DMD Fast Forward. Published on July 12, 2007 as doi:10.1124/dmd.107.015297




**Title: Differences in endogenous esterification and retention in the rat trachea between budesonide and ciclesonide active metabolite.**

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**Running title:** Esterification of budesonide versus ciclesonide in airways

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**Number of text pages:**

Number of tables: 1

Number of figures: 7 (incl. 2 multiple figures)

Number of words in the Abstract: 250

Number of words in the Introduction: 713

Number of words in the Discussion: 1830

Number of References: 47

**List of non-standard abbreviations used in the paper:**

GC, glucocorticosteroid

BUD, budesonide

CIC, ciclesonide

CIC-AM, ciclesonide-active metabolite

FP, fluticasone propionate

MF, mometasone furoate

BDP, beclomethasone dipropionate

BMP, beclomethasone monopropionate

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## ABSTRACT

The airway retention of inhaled glucocorticosteroids (GCs) depends largely on their lipophilicity. Inhaled budesonide (BUD) becomes highly lipophilic reversibly by the formation of esters, acting as a reservoir of active BUD. Ciclesonide (CIC) was also reported to form esters after hydrolysis to active metabolite (CIC-AM). We have investigated lipophilicity and airway retention of BUD, CIC/CIC-AM, fluticasone propionate (FP) and mometasone furoate (MF), and compared esterification of BUD and CIC-AM and its contribution to GC airway retention. Rat tracheas were preincubated with the esterification inhibitor cyclandelate or vehicle. A $^{3}$H-GC (~$10^{-7}$M: BUD, CIC, CIC-AM, FP, MF) was added for 20 min. After incubation, one half of trachea was used for analysis of GC uptake and the other to analyse GC release during 3 hours in drug–free medium. GC species in trachea halves were analysed by radiochromatography. At 20 min the uptake of BUD was similar to CIC/CIC-AM, however BUD-ester pool was 9-fold greater ($p<0.01$). BUD overall retention in trachea at 3 hours was greater than other GCs ($p<0.01$), and BUD-ester pool was 3-fold greater than CIC-AM-ester pool ($p<0.01$). Cyclandelate decreased the initial BUD- and CIC-AM-ester pools ($p<0.01$), and reduced the overall retention of BUD at 3 hours ($p<0.01$) but not of CIC-AM. Thus, BUD becomes esterified in the airways more promptly and to a greater extent than CIC-AM, and BUD esterification prolongs BUD airway retention. In contrast, airway retention of CIC-AM and CIC seems to be determined mainly by their lipophilicity, similarly to FP and MF that are not esterified.

**Introduction**

Lipophilicity is an important physicochemical property of inhaled glucocorticosteroids (GCs) affecting their tissue kinetics and activity. High lipophilicity increases both the GC uptake and retention in airway tissue (i.e. decelerates removal of GCs from airway tissue), and thereby enhances and prolongs anti-inflammatory efficacy of inhaled GCs in the airways (Brattsand,1997). On the other hand, high lipophilicity can be a disadvantage in the peripheral tissues leading to enhanced distribution and retention, prolonged terminal plasma half-life, and accumulation (Thorsson et al., 1997, and 2001; Lipworth and Jackson, 2000). Furthermore, highly lipophilic inhaled GCs have a low water solubility which may increase the GC fraction that is cleared by mucocilliary escalator before it is dissolved in airway fluid and absorbed into airway tissue and becomes available for cytosolic GC receptor (Edsbäcker, 2002). Therefore, the “ideal” inhaled GC might be one with “transformable” lipophilicity, that is, moderate lipophilicity in airway lumen, high lipophilicity when taken up into the airway tissue, and low lipophilicity during systemic distribution (Brattsand,1999).

Beclomethasone dipropionate (BDP), fluticasone propionate (FP) and recently introduced mometasone furoate (MF) are examples of highly lipophilic inhaled GCs. After absorption from the airway lumen, BDP is converted to a less lipophilic, active metabolite, beclomethasone monopropionate (BMP), whereas lipophilicity of FP and MF does not change during the passage from the airway lumen to systemic distribution. In contrast to these GCs, inhaled budesonide (BUD) has a moderate lipophilicity but in airway tissue it becomes esterified with fatty acids forming a highly lipophilic depot of reversible BUD esters that act as a reservoir of active BUD (Miller-Larsson et al., 1998).

After inhalation, more than 50% of total BUD retained in the rat and human bronchial, nasal and lung tissue is esterified at the carbon-21-hydroxyl group, primarily as BUD oleate (Miller-Larsson et al.,1998; Jendbro et al., 2001; Petersen et al., 2001; Thorsson et al., 1998; Maasen van den Brink et al., 2005). BUD esters are not active i.e. do not bind to glucocorticoid receptor (Wieslander et al., 1998) but they are hydrolysed slowly, gradually releasing active BUD (Wieslander et al., 1998; Miller-Larsson et al., 1998). The formation of BUD esters prolongs the retention and activity of a BUD pulse in rat fibroblast cell line (Wieslander et al., 1998, and 2000), and esterification of BUD in the airways likely explains the very well documented once daily efficacy of inhaled BUD (Campbell et al., 1991; Jones et al., 1994; Banov et al., 2001; Selroos et al., 2004). Theoretically, other GCs with hydroxyl group at carbon-21 can be esterified in a similar way to BUD. These include an endogenous GC – corticosterone and a synthetic GC – BMP. However, esterification of corticosterone seems not to play a role in corticosterone tissue kinetics and biological activity, probably because corticosterone esters are hydrolysed rapidly (Hochberg et al., 1991). Rapid hydrolysis may also explain why BMP esters were not found in rat airways and lungs (Miller-Larsson et al., 1998).

Recently, a new inhaled GC, ciclesonide (CIC), was introduced. CIC is inactive but it is hydrolysed to an active metabolite (CIC-AM) that was reported to form fatty acid esters in airway tissue. CIC-AM esters have been detected *in vitro* in human alveolar and nasal epithelial cells (Nave et al., 2005a, 2006a) and in rat and human lung slices (Nave et al., 2004, and 2006b). They were also detected after inhalation of CIC in rat and human lungs (Nave et al., 2005b; Watz et al., 2006). However, the experience from corticosterone suggests that formation of esters alone may not be sufficient to affect the tissue kinetics of the GCs. There is no information on the role of CIC-AM esterification on the retention of CIC-AM in the airways, and once daily efficacy of CIC (Chapman et al., 2005; Pearlman et al., 2005) may be due to other CIC and CIC-AM features, such as

high lipophilicity. For example, MF is reported to be effective at once daily inhalation (Nayak et al., 2000; Karpel et al., 2005) although it does not form esters.

Therefore, in this study, in a rat trachea model, we have compared airway retention of CIC/CIC-AM and CIC-AM esterification to those of BUD, and determined CIC and CIC-AM lipophilicity in relation to BUD. As a reference, and to validate the system, we have used FP and MF, which do not form esters.

DMD Fast Forward. Published on July 12, 2007 as DOI: 10.1124/dmd.107.015297
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## Material and Methods

### Materials

Male Brown Norway rats were supplied by Charles River (Wiga, Germany) or Harlan (Horst, Netherlands). $^{3}$H-labeled GCs (BUD, FP, MF, CIC, and CIC-AM) were supplied in 99.5% ethanol solutions by AstraZeneca R&D Lund, (Lund, Sweden) or by the GE Healthcare (Cardiff, UK). Concentration of radioactivity was 40-60 MBq/ml. Specific radioactivity was 1.5-1.6 TBq/mmol for BUD, CIC and CIC-AM, 0.21 TBq/mmol for FP and 0.96 TBq/mmol for MF. Concentration determined by liquid chromatography was $3x10^{-5}$ M for BUD, CIC and CIC-AM, $26x10^{-5}$ M for FP and $6x10^{-5}$ M for MF. Radiochemical purity was at least 98.0%. Cyclandelate was purchased from TCI Tokyo Kasei (Tokyo, Japan) or Sigma-Aldrich (Cat# C-9260) (Stockholm, Sweden).

### Determination of GC water solubility and lipophilicity

***Water solubility*.** The water solubility of GCs was determined by mixing 1 mg of the GC powder and 5 ml Milli-Q water. The slurry was sonicated for 5 min and left on a shaker for 17 h. The sample was then filtered through a 0.45 μm Millipore Millex HV filter (Millipore, Billerica, MA,USA). The concentration in the filtrate was determined by high performance liquid chromatography (HPLC) using a Supelcosil LC-18 column (Supelco Belefonte, PA, USA), 150x4.6 mm. The flow rate was 1.0 ml/min of a mixture of acetonitrile and water, 72/28 for CIC and 47/53 for all other GCs. The GC concentration was calculated using a reference standard for each GC. The limit of quantification at 254 nm was determined to 0.1 μg/ml.

In the analysis of water solubility of MF there was a second peak in the chromatogram, approximately 30% of the size of the MF, which may indicate a degradation of MF. However, the actual value measured for the MF solubility was 0.03 μg/ml, which was

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below the lowest standard value. For the purpose of this paper, the low limit of quantitation was set to 0.1 µg/ml and thus a possible stability problem of MF did not affect the result on water solubility reported.

***Lipophilicity***. The relative lipophilicity of GCs was estimated by the chromatographic capacity factor log k'(0). The capacity factor, k', was determined as $k'=(t_R-t_0)/t_0$ were $t_R$ is the retention time for the analyte and $t_0$ is the retention time for a non-retained analyte, e.g. $KNO_3$, in a given liquid chromatography system. The retention time for each analyte in the HPLC system described above for water solubility was determined for at least four different concentrations of acetonitrile in the range of 30-70%. For each analyte the log k' values were calculated and plotted against organic content giving linear relation; the correlation coefficient value ($R^2$) for was each analyte > 0.99. From this, the value of log k'(0), i.e. k' at 0% organic phase, was extrapolated. For structurally related compounds it has been shown that log k'(0) is a good representation of the log P value i.e. octanol/water distribution (Tsantili-Kakoulidou et al., 1993; Rothemund et al., 1994; Yamagami et al., 2002).

**Study design**

Study design is shown in Fig.1. Tracheal segments from Brown Norway rats were dissected (approximately 50 mg) and placed in cold standard oxygenated Krebs buffer. A lengthwise incision through the cartilage of the trachea was made and trachea was tied to a glass rod and immersed in 10 ml medium consisted of standard Krebs buffer with 0.2% glucose and 10% autologous rat plasma (to approximate the protein concentration of airway epithelial lining fluid; Robinson et al., 1989). Tracheas were preincubated in 37°C oxygenated medium (95% $O_2$/5% $CO_2$) with the esterification inhibitor cyclandelate or vehicle. After 15 min, a $^3$H-GC (50 µl) was added, and after further 20 min tracheas were collected, rinsed briefly in fresh buffer, gently and briefly dried on blotting paper to get rid of buffer droplets, cut lengthwise in half, and two halves were weighed. One half

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was frozen at -70°C, awaiting analysis of $^{3}$H-GC tissue uptake. The other half was placed in 10 ml of fresh drug-free medium for 3 hours to monitor the total radioactivity release from the trachea into medium. Tissue extracts were prepared from both trachea halves to determine $^{3}$H-GC tissue concentrations before (initial uptake) and after 3 hour-release. The total radioactivity of tissue extracts was measured by liquid scintillation counting, and GC species in tissue extracts were analysed by radiochromatography.

**$^{3}$H-GC preparation**

For all GCs except FP, 50 µl of $^{3}$H-GC stock solution in ethanol was added to 10 ml incubation medium. This resulted in final concentration of 1.1 x$10^{-7}$ – 2.1 x $10^{-7}$ M for BUD, CIC and CIC-AM, and 2.7-2.9 x $10^{-7}$ M for MF. For FP, 5 µl of $^{3}$H-FP ethanol solution together with 45 µl of 99.5% ethanol was used; this resulted in final concentration of 1.5-2.7 x $10^{-7}$ M of FP. After addition of cyclandelate or its vehicle, the final concentration of ethanol in incubation medium was 1%.

**Cyclandelate preparation**

Cyclandelate was suspended in 99.5% ethanol to a concentration of 55.3 mg/ml (2x$10^{-1}$ M) and 50 µl was added to 10 ml incubation medium giving $10^{-3}$ M as the final concentration. This concentration of cyclandelate as well as incubation time (15 min preincubation followed by 20 min incubation with $^{3}$H-GC) were titrated in pilot experiments to investigate whether cyclandelate can inhibit GC ester formation without exerting cytotoxic effects on epithelium in trachea. Histological sections of tracheas were examined using scanning electron microscopy and fluorescence analysis of DNA to investigate epithelial integrity and reveal any damage to epithelial cell nuclei. At the cyclandelate concentration and incubation time applied, normal appearance of epithelium was observed i.e. normal density of epithelial cells and evenly stained cell nuclei without chromatin fragmentation. The histological analysis was performed by Dr. Jonas Erjefält at Department of Experimental Medical Science at Lund University (Lund, Sweden).

**Release of GCs from trachea**

During 3 hour-release period, 200 µl samples were withdrawn from incubation medium (10 ml) at various time points and the volume was replaced with fresh medium. The radioactivity of withdrawn samples were analysed by liquid scintillation counting (200 µl in 10 ml Ultimagold scintillation cocktail, Packard, Groningen, Netherlands) in Tri-Carb Spectrometer 2200CA (Packard, Groningen, Netherlands). The accumulated release of radioactivity was calculated for each time point and expressed as percentage of total radioactivity initially present in trachea (calculated as a sum of released and remained radioactivity in trachea at 3 hours).

**Analysis of GC species in tracheal extracts**

Trachea extracts were prepared by microwave assisted extraction in 2 ml 99.5% ethanol in a microwave oven (Mars X or MES 1000, CEM Corporation, Matthews, NC) for 30 min at 90°C with a power of 13-17%. The efficiency of extraction measured with spiked control samples was nearly complete (>90%). The extracts were filtered and stored at (-20°C) before analysis. Total radioactivity was measured in 50 µl extracts by liquid scintillation counting in 10 ml of Ultimagold scintillation cocktail (Packard, Groningen, Netherlands) in Tri-Carb Spectrometer 2200CA (Packard, Groningen, Netherlands).

For analysis of GC species, 500 µl of extract was injected into the liquid chromatography system combined with on-line scintillation detection in Radiomatic Flo-One beta A500 radiochromatography detector (Packard, Meriden, CT). The reverse-phase column (Supelcosil LC-18-DB, 3.3 cm x 4.6 mm, 3 µm; Supelco Inc., Bellefonte, PA) was used with a three-phase ethanol gradient: phase A containing 5% ethanol and 0.1% acetic acid, phase B containing 95% ethanol and 0.1% acetic acid, phase C containing 0.1% acetic acid. The gradient was as follows: 0 - 7.6 min, 65% A/35% B; 7.6 - 15.2 min 15%

A/85% B; 15.2 – 20 min 100% B. The flow rate of phases A and B was 0.35 ml/min for the first 3 min, and 0.65 ml/min for phase C for the first 3 min and thereafter 1 ml/min of phases A and B. The recovery of the total radioactivity in this system versus liquid scintilaltion counting was 90-106%.

GC species were identified and confirmed in pilot experiments applying liquid chromatography and mass spectroscopy techniques using HP1100 LC system (Agilent Technologies, Waldbronn, Germany) in line with a QTOF mass spectrometer (Waters, Manchester, UK). The identification was based on comparison of chromatographic retention times of reference compounds and mass spectrometry data.

**Data Analysis**

The tissue concentration (Q) of GC species in the samples, expressed as picomoles/gram (pmol/g), was calculated from the following equation: Q = DPM x DF/(C x SA x W), where DPM is the mean number of disintegrations per minute (dpm) measured in the sample, DF is dilution factor (=4, total extract volume/LC injection volume), C = 2.22 x $10^6$ dpm per microCurie (µCi,) SA is the specific radioactivity of GC applied (µCi per picomoles), and W is the weight (grams) of tissue samples. As the $^3$H-GC concentrations in incubation medium differed somewhat between GCs (see Study Design), Q values were normalised to the concentration of 1.0x$10^{-7}$ M and expressed as pmol/g. When two or more species of a GC were detected, the sum of their Q values was calculated, and is referred here as the total tissue concentration of a GC in trachea.

**Statistical analysis**

Data are presented as arithmetic means ± SEM. Data were analysed by one-way analysis of variance ANOVA with significance level down to 1% using Astute software 1.5 (DDU Software, Leed, UK). Differences were considered significant at $p<0.05$.

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## Results

### Water solubility and lipophilicity of GCs

The relative lipophilicitiy and water solubility for the inhaled GCs: BUD, FP, MF and CIC/CIC-AM were determined and are presented in the Table 1.

### Tissue uptake and retention of GCs

Radiochromatographic analysis of trachea extracts revealed fractions of active GCs, GC esters, and parent GC (for CIC). In experiments with BUD or CIC-AM, unmodfied GCs and GC esters were detected (Fig. 2A-D). In experiments with CIC, three fractions were detected: parent CIC, CIC-AM and CIC-AM esters (Fig. 2E-F). Thus for BUD, CIC-AM and CIC, the total tissue concentration in trachea is represented by a sum of individual GC species. In experiments with FP and MF only unmodified GCs were detected (not shown).

GC uptake during 20 min incubation with solutions of BUD, MF, CIC and CIC-AM at 1-3 x $10^{-7}$ M, resulted in GC concentrations in tracheal tissue equal to 300-500 pmol/g which corresponds to approximately 200 pmol/g (200 nmol/l) when GC concentrations are normalised to 1.0 x$10^{-7}$ M (Fig. 3). Thus, at 20 min a 2-fold higher concentration of these GCs was attained in tracheal tissue than GC concentration present in the incubation medium. The exception was FP, which reached 5-fold higher tissue concentration than the level in the medium (Fig. 3). Uptake of FP into tracheal tissue was 2-fold higher than that of total BUD ($p<0.01$), whereas uptake of MF was 34% lower than that of total BUD ($p<0.05$). The total CIC/CIC-AM tracheal concentrations were 20-25% lower than that of BUD but these differences were not statistically significant (Fig. 3).

The retention of GCs in tracheal tissue was determined by the percentage of the total tracheal concentration of a GC remained after the 3 hour-incubation of trachea in GC-

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free medium (when GCs were released into the medium) compared with the initial tracheal concentration (measured at 20 min). The retention of BUD was significantly greater than that of other GCs tested; 34% as compared to 12% of FP, 13% of MF, and 23-26% of total CIC when trachea was incubated with CIC or CIC-AM ($p<0.01$ for all versus BUD; Fig. 3). We have shown earlier in this system that only the intact BUD (and not BUD esters) is released from trachea into incubation medium (Miller-Larsson et al., 1998). Similarly, only CIC and CIC-AM, but not CIC-AM esters, were detected in medium after incubation with human lung slices (Nave et al., 2006c).

Analysis of esterified species in trachea revealed that a much higher percentage of BUD was in the esterified form than for the CIC/CIC-AM (Fig. 4). After the 20 min incubation, BUD esters made up 46% of the total BUD content in trachea whereas CIC-AM esters made up 6-7% of the total CIC content ($p<0.01$ versus BUD) after both CIC or CIC-AM incubation. After the subsequent 3 hour-release period in GC-free medium, BUD esters made up 92% of the total BUD as compared to 52% or 67% of CIC-AM esters after incubation with CIC or CIC-AM, respectively ($p<0.01$ for both versus BUD). In the absolute amounts, the BUD-ester pool was 9-fold greater than the CIC-AM-ester pool after 20 min incubation ($p<0.01$), and 3-fold greater after the 3 hour-release period ($p<0.01$). These results show that BUD was esterified in trachea more promptly and to a greater extent than CIC-AM.

**Role of GC esterification for tissue uptake and retention of GCs**

The relationship between esterification and retention of BUD, CIC and CIC-AM was evaluated by radiochromatographic analysis of tracheas incubated with esterification inhibitor cyclandelate for 15 min before and during the 20 min incubation with GCs (examples of typical radiochromatograms are shown in Fig. 5). In the experiments involving CIC incubation, cyclandelate significantly inhibited hydrolysis of CIC to CIC-AM

(not shown), and this effect prevented any conclusions regarding the consequences of ester inhibition on CIC kinetics in trachea.

Incubation with cyclandelate significantly decreased the initial BUD-ester pool in trachea to 31% of vehicle-treated value ($p<0.01$), and increased the pool of BUD 1.8-fold ($p<0.01$) but it did not affect the total BUD uptake at 20 min (1.1-fold increase, $p=0.2$) (Fig. 6). Similarly, incubation of CIC-AM with cyclandelate significantly decreased CIC-AM esters to 6% of vehicle-treated value ($p<0.01$), and increased the pool of CIC-AM 2-fold ($p<0.01$), but it also significantly increased 1.9-fold ($p<0.01$) the total uptake of CIC-AM at 20 min (Fig. 6). The uptake of MF was not affected by cyclandelate, while the uptake of FP was reduced by 19%, but this difference was not statistically significant ($p=0.1$; data not shown).

Although cyclandelate was discontinued before the 3 hour-release period (and trachea was briefly washed in fresh buffer), cyclandelate accelerated and increased BUD release during 3 hour-incubation period in drug-free medium (without GC and cyclandelate). In consequence, the total BUD remained in trachea after this period was significantly lower than BUD remained under control conditions (i.e. without cyclandelate) (Fig. 7A and C). In contrast, cyclandelate did not affect the retention of CIC-AM (which forms esters) (Fig 7B and C) nor of FP and MF (which cannot form esters) (Fig. 7C). Under control conditions, the release of BUD from trachea was significantly slower than those for all other GCs tested, and cyclandelate abolished this difference (Fig. 7C). Thus, reduction of BUD-ester pool at 20 min significantly decreased total retention of BUD after 3 hour-release period. These results give evidence that BUD retention in tracheal tissue is prolonged due to formation of BUD esters.

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**Discussion**

We have demonstrated in a rat trachea *in vitro* model, the prolonged overall retention of BUD in tracheal tissue compared to more lipophilic FP, MF and CIC/CIC-AM. The prolonged retention of BUD was due to the endogenous formation of BUD fatty acid esters, as it was reduced to the level of other GCs when formation of BUD esters was inhibited. BUD was esterified more promptly and to a greater extent than CIC-AM. In contrast to BUD, the nearly complete inhibition of the intitial CIC-AM-ester pool did not reduce retention of CIC-AM. These results suggest that the airway retention of CIC-AM is not dependent on CIC-AM esterification but may be mainly determined by high lipophilicity of CIC-AM and parent CIC, similarly to FP and MF that are not esterified.

The uptake of BUD into rat tracheal tissue was roughly comparable to the uptake of either CIC or CIC-AM. The absolute total tissue concentration of these GCs reached in trachea after 20 min incubation was at the level of ~$10^{-7}$ M ($10^{-7}$ mol/kg). Similar tissue concentrations in rat trachea, and a 10-fold lower level in lung tissue, were earlier obtained *in vivo* after inhalation of BUD and FP (Miller-Larsson et al., 1998) and recently after inhalation of CIC (Watz et al., 2006). The ~$10^{-8}$ M concentration in lung tissue corresponds to the levels obtained in humans 1-4 hours after inhalation of high clinical doses (1-2 mg) of GCs (Van den Bosch et al., 1993; Esmailpour et al., 1997; Thorsson et al., 1998; Maassen van den Brink et al., 2005; Watz et al., 2006).

The experiments on *in vitro* uptake of GCs were conducted with 10% autologous rat serum in the incubation medium. This concentration was chosen to approximate the protein concentration present in airway lining fluid. The protein amount in airway lining fluid is difficult to measure, and it is known to increase in inflammation and certain airway diseases, but we decided to use a conservatively low value for these experiments (Robinson et al., 1989). This is an important technical consideration, because the tissue

uptake of lipophilic molecules with high protein binding may be impaired by binding to proteins in culture medium, or it may be increased by the use of low protein concentrations. CIC and CIC-AM bind to plasma proteins at ~99% (Rohatagi et al., 2005) as compared to 88% binding of BUD (Ryrfeldt et al., 1982). Accordingly, Jerre et al. (2006) have shown that uptake of CIC into cultured human airway epithelial cells depends strongly on protein concentration in culture medium. At physiologic protein concentration in airway lining fluid, represented by 10% serum in culture medium, CIC was poorly taken up into airway epithelial cells and its uptake over 2 hour incubation was 3-fold lower than of BUD. Nave et al. have reported higher uptake of CIC than BUD into human alveolar type II epithelial cell line and human lung slices *in vitro* (2005a and 2006b). This apparent discrepancy between our and the Nave et al. results are likely due to the very low protein concentration (0.1% bovine serum albumin) in the incubation medium in the Nave et al. study. In contrast, in an *in vivo* study where BUD or CIC were instilled intratracheally into rats, the uptake of BUD into both trachea and lung tissue was higher than CIC (Gullstrand et al., 2005). It is conceivable that besides the initial uptake, also the tissue retention of GCs with high protein binding can be increased by using too low protein concentration in the incubation medium in *in vitro* experiments.

Despite roughly equal uptake of BUD and CIC/CIC-AM into rat trachea in the present study, there were two major differences in tracheal tissue kinetics between BUD and CIC/CIC-AM. The first one was that a much larger proportion of BUD than of CIC-AM was esterified within the 20 min and remained esterified after the 3 hour-release period. Similarly, in an above mentioned *in vivo* study, where rats were instilled intratracheally with BUD or CIC, BUD retained in trachea tissue was esterified to a significantly greater extent than CIC-AM (and in contrast to BUD esters, CIC-AM esters were not detectable in lung tissue; Gullstrand et al., 2005). In the present study, to investigate CIC-AM esterification *per se*, independently of the efficiency of the transformation of CIC into CIC-

AM, we have incubated trachea with CIC or CIC-AM. In both cases the extent of CIC-AM esterification was very similar, showing that CIC was efficiently converted to CIC-AM in this trachea *in vitro* system.

The second major difference was detected by use of an inhibitor of esterification, cyclandelate. Esterification of BUD at carbon-21 was earlier shown to be dependent on co-enzyme A (CoA) and ATP and probably catalyzed by the ATP- and CoA-dependent microsomal acyl-CoA:cholesterol acyl transferase (ACAT) (Tunek et al., 1997). It was earlier shown that cyclandelate, an inhibitor of ACAT, decreased the amount of BUD esters and shortened the retention and activity of BUD pulse in rat fibroblast cell line (Wieslander et al., 2000). In the present study we have confirmed and extended these findings demonstrating that reduction of the initial BUD-ester pool by cyclandelate significantly decreased retention of BUD in rat tracheal tissue at 3 hours. Cyclandelate treatment also nearly completely blocked the initial CIC-AM-ester pool, however in contrast to BUD, it did not decrease the retention of CIC-AM in rat trachea at 3 hours. It was not expected that cyclandelate should affect tissue kinetics of FP or MF in trachea, and it had no effect on either their uptake or retention, which validates the system. The retention of FP and MF in trachea was similar to those of CIC and CIC-AM (Fig. 7). FP, MF, CIC and CIC-AM are all highly lipophilic GCs (Table 1), and high lipophilicity was shown to increase the airway retention of inhaled GCs (Brattsand, 1997). The very high lipophilicity of BUD esters (Table 1), rapidly formed in the airways, retards absorption of BUD into blood circulation and explains the prolonged airway retention of inhaled BUD. The major CIC-AM ester, oleate, was reported to be 5 times more lipophilic than BUD oleate (Nave et al., 2004). However, a slow rate of net formation of CIC-AM esters probably explains the finding that the blockage of the initial small pool of CIC-AM esters did not prolong CIC-AM retention in trachea in the present study. It seems that after

inhalation of CIC, CIC-AM retention is determined by parent CIC's and CIC-AM's lipophilicity, similary to retention of inhaled FP and MF.

Surprisingly, the presence of cyclandelate significantly increased nearly 2-fold the initial uptake of CIC-AM but not that of BUD or other GCs tested. This is despite that CIC-AM esters initially made up only 6-7% of the total CIC-AM in tracheal tissue while BUD esters made up nearly 50% of the total BUD. We do not have explanation for the increased uptake of CIC-AM by cyclandelate. Furthermore, we have expected that the blockage of BUD esterification will decrease BUD uptake as it was shown in rat fibroblast cell line (Wieslander et al., 2000). The finding that the BUD uptake into tracheal tissue was not dependent on BUD esterification and was roughly equal to that of the 2-4 times more lipophilic MF, CIC-AM and CIC, but lower than the 3-fold more lipophilic FP, suggests that besides lipophilicity there are other factors which influence GC uptake into airway tissue. For example, evidence is growing that cellular concentrations of GCs are regulated not only by GC passive diffusion, dependent on small size and high lipophilicity of GC molecules, but also via efflux transporters, such as P-glycoprotein. The efficiency of P-glycoprotein-mediated efflux of various GCs seems to depend on subtle differences in GC structure (Yates et al., 2003). Recent data in colon carcinoma cells suggest that BDP, MF and CIC-AM, but not FP, are substrates to P-glycoprotein (Cooray et al., 2006), whereas data on BUD are controversial (Cooray et al., 2006; Dilger et al., 2004). However, it is not yet known whether GC transporters operate in airway and lung cells.

The observed difference between BUD and CIC-AM rates of net ester formation are likely to result from equilibrium differences between ester synthesis and lipase/esterase-catalyzed hydrolysis. It cannot be excluded that these differences are due to different enzymes regulating esterification and/or hydrolysis of these two GCs, although ACAT seems to be involved in the esterification of both BUD and CIC-AM as cyclandelate

(ACAT inhibitor) reduced the initial formation of both BUD and CIC-AM esters. Furthermore, the hydrolysis of GC esters is a relatively stereoselective process (Hochberg et al et al., 1991), and hydrolysis of BUD esters may be decelerated by the steric hindrance of the acetal group at 16α- and 17α-carbons. Contrariwise, a rapid hydrolysis of enzymatically accessible corticosterone esters (Hochberg et al., 1991) and cortisol esters (Dr. A. Tunek, AstraZeneca, personal communication) may explain why little esters of corticosterone and cortisol are detected, and why biological activity of these endogenous GCs is not dependent on ester formation.

Rapid formation of BUD esters in the airways and their slow hydrolysis contribute not only to prolong airway retention and activity (resulting in once daily efficacy; Campbell et al., 1991; Jones et al., 1994; Banov et al., 2001; Selroos et al., 2004), but probably also to high airway selectivity (Edsbäcker and Jendbro, 1998; Miller-Larsson et al., 2000; Jendbro and Johansson, 2002). This is because: i) esterification of inhaled BUD in the airway tissue retards BUD absorption into blood circulation, and ii) BUD esterification occurs to a much lower extent in skeletal muscles (10-15%) than in the airways (more than 50%), and does not occur in plasma (Miller-Larsson et al., 1999; Jendbro et al., 2001). Importantly, the results of pharmacokinetic and pharmacodynamic modelling suggest that the faster the formation of esters in the airways and slower their hydrolysis, the greater the airway selectivity of inhaled GCs (Jendbro and Johansson, 2002). Indeed, we have earlier shown that after 4 days of repeated intratracheal instillation of BUD into rats, the total tissue concentration of BUD over 24 hours was 20-40-fold greater in trachea than in skeletal muscles and plasma, while respective ratios for CIC were 2-fold lower than for BUD (Gullstrand et al., 2005). Thus, although inhaled CIC is reported to have a high airway selectivity, according to the results of this study this is unlikely to be due to airway esterification of CIC-AM.

In summary, this study gives the evidence that prolonged retention of BUD in the airways is due to the rapid and extensive formation of highly lipophilic and reversible BUD-ester depot. In contrast, CIC-AM was esterified only slowly and to a lower degree than BUD, which may explain the lower retention of CIC-AM in the tracheal tissue and the finding that CIC-AM retention was not dependent on CIC-AM esterification in this rat trachea model. These results suggest that unlike inhaled BUD, retention of CIC-AM after inhalation of CIC is mainly determined by parent CIC's and CIC-AM lipophilicity - similarly to inhaled FP and MF which do not form esters.

**Acknowledgments**

The authors are grateful to Dr. Jonas Erjefält for histological analyses of tracheas, and to Hanna Falk Nilsson, Maria Lindahl and Amir Smailagic for their help with experiments. They also thank Dr. Ralph Brattsand, Dr. Staffan Edsbäcker, Jarl Ingelf and Elisabet Wieslander for helpful discussion.

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**Figure Legends**

**Figure 1.** Study design, briefly: Rat tracheas were preincubated in oxygenated medium with the esterification inhibitor cyclandelate or vehicle. After 15 min, a $^{3}$H-GC was added, and after further 20 min tracheas were collected and briefly washed (Sampling1) and cut lengthwise in two halves. One half was frozen at -70°C, awaiting analysis of $^{3}$H-GC tissue uptake. The other half was placed in fresh drug-free medium to monitor the total radioactivity release from the trachea into medium. After 3 hours, trachea was collected (Sampling 2). Tissue extracts were prepared from both trachea halves and GC species analysed by radiochromatography.

**Figure 2A-F**. Examples of typical radiochromatograms obtained from analysis of tracheas collected directly 20 min after incubation of tracheas with $^{3}$H-GC solutions, or after further 3 hour-incubation period in GC-free medium. Note different order of magnitude of scale for disintergration per minute (dpm) axes in A, C and E figures compared to B, D and F figures.

**Figure 3**. The total concentration of the GCs in tracheal tissue directly after 20 min incubation (grey bars) with $^{3}$H-GCs: BUD, FP, MF, CIC, or CIC-AM, and after further 3 hour-incubation period in GC-free medium (black bars). The tissue concentrations (pmol/g) are normalised to $1.0 \times 10^{-7}$ M GC concentration in incubation medium. Data shown as means ± SEM (n=3-12). Statistical comparisons within groups of 20 min or 3 hours: *=$p<0.05$, **=$p<0.01$ versus BUD at 20 min; ##=$p<0.01$ versus BUD after further 3 hours.

**Figure 4**. The concentrations of GC esters (open bars) in tracheal tissue after 20 min incubation with $^{3}$H-BUD, $^{3}$H-CIC or $^{3}$H-CIC-AM, and after further 3 hour-incubation period in GC-free medium. Concentrations of intact/active GCs (grey bars) and parent/inactive

CIC (black bars) are also shown. The tissue concentrations (pmol/g) are normalised to $1.0x10^{-7}$ M GC concentration in incubation medium. Data shown as means ± SEM (n=4-12). Statistical comparisons within groups of 20 min or 3 hours; $^{**}$=p<0.01 for comparison of CIC-AM esters versus BUD esters at respective times (20 min or 3 hours).

**Fig. 5A-B**. Examples of typical radiochromatograms obtained from analysis of tracheas collected after incubation with 15 min cyclandelate (CD) followed by 20 minute incubation with CD together with $^{3}$H-GC solutions. Note inhibition of GC esters by CD (compare with Fig. 2A and C )

**Figure 6**. The effect of esterification inhibitor cyclandelate (CD) on the concentration of GC esters (open bars) and intact GCs (grey bars) in tracheal tissue after 20 min incubation with $^{3}$H-BUD and $^{3}$H-CIC-AM. The tissue concentrations (pmol/g) are normalised to $1.0x10^{-7}$ M GC concentration in incubation medium. Data shown as means ± SEM (n=4-12); **=p<0.01, ##=p<0.01, §§=p<0.01 for comparison of GC esters, intact GC, and the total GC pool (intact GC + GC esters), respectively, with CD treatment versus without CD treatment.

**Figure 7A-C**.

Percentage of total tissue radioactivity retained in tracheal tissue during 3 hour-incubation in drug-free medium (figures A and B) in tracheas incubated earlier with $^{3}$H-GCs with esterification inhibitor cyclandelate (CD) or its vehicle. In (A): solide line = BUD, dotted line = BUD+CD, broken line = FP, broken/dotted line = MF; CD had no effect on FP and MF release and these data are not shown for clearness of the figure. In (B): solid line = CIC, broken line = CIC-AM, dotted line = CIC-AM+CD; the release experiments for the group CIC+CD are not shown because CD inhibited hydrolysis of CIC to CIC-AM. In (C): the comparison of areas (AUC) under retaining curves for GCs incubated without CD (grey bars) or with CD (black bars). Data shown as means ± SEM (n=3-12); for the figure

A and B clearness, SEM values are not shown for all curves. In (A): §§=p<0.01 for the total BUD retained under control conditions (i.e. without CD) in trachea after 3 hour-incubation in drug free-medium versus retained pools of FP and MF, and versus the total BUD retained after BUD+CD treatment. In (C): ##=p<0.01 for AUC comparisons versus BUD under control conditions (i.e. without CD); there are no statistically significant differences between AUC for GCs incubated with CD; **=p<0.01 for comparisons of AUC obtained with GC+CD versus the own control (significant difference only for BUD).

DMD Fast Forward. Published on July 12, 2007 as DOI: 10.1124/dmd.107.015297
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**Table 1.**

**Water solubility and relative lipophilicity[a] of GCs**

| | BUD | BUD oleate | FP | MF | CIC | CIC-AM[c] |
|---|---|---|---|---|---|---|
| **water solubility (µg/mL)** | **14** | **nd[b]** | **<0.1** | **<0.1** | **<0.1** | **7** |
| **relative lipophilicity (logk'(0))** | **2.6** | **6** | **3.1** | **2.9** | **3.2** | **3.0** |
| **normalised lipophilicity ($k'(0)_{BUD}$=1)** | **1** | **2500** | **3.2** | **2.0** | **4.0** | **2.5** |

[a] The relative lipophilicity of GCs was estimated by the chromatographic capacity factor log k'(0); k', was determined as $k'=(t_R-t_0)/t_0$ were $t_R$ is the retention time for the analyte and $t_0$ is the retention time for a non-retained analyte in a given liquid chromatography system.

[b] Not determined due to LC-UV detection limit; expected water solubility <<0.1 ug/ml.

[c] log k'(0) for CIC-AM oleate was not determined in this paper however, according to Nave et al. (2004) the lipophilicity of CIC-AM oleate is 5 times greater than that of BUD oleate as determined by log D value (octanol/phosphate buffer distribution).

**Figure 1.**

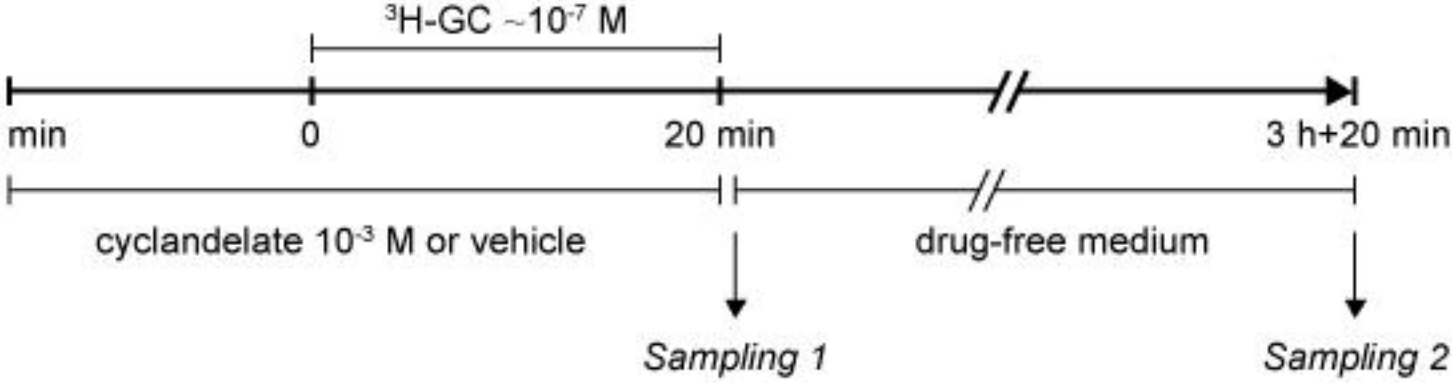

$^3$H-GC ~$10^{-7}$ M
-15 min
0
20 min
3 h+20 min
cyclandelate $10^{-3}$ M or vehicle
drug-free medium
*Sampling 1*
*Sampling 2*

**Figure 2.**

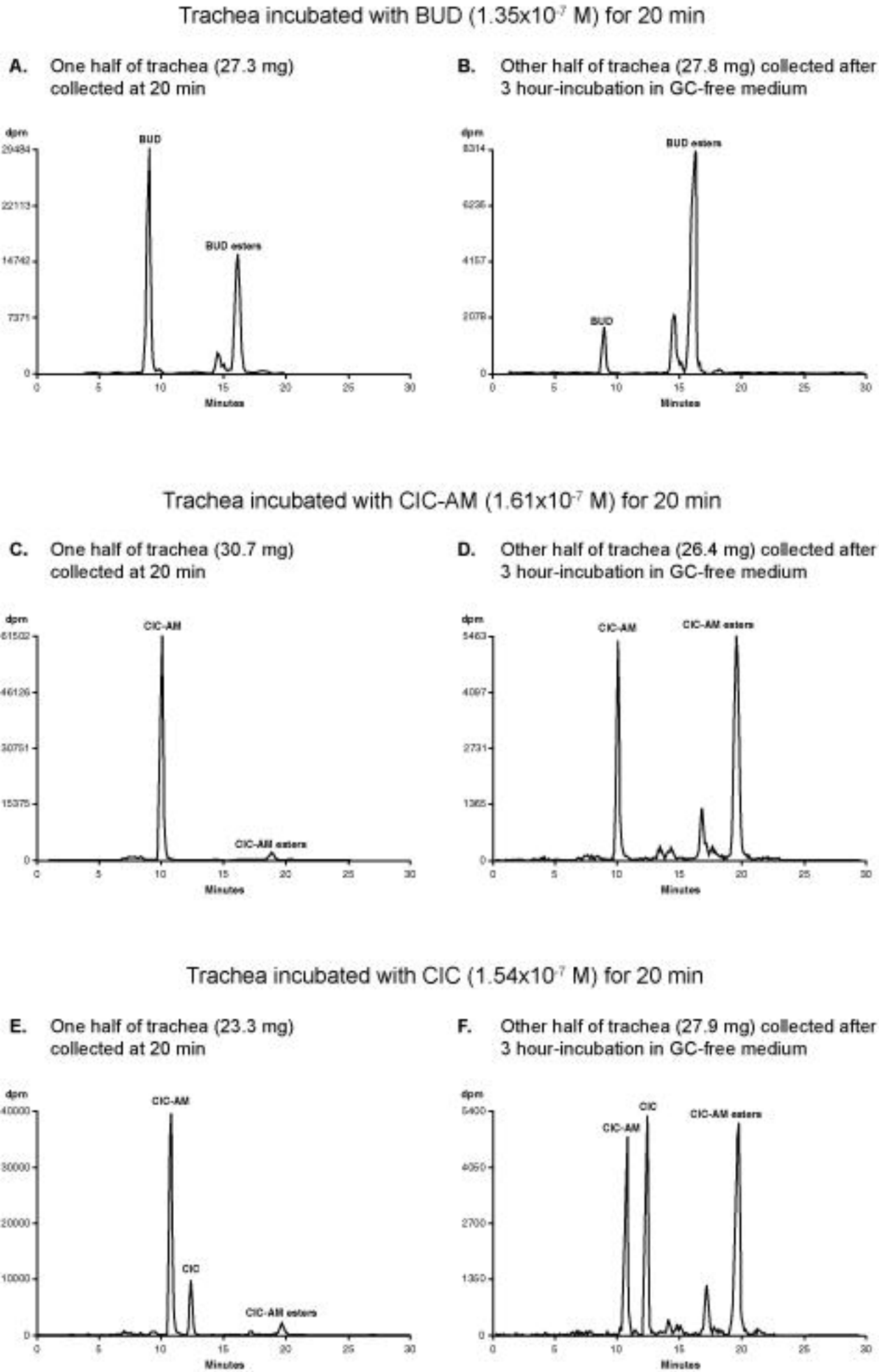


**Figure 3.**

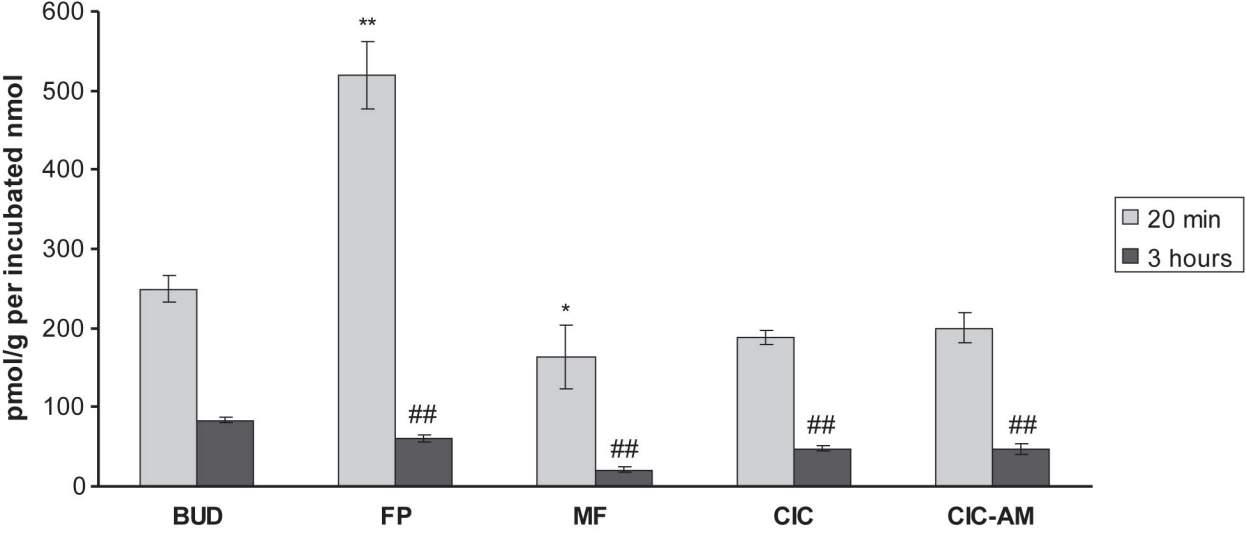

600
500
400
300
200
100
0
pmol/g per incubated nmol
**
*
##
##
##
##
20 min
3 hours
BUD
FP
MF
CIC
CIC-AM

**Figure 4.**

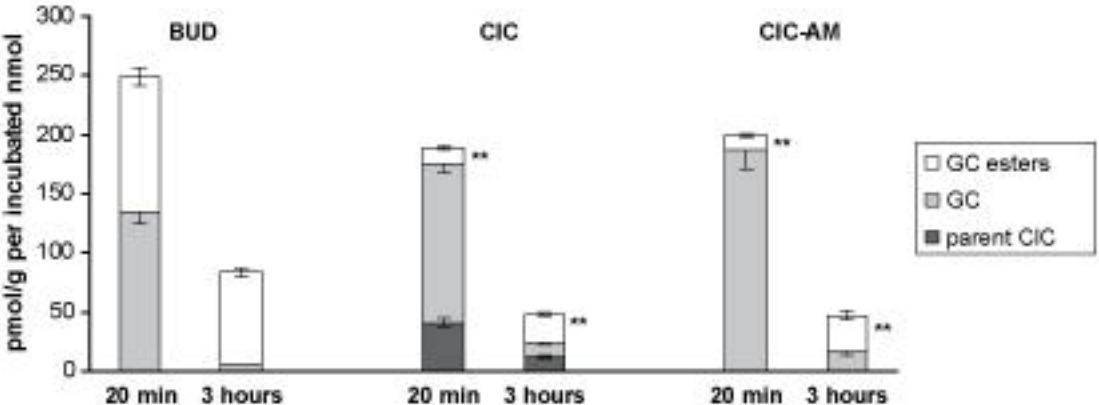
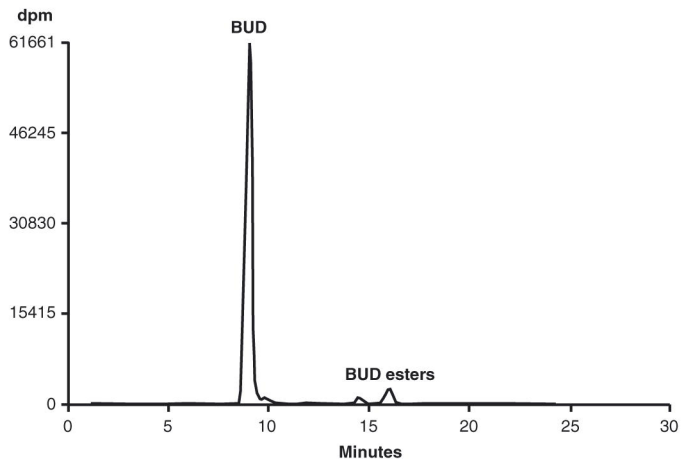

BUD
CIC
CIC-AM
pmol/g per incubated nmol
300
250
200
150
100
50
0
**
**
**
GC esters
GC
parent CIC
20 min
3 hours
20 min
3 hours
20 min
3 hours

**Figure 5.**

Tracheas preincubated with cyclandelate (CD) and further incubated with:

**A.** BUD ($1.63 \times 10^{-7}$ M) and CD for 20 min
One half of trachea (27.1 mg) collected at 20 min




**B.** CIC-AM ($1.69 \times 10^{-7}$ M) and CD for 20 min
One half of trachea (23.8 mg) collected at 20 min

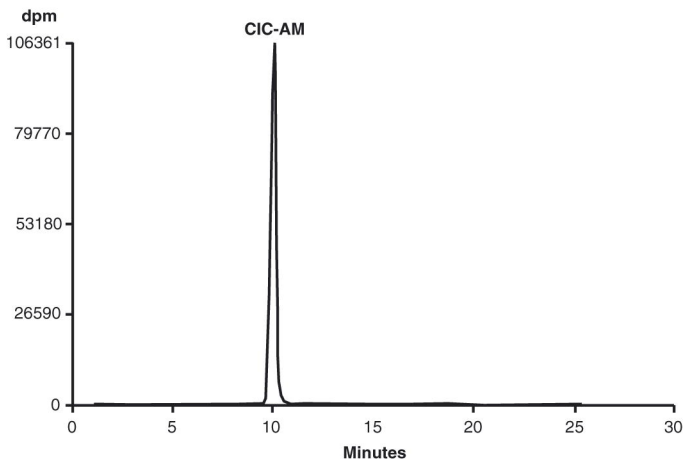


**Figure 6.**

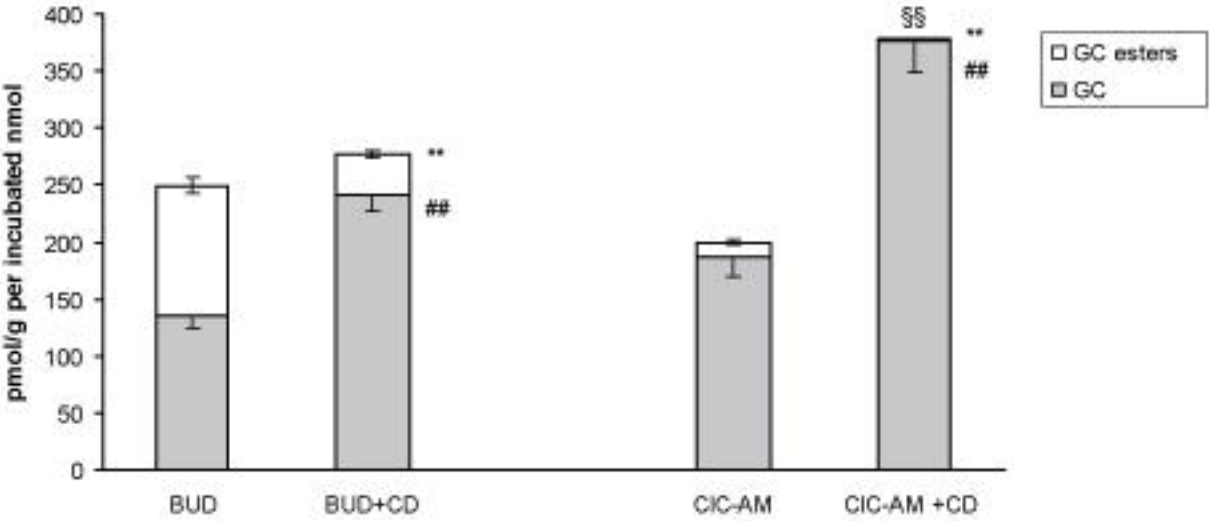

400
350
300
250
200
150
100
50
0
pmol/g per incubated nmol
BUD
BUD+CD
CIC-AM
CIC-AM +CD
**
##
§§
GC esters
GC

**Figure 7.**

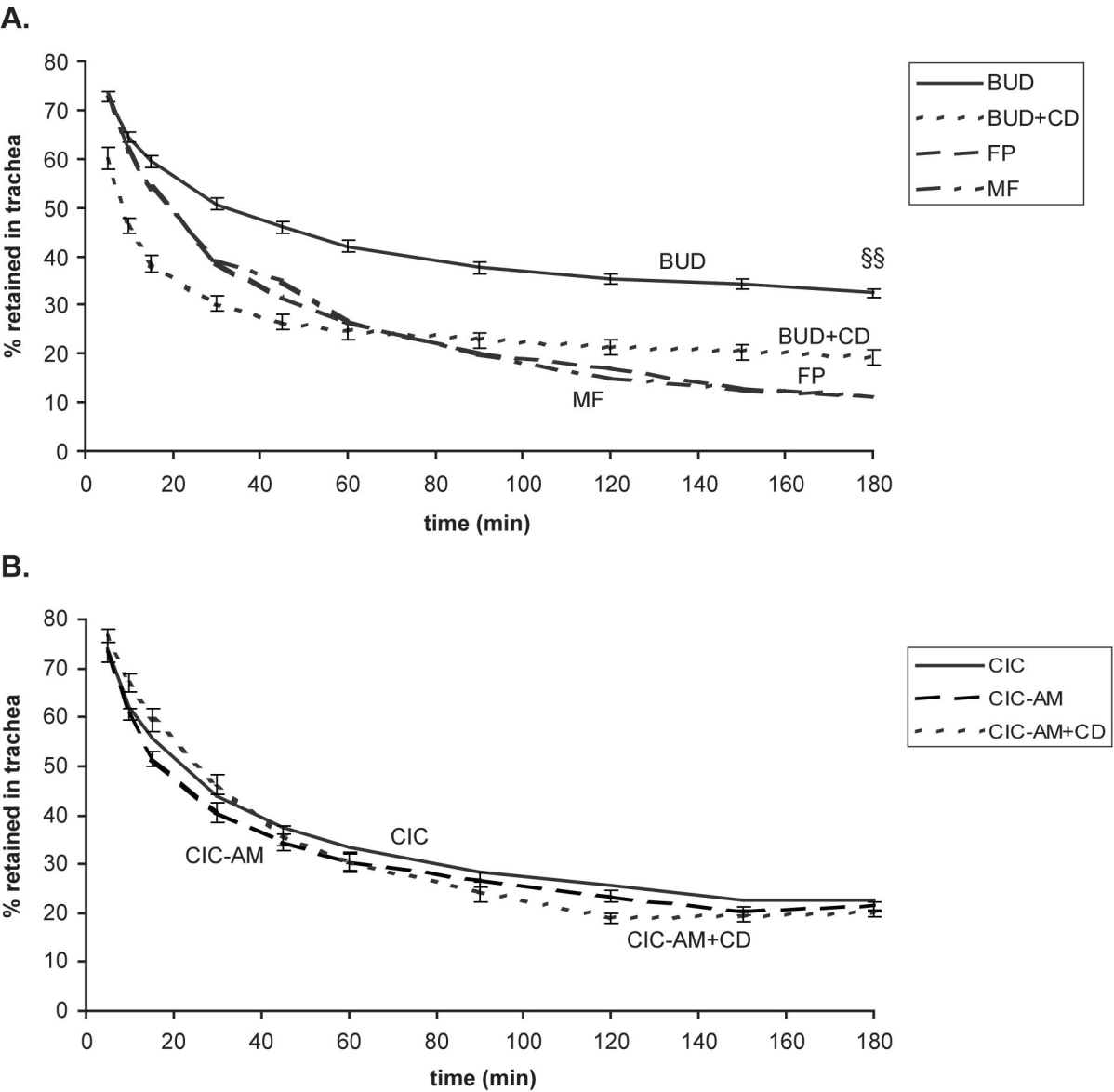
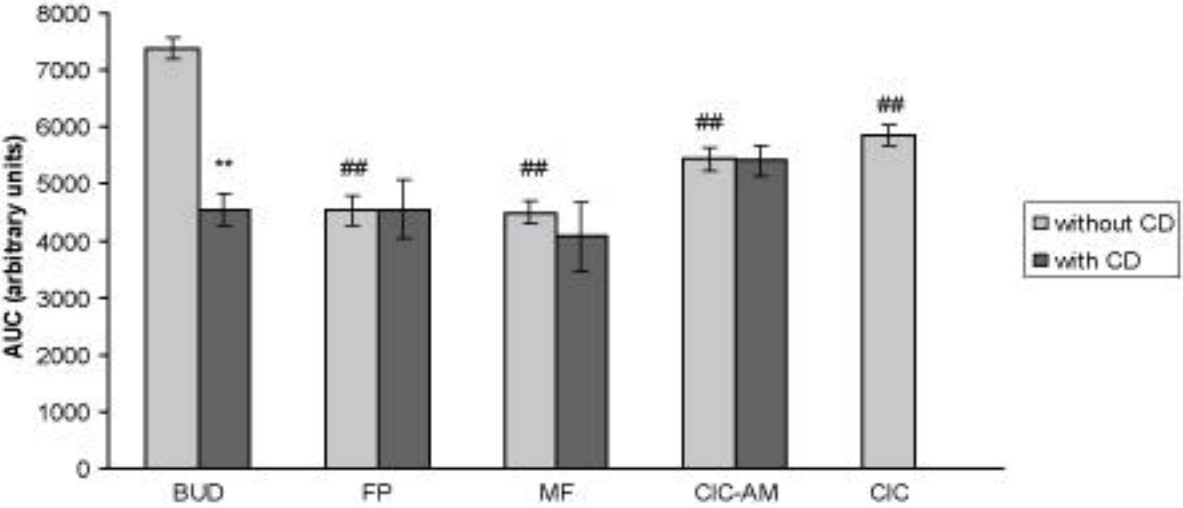

A.
% retained in trachea
time (min)
BUD
BUD+CD
FP
MF
§§
B.
% retained in trachea
time (min)
CIC
CIC-AM
CIC-AM+CD

**Figure 7.**

**C.**


8000
7000
6000
5000
4000
3000
2000
1000
0
AUC (arbitrary units)
**
##
##
##
##
BUD
FP
MF
CIC-AM
CIC
without CD
with CD