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

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SUPPORTING INFORMATION

METHODS

Dual-luciferase glucocorticoid receptor response element reporter assay. A549 cells (2×10^4 cells in 100 μL RPMI 1640 medium supplemented with 2% FCS) were transfected with a mixture of an inducible GR-responsive firefly luciferase reporter construct and a constitutively expressing *Renilla* luciferase construct (100 ng) using the CignalTM GRE Reporter Assay Kit (SABiosciences corp., Frederick, MD, USA) and the transfection reagent SureFECTTM (SABiosciences corp., Frederick, MD, USA) according to the manufacturer's instructions. The constitutively expressing *Renilla* luciferase construct served as an internal control for normalizing transfection efficiency and monitoring cell viability. A negative control composed

of a non-inducible firefly luciferase reporter construct and a constitutively expressing *Renilla* luciferase construct (100 ng) was carried out for each sample to exclude unspecific effects or spontaneous reporter activity. After 24 h (37 °C, 6% CO_2), the medium was changed to RPMI 1640 medium supplemented with 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin and the cells were allowed to recover for 16 h. Successful transfection was checked by fluorescence microscopy, monitoring the positive control, which constitutively expressed green fluorescent protein (GFP) along with firefly and *Renilla* luciferases. Then, the cells were treated with the test substances or vehicle (DMSO) for 6 h (37 °C, 6% CO2) and the incubation was stopped by cell lysis. For passive cell lysis and differential determination of the luciferases' activities, a dual-luciferase reporter assay system (Promega GmbH, Mannheim, Germany) was used according to the manufacturer's instructions. Luciferase activity was measured in a luminometer (Victor3 plate reader, Perkin Elmer LAS GmbH, Rodgau-Jügesheim, Germany).

Radiometric binding assay to human GR alpha. The affinity of KBA and KBA were tested against purified human GR alpha at concentrations of 10 nM, 100 nM, 1 μM, 10 μM and 100 μM in duplicate in a 96 well plate format. For validation [^{3}H]-labeled DEX alone was tested and its values fell within acceptable range of known reference samples. Dilutions were obtained in 10 mM Tris-HCl pH7.4, 1.5 mM EDTA and 10% glycerol. DEX in a concentration of 20 nM was mixed with lysate in a total volume of 50 μL. Bound and free [^{3}H]-DEX was separated by the addition of a charcoal suspension (2% charcoal, 0.5% dextran made up in 10 mM Tris-HCl pH 7.4, 1 mM EDTA). Plates were centrifuged and 20 μL of the supernatant was added to 100 μL of microscint and luminescence was detected on a Packard Topcount plate reader.

RESULTS

Functional analysis of BA-GR interaction in live cells. The lung epithelial carcinoma cell line A549 was chosen to be transiently transfected with the reporter or control constructs, since the GR is highly involved in transcriptional signaling in this cell line. Three approaches were performed to control proper transfection and readout of the luciferase activity. The first approach (positive control) contained constitutively expressing firefly and *Renilla* luciferase constructs and a constitutively expressing GFP construct. The second approach (reporter approach) contained the inducible GR responsive firefly luciferase reporter construct and a constitutively expressing *Renilla* luciferase construct. The third approach (negative control) resembled the second one, but contained a non-inducible firefly luciferase reporter lacking the transcriptional response element. The first approach was used to assure the successful transfection of the cells, which was checked by fluorescence microscopy for GFP in the intact cells and by assessing the activity of constitutively expressed firefly and *Renilla* luciferases after lysis of the cells (ratio of firefly to *Renilla* luciferase activity 0.713 ± 0.0496). Only part of the cells that were observed under the light microscope (see Figure SI-1A) could also be visualized by fluorescence microscopy (see Figure SI-1B) and thus were successfully transfected.

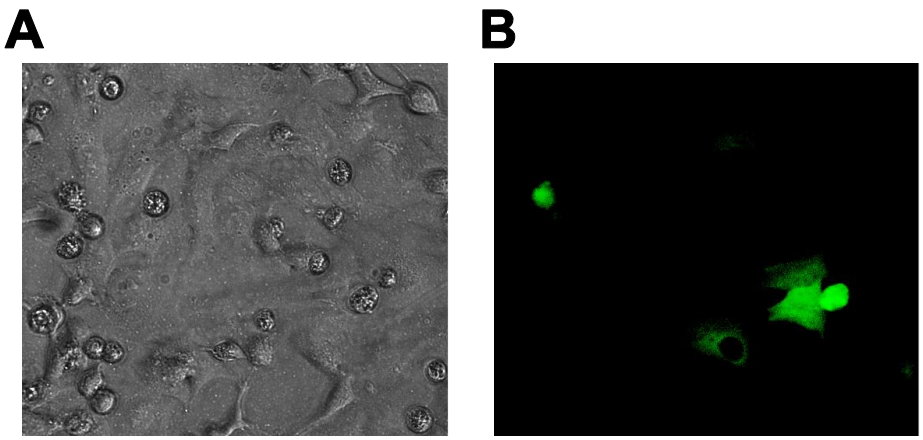

Figure SI-1. Expression of GFP in A549 cells after transfection with luciferase and GFP constructs. A549 cells were transfected with constructs constitutively expressing green fluorescent protein (GFP) and firefly and *Renilla* luciferases. After 24 h (37 °C, 6% CO2), medium was changed to RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin. After another 16 h, the same sector of cells was examined under a light microscope (**A**) or by fluorescence microscopy (**B**) with excitation at 470 nm and recording emission at 515 nm. Data are representative for at least three similar results.

After transfection and recovery of the cells, the test compounds were added and cells were further incubated for 6 h. For each reporter approach, one negative control was used containing the respective test compound to identify background reporter activity and unspecific effects that were not related to GR signaling. The incubation was stopped and the luciferases were released from the cells by passive cell lysis. The luciferases' activity was determined using a dual-luciferase reporter assay system. Constitutively expressed *Renilla* luciferase served as internal control to normalize fluctuations in transfection efficiency and effects of the test compounds on cell viability. As expected, dexamethasone significantly stimulated the relative luciferase activity about 7.5-fold (ratio of the reporter luciferase activity to the constitutive luciferase activity was related to the same ratio of the negative control) (see Figure SI-1). On the contrary, none of the BAs seemingly induced luciferase activity at a concentration of 30 μM. The neutral triterpene amyrin (30 μM) was ineffective in modulating the activity of the glucocorticoid receptor on the transcriptional response element as well.

Binding of BAs to the GR in a cell-free competition assay

In a competitive binding assay with DEX, AKBA and KBA displayed functional binding to GR-α at 10 and at 100 nM, but surprisingly at higher concentrations of the BAs (at 1, 10, and 100 μM) the binding was reversed (see Table SI-1). The failure of a GR-BA interaction at BA concentrations > 100 nM might be attributed to one or more epi-phenomena occurring when BAs and radio-labeled DEX are present together in the assay tube. Thus, the solubility of BAs is lower in aqueous solution than that of DEX (100 µM versus 1 mM, data not shown), and the critical micelle formation concentration (CMC) of structurally related natural substances lies in the lower μM range as reported in the literature, e.g., ginseng saponin´s CMC value falls within a range of 0.01 to 0.02 g / 100 mL,[75] which corresponds to a micromolar range assuming a molecular weight of 1000 D.[76] Hence, micelle formation cannot be the reason for the loss of potency of the BAs.

Table SI-1. Percentage inhibition values of BA compounds against human GR-alpha.

Concentration	AKBA	KBA	Dexamethasone
100 μM	19.1	32.1	--
10 μM	12.7	0.00	87.4
1 μM	5.62	2.20	85.6
100 nM	29.8	16.1	82.3
10 nM	20.5	13.2	48.1
1 nM	--	--	43.0

Table SI-1: Percentage inhibition values of compounds against human GR-alpha. The BAs were tested at the indicated concentrations in an assay containing 20 µM [3H]-dexamethasone, as described in Methods. Percentage values are an average of duplicate data points. The Z' value was 0.63 for this assay, thus passing our QC criteria of greater than 0.5.

The molar solubility of DEX and KBA was estimated in buffer at pH 7 as 10 vs. 50 µM (https://scifinder.cas.org/). The overall molecular lipophilicity of BAs, however, is much lower

than that of DEX (logP = 6 vs. 2; polar surface area 95 vs. 74 Å2). Despite their higher lipophilicity, the solubility of BAs is better than that of DEX and can be explained by the fact that BAs dissociates into carboxylate anions at physiological pH values. Hence, ceiling effects by limited solubility as reason for the differential potencies for GR interactions can also be discarded.

DISCUSSION

The anti-inflammatory effects of BAs might be attributed to interactions with multiple targets that require different effective concentrations. For instance, TNF-α production in LPS-stimulated human monocytes was suppressed at micromolar concentrations of AKBA.[5] Along these lines, AKBA acts as an inhibitor of NFκ-B activation in mice at 100 μmol/kg[77] and micromolar BA administration inhibited lymphocyte proliferation when triggered with LPS, phytohaemagglutinin, concanavalin and other mitogen-stimulating agents.[78] KBA and AKBA also interfere with pro-inflammatory lipid mediator formation involving 5-LO, COX-1 and mPGES-1 in the low micromolar range.[9,10] Together, the anti-inflammatory actions of BAs are a complex matter, where many targets may be involved with different mechanisms of action (see Table 1)[14].