

# CHRONIC OBSTRUCTIVE PULMONARY DISEASE

## Effect of cilomilast (Ariflo) on TNF- $\alpha$ , IL-8, and GM-CSF release by airway cells of patients with COPD

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**Background:** Inflammation in chronic obstructive pulmonary disease (COPD) is characterised by increased neutrophilic infiltration of the airways. Cilomilast, a novel selective phosphodiesterase 4 inhibitor in clinical development for COPD treatment, exerts anti-inflammatory effects. The ability of cilomilast to inhibit the release of neutrophil chemoattractants such as tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-8, and granulocyte-macrophage colony stimulating factor (GM-CSF) by bronchial epithelial cells and sputum cells isolated from 10 patients with COPD, 14 normal controls, and 10 smokers was investigated.

**Methods:** Bronchial epithelial cells obtained by bronchial brushing and sputum cells isolated from induced sputum samples were cultured for 24 hours in the presence or absence of cilomilast (1  $\mu$ M). After incubation the supernatants were harvested and the levels of mediators measured by ELISA. Chemotactic activity in supernatants was also measured using a Boyden chamber.

**Results:** TNF- $\alpha$  and IL-8 release by bronchial epithelial cells and sputum cells was higher in patients with COPD than in controls ( $p < 0.0001$ ) and smokers ( $p < 0.0001$ ). GM-CSF was only detectable in sputum cell supernatants and its level was higher in patients with COPD than in controls and smokers ( $p < 0.0001$ , respectively). Cilomilast significantly reduced TNF- $\alpha$  release by bronchial epithelial cells and sputum cells ( $p = 0.005$ ) and GM-CSF release by sputum cells ( $p = 0.003$ ), whereas IL-8 release was not statistically inhibited. Supernatants of sputum cells and bronchial epithelial cells treated with cilomilast significantly decreased neutrophil chemotaxis ( $p < 0.006$  and  $p < 0.008$ , respectively).

**Conclusions:** Cilomilast inhibits the production of some neutrophil chemoattractants by airway cells. This drug may play a role in the resolution of neutrophilic inflammation associated with COPD and cigarette smoke.

Inflammation in chronic obstructive pulmonary disease (COPD) is characterised by increased infiltration of neutrophils, lymphocytes, and macrophages into the airways.<sup>1</sup> Neutrophils play an important role in the pathogenesis of airway inflammation in COPD because of their ability to release a number of mediators including elastase, metalloproteases, and oxygen radicals which promote tissue inflammation and damage.<sup>2</sup> Although more direct evidence for the pathogenesis of neutrophilic inflammation in COPD is still lacking, it is likely that neutrophil accumulation in the airways of patients with COPD is driven by increased release of cytokines exerting a chemotactic effect on these cells. Among them, an important role may be played by tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 8 (IL-8).<sup>3,4</sup> In addition, TNF- $\alpha$  and IL-8 levels are increased in the airways of patients with COPD,<sup>5</sup> suggesting that these mediators may play an important role in the pathogenesis of the disease.

Granulocyte-macrophage colony stimulating factor (GM-CSF) is another mediator involved in the recruitment and activation of leucocytes.<sup>6</sup> We have previously shown that GM-CSF is expressed in the epithelium of subjects with chronic bronchitis<sup>7</sup> and that high levels of this mediator are released in severe asthmatic subjects with neutrophilic inflammation.<sup>8</sup> In addition, increased levels of GM-CSF have been found in the bronchoalveolar lavage fluid from subjects with chronic bronchitis during exacerbations.<sup>9</sup>

Intracellular cAMP seems to have a fundamental role, not only in smooth muscle relaxation but also in the modulation of the release of mediators by inflammatory cells.<sup>10</sup> Enhanced cAMP levels can lead to increased production of inflammatory mediators such as TNF- $\alpha$ , GM-CSF, and IL-8 in airway epithelial cells.<sup>11-14</sup>

Cilomilast (Ariflo, SB-207499) is an orally active second generation PDE4 inhibitor that may be effective in the treatment of COPD.<sup>15</sup> It was found to reduce the release of TNF- $\alpha$  and IL-4<sup>16</sup> and to block the recruitment of neutrophils into tissues as well as the production of LTB<sub>4</sub>.<sup>17,18</sup> However, to date no studies have evaluated the effects of cilomilast on airway cells isolated from subjects with COPD. We have therefore undertaken a study to investigate the spontaneous release of TNF- $\alpha$ , IL-8, and GM-CSF by bronchial epithelial cells and by sputum cells isolated from normal subjects and smokers, with or without COPD. The anti-inflammatory properties of cilomilast were also examined by assessing its ability to inhibit the release of TNF- $\alpha$ , IL-8, and GM-CSF by bronchial epithelial cells and sputum cells. Furthermore, to obtain a better understanding of the potential activity of cilomilast on neutrophilic inflammation in COPD, we analysed the chemotactic activity of sputum cell and bronchial epithelial cell supernatants recovered from cultured cells in the presence or absence of cilomilast.

### METHODS

#### Patients

The study was performed on three groups of subjects: 10 smokers with COPD (median age 63 years, range 52-74, 25-75% percentiles 56-71), 10 smokers without COPD (healthy smokers) (median age 41 years, range 33-76, 25-75% percentiles 33-59), and 14 control subjects (median age 50 years, range 22-77, 25-75% percentiles 40-70).

COPD was defined and classified according to the criteria reported by GOLD guidelines.<sup>19</sup> All subjects were smokers or ex-smokers (median 37.5 pack years, range 32.5-52). All

**Table 1** Assessment of inhibitory effects of different concentrations of cilomilast on mediators released by bronchial epithelial cells and sputum cells isolated from patients with COPD

	Bronchial epithelial cells				Sputum cells			
	Baseline	Cilomilast 0.1 $\mu$ M	Cilomilast 1 $\mu$ M	Cilomilast 10 $\mu$ M	Baseline	Cilomilast 0.1 $\mu$ M	Cilomilast 1 $\mu$ M	Cilomilast 10 $\mu$ M
TNF- $\alpha$	166 (165.2–201.2)	150 (138.7–161.2)	125.7 (111.3–131.9)	155 (147–164.7)	1742 (1476.5–1811)	1523 (1205.7–1529.7)	1020 (756.7–1155)	1652 (1350.5–1652.7)
GM-CSF	NA	NA	NA	NA	774 (686.2–831.7)	569 (532.2–707.7)	350 (233–548)	652 (575.5–748)

Results are expressed as median and 25–75th percentiles of three consecutive experiments.  
NA=not available because of undetectable levels of GM-CSF.

COPD patients with radiographic or CT evidence of emphysema were excluded. Healthy smokers (median 40 pack years, range 32–45) had pulmonary function within the normal range. Normal subjects (controls) had never suffered from asthma or chronic bronchitis or from any bronchial or respiratory tract infection during the month preceding the study. All were lifelong non-smokers and their pulmonary function was within the normal range.

Subjects were excluded if they had had a bronchial infection during the month preceding the study. No subject had received corticosteroids or theophylline in any form during the 2 months before the study. The study was approved by the local ethics committee and subjects gave their informed consent.

#### Isolation of bronchial epithelial cells

Bronchial epithelial cells were recovered by bronchial brushing as previously described.<sup>20</sup> Most of the patients were volunteers; two underwent bronchoscopy for diagnostic purposes. Briefly, the cells were pelleted by centrifugation (400g, 4°C, 10 minutes), washed three times, and resuspended in 1 ml RPMI at a final density of  $1 \times 10^5$  cells/ml. Slides prepared by cytocentrifugation (800g, 10 minutes, Cytospin, Shandon Southern Products, Runcorn, Cheshire, UK) of 200  $\mu$ l of the cell suspension were stained with May-Grunwald-Giemsa for differential cell counts and by immunocytochemistry for cytokeratin using an alkaline phosphatase, anti-alkaline phosphatase (APAAP) system (Dako, Versailles, France) to assess their epithelial origin. Negative controls were prepared using a mouse IgG1 MOPC cell line antibody (Organon Laboratories, West Chester, PA, USA) as the primary antibody.

#### Treatment of bronchial epithelial cells with cilomilast

Bronchial epithelial cells were cultured in the absence or presence of cilomilast (SB-207499 BULK, SmithKline Beecham, UK) 1  $\mu$ M for 24 hours at 37°C in a humidified 5% CO<sub>2</sub>. This concentration of cilomilast was determined from three consecutive dose-response curves in which the effects on the release of TNF- $\alpha$  and GM-CSF of three concentrations of cilomilast (0.1, 1 and 10  $\mu$ M) were assessed (table 1). At the end of the incubation period the cell viability was >90% as assessed by trypan blue exclusion and the cell supernatants were harvested and stored at –80°C for further analysis (TNF- $\alpha$ , IL-8, and GM-CSF) and chemotaxis assay.

#### Sputum induction and processing

Sputum induction and processing were performed according to the methods of Hargreave *et al* without modification.<sup>21</sup> Differential cell counts were performed on cytocentrifuge preparations stained with May-Grunwald-Giemsa. In all cases 400 cells were counted by two blind observers and the results are expressed as a percentage of the total cells.

#### Treatment of sputum cells with cilomilast

Sputum cells were resuspended at a concentration of  $1 \times 10^6$ /ml in RPMI 1640 + 10% heat inactivated (56°C, 30

minutes) FCS + 1% penicillin-streptomycin solution + 1 mmol/l L-glutamine (RPMI buffer). The sputum cells were cultured in the absence or presence of cilomilast 1  $\mu$ M for 24 hours at 37°C in a humidified 5% CO<sub>2</sub>. This concentration of cilomilast was determined on the basis of three consecutive dose-response curves in which the effects of three concentrations of the drug (0.1, 1 and 10  $\mu$ M) on the release of TNF- $\alpha$  and GM-CSF were assessed. Dose-response curve experiments with a higher number of concentration points were precluded by the limited number of cells. At the end of the incubation time the cell viability was >85%, as assessed by trypan blue exclusion, and supernatants were harvested and stored at –80°C for further analysis (TNF- $\alpha$ , IL-8, and GM-CSF) and chemotaxis assay.

#### Measurement of TNF- $\alpha$ , IL-8 and GM-CSF

The absolute values of TNF- $\alpha$ , IL-8, and GM-CSF in supernatants of bronchial epithelial cells and sputum cells were assessed using commercially available specific enzyme immunoassay kits (EIA). TNF- $\alpha$ , IL-8, and GM-CSF kits were purchased from R&D Systems (Minneapolis, USA) and their limits of detection were <0.18 pg/ml, 3 pg/ml, and 10 pg/ml, respectively.

#### Neutrophil chemotaxis assay

To lend further support to the hypothesis that cilomilast may have a role in decreased neutrophil migration within the airways of subjects with COPD, we evaluated the ability of supernatants of bronchial epithelial cells and sputum cells incubated in the presence or absence of cilomilast 1  $\mu$ M for 24 hours at 37°C in humidified 5% CO<sub>2</sub> to induce neutrophil chemotaxis. Neutrophils were obtained from the peripheral blood of healthy donors as previously described,<sup>22</sup> resuspended at a concentration of  $1 \times 10^6$ /ml in PBS buffer, and incubated with supernatants of bronchial epithelial and sputum cells for chemotaxis assay.

To determine how much of the supernatant chemotactic activity was due to IL-8 (whose activity was not affected by cilomilast), the sputum cell supernatants were cultured in four blocking experiments for 24 hours with or without cilomilast 1  $\mu$ M, then incubated for 30 minutes with an anti-IL-8 monoclonal antibody (Genzyme, Cambridge, MA, USA) in a concentration of 1 mg/ml at 37°C before loading the chemotaxis chamber.<sup>23</sup> Chemotaxis was performed using a 48-well microchemotaxis chamber (Costar; Neuro Probe, Cabin John, MD, USA) as previously described.<sup>22</sup> Briefly, neutrophils were loaded into the upper well and supernatants of induced bronchial epithelial and sputum cells were in the bottom chamber. The two wells were separated by a polycarbonate filter paper with a pore size of 3  $\mu$ m. The chamber was incubated at 37°C for 1 hour, after which the filter was fixed, stained, and mounted on a glass microscope slide (observed at 400 $\times$ ). The numbers of cells migrating beyond a certain depth into the filter were counted. Each experimental condition was performed in duplicate and 3–4 fields were assessed. The

**Table 2** Demographic characteristic of patients

	Control (C)	Smoker (S)	COPD	p value†	C v S*	C v COPD*	S v COPD*
n	14	8	10	NS			
Sex (M/F)	9/5	6/2	6/4				
Age (years)*	50 (40–70)	41 (33–59)	63 (56–71)	NS	NS	NS	NS
Smoke (% of current smokers)	0%	100%	70%				
Packs/year*	0	40 (32–45)	37.5 (32.5–52)				NS
FEV <sub>1</sub> (%)*	105 (100–110)	93 (90–103)	54 (50–62.5)	0.0001	p=0.009	p<0.0001	p<0.0001

Results are expressed as median with 25–75th percentiles in parentheses.

FEV<sub>1</sub>=forced expiratory volume in 1 second.

\*p value for non-parametric Bonferroni Dunn's post hoc test.

†p value for non-parametric Kruskal-Wallis test comparing the differences between the groups.

chemotactic activity of the supernatants was also evaluated and the results expressed as medians with 25–75th percentiles. The number of cells migrating spontaneously in the presence of RPMI 10% FCS under the same conditions as the supernatants (negative control) was subtracted from all measurements before data analysis.

### Statistical analysis

The results are expressed as median values with 25–75th percentiles. Statistical analysis was performed with a non-parametric Kruskal-Wallis test with Bonferroni Dunn's correction to assess differences between groups. Statistical analysis of the effects of bronchial epithelial and sputum cell supernatants cultured in the presence or absence of cilomilast was performed using the Wilcoxon test.

## RESULTS

### Demographic characteristics of patients

The differences in the median ages of the controls, healthy smokers, and subjects with COPD were not statistically significant. Forced expiratory volume in 1 second (FEV<sub>1</sub>) was lower in patients with COPD than in smokers ( $p<0.0001$ ; table 2). All patients with COPD had moderate disease (FEV<sub>1</sub> 43–75% predicted). Eight were classified as IIA moderate COPD (FEV<sub>1</sub> 50–75% predicted, median 58%, 25–75th percentiles 52–63%) and two as IIB moderate COPD (FEV<sub>1</sub> 43–45% predicted, median 44%, 25–75th percentiles 43–45%).

### Total and differential cell counts in sputum

The viability of sputum cells was similar in the three study groups. The percentage of squamous cells in sputum samples was not significantly different between controls and smokers, or between smokers and patients with COPD. The median total cell count was higher in COPD patients ( $9.7 \times 10^6$ /g sputum, 25–75th percentiles 2.5–17.2), than in control subjects ( $2.4 \times 10^6$ , 25–75th percentiles 1.9–3.0;  $p=0.0023$ , table 3). The percentage of macrophages was significantly higher in control subjects and smokers than in patients with COPD ( $p<0.0001$  and  $p=0.0002$ , respectively); the percentage of neutrophils

was significantly higher in patients with COPD than in controls and smokers ( $p<0.0001$  and  $p=0.0005$ , respectively); and the percentage of eosinophils was higher in patients with COPD than in controls ( $p=0.008$ ). No statistical differences were observed in lymphocytes and bronchial epithelial cells (table 3).

### Effects of cilomilast on TNF- $\alpha$ , IL-8, and GM-CSF release

Cultured bronchial epithelial cells isolated from patients with COPD released higher levels of TNF- $\alpha$  (165 (89–180) pg/ml) than cells isolated from smokers (65 (50–85) pg/ml) and control subjects (17 (12–25) pg/ml;  $p<0.0001$ ). In addition, bronchial epithelial cells isolated from smokers with COPD released higher levels of TNF- $\alpha$  than those isolated from controls ( $p<0.0001$ ; fig 1A). When bronchial epithelial cells isolated from COPD patients were cultured in the presence of cilomilast there was a significant inhibition of TNF- $\alpha$  release (116 (40–134) pg/ml;  $p=0.005$ ; fig 1B).

Cultured sputum cells isolated from patients with COPD released higher levels of TNF- $\alpha$  (1650 (1421–1720) pg/ml) than sputum cells isolated from smokers (404 (320–500) pg/ml) and control subjects (97.5 (90–115) pg/ml;  $p<0.0001$ ). In addition, sputum cells isolated from smokers released higher levels of TNF- $\alpha$  than cells isolated from controls ( $p=0.005$ ; fig 1C). Furthermore, when the sputum cells isolated from COPD patients were cultured in the presence of cilomilast there was a significant inhibition of TNF- $\alpha$  release (1230 (1020–1230) pg/ml;  $p=0.005$ , fig 1D).

Cultured bronchial epithelial and sputum cells isolated from patients with COPD released higher levels of IL-8 than cells isolated from smokers and controls (bronchial epithelial cells: 4423.5 (4325–4589) v 2200 (1350–2654) v 782 (670–900) pg/ml,  $p<0.0001$ , fig 2A; sputum cells: 4458.5 (4255–4677) v 1789 (1550–1980) v 990 (890–1224) pg/ml,  $p<0.0001$ , fig 2C).

Cilomilast did not significantly reduce IL-8 release by bronchial epithelial cells (baseline: 4423.6 (4325–4589) pg/ml; cilomilast: 4336.5 (4267–4526) pg/ml; % inhibition compared

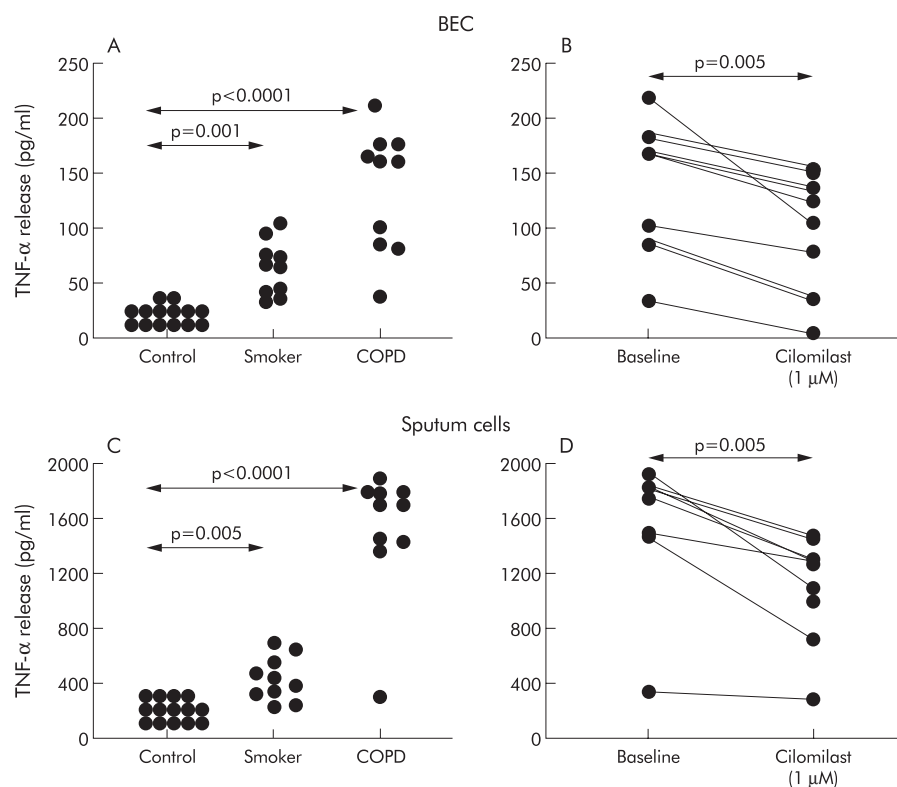
**Table 3** Cell analysis of sputum samples

	Control (C)	Smoker (S)	COPD	p value†	C v S*	C v COPD*	S v COPD*
Viability (%)	86.8 (67–89.3)	75.7 (73.4–83.3)	80.5 (74.6–87.8)	NS	NS	NS	NS
Squamous cells (%)	4.3 (2.9–8.0)	2.8 (1.1–4.9)	4 (1.2–5.4)	NS	NS	NS	NS
Total cell counts ( $10^6$ /g sputum)	2.4 (1.9–3.0)	5 (4.2–6.4)	9.7 (2.5–17.2)	NS	NS	p=0.0023	NS
Differential cell counts (%)							
Macrophages	83.1 (72.4–87)	57.2 (49.4–72.4)	25.2 (15.1–39.3)	$p<0.0001$	NS	$p<0.0001$	$p=0.0002$
Neutrophils	15.1 (12.7–26.0)	40.6 (25.8–47.0)	68.2 (54.4–81.0)	$p<0.0001$	NS	$p<0.0001$	$p=0.0005$
Lymphocytes	0.8 (0–1)	0.9 (0.3–1.2)	1 (0–1.7)	NS	NS	NS	NS
Eosinophils	0 (0–0.3)	0.9 (0.1–1.7)	1.9 (0.8–4.8)	$p<0.008$	NS	$p=0.008$	NS
Epithelial cells	0.4 (0.1–0.8)	0 (0–0.2)	0.2 (0–0.9)	NS	NS	NS	NS

Results are expressed as median values with 25–75th percentiles in parentheses.

\*p value for non-parametric Bonferroni Dunn's post hoc test.

†p value for non-parametric Kruskal-Wallis test comparing the differences between the groups.



**Figure 1** TNF- $\alpha$  release by (A) bronchial epithelial cells (BEC) and (C) sputum cells isolated from the airways of controls, smokers, and patients with COPD. The recovered bronchial epithelial cells and sputum cells were cultured for 24 hours at 37°C to test the release of TNF- $\alpha$  either spontaneously or after incubation with cilomilast 1  $\mu$ M (B and D). Statistical analysis was performed by the Mann-Whitney U test for comparisons between groups and by the Wilcoxon U test for comparisons between baseline and cilomilast.

with baseline 1.4% (0–3.3), fig 2B) and sputum cells (baseline: 4458.5 (4255–4677) v 4345 (4125–4581) pg/ml; % inhibition compared with baseline 3.1% (0–5.6), fig 2D) isolated from patients with COPD.

Bronchial epithelial cells from control subjects, smokers, and patients with COPD released very low or undetectable levels of GM-CSF. Cultured sputum cells isolated from patients with COPD (599.5 (454–657) pg/ml) released higher levels of GM-CSF than sputum cells isolated from smokers (294 (253–325) pg/ml) and control subjects (125 (100–150) pg/ml;  $p = 0.0002$  and  $p < 0.0001$ , respectively; fig 3A). Furthermore, cilomilast significantly inhibited GM-CSF release by sputum cells isolated from patients with COPD (322 (250–350) pg/ml;  $p = 0.005$ , fig 3B).

#### Effect of cilomilast on neutrophil chemotaxis assay

Supernatant fluids harvested from bronchial epithelial and sputum cells from patients with COPD recovered and cultured in the presence or absence of cilomilast were tested for their capacity to induce neutrophil chemotaxis. Supernatants recovered from bronchial epithelial cells incubated with cilomilast had significantly less chemotactic activity for neutrophils than supernatants from cells untreated with cilomilast (200.5 (150–221) v 130 (120–169) cells high power field;  $p = 0.008$ , fig 4A). Furthermore, supernatants recovered from sputum cells incubated with cilomilast had significantly lower chemotactic activity for neutrophils than those from cells untreated with cilomilast (198.5 (167–267) v 124 (119–172.5) cells high power field;  $p = 0.006$ , fig 4B).

In addition, blocking experiments showed that the combination of an anti-IL-8 antibody with cilomilast exerted a greater inhibitory effect on neutrophil chemotaxis than either the anti-IL-8 antibody or cilomilast alone (from 80 (75–115) to 149 (127.5–200) or 124 (119–172.5) cells high power field).

#### DISCUSSION

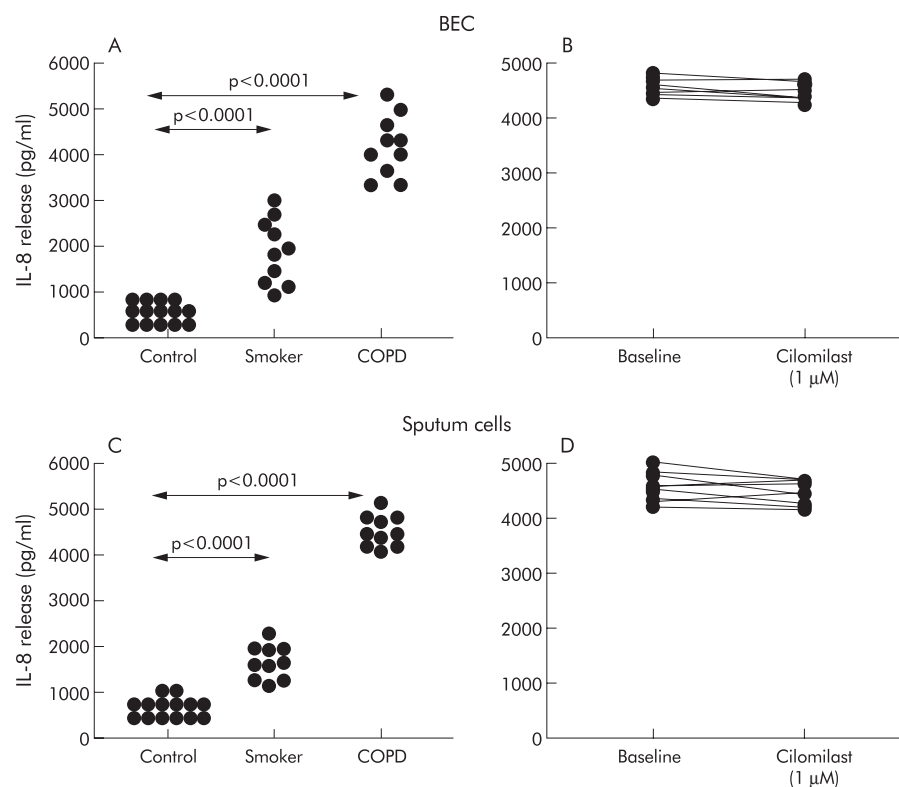
This study shows that cilomilast inhibits the release of TNF- $\alpha$  and GM-CSF by bronchial epithelial cells and sputum cells isolated from patients with COPD. In addition, cilomilast significantly reduced the chemotactic activity of supernatant fluids harvested from cultured bronchial epithelial and sputum cells of COPD patients.

The levels of cAMP play an important role in the modulation of the functional activation of several airway cells. Increased levels of cAMP have been shown to decrease the release of neutrophil chemotactic activity as well as neutrophil adhesion to bronchial epithelial cells via MAC-1.<sup>24</sup> In airway epithelial cells the rise in cAMP was found to be protective against oxidants<sup>25</sup> and to enhance the release of prostaglandin E2.<sup>13</sup> It is therefore conceivable that increases in the cellular concentration of cAMP not only mediate the relaxation of airway smooth muscle but also inhibit the activation of inflammatory cells.<sup>13, 26</sup>

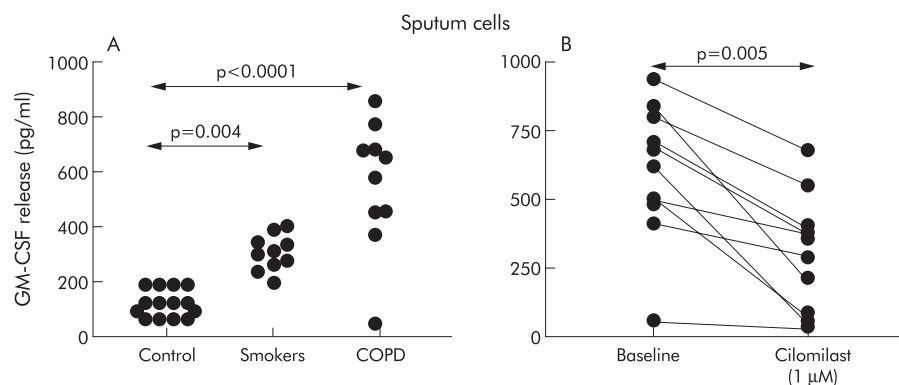
Inflammatory and immunomodulatory cells contain PDE4<sup>27</sup> and many functions of these cells are inhibited by selective PDE4 inhibitors.<sup>26, 28</sup> In addition, PDE4 seems to be the most important isoenzyme in both the microsomal and cytoplasmic compartments of airway cells.<sup>12, 13</sup>

Cilomilast is a second generation PDE4 inhibitor effective in the treatment of COPD. It has high selectivity for the cyclic AMP specific (or PDE4) isoenzyme. In vitro, cilomilast suppresses the activity of many proinflammatory and immune cells implicated in the pathogenesis of COPD and is highly active in animal models.<sup>16, 29</sup> In vitro studies have also suggested that cilomilast can inhibit antigen driven IL-5 production,<sup>28</sup> cytokine induced adhesion to endothelial cells,<sup>10</sup> and chemotaxis.<sup>29, 30</sup> However, most of these effects have been evaluated using cell lines or animal models and, to date, no studies have assessed the effects of cilomilast on airway cells isolated from patients with COPD.





**Figure 2** IL-8 release by (A) bronchial epithelial cells (BEC) and (C) sputum cells isolated from the airways of controls, smokers, and patients with COPD. The recovered bronchial epithelial cells and sputum cells were cultured for 24 hours at 37°C to test the release of IL-8 either spontaneously or after incubation with cilomilast 1 µM (B and D). Statistical analysis was performed by the Mann-Whitney U test for comparisons between groups and by the Wilcoxon U test for comparisons between baseline and cilomilast.

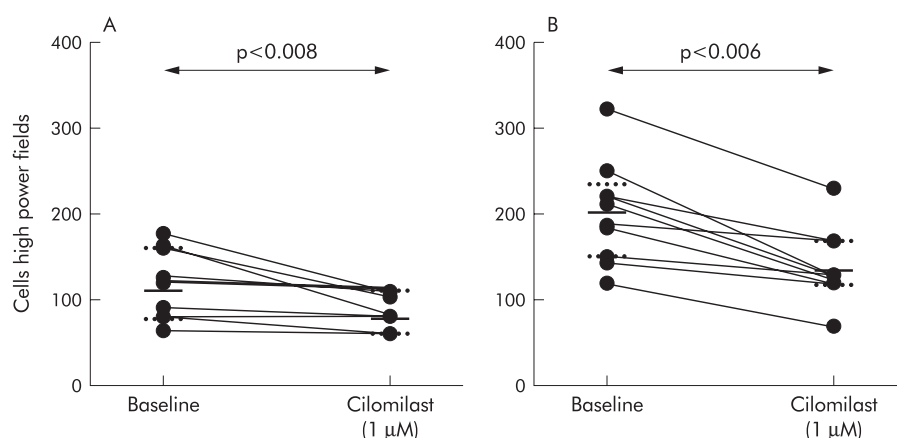


**Figure 3** GM-CSF release by sputum cells (A) isolated from the airways of controls, smokers, and patients with COPD. The recovered sputum cells were cultured for 24 hours at 37°C in the presence or absence of cilomilast 1 µM (B). Statistical analysis was performed by the Mann-Whitney U test for comparisons between groups and by the Wilcoxon U test for comparisons between baseline and cilomilast.

In this study sputum cells were cultured to assess their ability to release inflammatory mediators and to respond to a drug such as cilomilast. It should be noted that, in reality, drugs or stimuli usually target the various cell types present in the airways and rarely just a single cell population, so we used airway cells from COPD patients to confirm the ability of cilomilast in a concentration of 1 µM to influence the functional activation of airway cells. This concentration of cilomilast was chosen based on the results of the dose-response curve experiments as well as on previous evidence.<sup>31</sup> Cilomilast significantly inhibited the release of TNF-α and GM-CSF by both epithelial and sputum cells, while there was no inhibitory effect on IL-8 release.

It is unlikely that the heterogeneity of the cell population is the main cause of the different effects of cilomilast on mediator release and on the low or absent inhibitory effect on IL-8 as

similar results were obtained with cultured bronchial epithelial cells which were virtually pure. There are several possible explanations for the lack of inhibition by cilomilast on IL-8 release. It is possible that IL-8 release might have been inhibited by a higher concentration or a different incubation time. In addition, as IL-8 release by airway cells is regulated by complex intracellular pathways, it is possible that some of them may not be targeted by cilomilast. This hypothesis is supported by a previous study<sup>13</sup> which showed that enhanced cAMP levels in epithelial cells did not inhibit IL-8 release, suggesting that IL-8 release is not solely modulated by cAMP. Moreover, the extent of the inhibitory effect of cilomilast may differ according to the clinical severity of COPD—being greater in patients with severe COPD than in those with mild COPD in whom treatment with theophylline reduced sputum



**Figure 4** Neutrophil chemotactic activity of supernatants of (A) bronchial epithelial cells and (B) induced sputum cells stimulated in the presence or absence of cilomilast (1  $\mu$ M) for 24 hours at 37°C. Statistical analysis was performed by the Wilcoxon U test. The results are expressed as medians (horizontal bars) with 25th and 75th percentiles (dotted bars).

levels of IL-8 by 24% compared with baseline.<sup>32</sup> However, Cul-pitt *et al*<sup>32</sup> assessed the effects of theophylline on IL-8 levels in sputum supernatants obtained immediately after sputum processing while we evaluated the effects of cilomilast on IL-8 release from sputum cells cultured for 24 hours. Whether this effect reflects different modes of action of theophylline and cilomilast or whether it depends on the different profile of airway inflammation in moderate and severe COPD needs to be studied further.

The findings of this study enable us to define better the scientific rationale for the use of cilomilast in the treatment of COPD, and provide important information about the mechanisms by which it may affect the inflammatory process in the airways of these subjects. Interestingly, the inhibitory effect of cilomilast on TNF- $\alpha$  and GM-CSF release by airway cells was associated with a significant reduction in neutrophil chemotactic activity exerted by supernatants harvested from cultured bronchial epithelial cells and sputum cells incubated for 24 hours with the drug. It is also likely that cilomilast may exert a direct inhibitory effect on neutrophil chemotaxis, as has already been demonstrated on fibroblast migration.<sup>31</sup> It is of note that, although significant, the inhibitory effect on neutrophil chemotaxis was not complete. This might be attributed to the low inhibitory effect of cilomilast on IL-8 release by both bronchial epithelial and sputum cells, as well as to the presence of other chemotactic mediators for neutrophils such as LTB<sub>4</sub>.

Taken together, the results of the present study suggest that cilomilast has the potential to inhibit the development of neutrophilic inflammation in the airways of patients with COPD. Indeed, it is believed that neutrophil recruitment and activation is an important step in the pathogenesis of airway inflammation in this disease, as shown by the increased numbers of neutrophils in both central and peripheral airways<sup>33</sup> as well as by their distribution in the epithelium layer and within mucous glands. It is well known that neutrophil activation leads to the release of a wide variety of inflammatory mediators such as proteases and oxygen free radicals<sup>34</sup> that not only amplify the recruitment of inflammatory cells, but also cause damage to lung tissues.

It is therefore conceivable that drugs capable of inhibiting neutrophil recruitment and function may be a promising strategy in the treatment of COPD for several reasons. Firstly, so far very few drugs have been shown to inhibit neutrophil function as well as the release of mediators by airway cells isolated from patients with COPD.<sup>35</sup> Secondly, the effects of cilomilast on the release of inflammatory mediators further support the concept that this drug not only provides bronchodilatory effects but is also characterised by anti-

inflammatory properties which appear to be effective on neutrophils. Thirdly, the inhibitory effects of cilomilast on the release of neutrophil chemoattractants by bronchial and sputum cells isolated from patients with COPD suggest that these cells have a certain degree of responsiveness to this drug, a finding which has not always been observed with other drugs including corticosteroids.<sup>35</sup>

This study has indicated several anti-inflammatory effects of cilomilast on airway cells isolated from patients with COPD and supports its potential usefulness in the treatment of this disease.

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## LUNG ALERT .....

### Contamination of rhinovirus stocks with poliovirus type 1

▲ Davies M, Bruce C, Bewley K, *et al*. Poliovirus type 1 in working stocks of typed human rhinoviruses. *Lancet* 2003;**361**:1187–8

▲ Savolainen C, Hovi T. Caveat: poliovirus may be hiding under other labels. *Lancet* 2003;**361**:1145–6

One of the major successes of public health vaccination programmes has been the eradication of circulating poliovirus from the majority of countries in the world. Reintroduction of wild type poliovirus may occur following spread from sources of virus stored in virological and other laboratories for research purposes or from countries where polio remains endemic. It is therefore of obvious concern that the authors at the National Collection of Pathogenic Viruses (NCPV), whilst characterising stocks of rhinovirus from the MRC Common Cold Research Unit, found that five of 22 cell cultures had abnormal cytopathic effects when infected with putative rhinovirus. PCR and sequencing confirmed that four of these putative rhinovirus stocks (1A, 1B, 4 and 6) had similarities to polio type 1 sequences and were confirmed to contain poliovirus type 1 with likely contamination of two further serotypes. Genomic analysis of the poliovirus showed similarity to wild type virus, although the possibility that this was a vaccine strain that has undergone genetic drift remains. These findings should alert the medical and scientific communities to the risk of possible contamination of rhinovirus and, indeed, other virus stocks with poliovirus which has the ability to replicate in most cell lines and to overgrow more slowly replicating viruses. The authors advise that working stocks of virus should be checked regularly and eventually replaced with fresh virus from appropriate reference strains.

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