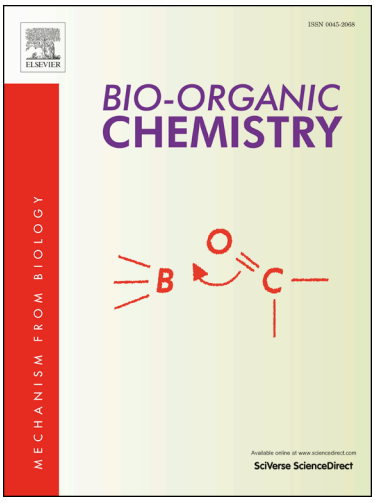

EGFR inhibitors from cancer to inflammation: Discovery of 4-fluoro-*N*-(4-(3-(trifluoromethyl)phenoxy)pyrimidin-5-yl)benzamide as a novel anti-inflammatory EGFR inhibitor

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EGFR inhibitors from cancer to inflammation: Discovery of 4-fluoro-*N*-(4-(3-(trifluoromethyl)phenoxy)pyrimidin-5-yl)benzamide as a novel anti-inflammatory EGFR inhibitor

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Abstract

EGFR inhibitors are well-known as anticancer agents. Quite differently, we report our effort to develop EGFR inhibitors as anti-inflammatory agents. Pyrimidinamide EGFR inhibitors eliciting low micromolar IC_{50} and the structurally close non-EGFR inhibitor urea analog were synthesized. Comparing their nitric oxide (NO) production inhibitory activity in peritoneal macrophages and RAW 246.7 macrophages indicated that their anti-inflammatory activity in peritoneal macrophages might be a sequence of EGFR inhibition. Further evaluations proved that compound **4d** significantly and dose-dependently inhibits LPS-induced iNOS expression and IL-1β, IL-6, and TNF-α production via NF-κB inactivation in peritoneal macrophages. Compound **4d** might serve as a lead compound for development of a novel class of anti-inflammatory EGFR inhibitors.

***Keywords*:**

EGFR inhibitors; Anti-inflammatory; Nitric oxide production; Cytokines production; Macrophages.

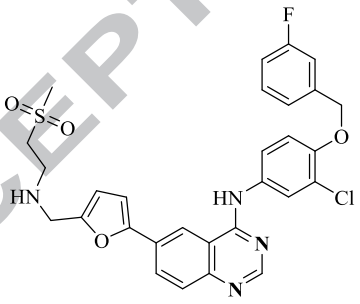
1. Introduction

Under physiological conditions, inflammation plays a protective role against exogenous and endogenous noxious stimuli such as infectious organisms or damaged tissues. However, under pathological conditions, inflammation contributes to the development of a vast array of diseases that share the presence of an inflammatory component in their pathogenetic process. Thus, inflammatory conditions are recognized as a part of the pathogenesis of sepsis, atherosclerosis, rheumatoid arthritis, neurodegenerative diseases, diabetes, liver diseases, lung diseases and inflammatory skin diseases [1].

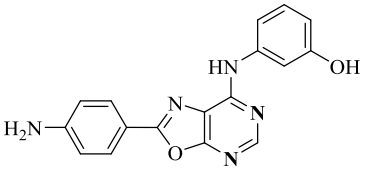
Epidermal growth factor receptor (EGFR) is a tyrosine kinase that has been studied extensively as a target for development of anticancer therapies [2-7]. Recently, the EGFR–ERK pathway has been shown to play a crucial role in several inflammatory conditions. A recent study unveiled that tris(2-chloroethyl)phosphate (TCEP) induces inflammatory response in HepG2 cancer cells through EGFR stimulation which could be counteracted through EGFR inhibition [8]. Also, EGFR activation was found to be required for the development of respiratory inflammation by respiratory syncytial virus (RSV) and by nontypeable Haemophilus influenzae (NTHi) [9, 10]. In addition, a crucial regulatory role for EGFR in thrombin-mediated inflammation was reported recently [11]. Furthermore, a study of obesity-related cardiovascular diseases showed that inhibition of EGFR resulted in attenuation of palmitic acid-induced inflammation in cardiac muscle cells [12]. Moreover, *in vivo* inhibition of EGFR in a spinal cord injury as a model of neuroinflammation resulted in decrease of inflammation and the secondary damage [13].

Macrophages are immune cells playing a pivotal role in development of inflammation [14]. Interestingly, recent studies showed potential roles for ligand stimulation of EGFR in

macrophage cells suggesting EGFR-dependent production of inflammatory cytokines and chemokines [15, 16]. It was shown that nitric oxide (NO), cytokines and chemokines production was attenuated in EGFR-deleted macrophages [16]. In addition, *in vivo* experiments using mice with EGFR deleted myeloid cells (the progenitor cells that differentiate into macrophages) or inhibiting the EGFR using a small molecule inhibitor resulted in significant reduction of atherosclerosis [17, 18]. Furthermore, *in vitro* EGFR inhibition decreased LPS-induced cytokine production by macrophages while *in vivo* EGFR inhibition attenuated LPS-triggered lung inflammation [18]. A logic consequence to the previously mentioned recent studies validating EGFR inhibition in macrophages as a promising target for development of anti-inflammatory therapeutics would be pursuing the development of EGFR inhibitors as anti-inflammatory agents. However, to the best of our knowledge, such effort has been reported yet despite the use of some EGFR inhibitor molecules in elucidating the biological role of EGFR activation in developing inflammation.



1
Lapatinib



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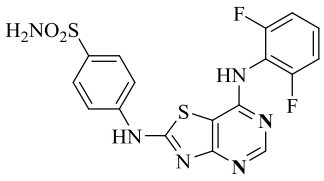

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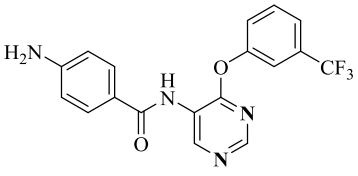


4a

Fig. 1. Reported pyrimidine-based EGFR inhibitors

Several fused pyrimidines-based small molecules were developed as antitumor EGFR inhibitors such as lapatinib (**1**, quinazoline scaffold, Fig. 1) [19], compound **2** (oxazolo[5,4-*d*]pyrimidine scaffold) [20] and compound **3** (thiazolo[4,5-*d*]pyrimidine scaffold) [21]. Despite the development of several unfused pyrimidines as EGFR inhibitors, only few reports exist of unfused pyrimidines bearing substituents in positions equivalent to that of fused pyrimidines **1**−**3**. Based on a cheminformatics approach, we have recently reported the discovery of a hit EGFR inhibitor small molecule compound **4a** [22]. Based on our previous report, we have synthesized 4-aryloxy-5-benzamidopyrimidine derivatives (**4**, Fig. 2) and the closely related 4-aryloxy-5-(3-phenylureido)pyrimidine derivative (**5**) in an attempt to develop anti-inflammatory EGFR inhibitors against the well-known inflammatory macrophage cells. According to our previous report, the amide analogs (**4**) would elicit EGFR inhibition while the closely related urea derivative (**5**) would not show a significant EGFR inhibition. The high structural similarity of urea derivative (**5**) to the EGFR active amide series (**4**) might allow us to employ it as a decoy to assess and elucidate the possible relation between the anticipated anti-inflammatory activities with EGFR inhibition of these compounds. The amide analogs (**4**) possessed two variably substituted phenyl moieties. On the other hand, the urea derivative (**5**) possessed methoxy substituents on both phenyl rings. Herein, we report our interesting results.

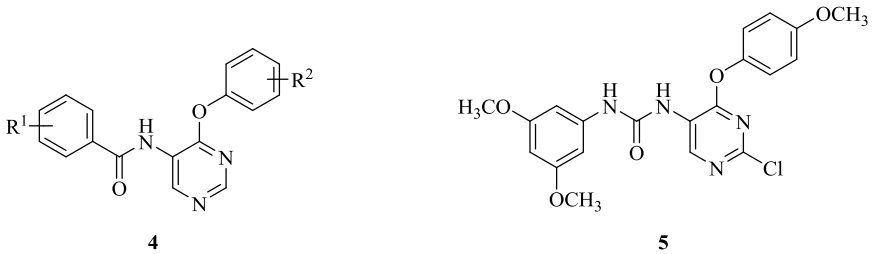
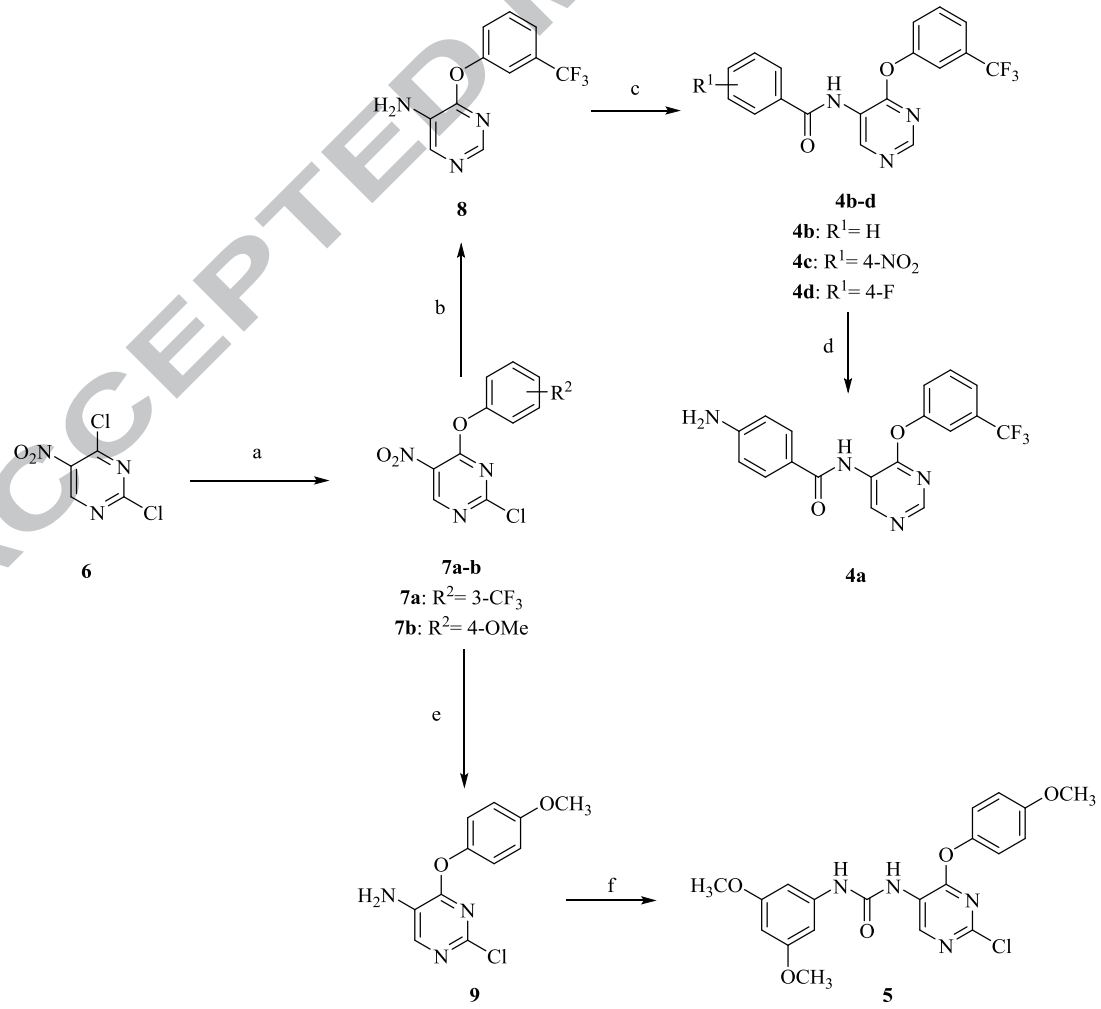


Fig. 2. Chemical structure of the synthesized compounds

2. Results and discussion

2.1. Chemical Synthesis

Synthesis of the target compounds **4a**–**d** and **5** was conducted as illustrated in Scheme 1. Aromatic nucleophilic substitution of the more reactive 4-chloro group of 2,4-dichloro-5-niropyrimidine (**6**) with substituted phenols afforded substituted phenoxy derivatives (**7a** and **7b**). Catalytic hydrogenation of the 4-(3-trifluoromethylphenoxy) derivative (**7a**) afforded the dehalogenated pyrimidin-5-amine derivative (**8**). Amide coupling of compound **8** with the appropriate benzoyl chloride derivative yielded the targeted compounds **4b**–**d**. A second catalytic hydrogenation of compound **4c** afforded compound **4a**. For preparation of compound **9**, reduction of the 5-nitro group of compounds **7b** was performed employing the dissolving metal reduction using iron/ammonium chloride. Refluxing 3,5-dimethoxyisocyante with **9** afforded the desired urea analog **5**.



Scheme 1. Reagents and conditions: (a) appropriate phenol derivative, aq. $NaHCO_3$, acetone, 0 °C to rt, 3 h; (b) H_2, 10% Pd/C, CH_3OH, rt, 6 h; (c) appropriate benzoyl chloride derivative, TEA, THF, 50 °C, overnight; (d) H_2, 10% Pd/C, CH_3OH, rt, 6 h; (e) iron powder, ammonium chloride, $EtOH/H_2O$, reflux, 1.5 h; (f) appropriate iso(thio)cyanate derivative, dichloromethane, 0 °C to 70 °C, overnight.

2.2. Assessment of NO production in LPS-induced macrophages

NO is a major inflammatory mediator that plays pivotal roles in inflammatory diseases [23-25].The exhaled NO was claimed as an inflammometer in inflammatory respiratory diseases such as asthma [26, 27]. In an asthma model, EGFR activation was found to trigger the inflammatory response and EGFR inhibition was more effective in counteracting inflammatory conditions than selective targeting of NF-κB, ERK1/2 or PI3Kδ pathways [28]. Interestingly, in some cancerous cells, it was found that EGFR activation is correlated with the expression of inducible nitric oxide synthase (iNOS) which is responsible for production of NO [29, 30].

In order to evaluate the impact of the synthesized compounds on NO production, we adopted the use of LPS-induced macrophages, which is a widely used as *in vitro* model of inflammation. We used two types of macrophage cells; mouse peritoneal macrophages and RAW 264.7 macrophages. Peritoneal macrophages are normal cell line possessing normal signaling pathways. Thus, it would produce a normal response. On the opposite, RAW 264.7 macrophages are transformed mouse leukemic macrophages possessing in which some signaling pathways are altered from normal. Accordingly, RAW 264.7 macrophages might show different responses from those of primary peritoneal macrophages [31]. Interestingly, it was reported that inhibition of EGFR in RAW 264.7 macrophages resulted in an enhancement of NF-κB activity which is the opposite to the expected inhibition of NF-κB as a downstream of EGFR [32]. Therefore, if the active mechanism for inhibition of inflammatory response by a molecule is mediated through EGFR inhibition in peritoneal

macrophages, it will not be reproduced in RAW 264.7 macrophages as of their altered EGFR signaling pathway. This might be exploited to verify the responsibility of EGFR for the elicited anti-inflammatory activity (if any).

The conducted assay employed 40 μM single dose of L-N^6-(1-Iminoethyl)lysine (L-NIL), a selective inhibitor of iNOS as a standard positive control [33]. The synthesized compounds were evaluated at the same 40 μM dose. To account for the non-specific reduction in NO production that may arise from probable cell death, both cell viability and the percent of increased NO production triggered by LPS (1 μg/mL) in presence, as well as, in absence of the tested compounds were determined simultaneously. The percent of the increased NO values were calculated relative to the measured increase in NO production triggered by LPS only-treated cells and the results were summarized in Table 1.

Table 1. Inhibition of nitric oxide (NO) production in macrophages and cell viability by the synthesized compounds

Comp.	R^1	R^2	Peritoneal macrophage			RAW 264.7 macrophages		
			% NO production [a]	% Cell Viability [b]	IC_{50} of NO production (μM)	% NO production [a]	% Cell Viability [b]	IC_{50} of NO production (μM)
4a	4-NH_2	3-CF_3	30.6	101.1	33.1	57.0	85.3	>40
4b	H	3-CF_3	18.5	103.8	25.2	65.7	83.7	>40
4c	4-NO_2	3-CF_3	25.0	106.3	27.1	57.0	57.8	ND
4d	4-F	3-CF_3	18.9	99.0	20.8	64.2	91.3	>40
5	3,5-diMeO	4-MeO	87.6	99.7	ND	63.6	69.0	>40
L-NIL	—	—	3.3	103.7	ND	22.8	85.5	ND

[a] Percent of the increase in NO production triggered by LPS in presence of a single dose of 40 μM concentration of the tested compound relative to the increase in NO induced by LPS only-treated sample. The results are the means of four independent assays.
[b] Cell viability of a single dose of 40 μM concentration of the tested compound in presence of LPS. The results are the means of four independent assays.

According to anti-inflammatory assay in peritoneal macrophages results, L-NIL (the positive standard) reduced the NO production increase to 3.3% of the level of increased NO

production measured in LPS only-treated cells while the measured peritoneal macrophage cells viability was around 100%. The elicited activity pattern of the amide series (**4a**−**d**) was consistent with their anticipated anti-inflammatory activity as EGFR inhibitors. They showed high inhibition of the NO production increase as well as excellent viability values (not less than 99%) indicating absence of cytotoxic effects over peritoneal macrophages. The previously identified EGFR inhibitor hit compound **4a** with 4-amino substituent on the left phenyl moiety elicited a low percent of remaining increase in the NO production (30.6%) with measured IC_{50} of 33.1 μM. The most active compound against NO production in peritoneal macrophages was compound **4d**, which possesses 4-fluoro substituent on the left phenyl moiety. It showed a percent of remaining increased NO production as low as 18.9% with a determined IC_{50} of 20.8 μM. Also, amide compounds **4b** and **4c** efficiently reduced the remaining increased NO production to 18.5 and 25.0% and elicited IC_{50} values of 25.2 and 27.1 μM, respectively, which is still comparable to that of the most active compound **4d**. Meanwhile, the decoy non-EGFR inhibitor (compound **5**) showed good viability similar to the EGFR inhibitors in peritoneal macrophages. Nevertheless, this decoy compound was almost inactive as NO production level remained elevated at 87.6% of the level of NO production of LPS-treated the negative control.

In contrast to the normal peritoneal macrophages, the elicited activity pattern in RAW 264.7 macrophages was completely different. First, RAW 264.7 macrophages viability values declined in presence of all of the evaluated compounds. Among the assayed amides (**4a**−**d**), compound **4c**, with 4-nitro substituent on the left phenyl moiety, showed the highest deterioration of the measured RAW 264.7 macrophages viability reducing it to 57.8%. This value is almost the same value of measured increase in NO production (Table 1). Accordingly, this measured lowering in the NO production increase by the derivative **4c** is

exclusively a non-specific lowering that arises from cell death. On the other hand, for **4a**, **4b** and **4d**, acceptable viability values (more than 80%) were elicited. Among them, **4d**, which possesses 4-fluoro substituent on the left phenyl moiety, showed the highest percent of cell viability. In contrast to their pronounced impact on NO production in primary peritoneal macrophages, all of amide compounds **4a**, **4b** and **4d** elicited a modest lowering of the increased NO production to the range of 65~57% of that of LPS only-treated sample and IC_{50} values more than 40 μM. The results showed that the decoy compound **5** showed high reduction of RAW 264.7 macrophages viability to 69.0%. As this value is very close to the measured 63.6% increase in NO production, such measured reduction in NO production is not real lowering but is a consequence of RAW 264.7 macrophages cells death.

In summary, the results of the performed cellular anti-inflammatory assay in macrophages showed significant differences in the elicited activities of the tested compounds between the normal peritoneal macrophages and the transformed peritoneal RAW 264.7 macrophages. This might arise from the fact that some signaling pathways are altered in the transformed cells. Therefore, using the transformed RAW 264.7 macrophages is not sufficient to anticipate the activity of tested compounds in normal macrophage cells. This result is in conform to recent reports [31]. The assay results presented EGFR inhibitors amide compounds **4a**−**d** as promising effective lead anti-inflammatory compounds in normal peritoneal macrophages, despite their lower activity in the transformed RAW 264.7 macrophage cells. Thus, the employed macrophages cells model is a critical factor that should be carefully considered, as it would affect the outcome of the assay.

2.3. EGFR inhibition as a mechanism of the elicited inhibition of NO production in LPS-induced macrophages

To probe the presence of a possible relation between EGFR inhibition and NO inhibition by the tested compounds, the compounds whose impact on EGFR kinase reaction is not known were subjected to an *in vitro* assay of EGFR kinase reaction inhibition. First, the inhibition percent at 100 μM concentration was conducted followed by determination of IC_{50} for the compounds that showed inhibition of EGFR activity at this concentration. The purpose of starting with such high concentration was to confirm that the used decoy compound **5** is completely devoid from any EGFR inhibition activity despite its close structural similarity with the amide series (**4**) compounds. It should be noted that the death of macrophage cells (if any occurs relative to the control) would contribute to the measured reduction in the production of NO. Thus, it is important to distinguish the nonspecific reduction that arises from cells death from the specific inhibition occurs due to modulation of the signaling pathways in living cells. This nonspecific inhibition should be eliminated from measured inhibition. Therefore, we excluded this nonspecific inhibition by subtracting the percentage of dead cells from the percentage of measured inhibition of NO production to calculate the specific inhibition of NO production corresponding to a real inhibition in living cells. Table 2 summarizes the results of EGFR kinase reaction inhibition assay and the calculated specific inhibition of NO production.

Table 2. Percentage inhibition and IC_{50} of EGFR kinase reaction and calculated specific reduction of the increased NO production in different macrophage cells

Comp.	% EGFR Inhibition at 100 μM	EGFR Inhibition. IC_{50} (μM)	Specific Inhibition in Peritoneal Macrophages[a]	Specific Inhibition in RAW 264.7 Macrophages[a]
4a	95.6	1.05	**70.5**	28.3
4b	90.9	2.37	**85.3**	18.0
4c	76.6	5.37	**81.3**	0.8
4d	88.5	2.98	**80.1**	27.1
5	4.1	ND	12.1	5.4

[a] Specific inhibition was calculated by subtracting the percentage of dead cells from the percentage of measured inhibition of NO production.

The kinase inhibition assay confirmed total absence of EGFR inhibition ability for decoy compound **5** even at the tested high concentration. Meanwhile, all of the four compounds belonging to the amide series (**5**) elicited high inhibition percent and low micromolar IC_{50} values (Table 2). All of the active EGFR inhibitors (**4a**−**d**) showed high specific inhibition in normal peritoneal macrophages while in RAW 264.7 macrophages, whose EGFR signaling pathway is altered, the specific inhibition was very low. This might be an indication that the observed anti-inflammatory activity in peritoneal macrophages is mediated mainly through inhibition of EGFR. On the other hand, no high specific inhibition was observed in RAW 264.7 macrophages probably because of their reported altered EGFR signaling pathway [32]. On the contrary to EGFR inhibitors series (**4**), the decoy non EGFR-inhibitor compound **5** did not show significant difference in inhibition of NO production in peritoneal macrophages versus RAW 264.7 macrophages.

In summary, these results might indicate that EGFR inhibition is, at least, the major mechanism mediating the anti-inflammatory activity of the amide compounds (**4a**−**d**). This was evident in the calculated high specific inhibition in peritoneal macrophages against the low specific inhibition in RAW 264.7 macrophages. This is consistent with the fact that RAW 264.7 macrophages are transformed cells with altered EGFR pathway [32]. This might be further bolstered as the structurally closely related non-EGFR inhibitor decoy compound (**5**) failed to show similar activity pattern.

2.4. EGFR inhibitor compound 4d inhibited production of multiple LPS-induced inflammatory mediators

To obtain a more detailed picture of the anti-inflammatory activities of the developed anti-inflammatory EGFR inhibitor compounds **4a**−**d** in peritoneal macrophages, the most potent compound **4d** was selected for conduction of further investigations. As shown in Fig. 3A, incubation of peritoneal macrophages with compound **4d** in absence of LPS did not increase NO production confirming no influence for compound **4d** on NO production in the absence of stimuli. As expected, LPS induced a high elevation in NO production (red bar). This LPS-induced NO production was attenuated by compound **4d** in a dose-dependent manner (dark blue bars). In the absence of LPS as well as in presence of only compound **4d**, iNOS expression level was not detectable confirming that compound **4d** does not induce iNOS expression (Fig. 3B). As known, LPS induced a iNOS high expression level in peritoneal macrophages. Western blotting revealed that compounds **4d** produced a significant and dose-dependent reduction of LPS-induced expression levels of iNOS as shown in Fig. 3B. This explains the measured NO production reduction by compound **4d**. In addition, further anti-inflammatory activities of compound **4d** via reduction of other inflammatory mediators production were explored. Accordingly, the effects of compound **4d** on the production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in peritoneal macrophages were assessed. As shown in Figs. 3C-E, all of the three cytokines IL-1β, IL-6, and TNF-α were barely detectable in absence of LPS as well as in presence of compound **4d** alone confirming that compound **4d** does not induce production of these cytokines. As expected, LPS induced high increase in the production levels of all of IL-1β, IL-6, and TNF-α. However, compound **4d** resulted in significant reductions in the production of IL-1β, IL-6, and TNF-α. These results suggest that the EGFR inhibitor compound **4d** might be a novel potential anti-inflammatory lead compound inhibiting the production of multiple inflammatory mediators including NO, IL-1β, IL-6, and TNF-α.

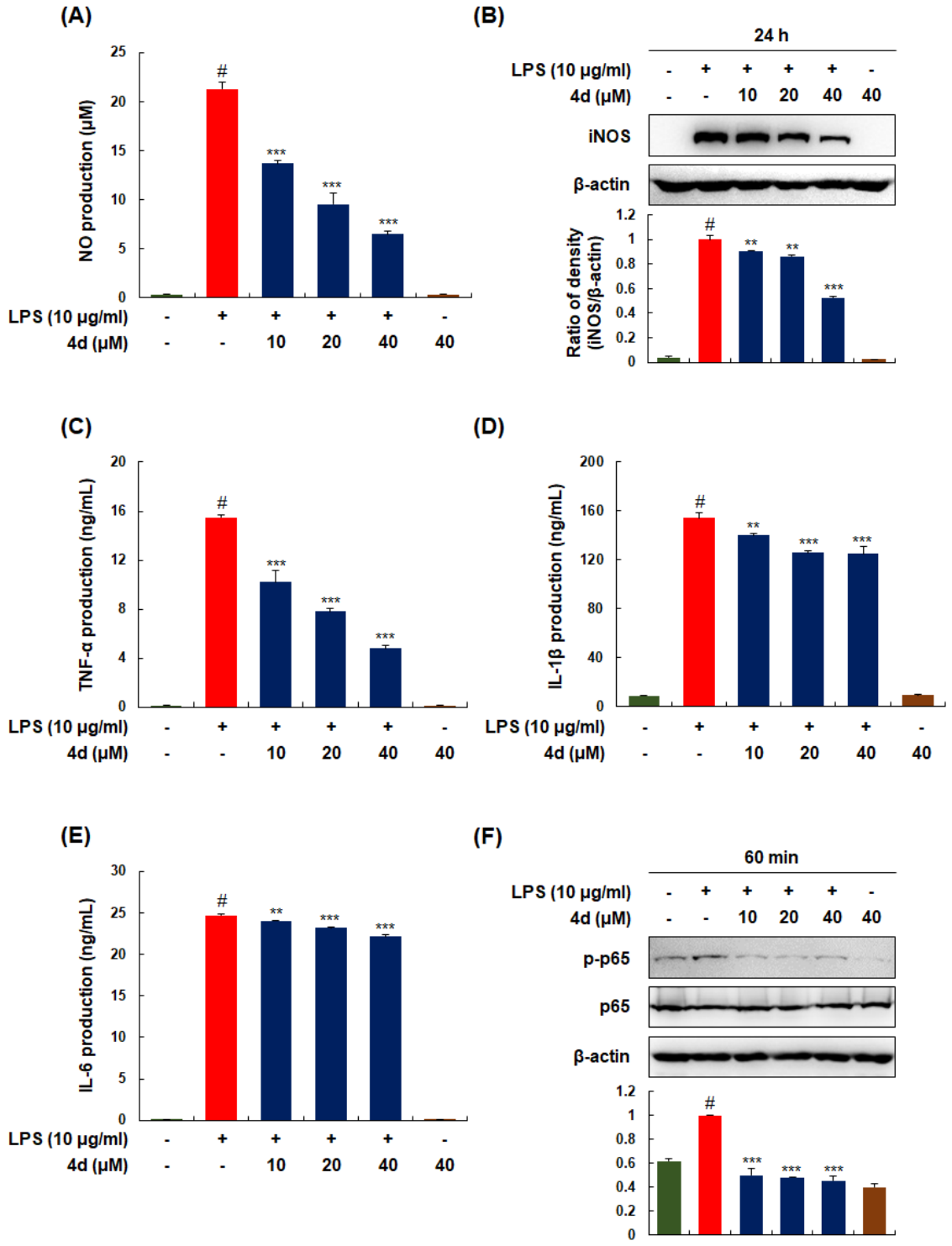


Fig. 3. Anti-inflammatory effects of compound **4d** in LPS-induced peritoneal macrophages; A) Following pretreatment with/without compound **4d** (10, 20, or 40 μM) for 1 h, cells were incubated with LPS (10 μg/mL) for 24 h. NO cellular levels in the culture media were determined by Griess reaction. B) Total cellular proteins were prepared from the cells treated with LPS (10 μg/mL) for 24 h in presence or absence of compound **4d** (10, 20, or 40 μM). The iNOS protein expression were estimated by Western blotting. C-E) Cells were incubated with/without compound **4d** (10, 20, or 40 μM) for 1 h, and then treated with LPS (10 μg/mL) for 24 h. The levels of TNF-α, IL-1β, and IL-6 were estimated in culture media by ELISA Kit. F) Total cellular proteins were prepared from the cell treated with compound **4d** (10, 20, or 40 μM) in presence or absence of LPS (10 μg/mL) for 1 h. $^{\#}P < 0.05$ *vs.* the control group; $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* the LPS-induced group.

2.5. EGFR inhibitor compound 4d attenuated NF-κB activation

NF-κB complex; typically a heterodimer of p65-p50 subunits, is the bottleneck of a signaling pathway involved in the development of inflammations and cancers [34]. It regulates the expression of several genes responsible for inflammatory mediators including cytokines as well as genes controlling cells' proliferation and survival [35, 36]. A recent report showed that EGFR signaling activates NF-κB in cancer cells [37]. Another recent report showed that activation of NF-κB by EGFR in cancer cells involves phosphorylation of p65 subunit of NF-κB complex [38]. In fact, it is known that phosphorylation of p65 is required for the activation of NF-κB and, thus, dephosphorylation of p65 results in reduction of NF-κB activity [39, 40]. The activation of NF-κB is critically required for the LPS-induced transcriptional regulation of inflammatory mediators in macrophages [34]. Therefore, we checked whether compound **4d** influence p65 phosphorylation and consequently reduces the production of the inflammatory mediators *via* inhibition of NF-κB activity. We measured phosphorylation of p65 in LPS-induced peritoneal macrophages in presence and absence of compound **4d** as shown in Fig. 3F. We found that treatment with compound **4d** significantly attenuated the LPS-induced phosphorylation of p65 subunit of NF-κB. This might provide an understanding of the molecular pathway for reduction of inflammatory mediators' production by EGFR inhibitor compound **4d** in peritoneal macrophage cells.

3. Conclusion

Recent studies showed that EGFR signaling is involved in inflammatory response in several inflammatory conditions. The normal inflammatory macrophage cells show EGFR-dependent production of inflammatory mediators. Nevertheless, the development of EGFR inhibitors as anti-inflammatory agents has not been reported yet. In this study, we have synthesized a series of amides (**4**) and structurally related urea derivative (**5**). While the amide derivatives

(**4**) exhibited EGFR inhibitory activity, the urea derivative (**5**) was devoid from any significant EGFR inhibition up to 100 µM concentration. These derivatives were employed in cellular assays for anti-inflammatory activity in LPS-induced macrophages. Two macrophage cell lines were employed, peritoneal macrophages and RAW 264.7 macrophages. While the EGFR signaling pathway is intact in normal peritoneal macrophages, the transformed RAW 264.7 macrophages have modified EGFR signaling. Thus, EGFR inhibitors might reduce production of inflammatory mediators in peritoneal macrophages but not in RAW 264.7 macrophages. This might help to confirm that the elicited anti-inflammatory activity is mediated through EGFR inhibition. On contrast, the profile of non-EGFR inhibitor decoy compound might be different from EGFR inhibitors. The results of the anti-inflammatory assay showed that all of amide series exhibited high specific anti-inflammatory response against LPS-stimulated production of NO in peritoneal macrophages through inhibition of EGFR. Among them, compound **4d** was the most potent eliciting IC_{50} value of 20.8 µM and showed high protection percent (18.9% remaining increased NO production at 40 µM). In addition, compound **4b** presented high protection against increased LPS-stimulated NO production (18.5% remaining increased NO production at 40 µM) and IC_{50} value of 25.2 µM. We selected the most potent compound **4d** to conduct further molecular mechanistic studies. Compound **4d** inhibited the production of multiple inflammatory cytokines in macrophage cells including IL-1β, IL-6, and TNF-α. Furthermore, the EGFR inhibitor compound **4d** attenuated NF-κB activation by dephosphorylating p65. It might be concluded that compound **4d** might serve as a lead compound for development of a class of novel EGFR inhibitors as anti-inflammatory therapeutics. Furthermore, this study highlighted that the use of RAW 264.7 macrophages as a sole screen to judge the anti-inflammatory activity may result in loss of molecules with potential anti-inflammatory activity if their mechanism of action is mediated through the altered pathways in the transformed RAW 264.7 macrophage cells.

4. Experimental

4.1.Chemistry

General

Reactions were performed under nitrogen atmosphere. Solvents and reagents have been purchased and used without further purification. NMR spectra were obtained on Bruker Avance 400 (400 MHz ^{1}H and 100 MHz ^{13}C NMR). High-resolution spectra were performed on Waters ACQUITY UPLC BEH C18 1.7 μ–Q-TOF SYNAPT G2-Si High Definition Mass Spectrometry. FTIR spectra were recorded on a Thermo Scientific NICOLET iS10 (attenuated total reflection, ATR) spectrometer. Purifications were done by column chromatography (Merck Silica Gel 60, 230–400 mesh). Reactions monitored by TLC (glass sheets pre-coated with silica gel 60 F_{254}). Compounds **7a**, **7b**, **8a**, **4a**−**c**, **9** and **5** have been synthesized as reported previously [22].

4.1.1. Synthesis of 4-fluoro-*N*-(4-(3-(trifluoromethyl)phenoxy)pyrimidin-5-yl)benzamide (4d)

To a solution of compound **8a** (1 mmol, 255.2 mg) in THF (20 mL), 4-fluorobenzoyl chloride (1 mmol, 0.12 mL) and TEA (1.4 mmol, 0.20 mL) were added and stirred at 50 °C overnight. The reaction was monitored by TLC (EA/*n*-Hex). After cooling, the organic solvent was evaporated in *vacuo* and the residue was extracted with ethyl acetate and water. The organic solvent was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, EA/*n*-Hex) to give compound **4d**. White solid, yield: 80%, mp: 92.3-94.0 °C, ^{1}H NMR (400 MHz, DMSO-d_6) δ = 10.36 (s, 1H), 9.06 (s, 1H), 8.63 (s, 1H), 8.09−8.12 (dd, *J* = 8.0 Hz, 4.0 Hz, 2H), 7.60−7.76 (m, 4H), 7.42 (t, *J* = 8.9 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ = 165.64, 164.85,

163.16, 161.30, 153.89, 153.21, 152.46, 132.09, 131.00, 130.78, 126.37, 122.35, 121.30, 118.81, 115.60, 115.38. HRMS (ES^+): *m/z* calculated for $C_{18}H_{11}F_4N_3O_2$: 378.0865 $[M+H]^+$. Found 378.0871. FTIR = 3476.84 (-NH), 1650.03 (-C=O) cm^{-1}.

4.2. EGFR Kinase assay

Kinase assays were performed at Reaction Biology Corporation using the 'HotSpot' assay platform as described in the supporting information [41].

4.3. Cellular assays

4.3.1. Cells' viability assay

The macrophage cells' viability assay was done using MTT-viability method as described in the supporting information.

4.3.2. Nitric oxide production assay

Nitrite levels in culture media were determined using Griess assay [42, 43] as described in the supporting information.

4.3.3. Determination of TNF-α, IL-1β and IL-6 production

TNF-α, IL-1β and IL-6 levels in cell culture media were determined by ELISA as described in the supporting information.

4.3.4. Western Blot Analysis

Western blot analysis was conducted according to known literature protocols [44] as described in the supporting information.

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Declaration of interest

The authors declare no conflict of interest

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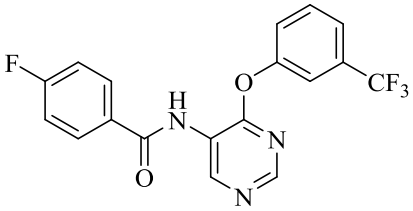
Highlights

- Compound **4d** is an anti-inflammatory EGFR inhibitor.
- Compound **4d** inhibits LPS-induced NO, IL-1β, IL-6 and TNF-α production.
- Compound **4d** inhibits iNOS expression and attenuates NF-κB activation.
- Compound **4d** is a novel lead anti-inflammatory EGFR inhibitor.

Graphical abstract



4d

EGFR Inhibitor

In peritoneal macrophages: Attenuates NO Production

Attenuates iNOS Expression

Attenuates IL-1β, IL-6 and TNF-α Production

Attenuates NF-κB