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Novel non-transcriptionally acting glucocorticoid receptor ligands that dissociate immunosuppression from effects on adipogenesis and muscle cell atrophy.

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Summary

The use of glucocorticoids as immunosuppressives is limited by important side effects such as induction of adipogenesis and development of muscle atrophy. Glucocorticoids bind to the glucocorticoid receptor (GR) resulting in the formation of a homodimer that translocates from the cytosol to the nucleus. In the nucleus these dimers bind to glucocorticoid response elements (GRE) on the DNA and mediate both transactivation and transrepression of target genes. In addition to this transcriptional regulation of cellular phenotype, there are also more rapid, non-transcriptionally mediated effects of glucocorticoids. We have previously shown that the GR is part of the T cell receptor (TCR) complex and that ligand binding results in dissociation of the GR from this complex and inhibition of canonical TCR signaling through LCK-PLCγ.[1, 2] The dissociation of this complex appears to play an important role in glucocorticoid mediated inhibition of T cell activation as stimuli that bypass LCK-PLCγ-signaling render T lymphocytes glucocorticoid resistant.[3] Here we aimed to develop a GR ligand that inactivates LCK-PLCγ-signaling without resulting in transcriptional regulation. Potential steroidal and non-steroidal candidates were identified using an in silico docking assay to predict GR affinity. Selected compounds where screened in in vitro for GRE mediated transcriptional regulation and their capacity to inhibit T cell activation. This approach led to the discovery of compounds S3.1 and S3.4, which lack a generic cortisol structure but bind the GR in T lymphocytes and inhibit LCK-PLCγ-dependent T cell proliferation without causing transcriptional modulation of GR target genes. In contrast to classical glucocorticoids, S3.1 and S3.4 do not induce adipogenesis and do not cause muscle cell atrophy in vitro. Our data show that it is possible to develop non-steroidal GR ligands that dissociate transcriptional from non-transcriptional effects and may have reduced side effects.

We started our search for a non-transcriptionally acting GR ligand with two rounds of in silico screening. In the first round, we identified ~18,000 steroidal and ~230,000 non-steroidal compounds based on their similarity to the structure of cortisol or gonane respectively. In the second round these compounds were virtually docked into the binding pocket of the GR in two distinct conformations and assigned a score that reflected their predicted binding potency. The top 30 steroidal and 50 non-steroidal compounds with the highest scores were then further screened using two in vitro assays. The first assay consisted of HEK-293 cells stably transduced with a GRE-luciferase construct to determine if the compounds were capable of inducing GR-dependent transcriptional regulation. To measure the ability of the compounds to inhibit T cell proliferation, primary human peripheral blood lymphocytes (PBL) were stimulated with phytohemagglutinin (PHA) or Staphylococcus aureus enterotoxin B (SEB) in the presence of 10 μM of each compound and proliferation was measured using a tritiated thymidine incorporation assay. PHA binds CD3 and activates canonical LCK-PLCγ signaling which can be almost completely inhibited by glucocorticoids.[4] In contrast, SEB activates both canonical TCR dependent LCK-PLCγ signaling and a non-canonical G protein-PLCβ dependent signaling pathway, which renders SEB stimulated lymphocytes partially steroid resistant.[3] We were therefore looking for a compound that would inhibit PHA dependent proliferation to a greater extent than SEB mediated proliferation. This strategy (for results of all compounds see Extended Data Table 1) resulted in the identification of two non-steroidal compounds, S3.1 and S3.4 (Fig. 1a-d) which did not result in GRE-mediated transactivation and inhibited PHA mediated proliferation to a greater extent than SEB mediated proliferation (Fig. 1e). To confirm binding of S3.1 and S3.4 to the GR, we performed a competition assay using [^{3}H]dexamethasone in human PBL (Fig. 1f). This showed that both compounds are low affinity ligands for the GR. The dose-response curve for inhibition of PHA mediated lymphocyte proliferation of both compounds was much lower than high affinity ligand dexamethasone but similar to that of hydrocortisone (Fig. 1g).

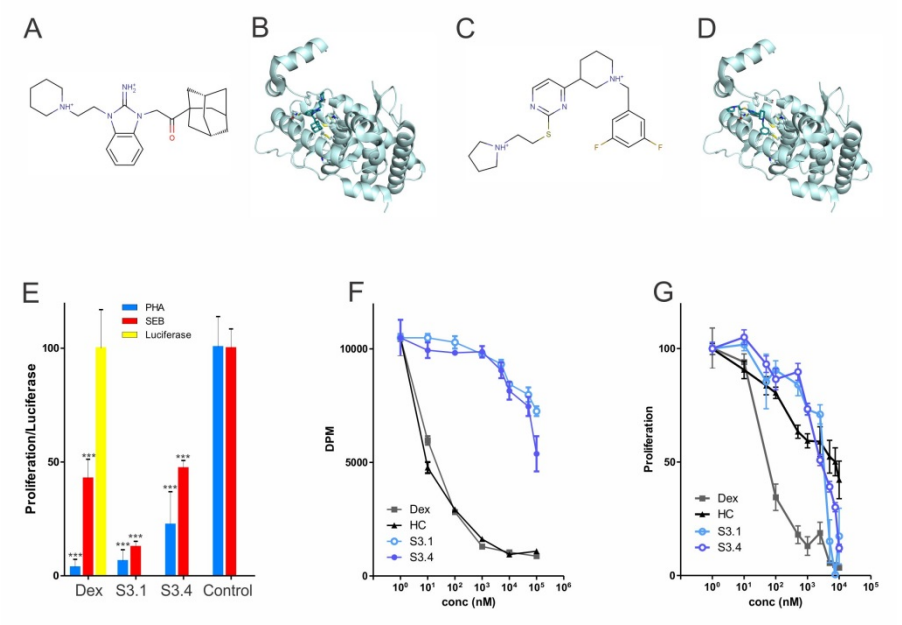

Figure 1. Identification of S3.1 and S3.4 as low affinity GR ligands that lack transcriptional activation but suppress T cell activation. **a**, structure formula of S3.1. **b**, 3D representation of S3.1 binding to the GR. **c**, structure formula of S3.4. **d**, 3D representation of S3.4 binding to the GR. **e**, HEK-293 cells stably transduced with a GRE-luciferase construct were treated with dexamethasone (Dex, 10 μm), S3.1 (10 μm) or S3.4 (10 μm), luciferase production (yellow bars) is shown relative to dexamethasone. Human PBL were treated with PHA (10 mg/ml, blue bars) or SEB (100 ng/ ml, red bars) in the presence or absence of dexamethasone, S3.1 and S3.4 and T cell proliferation was measured using tritiated thymidine, data are relative to PHA/SEB stimulated PBL in the absence of compound. **f**, competition assay using [^{3}H]dexamethasone in whole human PBL. S3.1 and S3.4 show low affinity for the GR compared to both dexamethasone (Dex) and hydrocortisone (HC). **g**, proliferation of PHA (10 mg/ml) stimulated human PBL relative to no compound in the presence of a concentration range of dexamethasone (Dex), hydrocortisone (HC), S3.1 and S3.4. Data in **e**-**g** are mean and standard error. *** = $P < 0.001$.

We previously found that the chaperone HSP90 is specifically complexed with both LCK and the GR in activated T cells in the absence of GR ligand. Binding of the GR by glucocorticoids dissociates this complex with resulting

inhibition of signaling downstream of LCK. We found that both S3.1 and S3.4 inhibited of the phosphorylation of downstream LCK signaling targets ZAP70 and LAT similar to dexamethasone (Fig. 2a). To examined if S3.1 and S3.4 would disrupt the interaction between the GR and LCK we performed an immunoprecipitation experiment on the endogenous proteins in primary human PBL. Immunopreciptation of GR showed that treatment of PBL with PHA resulted in increased interaction between the GR and LCK, this interaction was disrupted by dexamethasone as well as S3.1 and S3.4 (Fig 2b). Dissociation by S3.4 was as potent as that induced by dexamethasone. We next determined if S3.1 and S3.4 would also dissociate LCK from the chaperone HSP90, again by immunoprecipitating the endogenous proteins in PHA treated primary human PBL. Treatment with PHA increased the interaction between LCK and HSP90, which was reduced by both compounds (Fig. 2c). Again the dissociation induced by S3.4 was as potent as that induced by dexamethasone. Immunoprecipitation of LCK flowed by Western blot for HSP90 gave the same results (Fig. 2d).

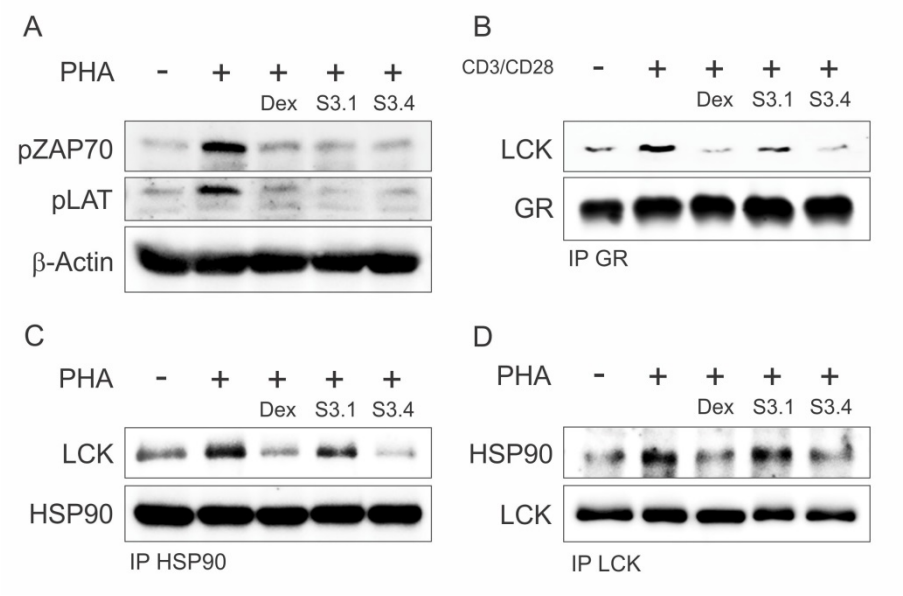

Figure 2. S3.1 and S3.4 dissociate the HSP90-GR-LCK complex. **a**, Dexamethasone (10 μm), S3.1 (10 μm) and S3.4 (10 μm) inhibit phosphorylation of downstream TCR signaling targets ZAP70 and LAT in PHA stimulated human PBL. **b**,

Immunoprecipitation of the GR in CD3/CD28 stimulated human PBL shows that dexamethasone (Dex, 10 μm), S3.1 (10 μm) and S3.4 (10 μm) dissociate the interaction of the GR and LCK in activated T cells. **c**, Immunoprecipitation of HSP90 in PHA stimulated human PBL shows that dexamethasone (Dex, 10 μm), S3.1 (10 μm) and S3.4 (10 μm) dissociate the interaction of the HSP90 and LCK in activated T cells. **d**, immunoprecipitation of LCK shows the same results.

Since both S3.1 and S3.4 do not induce GRE-mediated transcriptional modulation, we examined if the compounds induced nuclear translocation of the GR. We transiently transfected HCT116 cells that express no detectable GR, with a GFP labeled GR. Dexamethasone induced a potent nuclear translocation of the GR as expected (Fig. 3a). Of the two compounds, only S3.4 induced a modest level of nuclear translocation in GR-GFP transfected cells. This result was confirmed by detecting endogenous GR expression by Western blot in Hela cells after isolating nuclear and cytosolic fractions (Fig. 3b,c). Phosphorylation of residues near the N terminus of the GR such as serine 211 (S211) is important to regulate its transcriptional activity.[5] To investigate the phosphorylation status of the GR receptor at S211 after treatment, lysates from Hela cells were analyzed on Western blot (Fig. 3d). Treatment with neither S3.1 nor with S3.4 causes the GR to become phosphorylated. Similar results were obtained when using lysates from stimulated PBL (Fig. 3e).

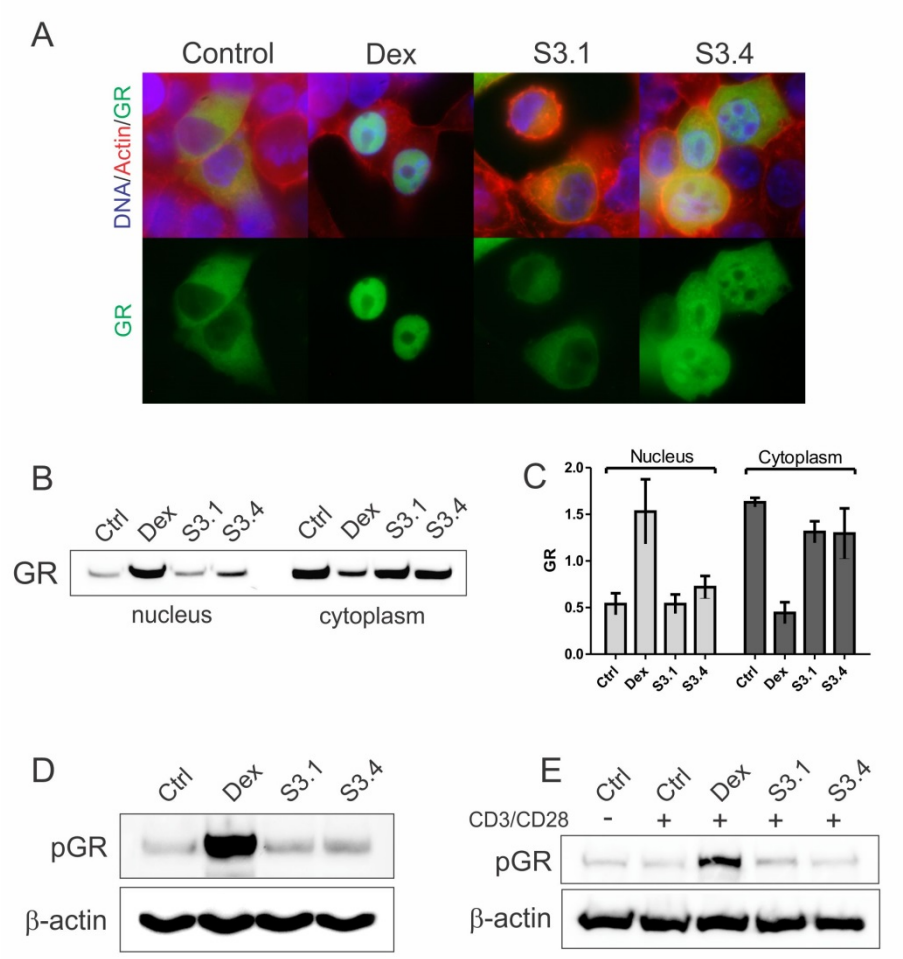

Figure 3. S3.1 and S3.4 do not cause nuclear translocation of phosphorylation of the GR. **a**, HCT116 cells were transfected with a GFP tagged GR and treated with either dexamethasone (Dex, 10 μm), S3.1 (10 μm) or S3.4 (10 μm). The localization of the GR was examined by immunofluoresence. **b**, Hela cells were treated with dexamethasone (Dex, 10 μm), S3.1 (10 μm) or S3.4 (10 μm) and the localization of the GR was determined by western blot in nuclear and cytosolic extracts. **c**, densiometric analysis of nuclear translocation of the GR in Hela cells as determined by western blots of three independent experiments. **d**, phosphorylation of the GR in Hela cells treated with dexamethasone, S3.1 and S3.4. **e**, phosphorylation of the GR in CD3/CD28 stimulated human PBL treated with dexamethasone, S3.1 and S3.4.

Thus, S3.1 and S3.4 are low affinity nonsteroidal GR ligands that inhibit T cell proliferation by dissociating the HSP90-LCK interaction without inducing nuclear translocation of the GR. It is not known to what extent side effects such as adipogenesis and muscle atrophy are dependent on non-transcriptional or transcriptional effects of the GR. We therefore examined the capacity of S3.1 and S3.4 to induce adipogenesis in 3T3-L1 cells, a well-established model of glucocorticoid induced adipogenesis. In these cells treatment with dexamethasone causes adipocyte formation that can be detected by significant accumulation of fatty acids in intracellular vacuoles (Fig. 4a,b). In contrast, treatment with S3.1 or S3.4 did not induce any adipogenesis at all.

Glucocorticoid induced muscle atrophy is caused by loss of muscle fiber thickness due to transactivation of myostatin, a negative modulator of skeletal muscle. To test if treatment with S3.1 or S3.4 caused a reduction in the diameter of muscle fibers a C2C12 myotube model was used. After establishment of the myotubes in culture, the cells were treated for 24 hours with S3.1, S3.4 or dexamethasone. Treatment with dexamethasone reduced the diameter of the myotubes significantly while treatment with S3.1 or S3.4 did not affect the myotubes and diameters remained comparable to the untreated control (Fig. 4c,d).

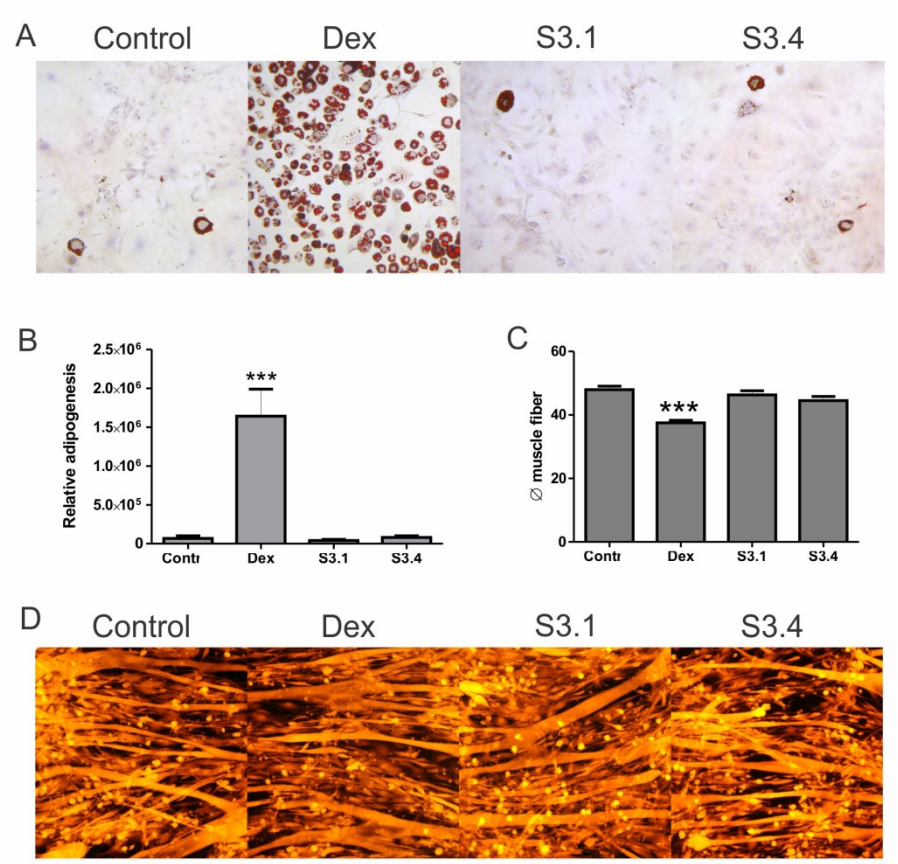

Figure 4. S3.1 and S3.4 do not induce adipogenesis and do not cause myotube atrophy. **a**, 3T3-L1 cells treated with dexamethasone (Dex, 10 μm), S3.1 (10 μm) or S3.4 (10 μm). **b**, quantification of induction of adipogenesis measuring oil red O positive pixels per high powerfield (200x magnification) using ImageJ software (n=4 per condition). **c**, quantification of muscle fiber thickness (n >250 per condition) in C2C12 cells treated with dexamethasone (Dex, 10 μm), S3.1 (10 μm) or S3.4 (10 μm). **d**, muscle fibers of C2C12 cells visualized using glutaraldehyde induced autofluorescence. Data in **b** and **c** are mean and standard error. *** = $P < 0.001$.

All steroid hormones have rapid non-transcriptionally mediated effects in addition to their classical transcriptionally mediated cellular responses.[6] We have previously shown that glucocorticoids inhibit TCR signaling non-transcriptionally by dissociating a TCR associated complex that contains

HSP90, LCK and the GR.[1-3, 7] Thus far it has not been possible to determine if non-transcriptionally mediated GR dependent effects are sufficient to cause inhibition of T cell signaling. Also, since no compounds were available thus far to specifically induce non-transcriptional GR mediated effects it was not known if side effects such as adipogenesis and muscle cell atrophy were dependent on transcriptional or non-transcriptional effects of glucocorticoids. The findings presented here show that it is possible to design GR ligands that specifically dissociate the GR from its cytosolic complex with HSP90 and LCK without causing phosphorylation or nuclear translocation of the GR. Such compounds therefore lack any of the transcriptionally mediated effects of GR bound by classical agonists such as dexamethasone that cause potent phosphorylation and nuclear translocation of the GR. The identification of S3.1 and S3.4 as non-transcriptionally acting GR ligands has allowed us to demonstrate that such compounds inhibit TCR signaling and T cell proliferation without causing effects on adipogenesis or myotube thickness in established in vitro models of these glucocorticoid induced side effects. Our approach shows that it is possible to dissociate GR mediated immunosuppression from unwanted side effects by generating ligands that dislocate the GR from the HSP90 complex but fail to induce nuclear translocation.

Material and Methods

In silico compound screen

We screened 9.2 million publically available compounds from the Zinc database (http://zinc.docking.org) in October 2009. In order to enhance the chance of finding chemicals with the desired effect we worked in parallel with two sets of compounds. The first set consisted of steroidal compounds that contained the four conjugated rings of cortisol (Extended data Fig. 1a), other naturally occurring steroid hormones (Extended data Fig. 1b,c) or synthetic GR agonists such as dexamethasone possessing two double bonds in the first ring (Extended data Fig. 1d). An exact match of a compound with the query skeleton was used as a selection criterion resulting in the identification of ~18,000 candidate compounds. The second

set of compounds comprised molecules that share some similarity to gonane which was quantified using the LINGO method that has been previously described[8] and has been implemented in the OEChem Tool Kit (OpenEye Scientific Software, Inc., Santa Fe, NM, USA). This second set consisted of a further ~230,000 non-steroidal compounds.

In the next round of *in silico* screening, all compounds selected in the first round were virtually docked to the GR binding pocket using the glide mode of the Schrödinger program suite (Schrödinger, LLC, New York). To cover the different conformational spaces in which the compounds could be docking we used four different structures of the GR (to be found at Protein Data Bank http://www.rcsb.org/pdb/home/home.do, under IDs 1M2Z, 1P93, 1NHZ and 3BQD). Structures 1M2Z and 1P93 describe the GR in a state bound by dexamethasone. Another 2 structures (1NHZ, 3BQD) describe the GR bound by an antagonist.

Compounds were finally sorted according to their Z-score values that reflect the probability of binding to the receptor. In order to test compounds capable of binding both known conformational states of the GR we selected the top 30 steroidal compounds and the top 50 non-steroidal compounds in each conformation for *in vitro* testing, resulting in a total of 80 compounds (Extended Data Table 1).

Antibodies and Reagents

Dexamethasone, hydrocortisone, IBMX, insulin, oil red O, glutaraldehyde and SEB were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Luciferase was purchased from Promega (Leiden, The Netherlands). PHA (PHA-M), phalloidin, SlowFade® Gold Antifade Reagent with DAPI and horse serum was purchased from Life Technologies (Bleiswijk, The Netherlands). Human anti-CD3 and anti-CD28 were purchased from Sanquin (Amsterdam, The Netherlands). Anti-phospho-GR (Ser211) #4161, anti-phospho-Zap70 (Tyr319)/Syk (Tyr352) #2701 and anti-phospho-LAT (Tyr171) #3581 were purchase from Cell Signaling Technologies (Leiden, The Netherlands). Anti-Actin (I-19) (sc-1616-R), HSP90α/β (H-114) (sc-7947), HSP 90α/β (N-17) (sc-1055), Lck (2102) (sc-

13), Lck (3A5) (sc-433), GR (E-20) (sc-1003) and GR (P-20) (sc-1002) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Cell Culture

Human PBLs were isolated from whole blood of healthy volunteers by ficoll-isopaque density-gradient centrifugation. After washing, monocytes were separated from lymphocytes by percoll density-gradient centrifugation. The lymphocytes were cultured in IMDM (Life Technologies, Verviers, Belgium) supplemented with 10% heat-inactivated FCS. For measurements of cell proliferation, cells were stimulated for 24 h. In all experiments, lymphocytes were stimulated with PHA (10 mg/ml) or SEB (100 ng/ ml). Proliferation was measured using a [^{3}H]thymidine-incorporation assay.

Glucocorticoid mediated transactivation was measured in HEK293 cells stably transduced with a luciferase gene behind a glucocorticoid responsive element (Panomics, Milano, Italy). The cells contain Luciferase activity was measured using the Luciferase Assay System from Promega (Leiden, the Netherlands) according to the manufacturers protocol.

Localization of the GR was investigated by transiently transfecting HCT116 colorectal cancer cells with a GR-GFP fusion construct using lipofectamine (Life Technologies, Bleiswijk, the Netherlands). Measurements of GR in nuclear fractions was performed in Hela cells. HCT116 and Hela cells were cultured in Dulbecco's Modified Eagle Medium (Life Technologies, Verviers, Belgium) supplemented with 10% heat-inactivated FCS.

Induction of adipogenesis was determined in 3T3-L1 mouse fibroblasts. Two days after the cells reached confluency the medium was replaced by DMEM containing 10% FCS, 1.6 μM insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and either 0.25 μM dexamethasone, 10 μm S3.1 or 10 μm S3.4. After two days the medium was replaced by DMEM containing 10% FCS and 1.6 μM insulin. One week later lipid vacuoles were visualized using oil red O.

GC induced muscle atrophy was investigated using C2C12 myotube model. C2C12 cells were allowed to reach 90% confluency at which point the medium was replaced by DMEM containing 2% horse serum and 1 µM insulin. To stop cells dividing the culture was treated with 4 µg/ml Arac (Sigma-Aldrich, Zwijndrecht, The Netherlands). After 1 week cells were treated for 24 hours with dexamethasone, S3.1 or S3.4 and fixed in glutaraldehyde. Glutaraldehyde causes autofluorescence which allows visualization under a fluorescent microscope.

Western Blot

Samples for Western blot were made by treating 5 x 10^6 lymphocytes for 10 minutes with 10 µM dexamethasone followed by 10 minutes stimulation. When using cell lines 20 µg of protein was loaded, as determined by BCA (Pierce, Etten-Leur, The Netherlands). Cells were pelleted and lysed in lysis buffer (0.1% NP40, 50 mM Tris HCl, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 200 µM Na3VO4, 10 mM NaF) containing protease inhibitors (Roche, Mannheim, Germany) for 20 minutes on ice. Samples were spinned down, sample buffer was added to the supernatant and heated to 95 °C for 5 minutes, cooled on ice and sonicated. Samples were blotted overnight at 4°C in buffer containing 20% methanol on PVDF membrane (Millipore, Bedford, USA). The membrane was blocked with 5% BSA for 1 hour at RT followed by overnight incubation with the primary antibody at 4°C.

Immunoprecipitation

For immunprecipitation experiments 10 x 10^7 cells were used per condition. Cells were pelleted and lyzed as described above. The supernatant was precleared by adding 10 µl Protein A/G UltraLink resin (Pierce Biotechnology, Rockford, USA) for 30 minutes at 4°C. Beads were spinned down and discarded. Samples were incubated with 2 µg primary antibody and 25 µl beads for 2 hours at 4°C. Beads were washed with PBS, sample buffer was added and heated to 95°C for 5 minutes.

Preparing Nuclear Extracts

Cells were grown in 10cm plates till approximately 80% confluency. After completing the treatment, medium was replaced with ice cold PBS and cells were scraped while on ice. Cells were spinned down, supernatant discarded and the pellet was resuspended in 200 µl of buffer 1 (25 mM Hepes, pH 7.9, 5 mM KCl, 0.5 mM MgCl2, 1 mM DTT, protease inhibitors). 200 µl Buffer 2 (Buffer 1 + 0.1% NP40) was added and tubes were rotated for 15 minutes at 4°C. Nuclei were spinned down for 1 minute at 2500 rpm at 4°C. Supernatant was transferred to new tube as cytosolic fraction. Pellet was washed in 1 ml ice cold PBS followed by resuspension in 400 µl of Buffer 3 (25 mM Hepes, pH 7.9, 350 mM Na, 400 mg sucrose, 0.05% NP40, 1 mM DTT, protease inhibitors) and 1 hour of rotation at 4°C. Tubes were spinned down and the supernatant transferred as the nuclear fraction. Equal volumes were loaded on gel.

Immunofluorescence

Subcellular localization of the GR was visualized by using a GR-GFP fusion construct which was transiently tranfected in HCT116 colorectal cancer cells. Actin was stained red using phalloidin-TRITC (Sigma, Zwijndrecht, The Netherlands) and the nucleus with DAPI included in the Slowfade mounting fluid (Life Technologies, Bleiswijk, The Netherlands).

Formation of lipid vacuoles during fibroblast-adipocyte differentiation was visualized by staining triglycerides and lipids with the lysochrome Oil Red O. The C2C12 myotubes were visualized using the autofluorescence caused by fixation with 2% glutaraldehyde.

Competition Assay

Binding of the compounds to the GR was tested in a whole cell in vitro competition assay using [3H]dexamethasone. Per condition 3 x 10^6 PBL were stimulated for 15 minutes with PHA at 37°C. Tubes were then incubated for one hour at room temperature with compound and [3H]dexamethasone. Samples were spinned down at 400 G and supernatant discarded. The cells were washed three times by resuspending in ice cold PBS and rotating for 20 minutes at 4°C. Radioactivity was

measured in a scintillation counter using Utima gold scintillation fluid (Perkin Elmer, Groningen, The Netherlands).

Quantification and Data Analysis

Measurement of the thickness of myotubes was done in arbitrary units using the measuring tool of Photoshop CS6 (Adobe Systems Benelux BV, Amsterdam, Nederland). Thickness of each myotube was measured at three different places. Quantification of the Oil Red O staining and densitometric measurements of Western blot were done using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA). For data and statistical analysis we used Excel 2010 (Microsoft, Redmond, WA, USA) and Graphpad Prism (Graphpad Software Inc, La Jolla, CA, USA).

Acknowledgements

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