Inhibition of the acetyltransferases p300 and CBP reveals a targetable function for p300 in the survival and invasion pathways of prostate cancer cell lines

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Abbreviations:

AR  androgen receptor
ChIP  chromatin immunoprecipitation
DHT  dihydrotestosterone
DMSO  dimethyl sulfoxide
ELISA  enzyme-linked immunosorbent assay
FOV  field of view
HAT  histone acetyltransferase
HDAC  histone deacetylase
IκBα  inhibitor of κB-α
KAT  lysine acetyltransferase
MMP  matrix metalloproteinase
NF-κB  nuclear factor-κB
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PI  propidium iodide
PSA  prostate-specific antigen
SD  standard deviation
SEM  standard error of the mean
TNF-α  tumor necrosis factor-α

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Abstract

Inhibitors of histone deacetylases have been approved for clinical application in cancer treatment. On the other hand, histone acetyltransferase inhibitors have been less extensively investigated for their potential use in cancer therapy. In prostate cancer the histone acetyltransferases and coactivators p300 and CBP are upregulated and may induce transcription of androgen-receptor responsive genes, even in the absence or in the presence of low levels of androgen receptor. To discover a potential anti-cancer effect of p300/CBP inhibition, we used two different approaches: i) downregulation of p300 and CBP by specific siRNA and ii) chemical inhibition of the acetyltransferase activity by a newly developed small molecule, C646. Knockdown of p300 by specific siRNA, but surprisingly not of CBP, led to an increase of caspase-dependent apoptosis involving both extrinsic and intrinsic cell death pathways in androgen-dependent and castration-resistant prostate cancer cells. Induction of apoptosis was mediated by several pathways including inhibition of androgen receptor function and decrease of the NF-κB subunit p65. Furthermore, cell invasion was decreased upon p300, but not CBP depletion and was accompanied by lower MMP-2 and -9 transcriptions. Thus, p300 and CBP have differential roles in the processes of survival and invasion of prostate cancer cells. Induction of apoptosis in prostate cancer cells was confirmed by the use of C646. This was substantiated by a decrease of androgen receptor function and downregulation of p65 impairing several NF-κB target genes. Taken together, those results suggest that p300 inhibition may be a promising approach for the development of new anti-cancer therapies.
Introduction

The histone acetyltransferases (HATs) CBP and p300 are important coactivators favoring transcription. Their involvement in various pathological processes has been extensively studied in the past (1). In fact, CBP and p300 are known as functional paralogues and their HAT domains show sequence identity of more than 90% (2). Both proteins are fundamental coactivators of a large number of transcription factors by remodeling chromatin. Moreover, p300 and CBP can bind to target proteins and regulate DNA binding affinity, transcriptional activation, protein-protein interactions, and stability of transcription factors, but also other cellular proteins by acetylation (3). For that reason, HATs are also named lysine acetyltransferases (KATs) (4).

In cancer, the role of p300 and CBP is not clear and may depend on the physiological background of the tumor. The fact that p300 is targeted by viral oncoproteins and is often found mutated or in a truncated form in breast, colorectal, and pancreatic cancers, indicate that p300 may act as a classical tumor suppressor (5). In prostate cancer, however, p300 and CBP clearly have oncogenic potential. Both p300 and CBP are coactivators of the androgen receptor (AR), the latter driving progression of prostate cancer by the regulating action of dihydrotestosterone (DHT) (6). Acetylation of AR has been shown to enhance coactivator binding and promote prostate cancer cell growth (7). Surgical or chemical androgen ablation is the first-line treatment of metastatic prostate cancer; however, after one to two years therapy eventually fails leading to the phenotype of castration-resistant prostate cancer. One of the mechanisms of castration resistance involves alterations in the balance between AR and its transcriptional coregulators (8). In fact, in advanced prostate cancer p300 and CBP are highly expressed and androgen deprivation leads to an upregulation of both proteins (9, 10). Moreover, it was postulated that p300 could regulate androgen-dependent genes in the
absence or in the presence of very low levels of AR (11). Interestingly, in several prostate
cancer cell lines the agonistic action of the anti-androgen hydroxyflutamide was enhanced
through CBP (12). Likewise, p300 mediates androgen-independent transactivation of the AR
by interleukin-6 (13), a cytokine which may facilitate prostate cancer progression through
stimulation of proliferation and angiogenesis and inhibition of apoptosis (14).

The p65 subunit of nuclear factor – κB (NF-κB) is a well-known target of p300 and CBP (15).
NF-κB is a transcriptional factor, which is frequently constitutively activated in cancer (16).
The prototypical NF-κB factor is a heterodimer of the p65 and p50 subunits belonging to the
NF-κB/Rel family. NF-κB is sequestered and kept inactive in the cytoplasm by inhibitor of
κB-α (IκBα). Upon pathway activation by different extracellular stimuli NF-κB shuttles to the
nucleus and transactivates hundreds of genes involved in different biological processes
including inflammation, proliferation, and cell survival (17). Besides their chromatin
remodeling function on κB elements, p300 and CBP also acetylate p65 itself. Recently, it has
been shown that the acetylation of lysine 310 of p65 impairs methylation of lysines 314 and
315, which is important for the ubiquitination and degradation of chromatin-associated p65
(18).

A variety of histone deacetylase (HDAC) inhibitors have been developed in the last years. In
fact, the U.S. Food and Drug Administration has licensed Vorinostat and Romidepsin for the
treatment of cutaneous T-cell lymphoma. Several other HDAC inhibitors are currently being
validated in clinical trials. Molecular mechanisms of HDAC inhibitors are induction of cell-
cycle arrest, differentiation, and tumor cell death. Induction of programmed cell death is
mediated by intrinsic and extrinsic apoptosis pathways, generation of reactive oxygen species,
inhibition of angiogenesis, and autophagy (19). On the other hand, the use of HAT inhibitors
as anti-cancer agents has received little attention, due to the lack of selective, potent, and cell
permeable inhibitors. Recently, a small molecule inhibitor of p300/CBP, C646, with these properties became available (20). Here we report on the potential use of p300/CBP inhibition in therapy of androgen-dependent and castration-resistant prostate cancer. To this end we selected two approaches: i) inhibition of p300 or CBP via protein downregulation with specific siRNAs and ii) chemical inhibition of p300/CBP’s HAT/KAT activity with the above mentioned compound.

Materials and Methods

Cell culture and authentication, transfections, and treatments

All cell lines were cultured in RPMI containing 10% FCS and 1% antibiotics and glutamax. LAPC-4 cells were supplemented with 100 nM DHT. Cell line authenticity was confirmed in August 2010 by short tandem repeat analysis following the procedure by Parson et al (21). Lipofectamine 2000 transfections were performed in serum- and antibiotics-free medium with 50 nM siRNA according to the manufacturer’s protocol (Invitrogen, Lofer, Austria). Target sequence for p300 was published previously (13), siControl and siCBP (On-target Plus Smart Pools) were purchased from Dharmacon (THP, Vienna, Austria). Six hours after transfection, medium was changed to full growth conditions. Cells were harvested 72 hours post-transfection. Since C646 was inhibited by serum (Fig. S1), treatment with the chemical inhibitors was performed for 24 hours in serum-free HITES medium (RPMI medium supplemented with 10 nM hydrocortisone, 10 nM estradiol, 5 μg/ml insulin, 100 μg/ml transferrin, and 30 nM sodium selenite).

Western Blot

Western Blot was performed as described before (22). For p300 and CBP 3-8% Tris/Acetate Gels and for p65, p50 and Bcl-xL 4-12% Bis/Tris Gels were used. Antibodies were purchased
from Santa Cruz Biotechnologies, Heidelberg, Germany (p300 C-20, CBP A-22, α-Tubulin),
Cell Signaling, Frankfurt, Germany (p65, p50), Chemicon/Millipore, Vienna (GAPDH), and
New England Biolabs, Frankfurt, Germany (Bcl-xL).

**Proliferation assays**

Cells were incubated for the last 16 hours with 37 kBq/well ³H-thymidine and DNA was
measured as described before (22).

**Apoptosis assays**

Cells were seeded in 6-wells and transfected with siRNA or treated with C37 or C646 on the
next day as described above. Cells were harvested and centrifuged. For flow cytometry cell
pellets were resuspended in propidium iodide (PI) buffer (0.2% Triton-X-100, 2 ng/ml Na-
Citrate, and 0.1 mg/ml PI) and cell cycle distribution was measured with a FACS Calibur
(Becton Dickinson, Heidelberg). For caspase 3/7, 9, and 8/10 activity assays cell pellets were
resuspended in 50 μl water and 5 μl of the cell suspension was mixed together with 20 μl of
PBS and 25 μl of the respective two-fold substrate (Caspase-Glo 3/7, 8, 9, Promega,
Mannheim, Germany). Another 5 μl of the cell suspension was used to quantify protein
concentration by the Bradford method. Luminescence was measured after twenty minutes
with Chameleon 5025 and values were normalized to protein amount. InSolution Caspase 8
(extrinsic pathway) or 9 (intrinsic pathway) inhibitors were purchased from Calbiochem
(VWR, Vienna) and used at a concentration of 2 nM. Expression of phosphatidylserine on cell
surfaces was corroborated using the PE Annexin V Apoptosis Detection Kit I (Becton
Dickinson) following the manufacturer’s protocol. Cell populations were analyzed with
FACS Calibur.

**PSA measurements**
Cells were incubated in serum-free medium for the last 24 hours prior to harvesting. Prostate-specific antigen (PSA) concentration was determined on an Advia Centaur XP Immunoassay System (Siemens, Munich, Germany). Cells were trypsinized and counted with a Casy Counter (Schärfe System, Reutlingen, Germany). PSA concentrations were normalized to cell number.

Quantitative PCR
Quantitative PCR was performed as described before (22). Taqman Primer/Probe mixes for p65, p50, Bcl-xL, and matrix metalloproteinase (MMP) -2 and -9 were purchased from Applied Biosystems (Vienna). Sybr Green Primers for p300 and CBP were published previously (23).

Scratch (wound healing) and Boyden chamber assays
Scratch and Boyden chamber assays were performed as described before (24). For invasion assays inserts were coated with Matrigel (Becton Dickinson) diluted 1:3 with serum-free medium.

Zymography
Cells were transfected as described above and after two days serum-free medium was added for the last 24 hours. Supernatant was collected and concentrated using Vivaspin 500 (VWR) to approximately 50 μl. Protein was quantified by the Bradford method and equal amounts of protein were loaded onto 10% Gelatin-Zymograms (Invitrogen). Gels were Coomassie-stained and decolored until clear bands indicating proteinase activity were visible.

Chromatin immunoprecipitation assays
Chromatin immunoprecipitations (ChIP) of p65 was performed with the ChIPAb+NFkB (RelA) antibody/primer set together with the EZ-Magna ChIP™ G kit (both from Millipore), according to the manufacturer’s instructions. AR ChIP was done on LNCaP and LAPC-4 cells that were starved for two days in RPMI containing 5% charcoal-stripped FCS. AR binding was induced with 1 nM R1881 together with C37 or C646 treatment for 4 hours. Cells were then fixed with 1% formaldehyde, lysed, and sonified to shear DNA. IP was performed with a mixture of anti-AR (Cell Signaling) and anti-AR (PG-21, Upstate/Millipore), and Protein A Sepharose CL-4B beads (GE Healthcare, Freiburg, Germany) overnight at 4°C, followed by four wash steps. Cross-linking was reversed by proteinase K digestion at 56°-65°C for 4 hours and DNA was purified with the ChIP DNA Clean & Concentrator Kit (Zymo Research, Anopoli, Eichgraben, Austria). Sybr Green qPCR was performed with the following primer pair of the PSA enhancer region: forward: 5´-GGG GTT TGT GCC ACT GGT GAG-3´; reverse: 5´-GGG AGG CAA TTC TCC ATG GTT C-3´. Values were normalized to input controls taken before IP step.

Statistical analysis

Student’s t-test was used to assess significant differences between siControl or C37-treated groups and the indicated treated group (unless identified otherwise in the figure legend) and were encoded as follows: * p<0.05; ** p<0.01; *** p<0.001.

Results

Specific downregulation of p300 by siRNA reduces proliferation and induces apoptosis.

Inhibition of p300 and CBP was carried out by siRNA transfections and was confirmed to be efficient in the castration-resistant cell line PC3 and in the androgen-sensitive cell line LNCaP three days after transfection (Fig. 1a). Quantitative PCR showed reduced mRNA levels of
p300 and CBP at 24, 48, and 72 hours after transfection and confirmed specificity of siRNAs (Fig. S2). To test whether inhibition of p300/CBP has anti-proliferative effects on prostate cancer cells, we measured \(^{3}\)H-thymidine incorporation on transfected PC3 and LNCaP cells (Fig. 1b). In PC3 depletion of both p300 and CBP resulted in a decreased proliferation, whereas LNCaP showed a significant decrease after p300 downregulation only. Moreover, decreased proliferation correlated with reduced PSA secretion in LNCaP cells, indicating a loss of transcriptional activity of the AR (Fig. 1c). Decreased \(^{3}\)H-thymidine incorporation can be a consequence of increased apoptosis. Therefore, we measured cell cycle distribution after staining the cells with PI (Fig. 1d). Quantification of the sub-\(G_1\) peak revealed that downregulation of p300 leads to an increase of apoptosis in both cell lines. Surprisingly, downregulation of CBP did not lead to an increase of apoptosis in either cell line. We concluded that expression of p300, but obviously not of CBP, is essential for the survival of PC3 and LNCaP cells.

**Induction of apoptosis by p300 depletion involves both extrinsic and intrinsic pathways.**

To determine whether induction of apoptosis by p300 depletion is a caspase-dependent mechanism, we measured activities of the extrinsic initiator caspases 8 and 10, the intrinsic initiator caspase 9, and the executioner caspases 3 and 7 (Fig. 2a). Downregulation of p300 led to a 13.8-fold and 4.2-fold increase in caspase 3/7 activity in PC3 and LNCaP, respectively. Again, downregulation of CBP had no effect on caspase 3/7 activity. Moreover, both extrinsic and intrinsic apoptotic pathways were activated to the same extent after p300 depletion (Fig. 2a). We confirmed the involvement of the extrinsic pathway by using specific inhibitors of caspases 8 and 9 (Fig. 2b). Indeed, inhibition of the extrinsic pathway by caspase 8 inhibitor led to a significant reduction of caspase 3/7 activity after p300 depletion in both cell lines. Repression of the intrinsic pathway resulted only in a minor reduction of caspase
3/7 activity. These findings show that apoptosis is induced primarily via the extrinsic pathway.

**Downregulation of p300 induces p65 degradation.**

Constitutive activation of NF-κB is a widely observed phenomenon in several cancer types including prostate cancer, as NF-κB regulates a variety of apoptotic antagonists (25). We hypothesized that in the absence of p300 proper signaling of NF-κB is inhibited, thereby rendering the prostate cancer cells sensitive to apoptotic stimuli. We analyzed expression levels of the two main NF-κB subunits p65 (Rel A) and p50 after treatment with specific siRNAs for p300 and CBP. Indeed, in p300-depleted cells the protein level of p65 was decreased and this reduction was accompanied by the appearance of a higher molecular weighted band (Fig. 3a). Moreover, mRNA levels of p65 were not affected significantly (Fig. 3a), which led to the conclusion that p65’s degradation rate is probably increased after p300 downregulation. Analysis of p50 showed slight upregulation of protein levels in both p300-depleted PC3 and p300- or CBP-depleted LNCaP (Fig. 3b), while p50 mRNA levels were not changed (Fig. 3b). Taken together, these data indicate that upon p300 depletion NF-κB signaling is impaired through p65 degradation.

**Inhibition of p300 decreases the invasive capacity of prostate cancer cells through a MMP-2 and -9 dependent mechanism.**

The metastatic cascade is a well-regulated molecular process underlying different steps, including epithelial–mesenchymal transition, invasion, resistance to anoikis, angiogenesis, transport through vessels, and outgrowth of secondary tumors (26). Due to p300/CBP’s interaction with a large number of pathways, we tested whether the two coactivators also interfere with metastatogenesis. Indeed, in scratch assays p300- or CBP-depleted PC3 cells showed impaired wound healing (Fig. 4a). A reduced migration was confirmed after p300 or
CBP downregulation by measuring transition of PC3 and LNCaP cells through 8 μm pores of a PET membrane in Boyden chambers assays (Fig. 4b). The same Boyden chambers were coated with matrigel and used to measure invasion (Fig. 4c). Interestingly, only p300 depletion resulted in a significant reduction of invasiveness in both cell lines showing again that p300 and CBP can regulate different cellular processes. MMPs are enzymes that degrade among others collagen and gelatin thereby regulating the tumor microenvironment. It has been shown that MMP-2 and -9 are expressed in prostate cancer (27, 28). Using gelatin-zymography we measured the activity of MMP-9. PC3 cells showed a reduced protease activity after p300 depletion. This was corroborated with a reduced transcription of the MMP-9 gene (Fig. 4d). The mRNA levels of MMP-2, a known activator of pro-MMP-9 (29), were also significantly decreased upon sip300 transfection. Interestingly, MMP-2 and -9 mRNA expressions in LNCaP were nearly undetectable (data not shown). Reduced invasion capacity of LNCaP cells was supported by a diminished number of cells undergoing migration, when comparing coated and uncoated Boyden chamber assays (Fig. 4b, c). Taken together, we conclude that both CBP and p300 play a role in regulating motility of prostate cancer cells. However, by regulating the expression of MMP-2 and -9, p300 appears to be most critical in impacting the molecular pathways of invasion.

The chemical p300 inhibitor C646 induces apoptosis in androgen-sensitive and castration-resistant prostate cancer cell lines by interfering with AR and NF-κB pathways.

To support our findings of reduced survival by inhibiting p300 with the siRNA approach, we tested whether chemical inhibition of p300 would also lead to these effects. The compound C646 specifically inhibits the acetyltransferase activities of p300 and CBP (20), whereas C37 is an analog control compound that lacks acetyltransferase inhibitory properties (Fig. S3). We tested the ability of C646 to induce apoptosis in two androgen-independent cell lines, PC3
and Du145, and two androgen-sensitive cell lines, LNCaP and LAPC-4. In caspase 3/7 activity assays, both PC3 and Du145 and in particular LNCaP showed an increase of apoptosis after treatment with 10 and 20 μM C646, but were insensitive to the control compound C37 (Fig. 5a). Interestingly, LAPC-4 showed only a marginal increase of caspase 3/7 activity at the highest concentration. For this reason 20 μM C646 was used in subsequent experiments. Apoptosis induction was confirmed by Annexin-V staining of C646-treated cells (Fig. 5b). Moreover, an induction of caspase 8/10 and caspase 9 activity after C646 treatment could be observed, showing again an involvement of both extrinsic and intrinsic pathways after inhibition of p300 (Fig. S4). Next, we analyzed whether AR and NF-κB pathways were affected after treatment with C646. In LNCaP and LAPC-4 PSA secretion was markedly reduced after treatment with 20 μM C646 (Fig. 6a). However, chromatin binding of AR was not affected by C646, suggesting that acetylation of AR is not a prerequisite for its DNA binding activity (Fig. S5). Furthermore, decreased expression levels of p65 were observed in all cell lines (Fig. 6b), which was associated with a decreased binding of p65 at the IκBα promoter (Fig. 6c). Bcl-xL, an anti-apototic member of the Bcl-2 family, which is regulated by NF-κB, was also decreased in all cell lines except LAPC-4, giving a possible explanation for the low sensitivity of LAPC-4 to apoptotic induction by C646 (Fig. 6b). Moreover, both MMP-2 and -9 mRNA expressions were significantly decreased in PC3 cells, showing that C646 may also interfere with cell invasion (Fig. 6d). Altogether, these results show that chemical inhibition of p300/CBP’s HAT/KAT function is leading to induction of apoptosis via distinct molecular pathways.

**Discussion**

**Differential functions of p300 and CBP.**
In the cellular processes of induction of apoptosis and invasion we found differential functions of p300 and CBP by using specific siRNAs. In the past, differential properties of p300 and CBP have been found in various cellular pathways. For example, Kawasaki and colleagues found that p300, but not CBP is needed for retinoic acid-induced differentiation of embryonal carcinoma F9 cells, whereas both proteins were required for the induction of apoptosis and G1-arrest (30). Furthermore, multilineage defects in hematopoietic differentiation were found in mice with a monoallelic inactivation of the CBP gene. However, these pathologies could not be observed in p300 heterozygous mice, suggesting that a full complement of CBP, but not p300 is required for normal hematopoietic differentiation (31).

For more examples on differential roles of p300 and CBP we refer to an interesting review by Kalkhoven (1). Recently, a genome-wide ChIP-Seq has been performed on cell cycle-synchronized cells with specific antibodies against p300 or CBP (32). Interestingly, significant differences in the levels and binding targets of both proteins were observed. Among processes differentially regulated by p300/CBP binding were also cell death and cell adhesion pathways. Taken together, those studies support our findings of differential effects of p300 and CBP.

**Induction of apoptosis by p300 inhibition through multiple pathways.**

Since it is known that p300 and CBP interfere with multiple pathways, we considered that induction of apoptosis is not restricted to one pathway. The AR is of particular importance in LNCaP and LAPC-4 cells that will stop growing after androgen depletion (33, 34). Knockdown of p300 decreased PSA secretion, which is an indicator of diminished AR activity. AR-regulated transcription may be impaired due to the lack of p300 as an important coactivator of AR. Furthermore, during prostate cancer progression from androgen-dependence to castration resistance, crosstalk between transduction pathways plays an important role in the ligand-independent activation of the AR (8). For example, it has been
shown that IL-6 activation of AR-dependent genes in the absence of androgens is mediated by p300 (13). Thus, a combined treatment of AR blockade (e.g. bicalutamide) for ligand-dependent and p300 inhibition for ligand-dependent and -independent activation of the AR could be of potential interest.

In the second pathway, we found that p300 depletion leads to a decreased p65 level, apparently through increased turnover of the protein. This is in line with the results of a previous study, where deacetylation of lysine 310 of p65 led to Set9-mediated methylation of p65 at lysines 314 and 315, which, in turn resulted in polyubiquitination and proteasomal degradation of chromatin-associated p65 (18). Although we were not able to identify the nature of the higher molecular weighted band recognized by the p65 antibody in Fig. 3a, it seems to be linked to the absence of p300 rather than to the inhibition of p300’s acetyltransferase activity, since it was not observable after treatment with C646. Constitutive activation of NF-κB can be found in many prostate cancer cell lines and frequently correlates with AR downregulation (35). It was proposed that NF-κB may compensate for the lack of androgen-induced AR activity by activating the expression of several AR target genes (35). Indeed, this possibility was demonstrated in LNCaP cells, where NF-κB activated the expression of PSA (36). Thus, compromised NF-κB signaling can confer a decrease in NF-κB- and AR-responsive genes, resulting in a lack of activated pro-survival pathways and rendering prostate cancer cells susceptible to apoptotic induction. Interestingly, degradation of p65 after p300 downregulation could also be seen in LNCaP cells that do not show constitutively activated NF-κB signaling (37). This correlated also with a lower apoptotic rate when comparing LNCaP to PC3 cells (Figs. 1d, 2a). Thus, p65 downregulation might be less important in LNCaP cell growth effects.
The involvement of the extrinsic pathway in induction of apoptosis after p300 inhibition was demonstrated by the use of caspase 8/10 activity assays and inhibitors of caspase 8 and/or 9. It is well known that the activation of the extrinsic pathway turns on the intrinsic pathway by the action of Bid (38), while transactivation of the extrinsic by the intrinsic pathway has not been demonstrated. Therefore, high caspase 9 activity could be induced by crosstalk with the extrinsic pathway. On the other hand, we have also found deregulation in the AR- and NF-κB-signaling pathways leading to a lack of pro-proliferative stimuli. Hence, deregulation of intracellular pathways sensitizes cells to apoptosis induction. We therefore hypothesize that both intrinsic and extrinsic pathways contribute to programmed cell death after p300 inhibition. Interestingly, HDAC inhibitors also activate both apoptotic pathways (19), although it seems that in this case the intrinsic one has a superior role over the extrinsic pathway.

**Decrease of invasion by p300 inhibition.**

Inhibition of tumor cell dissemination is of particular interest in prostate cancer, since most patients die from metastatic lesions, rather than from the primary tumor. In addition to the pro-apoptotic effect of p300 inhibition, we have found a decrease in cell migration and invasion. In fact, this reduction in migration and invasion is certainly also due to the increase of apoptotic cells. However, since we have found that p300 inhibition decreases expression of MMP-2 and 9, we conclude that p300 also interferes, as a distinct event, with the cellular pathways that regulate invasion. Interestingly, CBP depletion also led to a decrease in migration, but not invasion in either cell line. Thus, we concluded that both p300 and CBP are important for the regulation of migration, while only p300 is essential for invasion. Moreover, MMP-2 and -9 are known targets of NF-κB regulation (39). It has been shown that MMP-9 is downregulated in PC3 cells that have been modified to have low NF-κB activity by the introduction of a mutant form of IκBα (40). Hence, the decrease of p65 after p300 inhibition
may lead to lower NF-κB activity and MMP-9 expression. *Vice versa*, the high activity of NF-κB in untreated PC3 cells can explain the elevated expression of MMP-9, whereas in LNCaP cells, that have low NF-κB activity, MMP-2 and -9 were only faintly expressed. Since p300 inhibition could still decrease LNCaP invasion, we deduce that p300 also regulates other pathways important for invasion.

**Potential of chemical p300 inhibition.**

In addition to downregulation of p300/CBP by specific siRNAs we have used the small molecule C646, which has been shown to specifically inhibit the HAT/KAT domain of p300 and CBP (20). C646 is a competitive inhibitor with a $K_i$ of 400 nM and is the most potent p300/CBP inhibitor in live cells (20). We could observe similar effects of C646 on caspase activity, PSA secretion, and p65 expression levels compared to cells transfected with p300 siRNA. This led to the conclusion that apoptosis induction is dependent on the inhibition of p300’s HAT/KAT function. Moreover, the four examined cell lines showed varying sensitivity to C646, which leads to the conclusion that C646’s effects might be cell type-, time- and dose-dependent. Several other p300/CBP HAT inhibitors are known, e.g. the phytoextract curcumin (41). Indeed, it has been shown that curcumin is able to induce apoptosis and to potentiate TNF-α-induced apoptosis and suppress NF-κB activation in Du145 and LNCaP cells (42). However, curcumin’s role as acetyltransferase inhibitor has not been investigated in that study. Another example with p300/CBP HAT inhibiting abilities is plumbagin (43). Plumbagin induces apoptosis and inhibits invasion of prostate cancer cells by several mechanisms including blocking of NF-κB’s DNA-binding (44). Again, in this context the role of plumbagin as acetyltransferase inhibitor was not analyzed. In this sense, we provide a possible functional link between the anti-cancer properties of the above mentioned plant extracts and their p300/CBP HAT inhibiting properties.
Overall, our study shows that siRNA-mediated downregulation of p300 (but not of CBP) and its chemical inhibition by the small molecule C646 led to induction of caspase-dependent apoptosis in several androgen-dependent and castration-resistant prostate cancer cells. Furthermore, p300 depletion led to a decrease of migration and invasion and may thus inhibit tumor cell dissemination. Therefore, targeting p300 by HAT inhibitors may be a promising approach in therapy of prostate cancer.
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References


Figure Legends

**Figure 1: Differential effects of p300 and CBP on proliferation and survival of prostate cancer cell lines.**

PC3 and LNCaP were transfected with indicated siRNAs and grown in medium containing 10% FCS for additional 72h. (a) A representative Western Blot with corresponding densitometrical analysis is shown to demonstrate the efficiency of p300 and CBP downregulation. (b) Proliferation was assessed by $^3$H-thymidine incorporation. (c) PSA was measured from the 24h supernatant of LNCaP cells and concentration was normalized on cell number. (d) Cells were stained with PI and cells in sub-G$_1$-phase, indicating apoptosis, were quantified. (b-d) Values indicated are mean ± SEM, n≥3. RLU, relative light units.

**Figure 2: Apoptosis is induced through both extrinsic and intrinsic pathways in p300-depleted PC3 and LNCaP.**

PC3 and LNCaP were transfected with indicated siRNAs and grown in medium containing 10% FCS for additional 72h. (a) Activity of the executioner caspases 3/7 and the initiator caspases 9 (intrinsic) and 8/10 (extrinsic) were measured with the respective luminescent substrates. Statistical significances are calculated against the respective siControl-treated sample. (b) Either extrinsic or intrinsic or combination of both pathways were blocked by the indicated inhibitors and caspase 3/7 activity was measured. Statistical significances are calculated against DMSO and sip300-treated sample. (a,b) Values indicated are mean ± SEM, n≥3. RLU, relative light units.

**Figure 3: p300 depletion leads to decreased p65 expression levels.**

PC3 and LNCaP were transfected with indicated siRNAs and grown in medium containing 10% FCS for additional 72h. (a) Western Blot and quantitative PCR were performed with a
p65-specific antibody and primer/probe mix, respectively. (b) Western Blot and quantitative PCR were performed with a p50-specific antibody and primer/probe mix, respectively. (a, b) GAPDH was used as a reference in Western Blot experiments and in quantitative PCR assays values were normalized to HPRT1 and are indicated as fold regulation. No significant differences between mRNA levels were found. Values indicated are mean ± SEM, n≥3.

Figure 4: Downregulation of p300 decreases migration and invasion.
(a) A scratch assay was performed on a confluent layer of PC3 cells transfected 72h before as indicated. Pictures were taken at time points 0h and 9h. (b) PC3 and LNCaP cells were transfected as indicated, seeded on the next day in Boyden chambers, and grown for additional two (PC3) or four (LNCaP) days. Nuclei of migrated cells were stained with DAPI and cell number was assessed in five field of views (FOVs) by fluorescent microscopy with an automatic cell counter. (c) Assay was performed as in (b), except Boyden chambers were coated with a 1:3 dilution of Matrigel. (d) The gelatinase activity of MMP-9 was measured by zymography in the supernatant of PC3 cells that have been transfected 72h before as indicated. Quantitative PCR for MMP-2 and-9 mRNA expression was performed in parallel and values were normalized to HPRT1 expression. (b-d) Values indicated are mean ± SEM, n≥3.

Figure 5: The p300/CBP HAT inhibitor C646 induces apoptosis.
(a) PC3, LNCaP, Du145, and LAPC4 cells were treated with increasing concentrations of C646 or its control compound C37 in HITES medium for 24h as indicated. Activity of caspase 3/7 was measured by addition of a twice-concentrated luminescent substrate of caspase 3/7. (b) PC3, LNCaP, Du145, and LAPC4 cells were treated with 20 μM of C646 or its control compound C37 in HITES medium for 24h, as indicated. Apoptotic cells were
stained with PE Annexin-V and percentage of positive cells was determined by flow cytometry. (a, b) Values indicated are mean ± SEM, n≥3. RLU, relative light units.

Figure 6: The p300/CBP HAT inhibitor decreases PSA secretion and p65 expression levels.

(a) LNCaP and LAPC-4 cells were treated with 20 μM of C646 or its control compound C37 in HITES medium as indicated. After 24h, secreted PSA in the supernatant was measured and normalized to the respective cell number. (b) PC3, LNCaP, Du145, and LAPC-4 cells were treated with 20 μM of C646 or its control compound C37 in HITES medium as indicated. After 24h, cells were harvested and p65 and Bcl-xL expression were determined by Western Blotting, GAPDH served as a loading control. A representative Western Blot and ratio of densitometrically analyzed bands of all experiments are shown. (c) ChIP on the IκBa promoter with anti-p65 antibodies was performed on PC3 cells treated with 50 ng/ml TNF-α and 20μM C37 or C646, as indicated. A representative experiment performed in triplicate (Mean ±SD) is shown. (d) Quantitative PCR for MMP-2 and-9 mRNA expression was performed on PC3 cells treated with C37 or C646. Values were normalized to HPRT1 expression. (a, b, d) Values indicated are mean ± SEM, n≥3.
Figure 1, Santer et al., 2011
Figure 2, Santer et al., 2011
Figure 3, Santer et al., 2011
Figure 4, Santer et al., 2011
Figure 5, Santer et al., 2011
Figure 6, Santer et al., 2011