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ANTILIPOLYTIC PROPERTY OF CURCUMIN: MOLECULAR DOCKING AND KINETIC ASSESSMENT

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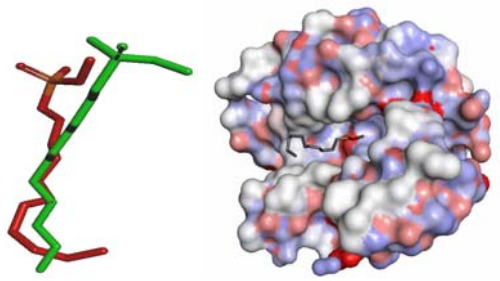
Curcumin inhibited efficaciously and dose dependently the pancreatic triacylglycerol lipase (PL) activity with an IC_{50} value of 7.3 μg/mL (19.8 μM). Its PL-catalysis inhibition propensities have been studied to draw further on structure-potency enhancement. Enzyme kinetics study proved that curcumin inhibits PL in a competitive manner. The observed first order rate constant values for Km and Vmax were 0.22 mM and 0.20 mM/min, respectively. The curcumin inhibitor constant (Ki) was 4.9 μM. Supported by docking studies, curcumin was found to fit within the binding pocket of PL via several attractive interactions with key amino acids and thereby identifying strategies for drug design. More broadly, the inferences from the present study validate the rigorous and robust use of curcumin as potentially useful template for the development of novel principles and selective lead compounds of metabolic syndrome and obesity pharmacotherapy.



INTRODUCTION

Turmeric, also known as *Curcuma longa* Linn. (Zingiberaceae), is a spice originally common in the kitchen and folk medicine for different ailments.[1-3] Curcumin is a yellowish phenolic spice derived from the rhizome of *C. longa*. It constitutes 2-5% by weight. Although some of the activities of *C. longa* can be mimicked by curcumin, other activities are curcumin-independent. Notably, it has been proven that curcumin is a highly pleiotropic molecule which can be a modulator of intracellular signaling pathways that control cell growth, inflammation, and apoptosis.[4] This culinary and medicinal food additive and coloring agent is a natural antioxidant with diverse pharmacological activities and is perhaps the most-studied phytoprinciple.[5-11]

Among multiple antioxidative herbal remedies, *C. longa* is linked to therapeutic amelioration of liver metabolism and maintaining of liver tissue integrity in diabetes.[12] Of note, emerging evidence of curcumin inhibitory potential of glycogen synthase kinase may be substantially involved.[13] Further favorable antiatherogenic propensities were ascribed to curcuminoids in hypercholesterolemic rabbits and type 2 diabetes patients, [14,15] in correlation with lowering cholesteryl ester transfer protein activity. Additional antihyperlipidemic effects were recognized for tetrahydrocurcumin in rats subjected to diabetogenic agents.[16] Recent studies have shown the pronounced role of dietary polyphenols as well as

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curcumin in the prevention of obesity and obesity-related chronic diseases via diverse molecular action mechanisms. [17-19]

Pancreatic lipase (PL) is a key enzyme in dietary triacylglycerol absorption, hydrolyzing triacylglycerols to 2-monoacylglycerol and fatty acids. Orlistat (tetrahydrolipstatin) interacts directly with lipase,[20] thus impairing the gastrointestinal fat absorption and achieving weight reduction.[21] With increased lipophilicity, the orlistat action mechanism of lipase inhibition was contributed by covalent bonding to the active site serine of the lipase. [22-24]

In the current study, the combination of pancreatic lipase (PL) enzyme kinetic measurements and the *in silico* docking studies were undertaken to investigate the highly specific interactions of PL with its natural inhibitor; curcumin, in an attempt to curtail obesity and its adverse health complications.

EXPERIMENTAL

Preparation of curcumin and orlistat for *in vitro* PL activity assay: Orlistat (10 mg, Sigma, USA) was dissolved in DMSO (10 mL). Using the stock solution of orlistat (1 mg/mL), six different working solutions with a concentration range of 0.625 - 20 µg/mL were prepared. Thereafter, 20 µL aliquot of each working solution was used in the reaction mixture to give a final concentration range of 0.0125 – 0.4 µg/mL. Curcumin (Sigma-Aldrich, USA) was initially dissolved in DMSO and subsequently prepared into eight different stock solutions (9.84 – 1250 µg/mL). Thereafter, 20 µL aliquot of each stock solution was used in the reaction mixture to give the following final concentration ranges (0.197 - 25 µg/ mL).

The assay procedure: Pancreatic lipase activity was determined by measuring the rate of release of *p*-nitrophenol (*p*-NP) from *p*-Nitro Phenol Butyrate (*p*-NPB). Enzyme assay was conducted by spectrophotometric method as per protocol obtained from Bustanji *et al*.[25] Subsequent determinations of inhibition of PL activity were undertaken for the tested curcumin in comparison to control evaluations, to calculate the concentration required for PL 50% inhibition (PL- IC_{50} value).

Kinetic studies of curcumin inhibitory properties of PL: Inhibition mode and Michaelis-Menten constant (Km), maximal velocity (Vmax) and Ki (the inhibitor constant) values were determined by Lineweaver-Burk plot analysis of the enzyme assay with various concentrations of substrate *p*-NPB (5.69, 2.85, 1.42, 0.71 and 0.36 mM) in the absence and presence of final different concentrations of curcumin (0.2714, 2.714 and 27.14 µM) from respective initial stock concentrations (13.57, 135.7 and 1357 µM).[26, 27]

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;[28] FRED (Version 2.1.2), OpenEye Scientific Software, (www.eyesopen.com), USA;[29] DS visualizer, Accelrsy Inc. (www.accelrys.com), USA.

Preparation of curcumin structure: The chemical structure of curcumin (Fig. 1) 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.[28] 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.

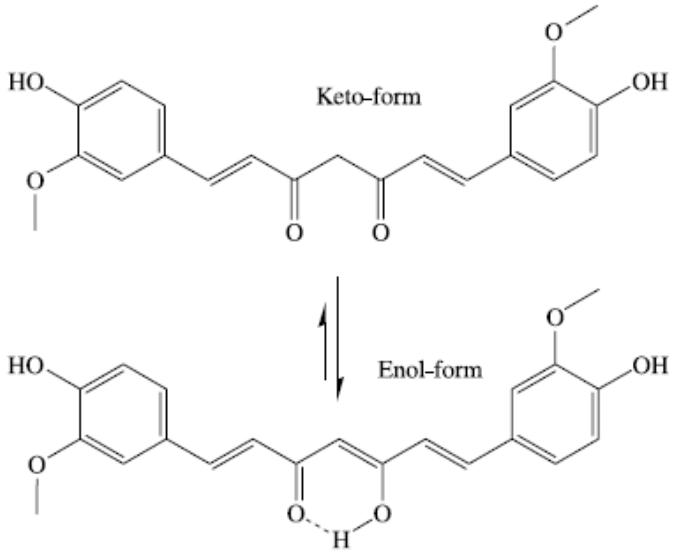

Fig. 1 – Molecular structure of curcumin.

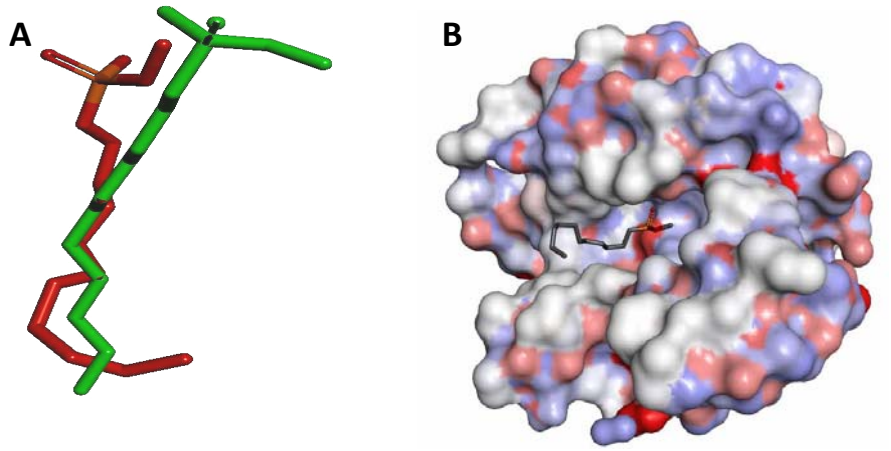

Fig. 2 – **(A)** Comparison between the docked pose (green) of the ligand MUP901 as produced by docking simulation and the crystallographic structure of this ligand (red) within the binding pocket of PL. **(B)** the solvent accessible surface area of the binding site of PL (1LPB) and the co-crystallized inhibitor (MUP901).

Docking experiment: The 3D coordinates of PL were retrieved from the Protein Data Bank (PDB code: 1LPB, resolution; 2.46 Å).[30] Hydrogen atoms were added to the protein using the DS visualizer templates for protein residues. The protein structure was utilized in subsequent docking experiments without energy minimization. The docking study was multiconformer database of one or more ligands, a target protein structure, and 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.[29] The conformational ensemble of curcumin generated using OMEGA software 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 (MUP901, Fig. 2A).[30] 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 2Å.

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 Chemgauss2 scoring function and the highest ranking poses were retained for evaluation. Chemgauss2 scoring function represents all atoms as smooth Gaussian functions. Hydrogen bonding interactions are the most significant chemical potentials accounted for by the Chemgauss2 scoring function. The Chemgauss2 function is the sum of the following potentials, all of which are based on smooth gaussian functions; 1) Shape based interactions between all heavy atoms. 2) Hydrogen bonding interactions based on favorable interactions between polar hydrogens and lone pairs and a mild repulsion between donor heavy atoms and acceptors (which tends to make the hydrogen bonds linear). 3) Aromatic ring interactions based on favorable interactions between aromatic atoms and the π- electron positions plus repulsive aromatic-atom to aromatic atom and π-electron to π-electron interactions.[30]

RESULTS AND DISCUSSION

Docking outcomes: We have started evaluating the possibility of curcumin binding within pancreatic lipase (PL) binding pocket via

computer-aided molecular modeling techniques 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. Accordingly, curcumin was docked into the binding pocket of PL (PDB code: 1LPB). Docking consists of two stages: (i) prediction of the conformation and pose of the bioactive compound into the binding cleft, and (ii) estimation of the tightness of target-ligand interactions (scoring) to guide conformational sampling.[13] The final docked conformations are selected according to their scores. The docking study was conducted utilizing the docking engine FRED.[29] FRED was recently reported to illustrate good overall performance, particularly in virtual high-throughput screening experiments. However, simulated molecular docking requires the user to provide FRED with an optimal set of parameters for the docking experiment (see Docking Experiment in Experimental). Those parameters differ according to the protein in question.

In our previous publication about pancreatic lipase (PL),[25] we have identified the optimal docking configuration and scoring function for PL, by extracting the ligand MUP901 (Fig. 2) from crystallographic structure of PL (PDB code: 1LPB)[30] and re-docking it again into the same protein (self-docking) via a variety of docking conditions and scoring functions. Chemgauss2 was found to yield the closest model to the crystallographic structure as shown in Fig. 2A.

Therefore curcumin was docked into the binding pocket of PL employing the same optimal docking parameters and scoring function (See Docking Experiment), and was predicted by simulated docking to bind within the active site of PL. Although curcumin exhibits ketone-enol tautomerization (Fig. 1A), there is compelling experimental and theoretical evidence that the enol structure is the most favored due to intra-molecular hydrogen bonding.[31] 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.[31] Therefore, only the enol form was employed in the docking study.

Fig. 3A shows how the co-crystallized structure binds within the binding pocket of PL While Fig. 3B shows how curcumin is predicted to bind within the same binding pocket. Several significant binding interactions can be observed between the docked curcumin and binding pocket of the PL, as shown in Fig. 3. Comparison of the docked poses of the curcumin (Fig. 3B) with the co-crystallized ligand within the binding site of PL (Fig. 3A) illustrates similarities in their binding profile. Both have potential hydrophobic interactions with the key amino acids Tyr-114 and Phe-77. However, the docked curcumin (IC_{50} value = 7.3 μg/mL) has of strong hydrogen bond in such a way that the enolic hydroxyl group of curcumin interacts with the carbonyl group of Phe-215. Moreover, it exhibits additional attractive interactions to those of the MUP901 due to hydrophobic interactions with LEU-213, ALA-178 and ALA-260 and strong aromatic stacking (π- π interaction) between both His-263 and His-151 imidazole (cationic NH) rings and electron rich phenolic ring in curcumin which stabilizes the ligand-protein complex and contributes to the very high affinity of curcumin. These additional interactions may explain the high potency exhibited by curcumin towards PL (see below).

Curcumin's PL inhibitory properties: The docking results were validated by evaluation of the *in vitro* PL inhibitory activity of curcumin. The antilipase activity of the tested concentrations of curcumin is given in Fig. 4. Curcumin showed dose dependent anti-pancreatic lipase activity with an IC_{50} value of 7.3 μg/mL (equivalent to 19.8 μM). The IC_{50} value of the standard orlistat is 0.2 μM which is comparable to cited IC_{50} values.[32, 33]

Enzyme kinetics studies revealed that the Km and Vmax of PL were 0.22 mM and 0.2 mM/min, respectively. The PL-curcumin complex showed no change in Vmax and showed increase of the Km. The Km rose to 0.31, 0.69 and 2.15 mM at the [curcumin] rising concentrations (0.2714, 2.714 and 27.14 μM) and Vmax almost remained unchanged. So the type of inhibition was competitive. The inhibitor constant (Ki) was calculated to be 4.9 μM. Lineweaver Burke plot is given in Fig. 5. It demonstrates the PL kinetic study of curcumin using *p*-NPB as the variable substrate.

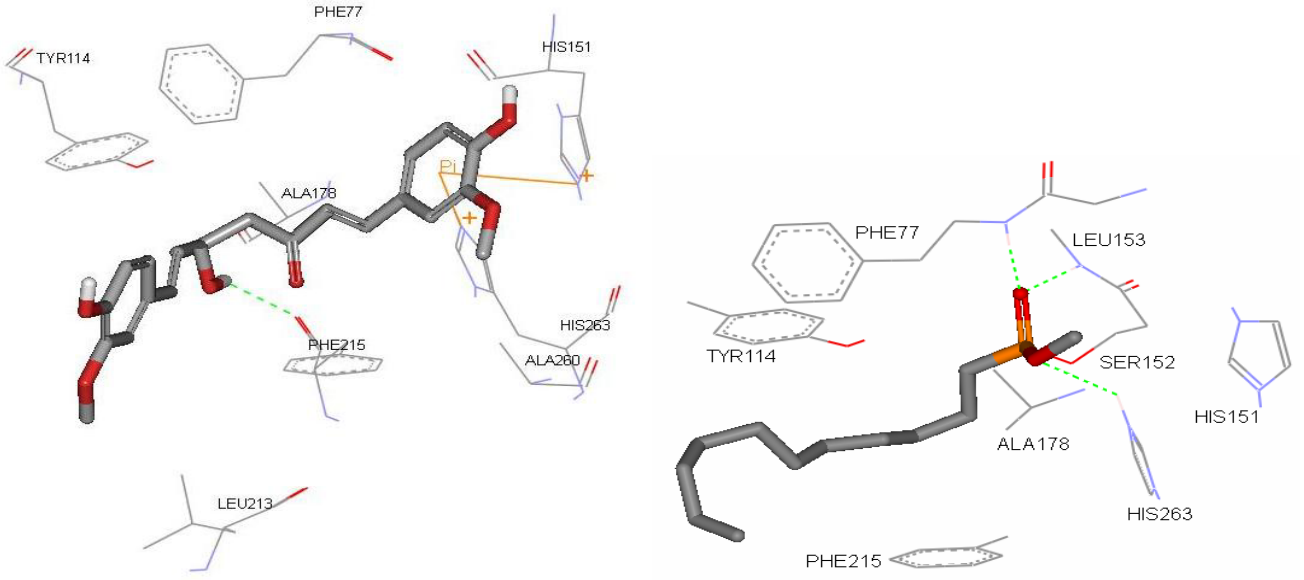

Fig. 3 – **(A)** Detailed view of the co-crystallized structure (MUP901) and the corresponding interacting amino-acids within the binding site of PL (PDB code: 1LPB). (**B**) Detailed view of the docked curcumin structure and the corresponding interacting amino acid moieties within the binding site of PL.

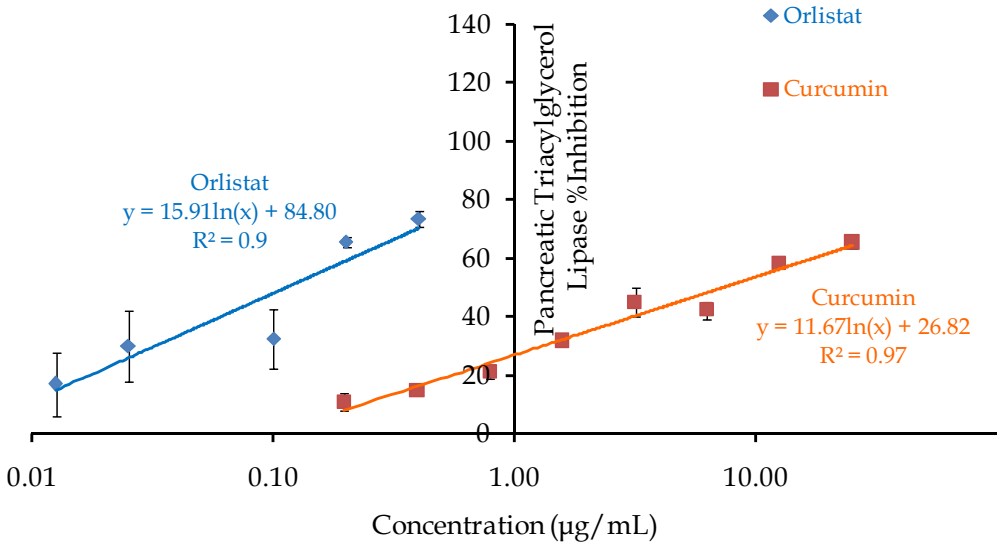

Fig. 4 – *In vitro* inhibitory effects of ascending concentrations of curcumin and orlistat on pancreatic triacylglycerol lipase activity. Results are mean ± SEM (n = 3 independent replicates).

There have been many reports of lipase inhibitors derived from natural materials, like phytic acid,[34] polyphenols and saponins,[35] sulfated triterpene saponins and saponins,[36, 37] as well as rosmarinic acid, chlorogenic acid, caffeic acid.[38] Impressively the basic protease-resistant peptide ε-polylysine, as a potent inhibitor of pancreatic lipase, could exert an antiobesity function in high fat diet fed mice by inhibiting intestinal absorption of dietary fat.[39] Taken together, natural products use a remarkably diverse set of mechanisms to covalently modify enzymes from distinct mechanistic classes, thus providing a wellspring of chemical concepts that can be exploited for the design of active site-directed proteomic probes.[40] Subsequent rational optimization of effective principles towards clinical leads with maximal *in vivo* efficacy devoid of adverse events is proving pivotal.[41]

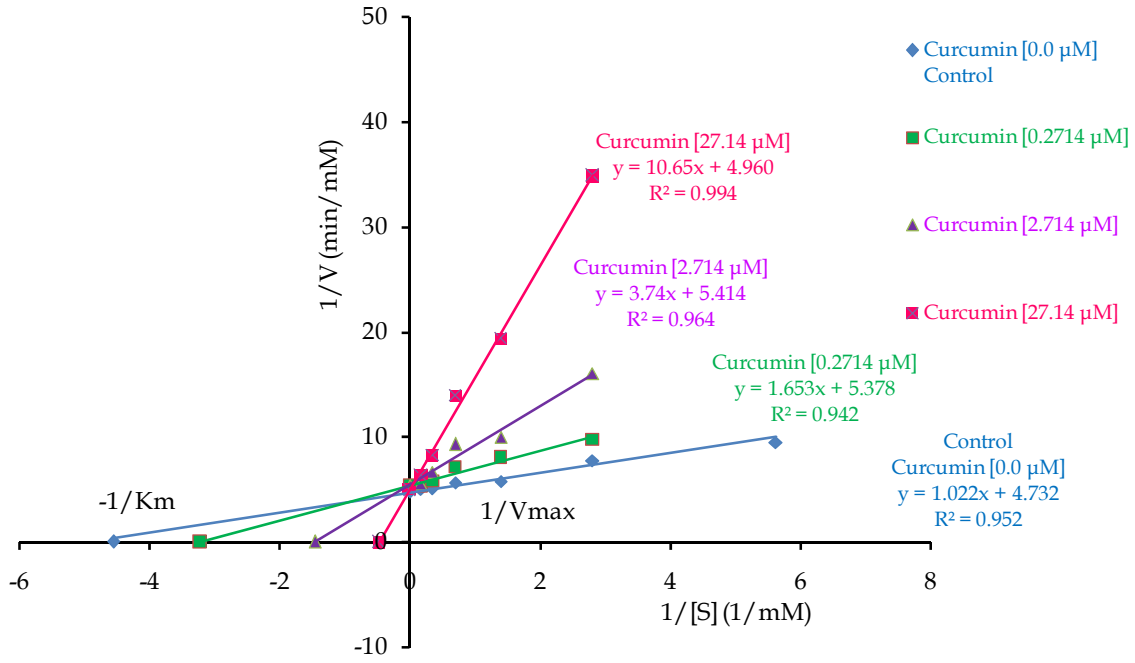

Fig. 5 – Lineweaver-Burk plot obtained by inhibition studies of various molar concentrations of curcumin against PL.

CONCLUDING REMARKS AND FUTURE DIRECTIVES

We have unequivocally proved through theoretical docking simulations and experimental evaluation that curcumin is a potent inhibitor of PL, which explains, at least partially, its established hypolipidimic properties. As combination therapy can substantially increase weight loss probabilities; [42] Combination of sodium-glucose cotransporter-2 (SGLT) inhibitor empagliflozin with orlistat can further improve the body-weight or body-fat loss of obese rats fed a cafeteria diet in comparison to orlistat alone.[43] This may suggestively indicate the success of oral hypoglycemic agents– curcumin combination in therapeutic/preventive strategies for the treatment of obese patients with prediabetes or type 2 diabetes. However, such speculations need more investigation.

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