RESEARCH ARTICLE

Curcumin Analogs PGV-1 and CCA-1.1 Induce Cell Cycle Arrest in Human Hepatocellular Carcinoma Cells with Overexpressed *MYCN*

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Received date: Nov 27, 2022; Revised date: Jan 5, 2023; Accepted date: Jan 6, 2023

Abstract

BACKGROUND: Liver cancer is the third leading mortality in cancer. Curcumin shows effective anticancer potency against various cancer including liver cancer. The synthesized curcumin analog compounds Pentagamavunone-1 (PGV-1) and Chemoprevention Curcumin Analog-1.1 (CCA-1.1) have been well studied in breast, leukemia, and colon cancer cells with better potency than curcumin itself, yet their cytotoxic activities were not known in liver cancer cells. Thus, this study was conducted to elevate the anticancer effect of these curcumin analogs against hepatocellular carcinoma (HCC) cells *in vitro*, specifically in *MYCN*-expressing cells, based on its cellular physiology.

METHODS: JHH-7 cells were used as the HCC cell model with high expression of *MYCN*. The viability of the cells was observed using trypan blue exclusion method while cell cycle profile and intracellular reactive oxygen species (ROS) levels were quantified by means of flow cytometry. Chromosomal staining with Hoechst was applied to determine the cell cycle arrest phase, whilst X-gal staining was used to assess the cellular senescence activity.

RESULTS: The result of current study presented that the growth inhibitory activity of PGV-1 as well as CCA-1.1 in JHH-7 cells was associated with the cell cycle arrest and cellular senescence. Both curcumin analogs PGV-1 and CCA-1.1 ultimately induced mitotic arrest ($p<0.001$) better than curcumin. Moreover, PGV-1 and CCA-1.1 similarly increased the senescent cells that partly mediated through ROS elevation. The transcription level of *MYCN* was not altered upon treatment with curcumin and its analogs in JHH-7 cells, suggesting that molecular mechanism of the inhibitory effect was independent from MYCN signaling.

CONCLUSION: Taken together, these observations revealed that both PGV-1 and CCA-1.1 potentially serve as multi-targeted curcumin-based compounds and lead to promising anti-hepatocellular cancer agents.

KEYWORDS: Curcumin analogs, hepatocellular carcinoma, mitotic arrest, MYCN

Indones Biomed J. 2023; 15(2): 141-9

Introduction

Pentagamavunone-1 (PGV-1) is a synthetic curcumin analog that is designed to improve the compound's stability, bioavailability, and biological activities, but maintain the safety indicated by minimal side effect to gastrointestinal tract and not toxic.(1) Extensive research on PGV-1 exhibits higher anticancer activity compared to that of curcumin for many different sorts of cancer include blood, breast, and colon cancer, both *in vitro* and *in vivo*. (2-8) The development of PGV-1's derivative, namely Chemoprevention Curcumin Analog-1.1 (CCA-1.), resulted in a more stable and soluble compound with similar or better anticancer activity compared to the parent compound. (9) The activities of CCA-1.1 that has been proven so far include delaying tumor progression *in vivo* while inhibiting cells' proliferation and inducing apoptosis *in vitro* against numerous cancer cell lines, including breast (5,6), colon (7,8), glioblastoma (10), and leukemia.(11) However, the activity of either PGV-1 or CCA-1.1 on liver cancer has not been reported yet.

Despite the incidence, liver cancer is included as the third leading mortality caused by cancer.(12) Virus infection, alcohol consumption, and high-fat diet all play a significant role in the development of liver cancer.(13,14) Hepatocellular carcinoma (HCC) is the most frequent category of liver cancer that accounts for up to 90% of the cases.(13) Overexpression of *MYCN* oncogene is associated with cancer cell development, tumor recurrence, and therapy resistance.(15,16) Identified firstly in neuroblastoma (15), increased expression of *MYCN* is also found in HCC.(14,17) MYCN belongs to the transcription factor superfamily and is a member of the MYC family, which induces transcription of many genes that involved in the cell cycle regulation. (18,19) Therefore, targeting MYCN may serve as a potential therapy approach in HCC.

In diverse types of cancer cells, PGV-1 and CCA-1.1 are not only have stronger cytotoxicity compared to that of curcumin, but also affect cells in terms of their physiology. Both compounds have proven to induce cell cycle arrest in the G2/M phase (2,3,5,7) with PGV-1 arrests cell in prometaphase specifically.(2) The two compounds are enable for apoptotic induction and raise the number of senescent cells that correlate with the intracellular reactive oxygen species (ROS) enhancement.(5,7) The ROS increase is caused by the inhibition of ROS metabolizing enzymes, enabling the specific effect on cancer cells but not healthy cells due to higher enzymatic activity in cancer cells.(2) Thus, either PGV-1 or CCA-1.1 could serve as potential successors of anticancer drug.

PGV-1 and CCA-1.1 have been reported stronger than curcumin in term of their anticancer potency in several cancer types (2-4), but no information in liver cancer yet. On the other hand, curcumin itself possesses anticancer potency for liver cancer.(20) This study was conducted to investigate the potency of PGV-1 and CCA-1.1 as anticancer agents in HCC expressing-*MYCN*. JHH-7 cells that highly express *MYCN* were used as the cell model.(14,17) Considering that PGV-1 and CCA-1.1 show stronger potency than curcumin in various cancer cells, their higher anticancer activity towards liver cancer expressing-*MYCN* is also expected. Since JHH-7 cells are endogenously expressed *MYCN*, which is believed to promote aggressiveness in HCC (14), hence the expression level was also measured in this study.

Methods

Compounds

The synthesis, purification, and characterization of PGV-1 and CCA-1.1 were handled by Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada as previously reported.(9) Curcumin was purchased from Sigma Aldrich (Cat No. C1386, St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) (Merck Millipore, Darmstadt, Germany) was used to dissolve each of tested compound with the final concentration during treatment was below 0.1%.

Cell Culture

The immortalized hepatocellular carcinoma JHH-7 cell was purchased from JCRB Cell Bank (Cat No. JCRB1031, Osaka, Japan) and cultured in a D-MEM culture medium with high glucose (Cat No. 044-29765, Wako, Osaka, Japan) with addition of penicillin-streptomycin (Cat No. 168-23191, Wako) and fetal bovine serum (FBS) (Cat No. SH30070.03, Hyclone, Logan, UT, USA). Cells were grown and stored in a humidified incubator with 5% of CO_2.

Determination of 50% Growth Inhibitory (GI_{50}) Score

The GI_{50} score was determined by the trypan blue exclusion assay as previously described.(2) Briefly, 2 x 10^4 cells were prepared into 48-well plates and grown for 24 h before incubated within a dilution series of curcumin, PGV-1, and CCA-1.1 (1; 10; 100; 250; 500; 1,000; 5,000; and 10,000 nM). After 96 h of incubation, cells were harvested and as much as 10 µL of cell suspension was mixed with equal

amount of 0.4% trypan blue (Wako) before inputting into the hemocytometer and counting the viable cells. The viable cells were then calculated for its percentage of cell viability and, further, for the GI_{50} of particular compounds.

As for the antiproliferative assay, a total of 2 x 10^4 cells were grown into a dish, treated with indicated doses of curcumin/PGV-1/CCA-1.1, and counted for viable cells throughout 5 days using trypan dye staining.(2)

Flow Cytometry-based Assays

Cells were grown in a 35-mm dish and incubated with each tested compound (5 µM curcumin, 2.5 µM PGV-1, or 2.5 µM CCA-1.1) for 24 h, then stained for the DNA content using propidium iodide (PI) solution (Sigma Aldrich) (with RNase and Triton X-100) and subjected to flow cytometry FACSCalibur (Beckton Dickinson, Franklin Lakes, NJ, USA). The cell distribution in each phase was generated in Cell Quest, and the percentage of each phase was plotted into a graph for treated and untreated groups.(21)

Briefly, 1 x 10^5 cells in a supplemented buffer (10% of FBS in phosphate-buffered saline, PBS) were pretreated with 20 µM with 2',7'-dichlorofluorescin diacetate (DCFDA) (Sigma Aldrich) for 30 min. Later, the tested compounds were added and stored in an incubator for designated intervals of time. Upon the end of incubation, mean fluorescence of the cells were measured in flow cytometer and plotted as a fold of untreated.(5)

Senescence Assay

After 24 h treatment, cells were PBS washed and incubated in fixation solution (4% p-formaldehyde) for 10 min. The solution was discarded and rinsed with PBS before incubating with 0.2% X-gal staining solution (Wako) for 24 h, then observed under an inverted microscope Olympus IX71 (Olympus, Tokyo, Japan). The quantification of senescent cells was processed through ImageJ (National Institute of Health, Bethesda, MD, USA) and determined as percentage of senescent cells against total quantified cells.(21)

Mitotic Spread Assay

Cells were grown and incubated for 24 h with curcumin or the analogs (PGV-1 and CCA-1.1). Following treatment, cells were washed with 1x PBS before being treated with 0.56% KCl, followed by fixation with a mixture of methanol and acetic acid (3:1 v/v). A drop of cell suspensions on microscope slides was air-dried before being stained with Hoechst33342 (Cell Signaling Technology, Danvers, MA, USA) and checked under the confocal microscope (LSM710, Zeiss, Jena, Germany). The documented images were counted for mitotic per total cells to calculate the mitotic index.(2)

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using the ISOGEN kit (Nippon Gene, Toyama, Japan) and reverse-transcribed into cDNA using the ReverTra Ace™ RT (Toyobo, Osaka, Japan) kit as per instructions. The RT-PCR was performed in a 96-well plate thermal cycler Veriti (Applied Biosystems, Waltham, MA, USA), and the PCR product was loaded in a 1.5% agarose gel within a linear range between the untreated and treated groups.(22) The quantified data were normalized to *GAPDH* expression for each sample from several independent experiments. The following oligonucleotide primers specific to human *MYCN* 5'-CCCTGAGCGATTCAGATGATGA-3' (sense) and 5'-ATGACACTCTTGAGCGGACG-3' (antisense); and human *GAPDH*, 5′-CCATCACCATCTTCCAGGAG-3′ (sense) and 5′-CCTGCTTCACCACCTTCTTG-3′ (antisense) were used.

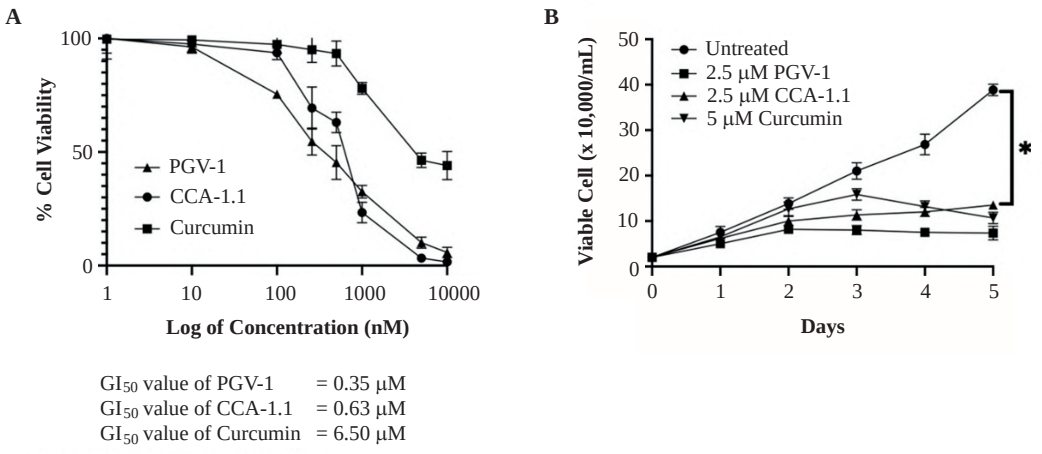
Statistical Analysis

All the presented data were demonstrated by the mean of three datasets±standard deviation (SD). The statistical analysis was carried out using the analysis of variance (ANOVA) (followed by the Dunnett test against the untreated group) with a confidence level of 95% and performed in GraphPad Prism software version 9.0 (GraphPad, San Diego, CA, USA).

Results

PGV-1 and CCA-1.1 Perform Better Cytotoxic Activity than Curcumin Against JHH-7 Cells

The cytotoxic assay was evaluated to determine the antiproliferative activity of PGV-1 and CCA-1.1 in comparison of curcumin in HCC JHH-7 cells. After 4 days of incubation, remarkable antiproliferative activity was observed in PGV-1 treatment based on the lowest GI_{50} score (0.35 µM), followed by CCA-1.1 with a GI_{50} value of 0.63 µM. The cytotoxic activity was more potent than curcumin which had a GI_{50} score of 6.5 µM, almost 10 times-fold than CCA-1.1 (Figure 1A). We later assessed the antiproliferative effect of each compound and counted for viable cells throughout five days. PGV-1 started to suppress the cells' growth on the third day and maintained



GI_{50} value of PGV-1 = 0.35 µM
GI_{50} value of CCA-1.1 = 0.63 µM
GI_{50} value of Curcumin = 6.50 µM

Figure 1. The cytotoxic effect of curcumin and its analogs (PGV-1 and CCA-1.1) in HCC JHH-7 cells. A: Percentage of cell viability after 96 h incubation of tested compounds in JHH-7 cells. B: The antiproliferative effect from curcumin and its analogs in JHH-7 cells. Trypan blue exclusion dye was selected to count the viable cells each day for 5 days. The data presented as mean±SD (n=3) (*p<0.05 against untreated).

total viable cells until the last day of observation. CCA-1.1 also inhibited cell proliferation, while curcumin decreased the viable cells after the third day (Figure 1B). Collectively, both synthetic analogs demonstrated better activity than curcumin to repress cancer cell growth, with PGV-1 being more sensitive to inhibit JHH-7 cell proliferation.

Curcumin and Its Analogs PGV-1 and CCA-1.1 Halt Cell Cycle Progression at the G2/M Phase in JHH-7 Cells

The cell cycle analysis was performed to check the distribution of the cell upon treatment with curcumin or its two analogs using flow cytometry (Figure 2A). All tested compounds significantly increased cell number arrested at the G2/M phase (Figure 2B). More than 80% of PGV-1-treated cells accumulated at G2/M, followed by CCA-1.1 (38.6% ± 0.38%) and curcumin (29.0% ± 0.67%, against 24.8% ± 0.67% in the untreated group). A few populations in curcumin-treated cells also increased at the S phase by 18.9% ± 0.71%, compared with untreated cells (13.8% ± 0.65%). Considering PGV-1 and CCA-1.1 promoted G2/M arrest, we used chromosomal Hoechst staining to determine whether G2 or M phase might be affected by these substances. The result displayed that both PGV-1 and CCA-1.1 caused mitotic arrest, as indicated by the nuclear membrane breakdown that allowed Hoechst to bind with nuclear DNA (Figure 2C). Curcumin-treated cells also presented mitotic cells, which confirm the inhibition during mitosis. The quantification of the mitotic cell revealed that PGV-1 showed the highest mitotic index score of 80 (Figure 2D), followed by CCA-1.1 with 39. This result corresponded with the initial cell cycle analysis. Thus, suggesting PGV-1 and CCA-1.1 induced mitotic arrest in JHH-7 cells.

Curcumin and Its Analogs, PGV-1 and CCA-1.1, Modulate Cellular ROS Level and Senescence in JHH-7 Cells

Next, we evaluated whether curcumin, PGV-1, and CCA-1.1 compounds affect cellular senescence in hepatocellular JHH-7 cells. The 24-h treatment with PGV-1 or CCA-1.1 significantly increased (p<0.01) the percentage of senescent cells by 16.8% and 13.5%, respectively. Yet, barely 8.1% of total cells were detected as positive-senescent cells when exposed to 5 µM curcumin (Figure 3A). Interestingly, some of the round-shaped cells, believed to be mitotic cells, were also presented as senescent cells (Figure 3B). Therefore, it might be expected that the cellular activity of CCA-1.1 in JHH-7 cells also affected the promotion of senescence.

We then checked the effect in ROS modulation at designated intervals (4, 6, and 24 h) exposed to the compounds. At 4 h, only curcumin significantly (p<0.0001) increased the intracellular ROS in JHH-7 cells. The ROS level in PGV-1 and curcumin-treated cells were higher than in the untreated group during 6 h. However, CCA-1.1-treated cells began to enhance elevated ROS levels after 24 h, while curcumin suppressed the level of cellular ROS (p<0.0001) (Figure 3C). Accordingly, the cellular senescence by curcumin analogs could be partly mediated by elevated intracellular ROS in JHH-7 cells.

The Effect of Curcumin, PGV-1, and CCA-1.1 in *MYCN* Expression in JHH-7 Cells

We evaluated whether curcumin and its analogs also affect MYCN expression. We further assessed the mRNA level of *MYCN* upon treatment of those compounds through RT-PCR. The 24 h treatment of PGV-1, CCA-1.1, and curcumin did

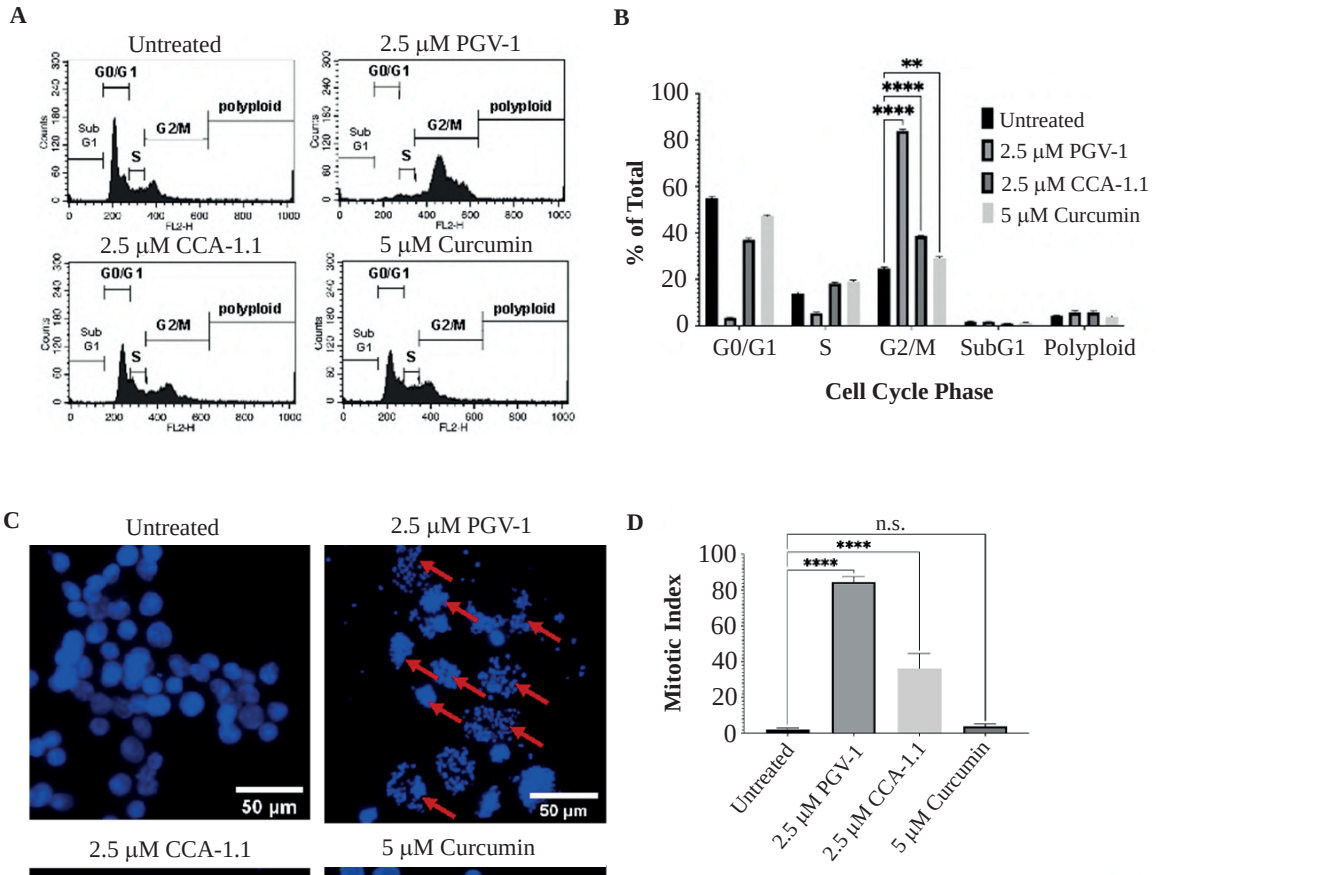


Figure 2. Curcumin analogs PGV-1 and CCA-1.1 induce mitotic arrest in HCC JHH-7 cells. A: Cell cycle profile upon 24 h incubation with PGV-1, CCA-1.1, and curcumin in JHH-7 cells. Cells were added with propidium iodide solution and subjected to a flow cytometer. B: Cells distribution throughout the cell cycle phase. C: The chromosomal Hoechst 33324 stain after 24 h treatment and observed under a confocal microscope. Red arrow indicates mitotic cells. D: The quantification of mitotic cells based on Hoechst staining in JHH-7 cells. The data presented as the mean±SD (n=3). The ANOVA was chosen to analyze the statistical difference between untreated and treated groups (n.s.: not significant; **p<0.01; ****p<0.0001).

not alter the transcription level of *MYCN* (Figure 4A), and the quantitative analysis also demonstrated an insignificant change in all-treated groups (Figure 4B). Hence, it was concluded that CCA-1.1 and curcumin treatment did not affect the *MYCN* transcription in JHH-7 cells.

Discussion

The anticancer activities of curcumin have been well-documented.(20) While curcumin promised the imminent effect of eliminating liver cancer cells, the challenge of developing curcumin remained a hurdle to achieving optimal results in therapy. Thus, synthesized curcumin analogs have been proposed to improve stability better than curcumin while remaining toxic to tumor cells. The current study used PGV-1 and CCA-1.1, which have been reported for their anticancer effects against breast and colorectal cancer cells.(9) The further cellular mechanisms mediated by these analogs include cell cycle arrest, induction to apoptosis and senescence, as well as intracellular ROS level modulation.(3-5,7) Looking for their potential, we tested for the cytotoxic effect of curcumin and its analogs in HCC JHH-7 cells. PGV-1 was the most prominent to inhibit JHH-7 cells' growth based on its GI_{50} score (0.35 µM), followed by CCA-1.1 with a GI_{50} value of 0.63 µM. These values were almost 10-fold higher than the GI_{50} value of curcumin, suggesting these curcumin analogs demonstrated better cytotoxic activity than curcumin itself. The loss of β-diketone from the modification of curcumin indeed

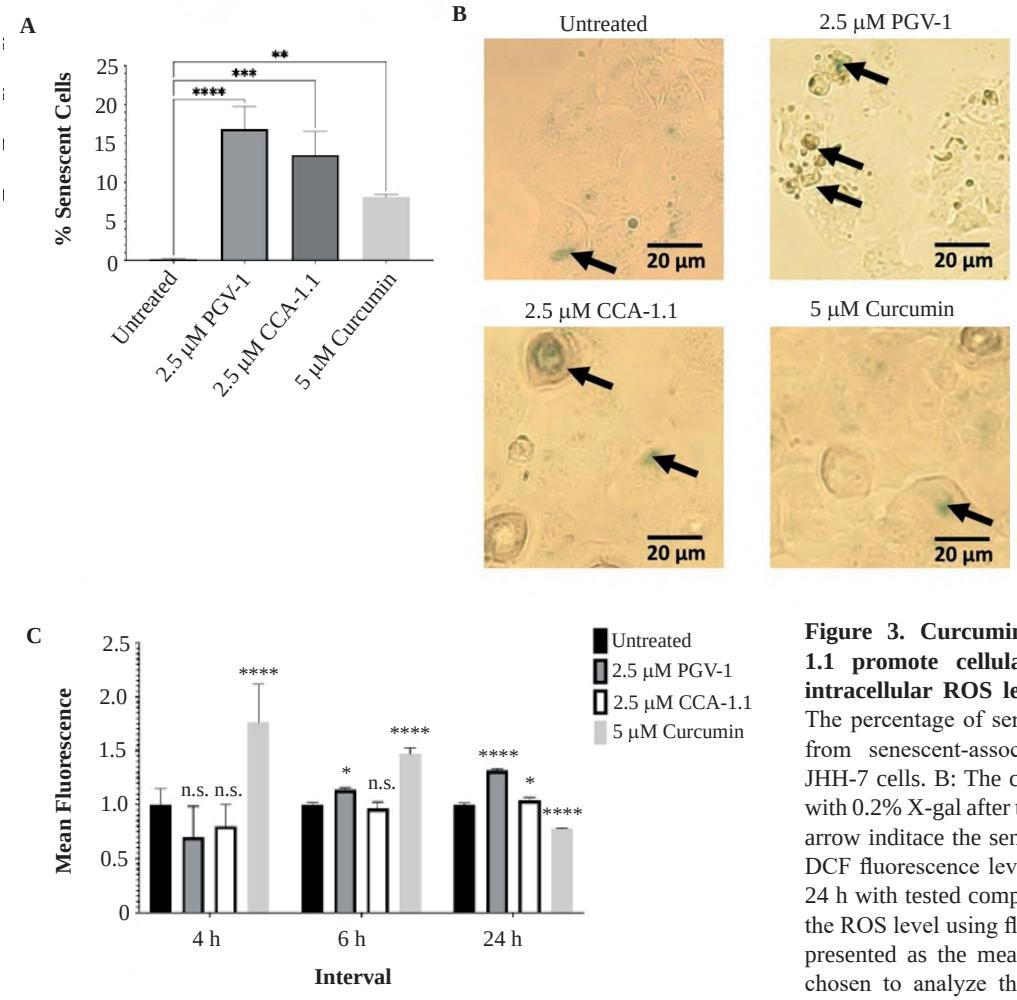


Figure 3. Curcumin analogs PGV-1 and CCA-1.1 promote cellular senescence and modulate intracellular ROS levels in HCC JHH-7 cells. A: The percentage of senescent cells after quantification from senescent-associated-β-galactosidase assay in JHH-7 cells. B: The cell morphology after incubation with 0.2% X-gal after treatment with compounds Black arrow inditace the senescent cells. C: The normalized DCF fluorescence level after incubation for 4, 6, and 24 h with tested compounds. Cells were measured for the ROS level using flow cytometry analysis. The data presented as the mean±SD (n=3). The ANOVA was chosen to analyze the statistical difference between untreated and treated groups (n.s.: not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

resulted in the higher cytotoxic activity of these compounds in cancer cells, as explained previously.(23) In addition, adding hydrophobic substituents such as -CH_3 in the benzyl rings has been associated with the increased antitumor activity of curcumin derivatives.(24) These reasons might be explained by the better cytotoxic effect of curcumin analogs PGV-1 and CCA-1.1 in JHH-7 cells.

Furthermore, PGV-1 and CCA-1.1 enhanced the JHH-7 cells to arrest significantly (p<0.001) in G2/M, with PGV-1 inducing the accumulation of G2/M cells by 83.9±0.8%, followed by CCA-1.1 by 38.6±0.3%. A quite different phenomenon was observed in 5 µM of curcumin in which exhibited no appreciable effect on the cell cycle distribution, similar to previous report.(25) Chromosomal staining later displayed that PGV-1 caused prometaphase arrest, while CCA-1.1 tended to induce prometaphase-metaphase arrest. This finding confirmed that both PGV-1 and CCA-1.1 particularly promote mitotic arrest in JHH-7 cells. The changes from diketone to monoketone curcumin analogs have been proven to enhance better cytotoxic effects than curcumin against cancer cells and a better inhibitory effect on G2/M arrest.(26) Only one change in the carbonyl substitution could affect cell cycle progression, and this might be why PGV-1 acted better than CCA-1.1 to promote mitotic arrest in JHH-7 cells. To some extent, the prolonged mitotic arrest could lead to a mitotic catastrophe and later induce cell death.(27) The molecular mechanism linked to the mitotic catastrophe phenomenon by curcumin analogs PGV-1 and CCA-1.1 is worth elucidating further.

Though not the only critical reason, the addition of exogenous ROS due to chemotherapy caused an imbalance of ROS homeostasis in the cancer cells themselves. In the case of curcumin, as with any other polyphenol, curcumin displays a biphasic effect (pro-oxidant and anti-oxidant) that depends on its concentration in cells. Five micromolar curcumin increased ROS generation in the early hours and reduced it significantly (p<0.0001) 24 h later. This might be because the concentration used in curcumin is not enough

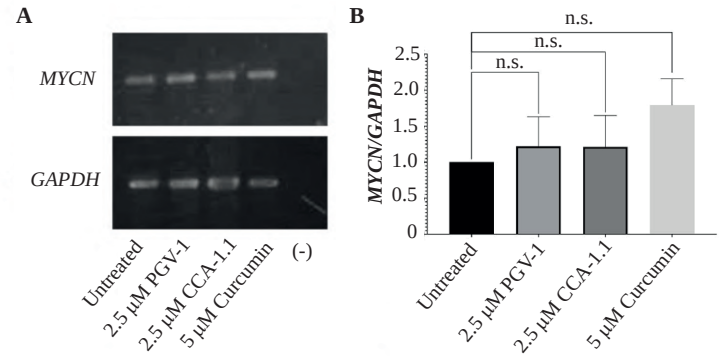


Figure 4. The effect of curcumin analogs PGV-1 and CCA-1.1 on transcription level of MYCN in HCC JHH-7 cells. A: mRNA level of *MYCN* upon treatment for 24 h and analyzed through RT-PCR. B: Relative mRNA level of *MYCN* after normalization with *GAPDH* housekeeping gene after RT-PCR analysis (n=3). The data presented as mean±SD. The ANOVA was chosen to analyze the statistical difference between untreated and treated groups (n.s.: not significant).

to maintain higher ROS production, which is also reflected by the prior result.(28) PGV-1 enhanced ROS generation earlier than CCA-1.1 in JHH-7 cells, and both compounds significantly (p<0.05 and p<0.001, respectively) elevated the intracellular ROS level. Since progression of mitosis is tightly controlled by cyclins and cyclin-dependent kinases (CDKs) and is also targeted to a redox control since ROS can affect the presence and activity of these proteins (29), we hypothesized that the cellular ROS generation by PGV-1 and CCA-1.1 partly mediated the mitotic arrest.

In addition to attributing the mitotic cycle, ROS imbalance can also trigger cellular senescence.(30) Senescence also appears during the G2/M transition with increased lysosomal β-galactosidase activity to some extent. (31) Cellular senescence can be triggered by the exogenous addition of ROS due to exposure to chemotherapeutic agents.(32) Thus, we checked whether these analogs also induced senescence in JHH-7 cells. The exposure to PGV-1 and CCA-1.1 drastically induced cellular senescence, which conformed with early findings in other cancer cells.(2,3,5,7) We also noticed that some of the mitotic cells in PGV-1-treated cells also appeared as senescent cells (marked by green colored-stain due to β-galactosidase activity). Numerous studies elaborated on the relationship between cell cycle arrest and senescence in the mitotic catastrophe. (33) Doxorubicin, for instance, exhibits a senescence-like phenotype (SLP) through mitotic catastrophe before inducing cell death in HCC cells.(34) Therefore, it is assumed that PGV-1 and CCA-1.1 also transiently induced cellular senescence before dying through mitotic catastrophe. Though, exploring the factors controlling the aspects of treatment-induced cell death through mitotic catastrophe by these curcumin analogs should add valuable findings for improving the efficacy of cancer chemotherapy.

The study of curcumin for its antitumor properties on MYCN-amplified neuroblastoma cells has been reviewed before (35), yet no further study that evaluated its effect on MYCN expression is found. Using JHH-7 hepatocarcinoma cells that endogenously expressed MYCN (14), there was an unchanged mRNA level of *MYCN* in curcumin-treated cells, and so did PGV-1 or CCA-1.1. This result indicated that these compounds might not alter *MYCN* on the transcription level, even these curcumin analogs, but rather stabilize the gene transcription. MYCN has been thoroughly studied for its connection with mitotic regulators. Aurora A kinase is one of the major mitotic kinases involved in stabilizing the MYCN.(36) In addition to that, the cyclin B1/CDK1 complex also has an activation binding site in MYCN, and once GSK3β also binds to MYCN, it will be recognized by the proteasome; thus, MYCN will be degraded.(37) Since PGV-1 and CCA-1.1 selectively induced mitotic arrest, further evaluation of whether these treatments affect the MYCN protein level in HCC could also be employed. Still, these initial studies at least presented scientific information on the anticancer activities from PGV-1 and CCA-1.1 as promising candidates for further exploration as chemotherapy for malignant hepatocellular carcinoma.

Conclusion

PGV-1 and CCA-1.1 compounds demonstrated stronger cytotoxic properties than curcumin against HCC JHH-7 cells and halted cell cycle progression in mitosis. Further assessment confirmed that PGV-1 and CCA-1.1 similarly induced cellular senescence that was partly mediated by higher intracellular ROS levels. Later, these curcumin analogs did not affect the transcription level of *MYCN* mRNA in JHH-7 cells.

Acknowledgements

Part of this work is funded by the "Doctoral Dissertation Research (*Penelitian Disertasi Doktor*/PDD)" program, the Ministry of Education, Culture, Research, and Technology

of Indonesia (Grant No. 1863/UN1/DITLIT/Dit-Lit/PT.01.03/2022). We also thank to "Partnership of Basic Research (P*enelitian Dasar Kemitraan*/PDK)" program (Grant No. 2023/UN1/DITLIT/Dit-Lit/PT.01.03/2022).

Authors Contribution

EM conceived the idea and conceptualization of the study. M and DN conducted the experiments and analyzed the data. M, DN, and MI composed the first draft of the research article. JK and RAS supervised and made substantial contributions to research conception. EM finalizes the manuscript. All authors read and approved the final manuscript.

References

1. Reksohadiprodjo MS, Timmerman H, Sardjiman, Margono SA, Martono S, Hakim LR, *et al.* Derivatives of benzylidene cyclohexanone, benzylidene cyclopentanone, and benzylidene acetone, and therapeutic uses thereof. US Patent 6,777,447 B2. 2004.
2. Lestari B, Nakamae I, Yoneda-Kato N, Morimoto T, Kanaya S, Yokoyama T, *et al.* Pentagamavunon-1 (PGV-1) inhibits ROS metabolic enzymes and suppresses tumor cell growth by inducing M phase (prometaphase) arrest and cell senescence. Sci Rep. 2019; 9: 14867. doi: 10.1038/s41598-019-51244-3.
3. Meiyanto E, Putri H, Larasati YA, Utomo RY, Jenie RI, Ikawati M, *et al.* Anti-proliferative and anti-metastatic potential of curcumin analogue, pentagamavunon-1 (PGV-1), toward highly metastatic breast cancer cells in correlation with ROS generation. Adv Pharm Bull. 2019; 9(3): 445-52.
4. Meiyanto E, Husnaa U, Kastian RF, Putri H, Larasati YA, Khumaira A, *et al.* The target differences of anti-tumorigenesis potential of curcumin and its analogues against HER-2 positive and triple-negative breast cancer cells. Adv Pharm Bull. 2021; 11(1): 188-96.
5. Novitasari D, Jenie RI, Wulandari F, Putri DDP, Kato J, Meiyanto E. A Curcumin like structure (CCA-1.1) induces permanent mitotic arrest (senescence) on triple negative breast cancer (TNBC) cells, 4T1. Res J Pharm Technol. 2021; 14(8): 4375-82.
6. Novitasari D, Jenie RI, Utomo RY, Kato J, Meiyanto E. CCA-1.1, a novel curcumin analog, exerts cytotoxic anti-migratory activity toward TNBC and HER2-enriched breast cancer cells. Asian Pac J Cancer Prev. 2021; 22(6): 1827-36.
7. Wulandari F, Ikawati M, Kirihata M, Kato J, Meiyanto E. A new curcumin analog, CCA-1.1, induces cell death and cell cycle arrest in WiDr colon cancer cells via ROS generation. J Appl Pharm Sci. 2021; 11(10): 99-105.
8. Wulandari F, Ikawati M, Kirihata M, Kato J, Meiyanto E. Curcumin Analogs, PGV-1 and CCA-1.1 Exhibit anti-migratory effects and suppress MMP9 expression on WiDr cells. Indones Biomed J. 2021; 13(3): 271-80.
9. Utomo RY, Wulandari F, Novitasari D, Lestari B, Susidarti RA, Jenie RI, *et al.* Preparation and cytotoxic evaluation of PGV-1 derivative, CCA-1.1, as a new curcumin analog with improved-physicochemical and pharmacological properties. Adv Pharm Bull. 2022; 12(3): 603-12.
10. Hermawan A, Wulandari F, Hanif N, Utomo RY, Jenie RI, Ikawati M, *et al.* Identification of potential targets of the curcumin analog CCA-1.1 for glioblastoma treatment: integrated computational analysis and in vitro study. Sci Rep. 2022; 12(1): 13928. doi: 10.1038/s41598-022-18348-9.
11. Jenie RI, Novitasari D, Putri DPP, Susidarti RA, Nakamae I, Yoneda-Kato N, *et al.* Different mitotic arrest targets of pentagamavunone-1 (PGV-1) and chemoprevention curcumin analog 1.1 (CCA-1.1) in leukemic K-562 cells. 2022; under review.
12. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, *et al.* Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021; 71(3): 209-49.
13. Llovet JM, Kelley RK, Villanueva A, Singal AG, Pikarsky E, Roayaie S, *et al.* Hepatocellular carcinoma. Nat Rev Dis Primer. 2021; 7: 6. doi: 10.1038/s41572-020-00240-3.
14. Qin XY, Suzuki H, Honda M, Okada H, Kaneko S, Inoue I, *et al.* Prevention of hepatocellular carcinoma by targeting MYCN-positive liver cancer stem cells with acyclic retinoid. Proc Natl Acad Sci. 2018; 115(19): 4969-74.
15. Suenaga Y, Kaneko Y, Matsumoto D, Hossain MS, Ozaki T, Nakagawara A. Positive auto-regulation of MYCN in human neuroblastoma. Biochem Biophys Res Commun. 2009; 390(1): 21-6.
16. Barone G, Anderson J, Pearson AD, Petrie K, Chesler L. New strategies in neuroblastoma: Therapeutic targeting of MYCN and ALK. Clin Cancer Res. 2013; 19(21): 5814-21.
17. Qin XY, Su T, Yu W, Kojima S. Lipid desaturation-associated endoplasmic reticulum stress regulates MYCN gene expression in hepatocellular carcinoma cells. Cell Death Dis. 2020; 11(1): 66. doi: 10.1038/s41419-020-2257-y.
18. Otto T, Horn S, Brockmann M, Eilers U, Schuttrumpf L, Popov N, *et al.* Stabilization of N-Myc is a critical function of Aurora A in human neuroblastoma. Cancer Cell. 2009; 15(1): 67-78.
19. Vaughan L, Clarke PA, Barker K, Chanthery Y, Gustafson CW, Tucker E, *et al.* Inhibition of mTOR-kinase destabilizes MYCN and is a potential therapy for MYCN-dependent tumors. Oncotarget. 2016; 7: 57525-44.
20. Soni VK, Ratre YK, Mehta A, Dixit AK, Dwivedi M, Shukla D, *et al.* Curcumin: a spice pigment against hepatic cancer. In: Nagaraju GP, Ahmad S, editors. Theranostics and Precision Medicine for the Management of Hepatocellular Carcinoma. Vol. 3. London: Academic Press; 2022, p. 141-59.
21. Larasati YA, Yoneda-Kato N, Nakamae I, Yokoyama T, Meiyanto E, Kato J. Curcumin targets multiple enzymes involved in the ROS metabolic pathway to suppress tumor cell growth. Sci Rep. 2018; 8: 2039. doi: 10.1038/s41598-018-20179-6.
22. Ito H, Nakamae I, Kato J, Yoneda-Kato N. Stabilization of fatty acid synthesis enzyme acetyl-CoA carboxylase 1 suppresses acute myeloid leukemia development. J Clin Invest. 2021; 131(12): e141529. doi: 10.1172/JCI141529.
23. Yin S, Zheng X, Yao X, Wang Y, Liao D. Synthesis and anticancer activity of mono-carbonyl analogues of curcumin. J Cancer Ther. 2013; 4(1): 113-23.
24. Tomeh M, Hadianamrei R, Zhao X. A Review of curcumin and its derivatives as anticancer agents. Int J Mol Sci. 2019; 20(5): 1033. doi: 10.3390/ijms20051033.
25. Hu A, Huang JJ, Zhang JF, Dai WJ, Li RL, Lu ZY, *et al.* Curcumin induces G2/M cell cycle arrest and apoptosis of head and neck squamous cell carcinoma in vitro and in vivo through ATM/Chk2/p53-dependent pathway. Oncotarget. 2017; 8(31): 50747-60.

26. Chainoglou E, Hadjipavlou-Litina D. Curcumin analogues and derivatives with anti-proliferative and anti-inflammatory activity: Structural characteristics and molecular targets. Expert Opin Drug Discov 2019; 14(8): 821-42.
27. Ianzini F, Mackey MA. Mitotic catastrophe. In: Gewirtz DA, Holt SE, Grant S, editors. Apoptosis Senescence Cancer. Totowa, NJ: Humana Press; 2007, p. 73-91.
28. Chen Q, Wang Y, Xu K, Lu G, Ying Z, Wu L, *et al*. Curcumin induces apoptosis in human lung adenocarcinoma A549 cells through a reactive oxygen species-dependent mitochondrial signaling pathway. Oncol Rep. 2010; 23(2): 397-403.
29. Verbon EH, Post JA, Boonstra J. The influence of reactive oxygen species on cell cycle progression in mammalian cells. Gene. 2012; 511(1): 1-6. doi: 10.1016/j.gene.2012.08.038.
30. Davalli P, Mitic T, Caporali A, Lauriola A, D'Arca D. ROS, cell senescence, and novel molecular mechanisms in aging and age-related diseases. Oxid Med Cell Longev. 2016; 2016: 3565127. doi: 10.1155/2016/3565127.
31. Gire V, Dulic V. Senescence from G2 arrest, revisited. Cell Cycle. 2015; 14(3): 297-304.
32. Liu J, Wang Z. Increased oxidative stress as a selective anticancer therapy. Oxid Med Cell Longev. 2015; 2015: 294303. doi: 10.1155/2015/294303.
33. Morelli MB, Amantini C, Santoni M, Soriani A, Nabissi M, Cardinali C, *et al*. Axitinib induces DNA damage response leading to senescence, mitotic catastrophe, and increased NK cell recognition in human renal carcinoma cells. Oncotarget. 2015; 6: 36245-59.
34. Eom YW, Kim MA, Park SS, Goo MJ, Kwon HJ, Sohn S, *et al*. Two distinct modes of cell death induced by doxorubicin: apoptosis and cell death through mitotic catastrophe accompanied by senescence-like phenotype. Oncogene. 2005; 24: 4765-77.
35. Zhai K, Brockmüller A, Kubatka P, Shakibaei M, Büsselberg D. Curcumin's beneficial effects on neuroblastoma: mechanisms, challenges, and potential solutions. Biomolecules. 2020; 10(11): 1469. doi: 10.3390/biom10111469.
36. Eberherr C, Beck A, Vokuhl C, Becker K, Häberle B, Von Schweinitz D, Kappler R. Targeting excessive MYCN expression using MLN8237 and JQ1 impairs the growth of hepatoblastoma cells. Int. J. Oncol. 2019; 54(5): 1853-63.
37. Vaughan L, Clarke PA, Barker K, Chanthery Y, Gustafson CW, Tucker E, *et al*. Inhibition of mTOR-kinase destabilizes MYCN and is a potential therapy for MYCN-dependent tumors. Oncotarget. 2016; 7: 57525-44.