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Recognition of chromatin by the plant alkaloid, ellipticine as a dual binder

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ABSTRACT

Recognition of core histone components of chromatin along with chromosomal DNA by a class of small molecule modulators is worth examining to evaluate their intracellular mode of action. A plant alkaloid ellipticine (ELP) which is a putative anticancer agent has so far been reported to function via DNA intercalation, association with topoisomerase II and binding to telomere region. However, its effect upon the potential intracellular target, chromatin is hitherto unreported. Here we have characterized the biomolecular recognition between ELP and different hierarchical levels of chromatin. The significant result is that in addition to DNA, it binds to core histone(s) and can be categorized as a 'dual binder'. As a sequel to binding with histone(s) and core octamer, it alters post-translational histone acetylation marks. We have further demonstrated that it has the potential to modulate gene expression thereby regulating several key biological processes such as nuclear organization, transcription, translation and histone modifications.

1. Introduction

Chromatin is the physiological template of eukaryotic genome. DNA-binding anticancer drugs actually target the chromatin template in cells. With the advent of the field, we have made an attempt to re-classify these small molecules as 'single binder' which interacts with DNA only or 'dual binder' which binds to both DNA and core histones [1–5]. Functionally the dual binders could affect the cellular chromatin organization, epigenetic landscape and consequent gene expression programs [1,5].

Ellipticine, ELP (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole) (Fig. 1A), a naturally occurring plant alkaloid is a putative anticancer agent. It is isolated from plants belonging to *Apocynaceae and Loganiaceae* families [6,7]. Research in the past few years have shown several targets and mechanisms of its biological action. Of these, interactions with DNA are well established, and include intercalation, topoisomerase II inhibition [8], bio-oxidation and adduct formation with DNA [9]. ELP inhibits some of the key cellular players such as c-Kit and AKT kinase [9] and induces activation of tumor suppressor p53 [9]. Our group has shown that ELP interacts with the human telomeric DNA sequence, $d(TTAGGG)_4$ and inhibits telomerase activity in MDAMB-231 breast cancer cell line extracts [10]. Here, we have studied the interaction of ELP with hierarchical levels of chromatin viz. long chromatin, chromatosome, nucleosome, chromosomal DNA and histone octamer. Our results show that ELP exhibits dual binding mode of interaction at the chromatin level [1–4,11], binding to both histones and DNA. At the structural level, ELP disrupts the integrity of histone octamer and causes DNA release from chromatosome. An enhancement in the hydrodynamic size of long chromatin ensues the association process.

Chromatin structure and function are influenced by post-translational modifications (PTMs) of histone(s) which impact gene expression [12]. These epigenetic modifications are reversible and serve as potential targets for drugs [13]. Interestingly, ELP was found to inhibit some of the important post-translational histone acetylation marks implicated in transcription activation. Histone modifications are key players in regulating chromatin states and

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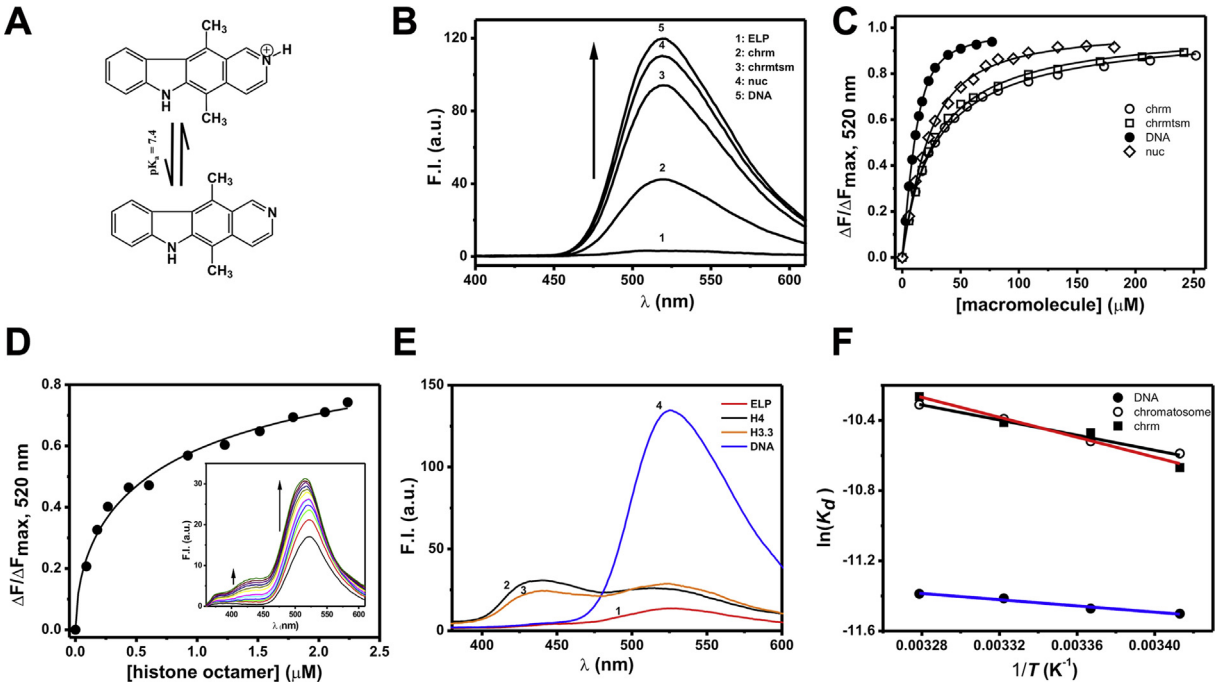

Fig. 1. **(A)** Chemical structure of ELP. **(B)** Emission spectra of 3 μM ELP in absence (curve 1) and in presence of 50 μM chromatosome (curve 2), long chromatin (curve 3), nucleosome (curve 4) and chromosomal DNA (curve 5) in 10 mM potassium phosphate, pH 6.8 containing 15 mM KCl at 25 °C. **(C)** Binding isotherms obtained by non-linear curve fitting analyses (○, long chromatin; □, chromatosome; ●, chromosomal DNA; ◊, nucleosome). **(D)** Binding isotherm for the interaction of ELP with core histone octamer in 10 mM potassium phosphate, pH 6.8 containing 2 M KCl at 25 °C. Inset shows the emission spectra of 2 μM ELP in presence of increasing concentrations of core octamer. **(E)** Emission spectra of 2 μM ELP in absence (curve 1) and in presence of 6 μM histone H4 (curve 2), H3.3 (curve 3) and chromosomal DNA (curve 4) in 10 mM Tris–HCl, pH 8.0 containing 150 mM NaCl at 25 °C. **(F)** van't Hoff plot to determine ΔH and ΔS for association of ELP with long chromatin (■), chromatosome (○) and chromosomal DNA (●). The linear best fits of the experimental data points are represented by the solid lines.

dynamics as well as in gene expression [14]. We have observed that the genes involved in several biological processes are differentially expressed upon ELP treatment. Summing up, histone-binding ability of ELP suggests an additional pathway of action of this biologically important molecule.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma Chemical Company, USA, unless otherwise specified.

2.2. Methods

The technical details and instrument specifications are provided under Supplementary Material.

2.2.1. Preparation of ELP stock

Stock solution of ELP was prepared in freshly distilled DMSO and the concentration was determined spectrophotometrically, using the molar extinction coefficient, $\varepsilon_{295} = 60{,}000\ M^{-1}\ cm^{-1}$ [10].

2.2.2. Preparation of chromatin samples

Chromatin, chromatosome, nucleosome and chromosomal DNA were isolated from rat liver following standard protocol [15] and mononucleotide concentrations of the samples were determined spectrophotometrically using the molar extinction coefficient (ε_{260}) of 6600 $M^{-1}\ cm^{-1}$.

Core octamer was prepared using the method described by Peterson and Hansen [16] and concentration of core octamer was determined using the molar extinction coefficient (ε_{230}) of 507,233 $M^{-1}cm^{-1}$.

2.2.3. Steady state fluorescence measurements

The fluorescence emission spectra of ELP (3 μM) were monitored in presence of chromatin components where macromolecule/ligand ratio was gradually increased. The binding isotherms were obtained from a plot of $\Delta F/\Delta F_{max}$ (at 520 nm, $\lambda_{ex} = 320$ nm) as a function of input macromolecule concentration. The apparent dissociation constant K_d, at a given temperature was determined using non-linear curve fitting analysis [17]. The thermodynamic parameters were determined from van't Hoff analysis [18].

2.2.4. Circular dichroism study

The circular dichroism spectra of chromatin components (50 μM of long chromatin, chromatosome, nucleosome, chromosomal DNA each and 3 μM core histone octamer) were recorded in absence and presence of ELP at 25 °C. The molar ellipticity of macromolecules was plotted against the wavelength. Convex constraint analysis (CCA) was performed to extract the basis spectra and their associated coefficients [19]. As controls, CD spectra of macromolecules were monitored in presence of DMSO (corresponding to the total ELP volume added).

2.2.5. Dynamic light scattering (DLS)

In order to investigate the effect of ELP on the hydrodynamic size, chromatin (120 μM) was treated with ELP in ligand: DNA base ratio of 0, 0.05, 0.10, 0.15 and 0.20 and the hydrodynamic diameters (Z_{av}) were monitored. Similarly, core histone octamer (3 μM) was treated with increasing concentrations of ELP (3, 6 and 9 μM) and the particle size was measured.

2.2.6. Cell culture

HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penstrep (10 μL/mL of medium) at 37 °C in 5% (v/v) CO_2.

2.2.7. Cell viability assay (MTT assay)

In order to check cell viability upon ELP treatment, MTT assay was performed as per standard protocol [20] with DMSO treated and 10 μM ELP treated HeLa cells (treatment time:15 h).

2.2.8. Histone acetyltransferase (HAT) assay

HAT assays were performed as per standard protocol [11,21] with 2 μg of histones (H3.1/H2B/H4) in the presence of ELP or DMSO (control). GCN5 was used as the HAT for H3K9Ac and CBP for other reactions.

2.2.9. RNA isolation and microarray analysis

Total RNA was isolated from ELP/DMSO treated HeLa cells. RNA samples in triplicates from different passages were processed for microarray analysis described under Supplementary Material.

2.2.10. qPCR analysis

Microarray data were validated by means of quantitative PCR (real-time PCR). The ΔΔCt method was used to analyze the data [22]. Expression levels were calculated as fold change over DMSO.

3. Results and discussion

3.1. Association of ELP with hierarchical levels of chromatin and histone octamer

The physical association of ELP with long chromatin, chromatosome, nucleosome, chromosomal DNA and core histone octamer was examined by means of steady state fluorescence spectroscopy. Binding of ELP with these macromolecules results in enhancement of its fluorescence quantum yield. Increase in emission intensity (Fig. 1B) may be due to one or more factors: a change in the local environment such as viscosity or hydrophobicity of the ligand molecule, alteration in its conformation and the enhanced excited state proton transfer [23,24]. ELP binds to chromatin components with different site sizes and the dissociation constants are in the micromolar range (Table 1). ELP also exhibits binding interactions with individual histones (H3 and H4, Fig. S1). The difference in fluorescence signature of ELP in case of histones and DNA (Fig. 1E) indicate that histones interact preferentially with the neutral form of ELP and DNA interacts with the protonated form (elaborated under supplementary material).

Comparative values of the relevant dissociation constants suggest that ELP has the highest affinity for core octamer followed by chromosomal DNA. The intermediate affinities for the other components might be ascribed to inherent DNA-histone interaction which competes with DNA-ELP and histone-ELP interactions. Higher affinity of nucleosome compared to chromatosome might be due to the presence of linker histone in chromatosome which impedes ELP-nucleosome interaction. When histone H1 is removed from chromatosome about 20 base pairs of DNA are exposed and hence accessible for ELP binding. Similar affinity of ELP for long chromatin and chromatosome indicates that ELP has no particular preference for linker DNA. Comparable binding parameters of ELP for chromatin obtained from different sources (rat liver and HeLa cell, Table 1) suggest that the interaction is independent of the nature of sources.

Notwithstanding the fact that Isothermal Titration Calorimetry is a better choice to get the binding associated thermodynamic parameters [25], we were constrained to adopt van't Hoff analysis due to solubility of ELP in DMSO. Volume percent of DMSO in the reaction mixture acts as a deterrent to obtain good thermograms in ITC measurements. Dissociation constants for the interaction of ELP with long chromatin, chromatosome and chromosomal DNA were estimated at four different temperatures to obtain an idea of the energetic scenario with alteration in the degree of DNA compaction. The magnitude of K_d increases with rise in temperature and the thermodynamic parameters evaluated using van't Hoff relationship are listed in Table 1. The binding reactions are characterized by a favorable contribution from both enthalpy and entropy. Release of water molecules and counter ions as a sequel to ligand binding serves as the entropy source. The sharp decline in the negative enthalpy from histone bound DNA (chromatin and chromatosome) to naked DNA implies that histones are involved in non-covalent interactions during association. Such trend was reported from the studies on the interaction of ethidium bromide and netropsin with chicken erythrocyte chromatin [26] and mithramycin and chromomycin with rat liver chromatin [27]. Enthalpy-entropy compensation during the association results in comparable free energy change.

Table 1
Binding parameters for the interaction of ELP with chromatin components obtained from spectrofluorimetric analysis.

Binding parameters for the interaction of ELP with rat liver chromatin components obtained from spectrofluorimetric titrations at 25 °C		
System	K_d (μM)	Stoichiometry (N)
long chromatin	28.0 ± 0.7	[a]10.2 ± 0.7
	[c]35.4 ± 0.4	[c]14.7 ± 0.3
chromatosome	28.3 ± 0.4	[a]11.9 ± 1.5
nucleosome	20.4 ± 0.7	[a]8.8 ± 0.5
chromosomal DNA	10.8 ± 0.6	[a]6.2 ± 0.6
core histone octamer	0.6 ± 0.05	[b]0.13 ± 0.02

Thermodynamic parameters for the association of ELP with rat liver long chromatin, chromatosome and chromosomal DNA obtained from van't Hoff analysis			
System	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	$\Delta G_{25\,°C}$ (kcal mol^{-1})
long chromatin	−5.64 ± 0.8	1.9 ± 0.3	−6.2 ± 0.8
chromatosome	−4.3 ± 0.4	6.5 ± 1.1	−6.2 ± 0.4
chromosomal DNA	−1.76 ± 0.1	16.8 ± 0.6	−6.8 ± 0.1

[a] N = DNA bases bound per ELP molecule.
[b] N = core histone octamer bound per ELP molecule.
[c] Source: HeLa cell.

3.2. ELP induced structural alteration of chromatin components

Effect of ELP on chromatin architecture was investigated by means of CD spectroscopy, DLS and gel assay. ELP, a planar aromatic molecule which is optically inactive exhibits positive induced band upon intercalation within DNA base pairs [10]. The CD spectra of chromatin, chromatosome, nucleosome and chromosomal DNA in absence of ELP agree well with literature reports [28,29]. Optically asymmetric environment of DNA and DNA-histone complexes (chromatin, chromatosome and nucleosome) gives rise to induced CD bands in the absorption region of ELP whose intensity increase in a concentration dependent manner (Fig. S2). When the CD spectra were subjected to CCA analysis, two basis spectra were obtained. In each case, the percentage population of component 1 (unbound macromolecule) decreases and that of component 2 (ELP-bound form) increases (Fig. S3). The observations fall in line with the analysis of the binding data. The histone octamer has a characteristic α-helical CD signature [30,31] which undergoes a notable alteration in presence of ELP (Fig. 2B) suggesting that ELP causes a major structural transition of histone octamer. Since ELP is soluble in DMSO, CD spectra of the macromolecules were

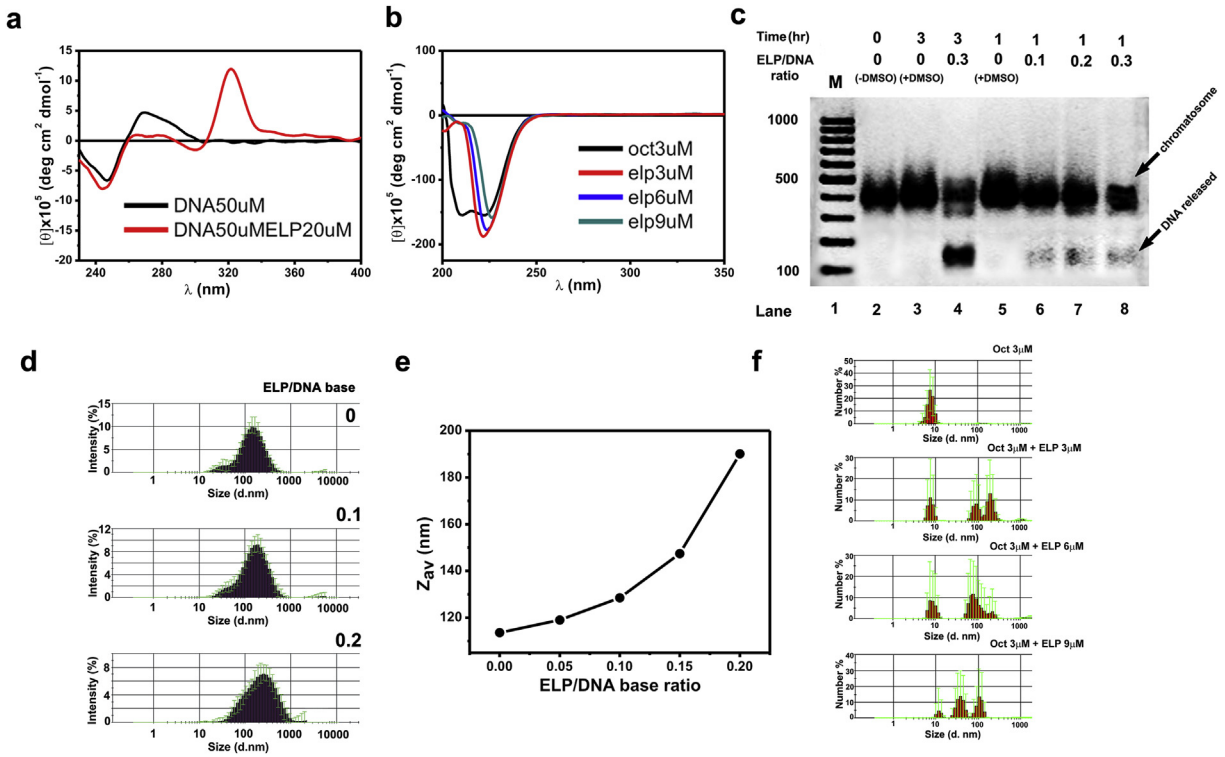

Fig. 2. CD spectra of **(A)** 50 μM chromosomal DNA and **(B)** 3 μM core histone octamer in absence and presence of ELP at 25 °C in 10 mM potassium phosphate, pH 6.8 containing 15 mM KCl in (A) and 2 M KCl in (B). **(C)** Chromatosome stability assay showing DNA release from chromatosome (120 μM) incubated with different concentrations of ELP for 1 and 3 h at 37 °C. Lanes 2, 3 and 5 served as negative controls. **(D)** Plot of intensity statistics against hydrodynamic size of chromatin (120 μM DNA base) alone and in presence of ELP at 25 °C obtained from DLS. Error bars indicate standard deviations. **(E)** Variation of hydrodynamic diameter (Z_{av}) of chromatin as a function of ligand/macromolecule ratio. **(F)** Plot of number statistics versus hydrodynamic size of core histone octamer (3 μM) in absence and presence of ELP at 25 °C.

monitored in presence of DMSO (representative figure for chromatin, Fig. S3) to rule out any artifact that may arise due to interference of DMSO. Percent volume of DMSO in the final mixture does not cause any alteration in the CD spectral shape or intensity of the macromolecules. So, the observed changes in CD spectra were attributed solely to ELP-macromolecule interaction.

Further evidence of the effect of ELP upon the structure of chromatin and histone octamer was obtained from DLS which provides an idea of the apparent size of a solvated dynamic particle. Among different hierarchical levels of chromatin assembly, long chromatin was chosen as a template for DLS since it is a multimer of nucleosomes where the extent of structural changes would be more apparent. Chromatin shows two peaks maximizing at 164.2 and 37.8 nm respectively which tend to merge in presence of ELP and at ELP/chromatin ratio of 0.2, the peak at 37.8 nm almost disappears and the peak at 164.2 nm shifts to 220 nm (Fig. 2D). The trend in Z_{av} values of chromatin (Fig. 2E) in absence and presence of ELP shows that the hydrodynamic size of chromatin is enhanced in presence of ELP (113.6 nm–190.3 nm). Experiments with histone octamer focus on ELP induced structural disruption. Fig. 2F reveals that ELP disrupts the integrity of core octamer. Existence of different populations in absence and presence of ELP demonstrates that the intact octamer dissociates into subunits which then form heterogeneous aggregates of different sizes on gradually increasing the ligand/octamer ratio. Any perturbation of chromatin or octamer structure due to presence of DMSO in the system was ruled out from appropriate control experiments (Fig. S4).

At the chromatosome level, binding of ELP leads to destabilization of the chromatosome. Incubation of chromatosome with ELP at 37 °C followed by analysis on an agarose gel leads to the appearance of a band around 200 bp indicating DNA release from chromatosome (~400 bp) (Fig. 2C). This provides another evidence for ELP induced disruption of histone-DNA complex triggering DNA release. Results from DLS and chromatosome stability assay corroborate the observations from CD experiments. It needs to be mentioned that the spectroscopic and other biophysical studies were performed at 25 °C where the disruption of chromatosome structure leading to DNA release was not observed within the experimental time frame.

3.3. *Alteration of histone PTMs by ELP and its effect upon global gene expression*

The dynamic chromatin architecture is regulated by several factors, one of which is histone PTM. Hence we were curious to check whether ELP, a potential histone binder, could alter epigenetic modifications like other dual binders [1,4,5,32]. In accordance with our speculation we have found that ELP inhibits some of the abundant acetylation marks of core histones implicated in transcription activation (H3K27Ac, H3K18Ac, H3K9Ac, H4K5Ac, H4K8Ac, H2BK12Ac) without any significant effect on $H3K9Me_3$, a transcription repression signature. Fig. 3 (A, B) shows the western blot analysis of DMSO treated (lane 1) and ELP-treated (lane 2) HeLa cells for each of the histone modification marks followed by their quantification. The cell survivability was not altered to a significant extent under this condition as evident from MTT assay (Fig. S5) reflecting that inhibition of global histone acetylation is not due to ligand induced secondary effect. *In vitro* HAT assays were performed with purified histone substrates. The results (Fig. S6) show that ELP induces repression in H3K18Ac, H3K9Ac and H3K27Ac without any detectable alteration in the acetylation levels of H4K5

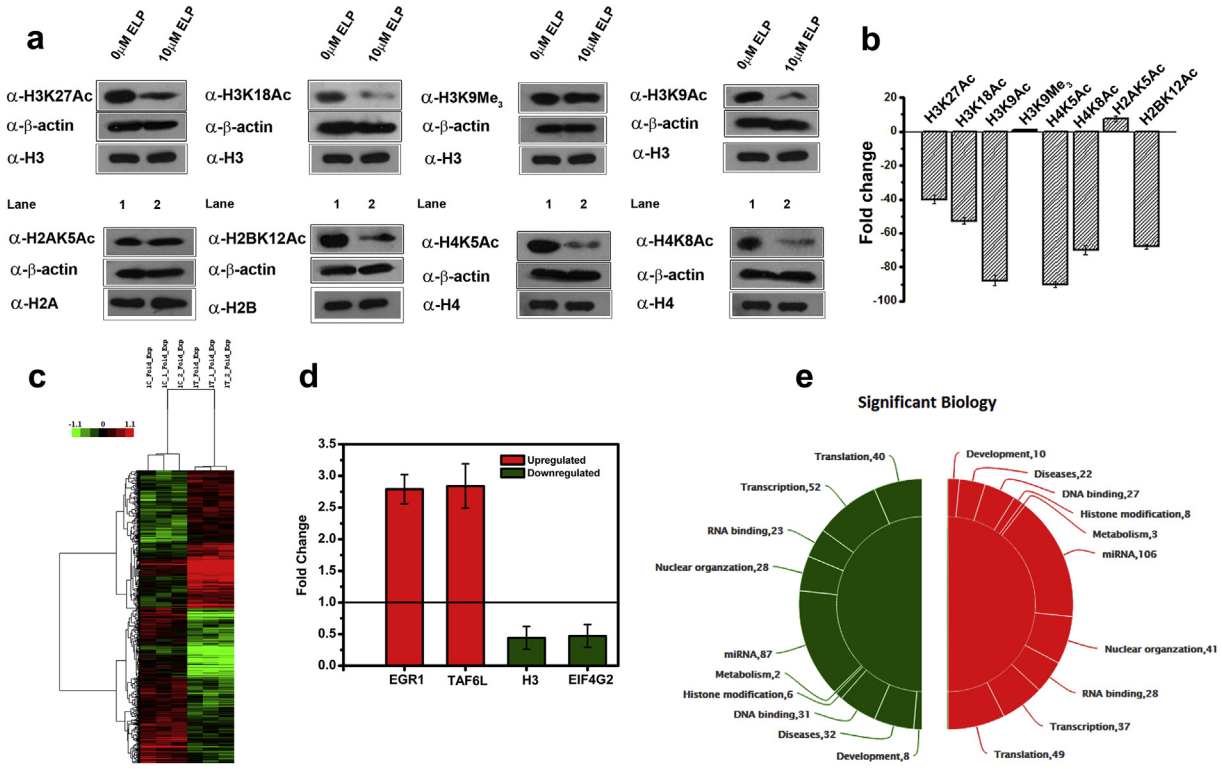

Fig. 3. (A) Effect of ELP on histone PTMs monitored by western blot analysis. β-actin and histones H3, H4, H2A, H2B served as loading controls. **(B)** Extent of ELP induced alteration of PTM levels quantified using Image J software after normalizing with respective histone loading controls. Fold change was calculated in origin. Error bars were estimated from three independent sets of experiments. **(C)** Unsupervised hierarchical clustering of differentially expressed genes showing distinct patterns of up and down regulated genes upon ELP treatment in comparison to DMSO treated samples. **(D)** Validation of candidate genes by qPCR. Fold change of mRNA level was quantified after normalization with respect to β-actin. Reference level is 1. Each bar is an average of 3 individual biological replicates. Error bars show standard deviations. **(E)** Pie chart representation of Significant Biological processes influenced by ELP. The numbers denote the differentially expressed genes in HeLa cell line upon ELP treatment. Green and red color codes represent downregulated and upregulated genes respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and H2BK12. So, histone H3 appears to be a preferential target for ELP both *in vitro* and *ex vivo*.

Imbalance in the basal level of the epigenetic signatures is often observed in tumorigenesis and non-toxic small molecules which can restore this balance are finding importance in drug designing and epigenetic therapy. Histone acetylation alters DNA/histone contacts and promotes chromatin disassembly [33]. The follow up events alter the local chromatin architecture and underlying gene expression profiles. Thus small molecules which perturb the histone acetylation status could affect global gene expression [1,4,5,32]. The ability of ELP to alter histone PTMs prompted us to examine its impact at the gene level employing microarray analysis. Gene expression data showed 853 transcripts upregulated and 960 transcripts downregulated with a fold change of $\geq$1.1 and pValue of $\leq$0.05 (Supplementary File 1) which is of statistical significance. Unsupervised hierarchical clustering of differentially expressed genes showed distinct pattern of up and downregulated transcripts upon ELP treatment (Fig. 3C). Over representation analysis of significant biological groups that harbor differentially expressed transcripts revealed various categories related to transcription, translation, nuclear organization, histone modification, cellular development and diseases (Fig. 3E and Supplementary File 2). Biological Analysis Network of enriched gene sets along with differentially expressed genes revealed key nodes (gene: function) and edges that are likely to be key regulatory circuits induced upon ELP treatment (Fig. S7).

A few candidate genes were selected for validation by qPCR (Fig. 3D). Transcriptional regulator *EGR1* (early growth response 1), a tumor suppressor [34] was found to be upregulated upon ELP treatment. Another upregulated candidate is transcription factor *TAF6L*, a component of the PCAF histone acetyltransferase complex [35]. *EIF4G2* (eukaryotic translation initiation factor 4 gamma 2) involved in regulation of translation and H3i (*HIST1H3I*) associated with nuclear organization [36,37] were downregulated in presence of ELP. It may be noted that ELP alters the differential expression of fewer number of genes compared to the dual binder mithramycin [5] obtained from bacterial sources [38] indicating that perhaps the plant alkaloids do not have significant off-target effects.

Summing up, we have characterized the association of ELP with hierarchical levels of chromatin and have shown that core histone(s) is another potential target for the ligand making it a dual binder [1–5] in the chromatin context. ELP induces structural perturbations at chromatin and chromatosomal levels and disrupts the integrity of core octamer. ELP also alters epigenetic signatures and affects global gene expression. This study suggests an additional mode of action of the plant alkaloid, ELP and hitherto unreported potential of the compound to function as an epigenetic modulator via the inhibition of acetylation of core histone, H3 as demonstrated both *in vitro* and *ex vivo*.

Conflict of interest

No conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.140.

Transparency document

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