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PGV-1 permanently arrests HepG2 cells in M phase and inhibits DMH-induced liver carcinogenesis in rats

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ABSTRACT

Pentagamavunone-1 (PGV-1) has been reported to eliminate cancer cell progression in the breast, blood, and colon. The current approach evaluates its antiproliferative effects and cellular activity against hepatocellular carcinoma cells (HCC). We used the HepG2 cells as an *in vitro* model for HCC, and PGV-1 was tested for its effects on cell viability, cell cycle modulation, senescence induction, reactive oxygen species (ROS) generation, and cell migration. Moreover, the ability of PGV-1 to prevent liver carcinogenesis was tested in 1,2-dimethylhydrazine- (DMH-) induced rats. PGV-1 irreversibly inhibited cell proliferation via mitotic arrest and cellular senescence. The ROS production was enhanced during the earlier hours of incubation with the compound. Later, PGV-1 significantly delayed the HepG2 cell migration and invasion. Furthermore, PGV-1 prevented steatohepatitis upon DMH administration and drastically reduced the Ki-67 expression in DMH-induced rat liver, indicating its ability to suppress aberrant liver cell proliferation. These findings add to the evidence that PGV-1 could be further developed pharmaceutically as a candidate for cancer therapy with a specific target on mitosis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the cancers with the highest propensity to be fatal. The challenge to overcome this type of cancer is significant because, besides being lethal, liver cancer often develops with molecular characteristics and is associated with steatohepatitis (inflammation of the liver with concurrent fat accumulation) (Cholankeril *et al*., 2017; Younossi *et al*., 2019). During the early stage of liver cancer, it can be successfully treated by surgical resection. However, in clinical practices, it is commonly found that HCC cases are diagnosed in the advanced stage; thus, the available options for treatment become limited. Currently, the available agents as the standard therapy for HCC are immunotherapy with antibodies and chemotherapy with kinase inhibitors. Immunotherapy, especially targeting *T*-cell activation for cancer, yields promising results and may extend the patient's life expectancy (Le Grazie *et al*., 2017; Sangro *et al*., 2020; Woller *et al*., 2021). In some cases, immunotherapy increases the quality of life of patients with breast cancer (Mina *et al*., 2019). However, it does not have positive outcomes in liver cancer, especially in liver metastases (Yu *et al*., 2021). Immunotherapy using macromolecules, such as antibodies, tends to be more expensive because it needs special preparation and handling (Riley *et al*., 2019). Thus, developing small molecules with specific targets is still challenging against HCC. Many small molecules targeting protein kinases have been developed and reported elsewhere in various stages, from early development to clinical studies. Sorafenib is one of the successful agents against

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HCC, which is a cellular kinase inhibitor that targets multiple cell surface receptors and the downstream pathways involved in tumor progression (Raza and Sood, 2014). However, sorafenib has some limitations and side effects, such as vomiting, nausea, bleeding, and immunosuppression (Li *et al*., 2015). In addition, this drug does not kill cancer stem cells effectively (Dong *et al*., 2017). These side effects may be due to the nonspecific targets of the drug since protein kinases are common regulators in normal cells, including proliferating normal cells. Therefore, the area of new small molecules is still open to be explored to obtain better properties and efficacy of therapy against HCC.

Here, we proposed a novel compound as a new candidate for HCC therapy, namely, pentagamavunone-1 (PGV-1) (Fig. 1A). This compound is a curcumin analog exhibiting anticancer properties against several cancer cells, including T47D, Michigan Cancer Foundation-7 (MCF-7), 4T1, MCF- 7/DOX, K562, and WiDr cell lines (Lestari *et al*., 2019; Meiyanto *et al*., 2019, 2018, 2014; Wulandari *et al*., 2020). Moreover, PGV-1 exerts a more potent antitumor effect than curcumin and other curcumin analogs (Meiyanto *et al*., 2021). The unique mechanism of PGV-1 involves the induction of cell cycle arrest in the prometaphase (Lestari *et al*., 2019), which is associated with increased intracellular reactive oxygen species (ROS) and senescent cells and interference with the centromere protein cell replication (Lestari *et al*., 2019; Meiyanto *et al*., 2019). Furthermore, PGV-1 induced cell cycle arrest arrest via the mitotic catastrophe mechanism through targeting aurora A kinase A (AURKA), polo-like kinase 1, and cyclin-dependent kinase 1 (CDK1) (Meiyanto *et al*., 2022). The PGV-1 activity is expected to suppress the growth of HCC cells and trigger cell cycle arrest in the mitotic phase. Compared to normal cells, PGV-1 selectivity suppressed the proliferation of cancer cells with fewer adverse effects (Lestari *et al*., 2019).

This study reports the activity of PGV-1 in suppressing the development of liver cancer cells *in vitro* and *in vivo*. We explored the effect of PGV-1 on the proliferative characteristic of HepG2 cells, including the cell cycle process as the major target of PGV-1, cellular senescence, and ROS generation. HepG2 is a liver cancer cell line derived from liver tissue characterized as epithelial-like cells, suitable to be used for this study. Furthermore, we investigated the potential activity of PGV-1 in inhibiting the migratory property of HepG2 cells and its ability to prevent carcinogenesis *in vivo* using the dimethylhydrazine-(DMH-) induced rat liver cancer model. All the results confirm the anticancer potential of PGV-1, especially against liver cancer, which remains a major challenge among cancer diseases.

MATERIAL AND METHODS

Chemicals

PGV-1 was obtained from the Cancer Chemoprevention Research Center, Universitas Gadjah Mada, Indonesia. 1,2-DMH was purchased from Santa Cruz Biotechnology.

Cell culture and animals

The hepatoblastoma HepG2 cells were purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB1054) and maintained in a Dulbecco's Modified Eagle Medium culture medium. For the *in vivo* assay, the experimental study was conducted on adult male Wistar rats (150–200 grams). The animals were purchased and kept in an animal house of the Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada, Indonesia. The animals were fed on standard pellets and water *ad libitum*. They were maintained at standard housing facilities with a temperature of 24°C, 50%–65% humidity, and 12 hours light/dark cycles. The protocol for this experiment was approved by the Ethical Committee of Universitas Gadjah Mada (00001/04/LPPT/I/2020).

The determination of the 50% inhibitory concentration (IC_{50}) score

Briefly, 2.5×10^3 cells were prepared in 96-well plates, grown for 24 hours before incubating with dilution series of PGV-1, and set for 24, 48, and 72 hours. At the end of the indicated time, cell counting kit-8 (CCK-8) (Dojindo, Japan) was added to the well and stored for 2 hours in an incubator before measuring the absorbance at 450 nm in a microplate reader (PerkinElmer). Then, the absorbance was processed to obtain the percentage of cell viability and calculated for IC_{50} of PGV-1 at the specified observation time.

Antiproliferative and drug washout assays

A total of 1×10^5 cells were grown in a dish, treated with indicated doses of PGV-1, and counted for both viable and dead cells for 5 days using trypan dye staining. In another set of experiments, 5×10^4 cells were cultured and incubated with PGV-1, and the cell number (viable and dead) was counted using trypan blue dye. After 3 days, the culture medium containing the compound was replaced with a fresh medium and incubated for the next 3 days.

Cell cycle assay

Cells were grown in a 35 mm dish and incubated with PGV-1, then stained for the DNA content using propidium iodide (PI) (Sigma) solution (with RNase and triton-×100), and subjected to flow cytometry (FACSCalibur). The cell distribution in each phase was processed in Cell Quest, and the percentage of each phase was plotted into a graph for treated and untreated groups.

Mitotic spread assay

Cells were grown on the coverslips and incubated with PGV-1 for 24 hours. Upon the end of the treatment, the cells were washed with 1 × phosphate buffer saline (PBS) before being fixed in 10% neutral buffered formalin (NBF) for 15 minutes. Coverslips were soaked in PBS twice, and 70% ethanol was added to the coverslips for 20 minutes at room temperature before rinsing with water for 2 minutes. Mayer's hematoxylin (Wako) was added to the coverslips for 20 minutes, then rinsed in water for 2 minutes. Afterward, 70% ethanol was added to the coverslip, followed by 1% eosin (in water) for 5 minutes. Eosin was discarded, and coverslips were gradually rinsed twice for 5 minutes in 70%, 96%, and 100% ethanol and xylol (Visagie *et al*., 2014). Lastly, coverslips were mounted in microscope slides and observed under phase contrast microscopy (Olympus).

In another experiment, the treated cells (with untreated cells as controls) were added to a hypotonic solution (0.56% KCl) and then fixated with a mixture of methanol: acetic acid (3:1 *v*/*v*). Cell suspensions were carefully dropped onto microscope slides and air-dried before being stained with Hoechst 33342 (Cell Signaling Technology) and observed under the confocal microscope (LSM710, Zeiss). The documented images were used

to count the number of mitotic cells per the total number of cells to determine the mitotic index.

ROS measurement level

Briefly, 1×10^5 cells in a supplemented buffer (10% FBS in PBS) were pretreated with 20 µM dichlorodihydrofluoroscein diacetate (DCFDA) (Sigma) for 30 minutes. Later, PGV-1 was added and stored in an incubator for designated intervals. Upon the end of incubation, cells were subjected to flow cytometry to measure the mean fluorescence and plotted as a fold of untreated cells.

Senescence assay

After PGV-1 treatment for 24 hours, cells were washed with PBS and replaced with fixation solution (glutaraldehyde and p-formaldehyde) for 10 minutes. The solution was discarded, rinsed with PBS before incubating with 0.2% X-gal (Wako) staining solution for 16 hours, and then observed under the light microscope. The quantification of senescent cells was processed through ImageJ and calculated as the percentage of senescent cells from total quantified cells.

Migration and invasion assay

The migration and invasion assays were conducted using CytoSelect™ 24-well cell migration and invasion assay (CBA-100-C, Cell Biolabs). Cells were seeded on the upper surface of migration and invasion inserts in a 300 µl starvation medium. The lower well of the migration plate was filled with 500 µl culture media with 10% FBS. Inserts were cultured in a humidified atmosphere (5% CO_2 at 37°C) for 24 hours for both the migration and invasion assays. The medium of the insert was aspirated, and nonmigratory/invasive cells were removed with a cotton-tipped swab. Cells were stained with cell stain solution, rinsed in dH_2O, and dried. Later, cells that had penetrated the insert pore were extracted, and absorbance at OD560 nm was measured using a microplate reader (SH-1000, Corona Electric).

Induction of steatohepatitis in liver rats

The animals were acclimated for a week before being assigned into four groups, with five rats in each group: untreated, DMH, DMH + PGV 10 mg/kgBW, and DMH + PGV-1 20 mg/kgBW. The rats were subcutaneously injected with 60 mg/kgBW DMH for 16 weeks, except for the untreated group. Treatment groups were orally administrated with PGV-1 (10 and 20 mg/kgBW) via oral gavage twice a week for 16 weeks. Rats were sacrificed under ketamine (KET-A-100, Agrovet Market) and xylazine (Interchemie) 2 weeks after all treatments were completed. The liver was collected and preserved in formalin before the histological and immunohistochemical examination.

Histological examination of liver

10% NBF was used to fix the liver. The liver of each group was rinsed in water, dehydrated and cleared in xylene, and impregnated in parablast. Paraffin blocks serial sections of 5 µm thickness were prepared and stained subsequently with Hematoxylin and Eosin (H&E) solution. The staining was performed and assessed by core facilities at the laboratory of pathology anatomy, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada. Liver damage was observed based on the macroscopic and microscopic appearance under histopathology observation using the microscope (Olympus) in the Department of Pathology, Faculty of Veterinary Medicine, Universitas Gadjah Mada. Moreover, for documentation purposes, the images were directly captured on the computer monitor using Optilab Viewer 3.0 and Image Raster 3.0 in the Laboratory of Pharmacology, Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada.

Immunohistochemical examination

Hepatocyte proliferative activity in the liver after DMH injection was observed using the biomarker Ki-67. Paraffin-embedded tissues were cut using a microtome to a thickness of 5 µm and placed on object glass. Slides were de-waxed and rehydrated with a gradient concentration of ethanol. After blocking with peroxide, the slides were incubated with Ki-67 primary antibody (Reagent Genie, CAB2094) for 90 minutes. This was followed by the procedure of secondary antibody application using the immunoperoxidase technique. Then, the Ki-67 positive cellular index was described as the presence of nuclear staining. All stained nuclei were scored as positive regardless of the intensity of staining. Cell counts were made at ×1,000 magnification using a conventional light microscope (Olympus BX53 Laboratory of Pharmacology, Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada) in 20 randomly selected fields. The percentage of cells expressing Ki-67 was determined by counting three different areas.

The statistical analysis

All the presented data were demonstrated by the mean of three datasets ± SD or SEM. The statistical analysis was determined using Student's *t*-test or analysis of variance (ANOVA) (followed by the Dunnett test against the untreated group) and performed in GraphPad Prism software (version 9.0). The significance mark of each data was interpreted on each figure.

RESULTS

Cytotoxic effect of PGV-1 on HepG2 cells

To understand the possible molecular mechanism of how PGV-1 exerts antitumor activity, we conducted *in vitro*-based experiments using the hepatoblastoma HepG2 cells. We monitored the cytotoxic effects for 24, 48, and 72 hours. The findings indicated few differences in the 24 and 48 hours growth patterns. While at low concentrations (below 1 µM) after 72 hours, it displayed a low decreasing percentage of viable cells (Fig. 1B). The IC_{50} values at 24 and 48 hours were essentially the same, which were 5 µM. However, the IC_{50} value for the 72 hours treatment was 0.3 µM (Fig. 1C). Then, for 5 days, we performed a proliferation assay using a range of concentrations that were either below or above the IC_{50} score (24 hours). The results illustrated that, at all concentrations, the viability of the cells was much lower than that of untreated cells (Fig. 1D). As for the number of dead cells observed, treatments with increasing concentrations of PGV-1 resulted in a noticeable increase in the number of dead cells (Fig. 1E). Therefore, it can be stated that PGV-1 considerably reduced the proliferation of HepG2 cells at a concentration of 2.5 µM (lower than IC_{50}); therefore, it can be used for further studies.

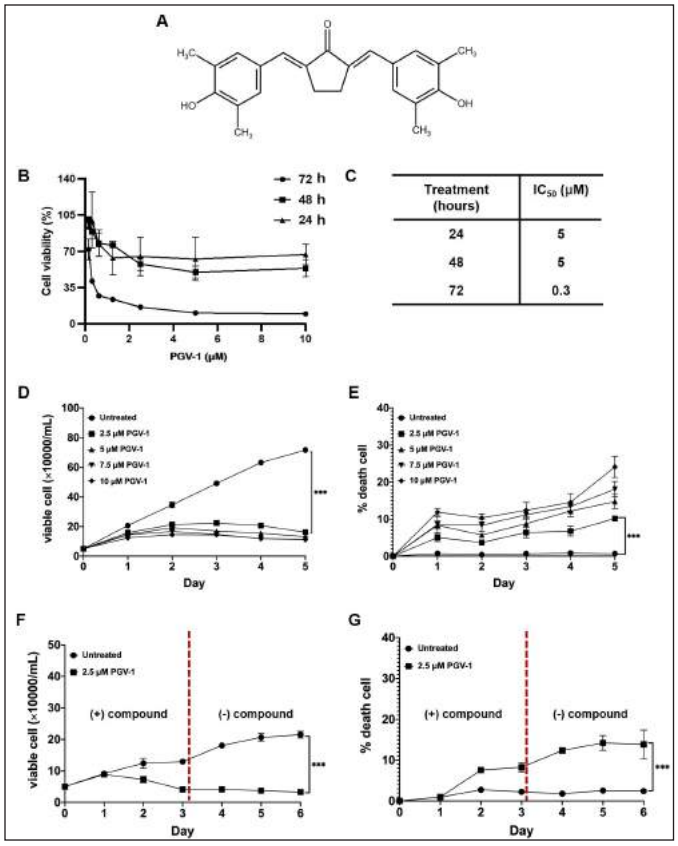

Figure 1. PGV-1 treatment irreversibly suppressed HepG2 cell proliferation. (A) The chemical structure of PGV-1. (B) PGV-1 demonstrated an antiproliferative effect in HepG2 cells. The viability of the cells was measured with a CCK-8 kit. Once the graph was plotted, the 50% inhibitory concentration (IC_{50}) was calculated as presented in (C). (D) PGV-1 suppressed the growth of HepG2 cells, followed by increasing dead cells (E) throughout 5 days of observation. The number of cells (viable and dead) was assessed by the trypan blue exclusion assay. (F) Cells were treated with PGV-1 and counted for viable and (G) dead cells using the trypan blue method. After 3 days, all the medium was replaced with fresh medium (without PGV-1). The number of cells (alive and dead) was counted for the remaining 3 days. The data presented as the average of three replications ± SEM. ANOVA was chosen to analyze the statistical differences between the untreated and PGV-1 groups (***p<0.001).

When PGV-1 was no longer present in the medium, it was essential to understand whether this effect was still maintained. In this experiment, we observed the viability of cells and the number of dead cells before changing the medium after 72 hours, and we counted them again for another 3 days. We used a concentration of 2.5 μM, which was the lowest concentration that still had a cytotoxic effect. In this experiment, we found that even though the medium was changed without adding PGV-1 again, the cell viability continued to decrease until the 6th day (Fig. 1F). This phenomenon was also the same when we observed the number of dead cells (Fig. 1G), which gradually increased on day 6 despite changing the medium at 24 hours without further PGV-1 administration. These findings implied that the cytotoxic effect of PGV-1 is lasting or irreversible, even at lower concentrations.

The effect of PGV-1 on HepG2 cell cycle progression

Cell proliferation is firmly related to the cell cycle program. Therefore, we examined whether the cytotoxic and antiproliferative effects of PGV-1 were associated to the modulating effects of cell cycle progression. In this experiment, we treated the cells with concentrations of 2.5 and 5 μM and observed them for 24, 48, and 72 hours using flow cytometry analysis (Fig. 2A). Surprisingly, PGV-1 triggered G2/M arrest in all treatments and observations, regardless of duration or concentration. G2/M appeared to be declining, especially after 72 hours, while the sub-G1 and polyploidy populations were increasing (Fig. 2B).

Further research into this phenomenon is intriguing since it relates to the potential for changes, particularly in the G2 phase or mitosis. As a result, we used Hoechst and H&E staining to later differentiate between mitosis and the G2 phase. With concentrations of 2.5 and 5 μM, cell accumulation increased in the mitosis phase, as indicated by the significant number of cells with no nuclear envelope (Fig. 2C–F). Based on these findings, a rise in the G2/M population in a flow cytometry analysis corresponded to the increase in the mitotic phase rather than that in the G2 phase. Therefore, it may be inferred that PGV-1 administration during the cell cycle increased the cell population during mitosis and perhaps even during prometaphase.

Senescence induction of PGV-1 on HepG2 cells

Cell cycle arrest due to cytotoxic agents generally results in physiological changes that lead to cellular senescence and apoptosis. This phenomenon was also observed when PGV-1 was applied to various cancer cells (Endah *et al*., 2022; Lestari *et al*., 2019). In this regard, we further examined the effect of PGV-1 on HepG2 cells in terms of cellular senescence induction. The concentrations used in this assay were the same as those in the cell cycle assay. The observations were also carried out at the same time course, 24 hours. The results confirmed that PGV-1 at 2.5 and 5 μM concentrations significantly increased the incidence of cellular senescence (Fig. 3A and B). This effect of PGV-1 on increased senescence was also associated with increased ROS. In this experiment, PGV-1 with a concentration of 2.5 μM increased the ROS level significantly, especially at the earlier observation times (2 and 4 hours) (Fig. 3C). This increase in cellular ROS was also similarly presented with the treatment with peroxide. Overall, these findings would support previous studies on the role of PGV-1 in inducing cellular senescence of cancer and its cytotoxic activity.

Antimigratory effect of PGV-1 on HepG2 cells

To complete the exploration of PGV-1 as an anticancer, especially liver cancer, we further tested the effect of suppression of PGV-1 on the aggressive nature of HepG2 cells based on the invasion and migration phenomenon. In this experiment, we used the Boyden chamber technique with PGV-1 concentrations of 0.5 and 1 μM (Fig. 4A). The concentrations used in this study were far below the IC_{50} value to prevent cell death or significant growth inhibition in the HepG2 cells. We found that PGV-1 at concentrations of 1 μM significantly inhibited HepG2 cell migration but not invasion (Fig. 4B). This migration inhibition against HepG2 cells complemented the cytotoxic effect of PGV-1, suggesting that it could be developed as a potential anticancer agent.

Effect of PGV-1 on DMH-induced cellular damage of rat liver

Next, we challenged the effect of PGV-1 on liver cancer *in vivo*. We used DMH-induced liver cancer rats in this

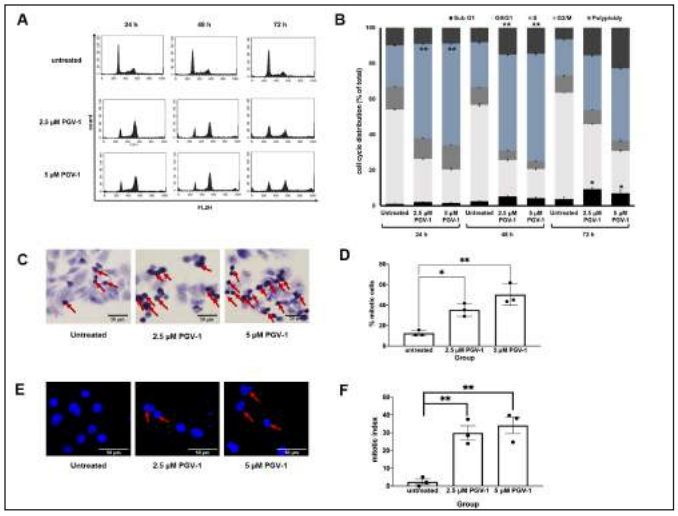

Figure 2. PGV-1 treatment enhanced the accumulation of mitotic arrest cells on HepG2 cells. (A) Cells (untreated and PGV-1-treated groups) were stained with PI solution and subjected to a flow cytometer. (B) The cell cycle phase distribution was plotted into a bar graph based on the incubation period. (C) The mitotic cells after PGV-1 treatment for 24 hours were observed after consecutively stained with H&E, with the red arrow indicating mitotic cells. (D) The quantification of mitotic cells after HE stains. (E) Hoechst staining was applied to cells in mitotic spread assay, and cells were monitored under the confocal microscope. Cells with the red arrow are marked as prometaphase cells. (F) The calculation of the mitotic index was determined based on the quantification of mitotic cells per total cell—the data presented as the average of 3 different field of view ± SEM. ANOVA was used to analyze the statistical differences between the untreated and PGV-1 groups (*$p < 0.05$; **$p < 0.01$).

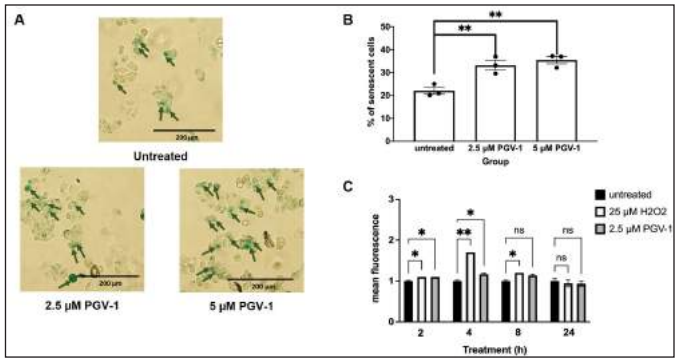

Figure 3. PGV-1 induced cellular senescence and enhanced ROS generation in HepG2 cells. (A) The SA-β-gal assay was chosen to evaluate the effect of PGV-1 after 24 hours in the aspect of cellular senescence. The senescent cells were marked by the green arrow. (B) The positive-senescent cells were counted along with the total cells and calculated as % of senescent cells. (C) Cells were pretreated with 20 μM DCFDA before exposure to PGV-1 and H_2O_2. Treated cells were subjected to a flow cytometer at indicated times (2, 4, 8, and 24 hours), and the fluorescence was normalized against the untreated group. The graphs were displayed as the average of three replications ± SEM. ANOVA was selected to analyze the statistical differences between the untreated and PGV-1 groups (ns, not significant; *$p < 0.05$; **$p < 0.01$).

experiment. The focus of our investigation here is not only on the incidence of liver cancer but also on the carcinogenesis process that occurs because of the DMH-induced damage to liver. DMH is a potent carcinogenic agent that induces cancer in several organs, especially colon cancer, and has been known to cause liver cell damage and steatohepatitis incidence. Technically, we used DMH induction with a concentration of 60 mg/kgBW subcutaneously as described in the method and used PGV-1 concentrations of 10 and 20 mg/kgBW orally (Lestari *et al*., 2019).

Furthermore, we also found that administration of DMH showed signs of steatohepatitis in the liver, as evidenced by the appearance of white nodules in the macroscopic DMH-treated liver (Fig. 5A). Microscopically, we also found that DMH treatment caused cytoplasmic vacuolation and centrilobular necrosis in hepatocyte, indicating liver cell damage (Fig. 5B). From this result, we also revealed that the DMH-treated liver showed a higher density of cells than that in the untreated liver.Meanwhile, the oral administration of PGV-1 at doses of 10 and 20 mg/kg BW did not show any macroscopic white nodules. No changes were observed in the histology or microscopic appearance of the liver after DMH induction, which might result in the inhibition of steatohepatitis occurrence. In addition, DMH-induced liver also showed an increase in the proliferation of liver cells through Ki67 staining (Fig. 6A). Furthermore, we also found that PGV-1 treatment significantly reduced the proliferation of liver cells ($p < 0.001$) due to DMH induction (Fig. 6B). These results support the discovery of the anticancer effect of PGV-1, which has been shown before *in vitro* on liver cancer, thus indicating its potential as a liver cancer agent.

DISCUSSION

PGV-1 has shown promise as an anticancer agent, with breast cancer being the primary target, supported by several different molecular mechanisms (Meiyanto *et al*., 2021). This study further supported PGV-1's potential as an anticancer agent for liver cancer, which was carried out *in vitro* and *in vivo*. The scope of this study covered the physiological effects, which provide essential information regarding the antiproliferative effects of PGV-1 on HepG2 cells. This investigation is a preliminary study to confirm the potential of PGV-1 as a chemotherapy candidate for liver cancer and whether it is not associated with MYCN.

We started this exploration of PGV-1 with an antiproliferative assay on HepG2 cells. Undoubtedly, PGV-1 is a potent cytotoxic effect on HepG2 cells, as indicated by the low IC_{50} value (5 μM) after 24 hours of treatment. Interestingly, this cytotoxic effect appeared to significantly increase 72 hours after PGV-1 administration, with an IC_{50} value of 0.3 μM. This value was relatively lower than that of PGV-1 on 4T1 (Meiyanto *et al*., 2019) or K562 cells (Lestari *et al*., 2019), which indicated that HepG2 was reasonably sensitive to PGV-1. Though there have been some techniques to assess the effect of PGV-1 on those cells, PGV-1 demonstrated an antiproliferative effect across cancer cell lines. Furthermore, the proliferation assay revealed that 2.5 μM PGV-1 could effectively suppress HepG2 cell growth. More interestingly, PGV-1 maintained the suppressive effect even after its removal from the medium, proving that the compound had irreversible activity on HepG2 cell proliferation. This phenomenon showed the possibility of PGV-1 remaining in the cells despite a drug efflux mechanism, which is essential to overcoming cancer cell resistance. The cellular mechanism of this irreversible effect of PGV-1 within cancer cells should be challenging to explore in future studies.

Many physiological processes of cells can be linked to the cytotoxic or antiproliferative mechanisms of anticancer agents.

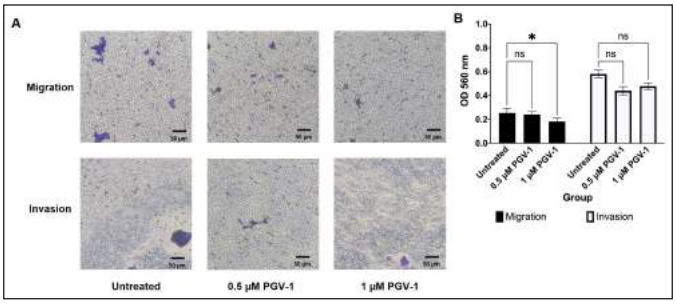

Figure 4. PGV-1 inhibited HepG2 cells' migration and invasion. (A) The migration and invasion ability of HepG2 cells treated with PGV-1 was determined using a Cytoselect™ kit. Cellular images of cells that had passed through the membrane were captured using an inverted microscope. (B) After 24 hours of culture in the chamber, cells that got through the insert pore were extracted, and absorbance at OD560 nm was measured. Data were displayed as the mean ± SEM ($n = 3$) (ns = not significant; *$p < 0.05$).

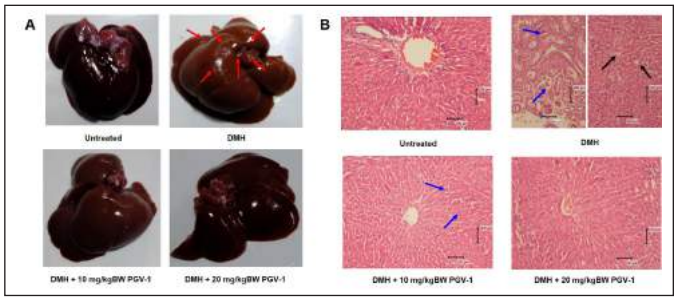

Figure 5. Effect of PGV-1 on DMH-induced cellular damage of mice liver. (A) Macroscopic appearance of the liver after treatment with the respected compound. (B) Microscopic HE stains showing the liver after treatment with the compound. Red arrows indicate steatohepatitis as indicated by blunt edge of hepatic lobe. The black arrow marks vacuolation, while the blue arrow marks necrosis.

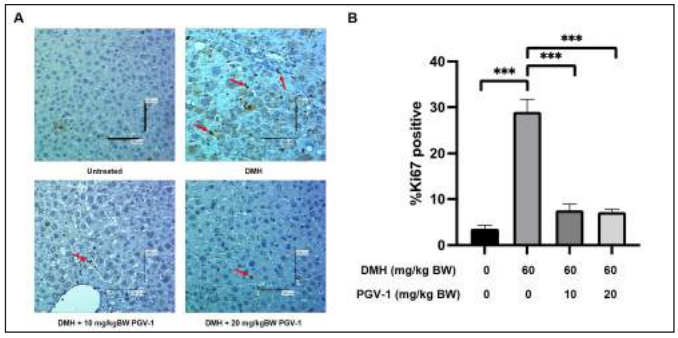

Figure 6. The histologic analysis of Ki-67 expression in liver sections from mice. (A) Immunohistochemical staining for Ki-67. Ki-67-positive cells were shown with the brown nuclear pattern (red arrow) (scale bar = 100 µm). (B) The quantitative analysis of Ki67-positive cells in control and treated groups. Data were averaged from 3 fields of view ± SD (***$p < 0.001$).

We discovered that the cytostatic effect of PGV-1 was closely related to cell cycle (mitotic) arrest, the induction of cellular senescence, and intracellular ROS generation. This phenomenon is typical of PGV-1, which has also been found in other cells, such as 4T1 (Meiyanto *et al*., 2019) and K562 cells (Lestari *et al*., 2019). Intriguingly, the increase in intracellular ROS was not a significant reason for the cytotoxic effect of PGV-1 because this effect occurred at the earlier hours (2 to 4 hours) after treatment. According to the cell cycle profile, the cytostatic effect of PGV-1 on HepG2 cells appeared to be caused by its activity on the cell cycle progression by G2/M arrest, particularly in prometaphase. This finding is in agreement with a prior study on leukemic K562 cells, which reported that PGV-1 promoted prometaphase arrest (Lestari *et al*., 2019). We concluded that PGV-1-treated cells were arrested similarly in cell cycle machinery despite being tested across different cancer cell lines. Molecular studies in triple-negative breast cancer showed that PGV-1 caused the mitotic catastrophe, which was associated with aurora A kinase and cyclin B1 activities (unpublished data). Bioinformatics and molecular docking studies concluded that PGV-1 was predicted to interact with mitotic regulator proteins (CDK1, Aurora A, and WEE1), which indicated that PGV-1 activity in mitosis could be mediated by these proteins (Meiyanto *et al*., 2022). The specific mechanism of PGV-1 during mitosis should be investigated further using the HCC model. Nevertheless, this antiproliferative effect provided vital evidence of the potential of PGV-1 application as a chemotherapy agent for liver cancer.

Exploration of the potential antineoplastic drug requires more than just investigating its cytotoxic and proliferative effects because cancer cells have various features that make them malignant. Cell migration and invasion are essential characteristics of cancer cells in promoting metastasis (Fares *et al*., 2020). Therefore, we investigated this potential further by testing its ability to inhibit cell migration. PGV-1 significantly inhibited the migration of HepG2 cells at low concentrations (1 µM). This outcome was consistent with the findings of the antimigratory effect of PGV-1 through wound healing assay on other cancer cells, such as 4T1 (Meiyanto *et al*., 2019) and T47D cells (Wulandari *et al*., 2020). Moreover, PGV-1 inhibited matrix metalloproteinase-9 expression and activity in metastatic breast cancer cells (Meiyanto *et al*., 2019). These results support the notion that PGV-1 has excellent potential as an antimetastatic agent for cancer cells with malignant properties, such as HCC.

Since PGV-1 has been shown to have unique activities on cellular and physiological levels in HepG2 cells, we assessed the chemopreventive effects of PGV-1 using rodents. Adult Wistar rats were selected as a model to demonstrate the impacts of PGV-1 in mammals. We used DMH injection since this compound selectively targets colon tumor formation (Rosenberg *et al*., 2009); the metabolism of DMH induces oxidative stress and binds the DNA, leading to hepatocarcinoma (Swenberg *et al*., 1979). Thus, this carcinogen has been commonly chosen as a representative to assess the effect of the chemopreventive agent on hepatic molecular status during carcinogenesis steps (Vinothkumar *et al*., 2014). We found that PGV-1 reduced steatohepatitis incidence in DMH-induced rats. Steatohepatitis is an inflammation of the liver cells that interferes with liver function and is an important marker of liver cancer carcinogenesis (Cholankeril *et al*., 2017; Younossi *et al*., 2019). This evidence indicated that PGV-1 could act systematically to inhibit hepatic damage caused by carcinogenic agents. In addition, PGV-1 treatment induced significantly lower Ki-67 expression in the DMH-induced group. The Ki-67 labeling has been prominently used for cancer diagnostics as an indicator of active cell proliferation and is closely related to the prognosis of HCC (Moussa *et al*., 2018). Moreover, *MKI67* (Ki-67 gene coding) is maximally expressed during the G2/M phase (Uxa *et al*., 2021).

Hence, it could be interpreted that the activity of PGV-1 halts the progression of cancer cells (proliferation) on mitosis and could also suppress the early carcinogenesis of liver cancer. The findings of this *in vivo* study supported a prior result that PGV-1 inhibited the growth of K562 cells (Lestari *et al*., 2019) and breast cancer in a xenograft mouse model (Meiyanto *et al*., 2021) with no noticeable side effects. These findings highlighted the importance of PGV-1 in mammalian systems with direct targets on the liver. As a result, PGV-1 is effective when administered systemically to suppress tumor growth (carcinogenesis).

Our current results suggest that PGV-1 remarkably inhibits the liver cancer cells' proliferation and halts cell migration and invasion that may correlate to its target during mitosis. Furthermore, PGV-1 also seemed to prevent carcinogenesis in the liver upon DMH administration in the rats' model. Meanwhile, previous studies using the cancer cell-derived xenograft through subcutaneous implantation (Lestari *et al*., 2019; Meiyanto *et al*., 2021) were still unable to describe the pharmacodynamics of the drug toward the target (liver). Therefore, conducting a comprehensive evaluation of PGV-1 through this study can be a promising first step in developing PGV-1 as an anticancer drug throughout preclinical and clinical follow-up studies. Further investigation of the molecular mechanism given by PGV-1 on cancer cells should be focused on its mechanism related to MYCN/NCYM signaling in HCC since MYCN serves as an essential oncogene for the progression of neuroblastoma, and recently, it has also been associated with the malignancy of HCC. At last, PGV-1 should be developed as an anticancer agent by preparing the compound into a suitable formula that is easy to use and safe for consumption by liver cancer patients.

CONCLUSION

PGV-1 showed potent and permanent cytotoxic effects on HepG2 cells by inducing cell cycle arrest in mitosis, cellular senescence, and formation of ROS. This permanent cytotoxic activity may be correlated with its antimetastatic activity by inhibiting the migration and invasion of HCC cells, preventing steatohepatitis, and significantly reducing cell proliferation attributed to liver carcinogenesis in DMH-induced rats.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/ guidelines.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare relevant to the content of this article.

ETHICAL APPROVAL

The experimental protocol for animal use for this study was approved by the Ethics Committee of UGM, Indonesia (No. 00001/04/LPPT/I/2020).

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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