Journal of Applied Biological Sciences
E-ISSN: 2146-0108, 16(1): 102-114, 2022
DOI: https://doi.org/10.71336/jabs.968
Research Article

IN VITRO AND *IN SILICO* CYTOTOXICITY EVALUATION OF SOME ISATIN MANNICH BASES ON HUMAN MELANOMA CELLS

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(Received 28th June 2021; accepted 16th August 2021)

ABSTRACT. Mannich bases tend to suppress cell proliferation in damaged tissues because of particular metal chelation properties. Amine components of isatin mannich bases - piperidine (P1), morpholine (P2) and N-methyl piperazine (P3) – were evaluated for their cytotoxicity potentials on melanoma cells. P1, P2 and P3 products were purified using crystallization and characterized by NMR. Human melanoma cells (G361) were produced in DMEM medium including 10% FBS, 1% penicillin/streptomycin at 37°C and 5% CO_2 conditions. Compounds were applied to medium containers separated to 24 pieces plates as $1x10^5$ melanoma cells/well for 24 hours. Expression levels of caspase-3, p53 and β-actin were investigated from RNA samples by qRT-PCR. Mannich bases were efficient at 20, 20 and 50 µg/ml concentrations for P1, P2 and P3, respectively. P2 (20 µg/ml) showed the highest cytotoxic effect with 92 percent. The most significant increase in p53 gene expression was carried out by P2 product with 6.78 fold compared to control group. P2 also upregulated caspase-3 expression by 9.72 fold. Newly synthesized Mannich bases, especially P2, were found to have antitumor potential. Moreover, molecular docking studies revealed that P2 is a potent allosteric activator of caspase-3. However, there is need for in vivo trials and extensive researches to fully elucidate the molecular bioefficacies of these molecules.

Keywords: *Apoptosis, cytotoxicity, isatin mannich bases, melanoma, molecular docking.*

INTRODUCTION

Cancer is a multi-step process in which they undergo metabolic behavioral changes leading to excessive and timeless proliferation of cells. Skin cancer is a type of cancer characterized by very little apoptosis or proliferation of too many cells in the epidermis. Melanoma is a kind of skin cancer that occurs in the melanocytes that give the skin color. Although melanoma is less common than other skin cancer types, it is more dangerous. If it can't be diagnosed in the early phases of the disease, it is more likely to spread to the

How to Cite: Isgor, M. M., Kucukgul, A., Karaman, M., Ozmen Ozgun, D., Yanik, T., Gul, H. I., & Bulgurcu, M (2022). In Vitro and In Silico Cytotoxicity Evaluation of Some Isatin Mannich Bases on Human Melanoma Cells. *Journal of Applied Biological Sciences*, 16(1): 101–113.

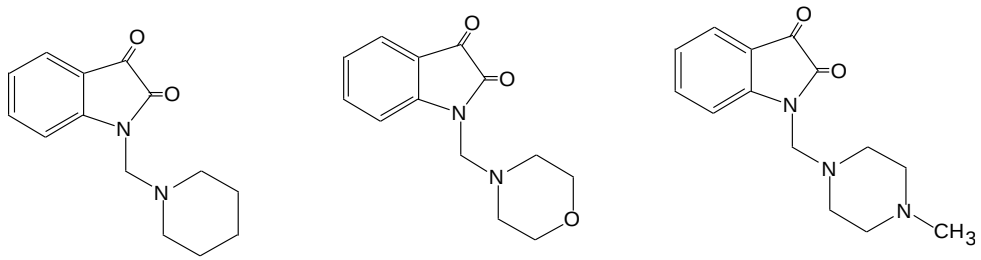
other part of the body and shows rather poor prognosis [1,2]. Tumor suppressor protein (p53) causes a large number of gene expressions in response to various stimuli through transcriptional pathways. It is a DNA-binding protein in which it proceeds as a transcriptional regulator and plays an important role in maintaining genome stability and regulating cell cycle, apoptosis, cell differentiation and senescence [3,4]. With this feature, p53 plays a critical role in anticancer approaches.

Some new synthetic molecules in organic chemistry have been reported to be effective against melanoma cancer in in vitro studies [5]. Currently used melanoma drugs do not appear to be effective in the treatment of the disease and have lower treatment rates [6]. Therefore, it is still important to investigate new synthetic molecules alone or in combination with other medicines as cancer-preventing agents for melanoma treatment. Mannich bases are the final products of the Mannich reaction and they are the compounds in beta-amino ketone structure [7]. They are known to exhibit many biological activities such as anti-inflammatory [8], antiviral [9], antibacterial [10], antifungal [11] and anticancer [12,13]. Mannich bases having an aminoalkyl chain are important molecules used for the synthesis of a wide variety of medicinal potential agents. Compounds having both a ketonic Mannich base and an activated unsaturated carbon-carbon double bond in its structure result in the release of cytotoxic agents lead to toxic effects in tumor cells much more than normal cells [14]. Thus, in this study, it was aimed to investigate the *in vitro* anticancer potentials of some newly synthesized isatin derivatives of Mannich bases on human melanoma cells.

MATERIALS AND METHODS

Synthesis of the molecules

Isatin mannich base derivatives (P1-3) were used in the study. These molecules were synthesized according to the method previously used [15]. NMR spectra of the molecules were obtained using a Varian Mercury Plus spectrometer (Varian Inc., Palo Alto, CA). The melting points of molecules were identified with the help of an Electrothermal 9100 (IA9100, Bibby Scientific Limited, Staffordshire, UK).



P1 **P2** **P3**

Fig. 1. *Molecular structures of isatin mannich bases (Özgün et al., 2016).*

Cell viability assessment

Upon exposure to mannich bases (P1-3) for 24 hours, cell survival was quantified by the colorimetric MTT assay (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Fluka, USA). G361 cells were seeded in a 12-well flat-bottom plate in triplicate

and cultured in media containing mannich bases, which was diluted in culture medium. Following culture at 37ºC, 1 ml/well of MTT (1 mg/ml) was added, followed by incubation for an additional 90 min for each experiment. The viable cells produced a dark blue formazan product, whereas no such staining was formed in the dead cells. The resulting formazan product was dissolved in 100 µl/well of acid-isopropanol, and absorbance read at 570 nm. The cell viability was calculated by the normalization of optical densities (OD) to the negative control.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

qRT-PCR analysis was performed in a qPCR system (Bio- RAD, CFX96 Touch real-time PCR, South Korea). Total RNA from G361 cells were extracted using TRIZOL reagent (Sigma, USA) according to the manufacturer's instructions. 1 mg of total RNA was reverse transcribed in a reaction volume of 20 µl using reverse transcriptase kit (Fermentas, EU). 1 ml of each cDNA was used as templates for amplification using SYBER Green PCR amplification reagent and gene-specific primers. The human primer sets obtained from Thermo Electron Corporation (Germany). Caspase-3 forward: 5'-ACA TGG CGT CAT AAA ATA C-3', reverse: 5'-CAC AAA GCG ACT GGA TGA AC-3', p53 forward: 5′-TGC CCT ATG AGC CGC CTG AG-3', reverse: 5′-CTG AAG GGT GAA ATA TTC TCC-3′. The amount of RNA was normalized to β-actin amplification in a separate reaction. β-actin forward: 5'-CAT CGT CAC CAA CTG GGA CGA C-3', reverse: 5'-CGT GGC CAT CTC TTG CTC GAA G-3'. β-Actin was used as endogenous control, and each sample was normalized on the basis of its β-actin content.

Statistical analysis

The one-way analysis of variance (ANOVA) and post hoc Duncan tests were performed on the data to examine the differences among groups using the SPSS statistical software package. The results are expressed as the average ± standard error (SE). A value of $p<0.05$ was considered significant.

In silico studies

Molecular docking studies were carried out using the Small Drug Discovery Suites version 2019-4 (Schrödinger, LLC, USA). In order to detect possible activation mechanism on caspase-3 of mannich bases, the crystal structures of zinc-bounded inactive caspase-6 enzyme, which is highly homologous to caspase-3, was used because crystal structure for zinc-bound procaspase-3 has not been determined till now [16]. X-ray crystal structures of caspase-6 enzyme (PDB code: 4FXO) was obtained from the Protein Data Bank (www.rcsb.org) and prepared using protein preparation wizard module [17] with several steps including assigning bond order and charges, adding hydrogen atoms, filling missing side chains, ionizing amino acids, minimizing energy and optimizing geometry, respectively [18, 19]. All water molecules were not removed from the enzyme structure because the water molecules in the active site play an important role in the activation/inactivation of the enzyme.

In order to use at docking process, the 3D structure of PAC-1, which is an activator of caspase-3, was downloaded from PubChem and the 3D structure of mannich bases were produced using 2D sketching module. Correct molecular geometries and protonation state at 7.0 ± 2.0 of the ligands were prepared using LigPrep module [20].

Prepared mannich bases were docked by induced-fit docking method using fully flexible receptor/ligand protocol [21]. Grid box was generated around the selected residues in the allosteric inhibitory site of the enzyme. After that, side chains were automatically trimmed based on B-factor; closest residues to the ligand were refined within 3.4 Å of ligand pose in prime refinement. Following the docking process, the types of interactions and interacted residues for best-scored compound results were analyzed [22,23].

RESULTS AND DISCUSSION

According to the cell viability results, it was determined that at each applied concentration of P1 (10, 20, and 50 µg/ml) caused cell loss by 16%, 41% and 61%, respectively, compared to the control group. In the viability analyses, P2 treated with 10, 20, and 50 µg / ml concentrations was found to cause cell loss of 63%, 81% and 92%, respectively, in the cells compared to the control group. Another viability test result was analyzed by applying different concentrations of mannich base 3 to the cells for 24h. Different concentrations of P3 (10, 20, and 50 µg/ml) resulted in cell losses of 0.6%, 8% and 11%, respectively, compared to the control group (Figure 2).

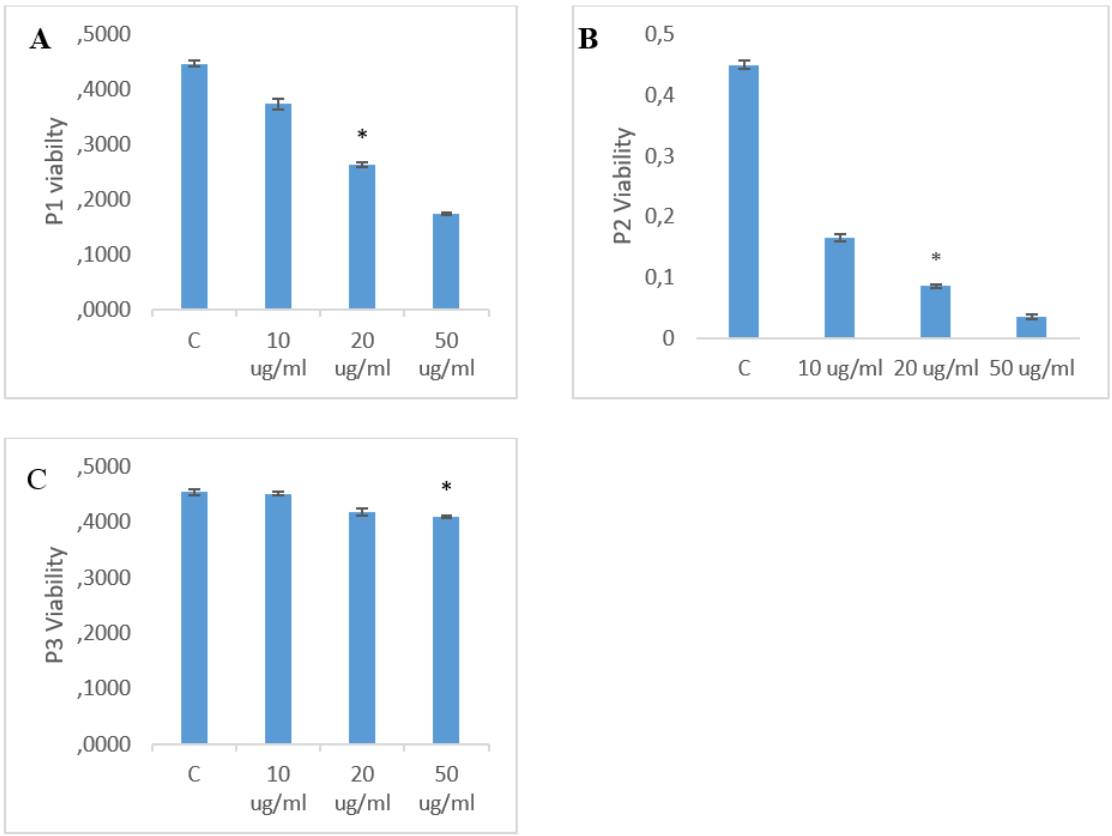


Fig. 2. *The effects of compounds P1, P2 and P3 on cell viabilities.* ***(A)*** *Concentration dependent effect of P1 on human melanoma cell viability. Cells were incubated for 24 h at different concentrations of P1 (10, 20 and 50 µg/ml).* ***(B)*** *Concentration dependent effect of P2 in melanoma cells.* ***(C)*** *Effect of P3 at different concentrations (10, 20 and 50 µg/ml) on cell viability of G361 cells (n=6). *p<0.05 versus control group.*

Caspase-3 and p53 gene expression levels were investigated by qRT-PCR using specific primers as a result of application of Mannich bases at determined concentrations (P1-20 µg/ml, P2-20 µg/ml and P3-50 µg/ml) in melanoma cells for 24 hours. Another aim of the study was to investigate the efficacy of P1-3 on apoptosis. According to the obtained data, P1, P2 and P3 compounds upregulated relative expression of caspase-3 by 2.18, 9.72 and 1,05 fold, respectively (figure 3) according to the control group. In addition, p53 transcription levels were found to be upregulated by 1.44, 6.78 and 1.01 fold, respectively (figure 4).

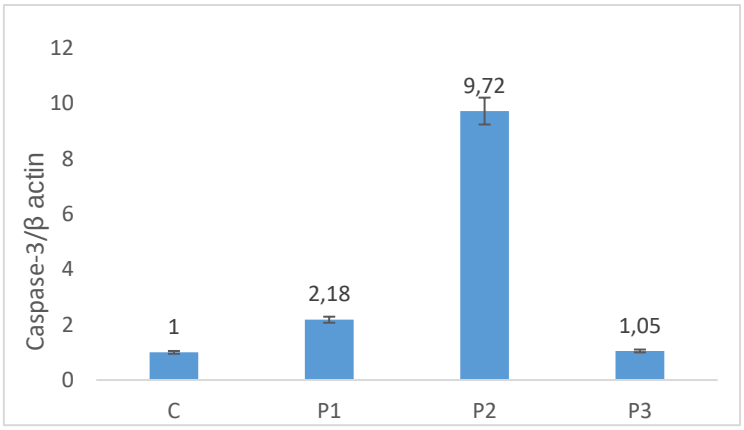


Fig. 3: *Relative gene expression levels of caspase-3 in G361 cells. Effective concentration of molecules were used (P1: 20 µg/ml, P2: 20 µg/ml, P3: 50 µg/ml). The results were normalized with housekeeping gene, β actin. The data is represented as mean ± SEM.*

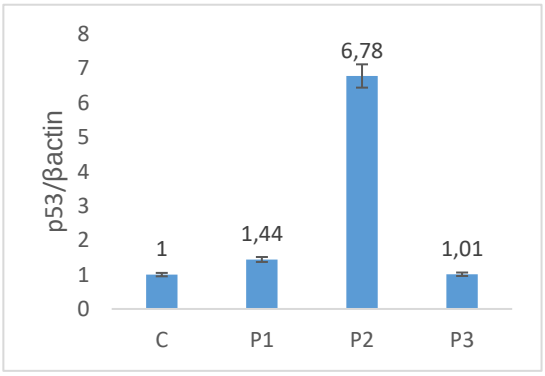


Fig. 4. *Analysis of p53 relative gene expression levels by qRT-PCR technique. Effective concentration of molecules were used (P1: 20 µg/ml, P2: 20 µg/ml, P3: 50 µg/ml). β-Actin was used as housekeeping gene. The data is represented as mean ± SEM.*

In order to identify possible activation mechanism of mannich bases, they were docked into the zinc-bonded inactivated caspase-6 enzyme and analysed their binding affinity and binding mode. Beside of the mannich bases, PAC-1 was docked into the enzyme and its docking results were used for comparison with those of mannich bases. After the

docking process, pose with highest Glide Emodel in the negative direction was selected as a past pose for PAC-1 and each base. The binding affinity of the best-posed ligands was evaluated based on their Glide Scores presented in Table 1. Glide scores of PAC-1, P1, P2, and P3 were calculated as -7.076, -4.365, -5.142, and -3.942 kcal/mol, respectively. Interaction modes of the best-posed ligands were presented in Figure 5 and 7. According to the figures, PAC-1 and P2 interacted with Zn atom and its coordinated Lys36 and Glu244 residues.

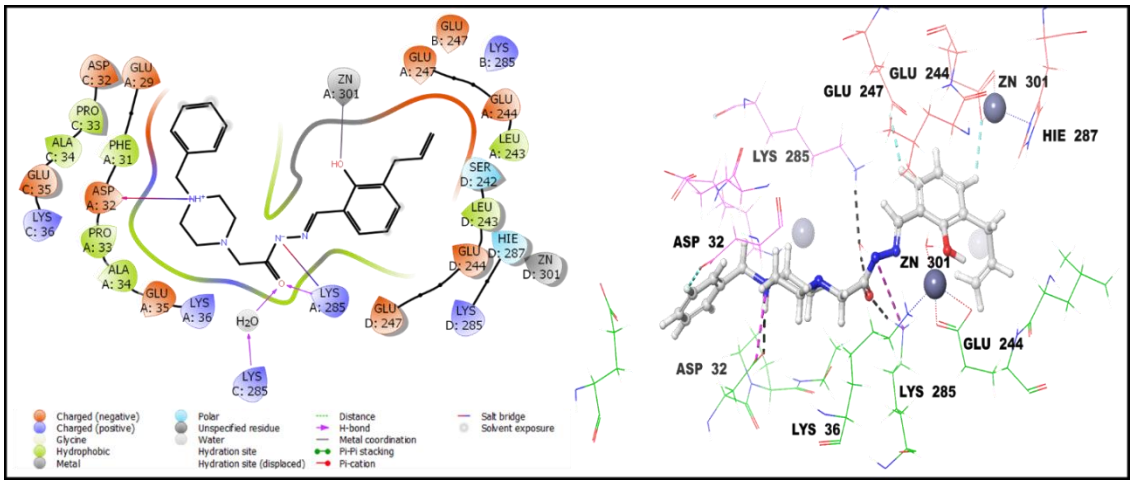


Fig. 5. *2D molecular docking model of PAC-1 into caspase-6.*

Malign melanoma is a fatal skin cancer type and characterized by metastasis that rapidly spreads to other parts of the body, although it is less common than other skin cancers [24, 25]. Existing clinical chemotherapy agents such as cisplatin and nitrogen mustard offer improved treatment options for melanoma patients, but they continue to be problematic especially due to serious side effects such as bone marrow and immunosuppression [26].

In this study, mannich base derivatives (P1-3) in melanoma cells showed dose-dependent cytotoxic effects at different concentrations. Especially P2 has been found to have strong cytotoxic effect. P3 did not show significant cytotoxic effect. Recent studies on the anticancer activity of isatin mannich bases revealed that 1,2,4-Triazoles mannich bases exhibited anticancer activity with cytotoxic effect in different melanoma cells [27]. In addition, Rana et al. [28] reported that isatin derived spirocyclic compounds exhibit cytotoxic effects due to by providing TNF-alpha stimulation and NFkB activation in cancer cells. In addition, Kucukoglu et al. [29] investigated the effects of mannich base hydrazone derivatives on hepatoma and breast cancer cells. According to their results obtained, P7, one of the synthesized molecules, showed a strong cytotoxic effect by affecting the different complexes of the mitochondrial electron transport chain and decreasing the oxygen consumption dose-dependently. Likewise, in our experiment, P1 and P2 of the synthesized molecules were found to have cytotoxic effect on human melanoma cells. Especially P2 compound significantly reduced the viability of the melanoma cells and showed strong antiproliferative effect (Figure 6).

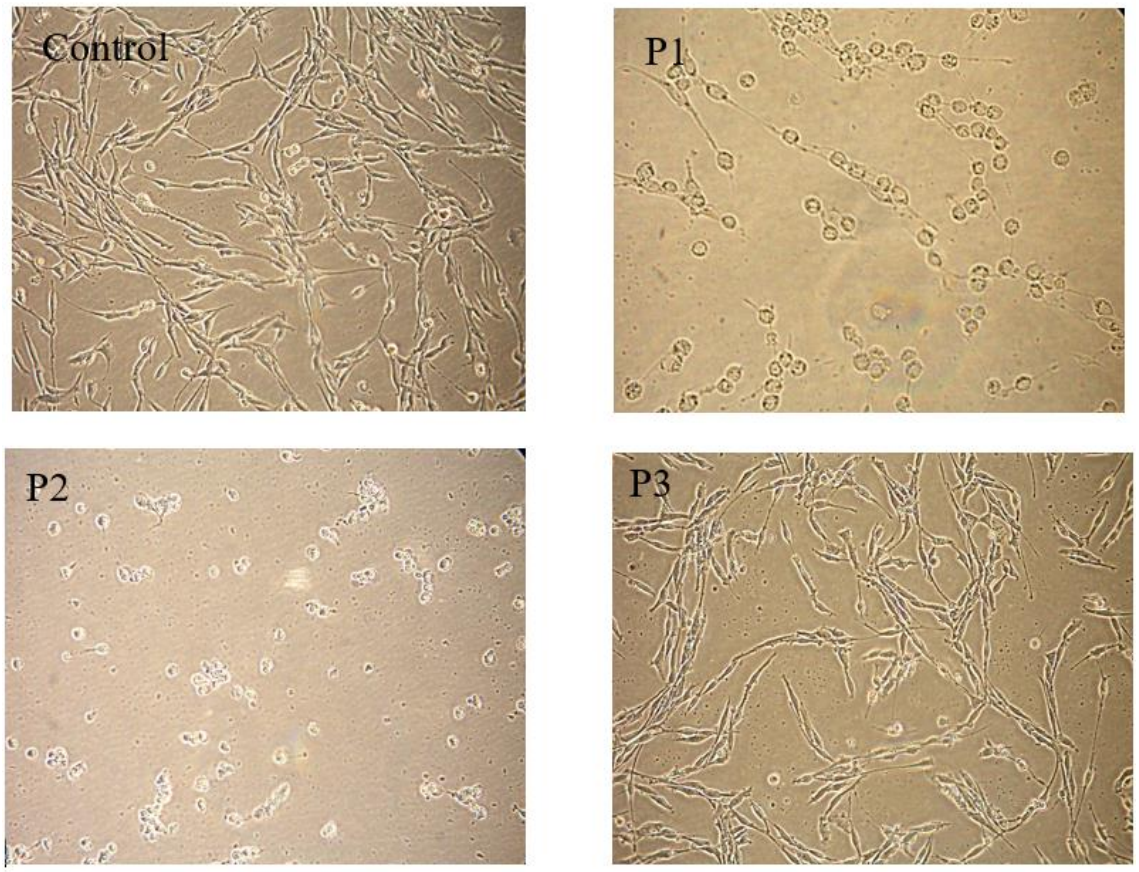


Fig. 6. *Images of human melanoma cells. Cells were photographed by 10-X lens after the application of P1 (20µg/ml), P2 (20µg/ml) and P3 (50µg/ml) (Scale bar 50 nm).*

It is thought that the P2 compound is responsible for this cytotoxic effect owing to the contained morpholine ring in its chemical structure. It is known that compounds containing the basic morpholine ring have pharmacological effects in a very wide range. Some compounds containing dimorpholin derivatives have been reported to trigger various morphological changes in colon and breast cancer and induce apoptosis by stimulating PI3K / Akt / mTOR pathway [30]. However, the other molecule P3 in our study was found to have weak cytotoxic effect according to P1 and P2.

Caspases are important mediators of programmed cell death and proteolytic activation of the caspase cascade is a characteristic event in initiation of apoptosis [31]. Caspase-3 is known to be an effector caspase associated with the initiation of the death cascade and is therefore an important marker in the cell's apoptotic signaling pathway [32]. Some isatin-quinazoline hybrid compounds found to have cytotoxic effects of on different cancer cell lines and this compounds gave rise to intrinsic apoptosis due to increase in caspase-3 levels in HepG2 cell line, a hepatocellular carcinoma, even at very low concentrations [33]. Vedarethinam et al. [34] reported that the mannich base derivative compounds they produced upregulated caspase-3 expression in HepG2 cells, induced apoptosis and showed cytotoxic effect. Szebeni et al. [35] found that the achiral curcumin mannich base derivative compounds exerted cytotoxic effects on apoptosis induction due to an increase in caspase-3 activity particularly in a pancreatic cancer cell line. Gupta et al. [36] showed that some mannich base derivatives exerted cytotoxic effects on colon,

lung and breast cancer by activating caspase-3 and leading to apoptosis. Likewise, all three molecules in our study affected caspase-3 levels in melanoma cells. From these molecules, P2 showed strong caspase-3 activator effect.

The p53 tumor suppressor gene plays a central role in providing cellular responses to various signals such as DNA damage, hypoxia, and oncogenic activation and acts as a transcriptional regulator by controlling cell proliferation [37]. Inactivation or at least partial suppression of the p53 tumor suppressor pathway is thought to occur in almost all human cancers, and inactivation of p53 mutations is the most common genetic alteration in malignancies [38,39]. It has been reported that the frequency of p53 mutations in the melanoma is significantly lower than in other tumors. It has also been shown that p53 function is largely suppressed by many mechanisms in the melanoma [40,41]. In these cancers with suppressed wild-type p53 function, the restoration of p53 activity appears to be an important therapeutic strategy for melanoma and wider cancer therapies [42]. In this context, based on current literature studies it has been reported that synthetic isatin Schiff base derivatives exert strong p53 stimulatory effect in *in silico* and *in vivo* studies, by disrupting the interaction of p53 with proteins that provide ubiquitin dependent degradation [43]. Also, gold (I) N-heterocyclic carbene compounds were reported to induce ROS and p53-dependent apoptosis in the cells by regulating p53, p21, NF-κB, VEGF and MMP-9 proapoptotic factors in melanoma tumor cells (B16F10) *in vitro* and thus show a cytotoxic effect [44]. In accordance with the results obtained in the literature, three of our synthesized molecules were found increase p53 levels. Similar to the results we obtained in caspase-3, P2 was found to have a significantly more effective p53 activating effect. It is thought that these molecules used in the study triggered the mechanism of apoptosis in melanoma cells by activating the expressions of caspase-3 and increasing p53 levels and thus showed antiproliferative effect.

Transition metals, such as zinc and copper lead to inhibition of caspases [45]. For this reason, they suppress apoptosis at their physiologically relevant concentrations [46]. It has been reported that zinc chelators induced apoptosis by activating zinc-suppressed caspases [47,48]. Vela´zquez-Delgado and Hardy [49] have revealed the underlying mechanism of zinc-mediated caspase-6 inactivation. According to their results, Zn atom inhibited caspase-6 by interacting with a site outside of the active sites. The interactions have arisen from the tetrahedral coordination between Zn atom and the enzyme's residues including Lys36, Glu244, and His287. Water molecules in the allosteric site are also important at zinc-mediated inhibition. Huan et al. [16] have indicated that PAC-1 perturbed the tetrahedral coordination by interacted Zn atom, Lys36 and water molecule in the allosteric site. Another study, it has been calculated as -5.03 kcal/mol binding affinity of PAC-1 against caspase-6 [50]. According to our docking calculation, the binding affinity of PAC-1 was -7.076 kcal/mol. Figure 5 has shown that PAC-1 caused perturbation of the tetrahedral coordination by constructing metal coordination with Zn atom and hydrogen bonds, and salt bridges with different subunits of the enzyme. After this finding, mannich bases were docked with the same way and the docking results were compared with those of PAC-1. The binding affinities of mannich bases were presented in Table 1.

Table 1. *Glide scores of isatin mannich bases and PAC-1.*

Compounds	Glide Score (kcal/mol)	Glide Emodel (kcal/mol)
P1	-4.365	-45.600
P2	-5.142	-58.374
P3	-3.942	-45.032
PAC-1*	-7.076	-103.421

*Standard activator for caspase-6.

According to the scores, it has been detected that P2 exhibited highest activator properties than the others. The finding was wholly compatible with *in vivo* results. For this reason, we have concentrated on describing the possible activation mechanism of P2. The compound has constructed metal coordinations with Zn ion of A and B subunits and also hydrogen bond with water molecule into the allosteric site. The interactions were very similar to those of PAC-1. However, PAC-1 has interacted very closely with them and consequently, it has exhibited strong activator properties. P2 formed hydrogen bonds with A:Lys36 (water bridge), B:Lys36, and B:Lys235 residues. Moreover, it has contacted with D:Lys 285 residues through π-cation interaction as seen in Figure 7-b-e.

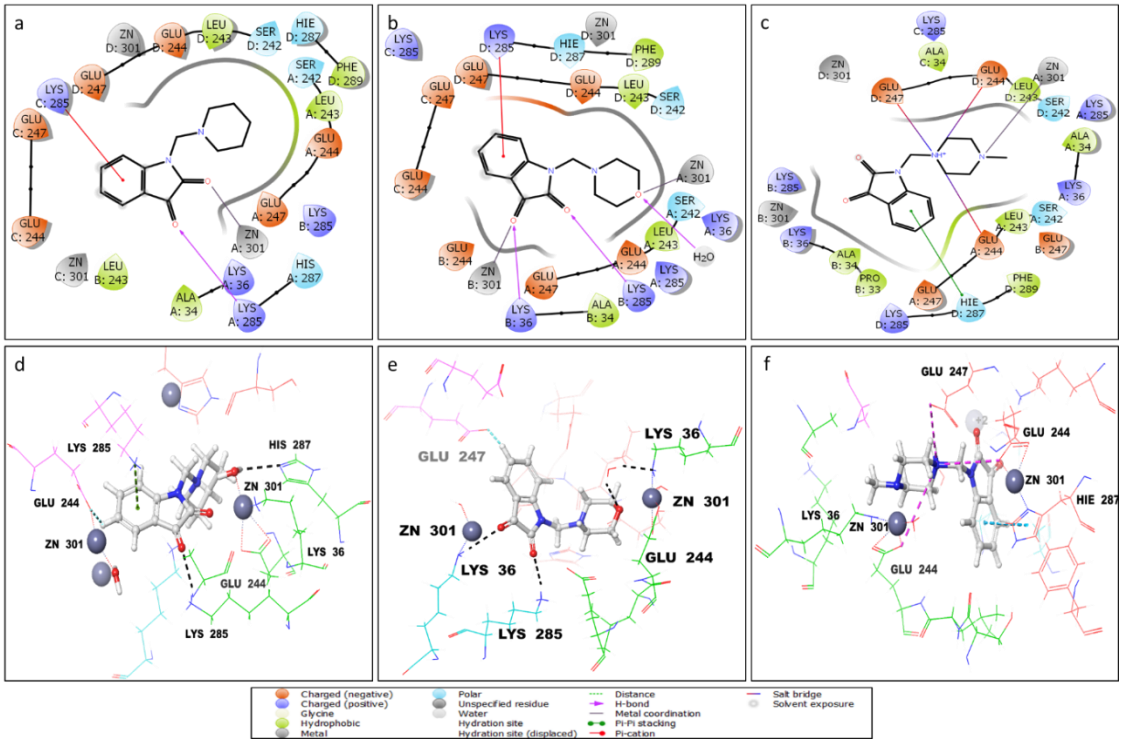


Fig. 7. *2D and detailed binding mode of (a) P1, (b) P2, and (c) P3 into caspase-6. Mannich bases were represented white ball-stick, A subunit was represented as green amino acids, B subunit was represented as turquoise amino acids, C subunit was represented as purple amino acids, and D subunit was represented as red aminoacids.*

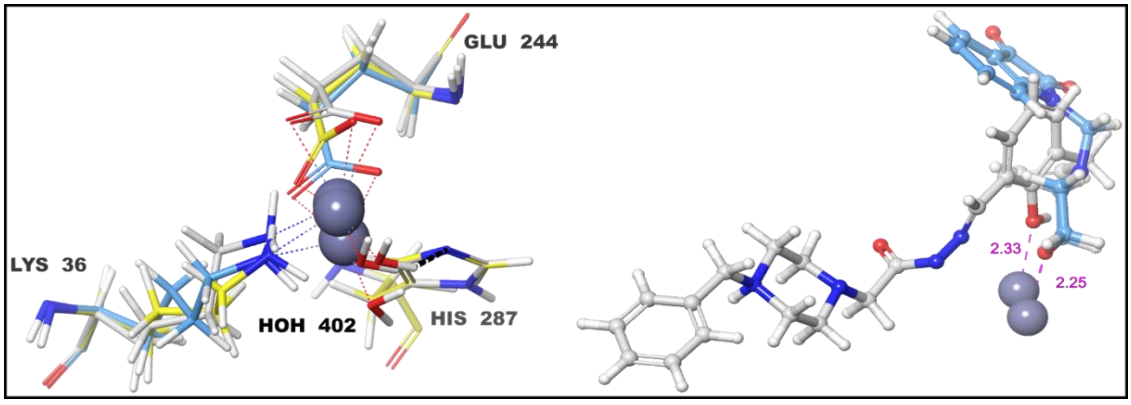


Fig. 8. *Structural changing of key amino acids of enzyme bonded inhibitor and activator. Caspase6$_{native}$ is represented as thick tube, Caspase6$_{PAC-1}$ as white thick tube, and Caspase6$_{P2}$ as yellow ribbon and thick tube.*

We have expected that the compound would construct more than one coordination with Zn ion due to oxygens of the indoline-2,3-dione moiety. However, P2 has activated the enzyme by interacting with mostly subunit residues. Figure 8 shows how the activators affect the structure of Zn-inactivated caspase-6. According to the figure, PAC-1 and P2 have undermined the geometry of tetrahedral coordination built by Zn ion, Lys36, Glu244, and His287. P2 are probably more effective than the other mannich bases due to its morpholine moiety.

CONCLUSION

Isatin mannich derivatives used in the study were found to show antiproliferative effect as a result of all the vitality tests. In addition, it has been found that these molecules induce apoptosis by inducing caspase-3 and p53 gene expression levels. Of these molecules, P2 showed more activity than the other two molecules. Moreover, molecular docking studies revealed that P2 compound is a potent allosteric activator of caspase-3, and morpholine moiety plays a central role at the enzyme activation. This study has brought out potential hits for the design of novel caspases activators. However, advanced molecular techniques and *in vivo* studies are needed to fully elucidate the mechanism underlying this activity of molecules.

Conflict of Interest: The authors declare no potential conflicts of interest with regard to this research, authorship, and/or publication of this article.

Authorship Contributions: Concept: M.M.I., A.K., M.K., D.O.O., T.Y., H.I.G., M.B., Design: A.K., Data Collection or Processing: M.M.I., A.K., Analysis or Interpretation: M.M.I., A.K., M.K., D.O.O., T.Y., H.I.G., M.B., Literature Search: A.K., M.M.I., M.B., Writing: M.M.I.

Financial Disclosure: This research received no grant from any funding agency/sector

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