $C_{15}H_{13}N_5O$ (C, H, N).

*6.1.56. 8-(2-Benzo[b]thienyl)-2-methyl-4-(N-methylamino)pyrazolo[1,5-a]-1,3,5-triazine (**18j**)*

Prepared from **17j** using the procedure described for **14a**. Compound **18j** was obtained (58%) as colorless prisms: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.70 (s, 3H, CH_3), 3.29 (d, $J = 5.1$, 3H, CH_3), 6.55 (q, $J = 5.1$, 1H, NH), 7.26–7.44 (m, 2H, ArH), 7.78–7.89 (m, 3H, ArH), 8.22 (s, 1H, 7-H); *m/z* 296 $(M + H)^+$. Anal. Found: $C_{15}H_{13}N_5S$ (C, H, N).

*6.1.57. 8-(1-Hydroxypropyl)-2-methyl-4-(N-methylamino)pyrazolo[1,5-a]-1,3,5-triazine (**19**)*

Prepared from **12a** using the procedure described for **14a**. Compound **19** was obtained (81%) as colorless crystals: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.02 (t, $J = 7.5$, 3H, CH_3), 1.90–2.04 (m, 2H, CH_2), 2.47 (s, 3H, CH_3), 2.94 (d, $J = 4.4$, 1H, OH), 3.25 (q, $J = 5.1$, 3H, CH_3), 4.94–5.03 (m, 1H, CH), 6.52 (br s, 1H, NH), 7.91 (s, 1H, 7-H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 10.50, 26.30, 27.60, 31.16, 67.57, 112.58, 143.37, 146.37, 149.38, 163.61; *m/z* 222 $(M + H)^+$. Anal. Found: $C_{10}H_{15}N_5O$ (C, H, N).

6.2. Molecular modeling

Molecules were sketched in Sybyl 6.8 sketcher [20], and Concord 4.05 [24] was used to generate 3D structures. They were minimized in the Tripos force field [21] with 20 Simplex [25] iterations as initial optimization step. Powell method [26] was applied with a termination gradient of 0.05 kcal mol^{-1} Å^{-1}. The maximum iterations were set to 1000. No charge was used. The minimized structures were submitted to AM1 [22] optimization in singlet state with zero net charge and with a time limit of 3600 s as found in Mopac [23] implemented in Sybyl. Normal convergence and full optimization were selected. The keywords were DENSITY LOCAL VECT MULLIK AM1 PI BONDS GRAPH T=3600. Molcad [27] surfaces were calculated using the Fast Connolly method with a probe radius of 1.4 Å as found in Sybyl 6.8, and the electrostatic potential [28] was derived from

the AM1 charges calculated previously with Mopac and mapped onto the Connoly surfaces. Surface area was estimated by the Molcad Surface Property Area utilities. Isopotential curves are drawn in the contour surface tools available in Sybyl.

6.3. Pharmacology

6.3.1. PDE inhibition

PDE1, -3, -4 and -5 were isolated from the media layer of bovine aorta according to a modification of the previously reported method [31]. PDE2 was isolated from cultured bovine aortic endothelial cells [32]. PDE activities were measured by the two-step assay previously described [33] at a [^{3}H]-cAMP or [^{3}H]-cGMP concentration of 1 μM as substrate in a buffer solution of 50 mM of Tris–HCl, at pH 7.5, containing 2 mM of magnesium acetate, 1 mg mL^{-1} of BSA. PDE1 was assayed at 1 μM cGMP in calmodulin activated state (18 nM calmodulin with 10 μM $CaCl_2$). PDE2 was evaluated at 1 μM cAMP + 1 mM EGTA in activated state (in presence of 5 μM cGMP). PDE3 and PDE4 were assayed at 1 μM cAMP + 1 mM EGTA. To prevent the influence of reciprocal cross-contamination between PDE3 and PDE4, the studies were always carried out in the presence of 50 μM rolipram for PDE3 and in the presence of 50 μM cGMP for PDE4. PDE5 activity was measured at 1 μM cGMP in the presence of 1 mM of EGTA. PDE inhibitors were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 1%. At this concentration, DMSO had no significant effect on PDE activity. The concentration of drugs that produced 50% inhibition of substrate hydrolysis (IC_{50}) was calculated by nonlinear regression analysis from concentration–response curves and included different concentrations of inhibitors. The results represent the mean of three determinations obtained for three different enzymatic preparations. The experimental error is about 15%.

6.3.2. Subjects

Healthy donors who had no clinical history were selected for this study. This work was approved by the local ethics committee (CCPPRB d'Alsace no. 1), and carried out according to national guidelines.

6.3.3. Peripheral blood mononuclear cells

Healthy subjects PBMCs were separated as previously described [34]. Briefly, peripheral blood diluted with Hank's balanced salt solution, Ca^{2+} and Mg^{2+} free, containing 100 IU heparin per mL was layered over Histopaque-1077 (Sigma, St. Louis, MO, USA), and centrifuged for 30 min at 400 g (20 °C). Cells harvested from the interface were washed three times in HBSS-CMF and resuspended at a final concentration of 2×10^6 mL^{-1} in a culture medium. Human PBMCs were incubated with increasing doses of the tested drugs ranging from 10^{-8} M to 10^{-5} M, with or without activation by lipopolysacharide from *Salmonella abortus equi* (Sigma, L'Isle d'Abeau Chesnes, France) in 24 well culture plates (Falcon, Poly Labo, Strasbourg, France) for 24 h at 37 °C in a humidified 5% CO_2/95% air atmosphere. After incubation, supernatants were removed and stored at −80 °C until ELISA. Cell viability was assessed by the Trypan blue exclusion test.

6.3.4. Immunoassays for TNFα

Culture supernatants were assayed with two-site ELISAs specific for human interleukin: TNFα antibodies (Antibody Solutions, Half Moon Bay, CA, USA). Quantitative evaluation of TNFα secreted by PBMC's was achieved by ELISA using conditions as previously described [35]. Polyvinyl chloride plates (Costar, #2596) were coated with 50 μL per well of antibodies (15 μg mL^{-1}) and incubated overnight at 4 °C. After the usual wash and non specific saturation steps, 25 μL of standard or sample was added to 25 μL of biotinylated monoclonal antibody (2 μg mL^{-1}) for 2 h at room temperature. Following washing steps, 50 μL of peroxidase streptavidin dilution (1:3000 in PBS) were added (1 h at room temperature). A colorimetric reaction (O.D. at 450 nm) using *O*-phenylenediamine as peroxidase substrate was performed ensuing four washing steps. Concentrations (pg mL^{-1}) of unknown samples were computed by interpolation with a standard curve run on each plate using four parameters logistics analysis. Standard human recombinant protein, hr-TNFα, was purchased from R&D Systems Europe (Abingdon, UK).

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2007.05.016.

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