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Interference of Boswellic Acids with the Ligand Binding Domain of the Glucocorticoid Receptor

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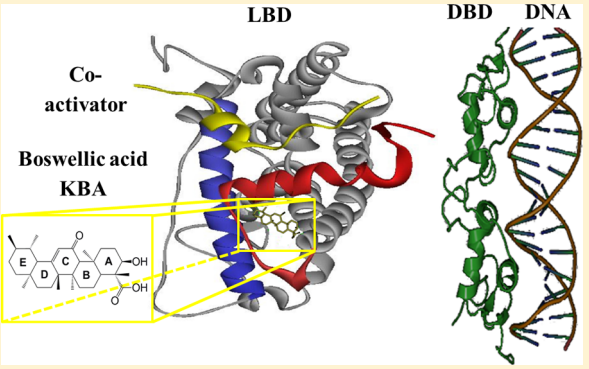
Supporting Information

ABSTRACT: Boswellic acids (BAs) possess anti-inflammatory properties in various biological models with similar features to those of glucocorticoids (GCs), such as suppression of the release of pro-inflammatory cytokines. Hence, the molecular mechanism of BAs responsible for their anti-inflammatory features might be attributable to interference with the human glucocorticoid receptor (GR). Due to obvious structural similarities with GCs, we conducted pharmacophore studies as well as molecular docking simulations of BAs as putative ligands at the ligand binding site (LBS) of the GR in distinct functional states. In order to verify receptor binding and functional activation of the GR by BAs, radiometric binding assays as well as GR response element-dependent luciferase reporter assay were performed with dexamethasone (DEX) as a functional positive control. With respect to the observed position of GCs in GR crystal complexes in the active antagonist state, BAs docked in a flipped orientation with estimated binding constants reflecting nanomolar affinities. For validation, DEX and other steroids were successfully redocked into their crystal poses in similar ranges as reported in the literature. In line with the pharmacophore and docking models, the BAs were strong GR binders (radiometric binding assay), albeit none of the BAs activated the GR in the reporter gene assay, when compared to the GC agonist DEX. The flipped scaffolds of all BAs dislodge the known C-11 function from its receiving amino acid (Asn564), which may explain the silencing effects of receptor-bound BAs in the reporter gene assay. Together, our results constitute a compelling example of rigid keys acting in an adaptable lock qualifying as a reversed induced fit mechanism, thereby extending the hitherto published knowledge about molecular target interactions of BAs.



INTRODUCTION

Boswellic acids (BAs) are pentacyclic triterpenes being pharmacologically active constituents of the oleo gum resin of the *Boswellia* species traditionally known as frankincense or olibanum. Büchele and Simmet have identified 12 different pentacyclic triterpenes including boswellic acids (BAs) in the resin of frankincense.[1] Over thousands of years, frankincense has been used in different cultures as a disinfectant, to refresh the air and combat unpleasant odors, and for ceremonial purposes. The resin has also been used as a valuable remedy to treat a variety of diseases.

During the latter part of the 20th century, the resin received scientific interest as an anti-inflammatory phytomedicine, and extracts of the resin have been applied to treat a variety of chronic inflammatory and autoimmune diseases.[2−4] In fact, numerous clinical studies have demonstrated the effectiveness of frankincense preparations in the treatment of asthma, inflammatory bowel diseases, cancer, and arthritis.[2,4] Pharmacological studies revealed inhibitory effects of frankincense extracts on the arachidonic acid cascade, i.e., synthesis of prostaglandins (inhibition of cyclooxygenase (COX) and microsomal prostaglandin E_2 synthase (mPGES)-1) and leukotrienes (inhibition of 5-lipoxygenase (5-LO)). Moreover, extracts of the resin and some of its active principles inhibited cytokine release from monocytes, macrophages, and lymphocytes, including the proinflammatory interleukins (IL-1, IL-2, IL-6) as well as INF-γ and TNF-α.[3,4]

The pentacyclic triterpenes BAs are believed to be the pharmacologically active key players of frankincense preparations, and there is accumulating evidence for anti-inflammatory and antitumorigenic effects of BAs in cellular and animal models.[4] The molecular mechanism of BAs responsible for these pharmacological effects has been attributed to signaling

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pathways including the NFκB route,[5] the MAPK pathway,[6] and Ca^{2+} signaling[7] as well as targeting of inflammatory related proteins such as 5-LO,[8] COX-1,[9] mPGES-1,[10] platelet-type 12-lipoxygenase (p12-LO),[11] cathepsin G,[12] and human leukocyte elastase (HLE;[13] see Table 1).[14]

Table 1. Possible Targets Directly or Indirectly Modulated by Boswellic Acids

type of BA	molecular target (inhibition)	references
β-BA, AβBA, KBA, AKBA	mPGES-1	10
AKBA	5-LO, TNF-α, MAPK, IκBα→ NFκB	15
AKBA	STAT-3	16
AKBA	VEGF-R2	17
AKBA	5-LO, 12-LO, COX-1, HLE	8, 9, 11, 13
β-BA, AβBA, KBA, AKBA	cathepsin G	12
AαBA, AβBA, AKBA	topoisomerase I and IIa	18
β-BA, KBA, AKBA	CYP 2C8/2C9/3A4	19
AαBA, AKBA	IKK α/β	5, 20

The clinical application of frankincense extracts widely coincides with that of glucocorticoids (GCs), and structural similarities between GCs and BA are obvious. This observation suggested that BAs could act as ligands of the glucocorticoid receptor (GR). The GR contributes to the immunosuppressive and anti-inflammatory effects of GC with distinct molecular mechanisms.[21−23] Thus, we attempted to align the structures of BAs with those of GCs. Our approach is derived from structure−activity relationships (SARs) that assume that structural similarities of drugs reflect a common molecular mechanism of action.[24]

The initial GR complex constitutes a ligand-activated transcription factor present in the cytoplasm in an inactive state.[25] Upon GC binding, the receptor complex undergoes rearrangements in the subunit composition and conformational changes occur, allowing the active receptor complex to expose its nuclear localization signal. In the subsequent translocation step, the GR with bound GC in its active state is transported into the nucleus where it binds as a homodimer to the GR response element (GRE) located in the promoter region of target genes and thereby specifically modulates the expression of these genes (transactivation).[26] On the other hand, transrepression (negative regulation of gene expression) is preceded by the recruitment of repressing cofactors.[25−28]

The GR belongs to the steroid hormone receptors, a subfamily of the nuclear receptors (NR) sharing a common protein fold with three layers of α-helices.[29] They also share a general DNA-binding domain, a hinge-region as a linker to the ligand binding domain (LBD) with its deeply buried hormone binding pocket, and a coregulator-binding site at its surface.[29] The size of the ligand cavity varies according to the size of the rigid steroidal and nonsteroidal ligands.[30,31] This behavior belongs to the general induced fit mechanism: upon ligand binding, the receptor undergoes conformational changes in an adaptive manner as an individual response to a given rigid ligand structure which in turn increases the binding affinity. In addition, the LBD embraces another adaptive region spanning helices H11 and H12 at the C-terminus of the LBD which is engaged in regulation of downstream signaling in a ligand-dependent manner.[32] Given this particular GR flexibility, the canonical classification of a GC ligand as either an agonist or an antagonist becomes illusive, as illustrated by mifepristone (RU-486), which shows downstream effects as both an agonist and a passive or active antagonist at the related progesterone receptor, a highly GR-related NR.[29,33] In contrast to the anti-inflammatory and immunosuppressive mechanisms of agonistic GCs, active (or passive) antagonism means that upon ligand binding, the otherwise dormant cytoplasmic GR moves (does not move) into the nucleus. There, according to its differential conformation which is a ligand- and coactivator/corepressor-dependent step, the ligand-occupied GR binds to the nuclear GRE with different degrees of affinity for specific transcriptional activities. These processes can then induce or repress downstream gene expression of pro- and anti-inflammatory enzymes and cytokines in the cells.[34]

With respect to their action on factors of the immune system, GCs as well as BAs exhibit inhibitory effects on the transcription factor NFκB and the pro-inflammatory cytokines INF-γ and TNF-α,[4,26] suggesting that BAs and GC act at a common target receptor, i.e., the GR. Since the GC-bound GR can regulate gene expression by physically interacting with NFκB (transrepression),[26] we investigated whether BAs can interact with the GR, supported by the structural similarities between GCs and BAs.

METHODS

Setup of Computational Docking Studies. Over 2000 poses in the LBD of the human GR were calculated with *Autodock* under *Autodock Tools* software (AD4 vers. 4.2, ADT vers. 1.5.2).[35,36] Prior to docking with the Lamarckian genetic algorithm (LGA), Gasteiger charges were added to the protein and the ligand, respectively.[37] To generate equal start conditions (for grid box evaluation), all ligands (KBA, βBA, αBA, DEX, deacylcortivazol (DAC), estradiol) and LBDs (PDB codes: 1NHZ, 1P93, 3H52, 3BQD, 1M2Z, 2B23, 3CLD, 2K22, 2K23, 3E7C) were subjected to an alignment procedure against DAC or its LBD.[29,31−33,38−43] Since the scaffolds of BAs and GCs are totally rigid with only a few rotatable bonds attached to them, the (default) setup values could be optimized: t-step 0.5 (2.0); translation step/Å q-step 15.0 (50.0); and quaternion step/deg d-step 15.0 (50.0). The side length of the evaluation cube was widened to 45 Å, which is 5 Å more than twice the length of the largest ligand (DAC) and was ligand-centered. Elitism was set to 3 in order to keep promising solutions alive. Other grid parameters are left as default values. For preparing the parameter files, we used the following LGA values: population size = 150; number of energy evaluations = 250 000 and 2 500 000; rate of gene mutation = 0.02; rate of crossover = 0.8; maximum number of generations = 27 000; number of runs = 256; and (unbiased) random positions were defined as start conformations. Clustering of docked results was done at 2.5 Å RMSD.

Computational Implications and Limitations. Our choice of software has been based on our experience and literature evidence.[35−37,43−49] The simulation problem is amenable to AD4 for the following nine reasons. In particular, well-documented improvements were achieved by the authors of AD3/4 for protein kinases, which are also driven by large hydrophobic/entropic forces.[50]

The AD4 study describes the pharmacophore features for GR docking: (1) The training set embraces all the types of interactions seen between GC and GR. (2) In principle, AD4 is calibrated to handle subtle energetic differences, that is, the interrelatedness of polar and nonpolar interactions (enthalpies vs. entropic contribution estimations). (3) The LBD has a

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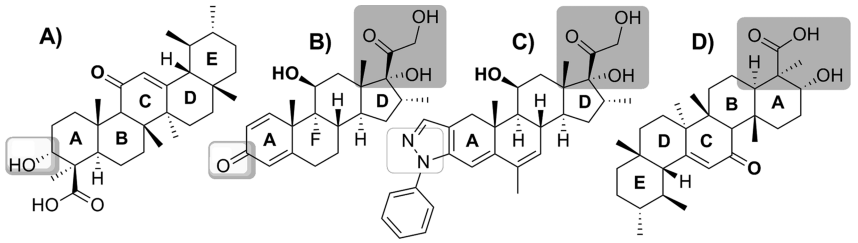

Figure 1. Molecular structures of 11-keto-β-boswellic acid (KBA) in canonical (A) and flipped (D) orientations. Dexamethasone (B) is a weaker GR binder than deacylcortivazol (DAC) (C) containing an additional *N*-phenyl heterocyclic ring fused at positions C-2 and C-3 of ring A. Docking results confirmed the SAR-predicted flipped pose of KBA (D) in the BS cavity with its additional pocket: ring E of BAs (D) or *N*-phenyl-pyrazole of DAC (C). The conventional or canonical superimposition (A, B, C) aligns the C-3 or C-11 hydroxyl groups with the C-3 or C-11 keto groups, respectively (A, B: light gray boxes and bold face). Only in the flipped binding mode (D) does KBA bind tightly (nanomolar K_m) to GR. The C-3 hydroxyl and C-4 carboxyl substituents on ring A of KBA overlap with the corresponding keto function on ring D of GCs (C, D: gray boxes).

pronounced hydrophobic area in a deeply buried pocket near the domain core. The nonpolar ligand scaffolds are decorated with few oxygen functionalities for hydrogen bonding. (4) AD4 is designed to evaluate binding affinities for chemically similar ligands to those found in its calibration set: here, we used camphor, which highly resembles GCs and BAs with their rigid hydrocarbon ring systems and few exocyclic oxygen atoms. (5) A further asset is the fact that the GCs are amenable to redocking for validation of AD4 based on their observed poses in published crystal complexes with GR in different activation states. (6) In principle, AD4 is biased in that richer scaffold decoration (very large ligands with many more functional groups) will end up showing stronger interactions (overestimation bias) in the same way that shallow binding depressions are not favored over deep grooves.[51] The latter is the case here. (7) The binding site of GR is a deeply buried groove in the LBD, so no surface effects (i.e., flaws through nonexisting residues) are produced. Upon inspection of the known LBD of NR, it was concluded that no explicit water is necessary for binding simulations.[29,31−33,38−42] (8) The resulting energetic parameters and quantities are relative values to permit comparison between ligands and are not absolute (physical) values (to be compared with experimental data). (9) The concept of rigid ligands and an adaptable GR presents another challenge because docking algorithms assume the reverse; i.e., the ligand changes conformation while the receptor backbone is held rigid (although sometimes side chains rotations are taken into account).

Different conformations of the NRs are induced by ligand binding. The ligands have rigid scaffolds and impose changes in receptor conformations leading to differential GR states in crystal complexes.[32,33] However, only the conformation of the ligand is allowed to change fully and freely, and typically only a limited number of selected side chain conformations can also vary, but not the receptor backbone.[35] As a practical workaround, rigid body docking was conducted with a large set of NRs with distinct state-dependent conformations.[33] The study was designed to evaluate all hitherto known GR states and to inspect other conformations, e.g., the C-terminal region of helices H11 and H12 of the GR and the estrogen receptor (ER) as well as the larger LBD cavity.[29,31−33,38]

RESULTS

Structure−Activity Analysis between Boswellic Acids and Glucocorticoids. We analyzed the theoretical interactions between the GR in different states with two types of ligands: (i) in complex with DEX[38] or DAC[31] and (ii) in complex with α- and β-series of BA[12] by means of computed docking simulations.[35,36,43]

Prior to docking, a formal SAR study (drawings on paper) suggested the flipped orientation of BAs, so that the C-3 hydroxyl group or the C-4 carboxyl decoration on ring A of the pentacyclic scaffold of BA can greatly overlap with the C20-keto function of the GC scaffold (see Figure 1). In this orientation, both scaffolds do not coincide with the cis/trans pattern between their rings but are aligned.

In addition, a meticulous inspection of the LBD[29,31,33,38] clearly shows a "bifacial" deeply buried cleft to accommodate "bifacial" ligands, that is, one-half of both the binding site (BS) and the ligand is pronouncedly hydrophobic whereas the other half is more hydrophilic in nature. The BS in the LBD of GR revealed a higher hydrophobicity around steroidal ring A, which is actually addressed by an additional bicyclic system attached to ring A of DAC, which displayed the highest receptor affinity of all investigated ligands.[32]

In the next step, we conducted a manual docking of BAs into the LBD of GR with VEGA ZZ.[43] Based on intuitive grounds, we gained insights into how both α- and β-series of BA can occupy this pocket through their dimethyl substituents only through the flipped orientation. At this stage, we also evaluated the influence of the reported extra space opened by side chain rotation of two amino acids (Gln570 and Arg611) in the DAC-bound GR in the active state.[31] Since pentacyclic BAs are of comparable size to DAC (extra rings), as compared to canonical DEX (tetracyclic steroid), we merged this extra space into all LBD for its use during fully automated docking.[33]

Docking of Boswellic Acids and Glucocorticoids into the GR. Despite the limitations of computational approaches,[24,48,49,52] SAR studies,[52] and docking,[51] our studies of BAs lend detailed insight into their putative binding modes when related to the crystal structures of complexes of GC with GR in different states.[29,31,33,38]

Docking conditions were validated by successful back-docking of DEX and DAC into their crystal structure poses.[31,38] During blind docking simulations, BAs behave as weak binders in a pose reflecting a canonical superimposition of KBA onto GCs, i.e., aligning the C-3 hydroxyl of KBA with the C-3 carbonyl of GC (see Figure 2). Scoring estimated a ∼1000-fold affinity gain of BAs under a flipped binding mode which is also the most populated cluster (over 90% at RMSD of 2.5 Å). The canonical pose cluster shows only a few hits. The scaffold of the flipped pose of KBA lies over the DAC structure in an almost perfect match: its plane tilts slightly (30°) on an imaginary axis spanning from ring A to E. Specifically, the

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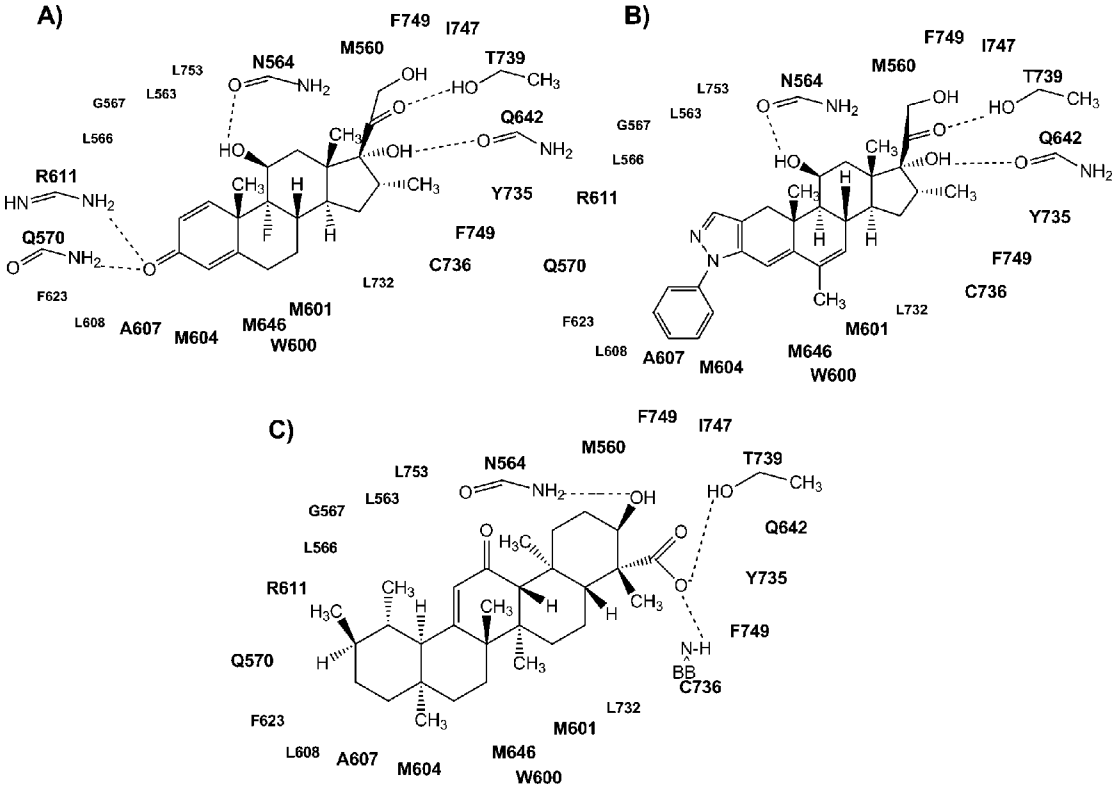

Figure 2. Binding site representation with the analysis of interacting residues with DEX (A), DAC (B), and fitted KBA (C). The ligand specific pattern of hydrogen bonding (dashed lines) is established by a few oxygen atoms and backbone or side chain hetero atoms. In good keeping with other NR complexes, water molecules do not mediate the lipophilic ligand binding at the extremely hydrophobic site.[29,31−33,38−43]

additional space required to accommodate the *N*-phenyl heterocyclic substitution of ring A on DAC in the binding site was largely filled by the methyl-substituted "extra" ring (E) of KBA. DAC has been shown to bind 40-fold more tightly to GR than DEX and 200-times more than the natural GR ligand cortisol.[31] Thanks to the successful back docking (for validation) of the strong binder DAC, the calculated strong binding of KBA in the flipped orientation may be viewed as highly trustworthy, also because the flipped KBA occupies the same position. The aforementioned extra space required in the binding groove is created by the amino acid rearrangements reported in detail by Suino-Powell et al.[31] The extra space is also the pivotal prerequisite for successful BA binding.

All BAs were docked into the ligand binding site of GR in the agonist state. The crystal complex of DAC and GR opens an additional pocket to accommodate even larger ligands than steroidal DEX (see Figures 1 and 2). The extra space is provided by side chain rearrangements (Arg611, Glu570).[31]

In one model, α-BA could not dock in the flipped mode because of Van-der-Waals repulsion of its β-methyl group at C-20 by the side chain of Leu608. The carboxyl and methyl groups at C-4 on ring A of BAs clash into residue Met604 despite such rearrangements.[31] In addition, the α-positioned methyl on C-20 collides with Gln642. In another model, α-BA binds under the condition of side chain rearrangements at the BS.

The Functionality of the C-11 Keto or Hydroxyl Groups on the Steroidal and Pentacyclic Scaffolds. In the flipped mode, the C-11 keto group of KBA is reorientated (30° tilted plane) and cannot contact Asn564, which is crucial for agonist signal transduction of GCs. Only the hydrogen bond donor hydroxyl group at C-11 of the GCs (see Figure 3)

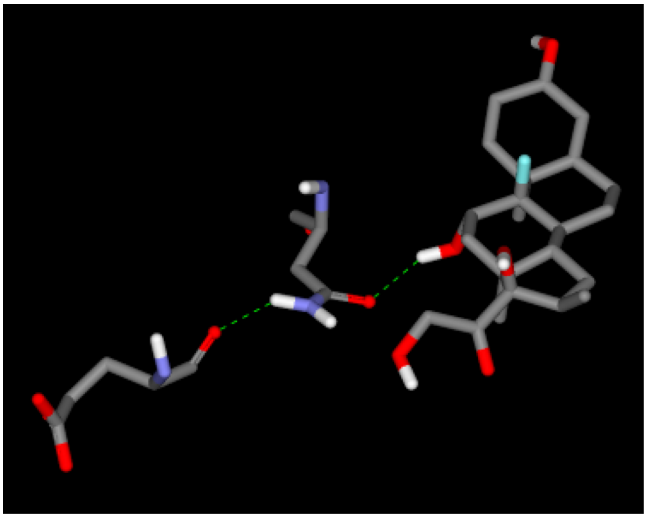

Figure 3. The signaling triad between triggering steroidal ligand with its C-11 hydroxyl group (right) forming a hydrogen-bond (green dashed lines) to Asn565 (center) and adjacent Glu748 (left) to induce the helical shift to further promote coactivator attachment to the nuclear transcription factor complex prior to gene segment reading. Note: Glu748 is positioned at the N-terminal kink of helix 12 (Pro750 to Glu760).

can attach to the acceptor carbonyl function of the amide side chain of Asn564. The keto group is not equivalent, because it is not a hydrogen-bond donor. In theory, the omega (terminal) functionality of the asparagine side undergoes amide−imidic acid tautomerism, but only the imidic acid tautomeric form of Asn564 (HO−C═NH) can form a hydrogen bond to the C-11

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keto group. Maybe, the imide acts as a hydrogen-bond donor and the C-11 keto group acts as its acceptor. However the amide tautomeric form of the Asn564 side chain (O═C−NH_2) is consolidated by another hydrogen bond between its terminal amino group (−NH_2) with Glu748, which lies at the *N*-terminal kink of helix 12 (ending in Glu760). In addition, the imidic tautomeric form has never been observed in polypeptide structures. As noted by Shimoni and Glusker, "In asparagine and glutamine side chains, there are two hydrogen bond donors in the same group, −NH_2, and a hydrogen bond acceptor, >C═O, which can accept one or two hydrogen bonds."[53]

The SAR study describes the following six pharmacophore features (see corresponding Figures 1D, 2C and 5): (1) Only in flipped poses do the C-3 hydroxyl and C-4 carboxyl substituents on ring A of BAs overlap with the relevant ketol function on ring D of GCs (see Figure 4). In this flipped pose, the carboxylic acid group of BAs is engaged in a hydrogen-bond network similar in strength and extension to that of GCs, regardless of the dissociation state. (2) BAs dissociate almost totally into monoanions (−COO^-) adopting a strongly polar hydrogen bond network, formally preserving docked poses of neutral BAs (−COOH). (3) The C-11 hydroxyl (bold face) on ring C of GCs is a known hydrogen-bond donor for triggering transactivation (see Figure 4).[31,33] (4) Modeling suggests that even 11-keto BAs cannot interact with Asn564—neither in the canonical nor in the flipped poses (see Figures 4 and 5). (5) This finding is supported by parallel docking of two natural GR binders based on literature observations of inactive cortisone (C-11 ketone) versus active cortisol hormone (C-11 hydroxyl group).[54] (6) The binding site at the LBD of GR possesses a more hydrophobic pocket around steroid ring A and an area with both hydrogen bond donor and acceptor residues to accommodate the more polar rings of steroids and A of BAs (see Figure 6, cf. Figures 2A and 2C).

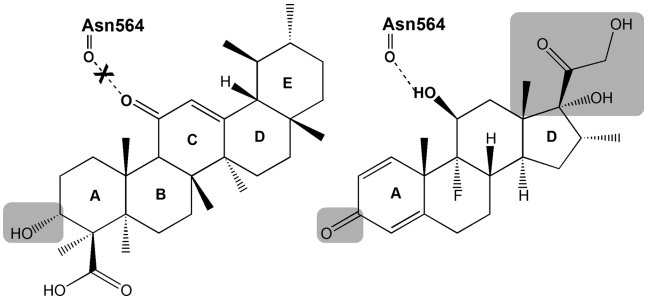

Figure 4. Display of the canonical alignment: pairwise superimposition of rings (A:A, B:B ... D:D) of KBA (left) and DEX (right). The boxes mark structural issues dealt with within the text.

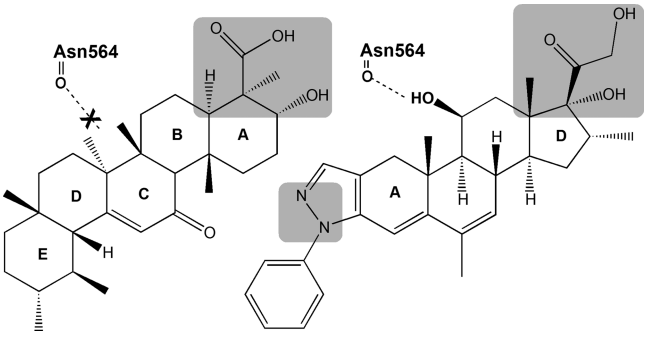

Figure 5. Schematic display of KBA and DAC. Docked KBA (left) in its flipped orientation can be compared to the orientation of the crystal structure of DAC (right). KBA contains an extra (fifth) ring which occupies the additional space of the *N*-phenyl heterocyclic ring fused at positions C-2 and C-3 of ring A of DAC.

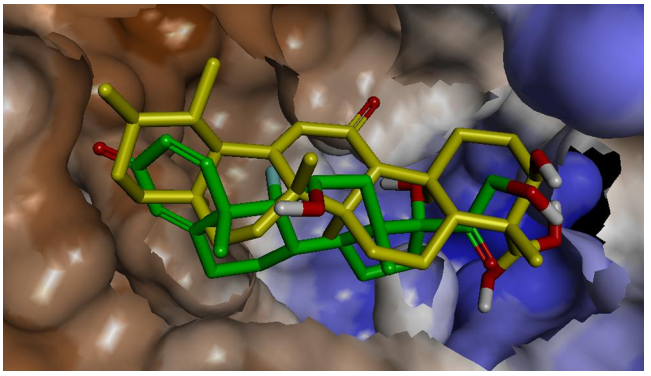

Figure 6. Computer-aided molecular modeling. Crystallographically observed binding mode of dexamethasone (green sticks) and the flipped docking pose of 11-keto β boswellic acid (yellow sticks) as strong binders to the ligand binding domain of the glucocorticoid receptor. The crystal pose (PDB code: 1M2Z) was regained through back-docking from random start positions in order to validate the blind docking of KBA and AKBA, in view of possible allosteric sites.[38,55] The shaded cavity surface of the ligand binding site is color-coded according to the side chain properties of hydrophilicity (bluish) or hydrophobicity (brownish). White/gray cavity surface color marks in between "neutral" zones. Red (light blue) colors mark the oxygen (flourine) atoms on the stick models. Hydrogen atoms are omitted for better viewing.

Functional Consequences of BA-GR Interactions. Given the structural similarities to GC, BAs may modulate their anti-inflammatory effects as functional ligands of the GR. It is most likely that BAs will bind as strongly as or even stronger than GCs to the GR, and the extra E ring will enhance binding affinities, as reported for DAC.

As an extension of our computer-generated models, we tested BAs in two biological assays, i.e., a GR competitive binding assay (cell-free) as well as in live cells using a GRE luciferase reporter gene assay (see Supporting Information, SI). Of interest, at rather low concentrations of BAs (10 and 100 nM), a weak binding/competition with DEX was evident, but this effect declined at higher concentrations (1−100 μM) for unknown reasons. This is in line with the failure of the BAs to induce the reporter gene activity in live cells at high concentrations (30 μM; see SI). Obviously, the structural analogy of the BAs and GCs did not suffice to fulfill the stringent structural requirements for full GR transactivation. However, this finding does not exclude a possible interference of BAs with nongenomic effects of the GR, which are also accepted as major mechanisms for the anti-inflammatory activity of GC (although still poorly understood).

All docking simulations demonstrated that the flipped orientation of the pentacyclic scaffold is the most likely pose (EDCB equivalent to ABCD systems). Docking solutions showed an over 90% preference for flipped poses as having the strongest affinities over other binding modes. In flipped poses, the oxygen decoration at the C-11 position on the pentacyclic scaffold did not interact with amino acid Asn564. The C-11 hydroxyl group, however, constitutes a pivotal pharmacophore feature for steroidal hormones and structurally related drugs

with agonistic action, e.g., inactive cortisone (C-11 keto function) versus active cortisol (C-11 hydroxyl group).

The present simulations also infer that the C-11 substituents do not influence the binding strength to the NRs. Estimated affinities showed that a ligand (estrogen) without an oxygen atom at C-11 is an equally strong binder as its theoretical congeners with keto or hydroxyl groups and compared to observed affinities from the PDB (data not shown).[32] Crystallography revealed that steroids bind in a remarkably uniform way in the highly conserved binding site of the LBD.[29,31−33,38,39]

A comparison between the crystal complexes of GC-GR and the computed complexes of BAs revealed that the C-11 substituent plays a signaling role only in GR binders with a steroid scaffold (GCs), but not with pentacyclic systems (BAs). Bardoxolone methyl or RTA 402, a new synthetic pentacyclic triterpene without an oxygen substitution at C-11, serves as a proof-of-principle for the biological role and binding strength of the oxygen group at C-11.[56]

In a more general view, the NR binders are extremely rigid and are chemically uniform while the NRs are notably flexible proteins, sharing a highly conserved binding site. Hence, ligand binding can induce conformational changes (e.g., at helix H11/loop/H12).[29,32] This alteration of structure leads to ligand-dependent gene regulation; i.e., the liganded NRs act as transcription factors that modulate expression of proteins in differential ways.[57,58] Depending on the nature of the ligand bound to the LBD, the pharmacological activity varies: agonism and active or passive antagonism (with or without transfer to nucleus) have all been experimentally demonstrated.[29,33] The same ligand can act in different ways, too: e.g., the activity of selective ER modulators (SERMs) can depend on the tissue or metabolic states. By changing protein expression, SERMs can act more as modulators than as mere blockers or stimulators.[59] The results of the present study suggest that the mechanistic behavior of BA−GR complexes resembles that of SERMs. Their agonistic and antagonistic estrogenic activities may depend on the cell types or the tissues in which they function. Moreover, our findings are consistent with reports that examined selective ER ligands with reduced activation of transcriptional activity in an estrogen response element (ERE)-luciferase assay relative to the natural ligand estradiol. Thus, the ligands strongly suppressed NFκB-dependent transcription of an NFκB-dependent luciferase reporter. On a theoretical molecular level, the study explains how the ER ligand binding contributes to the selective NFκB signaling, in contrast to the activation of the ERE-dependent transcription pathway.[32] ER ligands also displayed strong suppression of TNFα-induced inflammatory gene expression by suppression of an NFκB-dependent luciferase reporter in heterologous systems demonstrating the mechanistic similarities in hormone-receptor recognition for estradiol and cortisol.

Both natural and synthetic BAs may act as nonsteroidal dissociated GR modulators, some of which are under corporate and academic development.[56,60−62] The term "dissociated" refers to the proposed ability of a drug to uncouple negative side effects through selective gene activation (of anti-inflammatory cytokines) and repression (of proinflammatory cytokines).[33,63−67] Our experimental and modeled findings support a possible engagement of all BAs as direct GR modulators that favor transrepression over transactivation.[33] One may speculate that docked BAs may function as active agonists by inducing a new GR state (i.e., induced fit).[25,26,32,33,68−72]

GR ligands that promote the negative regulatory action of the receptor by reducing the positive regulatory function should result in improved therapeutic potential, i.e., fewer side effects than GC.[25] Transactivation and transrepression are two forms of differential upstream reading via the NR response elements, resulting in a ligand-specific expression of cytokines. For instance, the clinical picture of undesired side effects during GC treatment is ascribed to transactivation due to the presence of pro-inflammatory cytokines triggered by GCs as GR ligands.[73,74] BAs were shown to down-regulate inflammatory reactions by interfering with the activation of the NFκB family of transcription factors. In biochemical experiments, BAs directly inhibited IKK conveying inhibition to NFκB and subsequently down regulating of TNFα expression in human monocytes.[5] Also the GR, upon ligand binding, can regulate gene expression independently of GRE binding, by physically interacting with NFκB (transrepression).[26] It is conceivable that BAs could replace GCs at the BS of GR. They may induce conformational changes not only in the LBD but also in the DNA binding domain, if compared to GC-GR complexes. The distinct domain rotations allow for specific differences in the reading of gene segments by the response elements. The literature refers to transrepression under NFκB mediation. Clinically, treatment with BAs may result in a milder, better tolerated anti-inflammatory therapy, as witnessed during centuries of Indian traditional medicine (*Salai guggal*, an Aryuveda remedy). As active antagonists or modulators at the BS of GR, they may perform better for producing a more favorable "cocktail" of cytokines than seen with GCs.

■ CONCLUSIONS

The hypothesis that BAs may act on the classical GR-mediated transcription of GC-responsive genes was assessed in this study. Computed evidence was generated, which sheds light on the plausible binding modes of BAs in the cleft of the LBD of human GR. The molecular simulation suggested a flipped orientation of BAs rather than a canonical atomwise superposition between GCs and BAs. This formal 180° rotation of the pentacyclic scaffold allows the vicinal C-3 hydroxyl and C-4 carboxyl decorations on ring A to overlap with the C-20 keto function of GCs. The flipped scaffold of BAs follows the extended steroidal system of DAC and thereby occupies the same cavities at the binding site. In the flipped orientation, BAs constitute strong binders to the GR like DAC. Mechanistically, the flipped orientation turns the C-11 function of BAs (same position but 30° tilted scaffolds) away from Asn564. Since a hydrogen bond between the ligand and Asn564 cannot be established, the biological signal triggering is suppressed (active antagonism), all of which is reflected by the GR response element luciferase reporter assay with DEX as an agonistic positive control. In fact, we found no experimental evidence for a functional role of BAs on the GR-mediated gene expression, but still at low concentrations (10 and 100 nM) the competition with DEX at the GR was evident in a cell-free binding assay.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details of reporter and binding assays and their results and further discussion. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ABA, 3-*O*-acetyl-β-boswellic acid; AKBA, 3-*O*-acetyl-11-keto-β-boswellic acid; BA(s), boswellic acid(s); BS, binding site; DAC, deacylcortivazol (formerly: DAY); DEX, dexamethasone; ER, estrogen receptor; GC(s), glucocorticoid(s); GR, glucocorticoid receptor; GR-LBD, glucocorticoid receptor ligand-binding domain; GRE, glucocorticoid response element; KBA, 11-keto-β-boswellic acid; LBD, ligand binding domain; NCoR, nuclear receptor corepressor (3H52.pdb); NF-κB, nuclear factor κ-B; NR, nuclear receptor; SAR, structure−activity relationships; VDW, van der Waals contacts of atoms

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