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Chapter 5

C118335 antagonizes glucocorticoid receptor-dependent effects on gene expression and fear memory consolidation

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In preparation
Abstract

Blockade of glucocorticoid effects may be relevant for various disease conditions characterized by excess of glucocorticoid levels, such as Cushing’s disease and psychotic depression. However, classical antagonists such as RU486, which also binds the progesterone receptor (PR), may not be sufficiently selective for the glucocorticoid receptor (GR). In addition, RU486 may lead to disinhibition of the HPA axis, thus resulting in higher glucocorticoid levels that counteract its antagonism. Here we investigated the functional profile of a novel selective GR ligand (C118335). C118335 does not bind to the PR, but retains modest affinity for the mineralocorticoid receptor. Our results showed that C118335 induced a unique GR-coregulator interaction profile with preferential recruitment of the Steroid receptor coactivator-1a nuclear receptor box. C118335 antagonized the effects of corticosterone on SGK-1 and FKBP5 expression in the CA1-CA2 region of the hippocampus and attenuated memory consolidation in an inhibitory avoidance test. Finally, we did not find disinhibition of the HPA axis after treatment with C118335. In conclusion, we offer here a proof-of-principle for the efficacy of this compound, which shows a more selective antagonistic profile and may be of interest for the treatment of the effects of hypercortisolemia.
Introduction

Orchestration of appropriate responses to stressors is indispensable for survival. In neuroendocrine realm such responses are largely mediated by the HPA axis and glucocorticoids (1, 2). However, if glucocorticoid responses are excessive or prolonged, vulnerability to psychopathology is enhanced (e.g. major depressive disorder) (1). In such cases, antagonism of the glucocorticoid receptor (GR) may be of therapeutic interest (3). GR shows a widely distributed expression pattern and is involved among others in neuroendocrine negative feedback regulation (4) and learning and memory processes (5, 6).

In order to mediate glucocorticoid effects on transcription, the GR, similarly to other nuclear receptors, needs to interact with other proteins, among which several classes of transcriptional coregulators. To date, several hundred coregulators that interact with nuclear receptors have been discovered. They differ in their expression patterns in the brain, as well as in their affinity for different ligand-bound nuclear receptors (7). The differences in expression patterns of nuclear receptors and coregulators in different brain tissues may be the basis for the gene- and tissue-specific effects of glucocorticoids that are often observed in different contexts (8, 9). This variability of nuclear receptor-coregulator interactions may also offer a new approach for neuropharmacological intervention in psychopathology.

Due to the pleiotropic effects of cortisol and corticosterone on diverse processes, full antagonism may not always be desirable, as it may block the pathogenic as well as the beneficial effects of these naturally occurring glucocorticoids. Moreover, the classical GR antagonist RU486 is not specific for the GR, but can also bind the progesterone receptor (PR), resulting in serious adverse effects (10). Finally, full GR antagonism also blocks the negative feedback loop of the HPA axis, thus resulting in even higher levels of circulating glucocorticoids, which may still exert effects via the other receptor of glucocorticoids in the brain, the mineralocorticoid receptor (MR) (11, 12). Therefore, there have been continuous attempts to develop ligands with the highest possible specificity for GR that can also target specific GR-dependent pathways (13-16).

Here, we investigated the effects on the brain of a novel GR ligand (C118335) that shows selectivity for GR over AR and PR, but with modest affinity for MR (17). We studied the effects of this compound on SGK-1, BDNF and FKBP5 expression in the CA1-CA2 region of the hippocampus and the dorsal striatum, stress-related behavior and regulation of the HPA axis. Gene selection was based on known GR-targets and on the involvement of these genes in GR signaling. We found that C118335 had antagonistic effects on glucocorticoid-induced SGK-1 and FKBP5 expression in the brain, and showed mild suppression of the HPA axis after stress. In line with the gene expression findings, it showed antagonistic effects on memory consolidation of an inhibitory avoidance response.
Methods

Peptide interaction profiling: Interactions between the GR ligand binding domain (LBD) and coregulator NR-boxes were determined on a MARCoNI assay. The method has been previously described, in detail, elsewhere (9, 18). Briefly, each array was incubated with a reaction mixture of 1 nM GST-tagged GR-LBD, ALEXA488-conjugated GST-antibody, and buffer F (PV4689, A-11131, and PV4547; Invitrogen, Bleiswijk, the Netherlands) and vehicle (2% DMSO in water), Dexamethasone (DEX; 1 μM), RU486 (1 μM), or C118335 in various concentrations. Incubation was performed at 20 °C in a PamStation96 (Pamgene International, Den Bosch, the Netherlands). GR binding to each peptide on the array, reflected by fluorescent signal, was quantified by analysis of .tiff images using BioNavigator software (Pamgene International).

Animals: 10-14 week old male Sprague-Dawley rats were used. The rats were group housed with food and water available ad libitum under a 12:12 dark:light regime. For gene expression studies, 5-7 rats per group were injected subcutaneously with vehicle (90% PEG, 10% DMSO), C118335 (100 mg/kg), followed 30 mins later by an injection of corticosterone (3 mg/kg) or vehicle. Three hours after the second injection animals were sacrificed by an intraperitoneal injection of overdose Euthasol (ASTfarma, Oudewater, the Netherlands) followed by decapitation. Their brains were harvested and snap frozen in isopentane on dry ice and subsequently stored at -80 °C. Trunk blood was also collected in EDTA coated tubes, centrifuged and plasma collected and stored at -20 °C until further processing. All experiments were carried out in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC) and experiments were approved by the Local Committees for Animal Health, Ethics, and Research of the Dutch universities involved (DEC protocol: 12167).

Radioimmunoassay: Plasma corticosterone levels were determined with Radioimmunoassays using 125I RIA kits (MP Biochemicals, Santa Ana, CA, USA) as per the manufacturer’s instructions.

Punching: 200 μm thick sections were taken on a Leica 3050 cryostat (Rijswijk, the Netherlands) and mounted on uncoated glass slides (Menzel-Gläser, Braunschweig, Germany). Subsequently tissue was punched out from the caudate putamen and the CA1-CA2 region of the dorsal hippocampus using appropriate Harris Uni-core punching needles (Tedpella, Redding, CA, USA).

RNA isolation, cDNA synthesis and qPCR: The samples were homogenized on a TissueLyser II (Retsch Qiagen, Haan, Germany) in 1 ml Trizol, centrifuged and 200 μl of chloroform (Merck KGaA, Darmstadt, Germany) was added to each sample. After centrifugation, the aqueous phase (top phase) was taken and 5 μl of 5 mg/ml linear acrylamide (Ambion, Austin, USA), as a carrier, and 500 μl isopropyl alcohol (Merck KGaA, Darmstadt, Germany) were added, followed by centrifugation and removal of the supernatant. Then, the RNA pellet was washed twice with 75% ethanol (Merck KGaA, Darmstadt, Germany), air-dried and dissolved in demineralized H₂O. The purity and concentration of the RNA samples
were measured on the Nanodrop 1000 (Isogen Life Science, De Meern, The Netherlands). The integrity of the samples was measured on Standardsens chips on a Bio-Rad experion system (Hercules, USA).

For cDNA synthesis, RNA samples were pretreated with DNase (Promega, Madison, USA) to remove potential genomic DNA contamination according to the manufacturer’s specifications. For the incubation a MyCycler™ Thermal Cycler (Bio-Rad, Hercules, USA) was used. Subsequently, cDNA was synthesized with iScript cDNA Synthesis Kit (Bio-Rad). Four µl 5x iScript reaction mix, 1 µl iScript reverse transcriptase (RT) and 5 µl nuclease free water were added to each DNase pretreated sample. A control sample without RT treatment was also included in which the 1 µl RT was replaced by 1 µl nuclease free water. The samples were placed in a MyCycler™ Thermal Cycler and incubated for 5 minutes at 25°C, 30 minutes at 42°C and 5 minutes at 85°C (11).

Quantitative polymerase chain reaction (qPCR) was performed to measure gene expression in the different brain regions. The efficiency of the used primers was first measured for each gene in each region. To perform the qPCR the FC FastStartDNA MasterPlus SYBR Green I (Roche Applied Science, Basel, Switzerland) kit was used. 2.5 µl per cDNA sample was added to a mix of 2 µl 5x Sybr green mix, 0.5 µl 10 µM of both the forward and reverse primers (table 1) and 4.5 µl DEPC H$_2$O to a total volume of 10 µl. For the reactions 20 µl LightCycler Capillaries (Roche) were used placed in a LightCycler Sample Carousel 2.0 (Roche). The carousel was centrifuged on a LC Carousel Centrifuge 2.0 (Roche), subsequently placed in a LightCycler 2.0 (Roche) to perform qPCR. All samples were measured in duplicate. The samples were incubated for 10 minutes at 95°C, followed by 45 replication cycles (10 seconds denaturation at 95°C, 10 seconds annealing at 60°C and 10 seconds elongation at 72°C) and finally a melting curve was made (65°C to 95°C, 0.1°C/s).

**Inhibitory avoidance:** One-trial inhibitory avoidance training and retention was performed as has been described elsewhere (19), using single-housed male Wistar rats (10-14 weeks of age; Charles River) and a foot-shock intensity of 0.38 mA for 1 s. C118335 (20 or 80 mg/kg) or corticosterone (1 mg/kg) was dissolved in DMSO and administered (100 µl, s.c.) immediately after the training trial, to prevent interference with memory acquisition. Retention was tested 48 h later. A shorter latency to enter the former shock compartment with all four paws (maximum latency of 600 s) was interpreted as weaker memory.

**Statistical analysis:** To analyze the levels of Cort in the trunk blood a t-test with a significance level of P<0.05 between the vehicle and the C118335 treated group was used. In order to determine whether treatment with corticosterone increased the corticosterone-circulating levels a two-way ANOVA was used. For the analysis of the Ct values from the qPCR the mathematical model from Pfaffl (20) was used. Tubulin and ACTB were used as reference (housekeeping) genes. The geometric mean of these two genes was used as the reference value. The Grubbs’ outlier test was conducted and outliers were excluded from the analysis. The values were analysed by one-way ANOVA followed by Tukey’s post-hoc test with a significance level of P<0.05. In the inhibitory avoidance test the Kruskal-Wallis test was used followed by Dunn’s test.
Figure 1. C118335 did not induce as many GR-LBD – coregulator peptide interactions as dexamethasone. However, it induced partial recruitment of the SRC-1a specific NR box-IV (NCOA1-1421-1441). A. Overview of the ligand-induced interactions between GR-LBD and coregulator motifs after treatment with DMSO, the classical antagonist RU486, the novel GR ligand C118335 and dexamethasone. B. C118335 induced GR-LBD – SRC-1 NR-box IV interactions in a dose-dependent manner, while it did not induce considerable GR-LBD- SRC-1 NR-box I (NCOA1-620-643) interactions at any concentration. C. C118335 induced significantly stronger interactions between GR-LBD and SRC-1 NR-box IV than DMSO, though not as strong as the dexamethasone induced interactions (one-way ANOVA, p<0.0001, F(3,15) = 168.6, tukey’s post hoc test: ***, p<0.001 compared to DMSO group; #, p<0.001 compared to RU486 group. D. C118335 did not induce SRC-1 NR-box I –GR-LBD interaction, unlike dexamethasone: One-way ANOVA: p<0.0001, F(3,15)= 227.6, tukey’s post-hoc test: ***, p <0.001 compared to DMSO group, #, p<0.001 compared to RU486 group, $, p<0.001 compared to C118335 group. E. C118335 did not induce interactions with the corepressor motif NCOR1-2251-2273 : One-way ANOVA : p< 0.0001, F(3,15) = 16.89, tukey’s post hoc test: ***, p<0.001 compared to DMSO group; #, p<0.001 compared to RU486 group.
Results

**C118335 induces a unique GR-LBD – coregulator interaction profile:** C118335 generally did not induce as many interactions as dexamethasone (Figure 1a). However, it selectively recruited a number of NR boxes, such as the SRC-1 NR-box IV in a dose-dependent manner (Figure 1b, c), but to a lesser extent than dexamethasone (Figure 1c). On the other hand, C118335 did not induce interactions between the GR-LBD and SRC-1 NR-box I, unlike dexamethasone (Figure 1d) and in contrast to RU486, it did not recruit corepressor motif NCOR1 2251-2273 (Figure 1e). This suggests that the compound will act as an antagonist on most processes that depend on the coregulators represented at the array, but may show substantial partial agonism for others.

**Trunk blood corticosterone levels:** Animals treated with C118335 had significantly lower corticosterone plasma levels than controls (Figure 2a). As expected corticosterone-treated animals had higher corticosterone plasma levels than the respective control treated groups (Figure 2b). Of relevance for the interpretation of the gene expression data, vehicle animals also had relatively high levels of plasma corticosterone.

**C118335 attenuates the corticosterone-induced upregulation of FKBP5 and SGK-1 but enhances BDNF expression, in the CA1-CA2 region of the hippocampus:** Treatment with corticosterone in the absence of other ligands resulted in a strong upregulation of FKBP5 expression in the CA1-CA2 region of the hippocampus. Treatment with C118335 resulted in suppression of the corticosterone-induced FKBP5 upregulation (Figure 3a). Similarly,

<table>
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C118335 resulted in decreased expression of SGK-1 regardless of glucocorticoid treatment in these adrenally intact animals (Figure 3b). C118335 increased BDNF expression in the hippocampus, but this effect was blocked by corticosterone treatment (Figure 4).

**C118335 attenuates the corticosterone-induced upregulation of SGK-1 in the striatum, but it had no effect on FKBP5:** C118335 had no effect on FKBP5 expression (Figure 5a). However, SGK-1 expression was upregulated in the striatum after treatment with corticosterone, an effect which was blocked by pretreatment with C118335 (Figure 5b).

Figure 2. A. Endogenous corticosterone levels after treatment with vehicle or C118335. Rats treated with C118335 had lower plasma corticosterone levels compared to vehicle (two-tailed t-test: $t_{(10)} = 2.346$, $p=0.04$). B. Two-way ANOVA revealed a Glucocorticoid treatment effect, where treatment with corticosterone increased circulating corticosterone plasma levels ($F_{(1,21)} = 14.71$, $p=0.001$). N = 5-6 per group. Bonferroni post-hoc test: ***, $p<0.001$. No Compound effect was found in this analysis.

Figure 3: FKBP5 and SGK-1 expression in the hippocampus. A. C118335 can block the corticosterone-induced upregulation of FKBP5 in the hippocampus. There was a significant Glucocorticoid treatment effect ($F_{(1,20)} = 16.56$, $p<0.001$), a significant Compound treatment effect ($F_{(1,20)} = 16.23$, $p<0.001$) and a significant Glucocorticoid X Compound interaction ($F_{(1,20)} = 7.301$, $p<0.05$). B. C118335 downregulated SGK-1 expression in the hippocampus regardless of glucocorticoid treatment. A compound effect was observed ($F_{(1,20)} = 25.71$, $p<0.001$) and a marginally non-significant interaction effect ($F_{(1,20)} = 4.127$, $p<0.06$). Bonferroni post-hoc test, ***, $p<0.001$, n=5-6 per group.
Figure 4: C118335 had effects on BDNF expression in the hippocampus. C18335 treatment resulted in upregulation of BDNF expression, but this effect was blocked by treatment with glucocorticoids. A significant Glucocorticoid treatment effect was found ($F_{(1,20)} = 5.093, p<0.05$). The Compound effect was marginally non-significant ($F_{(1,20)} = 4.286, p = 0.052$). Bonferroni post-hoc test: *, $p<0.05$, n=5-6 per group.

Figure 5: SGK-1 expression in the striatum. A. No Compound effect was found on FKBP5 expression in the striatum ($F_{(1,21)}=0.003, p>0.95$). However, a trend towards a Glucocorticoid treatment effect was found ($F_{(1,21)}=3.274, p=0.084$). B. C118335 treatment prevented the corticosterone-induced upregulation of SGK-1. There was a significant Glucocorticoid treatment effect ($F_{(1,20)} = 8.197, p<0.01$) and a significant Compound effect ($F_{(1,20)} = 8.295, p<0.01$).

**C118335 resulted in decreased memory consolidation in an inhibitory avoidance test:** To determine the effect of C118335 on stress-related behavior we used an inhibitory avoidance paradigm. In order to examine the potential dose responsiveness, we used two doses of the compound (20 and 80 mg/kg). Our results showed that immediate post-training treatment with C118335 resulted in decreased latency to enter the dark compartment in testing two days later only when the higher dose was administered. Treatment with 20 mg/kg C118335 had no effect on the latency to enter the dark compartment at re-exposure to the task (Figure 6).
Blocking the undesired effects of glucocorticoids, in both the brain and the periphery, may be of relevance for a number of conditions such as Cushing’s disease and psychotic depression (21-24). However, the available antagonists are, however, not specific for GR and their use may be accompanied by adverse effects that decrease their therapeutic potential. RU486, for instance, binds also the progesterone receptor and can induce abortion. Moreover, it disinhibits the HPA axis resulting in even higher cortisol levels, thus counteracting its effects (25). Therefore, it is necessary to search for more specific GR ligands in order to minimize potential side effects.

A possible level of regulation of nuclear receptor function arises from modulation of nuclear receptor-coregulator interactions (7). Recently, we characterized the selective GR modulator C108297 in a wide array of tests (9). This compound behaved both as agonist and antagonist depending on the context and brain region. Its effects were, at least to some extent, attributed to the unique profile of GR-coregulator interactions it could induce (9).

In this study we investigated the effects of the novel GR ligand C118335 on glucocorticoid dependent gene expression in vivo and on fear memory consolidation in an inhibitory avoidance test. In contrast to RU486, this compound does not bind to the progesterone or the androgen receptor, but it retains some affinity for the MR (17). Our findings suggest a strong antagonist profile of C118335, both in gene expression and inhibitory avoidance. Interestingly, however, treatment with C118335 did not disinhibit the HPA axis.

Figure 6: C118335 showed an antagonist effect in an inhibitory avoidance test only at a higher dose (80 mg/kg). The lower dose of 20 mg/kg did not have an effect on the consolidation in the inhibitory avoidance test (Kruskal-Wallis test, Kruskal-Wallis statistic =18.63, p<0.001, n=9-11 per group, ***, Dunn’s post-hoc test, p<0.001).

Discussion
C118335 induced only a modest subset of the dexamethasone-induced GR-LBD - coregulator interactions, with about 50% efficacy for the strongest interactions. Interestingly, however, it recruited the SRC-1a specific NR box (SRC-1 NR-box IV) in a dose-dependent manner. SRC-1a potentiates repression of crh promoter activity in vitro (26), while it may also be necessary for appropriate crh expression regulation by glucocorticoids in the PVN, as well (8). In the current study using adrenally intact rats it is not possible to discriminate between pure antagonistic effects and partial agonism relative to endogenous corticosterone. In contrast to RU486, C118335 did not recruit any corepressor motifs. This suggests differences in the mode of action of C118335 compared to RU486 and the lack of corepressor recruitment may prevent the abrogation of all GR-mediated effects, thus it may lack some of the RU486-associated adverse effects.

Despite the high corticosterone plasma levels of the vehicle group in the present study, additional exogenous corticosterone treatment further increased the corticosterone levels and this increase was accompanied by induced changes in gene expression in the brain. On the other hand, the high corticosterone levels may have masked potential agonistic properties of C118335. C118335 treatment resulted in blockade of corticosterone-induced upregulation of FKBP5 and SGK-1. Both genes are GR-target genes, but also play an important role in mediation of the transcriptional effects of GR. SGK-1 may prolong the GR effects even in the absence of glucocorticoids, while it has been found increased in depressed patients (27). Here we found downregulation of SGK-1 expression below basal levels, which may be indicative of an effect at two levels: a direct effect on SGK-1 expression regulation by GR and an indirect effect on transcriptional activity due to decreased SGK-1 expression (27). On the other hand, FKBP5 may have inhibitory activity on GR function and it is involved in the ultrashort intracellular negative feedback loop of GR activity (28, 29). Although these two target genes exert opposite actions on GR signaling, the fact that the corticosterone-induced upregulation of both was blocked may indicate an overall dampening of the transcriptional effects of GR. Nevertheless, SGK-1 and FKBP5 may also be regulated in a brain region-specific fashion, thus making predictions of the net GR-dependent transcriptional outcome difficult.

BDNF is another gene regulated by glucocorticoids (30-32), also involved itself in GR signaling (32-35). C118335 treatment upregulated BDNF expression in the hippocampus, however, it was not enough to counteract the effect of higher corticosterone levels. The lack of efficacy of corticosterone treatment may reflect the relatively high endogenous corticosterone levels, which may have led to low BDNF expression levels (9, 36). The observed upregulation of BDNF expression in the hippocampus may be of relevance for psychopathology, as similar effects have been reported after treatment with antidepressants in rodents and humans (37-39).

The effects on glucocorticoid levels may be explained by the effects of C118335 on GR-coregulator interactions. C118335 preferentially recruited the SRC-1a-specific NR-box. SRC-1a potentiates the repression of the crh promoter and may be involved in the regulation of CRH expression by glucocorticoids in the PVN by the GR (8, 26). Interestingly, in studies in SRC-1 KO mice it has been shown that SRC-1 is involved in negative feedback of the HPA axis at the pituitary and the PVN (8, 40). Considering the relative abundance of the two SRC-1 splice variants, these effects are likely mediated by SRC-1a (41). It remains to be seen whether
this is relevant (and to which extent) in conditions of chronic stress or prolonged hypercortisolemia.

There was a clear antagonist effect of C118335 on fear memory consolidation in an inhibitory avoidance test. This was in line with previous findings, as immediate post-training GR antagonism has been shown to interfere with memory consolidation. The considerable effect of C118335 treatment observed here may also be related to the downregulation of SGK-1 expression below basal levels. Finally, the expected weak antagonism on MR may also be relevant to the effects of C118335 on memory consolidation.

In conclusion, we offer here a proof-of-principle for the efficacy of a novel GR antagonist which, in contrast to RU486, does not bind the progesterone receptor and induces a distinct GR-LBD – coregulator motif interaction profile. Therefore, C118335 may have an interesting novel therapeutic potential in the treatment of hypercortisolemia-induced psychopathology.

Acknowledgments

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Conflict of interest

HH and is employed by Corcept Therapeutics, and made C118335 available. Corcept financed part of the costs of the experiments. RH is employed by Pamgene Int, who made MARCoNI arrays available for this study.
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