Chapter II
Chapter II. Synthesis, characterization and biological activity of small molecule activators of p300 HAT activity

2.1 Introduction

A precise organization of chromatin is essential for all DNA templated phenomenon inside the cell. The dynamic alteration of chromatin structure acts as a key regulatory switch in cellular physiology. The posttranslational modification of chromatin proteins confers the dynamic nature of chromatin. Specific amino acids within the histone tail and nonhistone proteins are the sites for variety of modifications, including phosphorylation, acetylation, and methylation. Among these, reversible acetylation of histones and nonhistones play pivotal role in the regulation of gene expression. Dysfunction of histone acetyltransferases and deacetylases is often associated with manifestation of several diseases including cancer, cardiac hypertrophy, asthma and diabetes (Backs, J., et. al., 2006, Barnes, P. J., et. al., 2005 & Gray, S. G. et. al., 2005). Analysis of colorectal, gastric, and epithelial cancer samples show that in several instances, there is a mis-sense mutation as well as deletion mutation in the p300 gene and loss of p300 histone acetyltransferase enzymatic activity (Muraoka, M., et. al., 1996). Mutations in HATs cause several other disorders apart from cancer. The degradation of p300/CBP is also found to be associated with certain neurodegenerative diseases (Cong, S. Y., et. al., 2005). These observations lead to develop several therapeutic approaches based on histone deacetylase inhibitors (Espino, P. S. et. al., 2005 & Yeow, W. S. et. al., 2006). The overall nonspecific nature of HDAC (histone deacetylases) inhibitors and its undesired effect on global gene expression inspired us to look for HAT activators.
However, very few small molecule modulators of histone acetyltransferases are known so far. Availability of recombinant HATs made it possible to isolate/synthesize and evaluate HAT inhibitors.

2.2. Diseases linked to loss of Histone Acetyltransferase activity

Acetylation of histones is a reversible process. The balance between acetylation and deacetylation is one of the key factors for the regulation of gene expression. Hyperacetylation of histones associated with normally silenced genes or deacetylation of histones associated with actively transcribed genes may lead to several disorders. Acetylation-associated disorders may occur through i) hyperacetylation and derepression of normally repressed promoters; ii) the hypoacetylation and repression of genes necessary for cell viability, and iii) mistargeting of HAT and HDAC activity. Mutation, mistargeting and translocation (formation of fusion proteins) of the histone acetyltransferases cause aberrant acetylation.

2.2.1. Mutation in HATs

Rubinstein–Taybi Syndrome (RTS) is a developmental haploinsufficiency disorder, which is marked by mental retardation, craniofacial defects, broad big toes and thumb. These patients are highly susceptible to cancer. Mutation in the cebp locus results in RTS. It was found that a single mutation at the plant homeodomain (PHD)-type zinc finger in the HAT domain of cebp causes this syndrome. The mutation alters a conserved finger amino acid residue E to K at position 1278. Interestingly, this mutation in cebp also abolishes its histone acetyltransferase activity (Kalkhoven, E. et. al., 2003).
2.2.2. Mistargeting of HATs – Sequestration and Degradation Manifested in Neurodegenerative Disease. Spinal and Bulbar Muscular Atrophy (SBMA) is an inherited neurodegenerative disease caused by CAG repeat expansion in the androgen receptor gene. This results in an expanded polyglutamine tract, which confers a novel, toxic function to the affected protein. The transcriptional activator CBP (Cyclic AMP Response Element Binding Protein) is incorporated into nuclear inclusion bodies formed by polyglutamine-containing proteins. Free CBP levels are reduced in cells expressing expanded polyglutamine, though the CBP mRNA level is not affected. Over expression of CBP can rescue cells from polyglutamine-mediated toxicity (Cong, S. Y., et. al., 2005).

2.2.3. Huntington’s Disease: This disease is a fatal neurodegenerative disease with a late onset and is inherited in a dominant fashion. It is also associated with increase in the length of a CAG triplet repeat present in the huntingtin gene located on chromosome 4p16.3. Mutant huntingtin protein from human brain cells is more resistant to proteolysis than normal huntingtin, which leads to aggregation and toxicity through the sequestration of important targets, including normal huntingtin protein. Polyglutamine-containing domain of huntingtin directly binds to the acetyltransferase domains of CBP and PCAF (p300-CBP Associated Factor) and precipitates them from the cellular environment. The PolyQ-huntingtin also inhibits acetyltransferase activity of p300, CBP and PCAF in vitro.

2.3. Modulators of histone acetyltransferase activity

Unlike HDAC inhibitors, the number of HAT modulators (activators and inhibitors) discovered so far is scanty. The first group of synthetic HAT inhibitors to be described
was the Peptide Co-A conjugates. Of these Lys Co-A (a competitive inhibitor) was specific for p300 while and H3 Co-A20 was specific for PCAF (Lau, O. D., et. al., 2000) Recently, γ-butyrolactones were discovered as small-molecule inhibitors of human Gcn5 HAT activity (Biel. M., et. al., 2004).

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<th>SMALL MOLECULES</th>
<th>ENZYME</th>
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<tr>
<td>Lys-CoA</td>
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<td>PCAF</td>
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<td>Anacardic acid</td>
<td>p300 and PCAF</td>
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<tr>
<td>Garcinol</td>
<td>p300 and PCAF</td>
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<td>γ-butyrolactones</td>
<td>Gcn5</td>
<td>100 μM</td>
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<td>Isothiazolones</td>
<td>p300 and PCAF</td>
<td>1-50 μM</td>
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<td>CTPB</td>
<td>p300</td>
<td>Activator</td>
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Table 2.1: HAT modulators. Various small molecules of synthetic and natural origin and the target enzyme.

A group of compounds having isothiazolone structure were shown to have inhibitory activity on both p300 and PCAF (Stimson, L., et. al., 2005). Screening of inhibitors for HAT activity from natural sources was successful with the discovery of Anacardic acid from cashew nut-shell liquid which was found as a potent inhibitor of p300 and PCAF. Interestingly, CTPB (N-[4-chloro-3-trifluoromethyl-phenyl]-2-ethoxy-6-pentadecyl-
benzamide), an amide derivative of Anacardic acid activates p300 HAT activity in vitro. Unfortunately, these compounds were found to be cell impermeable (Balasubramanyam, K., et. al., 2003). Garcinol extracted from the fruit rind of *Garcinia indica* and Curcumin were also found to be potent inhibitors of HAT activity. While Garcinol is non specific (inhibits both p300 and PCAF), Curcumin was found to be a p300 specific HAT inhibitor (Balasubramanyam, K., et. al., 2004). Recently, we have found that LTK-14 a derivative of isogarcinol was found to be a potent, specific p300 HAT inhibitor. It is important to note that LTK-14 is less toxic as compared to that of the parent compound.

2.4. HAT Modulators as Therapeutics of the Future

As suggested in recent reviews, the development of small molecular weight HAT inhibitors and activators as therapeutic agents is the next step, following the HDAC inhibitors. Recently discovered molecules Anacardic acid, CTPB, Garcinol and Curcumin are successful efforts in this direction. However, the preference would be for a more specific and cell permeable inhibitor. Synthesis of novel compounds similar to these known modulators in conjunction with continued search for new molecules could lead to the development of potential drugs from HAT modulators. The HAT inhibitors/activators along with HDAC inhibitors could be very useful in anti-HIV drug research also. The HAT activator molecule could be an excellent alternative to HDAC inhibitor, considering their analogous function, in causing hyperacetylation. The small molecule modulator of HATs thus should open up new possibilities to design better therapeutics in future.
2.5. Materials and Methods

2.5.1. Organic solvents

The organic solvents used were Methanol-AR grade (E-Merck), Chloroform-AR grade (E-Merck), Hexane-AR grade (E-Merck), Benzene-AR grade (Ranbaxy), Toluene-AR grade (E-Merck), Ether-AR grade (Ranbaxy), Acetonitrile-AR grade (E-Merck), Dimethyl sulphoxide-AR grade (Ranbaxy).

2.5.2. Reagents

All the chemicals were obtained from standard commercial sources unless otherwise indicated, acetic anhydride, hydroxyl ammonium sulphate, sodium acetate, sodium hydroxide, potassium hydroxide, potassium bicarbonate, sodium bicarbonate, sodium chloride (E-merck), silica gel-923 (E-merck), potassium carbonate (E-merck), N',N'-dimethylformamide (DMF) (E-merck).

2.5.3. Analytical techniques

(i) Thin-Layer Chromatography (TLC)

TLC was performed on aluminum-backed silica gel plates (Merck, DC-Alufolien Kieselgel 60 F254) and the spots were visualized by UV light. The following mobile phases were employed for TLC: chloroform-hexane (1:1), benzene-hexane (1:1), ether-hexane and (9:1), ethylacetate-hexane (1:1), acetonitrile-methanol (1:1), methanol-water (7:3), benzene-ether (1:1). Column chromatography was carried out using silica gel (BDH, 60-120 mesh).
(ii) Determination of Melting Point

Melting points of the compounds were determined on a VEEGO-VMP-D melting point apparatus and are uncorrected.

2.5.4. Instrumentation

The instrumental techniques employed for the characterization of the newly synthesized compound includes, \(^1\)H NMR and Mass spectrometry analysis. The details of the instrumentation are briefly given below.

(i) \(^1\)H NMR Spectra

\(^1\)H nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz on a Bruker AC 400 spectrometer. The chemical shifts are reported in (ppm) units relative to the internal reference tetramethylsilane (Me\(_4\)Si).

(ii) Mass spectrometry

Mass spectrometry was done using Bruker instrument.

2.5.5. Synthesis of CTPB and CTB derivatives

General procedure for the synthesis of Anacardic acid and salicylic acid derivatives 6 (a-r).

Alkylation of Anacardic acid and salicylic acid with diethyl sulfate, dimethyl sulfate and isopropyl iodide using potassium carbonate as a base and acetone as a solvent resulted the dialkylated derivatives 2 (a-r). Dialkylated compounds were de-protected into the respective acids 3 (a-r) using potassiumtertiarybutoxide and DMSO. The de-protected acids 3 (a-r) on treatment with thionyl chloride in the presence of catalytic amount of
DMF yielded corresponding acid chlorides 4 (a-r). The resultant acid chlorides were used for next step of reactions without purification. Compounds 4 (a-r) were condensed with different amino benzotrifluorides in dichloromethane in the presence of triethylamine as acid scavenger to yield compounds 6 (a-r) (Kiong, L. S., et. al., 1942, Chang, F. C., et. al., 1964 & Shobha, S. V., et. al., 1991). The obtained products were purified by column chromatography. MP, TLC, NMR and mass spectrum analysis confirmed the formation of anacardic acid and salicylic acid derivatives (Scheme 1).

\[ \begin{align*}
\text{R} & \quad \text{COOH} \\
\text{H} & \quad \text{OR, A^OR, ^-OR, 1(M) 2(a^)} \\
\text{OH} & \quad \text{CI} \\
\text{R} & \quad \text{P} \\
\text{OR,H} & \quad \text{w -/OR, 6(w)} \\
\end{align*} \]

Scheme 1. a. Diethyl Sulfate, Dimethyl sulfate, Isopropyl Iodide, K$_2$CO$_3$, acetone reflux with Potassium tertiary butoxide b. DMSO at 80°C. c. SOCl$_2$, Dichloro methane, Dimethyl formamide. d. Triethyl amine, Dichloro methane. R = pentadecane, R1 = Ethyl/methyl/isopropyl, R2 = -Cl/-CN/-NO$_2$.

To a solution of salicylic acid in acetone was added diethyl sulfate (2 equiv.), potassium carbonate (3 equiv.). The reaction mixture was refluxed for three hours to give the compounds 2 (s-v). 2-ethoxy benzoate was treated with potassium tertiary butoxide in the presence of dimethyl sulfoxide to give 2-ethoxy benzoic acid 3 (s-v). 2-ethoxy benzoic
acid upon treatment with thionyl chloride in the presence of catalytic amount of DMF yielded 2-ethoxy benzoyl chloride 4 (s-v). The compounds 4 (s-v) were condensed with different amino-benzotri fluorides in dichloro methane in the presence of triethyl amine as acid scavenger to yield 6 (s-v). The obtained products were purified by recrystallization and column chromatography (Scheme 2).

![Chemical diagram]

*Scheme 2.*

- **e.** Diethyl sulfate, K2CO3, acetone.
- **f.** DMSO 80°C.
- **g.** SOCl2, Hexane.
- **h.** Triethyl Amine.

**Dimethyl formamide, reflux.**

**h.** Triethyl Amine.

### 2.5.6. Gel Electrophoresis for separating proteins

Different proteins were resolved according to their molecular weights using SDS - PAGE. The separating (or resolving) gels were made with different percentages of acrylamide (30% Stock solution of acrylamide : bis-acrylamide :: 29:1), 0.375 M Tris-HCl (pH-8.8), 0.1% SDS, 0.1% APS and TEMED. The components of stacking gel include 5% acrylamide, 0.375 M Tris-HCl (pH-6.8), 0.1% SDS, 0.1% APS and TEMED. Protein samples were made in 5X SDS sample buffer (for 1X- 50 mM Tris-HCl pH 6.8, 100 mM...
DTT, 0.1% Bromophenol blue, 10% Glycerol), heated at 90°C for 3 min. before loading into the gel. The gel was electrophoresed in Tris-glycine electrophoresis buffer (25 mM Tris-HCl, 250 mM Glycine (pH-8.3), 0.1% SDS). The gel was stained in Commassie Brilliant Blue (CBB) (45% Methanol, 10% glacial Acetic acid, 0.25% CBB), followed by destaining in Destaining solution (30% Methanol, 10% glacial Acetic acid).

2.5.7. Estimation of Proteins

The estimation of proteins was done with the Biorad Protein Estimation Reagent as per manufacturer's protocol, by measuring absorbance at 595 nm (A_{595}).

2.5.8. Culturing Insect cells

To express and purify human proteins using respective baculoviruses the *Spodoptera frugiperda* ovarian cell line Sf21 was cultured in TC100 medium (Sigma), supplemented with 0.1% Pluronic F-68 solution (Sigma), 10 μg/ml Gentamycin (Sigma) and 10% FBS (Hyclone) at 27°C in a BOD incubator. After confluency, the cells were dislodged from the substratum using a cell scraper and sub-cultured in a 1:3 ratio.

2.5.9. Purification of p300 from Sf21 cells

150 mm tissue culture plates of Sf21 cells grown in TC100 medium with 10% FBS were infected with freshly amplified p300 baculovirus. 70 hours post infection the cells were harvested at 4°C, washed in cold phosphate buffered saline (PBS) and pelleted (2000 rpm, 10 min at 4°C). The cell pellet is resuspended in homogenization buffer (10mM Tris-HCl,
pH 7.5, 0.5M NaCl, 10% glycerol (v/v), 0.1% NP40 (v/v), 15 mM imidazole, 2 mM 2-mercaptoethanol, 2 mM PMSF, 50 µg leupeptin, 50 µg aprotinin) and homogenized in Dounce’s homogenizer (4 x 3 strokes). The resulting whole cell lysate was spun for 20 min. at 16,000 rpm, 4°C. The supernatant was collected into a new tube and mixed with required amount of Ni-NTA beads (pre-equilibrated in homogenization buffer) and incubated for 2 hrs. After incubation the resin was washed in batch, 5 to 6 times with wash buffer (10 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 10% glycerol (v/v), 0.2% NP40 (v/v), 15 mM imidazole, 2 mM 2-mercaptoethanol, 2 mM PMSF). Protein was eluted in batch two to three times with elution buffer (10 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 10% glycerol (v/v), 0.1% NP40 (v/v), 250 mM Imidazole, 2 mM 2-mercaptoethanol, 2 mM PMSF), aliquoted and frozen in liquid nitrogen and stored at −80°C. The proteins were resolved on an 8% polyacrylamide gel and the activity was determined by HAT filter binding and Gel assays.

Figure 2.1: p300 protein profile. 1 µg of p300 resolved on 8% SDS-polyacrylamide gel.
2.5.10. Culturing Mammalian cells

The human embryonic kidney cell line HEK293T cells were grown in the commercial Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma), supplemented with L-Glutamine (Sigma), Penicillin-Streptomycin-Amphotericin B (Sigma) and 10% Foetal Bovine Serum (FBS) (Hyclone) according to manufacturer’s protocol. The cells were grown at 37°C in a 5% CO₂ supply and an 80% relative humidity in a CO₂ incubator. After 70% confluency is attained, the cells were dislodged from the substratum by 0.025% Trypsin-EDTA (Sigma) for 5-10 mins. followed by inactivation using FBS, and sub-cultured in 1:2 ratio.

2.5.11. Purification of Core histones from HeLa nuclear pellet

Human core histones were purified from the HeLa nuclear pellet. The HeLa nuclear pellet was resuspended in Buffer A (100 mM potassium phosphate buffer (pH 6.7), 0.1 mM EDTA, 10% Glycerol, 0.1 mM PMSF, 0.1 mM DTT and 630 mM NaCl). The suspension was homogenized in a Dounce’s homogenizer (Wheaton) with Pestle B for 30 min. on ice. The suspension was clarified at 14,000 rpm for 20 min. at 4°C. The supernatant was transferred to a fresh tube and 0.5 g of Hydroxyapatite BioGel HTP (BioRad) (presoaked in 10 mM potassium phosphate buffer pH 6.7) was added per ml of nuclear pellet taken. This was incubated at 4°C for 3 hrs. on a rotary shaker. The resin was washed with 25 ml of buffer A with 630 mM NaCl on a centrifuge. The Hydroxyapatite was then packed into a column, and washed overnight with Buffer A containing 630 mM NaCl. The core histones were eluted in Buffer A containing 2 M NaCl in batches of 500 μl. The different fractions were then analyzed for protein content.
using 15% SDS-PAGE and peak fractions pooled together. The pooled fractions were then dialyzed against BC100 and stored at -80°C.

![Image of SDS-PAGE gel](image)

*Figure 2.2: Core histones profile. 1.4 µg of core histones resolved using 15% polyacrylamide gel electrophoresis.*

2.5.12. HAT Assay

(i) **HAT Filter binding assay:** 800 ng of core histones (substrate) were incubated in HAT assay buffer (50 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol, 1 mM DTT, 1 mM PMSF, 0.1 mM EDTA, pH 8.0, 10 mM sodium butyrate) at 30°C for 10 min. in the presence or absence of compound followed by the addition of 0.98 µM [³H]-Acetyl coenzyme A and were further incubated for 10 min. The final reaction volume was 30 µl. The reaction mixture was then blotted onto P-81 (Whatman) filter paper and allowed to bind for at least 30 min. The filter papers were then washed with bicarbonate buffer 3 times (10 min. each) on a rocker and dried. The dried filter papers were soaked in scintillation fluid (POP and POPOP in toluene) and radioactive counts were recorded on a Wallac 1409 Liquid Scintillation Counter.
(ii) **HAT Gel fluorography assay:** Core histones (1600 ng) were used and HAT assay was done as described above. The reaction mixture is subjected to 25% TCA precipitation. The precipitate was washed with acetone, dried and resolved using 15% SDS-PAGE and processed for fluorography. (Fluorography – The gel was stained using commassie and destained. The destained gel is dehydrated in DMSO for 30 min. twice and then soaked in Fluorography solution (25% PPO in DMSO) for 30 min. and then hydrated for 30 min. The gel is dried and autoradiography is done at -80°C).

**2.5.13. Circular Dichroism**

p300 (300 μg/ml) was dialyzed in buffer containing 10 mM Tris-HCl, 10% glycerol, 100 mM NaCl and 2 mM β-mercaptoethanol for 10 hrs. to remove the detergents and imidazole used during purification steps. p300 is incubated with either DMSO or CTB (200 μM) for 10 min and CD spectra was recorded at room temperature (27°C) in a JASCO model J715 spectropolarimeter.

**2.5.14. Acid extraction of core histones from mammalian cells**

Human Embryonic Kidney cells (HEK293T) treated with various compounds were harvested by trypsinization and washed with PBS. The cell pellet was washed with Buffer A (150 mM KCl, 20 mM HEPES pH 7.9, 0.1 mM EDTA, 2.5 mM MgCl2, 10 volumes) and resuspended in ice-cold Buffer B (150 mM KCl, 20 mM HEPES pH 7.9, 0.1 mM EDTA, 2.5 mM MgCl2, 250 mM sucrose, 1% v/v Triton X-100, 10 volumes) and allowed for lysis to occur for 1 hr. at 4°C. The nuclei were spun down by centrifugation (10000 rpm, 4°C, 30 min.). The pellet containing the nuclei was resuspended in buffer A
and 1 M HCl was added to a final concentration of 0.25 M and the samples were incubated at 4°C for 1 hr. with intermittent mixing followed by centrifugation at 12000 rpm for 20 min at 4°C to obtain acid soluble fraction. The proteins were precipitated using 25% TCA, washed with acetone, dried and dissolved in required amount of water.

2.5.15. Western Blotting

The core histones extracted from the treated cell pellet were resolved using a 12% SDS-PAGE. The gel was incubated for a period of 15 min. in Transfer Buffer (25 mM Tris, 192 mM Glycine, 0.038% SDS and 20% MeOH). As per the dimensions of the gel, PVDF membrane was cut, activated in Methanol, and kept in transfer buffer for a period of 10 min. Proteins were transferred from the gel to the membrane using a semidry western transfer apparatus (Biorad) at 25 V, for 40 min. The membrane was blocked in 5% skimmed milk solution in PBS for a minimum period of 5 hrs. Primary and HRP-conjugated Secondary antibody was made in 2.5% skimmed milk solution (in PBS containing 0.05% Tween20). After blocking, the blot was incubated with Primary antibody for a period of 3 hrs. at 4°C, followed by washes in PBS containing 0.05% Tween20 for a period of 15 mins, 4 times at room temperature. This was followed by incubation with Secondary antibody for a period of 3 hrs at 4°C, followed by similar set of 4 washes at room temperature. The blot was developed using the Pierce Super Signal West Pico Chemiluminiscence Kit, as per the manufacturer’s protocol. The X-Ray films (TMX-Kodak) were exposed to the membranes for different time points and developed using GBX-Developer-Fixer Kit (Sigma).
2.5.16. Transient transfection

Mammalian cells (HEK293T/H1299) were seeded, and grown overnight in 10% FBS supplemented DMEM medium (without Penicillin-Streptomycin-Amphotericin B). Prior to transfection, the medium was replaced with fresh DMEM without antibiotics and FBS. The amount of constructs taken was in accordance with manufacturer’s protocol, maintaining 1:1 ratio of µg of DNA: µl of Lipofectamine 2000 (Invitrogen). The constructs PG13Luc, p53 and Lipofectamine were incubated for a period of 20 min. to ensure Lipofectamine-DNA complex formation and added on the cells drop wise. After 6 hrs. the medium was replaced by 10% FBS supplemented DMEM medium (without antibiotic), compounds were added and further incubated for 18 hrs.

2.5.17. Luciferase assay

Luciferase assays were done after 24 hrs. of incubation using Luciferase Assay kit (Promega). After incubation the cells were lysed with 200 µl of Lysis buffer (Promega) and lysate was cleared by centrifugation for 1 min. at 4°C. 10 µl lysate was then mixed with 10 µl of luciferase assay substrate (Promega) and luciferase activity was measured in Liquid Scintillation counter using ^14C window.
2.6. Results

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*Table 2.2: Derivatives of CTPB and CTB.*
2.6.1. Characterization of CTPB and CTB derivatives

The yield, melting point and $^1$H NMR results of the compounds are as follows:

1. **N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide (6a).**
   Yield: 88% (650 mg), white solid. MP: 59-61 °C
   $^1$NMR(DMSO-d$_6$) δ: 11.01(s, 1H, NH), 8.41(s, 1H, Ar-H), 8.13(m, 2H, Ar-H), 7.35 (t, 1H, Ar-H), 6.98(d, 1H, Ar-H), 6.91(d, 1H, Ar-H), 4.51(q, 2H, CH$_2$-CH$_3$), 1.51(m, 3H, CH$_3$-CH$_2$), 1.0-1.31(m, 25H, alkyl), 0.85 (t, 3H, CH$_3$-CH$_2$); MS (EI) m/z: [M+] 540.4, 373.6, 345.6, 161.1.

2. **N-(4-cyano-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide (6b).**
   Yield: 83% (580 mg), white solid. MP: 98-100 °C
   $^1$NMR(DMSO-d$_6$) δ: 11.01(s, 1H, NH), 8.41(s, 1H, Ar-H), 8.13(m, 2H, Ar-H), 7.35 (t, 1H, Ar-H), 6.98(d, 1H, J=8.2, Ar-H), 6.91(d, 1H, J=7.4 Hz, Ar-H), 4.51(q, 2H, CH$_2$-CH$_3$), 1.51(m, 3H, CH$_3$-CH$_2$), 1.0-1.31(m, 25H, alkyl), 0.85 (t, 3H, CH$_3$-CH$_2$); MS (EI) m/z: [M+] 545.5, 359.9, 149.1, 136.1

3. **N-(4-nitro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide (6c).**
   Yield: 74 % (530 mg), pale yellow solid. MP: 93-95 °C
   $^1$NMR(DMSO-d$_6$) δ: 11.15(s, 1H, NH), 8.4 (s, 1H, Ar-H), 8.16-8.29(m, 2H, J=9 Hz, Ar-H), 7.35 (t, 1H, Ar-H), 6.99(d, 1H, J=8.3Hz, Ar-H), 6.91(d, 1H, J=7.7 Hz, Ar-H), 4.1(q, 2H, CH$_2$-CH$_3$), 1.51(m, 3H, CH$_3$-CH$_2$), 1.01-1.42(m, 25H, alkyl), 0.85 (t, 3H, CH$_3$-CH$_2$); MS (EI) m/z: 545.5 [M+] 359.9, 149.1, 136.1

4. **N-(4-chloro-3-trifluoromethyl-phenyl)-2-methoxy-6-pentadecyl-benzamide (6d).**
   Yield: 91 % (650 mg), white solid. MP: 79-81 °C
1NMR(DMSO-d$_6$) δ: 11.14(s, 1H, NH), 8.42 (s, 1H, Ar-H), 8.15(m, 2H, Ar-H), 7.38 (t, 1H, Ar-H), 7.01(d, 1H, Ar-H), 6.92(d, 1H, Ar-H), 3.8(s, 3H, OCH$_3$), 1.52(m, 3H, CH$_3$-CH$_2$), 1.0-1.41(m, 25H, alkyl), 0.82 (t, 3H, CH$_3$-CH$_2$); MS (EI) m/z: 540.4 [M$^-$], 373.6, 345.6, 161.1

5. N-(4-cyano-3-trifluoromethyl-phenyl)-2-methoxy-6-pentadecyl-benzamide (6e). Yield: 84% (590 mg), white solid. MP: 96-98 °C

1NMR(DMSO-d$_6$) δ: 11.14(s, 1H, NH), 8.42 (s, 1H, Ar-H), 8.15(m, 2H, Ar-H), 7.38 (t, 1H, Ar-H), 7.01(d, 1H, J=8.3 Hz, Ar-H), 6.92(d, 1H, J=7.6 Hz, Ar-H), 3.8(s, 3H, OCH$_3$), 1.52(m, 3H, CH$_3$-CH$_2$), 1.0-1.41(m, 25H, alkyl), 0.82 (t, 3H, CH$_3$-CH$_2$); MS (EI) m/z: 531.8 [M$^-$], 391.5, 161.1, 149.2

6. N-(4-nitro-3-trifluoromethyl-phenyl)-2-methoxy-6-pentadecyl-benzamide (6f). Yield: 72% (520 mg), pale yellow solid. MP: 100-102 °C

1NMR(DMSO-d$_6$) δ: 11.28(s, 1H, NH), 8.4 (s, 1H, Ar-H), 8.15-8.28(m, 2H, Ar-H), 7.38 (t, 1H, Ar-H), 7.01(d, 1H, Ar-H), 6.92(d, 1H, Ar-H), 3.81(s, 3H, OCH$_3$), 1.52(m, 2H, CH$_3$-CH$_2$), 1.0-1.41(m, 25H, alkyl), 0.82 (t, 3H, CH$_3$-CH$_2$); MS (EI) m/z: 551.8 [M$^-$], 391.5, 161.1, 149.2

7. N-(4-chloro-3-trifluoromethyl-phenyl)-2-isopropoxy-6-pentadecyl-benzamide (6g). Yield: 86% (600 mg), white solid. MP: 61-63 °C

1NMR(DMSO-d$_6$) δ 10.63(s, 1H, NH), 8.34 (d, 1H, J=8.2 Hz, Ar-H), 7.95 (dd, 1H, J=10.5, Hz, Ar-H), 7.69 (d, 1H, Ar-H), 7.31(t, 1H, Ar-H), 6.97(d, 1H, J=8.3Hz, Ar-H), 6.87(d, 1H, J=7.56 Hz, Ar-H), 4.51(m, 1H, OCH-(CH$_3$)$_2$), 1.51(m, 2H, Ar-CH), 1.1-1.35(m, 25H, alkyl tail), 0.85 (t, 3H, CH$_3$-CH$_2$); MS (EI) m/z: 569.5 [M$^-$], 371.3
8. \(N\)-(4-cyano-3-trifluoromethyl-phenyl)-2-isopropoxy-6-pentadecyl-benzamide (6h).  
Yield: 81.8% (560 mg), light yellow solid. MP: 100-103 °C  
\(\text{^1}N\text{MR(DMSO-d_6)} \delta: \) 11(s, 1H, NH), 8.41 (d, 1H, Ar-H), 8.12-8.29 (m, 2H, Ar-H), 7.34(t, 1H, Ar-H), 7.01(d, 1H, Ar-H), 6.88(d, 1H, Ar-H), 4.51(m, 1H, OCH-(CH_3)_2, 2.5(s, 6H, (CH_3)_2CH), 1.0-1.35(m, 25H, alkyl), 0.81 (t, 3H, CH_3-CH_2); MS (EI) m/z: 560.1 [M^+], 517.8, 446.7, 373.7, 147.2

9. \(N\)-(4-nitro-3-trifluoromethyl-phenyl)-2-isopropoxy-6-pentadecyl-benzamide (6i).  
Yield: 70% (500 mg), light yellow solid. MP: 86-89 °C  
\(\text{^1}N\text{MR(DMSO-d_6)} \delta: \) 11(s, 1H, NH), 8.41 (d, 1H, Ar-H), 8.12-8.29 (m, 2H, Ar-H), 7.34(t, 1H, Ar-H), 7.01(d, 1H, Ar-H), 6.88(d, 1H, Ar-H), 4.51(m, 1H, OCH-(CH_3)_2, 2.5(s, 6H, (CH_3)_2CH), 1.0-1.35(m, 25H, alkyl), 0.81 (t, 3H, CH_3-CH_2); MS (EI) m/z: 580.1 [M^+], 580.1, 537.9, 33.6

10. \(N\)-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-benzamide (6j). Yield: 90% (850 mg), white solid. MP: 109-111 °C  
\(\text{^1}N\text{MR(DMSO-d_6)} \delta: \) 11.01(s, 1H, NH), 8.41(s, 1H, Ar-H), 8.13(m, 2H, Ar-H), 7.35(t, 1H, Ar-H), 6.98(d, 1H, Ar-H), 6.91(d, 1H, Ar-H), 4.51(q, 2H, CH_2-CH_3), 1.51(m, 3H, CH_3-CH_2), 0.85 (t, 3H, CH_3-CH_2); MS (EI) m/z: 391.1 [M^+], 344.4, 149.0 121.0 102.1

11. \(N\)-(4-cyano-3-trifluoromethyl-phenyl)-2-ethoxy-benzamide (6k). Yield: 77% (700 mg), light yellow solid. MP: 150-153 °C  
\(\text{^1}N\text{MR(DMSO-d_6)} \delta: \) 11.01(s, 1H, NH), 8.41(s, 1H, Ar-H), 8.13(m, 2H, Ar-H), 7.35(t, 1H, Ar-H), 6.98(d, 1H, Ar-H), 6.91(d, 1H, Ar-H), 4.51(q, 2H, CH_2-CH_3), 1.51(m, 3H, CH_3-CH_2), 0.85 (t, 3H, CH_3-CH_2)
12. *N*-\((4\text{-nitro-3-trifluoromethyl-phenyl})\)-2-ethoxy-benzamide (6l). Yield: 78.8% (760 mg), light yellow solid. MP: 65- 68 °C

\[^1\text{NMR}(\text{DMSO-d}_6) \delta 11.15 (s, 1H, \text{NH}), 8.4 (s, 1H, \text{Ar-H}), 8.16-8.29 (m, 2H, \text{Ar-H}), 7.35 (t, 1H, \text{Ar-H}), 6.99 (d, 1H, \text{Ar-H}), 6.91 (d, 1H, \text{Ar-H}), 4.1 (q, 2H, \text{CH}_2\text{-CH}_3), 1.51 (m, 3H, \text{CH}_3\text{-CH}_2), 0.85 (t, 3H, \text{CH}_3\text{-CH}_2)\]

13. *N*-\((4\text{-chloro-3-trifluoromethyl-phenyl})\)-2-methoxy-benzamide (6m). Yield: 87% (850 mg), white solid. MP: 160- 165 °C

\[^1\text{NMR}(\text{DMSO-d}_6) \delta: 11.14 (s, 1H, \text{NH}), 8.42 (s, 1H, \text{Ar-H}), 8.15 (m, 2H, \text{Ar-H}), 7.38 (t, 1H, \text{Ar-H}), 7.01 (d, 1H, \text{Ar-H}), 6.92 (d, 1H, \text{Ar-H}), 3.8 (s, 3H, \text{OCH}_3), 1.52 (m, 3H, \text{CH}_3\text{-CH}_2), 1.0-1.41 (m, 25H, alkyl), 0.82 (t, 3H, \text{CH}_3\text{-CH}_2)\]

14. *N*-\((4\text{-cyano-3-trifluoromethyl-phenyl})\)-2-methoxy-benzamide (6n). Yield: 79 % (750 mg), white solid. MP: °C

\[^1\text{NMR}(\text{DMSO-d}_6) \delta: 11.14 (s, 1H, \text{NH}), 8.42 (s, 1H, \text{Ar-H}), 8.15 (m, 2H, \text{Ar-H}), 7.38 (t, 1H, \text{Ar-H}), 7.01 (d, 1H, \text{Ar-H}), 6.92 (d, 1H, \text{Ar-H}), 3.8 (s, 3H, \text{OCH}_3), 1.52 (m, 3H, \text{CH}_3\text{-CH}_2), 0.82 (t, 3H, \text{CH}_3\text{-CH}_2)\]; MS (EI) m/z: 321.4 [M]^+, 189.2, 161.1, 149.1

15. *N*-\((4\text{-nitro-3-trifluoromethyl-phenyl})\)-2-methoxy-benzamide (6o). Yield: 80% (800 mg), yellow solid. MP: 80- 83 °C

\[^1\text{NMR}(\text{DMSO-d}_6) \delta: 11.28 (s, 1H, \text{NH}), 8.4 (s, 1H, \text{Ar-H}), 8.15-8.28 (m, 2H, \text{Ar-H}), 7.38 (t, 1H, \text{Ar-H}), 7.01 (d, 1H, \text{Ar-H}), 6.92 (d, 1H, \text{Ar-H}), 3.81 (s, 3H, \text{OCH}_3), 1.52 (m, 2H, \text{CH}_3\text{-CH}_2), 0.82 (t, 3H, \text{CH}_3\text{-CH}_2)\]

16. *N*-\((4\text{-chloro-3-trifluoromethyl-phenyl})\)-2-isopropoxy-benzamide (6p). Yield: 81 % (740 mg), colorless solid. MP: 88-92 °C
$^1$NMR(DMSO-d$_6$) $\delta$: 10.63 (s, 1H, NH), 8.34 (d, 1H, Ar-H), 7.95 (dd, 1H, Ar-H), 7.69 (d, 1H, Ar-H), 7.31 (t, 1H, Ar-H), 6.97 (d, 1H, Ar-H), 6.87 (d, 1H, Ar-H), 4.51 (m, 1H, O-CH(CH$_3$)$_2$), 1.51 (m, 2H, Ar-CH), 0.85 (t, 3H, CH$_3$-CH$_2$)

17. $N$-(4-cyano-3-trifluoromethyl-phenyl)-2-isopropoxy-benzamide (6q). Yield: 81% (740 mg), white solid MP: 156-160 °C

$^1$NMR(DMSO-d$_6$) $\delta$: 11 (s, 1H, NH), 8.41 (d, 1H, Ar-H), 8.12-8.29 (m, 2H, Ar-H), 7.34 (t, 1H, Ar-H), 7.01 (d, 1H, Ar-H), 6.88 (d, 1H, Ar-H), 4.51 (m, 1H, OCH-(CH$_3$)$_2$), 2.5 (s, 6H, (CH$_3$)$_2$CHO), 0.81 (t, 3H, CH$_3$-CH$_2$)

18. $N$-(4-nitro-3-trifluoromethyl-phenyl)-2-isopropoxy-benzamide (6r). Yield: 73.7% (650 mg), white solid MP: 58-63 °C

$^1$NMR(DMSO-d$_6$) $\delta$: 11 (s, 1H, NH), 8.41 (d, 1H, Ar-H), 8.12-8.29 (m, 2H, Ar-H), 7.34 (t, 1H, Ar-H), 7.01 (d, 1H, Ar-H), 6.88 (d, 1H, Ar-H), 4.51 (m, 1H, OCH-(CH$_3$)$_2$), 2.5 (s, 6H, (CH$_3$)$_2$CHO), 0.81 (t, 3H, CH$_3$-CH$_2$)

Table 2.3: CTB derivatives
2.6.2. CTPB (6a) and CTB (6j) Activate p300 HAT Activity.

Recently, CTPB was discovered as the first known small molecular activator of histone acetyltransferase p300. However, the structural basis and the mechanism of HAT activation by CTPB were not elucidated. To understand the chemical entities involved in the activation of p300, we have synthesized several derivatives of trifluoromethyl benzamide and have tested their activity using highly purified assay system comprising human core histones from HeLa nuclear pellet and full-length p300 enzyme expressed in the baculovirus infected Sf21 insect cell line (Figure 2.1 and 2.2). First, we investigated the role of pentadecyl hydrocarbon side chain of CTPB in the HAT activation. Incubation of p300 with increasing concentration (10-300 μM) of CTPB (6a) resulted in a dose-dependent enhancement of p300 HAT activity (~4 fold at 250 μM) as estimated by filter-binding HAT assay (Figure 2.3A compare lane 2 vs. lanes 3-9). Interestingly, CTB (6j), which lacks pentadecyl hydrocarbon chain, also enhanced p300 HAT activity of p300 in a dose dependent manner similar to CTPB but with a small but consistently higher fold of activation. (addition of 250 μM CTPB showed 10792.37 CPM while CTB showed 13887.17 CPM) (Figure 2.3B compare lane 2 vs. lanes 3-9).
Figure 2.3 Dose dependent HAT filter binding assay. HAT assays were done using 10 – 300 μM concentration of either CTPB or CTB with DMSO as control.

In order to visualize the activation of HAT activity by CTPB and CTB, duplicate reactions were subjected to HAT gel fluorography assay. In agreement with the filter-binding data, the results show that increasing concentrations of the small molecule modulators enhance the HAT activity of p300 (Figure 2.3C & D compare lane 2 vs. lanes 3-9).

C

| Core histones | + | + | + | + | + | + | + | + |
| [3H]Acetyl-CoA | + | + | + | + | + | + | + | + |
| p300          | + | + | + | + | + | + | + | + |
| DMSO          | - | + | + | + | + | + | + | + |
| CTPB (µM)     | - | - | 10 | 50 | 100 | 150 | 200 | 250 | 300 |

Autoradiogram

Coommassie
**Figure 2.3: Dose dependent HAT-gel fluorography assays.** HAT assays were done by incubating core histones and p300 in the presence of 10 – 300 μM concentration of either CTPB or CTB with DMSO as a control.

We further characterized the nature of activation by enzyme kinetics analysis in the presence of CTPB and CTB. The rates of acetylation reaction at different concentrations of the activators (and its absence) were recorded with increasing concentrations of $[^{3}\text{H}]$ acetyl-CoA and a constant amount of core histones as well as with increasing concentrations of histones and constant amount of $[^{3}\text{H}]$acetyl-CoA. Lineweaver Burk (double reciprocal) plot of $1/\text{CPM}$ versus $1/[^{3}\text{H}]$acetyl-CoA and $1[/\text{core histones}]$ shows that in the presence of CTPB/CTB, $K_m$ decreases while $V_{max}$ and $K_{cat}$ increase with increasing concentration of $[^{3}\text{H}]$acetyl-CoA and constant amount of core histones (Figure 2.4A & B). Interestingly, with increasing concentration of core histones and constant amount of $[^{3}\text{H}]$acetyl-CoA, all the three kinetic parameters $K_m$, $V_{max}$, and $K_{cat}$ increased significantly (Figure 2.4 C & D). As observed in the filter-binding assay,
kinetic analyses also suggests that at 200 μM and 250 μM concentrations CTB is a better activator of p300 HAT activity as compared to CTPB.

Figure 2.4: Kinetics of p300 HAT activation by CTPB (6a) and CTB (6j): A and B depict the Lineweaver Burk (LB) plot representation of 6a and 6j effect on p300 HAT activity at a fixed concentration of core histones (8 pM) and increasing concentration of $[^3]H$acetyl-CoA in the presence (200 and 275 μM) or absence of 6a and 6j. C and D show LB plot of the effect on 6a and 6j on p300 mediated acetylation of core histones (200-275 μM) at fixed concentration of $[^3]H$acetyl-CoA (275 nM) and increasing concentrations of histones (0.033-0.165 μM). The results were plotted using the Graph Pad Prism software.
2.6.3. Context-Based Requirement of Pentadecyl Hydrocarbon Chain in CTPB Derivatives to Enhance the HAT Activity.

The structural basis of CTPB and CTB to enhance the HAT activity was further investigated using several derivatives of these two classes of compounds. First, several derivatives of CTPB were synthesized keeping the pentadecyl hydrocarbon chain intact. The ethyl and -Cl groups were substituted by methyl or isopropyl and -NO2 or -CN groups, respectively, and were used in HAT assays (Table 2.2). The results show that all the compounds 6a-6i could enhance the p300 HAT activity with varying degree at 200 μM concentration. (Table 2.2 and Figure 2.5A, compare lane 2 vs. lanes 3-11). The results of the filter-binding assay were further confirmed by HAT gel fluorography assay (Figure 2.5B compare lane 2 vs. lanes 3-11).
Figure 2.3: Dose dependent HAT-gel fluorography assays. HAT assays were done by incubating core histones and p300 in the presence of 10 – 300 μM concentration of either CTPB or CTB with DMSO as a control.

We further characterized the nature of activation by enzyme kinetics analysis in the presence of CTPB and CTB. The rates of acetylation reaction at different concentrations of the activators (and its absence) were recorded with increasing concentrations of $[^3]$H acetyl-CoA and a constant amount of core histones as well as with increasing concentrations of histones and constant amount of $[^3]$H acetyl-CoA. Lineweaver Burk (double reciprocal) plot of $1/\text{CPM}$ versus $1/[[^3]\text{H} \text{acetyl-CoA}]$ and $1/[	ext{core histones}]$ shows that in the presence of CTPB/CTB, $K_m$ decreases while $V_{\text{max}}$ and $K_{\text{cat}}$ increase with increasing concentration of $[^3]$H acetyl-CoA and constant amount of core histones (Figure 2.4A & B). Interestingly, with increasing concentration of core histones and constant amount of $[^3]$H acetyl-CoA, all the three kinetic parameters $K_m$, $V_{\text{max}}$, and $K_{\text{cat}}$ increased significantly (Figure 2.4 C & D). As observed in the filter-binding assay,
2.6.4. Relative Position of -CF3 and -Cl Is Crucial for p300 HAT Activity Enhancement. To find out the specific positions of functional group required for the enzyme activation, we have synthesized different derivatives of CTB, containing altered positions of -CF3 and -Cl in the benzamide ring (Table 2.3). The activity of CTB (6j),
which significantly induces the activity of p300 at 200 μM concentration, was taken as control (Figure 2.7A lane 3). It was observed that although the p-chloro-o-trifluoromethyl benzamide (6s) and o-chloro-p-trifluoromethyl benzamide (6u) could enhance the HAT activity at 200 μM concentration the fold of activation decreased substantially especially for 6u (Figure 2.7A, compare lanes 4 and 6). Most interestingly, we observed that m-chloro-o-trifluoromethyl benzamide (6t) and o-chloro-m-trifluoromethyl benzamide (6v) completely lost the ability to enhance the p300 HAT activity (Figure 2.7A, compare lanes 5 and 7). In agreement with the filter-binding assay, HAT gel fluorographic assay also showed that m-trifluoromethyl and p-chloro positions are essential for the enzyme activation (Figure 2.7B). These results suggest that the position of -CF3 and -Cl group is very crucial to induce the activity of p300.
Figure 2.7: The relative position of –CF3 and –Cl in CTB are crucial for HAT activation. HAT filter binding (A) and gel fluorography assays (B) were done with various CTB derivatives (200 μM) in which the relative position of –CF3 and –Cl positions are changed.

2.6.5. CTB could induce changes in the structure of p300

To confirm the perturbation of backbone conformation of p300 upon compound treatment, CD-spectroscopy of p300 and the compound complex was performed. The data shows that although marginal alteration in the CD spectra could be observed in the 220-230 nm range, a dramatic change could be observed in lower wavelength (200-220 nm range).
Figure 2.8: The circular dichroism spectra of (i) p300 in presence of buffer containing DMSO (represented in blue) and (ii) p300 in presence of CTB (red). p300 (30 µg/ml in 10 mM Tris-HCl, 10% glycerol, 100 mM NaCl and 2 mM β-mercaptoethanol) was incubated either with DMSO or 200 µM of CTB for 5 minutes and the CD spectra was recorded at room temperature (27°C) in a JASCO model J715 spectropolarimeter.

2.6.6. Compounds 6c and 6q can enhance acetylation of histones and nonhistone substrates in vivo.

All the derivatives of CTPB and CTB which could activate p300 HAT activity in vitro were screened for their activity in vivo. Due to the insoluble nature most of the compounds could not be tested. But the compounds 6c and 6q which were sparingly soluble in the culture media were tested on the mammalian cells for their ability to induce hyperacetylation by activating the HAT activity in vivo. After the treatment of the cells the histones were extracted, estimated and western blotting analysis was done using
antibodies against histone H3 (loading control) and acetylated histones. As a positive control TSA (HDAC inhibitor) was taken which could induce hyperacetylation (Figure 2.9 lane 5) along with untreated and DMSO treated cells as controls (Figure 2.9 lanes 1 & 2). Compounds 6c and 6q could very weakly induce hyperacetylation, which could be because of their miscibility in aqueous medium and cell permeability (Figure 2.9 lanes 3 & 4).

![Western blotting analysis](image)

**Figure 2.9:** Western blotting analysis shows induction of acetylation in vivo by 6c and 6q. Acid extracted histones (500 ng) were resolved on a 15% polyacrylamide gel and western blotting analysis was done with α-acetyl lysine (upper panel) and histone H3 (lower panel) antibodies.

Apart from histones p300 can acetylate many other nonhistone substrates of which p53 is very well known(ref). Acetylation of p53 is known to enhance its DNA binding ability and thereby its transcriptional activity. Thus by inducing acetylation of ectopically expressed p53 by various compounds the *in vivo* effect of the compounds can be studied.
Mammalian cells (H1299 (p53 -/-) cells) were transfected with pG13Luc and p53 constructs followed by treatment with compound 6q and Sodium Butyrate (NaBu, a known HDAC inhibitor) as a positive control. The results show that compound 6q could enhance the acetylation of p53 as shown by its transcriptional activity in a dose dependent manner (Figure 2.10) and a maximum of ~2 fold could be achieved at 10 μM concentration.

![Bar graph showing dose dependent induction of acetylation by 6q.](image)

**Figure 2.10: Dose dependent induction of acetylation by 6q.** After transfection with PG13Luc (reporter vector) and p53 (activator) constructs the cells were treated with increasing concentrations of compound 6q (lane 1, untreated; lane 2, DMSO treated; lanes 3-7, 2, 5, 10, 15 and 20 μM) and NaBu (lane 8). The lysates were subjected to luciferase assay and the counts/30 seconds are plotted.

2.7. Discussion

Anacardic acid from CNSL (Cashew Nut Shell Liquid) was discovered as the first natural, nonspecific histone acetyltransferase inhibitor.15 Surprisingly, the amide derivative of
anacardic acid was found to specifically enhance the HAT activity of p300 in vitro. However, the mechanism of activation of the p300 HAT activity as well as the functional moiety of the compound responsible for the enhancement of activity was not known. Here, we report the synthesis of CTB (6j) and the evaluation of the enhancement of p300 HAT activity. We have also synthesized several derivatives of CTPB (6a-i) and CTB (6j-r) (Figure 2.11). To see whether the long carbon chain in 6a is responsible for the activation, we synthesized 6j. Both 6a and 6j enhanced the HAT activity of p300. Interestingly, we found that at similar concentrations of 6a and 6j, the latter is slightly better activator of p300.

Figure 2.11: Comparison of CTPB and CTB derivatives. The relative fold activation of HAT activity of p300 by different derivatives of CTPB and CTB is shown in comparison with DMSO (control).

To confirm the perturbation of backbone conformation of p300 upon compound treatment, we have also performed CD-spectroscopy of p300 and the compound complex. The data showed that although marginal alteration in the CD spectra could be observed in the 220-230 nm range, a dramatic change could be observed in lower wavelength (200-
220 nm range). Though these molecules are hydrophobic, they have regions for strong hydrogen bonding as well as hydrophobic interactions. These would be the trigger for the change in the p300 structure. It is well-known that the most hydrophobic region of the p300 is close to the HAT domain of the protein. Hence, the molecules would be causing structural changes in the HAT domain itself and thereby activate the p300 HAT activity. Significantly, in case of 6j derivatives, only three compounds (6p, 6q, and 6r) with isopropyl side chain could stimulate the p300 HAT. These results argue for the requirement of pentadecyl hydrocarbon chain under certain context. In search of chemical basis of activation, further we altered the relative position of -CF3 and -Cl in CTB. Interestingly, we found that the activator effect is reduced in 6s and is further reduced in 6u, while 6t and 6v have completely lost the enhancement activity. These results confirm that the relative positions of -CF3 and -Cl in CTB are important for activation of the protein. It is important to observe that in the case of 6t there is no functional group in the para position of the phenyl ring of CTB. The HAT activity induced by 6s is relatively low as compared to 6j but is much larger than 6u. Therefore, the presence of the -CF3 group at the meta position also plays an important role in the activation. It can be inferred that substitution at the -meta and -para positions in the phenyl group of CTB with strong electronegative group, such as -F, may lead to stronger activation of p300 HAT activity. Owing to the poor solubility and cell permeability these activators are very less potent in vivo. Further derivatization could lead to discovery of several cell permeable HAT activators which could be used as alternatives to HDAC inhibitors or in combination with HDAC inhibitors. This report thus describes the identification of chemical entities
essential to activate p300 HAT activity by the only known small molecule activator, CTPB and partially the mechanism of activation.