

Helenalin Acetate, a Natural Sesquiterpene Lactone with Anti-inflammatory and Anti-cancer Activity, Disrupts the Cooperation of CCAAT Box/Enhancer-binding Protein β (C/EBP β) and Co-activator p300*

Received for publication, July 13, 2016, and in revised form, October 27, 2016 Published, JBC Papers in Press, November 1, 2016, DOI 10.1074/jbc.M116.748129

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Edited by Joel Gottesfeld

Recent work has demonstrated pro-oncogenic functions of the transcription factor CCAAT box/enhancer-binding protein β (C/EBP β) in various tumors, implicating C/EBP β as an interesting target for the development of small-molecule inhibitors. We have previously discovered that the sesquiterpene lactone helenalin acetate, a natural compound known to inhibit NF- κ B, is a potent C/EBP β inhibitor. We have now examined the inhibitory mechanism of helenalin acetate in more detail. We demonstrate that helenalin acetate is a significantly more potent inhibitor of C/EBP β than of NF- κ B. Our work shows that helenalin acetate inhibits C/EBP β by binding to the N-terminal part of C/EBP β , thereby disrupting the cooperation of C/EBP β with the co-activator p300. C/EBP β is expressed in several isoforms from alternative translational start codons. We have previously demonstrated that helenalin acetate selectively inhibits only the full-length (liver-enriched activating protein* (LAP*)) isoform but not the slightly shorter (LAP) isoform. Consistent with this, helenalin acetate binds to the LAP* but not to the LAP isoform, explaining why its inhibitory activity is selective for LAP*. Although helenalin acetate contains reactive groups that are able to interact covalently with cysteine residues, as exemplified by its effect on NF- κ B, the inhibition of C/EBP β by helenalin acetate is not due to irreversible reaction with cysteine residues of C/EBP β . In summary, helenalin acetate is the first highly active small-molecule C/EBP β inhibitor that inhibits C/EBP β by a direct binding mechanism. Its selectivity for the LAP* isoform also makes helenalin acetate an interesting tool to dissect the functions of the LAP* and LAP isoforms.

The CCAAT box/enhancer-binding protein β (C/EBP β),² a highly conserved member of the family of basic-region leucine

zipper transcription factors, is involved in the control of differentiation and proliferation of various cell types, including adipocytes, keratinocytes, mammary epithelial cells, and myelomonocytic cells (1, 2). In accordance with its multiple roles, the activity of C/EBP β is highly regulated on the transcriptional as well as post-transcriptional levels. Based on alternative translation initiation, the C/EBP β mRNA is translated into several isoforms of C/EBP β , two of which (LAP* and LAP) function as transcriptional activators, whereas the third isoform (liver-enriched inhibitory protein (LIP)) is a transcriptional inhibitor (3–5). The full-length isoform of C/EBP β (LAP*) and the slightly shorter LAP isoform differ by only 21 N-terminal amino acids, whereas LIP lacks most of the N-terminal sequences that harbor the transactivation domain. The relative expression levels of the different isoforms are controlled by short upstream open reading frames, and their deregulation has been implicated in tumor development, for example of breast cancer (5–7). In addition to the differential isoform expression, C/EBP β is regulated by various post-translational modifications, including phosphorylation (8–10), acetylation (11–13), methylation (14, 15), and sumoylation (16).

Recent work has shown that deregulation of C/EBP β plays a crucial role in the development of certain tumors. Increased expression of C/EBP β has been demonstrated in tumors of the colon, kidney, stomach, prostate, and the ovaries (6, 17–19). In many cases, high C/EBP β expression correlates with increased malignancy and invasiveness of the tumor cells. In glioblastoma, high C/EBP β expression correlates with a poor prognosis (20–22) and, together with STAT3, plays a key role in establishing a mesenchymal gene expression signature that is responsible for the aggressiveness of high-grade glioblastomas (23). In the hematopoietic system, C/EBP β plays pro-oncogenic roles in anaplastic lymphoma kinase (ALK)-positive anaplastic large cell lymphoma (ALCL) (24, 25), where C/EBP β has been identified as a crucial downstream target of oncogenic anaplastic lymphoma kinase signaling. Finally, our own work has recently suggested a pro-oncogenic role of C/EBP β , possibly in conjunction with c-Myb, in acute myeloid leukemia (26).

* This work was supported by the Deutsche Krebshilfe. This work was also supported by a fellowship from the Graduate School of Chemistry at the University of Münster (GSC-MS) (to S. M. H.). The authors declare that they have no conflicts of interest with the contents of this article.

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² The abbreviations used are: C/EBP β , CCAAT box/enhancer-binding protein β ; MST, microscale thermophoresis; BMCC-Biotin, 1-biotinamido-4-[4'-(maleimidomethyl) cyclohexanecarboxamido]butane; p, phosphorylated;

LAP, liver-enriched activating protein; LIP, liver-enriched inhibitory protein.

C/EBP β is therefore emerging as an interesting target for the development of small-molecule inhibitors.

We have recently identified the sesquiterpene lactone helenalin acetate, a natural compound from *Arnica montana*, as the first highly active small-molecule inhibitor of C/EBP β (26). The initial characterization of its inhibitory activity has shown that helenalin acetate inhibits the LAP* isoform but not the LAP isoform of C/EBP β or the related transcription factor C/EBP α , and that helenalin acetate exerts anti-proliferative effects in acute myeloid leukemia cells but not in normal hematopoietic progenitor cells. Overall, these findings have suggested that small-molecule C/EBP β inhibitors might have therapeutic potential. Here, we have examined the inhibitory mechanism of helenalin acetate in more detail. We show that helenalin acetate binds to the N-terminal domain of C/EBP β and thereby disrupts its cooperation with the co-activator p300. Interestingly, helenalin acetate does not bind to the LAP isoform, explaining why it is not inhibited by helenalin acetate.

Results

Helenalin Acetate Is a Potent Small-molecule Inhibitor of C/EBP β —Helenalin and its acetate ester helenalin acetate have anti-inflammatory activities and were previously shown to inhibit the transcription factor NF- κ B, a key regulator of inflammation (27, 28). It is therefore thought that the anti-inflammatory activities of helenalin and its derivatives are due to their ability to inhibit the activity of NF- κ B. We have recently identified helenalin acetate as the first highly potent small-molecule inhibitor of C/EBP β (26). That helenalin acetate targets NF- κ B as well as C/EBP β prompted us to compare its inhibitory activity for both transcription factors, in particular because C/EBP β has also been implicated in inflammatory processes (29). Because our previous work has focused on chicken C/EBP β , we first assessed the effect of helenalin acetate on human C/EBP β , using reporter experiments with an expression vector for human C/EBP β and a C/EBP β -responsive luciferase reporter gene. Similar to the inhibition of chicken C/EBP β , submicromolar concentrations of helenalin acetate strongly inhibited the activity of human C/EBP β (Fig. 1A). Western blotting confirmed that the inhibition was not caused by decreased amounts of C/EBP β . To monitor the activity of NF- κ B, we measured the TNF α -dependent stimulation of a luciferase reporter gene that contains several NF- κ B sites (Fig. 1B). Helenalin and helenalin acetate showed partial inhibitory effects at concentrations of 4 μ M. We estimated the EC₅₀ concentrations for the inhibition of NF- κ B to lie between 4 and 5 μ M for both compounds, in accordance with published data (30). These experiments show that the concentrations necessary to inhibit the two transcription factors differ by a factor of 10 or more, clearly demonstrating that helenalin acetate is a significantly more potent inhibitor of C/EBP β than of NF- κ B.

We also examined the effect of helenalin acetate on the conversion of 3T3-L1 pre-adipocytes into fat cells as a well characterized model of a physiological C/EBP β -dependent differentiation process (31, 32). A number of studies have established that C/EBP β plays a key role in the initiation of the 3T3-L1 adipocyte differentiation program by inducing a cascade of gene activations, leading to fat cells, which are characterized by

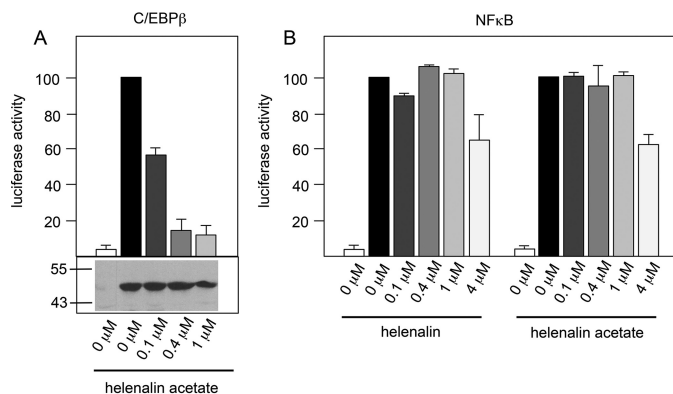


FIGURE 1. Helenalin acetate is a more potent inhibitor of C/EBP β than of NF- κ B. A, QT6 fibroblasts were transfected with the C/EBP-inducible luciferase reporter gene p-240luc, pCMV β , and expression vectors for human C/EBP β . Cells were treated for 12 h with the indicated concentrations of helenalin acetate and then analyzed for luciferase activity. The columns show the average luciferase activity normalized against the β -galactosidase activity. Error bars show the S.D. Aliquots of the cell extracts were used to visualize the amounts of C/EBP β by Western blotting (bottom panel). Numbers on the left side refer to molecular weight markers. B, Hek293T cells transfected with the NF- κ B-dependent luciferase reporter gene pGL2-6 \times NF κ B-luc and pCMV β were treated with 10 μ g/ml TNF α and the indicated concentrations of helenalin acetate or helenalin. Cells were analyzed as in panel A.

the appearance of lipid droplets in the cytoplasm. In the light microscope, these droplets appear as bright structures in the cytoplasm (Fig. 2A, top panels). Fig. 2A shows that 0.1 μ M helenalin acetate already inhibited the differentiation significantly, whereas 1 μ M resulted in virtually complete inhibition, as assessed by microscopic inspection of the cells and by staining with the lipid dye Oil-Red-O. This result is in accord with helenalin acetate being a potent inhibitor of C/EBP β . Fig. 2B shows differentiated cells at higher magnification, demonstrating the presence of lipid drops in the cytoplasm, which appear as bright vesicles. As shown by the Western blots in Figs. 1A and 2C, the inhibition of C/EBP β by helenalin acetate was not accompanied by a decrease of the amount of C/EBP β , indicating that the activity and not the expression of C/EBP β is inhibited.

The Presence of Reactive Groups Is Not Sufficient to Explain the Inhibitory Activity of Helenalin Acetate on C/EBP β —To gain insight into the structural features of helenalin acetate that contribute to its inhibitory activity, we first examined the activity of helenalin and several ester derivatives of helenalin. As a read-out of C/EBP β activity, we analyzed the expression of the endogenous C/EBP-inducible MRP126 gene by Northern blotting, using S17 mRNA as a loading control. The MRP126 gene is not expressed in fibroblasts; however, its expression is induced when the cells are transfected with an expression vector for C/EBP β (10, 26). Analysis of the increase of MRP126 mRNA levels caused by C/EBP β expression is therefore a convenient method to assess the activity of C/EBP β . In Fig. 3A, the induction of MRP126 expression by C/EBP β is evident by comparing MRP126 expression in the first and last lane. The other lanes in Fig. 3A illustrate the inhibitory effects of helenalin and different helenalin esters on C/EBP β activity. Helenalin acetate was the most potent C/EBP β inhibitor of these compounds. Helenalin was only slightly less active, whereas the inhibitory activities of helenalin isovalerate and helenalin 2- and 3-methyl butyrates were significantly lower. Notably, the amount of C/EBP β

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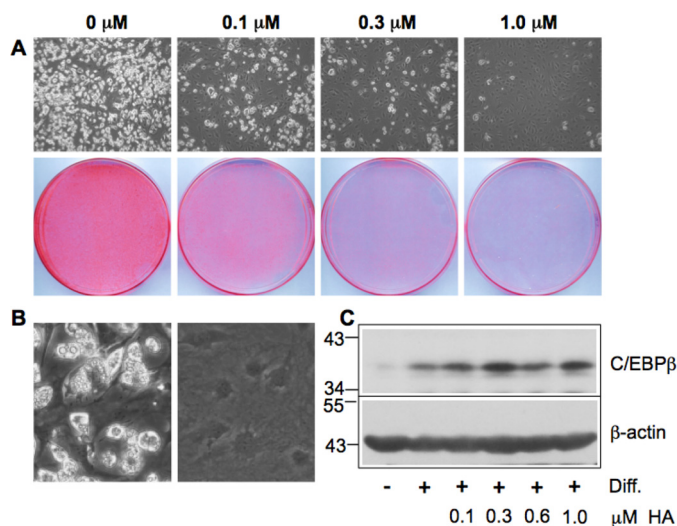


FIGURE 2. Helenalin acetate inhibits adipocyte differentiation. A, 3T3-L1 cells were induced to differentiate for 7 days in the absence or presence of the indicated concentrations of helenalin acetate. The *top panels* show microscopic pictures of the cells at low magnification with differentiated cells appearing white due to the presence of lipid drops in the cytoplasm. The *bottom panels* show staining of the cells with Oil-Red O. The same number of cells was plated in each case. B, microscopic pictures of differentiated and undifferentiated 3T3-L1 cells at higher magnification. C, Western blotting analysis of C/EBP β and β -actin expression in undifferentiated (–) and differentiated (+) 3T3-L1 cells treated with the indicated concentrations of helenalin acetate. Numbers on the left refer to molecular weight markers. Diff., differentiation.

(shown in Fig. 3A, *bottom panel*) was not significantly affected by any of the compounds.

Helenalin acetate contains two α,β -unsaturated carbonyl groups that are highlighted in Fig. 3B. These electrophilic groups are known to undergo Michael-type addition reactions with nucleophiles, such as the thiol groups of cysteine residues, leading to the formation of covalent adducts (33, 34). For example, helenalin and helenalin acetate have been shown to inhibit the DNA binding activity of transcription factor NF- κ B by covalent modification of Cys-38 of the p65 subunit of NF- κ B (27, 28). To address the relevance of these reactive groups for the inhibition of C/EBP β , we examined the inhibitory potential of two related sesquiterpene lactones, chamissonolide and 11 α ,13-dihydrohelenalin acetate, each of which lack one of the reactive groups of helenalin acetate, as shown by the comparison of their structures. Both compounds were virtually inactive when compared with helenalin acetate (Fig. 3B), strongly suggesting that the inhibitory activity of helenalin acetate might depend on the presence of both reactive groups. However, that the presence of the reactive groups alone cannot explain the inhibitory activity of helenalin acetate is demonstrated by the fact that even high concentrations (up to 10 μ M) of cyclopentene-1-one and α -methylene- γ -butyrolactone, each of which corresponds to one of the reactive partial structures of helenalin acetate, had no inhibitory effect at all (Fig. 3B). Because of the presence of α,β -unsaturated carbonyl groups, both compounds have an inherent ability to react with nucleophilic groups to undergo Michael-type addition reactions, well known in organic chemistry. We also combined both compounds to mimic helenalin acetate, but even at the highest concentration (10 μ M each), no inhibition was observed (data not

shown). Thus, it appeared that the specific spatial structure of helenalin acetate and the correct relative orientation of the two enone groups are important for its inhibitory activity and that the presence of these reactive groups alone cannot account for its ability to inhibit the activity of C/EBP β . This is also supported by the comparison of the inhibitory activity of helenalin and mexicanin-I. We have previously shown that the inhibitory activity of helenalin is significantly higher than that of mexicanin-I (26). Both compounds are stereoisomers, which means that they both have the same reactive groups in a slightly different three-dimensional framework. The fact that their inhibitory activities differ strongly underlines the notion that the specific spatial structures of these molecules rather than the mere presence of reactive groups determines their inhibitory activity.

*The Inhibition of C/EBP β by Helenalin Acetate Does Not Involve Irreversible Covalent Reaction with Cysteine Residues—*Our data suggested that the presence of both potentially reactive sites in the specific three-dimensional structure of helenalin acetate determines its inhibitory activity. We were therefore interested to know whether the cysteine residues of C/EBP β , which are the likely candidates for covalent attack, are involved in the inhibitory mechanism of helenalin acetate. We have previously shown that helenalin acetate inhibits only the LAP* isoform, but not the LAP isoform, which lacks the first 21 N-terminal amino acids of C/EBP β (26). This strongly suggested that helenalin acetate targets the N-terminal part of C/EBP β , which contains cysteine residues at positions 11 and 33. To study the role of these residues, we mutated them individually or in combination to alanine and analyzed the sensitivity of the resulting proteins toward helenalin acetate. Surprisingly, Fig. 4A shows that the cysteine mutants have somewhat increased basal activity. We do not know the reasons for this increase; however, the cysteines in C/EBP β have been shown to form disulfide bridges (35), whose disruption by the mutations might affect the structure of the protein. The N-terminal part of C/EBP β has also been shown to bind to inhibitory factors (14, 36), whose binding might be weakened by the loss of the cysteine residues. In any case, Fig. 4A shows that individual mutation of either Cys-11 or Cys-33 had no effect on the sensitivity of the protein toward helenalin acetate, whereas the combination of both mutations slightly reduced its sensitivity. We confirmed this finding using the expression of the endogenous C/EBP-inducible MRP126 gene as read-out for C/EBP β activity (Fig. 4B). Because the C11A/C33A double mutant was still inhibited by helenalin acetate, we generated a completely cysteine-free mutant of C/EBP β (referred to as CallA) to investigate whether the other cysteines are responsible for the residual sensitivity toward helenalin acetate. However, the sensitivity of the cysteine-free C/EBP β was not decreased more than that of the C11A/C33A double mutant (Fig. 4B).

To investigate whether cysteines 11 and 33 undergo covalent modification by helenalin acetate, we generated C/EBP β mutants containing only Cys-11 or Cys-33 or both of them. These mutants, wild-type C/EBP β and the cysteine-free C/EBP β , were then expressed in fibroblasts in the absence or presence of helenalin acetate. Cell extracts were then incubated with the cysteine-specific biotinylation reagent BMCC-Biotin (1-biotinamido-4-[4'-(maleimidomethyl) cyclohexanecarbox-

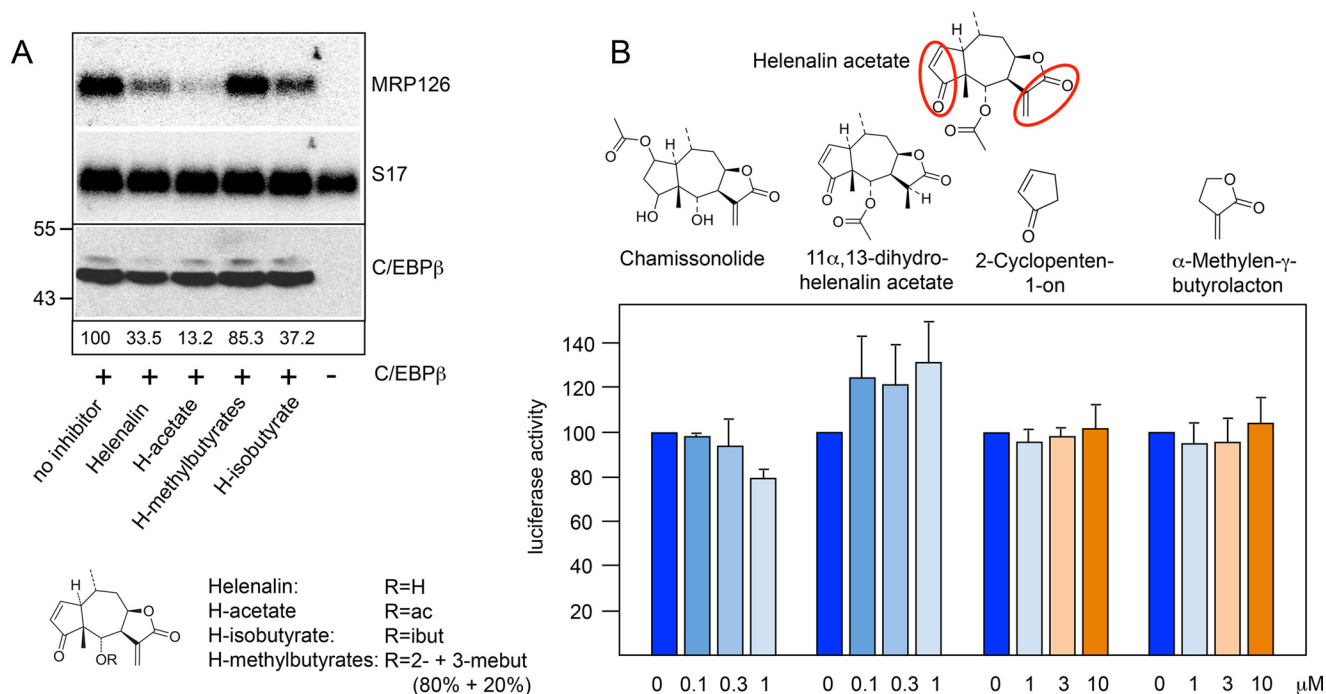


FIGURE 3. Inhibitory activities of different sesquiterpene lactones. *A*, QT6 cells were transfected with expression vector for chicken C/EBP β and treated with 1 μ M of the indicated helenalin esters or left untreated, as indicated below the lanes. The last lane shows untransfected cells, which serve as control. Cells were harvested after 12 h and analyzed by Northern blotting for expression of MRP126 and S17 mRNAs (*top* and *middle* panels) and by Western blotting for the expression of C/EBP β (*bottom* panel). Numbers below the lanes indicate the relative amounts of MRP126 mRNA determined by quantification with a phosphor image analyzer. *ac*, acetate; *ibut*, isobutyrate; *mebut*, methylbutyrate. *B*, the structure of helenalin acetate is shown at the *top*. Reactive α,β -unsaturated carbonyl groups are marked with red circles. The structures of chamissonolide, 11 α ,13-dihydrohelenalin acetate, 2-cyclopenten-1-one and α -methylene- γ -butyrolactone are shown below. Reporter assays were performed with QT6 fibroblasts transfected with the C/EBP-dependent luciferase reporter gene p-240luc, expression vector for chicken C/EBP β and pCMV β . Cells were cultivated for 12 h with the indicated concentrations of the compounds shown above and analyzed for luciferase and β -galactosidase activities as in Fig. 1. Error bars show the S.D.

amido]butane, Thermo Scientific), followed by binding of biotinylated proteins to streptavidin beads and Western blotting. We reasoned that if helenalin acetate binds in an irreversible covalent manner to Cys-11 or -33 *in vivo*, these residues would no longer be available for subsequent biotinylation *in vitro*. Fig. 4C shows that all C/EBP β mutants containing one or more cysteine residues were bound to streptavidin beads, whereas the cysteine-free C/EBP β was not bound, indicating that the streptavidin pull-down was specific. Importantly, treatment with helenalin acetate did not decrease the binding to the streptavidin beads for any of the constructs, indicating that Cys-11 and -33 were not covalently modified by helenalin acetate. Together with the finding that a completely cysteine-free C/EBP β was still inhibited by helenalin acetate, we concluded that helenalin acetate inhibits C/EBP β by a mechanism that does not involve covalent modification of cysteine residues.

Helenalin Acetate Binds to the N-terminal Part of C/EBP β —The finding that helenalin acetate does not inhibit C/EBP β by alkylation of cysteine residues prompted us to explore whether helenalin acetate inhibits C/EBP β by direct binding. The fact that the stereoisomers helenalin and mexicanin-1 differ significantly in their C/EBP β inhibitory activity (26) underscores the idea that the three-dimensional structure of helenalin acetate is crucial for its inhibitory activity, consistent with a stereospecific binding mechanism. To examine the binding of helenalin acetate to C/EBP β , we employed microscale thermophoresis (MST), a biophysical method to characterize molecular interactions. Thermophoresis is based on the directed movement of

molecules along a temperature gradient, which is sensitive to changes of the molecule/solvent interface caused by molecular interactions and, thus, permits measurement of binding constants for biomolecular interactions, such as protein-protein or protein-small-molecule interactions (37). To detect binding of helenalin acetate to C/EBP β by microscale thermophoresis, a constant amount of a cell extract containing GFP-LAP* was titrated with increasing amounts of helenalin acetate. Fig. 5A shows the result of a representative thermophoresis experiment as an example. Based on the quantification of replicate binding experiments, we have previously reported that helenalin acetate is able to bind to GFP-LAP* with an approximate dissociation constant of less than 1 μ M, in accordance with the strong inhibition of C/EBP β activity by helenalin acetate seen in Fig. 5C and reported before (26). Interestingly, GFP-LAP showed no binding of helenalin acetate, consistent with the finding that helenalin acetate inhibits the LAP* isoform but not the LAP isoform of C/EBP β (26). These experiments therefore suggested that helenalin acetate binds to the N-terminal domain of LAP*. To further explore the specificity of the binding of helenalin acetate to C/EBP β , we performed MST experiments with a mutant of C/EBP β (C/EBP β -mut1) that is significantly less sensitive to helenalin acetate than wt C/EBP β . In this mutant, a highly conserved FYY amino acid motif is replaced by alanine residues. As shown in Fig. 5C, the activity of the mutant C/EBP β was only marginally affected by helenalin acetate. MST experiments showed that the reduced sensitivity correlated with reduced binding of helenalin acetate, providing

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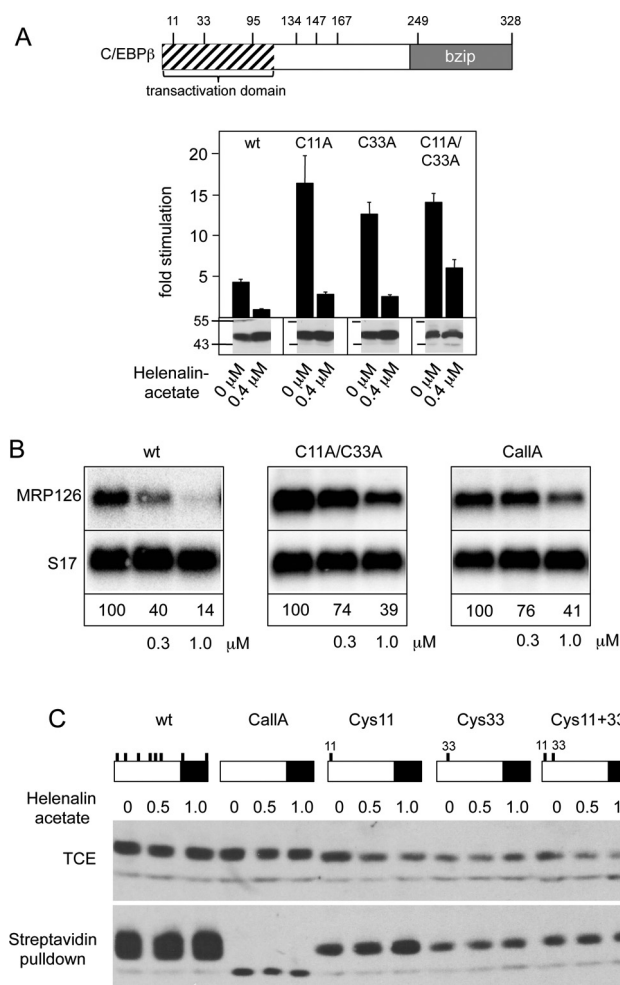


FIGURE 4. Inhibition of C/EBP β activity by helenalin acetate is not due to the alkylation of cysteine residues of C/EBP β . *A*, the positions of cysteine residues of C/EBP β are shown schematically at the top. The panel below illustrates reporter assays with C/EBP β mutants lacking Cys-11 or Cys-33 or both. Reporter assays were performed as in Fig. 1*A*. Aliquots of the cell extracts were used to visualize the amounts of C/EBP β (bottom panels). Error bars show the S.D. *B*, QT6 cells were transfected with expression vectors for wild-type or C/EBP β mutants lacking cysteines 11 and 33 (C11A/C33A) or all cysteines (CallA) and treated with the indicated concentrations of helenalin acetate. Cells were harvested after 12 h and analyzed by Northern blotting for expression of MRP126 and S17 mRNAs. The numbers below the blots indicate the relative expression levels of MRP126 mRNA. *C*, QT6 cells were transfected with expression vectors for the different C/EBP β mutants (containing only the indicated cysteine residues) and treated for 12 h with or without helenalin acetate, as indicated. Cell extracts were then subjected to biotinylation with BMCC-Biotin, followed by incubation with streptavidin agarose. Total cell extracts (TCE) and bound proteins were analyzed by SDS-PAGE and Western blotting, using antibodies against C/EBP β . Numbers on the side of the Western blots in *A* and *C* refer to molecular weight markers.

further evidence that helenalin acetate inhibits C/EBP β by direct binding to the N-terminal region of C/EBP β (Fig. 5*B*). Presumably, the amino acid replacements cause changes of the structure of the N-terminal domain of the mutant protein that decrease the binding of helenalin acetate. We also measured the binding of helenalin acetate to the C11A/C33A mutant of C/EBP β and found that binding occurred with reduced affinity (Fig. 5*B*). This provides a plausible explanation for the observation that the C11A/C33A mutant is less sensitive toward helenalin acetate than the wild-type protein (Fig. 4*A*). Finally, we analyzed the binding of the inactive compound 11 α ,13-dihy-

drohelenalin acetate to GFP-LAP*. Again, MST experiments showed very weak binding, consistent with the lack of inhibition of LAP* by this compound (Fig. 5*B*). Taken together, these experiments strongly support the notion that helenalin acetate inhibits C/EBP β mainly by specific binding to the N-terminal domain of the protein. We noted that the activity of C/EBP β -mut1 was slightly inhibited at high concentrations of helenalin acetate, although no binding was detected in the MST experiment. We therefore do not exclude the possibility that inhibition of C/EBP β at higher concentrations of helenalin acetate could be due to additional effects on other proteins, such as p300 or other proteins binding to C/EBP β .

The three-dimensional structure of the N-terminal part of C/EBP β is not known so far. The exact location of the binding site for helenalin acetate within C/EBP β is therefore a matter of speculation. As helenalin acetate does not bind to the LAP isoform, the first 21 amino acids of LAP* are required for binding. On the other hand, mutations (such as mut1) that affect the amino acid sequence of the N-terminal part of the LAP isoform also disrupt the binding. We therefore think that the binding site for helenalin acetate overlaps the N-terminal parts of the LAP* and LAP isoforms.

Expression of p300 Reduces the Inhibitory Effect of Helenalin Acetate—Because the N-terminal domain of C/EBP β has been implicated in interactions with several other proteins (10, 15, 38, 39), we considered the possibility that the binding of helenalin acetate inhibits C/EBP β activity by disturbing the cooperation of C/EBP β with its interaction partners. The co-activator p300 has been identified as one of the key interaction and cooperation partners of C/EBP β (38). To examine whether helenalin acetate interferes with the cooperation of C/EBP β and p300, we first investigated whether increased expression of p300 counteracts the inhibition by helenalin acetate. Underlying this experiment is the assumption that if helenalin acetate competes with p300 for binding to C/EBP β , overexpression of p300 might rescue C/EBP β activity. Fig. 6*A* shows that transfection of an expression vector for p300 indeed partially reversed the inhibitory effect of helenalin acetate on the activity of C/EBP β , consistent with a competition model.

We have previously examined the interaction of p300 with C/EBP β in detail and showed that it is mediated by the Taz2 domain of p300 (38). In these studies, we also showed that a deletion mutant of p300 lacking the Taz2 domain does not stimulate C/EBP β activity, demonstrating that the Taz2 domain is the relevant part of p300 that is responsible for the cooperation of p300 and C/EBP β . According to a recent model of the interaction of the Taz2 domain with the transcription factor C/EBP ϵ (40), the Taz2 domain of p300 interacts with two stretches of amino acids that are conserved among C/EBP family members and are located at positions 68–80 and 115–123 of C/EBP β . Because C/EBP ϵ lacks the LAP*-specific N-terminal sequences of C/EBP β , it remained open in this model whether these sequences also bind to the Taz2 domain. To address whether this is the case, we performed two-hybrid experiments with VP16-p300 (encompassing the Taz2 domain) and Gal4-C/EBP β fusion proteins containing various parts from the N terminus of C/EBP β . Fig. 6*B* shows that the LAP*-specific N-terminal 21 amino acids on their own did not interact with the

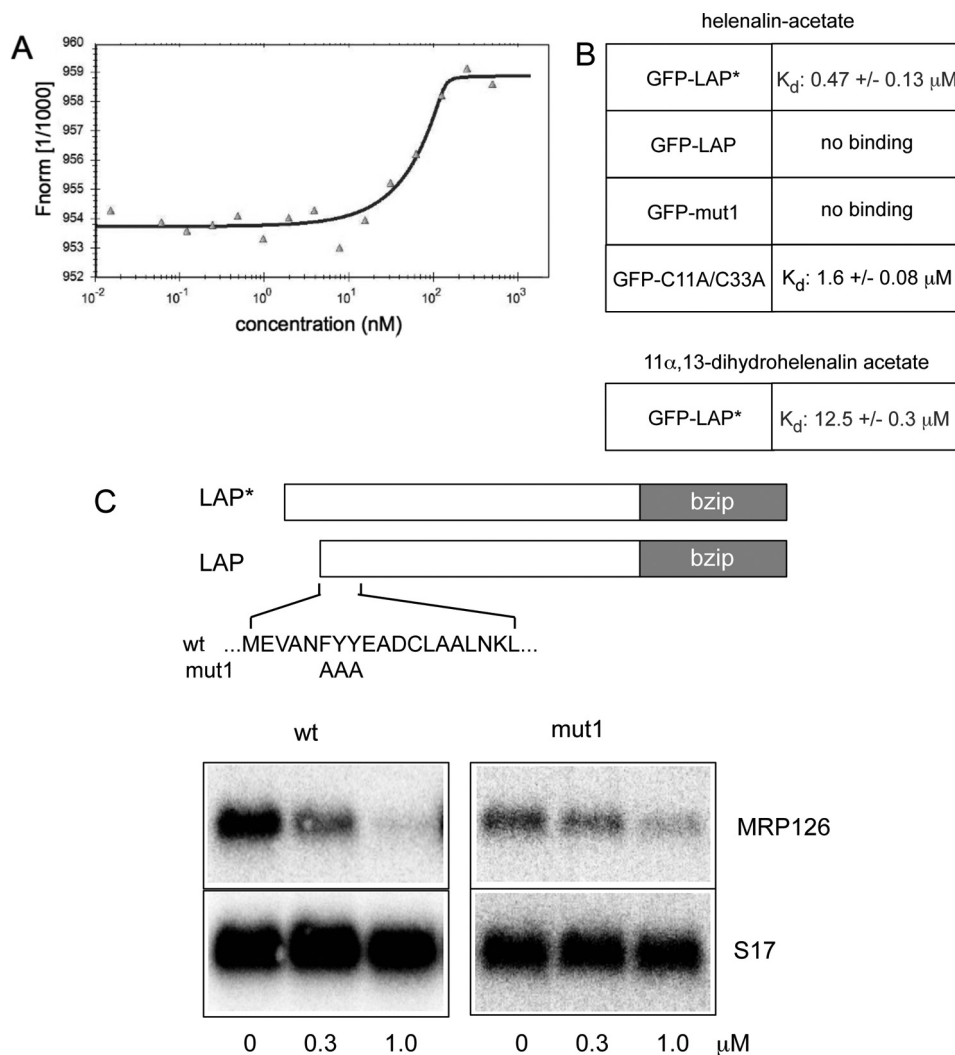


FIGURE 5. Helenalin acetate binds to the N-terminal domain of C/EBP β . *A*, analysis of the binding of helenalin acetate to C/EBP β by microscale thermophoresis. The result of a single MST experiment is shown. Constant amounts of extract from QT6 cells transfected with GFP-C/EBP β were titrated with helenalin acetate from 1.4 nM to 50 μ M. The normalized fluorescence (F_{norm} 1/1000) was plotted against the concentration of helenalin acetate. *B*, summary of K_d values for binding of helenalin acetate or 11 α ,13-dihydrohelenalin acetate to wt C/EBP β or the indicated C/EBP β mutants. The K_d values \pm S.D. were determined from several independent experiments. *C*, QT6 cells were transfected with expression vectors for wild-type or mutant C/EBP β and treated with the indicated concentrations of helenalin acetate. Cells were harvested after 12 h and analyzed by Northern blotting for expression of MRP126 and S17 mRNAs. The amino acid replacements in C/EBP β -mut1 are indicated at the top.

Taz2 domain; however, when additional sequences were included, binding to the Taz2 domain was observed. Thus, in addition to the p300 binding regions at positions 68–80 and 115–123, the N-terminal part of C/EBP β also interacts with the Taz2 domain.

To confirm this interaction by an independent approach, we performed *in vitro* pulldown experiments using bacterially expressed GFP-Taz2 and several GST-C/EBP β fusion proteins. As expected, GFP-Taz2 bound strongly to full-length C/EBP β or a shorter construct containing only amino acids 1–200 of C/EBP β , fused to GST, both of which contain the known interaction sites for the Taz2 domain at amino acids 68–80 and 115–123, whereas no binding was detected to GST alone (Fig. 6C, lanes 1–3). Importantly, a GST-C/EBP β fusion protein containing only amino acids 1–50 of C/EBP β also interacted with the Taz2 domain, although less strongly than full-length C/EBP β , corroborating the result of the two-hybrid experiment (Fig. 6C, lane 4). Both experiments therefore demonstrate that

the N-terminal part of C/EBP β also harbors a binding site for the Taz2 domain. The fact that only bacterially expressed proteins were used in the pulldown experiment also indicates that the interaction occurs in the absence of other eukaryotic proteins and, hence, presumably is a direct protein-protein interaction.

We were therefore interested to investigate whether the N-terminal part of C/EBP β contributes to the cooperation of C/EBP β with p300. To address this question, we performed reporter experiments with full-length and N-terminally truncated versions of C/EBP β carrying additional mutations in one of the conserved p300 interaction sites (referred to as mut3a). Fig. 6D shows that the mut3a version of full-length C/EBP β was still stimulated by co-transfection of a p300 expression vector, although less strongly than the wild-type construct, whereas the mut3a version of C/EBP β - Δ N33 was completely inactive and was not stimulated by co-expression of p300. This indicated that the N-terminal part of C/EBP β is crucial for the

Helenalin Acetate Inhibits C/EBP β Activity

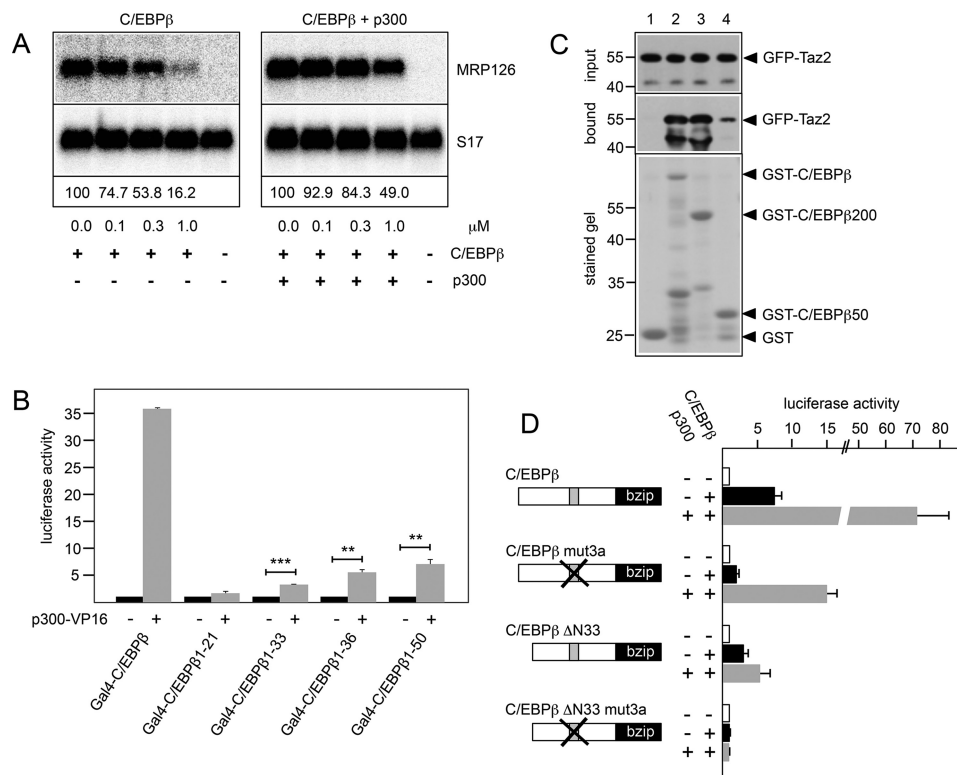


FIGURE 6. Helenalin acetate disrupts the cooperation of C/EBP β and p300. *A*, QT6 cells transfected with expression vectors for C/EBP β and p300, as indicated. Cells were treated with the indicated concentrations of helenalin acetate, harvested after 12 h, and analyzed by Northern blotting for expression of MRP126 and S17 mRNAs. The numbers below the blots indicate the relative expression levels of MRP126 mRNA. *B*, QT6 fibroblasts were transfected with the Gal4-dependent reporter gene pG5E4-38Luc, expression vectors for p300-VP16 and the indicated Gal4-C/EBP β proteins. Cells were harvested after 12 h and analyzed for luciferase and β -galactosidase activities. The columns show the average luciferase activity normalized against the β -galactosidase activity. Error bars show the S.D. **, $p < 0.01$; ***, $p < 0.001$. *C*, *in vitro* pulldown experiment. Glutathione-Sepharose beads loaded with equivalent amounts of bacterially expressed GST (lane 1), GST-C/EBP β (lane 2), GST-C/EBP β (1–200) (lane 3), and GST-C/EBP β (1–50) (lane 4) were incubated with identical amounts of a bacterially expressed GFP-Taz2 fusion protein. Bound GFP-Taz2 and aliquots of the input samples were analyzed by Western blotting with antibodies against GFP. The bottom panel shows a stained gel to confirm loading of the Sepharose beads with equivalent protein amounts. Numbers on the left side refer to molecular weight markers. *D*, QT6 cells were transfected with the C/EBP-dependent luciferase reporter gene p-240Luc, the β -galactosidase expression vector pCMV β , expression vectors for p300, and the indicated C/EBP β constructs. Reporter gene activities were analyzed as in *B*. Error bars show the S.D.

cooperation with p300. Furthermore, comparison of the stimulation of the activity of the full-length and truncated wild-type forms of C/EBP β by p300 clearly showed that the N-terminal part of C/EBP β significantly enhances the ability to cooperate with p300. Taken together, it is evident that the N-terminal sequences of C/EBP β significantly enhance its ability to cooperate with p300.

Helenalin Acetate Disrupts the Interaction between the Taz2 Domain of p300 and the N-terminal Domain of C/EBP β —The experiments described above have revealed an interaction of the Taz2 domain with the N-terminal part of C/EBP β that plays a critical role in the cooperation of C/EBP β and p300. Importantly, this region of C/EBP β does not encompass the previously known p300 binding sites at amino acid positions 68–80 and 115–123 of C/EBP β . Hence, our experiments have revealed a novel interaction between C/EBP β and the Taz2 domain of p300. We were therefore interested to see whether this interaction is affected by helenalin acetate. Fig. 7A shows that helenalin acetate clearly diminished the interaction of VP16-p300 with Gal4-C/EBP β (1–50), indicating that it disrupts the interaction between the N-terminal domain of C/EBP β and p300. Interestingly, helenalin acetate did not disrupt the interaction of VP16-p300 with a fusion protein of Gal4 and full-length C/EBP β (Fig. 7A). Presumably, the p300 binding regions in the

center of C/EBP β are sufficient for the overall interaction of C/EBP β and p300. Taken together, our data suggest the model illustrated in Fig. 7B. In this model, the Taz2 domain interacts with three distinct regions of C/EBP β , one of which is disrupted by helenalin acetate. As depicted in this model, helenalin acetate might affect the arrangement (and, presumably, the function) of the N-terminal sequences of C/EBP β in the C/EBP β -p300 complex.

Discussion

By using an engineered cell line with a GFP-based Myb-inducible reporter gene as a tool to search for inhibitors of the transcription factor c-Myb, we have recently identified helenalin acetate as a highly active small-molecule inhibitor of C/EBP β (26). Together with a variety of structurally related compounds, helenalin acetate was initially thought to be a Myb inhibitor. However, further work showed that helenalin acetate does not inhibit Myb but instead inhibits C/EBP β . This unintentional discovery of a C/EBP β inhibitor instead of a Myb inhibitor can be explained by the fact that the cis-elements that drive the Myb-dependent reporter gene in this cell line are derived from the myeloid-specific Myb target gene *mim-1*, which is activated cooperatively by Myb together with C/EBP β or other C/EBP transcription factors (41–43). Like direct Myb

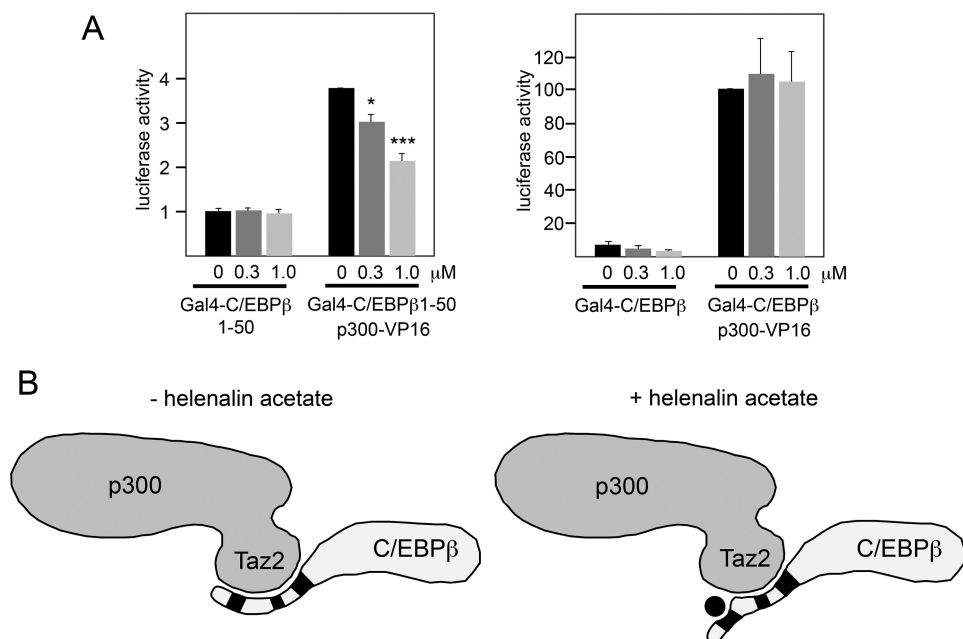


FIGURE 7. Helenalin acetate disrupts the interaction between the Taz2 domain and the N-terminal domain of C/EBP β . *A*, QT6 fibroblasts were transfected with expression vectors for p300-VP16 and the indicated Gal4-C/EBP β proteins. Cells were additionally treated without or with helenalin acetate, as indicated. Cells were harvested after 12 h and analyzed for luciferase and β -galactosidase activities. The columns show the average luciferase activity normalized against the β -galactosidase activity. Error bars show the S.D. *, $p < 0.05$; ***, $p < 0.001$. *B*, model describing the influence of helenalin acetate on the C/EBP β -p300 interaction. The Taz2 domain of p300 is highlighted. The black boxes mark the binding sites for the Taz2 domain in the N-terminal part and between amino acids 68–80 and 115–123 of C/EBP β . Helenalin acetate is shown as a black circle. Binding of helenalin acetate to the N-terminal region of C/EBP β displaces this region from the Taz2 domain of p300 while leaving the other interactions intact.

inhibitors, compounds that decrease the activity of C/EBP β will therefore also reduce the expression of the reporter gene. We have recently investigated a series of almost 70 structurally related sesquiterpene lactones with the GFP-based reporter system to establish structure-activity relationships to explain their inhibitory effects on activity of the Myb-dependent reporter gene (44). On the grounds of these considerations, it may be hypothesized that these compounds act by related mechanisms in which C/EBP β rather than c-Myb is involved as a major target.

There is now strong evidence for pro-oncogenic roles of C/EBP β in certain tumors, which has raised interest in C/EBP β as a potential drug target. Because helenalin acetate is the first highly active small-molecule inhibitor of C/EBP β , we have characterized its inhibitory activity in more detail. Helenalin acetate and helenalin are well known for their anti-inflammatory activities, which have been attributed to their ability to inhibit the pro-inflammatory transcription factor NF- κ B. One important result of our work is that both compounds are actually significantly more active as inhibitors of C/EBP β than of NF- κ B. Like NF- κ B, C/EBP β also plays major roles in inflammatory processes, for example by controlling the transcription of various pro-inflammatory genes, including IL-1, IL-6, IL-8, TNF α , NO synthase, and others (29). Furthermore, knock-out of C/EBP β in mice leads to defects in their innate, humoral, and cellular immunity, further pointing to important functions of C/EBP β in immune and inflammatory responses. That helenalin and helenalin acetate inhibit C/EBP β significantly more potently than NF- κ B suggests that the inhibition of C/EBP β also contributes to the anti-inflammatory activities of helenalin and helenalin acetate.

The main aim of this study was to explore the mechanism that is responsible for the inhibition of C/EBP β by helenalin acetate. Our initial characterization of its inhibitory activity had shown that helenalin acetate selectively inhibits the LAP* isoform of C/EBP β . Neither the LAP isoform nor the related transcription factor C/EBP α was significantly inhibited, which has indicated that the N-terminal domain of LAP* plays a key role in the inhibitory mechanism (26). Helenalin acetate contains reactive groups that are able to form covalent adducts with cysteine residues, as reported in case of NF- κ B. However, in contrast to the inhibition of NF- κ B, our data suggest that helenalin acetate inhibits C/EBP β by a non-covalent binding mechanism. Although mutation of Cys-11 and -33 in the N-terminal domain of C/EBP β renders the protein somewhat more resistant to inhibition, these cysteines are not covalently modified by helenalin acetate. Rather, the analysis of the inhibitory potential of several related compounds strongly supports the notion that the specific three-dimensional structure of helenalin acetate, and not the mere presence of reactive groups, is responsible for its inhibitory activity. Support for a non-covalent inhibitory mechanism also comes from microscale thermophoresis measurements, which have allowed us to determine a binding constant for the interaction of helenalin acetate and full-length C/EBP β . These measurements have confirmed that only the LAP* but not the LAP isoform binds helenalin acetate, which is consistent with the fact that only LAP* is inhibited by helenalin acetate. Furthermore, these experiments have revealed that a mutation of the conserved FYY motif in the N-terminal domain of C/EBP β decreases the sensitivity toward helenalin acetate and also reduces its binding to C/EBP β . Likewise, the C11A/C33A mutant, which is less sensitive, shows

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decreased binding to helenalin acetate. Thus, the reduced sensitivity due to mutation of the FYY motif and the replacement of Cys-11 and -33 by alanine most likely reflects structural alterations of the N-terminal domain of C/EBP β that reduce the affinity for helenalin acetate. Taken together, our results strongly argue that a stereospecific binding mechanism of helenalin acetate is responsible for the inhibition of C/EBP β .

Our data indicate that the binding of helenalin acetate to C/EBP β disturbs its cooperation with the co-activator p300. As shown before, binding of p300 to C/EBP β is mediated by the Taz2 domain of p300 and two conserved stretches of amino acids located at positions 68–80 and 115–123 of C/EBP β , which form a tight complex with the Taz2 domain (38, 40). We have now demonstrated by two-hybrid and by GST pull-down experiments that the N-terminal part of C/EBP β is also involved in interactions with the Taz2 domain of p300 and, furthermore, that this part of C/EBP β contributes significantly to the cooperation with p300 (Fig. 6, B–D). Importantly, the two-hybrid experiments have shown that helenalin acetate disrupts this interaction (Fig. 7A). Interestingly, helenalin acetate disrupts the interaction of the Taz2 domain only with the N-terminal part of C/EBP β but not with the full-length protein. Overall, these findings suggest the model, illustrated in Fig. 7B, in which helenalin acetate binds to the N-terminal region of C/EBP β and thereby disturbs the interaction of this part of C/EBP β with p300 while leaving the interaction of p300 with the binding sites at positions 68–80 and 115–123 of C/EBP β intact. This suggests that helenalin acetate influences how the N-terminal sequences of C/EBP β are arranged in the C/EBP β -p300 complex. How this leads to the inhibition of C/EBP β activity is presently not known. It is possible that the altered conformation of the N-terminal part of C/EBP β in the C/EBP β -p300 complex affects other protein-protein interactions or leads to a different pattern of covalent modifications in the N-terminal part of C/EBP β . For example, the acetylation of a specific lysine residue in the N-terminal part of C/EBP β by p300, Lys-39, is known to exert a major effect on the activity of C/EBP β (45). Moreover, the N-terminal domain of C/EBP β has been implicated in the binding of other proteins, including the SWI/SNF chromatin remodeling complex (39), the Mediator complex (15), and protein kinase Hipk2 (10). It is therefore possible that the inhibitory mechanism of helenalin acetate is even more complex and also involves inhibition of further protein-protein interactions or post-translational modifications of the N-terminal domain of LAP*.

As mentioned above, we have recently used the GFP-based Myb-inducible reporter system to perform a structure-activity study of the inhibitory activities of a variety of almost 70 sesquiterpene lactones, including helenalin acetate as one of the strongest inhibitors (44). Based on the inhibition of c-Myb-dependent reporter gene activity by this set of related compounds, a thorough analysis of the influence of molecular structure on inhibitory potency was carried out. A rather straightforward correlation was observed between the compounds' inhibitory activity and their potential ability to act as Michael acceptors (*i.e.* the number of structural elements in the molecule that are potentially able to undergo covalent reactions with nucleophilic partial structures of proteins). Interestingly, the potential

reactivity alone did not fully explain the level of activity, but it was found that the steric orientation of the reactive centers played an important role. The resulting quantitative structure-activity relationship (QSAR) model, giving a statistically sound, reasonable, and plausible explanation for the differences in activity between the various compounds, is in partial agreement with the observation of the present study that the mere presence of reactive structure elements does not suffice to explain high activity. However, the present study clearly shows that helenalin acetate does not bind in an irreversible covalent manner to C/EBP β , and this finding, at first sight, appears contradictory to the structure-activity relationship requiring the presence of more than one reactive partial structure as a prerequisite for high activity. However, it may simply indicate that, as stated above, apart from a non-covalent binding mechanism to C/EBP β , there may be further effects related to covalent binding to other proteins involved in the overall observed activity that remain yet to be elucidated. From the fact that a statistically relevant common structure-activity relationship exists for all investigated sesquiterpene lactones, it may be deduced that they all share a common mechanism of action. Experiments investigating a broader structural variety of sesquiterpene lactones for their binding to and inhibition of C/EBP β may provide important evidence for the elucidation of the full mechanism of inhibition of the transcriptional machinery under study and will also shed further light on the molecular reasons for their anti-inflammatory and anti-tumoral activity. Studies in this direction are therefore under way.

Overall, our work has identified helenalin acetate as the first highly potent small-molecule inhibitor of C/EBP β . We envision helenalin acetate as a useful tool to explore the role of C/EBP β in normal and in tumor cells. Because it acts primarily on the LAP* isoform of C/EBP β , helenalin acetate will also be useful to dissect the functions of the LAP* and LAP isoforms.

Experimental Procedures

Cells—QT6 is a line of quail fibroblasts, and 3T3-L1 is a mouse pre-adipocyte line whose differentiation into fat cells was induced by published procedures (31). All cell lines were free of mycoplasma contamination.

Sesquiterpene Lactones—Helenalin, helenalin acetate, helenalin isobutyrate, helenalin methylbutyrates, 11 α ,13-dihydro-helenalin acetate, and chamissonolide were isolated from *Arnica* species (44). 2-Cyclopentene-1-one and α -methylene- γ -butyrolactone were obtained from Sigma-Aldrich. The purity of all compounds was >90% by HPLC and/or ¹H NMR. 10 mM stock solutions were prepared in DMSO. Human recombinant TNF α was obtained from Biomol.

Transfections—QT6 fibroblasts were transfected by calcium phosphate co-precipitation, and reporter gene activities were analyzed as described (46). The C/EBP-inducible luciferase reporter gene p-240Luc and the Gal4-dependent reporter gene pG5E4-38Luc have been described before (38, 47). The NF- κ B luciferase reporter gene pGL2-6 \times NF κ B-luc was obtained from C. Scheidereit. The β -galactosidase reporter gene pCMV β was from Clontech (Heidelberg, Germany). All reporter studies were done in at least 3 independent experiments, with duplicate measurements in each experiment. Expression vectors for

chicken and human C/EBP β have been described (48, 49). The expression vector for human C/EBP β was kindly provided by G. Steger. Point mutants of chicken C/EBP β carrying replacements of individual or all cysteine residues by alanine or the FYY motif (mut1) were generated by PCR using appropriate primers. pEYFP-N1-C/EBP β wild type, C11A/C33A, or mut1 and pEYFP-N1-C/EBP β - Δ N21 were generated by cloning the full-length or N-terminally truncated coding region of chicken C/EBP β between the EcoRI and BamHI sites into pEYFP-N1. The C/EBP β stop codon was removed and replaced by a BamHI site. pVP16/p300 and a full-length Gal4-C/EBP β construct have been described (48). Gal4-C/EBP β (1–21), (1–33), (1–36), and (1–50) were generated by PCR amplification of the indicated parts of the chicken C/EBP β coding region and cloning them downstream of the Gal4 coding region. Similarly, the GST-C/EBP β (1–50) was generated by cloning the PCR-amplified coding region for amino acids 1–50 of chicken C/EBP β into pGex-6P2. C/EBP β -mut3a, C/EBP β - Δ N33, and C/EBP β - Δ N33-mut3a have been described (48). C/EBP β -mut3a carries amino acid substitutions in one of the conserved p300 interaction sites, changing the amino acid sequence at positions 101–107 (of chicken C/EBP β) from DLFSDFL to AAASAAA. The expression vector for full-length human p300 was obtained from R. Eckner. The bacterial expression vector for a GFP-Taz2 fusion protein was generated by subcloning the PCR-amplified p300 coding sequences for amino acids 1710–1891 between the BamHI and NotI of the bacterial expression vector pGV82 (gift from H. Mootz, Institute for Biochemistry, University of Münster). Human recombinant TNF α was obtained from Biomol. The expression of the endogenous MRP126 and ribosomal protein S17 mRNAs was analyzed as described before (10).

Microscale Thermophoresis—To measure the dissociation constant for the interaction of helenalin acetate with C/EBP β , extracts of QT6 cells transfected with YFP-C/EBP β or YFP-C/EBP β - Δ N21 were prepared in 50 mM Hepes, pH 7.4, 120 mM sodium chloride, 1 mM EDTA, 6 mM EGTA, and 0.5% Nonidet P-40. Concentrations of helenalin acetate ranging from 1.4 nM to 50 μ M were combined with constant amounts of cell extract, incubated for 1–2 h at room temperature, and filled in capillaries to perform thermophoresis measurements in a NanoTemper Monolith (NT.015) instrument. Thermophoresis was performed at 1475 nm \pm 15 nm. Data from several independent experiments were normalized to ΔF_{norm} [%] ($10 \times (F_{\text{norm}}(\text{bound}) - F_{\text{norm}}(\text{unbound}))$) or fraction bound (ΔF_{norm} [%]/amplitude) to calculate the K_d value.

Biotinylation of Cell Extracts—Transfected cells were lysed in ELB buffer (120 mM NaCl, 50 mM Tris/HCl, pH 7.4, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM sodium pyrophosphate, 1 mM PMSF, 0.5% Nonidet P-40, 1 ng/ml aprotinin, 0.2 ng/ml leupeptin, 1 ng/ml pepstatin). After removal of input samples, 450 μ l of the cell extract were mixed with 50 μ l of 8 μ M EZ-LinkTM BMCC-Biotin (Thermo Scientific) dissolved in DMSO. The solution was first kept on ice for 4 h and then incubated with Streptavidin-agarose beads overnight with constant agitation. After washing the beads with ELB buffer, bound protein and input samples were analyzed by SDS-PAGE and Western blotting.

In Vitro GST Pulldown—Purification of GST fusion proteins and *in vitro* GST pulldown experiments were performed as described before (10).

Western Blotting—SDS-PAGE and Western blotting were performed by standard procedures using antibodies against chicken (43) or mouse (Santa Cruz Biotechnology, H-7) C/EBP β . Antibodies against β -actin were obtained from Sigma-Aldrich (AC-15). Samples of extracts from transfected cells (Figs. 1A, 3A, and 4A) were normalized to the corresponding transfection efficiency. Protein samples from untransfected cells (Fig. 2C) were loaded as equal aliquots.

Author Contributions—A. J. and K.-H. K. designed and performed experiments, analyzed data, and wrote the manuscript. S. S. and S. M. H. designed and performed experiments and analyzed data. T. J. S. designed experiments, analyzed data, and wrote the manuscript.

Acknowledgments—We thank B. Berkenfeld and P. Pieloch for technical assistance and H. Mootz and C. Scheidereit for plasmids.

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