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Influence of the C-5 substitution in polysubstituted pyrimidines on inhibition of prostaglandin E_2 production

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Abstract: As a part of a broader structure-activity relationship study of substituted 2-aminopyrimidines the influence of the C-5 substitution on inhibition of prostaglandin E_2 (PGE_2) production was studied. Thirty compounds were prepared starting from the corresponding 2-amino-4,6-dichloropyrimidines using Suzuki cross-coupling. It was shown previously that 2-amino-4,6-dichloropyrimidines with smaller C-5 substituent (hydrogen and methyl) were devoid of significant activity, while 5-butyl derivatives exhibited prominent potency. In this study, on the other hand, both monoaryl- and bisarylpyrimidines were potent inhibitors of PGE_2 production regardless the length of the C-5 substituent (hydrogen, methyl, n-butyl). Moreover, the shorter the C-5 substituent the higher potency to inhibit PGE_2 production was observed. 2-Amino-4,6-diphenylpyrimidine was the best inhibitor of PGE_2 production with IC_{50} = 3 nM and no cytotoxicity. The most potent inhibitors deserve further preclinical evaluation as potential anti-inflammatory agents.

Keywords: pyrimidines; Suzuki-Miyaura reaction; prostaglandin E_2; inhibitor

1. Introduction

Prostaglandins (PGs) are lipid mediators and end products of fatty acid metabolism, that are produced from the key precursor, arachidonic acid (AA), *via* the cyclooxygenase (COX)

pathway: either by constitutively expressed COX-1 or by, in response to cell-specific stimuli, highly inducible COX-2.[1-4]

Prostaglandin E_2 (PGE_2) is a naturally occurring eicosanoid derived from AA, produced by an action of COX enzymes and prostaglandin E synthases (PGES).[5,6] PGE_2, as a lipid mediator, is known to regulate various important proceses including inflammation, pain and pyrexia.[7] Nonsteroidal anti-inflammatory drugs (NSAIDs)[1,8-10] are agents that, by inhibiting the activity of COX enzymes (and thus inhibiting PGs production), efficiently reduce pain and fever, prevent blood clots, and at higher doses, decrease inflammation. The traditional (non-selective) NSAIDs belong to the most widely used drugs around the world, but they carry the risk of serious gastrointestinal (GI), as well as cardiovascular (CV) and renal adverse effects.[8-10] In search for safer medication, COX-2 selective inhibitors (coxibs)[11,12] were developed with reduced upper GI toxicity, nevertheless still exhibiting CV adverse events.[10,13] Thus, there is a persisting need for development of novel PGE_2 production inhibitors lacking the negative side effects of currently used therapeutics.

A pyrimidine moiety represents one of the most important medicinal chemistry scaffolds.[14,15] Numerous compounds based on the pyrimidine scaffold are known to exhibit anti-inflammatory activity,[16-19] as well as properties immunomodulating,[20] anti-leishmanial,[21] antibacterial,[22-24] antifungal,[23] analgesic,[25] anticonvulsant,[26] and anticancer.[27-31]

Within previous extensive structure-activity relationship studies, we discovered a series of polysubstituted pyrimidines as potent inhibitors of immune-activated nitric oxide (NO) production,[32-36] as well as inhibitors of production of prostaglandin E_2 (PGE_2).[34-36] It has been demonstrated, that in the case of 2-amino-4,6-dichloropyrimidines, at least a 3-carbon-long aliphatic group in the C-5 position of the pyrimidine moiety was required to inhibit PGE_2 production substantially, while derivatives bearing either hydrogen or methyl in the C-5 position were not PGE_2 production inhibitors.[34] Based on these results novel series of compounds was prepared, 2-amino-4,6-di(hetero)arylpyrimidines having a *n*-butyl group in the C-5 position, as an optimal substituent.[36] Several compounds in this series were found to be low micromolar dual inhibitors of NO and PGE_2 production.[36] Since non-toxic small-molecule inhibitors of PGE_2 production may have great therapeutic potential in treatment of serious inflammatory diseases, we decided to verify the influence of the C-5 substitution in the series of selected 2-amino-4-aryl-6-chloropyrimidines and 2-amino-4,6-diarylpyrimidines.

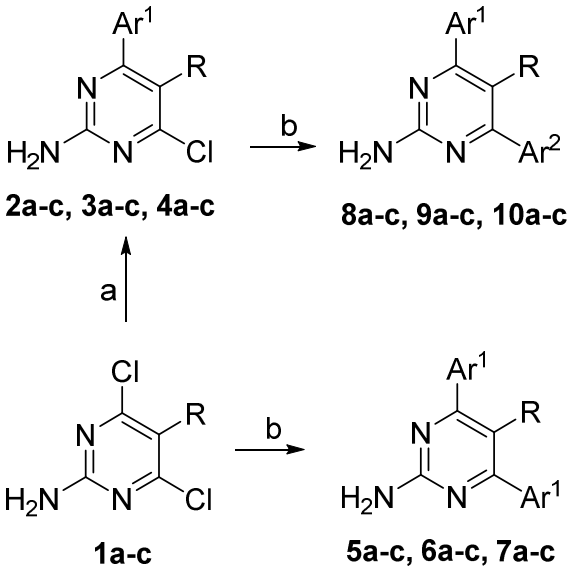
In this work, we prepared novel types of symmetrical and unsymmetrical 2-amino-4,6-diarylpyrimidines (where aryl is either phenyl, *p*-tolyl or 4-methoxylphenyl) bearing in the C-5 position either hydrogen, methyl or *n*-butyl group. The prepared compounds were subsequently tested for their ability to inhibit PGE_2 production in mouse peritoneal cells.

2. Results and discussion

2.1. Synthesis

Starting 2-amino-4,6-dichloropyrimidines **1a**-**1c** (Scheme 1) were prepared according to the previously published procedure.[32] Compounds **1a**-**1c** were subsequently treated with commercially available arylboronic acids (phenyl-, *p*-tolyl-, and 4-methoxyphenylboronic acid) under Suzuki-Miyaura cross-coupling conditions.[37] Depending on the reaction conditions,[36] we were able to prepare both monoarylated products **2**-**4** (Method A: $Pd(PPh_3)_4$, Na_2CO_3, toluene/ethanol (3:1), 80 °C, 12 h) or symmetrical diarylated products **5**-**7** (Method B: $Pd(PPh_3)_4$, Cs_2CO_3, dioxane/water (4:1), 100 °C, 12 h).

Treatment of 2-amino-4-aryl-6-chloropyrimidines **2**-**4** with another arylboronic acid using the latter conditions (Method B) afforded unsymmetrical 4,6-diarylpyrimidine analogues **8**-**10** (Scheme 1). Structures of all prepared compounds are summarized in Table 1.



Scheme 1. General synthesis of target polysubstituted pyrimidines. Ar^1, Ar^2 – phenyl, *p*-tolyl, 4-methoxyphenyl; R – hydrogen, methyl, *n*-butyl. Reaction conditions: a) arylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, toluene/ethanol (3:1), 80 °C, 12 h; b) arylboronic acid, $Pd(PPh_3)_4$, Cs_2CO_3, dioxane/water (4:1), 100 °C, 12 h.

Table 1. List of the prepared compounds. Me, methyl; Bu, *n*-butyl; Ph, phenyl; Tol, *p*-tolyl; MeOPh, 4-methoxyphenyl.

Compound	R	Ar^1	Ar^2
1a	H	-	-
1b	Me	-	-
1c	Bu	-	-
2a	H	Ph	-
2b	Me	Ph	-
2c	Bu	Ph	-
3a	H	Tol	-
3b	Me	Tol	-
3c	Bu	Tol	-
4a	H	MeOPh	-
4b	Me	MeOPh	-
4c	Bu	MeOPh	-
5a	H	Ph	-

5b	Me	Ph	-
5c	Bu	Ph	-
6a	H	Tol	-
6b	Me	Tol	-
6c	Bu	Tol	-
7a	H	MeOPh	-
7b	Me	MeOPh	-
7c	Bu	MeOPh	-
8a	H	Tol	Ph
8b	Me	Tol	Ph
8c	Bu	Tol	Ph
9a	H	MeOPh	Ph
9b	Me	MeOPh	Ph
9c	Bu	MeOPh	Ph
10a	H	MeOPh	Tol
10b	Me	MeOPh	Tol
10c	Bu	MeOPh	Tol

2.2. Biological evaluation

All prepared polysubstituted pyrimidines were evaluated for their ability to inhibit production of PGE_2 *in vitro*, using C57BL6 mouse peritoneal cells. High output PGE_2 production was activated by lipopolysaccharide (LPS) and assayed at the interval of 5 h of culture. Compounds (as 50 μM solutions) were applied concomitantly with LPS. The effects of pyrimidines were expressed as a percent change related to the response of LPS-stimulated control cells. With the exception of 2-amino-4,6-dichloropyrimidine analogues bearing hydrogen or methyl at C-5 position (**1a** and **1b**, respectively), all tested compounds were discovered to be potent inhibitors of PGE_2 production (Fig. 1A). The extent of the inhibition of PGE_2 production clearly depended on the character of the C-5 substituent. Contrary to the previously studied 2-amino-4,6-dichloropyrimidine analogues,[32] the current series of 2-amino-4-arylpyrimidines and 2-amino-4,6-diarylpyrimidines no longer required prolonged aliphatic chain in the C-5 position to exert

their biological activity. The most potent pyrimidines were those bearing hydrogen at C-5 position (i.e. compounds **2a**, **3a**, **4a**, **5a**, **6a**, **7a**, **8a**, **9a**, and **10a**), while prolongation of the substituent in this position led to attenuation of the capability to reduce PGE_2 production (Fig. 1B). Namely, analogues bearing methyl in the C-5 position are statistically less efficient to inhibit PGE_2 production compared to those bearing hydrogen at that position and the compounds bearing *n*-butyl group in the C-5 position are even less effective in comparison to the 5-methyl derivatives (Fig. 1B).

For the selected potent compounds, the IC_{50} values were also determined in comparison with two commonly used NSAIDs as control compounds, indomethacin[38-40] and aspirin[40,41] (Table 2). The compounds were found to be potent at low micromolar to submicromolar levels. The most effective inhibitors of PGE_2 production were compounds **5a, 6a**, **8a**, and **9a** with IC_{50}s in the range of 3-33 nM (Table 2). Again, all of them were the C-5 unsubstituted analogues, i.e. derivatives with hydrogen at the C-5 position.

Most importantly, the high inhibitory activity of the studied compounds cannot be explained as a result of possible cytotoxic effects, as no changes in viability of cells could be detected in the presence of the compounds (Table 2). Cell death was analyzed in the same cells using the lactate dehydrogenase (LDH) assay which is based on the determination of LDH activity released from the cytosol of damaged cells into the supernatant.

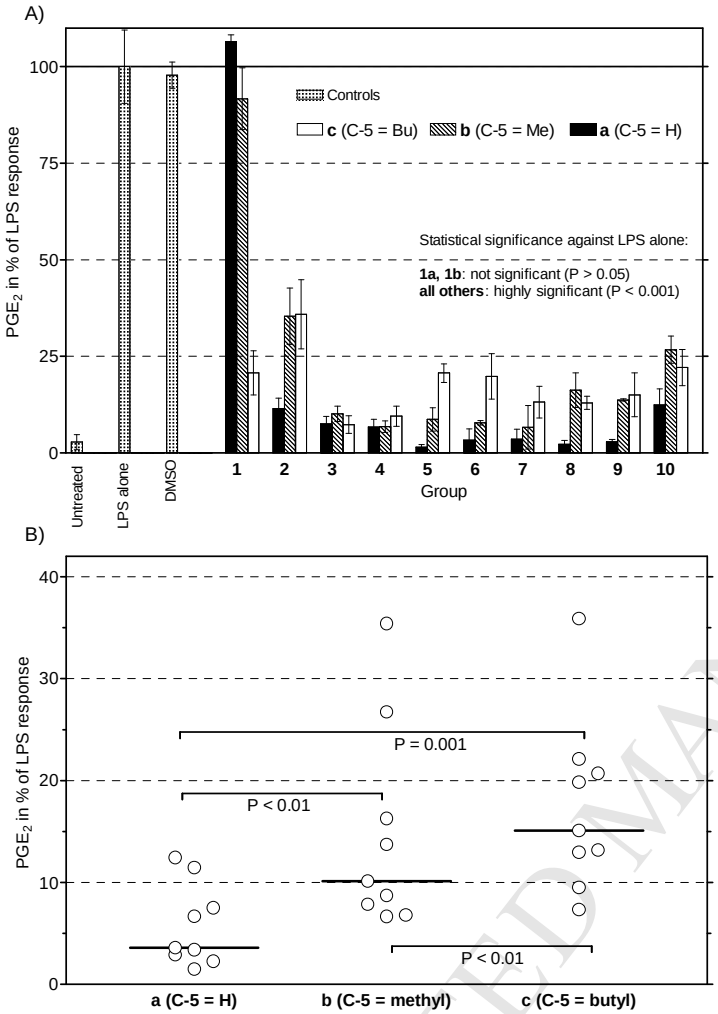


Figure 1. Effects of individual pyrimidine derivatives (50 μM solutions) (A) and medians of grouped compounds according to the C-5 substituents (B) on *in vitro* production of PGE_2 in mouse peritoneal cells. Bars are means ± S.E.M. that were obtained by averaging results of two to four experiments for each compound.

Previously reported data[36] suggested that post-translation mechanism(s) are plausible explanation for the PGE_2-inhibitory action of the pyrimidine derivatives. Their interaction with activity of enzymes involved in biosynthesis of PGE_2 is under current investigation.

Table 2. Concentration of selected pyrimidines inhibiting PGE_2 production in C57BL6 mouse peritoneal cells by 50% (IC_{50}), and effects on cell viability of the same cell type evaluated as a percent change against untreated control cells.

Compound	IC_{50} (μM)	Viability (%)
1c	5.34 (4.65-6.12)[a]	99.82±1.33[b]
3a	2.32 (1.46-3.42)	102.6±0.4
3c	1.86 (1.27-2.74)	101.4±1.7
4a	2.79 (1.98-3.92)	103.2±0.7
4c	2.16 (1.87-2.49)	96.6±1.8
5a	0.003 (0.002-0.004)	97.4±0.9
6a	0.033 (0.024-0.046)	102.8±1.2
7a	0.119 (0.068-0.202)	101.7±0.7
7c	3.71 (2.81-4.90)	99.1±0.6
8a	0.009 (0.007-0.01)	103.1±0.7
8c	2.46 (1.91-3.16)	102.2±0.6
9a	0.009 (0.005-0.016)	99.9±1.7
9c	3.40 (2.55-4.55)	102.0±0.9
indomethacin	0.005 (0.003-0.007)	n.d.[c]
aspirin	4.08 (3.09-5.39)	n.d.
DMSO	n.d.	98.9±1.6

[a]95% limits of confidence; [b] ± S.E.M. Results represent means of two identical experiments. [c]n.d. = not determined.

3. Conclusion

In summary, we have identified novel potent and non-toxic inhibitors of prostaglandin E_2 (PGE_2) production using C57BL6 mouse peritoneal cells. The selected monoaryl- and diarylsubstituted 2-aminopyrimidines exhibited single-digit micromolar to submicromolar potency to inhibit PGE_2 production. By contrast to the previously studied 2-amino-4,6-dichloropyrimidines, herein reported arylpyrimidines were most potent inhibitors when bearing hydrogen atom in the C-5 position. At least twelve of the prepared compounds were more potent than still popular aspirin. 2-Amino-4,6-diphenylpyrimidine was the most potent inhibitor in the whole series, with IC_{50} = 3 nM and no observed cytotoxicity. This compound was shown to be

equipotent to indomethacin, a nonsteroidal anti-inflammatory drug commonly used as a prescription medication to treat inflammation symptoms. Indomethacin, as an inhibitor of both COX-1 and COX-2, nevertheless, exhibits many serious adverse effects and superior drugs are needed. Although the exact mode of action of the reported compounds remains to be elucidated, the preliminary findings encourage further preclinical studies of the selected candidates as potential anti-inflammatory agents.

4. Experimental section

4.1. Chemistry

Unless otherwise stated, solvents were evaporated at 40 °C/2 kPa and the compounds were dried over P_2O_5 at 2 kPa. ^{1}H NMR and ^{13}C NMR were recorded using Bruker Avance 400 MHz spectrometer operating at 9.39 T in DMSO-*d6* solution. The ^{1}H and ^{13}C spectra were acquired at 400.00 MHz and 100 MHz, respectively. Chemical shifts (δ) are reported in ppm and interaction constants (*J*) in Hz. The spectra were referenced to tetramethylsilane (TMS) or to the residual solvent signal. High-resolution mass spectra of the products were obtained using GCT Premier (Waters, USA) spectrometer for electron ionization (EI) and LTQ Orbitrap XL (Thermo Fisher Scientific) spectrometer for electrospray ionization (ESI). Starting 2-amino-4,6-dichloropyrimidines were prepared according to the published procedure.[32] Purity of the prepared compounds was ≥95%; the purity was determined by the combination of HPLC (H_2O/CH_3CN,linear gradient), NMR, and HR-MS.

4.1.1. Method A. General procedure for synthesis of 2-amino-4-aryl-6-chloropyrimidines 2a-c, 3a-c, 4a-c. Starting 2-amino-4,6-dichloropyrimidine derivative (1.36 mmol), aryl boronic acid (1.51 mmol), tetrakis(triphenylphosphine) palladium (58 mg, 0.05 mmol) and sodium carbonate (213 mg, 2.01 mmol) were suspended in a toluene/ethanol mixture (3:1, 50 mL). The reaction was heated to 80 ^{o}C for 12 h. After cooling to room temperature, the solid was filtered off and the solvents were removed *in vacuo*. The residue was dissolved in ethyl acetate (100 mL) and adsorbed on silica gel. After removal of organic solvent *in vacuo* the product was purified on silica gel column chromatography using a hexane/ethyl acetate mixture (4:1) as the eluent and

subsequently on C-18 reverse chromatography column using water/methanol eluent (0-100% gradient).

4.1.1.1. 4-Chloro-6-phenylpyrimidin-2-amine (2a). Yield 76%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 8.13 – 8.08 (m, 2H), 7.57 – 7.48 (m, 3H), 7.26 (s, 1H), 7.19 (s, 2H). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) δ 166.45, 163.99, 161.60, 136.33, 131.57, 129.22, 127.52, 105.15. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{10}H_8ClN_3$: 205.0407, found: 205.0406.

4.1.1.2. 4-Chloro-5-methyl-6-phenylpyrimidin-2-amine (2b). Yield 50%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.56 – 7.42 (5 H, m), 6.89 (2 H, s), 2.11 (3 H, s). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 168.27, 161.88, 161.51, 138.74, 129.49, 129.04, 128.52, 113.57, 15.70. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{11}H_{10}ClN_3$: 219.0563, found: 219.0566.

4.1.1.3. 5-Butyl-4-chloro-6-phenylpyrimidin-2-amine (2c). Yield 50%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.53 – 7.45 (3 H, m), 7.44 – 7.40 (2 H, m), 6.93 (2 H, s), 2.49 – 2.42 (2 H, m), 1.42 – 1.28 (2 H, m), 1.14 (2 H, q, *J* = 7.2 Hz), 0.72 (3 H, t, *J* = 7.3 Hz). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 169.08, 161.56, 161.41, 139.00, 129.18, 128.54, 128.42, 118.41, 31.64, 27.96, 22.28, 13.82. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{14}H_{16}ClN_3$: 261.1033, found: 216.1031.

4.1.1.4. 4-Chloro-6-(p-tolyl)pyrimidin-2-amine (3a). Yield 41%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 8.01 (d, *J* = 8.3 Hz, 2H), 7.32 (d, *J* = 8.0 Hz, 2H), 7.22 (s, 1H), 7.14 (s, 2H), 2.37 (s, 3H). **^{13}C NMR NMR** δ_C (100 MHz, DMSO-d_6) 166.36, 163.96, 161.50, 141.54, 133.56, 129.81, 127.47, 104.77, 21.44. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{11}H_{10}ClN_3$: 219.0563, found: 219.0565.

4.1.1.5. 4-Chloro-5-methyl-6-(p-tolyl)pyrimidin-2-amine (3b). Yield 32%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.43 (2 H, d, *J* = 8.2 Hz), 7.38 – 7.20 (2 H, m), 6.87 (2 H, s), 2.37 (3 H, s), 2.12 (3 H, s). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 168.19, 161.50, 161.22, 161.01, 139.07, 135.90, 129.10, 113.48, 21.38, 15.80. **HRMS** (ESI) *m/z* $[M+H]^+$ calcd for $C_{12}H_{13}ClN_3$: 234.0793, found: 234.0793.

4.1.1.6. 5-Butyl-4-chloro-6-(p-tolyl)pyrimidin-2-amine (3c). Yield 7%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.33 (2 H, d, *J* = 8.2 Hz), 7.28 (2 H, d, *J* = 7.9 Hz), 6.91 (2 H, s), 2.49 – 2.43 (2 H, m), 2.37 (3 H, s), 1.46 – 1.29 (2 H, m), 1.23 – 1.08 (2 H, m), 0.75 (3 H, t, *J* = 7.3 Hz). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 169.00, 161.57, 161.39, 138.65, 136.18, 129.06, 128.46, 118.38, 31.69, 28.06, 22.33, 21.35, 13.89. **HRMS** (ESI) *m/z* $[M+H]^+$ calcd for $C_{15}H_{19}ClN_3$: 276.1262, found: 276.1262.

4.1.1.7. 4-Chloro-6-(4-methoxyphenyl)pyrimidin-2-amine (4a). Yield 34%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 8.11 – 8.06 (m, 2H), 7.20 (s, 1H), 7.09 (s, 2H, NH_2), 7.08 – 7.02 (m, 2H), 3.84 (s, 3H). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 165.98, 163.89, 162.17, 161.35, 129.21, 128.58, 114.55, 104.25, 55.84. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{11}H_{10}ClN_3O$: 235.0512, found: 235.0514.

4.1.1.8. 4-Chloro-6-(4-methoxyphenyl)-5-methylpyrimidin-2-amine (4b). Yield 44%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.51 (2 H, d, *J* = 8.8), 7.03 (2 H, d, *J* = 8.8), 6.84 (2 H, s), 3.82 (3 H, s), 2.15 (3 H, s). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 167.76, 161.86, 161.46, 160.35, 130.95, 130.84, 113.86, 113.30, 55.70, 15.94. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{12}H_{12}ClN_3O$: 249.0669, found: 249.0673.

4.1.1.9. 5-Butyl-4-chloro-6-(4-methoxyphenyl)pyrimidin-2-amine (4c). Yield 52%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.51 (2 H, d, *J* = 8.9), 7.12 (2 H, d, *J* = 8.9), 6.99 (2 H, s), 3.91 (3 H, s), 2.66 – 2.53 (2 H, m), 1.56 – 1.39 (2 H, m), 1.34 – 1.20 (2 H, m), 0.86 (3 H, t, *J* 7.3). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 168.58, 161.60, 161.38, 160.08, 131.29, 130.12, 118.30, 113.89, 55.66, 31.69, 28.11, 22.33, 13.90. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{15}H_{18}ClN_3O$: 291.1138, found: 291.1136.

4.1.2. Method B. General procedure for synthesis of symmetrical 4,6-diarylpyrimidines 5a-c, 6a-c, 7a-c. Starting 2-amino-4,6-dichloropyrimidine derivative (1.36 mmol), boronic acid (2.05 mmol), tetrakis(triphenylphosphine)palladium (58 mg, 0.05 mmol) and cesium carbonate (213 mg, 2.01 mmol) were dissolved in a dioxane/water mixture (4:1, 50 mL). The reaction was heated to 100 ^{o}C for 12 h. After cooling to room temperature the solvents were removed *in*

vacuo. The residue was dissolvedin ethyl acetate (100 mL) and adsorbed on silica gel. After removal of the organic solvent *in vacuo* the product was purified on silica gel column chromatography using a hexane/ethyl acetate mixture (4:1) as the eluent and subsequently on C-18 reverse chromatography column using water/methanol eluent (0-100% gradient).

4.1.2.1. 4,6-Diphenylpyrimidin-2-amine (5a). Yield 81%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 8.26 – 8.19 (m, 4H), 7.71 (s, 1H), 7.53 (p, J = 3.8, 3.3 Hz, 6H), 6.76 (s, 2H). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) δ 165.32, 164.48, 137.81, 130.90, 129.07, 127.44, 102.30. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{16}H_{13}N_3$: 247.1109, found: 247.1108.

4.1.2.2. 5-Methyl-4,6-diphenylpyrimidin-2-amine (5b). Yield 44%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.63 – 7.54 (4 H, m), 7.52 – 7.39 (6 H, m), 6.51 (2 H, s), 2.04 (3 H, s). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 167.54, 161.90, 139.73, 129.20, 129.04, 128.43, 113.28, 16.72. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{17}H_{15}N_3$: 261.1266, found: 261.1269.

4.1.2.3. 5-Butyl-4,6-diphenylpyrimidin-2-amine (5c). Yield 69%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.55 – 7.39 (10 H, m), 6.52 (2 H, s), 2.49 – 2.42 (2 H, m), 1.02 – 0.91 (2 H, m), 0.88 – 0.76 (2 H, m), 0.45 (3 H, t, J = 7.3). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 168.08, 161.49, 158.68, 140.10, 128.70, 128.62, 128.45, 118.60, 32.24, 26.92, 22.02, 13.55. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{20}H_{21}N_3$: 303.1735, found: 303.1740.

4.1.2.4. 4,6-Di-p-tolylpyrimidin-2-amine (6a). Yield 37%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 8.13 (4H, d, J = 8.2 Hz), 7.65 (1 H, s), 7.33 (4H, d, J = 7.9 Hz), 6.67 (2H, s), 2.39 (s, 6H). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 165.06, 164.40, 140.61, 135.07, 129.65, 127.34, 101.57, 21.43. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{18}H_{17}N_3$: 275.1422, found: 275.1420.

4.1.2.5. 5-Methyl-4,6-di-p-tolylpyrimidin-2-amine (6b). Yield 40%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.49 (4 H, d, J = 7.8), 7.28 (4 H, d, J = 7.8), 6.43 (2 H, s), 2.37 (6 H, s), 2.05 (3 H, s). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 167.39, 161.89, 138.46, 136.93, 129.26, 128.95, 115.46, 113.22, 21.37, 16.99. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{19}H_{19}N_3$: 289.1579, found: 289.1574.

4.1.2.6. 5-Butyl-4,6-di-p-tolylpyrimidin-2-amine (6c). Yield 49%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.38 (4 H, d, *J* 8.2), 7.27 (4 H, d, *J* = 7.7), 6.45 (2 H, s), 2.54 – 2.46 (2 H, m), 2.37 (6 H, s), 1.03 – 0.89 (2 H, m), 0.89 – 0.77 (2 H, m), 0.47 (3 H, t, *J* = 7.3). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 167.96, 161.48, 137.97, 137.38, 128.97, 128.65, 118.61, 32.22, 27.03, 21.36, 13.66. δ_C (101 MHz, DMSO-d_6) 167.96, 161.48, 153.31, 137.98, 137.38, 128.96, 128.65, 118.63, 32.22, 27.03, 22.01, 21.35, 13.66. **HRMS** (ESI) *m/z* $[M+H]^+$calcd for $C_{22}H_{26}N_3$: 332.2121, found: 332.2122.

4.1.2.7. 4,6-Bis(4-methoxyphenyl)pyrimidin-2-amine (7a). Yield 25%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d6) 8.22 – 8.17 (m, 4H), 7.60 (s, 1H), 7.09 – 7.04 (m, 4H), 6.58 (s, 2H), 3.85 (s, 6H). **^{13}C NMR** δ_C (100 MHz, DMSO-d6) 164.55, 164.27, 161.59, 130.23, 128.94, 114.35, 100.71, 55.78. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{22}H_{17}N_3O_2$: 307.1321, found: 307.1320.

4.1.2.8. 4,6-Bis(4-methoxyphenyl)-5-methylpyrimidin-2-amine (7b). Yield 33%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.57 (4 H, d, *J* = 9.0), 7.03 (4 H, d, *J* = 9.0), 6.38 (2 H, s), 3.82 (6 H, s), 2.10 (3 H, s). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 166.94, 161.84, 160.00, 132.06, 130.93, 113.73, 113.00, 55.66, 17.36. **HRMS** (ESI) m/z $[M+H]^+$ calcd for $C_{19}H_{20}N_3O_2$: 322.1550, found: 322.1551.

4.1.2.9. 5-Butyl-4,6-bis(4-methoxyphenyl)pyrimidin-2-amine (7c). Yield 14%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.45 (4 H, d, *J* = 8.6), 7.02 (4 H, d, *J* = 8.8), 6.41 (2 H, s), 3.81 (6 H, s), 2.61 – 2.44 (2 H, m), 1.02 – 0.91 (2 H, m), 0.91 – 0.80 (2 H, m), 0.49 (3 H, t, *J* = 7.2). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 167.57, 161.48, 159.66, 132.60, 130.22, 118.57, 113.78, 55.61, 32.14, 27.14, 22.02, 13.71. **HRMS** (ESI) m/z $[M+H]^+$ calcd for $C_{22}H_{26}N_3O_2$: 364.2020, found: 364.2019.

4.1.3. Method C. General procedure for synthesis of unsymmetrical 4,6-diarylpyrimidines 8a-c, 9a-c, 10a-c. Starting 2-amino-4-aryl-6-chloropyrimidine derivative (1.36 mmol), boronic acid (1.51 mmol), tetrakis(triphenylphosphine)palladium (58 mg, 0.05 mmol) and cesium carbonate (213 mg, 2.01 mmol) were dissolved in a dioxane/water mixture (4:1, 50 mL). The reaction was

heated to 100 ^{o}C for 12 h. After cooling to room temperature the solvents were removed *in vacuo*. The residue was dissolved in ethyl acetate (100 mL) and adsorbed on silica gel. After removal of the organic solvent *in vacuo* the product was purified on silica gel column chromatography using a hexane/ethyl acetate mixture (4:1) as the eluent and subsequently on C-18 reverse chromatography column using water/methanol eluent (0-100% gradient).

4.1.3.1. 4-Phenyl-6-(p-tolyl)pyrimidin-2-amine (8a). Yield 75%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 8.24 – 8.19 (m, 2H), 8.14 (d, J = 8.2 Hz, 2H), 7.68 (s, 1H), 7.55 – 7.50 (m, 3H), 7.33 (d, J = 7.7 Hz, 2H), 6.71 (s, 2H), 2.39 (s, 3H). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 165.21, 165.17, 164.44, 140.69, 137.88, 135.01, 130.83, 129.67, 129.05, 127.41, 127.37, 101.93, 21.44. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{17}H_{15}N_3$: 261.1266, found: 261.1269.

4.1.3.2. 5-Methyl-4-phenyl-6-(p-tolyl)pyrimidin-2-amine (8b). Yield 59%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.57 (2 H, d, J = 6.1), 7.52 – 7.42 (5 H, m), 7.29 (2 H, d, J = 8.1), 6.47 (2 H, s), 2.37 (3 H, s), 2.04 (3 H, s). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 167. 52, 167.47, 161.89, 139.80, 138.51, 136.86, 129.25, 129.20, 129.01, 128.96, 128.41, 113.25, 21.38, 16.86. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{18}H_{17}N_3$: 275.1422, found: 275.1425.

4.1.3.3. 5-Butyl-4-phenyl-6-(p-tolyl)pyrimidin-2-amine (8c). Yield 52%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.53 – 7.42 (5 H, m), 7.39 (2 H, d, J = 8.1), 7.27 (2 H, d, J = 7.8), 6.49 (2 H, s), 2.49 – 2.45 (2 H, m), 2.37 (3H, s), 1.02 – 0.89 (2 H, m), 0.89 – 0.74 (2 H, m), 0.46 (3 H, t, J = 7.2). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 168.05, 167.99, 161.49, 140.18, 138.03, 137.29, 130.14, 128.98, 128.65, 128.43, 118.61, 115.46, 32.24, 26.98, 22.02, 21.35, 13.60. **HRMS** (EI) *m/z* $[M]^+$ calcd for $C_{21}H_{23}N_3$: 317.1892, found: 317.1895.

4.1.3.4. 4-(4-Methoxyphenyl)-6-phenylpyrimidin-2-amine (9a). Yield 47%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d6) 8.23 – 8.18 (m, 4H), 7.66 (s, 1H), 7.55 – 7.47 (m, 3H), 7.09 – 7.03 (m, 2H), 6.68 (s, 2H), 3.84 (s, 3H). **^{13}C NMR** δ_C (100 MHz, DMSO-d6) 164.83, 164.68, 161.53, 138.26, 130.57, 130.42, 128.95, 128.92, 127.33, 114.31, 100.21, 55.77. **HRMS** (EI) m/z $[M]^+$ calcd for $C_{17}H_{15}N_3O$: 277.1215, found: 277.1217.

4.1.3.5. 4-(4-Methoxyphenyl)-5-methyl-6-phenylpyrimidin-2-amine (9b). Yield 49%, white solid.**^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.62 – 7.54 (4 H, m), 7.51 – 7.41 (3 H, m), 7.03 (2 H, d, J = 8.8), 6.45 (2 H, s), 3.82 (3 H, s), 2.07 (3 H, s). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 167.51, 166.98, 161.87, 160.03, 139.86, 131.92, 130.91, 129.23, 128.98, 128.40, 113.76, 113.14, 55.66, 17.04. **HRMS** (ESI) m/z $[M+H]^+$ calcd for $C_{18}H_{18}N_3O$: 292.1444, found: 292.1446.

4.1.3.6. 5-Butyl-4-(4-methoxyphenyl)-6-phenylpyrimidin-2-amine (9c). Yield 92%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.54 – 7.37 (7 H, m), 7.02 (2 H, d, J = 8.8), 6.48 (2 H, s), 3.81 (3 H, s), 2.57 – 2.46 (2 H, m), 1.07 – 0.92 (2 H, m), 0.90 – 0.74 (2 H, m), 0.47 (3 H, t, J = 7.2). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 168.07, 167.58, 161.49, 159.70, 140.26, 132.43, 130.20, 128.64, 128.42, 118.58, 113.80, 55.61, 32.20, 27.03, 22.02, 13.63. **HRMS** (ESI) m/z $[M+H]^+$ calcd for $C_{21}H_{24}N_3O$: 334.1914, found: 334.1914.

4.1.3.7. 4-(4-Methoxyphenyl)-6-(p-tolyl)pyrimidin-2-amine (10a). Yield 89%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 8.20 (2 H, d, J = 9.0), 8.12 (2 H, d, J = 8.3), 7.62 (1 H, s), 7.33 (2 H, d, J = 7.8), 7.06 (2 H, d, J = 9.0), 6.61 (2 H, s), 3.85 (3 H, s), 2.39 (3 H, s). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 164.90, 164.73, 164.34, 161.65, 140.53, 135.15, 130.16, 129.63, 128.98, 127.32, 114.38, 101.16, 55.79, 21.43. **HRMS** (ESI) m/z $[M+H]^+$ calcd for $C_{18}H_{18}N_3O$: 292.1444, found: 292.1445.

4.1.3.8. 4-(4-Methoxyphenyl)-5-methyl-6-(p-tolyl)pyrimidin-2-amine (10b). Yield 43%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.57 (2 H, d, J = 8.8), 7.49 (2 H, d, J = 8.3), 7.29 (2 H, d, J = 7.8), 7.03 (2 H, d, J = 8.8), 6.40 (2 H, s), 3.82 (3 H, s), 2.38 (3 H, s), 2.08 (3 H, s). **^{13}C NMR** δ_C (100 MHz, DMSO-d_6) 167.41, 166.93, 161.86, 160.01, 138.45, 137.00, 132.00, 130.91, 129.28, 128.94, 113.74, 113.11, 55.66, 21.38, 17.18. **HRMS** (ESI) m/z $[M+H]^+$ calcd for $C_{19}H_{20}N_3O$: 306.1601, found: 306.1602.

4.1.3.9. 5-Butyl-4-(4-methoxyphenyl)-6-(p-tolyl)pyrimidin-2-amine (10c). Yield 36%, white solid. **^{1}H NMR** δ_H (400 MHz, DMSO-d_6) 7.45 (2 H, d, J = 8.8), 7.38 (2 H, d, J = 8.3), 7.27 (2 H, d, J = 7.6), 7.02 (2 H, d, J = 9.0), 6.42 (2 H, s), 3.81 (3 H, s), 2.56 – 2.48 (2 H, m), 2.37 (3 H, s), 1.02 – 0.91 (2 H, m), 0.90 – 0.79 (2 H, m), 0.49 (3 H, t, J = 7.2). **^{13}C NMR** δ_C (100 MHz,

DMSO-d_6) 166.75, 161.49, 159.69, 159.25, 137.96, 137.45, 132.54, 130.21, 128.96, 128.67, 118.60, 113.78, 55.62, 32.17, 27.08, 22.01, 21.35, 13.68. **HRMS** (ESI) m/z $[M+H]^+$ calcd for $C_{22}H_{26}N_3O$: 348.2070, found: 348.2071.

4.2. Biology

Stock solutions (200 mM) of pyrimidine analogues were prepared in tissue-culture tested dimethylsulfoxide (DMSO, Sigma-Aldrich). Required working solutions were freshly obtained by the dilution in complete RPMI-1640 culture medium. It contained 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 50 µg/ml gentamicin, and 5 x 10^{-5} M 2-mercaptoethanol (all Sigma-Aldrich). Effects of studied pyrimidine analogues on production of prostaglandin E_2 (PGE_2) were evaluated *in vitro*, using mouse (C57BL6, Charles River Deutschland, Sulzfeld, Germany) peritoneal cells. Animals, killed by cervical dislocation, were i.p. injected with 8 ml of sterile saline. Pooled peritoneal cells collected from mice (n = 5-8 in individual experiments) were washed, re-suspended in complete culture medium, and seeded into 96-well round-bottom microplates (Costar) in 100-µl volumes, 2 x 10^5 cells/well. Cultures were maintained at 37 °C, 5% CO_2 in humidified incubator (Sanyo Electric Biomedical, Osaka, Japan). Possible effects of the vehiculum (DMSO) were evaluated using the 0.025% dilution corresponding to the highest molarity of tested pyrimidines (i.e. 50 µM). This amount of DMSO was found to be devoid of any interference with the PGE_2 and viability assays (see Fig. 1A, and Table 2, respectively). Experimental protocols were approved by the institutional ethics committee.

4.2.1. PGE_2 and cytotoxicity assays in mouse C57BL6 peritoneal cells. The cells were cultured in presence of test compounds applied concomitantly with lipopolysaccharide (LPS from *E. coli* 0111:B4, Sigma; 10 ng/ml). Amount of PGE_2 was determined at the interval of 5 h of culture using ELISA kit (R&D Systems), following manufacturer instruction. The effects of compounds were evaluated as a percentage of LPS-induced (control) concentrations of PGE_2.

4.2.3. LDH assay. Viability of cells was analyzed using the lactate dehydrogenase (LDH) assay. It is based on the determination of LDH activity released from the cytosol of damaged cells into the supernatant. The cell supernatants were harvested at the interval of 5 h of culture, diluted 1:1

and mixed with an aliquot of the LDH kit. After 30-min incubation in the dark at ambient temperature, the reaction was stopped with 2 N HCl. Differences between absorbances at 492 and 690 nm were evaluated. The percentage cytotoxicity of test samples was related to the control samples and to the samples with 100% dead cells evoked by 1% Triton, according to the formula: [(exp. value – control value) / (Triton value – control value)] x 100. All control and experimental variants were run in triplicate.

4.2.4. Statistical analysis. Analysis of variance (ANOVA) with subsequent Bonferroni´s multiple comparison test, Wilcoxon signed rank test for comparison of medians, and graphical presentation of data were done using the Prism program (GraphPad Software, San Diego, CA).

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Abbreviations

COX, cyclooxygenase; LPS, lipopolysaccharide; NSAIDs, nonsteroidal anti-inflammatory drugs; NO, nitric oxide; PGE_2, prostaglandin E_2.

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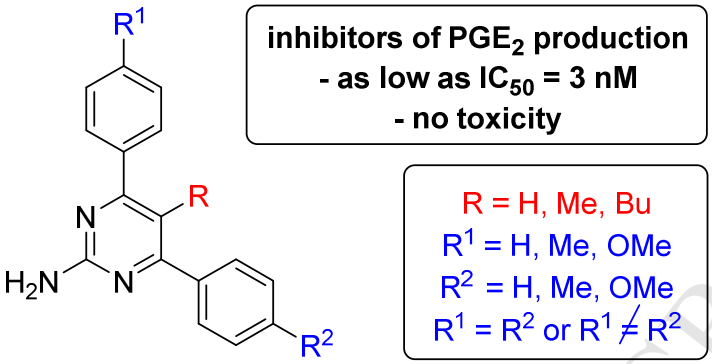
Graphical abstract



Highlights

• Synthesis of polysubstituted pyrimidines using Suzuki-Miyaura cross-coupling reaction

• Potent inhibitors of prostaglandin E_2 production

• No cytotoxicity observed at the concentrations tested

• Potential anti-inflammatory agents