

# Identification of Novel in vitro Test Systems for the Determination of Glucocorticoid Receptor Ligand-Induced Skin Atrophy

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## Key Words

In vitro test systems · Glucocorticoid receptor ligand · Skin atrophy

## Abstract

Topical glucocorticoids (GCs) demonstrate good anti-inflammatory effects but are limited by their side effect potential, with skin atrophy being the most prominent one. Thus, determining the atrophogenic potential of novel compounds is important. The aim of this study was to establish an in vitro skin atrophy model. A screening cascade was applied and GCs with a known atrophogenic potential were used as tool compounds. Five rodent and human cutaneous cell types/cell lines and 2 human skin equivalents were tested. Known and suspected atrophy markers related to collagen metabolism and epidermal thickness were measured. Altogether, a combination of 7 different cellular assays with up to 16 markers each were investigated. A reproducible, more than 2-fold, regulation of the candidate markers by dexamethasone or clobetasol was found for: (a) matrix metalloproteinase (*MMP*) 1, 2, 3 and 9 expression in human keratinocytes, (b) *COL1A1* and *COL3A1* expression in 3T3 fibroblasts, and (c) epidermal thickness, collagen and MMP synthesis in the full-thickness skin model (FTSM). These 3 models were further investigated with a panel of 4–5 GCs, demonstrating dose dependency and correlation with the

atrophogenic potential of the tool compounds, qualifying them as potentially suitable. Finally, the predictability of these models for the in vivo situation was analyzed, testing a novel selective GC receptor agonist (SEGRA) in comparison to clobetasol. The results from the in vitro models suggested less atrophogenic effects for the SEGRA compound, which indeed was confirmed in the *hr/hr* rat skin atrophy model. In conclusion, a combination of 3 in vitro models based on 3T3 cells, human keratinocytes and FTSM with several readouts is recommended to determine atrophogenicity of GC receptor ligands. Further experiments are necessary to eventually reduce this panel and to demonstrate the true predictability for the clinic.

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## Introduction

Glucocorticoids (GCs) are the most widely used drugs for topical therapy of inflammatory skin diseases. Besides their desired anti-inflammatory effects, however, GCs may also induce adverse effects. Among those, skin atrophy is frequently observed and due to its irreversibility a particularly serious condition after topical long-term GC therapy [1]. Topical GCs are classified according to their potencies from weak to very strong [2] – considering both their therapeutic and side effect potential (ta-

ble 1). The therapeutic index (TIX), more recently introduced for topical GCs, indicates the ratio of beneficial and adverse effects of several GCs [3]. Optimization of the benefit/risk ratio is a main challenge for the development of novel glucocorticoid receptor (GR) agonists [4].

Skin atrophy is characterized by a profound loss in skin thickness and elasticity causing transparency of skin, a cigarette paper-like consistency, an increased fragility, and a telangiectatic surface [5–7]. It is accompanied by an increased permeability and transepidermal water loss [8], which indicates a disrupted barrier function of the skin [9, 10]. Histopathologically, flat dermal-epidermal junctions [5], reduced thickness of the epidermis [7], decreased size of keratinocytes [8], reduced number of fibroblasts [8, 11], rearrangement of the geometry of the dermal fibrous network [12], and a diminution of dermal collagen [13, 14] are found. The GC effects are mediated via the GR and involve many different molecular mechanisms including activation and repression of gene expression. However, the detailed underlying molecular mechanisms leading to skin atrophy are only partially understood (reviewed in Schoepe et al. [15] and Schäcke et al. [16]).

To determine the atrophy risk of topical GCs, predictive preclinical test systems are important. The currently preferred standard model for GC-induced skin atrophy in basic and pharmaceutical research is the model of hairless OFA *hr/hr* rats [17, 18]. In vivo models, however, are not favorable experimental models due to many reasons including: aspects of animal protection, their high requirement of labor, long duration, and compound amount needed. In contrast, in vitro test systems in general are fast, rather inexpensive, and feasible with limited amounts of compounds. They are, therefore, highly attractive, especially in early drug discovery and may also allow medium or even high throughput compound screening. So far, however, just a few approaches have been described, that to some extent may allow the estimation of GC atrophogenic potential in vitro [15]. Classically, those tests assess proliferation of cutaneous cells [19], or collagen synthesis in primary human fibroblasts [20]. However, no 'perfect'/validated in vitro test system for the reliable determination of GC atrophogenic potential has been described so far and to our knowledge no such systems are in routine use in drug discovery, although the identification of compounds with anti-inflammatory effects such as GCs but with less side effects is within the focus of numerous programs within the pharmaceutical industry [21].

The goal of this study was the identification of in vitro test systems that had to be efficient, robust, and econom-

ical and at the same time lead to reliable results with high predictive values. Such a suitable test system consists of two parts, an in vitro assay and readout parameters. We explored rodent and human cutaneous cells grown in monolayer culture and human cutaneous cells grown as full-thickness skin equivalents together with numerous markers as potential readout parameters. Whereas monolayer cell culture systems are well established in pharmacology and toxicology, full-thickness skin models (FTSMs) have been introduced more recently. They offer in vivo like characteristics of the skin such as stratification, homeostasis, expression and location of specific differentiation markers [22, 23]. As the key result, we propose here a panel of 3 systems for the estimation of the atrophogenic potential of GR ligands in vitro.

## Materials and Methods

### Cell Cultures

#### Tests with Adherent Cells

3T3 mouse embryonic fibroblast cell line (ECACC, Taufkirchen, Germany) and the immortalized human keratinocyte cell line HaCaT were maintained in DMEM (Gibco, Eggenstein, Germany) supplemented with 10% heat-inactivated FCS (Gibco). Primary rat fibroblasts were cultivated in fibroblast growth medium (Provitro, Berlin, Germany) containing 10% FCS. Normal human dermal fibroblasts and normal human epidermal keratinocytes (NHEK) from female adult donors were purchased from Provitro. Primary cells of passages 3–7 were used for the experiments. Primary human cells were maintained in fibroblast growth medium supplemented with 10% heat-inactivated FCS (Provitro) or in serum-free keratinocyte growth medium (Provitro). Additionally, both media were supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml) (Provitro). To determine the effect of GCs on mRNA expression of several genes (see below), fibroblast cells and lines were seeded at a density of  $2.1 \times 10^4$  cells/cm<sup>2</sup>, whereas the smaller keratinocyte cells and cell lines were seeded at  $5.2 \times 10^4$  cells/cm<sup>2</sup> in cell culture plates (Costar, Bodenheim, Germany). Test media for 3T3 cells were supplemented with 5% charcoal-treated serum instead of FCS. Test medium for NHEK was hydrocortisone free. After a 24-hour adhesion time, cells were treated with 0.1% DMSO with or without different GCs (table 1). Cells were treated for 2, 4, 6 and 24 h with GCs. Then, cells were lysed in RNA lysis buffer (Applied Biosystems, Darmstadt, Germany) and compound effects on mRNA expression were measured by RT-PCR as indicated below.

#### Isolation of Primary Rat Fibroblasts

Ears of adult *hr/hr* rats were disinfected with 70% ethanol and washed in 4% antibiotic-antimycotic solution (400 U/ml penicillin, 400 µg/ml streptomycin and 1 µg/ml amphotericin; Gibco). The front and back sides of the ears were separated under aseptic conditions with a forceps and the dermis side carefully laid flat in a sterile cell culture plate. Ear sheets were incubated overnight at 4°C in PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> with 2.4 U/ml dispase II

**Table 1.** Tool compounds used

GR ligand	Class	TIX	Provider
Glucocorticoid			
Hydrocortisone	I	1	Sigma-Aldrich, Schnelldorf, Germany
Prednicarbate	II	2	Synthesized by Bayer Schering Pharma
Mometasone-17-furoate	III	2	Synthesized by Bayer Schering Pharma
Betamethasone-17-valerate	III	1, 2	Sigma-Aldrich
Clobetasol-17-propionate	IV	1, 5	Sigma-Aldrich
SEGRA			
Compound A	–	–	Synthesized by Bayer Schering Pharma

Class based on potency according to Niedner [2]: I = mild, IV = superpotent. Therapeutic index (TIX) according to Luger et al. [3]: 1 ≤ 2 relation between desired and adverse effect is equal, 2–3 means GC with improved benefit/risk ratio. Modified from Schoepe et al. [15].

(Roche, Mannheim, Germany) to separate the epidermis from the dermis using a forceps. The dermis was incubated with sterile filtered 0.2% collagenase type IA (Sigma, Deisenhofen, Germany) in PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> for 1 h at 37°C with constant shaking until the solution became opaque. After filtration through a 100-µm cell strainer (BD Biosciences, Heidelberg, Germany) cells were centrifuged, cell pellet was washed two times with fibroblast growth medium (Provitro) containing 10% FCS and cells were seeded in cell culture flasks (3–8 × 10<sup>4</sup> cells/cm<sup>2</sup>).

#### Tests with Human Skin Equivalents

Two different commercially available human FTSMs were used. The FTSM (Phenion, Düsseldorf, Germany) was placed on filter paper on top of preplaced metal support in 5 ml hydrocortisone-free FTSM medium in a 6-well plate and adapted to cell culture conditions (37°C, 5% CO<sub>2</sub>, max. humidity). The Advanced Skin Test 2000 (AST-2000, CellSystems, St. Katharinen, Germany) was placed in 1 ml hydrocortisone-free AST medium (CellSystems) in a 6-well plate and adapted to cell culture conditions (37°C, 5% CO<sub>2</sub>, max. humidity). Test compounds were dissolved in corresponding culture medium (final concentration 0.01% DMSO with or without test compounds) and applied into the medium. The medium was replaced daily by fresh and prewarmed medium. Skin equivalents were exposed to the compounds for 24 h up to 12 days. After compound exposure, skin models were washed twice with PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> and were prepared for histological analysis. For compartment separation, AST-2000 epidermis was carefully detached from the dermis with a forceps, while FTSMs were washed twice with PBS with Ca<sup>2+</sup>, Mg<sup>2+</sup>, incubated in thermolysin solution (0.5 mg/ml thermolysin in 33 mM KCl, 50 mM NaCl, 5 mM CaCl<sub>2</sub>, 0.01 M HEPES) for 2 h at 4°C. For molecular analysis of mRNA and protein expression (see below), the epidermis and the dermis were analyzed separately.

#### Animal Models

Female Wistar and juvenile, hairless rats (*hr/hr*) were obtained from Charles River, Berlin, Germany. Rats were housed and experimental procedures were performed according to institutional guidelines: animals had access to food and water ad libitum. All animal studies were approved by the competent authority for la-

bor protection, occupational health, and technical safety for the state and city of Berlin, Germany, and were performed in accordance with the ethical guidelines of Bayer Schering Pharma AG.

#### Skin Atrophy in *hr/hr* Rats

To investigate induction of skin atrophy *in vivo*, 75 µl of vehicle [ethanol/isopropyl myristate (95:5 v/v)] or test compound [clobetasol-17-propionate (CB), selective GC receptor agonist compound A (SEGRA A)] were applied daily on a marked area of 9 cm<sup>2</sup> [18] for 10 days to dorsal skin of *hr/hr* rats (120–140 g, n = 6). At the end of the experiment, skin thickness was determined by using a specifically designed dial thickness gauge [18], animals were sacrificed and 5-mm punch biopsies of treated skin were taken for gene expression analysis (see below).

#### Irritant Contact Dermatitis in Wistar Rats

Test compounds (CB, SEGRA A) or vehicle were topically applied with the croton oil solution (6%) in ethanol/isopropyl myristate (95:5 v/v) to both ears of Wistar rats (80–100 g, n = 10). After 24 h, rats were sacrificed and the weight of 10 mm ear punch biopsies was determined as an overall readout of inflammation. Peroxidase activity as a parameter for granulocyte infiltration was analyzed in ear homogenates [18].

#### Tool Compounds

For testing the effects of standard GCs in pilot studies cells grown in monolayer culture were treated with 1 µM dexamethasone (Sigma), whereas skin models were treated with 0.1 µM CB. In evaluation experiments, cells and skin models were treated with a panel of tool compounds consisting of GR ligands from different classes and with a different TIX, i.e., GC hydrocortisone (HC), prednicarbate (PC), mometasone furoate (MF), and CB as well as SEGRA A (table 1) were used. This compound belongs to the family of nonsteroidal GR-selective compounds showing a dissociated molecular action [18]. The compounds were applied to cells grown in monolayer culture with the concentrations indicated. For the experiments with the skin equivalents as well as for the animal experiments, concentrations of the test compounds that achieve an equal anti-inflammatory effect were determined and subsequently applied.

**Table 2.** Primer (Assays-on-Demand™, purchased from Applied Biosystems) used for TaqMan RT-PCR

Gene	Name	Human	Rat	Mouse
<i>HPRT</i>	hypoxanthine-guanine phosphoribosyltransferase	Hs99999909_m1	Rn01527840_m1	Mm00446968_m1
<i>COL1A1</i>	collagen type I, alpha 1	Hs00164004_m1	Rn00801649_g1	Mm00801666_g1
<i>COL3A1</i>	collagen type III, alpha 1	Hs00164103_m1	Rn01437683_m1	Mm00802331_m1
<i>COL4A1</i>	collagen type IV, alpha 1			Mm00802372_m1
<i>COL5A1</i>	collagen type V, alpha 1			Mm00489342_m1
<i>COL7A1</i>	collagen type VII, alpha 1			Mm00483818_m1
<i>P4HA1</i>	prolyl-4-hydroxylase, alpha 1	Hs00168575_m1	Rn00597082_m1	Mm00803137_m1
<i>P4HA2</i>	prolyl-4-hydroxylase, beta	Hs00168586_m1	Rn00564459_m1	Mm00477940_m1
<i>P4HB</i>	prolyl-4-hydroxylase, beta	Hs00168586_m1	Rn00564459_m1	Mm01243184_m1
<i>MMP1</i>	matrix metalloproteinase 1	Hs00233958_m1		
<i>MMP2</i>	matrix metalloproteinase 2	Hs00234422_m1		
<i>MMP3</i>	matrix metalloproteinase 3	Hs00233962_m1		
<i>MMP9</i>	matrix metalloproteinase 9	Hs00234579_m1		

#### Determination of mRNA Expression

Total RNA of cells grown in monolayer cell culture was extracted using an ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocol. Total RNA from homogenized epidermis and dermis from skin models and from homogenized rat whole skin was isolated using the Qiagen RNeasy Kit (Hilden, Germany) according to the manufacturer's protocol. RNA was quantified using an Agilent 2100 BioAnalyzer (Applied Biosystems) and the RNA 6000 Nano Assay Kit (Ambion Biotechnology, Cambridgeshire, UK). Two hundred and fifty nanograms of total RNA was transcribed to complementary DNA using TaqMan® Reverse Transcription Reagents Kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative RT-PCR was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) [24] according to the manufacturer's protocol. PCRs contained 0.5 µl of complementary DNA template, 6.25 µl quantitative PCR Mastermix Plus without UNG (Eurogentec), 5.15 µl RNase-free water (Gibco) and 0.62 µl Assay-on-Demand™ (Applied Biosystems) for specific gene (table 2). Hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) was used as an endogenous reference gene. The reactions were carried out at 50°C for 2 min, 95°C for 10 min, and then 95°C for 15 s and 60°C for 1 min for 40 cycles. Standard curves (log of template dilution versus Ct value) for each gene-specific primer set were used to determine relative mRNA content for each target gene. Triplicate values obtained from each gene-specific PCR were used to determine a relative baseline for each of the experimental groups. The relation between GC and vehicle treated is given in percent (vehicle = 0%).

#### Determination of Protein Concentration

For the measurement of protein secretion into the supernatants, the following commercially available ELISAs were used: Metra CICP EIA Kit (Quidel, Marburg, Germany), MS6000 Human MMP 3-Plex Ultra-Sensitive Kit for the detection of MMP1 and MMP3 (Meso Scale Discovery), MA6000 Human IL-8 Base Kit (Meso Scale Discovery) and Human Proinflammatory II 4-Plex Kit for the detection of IL-1β, IL-6, IL-8 and TNF-α (Meso

Scale Discovery) [25]. Supernatants of compound-treated FTSMs were collected and prepared for detection according to the manufacturer's protocol.

#### Histological Analysis

The epidermal thinning of FTSMs was assessed by histological analysis [26]. Briefly, models were fixed in 4.5% formalin for 24 h, then dehydrated and embedded in paraffin. Sections of 5-µm thickness were obtained by using a Leica RM2125 rotary microtome (Leica Microsystems, Wetzlar, Germany). For analysis of epidermal architecture, sections were stained with hematoxylin/eosin [26]. The number of epidermal cell layers was visually determined in 5 areas of 3 nonconsecutive sections per single FTSM using hematoxylin/eosin-stained FTSM sections with a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) with Meta Vue program (Visitron, Puchheim, Germany).

#### Statistical Analysis

For in vitro models with monolayer cells, statistical analysis was performed using the paired t test after the Wilcoxon test (SigmaStat 3.0, SPSS GmbH, Munich, Germany). The potency, i.e. the concentration of the compound to reduce the expression by 50% (IC<sub>50</sub>), was calculated for each compound and compared between each other. For compound-induced effects in FTSMs, differences between groups were assessed by calculating the Fieller confidence interval [27]. For that it was to be assumed that gaussian distributions with equal variances are a reasonable model for the original scale of measurement. By event replication, more information was obtained. To calculate the Fieller confidence interval, the mean inhibition was estimated with the lower and upper 0.95 confidence limit.

Differences between groups in the in vivo experiments were assessed with the Mann-Whitney U test (SigmaStat 3.0) [17]. Outliers were determined according to Dixon's r<sub>10</sub> outlier test [28].

Data are shown as mean values ± standard deviation or as Fieller confidence interval; p values ≤ 0.05 were considered as statistically significant.

**Table 3.** GC-dependent regulation of readout parameters in cutaneous cell systems grown in monolayer

Parameter	Keratinocytes		Fibroblasts		
	HaCaT cells (n = 3)	NHEK (n = 5)	3T3 cells (n = 5)	rat fibroblasts (n = 3)	human fibroblasts (n = 3)
<i>COL1A1</i> mRNA	-	-	<b>-71 ± 2</b>	-12 ± 20	-10 ± 20
<i>COL3A1</i> mRNA	-	-	<b>-69 ± 6</b>	-10 ± 10	-10 ± 10
<i>COL4A1</i> mRNA	-	-	+110 ± 50	-	-
<i>COL5A1</i> mRNA	-	-	-20 ± 10	-	-
<i>COL7A1</i> mRNA	-	-	-30 ± 10	-	-
<i>P4HA1</i> mRNA	-	-	-27 ± 7	-23 ± 10	-13 ± 6
<i>P4HA2</i> mRNA	-	-	-18 ± 6	+70 ± 22	-35 ± 39
<i>P4HB</i> mRNA	-	-	-11 ± 8	+26 ± 38	-4 ± 25
<i>MMP1</i> mRNA	-	<b>-76 ± 3</b>	-	-	-
<i>MMP2</i> mRNA	-41 ± 7	<b>-66 ± 3</b>	-	-	-
<i>MMP3</i> mRNA	-	<b>-75 ± 8</b>	-	-	-
<i>MMP9</i> mRNA	-35 ± 14	<b>-65 ± 3</b>	-	-	-

HaCaT cells, primary human keratinocytes (NHEK), mouse 3T3 fibroblasts, primary rat fibroblasts or primary human fibroblasts were treated with 0.1% DMSO and with or without 1 μM dexamethasone for 24 h. mRNA expression was determined by quantitative RT-PCR as described in Materials and Methods. Relative regulation compared to DMSO control (%) is given as mean ± SD, DMSO control equates 0%. - = Not determined. Parameters in assays shown in bold were considered as candidate test systems for further evaluation.

## Results

Pilot experiments were designed to prescreen the number of test systems containing different cellular assays and multiple readout parameters in order to identify a reduced number of candidates entering further evaluation. Test systems fulfilling the criteria for further investigations were then applied for a prospective application.

### Pilot Studies

It is known that the risk of developing skin atrophy besides the strength of the used GC and the duration of the treatment increases with rising concentrations of the applied GC [1, 29]. Therefore, the test systems were screened with a high-dosed classical GC for a reproducible, more than 2-fold, regulation. Since GC-induced skin atrophy is considered to be caused by effects on keratinocytes and fibroblasts, the corresponding cells were investigated. Mouse 3T3 fibroblasts, primary rat dermal fibroblasts, normal human dermal fibroblasts, the immortalized human keratinocyte cell line (HaCaT), and NHEK as well as the human organotypic 3-dimensional cultures AST-2000 and FTSM were used. As readout parameters mRNA expression of collagen metabolism, i.e. collagens and prolyl-4-hydroxylase subunits (fibroblasts) and

MMPs (keratinocytes) were analyzed. In the case of full-thickness skin equivalents, epidermal thickness was analyzed additionally.

Whereas the investigated readout parameters were not reproducibly regulated more than 2-fold (≤ -50% inhibition) compared to vehicle in HaCaT cells, rat fibroblasts, human fibroblasts (table 3) and AST-2000 (table 4), 3 in vitro test systems met the screening criteria: (1) *COL1A1* and *COL3A1* mRNA expression in 3T3 fibroblasts, (2) mRNA expression of *MMP1*, *MMP2*, *MMP3* and *MMP9* in NHEK (table 3) and (3) epidermal thickness, *COL1A1*, *COL3A1*, *MMP1* and *MMP3* mRNA expression in FTSM (table 4). These 3 test systems were further investigated in the evaluation studies.

### Evaluation Studies

Despite the dose, the class (potency) and in the case of GCs from one class the TIX correlates with the atrophogenic potential of GCs [1, 3]. Thus, the 3 test systems meeting the screening criteria were further characterized for (1) dose-dependent regulation of readout parameter, (2) correlation with the class of topical GCs and (3) the correlation of TIX (in the case of cells grown in monolayer culture). The cells were incubated with ascending concentrations of at least 4 of the GCs listed in table 1.

**Table 4.** GC-dependent regulation of readout parameters in skin equivalents

Parameter	AST-2000 (n = 3)		FTSM (n = 2)	
	epidermis	dermis	epidermis	dermis
<i>COL1A1</i> mRNA	–	–23 ± 20 (8 days)	–	<b>–81 ± 8 (12 days)</b>
<i>COL3A1</i> mRNA	–	–4 ± 17 (8 days)	–	<b>–80 ± 7 (12 days)</b>
<i>P4HA1</i> mRNA	–	–	–	–12 ± 5 (1 day)
<i>P4HA2</i> mRNA	–	–	–	–12 ± 6 (1 day)
<i>P4HB</i> mRNA	–	–	–	+9 ± 8 (1 day)
<i>MMP1</i> mRNA	–23 ± 39 (1 day)	–	–82 ± 23 (1 day)	–86 ± 2 (1 day)
	–55 ± 23 (8 days)	–	<b>–98 ± 14 (12 days)</b>	<b>–96 ± 7 (12 days)</b>
<i>MMP2</i> mRNA	+75 ± 74 (1 day)	–	–11 ± 49 (1 day)	+12 ± 5 (1 day)
	+345 ± 622 (8 days)	–	+4 ± 32 (12 days)	–8 ± 7 (12 days)
<i>MMP3</i> mRNA	–72 ± 9 (1 day)	–	–26 ± 37 (1 day)	–87 ± 21 (1 day)
	–37 ± 13 (8 days)	–	n.d. (12 days)	<b>–99 ± 13 (12 days)</b>
<i>MMP9</i> mRNA	–27 ± 92 (1 day)	–	–20 ± 12 (1 day)	–49 ± 38 (1 day)
	–59 ± 43 (8 days)	–	–17 ± 7 (12 days)	+26 ± 12 (12 days)
Epidermal thickness	n.d.	–	<b>–75 ± 4 (12 days)</b>	–

AST-2000 and FTSM were treated daily for the indicated time with 0.01% DMSO or with 0.1 μM CB. mRNA expression was detected by quantitative RT-PCR as described in Materials and Methods, epidermal thickness was detected by counting keratinocyte cell layers. Relative regulation compared to DMSO (%) is given as mean ± SD, DMSO control equates 0%. n.d. = Not detected; – = not determined. Parameters in assays shown in bold were considered as candidate test systems for further evaluation.

### Monolayer Cultures

**Dose Dependency.** Treatment with increasing concentrations of CB resulted in an increased inhibition of collagen mRNA expression in 3T3 mouse fibroblasts. Application of CB at concentrations ≤0.1 nM had no effects on *COL1A1* mRNA expression, whereas the maximum inhibitory effect of 63% was reached at concentrations ≥10 nM (fig. 1a). Similar results were found for *COL3A1* mRNA expression in 3T3 cells (data not shown).

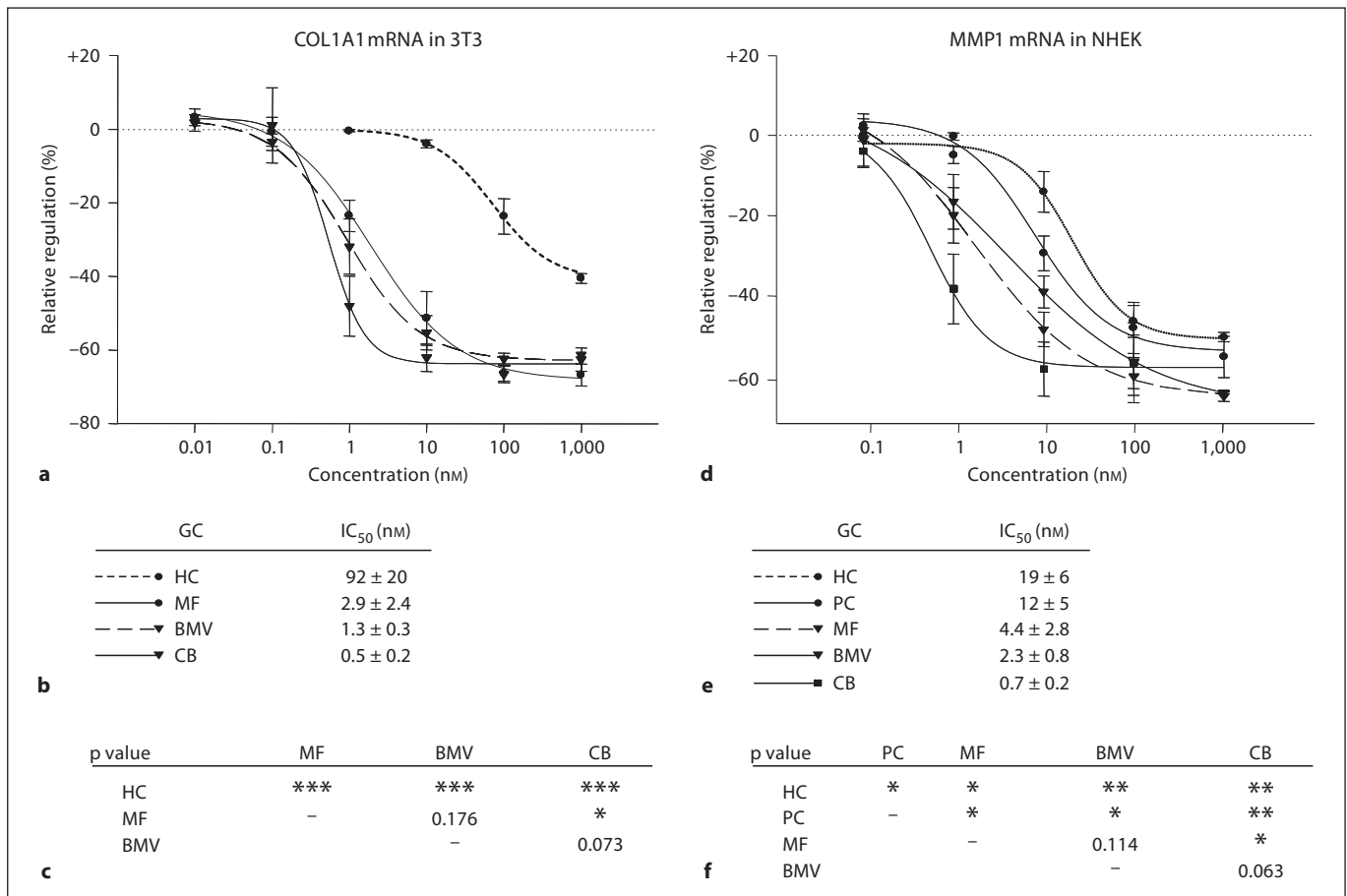
In NHEK, increasing concentrations resulted in an increased inhibition of *MMP1* mRNA expression (fig. 1d) with a maximum inhibitory effect of 54% at 10 nM CB. Similar results were found for *MMP2*, *MMP3*, and *MMP9* mRNA expression (data not shown).

**Correlation with GC Classification.** In 3T3 cells, the class I, III and IV GCs, HC, MF and CB displayed distinct potencies regarding the inhibition of *COL1A1* mRNA expression with the following potencies: IC<sub>50</sub> values of 92, 1.3, and 0.5 nM, respectively (fig. 1b). The weak GC HC not only displayed less potent activities but also a reduced efficacy of about 40% decrease in *COL1A1* mRNA expression compared to the stronger GCs MF and CB (65%) at a concentration of 1 μM (fig. 1a). Similar results were found for *COL1A3* mRNA expression (data not shown).

In NHEK, HC, PC, MF and CB inhibited *MMP1* mRNA expression with distinct potencies of 19, 12, 4.4 and 0.7 nM, respectively (fig. 1e). These results are in accordance with the classification of topical GCs. The IC<sub>50</sub> of these GCs differed significantly from each other (fig. 1c, f). Similar results were found for *MMP2*, *MMP3*, and *MMP9* mRNA expression (data not shown).

**Correlation with TIX.** Although of the same GC class, betamethasone-17-valerate (BMV) is known to show a higher atrophy potential than MF in human skin [3]. In 3T3 cells, BMV was more potent (1.3 nM) than MF (2.9 nM) (fig. 1b). Similarly, BMV (IC<sub>50</sub> of 2.3 nM) was about 2-fold more potent than MF (IC<sub>50</sub> of 4.4 nM) in NHEK inhibiting *MMP1* mRNA expression (fig. 1e). Although these differences were not statistically significant (fig. 1c, f), it suggests that a differentiation of GCs from the same class may be possible in this assay. Similar results were found for *MMP2*, *MMP3*, and *MMP9* mRNA expression in NHEK and *COL1A3* mRNA expression in 3T3 cells (data not shown).

Taken together, the inhibition of *COL1A1* and *COL3A1* mRNA expression in 3T3 mouse fibroblasts and *MMP1*, *MMP2*, *MMP3* and *MMP9* mRNA expression in NHEK correlated with dose and GC class. Moreover, there were hints for correlation with the TIX.



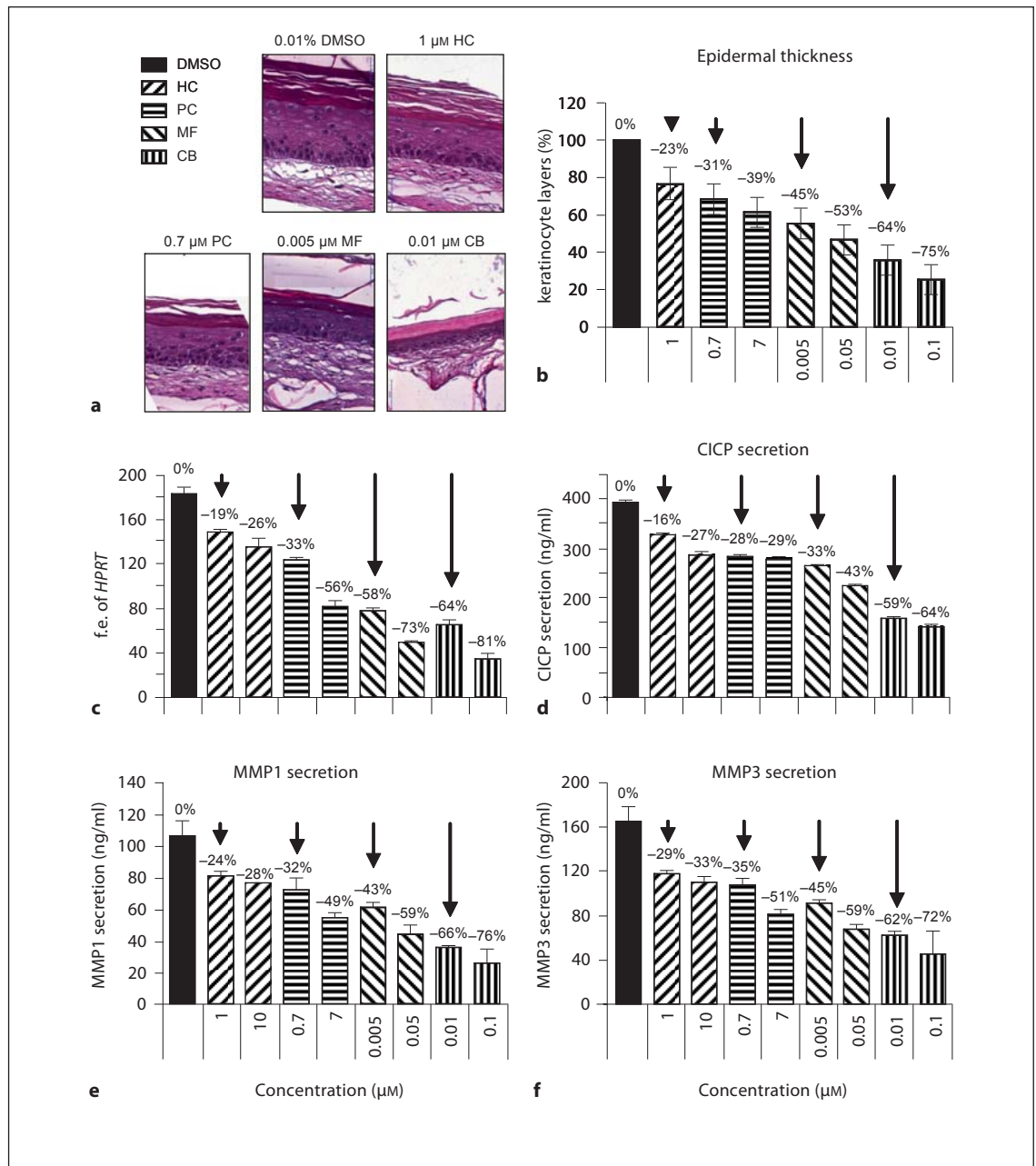
**Fig. 1.** GC treatment of 3T3 fibroblasts and NHEK. After adhesion over night, cells were treated with an ascending concentration of the classical GCs for 24 h. mRNA expression of *COL1A1* was determined in 3T3 fibroblasts, and *MMP1* mRNA expression was determined in NHEK by RT-PCR as described in Materials and Methods. The regulation relative to 0.1% DMSO control

(= 0%) is given in percent (**a, d**) and the resulting potency IC<sub>50</sub> of GC-treated versus DMSO-treated cells is shown as mean ± SD (n = 5; **b, e**). Results of the paired t test to estimate variations of GC treatment between each other are given as p values (\* p ≤ 0.5, \*\* p ≤ 0.01, \*\*\* p ≤ 0.001; **c, f**).

#### Full-Thickness Skin Equivalents

The skin equivalent FTSM was investigated for dose dependency and correlation with GC classification measuring (a) epidermal thickness, (b) collagen, and (c) MMP synthesis. For a meaningful interpretation of the data anti-inflammatory equi-effective concentrations needed to be determined. Comparable anti-inflammatory effects of GCs in FTSM were detected at the following concentrations: 1 μM HC, 0.7 μM PC, 0.005 μM MF and 0.01 μM CB by measuring the GC-dependent inhibition of pro-inflammatory cytokine secretion (table 5). Hence, these concentrations were considered as anti-inflammatory equi-effective and the potential atrophogenic effects of the GCs in FTSM were determined at these and at 10-fold higher concentrations.

**Dose Dependency.** GC treatment reduced epidermal thickness statistically significantly. The higher dose of CB, MF, and PC always led to a stronger epidermal thinning on day 12 (fig. 2b) compared to the 10 times lower doses. Also the higher concentrations of the GCs reduced the mRNA expression of *COL1A1* (and *COL1A3*, not shown) more than the 10-fold lower concentrations (fig. 2c) used. Secretion of C-terminal propeptide of collagen type I (CICP) was found to be dose-dependently inhibited by all GCs tested (fig. 2d), suggesting not only an inhibited synthesis but also a reduced formation of collagen type I. *MMP1* mRNA expression showed a slight tendency for a dose-dependent downregulation after the 12-day treatment with CB in the epidermal layer (91% by 0.01 μM CB; 98% by 0.1 μM CB) but not in the dermis (96% by both



**Fig. 2.** GC treatment of the FTSM. Increasing concentrations of GCs were daily added to growth medium. Medium was changed daily. Hematoxylin/eosin-stained histological sections show effects of GCs on the epidermis on day 12. Scale = 50  $\mu\text{m}$  (a). The number of keratinocyte layers was determined at 5 different areas on 3 nonconsecutive sections. According to Fieller, statistical analysis of epidermal cell layers was performed (see Materials and Methods) and resulted in mean inhibition  $\pm$  upper and lower confidence level (n = 2) (b). Collagen synthesis was analyzed after

12 days of treatment. mRNA expression of *COL1A1* (c) was detected in the dermal compartment by RT-PCR. Expression level of mRNA is given as fold expression (f.e.) relative to the house-keeping gene *HPRT*. Secretion of C1CP was determined in medium by ELISA (d). MMP1 (e) and MMP3 (f) secretion into the supernatant was quantified on day 3 by MesoScale. Mean  $\pm$  SD is given for collagen and MMP synthesis based on duplicates in one experiment with the FTSM. Arrows indicate anti-inflammatory equi-effective concentrations of GCs.



concentrations). No epidermal *MMP3* mRNA expression was detectable on day 12 (table 4), whereas the dermal *MMP3* mRNA expression was already reduced to the maximum by CB at the lower concentration (99% by both concentrations of CB). At the protein level, both MMP1 and MMP3 secretion was dose-dependently reduced after a 3-day incubation with all 4 GCs tested (fig. 2e, f).

**Correlation with GC Classification.** Application for 12 days of 1  $\mu\text{M}$  HC, 0.7  $\mu\text{M}$  PC, 0.005  $\mu\text{M}$  MF and 0.01  $\mu\text{M}$  CB reduced the number of keratinocyte layers by about 23, 31, 45 and 64%, respectively, relative to control (fig. 2a, b). Nonoverlapping Fieller confidence intervals (see Materials and Methods) indicated significant differences between the GCs (fig. 2b).

*COL1A1* mRNA expression was inhibited by anti-inflammatory equi-effective doses of HC, PC, MF and CB of about 19, 33, 58 and 64%, respectively, relative to control (fig. 2c). Similar results were obtained for the repression of *COL3A1* mRNA expression (data not shown) and for C1CP protein secretion (fig. 2d).

MMP1 synthesis was repressed in a compound-specific manner that corresponded to the GC class. So, in the epidermal layer, *MMP1* mRNA expression was inhibited by HC, PC, MF and CB at anti-inflammatory equi-effective concentrations of about 7, 58, 83 and 91%, respectively, relative to control. Inhibition of MMP1 and MMP3 protein secretion correlated with the GC class (fig. 2e, f).

In summary, the expression of *MMP1* and *MMP3* mRNA in the dermal layer of the FTSM was not found to be regulated dose dependently or compound class specifically. In contrast, the regulation of epidermal thickness, dermal *COL1A1* and *COL3A1* and epidermal *MMP1* expression after 12 days, as well as C1CP, MMP1 and MMP3 protein secretion into culture medium after 3 days were found to be dose dependent and correlating with the class of GC potency.

#### First Prospective Application

The evaluated in vitro test systems with 3T3 cells, NHEK and FTSM were used to measure effects of a SEGRA with hitherto unknown atrophogenicity to predict its corresponding side effect potential. SEGRA compounds are hypothesized to display less atrophy but similar anti-inflammatory effects as classical GCs [21]. The superpotent GC CB was used as reference. To determine the validity of the prediction, the skin thinning effect of the compounds was determined in vivo subsequently.

In 3T3 cells, the SEGRA A displayed an  $\text{IC}_{50}$  of about 1 nM, while CB showed an  $\text{IC}_{50}$  of 0.5 nM in the inhibition

**Table 5.** Compound-dependent regulation (%) of pro-inflammatory interleukin concentration in medium of skin-equivalent FTSM

Compound	Conc. nM	IL-1 $\beta$	IL-6	IL-8
Glucocorticoid				
Hydrocortisone	<b>1</b>	-64 $\pm$ 2	-44 $\pm$ 6	-69 $\pm$ 6
	10	-64 $\pm$ 3	-53 $\pm$ 4	-75 $\pm$ 7
Prednicarbate	<b>0.7</b>	-65 $\pm$ 4	-44 $\pm$ 4	-70 $\pm$ 1
	7	-71 $\pm$ 5	-63 $\pm$ 7	-79 $\pm$ 1
Mometasone-17-furoate	<b>0.005</b>	-67 $\pm$ 2	-46 $\pm$ 6	-68 $\pm$ 5
	0.05	-75 $\pm$ 2	-50 $\pm$ 2	-77 $\pm$ 0
Clobetasol-17-propionate	<b>0.01</b>	-67 $\pm$ 0	-47 $\pm$ 4	-74 $\pm$ 1
	0.1	-85 $\pm$ 5	-61 $\pm$ 1	-86 $\pm$ 7
SEGRA				
Compound A	<b>0.1</b>	-66 $\pm$ 4	-47 $\pm$ 2	-72 $\pm$ 2
	1	-82 $\pm$ 2	-59 $\pm$ 4	-83 $\pm$ 1

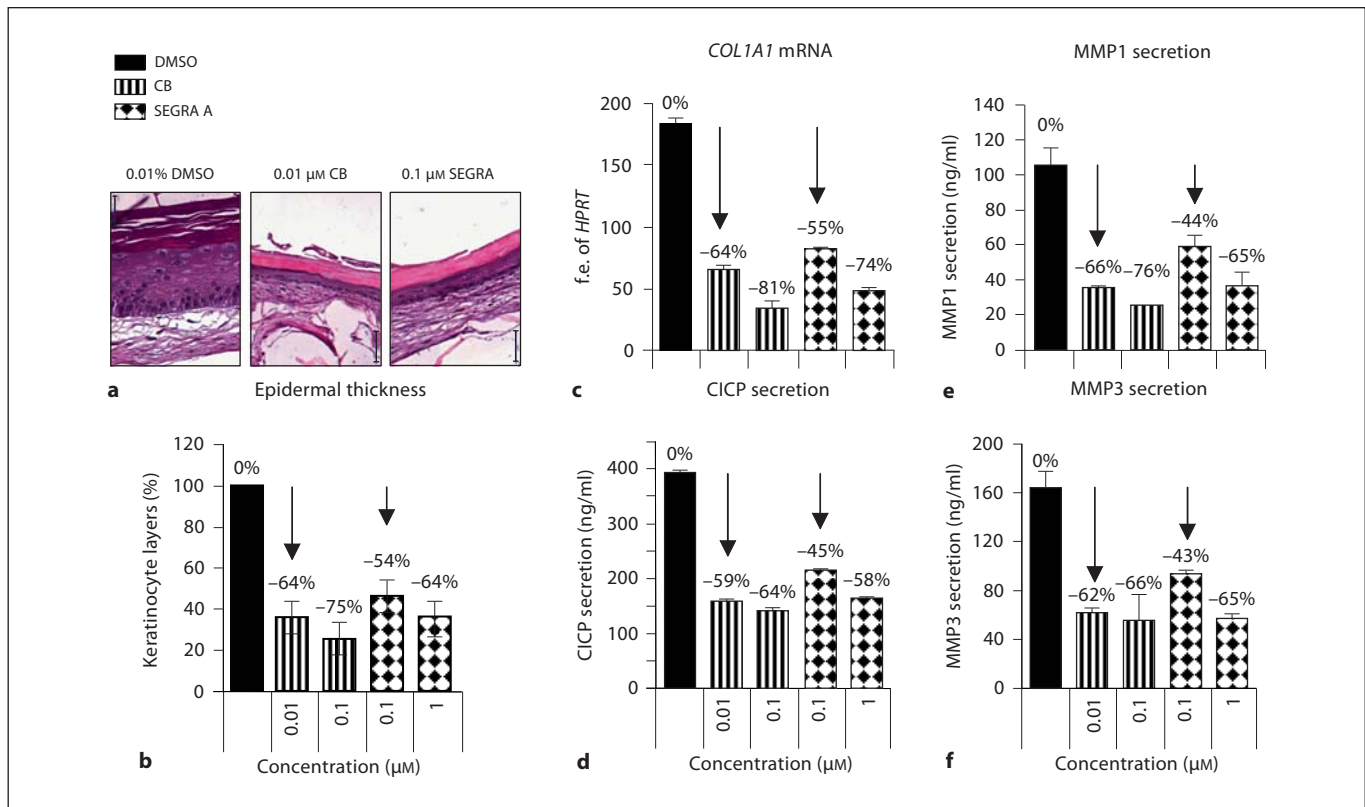
FTSMs were treated daily for 3 days with 0.01% DMSO or with compounds with different concentrations. Concentration of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6 and IL-8 was detected in medium as described in Materials and Methods. Concentrations in bold are considered as anti-inflammatory equi-effective.

of *COL1A1* expression (table 6). Similar results were obtained for the inhibition of *COL3A1* mRNA expression. SEGRA A displayed a 2.3- to 2.6-fold significantly weaker potency for the inhibition of collagen mRNA expression than CB. In NHEK, SEGRA A exhibited a weaker potency in the inhibition of *MMP1*, *MMP2*, *MMP3* and *MMP9* compared to CB (table 6).

The 3-dimensional in vitro model (FTSM) was treated with anti-inflammatory equi-effective and additionally 10 times higher concentrations of the compounds (table 5). At anti-inflammatory equi-effective concentrations, all atrophogenic parameters tested were more strongly affected by CB compared to SEGRA A. Figure 3 shows the data for the epidermal layers, C1CP (-59% by 0.01  $\mu\text{M}$  CB vs. -45% by 0.1  $\mu\text{M}$  SEGRA A relative to control) and MMP1 secretion (-66% by CB vs. -44% by SEGRA A). Similarly, SEGRA A reduced the *COL1A1* mRNA expression by about 55% whereas CB treatment led to an inhibition of 64%.

Thus, the results from the 3T3 fibroblasts, NHEK and FTSM models all predict a slightly lower skin atrophy potential of SEGRA A compared to CB.

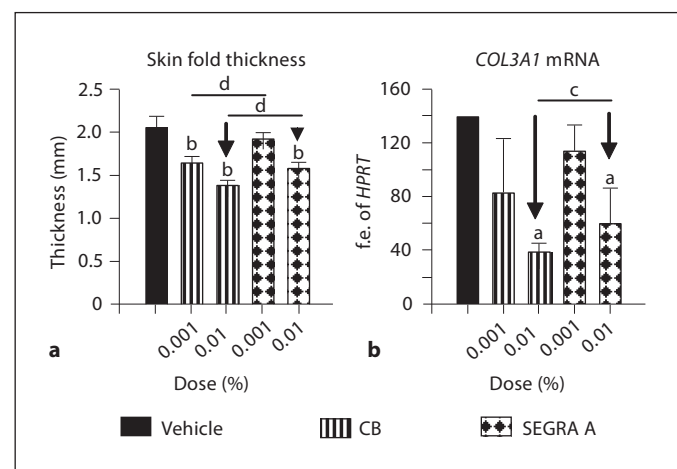
In a last step, the atrophogenicity of SEGRA A in relation to CB was determined in the *hr/hr* rat skin atrophy model. The croton-oil-induced inflammation of Wistar



**Fig. 3.** SEGRA treatment of the FTSM. Skin models were incubated with culture medium supplemented with SEGRA A or CB dissolved in 0.01% DMSO. Medium was changed daily. Epidermal thickness was determined on day 12 by counting the number of keratinocyte layers at 5 different areas on 3 nonconsecutive histological hematoxylin/eosin-stained sections (a). According to Fiebler, statistical analysis of epidermal cell layers was performed (see Materials and Methods) and resulted in mean inhibition  $\pm$  upper and lower confidence level ( $n = 2$ ) (b). Collagen synthesis was analyzed after 12 days of treatment. mRNA expression of

*COL1A1* (c) was detected in the dermal compartment by RT-PCR. Expression level of mRNA is given as fold expression (f.e.) relative to the house-keeping gene *HPRT*. Concentration of C1CP was determined in medium by ELISA (d), while MMP1 (e) and MMP3 (f) secretion into medium was analyzed on day 3 by MesoScale. Mean  $\pm$  SD of duplicates from one experiment with the FTSM. Arrows indicate anti-inflammatory equi-effective concentrations of test compounds. Inhibition by compound treatment relative to control is given in percent.

**Fig. 4.** Topical application of SEGRA A on *hr/hr* rat skin. After daily topical treatment for 10 days, skinfold thickness (a) was measured with a special gauge as described in Materials and Methods. Additionally, punch biopsies of the skin were taken and used for mRNA expression analysis of *COL3A1* (b). The mean  $\pm$  SD of skin thickness and fold expression (f.e.) of *HPRT*, respectively, is given of 6 animals per group. The Mann-Whitney test was used to estimate the variation of compound treatment and vehicle control (<sup>a</sup>  $p \leq 0.05$ ; <sup>b</sup>  $p \leq 0.01$ ) and to calculate variations of compound treatments between each other (<sup>c</sup>  $p \leq 0.05$ ; <sup>d</sup>  $p \leq 0.01$ ). Arrows indicate anti-inflammatory equi-effective concentrations of compounds tested in croton oil-induced inflammation of Wistar rat ear (data not shown).



**Table 6.** Comparison of SEGRA compound A and CB in 3T3 and NHEK fibroblasts test systems

Inhibition of mRNA expression	CB IC <sub>50</sub> nM	SEGRA A IC <sub>50</sub> , nM	Ratio SEGRA:CB	p value
<i>COL1A1</i> in 3T3 cells	0.5 ± 0.2	1.1 ± 0.4	2.3	0.012
<i>COL3A1</i> in 3T3 cells	0.4 ± 0.2	0.9 ± 0.3	2.6	0.010
<i>MMP1</i> in NHEK	0.7 ± 0.2	2.0 ± 1.5	2.9	0.063
<i>MMP2</i> in NHEK	0.7 ± 0.6	1.6 ± 0.8	2.3	0.017
<i>MMP3</i> in NHEK	0.3 ± 0.02	0.7 ± 0.1	2.0	<0.001
<i>MMP9</i> in NHEK	0.6 ± 0.1	0.9 ± 0.1	1.4	0.063

Cells were treated for 24 h with increasing concentrations of CB or SEGRA compound A. In 3T3 fibroblasts, the mRNA expression of collagens was detected by RT-PCR. In NHEK, *MMP* mRNA expression was determined as described in Materials and Methods. IC<sub>50</sub> (nM) is shown as mean ± SD (n = 5). The ratio between SEGRA and CB was calculated by dividing the mean IC<sub>50</sub> values. p values were calculated by using a paired t test to estimate variations of IC<sub>50</sub> of CB vs. SEGRA compound A.

rat ear displayed for 0.01% CB and 0.01% SEGRA A an equal anti-inflammatory efficacy (–119% by CB, and –117% by SEGRA A). For the determination of skin atrophy, *hr/hr* rats were treated daily for 10 days with SEGRA A and CB at these anti-inflammatory equi-effective and 10 times lower doses. At anti-inflammatory equi-effective doses, SEGRA resulted in a 23% decrease in skin thickness relative to the vehicle control, while CB resulted in a 33% decrease in skin thickness (fig. 4a). At 10 times lower concentrations, the difference was even more pronounced; SEGRA A did not decrease skin thickness statistically significantly relative to vehicle control, while CB exhibited a 20% decrease in skin thickness. These results were supported by collagen mRNA expression data. SEGRA A was less efficient in repressing *COL1A1* and *COL3A1* (fig. 4b) mRNA expression than the reference compound CB.

Thus, the validity of prediction based on the 3 in vitro test systems for the rodent in vivo situation was demonstrated.

## Discussion

We identified 3 in vitro test systems as suitable to determine the atrophogenic potential of GR ligands: (1) *COL1A1* and *COL3A1* mRNA expression in 3T3 mouse fibroblasts, (2) *MMP1*, *MMP2*, *MMP3* and *MMP9* mRNA expression in NHEK, and (3) epidermal thickness as well as collagen and MMP synthesis in the FTSM. Results ob-

tained in these test systems comply with the known clinical effects of GC-induced skin atrophy. So they were regulated by GCs in a dose-dependent manner and correlated well with the GC classification based on potency. Additionally, the monolayer test systems correlated with the different atrophogenic potential of 2 GCs from the same class of topical GCs but with a different TIX. Moreover, by application of a novel SEGRA compound with hitherto unknown atrophogenicity, the predictability of the 3 in vitro models for the rodent in vivo situation was demonstrated in a prospective setting.

The test systems were identified in a ‘screening approach’. So in a first step, candidate test systems were tested just with 1 potent GC at 1 high concentration at 1 point in time and the experiment was just repeated once or twice. The numerous test systems resulted from the multiple combinations of potential cellular assays containing human or rodent cutaneous (epidermal and dermal) cells with many potential readout parameters. A considerable (–50%) regulation was a precondition for further investigations of these test systems in the following evaluation experiments. This is a meaningful approach to reduce the number of candidates, frequently applied in drug discovery, for example for lead compound identification. In fact, by using this approach, we have been able to reduce the number of test systems by almost 2/3. It is important to notice, however, that our data do not prove that certain test systems found not to be qualified in the pilot experiments are fully devaluated. They may represent false-negative results and the test systems may be suited, for example under different conditions.

None of the suited in vitro test systems identified may be fully sufficient for reliable prediction of the atrophogenic potential in men so far. On the one hand, the level of differentiation for individual results with a single assay/readout was not so high when investigating GCs from different classes and with different TIX, indeed partially no statistically significant differences were found. Applying a combination of tests, however, should give more certainty. On the other hand, all test systems used have their advantages and disadvantages. Based on our experiences, we recommend to work with the following cascade of test systems.

First the monolayer cell cultures (NHEK, 3T3 cells) should be applied, where approximately 10–15 compounds at 5–6 concentrations can be easily screened within 3 days by 1 investigator. Although these monolayer cell systems have many advantages such as being robust, inexpensive, and easily reproducible, they have major limitations. (1) They show the characteristics of 1 cell type only and thus

do not reflect the complex interactions of different cell types representative of the skin. (2) Tissue thinning, the main clinical readout parameter for atrophy, cannot be detected in monolayer cultures. (3) The cells are growing in a large excess of growth medium and therefore, topical application of compounds is not possible. Likewise, it is not possible to test topical formulations in monolayer cell systems, although it is known that the formulation impacts the atrophogenic potential of a GR ligand. (4) For a reasonable interpretation of the results, the ratio between the anti-inflammatory and the side effect potential is important [18]. This cannot be determined in individual monolayer cell cultures, since these nonimmune cells do not represent the primary cellular target for the anti-inflammatory effects and it is questionable whether it can be translated 1:1 from results in other cellular test systems, e.g. based on leukocytes [21].

To reduce these limitations, favorable compounds can subsequently be tested in 3-dimensional *in vitro* test systems like the FTSM. As true human skin equivalents they are considered to reflect the human situation more closely [23]. Full-thickness skin equivalents in general might be useful models for dermatological investigations as they consist of (a) human neonatal fibroblasts growing in a 3-dimensional matrix, (b) a functional basal lamina and (c) human neonatal keratinocytes differentiated in a real epidermis characterized by different strata [26]. Besides the complex fibroblast-keratinocyte and cell-matrix interaction, skin equivalents allow topical drug administration. Recently, Zöller et al. [26] tested topical surface application of GC-containing creams of airlifted FTSMs for the first time and were able to determine their atrophogenic potential. Our data reported here with the application of the compounds into the culture media are fully in line with this report. An advance of the FTSM is that a clinically relevant readout parameter, the epidermal thinning, can be determined directly. Additional important surrogates, such as the inhibition of collagen and MMP synthesis, are measurable within the same model, in the same experiment. Also, the anti-inflammatory potential (represented by suppression of pro-inflammatory cytokine production in skin-derived cells) can be determined head to head to the atrophogenic potential, which should be of tremendous value predicting the clinically relevant TIX. Very recently, it has also been possible to generate skin equivalents for cancer research such as primary cutaneous human squamous cell carcinomas [30]. This fact underlines the potential of 3-dimensional skin models. Current disadvantages of the system presented in our study, however, represent the high amount of labor and time

needed for the corresponding experiments (about 2 weeks), the high costs (about 50-fold higher compared to monolayer systems), the limited throughput and the lack of infiltrating immune cells. Based on our experiences, 1 investigator can handle up to 60–70 samples allowing tests of approximately 6–8 compounds with 2 concentrations in duplicates for histology and molecular biological investigations. Handling, time and compound consumption may be further optimized, for example by a medium change on every second or third day as opposed to every day or by using special inserts instead of metal supports plus filter paper, which needs to be tested. However, we do not expect that the full-thickness skin equivalents are suited as first-line assays in the near future.

Better *in vitro* test systems, including those presented here, can reduce the number of animal experiments, which is highly desired not just for animal protection but also for economic reasons. It is unlikely, however, that *in vitro* test systems can completely replace *in vivo* models. The situation *in vivo* is much more complex than it can be mirrored *in vitro*. At least in a final step, before entering the clinic, a minimized number of drug candidates have to be tested in an appropriate *in vivo* model, which are also required for regulatory aspects. To determine the skin atrophy potential of novel compounds, the *hr/hr* hairless rat is used [18]. An additional advantage of this *in vivo* model compared to the *in vitro* models is the possibility to detect systemic side effects of GCs, like atrophy of the thymus, spleen or adrenal glands [18].

In summary, we described 3 *in vitro* test systems for the prediction of skin atrophy. Further investigations are necessary to demonstrate the ultimate clinical relevance of these models, i.e. the true predictability for the clinical situation. For this, many known GCs and novel GR ligands should be tested comprehensively *in vitro*, in animal models and in humans. Despite this, the use of the 3 *in vitro* test systems should to some extent help to reduce the number of animal experiments, to identify novel drugs with weaker atrophogenicity at reasonable costs and to decrease the risk of compound failure in clinical development.

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