Short Communication

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Future Medicinal Chemistry

Synthetic corticosteroids as tryptophan hydroxylase stabilizers

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Background: Clinically, corticosteroids are used mainly for their immune-modulatory properties but are also known to influence mood. Despite evidence of a role in regulating tryptophan hydroxylases (TPH), key enzymes in serotonin biosynthesis, a direct action of corticosteroids on these enzymes has not been systematically investigated. **Methodology & results:** Corticosteroid effects on TPHs were tested using an *in vitro* assay. The compound with the strongest modulatory effect, beclomethasone dipropionate, activated TPH1 and TPH2 with low micromolar potency. Thermostability assays suggested a stabilizing mechanism, and computational docking indicated that beclomethasone dipropionate interacts with the TPH active site. **Conclusion:** Beclomethasone dipropionate is a stabilizer of TPHs, acting as a pharmacological chaperone. Our findings may inspire further development of steroid scaffolds as putative antidepressant drugs.

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Keywords: aromatic amino acid hydroxylases • enzyme assay • molecular docking analysis • pharmacological chaperone • serotonin • stabilizer

Corticosteroid drugs have been widely used in clinical practice since the 1950s. They are highly effective in treating a wide variety of acute inflammatory diseases [1], including asthma, chronic obstructive pulmonary disorder [2], systemic lupus erythematosus [3], cancer [4], rheumatoid arthritis [5], and autoimmune pancreatitis [6]. Their anti-inflammatory action also has beneficial effects in patients with acute respiratory distress syndrome (e.g., as a consequence of COVID-19) [7]. However, there is a wide range of risks associated with the use of corticosteroids, including immune suppression, metabolic disturbances, and tissue damage. Moreover, an increased risk of neuropsychiatric disorders has been associated with these medications [8–10]. These psychiatric complications are varied and include mania, psychosis, delirium, depression [11,12], insomnia and restlessness [13], obsessive–compulsive disorder, reversible dementia-like cognitive changes and impaired concentration and memory [8,14,15]. More than 20% of patients who develop psychiatric side effects while taking corticosteroids require pharmacological treatment for these symptoms. However, most of these side effects are reversible upon discontinuation of corticosteroid medication [16]. Studies also point to disturbances of food intake, with 70% of patients reporting increased appetite and body weight in connection with cortisone use [1]. Interestingly, several reports indicate that corticosteroid treatment can also be associated with mood elevation, increased satisfaction and optimism and a reduced sense of anxiety known as 'steroid euphoria' [14,17].

The association between corticosteroid use and neuropsychiatric symptoms was first described more than 6 decades ago [18], but the underlying mechanisms behind the psychiatric complications associated with the clinical administration of corticosteroids are still not well understood. Serotonin (5-HT), which is implicated in the control of a wide variety of physiological functions in the CNS and the peripheral nervous system (PNS), has been suggested as one possible link between corticosteroid use and mood elevation [19]. The dysregulation of this neurotransmitter is believed to play an important role in the development of several psychiatric conditions, notably including disturbances of mood and satiety [20]. The tryptophan hydroxylases (TPHs) are key enzymes

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responsible for the rate-limiting step in serotonin biosynthesis and are expressed as two subtypes, TPH1 and TPH2, which have different patterns of expression, kinetics and regulatory properties. These two enzymes are members of the iron- and pterin-dependent aromatic amino acid hydroxylase family, which also includes tyrosine hydroxylase (TH) and phenylalanine hydroxylase (PAH). TPH1 is mainly expressed in nonneuronal serotonergic tissues such as enterochromaffin cells of the gut, pancreas, fat, heart and lung, as well as in the pineal gland, whereas TPH2 is the predominant subtype in the rest of the CNS [21]. Interestingly, TPH2 mRNA and protein levels in the dorsal raphe nucleus (DRN), the main source of CNS serotonin, have been shown to be regulated by steroid hormones, with consequent effects on central serotonin synthesis [22–25]. Direct modulators or stabilizers (pharmacological chaperones) of TPH activity have not been reported to date but have been proposed as potential future antidepressants [26,27].

The relation between corticosteroid signaling and serotonergic transmission in the brain is complex, and the final outcome may be influenced both by the developmental stage of the organism and the time frame of the study. For example, acute and subchronic (4 days) treatment with corticosterone or dexamethasone was found to decrease serotonin levels in rodent brain, whereas chronic (12 days) treatment was not associated with any significant change [25,28]. Conversely, other rat studies showed that glucocorticoid signaling is necessary for the developmental rise of TPH activity in the midbrain [29,30]. In agreement with the latter mechanism, dexamethasone treatment elicited an increase of tryptophan hydroxylase activity in the midbrain of adrenalectomized rats [31].

Despite extensive studies of the relationship between the hypothalamic-pituitary-adrenal axis and serotonin synthesis and multiple reports suggesting the involvement of corticosteroids in regulating the TPHs, the direct action of synthetic corticosteroids on the enzymatic activities of the TPHs has hitherto not been reported. In the present study, we investigated the effect of clinically used corticosteroids; beclomethasone dipropionate, dexamethasone, cortisone, prednisolone and betamethasone, on the activity of TPH1 and TPH2.

Materials & methods

Materials

Reagents and compounds were purchased from Sigma-Aldrich (MO, USA) with a purity of at least 95%. Chromatography materials for enzyme purification and enzymatic activity assays were purchased from Amersham Biosciences, GE Healthcare (IL, USA), and 6R-L-erythro-5,6,7,8-tetrahydrobiopterin (BH4) was purchased from Schircks Laboratories (Bauma, Switzerland).

Enzymatic activity assay

Human doubly truncated TPH1 (ΔNH102-ΔCOOH402) with a 6-His C-terminal fusion and wild-type (WT) TPH2 with an N-terminal 6-His-maltose-binding protein (MBP) were overexpressed in BL21(DE3) *Escherichia coli* cells. Human WT TH was expressed and isolated in *E. coli* BL21(DE3) pLysS as described previously [32]. Purified human PAH was expressed and isolated in the *E. coli* strain BL21-Codon Plus (DE3) RIL, as described by Flydal *et al.* [33]. After affinity purification and removal of the fusion partners, the proteins were further purified as described previously [32–34]. TPH1 and TPH2 were used for enzymatic activity assays at 37°C in a standard reaction mixture (100 µl final volume) containing 40 mM Na-Hepes (pH 7.0), 0.05 mg/ml catalase, 10 µM ferrous ammonium sulfate and 20 µM L-tryptophan (L-Trp). The enzymatic reaction was initiated by adding 200 µM BH4 and 2 mM DTT (final concentrations) and stopped by precipitation with 2% (v/v) acetic acid in ethanol. The activity assays for PAH, and TH were performed as described previously [34,35]. Formation of product (5-hydroxy-tryptophan, 5-OH-Trp; L-3,4-dihydroxyphenylalanine, L-DOPA; or tyrosine) was determined by high-performance liquid chromatography with fluorometric detection, essentially as described previously [32,36–38].

Differential scanning fluorimetry

SYPRO Orange was utilized at 1000x dilution to monitor protein unfolding using the Lightcycler 480 Real-Time PCR System (Roche Applied Science, Penzberg, Germany) with the 384-well format. Enzyme was diluted to a final concentration of 0.075 mg/ml in 20 mM Na-Hepes (pH 7.0) buffer with 200 mM NaCl, and compounds were added to a final concentration of 200 µM. Control experiments with 2% DMSO were performed in the absence of ligand. The samples were incubated at room temperature for 30 min before measurements were started. The thermal shift curves were recorded in the presence and absence of compounds from 20 to 95°C with four acquisitions per °C.

Measurement of thermal inactivation rates

TPH1 enzyme was incubated at 37°C with 100 µM beclomethasone dipropionate or 1% DMSO (vehicle control) in the assay mixture for different time periods (0, 5, 10, 15, 20, 25 or 30 min). Thereafter, BH4 was added and the TPH1 enzyme activity in each sample was determined as described earlier.

Molecular docking

Molecular docking was performed with Glide which is part of the Schrodinger program package (Schrödinger Release 2020–1: Glide, Schrödinger, LLC, NY, USA, 2020). The 'Induced Fit Docking' (IFD) protocol was used to flexibly dock beclomethasone dipropionate into TPH1. Coordinates used for docking were those of human TPH1 in complex with its BH4 cofactor (Protein Data Bank [PDB] identification code: 1MLW). Sidechains of protein pocket-residues were reoriented to accommodate beclomethasone dipropionate to optimize calculated interaction energies. Cofactor and water molecules were removed from the protein structure before docking. Two binding pockets were defined for docking to TPH1: first, the cofactor pocket, defined by the center of the cofactor in the crystal structure, and second, the postulated allosteric pocket, where the center of the docking grid box was defined as the center of residues that form close contacts with allosteric ligands as described by Petrassi *et al.* [39], i.e.; residues 190, 280, 283–286, 289, 293, 311–312, 315–316, 321, 330, 354, 376, 378–379, 382 and 386.

Data analysis

Data analysis was done in Graphpad Prism 8 (Prism Software, CA, USA). Data obtained from independent measurements are presented as mean ± standard error of the mean (SEM). EC_{50} values were calculated using nonlinear regression, fitting the following Equation 1 to the data:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10(\text{Log}EC_{50} - X)) \quad \text{(Eq. 1)}$$

where Y is the response as a fraction of 1, X is the logarithm of ligand concentration, Top is the maximum response and Bottom is the minimum response in the presence of ligand.

Results & discussion

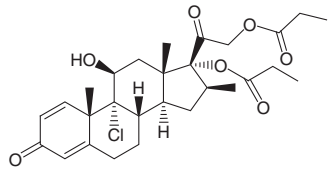
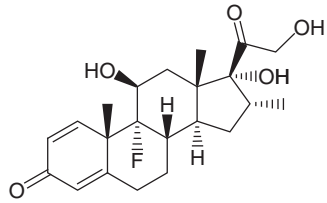
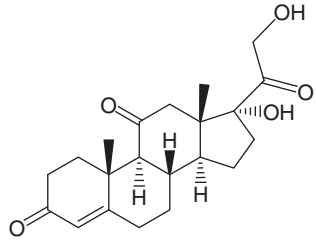
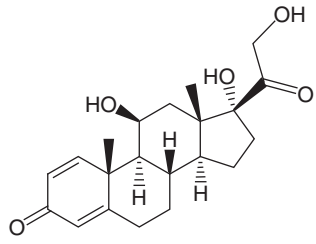
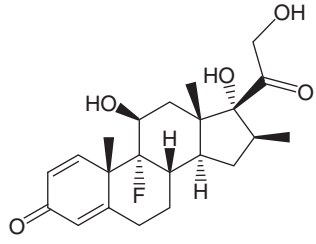
Enzymatic activity assay of corticosteroids acting on TPH1 & TPH2

Early studies demonstrated that the effect of dexamethasone to increase tryptophan hydroxylase content in adrenalectomized rat midbrain [31] correlated with an increase in TPH protein expression. In contrast, later investigations, following the cloning of TPH2, went on to show that in intact animals, glucocorticoids reduce TPH2, but not TPH1, mRNA and protein expression in the raphe nuclei, along with a consequent decrease in 5-OH-Trp formation in brain [23]. Whereas earlier studies have suggested no effect of the main endogenous rodent corticosteroid, corticosterone (cortisol playing a corresponding role in humans), on TPH activity at 100 µM [22], there have, to the best of our knowledge, been no reports on the putative direct effects of synthetic corticosteroids on the stability and activity of the TPHs.

Thus, we performed enzymatic activity assays of TPH1 and TPH2 in the presence or absence of clinically used corticosteroids (beclomethasone dipropionate, dexamethasone, cortisone, prednisolone and betamethasone), employing high-performance liquid chromatography with fluorometric detection to quantify conversion of the TPH substrate, L-Trp, into the product, 5-OH-Trp. First, each corticosteroid was tested at a concentration of 100 µM. All five compounds were found to increase product formation by both TPH1 and TPH2, with beclomethasone dipropionate showing the strongest effect, increasing product formation by about twofold (to 220.6 ± 10.4% and 198.2 ± 12.7% of control, respectively), whereas prednisolone had the weakest effect (see Table 1).

Apart from mood changes, administration of corticosteroids is not infrequently associated with other psychiatric side effects, such as psychosis, especially at high doses. Although the mechanisms behind these complications are debated, both serotonin and dopamine have been implicated in the pathophysiology of other types of psychosis, such as in schizophrenia, dementia and Parkinson disease [40]. The rate-limiting step in dopamine biosynthesis, the hydroxylation of L-tyrosine to L-DOPA, is catalyzed by TH, which, as mentioned earlier, also belongs to the aromatic amino acid hydroxylases (AAAHs) and is upregulated in schizophrenia [41]. Given the connection of dopamine and psychosis, we also studied the effects of the five glucocorticoids on TH catalytic activity at 100 µM. However, none of the tested compounds had any appreciable effect on L-DOPA formation (Table 1).

Table 1. Enzymatic activity assay of corticosteroid effects on human doubly truncated TPH 1 (Δ NH102-Δ COOH402), wild-type TPH2 and TH.

Name	Structure	TPH1	TPH2	TH
Beclomethasone dipropionate		220.6 ± 10.4%	198.2 ± 12.7%	99.4 ± 0.7
Dexamethasone		124.0 ± 20.9%	149.5 ± 8.6%	100.5 ± 0.5
Cortisone		143.2 ± 5.1%	138.7 ± 4.7%	98.8 ± 0.7
Prednisolone		119.9 ± 6.5%	111.4 ± 2.8%	100.9 ± 1.0
Betamethasone		150.0 ± 7.6%	145.3 ± 11.6%	99.8 ± 0.8

Compounds were tested at 100 μM and vehicle (1% DMSO) was used in control experiments. Values shown are percentages of enzyme activity (% of control) in the presence of the tested compounds. Data represent mean ± standard error of the mean from three independent experiments.

Potency & selectivity of beclomethasone dipropionate at TPH1 & TPH2 in the enzymatic activity assay

Because the greatest effects in the enzyme activity assay were observed with 100 μM beclomethasone dipropionate, we subjected this compound to further analyses. Due to the highly conserved active site of AAAHs, developing ligands selective for any one of these enzymes remains a considerable challenge [42–44]. Therefore, it was relevant to determine the potencies of beclomethasone dipropionate at each of the AAAHs. *In vitro* activity assays revealed that beclomethasone dipropionate activates TPH1 and TPH2 with similar potency (TPH1(ΔNH102-ΔCOOH402), EC_{50} = 3.97 ± 0.25 μM; full-length TPH2, EC_{50} = 10.06 ± 0.16 μM; see Figure 1), whereas the potency to

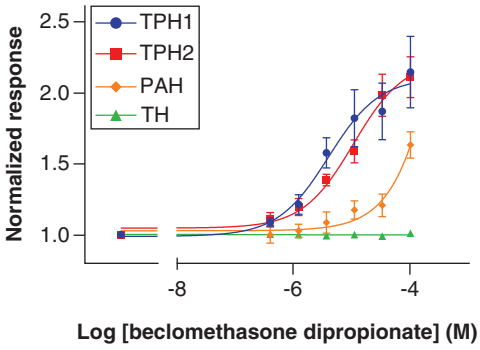


Figure 1. Concentration-dependence of the effects of beclomethasone dipropionate in enzymatic activity assays using TPH1, TPH2, PAH, and TH. Doubly truncated TPH1 (ΔNH102-ΔCOOH402) was used, whereas the other enzymes were full length. Data represent means ± standard error of the mean of three separate experiments performed in duplicate.

activate PAH was at least 60-fold lower (Figure 1). As already mentioned, no activation was seen with TH. Further characterization of the binding mode of beclomethasone dipropionate may provide useful insights for the future development of more selective and potent TPH activators as potential antidepressants.

Effect of beclomethasone dipropionate on TPH1 thermal stability

Some AAAH binders have previously been found to act as pharmacological chaperones, protecting the enzyme from thermal denaturation by transiently binding to the active site [45]. To study whether a similar mechanism might apply to beclomethasone dipropionate when acting at TPH1, we studied this compound in two thermal stability assays. First, differential scanning fluorimetry was used to characterize the effect of beclomethasone dipropionate on the thermal stability of TPH1. The fluorescent dye SYPRO Orange was used to measure the melting temperature (Tm) of the protein by recording the shift in the midpoint denaturation temperature (ΔTm) upon addition of beclomethasone dipropionate. ΔTm was determined as $\Delta Tm = Tm - Tm_{ref}$, where Tm_{ref} is the mean control value in the presence of 2% DMSO [37,38]. Beclomethasone dipropionate was found to increase the thermal stability of TPH1 (ΔTm = 7.2; see Figure 2A).

A second thermostability assay, measuring the effect of beclomethasone dipropionate on TPH1 catalytic activity, was also performed: TPH1 was incubated at 37°C for various periods of time in the presence or absence of beclomethasone dipropionate and assayed for activity. Under control conditions (1% DMSO), TPH1 lost more than 80% of its initial activity after 30-min incubation, compared with a 30% loss when incubated with beclomethasone dipropionate (Figure 2B).

Thus, the data support thermal stabilization of the enzyme by beclomethasone dipropionate as the mechanism behind the increase in product formation observed in the presence of this compound (Table 1 & Figure 1). Here, we focused on characterization of beclomethasone dipropionate action at doubly truncated TPH1 because of the higher stability of this protein compared to full-length TPH1 and TPH2, thus making it more amenable to time-resolved studies, such as that shown in Figure 2B. However, corticosteroid-induced enhancement of activity was observed also for TPH2, which is known to share a highly conserved active site with TPH1 [46]. We would thus expect the mechanism of action of the corticosteroids to be similar at both TPH isoforms, an assumption that is supported by the observation that the efficacies of the various steroid compounds were similar, overall, at TPH1 and TPH2 (Table 1).

Docking studies of beclomethasone dipropionate binding to TPH1

Beclomethasone dipropionate was docked to the active site and a postulated regulatory site [39] of TPH1 and was found to have the highest affinity to the active site. The predicted binding mode of beclomethasone dipropionate overlaps partly with both the substrate and cofactor in the active site (Figure 3). Docking scores (crude estimates of free energies of binding, where more negative numbers indicate higher predicted binding affinity) for binding to the active site (-7.4) and to the regulatory site (-4.1) indicated that beclomethasone dipropionate may preferentially bind to the active site in the low μM range.

These computational data, together with the experimental thermal stability experiments, support the notion that beclomethasone dipropionate might act as a pharmacological chaperone at TPH1. Pharmacological chaperones stabilize and protect target proteins against thermal denaturation, thus slowing their degradation time course [43,45], and are often found to be weak inhibitors of enzyme activity [47], as would be expected of the active site binder beclomethasone dipropionate. The net effect of active site pharmacological chaperones depends on

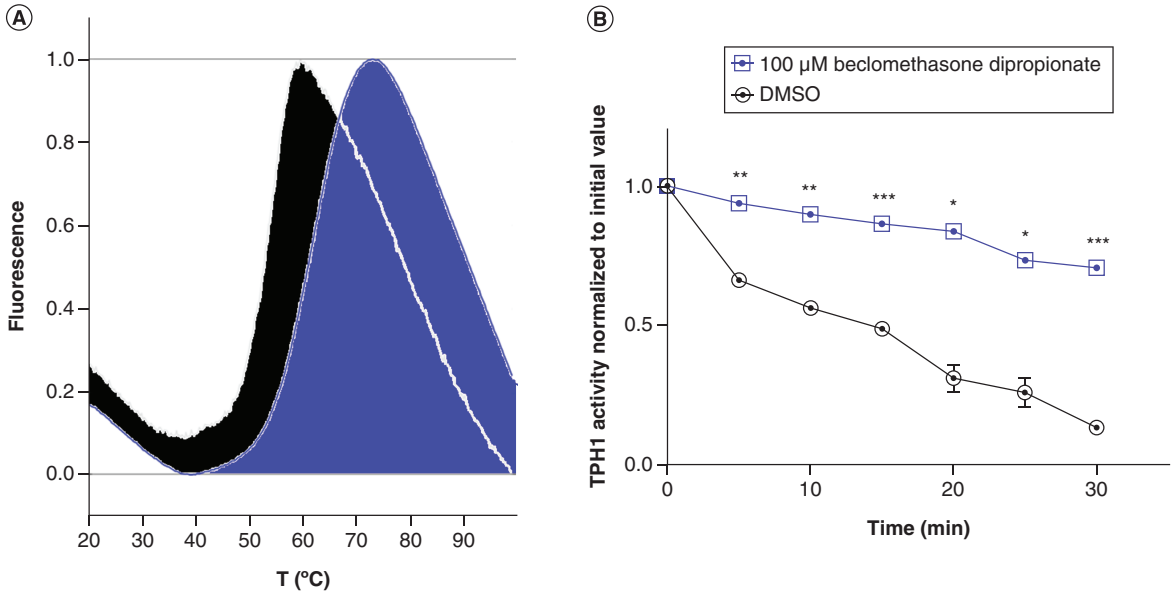


Figure 2. Effects of beclomethasone dipropionate on TPH1 thermal stability. (A) Results of a differential shift fluorimetry assay to evaluate TPH1 stability in the presence and absence of beclomethasone dipropionate. Human doubly truncated TPH1 (ΔNH102-ΔCOOH402) was used for screening. ΔTm = 7.2. **(B)** The effect of beclomethasone dipropionate on TPH1 functional stability. TPH1 (75 µg/ml) was incubated at 37°C in 40 mM NaHepes (pH 7.0) in the presence of beclomethasone dipropionate or vehicle control (1% DMSO). The graph represents data from three independent experiments, each performed in duplicate. A two-way repeated-measures analysis of variance was performed with time and treatment (beclomethasone dipropionate or vehicle control) as factors, comparing the 30-min time course, and found significant main effects of time, $F(1.309, 5.236) = 167.9$, $p < 0.001$, and treatment, $F(1, 4) = 473.4$, $p < 0.001$, as well as a significant interaction between time and treatment, $F(6, 24) = 41.02$, $p < 0.001$. Asterisks indicate Sidak posttests performed for each time point.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

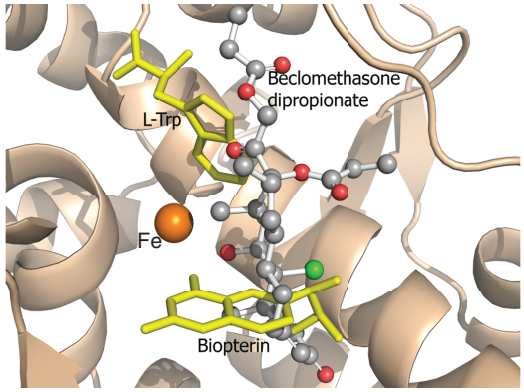


Figure 3. Docking of beclomethasone dipropionate to the active site of TPH1. Beclomethasone dipropionate is shown as a ball and stick representation with carbons colored grey while the biopterin cofactor is shown in yellow sticks. The active site iron is shown as an orange sphere. The L-tryptophan (L-Trp) substrate is also superimposed in yellow sticks (PDB identification code: 3E2T). Beclomethasone dipropionate partially overlaps with the binding pockets of both the cofactor (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH4) and L-Trp when docked into the active site of TPH1.

the balance between enzyme activity enhancement and inhibition [48]. The affinities of synthetic corticosteroids for their cognate target, the glucocorticoid receptor, are in the nanomolar range [49,50], whereas the actions of beclomethasone dipropionate as an activator/stabilizer of TPH1 and TPH2, as described here, only become apparent at micromolar concentrations. Glucocorticoid receptor activation by corticosteroids has opposite effects on TPH2 activity by downregulating its mRNA expression and consequently decreasing central 5-HT synthesis [25]. Thus, the direct, stabilizing interactions with the TPHs probably only take place at the higher end of therapeutic dosages, which is also when psychiatric side effects are more likely to occur [10]. Corticosteroid use has been associated with depression [8], a disorder that has been putatively linked to reduced levels of 5-HT and thus would be in agreement with glucocorticoid receptor-mediated downregulation of TPH2 expression [25]. However, the most frequently reported corticosteroid-induced neuropsychiatric adverse effect is mania [8]. Interestingly, mania is also a known

side effect of serotonergic antidepressants [51,52] and could thus, potentially, correspond to the TPH2-stabilizing actions of corticosteroids reported here. It is apparent from the clinical literature referenced here that there is a great interindividual variability of the response (including side effects) to corticosteroid therapy, and the impact of corticosteroid treatment on central 5-HT may depend on a number of factors, including drug dosage and metabolic parameters.

In light of the many potentially undesirable effects of glucocorticoid receptor activation, including immunological and metabolic consequences and the opposing actions on TPH2 expression just described, it should be emphasized that we do not consider existing glucocorticoids as therapeutically viable stabilizers of TPH2 activity. Nevertheless, the present results suggest that steroid scaffolds may be useful starting points for the development of more potent and selective TPH2 stabilizers, which would be highly interesting as potential antidepressant drug candidates [26,27].

Conclusion

In summary, we have reported on the enhancing properties of five clinically used corticosteroids on two key enzymes for the biosynthesis of serotonin, TPH1 and TPH2, using an *in vitro* enzymatic assay. The compound with the strongest modulatory effect, beclomethasone dipropionate, activated TPH1 and TPH2 with micromolar potency and high selectivity over the other AAAHs, PAH and TH. Thermostability assays supported thermal stabilization of TPH as a possible mechanism of action. Computational docking studies were in agreement with the hypothesis that beclomethasone dipropionate interacts with the active site binding pocket of TPH1. Taken together, these data suggest that beclomethasone dipropionate may act as a pharmacological chaperone of TPH1. The serotonergic action of the corticosteroids described here may be relevant for understanding the mood elevation associated with administration of these drugs.

Future perspective

The finding that beclomethasone dipropionate activates TPH1 and TPH2 and shows selectivity over the other two AAAHs might inspire the design of future compounds with more specific and potent action as TPH2 stabilizers. Direct activators of brain serotonin synthesis represent a novel class of potential antidepressants and an attractive opportunity for medicinal chemists. However, no TPH2 activators have hitherto been described in the literature. Thus, we hope that the present report may stimulate future exploration of steroid scaffolds as pharmacological chaperones of the AAAHs. In future investigations, it will be important to determine the relevance of TPH stabilization and activation for the *in vivo* actions of glucocorticoids.

Summary points

- Corticosteroids are widely used in the clinic to modify immune-mediated responses and to substitute for endogenous adrenal production.
- The administration of corticosteroids is associated with mood elevation, increased satisfaction and optimism and a reduced sense of anxiety.
- Serotonin is implicated in development of several psychiatric conditions, including disturbances of mood and satiety.
- Corticosteroids are known to regulate the activity of tryptophan hydroxylases (TPH), key enzymes in serotonin biosynthesis, but the direct action of synthetic corticosteroids on the enzymatic activities of the TPHs has not been reported.
- Here we present the direct effects of five corticosteroids on the activities of two key enzymes for the biosynthesis of serotonin: TPH1 and TPH2.
- Beclomethasone dipropionate, the most efficacious of the tested compounds, increased the activity of both TPHs in micromolar range and exhibited selectivity over the other AAAHs.
- Thermostability assays suggested a stabilizing action of beclomethasone dipropionate, and docking studies support its binding to the active site.
- Steroid scaffolds may represent promising leads for developing TPH2 stabilizers as potential antidepressants in the future.

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Papers of special note have been highlighted as: • of interest; •• of considerable interest

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