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Curcumin as an Antitumor Agent: Targeting Cancer Stem Cell Markers in Glioblastoma through *In Silico* and *In Vitro* Approaches

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Abstract

Glioblastoma is prevalent and aggressive primary malignant brain tumor in adults with an annual incidence rate approximately 0.59 to 5 cases per 100,000 individuals. Curcumin, a bioactive compound from *Curcuma longa*, holds promise as a potential antitumor agent for use in drug or functional food development. This study aimed to analyze the *in silico* and *in vitro* antitumor potency of curcumin. Molecular docking was conducted to assess curcumin's interactions with tumor-related proteins *SOX-2*, *OCT-4*, and *IGFBP2*. *In vitro* experiments involved quantifying the *SOX-2* and *OCT-4* genes expression in glioblastoma (GBM) cells treated with curcumin using qRT-PCR method. Molecular docking revealed that curcumin binds effectively to all target proteins, with *SOX-2* and *OCT-4* exhibiting the lowest binding energy also the highest number of interactions, indicating stronger binding. *In vitro* analysis demonstrated that curcumin significantly downregulated the *SOX-2* and *OCT-4* expression in GBM cells, surpassing the effects of temozolomide, a standard cancer treatment. These findings highlight curcumin's potential as an antitumor agent by targeting cancer stem cell markers and pathways. This study provides foundational evidence for the future development of curcumin-based drugs or functional foods aimed at cancer therapy.

Keywords: Antitumor, Curcumin, IGFBP2, *In silico*, *In vitro*, Glioblastoma, OCT-4, SOX-2

Introduction

Glioblastoma represents the most common and highly aggressive form of primary malignant brain tumor found in adults. International research indicates an annual incidence rate varies across different regions, approximately 0.59 to 5 cases per 100,000 individuals, with some studies reporting an increasing trend in incidence over time [1]. Glioblastoma exemplifies the challenges of managing malignancies with poor prognoses and limited therapeutic options. This urgency underscores the need for innovative approaches targeting the underlying mechanisms of tumor progression, particularly focusing on cancer stem cells (CSCs), that involved in metastasis, tumorigenesis, and therapy resistance.

Among the key markers of CSCs, SRY-Box Transcription Factor 2 (SOX-2) and Octamer-binding transcription Factor 4 (OCT-4) are critical factors of transcription that regulate pluripotency and self-renewal, making them essential for CSCs survival and proliferation. High expression of these markers has been linked to aggressive tumor behavior and resistance to conventional therapies [2,3]. Additionally, Insulin-like Growth Factor Binding Protein 2 (IGFBP2) has a pivotal role in modulating the tumor microenvironment by regulating growth factor availability, thereby promoting tumor growth and metastasis [4]. In addition, IGFBP2 acts as a hub of an oncogenic network [5]. These proteins represent promising therapeutic targets, as their

inhibition could impair CSC maintenance and limit tumor progression.

Curcumin is a natural compound derived from turmeric plant (*Curcuma longa* L.), has received attention for its biological activities, such as anticancer, anti-inflammatory, and antioxidant activities [6,7]. Curcumin modulates various immune regulators, which play a key role in its anticancer properties. Curcumin is also capable of influencing both the expression and activity of immune cytokines [8]. Curcumin shows potential as an anticancer agent by inhibiting protein aggregation and misfolding, preventing cancer cell growth, also triggers apoptosis and inhibits angiogenesis [9]. Research demonstrated that curcumin reduced cell proliferation and eradicated by 50 % and reduced spheroid size in CSC subpopulations. Additionally, curcumin reported to exhibit cancer-preventive effects by inhibiting tumor development and functioning as a chemopreventive agent. [10]. Nonetheless, the use of curcumin to downregulate SOX-2 and OCT-4 expression in stemness of tumor cell has not been reported before.

This study investigates the antitumor potential of curcumin through molecular docking simulations and the gene expression analysis. Molecular docking explore the binding interactions between curcumin and its protein targets (SOX-2, OCT-4, and IGFBP2), providing insights into the molecular mechanisms underlying its activity. Concurrently, qRT-PCR assays measure changes in SOX-2 and OCT-4 gene expression in glioblastoma cells treated with curcumin, showing its effect on transcriptional regulation. By combining computational and experimental methodologies, this research aims to uncover therapeutic strategies that target CSC-associated pathways and improve outcomes for patients with glioblastoma.

Materials and methods

Preparation of curcumin

Curcumin was acquired from Plamed Green Science Limited, China. The formulation was conducted following the method [11]. After that, curcumin was initially diluted in 100 % dimethyl sulfoxide (DMSO), and then further diluted resulting in a final concentration containing 1 % DMSO.

Molecular docking

Molecular docking was utilized to present the curcumin potential as antitumor. Proteins target that used were related to inflammatory pathway and, including SOX-2, OCT-4, and IGFBP2. Proteins target were derived from RCSB (rcsb.org/) with PDB ID 8BX2 and 2H7T, respectively then prepared in Autodock 4.2 [12] by discarding water and native ligand. Molecular docking was performed in AutoDock Vina [13]. Proteins target was redocking with native ligand to validated the binding site. The results of molecular docking was binding affinity value from protein and compound interactions. Visualization of the 3D and 2D interaction were done in Discovery Studio 2021 [14].

GBM cells culture

Glioblastoma multiforme (GBM) cell line (ATCC HTB-14) - U87 was obtained from Aretha Medika Utama, BBRC, Indonesia. Cells were removed from liquid nitrogen tanks (−196 °C) and thawed at 37 °C for 2 min. The cells were grown in Dulbecco's modified Eagle's culture medium (Biowest, L0103-500) culture medium consist of 10 % (v/v) Fetal Bovine Serum (Biowest S1810-500), 1 % Antibiotic-antimycotic (Biowest, L0010-100), 1 % amphotericin B (Biowest, L0009-050), 1 % MEM vitamins 100x (Biowest, X0556-100), and 0.1 % gentamicin (Gibco, 12750060) [15].). The incubation took place in a humidified atmosphere with 5 % CO_2 at 37 °C, with a regular medium replacement every 2 days. The confluent cells were collected for subsequent analysis by using Trypsin 0.25 % EDTA (Biowest, L0931-500) [16,17]. The cells were then plated onto a 6-well-plate for treatment, which was divided into 6 groups, namely: 1) untreated GBM cells; 2) GBM cells + DMSO 1 %; 3) GBM cells + Temozolomide (TMZ) 300 µM; 4) GBM cells + Curcumin 50 µg/mL; 5) GBM cells + Curcumin 100 µg/mL; 6) GBM cells + Curcumin 200 µg/mL.

Quantification of SOX-2 and OCT-4 genes expression

GBM cells treated with 50, 100, and 200 µg/mL curcumin. The cells were prepared for RNA isolation and cDNA synthesis for further analysis. RNA isolation was performed with Direct-zol RNA Miniprep Plus Kit (R2073, Zymo). cDNA synthesis was conducted using iScript Reverse Transcription Supermix (170 - 8841, Bio-Rad), as described in the manufacturer's instructions. cDNA was combined with nuclease free water (R0581, Thermocientific), primers (Macrogen), and SsoFast Evagreen Supermix (172 - 5200, Bio-Rad). The SOX-2 and OCT-4 genes expression were assessed with qRT-PCR (PikoReal 96, Thermo Scientific) and

SsoFast Evagreen Supermix (G8830A, Agilent). The qPCR protocol included pre-denaturation at 95 °C for 5 min; denaturation with 40 cycles at 95 °C for 30 s, annealing with 40 cycles at 63 °C for 40 s, and elongation at 72 °C for 60 s. Primer sequences that used are detailed in **Table 1** [15,18].

Table 1 Primer sequences.

Gen	Forward (5′-3′)	Reverse (5′-3′)	Product Length (bp)	Reference
GADPH	GCCAAAAGGGTCATCATCTC	TGAGTCCTTCCACGATACCA	178	NM_001357943.2
SOX-2	CAACCAGAAAAACAGCCCGG	TGTGCATCTTGGGGTTCTCC	170	NM_003106.4
OCT-4	GAAGCCTTTCCCCCTGTCTC	CCCTGTCCCCCATTCCTAGA	89	NM_001285986.2

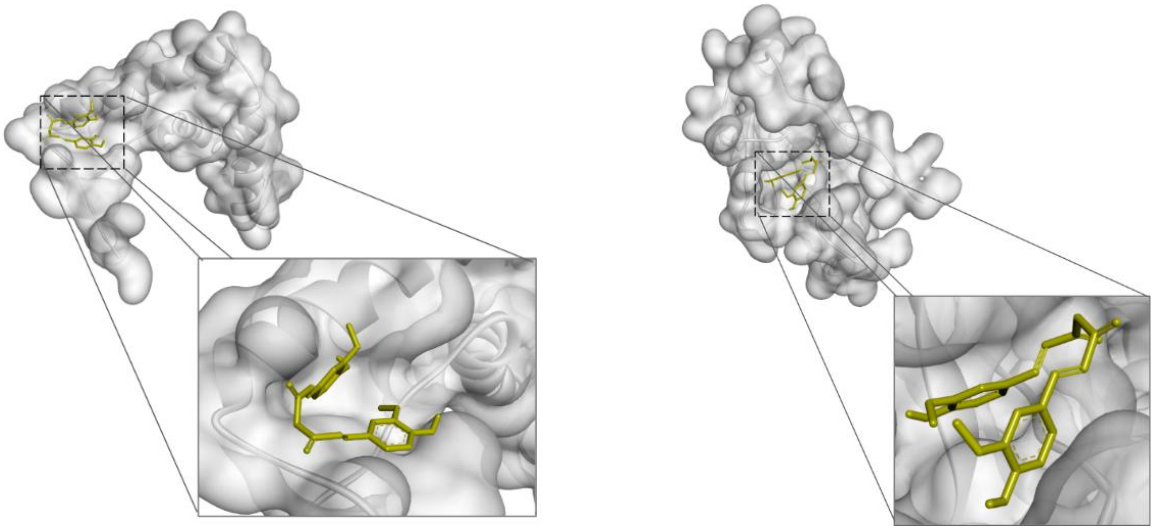
Statistical analysis

The data was analysed with IBM SPSS Statistics (version 20.0; SPSS Inc; USA). The data was performed normality test (Shapiro-Wilk) and homogeneity test (Levene), then data analysis was performed through one-way analysis of variance (ANOVA) ($p \leq 0.05$), then followed by Tukey HSD post test for normally distributed and homogenous data. The p value was ≤ 0.05 [19].

Results and discussion

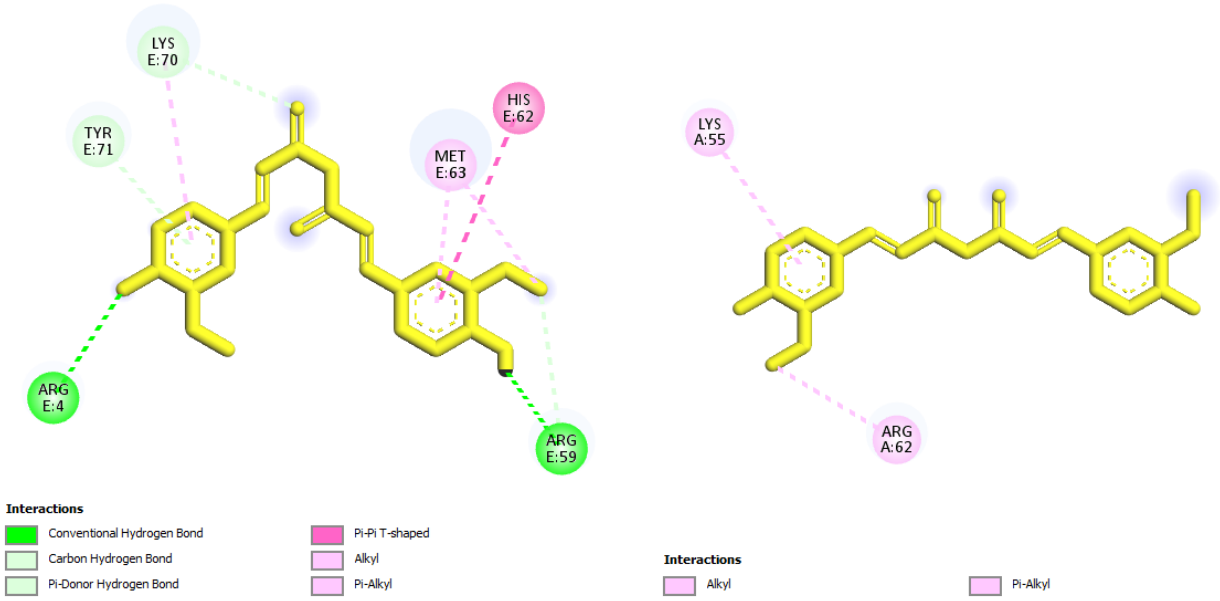
Molecular docking

Curcumin was analyzed in molecular docking against SOX-2, OCT-4, and IGFBP2 proteins. Curcumin was successfully docked with all target proteins. Molecular docking results visualized in 3D showed the test compounds successfully bind with the protein active site (**Figure 1**). 2D visualization demonstrated protein-ligand interaction, green circles indicated hydrogen bond and pink or purple circles indicated hydrophobic interaction (**Figure 2**). SOX-2 & OCT-4 possessed lower binding affinity values and more protein-ligand interaction compared to IGFBP2 (**Table 2**). The lower binding affinity value indicated stronger protein-ligand interaction.



(A) (B)

Figure 1 3D Visualization of molecular docking results. Yellow compound represented Curcumin against (A) SOX-2 & OCT-4, (B) IGFBP2.



(A) (B)

Figure 2 2D Visualization of molecular docking results. Yellow compound represented Curcumin against (A) SOX-2 & OCT-4, (B) IGFBP2.

Table 2 Binding affinity value and amino acid residue from molecular interaction.

Compound	Protein target	Binding affinity	Hydrogen bond	Hydrophobic Interaction
Curcumin	SOX-2 & OCT-4	-5,7 kcal/mol	ARG4, ARG59, LYS70, TYR71	HIS62, ARGH59, MET63, LYS70
	IGFBP2	-5,3 kcal/mol	-	ARG62, LYS55

Molecular docking studies have become an essential tool in drug discovery, particularly for understanding the interactions between small molecules and their protein targets. These proteins are critical in maintaining cancer stem cell properties and promoting tumor progression, making them relevant targets for therapeutic intervention [20]. The results of the molecular docking simulations indicate that curcumin exhibits lower binding affinities towards SOX-2 and OCT-4 compared to IGFBP2. However, the interactions observed with SOX-2 and OCT-4 are characterized by a higher number of hydrogen bonds and hydrophobic interactions, which could enhance the stability of the curcumin-protein complexes [21]. Hydrogen bonds are crucial for the specificity of ligand-receptor interactions, while hydrophobic interactions contribute to the overall binding affinity and the complex stability [22]. The presence of multiple hydrogen bonds suggests that curcumin may effectively modulate the activity of these transcription factors, potentially disrupting their roles in maintaining cancer stemness and promoting tumor growth [23].

The implications of these findings are significant, as they highlight the multifaceted interactions of curcumin with critical proteins involved in cancer biology. By targeting SOX-2 and OCT-4, curcumin may suppress the self-renewal cancer stem cells capabilities, thereby reducing tumor recurrence and enhancing the efficacy of existing therapies [24]. Furthermore, the interactions with IGFBP2 may provide an additional mechanism through which curcumin can exert its antitumor effects, potentially leading to a more comprehensive therapeutic strategy against various malignancies [25].

SOX-2 and OCT-4 genes expression

Curcumin treatment in GBM cells was downregulated SOX-2 and OCT-4 genes expression significantly (**Figure 3**). Each concentration decreased the SOX-2 gene expression significantly lower compared with negative control. Curcumin 100 and 200 µg/mL decreased the OCT-4 gene expression significantly lower compared with negative control. Comparison control was able to upregulated SOX-2 and

OCT-4 gene expression significantly compared with negative control, vehicle control, and curcumin treatment.

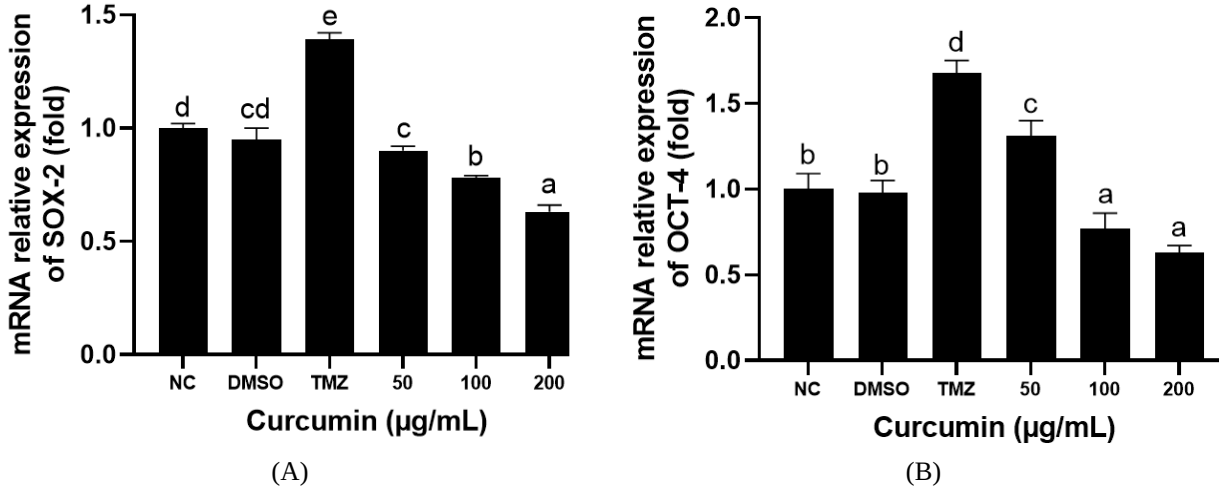


Figure 3 Effect of curcumin towards SOX-2 and OCT-4 gene expressions in GBM cells. (A) SOX-2; (B) OCT-4. Data is represented as mean ± SD. Treatment negative control (NC): untreated GBM cells; vehicle control, (DMSO): GBM cells + DMSO 1 %; positive control, (TMZ): GBM cells + TMZ 300 µM, (50): GBM cells + Curcumin 50 µg/mL, (100): GBM cells + Curcumin 100 µg/mL, (200): GBM cells + Curcumin 200 µg/mL. Significant statistical differences were determined using Tukey's HSD post hoc test ($p \leq 0.05$), then presented by different letters (a, b, c, cd, d, e) in SOX-2 and (a, b, c, d) in OCT-4 gene expressions.

TMZ is commonly used alongside radiotherapy as a first-line treatment for cancer or high-grade gliomas [26]. TMZ is effective in eliminating a subset of GBM cells, the remaining cells have the capacity to adapt and develop resistance mechanisms. The TMZ and cyclopamine combination has been shown to upregulate the SOX-2 and OCT-4 expression in GBM cells. These findings suggest that, although TMZ initially targets tumor cells, its treatment may inadvertently enhance stemness properties, potentially contributing to therapeutic resistance and tumor recurrence [27].

Curcumin treatment significantly downregulated the SOX-2 and OCT-4 genes expression in GBM cells to provide compelling evidence for the curcumin potency as a therapeutic agent in targeting cancer stem cell properties. The downregulation of these genes, which are critical markers of stemness and pluripotency, suggests that curcumin may inhibit the maintenance of the cancer stem cell phenotype, thereby reducing tumor aggressiveness and potential recurrence. The use of curcumin to downregulate expression of SOX-2 and OCT-4 in stemness of tumor cell is not previously reported

SOX-2 and OCT-4 expressions are often linked to the maintenance of undifferentiated states and the aggressive behavior of tumors [28]. The align with previous studies that curcumin has been shown to exhibit anti-cancer effects by modulating multiple signaling pathways, which may include the downregulation of stemness-related genes [29]. The upregulation of SOX-2 and OCT-4 promotes not only tumor initiation and development, but also enhances the cell's ability to evade therapeutic interventions [28]. This resistance is particularly concerning in glioblastoma, where the expression of these markers has been linked to the aggressive nature of the disease and its propensity for recurrence [27]. Despite this study showed satisfactory results, there were several obstacles encountered during the research process, especially at the cell culture stage. The main obstacles include the difficulty in maintaining optimal cell culture conditions and the time required to wait for the cells to reach the desired confluence level.

Conclusions

Curcumin demonstrates potential as an antitumor agent based on both *in silico* and *in vitro* analyses. Molecular docking results revealed that curcumin successfully binds to SOX-2, OCT-4, and IGFBP2. *In vitro* experiments using qRT-PCR confirmed that

curcumin treatment effectively downregulated the SOX-2 and OCT-4 mRNA expression levels in glioblastoma cells, particularly at higher concentrations. This study emphasizes the potential of curcumin for therapeutic agents in glioblastoma and other cancers.

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