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Pentagamavunone-1 Suppresses MYCN-Positive HuH-7 Cancer Cell Growth Via Mitotic Arrest *In Vitro*

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ABSTRACT

High expressions of MYCN is usually found in progressive liver cancer. Herein, we examined the possible effect of pentagamavunone-1 (PGV-1) on the MYCN-positive human hepatocellular carcinoma cell line *in vitro* based on cellular physiology and using the MYCN-positive cell line, HuH-7, as a cell model. The cytotoxic impact of PGV-1 was distinguished through the trypan blue exclusion technique. Flow cytometry was used to assess cell cycle alterations and changes in the level of intracellular reactive oxygen species (ROS). The cellular morphology of the cells was evaluated with Hoechst and X-gal staining while the level of MYCN was identified using western blotting. The results demonstrated that PGV-1 was cytotoxic to HuH-7 cells with a GI_{50} of 0.36 μM, which was much lower than that of sorafenib and peretinoin (GI_{50} of 2.07 and 45.6 μM, respectively). PGV-1–induced growth inhibition exhibited a concentration-dependent relationship, even after removal of the drug. Flow cytometry confirmed a cell cycle arrest at the G2/M phase in addition to increased intracellular ROS levels the Hoechst staining showed chromosomal condensation in the mitotic phase, particularly in prometaphase. The X-gal staining confirmed that the PGV-1–treated cells undergo senescence. While PGV-1 treatment was not proven to decrease MYCN expression in HuH-7 cells, overall, these results imply that PGV-1 is still a potential agent to inhibit MYCN-positive liver cancer cells via mitotic arrest.

Keywords: PGV-1, hepatocellular carcinoma, HuH-7, mitotic arrest, MYCN

INTRODUCTION

The upregulation of oncogene expression may promote normal cells to develop into tumor cells, primarily in the growth regulatory system leading to clonogenic cancer development (Hanahan, 2022). *MYCN* is included in a *MYC* proto-oncogene family and is significantly involved in tumor growth (Mathsyaraja & Eisenman, 2016; Suenaga *et al.*, 2009). Amplifications of the gene are frequently associated with a high risk of disease and a poor prognosis (Agarwal *et al.*, 2023; Dzieran *et al.*, 2018). The expressed protein, MYCN, is a transcription factor that belongs to a larger class of proteins which contains a basic-region/helix-loop-helix/leucine-zipper (bHLHZip) important for protein dimerization and sequence-specific DNA

binding (Gherardi *et al.*, 2013). As a transcription factor, MYCN promotes tumor expansion through the upregulation of several oncogenes that are associated either with cell survival or cell division (Montemurro *et al.*, 2019). Initially, MYCN was identified in neuroblastoma (Raieli *et al.*, 2021; Suenaga *et al.*, 2009). However, subsequent discoveries revealed the presence of MYCN expression in hepatocellular carcinoma as well (Qin *et al.*, 2018, 2020), thus making this gene a potential therapeutic target (Ruiz-Pérez *et al.*, 2017).

The incidence and mortality from liver cancer are predicted to increase worldwide (Burton *et al.*, 2021; Petrick *et al.*, 2020; Valery *et al.*, 2018). Hepatocellular carcinoma (HCC) shows a highest incidence among liver cancers (75%–85% of all liver cancer cases) (McGlynn *et al.*, 2021). The principal factors contributing to HCC are the presence of hepatitis B and C viruses, coupled with alcohol consumption, followed by other factors such as nonalcoholic steatohepatitis (Llovet *et al.*, 2021; Singh *et al.*, 2018; Suresh *et al.*, 2020). Although the detection of new cases of liver cancer is not as high as that of breast or lung cancers, the death rate caused by this cancer is the third highest after that of lung and colorectal cancers (Sung *et al.*, 2021). The low prognosis, as well as limited treatment options, contribute to mortality due to liver cancer. Until now, sorafenib has been used as the main treatment of HCC (Keating, 2017). However, sorafenib resistance can occur in patients with advanced and metastatic HCC (Chen *et al.*, 2022).

Research has demonstrated the efficacy of pentagamavunone-1 (PGV-1) as an anticancer and chemotherapeutic agent. PGV-1 is a curcumin derivative synthesized to overcome the limitations of curcumin (Utomo *et al.*, 2022). PGV-1 more effectively reduces the growth of leukemic cells, than either curcumin or imatinib (Lestari *et al.*, 2019). The superiority of PGV-1 is also shown in treating luminal metastatic cancer (Meiyanto *et al.*, 2019), including colorectal cancer, even in animal studies (Lestari *et al.*, 2019; Wulandari *et al.*, 2023). Inducing mitotic arrest at prometaphase and enhancing the production of reactive oxygen species (ROS) are considered to be the key mechanisms whereby PGV-1 hampers cancer growth (Lestari *et al.*, 2019). Additionally, the expression of β-galactosidase in senescent cells is characteristic of PGV-1 treatment on cancer cells that possibly correlates with evidence for apoptosis (Meiyanto *et al.*, 2019). Through bioinformatics studies, recent research also demonstrated the potential of PGV-1 and its derivative, CCA-1.1, in glioblastoma therapy (Hermawan *et al.*, 2022; Hermawan & Putri, 2021).

New findings using MYCN-positive cell lines show that PGV-1 is cytotoxic to the high MYCN-positive JHH-7 cell line (Moordiani *et al.*, 2023) as well as to the low MYCN-expressing HCC HepG2 cell line (Novitasari, Kato, *et al.*, 2023). However, a more comprehensive understanding of this compound needs further investigation using other MYCN-positive liver cancer cells (Eberherr *et al.*, 2019; Yu *et al.*, 2020). Unlike the other two cell lines previously mentioned, the nature of HuH-7 cells is nonviral and derived from well-differentiated HCC (Fukuyama *et al.*, 2021), making this research more challenging.

MATERIALS AND METHODS

Compounds

PGV-1 was synthesized by the Cancer Chemoprevention Research Center, UGM, Indonesia (Utomo *et al.*, 2022). Dimethyl sulfoxide (Merck Millipore, Darmstadt, Germany) was used as the solvent, with a maximum ultimate concentration of 0.1%. Sorafenib and peretinoin were acquired from Sigma (Missouri, USA).

Cell culture and reagents

The HuH-7 cell line sourced from the Nara Institute of Science and Technology in Japan, specifically the Laboratory of Tumor Cell Biology, was obtained from the Japanese Collection of Research Bioresources Cell Bank. Cells were grown in complete media, comprising Dulbecco's modified Eagle medium (Gibco, USA) enriched with 10% fetal bovine serum (FBS) and 100 U/mL penicillin–streptomycin. Cells were cultured at 37°C in a humid environment with 5% CO_2.

Cytotoxicity assay

The trypan blue exclusion technique was employed to ascertain the 50% growth inhibitory (GI_{50}) score (Lestari *et al.*, 2019). The cellular suspension was evenly distributed across a 48-well plate, with each well receiving 2×10^4 cells. Following a 24-h incubation period, adherent cells were treated with varying concentrations of PGV-1, sorafenib, or peretinoin through serial dilution. After 96 h, culture media were aspirated, and cells were washed with phosphate-buffered saline (PBS; Sigma Aldrich, Missouri, USA). Cells were then trypsinized and collected as a cell suspension by adding media. A 10-mL cell suspension was mixed

with an equivalent volume of 0.4% trypan blue solution (Sigma Aldrich). The mixture was incubated for 3 min at room temperature, followed by conducting viable cell counts using an inverted microscope (Olympus CKX41). The percentage of viable cells was plotted versus the concentration to attain the linear regression value, which was converted to the GI_{50} value using Excel MS Office 2016 (Moordiani *et al.*, 2023).

Cell persistence viability assay

The same number of cells used in the cytotoxicity assay was grown in a 48-well plate. Subsequently, cells were treated with PGV-1 for 72 h followed by removal of the media and washing with PBS. Treated media were refreshed with fully supplemented culture media without PGV-1. Cell viability was then measured via trypan blue staining every 24 h for 6 days after the removal of treated media.

Cell cycle assay

Cell cycle patterns were assessed through flow cytometry analysis (Novitasari, Kato, *et al.*, 2023). Briefly, cells (1×10^5) were treated with compounds for 24, 48, and 72 h, after which they were stained with propidium iodide (PI). The DNA content was evaluated using a BD FACSCalibur flow cytometer. Flow cytograms were then generated and quantified using BD FACSstation software.

Mitotic spread assay

HuH-7 cells were grown until reaching 70%–80% confluency. After 24-h exposure to PGV-1, cells were washed with PBS followed by treatment with 0.56% KCl. Subsequently, cells were fixed using a methanol–acetic acid solution at a 3:1 ratio. The fixed cell suspension was dispensed onto microscope slides, allowed to air dry, and then stained with Hoechst 33342 obtained from Cell Signaling Technology (Danvers, Massachusetts, USA). The cell morphology was examined and documented with a confocal microscope (LSM710, Zeiss, Jena, Germany). The mitotic index was determined by dividing the number of mitotic events by the total cell count (Lestari *et al.*, 2019).

Senescence assay

Cells were cultured in wells containing a coverslip, seeded at the same cell density used for the mitotic spread assay, and exposed to PGV-1 for 24 h. Following treatment, cells were fixed in a 4% paraformaldehyde solution and were subsequently stained with X-gal (0.2% solution; Wako) for 24 h. The examination was conducted using an inverted microscope (Olympus IX71, Tokyo, Japan). Afterward, the number of senescent cells was evaluated using ImageJ (freely available software by NIH, USA) and represented as the proportion of β-galactosidase–positive cells in the total quantified cell count (Wulandari *et al.*, 2020; Ahlina *et al.*, 2022).

ROS modulation assay

HuH-7 cells were resuspended in a buffer supplemented with 10% FBS/PBS and preincubated with 20 μM 2′,7′-dichlorofluorescin diacetate (Sigma) for 30 min. Cells were then exposed to PGV-1 and subsequently assessed using flow cytometry at specific time intervals. The average fluorescence intensity of the treated cells was graphed relative to the signal obtained from untreated cells (Wulandari *et al.*, 2020).

Western blot analysis

The level of N-MYC expression was investigated by immunoblotting as previously described (Novitasari, Jenie, *et al.*, 2023). Briefly, HuH-7 cells (2×10^6) were sown into 10-cm tissue culture dishes and grown overnight before treatment with PGV-1. After a 24-h treatment, HuH-7 cells were harvested, lysed in lysis buffer, and centrifuged at 12,000× g at 4°C for 15 min. The supernatant was gathered, and the protein content was assessed. Cell lysates were normalized to the protein concentration and separated using a 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis. Following electrophoresis, the separated proteins were transferred onto polyvinylidene difluoride membranes (Immobilon, Merck, Germany), followed by blocking with a blocking solution at room temperature for 1 h. The membrane was then incubated at 4°C overnight incubation with the N-MYC antibody (sc-142, Santa Cruz Biotechnology, USA) diluted 1:250 in the Can Get Signal solution (NKB-101, Toyobo, Japan). γ-Tubulin antibody (#3700, Santa Cruz Biotechnology, USA) diluted 1:5,000 in 5% of bovine serum albumin was used as a housekeeping protein. After the initial antibody incubation, the membrane was rinsed with PBS-Tween and then exposed for 1 h to the secondary antibody at room temperature. The secondary antibody used for N-MYC was anti-mouse IgG (#NA931V), whereas, for tubulin, this was a protein-A HRP-linked antibody (#NA9120V) (Cytiva, UK).

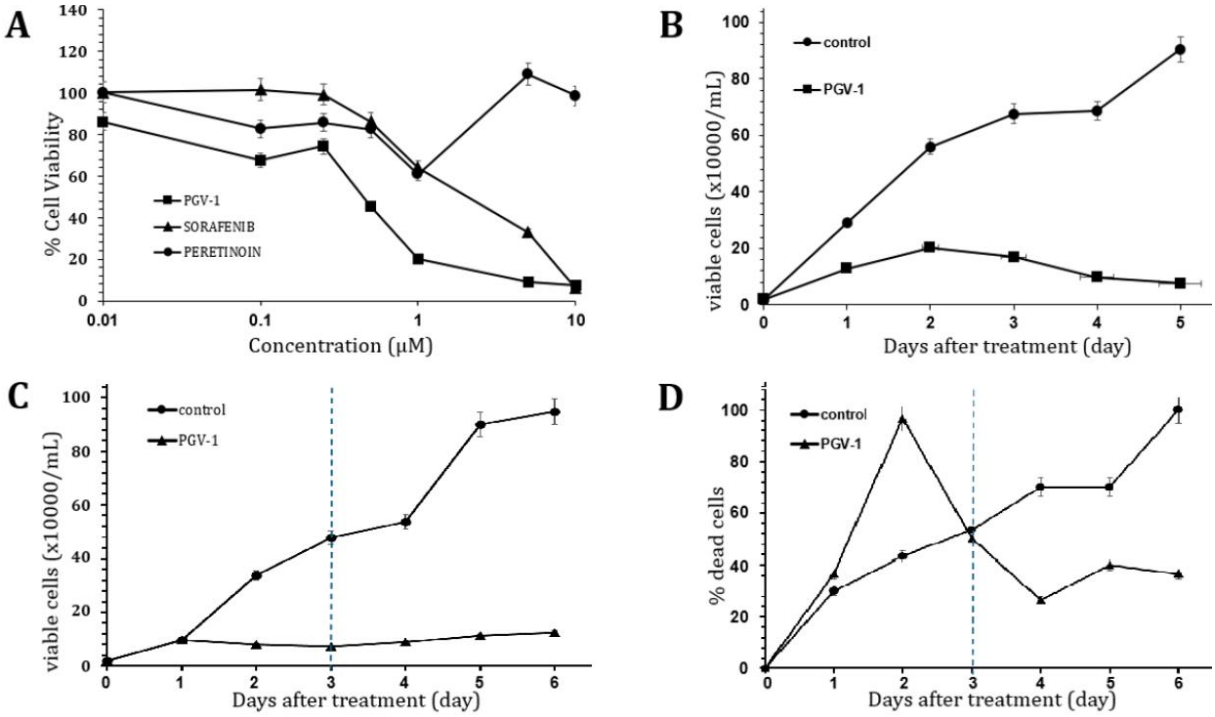

Figure 1. The cytotoxic and anti-proliferative effects of PGV-1 against HuH-7 cells. (A) The GI_{50} of PGV-1 was determined after four days of incubation. (B) HuH-7 cells (2 x 10^4 cells/mL) were treated with 2 μM PGV-1 for 6 days and enumerated everyday using trypan blue exclusion method. (C) HuH-7 cells were treated with 2 μM PGV-1 for 3 days, washed on the third day and had a fresh media without the compound. The counts of viable (C) and dead cells (D) were recorded at specified intervals. Data are displayed as mean ± standard deviation (SD) (n = 3), 95% confidence interval or percentage.

The immunoreactive signals were detected through chemiluminescence (GE Life Science, UK) and captured on autoradiography film (Fujifilm, Japan). Protein bands were enumerated using ImageJ software. Following normalization to tubulin, the N-MYC expression level in the group treated with PGV-1 was contrasted with the untreated group.

Data analysis

Graphs were plotted with Excel (version 16.69.1) on a MacBook Air (M2, 2022). The significance at a 95% confidence level was evaluated using the student's T-test.

RESULTS AND DISCUSSION

PGV-1 is cytotoxic to HuH-7 cells

PGV-1 showed significant cytotoxicity against HuH-7 cells, increasing with concentration, with a GI50 value of 0.36 μM (Figure 1A). Compared with sorafenib and peretinoin, which had a GI_{50} of 2.07 and 45.6 μM, respectively, PGV-1 demonstrated a stronger cytotoxic effect. The cell proliferation in untreated and PGV-1 groups illustrated the inhibitory effect on proliferation induced by PGV-1 (Fig. 1B). PGV-1 could inhibit the growth of HuH-7 cells until day 5 while the control group continued to grow well. We treated the cell for up to 3 days, then changed the media without treatment. After the third day, the average live and dead cells of the sample group were around 1.2 × 10^5 cells/mL (Fig. 1C) and 35% (Figure 1D), respectively. On the contrary, the untreated group had around 8 × 10^5 cells/mL and 80% of survived and deceased cells. This indicates that cells were prevented from growing even after the drug was withdrawn.

PGV-1 induces mitotic arrest of HuH-7 cells

Flow cytometry was used to examine alterations in the cell cycle of HuH-7 cells following treatment with PGV-1. The flow cytogram showed a shift in the cell distribution between the untreated and PGV-1–treated cells. PGV-1 treatment shifted the cell population into the G2/M phase (Fig. 2A

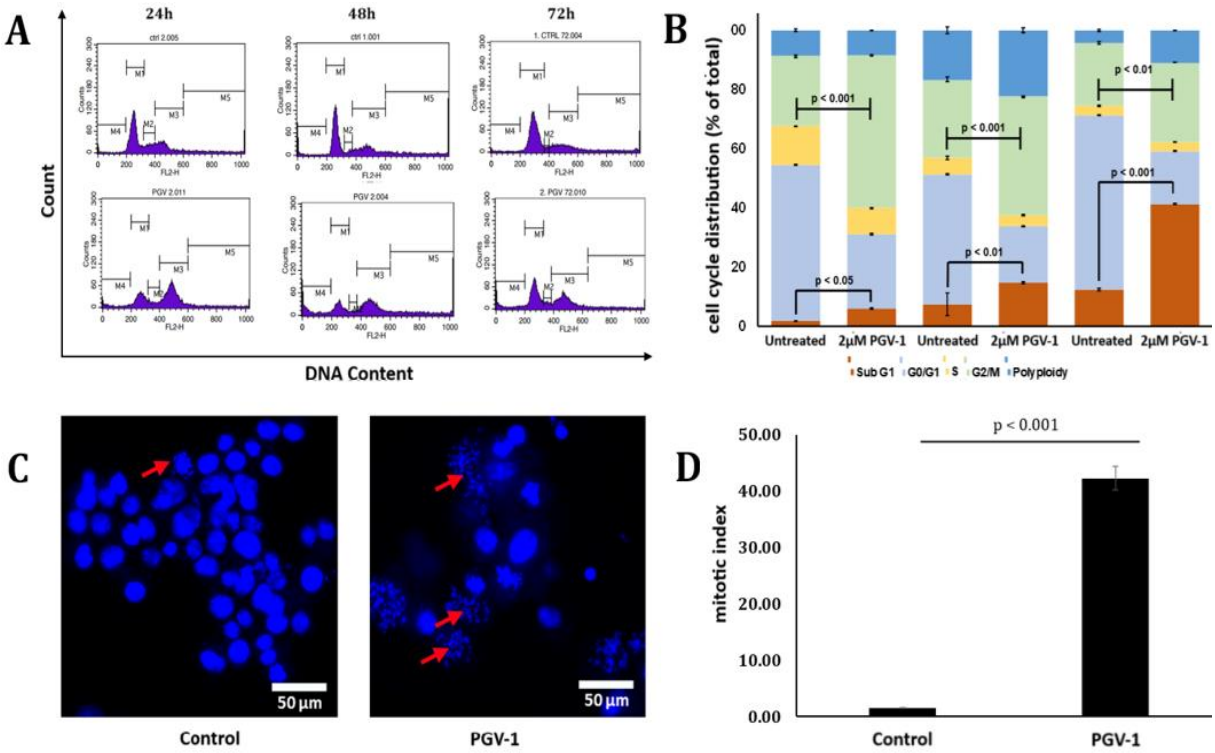

Figure 2. Effect of PGV-1 on the tumor cell cycle *in vitro*. (A) A 2 μM PGV-1 was applied to HuH-7 cells (2 x 105 cells/mL) for the time course as indicated and read the DNA content using flow cytometer after staining with propidium iodide to generate the flow cytogram. (B) The cell distributions were represented in quantitative histograms generated from three independent experiments. (C) Mitosis evidence of HuH-7 cells due to PGV-1 for 24 h appeared as Chromosomal condensation after being stained with Hoechst 33342 under confocal microscopy. The red arrows show the prometaphase, metaphase, or mitosis evidence. (D) Value of mitotic index, shown as mean ± SD.

and 2B) and thus gradually caused cell death (from 6.1% ± 0.46% at 24 h to 14.82% ± 0.55% at 48 h and 41.43% ± 0.33% at 72 h, represented by the subG1 population).

Additionally, the frequency of mitosis was determined to detect the most accumulation of mitotic arrests in the cell cycle. Cells were evaluated using the mitotic spread assay following treatment with PGV-1 (2 μM) for 24 h. A deteriorating nuclear membrane indicated that the envelope was degraded, thus enabling the Hoechst stain to bind nuclear DNA (Figure 2C). This phenomenon confirmed that mitosis was inhibited. Consequently, cells treated with PGV-1 had a mitotic index value of 42.24, which was >27-fold more than that of the untreated group (Figure 2D). Thus, we observed that PGV-1 induced chromosomal condensation, suggestive of prometaphase characteristics.

PGV-1 stimulated cellular senescence and the generation of ROS in HuH-7 cells

The effect of PGV-1 on HuH-7 cells was also evident at the cellular level. Cells treated with PGV-1 displayed signs of senescence as indicated by green staining (Figure 3A). The number of senescent-positive cells in the treated group was more than three-fold higher than in the untreated group (Figure 3B). Moreover, the intracellular ROS level increased after 24 h of PGV-1 exposure (Figure 3C).

MYCN expression in PGV-1–treated HuH-7 cells

MYCN plays a pivotal role in tumorigenesis by acting as a transcription factor that promotes cell proliferation and survival (Liu *et al.*, 2021). HuH-7 cells are MYCN positive, and we therefore assessed the influence of PGV-1 on the expression of MYCN protein. The expression of MYCN was not significantly altered in

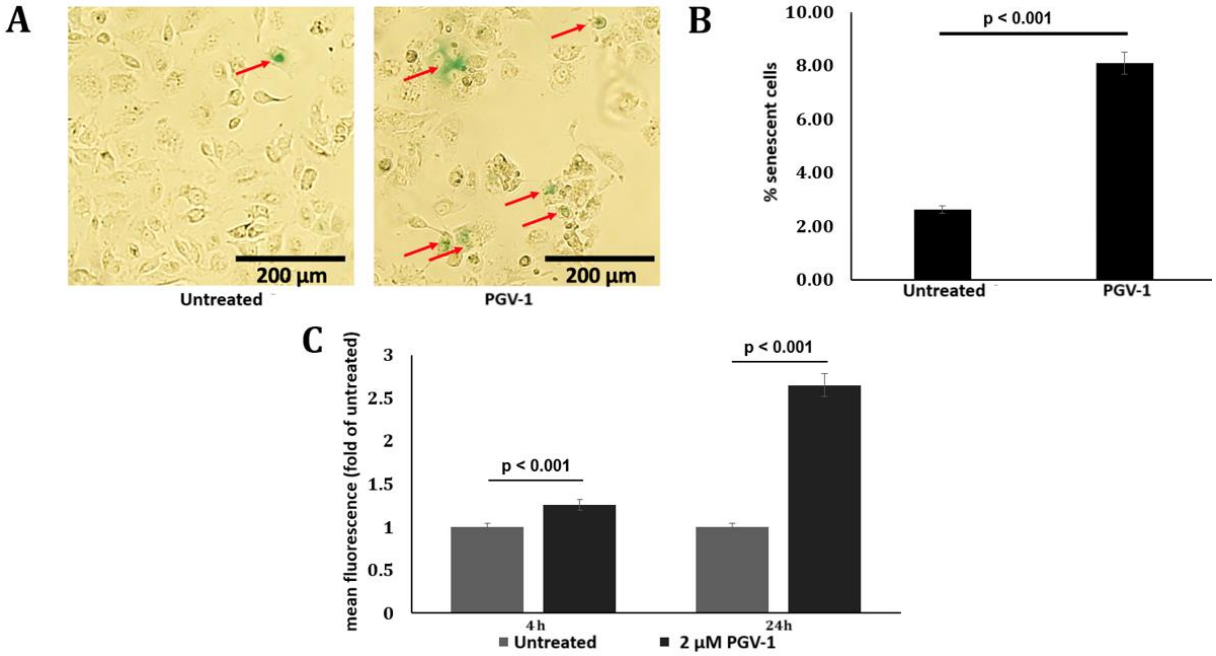

Figure 3. PGV-1 promotes cellular senescence and modulates intracellular ROS levels in HuH-7 cells. The 60% confluence cells were treated with PGV1 for 24 h followed by SA-b-gal staining (A) then the stained positive cells were counted to give % senescent cells (B). Intracellular ROS expressions were analysed using Flow cytometry and presented as mean fluorescence (C).

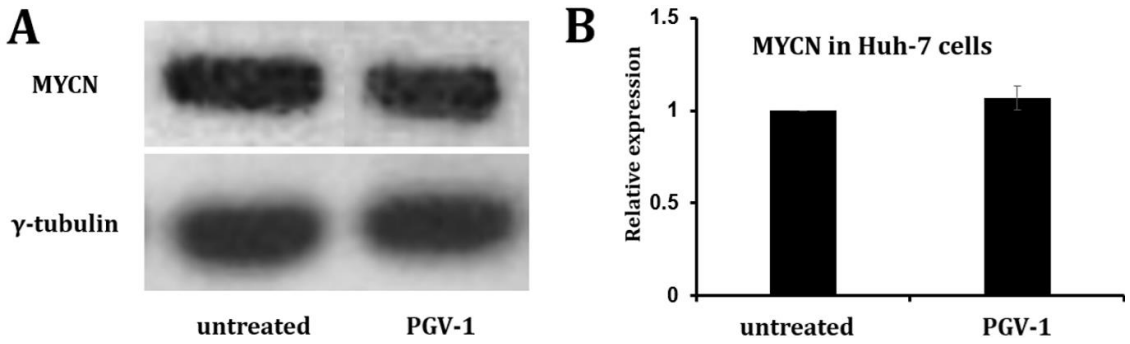

Figure 4. The effect of PGV-1 on the protein level of MYCN in HuH-7 cells. (A) Western blot analysis shows increased MYCN in PGV-1-treated HuH-7 cells. (B) The bar chart depicts the mean relative signal intensity of the PGV-1-treated group for three experiments (error bars indicate the standard error of the mean (SEM) of three separate experiments).

PGV-1–treated cells (Figure 4). Thus, no sufficient evidence was found for cell cycle inhibition associated with MYCN signaling.

Overall, these results demonstrated the effect of PGV-1 against MYCN-positive HCCs with the same specific effect of this compound to arrest cancer cells at mitosis/prometaphase. HCC is a frequent type of liver cancer, and recent research shows that an amplification and overexpression of MYCN in HCC supports the aggressiveness of the tumor (Suenaga *et al.*, 2021). We expect that the current study will become an effective alternative treatment for MYCN-expressing liver cancer.

Regarding cytotoxicity, PGV-1 shows a stronger effect than sorafenib and peretinoin as indicated by the GI_{50} values, which are 5–100-fold lower. Unlike peretinoin, PGV-1 is akin to sorafenib in exhibiting a dose-dependent effect. This agrees with other research that demonstrated that sorafenib reduced the viability of HuH-7 cells (Baek *et al.*, 2020; Garten *et al.*, 2019; Wei *et al.*, 2015). Although sorafenib resistance was found in HCC HuH-7 cells (Zhang *et al.*, 2020), other studies have shown that HuH-7 resistance to sorafenib can be overcome by increasing cellular uptake of sorafenib either through combination with other compounds such as camptothecin (Elkateb *et al.*, 2023) or by suppressing certain pathways (Xiao *et al.*, 2023; Zhou *et al.*, 2023).

However, another study in insulin resistance–based hepatocarcinogenesis revealed that peretinoin as an acyclic retinoid in combination with an angiotensin-II receptor blocker effectively inhibited the growth of HuH-7 cells (Nishimura *et al.*, 2018). Moreover, peretinoin has been particularly used for secondary chemoprevention, especially in HCC with curative surgical therapy (Woo *et al.*, 2021).

The effects of PGV-1 have been well documented to target cell cycle progression in cancer cells. PGV-1 appears to target proteins involved in mitosis in K562 leukemia cells (Meiyanto *et al.*, 2022). PGV-1 also prompted the halting of the cell cycle in the G2/M phase in MCF-7, WiDr, and 4T1 cells (Meiyanto *et al.*, 2019). PGV-1 suppression of 4T1 cell mechanisms also involved mitotic catastrophe, senescence induction, and an increase in the intracellular ROS level (Hasbiyani et al., 2021; Meiyanto et al., 2019; Musyayyadah et al., 2021). Therefore, the results of this study, indicating that PGV-1 caused cell cycle arrest in the G2/M phase, induced mitotic arrest, and elevated intracellular ROS levels, are consistent with these prior research findings.

Similar results were found in liver cancer cell lines JHH-7 and HepG2 (Moordiani *et al.*, 2023; Novitasari, Kato, *et al.*, 2023). Both JHH-7 and HepG2 are HBV+ HCC and differentiated HCC cells, respectively, while HuH-7 cells are well-differentiated HCC cells (Fukuyama *et al.*, 2021). Generally, these characteristics correspond to the different histological grades of HCC (Okuno *et al.*, 2020). Studies in JHH-7, HepG2, and HuH-7 cells revealed that PGV-1 can affect all types of HCC cells. However, further study is needed into the molecular target mechanisms underlying the PGV-1 inhibition of high MYCN-liver cancer cells, including the possibility of suppressing proteins involved in the cell cycle. Subsequently, considering that PGV-1 is a potential candidate for HCC therapy, the development of the compound itself into new analogs or pharmaceutical formulations is expected to accelerate the process.

CONCLUSION

PGV-1 had a growth-inhibitory effect on HuH-7 cells *in vitro* with a GI_{50} of 0.36 μM and suppressed growth in a dose-dependent manner, which continued after drug removal. Flow cytometry analysis validated that the cycle progression of HuH-7 cells seemed to be halted in the G2/M phase, and alterations were observed in intracellular ROS levels. Moreover, the chromosomal staining assay showed that the PGV-1 treated cells accumulated mostly at prometaphase and that cellular senescence was promoted. Although PGV-1 treatment was not proven to suppress the N-MYC expression, overall, these results imply that PGV-1 shows promise as a candidate for inhibiting liver cancer cells that are MYCN-positive via mitotic arrest.

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CONFLICT OF INTEREST

The authors assert the absence of any conflicts of interest.

REFERENCES

Agarwal, P., Glowacka, A., Mahmoud, L., Bazzar, W., Larsson, L.-G., & Alzrigat, M. (2023). MYCN amplification is associated with reduced expression of genes encoding γ-Secretase complex and NOTCH signaling components in neuroblastoma. *International Journal of Molecular Sciences*, *24*(9). https://doi.org/10.3390/ijms24098141

Ahlina, F. N., Anggriani, L., Salsabila, I. A., & Jenie, R. I. (2022). Bioactivity of black cumin oil on the senescence of HER-2-overexpressing breast cancer cells. *Malaysian Applied Biology*, *51*(1

SE-Research Articles), 91–98. https://doi.org/10.55230/mabjournal.v51i1.2008

Baek, S. Y., Hwang, U. W., Suk, H. Y., & Kim, Y. W. (2020). Hemistepsin A inhibits cell proliferation and induces G0/G1-Phase arrest, cellular senescence and apoptosis via the AMPK and p53/p21 signals in human hepatocellular carcinoma. In *Biomolecules* (Vol. 10, Issue 5). https://doi.org/10.3390/biom10050713

Burton, A., Tataru, D., Driver, R. J., Bird, T. G., Huws, D., Wallace, D., Cross, T. J. S., Rowe, I. A., Alexander, G., & Marshall, A. (2021). Primary liver cancer in the UK: Incidence, incidence-based mortality, and survival by subtype, sex, and nation. *JHEP Reports : Innovation in Hepatology*, *3*(2), 100232. https://doi.org/10.1016/j.jhepr.2021.100232

Chen, X., Yang, G., Guo, X., Zhang, J., Sun, W., Liu, D., Wang, H., & Liu, S. (2022). DJ-1/FGFR-1 Signaling pathway contributes to sorafenib resistance in hepatocellular carcinoma. *Oxidative Medicine and Cellular Longevity*, *2022*, 2543220. https://doi.org/10.1155/2022/2543220

Dzieran, J., Rodriguez Garcia, A., Westermark, U. K., Henley, A. B., Eyre Sánchez, E., Träger, C., Johansson, H. J., Lehtiö, J., & Arsenian-Henriksson, M. (2018). MYCN-amplified neuroblastoma maintains an aggressive and undifferentiated phenotype by deregulation of estrogen and NGF signaling. *Proceedings of the National Academy of Sciences*, *115*(6), E1229–E1238. https://doi.org/10.1073/pnas.1710901115

Eberherr, C., Beck, A., Vokuhl, C., Becker, K., Häberle, B., Von Schweinitz, D., & Kappler, R. (2019). Targeting excessive MYCN expression using MLN8237 and JQ1 impairs the growth of hepatoblastoma cells. *International Journal of Oncology*, *54*(5), 1853–1863. https://doi.org/10.3892/ijo.2019.4741

Elkateb, A. S., Nofal, S., Ali, S. A., & Atya, H. B. (2023). Camptothecin sensitizes hepatocellular carcinoma cells to sorafenib- induced ferroptosis via suppression of Nrf2. *Inflammation*, *46*(4), 1493–1511. https://doi.org/10.1007/s10753-023-01823-4

Fukuyama, K., Asagiri, M., Sugimoto, M., Tsushima, H., Seo, S., Taura, K., Uemoto, S., & Iwaisako, K. (2021). Gene expression profiles of liver cancer cell lines reveal two hepatocyte-like and fibroblast-like clusters. *PLoS ONE*, *16*(2 February), 1–17. https://doi.org/10.1371/journal.pone.0245939

Garten, A., Grohmann, T., Kluckova, K., Lavery, G. G., Kiess, W., & Penke, M. (2019). Sorafenib-induced apoptosis in hepatocellular carcinoma is reversed by SIRT1. *International Journal of Molecular Sciences*, *20*(16). https://doi.org/10.3390/ijms20164048

Gherardi, S., Valli, E., Erriquez, D., & Perini, G. (2013). MYCN-mediated transcriptional repression in neuroblastoma: the other side of the coin. *Frontiers in Oncology*, *3*. https://www.frontiersin.org/journals/oncology/articles/10.3389/fonc.2013.00042

Hanahan, D. (2022). Hallmarks of cancer: new dimensions. *Cancer Discovery*, *12*(1), 31–46. https://doi.org/10.1158/2159-8290.CD-21-1059

Hasbiyani, N. A., Wulandari, F., Nugroho, E. P., Hermawan, A., & Meiyanto, E. (2021). Bioinformatics analysis confirms the target protein underlying mitotic catastrophe of 4T1 cells under combinatorial treatment of PGV-1 and galangin. In *Scientia Pharmaceutica* (Vol. 89, Issue 3). https://doi.org/10.3390/scipharm89030038

Hermawan, A., & Putri, H. (2021). Systematic analysis of potential targets of the curcumin analog pentagamavunon-1 (PGV-1) in overcoming resistance of glioblastoma cells to bevacizumab. *Saudi Pharmaceutical Journal*, *29*(11), 1289–1302. https://doi.org/https://doi.org/10.1016/j.jsps.2021.09.015

Hermawan, A., Wulandari, F., Hanif, N., Utomo, R. Y., Jenie, R. I., Ikawati, M., & Tafrihani, A. S. (2022). Identification of potential targets of the curcumin analog CCA-1.1 for glioblastoma treatment : integrated computational analysis and in vitro study. *Scientific Reports*, *12*(1), 13928. https://doi.org/10.1038/s41598-022-18348-9

Keating, G. M. (2017). Sorafenib: a review in hepatocellular carcinoma. *Targeted Oncology*, *12*(2), 243–253. https://doi.org/10.1007/s11523-017-0484-7

Lestari, B., Nakamae, I., Yoneda-Kato, N., Morimoto,

T., Kanaya, S., Yokoyama, T., Shionyu, M., Shirai, T., Meiyanto, E., & Kato, J. (2019). Pentagamavunon-1 (PGV-1) inhibits ROS metabolic enzymes and suppresses tumor cell growth by inducing M phase (prometaphase) arrest and cell senescence. *Scientific Reports*, *9*(1), 14867. https://doi.org/10.1038/s41598-019-51244-3

Liu, R., Shi, P., Wang, Z., Yuan, C., & Cui, H. (2021). Molecular mechanisms of MYCN dysregulation in cancers. In *Frontiers in Oncology* (Vol. 10, pp. 1–12). https://doi.org/https://doi.org/10.3389/fonc.2020.625332

Llovet, J. M., Kelley, R. K., Villanueva, A., Singal, A. G., Pikarsky, E., Roayaie, S., Lencioni, R., Koike, K., Zucman-Rossi, J., & Finn, R. S. (2021). Hepatocellular carcinoma. *Nature Reviews. Disease Primers*, *7*(1), 6. https://doi.org/10.1038/s41572-020-00240-3

Mathsyaraja, H., & Eisenman, R. N. (2016). Parsing Myc Paralogs in oncogenesis. *Cancer Cell*, *29*(1), 1–2. https://doi.org/https://doi.org/10.1016/j.ccell.2015.12.009

McGlynn, K. A., Petrick, J. L., & El-Serag, H. B. (2021). Epidemiology of hepatocellular carcinoma. *Hepatology*, *73*(S1), 4–13. https://doi.org/https://doi.org/10.1002/hep.31288

Meiyanto, E., Novitasari, D., Utomo, R. Y., Susidarti, R. A., Putri, D. D. P., & Kato, J. Y. (2022). Bioinformatic and molecular interaction studies uncover that CCA-1.1 and PGV-1 differentially target mitotic regulatory protein and have a synergistic effect against leukemia cells. *Indonesian Journal of Pharmacy*, *33*(2), 225–233. https://doi.org/10.22146/ijp.3382

Meiyanto, E., Putri, H., Arum Larasati, Y., Yudi Utomo, R., Istighfari Jenie, R., Ikawati, M., Lestari, B., Yoneda-Kato, N., Nakamae, I., Kawaichi, M., & Kato, J.-Y. (2019). Anti-proliferative and anti-metastatic potential of curcumin analogue, pentagamavunon-1 (PGV-1), toward highly metastatic breast cancer cells in correlation with ROS generation. *Advanced Pharmaceutical Bulletin*, *9*(3), 445–452. https://doi.org/10.15171/apb.2019.053

Montemurro, L., Raieli, S., Angelucci, S., Bartolucci, D., Amadesi, C., Lampis, S., Scardovi, A. L., Venturelli, L., Nieddu, G., Cerisoli, L., Fischer, M., Teti, G., Falconi, M., Pession, A., Hrelia, P., & Tonelli, R. (2019). A novel MYCN-specific antigene oligonucleotide deregulates mitochondria and inhibits tumor growth in MYCN-amplified neuroblastoma. *Cancer Research*, *79*(24), 6166–6177. https://doi.org/10.1158/0008-5472.CAN-19-0008

Moordiani, Novitasari, D., Susidarti, R. A., Ikawati, M., Kato, J. Y., & Meiyanto, E. (2023). Curcumin analogs PGV-1 and CCA-1.1 induce cell cycle arrest in human hepatocellular carcinoma cells with overexpressed MYCN. *Indonesian Biomedical Journal*, *15*(2), 141–149. https://doi.org/10.18585/inabj.v15i2.2147

Musyayyadah, H., Wulandari, F., Nangimi, A. F., Anggraeni, A. D., Ikawati, M., & Meiyanto, E. (2021). The growth suppression activity of diosmin and PGV-1 co-treatment on 4T1 breast cancer targets mitotic regulatory proteins. *Asian Pacific Journal of Cancer Prevention : APJCP*, *22*(9), 2929–2938. https://doi.org/10.31557/APJCP.2021.22.9.2929

Nishimura, N., Kaji, K., Kitade, M., Aihara, Y., Sato, S., Seki, K., Sawada, Y., Takaya, H., Okura, Y., Kawaratani, H., Moriya, K., Namisaki, T., Mitoro, A., & Yoshiji, H. (2018). Acyclic retinoid and angiotensin-II receptor blocker exert a combined protective effect against diethylnitrosamine-induced hepatocarcinogenesis in diabetic OLETF rats. *BMC Cancer*, *18*(1), 1164. https://doi.org/10.1186/s12885-018-5099-6

Novitasari, D., Jenie, R., Kato, J. Y., & Meiyanto, E. (2023). Chemoprevention curcumin analog 1.1 promotes metaphase arrest and enhances intracellular reactive oxygen species levels on TNBC MDA-MB-231 and HER2-positive HCC1954 cells. *Research in Pharmaceutical Sciences*, *18*(4), 358–370. https://doi.org/10.4103/1735-5362.378083

Novitasari, D., Kato, J. ya, Ikawati, M., Putri, D. D. P., Wulandari, F., Widyarini, S., Zulfin, U. M., Salsabila, D. U., & Meiyanto, E. (2023). PGV-1 permanently arrests HepG2 cells in M phase and inhibits DMH-induced liver carcinogenesis in rats. *Journal of Applied Pharmaceutical Science*, *13*(8), 204–211. https://doi.org/10.7324/JAPS.2023.131550

Okuno, M., Newhook, T. E., Joechle, K., Kawaguchi, Y., Bellis, M. De, Tzeng, C. D., Chun, Y. S., Aloia,

T. A., Shindoh, J., Kaseb, A. O., & Vauthey, J. (2020). Characteristics of atypical large well-differentiated hepatocellular carcinoma : a specific subtype of hepatocellular carcinoma? *International Hepato-Pancreato-Biliary Association*, *22*(4), 545–552. https://doi.org/10.1016/j.hpb.2019.08.012

Petrick, J. L., Florio, A. A., Loomba, R., & McGlynn, K. A. (2020). Have incidence rates of liver cancer peaked in the United States? *Cancer*, *126*(13), 3151–3155. https://doi.org/https://doi.org/10.1002/cncr.32794

Qin, X.-Y., Su, T., Yu, W., & Kojima, S. (2020). Lipid desaturation-associated endoplasmic reticulum stress regulates MYCN gene expression in hepatocellular carcinoma cells. *Cell Death & Disease*, *11*(1), 66. https://doi.org/10.1038/s41419-020-2257-y

Qin, X.-Y., Suzuki, H., Honda, M., Okada, H., Kaneko, S., Inoue, I., Ebisui, E., Hashimoto, K., Carninci, P., Kanki, K., Tatsukawa, H., Ishibashi, N., Masaki, T., Matsuura, T., Kagechika, H., Toriguchi, K., Hatano, E., Shirakami, Y., Shiota, G., … Kojima, S. (2018). Prevention of hepatocellular carcinoma by targeting MYCN-positive liver cancer stem cells with acyclic retinoid. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(19), 4969–4974. https://doi.org/10.1073/pnas.1802279115

Raieli, S., Di Renzo, D., Lampis, S., Amadesi, C., Montemurro, L., Pession, A., Hrelia, P., Fischer, M., & Tonelli, R. (2021). MYCN drives a tumor immunosuppressive environment which impacts survival in neuroblastoma. *Frontiers in Oncology*, *11*, 625207. https://doi.org/10.3389/fonc.2021.625207

Ruiz-Pérez, M. V., Henley, A. B., & Arsenian-Henriksson, M. (2017). The MYCN protein in health and disease. In *Genes* (Vol. 8, Issue 4). https://doi.org/10.3390/genes8040113

Singh, A. K., Kumar, R., & Pandey, A. K. (2018). Hepatocellular carcinoma: causes, mechanism of progression and biomarkers. *Current Chemical Genomics and Translational Medicine*, *12*, 9–26. https://doi.org/10.2174/2213988501812010009

Suenaga, Y., Einvik, C., Takatori, A., & Zhu, Y. (2021). Editorial: Molecular mechanisms and treatment of MYCN-driven tumors. In *Frontiers in oncology* (Vol. 11, p. 803443). https://doi.org/10.3389/fonc.2021.803443

Suenaga, Y., Kaneko, Y., Matsumoto, D., Hossain, M. S., Ozaki, T., & Nakagawara, A. (2009). Positive auto-regulation of MYCN in human neuroblastoma. *Biochemical and Biophysical Research Communications*, *390*(1), 21–26. https://doi.org/10.1016/j.bbrc.2009.09.044

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, *71*(3), 209–249. https://doi.org/10.3322/caac.21660

Suresh, D., Srinivas, A. N., & Kumar, D. P. (2020). Etiology of hepatocellular carcinoma: special focus on fatty liver disease. *Frontiers in Oncology*, *10*, 601710. https://doi.org/10.3389/fonc.2020.601710

Utomo, R. Y., Wulandari, F., Novitasari, D., Lestari, B., Susidarti, R. A., Jenie, R. I., Kato, J., Sardjiman, S., & Meiyanto, E. (2022). Preparation and cytotoxic evaluation of PGV-1 derivative, CCA-1.1, as a new curcumin analog with improved-physicochemical and pharmacological properties. *Advanced Pharmaceutical Bulletin*, *12*(3), 603–612. https://doi.org/10.34172/apb.2022.063

Valery, P. C., Laversanne, M., Clark, P. J., Petrick, J. L., McGlynn, K. A., & Bray, F. (2018). Projections of primary liver cancer to 2030 in 30 countries worldwide. *Hepatology (Baltimore, Md.)*, *67*(2), 600–611. https://doi.org/10.1002/hep.29498

Wei, J., Meng, F., Qu, K., Wang, Z., Wu, Q., Zhang, L., Pang, Q., & Liu, C. (2015). Sorafenib inhibits proliferation and invasion of human hepatocellular carcinoma cells via up-regulation of p53 and suppressing FoxM1. *Acta Pharmacologica Sinica*, *36*(2), 241–251. https://doi.org/10.1038/aps.2014.122

Woo, H. Y., Yoo, S. Y., & Heo, J. (2021). Peretinoin, an acyclic retinoid, for the secondary prevention of hepatocellular carcinoma. In *Molecules* (Vol. 26, Issue 2). https://doi.org/10.3390/molecules26020295

Wulandari, F., Ikawati, M., Novitasari, D., Kirihata, M., Kato, J.-Y., & Meiyanto, E. (2020). New curcumin analog, CCA-1.1, synergistically improves the antiproliferative effect of doxorubicin against T47D breast cancer cells. *Indonesian Journal of Pharmacy*, *31*(4), 244-

256. https://doi.org/10.22146/ijp.681.

Wulandari, F., Ikawati, M., Widyarini, S., Kirihata, M., Novitasari, D., Kato, J.-Y., & Meiyanto, E. (2023). Tumour-suppressive effects of curcumin analogs CCA-1.1 and Pentagamavunone-1 in colon cancer: In vivo and in vitro studies. *Journal of Advanced Pharmaceutical Technology & Research*, *14*(4), 317–324. https://doi.org/10.4103/JAPTR.JAPTR_315_23

Xiao, Y., Xu, Z., Cheng, Y., Huang, R., Xie, Y., Tsai, H.-I., Zha, H., Xi, L., Wang, K., Cheng, X., Gao, Y., Zhang, C., Cheng, F., & Chen, H. (2023). Fe3+-binding transferrin nanovesicles encapsulating sorafenib induce ferroptosis in hepatocellular carcinoma. *Biomaterials Research*, *27*(1). https://doi.org/10.1186/s40824-023-00401-x

Yu, J., Ou, Z., Lei, Y., Chen, L., Su, Q., & Zhang, K. (2020). LncRNA MYCNOS facilitates proliferation and invasion in hepatocellular carcinoma by regulating miR-340. *Human Cell*, *33*(1), 148–158. https://doi.org/10.1007/s13577-019-00303-y

Zhang, Z., He, C.-Z., Qin, Y.-Q., Liao, J.-J., Huang, S.-T., Mo, S., Li, H.-M., & Lin, J.-Y. (2020). Exploring the mechanism of resistance to sorafenib in two hepatocellular carcinoma cell lines. *Aging*, *12*(23), 24255–24269. https://doi.org/10.18632/aging.104195

Zhou, S., Xu, H., & Wei, T. (2023). Inhibition of stress proteins TRIB3 and STC2 potentiates sorafenib sensitivity in hepatocellular carcinoma. *Heliyon*, *9*(6), e17295. https://doi.org/https://doi.org/10.1016/j.heliyon.2023.e17295