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RESEARCH ARTICLE

Heat-solubilized curry spice curcumin inhibits antibody–antigen interaction in *in vitro* studies: A possible therapy to alleviate autoimmune disorders

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Chronic and complex autoimmune diseases, currently treated palliatively with immunosuppressives, require multi-targeted therapy for greater effectiveness. The naturally occurring polyphenol curcumin has emerged as a powerful ''nutraceutical'' that interacts with multiple targets to regress diseases safely and inexpensively. Up to 8 g/day of curcumin for 18 months was non-toxic to humans. However, curcumin's utility is limited by its aqueous insolubility. We have demonstrated a heat-mediated 12-fold increase in curcumin's aqueous solubility. Here, we show by SDS-PAGE and surface plasmon resonance that heat-solubilized curcumin binds to proteins. Based on this binding we hypothesized that heat-solubilized curcumin or turmeric would prevent autoantibody targeting of cognate autoantigens. Heat-solubilized curcumin/turmeric significantly decreased binding of autoantibodies from Sjögren's syndrome (up to 43/70%, respectively) and systemic lupus erythematosus (up to 52/70%, respectively) patients as well as an animal model of Sjögren's syndrome (up to 50/60%, respectively) to their cognate antigens. However, inhibition was not specific to autoimmunity. Heat-solubilized curcumin/turmeric also inhibited binding of commercial polyclonal anti-spectrin to spectrin (50/56%, respectively). Thus, we suggest that the multifaceted heat-solubilized curcumin can ameliorate autoimmune disorders. In addition, the non-toxic curcumin could serve as a new protein stain in SDS-PAGE even though it is less sensitive than the Coomassie system which involves toxic chemicals.

Keywords:
Autoimmunity / Curcumin / Protein staining / SDS-PAGE / Solubility

1 Introduction

Treatments for chronic diseases need multi-targeted therapy. Immunosuppressives have been the main therapeutic choice for chronic and complex diseases such as Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE) and treatment is largely palliative [1, 2]. SS is characterized by dry eyes and dry mouth, the presence of anti-Ro 60/anti-La autoantibodies in up to 90% of patients and a highly significant over representation of lymphoma compared with the normal population [3]. SLE is also a multisystem disorder distinguished by antibodies to a variety of self-proteins, often affecting kidney function and involving premature atherosclerosis. Anti-Ro autoantibodies are present in up to 50% of SLE patients [4].

The naturally occurring phytochemical curcumin (CU), the most active component in the Indian curry spice turmeric (TU) (*Curcuma longa*), has emerged as a ''nutraceutical'' that can interact with multiple targets to regress diseases safely and inexpensively. In addition to inhibiting tumorigenesis, metastasis, platelet aggregation,

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Abbreviations: CU, curcumin; **HNE,** 4-hydroxy-2-nonenal; **MAP,** multiple antigenic peptide; **SLE,** systemic lupus erythematosus; **SPR,** surface plasmon resonance; **SS,** Sjögren's syndrome; **TU,** turmeric

inflammatory cytokine production, cataract formation, inflammatory bowel disease and myocardial infarction, CU has been shown to lower cholesterol, suppress diabetes, enhance wound healing, modulate multiple sclerosis and Alzheimer's disease and block HIV replication [5–9]. Although 12 g/day was found to be non-toxic to humans, its insolubility limits its biological utility. Our laboratory has shown that heat treatment increased CU's solubility 12-fold in water without affecting its integrity. Moreover, heat-solubilized CU inhibited 4-hydroxy-2-nonenal (HNE) mediated oxidative modification of a protein substrate by 80%.

Since we were able to heat solubilize CU in water, we were able to find out that aqueous CU stained proteins on SDS-PAGE. Based on this observation, that heat-solubilized CU could bind to proteins on SDS-PAGE, we hypothesized that it would bind to antigen and/or antibody and thus inhibit autoantibody–antigen interaction. Such an intervention would be of therapeutic potential to prevent autoantibody targeting of cognate antigens and thus ameliorate autoimmune disorders such as SS and SLE.

2 Materials and methods

2.1 Instrumentation and materials

The BIAcore instrument, part of Oklahoma Medical Research Foundation (OMRF) Core Facility, was from GE Healthcare (Piscataway, NJ, USA). Carboxy methylated dextran-5 sensor chips (research grade), P20 surfactant and amine coupling kit were also from GE Healthcare. Serocluster ''U'' vinyl ELISA plates were from Costa (Cambridge, MA, USA).

Purified Ro 60 (SS-A) was purchased from Immunovision (Springdale, AK, USA). Recombinant human La (SS-B) was a gift from Dr. Michael Bachmann, Dresden, Germany. A peptide spanning the Ro 60 [10, 11] sequence 273–289 (LQEMPLTALLRNLGKMT) tagged with a terminal cysteine was synthesized as a multiple antigenic peptide (MAP) [12] and a linear peptide at the Molecular Biology Resource Facility, University of Oklahoma Health Sciences Center by a manual stepwise solid-phase procedure. CU and phosphate buffered saline tablets were from Sigma Chemical Company (St. Louis, MO, USA). TU was purchased from a local grocery. Alkaline phosphatase conjugates were from Jackson Laboratories (Bar Harbor, ME, USA). All other chemicals were reagent grade.

2.2 Patients

Primary SS and SLE sera were kindly provided by Dr. Tom Gordon, Flinders Medical Institute, Adelaide, Australia and the Lupus Family Registry and Repository, OMRF, Oklahoma City, OK, USA, respectively.

2.3 Heat-solubilization of CU and TU

CU or TU (5 mg/mL) was solubilized as described previously [13]. Essentially hot distilled water was added to either CU or TU at 5 mg/mL (1 m hot distilled water/5 mg CU or TU) and further boiled for 10 min and centrifuged twice. The supernatant was used to test inhibition of binding of anti-Ro 60 or anti-Ro 273 peptide antibodies binding to their cognate antigen by ELISA.

2.4 Mice immunization

Ten BALB/c mice were used in this experiment. Five mice were immunized (day 1) with 50 μg of a linear peptide (273–289 amino acid sequence from the Ro 60 autoantigen) emulsified in Freund's Complete Adjuvant. Five mice were administered saline in Freund's Complete Adjuvant. Subsequent boosts were in Freund's Incomplete Adjuvant. The first, second and third boosts were given on day 7, 27 and 60, respectively [14]. The research was approved by the Institutional Review Board and was conducted in accordance with the internationally accepted principles for laboratory animal use.

2.5 Ro 60 or Ro 273 MAP ELISA

ELISA was carried out as mentioned earlier with modifications. Purified Ro 60 or Ro 60 MAP was coated at 5 μg/well at room temperature for 2 h. The plate was washed twice with phosphate buffered saline and tapped dry. Heat-solubilized CU (7.4 μg/mL) or TU (15.5 μg/mL) was added to each well (100 μL) except those wells receiving uninhibited sera samples. Purified water (100 μL) was added to each of the wells to which only sera was to be added (uninhibited samples). In individual microcentrifuge tubes, 2.4 μL of each serum sample were added to 40 μL of heat-solubilized CU (7.4 μg/mL) or TU (15.5 μg/mL). The plate and the sera samples with or without CU/TU were incubated overnight at 4°C. The wells were washed twice with PBST. The wells were then blocked with 3% milk/PBST for 2 h at room temperature. The sera samples with or without CU/TU were diluted to required dilution (1:100 for human samples; 1:1000 for mice or rabbit samples) with diluent and each sample was added in quadruplicate at 100 μL/well. The ELISA was developed as described previously [10].

2.6 Staining of proteins by CU on SDS-PAGE

Unstained molecular weight standards, BSA, HeLa cell antigens, La and Ro 60 autoantigens were resolved by SDS-PAGE, fixed with 25% methanol and 10% acetic acid for 10 min, rinsed with water and stained with heat-solubilized CU for 30 min. The CU-stained gel was visualized by ultraviolet light using a UVP BioDoc-It™ system.

2.7 Surface plasmon resonance

The BIAcore system was used to measure surface plasmon resonance (SPR). This system involves a sensor, in contact with a microfluidic cartridge. Ro MAP 273 was immobilized to a carboxy-methylated dextran matrix attached to the sensor surface. The other surface of the sensor, coated with a thin gold film faces the optical system. The SPR detector responds to refractive index changes in the vicinity of sensor surface as the Ro MAP 273 interacts with its ligand in fluid phase. The sensor surface was prepared essentially as reported earlier [10, 11]. A mock surface was also prepared similarly, but without the Ro MAP 273, to check the binding of CU to the matrix. Typically, a single ligand surface was used for several analyses. CU, prepared at 5 mg/mL (heated), was analyzed over the sensor surface to check for binding to Ro MAP.

3 Results and discussion

In this study, we used sera of patients with primary SS or SLE, sera from an Ro 273 peptide induced animal model of SS [14] and a commercial polyclonal anti-spectrin antibody in order to assess the ability of CU or TU to inhibit binding of these antibodies to their respective antigens, namely Ro 60, Ro 273 MAP or human spectrin.

Anti-Ro 60 antibodies from SS (Fig. 1) bound significantly to Ro 60 whereas the controls did not. Similar results were obtained with sera for SLE patients (data not shown). Ro 60 binding by anti-Ro 60 positive SS sera was inhibited 24–43% ($35 \pm 5.8\%$; $n = 9$) by heat-solubilized CU (Fig. 1A). However, when heat-solubilized TU was used the binding was inhibited up to 74% ($58\% \pm 13.5$; $n = 9$) (Fig. 1B). Similarly, Ro 60 binding by anti-Ro 60 positive SLE sera was inhibited by CU or TU by 36–52% (43 ± 6.6; $n = 4$) or 61–70% (65 ± 4; $n = 4$), respectively (data not shown).

Sera from an SS mouse model [14], obtained by immunizing with a Ro 60 peptide (Ro 273–289) bound significantly to the Ro MAP 273 whereas sera from Freund's immunized mice did not bind. CU inhibited binding of anti-Ro 273 antibodies to Ro MAP 273 by 35–50% ($46\% \pm 8.9$; $n = 5$) (Fig. 2A) whereas TU inhibited by 55–60% ($58\% \pm 2.7$; $n = 5$) (Fig. 2B). The inhibition of binding to Ro 60 or Ro 273 MAP, respectively, by CU or TU was highly significant (up to $p < 0.0000001$) (Figs. 1 and 2).

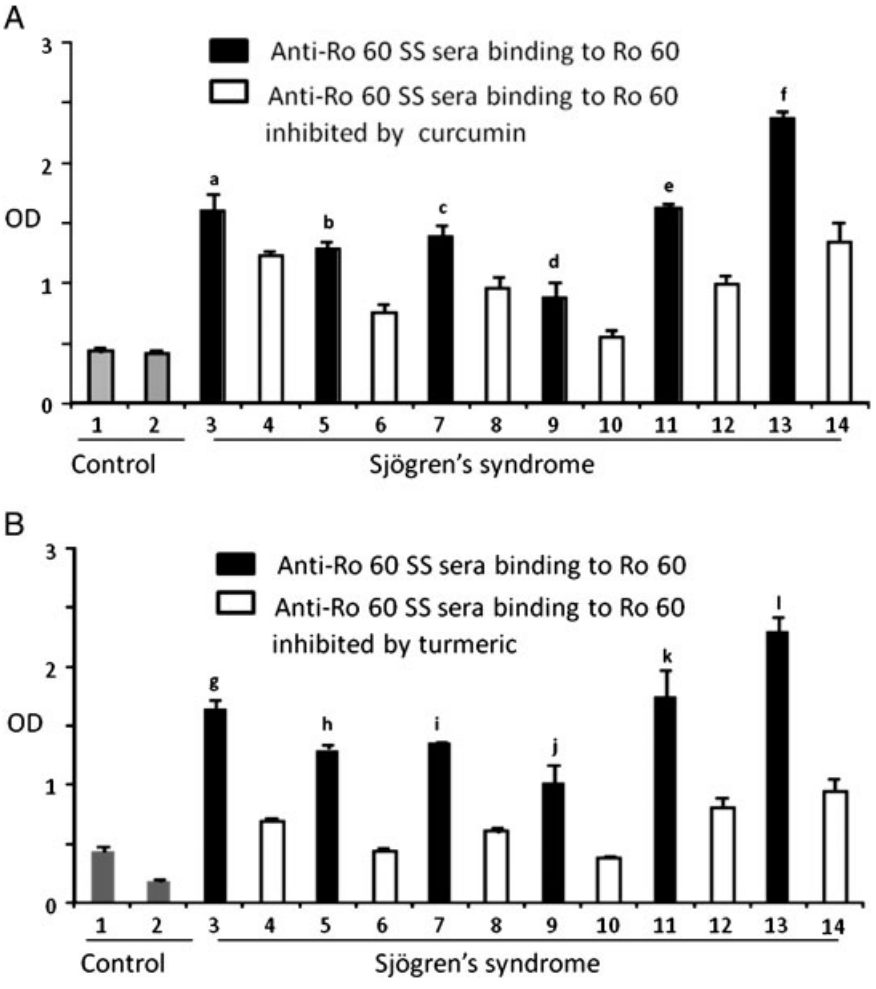

Figure 1. Binding of sera from SS patients containing anti-Ro 60 antibody to Ro 60 autoantigen by ELISA. ELISA was carried out as mentioned in Section 2. Panel A: Binding of sera from SS patients to solid phase Ro 60 antigen and inhibition of binding to the antigen by heat-solubilized CU. Control refers to normal human sera. Panel B: Binding of sera from SS patients to solid phase Ro 60 antigen and inhibition of binding to the antigen by heat-solubilized TU. Control refers to normal human sera. Values are means $\pm$ standard deviation for four determinations for each sample. [a] $p < 0.002$; [b] $p < 0.0001$; [c] $p < 0.001$; [d] $p < 0.000001$; [e] $p < 0.0001$; [f] $p < 0.0001$; [g] $p < 0.0001$; [h] $p < 0.0000001$; [i] $p < 0.0000001$; [j] $p < 0.00015$; [k] $p < 0.0001$; [l] $p < 0.00001$.

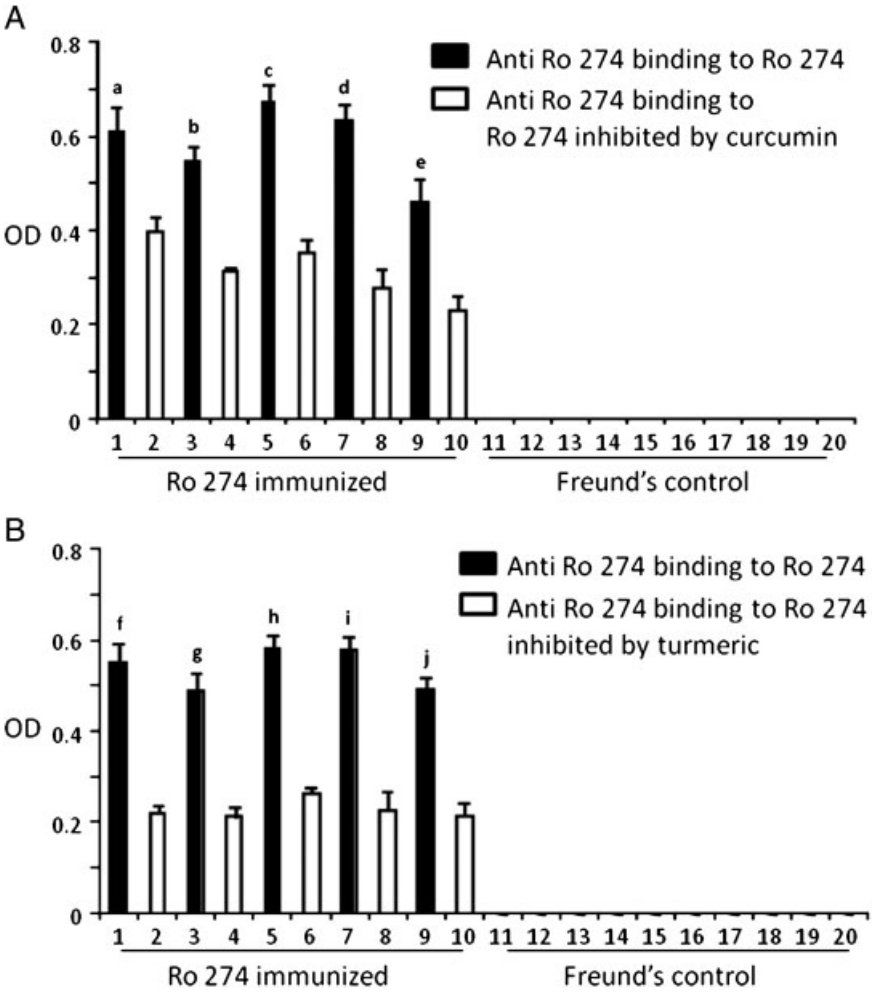

Figure 2. Binding of sera containing anti-Ro 273 peptide antibody from an animal model of SS to Ro 273 multiple antigenic peptide MAP by ELISA. ELISA was carried out as mentioned in Section 2. Panel A: Binding of sera from mice immunized with Ro 273 peptide or Freund's adjuvant to solid phase Ro 273 MAP antigen and inhibition of binding to the antigen by heat-solubilized CU. Panel B: Binding of sera from mice immunized with Ro 273 peptide or Freund adjuvant to solid phase Ro 273 MAP antigen and inhibition of binding to the antigen by heat-solubilized TU. Values are means $\pm$ standard deviation for four determinations for each sample. [a] $p<0.0004$; [b] $p<0.00001$; [c] $p<0.00001$; [d] $p<0.0001$; [e] $p<0.0003$; [f] $p<0.00001$; [g] $p<0.000001$; [h] $p<0.00001$; [i] $p<0.000012$; [j] $p<0.00001$.

In order to determine the protein binding specificity of CU, we stained molecular weight standards (purchased as unstained protein molecular weight standards), BSA, La and Ro 60 autoantigens separated by SDS-PAGE, with heat-solubilized CU. CU bound non-specifically to an array of 15 different proteins derived from a set of unstained molecular weight standards when each individual protein amounts was 500 ng or more. However, these proteins could also be stained with Coomassie Brilliant Blue, even at levels as low as 100 ng. In total 200 ng of Ro 60 or recombinant La autoantigen was not stained by CU, while it could be stained with CBB. Thus, there was no preferential staining of proteins with CU and the staining sensitivity was four to fivefold less compared with that seen with Coomassie (Fig. 3). To extend this information, we also determined the binding of heat-solubilized CU to MAP by SPR. The result of this study showed that CU bound to the Ro MAP 273 (Fig. 4).

Since CU binds to proteins non-specifically, we hypothesized that it would inhibit other antibody–antigen interactions as well and that it will not be restricted to autoimmune specificities. Purified human spectrin was used as the solid-phase antigen and binding of commercial anti-spectrin to spectrin in the presence and absence of CU or TU was determined by ELISA. Rabbit anti-human spectrin antibodies bound to spectrin with an optical density of 2.57, whereas the rabbit control antibody did not bind. Binding of anti-spectrin to spectrin was inhibited 50% by CU and by 56% by TU. Control antibody did not bind to spectrin with or without CU/TU (Fig. 5).

The most important limitation in using CU for *in vitro* studies or therapeutic purposes is its insolubility in water and consequently its poor bioavailability. Investigators have demonstrated [5] no detectable CU or CU metabolites in the blood or urine after patients with advanced colorectal cancer were administered 440–2200 mg of curcuma extract *per* day (36–180 mg of CU) for up to 29 days. Others have [6] shown that the peak concentration of CU in the serum following administration of 4, 6 and 8 g of CU were 0.51, 0.64 and 1.77 μM, respectively. These authors also found that doses below 4 mg were barely detectable. Lao *et al.* [15] report finding no CU in the serum of volunteers given 0.5, 1.0, 2.0,

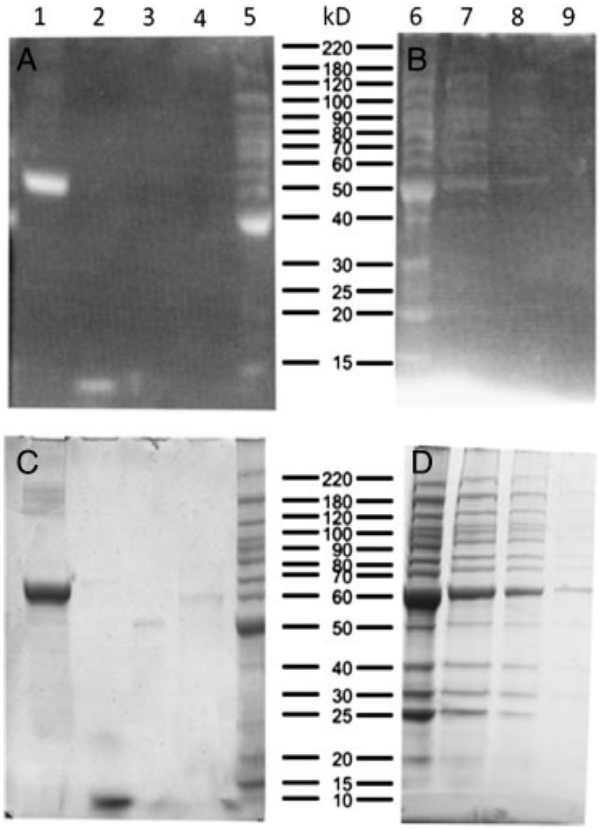

Figure 3. SDS-PAGE analysis of proteins stained by CU and CBB. (A) Lane 1 – BSA; lane 2 – Ro 273 MAP; lane 3 – La; lane 4 – Ro 60; lane 5 – unstained molecular weight standards stained with CU. (B) Lane 6 – unstained molecular weight standards (20 μL) stained with CU; lane 7 – unstained molecular weight standards (10 μL) stained with CU; lane 8 – unstained molecular weight standards (5 μL) stained with CU; lane 9 – unstained molecular weight standards (2.5 μL) stained with CU. (C) Same as (A) but stained with CBB. (D) Same as (B) but stained with CBB.

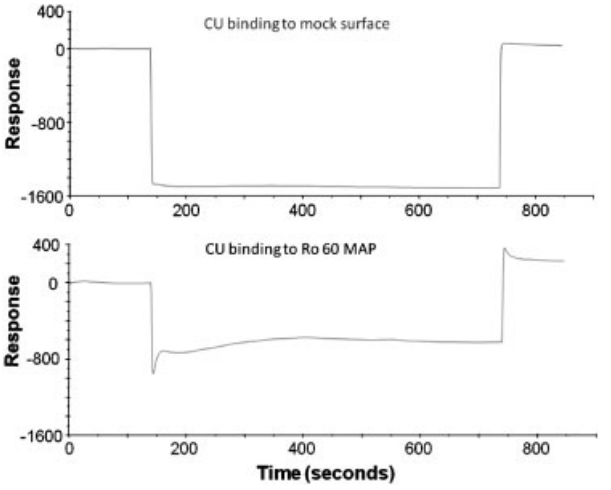

Figure 4. SPR studies of CU binding to Ro MAP 273. Top: CU analyzed over a mock sensor surface. Bottom: CU analyzed over Ro MAP 273 coupled to the sensor surface. The dip in the sensorgram seen as CU passes over the sensor surface is owing to the differential refractive index of the heat-solubilized CU compared with the PBST used as the running buffer.

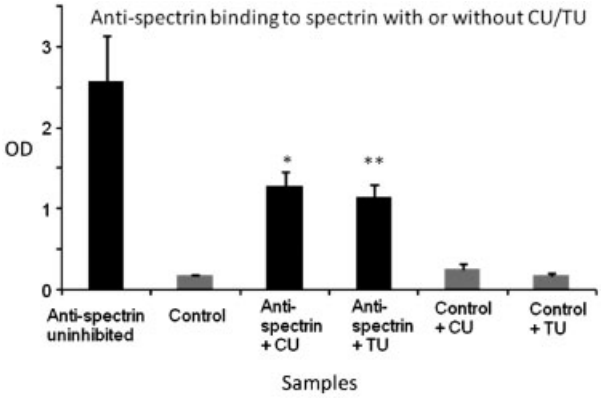

Figure 5. Antispectrin antibodies binding to solid-phase human spectrin in the presence or absence of CU or TU. Samples used are given on the *x*-axis while optical density is given on the *y*-axis. Sample 1: Antispectrin antibodies binding to spectrin in the absence of CU or TU. Sample 2: Control antibody binding to spectrin. Sample 3: Antispectrin binding to spectrin in the presence of CU. Sample 4: Antispectrin binding to spectrin in the presence of TU. Sample 5: Control antibody binding to spectrin in the presence of CU. Sample 6: Control antibody binding to spectrin in the presence of TU. * $p = 0.0043$; ** $p = 0.0025$.

4.0, 6.0, or 8.0 g CU. However, these authors found that CU levels reached 50.5 and 51.2 ng/mL sera by 4 h in two subjects administered 10 and 12 g of CU, respectively. Yet another study [9] showed that only about 22–41 ng/mL were detectable in plasma even when 8 g CU/day was given orally. Consequently, any method that aims to improve CU's solubility in water would be immensely useful to investigators attempting to find therapeutic advances to several debilitating and terminal illnesses.

We have shown a 12-fold increase in solubility of CU and a threefold increase in the solubility of TU by boiling a solution of CU/TU in water for 10 min. Profiling of the heat-extracted CU with matrix assisted laser desorption ionization mass spectrometry and spectrophotometry (400–700 nm) indicated no heat-mediated disintegration of CU [13, 16]. By using an ELISA that involved HNE modification of a solid-phase antigen substrate [17], the heat-solubilized CU was found to inhibit HNE-protein modification by 80%. We have also shown that CU solubilized with mild alkali-inhibited HNE-protein modification significantly [18]. Thus, inhibition of HNE modification may be a mechanism by which CU exerts its effect in many disorders [13, 18].

We believe that one solution to this bioavailability problem would be to increase the solubility of CU before oral administration to patients. Thus, heat-solubilized CU should be considered in clinical trials involving CU since

CU's full pharmacological potential is limited owing to its extremely limited water solubility [19].

An earlier study showed that 90% of CU dissolved in 0.1 M phosphate buffer (pH 7.2) was broken down in 30 min [20]. We stored the heat-solubilized CU or TU at 4°C for 12 or 72 h and measured the optical density at 405 nm as described earlier [13] following centrifugation at 16 000 × *g* [13]. The level of heat-solubilized CU was found to decrease only 47% in 12 h and 67% in 72 h. However, there was only a 17 and 25% decrease in the corresponding TU samples.

Barik *et al.* [21] showed that CU binds very strongly to HSA, with binding constants in the order of 10^4–10^5/M, thus raising the possibility that it could be used as a carrier for CU *in vivo*. Recently Liu *et al.* [22] demonstrated that CU binds to the CDRs of Fab of intravenous Ig, with binding constants of only 10^6–10^8/M raising the possibility that intravenous Ig could also serve to transport CU. Since albumin binds stronger to CU compared with intravenous Ig, there is a competition for binding between albumin and intravenous Ig.

Studies in humans and animal models have shown that CU ameliorates autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, psoriasis and inflammatory bowel disease by regulating inflammatory cytokines such as IL-1beta, IL-6, IL-12, TNF-α and IFN-ã and associated JAK-STAT, AP-1 and NF-κB signaling pathways in immune cells [7]. Our results show that inhibition of antibody–antigen interaction could be another mechanism for ameliorating autoimmune disorders, along with CU's ability to inhibit oxidative modification of proteins.

Oxidative damage and oxidative modification of proteins have been implicated in SLE and other autoimmune disorders [23, 24]. By binding to proteins CU might prevent the oxidative modification of proteins induced by oxidative stress. CU solubilized by heat in water [13, 16, 19, 25–27] is a plausible reagent for use in both *in vitro* and *in vivo* experiments as opposed to the use of CU solubilized in DMSO [28] the most commonly used solubilizing agent.

Investigators have recently used CU solubilized in DMSO (final concentration of 0.1%) to show that it could attenuate the toxicity of acrylamide on HepG2 cells [29]. DMSO has been used as a solvent for chemotherapeutic drugs and it has been used to treat rheumatic, pulmonary, gastrointestinal, neurological, urinary and dermatological disorders owing to its anti-inflammatory properties [30]. The effects of DMSO on the outcomes of such studies are not completely clear yet. The DMSO levels reported to be safe varies considerably. Adverse effects of DMSO on the neuronal system have been reported. DMSO has been shown to induce apoptosis in a widespread manner in developing mouse cells at all ages tested, as well as induce neuronal loss at 0.5 and 1% [31]. Investigators have shown that DMSO accumulated in brain and was found to increase the metabolic rate. There is no practical concentration of DMSO that can be used in metabolic experiments without effect [32]. Our approach of heat solubilizing CU in water appears to be a simple and safe solution for overcoming the solubility problem associated with this polyphenol.

We obtained higher inhibition with TU extract than with the purified CU suggesting that some curcuminoid is lost in the purification process. Heat-solubilized CU/TU could prove useful as a therapeutic intervention in SS or SLE to suppress autoantibody–antigen interaction, to inhibit oxidative damage and thus reduce severity of disease manifestation. In addition, heat-solubilized CU/TU could be used to stain proteins on SDS-PAGE gels as an alternative to heat-mediated staining of proteins with Coomassie [33]. The advantage here is that CU is non-toxic, although it is less sensitive than Coomassie staining.

There are some limitations to this study. First, the results reported here are based on *in-vitro* experiments. Plans are afoot to study the *in vivo* effects of heat-solubilized CU/TU in our experimental animal model of SS and possibly in spontaneous animal models of SLE. This will enable us to determine if the administration of heat-solubilized CU would increase bioavailability. If heat-solubilized CU does increase bioavailability it would be of interest to see if it would suppress autoimmunity along the line seen with induction of tolerance with oral feeding of Ro 60 autoantigen in experimental SS [34].

Second, the implications of the more or less promiscuous binding of CU by proteins may extend beyond the interference of antibody–antigen interactions, protein gel staining or intravenous Ig. It is possible that this could affect antibody interactions to non-self antigens, clearance of pathogens by antibodies, response to vaccination, or response to therapeutic antibodies employed in cancer or autoimmune diseases. The encouraging aspect of studies with non-heat-solubilized CU thus far (up to 12 g/CU/day in some studies) is that most studies show highly significant beneficial effects [5–9, 13, 15, 18, 20, 22, 35, 36] without any significant adverse effects. We hypothesize that heat-solubilized CU/TU would behave in this manner as well without significant adverse side effects as opposed to using DMSO.

Since Ro 60 autoantigen, is a major target of autoantibodies in patients suffering from rheumatic diseases [4, 10, 11, 23], we hypothesize that the use of heat-solubilized TU/CU (especially in times of flare in disease) may be a better therapeutic approach compared with non-heat-solubilized TU/CU (CU is practically insoluble in water maintained at room temperature) to ameliorate these diseases. The result of this study shows that heated CU retains its ability to bind autoantigens. In addition, it will be uncomplicated to administer specific quantities of heat-solubilized CU/TU in cooked food (especially omelette, since albumin binds CU strongly) or in infusions (after cooling and centrifugation or filtration to remove insoluble CU) to patients with autoimmune diseases.

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The authors have declared no conflict of interest.

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