Supporting Information Contents Page

Potential Antiosteoporotic Natural Products as Lead Compounds that Inhibit 17*β*-Hydroxysteroid Dehydrogenase Type 2

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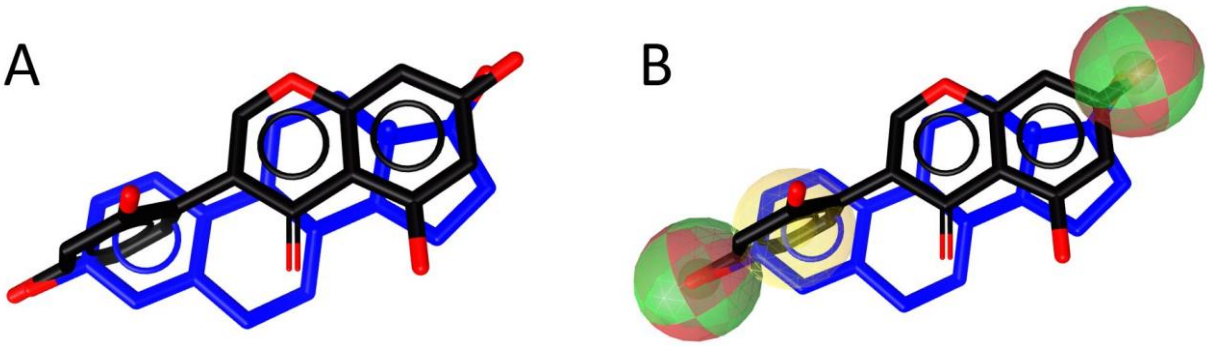
Contents:

Figure S1. Comparison of estradiol and 2'-hydroxygenistein



3D Alignment of estradiol (blue) and the flavonoid 2'-hydroxygenistein (black) (A). The aligned molecules share the positions of hydrogen bond donors and acceptors (green and red spheres) as well as a hydrophobic area (yellow sphere), which are essential for target recognition and biological activities (B). The alignment was generated by calculating a ligand-based, shared feature pharmacophore model in LigandScout 4.1 (inteligand GmbH, Vienna, Austria).

Virtual screening of databases

In total, the in-house natural product databases contained 439 compounds, whereas the Sigma-Aldrich database consisted of over 67 000 (mostly synthetic) molecules. The databases, their size, and the virtual screening hits from each database are given in Table S1. Because the 17β-HSD2 inhibitor pharmacophore models were initially based on the size and features of synthetic chemicals, they were rather restrictive towards natural compounds that can differ from the so-called

drug-like synthetic chemicals. Therefore, for all screenings except for the Sigma-Aldrich database, one omitted feature was allowed.

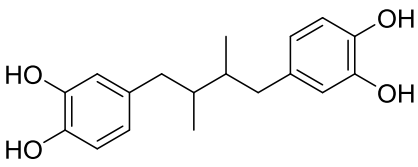
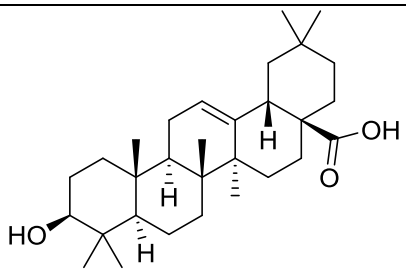
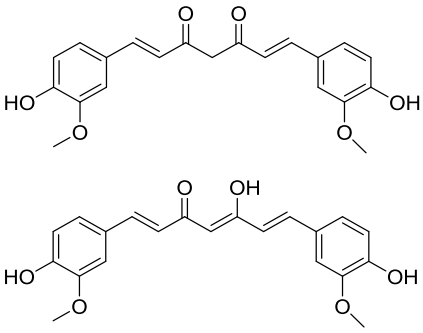
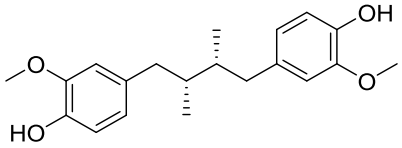
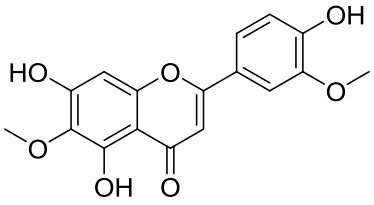
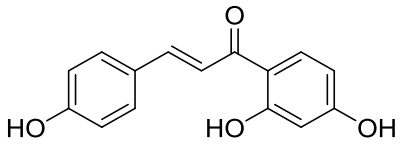
Table S1. Databases Used for Virtual Screening and the Number of Hits Obtained

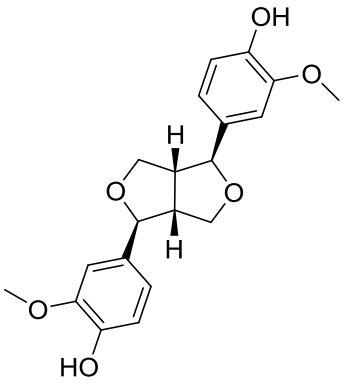
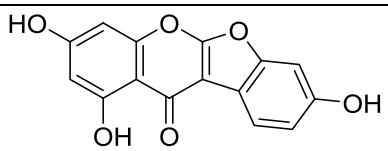
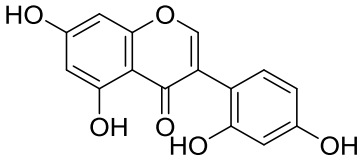
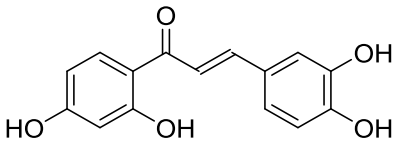
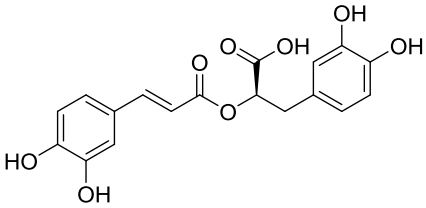
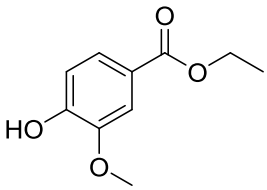
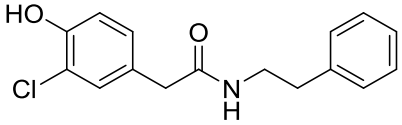
database	organization	entries	hits model 1 without / with one omitted feature	hits model 2 without / with one omitted feature	tested compounds
Davis	Griffith University	352	10 / 64	17 / 79	18
Atanasov	University of Vienna	51	1 / 13	0 / 11	12
Krenn	University of Vienna	13	0 / 0	0 / 3	2
Sigma	Sigma-Aldrich	67 748	120 / not screened	not screened	3
Waltenberger	University of Innsbruck	23	0 / 12	2 / 9	1

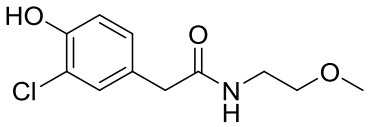
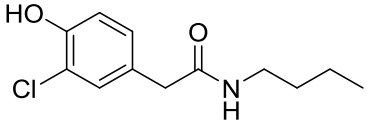
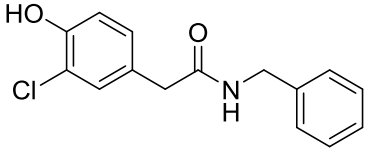
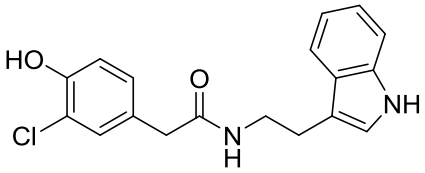
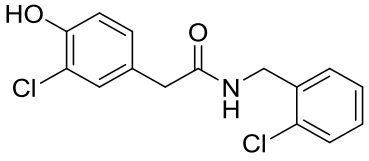
For the final selection of the test compounds, especially the availability and analytical quality of the hits was taken into account. Among synthetic compounds, the Sigma-Aldrich hit list contained only few natural products, and the focus was set on them. Only three compounds not contained in the other databases were selected for biological testing.

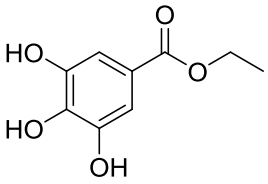
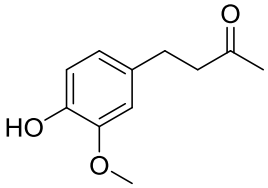
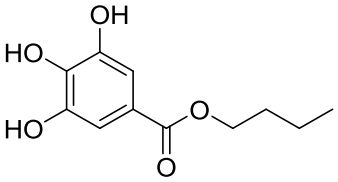
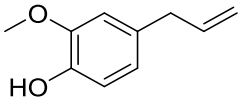
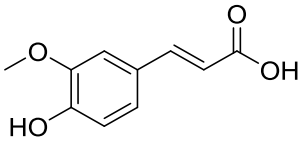
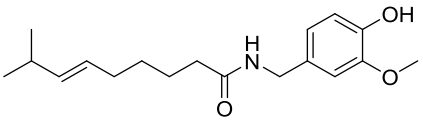
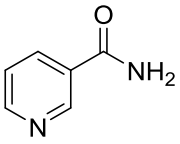
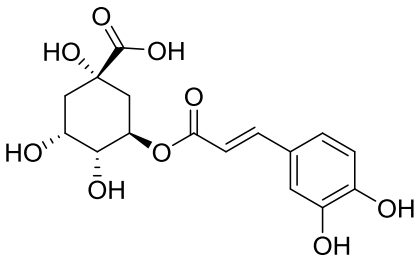
Table S2. All Selected Compounds and Their Activities

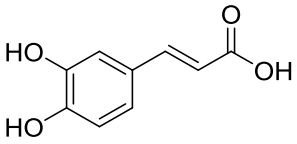
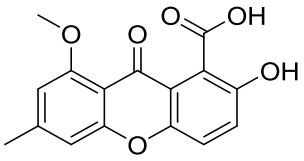
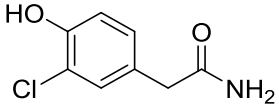
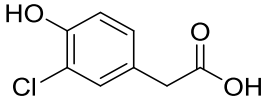
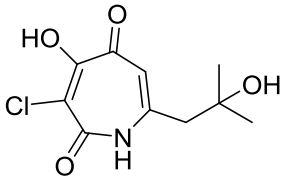
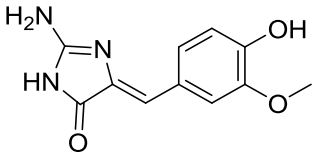
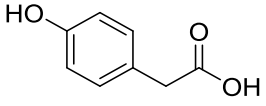
compound	structure	database	remaining activity at 20 µM (% of control)	IC_{50}

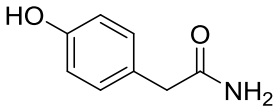
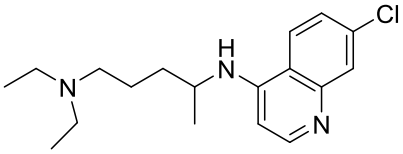
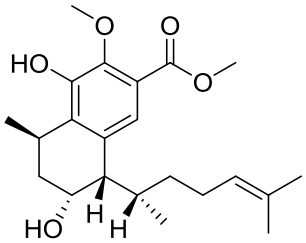
nordihydroguaiaretic acid (**1**)		Atanasov		0.38 ± 0.04 µM
oleanolic acid (**2**)		Atanasov	49 ± 6%	
curcumin (**3**)		Atanasov		1.73 ± 0.2 µM
(-)-dihydro-guaiaretic acid (**4**)		Davis		0.94 ± 0.02 µM
jaceosidin (**5**)		Davis		9.3 ± 2.3 µM
isoliquiritigenin (**6**)		Davis		0.36 ± 0.08 µM

pinoresinol (**7**)		Walten-berger	42 ± 5%	
lupinalbin A (**8**)		Krenn		1.52 ± 0.15 µM
2'-hydroxy-genistein (**9**)		Krenn		2.03 ± 0.37 µM
butein (**10**)		Sigma		7.3 ± 2.70 µM
rosmarinic acid (**11**)		Sigma		3.72 ± 0.17 µM
ethyl vanillate (**12**)		Sigma		1.28 ± 0.26 µM
2-(3-chloro-4-hydroxyphenyl)-*N*-		Davis		1.57 ± 0.16 µM

phenethylacetamide (**13**)				
2-(3-chloro-4-hydroxyphenyl)-*N*-(2-methoxyethyl) acetamide (**14**)		Davis	37 ± 3%	
N-butyl-2-(3-chloro-4-hydroxyphenyl) acetamide (**15**)		Davis	33 ± 6%	
N-benzyl-2-(3-chloro-4-hydroxyphenyl) acetamide (**16**)		Davis		3.42 ± 0.74 µM
N-(2-(1H-indol-3-yl)ethyl)-2-(3-chloro-4-hydroxyphenyl) acetamide (**17**)		Davis		0.98 ± 0.24 µM
2-(3-chloro-4-hydroxyphenyl)-*N*-(2-chlorobenzyl) acetamide (**18**)		Davis		0.78 ± 0.16 µM

ethyl gallate (**S1**)		Atanasov	60 ± 6%	
vanillyl acetone (**S2**)		Atanasov	106 ± 9%	
butyl gallate (**S3**)		Atanasov	56 ± 5%	
eugenol (**S4**)		Atanasov	94 ± 11%	
ferulic acid (**S5**)		Atanasov	79 ± 10%	
capsaicin (**S6**)		Atanasov	53 ± 6%	
nicotinamide (**S7**)		Atanasov	106 ± 12%	
chlorogenic acid (**S8**)		Atanasov	97 ± 10%	

caffeic acid (**S9**)		Atanasov	58 ± 13%	
2-hydroxy-8-methoxy-6-methyl-9-oxo-9H-xanthene-1-carboxylic acid (**S10**)		Davis	64 ± 5%	
2-(3-chloro-4-hydroxyphenyl) acetamide (**S11**)		Davis	80 ± 10%	
2-(3-chloro-4-hydroxyphenyl) acetic acid (**S12**)		Davis	88 ± 7%	
pestalactam A (**S13**)		Davis	85 ± 11%	
polyandrocarpamine A (**S14**)		Davis	101 ± 7%	
2-(4-hydroxyphenyl) acetic acid (**S15**)		Davis	108 ± 5%	

2-(4-hydroxyphenyl) acetamide (**S16**)		Davis	110 ± 10%	
(±)-chloroquine (**S17**)		Davis	111 ± 13%	
methyl (5*R*,7*R*,8*S*)-4,7-dihydroxy-3-methoxy-5-methyl-8-((*S*)-6-methylhept-5-en-2-yl)-5,6,7,8-tetrahydronaphthalene-2-carboxylate (**S18**)		Davis	59 ± 7%	

Discussion on the hit curcumin

Curcumin is a compound that has previously been recognized to interfere with AlphaScreen-based high throughput assays in protein-protein-interaction inhibitor studies and is therefore classified as a so-called PAINS.[1-3] Several studies reported the presence of PAINS also amongst natural products, which definitely needs to be considered in in vitro studies. [1-3] However, 87 approved and clinically useful drugs bear chemical structures typical for PAINS,[4] amongst them many antifungals. Just because of their possible interference with in vitro activity detection, these drugs can still be of clinical value. There is currently a lively ongoing discussion among scientist to which

extent these PAINS should be excluded from further research. Much more data is needed to finally provide directions for the use of PAINS filters[4].

Besides these general aspects of PAINS, there are of course many arguments about curcumin itself as a hit. We are aware of the compound's many reported bioactivities observed in in vitro studies and additionally of its very poor bioavailability. However, this study is a proof of concept study identifying many natural products inhibiting 17β-HSD2 when there is direct access to the binding site and we categorize curcumin as one of them. Why? A very recent analysis of curcumin data suggested 6 criteria to distinguish unspecific effects by the compound from specific, target-based activity.[5] We had a look at these criteria and address them point-by-point:

Criterion 1. Look for evidence of compound stability in assay buffer/media, including when molecular models are invoked as supporting evidence of target engagement.

In our test system, curcumin seemed to be stable. We observed no significant changes in inhibition in the experimental repetition also when the incubation time was prolonged. Actually, the assay duration is only 10 minutes and the compound has direct access to the binding site when using cell lysates. Additionally, IC_{50} determinations in weeks 1 and 3 of the experiments yielded the same values. Therefore, also thawing-freezing cycles did not seem to affect the stability of curcumin.

Criterion 2. Look for the presence of detergent and thiol-scavenging reagents in biochemical assays to mitigate the impact of chemical aggregation and nonspecific thiol reactivity. Are/were any additional counterscreens performed to rule out these phenomena?

We added 0.1% Triton-X100 to the reaction mixture as suggested by McGovern et al.[6] The calculated IC_{50} values were comparable with those obtained in the absence of the detergent. Thiol reactivity would alter the enzymes activities. Also this has not been observed.

Criterion 3. Examine the selectivity data. What are the magnitudes of any observed selectivity? Are these significant? Can any selectivity be explained by differential target susceptibilities to nonspecific interference modalities like thiol reactivity? Can any apparent selectivity be explained by the assay conditions, such as target or total protein concentration?

Both assays on 17β-HSD2 and 17β-HSD1 were performed under the same conditions. Only: the lysates were diluted a bit differently by addition of more or less buffer. However, one would rather expect stronger inhibition in the more diluted lysates because of lower unspecific binding of curcumin if this were the case. On the contrary, 17β-HSD2 was inhibited more potently in the more diluted lysate. So actually, the inhibitory potency difference would be expected to be even larger if it was unspecific.

Criterion 4. Examine the potency of the compound. At those concentrations, would there be any expected aggregation or off-target effects? And if so, can one make meaningful conclusions about specific pathways and target engagement? Does the stoichiometry make sense?

As mentioned above, no aggregation effects were observed. The assays are specifically measuring 17β-HSD1 and 17β-HSD2 inhibition and were conducted under very similar conditions. Off-target effects are not to be expected in these assays.

Criterion 5. Evaluate the methods to confirm target engagement. Look for biophysical orthogonal methods for support of target engagement (e.g., SPR, ITC, CETSA), not solely phenotypic assays.

Does not apply, because we did not use phenotypic assays.

Criterion 6. Carefully examine the detection method for determining the concentration of curcumin present in an assay. What biophysical method is/was used for detection? Can likely degradation products or metabolites have a similar response and/or explain the data/hypothesis?

Because HEK cells don't express drug-metabolizing CYPs, they are hardly metabolically active. Additionally, in our assays, we have to add the respective cofactor for enzyme activity. Untransfected HEK lysates did not show any activity. Inhibition was not altered by different incubation times, there were no color changes and also no precipitation before, during or after the assay. For all these reasons, a direct inhibition of the enzymes by the unmetabolized test compounds can be expected.

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