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In-silico and *in-vitro* evaluation of docetaxel and berberine as potential p53 modulating apoptotic inducers in oral squamous cell carcinoma

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ABSTRACT

Objective: To investigate the interaction of p53 with docetaxel and berberine and their anticancer activities against oral squamous cell carcinoma.

Methods: The interaction between p53 with docetaxel and berberine was investigated and their mechanisms of action against oral squamous cell carcinoma were studied. Toxicity studies were performed to determine any toxic impact of the drugs on the vital organs of tested animals.

Results: *In silico* results revealed the molecular interaction of docetaxel and berberine with p53 and the molecules were found to be potential p53 inducers. Docetaxel and berberine inhibited the proliferation of cancer cells in a concentration-dependent manner. Flow cytometry analysis revealed that docetaxel and berberine at IC_{50} concentrations upregulated the expression of p53 in oral squamous cell carcinoma cells, thus triggering apoptotic cell death. In addition, no toxicity was observed in the liver and kidney tissues of mice after docetaxel and berberine treatment.

Conclusions: Docetaxel and berberine significantly suppressed the proliferation of oral cancer cells by activating p53 expression and causing apoptotic cell death. Both compounds can be potential agents for the treatment of oral cancer, with little to no toxicity at the tissue level.

KEYWORDS: Docetaxel; Berberine; PC-3; OVCAR-3; OSCC; SCC-29B; p53; Anticancer; Apoptosis

1. Introduction

Cancer is a large group of diseases that has a significant impact on human health, therefore the development of appropriate measures for prevention and treatment of cancer is crucial. Cancer, as one of the most significant obstacles to human survival, is mostly responsible for high mortality rates. Invasive surgical procedures

Significance

Docetaxel and berberine are important phytocompounds synthesized naturally. The current study exhibited the interaction of the two compounds with p53 in oral cancer cell lines. Docetaxel and berberine inhibited oral cancer cells by inducing p53 expression and apoptotic cell death in oral cancer cells with no toxicity observed. Therefore, both compounds could be potent agents for anti-cancer therapeutics.

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along with the use of radiotherapy or chemotherapeutic agents and sometimes the combination of both are still one of the most preferred approaches to fighting cancer. However, they always come with a risk of increased drug resistance and possible damage to vital organs. Targeted therapy including immunotherapy and metabolic therapy has also been utilized to treat various forms of cancer while researches on new therapeutic strategies are still in progress[1,2]. The Global Cancer Observatory has estimated 10 million deaths and 19.3 million new cases of cancer in 2020. Global rise in the incidence of cancer cases necessitates the development of novel, effective, and safe therapeutic drugs for treatment[3].

Mutations in some oncogenes and tumor suppressor genes are linked to human cancer at different stages, from carcinogenesis to tumor development, recurrence, and metastasis[4]. Proto-oncogenes typically regulate the division of cells; however, molecular changes such as gain-of-function mutations, duplication, and overexpression could activate oncogenes, resulting in abnormal proliferation of cells and eventually cancer[5]. Tumor suppressor genes, on the other hand, are "helpful" genes that generally inhibit cell proliferation and development[6]. Tumor suppressors are inhibited when loss-of-function mutations and deletions of tumor suppressor genes occur, which causes uncontrolled cell division and proliferation, thus leading to cancer. The balance between oncogenes and tumor suppressor genes is crucial for cancer development. Inhibition of the oncogenes and activation of the tumor suppressor genes are potential ways of preventing or treating cancer.

The p53, a vital protein of multicellular organisms which protects from gene mutation and cancer, is a key tumor suppressor in humans and has developed as a vital indicator of cellular responses. It is also known as the 'protector of the genome' because of its capacity to restore damaged DNA under certain physiological conditions. Under irreversible conditions wherein restoration to the damaged DNA is not possible, the p53 can bring about apoptosis in the targeted cell thus leading to irreversible cell elimination[7]. It is also one of the most common mutated genes in oral cancer that leads to the loss of function. Under normal conditions, p53 expression is low and it tends to bind with MDM2 thus forming a complex in the cytoplasm. Cellular stresses generally disrupt the p53-MDM2 complex leading to the phosphorylation of the *p53* gene. Mutation in p53 can lead to drug resistance. In locally advanced cases of oral cancer, concurrent chemoradiotherapy achieves a higher success rate thus increasing the survival of patients[8].

Natural therapeutics derived from plants have revealed substantial cancer chemopreventive and chemotherapeutic activities in both preclinical and clinical investigations. Many natural products target oncogenes and tumor suppressors to counter cancer. Most anticancer natural products have multiple targets than synthetic anticancer drugs that are developed for a specific molecular target, such as the target to oncogene as well as the p53 tumor suppressor gene[9–11]. Several natural products have been reported to target the tumor suppressor gene, including chalcones, genistein, curcumin, sesquiterpenoids, ginsenosides, *etc*[10].

However, synthetic drugs like taxanes and their semi-synthetic derivatives have a great potential for promoting the synthesis of tumor suppressor genes. Taxanes as microtubule-stabilizing agents demonstrated anticancer properties in a variety of human cancers[12]. Docetaxel, known as taxotere, is a semi-synthetic derivative taxane and a microtubule-targeted chemotherapeutic agent which arrests cell cycle progression in dividing cells, leading to apoptotic cell death. It has been used as a single agent and also as a combination with other chemotherapeutic drugs for the treatment of various cancers. Docetaxel has a stronger affinity for tubulin[13] and demonstrates better anticancer activity than paclitaxel and other taxanes[14]. It has been approved for use in the treatment of lung, breast, and prostate cancers[15]. Studies have confirmed the role of docetaxel in the induction of p53 phosphorylation. A combination of docetaxel with *p53* gene therapy in cancer patients has been found to be useful[16]. The p53 tumor suppressive effects of the therapy were found to be enhanced suggesting a vital role of docetaxel in oral cancer[17].

Berberine, an isoquinoline alkaloid derived from the rhizomes of *Coptis chinensis* and *Hydrastis canadensis*, has been used to treat diarrhea and bacterial infections for centuries[18,19]. Recent research revealed the therapeutic potential of berberine to reduce glycemic index and type-2 diabetes[20,21]. The anti-tumor potential of the phytochemical has also been reported[22]. Berberine can suppress cancer development in several ways such as inhibition of cell cycle progression, induction of apoptosis, promotion of cellular autophagy, suppression of cell invasion and proliferation, the expression of micro-RNA, and telomerase activity, and hindrance in the tumor microenvironment. Berberine is now being utilized extensively in fundamental research and clinical studies[23–26]. Furthermore, it has been found to cause mitochondrial dysfunction by inhibiting respiration in the mitochondria[27]. Berberine induced changes in the mitochondrial membrane resulting in the release of cytochrome c, thus leading to the formation of reactive oxygen species (ROS) and cell apoptosis. Ho *et al*. also found that berberine could induce apoptosis in oral squamous carcinoma cell lines SCC-4, and HSC-3 by activation of the caspase pathway, disruption of mitochondrial membrane proteins, the release of cytochrome c, ROS production, and cell cycle arrest[28].

Molecular docking simulations can predict the binding interplay with the target template which provides preliminary clues for the suitability of the molecule toward the target. In drug repurposing, computational tools were found useful for the virtual screening of small and macromolecules to determine the mechanism underlying the target[29]. Currently, machine learning techniques are being used to predict the interactions of biomolecular systems, atoms, and proteins, and have now become a prominent tool in both commercial

and academic sectors[30,31]. Therefore, in this study, the molecular docking simulation with p53 protein was carried out to observe the bindings of the docetaxel and berberine molecules with the binding site of the target. To evaluate its binding affinity and its mechanism, *in vitro* experiments have also been carried out in our current study. Moreover, the toxicity of both compounds to the liver and kidney was evaluated.

2. Materials and methods

2.1. Ligand–optimizations and ADMET analysis

Docetaxel and berberine were sketched using Chem Draw Ultra 8.0 Software. Both ligands were optimized by using B3YLP/G parallel to the Gaussian approximations in a solvated medium. The higher occupied and lower unoccupied atomic orbitals (HOMO and LUMO) were observed after terminations by ORCA 4.2 Linux 64-bit Software. The HOMO-LUMO energy gap, hardness, softness, ionization energy, electrophilic energy, electron affinity, and electronegativity were calculated to determine the stability and reactivity of the ligand[32,33]. The SWISSadme, pkCSM, and MolSoft L.L.C servers[34] were used to calculate the molecular properties, drug-likeness, and ADMET properties. The two-dimensional structure files were converted into SMILES by using open babel. Further SMILES were used as inputs to obtain the ADMET properties and drug-likeness score of each ligand.

2.2. Assessment of molecular docking simulations

Molecular dockings were carried out using (MOE09) Software. 4HFZ (a MDM2/p53 peptide complex) was retrieved from the online RBCS database. The binding site of p53 protein (*Homo sapiens*, NCBI ID-MMDBID: 11222; PDB ID-4HFZ) was predicted by the CASTp 3.0 available online at http://cast.engr.uic.edu[30] and the amino acids occupying the binding site were found to be LYS31, PRO32, LEU33, LEU34, ASP84, LEU85, ARG105, ASN106, in chain A, and GLY59, MET62, THR63, and ARG65 in chain C (Figure 1A). Both chains played a pivotal role in the bindings of the ligand during molecular dockings. The amino acid sequence annotations are represented in Figure 1B. The amino acids occupying the binding sites were located at the interface of chain A and C. The protein was prepared by removing water molecules, partial charges were added, and hydrogens were incorporated and then minimized using MMFF94x forcefield[31]. The electrostatic functional form with a distance cut-off of 6.0 A was used to truncate the electrostatic interaction. The dielectric constant was set at the gas phase. Van der waals functional forms (SCWRL form and Lennard-Jones form) were used to simulate the repulsive and attractive part during simulations. The site finder tool was used to select the binding site. Berberine (Source: Pubchem, Compound CID: 2353) and docetaxel (Source: Pubchem, Compound CID: 148124) were selected for the estimation of the free-binding energy with the corresponding protein. The triangle matcher algorithm was used for docking. The London dG module was used to generate the docking poses and the final docking poses were minimized in a binding site. The LigX module was used to find the interactions attained by the protein and ligands.

2.3. In vitro experimental evaluation

2.3.1. Cell culture and in vitro cell proliferation assay

Sigma-Aldrich and Invitrogen provided all of the chemical reagents and mediums utilized in the cell culture. Oral squamous cell carcinoma cell lines (SCC-29B) were acquired from the cell repository National Center for Cell Science in Pune, Maharashtra.

2.3.2. Anticancer evaluation by using oral squamous cell carcinoma cell lines

Preliminary tests were conducted on three cell lines including PC-3, OVCAR-3, and SCC-29B. Since comparatively promising results were obtained in oral squamous cell carcinoma cell line (SCC-29B), SCC-29B cells were used for further studies. The oral squamous cell carcinoma cell lines were used in an *in vitro* cell proliferation experiment for anticancer purposes. In a 96-well

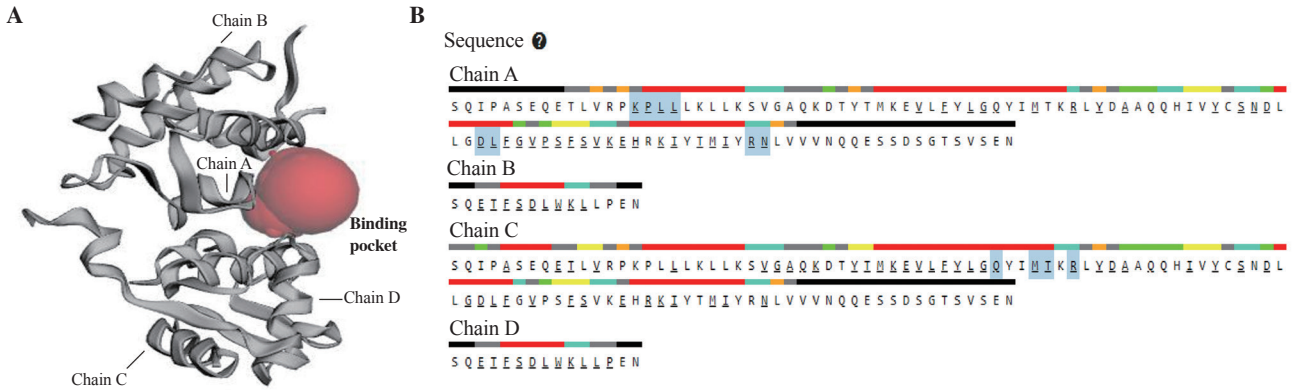

Figure 1. (A) The binding pocket of 4HFZ is represented by VDW contact in the ball-shaped annotation. (B) The p53 protein sequence.

culture plate supplied with complete media (RPMI-1640) and 1% penicillin/streptomycin, cells were seeded at a density of 4×10^3 cells per well and incubated in a CO_2 incubator at 37℃ and 5% CO_2. Subsequently, cells were treated with docetaxel (0.5-100 μM) or berberine (10-150 μM) and incubated for 72 h. The sulphorhodamine B test was used to determine cell viability, and the absorbance was measured at 510 and 564 nm, respectively using a Bio Red 96 well plate reader. Using an online IC_{50} value calculator, the IC_{50} value was determined from plate reader data (AAT Bioquest, Inc., Sunnyvale, CA, USA)[29,35,36].

2.3.3. Detection of apoptosis and p53 accumulation in cancer cells

Cellular apoptosis was determined by fluorescence microscopy using Hoechst staining. Oral squamous cells were grown on 6-well plates and treated with docetaxel and berberine at an IC_{50} for 72 h. After incubation, the plates were fixed in 3% *p*-formaldehyde and washed with phosphate-buffered saline (PBS), stained with 10 μg/mL of Hoechst, and washed after 10 min by using PBS to remove the unbound stain. For detection of apoptosis, 4',6-diamidino-2-phenylindole (DAPI) stain at 10 μg/mL was also used in both treated and untreated oral cancer cells. Images were captured using a confocal microscope (Carl Zeiss). Apoptotic cells were identified by their shape. In comparison to untreated cells, nuclear condensation, membrane bleb formation, and apoptotic bodies were seen. For fluorescence imaging, the cells grown on a 6-well plate with a coverslip were fixed with 3% *p*-formaldehyde and stained with the anti-p53 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), permeabilized with Triton X-100, followed by the addition of secondary antibody conjugated with TRITC and viewed using the same confocal microscope[37,38].

2.3.4. Apoptosis detection by flow cytometry analysis

For induction of apoptosis, cancer cells were treated with IC_{50} concentration of docetaxel and berberine separately and incubated for 48 h. After inducing apoptosis, cells were labeled by Hoechst 33342 (15 μg/mL) for 45 min. With 30 000 events recorded, Hoechst 33342 was stimulated by UV or 405 nm lasers, and emission was captured at 440/40 nm[39].

2.3.5. Detection of p53 expression by flow cytometry analysis

Oral squamous cancer cells were treated with IC_{50} concentration of docetaxel and berberine and incubated for 48 h followed by fixing. The permeabilized cells were treated with anti-p53 MoAb (5 μL) tagged with FITC for 20 min (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by two washes with Tween 20/PBS, resuspended in 500 μL of 1% formaldehyde/PBS, and analyzed by FC. As a negative control, isotype-matched MoAb was employed in each test (IgG 1-FITC). By using BD FACS Fluorescence-Activated Cell Analyzer with software BD FACS Diva 8.0.2, a total of 25 000 events per sample were used to collect by setting forward to scatter (FSC) and side scatters (SSC) gated sets around the blastic cell population and mean fluorescence for p53 were measured[40].

2.3.6. Toxicity analysis

Nude mice aged 7 to 10 weeks were kept in the Animal Care Facility. Mice were kept in a controlled environment of (23±20)℃ with a 12 h light-dark cycle. Food and water were freely available. The experimental animal was randomly divided into 4 groups with 5 animals in each group. Group 1 (control) was given a daily gavage of vehicle solution (acidified water, pH 4.0) only, whereas groups 2-4 were treated with 5, 20, and 100 μM of docetaxel/day or 50, 200, and 500 μM of berberine/day. On day 30, animals were anesthetized with ketamine (20 mg/kg *i.p.*) at the end of the study. The animals were sacrificed after anesthetization and the liver and kidney were excised and histologically examined. Tissues were paraffin-embedded, sectioned, and stained with hematoxylin and eosin. To assess toxicity, the tissues were examined under a confocal microscope (Carl Zeiss)[41].

2.4. Statistical analysis

The data were expressed as mean±standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) using Sigma Plot version 14.0 (Systat Software Inc.). $P<0.05$ was considered a significant difference.

2.5. Ethical statement

The Animal Ethics Committee of the SOA University of Bhubaneswar approved all the experimental procedures (Protocol IAEC/SPS/SOA/18/2019). Experiments were carried out at a lab with registration number 1171/Po/Re/S/08/CPCSEA.

3. Results

3.1. Molecular orbital analysis of the ligand

The ligands were optimized to determine HOMO and LUMO, and global quantum parameters. The energy gap (ΔE_{gap}) between HOMO and LUMO provides clues about the behavior of the molecule and is calculated by the following equations given in Supplementary Table 1. The frontal orbitals determined that berberine emerges as a more reactive molecule than docetaxel and the stability was found to be comparably determined from global electrophilicity parameters

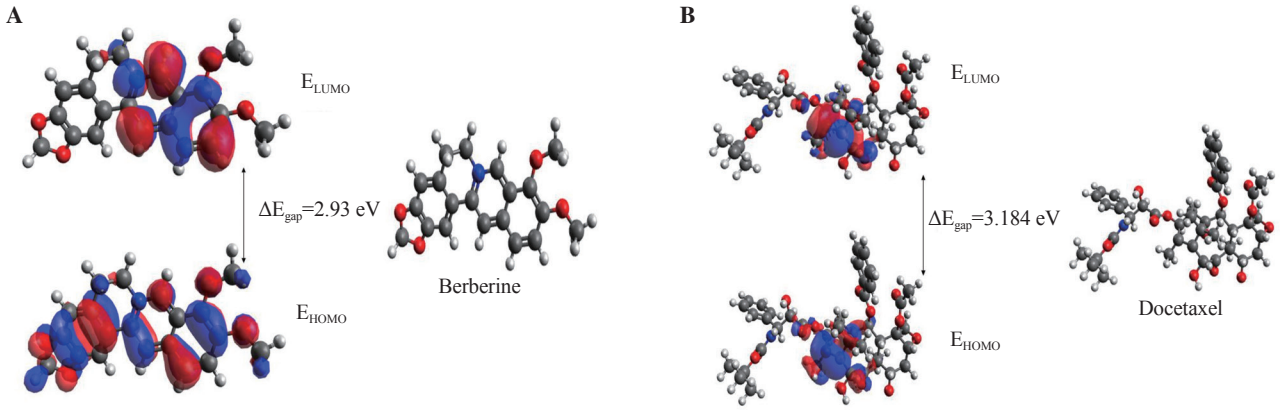

Figure 2. The higher occupied and lower unoccupied atomic orbitals (HOMO and LUMO) of (A) berberine and (B) docetaxel with an energy bandgap.

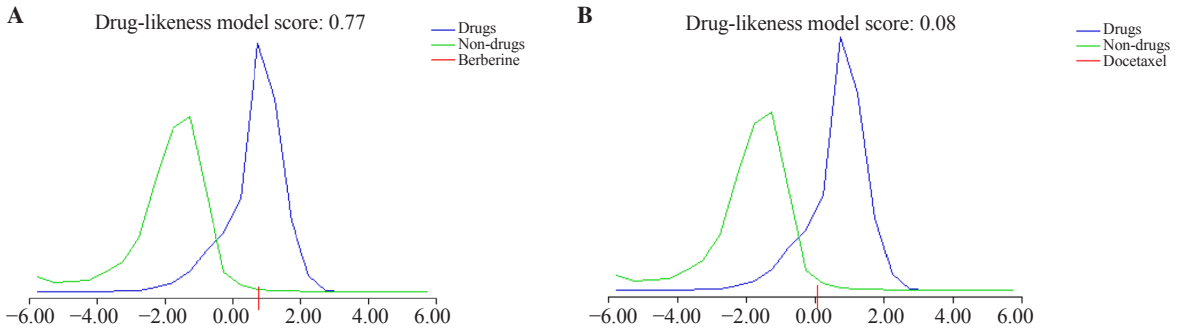

Figure 3. Drug likeness of (A) berberine and (B) docetaxel.

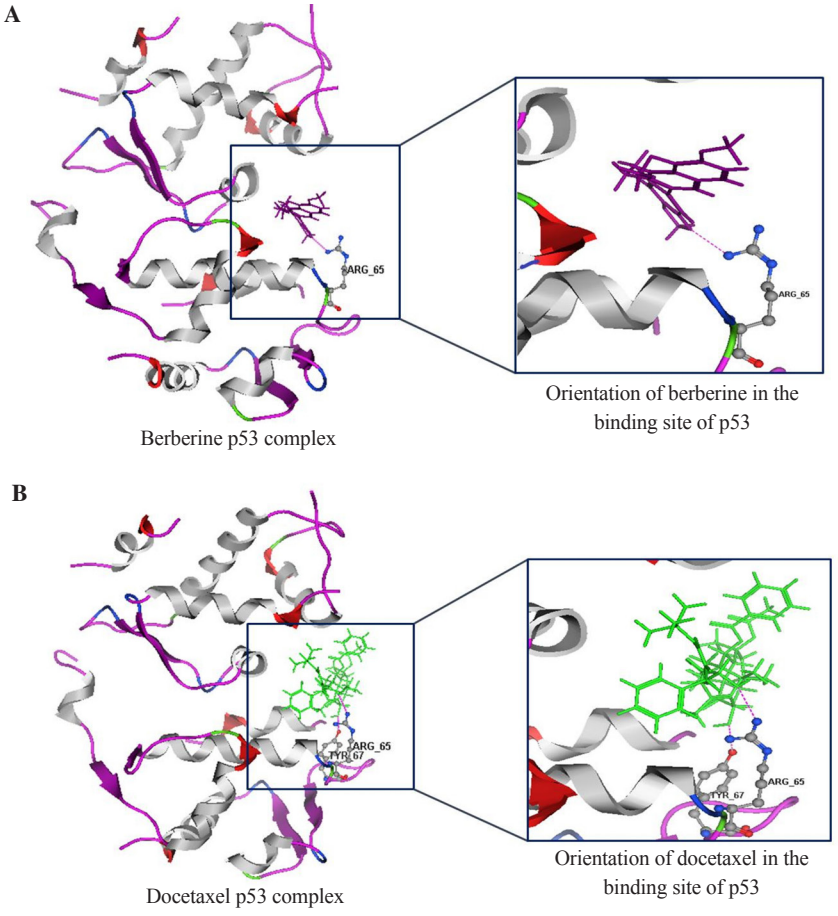

Figure 4. The molecular interactions of (A) berberine and (B) docetaxel with ARG65 in the binding site of p53 protein.

(Figure 2). The water solubility of berberine and docetaxel was found to be −3.973 and −3.258, respectively, illustrating both the molecules have similar water solubility and are comparable. The intestinal absorption was found higher at 97.147 and 100.000 for berberine and docetaxel (Supplementary Table 2). Both molecules were not hERG-Ⅰ and hERG-Ⅱ inhibitors and did not show skin sensitization and renal toxicity. The drug-likeness of berberine was found to be 0.77 which was higher than docetaxel's 0.08 out of a score of 1 (Figures 3A and B).

3.2. Molecular docking analysis

Berberine exhibited interactions with ARG65 of the C chain in the binding site (Figure 4A & Supplementary Table 3). The binding affinity and binding score were found to be −11.07 kcal/mol and 44.6%, respectively. However, the binding interactions exhibited by docetaxel with ARG65 were found between the atom and O of the ligand. The binding affinity was found to be −10.56 kcal/mol and the binding score was observed to be 42.3%. Besides this interaction, another interaction of docetaxel was observed with TYR67 and the score was found to be 66.8%. It was worth to mention the TYR67 did not occupy the binding site of p53 protein but exhibited interactions with aforementioned amino acid (Figure 4B & Supplementary Table 3). Both compounds showed better interactions with the binding site. The hydrophobic interactions played a pivotal role in the present case.

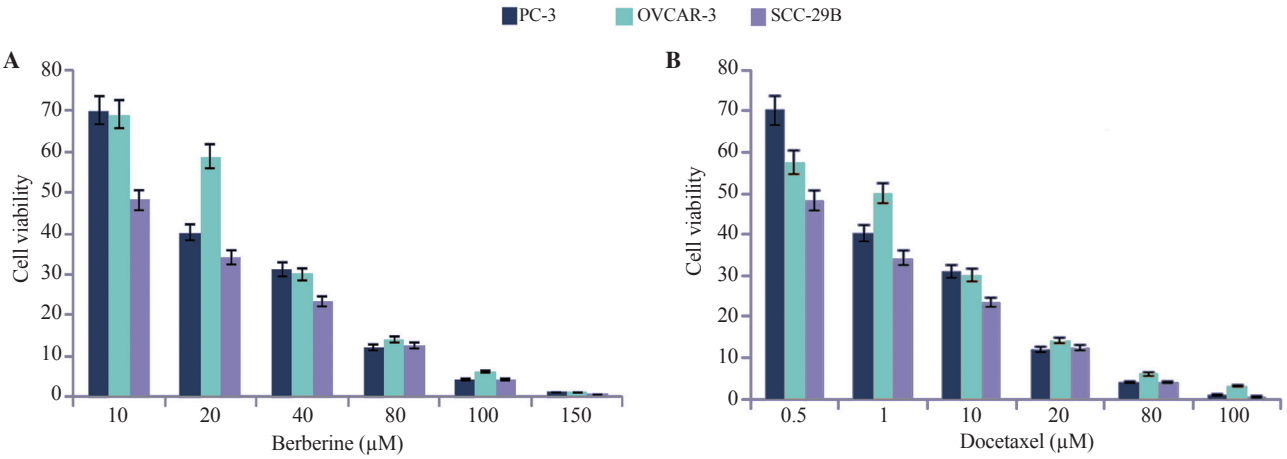

Figure 5. Inhibitory effects of (A) berberine and (B) docetaxel on cell proliferation of prostate cancer PC-3 cells, ovarian cancer OVCAR-3 cells, and oral squamous cell carcinoma SCC-29B cells.

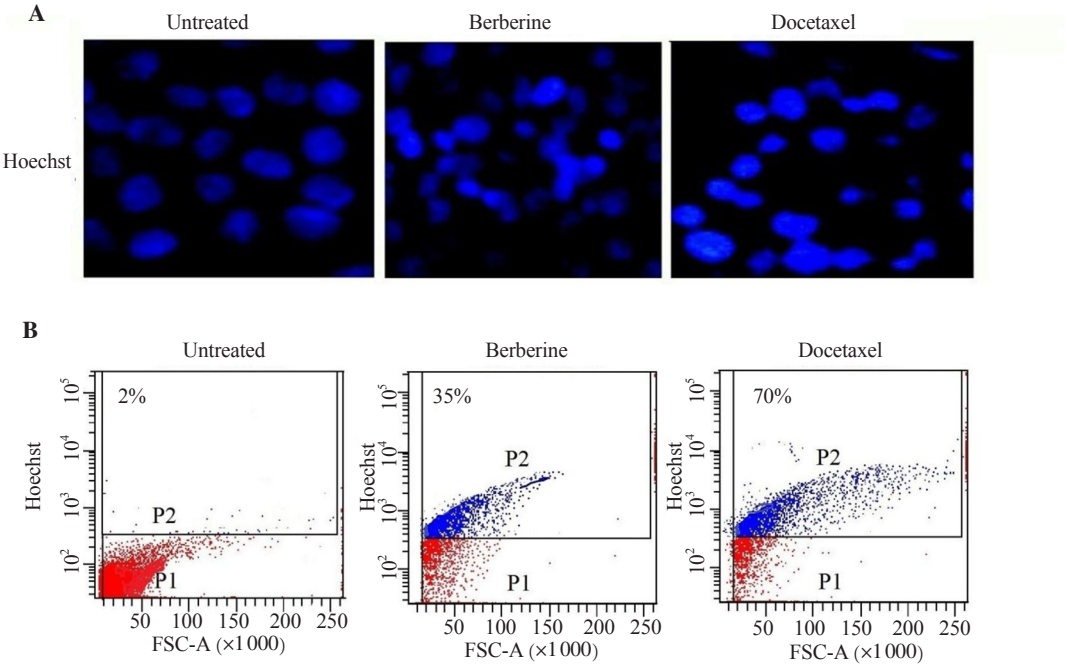

Figure 6. Detection of apoptosis in SCC-29B cells treated with docetaxel or berberine at IC_{50} concentrations. (A) Fluorescent images. Apoptosis was determined by confocal microscopy using Hoechst (10 μg/mL) staining. (B) Flow cytometry analysis (magnifications 200×; scale bar: 20 μm).

3.3. Inhibition of cell proliferation by docetaxel and berberine

Docetaxel inhibited the proliferation of all cancer cells in a concentration-dependent manner with IC_{50} values of (0.7±0.3) μM for PC-3, (1.0±0.5) μM for OVCAR-3, and (0.4±0.2) μM for SCC-29B. Berberine also showed inhibitory effects against these three cell lines with IC_{50} values of (30.0±2.5) μM for PC-3, (25.0±1.9) μM for OVCAR-3, and (7.0±1.2) μM for SCC-29B (Figures 5A & B).

3.3.1. Berberine and docetaxel trigger apoptotic cell death in SCC-29B

The induction of apoptotic cell death in SCC-29B cells by treatment with docetaxel and berberine was investigated by FACS and fluoroscence microscopy using fluorescent dyes. As shown in Figure 6, docetaxel and berberine prominently induced apoptosis in SCC-29B cells compared with the untreated cells [(70.0±2.9)% for docetaxel and (35.0±2.6)% for berberine *vs.* (2.0±0.3)%].

3.3.2. Expression of p53 in berberine and docetaxel-treated SCC-29B

As shown in Figure 7, both docetaxel and berberine markedly upregulated p53 expression in SCC-29B cells compared with the untreated cells [(64.0±3.5)% for docetaxel and (38.0±2.3)% for berberine *vs.* (14.0±1.2)%].

3.4. Toxicity effect of berberine and docetaxel

Administration of berberine (50 μM, 200 μM, and 500 μM) and docetaxel (5 μM, 20 μM, and 100 μM) resulted in no significant toxicity to mice. There was also no observed damage to the kidney and liver tissues. Histological examination of the liver and kidney at different doses showed insignificant changes when compared to the control group (Figures 8A & B). Moreover, no fatalities were detected during the treatment period.

4. Discussion

The increase in cancer incidence and emergence of drug resistance in cancer cells are major obstacles to chemotherapeutic effectiveness[42,43]. The wild-type p53 plays a key role in chemotherapeutic drug susceptibility. Loss of function and mutation in wild-type p53 has been shown to increase drug resistance. Studies illustrate that wild-type p53 modulates drug sensitivity in various cancer cells on an individual basis. The more we discover about the wild-type p53 reaction to drug exposure and cellular response modulation, the easier we will be able to treat malignancies depending on their p53 status. Increased expression of the *MDR-1* gene is the most prevalent change in drug transport. The role of wild-type p53 in MDR-1 regulation and the mechanism of the p53

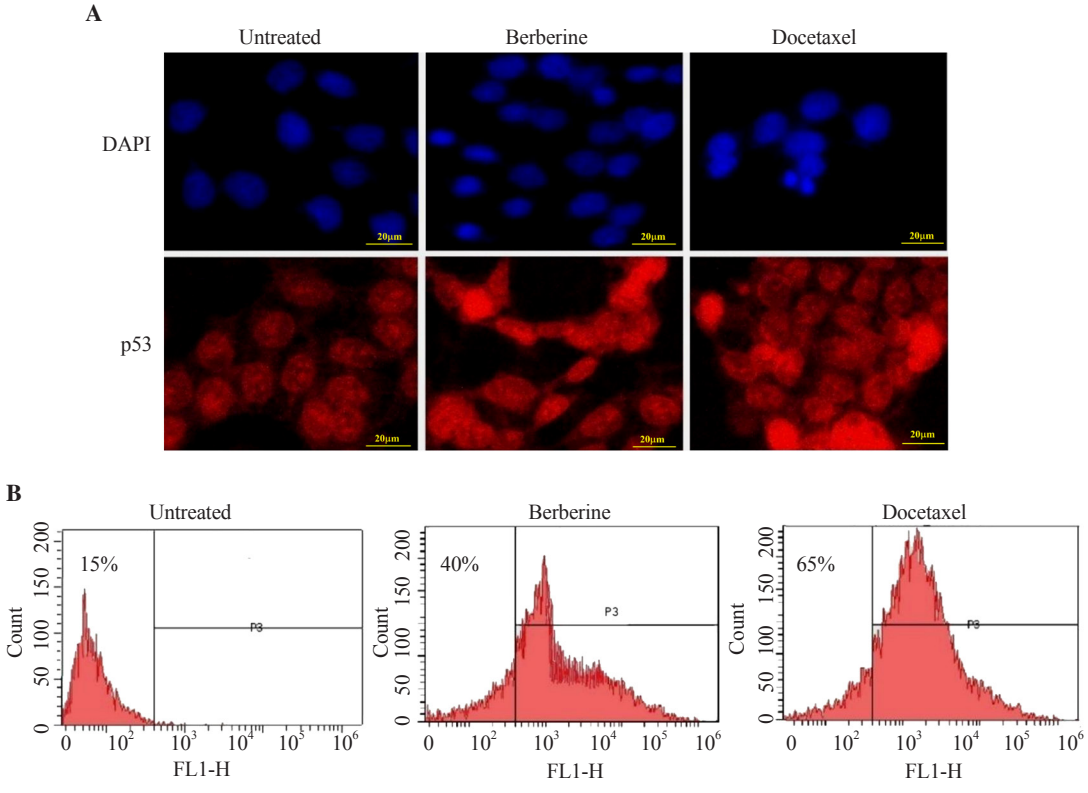

Figure 7. Detection of p53 expression in SCC-29B cells treated with docetaxel or berberine treated at IC_{50} concentrations. (A) Fluorescent images. (B) Flow cytometry analysis for p53 expression. SCC-29B cells were treated with IC_{50} concentration of docetaxel and berberine followed by treatment with anti-p53 MoAb (5 μL) tagged with FITC (magnifications 200×; scale bar: 20 μm).

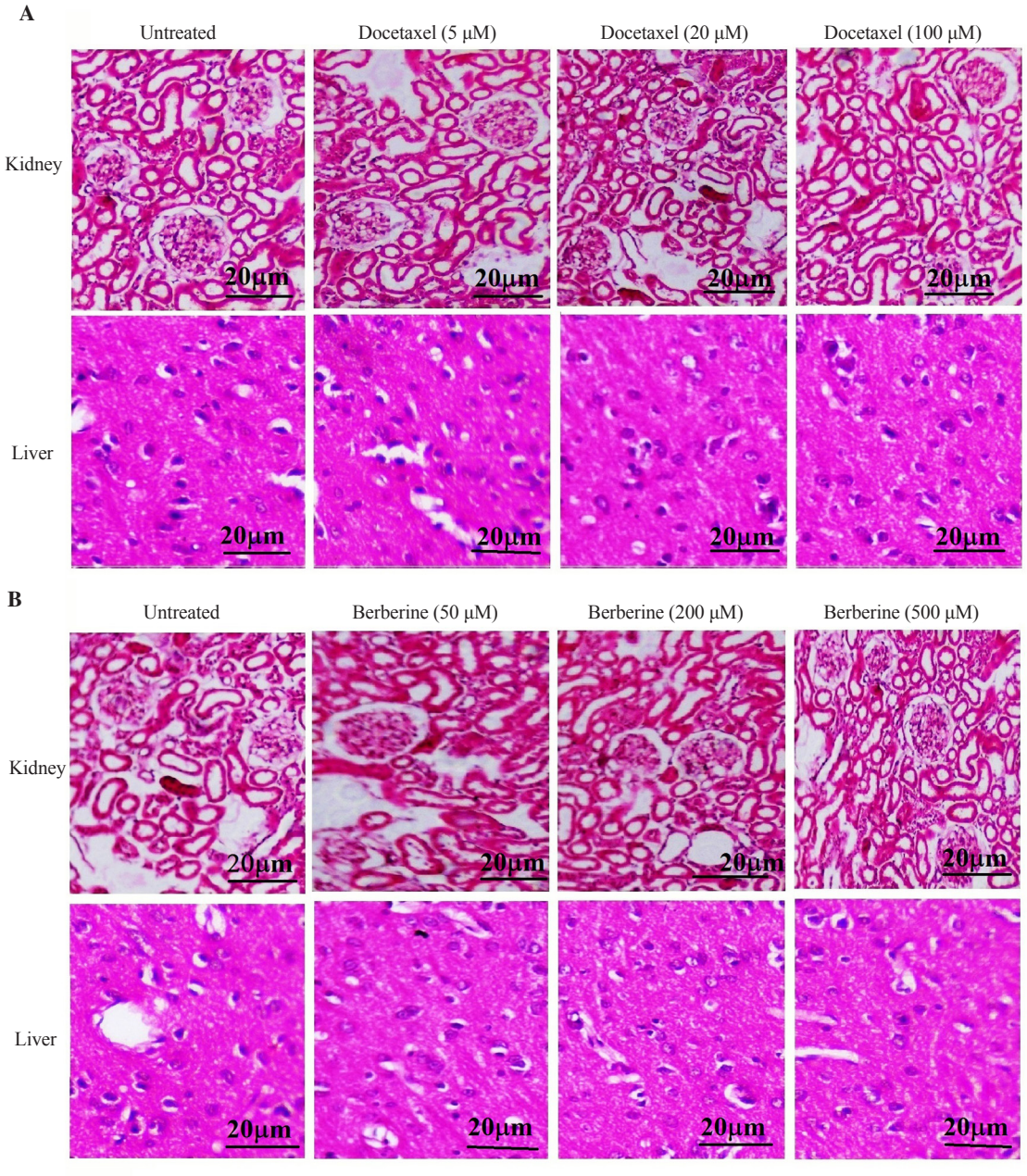

Figure 8. Histological results of the kidney and liver tissues in mice treated with (A) docetaxel or (B) berberine (magnifications 200×; scale bar: 20 µm). The liver of docetaxel- or berberine-treated mice shows normal hepatic lobular architecture. The kidneys of docetaxel- or berberine-treated mice show normal glomeruli, proximal and distal tubules, interstitium, and blood vessels.

response element in MDR-1 has been thoroughly investigated[44]. The MDR-1 promoter is stimulated by mutant p53 protein, while MDR-1 is repressed by wild-type p53. There are various targeting strategies to reactivate mutant p53 which include virus-mediated gene therapy, targeting modulator protein for wild-type p53, and reactivation of mutant p53 by drug molecules and peptides. Among the techniques, reactivating mutant p53 by drug molecules is most convenient. Several compounds have been screened for this purpose and have shown promising outcomes. Azacitidine has targeted patients with acute myeloid leukemia. Aprea-Met, a molecule developed by Aprea therapeutics has been shown to covalently bind to both wild-type and mutant p53. Studies also revealed that the addition of the drugs to mutant p53 reactivates its wild-type function and leads to apoptotic cell death in cancer cells. Cells with p53-mediated cell cycle arrest can repair damaged DNA. Cells re-enter the regular cell cycle when DNA repair is completed. When cells have substantial DNA damage, p53 activates its pro-apoptotic function, causing them to die and thus preventing the transmission of damaged DNA to daughter cells. As a result, p53 is capable of maintaining genetic integrity. Chemo-resistant phenotypes have been observed in cancers with p53 mutations, showing that p53 is involved in the maintenance of DNA damage response. As a result,

developing a novel strategy to minimize the deleterious effect of mutant p53 on wild-type p53 is critical for effective treatment. In the current study, we have investigated the molecular interplay of berberine and docetaxel with p53. 4HFZ was retrieved from the online protein database, and the structural analysis was further done by CASTp server. In 4HFZ, p53 proteins have symmetrical chains A-C and B-D and the binding site was marked as VdW contact. Both berberine and docetaxel also exhibited bindings with ARG65 of 4HFZ which demonstrates they are a potent p53 inducer. The drug-likeness score was observed to be higher in berberine as compared to docetaxel. The global electrophilicity investigations revealed berberine is a higher reactive molecule and exhibits better stability. In addition, the antiproliferative effect of docetaxel and berberine against three different cell lines prostate cancer (PC-3), ovarian cancer (OVCAR-3), and oral squamous cell carcinoma (SCC-29B) was observed. As both compounds have shown great potential against SCC-29B, further studies have been carried out with SCC-29B. To check the apoptotic potential of the compounds, apoptosis was induced on SCC-29B cells and evaluated both qualitatively and quantitatively by using confocal microscopy and flow cytometry. A higher number of apoptotic cells were found in docetaxel- or berberine-treated cells as compared to untreated cells with changes in cellular and nuclear morphology. To further investigate the mechanism of apoptosis, we performed experiments to determine the expression of p53. The expression of p53 was found to be higher in both berberine- and docetaxel-treated SCC-29B cells as compared to untreated cells. Furthermore, no significant toxicity or lethality was observed in treated mice. However, there are limitations in both *in silico* and uncertainty in cellular models. Before clinical trials, their capacity to prevent cancer growth in animal models and other drug safety factors should be taken into consideration.

In conclusion, docetaxel and berberine had significant *in vitro* anticancer activity against oral squamous cell carcinoma and caused cell death by p53 mediated apoptotic pathway. However, docetaxel showed more significant antitumor activity than berberine against oral squamous cell carcinoma. The results also indicated that the compounds could be suitable candidates to target p53, which is implicated in the induction of cellular death in cancer.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

Both RKM and DD developed the theoretical concept. SS, RKM and DD carried out the experimental work. SAM and BN contributed to *in silico* work. PKN analyzed the data. MCH and GR contributed to the final version of the manuscript. SKS supervised the project.

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