Exploration of mechanisms in nutriepigenomics: Identification of chromatin-modifying compounds from *Olea Europaea*

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Abstract
Chemical modification of histones represents an important epigenetic mechanism critical for DNA metabolism including, transcription, replication and repair. A well-known example is maintenance of histone acetylation status by the opposing actions of histone acetyltransferase and histone deacetylase enzymes which add and remove acetyl groups on lysine residues on histone tails, respectively. Similarly, histone methyltransferase and histone demethylase enzymes are responsible for adding and removing methyl groups on histone tails, respectively. Further, there is accumuluated evidence indicating a histone code where combinations of different chemical modifications on histone tails act in concert to regulate DNA metabolic events. Although numerous compounds have been developed to specifically alter the function of chromatin modifying enzymes (for example, histone deacetylase inhibitors are relatively well-investigated), we are only at the early stages of understanding the epigenetic effects of dietary compounds. Here we used in silico molecular modeling approaches combined with known experimental affinities for controls, to identify potential chromatin modifying compounds derived from Olea Europaea. Our findings indicate that various compounds derived from Olea Europaea have the ability to bind to the active site of different chromatin modifying enzymes, with an affinity analogous or higher than that for a known positive control. Further, we initiated the process of validating targets using in vitro binding and enzyme activity inhibition assays and provide initial findings of potential epigenetic effects in a clinical context. Overall, our findings can be considered as the first instalment of a comprehensive endeavour to catalogue and detail the epigenetic effects of compounds derived from Olea Europaea.

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Introduction

Epigenetics which literally translates to as “above” or “upon” genetics, is broadly understood to involve mechanisms resulting in heritable changes in gene expression that are not due to changes in the underlying DNA sequence. Therefore, if the sum of genes in a cell makes up the genome of that cell, the epigenome is considered as the totality of epigenetic processes involved in regulating gene expression which are not associated with changes in the gene sequence. Further, the large-scale and systematic investigation of the epigenome, predominantly via experiments using next generation sequencing technologies (NGS), refers to epigenomics. Epigenomic analyses have been typified by the Encyclopedia of DNA Elements (ENCODE) project and are poised to be extended by the US National Institutes of Health, Roadmap Epigenomics Project [1-3]. For example, chemical modification of DNA with a methyl group (or DNA methylation) is a well-known epigenetic mechanism and has been widely investigated using NGS [4, 5]. Indeed, aberrant DNA methylation has now been associated with various malignancies and a host of other chronic diseases.

Similarly, aberrant chemical modifications on chromatin have been linked to disease. Briefly, the approximately 2 metres of DNA in each cell is organized into functional units known as nucleosomes (Figure 1) [6-8]. Each nucleosome consists of approximately 146 base pairs of DNA wrapped around two copies of each of the four core histones, H2A, H2B, H3 and H4. Predominantly the lysine residues of the protruding histone tails of the core histones undergo chemical modification, of which acetylation and methylation are amongst the most-investigated. The acetylation status of histones is regulated by the opposing actions of histone acetyltransferases (HAT) which add acetyl groups to lysine residues on core histones and histone deacetylase enzymes (HDAC) which remove acetyl groups [9].

Numerous enzymes have been identified to possess HAT activity and are classified into the major Gcn5-related N-acetyltransferase and MYST families with the p300/CBP, nuclear receptor coactivators representing another important class [10]. In turn, the 18 mammalian HDAC enzymes are categorized into two major groups on the basis of their homology to yeast counterparts, 1) the metal-dependent classical HDAC enzymes which include (class I: HDACs, 1, 2, 3
Figure 1. 3D chemical structure of a nucleosome core particle with DNA fragment (146 base pairs) wrapped around the histone core (PDB ID: 1AOI). Nucleosomes are comprised of two sets of each of the four core histones with histone tails (H3 tail depicted—not to scale) protruding from the structure enabling chemical modification. Acetylation status is maintained by the opposing actions of histone acetyltransferases (HAT) which add acetyl groups to lysine residues and histone deacetylases (HDACs) which remove acetyl groups. Similarly, histone methylases control methylation levels, where histone methyltransferase (HMT) add methyl groups to residues whilst histone demethylases (HD) remove methyl groups.

and 8; class IIa: HDACs 4, 5, 7 and 9; class IIb: HDACs 6 and 10; class IV: HDAC11) and 2) the sirtuins 1-7 which are nicotinamide adenine dinucleotide-dependent [9, 11, 12]. Similarly, histone methylation status is maintained by histone methyltransferases (HMT) which add methyl groups and include histone-lysine N-methyltransferases (divided into SET [Su(var)3-9, Enhancer of Zeste, Trithorax] domain containing or non-SET domain containing enzymes), and histone-arginine N-methyltransferases and histone demethylases (HDM) which remove methyl groups from core histones [10]. Importantly, it has been demonstrated that combinations of patterns of differential histone acetylation and methylation regulate work in concert to control chromatin architecture and gene expression providing evidence for the “histone code” hypothesis [13, 14]. This adds a layer of complexity to the genome with such a vast array of possible chemical modifications. To provide a general example, histone acetylation and methylation of histone 3 on lysine 4 is associated with active transcription whereas deacetylation and methylation of histone 3 on lysine 9 is associated with inactive chromatin.

Diverse classes of chromatin modifying compounds have now been developed for their clinical potential, particularly for various malignancies, either as standalone therapeutics or combined with conventional therapies. Cases in point include the HDACi suberoylanilide hydroxamic acid (SAHA, Vorinostat, Zolinza; Merck) and depsipeptide (Romidepsin, Istodax; Celgene) which, were the initial HDACi approved by the US Food and Drug Administration (FDA) for the treatment of advanced cutaneous T-cell lymphoma [15]. In addition, there is excitement regarding the potential of diet and regulation of the epigenome, necessitating research in the new field of nutriepigenomics, with an accumulating list of dietary compounds that have been shown to alter the chromatin landscape and gene expression [16]. Although still at very early stages, well-known examples of dietary chromatin modifying compounds include the active constituent of turmeric, curcumin which has been identified to be an HDACi, as well as the red wine phenol, resveratrol which activates the class III HDAC, Sirt1 [17, 18].

Here, we explored the epigenome-modifying potential of a selected group of compounds derived from Olea Europaea. Our selection was based on the ability to easily quantitate the chosen compounds from standard olive pomace extractions by high performance liquid chromatography (HPLC), which represents distinct advantages for further characterization. For this first instalment, our partial list of compounds, includes hydroxytyrosol and oleuropein, which have extensively been shown to have wide-ranging beneficial health effects, and oleocanthal which has been shown to be a potent inhibitor of cyclooxygenase enzymes (Cox-1 and 2) and therefore, associated with pain relief [19, 20]. Our investigations predominantly involve in silico evaluation of the potential of Olea Europaea-derived compounds to bind in the active domains of target chromatin-modifying enzymes including HATs, HDACs and histone demethylases. The epigenetic targets were chosen on the basis of the lists compiled by commercial provider Reaction Biology Corp. An important advantage of this strategy is the public availability of known IC\(_{50}\) values or % inhibition values for positive controls for each of the target proteins, which can inform our molecular modelling in an iterative process. With a focus on hydroxytyrosol, our work includes experimental validation of an epigenetic target and an analysis of modulation of HDAC6 in the context of a diabetic wound case study.

Materials and Methods

In silico molecular docking
Protein and ligand structure preparation

Chemical structures for olive bioactive compounds were obtained from the NCBI PubChem Compound database [21] as sdf. files, and then converted to pdb. format with OpenBabel [22], as listed in Table 1. The experimental coordinates of the epigenetic structures were obtained from the Protein Data Bank (PDB) [23], where available, as listed in Table 2. All PDB crystal structures used had a resolution of less than 3.00Å, and all water molecules were removed, as well as any exogenous ligands prior to docking. The first protein chain (chain A) was used for subsequent docking calculations, and where present Zn atoms were retained in the structures. A comparative (homology) model of several HDACs and HATs (where their respective x-ray crystallographic structure was not available) were constructed using the SWISS-MODEL ExPASy server, where the human FASTA sequence for each protein was obtained from the UniProt database [24] (ID listed in Table 2). Three models were generated by the server, and the top ranked GMQE model was selected for each epigenetic target.

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<td>SIRT5</td>
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Protein-ligand docking calculations
The molecular docking program AutoDock Vina (The Scripps Research Institute, California, USA) was used to generate energetically favourable binding sites for the phenolic ligands onto the chosen epigenetic targets. AutoDock Vina combines the use of knowledge-based and empirical scoring functions, where the program draws on experimental affinity measurements and conformational predictions of the receptor-ligand complex. The binding pose of the ligand is then ranked based on an energy scoring function [25]. Epigenetic targets and ligand pdb. files were processed using PyRx (The Scripps Research Institute, California, USA) to create their corresponding pdbqt. files for use with AutoDock Vina. Rotatable torsions of the ligands were activated and all epigenetic protein structures were assumed rigid. The docking site of the ligand was defined by establishing a cube at the geometrical centre of the protein active or binding site with the dimensions for each macromolecule listed in Table 2. The best binding pose was saved, and its corresponding binding affinity value for each complex. The binding poses were then visualised and analysed using VMD version 1.9.2 [26].

Demethylase inhibition assay
The inhibitory activity of hydroxytyrosol against lysine specific demethylase 1 (LSD1) was estimated by the commercial provider, Reaction Biology Corp (RBC) (Pennsylvania, USA). Briefly, hydroxytyrosol was tested in ten doses with a three-fold serial dilution starting at 100μM. Similarly, the positive control compound, tranylcypromine was tested in ten doses
with a three-fold serial dilution starting at 100µM. A fluorescence coupling enzyme assay based on the production of FAD-dependent H₂O₂ as a result of demethylase activity of LSD1, using 10µM histone H3(1-21) K4me2 peptide as a substrate, was measured by coupling with horse radish peroxidase and Amplex Red. The IC₅₀ values for each compound were then calculated.

Immunofluorescence staining of epidermal tissue sections

Tissue collection and processing
Six tissue samples were obtained through therapeutic debridement procedures over several weeks, at Ashwood Medical Group, Melbourne, Australia with informed consent. The samples were collected from a 79 year old male patient whom suffered from a chronic diabetic venous ulcer of approximately 10cm², located on the right inferolateral lower leg. The patient was treated with a commercial topical antimicrobial spray, containing the Olivamine 10° formulation, which includes a hydroxytyrosol containing olive extract. Sections were paraffin embedded by Gribbles Pathology Veterinary, and subsequently, sections of 4µm were prepared at a clearance angle of 5° using the Leica Microsystems microtome.

Immunofluorescence
Expression of HDAC6 and acetylated α-tubulin were examined in the skin samples using anti-HDAC6 (1:500) (Epitomics, California, U.S.A) and anti-acetylated α-tubulin (1:250) antibodies. Samples were deparaffinised in xylene and hydrated in three washes of graded ethanol. Slides were subsequently microwaved on high power for 1 minute and low power for 10 minutes before left to cool at room temperature for 20 minutes. Slides were then equilibrated with two washes in 0.1% Tween 20 with phosphate buffered saline (PBS) for 15 minutes on an orbital mixer at room temperature. Tissues on the slide were circled with a mini pap pen (Invitrogen, California, U.S.A) and were blocked with 100µL of 1% bovine serum albumin (BSA (v/v)) for 1 hour and blotted. Subsequently, 100µL of primary antibody in 1% BSA was added and slides were incubated overnight in a 4°C humidified chamber. Slides were then washed 3 times in 0.1% Tween 20 PBS for 10 minutes at room temperature on an orbital mixer. Secondary antibody (100-300µL) anti-mouse 488 (1:500) and anti-rabbit 546 (1:500) in 1% BSA was added for a 1 hour incubation in a dark humid chamber on an orbital mixer at room temperature. TOPRO-3 (100-300µL; 1:1000) in PBS was the added for a further 10 minutes. Slides were then washed twice for 10 minutes in PBS on an orbital mixer. Excess PBS was blotted off the slides and samples were cover slipped with Permount before left to dry in a dark humid chamber for three days. Skin sections were examined and images were obtained using the A1r Nikon confocal microscope from Monash Micro Imaging set at x60 objective with oil. A Z-stacked imaged was acquired with a step size of 0.25µm.

Results

In silico docking studies and in vitro inhibition of LSD1 by hydroxytyrosol
Molecular docking studies were performed on the 14 bioactive compounds to obtain insight into their binding pose onto the epigenetic target enzymes. In turn, the calculated free energy of binding (ΔG) for each complex was also predicted. The binding affinities obtained for the bioactive compounds to each epigenetic target is presented in Figure 2. The values indicate the binding energy upon docking of the ligand to the known active site or binding site of the protein, as stipulated in the literature. The results demonstrate clear trends within the protein groups, and among the global dataset. Firstly, there appears to be an increase in binding affinity following docking with the higher weighted compounds, especially from apigenin through to oleuropein which all possess more than one phenyl ring. Secondly, there appears to showcase a more homogenous spread of binding affinities, with some interesting higher docking affinities seen with the lower weight compounds hydroxytyrosol and cinnamic acid. Upon close inspection of the docked ligands to the above epigenetic targets, it was noticed that histidine residues were directly involved in ligand interactions among HDAC 4, 6, 7 and 11 as well as within LSD1 (Figure 3).

The findings from the LSD1 demethylase profiling indicated inhibition of the enzyme by hydroxytyrosol with an IC₅₀ of 3.57µM demonstrating more potent activity than the positive control and well-known LSD1 inhibitor, tranylcypromine (IC₅₀ =17.7µM).

Immunofluorescence expression of HDAC6 in a chronic diabetic ulcer
The level of HDAC6 and acetylated α-tubulin expression was characterised in tissue sections obtained from the peri wound of a diabetic ulcer treated with a commercial topical product, containing the Olivamine 10° formulation, which includes a hydroxytyrosol containing olive extract. The findings illustrate the time-dependent increase in expression of HDAC6 within the tissue sections examined, without a concomitant change in expression of acetylated α-tubulin from week 1 to week 3, Figure 5.
**Figure 2.** Heat map depicting free energy of binding (ΔG) for each complex. Red corresponds to the weakest binding affinity whilst green indicates strongest binding values. The selected bioactive compounds are in ascending order, based on molecular weight.
Figure 3. 3D structure of epigenetic targets complexed with hydroxytyrosol (HT), cinnamic acid (CA) or oleocanthal (OL). Depicts the binding mode of the highest bound affinity for each compound, and shows residues directly involved in the interaction, within 3.5Å of the ligand.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (μM)</th>
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<tbody>
<tr>
<td>Hydroxytyrosol</td>
<td>3.57</td>
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<tr>
<td>Tranylcypromine</td>
<td>17.7</td>
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Figure 4. LSD1 demethylase activity and binding of hydroxytyrosol and the control compound, tranylcypromine. (A) IC$_{50}$ values for the two compounds, as produced by RBC. (B) 3D structure LSD1 (PDB ID: 2DW4) complexed with hydroxytyrosol (orange) and tranylcypromine (silver). Depicts the binding mode of the highest bound affinity for each compound, and shows residues directly involved in the interaction, within 3.5Å of the ligand.

Discussion

Using molecular docking, we propose models of the interactions between chosen epigenetic targets and bioactive olive ligands within the known active sites of the proteins. Firstly, our results generally indicate an increase in binding affinity in correlation with a higher ligand molecular weight. Similar patterns regarding molecular weight have emerged in a range of docking studies, due to limitations of unique algorithms in various programs [28-30]. Nevertheless, in general and in our case, false positives represent a better outcome than missing potential interactions. Overall, our studies identify specific interactions of the ligand and surrounding residues providing important insights into the potential unique ligand binding positions. Inspection of the binding modes obtained from AutoDock Vina, it was found that the various bioactive ligands had effectively bound to the known active sites of the proteins, and specific residue interactions of the docked compounds were shown to be essential in effective binding (Figures 2-4). The active site containing the histidine residues directly involved in proton transfer of HDACs [31] is found to interact with the bioactive compounds HT, CA and OL among the HDACs 4, 6, 7, 11 and SIRT1. This indicates that the observed binding modes of these complexes, is probably an accurate representation of the ligands as inhibitors, and may be the cause for the relatively high ΔG values observed.

Given the relatively high binding affinity of hydroxytyrosol to LSD1 in comparison to the other single phenyl-group compounds, further analysis of the binding mode of hydroxytyrosol and a known inhibitor was conducted for LSD1. Briefly, the discovery of LSD1 led to the identification of the first histone demethylase that specifically removes methyl groups from lysine 4 of histone H3 [32]. In vivo, LSD1 is a key histone modifier that maintains gene expression in human...
Figure 5. Level of expression of HDAC6 and acetylated α-tubulin, as detected by immunofluorescence staining. Bar=200µm, 60x magnification.

It has also been shown to be overexpressed in many cancer types [34], and its inhibition has been shown to mitigate cellular proliferation and invasion of neoplastic cells. As a histone demethylase, the catalytic centre of LSD1 is formed of both FAD-binding and substrate binding subdomains [35]. From our findings, assessment of the binding mode generated by AutoDock Vina demonstrated direct binding of hydroxytyrosol into the catalytic core of the enzyme, interacting with the residues of the substrate binding subdomain (Figure 4). The binding of LSD1 and hydroxytyrosol suggests the possibility of π-π interactions between Tyr807, Phe560 and hydroxytyrosol (see Figure 4), as well as cation-π interactions between His812 and hydroxytyrosol, which may serve to stabilise bonds. In conjunction, the binding of the putative LSD1 inhibitor, tranylcypromine, was found to have a different binding position to hydroxytyrosol with fewer interactions with surrounding residues (see Figure 4). X-ray crystallography coordinates of LSD1 bound to tranylcypromine indicate the two residues intimately bound to the inhibitor found in our results, are likewise found in the confirmatory X-ray structure [36]. This demonstrates the binding mode predicted from our findings, may be a likely binding site of the inhibitor. Interestingly, the commercial demethylase assay found the predicted IC50 for LSD1 inhibition by hydroxytyrosol to be lower than the positive control tranylcypromine (see Figure 4). However, although in the positive range, hydroxytyrosol was not predicted to be the most potent LSD1 inhibitor calculated by our molecular docking studies compared with several other compounds of higher molecular weight and those ligands containing multiple phenyl rings. This highlights that predicting protein-ligand interactions is inherently challenging owing to the many forces that contribute to these interactions [37].

Access to a specialised wound clinic provided us the opportunity to obtain human tissue samples that allow for the elucidation of the expressional changes of epigenetic targets in vivo. In an heterogeneous group of patients, the presentation of a diabetic patient with an associated ulcer is commonplace, and suitable samples were obtained from one such patient treated at the clinic. The adverse effects of diabetes is orchestrated by hyperglycaemia and the resultant oxidative stress on endothelial health increases the risk of serious long-term complications, including the formation of...
diabetic leg and foot ulcers [38]. Several studies have shown that epigenetic changes play a role in diabetic complications [39-41]. Specifically, underlying differences in the cellular phenotype of fibroblasts obtained from diabetic ulcers has been shown to be influenced by significantly lower global DNA methylation patterns as compared to fibroblasts from non-diabetic wounds [42]. Activation and persistence of the proinflammatory M1 macrophage in diabetic wound tissue has also been indicated to be a result of altered H3K27 demethylase (KDM6B) activity [43].

Following the relatively high binding affinity obtained from hydroxytyrosol bound to HDAC6, we further examined the change in HDAC6 and acetylated α-tubulin expression from tissue obtained from a diabetic wound, treated with a commercial topical antimicrobial, containing the Olivamine 10® formulation, which includes a hydroxytyrosol-containing olive extract (Figure 5). The results showed an increase in expression of HDAC6 without any quantitative change in the acetylated α-tubulin levels. HDAC6 is an α-tubulin deacetylase and its over-expression has been demonstrated to promote chemotactic cell movement [44]. This indicates that although the expressional increase in HDAC6 is apparent over the time course, the analogous α-tubulin deacetylase activity of HDAC6 is not pronounced. Little work has been conducted on the effect of HDAC6 expression on wound healing, however a study on corneal epithelial cells, has shown the enhancement of HDAC6 activity results in the deacetylation of α-tubulin, effectively increasing cell migration [45, 46]. Indeed, given the dynamic and transient nature of protein acetylation, our findings require further clarifications with much greater n numbers and time points. Further, whether the expressional increase in HDAC6 within the tissue was due to hydroxytyrosol specifically or the synergistic effect of the Olivamine 10® formulation must be further explored.

Our in silico findings indicate that the predicted IC₅₀ for LSD1 inhibition by hydroxytyrosol is lower than the positive control tranicypromine. However, it should be noted that although in the positive range, hydroxytyrosol was not predicted to be the most potent LSD1 inhibitor on the basis of molecular docking compared with various compounds of higher molecular weight. This highlights the need to refine our in silico methodologies and to utilise algorithms designed to factor for differences in molecular weight. Given the above observations however, on the basis of the molecular docking prediction, we may assume a high inhibition activity of hydroxytyrosol against HDAC6, which, nevertheless must be validated both in vitro and in vivo. These observations are interesting in the context of our case study results which indicated a significant increase in the expression of HDAC6 with time without a concomitant change in the expression of acetylated α-tubulin. Taken together, reasonable hypotheses may be that 1) the increased expression of the enzyme in the wounded skin could be a compensatory response to inhibition by the olive-derived, hydroxytyrosol containing components of the formulation and 2) the potent inhibition of HDAC6 by components of the formulation supersedes the increased expression of the HDAC6 enzyme and hence the lack of significant changes in the acetylation of α-tubulin.

Conclusion

Overall, we provide the first instalment for the identification of potential chromatin modifying compounds derived from Olea Europaea. Our combined in silico and in vitro observations highlight various compounds that may regulate the function of enzymes that write (HATs) and erase (HDACs and HDM) chemical modifications on chromatin. A pertinent example arising from the current work is the finding that the extensively investigated phenolic compound, hydroxytyrosol, may have activity as an inhibitor of HDAC6 and LSD1. When considering our in silico findings, we observe a trend indicating that molecular weight is an important determinant of a positive result in terms of binding the target domain of the enzymes. This is a known caveat in the field and future work will aim at eliminating this bias. Our approach will involve both using alternative docking software with varying algorithms and if required developing our own algorithms to further consolidate the in silico and in vitro findings in an iterative process. Furthermore, we aim to extend our work to incorporate further chromatin modifying enzymes (for example, histone methyltransferases) and enzymes involved in DNA methylation. Finally, we plan to extend our database of compounds derived from Olea Europaea for exploration using our methodologies.

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Conflicts of interest

Epigenomic Medicine at Baker IDI Heart and Diabetes Institute and N.B.R are funded by McCord Research.

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