European Journal of Pharmacology xxx (xxxx) xxxx

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Full length article

Novel phosphodiesterases inhibitors from the group of purine-2,6-dione derivatives as potent modulators of airway smooth muscle cell remodelling

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ARTICLE INFO

Keywords:
Phosphodiesterases inhibitors
cAMP
ASMC hypertrophy
ASMC hyperplasia
Airway remodelling

ABSTRACT

Airway remodelling (AR) is an important pathological feature of chronic asthma and chronic obstructive pulmonary disease. The etiology of AR is complex and involves both lung structural and immune cells. One of the main contributors to airway remodelling is the airway smooth muscle (ASM), which is thickened by asthma, becomes more contractile and produces more extracellular matrix. As a second messenger, adenosine 3′,5′-cyclic monophosphate (cAMP) has been shown to contribute to ASM cell (ASMC) relaxation as well as to anti-remodelling effects in ASMC. Phosphodiesterase (PDE) inhibitors have drawn attention as an interesting new group of potential anti-inflammatory and anti-remodelling drugs. Recently, new hydrazide and amide purine-2,6-dione derivatives with anti-inflammatory properties have been synthesized by our team (compounds **1** and **2**). We expanded our study of their PDE selectivity profile, ability to increase intracellular cAMP levels, metabolic stability and, above all, their capacity to modulate cell responses associated with ASMC remodelling. The results show that both compounds have subtype specificity for several PDE isoforms (including inhibition of PDE1, PDE3, PDE4 and PDE7). Interestingly, such combined PDE subtype inhibition exerts improved anti-remodelling efficacies against several ASMC-induced responses such as proliferation, contractility, extracellular matrix (ECM) protein expression and migration when compared to other non-selective and selective PDE inhibitors. Our findings open novel perspectives in the search for new chemical entities with dual anti-inflammatory and anti-remodelling profiles in the group of purine-2,6-dione derivatives as broad-spectrum PDE inhibitors.

1. Introduction

Airway remodelling (AR) is a collective term for pathological structural alterations in the airways of patients with obstructive lung diseases such as asthma or chronic obstructive pulmonary disease (COPD) (Bergeron et al., 2010; Fehrenbach et al., 2017; Hogg et al., 2004; James and Wenzel, 2007). AR leads to thickening of the airway wall and consequently leads to airflow obstruction, thereby causing breathing disorders and reduced airway patency (Aoshiba and Nagai, 2004; Jones et al., 2016). Nearly all airway structural cells as well as a number of infiltrating inflammatory cells play an active, concerted role in AR. Structural changes which occur in the airways in asthma and COPD patients are currently not the target of any available treatment for these diseases, although a recent study has shown promising effects of the novel prostaglandin D2 receptor antagonist fevipiprant (Saunders et al., 2019). According to the Global Initiative for COPD and Asthma, the main therapeutic strategies are based on administration of inhaled or systemic corticosteroids, β2-agonists, anti-leukotrienes or cromones (Global Initiative for Asthma, 2018; Global Initiative for Chronic Obstructive Lung Disease, 2019). However, although these drugs inhibit inflammation and promote bronchodilation, they do not substantially affect AR (Beckett and Howarth, 2003; Bergeron et al., 2010; Durrani et al., 2011; Nayak et al., 2018).

Airway smooth muscle cells (ASMC) play a key role in AR as a result

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https://doi.org/10.1016/j.ejphar.2019.172779
Received 17 September 2019; Received in revised form 24 October 2019; Accepted 4 November 2019

of ASMC proliferation (hyperplasia), increased cell size (hypertrophy), increased extracellular matrix deposition and augmented cell migration (Bentley and Hershenson, 2008; Chung, 2005; Salter et al., 2017; Zuyderduyn et al., 2008). It has been shown that increased ASM mass is associated with the severity of asthma and COPD (Baraldo et al., 2012; Doeing and Solway, 2013; Hogg et al., 2004; James et al., 2012; Yan et al., 2018). Increased ASMC proliferation has also been described in these diseases (Johnson et al., 2001; Michaeloudes et al., 2017; Perry et al., 2011). Several different growth factors and cytokines may be responsible for evoking an ASMC remodelling response (Gawaziuk et al., 2007; Michaeloudes et al., 2017). Among others, a special role is attributed to transforming growth factor type β (TGF-β) which activates genes responsible for the expression of profibrotic proteins. A large body of evidence suggests an increased intracellular level of adenosine or guanosine 3′,5′-cyclic monophosphate (cAMP or cGMP) may lead to an inhibition of immune and lung structural cells' pro-inflammatory response (Oldenburger et al., 2012; Raker et al., 2016). Among other factors, high levels of cAMP and cGMP have been shown to contribute to ASMC relaxation as well as have suppressive effects on proliferation, extracellular matrix production and cell migration (Billington et al., 2013). As phosphodiesterases (PDEs) are one of the most important players that bring about a decrease in cAMP and/or cGMP intracellular levels, targeted, anti-PDEs therapy has been put forth as a promising approach for treating patients with asthma and COPD (Global Initiative for Asthma, 2018; Global Initiative for Chronic Obstructive Lung Disease, 2019). This may be especially important for corticosteroids-resistant patients, a group that is particularly in need of effective pharmacotherapy for asthma.

Recently, our team synthesized new hydrazide and amide derivatives of 1,3-dimethylpurine-2,6-dione (theophylline) (compounds **1** and **2**, Fig. 1A and B). Pharmacological studies of their biological activity have revealed their strong anti-inflammatory properties. Both compounds decreased the tumor necrosis factor α (TNF-α) concentration in rats (Chłoń-Rzepa et al., 2018a, 2018b). This effect was caused, at least in part, by PDE4B and 7A inhibition (Chłoń-Rzepa et al., 2018b, 2018a). In the current study, we aimed to investigate compounds **1** and **2** in the context of their potential to limit ASMC remodelling responses. First, the importance of studying the PDE selectivity profile of the compounds was demonstrated. We also examined their metabolic stability and tested their ability to raise the intracellular cAMP. Finally, we used FBS and TGF-β1-treated ASMC cultured in the presence of compounds **1** or **2** to study their effects on ASMC remodelling and compare their efficacy against several PDE inhibitors.

2. Materials and methods

2.1. Study compounds

Compound **1** (4-(8-(benzylamino)-1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1*H*-purin-7-yl)-*N*'-(2,3,4-trihydroxybenzylidene) butanehydrazide) and compound **2** (4-(8-butoxy-1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1*H*-purin-7-yl)-*N*-(5-*tert*-butyl-2-hydroxyphenyl)butanamide) were used in the study. They were synthesized according to the multistep procedure as described previously (Chłoń-Rzepa et al., 2018b, 2018a). Briefly, to obtain compound **1**, 8-bromo-1,3-dimethyl-2,3,6,7-tetrahydro-1*H*-purine-2,6-dione was treated with benzylamine in refluxing 2-methoxyethanol. Next, the obtained 8-benzylamine derivative was alkylated at the 7-position by treatment with ethyl 4-bromobutyrate in the presence of potassium carbonate (K_2CO_3) and a catalytic amount of benzyltriethylammonium chloride (TEBA) in refluxing acetone. The treatment of the obtained ester with hydrazine hydrate in anhydrous ethanol gave corresponding hydrazide. In the final step, the obtained hydrazide was condensed with 2,3,4-trihydroxybenzaldehyde in methanol in the presence of a catalytic amount of hydrochloric acid (HCl). To obtain compound **2**, 8-bromo-1,3-dimethyl-2,3,6,7-tetrahydro-1*H*-purine-2,6-dione was alkylated at the 7-position with ethyl 4-bromobutanoate in the presence of K_2CO_3 and a catalytic amount of TEBA in refluxing acetone. Next, the obtained ester was treated with sodium 1-butoxide in butan-1-ol, then hydrolysed using potassium hydroxide in water-acetone mixture and acidified with concentrated HCl to obtain the corresponding acid. In the final step, the obtained acid was condensed with 2-amino-4-(*tert*-butyl) phenol in *N*,*N*-dimethylformamide in the presence of di (1*H*-imidazole-1-yl)methanone. Both evaluated compounds were dissolved in dimethylsulfoxide (DMSO, PAN-Biotech, Germany). Reference substances: theophylline (1,3-dimethyl-7H-purine-2,6-dione), 1-methyl-3-(2-methylpropyl)-7H–purine-2,6-dione (IBMX), BRL-50481, KW-3920, KW-6002, roflumilast, cilomilast and forskolin (Sigma Aldrich, St. Louis, MO, USA) as well as vinpocetine, milrinone and rolipram (Cayman Chemical, Ann Arbor, MI, USA), were dissolved in DMSO and culture medium according to the manufacturer's protocol. The final DMSO concentration in culture medium did not exceed 0.9%. Vehicle control was performed in all experiments. No harmful effects of DMSO were demonstrated at the applied concentrations and incubation time for ASMCs. No changes in ASMCs' morphology, viability and proliferation, even at longer culture time with DMSO were observed. In addition, mixtures of selective PDE inhibitors, MIX 1 and MIX 2 were prepared by combining vinpocetine, milrinone, roflumilast and BRL-50481 in 1:1:1:1 and 1:0.2:2:6 ratios, respectively.

2.2. In vitro PDE inhibition assay

The PDE inhibitory activity of compounds **1** and **2** was evaluated using the PDE-Glo™ Phosphodiesterase Assay (Promega Corporation, Madison, WI, USA). Human recombinant PDEs were purchased from SignalChem, Richmond, Canada. The optimal amount of PDE in the reaction mixture and the optimal PDE reaction time was determined individually for each enzyme subtype according to the manufacturer's instructions. Aliquots of 1X PDE-Glo Reaction buffer (6.5 µl) containing appropriate amounts of purified human recombinants PDE1B, PDE2A,

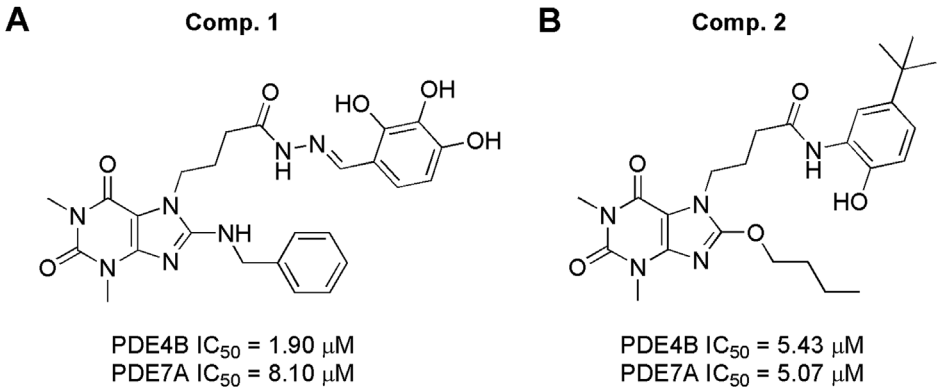

Fig. 1. Compounds 1 and 2 as PDE4B and 7A inhibitors. Chemical structures of 4-(8-(benzylamino)-1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1*H*-purin-7-yl)-*N*'-(2,3,4-trihydroxybenzylidene) butanehydrazide - compound **1** (A) and 4-(8-butoxy-1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1*H*-purin-7-yl)-*N*-(5-*tert*-butyl-2-hydroxyphenyl) butanamide – compound **2** (B) are presented.

PDE3A, PDE4D, PDE5A, or PDA10A were added to a 384 well plate (Thermo Scientific, USA). Both tested compounds were dissolved in DMSO and a serial dilution using 1X PDE-Glo Reaction buffer was performed. After the addition of 1 µl of diluted inhibitors to each well, 2.5 µl of cAMP solution was added to initiate the reaction. In the case of PDE2A and PDE5A, 2.5 µl of cGMP solution was used instead of the cAMP solution. After an appropriate incubation time at 30 °C, 2.5 µl of PDE-Glo™ Termination Buffer and 2.5 µl of PDE-Glo™ Detection Solution were added to each well. After 20 min of incubation, 10 µl of Kinase-Glo® Reagent was pipetted into each well and 10 min later luminescence was measured using a microplate luminometer (POLARstar Omega, BMG LABTECH, Ortenberg, Germany). All data were expressed as the mean of the percentage of the relative PDE activity from three independent determinations (± S.D.). IC_{50} values were obtained by non-linear regression using Phoenix WinNonlin v. 7.0 (Certara, USA).

2.3. Metabolic stability in human liver microsomes

Glucose-6-phosphate, NADP+, glucose-6-phosphate dehydrogenase, buffers and human liver microsomes (HLM) were supplied by Sigma Aldrich (St. Louis, MO, USA). Pentoxifylline (PTX) was obtained from Polpharma (Kraków). Reaction mixtures, consisting of tested drugs (20 µM), HLM (0.4 mg protein/ml) in phosphate buffer (pH = 7.40; 0.1 M) were preincubated for 10 min, at 37 °C on an Eppendorf ThermoMixer®. Next, NADPH-regenerating system (NADP+, glucose-6-phosphate, glucose-6-phosphate dehydrogenase in phosphate buffer) was added to initiate the reaction. Tests without NADPH-regenerating system were conducted in parallel and incubations were conducted in duplicates. Total volume of the reaction mixture was 250 µl. At different time points an internal standard (PTX, 20 µM) was added, and the reaction was quenched with ice-cold methanol. Tubes were transferred for 1 h to −20 °C. Samples were then centrifuged for 10 min at 15,000 g and the obtained supernatants subjected to ultra performance liquid chromatography coupled with mass spectrometer analysis. *In vitro* half-life ($t_{1/2}$) was assessed from the slope of linear regression of ln% parent compound remaining versus the incubation time. Then intrinsic clearance (CL_{int}) was calculated as described previously (Singh and Solanki, 2012).

2.4. ASMC culture

Human ASMC (n = 3 donors) immortalized by stable expression of human telomerase reverse transcriptase (hTERT) and isolated from two donors, was used in the study. The entire procedure of obtaining ASMC has been described in detail by Gosens et al. (2006). Cells were maintained between passages 21–28 on Petri dishes in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Scientific, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Gibco, Thermo Scientific, USA) and an antibiotics mixture (Penicillin, Streptomycin, Amphotericin B; Gibco, Thermo Scientific, USA). Cells were cultured in standard conditions (5% CO_2, 37 °C, 95% humidity). ASMC were serum-deprived for all experiments in DMEM supplemented with ITS-A (Insulin-Transferrin-Selenium–Sodium Pyruvate Solution, Gibco, Thermo Scientific, USA) and the antibiotics mixture. Compounds **1** and **2** were added at final concentrations from 1 µM to 50 µM, 1 h before FBS or TGF-β_1 (2 ng/ml, BD Biosciences, USA) treatment.

2.5. ASMC proliferation

The effect of compounds **1** and **2** on ASMC proliferation rate was evaluated by using an alamarBlue® Assay. Cells were seeded in 24-well plates (3×10^4 cells/well) in standard culture medium. After 24 h of culture they were serum-starved for 3 days to induce cell cycle arrest. Compounds **1** and **2** (1–50 µM) and reference substances, roflumilast and cilomilast (1–10 µM) were added 1 h prior to stimulation of ASMC growth with FBS (10%, vol/vol) for an additional 48 h. Proliferation assays were performed by incubation of the cells with alamarBlue® reagent (Invitrogen, Life Technologies, USA) and dissolved in Hank's Balanced Salt Solution (HBSS, 10% vol/vol; Gibco, Thermo Scientific, USA) for 30 min at 37 °C. After the observed colour change, the supernatant was transferred to 96-well plates and the absorbance was measured at 570 and 600 nm. The percent difference in reduction between treated and control cells was calculated according to the manufacturer's protocol.

2.6. RNA isolation and quantitative PCR

Real-time polymerase chain reaction (qPCR) was used to detect and quantify the expression of specific human genes: *ACTA2, CNN1, COL1A1, FN1,* and *VCAN*. ASMC were seeded in 6-well plates (2×10^5 cells/well) in standard culture medium. After 24 h of culture they were serum-starved for 3 days to induce cell cycle arrest. The cells were incubated with the study compounds and TGF-β_1 for 24 h and total RNA was extracted after 24 h incubation using the TRI Reagent® Solution (Applied Biosystems, Foster City, California, USA) according to the manufacturer's protocol and RNA concentration was determined with a NanoDrop spectrophotometer (ThermoFisher, Breda, the Netherlands). Equal amounts of total RNA were reversely transcribed using the Reverse Transcription System (Promega Corporation, Madison, WI, USA). Real-time PCR assays were performed using the PIXO™ Real-Time PCR System (Helixis, Carlsbad, California, USA) and SYBR® Green Mastermix (Roche Diagnostics, Almere, the Netherlands). PCR cycling was performed with denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s and an extension at 72 °C for 30 s. Specific primers used to quantify the transcripts are listed in Table 1. The relative abundance of specific mRNA transcripts was estimated based on cycle threshold (C_T) value and recalculated against the endogenous reference 18S ribosomal RNA gene using the ΔC_T method. All samples were run in duplicates.

2.7. Immunoblotting

Western Blot analysis was performed according to standard protocol. Briefly, cells were seeded in 6-well plates (2×10^5 cells/well) in standard culture medium. After 24 h of culture they were serum-starved for 3 days to induce cell cycle arrest. The cells were incubated with the study compounds and TGF-β_1 for 48 h and then lysed in RIPA buffer (RIPA Lysis and Extraction Buffer, Thermo Scientific, USA) and supplemented with protease inhibitors (Pierce Protease Inhibitor Tablets, Thermo Scientific, USA). Protein concentrations were measured using a Bradford Reagent. Equal amounts of protein (15 µg of total protein) were separated using 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Hybond™, GE™ Healthcare, USA), at a constant voltage (35 V) in a transfer buffer at 4 °C. Blots were

Table 1
List of primers used for detection of specific human genes.

Gene	Primers	
	5'→3' forward	5'→3' reverse
α-SMA *ACTA2*	CCG GGA GAA AAT GAC TCA AA	GAA GGA ATA GCC ACG CTC AG
calponin-1 *CNN1*	ACA TTT TTG AGG CCA ACG AC	CTC CCA CGT TCA CCT TGT TT
collagen Iα1 *COL1A1*	AGC CAG CAG ATC GAG AAC AT	TCT TGT CCT TGG GGT TCT TG
fibronectin I *FN1*	TCG AGG AGG AAA TTC CAA TG	ACA CAC GTC CAC CTC ATC AT
versican *VCAN*	GGG AAC CTG GTG AAG AAA CA	CTT CCA CAG TGG GTG GTC TT
18S rRNA	CGC CGC TAG AGG TGA AAT TC	TTG GCA AAT GCT TTC GCT C

washed with TBST buffer (150 mM NaCl, 50 mM Tris, 0.05% Tween 20), blocked with 5% skim milk in TBST and incubated overnight at 4 °C with the appropriate primary antibody (mouse monoclonal anti-α-smooth muscle actin antibody, clone 1A4, dilution 1:1000, #A2547; mouse monoclonal anti-collagen type I antibody, dilution 1:1000, #SAB4200678; mouse monoclonal anti-GAPDH antibody, dilution 1:5000, #G8795, all from Sigma Aldrich, St. Louis, MO, USA). After incubation with primary antibodies, membranes were washed 3 times with TBST buffer and incubated with secondary, rabbit anti-mouse IgG (whole molecule)-peroxidase antibodies (#A9044, dilution 1:3000, Sigma Aldrich, St. Louis, MO, USA). The protein bands were visualized using an enhanced chemiluminescence method (SuperSignal™ West Pico PLUS Chemiluminescent Substrate, Thermo Scientific, USA) with a Chemi Doc Camera (BioRad, Hercules, CA, USA). Relative Optical Density (ROD) was measured according to NIH guidelines with ImageJ Software.

2.8. Migration assay

Migration assay was performed using 6.5-mm Transwell culture plates with an 8.0-μm pore polycarbonate membrane (Corning Incorporated, USA). ASMC were serum-starved and following harvesting were seeded (1.5×10^3 cells/well) in the upper chamber in serum-free culture medium supplemented with or without compounds **1** and **2** or reference drugs. The lower compartment was filled with the same culture medium both with and without studied compounds and after 1 h TGF-β_1 was added to the wells. After 24 h of incubation the non-migrated cells were removed from the upper face of the membranes using a cotton swab. The remaining ASMC were fixed with 4% paraformaldehyde solution (Sigma Aldrich, St. Louis, MO, USA) and stained with 0.5% crystal violet solution (Sigma Aldrich, St. Louis, MO, USA) for 10 min. The number of migrated cells on the lower face of the filter was counted under an inverted microscope (Nikon Eclipse TS 100) using 10 randomly selected fields of view.

2.9. ELISA

Intracellular cAMP level was measured using a colorimetric cAMP ELISA Kit according to the manufacturer's protocol (Cell Biolabs, INC., San Diego, CA, USA). ASMC were seeded in 6-well plates (2×10^5 cells/well) in standard culture medium. After 24 h of culture they were serum-starved for 2 h in HBSS. The study compounds and reference substances were added to the cell culture and incubated for 30 min. Whole cell lysates were used to quantify cAMP level. Total protein content was measured by Bradford Assay and final cAMP concentrations were normalized to total protein content in each sample. The experiment was run twice in duplicates.

2.10. Data analysis

Unless indicated otherwise, statistical analysis was performed with a GraphPad Prism (GraphPad Software, Inc., San Diego, California, USA). The comparison of parameters between experimental conditions was performed using a non-parametric Wilcoxon test for paired data. Values presented in the graphs correspond to mean ± standard error of the mean (S.E.M.). Statistical significance was assumed at the level of $P < 0.05$.

3. Results

3.1. Compounds 1 and 2 are non-selective but strong inhibitors of selected PDE isoforms

Our previous research has shown that compounds **1** and **2** possess strong anti-inflammatory properties and are inhibitors of PDE4B and 7A isoenzymes (Fig. 1A and B, Fig. 2C and D, Table 2). To expand on this, we determined their ability to inhibit other key PDE isoforms. Compounds **1** and **2** could both inhibit the activity of PDE1B, 2A, 3A, 4D, and 10A isoenzymes (Fig. 2A and B, Table 2). Their potency, expressed as the IC50 value, was comparable to commercially available inhibitors of individual PDE isoforms and greater than those that of other pan-PDE inhibitors – theophylline and IBMX (Table 2). Both compounds showed particularly strong activity against the 1B and 3A isoforms (Fig. 2A and B). The IC50 for compounds **1** and **2** against PDE1B inhibition was 0.07 and 2.69 μM, respectively, and in the case of PDE3A, 0.25 and 0.184 μM, respectively (Table 2). Both compounds showed virtually no activity against PDE5A. For compound **1**, the PDE inhibitory potency increased according to the following sequence: 1B > 3A > 4B, 7A, 2A, 4D, 10A > 5A. In turn, for compound **2,** this was as follows: 3A > 1B, 4B, 7A > 2A, 10A, 4D > 5A. As the PDE4D isoform is considered responsible for the inhibitors' side effects (Hatzelmann et al., 2010; Kawamatawong, 2017), it is also worth noticing the reduced potential of both compounds for inducing nausea (compounds **1** and **2** have higher IC50 for PDE4D in comparison to roflumilast and cilomilast which exhibit PDE4D activity at a nano-molar level).

3.2. Compounds 1 and 2 elevate the intracellular cAMP level

As the selected purine-2,6-dione derivatives are strong pan-PDE inhibitors, for the next step we decided to verify their potential impact on intracellular cAMP levels. Both compounds significantly increased the levels of this second messenger in ASMC (Fig. 3). Their effect was comparable to another pan-PDE inhibitor – IBMX, as well as to a known adenylyl cyclase activator – forskolin. Interestingly, the applied selective PDE inhibitors: vinpocetine (PDE1 inhibitor), milrinone (PDE3 inhibitor) roflumilast and cilomilast (PDE4 inhibitors) and BRL-50481 (PDE7 inhibitor) also slightly increased cAMP levels but their effect was less pronounced (Fig. 3). As we wanted to confirm the additive effect of pan-PDE inhibitors we also used the mixtures of selective PDE1, PDE3, PDE4, and PDE7 inhibitors. We tested two mixtures, the first contained equal amounts of individual inhibitors and the second comprised amounts of inhibitors in a ratio dependent on the experimental activity of compounds **1** and **2** against individual PDE isoforms. Both blends caused an effect similar to the effect of purine-2,6-derivatives on intracellular cAMP (Fig. 3).

3.3. Compounds 1 and 2 are metabolically stable in human liver microsomes

The metabolic stability of compounds **1** and **2** was quantified by monitoring their disappearance over time in HLM supplemented with an NADPH-regenerating system. Both compounds were determined to be metabolically stable with CL_{int} of 2.78 and 13.02 μL/min/mg for compounds **1** and **2,** respectively (Table 3). Two metabolites of compound **2** were found: compound **2-M1** resulting from the hydroxylation of the parent compound and compound **2-M2** – a product of hydroxylation and simultaneous oxidation of the methyl group at the *tert*-butyl substituent to carboxylic acid. No metabolites of compound **1** were detected.

3.4. Compounds 1 and 2 inhibit ASMC proliferation

To investigate the potential effect of compounds **1** and **2** on ASMC responses associated with remodelling, we first determined their impact on cell proliferation. None of the selective reference PDE inhibitors (PDE1 – vinpocetine, PDE3 – milrinone, PDE4 – roflumilast/cilomilast, PDE7 – BRL50-481) reduced ASMC proliferation (Fig. 4A). At higher concentrations, a slight decrease in cell proliferation was observed when pan-PDE inhibitors, such as theophylline or IBMX were used (Fig. 4B). However, a significant reduction in the proliferation rate was observed for compounds **1** and **2**. We demonstrated that in the range of lower concentrations (1–10 μM), both compounds caused dose-

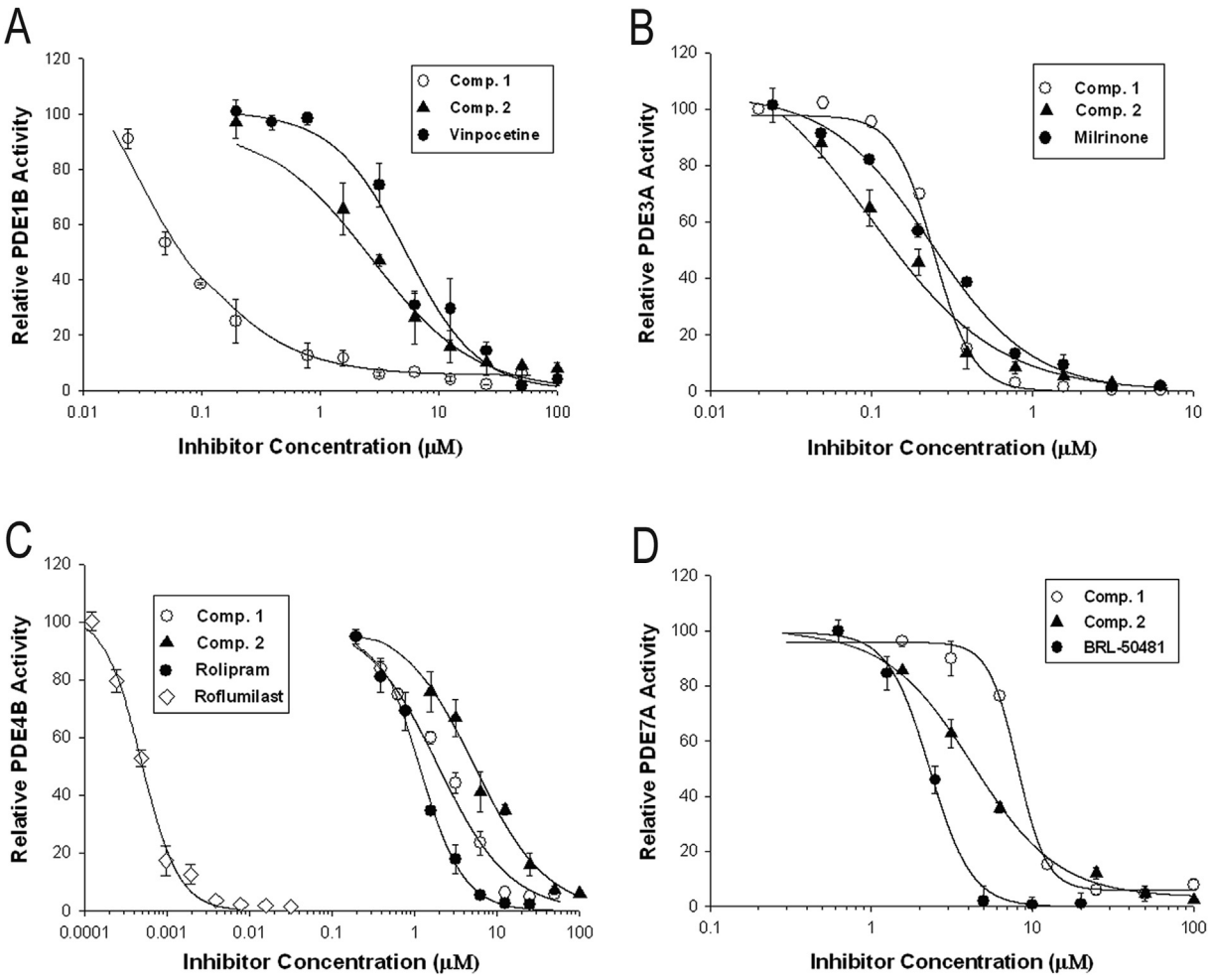

Fig. 2. Compounds 1 and 2 PDE inhibitory activity. Plots represent curves obtained during determination of IC_{50} for compounds **1** and **2** against PDE1B (A), PDE3A (B), PDE4B (C), and PDE7A (D). IC_{50} values obtained for compound **1** against PDE4B and 7A were published previously (Chłoń-Rzepa et al., 2018b, 2018a).

Table 2
PDEs' selectivity profile of compounds **1** and **2** in comparison to reference compounds.

PDE isoform	IC_{50} [μM]					
	1	2	Theophylline	IBMX	Reference compounds	
1B	0.07	2.69	> 1000	35.70	Vinpocetine	10.06
2A	9.16	10.89	553.77	9.22	EHNA	4.05
3A	0.25	0.18	185.95	0.71	Milrinone	0.23
4D	10.70	13.90	> 1000	–	Rolipram	4.27
5A	> 200	103.88	> 1000	45.09	Zaprinast	6.98
10A	27.04	13.84	227.49	1.16	Papaverine	0.04
4B	1.90[a]	5.43[a]	> 1000	46.60[b]	Rolipram	1.10[a]
					Roflumilast	0.00049
7A	8.10[a]	5.07[a]	> 1000	77.70[b]	BRL-50481	2.10[a]

Values were published previously -.
[a] (Chłoń-Rzepa et al., 2018a, 2018b).
[b] (Świerczek et al., 2017).

dependent inhibition of ASMC proliferation (Fig. 4B). At a concentration of 10 μM, the percent decrease in ASMC proliferation expressed as the number of cells relative to the control, was 24% and 38% for compounds **1** and **2**, respectively. At higher applied concentrations, compounds **1** and **2** caused further reduction of ASMC proliferation. Compound **2** was particularly active in these ranges, as at a 50 μM concentration, it caused a nearly complete reduction in ASMC proliferation, in comparison to the control. A similar effect was observed for the mixtures of individual PDE inhibitors (Fig. 4B). Both compounds substantially limited ASMC proliferation, thus confirming the importance of inhibition of PDEs 1, 3, 4, and 7 for the observed effect. To rule out the involvement of adenosine receptors antagonism in the effects, we also examined the effect of adenosine receptor antagonists - A1 (KW-3920) and A2A (KW-6002) on ASMC proliferation. Both compounds had no effect on cell proliferation whatsoever (Fig. 4C).

3.5. Compounds 1 and 2 repress TGF-β_1-induced ASMC contractile phenotype marker expression

TGF-β_1 stimulation *in vitro* of cultured ASMC promotes a contractile phenotype which can be characterized by increased expression of contractile genes such as *ACTA2* (α-SMA) and *CNN1* (calponin 1) (Kumawat et al., 2016; Schuliga et al., 2013). We found that compounds **1** and **2** both strongly reduced TGF-β1-induced *ACTA2* and *CNN1* genes' expression in ASMC (Fig. 5A and B). Both compounds caused a significant and concentration-dependent decrease of α-SMA and calponin-1 transcript levels. The ability of both compounds **1** and **2** to reduce TGF-β_1-induced contractile gene expression was substantially stronger than the effectiveness of reference substances, roflumilast and cilomilast. At a concentration of 10 μM, compounds **1** and **2** were almost three times more efficacious in inhibiting α-SMA expression than roflumilast and cilomilast. For calponin-1 expression, compounds **1** and **2** were respectively two and three times more efficacious than roflumilast or cilomilast. We also performed immunoblotting, which confirmed the inhibitory effects of compounds **1** and **2** on TGF-β_1-

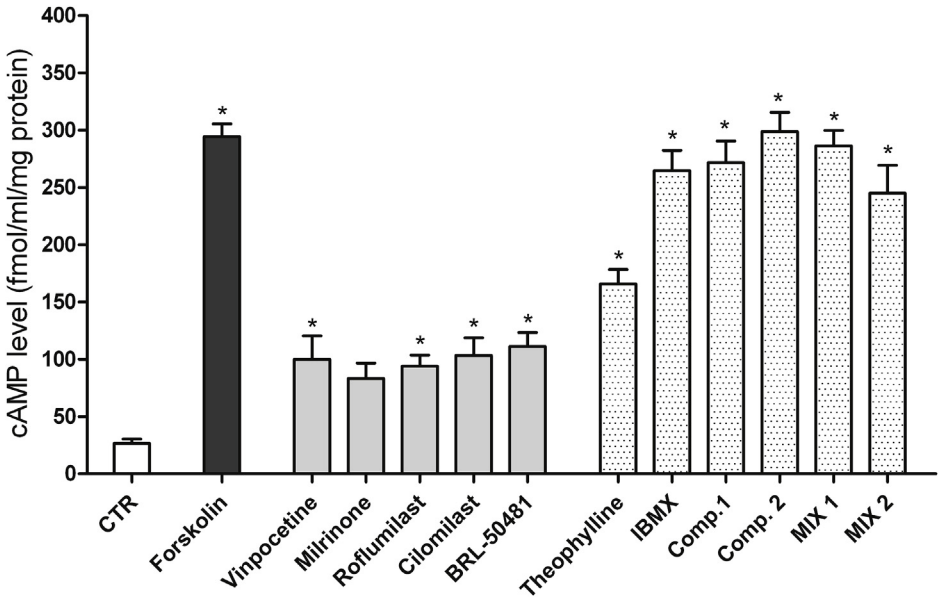

Fig. 3. Compounds 1 and 2 elevate intracellular cAMP levels in ASMC. ASMC (n = 2 cell lines) were cultured in DMEM with 10% FBS for 24 h, then serum deprived in HBSS for 2 h. All compounds were added for 30 min at 10 μM. Intracellular cAMP levels were measured according to the manufacturer's protocol. Each bar represents the mean cAMP level (fmol/ml/mg of protein; from 4 independent repeats) ± S.E.M. *P < 0.05 (in reference to control).

induced α-SMA protein level (Fig. 5C).

3.6. Compound 2 diminishes TGF-β_1-induced extracellular matrix components in ASMC

As the studied compounds effectively reduced the level of contractile proteins in ASMC, in the next experiments we investigated whether they could also affect the level of ECM components synthesized by these cells. To address this issue we estimated the level of *COL1α1* (collagen Iα1), *FN1* (fibronectin 1), and *VCAN* (versican) gene expression. Our experiments showed divergent effects of compounds **1** and **2** on the ECM components' transcript levels in TGF-β_1-induced ASMC (Fig. 6). Whereas compound **2** was able to limit the expression of both *COL1α1* and *FN1*, compound **1** only inhibited the *FN1* transcript level (Fig. 6A and B). Roflumilast and cilomilast showed a limited impact on the TGF-β_1-induced ECM gene expression. Versican expression seemed to be less affected under the action of both compounds as well as reference substances (Fig. 6C). The decrease in this proteoglycan transcript level was only observed for compound **2** at the highest applied concentration. To confirm these results, immunoblot analyses were also performed. The experiments showed that both test compounds and reference substances were able to diminish TGF-β_1-induced collagen levels in ASMC (Fig. 6D). However, in this case the action of especially compound **2** was most profound.

3.7. Compounds 1 and 2 attenuate TGF-β_1-induced ASMC migration

We further analyzed if our compounds may cause disturbances of TGF-β_1-induced ASMC migration. As demonstrated in Fig. 7A and B, treatment of ASMC with compounds **1** and **2** in the presence of TGF-β1 decreased the migrated cell number by 42% and 47%, respectively. Roflumilast and cilomilast also reduced ASMC migration, and in this instance their effect was stronger than that of compounds **1** and **2** (75% and 74% respective decrease in migrated cell number, Fig. 7A and B).

4. Discussion

Asthma and COPD are widespread diseases in both developed and developing countries with increasing morbidity and mortality (Global Initiative for Asthma, 2018; Global Initiative for Chronic Obstructive Lung Disease, 2019). Current pharmacotherapy guidelines include numerous drugs ranging from bronchodilators and corticosteroids to targeted anti-inflammatory therapeutics such as monoclonal antibodies (Durham et al., 2016; Global Initiative for Asthma, 2018; Global Initiative for Chronic Obstructive Lung Disease, 2019). Unfortunately, most of such drugs essentially do not affect airway remodelling or only slightly inhibit individual characteristics of airway remodelling (Beckett and Howarth, 2003; Durrani et al., 2011; Nayak et al., 2018). Therefore, the search to find new chemical entities that could become anti-remodelling drugs in the future is extremely urgent (Fromer, 2011; Mullane, 2011). Here, we describe a novel class of PDE inhibitors with improved anti-remodelling efficacies against several ASMC induced responses such as proliferation, contractility, ECM protein expression and migration.

Theophylline (1,3-dimethylpurine-2,6-dione), a non-selective PDE family inhibitor has been available for the treatment of asthma or COPD for almost 100 years. Although it has a good anti-inflammatory profile, it is currently rarely used in clinical practice, mainly due to its narrow therapeutic window and side-effects (Barnes, 2013a). To improve the therapeutic use of PDE inhibition, numerous studies have aimed to identify substances that might be used for the treatment of asthma or COPD with more selective PDE inhibition profiles, especially with respect to the PDE4 family (Kawamatawong, 2017; Mulhall et al., 2015; Page, 2014). This isoform is expressed in inflammatory cells and lung structural cells which are both involved in the pathogenesis of respiratory diseases (Mulhall et al., 2015; Page, 2014). Pharmacological

Table 3
Metabolic stability of compounds **1** and **2** in HLM.

Compound	Retention time [min]	m/z	CL_{int} [μl/min/mg]	Metabolic reaction
1	4.86	522.3	2.78	–
2	7.82	486.4	13.02	
2-M1	5.52	531.3	–	hydroxylation; oxidation of methyl group at the *tert*-butyl substituent to carboxylic acid
2-M2	5.97	502.3	–	hydroxylation

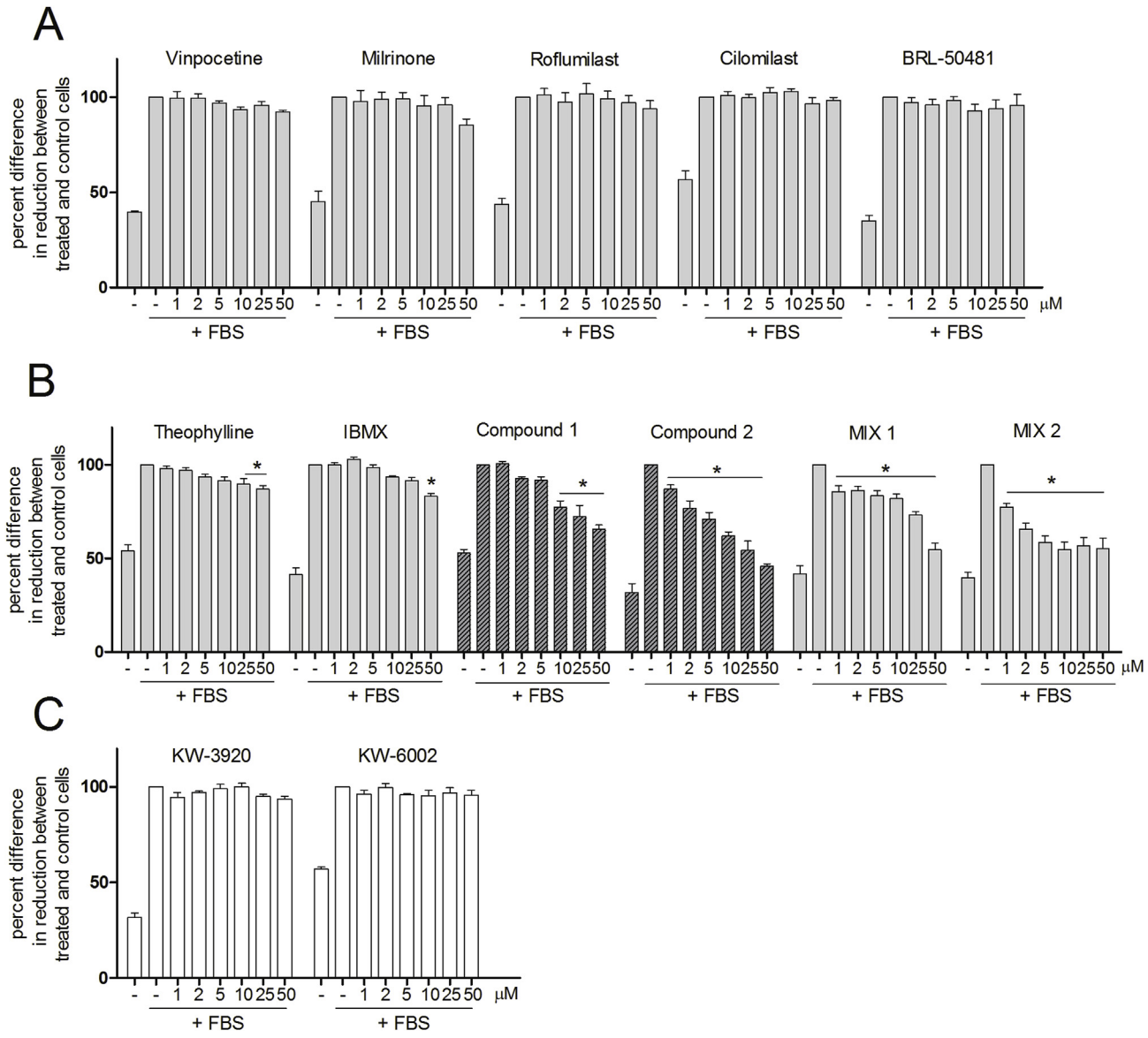

Fig. 4. Compounds 1 and 2 reduce ASMC proliferation. ASMC (n = 3 cell lines) were cultured in DMEM with 10% fetal bovine serum (FBS) for 24 h, then serum deprived in DMEM with ITS for 72 h. Compounds **1** and **2**, reference inhibitors (PDE1 – vinpocetine, PDE3 – milrinone, PDE4 – roflumilast/cilomilast, PDE7 – BRL-50481, theophylline, IBMX), adenosine receptor antagonists (KW-3920, KW-6002) and PDE inhibitors mixtures were added at increasing concentrations (1–50 µM) 1 h before FBS treatment. ASMC proliferation was measured after 48 h of incubation. Each bar represents mean percentile differences in alamar®Blue reduction between treated and control cells (± S.E.M.). MIX 1 and MIX 2 - the final concentration of all inhibitors in the mixture is included in the X axis. Experiments were run in duplicates for each ASMC culture.*P < 0.05 (in reference to control).

inhibition of PDE4 increases intracellular cAMP level and consequently causes anti-inflammatory effects (Raker et al., 2016). Although the use of inhibitors of a single PDE markedly improved the safety of using compounds from this group, their effectiveness is suboptimal. Recent findings indicate that dual inhibitors (PDE1-PDE4, PDE3-PDE4, or PDE4-PDE7) may be more effective in preventing changes related to AR (Maurice et al., 2014). This provides a rationale for discovering molecules with a broader spectrum of PDE inhibition.

Our studies have shown that novel purine-2,6-dione derivatives are non-selective, however strong PDE inhibitors. We have demonstrated that they can significantly inhibit PDE4 as well as PDE1, PDE3 and PDE7 isoenzymes, which are accompanied by an elevated intracellular cAMP level. Although the role of PDE4 in asthma and COPD is well established, the functional roles of PDE1, PDE3 or PDE7 sub-families in airways disease are less known. All 3 of these isoforms are expressed in bronchial epithelium, airway smooth muscle cells, pulmonary fibroblasts as well as inflammatory cells (Halpin, 2008; Oldenburger et al., 2012). Several studies indicate that PDE3 and PDE7 inhibitors augment the anti-inflammatory effects of PDE4 inhibitors (Maurice et al., 2014; Page, 2014). It is believed that a broader spectrum of activity with simultaneous strong action not only improves the anti-inflammatory properties of compounds but also provides a better safety profile. For example, theophylline also exhibits activity towards different PDE isoforms but inhibits them at much higher concentrations than compounds **1** and **2** (Hatzelmann et al., 2010). Our aim was to obtain compounds which due to their activity will be strong, second generation, pan-PDE inhibitors. Compounds **1** and **2** have relatively low IC50 values against individual PDE isoenzymes, including PDE1, PDE3, PDE4 and PDE7, which seems to be extremely important for the observed pharmacological effects. These characteristics distinguish them from the group of existing pan-PDE inhibitors, such as theophylline or IBMX. It is worth emphasizing that in spite of adverse effects, theophylline is still used in difficult cases to treat asthma. Therefore, the search for new compounds with similar properties but with a preferential activity

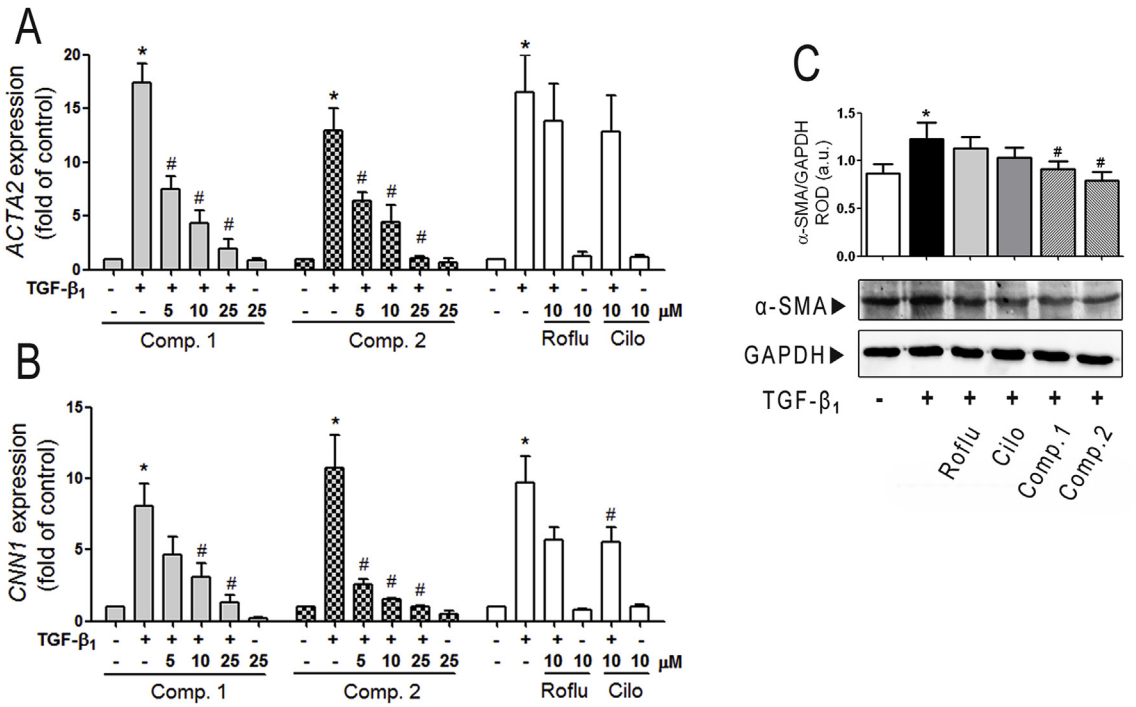

Fig. 5. Compounds 1 and 2 repress TGF-β$_1$-induced contractile markers in ASMC. ASMC (n = 3) were cultured in DMEM with 10% FBS for 24 h, then serum deprived in DMEM with ITS for 72 h. Compounds **1** and **2** and reference substances (roflumilast and cilomilast) were added at increasing concentrations (1–25 μM) 1 h before TGF-β$_1$ (2 ng/ml) treatment. Expression of *ACTA2* (A) and *CNN1* (B) genes in comparison to 18S rRNA was quantified by qPCR, after 24 h of incubation. Values represent means (± S.E.M.) of three ASMC cultures run in duplicates. (C) Representative immunoblot and densitometry analysis of α-SMA protein in ASMC cultured in the presence of compounds **1**, **2**, roflumilast or cilomilast, and TGF-β$_1$ was performed. ROD was measured and compared to GAPDH level. Each bar represents the mean value (± S.E.M.) obtained from three ASMC cultures run in duplicates. *P < 0.05 - in reference to control, #P < 0.05 - in reference to TGF-β$_1$-treated ASMC.

profile is important. The strong activity of novel purine-2,6-dione derivatives against different PDE isoforms makes them an interesting alternative to theophylline, a broad spectrum PDE inhibitor and roflumilast or cilomilast, which only inhibit the PDE4 family (Hatzelmann et al., 2010).

As the studies of metabolic stability carried out in the HLM model showed favourable metabolic profiles of compounds **1** and **2** we evaluated their ability to modulate cell responses associated with ASMC remodelling. ASMC were used in the study as their hyperplasia and hypertrophy is described in AR and they are a good experimental model to test the efficiency of possible anti-fibrotic compounds (Baroffio et al., 2008). Our experiments also confirmed the strong and prominent profibrotic response of TGF-β in ASMC. This growth factor activates ASMC expression of certain genes linked to the remodelling response phenotype (e.g. *ACTA2, CNN1, COL1α1*, *FN1*). Studies by Burgess and coworkers have shown that roflumilast may reduce TGF-β-induced expression of CTGF and ECM proteins in ASMC (Burgess et al., 2006). Our research confirms the potential anti-fibrotic properties of PDE4 inhibitors, although purine-2,6-dione derivatives appear to have preferable activity. These strong, non-selective PDE inhibitors were determined to be more potent in reducing FBS or TGF-β-induced ASMC remodelling response. This was expressed by a significant limitation of ASMC proliferation and expression of profibrotic and ECM proteins genes. Our observations relate to control ASMC but we would expect similar effects in asthmatic airway smooth muscle cells as the PDE inhibitors provide a functional antagonism response, not a mediator blocking response. Therefore, the effect is expected to occur for other growth stimuli or growth conditions as well. Conducted studies have shown compounds **1** and **2** have a slightly weaker effect on TGF-β-induced ASCM migration when compared to the selective PDE4 inhibitors roflumilast and cilomilast. This may be related to the high cAMP and PDE compartmentalization in the cells (Zuo et al., 2019a, 2019b). Possibly, the broader spectrum activities of compounds **1** and **2** lead to a different activation of intracellular signalling compartments (including those responsible for the migration process) than roflumilast or cilomilast. For some responses, this may result in more efficacy, for others (e.g. responses heavily dependent on PDE4) this difference may be less profound. Comparison of the observed activities of compounds **1** and **2** to PDE4-selective inhibitors suggests that inhibition of PDE1, PDE3 and/or PDE7 isoforms may also be involved in reducing TGF-β-induced remodelling responses in ASMC. For the inhibition of TGF-β functional responses, these isoforms may contribute directly, or work together by enhancing the functional effects associated with the inhibition of PDE4 isoforms. As the selective PDE inhibitors can act synergistically in reducing inflammation or AR (Maurice et al., 2014) this contention seems to be plausible.

The mechanisms of action of the compounds from the methylxanthine group include not only the inhibition of intracellular PDEs but also the binding and blocking of adenosine receptors. It is known that caffeine, theophylline and IBMX are antagonists of adenosine receptors (Müller and Jacobson, 2011). From previous literature reports and structure-activity relationship (SAR) studies it appears that in order to be a strong antagonist of adenosine receptors (stronger than theophylline), the compound should have large, complex and strongly lipophilic substituents, e.g. phenyl or phenylethyl, at position 8 of the purine-2,6-dione core (Müller and Jacobson, 2011). Also, tricyclic structures with a third ring added to xanthine are preferential (Müller and Jacobson, 2011). Our compounds have less lipophilic benzylamine or butoxy substituents at position 8 of the purine-2,6-dione compared to phenyl or phenylethyl. Moreover, the obtained research results showed that the mechanism of adenosine A1 and A2A receptor blockade did not play a significant role in the changes associated with ASMC remodelling, although additional mechanisms of action of the compounds studied cannot be excluded. From the preliminary studies, it also follows

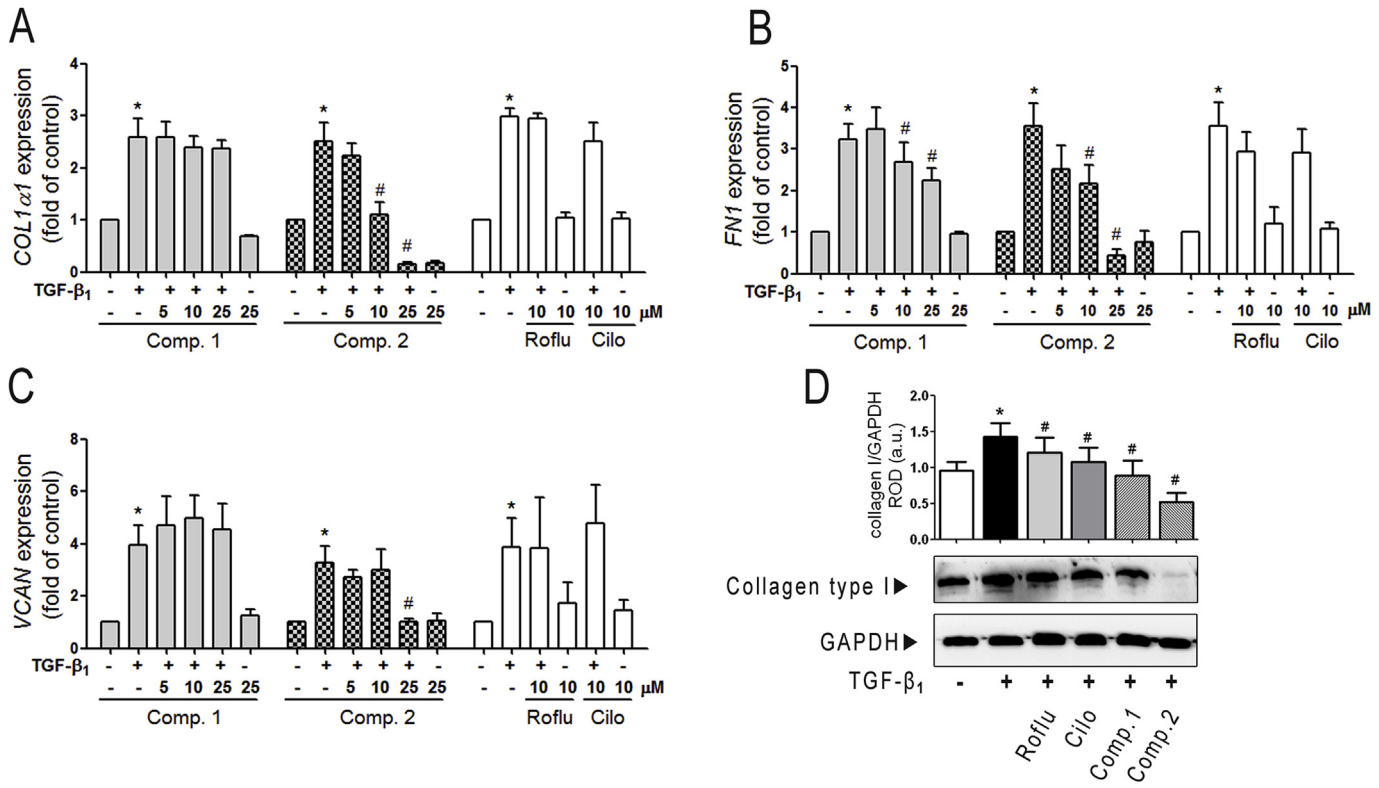

Fig. 6. Compound 2 reduces TGF-β_1-induced extracellular matrix components in ASMC. ASMC (n = 3) were cultured in DMEM with 10% FBS for 24 h, then serum deprived in DMEM with ITS for 72 h. Compounds **1** and **2** and reference substances (roflumilast and cilomilast) were added at increasing concentrations (1–25 μM) 1 h before TGF-β_1 (2 ng/ml) treatment. Expression of *COL1α1* (A), *FN1* (B) and *VCAN* (C) genes in comparison to 18S rRNA was quantified by qPCR, after 24 h of incubation. Values represent means (± S.E.M.) of three ASMC cultures run in duplicates. (D) Representative immunoblot and densitometry analysis of collagen type I protein in ASMC cultured in the presence of compounds **1** and **2**, roflumilast or cilomilast, and TGF-β_1. ROD was measured as compared to GAPDH level. Each bar represents the mean value (± S.E.M.) obtained from three ASMC cultures run in duplicates. *P < 0.05 - in reference to control, #P < 0.05 - in reference to TGF-β_1-treated ASMC.

that compounds **1** and **2** do not regulate the catalytic activity of adenylate kinases (unpublished data).

In the applied experimental model, both tested compounds demonstrated the ability to reduce TGF-β-induced ASMC remodelling response, although compound **2** was more potent. We observed this activity despite the fact that both compounds showed a comparable inhibitory effect on selected PDE isoforms. We can therefore exclude that the superior properties of compound **2** correspond to the preferable inhibition profile of individual PDE isoforms. The predominant activity of compound **2** in limiting ASMC remodelling response may be associated with stronger anti-inflammatory properties. In pharmacological studies, compound **2** was shown to possess a greater ability to inhibit lipopolysaccharide-induced TNF-α secretion in rats (Chłoń-Rzepa et al., 2018a, 2018b). Another reason for the stronger activity of compound **2** is its metabolic stability. The obtained CL_{int} values indicate that the compounds were similar in terms of biotransformation, although in the case of compound **2**, two metabolites were formed. The direction of metabolism we suggested indicates the oxidation of a methyl group at the *tert*-butyl substituent, which is a characteristic reaction for this moiety. Analogical phenomenon was observed for the metabolism of terfenadine, an antihistaminic drug. The metabolite formed as a result of the oxidation of the *tert*-butyl group in the parent structure, turned out to have similar properties and became a new individual antihistaminic drug (Terrien et al., 1999). That is why we cannot exclude that compound **2** metabolites also have similar properties that can additionally enhance compound **2** activity. Finally, it is worth emphasizing that compound **2** possesses additional activity towards transient receptor potential cation channel, subfamily A, member 1 (TRPA1) (Chłoń-Rzepa et al., 2018b). Recent reports indicate that these receptors may play an important role in the inflammatory response and airway hyperresponsiveness in asthma or COPD (Jha et al., 2015; Mukhopadhyay et al., 2016). The hypothesis that amplification of anti-inflammatory and anti-fibrotic properties may be achieved by simultaneous inhibition of selected PDEs and TRPA1 channels can undoubtedly be an interesting direction for further studies. Future perspectives also include research into the therapeutic properties of purine-2,6-dione derivatives. At a later point, we plan to involve mouse models of chronic allergic asthma by which verification of the compounds' effectiveness will be conducted.

5. Conclusion

Currently, the state of the art in the search for new drugs is directed at re-using known structures and improving and modifying them to design new ones. Research on theophylline derivatives fits this trend very well. Although methylxanthines are currently not the drugs of choice in the treatment of asthma or COPD, nevertheless, they are an interesting and valuable group of substances with anti-inflammatory and anti-fibrotic properties. The new theophylline derivatives - PDE inhibitors, may find particular application in the treatment of cases of severe asthma and COPD resistant to standard therapy with corticosteroids (Barnes, 2013a). It is believed that one of the mechanisms responsible for resistance to corticosteroids in this group of patients may be linked to histone deacetylase dysfunction through its reduced activity and expression (Barnes, 2013b). As mentioned, theophylline and its derivatives can activate various signalling pathways in cells and one of them is histone deacetylase activation. Undoubtedly, this is another important reason to search for new drugs for treatment of asthma or

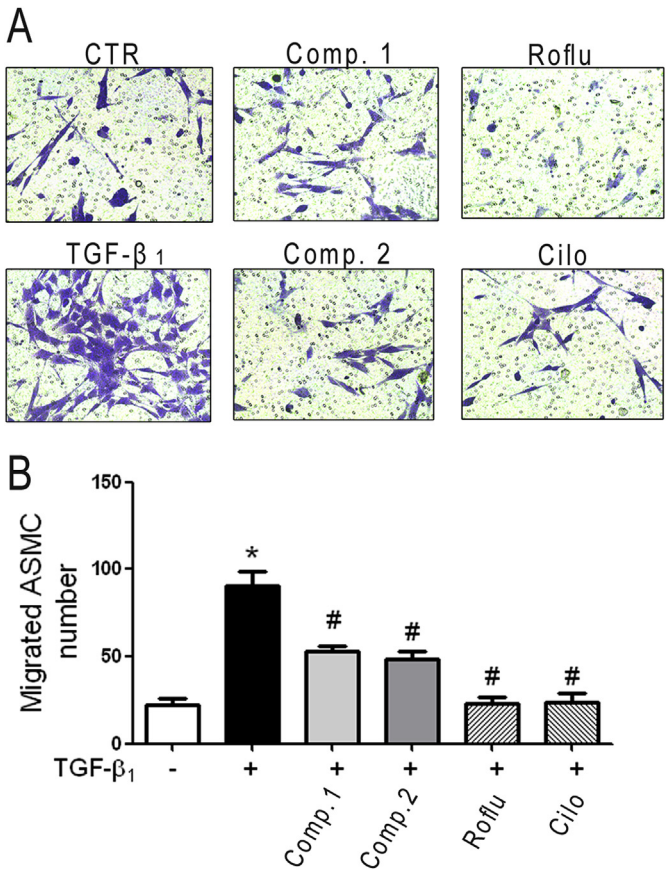

Fig. 7. Compounds 1 and 2 attenuate TGF-β_1-induced ASMC migration. ASMC (n = 3) were cultured in serum-free DMEM with ITS for 24 h and then seeded in a Transwell upper chamber in the presence of compounds **1** and **2**, roflumilast or cilomilast (10 μM). After 1 h TGF-β_1 was added to the lower compartment and cells were left for 24 h of incubation. (A) Migrated ASMC were visualized with crystal violet staining. Representative images were taken using a Nikon inverted microscope. (B) Cells from 10 different fields of view were counted. Values represent means (± S.E.M.) of three ASMC cultures run in duplicates. *P < 0.05 - in reference to control, #P < 0.05 - in reference to TGF-β_1-treated ASMC.

COPD in this group of purine-2,6-dione derivatives. Our results have shown for the first time that compounds having activity against several isoenzymes from the PDE family of non-selective but strong inhibitors, may have better properties than single or dual ones. This supports the hypothesis that multifunctional drugs are the future of targeted asthma or COPD therapy (Page and Cazzola, 2014). Further research to investigate the anti-fibrotic properties in the group of 7,8-disubstituted purine-2,6-dione derivatives is needed.

Acknowledgements

This work was supported by the National Science Centre, Poland, funded grants No. UMO-2017/27/B/NZ7/01633 and No. UMO-2014/15/B/NZ7/00885. We acknowledge the support of the European Respiratory Society, Fellowship STRF 2017.

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