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Novel selective glucocorticoid receptor agonists (SEGRAs) with a covalent warhead for long-lasting inhibition

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ABSTRACT

The synthesis and in vitro properties of six analogues of the selective glucocorticoid receptor (GR) agonist GSK866, bearing a warhead for covalent linkage to the glucocorticoid receptor, is described.

Glucocorticosteroids are an established class of medicines that are still successfully used to treat inflammatory and auto-immune diseases. However, the main disadvantage of these compounds, especially at high dosing levels and long-term usage, is the tendency to induce skin atrophy, tachyphylaxis and the occurrence of a rebound effect upon discontinuation of treatment.[1] For this purpose, the search towards more selective glucocorticoid receptor agonists (SEGRAs) is still an active research domain, with GSK866 and mapracorat being the prototypes of this class of compounds (Fig. 1).[2] Both glucocorticosteroids and selective glucocorticoid receptor agonists show a pharmacological effect by binding to and activating the glucocorticoid receptor (GR). However, in contrast to the glucocorticosteroids – which pharmacological effects arises from the activation of both a transactivation and transrepression pathway – SEGRAs selectively activate the GR and exert their pharmacological effect by activation of only the transrepression pathway.

Over the past years, we have been involved in the synthesis of close analogues of GSK866 with the intention to search for covalent inhibitors of the GR. The rationale behind the covalent binding approach is to identify GR agonists for topical application and that could have a long-lasting effect on the GR but without the potential systemic side-effects that common SEGRA compounds still have. Having a covalent linkage between the agonist and the GR might potentially reduce systemic release of the compound with less systemic side-effects as a result. This proof-of-concept study builds further upon the ideas that emerged from the recent development of covalent-binding kinase inhibitors,[3,4] with the underlying idea to develop long-lasting GR agonists with applicability in the anti-inflammatory domain.

The crystal structure of the ligand-binding domain of the GR has been solved in complex with GSK866 (pdb-code 3E7C),[5] and this structure shows the location of a cysteine residue (Cys_{643}) in close proximity of the dichorophenyl group of GSK866 (Fig. 2). Complexes of the GR with other SEGRA compounds are available from the PDB (Fig. 1),[6] but we have selected GSK866 as a synthetic starting point for the design of covalent analogues due to synthetic feasibility reasons for the introduction of the covalent warhead, and also because of the fact that molecular graphics analyses were favorable in terms of this compound to be used as a starting template structure. The close presence of such cysteine residue has prompted us to investigate the possibility of modifying GSK866 by introducing a reactive warhead into the structure, with the possibility of forming a covalent linkage between protein structure and ligand upon binding. Supported by molecular graphics-based

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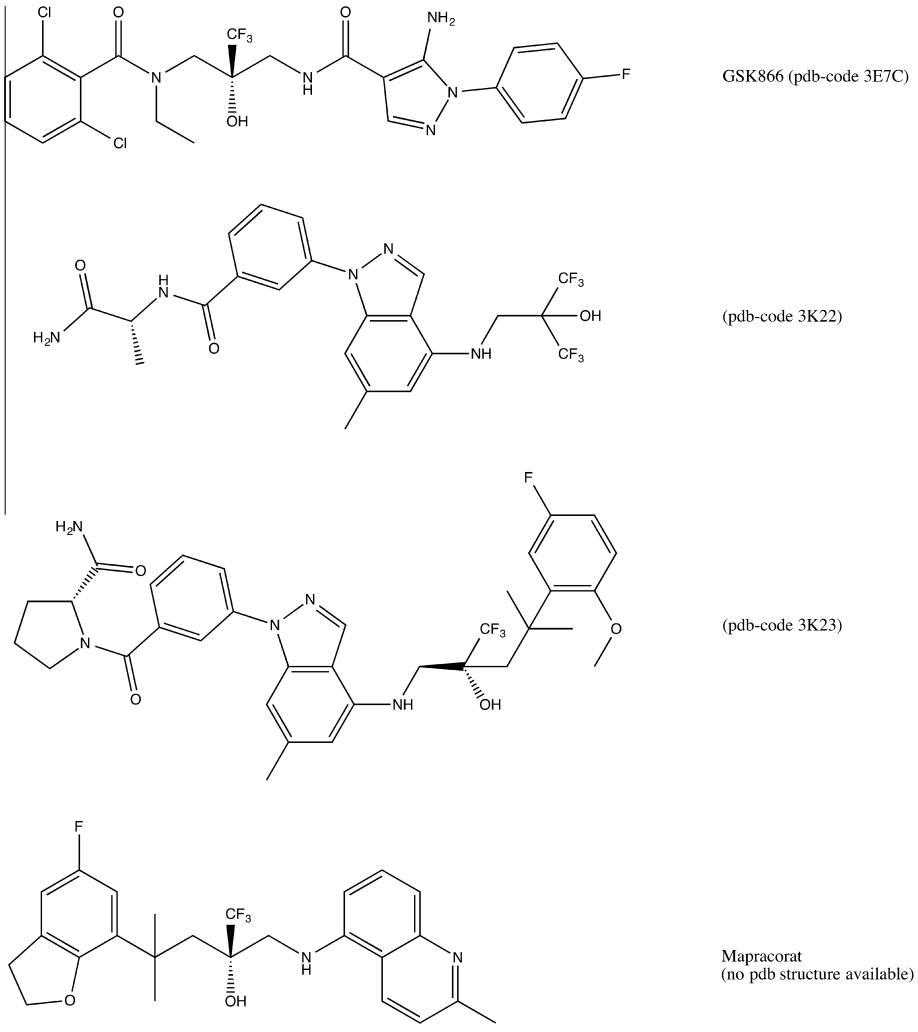

Figure 1. The structure of GSK866 and two other SEGRA analogues of which the crystal structure of the complex with the GR ligand-binding domain is available from the PDB (entry codes are given in parentheses). The structure of mapracorat is also shown.

qualitative modeling, six such close analogues of GSK866 were synthesized and their biochemical properties were evaluated. The structures are shown in Figure 3.

The main building block **11** was synthesized according Scheme 1, starting from the commercially available 1,3-bis(benzyloxy)propan-2-ol (**6**), followed by a Corey-Kim oxidation to the corresponding ketone **7**.[7] Conversion of the ketone into the α-trifluoromethyl-α-tosyloxymethyl epoxide **8** was done according the method as described by Keeling and coworkers.[8] Nucleophilic substitution, initially with *N*-benzylethanamine and subsequently with ammonia in methanol, yielded compound **9**.[9] Coupling of **9** with **13** finally yielded **10**, which was then debenzylated into **11** by means of hydrogenolysis.[5]

Synthesis of the target compound **2** (Scheme 2) was achieved by coupling **11** with 2,5-dioxopyrrolidin-1-yl 3-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)propanoate (**16**). This latter was prepared from the reaction between furan-1,5-dione (**14**) and 3-aminopropanoic acid (**15**).[10]

Compound **3a** was prepared from **11** through coupling with *N*-*Z*-(D)-proline,[11] deprotected by means of Pd/C-catalyzed hydrogenolysis, and finally converted into the target structure after nucleophilic addition–elimination with chloroacetyl chloride. Treatment of **18** with acryloylchloride yielded compound **3b** (Scheme 3).

Compound **4a** was synthesized by coupling **11** with the commercially available 2,6-dichloro-3-nitrobenzoic acid (**19**) to yield **20**, followed by reduction to the aniline **21** and nucleophilic addition–elimination with chloroacetyl chloride to yield the final product **4a** (Scheme 4). Compound **4b** was prepared by coupling structure **11** with **24** (from a mixture of **24** and **25**); compound **24** was synthesized from the commercially available **19**, reduction to the corresponding aniline **22,** and conversion into **24** by means of nucleophilic addition–elimination reaction with chloroacetyl chloride (generating also **25** resulting from an electrophilic addition side-reaction) (Scheme 5).

Synthesis of **5** was accomplished in three steps, starting from **11** by coupling the secondary amine to the commercially available N-Z-(R)-(+)-pipecolinic acid to yield **26**, removal of the benzyl carbamate group by means of Pd/C-catalyzed hydrogenolysis, and finally a nucleophilic addition–elimination reaction with chloroacetylchloride to yield the target compound **5** in moderate yields (Scheme 6).

Cellular inhibitory activity of the GSK866 analogues was determined using a human GR receptor radioligand binding assay using 1.5 nM [^3H]-dexamethasone as radioligand.[12] IM-9 cells were used as the source of the soluble GR. Assays were performed at Cerep.[13] The results are given in Table 1. Only compounds **4a** and **4b** displayed significant specific binding to the receptor, suggesting

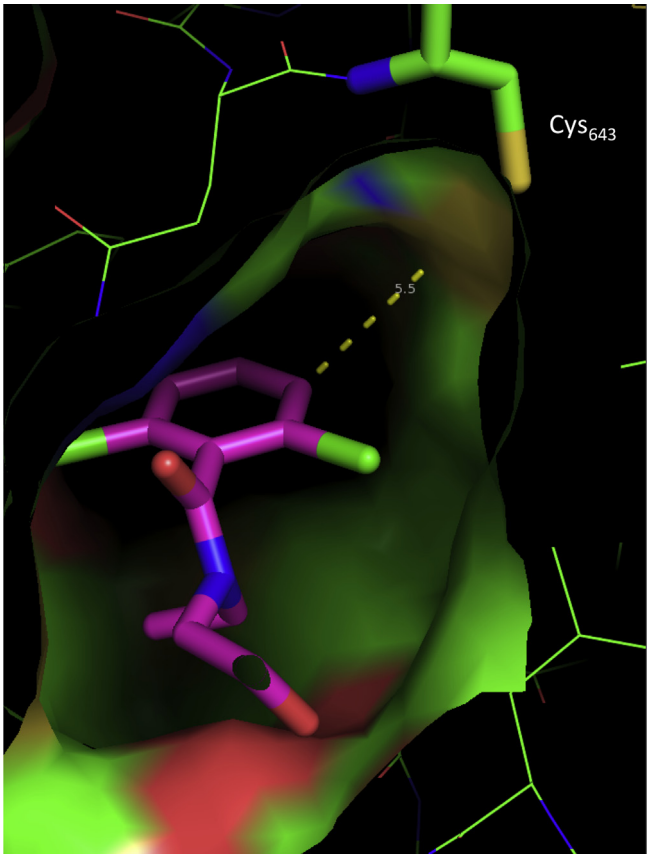

Figure 2. Close-up in the ligand-binding pocket of the ligand-binding domain of the glucocorticoid receptor.[5] Shown with purple-colored carbon atoms is the dichlorophenyl ring of GSK866, with the yellow dotted line indicating the distance between this ring and the thiol group of Cys_{643}.

pivotal importance of the dichlorophenyl ring in target recognition, an observation which is in agreement with the reported SAR around the GSK866 series.[9]

In order to investigate whether compounds **4a** and **4b** bind to the GR in an irreversible manner, a wash-out experiment was set up using dexamethasone mesylate and cortisol. Dexamethasone mesylate has been reported as an irreversibly binding covalent ligand of the receptor and served as a positive control for covalent modification.[14] Compared to the corresponding C-21 mesylates of cortisol and deacylcortivazol, dexamethasone mesylate has been reported to be the most useful derivative due to its favorable balance of high receptor affinity and predominantly irreversible antiglucocorticoid activity.[15,16] Cortisol served as a positive control of a non-covalent binder. The protocol was based on the method as described by Simons and Thompson.[17] After initial incubation of the compounds with the receptor and subsequent removal of unbound compounds, the ability of the receptor to bind [^3H]-dexamethasone added in excess was assessed. The IM-9 cell-line cytosolic preparations (240 μg/assay) were pre-incubated for six hours with **4a**, **4b**, dexamethasone mesylate and cortisol at a concentration chosen as to occupy approximately 80% of the receptors. For **4a**, **4b** and dexamethasone mesylate the concentration tested was 100 nM, and for cortisol this was 60 nM. After incubation, free compounds were removed by activated charcoal followed by centrifugation. Subsequently, an excess (3 nM) of [^3H]-dexamethasone was added a second incubation was performed. Binding capacity of [^3H]-dexamethasone was quantified at several incubation times (1 h, 2 h, 3 h, 4 h, 16 h, 24 h). The results are given in Table 2 ('Initial experiments').

The kinetics of the reaction were considered to be rapid due to the fact that after one hour the maximum binding was already attained (data not shown). For cortisol, binding to the GR was totally recovered, confirming that cortisol binds reversibly to the receptor. However, although the fact that compounds **4a** and **4b** were designed as irreversible binders to the GR, we could not confirm this covalent linkage in the present experiments as the

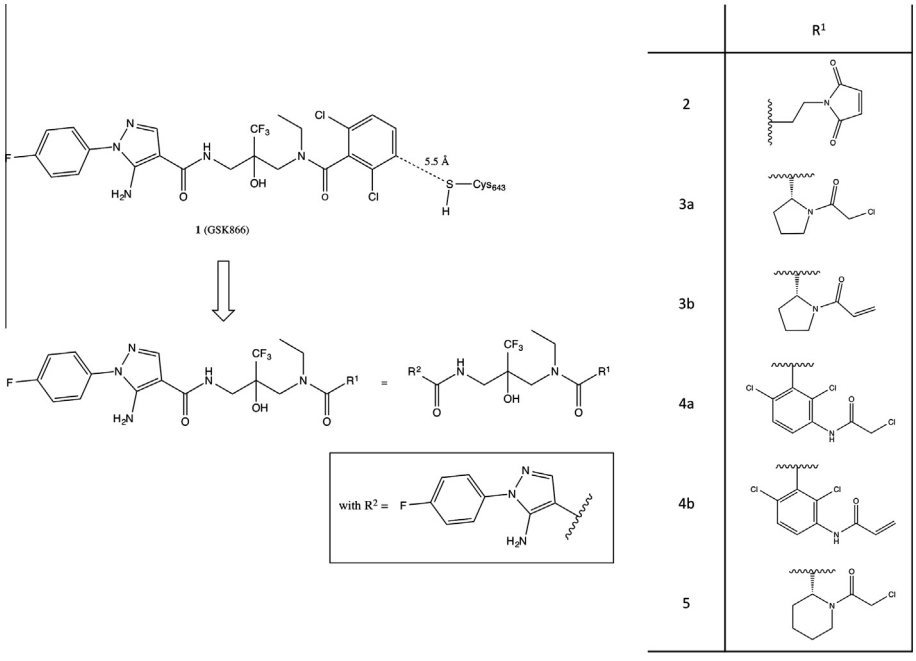

Figure 3. Design of novel GSK866 (**1**) analogues with the dichlorophenyl substituent replaced by reactive groups to form a covalent bond with the thiol group of Cys_{643} of the ligand-binding domain of the GR (**2**–**5**). Proposed docking poses of **4a** and **4b** in the receptor are shown in Figure S1 (Supplementary Material).

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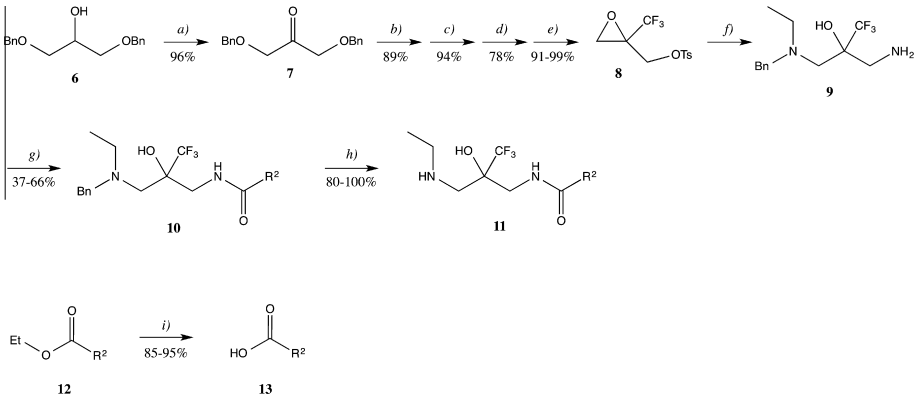
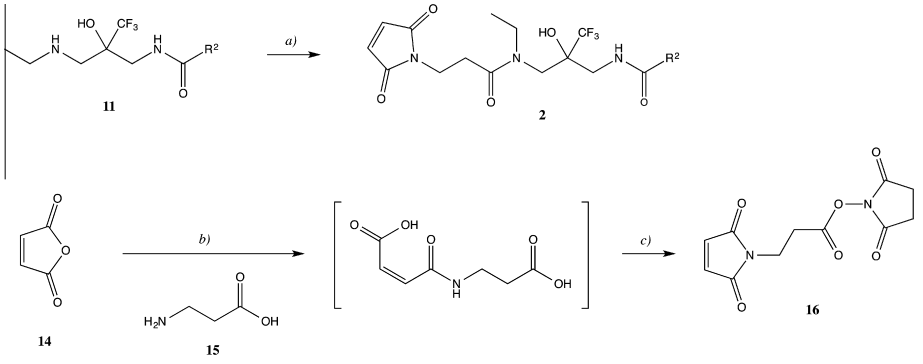
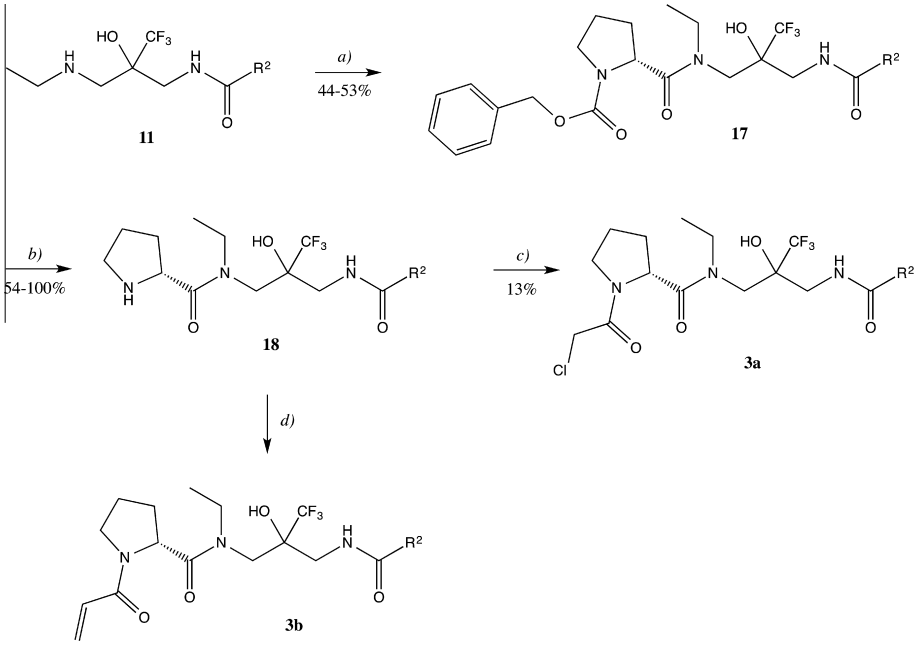


Scheme 1. Synthesis of **11**. Reagents and conditions: (a) NCS, Me_2S, Et_3N, toluene; (b) $TMSCF_3$, Bu_4NF, THF; (c) H_2, Pd/,; EtOH; (d) TsCl, pyridine; (e) K_2CO_3, DCM; (f) $PhCH_2NHEt$, dioxane, N_2, followed by NH_3 in MeOH; (g) **13**, HATU, DMF; (h) H_2, EtOH, Pd/C; (i) LiOH, $EtOH/H_2O$, reflux, then acidification.

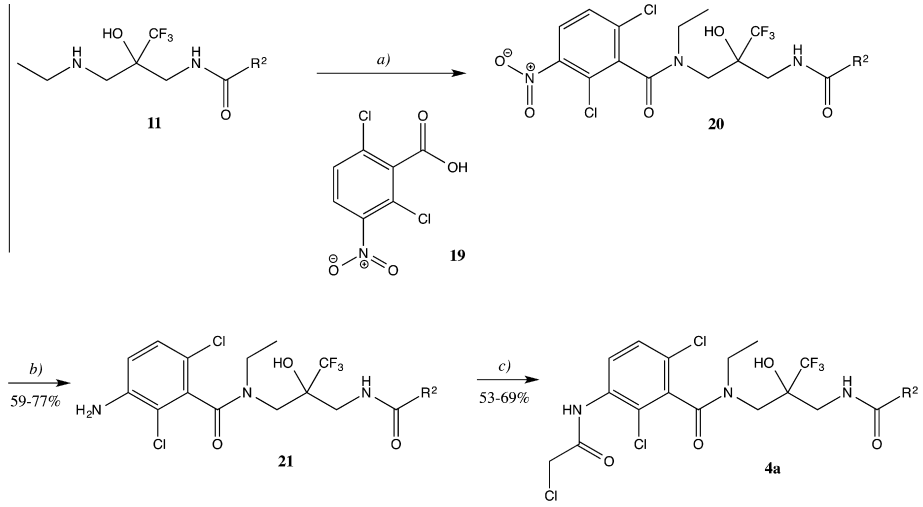


Scheme 2. Synthesis of **2**. Reagents and conditions: (a) **16**; DMF, rt; (b) DMF, rt; (c) DCC, NHS.

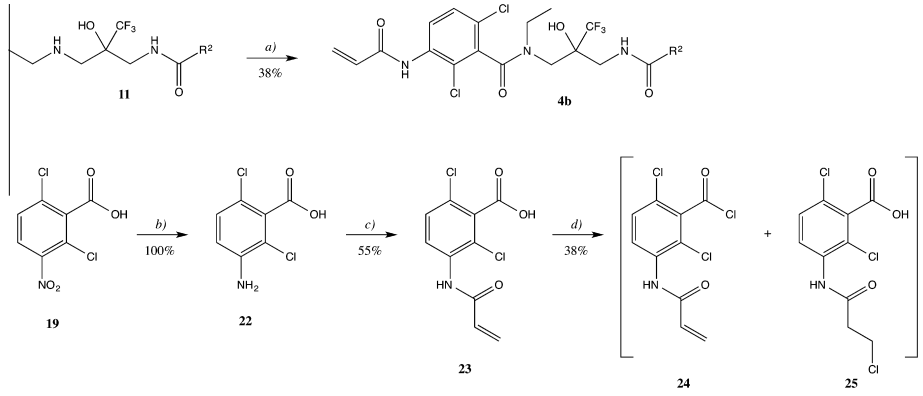


Scheme 3. Synthesis of **3a** and **3b**. Reagents and conditions: (a) N-Z-(D)-proline, HATU, DIPEA, DMF, rt; (b) Pd/C, H_2, MeOH, rt; (c) chloroacetylchloride, DIPEA, DCM; (d) acryloylchloride, DIPEA, DCM.

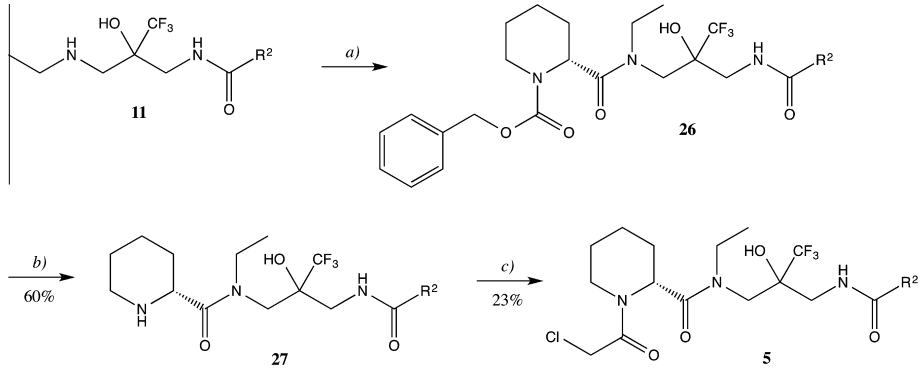
recovery was complete for **4a** and significant for **4b** (although not complete: 74% vs 21%). Strangely, we could not confirm the published results of dexamethasone mesylate as being a covalent binder (according to reference 10, the pre-incubation with dexamethasone mesylate reduced the ability of the receptor by 75% to bind [^3H]-dexamethasone), as in our hands the recovery



Scheme 4. Synthesis of **4a**. Reagents and conditions: (a) first $SOCl_2$, benzene, reflux 1 h, then DCM; (b) Zn, NH_4Cl/THF; (c) chloroacetylchloride; DCM, $NaHCO_3$.



Scheme 5. Synthesis of **4b**. Reagents and conditions: (a) **24**, first $SOCl_2$, benzene, DrySyn 100 °C, then 1 equiv NEt_3, DCM, −50 °C, overnight; (b) Zn, NH_4Cl/THF; (c) acryloylchloride, DCM, then 0.5 M NaOH, then 1 M HCl; (d) $SOCl_2$, benzene.



Scheme 6. Synthesis of **5**. Reagents and conditions: (a) N-Z-(R)-(+)-pipecolinic acid, HATU, DIPEA, DMF, rt, overnight; (b) Pd/C, H_2, MeOH, rt, overnight; (c) chloroacetylchloride, DIPEA, DCM.

of dexamethasone mesylate was almost complete. Potential causes could have been (1) the presence of β-mercaptoethanol in the incubation buffer solutions used by Cerep, (2) a decomposed form of dexamethasone mesylate in which the mesylate group would be cleaved prior to performing the experiments, or (3) differences in the protein sequence of the GR depending on the species from which the protein has been extracted. To exclude the first two possibilities, all experiments were repeated using buffer solutions without β-mercaptoethanol, and also using a new batch of dexamethasone mesylate (the original batch was supplied by ARC,[18] the second batch was synthesized from dexamethasone according a method as described by Simons and coworkers[19]). Results are shown in the second part of Table 2 ('Replication experiments') and confirm the earlier findings. The two different batches of

Table 1
Specific binding measured as % inhibition of control. All experiments were performed in duplicate

	% inhibition of control	
	1 μM ligand concentration	0.1 μM ligand concentration
18	−20%	
3b	−23%	
27	−12%	
4a	92%	78%
4b	96%	74%
5	−29%	

dexamethasone mesylate produce the same results, in the sense that a total recovery of GR binding was observed without blockage of the receptor by the compound. In addition, β-mercaptoethanol does not seem to influence the reversibility of dexamethasone mesylate as well as compounds **4a** and **4b**.

The dexamethasone mesylate binding experiments performed by Simons and coworkers[16,17] were performed on the glucocorticoid receptor extracted from rat hematoma tissue culture cells, while the present experiments are performed on human IM-9 cell-line cytosolic preparations. In the work done by Simons, degradation experiments identified Cys_{656} as the target for

Table 2
Specific binding measured as % inhibition of control, before and after wash-out at given time points. All experiments were performed in duplicate. 'Dex-mes' stands for dexamethasone mesylate. For the replication experiments, buffer solutions were devoid of β-mercaptoethanol and a new batch of dexamethasone mesylate was used

	% inhibition of control							
	Before wash-out	1 h	2 h	3 h	4 h	16 h	24 h	Average (1–24 h)
				Initial experiments				
Cortisol	69%	3%	6%	4%	−1%	5%	12%	5%
Dex-mes	93%	7%	3%	7%	−14%	13%	23%	6%
4a	78%	−4%	−16%	−19%	−28%	1%	2%	−11%
4b	74%	21%	17%	24%	6%	31%	%	21%
				Replication experiments				
Cortisol	69%	−16%	−15%	−23%	−12%	16%	−8%	−10%
Dex-mes	93%	3%	−11%	−2%	3%	46%	13%	9%
4a	78%	−1%	−8%	−6%	0%	37%	8%	5%
4b	74%	−14%	−33%	−22%	−10%	−5%	−2%	−14%

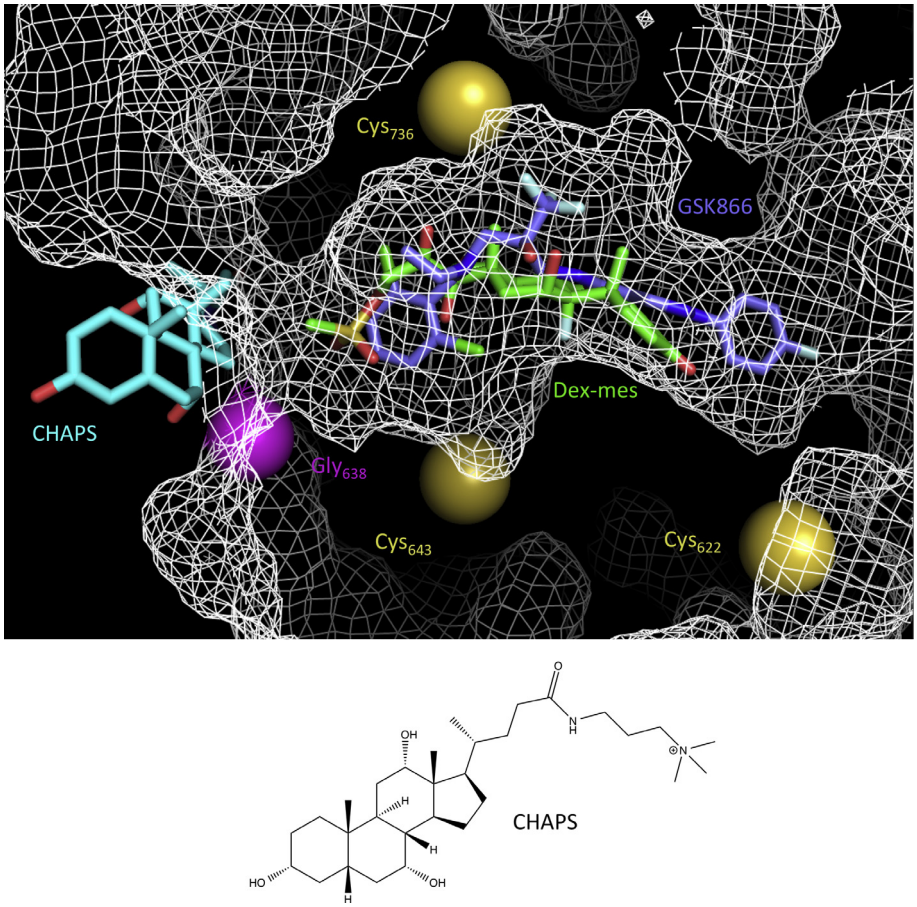


Figure 4. Comparison of the binding of dexamethasone mesylate ('Dex-mes', green-colored carbon atoms) and GSK866 (purple-colored carbon atoms) projected into the crystal structure of the GSK866. Also shown is the binding of CHAPS[21] (cyan-colored carbon atoms) as observed in the structure of the dexamethasone complex. The three cysteine residues which are located in the vicinity of the ligand-binding pocket are highlighted by yellow spheres. Finally, Gly_{638} is indicated by a purple sphere. In the sequence of the human and rat GR, this glycine is replaced by a cysteine. The docking pose of dexamethasone mesylate was generated starting from the crystal structure of dexamethasone in complex with the ligand-binding domain of the GR receptor,[20] and manually adding the mesylate sidechain whilst keeping the conformation of this sidechain in a low energy conformation.

dexamethasone mesylate covalent binding.[16] In the crystal structure of the human GR with GSK866,[5] this cysteine has been mutated to a Gly_{638} and is located in close proximity to the dichlorophenyl ring of GSP866 and to the C-21 atom of the dexamethasone ligand (Fig. 4).[20] Interestingly, the crystal structure of the GR/dexamethasone complex reveals binding of second steroid-like molecule (CHAPS, see Fig. 4), also located in very close proximity of this Cys_{656}, indicating that alternative binding modes are observable in the case of the GR receptor. It might therefore be possible that the discrepancy with the work of Simons and coworkers[16,17] could be attributed to undocumented differences in experimental conditions, thereby exploring alternative binding modes of which the details are not yet understood.

The current experiments cannot exclude, nor confirm, that dexamethasone mesylate can bind covalently to another cysteine residue not necessary located in the ligand-binding pocket. In preliminary and unpublished mass spectroscopy experiments, we identified dexamethasone mesylate to bind covalently to Cys_{622}, but further research around the exact mechanism is needed to understand and confirm these findings.[22] Initial docking models of dexamethasone mesylate in the ligand-binding domain of the GR do not reveal a close contact between the mesylate group and Cys_{622} or Cys_{643}, respectively, but rather a potential contact with Cys_{736} (see Fig. S2 of the Supplementary Material). However, more refined docking studies as well as elongated molecular dynamics simulations will be essential to validate these hypotheses.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.08.091.

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21. 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
22. Molecular graphics analysis of the crystal structure of the GR binding domain in complex with GSK866 confirms the location of Cys_{622} in proximity of the GSK866 ligand. However, while Cys_{643} is positioned in close proximity of the 2,6-dichlorophenyl sidechain of GSK866 (see Fig. 2), Cys_{622} is located along the 4-fluorophenylpyrazole side of GSK866 with the thiol group pointing outwards into the solvent. A close-up on GSK866 and both cysteines is shown in Figure S3 of the Supplementary Material.