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Research Article

Drug Repurposing of Loratadine as a DNMT1 Inhibitor: Comparative Evaluation with Cisplatin in Patient-Derived Oral Squamous Cell Carcinoma Cells

Nooria Siddiqui[1], Aiman Shoaib[1], Ubaid Khan[1,,2], Muhammad Zubair[3], Shafaq Zafar[1,4,5]*

[1]Institute of Pharmaceutical Sciences, Khyber Medical University, Peshawar, Pakistan
[2]Department of Orthodontics, Sardar Begum Dental Hospital, Gandhara University. Peshawar, Pakistan
[3]Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Pakistan
[4]Department of Pharmacology, Peshawar Medical College, Peshawar, Pakistan
[5]Riphah International University, Islamabad, Pakistan.
***Correspondence:** dr.shafaqzafar@gmail.com

Abstract

Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer, with a low overall survival rate due to altered epigenetic regulation, specifically DNA hypermethylation mediated by DNA methyltransferase 1 (DNMT1). Inhibitors of DNMT1 are promising compounds for cancer treatment and research. In this study, repurposed drugs, including Loratadine, were virtually screened through molecular docking to select the drug with the highest interaction with the DNMT1 protein. Loratadine demonstrated maximum drug-like characteristics, making it a promising treatment option for OSCC patients. Furthermore, for experimental validation, we investigated the cytotoxicity of Loratadine, Cisplatin, and their combination against primary oral squamous cell carcinoma cells. The MTT assays revealed that Loratadine and Cisplatin each had a time and dose-dependent inhibitory impact, with 50% inhibitory concentrations (IC_{50}) of 86.78 µM and 67.05 µM, respectively. The combination of both drugs had an additive effect against OSCC cells, with an IC50 of 52.05 µM. However, the apoptosis assay results revealed that treatment with loratadine had no significant effect on cell viability in OSCC. These findings suggest that Loratadine likely has anti-cancer effects but kills or inhibits cells at various dosages, independently of apoptosis. Further mechanistic studies are needed.

Keywords: Loratadine, Cisplatin, Drug repurposing, OSCC, DNMT1, Molecular Docking

1. Introduction

Oral squamous cell carcinoma (OSCC) is an important subtype of head and neck squamous cell cancer (HNSCC) (Kumari et al. 2022). It is one of the six most common oral malignancies, with over 500,000 cases reported each year (Giovannacci et al. 2016). OSCC is responsible for more than 90% of oral cancer cases and has the highest mortality rate globally (Shahaf Givony 2020), with a low overall survival rate of OSCC patients at advanced stages (Le Campion et al. 2017) . OSCC's cell of origin is the oral keratinocyte. OSCC, like any other cancer, is caused by DNA mutations, which are often spontaneous but can be accelerated by exposure to various mutagens, including chemical, physical, and microbiological agents. The different mutations in the DNA can transform a normal keratinocyte into a pre-malignant or potentially malignant keratinocyte, characterized by its ability to multiply in a less-controlled manner than normal (Scully and Bagan 2009).

The majority of oral cancer instances are connected to heavy alcohol use, tobacco use (both smoked and smokeless), high-risk human

papillomavirus (HPV) infection, and poor oral hygiene(Lars Sand1 et al. 2014). Other factors, such as dietary micronutrient deficiency, environmental factors, may also increase the risk of oral cancer development (Petti 2009). Evidence from a recent review article indicates that there is a direct correlation between Naswar use and Pakistan's oral cancer incidence. Additionally, there are indications of an exposure-response relationship between Naswar use and oral cancer. In Khyber Pakhtunkhwa, where the use of Naswar is prevalent, the odds ratio for oral cancer is significantly higher. Naswar accounts for over 60% of oral cancer cases in Khyber Pakhtunkhwa (Khan et al. 2019).

OSCC progression is driven by genetic and environmental factors, causing alterations in oncogenes and tumor suppressor genes. These lead to genomic instability through mutations and epigenetic changes (Goon et al. 2009). DNA methylation of 5′ CpG islands is a key mechanism in the inactivation of tumor suppressor genes, commonly observed in neoplasms (Issa 1999, Stephen B. Baylin 2001). DNMTs transfer a methyl group from SAM (S-adenosylmethionine) to cytosine, producing SAH (S-adenosylhomocysteine), a strong inhibitor of methylation (Jurkowska, Jurkowski, and Jeltsch 2011, Martha J Shrubsole1 et al. 2015). This epigenetic modification affects genes involved in angiogenesis, apoptosis, DNA repair, and cell cycle regulation (Issa 1999, Stephen B. Baylin 2001), contributing to the development and progression of head and neck cancers, including OSCC (Kazuhiro Ogi et al. 2002, Nakahara et al. 2000).

The recent development of DNMT inhibitors like decitabine (5-aza-2-deoxycytidine) and azacitidine (5-azacytidine), which the Food and Drug Administration (FDA) has approved for myelodysplastic syndrome (MDS), offers encouraging prospects for novel anticancer treatments. Both drugs have the potential to reverse epigenetic changes responsible for tumor progression (Fenaux 2005).

Combined computational and experimental methods have advanced the discovery of new therapeutic molecules (Jing Tang1 et al. 2014). Molecular docking is a contemporary bioinformatics technique used to predict ligand-target interactions and binding affinity, thus aiding in drug discovery and repurposing (18). Drug repurposing offers a faster and cost-effective alternative to traditional drug development by finding new uses for existing medications (Ashburn and Thor 2004).

Antihistaminic medications, which work by blocking H1 receptors, are commonly used to treat allergies and have shown anti-cancer properties alone or with chemotherapeutic treatments. Loratadine, among them, has demonstrated anti-proliferative and G2/M phase cell cycle arrest in colon cancer. In addition, various antihistaminic medications, including loratadine, desloratadine, ebastine, and terfenadine, are tested on different cancer cell lines, including breast, prostate, and non-small cell lung cancer. Therefore, loratadine is a promising candidate for drug repurposing in cancer therapy, as it is safe and well tolerated, with increasing evidence supporting its effectiveness against various tumors (Trybus and Trybus 2024).

In the current investigation, the repurposed anti-histamine Loratadine was explored as a new therapeutic target against OSCC by evaluating its binding affinity with DNMT1 and its anticancer effects on OSCC cell lines. *In vitro* tests assessed the potential of loratadine alone and in combination with cisplatin, aiming to improve clinical outcomes and investigate its antiapoptotic activity.

2. Methods & Materials

2.1. Computational Analysis

The crystallographic structure of human DNMT1 (PDB ID: 4WXX) bound to SAH was retrieved from the RCSB Protein Data Bank

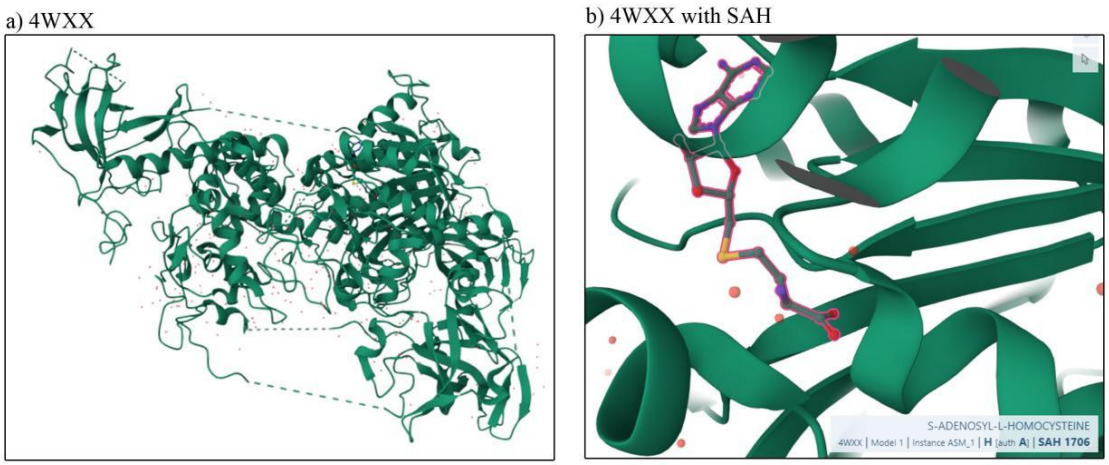

Figure 1: a) 4WXX is DNMT1 Protein wherein the red dots are water molecules whereas b) 4WXX with SAH shows DNMT1 Protein whereupon SAH Ligand is attached.

(PDB) (*https://www.rcsb.org/*) (Vaishnav et al. 2020). Water molecules and ligands bound to the protein's active site were removed using the Autodock tool and Discovery Studio. To simplify analysis and conserve resources, a single peptide chain of DNMT1 (PDB ID: 4WXX) was selected using "PyRx" software to eliminate complexity, thereby reducing the time and resources spent on experimentation. This structure was saved in a .pdb file. Through existing literature, the repurposed medicine Loratadine was selected for the docking investigation. The ligand molecule was obtained through PubChem (*https://pubchem.ncbi.nlm.nih.gov/*) in 3D structure as ".sdf" files, and its structure was transformed to ".pdb" format using "PyMOL". PyRx software was used for molecular docking investigations. The ligand grid box was configured to have the maximum dimension enclosing the SAH binding site. The number of binding modes was set to default, the search exhaustiveness was set to 8, and the maximum energy difference was 3 kcal/mol. Protein-ligand complexes were prepared using the CHIMAERA software. Biovia Discovery Studio Visualizer 2020, was used to understand the binding between the docked ligand and protein. The interaction between individual amino acids in the protein and the ligand was then investigated and compared to that of the co-ligand. Furthermore, the ligand was tested in accordance with Lipinski's rule of five. The Lipinski's rule of five was analyzed utilizing the online web server Swiss ADME http://www.swissadme.ch/ (Daina, Michielin, and Zoete 2017, Vaishnav et al. 2020).

2.2. *In-vitro* Drug-Induced Cytotoxicity Assessment

2.2.1. Cell Line Generation and Maintenance

Fresh tissue samples were collected from the Department of Oral and Maxillofacial Surgery at the Hayatabad Medical Complex, Peshawar. The OR-30 cell lines were generated in the Cancer Cell Culture and Precision Oncomedicine Lab (CCCPOL) at IBMS, KMU, Peshawar. A cytologically diagnosed excisional biopsy sample of a 74-year-old male patient with moderately differentiated squamous cell carcinoma of the oral cavity was utilized for cell line generation. Cell lines were grown in 90% DMEM-F12 medium (Gibco), supplemented with 9% fetal bovine serum (Gibco) and 1% penicillin-streptomycin antibiotic (Gibco) (Maggioni et al. 2013). Cells were incubated in a T-25 flask at 37°C in a humidified environment with 5% CO2. The flask's confluency was examined with a phase contrast microscope, and media changes were made as needed.

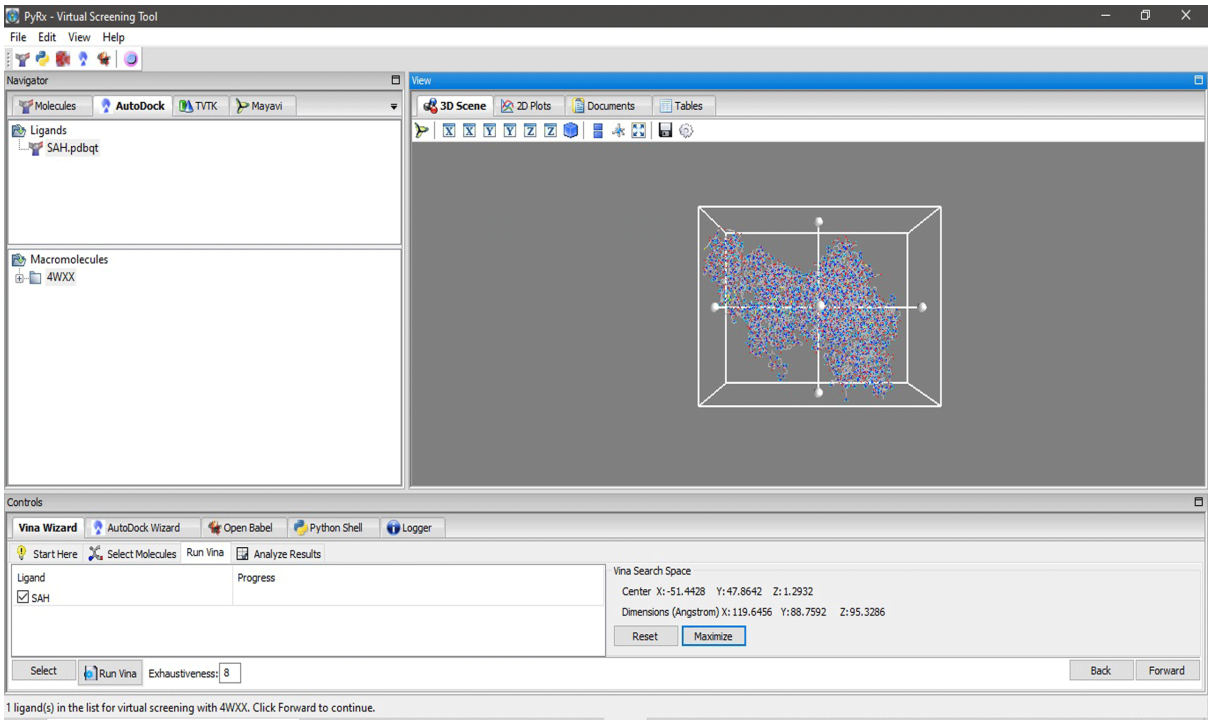

Figure 2: PyRx Tool grid box displaying all dimensions and coordinates. This figure shows the PyRx screen before docking commences. In the left AutoDock tab, ligand SAH and macromolecule 4WXX have been shown and specified to PyRx for docking. The right of the image depicts 4WXX Protein enclosed by a grid with default dimensions shown in the bottom right.

2.2.2. Cell Viability Assay

Primary OSCC cells were seeded at 10,000 cells per well in 96-well plates for the MTT experiment and cultivated for 24 hours to reach confluence. The cells were then cultured in media containing the relevant drug doses for 24 hours (Nakaoka et al. 2014). The cells were treated with Loratadine and Cisplatin alone and in combination to achieve an additive effect. Drugs were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 3mg/ml and then diluted with DMEM medium to prepare the working concentration. The highest and lowest drug concentrations were 400 µM and 6.25 µM, respectively. A triple reaction needed a final volume (V2) of 1500 µL and an initial drug concentration (C2) of 400 µM. Following a 24-hour period, the media was taken out, and the cells were then incubated for four hours at 37°C with MTT (Acros Organics Thermofisher). After four hours, DMSO was used to dissolve the formazan crystals, and the plate was incubated for fifteen minutes. Then, using an ELISA reader (BioTex USA), the absorbance of each treatment was determined at 630 nm. The untreated cells used as controls represented 100% viability. GraphPad Prism 5.0 software (GraphPad Software) was used to plot cell viability against drug concentration in a dose-dependent graph. The IC_{50}, or the drug concentration that resulted in 50% cell death, was then determined (Aldawsari et al. 2016).

2.2.3. Cell Apoptosis Assay

Cellular apoptosis was assessed with the Annexin V-FITC Muse Apoptosis Detection Kit according to the manufacturer's instructions. Six-well culture plates were seeded with 1.5×105 OSCC cells per well, and the plates were incubated for 24 hours at 37 °C (Nakaoka et al. 2014). Using untreated cells as controls, the cells were exposed to varying doses of cisplatin and loratadine for a full day at their IC_{50} values.

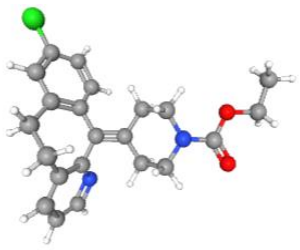	PubChem CID-3957 MW - 382.88 g/mol MF- $C_{22}H_{23}ClN_2O_2$

Table 1: 3D structure of loratadine.

S.NO.	LIGAND	BINDING AFFINITY	RMSD/UB	RMSD/LB
1.	**SAH**	-7.2	0	0
2.	**Loratadine**	-8	0	0

Table 2: The ligand-protein molecule binding affinity, RMSD lower bound, and RMSD higher bound

Trypsin was used to harvest the cells after incubation, followed by two cold PBS washes and suspension in binding buffer containing Annexin V and PI. The presence of apoptotic cells was examined using a flow cytometer following a 15-minute dark incubation period. P-negative and Annexin V-FITC-positive cells were shown to be apoptotic (Maggioni et al. 2013).

3. Results

3.1. Computational Analysis

The target protein's DNMT1 structure was 4WXX (RCSB PDB ID via URL *http://www.rcsb.org/structure/4WXX*), which was acquired from PDB. This structure is in combination with S-Adenosyl Homocysteine (SAH) (Figure 1). The position of this co-factor, SAH, on the protein determined the ligand binding site.

Before structural optimization, analysis of the target-ligand interaction between SAH and 4WXX revealed that the amino acids that interacted with DNMT1 were VAL A:1580, HOH A:1966, HOH A:1861, LEU A:1151, GLY A:1190, GLU A:1266, ASN A:1578, ALA A:1579, GLY A:1149, ASP A:1143, CYS A:1148, GLY A:1147, HOH A:205, GLY A:1223, SER A:1146, GLU A:1168, TRP A:1170, PHE A:1145, ILE A:1167, MET A:1169, GLU A:1189, CYS A:1191, ASP A:1190, HOH A:1918, LEU A:1247, and PRO A:1225. The finest redocking run of SAH to 4WXX with PyRx got an RMSD value of 0. For this run, the parameters were exhaustiveness = 8, center_x = -51.4459, center_y = 47.8174, center_z = 1.2809, size_x = 119.645609243, size_y = 88.7592364609, size_z = 95.3285808563 (Figure 2).

We extracted the structure of the candidate compound Loratadine from the PubChem database and used 4WXX crystal protein structures for molecular docking. Table 1 shows the structure of loratadine and its chemical name.

Loratadine is a Benzocycloheptapyridine that is 6,11-dihydro-5H-benzo[5,6]cyclohepta[1,2-b]pyridine modified with a chloro group at position 8 and a 1-(ethoxycarbonyl) piperidin-4-ylidene group at position 11. It is an H1-receptor antagonist often used to treat allergic diseases. It

Amino Acid	Reference ligand		Loratadine
	ATTACHED SAH	DOCKED SAH	
GLY A:1147	✓	✓	✓
GLY A:1223	✓	✓	✓
GLU A:1168	✓	✓	✓
TRP A:1170	✓	–	✓
PHE A:1145	✓	✓	✓
MET A:1169	✓	–	✓
PRO A:1225	✓	✓	✓
SER A:520	–	✓	✓
GLN A:573	–	✓	✓
GLN A:1227	–	✓	✓
ARG A:1574	–	✓	✓
ASN A:1578	–	✓	✓
ASP A:565	–	✓	✓
GLY A:568	–	✓	✓
GLU A:572	–	✓	✓
PRO A:574	–	✓	✓
ILE A:466	–	✓	✓
ALA A:599	–	✓	✓
ARG A:600	–	✓	✓
PHE A:469	–	✓	✓
ILE A:506	–	✓	✓
LYS A:510	–	✓	✓
SER A:509	–	✓	✓
VAL A:513	–	✓	✓
GLN A:560	–	✓	✓
TYR A:564	–	✓	✓
ALA A:567	–	✓	✓
SER A:563	–	✓	✓
SER A:570	–	✓	✓

Table 3: Reference target-ligand interactions and the docking outcome of loratadine

functions as a geroprotector, an H1 receptor antagonist, an anti-allergic agent, and a cholinergic antagonist. It contains an ethyl ester, a N-acylpiperidine, a tertiary carboxamide, an organochlorine molecule, and benzocycloheptapyridine.

Docking a drug candidate to a protein resulted in nine ligand poses in protein-ligand complexes, with the protein remaining in a fixed position and the ligand adopting diverse conformations. The software calculated a binding score for each ligand conformation/pose. The target protein docks with loratadine, exhibiting a higher binding energy than the endogenous molecule (SAH). Following docking, the binding sites were analyzed for their lowest binding energies; the lower the binding energy, the higher the ligand's affinity to the target protein. Table 2 shows the results of the docking of the compound exhibiting the lowest binding energy.

Loratadine acted on all amino acids with which SAH interacted, according to an analysis of all target-ligand interactions. Table 3 displays the findings of all target-ligand analysis. Loratadine binds to the exact binding location as the already known SAH inhibitor.

The screened repurposed drug Loratadine was then evaluated for drug likeness properties using the SwissADME online web server. Furthermore, it was screened based on the qualifying Lipinski Rule of Five, as shown in Table 4. It was discovered that Loratadine had the lowest binding energy with the protein molecule and also met the Lipinski rule of five.

3.2. Cell Viability by MTT Assay

A cell viability experiment assessed Loratadine's cytotoxic potential at doses ranging from 6.25 to 400 μM over 24 hours. Figure 3a shows the concentration of drugs in μM on the x-axis and the survival fraction of cancer cells in percentages on the y-axis. In Figure 3b, the blue bar represents the concentration of loratadine alone, and at 50% viable cells, it was 86.78 μM. The red bar represents the concentration of cisplatin, and at 50% viable cells, it was 62.05 μM, while the green bar represents the concentration of the combination of both loratadine and cisplatin, which was 52.05 μM. Using GraphPad Prism 9, the results were presented as mean ± standard error of the mean (SEM). At $p < 0.05$ (*), the data proved significant. A two-way ANOVA was utilized in cytotoxicity experiments to ascertain how concentration and time affected cell death.

These results in Figure 3 demonstrate that loratadine has a dose-dependent effect on OSCC cell lines. Loratadine's IC_{50} was determined as 86.78 μM. A comparable plot was developed to test the effect of Cisplatin on OSCC cell lines and to determine the Cisplatin IC_{50} over 24 hours using the same concentration range. Cisplatin treatment alone resulted in a dose-dependent reduction in cell count. Cisplatin's cytotoxic impact is confirmed in Figure 3, with an IC_{50} of 67 μM. Following that, we investigated if co-treatment with cisplatin and Loratadine had a synergistic effect on cell proliferation. The combination therapy with both medicines inhibited cell growth more effectively than either treatment delivered alone Figure 3, with an IC_{50} of 62 μM. At each concentration, the cisplatin/Loratadine combination therapy inhibited growth more effectively than cisplatin alone.

3.3. Flow Cytometry Analysis

The antiapoptotic effect of loratadine and cisplatin was determined using PI/Annexin V staining followed by flow cytometry. Cells were treated for 24 hours with Cisplatin 13 μM and Loratadine 25 μM before being processed for flow cytometry with Annexin V-FITC and PI, as described in the Methods section. Figure 4(a) demonstrates that cells treated with Loratadine exhibit 0.11% early apoptosis and 0.14% late apoptosis, with 9.21% of necrotic cells and a proportion of live cells exceeding 90.54%. Figure 4(b) shows that the necrosis of Cisplatin is three

Compound Name	Molecular weight	Hydrogen bond donor	Hydrogen bond acceptor	Partition coefficient MlogP	Violation
Loratadine	382.88g/mol	0	3	4.51	Yes, 0

Table 4: Drug likeliness Property Analysis

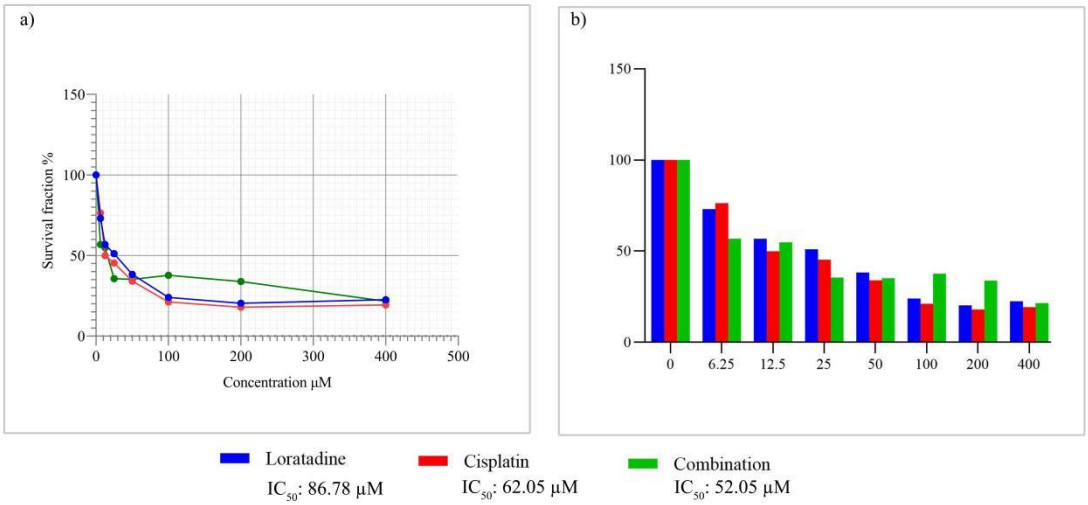

Figure 3: MTT assay presenting the percentages of cell viability, and the IC_{50} of Loratadine, Cisplatin, and Loratadine/cisplatin in combination.

times greater than that of Loratadine. The majority of control cells are necrotic (68.85%) (found in the upper left quadrant), with a small proportion of live cells too (16.74%) in the lower left quadrant of Figure 4 (c). The results are summarized in Figure 4 (d).

4. Discussion

In this study, we conducted a comprehensive *in silico* analysis revealing that the repurposed antihistamine medication loratadine can preferentially inhibit DNMT1 and has cytotoxic effects against OSCC. To investigate Loratadine's potential to interact with the catalytic site of DNMT enzymes *in silico*, we performed molecular docking studies, assessing Loratadine's ability to exert binding interactions with critical amino acid residues in the enzyme's active site. We investigated the binding locations of SAH when it was already attached to the protein and after it was re-docked.

Our findings demonstrated the binding modes of loratadine within the human DNMT enzyme binding sites. Additionally, we found that loratadine has the minimum binding energy, indicating the strongest interaction with our target protein and, consequently, the highest inhibitory and anti-proliferative activity. Furthermore, visualization indicated that loratadine interacts with the receptor through twenty-nine amino acids. The Loratadine structure docked into the active site of DNMT1,

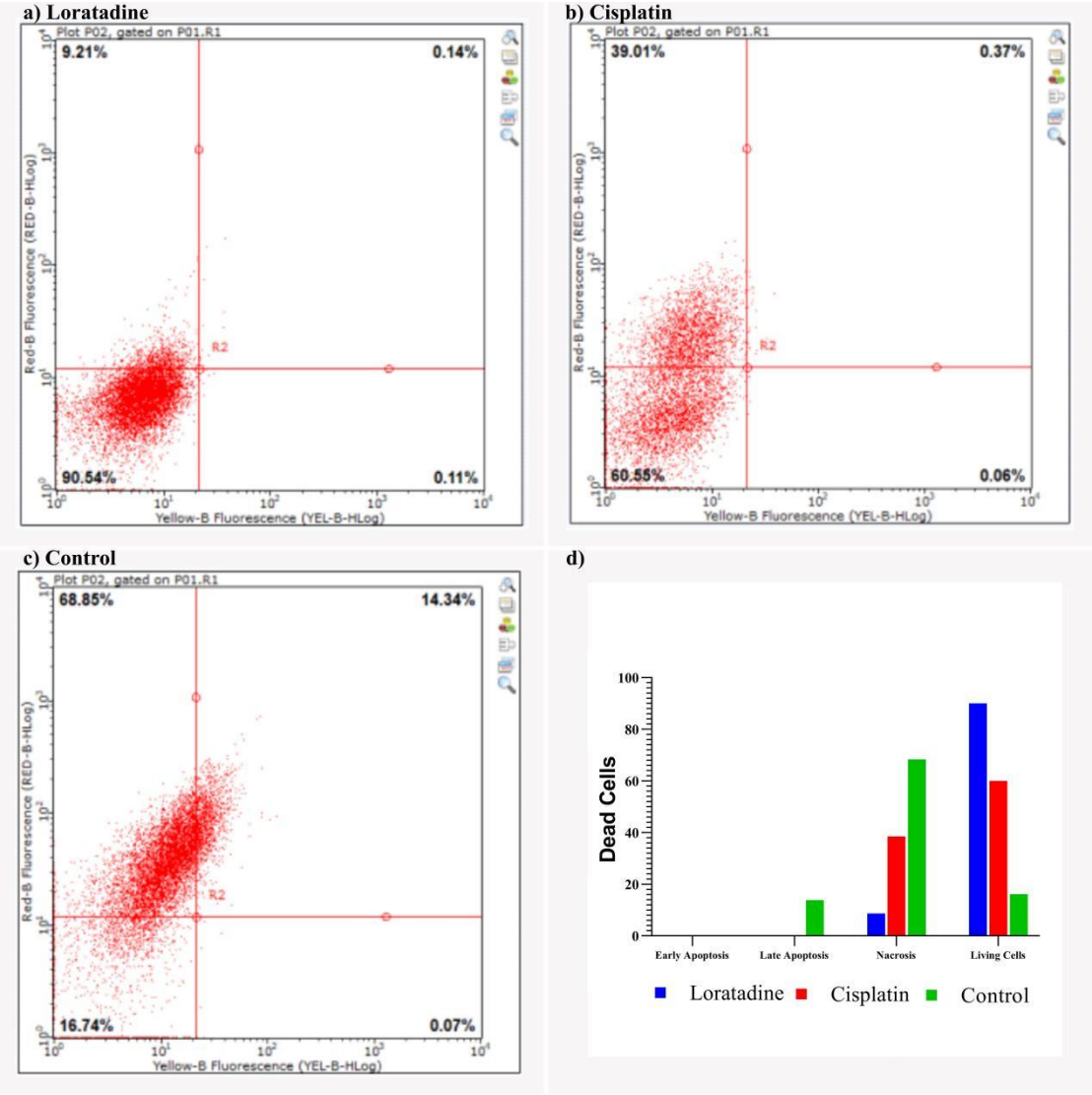

Figure 4: Annexin V/Propidium iodide apoptotic/necrotic assay for OSCC cells cultured with (a) Loratadine (b) Cisplatin (c) Untreated cells, as determined by Flow cytometry d) Percentages of cell death due to early apoptosis, late apoptosis, and necrosis for the different treatments.

resulting in binding modes and a docking score of -8.2 kcal/mol, comparable to the cofactor inhibitor SAH (docking score = -7.1 kcal/mol). Several similar amino acid interactions with DNMT1 protein were shown by Loratadine and SAH.

Furthermore, MTT assay was utilized to determine lortatadine's cytotoxic effects on OSCC cells *in vitro*. Literature has shown that loratadine has anticancer activity in various cancers. In 2006, it was thoroughly tested for its cytotoxic activity against human colon cancer cells (COLO 205). Loratadine inhibited COLO 205 growth by inducing cell cycle arrest in the G2/M phase and caspase-9-mediated cell death. Soule *et al.* investigated loratadine from a different perspective in 2010. Their idea was based on Chen et al.'s 2006 findings: Loratadine may affect the radiosensitivity of human prostate, glioblastoma, and colon cancer cell lines because it induces cell cycle arrest in the G2/M phase, which is a radiation-sensitive phase. They concluded that cells pre-treated with Loratadine significantly boost radiation-

induced cytotoxicity and directly promote DNA damage in different types of human cancer cells (Chen et al. 2006). Furthermore, in 2016, Ellegaard et al conducted a comprehensive study regarding the effect of several antihistamines on non-small cell lung cancer (NSCLC). They discovered that loratadine not only reduced cancer mortality but also sensitized NSCLC to chemotherapy and reversed resistance (Ellegaard et al. 2016).

In this study, we also investigated the effect of loratadine and its combination with Cisplatin on OSCC cell lines. Our results demonstrated that loratadine has a significant cytotoxic effect on OSCC cell lines, with an IC_{50} of 86.78 μM. The combined therapy of loratadine and cisplatin shows a notable inhibitory impact. With an IC_{50} of 52.05 μM, it exhibits a more potent cytotoxic effect than loratadine alone, suggesting a potential therapeutic benefit at a lower dose. Given that loratadine has a more significant inhibitory effect when combined with Cisplatin, it is clear that this combination enhances the drug's effectiveness.

Apoptotic experiments were performed utilizing an Annexin flow cytometry assay to determine Loratadine's mechanism of action. Unlike previous research, which indicates loratadine's anticancer potential in hepatocellular carcinoma cell lines (Adly 2018), our results suggest that loratadine therapy has no discernible impact on cell viability in terms of apoptotic levels against OSCC cells, indicating that the difference in cell type also has an effect. This suggests that the mechanism of action of Loratadine may not be mediated by apoptosis in oral cancer cells derived from our population. As a result, the loratadine apoptosis assay results could not be validated. In addition, the Cisplatin-treated cells stained positive for PI, indicating necrotic cell death. To verify the conclusion as mentioned earlier, however, real-time PCR and western blotting as well as TUNEL assay are required for the expression analysis of apoptotic markers/genes, such as caspase 8, caspase 9, caspase 3, and necrotic markers, such as HMGB1 or High Mobility Group Protein B1.

5. Conclusions

Loratadine can be a promising DNMT1 inhibitor as it has shown strong binding affinity and no Lipinski violation. It also demonstrated anticancer activity against primary OSCC cell lines. Its combination with cisplatin may enhance chemosensitivity in OSCC, warranting further *in vivo* and clinical investigations to validate its therapeutic potential.

Conflict of Interest

The authors declare that they have no competing interests.

Funding

No separate funding was received for this project.

Study Approval

This study was approved by the ethical committee of Khyber Medical University, Peshawar, Pakistan.

Consent Forms

Consent forms are available from the authors.

Data Availability

All the data related to this study are available from the authors.

Authors Contribution

NS performed data curation, formal analysis, investigation, methodology, writing review, and editing, AS performed data curation, formal analysis, investigation, methodology, software, and writing original draft, UK ran the software, writing review, and editing, MZ performed experiments, writing review, and editing, SZ was responsible for conceptualization, supervision, writing review and editing, visualization, and validation.

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