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Synthesis and *in vitro* evaluation of anti-inflammatory, antioxidant, and anti-fibrotic effects of new 8-aminopurine-2,6-dione-based phosphodiesterase inhibitors as promising anti-asthmatic agents

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ABSTRACT

Phosphodiesterase (PDE) inhibitors are currently an extensively studied group of compounds that can bring many benefits in the treatment of various inflammatory and fibrotic diseases, including asthma. Herein, we describe a series of novel *N'*-phenyl- or *N'*-benzylbutanamide and *N'*-arylidenebutanehydrazide derivatives of 8-aminopurine-2,6-dione (**27**–**43**) and characterized them as prominent pan-PDE inhibitors. Most of the compounds exhibited antioxidant and anti-inflammatory activity in lipopolysaccharide (LPS)-induced murine macrophages RAW264.7. The most active compounds (**32**–**35** and **38**) were evaluated in human bronchial epithelial cells (HBECs) derived from asthmatics. To better map the bronchial microenvironment in asthma, HBECs after exposure to selected 8-aminopurine-2,6-dione derivatives were incubated in the presence of two proinflammatory and/or profibrotic factors: transforming growth factor type β (TGF-β) and interleukin 13 (IL-13). Compounds **32**–**35** and **38** significantly reduced both IL-13- and TGF-β-induced expression of proinflammatory and profibrotic mediators, respectively. Detailed analysis of their inhibition preferences for selected PDEs showed high affinity for isoenzymes important in the pathogenesis of asthma, including PDE1, PDE3, PDE4, PDE7, and PDE8. The presented data confirm that structural modifications within the 7 and 8 positions of the purine-2,6-dione core result in obtaining preferable pan-PDE inhibitors which in turn exert an excellent anti-inflammatory and anti-fibrotic effect in the bronchial epithelial cells derived from asthmatic patients. This dual-acting pan-PDE inhibitors constitute interesting and promising lead structures for further anti-asthmatic agent discovery.

1. Introduction

Asthma is one of the commonly occurring, heterogeneous, chronic diseases of the respiratory tract, characterized by wheezing, shortening of breath, tightness in the chest, and persistent cough [1]. Asthma is considered an inflammatory disease, but it is known that inflammation in the airways is accompanied by a number of structural changes, generally referred to as airway remodeling (AR) [2,3]. At the cellular level, AR manifests mainly by airway smooth muscle cell hyperplasia and hypertrophy, an increased number of fibroblasts and myofibroblasts, enhanced synthesis of extracellular matrix proteins, excessive vascularization, and mucus hyperproduction [4]. However, the disease process begins in the airway epithelium, which is crucial for asthma development and progression [5,6]. Airway epithelial cells represent the interface between the outer and inner airway microenvironment. This tight barrier underlying lung tissue homeostasis, may be damaged in

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response to many factors which trigger disease exacerbation, such as pathogens (respiratory tract infections), allergens, and air pollutants. The loss of epithelial barrier function is one of the reasons for immune cells recruitment and increased secretion of proinflammatory cytokines, chemokines, and growth factors [7]. The released asthmatic inflammation factors are responsible for further structural changes in the epithelium, including interleukin-13 (IL-13)-induced goblet cell metaplasia or transforming growth factor β (TGF-β)-induced mesenchymal transition and proliferation [8]. Thus, damaged epithelial cells with impaired repair potential represent a key player in airway inflammation and the potential target of drugs for asthma treatment and prevention [7,9].

Pharmacotherapy of asthma is based mainly on inhaled or oral glucocorticosteroids, short and long-acting β-agonists, and anti-IgE treatment [1]. Their primary function is to reduce inflammation and exert a bronchodilator effect to improve asthma control. Only some of them may have a slight influence on airway remodeling [10,11]. Despite extensive research, there is still no drug available that specifically targets AR. Further concerns are related to severe asthma pharmacotherapy, or corticosteroid hyperresponsiveness /resistant patients [12,13]. It is also known that prolonged treatment with high doses of oral glucocorticosteroids can cause various side effects, including infections, as well as gastrointestinal, bone and muscle, cardiovascular, metabolic, psychiatric and ocular complications [14]. Hence, the need to search for new therapeutic possibilities is still highly relevant. Recently, it has been proven that inhibition of selected phosphodiesterases (PDEs) can affect the airways through anti-inflammatory, bronchodilator, and anti-fibrotic actions [15]. A well-known, nonselective PDE inhibitor – theophylline - has long been used in some difficult cases of asthma [16]. Therefore, it is not surprising that the group of PDE inhibitors is widely studied in the context of potential anti-asthmatic drugs with dual anti-inflammatory and anti-fibrotic profile.

PDEs represent a large group of enzymes causing the breakdown of secondary signal transducers in cells: adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP). There are two main reasons why there is a strong interest in PDE inhibitors as potential drugs: 1) it has been concluded that elevated cAMP or cGMP levels may be responsible for anti-inflammatory properties of the drugs, and 2) in many types of cells involved in inflammation, an increased expression of PDEs has been found. PDE inhibitors can prevent excessive cAMP and cGMP degradation, simultaneously balancing the overexpression of individual PDEs in cells.

Our recent research demonstrated that pan-PDE inhibitors (that is, compounds that are able to inhibit simultaneously different PDE isoforms), purine-2,6-dione derivatives (compounds **I** – **III**; Fig. 1), can significantly reduce AR-related processes in TGF-β_1-induced human lung fibroblasts and airway smooth muscle cell [17,18]. These compounds increased the intracellular cAMP level, which resulted in the reduction of cells proliferation, migration, contraction, and expression of AR marker genes [17,18]. The preferential activity of **I** - **III** prompted us to design and synthesize new purine-2,6-dione-based compounds, namely, *N*'-phenyl- or *N*'-benzylbutanamide and *N*'-arylidenebutanehydrazide derivatives of 8-aminopurine-2,6-dione (Fig. 1). The proposed new series comprise close analogues of the above mentioned compounds **I** - **III** modified in terms of hydrophobic fragment in position 8 and kind of substitution in the phenyl group in amide or hydrazide moieties.

According to the results of molecular modeling studies, a substituent in position 8 of purine-2,6-dione containing hydrogen bond acceptor or donor/acceptor as well as hydrophobic part was crucial for PDE4/7 inhibition. The design series with different amine moieties (arylalkylamine or cyclic amine) was expected to fit structural requirements for these biological targets. Here, the newly obtained compounds were tested for the ability to inhibit selected PDE isoforms. To verify their anti-inflammatory and antioxidant potential, we performed a series of experiments with lipopolysaccharide (LPS)-induced murine macrophage cell line. Considering the central role of the airway epithelium in asthma, including inflammation exacerbation, induction of AR, and finally disease propagation, we decided to verify the effect of several the most active 8-aminopurine-2,6-dione derivatives on IL-13 or TGF-β-induced responses in human bronchial epithelial cells isolated from respiratory mucosa of mild asthmatics.

2. Results and discussion

2.1. Chemistry

The designed *N*'-phenyl- and *N*'-benzylbutanamides (**27**–**37**) and *N*'-arylidenebutanehydrazides (**38**–**43**) of 8-aminopurine-2,6-dione were

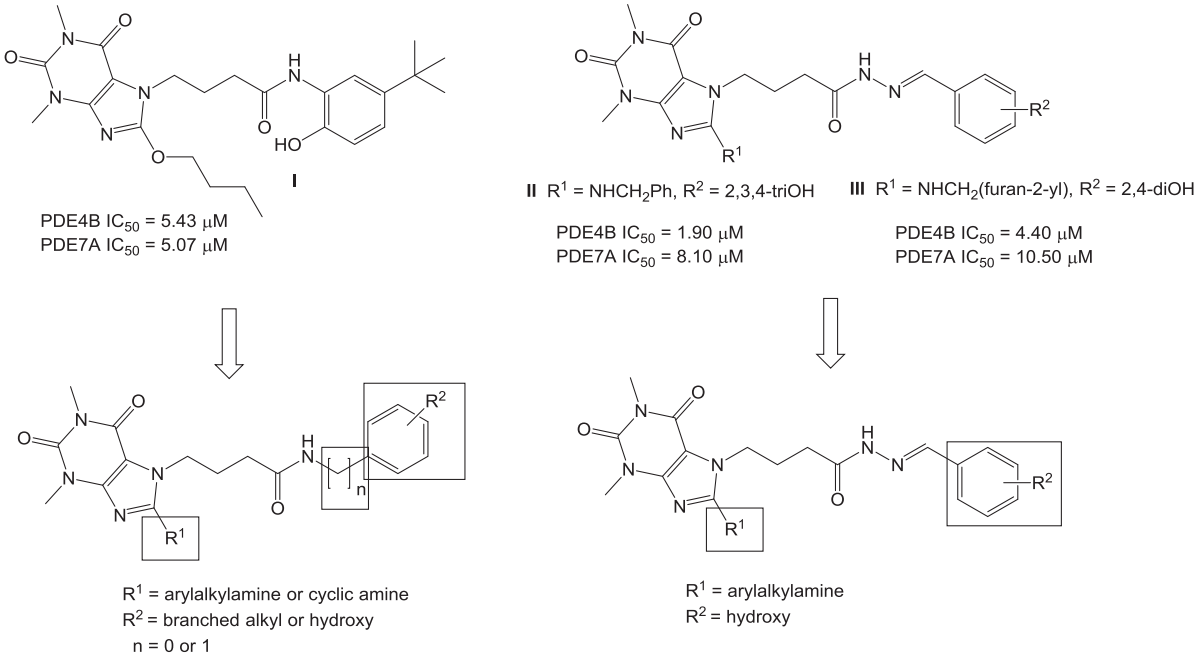


Fig. 1. Design of novel 8-aminopurine-2,6-dione derivatives by structural modification of the most active compounds **I** - **III** from our previous studies [17,18].

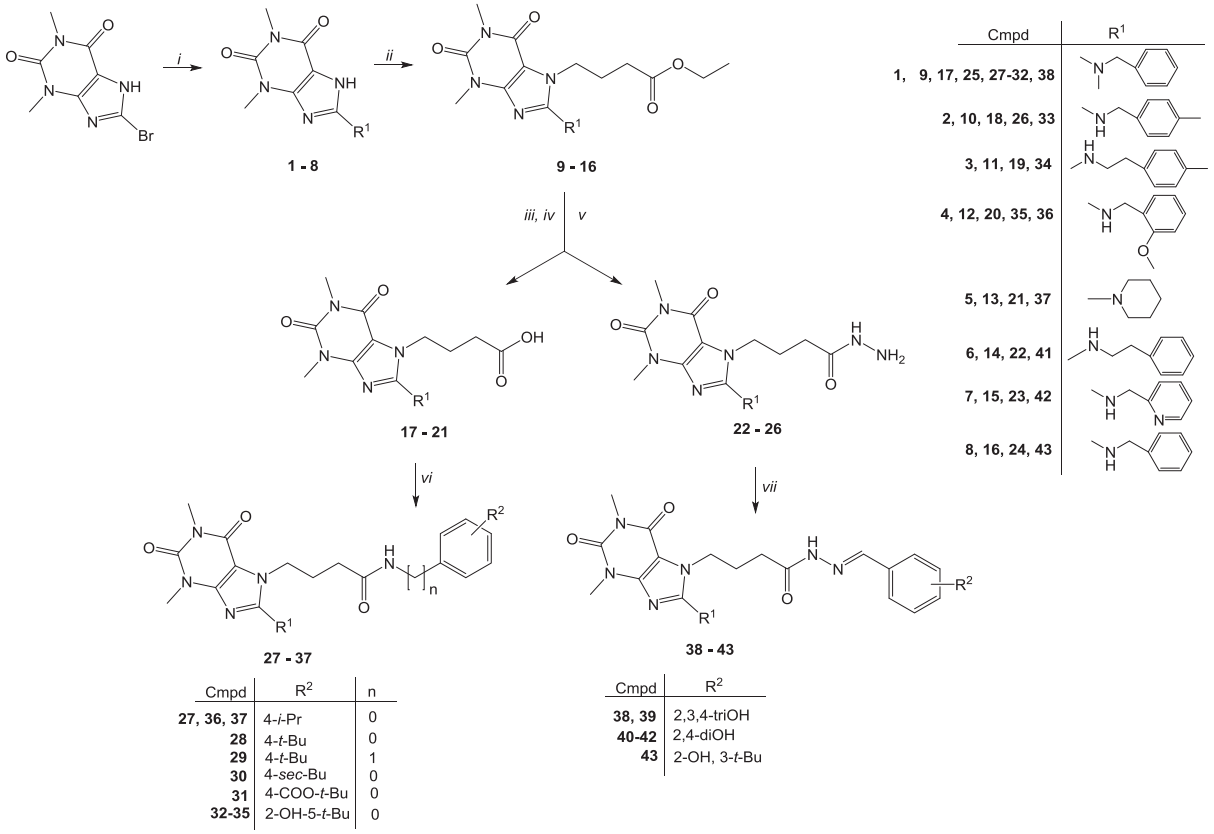
synthesized according to a multistep procedure presented in Scheme 1. In the first step, previously obtained 8-bromo-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (8-BrTHEO) [19] was treated with appropriate amine in refluxing 2-methoxyethanol to give 8-arylalkylamine or 8-piperidine derivatives (**1**–**8**). Next, the obtained 8-amine derivatives were alkylated in the 7-position with ethyl 4-bromobutyrate in the presence of K_2CO_3 and catalytic amount of TEBA in refluxing acetone to give ethyl esters **9**–**16**. In the next step, ester **9**, **10**, **11**, **12**, or **13** was subsequently hydrolysed using KOH and then acidified with concentrated HCl to obtain the corresponding acids **17**, **18**, **19**, **20**, or **21**. In the final step, the synthesized acids were coupled with different aniline or benzylamine derivatives in the presence of di(1*H*-imidazol-1-yl)methanone (CDI), yielding amides **27**–**37**. The treatment of esters **9**, **10**, **14**, **15,** or **16** with hydrazine hydrate in anhydrous ethanol gave appropriate hydrazide **22**–**26**. The synthesis of the final *N*'-arylidenebutanehydrazides (**38**–**43**) was based on the condensation reaction of the corresponding nonsubstituted hydrazide with various aromatic aldehydes in methanol in the presence of a catalytic amount of HCl. Compounds **38**–**43** were obtained as mixtures of *E* and *Z* isomers as was indicated by the results of ^{1}H NMR analysis.

2.2. Biological evaluation

2.2.1. Phosphodiesterase inhibition and structure-activity relationships

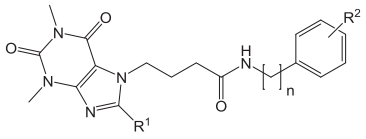
PDE4B is present in many immune and structural cells involved in asthma pathogenesis [15] and it plays an essential role in type 2 helper (Th_2) cell activation and dendritic cell recruitment during airway inflammation [20]. Many of the selective PDE4 inhibitors are currently being extensively studied for the treatment of lung diseases, and some of them, such as roflumilast, are already used in the clinic for COPD therapy [21]. The anti-inflammatory properties of PDE4 inhibitors may be further enhanced by inhibiting other PDE isoforms including 7A [15,22,23]. Thus, the first step of biological evaluation was *in vitro* screening of the new 8-aminopurine-2,6-dione derivatives **27**–**43** to assess their ability to inhibit human recombinant PDE4B and 7A isoenzymes.

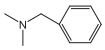
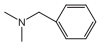
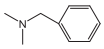
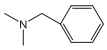
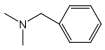
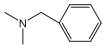
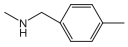
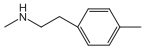
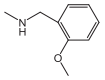
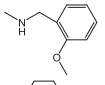
Generally, numerous of the newly synthesized derivatives showed inhibitory activity against PDE4B and/or 7A isoforms (Tables 1 and 2). Compounds **32**, **35**, **38**, and **39** possessed mono-digit micromolar IC_{50} values for PDE4B, thus, they were about 10 times higher than that of rolipram, used as a reference PDE4 inhibitor. At the same time, the most active compounds **31**, **38**, **39,** and **41** were slightly weaker PDE7A inhibitors in comparison with BRL-50481, a well-known PDE7 inhibitor. The obtained results allowed to conclude some structure–activity relationships (SAR) within the two evaluated series of compounds. First, we focused on the impact of the kind of amine substituent at the 8-position of the purine-2,6-dione core. Among the compounds **32**–**35** with the same 2-OH, 5-*t*-Bu substituent in the phenyl group, the benzyl(methyl) amine or 2-methoxybenzylamine substituent was preferable for inhibitory activity against PDE4B isoenzyme. The SAR explored around a amide benzene ring in the series of anilide derivatives with benzyl (methyl)amine at the 8-position of purine-2,6-dione showed that 4-COO-*t*-Bu substituent (**31**) provided the most interesting dual PDE4B/7A inhibition. Moreover, the results represented in Table 1 demonstrated that the replacement of anilide fragment (**28**) by benzylamide one (**29**) slightly increased PDE7A inhibitory activity. The introduction of a hydrazide fragment instead of the amide moiety enhanced PDE4B/7A inhibitory activity as was evidenced by counterparts with the same 8-amino substituents (e.g., **29**–**32** *vs*. **38** and **33** *vs*. **39**). In the series of arylbutanehydrazides, the 2,3,4-tri-OH substitution in the phenyl group was the most preferable for both PDE4 and PDE7 inhibition (**38** and **39**) (Table 2). The 2,4-dihydroxy analogs were 5–10 fold less active PDE4B



Scheme 1. Synthesis of *N*'-phenyl- and *N*'-benzylbutanamides (**27**–**37**) and *N*'-arylidenebutanehydrazides (**38**–**43**) of 8-aminopurine-2,6-dione. Reagents and conditions: (*i*) amine, 2-methoxyethanol, reflux; (*ii*) ethyl 4-bromobutyrate, K_2CO_3, TEBA, acetone, reflux; (*iii*) KOH, acetone/H_2O, reflux; (*iv*) conc. HCl; (*v*) hydrazide hydrate, anhydrous ethanol, reflux; (*vi*) aniline or benzylamine derivative, DMF, CDI, rt; (*vii*) benzaldehyde derivative, methanol, HCl cat., rt.

Table 1
The structures and PDE4B and PDE7A inhibitory activities of *N*'-phenyl- and *N*'-benzylbutanamides of 8-aminopurine-2,6-dione.

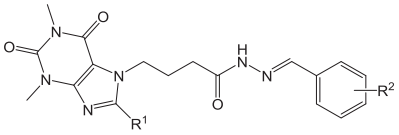


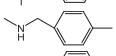
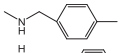
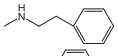
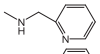
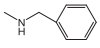
Cmpd	R^1	R^2	n	PDE4B IC_{50} [μM][a]	PDE7A IC_{50} [μM][a]
27		4-*i*-Pr	0	71.19	67.11
28		4-*t*-Bu	0	>200	51.64
29		4-*t*-Bu	1	>200	28.85
30		4-*sec*-Bu	0	123.96	81.63
31		4-COO-*t*-Bu	0	35.90	12.14
32		2-OH, 5-*t*-Bu	0	4.31	113.30
33		2-OH, 5-*t*-Bu	0	161.41	150.90
34		2-OH, 5-*t*-Bu	0	42.13	135.10
35		2-OH, 5-*t*-Bu	0	5.22	151.00
36		4-*i*-Pr	0	102.33	124.40
37		4-*i*-Pr	0	106.10	86.54
Rolipram	–	–	–	0.38	–
BRL-50481	–	–	–	–	4.29
Theophylline[b]	–	–	–	>1000	>1000

[a] IC_{50} values based on the results of three independent experiments.
[b] Values were published previously [17].

and PDE7A inhibitors, respectively. On the other hand, the kind of arylalkylamine group at the 8-position of purine-2,6-dione was essential for the PDE4B/7A activity. The replacement of the phenylethylamine moiety by the pyridin-2-yl-amine group had a negative impact on both PDE4B and PDE7A inhibition (**41** *vs.* **42**). Disappointingly, the 2-OH, 3-*t*-Bu substituent in the phenyl group led to a loss of activity toward both evaluated enzymes. The above-mentioned observations suggested that both the 8-arylalkylamine substituent and the kind of phenyl substitution at the 7-position of purine-2,6-dione had influence on the inhibitory properties toward the evaluated isoenzymes. The important effects of

Table 2
The structures and PDE4B and PDE7A inhibitory activity of *N*'-arylidenebutanehydrazides of 8-aminopurine-2,6-dione.



Cmpd	R^1	R^2	PDE4B IC_{50} [μM][a]	PDE7A IC_{50} [μM][a]
38		2,3,4-triOH	2.81	10.30
39		2,3,4-triOH	5.10	16.50
40		2,4-diOH	26.18	178.60
41		2,4-diOH	15.25	16.89
42		2,4-diOH	105.57	> 200
43		2-OH, 3-*t*-Bu	> 200	> 200
Rolipram	–	–	0.38	–
BRL-50481	–	–	–	4.29
Theophylline[b]	–	–	>1000	>1000

[a] IC_{50} value based on the results of three independent experiments.
[b] values were published previously [17].

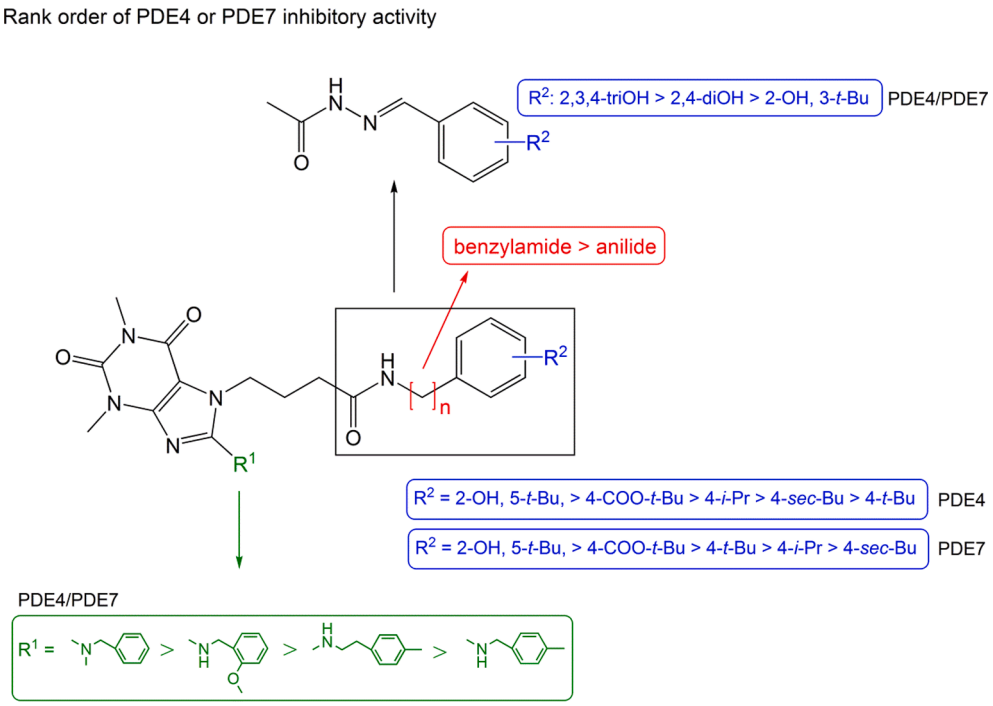


Fig. 2. Summary of the SAR studies of *N'*-phenyl- and *N'*-benzylbutanamides and *N'*-arylidenebutanehydrazides as PDE4B and/or PDE7A inhibitors.

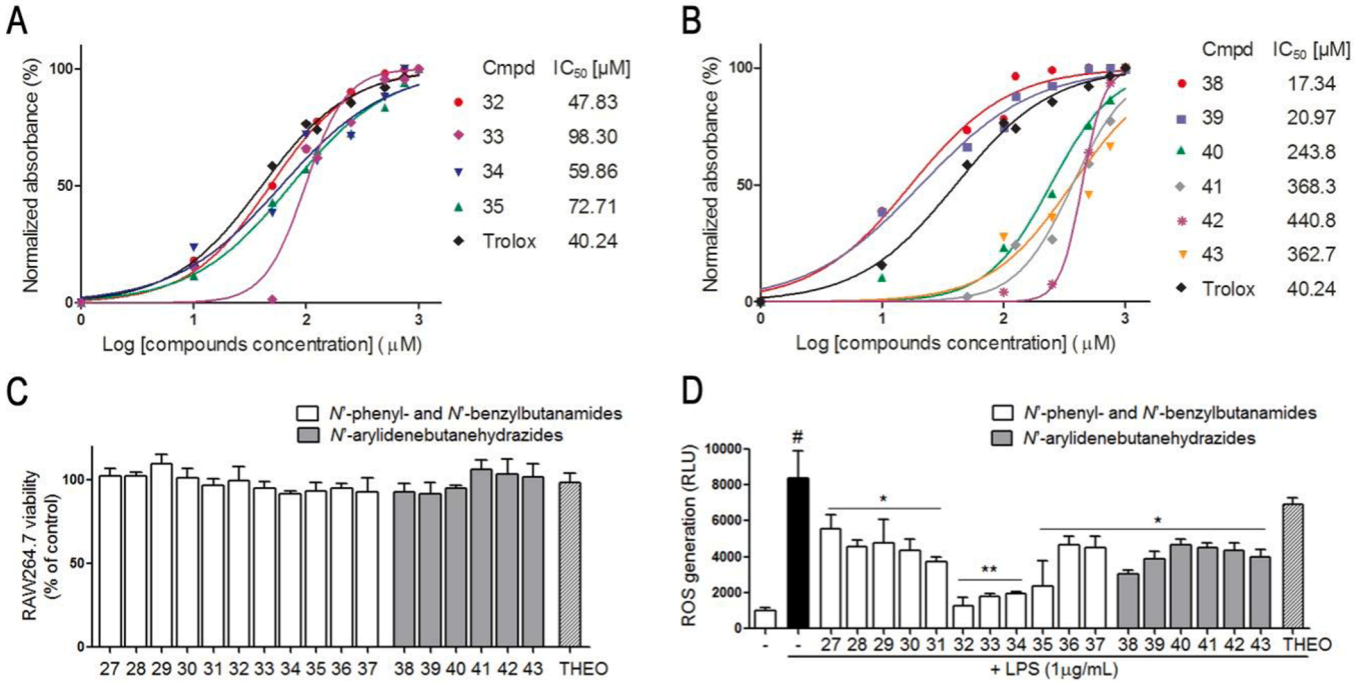


Fig. 3. Antioxidant properties of 8-aminopurine-2,6-dione derivatives. *N'*-phenyl- or *N'*-benzylbutanamide (**27**–**37**) (A) and *N'*-arylidenebutanehydrazide (**38**–**43**) (B) antioxidant ability was screened by the DPPH assay. The calculated IC_{50} values for the selected compounds are included in the graphs. (C) RAW264.7 cells were cultured in the presence of **27**–**43** (10 μM) or theophylline (10 μM) and cell viability was determined after 24 h of incubation. Each bar represents the mean (±S.E.M.) of 3 independent experiments. (D) RAW264.7 cells were cultured in the presence of **27**–**43** (10 μM) or theophylline (10 μM) 1 h before LPS (1 μg/mL) treatment. The level of ROS production was determined after 24 h of incubation. Values represent means (±S.E.M.) of 4 repeats. The results were considered statistically significant at the p level of 0.05 (*) or 0.005 (**), against control (#) and LPS (*).

some structural modifications on PDE4B/7A inhibitory activity were summarized in Fig. 2. The advantage of new compounds over theophylline as PDE4B/7A inhibitors is evident. This anti-asthmatic drug exerted considerably lower inhibitory activity toward the evaluated enzymes as expressed by significantly higher (above 1 mM) IC_{50} values (Tables 1 and 2).

Knowing that 8-aminopurine-2,6-dione derivatives showed differentiated PDE4B/7A inhibitory activity (Tables 1 and 2), in the next step, we tested whether the obtained IC_{50} values for the inhibition of these isoforms correlate with the basic antioxidant and anti-inflammatory

properties of the investigated compounds. To verify this, we performed a series of *in vitro* experiments for all newly synthesized derivatives (**27**–**43**). The aim of this approach was to select the most promising compounds for a detailed research of their anti-asthmatic properties.

2.2.2. *Antioxidant and anti-inflammatory screening of new 8-aminopurine-2,6-dione derivatives (27–43)*

It is widely accepted that the beneficial effects of PDE inhibitors in asthma mainly depend on their broad anti-inflammatory activity against numerous airway as well as inflammatory cells [22,23]. On the other hand, it is known that airway inflammation in asthma can also be activated in response to an oxidative imbalance between the production of reactive oxygen species (ROS) and the availability of antioxidants or radical scavengers [24]. Therefore, the maintenance of chronic inflammation in the airways microenvironment is largely dependent on ROS overproduction and the interdependence of the two processes by positive feedback [24,25]. Considering the above premises, in the next part of the study we verified both the antioxidant and anti-inflammatory properties of new 8-aminopurine-2,6-dione derivatives (**27**–**43**). An antioxidant, based on electron-transfer assay was performed with the artificial free radical − 2,2-diphenyl-1-picryl-hydrazyl hydrate (DPPH). The obtained IC_{50} values for compounds that represented activity in DPPH assay are shown in Fig. 3A and B, for *N*'-phenyl- or *N*'-benzylbutanamides and *N*'-arylidenebutanehydrazides of 8-aminopurine-2,6-dione, respectively. The antioxidant activity of compounds **32**–**35** was comparable to Trolox, a known antioxidant used as a positive control (Fig. 3A). Interestingly, for compounds **38** and **39**, the free radical scavenging ability was even better than that of Trolox (Fig. 3B), while for theophylline alone it was undetectable. Moreover, it was observed that the antioxidant activities of 8-aminopurine-2,6-dione derivatives were detectable for compounds with hydroxy groups within the phenyl ring (**32**–**35** and **38**–**43**). Since it was previously shown that the antioxidant activity related to DPPH scavenging activity can be characteristic of phenolic acids or other phenolic compounds with hydroxy groups [26,27], it cannot be ruled out that the antioxidant activity of some derivatives demonstrated in the DPPH assay may arise from their characteristic structure within the R^2 substituent. With the above in mind as well as knowing that the antioxidant or anti-inflammatory response in biological systems may depend on many different processes, for further research, we engaged an *in vitro* cellular model. We used the well-known and widely adapted model of RAW264.7 murine macrophages. To better mimic the inflammation, cells were activated by LPS isolated from *E. coli* bacteria. The safety of 8-aminopurine-2,6-dione derivatives against murine macrophages was confirmed by the MTT assay (Fig. 3C). None of the tested compounds changed the viability of RAW264.7 cells. Following this, we analyzed the presence of free radicals in LPS-induced murine macrophages cultured in the presence of tested derivatives. A bioluminescent assay that measures the level of hydrogen peroxide (H_2O_2), as one of ROS, revealed a significant but differentiated effect of individual compounds on activated RAW264.7 cells, thus confirming their antioxidant properties (Fig. 3D). Compounds **32**–**35** from the *N*'-phenyl- or *N*'-benzylbutanamide group and compound **38** from the *N*'-arylidenebutanehydrazide group, revealed the best properties of reducing the H_2O_2 level in LPS-induced murine macrophages (Fig. 3D).

Theophylline, a nonselective PDE inhibitor, also lowered ROS levels in the LPS-induced RAW264.7 cells, but 8-aminopurine-2,6-dione derivatives showed a stronger antioxidant effect. Similar results were obtained by Constantin *et al.*, who also demonstrated the preferential antioxidant properties of another group of 1,3-dimethylxanthine derivatives with the thiazolidine-4-one scaffold in relation to theophylline [28]. Oxidative stress may reduce the anti-inflammatory effects of corticosteroids and therefore may contribute to the relative insensitivity to corticosteroid therapy in asthma. Currently, the treatment of corticosteroid-resistant asthma appears to be a significant therapeutic challenge. It is known that theophylline may reduce oxidative stress during asthma [29]. In addition, Marwick *et al.* showed that theophylline, thanks to its antioxidant properties, can also restore the corticosteroid inhibition of proinflammatory mediators and histone acetylation in cells exposed to the oxidant [30]. As the novel 8-aminopurine-2,6-dione derivatives have stronger antioxidant properties than theophylline, they can also be considered as a beneficial alternative supporting corticosteroid therapy.

Macrophages activated by LPS can produce not only ROS, but also diverse proinflammatory molecules including nitric oxide (NO), tumor necrosis factor type alpha (TNF-α), or interleukin 6 (IL-6). Therefore, in the next stage of 8-aminopurine-2,6-dione derivatives screening, we focused on their anti-inflammatory activity. As indicated in Fig. 4, stimulation of murine macrophages with LPS resulted in a significant 22.5-fold, 29.5-fold, and 14.7-fold increase (in comparison to control) in NO (A), TNF-α (B), and IL-6 (C) levels, respectively. An addition of *N*'-phenyl- or *N*'-benzylbutanamide and *N*'-arylidenebutanehydrazide derivatives of 8-aminopurine-2,6-dione led to a significant reduction in the level of all tested mediators in LPS-induced RAW264.7 supernatants. The most diverse effect was observed in the case of NO level (Fig. 4A). Compound **35** from the *N*'-phenyl- or *N*'-benzylbutanamide group and compound **38** from the *N*'-arylidenebutanehydrazide group caused a significant inhibition of LPS-induced NO production in murine macrophages from 45.06 μM (observed after LPS treatment) to 7.34 μM and 14.66 μM, respectively. As for TNF-α and IL-6, all 8-aminopurine-2,6-dione derivatives showed a similar potency in reducing LPS-induced

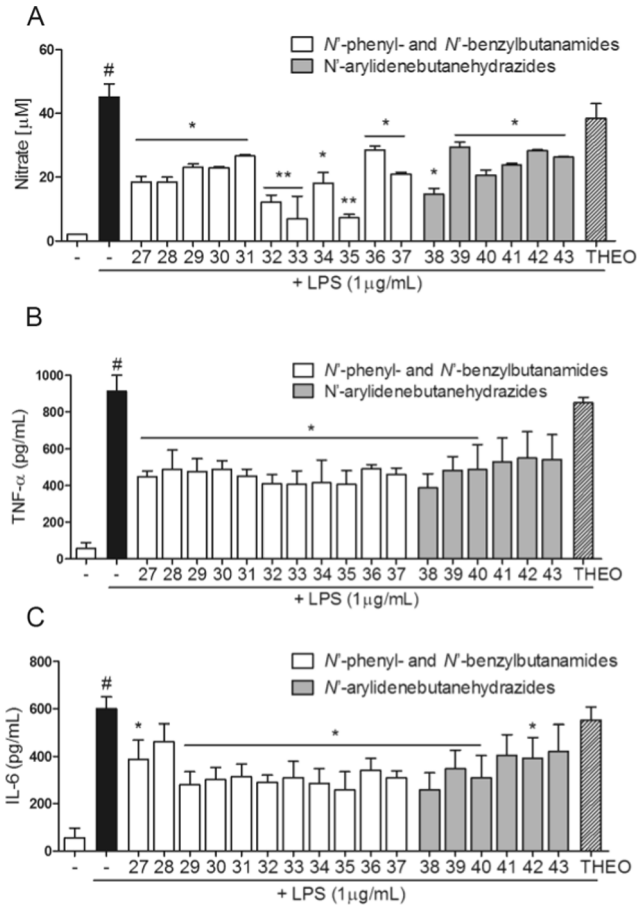


Fig. 4. Anti-inflammatory properties of 8-aminopurine-2,6-dione derivatives. RAW264.7 cells were cultured in the presence of **27**–**43** (10 μM) or theophylline (10 μM) 1 h before LPS (1 μg/mL) treatment. The level of NO, TNF-α, and IL-6 production was determined after 24 h of incubation. Values represent means (±S.E.M.) of 3 independent experiments run in duplicate. The results were considered statistically significant at the p level of 0.05 (*) or 0.005 (**), with control (#) and LPS (*).

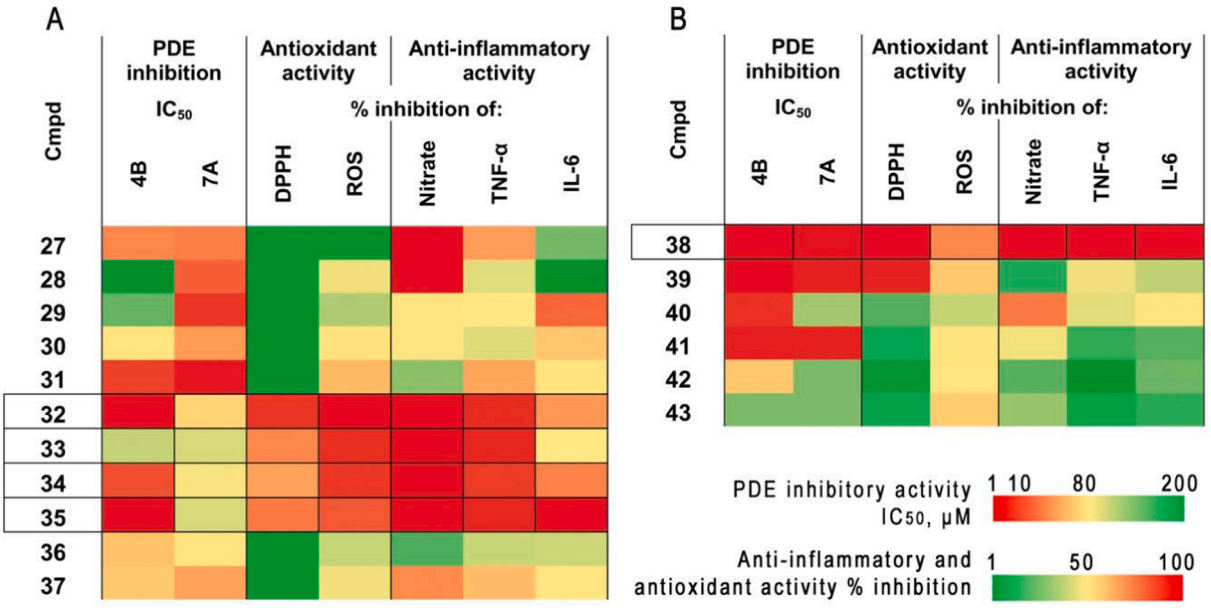


Fig. 5. Graphical comparison of 8-aminopurine-2,6-dione derivatives biological activity screening results. The heat map representing *N*'-phenyl- and *N*'-benzyl-butanamide (A) and *N*'-arylidenebutanehydrazide (B) derivatives of 8-aminopurine-2,6-dione antioxidant, and anti-inflammatory properties and PDE4B and 7A inhibitory activity. The red boxes represent the strongest PDE inhibitor activity and the highest reduction in oxidative stress and inflammation.

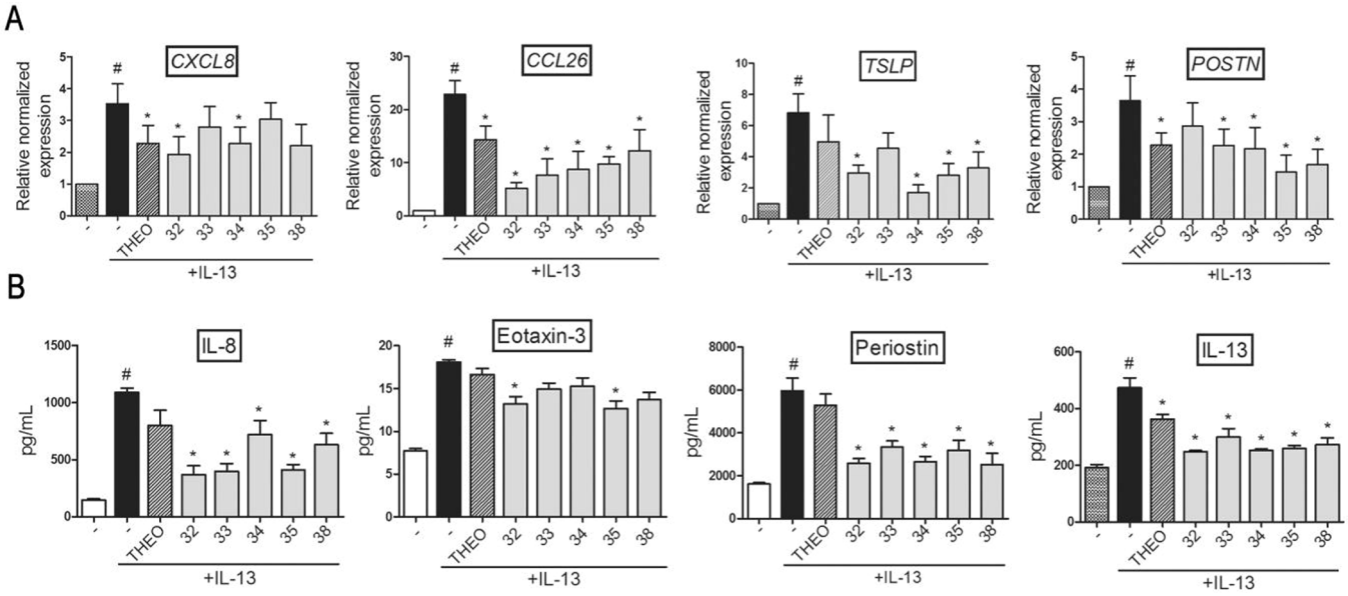


Fig. 6. Anti-inflammatory effects of selected 8-aminopurine-2,6-dione derivatives in IL-13-induced human bronchial epithelial cells. HBECs were cultured in the presence of **32**–**35**, **38**, and theophylline (10 μM) 1 h before IL-13 (1 ng/mL) treatment. (A) Expression of *CXCL8*, *CCL26*, *TSLP*, and *POSTN* genes in comparison to *18S rRNA* was quantified by qPCR after 24 h of incubation. (B) The levels of IL-8, eotaxin-3, periostin, and IL-13 in HBEC supernatant were determined by Luminex assay after 48 h of incubation. Values represent means (±S.E.M.) of three different HBEC cultures run in duplicates. The results were considered statistically significant at the p level of 0.05, against control (#) and IL-13 (*).

secretion of these components to the extracellular space (Fig. 4B and C). An almost two-fold decrease in LPS-induced TNF-α levels was observed in supernatants collected from macrophages treated with all 8-aminopurine-2,6-dione derivatives. The obtained results confirmed the varied and strong anti-inflammatory properties of all tested derivatives over theophylline.

As summarized on the heat map (Fig. 5), the most preferential activity (preferred IC_{50} values with simultaneous antioxidant and/or anti-inflammatory activity) was attributed to **32**–**35**, and **38** derivatives. They revealed satisfactory properties as PDE inhibitors, but also significant antioxidant and/or anti-inflammatory activity *in vitro*. Although compounds **31** and **39**–**41** were good inhibitors of PDE4B and 7A, they did not show any significant biological activity in the LPS-induced murine macrophage model. Taking into account not only the PDE4B/7A inhibitory activity, but also the antioxidant and anti-inflammatory properties from the group of analyzed derivatives, only **32**–**35** from the *N*'-phenyl- or *N*'-benzylbutanamide group and **38** from the *N*'-arylidenebutanehydrazide group of 8-aminopurine-2,6-dione derivatives were selected for a detailed *in vitro* research of their anti-asthmatic properties in human bronchial epithelial cells isolated from asthmatic patients.

2.2.3. *Anti-inflammatory and anti-fibrotic effects of **32**–**35** and **38** in human bronchial epithelial cells*

Bronchial epithelium plays a central role in asthma pathogenesis by increasing inflammation and initiating multicellular events, finally leading to airway remodeling [5,6,8]. This is because the triggered epithelial cells recruit and activate a variety of immune cells, which serve as whole cascade amplifiers. The factors/substances secreted by both types of cells then stimulate mesenchymal effector cells (fibroblasts and smooth muscle cells), responsible for inducing and enhancing airway remodeling [31]. Therefore, to determine the utility of 8-aminopurine-2,6-dione derivatives as anti-asthmatic agents, we performed a series of experiments in human bronchial epithelial cells (HBECs). Cells were isolated from tracheobronchial brush biopsies sampled from patients with moderate asthma (n = 3). To better reflect the conditions to which HBECs are exposed during chronic inflammation in the airways, we stimulated the cells with a type 2 (T2) proinflammatory cytokine - IL-13 or with a profibrotic growth factor - TGF-β. IL-13, in addition to IL-4 and IL-5, is one of the major cytokines characteristic for T2 inflammatory phenotype of asthma [32,33]. In turn, TGF-β is overexpressed in asthmatic airways where is responsible for mediating AR-related changes in a variety of mesenchymal cells [34,35].

We initially investigated the anti-inflammatory potential of 8-aminopurine-2,6-dione derivatives. To achieve this goal, we selected several proinflammatory mediators (*CXCL8*/IL-8, *CCL26*/eotaxin-3, *TSLP* (Thymic stromal lymphopoietin), and *POSTN*/periostin) and verified the effect of **32**–**35** and **38** on their transcript levels (Fig. 6A) and presence in supernatants collected from IL-13-induced HBEC cultures (Fig. 6B). All above-mentioned cytokines are produced by bronchial epithelial cells in asthma and substantially contribute to airway inflammation: IL-8 is significantly increased in uncontrolled asthma and participates in neutrophilic inflammation [36,37]; eotaxin-3 enhances the attraction of eosinophils to the asthmatic lungs [38]; TSLP is a biomarker and a new therapeutic target in asthma [39,40]; periostin, a matricellular protein is involved in both eosinophilic inflammation and AR [41]. As expected, IL-13 significantly elevated expression of the studied genes (Fig. 6A) and the level of proinflammatory mediators in HBEC culture supernatants (Fig. 6B). The obtained data showed that the selected 8-aminopurine-2,6-dione derivatives exerted a strong inhibitory effect on the IL-13-induced *CXCL8*, *CCL26*, *TSLP*, and *POSTN* transcript levels (Fig. 6A), as well as IL-8, eotaxin-3, and periostin secretion (Fig. 6B) in HBECs. Our data revealed a significantly higher anti-inflammatory potential of the tested derivatives over the parent structure - theophylline. Depending on the analyzed inflammatory target, the selected 8-aminopurine-2,6-dione derivatives represented differential activity. The strongest inhibitory effect of the evaluated compounds was observed on *CXCL8* and *TSLP* transcripts and IL-8 and periostin supernatant levels. Since IL-13 is involved in inducing both AR and inflammation, but is also overproduced in severe asthma [42], we verified itself the level in HBEC supernatants. Treatment with 8-aminopurine-2,6-dione derivatives resulted in a decrease of IL-13 levels in activated HBECs. The obtained results confirmed that the selected 8-aminopurine-2,6-dione derivatives represent comparable anti-inflammatory activity in HBECs. Slightly stronger anti-inflammatory properties may be concluded for **32**–**35** derivatives containing in the phenyl ring of R^2 substituents, the 2-OH, 5-

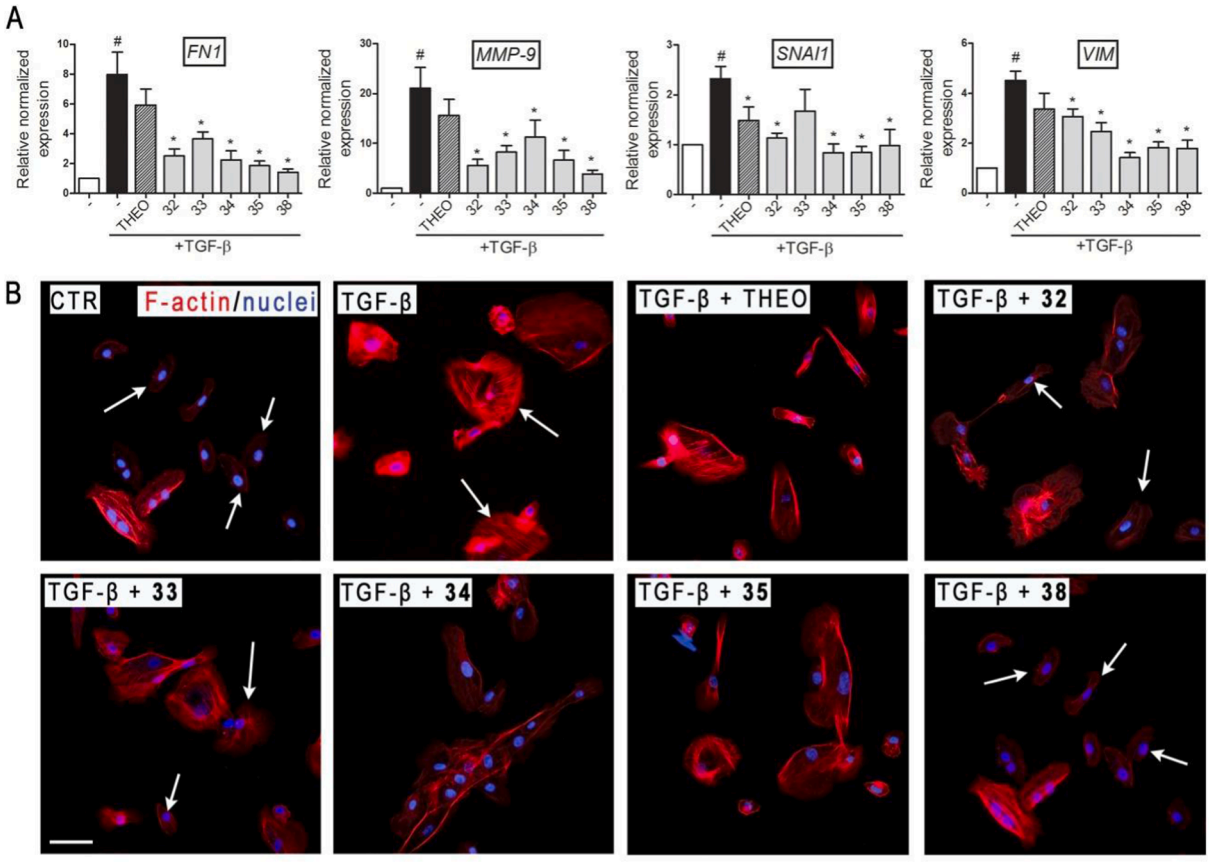


Fig. 7. Anti-fibrotic effects of selected 8-aminopurine-2,6-dione derivatives in TGF-β-induced human bronchial epithelial cells. HBECs were cultured in the presence of **32**–**35**, **38**, and theophylline (10 μM) 1 h before TGF-β (5 ng/mL) treatment. (A) Expression of *FN1*, *MMP-9*, *SNAI1*, and *VIM* genes in comparison to *18S rRNA* was quantified by qPCR after 24 h of incubation. Values represent means (±S.E.M.) of three different HBEC cultures run in duplicate. The results were considered statistically significant at the p level of 0.05, with control (#) and TGF-β (*). (B) Actin cytoskeleton was visualized in HBECs with TRITC-labeled phalloidin staining, after 48 h incubation. Nuclei were counterstained with Hoechst 33,342 dye. Microphotographs were taken using Leica DMiL LED Fluo microscope, 40 × objective, bar = 50 μm.

Table 3
Compounds **32**–**35** and **38** as pan-PDE inhibitors.

Comp	PDE - IC_{50} [μM][a]									
	1A	1B	3A	3B	4A	4B	4D	5A	7A	8A
32	>200	175.36	0.22	0.065	2.67	4.31	72.41	11.95	113.30	5.71
33	>200	>200	0.07	0.027	3.59	161.41	>200	11.12	150.90	>200
34	85.27	134.0	18.21	2.31	19.13	42.13	127.97	>200	135.10	171.09
35	>200	>200	0.99	0.275	1.68	5.22	>200	53.21	151	>200
38	16.32	10.89	0.27	0.055	1.31	2.81	1.88	25.42	10.30	0.17
Theophylline[b]	N/D	>1000	185.955	N/D	N/D	>1000	>1000	>1000	>1000	N/D
Reference	Vinpocetine		Milrinone		Rolipram			Tadalafil	BRL-50481	Dipyridamole
	27.93	20.66	0.11	0.064	0.18	0.38	7.58	0.32	4.29	8.26

[a] IC_{50} value based on the results of three independent experiments.
[b] values were published previously [17]; N/D – no data.

t-Bu residues. However, **38**, as a sole representative of *N*'-arylidenebutanehydrazide derivative of 8-aminopurine-2,6-dione, also markedly inhibited IL-13-induced inflammatory processes in HBECs.

The demonstrated anti-inflammatory properties of 8-aminopurine-2,6-dione derivatives prompted us to further evaluation of the compounds profile in terms of anti-fibrotic activity. Thus, we examined the expression of profibrotic genes, including fibronectin (*FN1*), metalloproteinase-9 (*MMP-9*), SNAIL transcription factor (*SNAI1*) and vimentin (*VIM*). Fibronectin, an ECM protein, is overproduced in asthma and drives both inflammation and AR [43]. Another fibrotic factor found in excess in asthmatic airways, is metalloproteinase-9, an ECM component disrupting its structure and combining multiple inflammatory and remodeling effects in the extracellular space [44,45]. In turn, overexpression of SNAIL1 and vimentin is one of the symptoms of HBEC epithelial-mesenchymal transition (EMT), a process promoting airway remodeling in asthma [46,47]. TGF-β, a prominent profibrotic factor, significantly increased the expression of all analyzed genes in HBECs (Fig. 7A). The strong profibrotic effect of TGF-β on bronchial epithelial cells was reversed by both theophylline and 8-aminopurine-2,6-dione derivatives (Fig. 6A). However, while theophylline only slightly decreased TGF-β-induced *FN1*, *MMP-9*, *SNAI1*, and *VIM* expression, **32**–**35** and **38** exerted a strong anti-fibrotic effect. The expression of all tested genes was significantly reduced under the influence of selected 8-aminopurine-2,6-dione derivatives, but the most prominent anti-fibrotic activity was attributed to **32**, **35,** and **38**. This was especially marked for TGF-β-induced *MMP-9* expression, where treatment with **32**, **35,** and **38** resulted in a 3.8-fold, 3.2-fold, and 5.5-fold decrease in the level of this transcript, respectively. The performed gene expression analysis confirmed the inhibitory activity of 8-aminopurine-2,6-dione derivatives against the TGF-β profibrotic action. To support this finding, we further stained F-actin filamentous in HBECs. Actin cytoskeleton rearrangement is one of EMT markers in epithelial cells [48]. As indicated on microphotographs (Fig. 7B), the TGF-β-treated HBECs spread out and flattened in shape, in addition, much more visible F-actin fibers could be observed in their cytoplasm. Contrary, the treatment with 8-aminopurine-2,6-dione derivatives resulted in a reversal of TGF-β-induced actin cytoskeleton organization. As indicated by the arrows, many cells returned to their original size and the number of visible actin microfibers was decreased. This effect was most pronounced for **32** and **38** treated HBECs. The observed rearrangement of the actin cytoskeleton induced by individual compounds confirmed the advantage of 8-aminopurine-2,6-dione derivatives over the prototype drug - theophylline.

*2.2.4. Inhibitory activity of **32**–**35** and **38** against selected asthma-related PDEs*

Our earlier studies performed on the group of purine-2,6-dione derivatives showed that these compounds are pan-PDE inhibitors having a favorable in relation to asthma pathophysiology/treatment, PDE inhibitory profile [17,18,49,50]. Compounds **32**–**35** and **38** obtained in the current study revealed very promising anti-inflammatory and anti-fibrotic properties in HBECs derived from patients with bronchial asthma, compared to the reference drug, theophylline. To explain the reason of their superiority over the parent structure, we also performed an extended analysis of their inhibition profile against other members of PDE family, including 1A, 1B, 3A, 3B, 4A, 4D, 5A, and 8A, important for the desired anti-inflammatory and anti-fibrotic properties [15,22,23]. PDE1 plays an important role in smooth muscle cells and vascular smooth muscle cell remodeling [22]. It has been shown to regulate smooth muscle cells proliferation as well as TGF-β-dependent myofibroblast differentiation [22,51]. In turn, PDE3 inhibition is responsible for airway smooth muscle relaxation and, thus, mediating bronchodilator effects [15,22,23]. Recently, it has been also concluded that PDE8 may represent a novel therapeutic target to modulate airway smooth muscle cell responsiveness and airway remodeling [52]. Our experiments revealed **32**–**35** and **38** as prominent PDE3A, PDE3B, and PDE4A inhibitors (Table 3). The calculated IC_{50} values for the selected 8-aminopurine-2,6-dione derivatives against these isoforms were close to the values of PDE3 selective inhibitor used in the study, milrinone, and quite close (only one order of magnitude lower) to the value of a strong and selective PDE4 inhibitor, rolipram. The tested compounds showed a differential activity against PDE1 and only **34** and **38** were able to inhibit this isoform. Interestingly, it turned out that compounds **32** and **38** were strong PDE8 inhibitors. As demonstrated in Table 3, **32** inhibited PDE8A comparably to dipyridamole, while the IC_{50} value of **38** was an order of magnitude lower than that of the reference drug. We also tested the activity of the compounds against PDE5 and found that **32**, **33**, **35**, and **38** are inhibitors of this cGMP-selective isoform. Finally, we verified the activity of selected 8-aminopurine-2,6-dione derivatives against PDE, the inhibition of which is responsible for the potential side effects of PDE inhibitors, that is, PDE4D [53]. Except compound **38**, **32**–**35** derivatives exhibited low PDE4D inhibitory properties, indicating that they may be devoid of adverse emetogenic effects resulting from PDE4D blockade [53]. Regardless of the IC_{50} values presented in Table 3, compounds **32**–**35** and **38** had much stronger inhibitory properties on individual PDEs than the prototype drug theophylline. Therefore, it can be summarized that 8-aminopurine-2,6-dione derivatives are promising representatives of the pan-PDE inhibitors that may be much more potent in the treatment of asthma and related inflammatory lung diseases than the nonselective prototype purine-2,6-dione core structure, theophylline.

Recently, there have been many reports indicating the benefits of using selective or dual PDE inhibitors in reducing the asthmatic phenotype of bronchial epithelial cells [54–59]. However, our data obtained in HBECs clearly indicate that pan-PDE inhibitors constitute an interesting alternative when targeting a therapeutic possibility in asthmatic airway epithelium. These results are of particular interest knowing that different isoforms are present in the bronchial epithelium [60,61] and, furthermore, changes in the expression of these isoforms during disease progression cannot be excluded. Several reasons for the promising activity of pan-PDE inhibitors in bronchial epithelium can be distinguished: 1) PDE expression is diverse and cell-specific as well as PDE intracellular localization is compartmentalized, so by targeting

different isoforms a multidirectional effect can be achieved; 2) proinflammatory and profibrotic intracellular signaling pathways can be controlled by different isoforms; and finally, 3) by using pan-PDE inhibitors, it is possible to lower the therapeutic dose while maintaining a similar activity as with selective ones at higher doses, which also means minimizing resulting final side effects. Theophylline is currently the only nonselective PDE inhibitor that affects multiple isoforms and is approved for asthma therapy. Unfortunately, due to its side effects, narrow therapeutic index, nonlinear pharmacokinetics, and the availability of other drugs, its use in the clinic is now rather occasional. However, some reports pointing to a preferential role of theophylline in the context of severe asthma cases or patients resistant to standard pharmacotherapy have been published [16,62]. It was suggested that theophylline administrated even at low doses, can restore the benefits of glucocorticosteroids [30,62–64]. This sheds new light on the group of pan-PDE inhibitors and their potential supportive effects in asthma therapy. The presented data clearly demonstrate that the structural modifications in positions 7 and 8 of the purine-2,6-dione significantly increase the preference of the new derivatives to particular PDE isoenzymes and are responsible for their efficiency as pan-PDE inhibitors. Both modifications are responsible for this effect, but substitutions in positions 7 and 8 of the purine-2,6-dione core with hydroxy and *N*-methylbenzylamine groups, respectively, seem to be particularly important. Together, an *in vitro* data on 8-aminopurine-2,6-dione derivatives activity in HBECs indicate that these compounds represent comprehensive and multifunctional agents that can be considered as promising drug candidates in asthma treatment.

3. Conclusions

In this study, novel *N*'-phenyl- or *N*'-benzylbutanamides and *N*'-arylidenebutanehydrazides of 8-aminopurine-2,6-dione were designed and their synthesis was described. These compounds were revealed to be effective pan-PDE inhibitors, with preferential activity against PDE3, PDE4, PDE7, and PDE8. Furthermore, the 8-aminopurine-2,6-dione derivatives represented prominent anti-inflammatory, antioxidant, and potential anti-fibrotic activity. Thus, based on this study results, we suggest that 8-aminopurine-2,6-dione derivatives are promising multifunctional agents with possible use in asthma therapy. They target key gene expression in airway epithelium, leading to inflammation and remodeling related changes reduction. The obtained results indicate that **32**–**35** compounds from the *N*'-phenyl- or *N*'-benzylbutanamide group and compound **38** from the *N*'-arylidenebutanehydrazide group of 8-aminopurine-2,6-dione are suitable candidates for further preclinical testing, including, *e.g.*, a detailed EMT study or *in vivo* animal asthma models. Consequently, an interesting dual anti-inflammatory and anti-fibrotic profile of the 8-aminopurine-2,6-dione derivatives can be a base for the development of further pan-PDE inhibitors for use in asthma therapy.

4. Experimental section

4.1. Chemistry

All reagents and solvents were purchased from Sigma Aldrich, TCI Europe, or Fluorochem. Thin layer chromatography was performed on Merck silica gel 60 F_{254} aluminium sheets (Merck, Darmstadt, Germany) with the following solvents: (A) dichloromethane/methanol (9.5:0.5, v/v), (B) dichloromethane/methanol (9.0:1.0, v/v) or (C) dichloromethane/methanol (5.0:5.0, v/v). Spots were detected based on their absorption under UV light ($\lambda = 254$ nm). Column chromatography was performed on Merck silica gel 60 (63–200 mm) with the solvent: A or B.

^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM-ECZR500 RS1 (ECZR version) at 500 MHz and 126 MHz, respectively, using TMS (0.00 ppm) as an internal standard, as well as $CDCl_3$ or DMSO-d_6 as solvents. Chemical shifts were expressed in δ (ppm) and the coupling constants J in Hertz (Hz) and the splitting patterns are designated as follows: s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), quint (quintet), and m (multiplet).

LC/MS analyses were performed on Waters Acquity TQD apparatus with an eλ DAD detector. For mass spectrometry, ESI^+ (electrospray positive) ionization mode was used. UV spectra were obtained in the range of 200–700 nm. For establishing the purity of the compounds, UV chromatograms were used. The UPLC/MS purity of all investigated compounds was determined to be over 95%.

Melting points (mp) were determined on a Büchi apparatus and are uncorrected. Elemental analysis was performed with Elementar Vario EL III apparatus. UV, MS, and ^{1}H NMR spectra of all final compounds **27**–**43** were attached in the supplementary material.

4.1.1. Synthesis of intermediates

The intermediates: 8-amino derivatives **1**–**8**, esters **9**–**16**, acids **17**–**21**, and hydrazides **22**–**26** were prepared starting from 8-bromo-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (8-BrTHEO) following the process shown in Scheme 1 according to the previously reported manner [49,50]. The following compounds: 8-(benzyl(methyl)amino)-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (**1**) [65]. 1,3-Dimethyl-8-(piperidin-1-yl)-3,7-dihydro-1*H*-purine-2,6-dione (**5**) [66]. The following compounds: 1,3-dimethyl-8-(phenethylamino)-3,7-dihydro-1*H*-purine-2,6-dione (**6**), 1,3-dimethyl-8-((pyridin-2-ylmethyl)amino)-3,7-dihydro-1*H*-purine-2,6-dione (**7**), 8-(benzylamino)-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (**8**), ethyl 4-(1,3-dimethyl-2,6-dioxo-8-(phenethylamino)-1,2,3,6-tetrahydro-7*H*-purin-7-yl)butanoate (**14**), ethyl 4-(1,3-dimethyl-2,6-dioxo-8-((pyridin-2-ylmethyl)amino)-1,2,3,6-tetrahydro-7*H*-purin-7-yl)butanoate (**15**), ethyl 4-(8-(benzylamino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7*H*-purin-7-yl)butanoate (**16**), 4-(1,3-dimethyl-2,6-dioxo-8-(phenethylamino)-1,2,3,6-tetrahydro-7*H*-purin-7-yl)butanehydrazide (**22**), 4-(1,3-dimethyl-2,6-dioxo-8-((pyridin-2-ylmethyl)amino)-1,2,3,6-tetrahydro-7*H*-purin-7-yl)butanehydrazide (**23**), 4-(8-(benzylamino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7*H*-purin-7-yl)butanehydrazide (**24**) were reported previously [67–69].

*4.1.1.1. 1,3-Dimethyl-8-((4-methylbenzyl)amino)-3,7-dihydro-1H-purine-2,6-dione (**2**).* From 8-BrTHEO in 75% yield; mp 238–239 °C; $R_f = 0.34$ (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 2.22 (s, 3H, CH_3), 3.13 (s, 3H, N^{1}CH_3), 3.30 (s, 3H, N^{3}CH_3), 4.35 (d, $J = 6.66$ Hz, 2H, NHCH_2), 7.08 (d, $J = 7.70$ Hz, 2H, 3,5-Ph), 7.18 (d, $J = 8.00$ Hz, 2H, 2,6-Ph), 7.62 (t, $J = 6.62$ Hz, 1H, N*H*CH$_2$), 11.52 (s, 1H, N^7*H*). LC/MS: *m/z* calc. 300.14, found 300.21; Formula $C_{15}H_{17}N_5O_2$; MW 299.32.

*4.1.1.2. 1,3-Dimethyl-8-((4-methylphenethyl)amino)-3,7-dihydro-1H-purine-2,6-dione (**3**).* From 8-BrTHEO in 59% yield; mp 214–215 °C; $R_f = 0.46$ (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 2.22 (s, 3H, CH_3), 2.73 (t, $J = 7.55$ Hz, 2H, NHCH$_2$CH_2), 3.15 (s, 3H, N^{1}CH_3), 3.31 (s, 3H, N^{3}CH_3), 3.34–3.41 (m, 2H, NHCH_2CH$_2$), 7.06 (d, $J = 7.80$ Hz, 2H, 2,6-Ph), 7.10 (d, $J = 8.00$ Hz, 2H, 3,5-Ph), 7.14 (t, $J = 5.98$ Hz, 1H, N*H*CH$_2$CH$_2$), 11.46 (s, 1H, N^7*H*). LC/MS: *m/z* calc. 314.16, found 314.15; Formula $C_{16}H_{19}N_5O_2$; MW 313.35.

*4.1.1.3. 8-((2-Methoxybenzyl)amino)-1,3-dimethyl-3,7-dihydro-1H-purine-2,6-dione (**4**).* From 8-BrTHEO in 83% yield; mp 254–256 °C; $R_f = 0.37$ (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 3.14 (s, 3H, N^{1}CH_3), 3.30 (s, 3H, N^{3}CH_3), 3.77 (s, 3H, OCH_3), 4.38 (d, $J = 6.30$ Hz, 2H, NHCH_2), 6.86 (t, $J = 7.45$ Hz, 1H, 4-Ph), 6.95 (d, $J = 8.02$ Hz, 1H, 3-Ph), 7.15 (d, $J = 6.87$ Hz, 1H, 6-Ph), 7.20 (t, $J = 8.00$ Hz, 1H, 5-Ph), 7.33 (t, $J = 6.59$ Hz, 1H, N*H*CH$_2$), 11.47 (s, 1H, N^{7}N*H*). LC/MS: *m/z* calc. 316.14, found 316.21; Formula $C_{15}H_{17}N_5O_3$; MW 315.33.

*4.1.1.4. Ethyl 4-(8-(benzyl(methyl)amino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanoate (**9**).* From **1** in 73% yield; R_f

= 0.78 (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.09 (t, J = 7.16 Hz, 3H, COOCH$_2$C*H*$_3$), 1.93 (quin, J = 7.02 Hz, 2H, CH$_2$C*H*$_2$CH$_2$), 2.17 (t, J = 7.16 Hz, 2H, CH$_2$CH$_2$C*H*$_2$), 2.83 (s, 3H, CH$_2$N-C*H*$_3$), 3.16 (s, 3H, N^{1}C*H*$_3$), 3.33 (s, 3H, N^{3}C*H*$_3$), 3.94 (q, J = 6.87 Hz, 2H, COOC*H*$_2$CH$_3$), 4.14 (t, J = 7.16 Hz, 2H, C*H*$_2$CH$_2$CH$_2$), 4.43 (s, 2H, C*H*$_2$N-CH$_3$), 7.22–7.35 (m, 5H, 2,3,4,5,6-Ph). LC/MS: *m/z* calc. 414.21, found 414.30; Formula $C_{21}H_{27}N_5O_4$; MW 413.48.

4.1.1.5. Ethyl 4-(1,3-dimethyl-8-((4-methylbenzyl)amino)-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanoate ***(10)****.* From **2** in 66% yield; mp 166–167 °C; R_f = 0.67 (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.09 (t, J = 7.20 Hz, 3H, OCH$_2$C*H*$_3$), 1.86 (quin, J = 7.20 Hz, 2H, CH$_2$C*H*$_2$CH$_2$), 2.19–2.27 (m, 5H, CH$_2$CH$_2$C*H*$_2$ and C*H*$_3$), 3.13 (s, 3H, N^{1}C*H*$_3$), 3.29 (s, 3H, N^{3}C*H*$_3$), 3.94 (q, J = 7.09 Hz, 2H, OC*H*$_2$CH$_3$), 4.03 (t, J = 6.80 Hz, 2H C*H*$_2$CH$_2$CH$_2$), 4.46 (d, J = 5.23 Hz, 2H, NHC*H*$_2$), 7.09 (d, J = 7.80 Hz, 2H, 3,5-Ph), 7.21 (d, J = 8.00 Hz, 2H, 2,6-Ph), 7.51 (t, J = 5.58 Hz, 1H, N*H*CH$_2$). LC/MS: *m/z* calc. 414.21, found 414.44; Formula $C_{21}H_{27}N_5O_4$; MW 413.47.

4.1.1.6. Ethyl 4-(1,3-dimethyl-8-((4-methylphenethyl)amino)-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanoate ***(11)****.* From **3** in 44% yield; mp 154–155 °C; R_f = 0.65 (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.11 (t, J = 7.10 Hz, 3H, OCH$_2$C*H*$_3$), 1.82 (quint, J = 7.10 Hz, 2H, CH$_2$C*H*$_2$CH$_2$), 2.15–2.23 (m, 5H, CH$_2$CH$_2$C*H*$_2$ and C*H*$_3$), 2.80 (t, J = 7.34 Hz, 2H, NHCH$_2$C*H*$_2$), 3.13 (s, 3H, N^{1}C*H*$_3$), 3.32 (s, 3H, N^{3}C*H*$_3$), 3.42–3.51 (m, 2H, NHC*H*$_2$CH$_2$), 3.91–4.02 (m, 4H, C*H*$_2$CH$_2$CH$_2$ and OC*H*$_2$CH$_3$), 7.02–7.11 (m, 5H, 2,3,5,6-Ph and N*H*CH$_2$CH$_2$). LC/MS: *m/z* calc. 428.22, found 428.39; Formula $C_{22}H_{29}N_5O_4$; MW 427.50.

4.1.1.7. Ethyl 4-(8-((2-methoxybenzyl)amino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanoate ***(12)****.* From **4** in 79% yield; R_f = 0.67 (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.09 (t, J = 7.16 Hz, 3H, COOCH$_2$C*H*$_3$), 1.88 (quint, J = 7.30 Hz, 2H, CH$_2$C*H*$_2$CH$_2$), 2.26 (t, J = 7.73 Hz, 2H, CH$_2$CH$_2$C*H*$_2$), 3.13 (s, 3H, N^{1}C*H*$_3$), 3.27 (s, 3H, N^{3}C*H*$_3$), 3.78 (s, 3H, OC*H*$_3$), 3.95 (q, J = 7.06 Hz, 2H, COOC*H*$_2$CH$_3$), 4.07 (t, J = 6.87 Hz, 2H, C*H*$_2$CH$_2$CH$_2$), 4.50 (d, J = 5.73 Hz, 2H, NHC*H*$_2$), 6.87 (t, J = 7.45 Hz, 1H, 4-Ph), 6.96 (d, J = 8.02 Hz, 1H, 3-Ph), 7.16–7.26 (m, 2H, 5,6-Ph), 7.32 (t, J = 5.73 Hz, 1H, N*H*CH$_2$). LC/MS: *m/z* calc. 430.21, found 430.25; Formula $C_{21}H_{27}N_5O_5$; MW 429.48.

4.1.1.8. Ethyl 4-(1,3-dimethyl-2,6-dioxo-8-(piperidin-1-yl)-1,2,3,6-tetrahydro-7H-purin-7-yl)butanoate ***(13)****.* From **5** in 65% yield; R_f = 0.79 (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.12 (t, J = 7.16 Hz, 3H, COOCH$_2$C*H*$_3$), 1.48–1.55 (m, 2H, 4,4-Pip), 1.56–1.64 (m, 4H, 3,3,5,5-Pip), 1.95 (quint, J = 6.87 Hz, 2H, CH$_2$C*H*$_2$CH$_2$), 2.19 (t, J = 6.87 Hz, 2H, CH$_2$CH$_2$C*H*$_2$), 3.03–3.13 (m, 4H, 2,2,6,6-Pip), 3.16 (s, 3H, N^{1}C*H*$_3$), 3.33 (s, 3H, N^{3}C*H*$_3$), 3.97 (q, J = 7.45 Hz, 2H, COOC*H*$_2$CH$_3$), 4.02 (t, J = 7.16 Hz, 2H, C*H*$_2$CH$_2$CH$_2$). LC/MS: *m/z* calc. 378.21, found 378.21; Formula $C_{18}H_{27}N_5O_4$; MW 377.45.

4.1.1.9. 4-(8-(Benzyl(methyl)amino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanoic acid ***(17)****.* From **9** in 73% yield; mp 69–71 °C; R_f = 0.67(C); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.90 (quin, J = 7.16 Hz, 2H, CH$_2$C*H*$_2$CH$_2$), 2.06–2.15 (m, 2H, CH$_2$CH$_2$C*H*$_2$), 2.83 (s, 3H, CH$_2$N-C*H*$_3$), 3.16 (s, 3H, N^{1}C*H*$_3$), 3.33 (s, 3H, N^{3}C*H*$_3$), 4.12 (t, J = 7.16 Hz, 2H, C*H*$_2$CH$_2$CH$_2$), 4.43 (s, 2H, C*H*$_2$N-CH$_3$), 7.22–7.36 (m, 5H, 2,3,4,5,6-Ph), 12.14 (br.s., 1H, COO*H*). LC/MS: *m/z* calc. 386.18, found 386.26; Formula $C_{19}H_{23}N_5O_4$; MW 385.42.

4.1.1.10. 4-(1,3-Dimethyl-8-((4-methylbenzyl)amino)-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanoic acid ***(18)****.* From **10** in 97% yield; mp 204–205 °C; R_f = 0.76 (C); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.81 (quint, J = 7.10 Hz, 2H, CH$_2$C*H*$_2$CH$_2$), 2.08 (t, J = 7.48 Hz, 2H, CH$_2$CH$_2$C*H*$_2$), 2.22 (s, 3H, C*H*$_3$), 3.13 (s, 3H, N^{1}C*H*$_3$), 3.28 (s, 3H, N^{3}C*H*$_3$), 4.01 (t, J = 6.87 Hz, 2H, C*H*$_2$CH$_2$CH$_2$), 4.42–4.48 (m, 2H, NHC*H*$_2$), 7.08 (d, J = 7.80 Hz, 2H, 3,5-Ph), 7.21 (d, J = 7.90 Hz, 2H, 2,6-Ph), 8.11 (brs., 1H, N*H*CH$_2$). LC/MS: *m/z* calc. 386.18, found 386.39; Formula $C_{19}H_{23}N_5O_4$; MW 385.41.

4.1.1.11. 4-(1,3-Dimethyl-8-((4-methylphenethyl)amino)-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanoic acid ***(19)****.* From **11** in 87% yield; mp 204–205 °C; R_f = 0.73 (C); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.78 (quint, J = 7.32 Hz, 2H, CH$_2$C*H*$_2$CH$_2$), 2.08–2.16 (m, 2H, CH$_2$CH$_2$C*H*$_2$), 2.21 (s, 3H, C*H*$_3$), 2.80 (t, J = 7.34 Hz, 2H, NHCH$_2$C*H*$_2$), 3.13 (s, 3H, N^{1}C*H*$_3$), 3.33 (s, 3H, N^{3}C*H*$_3$), 3.41–3.51 (m, 2H, NHC*H*$_2$CH$_2$), 3.96 (t, J = 6.87 Hz, 2H, C*H*$_2$CH$_2$CH$_2$), 6.99–7.11 (m, 5H, 2,3,5,6-Ph and N*H*CH$_2$CH$_2$), 12.08 (brs., 1H, O*H*). LC/MS: *m/z* calc. 400.19, found 400.41; Formula $C_{20}H_{25}N_5O_4$; MW 399.44.

4.1.1.12. 4-(8-((2-Methoxybenzyl)amino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanoic acid ***(20)****.* From **12** in 59% yield; mp 202–204 °C; R_f = 0.57 (C); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.85 (quint, J = 7.30 Hz, 2H, CH$_2$C*H*$_2$CH$_2$), 2.19 (t, J = 7.73 Hz, 2H, CH$_2$CH$_2$C*H*$_2$), 3.13 (s, 3H, N^{1}C*H*$_3$), 3.27 (s, 3H, N^{3}C*H*$_3$), 3.78 (s, 3H, OC*H*$_3$), 4.06 (t, J = 6.87 Hz, 2H, C*H*$_2$CH$_2$CH$_2$), 4.50 (d, J = 5.73 Hz, 2H, NHC*H*$_2$), 6.87 (t, J = 7.45 Hz, 1H, 4-Ph), 6.95 (d, J = 8.02 Hz, 1H, 3-Ph), 7.17–7.25 (m, 2H, 5,6-Ph), 7.33 (t, J = 5.44 Hz, 1H, N*H*CH$_2$), 12.09 (brs., 1H, COO*H*). LC/MS: *m/z* calc. 402.18, found 402.21; Formula $C_{19}H_{23}N_5O_5$; MW 401.42.

4.1.1.13. 4-(1,3-Dimethyl-2,6-dioxo-8-(piperidin-1-yl)-1,2,3,6-tetrahydro-7H-purin-7-yl)butanoic acid ***(21)****.* From **13** in 82% yield; mp 101–103 °C; R_f = 0.61 (B); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.45–1.54 (m, 2H, 4,4-Pip), 1.56–1.63 (m, 4H, 3,3,5,5-Pip), 1.91 (quint, J = 7.02 Hz, 2H, CH$_2$C*H*$_2$CH$_2$), 2.04–2.12 (m, 2H, CH$_2$CH$_2$C*H*$_2$), 3.05–3.12 (m, 4H, 2,2,6,6-Pip), 3.15 (s, 3H, N^{1}C*H*$_3$), 3.33 (s, 3H, N^{3}C*H*$_3$), 4.01 (t, J = 6.87 Hz, 2H, C*H*$_2$CH$_2$CH$_2$), 12.14 (brs., 1H, COO*H*). LC/MS: *m/z* calc. 350.18, found 350.21; Formula $C_{16}H23N_5O_4$; MW 349.38.

4.1.1.14. 4-(8-(Benzyl(methyl)amino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanehydrazide ***(25)****.* From **9** in 61% yield; mp 134–136 °C; R_f = 0.40 (B); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.84–1.96 (m, 4H, N^7-CH$_2$C*H*$_2$C*H*$_2$), 2.83 (s, 3H, N(C*H*$_3$)CH$_2$), 3.16 (s, 3H, N^1-CH$_3$), 3.31 (s, 3H, N^3-CH$_3$), 3.99–4.16 (m, 4H, N^7-C*H*$_2$CH$_2$CH$_2$, NHN*H*$_2$), 4.44 (s, 2H, N(CH$_3$)C*H*$_2$), 7.21–7.27 (m, 1H, 4-Ph), 7.28–7.35 (m, 3H, 2,3,5,6-pH), 8.91 (s, 1H, N*H*NH$_2$); LC/MS: *m/z* calc. 400.21, found 400.14; Formula $C_{19}H_{25}N_7O_3$; MW 399.46.

4.1.1.15. 4-(1,3-Dimethyl-8-((4-methylbenzyl)amino)-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanehydrazide ***(26)****.* From **10** in 79% yield; mp 194–195 °C; R_f = 0.50(B); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.80–1.97 (m, 2H, CH$_2$C*H*$_2$CH$_2$), 2.23 (br. s., 3H, C*H*$_3$), 2.46 (s, 2H, CH$_2$CH$_2$C*H*$_2$), 3.13 (brs., 3H, N^{1}C*H*$_3$), 3.25 (s, 3H, N^{3}C*H*$_3$), 4.01 (brs., 2H, C*H*$_2$CH$_2$CH$_2$), 4.28 (brs., 2H, NHN*H*$_2$), 4.46 (brs., 2H, NHC*H*$_2$), 7.09 (brs., 2H, 3,5-Ph), 7.20 (br. s., 2H, 2,6-Ph), 7.64 (brs., 1H, N*H*CH$_2$), 8.98 (brs., 1H, N*H*NH$_2$). LC/MS: *m/z* calc. 400.21, found 400.35; Formula $C_{19}H_{25}N_7O_3$; MW 399.45.

4.1.2. General procedure for the synthesis of variously substituted 4-[8-amino 1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-7-yl]-N-phenylbutanamides ***(27–37)***

1 eq. of the appropriate acid **17**–**21** (2.5 mmol) was dissolved in 5 mL of DMF and 1.5 eq. CDI was added. Then the mixture was stirred at room temperature for 30 min, and afterwards 1 eq. of the respective aniline or benzylamine derivative was added. Stirring was continued for 3 days. Afterwards, 2–3 drops of water were added and the mixture was evaporated under reduced pressure. The product was precipitated out with the access of cold water and purified by crystallization from methanol or propan-2-ol.

*4.1.2.1. 8-(Benzyl(methyl)amino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)butanamide **(27)**.* From **17** in 85% yield; UPLC/MS purity 100%; mp 152–154 °C; R_f = 0.52 (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.13 (d, J = 6.87 Hz, 6H, CH(CH_3)$_2$), 1.96–2.05 (m, 2H, CH$_2$CH_2CH$_2$), 2.19 (t, J = 6.90 Hz, 2H, CH$_2$CH$_2$CH_2), 2.74–2.81 (m, 1H, CH(CH$_3$)$_2$), 2.82 (s, 3H, NCH_3), 3.14 (s, 3H, N^{1}CH_3), 3.30 (s, 3H, N^{3}CH_3), 4.18 (t, J = 7.16 Hz, 2H, CH_2CH$_2$CH$_2$), 4.43 (s, 2H, NCH_2), 7.09 (d, J = 8.60 Hz, 2H, 3,5-Ph'), 7.19–7.32 (m, 5H, 2,3,4,5,6-Ph), 7.40 (d, J = 8.60 Hz, 2H, 2,6-Ph'), 9.72 (s, 1H, CONH). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 24.5, 25.5, 27.9, 29.9, 33.3, 33.4, 39.4, 45.1, 57.8, 104.1, 119.6, 126.8, 127.9, 128.5, 129.0, 137.5, 137.6, 143.5, 148.0, 151.4, 154.0, 157.2, 170.3. LC/MS: *m/z* calc. 503.28, found 503.17; Anal calcd. for $C_{28}H_{34}N_6O_3$ (502.61): C, 66.91; H, 6.82; N, 16.72. Found: C, 66.78; H, 6.80; N, 16.68.

*4.1.2.2. 4-(8-(Benzyl(methyl)amino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-(tert-butyl)phenyl)butanamide **(28)**.* From **17** in 79% yield; UPLC/MS purity 98.88%; mp 147–149 °C; R_f = 0.54 (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.21 (s, 9H, (CH_3)$_3$), 1.93–2.07 (m, 2H, CH$_2$CH_2CH$_2$), 2.20 (t, J = 6.90 Hz, 2H, CH$_2$CH$_2$CH_2), 2.82 (s, 3H, NCH_3), 3.14 (s, 3H, N^{1}CH_3), 3.30 (s, 3H, N^{3}CH_3), 4.18 (t, J = 7.16 Hz, 2H, CH_2CH$_2$CH$_2$), 4.43 (s, 2H, NCH_2), 7.18–7.33 (m, 7H, 2,3,4,5,6-Ph, 3,5-Ph'), 7.41 (d, J = 8.59 Hz, 2H, 2,6-Ph'), 9.73 (s, 1H, CONH). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 25.5, 27.9, 29.9, 31.7, 33.3, 34.5, 39.4, 45.1, 57.8, 104.1, 119.3, 125.7, 127.9, 128.5, 129.0, 137.2, 137.6, 145.7, 147.9, 151.4, 154.0, 157.2, 170.3. LC/MS: *m/z* calc. 517.29, found 517.39; Anal calcd. for $C_{29}H_{36}N_6O_3$ (516.63): C, 67.42; H, 7.02; N, 16.27. Found: C, 67.19; H, 6.99; N, 16.22.

*4.1.2.3. 4-(8-(Benzyl(methyl)amino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-(tert-butyl)benzyl)butanamide **(29)**.* From **17** in 86% yield; UPLC/MS purity 96.95%; R_f = 0.41 (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.19 (s, 9H, C(CH_3)$_3$), 1.83–1.99 (m, 2H, CH$_2$CH_2CH$_2$), 1.99–2.12 (m, 2H, CH$_2$CH$_2$CH_2), 2.82 (s, 3H, NCH_3), 3.16 (s, 3H, N^{1}CH_3), 3.32 (s, 3H, N^{3}CH_3), 4.00–4.22 (m, 4H, CH_2CH$_2$CH$_2$, CONHCH_2), 4.43 (s, 2H, NCH_2), 6.98–7.17 (m, 2H, 3,5-Ph), 7.17–7.41 (m, 7H, 2,3,4,5,6-Ph', 2,6-Ph), 8.24 (brs., 1H, CONH). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 26.0, 27.9, 29.9, 31.7, 32.4, 34.6, 39.4, 42.4, 45.2, 57.7, 104.0, 125.5, 127.7, 127.9, 128.5, 129.0, 137.0, 137.7, 148.0, 149.7, 151.5, 154.0, 157.1, 171.5. LC/MS: *m/z* calc. 531.30, found 531.28; Anal calcd. for $C_{30}H_{38}N_6O_3$ (530.66): C, 67.90; H, 7.22; N, 15.84. Found: C, 67.59; H, 7.37; N, 16.01.

*4.1.2.4. 4-(8-(Benzyl(methyl)amino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-(sec-butyl)phenyl)butanamide **(30)**.* From **17** in 92% yield; UPLC/MS purity 98.46%; mp 180–182 °C; R_f = 0.57 (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 0.70 (t, J = 7.16 Hz, 3H, CH_3CH$_2$(CH)CH$_3$), 0.77–0.90 (m, 1H, CH$_3$CH$_2$(CH)CH$_3$), 1.11 (d, J = 6.87 Hz, 3H, CH$_3$CH$_2$(CH)CH_3), 1.41–1.54 (m, 2H, CH$_3$CH_2(CH)CH$_3$), 1.94–2.07 (m, 2H, CH$_2$CH_2CH$_2$), 2.14–2.26 (m, 2H, CH$_2$CH$_2$CH_2), 2.81 (s, 3H, NCH_3), 3.14 (s, 3H, N^{1}CH_3), 3.29 (s, 3H, N^{3}CH_3), 4.18 (t, J = 6.87 Hz, 2H, CH_2CH$_2$CH$_2$), 4.42 (s, 2H, NCH_2), 7.04 (d, J = 8.00 Hz, 2H, 3,5-Ph), 7.16–7.34 (m, 5H, 2,3,4,5,6-Ph'), 7.41 (d, J = 8.00 Hz, 2H, 2,6-Ph), 9.73 (s, 1H, CONH). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 12.6, 22.4, 25.5, 27.9, 29.9, 31.1, 33.3, 39.3, 40.9, 45.1, 57.8, 104.1, 119.6, 127.4, 127.9, 128.5, 129.0, 137.5, 137.6, 142.2, 148.0, 151.4, 154.0, 157.2, 170.3. LC/MS: *m/z* calc. 517.29, found 517.26; Anal calcd. for $C_{29}H_{36}N_6O_3$ (516.63): C, 67.42; H, 7.02; N, 16.27. Found: C, 67.29; H, 6.98; N, 16.21.

*4.1.2.5. Tert-butyl 4-(4-(8-(benzyl(methyl)amino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanamido)benzoate **(31)**.* From **17** in 87% yield; UPLC/MS purity 95.02%; mp 234–236 °C; R_f = 0.46 (A); ^{1}H NMR (500 MHz, CDCl$_3$-*d*) δ ppm 1.57 (s, 9H, (CH_3)$_3$), 2.18–2.28 (m, 2H, CH$_2$CH_2CH$_2$), 2.37–2.44 (m, 2H, CH$_2$CH$_2$CH_2), 2.93 (s, 3H, CH$_2$N-CH_3), 3.40 (s, 3H, N^{1}CH_3), 3.52 (s, 3H, N^{3}CH_3), 4.21 (t, J = 7.45 Hz, 2H, CH_2CH$_2$CH$_2$), 4.44 (s, 2H, CH_2N-CH$_3$), 7.25–7.37 (m, 5H, 2,3,4,5,6-Ph), 7.66 (d, J = 8.60 Hz, 2H, 2,6-Ph'), 7.92 (d, J = 8.60 Hz, 2H, 3,5-Ph'), 8.50 (brs., 1H, CONH). ^{13}C NMR (126 MHz, CDCl$_3$-*d*) δ ppm 14.1, 23.0, 25.9, 28.1, 28.3, 29.9, 39.9, 44.8, 58.4, 80.9, 104.5, 118.9, 127.9, 128.0, 128.8, 129.6, 130.6, 136.8, 142.1, 148.5, 151.7, 154.8, 157.3, 165.5 LC/MS: *m/z* calc. 561.28, found 561.26; Anal calcd. for $C_{30}H_{36}N_6O_5$ (560.64): C, 64.27; H, 6.47; N, 14.99. Found: C, 63.98; H, 6.54; N, 14.83.

*4.1.2.6. 4-(8-(Benzyl(methyl)amino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(5-(tert-butyl)-2-hydroxyphenyl)butanamide **(32)**.* From **17** in 73% yield; UPLC/MS purity 99.47%; mp 164–166 °C; R_f = 0.59 (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.17 (s, 9H, (CH_3)$_3$), 1.95–2.04 (m, 2H, CH$_2$CH_2CH$_2$), 2.30 (t, J = 7.16 Hz, 2H, CH$_2$CH$_2$CH_2), 2.84 (s, 3H, NCH_3), 3.16 (s, 3H, N^{1}CH_3), 3.30 (s, 3H, N^{3}CH_3), 4.19 (t, J = 7.16 Hz, 2H, CH_2CH$_2$CH$_2$), 4.44 (s, 2H, NCH_2), 6.72 (d, J = 8.59 Hz, 1H, 3-Ph'), 6.92 (dd, J = 8.31, 2.58 Hz, 1H, 4-Ph'), 7.19–7.34 (m, 5H, 2,3,4,5,6-Ph), 7.58–7.65 (m, 1H, 6-Ph'), 9.26 (s, 1H, OH), 9.44 (s, 1H, CONH). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 25.7, 27.9, 29.9, 31.9, 33.0, 34.3, 39.4, 45.1, 57.7, 104.0, 116.2, 120.0, 122.1, 126.1, 127.9, 128.5, 129.0, 137.6, 141.7, 146.2, 148.0, 151.5, 154.0, 157.2, 171.4. LC/MS: *m/z* calc. 533.29, found 533.28; Anal calcd. for $C_{29}H_{36}N_6O_4$ (532.63): C, 65.39; H, 6.81; N, 15.78. Found: C, 65.27; H, 6.85; N, 15.72.

*4.1.2.7. N-(5-(tert-butyl)-2-hydroxyphenyl)-4-(1,3-dimethyl-8-((4-methylbenzyl)amino)-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanamide **(33)**.* From **18** in 58% yield; UPLC/MS purity 100%; mp 216–217 °C; R_f = 0.64 (B); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.17 (s, 9H, (CH_3)$_3$), 1.88–2.00 (m, 2H, CH$_2$CH_2CH$_2$), 2.21 (s, 3H, CH_3), 2.35 (t, J = 7.40 Hz, 2H, CH$_2$CH$_2$CH_2), 3.14 (s, 3H, N^{1}CH_3), 3.28 (s, 3H, N^{3}CH_3), 4.09 (t, J = 6.30 Hz, 2H, CH_2CH$_2$CH$_2$), 4.48 (d, J = 5.00 Hz, 2H, NHCH_2), 6.73 (d, J = 8.23 Hz, 1H, 3-Ph'), 6.93 (d, J = 7.52 Hz, 1H, 4-Ph'), 7.07 (d, J = 7.20 Hz, 2H, 3,5-Ph), 7.22 (d, J = 7.10 Hz, 2H, 2,6-Ph), 7.52–7.62 (m, 2H, NHCH$_2$ & 6-Ph'), 9.29 (s, 1H, CONH), 9.43 (s, 1H, OH). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 21.2, 25.7, 27.8, 29.8, 31.9, 33.0, 34.3, 42.4, 46.1, 102.3, 116.2, 120.2, 122.2, 126.0, 127.8, 129.4, 136.5, 137.1, 141.8, 146.4, 148.9, 151.5, 153.2, 154.0, 171.8. LC/MS: *m/z* calc. 533.28, found 533.28; Anal calcd. for $C_{29}H_{36}N_6O_4$ (532.63): C, 65.39; H, 6.81; N, 15.78. Found: C, 65.25; H, 6.78; N, 15.85.

*4.1.2.8. N-(5-(tert-butyl)-2-hydroxyphenyl)-4-(1,3-dimethyl-8-((4-methylphenethyl)amino)-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanamide **(34)**.* From **19** in 46% yield; UPLC/MS purity 100%; mp 254–256 °C; R_f = 0.66 (B); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.17 (s, 9H, (CH_3)$_3$), 1.88 (quin, J = 7.10 Hz, 2H, CH$_2$CH_2CH$_2$), 2.18 (s, 3H, CH_3), 2.31 (t, J = 7.45 Hz, 2H, CH$_2$CH$_2$CH_2), 2.81 (t, J = 7.16 Hz, 2H, NHCH$_2$CH_2), 3.14 (s, 3H, N^{1}CH_3), 3.30 (s, 3H, N^{3}CH_3), 3.47 (q, J = 6.40 Hz, 2H, NHCH_2CH$_2$), 4.03 (t, J = 6.41 Hz, 2H, CH_2CH$_2$CH$_2$), 6.72 (d, J = 8.38 Hz, 1H, 3-Ph'), 6.93 (d, J = 8.30 Hz, 1H, 4-Ph'), 7.00–7.09 (m, 4H, 2,3,5,6-Ph), 7.12 (t, J = 5.00 Hz, 1H, NHCH$_2$CH$_2$), 7.59 (s, 1H, 6-Ph'), 9.28 (s, 1H, CONH), 9.43 (s, 1H, OH). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 21.1, 25.6, 27.8, 29.8, 31.9, 32.9, 34.3, 35.3, 42.3, 44.8, 102.1, 116.2, 120.2, 122.2, 126.0, 129.2, 129.4, 135.5, 136.8, 141.8, 146.4, 149.1, 151.5, 153.2, 153.9, 171.8. LC/MS: *m/z* calc. 547.30, found 547.37; Anal calcd. for $C_{30}H_{38}N_6O_4$ (546.66): C, 65.91; H, 7.01; N, 15.37. Found: C, 65.76; H, 6.98; N, 15.43.

*4.1.2.9. N-(5-(tert-butyl)-2-hydroxyphenyl)-4-(8-((2-methoxybenzyl)amino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanamide **(35)**.* From **20** in 83% yield; UPLC/MS purity 100%; mp 178–180 °C; R_f = 0.52 (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.17 (s, 9H, (CH_3)$_3$), 1.91–2.00 (m, 2H, CH$_2$CH_2CH$_2$), 2.37 (t, J = 7.45 Hz, 2H, CH$_2$CH$_2$CH_2), 3.14 (s, 3H, N^{1}CH_3), 3.26 (s, 3H, N^{3}CH_3), 3.78 (s, 3H, OCH_3), 4.13 (t, J = 6.59 Hz, 2H, CH_2CH$_2$CH$_2$), 4.51 (d, J = 5.16 Hz, 2H,

NCH_2), 6.72 (d, J = 8.59 Hz, 1H, 3-Ph'), 6.86 (t, J = 7.45 Hz, 1H, 5-Ph), 6.90–6.98 (m, 2H, 3-Ph, 4-Ph'), 7.19 (t, J = 7.16 Hz, 1H, 4-Ph), 7.25 (d, J = 7.45 Hz, 1H, 6-Ph), 7.40 (t, J = 6.01 Hz, 1H, N*H*), 7.56 (d, J = 2.29 Hz, 1H, 6-Ph'), 9.30 (s, 1H, O*H*), 9.41 (s, 1H, CON*H*). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 25.8, 27.8, 29.8, 31.9, 33.0, 34.3, 41.5, 42.5, 55.8, 102.3, 111.0, 116.3, 120.3, 120.7, 122.2, 126.0, 127.4, 128.2, 128.6, 141.8, 146.4, 149.0, 151.5, 153.2, 154.1, 157.2, 171.8. LC/MS: *m/z* calc. 549.28, found 549.30; Anal calcd. for $C_{29}H_{36}N_6O_5$ (548.63): C, 63.49; H, 6.61; N, 15.32. Found: C, 63.67; H, 6.58; N, 15.27.

4.1.2.10. N-(4-isopropylphenyl)-4-(8-((2-methoxybenzyl)amino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanamide ***(36)****.* From **20** in 81% yield; UPLC/MS purity 98.48%; mp 197–199 °C; R_f = 0.37 (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.12 (dd, J = 6.87, 1.72 Hz, 6H, CH(CH_3)$_2$), 1.90–2.00 (m, 2H, CH$_2$CH_2CH$_2$), 2.26 (t, J = 7.45 Hz, 2H, CH$_2$CH$_2$CH_2), 2.77 (quint, J = 6.87 Hz, 1H, C*H*(CH$_3$)$_2$), 3.12 (m, 3H, N^{1}CH_3), 3.26 (m, 3H, N^{3}CH_3), 3.78 (m, 3H, OCH_3), 4.12 (t, J = 6.30 Hz, 2H, CH_2CH$_2$CH$_2$), 4.51 (d, J = 5.73 Hz, 2H, NCH_2), 6.82–6.89 (m, 1H, 5-Ph), 6.95 (d, J = 8.02 Hz, 1H, 3-Ph), 7.09 (d, J = 6.87 Hz, 2H, 3,5-Ph'), 7.17–7.28 (m, 2H, 4,6-Ph), 7.36–7.45 (m, 3H, 3-Ph, 2,6-Ph'), 9.77 (s, 1H, O*H*); ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 24.5, 25.6, 27.7, 29.8, 33.4, 41.5, 42.5, 55.8, 102.3, 111.0, 119.7, 120.6, 126.8, 127.4, 128.3, 128.6, 130.0, 137.5, 143.6, 149.0, 151.5, 153.2, 154.1, 157.2, 170.8. LC/MS: *m/z* calc. 519.27, found 519.25; Anal calcd. for $C_{28}H_{34}N_6O_4$ (518.61): C, 64.85; H, 6.61; N, 16.20. Found: C, 65.11; H, 6.64; N, 16.13.

4.1.2.11. 4-(1,3-Dimethyl-2,6-dioxo-8-(piperidin-1-yl)-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)butanamide ***(37)****.* From **21** in 76 yield %; UPLC/MS purity 100%; mp 180–182 °C; R_f = 0.46 (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.12 (d, J = 6.87 Hz, 6H, CH(CH_3)$_2$), 1.40–1.50 (m, 2H, 5,5-Pip), 1.50–1.62 (m, 4H, 3,3,5,5-Pip), 2.02 (quint, J = 6.87 Hz, 2H, CH$_2$CH_2CH$_2$), 2.19 (t, J = 6.87 Hz, 2H, CH$_2$CH$_2$CH_2), 2.71–2.83 (m, 1H, C*H*(CH$_3$)$_2$), 3.06–3.13 (m, 4H, 2,2,6,6-Pip), 3.15 (s, 3H, N^{1}CH_3), 3.29 (s, 3H, N^{3}CH_3), 4.04 (t, J = 7.16 Hz, 2H, CH_2CH$_2$CH$_2$), 7.09 (d, J = 8.60 Hz, 2H, 3,5-Ph), 7.41 (d, J = 8.60 Hz, 2H, 2,6-Ph), 9.71 (s, 1H, CON*H*). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 24.1, 24.5, 25.4, 25.6, 28.0, 29.9, 33.2, 33.4, 45.1, 51.6, 104.2, 119.5, 126.8, 137.5, 143.4, 147.9, 151.4, 154.2, 157.3, 170.3. LC/MS: *m/z* calc. 467.28, found 467.34; Anal calcd. for $C_{25}H_{34}N_6O_3$ (466.58): C, 64.36; H, 7.34; N, 18.01. Found: C, 64.24; H, 7.31; N, 17.96.

4.1.3. General procedure for the synthesis of N'-arylidene-4-[8-arylalkylamino-1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-7-yl] butanehydrazides ***(38–43)***

A mixture of compounds **22**, **23**, **24**, **25,** or **26** (1 mmol) and 2,3,4-trihydroxy-, 2,4-dihydroxy-, or 2-hydroxy-3-*tert*-butyl-benzaldehyde (1 mmol) was stirred in 10 mL of methanol in the presence of a catalytic amount of HCl (2 drops of conc. acid) at room temperature for 3 days. Afterward, water was added and the resultant precipitate of the product was filtered and purified by crystallization from methanol or by silica gel chromatography eluted with solvent A.

4.1.3.1. (E,Z)-4-(8-(Benzyl(methyl)amino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N'-(2,3,4-trihydroxybenzylidene)butanehydrazide ***(38)****.* From **25** in 69% yield; UPLC/MS purity 100%; mp 234–236 °C; R_f = 0.49 (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.91–2.05 (m, 2H), 2.05–2.15 (m, 1H), 2.42 (t, J = 7.16 Hz, 1H), 2.84 (s, 3H), 3.13 (s, 1H), 3.16 (s, 2H), 3.31 (s, 3H), 4.11–4.24 (m, 2H), 4.44 (s, 1H), 4.45 (s, 1H), 6.28–6.34 (m, 1H), 6.68 (d, J = 8.59 Hz, 1H), 6.75 (d, J = 8.59 Hz, 1H), 7.19–7.26 (m, 1H), 7.26–7.36 (m, 4H), 8.02 (s, 1H), 8.08 (s, 1H), 9.39 (brs., 1H), 11.08 (s, 1H), 11.36 (brs., 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 30.1, 32.7, 34.7, 35.7, 44.2, 44.2, 49.8, 62.5, 108.8, 116.0, 132.7, 133.2, 133.2, 133.8, 137.9, 137.9, 142.4, 152.6, 152.8, 153.8, 156.2, 158.8, 162.0, 172.2. LC/MS: *m/z* calc. 536.22, found 536.14; Anal calcd. for $C_{26}H_{29}N_7O_6$ (535.55): C, 58.31; H, 5.46; N, 18.31. Found: C, 58.13; H, 5.48; N, 18.33.

4.1.3.2. (E,Z)-4-(1,3-Dimethyl-8-((4-methylbenzyl)amino)-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N'-(2,3,4-trihydroxybenzylidene)butanehydrazide ***(39)****.* From **26** in 49% yield; UPLC/MS purity 100%; mp 154–155 °C; R_f = 0.49 (B); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.91 (quint, J = 6.90 Hz, 2H), 2.15 (t, J = 7.70 Hz, 2H), 2.21 (s, 3H), 3.09–3.15 (m, 3H), 3.29 (s, 3H), 4.08 (t, J = 6.44 Hz, 2H), 4.48 (d, J = 5.44 Hz, 2H), 6.32 (d, J = 8.38 Hz, 1H), 6.68 (d, J = 8.52 Hz, 1H), 6.77 (d, J = 8.50 Hz, 1H), 7.04–7.12 (m, 2H), 7.18–7.23 (m, 2H), 7.57 (t, J = 6.00 Hz, 1H), 8.04 (s, 1H), 8.10 (s, 1H), 8.39 (s, 1H), 9.22–9.65 (m, 1H), 11.05 (s, 1H), 11.27–11.44 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 21.2, 25.5, 27.8, 29.8, 31.1, 42.4, 46.1, 102.2, 108.1, 111.3, 118.9, 121.5, 127.8, 129.4, 133.2, 136.5, 137.1, 143.8, 147.9, 149.0, 149.1, 151.5, 153.2, 154.0, 167.8, 173.1. LC/MS: *m/z* calc. 536.22, found 536.27; Anal calcd. for $C_{26}H_{29}N_7O_6$ (535.55): C, 58.31; H, 5.46; N, 18.31. Found: C, 58.19; H, 5.48; N, 18.24.

4.1.3.3. (E,Z)-N'-(2,4-dihydroxybenzylidene)-4-(1,3-dimethyl-8-((4-methylbenzyl)amino)-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)butanehydrazide ***(40)****.* From **26** in 55% yield; UPLC/MS purity 96.26%; mp 252–253 °C; R_f = 0.55 (B); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.91 (quint, J = 7.50 Hz, 2H), 2.14 (t, J = 7.90 Hz, 2H), 2.21 (s, 3H), 3.10–3.14 (m, 3H), 3.29 (s, 3H), 4.05–4.13 (m, 2H), 4.47 (d, J = 5.66 Hz, 2H), 6.24 (d, J = 2.29 Hz, 1H), 6.29 (dd, J = 8.41, 2.33 Hz, 1H), 7.04–7.11 (m, 2H), 7.17–7.23 (m, 3H), 7.26 (d, J = 8.00 Hz, 1H), 7.38 (d, J = 8.40 Hz, 1H), 7.52–7.61 (m, 1H), 8.05 (s, 1H), 8.14 (s, 1H), 9.75 (s, 1H), 9.88 (s, 1H), 10.00 (s, 1H), 11.00 (s, 1H), 11.27 (s, 1H), 11.36 (s, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 21.2, 25.6, 27.8, 29.8, 31.1, 42.4, 46.1, 102.2, 103.1, 108.1, 111.0, 127.9, 129.4, 131.7, 136.5, 137.1, 142.6, 148.0, 149.0, 151.5, 153.2, 154.0, 158.6, 159.8, 161.1, 167.8. LC/MS: *m/z* calc. 520.23, found 520.38; Anal calcd. for $C_{26}H_{29}N_7O_5$ (519.55): C, 60.11; H, 5.63; N, 18.87. Found: C, 60.41; H, 5.60; N, 18.79.

4.1.3.4. (E,Z)-N'-(2,4-dihydroxybenzylidene)-4-(1,3-dimethyl-2,6-dioxo-8-(phenethylamino)-1,2,3,6-tetrahydro-7H-purin-7-yl)butanehydrazide ***(41)****.* From **22** in 47% yield; UPLC/MS purity 98.97%; mp 267–269 °C; R_f = 0.76 (B); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.78–1.97 (m, 2H), 2.12 (t, J = 7.60 Hz, 2H), 2.88 (t, J = 6.70 Hz, 2H), 3.15 (s, 3H), 3.35 (s, 3H), 3.45–3.60 (m, 2H), 4.05 (t, J = 6.70 Hz, 2H), 6.18–6.36 (m, 2H), 7.09–7.31 (m, 7H), 8.07 (s, 1H), 8.17 (s, 1H), 10.02 (s, 1H), 11.04 (s, 1H), 11.31 (s, 1H), 11.38 (s, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 25.5, 27.8, 29.8, 31.1, 35.8, 42.3, 44.7, 102.1, 102.2, 103.1, 108.1, 111.0, 126.6, 128.8, 129.3, 131.7, 139.9, 148.0, 149.1, 151.5, 153.2, 153.9, 159.8, 161.1, 167.9, 173.3. LC/MS: *m/z* calc. 520.23, found 520.25; Anal calcd. for $C_{26}H_{29}N_7O_5$ (519.55): C, 60.11; H, 5.63; N, 18.87. Found: C, 60.40; H, 5.61; N, 18.95.

4.1.3.5. (E,Z)-N'-(2,4-dihydroxybenzylidene)-4-(1,3-dimethyl-2,6-dioxo-8-((pyridin-2-ylmethyl)amino)-1,2,3,6-tetrahydro-7H-purin-7-yl)butanehydrazide ***(42)****.* From **23** in 42% yield; UPLC/MS purity 96.87%; mp 219–220 °C; R_f = 0.45 (B); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.90–2.04 (m, 2H), 2.20 (t, J = 8.10 Hz, 2H), 3.15 (s, 3H), 3.27 (s, 3H), 4.08–4.22 (m, 2H), 4.64 (d, J = 5.27 Hz, 2H), 6.24–6.27 (m, 1H), 6.27–6.33 (m, 1H), 7.18–7.30 (m, 2H), 7.33–7.41 (m, 1H), 7.64–7.79 (m, 2H), 8.06 (s, 1H), 8.15 (s, 1H), 8.43–8.54 (m, 1H), 9.81 (s, 1H), 9.94 (s, 1H), 10.05 (s, 1H), 11.03 (s, 1H), 11.30 (s, 1H), 11.41 (s, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 25.6, 27.7, 29.3, 29.7, 31.1, 42.6, 48.1, 101.6, 103.1, 108.1, 110.9, 125.1, 127.5, 131.7, 138.0, 148.1, 149.3, 149.9, 151.6, 153.0, 154.5, 159.8, 161.1, 167.9. LC/MS: *m/z* calc. 507.20, found 507.09; Anal calcd. for $C_{24}H_{26}N_8O_5$ (506.51): C, 56.91; H, 5.17; N, 22.12. Found: C, 56.68; H, 5.15; N, 22.01.

*4.1.3.6. (E,Z)-4-(8-(Benzylamino)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N'-(3-(tert-butyl)-2-hydroxybenzylidene)butanehydrazide (**43**).* From **24** in 51% yield; UPLC/MS purity 100%; mp 135–137 °C; $R_f = 0.26$ (A); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.33 (s, 9H), 1.95 (quin, $J = 7.20$ Hz, 2H), 2.21 (t, $J = 7.73$ Hz, 2H), 3.14 (s, 3H), 3.29 (s, 3H), 4.11 (t, $J = 6.73$ Hz, 2H), 4.54 (d, $J = 5.73$ Hz, 2H), 6.81 (t, $J = 7.66$ Hz, 1H), 7.14–7.36 (m, 7H), 7.55–7.70 (m, 1H), 8.09 (s, 1H), 8.21 (s, 1H), 11.68 (s, 1H), 12.25 (s, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 25.4, 27.8, 29.7, 29.8, 31.1, 35.0, 42.4, 46.4, 102.3, 118.1, 119.2, 127.5, 127.9, 128.8, 129.9, 136.8, 140.1, 149.0, 149.6, 151.5, 153.3, 154.0, 157.3, 168.2. LC/MS: *m/z* calc. 546.28, found 546.37; Anal calcd. for $C_{29}H_{35}N_7O_4$ (545.63): C, 63.84; H, 6.47; N, 17.97. Found: C, 63.81; H, 6.49; N, 18.02.

4.2. PDE inhibition

The IC_{50} of the tested compounds were determined for hrPDE 1A, 1B, 3A, 3B, 4A, 4B, 4D, 5A, 7A, and 8A (SignalChem, Richmond, Canada) using the PDE-Glo™ catalytic activity assay with a luminescence detection (Promega Corporation, Madison, WI, USA). The assay was performed on 384-well plates according to the manufacturer's protocol with minor modifications. At first, solutions of the PDEs were prepared by diluting their respective stock solutions in the PDE-Glo™ Reaction Buffer (RB) and added to the wells (6.5 μL). The concentrations of each PDE were selected so that nearly all substrate was reacted completely in the wells without any inhibitor. Stock solutions of all tested compounds were prepared by dissolving them in DMSO at a concentration of 10 mM. Then, the tested compounds were diluted serially in DMSO. Finally, serial dilutions of the compounds were diluted in RB (1:5, v/v), and transferred to the respective wells (1 μL). For each inhibitor concentration, three independent measurements were performed. The mixture of the PDE and compound dilutions was incubated for 10 min at 30 °C on a heated plate shaker (Grant Instruments, Cambridge, United Kingdom). The reaction was started by the addition of 2.5 μL of a 0.2 μM cAMP solution (for PDE 1A, 1B, 3A, 3B, 4A, 4B, 4D, 7A, and 8A) or a 20 μM cGMP solution (for PDE5A) to the respected wells and the plate was incubated for 10 or 20 min at 30 °C on a heated plate shaker. For each plate, respective control reactions were performed: no-substrate, no-enzyme negative control reaction; no-enzyme positive control reaction containing substrate; and no-inhibitor positive control reactions containing both substrate and PDE. After the incubation, the reaction was stopped by adding of 2.5 μL of the PDE-Glo™ Termination Buffer. A volume of 2.5 μL of the PDE-Glo™ Detection Buffer was transferred to each well and the plate was incubated for 20 min at room temperature. Finally, an aliquot of 10 μL of the PDE-Glo Kinase® Reagent was added to the wells. The plate was incubated for 10 min at room temperature and luminescence was measured with a plate-reading luminometer (BMG Labtech, Ortenberg, Germany). After the addition of each reagent, the plate was centrifuged briefly on a plate centrifuge (Benchmark Scientific, Sayreville, NJ, USA). To assess IC_{50}, the resulting data were expressed as percent of an uninhibited control and plotted against inhibitor concentration. The IC_{50} values were estimated by nonlinear regression using ADAPT 5 software (BMSR, Los Angeles, CA, USA).

4.3. DPPH free radical scavenging assay

Free radical scavenging activity of the test compounds was assessed by microplate assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH) [70]. Stock solutions (20 mM) of the tested compounds and Trolox (positive control) were prepared by dissolving in DMSO (PAN-Biotech, Germany). Then, the working mixture was prepared by combining solutions of the tested compounds (final concentration range: 10–1000 μM) and DPPH methanolic solution (150 μM). DMSO was used as a blank sample. Next, the plates were incubated at room temperature in the dark with shaking for 30 min, then the absorbance was measured at 517 nm (SpectraMax iD3 microplate reader, Molecular Devices). All samples were run 2 times, in triplicate. The ability of the compounds to scavenge DPPH radical was calculated from the following formula: Scavenging of DPPH (%) = $((A_c - A_s)/A_c) \times 100\%$, where A_c is the mean absorbance of the negative control (DPPH solution with DMSO), A_s is the mean absorbance of the tested compounds in the presence of DPPH.

4.4. Compound preparation for cell-based assays

All newly synthesized compounds were dissolved in DMSO for cell-based assays. Working concentrations of the tested compounds were prepared by diluting in reference culture medium (the final DMSO concentration did not exceed 0.5% and was not harmful to the cells, which did not change their morphology and viability). An aqueous solution of theophylline (1,3-dimethylpurine-2,6-dione) were also used in the experiments.

4.5. In vitro cell culture

The murine macrophages cell line - RAW 264.7 (ATCC® TIB-71™, Manassas, VA, USA) was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and pen-strept (penicillin, streptomycin; Gibco, Thermo Fisher Scientific, Waltham, MA, USA). To induce proinflammatory phenotype, murine macrophages were stimulated with lipopolysaccharide (LPS, Sigma Aldrich, St. Louis, MO, USA) at 1 μg/mL.

In this study, we used cryopreserved human bronchial epithelial cells (HBECs) isolated from tracheobronchial brushings sampled from asthma patients (n = 3; moderate disease, non-smokers, females, aged 36–64 years) at the Department of Internal Medicine of the Jagiellonian University Medical College, Krakow. Asthma diagnosis was assessed according to GINA guideline (https://ginasthma.org/). Cells were retrieved during our earlier project, with the sampling procedure approved by Jagiellonian University Bioethics Committee (KBET/68/B/2008) and informed written consent obtained from each participant. Cells were cultured in collagen coated flasks (collagen type IV, Sigma Aldrich, St. Louis, MO, USA) in Bronchial Epithelial Cell Growth Medium (BEGM™, Lonza, Basel, Switzerland) supplemented with Bronchial Epithelial Cell Growth Medium BulletKit™ (Lonza, Basel, Switzerland). To induce proinflammatory phenotype, HBECs were stimulated with interleukin-13 (IL-13, R&D Systems, Inc., Minneapolis, MN, USA) at 1 ng/mL or TGF-β_1 (BD Biosciences, San Jose, CA, USA) at 5 ng/mL.

4.6. MTT assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich, St. Louis, MO, USA) assay. Cells were seeded into 96-well plates and cultured in the presence of tested compounds (10 μM) for 24 h. The MTT (final concentration 0.5 mg/mL) was added to each well for 4 h and then formazan crystals were dissolved in DMSO. The absorbance was measured at 570 nm (SpectraMax® iD3, Molecular Devices, San Jose, CA, USA) and the experiment was performed 3 times in duplicates.

4.7. Ros-Glo H_2O_2 assay

The level of hydrogen peroxide in cell culture was measured by Ros-Glo H_2O_2 assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Briefly, cells were seeded into 96-well plates and incubated overnight to allow adhesion. The next day, cells were pre-incubated with the tested compounds (10 μM) for 1 h and then stimulated with LPS. 6 h before the end of the 24 h long incubation, the H_2O_2 substrate was added to the wells. Relative luminescence units have been recorded using a multifunctional plate reader (SpectraMax® iD3,

Molecular Devices, San Jose, CA, USA). The experiment was performed in quadruplicates.

4.8. Determination of NO level

The nitrate concentration in the cell culture supernatant was measured by Griess Reagent System, according to the manufacturers' instructions (Promega Corporation, Madison, WI, USA). Briefly, cells were seeded into 24-well plates and incubated overnight to allow adhesion. The next day, cells were pre-incubated with the tested compounds (10 μM) for 1 h and then stimulated with LPS (1 μg/mL). After 24 h long incubation, 50 μL of cell culture supernatant was transferred to a new plate and then the Griess reaction was performed. The absorbance was measured at 540 nm and the concentration of nitrate was calculated using a nitrate standard reference curve (range 1.56–100 μM). The experiment was performed 3 times in duplicates.

4.9. Quantitative PCR

Quantitative polymerase chain reaction (qPCR) was performed to assess the expression level of selected genes. Cells were preincubated with the study compounds (10 μM) for 1 h, followed by 24 h incubation with IL-13 or TGF-β, and then suspended in TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The total RNA was extracted using Total RNA Mini Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol. RNA concentration was measured with a BioSpectrometer® basic (Eppendorf), and equal amounts of total RNA (about 1 μg) were reverse-transcribed using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA, USA). qPCR assays were performed using the CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA, USA) and TaqMan® Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA) with specific probes for: *CXCL8* (Hs00174103_m1), *TSLP* (Hs00263639_m1), *CCL26* (Hs00171146_m1), *POSTN* (Hs01566750_m1), *VIM* (Hs00958111_m1), *FN1* (Hs01549976_m1), *SNAI1* (Hs00195591_m1), and *MMP9* (Hs00957562_m1). qPCR cycling (40x) was performed with denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s. The relative abundance of specific mRNA transcripts was estimated based on the cycle threshold (Ct) values and recalculated against the endogenous reference *18S* (Hs99999901_s1) ribosomal RNA using the ΔCt method. The experiments were run 3 times in duplicates.

4.10. Immunocytochemical assays

Enzyme-linked immunosorbent assay (ELISA) was used to determine the proinflammatory cytokines in murine macrophage supernatants using commercial Mouse IL-6 DuoSet ELISA and Mouse TNF-α DuoSet ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA). Briefly, RAW264.7 cells were cultured in the presence of tested compounds (10 μM) 1 h before LPS treatment. The TNF-α and IL-6 production was determined in cell culture supernatants after 24 h of incubation, according to the manufacturers' instructions.

Levels of cytokines in HBEC supernatant were measured by Luminex method using predesigned multiplex panels (R&D Systems, Inc., Minneapolis, MN, USA) with Magpix detection platform (Luminex Corp., Austin, TX, USA) and xPonent software (Luminex). Briefly, HBECs were cultured in the presence of tested compounds (10 μM) 1 h before IL-13 treatment. The IL-8, eotaxin-3, periostin, and IL-13 production was determined in cell culture supernatants after 48 h of incubation, according to the manufacturer's instructions.

4.11. F-actin visualization

F-actin organization in HBECs was determined by fluorescence microscopy. Cells were seeded on collagen coated glass coverslips. Tested compounds (10 μM) were added 1 h before TGF-β treatment. After 48 h of incubation, cells were fixed in 4% formaldehyde solution, permeabilized, and then TRITC-labelled phalloidin solution (Sigma Aldrich, St. Louis, MO, USA) was added for 1 h. Nuclei were counterstained with Hoechst 33,342 dye (Thermo Fisher Scientific, Waltham, MA, USA). Slides were mounted in ProLong™ Glass Antifade Mountant (Invitrogen, Carlsbad, CA, USA) and analysed using Leica DMiL LED Fluo microscope (×40 objective) equipped with LAS-X Software (Leica Microsystems GmbH, Wetzlar, Germany). Experiments were run 3 times and several randomly selected fields of view were analysed at the same fluorescent time exposure and in a blind-folded manner.

4.12. Statistical analysis

Statistical analysis was performed with the GraphPad Prism (GraphPad Software, Inc., San Diego, California, USA). The comparisons between experimental conditions were performed using a nonparametric Wilcoxon test for paired data and a nonparametric Mann-Whitney *U* test (for qPCR data). Values presented in the graphs correspond to the mean ± standard error of the mean (S.E.M.). $P < 0.05$ was considered statistically significant.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2021.105409.

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