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Complexes of Pentagamavunon-1 and Lactosylated Albumin for Enhancing the Cytotoxic Effect on Hepatocellular Carcinoma

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ABSTRACT

Pentagamavunon-1 (PGV-1) has potential as an anticancer drug for hepatocellular carcinoma (HCC) but has limitations in terms of solubility. This study aimed to enhance its solubility and cytotoxicity against liver cancer cells. Complexes were developed by combining bovine serum albumin (BSA) with lactose, aiming to improve the bioavailability of PGV-1 as an anticancer agent. These complexes were further modified through lactosylation, producing PGV-1–loaded lactosylated BSA (PGV-1 + BSA + Lac), to increase their cytotoxic effects on HCC cells. The glycation of BSA with d-lactose was conducted under dry-heat conditions at 60°C for 0, 30, 60, 120, or 240 min. The extent of BSA glycation was monitored by assessing the availability of free peptides and examining the molecular weight profile. A straightforward formulation was proposed involving a complex of BSA lactosylated with PGV-1. The solubility of PGV-1 in aqueous solutions was assessed with varying amounts of BSA. Cytotoxicity was evaluated through an MTT assay using the HLF cancer cell line. The results revealed distinct variations in BSA conjugation that were dependent on the duration of heating. Modified BSA exhibited reduced peptide availability and slower migration in SDS-PAGE. Notably, PGV-1 demonstrated significantly higher solubility when combined with BSA + Lac compared with PGV-1 + BSA. The IC_{50} value for PGV-1 + BSA + Lac in HLF cells (0.008 ± 0.002 μM) was lower than that for PGV-1 (0.437 ± 0.002 μM) and PGV-1 + BSA (0.631 ± 0.002 μM), highlighting the potent inhibitory effect of PGV-1 + BSA + Lac on HLF cell proliferation. Thus, PGV-1 + BSA + Lac complexes may be promising candidates for targeted PGV-1 delivery in HCC therapy.

Keywords: PGV-1, albumin, glycoprotein, lactosylated, HCC

INTRODUCTION

Pentagamavunon-1 (PGV-1), a novel anticancer candidate that is chemically similar to curcumin, is synthesized from cyclopentanone, 4-hydroxy-3,5-dimethylbenzal-dehyde, and hydrochloric acid (Sardjiman et al., 1997). PGV-1 inhibits cancer cells by inducing cell cycle arrest, promoting apoptosis, reducing inflammation, interfering with specific cancer-related signaling pathways, inhibiting angiogenesis, and possibly interacting with cancer-specific receptors, depending on the cancer type (Lestari et al., 2019; Meiyanto et al., 2018). PGV-1 also inhibits enzymes that metabolize reactive oxygen species (ROS), leading to increased ROS levels and resulting in cell senescence (Meiyanto, et al., 2022). However, the low solubility of PGV-1 in water (logP = 5.161) limits its potential to reach effective tissue

concentrations (Utomo et al., 2022). Inadequate drug solubility remains a significant challenge in drug research and development, causing unpredictable absorption and poor oral bioavailability (Bellantone, 2014). This solubility issue substantially hinders the development of drug formulations, including parenteral, topical, and oral forms, leading to slow dissolution rates and compromised drug absorption. Approximately 60%–70% of drugs in development have poor water solubility, with >40% being nearly insoluble (Bosselmann & Williams, 2011). Although PGV-1 has proven effective in in vitro testing, the low solubility reduced the bioavailability, thereby limiting the absorption and distribution of the drug to target tissues. Therefore, a PGV-1 formulation is required that it is more soluble and stable.

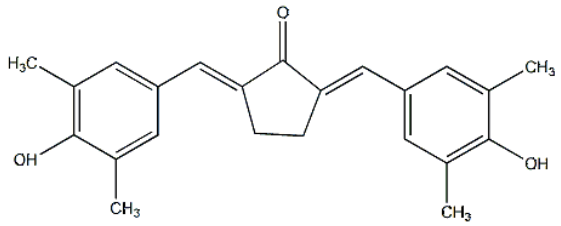


Figure 1. Chemical Structure of PGV-1

Complexes using water-soluble carriers have been developed as a solution to this issue. Protein-based complexes, particularly those involving albumin, are effective because of the abundance of carboxylic and amino groups on the surface of the protein, enabling strong interactions with drug compounds, such as PGV-1 (Fu et al., 2015; Nosrati et al., 2018). Albumin, which is biodegradable, lacks antigenicity, and is easily produced, enhances solubility by forming reversible binding complexes with ligands, enabling molecules to circulate at higher concentrations than their intrinsic solubility would allow (Ghuman et al., 2005; Urien et al., 2001). Albumin has two primary binding sites characterized by positive charges, facilitating the binding of anionic molecules, and additionally possesses multiple secondary binding sites, broadening the range of molecules that can be bound (Evans, 2002). The ketone group and molecular weight (MW) of 348.43 g/mol of PGV-1 indicate a substantial drug-loading capacity when complexed with albumin while minimizing tissue irritation and toxicity. Furthermore, the large size of albumin nanoparticles allows for incorporating a significant amount of medication into the particle matrix (Jithan et al., 2011).

The use of glycoprotein–drug complexes has gained prominence for in vitro cytotoxicity against hepatocellular carcinoma cells (HCCs), primarily because of their affinity with asialoglycoprotein receptors (ASGPRs) (Singh et al., 2017). Glycoproteins often function as receptors on the cell surface, allowing the cell to receive signals from the external environment, including hormones, neurotransmitters, and growth factors. Enzymes on the cell membrane can interact with glycoproteins to activate or deactivate specific signaling pathways (Widjaja et al., 2023). Glycoproteins are formed through the enzymatic linkage of sugars to specific amino acids. N-glycans are covalently attached to asparagine residues within a polypeptide chain while O-glycans are commonly attached to hydroxyl groups of serine, threonine, or tyrosine residues (Ledesma et al., 2008). Given the inherent affinity of ASGPRs for sugars, these glycosylated proteins are expected to target the specific ASGPRs and enhance the cellular uptake of PGV-1. Nevertheless, a crucial challenge is determining whether the protein glycation complex with PGV-1 exhibits superior cytotoxic activity than free PGV-1. Therefore, this study evaluated the cytotoxic effects of a BSA–PGV-1 formulation on liver cancer cells by using the HCC cell line as an in vitro model.

MATERIALS AND METHODS

PGV-1 was synthesized, purified, and characterized by the Cancer Chemoprevention Research Center at the Faculty of Pharmacy, Gadjah Mada University (CCRC-UGM) as previously reported (Meiyanto et al., 2019). BSA and d-lactose were purchased from Sigma Aldrich Chemicals Co. (St Louis, MO, USA). Phosphate buffer saline (PBS) (1×, pH 7.4) was obtained from Fujifilm (Wako, Osaka, Japan). Broad range markers were obtained from SMOBIO Tech. Inc. (Hsinchu City, Taiwan). Dimethyl sulfoxide (DMSO) was sourced from Merck Millipore, Darmstadt, Germany. The Dulbecco modified Eagle medium (DMEM) with high glucose and penicillin–streptomycin was procured from Wako, Osaka, Japan, and fetal bovine serum (FBS) from Hyclone, Logan, UT, USA. All other reagents were of analytical grade.

Glycation

Glycation procedures were performed as previously described (Ledesma et al., 2008). Briefly, 100 mg BSA in 10.0 mL of PBS (0.2 M, pH 7.4) was combined with 300 mg of lactose. Samples were frozen at −40°C, freeze-dried, and then heated at 50°C for 0, 30, 60, 120, and 240 min.

Free Peptide assessment

Untreated BSA was used as the control. The quantification of free peptide was conducted by fusing the Bradford method outlined by Asyhari et al. (2018). For sample preparation, 5 mL of Bradford reagent was used to dissolve each sample, with their concentrations adjusted to match 15 ppm BSA. Control samples included unglycated and unheated BSA. Solutions were vigorously mixed, and their absorbance was subsequently measured at 595 nm. The calculation of free peptide was determined using the following formula:

$$\text{Free peptide (\%)} = \frac{\text{C0-C1}}{\text{C0}} \text{ x } 100\%$$

Where C0 is the free peptide concentration of the control, and C1 is the free peptide concentration of the sample. The BSA standard curve equation was $y = 0.0309 x + 0.0619$ ($r^2 = 0.9939$).

Gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as previously described (Weber & Osborn, 1969). Glycoconjugates from each treatment were separated using 10% SDS-PAGE under reducing conditions. Samples (10 μL) were mixed with 5× SDS-PAGE–loading buffer (40 μL) and heated for 5 min at 95°C (Laemmli, 1970). Each sample (15 μL) was then loaded into the gel wells, with a protein marker (5 μL) in one well. Electrophoresis was conducted over 180 min at 100 volts when the dye migrated approximately 0.5 cm from the bottom. Subsequently, the gels were stained with Coomassie Brilliant Blue R for 30 min and then destained in water: acetic acid: methanol (8: 1: 1) for 24 h until blue protein bands became visible.

Solubility studies

Solubility studies were conducted to determine the aqueous solubility of PGV-1. PGV-1 (1 mg) was added to BSA (1 mL) (PGV-1 + BSA) and BSA-lactose (PGV-1 + BSA + Lac) solutions in PBS, with progressive concentrations (0, 10, 20, 40, 80, and 160 μM). This step aimed to explore the relationship between PGV-1 solubility and the concentration of the BSA solution. Samples were gently stirred at 25°C for 24 h to attain solubility equilibrium. Samples were then centrifuged at 10,000 rpm for 3 min, and the drug concentration was measured through UV-Vis spectrophotometric analysis (Khoder et al., 2016).

Thin layer chromatography

The stability of PGV-1 in an aqueous medium was monitored via thin layer chromatography (TLC) employing silica gel 60 F254 (Merck, Darmstadt, Germany) as the stationary phase and a mobile phase of n-hexane: ethyl acetate (2:1). The TLC analysis was conducted over 7 days to ascertain whether the PGV spots underwent any alterations.

Cell culture

The HCC cell line, HLF, was provided by Chiba Cancer Center Research Institute (CCCRI), Japan, and stored in CCRC-UGM based on the eAsia collaborative program 2023. Cryopreserved cells were thawed at room temperature until they transitioned into a liquid state, after which they were cultured in a 10-cm tissue culture dish in DMEM (Gibco, New York, USA), supplemented with 10% FBS (Gibco) and 1.5% penicillin–streptomycin (Gibco).

Cytotoxic assay

Cells were grown in 96-well plates at 1×10^4 cells per well and divided into untreated and treated groups. A range of curcumin or PGV-1 concentrations was diluted in culture media. Following a 24-h incubation (37°C, 5% CO_2), the culture medium was aspirated, and cells were rinsed with PBS. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma) at 5 mg/mL in PBS was further diluted in DMEM to a ratio of 1:9. Subsequently, 100 μL of this reagent was introduced per well. Following a 3-h incubation, the reaction was halted by adding 10% SDS in 0.01 N HCl. The plate was then left to incubate overnight at room temperature in the dark. Afterward, the dish was agitated for 10 min to ensure the complete dissolution of the purple formazan salt. The absorbance of purple formazan was determined at 550 nm using an ELISA Reader (Bio-Rad 680XR). The viability of HLF cells was calculated as follows (Mosmann, 1983):

$$\text{Viability cell (\%)} = \frac{\text{Abs of treated well}}{\text{Abs of cell control}} \text{ x } 100\%$$

The IC_{50} value was calculated through probit analysis, using data related to cell viability and the logarithm of PGV-1, PGV-1 + BSA, and PGV-1 + BSA + Lac concentrations.

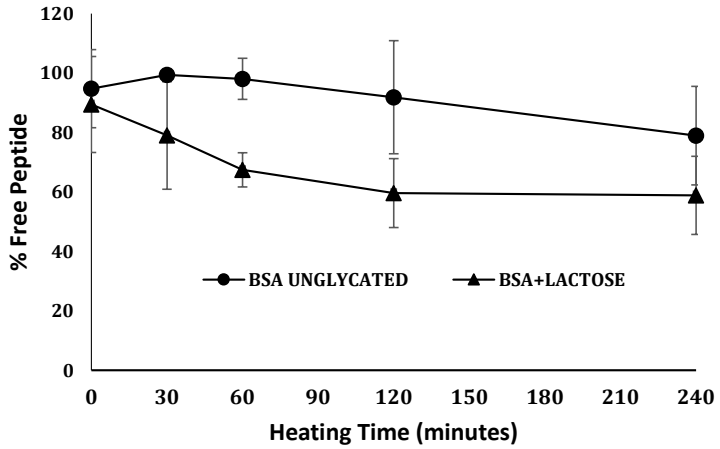


Figure 2. Available free peptide of untreated (without carbohydrates) and glycated BSA with lactose at 50°C for 0, 30, 60, 120, and 240 min.

Statistical analysis

Data are presented as mean values accompanied by the standard error of the mean (SEM). Statistical analysis was performed via analysis of variance (ANOVA), followed by the Dunnett test for comparison against the untreated group, with a 95% confidence level. Data were analyzed using Microsoft Excel 2019.

RESULTS AND DISCUSSION

Sugar loading onto albumin peptides

Herein, we synthesized glycoprotein complexes using the Maillard reaction, where the BSA peptide interacted with the carbonyl groups of carbohydrates, specifically lactose. This degree of conjugation between the peptide and carbohydrate depended on the heating time and displayed selectivity toward specific peptide sequences. The assessment of free peptide glycation, measured using the Bradford test (Figure 2).

Glycation occurs when the carbonyl groups of lactose form covalent bonds with the free peptide groups in BSA, forming Schiff bases, which then undergo Amadori rearrangement (Bu et al., 2015). The Bradford assay is used to measure the concentration of free peptides based on the principle of electrostatic interaction between peptide residues and sulfonate groups of the Coomassie Brilliant Blue G-250 dye. The assay measures the difference in free peptide concentration between a control sample containing unglycated BSA and a sample with BSA and lactose (BSA + Lac). The percentage of free peptides significantly decreased during 120 min of heating in the BSA + Lac mixture (Figure 2). This decrease was directly proportional to the heating duration, indicating that glycation progresses over time. The use of the Bradford assay is important as this quantifies the extent of glycation by comparing the levels of free peptides in different samples. This comparison allows an understanding of how glycation affects the protein structure and the potential implications for subsequent biochemical processes.

In the early stages of the glycation process, free peptides on the surface of the protein molecule are the first to bind with the sugar, followed by the internal free peptides (Lagemaat et al., 2007). The decrease in free peptide levels signifies a Maillard reaction between the free BSA peptides and lactose. This process continues until the peptide residues become depleted as they bind with sugar, which ultimately terminates the glycation process.

MW profile analysis

The SDS-PAGE analysis revealed significant variations in the migration patterns of glycated BSA (Figure 3). Bands corresponding to BSA + Lac displayed broader and slower migration than untreated BSA, indicating that glycated samples contained various protein molecules with differing levels of attached sugar residues. In Lane S, a distinct band with a high density, approximately 66 kDa in size, was observed for unmodified BSA (Figure 2, Lane S). Conversely, a new BSA + Lac band had an approximate MW of 70 kDa (Figure 2, Lanes 1–5, respectively), surpassing that of BSA. These results suggest that the increased MW of Lac-BSA may be attributed to the number of lactose molecules (MW = 360.31) conjugated to BSA.

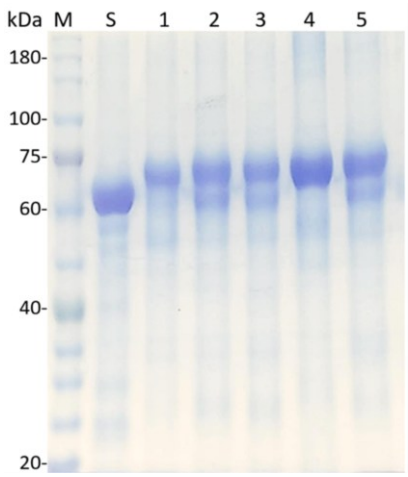


Figure 3. Electrophoretic analysis of glycated BSA (BSA-Lac). SDS-PAGE in 10% gel of (M) molecular mass markers; (S) untreated BSA (without heating and carbohydrates); (1–5) BSA-Lac heated at 50°C for 0, 30, 60, 120, and 240 min respectively.

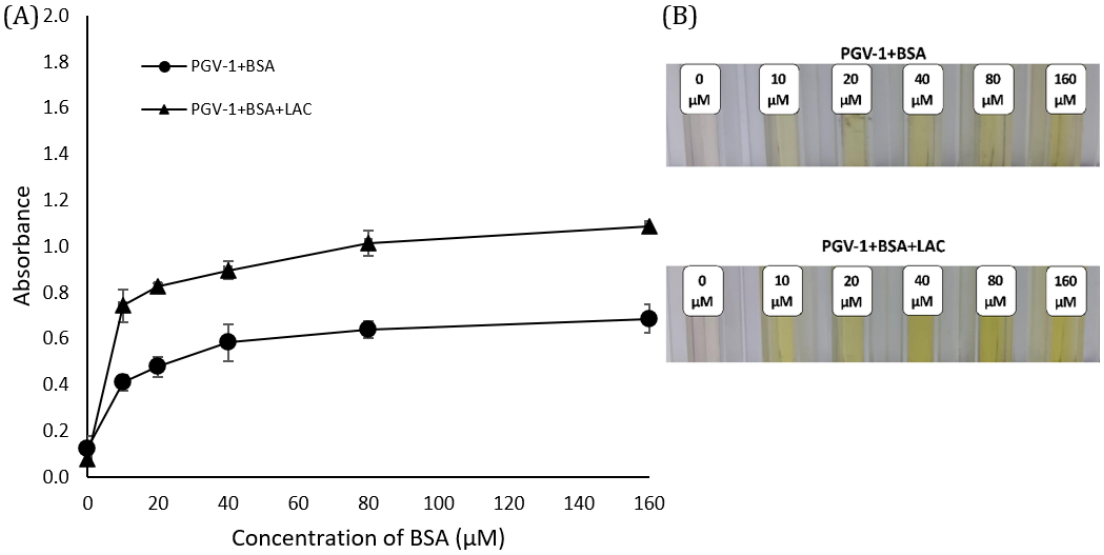


Figure 4. Profile of PGV-1 solubility in BSA and BSA + Lac solution at concentrations (0, 10, 20, 40, 80, and 160 μM) equivalent to BSA. A: PGV-1 absorbance in water solutions as a function of the BSA concentration (μM). B: PGV-1 water solutions in the presence of BSA in serial concentrations.

Solubility and stability studies

Solubility is a crucial factor in ensuring the adequate bioavailability of a drug in a biological system mainly comprising water. We aimed to evaluate the improved solubility of PGV-1 achieved through complexation with a lactose-conjugated protein. The complexation with BSA significantly enhanced the solubility of PGV-1 and was visually evident in the solutions as indicated by a more intense yellow color. With increasing concentrations of BSA, more PGV-1 could dissolve, producing a more pronounced yellow color in the resulting solution (Figure 4b).

BSA has hydrophobic regions that can interact with PGV-1 molecules and possesses hydrophobic properties. These interactions help stabilize the complex by reducing the exposure of the hydrophobic regions to the aqueous environment.

The TLC analysis showed that no additional spots were present in samples 1,2, and 3 (Figure 5).

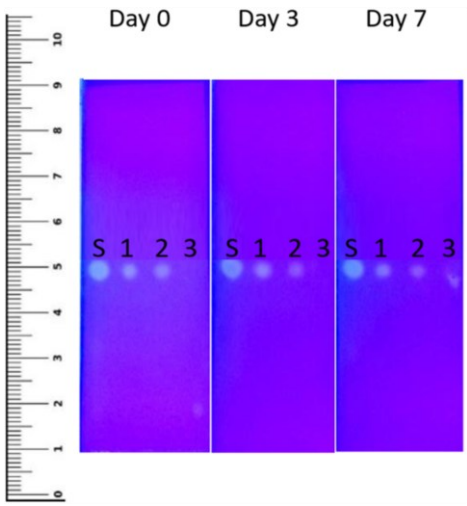
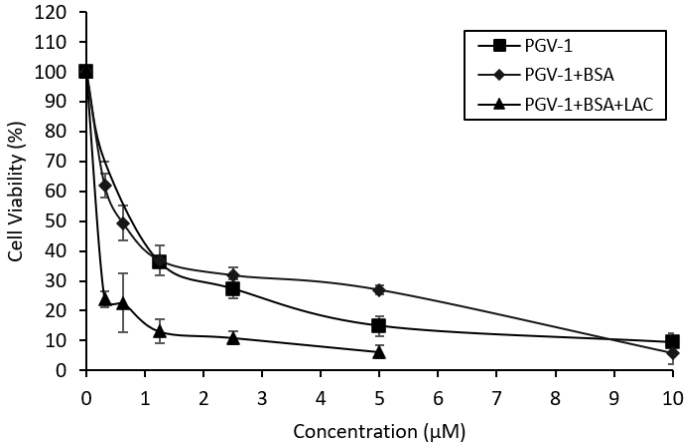


Figure 5. TLC analysis (UV 366 nm) for 7 days. S: Standard of PGV-1 in ethanol; Samples in PBS (1×, pH 7,4). 1: PGV-1 + BSA + Lac; 2: PGV-1 + BSA; 3: PGV-1.

(A)



(B)

Sample	IC_{50} (µM)	Cytotoxic Effect (Sharma & Singh, 2010)
PGV-1	0.437±0.002	Highly Active
PGV-1+BSA	0.631±0.002	Highly Active
PGV-1+BSA+Lac	0.008±0.002*	Highly Active

Data expressed as mean±SD as triplicate.

Figure 6. Cytotoxic effects of PGV-1, PGV-1 + BSA, PGV-1 + BSA + Lac in HLF cells. A: Percentage of cell viability after 24 h incubation of tested compounds in HLF cells. B: The IC_{50} of PGV-1, PGV-1 + BSA and PGV-1 + BSA + Lac. Data presented as mean ± SD (n = 3) (*$p < 0.05$ against untreated).

This absence of additional spots was visually confirmed by inspecting the sample tracks, which did not reveal any extra spots. However, the intensity of the spots suggested a disparity in concentration between the PGV-1 + BSA and PGV-1 + BSA + Lac samples, with the spots in the PGV-1 + BSA + Lac samples appearing denser. Notably, PGV-1 did not display significant spots in aqueous media, possibly because of its deficient concentration.

Cytotoxicity

We then evaluated the anticancer effectiveness of glycoprotein complexes and PGV-1 against HLF cells. Although several studies have explored the anticancer properties of PGV-1, the antiproliferative and antimetastatic attributes in HLF cells have yet to be investigated. The cytotoxicity of PGV-1 + BSA + Lac as a targeted anticancer agent was assessed by treating HLF cells with various doses of PGV-1, PGV-1 + BSA, and PGV-1 + BSA + Lac for 24 h (Figure 6).

The cytotoxic assay conducted on HLF cells revealed that the IC_{50} values for PGV-1, PGV-1 + BSA, and PGV-1 + BSA + Lac were all <10 µM. Interestingly, PGV-1 + BSA + Lac exhibited a higher cell inhibition rate than PGV-1, which may be

because of the amount of PGV-1 within the cells. Notably, PGV-1 + BSA + Lac demonstrated a more substantial inhibitory effect on cancer cells than PGV-1 + BSA. The IC_{50} values of PGV-1 + BSA + Lac (0.008 ± 0.002 μM) in HLF cells were significantly lower than those of PGV-1 (0.437 ± 0.002 μM) and PGV-1 + BSA (0.631 ± 0.002 μM), underscoring the potent inhibitory impact of PGV-1 + BSA + Lac on HLF cell proliferation.

The results suggest that the glycoprotein complexes developed in this study have the potential to act as an efficient delivery system for PGV-1 in treating liver cancer. Thus, this study provides valuable data for the practical use of glycoprotein complexes to enhance the potential of PGV-1 in cancer treatment. Our results affirm that the PGV-1 + BSA + Lac complexes exhibit high chemotherapeutic properties in the context of liver cancer compared with those of free PGV-1. This efficacy is attributed to their enhanced solubility and antiproliferative effects on cancer cells. Consequently, more PGV-1 molecules are available at the cancer site to exert a cytotoxic effect. In addition, PGV-1 + BSA + Lac is suspected to specifically target receptors (ASGPR) expressed by cancer cells, allowing this complex to deliver the drug directly to cancer cells more effectively than the noncomplexed PGV-1 and increase selective cytotoxicity against cancer cells.

The research aimed to develop a glycoprotein-PGV-1 complex to enhance the solubility and effectiveness of PGV-1 as a liver-targeted anticancer agent. PGV-1 has low water solubility, limiting its ability to dissolve effectively in an aqueous environment. This consequently poses challenges when using PGV-1 as a therapeutic agent. Albumin, especially in the form of BSA and lactosylated albumin (BSA + Lac), has established itself as a valuable material for creating drug complexes, particularly for drugs that suffer from poor water solubility and stability (Lou et al., 2014). Notably, albumin-based complexes such as BSA and BSA + Lac have attracted considerable attention because of their capacity to effectively carry a wide range of drugs while being well-tolerated with minimal side effects (Zhang et al., 2004; Zu et al., 2009). Therefore, our choice of carriers for delivering PGV-1 in chemotherapy included albumin, specifically BSA and BSA + Lac.

Our study illustrates the positive impact of increasing the amount of BSA and BSA + Lac on the water solubility of PGV-1. BSA solutions exhibit a low buffer capacity within a pH range of 5.2 to 7. Although a phosphate buffer was used to enhance the solubility of PGV-1 solubility by forming salt bridges, this did not significantly enhance PGV-1 solubility. Conversely, adding albumin solutions, notably when lactose was bound to albumin, substantially improved the solubility of PGV-1. This suggests that the increased solubility of PGV-1 in albumin solutions is not solely dependent on the dissociation of PGV-1 molecules but involves other mechanisms, such as the formation of salt bridges and binding complexes (Curry, 2002; Ghuman et al., 2005). These mechanisms encompass interactions, such as hydrophobic contacts and hydrophilic interactions, as well as salt bridges, which may contribute to the binding process of PGV-1 + BSA and PGV-1 + BSA + Lac (Bogdan et al., 2008; J. Zhang et al., 2012). The enhanced solubility of PGV-1 was more pronounced in BSA + Lac than in BSA alone, possibly because of the higher MW as confirmed by SDS-PAGE results. However, further research is needed to elucidate the exact binding mechanism of PGV-1 with the BSA-conjugated lactose.

The cell uptake of PGV-1 is a critical factor affecting its cytotoxicity, and the improved dispersion of PGV-1 within the BAS + Lac complex enhanced cellular uptake. Cancer cells exhibit heightened glucose consumption compared with normal cells, increasing glucose transport across the cell membrane. Cancer cells often overexpress members of the GLUT family, typically found in noncancerous tissues, to meet their energy demands for uncontrolled growth (Adekola et al., 2012). Thus, BSA + Lac may aid the penetration of PGV-1 into cells via these receptors.

Cells use endocytosis to continually internalize molecules from their surroundings into specific intracellular compartments. The efficacy of this process is augmented when cell receptors for foreign substances exhibit a robust binding affinity. This receptor-mediated endocytosis has been employed to enhance drug delivery to specific cell types, such as ASGPR, for targeting hepatocytes (Kim et al., 2012; Perales et al., 1994). These findings underscore the substantial potential of the glycoprotein complex with PGV-1, making this a promising candidate for further development. Furthermore, this formula can be adapted for other glycoproteins and tested on various cancer cells.

Endocytosis is how cells continually internalize molecules from their surroundings into specific intracellular compartments. The efficacy of this process is augmented when cell receptors for foreign substances exhibit a robust binding affinity. This type of internalization, known as receptor-mediated endocytosis, has been employed to

enhance drug delivery to specific cell types, such as ASGPR, for targeting hepatocytes (Kim et al., 2012; Perales et al., 1994). These findings underscore the substantial potential of the glycoprotein complex with PGV-1, making it a promising candidate for further development. Furthermore, this formula can be adapted for other glycoproteins and tested on various cancer cells.

CONCLUSION

This study obtained a PGV-1 glycoprotein complex by the chemical reaction between Lac and BSA peptide groups. The solubility of PGV-1 was found to be dependent on the concentration of BSA. PGV-1 + BSA + Lac demonstrated more potent cytotoxic properties than PGV-1 + BSA and PGV-1 against HLF cells.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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