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ORIGINAL ARTICLE

Selective Activator of the Glucocorticoid Receptor Compound A Dissociates Therapeutic and Atrophogenic Effects of Glucocorticoid Receptor Signaling in Skin

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Background: Glucocorticoids are effective anti-inflammatory drugs widely used in dermatology and for the treatment of blood cancer patients. Unfortunately, chronic treatment with glucocorticoids results in serious metabolic and atrophogenic adverse effects including skin atrophy. Glucocorticoids act via the glucocorticoid receptor (GR), a transcription factor that causes either gene transactivation (TA) or transrepression (TR). Compound A (CpdA), a novel non-steroidal GR ligand, does not promote GR dimerization and TA, retains anti-inflammatory potential but induces fewer metabolic side effects compared to classical glucocorticoids when used systemically. As topical effects of CpdA have not been well studied, this work goal was to compare the anti-inflammatory and side effects of topical CpdA and glucocorticoids and to assess their effect on GR TA and TR in keratinocytes.
Methods: We used murine immortalized keratinocytes and F1 C57BlxDBA mice. Effect of glucocorticoid fluocinolone acetonide (FA) and CpdA on gene expression in keratinocytes in vitro and in vivo was evaluated by reverse transcription-PCR. The anti-inflammatory effects were assessed in the model of tumor promoter 12-*O*-tertradecanoyl-acetate (TPA)-induced dermatitis and in croton oil-induced ear edema test. Skin atrophy was assessed by analysis of epidermal thickness, keratinocyte proliferation, subcutaneous adipose hypoplasia, and dermal changes after chronic treatment with FA and CpdA.
Results: In mouse keratinocytes in vitro and in vivo, CpdA did not activate GR-dependent genes but mimicked closely the inhibitory effect of glucocorticoid FA on the expression of inflammatory cytokines and matrix metalloproteinases. When applied topically, CpdA inhibited TPA-induced skin inflammation and hyperplasia. Unlike glucocorticoids, CpdA itself did not induce skin atrophy which correlated with lack of induction of atrophogene regulated in development and DNA damage response 1 (REDD1) causatively involved in skin and muscle steroid-induced atrophy.
Conclusions: Overall, our results suggest that CpdA and its derivatives represent novel promising class of anti-inflammatory compounds with reduced topical side effects.
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Key Words: Glucocorticoid receptors, Selective glucocorticoid receptor activator, Skin, Atrophy, Inflammation

INTRODUCTION

Glucocorticoids are among the most effective anti-inflammatory drugs. They are also extensively used as anti-cancer drugs, especially for the treatment of patients with hematological malignancies.[1] In animal models glucocorticoids decrease/prevent skin carcinogenesis mostly by blocking skin inflammation.[2]

Unfortunately, prominent therapeutic effects of glucocorticoids are often accompanied by numerous metabolic and atrophogenic adverse effects including skin atrophy that develops after steroid topical application.[1,2-5]

Glucocorticoid effects are mediated by their receptor (glucocor-

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ticoid receptor, GR), a well known transcription factor.[6-10] Upon glucocorticoid binding, GR undergoes phosphorylation, dimerization and translocates to the nucleus, where it regulates gene expression positively (transactivation, TA) or negatively (transrepression, TR). TA in most cases requires GR-homodimer binding to palindromic glucocorticoid-responsive elements (GRE). The TR mechanisms are more diverse and include tethering of GR monomer to other transcription factors including major pro-inflammatory factors NF-κB and activator protein 1 (AP-1); binding of GR to negative GREs; alteration of the composition of the transcription-initiating enhanceosome.[1,6,9-12]

The blockage of pro-inflammatory transcription factors by glucocorticoids via negative protein-protein interactions with GR is still viewed as a central molecular component of the therapeutic effects of glucocorticoids, even though the recent data showed that the GR TA is important for its complete anti-inflammatory potential.[1,6,11,13,14] For example, GR tethering to NF-κB and some other transcription factors results in inhibition of a wide variety of pro-inflammatory genes including inflammatory cytokines, adhesion molecules, and matrix metalloproteinases (MMPs).[4,6,9,10,14-16] Our previous work showed that GR TR is also very important for anti-cancer effects of GR signaling.[1,2] On the other hand, GR dimerization and gene activation significantly contribute to metabolic and atrophogenic adverse effects of glucocorticoids.[1,3,4,13,14,17] Thus, alternative ligands that inhibit GR dimerization/GR TA are expected to have a significantly improved therapeutic index compared to classical glucocorticoids.[1,18-20]

Skin atrophy affects all skin compartments and compromises the barrier function of the skin.[2-5] The molecular mechanisms of steroid-induced skin atrophy are poorly understood. We recently discovered that one of the GR target genes in skin – regulated in development and DNA damage responses 1 (REDD1) is causatively involved in steroid atrophy.[17] REDD1 is an important negative mTOR regulator, and also acts as atrophogene in muscle.[21,22] In this work, we for the first time used REDD1 as a molecular marker of skin atrophy in vivo.

There has been an extensive search for alternative GR ligands called selective GR activators (SEGRA) that retain therapeutic activity of classical glucocorticoids but have fewer side effects. Recently several SEGRA that do not induce GR TA have been designed or selected by the screening of chemical libraries by Pfizer, Abbott/Ligand and Bayer Schering Pharma, and some of them such as maprakorat went to clinical trials.[4,11,18-20,23]

In addition, there is a growing interest to natural products that behave as SEGRA. One of novel GR ligands is 2-(4-acetoxyphenyl)-2-chloro-N-methylethylammonium-chloride, also called compound A (CpdA). CpdA is a synthetic analogue of aziridine precursor from the Namibian shrub *Salsola tuberculatiformis* Botschantzev.[1,24] We showed that CpdA acts as GR ligand: it strongly competed with glucocorticoids for GR binding, and induced GR nuclear translocation.[1,25-28] However, unlike classical glucocorticoids, CpdA did not induce GR phosphorylation or dimerization, and did not efficiently induce GR TA.[1,25-28] At the same time, CpdA strongly inhibited pro-inflammatory gene expression, was very effective in counteracting inflammation in numerous animal models when used systemically, and exerted strong anti-cancer effects in vitro and in vivo.[1,25-30] Importantly, in contrast to glucocorticoids CpdA had fewer metabolic side effects related to glucose control, maintenance of the hypothalamo-pituitary-adrenal (HPA) axis bone metabolism, and muscle atrophy.[1,25,26,29-31]

The studies of topical CpdA effects in skin have been limited, and yielded controversial results.[5,32] In addition, the effects of CpdA on GR activity in keratinocytes have not been investigated. As effect of GR ligands on gene expression strongly depends on the cell type,[12,33,34] in the presented work, we assessed CpdA effects on gene expression in keratinocytes in vitro and in vivo. We also evaluated the atrophogenic and anti-inflammatory effects of topical CpdA in comparison to fluocinolone acetonide (FA) using model of contact dermatitis induced by tumor promoter and irritant 12-*O*tetradecanoylphorbol-13-acetate (TPA).

We found that CpdA did not activate GR-dependent genes including atrophogene regulated in development and DNA damage response 1 (REDD1). At the same time, CpdA mimicked the inhibitory effect of glucocorticoids on the expression of key anti-inflammatory genes; inhibited skin inflammation and hyperplasia induced by TPA; but did not induce skin atrophy. This suggested good dissociation of therapeutic/atrophogenic side effects in case of topical application of this SEGRA.

MATERIALS AND METHODS

1. Chemicals

FA, TPA, Croton oil (CO) and all other chemicals unless stated otherwise were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). CpdA was synthesized as previously described.[28]

2. Keratinocyte cell cultures

3PC murine immortalized keratinocytes[35] were cultured in Eagle's minimal essential medium (Cellgro; Mediatech, Inc., Manassas, VA, USA) containing 4% FBS (Cellgro; Mediatech, Inc.), 0.05 mM Ca^{2+}, and growth factors as described.

3. Luciferase assay

The *Firefly* Luciferase glucocorticoid-responsive reporters TAT-Luciferase (Luc) and MMTV-Luc, and NF-κB reporter × 3 κB-Luc were described previously.[28] The transfection efficacy was normalized using co-transfections with pRL-CMV-*Renilla* luciferase (RL) under minimal promoter (Promega, Madison, WI, USA). 3PC cells were transfected in 24-well plates (at least three wells/experimental group) using Plus Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Each well contained 0.33 μg of the plasmid DNA. Twenty four hours after transfection, cells were treated with vehicle (0.01% acetone), CpdA (10^{-6}-1^{0-5} M) or FA (10^{-6} M) for 24 hours. To activate NF-κB, we used TNF-α (10 ng/mL; R D Systems, Minneapolis, MN, USA). Cells were treated with TNF-α and GR ligands simultaneously for 24 hours. The *Firefly* and RL activity was measured using TD20/20 Luminometer (Turner Biosystems, Sunnyvale, CA, USA) following the protocol for Dual Luciferase reporter assay (Promega).

4. RNA isolation and reverse transcription-PCR

3PC cells at 70% confluence were treated with CpdA (10^{-6}-10^{-5} M), FA (10^{-7}-10^{-6} M) or 0.01% acetone for 8 to 24 hours (3PC cells). Animals were treated topically with TPA, CpdA or FA as described below. Skin was harvested and epidermis was mechanically isolated from dermis by scraping as described.[36] Total RNA was isolated from cells or epidermis by Tri-Reagent (Sigma-Aldrich) according to the manufacturer's protocol. The gene expression levels were assessed in samples of epidermis from individual animals (3-4 animals/ group) using two-step reverse transcription-PCR. Reverse transcription was performed using 1 μg RNA, random hexamers and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) followed by PCR with Taq DNA polymerase (Promega) using specific primers designed with NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Amplified PCR products were run on 1.5% agarose gels, digitally acquired on a ChemiDoc XRS™ image documentation system and quantitated with Quantity One software (Bio-Rad, Hercules, CA, USA). The relative fold change was calculated by normalized to GAPDH, a reference housekeeping gene. Statistical analysis was performed as described below.

PCR primers used were Sgk1: forward, 5′- AGC CTC TCC AGT TGA AAC CA -3′, reverse, 5′- GAA TCC ACA GGA GGT GCA TAG -3′, Gilz: forward, 5′- CAT GGA GGT GGC GGT CTA TCA -3′, reverse, 5′- GCG TCT TCA GGA GGG TGT TCT -3′, Fkbp51: forward, 5′- TGA GGG CAC CAG TAA CAA TGG -3′, reverse, 5′- CAA CAT CCC TTT GTA GTG GAC AT -3′, Mmp3: forward, 5′- CGT GGT ACC CAC CAA GTC TAA -3′, reverse, 5′- CCT TGA GTC AAC ACC TGG AAA -3′, Mmp9: forward, 5′- CTT TGA GTC CGG CAG ACA AT -3′, reverse, 5′- TGC CTG TGT ACA CCC ACA TT -3′, Mmp13: forward, 5′- GAA GTG TGA CCC AGC CCT ATC -3′, reverse, 5′- GTC TTC CCC GTG TTC TCA AAG -3′, Il1α: forward, 5′-CGA AGA CTA CAG TTC TGC CAT T -3′, reverse, 5′- GAC GTT TCA GAG GTT CTC AGAG -3′, Il1β: forward, 5′- TCC TGA ACT CAA CTG TGA -3′, reverse, 5′- CCA GCA GGT TAT CAT CAT -3′, Il6: forward, 5′- CCA AGA GGT GAG TGC TTC CC -3′, reverse, 5′- CTG TTG TTC AGA CTC TCT CCC T -3′; Ddit4 (Redd1): forward, 5′- GGGCCGGAGGAAGACTCCTCATA-3′, reverse, 5′-CTGTATGCCAGGCGCAG GAGTTC-3′. Gapdh: forward, 5′- CAA CTT TGG CAT TGT GGA AGG -3′, reverse, 5′- ACA CAT TGG GGG TAG GAA CAC -3′.

5. Animal treatments

We used F1 C57BlxDBA mice (B6D2; Jackson Laboratory, Bar Harbor, ME, USA) that are sensitive to contact dermatitis induced by TPA,[37] and well characterized in terms of their response to topical glucocorticoid FA.[38] All animal experiments were performed in compliance with Animal Care and Use Committee protocol approved by the Northwestern University Animal Care and Use Committee.

1) Contact dermatitis test

Back skin of 7 to 8 weeks old animals (3-4 animals per group) was shaved, and treated three days later with TPA (2 μg/animal) after 1 hour pretreatment with vehicle (acetone), CpdA (20-40 μg) or FA (2 μg). All compounds were applied in 200 μL of acetone topically. Control animals were treated with acetone only. Effects of CpdA and FA on TPA-induced inflammation and proliferation in back skin, were evaluated 24 hours after TPA application. To assess proliferation, animals were injected i.p. with bromodeoxyuridine (BrdU; Sigma-Aldrich; 50 μg/g of animal weight) 1 hour before skin was harvested as in.[36]

2) Ear edema test

We used the standard ear edema test to evaluate anti-inflammatory effect of GR ligands.[5,17,19,39-44] To induce edema, CO (2.5 % solution in acetone) was applied to the back of the right ear lobe of each mouse, and solvent was applied to the left ear lobe as an internal control. Animals were pretreated with solvent (acetone), CpdA (20 μg) or FA (1 μg) 1 hour before the application of irritant. All compounds were applied in 10 μL acetone. Animals were sacrificed 9 hours after CO application when maximum swelling was achieved,[17] and wet weight of 4 mm ear punches was determined as the major read-out of inflammation as in.[17]

3) Induction of skin atrophy

To evaluate atrophogenic effects, FA (2 μg/animal) or CpdA (20-100 μg/animal) were applied to the back skin of B6D2 animals every 24 hours for four consecutive days. We showed previously that this regimen of treatment induces similar skin atrophy in B6D2 mice as FA chronic application for two weeks.[38]

6. Histological analysis and immunostaining

The skin samples were fixed in formaldehyde, embedded in paraffin, and stained with H&E; Masson's trichrome to evaluate changes in dermal collagen fibers; or with anti-BrdU antibodies (BD Biosciences, San Jose, CA, USA) to determine proliferation as described.[38]

7. Morphometric analysis.

Quantitation of the epidermal width (as the readout for skin thinning) and dermal cellularity (as the readout for inflammation) were performed in dorsal skin sections stained with hematoxylin and eosin. At least 20 individual fields per slide with at least three skin samples from individual animals in each experimental group were counted using Axioplan2 microscope software (Carl Zeiss, Oberkochen, Germany). The epidermal thickness and dermal cellularity in treated animals are presented as the percentage of those parameters in control animals.

To assess keratinocyte proliferation, the number of proliferating (BrdU-positive) and total basal keratinocytes was evaluated in each skin sample, in 25 individual fields of view under the microscope. Number of BrdU-positive cells is presented as percent of total number of basal keratinocytes.

8. Statistical analysis

All experiments were repeated at least three times. Data is presented as the means values ± SEM that were determined using the commercial software Excel 2007 (Microsoft, Redmond, WA, USA). The treatment effects in each experiment were compared by one-way ANOVA using the GraphPad statistical software package (GraphPad software, La Jolla, CA, USA) and Tukey post-test. Differences between groups were considered significant at $P < 0.05$.

RESULTS

1. Effect of compound A on gene expression in keratinocytes in vitro and in vivo

We compared GR activation by CpdA and the glucocorticoid fluocinilone acetonide (FA)–a medium-potency corticosteroid frequently used in dermatology[5] in vitro, in 3PC mouse non-transformed keratinocytes expressing functional GR,[35] and in vivo, in mouse skin. Both, in vitro and in vivo CpdA was applied at previously reported effective concentrations[25-30] which were 5-10-fold higher compared to glucocorticoids possibly reflecting lower GR affinity.[1]

In 3PC keratinocytes, CpdA acted as partial GR antagonist: it did not activate GR in Luciferase assay with glucocorticoid-responsive MMTV-Luciferase and TAT-Luciferase reporters (Fig. 1A), and did not activate well-known GR target genes Fkbp51, also called Fkbp5 (FK506 binding protein 5), Gilz (glucocorticoid-induced leucine zipper)[40,41] and Sgk1 (serum/glucocorticoid regulated kinase 1) (Fig. 1C-1).[42,43]

At the same time, CpdA significantly, even though weaker than classical GR antagonist RU486, inhibited FA-induced GR activation (Fig. 1A). The GR TR was assessed by the inhibitory effect on NF-κB activation by TNF-α using previously described × 3 κB-Luc reporter[25,28] and by the effect on the expression of endogenous MMP genes negatively regulated by glucocorticoids.[4,6,9,10] Both GR ligands significantly inhibited TNF-α–induced NF-κB. Luciferase (Fig. 1B) and the expression of MMPs (Fig. 1C-2).

Our recently published DNA arrays suggested that basal expression levels of many cytokines and MMPs were only weakly affected by FA in mouse skin (GEO Submission GSE59151). Thus, in vivo, we compared the effects of FA and CpdA on gene expression in epidermis after skin was topically treated with irritant/tumor promoter TPA to induce pro-inflammatory genes.

Interestingly, the expression of the direct GR targets such as Fkbp51 and Gilz was significantly inhibited by TPA in epidermis (Fig. 2D-2). While CpdA did not modulate TPA-induced changes, animal pretreatment with FA resulted in complete reversal of TPA effect on Fkbp51 and Gilz expression. The inhibitory effects of CpdA and FA on Il-6, Il-1α, Il-1β, Mmp3 and Mmp13 expression induced by TPA were overall similar (Fig. 2D-1). However, quantitatively the TR effect of FA was stronger, especially in context of Il-1α/β, and Mmp3 genes. Overall, the in vivo results confirmed the dissociated ligand profile of CpdA that was revealed in keratinocytes in vitro.

2. Topical compound A exerts anti-inflammatory activity in skin.

Next, we evaluated CpdA effect on inflammation and keratinocyte proliferation using a model of contact dermatitis induced by topical TPA in skin of B6D2 mice. Following previous experimental design,[25,26,29] we applied to skin glucocorticoid FA at efficient anti-inflammatory and anti-proliferative dose 2 μg,[36,38,44]

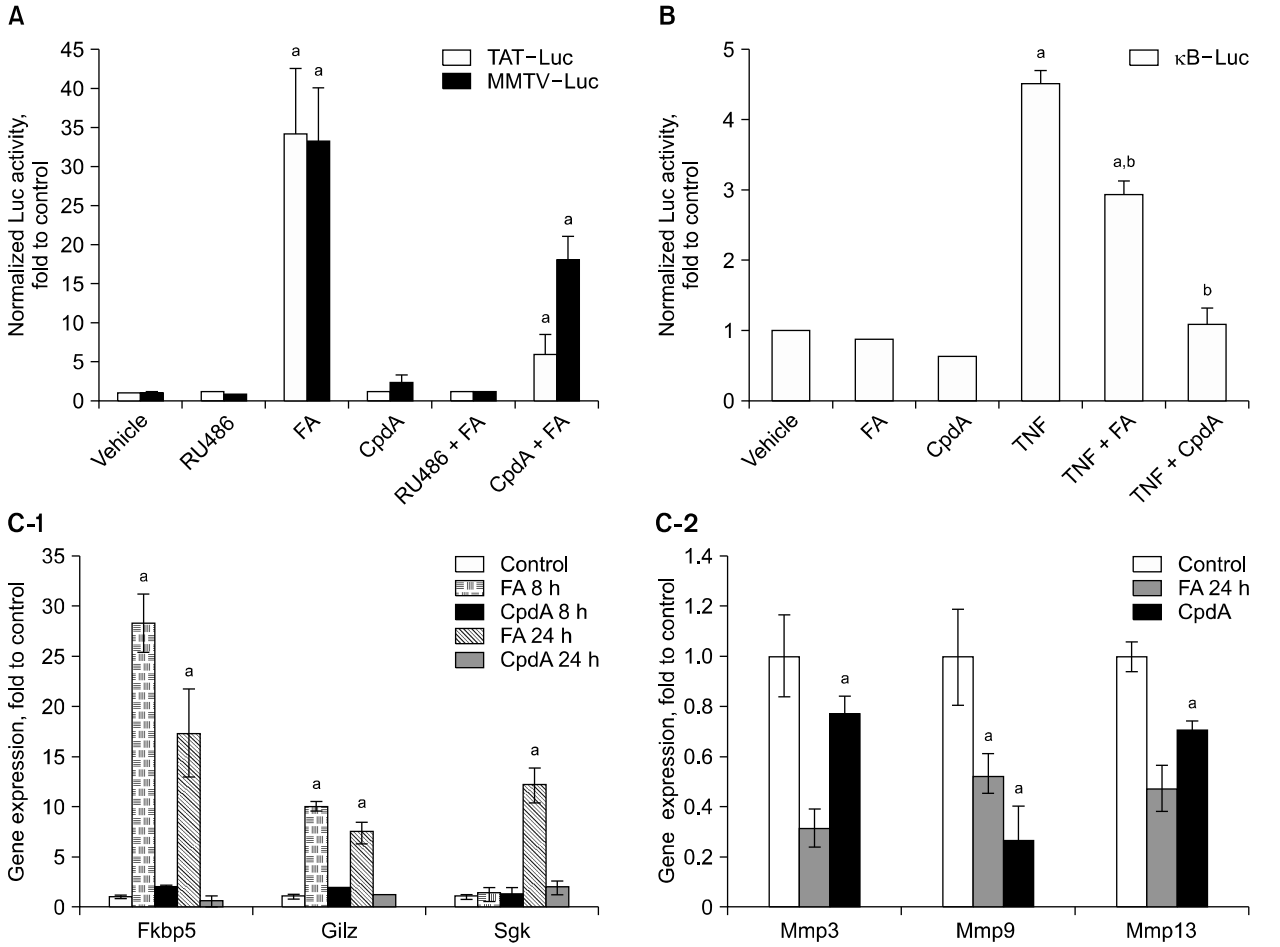

Figure 1. Compound A (CpdA) acts as selective glucocorticoid receptor (GR) modulator in mouse keratinocytes in vitro. (A) 3PC non-transformed mouse keratinocytes were transfected with TAT-*Firefly* Luc (FL) or MMTV-FL reporters, and *Renilla* Luc (RL) reference reporter. Cells were treated with 0.01% acetone (vehicle), GR agonist FA (10^{-6} M), GR antagonist RU486 (10^{-5}M), CpdA (10^{-5}M), and their combinations for 24 hours. (B) 3PC cells were transfected with κB × 3-FL and RL reference reporters, pretreated with 0.01% acetone, CpdA (10^{-5} M) or FA (10^{-6} M) for 24 hours, and treated with TNF-α (10 ng/mL) for 6 hours to activate NF-κB. (A, B) FL activity was normalized against RL activity to equalize for transfection efficacy. The results of one representative experiment (three wells/experimental group) are shown as fold of Luciferase induction compared to control (mean ± SEM). [a]Statistically significant differences in comparison to vehicle treatment; [b]statistically significant differences in comparison to TNF-α treatment ($P < 0.5$). (C-1, C-2) 3PC cells were treated with 0.01% acetone, 10^{-6} M FA, 10^{-5} M CpdA for 8 to 24 hours, and the expression of target genes was assessed by reverse transcription-PCR. The mRNA expression analysis was performed with ChemiDoc XRS^{TM} image documentation system and quantitated with Quantity One software. The results of one representative experiment (three dishes/experimental group) are shown as relative fold change normalized to a housekeeping gene GAPDH. [a]Statistically significant differences in comparison to vehicle treatment ($P < 0.5$). Luc, luciferase; FA, fluocinolone acetonide; Fkbp5, FK506 binding protein 5; Sgk, serum/glucocorticoid regulated kinase.

and CpdA at doses 20 to 40 μg, and in some cases, 100 μg/animal.

As expected, a single application of TPA on the dorsal skin resulted in skin inflammation characterized by typical extensive leukocyte infiltration, combined with increased keratinocyte proliferation and epidermal hyperplasia (Fig. 2A~2C). Short (1 hr) pretreatment with FA or CpdA resulted in notably reduced skin inflammation, as evaluated by gross skin morphology and quantitative analysis of dermal infiltration with leukocytes: FA reduced the effect of TPA on dermal infiltration by 55 % and CpdA by 21% (Fig. 2A and 2B).

In addition, we assessed FA and CpdA effect on CO-induced ear edema – one of the tests of choice to study the effect of new anti-inflammatory compounds.[5,19,17,39] The anti-inflammatory effect of GR ligands was quantitatively assessed by ear weight 9 hours after application of irritant when ear swelling in B6D2 mice reaches its maximum.[17] The pretreatment with FA or CpdA reduced the effect of TPA on ear edema by 35% and 25% accordingly.

The cutaneous inflammatory response to TPA was accom-

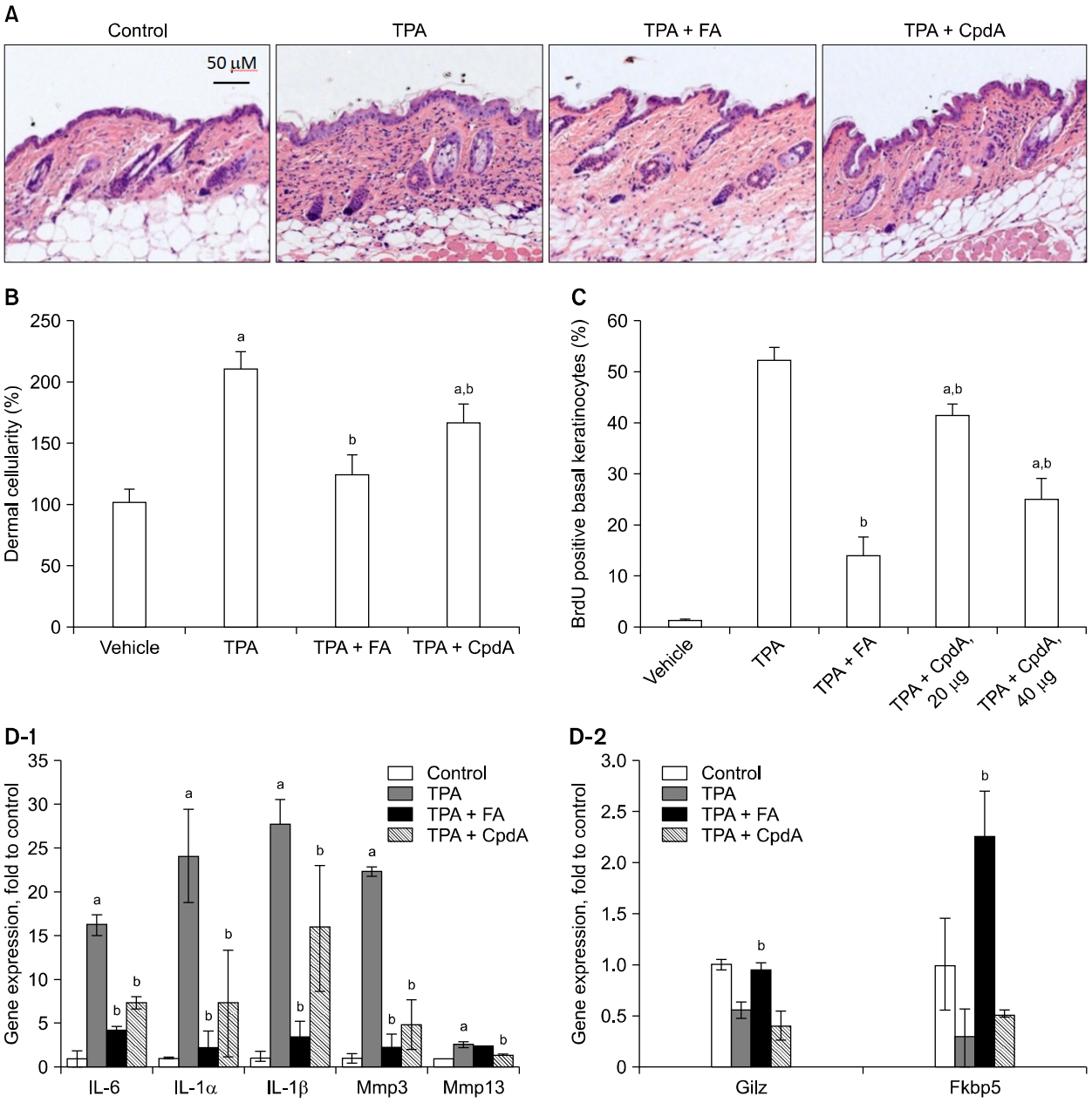

Figure 2. Inhibitory effect of compound A (CpdA) on the expression of inflammatory cytokines, contact dermatitis and epidermal hyperplasia induced by tumor promoter 2-*O*-tetradecanoylphorbol-13-acetate (TPA). B6D2 mice were pre-treated topically with acetone, fluocinolone acetonide (FA) (2 μg), or CpdA (40 μg) for 1 hour, and treated with acetone (control) or TPA (2 μg) for 24 hours. (A) H&E staining of formalin-fixed, paraffin-embedded skin sections. (B) Quantitative analysis of dermal cellularity (as the readout for inflammation) was performed in dorsal skin sections as described in Materials and Methods. The dermal cellularity in treated animals is presented as % to control animals. (C) Quantitative analysis of keratinocyte proliferation. The number of bromodeoxyuridine (BrdU)-positive keratinocytes is presented as % to the total number of basal keratinocytes in interfollicular epidermis. (D-1, D-2) Reverse transcription-PCR analysis of gene expression. Mice were pretreated with acetone, CpdA or FA as above, and treated with acetone (control) or TPA (2 μg) for 8 hours. RNA was extracted from dorsal epidermis harvested from individual animals and used for RT-PCR analysis of target genes. The mRNA expression analysis was performed with ChemiDoc XRS^{TM} image documentation system and quantitated with Quantity One software. The results of one representative experiment (three animals/experimental group) are shown as relative fold change normalized to a housekeeping gene GAPDH. (B, C, D-1, D-2) The mean ± SD were calculated for three individual skin samples in one representative experiment. [a]Statistically significant differences ($P < 0.05$) in comparison to vehicle treatment; [b]statistically significant differences in comparison to TPA treatment. IL, interleukin; Gilz, glucocorticoid-induced leucine zipper; Fkbp5, FK506 binding protein 5.

panied by epidermal hyperplasia. CpdA inhibited keratinocyte proliferation induced by TPA by 25% to 45% in a dose-dependent manner (Fig. 2C), which consequently resulted in inhibition of TPA-induced epidermal hyperplasia. A further increase of CpdA dose to 100 μg/mouse did not result in the increased TPA counteraction by CpdA (data not shown).

3. CpdA does not induce skin atrophy

In humans and mice, topical glucocorticoids induce skin atrophy that affects all skin compartments.[2-5,17,38,45]

We used continuous regiment of treatment to compare the effects of GR ligands on skin hypoplasia. After four daily applications of FA (2 μg/animal), keratinocyte proliferation was completely blocked, and the epidermal thickness was reduced by 45%-50% (Fig. 3A and 3C). FA also affected collagen fiber packaging and orientation, and induced severe atrophy of subcutaneous fat as evident in sections stained by both H&E and Mason's trichrome (Fig. 3A).

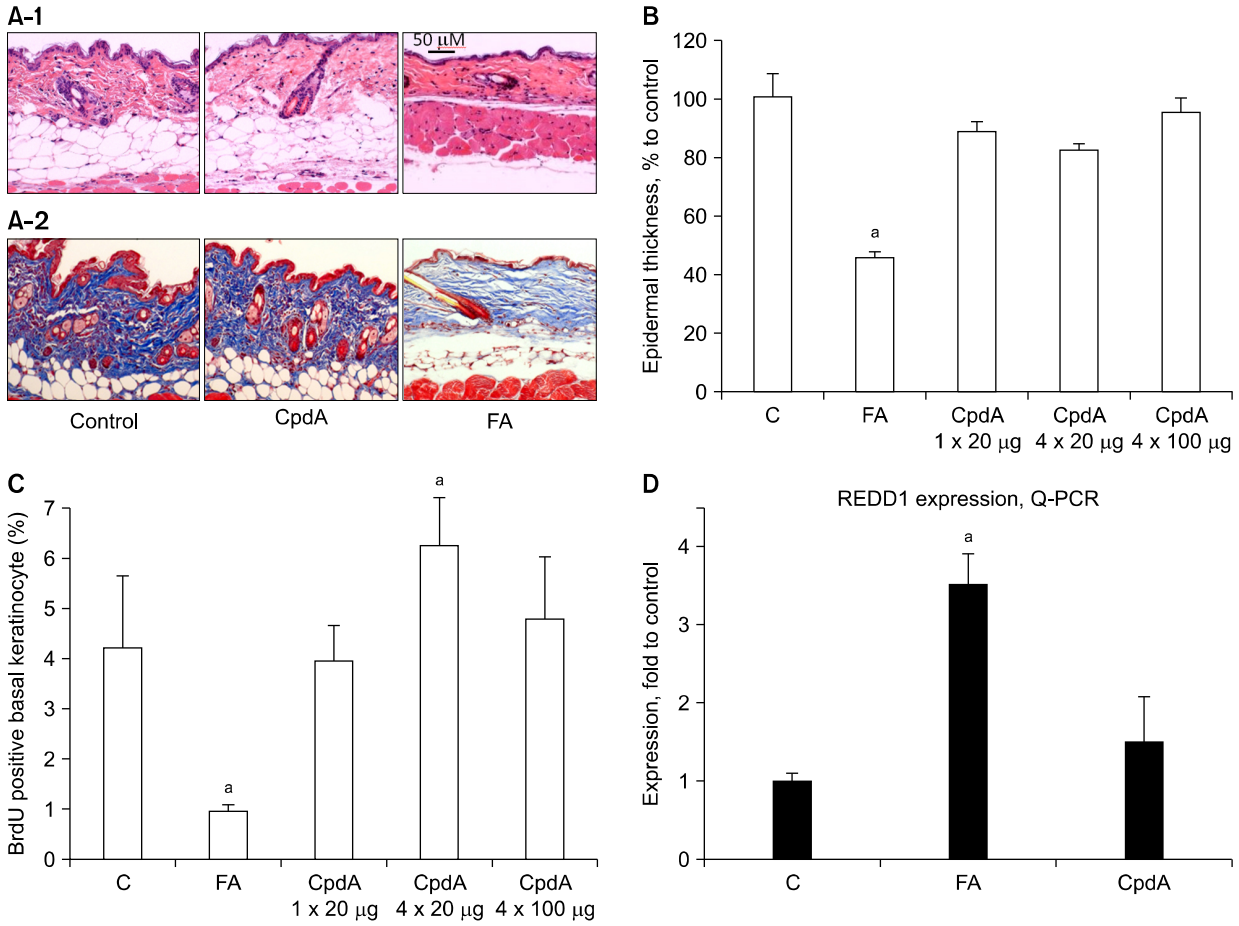

Figure 3. Compound A (CpdA) does not activate the expression of atrophogene regulated in development and DNA damage response 1 (REDD1) and does not induce skin atrophy. CpdA (20-100 μg/animal), or vehicle (200 μL acetone) were applied to the back skin of B6D2 animals every 24 hours for one (×1) or four (×4) days as indicated. Fluocinolone acetonide (FA) (2 μg/animal) was applied every 24 hours for four days. (A) Formalin-fixed, paraffin-embedded skin was stained with H&E (A-1) or Masson's trichrome (A-2). (B) Effect of FA and CpdA on epidermal thickness in B6D2 mice. Quantification of the epidermal thickness was performed in dorsal skin sections as described in Materials and Methods. The epidermal thickness in treated animals is presented as % to control animals. (C) Effect of FA and CpdA on basal keratinocyte proliferation in B6D2 animals. The number of bromodeoxyuridine (BrdU)-positive keratinocytes is presented as % to the total number of basal keratinocytes in interfollicular epidermis. (D) Effect of FA and CpdA on the expression of REDD1 in epidermis. B6D2 mice were treated once topically with FA and CpdA and solvent (as above). Three animals per experimental group were used. Twenty-four hours after application, total RNA was extracted from dorsal epidermis and used for RT-PCR analysis of REDD1 expression. The mRNA expression analysis was performed with ChemiDoc XRS™ image documentation system and quantitated with Quantity One software. The results of one representative experiment (three animals/experimental group) are shown as relative fold change normalized to a housekeeping gene GAPDH. (B~D) The mean ± SD were calculated for three individual skin samples in one representative experiment. [a]Statistically significant differences ($P < 0.05$) in comparison to vehicle treatment. C, control.

In striking contrast, CpdA (20-100 μg) did not significantly affect keratinocyte proliferation after one or two applications, and even modestly increased it after longer treatments (Fig. 3B). This pro-proliferative effect of CpdA in keratinocytes may reflect the partial inhibition of endogenous GR signaling due to CpdA antagonistic effect on GR activity (Fig. 1).

We recently showed that REDD1 plays a causative role in skin atrophy induced by glucocorticoids.[17] Interestingly, the inability of CpdA to induce skin atrophy and epidermal hypoplasia correlated with weak, borderline induction of REDD1 by CpdA (Fig. 3D).

DISCUSSION

Traditionally, the focus in the search for novel dissociated GR ligands has been on the compounds for systemic use.[4,20,23] Consequently, both therapeutic and side effects of selective GR modulators in skin have not been well investigated. The major goals of our study were to determine the effects of the novel GR modulator, CpdA, on gene expression in keratinocytes, and to evaluate the anti-inflammatory and atrophogenic effects of topical CpdA applications in vivo.

Our previous studies revealed the remarkable capability of CpdA to shift GR function towards TR due to inefficient GR dimerization and phosphorylation at S211 required for the maximal GR TA; and/or selective recruitment of transcriptional co-repressors, such as NCoR and SMRT to GR after CpdA exposure.[1,25-27,29] We showed here that CpdA did not significantly activate glucocorticoid-responsive endogenous genes in keratinocytes. Even more, CpdA acted as a partial GR antagonist when keratinocytes were treated with CpdA + FA. At the same time, CpdA was proficient in TR of whole cohort of endogenous genes related to inflammation, such as interleukins and MMPs both in vitro and in vivo. It is well accepted in the field that the mechanisms underlying GR TA and GR TR are different. GR TA is mostly mediated via binding of GR homodimer to the glucocorticoid responsive elements in gene promoters/enhancers, while GR TR is in many cases mediated via binding of monomeric GR to other transcription factors such as NF-κB.[1,4,5,10,11] It is possible that the unparalleled capability of CpdA to dissociate GR TA and TR could be explained by inability of CpdA to induce GR dimerization upon binding to the receptor. The inhibitory effect of CpdA on interleukin (IL)-6, IL-1β, and Mmp3 expression was weaker compared to the effect of glucocorticoid FA (Fig. 1 and 2). It is known that these pro-inflammatory genes are regulated by both NF-κB and AP1.[9,10,15] Interestingly, in our recent paper,[46] we showed that at least in some cell types CpdA has a unique capability to selectively inhibit NF-κB but not AP-1 activity, which may explain the weaker TR effect of CpdA in context of genes regulated by AP-1.

We demonstrated here that CpdA has an anti-inflammatory potential when used topically in a mouse model of contact dermatitis. In our studies, we followed the design of our previous experiments with systemic CpdA delivery,[25,26] and used 10-20 fold higher doses of topical CpdA compared to glucocorticoid FA. Overall, in topical treatments the anti-inflammatory effect of CpdA was very consistent, but less pronounced than effects of the glucocorticoid. As systemic treatment with CpdA resulted in strong, comparable to glucocorticoids anti-inflammatory activity,[25,26,29,30] it is possible that moderate effect of topical CpdA could reflect its incomplete penetration through the stratum corneum–an important step that defines steroid potency.[47]

It was reported recently that topical CpdA at very high, milligram range of doses could aggravate the pro-inflammatory and pro-proliferative effect of TPA possibly due to the induction of c-jun and some cytokines including IL-6.[32] Those in vivo data are in line with the findings in our recent article[46] demonstrating that CpdA can in some cell types enhance AP-1 activity and consequently can increase expression of AP-1-dependent genes including c-Jun, IL-6, and Mmp-3. Moreover, in the same study, we found that in contrast to glucocorticoids, CpdA failed to block up-stream JNK kinase activation and c-Jun phosphorylation activated by TPA, TNF-α, and other inducers. Overall, the data by Kowalczyk et al.[32] indicate potential side effects of high doses of topical CpdA, outside of its therapeutic window. Interestingly, Schoepe et al.,[5] used low but still therapeutically active doses of topical CpdA (0.01%-0.001%) in hairless rats, and did not observe pro-proliferative CpdA activity. Thus, our results and literature data suggest the beneficial effect of lower therapeutic doses of CpdA, and indicate that use of high topical doses of CpdA should be avoided.

One of the most prominent adverse effects of topical glucocorticoids is skin atrophy.[2-5,17,38,45] In our work we mostly focused on the epidermal compartment, and found that in contrast to glucocorticoid FA, CpdA did not induce epidermal hypoplasia. We recently discovered that induction of REDD1, a stress-inducible inhibitor of mTOR complex 1, by glucocorticoids, plays a critical role in skin atrophy.[17] In this study, we for the first time revealed a strong correlation between REDD1 induction in epidermis by GR ligands and animal sensitivity to epidermal hypoplasia. Overall, our data suggest that REDD1 has a potential as a reliable surrogate marker for steroid-induced skin atrophy.

In conclusion, our work confirmed unique ligand properties of

GR selective activator CpdA in keratinocytes, provided proof of principle that anti-inflammatory and atrophogenic effects of GR signaling could be dissociated, and suggested the potential clinical applications of CpdA and its derivatives as novel topical anti-inflammatory compounds with reduced side effects for the treatment of dermatological patients. Taking into consideration the important role of inflammation in development of skin cancer[48,49] and proven anti-cancer properties of CpdA,[1] CpdA and its derivatives could be considered for skin cancer treatment and prevention at the doses that do not affect keratinocyte proliferation.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

REFERENCES

1. Lesovaya E, Yemelyanov A, Swart AC, Swart P, Haegeman G, Budunova I. Discovery of compound A: a selective activator of the glucocorticoid receptor with anti-inflammatory and anti-cancer activity. Oncotarget 2015;6:30730-44.
2. Chebotaev D, Yemelyanov A, Budunova I. The mechanisms of tumor suppressor effect of glucocorticoid receptor in skin. Mol Carcinog 2007;46:732-40.
3. Schoepe S, Schäcke H, May E, Asadullah K. Glucocorticoid therapy-induced skin atrophy. Exp Dermatol 2006;15:406-20.
4. Schäcke H, Rehwinkel H, Asadullah K, Cato AC. Insight into the molecular mechanisms of glucocorticoid receptor action promotes identification of novel ligands with an improved therapeutic index. Exp Dermatol 2006;15:565-73.
5. Schoepe S, Schäcke H, Bernd A, Zöller N, Asadullah K. Identification of novel in vitro test systems for the determination of glucocorticoid receptor ligand-induced skin atrophy. Skin Pharmacol Physiol 2010;23:139-51.
6. Barnes PJ. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin Sci (Lond) 1998;94:557-72.
7. Adcock IM. Glucocorticoid-regulated transcription factors. Pulm Pharmacol Ther 2001;14:211-9.
8. Beato M, Herrlich P, Schütz G. Steroid hormone receptors: many actors in search of a plot. Cell 1995;83:851-7.
9. Necela BM, Cidlowski JA. Mechanisms of glucocorticoid receptor action in noninflammatory and inflammatory cells. Proc Am Thorac Soc 2004;1:239-46.
10. De Bosscher K, Vanden Berghe W, Haegeman G. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. Endocr Rev 2003;24:488-522.
11. Baschant U, Lane NE, Tuckermann J. The multiple facets of glucocorticoid action in rheumatoid arthritis. Nat Rev Rheumatol 2012;8:645-55.
12. Ramamoorthy S, Cidlowski JA. Exploring the molecular mechanisms of glucocorticoid receptor action from sensitivity to resistance. In: Maghnie M, Loche S, Cappa M, Ghizzoni L, Lorini R, eds. Hormone Resistance and Hypersensitivity. From Genetics to Clinical Management. Endocr Dev 24. Basel, Karger, pp 41-56, 2013.
13. De Bosscher K, Beck IM, Haegeman G. Classic glucocorticoids versus non-steroidal glucocorticoid receptor modulators: survival of the fittest regulator of the immune system? Brain Behav Immun 2010;24:1035-42.
14. Nixon M, Andrew R, Chapman KE. It takes two to tango: dimerisation of glucocorticoid receptor and its anti-inflammatory functions. Steroids 2013;78:59-68.
15. Tuckermann JP, Reichardt HM, Arribas R, Richter KH, Schütz G, Angel P. The DNA binding-independent function of the glucocorticoid receptor mediates repression of AP-1-dependent genes in skin. J Cell Biol 1999;147:1365-70.
16. Johansson N, Airola K, Grénman R, Kariniemi AL, Saarialho-Kere U, Kähäri VM. Expression of collagenase-3 (matrix metalloproteinase-13) in squamous cell carcinomas of the head and neck. Am J Pathol 1997;151:499-508.
17. Baida G, Bhalla P, Kirsanov K, Lesovaya E, Yakubovskaya M, Yuen K, et al. REDD1 functions at the crossroads between the therapeutic and adverse effects of topical glucocorticoids. EMBO Mol Med 2014;7:42-58.
18. Schäcke H, Schottelius A, Döcke WD, Strehlke P, Jaroch S, Schmees N, et al. Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects. Proc Natl Acad Sci U S A 2004;101:227-32.
19. Schäcke H, Zollner TM, Döcke WD, Rehwinkel H, Jaroch S, Skuballa W, et al. Characterization of ZK 245186, a novel, selective glucocorticoid receptor agonist for the topical treatment of inflammatory skin diseases. Br J Pharmacol 2009;158:1088-103.
20. Hu X, Du S, Tunca C, Braden T, Long KR, Lee J, et al. The antagonists but not partial agonists of glucocorticoid receptor ligands show substantial side effect dissociation. Endocrinology 2011;152:3123-34.
21. Shimizu N, Yoshikawa N, Ito N, Maruyama T, Suzuki Y, Takeda S, et al. Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle. Cell Metab 2011;13:170-82.
22. Britto FA, Begue G, Rossano B, Docquier A, Vernus B, Sar C, et al. REDD1 deletion prevents dexamethasone-induced skeletal muscle atrophy. Am J Physiol Endocrinol Metab 2014;307:E983-93.
23. López FJ, Ardecky RJ, Bebo B, Benbatoul K, De Grandpre L, Liu S, et al. LGD-5552, an antiinflammatory glucocorticoid receptor ligand with reduced side effects, in vivo. Endocrinology 2008;149:2080-9.
24. Swart P, Swart AC, Louw A, van der Merwe KJ. Biological activities of the shrub Salsola tuberculatiformis Botsch: contraceptive or stress alleviator? Bioessays 2003;25:612-9.
25. De Bosscher K, Vanden Berghe W, Beck IM, Van Molle W, Hennuyer N, Hapgood J, et al. A fully dissociated compound of plant origin for inflammatory gene repression. Proc Natl Acad Sci U S A 2005;102:15827-32.

26. Dewint P, Gossye V, De Bosscher K, Vanden Berghe W, Van Beneden K, Deforce D, et al. A plant-derived ligand favoring monomeric glucocorticoid receptor conformation with impaired transactivation potential attenuates collagen-induced arthritis. J Immunol 2008;180:2608-15.
27. Robertson S, Allie-Reid F, Vanden Berghe W, Visser K, Binder A, Africander D, et al. Abrogation of glucocorticoid receptor dimerization correlates with dissociated glucocorticoid behavior of compound a. J Biol Chem 2010;285:8061-75.
28. Yemelyanov A, Czwornog J, Gera L, Joshi S, Chatterton RT Jr, Budunova I. Novel steroid receptor phyto-modulator compound a inhibits growth and survival of prostate cancer cells. Cancer Res 2008;68:4763-73.
29. van Loo G, Sze M, Bougarne N, Praet J, Mc Guire C, Ullrich A, et al. Antiinflammatory properties of a plant-derived nonsteroidal, dissociated glucocorticoid receptor modulator in experimental autoimmune encephalomyelitis. Mol Endocrinol 2010;24:310-22.
30. Reber LL, Daubeuf F, Plantinga M, De Cauwer L, Gerlo S, Waelput W, et al. A dissociated glucocorticoid receptor modulator reduces airway hyperresponsiveness and inflammation in a mouse model of asthma. J Immunol 2012;188:3478-87.
31. Rauner M, Goettsch C, Stein N, Thiele S, Bornhaeuser M, De Bosscher K, et al. Dissociation of osteogenic and immunological effects by the selective glucocorticoid receptor agonist, compound A, in human bone marrow stromal cells. Endocrinology 2011;152:103-12.
32. Kowalczyk P, Kowalczyk MC, Junco JJ, Tolstykh O, Kinjo T, Truong H, et al. The possible separation of 12-O-tetradecanoylphorbol-13-acetate-induced skin inflammation and hyperplasia by compound A. Mol Carcinog 2013;52:488-96.
33. Chebotaev D, Yemelyanov A, Zhu L, Lavker RM, Budunova I. The tumor suppressor effect of the glucocorticoid receptor in skin is mediated via its effect on follicular epithelial stem cells. Oncogene 2007;26:3060-8.
34. Sevilla LM, Bayo P, Latorre V, Sanchis A, Pérez P. Glucocorticoid receptor regulates overlapping and differential gene subsets in developing and adult skin. Mol Endocrinol 2010;24:2166-78.
35. Spiegelman VS, Budunova IV, Carbajal S, Slaga TJ. Resistance of transformed mouse keratinocytes to growth inhibition by glucocorticoids. Mol Carcinog 1997;20:99-107.
36. Budunova IV, Carbajal S, Kang H, Viaje A, Slaga TJ. Altered glucocorticoid receptor expression and function during mouse skin carcinogenesis. Mol Carcinog 1997;18:177-85.
37. Pérez P, Page A, Bravo A, Del Río M, Giménez-Conti I, Budunova I, et al. Altered skin development and impaired proliferative and inflammatory responses in transgenic mice overexpressing the glucocorticoid receptor. FASEB J 2001;15:2030-2.
38. Chebotaev DV, Yemelyanov AY, Lavker RM, Budunova IV. Epithelial cells in the hair follicle bulge do not contribute to epidermal regeneration after glucocorticoid-induced cutaneous atrophy. J Invest Dermatol 2007;127:2749-58.
39. Budunova IV, Kowalczyk D, Pérez P, Yao YJ, Jorcano JL, Slaga TJ. Glucocorticoid receptor functions as a potent suppressor of mouse skin carcinogenesis. Oncogene 2003;22:3279-87.
40. Park KK, Ko DH, You Z, Heiman AS, Lee HJ. Synthesis and pharmacological evaluations of new steroidal anti-inflammatory antedrugs: 9alpha-Fluoro-11beta,17alpha,21-trihydroxy-3,20-dioxo-pregna-1,4-diene-16alpha-carboxylate (FP16CM) and its derivatives. Steroids 2006;71:83-9.
41. Cannarile L, Cuzzocrea S, Santucci L, Agostini M, Mazzon E, Esposito E, et al. Glucocorticoid-induced leucine zipper is protective in Th1-mediated models of colitis. Gastroenterology 2009; 136:530-41.
42. Wang JC, Derynck MK, Nonaka DF, Khodabakhsh DB, Haqq C, Yamamoto KR. Chromatin immunoprecipitation (ChIP) scanning identifies primary glucocorticoid receptor target genes. Proc Natl Acad Sci U S A 2004;101:15603-8.
43. Itani OA, Liu KZ, Cornish KL, Campbell JR, Thomas CP. Glucocorticoids stimulate human sgk1 gene expression by activation of a GRE in its 5'-flanking region. Am J Physiol Endocrinol Metab 2002;283:E971-9.
44. Yemelyanov A, Bhalla P, Yang X, Ugolkov A, Iwadate K, Karseladze A, et al. Differential targeting of androgen and glucocorticoid receptors induces ER stress and apoptosis in prostate cancer cells: a novel therapeutic modality. Cell Cycle 2012;11:395-406.
45. Woodbury R, Kligman AM. The hairless mouse model for assaying the atrophogenicity of topical corticosteroids. Acta Derm Venereol 1992;72:403-6.
46. De Bosscher K, Beck IM, Dejager L, Bougarne N, Gaigneaux A, Chateauvieux S, et al. Selective modulation of the glucocorticoid receptor can distinguish between transrepression of NF-κB and AP-1. Cell Mol Life Sci 2014;71:143-63.
47. Wiedersberg S, Leopold CS, Guy RH. Bioavailability and bioequivalence of topical glucocorticoids. Eur J Pharm Biopharm 2008;68:453-66.
48. Afaq F, Katiyar SK. Polyphenols: skin photoprotection and inhibition of photocarcinogenesis. Mini Rev Med Chem 2011;11: 1200-15.
49. Rundhaug JE, Fischer SM. Cyclo-oxygenase-2 plays a critical role in UV-induced skin carcinogenesis. Photochem Photobiol 2008; 84:322-9.