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Hsp Inhibitor is Affective Against Adenocarcinomic Human Alveolar Basal Epithelial Cells Through Modulating ERK/MAPK Signaling Pathway

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The extracellular signal-regulated kinase (ERK) - mitogen-activated protein kinase (MAPK) pathway regulates cell proliferation, differentiation, and apoptosis. Heat Shock Protein 90 (HSP90) is required to activate proto-oncogenic protein kinases and promotes tumor growth through anti-apoptotic effects on A549-non-small cell lung cancer (NSCLC). Therefore, deregulation of the ERK-MAPK pathway and abnormal expression of HSP90 are reasonably frequent events in NSCLC. In this study, novel perimidine-pyrazole compounds employed to block ERK-MAPK deregulation through inhibiting HSP dependent cancer cell survival mechanisms. A set of perimidine-pyrazole derivatives effects was monitored on NSCLC cell line. Array experiments performed to understand the effect of the compounds on signaling pathways and results were analyzed by gene enrichment analysis. Further, senescence and apoptosis experiments were performed to support the enrichment results along with in silico methods to determine perimidine-pyrazole/HSP interactions. Treatment of NSCLC cells with perimidine-pyrazole derivatives displayed cancer-inhibitory, pro-senescent and pro-apoptotic effects on NSCLC cells through ERK/MAPK pathway and these compounds are promising templates for designing anticancer drugs

Introduction

Lung cancer is one of the most common causes of cancer-related deaths worldwide. Elucidating the molecular mechanisms of lung cancer is of great importance in designing innovative therapies for patients. NSCLC is a complex process and aberrant expression of MAPK is a relatively common event in NSCLC. The MAPK pathway plays an important role in cell proliferation, differentiation, and apoptosis. Increasing evidence supports the deregulation of the MAPK pathway and its association with various types of malignancies, including NSCLC.[1]

MAPK signalling pathway is a novel target for cancer drug studies. PD-184352 was designed to inhibit downstream effector MEK. Although preclinical and phase I clinical trials determined its inhibitory function, the compound was found inefficient by phase II clinical trials, resulting in the withdrawal of the compound.[2,3] However, PD0325901 MEK inhibitor displayed better therapeutic and pharmaceutical activity in NSCLC.[2,4] Additionally, LGX818 targets proto-oncogene B-Raf (BRAF) and inhibits malignant cell lines through blocking ERK phosphorylation and inhibits RAF kinase and mTOR.[5,6] NSCLC patients can now receive approval for treatment with BRAF and MEK inhibitors.[7]

Several small molecules are in phase trials for MAPK pathway at NSCLC treatment. Hub genes on MAPK pathway targeted; for Ras (AMG-510-Phase III; MRTX849-Phase I/II; Rigosertib-Phase I/II), for Raf (Dabrafenib + Trametinib-Approved), for MEK1/2 (RO5126766-Phase I; Trametinib + Dabrafenib-Approved; Mirdametinib + Palbociclib-Phase II; AZD6244-Phase II), for ERK1/2 (Ulixertinib Phase I/II) distinct small molecules designed.

The MAPK pathway, which is commonly suppressed in cancer, presents a viable target for innovative cancer therapy. It is crucial for appropriate drugs to be specific to the target and potentially less harmful compared to traditional cancer treatments. EGFR inhibitors effectively bind to EGFR, thereby inhibiting its overexpression and proliferation in NSCLC by

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reducing the binding of alternative ligands. Both gefitinib and erlotinib, FDA-approved EGFR inhibitors, have the potential to be utilized in the treatment of NSCLC. Trametinib and cobimetinib, two MEK inhibitors, have received approval from the EMA and FDA. In a pre-clinical study, trametinib demonstrated inhibition of tumor growth in a xenograft model. Cobimetinib, on the other hand, is an extremely selective and potent MEK inhibitor that has shown significant activity in xenograft models using KRAS- and BRAF-mutated cell lines in vivo. Trametinib and dabrafenib have been granted breakthrough designation by the FDA for BRAF-mutant NSCLC, and their combination has also been authorized. The combination of MEK and BRAF inhibitors has provided a new therapeutic option.[8]

However, no specific single compound approved for NSCLC, rather combinatory drugs were approved. Further, single treatments had promising efficiancy initially but the effect of the compound gradually abolished by drug resistance.[9–11] This combined action may depend on nature of MAPK signaling which consists of three-kinase cascades (The kinase cascades are MAPKKK upstream kinase, MAPKK middle kinase, and MAPK. MAPKs are protein kinases (i.e JNK, ERK-MAPK)). Further, the pathway involves in cell survival, differentiation, proliferation, metastasis and apoptosis in which the three-cascades may cross talk with other signalling pathways and form multiple targets to be suppressed for cancer treatment.

This current research determined MAPK involvement in immune system and in senesence as revealed by array experiments.[12] Therefore, understanding molecular mechanism of cancer biology has allowed to determine novel targeted approaches for NSCLC treatment. Moreover, MAPK cascades are crucial in NSCLC and kinase proteins are substrate for HSP90. HSPs regulate numerous signal-transduction and receptor regulatory kinases. Key results from the experiments are supported by literature. Thus, main findings are:

i. inhibition of deregulated kinase pathway through HSP90 inhibition is an innovative drug targeting approach.[13,14]
ii. since metabolism of cancer cell is higher and fully folded proteins are required for the protein biochemical functions, cancer cells overexpress HSPs to assist oncogene protein folding, along with modification and transport.[15] However due to hepato-toxicity and poor solubility, HSP-inhibitor formulations are poorly tolerated up till now.[16] HSP inhibitors are disappointing and novel-target specific designs are required. Results indicated that perimidine-pyrazole template seems a fine structure for deregulated MAPK pathway.
iii. HSPs are essential for tumor cell survival by regulating substrate protein homeostasis. HSP70 can inhibit apoptosis and HSP90 regulates ERK-MAPK pathway; thus, HSP70 and HSP90 regulate NSCLC growth.[17,18] Dual HSP inhibitor (inhibition of both HSP90 and it's functionally complementer structure HSP70) may provide efficient cancer treatment. Pyrazoles in our earlier work displayed dual HSP70 and HSP90 inhibition.[19,20]
iv. p53 protein can induce senescence followed by apoptosis in cells with unrepairable damage. Increase expression of MYC, p53, p16 and decrease expression levels of cyclin D1 and CDK4 supports senescence character as determined by array experiments. The senescence A549 cells are driven to apoptosis by perimidine-pyrazole compound 7 (**PP7**).[21,22]
v. senesence induces an unfolded protein response in the endoplasmic reticulum because of an accumulation of unfolded proteins, resulting in proteotoxic impairment of cell function. Cells can undergo acute senescence where senescent cells are permanently withdrawn from the cell cycle. Acute senescence cells suppress tumorigenesis and the immune system clear these cells. However, perturbation of the immune system, as monitored in certain cancer cells, may not kill senescent cells and this may contribute to tumorigenesis.[23] The compound **PP7** created response in immune system genes: a. Cytokine signalling; b. Interleuking-4 and interleukin-13 signalling; c. Toll-like receptor cascades; d. Signalling by interleukins; e. Growth hormone receptor signalling; f. Interferon signalling; g. JNK signalling, and h. MAPK. Thus, the compound **PP7** not only inhibits HSPs but also induce immune system and these help suppression.
vi. The **PP7** compound derives senescent NSCLC cells to apoptosis and this is recently described process in cellular death.[24–26]

Therefore, to inhibit deregulated MAPK-ERK and employ so called acute senesence innovative perimidine-pyrazole compounds examined. Preliminary results indicated the compounds potential effectiveness. Pyrazole moiety used as dual HSP70 and HSP90 inhibitor functional group as identified in our previous work and the inhibitor was patented.[24,27]

This study focuses on changes in the MAPK signaling at the molecular level in the presence of PP inhibitors. Some of the clinical drugs resist NSCLC patients over time in the treatment. Therefore, treatment requires innovative inhibitors for novel drugs.[28,29] There are several promising compounds, but designing pharmacologically active agents from existing backbones/functional moieties with potential medical properties is interesting. Pyrazoles are well studied and exist in pharmaceutically active compounds. Pyrazole scaffold has been used in anticancer, antiinflammatory, antibacterial, antifungal, and analgesic agents.[29] Furthermore, perimidines are interesting N-heterocycle class agents in medical sciences. Perimidines interactions with distinct proteins make them susceptible for drug templates.[30] Preliminary experiments in *in silico* studies provided an insight about the functionality of pyrazole-perimidines combinations. Therefore, a set of these agents synthesized and tested on NSCLC cells and A549 human lung adenocarcinoma cell lines was selected to monitor the inhibition of proliferative pathways (Figure 1).

In the current study, we sought to characterize HSP70 and HSP90 as potent regulators of NSCLC growth. Our results indicate that differential expression of HSP70 and HSP90 is associated with the malignant phenotype of NSCLC cell lines and plays an important regulatory role in NSCLC cell proliferation. Moreover, a specific inhibitor of HSP70 and HSP90, **PP7**, significantly inhibits NSCLC proliferation.

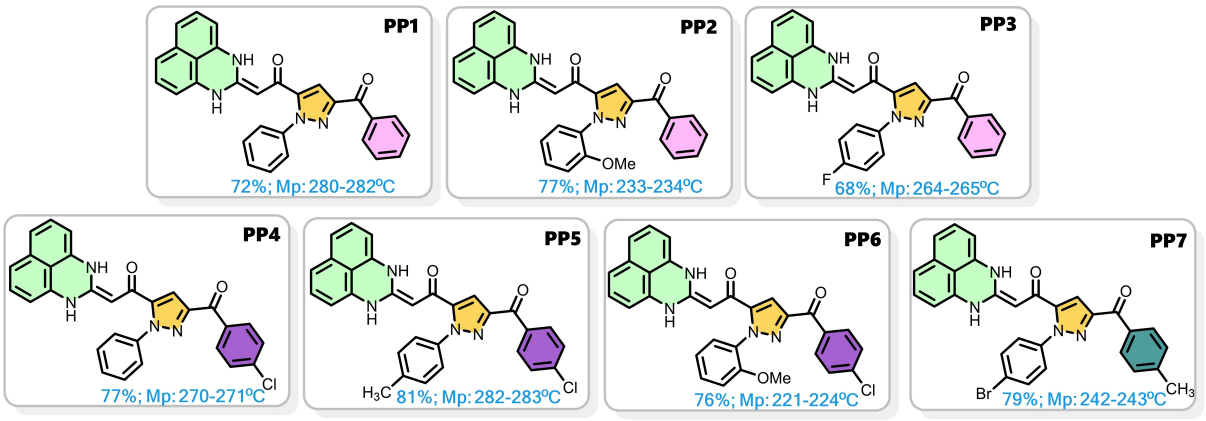

Figure 1. Perimidine-pyrazole compounds.

HSP90 and HSP70 play a key role in folding substrate protein signaling associated oncogene folding to make these proteins functional during proliferation and metastasis.[31–33] HSP90 and HSP70 are known to be involved in the proliferation and survival of cancer cells as well as resistance to anticancer drugs.[34] Cancer stem cells (CSCs) cause tumor recurrence and HSP system plays an essential role in several oncogenic proteins as well as in CSCs protein activity.[35,36] CSCs activates transcriptional activation of HSP system proteins.[37] MYC, NANOG, SOX2, and POUF5F1 genes are well known for their roles in CSCs pluripotency. Perturbation of HSP system through perimidine-pyrazole compound decreases the expression levels of NANOG. These genes activate genes related to proliferation and repress genes related to differentiation.[38] Pathway analysis revealed STAT3, SMAD2, SMAD4, and ICLF4 expression alterations as a result of a decrease in expression of pluripotency genes. CSCs presumably resist HSP system-related inhibition, but overall, the NSCLC cells are driven to apoptosis. Hence, targeting HSP70 and HSP90 may affectively eliminate both CSCs and non-CSCs for cancer treatment through both senescence and apoptosis.

This study employed perimidine-pyrazole compound **PP7** to induce immune response and HSPs at NSCLC cells and treatment with perimidine-pyrazole derivative displayed cancer-inhibitory, pro-senescent and pro-apoptotic effects on NSCLC cells through ERK/MAPK pathway.

Results and Discussion

Cell Viability Assay: Compound **PP7** displayed significant inhibition effect while rest of the PP compounds exerted their effect over 20 μM after 24 hours. Substitute halogen groups (F, Cl), particularly heavier bromine, were used to understand the influence of interaction and compound selectivity. Fluorine is small and highly electronegative (**PP3**) and proposed to have profound antineoplastic activity but the compound activity is optimum at higher concentration. Further, substitution of fluorine with a methyl group (**PP1**) displayed lower activity as that of fluorine. **PP7** has bromine at the same position and this small molecule has apoptotic and potential antineoplastic activity. Thus, larger bromine in a key position result in the optimum pharmaceutical effect (Figure 2).

RT-qPCR Experiments: A signaling PCR array was employed to understand the effect of **PP**s on A549 cell line. Expression alterations of these genes were used to elucidate key pathways (Figure 3). Compound **PP7** has a profound effect on A549 cell line (Figure 4). **PP7** has multiple effects on the cell line. **PP7** induces senescence through C/EBPβ and immune response by ERK/MAPK. The compound blocks STAT3 and SMAD 2–4 to halt cell cycle and cell growth, respectively. Tumor suppressor FOXO1 expression decreases as a result of oxidative stress and the inhibitor blocks this effect also. Furthermore, **PP7** represses NF-KB to interfere with HSP dependent survival mechanism. Overall, the process leads A549 cells to apoptosis.

The gene expression difference revealed that **PP7** decreases the expression of survival HSP gene expression. Thus, interaction of the compound with HSP70 and HSP90 ATPase and substrate binding domains showed that binding is optimum for HSP70 ATPase domain (1S3X), HSP90α N-terminal ATP binding (1YC4) and Hsp90β N-terminal domain (6 N8Y) but not for HSP70 substrate binding domain (4WV5) (Figure 5). However,

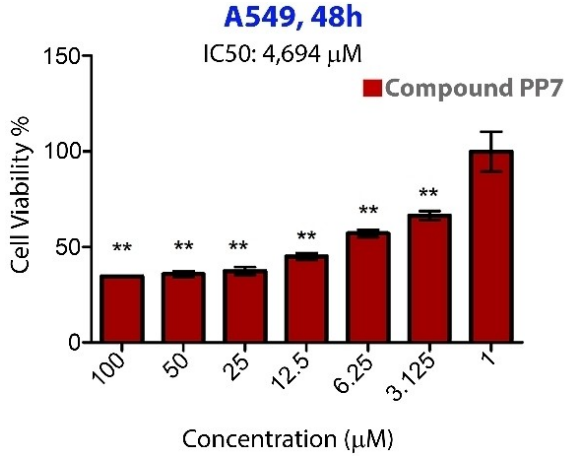

Figure 2. Cytotoxicity of compound **PP7** on A549 cell line.

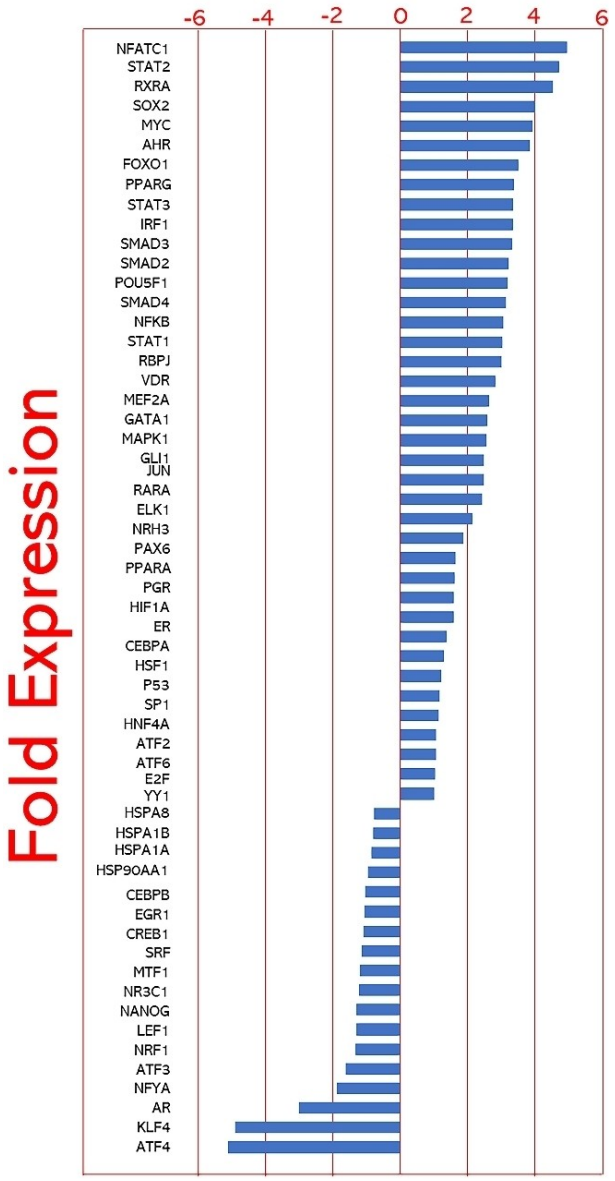

Figure 3. A set of genes to understand signaling pathways in the presence of perimidine-pyrazole compound (**PP7**) antineoplastic effect. PP7 treated and untreated cells normalized against housekeeping genes. Then, PP7 dependent expression is compared to untreated cells. Fold expression differences is given in the figure.

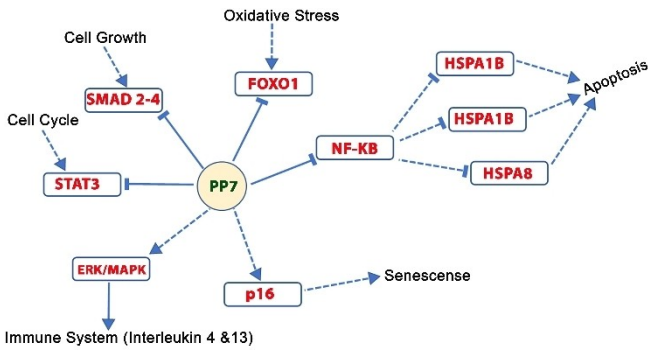

Figure 4. Pathways involved in **PP7** affect on A549 cell line.

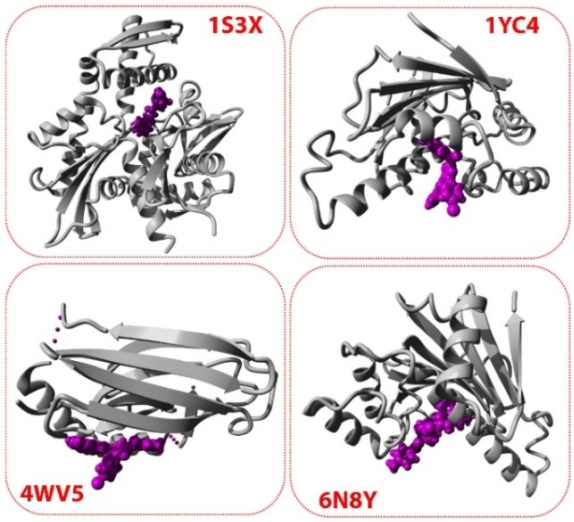

Figure 5. Molecular docking studies of **PP7** with HSP70/HSP90.

PP7 repressed HSPA8 (Heat Shock Cognate 70) gene expression as indicated by qPCR experiments.

PP7 inhibits ERK/MAPK cell signalling pathway and induces cells to apoptosis and senesence. ERK-MAPK pathway is a critical intermediary for cell proliferation, differentiation, and survival. The elevated expression of Dnmt-1 has been reported in carcinomas of the colon, lung and prostate. **PP7** inhibit Dnmt-1 marker (Figure S1). Thus, **PP7** afficiently inhibit A549 cells by ERK/MAPK pathway.

The 3D structure 1S3X at 1.84 Å resolution, 4WV5 at 2.04 Å resolution, 1YC4 at 1.81 Å resolution, and 6 N8Y at 1.55 Å resolution were downloaded from PDB (Protein Data Bank). The binding energies and dissociation constant (KD) values were calculated by YASARA. HSP70/PDB: 1S3X; binding energy −10.61 kcal/mol and KD value 16505.77 [pM], HSP70/4WV5; binding energy −8.28 kcal/mol and KD value 843881.50 [pM], HSP90/PDB: 1YC4; binding energy −8.96 kcal/mol and KD value 267814.96 [pM], HSP90/PDB: 6 N8Y; binding energy −9.35 kcal/mol and KD value 139132.01 [pM]. Furthermore, as shown in Figure 5, the optimum poses of the PP7 molecule in the active site of HSP70/PDB: 1S3X, HSP70/4WV5, HSP90/PDB: 1YC4, HSP90/PDB: 6 N8Y could be observed.

Thus, the compound is proposed to be an ATPase domain inhibitor and this mechanism of action perturbs cancer cell survival since HSP70 and HSP90 are antiapoptotic structures. Since HSPs play key role in senescence and as revealed by gene enrichment analysis, supportive experiment performed (Figure 6). Further, to support cytotoxicity and pathway analysis results, flow cytomety experiments were performed (Figure 7).

The perimidine-pyrazole compounds displayed apoptotic properties compared to that of untreated cells. Minor percentage of cells goes to necrosis (Q1) and viable cell percentage in the presence of inhibitor significantly decreases (Q3) but both early and late apoptotic cell proportion (Q2 and Q4) increases in the presence of **PP7**.

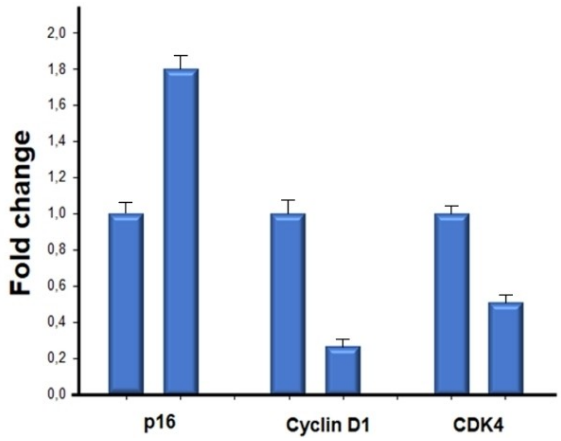

Figure 6. Senescence properties of perimidine-pyrazole compound. **PP7** untreated fold change is unity and PP7 effect on expression differenes of p16, Cyclin D1 and CDK4 is compared to this normalized fold change. P16 (cyclin dependent kinase inhibitor 2 A) increases its expression while a decrease in cyclin D1 and CDK4 is observed. The data indicates senesence of A549 cells.

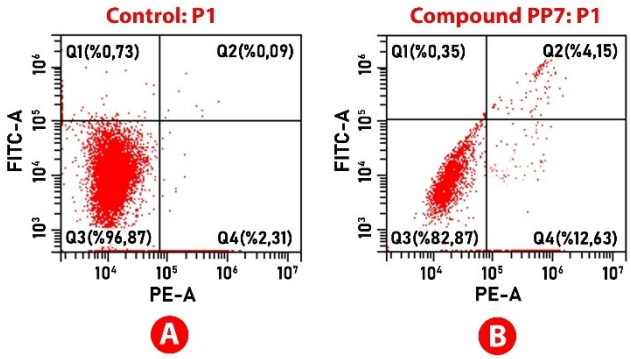

Figure 7. Flow cytometry experiments of A549 cell line in the absence (A) and in the presence of compound **PP7** (B).

Senescence and cell death are fail-safe mechanisms protecting against tumorigenesis. Both these forms of cellular response could be induced in cancer cells, thus suppressing tumor progression. Therefore, to fully understand the chemotherapeutic effects, not only the symptoms of cell death, but also of senescence should be evaluated.

A novel method in tumor biology is to halt proliferation and kill the tumor cells. Senescence cancer cells halt the cell cycle and induction of immune system destroy these cells through apoptosis. Developing senolytics (drugs that specifically kill senescent cells) or suppress the senescence-associated secretory phenotype are the major pathways for this novel method.[39] This study can provide a new HSP inhibitor-**PP7**. Cells in senescence lose proliferative capacity with increased metabolic activity but resist to apoptosis.[40] Senescent cells release proinflammatory cytokines, chemokines with the senescence-associated secretory phenotype and the process adversely affects neighboring cells.[41] This is a especially problematic dogma for tumor cells. Designing a drug to elicit clearance of senescence cells and even trigger an immune response may provide the therapeutic benefits of reducing the senescent cell burden. For this purpose, HSP dependent regulatory pathway to inhibit senescent cell survival perimidine-pyrazole compound screening (Figure 1) identified a hit, **PP7**. During the last decades, it has become apparent that not only apoptosis, but also other modes of cell death, as well as senescence determine the outcome of cancer therapy. The compound is promising in this sense and provides an innovative method for cancer therapy since the compound effects not only senescence but also immune system and pluripotency genes. Potential combination of this drug candidate alone or with clinical drugs may initiate highly effective cancer treatment processes.

Conclusions

Although acute senescence function to avoid malignant transformation, the immune system clears these senescence cells, but chronic senescence promotes cancer development. Perimidine-pyrazole compound (**PP7**) triggers immune response through ERK/MAPK. Further, p53/p21 induces senescence and the compound inhibits NF-κB activation that triggers cell death resistance along with HSPs. All these processes ultimately lead apoptosis of lung cancer cells. Therefore, this promising HSP inhibitor can be used as NSCLC therapeutic agent.

Experimental Section

Cell Viability Assay: Viability analysis was performed with PP inhibitors in A549 cell line (ATCC). 24 hours prior to inhibitor addition, the cell lines was seeded (5,000 cells/well) in 96-well culture plates. Cells incubated for 48 hours after the inhibitor (100-1 μM) were added. Cell viability was determined using the MTT assay kit (Sigma) according to the manufacturer's instructions. The IC50 is defined as the concentration that causes a 50% reduction in absorbance relative to the negative control. The IC50 value was determined by nonlinear regression analysis using Sigma Plot (Systat Software).

RT-qPCR Experiments: Previously established qPCR experiments were employed by using drug/compound treated cells and compared to that of untreated (Analytik Jena QTower3,Germany). Trizol reagent (Invitrogen, U.S.A.) was used to extract total RNA in strict accordance with the manufacturer's instructions. Total RNA samples with good quality were subjected to reverse transcription to obtain cDNA. cDNA was tested by routine PCR before qRT-PCR. SYBR Green Real-Time PCR Master Mix (SensiFAST, Bioline, U.S.A.) was used to prepare PCR reaction systems. Primers of signal pathway array, resistant gene sets, CSC Marker genes along with four control genes were bought from Merck Millipore, U.S.A..[42] Parameters of PCR reactions were: 5 min at 95○C, then 40 cycles of 15 sec at 95○C and 30 sec at 60○C. Data normalization was performed using 2-ΔΔCT method. Data were evaluated by Reactome analysis (https://reactome.org/PathwayBrowser/#TOOL=AT) and Pathway Mapper (https://www.pathwaymapper.org).[43–45]

Apoptosis Assay: The FITC-Annexin staining method was used to demonstrate apoptosis or necrosis content of the cells. In the staining method, cells centrifuged at 130 g for 5 minutes and washed twice with ice-cold PBS. Then it was suspended in 1X Annexin-V binding solution at a cell concentration of 1×106 cells/

ml. Then, 200 µl of the cell solution was transferred to the tubes and 3 µl of Annexin-V FITC + 2 µl propidium iodide mixture was added to each tube. After 15 minutes of incubation at room temperature, on ice in the dark. The percentage of viable, apoptotic, and necrotic cells was analyzed by flow cytometry (Cytoflex-Beckman Coulter).[46]

Molecular Docking: YASARA software version 20.8.23[47] was used to perform molecular docking of the **PP7** molecule with various selected targets and visualization of the docked complexes. Molecular docking of **PP7** has been performed to compare its theoretical and experimental binding affinities with HSP70 ATPase domain (PDB ID: 1S3X), HSP70 substrate binding domain (PDB ID: 4WV5), HSP90alpha (PDB ID: 1YC4) and HSP90beta (PDB ID: 6 N8Y). The 2D structure of the PP7 molecule was drawn using Chem3Draw Ultra 16.0.1.2 software and was converted into a 3D structure using a PyRX.

Compounds: Perimidine-pyrazole compounds were synthesized as described earlier.[48] Target compounds are obtained after four steps of reaction, starting from acetophenone derivatives. As reagents, dimethyl formamide dimethyl acetal (DMFDMA) was used in the first step, aryl diazonium salts in the second step, ethyl 4-chloro-3-oxobutanoate in the third step, and 1,8-naphthalenediamine in the last step. The yields of perimidine-pyrazole compounds are in the range of 68–81 %. Derivatization of the molecules was carried out using different acetophenones or aryl diazonium salts.

Author Contributions

All authors have made essential contributions to this study. İK, YT: Conceptualization, Investigation, Writing - original draft, Writing - review & editing. YT: Reviewing and Supervision. ST, LT, ENYT Biological Activity Studies, ST: Molecular Docking studies. MG, İK: Synthesis. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

All date were availability.

Keywords: Senescence · Apoptosis · Perimidine-pyrazole · NSCLC · A549 · HSP

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