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Identification of dissociated non-steroidal glucocorticoid receptor agonists

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Abstract—A new series of ligands for the glucocorticoid receptor (GR) is described. SAR development was guided by docking **3** into the GR active site and optimizing an unsubstituted phenyl ring for key interactions found in the steroid A-ring binding pocket. To identify compounds with an improved side effect profile over marketed steroids the functional activity of compounds was evaluated in cell based assays for transactivation (aromatase) and transrepression (IL-6). Through this effort, **36** has been identified as a partial agonist with a dissociated profile in these cell based assays.

The glucocorticoid receptor is a member of the nuclear receptor superfamily of intracellular receptors that also include the mineralocorticoid (MR), progesterone (PR), estrogen (ER), and androgen (AR) receptors.[1,2] Glucocorticoids (GCs), for example, cortisol, are endogenous hormones that play an important role in homeostasis. They also participate in the resolution of inflammatory conditions by suppressing a variety of immune and inflammatory functions by inhibition of inflammatory cytokines such as IL-1, IL-2, IL-6, and TNF-α, as well as the expression of adhesion molecules on endothelial cells.[3] The anti-inflammatory effects of endogenous steroids prompted the development of synthetic glucocorticoids such as prednisolone and dexamethasone (Fig. 1).[4] In addition to their potent anti-inflammatory effects endogenous GCs initiate gluconeogenesis, catabolism of proteins, play a role in electrolyte and water balance, reduce calcium absorption, and inhibit osteoblast function.[5] Side effects associated with glucocorticoid therapy, or corticosteroid excess, occur as a result of homeostatic disruption and include alterations in fluid and electrolyte balance, edema, weight gain, hypertension, muscle weakness, diabetes, and/or steroid induced osteoporosis.[6] Furthermore, cross-reactivity of GCs with other nuclear receptors, especially MR and PR, may also lead to a number of side effects. A mechanism by which GCs exhibit their effect has been proposed.[7–9] Transgenic mice expressing a dimerization-deficient GR exhibit reduced levels of GR mediated transcriptional activation (transactivation) of genes with glucocorticoid response elements (GREs) and it is upregulation of these genes that is believed to be the predominant side effect pathway. Transcriptional repression of gene expression (transrepression) driven by inhibition of pro-inflammatory transcription factors such as NF-κB remains intact and thus GCs retain their beneficial anti-inflammatory effects. Therefore, the identification of selective immunosuppressive GR ligands that can preferentially transrepress immune genes while exhibiting reduced levels of transactivation of metabolic genes (a dissociated ligand) may offer a therapeutic advantage over the currently marketed glucocorticoids.[10–12]

Keywords: Glucocorticoid receptor; Glucocorticoid receptor agonist, Anti-inflammatory; IL-6, Aromatase.
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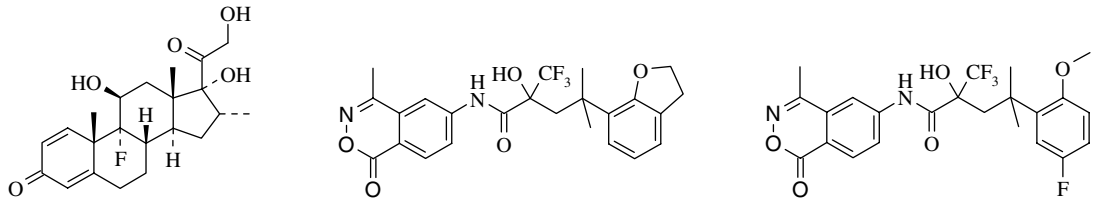


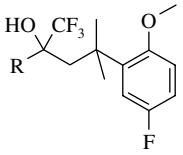
Dexamethasone ZK216348, (+)-enantiomer Compound **1**

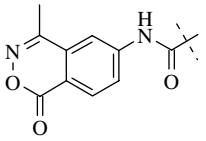
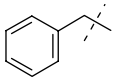
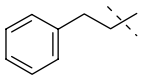
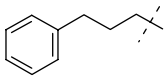
Figure 1. Synthetic glucocorticoid agonists.

Recently, the identification of dissociated GC ligands has been reported by several drug discovery groups.[13–22] During the course of our work, ZK216348 was disclosed as a dissociated GC ligand and an earlier analog in this series is represented by compound **1** (Fig. 1).[23] Recently, we reported the effect of replacing the trifluoromethyl group on binding and agonist activity with respect to compound **1**.[24] We now disclose the results of modifications to the amide and the attached heteroaryl group (benzoxazinone) of these potent GR agonists and the effect of these simplifications on binding potency, nuclear receptor selectivity, and agonist activity.[25,26]

Initially, we focused on preparing compounds **2**–**5** (Table 1) where R is a phenyl group with various linker lengths. The rational for this approach was based on the SAR within the amide series (compound **1**) which demonstrated that the reduced amide retained GR binding potency suggesting the possibility of additional changes to the amide linker.[23] For simplicity, we chose to replace the benzoxazinone hetereocycle with a phenyl group and planned to develop future SAR with respect to substitution on the phenyl ring. The IC_{50} values for binding to GR, MR, and PR were determined by using a fluorescence polarization competitive binding assay.[27] The GR and MR assays measure the ability of test compounds to compete with tetramethyl rhodamine (TAMRA)-labeled dexamethasone. The PR binding assay was similarly run using TAMRA-labeled mifepristone. To assess the functional activity of new ligands we employed two cellular assays. The first assay, an indicator of anti-inflammatory activity (transrepression), measures the inhibition of IL-6 production in human foreskin fibroblasts (HFF) in response to stimulation by pro-inflammatory cytokine IL-1.[28] It is known that circulating IL-6 levels increase during an inflammatory response and IL-6 production can be inhibited by glucocorticoids such as dexamethasone. The second assay, an indicator of side effect potential (transactivation), measures the upregulation of aromatase, an enzyme responsible for the conversion of testosterone

Table 1. Identification of optimal linker length



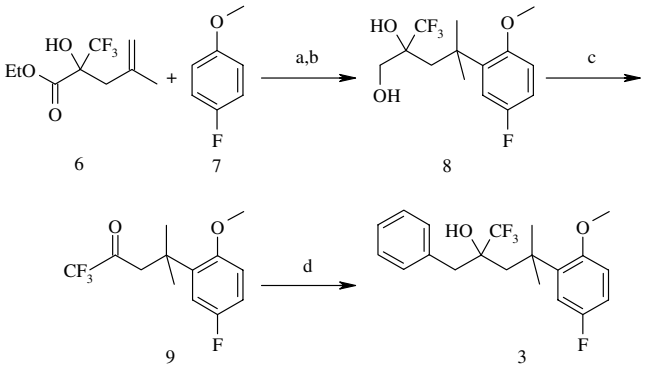
Compound	R	GR IC_{50} (nM)	PR IC_{50} (nM)	MR IC_{50} (nM)
Dexamethasone	—	3	>2000	33
1		8	22	130
2		>1600	>1600	1600
3		610	>1300	>1300
4		>1300	>1300	>1300
5		1125	>740	>740

to estradiol.[29] It is known that glucocorticoids upregulate aromatase production in HFF cells.[30] In both assays potency and efficacy are measured and the efficacy is expressed as a percentage of the maximum response observed with dexamethasone treatment. All compounds in this paper were tested in the assays as racemates.

Our initial studies established the optimal linker length for this series. Although analogs **2**–**5** demonstrated a loss in GR binding potency when compared to compound **1** we viewed compound **3**, where R = $PhCH_2$ (GR IC_{50} = 610 nM), as a potential starting point for further optimization due to the identification of a new linker (–C(O)NH– to $–CH_2–$) and potential for functionalization of the left-hand side phenyl which replaced the complex heterocycle found in compound **1**. Next, we explored the effect of substitution of the left-hand side phenyl ring with a goal of improving GR binding, nuclear selectivity and obtaining cellular potency. To guide our early SAR in this program, we developed a homology model derived from a X-ray crystal structure of progesterone bound to the human progesterone receptor and later refined this model with the aid of a report of a X-ray co-crystal structure of dexamethasone in the GR-LBD.[31,32]

A proposed binding pose of compound (*S*)-**3** revealed (Fig. 2b) a number of interesting features.[33,34] The central hydroxyl group of (*S*)-**3** forms a key H-bond with Asn564 mimicking the interaction seen with the 11β-hydroxyl group of dexamethasone. The methoxyfluorophenyl group extends above the plane of the steroid (D-ring region) forming a potential π-stacking network with Phe623 and the unsubstituted phenyl ring of **3** (steroid A-ring region). Finally, the 3-keto group of dexamethasone forms hydrogen bonds to both Arg611 and Gln570 which, due to the lack of appropriate functionality, is not possible for compound **3** thus establishing our main focus of SAR described herein.

The synthesis of the compounds described in the present work has been reported elsewhere.[26] Racemic analogs of compound **3** were prepared according to Scheme 1. A



Scheme 1. Reagents and condition: (a) $AlCl_3$; (b) $LiAlH_4$, THF; (c) $NaIO_4$, MeOH; (d) $PhCH_2Br$, Mg, THF, Δ.

Friedel–Crafts reaction with olefin **6** and *p*-fluoroanisole using aluminum chloride afforded an ester which, upon reduction with lithium aluminum hydride in THF, furnished diol **8**. Oxidative cleavage of vicinal diol **8** provided the key intermediate, trifluoromethyl ketone **9**. A mixture of **9**, benzyl bromide, and Mg in THF was warmed at 65 °C in a sealed tube to afford **3**.

With chemistry in hand amenable for reacting ketone **9** with various benzyl bromides, we explored the effects of substitution on the left-hand side aromatic ring (compound **3**, R = CH_2Ph) via incorporation of lipophilic or polar groups as shown in Table 2.

Incorporation of lipophilic groups, such as methyl and halogen, resulted in a modest improvement in GR binding potency. The 3-methylphenyl compound **11** was equipotent to **3**, but more potent than the 2- or 4-methyl analogs **10** and **12**. Introduction of chlorine or bromine in the 2-position gave compounds **14** and **15**, resulting in a three- to sixfold increase in binding potency, respectively. In general, the 2-chloro analog was more potent than the 3- and 4-chloro analogs **16** and **18**. The lipophilic naphthyl analog **22** which fills the steroid A-ring binding pocket to a greater extent than compound **3** (Fig. 2b) also demonstrated improved GR binding.

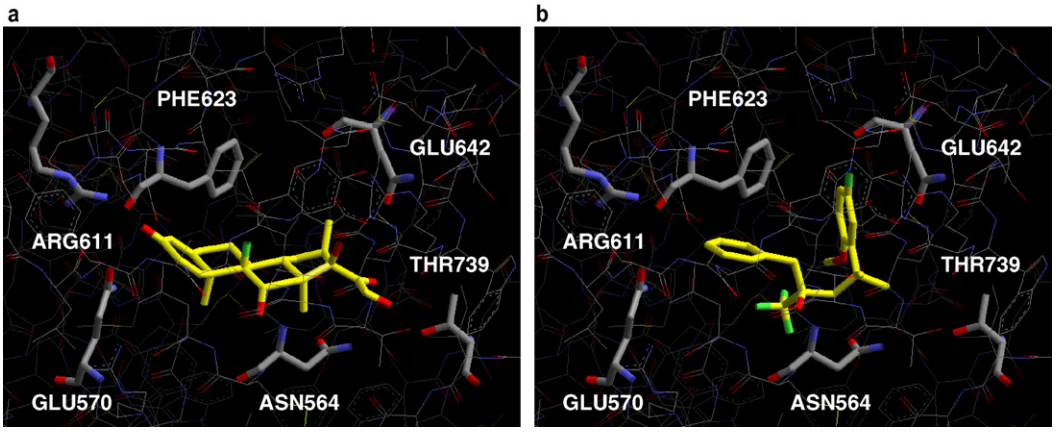
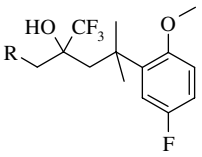


Figure 2. (a) Co-complex X-ray structure of dexamethasone in the GR-LBD.[32] (b) Docking results for *S*-**3** into the GR-LBD using the GR-LBD/dexamethasone co-complex X-ray structure.[33,34]

Table 2. Mono-substituted phenyl and naphthyl analogs



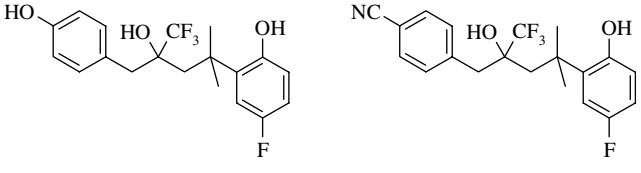
Compound	R	GR IC_{50} (nM)	PR IC_{50} (nM)	MR IC_{50} (nM)
3	Phenyl	610	>1300	>1300
10	2-Methylphenyl	855	>1130	>1130
11	3-Methylphenyl	580	>1050	>1050
12	4-Methylphenyl	945	>2000	>2000
13	2-Fluorophenyl	370	>2000	1350
14	2-Chlorophenyl	101	>2000	715
15	2-Bromophenyl	205	>512	>512
16	3-Chlorophenyl	550	>2000	>2000
17	3-Cyanophenyl	81	>2000	400
18	4-Chlorophenyl	340	>2000	1800
19	4-Methoxyphenyl	1250	>1420	>1420
20	4-Formylphenyl	20	>2000	650
21	4-Cyanophenyl	30	345	510
22	1-Naphthyl	120	>2000	1400
23	2-Naphthyl	275	>2000	>2000

Consistent with Figure 2b, further improvements in binding potency were obtained by incorporation of polar groups in the 4-position of the left-hand side phenyl ring which could engage the Arg611/Gln570 pair. For example, introduction of either a formyl or a cyano group, compounds **20** and **21**, respectively, generated highly potent and selective GR ligands. Interestingly, the 4-methoxyphenyl analog **19** was considerably less potent then **20** and **21** potentially due to steric constraints. Indeed, exhaustive demethylation of compound **19** gave the 4-hydroxyphenyl analog **24** (Fig. 3) (GR IC_{50} = 43 nM) which was 29-fold more potent than **19**. In comparison, compound **25** (Fig. 3) is only twofold more potent than methylated analog **21**. These results are supported by the docking results for **24** in the GR-LBD (not shown) which positions the 4-OH group of **24** within H-bonding distance to either the Arg611 or the Gln570. While we observed the largest increase in binding potency with polar groups in the mono-substituted phenyl series (compound **21**), we were unable to build in the desired agonist activity. Our goal now focused on incorporation of additional polar and lipophilic groups to obtain agonist activity.

Thus, we prepared a series of di- and tri-substituted phenyl and mono-substituted naphthyl analogs as shown in Table 3. Our focus was on preferred groups identified in Table 2. The introduction of two lipophilic groups provided potent binding to GR, for example, compound **26** has a GR binding IC_{50} of 55 nM. Furthermore, **26** demonstrated weak but non-dissociated activity in the IL-6 and aromatase cellular assays (Table 4). Replacement of one of the methyl groups of **26** with a chlorine atom gave analog **33** which was equivalent to **26** in binding potency and non-dissociated cellular activity. However, replacement of both methyl groups with chlorine atoms, to give analog **30**, resulted in a complete loss of cellular activity yet analog **30** was equipotent to both **26** and **33** in the GR binding assay. The dichloro analogs **27**–**30** were all more potent than the mono 3- and 4-chlorophenyl analogs **16** and **18** in the GR binding assay. However, the 2,6-dichlorophenyl analog **31** was less potent than the 2-chlorophenyl analog **14** in the GR binding assay. The decrease in binding potency of analog **31** compared to dichloro analogs **27**–**30** is difficult to explain, it is unlikely to be attributed to a change in torsion angle about the phenyl ring since the 2-methyl-1-naphthyl analog **39** retained binding potency.

Analogs **36**, **38**, **40**, and **41** which incorporated both polar and lipophilic groups demonstrated improved activity in the IL-6 assay (Table 4). Interestingly, we have observed that the substitution pattern on the phenyl ring is critical to achieving agonist activity and that relativity small structural modification can have a large impact on cellular and dissociated cellular activity. For example, 2-fluoro-4-cyanophenyl analog **35** and 2-chloro-4-cyanophenyl derivative **36** both bind to GR with equal potency however in the functional assay for transrepression only **36** demonstrates IL-6 activity (Table 4). Furthermore, replacement of the cyano group in **36** with a formyl group (analog **32**) also resulted in a loss of functional activity while binding potency was retained. It has been reported that small structural changes to ligands which bind to GR, AR, and PR have resulted in antagonist/agonist switching.[14,35,36]

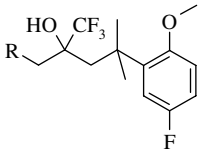
Not surprisingly, docking studies would suggest that the 4-cyano-1-naphthyl analog **38** could engage the Arg611 and Gln570. Indeed, **38** demonstrated improved GR binding when compared to the 1-naphthyl analog **22**. More importantly, compound **38** was potent in the functional assays (Table 4) while the 2- and 4-methyl naphthyl analogs **37** and **39** which were equipotent in the binding assay failed to display agonist activity. Obviously, binding potency alone is not a predictor of agonist activity for these structurally similar ligands. These studies suggest that potent agonist activity for this class of ligands is best achieved by fulfilling both a lipophilic (space filling) and polar (hydrogen bonding) component in the steroid A-ring pocket. In comparison, the mono-substituted 4-cyanophenyl analog **21** achieved low nanomolar potency in the GR binding assay; however introduction of the polar cyano group was not sufficient to achieve agonist activity. However, introduction of additional lipophilicity, the 3,5-dimethyl substitution, along with the cyano group afforded analog **40** which displayed single digit nanomolar activity in the IL-6 assay.



Compound 24 Compound 25

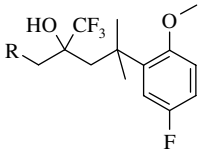
Figure 3. Demethylation of compounds **19** and **21**.

Table 3. Di- and tri-substituted phenyl and mono-substituted naphthyl analogs



Compound	R	GR IC_{50} (nM)	PR IC_{50} (nM)	MR IC_{50} (nM)
26	3,5-Dimethylphenyl	55	>354	220
27	2,3-Dichlorophenyl	76	1300	900
28	2,4-Dichlorophenyl	68	665	875
29	2,5-Dichlorophenyl	45	>2000	535
30	3,5-Dichlorophenyl	60	>2000	760
31	2,6-Dichlorophenyl	580	>2000	1100
32	2-Chloro-4-formylphenyl	19	1800	345
33	3-Chloro-5-methylphenyl	46	>2000	940
34	3-Chloro-5-cyanophenyl	27	>2000	730
35	2-Fluoro-4-cyanophenyl	22	280	500
36	2-Chloro-4-cyanophenyl	17	140	320
37	4-Methyl-1-naphthyl	58	1200	1050
38	4-Cyano-1-naphthyl	21	325	430
39	2-Methyl-1-naphthyl	64	>2000	450
40	4-Cyano-3,5-dimethylphenyl	11	390	300
41	2-Cyano-3,5-dimethylphenyl	8	1800	250

Table 4. Transrepression (IL-6 agonism) and transactivation (aromatase) data for selected analogs



Compound	GR IC_{50} (nM)	IL-6 EC_{50} (nM)	IL-6 % efficacy	Aromatase EC_{50} (nM)	Aromatase % induction
Dexamethasone	3	0.5	100	2	100
1	8	>2000	20	>2000	0
21	30	>2000	0	>2000	0
26	55	280	55	285	80
33	46	125	79	245	80
34	27	>2000	46	4750	53
35	22	>2000	40	660	15
36	17	20	60	30	20
37	58	>2000	9	nt	nt
38	21	70	92	260	103
40	11	4	95	8	115
41	8	40	83	250	75

nt, not tested.

The following observations were made regarding GR, PR, and MR selectivity. In general, most compounds showed the following relative potency in the binding assays GR > MR > PR ≫ ER (data not shown). Compound **41** was the most selective for GR over PR (225-fold). In comparison, a close analog **40** which differs only in the placement of the cyano group is less selective toward GR (35-fold). Presumably, the 4-cyano group which was designed to interact with the Arg611/Gln570 in GR can also interact with these conserved residues in PR and MR. ER activity was not detectable at 2 μM for compounds reported in Tables 1–4.

Finally, we compared both the potency and efficacy of our compounds in the IL-6 (transrepression) and aromatase (transactivation) assays to determine if they demonstrated dissociated cellular activity. Compound **36** was equipotent in both of these cellular assays (Table 4) and although not a full agonist in the transrepression assay (60%), **36** did demonstrate a separation of activity based on efficacy inducing aromatase at a very low level (20%) compared to dexamethasone. Thus, **36** represents a novel GR ligand that is dissociated based on efficacy. Compound **40** was the most potent GR agonist in the cellular assays; however, improved cellular potency and efficacy did not result in an improved dissociation

profile. Movement of the cyano group to the 2-postion gave compound **41** which binds to GR with equal potency compared to **40**, however **41** was not as potent and efficacious as **40** in the cellular assays. Compound **41** is however sixfold more potent in the transrepression assay, while maintaining similar efficacy, compared to the transactivation assay. Thus, compound **41** is a novel GR ligand that is dissociated based upon potency.

In conclusion, we have described a new class of non-steroidal glucocorticoid receptor ligands. We have proposed a binding mode for this scaffold which was used to guide and is supported by our SAR studies. This model suggests that the methoxyfluorophenyl ring extends above and sits over the dexamethasone D-ring binding region while the benzyl group adjacent to the chiral center occupies the A-ring portion of the binding pocket. We have shown that GR binding potency can be improved via the introduction of either lipophilic or polar groups to the phenyl group that occupies the steroid A-ring binding site. However, to improve the functional activity of this scaffold the introduction of both polar and lipophilic groups is required. Finally, it should be noted that small structural changes can alter functional activity and dissociated functional activity in unpredictable ways. Taken all together, these observations have made this series highly attractive for further studies and will be subject of future reports.

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25. This work was previously disclosed in part: Kuzmich, D.; Kirrane, T.; Bekkali, Y.; Zindell, R.; Beck, L.; Nabozny, J.; Proudfoot, J.; Nelson, R.; Shih, S.-K.; Capolino, A.; Paw, Z.; Reilly, P.; Deleon, R.; Thomson, D. In *Inflammation Research Association, 12th International Conference*, October 3–7, Bolton Landing, NY, USA., Poster A039, 2004.
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27. GR, PR, and MR binding assays were performed in a fluorescence polarization format that measures competition for binding to the nuclear receptor, present in lysates of baculovirus-infected insect cells, between a test compound and a fluorescently labeled receptor ligand, or probe. IC_{50} values were determined by fitting the fluorescence polarization signal data to a 4-parameter logistic equation. All IC_{50} values shown represent means of at least two independent determinations. Repeated testing of reference compounds in these assays demonstrated typical IC_{50} standard deviations of 20–40% about the mean. The ER fluorescence polarization competitive binding assays were performed

according to the protocol provided with the Panvera kit P-2614.
28. Human foreskin fibroblasts are stimulated with 1 ng/mL recombinant human IL-1 in the presence of test compound. After 24 h, the degree of GR agonist activity (transrepression) is determined by measuring IL-6 in the tissue culture media and calculating EC_{50} values. Top concentration in the assay was 2 μM.
29. The assay measures the ability of test compounds to induce aromatase activity in human foreskin fibroblasts, as indicated by the production of β-estradiol in the presence of exogenously added testosterone. β-Estradiol levels were quantitated in the tissue culture media 18–24 h following test compound and testosterone addition, and test compound EC_{50} values were determined by fitting data from duplicate 11-point concentration–response curves to a 4-parameter logistic equation. For test compound comparison, dexamethasone was considered to possess 100% efficacy in this assay. All EC_{50} values shown represent means of at least two independent determinations. Repeated testing of reference compounds in these assays demonstrates typical EC_{50} standard deviations of 30–50% about the mean.
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33. Recently we reported the absolute stereochemistry of the more potent enantiomer in our series, see: Lee, T. W.; Proudfoot, J. R.; Thomson, D. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 654.
34. (*S*)-Enantiomer of compound **3** was docked into the GR-LDB pocket using the GR-LBD/dexamethasone co-crystal structure deposited in the RCSB protein data bank (PBD ID 1P93) to afford Figure 2b. The docking studies were done using Cerius2, LigandFit. The docking site was defined using dexamethasone and enlarged to fill available space. The energy grid was calculated with a distance-dependent dielectric constant of 1.0 and a non-bonded cutoff distance of 10.0 Å and the grid was extended 5.0 Å from the site. Docking was performed with a flexible ligand. Clustering of the docked ligands was performed using complete linkage with a distance threshold of 1.5 Å. Site partitioning was used with one partition.
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