In Vitro Anti-Cancer Activity of Native Curcumin and “Protein-Curcumin” Systems: A Perspective on Drug-Delivery Application

Mohammad Reza Ashrafi Kooshka, Kamran Mansouri, Mohammad MostafaNadi, Sina Khodarahmi, Reza Khodarahmi*

*Corresponding Author: Reza Khodarahmi, E-mail: rkhodarahmi@mbrc.ac.ir; rkhodarahmi@kums.ac.ir
Copyright © 2013 by Kermanshah University of Medical Sciences

ABSTRACT

Curcumin is a natural polyphenolic compound with anti-cancer, anti-inflammatory, and anti-oxidation properties. Low water solubility and rapid hydrolytic degradation are two challenges that limit the use of curcumin as a therapeutic agent. In the current study, the role of the native/modified forms of bovine serum albumin (BSA) and casein, as food-grade biopolymers and safe drug delivery systems, on the physical and biological activity of curcumin have been surveyed. Analyses of quenching of proteins fluorescence by curcumin indicated that chemical modification decreased the binding affinity of curcumin toward albumin whereas, it significantly increased for casein and the average number of binding sites also doubled in the modified casein. Measurement of cell viability using LDH assay showed that cytotoxicity of protein-bound curcumin is higher than free curcumin. Moreover, in the presence of native proteins, curcumin revealed elevated in vitro anti-cancer activity (against MCF7 and SKNMC) compared to modified forms. It appears that BSA and casein as protein vehicles are useful tools to increase both food quality and the bioavailability of curcumin as a health promoting agent. However, results imply that the chemical modification of proteins cannot improve the anti-cancer activity of curcumin despite increasing of their binding affinity.
Introduction

According to the International Food Information Council (IFIC) “functional food” is defined as “food that has health promotion properties beyond basic nutrition”. In this regard, different phytochemicals especially polyphenols have attracted the attention of scientists and extensive research has been carried out to devise novel methods to incorporate the functional ingredients into foods. Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is the best characterized plant-derived polyphenol showing chemopreventive and safety activities against malignancy[1]. This yellow-orange natural compound which is the main component of Curcuma longa rhizomes (Fig. 1), has been consumed as dye and food additive for a long time in Asia[2].

Fig. 1. (Top, Left) Curcuma Longa with flower and rhizome. Turmeric (Curcuma longa L.), belonging to the family of Zingiberaceae, is a perennial herb native to India where its rhizome is used as a yellow colorant curry spice and traditional medicine. (Bottom) Turmeric color at different conditions and dried pure curcumin. (Top, Right) Chemical structure of turmeric constituent compounds. The active principle in turmeric was identified as a group of polyphenolic compounds, namely curcumin (74-78%), demethoxycurcumin (15-18%) and bisdemethoxycurcumin (4-6%) commonly referred to as “curcumin”.

Anti-cystic fibrosis, anti-oxidant and wound healing properties[3,4]. It is of special importance among multitarget components in cancer chemotherapy. Today, it is also used in curry as spice, and as preservative and industrial food dye (E-100).

In the past decade, scientific studies have revealed other medicinal effects of curcumin including anti-proliferative, anti-angiogenic, anti-inflammatory, due to low intrinsic toxicity of curcumin for healthy (normal) cells, several clinical trials are either underway or have been completed with an aim to develop curcumin into a treatment agent[2,5,6]. Preclinical studies on curcumin have shown its capability for carcinogenesis inhibition in various cell lines originated from cervix, stomach, breast, liver, ovary, bone marrow, colon, oral epithelium, prostate, and pancreas (See Fig. 2). Curcumin is extremely safe even at very high doses of 8–12 g/day[5,6]. But, from the chemical viewpoint, there are several challenges that must be overcome for curcumin to become a routine treatment agent: Despite the safety, efficacy and well-tracked mechanisms of action, curcumin has a low aqueous solubility (approximately 11 ng/mL), which significantly limits its bioavailability in vivo[4-7]. On the other hand, in aqueous medium, curcumin undergoes rapid degradation by hydrolysis (even at physiological pH) followed by molecular fragmentation within 30 min[7] (see Fig. 2). Moreover, the recent studies illustrated that the β-diketone moiety seems to be a specific substrate of a series of aldo-ketoreductases and can be rapidly decomposed in vivo[8].

Study on the curcumin chemical degradation is an interesting research topic due to the effects of its degradation products on human health and also possible changes in the color and organoleptic properties of food products in which curcumin is used as an additive[3,9]. Various approaches have been applied to increase water solubility, stability and bioavailability of curcumin (as well as its controlled delivery at or around cancer tissues) such as emulsification, chemical modification, and encapsulation in polymer nanoparticles, cyclodextrins, hydrogels and nanogels, polymeric and surfactants micelles, lipid bilayers, liposome/phospholipid, solid lipid nanoparticles, polymer conjugates, self-assemblies, and vesicles and other delivery systems (See Fig. 3)[10]. For instance, the water solubility of curcumin in cyclodextrin-based or chemically modified systems can reach about 2-200 mg/ml[11,12]. However, each delivery system may have cytotoxic effects on some normal tissues and cells and/or display limited influence on curcumin stability.
Drug delivery systems based on food proteins hold much promise because of their high nutritional value and excellent functional properties, including emulsification, gelation, foaming and water binding capacity as well as their applications as ingredients in the food industry. Systems based on proteins including gelatin, collagen, casein, albumin and whey protein have been studied for delivering drugs, nutrients, bioactive peptides and probiotic organisms. The main aim of the current study is to introduce albumin and casein (See Fig. 4) as food-grade amphiphilic materials to interact with curcumin, since serum albumins and caseins are commonly used as transporting vehicles for proteins, hormones, drugs, and diagnostic agents [13]. Casein micelles are relatively easy to prepare, biodegradable, and have potential for high drug loading capacity. Serum albumin is the most abundant of the proteins, circulated several times in the blood [14]. Both casein micelles and albumin delivery systems have been previously employed for administration of several hydrophobic drugs [15, 16]. Beta-casein is also an amphiphilic self-assembling protein that helps to solubilize curcumin more efficiently in aqueous solution [17]. This micellar curcumin solubility is $7.7 \times 10^{-5}\text{mol/l}$, whereas that of curcumin is only $2.99 \times 10^{-8}\text{mol/l}$. This suggests at least a 2500-fold enhancement in the solubility of curcumin.

In the literature, there are a few reports on the interaction of curcumin with casein and albumins [2, 18] and its potential delivery to tumor cells [2], implying that these proteins have the ability to solubilize and stabilize curcumin in aqueous
medium \[2, 4, 17, 19\]. This implication is consistent with the results by Wang et al., indicating that degradation of curcumin is suppressed in the presence of serum \[7\]. Since proteins are generally amphiphilic polymers, we hypothesize that albumin and casein (micelle) are able to bind/encapsulate curcumin and improve its anti-cancer activity.

Fig. 4. (Left) One of proposed representations of the model of casein micelles. (Right) Ribbon representation of BSA. The locations of drug binding sites (Sudlow I and Sudlow II) are indicated. The protein secondary structure is shown as ribbon with the sub-domains colour-coded as follows: IA, blue; IB, skyblue; IIA, green; IIB, yellow; IIIA, orange; IIIB, red.

Chemical modification induces structural changes in proteins and exposure of previously buried hydrophobic patches or rendering groups prone to salt bridge and H-bond formation, affecting the binding of hydrophobic and hydrophilic/charged compounds, respectively\[20, 21\]. In this way, proteins were chemically modified to increase their binding affinity for curcumin. We demonstrated that stability enhancement of curcumin in the presence of proteins, originates in part from its stronger binding (high $K_b$) to the protein. Finally, bound, encapsulated (and stabilized) curcumin exhibited increased anti-cancer activity \textit{in vitro}.

Materials and Methods

Materials

Bovine serum albumin (essentially free fatty acids) fraction V was from Applichem, pure curcumin was from Merck (Darmstadt, Germany) and whole casein was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). The other reagents were from analytical grade. Curcumin stock (1.5 mM) was prepared in DMSO and in all experiments, DMSO final concentration was less than 1%. All of the experiments were carried out in 50 mM sodium phosphate, pH 7.0 as the buffer at room temperature in triplicate.

BSA modification with acetic anhydride

To increase protein solubility and obtain high modification yield, BSA solution (10% w/v) was made in doubly-distilled water at 30°C and the pH was adjusted to 7.5-8.5. Acetic anhydride was added to the solution at the level of 0.5 g/g of protein over 30-90 min \[22\]. Then it was dialyzed against 50 mM phosphate buffer pH 7.0 for 24 h and the buffer was exchanged four times at 6 h intervals.

Casein modification with glyceraldehyde

10 ml of casein concentrate (150 µM) was incubated overnight at 37 °C in glyceraldehyde aqueous solution (3 mM) which was buffered with 50 mM phosphate buffer pH 7.0. At the end, 100 µl of a 0.1 M NaBH4 solution in 0.1 N NaOH was added and the mixture was further incubated for 1 h. After cooling under room temperature condition, the modified proteins were dialyzed against 50 mM phosphate buffer pH 7.0 for 24 h and the buffer was exchanged four times at 6 h intervals \[23\]. Free amino groups in the proteins were assessed by the TNBS method for determination of the lysine modification extent, as described elsewhere \[24\].

Determination of binding constant and number of binding sites

Fluorescence quenching technique was used to study the binding of curcumin to the proteins. Different concentrations of curcumin (0-10 µM) were equilibrated with 1 µM protein in a 1 cm path-length quartz cuvette (3.0 ml). Fluorescence measurements were performed with a Cary Eclipse (Varian, Australia) Spectrofluorimeter at 293 K. After excitation at 290 nm, emission spectra were recorded from 300 to 450 nm (excitation/emission slit widths were 5/10 nm). The background fluorescence was subtracted from each emission spectrum.

The modified Stern-Volmer equation can be used for the relationship explanation between the extent of fluorescence quenching of the macromolecule and the quencher concentration (Eq. 1). Since this equation is correct that a small molecules bind independently to a set of equivalent sites on a fluorescent macromolecule.
In vitro anti-cancer activity of native curcumin …

\[
\log \left( \frac{(F_0 - F)}{F} \right) = \log K_b + n \log [Q]
\]

(1)

Where \( K_b \) is the binding constant of the quencher with the fluorescent macromolecule, \( n \) is the average number of binding sites per macromolecule and \([Q]\) is the quencher (curcumin) concentration. \( F_0 \) and \( F \) represent the fluorescence intensities of the macromolecule in the absence and presence of a quencher, respectively \[25\]. By plotting \( \log \left( \frac{(F_0 - F)}{F} \right) \) vs. \( \log [Q] \), the values of \( K_b \) and \( n \) were gained from the \( y \)-intercept and slope of the plot, respectively.

**In vitro cytotoxicity assay**

MCF7, HUVEC and SKNMC cells were cultured in DMEM in addition to 5% FBS by 1% antibiotic/antimycotic agents. The cells were cultured in 96-well plates at 0.01 million cells/well/0.3 mL with at least two replicates for 24 h in a humidified CO2 incubator at 37°C and 5% CO2 in the air. Adequate amount of free and premixed protein-bound curcumin (curcumin to protein ration is 1:5) was added to the designated wells. The control wells were considered with free carrier proteins in culture medium. Cell viability was checked by LDH cytotoxicity assay kit. IC\textsubscript{50} values were calculated by GraphPad Prism v.5 (San Diego, California). Data were analyzed through one-way ANOVA followed by Tukey's Multiple Comparison Test using the same software. All data are presented as mean±SD with statistical significance defined as \( P \leq 0.05 \).

**Results and Discussion**

Curcumin, with considerable pharmaceutical activity, is a hydrophobic polyphenolic compound with low water solubility and stability \[1, 7\]. Hence, curcumin requires a convenient carrier system to deliver to different parts of the body. Recently, various systems such as encapsulation in nanoparticles, cyclodextrins, micelles, and proteins are being expanded for drug delivery investigations \[4, 10\]. In this study, the interaction of curcumin with the native and modified forms of two important proteins, serum albumin and milk casein, and their effects on anti-cancer activity have been studied.

**Interaction between curcumin and proteins**

Fluorescence quenching of macromolecules presents useful details about ligand binding to specific sites on macromolecule \[26\]. So, fluorescence quenching experiments of the native/modified proteins were carried out at 293 K. The Native and modified proteins exhibits a strong fluorescence emission at 344 nm on excitation at 290 nm. Addition of curcumin to the proteins causes significant quenching of their fluorescence intensity with the formation of a new peak at 510 nm. This indicates an interaction between the ligand and the protein and also suggests that curcumin is located at vicinity of the proteins tryptophans \[27\].

According to modified Stern-Volmer equation (Eq. 1), plot of \( \log \left( \frac{(F_0 - F)}{F} \right) \) vs. \( \log [\text{curcumin}] \) gives the values of the binding constant \( (K_b) \) and average number of binding sites \( (n) \) of the curcumin-protein complex (Fig. 5) \[25, 27\]. The value of \( K_b \) is essential for estimation of efficiency of the drug carrier systems; since weak binding may result in a short half-life/rapid eliminate or poor distribution, while strong binding can decrease the concentrations of free drug in body fluids \[27, 28\]. Furthermore, in the case of curcumin, strong binding can help to its protection against degradation \[4, 19\].

The values of \( K_b \) and \( n \) were calculated from linear equation of modified Stern-Volmer curves (Fig. 5) and were presented in Table 1. The proteins exhibit high binding constants on the order of \( 10^{-6} \text{ M}^{-1} \). The values of the binding constants for the association of curcumin to the native forms of BSA and casein are in good agreement with the reported values in the previous studies \[2, 18\].

**Fig. 5.** Double-logarithm plot for fluorescence quenching of native (●) and modified (○) forms of BSA and native (▲) and modified (△) forms of casein by curcumin at 293 K in 50 mM sodium phosphate buffer pH 7.0.\( K_b \) and \( n \) is obtained from the \( y \)-intercept and slope of the plot, respectively.
Table 1. Binding constants \( (K_b) \) and average number of binding sites \( (n) \) for interaction of curcumin with native and modified forms of BSA and casein at 293 K. Values are expressed as mean±SD from at least three independent experiments.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Linear equation</th>
<th>( K_b \times 10^5 ) (M(^{-1}))</th>
<th>( n )</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native BSA</td>
<td>( y = 1.175x + 6.268 )</td>
<td>25.044±0.751</td>
<td>0.971±0.029</td>
<td>0.990</td>
</tr>
<tr>
<td>Modified BSA</td>
<td>( y = 1.177x + 6.398 )</td>
<td>18.570±1.114*</td>
<td>1.173±0.070</td>
<td>0.996</td>
</tr>
<tr>
<td>Native casein</td>
<td>( y = 1.210x + 6.128 )</td>
<td>13.434±0.537</td>
<td>1.215±0.048</td>
<td>0.992</td>
</tr>
<tr>
<td>Modified casein</td>
<td>( y = 1.925x + 9.532 )</td>
<td>34048.658±510.730**</td>
<td>1.937±0.029*</td>
<td>0.982</td>
</tr>
</tbody>
</table>

* \( P<0.05 \) versus native protein; ** \( P<0.01 \) versus native protein.

As it can be observed at Table 1, \( K_b \) decreases upon modification indicating lower stability of the curcumin-modified BSA complex. However, opposite observations were made in the case of casein and \( K_b \) increased 2600-fold upon glycation-like modification. Mohammadi et al. suggested that hydroxyl phenolic groups have important role in the curcumin binding process \[18\]. Hence, the modified casein with novel hydroxyl groups is probably prone to H-bond formation with hydroxyl phenolic groups of curcumin. Moreover, the \( n \) values for the native/modified BSA and the native casein are around unity, which shows the existence of only one binding site both on the native and modified albumin, whereas \( n \) is doubled for the modified casein. These findings may be suggested that the native albumin and the modified casein as a natural biopolymer are good candidates for a suitable drug carrier rather than the respective forms. To assessment of this assumption, the biological activities (anti-cancer efficacy) of the free and the protein-bound curcumin systems were also surveyed.

**Cytotoxic effects of the free and the protein-bound curcumin against cancerous cells**

Several types of carriers have been found to be suitable for the encapsulation or loading of curcumin to improve its effects in cancer therapeutics. The characteristics of these curcumin (nano) formulations can be tailored according to the specific requirement for inducing cellular death by various mechanisms \[10\]. The interaction of curcumin with different biomacromolecules could have strong consequences on its activity in biological systems \[29\]. According to previous reports on cytotoxicity effects of curcumin on cancer cells \[2, 29, 30\] and to evaluate the efficacy of protein delivery systems, two cancerous cell lines of human origin (MCF7, and SKNMC) were exposed to a number of equivalent concentrations of the free and the protein-bound curcumin. After treatment for 24 h, the cell viability/cytotoxicity was quantified using LDH assay (Fig. 6). Also, \( IC_{50} \) values for cytotoxicity of the free and different protein-bound curcumin systems were calculated using GraphPad Prism v.5 and showed in Fig. 7. Whereas safe toxicological profiles of the various curcumin (nano) formulations and their efficacy in the cell line models highlight their potential for evaluation in \textit{in vivo} models, cytotoxicity of DMSO and/or protein alone and effect of the free and different protein-bound curcumin systems on normal cell line (HUVEC) were measured. The DMSO/protein alone show comparable cell viability to untreated cells indicating that they have no harmful effect on the cells. Moreover, as can be seen at Fig. 7, the protein-bound curcumin exhibited lower cytotoxic effects on the normal HUVEC cells compared to cancerous cells. Regardless of vehicle type, the MCF7 and SKNMC cell lines showed different behavior toward curcumin uptake. So that, the MCF7 cell line was more sensitive to curcumin than the SKNMC cell line (See Fig. 7). Hence, depend on disease circumstances can be choose a convenient protein-curcumin delivery system.
As demonstrated in Fig. 6, both free curcumin and “curcumin-protein” systems showed dose-dependent cytotoxicities on the MCF7 cells. Especially at higher concentrations, the anti-cancer activity of the “curcumin-protein” systems was significantly higher than the (DMSO-dissolved) free curcumin, suggesting that the “curcumin-protein” systems are more effective to protect...
curcumin and deliver it to cancer cells (See Fig.7). Beside, our understanding from the available literature is that the use of curcumin (nano)formulations in chemotherapy for cancer treatment is a facile modality that improves existing curcumin therapies by targeting tumors and by reducing the dose required[10]. In recognition of this, the IC\textsubscript{50} value of free curcumin is 24.13 μM, whereas it decreased to 6.65 μM in the presence of the native BSA.

According to Fig.7, among the “curcumin-protein” systems, the “BSA-curcumin” systems possessed higher activities than those bound to the caseins. Additionally, in agreement with binding characteristic data, curcumin in the presence of the native (not the modified) albumin displayed higher cell-killing activity indicating that binding affinity of curcumin toward proteins may play an important role in its cytotoxic properties. High binding affinity may results in more preservation effect against degradation [4, 19]. The increased stability enhances lifetime and gives an additional opportunity to intact/functional curcumin to be up taken by target cells. Moreover, binding of curcumin to proteins may also lead to its controlled release. This can affect the distribution and pharmacokinetics of curcumin. Unlike, the modified casein has lower cytotoxicity toward cancerous cells compared to the native form in spite of its very high binding constant (See Table 1). It seems that tight binding of curcumin to the modified casein decreased its free functional concentration regardless of more predictable protection from degradation [27, 28].

**Conclusion**

In brief, anti-cancer activity of the “native protein-curcumin” systems are slightly higher than that of the “modified proteins-curcumin” systems (P< 0.001), suggesting that the “native protein-curcumin” complexes are more effective tools for curcumin delivery to cancer cells. This laboratory suggest that mixing of curcumin with milk or its consumption as albumin containing capsules may lead to its enhanced stability and bioavailability. Furthermore, enhanced permeation and retention (ESR) of albumin within tumor cells may highlight its application as an important drug delivery system. However, human trials need to be conducted to establish their effectiveness in clinical applications as an improved therapeutic modality for cancer treatment.

**Abbreviations Used**

SA, serum albumin; BSA, bovine serum albumin; TNBS, 2,4,6-trinitrobenzenesulfonic acid; LDH, lactate dehydrogenase; MCF7, a breast cancer cell line; SKNMC, a human neuronal epithelioma cell line; HUVEC, human umbilical vein endothelial cell.

**Funding**

This work is sponsored by the Research Council of Kermanshah University of Medical Sciences, Kermanshah, Iran.

**Conflict of interest**

Authors certify that no actual or potential conflict of interest in relation to this article exists.

**References**

In vitro anti-cancer activity of native curcumin ...