RESEARCH ARTICLE

GSH-conjugation Reduces PGV-1 Cytotoxicity and Its Ability in Downregulating N-Myc, β-catenin, and p62 Protein in Huh-6 Cells

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Abstract

BACKGROUND: Pentagamuvone-1 (PGV-1), a synthetic curcumin analogue, exhibits potent anticancer activity against Hepatocellular Carcinoma (HCC) by disrupting cell cycle regulation and downregulating key oncogenes such as N-Myc. Numerous studies have examined the role of glutathione (GSH) conjugation in modulating the anticancer properties of curcumin and its analogues. In contrast, the impact of PGV-1 metabolism, particularly GSH conjugation, and its implications for anticancer efficacy have not yet been elucidated. This study was performed to prepare GSH-conjugated PGV-1 (PGV-1-$(GSH)_2$) as the model of PGV-1 metabolite and evaluate its potential distinct cytotoxicity on Huh-6 cells.
METHODS: PGV-1 was synthesized via an acid-catalyzed reaction between 4-hydroxy-3,5-dimethylbenzaldehyde and cyclopentanone while PGV-1-$(GSH)_2$ was obtained through reflux at 70°C for 2 hours. The cytotoxic effects of PGV-1 and PGV-1-$(GSH)_2$ on Huh-6 and JHH4, two HCC cells, were assessed using a cell counting kit-8 (CCK-8) assay, while immunoblotting was performed to evaluate their impact on N-Myc and its downstream protein such as β-catenin, and p62.
RESULTS: PGV-1-$(GSH)_2$ was prepared through GSH conjugation of PGV-1 in orange color solution, as confirmed by Electrospray Ionization Mass Spectrometry (ESI-MS), Fourier Transform Infrared Spectroscopy (FT-IR), and Nuclear Magnetic Resonance (NMR) analysis. Cytotoxicity assays revealed that PGV-1-$(GSH)_2$ exhibited less potent anticancer activity against HCC cells than PGV-1. GSH conjugation also decreased the ability of PGV-1 in downregulating the N-Myc, β-catenin, and p62 protein level.
CONCLUSION: The prepared PGV-1-$(GSH)_2$ reduces the cytotoxicity of PGV-1 and its ability on downregulating N-Myc, β-catenin, and p62 in Huh-6 cells. These findings highlight the need for further exploration about the study of PGV-1 metabolism which could affect the anticancer efficacy against HCC.
KEYWORDS: curcumin, PGV-1, GSH, HCC, N-Myc

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Introduction

Hepatocellular carcinoma (HCC) remains a significant global health challenge, representing the majority of primary liver cancer cases worldwide. It is associated with high mortality rates due to its aggressive progression and limited therapeutic options.(1) Natural compounds and their derivatives have gained considerable attention as potential anticancer agents, with curcumin, a polyphenolic compound derived from *Curcuma longa*, being one of the most extensively studied.(2,3) However, due to its instability and the requirement of high dose to achieve anticancer effect, various structural modifications of curcumin have

been explored to enhance its potency and pharmacokinetic properties.(4–6) Pentagamavunone-1 (PGV-1), a synthetic curcumin analogue, has demonstrated potent anticancer activity compared to curcumin, particularly against HCC cells.(7–10) PGV-1 is known to be able to maintain its anticancer effects, and selectively target cancer cells.(11) PGV-1 exerts its cytotoxic effects through dysregulation of cell cycle machinery, including prometaphase arrest, spindle microtubule rearrangement, and downregulation of several protein.(12–15) PGV-1 also downregulates one of the main oncogenes in HCC, N-Myc, resulting in persistent cytotoxic effects.(10) N-Myc positively correlates with β-catenin by regulating Wnt/β-catenin signalling to promote neuroblastoma cell survival.(16) In addition, high amplification of the *MYCN* gene in neuroblastoma is associated with downregulation of p62, resulting in high autophagic activity that supports carcinogenesis.(17) Collectively, PGV-1 possesses a promising therapeutic agent targeting key oncogenic pathway in HCC.

Curcumin undergoes extensive metabolism *in vivo*, which significantly reduces its systemic bioavailability and therapeutic efficacy. Curcumin underwent several metabolisms including oxidation, reduction, glucuronidation, sulfation, and glutathione (GSH) conjugation.(18,19) GSH conjugation on curcumin via glutathione S-transferase (GST)-mediated reactions has been demonstrated in incubation with purified human GST, human intestinal cell, and Caco2 cells.(19,20). The olefin moiety of curcumin forms a covalent bond with sulfhydryl group of GSH through Michael addition reaction, which is facilitated by the electrophilic nature of α,β-unsaturated carbonyl presents in curcumin.(15) This reaction results in the formation of glutathionylated curcumin derivatives, which influences its pharmacokinetics and therapeutic efficacy.(16) On the other hand, the olefin structure of curcumin and its analogues play a critical role in its anticancer mechanisms by facilitating interactions with cellular targets and enhancing its biological activity.(17) While GSH conjugation generally serves as a protective mechanism against electrophilic xenobiotics, it can also modulate drug response by altering cellular redox balance and affecting drug-induced cytotoxicity.(18) At low concentration, curcumin possesses as antioxidant effect and GSH inducer, while at high concentration curcumin demonstrated pro-oxidant effect which inhibits the GSH activity.(21)

GSH has been known for its relation to free radical production.(22) Elevated GSH level has been observed in HCC, contributing to the cellular defence against oxidative stress induced by therapeutic compounds, including curcumin and its analogues.(23,24) Given the crucial role of GSH conjugation in cancer cell survival, investigating the effects of GSH-conjugation on curcumin and its analogues is essential for optimizing their therapeutic utility.

Several studies have explored the impact of GSH conjugation on curcumin and its analogues, revealing both beneficial and detrimental effects in the context of anticancer activity. On the one hand, GSH conjugation may enhance the solubility and stability of curcumin derivatives, potentially improving cellular uptake and distribution.(25) On the other hand, excessive GSH conjugation may lead to rapid clearance and diminished cytotoxic efficacy, limiting the therapeutic potential of these compounds.(26) In particular, the GSH-conjugation with curcumin analogues, such as PGV-1 warrants further investigation to determine possible enhancements or reductions of their cytotoxicity.

Since the influence of PGV-1 metabolism, particularly its conjugation with GSH on its anticancer effectiveness has not yet been investigated, this study was performed to prepare GSH-conjugated PGV-1 (PGV-1-$(GSH)_2$) as the model of PGV-1 metabolite and evaluate its potential distinct cytotoxicity on Huh-6 cells especially on modulation of *MYCN* signalling, including N-Myc, β-catenin, and p62 in protein level. The olefin moiety present in PGV-1 allows for the introduction of GSH through a Michael addition reaction, facilitating the formation of conjugated derivatives. Change of cytotoxic activity also could be expected from the PGV-1-$(GSH)_2$ especially in correlation to modulation of *MYCN* signaling pathway.

Methods

Preparation of PGV-1-$(GSH)_2$

The synthesis of PGV-1 was performed following a previously established protocol (27), and the GSH conjugation was carried out according to an earlier described procedure (28). The 4-hydroxy-3,5-dimethylbenzaldehyde (2 eq) was mixed with cyclopentanone (1 eq) under acidic condition with concentrated HCl. The reaction mixture was standing for 11 days at room temperature. The final product was obtained through sequential washing using acetic acid:water (1:1), cold water, and chloroform to get the PGV-1. Further reaction between PGV-1 (1 eq) and GSH proceeded through reflux at 70°C for 2 hours. The completion of GSH-conjugation reaction of PGV-1 was monitored by Thin Layer Chromatography (TLC) and the resulting product was structurally characterized using Electrospray Ionization Mass Spectrometry (ESI-

MS), Fourier Transform Infrared Spectroscopy (FT-IR), and Nuclear Magnetic Resonance (NMR) analysis. The preparation of PGV-1-$(GSH)_2$ was performed based on previous methods with slight modifications.(20,21,23)

Cell Culture

Huh-6 and JHH4 were grown in Dulbecco's modified eagle medium (DMEM) supplemented in fetal bovine serum (FBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium bicarbonate, 150 IU/mL penicillin, 150 µg/mL streptomycin, and 1.25 µg/mL amphotericin B. During the culture process, all cell lines were maintained in an incubator at 37°C with 5% CO_2 and were used for biological assay after two passages. The research protocol on this study has been approved by the Ethical Committee of Faculty of Dentistry, Universitas Gadjah Mada (No. 226/UN1/KEP/FKG-RSGM/EC/2023).

Cytotoxicity Assay

The procedure for cytotoxicity assay followed the previous published work.(12) Cells were seeded at a density of 8×10^3 cells/well in a 96-well plate and cultured overnight. The cells were treated with varying concentrations (0.1; 0.5; 1;5; 10; 50 µM) of PGV-1 and PGV-1-$(GSH)_2$ compounds and incubated for 72 hours. The solution stock of PGV-1 and PGV-1-$(GSH)_2$ was prepared in dimethyl sulfoxide (DMSO) and MiliQ water, respectively. Cell viability was assessed using the cell counting kit-8 (CCK-8) reagent, and absorbance was measured using a microplate reader at a wavelength of 595 nm.

Immunoblotting for N-Myc, p62, and β-catenin Analysis

Protein expression was analysed using Abby capillary western blot system (ProteinSimple, San Jose, CA, USA) following manufacturer's protocol with supplied reagent kit. Cell lysis was performed using ice-cold radioimmunoprecipitation assay (RIPA) buffer, followed by brief sonication. The lysates were then centrifuged at 10,000 g for 10 minutes to separate cellular debris. The resulting supernatant were collected and mixed with fluorescent master mix containing sample buffer, a fluorescence standard, and dithiothreitol (DTT), followed by boiling as the denaturation step. The samples, antibodies, and reagents were loaded into the designated wells of the plates as specified in the system module. The antibodies used in this study included: anti-N-Myc (1:1000 dilution) (Cat #9405; Cell Signaling Technology, Danvers, MA, USA), anti-p62 (1:1000 dilution) (PM045; MBL Life Science, Tokyo, Japan), and anti-β-catenin (1:1000 dilution) (Cat #9587; Cell Signaling Technology), and anti-tubulin (1:4000 dilution) (Cat #2144S; Cell Signaling Technology). Protein detection was determined automatically in the instrument based on peak area values.

Data Analysis

Each experiment was conducted in triplicate, with a minimum of three replications, and analysed statistically using student t-test or ANOVA with a confidence level of >95%.

Results

Preparation of PGV-1-$(GSH)_2$

The preparation of PGV-1-$(GSH)_2$ was successfully performed as illustrated in Figure 1. Condensation reaction between an aldehyde compound, 4-hydroxy-3,5-dimethylbenzaldehyde (**1**) with a ketone compound, cyclopentanone (**2**) in acidic conditions produced PGV-1 compound in 48% yield. Conjugation of glutathione (**3**) with PGV-1-$(GSH)_2$ under reflux condition produced the final product shown as orange colour solution.

Structure elucidation using the ESI-MS of PGV-1-$(GSH)_2$ showed the presence of a peak at 961.1771 m/z, which confirmed the formation of PGV-1-$(GSH)_2$ (Figure 2). The base peak at 948.5284 m/z was likely generated through further ionization, attributed to the compounds which contained multiple carboxylate, amide, amine, and phenolic hydroxyl groups, which readily underwent hydrogen loss. The low intensity of detected m/z in the

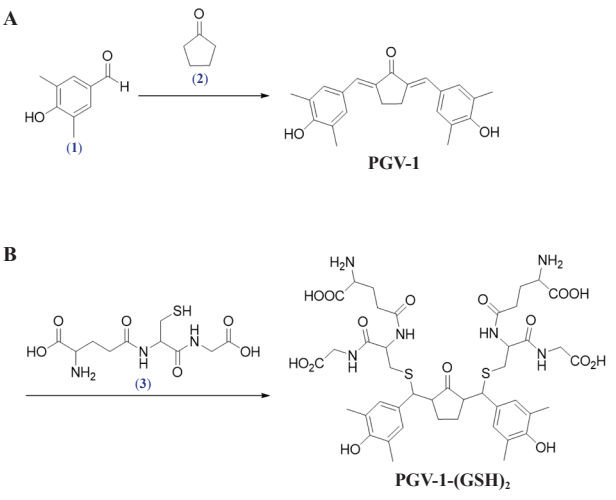

Figure 1. Synthetic scheme for the preparation of PGV-1-$(GSH)_2$. A: Reaction condition of concentrated HCl for 11 days; (1): 4-hydroxy-3,5-dimethylbenzaldehyde; (2): cyclopentanone. B: Reaction condition of MeOH, H_2O, 70°C for 2 hours

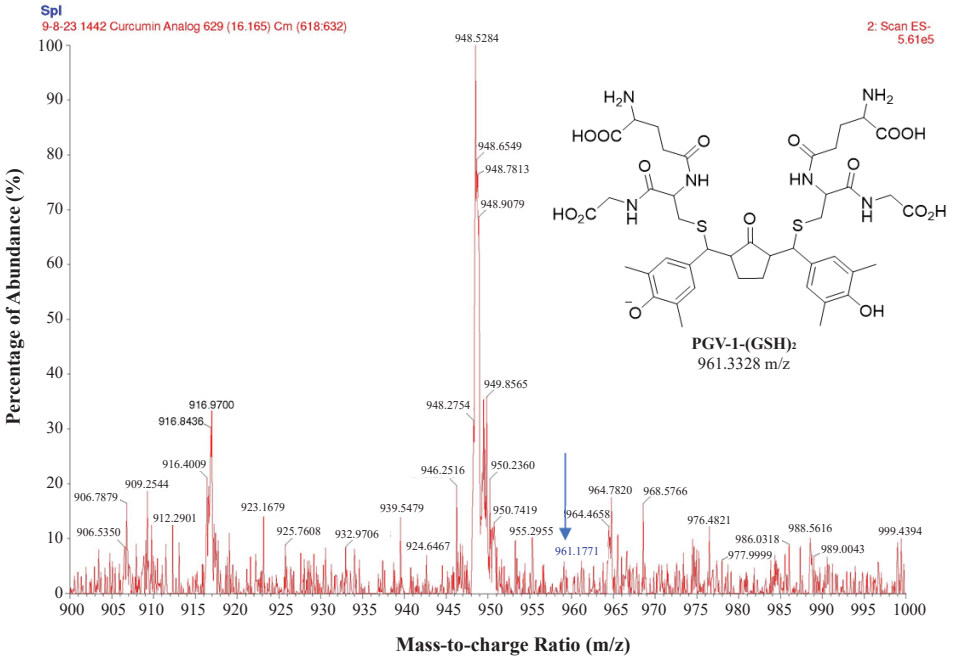

Figure 2. High resolution MS profile of PGV-1-(GSH)$_2$. The PGV-1 and PGV-1-(GSH)$_2$ were diluted in methanol grade LC-MS then subjected to ESI-MS. Identified product of PGV-1-(GSH)$_2$ was shown in blue arrow.

sample suggested that the PGV-1-(GSH)$_2$ was presented in low abundance.

The infrared (IR) spectrum of PGV-1 displayed a broad absorption band between 2500-3600 cm^{-1}, corresponding to overlapping signals from carboxylate, amide, amine, and thiol groups (Figure 3). Additionally, the broad bands at 1591 and 1598 cm^{-1} likely resulted from overlapping carbonyl stretching vibrations of amide and carboxyl groups. In contrast, the IR spectrum of PGV-1 revealed characteristics peaks for phenolic hydroxyl group (3336 cm^{-1}), conjugated ketones (1650 cm^{-1}), stretching and bending of aromatic or aliphatic -CH groups at ~3000 and 1640 cm^{-1}, respectively. These spectral differences suggested that PGV-1-(GSH)$_2$ was formed.

The conclusion was further supported by the absence of proton signals at δ 7-8 in the ^{1}H-NMR spectrum (Figure 4). Instead, signals at δ 3.642-1.937 correspond to methine (-CH) and methylene (CH$_2$) protons of GSH. The signals for amide (-CONH), amine (-NH$_2$, -NH-), carboxyl (-COOH), and phenolic hydroxyl (-OH) protons were absent, likely due to exchange with deuterium from the D$_2$O solvent. The ^{13}C-NMR spectrum further corroborated this, with four signals at δ 176.8-171.8 corresponding to a carboxyl and an amide carbonyl group of glutathione, while signals at δ 54.2-25.3 rose from methine and methylene groups (Figure 5). Notably, neither the IR nor the NMR spectra exhibited signals corresponding to the aromatic or olefinic groups of PGV-1 likely due to its poor solubility in D$_2$O.

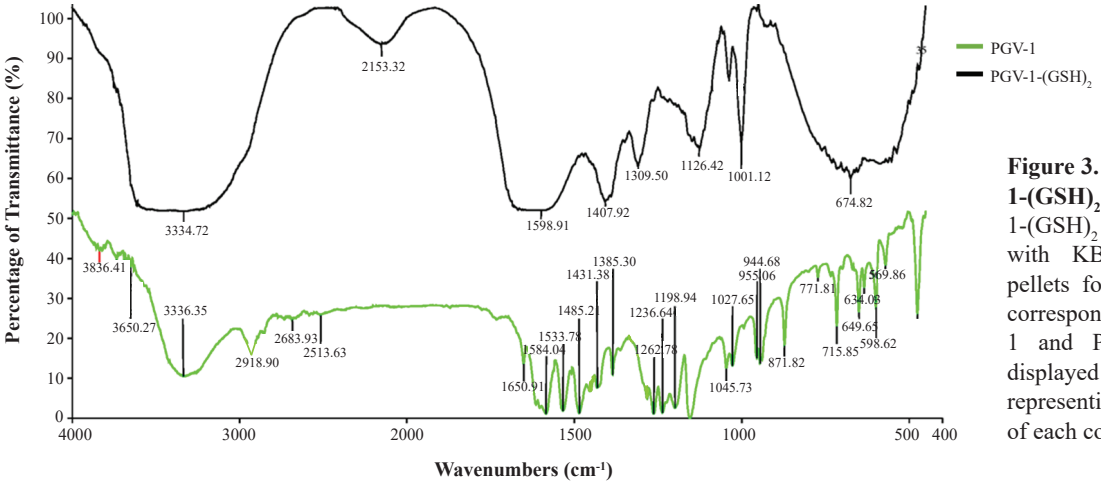

Figure 3. FTIR profile of PGV-1-(GSH)$_2$. The PGV-1 and PGV-1-(GSH)$_2$ were homogenized with KBr and pressed into pellets for FT-IR analysis. The corresponding spectra of PGV-1 and PGV-1-(GSH)$_2$ spectra displayed characteristic peaks representing the functional group of each compound.

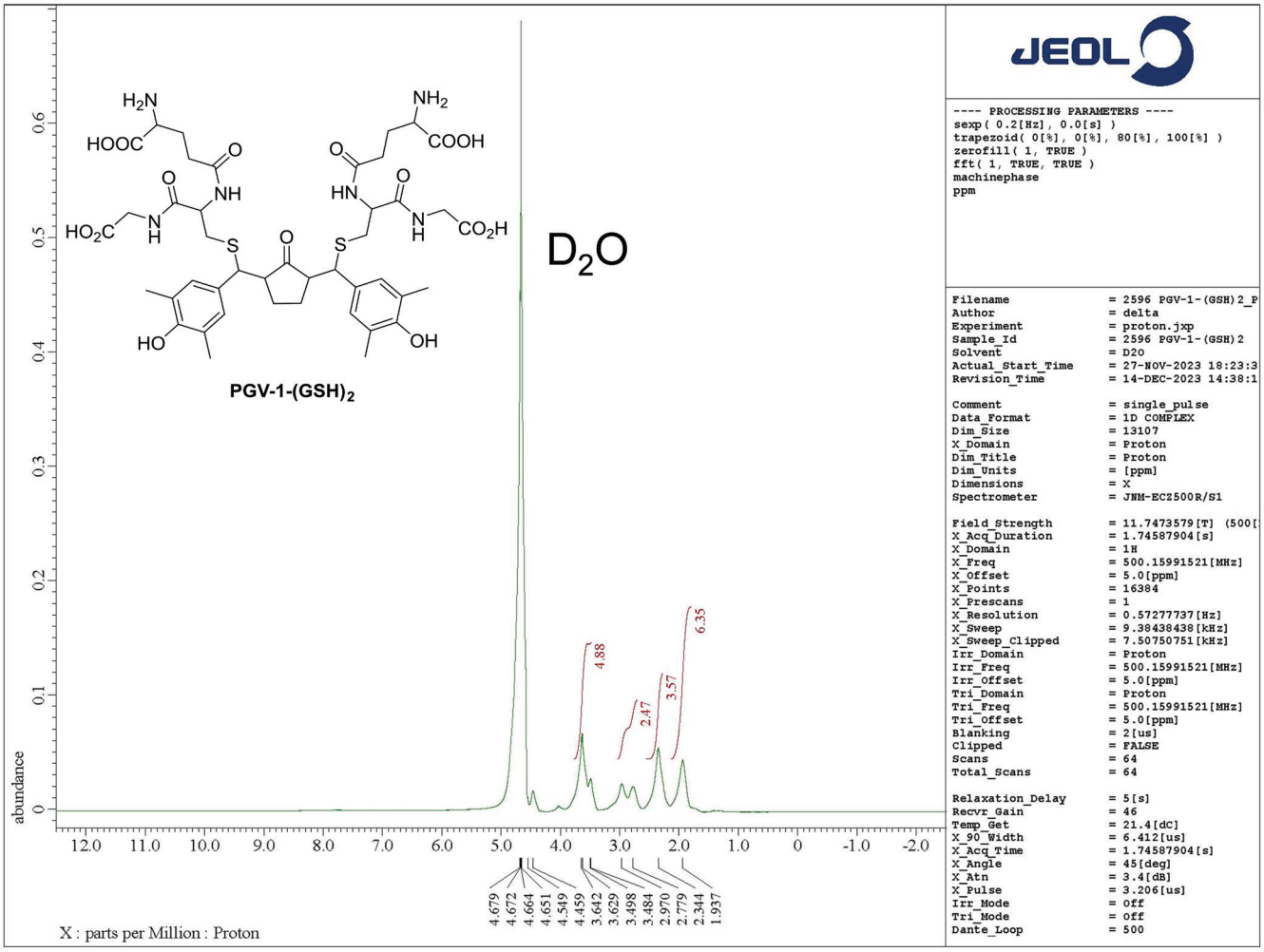

Figure 4. ^{1}H-NMR profile of PGV-1-(GSH)$_2$.

This suggested that glutathione conjugation improved the aqueous solubility of PGV-1.

Cytotoxic Effect of PGV-1 and PGV-1-(GSH)$_2$ against HCC Cells

Cytotoxicity evaluation serves as the primary assessment of potential anticancer agents by determining their ability to reduce cell viability. This study utilized Huh-6 cells as a model of HCC with high *MYCN* expression and JHH4 as the model of low-expressed *MYCN* HCC. The dose-response curves demonstrated that PGV-1 and PGV-1-(GSH)$_2$ exerted different cytotoxic effects on the cells. PGV-1 exhibited as a more potent dose-dependent reduction in cell viability against Huh-6 and JHH-4 cells with IC_{50} value of 2.41±1.94 µM and 2.15±0.29 µM, respectively (Figure 6A). In contrast, PGV-1-(GSH)$_2$ demonstrated lower cytotoxicity toward both Huh-6 and JHH4 cells with IC_{50} >50 µM (Figure 6A). Microscopic analysis further supported these findings, showing significant morphological alterations, including cell shrinkage and detachment upon treatment at 5 µM of PGV-1, whereas PGV-1-(GSH)$_2$ induced milder changes (Figure 6B, Figure 6C). Collectively, these results indicated that PGV-1-(GSH)$_2$ possessed lower cytotoxicity than PGV-1 in HCC cells.

Effect of PGV-1 and PGV-1-(GSH)$_2$ toward *MYCN* Signaling Protein

MYCN signaling plays a critical role in HCC progression by regulating cell proliferation, differentiation, and survival, making them key therapeutic targets. In this study, western blot analysis demonstrated that treatment with PGV-1 but not PGV-1-(GSH)$_2$, resulted in a reduction of N-Myc protein level and its downstream, β-catenin compared to untreated control, suggesting a suppressive effect on *MYCN*-driven oncogenic signaling (Figure 7A, Figure 7B). PGV-1, but not PGV-1-(GSH)$_2$, also downregulated p62 while PGV-1-(GSH)$_2$ showed a relatively weaker effect, suggesting the possible suppression of autophagy

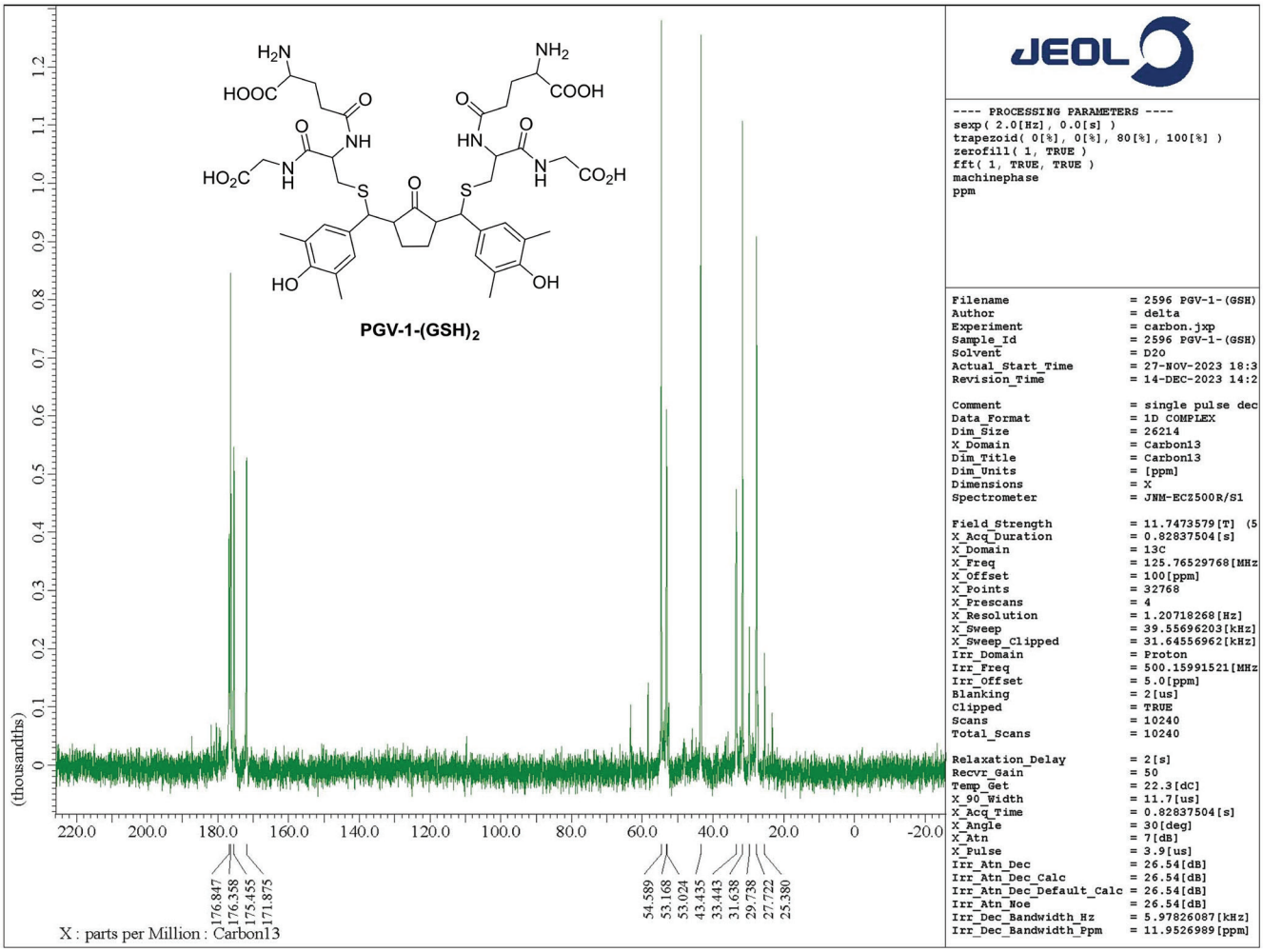

Figure 5. ^{13}C-NMR profile of PGV-1-(GSH)$_2$.

degradation by PGV-1 due to GSH conjugation (Figure 7A, Figure 7B). Collectively, these findings suggested that PGV-1 effectively downregulated N-Myc, β-catenin, and p62. However, PGV-1-(GSH)$_2$ suppressed the modulation ability of PGV-1, which could affect its anticancer potential against HCC.

Discussion

Several reports demonstrate GSH conjugation as a widely recognized metabolic modification that influences the bioavailability and therapeutic potential of curcumin and its analogues. In rat model, curcumin is predominantly accumulates in intestine rather than in plasma, where GSH conjugation occurs more prominently.(20,29) This conjugation influences the bioavailability of curcumin and may potentially affects its biological activity.(26) Therefore, the evaluation of GSH-conjugated forms of curcumin analogues, including PGV-1 was critically needed, particularly for their application in drug development. This study successfully achieved PGV-1-(GSH)$_2$ through a Michael addition reaction between PGV-1 and GSH under reflux conditions at 70^{o}C for 2 hours, which is significantly shorter than 24 hours to several day durations reported for other curcumin analogues-based GSH conjugates. (28,30) However, the ESI-MS spectra showed the low abundance of the targeted peak suggested to the instability of GSH conjugation on PGV-1. This phenomenon was in compromise with the GSH-conjugated curcumin which gradually decreased the detectable peak during the Liquid Chromatography-Mass Spectrometry (LC-MS) analysis. (26) The olefin moiety in PGV-1 plays a critical role in facilitating this reaction due to its electrophilic nature, allowing efficient covalent bonding with nucleophilic sulfhydryl group of GSH.(20) Previous studies have shown that the conjugation of curcumin analogues often leads to the formation of water-soluble compounds, improving

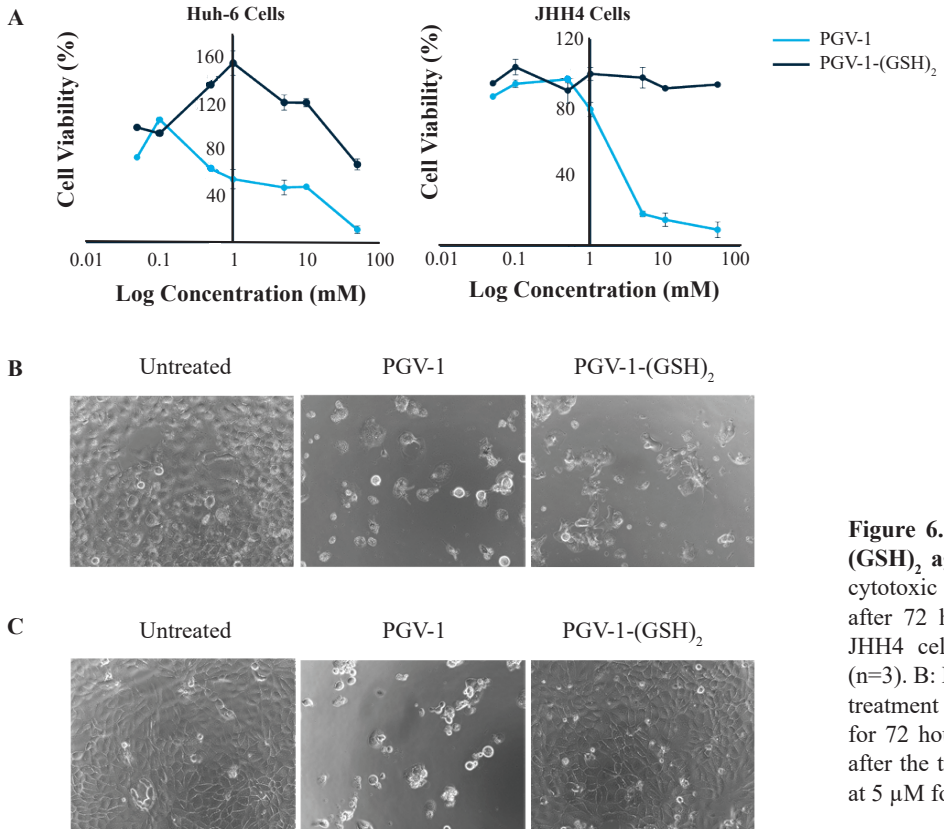

Figure 6. Cytotoxicity of PGV-1 and PGV-1-(GSH)$_2$ against HCC cells. A: Dose-dependent cytotoxic effects of PGV-1 and PGV-1-(GSH)$_2$ after 72 hours of treatment against Huh-6 and JHH4 cells. Data was expressed in mean±SE (n=3). B: Morphology of the Huh-6 cells after the treatment of PGV-1 and PGV-1-(GSH)$_2$ at 5 µM for 72 hours. C: Morphology of the JHH4 cells after the treatment of PGV-1 and PGV-1-(GSH)$_2$ at 5 µM for 72 hours.

solubility but sometimes reducing cytotoxic potency.(28,30) The successful and rapid preparation of PGV-1-(GSH)$_2$ in this study suggest that modifying the olefin moiety could be a strategic approach to control reaction efficiency and stability in further curcumin analogues design.

The pro-oxidant properties of PGV-1 primarily drive its cytotoxicity by inducing oxidative stress and disrupting cellular redox homeostasis. Pro-oxidant properties of PGV-1 act more rapidly than curcumin, exhibiting significant effects less than 12 hours of treatment against cancer cells.(13,31) Additionally, PGV-1 demonstrated binding affinity to several ROS metabolizing enzymes with distinct characteristics that differentiate its mechanism of action than curcumin.(13,32) The conjugation of PGV-1 with GSH introduces an additional redox-regulating factor, as GSH serves as a key antioxidant that neutralizes reactive oxygen species (ROS) and mitigates oxidative stress-induced cytotoxicity.(33) The observed decreased in cytotoxic potency of PGV-1-(GSH)$_2$ compared to PGV-1 suggested that the presence of GSH counteracts the oxidative stress-mediated cell death mechanism. This phenomenon is consistent with previous findings, where GSH treatment

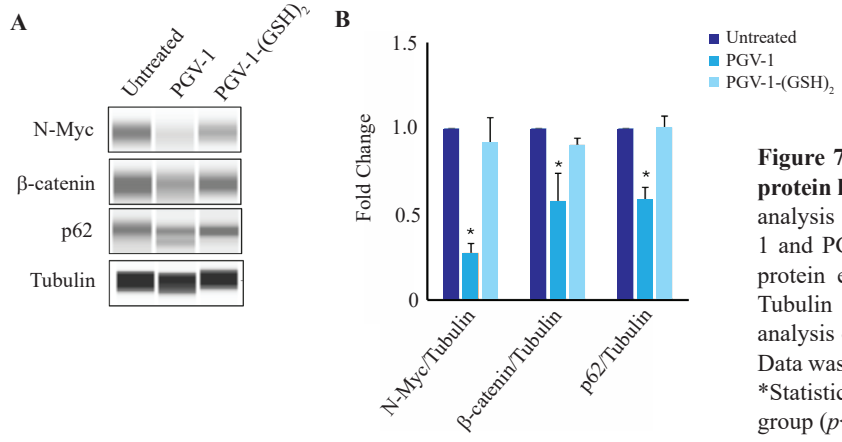

Figure 7. Modulation of PGV-1 and PGV-1-(GSH)$_2$ on protein level of *MYCN* signaling protein. A: Western blot analysis was performed on Huh-6 cells treated with PGV-1 and PGV-1-(GSH)$_2$ at 5 µM for 24 hours to assess the protein expression level of N-Myc, β-catenin, and p62. Tubulin served as a loading control. B: Densitometric analysis of N-Myc, β-catenin, and p62 normalized tubulin. Data was presented as the mean±SEM relative to untreated. *Statistically significant difference compared to untreated group (p<0.01, analyzed with Student's t-test).

attenuated the ROS elevation of PGV-1 in K562 cells. (13) In addition, low concentration of GSH conjugated curcumin analogues slightly increased the percentage of cell survival in MDA-MB-435 cells, suggesting a reduction in cytotoxicity.(28) Similarly, a cytotoxicity study of GSH-conjugated EF25 indicated a reduced cytotoxic effect, as reflected by a higher IC_{50} value at 24 hours of treatment. (30) These findings emphasize the dual role of GSH in both detoxification and drug resistance, highlighting the need for strategies to modulate GSH metabolism to maximize the therapeutic efficacy of curcumin-based anticancer agents.

The *MYCN* signaling pathway plays a pivotal role in HCC progression, regulating oncogene expression and promoting cell proliferation. PGV-1 effectively suppressed N-Myc protein level, indicating its potential as a therapeutic agent targeting *MYCN*-driven HCC.(6–9) Additionally, our study revealed the role of PGV-1 on downregulation of β-catenin, a promotor of proliferation through Wnt/β-catenin signaling.(29) Wnt/β-catenin signaling plays a critical role in HCC by promoting tumor progression cancer stem cells (CSC) maintenance, which can be renewed in undifferentiated state (34), with N-Myc expression positively correlating with the activated Wnt/β-catenin signaling.(35) The autophagy flux marker p62 was also suppressed by PGV-1; however, this effect was counteracted by GSH conjungation. Amplification of *MYCN* induced autophagy under stress which was mediated by p62 protein to promote tumor survival and therapeutic resistance.(30) The role of p62 is more directly correlated autophagy compared to other autophagy markers such as LC3, ATG, and Beclin, as it reflected the occurrence of autolysosomal degradation, which lead to a reduction of p62.(36) However, the diminished effect of PGV-1-$(GSH)_2$ on N-Myc and its downstream proteins suppression suggested that the olefin moiety in PGV-1 was crucial for its interaction with *MYCN*-related proteins. The loss of this interaction upon GSH conjugation could reduce its ability to interfere with oncogenic signaling, thereby lowering its anticancer potency. The absence of olefin moiety as found in Tetrahydropentagamavunone-1 (THPGV-1) also decreased cytotoxicity of PGV-1 against HeLa, T47D, and WiDr cells.(31) This observation highlights the importance of structural integrity in curcumin analogues and suggests that preserving the olefin functionality could be essential for maintaining their therapeutic activity.

The findings from this study provided critical insights into the comparative cytotoxic effect of PGV-1 and its GSH-conjugated form, which may have important implications for clinical applications. GSH levels are significantly elevated in HCC, playing a crucial role in cancer progression and respond to pro-oxidant anticancer agents.(28) Research has shown that GSH level could double in HCC tissues compared to adjacent normal liver tissues, which is associated with increased expression of GSH synthetic enzymes, such as γ-glutamylcysteine synthetase (GCS) and glutathione synthesis (GS).(32) Given that GSH metabolism is a common pathway for many curcumin analogues, understanding its impact is essential for designing more effective derivatives with enhanced stability and anticancer activity.(15) Future research should explore strategies to modulate GSH interaction, either by inhibiting GST-mediated conjugation or by modifying the chemical structure of curcumin analogues to balance metabolic stability and anticancer potency. These findings emphasize the need for careful consideration of metabolic modifications in drug design to optimize the clinical potential of curcumin-based therapeutics for HCC treatment.

Conclusion

This study demonstrates the preparation of PGV-1-$(GSH)_2$ as the possible metabolites of PGV-1. The GSH-conjugation reduces the cytotoxicity of PGV-1 against Huh-6 cells including the downregulation of several protein such as N-Myc, β-catenin, and p62. Given that GSH conjugation is a one of the metabolic pathways for curcumin analogues, its impact on PGV-1 particularly the anticancer efficacy requires further exploration.

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Authors Contribution

RYU contributes to designing research, collecting data, and analyzing the results. EM and RAS conceptualize the research and analyze the results. All authors finalize the manuscript.

Conflict of Interest

The authors declare no conflicts of interest or competing interests related to the content of this manuscript.

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