Potency of Phytocompounds as Hydroxychavicol and Plumbagin in Combination to Fight Against Leukemia via Activation of MAPK-Mediated Apoptotic Pathway: An In vitro Approach

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ABSTRACT

Plants provide natural molecules as an effective agent for phytomedicine. Anticancer properties of natural products are well described. Several studies on hydroxychavicol (HCH), and plumbagin (PLB), which are found in Piper betle leaf and Plumbago sp. respectively, are evidenced as anti-carcinogenic effect on chronic myeloid leukemic (CML) cells through increased reactive oxygen species (ROS). An attempt was taken to determine the efficacy of a new combination of HCH and PLB on CML cells (K562) and the mechanism of apoptosis thereon. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the dose for individual and combination treatments of HCH and PLB against leukemic cells, (K562). Apoptotic activity was assessed through flow cytometric analysis with annexin V-FITC/propidium iodide staining. Immunoblotting were also performed on cell extracts before and after the treatments. Levels of ROS and nitric oxide (NO) in K562 cells were measured by DCF-DA and DAF-FM staining, respectively. In addition, p38 and JNK siRNA transfection were performed to assess the role of mitogen-activated protein kinase (MAPK) pathways on apoptosis. Combination treatments of HCH (16 µM) and PLB (0.5 µM) showed synergistic effects on reducing viability and increased cellular apoptosis of K562 cells. Combined treatments showed elevated reactive oxygen species (ROS) and NO levels than individual treatments of HCH and PLB. Moreover, decreasing the ROS generations by antioxidants/catalase reversed cell deaths and increased viability. Immunoblotting of MAPK pathways components showed reduction of pERK levels, while upregulation pJNK and pP38 levels upon HCH+PLB treatments. Furthermore, silencing of JNK and/or P38 rescued the K562 cells from deaths. The present study indicates combination treatments of HCH and PLB act as a better therapeutic against CML by promoting MAPK-mediated apoptosis via increased oxidative and nitrosative stress. This in vitro approach is the first report describing the mechanism of action of HCH/PLB to fight against Leukemia via interaction with phosphorylated P38.

INTRODUCTION

A hemato-proliferative neoplasm is designated as chronic myeloid leukemia (CML), which is indicated by uncontrolled cell divisions especially myeloid cells in the bone marrow.[1] According to Deininger et al., CML occurs through a reciprocal translocation between two chromosomes viz. 9 and 22, culminating through the genesis of the oncogene like bcr-abl. It was estimated approximately 90% of CML patients have shortened chromosome called "Philadelphia chromosome".[2,3]

It is evidenced from an earlier study that many natural products such as polyphenolic acids, flavonoids, alkaloids, terpenoids, polyketides, saponins and lignans have capacity to inhibit cell proliferation during the development of CML through the induction of apoptosis.[3]

A natural phenolic compound, Hydroxychavicol (HCH) is found in Piper betle leaves with potential anti-mutagenic properties.[4] Previous studies have shown its protective efficacy against multiple cancer cells including oral cancer,[5] prostate cancer and chronic myeloid lymphoma.[6]

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It has been established that HCH was found to induce apoptosis in CML cells through ROS-mediated pathways and activation of mitogen-activated protein kinase (MAPK) pathways.[7]

Another phytocompound, namely Plumbagin (PLB), is a naphthoquinone derived from the plant belonging to genus Plumbago and has been reported having anti-tumor effect against different cancers.[8] This compound is also found in the plants belonging to genera Drosera, Nepenthes, etc.[9,10] Moreover, an early study has shown PLB to have cytotoxic effects on cancer cells.[11] It is well known that this compound affecting a specific signaling mechanism for induction of apoptosis.[12,13] On the other hand, it suppresses the transformation growth factor-β-induction and expression of pro-fibrotic targeted-gene along with the proliferation of fibroblast cell lines.[14]

Further, to prevent carcinogenic effects, it is important to understand the pathways of MAPK because this plays a critical role in cell proliferation, apoptosis, and stress-mediated responses in mammalian cells.[15,16] MAPK consists of three major pathways viz. ERK (extracellular regulated kinase), JNK (c-Jun N-terminal kinase) and P38 pathway. Although the pathway of ERK1/2, is implicated in cell differentiation and proliferation, the JNK and P38 pathways are promoted by cellular stimuli and stress, such as inflammation and oxidative stress. Intracellular oxidants, within the limited threshold, are critical regulators of many cellular functions, whereas above threshold level they may cause damage to nucleic acids, proteins, and lipids.[17,18] Higher levels of ROS are associated with dysregulated redox homeostasis and are partly responsible for the increased proliferation of tumor cells. However, as cancer cells are more prone to cellular damage, further alleviating oxidative stress by chemotherapeutic agents leads to targeted apoptosis of malignant cells.[19,20] An induction of reactive oxygen species/reactive nitrogen species (ROS/RNS), directly and/or indirectly, plays a critical role in the activation of MAPK signaling.[21] Moreover, oxidative stress promotes ASK-1 mediated activation of JNK and P38, and it was documented earlier that P38alpha MAPK acts as a sensor of ROS in tumorigenesis.[22] Additionally, intracellular accumulation of H₂O₂ promotes sustained activation of JNK by inactivating MAPK phosphatase.[23] While JNKs and their downstream targeted JNK have been implicated in H₂O₂ induced apoptosis.[24]

Interestingly, the anti-carcinogenic effect HCH and PLB in an individual efficacy has been reported; however, combining these two phytocompounds after extraction is the first-time endeavor as anti-CML. Herein, we are attempting to know the efficacy of combining two phytochemicals HCH and PLB on CML using K562 cell model and the underlying mechanism for activation of apoptosis.

**Material and Methods**

**Reagents and Antibodies**

As per the protocol of Chakraborty et al.[7] and Chaudhuri et al.,[25] Hydroxychavicol were synthesized. Different chemicals of AR grade were purchased and used in the present study. N-acetyl-L-cysteine (NAC), 4′,6-diamidino-2-phenylindole (DAPI), 2′,7′-dichlorofluorescin diacetate (DCF-DA), dihydroethidium (DHE) were purchased from Calbiochem, San Diego, CA, USA; poly ethylene glycol conjugated catalase (PEG-cat) was purchased from Sigma Aldrich, St. Louis, MO, USA; Plumbagin, Propidium Iodide (PI) were purchased from MP Biomedicals, Santa Anna, CA, USA; and MTT (3-{4,5-Dimethylthiazol-2-yl}-2,5-diphenyltetrazolium bromide) was purchased from USB corporation, Cleaveland, OH, USA. Antibodies specific to cleaved caspase 3, cleaved caspase 7, cleaved caspase 8, cleaved caspase 9, cleaved PARP, antibodies to phospho JNK (pJNK, Thr183/Tyr185), pP38 (Thr180/Tyr182), pERK1/2 (Thr 202/Tyr204) were purchased from Cell Signaling Technology, Danver, MA, USA. Different antibodies for JNK1, P38, ERK1, Bax, Bid, Bcl-xL, and Actin were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

**Culture of Cell Lines**

CML cell line i.e. K562 was purchased from American Type Culture Collection (Manassas, VA, USA) and was cultured as per protocol of Lozzio and Lozzio. [26] RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin G, 100 μg/mL streptomycin (from Life Technologies) was used as per the study of Chaudhuri et al.[25]

**Assessment of Cell Viability**

Cells were incubated with varying concentration of HCH and PLB either individually or in combination. Cell viability was conducted by using MTT assay as per the earlier study.[26]

**Assessment of Apoptosis**

The apoptotic assay was performed using 24 well plates in which 1×10⁵ cells were plated. The apoptosis rate was estimated after treatment with the phytocompounds followed by staining with FITC conjugated annexin V and PI. The analysis was done in FACS Aria (Model: Becton Dickinson, San Jose, USA).[27]

**Measurement of Intracellular ROS**

Intracellular ROS were estimated after staining with DCF-DA, following the study of Rakshit et al.[28] Briefly, treated and without treated K562 cells were incubated for 20 minutes at 37°C with 10 mM DCF-DA, washed with PBS and measured in a flow cytometer (Fluorescence-activated cell sorter).
Estimation of Intracellular Nitric Oxide
For measurement of intracellular nitric oxide (NO), treated and without treated cells were incubated with the cell-permeable dye DAF-FM (3 mM) for 45 minutes at 37°C and cells were washed with PBS and measured in a flow cytometer as per the protocol of Dias et al.[29]

Immunoblotting Assay
The immunoblotting was carried out as per Chaudhuri et al.,[25] the lysis of cells was performed by incubating the cells in radioimmunoprecipitation assay (RIPA) buffer, followed by sonication and centrifugation. About 50 µg total proteins from the cell free extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) containing 8–12% polyacrylamide, transferred to polyvinylidene fluoride (PVDF) membrane and probed with specific antibodies. Blots were studied through chemiluminescence method by using ECL chemiluminescence kit developed by GE Healthcare Biosciences, PA, USA.

Assessment of siRNA Knockdown
The cells (K562) were transfected with untreated siRNA or treated siRNA (purchased from Dharmacon, Laffayette, CO, USA). Transfection reagents (purchased from Santa Cruz Biotechnology) were used for their respective set of siRNAs. Transfections were carried out for 48 hours.[25]

Immunofluorescence Study for Protein Expression
Treated and untreated cells in confocal dishes were first fixed with 4% paraformaldehyde, and then blocking was performed using 5% BSA in TBS (20 mM TrisHCl, pH 7.4 containing 150 mM NaCl) for 2 hours at room temperature followed by the incubation overnight at 4°C in primary antibody solutions (1:500 dilutions in TBS with 1% BSA) in a humid chamber. The cells were washed thrice with TBST (20 mM TrisHCl, pH 7.4 containing 150 mM NaCl and 0.025% Triton X-100) followed by incubation with FITC conjugated secondary antibody (purchased from Santa Cruz Biotechnology, USA) solution (1:200 dilutions in TBS containing 1% BSA) for 1-hour at room temperature. Finally, cells were washed three times with TBST, followed by counter staining with DAPI. The images were observed in STED confocal microscope.

Statistical Analysis
Data were expressed as mean ± SD (triplicate experiments). Protein band intensities were quantified by densitometric analysis using Lab image (version 2.7.1, Kapelan GmbH, Germany) tool. The statistical analysis was performed using GraphPad Instat-3 tool (version 3.06, San Diego, California, USA). Comparison between groups was performed using one-way analysis of variance (ANOVA). The p < 0.05 was considered as statistically significant.

Results
Combination of HCH and PLB Induced Apoptosis in K562 Cells
The present investigation is a combination approach of HCH with PLB to investigate the cytotoxic effects on CML, specifically on K562 cells (Fig. 1A). MTT assay showed that individual treatments of HCH and PLB reduce cancer cell viability in a dose-dependent manner. With a relatively lower dose range (0.5–2 µM), PLB was more effective in reducing K562 cells’ viability than HCH (8–32 µM). Furthermore, combining both compounds showed a synergistic effect in reducing K562 cell viability (Fig. 1A). The morphology of the cells was healthy and round during normal growth but became disintegrated and fragmented with HCH (16 µM) or PLB (0.5 µM) treatments. However, combination treatments resulted in shrunken and apoptotic cells (Fig. 1B).

The reduction in viability was attributed to increased induction of apoptosis in K562 cells. Cell sorting analysis with Annexin V-propidium iodide staining showed that independent treatments of HCH (16 µM) and PLB (0.5 µM) induced significant apoptosis of K562 cells. However, a combination of both compounds (HCH 16 µM with PLB 0.5 µM) promoted both early and late apoptosis responses (Fig. 1C). Changing the combination doses by increasing any or both the compounds further increased cellular deaths in the cancer cell population (Suppl. Fig. 1).

Combination Therapy Induced Synergistic Oxidative and Nitrosative Stress Responses
Elevated oxidative stress in cancer cells is one of the major factors leading to cell death upon chemotherapeutic treatments. Therefore, we measured cellular ROS and
NO production levels upon HCH and PLB treatments. Individual treatments of HCH and PLB showed a significant increase in ROS productions (DCF-DA fluorescence) for both treatments. Interestingly, PLB showed higher oxidative responses than individual HCH treatments. The combination of both drugs showed a synergetic effect and exhibited significantly higher levels of ROS production in cancer cells, than the individual treatments (Fig. 2A).

HCH individually at a dose of 16 µM, failed to increase the NO levels (DAF-FM fluorescence) in K562 cells. However, PLB treatments showed an increased NO level, and combination therapy significantly elevated NO level as compared to individual treatments (Fig. 2B). Our data indicated that HCH only increased oxidative stress at the given dose, whereas PLB induced both oxidative and nitrosative stress in K562 cells. During combined treatments K562 cells respond to the additive effect of both oxidative and nitrosative stress, which promotes increased cellular deaths.

To revalidate the importance of ROS on cancer cell viability, K562 cells were treated with N-acetyl cysteine (NAC), an antioxidant, along with HCH and PLBs. The apoptosis of K562 cells induced by HCH and PLB, individually or in combinations, was completely reversed with NAC treatment, indicating the relevance of increased oxidative stress on cancer cell viability and death (Fig. 2C).

**Mechanism of Cellular Deaths for Combination Treatments on K562 Cells**

To understand the mechanism of cancer cell death through apoptosis by HCH and PLB treatments, the levels of different apoptotic markers were assessed through immunoblotting. PARP is an important regulator of DNA repair and energy metabolism, and cleaved levels of PARP indicate serious stress and damage to cells leading to apoptosis. Although individual treatments of HCH and PLB did not induce any cleaved PARP, combinatorial treatments showed significant elevation in the cleaved PARP levels (Fig. 3A).

Bax is a proapoptotic molecule that was increased with HCH and PLB treatments and further elevated with combinatorial treatments. Similar to Bax, another proapoptotic molecule BID showed upregulated levels with HCH+PLB treatments as compared to individual treatments. Bcl-XL levels did not change with individual or combination treatments of HCH and PLB in K562 cancer cells (Fig. 3A, B). Moreover, cleaved caspase3 levels were elevated with HCH+PLB treatments. The levels of cleaved caspase-7 were significantly elevated with individual treatments; however, significant changes were observed when the drugs were applied in combination (Fig. 3C and 3D).

Interestingly, caspase-9, an initiator caspase for intrinsic apoptotic pathways, showed increased cleaved caspase-9 with PLB treatments, but not with HCH treatments. This suggested that PLB acts principally through mitochondria-mediated intrinsic apoptotic pathways. Moreover, the activation of intrinsic pathway by PLB, both individually and combinatorically, upregulates more executioner caspases, such as caspase-7. Increased levels of cleaved caspase-8 with HCH+PLB treatments also indicated activation of extrinsic apoptotic pathways in K562 cells (Fig. 3C and 3D).

**Role of MAP Kinase Pathways in Promoting K562 Cell Deaths**

MAPK plays a critical role in cell survival, proliferation, and stress-mediated responses. It was estimated the levels of
MAPK pathway protein after individual and combination HCH and PLB treatments. Vehicle-treated cancer cells showed increased pERK levels, which were reduced upon individual HCH and PLB treatments, and even further downregulated with the combination treatments (Fig. 4A and 4B). On the contrary, to pERK levels, pJNK and pP38 levels showed increased responses with the treatments. Although individual HCH and PLB treatments failed to increase pJNK/JNK levels enough, combination therapy showed significantly elevated pJNK/JNK, pP38/P38 levels also showed a significant increase with individual and combination PLB treatments but did not show any upregulations with only HCH treatments (Fig. 4A and 4B).

Quenching Oxidative Stress Suppresses Apoptosis in K562 Cancer Cells

To investigate the importance of oxidative stress on MAPK activation in K562 cells upon HCH+PLB treatments, we used a known oxidative quencher, PEG-Catalase (500 U/mL) with or without the combinational therapy for 24 hours. The cell extract was used to immunoblot for MAPK markers JNK and P38, and we found that co-treatment of PEG-Cat suppressed the upregulation of phosphorylated pJNK and pP38 levels (Fig. 4C), suggesting oxidative-stress mediated MAPK activation in K562 cancer cells. Interestingly, catalase treatments also attenuated the increased levels of proapoptotic marker BAX, which further indicated oxidative-stress mediated proapoptotic mechanism for the cancer cells. (Fig. 4D).

JNK and P38 MAP Kinase Promotes HCH+PLB-Induced Apoptosis on K562 Cells

To identify which MAPK member is responsible for inducing HCH+PLB-mediated CML cell death, we used small interfering RNAs against P38 and JNK (Fig. 5A and 5B). Control scrambled RNA was used further as vehicle control on K562 cells. Silencing of P38 or JNK was confirmed with immunoblotting and subjected to apoptosis assay with or without HCH+PLB treatments after 18 hours treatments. We immunoblotted BAX from P38 and JNK knockdown cells treated with HCH+PLB (Fig. 5A). Similar to an earlier study, the levels of BAX upregulated with HCH+PLB, however, attenuated with JNK silencing. Interestingly, in P38 silencing, HCH+PLB treatments completely failed to upregulate the BAX levels suggesting its invaluable role in BAX-mediated cellular apoptosis in K562 cancer cells (Fig. 5B).

Not much changes in apoptotic cells were observed with only the silencing of the MAPK members (vehicle treatments). However, with HCH+PLB treatments, the knockdown cells showed significant protection against cellular apoptosis (Fig. 5C and 5D). Upon the combination treatments, the P38 or JNK gene silencing resulted in almost 2-3 folds decreased apoptosis of the cancer cell populations compared to the respective control siRNA treatments (Fig. 5C and 5D).

Expression Pattern of Cleaved PARP and Phospho P38 through Immunofluorescence

The investigation was done on the localization and expression pattern of cleaved-PARP and phosphor-P38 in the K562 cells with or without treatment with compounds for 18 hours treatments. An increased level of cleaved PARP indicates DNA damage and apoptosis. Its elevated

Fig. 4: Assessment of MAPK pathways through oxidative damages and CML (K562) cell deaths. (A) Cultured K562 cells through graded concentrations of HCH and PLB for 18 hours and immunoblotting for 18 hours (A-B) phosphorylated and total ERK, JNK and P38. (B) Densitometric data represents ratio for phosphorylated/total protein levels (mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.001). (C) K562 cells were subjected to combination treatments with or without PEG-catalase for 18 hours and cell extracts were immunoblotted for phosphorylated and total JNK and P38 and (D) Bax densitometric analysis for Bax levels were normalized against β-actin values for 18 hours treatments (mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.001).

Fig. 5: Combination therapy for JNK and P38 MAPK CML (K562) cells. SiRNA were used for knockdown of JNK1 and P38 genes in K562 cells (either alone or in combination) for 18 hours treatments. (A) Immunobloting assay for JNK and P38 to confirm the knockdowns. (B) Analysis of Bax level by immunobloting after 18 hours treatments. (C-D) Flow cytometry assay (annexin V/PI binding) for JNK1 and P38 silenced cells (either alone or in combination) for 18 hours.
HCH and PLB in combination to combat leukemia.

Expression was observed in treatment groups compared to that of control (Fig. 6A). However, phospho P38 has to dephosphorylate before it migrates to the nucleus and act as a transcription factor. We observed increased phospho P38 expression and nuclear degradation in treated cells compared to untreated (Fig. 6B).

**DISCUSSIONS**

Among several phenolic compounds, HCH is well-known phytocompound present in the aqueous extract of the *Piper betle* leaf. [4] Recent studies on HCH provided considerable evidence of its anti-mutagenic and anti-carcinogenic effects, [6] while the mechanism of action yet to be explained. The present study revealed that HCH exerts an anti-cancer effect on CML (K562/B cell lymphoma cell line) by reducing cell viability and increased apoptosis. On the other hand, PLB with a relatively lower dose range has more cytotoxic effects on cancer cells, probably because it is a non-competitive p300 inhibitor and can affect specific signaling mechanisms to induce apoptosis. [4] The cytotoxicity in CML cells may cause from an increased generation of free radicals, ROS and NOS, and subsequent cellular stresses.

Oxidative stress has resulted where ROS-mediated damage of nucleic acids, proteins, and lipids occurs and is implicated in the pathogenesis of several diseases. [30,31] Nitrosative stress, induced by increased NO levels, can have pleiotropic effects on cancers. [32] Nitrosylation of proteins is associated with cellular stress and metabolism, apoptosis, protein phosphorylation, and transcriptional regulations. [33] A low level of ROS is required for the cell signaling pathway in regulating cellular events, while above threshold level is cytotoxic for cells. HCH promotes increased ROS production failed to induce sufficient nitric oxide levels in CML cells. In addition, PLB, however, induced both ROS and NO-mediated stress to induce apoptosis in CML cancer cells. Combination therapy of HCH+PLB showed significantly higher oxidative (ROS) and nitrosative (NO) stress levels than individual treatments and has observed higher efficacy in damaging and inducing apoptosis of CML cells. Moreover, inhibition of oxidative burden by PEG-catalase suppressed the increased levels of BAX and apoptosis, which signifies the importance of oxidative stress in regulating apoptosis of CML cells.

In the present study combination treatments indicated increased proapoptotic molecules in CML cells. Moreover, increased cleaved PARP, a marker of depleted energy in cells [34] and late stages of apoptosis, [35] indicated combination therapy’s effectiveness. Because PLB, in combination or alone, induces increased cleaved caspase 7 and 9, we speculate that PLB induces primarily mitochondria-mediated intrinsic apoptotic pathways. Increased cleaved caspase-8 in HCH+PLB treatments also indicated the involvement of extrinsic apoptotic pathways in apoptosis of CML cells. Furthermore, ROS-NO interplay can be a critical regulator FLIP, down-regulation, which involves Fas-induced apoptosis. [36]

According to different studies, MAPK pathway is an essential regulator for cell survival, proliferation, and stress responses. [15,16] ERK becomes activated (phosphorylated) by external stimuli such as growth factors and cytokines and phosphorylates an array of signaling molecules (including other kinases), and proliferative transcriptional factors for cell survival. [37] We observed, the levels of pERK/ERK were suppressed by individual and combination treatments of HCH+PLB, which suggested synergistic inhibition on cell growth. Furthermore, increased phosphorylation of P38 and JNK in combination treatment indicated an association of stress responses in cancer cells. Attenuation of the oxidative burden by PEG-catalase reduced apoptosis along with the levels of P38 and pJNK, suggesting cooperative relationship in-between. Silencing of JNK or P38 reversed cell death to a similar extent, suggesting the involvement of both JNK and P38 pathways in promoting cytotoxicity of CML cancer cells in combination therapy.
CONCLUSION

In conclusion, the present study revealed that a combination treatment of HCH and PLB exerted better anti-cancer effects on chronic myeloid leukemia cells through increased cellular stress-mediated apoptosis via activation of JNK and P38 MAPK pathways. Our study also suggests using these phytochemicals as potent anti-carcinogenic compounds.

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Supplementary Fig. 1: Different combination treatments with HCH and PLB induces apoptosis in leukemic cells. (A) Flow cytometry assay (annexin V/PI binding) with K562 cells after 24 hours of treatment (either alone or in combination).