



HAL
open science

Human CREBBP acetyltransferase is impaired by etoposide quinone, an oxidative and leukemogenic metabolite of the anticancer drug etoposide through modification of redox-sensitive zinc-finger cysteine residues

Wenchao Zhang, Jérémy Berthelet, Christina Michail, Linh-Chi Bui, Panhong Gou, Rongxing Liu, Romain Duval, Justine Renault, Jean-Marie Dupret, Fabien Guidez, et al.

► To cite this version:

Wenchao Zhang, Jérémy Berthelet, Christina Michail, Linh-Chi Bui, Panhong Gou, et al.. Human CREBBP acetyltransferase is impaired by etoposide quinone, an oxidative and leukemogenic metabolite of the anticancer drug etoposide through modification of redox-sensitive zinc-finger cysteine residues. *Free Radical Biology and Medicine*, 2021, 162, pp.27 - 37. 10.1016/j.freeradbiomed.2020.11.027 . hal-03492863

HAL Id: hal-03492863

<https://hal.science/hal-03492863>

Submitted on 15 Dec 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

Human CREBBP acetyltransferase is impaired by etoposide quinone, an oxidative and leukemogenic metabolite of the anticancer drug etoposide through modification of redox-sensitive zinc-finger cysteine residues

Wenchao ZHANG¹, Jérémy BERTHELET^{1,2}, Christina MICHAIL¹, Linh-Chi BUI¹, Panhong GOU³, Rongxing LIU¹, Romain DUVAL⁴, Justine RENAULT¹, Jean-Marie DUPRET¹, Fabien GUIDEZ³, Christine CHOMIENNE^{3,5} and Fernando RODRIGUES LIMA¹

¹ Université de Paris, BFA, UMR 8251, CNRS, F-75013 Paris, France

² Université de Paris, CEDC, UMR 7216, CNRS, F-75013 Paris, France

³ Université de Paris, Institut de Recherche Saint-Louis, UMRS 1131, INSERM, F-75010 Paris, France

⁴ Université de Paris, BIGR, UMRS 1134, INSERM, F-75015 Paris, France

⁵ Service de Biologie Cellulaire, Assistance Publique des Hôpitaux de Paris (AP-HP), Hôpital Saint Louis, Paris, France.

Correspondence and request for materials should be addressed to FRL (fernando.rodrigues-lima@u-paris.fr)

ABSTRACT

Etoposide is an extensively prescribed anticancer drug that, unfortunately, causes therapy-related leukemia. The mechanisms by which etoposide induces secondary hematopoietic malignancies are poorly documented. However, etoposide-related leukemogenesis is known to depend on oxidative metabolites of etoposide, notably etoposide quinone, that can react with protein cysteine residues such as in topoisomerases II. CREBBP is a major histone acetyltransferase that functions mainly as a transcriptional co-activator. This epigenetic enzyme is considered as a tumor suppressor that plays a major role in hematopoiesis. Genetic alterations affecting CREBBP activity are highly common in hematopoietic malignancies. We report here that CREBBP is impaired by etoposide quinone. Molecular and kinetic analyses show that this inhibition occurs through the rapid and covalent ($k_{inhib}=16.10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$) adduction of etoposide quinone with redox sensitive cysteine residues within the RING and PHD Zn²⁺-fingers of CREBBP catalytic core leading to subsequent release of Zn²⁺. In agreement with these findings, experiments conducted in cells and in mice treated with etoposide showed irreversible inhibition of endogenous CREBBP activity and decreased H3K18 and H3K27 acetylation. As shown for topoisomerases II, our work thus suggests that the leukemogenic metabolite etoposide quinone can impair the epigenetic CREBBP acetyltransferase through reaction with redox sensitive cysteine residues.

KEYWORDS

CREBBP, quinone, oxidative metabolite, inhibition, zinc-finger cysteines, histone acetylation

INTRODUCTION

Lysine acetyltransferase (KAT) enzymes acetylate histone and non-histone proteins through the transfer of an acetyl group from acetyl-coenzyme A (AcCoA) to specific lysine residues (1,2). CREB binding protein (CREBBP, also known as CBP or KAT3A) is a major mammalian KAT enzyme that functions as a transcriptional co-activator notably through acetylation of histones (in particular H3K18 and H3K27) and transcription factors (such as p53) (3-5). CREBBP is a tumor suppressor that is important in normal development. Germline loss-of-function mutations in *CREBBP* cause Rubinstein-Taybi syndrome, a developmental disorder that includes predisposition for leukemia (6). CREBBP is a critical regulator of normal hematopoiesis and alteration of the acetyltransferase activity of the enzyme contributes to lymphomagenesis and leukemogenesis (7-11). Moreover, loss of function mutations in *CREBBP* are indeed common in lymphoma and leukemia and are associated with aberrant acetylation of histone H3 (and non-histone proteins (9,10,12-14). Multiple chromosomal translocations associated with hematological malignancies involve the KAT domain of CREBBP resulting in fusion proteins that contribute to leukemogenesis through abnormal histone acetylation (7,15,16). The crucial role of CREBBP in normal and malignant hematopoiesis is further underlined by different mouse models that show that lack of functional CREBBP leads to serious hematological defects (17-19).

Etoposide is a semi-synthetic podophyllotoxin derivative widely used for the treatment of many types of cancer. This chemotherapeutic drug acts as a topoisomerase II poison that inhibits the enzyme's ability to ligate DNA, resulting in an accumulation of double-strand breaks and subsequent apoptosis (20). Although etoposide is commonly prescribed as a front-line treatment of a variety of human malignancies, this chemotherapeutic drug is also known to cause secondary leukemia (21-23). However, the molecular basis underlying etoposide-induced leukemogenesis is not well-defined. In humans, etoposide is readily metabolized by CYP3A4 into etoposide catechol which is further oxidized by cellular oxidases/peroxidases into the reactive metabolite etoposide quinone (24-26). Several studies have implicated oxidative etoposide metabolites, in particular etoposide quinone, as the primary contributors to the leukemogenic process (27-31). Notably, etoposide quinone has been shown to react with redox sensitive cysteine residues of topoisomerase II enzymes (28-30). In addition, efficacy of etoposide quinone against type II topoisomerases was found to be considerably higher than that of etoposide thus emphasizing the key role of this oxidative metabolite in the biological

effects of etoposide and putting forward impairment of topoisomerase II functions by etoposide quinone as a major mechanism in etoposide-induced leukemia (28-30). Nevertheless, reaction of etoposide quinone with additional cellular proteins besides topoisomerase II inhibition have been suggested (32-36). In particular, recent studies indicate that exposure to etoposide may have epigenetic effects unrelated to topoisomerases II (37-40). In line with these observations, we report herein molecular and kinetic evidence showing that the histone acetyltransferase CREBBP is irreversibly impaired by etoposide quinone, the major oxidative metabolite of etoposide. We found that this inhibition occurs through the rapid adduction by etoposide quinone of redox-sensitive cysteine residues located in the PHD/RING Zn²⁺-fingers of CREBBP and known to be crucial for its acetyltransferase activity (41-43). Experiments in hematopoietic cells and in mice further confirm that exposure to etoposide irreversibly impairs CREBBP acetyltransferase activity with concomitant decrease in cellular histone H3 acetylation. Overall, our data indicate that, in addition to the alteration of topoisomerase II functions, etoposide quinone can impair CREBBP acetyltransferase by reacting with key cysteine residues within the catalytic core of the enzyme. Inhibition of a key epigenetic enzyme such as CREBBP by etoposide quinone may be an additional mechanism contributing to etoposide-dependent leukemogenesis.

MATERIALS AND METHODS

Chemicals and reagents

Etoposide and etoposide quinone were from Toronto Research Chemicals (Canada). Recombinant human H3.1 histone was from Actif Motif (Belgium). Antibodies used in this paper are: H3K18Ac (9675S, Cell Signaling), H3K27Ac (ab4729, Abcam, UK), H3 (3638S, Cell Signaling, France), CREBBP (A22, Santa Cruz, Germany), 6xHis (H1029, Sigma-Aldrich, France), secondary anti-rabbit (A1949, Sigma-Aldrich, France) and secondary anti-mouse (31430, ThermoFisher, France). All other chemicals, reagents and cells were from Sigma-Aldrich (France) otherwise noted.

Protein expression and purification

cDNA sequences from human CREBBP encoding residues 1096-1751 (CREBBP catalytic core), 1205-1279 (CREBBP RING domain) and 1280-1321 (CREBBP PHD domain) were cloned into pET28a expression vector and transformed into competent *E.coli* C41 (DE3). Bacteria were grown in Luria-Broth (LB) medium containing 30 µg/ml kanamycin and 20 µg/ml chloramphenicol. Recombinant protein expressions were induced by 0.5 mM of IPTG and the cultures were shaken overnight at 16 °C. Bacteria pellets were resuspended in buffer A (20 mM Tris-HCl, 300 mM NaCl, 1% Triton X-100, 0.2 mM β-mercaptoethanol (β-ME), 1 mg/ml lysozyme, protease inhibitors, pH 8.0) and lysed for 30 min at 4°C under agitation. The lysate was then sonicated for 10 min (10 sec ON, 20 sec OFF, 20% power) and the soluble fraction was clarified by centrifugation (15000g, 4°C, 30 min). The supernatant was incubated with His-Select Nickel Affinity Gel beads for 2h on ice under agitation in presence of 10 mM imidazole. The proteins were eluted with buffer B (20 mM Tris-HCl, 300 mM NaCl, pH 8.0) containing 300 mM imidazole and buffer exchanged with a desalting column (PD-10) to buffer C (Tris-HCl 20 mM, NaCl 150 mM, 10% glycerol pH 8.0). Protein purity was assessed by SDS PAGE followed by Coomassie staining. Proteins were stored at -80 °C.

CREBBP acetyltransferase activity assay

CREBBP acetyltransferase activity was assayed as previously described (44). CREBBP catalytic core was incubated with 100 µM AcCoA, 30 µM H3K18 or H3K27 peptide, 1 mM DTT in 100 µl of assay buffer (25 mM Tris-HCl pH 8.0) for 20 minutes at room temperature. The

reaction was stopped with 100 μ l of HClO_4 (15% v/v in water) and 20 μ l aliquots of the mixture were injected in a reverse phase ultra-fast liquid chromatography system (RP-UFLC, Shimadzu, France). Peptides were separated under isocratic conditions (80% acetonitrile, 20% water, 0.12% TFA, flow rate of 0.6 ml/min) on a Shim-pack XR-ODS column at 40 °C. H3K18 and H3K27 substrate peptides and their acetylated product were detected by fluorescence (excitation: 485 nm, emission: 530 nm) and quantified by integration of the peak fluorescence areas.

Effects of etoposide quinone on CREBBP acetyltransferase activity

CREBBP catalytic core (500 nM) was incubated for 10 min at room temperature in the presence of different concentrations (0, 5, 10, 20, 40 μ M) of etoposide quinone (EQ) or the parent drug (ET) in 20 μ l assay buffer. Then, 80 μ l of assay buffer (100 μ M AcCoA, 30 μ M H3K18 or H3K27 peptide, final concentrations) were added to the treated enzyme mixture and CREBBP acetyltransferase activity was assayed for 20 minutes at room temperature as described above.

Effects of reducing agents and buffer exchange on etoposide quinone-inhibited CREBBP

CREBBP catalytic core (500 nM) was first incubated with etoposide quinone (40 μ M) or etoposide (ET) for 10 minutes at room temperature. Samples were either buffer exchanged using a desalting column (PD SpinTrap G-25, GE Healthcare, France) to remove free etoposide quinone in solution or further incubated with 10 mM DTT. Protein concentration was assessed after buffer exchange using Bradford assay (Bio-Rad, France). Residual acetyltransferase activity of CREBBP was assayed as described above.

Kinetic analysis of CREBBP inhibition by etoposide quinone

CREBBP catalytic core (500 nM) was incubated in the presence of different concentrations of etoposide quinone (0, 1, 2.5, 5, 7.5, 10 μ M) in assay buffer. Every 30 seconds, an aliquot of the solution was taken and diluted 10 times in assay buffer to stop the reaction. Residual activity of CREBBP was then measured by UFLC as described above. The data were analyzed as described previously for irreversible inhibitors under pseudo first order conditions using Prism Graphpad and Excel softwares (45). The rate of inhibition of CREBBP by etoposide quinone can be described as follows:

$\text{Ln}(\% \text{ residual CREBBP activity}) = -k_{obs} \times t$, where k_{obs} is apparent first-order inhibition rate constant, and t is time). For each etoposide concentration, the k_{obs} values can be extracted

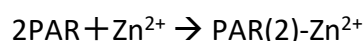
from the slopes of the natural logarithm of the percentage of CREBBP activity as a function of time. The second order rate constant (k_{inhib}) was obtained from the slope of k_{obs} values plotted as a function of etoposide concentration ($k_{inhib}=k_{obs}/[\text{etoposide quinone}]$).

Fluorescein-conjugated iodoacetamide (IAF) labeling and nitro blue tetrazolium (NBT) staining

CREBBP catalytic core (10 μg), CREBBP RING (10 μg) or CREBBP PHD (10 μg) domains were incubated in the presence of increasing concentrations (0, 5, 10, 20, 40 μM) of etoposide quinone for 10 min at room temperature in assay buffer. For IAF labelling of free CREBBP protein cysteines, aliquots were incubated in the presence of 50 μM fluorescein-conjugated iodoacetamide (IAF) for 10 min at 37 °C. The labelling reaction was stopped by the addition of 10 μl sample buffer containing 200 mM β -mercaptoethanol prior to SDS-PAGE and transfer onto a nitrocellulose membrane. IAF fluorescence was detected using a Fujifilm LAS 4000 detection system ($\lambda_{exc}=485$ nm, $\lambda_{em}=530$ nm). For detection of etoposide quinone adducts on CREBBP proteins, NBT staining was carried out as reported by (46). Briefly, aliquots of proteins were first separated by SDS-PAGE and transferred onto a nitrocellulose membrane prior to incubation with 2 M potassium glycinate, 0.6 mg/ml NBT, pH 10.0 at room temperature for 30 min in the dark.

Zn²⁺ release analysis

CREBBP RING (5 μM) or CREBBP PHD (5 μM) domains were incubated in the presence or absence of etoposide quinone (40 μM) in assay buffer containing 100 μM 4-(2-pyridylazo) resorcinol (PAR). PAR reacts with free Zn²⁺ in solution according to the scheme:



The PAR(2)-Zn²⁺ complex has an absorption peak around 490 nm. The reaction was initiated by addition of etoposide quinone and monitored by reading the absorbance of the solution at 490 nm using a microplate reader (Biotek Instruments, France).

Mass spectrometry analysis

CREBBP catalytic core (10 μM) was incubated with 40 μM EQ for 20 min at room temperature in assay buffer. The reaction was stopped with 10 mM DTT and diluted 20 times in assay buffer. Samples were then incubated overnight at 37 °C with trypsin (Promega, France) at

12.5 ng/ μ l in 25 mM ammonium bicarbonate pH 8.0. The supernatant containing peptides was acidified with formic acid (FA), desalted on C18 tips (Pierce C18 tips, Thermo Scientific, France), and eluted in 10 μ l 70% ACN, 0.1% FA. Desalted samples were evaporated using a SpeedVac then taken up in 10 μ l of buffer A (water, 0.1% FA) and 5 μ l were injected on a nanoLC HPLC system (Thermo Scientific, France) coupled to a hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, France). Peptides were loaded on a reverse phase C18 μ -precolumn (C18 PepMap100, 5 μ m, 100A, 300 μ m i.d.x5 mm) and separated on a C18 column (EASY-spray C18 column, 75 μ m i.d.x50 cm) at a constant flow rate of 300 nl/min, with a 120 min gradient of 2 to 40% buffer B (buffer B: 20% water, 80% ACN, 0.1% FA). MS analyses were performed by the Orbitrap cell with a resolution of 120.000 (at m/z 200). MS/MS fragments were obtained by HCD activation (collisional energy of 28%) and acquired in the ion trap in top-speed mode with a total cycle of 3 seconds. The database search was performed against the Swissprot database and the Homo sapiens taxonomy with Mascot v2.5.1 software with the following parameters: tryptic peptides only with up to 2 missed cleavages, variable modifications: cysteine etoposide quinone and methionine oxidation. MS and MS/MS error tolerances were set respectively to 7 ppm and 0.5 Da. Peptide identifications were validated using a 1% False Discovery Rate (FDR) threshold obtained by Proteome Discoverer (version 2.2, Thermo Scientific, France) and the percolator algorithm. The candidate sequences modified by etoposide quinone were manually inspected for *de novo* sequencing.

Bioactivation of etoposide into etoposide quinone by a myeloperoxidase (MPO)/H₂O₂ system

Etoposide was bioactivated into etoposide quinone *in vitro* using a myeloperoxidase/H₂O₂ system as described previously (31). Briefly, etoposide (100 μ M), H₂O₂ (200 μ M) and myeloperoxidase (MPO) (5 units/ml) were co-incubated in 1 ml of 100 mM sodium acetate (pH 6.5) for 30 min at 37°C. Etoposide quinone formation was detected by spectrophotometry (UV-1650PC, Shimadzu, France). Inactive MPO (heated for 30 min at 95 °C) was used as a negative control. 20 μ l of bioactivated etoposide were mixed with 30 μ l CREBBP (0.5 μ M final) in assay buffer and incubated 20 minutes at room temperature. Then, 50 μ l of assay buffer containing 30 μ M H3K18 peptide, 100 μ M AcCoA and 1 mM DTT (final concentrations) were added to the solution and CREBBP activity was assessed by UFLC as described above.

Detection of H3 acetylation by Dot blot

CREBBP (500 nM) was first incubated with etoposide quinone or etoposide (40 μM) in 20 μl assay buffer for 10 minutes at room temperature. Then, 80 μl of assay mix (100 μM AcCoA, 300 ng recombinant human H3) were added and further incubated for 20 minutes at room temperature. Samples (corresponding to 200 and 100 ng H3) were dot-blotted using a Dot-Blot apparatus (Bio-Rad, France) onto nitrocellulose membranes (0.1 μm). Detection of H3K18 and H3K27 acetylation was carried out by western blotting using specific antibodies. The membranes were then stripped and reprobed with anti-H3 antibody.

Western blot

Samples were separated by SDS-PAGE prior to transfer onto a nitrocellulose membrane (0.45 μm , GE Healthcare, France) for 2 h 30 min at 4°C. Membranes were blocked in 5% non-fat milk in PBS 0.05% Tween-20 (PBST) for 2h and incubated overnight with primary antibody in 1% non-fat milk PBST at 4 °C. The next day, membranes were washed 5 times with PBST and incubated with secondary antibody for 2 h at room temperature. Membranes were then washed 5 more times and the signal was detected by chemiluminescence using ECL Prime on an Amersham Imager 600 detection system (GE Healthcare, France).

Cellular culture and treatment with etoposide

K562 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1 mM L-glutamine at 37 °C, 5% CO₂. K562 cells (10.10⁶ cells) were washed 3 times with RPMI 1640 medium and then treated with 40 μM etoposide in RPMI 1640 medium containing 1 mM L-glutamine for 24 hours at 37 °C, 5% CO₂. Cells were then washed with PBS before use.

Acid extraction of histones

K562 cells in 100 mm petri dishes or bone marrow from mice hind limb long bones were lysed for 30 minutes at 4 °C with 400 μl lysis buffer (PBS pH 7.5, 0.5% Triton X-100, 3.3 μM trichostatin A (TSA) and protease inhibitors) and then centrifuged (15000 g) for 10 minutes at 4°C. The pellet (nucleus enriched fraction) was incubated overnight at 4 °C in 400 μl 0.2 M HCl. The next day, the acid extract was centrifuged (10 minutes at 6500 g, 4°C) and the supernatant (histone enriched fraction) was taken. Protein concentration was determined using Bradford

reagent (Bio-Rad, France). 10 µg of histone extract were separated by SDS-PAGE (18%), transferred onto a nitrocellulose membrane and detected by western-blot using H3K18Ac, H3K27Ac or H3 antibodies. Grayscale quantitative analysis of Western blots was performed using ImageJ software.

Immunoprecipitation of endogenous CREBBP

K562 cells ($10 \cdot 10^6$ cells) or mice bone marrow from hind limb long bones were lysed with lysis buffer (PBS pH 7.5, 1% Triton X-100, 3.3 µM trichostatin A, 100 µM DTT and protease inhibitors) for 30 min on ice and then centrifuged for 15 minutes (15000 g) at 4°C. The supernatant was taken and the concentration of proteins in the extracts was determined by the Bradford reagent (Bio Rad, France). CREBBP was immunoprecipitated from 2.5 mg total protein extracts by an overnight incubation (under rocking conditions) with 1 µg of anti-CREBBP antibody (Santa Cruz, Germany) at 4°C. The following day, samples were rocked for 2 hours at 4°C with 25 µl protein A agarose beads (Santa Cruz, Germany). The beads were washed 3 times with assay buffer and then incubated at room temperature with 60 µl of assay buffer containing 30 µM H3K18 fluorescent peptide, 100 µM AcCoA and 1 mM DTT. After 1 hour of incubation, beads were sedimented by centrifugation and 50 µl of the supernatant were taken and mixed with 50 µl of HClO₄ (15% v/v in water) to determine CREBBP activity by UFLC. Beads were then washed with PBS and incubated with sample buffer containing 200 mM β-ME for 5 min at 100 °C prior to SDS-PAGE and Western-blot analysis. Grayscale quantitative analysis of Western blots was performed using ImageJ software.

Treatment of mice with etoposide

C57BL/6 mice (12 weeks old female) were obtained from Janvier (Le Genest, France) and bred in pathogen-free conditions in the animal facility of the Institut de Recherche Saint-Louis IRSL (Hôpital Saint-Louis, Université de Paris). Mice (n=6) were treated overnight by intraperitoneal injection of 100 mg/Kg etoposide in DMSO. Controls (n=6) were carried out with vehicle only. The following day, the hind limb long bones were taken and washed 3 times with cold PBS. Bone marrow was flushed from the bones with PBS and the bone marrow cells ($5 \cdot 10^7$ cells/mice) were lysed with lysis buffer (PBS pH 7.5, 1% Triton X-100, 3.3 µM TSA, 1 mM DTT and protease inhibitors) for 30 min on ice under agitation. The supernatant was then used for immunoprecipitation of CREBBP and the pellet for histone extraction as described above. ChIP

qPCR experiments were carried out as described below. All the animal procedures performed were approved by the local Committee on the use and care of Animal.

Chromatin immunoprecipitation (ChIP)

K562 cells ($10 \cdot 10^6$ cells) or bone marrow cells ($5 \cdot 10^7$ cells/mice) were fixed with 1% formaldehyde for 10 min at 37 °C. The reaction was stopped with the addition of 0.125 M glycine for 5 min at room temperature. Cells were then proceeded for ChIP using ChIP-IT® Express Chromatin Immunoprecipitation Kit (Active Motif, USA) according to manufacturer instructions. Anti-H3K18Ac, anti-H3K27Ac, and anti-H3 were used. Rabbit IgG were used as a control. Immunocomplexes were isolated with protein-A-agarose beads (Santa Cruz, Germany). DNA was isolated by phenol/chloroform extraction followed by ethanol precipitation. The primers used for qPCR were:

Human IL6_F : CAGCAGCCAACCTCCTCTAA
Human IL6_R : CTGTGAGCGGCTGTTGTAGA
Human MDM2_F : CGGAAAGATGGAGCAAGAAG
Human MDM2_R : CCCTCAAGACTCCCCAGTTT
Mouse MDM2_F : AGATCTGACGCCCTCTTCTG
Mouse MDM2_R : GCAGCTTCAGTCCCGATTTA
Mouse COPS2_F : CATCGCACATGAAATCATCC
Mouse COPS2_R : CACAACTGTCGCTCAGGAA

Statistical analysis

Values are presented as mean \pm standard deviation (SD) and analyzed by either *t*-test or one-way analysis of variance (ANOVA) using Prism GraphPad 5 followed by two-tailed Tukey test. A threshold of $p < 0.05$ was used to consider differences as statistically significant.

RESULTS

Etoposide quinone impairs the acetylation of H3K18 and H3K27 by CREBBP *in vitro*

The leukemogenic properties of etoposide resides, at least in part, on its metabolic bioactivation in hematopoietic cells into oxidative quinone metabolites, most notably etoposide quinone which is known to react with cysteine residues and be a strong inhibitor of nuclear topoisomerases II (24,28,29,47). We asked whether etoposide quinone could affect other nuclear enzymes whose alteration could contribute to leukemogenesis. Interestingly, the histone acetyltransferase CREBBP is known to be a major regulator of hematopoiesis and impairment of its activity is linked to the development of hematological malignancies (7). In addition, CREBBP activity is known to rely on redox-sensitive cysteine zinc finger within the catalytic core of the enzyme (41-43). To test the effects of etoposide quinone on human CREBBP HAT activity, the purified recombinant catalytic core domain of the enzyme (residues 1096 to 1700) (48) (Supplementary Figure 1) was incubated with increasing concentrations of the metabolite or the parent drug prior to measurement of the HAT activity of CREBBP using fluorescent histone H3 peptides as substrates (44). We found that contrary to etoposide, etoposide quinone was able to inhibit CREBBP HAT activity in a dose-dependent manner with IC_{50} values in the low micromolar ranges (below 10 μ M) for both H3K18 and H3K27 peptides (Figure 1A and Supplementary Figure 2). Consistently, assays carried out with recombinant histone H3 confirmed that etoposide quinone but not etoposide inhibits CREBBP HAT activity (Figure 1B). No significant effects of etoposide on CREBBP activity were observed (even at concentrations of etoposide >200 μ M (data not shown) further confirming that CREBBP HAT activity is more sensitive to etoposide quinone than to the parent drug etoposide.

Inhibition of CREBBP by etoposide quinone is fast and irreversible

Etoposide quinone has been shown to be an irreversible and fast inhibitor of human topoisomerases II at micromolar concentrations (29,30). To further analyze the inhibition mechanism of CREBBP by etoposide quinone, the enzyme was incubated with etoposide quinone and subsequently subjected to buffer exchange prior to measurement of residual HAT activity. As shown in Figure 2A, the activity of CREBBP could not be recovered by buffer exchange after inhibition by etoposide quinone. Neither could the activity be restored by addition of the reducing agent DTT (Figure 2B). To further analyze the inhibition mechanism of

CREBBP by etoposide quinone, we performed kinetic experiments under pseudo-first order conditions (Figure 2C and 2D) (45). As shown in Figure 2C, the semilogarithmic plots of percentage of residual activity against time for various concentrations of etoposide quinone indicated that the enzyme was inhibited in a time- and dose-dependent manner thus confirming the irreversible nature of the inhibition. The second-order rate constant of inhibition (k_{inhib}) was determined by plotting the k_{obs} values obtained from the semilogarithmic plots against etoposide quinone concentration and was found to be equal to $16.10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Figure 2D). Overall, these data show that etoposide quinone inhibits CREBBP rapidly and irreversibly similar to etoposide quinone-dependent inhibition of human topoisomerases II (29,30).

Etoposide quinone forms covalent adducts with PHD/RING Zn²⁺-finger cysteines of the catalytic core of CREBBP

Etoposide quinone has been previously shown to covalently inhibit human topoisomerases II by reaction with cysteine residues (30). Moreover, quinone species, such as etoposide quinone, are known to be electrophilic chemicals that can readily react covalently with nucleophilic amino acids in proteins, most notably redox-sensitive cysteine residues (36,49-51). The formation of these covalent quinone-protein adducts is considered as a major mechanism for the biological and toxicological effects of quinone species (51-54). We carried out mass spectrometry analyses to investigate whether amino acids found within the catalytic core of human CREBBP could react with etoposide quinone and form such covalent adducts. LC-MS/MS analysis of tryptic digests of etoposide quinone-inhibited CREBBP allowed the identification of two peptides derived from the RING and PHD Zn²⁺-finger domains with mass increases of 572.15 Da (corresponding to the mass of an etoposide quinone molecule) at cysteine residues 1220 and 1284, respectively (Figure 3, upper and lower panel for RING and PHD sequences, respectively). To further confirm that etoposide quinone can react covalently with cysteine residues within the RING and PHD domains of the enzyme, we incubated purified recombinant RING and PHD proteins (aa 1205-1279 and 1280-1321, respectively; Supplementary Figure 1) with etoposide quinone prior to quinone-specific nitro blue tetrazolium (NBT) staining and protein cysteine labeling by fluorescein-iodoacetamide (IAF) (34,46,55). As shown in Figure 4A, etoposide-quinone was able to form covalent adducts with both RING and PHD domains as evidenced by NBT staining of RING and PHD proteins treated

with etoposide quinone. Covalent adduction of etoposide quinone on the two protein domains was accompanied with the concomitant loss of cysteine labeling by IAF thus supporting that quinone adducts occurred on reactive cysteine residues in these two domains. Zn²⁺-bound cysteines found in Zn²⁺-fingers are known to be redox sensitive and to readily react with electrophilic agents such as quinone species leading to covalent modification of these residues and to subsequent ejection of Zn²⁺ (56,57). We thus used the metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR) to monitor the release of Zn²⁺ from RING and PHD Zn²⁺-fingers upon exposure to etoposide quinone (58). As shown in Figure 4B, exposure to etoposide quinone led to the rapid ejection of Zn²⁺ from both RING and PHD domains. Altogether, the data reported above indicate that etoposide quinone covalently reacts with CREBBP catalytic core forming adducts with cysteine residues found within the RING and PHD Zn²⁺-fingers, leading to the subsequent release of Zn²⁺.

CREBBP activity is irreversibly inhibited in human hematopoietic cells and in mice exposed to etoposide

Upon administration, etoposide is readily metabolized into reactive oxidative compounds in cells, most notably etoposide quinone, that covalently binds to redox sensitive cysteine residues of proteins (25,59-62). The biotransformation of etoposide into etoposide quinone is mainly due to cellular oxidative enzymes such as CYP3A4 or peroxidases (24,63). To confirm that etoposide quinone generated from biotransformation of etoposide can indeed inhibit CREBBP, we took advantage of the previously described *in vitro* peroxidase/H₂O₂ system, that mimics the cellular oxidative bioactivation of etoposide into etoposide quinone (31,47). As shown in Supplementary Figure 3, etoposide oxidized by a functional peroxidase/H₂O₂ system was able to irreversibly inhibit CREBBP activity and lead to the formation of protein-quinone adducts (NBT staining) as well as loss of cysteine thiol labeling by IAF. In contrast, when a non-functional peroxidase/H₂O₂ system was used to metabolize etoposide, we did not observe inhibition nor formation of quinone adducts on cysteine residues were observed (Supplementary Figure 3). These findings are in agreement with our results described above and confirm that, contrary to etoposide, its quinone metabolite can readily impair CREBBP HAT activity through formation of irreversible covalent adducts on redox sensitive cysteine residues.

As we mentioned above, metabolization of etoposide into oxidative metabolites, such as etoposide quinone, is prominent in hematopoietic cells treated with this chemotherapeutic

drug and has been implicated in the leukemogenic processes (24,59,63). We thus tested whether the inhibition of CREBBP HAT activity could occur in a cellular context. To this end, K562 cells were exposed to etoposide at concentrations that do not significantly impact cell viability (Supplementary Figure 4) (64). The endogenous CREBBP enzyme was then immunoprecipitated and assayed for HAT activity. As shown in Figure 5A, endogenous CREBBP HAT activity was irreversibly inhibited in cells exposed to etoposide (~60% residual activity). Remarkably, we also found that the acetylation of histone H3 (H3K18Ac and H3K27Ac) in cells was also reduced upon etoposide treatment (Figure 5B). Consistent with the loss of acetylation mark on histone H3, ChIP-qPCR experiments revealed decreased levels of H3K18Ac and H3K27Ac on the promoters of *IL6* and *MDM2* genes (known to be acetylated and regulated by CREBBP, (10,65-67) (Figure 5C). Further investigations carried out on mice (C57BL/6J, n=6) treated with a clinically-relevant dose of etoposide *via* intraperitoneal injection (100 mg/kg) (68) revealed a decrease in H3K18 and H3K27 acetylation levels in histones isolated from the bone marrow cells of treated mice (Figure 6A). In line with these data, the intrinsic HAT activity of immunoprecipitated CREBBP was significantly reduced in etoposide-treated mice (Figure 6A and 6B). Furthermore, ChIP-qPCR experiments performed on chromatin extracted from mice bone marrow cells were in agreement with the previous results showing that H3K18 and H3K27 acetylation was decreased on the promoters of *COPS2* and *MDM2* genes of etoposide-treated mice (Figure 6C). Taken together, these results are consistent with the data obtained with recombinant CREBBP and support the notion that metabolic activation of etoposide into oxidative reactive metabolites, notably etoposide quinone, leads to impairment of CREBBP HAT activity with subsequent aberrant histone acetylation.

DISCUSSION

Despite its strong potential as a chemotherapeutic drug, etoposide is well-known to be associated with the development of treatment-related hematopoietic malignancies (22). The molecular events underlying etoposide-dependent leukemogenesis remain poorly understood, recent studies suggest nonetheless that etoposide quinone, a reactive oxidative metabolite of etoposide, could be a major player in the leukemogenic process, notably through interactions with topoisomerase II enzymes (28-30). However, as a reactive electrophilic species etoposide quinone likely exerts its toxic effects through alteration of other cellular protein targets (24,32,34,35,50,51). In addition, recent studies suggest that exposure to etoposide could lead to epigenetic alterations, notably affecting histones, that may contribute to the deleterious effects of the drug (37,38). In this work, we show that the leukemogenic oxidative metabolite of etoposide, etoposide quinone, is able to impair the activity of the histone acetyltransferase CREBBP. Remarkably, this histone acetyltransferase is recognized as a tumor suppressor that plays a crucial role in the homeostasis of the hematopoietic system (7,11,17,19,69). Moreover, mutations and other molecular genetic abnormalities of CREBBP that alter its acetyltransferase activity are commonly observed in leukemia and lymphoma (7,9,10,14). Interestingly, it has been shown that the histone acetyltransferase p300, the close homologue of CREBBP, is irreversibly inhibited by quinone species such as hydroxynaphthoquinones (70,71). Our in depth enzymatic analyses indicate that the inhibition of CREBBP by etoposide quinone is irreversible and takes places rapidly with a second-order inhibition rate constant of $16 \cdot 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ consistent with the value found for the irreversible inhibition of topoisomerase II α by etoposide quinone ($28 \cdot 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$) (29). Interestingly, topoisomerases II are known biological targets of etoposide quinone and impairment of these nuclear enzymes by etoposide quinone have been suggested to contribute to etoposide-related leukemogenesis (28-30). The biological and toxicological effects of numerous quinone species, such as etoposide quinone, are known to depend on their capacity to react rapidly and covalently with protein cysteine residues, to form irreversible adducts that impact protein structure and/or functions (50,51). Consistently, the irreversible inhibition of topoisomerase II α by etoposide quinone has been suggested to rely on quinone adduction of several reactive cysteine residues of the enzyme (29,72). In a similar manner, our mass spectrometry and labeling experiments showed that etoposide quinone was indeed able to form covalent adducts with two cysteine residues within the RING

and PHD domains of the CREBBP catalytic core (Figures 3 and 4). The RING and PHD domains are each constituted of a redox sensitive C4 Zn²⁺-fingers (4 cysteines coordinating the Zn²⁺ atom) that are known to be an integral part of the CREBBP catalytic core and to be essential for its acetyltransferase activity (41,42,48). Of importance, the two cysteines we identified (Cys1220 and Cys1284) belong, to the RING and PHD Zn²⁺-fingers, respectively where they coordinate the Zn²⁺ atom. Importantly, it was previously shown that mutation of Cys1284 to Ala results in CREBBP inactivation, thus supporting our findings (41). Moreover, comparatively to our findings, it has been shown that Zn²⁺-bound cysteines in Zn²⁺-fingers readily react with quinone species to form covalent adducts that can lead to Zn²⁺ ejection and subsequent alteration of protein function (56,57). More generally, release of Zn²⁺ from C4 Zn²⁺-fingers (such as those in the RING and PHD domains of CREBBP) and their subsequent structural alterations are commonly observed when Zn²⁺-finger cysteines are adducted by electrophilic chemicals (56,57). We indeed observed that reaction of etoposide-quinone with RING and PHD Zn²⁺-finger cysteines of CREBBP led to Zn²⁺ ejection (Figure 4). Interestingly, it has been reported recently that certain quinones species, were able to react with a Zn²⁺-finger in the catalytic domain of the histone demethylase KDM4A and to induce similar Zn²⁺ ejection (73). In line with these observations, our results strongly suggest that impairment of CREBBP activity is likely due to the adduction of the bulky etoposide quinone molecule on these critical RING and PHD Zn²⁺-finger cysteine residues, resulting in subsequent release of Zn²⁺ and alteration of the RING/PHD structures.

As shown for other well-known chemical leukemogens such as benzene, the leukemogenic effects of etoposide are increasingly recognized as relying on the metabolic activation of etoposide into reactive oxidative metabolites in the bone marrow, in particular etoposide quinone (24-26,30,31). Recent studies indicate that etoposide quinone is more potent at impairing topoisomerase II functions than the parent drug etoposide, thus supporting a major role for this quinone metabolite on the topoisomerase II-dependent leukemogenic process of etoposide (28,30). In accordance with these observations, we were unable to find any significant effect of etoposide on purified recombinant CREBBP activity *in vitro* in contrast to etoposide quinone. This is in agreement with the electrophilic properties of the quinone metabolite which prompt this species to react covalently with redox sensitive Zn²⁺-finger cysteines (50,51,56). As stated above, etoposide is readily oxidized in hematopoietic cells into etoposide quinone by cellular cytochromes P450 and peroxidases (24,25,63). In line with the

data obtained with recombinant purified CREBBP, exposure of hematopoietic cells to etoposide led to the inhibition of the endogenous enzyme with concomitant decrease in histone H3K18 and H3K27 acetylation (Figure 5). Further experiments with mice exposed to etoposide confirmed inhibition of CREBBP acetyltransferase activity and decreased H3K18 and H3K27 acetylation (Figure 6). Interestingly, a similar decrease in histone H3 acetylation was recently reported in a human neuroblastoma cell line exposed to etoposide (39).

Overall, our study shows that, in addition to the alteration of topoisomerase II functions, etoposide quinone, a leukemogenic oxidative metabolite of etoposide can impair CREBBP acetyltransferase by reacting with key cysteine residues within the catalytic core of the enzyme.

ACKNOWLEDGMENTS

This work was supported by running grants from Université de Paris and CNRS, and by grants from ANSES (Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail) and ITMO Cancer (plan Cancer-Environnement). WZ, PG and RL are supported by China Scholarship Council PhD fellowships. CM is supported by a PhD fellowship from Université de Paris (ED BioSPC). We thank the technical platform "BioProfiler" (BFA, CNRS UMR 8251 Unit, Université de Paris) for provision of UFLC facilities. We thank Dr. O. Brookes and Dr. C. Mathieu for English language proofreading and comments. We thank Dr. F. Busi for help with PCR primers design and ordering.

CONFLICT OF INTEREST

None declared

ABBREVIATIONS

CREBBP, CREB Binding Protein; KAT, lysine acetyltransferase; NBT, nitroblue tetrazolium; IAF, iodoacetamide-fluorescein

REFERENCES

1. Chi, P., Allis, C.D. and Wang, G.G. (2010) Covalent histone modifications--miswritten, misinterpreted and mis-erased in human cancers. *Nature reviews. Cancer*, **10**, 457-469.
2. Verdin, E. and Ott, M. (2015) 50 years of protein acetylation: from gene regulation to epigenetics, metabolism and beyond. *Nature reviews. Molecular cell biology*, **16**, 258-264.
3. Gu, W. and Roeder, R.G. (1997) Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell*, **90**, 595-606.
4. Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H. and Nakatani, Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell*, **87**, 953-959.
5. Dancy, B.M. and Cole, P.A. (2015) Protein lysine acetylation by p300/CBP. *Chemical reviews*, **115**, 2419-2452.
6. Roelfsema, J.H. and Peters, D.J. (2007) Rubinstein-Taybi syndrome: clinical and molecular overview. *Expert reviews in molecular medicine*, **9**, 1-16.
7. Dutta, R., Tiu, B. and Sakamoto, K.M. (2016) CBP/p300 acetyltransferase activity in hematologic malignancies. *Molecular genetics and metabolism*, **119**, 37-43.
8. Garcia-Ramirez, I., Tadros, S., Gonzalez-Herrero, I., Martin-Lorenzo, A., Rodriguez-Hernandez, G., Moore, D., Ruiz-Roca, L., Blanco, O., Alonso-Lopez, D., Rivas, J.L. *et al.* (2017) Crebbp loss cooperates with Bcl2 overexpression to promote lymphoma in mice. *Blood*, **129**, 2645-2656.
9. Mullighan, C.G., Zhang, J., Kasper, L.H., Lerach, S., Payne-Turner, D., Phillips, L.A., Heatley, S.L., Holmfeldt, L., Collins-Underwood, J.R., Ma, J. *et al.* (2011) CREBBP mutations in relapsed acute lymphoblastic leukaemia. *Nature*, **471**, 235-239.
10. Pasqualucci, L., Trifonov, V., Fabbri, G., Ma, J., Rossi, D., Chiarenza, A., Wells, V.A., Grunn, A., Messina, M., Elliot, O. *et al.* (2011) Analysis of the coding genome of diffuse large B-cell lymphoma. *Nature genetics*, **43**, 830-837.
11. Zhang, J., Vlasevska, S., Wells, V.A., Nataraj, S., Holmes, A.B., Duval, R., Meyer, S.N., Mo, T., Basso, K., Brindle, P.K. *et al.* (2017) The CREBBP Acetyltransferase Is a Haploinsufficient Tumor Suppressor in B-cell Lymphoma. *Cancer discovery*, **7**, 322-337.
12. Hashwah, H., Schmid, C.A., Kasser, S., Bertram, K., Stelling, A., Manz, M.G. and Muller, A. (2017) Inactivation of CREBBP expands the germinal center B cell compartment, down-regulates MHCII expression and promotes DLBCL growth. *Proceedings of the National Academy of Sciences of the United States of America*, **114**, 9701-9706.
13. Horton, S.J., Giotopoulos, G., Yun, H., Vohra, S., Sheppard, O., Bashford-Rogers, R., Rashid, M., Clipson, A., Chan, W.I., Sasca, D. *et al.* (2017) Early loss of Crebbp confers malignant stem cell properties on lymphoid progenitors. *Nature cell biology*, **19**, 1093-1104.
14. Inthal, A., Zeitlhofer, P., Zeginigg, M., Morak, M., Grausenburger, R., Fronkova, E., Fahrner, B., Mann, G., Haas, O.A. and Panzer-Grumayer, R. (2012) CREBBP HAT domain mutations prevail in relapse cases of high hyperdiploid childhood acute lymphoblastic leukemia. *Leukemia*, **26**, 1797-1803.
15. Qian, M., Zhang, H., Kham, S.K., Liu, S., Jiang, C., Zhao, X., Lu, Y., Goodings, C., Lin, T.N., Zhang, R. *et al.* (2017) Whole-transcriptome sequencing identifies a distinct subtype of acute lymphoblastic leukemia with predominant genomic abnormalities of EP300 and CREBBP. *Genome research*, **27**, 185-195.

16. Yang, X.J. (2004) Lysine acetylation and the bromodomain: a new partnership for signaling. *BioEssays : news and reviews in molecular, cellular and developmental biology*, **26**, 1076-1087.
17. Kung, A.L., Rebel, V.I., Bronson, R.T., Ch'ng, L.E., Sieff, C.A., Livingston, D.M. and Yao, T.P. (2000) Gene dose-dependent control of hematopoiesis and hematologic tumor suppression by CBP. *Genes & development*, **14**, 272-277.
18. Rebel, V.I., Kung, A.L., Tanner, E.A., Yang, H., Bronson, R.T. and Livingston, D.M. (2002) Distinct roles for CREB-binding protein and p300 in hematopoietic stem cell self-renewal. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 14789-14794.
19. Zimmer, S.N., Zhou, Q., Zhou, T., Cheng, Z., Abboud-Werner, S.L., Horn, D., Lecoche, M., White, R., Krittsov, A.V., Armstrong, S.A. *et al.* (2011) Crebbp haploinsufficiency in mice alters the bone marrow microenvironment, leading to loss of stem cells and excessive myelopoiesis. *Blood*, **118**, 69-79.
20. Baldwin, E.L. and Osheroff, N. (2005) Etoposide, topoisomerase II and cancer. *Current medicinal chemistry. Anti-cancer agents*, **5**, 363-372.
21. Mistry, A.R., Felix, C.A., Whitmarsh, R.J., Mason, A., Reiter, A., Cassinat, B., Parry, A., Walz, C., Wiemels, J.L., Segal, M.R. *et al.* (2005) DNA topoisomerase II in therapy-related acute promyelocytic leukemia. *The New England journal of medicine*, **352**, 1529-1538.
22. Pendleton, M., Lindsey, R.H., Jr., Felix, C.A., Grimwade, D. and Osheroff, N. (2014) Topoisomerase II and leukemia. *Annals of the New York Academy of Sciences*, **1310**, 98-110.
23. Smith, M.A., Rubinstein, L., Anderson, J.R., Arthur, D., Catalano, P.J., Freidlin, B., Heyn, R., Khayat, A., Krailo, M., Land, V.J. *et al.* (1999) Secondary leukemia or myelodysplastic syndrome after treatment with epipodophyllotoxins. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, **17**, 569-577.
24. Atwal, M., Lishman, E.L., Austin, C.A. and Cowell, I.G. (2017) Myeloperoxidase Enhances Etoposide and Mitoxantrone-Mediated DNA Damage: A Target for Myeloprotection in Cancer Chemotherapy. *Molecular pharmacology*, **91**, 49-57.
25. Kagan, V.E., Kuzmenko, A.I., Tyurina, Y.Y., Shvedova, A.A., Matura, T. and Yalowich, J.C. (2001) Pro-oxidant and antioxidant mechanisms of etoposide in HL-60 cells: role of myeloperoxidase. *Cancer research*, **61**, 7777-7784.
26. Relling, M.V., McLeod, H.L., Bowman, L.C. and Santana, V.M. (1994) Etoposide pharmacokinetics and pharmacodynamics after acute and chronic exposure to cisplatin. *Clinical pharmacology and therapeutics*, **56**, 503-511.
27. Felix, C.A., Walker, A.H., Lange, B.J., Williams, T.M., Winick, N.J., Cheung, N.K., Lovett, B.D., Nowell, P.C., Blair, I.A. and Rebbeck, T.R. (1998) Association of CYP3A4 genotype with treatment-related leukemia. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 13176-13181.
28. Gibson, E.G., King, M.M., Mercer, S.L. and Deweese, J.E. (2016) Two-Mechanism Model for the Interaction of Etoposide Quinone with Topoisomerase IIalpha. *Chemical research in toxicology*, **29**, 1541-1548.
29. Jacob, D.A., Mercer, S.L., Osheroff, N. and Deweese, J.E. (2011) Etoposide quinone is a redox-dependent topoisomerase II poison. *Biochemistry*, **50**, 5660-5667.
30. Smith, N.A., Byl, J.A., Mercer, S.L., Deweese, J.E. and Osheroff, N. (2014) Etoposide quinone is a covalent poison of human topoisomerase IIbeta. *Biochemistry*, **53**, 3229-3236.

31. Vlasova, I., Feng, W.H., Goff, J.P., Giorgianni, A., Do, D., Gollin, S.M., Lewis, D.W., Kagan, V.E. and Yalowich, J.C. (2011) Myeloperoxidase-dependent oxidation of etoposide in human myeloid progenitor CD34+ cells. *Molecular pharmacology*, **79**, 479-487.
32. Alegria, A.E., Sanchez-Cruz, P., Kumar, A., Garcia, C., Gonzalez, F.A., Orellano, A., Zayas, B. and Gordaliza, M. (2008) Thiols oxidation and covalent binding of BSA by cyclolignanic quinones are enhanced by the magnesium cation. *Free radical research*, **42**, 70-81.
33. Montecucco, A., Zanetta, F. and Biamonti, G. (2015) Molecular mechanisms of etoposide. *EXCLI journal*, **14**, 95-108.
34. Nian, Q., Berthelet, J., Zhang, W., Bui, L.C., Liu, R., Xu, X., Duval, R., Ganesan, S., Leger, T., Chomienne, C. *et al.* (2019) T-Cell Protein Tyrosine Phosphatase Is Irreversibly Inhibited by Etoposide-Quinone, a Reactive Metabolite of the Chemotherapy Drug Etoposide. *Molecular pharmacology*, **96**, 297-306.
35. Rojas, E., Mussali, P., Tovar, E. and Valverde, M. (2009) DNA-AP sites generation by etoposide in whole blood cells. *BMC cancer*, **9**, 398.
36. Sinha, B.K. and Myers, C.E. (1984) Irreversible binding of etoposide (VP-16-213) to deoxyribonucleic acid and proteins. *Biochemical pharmacology*, **33**, 3725-3728.
37. Chamani, E., Rabbani-Chadegani, A. and Zahraei, Z. (2014) Spectroscopic detection of etoposide binding to chromatin components: the role of histone proteins. *Spectrochimica acta. Part A, Molecular and biomolecular spectroscopy*, **133**, 292-299.
38. Gill Super, H.J. (2015) A role for epigenetics in the formation of chromosome translocations in acute leukemia. *Cancer genetics*, **208**, 230-236.
39. Groh, T., Hrabeta, J., Khalil, M.A., Doktorova, H., Eckschlager, T. and Stiborova, M. (2015) The synergistic effects of DNA-damaging drugs cisplatin and etoposide with a histone deacetylase inhibitor valproate in high-risk neuroblastoma cells. *International journal of oncology*, **47**, 343-352.
40. Bagheri-Sereshki, N., Hales, B.F. and Robaire, B. (2016) The Effects of Chemotherapeutic Agents, Bleomycin, Etoposide, and Cisplatin, on Chromatin Remodeling in Male Rat Germ Cells. *Biology of reproduction*, **94**, 81.
41. Kalkhoven, E., Teunissen, H., Houweling, A., Verrijzer, C.P. and Zantema, A. (2002) The PHD type zinc finger is an integral part of the CBP acetyltransferase domain. *Molecular and cellular biology*, **22**, 1961-1970.
42. Kalkhoven, E., Roelfsema, J.H., Teunissen, H., den Boer, A., Ariyurek, Y., Zantema, A., Breuning, M.H., Hennekam, R.C. and Peters, D.J. (2003) Loss of CBP acetyltransferase activity by PHD finger mutations in Rubinstein-Taybi syndrome. *Human molecular genetics*, **12**, 441-450.
43. Delvecchio, M., Gaucher, J., Aguilar-Gurrieri, C., Ortega, E. and Panne, D. (2013) Structure of the p300 catalytic core and implications for chromatin targeting and HAT regulation. *Nature structural & molecular biology*, **20**, 1040-1046.
44. Duval, R., Fritsch, L., Bui, L.C., Berthelet, J., Guidez, F., Mathieu, C., Dupret, J.M., Chomienne, C., Ait-Si-Ali, S. and Rodrigues-Lima, F. (2015) An acetyltransferase assay for CREB-binding protein based on reverse phase-ultra-fast liquid chromatography of fluorescent histone H3 peptides. *Analytical biochemistry*, **486**, 35-37.
45. Copeland, R.A. (2005) Evaluation of enzyme inhibitors in drug discovery. A guide for medicinal chemists and pharmacologists. *Methods of biochemical analysis*, **46**, 1-265.
46. Paz, M.A., Fluckiger, R., Boak, A., Kagan, H.M. and Gallop, P.M. (1991) Specific detection of quinoproteins by redox-cycling staining. *The Journal of biological chemistry*, **266**, 689-692.

47. Kagan, V.E., Yalowich, J.C., Borisenko, G.G., Tyurina, Y.Y., Tyurin, V.A., Thampatty, P. and Fabisiak, J.P. (1999) Mechanism-based chemopreventive strategies against etoposide-induced acute myeloid leukemia: free radical/antioxidant approach. *Molecular pharmacology*, **56**, 494-506.
48. Park, S., Stanfield, R.L., Martinez-Yamout, M.A., Dyson, H.J., Wilson, I.A. and Wright, P.E. (2017) Role of the CBP catalytic core in intramolecular SUMOylation and control of histone H3 acetylation. *Proceedings of the National Academy of Sciences of the United States of America*, **114**, E5335-E5342.
49. Elgawish, M.S., Kishikawa, N., Ohyama, K. and Kuroda, N. (2015) Characterization of quinone derived protein adducts and their selective identification using redox cycling based chemiluminescence assay. *Journal of chromatography. A*, **1403**, 96-103.
50. Bolton, J.L. and Dunlap, T. (2017) Formation and Biological Targets of Quinones: Cytotoxic versus Cytoprotective Effects. *Chemical research in toxicology*, **30**, 13-37.
51. Shu, N., Lorentzen, L.G. and Davies, M.J. (2019) Reaction of quinones with proteins: Kinetics of adduct formation, effects on enzymatic activity and protein structure, and potential reversibility of modifications. *Free radical biology & medicine*, **137**, 169-180.
52. Bolton, J.L., Trush, M.A., Penning, T.M., Dryhurst, G. and Monks, T.J. (2000) Role of quinones in toxicology. *Chemical research in toxicology*, **13**, 135-160.
53. Wang, X., Thomas, B., Sachdeva, R., Arterburn, L., Frye, L., Hatcher, P.G., Cornwell, D.G. and Ma, J. (2006) Mechanism of arylating quinone toxicity involving Michael adduct formation and induction of endoplasmic reticulum stress. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 3604-3609.
54. Klopčič, I. and Dolenc, M.S. (2019) Chemicals and Drugs Forming Reactive Quinone and Quinone Imine Metabolites. *Chemical research in toxicology*, **32**, 1-34.
55. Wu, Y., Kwon, K.S. and Rhee, S.G. (1998) Probing cellular protein targets of H₂O₂ with fluorescein-conjugated iodoacetamide and antibodies to fluorescein. *FEBS letters*, **440**, 111-115.
56. Quintal, S.M., dePaula, Q.A. and Farrell, N.P. (2011) Zinc finger proteins as templates for metal ion exchange and ligand reactivity. Chemical and biological consequences. *Metallomics : integrated biometal science*, **3**, 121-139.
57. Lee, H., Komano, J., Saitoh, Y., Yamaoka, S., Kozaki, T., Misawa, T., Takahama, M., Satoh, T., Takeuchi, O., Yamamoto, N. *et al.* (2013) Zinc-finger antiviral protein mediates retinoic acid inducible gene I-like receptor-independent antiviral response to murine leukemia virus. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 12379-12384.
58. Miyamoto, K. and Togiya, K. (2010) The creation of the artificial RING finger from the cross-brace zinc finger by alpha-helical region substitution. *Biochemical and biophysical research communications*, **394**, 972-975.
59. Haim, N., Nemec, J., Roman, J. and Sinha, B.K. (1987) Peroxidase-catalyzed metabolism of etoposide (VP-16-213) and covalent binding of reactive intermediates to cellular macromolecules. *Cancer research*, **47**, 5835-5840.
60. Zheng, N., Felix, C.A., Pang, S., Boston, R., Moate, P., Scavuzzo, J. and Blair, I.A. (2004) Plasma etoposide catechol increases in pediatric patients undergoing multiple-day chemotherapy with etoposide. *Clinical cancer research : an official journal of the American Association for Cancer Research*, **10**, 2977-2985.

61. Fan, Y., Schreiber, E.M., Giorgianni, A., Yalowich, J.C. and Day, B.W. (2006) Myeloperoxidase-catalyzed metabolism of etoposide to its quinone and glutathione adduct forms in HL60 cells. *Chemical research in toxicology*, **19**, 937-943.
62. Lovett, B.D., Lo Nigro, L., Rappaport, E.F., Blair, I.A., Osheroff, N., Zheng, N., Megonigal, M.D., Williams, W.R., Nowell, P.C. and Felix, C.A. (2001) Near-precise interchromosomal recombination and functional DNA topoisomerase II cleavage sites at MLL and AF-4 genomic breakpoints in treatment-related acute lymphoblastic leukemia with t(4;11) translocation. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 9802-9807.
63. Zhuo, X., Zheng, N., Felix, C.A. and Blair, I.A. (2004) Kinetics and regulation of cytochrome P450-mediated etoposide metabolism. *Drug metabolism and disposition: the biological fate of chemicals*, **32**, 993-1000.
64. Mc Gee, M.M., Campiani, G., Ramunno, A., Fattorusso, C., Nacci, V., Lawler, M., Williams, D.C. and Zisterer, D.M. (2001) Pyrrolo-1,5-benzoxazepines induce apoptosis in chronic myelogenous leukemia (CML) cells by bypassing the apoptotic suppressor bcr-abl. *The Journal of pharmacology and experimental therapeutics*, **296**, 31-40.
65. Barber, M.F., Michishita-Kioi, E., Xi, Y., Tasselli, L., Kioi, M., Moqtaderi, Z., Tennen, R.I., Paredes, S., Young, N.L., Chen, K. *et al.* (2012) SIRT7 links H3K18 deacetylation to maintenance of oncogenic transformation. *Nature*, **487**, 114-118.
66. Liu, Y., Xing, Z.B., Zhang, J.H. and Fang, Y. (2013) Akt kinase targets the association of CBP with histone H3 to regulate the acetylation of lysine K18. *FEBS letters*, **587**, 847-853.
67. Sriraman, A., Dickmanns, A., Najafova, Z., Johnsen, S.A. and Dobbstein, M. (2018) CDK4 inhibition diminishes p53 activation by MDM2 antagonists. *Cell death & disease*, **9**, 918.
68. Marchetti, F., Bishop, J.B., Lowe, X., Generoso, W.M., Hozier, J. and Wyrobek, A.J. (2001) Etoposide induces heritable chromosomal aberrations and aneuploidy during male meiosis in the mouse. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 3952-3957.
69. Chan, W.I., Hannah, R.L., Dawson, M.A., Pridans, C., Foster, D., Joshi, A., Gottgens, B., Van Deursen, J.M. and Huntly, B.J. (2011) The transcriptional coactivator Cbp regulates self-renewal and differentiation in adult hematopoietic stem cells. *Molecular and cellular biology*, **31**, 5046-5060.
70. Ravindra, K.C., Selvi, B.R., Arif, M., Reddy, B.A., Thanuja, G.R., Agrawal, S., Pradhan, S.K., Nagashayana, N., Dasgupta, D. and Kundu, T.K. (2009) Inhibition of lysine acetyltransferase KAT3B/p300 activity by a naturally occurring hydroxynaphthoquinone, plumbagin. *The Journal of biological chemistry*, **284**, 24453-24464.
71. Vasudevarao, M.D., Mizar, P., Kumari, S., Mandal, S., Siddhanta, S., Swamy, M.M., Kaypee, S., Kodihalli, R.C., Banerjee, A., Naryana, C. *et al.* (2014) Naphthoquinone-mediated inhibition of lysine acetyltransferase KAT3B/p300, basis for non-toxic inhibitor synthesis. *The Journal of biological chemistry*, **289**, 7702-7717.
72. Bender, R.P., Ham, A.J. and Osheroff, N. (2007) Quinone-induced enhancement of DNA cleavage by human topoisomerase IIalpha: adduction of cysteine residues 392 and 405. *Biochemistry*, **46**, 2856-2864.
73. Jayatunga, M.K., Thompson, S., McKee, T.C., Chan, M.C., Reece, K.M., Hardy, A.P., Sekirnik, R., Seden, P.T., Cook, K.M., McMahan, J.B. *et al.* (2015) Inhibition of the HIF1alpha-p300 interaction by quinone- and indandione-mediated ejection of structural Zn(II). *European journal of medicinal chemistry*, **94**, 509-516.

FIGURE LEGENDS

Figure 1. Etoposide quinone inhibits CREBBP acetyltransferase activity

(A) Purified CREBBP catalytic core (aa 1096-1700) was incubated with increasing concentrations of etoposide quinone (EQ), etoposide (ET) or vehicle (Ctrl) for 20 min at room temperature. Residual CREBBP acetyltransferase activity was then measured by UFLC using H3K18 and H3K27 fluorescent peptides. Results are means of three independent experiments. Error bars indicate S.D. values. ** $p < 0.05$ or *** $p < 0.001$ compared with control (Ctrl).

(B) CREBBP catalytic core was first pre-incubated with 40 μM etoposide quinone (EQ) or etoposide (ET) for 20 min at room temperature prior to further incubation with recombinant histone H3 and acetyl-Coenzyme A (AcCoA) for another 20 min at room temperature. Samples were dot-blotted and H3K18 (left panel) and H3K27 (right panel) acetylation was detected using specific antibodies. Dot-blots were quantified using ImageJ software. Results are means of three independent experiments. Error bars indicate S.D. values. *** $p < 0.001$ compared with control (no EQ or ET).

Figure 2. Inhibition of CREBBP acetyltransferase activity by etoposide quinone is irreversible and fast

(A) CREBBP catalytic core was incubated with 40 μM etoposide quinone (EQ) or vehicle (Ctrl) for 20 min at room temperature. Samples were then buffer exchanged using PD-10 columns and CREBBP residual activity assayed by UFLC using H3K18 fluorescent peptide. Results are means of three independent experiments. Error bars indicate S.D. values. *** $p < 0.001$ compared with control.

(B) CREBBP catalytic core was pre-incubated with 40 μM etoposide quinone (EQ) for 20 min at room temperature followed by incubation with 10 mM DTT for 20 min at room temperature. Samples were then assayed for CREBBP activity by UFLC using H3K18 fluorescent peptide. Results are means of three independent experiments. Error bars indicate S.D. values. *** $p < 0.001$ compared with control.

(C) CREBBP catalytic core was incubated with different concentrations of etoposide quinone (0-10 μM). Aliquots were taken every 30s and residual acetyltransferase activity was measured by UFLC using H3K18 fluorescent peptide. For each etoposide quinone concentration the natural logarithm (\ln) of the percentage (%) of CREBBP residual activity was plotted as a function of time. The apparent first order inhibition constants (k_{obs}) were calculated from the linear regressions (slopes). Results are means of three independent experiments. Error bars indicate S.D. values.

(D) The k_{obs} values were plotted against etoposide quinone (EQ) concentrations and the second order inhibition constant (k_{inhib}) was extracted from the slope. Results are means of three independent experiments. Error bars indicate S.D. values.

Figure 3. Mass spectrometry analyses of etoposide quinone adducts on CREBBP catalytic core

CREBBP catalytic core was incubated with etoposide quinone (40 μM) for 20 min at room temperature. The samples were reduced with DTT and alkylated with NEM prior to trypsin digestion and liquid chromatography-MS/MS analysis. A control without etoposide quinone was done in the same conditions. Mass spectra showing presence of an etoposide quinone

adduct on cysteine 1220 of the RING Zn²⁺-finger (Upper panels) and on cysteine 1284 of the PHD Zn²⁺-finger (Lower panels) are shown. The sequences of the peptides are displayed on each panel in addition to the etoposide quinone adducts (EQ) on the cysteine residues.

Figure 4. Modification of cysteine residues of the RING and PHD domains of CREBBP by etoposide quinone and subsequent Zn²⁺ release

(A) Purified RING (aa 1205-1279) (top panel) and PHD (aa 1280-1321) (bottom panel) domains were incubated with increasing concentrations of etoposide quinone (EQ, 0-40 μM) or 40 μM etoposide (ET) for 20 min at room temperature. For the quinone-specific nitro blue tetrazolium (NBT) staining, samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Etoposide quinone adducts on the RING and PHD proteins were then detected using NBT as described in Materials and Methods. Ponceau red staining is shown as a loading control. For the iodoacetamide-fluorescein (IAF) labelling, samples were incubated with 10 μM IAF for 10 min at room temperature, separated by SDS-PAGE and transferred onto nitrocellulose membranes. Cysteine residues labelled by IAF were detected by fluorescence ($\lambda_{exc}=485$ nm, $\lambda_{em}=530$ nm). Ponceau red staining is showed as a loading control.

(B) Purified RING and PHD domains were incubated with or without etoposide quinone (40 μM) in the presence of 100 μM PAR. The absorbance of PAR-Zn²⁺-complexes (490 nm) was followed as a function of time at room temperature using a plate reader. Etoposide quinone had no effect on PAR absorbance (data not shown).

Figure 5. Exposure of K562 cells to etoposide leads to inhibition of CREBBP and to the loss of H3K18 and H3K27 acetylation

(A) K562 cells were exposed to 40 μM etoposide (ET) or vehicle (Ctrl) for 24h, cells were lysed and cellular CREBBP was isolated by immunoprecipitation. Immunoprecipitated CREBBP activity was measured by UFLC. In parallel, CREBBP protein content in immunoprecipitates was determined by Western-blot (Right panel). Results are means of three independent experiments. Western blot is representative of three independent experiments. Error bars indicate S.D. values. ** $p<0.01$ compared with control (Ctrl).

(B) Histones were extracted from the cell lysates and H3K18 and H3K27 acetylation was detected by Western blot. Quantification of H3 acetylation in Western blots was carried out using ImageJ software. Results are means of three independent experiments. Western blot is representative of three independent experiments. Error bars indicate S.D. values.* $p<0.05$ compared with control (Ctrl), *** $p<0.001$.

(C) Chromatin immunoprecipitation (ChIP) was conducted on cells chromatin using anti-H3K18ac and H3K27ac histone marks as described in *Materials and Methods*. RT-qPCR experiments were subsequently carried out on *IL6* and *MDM2* genes using primers specific for their promoters. Results are means of three independent experiments. Error bars indicate S.D. values.*** $p<0.001$ compared with control (Ctrl).

Figure 6. Exposure of mice to etoposide inhibits CREBBP activity and H3K18 and H3K27 acetylation in bone marrow cells.

(A) C57BL/6J mice (n=6) were treated with 100 mg/kg etoposide (Tn) or vehicle (Cn) for 24h. Bone marrow was isolated from hind limb long bone. Histones were extracted from cell lysates and H3K18 and H3K27 acetylation was detected by Western blot (Left panels). Quantification of H3 acetylation in Western blots was carried out using ImageJ software (Right panel). Results are

means of the six treated (Tn) and non-treated (Cn) mice. Error bars indicate S.D. $**p<0.01$ compared with control (Ctrl), $***p<0.001$ compared with Ctrl.

(B) CREBBP was first immunoprecipitated from the bone marrow cell lysates and its acetyltransferase activity of the immunoprecipitates was measured by UFLC. In parallel, CREBBP protein content in the immunoprecipitates was determined by Western-blot (Figure 6A, Left panel). Results are means of the six treated (Tn) and non-treated (Cn) mice. Error bars indicate S.D. $***p<0.01$ compared with control (Ctrl).

(C) Chromatin immunoprecipitation (ChIP) was carried on bone marrow cells chromatin using antibodies against the H3K18Ac and H3K27Ac histone marks as described in *Materials and Methods*. RT-qPCR experiments were subsequently carried out on murine *COPS2* and *MDM2* genes using primers specific for their promoters. Results are means of the six treated (Tn) and non-treated (Cn) mice. Error bars indicate S.D. values. $**p<0.01$ compared with control (Ctrl), $***p<0.001$ compared with Ctrl.

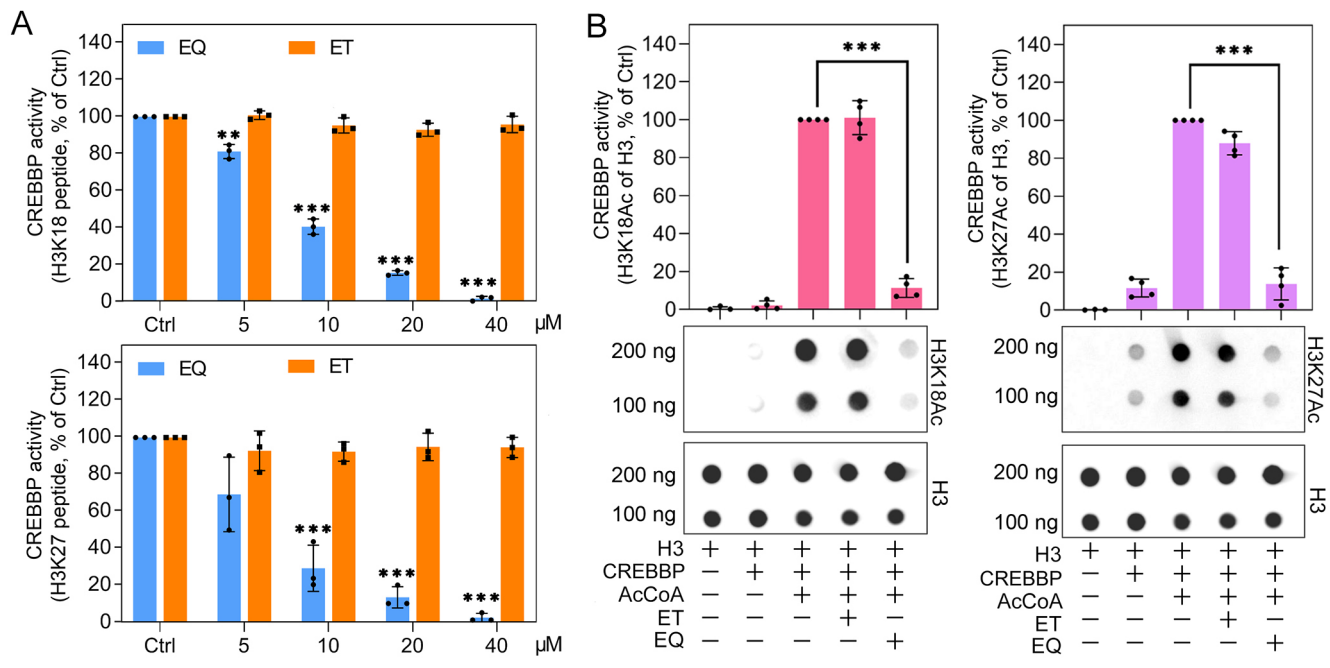


Figure 1

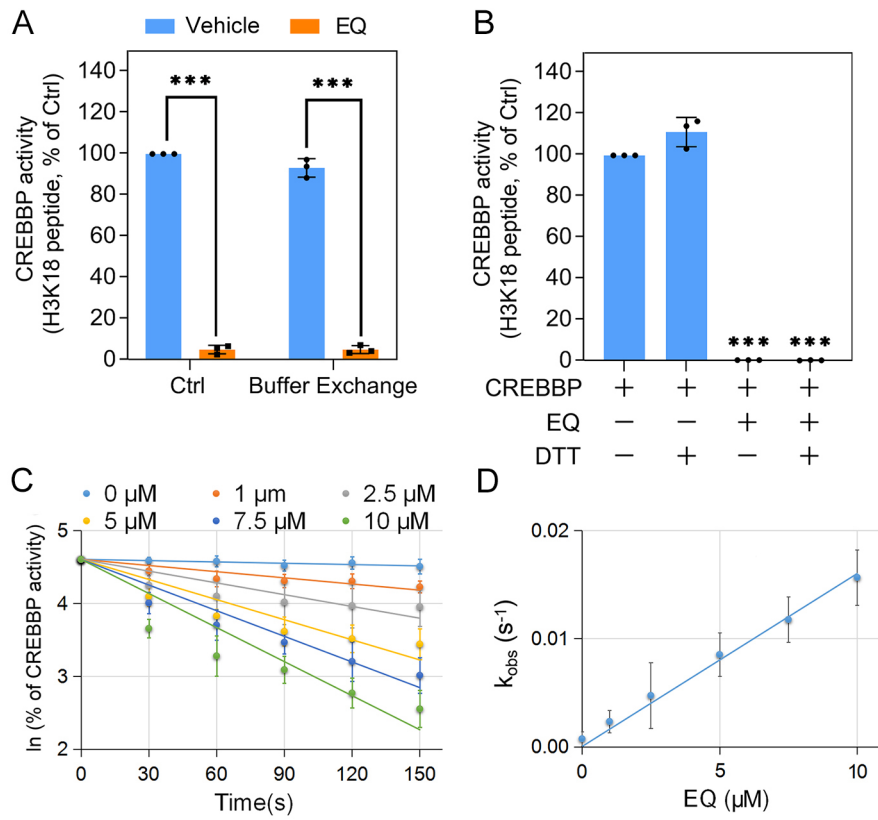


Figure 2

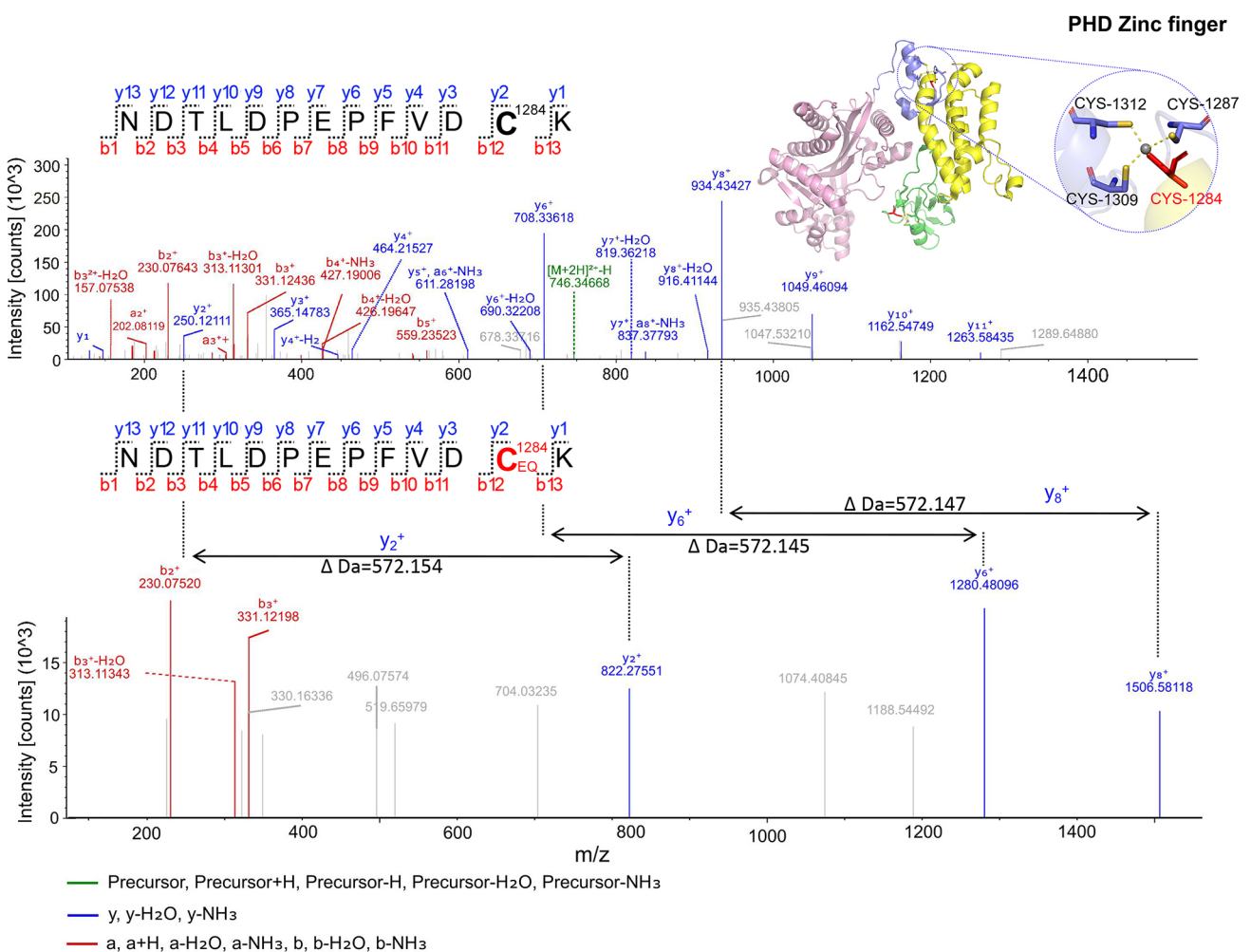
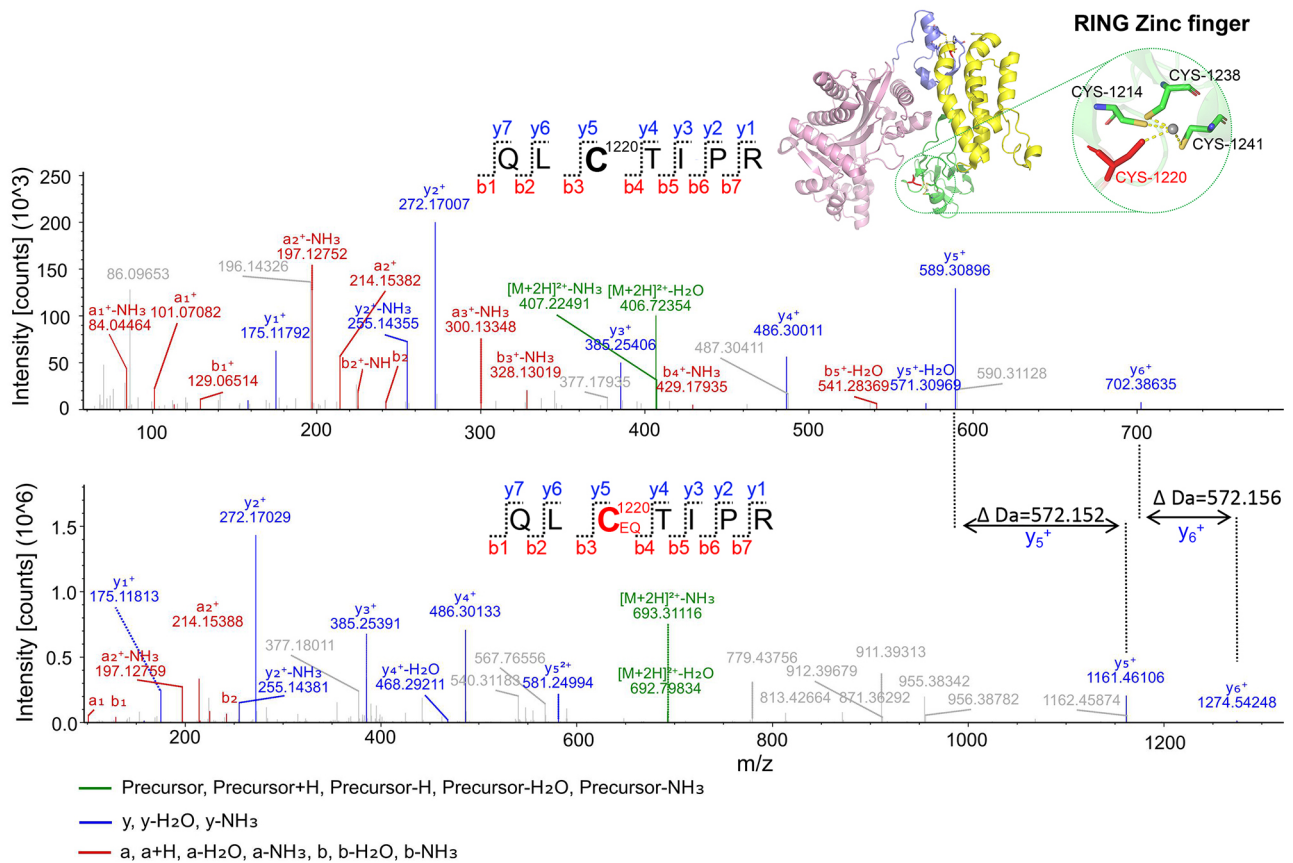


Figure 3

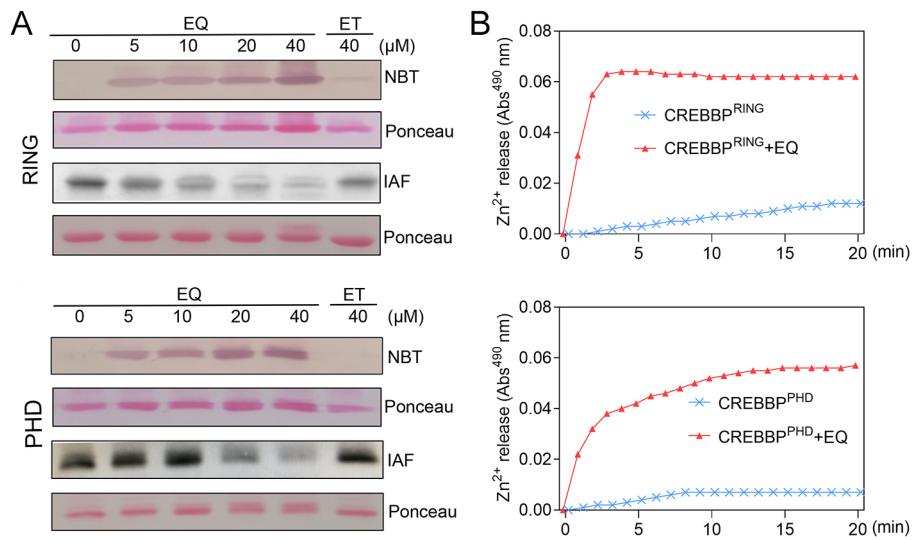


Figure 4

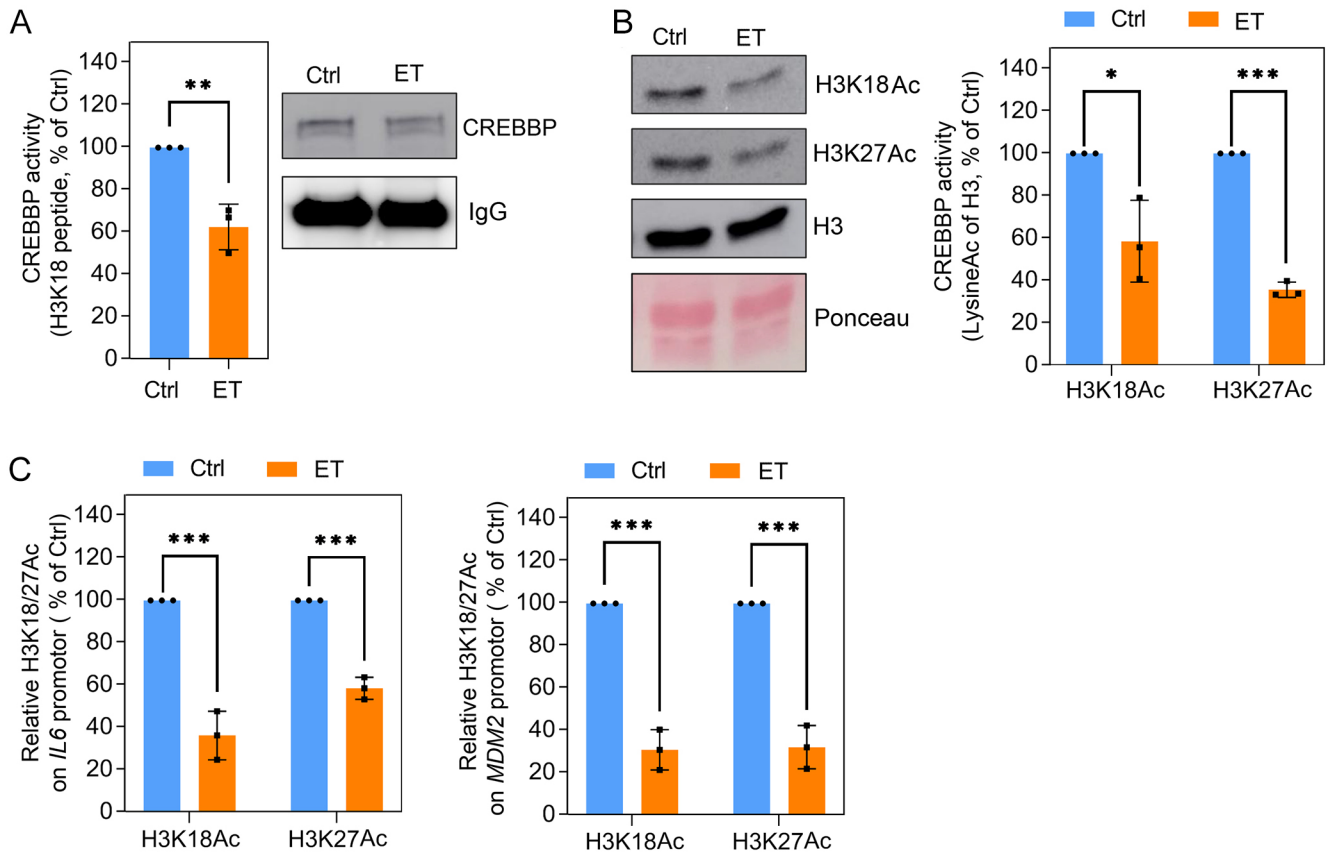


Figure 5

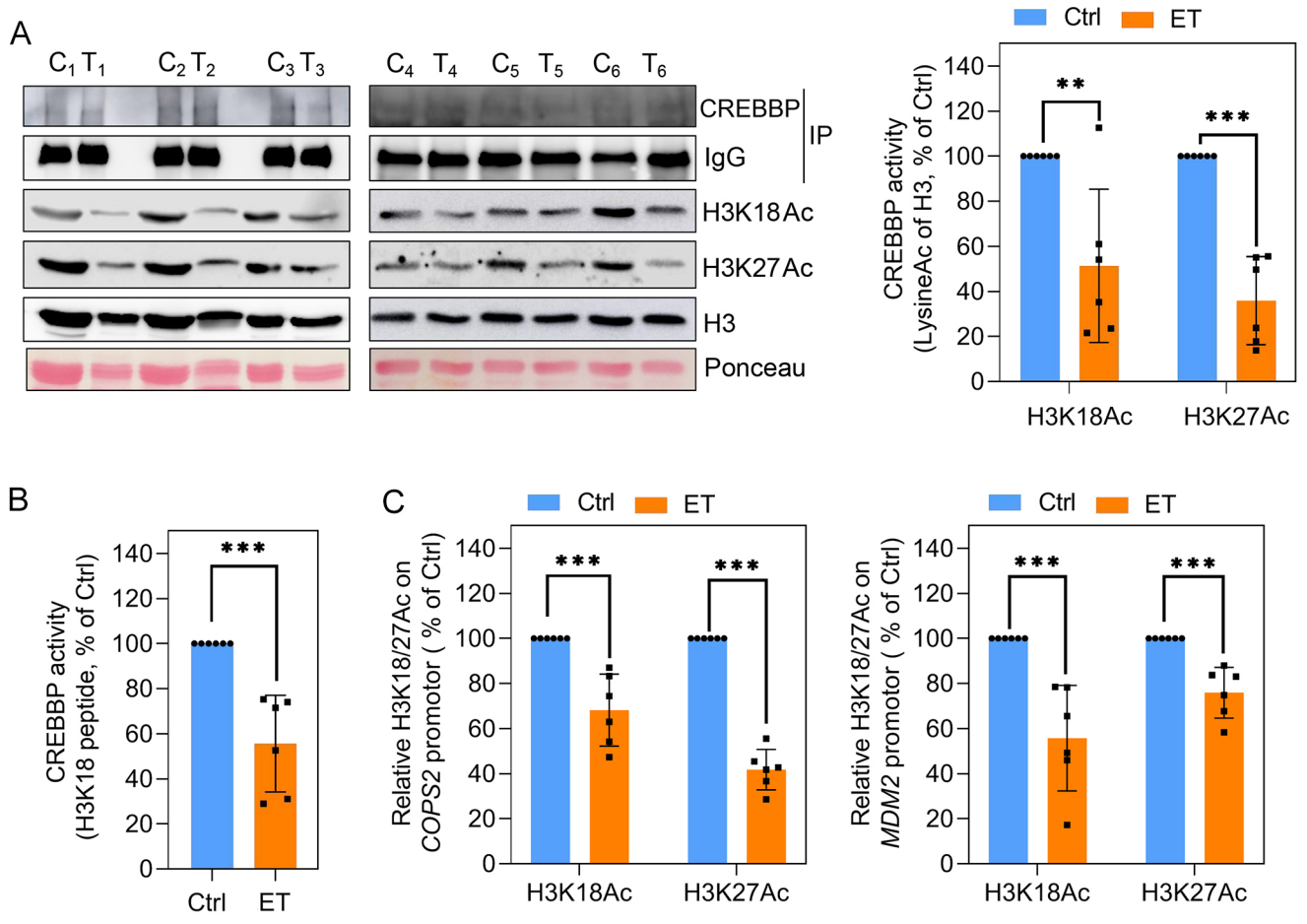


Figure 6

