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Inhibition of glycogen synthase kinase by curcumin: Investigation by simulated molecular docking and subsequent *in vitrolin vivo* evaluation

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Abstract

Curcumin was investigated as an inhibitor of glycogen synthase kinase-3β (GSK-3β) in an attempt to explain some of its interesting multiple pharmacological effects, such as its anti-diabetic, anti-inflammatory, anti-cancer, anti-malarial and anti-alzheimer's properties. The investigation included simulated docking experiments to fit curcumin within the binding pocket of GSK-3β followed by experimental *in vitro* and *in vivo* validations. Curcumin was found to optimally fit within the binding pocket of GSK-3β via several attractive interactions with key amino acids. Experimentally, curcumin was found to potently inhibit GSK-3β (IC50 = 66.3 nM). Furthermore, our *in vivo* experiments illustrated that curcumin significantly increases liver glycogen in fasting Balb/c mice. Our findings strongly suggest that the diverse pharmacological activities of curcumin are at least partially mediated by inhibition of GSK-3β.

Keywords: *Curcumin, glycogen synthase kinase, docking simulation, diabetes, inflammation*

Abbreviations: *GSK-3β, Glycogen synthase kinase-3β; NF-κB, Nuclear factor kappa B; PBS, Phosphate buffer saline*

Introduction

Curcumin, the curry flavoring and coloring agent, is the principal curcuminoid constituent of the spice turmeric [1]. Curcumin, a symmetrical molecule, exists in two tautomeric forms: ketone and enol (Figure 1a). However, the enol form is the most abundant [2,3]. Traditionally, curcumin is widely used as spice, flavoring, coloring agent and in traditional medicine [1].

For the last few decades, extensive research has been carried out to investigate the pharmacological actions of curcumin. In fact, curcumin has shown wide range of pharmacological activities including anti-inflammatory [4,5], hypoglycemic [6,7], anti-oxidant [8], wound healing [9], anti-microbial effects [10], improvement of defects associated with cystic fibrosis [11] and reducing the destructive beta-amyloid in the brains of Alzheimer's disease animal models [12]. Recently, curcumin and curcumin derivatives have shown potent anti-malarial activities [13]. Chronic treatment with curcumin attenuates significantly renal dysfunction and oxidative stress in diabetic animals [6]. Furthermore, curcumin demonstrates potent anti-cancer and chemopreventive activities [14–16]. In fact, many clinical trials using curcumin as anti-cancer are underway [17]. A recent one [18] reports a significant effect in a Phase II clinical trial involving a small group of patients with advanced pancreatic cancer. Previously, it was reported that curcumin has chemopreventive activity in patients with pre-malignant lesions (phase I clinical trials) [19].

Curcumin potently inhibits a multitude of key oncogenic signaling factors including histone acetyl-transferase activity [20,21], cycloxygenase-2 [22], ornithine decarboxylase [23], c-Jun/AP-1 [24], and NF-κB [25].

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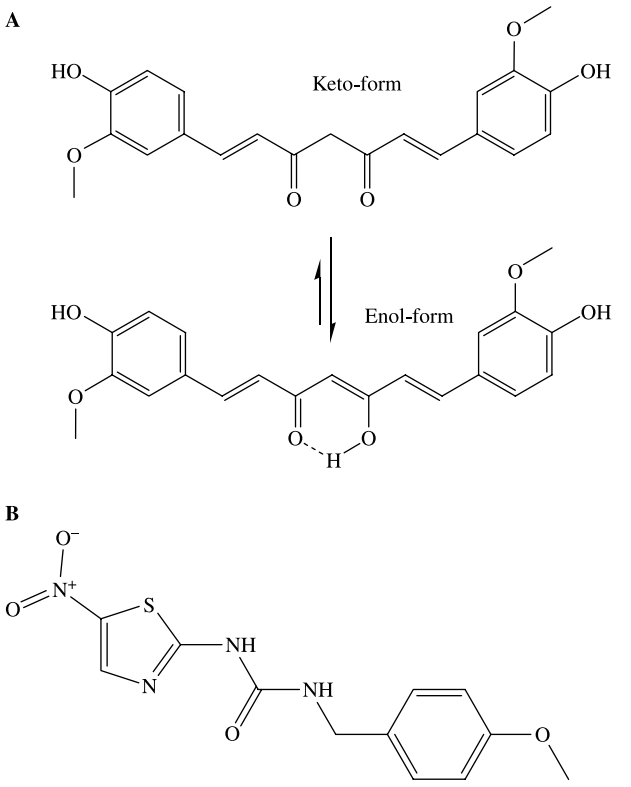

Figure 1. (A) Molecular structure of curcumin, (B) Molecular structure of co-crystallized structure AR-A014418 [39].

Although curcumin has been used for centuries as a traditional medicine to treat different inflammatory disorders, its anti-inflammatory mechanism is still not very well defined [5]. One of the suggested anti-inflammatory mechanisms of curcumin is suppressing the activation of nuclear factor kappa B (NF-κB) induced by inflammatory stimuli [25]. NF-κB activation is tightly regulated by many factors including IκB and glycogen synthase kinase (GSK-3) [26].

Although the anti-inflammatory actions of curcumin were traced to its suppressive actions on NF-κB through enhancing the stability of the natural inhibitor IκB [27], its diverse pharmacological properties hint to a direct inhibitory action on GSK-3, a key regulatory enzyme upstream and downstream to many other regulatory factors.

GSK-3, a multi-tasking serine/threonine kinase, was initially described as a key enzyme involved in glycogen metabolism by phosphorylating, and thus inhibiting, glycogen synthase [28]. However, recent studies have indicated that GSK regulates the functions of diverse proteins implicated in many critical biological processes including glucose metabolism, tumorigenesis, inflammation, gene expression, cell survival, and Alzheimer disease [29–33]. Interestingly, a homologous GSK-3 is expressed in *Plasmodium falciparum* and was evaluated as a potential screening target for the identification of antimalarial agents [34].

GSK-3 exists in two closely related isoforms; GSK-3α (51 kDa) and GSK-3β (47 kDa), both isoforms have nearly identical biochemical functions and substrate affinities [35]. However, GSK-3β inhibitors were recently evaluated for the treatment of many disease, e.g., type II diabetes, cancer, Alzheimer's disease, mood disorders, bipolar disorders, stroke, and chronic inflammatory [29–33]. Presently, there are several small molecule GSK-3β inhibitors in clinical trials for the treatment of type II diabetes [36].

The wide range of pharmacological activities observed for curcumin, combined with the multi-tasking properties of GSK-3β prompted us to evaluate the possibility that curcumin directly binds and inhibits GSK-3β. Findings from this study should help in understanding the mechanism of action of curcumin; furthermore, it should provide new insights towards the discovery of new potent and safe GSK-3β inhibitors.

The current study commenced by computer-aided molecular docking of curcumin into the binding pocket of GSK-3β in order to reach to preliminary conclusions about curcumin/GSK-3β binding energetics. Eventually, curcumin was tested *in vitro* against human recombinant GSK-3β, and in Balb/c mice to evaluate its glycogen-sparing potential.

Materials and methods

Materials

All of the chemicals used in these experiments were of reagent grade and obtained from commercial suppliers: Recombinant GSK-3β (from Upstate Biotechnology, USA); Tau [pS396] phosphoELISA kit (Biosource, USA); Assay buffer components (HEPES; $MgCl_2$ and EDTA, Biosource, USA); Curcumin (SIGMA, USA); ATP (SIGMA, USA); heparin (Sandoz GmbH, Austria); Anthrone (SIGMA, USA); Thiourea (Sigma, USA).

Molecular modeling

Software and hardware. The following software packages were utilized in the present research.

- CS ChemDraw Ultra 7.01, Cambridge Soft Corp. (http://www.cambridgesoft.Com), USA.
- OMEGA (Version 2.1.0), OpenEye Scientific Software (www. eyesopen.com), USA [37].
- FRED (Version 2.1.2), OpenEye Scientific Software, (www.eyesopen.com), USA [38].
- DS visualizer, Accelrsy Inc. (www.accelrys.com), USA.

Preparation of curcumin structure. The chemical structure of curcumin (Figure 1a) was sketched in Chemdraw Ultra (7.01) and saved in MDL molfile format. Subsequently, an ensemble of energetically

accessible curcumin conformers was generated using OMEGA2 software [37]. OMEGA builds initial models of structures by assembling fragment templates along sigma bonds. Input molecules graphs are fragmented at exocyclic sigma bonds, and carbon to heteroatom acyclic (but not exocyclic) sigma bonds. Conformations for the fragments are retrieved from pre-generated libraries within the software. Once an initial model of a structure is constructed, OMEGA generates additional models by enumerating ring conformations and invertible nitrogen atoms. The generated conformers are saved in SD format.

Docking experiment. The 3D coordinates of GSK-3β were retrieved from the Protein Data Bank (PDB code: 1Q5K, resolution = 1.94 Å) [39]. Hydrogen atoms were added to the protein using the DS visualizer templates for protein residues. The docking study was conducted in the presence of crystallographically explicit water molecules. The drug was docked into the binding site of GSK-3β employing FRED software [38]. This docking engine takes a multiconformer database of one or more ligands, a target protein structure, a box defining the active site of the protein based on the co-crystallized ligand and several optional parameters as input. The ligand conformers and protein structure are treated as rigid during the docking process. FRED's docking strategy is to exhaustively score all possible positions of each ligand in the active site [38]. The exhaustive search is based on rigid rotations and translations of each conformer. Therefore, it avoids sampling issues associated with stochastic methods. The conformational ensemble of curcumin generated using OMEGA software and stored in SD format was used as input in the FRED software.

We employed the docking settings that succeeded in reproducing the experimental pose of the co-crystallized ligand (AR-A014418, Figure 1b) [39]. Accordingly, the following FRED parameters were employed:

- *Addbox*: An optional parameter that adjusts the geometry of the box defining the active site by extending each edge of the box by the specified number of Angstroms. The binding site in the current docking experiment was generated from the co-crystallized ligand with the targeted protein. The box defining the active site was expanded to 4 Å.
- *Num_poses*: This parameter specifies the number of poses to be returned by the exhaustive rigid body search. In the current docking experiment, the number of poses to be returned by exhaustive search was set to the maximum value of 1000.
- *Num_alt_poses*: Only the top-ranking poses will be selected from the list and scored by the scoring functions specified by the user. This flag specifies how many alternate poses, in addition to the top pose, will be presented for consensus scoring. In the current experiment the number of alternative poses to be retrieved was set to 10.
- The docked poses were scored by the Chemscore [38] scoring function and the highest ranking poses were retained for evaluation. ChemScore is an empirical scoring function that estimates the binding affinity of ligands in receptor complexes [40]. It includes interactions between: (1) lipophilic atoms; (2) hydrogen bond donors and acceptors and (3) hydrogen bond acceptors and metals and (4) an additional contribution is calculated based on the number of frozen rotatable bonds in the ligand.

In vitro *GSK-3β assay and determination of curcumin inhibitory IC_{50} value*

Recombinant GSK-3β (Upstate Biotechnology, USA) was dissolved in a buffer solution (pH 7.2) containing the following: 40 mM HEPES; 5 mM MgCl2; 5 mM EDTA; 100 μM ATP and 50 μg/mL heparin to reach a final enzymatic solution of 10 pg/mL. Subsequently, 50 μL aliquots of the enzymatic solution were pipetted into 0.5 mL vials. Thereafter, appropriate volumes of the curcumin's stock solutions of DMSO were pipetted into the enzymatic solution to yield a final concentration of 10 μM, 1 μM, 100 nM and 10 nM after completion to 75 μL with the buffer solution. Curcumin was incubated with the enzyme over 30 min at room temperature. Then 25 μL of 2000 pg/mL Tau protein solution in HEPES were added to give final Tau protein concentration of 500 pg/mL. This mixture was incubated over 1 h at room temperature.

The detection of Tau phosphorylation was performed as follows [32,33,41]: The GSK-3β reaction mixtures were diluted 1:1 with sodium azide aqueous solution (15 mM) to achieve a final Tau protein concentration of 250 pg/mL. Then 100 μL aliquots of this solution were pipetted into the wells of the Tau [pS396] phosphoELISA kit (Biosource, USA). Subsequently, the wells were incubated for 2 h at room temperature. Then they were aspirated and washed (with the washing solution provided in the kit). Thereafter, 100 μL aliquots of rabbit detector antibody solution were pipetted in the wells and incubated for 1 h at room temperature. Thereafter, the wells were aspirated and washed with the wash buffer. Then, 100 μL aliquots of goat (polyclonal) anti-rabbit IgG-HRP were added to the wells and incubated for 30 min at room temperature. Subsequently, the wells were aspirated and washed with the wash buffer. Finally, 100 μL TMB substrate chromogen solution aliquots were added to each well and incubated for 20-30 min.

After the termination of the HRP reaction in each well, the absorbencies were measured spectrophotometrically at λ of 450 nm. Inhibition of recombinant GSK-3β by curcumin was calculated as a percent activity of the uninhibited enzyme control. Samples and blanks were prepared in duplicates. A standard GSK-3β inhibitor, TDZD-8, (Biosource, USA) was included as enzyme activity control for screening inhibition. TDZD-8 is a well known thiadiazolidinones derivative employed as standard GSK-3β inhibitor [42].

In vivo *evaluation*

Preparation of curcumin doses. The effect of curcumin on liver glycogen content of fasting Balb/c mice was investigated. Curcumin suspension was prepared in 0.5% carboxy methylcellulose in adjusted phosphate buffer saline (pH 7.2). For control animals, the vehicle without curcumin was used.

Three doses of curcumin were used: 15, 30 and 60 mg/kg. These doses are within the reported effective dose range in animal studies [6]. All curcumin doses were injected i.p. (ca. 0.20 mL) to yield the desired dosage after normalization to mouse weight (ca. 20 gm).

Determination of liver glycogen. All animal experiments comply with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

12-week old male Balb/c mice of average weight of 20 gm were used for the investigations. The animals were randomized and fed *ad libitum* with standard feed and water except when fasting was needed in the course of the study.

All animals were housed in the same conditions and separated randomly in five-mouse groups. Four groups were used: one group was injected i.p. with the vehicle without curcumin as negative control. The other three groups were injected with the three escalating doses of curcumin (mentioned earlier). In the day of the experiment, food and water were removed four hours before the injection. Three hours after the curcumin administration, the animals were sacrificed by cervical dislocation and their livers were removed immediately for glycogen determination. Liver glycogen content was determined quantitatively following reported procedure [43]. Briefly: livers were removed immediately after scarifying the animals and were homogenized by a blender (Ultra-Turrax, Janke & Kunkel GmbH & CoKg, Germany) with appropriate volume (10 mL) of 5% trichloroacetic acid over 5 min. The homogenate was centrifuged (Hettich zentrifugen, Germany) at 3000 r.p.m. for 5 min. The supernatant fluid was taken and filtered using acid-washed filter paper and the residues were homogenized again with another volume of 5% trichloroacetic over 1-3 min to insure better extraction of glycogen. The filtrate was made up to 50 mL with 5% trichloroacetic acid and mixed thoroughly. The glycogen of 1.0 mL of this filtrate was precipitated using ethanol (95%, 5 mL), incubated in water bath (Raypa water bath, Germany) at 37-40°C for 3 h and centrifuged at 3000 r.p.m. for 15 min. The clear liquid is gently decanted from the packed glycogen and the tubes were allowed to drain in an inverted position for 10 min. The glycogen was dissolved in distilled water (2 mL) and mixed with 10 mL of the anthrone reagent (0.05% anthrone, 1.0% thiourea in 72% sulfuric acid). The mixture was incubated in boiling water over 30 min, subsequently, the absorbance was measured λ 620 nm by spectrophotometer (Spectroscan 80D- UV-VIS spectrophotometer, USA). Blank and standard solution were prepared by adding 10 ml of anthrone reagent to 2 ml water and to 2 ml of glucose solution containing 0.1 mg of glucose in saturated benzoic acid, respectively. The liver glycogen content is estimated using the following formula:

$$\text{Amount (mg) of Glycogen Liver Tissue} = \frac{\text{DU}}{\text{DS}} \times \frac{\text{Volume of Extract (mL)}}{\text{Weight of Liver Tissue (gm)}} \times 0.09$$

Where, DU is the absorbance of the unknown sample, DS is the absorbance of the standard.

Results and discussion

The curry flavoring and coloring agent curcumin has shown a broad range of pharmacological effects in cells and animals: anti-inflammatory, anti-oxidant, anti-alzheimer, anti-malarial, wound healing, chemopreventive, diabetes, anti-cancer and others.

In spite of a centuries long history of folk medical use, large quantities in the food supply and many studies attempting to pin down its effects at the molecular level, the field is still uncertain as to how the apparently multiple targets of curcumin cooperate or synergize to exert its effects. Furthermore, the fact that many GSK-3β inhibitors have arisen as promising drugs for several diseases such as diabetes type II, cancer, Alzheimer disease and chronic inflammatory disease [29–33] encouraged us to evaluate possible inhibitory effect for curcumin on GSK-3β.

Our efforts started by evaluating the possibility of curcumin binding within GSK-3β binding pocket employing *in-silico* docking techniques. Simulated molecular docking is basically a conformational sampling procedure in which various docked poses/conformations are explored to identify the correct one. This process can be a very challenging problem given the degree of conformational flexibility at the ligand-macromolecular level [44,45]. Docking consists of two

parts: (i) prediction of the conformation, orientation and position (pose) of the bioactive compound into the binding pocket, and (ii) estimation of the tightness of target-ligand interactions (scoring) to guide conformational sampling [46]. The final docked conformations are selected according to their scores. We decided to conduct the docking study utilizing the docking engine FRED [47]. FRED was recently reported to illustrate good overall performance, particularly in virtual high-throughput screening experiments. It employs rigid body minimization (translational and rotational movements) to optimize binding interactions of rigid conformational ensembles of the docked molecule, i.e., it avoids the computationally intensive stochastic conformational sampling performed by other docking engines [47]. The resulting poses were scored employing ChemScore scoring function [40].

However, simulated molecular docking requires the user to provide FRED with an optimal set of parameters for the docking experiment (**Docking Experiment** in **Materials and Methods**). Those parameters differ according to the protein in question. Therefore, to identify the optimal docking configuration and scoring function for GSK-3β, we extracted the ligand AR-A014418 (Figure 1b) from one of the published crystallographic structures of GSK-3β (Protein Databank code: 1Q5K) [39] and redocked it again into the same protein (self-docking) *via* a variety of docking conditions and scoring functions. ChemScore was found to yield the closest model to the crystallographic structure as shown in Figure 2a. Subsequently, curcumin was docked into the binding pocket of GSK-3β employing the same optimal docking parameters and scoring function (Figure 3).

Although curcumin exhibits ketone-enol tautomerization (Figure 1a), there is compelling experimental and theoretical evidence that the enol structure is the most favored due to intra-molecular hydrogen bonding [2]. Furthermore, in a recent study, NMR experiments demonstrated that enol-form of curcumin is essentially the only form that exists in a variety of solvents including buffered aqueous solutions in pH range of 3 to 9 [3]. Therefore, only the enol form was employed in the docking study.

Figure 3 shows how curcumin is predicted to bind within the binding pocket of GSK-3β. Several significant binding interactions can be observed within

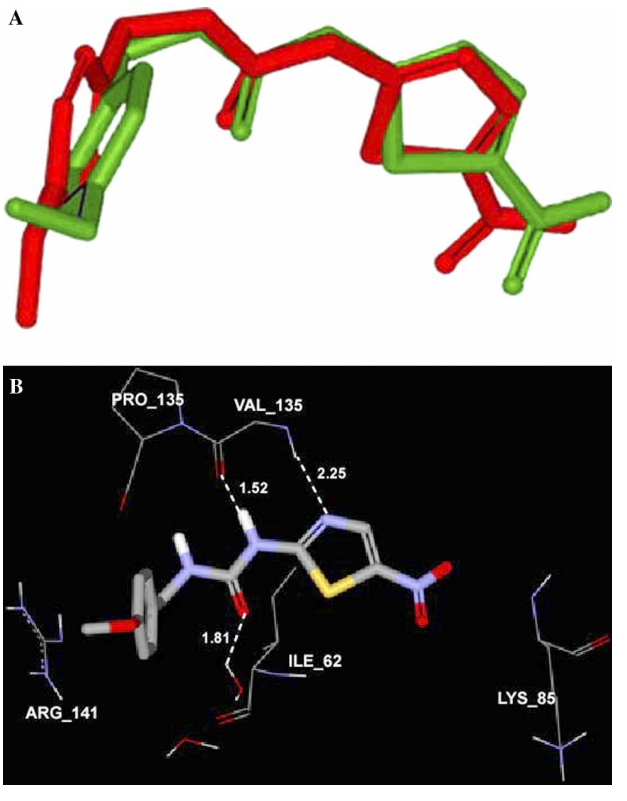

Figure 2. (A) Comparison between the docked pose (green) of the ligand AR-A014418 as produced by docking simulation and the crystallographic structure of this ligand (red) within the binding pocket of GSK-3β. (B) Detailed view of the co-crystallized structure (AR-A014418) and the corresponding interacting amino-acids within the binding site of GSK-3β.

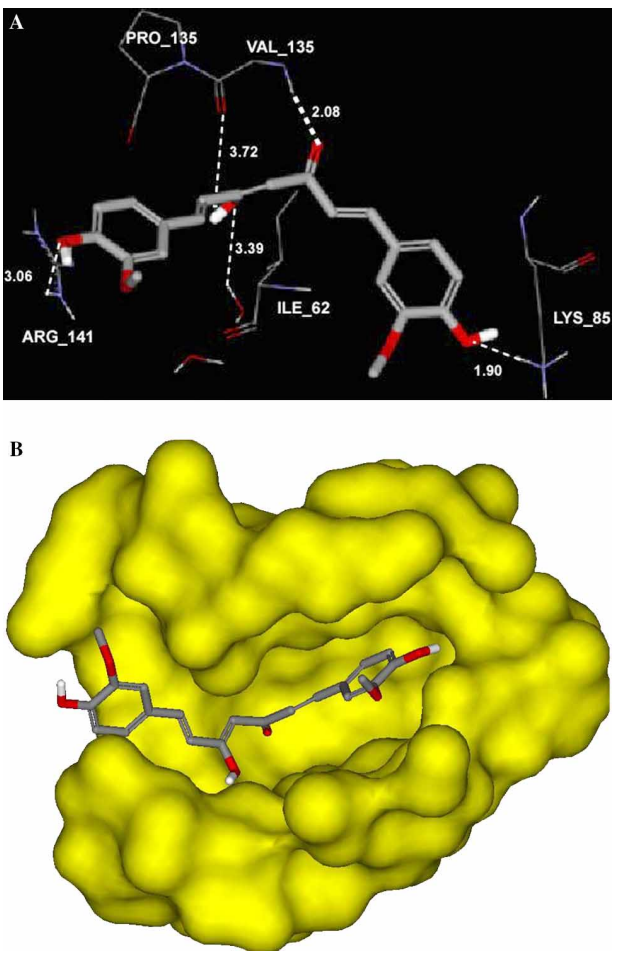

Figure 3. (A) Detailed view of the docked curcumin structure and the corresponding interacting amino-acid moieties within the binding site of GSK-3β. (B) Curcumin structure docked into the water-accessible surface (probe diameter 1.4 Å) within the active site of GSK-3β [39].

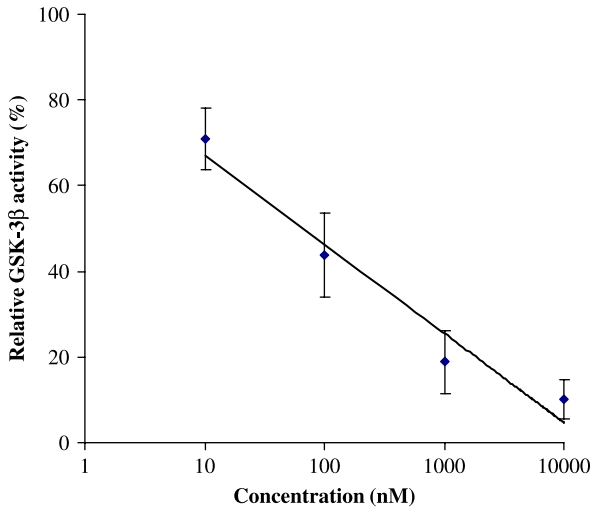

Figure 4. Effect of curcumin on the relative activity of GSK-3β. Data are expressed as means of duplicates ± standard error of the measurements.

the docked complex, as in Figure 3a. Clearly from the figure, the conjugated enol-ketone system of curcumin is hydrogen bonded to VAL-135, in such a way that the enolic hydroxyl group of curcumin interacts with the amidic carbonyl of VAL-135 (at a separating distance of 3.72 Å), while the conjugated ketone of curcumin is hydrogen-bonded to the NH of the same amino acid residue (at a separating distance of 2.08 Å). Furthermore, the enolic oxygen atom of curcumin seems to interact with IlE-62 via two bridging water molecules. Moreover, the electron rich phenolic ring in curcumin seems to interact via charge transfer with the positively charged guanidino of ARG-141. Interestingly, similar attractive interactions can be seen in the crystallographic image of GSK3β/AR-A014418 complex (Figure 2b), where

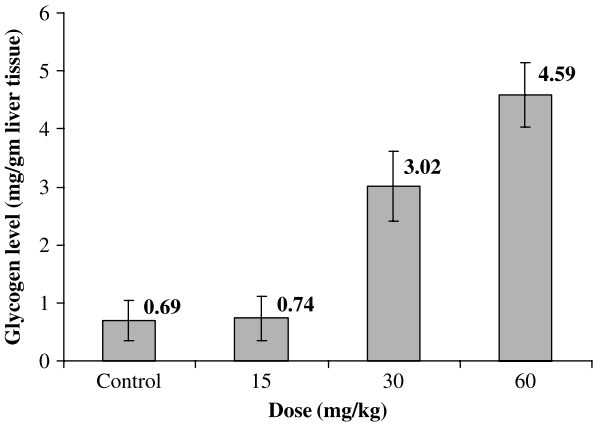

Figure 5. Liver glycogen reserves in fasting 12 week old male Balb/c mice three hours after i.p. administration of vehicle control, 15, 30 and 60 mg/kg of curcumin normalized to mice weights (20 gm). Each result represents the average glycogen level from 5 mice. Error bars indicate the standard deviations of the measurements.

the thiazole heterocyclic nitrogen and the urea NH of AR-A014418 are hydrogen-bonded to the amidic NH and carbonyl groups of VAL-135, respectively. On the other hand, the urea carbonyl of AR-A014418 is bridged to ILE-62 via two water molecules, reminiscent to the interaction of the enolic oxygen of curcumin with ILE-62. Finally, the electron-rich methoxylated phenyl of AR-A014418 seems to interact favorably with the guanidino of ARG-141 via charge transfer interaction similar the interaction of the methoxy-hydroxy phenyl of curcumin and the same amino acid residue.

However, the docked curcumin exhibits additional attractive interactions to those of AR-A014418: One of the phenolic hydroxyls of curcumin is hydrogen-bonded to the ε-nitrogen of LYS-85 (separating distance of 1.90 Å), while the other phenolic hydroxyl is hydrogen-bonded to the guanidino of ARG-141 (separating distance of 3.06 Å). Finally, the enolic α-carbon of curcumin seems to share a hydrophobic interaction with the isobutyl side chain of ILE-62. These additional interactions may explain the high potency exhibited by curcumin towards GSK-3β (see below).

To validate the docking results, we decided to evaluate the inhibitory actions of curcumin *in vitro* and *in vivo*. The *in vitro* activity was expressed as the concentration of curcumin that inhibited GSK-3β activity by 50% (IC_{50}) using tau protein as substrate [32,33,41]. Unsurprisingly, curcumin illustrated potent inhibitory action against GSK-3β with an IC_{50} value of 66.3 nM. Figure 4 shows the effect of different concentrations of curcumin on the relative activity of GSK-3β. The validity of the test was established by testing the inhibitory action of the standard inhibitor TDZD-8 [42] on GSK-3β, which showed an IC_{50} value of 1.5 μM that is comparable to the published value [42].

Furthermore, curcumin significantly increased liver glycogen reserves in fasting Balb/c mice in a dose-dependent manner. Figure 5 shows the responses of liver glycogen reserves to i.p. administered curcumin at doses 15, 30, 60 mg/kg normalized to mice weight. As apparent from this figure, a significant elevation of liver glycogen (6.5 folds increase) was observed with the highest dose of curcumin (60 mg/kg).

Glycogen metabolism is highly regulated process that is controlled by a balance between various kinases and phosphatases [48]. GSK-3β plays an essential role in controlling glycogen synthesis through phosphorylation and thus inhibition of the activity of glycogen synthase which is a key metabolic enzyme in the glycogen synthesis pathway. Therefore, inhibitors of GSK-3β could improve glucose disposal via increasing the rate of glycogen synthesis in the liver and skeletal muscles.

In our study, the significant increase in liver glycogen reserves caused by curcumin may be attributed, at least partially, to its inhibitory action

of GSK-3β, a hypothesis that was supported by the initial docking studies.

Moreover, we believe that the potent anti-GSK-3β of curcumin is at least partly responsible for its established antimalarial properties. Plasmodial GSK is highly homologous to human GSK-3β [34] while human GSK-3β is involved in many signaling pathways. Inhibition of GSK-3β was recently suggested as a new molecular mechanism for hydroxychloroquine [33], a well known anti-malarial and anti-inflammatory drug. Interestingly, the reported hypoglycemic effects of hydroxychloroquine [49] can be also explained by its potent inhibitory action of GSK.

Conclusion

We have unequivocally proved through theoretical docking simulations and experimental evaluation that curcumin is a potent inhibitor of GSK-3β, which explains, at least partially, the diverse pharmacological activities reported for curcumin: antidiabetic, anticancer, antiinflammatory, anti-malarial and anti-Alzheimer.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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