# Human Receptor Kinetics, Tissue Binding Affinity, and Stability of Mometasone Furoate

ANAGNOSTIS VALOTIS,[1] KONRAD NEUKAM,[2] OLAF ELERT,[2] PETRA HÖGGER[1]

[1]Institut für Pharmazie und Lebensmittelchemie, Bayerische Julius-Maximilians-Universität, Am Hubland, 97074 Würzburg, Germany

[2]Klinik und Poliklinik für Herz- und Thoraxchirurgie, Bayerische Julius-Maximilians-Universität, Würzburg, Germany

*Received 24 September 2003; revised 23 December 2003; accepted 29 December 2003*

**ABSTRACT:** Mometasone furoate (MF) is a topically used glucocorticoid with high anti-inflammatory potency. In contrast to the wealth of data derived from clinical studies, information about the molecular pharmacology of the compound is lacking or contradictory. Thus, we elucidated the characteristics of receptor binding kinetics and receptor affinity in a bioassay. Metabolite formation was determined in human plasma and lung tissue as well as binding affinity to human lung tissue. Fast and extensive association of MF to the human glucocorticoid receptor was observed while the dissociation of the MF–receptor complex was faster compared to fluticasone propionate (FP). The relative receptor affinity of MF was calculated as 2200 (dexamethasone = 100, FP = 1800) and confirmed in a bioassay measuring the induction of the glucocorticoid regulated protein CD163 in human monocytes. In plasma and human lung tissue MF formed a 9,11-epoxy degradation product. The binding affinity of MF to human lung tissue was low compared to FP due to fast redistribution from tissue into plasma. These molecular pharmacological properties are in accordance with clinical data. © 2004 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 93:1337–1350, 2004

**Keywords:** mometasone furoate; fluticasone propionate; receptor kinetics; receptor affinity; tissue affinity; stability; degradation products

## INTRODUCTION

Mometasone furoate (MF) is a highly potent glucocorticoid for topical application. It has been established for the treatment of asthma,[1] allergic rhinitis,[2] and various skin diseases.[3] In clinical asthma studies, the potency of MF appeared to be comparable to fluticasone propionate (FP).[4]

For topically applied glucocorticoids, it is favorable to combine high local efficacy with a low systemic exposure. Therefore, a high receptor affinity and a high retention in the target tissue should be paralleled by rapid and complete hepatic metabolism of the corticosteroids to inactive derivatives. Like FP, MF is a glucocorticoid with a high affinity for the specific glucocorticoid receptor. There has been some discussion about the systemic bioavailability of MF after inhalation, which was reported to be <1%.[5] However, the validity of the conclusion has been questioned.[6,7]

The relative receptor affinity (RRA) of MF has been determined by competition assays with transfected COS-1 cells.[8] The relative affinity to the recombinant glucocorticoid receptor was calculated to be 1235, with a binding affinity of dexamethasone defined as 100. In the same experimental setting the RRA of FP was 813, budesonide displayed a RRA of 258 while triamcinolone acetonide had a RRA of 164. In earlier studies with partially purified glucocorticoid receptors from human lung tissue a RRA of 1800

*Correspondence to*: Petra Högger (Telephone: +49 931 888 5468; Fax: +49 931 888 5494;
E-mail: hogger@pzlc.uni-wuerzburg.de)

Journal of Pharmaceutical Sciences, Vol. 93, 1337–1350 (2004)

was found for FP, 935 for budesonide and 361 for triamcinolone acetonide.[9] Although the absolute values for RRAs might vary to some degree depending on the competitor,[10] the relation of the RRAs to each other reported by Smith and Kreutner[8] was surprising. An alternative method to competition assays is the more precise kinetic method elucidating the association and dissociation rate constants of the glucocorticoid to the receptor. The RRAs can then be calculated based on the equilibrium dissociation rate constants.

The transcriptional activation of a reporter gene revealed that very low concentrations of 0.07 nM MF induced transactivation ($EC_{50}$), while 0.32 nM of FP were needed for the same effect.[8] These results are in contrast to the conclusions of a recent study that demonstrated equivalent transcriptional potencies of MF and FP.[11]

Glucocorticoids' relative potencies can be also precisely determined in *ex vivo* bioassays. The glucocorticoid-induced upregulation of the monocyte specific membrane protein CD163 correlates well with the corticosteroids' potencies.[12] The CD163 protein has been shown to have anti-inflammatory properties itself.[13,14] Relative glucocorticoid potencies are also reflected by their relative immunosuppressive effects that can be determined in *ex vivo* assays.[15]

The binding and retention of MF in human lung tissue has not been elucidated in detail yet. The tissue affinity of an inhaled glucocorticoid is an important compound property because the rate and amount of the corticosteroid, which is redistributed from the lung tissue into systemic circulation, determines undesired drug effects. There has been no information about the stability of MF in human lung tissue or human plasma. A variety of metabolites with binding affinity to the rat glucocorticoid receptor has been identified earlier,[16] but it is not clear if any of these derivatives can be generated locally in therapeutic target tissues and if those metabolites display binding affinity to the human glucocorticoid receptor. In human plasma, unidentified degradation products of MF were detected.[17] Further experiments revealed a pH-dependent degradation of MF in aqueous systems.[18] In rat tissues and fluids metabolism and degradation was observed.[19] However, no information is available about the stability or metabolism of MF in human tissues of therapeutic relevance such as human lung tissue. Furthermore, it is not clear yet which degradation products are formed in human plasma.

In the present study we addressed the open questions concerning receptor binding kinetics, tissue affinity, and stability of MF in human lung tissue and plasma.

## METHODS AND MATERIALS

### Chemicals and Reagents

[$^3$H]-Dexamethasone was obtained from Amersham (Freiburg, Germany), dexamethasone was purchased from Merck (Darmstadt, Germany). [$^3$H]-Fluticasone propionate, [$^3$H]-mometasone furoate, fluticasone propionate (FP), mometasone furoate (MF), 9,11-epoxy mometasone furoate and beclomethasone dipropionate (BDP) were generous gifts from GlaxoSmithKline (Greenford, England). Budesonide was purchased from AB Draco (Lund, Sweden). Amcinonide was obtained from Cyanamid (Wolfratshausen, Germany). Dimethyl-2-2-dichlorvinylphosphate (dichlorvos) was purchased from Riedel de Haën (Seelze, Germany), DL-dithiothreitol (DTT) from Sigma-Aldrich-Chemie (Taufkirchen, Germany). Complete$^{TM}$ (combination of different protease inhibitors) was obtained from Roche Applied Science (Mannheim, Germany), Norit A from Serva (Heidelberg, Germany). Diethylether (HPLC grade) and trifluoroacetic acid (TFA, spectroscopic grade) were purchased from Fluka (Buchs, Switzerland) and acetonitrile (ACN, HPLC gradient grade) from Fisher Scientific, (Schwerte, Germany). Water from a Millipore water purification unit was used. All other chemicals were obtained from E. Merck (Darmstadt, Germany).

### Buffer Solutions

Buffer Solution A contained 10 mM TRIS, 10 mM $NaMoO_4$, 30 mM NaCl, 10% glycerol, 4 mM DTT, 5 mM dichlorvos, and 1 mM Complete$^{TM}$ (protease inhibitor). Krebs-Ringer-HEPES buffer (pH 7.4) consisted of 118 mM NaCl, 4.84 mM KCl, 1.2 mM $KH_2PO_4$, 2.43 mM $MgSO_4$, 2.44 mM $CaCl_2 \cdot 2H_2O$, and 10 mM HEPES. For stability studies of MF phosphate buffers were used: 0.1 M (pH 5.4), 0.2 M (pH 7.4), and 0.2 M buffer (pH 9.0).

### Antibodies

The monoclonal antibody anti CD163 was purchased from Bachem (Weil am Rhein, Germany). The FITC (flourescein-isothiocyanat)-labeled sec-

ondary antibody goat-antimouse $IgG_1$ and the control mouse $IgG_1$ were purchased from Dianova (Hamburg, Germany).

### Isolation of Blood Monocytes

Monocytes were isolated from pooled buffy coats (Blutbank Münster, Germany) by Ficoll-Paque (Pharmacia, Freiburg, Germany) and subsequent Percoll (Pharmacia) density gradient centrifugation. The monocytes purity was >90%. Monocytes were cultured at a density of $2 \times 10^8$ cells/mL in hydrophobic Teflon bags (Hereaus, Hanau, Germany) in McCoy's 5a medium (Biochrom, Berlin, Germany) supplemented with 20% fetal calf serum.

### FACS-Analysis

Monocytes were cultivated for 2 days in presence of different concentrations of the respective glucocorticoid. Cells were washed with cold phosphate-buffered saline (PBS, pH 7.4) and incubated with 1% bovine serum albumine (BSA) for 30 min at 4°C. Then cells were washed and incubated with optimal antibody concentration (5–10 μg/mL) for 45 min at 4°C. Subsequently, monocytes were washed with PBS and incubated with FITC-labeled secondary antibody goat-antimouse $IgG_1$ in 1% BSA for 30 min at 4°C. Propidium iodide was added for the last 3 min of incubation to determine cell viability and exclusion of dead cells. The fluorescence intensity of $10^4$ cells was measured by FACS (fluorescence activated cell sorter)-analysis (FACscan, Becton-Dickinson, Heidelberg, Germany). The parameters used were 488 nm excitation wavelength, 250 mW, and logarithmic amplification. The antigen density and the number of RM3/1 positive cells were obtained from the main fluorescence channel using Lysis Software (Becton Dickinson).

### Source and Handling of Human Specimen

Human lung tissue resection material was obtained from patients with bronchial carcinomas who gave informed consent. Cancer-free tissue was used for the experiments. None of the patients was treated with glucocorticoids for the last 4 weeks prior to surgery. Tissue samples were used immediately for tissue metabolism studies to retain full enzymatic activity. For other experiments, tissue samples were shock frozen in liquid nitrogen after resection and stored at −70°C until usage. To collect sufficient material for the experiments, tissue samples of three or more patients were pooled. Tissue was washed in Krebs-Ringer-HEPES buffer (pH 7.4) and sliced into pieces of 1 $mm^3$. For each binding experiment approximately 0.5 g of lung tissue was used.

Plasma samples were obtained from healthy volunteers who gave informed consent. Samples were used immediately for metabolism studies to retain full enzymatic activity. For desorption and other experiments, plasma samples were shock frozen in liquid nitrogen and stored at −70°C until usage.

### Preparation of Lung Cytosol for Receptor Binding Experiments

Human lung tissue was deep frozen immediately after resection and stored in liquid nitrogen. Frozen tissue was pulverized and homogenized in three aliquots buffer solution A with an Ultra Turrax mixer (Janke and Kunkel, Staufen, Germany) in an ice bath. Thereafter, the diluted cytosol was centrifuged for 1 h at $105{,}000 \times g$ at 4°C (Ultracentrifuge L8-55 M, Beckman Instruments Irvine, CA). The cytosol was stored in aliquots at −80°C. The protein concentration of the cytosol was determined according to the method of Lowry et al.[20] Concentration of glucocorticoid receptors in the cytosol was 30–60 fmol/mg protein.

### Kinetics of Receptor Binding of Glucocorticoids

The receptor binding experiments were performed according to the procedure described earlier.[10] Radiochemical purity of the labeled glucocorticoids was demonstrated by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). Scintillation counting was performed with a Rackbeta 1214 LKB from Wallac (Freiburg, Germany) using Emulsifier-Safe™ from Packard Bioscience (Groningen, The Netherlands).

### Relative Induction of CD163 Expression (Relative Antigen Expression)

For determination of CD163 expression, peripheral blood monocytes were incubated for 1 day with different glucocorticoids, as described previously.[12] Various concentrations were used for each glucocorticoid, antigen expression was evaluated by FACscan analysis. After logit-log transformation

of percentage difference of the number of CD163 positive cells compared to nonstimulated control cells (Y values) the log concentration of glucocorticoid (x-axis) was plotted versus logit (Y). Intersection with the x-axis is found by linear regression, and gives the concentration of glucocorticoid necessary to induce a 50% increase of CD163 density. Relative antigen expression induced by glucocorticoids was calculated with reference to dexamethasone. The relative antigen expression induced by dexamethasone was set 100.

### Stability of Mometasone Furoate (MF) in Human Lung Tissue/Plasma *In Vitro*

MF (0.3 μg/mL) was incubated in 10 mL Krebs-Ringer-HEPES buffer with lung tissue pieces or in 20 mL freshly obtained human plasma at 37°C shielded from light in a thermostatically controlled shaking water bath GFL 1083 (Burgwedel, Germany). At time intervals 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h, samples of 1.0 mL were taken and immediately stored at −20°C until analysis.

### pH-Dependent Degradation of MF in Buffered Solutions

The pH-dependent stability of MF in comparison to beclomethasone dipropionate (BDP) was tested in phosphate buffers (0.1 M pH 5.4, 0.2 M pH 7.4, and 0.2 M pH 9.0) yielding an initial concentration of 0.3 μg/mL. Samples were incubated at 37°C for 24 h in a shaking water bath shielded from light. At time intervals 0, 1, 2, 3, 4, 6, 12, and 24 h samples of 1.0 mL were removed and immediately stored at −20°C until analysis.

### Adsorption of Glucocorticoids to Lung Tissue

Lung tissue pieces were suspended under gentle shaking for 1 h at 37°C in 20 mL Krebs-Ringer-HEPES buffer containing 0.3 μg/mL of MF or FP. 2.0 mL samples were taken after 5, 10, 20, 30, 45, and 60 min and stored at −20°C until analysis. The volume withdrawn was replaced with fresh glucocorticoid-free buffer of 37°C. Only glass lab ware was used for these experiments to avoid any nonspecific binding effects of the highly lipophilic compounds to plastic material. For control, blank samples with glucocorticoid-containing buffer, but no tissue, were incubated under the same experimental conditions (1 h at 37°C) and analyzed for nonspecific adsorption of the corticosteroids to the glass tubes.

### Desorption of Glucocorticoids from Lung Tissue

To determine desorption, lung tissue (1.0 g) was saturated with glucocorticoids for 1 h at 37°C by shaking in 40 mL: Krebs-Ringer-HEPES buffer containing 0.3 μg/mL of the respective glucocorticoid. After incubation tissue was washed with 2 mL buffer and transferred into 10.0 mL human plasma (37°C). Again, only glass lab ware was used for these experiments to exclude any nonspecific binding effects of the highly lipophilic compounds to plastic material. The tissue suspension was gently shaken in a thermostatically controlled shaking water bath. Samples of 1.0 mL were taken after 3, 6, 9, 15, 30, 45, and 60 min. The volume was replaced with fresh plasma of 37°C. Samples were stored at −20 °C until further analysis.

### Sample Preparation, HPLC Conditions, and Data Analysis

Samples of 1.0 (tissue desorption/stability) or 2.0 mL (tissue adsorption) were mixed with 0.1 mL internal standard solution and extracted twice with 3 mL diethylether for 30 min, using a roller mixer, followed by centrifugation at 3000 rpm (20°C) for 5 min. The organic phase was separated and evaporated to dryness under a gentle stream of nitrogen at 25°C. The resulting residue was reconstituted in 0.2 mL mobile phase. Internal standard (IS) was amcinonide 0.4 μg/mL (tissue binding studies) or dexamethasone 0.4 μg/mL (stability studies). Linearity was given from 10–500 ng/mL glucocorticoid, coefficients of correlation of the calibration curves were at least 0.99.

The HPLC system was a Waters HPLC (Milford, MA) consisting of an 1525 binary pump, an 717plus autosampler, and 2487 dual wavelength absorbence detector set at the detection wavelength of 254 nm. Data collection and integration were accomplished using Breeze™ software version 3.2. Analysis was performed on an Symmetry $C_{18}$ column (150 × 4.6 mm I.D., 5 μm particle size, Waters, MA). Typically, 20 μL of sample were injected and separated at a flow rate of 1 mL/min. Gradient elution was performed using water (containing 0.2% (v/v) acetic acid) and ACN, starting at 60:40 (v/v) water/ACN increasing linearly to 29:71 (v/v) water/ACN by 30 min. The assay was accurate and reproducible. The lower limit of quantitation was 10 ng/mL for all glucocorticoids.

### HPLC-ESI-MS-MS

Mometasone furoate (MF) and its decomposition product (MF-9,11-epoxide) were isolated according to the liquid–liquid extraction method as described above. The HPLC-ESI-MS-MS analysis of MF and MF-9,11-epoxide was performed on a Finnigan TSQ 7000 triple-stage quadrupol mass spectrometer (Finnigan MAT, Bremen, Germany) equipped with a Finnigan electrospray ionization interface (ESI) source operated in positive ion mode. Data acquisition and evaluation were conducted on a Personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) and ICIS 8.1 software (Finnigan MAT). Nitrogen served both as sheath and auxiliary gas; argon (Ar) served as collision gas. For ESI-MS-MS analysis electrospray ionisation parameters were optimized as follows: temperature of the heated capillary (200°C) serving simultaneously as repeller electrode (30 V), electrospray capillary voltage (3.5 kV), sheath gas (60 p.s.i.; 1 p.s.i. = 6894.76 Pa) and auxiliary gas (10 units). Positive ions were detected scanning from 155 to 1000 amu with a total scan duration of 1.0 s. MS-MS experiments were performed at a collision gas pressure of 267 mPa Ar and a collision energy of 30 eV, scanning a mass range from 10 to 600 amu with a total scan duration of 3.0 s.

For HPLC-ESI-MS-MS chromatographic separation was performed on an Eurospher 100 C18 column (150 × 2.0 mm i.d., 5 μm) (Knauer, Berlin, Germany) with a binary gradient delivered by an Applied Biosystems 140b pump. Solvent A was 0.05% (v/v) TFA in water, solvent B was 0.05 % (v/v) TFA in ACN. The flow-rate was 200 μL/min, the volume of injection was 5 μL.

#### *MF*

ESI-MS, $[M+H]^+$ $m/z$ 521; ESI-MS/MS (precursor ion $m/z$ 521, 30 eV, 267 mPa Ar), $m/z$ 521 (10), 503 (40), 410 (10), 391 (40), 373 (25), 355 (95), 301 (65), 278 (95), 263 (90), 171 (55).

#### *MF-9,11-Epoxide*

ESI-MS,$[M+H]^+$ $m/z$ 485; ESI-MS/MS (precursor ion $m/z$ 485, 30 eV, 267 mPa Ar), $m/z$ 485 (15), 467 (20), 373 (20), 355 (75), 301 (35), 278 (100), 263 (50), 171 (35).

### $^{13}$C- and $^{1}$H-NMR

Mometasone furoate (MF) and its decomposition product (MF-9,11-epoxide) were isolated according to the liquid–liquid extraction method as described above. NMR-spectroscopic data were acquired using a Bruker Avance 400 MHz NMR spectrometer, equipped with XWIN-NMR software (Version 3.0 Bruker Analytik GmbH, Rheinstetten, Germany). $^{1}$H-NMR spectra were obtained at 400.13 MHz and $^{13}$C spectra at 100.62 MHz. $CDCl_3$ was used as a solvent, tetramethylsilane (TMS) as an internal standard. $^{1}$H- and $^{13}$C-NMR data of MF and MF-9,11-epoxide were determined as follows.

#### *MF*

$^{1}$H-NMR: 0.98 (3H, d, $J = 7.08$ Hz, $C_{16}-CH_3$), 1.14 (3H, s, $C_{13}-CH_3$), 1.32 (1H, m, $H_{14}$), 1.68 (3H, s, $C_{10}-CH_3$), 1.72 (2H, dd, $J = 2.27$ Hz, 14.12 Hz, $C_{12}$), 1.94–1.76 (3H, m, $H_{15}$ (+1H from $C_7$), 2.42 (1H, dd, $J = 14.64$ Hz; 3.54 Hz, (2H from $C_7$) (split), 2.66 (3H, m, $H_8$, and $H_6$), 2.91 (1H, dd, $J = 14.4$ Hz; 3.29 Hz, $C_{11}-OH$), 3.46 (1H, m, $C_{16}$), 4.13 (2H, s, $H_{21}$), 4.63 (1H, s, $H_{11}$), 6.12 (1H, s, $H_4$), 6.36 (1H, dd, $J = 1.77$ Hz, $H_{4'}$), 7.17 (1H, d, $J = 2.78$ Hz,$H_1$), 7.24 (1H, d, $J = 2.78$ Hz, $H_{3'}$), 7.63 (1H, d, $J = 1$ Hz, $H_{5'}$). $^{13}$C-NMR: δ 196.8 $C_{20}$, 186.3 $C_3$, 165.2 $C_5$, 158.1 $C_{OCO}$, 151.5 $C_1$, 147.7 $C_{5'}$, 143.0 $C_{2'}$, 129.8 $C_2$, 125.3 $C_4$, 119.6 $C_{4'}$, 112.3 $C_{3'}$, 97.4 $C_{17}$, 82.8 $C_9$, 75.2 $C_{11}$, 49.8 $C_{10}$, 48.8 $C_{13}$, 44.7 $C_{21}$, 43.7 $C_{14}$, 36.7 $C_{12}$, 35.8 $C_{16}$, 34.5 $C_8$, 32.9 $C_{15}$, 30.6 $C_6$, 27.2 $C_7$, 24.5 $C_{19}$, 17.7 $C_{18}$, 16.7 $C_{22}$.

#### *MF-9,11-Epoxide*

$^{1}$H-NMR: 0.93 (3H, d, $J = 7.07$ Hz, $C_{16}-CH_3$), 1.02 (3H, s, $C_{13}-CH_3$), 1.48–1.39 (2H, m, $H_{14}$) (not clear), 1.46 (3H, s, $C_{10}-CH_3$), 1.73 (1H, q, $J = 11.70$ Hz, $H_{15}$), 1.86 (1H, bs, $J = 11.12$ Hz; 2.02 Hz, $H_{14}$), 2.06 (1H, dd, $J = 14.64$ Hz; 2.02 Hz, $C_{12}$), 3.30 (1H, s, $H_{11}$), 3.39 (1H, m, $C_{16}$), 4.11 (2H, s, $H_{21}$), 6.22 (1H, s, $H_4$), 6.25 (1H, dd, $J = 1.77$ Hz, $H_2$), 6.57 (1H, dd, $J = 1.77$ Hz, $H_{3'}$), 6.62 (1H, d, $J = 9.85$ Hz, $H_1$), 7.26 (1H, dd, $J = 1.48$ Hz, $H_{4'}$), 7.66 (1H, d, $J = 1.0$ Hz, $H_{5'}$). $^{13}$C-NMR: δ 196.3 $C_{20}$, 186.3 $C_3$, 164.7 $C_5$, 158.0 $C_{OCO}$, 151.9 $C_1$, 147.5 $C_{5'}$, (142.8 $C^*_{2'}$), 128.3 $C_2$, 125.0 $C_4$, 119.9 $C_{4'}$, 112.3 $C_{3'}$, 97.6 $C_{17}$, 66.1 $C_9$, 62.7 $C_{11}$, 48.8 $C_{14}$, 47.9 $C_{10}$, 45.1 $C_{21}$, 44.0 $C_{13}$, 32.5 $C_{12}$, 35.6 $C_{16}$, 34.5 $C_8$, 31.3 $C_{15}$, 31.1 $C_6$, 29.6 $C_7$, 23.6 $C_{19}$, 17.7 $C_{18}$, 16.7 $C_{22}$.

## RESULTS

### Receptor Binding Kinetics and Relative Receptor Affinity of Mometasone Furoate (MF)

Specific binding of MF to the human lung glucocorticoid receptor occurred rapidly and to a

**Table 1.** Results of the Kinetic Binding Experiments of Dexamethasone (Dexa), Budesonide (Bud), Fluticasone Propionate (FP), and Mometasone Furoate (MF) to the Human Lung Glucocorticoid Receptor

| Glucocorticold | Association $k_1 \times 10^5$ (L/mol/min) | Dissociation $k_{-1} \times 10^{-4}$ (L/min) | $k_d$ (nmol/L) | RRA | $t_{1/2}$ (h) |
|---|---|---|---|---|---|
| Dexa | $10.53 \pm 0.35$ | $94.67 \pm 5.43$ | $8.80 \pm 0.41$ | $100 \pm 5$ | $1.23 \pm 0.04$ |
| FP | $21.17 \pm 0.56$ | $10.73 \pm 0.65$ | $0.51 \pm 0.03$ | $1775 \pm 130$ | $10.82 \pm 0.64$ |
| MF | $29.46 \pm 1.10$ | $11.82 \pm 0.31$ | $0.41 \pm 0.03$ | $2244 \pm 142$ | $9.83 \pm 0.53$ |
| Bud | 18.9 | 25.0 | 1.32 | 855 | 4.6 |

Values given represent mean and mean deviation of the mean of at least four experiments. Binding data of Bud were derived from our previous experiments (Ref. 24). Abbreviations: $k_1$, association rate constant; $k_{-1}$, dissociation rate constant; $k_d$, equilibrium dissociation rate constant; RRA, relative receptor affinity; $t_{1/2}$, calculated half life of the receptor complex ($t_{1.2} = \ln 2/k_{-1}$).

higher extend compared to FP (FP) and the reference glucocorticoid dexamethasone. Thus, the calculated association rate constant MF was higher than the association rate constant of FP (Table 1). In contrast, the dissociation from the human glucocorticoid receptor was faster for MF compared to FP. The dissociation rate constants were assayed in duplicates and the mean was used to calculate the equilibrium dissociation rate constants $k_d$. The $k_d$ of MF was determined as 0.41 nmol/L, the $k_d$ of FP was 0.51 nmol/L and the $k_d$ of dexamethasone was 8.80 nmol/L (Table 1). The equilibrium dissociation rate constants of FP and dexamethasone were consistent with results obtained earlier.[10] The calculated half-lives of the receptor complexes confirmed previous results with 1 h for dexamethasone and about 11 h for FP. The half-life of the MF receptor complex revealed to be about 10 h. Based on the kinetic constants the relative receptor affinity (RRA) of MF was calculated as 2200.

## Induction of the Glucocorticoid-Regulated Anti-inflammatory Protein CD163 by MF

The intrinsic activity of MF in comparison to other glucocorticoids was determined by evaluation of the induction of the glucocorticoid-regulated protein CD163. Freshly isolated human monocytes were stimulated with different concentrations of glucocorticoids and induction of the membrane-bound CD163 was determined by FACS analysis. Glucocorticoid concentrations that induced a 50% increase in CD163 density at the monocytes' membrane were calculated and compared to reference glucocorticoids as described earlier.[12] Three series of experiments were performed; an example of a representative result is given in Figure 1. Lowest concentrations of MF and FP were necessary to induce a 50% increase in antigen density, while significantly higher concentrations of budesonide were required for the same effect. As expected, highest concentrations of dexamethasone had to be added to the monocytes' cultures to observe a half-maximal induction of CD163 expression.

The relationship between the relative receptor affinity of dexamethasone, budesonide, FP, and MF and their *in vitro* activity defined as induction of CD163 was determined (Fig. 2). A statistically significant correlation was revealed between both descriptors with coefficients of correlation $r > 0.99$ ($p = 0.01$). This plot was calculated based on a relative receptor affinity of 2200 for MF that was derived from the kinetic experiments. If the same plot was calculated with data for relative receptor affinity and protein induction of dexamethasone, budesonide, and FP only, the correlation was still

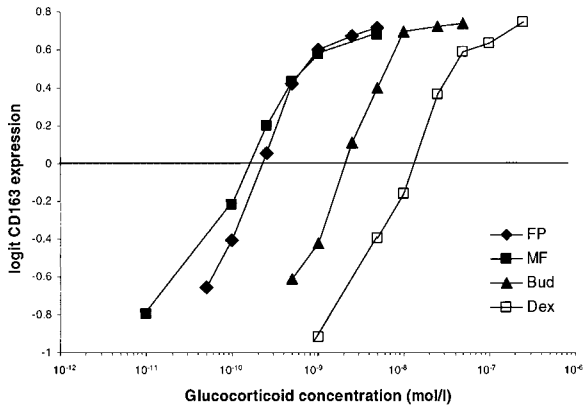


**Figure 1.** Dose–response curves of glucocorticoids upregulating the expression of the anti-inflammatory protein CD163. The density of CD163 expression on the membrane surface of human monocytes was determined by FACS analysis and logit transformed. FP: fluticasone propionate, MF: mometasone furoate, Bud: budesonide, Dex: dexamethasone.

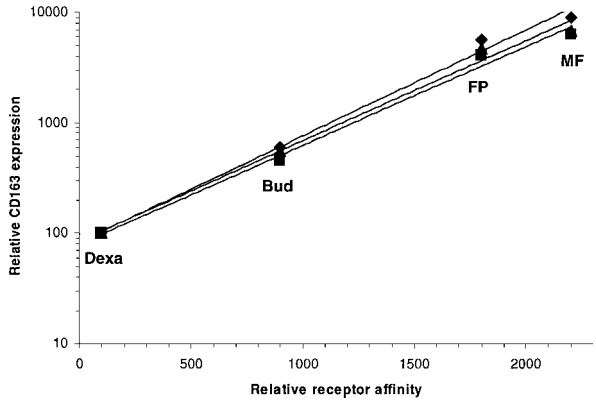


**Figure 2.** Interrelationship between the relative receptor affinity of glucocorticoids and their potency in upregulation CD163 expression. Three series of experiments were performed to determine the relative protein induction, and results were correlated with the relative receptor affinities of the respective glucocorticoids. Coefficients of correlation were better than 0.99 for each series ($p = 0.01$).

better than $r > 0.99$ ($p = 0.05$, figure not shown). In this case, the relative receptor affinity of MF could be determined solely based on the experimental data on its relative antigen induction. For the three series of experiments, the calculated mean relative receptor affinity of MF is $2004 \pm 20$. This confirms the affinity determined by receptor kinetics.

### Stability of MF in Human Lung Tissue and Plasma

The decomposition of MF in the presence of human lung tissue was monitored over a period of 24 h at an incubation temperature of 37°C (Fig. 3, upper panel). Samples of the tissue-free supernatant were analyzed for possible degradation products. The initial steep decrease of MF concentration indicated the binding to the lung tissue pieces. This binding to tissue structures should be completed after about an hour, as demonstrated in the tissue binding experiments. However, there is steady decrease in MF concentration in the incubation supernatant that slows down after 2 h but continues until the end of the incubation period. Only small new peaks below the limit of quantitation were observed in the HPLC chromatograms. The addition of the esterase inhibitor dichlorvos to the incubation mixture did not have any effect on the concentration of the MF parent compound. Thus, no nonspecific esterase-catalyzed hydrolysis of MF occurred in

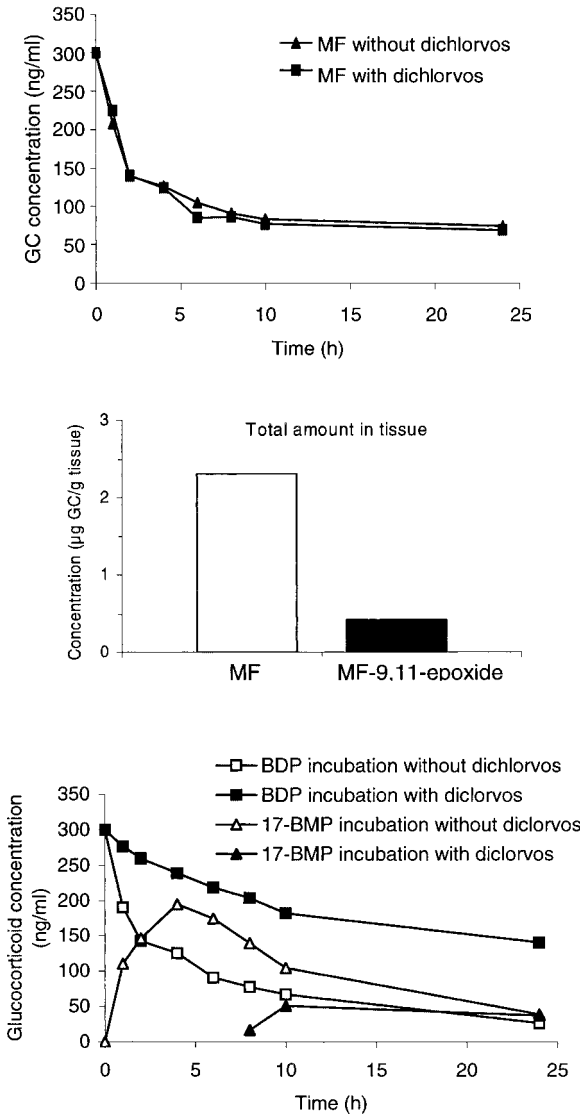


**Figure 3.** Stability of glucocorticoids in human lung tissue suspensions of 37°C over 24 h. Symbols represent the mean of two independent series of experiments. One incubation mixture contained the esterase inhibitor dichlorvos to determine a potential esterase mediated decomposition of the parent compound. Upper panel: stability of mometasone furoate (MF) with and without addition of dichlorvos in lung tissue supernatant. Middle panel: total amounts of compounds extracted from the tissue after termination of the incubation period. Lower panel: stability of beclomethasone dipropionate (BDP). Without dichlorvos rapid enzyme-mediated degradation of BDP occurred, and the metabolite beclomethasone-17-monopropionate (17-BMP) was detected.

the presence of human lung tissue. Extraction and analysis of the lung tissue after the 24-h incubation period revealed 2.31 µg MF per gram tissue and 0.42 µg/g of a decomposition product

that was later identified as MF-9,11-epoxide (Fig. 3, middle panel). The enzymatic integrity of the lung tissue was demonstrated in a simultaneously performed control experiment with beclomethasone dipropionate (BDP) (Fig. 3, lower panel). Without the addition of the esterase inhibitor dichlorvos BDP was rapidly hydrolyzed and the main metabolite beclomethasone-17-monopropionate (17-BMP) was detectable at high concentrations. Dichlorvos inhibited the decomposition of BDP and delayed the formation of 17-BMP up to 10 h of incubation.

The incubation of MF in fresh human plasma at 37°C revealed the formation of a major degradation product (Fig. 4). This decomposition product was not generated by esterase activity because the amounts detected were the same in the presence and absence of dichlorvos. As in lung tissue, the decomposition product was later identified as MF-9,11-epoxide. Interestingly, in plasma obviously occurred some esterase-dependent degradation after 10 h of incubation. Although higher concentrations of MF parent compound were detected in the presence of dichlorvos, no additional peak was identified in the HPLC chromatograms with the chosen chromatographic conditions. The enzymatic integrity of the fresh plasma was demonstrated in a simultaneously performed control experiment with BDP. Without addition of dichlorvos BDP was rapidly degraded in plasma as it was seen in tissue before.

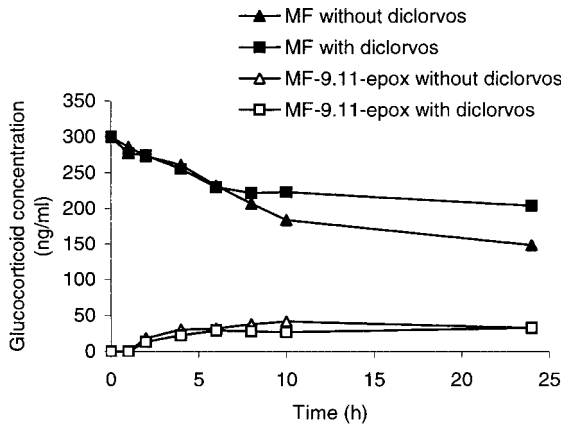


**Figure 4.** Stability of mometasone furoate (MF) in plasma of 37°C over 24 h. Symbols represent the mean of three independent series of experiments. One incubation mixture contained the esterase inhibitor dichlorvos to determine a potential esterase-mediated decomposition of the parent compound. Formation of the 9,11-epoxy MF degradation product was detected.

### Identification of a Major MF Degradation Product

HPLC-ESI-MS analysis revealed molecular ions $m/z$ 521 $[M+H]^+$ for MF and $m/z$ 485 $[M+H]^+$ for MF-9,11-epoxide. The product ion spectra as obtained by collision-induced dissociation showed similar fragmentation patterns. Spectra were consistent with those of authentic MF or authentic MF-9,11-epoxide, respectively.

Further confirmation of the structure assignment was derived from the HPLC-MS/MS analysis of the MF decomposition product, which was isolated and intensively characterized after chromatographic purification by NMR spectroscopy including DEPT, HH-COSY, HMBC, and HMQC experiments. Except for the $^1$H- and $^{13}$C-NMR data, literature information was available.[21] $^1$H- and $^{13}$C-NMR data were consistent with authentic MF and authentic MF-9,11-epoxide standards. The $^1$H-NMR data of MF and MF-9,11-epoxide are shown in Figure 5. Thus, the formation of MF-9,11-epoxide was clearly proved by HPLC-MS/MS and NMR spectroscopy.

### pH-Dependent Formation of the Degradation Product MF-9,11-Epoxide

For further characterization of the formation of the MF degradation product MF-9,11-epoxide we determined the pH dependency of its formation. For comparison, we monitored the degradation of beclomethasone dipropionate (BDP) that shares the structural similarity of a 9α-chlorine substitution and is known to form a 9,11-epoxide.[22,23] Degradation was monitored over 24 h at pH 5.4, pH 7.4, and pH 9.0 (Fig. 6, $n = 6$ for each data point). At pH 5.4 neither MF nor BDP formed 9,11-epoxides over 24 h. In contrast, at the physiological pH of 7.4 MF-9,11-epoxide was detectable after 1 h of incubation and rapidly increasing concentrations were found up to 24 h. After 12 h, equivalent amounts of MF parent compound and its degradation product MF-9,11-epoxide were present in the incubation mixture. On the contrary, BDP-9,11-epoxide was detected after 2 h of incubation at pH 7.4 and formation of the 9,11-epoxide was significantly accelerated up to 24 h. The much more rapid epoxide formation for MF was confirmed by the degradation experiments at pH 9.0. Immediate rapid formation of MF-9,11-epoxide was seen and the MF parent compound was no longer detectable after 3 h. Only trace amounts of BDP-9,11-epoxide were found after 1 h and the BDP parent

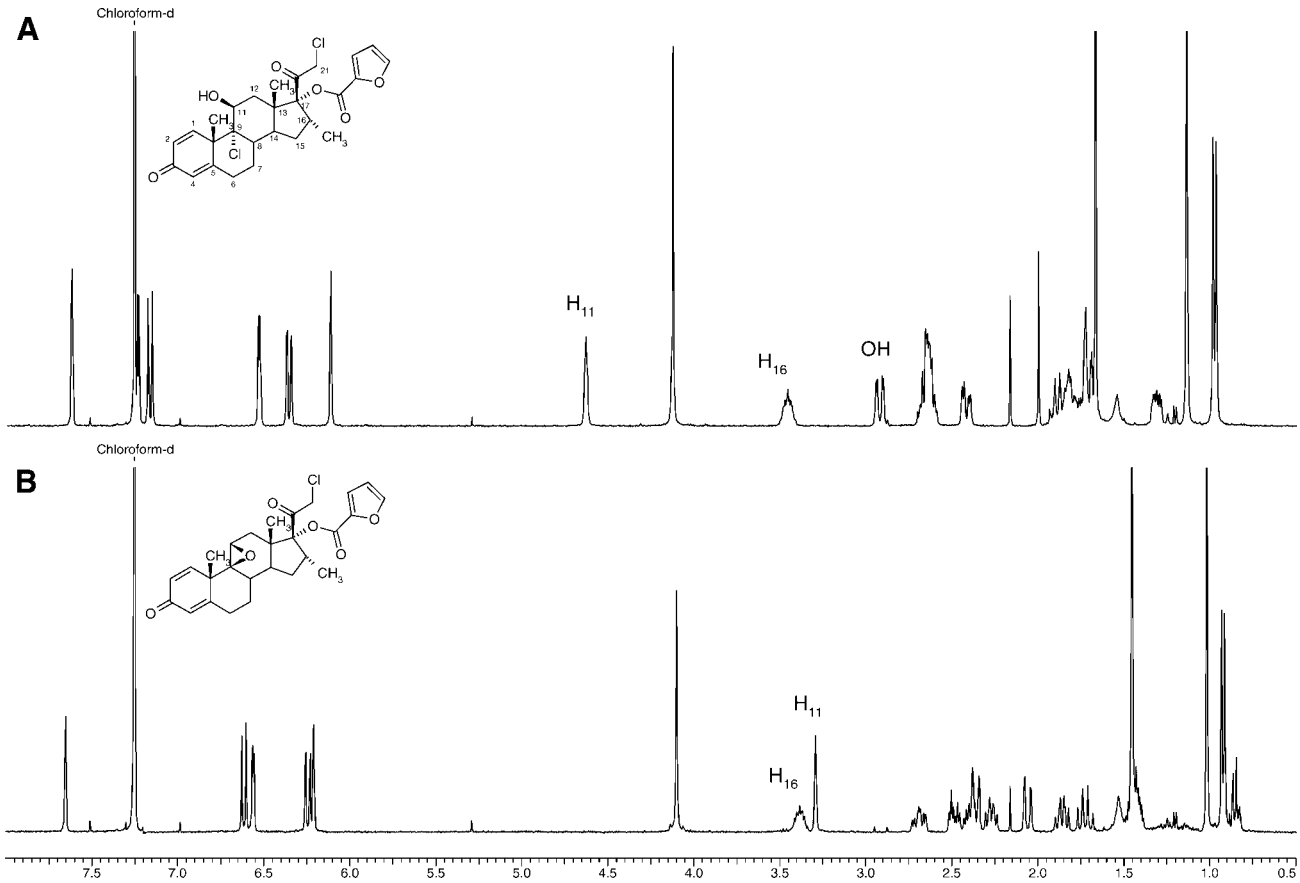


**Figure 5.** $^{1}$H-NMR data of mometasone furoate (A) and its degradation product 9.11-epoxy mometasone furoate (B).

compound remained detectable over 24 h of incubation.

### Tissue Affinity of MF

The binding affinity of MF in comparison with FP to human lung tissue was determined in separate adsorption and desorption experiments. A control experiment with glucocorticoid-containing buffer solutions that was incubated in parallel under identical conditions revealed that there was no nonspecific binding of neither MF nor FP to the glass incubation vials (data not shown). Adsorption of MF to lung tissue occurred fast, and was completed after about 20 min (Fig. 7, upper panel). The binding curves of MF to the tissue are almost super imposable with the adsorption curve obtained with FP. Thus, both glucocorticoids displayed a comparable *in vitro* binding affinity to human lung tissue at 37°C. In contrast, the desorption curves revealed clear differences between both compounds (Fig. 7, lower panel). Although MF was rapidly redistributed from the lung tissue into human plasma, the dissociation of FP was considerably slower, and lower concentrations were liberated into plasma. Consequently, the concentration of MF remaining in human lung tissue after 1 h incubation in human plasma is surprisingly low compared to FP (Fig. 8).

## DISCUSSION

Mometasone furoate (MF) has been introduced as a topical glucocorticoid for the treatment of asthma, allergic rhinitis, and inflammatory skin disorders.[1–3] Although MF demonstrated high anti-inflammatory activity in clinical studies, surprisingly little precise information is available on basic molecular characteristics of the compound such as receptor binding kinetics, formation and activity of metabolites, or degradation

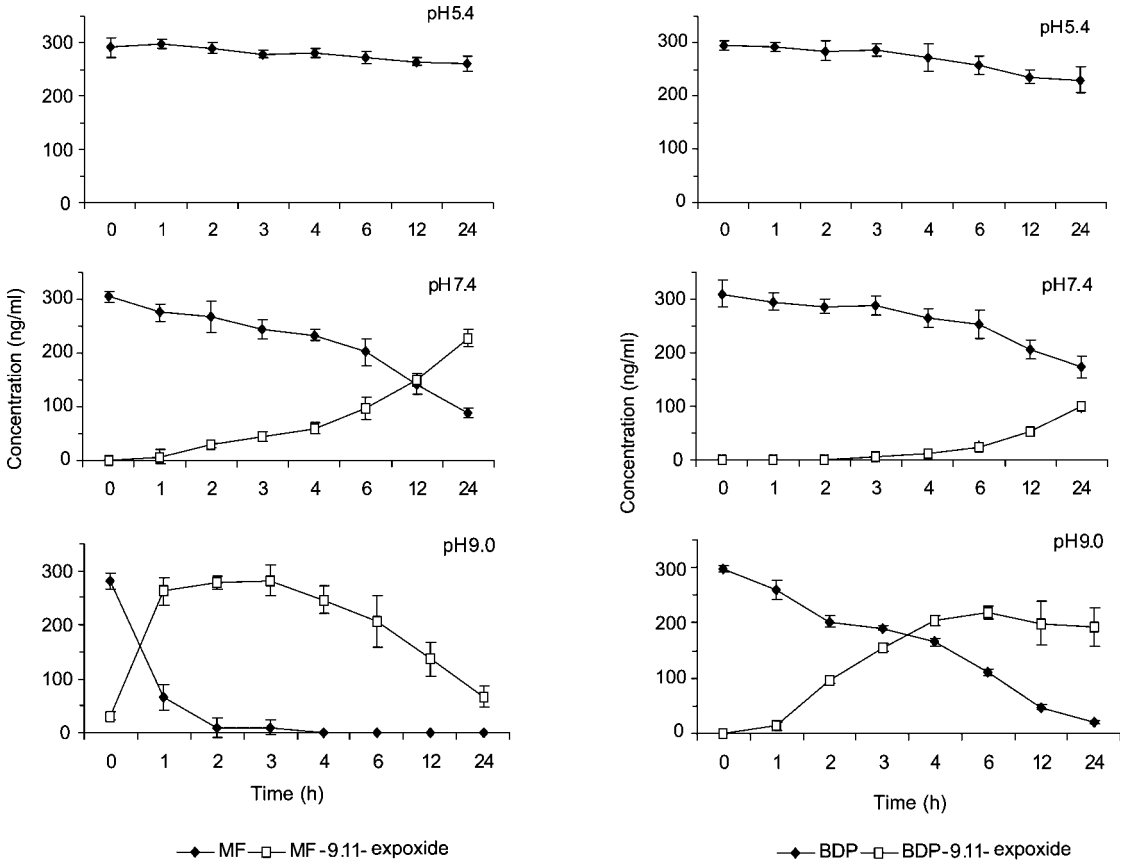


**Figure 6.** Determination of pH-dependent degradation of mometasone furoate (MF) and beclomethasone dipropionate (BDP) in buffer solutions of pH 5.4, pH 7.4, and pH 9.0 at 37°C over 24 h. Each data point represents the mean and standard deviation of six independent experiments. Both glucocorticoids formed 9,11-epoxy degradation products.

products in humans and tissue binding affinity. In the present study we supplement some of the lacking information and present new aspects of inherent molecule attributes that might be relevant for its clinical use.

The potency of glucocorticoids is directly related to their agonistic receptor affinity. For the clinical efficacy of inhaled or intranasally applied glucocorticoids the dose, percentage of lung deposition, and pulmonary residence time determine the therapeutic airway effects additionally to the compound's potency. The relative receptor affinity (RRA) of MF has been elucidated by competition assays in COS cells transfected with the human glucocorticoid receptor.[8] The results of these experiments suggested an RRA relationship of 1:3 for budesonide and FP. This was surprising because a 1:2 receptor affinity ratio was repeatedly determined earlier.[9,10,24] This relative potency of budesonide and FP of 1:2 is supported by clinically recommended doses for equivalent efficacy.[25] Based on the results of Smith and Kreutner, a receptor affinity relationship of 1:1.5 has to be assumed for FP and MF.[8] This, however, is in contrast to clinically observed effects that suggested equipotency of MF and FP in the treatment of asthma[4] and allergic rhinitis.[26] In the present study, we investigated the RRA of MF and FP by a kinetic receptor assay that involves separate determination of association and dissociation rate constants. Especially for glucocorticoids with very high receptor affinities the more complex kinetic receptor assays are superior to competition assays and give results of higher precision.[10] Employing this assay we confirmed the RRA of 1800 for FP and determined a RRA of 2200 for MF. This translates into a receptor affinity relationship of 1:1.2 for FP:MF, which is consistent with the clinically observed potency of both compounds.

The receptor kinetics of MF revealed some unexpected characteristics. Glucocorticoids such as dexamethasone, methylprednisolone, or triam-

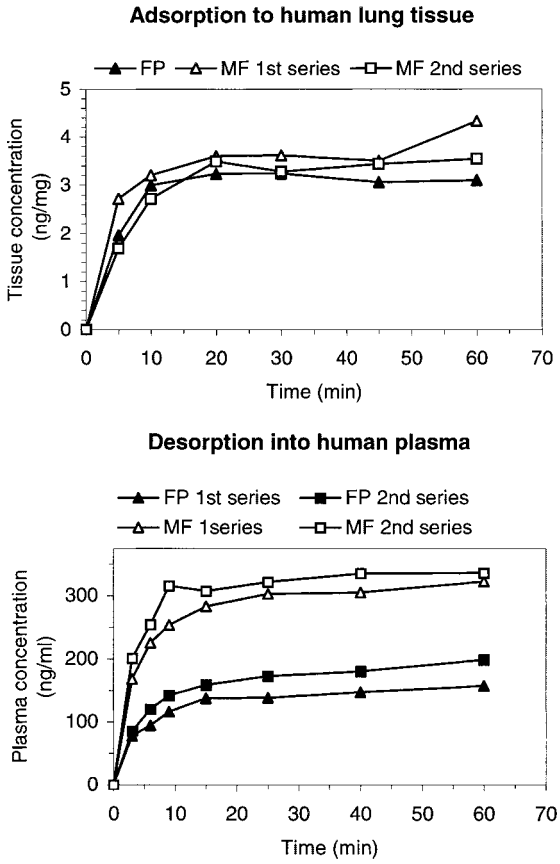


**Figure 7.** Adsorption of fluticasone propionate (FP) and mometasone furoate (MF) to human lung tissue pieces over 1 h at 37°C (upper panel) and time course of desorption of fluticasone propionate (FP) and mometasone furoate (MF) from human lung tissue pieces into human plasma at 37°C (lower panel).

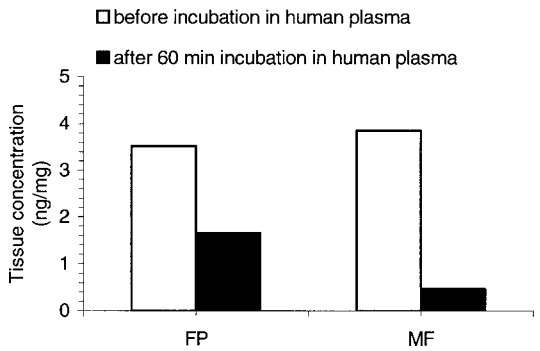


**Figure 8.** Comparison of concentrations of fluticasone propionate (FP) and mometasone furoate (MF) in human lung tissue. The first column represents the compound concentration in tissue before incubation in human plasma. The second column displays the glucocorticoid concentration remaining in the lung tissue after one hour incubation in human plasma at 37°C.

cinolone acetonide have comparable association rate constants while they exhibit clearly different dissociation rate constants.[27] Thus, the different receptor affinities of these compounds are mainly determined by their dissociation behavior. This was assumed to be a universal principle applicable to all corticosteroids. This view was challenged, however, when the kinetic constants of glucocorticoids with significantly higher receptor affinity such as FP were resolved.[10] Besides the expected slow dissociation from the receptor an unexpected fast and extensive binding to the receptor was discovered. Thus, both the association and dissociation rate constants of FP were considerably different from other glucocorticoids and both contributed to the resulting high receptor affinity. The receptor binding kinetics of MF now discloses another variation of the issue. The higher affinity of MF to the human lung glucocorticoid receptor is predominantly determined by its high association rate constant while its dissociation rate is slightly faster compared to FP. Consequently, the calculated half-lives of the glucocorticoid receptor complex are higher for FP with 11 h than for MF with 10 h. The calculated half-life of the FP–receptor complex in agreement with our previous results.[10]

The RRA should translate directly into the measured potency of these topically applied glucocorticoids. Smith and Kreutner evaluated ligand-induced transcriptional activation of a reporter gene and found an $EC_{50}$ 0.07 nM for MF, 0.32 nM for FP, and 1.2 nM for budesonide, which suggests vast differences between the individual compounds.[8] In contrast, Roumestan et al. recently reported equivalent transcriptional activities of MF and FP.[11] In the present study, we determined the glucocorticoid concentrations necessary to induce a 50% upregulation of the anti-inflammatory protein CD163 on the membrane surface of human monocytes. We calculated effective concentrations of, for example, 0.144 nM for MF, 0.227 nM for FP, 2.12 nM for budesonide, and established a statistically significant exponential interrelationship between the RRA and the relative protein expression (Fig. 2). Using this correlation for predicting the RRA of MF solely based its efficacy in CD163 upregulation we calculated a mean RRA of 2004. This calculated receptor affinity derived from the *in vitro* potency of MF is impressively coherent with the experimentally determined affinity to the human glucocorticoid receptor.

So far, there has been no published data on the stability of MF in the therapeutic target tissue, for

example, human lung. There was some information about so far unidentified degradation products of MF in human plasma and simulated lung fluid.[17] In aqueous buffer solutions degradation pathways such as epoxide formation, hydrolysis, and ester group migration were observed.[18] In rat extrahepatic tissues and fluids only slow degradation of MF into four products was reported.[19] We aimed at the monitoring of MF stability in human specimen over a time course of 24 h. We assumed that any degradation products observed after this time period would be not relevant *in vivo*. A selective and sensitive HPLC assay was developed. The lower limit of quantitation was 10 ng/mL, which is an order of magnitude more sensitive than the assay described earlier.[17] In fresh human lung tissue with unimpaired enzymatic activity we observed binding of the MF to the tissue pieces, but no degradation products were identified in the incubation supernatant. Esterase-mediated decomposition of MF was not seen in this specimen. However, extraction and analysis of the tissue pieces after the incubation period revealed formation of significant amounts of 9,11-epoxy MF, while no other degradation were detectable.

In accordance with the results of Teng et al.[17] decomposition was observed in freshly obtained human plasma. One degradation product that was detectable early in our plasma samples was again identified as 9,11-epoxy MF. No additional degradation products were identified under the chosen chromatographic conditions, the formation of 6-OH-MF and mometasone were excluded by injection of the respective control compounds. The 9,11-epoxy-MF was formed via a nonenzymatic pathway by elimination of HCl. The pH dependency of MF degradation in aqueous systems has been described recently,[18] and can be confirmed by our own observations. In a control experiment, we monitored the degradation of beclomethasone dipropionate (BDP). BDP shares the structural feature of a 9α-chlorine substitution with MF. This substitution is obviously prone to elimination reactions, while molecules with a 9α-fluorine such as dexamethasone remained stable over hours under alkaline conditions (data not shown). The formation of 9,11-epoxy BDP has been described earlier.[22,23] We now provide data on the comparison of the decomposition kinetics of BDP and MF. Degradation of MF started early and was much faster than seen with BDP.

Although significant and steadily increasing amounts of 9,11-epoxy MF were detectable in buffer of pH 7.4, this was not observed in the plasma sample or lung tissue supernatant. In plasma, 9,11-epoxy MF was detected 1 h later than in buffer and the concentration of this degradation product remained stable after 3 h of incubation. Simultaneously, however, the decomposition of the parent compound MF advanced steadily. It must be assumed that 9,11-epoxy MF was subject to follow-up reactions, and that the stable concentrations in the incubation mixture reflected a steady-state balance between formation and further reaction of this compound. Similarly, a steady decomposition of the parent compound MF was observed in the lung tissue supernatant while no increasing concentration of a decomposition product was detectable. The lung tissue contained 9,11-epoxy MF and MF in a 1:5 ratio after the end of the incubation period. Again, it cannot be excluded that earlier formed 9,11-epoxy MF was subject to follow-up reactions. Generally, epoxides are reactive moieties that tend to bind covalently to other molecules or structural elements. 9,11-Epoxy MF might react with, for example, amino groups of plasma or tissue proteins. In this case, the adduct would be no longer extractable by organic solvents, and hence, not detectable any more. Indeed, in first experiments with authentic 9,11-epoxy MF we determined that $9.14 \pm 2.3\%$ was irreversibly bound in the presence of plasma after 3 h of incubation at 37°C. Even higher rates of $16.6 \pm 0.6\%$ were no longer extractable after incubation with lung tissue (data not shown). A possible formation of covalent MF protein adducts, however, raises the possibility of a latent allergen formation with MF serving as hapten. This should be explored in more detail.

The final aspect we evaluated in the present study is the tissue binding affinity of MF in comparison to FP. It is favorable for an inhaled glucocorticoid to display a high affinity to lung tissue because the redistribution into systemic circulation is slowed down. The liberation of low concentrations of glucocorticoids into plasma over a prolonged period of time seems to reduce the incidence of adverse effects.[28] It has been demonstrated earlier that FP has a pronounced affinity to human lung and nasal tissue (Ref. 29, and references therein). Notably, our *in vitro* data were impressively confirmed in clinical studies we participated in.[30,31] These *in vivo* studies evaluated lung and nasal tissue concentrations after pulmonary or intranasal application of glucocorticoids. Thus, these *in vitro* binding assays seem to be suitable to predict the *in vivo* tissue binding and redistribution characteristics of topically applied

glucocorticoids. Therefore, we now determined the adsorption of MF and FP to human lung tissue and monitored the desorption process from the tissue pieces into human plasma of 37°C. The time course and extent of nonspecific binding of MF to lung tissue was comparable to the binding of FP. This was an anticipated result because FP and MF both are highly lipophilic molecules and this physicochemical property is an important determinant for tissue affinity. However, the desorption experiments revealed an unexpected redistribution behavior of MF from lung tissue into plasma. Although low amounts of FP were liberated into plasma, considerably higher concentrations of MF were desorbed from the tissue and detectable in plasma. Comparing the tissue concentrations of MF and FP after 60 min incubation of the saturated lung tissue in human plasma of 37°C MF is found at only one-third of the FP concentration in tissue. Thus, the tissue affinity of MF is significantly lower compared to FP. After inhalative application of MF and FP clearly higher concentrations of MF should be detected in the systemic circulation.

This result is especially surprising in the context of the ongoing discussion about the systemic bioavailability of MF. It has been suggested that the systemic exposure after inhalation of MF is about 1% after a single dose of 400 μg.[5] However, higher plasma concentrations were measured after repetitive inhalative dosing and suppression of the HPA axis was noted.[32] Thus, the low pulmonary bioavailability of MF has been questioned.[7,33] Based on our observation of a rather low *in vitro* tissue affinity of MF and the formation of a degradation product we support the latter view. We predict a considerable *in vivo* pulmonary bioavailability and substantial decomposition of MF with one or more (re)active metabolites. This property of MF seems to be different from other topically applied glucocorticoids such as budesonide or FP, which do not form active metabolites. According to early investigations of Isogai et al. multiple metabolites and related compounds of MF display binding activity to the glucocorticoid receptor.[16] Among these compounds 9,11-epoxy MF revealed an about 10-fold lower binding affinity to the rat glucocorticoid receptor compared to MF parent compound. This would translate into a significant receptor affinity of about 220, which is still about twice as high as for dexamethasone. The 6β-OH MF, a metabolite that is most probably generated in the liver,[19] should display an even higher residual binding affinity to the glucocorticoid receptor than 9,11-epoxy MF.[16] This issue should be clarified by a clinical trial measuring the MF parent compound as well as metabolites and degradation products. Furthermore, it would be essential to determine the binding affinity of all metabolites and degradation products identified *in vivo*.

## ACKNOWLEDGMENTS

We would like to thank Roswitha Skrabala for expert technical assistance. Parts of this study were supported by Fonds der Chemischen Industrie (FCI) and by GlaxoSmithKline.

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