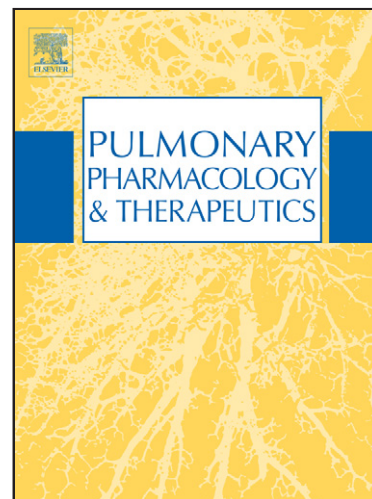


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Carl G. A. Persson, Olov Sterner

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## Discovery of a potent and long-acting bronchorelaxing capsazepinoid, RESPIR 4-95

Staffan Skogvall<sup>a</sup>, María F. Dalence-Guzmán<sup>b,c</sup>, Magnus Berglund<sup>b</sup>, Katrin Svensson<sup>a</sup>,

Admira Mesic<sup>a</sup>, Per Jönsson<sup>d</sup>, Carl G. A. Persson<sup>e,\*</sup> and Olov Sterner<sup>b</sup>

<sup>a</sup>Respiratorius AB, Magistratsvägen 10, 226 43 Lund, Sweden

<sup>b</sup>Department of Organic Chemistry, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden

<sup>c</sup>Centro de Tecnología Agroindustrial, Facultad de Ciencias y Tecnología, Universidad Mayor de San Simón, Cochabamba, Bolivia

<sup>d</sup>Department of General Thoracic Surgery, Lund University Hospital, SE-221 85 Lund, Sweden

<sup>e</sup>Department of Clinical and Experimental Pharmacology, Lund University Hospital, University of Lund, SE-221 00 Lund, Sweden

\* Corresponding author: Tel. +46 702239446; e-mail: carl.persson@med.lu.se

### Abstract

**Background:** Current drugs including beta-agonists have limited smooth muscle relaxant effects on human small airways. Yet this is a major site of obstruction in asthma and COPD.

**Objective:** This study explores human small airway relaxant effects of RESPIR 4-95, a novel chemical analogue (capsazepinoid) to capsazepine. Capsazepine was recently shown to relax small airways in a way which was independent of its TRPV<sub>1</sub> antagonism and independent of current bronchodilator drug mechanisms.

**Method:** In vitro preparations of human small airways, 0.5-1.5 mm in diameter and responding with reproducible contractions to LTD<sub>4</sub> for 12 h, were used.

**Results:** RESPIR 4-95 reversibly prevented LTD<sub>4</sub>-induced contractions as well as relaxed the established tonic contraction by LTD<sub>4</sub>. RESPIR 4-95 exhibited marked improvements over the reference capsazepinoid, capsazepine, by being 10 times more potent, exhibiting twice as long duration

of action after wash-out (9 h), and inhibiting equally well LTD<sub>4</sub>-, histamine-, PGD<sub>2</sub>-, and acetylcholine-induced contractions. RESPIR 4-95 was distinguished from L-type calcium channel antagonist nifedipine by its greater efficacy and potency and by exhibiting increased relaxant effect by repeated exposures. Furthermore, RESPIR 4-95 was more efficacious and longer acting than the long-acting beta agonist formoterol.

Conclusion: Efficacy, potency, duration of action, and inexhaustibility of its relaxation of human small airways make RESPIR 4-95 an interesting lead compound for further developments aiming at drug treatment of small airways obstruction in asthma and COPD. Further work is warranted to unveil the molecular biology behind its relaxant actions.

**Keywords:** Capsazepinoids, RESPIR 4-95, bronchorelaxant, COPD, asthma, human small airways

## 1. Introduction

We recently showed that the TRPV<sub>1</sub> antagonist capsazepine and several chemically related capsazepine analogues (capsazepinoids) relaxed well human small airway preparations [1]. The compounds inhibited or attenuated the bronchoconstriction evoked by contractile mediators including leukotriene D<sub>4</sub> (LTD<sub>4</sub>), and also relaxed the tonic phase of already contracted preparations. These features were surprising because previous work involving animal experiments had not identified any general bronchorelaxing properties with capsazepine [2, 3]. Although the mechanism of action behind the relaxation could not be determined, the data suggested that the capsazepinoids represented a novel class of highly efficacious human small airways relaxants [1].

Human small airways are defined as those airways of less than 2 mm internal diameter. Reflecting the branching pattern of human bronchi the small airways are numerous. Consequently the small airways collectively contribute much less than the large airways to the total resistance to air flow in the tracheobronchial tree [4]. As a “silent zone” obstructive disease processes in the small airways can develop into advanced stages before symptoms become evident and before common measurements of

airway resistance show loss of function [4]. This aspect together with observations of small airway pathology [4, 5, 6] have led to a strong focus on the small airways as a major site of inflammation and remodelling in asthma and chronic obstructive pulmonary disease (COPD). However, the human small airways are also the most muscular airways [4] and as isolated preparations they are at least as sensitive to contractile mediators as the large airways [7, 8]. Mechiche et al [8] working with human isolated airways actually demonstrated that the cysteinyl leukotrienes are 30 times more potent in contracting small airways as compared to large airways. Furthermore, it appears that the currently most effective bronchodilators, the beta agonists, are weak relaxants of human small airways in vitro [1, 9] and in vivo [10]. Inferentially, there is an important and also unmet medical need for drugs that relax human small airways.

We report here that our continued exploratory structure-activity studies involving a sensitive and robust preparation of human excised small airways have identified a capsazepinoid, RESPIR 4-95 (figure 1), with potentially important properties. Its superiority over the reference compound, capsazepine, concerns central features such as potency, duration of action and efficacy against different contractile mediators. The potential value of RESPIR 4-95 is further evident by the present comparison with the properties of a major long-acting beta agonist in human small airway preparations.

Figure 1.

## 2. Materials and Methods

### 2.1. *Preparation and experimental chamber*

In accordance with procedures approved by Lund ethical committee, human lung tissue was obtained from patients undergoing lobectomy due to lung carcinoma and treated as described previously [1]. In short, lung tissue was placed in a dissection bowl, an airway was identified and a bronchial preparation from a bronchus with a diameter of 0.5-1.5 mm was obtained. This was mounted in the experimental chamber for force measurements. The experimental chamber was kept at 37°C and was continuously perfused with physiological saline solution. The force development of the preparation was registered on a computer. After mounting in the chamber and a period of adjustment, the preparation was contracted with LTD<sub>4</sub> and stretched repeatedly until it exerted a force of 1.2 mN. This was, unless otherwise noted,

followed by 2 cycles with control contractions consisting of 60 min relaxation with LTD<sub>4</sub>-free PSS and 30 min contraction with LTD<sub>4</sub>. If these two consecutive LTD<sub>4</sub> contractions differed less than 15%, the preparations were considered to be stable and the experiments begun. At the end of all experiments, the preparations were exposed to a Ca<sup>2+</sup>-free solution, to establish the baseline tension level.

## 2.2. *Experimental protocols*

### 2.2.1. *Dose response relationships*

Dose response relationship for RESPIR 4-95 was determined by having different concentrations of this drug (0.1  $\mu$ M, 1  $\mu$ M, 10  $\mu$ M and 100  $\mu$ M) present during the whole cycle (1 h of LTD<sub>4</sub>-free PSS followed by 30 minutes of 10 nM LTD<sub>4</sub>). The dose-response curve was obtained in a non-cumulative way and each preparation was used for determination of the inhibitory effect of a single RESPIR 4-95 concentration.

In some experiments, the preparations were contracted with histamine (10  $\mu$ M), prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) (10  $\mu$ M) or acetylcholine (ACh) (100  $\mu$ M) producing contractions of a similar magnitude to 10 nM LTD<sub>4</sub>. When examining the effects of high external KCl, 60 mM was used. Inhibitory effects of a single concentration of RESPIR 4-95 (10  $\mu$ M) were then evaluated as described above.

### 2.2.2. *Onset of relaxation*

The onset of RESPIR 4-95-induced relaxations was examined in preparations contracted by LTD<sub>4</sub> (10 nM). When the contraction had levelled off at a plateau, RESPIR 4-95 (10  $\mu$ M) was added in the continuous presence of LTD<sub>4</sub>. After 1 h exposure to RESPIR 4-95 preparations were exposed to Ca<sup>2+</sup>-free solution to find the baseline level.

### 2.2.3. *Duration and reversibility of the relaxations*

After one control contraction, the preparations were exposed to RESPIR 4-95 (10  $\mu$ M) during the next cycle with 60 min LTD<sub>4</sub>-free PSS followed by 30 min LTD<sub>4</sub> (10 nM). Thereafter, preparations were exposed to 8 RESPIR 4-95-free cycles with LTD<sub>4</sub>-free PSS followed by LTD<sub>4</sub>. Capsazepine was tested in a similar way, except that the capsazepine treatment was followed by only 4 control cycles.

#### 2.2.4. Tests of tachyphylaxis

After 1 control cycle the preparations were exposed to RESPIR 4-95 (10  $\mu$ M) for 15 min of the LTD<sub>4</sub> free period. This was followed by 45 min of PSS without RESPIR 4-95 and 30 min exposure to LTD<sub>4</sub>. Thereafter, 3 more RESPIR 4-95-free cycles were performed, followed by 15 min exposure to RESPIR 4-95 followed by 45 min of PSS without RESPIR 4-95 and 30 min exposure to LTD<sub>4</sub>.

#### 2.2.5. Comparison with $\beta_2$ -agonists

Dose response curves for  $\beta_2$ -agonists were performed in a cumulative way. After stretch and 1 h PSS, LTD<sub>4</sub> (10 nM) was added. When the contraction had remained stable at a plateau for 1 h,  $\beta_2$ -agonists in gradually higher concentrations were added. Terbutaline was tested in the concentration 10 nM – 1 mM, Salbutamol 1 nM -1 mM and formoterol 0.1 nM – 10  $\mu$ M. The drug was administered until the relaxation had levelled off, or in 15 min in case of no effect. Dose response curve for RESPIR 4-95 was obtained in a non-cumulative way, as described above.

#### 2.2.6. Tests with atropine and propranolol

After stretch and 1 h PSS, preparations were exposed to LTD<sub>4</sub> (10 nM). When the contraction had been stable for 1h, atropine (1  $\mu$ M) and propranolol (10  $\mu$ M) were added for 30 min. This was followed by addition of RESPIR 4-95 (10  $\mu$ M) in the continuous presence of LTD<sub>4</sub>, atropine and propranolol. When the RESPIR 4-95-induced relaxation had levelled off, the experiment was concluded by addition of Ca<sup>2+</sup>-free solution.

#### 2.2.7. Comparison between relaxations by RESPIR 4-95 and formoterol

Determination of the relaxing effect by RESPIR 4-95 was performed as described in “Duration and reversibility of the relaxations” above, except that only 6 control cycles after the cycle with RESPIR 4-95 were performed. Determination of the relaxing effect by formoterol was performed as for RESPIR 4-95 except that 10  $\mu$ M formoterol was used instead.

#### 2.2.8. Test of any signs of exhaustion of the inhibitory effect of RESPIR 4-95 - Comparison with voltage operated calcium antagonists

After one control contraction, preparations were exposed to RESPIR 4-95 (0.1, 1 or 10  $\mu\text{M}$ ) or nifedipine (0.1, 1 or 10  $\mu\text{M}$ ) continuously during the following 4 cycles.

### 2.2.9. Test of involvement of nitric oxide (NO) in the relaxation by RESPIR 4-95

The preparations were exposed to the nitric oxide synthase inhibitor L-NAME (0.1 mM) during one test cycle. During the following cycle, both L-NAME and RESPIR 4-95 (10  $\mu\text{M}$ ) were added.

## 2.3. Solutions and chemicals

### 2.3.1. PSS

PSS contained (in mM): 117 NaCl, 4.88 KCl, 0.60  $\text{MgSO}_4$ , 25.0  $\text{NaHCO}_3$ , 5.23 glucose, and 1.60  $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ . The PSS during the experiments was bubbled with 94%  $\text{O}_2$  and 6%  $\text{CO}_2$ , giving a pH of 7.40. All chemicals were purchased from Sigma Aldrich.

### 2.3.2. $\text{Ca}^{2+}$ -free solution

In the end of all experiments, the preparations were exposed to  $\text{Ca}^{2+}$ -free PSS in order to find the level of passive tension.  $\text{Ca}^{2+}$ -free solution contains all of the chemicals in PSS except for  $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ . Also, 2 mM EGTA was added to bind any remaining  $\text{Ca}^{2+}$ .

### 2.3.3. Chemicals

All substances are prepared as stock solutions dissolved in the vehicles water, ethanol or DMSO. Leukotriene  $\text{D}_4$  ( $\text{LTD}_4$ ; Cayman Chemical): 0.1 mM in ethanol, Capsazepine (Tocris Bioscience): 0.1 M in ethanol, Histamine (Sigma Aldrich) 0.1 M in water,  $\text{PGD}_2$  (Sigma Aldrich) 0.1 M in ethanol, ACh (Sigma Aldrich) 1 M in water, Atropine (Sigma Aldrich) 10 mM in water, Propranolol (Sigma Aldrich) 0.1 M in water, Nifedipine (Tocris Bioscience) 0.1 M in DMSO,  $\text{N}^G$ -nitro-L-arginine methyl ester (L-NAME) (Tocris Bioscience): 0.1 M in water, RESPIR 4-95 synthesized as below, 0.1 M in ethanol.

Vehicle test: During experiments with substances that were dissolved in ethanol or DMSO, vehicle was added when the preparations were not exposed to the test substance so that the vehicle is continuously present in the bath, in order to exclude any influence by the vehicle.

#### 2.3.4. Synthesis of RESPIR 4-95

Commercially available 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride, (1.0 eq.) was suspended in glacial acetic acid, and  $\text{SO}_2\text{Cl}_2$  (2.2 eq.) was added dropwise. After stirring for 2.5 hours, the mixture was concentrated under vacuum affording 5,8-dichloro-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (79%). This compound was dissolved in HBr (48% in  $\text{H}_2\text{O}$ ). The mixture was heated to  $105^\circ\text{C}$  for 5 hours and then concentrated, affording 5,8-dichloro-1,2,3,4-tetrahydroisoquinoline-6,7-diol hydrobromide in quantitative yield as a gray solid. This salt was dissolved in anhydrous dimethylformamide and triethylamine (3.0 eq.) was added. This mixture was stirred for 15 minutes and then 2-(4-chlorophenyl)ethyl isothiocyanate (1.2 eq.) was added. This mixture was stirred for additional 4 hours and then concentrated. The residue was dissolved in ethyl acetate and washed with water. The organic phase was dried ( $\text{MgSO}_4$ ) and concentrated. Purification was done by flash column chromatography (silica, petroleum ether / ethyl acetate 60:40 +1% acetic acid) affording RESPIR 4-95 (51%) as a pale yellow solid. mp:  $83-86^\circ\text{C}$   $^1\text{H}$ -NMR  $\delta$  2.77 (t,  $J=5.8$  Hz, 2H), 2.93 (t,  $J=7.4$  Hz, 2H), 3.82 (t,  $J=7.4$  Hz, 2H), 3.95 (t,  $J=5.8$  Hz, 2H), 4.85 (s, 2H), 7.20 (m, 4H).  $^{13}\text{C}$ -NMR  $\delta$  27.1, 35.5, 45.8, 47.9, 49.3, 118.4, 120.2, 124.2, 125.8, 129.4, 129.4, 131.5, 131.5, 133.0, 139.5, 142.6, 142.9, 182.5. HRESI-MS calculated for  $\text{C}_{18}\text{H}_{18}\text{Cl}_3\text{N}_2\text{OS}$  (M+H) 431.0154, found 431.0162.

HRESI-MS spectrum was recorded with a Micromass Q-TOF Micro spectrometer. NMR spectra (in  $\text{CD}_3\text{OD}$ ) were recorded with a Bruker DRX 400 spectrometer at 400 MHz ( $^1\text{H}$ ) and at 100 MHz ( $^{13}\text{C}$ ). Chemical shifts are given in ppm relative to TMS using the residual  $\text{CD}_2\text{HOD}$  peak in  $\text{CD}_3\text{OD}$  solution as internal standard (3.32 and 49.0 ppm, respectively relative to TMS).

#### 2.4. Statistics

All test values are given as mean value  $\pm$  standard error of the mean. Tests of statistical significance were performed using the Anova test. When evaluating the statistical significance of relaxations by capsazepinoids, the test contraction was compared to the third contraction in control experiments (fig. 5a). Differences in efficacy of relaxant responses were made between levels of maximum relaxant effects of individual drugs.



### 3. Results

#### *3.1 Comparison of the bronchorelaxing properties of RESPIR 4-95 and capsazepine*

##### *3.1.1 Dose response relationships*

Human small airway preparations exposed to RESPIR 4-95 showed dose-dependent inhibitions of LTD<sub>4</sub>-contractions (Fig 2). The EC<sub>50</sub> for RESPIR 4-95 was ~2  $\mu$ M while, as previously shown [1], the EC<sub>50</sub> for capsazepine is about 15  $\mu$ M, demonstrating that the relaxing potency for RESPIR 4-95 was almost 10 times higher than for capsazepine.

Fig 2.

##### *3.1.2 Development of relaxation*

As demonstrated in preparations continuously exposed to LTD<sub>4</sub>, producing a sustained tonic contraction, the relaxation by either RESPIR 4-95 or capsazepine developed slowly. In agreement with previous observations with capsazepine [1], it took about one hour of exposure to RESPIR 4-95 (10  $\mu$ M) to get ~80% relaxation (Fig 3).

Fig 3.

##### *3.1.3 Effects on contractions evoked by different contractile agonists*

The inhibitory effect of RESPIR 4-95 (10  $\mu$ M) was of a similar magnitude whether the bronchi were contracted with LTD<sub>4</sub> (10 nM), histamine (10  $\mu$ M), PGD<sub>2</sub> (10  $\mu$ M), ACh (100  $\mu$ M), or high extra cellular KCl (60 mM) (Fig 4) indicating that RESPIR 4-95 is fully effective as a functional antagonist to contractile mediators in human small airways. This emerges as an improvement over capsazepine that was relatively less effective as relaxant of ACh-induced contractions [1].

Fig 4.

### 3.1.4 Duration of the inhibitory effect

The inhibition of LTD<sub>4</sub>-induced contractions by RESPIR 4-95 was very long lasting (Fig 5a). After exposure to RESPIR 4-95 (10  $\mu$ M) for merely one contractile cycle also the following six cycles with LTD<sub>4</sub>-contractions (obtained during further 9 h) were significantly inhibited (Fig 5a). Interestingly, the relaxant effect continued to increase for about an hour after the drug had been removed from the bathing solution (Fig 5a). The duration of action of RESPIR 4-95 (10  $\mu$ M) was markedly longer than that exhibited by capsazepine also when the latter compound was given in the high concentration of 100  $\mu$ M (Fig 5b and 5c). Yet the RESPIR 4-95-induced relaxation exhibited complete reversibility (Fig 5a).

Fig 5.

## 3.2 Comparison with $\beta_2$ -agonists

### 3.2.1 Potency and efficacy

Dose-response curves for terbutaline and salbutamol revealed that these classical  $\beta_2$ -agonists had relatively poor relaxing effect in human small airways (Fig 6). Terbutaline had an EC<sub>50</sub> ~5  $\mu$ M and a maximum relaxation of ~50% at 1 mM. Salbutamol similarly had an EC<sub>50</sub> ~1  $\mu$ M and a maximum relaxation of ~40% at 1 mM. Formoterol was more potent, with EC<sub>50</sub> ~40 nM, but still produced a maximum relaxation of only ~60% at 10  $\mu$ M. RESPIR 4-95 had an EC<sub>50</sub> ~2  $\mu$ M but produced ~90% relaxation already at a concentration of 10  $\mu$ M. Thus, the relaxing potency of RESPIR 4-95 is comparable to that of classical bronchorelaxants for inhalation treatment, but the efficacy of RESPIR 4-95 is superior (cf terbutalin:  $p < 0.01$ , salbutamol:  $p < 0.001$ ). Formoterol has a higher potency but a lower efficacy ( $p < 0.05$ ) than RESPIR 4-95 (Fig 6; see also Figures 7 and 8).

Fig 6.

### 3.2.2 Importance of muscarinic and adrenergic receptors for RESPIR 4-95 relaxation

To examine if the relaxing effect by RESPIR 4-95 to any degree is caused by stimulation of  $\beta_2$ -adrenoceptors or by blockade of muscarinic receptors, LTD<sub>4</sub>-contracted preparations were exposed to RESPIR 4-95 (10  $\mu$ M) in the presence of atropine (1  $\mu$ M) and propranolol (10  $\mu$ M). Neither blocking

agent affected the leukotriene-induced contractions (Fig 7). RESPIR 4-95 caused also in this situation a full relaxation which levelled off at  $\sim 94 \pm 2\%$  relaxation ( $n=8$ ). Also, preparations with relatively weak formoterol-induced relaxations displayed a full relaxation at exposure to RESPIR 4-95 (Fig 7).

Fig 7.

### 3.2.3 Further comparison with formoterol

Formoterol given at the same concentration as RESPIR 4-95 ( $10 \mu\text{M}$ ) produced less and shorter-lasting (3 cycles, 4.5 h) inhibition of the  $\text{LTD}_4$ -induced bronchoconstriction (Fig 8). Both formoterol- and RESPIR 4-95-induced relaxations were reversible (Fig 5a and 8).

Fig 8.

### 3.2.4. Test of involvement of nitric oxide (NO) in the relaxation by RESPIR 4-95

In separate experiments effects of presence and absence of the NO synthesis inhibitor L-NAME on relaxant responses were examined. RESPIR 4-95  $10 \mu\text{M}$  alone gave a remaining contraction of  $13.94 \pm 2.68\%$  ( $n=16$ ), while RESPIR 4-95  $10 \mu\text{M}$  given to preparations pretreated with L-NAME  $0.1 \text{ mM}$  exhibited a remaining contraction of  $25.00 \pm 13.01\%$  ( $n=3$ ). The results did not differ significantly ( $p>0.1$ ).

### 3.3 Increased effect with time of RESPIR 4-95 – comparison with nifedipine

In order to examine if RESPIR 4-95 displays tachyphylaxis, the preparations were first exposed to  $10 \mu\text{M}$  RESPIR 4-95 for 15 minutes, which resulted in a relaxation by  $\sim 60\%$  (Fig 9). After this short time exposure, the RESPIR 4-95-induced inhibition of  $\text{LTD}_4$  contractions largely disappeared after 3 control cycles. Then a renewed 15 min exposure to RESPIR 4-95 produced a relaxation which even appeared greater than the initial RESPIR 4-95-induced relaxation ( $n=5$ ). Thus, the inhibitory action of RESPIR 4-95 did not exhibit tachyphylaxis, rather the opposite. This finding agrees with the increasing relaxant effect that follows during the first hours after a single  $1.5 \mu\text{M}$  exposure of the small airways to RESPIR 4-95 (Fig 5a). The observation of a greater efficacy with time was further explored by continuous presence of

the drug in experiments comparing RESPIR 4-95 and the voltage operated calcium channel antagonist nifedipine.

Fig 9.

It is well-established that calcium is necessary for contraction in smooth muscle fibers and that voltage operated L-type calcium channel antagonists, such as verapamil or nifedipine, can relax many types of smooth muscle effectively [11]. However, it has also been shown that airway smooth muscle is unexpectedly insensitive to the relaxing effect of this type of substances [12, 13], which has led to the conclusion that airway smooth muscle has other types of calcium channels that are mainly responsible for calcium entry [14]. Here the relaxing effect of RESPIR 4-95, particularly its efficacy by prolonged presence in the organ bath, was compared with the effect of a major voltage operated L-type calcium channel antagonist, nifedipine [15]. Thus, preparations were exposed to 4 contractile LTD<sub>4</sub>-cycles with different concentrations of test substance being present continuously for the entire period (6 h). The relaxation by the continuously present RESPIR 4-95 was increasing with time and eventually amounted to 30% (0.1  $\mu$ M), 70% (1  $\mu$ M), and 95% (10  $\mu$ M) (Fig 10). Nifedipine did not exhibit such increased relaxations but ended with a ~50% relaxation to 10  $\mu$ M of nifedipine. The tendency at a gradually increased relaxation was also found with several other capsazepinoids (unpublished results), suggesting that this is a class effect.

Fig 10.

#### 4. Discussion

This study demonstrates that small chemical modifications of the reference compound capsazepine can significantly improve its relaxant features. Thus, we show here that RESPIR 4-95 exhibits promising properties including a very long, yet reversible, duration of relaxation in human small airway preparations in spite of continuous renewal of the bathing fluid. RESPIR 4-95 is more potent than capsazepine, even matching the potency of classical inhaled  $\beta$ 2 agonists. RESPIR 4-95 also prevents equally well the contractile effect of several mediators implicated in the pathophysiology of asthma and COPD including acetylcholine, leukotriene D<sub>4</sub>, and prostaglandin D<sub>2</sub>. Perhaps most importantly

RESPIR 4-95, which is not active on beta receptors, exhibits a greater efficacy and a longer duration of action than the potent and long-acting beta agonist formoterol. These data are of interest in view of the role of small airways obstruction in the pathogenesis of asthma and COPD and the need for improved treatment of these chronic obstructive pulmonary diseases.

#### Human small airways methodology

Different factors have been considered in development of the present human small airway set-up. Thus, our experimental chambers allowed a constant physiological milieu and the continuous perfusion of the chambers that was employed here removed any force artefacts otherwise occurring when the solution is changed in the bath. These considerations likely contributed to making the present preparations stable and responsive to drugs for more than 12 hours. During the present examinations we have used LTD<sub>4</sub> as the standard mode of contraction. Also, when other inflammatory mediators (histamine, PGD<sub>2</sub>) or acetylcholine were used to contract the bronchi reproducible results, similar to the repeatability of LTD<sub>4</sub>-induced contractions, were obtained.

#### Comparison with other bronchodilators

The relaxing effect of  $\beta$ 2-agonists in the present small bronchial preparation was limited. Quite high concentrations (1 mM of terbutaline and salbutamol and almost 1  $\mu$ M of formoterol) were needed to obtain a 50% relaxation of a control contraction and it was impossible to get a full relaxation using these beta agonists. In contrast, our preparations relaxed fully in response to RESPIR 4-95 or by removing calcium from the PSS. Thus, the weak bronchorelaxing effect by  $\beta$ 2-agonists reflects a property of these drugs and not inability of the small airways to relax. Interestingly, even direct application of a potent beta agonist (isoprenaline) through a wedged bronchoscope could not reduce histamine-induced contraction of the small airways *in vivo* in asthmatic individuals [10].

In clinical studies formoterol has been demonstrated to be long-acting and suitable for twice daily treatment in asthma [16]. In guinea-pig isolated tracheal preparations formoterol has also been demonstrated to have a longer duration of action than salbutamol [16]. The efficacy and the duration of action of RESPIR 4-95 suggest the possibility that this compound may have advantages over formoterol in maintenance treatment of obstructed small airways. Indeed, while the efficacy of RESPIR 4-95 was consistently high the maximum effect of formoterol was both less and varied between preparations (see

Figure 8), suggesting the possibility that there are subgroups of patients where RESPIR 4-95 could exhibit particularly great advantages over beta agonists. The long duration of RESPIR 4-95, extending beyond 9 h, is remarkable in view of the fact that the present organ baths were continuously perfused with drug-free solution at a high rate allowing quick clearance (95% change of PSS within 10 min) of RESPIR 4-95 unless it was firmly bound to its active site. Given under equal conditions and at the same concentration as RESPIR 4-95 formoterol exhibited an efficacy and a duration of action which were both about half those of RESPIR 4-95. However, a slow onset of action would make RESPIR 4-95, and similarly acting capsazepinoids, less suited for rescue bronchorelaxing treatment in acute episodes of obstructive pulmonary disease. The potency of RESPIR 4-95 appears sufficient for a bronchorelaxant to be employed as an inhaled drug for local treatment.

### Mechanism of action

The mechanism responsible for the powerful relaxing effect by RESPIR 4-95 is still unclear. However, several mechanisms can be excluded. First, our experiments show that propranolol and atropine are unable to reduce the relaxing effect of RESPIR 4-95; further, preparations with poor relaxation to  $\beta_2$ -agonists, or to calcium antagonists such as nifedipine, relax fully by RESPIR 4-95. This demonstrates that  $\beta_2$ -receptor activation and anti-cholinergic effects are not involved and that RESPIR 4-95 also differ from nifedipine-like compounds [1]. Second, exposure to the nitric oxide synthase inhibitor L-NAME (0.1 mM) does not reduce capsazepinoid-induced relaxation, likely excluding that NO is responsible for the relaxation. Third, RESPIR 4-95 is not a simple pharmacological antagonist (such as anti-cholinergic, anti-histamine or leukotriene receptor antagonist) because it causes a general bronchorelaxation independent of contracting agents. Fourth, we can exclude that RESPIR 4-95 acts by blocking the TRPV<sub>1</sub> receptor, because several well-established TRPV<sub>1</sub>-antagonists, given in high concentrations, lacked relaxing effect in the present set-up of human small airways [1]. However, it cannot be excluded that blocking of another TRP receptor is involved. Several types of TRP channels are known and TRPC channels are believed to be important for regulating calcium influx in airway smooth muscle [14]. In agreement with the property of RESPIR 4-95, inhibition of calcium influx would cause a general relaxing effect for all contracting agents. Also, TRPC<sub>1</sub>, TRPC<sub>4</sub> and TRPC<sub>6</sub> have been shown to be expressed at mRNA level in human airway smooth muscle [17]. Interestingly, preliminary data (unpublished observations) showed that high concentrations of the putative TRPC channel blocker 2-APB (2-aminoethoxydiphenyl borate) [18], relaxed the present preparations and that pre-treatment with 2-APB reduced the relaxing effect by a subsequent exposure to RESPIR 4-95. Hence, a shared mechanism by these two compounds is possible. However, further studies are

warranted to assess the mechanism of action of capsazepinoid-induced relaxation of human small airway smooth muscle.

#### Effects on small airways warranted in treatments of COPD and asthma

Little is currently discussed about any need for relaxation of human small airways. Rather it may be believed that the small airways obstruction is irreversible and, hence, that treatment must exclusively be directed towards reducing inflammation and remodelling. Apparently supporting this latter view *in vivo* data obtained in asthmatics suggest that current bronchodilators may not be very effective on small airways [10]. However, the present data as well as previous observations in human small airway preparations [1, 9], demonstrating weak relaxant efficacy of beta agonists, suggest the alternative possibility that the current bronchodilators cannot entirely show the therapeutic potential of peripheral airways relaxation. The properties of RESPIR 4-95 exhibiting complete and reversible relaxation of human small airways may thus represent a novel possibility of fulfilling a medical need for effective small airways relaxation.

## **5. Conclusion**

The present data support the notion that capsazepinoids potentially may become a useful addition to current bronchorelaxing drugs. Specifically, the efficacy, the duration of action, and the inexhaustibility of the bronchorelaxing effect of RESPIR 4-95 make it an interesting lead compound for further developments aiming at treatment of human small airway obstruction in COPD and asthma.

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Fig 1. Chemical structure of capsazepine and RESPIR 4-95 (1,2,3,4-tetrahydroisoquinoline). RESPIR 4-95 shares the major structural features with capsazepine, but has a saturated fused six-membered ring instead of the seven-membered ring of capsazepine, and two chlorines in the A-ring.

Fig 2. Dose response relationships for RESPIR 4-95 and capsazepine. RESPIR 4-95 has almost 10 times higher relaxing potency than capsazepine.

Fig 3. An original recording of a human small bronchus exposed to RESPIR 4-95 (10  $\mu$ M) for 1 h in the continuous presence of LTD<sub>4</sub>.

Fig 4. Inhibitory effect of RESPIR 4-95 (10  $\mu$ M) on different contractile agonists. RESPIR 4-95 inhibited equally well the effects of all contractile substances.

Fig 5. Inhibition of sequential LTD<sub>4</sub>-induced contractions by (a) RESPIR 4-95 (10  $\mu$ M), (b) capsazepine (10  $\mu$ M) and (c) capsazepine (100  $\mu$ M). The RESPIR 4-95-induced relaxation is highly significant for 6 additional cycles (9 h) following wash-out of the drug, while the relaxation after the 100  $\mu$ M capsazepine exposure is only significant for up to 3 cycles (\*\*( $p < 0.01$ );\*\*\*( $p < 0.001$ )).

Fig 6. Dose response relationships for RESPIR 4-95 and  $\beta_2$ -agonists. RESPIR 4-95 has a greater relaxing efficacy than the  $\beta_2$ -agonists.

Fig 7. Bronchorelaxing effect by RESPIR 4-95 in a preparation pre-treated with atropine and propranolol. An initial cumulative dose-response curve for formoterol revealed that it produced only weak relaxations in this human small bronchus even at the very high concentration of 10  $\mu$ M. After addition of atropine (1  $\mu$ M) and propranolol (10  $\mu$ M) the preparation was once again exposed to 10  $\mu$ M formoterol which then did not cause any relaxation, confirming that the  $\beta_2$ -adreno receptors were blocked. A subsequent exposure to RESPIR 4-95 (10  $\mu$ M) caused a full relaxation, demonstrating that RESPIR 4-95-induced relaxations are not mediated by  $\beta_2$ -adreno receptors.

Fig 8. Bronchorelaxing effect by RESPIR 4-95 and formoterol. One cycle of formoterol exposure gave a significant relaxation compared to the control contraction lasting for 3 cycles (4.5 h) (+( $p<0.05$ ); ++( $p<0.01$ ); +++( $p<0.001$ ) represents significance for formoterol relaxation versus control contraction). Exposure for one cycle (1.5 h) of RESPIR 4-95 or formoterol (both 10  $\mu$ M) was followed by 6 additional cycles without test substance. RESPIR 4-95 gave much stronger relaxations than formoterol during the whole experiment (7 cycles, 10.5 h), and this difference was highly significant (\*\*( $p<0.01$ );\*\*\*( $p<0.001$ )).

Fig 9. Inhibition of LTD<sub>4</sub>-contractions by two subsequent exposures to RESPIR 4-95 (10  $\mu$ M) for 15 min. The second RESPIR 4-95-induced relaxation was not significantly different to the first ( $p=0.17$ ,  $n=5$ ).

Fig 10. Bronchorelaxing effect by RESPIR 4-95 and nifedipine given continuously for 4 cycles (6 h). The relaxation by RES 4-95 increases over time, which is not seen during nifedipine exposure.

