Generation of a Selective Small Molecule Inhibitor of the CBP/p300 Bromodomain for Leukemia Therapy

Sarah Picaud1,2, Oleg Fedorov1,3, Angeliki Thanasopoulou4, Katharina Leonards4, Katherine Jones5, Julia Meier1,3, Heidi Olzschka6, Octavio Monteiro1,3, Sarah Martin1,3, Martin Philipott1,3, Anthony Tumber1,3, Panagis Filippakopoulos1,2, Clarence Yap3, Christopher Wells1,3, Ka Hing Che7, Andrew Bannister7, Samuel Robson7, Umesh Kumar6, Nigel Par5, Kevin Lee5, Dave Lugo8, Philip Jeffrey8, Simon Taylor9, Matteo L. Vecellio3, Chas Bountra1, Paul E. Brennan1,3, Alison O’Mahony9, Sharlene Velichko9, Susanne Müller1,3, Duncan Hay3, Danette L. Daniels10, Marjeta Urh10, Nicholas B. La Thangue6, Tony Kouzarides7, Rab Prinjha5, Jürg Schwaller4, and Stefan Knapp1,3

Abstract

The histone acetyltransferases CBP/p300 are involved in recurrent leukemia-associated chromosomal translocations and are key regulators of cell growth. Therefore, efforts to generate inhibitors of CBP/p300 are of clinical value. We developed a specific and potent acetyl-lysine competitive protein–protein interaction inhibitor, I-CBP112, that targets the CBP/p300 bromodomains. Exposure of human and mouse leukemic cell lines to I-CBP112 resulted in substantially impaired colony formation and induced cellular differentiation without significant cytotoxicity. I-CBP112 significantly reduced the leukemia-initiating potential of MLL-AF9+ acute myeloid leukemia cells in a dose-dependent manner in vitro and in vivo. Interestingly, I-CBP112 increased the cytotoxic activity of BET bromodomain inhibitor JQ1 as well as doxorubicin. Collectively, we report the development and preclinical evaluation of a novel, potent inhibitor targeting CBP/p300 bromodomains that impairs aberrant self-renewal of leukemic cells. The synergistic effects of I-CBP112 and current standard therapy (doxorubicin) as well as emerging treatment strategies (BET inhibition) provide new opportunities for combinatorial treatment of leukemia and potentially other cancers. Cancer Res; 75(23); 5106–19. © 2015 AACR.

Introduction

The cyclic AMP responsive element binding protein (CREB)-binding protein (CBP; CREBBP) and p300 (adenovirus E1A-associated 300-kD protein) are two closely related and evolutionary conserved histone acetyl transferases [HAT; ref. 1]. CBP/p300 function as transcriptional regulators by acetylating histone tails and other nuclear proteins. They also act as scaffolds recruiting transcription factors to transcriptional active loci through a large diversity of protein interaction domains. The HAT activity of CBP is regulated by autoacetylation of a basic surface loop region that competes with substrate binding and by the presence of a RING domain located adjacent to the catalytic HAT domain (2). Interestingly, CBP/p300-mediated acetylation creates binding sites for the acetyl-lysine–specific CBP/p300 bromodomain, which is required for proper substrate targeting, resulting in a positive feedback loop and maintenance of CBP/p300 enzymatic activity (2). Homozygous loss of either Cbp or p300 leads to embryonic lethality in utero in mice due to developmental defects including impaired hematopoiesis (3, 4). Studies in heterozygous mice have characterized Cbp as an essential regulator of hematopoietic stem cell (HSC) self-renewal. Likewise, conditional ablation of Cbp in adult mice altered differentiation, quiescence, apoptosis, and self-renewal of adult HSCs (5).

CBP/p300 has been functionally linked to the development of multiple human cancers, including solid tumors and hematologic...
CBP/p300 has been detected in several oncogenic fusions in leukemia involving either the MOZ acetyltransferase or the mixed lineage leukemia (MLL) gene product promoting cell proliferation (7, 8). The most prevalent is the chromosomal translocation t(11;16)(q23;p13) associated with mostly therapy-related acute myeloid leukemia (AML) or myelodysplastic syndromes resulting in a fusion protein that contains the bromodomains of CBP and parts of MLL (8, 9). CBP/p300 was also proposed to act as transcriptional coactivator of other leukemicogenic proteins such as the NUP98-HOXA9 fusion (10). Recently, it has been shown that p300 interacts with the AML1-ETO fusion protein, present in over 20% of human AML, and that it regulates transcription of multiple AML1–ETO target genes including Idl (inhibitor of DNA binding 1), the cell-cycle inhibitor p21 and Egr1 (early growth response 1) that are drivers of self-renewal of hematopoietic stem/progenitor cells (11). Furthermore, inhibition of p300 abrogates acetylation of AML1-ETO and impaired clonogenic growth in vitro and leukemic transformation in vivo (12).

The multidomain organization of CBP/p300 has prompted several inhibitor development projects. The HAT activity has been targeted by natural compounds that mostly lacked specificity (13). The most potent HAT inhibitor developed so far is C646, which has low micromolar activity (14). C646 inhibits growth of tumor cell lines and promotes cellular senescence and reduced proliferation of leukemic cells and AML patients (15). In addition, "ischemin" and a number of nonspecific fragments with micromolar potency have been reported to target the CBP bromodomain (16, 17).

Here, we report a selective and highly potent chemical probe compound targeting the bromodomains of CBP/p300. Bromodomains are protein interaction domains with predicted good druggability (18), which has been demonstrated by potent and selective inhibitors developed for the BET (bromo and extracellular domain) family of transcriptional regulators (19–22). We developed the acetyl-lysine mimetic oxazepine inhibitor I-CBP112 that binds to CBP/p300 with nanomolar affinity and good selectivity. In leukemia, we found that I-CBP112 impaired the disease-initiating self-renewal leukemic cells in vitro and in vivo without causing significant cytotoxicity.

Materials and Methods

Protein purification

cDNA encoding human bromodomains were cloned, expressed, and purified as previously described (19). For purification of in vivo biotinylated protein expression, the same construct boundaries (e.g., CBP residues R1081-G1198) were bromodomain subcloned into pNIC-BIO1 vector, a derivative from pNIC28-Bsa4 vector (Gene Bank: EF198106) containing a 10 His-tag and TEV protease cleavage site at the N-terminus and an in frame biotinylation sequence (SSKGCGGLNDIFEAQKIEWHE) inserted at the C-terminus. The constructs were transformed into BL21 (DE3)-R3-BirA cell line (BL21 derivative coexpressing BirA using a pACYC coexpression vector). Cells were grown overnight at 37°C in 10 mL of Luria-Bertani medium with 50 μg/ml kanamycin and 34 μg/ml chloramphenicol. The start-up culture was diluted 1:1000 in fresh medium and cell growth was allowed at 37°C to an optical density of about ~1.0 (OD600) before the temperature was decreased to 25°C. D-Biotin was dissolved into 10 mM bicine pH8.3 and added to the culture at 500 μM final. The protein expression was induced for 8 hours at 25°C with 50 μM isopropyl-β-D-thiogalactopyranoside. Proteins were purified using Ni-affinity chromatography and size exclusion chromatography.

Thermal shift assay

Thermal melting experiments were carried out using an Mx3005p Real-Time PCR machine (Stratagene) as described (23). Proteins were buffered in 10 mM HEPES pH 7.5, 500 mM NaCl, and assayed in a 96-well plate at a final concentration of 2 μM in 20 μL volume. Compounds were added at a final concentration of 10 μM. SYPRO Orange (Molecular Probes) was added as a fluorescence probe at a dilution of 1:1000.

AlphaScreen assay

Assays were performed as described previously (24), with minor modifications from the manufacturer’s protocol (PerkinElmer). A 1:1 point 1:2.5 serial dilution of the ligands was prepared over the range of 5000–0 μM and 0.1 μL transferred to low-volume 384-well plates filled with 5 μL of the assay buffer (ProxiPlate-384 Plus, PerkinElmer), followed by 7 μL of biotinylated peptide H-ALREIRRYQK(ac)STELLIRKLK(biotin)-OH and His-tagged protein to achieve final assay concentrations of 50 nM. Plates were sealed and incubated for a further 30 minutes before the addition of 8 μL of the mixture of streptavidin-coated donor beads (12.5 μg/ml) and nickel chelate acceptor beads (12.5 μg/ml) under low light conditions.

Cell culture and reagents

Human cell lines (MOLM13, MONOMAC, THP1, KOCI45, RS4;11, SEM, MV4;11, KOCI44, KOPN8, KASUMI-1, MUITZ25, HL60, K562, PL21, REH, U937) were obtained from ATCC and the Leibnitz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (www.dsmz.de). Cell lines were cultured in RPMI-1640 medium (Sigma) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin (Gibco). Synthesis of I-CBP112 and related analogues will be described elsewhere. The inhibitor was dissolved in DMSO at a concentration of 10 mM and diluted further in culture medium RPMI-1640 immediately before use.

I-CBP112 in an in vivo model of MLL-AF9+ leukemia

Leukemic blasts expressing MLL-AF9 were treated in liquid culture with 5 μM of I-CBP112 for 3 days. Control cells were exposed to the corresponding concentration of the DMSO vehicle. The 50 × 103 treated cells were then transplanted into sublethally irradiated (600 Rad) syngeneic mice via tail vein injection. Upon the development of signs of disease, the mice were sacrificed and analyzed. All experiments were done according to Swiss laws for animal welfare and approved by the Swiss Cantonal Veterinary Office of Basel (Basel, Switzerland).

RNA preparation and gene expression profiling

KASUMI-1, SEM, and MOLM13 cells were treated with 3 μM of I-CBP112 or the DMSO vehicle for 4 days. On the third day of treatment, the cells were replated in fresh medium with or without the inhibitor. Total RNA was extracted using the RNA extraction kit (Macherey-Nagel, GmbH & Co.) according to the manufacturer’s protocol. Primers for q-PCR and chromatin immunoprecipitation (ChIP) analysis are provided in the Supplementary Methods.
Peripheral blood samples were collected with informed consent from patients with newly diagnosed or relapse AML. Mononuclear cells were separated by Ficoll-Histopaque and frozen in 10% DMSO. Cells were then used for colony formation assays, as described above.

Drug synergism
KASUMI-1, SEM, and MOLM13 cells were plated in a 9-well plate and treated with iCBP112 combined with doxorubicin or IQ1 in cytotoxicity assays. Eight different concentrations of iCBP112 and 15 of IQ1 and doxorubicin were used in ratios from 8:1 to 1:16 (Supplementary Fig. 12A). As 1X, we set

**Figure 1.**
*In vitro* characterization of I-CBP112. A, chemical structure of I-CBP112 and the BET inhibitor I-BET762. B, selectivity of I-CBP112 assessed by temperature shift data. Temperature shifts are shown as spheres as indicated in the figure and were mapped to the phylogenetic tree of the bromodomain family. Screened targets are labeled in black and targets that were not screened are shown in gray. C, isothermal titration calorimetry titration for the bromodomain of CBP (a), p300 (b), and bromodomains BRD4(1) (c), and BRD4(2) (d), respectively. Shown are raw titration heats (top) as well as binding isotherms shown as normalized binding heats (bottom). Nonlinear least squares fits to the data are shown as solid lines and fitted parameters are included in Supplementary Table S2. D, concentration dependent sensograms (BLI) of I-CBP112 binding to CBP.
approximately the IC_{50} concentration. After excluding the concentrations that gave either no effect or extreme effect for the single treatment (Supplementary Fig. 13), we ran the CompuSyn software and calculated the combination index (CI) value using the Chou-Talalay method, where CI<1 indicates synergism.
Crystallization and structure solution

Aliquots of the purified proteins were set up for crystallization using a mosquito crystallization robot (TTP Labtech). Coarse screens were typically set up onto Greiner 3-well plates using three different drop ratios of precipitant to protein per condition (100 ± 50 nL, 75 ± 75 nL, and 50 ± 100 nL). All crystallizations were carried out using the sitting drop vapor diffusion method at 4°C. CBP crystals with I-CBP112 (2 mM final concentration) were grown by mixing 200 nL of the protein (8.6 mg/ml) with 100 μL of reservoir solution containing 0.1 M MgCl₂, 0.1 M MES pH 6.0, 20% PEG 6K, and 10% ethylene glycol. Crystals were cryo-protected using the well solution supplemented with additional ethylene glycol and were flash frozen in liquid nitrogen. Data were collected at diamond beamline I04 at a wavelength of 1.0121 Å. Phases were obtained by molecular replacement using an ensemble of known bromodomain models (PDB IDs 2OSS, 2OUO, 2GRC, 2OO1, 3DAI, 3D7C, 3DWY). The models and structure factors have been deposited with PDB accession codes: 4NR6. Additional experimental details are available in Supplementary Data.

Results

Discovery of I-CBP112

On the basis of the reported weak CBP/p300 bromodomain binding activity of BET inhibitors such as JQ1 and I-BET762 we analyzed the CBP/p300 activity of a series of related compounds that all contained a benzo-oxazepine core structure (Fig. 1A). Hit expansion at several positions led to I-CBP112 with good activity against CBP/p300. The description of the structure activity relationship (SAR) of this compound class as well as the synthesis of I-CBP112 and its intermediates will be reported elsewhere.

I-CBP112 is a potent and selective CBP/p300 inhibitor

We used temperature shift assays for the characterization of I-CBP112 potency and selectivity within the bromodomain family. This assay monitors binding of an inhibitor to a protein based on the magnitude of temperature stabilization (23). I-CBP112 showed significant stabilization of CBP/p300 melting temperatures (7.8°C and 8.6°C, respectively) while ΔTm values were low for 41 other members of the human bromodomain family suggesting excellent selectivity of the developed inhibitor (Fig. 1B; Supplementary Table S1). We investigated I-CBP112 selectivity further using biolayer interferometry (BLI) as an additional selectivity screening technology. Similar to the related surface plasmon resonance, this technology measures interactions of ligands with biomolecules immobilized on a surface monitoring changes in refractive index. To confirm the selectivity of I-CBP112, we screened a panel of 42 representative bromodomains that were homogenously biotinylated in vivo during recombinant expression. BLI confirmed the excellent selectivity measured by temperature shift experiments identifying only bromodomains of the BET family members as having weak off-target activity (Supplementary Fig. S1). Finally, we determined affinity of I-CBP112 for CBP/p300 in solution using isothermal titration calorimetry.
Figure 4.
BioMAP data for I-CBP112 and effects on mouse MLL-CBP-immortalized bone marrow cells. A, BioMAP data of four hematopoietic coculture systems (Sag and LPS, peripheral blood mononuclear cells cultured with venular endothelial cells; BT, B cells and peripheral blood mononuclear cells; IMphg, venular endothelial cells cocultured with M1 macrophages) treated with JQ1 or I-CBP112. The full BioMAP and explanation of the monitored marker proteins is shown in Supplementary Fig. S2. B, correlation of the phenotypic response of the panBET inhibitors JQ1, PFI-1, IBET-151, CBP30, and I-CBP112. Responses to compound inhibition above the selected threshold (Pearson $r^2 > 0.6$) are represented by connected dots. C, cytotoxicity (WST1 assay) of I-CBP112 on murine bone marrow cells immortalized by retroviral expression of the MLL-CBP fusion oncogene. Shown is the mean percentage normalized to vehicle-treated control; error bars represent $\pm$SD; $P$ values were calculated by using ANOVA and Dunnett multiple comparison; $^{* *}$, $P < 0.01$; $^{* * *}$, $P < 0.0001$; $n = 4$. D, clonogenic growth and replating assay of mouse MLL-CBP immortalized bone marrow-derived murine progenitors in methylcellulose, treated with I-CBP112. Shown is the mean percentage normalized to vehicle-treated control; error bars represent $\pm$SD; $P$ values were calculated by using ANOVA and Dunnett multiple comparison; $^{* *}$, $P < 0.01$; $^{* * *}$, $P < 0.0001$; $n = 2$. E, morphologic changes of MLL-CBP immortalized progenitors on cellular and colony level upon exposure to increasing concentrations of CBP112 (left). Percentage of blasts and differentiated cells scored on cytospots. Shown is the mean percentage normalized to vehicle-treated control; error bars represent $\pm$SD; $P$ values were calculated by using ANOVA and Dunnett multiple comparison; $^{* * *}$, $P < 0.0001$; $n = 20$ scored high-magnification (>60X) fields (right).
(ITC), which resulted in determination of a dissociation constant ($K_d$) of $151 \pm 6$ nmol/L and $167 \pm 8$ nmol/L for CBP and p300, respectively (Fig. 1C; Supplementary Table S2). Dose-dependent BLI experiments resulted in a comparable $K_d$ value for CBP ($K_d = 142$ nmol/L; Fig. 1D). The most significant off-target activity in our selectivity screen was against BRD4, which showed a $\Delta T_m$ of 2.1°C and 0.6°C for the first [BRD4(1)] and second [BRD4(2)] bromodomain, respectively. ITC titrations resulted in the determination of $K_d$s of 5.6 nmol/L and 20 nmol/L for BRD4(1) and BRD4(2), demonstrating 37 and 132 fold selectivity, respectively (Fig. 1C; Supplementary Table S2).

To assess selectivity of I-CBP112 outside the bromodomain family, we screened both stereoisomers against a commercial selectivity panel including 104 binding assays monitoring binding to nuclear receptors, ion channels, and 32 enzyme assays (including 10 kinases, 9 proteases, and 5 phosphodiesterases). Both I-CBP112 stereoisomers showed similar behavior when screened against this panel that revealed only weak interaction with two GPCRs (ø1A and 5HT1) in the low micromolar region (Supplementary Table S3). The S-isomer was selected as chemical probe compound and was used in all subsequent cellular assays.

**I-CBP112 is an acetyl-lysine competitive inhibitor**

To establish a functional assay we studied the specificity of the CBP bromodomain towards histone peptide target sequences by synthesizing acetylated and nonacetylated histone peptides on a SPOT cellulose array (19). We found that the CBP bromodomain did not bind nonacetylated control peptides confirming high specificity for the acetyl-lysine modification (Fig. 2A). The peptide screening data revealed strong interaction of CBP with the histone-3 acetylated lysine 56 (H3K56ac), a CBP HAT substrate site, as well as interaction with H3K36ac and H3K79ac and H4K12ac and H4K44ac. Polyacetylation seemed to increase binding for some of the histone H4 acetylation marks. However, ITC data showed that the CBP bromodomain had similar affinities for mono- and diacetylated peptides and the determined stoichiometries of 0.5 for polyacetylated peptides suggested that two bromodomains simultaneously bound one diacetylated peptide supporting a canonical binding model in which one acetyl-lysine interacted with one CBP bromodomain (Fig. 2B; Supplementary Table S4). The strongest interaction was observed with H3K56ac ($K_d = 13.8$ nmol/L), which was used for the development of an Amplified Luminescence Proximity Homogeneous Assay (AlphaScreen; ref. 24). However, we find it likely that additional interaction with acetylation sites other than H3K56ac may occur in the context of intact chromatin in cells. The developed assay demonstrated that I-CBP112 is an acetyl-lysine–competitive inhibitor displacing H3K56ac from the CBP-binding site with an IC$_{50}$ value of 170 nmol/L (Fig. 2C).

Co-crystallization of the R-isomer of I-CBP112 with the bromodomain of CBP confirmed the acetyl-lysine mimetic nature of this inhibitor. The structure of the complex was refined at 1.6 Å resolution (Supplementary Table S5). The inhibitor was well defined by electron density allowing assignment of its binding mode (Fig. 2D). As expected, the carbonyl group branching off the oxazepine ring acted as an acetyl-lysine mimetic moiety forming a hydrogen bond with the conserved asparagine (N1168 in CBP) as well as a water-mediated hydrogen bond with Y1125. The five water molecules that are typically present in bromodomain inhibitor and substrate complexes (25) were also conserved in the I-CBP112/CBP complex (Fig. 2E). Surprisingly, I-CBP112 bound with an induced fit binding mode, leading to an outward movement of the side chain of R1173. This movement creates a deep pocket that accommodates the dimethoxyphenyl ring system forming an aromatic stacking interaction with the R1173 side chain (Fig. 2F).

CBP remains bound to chromatin in the presence of I-CBP112

We next investigated whether I-CBP112 could dissociate CBP from chromatin using a fluorescent recovery after photo bleaching (FRAP) assay (26). Full-length CBP contains a large number of protein and DNA interaction domains (Fig. 3A). FRAP measurements using a GFP-tagged full-length CBP construct did not lead to decreased recovery times when treated with I-CBP112, or when a conserved key residue for acetyl-lysine interaction (N1168) was mutated to phenylalanine (N1168F), indicating that full-length CBP cannot be displaced from chromatin by bromodomain inhibitors (Fig. 3B). We used the pan-HDAC inhibitor SAHA to increase chromatin acetylation levels. In agreement with FRAP data using I-CBP112 and site-directed mutants, SAHA treatment did not significantly influence fluorescent recovery times of full-length CBP, suggesting that tight chromatin association of CBP is not exclusively acetylation-dependent and is primarily mediated by interaction domains other than the bromodomain. To verify that I-CBP112 bound to the CBP bromodomain in nuclei of cells, we cloned the CBP bromodomain fused to a nuclear localization signal and a GFP tag. The assay window obtained for this construct was small, which was expected considering the low affinity of isolated bromodomains with acetylated histone tails. However, expression of three bromodomains (3xCBP$^{\text{BRD}/\text{GFP}}$) fused in frame by a linker region resulted in a GFP fusion protein that showed significant increase in FRAP signal after SAHA treatment and provided a sufficient assay window when compared with the N1168F-mutant construct. Exposure to 1 µmol/L I-CBP112 significantly reduced FRAP signal after SAHA treatment demonstrating that I-CBP112 can indeed target the CBP bromodomain in the nucleus and is capable of competing with acetyl-lysine–mediated interactions of the CBP bromodomain in cellular environments (Fig. 3C). Representative data of beached nuclei as well as normalized fluorescent recovery data are shown in Supplementary Fig. S2. We confirmed these observations by a bioluminescence resonance energy transfer (BRET) assay using NanoLuc Luciferase fusions of full-length CBP and the isolated CBP bromodomain as well as Halo-tagged Histone H3.3, which was readily incorporated into chromatin (Fig. 3D), as BRET pairs. In agreement with our FRAP experiments, dose response experiments revealed that I-CBP112 had no significant effect on the recruitment of full-length CBP to chromatin, whereas a construct containing the bromodomain was readily replaced with a cellular IC$_{50}$ value of 600 ± 50 nmol/L (Fig. 3E) confirming cellular activity of I-CBP112 inhibiting acetylation-dependent CBP bromodomain-mediated interactions.

Phenotypic responses of CBP/p300 bromodomain inhibition

To get insight on the wider consequences of CBP/p300 bromodomain inhibition in cellular systems, we exposed a panel of 12 stimulated primary human cocultured cell types (BioMAP) with I-CBP112 and monitored a broad array of protein biomarkers. This unbiased approach has been well established to assess target-specific phenotypic responses by proving a phenotypic fingerprint of an inhibitor based on a large number of marker...
proteins (27). For I-CBP112, the analysis of BioMAP data revealed a modest but robust response on the expression of some of the measured protein markers. Comparison with fingerprints generated by well-established BET bromodomain inhibitors, I-CBP112 revealed a very distinct phenotypic response (Fig. 4A and Supplementary Fig. S3). Some effects of cytokine expression were shared between BET and CBP/p300 inhibitors. Expression of the anti-inflammatory cytokine IL10 or the vascular cell adhesion protein 1 (VCAM1/CD106) for instance was downregulated by both I-CBP112 as well as the BET inhibitor IQ1. In contrast to BET inhibition, I-CBP112 had no effect on expression levels of a number of marker proteins including for instance the chemokine (C-C motif) ligand 2 (CCL2) or the cell adhesion molecule Selectin (CD62). In addition, we observed a number of differential responses comparing both inhibitors. Secreted IgG was for instance strongly downregulated by the BET inhibitor JQ1, but it was upregulated by I-CBP112. Using statistical methods comparing profile similarity, we found that the BET bromodomain inhibitors IQ1, PFI-1, and I-BET151 clustered closely together (Pearson ≥ 0.6) in agreement with their reported selectivity for

Figure 5.
I-CBP112 impairs self-renewal and disease induction of murine MLL-AF9-driven acute leukemia. A, schematic outline of the used mouse model. Transplanting bone marrow cells retrovirally expressing the MLL-AF9 fusion oncogene leads to AML. Leukemic blasts from diseased mice were ex vivo treated with 5 μmol/L of I-CBP112 or DMSO vehicle for 3 days and transplanted into syngeneic secondary recipients. B, changes on cell morphology and colony formation of murine MLL-AF9 leukemic blasts treated with I-CBP112 at different concentrations as indicated in the figure (left). Percentage of blasts and differentiated cells scored on cytospots. Shown is the mean percentage normalized to vehicle-treated control; error bars represent ±SD. P values were calculated by using ANOVA and Dunnett multiple comparison; ***, P < 0.0001; n = 20 scored high-magnification (>60) fields (right). C, clonogenic growth and replating assay of leukemic MLL-AF9 blasts treated with I-CBP112. Concentration as indicated in the figure. Shown is the mean percentage normalized to vehicle-treated control; error bars represent ±SD. P values were calculated by using ANOVA and Dunnett multiple comparison; ***, P < 0.0001; n = 2. D, cytotoxicity (WST1 assay) of leukemic MLL-AF9 blasts treated with I-CBP112. Shown is the mean percentage normalized to vehicle-treated control; error bars represent ±SD. P values were calculated by using ANOVA and Dunnett multiple comparison; ***, P < 0.0001; n = 4. E, extreme limiting dilution analysis (ELDA). Shown is a log-fraction plot of the limiting dilution model fitted to the data. The slope of the line represents the log-active cell fraction. The dotted lines indicate the 95% confidence interval. The data value with zero negative response at highest dose (10 plated cells) is represented by a downpointing triangle. The estimated stem cell frequency decreased from 1/2.3 to 1/13. Statistical significance was assessed by an overall test for differences with χ2/218.3 and DF 1 showing P < 0.00001. F, Kaplan–Meier diagram showing significantly extended survival of mice injected with different number of I-CBP112-treated cells (dotted lines) compared with vehicle-treated controls (solid lines). The number of injected cells were 10,000 cells (red) and 500 cells (magenta). P values were calculated using log-rank (Mantel–Cox) test; n = 4/5 per group. Survival data for injections of 50,000 cells and 5,000 cells have been included in the Supplementary Data.
Figure 6.
I-CBP112 impairs the clonogenic growth of human leukemic cells and sensitizes them to BET inhibition and doxorubicin. A, clonogenic activity of three human leukemic cell lines in methylcellulose exposed to different doses of I-CBP112. Shown is the mean percentage normalized to vehicle treated; error bars, ±SD. *P values were calculated by using ANOVA and Dunnett multiple comparison; **, *P < 0.01; ***, *P < 0.001; ****, *P < 0.0001; n = 3. (Continued on the following page.)
BET bromodomains. I-CBP112 in contrast showed a distinct phenotypic response (Fig. 4B). Interestingly, another bromodomain inhibitor that we developed recently, CBP30, that has potent in vitro activity for the CBP/p300 bromodomain but also significant BET activity (28) clustered with I-CBP112 at low concentration but showed a clear BET bromodomain signature at 10 μmol/L as expected from its dual activity. Thus, exposure of the BioMAP primary cell panel to I-CBP112 resulted in a unique response on cytokine and marker protein expression.

I-CBP112 impairs clonogenic growth of immortalized murine bone marrow cells

Next, we aimed to explore the anticancer potential of I-CBP112 in a CBP/p300-dependent transformation model. Chromosomal translocations have been observed in patients after therapy with topoisomerase inhibitors leading to MLL-CBP fusion proteins that include the bromodomain. Expression of MLL-CBP has been shown to immortalize mouse bone marrow cells in vitro but it has a limited capacity of inducing leukemia in vivo (29). To address the effect of I-CBP112 on MLL-CBP–driven transformation we therefore established an immortalized hematopoietic cell line by retroviral expression of the MLL-CBP fusion in lineage marker depleted mouse bone marrow cells and expansion by two rounds of replating in methylcellulose. Interestingly, exposure of these cells with I-CBP112 did not affect cell survival and significant cytotoxicity was only observed at high inhibitor concentrations and after prolonged exposure (Fig. 4C). However, exposure to the inhibitor significantly reduced clonogenic growth of MLL-CBP immortalized cells in methylcellulose, which became even more apparent upon replating of the cells (Fig. 4D). I-CBP112 exposure resulted in morphologic changes from large round compact, so-called class I type, to smaller and more loosely shaped colonies. Wright–Giemsa–stained cytopsots of treated cells harvested from methylcellulose revealed morphologic changes, indicating differentiation of MLL-CBP–immortalized cells (Fig. 4E).

I-CBP112 pretreatment of MLL-AF9™ murine AML blasts reduced the number of LICs and delayed induction of the disease upon transplantation into irradiated recipients

We next studied the efficacy of I-CBP112 on leukemia cells isolated from mice transplanted with bone marrow retrovirally expressing fusion oncogenes (Fig. 5A; ref. 30). Treatment of primary murine MLL-AF9™ leukemic blasts with I-CBP112 significantly impaired self-renewal in clonogenic replating assays in methylcellulose without significant cytotoxicity and induced significant morphologic cellular differentiation (Fig. 5B–D). Similar results were observed in bone marrow cells in vitro immortalized by expression of the NUP98-HOXA9 or the MLL-AF9 fusion (Supplementary Fig. S4). The observation of reduced in vitro clonogenic growth (Fig. 5C) and absence of general toxicity (Fig. 5D) suggested that I-CBP112 might selectively reduce the number of leukemia-initiating cells (LIC) often also referred as leukemic stem cells. We therefore addressed the stem frequency of MLL-AF9–expressing blasts in methylcellulose by extreme limiting dilution analysis (ELDA; ref. 31) and found that treatment of the cells with 5 μmol/L I-CBP112 for 3 days resulted in a significant decrease in the estimated stem cell frequency (Fig. 5E).

In contrast to MLL-CBP, the MLL-AF9–driven mouse AML can be efficiently propagated by transplantation into irradiated recipient mice (30), making it an ideal model to study the antileukemic potential of CBP/p300 bromodomain inhibition in vivo. We first carried out a pharmacokinetic study in mice to assess suitability of I-CBP112 for progression into xenograft models. Intraperitoneal administration of the compound at 3 mg/kg yielded moderate systemic exposure of the compound with a mean Cmax of 590 ng/mL [1.26 ± 0.74 μmol/L (n = 3)], an AUC of 384 ng·h/mL, and a half-life of 0.6 ± 0.1 hours. Intravenous administration (1.2 mg/kg) revealed high blood clearance, moderate volume distribution, and comparison with intraperitoneal dosing suggested that I-CBP112 was bioavailable P%: 114 ± 63 (AUCs/Dp)/[AUCc/Dp].

Because of the modest pharmacokinetic properties of I-CBP112, we used a pretreatment scheme to obtain a preliminary evaluation of I-CBP112 in vivo activity (Fig. 5A). I-CBP112 exposure of the cells for 3 days (5 μmol/L) in liquid cultures led to minor changes of expression of cell surface markers, only c-Kit/CD117 and granulocyte marker Gr-1 were slightly decreased (Supplementary Fig. S5).

We then compared disease initiation in vivo by transplanting I-CBP112 or DMSO pretreated MLL-AF9™ AML cells in limited dilutions into secondary recipients. We observed that in doses from 5–50 × 10⁴ cells, mice receiving treated cells developed the disease significantly later. Transplantation of as little as 500 control cells induced AML in all recipients after 50 days. In contrast, only 1 mouse transplanted with 500 I-CBP112–treated cells developed the disease only after a long latency (Fig. 5F and Supplementary Fig. S6). Collectively, these data demonstrate that exposure of murine MLL-AF9™ leukemic blasts to the I-CBP112 CBP/p300 bromodomain inhibitor significantly reduced colony formation in vitro, as measured in the ELDA assay, and delayed induction of AML upon transplantation into irradiated recipients.

I-CBP112 impairs the clonogenic growth of human leukemic cells

To corroborate the observations made in the mouse model in the human disease, we screened a panel of 18 human leukemic cell lines for their sensitivity to I-CBP112. On the basis of the cancer cell line encyclopedia (CCLE), none of these cell lines harbor any mutations in the coding region of CBP or EP300 (Supplementary Table S4). Similar

(Continued)
to our observation on primary mouse leukemic blasts, we found that I-CBP112 did not cause immediate cytotoxic effects (Supplementary Figs. S7–S12). However, I-CBP112 significantly reduced the clonogenic growth in all 12 human cell lines that form visible colonies in methylcellulose (Supplementary Fig. S8). We selected three leukemic cell lines with distinct genetic lesions: KASUMI-1 (AML1-ETO+), SEM (MLL-AF4+), and MOLM13 (MLL-AF9+/FLT3-ITD+) for more detailed studies. I-CBP112 significantly impaired clonogenic growth of all three lines in a dose-dependent manner (Fig. 6A). I-CBP112 treatment resulted in dose-dependent G1 arrest of the cell cycle. Time-dependent analysis of KASUMI-1 cells showed a prominent G1 arrest at day 2 and 3 that was even more pronounced at day 6 (Fig. 6B). Prolonged exposure of cells to increasing concentrations of the compound in liquid culture resulted in signs of apoptosis (as measured by 7-AAD/AnnexinV staining) in KASUMI-1 cells at day 5, whereas MOLM13 and SEM were less sensitive (Supplementary Figs. S9–S11).

We also tested the effects of I-CBP112 on clonogenic growth of primary human cells. Exposure of cells from five different AML patients to I-CBP112 showed a dose-dependent reduction in the number of colonies and cells in methylcellulose. Similar to the murine and human leukemic cell lines, I-CBP112–treated primary AML cells showed morphological signs of differentiation (Fig. 6C). In contrast, I-CBP112 exposure of peripheral CD34+ cells from three healthy donors did not affect the colony number in methylcellulose. However, the size of the colonies was reduced, thus resulting in a statistically not significant tendency of lower overall cell numbers (Fig. 6D and Supplementary Fig. S12). These observations suggest that inhibition of the CBP/p300 bromodomain may have therapeutic potential in AML by suppressing self-renewal.

I-CBP112 sensitizes human leukemic cells to BET inhibition and doxorubicin

CBP/p300 and the BRD4 BET protein often colocalize on active promoters (32). However, in contrast to inhibition of BET bromodomains, blocking CBP/p300 BRDs by I-CBP112 resulted only in limited cytotoxicity. We therefore wondered whether exposure of combinations of both BRD inhibitors might show synergistic effects. In parallel, we also tested whether I-CBP112 might enhance cytotoxicity of doxorubicin, a clinically widely used genotoxic agent. We tested 8 different concentrations of I-CBP112 ranging from 0.1875 µmol/L to 24 µmol/L in combination with 15 different concentrations of JQ1 and doxorubicin, respectively, in ratios ranging from 8:1 to...
I-CBP112 modulates transcription of key inflammatory genes

To address the transcriptional consequences of exposure to I-CBP112, we performed microarray gene expression studies. We compared the gene expression signatures from KASUMI-1, MOLM13, and SEM leukemia cell lines that have been exposed to I-CBP112 for 4 days in liquid culture, a time point where cell-cycle alterations become detectable without any pronounced effects on cellular viability (Supplementary Fig. S7). Exposure of doxorubicin increased H2AX phosphorylation and foci that were not further increased by the combination with I-CBP112 (Supplementary Fig. S14). The combination of BET and CBP/p300 BRD inhibitors is of particular interest as both target subfamilies could be simultaneously targeted using a single agent with dual activity.

4-day exposure to I-CBP112, suggesting that bromodomain inhibition affects also p300/CBP HAT activity at regulated gene promoters.

Discussion

High-resolution crystal structures allowed to rationally design several small molecules that selectively interact with high affinity to bromodomains of the BET family (19–22). BET inhibitors of the benzodiazepine scaffold gave rise to the inhibitor I-CBP112, the first selective and potent inhibitor developed for CBP/p300 bromodomains. Together with the HAT inhibitor C646 and inhibitors targeting other protein interaction domains the developed chemical probe extends the set of tool molecules targeting this key chromatin protein. Several studies demonstrated that BET inhibitors potentially impaired the growth of cells originating from hematologic and solid cancers (22, 27, 36, 37). Analysis of a large number of different cell lines and primary murine and human cells revealed that I-CBP112 selectively impaired aberrant leukemic self-renewal. In contrast, BET inhibitors, such as IQ1 or PFI-1 potently impaired proliferation, survival, and self-renewal not only of cancer cells but also activated immune cells. This wide window of cellular activity might result from blocking interaction of BRD4 with a large number of super enhancer elements regulating multiple transcription factors including the Myc oncogene (38, 39). Not surprisingly, conditional ablation of BRD4 in vivo resulted in a severe phenotype due to stem cell depletion, for example, in the small intestine (40). Conditional ablation of CBP in the hematopoietic system of adult mice led to an increase in differentiation, quiescence, apoptosis, and defects in adult HSC maintenance and self-renewal (5). Comparison with the observed cellular phenotypes induced by I-CBP112 observed in this study suggests that chemical inhibition of the CBP/p300 bromodomain phenocopies some but not all of the cellular effects observed in knockout studies including most remarkably defects in self-renewal and increased differentiation of immortalized hematopoietic cells as well as primary leukemic blasts. Reduction of colony formation by MLL-AF9–immortalized cells as shown by the ELDA assay in vitro and impaired disease induction by I-CBP112–treated leukemic blasts after transplantation strongly suggest significant activity of the compound on aberrant self-renewal of leukemic cells.

Domain-specific functions have recently been addressed in a systematic p300 deletion study in which loss of the p300 KIX or CH1 domains have been reported to cause profound defects in hematopoiesis, whereas deletion of other domains including the p300 bromodomain lead to lineage-restricted effects (41). Deletion of the bromodomain only modestly impaired p300 hematopoietic function in B cells but the bromodomain was required during T-cell development. Thus, the bromodomain interaction module may only be required for a subset of albeit critical fraction of CBP/p300 functions. Indeed, our FRAP and BRET experiments demonstrated that wild-type full-length CBP/p300 or mutants that lack the bromodomain interaction with acetylated histones remained bound to chromatin in the presence of I-CBP112. The lack of full displacement of CBP/p300 from chromatin may explain the modest effects of I-CBP112 on gene transcription in our microarray study that showed few genes upregulated greater than 2-fold after treatment with I-CBP112. The observed modest changes in gene transcription are however consistent with expression studies carried out using genetic deletion of CBP (5, 42, 43).
The small number of commonly regulated genes in all three cell lines tested was surprising, suggesting a strong context dependence of CBP/p300–regulated transcriptional events.

The role of CBP/p500 in human leukemogenesis is complex. Conditional knock-in of MLL-CBP in mice revealed that the fusion protein selectively expanded granulocyte/macrophage progenitors and enhanced their self-renewal/proliferation (44). In the MLL-CBP fusion, the bromo/HAT domain is maintained and required for the transforming activity of this oncogene (29). CBP/p300 was also found to regulate the transcriptional activity of the AML1-ETO fusion driving AML-M2 by posttranslational modification (12). CBP/p300 also seem to cooperate with the c-MYB proto-oncogene in cellular transformation by different leukemia-associated fusions including AML1-ETO, AML1-ETO9a, MLL-ENL, and MLL-AF9 (45). These observations suggest that the block of aberrant self-renewal in leukemic cells mediated by I-CBP112 might be the consequence of multiple activities influenced by the driver fusion oncogenes and multiple protein–protein interactions of CBP/p300. It has been estimated that CBP/p300 physically or functionally interact with close to 400 proteins acting as a coactivator of many transcription factors including NF-kB, HIF-1α, p53, or β-catenin that are known key regulators of leukemogenesis (46). It is likely that some of these interactions are influenced by the bromodomains. The p300 bromodomain for instance, has been reported to be required for recognition of specific acetylated lysine residues in p53 (47–49). We have recently observed that p53-dependent induction of the cell-cycle inhibitor p21 by genotoxic stress might be dependent on the CBP/p300 BRD. Interestingly we also found regulation of some p53 target genes at early time points in I-CBP112–treated KasumI-1 leukemic cells (Supplementary Fig. S15). Hence, we found that the combination of I-CBP112 with doxorubicin resulted in significantly cytotoxicity in leukemic cells. Interestingly, we also saw also synergy between the BET inhibitor IQ1 and I-CBP112. Previous studies have shown synergistic cytotoxic activity of BET inhibitors with some HDAC inhibitors such as SAHA or LBH-589 in Mfc induced murine lymphoma (50). Thus, small molecules CBP/p300 bromodomain inhibitors may have not only the potential to improve current therapies by selectively targeting aberrant self-renewal linked to disease relapse but also to enhance protocols combining BET inhibitors and traditional chemotherapy and suggest to further explore dual inhibitors targeting CBP/p300 and BET family bromodomains for their anticancer potential. Several compounds with dual BET and CBP/p300 activity have been developed in our laboratory demonstrating that selective targeting bromodomains of both bromodomain subfamilies is feasible using a single agent.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Picaud, O. Fedorov, A. Thanasopoulos, K. Leonards, K. Jones, H. Olszach, S. Martin, A. Tumber, P. Filippakopoulos, K.H. Che, D. Lugo, S. Taylor, A. O’Mahony, S. Velichko, D.L. Daniels, N.B. La Thangue, J. Schwaller.


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Leonards, J. Meier, O. Monteiro, C. Yapp, N. Parr, C. Bountra, S. Muller.


Other (made the figures displayed in the article): S. Picaud, O. Fedorov.

Other (chemistry director with input and oversight of medicinal chemistry strategy and synthesis for GSK contribution): N. Parr.

Other (discovery and characterization of the CBP inhibitor): R. Priinjha.

Acknowledgments

The authors thank S. Iuge and U. Schneider for providing technical support.

Grant Support

The SGC is a registered charity (number 1097737) that receives funds from the Canadian Institutes for Health Research, the Canada Foundation for Innovation, Genome Canada, GlaxoSmithKline, Pfizer, Eli Lilly, the Novartis Research Foundation, Takeda, Bayer, the Ontario Ministry of Research and Innovation, and the Wellcome Trust (092809/Z/10/Z2). P. Filippakopoulos and S. Picaud are supported by a Wellcome Trust Career Development Fellowship (095751/Z/11/Z2). The work of the laboratory of J. Schwaller is supported by the Swiss National Science Foundation (SNSF-3100A3-130661/1), the Swiss Cancer League (KFS-3019-08-2012) and the Gertrude Von Meissner Foundation (Basel, Switzerland).

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Received January 28, 2015; revised July 17, 2015; accepted August 7, 2015; published OnlineFirst November 9, 2015.

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Sarah Picaud, Oleg Fedorov, Angeliki Thanasopoulou, et al.

Cancer Res 2015;75:5106-5119. Published OnlineFirst November 9, 2015.

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