Naphthoquinone-mediated Inhibition of Lysine Acetyltransferase KAT3B/p300, Basis for Non-toxic Inhibitor Synthesis*

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Background: 1,4-Naphthoquinone analogs, such as plumbagin, are toxic compounds due to their redox cycling and thiol-reactive properties.

Results: The p300 inhibitor PTK1, a plumbagin derivative with greatly reduced toxicity, was synthesized and characterized.

Conclusion: PTK1 is a reversible, non-competitive inhibitor of p300 KAT activity with reduced toxicity.

Significance: These studies provide insight into naphthoquinone-mediated KAT inhibition and describe the synthesis of a therapeutically important, non-toxic inhibitor.

Hydroxynaphthoquinone-based inhibitors of the lysine acetyltransferase KAT3B (p300), such as plumbagin, are relatively toxic. Here, we report that free thiol reactivity and redox cycling properties greatly contribute to the toxicity of plumbagin. A reactive 3rd position in the naphthoquinone derivatives is essential for thiol reactivity and enhances redox cycling. Using this clue, we synthesized PTK1, harboring a methyl substitution at the 3rd position of plumbagin. This molecule loses its thiol reactivity completely and its redox cycling ability to a lesser extent. Mechanistically, non-competitive, reversible binding of the inhibitor to the lysine acetyltransferase (KAT) domain of p300 is largely responsible for the acetyltransferase inhibition. Remarkably, the modified inhibitor PTK1 was a nearly non-toxic inhibitor of p300. The present report elucidates the mechanism of acetyltransferase activity inhibition by 1,4-naphthoquinones, which involves redox cycling and nucleophilic adduct formation, and it suggests possible routes of synthesis of the non-toxic inhibitor.

Over the past decade, several small molecule modulators of chromatin-modifying enzymes have been discovered (1, 2). These molecules include specific and nonspecific inhibitors of histone deacetylases, histone acetyltransferases (now known as lysine acetyltransferases or KATs),3 histone methyltransferases, and specific activators of the lysine acetyltransferases 3B/3A (p300/CBP). 1,4-Naphthoquinone derivatives are naturally occurring compounds that are present in the Plumbago and Diospyros plant genera and have a variety of biological activities (3). Our laboratory previously reported that one of the 1,4-naphthoquinones, plumbagin, is a potent inhibitor of the KAT p300. Plumbagin specifically inhibits p300-mediated p53 acetylation but not the p53 acetylation by the lysine acetyltransferase KAT2B (p300/CBP-associated factor) (4). This study described for the first time that a structural entity (a hydroxyl group at the 5th position of plumbagin) is required for the inhibition of acetyltransferase activity. However, naphthoquinone derivatives are relatively toxic molecules, and their efficacy and utility in vivo has been limited due to this characteristic (5–8). The aim of the present study is to understand the mechanism of KAT inhibition as well as the chemical entity responsible for its cytotoxicity and, thus, to synthesize a non-toxic KAT inhibitor.

Among the different small molecule KAT inhibitors known to date, Lys-CoA was the first to be discovered as a p300 acetyltransferase-specific inhibitor (9). The catalytic mechanisms of the enzyme have been investigated from the co-crystal structural analysis of the p300 KAT domain and Lys-CoA (10). Lys-CoA interacts extensively with the acetyltransferase domain, particularly in the hydrophobic tunnel. Lys-CoA-mediated inhibition supports a Theorell-Chance model rather than a standard ordered binding, ternary complex, or ping-pong mechanism. Based on the residues that Lys-CoA binds within the hydrophobic tunnel, a new enzyme-inhibitory scaffold, C646, has been synthesized by the same group (11). Over the years, we have discovered a few naturally occurring, small molecule KAT inhibitors (4, 12–16). Our investigations have

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revealed that there are pockets in the p300 acetyltransferase KAT domain, other than the hydrophobic tunnel, where these small molecules may bind and cause enzyme inhibition (4, 17). These p300 inhibitors, such as garcinol, plumbagin, and the p300-specific garcinol derivative LTK14, have at least one binding site within the KAT domain (17). A docking analysis with plumbagin has shown that binding may not occur in the hydrophobic tunnel of the KAT domain, suggesting that other binding pockets might exist (4). Although the mechanisms of action for these small molecule inhibitors have been investigated in terms of enzyme binding and kinetics, the chemical nature of these small molecules has received much less attention. Notably, most KAT inhibitors consist of hydroxyl groups, leading to speculation that the -OH groups could facilitate enzyme-small molecule interactions and thereby KAT inhibition (4). In this respect, we have previously reported that the activity of plumbagin can be ascribed to the hydrogen bonding between the hydroxyl group and Lys-1358 in the KAT domain (4). However, plumbagin is known to react with free -SH (thiol) groups available in the intracellular milieu, including glutathione, and is also involved in redox cycling. These chemical properties of 1,4-naphthoquinones, such as plumbagin, may be the cause of their cytotoxicity and may influence their KAT-inhibitory activity. The toxicity also hampers their utility (5–8). Therefore, we are interested in investigating the role of the chemical nature of plumbagin and other related 1,4-naphthoquinone analogs in KAT inhibition and cytotoxicity with the ultimate goal of synthesizing a non-toxic, reversible inhibitor.

Our results suggest that the major mechanism of plumbagin-mediated KAT inhibition is through irreversible protein interactions. However, the cytotoxicity of plumbagin analogs is due to their ability to generate reactive oxygen species as well as their reactivity to thiols. The structure-function relationships of these 1,4-naphthoquinones lead us to the conclusion that the structural moieties responsible for KAT inhibition and those responsible for toxicity do not overlap and can be delineated. Based on these observations, we have synthesized a new molecule that does not have free thiol reactivity and has less redox cycling potential but retains KAT inhibitory activity. Thus, this molecule could potently reduce histone acetylation in cell-based assays with greatly decreased toxicity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Treatments, and Immunoblotting—SHSY-5Y** (human neuroblastoma) and HEK293 (human embryonic kidney) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C and 5% CO₂. p300 inhibition is through irreversible protein interactions. However, the cytotoxicity of plumbagin analogs is due to their ability to generate reactive oxygen species as well as their reactivity to thiols. The structure-function relationships of these 1,4-naphthoquinones lead us to the conclusion that the structural moieties responsible for KAT inhibition and those responsible for toxicity do not overlap and can be delineated. Based on these observations, we have synthesized a new molecule that does not have free thiol reactivity and has less redox cycling potential but retains KAT inhibitory activity. Thus, this molecule could potently reduce histone acetylation in cell-based assays with greatly decreased toxicity.

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**Cell Viability Assay**—For quantitation of cell viability, we performed a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. SHSY-5Y cells were seeded in 96-well plates (5000 cells/well). After 12 h, naphthoquinone derivatives were resuspended in the culture medium and added to the wells at the desired concentrations. After 24 h, the compound-containing medium was removed, and equal volumes of fresh medium were added to each well along with 20 μl of MTT (Sigma; 5 mg/ml in PBS). As expected, MTT was reduced to purple-colored formazan only in viable cells. After incubation for 4 h, DMEM was removed by syringe, and the formazan crystals were redissolved in 200 μl of DMSO. The plate was incubated at 37 °C for 5 min. The color was quantified with an ELISA plate reader (Versa Max ELISA plate reader, Molecular Devices, 595-nm wavelength).

**Detection and Measurement of ROS**—HEK293 and HeLa cells were grown in their respective media to 75% confluence. For fluorescence-based images, cells were grown on coverslips coated with poly-l-lysine. Cells were washed with PBS and treated with 5 μM CM-H2DCFDA (Invitrogen) for 20 min at 37 °C under 5% CO₂. The cells were then treated with different concentrations of compounds for the indicated times. Later, the cells were trypsinized and resuspended in PBS, and the fluorescence intensity was measured by flow cytometry. The sample was excited at 485 nm, and fluorescence was measured at 530 nm. For imaging, coverslips containing cells were washed with PBS and mounted onto a glass slide using 70% glycerol as a mountant. Images were captured using a Zeiss Axioskop 2 Plus fluorescence microscope.

**Lysine Acetyltransferase Assay (Filter-binding Assay)**—Lysine acetyltransferase assays were performed as described previously (9). In brief, 800 ng of highly purified HeLa core histones was incubated in HAT assay buffer at 30 °C for 10 min with baculovirus-expressed recombinant p300 or CBP in the presence or absence of select compounds. This procedure was followed by the addition of 1.0 μl of 3.6 Ci/mM [3H]acetyl-CoA (PerkinElmer Life Sciences). This mixture was further incubated for 10 min. The reaction mixture was then kept on ice for 5 min to stop the reaction, spotted onto P81 (Whatman) filter paper, and dried. Scintillation counts were recorded on a Wallac 1409 liquid scintillation counter. All assays were performed in replicates, and the average was plotted, with error bars indicating the S.D.

**DTNB Assay and UV-visible Absorption/Emission Spectrometry**—DTNB solution (3 mg/ml) was prepared in potassium phosphate buffer (100 mM, pH 6.8). NAC, DTT, and 1,4-naphtho-
quinone derivatives were dissolved in 100 mM DMSO. Initially, increasing concentrations of NAC and DTT (25–150 μM) were added to 100 μl of phosphate buffer. 1,4-Naphthoquinone derivatives were subsequently added to make a final concentration of 50 μM. An incubation time of 5 min at room temperature was followed by the addition of 100 μl of DTNB solution, maintaining the total reaction volume as 200 μl in all cases. The reaction was allowed to proceed for 5 min at room temperature, and the absorbance of the solution was analyzed at 412 nm using a Versa Max ELISA plate reader (Molecular Devices). For performing UV-visible absorption spectrometry, 1,4-naphthoquinone derivatives (1 mM in DMSO) were diluted in 1 ml of 100 mM potassium phosphate buffer (pH 6.8) or 100 mM HEPES buffer (pH 7.5) and were scanned for UV-visible absorption from 200 to 600 nm. Subsequently, NAC (1 mM in DMSO) was added, wherever necessary, to the buffer containing the derivatives, and scanning was repeated. The absorption spectrum was read using a spectrophotometer (U-2010, Hitachi). The same sets of solutions were also used for measuring the excitation wavelength and emission spectra using FluoroMax-3 (Horiba Jobin Yvon).

Kinetic Characterization—Lysine acetyltransferase assays were carried out with an ectopically expressed baculoviral construct encoding full-length p300, in the absence or presence of different concentrations of the inhibitor PTK1 (0, 15, 20, 25, 35, and 45 μM). The enzyme is known to catalyze a bisubstrate reaction comprising two substrates, namely core histone and [3H]acetyl-CoA. Therefore, a kinetic analysis was performed on two different combinations. The enzyme activity was determined at increasing concentrations (1–8 μM) of [3H]acetyl-CoA while keeping the concentration of core histones constant at 1.7 mM. In the second combination, the [3H]acetyl-CoA concentration was kept constant at 1.6 μM while the core histone concentrations were varied from 5 to 70 nm. The reaction velocity was determined as a function of the incorporation of radioactivity and was expressed as counts/min (cpm). All of the experiments were conducted in triplicate, and the values were within 15% of the error range. The Michaelis-Menten plot was generated using the model, \( y = V_{max} \times [S]/(K_m + [S]) \). The \( K_m \) was determined by fitting to a straight line by the least square method in a Lineweaver-Burk plot using GraphPad prism version 5.0 software.

Reversibility Studies—The reversibility of the inhibitors was established by the time-dependent dilution and “drop dialysis” methods. For dialysis, the enzyme p300 was incubated with inhibitors for 30 min, and the mixture was dialyzed at 4 °C against 2× HAT assay buffer using 0.05-μm Millipore “V series membrane” to remove the inhibitors. The residual activity was assayed and compared with the predialysis activity. For time-dependent dialysis-based experiments, full-length p300 was preincubated in 1× HAT assay buffer at pH 8.0 with either enzyme alone or with increasing concentrations (25–100 μM) of inhibitors with varying preincubation times (1–15 min). Aliquots of an appropriate volume were taken from each reaction mixture to make it final 1:25 dilution in every case. From each reaction mixture, the residual activity was measured as a percentage of the control and plotted against the preincubation time. Semilogarithmic plots were constructed, and the data were fit using the semilog line module of GraphPad prism version 5.0. The rate of inactivation was measured by fitting to the equation, \( y = y \text{ intercept + slope} \times \log(x) \), where \( x \) is logarithmic, \( y \) is linear, and the rate is equivalent to the slope equal to the apparent inactivation constant (\( k_{obs} \) per min) at each inhibitor concentration.

Dynamic Light Scattering—The measurements were performed on a Zetasizer Nano S particle analyzer (Malvern Instruments) with a helium-neon laser (632.8 nm) light source that utilizes 4-milliwatt power at the same wavelength. Scattered light from the samples was collected at an angle of 173°. The built-in software generates a correlation curve from the intensity autocorrelation function. The intensity-weighted mean hydrodynamic diameter, or \( Z_{aw} \) diameter, of the sample was obtained from the cumulants analysis of the correlation curve. To study the effect of plumbagin, PTK1, and juglone on p300 oligomerization and compare their adduct-forming abilities, 5 μM p300 in 10 mM Tris-HCl at pH 7.5 containing 150 mM NaCl was treated with increasing concentrations (25–100 μM) of the three compounds, and the \( Z_{aw} \) diameters of treated and untreated p300 were monitored by dynamic light scattering. Ten measurements (10 runs for each measurement) were recorded for each sample at 25 °C. Because the compounds are dissolved in DMSO, in control experiments, p300 was treated with an equal volume of DMSO without the compound(s), and the hydrodynamic parameters mentioned above were checked to confirm the absence of any alteration.

Surface-enhanced Raman Spectroscopy (SERS) Analysis—SERS spectra were recorded in a Raman system using 180° back-scattering geometry and a 632.8-nm helium-neon laser (model 30995, Research Electro Optics, Inc.) as a Raman excitation source (18, 19). The spectrometer consists of a monochromator (HORIBA JOBIN YVON, iHR 320) and a Peltier-cooled CCD (Andor iDus). A holographic 1800 grooves mm⁻¹ grating was used along with the 200-μm spectrograph entrance slit setting, providing ~3 cm⁻¹ resolution. For SERS studies of protein, a 60× infinity-corrected objective (Nikon Plan Apo; numerical aperture 0.9) was used. The laser power used at the sample was 5 milliwatts. Samples were prepared by concentrating the silver nanoparticles to 10 times, and a concentrated nanoparticle solution/analyte ratio of 4:1 was maintained. The typical accumulation time of 30 s was used. The SERS spectrum was smoothed using a standard five-point fast Fourier transform filtering technique, as shown in Fig. 9D. The silver solution was prepared as reported previously (20). Initially, 45 mg of AgNO₃ was dissolved in 250 ml of water, and the solution was brought to a boil. A solution of 1% sodium citrate (5 ml) was then added to the AgNO₃ solution under vigorous stirring, and boiling was continued for ~60 min. The plasmon absorption maximum was located at 420 nm.

Docking Studies—The crystal structure of p300 KAT domain was extracted from the Protein Data Bank using the code 3BIY. The structure of the inhibitor PTK1 was optimized using the GAUSSIAN 03 program (21). Docking analysis was performed with Autodock version 4.2 using a Lamarckian genetic algorithm. A 5-grid spacing of 0.375 was used, and docking was performed with a flexible ligand with 100 Genetic Algorithm runs, a population size of 300, 25 × 10⁵ evaluations, and a max-
imum of 27,000 generations (22). The analysis of conformations was conducted based on clusters generated using MGL Autodock tools version 1.5.4. Figures were created using the PyMOL molecular graphics system (23).

Synthesis of 5-Hydroxy-2,3-dimethylnaphthalene-1,4-dione (PTK1)—PTK1 was synthesized as reported elsewhere (24). Plumbagin was isolated in our laboratory from *Plumbago rosea* as reported previously (4). PTK1 was synthesized by mixing plumbagin (1 mM), acetic acid (12 mM) as a radical source, and benzene (80 ml) as the solvent. The reaction mixture was cooled over dry ice and methanol until the solvent was frozen. The flask was degassed *in vacuo* for 15 min and filled with nitrogen. This degassing process was repeated three times. After adding lead tetra-acetate (3 mM), the reaction mixture was refluxed under a nitrogen atmosphere for 24 h. Upon completion, the reaction was quenched with water to decompose the excess lead tetra-acetate. The organic layer was dried over anhydrous Na$_2$SO$_4$ and evaporated. The obtained crude product was purified by column chromatography using hexane/ethyl acetate (8:5:1.5).

$^1$H NMR and HRMS characterization of PTK1 were performed as described previously (15). The chemical structure was confirmed by following parameters: Orange Needles, yield 59%, $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 2.17 (s, 6H, 2 CH$_3$), 7.52–7.64 (m, 3H, Ar-3H), 12.16 (s, 1H, OH). HRMS (m/z): calculated for C$_{12}$H$_{10}$O$_3$, 203.0663; found 203.0681.

**Chemicals and Instruments**—All of the chemicals were purchased from Sigma-Aldrich. Mass spectrometry was performed with a high resolution mass spectrometer (Agilent Technologies), and NMR spectra were obtained from a Bruker (400-MHz) NMR spectrometer.

**RESULTS**

*N*-Acetyl Cysteine Blocks Plumbagin-mediated KAT Inhibition as Well as Its Pleiotropic Effects in Vivo—Plumbagin is a known redox-cycling and free thiol-reactive molecule (25–27). Both of these properties influence its KAT-inhibitory properties in cells. Because there are earlier reports that are contradictory with respect to the role of ROS in histone acetylation (28–30), we investigated the potential effect of ROS generation in KAT-inhibitory activity and cytotoxicity. To do this, we treated the mammalian cell lines SH-SY5Y, HEK293, and HeLa S3 with plumbagin in the presence of the free radical scavenger NAC. As observed from the images of cells photographed after 6 h of treatment, NAC completely negated plumbagin-induced cell stress (Fig. 1A). Plumbagin induced DNA damage, presumably,
Redox Cycling and Thiold Reactivity in p300 Inhibition

A variety of analogs of 1,4-naphthoquinone (Fig. 3A) were procured, and in vitro enzyme assays were performed with these compounds in the presence or absence of NAC (Fig. 4A). These small molecules vary substantially in their reactive potential at the 3rd position. It was observed that all of the analogs with a chemically reactive 3rd position (1,4-naphthoquinone, menadione, and 5-hydroxy-1,4-naphthalenedione (juglone) but not lawsone) inhibited p300 activity, albeit to varying extents. Lawsone did not show significant inhibition even at 100 μM. Although the inhibitory activities of thiol-reactive molecules such as 1,4-naphthoquinone were abrogated upon the addition of NAC (similar to the abrogation of plumbagin activity), this was not the case for juglone. Lawsone could not inhibit p300 significantly, and there was no further change after the addition of NAC (Fig. 4A). Considering that the hydroxyl group of plumbagin is crucial for p300 inhibition, it was interesting to note that other analogs without hydroxyl groups, such as 1,4-naphthoquinone, also inhibited p300. However, 1,4-naphthoquinone possesses a reactive 3rd position that would enable reactivity to thiols. Thus, we presumed that the free thiol reactivity of these analogs with cysteines could influence their inhibitory activity. To correlate KAT inhibition and free thiol reactivity, we carried out a colorimetric assay for free thiol estimation using DTNB.

due to the generation of ROS. DNA damage is marked by the predominance of histone H2AX phosphorylation (H2AXγ). We observed that 20 μM plumbagin drastically induced the level of H2AXγ (Fig. 1B, lane 6). Consistent with the absence of any visible cellular stress, pretreatment for 2 h with 2 mM NAC almost completely inhibited the plumbagin-induced DNA damage, as revealed by the near absence of H2AXγ even at an 80 μM concentration in SH-SY5Y and HeLa S3 cells (Fig. 1B, lanes 3–5). Remarkably, the KAT-inhibitory potential of plumbagin was completely abrogated in the presence of NAC (Fig. 1B, lane 5 versus lane 6). Considering that the hydroxyl group of plumbagin is crucial for p300 inhibition, it was interesting to note that other analogs without hydroxyl groups, such as 1,4-naphthoquinone, also inhibited p300. However, thiol reactivity of the molecule could influence the inhibition of acetyltransferase activity. We addressed this possibility through structure–activity relationship analysis. A variety of analogs of 1,4-naphthoquinone (Fig. 3A) were procured, and in vitro enzyme assays were performed with these compounds in the presence or absence of NAC (Fig. 4A). These small molecules vary substantially in their reactive potential at the 3rd position. It was observed that all of the analogs with a chemically reactive 3rd position (1,4-naphthoquinone, menadione, and 5-hydroxy-1,4-naphthalenedione (juglone) but not lawsone) inhibited p300 activity, albeit to varying extents. Lawsone did not show significant inhibition even at 100 μM. Although the inhibitory activities of thiol-reactive molecules such as 1,4-naphthoquinone were abrogated upon the addition of NAC (similar to the abrogation of plumbagin activity), this was not the case for juglone. Lawsone could not inhibit p300 significantly, and there was no further change after the addition of NAC (Fig. 4A). Considering that the hydroxyl group of plumbagin is crucial for p300 inhibition, it was interesting to note that other analogs without hydroxyl groups, such as 1,4-naphthoquinone, also inhibited p300. However, 1,4-naphthoquinone possesses a reactive 3rd position that would enable reactivity to thiols. Thus, we presumed that the free thiol reactivity of these analogs with cysteines could influence their inhibitory activity. To correlate KAT inhibition and free thiol reactivity, we carried out a colorimetric assay for free thiol estimation using DTNB.
The assay is based on the fact that the aromatic disulfide (DTNB) reacts with aliphatic thiol groups to form a mixed disulfide and 2-nitro-5-thiobenzoate. DTNB has negligible absorbance, but when it reacts with -SH groups under mild alkaline conditions (pH 7–8, physiological buffers), the 2-nitro-5-thiobenzoate anion (TNB\(_2^–\)) gives an intense yellow color at 412 nm. Thus, if NAC covalently bound to any of the analogs of 1,4-naphthoquinone, the abundance of the free thiol group would be reduced, which would be reflected as a decrease in the absorbance. The NAC-mediated increase in absorbance was reduced by 1,4-naphthoquinone, plumbagin, and menadione at 50 \(\mu\)M, in all lanes containing NAC (Fig. 4B).

An electron-donating group (hydroxyl group) occurs at the 5th position of plumbagin and is conjugated with the carbonyl group at the 4th position, which results in a diminished \(\pi\)-electron cloud around the C2\(\rightarrow\)C3 double bond. This configuration leads to preferential nucleophilic attack at the 3rd position, and the structure is subsequently stabilized by the methyl group present at the 2nd position. In the absence of the electron-donating group (i.e. the methyl group), the symmetry would result in nucleophilic attack at either the 2nd or the 3rd position, as in the case of 1,4-naphthoquinone. However, in the case of menadione, which does not possess the hydroxyl group, the preferential position of nucleophilic attack is at the 3rd position because the 2nd position is occupied by a methyl group. The absence of a hydroxyl group at the 5th position coupled with a single site for nucleophilic addition is probably the reason that menadione is a weaker inhibitor than 1,4-naphthoquinone or plumbagin (Fig. 4A). In the case of juglone, the preferential mode of nucleophilic attack is at the 3rd position. However, the intermediate thus obtained is not as stable as that of plumbagin, and hence it results in poor affinity toward a nucleophilic attack, which can be correlated with a lack of a decrease in absorbance (Fig. 4B). In the case of lawsone, the 3rd position is highly nucleophilic in nature, which results in a decrease probability of NAC reacting with lawsone (Fig. 3B). Reactivity of 1,4-naphthoquinones to the free thiol group was also investigated using DTT, a different source of free thiol (Fig. 4C). Absorbance at 412 nm increased with increasing concentration of DTT. However, the increase was absent when 1,4-naphthoquinone, plumbagin, and menadione were present in the mixture at 50 \(\mu\)M. Consistent with previous observations, derivatives, such as lawsone and juglone, that do not react with NAC also do not alter the absorbance mediated by DTT. Lawsone and juglone did not react to DTT, whereas 1,4-naphthoquinone turned out to be the most reactive in terms of free thiol reactivity, followed by plumbagin and menadione (Fig. 4C). Because redox cycling could also influence the DTNB assay, direct reactivity of NAC with various derivatives of 1,4-naphthoquinone was confirmed by the UV-visible absorption spectra of these derivatives.
compounds in the absence and presence of NAC (Fig. 5, A–C, left panels). The absorption spectra of plumbagin at 100 µM concentration showed an intense absorbance band at 271 and 420 nm. Upon the addition of NAC, the absorption spectrum showed a hypochromic shift to 246 and 426 nm. In the case of 1,4-naphthoquinone, two absorption bands were observed at 251 and 347 nm. The addition of NAC shifted the absorption bands toward a higher wavelength (i.e., 266 and 451 nm). Similarly, in the case of menadione, the UV-visible spectrum showed three bands at 251, 265, and 345 nm, which upon the addition of NAC again shifted toward higher wavelengths of 260, 341, and 438 nm, respectively. These results indicated that the π-electron cloud is disturbed around the compounds due to the addition of NAC, which may be a result of NAC reacting directly with these molecules. The result of NAC reacting with the various derivatives of plumbagin was further reflected in the changes in the fluorescence emission spectra of these molecules (Fig. 5, A–C, right panels). Plumbagin exhibited an emission band at ~449 nm. The intensity of the emission spectra showed a drastic increase in intensity upon the addition of NAC. A highly intense emission band was also observed at 505 nm. In the case of 1,4-naphthoquinone, an intense emission band was observed at ~438 nm, which disappears completely upon the addition of NAC. Similarly, in the case of menadione, the emission band at 435 nm disappeared completely upon the addition of NAC. These results clearly indicate that the direct reaction of NAC with 1,4-naphthoquinone derivatives results in a drastic change in the electronic environment around the molecules, thereby altering their emission and absorption spectra. These results, in addition to those obtained with the DTNB assay, clearly indicate that NAC reacts with these molecules through its free thiol at the 3rd position of the molecules. Additionally, these molecules, except juglone, react with NAC and thereby inhibit KAT activity.

**PTK1, a Plumbagin Analog, Inhibits KAT Activity Independently of Free Thiol Reactivity**—Based on earlier observations, we hypothesized that, if the reactivity at the 3rd position could be nullified while maintaining the hydroxyl group at the 5th position, then it could be possible to synthesize a non-toxic KAT inhibitor. Thus, we synthesized PTK1, with a methyl substitution at the 3rd position of plumbagin (Fig. 6A). Remarkably, it was observed that the ability of PTK1 to inhibit KAT was insensitive to the presence of NAC in vitro (Fig. 6B). As expected, in the DTNB-based assay for free thiol, the presence of PTK1 did not significantly affect free thiol abundance in the reaction containing NAC (Fig. 6C). Fluorescence emission as well as the UV-visible absorption spectrum of PTK1 were analyzed to investigate any covalent interaction with free thiol (Fig. 6D). Unlike other analogs, the PTK1 absorption band at 249 nm was not affected at all upon the addition of NAC. PTK1 showed no
fluorescence emission spectra, and consequently, the changes with NAC addition could not be monitored. Moreover, this derivative exhibited the same in vitro KAT inhibition potency as the parent compound plumbagin (data not shown). To ascertain the inhibition of histone acetylation in cells, we performed immunoblotting with lysates of SH-SY5Y, HEK293, and HeLa S3 cells treated with PTK1 at different concentrations using an H3K9 acetylation antibody. We observed a dose-dependent decrease in acetylation after 24 h of treatment (Fig. 6).

To investigate and compare the reversibility of the plumbagin and PTK1-mediated inhibition, we performed dialysis in vitro.

**PTK1 Is a Non-competitive and Reversible Inhibitor of p300**

The kinetic analysis of the PTK1-mediated inhibition of full-length p300 acetyltransferase revealed a noncompetitive pattern with both of its substrates (i.e., core histones and [3H]acetyl-CoA) (Fig. 7, A and B). The Lineweaver–Burk plots (Fig. 7, A and B, left panels) at a fixed substrate concentration revealed that apparent K_m values (1.47 ± 0.2 μM for acetyl-CoA and 33.3 ± 2 nm for core histone) remained constant while the V_max of the enzyme decreased with increasing concentration of PTK1 (from 8476 ± 268.2 cpm without inhibitors to 3184 ± 125.6 cpm with 45 μM PTK1 for acetyl-CoA and from 4732 ± 36.52 cpm without inhibitors to 2077 ± 27.16 cpm with 45 μM PTK1 for core histone). We also observed a significant increase in both the slope (K_m/V_max) and the y intercept (when x = 0.0) by the same factor (2.7-fold for acetyl-CoA and 2.5-fold for core histone), which distinguishes between inhibition by classical noncompetitive kinetics and mixed kinetics. The slopes (K_m/V_max) obtained were further plotted against inhibitor concentrations to determine the K_i (inhibitor constant) value. The K_i of PTK1 was determined to be 23 μM for acetyl-CoA (R^2 > 0.98; p < 0.0001) and 30 μM for core histone (R^2 > 0.98; p = 0.0002) (Fig. 7, A and B, middle panels). To further validate the steady-state kinetics data, they were also analyzed by a secondary plot (Fig. 7, A and B, right panels), where 1/apparent maximum velocities (1/V_app) from Lineweaver-Burk plot were plotted against the PTK1 concentrations, and the K_i values were found to be 23 and 30 μM for acetyl-CoA and core histones, respectively. The identical K_i and K'-i values clearly suggest a purely non-competitive mechanism of inhibition. Additionally, the values are comparable with the inhibition constant of plumbagin (20–25 μM) (4) and strengthen the hypothesis that PTK1 possesses the same inhibition potency in vitro as the parent plumbagin molecule. These findings are not surprising, considering that PTK1 is a methylated derivative of plumbagin, and the parent molecule inhibits full-length p300 as well as the minimal KAT domain by a noncompetitive mechanism (4). Bisubstrate enzymes, such as p300, follow an ordered substrate binding mechanism (31). The non-competitive inhibitor PTK1 could bind to the free enzyme as well as to the substrate-bound form, resulting in an abortive ternary complex. The possibility of a conformational change due to allosteric binding also cannot be ruled out. In addition, PTK1 may have multiple or partially overlapping binding sites in the biologically relevant full-length p300.

To investigate and compare the reversibility of the plumbagin and PTK1-mediated inhibition, we performed dialysis in...
addition to dilution-based time course inhibition assays (Fig. 8). In these assays, an indication of reversibility is the independence of inhibition with respect to time. Perfectly reversible inhibitors reach rapid equilibrium with the enzyme, and inhibition does not depend on time. Plumbagin exhibited perfect time-dependent inhibition at a range of concentrations (25–100 μM) with 1–15 min of preincubation. Inhibition markedly increased with the time of incubation with the inhibitor. The inactivation rate constant (k_\text{obs}) value was determined from the fitted slope of the plots; for plumbagin, it was 4 × 10^{-2}/min (Fig. 8A, left). This type of inhibition is usually the result of the irreversible binding of the inhibitor with the enzyme, potentially due to the formation of covalent adducts. In the case of plumbagin, it could be due to the complex-forming ability of selective thiol residues in the protein. The irreversibility of plumbagin-mediated inhibition was further confirmed by the rapid “drop dialysis” method, in which dialysis of plumbagin was ineffective in restoring the native activity of the enzyme (Fig. 8B). This scenario changed when PTK1 was used; we observed minimal time-dependent decrease when compared with the without inhibitor control (k_\text{obs} = 9 × 10^{-3}/min) (Fig. 8A, right). Moreover, the inhibition of PTK1 was completely reversible because complete activity could be recovered after determining the residual activity of the dialyzed enzyme, which was free from PTK1 (Fig. 8B). These data suggest that the mechanism of inhibition of p300 by plumbagin is irreversible, whereas for PTK1, it is predominantly reversible. Importantly, when these assays were conducted with the minimal p300 KAT domain (Fig. 8C), both small molecules behaved in a totally reversible fashion with no time dependence and completely regained their activity upon dilution (Fig. 8D). This result also suggested that the susceptibility to redox cycling and free thiol reactivity resides in residues outside the KAT domain, presumably in the cysteine- and histidine-rich domains of the full-length enzyme.

The Binding Sites for Plumbagin and PTK1 Are Probably the Same within the KAT Domain—It was previously found that plumbagin could bind to only one site within the KAT domain of p300, namely, the Lys-1358 residue, by isothermal titration calorimetry, molecular docking, and mutation studies (4). A detailed kinetic analysis also revealed a non-competitive mechanism of inhibition of both the full-length protein and the KAT domain. To assess whether the binding site for PTK1 was similar to that of plumbagin, we performed molecular docking using Autodock software (Fig. 9A). The docking study with PTK1 and the p300 KAT domain showed hydrogen bond formation with residue Lys-1358 (Fig. 9B). The residues involved in the interaction were Lys-1358, Arg-1356, Thr-1296, Leu-1298, Glu-1380, Asp-1614, and Asp-1616 (Fig. 9C). This docking model is consistent with the aforementioned report (4) in
which it was shown that the -OH group of plumbagin, which has a structure similar to that of PTK1 (without the methyl group at C3), is important because plumbagin forms a hydrogen bond with Lys-1358 through this group. It had also been shown that alkyl substitution at the -OH position could not inhibit KAT activity. Additionally, Lys-1358 was found to be absolutely critical for KAT activity. A hydrogen bond is formed with the Arg-1356 residue, and, along with the hydrogen bond formed with Lys-1358, it could facilitate interactions with the other four residues. The docking of PTK1 with the p300 KAT domain suggested that hydrogen bonding through the -OH group of the molecule to the Lys-1358 residue could result in the inhibition of KAT activity. Therefore, we observed that PTK1 probably bound to the same region as plumbagin and possibly followed a similar mechanism for inhibition of KAT activity. The docking results are consistent with this conclusion but certainly not conclusive.

Plumbagin Induces p300 Structural Alterations by Inducing Thiol Cross-links and Adduct Formation—Plumbagin and 1,4-naphthoquinone share a reactive 3rd position, which could induce both redox cycling and covalent thiol adduct formation. Redox cycling in vitro can induce many changes in proteins. The induction of thiol cross-links between cysteine residues is one such effect. p300 has three cysteine- and histidine-rich regions (CH1, CH2, and CH3) and therefore is likely to be affected by redox states. CH2 and CH3 are adjacent to the KAT domain. It is possible that redox cycling induces disulfide bonds in an intermolecular or intramolecular fashion, thereby contributing to KAT inhibition. Full-length p300 is a 300-kilodalton protein (Fig. 10A), and induction of such cross-links can result in dimerization or oligomerization, which in turn result in a drastic increase in the protein size. This size increase can be observed as reduced mobility in SDS-PAGE (without DTT). As expected, after preincubation with plumbagin analogs (25 or 100 μM), there is a variable decrease in mobility (Fig. 10B, lane 1 versus lanes 2–5). The decrease in mobility correlates with increased protein sequestration at the interface of the stacking gel and the resolving gel as observed upon Coomassie Blue staining. This sequestration was due to a drastic increase in size, which was possibly a result of dimer-/oligomerization by thiol cross-links. Interestingly, PTK1 and juglone induced dimer-/oligomerization to a lesser extent than plumbagin at both concentrations. A reactive 3rd position probably enhances redox cycling to some extent. As expected, the presence of DTT greatly reduced the dimer-/oligomerization. It is possible that dimer-/oligomerization induced by redox cycling of 1,4-naphthoquinones contributes to KAT inhibition. PTK1 therefore possesses a limited ability to induce p300 intra-/intermolecular thiol cross-links. The thiol-cross-linking abilities of the three compounds were also compared by a dynamic light scattering experiment. The results suggest the following features (Fig. 10C). Plumbagin induced oligomerization of p300 to a greater extent relative to PTK1 and juglone, because the $Z_{av}$ diameter of untreated p300 (99.9 nm) increased to 161.1 nm in the pres-

FIGURE 7. PTK1 is a noncompetitive inhibitor of p300. A, kinetics of p300 KAT inhibition by PTK1 as represented by a Lineweaver-Burk plot (top left). The concentration of core histones has been kept constant at 1.7 μM while increasing the concentration of [3H]acetyl-CoA from 1 to 8 μM either in the absence (no inhibitor) or increasing concentrations of PTK1 (15, 20, 25, 35, and 45 μM). In order to determine the $K_i$ of PTK1 for acetyl-CoA, the slope ($K_m/V_{max}$) of the straight lines obtained from the Lineweaver-Burk plot is plotted against PTK1 concentrations (top middle). The apparent $V_{max}$ values from the Lineweaver-Burk plot are plotted against PTK1 concentrations (top right). B, inhibition of p300 KAT activity by PTK1 at a fixed concentration of [3H]acetyl-CoA (1.6 μM) and increasing concentrations of core histones (5–70 nM) either in the absence (no inhibitor) or increasing concentrations of PTK1 (15, 20, 25, 35, and 45 μM). Data are represented by a Lineweaver-Burk plot (bottom left). In order to determine the $K_i$ of PTK1 for core histone, the slope ($K_m/V_{max}$) of the straight lines obtained from the Lineweaver-Burk plot is plotted against PTK1 concentrations (bottom middle). The apparent $V_{max}$ values from the Lineweaver-Burk plot are also plotted against PTK1 concentrations. (bottom right). Error bars, S.D.
ence of 25 μM plumbagin (Fig. 10C, top). The further addition of plumbagin (100 μM) did not cause any significant change in the Zav diameter of p300. However, in the presence of 25 μM PTK1 or juglone, Zav was found to be ~129 nm. This value increased to ~129 nm in the presence of these compounds at 100 μM. The Zav diameter trends clearly reflected the differential cross-linking abilities of plumbagin versus PTK1 and juglone (Fig. 10C, top and bottom). The statistical analysis also supported the aggregation of p300 in the presence of the three compounds (data not shown). However, the extent of aggregation follows the following order: plumbagin > PTK1 ~ juglone. These results further corroborate the findings from the gel electrophoresis data.

To understand the structural changes in p300 (KAT3B) induced by plumbagin and PTK1, we used SERS analysis, which has been a very effective and quick technique used to look for gross protein structure. SERS is a unique approach to monitor structural changes in enzymes such as p300 upon the binding of...
ligands (32–34). These structural changes alter the orientation of the rings of aromatic amino acids with respect to the nanoparticle surface and thus alter the enhancement of different ring modes of these amino acids. In addition, the binding of the ligand can distance the metal nanoparticle’s surface from that of the protein, resulting in a reduction in intensity or the disappearance of the associated Raman modes. In cases where ligands do not bind to the protein, the SERS spectrum of the protein does not show any changes. SERS is a very sensitive method, and the spectra of the proteins depend on the distance and orientation of the protein on the surface of the nanoparticles. Therefore, this technique gives us insight into the structural changes upon the binding of ligands to proteins (35). The spectra of baculo-expressed full-length p300 (Fig. 10D) treated with plumbagin or PTK1 underwent such changes (Fig. 10D).

![Figure 10](image-url)

**FIGURE 10.** p300 undergoes thiol cross-links and adduct formation under redox stress in vitro. A, purification profile of recombinant full-length baculo-expressed p300. The protein was loaded in 8% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue stain. B, p300 was incubated with various 1,4-naphthoquinone analogs with 25 μM (I) and 100 μM (II) for 10 min at 30 °C and subjected to SDS-PAGE without DTT. The gel was stained with Coomassie Brilliant Blue, including the stacking/resolving interface. The heavy black arrow indicates oligomeric p300 in the presence of plumbagin (lane 2). C, dynamic light scattering to study the influence of plumbagin, PTK1, and juglone on p300 dimer/oligomerization. The intensity statistics of 10 measurements each are plotted for p300 (5 μM) alone and in the presence of 25 μM plumbagin, PTK1, and juglone, respectively (top). The Zav diameters are listed on each panel. Shown are Zav diameters for p300 (5 μM) treated with 25, 50, and 100 μM plumbagin, PTK1, and juglone, with error bars indicating S.D. (bottom). The measurements were performed at 25 °C. D, SERS spectra of p300 alone (black), p300 with PTK1 (blue), and p300 with plumbagin (red).
structure of the protein. For both PTK1 and plumbagin, there were changes in the intensities of the bands in the 1150–1550 cm\(^{-1}\) region that were related to the aromatic amino acids tryptophan, tyrosine, phenylalanine, and histidine, whose orientations with respect to the nanoparticle surface changed upon binding of the molecules to the protein. In the case of the p300 protein, we observed the stretching vibrations of C-COO\(^{-}\) as a doublet at 910–941 cm\(^{-1}\). The presence of this doublet indicated that the protein interacted with the silver nanoparticles predominantly through the carboxylate groups. However, the intensity of the doublet decreased in the case of PTK1 and almost completely disappeared in the case of plumbagin. Plumbagin also possesses an intrinsic tendency to bind to free thiol groups on the surface of the protein through nucleophilic addition to the C3 carbon. This result was confirmed by the appearance of a peak at 682 cm\(^{-1}\), which corresponds to the \(\nu_1(C-S-C)\) mode (37). This mode was absent in free protein and protein-PTK1 spectra. The appearance of this mode specifically when plumbagin was applied to p300 gave absolute evidence that protein-small molecule thiol adducts were being formed. The nonspecific binding of plumbagin to the protein surface could also prevent nanoparticles from binding to the protein or change the binding site, thereby causing an overall reduction in intensities of different modes like the C-COO\(^{-}\) stretching modes.

PTK1 Has a Greatly Improved Toxicity Profile—Based on the observation that 1,4-naphthoquinone derivatives reacted with NAC at their free 3rd position, we hypothesized that this mode of reaction between the molecules and various free thiols present within the cell could explain their high toxicity. We found that the application of plumbagin and juglone but not PTK1 at different concentrations in HeLa S3 and HEK293 cells induced drastic changes (Fig. 11A). Thus, when there was an excess of...
Redox Cycling and Thiol Reactivity in p300 Inhibition

Small molecule modulators of lysine acetyltransferases have recently received enthusiastic attention because they may help us to understand in vivo KAT function. They also possess tremendous potential as molecules for therapy. Here, we have described our effort to understand the mechanism of action of a class of KAT inhibitors (naphthoquinones), some of which interact with thiols in addition to the KAT domain of p300. The detailed functional analyses of these molecules led to the identification of the chemical entity responsible for KAT inhibition and cellular toxicity. Based on this information, we have synthesized a new inhibitor, PTK1, which does not react with thiols and inhibits KAT’s in a non-competitive and completely reversible fashion. In comparison with other analogs, it exhibits reduced ROS generation. Therefore, PTK1 is a non-toxic KAT inhibitor.

Plumbagin is an antiproliferative and cytotoxic compound (38). We have investigated the mechanism responsible for toxicity and have identified that the reactive 3rd position is essential for toxicity. Furthermore, through its free thiol reactivity, plumbagin influences p300/CBP KAT inhibition. The contribution of free thiol reactivity to KAT inhibition has also been suggested for the isothiazone-mediated inhibition of p300/CBP-associated factor and p300 (39). We previously reported that the inhibition of KAT activity by plumbagin is due to hydrogen bonding of the single hydroxyl group at the 5th position to a critical lysine residue within the KAT domain of p300 (4). This concept has been further strengthened by the observation that one of the analogs, juglone (5-hydroxy-2-methyl-1,4 naphthoquinone), which possesses only one hydroxyl group and does not possess thiol reactivity, greatly inhibits the KAT activity of p300. Furthermore, our data clearly suggest that the abilities of plumbagin with regard to redox cycling and the formation of adducts with the enzyme result in the toxicity of this molecule.

Based on this structure-function information about plumbagin, we synthesized a derivative, PTK1 that, because of a methyl substitution at the 3rd position, does not react with thiols. PTK1 possesses a hydroxyl group at the 5th position, which probably maintains its KAT-inhibitory activity. Although PTK1 showed an IC50 value similar to that of plumbagin in vitro, its inhibition of H3K9 acetylation in the cell lines was approximately half that of plumbagin. We propose two possibilities for this differential efficiency of PTK1 and plumbagin in the cellular system: 1) cellular permeability is low for PTK1 compared with plumbagin; 2) because plumbagin forms an adduct with the enzyme, it inhibits the enzyme’s activity more strongly than does PTK1. ROS generated by plumbagin could also induce thiol cross-links in the enzyme, thus reducing its activity in the cellular context.

NAC is able to bind to a wide array of molecules, including 18β-glycyrrhetinic acid derivatives, and thus prevent apoptosis (40). Additionally, NAC prevents plumbagin-dependent inhibition of the NFκB pathway in a free-thiol-dependent manner via inhibition of the association between plumbagin and p65, a key subunit of the NFκB p50/p65 heterodimer complex (25). NAC and other free thiol-containing reducing agents, but not the non-free thiol-reducing agents, modify the immunomodulatory activity of plumbagin (26). Apoptosis induced by 1,4-naphthoquinone analogs, such as plumbagin, can be completely blocked by NAC but not by the non-free thiol-containing reducing agents (27). From our study, it is clear that NAC can completely abrogate the toxicity of plumbagin and its analogs not only by scavenging ROS (25, 26, 39) but also by reacting with them directly in a cellular system. Collectively, thiol donors, such as NAC and DTT, render the 1,4-naphthoquinones chemically inert.

In conclusion, we have addressed the chemical mechanisms that could be involved in influencing KAT inhibition by 1,4-naphthoquinone, and we have delineated thiol reactivity and redox cycling. Although both of these mechanisms influence KAT-inhibitory activity, they are probably subsidiary mechanisms. However, these chemical events are the main causes of cellular toxicity. We therefore synthesized the molecule PTK1, which has a modification at the 3rd position and chemically differs from plumbagin. PTK1, having no thiol-reactive property, has fewer pleiotropic and toxic effects but still retains the ability to inhibit KAT. Stability conferred by a methyl substitution decreases the redox-cycling and thiol-reactive tendencies of PTK1 compared with plumbagin, thus improving the toxicity profile of PTK1. Therefore, investigations into the role of the chemical properties of 1,4-naphthoquinone analogs in cellular toxicity and the inhibition of acetyltransferase p300 have led to the synthesis of PTK1, a less toxic inhibitor. However, further modifications of this molecule are essential to improve its potency in the nanomolar range.
Redox Cycling and Thiol Reactivity in p300 Inhibition

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