# A screening assay for Selective Dimerizing Glucocorticoid Receptor Agonists and Modulators (SEDIGRAM) that are effective against acute inflammation

Jolien Souffriau[1,2], Melanie Eggermont[1,2], Sara Van Ryckeghem[1,2], Kelly Van Looveren[1,2], Lise Van Wyngene[1,2], Evelien Van Hamme[3], Marnik Vuylsteke[4], Rudi Beyaert[1,2], Karolien De Bosscher[5,6], Claude Libert[1,2*]

[1] Center for Inflammation Research, VIB, Ghent, Belgium

[2] Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

[3] Bio Imaging Core, Center for Inflammation Research, VIB, Ghent, Belgium

[4] GNOMIXX ltd, Statistics for Genomics, Melle, Belgium

[5] Receptor Research Laboratories, Nuclear Receptor Lab, Medical Biotechnology Center, VIB, Ghent, Belgium

[6] Department of Biochemistry, Ghent University, Ghent, Belgium

*Corresponding author:

Claude.Libert@IRC.VIB-UGent.be

**Supplemental methods: definition of X, $\beta$, Z and $\nu$**

$$\log(\mu) = \eta = \mathbf{X}\beta + \mathbf{Z}\nu.$$

***Luciferase test:***

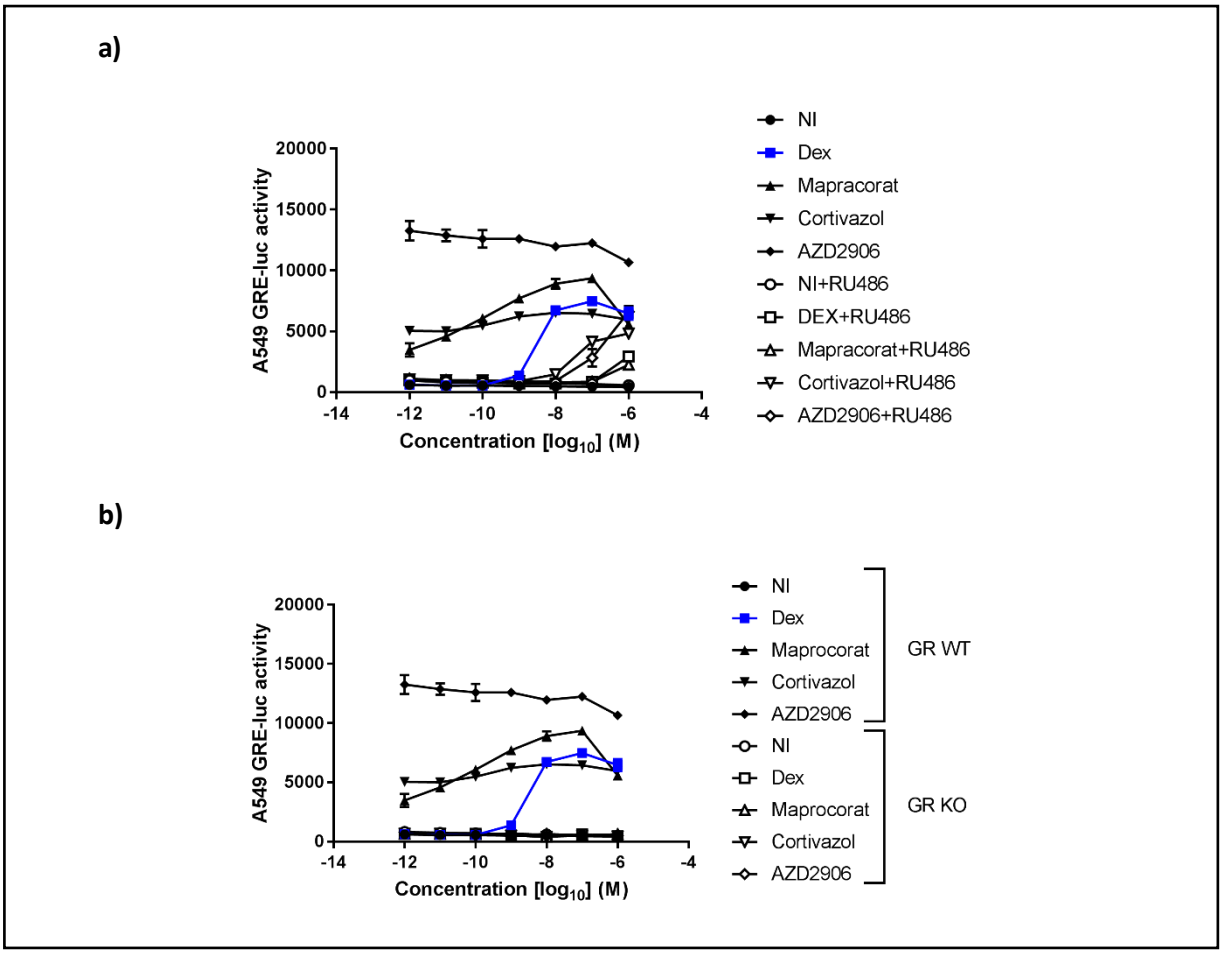
The matrix **X** is the design matrix for the fixed COMPOUND, CONCENTRATION and the COMPOUND.CONCENTRATION interaction terms, $\beta$ is the vector of corresponding regression coefficients, **Z** is the design matrix for the two random REPLICATE (representing the biological replicates) and PLATE terms with PLATE nested within REPLICATE, and $\nu$ is the corresponding vector of the random effects having a gamma distribution.

***Gene expression & ELISA test:***

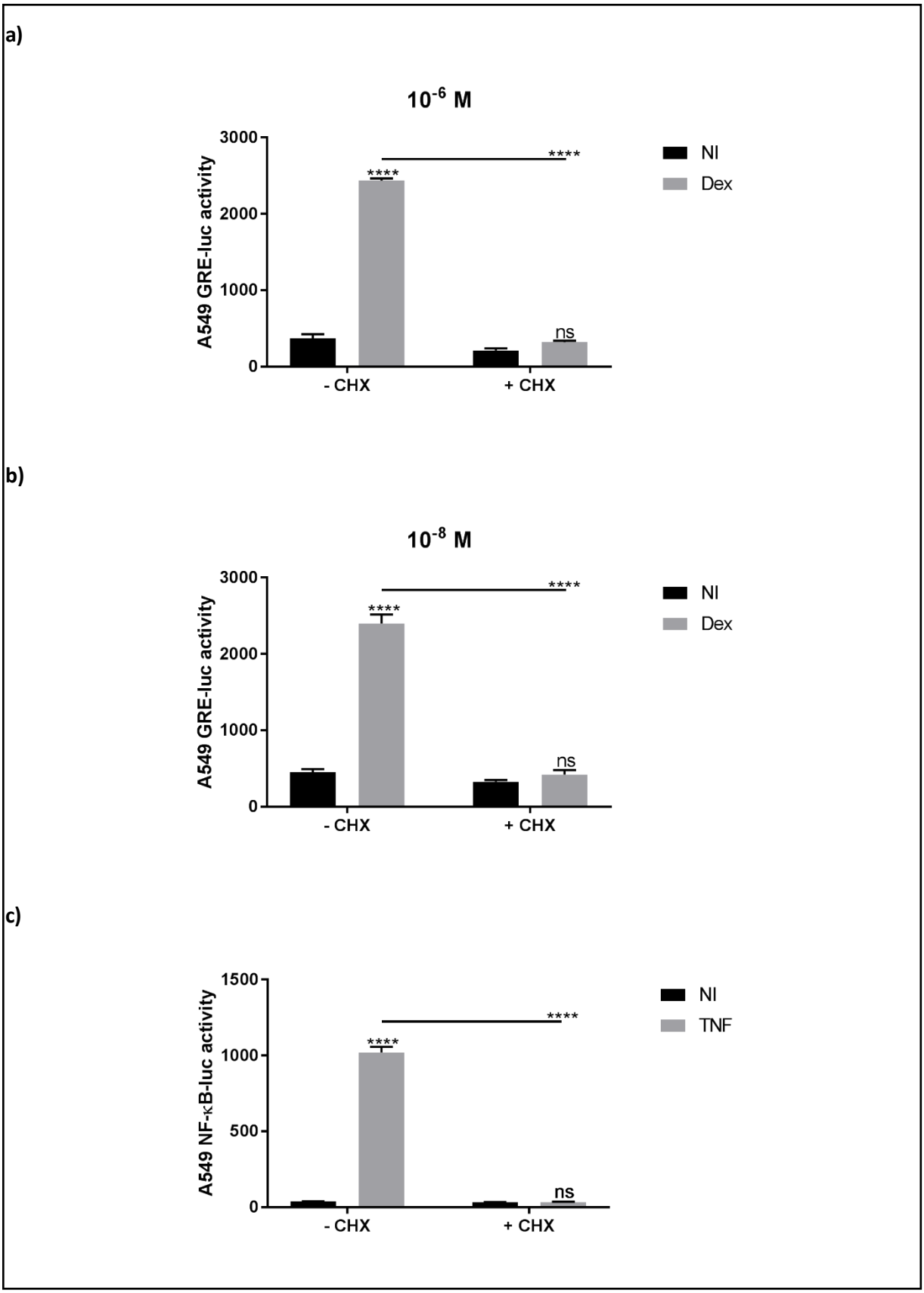
The matrix **X** is the design matrix for the fixed COMPOUND term, $\beta$ is the vector of corresponding regression coefficients, **Z** is the design matrix for the random REPLICATE term (representing biological replicates), and $\nu$ is the corresponding vector of the random REPLICATE effect having a gamma distribution.

***FRET test:***

The matrix **X** is the design matrix for the fixed COMPOUND term, $\beta$ is the vector of corresponding regression coefficients, **Z** is the design matrix for the random REPLICATE term (representing biological replicates), and $\nu$ is the corresponding vector of the random REPLICATE effect having a normal distribution.

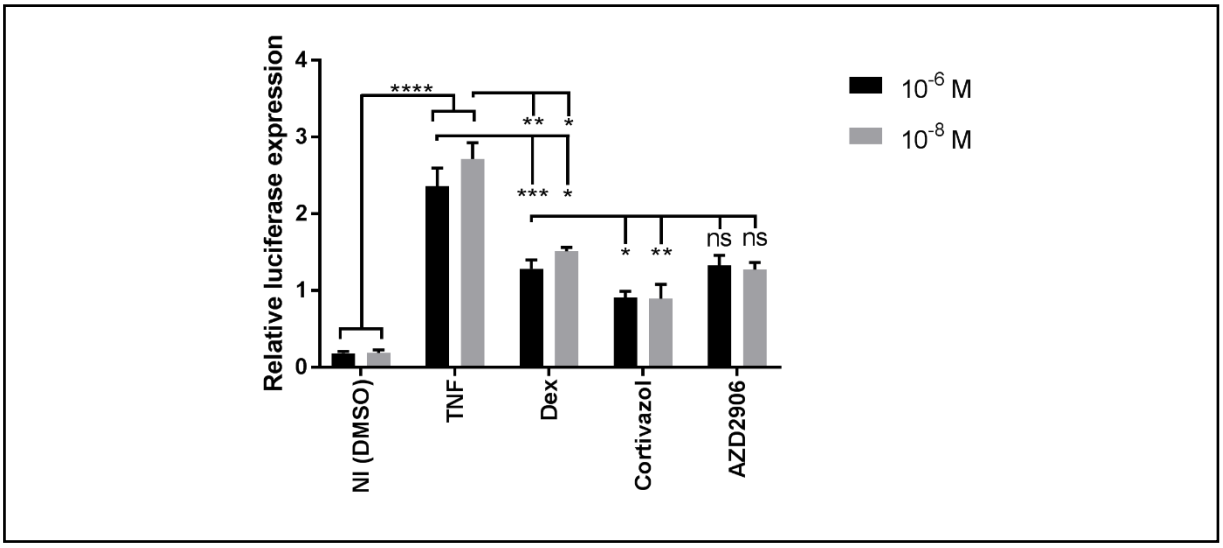



**Supplemental Figure S1. The GRE-luciferase signal induced by the compounds is glucocorticoid receptor (GR) dependent.** A549 GRE-luc cells were stimulated with a dilution series of compounds for 5 h, after which they were lysed and luminescence was read out. In **(a)** the cells were co-treated with $10^{-6}$ M RU486. In **(b)** A549 GR KO GRE-luc cells were used. NI: Non-Induced, Dex: Dexamethasone, WT: Wild-Type, KO: Knock-Out, GRE: Glucocorticoid Responsive Element.

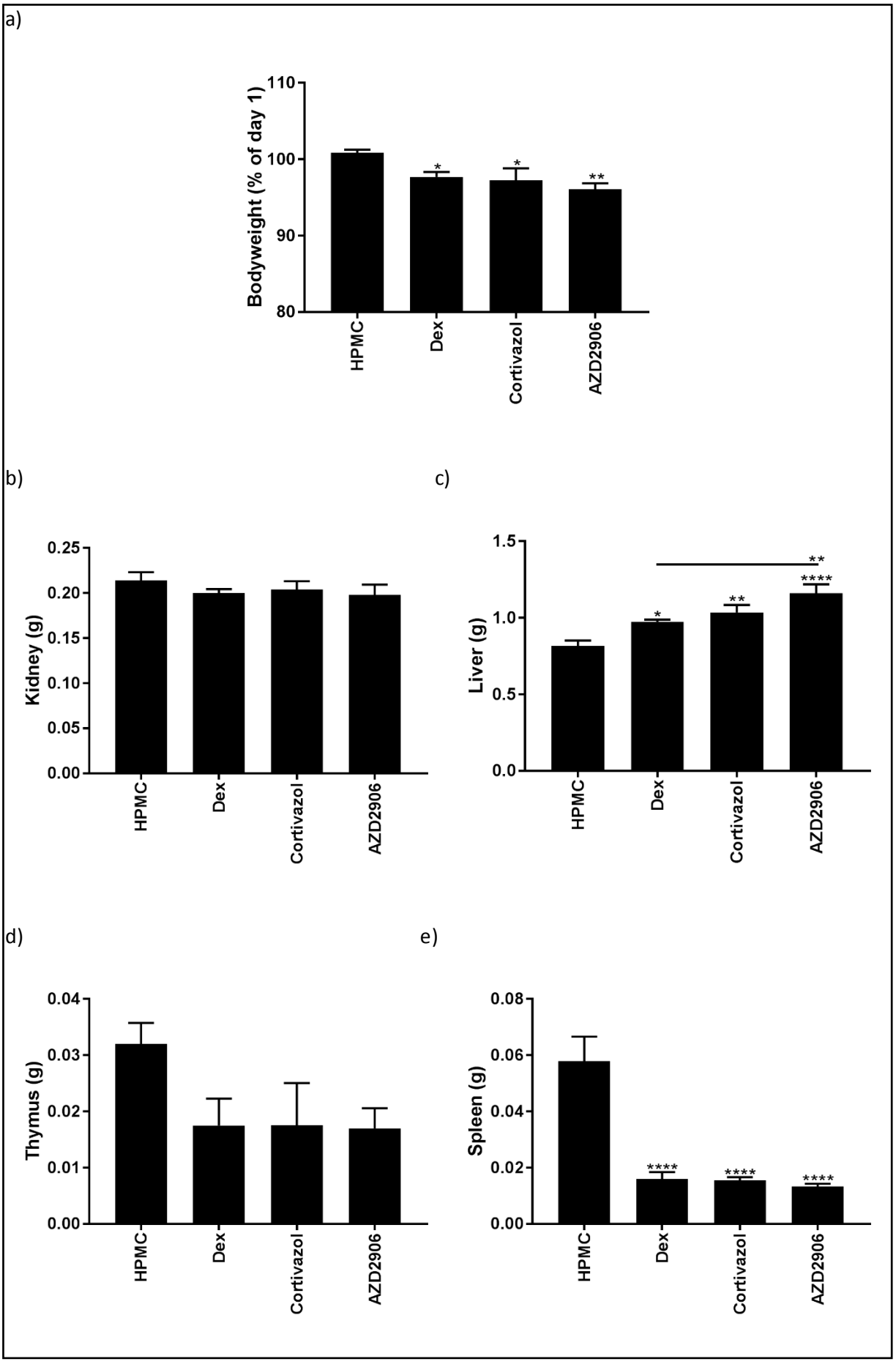



**Supplemental Figure S2. Luciferase activity confirms blocking of translation by cycloheximide.** A549 GRE-luc and NF-κB-luc cells were pretreated with or without 10 µG/ml cycloheximide (CHX, 30 min). CHX treatment in A549 GRE-luc was followed by a 3 h solvent control (NI) or Dex stimulation at **(a)** $10^{-6}$ M or **(b)** $10^{-8}$ M. **(c)** In A549 NF-κB-luc cells CHX treatment was followed by the solvent control for 1h

and subsequently cells were stimulated with 1000 U/ml human TNF for 2 h. The cells were lysed and luminescence was read out. Data are shown as mean ± SEM. Significant differences are calculated with a Two-way Anova. Statistical differences compared to NI (above error bars) or between the Dex or TNF stimuli (indicated with –) are given. **** represents a P-value <0.0001. Ns: Not significant, NI: Non-Induced, Dex: Dexamethasone, TNF: Tumor Necrosis Factor.




**Supplemental Figure S3. Comparison of maximal NF-κB luciferase transrepression over concentrations of GR dimer favouring compounds in absence of novel protein synthesis.** NF-κB-luciferase gene-expression in A549 NF-κB-luc cells pretreated with 10 µg/ml cycloheximide (CHX, 30 min), followed by stimulation with the compounds ($10^{-6}$ M and $10^{-8}$ M). 1 h later cells were challenged with 1000 U/ml human TNF for 2 h. Luciferase mRNA expression was measured with RT-qPCR. Data are shown as mean ± SEM. Significant differences are calculated with a Two-way Anova. *,**,*** or **** represent significant differences with P <0.05, 0.01, 0.001 and 0.0001 respectively. 3 experiments were pooled. Ns: Non-significant, NI: Non-Induced, TNF: Tumor Necrosis Factor, Dex: Dexamethasone.


a)
Bodyweight (% of day 1)
110
100
90
80
*
*
**
HPMC
Dex
Cortivazol
AZD2906
b)
Kidney (g)
0.25
0.20
0.15
0.10
0.05
0.00
HPMC
Dex
Cortivazol
AZD2906
c)
Liver (g)
1.5
1.0
0.5
0.0
*
**
**
****
HPMC
Dex
Cortivazol
AZD2906
d)
Thymus (g)
0.04
0.03
0.02
0.01
0.00
HPMC
Dex
Cortivazol
AZD2906
e)
Spleen (g)
0.08
0.06
0.04
0.02
0.00
****
****
****
HPMC
Dex
Cortivazol
AZD2906

**Supplemental Figure S4. Dex, Cortivazol and AZD2906 induce glucocorticoid side effects.** Female C57BL/6J mice were treated daily with 1 mg/kg Dex or an equimolar dose of Cortivazol or AZD2906 dissolved in 100 µl HPMC, by oral gavage, for 5 consecutive days. **(a)** Whole bodyweight was measured and represented as a percentage of the bodyweight of day 1. **(b)** Kidney, **(c)** liver, **(d)** thymus and **(e)** spleen were isolated and weighed 6 h after the last oral gavage on day 5. Significant differences are calculated with a One-way Anova test. Statistical differences compared to HPMC (above error bars) and Dex (indicated with –) are given. P-values < 0.05, 0.01 and 0.0001 are represented by *, ** or **** respectively. Non-significant differences are not indicated on the graph. N=5 mice per group. HPMC: Hydroxypropylmethylcellulose, Dex: Dexamethasone.

**Supplemental Table S5: Statistical significances A549 GRE luciferase test**

| | $10^{-6}$ M | | | $10^{-7}$ M | | | $10^{-8}$ M | | |
|---|---|---|---|---|---|---|---|---|---|
| | tvalue | t_Prob | | tvalue | t_Prob | | tvalue | t_Prob | |
| NI (DMSO) | -12,0194 | 0 | **** | -10.5178 | 0 | **** | -10.1074 | 0 | **** |
| Fosdagrocorat | -9.81095 | 0 | **** | -7.98583 | 3.33067E-15 | **** | -7.1263 | 1.775E-12 | **** |
| Prednisolone | -1.67533 | 0.094130019 | ns | -2.47463 | 0.013474763 | * | -4.9483 | 8.5516E-07 | **** |
| Mapracorat | -1.34209 | 0.179819598 | ns | 0.741652 | 0.458443248 | ns | 3.1998 | 0.00141136 | ** |
| Cortivazol | -1.50158 | 0.133467994 | ns | -0.31341 | 0.754021013 | ns | 0.4723 | 0.63676508 | ns |
| LGD5552 | -7.34966 | 3.66374E-13 | **** | -7.0909 | 2.26996E-12 | **** | -7.6088 | 5.5733E-14 | **** |
| AZD2906 | 4.67152 | 3.32704E-06 | **** | 5.970065 | 3.11913E-09 | **** | 7.7683 | 1.6875E-14 | **** |

A Hierarchical Generalized Linear Mixed Model (HGLMM) has been fitted to the luciferase activities measured at various concentrations of the compounds. T statistics (unpaired, two-tailed) were used to assess the significance of fixed compound and concentration effects estimated as differences (on the transformed scale) to Dex at a particular concentration set as reference. Estimated mean values were obtained as predictions from the HGLMM, formed on the log scale. T-values and P-values (t_Prob) for the concentrations $10^{-6}$ M, $10^{-7}$ M and $10^{-8}$ M (concentrations where Dex has its maximal activity) are represented in the table. P <0.05, 0.01, 0.001 and 0.0001 are represented with *,**,*** or **** respectively. Ns: Not significant. Experiments were repeated 6 times with at least 2 technical replicates included.

**Supplemental Table S6: Statistical significances A549 NF-κB luciferase test**

| | $10^{-6}$ M | | | $10^{-7}$ M | | | $10^{-8}$ M | | |
|---|---|---|---|---|---|---|---|---|---|
| | tvalue | t_Prob | | tvalue | t_Prob | | tvalue | t_Prob | |
| TNF | 4.093525 | 5.36968E-05 | **** | 5.061932 | 6.97285E-07 | **** | 5.31003 | 2.0399E-07 | **** |
| Fosdagrocorat | 1.698806 | 0.09031577 | ns | 2.534819 | 0.011720736 | * | 2.363495 | 0.0186934 | * |
| Prednisolone | 0.390697 | 0.696278242 | ns | 0.822702 | 0.411282717 | ns | 1.977445 | 0.04883856 | * |
| Mapracorat | 1.008059 | 0.314178525 | ns | 0.890265 | 0.373983976 | ns | 1.278091 | 0.20213207 | ns |
| Cortivazol | 0.08944 | 0.928787321 | ns | 0.398634 | 0.690425207 | ns | 0.417441 | 0.67663249 | ns |
| LGD5552 | 1.483195 | 0.138994568 | ns | 2.525585 | 0.012027034 | * | 3.725319 | 0.00023006 | *** |
| AZD2906 | -0.10878 | 0.913445047 | ns | -0.00309 | 0.99753567 | ns | -0.07995 | 0.93632506 | ns |

A Hierarchical Generalized Linear Mixed Model (HGLMM) has been fitted to the luciferase activities measured at various concentrations of the compounds. T statistics (unpaired, two-tailed) were used to assess the significance of fixed compound and concentration effects estimated as differences (on the transformed scale) to Dex at a particular concentration set as reference. Estimated mean values were obtained as predictions from the HGLMM, formed on the log scale. T-values and P-values (t_Prob) for the concentrations $10^{-6}$ M, $10^{-7}$ M and $10^{-8}$ M (concentrations where Dex has its maximal activity) are represented in the table. P <0.05, 0.01, 0.001 and 0.0001 are represented with *,**,*** or **** respectively. Ns: Not significant. Experiments were repeated twice with at least 2 technical replicates included.