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 and is the most common form of cancer of head and neck. The incidence and morbidity rate of Oral Cancer is increasing worldwide. Although treatment modalities have advanced, the survival rate of oral cancer patients has not improved significantly over the years. Thus, there arises a need for identification of new drug targets besides development of new and effective drugs for this disease. 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. Thus, HDAC6 could be a promising drug target for this disease. In this study, structure-based virtual screening was used to screen a library of 1539 natural compounds from NPACT (Naturally Occurring Plant based Anti-cancerous Compound-Activity-Target Database). Upon filtering and docking, top 30 hits were identified and two of them namely Camptothecin and Diosgenin were tested experimentally on oral squamous cell carcinoma cell lines for anti-proliferative effect. It was found that both these compounds exhibited inhibitory activity against the cancerous cells, thus validating our findings. Moreover, from virtual screening results, it was observed that top 29 hits namely Subtrifloralactone A, Diosgenin, Inophyllum E, Taiwanin E, Camptothecin and many others showed better binding energy than the standard HDAC inhibitor (Vorinostat), thus signifying that these compounds can be potential inhibitors of HDAC6 thus serving as promising leads for Oral Squamous Cell Carcinoma.

Keywords: Oral squamous cell carcinoma, Histone deacetylase inhibitors, Virtual screening, Molecular Docking, Cell line.

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Introduction

Traditional synthesis of new compounds employing combinatorial chemistry as well as high-throughput screening is a time-consuming and expensive process. However, on the other hand, screening small molecule databases for novel compounds represents an alternative process. Virtual screening is an alternative approach to computationally screen huge libraries of chemicals for compounds that counterpart targets of known structure, and experimentally test those that bind well [1]. Virtual screening, or Insilico 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 initial phase of drug discovery.

Oral cancer is a sub division of Head-and-Neck Squamous Cell Carcinoma (HNSCC). Oral Cancer contributes for more than 30% of all cancers, thus being one of the top three cancers in Indian Subcontinent. The annual incidence of Oral cancer is 3,00,000 cases, of which 62% are observed in the developing nations. As many as 145,000 deaths occur globally and 50,000 deaths in India annually. Majority of oral cancers are Oral Squamous Cell Carcinomas (OSCCs). It is the malignancy of the oral cavity. Risk factors include: chronic tobacco usage, excessive alcohol consumption, chronic inflammations, infection by human papilloma virus, betel quid chewing and genetic predisposition [2]. OSCC includes cancer of the tongue, lip, gingiva, buccal mucosa, floor of the mouth, palate, and the retromolar trigone. 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 the significance of early symptoms, or they might show ignorance towards the health implications.

Early detection of oral cancer could be one of the most efficient ways to decrease the high mortality rate of this disease. Over the years, although oral cancer treatment modalities have advanced, the survival rate of oral cancer patients is yet to improve significantly. The overall survival rate decreases as the carcinoma stage rises from 75 to 90% for Stage I to 10–22% for Stage IV. This marks the need for development of new and potential drugs in addition to finding new drug targets for this disease.

Over 3000 plants worldwide have been reported to possess anti-cancer properties. Globally, the incidence of the use of plant-derived products for cancer treatment is from 10% to 40%; reaching 50% in Asiatic patients. In traditional medicine, plants that are rich in a wide variety of secondary metabolites, such as terpenoids, flavonoids, tannins, alkaloids, phenols and quinones have been used to treat various infections and diseases.

Histone deacetylases (HDACs) are a promising class of anti-cancer drug targets which 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. HDAC6, a member of HDAC family, was found to be upregulated in oral squamous cell carcinoma and is known to increase in advanced stages of the cancer. Over-expression of HDAC6 is associated with tumor growth. Thus, selective inhibition of HDAC6 could be a promising approach 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 1539 plant-based natural compounds. Many hits were obtained of which several were novel inhibitors of HDAC6 and potential leads for Oral Cancer. Further, two of the procurable hits were tested for its inhibition activity *in vitro* by MTT assay to confirm its anti-cancer effects on the Oral Squamous Carcinoma Cell line (SCC-9).

Materials and Methods

Homology modelling

Due to non-availability of X-ray structure of complete sequence of 1215 residues for human HDAC6 during the time of study in 2016, 3D structure of the protein was predicted *via* homology modelling from its primary sequence. The amino acid sequence of human HDAC6 with Accession No. AAH69243.1 was retrieved in FASTA format from NCBI protein database. This was submitted to Swiss Model server. From the predicted result, top model with better QMEAN value (Qualitative Model Energy ANalysis), which is a default option in Swiss Model for the estimation of the best reliable model quality, was selected and was further validated by Ramachandran plot and Verify-3D.

Virtual screening

Identification of potential lead compounds was achieved by performing virtual screening of phytochemicals from NPACT (Naturally Occurring Plant based Anti-cancerous Compound-Activity-Target Database). 1539 anti-cancer plant-based natural compounds belonging to category 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 Lipinski guidelines for drug-likeness; partition coefficient logP ≤ 5, H-bond donors ≤ 5, H-bond acceptors ≤ 10 besides molecular weight ≤ 500. Finally, 676 compounds were subjected to docking.

Molecular docking

Molecular docking is a target-based drug design method. It was performed using AutoDock Vina module available in PyRx 0.8 software. Both the receptor and ligands were prepared and saved in .pdbqt format. During docking process, receptor was considered as rigid and ligand as flexible. The docking grid size was increased to accommodate the entire protein inside the grid with dimensions 53, 65 and 49A° (X, Y and Z).

The Lamarckian Genetic Algorithm (LGA) was used for screening for best possible conformers. During molecular docking, a maximum of 10 conformers were generated for each compound to predict best conformers. The population size was set to 150 and the individuals were initialized randomly. The number of energy evaluation was set to a maximum of 2500000, maximum number of top individuals that automatically survived was set to 1, with a mutation rate of 0.02 and a crossover rate of 0.80.

Short listing of potential leads

After docking, ten conformers were produced for each compound. Based upon the least binding affinity, the best pose was selected and the docked structures was visualized in PyMol (The PyMOL Molecular Graphics System, Version 1.8, Schrödinger, LLC) for studying the residue-ligand interactions.

Highest scoring compounds were determined from the docking result and a focussed 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 (FDA) approved HDAC inhibitor.

Cell lines and culture medium

In this study, oral squamous cancer cell line derived from human tongue (SCC-9) was obtained from the American Type Culture Collection (ATCC). Using Dulbecco's Modified Eagle's Medium (DMEM) the cells were cultured and supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml).

They were incubated in a humidified atmosphere of 5% CO2 at 37°C. The cell was dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS).

The viability of the cells was checked and centrifuged. Further, 50,000 cells/well of SCC-9 was seeded in a 96 well plate and incubated for 24 hours at 37oC, 5% CO2 incubator.

Statistical analysis

The percentage growth inhibition was calculated using the following formula:

% Inhibition=(OD of Control-OD of Sample/OD of Control) × 100

The concentration of Camptothecin needed to inhibit cell growth by 50% (IC50) values is generated from the dose-response curve.

IC50 values for MTT assay were derived from a non-linear regression analysis (curve fit) based on sigmoid dose response curve (variable) and computed using GraphPad Prism version 5.0 software.

Results and Discussion

Structure of human HDAC6 was predicted using homology modelling by submitting FASTA sequence of the protein with Accession No. AAH69243.1 to SWISS-MODEL server.

From the result, top model was selected (Template: 2vqw.1.A, Sequence identity: 47.35%) and it was further validated by Ramachandran plot and Verify-3D.

In Ramachandran plot, the number of residues in the favoured region was 93.6%, number of residues in allowed region was 5.6% and number of residues in outlier region was 0.8%, showing that it is a reliable structure [3].

From Verify 3D result, it was known that, 88.67% of the residues had an averaged 3D-1D score ≥ 0.2, which again shows that the structure is good. At least 80% of the amino acids must score ≥ 0.2 in the 3D/1D profile of Verify 3D, which in this case has satisfied. (Figure 1) shows the homology modelled structure of human HDAC6 as visualized in Chimera version 1.11.1.

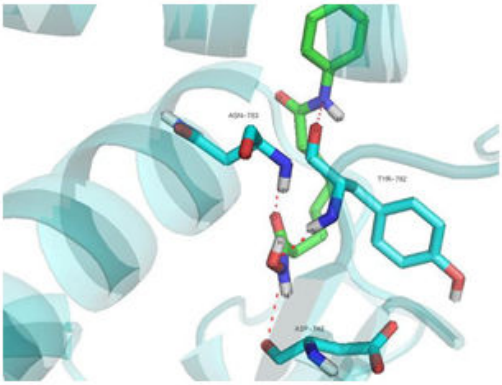

Figure 1. *Docked complex of Vorinostat (reference compound) and homology modelled protein.*

Out of 705 phytochemicals that satisfied Lipinski rule of 5, twenty-nine of them were excluded as they were duplicate entries.

Finally, docking was performed for remaining 676 compounds.

Their structures were downloaded in 3D SDF (Structure Data-file) from PubChem 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 [4]. The binding energy of all the docked complexes is given in (Table 1).

Table 1. *Camptothecin result on SCC-9 cell line.*

Sample name	Conc. (nM)	OD at 590 nm	% Inhibition
Camptothecin	Control	0.529	0
	15.6	0.502	5.12
	31.3	0.481	9.09
	62.5	0.432	18.4
	125	0.389	26.52
	250	0.302	42.95
	500	0.253	52.21
	1000	0.212	59.95

Protein-ligand complex between Camptothecin and homology modelled protein in which, SER-568 and ARG-561 were the interacting residues. The interacting residues of Vorinostat and homology modelled protein (i.e., ASN-783, ASP-742 and TYR-78). 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-beta0, 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 (> -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 Oral Squamous Cell Carcinoma (Table 2).

Table 2. *Diosgenin result on SCC-9 cell line.*

Sample name	Conc. µM	OD at 590 nm	% Inhibition
Diosgenin	Control	0.529	0
	6.25	0.514	2.91
	12.5	0.504	4.74
	25	0.475	10.2
	50	0.464	12.35
	100	0.457	13.68
	200	0.356	32.75
	400	0.334	36.91

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, 100 µg/ml [5]. Proliferation of these cells was significantly inhibited in a concentration-dependent manner for 24 hours. Camptothecin showed dose dependent inhibition

(60%) of growth of SCC-9 cells at 1 µM and IC50 value of 179 nM was obtained.

Conclusion

Various studies have reported that Diosgenin inhibits proliferation of cells and induces apoptosis in various human tumor cells such as prostate, breast, liver, colon, leukaemia and osteosarcoma. Although the pro-apoptotic and anti-cancer properties of Diosgenin are reported in various studies, its effect on Squamous Cell Carcinomas alone are not fully studied. In this study, Diosgenin, exhibited 37% inhibition at 400 µM concentration on SCC-9 cell line. Therefore, this study demonstrated the anti-cancer activity of Camptothecin and Diosgenin against SCC-9 cell lines, thus validating its inhibitory activity experimentally. In addition, remaining hits can be further examined for their anti-proliferative effects on oral cancer cells.

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