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Virtual screening for novel inhibitors of human Histone Deacetylase 6: Promising new leads for Oral Squamous Cell Carcinoma

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ABSTRACT

Over 90% of Oral Cancers are Oral Squamous Cell Carcinomas (OSCCs). Despite having advanced treatment modalities, there is no significant improvement in the survival rate of Oral Cancer patients over the years. Thus, there arises a need for the identification of new drug targets besides development of new and effective drugs for this disease. Since Histone Deacetylase 6 (HDAC6), a class IIB member of HDAC family, is known to be upregulated in this disease in addition to being associated with tumor growth, it can be considered as a promising drug target for this disease. In this study, a structure-based virtual screening strategy was used to screen a library of 1,539 natural compounds from Naturally Occurring Plant-based Anti-cancerous Compound–Activity–Target (NPACT) database. Upon filtering and docking, top 30 hits were identified and two of them, namely Camptothecin and Diosgenin, were tested experimentally on OSCCs cell lines for anti-proliferative effects. Both of these compounds exhibited inhibitory activity against Oral Cancer cells, therefore suggesting that they can be potential HDAC6 inhibitors, which can further serve as promising leads for OSCCs.

1. INTRODUCTION

Combinatorial chemistry and high-throughput screening are time-consuming and expensive methods for the synthesis of new compounds. The most suitable alternative is screening small molecule databases for novel compounds. Virtual screening involves computationally screening of huge libraries of chemicals for compounds that target counterparts of known structure, and experimentally test those that bind well. Virtual screening, or *in silico* screening, forms a new approach garnering high interest in the pharmaceutical industry as an easy, productive and cost-effective technology in pursuit of novel lead compounds for a specific target which is of utmost importance to the initial phase of drug discovery [1,2].

Structure-based virtual screening is based on the binding mode prediction along with the analysis of binding affinities of each compound by protein–ligand docking. This approach becomes tedious when the data set is huge. So, an alternative approach is to filter out unpromising compounds before docking. In this way, the data set can be restricted to drug-like compounds only. This filtering is based on appropriate property and sub-structural features. This method is quite effective in decreasing the data set used for docking to the order of 10^3–10^4 compounds from a huge library of compounds [3,4].

Oral Cancer is a sub-division of head-and-neck squamous cell carcinoma [5]. Oral Cancer contributes to more than 30% of all cancers, thus being one of the top three cancers in the Indian subcontinent. The annual incidence of Oral Cancer is ~300,000 cases, of which 62% are observed in developing nations. As many as 145,000 deaths occur globally and 50,000 deaths occur in India annually [6]. Majority of Oral Cancers are Oral Squamous Cell Carcinomas (OSCCs). It is the malignancy of the oral cavity [7]. Risk factors include chronic tobacco usage, excessive alcohol consumption, chronic inflammations, infection by human papilloma virus, betel quid chewing, and genetic predisposition [8]. OSCC mainly includes cancer of the tongue, lip, palate, gingiva, buccal mucosa, and floor of the mouth. OSCC is diagnosed often during the later stage of the disease as patients fail to seek medical help at the right time, either for the reason that they fail to realize

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the significance of early symptoms, or they might show ignorance toward the health implications [9,10].

Early detection of Oral Cancer could be one of the most effective ways to decrease the high mortality rate of this ailment [11]. Over the years, although Oral Cancer treatment modalities have advanced, the survival rate of Oral Cancer patients is yet to improve significantly [12,13]. The overall survival rate declines as the cancer stage rises from 75% to 90% for Stage I in contrast to 10%–22% for Stage IV [14]. This marks the necessity for the development of new and potential drugs in addition to finding new drug targets for this disease.

Over 3,000 plants globally are known to possess anti-cancer properties. The incidence of the use of plant-derived products for the treatment of cancer ranges from 10% to 40%, reaching 50% among Asiatic patients [15]. In traditional medicine, terpenoids, flavonoids, tannins, alkaloids, phenols, and quinones have been used to treat various infections and diseases as they are rich in secondary metabolites [16–18].

Histone deacetylases (HDACs) forms one of the promising classes of anti-cancer drug targets as they are capable of reversing abnormal epigenetic states related to cancer. Cell-cycle arrest, apoptosis, and differentiation are some of the cell type-specific effects that they elicit [19]. HDAC6, a member of the HDAC family, is known to be upregulated in OSCC and it increases during the advanced stages of the cancer. Overexpression of HDAC6 is associated with tumor growth. Thus, selective inhibition of HDAC6 could be a promising method for the treatment of oral cancer and has been, therefore, considered for this study.

In this study, virtual screening along with docking was employed to screen a library of 1,539 plant-based natural compounds. Many hits were obtained, of which several were novel inhibitors of HDAC6 and potential leads for oral cancer. Furthermore, two of the procurable hits were tested for its inhibition activity *in vitro* by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay to confirm its anti-cancer effects on the Squamous Carcinoma Cell line (SCC-9).

2. MATERIALS AND METHODS

2.1. Homology Modeling

Since the X-ray structure of a complete sequence of 1,215 residues for human HDAC6 was not available during the time of study in 2016, a 3D structure of the protein was predicted via homology modeling from its primary sequence. The amino acid sequence of human HDAC6, with accession no. AAH69243.1, was retrieved in FASTA file format from the NCBI protein database [20]. This was submitted to the SWISS-MODEL server [21]. From the predicted result, the top model with a better QMEAN (Qualitative Model Energy ANalysis) was selected and was further validated by Ramachandran plot and Verify-3D [22].

2.2. Virtual Screening

Identification of potential lead compounds was achieved by carrying out virtual screening of phytochemicals from the Naturally Occurring Plant-based Anti-cancerous Compound–Activity–Target (NPACT) database [23]. A total of 1,539 anti-cancer plant-based natural compounds belonging to categories of terpenoids, flavonoids, alkaloids, polyketides, lignans, polycyclic aromatic natural compounds, steroids, simple aromatic natural compounds, saponins, carbohydrates, organic chemicals, oxygen heterocycles, benzopyranoids, benzofuranoids, aliphatic natural compounds, amino acids, peptides, polypyrroles, and tannins were collected. Compounds were screened to fit using Lipinski guidelines for drug-likeness; partition coefficient logP was ≤ 5, hydrogen bond donors were ≤ 5, hydrogen bond acceptors were ≤ 10, besides molecular weight that was ≤ 500 [24]. Finally, 676 compounds were subjected to docking.

2.3. Molecular Docking

Molecular docking is a target-based drug design method [25]. It was carried out using the AutoDock Vina [26] module available in PyRx 0.8 software [27]. Both the receptor and ligands were prepared and saved in .pdbqt format. During the docking process, the receptor was considered rigid and the ligand flexible. Docking grid size was enlarged to accommodate the entire protein inside the grid box with dimensions of 53, 65, and 49A° (*X*, *Y*, and *Z*). Furthermore, Lamarckian genetic algorithm was used for searching best possible conformers. A maximum of 10 conformers were generated for each compound during the docking process.

2.4. Short Listing of Potential Leads

After docking, 10 conformers were produced for each compound. On the basis of the least binding affinity, the best pose was selected. These docked structures were visualized in PyMOL software (the PyMOL Molecular Graphics System, Version 1.8, Schrödinger, Inc) for studying the residue–ligand interactions [28]. The highest scoring compounds were determined from the docking result and a focused library was formed, which were considered for testing on cell lines, out of which Camptothecin and Diosgenin were chosen as they were procurable. The binding energy of all the compounds was compared with a reference drug molecule Vorinostat (IUPAC: N′-hydroxy-N-phenyloctanediamide), which is a Food and Drug Administration approved HDAC inhibitor.

2.5. Chemicals and Reagents for Experimental Validation

MTT powder (the solution was filtered through a 0.2 µm filter. It was then stored at 2°C–8°C), dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), –125 mM NaCl in 10 mM sodium phosphate buffer, CO_2 incubator, Tecan Plate reader were used.

2.6. Cell Lines and Culture Medium

The OSCC cell line derived from human tongue (SCC-9) was obtained from American Type Culture Collection for the study. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, and 0.05% glucose in PBS). Cell viability was checked. Finally,

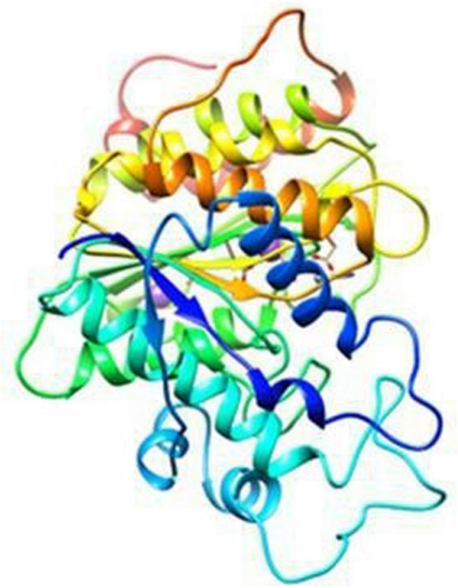

Figure 1. Homology modeled 3D structure of human HDAC6.

50,000 cells/well of SCC-9 were seeded in a 96-well plate and incubated for 24 hours at 37°C, 5% CO_2 incubator.

2.7. MTT Assay

5.0×10^5 cells of SCC-9 were seeded in 96-well plates with DMEM. Camptothecin was tested at different concentrations of 0, 15, 31, 62, 125, 250, 500, and 100 µg/ml in DMEM media. This was followed by incubation for 24 hours in a CO_2 incubator at 37°C. After incubation, the media was removed from the wells and 100 µl of the MTT reagent was added in each well and incubated again for 4 hours. Then, MTT reagent was removed and 100 µl DMSO was added to each well and gently shaken. Furthermore, absorbance or optical density (OD) was measured at 590 nm using a SpectraFluor Tecan plate reader and Camptothecin-treated cells were compared to untreated cells [29,30].

2.8. Statistical Analysis

The percentage growth inhibition was computed using the following formula:

$$\% \text{ Inhibition} = \text{OD of Control} - \text{OD of Sample}/\text{OD of Control}) \times 100$$

The concentration of Camptothecin needed to inhibit cell growth by 50% (IC_{50}) values is generated from the dose–response curve. IC_{50} values were derived from a nonlinear regression analysis based on sigmoid dose–response curve and visualized using GraphPad Prism version 5.0 software.

3. RESULTS AND DISCUSSION

3.1. Homology Modeling

The structure of human HDAC6 was predicted using homology modeling by submitting the FASTA sequence of the protein with accession no. AAH69243.1 to the SWISS-MODEL server. From

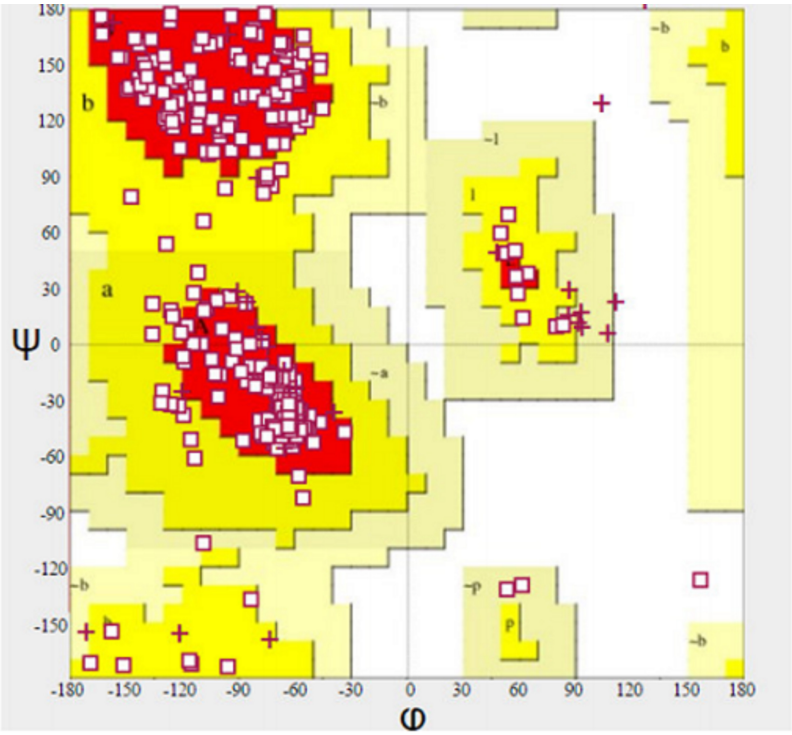

Figure 2. Ramachandran plot of homology modeled protein.

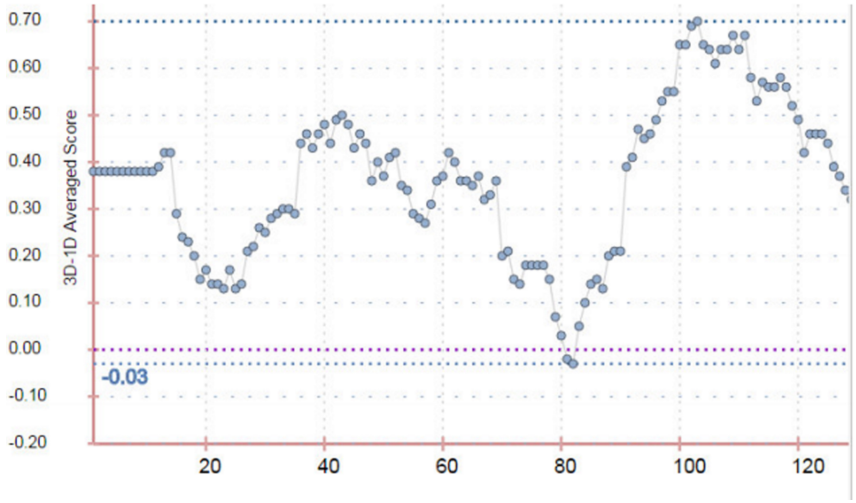

Figure 3. Verify-3D result of homology modeled protein.

the results, the top model was selected (template: 2vqw.1.A; sequence identity: 47.35%) and it was further validated by Ramachandran plot and Verify-3D. Figure 1 shows the homology modeled structure of human HDAC6 as visualized in Chimera version 1.11.1 [31].

In the Ramachandran plot (Figure 2), 93.6% of the residues were in the favored region, 5.6% were in allowed region, and 0.8% was in outlier region, thus showing that it is a reliable structure. From Verify-3D result (Figure 3), it was known that 88.67% of the residue had an average 3D-1D score of 0.2, which again showed that the structure is good. A minimum of 80% of the amino acids must have a score of 0.2 in the 3D/1D profile of Verify-3D, which in this case was satisfied.

3.2. Virtual Screening and Docking

Out of 705 phytochemicals that satisfied Lipinski rule of 5, 29 of them were excluded as they were duplicate entries. Finally, docking was carried out for the remaining 676 compounds. Their structures were downloaded in 3D structure data file (SDF format) from PubChem database [32] and were subjected to docking using AutoDock Vina in PyRx 0.8 in order to find optimal conformation of ligands and to understand the nature of interactions between them. Both receptor and ligand files were prepared in accordance with the format required by PyRx 0.8. Based upon the least binding affinity, the best pose was selected and the docked structures were visualized in PyMOL for detailed receptor–ligand interactions. The binding energy of all the docked complexes is given in Table 1.

Figure 4 shows the protein–ligand complex between Camptothecin and homology modeled protein.

From virtual screening results, it was observed that Subtrifloralactone A, Subtrifloralactone B, Subtrifloralactone E, Diosgenin, Inophyllum E, Alpha-Naphthoflavone, Taiwanin C, Subtrifloralactone F, Philadelphicalactone A, Subtrifloralactone C, Subtrifloralactone D, Remangilones C, Zhankuic acid C, Limonin, Tomatidenol, Withaphysacarpin, Subtrifloralactone G, 4-beta ,7-beta, 20R-trihydroxy-1-oxowitha-2,5-dien-22,26-olide, Diosmin, Silymarin, Sanguinarine, Taiwanin E, Cycloartobiloxanthone, Remangilones A, Tubulosine, Galbacin, Farnesiferol C, Withaferin A, and Camptothecin showed better binding energy (greater than –6.0 kcal/mol of the reference compound), thus signifying that these compounds can be potential HDAC6 inhibitors as well as potential drug candidates for OSCC.

3.3. Cell Line Study

To verify the possible anti-cancer effect of Camptothecin on oral squamous carcinoma cells, the sample was checked for its capability to inhibit cell growth on SCC-9 cancer cell lines with MTT assay at different concentrations of 0, 15, 31, 62, 125, 250, 500, and 100 µg/ml. Proliferation of these cells was significantly inhibited in a concentration-dependent manner for 24 hours, as shown in Figure 5. Camptothecin showed dose-dependent inhibition (60%) of the growth of SCC-9 cells at 1 µM and IC_{50} value of 179 nM was obtained (Table 2).

Various studies have reported that Diosgenin inhibits cell proliferation and induces apoptosis in various human tumor cells such as prostate, breast, liver, colon, leukemia, and osteosarcoma [33,34]. Although anti-cancer properties and pro-apoptotic effect of Diosgenin are reported in various studies, its effect on squamous cell carcinomas alone is not yet fully studied [35]. In this study, Diosgenin, exhibited 37% inhibition at 400 µM concentration on the SCC-9 cell line (Table 3).

Therefore, this study demonstrated the anti-cancer activity of Camptothecin and Diosgenin against SCC-9 cell lines, thus validating its inhibitory activity experimentally and indicating that it can be a potential inhibitor for Oral Cancer. In addition, remaining hits can be further examined for their anti-proliferative effects on Oral Cancer cells.

Table 1. Top hits resulted from docking.

Sl No.	Compound	PubChem ID	Binding energy (kcal/mol)
1	Subtrifloralactone A	10928536	–8.9
2	Subtrifloralactone B	10906541	–8.9
3	Subtrifloralactone E	21600009	–8.6
4	Diosgenin	99474	–8.5
5	Inophyllum E	455251	–8.3
6	Alpha-Naphthoflavone	11790	–8.2
7	Taiwanin C	363127	–8.2
8	Subtrifloralactone F	21600010	–8.2
9	Philadelphicalactone A	11038269	–8.1
10	Subtrifloralactone C	11027076	–8
11	Subtrifloralactone D	21600008	–8
12	Remangilones C	397856	–7.9
13	Zhankuic acid C	10838646	–7.9
14	Limonin	179651	–7.9
15	Tomatidenol	6453043	–7.9
16	Withaphysacarpin	44567005	–7.9
17	Subtrifloralactone G	21600011	–7.9
18	4-beta, 7-beta, 20R-trihydroxy-1-oxowitha-2,5-dien-22,26-olide	10950818	–7.9
19	Diosmin	5281613	–7.8
20	Silymarin	1548994	–7.8
21	Sanguinarine	5154	–7.8
22	Taiwanin E	493164	–7.8
23	Cycloartobiloxanthone	10342859	–7.8
24	Remangilones A	10503181	–7.7
25	Tubulosine	72341	–7.7
26	Galbacin	234441	–7.7
27	Farnesiferol C	11090246	–7.7
28	Withaferin A	265237	–7.6
29	Camptothecin	24360	–7.5
30	Vorinostat[a]	6918638	–6.0

Reference compound is indicated by '[a]'.

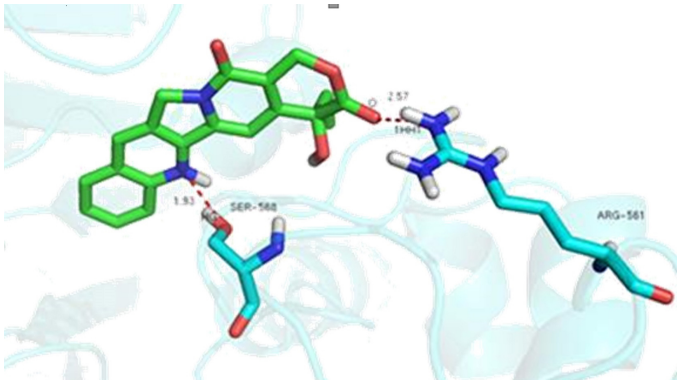

Figure 4. Docked complex of Camptothecin with homology modeled protein.

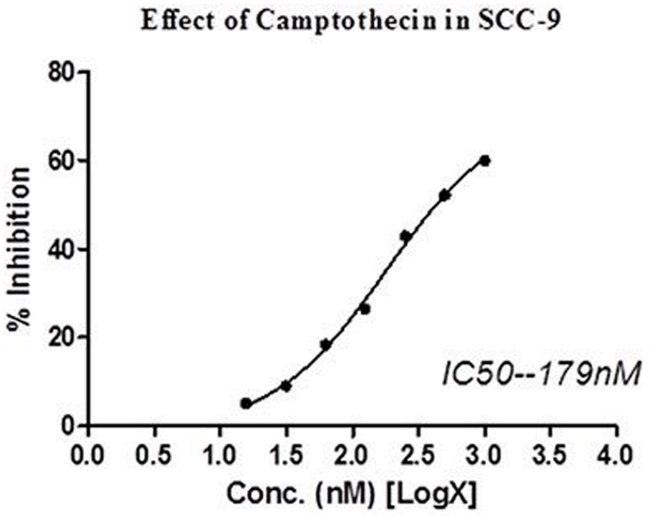

Figure 5. Dose-response curve.

Table 2. Camptothecin result on SCC-9 cell line.

Sample name	Conc. (nM)	OD at 590 nm	% Inhibition
Camptothecin	Control	0.529	0.00
	15.6	0.502	5.12
	31.3	0.481	9.09
	62.5	0.432	18.40
	125.0	0.389	26.52
	250.0	0.302	42.95
	500.0	0.253	52.21
	1000.0	0.212	59.95

Table 3. Diosgenin result on SCC-9 cell line.

Sample name	Conc. µM	OD at 590 nm	% Inhibition
Diosgenin	Control	0.529	0.00
	6.25	0.514	2.91
	12.5	0.504	4.74
	25	0.475	10.20
	50	0.464	12.35
	100	0.457	13.68
	200	0.356	32.75
	400	0.334	36.91

4. CONCLUSION

From virtual screening results, it was observed that Subtrifloralactone A, Subtrifloralactone B, Subtrifloralactone E, Diosgenin, Inophyllum E, Alpha-Naphthoflavone, Taiwanin C, Subtrifloralactone F, Philadelphicalactone A, Subtrifloralactone C, Subtrifloralactone D, Remangilones C, Zhankuic acid C, Limonin, Tomatidenol, Withaphysacarpin, Subtrifloralactone G, 4-beta ,7-

beta ,20R-trihydroxy-1-oxowitha-2,5-dien-22,26-olide, Diosmin, Silymarin, Sanguinarine, Taiwanin E, Cycloartobiloxanthone, Remangilones A, Tubulosine, Galbacin, Farnesiferol C, Withaferin A, and Camptothecin showed better binding energy (greater than –6.0 kcal/mol of the reference compound), thus signifying that these compounds can be potential HDAC6 inhibitors as well as potential drug candidates for OSCC. Also, hits namely Camptothecin and Diosgenin showed inhibitory activity against SCC-9 cell lines, thus indicating that it could be a potential drug candidate for Oral Cancer.

5. ACKNOWLEDGMENTS

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6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

7. FUNDING

There is no funding to report.

8. CONFLICT OF INTEREST

No conflicts of interest.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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