# Roflumilast *N*-oxide in combination with formoterol enhances the anti-inflammatory effect of dexamethasone in ASM cells

Brijeshkumar S. Patel[1], Md. Mostafizur Rahman[1], Gina Baehring[2], Dikaia Xenaki[3], Francesca Su-May Tang[2], Brian G. Oliver[2, 3, 4], and Alaina J. Ammit[2,4]

[1]Faculty of Pharmacy
University of Sydney
NSW Australia

[2]Woolcock Emphysema Centre and [3]Respiratory Cellular and Molecular Biology
Woolcock Institute of Medical Research
University of Sydney
NSW Australia

[4]Centre for Health Technologies and Molecular Biosciences
School of Life Sciences
Faculty of Science
University of Technology Sydney
NSW Australia

**Corresponding author:** Alaina J. Ammit Ph.D.
**Phone:** +61 2 91140368
**E-mail:** Alaina.Ammit@uts.edu.au

**Running title:** RNO does not impact the production of IL-8

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## Abstract

Roflumilast is an orally active phosphodiesterase 4 (PDE4) inhibitor approved for use in chronic obstructive pulmonary disease. Roflumilast *N*-oxide (RNO) is the active metabolite of roflumilast and has demonstrated anti-inflammatory impact *in vivo* and i*n vitro*. To date, the effect of RNO on the synthetic function of airway smooth muscle (ASM) cells is unknown. We address this herein and investigate the effect of RNO on $\beta_2$-adrenoceptor-mediated, cAMP-dependent responses in ASM cells *in vitro*, and whether RNO enhances steroid-induced repression of inflammation. RNO (0.001-1000 nM) alone had no effect on AMP production from ASM cells, and significant potentiation of the long-acting $\beta_2$-agonist formoterol-induced cAMP could only be achieved at the highest concentration of RNO tested (1000 nM). At this concentration, RNO exerted a small, but not significantly different, potentiation of formoterol-induced expression of anti-inflammatory mitogen-activated protein kinase phosphatase 1 (MKP-1). Consequently, tumor necrosis factor (TNF)-induced IL-8 secretion was unaffected by RNO in combination with formoterol. However, as there was the potential for PDE4 inhibitors and LABAs to interact with corticosteroids to achieve superior anti-inflammatory efficacy, we examined whether RNO, alone or in combination with formoterol, enhanced the anti-inflammatory effect of dexamethasone by measuring the impact on IL-8 secretion. While RNO alone did not significantly enhance cytokine repression achieved with steroids; RNO in combination with formoterol significantly enhanced the anti-inflammatory effect of dexamethasone in ASM cells. This was linked to increased MKP-1 expression in ASM cells, suggesting a molecular mechanism responsible for augmented anti-inflammatory actions of combination therapeutic approaches that include RNO.

**Keywords:** RNO, $\beta_2$-agonists, corticosteroids, MKP-1, interleukin 8, cAMP

## Introduction

Roflumilast *N*-oxide is the active metabolite of roflumilast; a phosphodiesterase 4 (PDE4) inhibitor that has been approved for the oral, once-daily treatment of severe chronic obstructive pulmonary disease (COPD) (1). PDEs are an enzyme superfamily with considerable importance in chronic respiratory diseases (2). In particular, the PDE4 isoform is known to inactivate the major bronchodilatory second messenger, cyclic adenosine monophosphate (cAMP) and together with $\beta_2$-agonists, PDE4 inhibitors can increase the amount of intracellular cAMP in airway smooth muscle (ASM) and reverse bronchospasm. However, there has been an emerging appreciation of the anti-inflammatory effects of these medicines (2, 3) that can also occur in a cAMP dependent manner (4). Utilizing primary cultures of airway smooth muscle (ASM) cells, we recently examined the ability of panel of PDE4 inhibitors to enhance cAMP production in response to the long acting $\beta_2$-agonist (LABA) formoterol (5). By increasing cAMP levels, PDE4 inhibitors in combination with formoterol inhibited pro-inflammatory cytokine production from ASM cells. This was due to upregulation of the cAMP-dependent, anti-inflammatory protein – mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1) (5).

Roflumilast has been shown to anti-inflammatory *in vitro* and *in vivo* (reviewed in (6)). It can have beneficial effects on its own in some experimental contexts (6), but can have positive interactions especially with LABAs-mediated impacts interactions that may prove to be therapeutically advantageous (7). To date, its effects on formoterol-induced cAMP production in ASM cells are unknown. Whether roflumilast acts on ASM cells in a manner similar to other PDE4 inhibitors (cilomilast, piclamilast, rolipram) (5) warrants further investigation. Accordingly, in this study we use the active metabolite of roflumilast responsible for >90% of the PDE4 inhibition (8), roflumilast *N*-oxide (abbreviated as RNO), and examine the impact on

cAMP production in ASM cells over a range of RNO concentration, in the absence and presence of a LABA (formoterol). We determine the effect on anti-inflammatory MKP-1 protein production and whether RNO affects neutrophil-chemoattractant cytokine IL-8 secretion from ASM cells. Finally, as there is the demonstrated potential of PDE4 inhibitors and LABAs to interact with corticosteroids to achieve superior anti-inflammatory efficacy (4, 7), we explore combination therapies *in vitro* and show that RNO, in combination with formoterol, enhances the repression of TNF-induced IL-8 by dexamethasone in ASM cells in a manner linked to MKP-1 upregulation.

## Material and Methods

### Cell culture

Ethical approval for the use of human bronchi (from patients undergoing surgical resection for carcinoma or lung transplant donors) was provided by the Human Research Ethics Committee of the University of Sydney and the Sydney South West Area Health Service and the. ASM cells were processed using established methods (9) and each experiment used a minimum of three different ASM primary cell lines.

### Chemicals

RNO was provided by Takeda. Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO), except for tumor necrosis factor (TNF) (R&D Systems).

### cAMP assay

Enzyme immunoassay was used to measure cAMP (cAMP EIA 581001: Cayman Chemical Company, Ann Arbor, MI).

### MKP-1 mRNA expression and protein upregulation

MKP-1 mRNA expression was quantitated by real-time RT-PCR and MKP-1 protein upregulation detected by Western blotting using methods outlined in our previous publication (5).

### ELISAs

IL-8 ELISAs were performed using kits from BD Biosciences Pharmingen, San Diego, CA.

**Statistical analysis**

Statistical analysis was performed using Student's unpaired *t* test or one-way ANOVA then Fisher's post-hoc multiple comparison test. *P* values $< 0.05$ were sufficient to reject the null hypothesis for all analyses.

## Results

### Effect of RNO (0.001-1000 nM) on formoterol-induced cAMP production in ASM cells

RNO is an inhibitor of PDE4 and in order to determine whether it enhances $\beta_2$-adrenoceptor-mediated responses in ASM cells in a similar manner to other PDE4 inhibitors (specifically cilomilast, piclamilast and rolipram (5)) we examined the impact of pretreating ASM cells with a range of concentrations of RNO (0.001-1000 nM) before stimulation with the long-acting $\beta_2$-agonist formoterol (10 nM). These experiments were performed alongside relevant controls, and as shown in Figure 1, increasing concentrations of RNO added alone (0.001-1000 nM) had no effect on cAMP production in the absence of $\beta_2$-agonist stimulation. In contrast, formoterol significantly increased cAMP production in ASM cells (in accordance with our earlier studies (5, 10)), and this could be significantly increased by pretreatment with RNO at the highest concentration tested, i.e. 1000 nM (Figure 1: $P<0.05$). Because RNO at 1000 nM increases formoterol-induced cAMP production, we chose this concentration for further investigation.

### Effect of RNO (1000 nM) on formoterol-induced MKP-1 mRNA expression and protein upregulation in ASM cells

Our previous studies in ASM cells have confirmed that stimuli that increase cAMP induce expression of the cAMP-dependent anti-inflammatory protein MKP-1 (5, 11-13). Because RNO (at 1000 nM) increased cAMP production in response to formoterol, we hypothesized that RNO will augment MKP-1 expression in response to the long-acting $\beta_2$-agonist in ASM cells. To test this, we pretreated ASM cells with 1000 nM of RNO and examined the impact on formoterol-induced MKP-1 mRNA expression (Figure 2A) and MKP-1 protein upregulation (Figures 2B and 2C), compared to relevant controls. While formoterol-induced MKP-1 mRNA and protein

upregulation, in accordance with our prior publications (5, 10-12), there was little impact of RNO on MKP-1 production. This is shown in Figure 2A, where although there was a small increase in formoterol induced-MKP-1 levels in the presence of 1000 nM RNO, this was not significantly different from formoterol alone. Moreover, there was no significant increase in formoterol-induced MKP-1 protein upregulation as shown by Western blotting (Figure 2B) or by densitometry (Figure 2C).

**RNO (1000 nM), alone or in combination with formoterol, does not repress TNF-induced IL-8 secretion**

MKP-1 is an anti-inflammatory protein that can repress synthetic function of ASM cells (14-17) Thus, it follows that since RNO did not increase formoterol-induced MKP-1, it is unlikely that RNO will not increase the anti-inflammatory capacity of formoterol in ASM cells. To confirm that this is indeed the case, we examined the impact of RNO (1000 nM) and formoterol (10 nM), alone or in combination, on the secretion of the neutrophil chemoattractant IL-8 induced by TNF. As shown in Figure 3, there was no effect of RNO and formoterol, alone or in combination, on unstimulated levels of IL-8. When cells were stimulated for 24 h with TNF (resulting in secretion of 23,219.9±6,682.9 pg/ml IL-8 (mean±SEM)), formoterol alone did not repress IL-8 secretion, in confirmation of our earlier study (12). Moreover, RNO (1000 nM), alone or in combination with formoterol, did not repress TNF-induced IL-8 secretion (Figure 3).

**RNO (1000 nM), in combination with formoterol, enhances repression of TNF-induced IL-8 secretion by dexamethasone in ASM cells in a manner linked to increased MKP-1 upregulation**

We then examined whether RNO can augment corticosteroid-mediated inhibition of cytokine expression and whether it is necessary for RNO to be in combination with $\beta_2$-agonist to exert anti-inflammatory impact. To test these questions, growth-arrested ASM cells were pretreated for 30 min with vehicle or 1000 nM RNO, then for 1 h with formoterol (10 nM), alone or in combination with dexamethasone (10 nM) before cells were stimulated for 24 h with TNF, compared to vehicle control. IL-8 protein secretion was detected by ELISA and as shown in Figure 4A, TNF-induced IL-8 secretion was inhibited by the corticosteroid dexamethasone ($P<0.05$). Notably, the only combination that resulted in a significantly greater repression of cytokine production above that achieved by dexamethasone alone were RNO in combination with formoterol ($P<0.05$). RNO alone did not increase dexamethasone-mediated cytokine repression. To examine whether this was due to an enhancement of MKP-1 upregulation under these conditions, MKP-1 protein was quantified by Western blotting. As shown in the representative Western blot (Figure 4B), MKP-1 protein levels were increased in cells treated with RNO + formoterol + dexamethasone, compared to TNF alone. This augmentation was shown to be significant by densitometric analysis ($P<0.05$: Figure 4C). Taken together these results suggest that RNO (1000 nM), in combination with formoterol, enhances the repression of TNF-induced IL-8 by dexamethasone in ASM cells in a manner linked to increased MKP-1 upregulation.

## Discussion

We are the first to examine the effect of RNO, the active metabolite of roflumilast, on cAMP production in ASM cells. We examined a range of RNO concentrations and only the highest concentration used (i.e. 1000 nM) had any significant effect on formoterol-induced cAMP. RNO alone did not increase cAMP production in ASM cells at any concentration examined. Given the relatively low efficacy as a cAMP-inducer in ASM cells, therefore RNO (at 1000 nM) only minimally increased formoterol-induced MKP-1 mRNA expression and protein upregulation and the impact was not significant. Consequently, TNF-induced IL-8 secretion was unaffected by RNO in combination with formoterol. However, cAMP and the corticosteroid dexamethasone can have additive effects on MKP-1 upregulation. Thus, we reasoned that RNO + formoterol may enhance corticosteroid-repressive effects in ASM cells via increased MKP-1 upregulation. This was supported by the demonstration that RNO, in combination with formoterol, enhances repression of TNF-induced IL-8 by dexamethasone in ASM cells in a manner linked to increased MKP-1 expression.

Over the past decade our *in vitro* studies have underscored the important role played by the MAPK deactivator MKP-1 in controlling airway inflammation in experimental models of chronic respiratory disease. We have delineated the molecular mechanisms underlying MKP-1 upregulation and confirmed its nature as an immediate early response gene (18) containing cAMP-responsive (19, 20) and novel glucocorticoid-responsive elements (21) within its 5'-untranslated region, by showing that MKP-1 can be upregulated by agents that stimulate cAMP in ASM cells (dibutyrl cAMP (22), forskolin (22), sphingosine 1-phosphate (22), prostaglandin $E_2$ (13), LABAs (formoterol (5, 10-12), salmeterol (12)), as well as steroids (dexamethasone (11, 14), fluticasone: (12, 14)). Together cAMP-elevating agents and steroids can act in an

additive manner to increase MKP-1 upregulation and we have shown this with steroids and LABA added in combination (11, 12 ).

More recently we have shown that PDE4 inhibitors, but not PDE3 inhibitors, can also increase MKP-1, in a manner dependent on their ability to increase cAMP (5). Herein we examine RNO, the active metabolite of the orally active PDE4 inhibitor roflumilast, and examined its impact on cAMP production, alone and in combination with formoterol. A broad concentration range was tested (0.001-1000 nM) because as RNO is PDE4-selective over this range. Notably, RNO had no effect on its own. When added with formoterol, RNO could only significantly increase formoterol-induced cAMP at the highest concentration tested (1000 nM). Consequently there was only a small but not statistically significant effect on formoterol-induced MKP-1 mRNA and protein upregulation. We do know however that there is cAMP dose-dependency to MKP-1 expression that was recently demonstrated because $PGE_2$ (a cAMP elevating agent in ASM cells (23)) increases MKP-1 mRNA expression in a concentration-dependent manner (13).

Thus, when we tested the impact of RNO + formoterol on the secretion of IL-8 in response to TNF, there was no anti-inflammatory impact. Given that we have previously shown an additive effect of cAMP-elevating agents with corticosteroids on MKP-1 upregulation (11, 12), we were intrigued to examine whether RNO in combination with formoterol increases the anti-inflammatory impact of dexamethasone on TNF-induced IL-8 in ASM cells. Indeed, this was the case, and while RNO alone did not significantly enhance cytokine repression achieved with steroids, RNO in combination with formoterol significantly enhanced the anti-inflammatory effect of dexamethasone in ASM cells. Collectively, the weight of previous evidence strongly suggests that the anti-inflammatory effects of RNO are due to increased MKP-1 upregulation. However, in this experimental context it is not possible to directly demonstrate MKP-1

dependence. To do this, two experimental approaches could be used (pharmacological inhibitors of MKP-1 or siRNA against MKP-1), but unfortunately, both have well-documented limitations. Firstly, one could utilise triptolide; the most commonly used pharmacological inhibitor of MKP-1 (24). We have previously used triptolide to block MKP-1 upregulation and demonstrate that MKP-1 restrains downstream MAPK signalling (15). But triptolide is a non-specific inhibitor and has other targets apart from MKP-1 (25); notably, these include NF-κB. As TNF induced IL-8 production in ASM cells is NF-κB-dependent (26), it is not possible to use triptolide to inhibit MKP-1 and demonstrate the involvement of MKP-1 in the impact of RNO plus formoterol on the TNF-induced IL-8 production. Secondly, MKP-1 can be knocked-down with. siRNA. But this approach cannot give clear-cut answers, because as we have previously published (12), MKP-1 is in an inter-dependent *ying-yang* relationship with another anti-inflammatory protein – TTP (tristetraprolin). TTP is an anti-inflammatory protein that induces the decay of mRNAs encoding several cytokines, including those that drive COPD pathogenesis, including IL-8 (27). Expression and function of TTP is highly regulated and amenable to rapid control in a mechanism akin to an on-off molecular switch. Importantly, it is controlled by p38 MAPK: its expression is p38 MAPK-dependent (28); and its mRNA destabilizing function is controlled by p38 MAPK/MK2-mediated phosphorylation on two key serine residues (S52 and S178) preventing the initiation of mRNA decay (29). We and others have underscored the importance of the MKP-1/p38/TTP regulatory network in the control of cytokine expression *in vivo* in MKP-1 and TTP transgenic mice (30, 31) and *in vitro* (16, 17, 32, 33). Thus, when we knockdown MKP-1 with siRNA, we cannot predict that we will observe an increase in cytokine production. This is because MKP-1 and TTP are intimately linked to control inflammatory pathways in a p38 MAPK-dependent manner. Importantly, the cytokine regulatory network controlled by MKP-

1/TTP ensures that pro-inflammatory cellular signalling can be switched-on and switched-off at the precisely the right time.

This report represents the first study examining the impact of RNO on ASM cells, although other cell types have been utilised in previous publications (reviewed in (6, 7)). Milara *et al.* (34, 35) have demonstrated the influence of RNO in improving the effect of corticosteroids. In bronchial epithelial cells from patients with COPD, RNO, when combined with dexamethasone, increased the expression of MKP-1 and enhanced the inhibitory effects on phospho-p38 MAPK and activity of transcription factors AP-1 and NF-κB (35). Additionally, Milara *et al.* (35) found that in neutrophils from COPD patients, RNO in combination with dexamethasone had an additive/synergistic anti-inflammatory effect. Moodley *et al.* (36) have used lung epithelial cells and primary cultures to demonstrate that PDE4 inhibitors (including roflumilast) augment the ability of formoterol to enhance transcriptional regulation (glucocorticoid response element (GRE) luciferase reporter activity) induced by steroids. Via a GRE-dependent molecular mechanism, roflumilast interacted with formoterol in a positive manner to enhance glucocorticoid-dependent transcription of anti-inflammatory genes, including as *GILZ* and *RGS2* (36). Although the gene for MKP-1 (*DUSP1*) was not examined in the study, our data concur with those from the Giembycz group as they support the concept that "these drugs together may impart clinical benefit beyond that achievable by an ICS alone, a PDE4 inhibitor alone, or an ICS/LABA combination therapy" (36). Although our *in vitro* experimental model of airway inflammation doesn't fully encapsulate the complexity of COPD, these studies, taken together, provide a molecular mechanism that may explain the benefits of adding roflumilast as an "add-on" therapy in severe COPD, where patients are likely taking LABA and inhaled corticosteroids.

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## Figure Legends

**Figure 1. Effect of RNO (0.001-1000 nM) on formoterol-induced cAMP production in ASM cells.** Growth-arrested ASM cells were pretreated for 30 min with vehicle or RNO (0.001-1000 nM) and cAMP production (pmol/ml) measured without and with stimulation with 10 nM formoterol for 15 min. Statistical analysis was performed using Student's unpaired *t* test or one-way ANOVA then Fisher's post-hoc multiple comparison test (where * denotes significant increase in cAMP production by formoterol, and § denotes significant potentiation of formoterol-induced cAMP by 1000 nM RNO ($P<0.05$)). Data are mean+SEM values from n=4 primary ASM cell cultures.

**Figure 2. Effect of RNO (1000 nM) on formoterol-induced MKP-1 mRNA expression and protein upregulation in ASM cells.** Growth-arrested ASM cells were pretreated for 30 min with vehicle or 1000 nM RNO, then for 1 h with vehicle or formoterol (10 nM). (A) MKP-1 mRNA expression was measured by RT-PCR (results expressed as a percentage of formoterol-induced MKP-1 mRNA expression). (B, C) MKP-1 protein was quantified by Western blotting, using α-tubulin as the loading control. Results are shown as (B) representative Western blots and (C) densitometric analysis (results expressed as a percentage of formoterol-induced MKP-1 protein upregulation). Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple comparison test (where * denotes significant induction of MKP-1 by formoterol ($P<0.05$)). Data are mean±SEM values from (A) n=8 and (B, C) n=3 primary ASM cell cultures.

**Figure 3. RNO (1000 nM), alone or in combination with formoterol, does not repress TNF-induced IL-8 secretion.** Growth-arrested ASM cells were pretreated for 30 min with vehicle or

1000 nM RNO, then for 1 h with vehicle or formoterol (10 nM), before stimulation for 24 h with TNF (10 ng/ml), compared to vehicle control. IL-8 secretion was measured by ELISA and results expressed as a percentage of TNF-induced IL-8 secretion. Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple comparison test (where * denotes significant increase in IL-8 secretion induced by TNF ($P$<0.05)). Data are mean + SEM values from n=13 primary ASM cell cultures.

**Figure 4. RNO (1000 nM), in combination with formoterol, enhances repression of TNF-induced IL-8 secretion by dexamethasone in ASM cells in a manner linked to increased MKP-1 upregulation.** Growth-arrested ASM cells were pretreated for 30 min with vehicle or 1000 nM RNO, then for 1 h with formoterol (10 nM), alone or in combination with dexamethasone (10 nM), before stimulation with TNF (10 ng/ml), compared to vehicle control. (A) After 24 h, IL-8 secretion was measured by ELISA and results expressed as a percentage of TNF-induced IL-8 secretion. Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple comparison test (where * denotes significant increase in IL-8 secretion induced by TNF, § denotes significant repression of TNF-induced IL-8 by dexamethasone, that is †significantly enhanced by RNO + formoterol ($P$<0.05). (B, C) After 1 h, MKP-1 protein was quantified by Western blotting, using α-tubulin as the loading control. Results are shown as (B) representative Western blots and (C) densitometric analysis (results expressed as a percentage of TNF-induced MKP-1 protein upregulation). Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple comparison test (where * denotes significant induction of MKP-1 by TNF, that is † significantly enhanced by RNO +

formoterol + dexamethasone ($P$<0.05)). Data are mean + SEM values from (A) n=4 and (B, C) n=3 and primary ASM cell cultures.