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Stable and Potent Analogues Derived from the Modification of the Dicarbonyl Moiety of Curcumin

Soumyananda Chakraborti,[†] Gopa Dhar,[†] Vishnu Dwivedi,[‡] Amlan Das,[§] Asim Poddar,[†] Gopal Chakraborti,[§] Gautam Basu,[∥] Pinak Chakrabarti,[†] Avadhesha Surolia,*[,⊥] and Bhabatarak Bhattacharyya*[,†]

[†]Department of Biochemistry, Bose Institute, P-1/12 CIT Scheme VIIM, Kolkata 700054, India

[‡]Centre for Molecular Medicine, National Institute of Immunology, New Delhi 110012, India

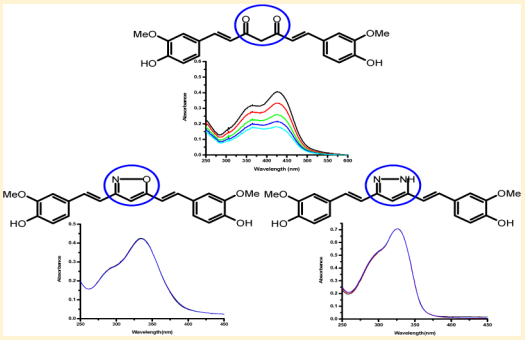
[§]Dr. B. C. Guha Centre for Genetic Engineering and Biotechnology, University of Calcutta, Kolkata 700019, India

[∥]Department of Biophysics, Bose Institute, P-1/12 CIT Scheme VIIM, Kolkata 700054, India

[⊥]Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India

Supporting Information

ABSTRACT: Curcumin has shown promising therapeutic utilities for many diseases, including cancer; however, its clinical application is severely limited because of its poor stability under physiological conditions. Here we find that curcumin also loses its activity instantaneously in a reducing environment. Curcumin can exist in solution as a tautomeric mixture of keto and enol forms, and the enol form was found to be responsible for the rapid degradation of the compound. To increase the stability of curcumin, several analogues were synthesized in which the diketone moiety of curcumin was replaced by isoxazole (compound **2**) and pyrazole (compound **3**) groups. Isoxazole and pyrazole curcumins were found to be extremely stable at physiological pH, in addition to reducing atmosphere, and they can kill cancer cells under serum-depleted condition. Using molecular modeling, we found that both compounds **2** and **3** could dock to the same site of tubulin as the parent molecule, curcumin. Interestingly, compounds **2** and **3** also show better free radical scavenging activity than curcumin. Altogether, these results strongly suggest that compounds **2** and **3** could be good replacements for curcumin in future drug development.



A large number of dietary agents from plant sources possess excellent medicinal properties. Curcumin, a yellow-colored compound isolated from the rhizomes of *Curcuma longa*, has been used for centuries as a dietary pigment, spice, and traditional medicine. It has been characterized as possessing a wide range of medicinal properties such as antibacterial, antifungal, antiviral, anti-inflammatory, and antiproliferative properties.[1,2] The question of how a single molecule prevents and acts as a medicine for so many different diseases, from bacterial infection to different types of cancers, has arisen. Although the mechanism of curcumin action is not well established, there are reports that demonstrate directly or indirectly that curcumin binds multiple proteins,[2] transcription factors,[3] co-activators and corepressors, etc.[4] Therefore, it appears that curcumin does not follow the single drug−single receptor concept when examined in cell studies. There are multiple targets for curcumin in cells, and multiple mechanisms of action are employed.[2,5] This recognition of multiple targets by curcumin in cells may give rise to nonspecific interactions, which in most cases are responsible for the toxicity of a drug. Surprisingly, unlike most chemotherapeutic agents, curcumin shows no toxicity, and it is believed that this unique property of curcumin is due to its cytotoxic activity being confined to cancer cells only.[6,7] It has been established that curcumin inhibits tumorigenesis by suppressing oncogenic cell proliferation, inducing apoptosis, and arresting cell cycle progression.[8,9]

The diverse bioactivities and unprecedented nontoxicity displayed by curcumin have made curcumin a subject of intense scientific scrutiny.[2,10] Unfortunately, *in vitro* studies of curcumin are severely limited because of (a) its rapid decomposition in presence of light and under solution conditions (pH and reducing agents) and (b) its low solubility and poor bioavailability.[10−12] An acute problem with curcumin is its poor solubility in an aqueous solution (20 μg/mL), which poses a severe limitation on the achievable concentration in a biological system. The solubility and bioavailability are interrelated, and studies have also shown that poor oral absorption is responsible for such a low bioavailability.[10] Curcumin is lipophilic in nature, and therefore, both the solubility and the bioavailability could be enhanced upon complexation with

Received: June 10, 2013
Revised: September 19, 2013
Published: September 24, 2013

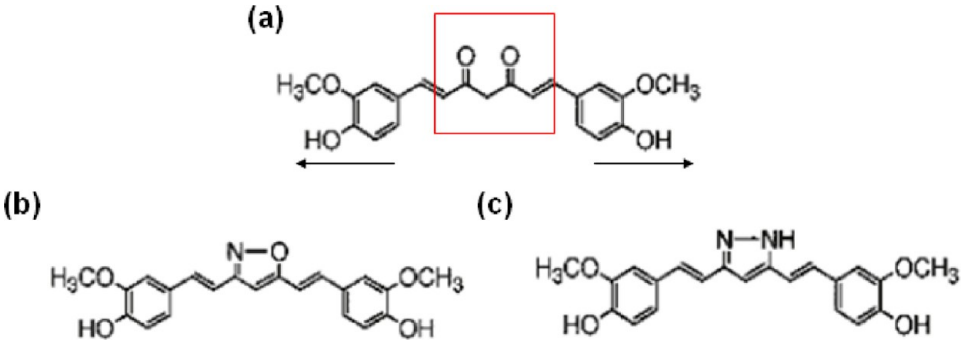


Figure 1. Chemical structures of (a) curcumin, (b) compound **2**, and (c) compound **3**.

cyclic oligosaccharide, cyclodextrin, and composite nanoparticles.[13−15] In one such study, black pepper alkaloid piperine (bioperine) was used to increase the bioavailability of curcumin.[16]

Curcumin undergoes rapid decomposition in the presence of light, in a physiological buffer solution (pH >7.4), and with the most frequently used reducing agents in biological samples. The chemical structure of curcumin has two *o*-methoxy phenols attached to two terminal positions symmetrically through an $\alpha\beta$-unsaturated β-diketone linker.[17] The molecule exhibits keto−enol tautomerism in solution under physiological pH conditions.[12,14,17] Evidence from both *in vitro* and *in vivo* studies showed that the enolic -OH moiety is responsible for the instability of curcumin.[11,12] It was reported previously that the presence of the β-diketone moiety may be essential for the biological activity of curcumin.[18,19] However, recent studies reported that curcumin derivatives without the β-diketone retained their antiproliferative activities.[20,21]

In our previous work[22] on structure−function studies of curcumin−tubulin interaction, we reported that curcumin analogues without a dicarbonyl moiety such as isoxazole or pyrazole can bind tubulin and inhibit tubulin self-assembly *in vitro*. Further studies of the mechanism of action of these new curcumin analogues led us to discover that they are very stable in light and under different pH conditions, including alkaline pH and reducing agents.

This investigation has been initiated with some specific questions in mind. (1) Is curcumin stable in a reducing environment? (2) Does any structural modification in curcumin make the molecule stable over varying pH and reducing conditions? (3) Do substituted curcumin analogues share the same molecular (protein) target with curcumin? To answer these, we have synthesized series of curcumin analogues and checked their stability and antioxidant activity using various spectroscopic methods. On the basis of the display of favorable properties, compounds **2** and **3** (Figure 1) were further screened for *in vitro* anticancer activity using serum-free cell culture medium, and eventually their *in vitro* molecular target was identified. Finally, an overall comparison has been made among compound **2**, compound **3**, and curcumin. Our results clearly suggest that compounds **2** and **3** are promising curcumin analogues that may find useful application (alone or combined with other anticancer drug) in anticancer therapeutics.

EXPERIMENTAL PROCEDURES

Materials. Curcumin (from *C. longa*, or turmeric), bovine serum albumin (97−99%, agarose gel electrophoresis), lysozyme, and lactalbumin were obtained from Sigma-Aldrich. TCEP [tris(2-carboxyethyl)phosphine] and DTT (dithiothreitol) were purchased from Sigma. Phosphate buffer solutions (50 mM) were prepared with neat water from a Millipore Milli-Q NANO pure water system, and the pH was adjusted to 7.4 with HCl. All other reagents were analytical grade and purchased from local vendors. Compounds **2** and **3** were prepared using a previously described protocol.[22,23]

UV−Visible Absorption Spectra of Curcumin. Absorbance readings were taken from 250 to 600 nm using a Shimadzu (UV−vis) spectrophotometer. In the experiments in which the degradation of curcumin was recorded, the UV−vis absorption spectra were collected for 20 min at 5 min intervals. Stock solutions of BSA, lysozyme, and lactalbumin, were prepared in phosphate buffer (pH 7.4). A stock solution of 10 mg/mL curcumin in DMSO (dimethyl sulfoxide) was prepared, and from that pool, a small quantity of curcumin was added to the reaction vessel to achieve a final concentration of 20 μM. Stock solutions of TCEP and DTT were prepared by simply dissolving each compound in water.

Tubulin Isolation and Estimation. Microtubular proteins were isolated from goat brains by two cycles of a temperature-dependent assembly−disassembly process. Pure tubulin was isolated from microtubular proteins by two additional cycles of temperature-dependent polymerization and depolymerization using 1 M glutamate buffer for assembly.[24] The assembly buffer consisted of 50 mM PIPES (pH 6.9), 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.5 mM GTP. The protein was stored at −70 °C. The protein concentration was determined by the Lowry method using bovine serum albumin as a standard. Tubulin preparations used in this study contained a natural mixture of isoforms.[25] Both calorimetry and fluorescence measurements were taken with this unfractionated tubulin, and therefore, the binding parameters obtained here are averages for the different isoforms.

Determination of Dissociation Constants. Dissociation constants (K_d) were estimated by titrating tubulin with compounds **2** and **3** and bovine serum albumin (BSA) with curcumin. The fluorescence spectra (λ_{ex} = 295 nm) of the protein−ligand mixtures were then analyzed using the equation[26]

$$F(Q_T) = F_0 + (F_\infty\text{-}F_0)\left\{\left[(K_d nC_0 + Q_T) - \sqrt{(K_d + nC_0 + Q_T)^2 - 4nC_0Q_T}\right]\Big/\left(2nC_0\right)\right\} \quad (1)$$

where F is a composite fluorescence intensity (defined later), subscripts 0 and ∞ denote ligand-free and ligand-saturated conditions, respectively, C_0 is the concentration of the protein,

dx.doi.org/10.1021/bi400734e | *Biochemistry* 2013, 52, 7449−7460

n is the number of binding sites, and Q_T is the total ligand concentration. Fluorescence spectra were recorded using a Hitachi F-3000 fluorescence spectrophotometer in a 0.5 cm path-length quartz cuvette. Unless otherwise stated, the raw fluorescence intensities were corrected for the inner filter effect using the equation[27]

$$F_{corr} = F_{obs}\ \text{antilog}[(A_{EX} + A_{EM})/2] \quad (2)$$

where F_{corr} and F_{obs} are the corrected and observed fluorescence intensities, respectively, and A_{EX} and A_{EM} are the absorbances at the excitation and emission wavelengths, respectively.

Molecular Modeling of the Compound 2−Tubulin and Compound 3−Tubulin Complexes. The crystal structure of $\alpha\beta$-tubulin (PDB entry 1SA0)[28] was utilized as template for docking studies. The energy-minimized three-dimensional atomic coordinates of compounds **2** and **3** were generated using corina (http://www.molecular-networks.com/products/corina). Docking models were obtained using PatchDock.[29] As compounds **2** and **3** are derivatives of curcumin and inhibit tubulin polymerization in a manner similar to that of curcumin, we hypothesized that they share the same binding site as curcumin, and hence, we modeled them in the curcumin binding site of tubulin. PyMol (http://www.pymol.org) was used for visualization.

Cell Culture and Maintenance. Human lung epithelium adenocarcinoma cells (A549) were maintained in DMEM supplemented with 1 mM L-glutamine, 10% fetal bovine serum, 50 μg/mL penicillin, 50 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were grown in tissue culture flasks until they were 80% confluent, followed by trypsinization with 1× trypsin and splitting. The morphology of control and treated cells was observed by an Olympus inverted microscope (model CKX41).

Cell Proliferation Inhibition Assay (MTT assay). The viability of A549 cells in the presence of varying concentrations of compounds **2** and **3** was assessed by an MTT assay.[30] Cultured A549 cells were grown in 96-well culture plates (1×10^4 cells per well). The cells were then treated with different concentrations of curcumin (0−50 μM), compound **2** (0−50 μM), and compound **3** (0−50 μM) and incubated in serum-free medium for 48 h. After incubation, 50 μL of an MTT (2 mg/mL) solution in PBS was added to each well. This was incubated until purple precipitate was visible. The absorbance was measured on an enzyme-linked immunosorbent assay reader (MultiskanEX, Lab systems, Helsinki, Finland) at a test wavelength of 570 nm and a reference wavelength of 650 nm.

Analysis of Apoptosis by Flow Cytometry. Apoptosis was measured using flow cytometry by annexin V (1 μg/mL) and propidium iodide (PI) (0.5 μg/mL) double staining following the method discussed in ref 30. Cultured A549 cells were grown to confluence (1×10^6) and incubated with different concentrations of curcumin (0−20 μM), compound **2** (0−20 μM), and compound **3** (0−20 μM) for 48 h in serum-free DMEM. Cells were then stained for 15 min at room temperature in the dark with fluorescein isothiocyanate (FITC)-conjugated annexin V (1 μg/mL) and propidium iodide (PI) (0.5 μg/mL) in a Ca^{2+}-enriched binding buffer. Analysis of apoptosis was performed using the Becton Dickinson FACS Caliber instrument, and the data were analyzed using Cell Quest from Becton Dickinson.

Preparation of Samples for Confocal Microscopy. Samples for confocal microscopy were prepared according to the protocol suggested by Das et al.[31] Cultured A549 cells were grown until the density reached 10^6 cells/mL and then treated with different concentrations of curcumin (0−20 μM), compound **2** (0−20 μM), and compound **3** (0−20 μM), for 24 h, under serum-free conditions. Subsequently, cells were washed twice with PBS, fixed with 2% paraformaldehyde, and incubated with a permeable solution (0.1% sodium citrate and 0.1% Triton) for 1 h. Cells were then mildly washed with PBS, and the nonspecific binding sites were blocked by incubating the cells with 5% BSA. Cells were then incubated with the anti-mouse monoclonal anti-α-tubulin antibody (1:200 dilutions, Sigma) followed by the anti-mouse rhodamine-conjugated IgG antibody (1:150 dilutions, GeNi) and DAPI (1 μg/mL). After incubation, cells were washed with PBS and viewed under a Zeiss LSM 510 Meta confocal microscope.

Antioxidant Activity of Curcumin (DPPH radical scavenging activity). The antioxidant activities of curcumin analogues were determined in terms of radical scavenging activity using the stable radical DPPH [di(phenyl)(2,4,6-trinitrophenyl)iminoazanium]. Variable amounts (10, 25, 50, and 100 μM) of each compound were taken separately, and 37.5 μL of a freshly prepared methanolic solution of DPPH (1 mM) was added to each of these solutions to make the final volume 750 μL. The samples were incubated for 15 min (in dark) followed by absorbance measurement. The decrease in absorbance at 517 nm was determined using a Shimadzu spectrophotometer. Methanol was used in this experiment as a blank. The absorbance of the DPPH radical without antioxidant, i.e., the control, was measured daily, and the concentration was calculated by applying the method described by Ferrari et al.[32]

RESULTS

Curcumin is an interesting molecule possessing palindromic structure with a dicarbonyl moiety at its center. The UV−visible absorption spectrum of curcumin displays an intense peak with an absorption maximum close to 427 nm, as shown in Figure 2a. Also, there is a shoulder around 350 nm. With time, the intensity of the spectra decreases significantly in phosphate buffer (pH 7.4); indeed, within 20 min of its incubation in phosphate buffer, curcumin lost more than 50% of its original intensity. Our observation was similar to the findings of Leung et al.[11] Dithiothreitol [DTT (Figure S1 of the Supporting Information)] has long been used as an effective reducing agent with a redox potential of −0.33 V at pH 7. Previously, Aaggarwal et al. have reported that upon treatment of DTT, curcumin loses its biological function.[33] They have also reported that DTT itself has no effect on TNF-induced NF-κB (a transcriptional activator and a key regulator of inflammatory signaling pathway) activation; however, the presence of DTT significantly reversed the inhibitory effects of curcumin upon NF-κB activation.[33] In a separate study, Jutooru et al. have found a similar effect of DTT.[34] These results surprised us because in both the cases a sufficient amount of serum was present in the medium yet curcumin failed to show its anticancer and anti-inflammatory activity. This observation has motivated us to investigate in molecular terms the effect of DTT on curcumin activity as most cellular compartments are reducing in nature. Our studies show that even a minute quantity of DTT is sufficient to accelerate the degradation of curcumin in buffer (Figure 2b). Further, it has been found that in the presence of DTT >80% of curcumin degradation occurs within the first 15 min of incubation.

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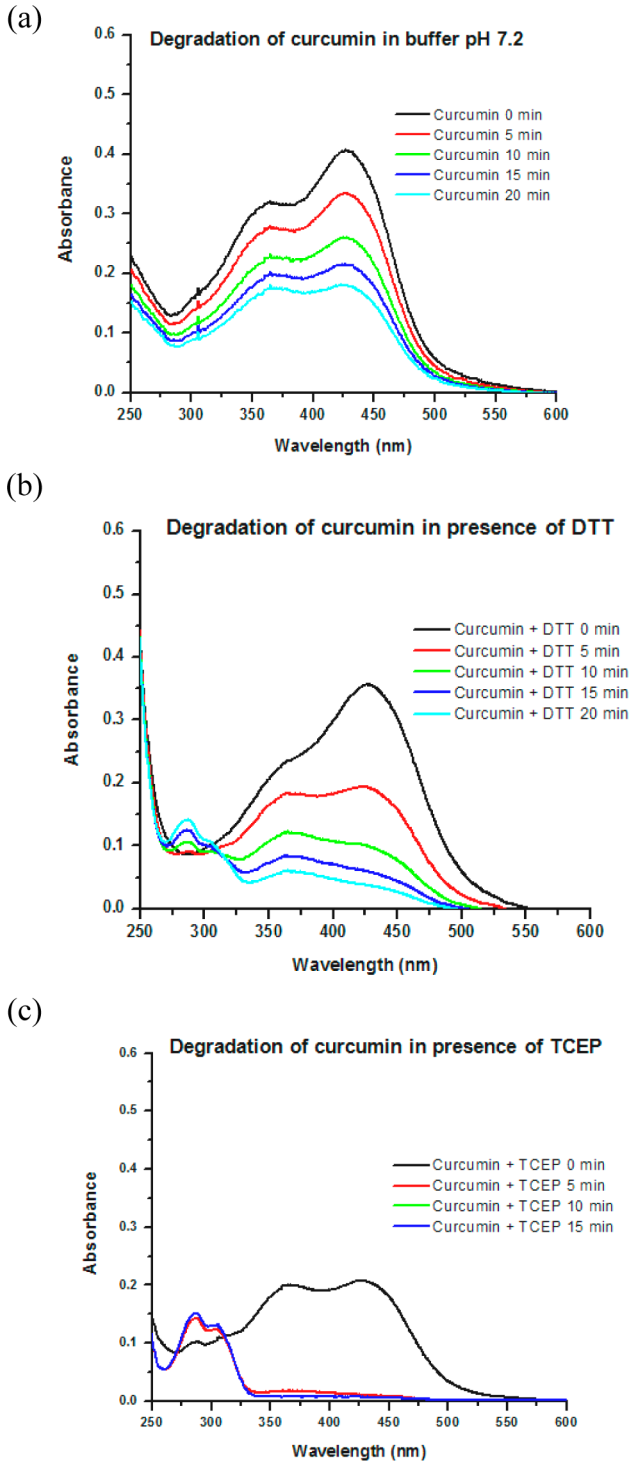

Figure 2. UV−vis absorption spectra of (a) curcumin in phosphate buffer (pH 7.4) and in the presence of (b) DTT and (c) TCEP.

Although DTT has been used as a reducing agent for a long time, it has some limitations; the molecule is prone to aerial oxidation, and as a result, it becomes ineffective after a certain period of time. Compared to DTT, TCEP [tris(2-carboxyethyl)phosphine (Figure S1 of the Supporting Information)] is a much stronger reducing agent and can withstand the aerial oxidation process.[35] The stability of curcumin was checked in the presence of TCEP. Interestingly, we observed a rapid degradation (much faster compared to that in the presence of DTT) of the molecule. This observation clearly suggests that the reduction potential of the medium is directly related to curcumin stability. It has been found that within 10 min of TCEP incubation, curcumin loses its spectral signature (Figure 2c); the rate of degradation is much faster with TCEP compared to that observed with DTT.

Effect of Different Proteins on Curcumin Stability. Experimental evidence has suggested that human serum albumin (HSA) and fibrinogen (both of which belong to the serum protein family) provide protection to curcumin from alkaline hydrolysis.[11] However, transferrin and immunoglobulin (which also belong to the same protein family) do not give that kind of shield.[11] Structural analysis has revealed that both HSA and fibrinogen possess a larger number of surface hydrophobic patches than the other two proteins. To verify whether surface hydrophobicity has any role in stabilizing curcumin, we selected three proteins with an increasing order of surface hydrophobicity. Among these proteins, α-lactalbumin has the highest surface hydrophobicity, followed by bovine serum albumin (BSA, which is a structural homologue of HSA with a >75% identical sequence) and then lysozyme. Surprisingly, we found that curcumin, bound to α-lactalbumin, is unstable and its degradation rate is comparable to that of lysozyme-bound curcumin (data not shown), whereas curcumin, bound to BSA, is exceptionally stable (Figure 3a). The difference in the stability of curcumin in the presence of lysozyme (or lactalbumin) and BSA may be explained by their differential binding affinities. The dissociation constant (K_d) of the curcumin−BSA complex was determined to be 1.0 ± 0.4 μM (Figure S2 of the Supporting Information), whereas the binding affinity of curcumin for lysozyme is ∼2 orders of magnitude lower (K_d = 833 μM),[36] indicating that a strongly bound curcumin in BSA leads to an increased stability. As we had already noted that DTT and TCEP accelerate the degradation of curcumin, we studied the effect of DTT and TCEP on the stability of BSA-bound curcumin. It was found that in the presence of DTT curcumin (bound to BSA) begins to degrade, and albumin is not capable of protecting the molecule (Figure 3b).

Search for a Stable and Potent Curcumin Analogue. To synthesize stable curcumin analogues, it is essential to know why curcumin is unstable. Solution studies at different pH values indicate that the β-diketone moiety of curcumin may result in its instability. On the basis of this hypothesis, Zhao et al.[37,38] have synthesized several monocarbonyl analogues of curcumin with enhanced stability. Structure−function studies indicate that curcumin needs both terminal methoxy phenyl groups for binding to tubulin. However, the half-curcumin molecule containing a monocarbonyl group does not bind tubulin, indicating that curcumin is a bifunctional ligand.[22] In compounds **2** and **3**, the diketone group of curcumin is replaced with an isoxazole or pyrozole ring; interestingly, even with this structural change, the molecules retained their antimitotic activity.[22] Curcumin stability is directly related to the presence of the β-diketone group, and our hypothesis is that if we can replace the β-diketone group with an isoxazole and pyrazole group the stability of the modified curcumin increases because the highly stable aromatic moiety does not break down upon deprotonation of the less acidic C_4-H. To prove our hypothesis, we have synthesized (1*E*,6*E*)-4,4-dimethyl-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-diones (4,4-dimethyl analogue of curcumin) and we observed that the compound is extremely stable under physiological pH and

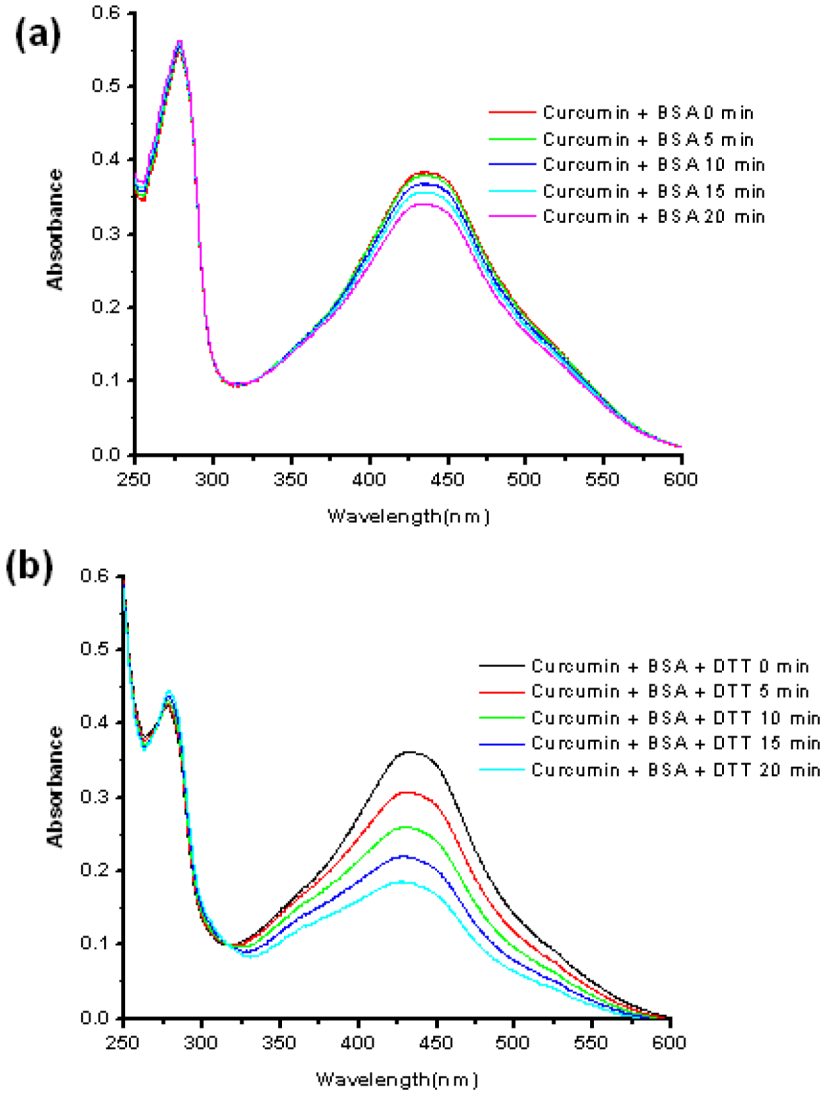

Figure 3. UV−visible absorption spectra of curcumin bound to (a) BSA and (b) BSA in the presence of DTT.

reducing conditions. Unfortunately, this compound was found to be inactive in inhibiting tubulin polymerization (data not shown). To prove our claim further, we have measured the stability of compounds **2** and **3** under physiological pH and reducing conditions. We observe that these molecules are stable at different pH conditions, including alkaline pH (data not shown). Interestingly, there is no significant degradation observed even after incubation for 1 h at physiological pH. To check the stability of compounds **2** and **3** under reducing conditions, we incubated these compounds with DTT and found no appreciable change in the absorption spectrum over time (Figure 4a,c). Finally, we repeated the same experiment with TCEP, as TCEP is a much stronger reducing agent than DTT; with TCEP, we also did not observe any appreciable change in the absorption spectrum (Figure 4b,d).

Fluorescence Properties of Compounds 2 and 3 and Their Binding Affinities for Tubulin. Fluorescence emission maxima of curcumin and compounds **2** and **3**, bound to tubulin, are around 500, 410, and 380 nm, respectively.[22] In all the cases, there is a slight blue shift relative to the free ligand fluorescence (with values of 510, 415, and 390 nm, respectively). It may be noted that the absorption maxima of the three ligands are around 427, 333, and 327 nm, respectively.[22] Thus, the Stokes shift, which indicates the difference in wavelength between the absorption and emission spectra, is quite similar for all three compounds (~70 nm). In addition, all three compounds exhibit a slight blue shift (5−10 nm) upon binding of tubulin, which is expected if the environment of the bound state is more hydrophobic than the aqueous environment.

On the basis of our earlier studies, we inferred that these compounds might share a common target (tubulin) as all of them inhibit tubulin polymerization in a similar manner. However, their exact mode of binding and the binding affinity for tubulin were not known. Binding of compound **2** was studied by monitoring the quenching of the intrinsic fluorescence of tubulin (λ_{ex} = 295 nm; λ_{em} = 330 nm), after the appropriate correction for the inner filter effect using eq 2 (compound **2** absorbs at 295 and 330 nm). The F(330 nm)/F_0(330 nm) ratio (F in eq 1), as a function of the total concentration of compound **2**, is shown in Figure 5a. The best fit to eq 1 yielded n = 1 (single binding site) and K_d = 5.9 ± 1.5 μM. In addition to absorbing at 295 and 330 nm, compound **3** also fluoresces significantly at 330 nm, precluding any reliable monitoring of the quenching of the intrinsic fluorescence of tubulin. Instead of intrinsic tubulin fluorescence, we monitored

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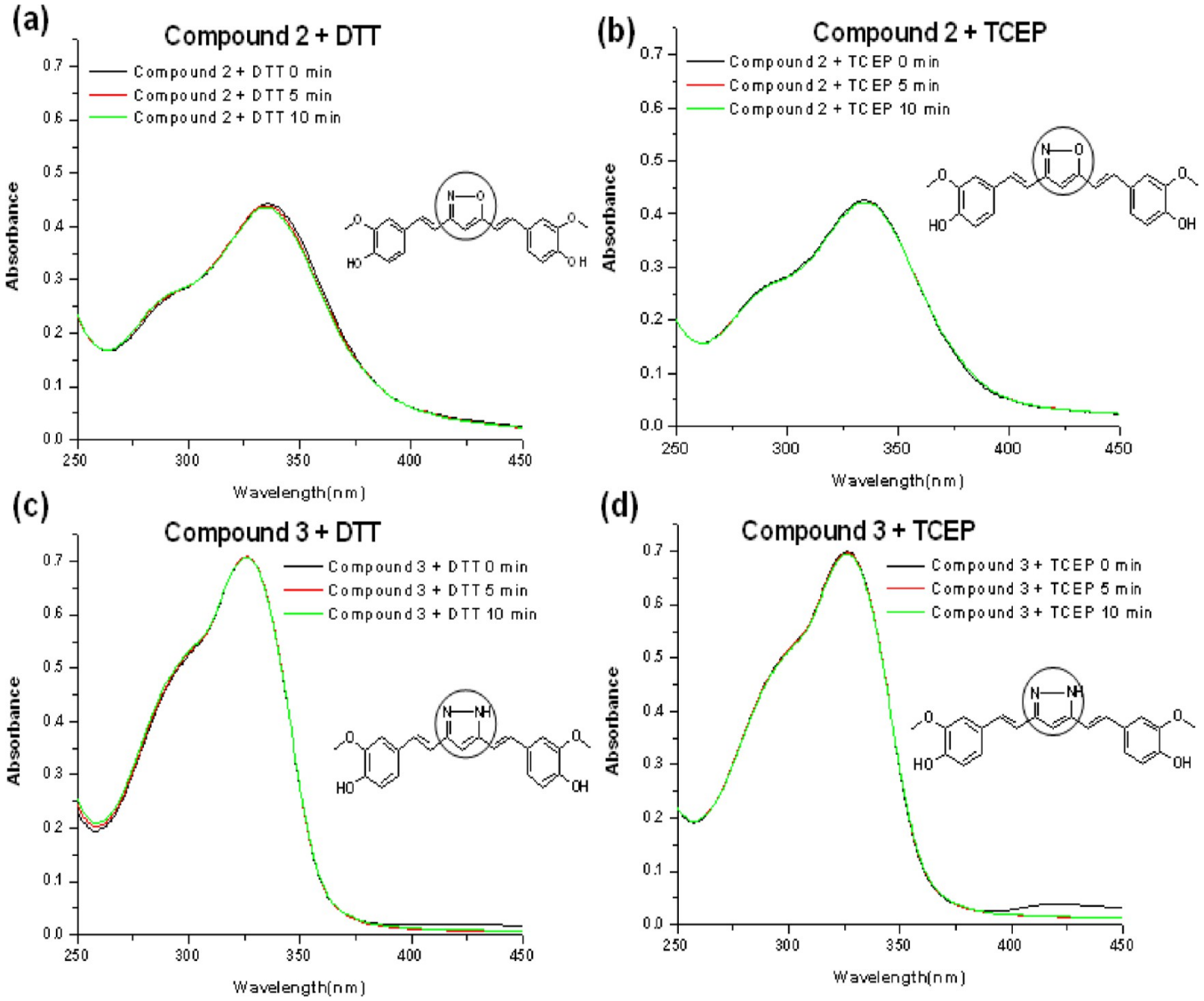

Figure 4. UV−visible absorption spectra of compound **2** in phosphate buffer (pH 7.4) in the presence of (a) DTT and (b) TCEP and compound **3** in (c) DTT and (d) TCEP.

the shape of the fluorescence spectra of compound **3** (λ_{ex} = 295 nm), as reflected in the F(365 nm)/F(380 nm) ratio (F in eq 1). Because the emission spectrum of compound **3** rides on the tail of the intrinsic tubulin emission spectrum, as the intrinsic tubulin fluorescence becomes quenched due to binding of compound **3**, the F(365 nm)/F(380 nm) ratio decreases in a manner that reflects the binding isotherm. The corresponding data and best fit to eq 1 are shown in Figure 5b, yielding n = 1 (single binding site) and K_d = 2.7 ± 0.2 μM. The affinity of compounds **2** and **3** for tubulin is similar to that of curcumin, as judged from the reported value of K_d (2.0 μM) for the curcumin−tubulin complex.[22] Using a molecular modeling technique, we then identified the binding sites of compounds **2** and **3** in tubulin, which is the same site as that of curcumin (interdimer interface) (Figure 6). In short, our results indicate that in spite of substitution (replacement of the diketone group with isoxazole and pyrazole) compounds **2** and **3** recognize tubulin in a manner (similar binding affinity) almost identical to that of curcumin. This information would be useful for designing a new generation of curcumin derivatives with higher stability.

Inhibition of the Proliferation of A549 Cells by Curcumin and Compounds 2 and 3 under Serum-Free Conditions. Our *in vivo* work demonstrated that compounds **2** and **3** are relatively stable compared to curcumin even in the absence of serum albumin. To determine whether these compounds are active (*in vitro*), we applied them in cell culture medium (devoid of serum) and measured their antiproliferative activity. Normally, FBS (fetal bovine serum) is used in cell culture medium as a growth promoter. FBS binds curcumin and shields it from external water molecules. As a result, curcumin behaves as a stable molecule in its presence in the cell culture medium. As FBS gives extra stability to curcumin, we preferred a cell culture medium devoid of albumin and investigated the antiproliferative activity of different curcumin analogues over time. The dietary antioxidant curcumin and its analogues inhibited the proliferation of A549 cells; however, when those cells are incubated for 48 h under serum-free conditions, their rate of killing is different. The MTT assay shows that both compounds **2** and **3** are much more potent than curcumin in serum-starved cell culture medium (Figure 7). When the cells were treated with curcumin,

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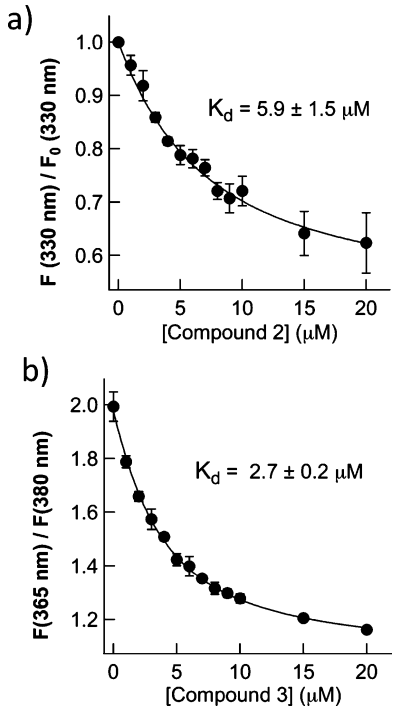

Figure 5. Determination of dissociation constants. (a) Normalized (to the fluorescence of ligand-free tubulin) fluorescence intensity of a tubulin/compound **2** mixture, monitored as a function of the concentration of compound **2** (excitation at 295 nm and emission at 330 nm). (b) Ratio of fluorescence intensity (excitation at 295 nm and emission at 365 and 380 nm) of a tubulin/compound **3** mixture as a function of the concentration of compound **3**. The solid lines indicate fits to eq 1 (best fits were obtained with $n = 1$, and corresponding K_d values are shown in each panel). All experiments were performed with 2 μM protein in 50 mM PIPES buffer (pH 7) at 25 °C. The extent of the inner filter correction for compound **2** was up to 2.34; such a correction was not needed for compound **3** as it involved monitoring the ratio of fluorescence values.

the IC_{50} (50% inhibitory concentration) value for A549 was found to be around 35 μM (Figure 7a), while those for compounds **2** and **3** were found to be 25 μM (Figure 7b,c).

Induction of Apoptosis in A549 Cells Treated with Curcumin and Compounds 2 and 3 under Serum-Free Conditions. To compare the apoptotic potentials of the analogues (compounds **2** and **3**) with the parent compound (curcumin), annexin V-FITC/propidium iodide (PI) double staining of the untreated and treated A549 cells was performed and the data were obtained flow cytometrically (Figure 8). Treatment of A549 cells with 10 and 20 μM curcumin resulted in the accumulation of 7 ± 0.5 and 16 ± 1% of the cells in the annexin V-positive region (early apoptotic), respectively, but when the A549 cells were treated with the same concentrations of compounds **2** and **3**, the apoptotic population increased to a significant level. At 10 and 20 μM compound **2**, 16 ± 2 and 31 ± 1.5% of the cells, respectively, were found to be apoptotic. Similarly, at the same concentration of compound **3**, 10 ± 0.6 and 25 ± 3% of the cells, respectively, were found to be apoptotic. Thus, the apoptotic potentials of the test compounds were found to decrease in the following order: compound **2** > compound **3** > curcumin.

Depolymerization of the Interphase Microtubules in A549 Cells Treated with Curcumin and Compounds 2 and 3 under Serum-Free Conditions. To investigate the effect of the curcumin and its analogues on the microtubule network under serum-free conditions, images of the interphase microtubules in the untreated and treated A549 cells were captured by a confocal microscope (LSM 510 Meta, Carl Zeiss, Inc.) (Figure 9). In the untreated A549 cells, regular networklike structure of the cellular microtubule was retained (Figure 9a), but when the cells were treated with 10 and 20 μM curcumin, for 24 h under the serum-free conditions, microtubules were found to be moderately depolymerized (Figure 9b,c). When the cells were further treated with similar concentrations of compounds **2** and **3**, the degree of depolymerization was found to increase by a considerable extent (Figure 9d–g). Hence, under serum-free conditions, compounds **2** and **3** were found to be much more effective than curcumin and induced a greater extent of depolymerization of the microtubule network.

Free Radical Scavenging Ability. The free radical scavenging ability of compounds **2** and **3** and curcumin was evaluated using the DPPH radical assay, which is one of the most widely used methods of determining antioxidant activity. Results, summarized in Figure 10, clearly demonstrate that compound **3** has the highest radical scavenging activity followed by compound **2** and curcumin. Previously, some studies of the antioxidant properties of curcumin[39] highlighted the importance of the transfer of a hydrogen atom from the keto to the enol moiety; however, in our case, this situation does not arise as compounds **2** and **3** cannot undergo keto–enol tautomerism to produce an enolic H atom, and we find that compound **3** has the highest antioxidant activity. It can thus be suggested that the enolic OH group is not solely responsible for the antioxidant activity of the curcumin scaffold. Previously, Selvam et al. showed that the pyrazole analogue of curcumin has a higher radical scavenging activity than curcumin.[40]

DISCUSSION

Curcumin has several limitations, which restrict its use as a potential drug for its anticancer activity and other diseases. One of the emerging problems associated with curcumin is its poor stability at physiological pH and under a reducing atmosphere of the reaction mixture. To make a stable curcumin analogue, scientists have applied different techniques, but success has remained elusive.[14,15,41,42] The literature also suggests that curcumin predominantly exists in the enol form in water and most of the known organic solvents. However, under some special circumstances, such as low pH, the keto form may also exist in equilibrium with the enol form.[14,43] The enol form is believed to be responsible for the degradation of curcumin. Although the exact mechanism of curcumin degradation is still uncertain, the accumulated evidence indicates that the presence or absence of the phenolic OH group does not play any significant role in the degradation process.[14] In its unmodified form, the β-unsaturated carbonyls in curcumin play the role of a good Michael acceptor and can undergo nucleophilic additions under biological conditions. These carbonyl groups form a diketone that exists in keto and enol tautomeric forms, with the latter being more stable both in the solid phase and in the solution phase. It can easily be deprotonated under mild alkaline conditions to yield an enolate moiety. Such facile tautomeric conversions are suspected to contribute to the rapid metabolism of curcumin. The degradation resulted in the formation of smaller phenolic compounds such as vanillin, ferulic acid, etc. Wang et al. have reported that curcumin

dx.doi.org/10.1021/bi400734e | Biochemistry 2013, 52, 7449–7460

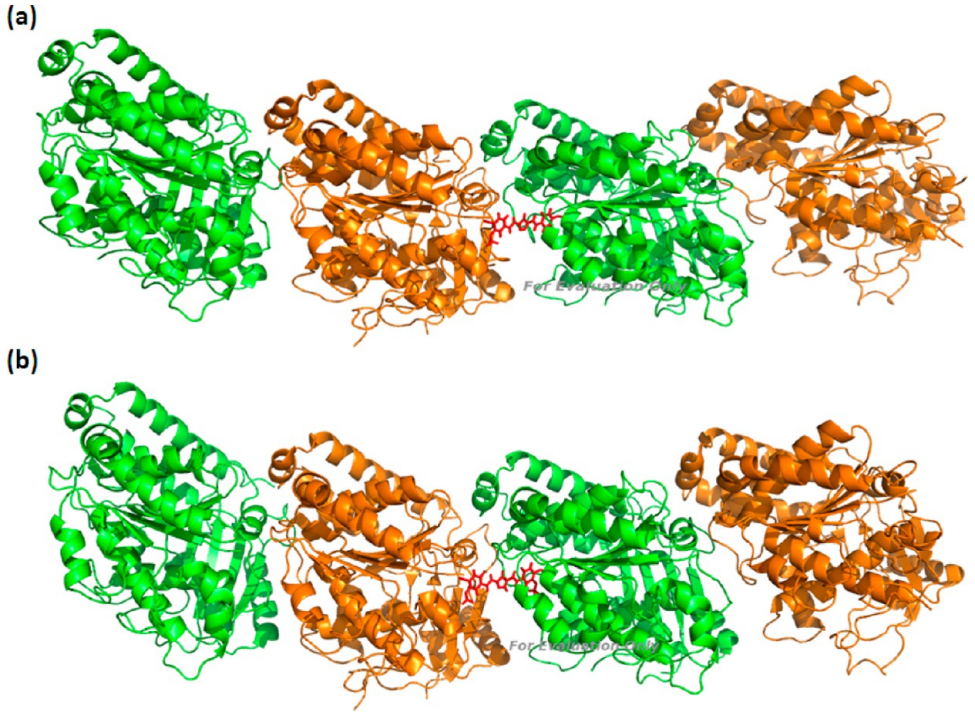

Figure 6. Molecular modeling of (a) compound **2** and (b) compound **3** bound to tubulin in the complex. The ligands (represented by red sticks) were placed between two $\alpha\beta$-heterodimers (α colored orange and β colored green).

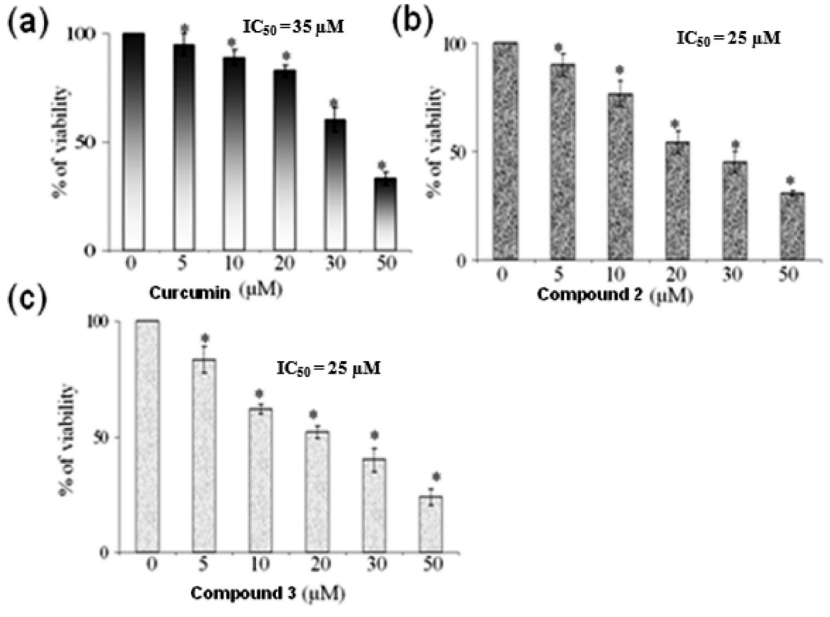

Figure 7. Inhibition of proliferation (i.e., loss of viability) of the A549 cells upon treatment with (a) curcumin, (b) compound **2**, and (c) compound **3**, under serum-free conditions. Cultured A549 cells were grown to a density of 1×10^4 cells/well in DMEM, and they were incubated with different concentrations of the compounds (0−50 μM), under serum-free conditions. Cell viability was assessed by the MTT assay, and data are represented as means ± standard error of the mean (*$P < 0.05$) (untreated cell) vs treated cells where $n = 4$.

degradation is pH-dependent.[12] They also proposed that the increased stability of curcumin at acidic pH values may be a contribution of the conjugated diene structure.

It has been further observed that among different proteins serum albumin is capable of protecting curcumin from alkaline hydrolysis. Although the surface hydrophobicity has a role in providing stability to curcumin, the affinity constant value suggests that a stronger binding to a protein shielding it from surrounding solvent molecules may also confer stability to the molecule. The crystal structures of lysozyme and α-lactalbumin are very similar and contain a large active site. Assuming the binding of curcumin to be around the active site, it is likely that there would be water molecules around it to conduct the nucleophilic attack leading to its breakdown. The standard binding pockets in BSA can sequester water molecules and thus protect curcumin. It has been found that in the presence of reducing agents (TCEP and DTT) the protection offered by albumin is considerably reduced (Figure 3). This phenomenon

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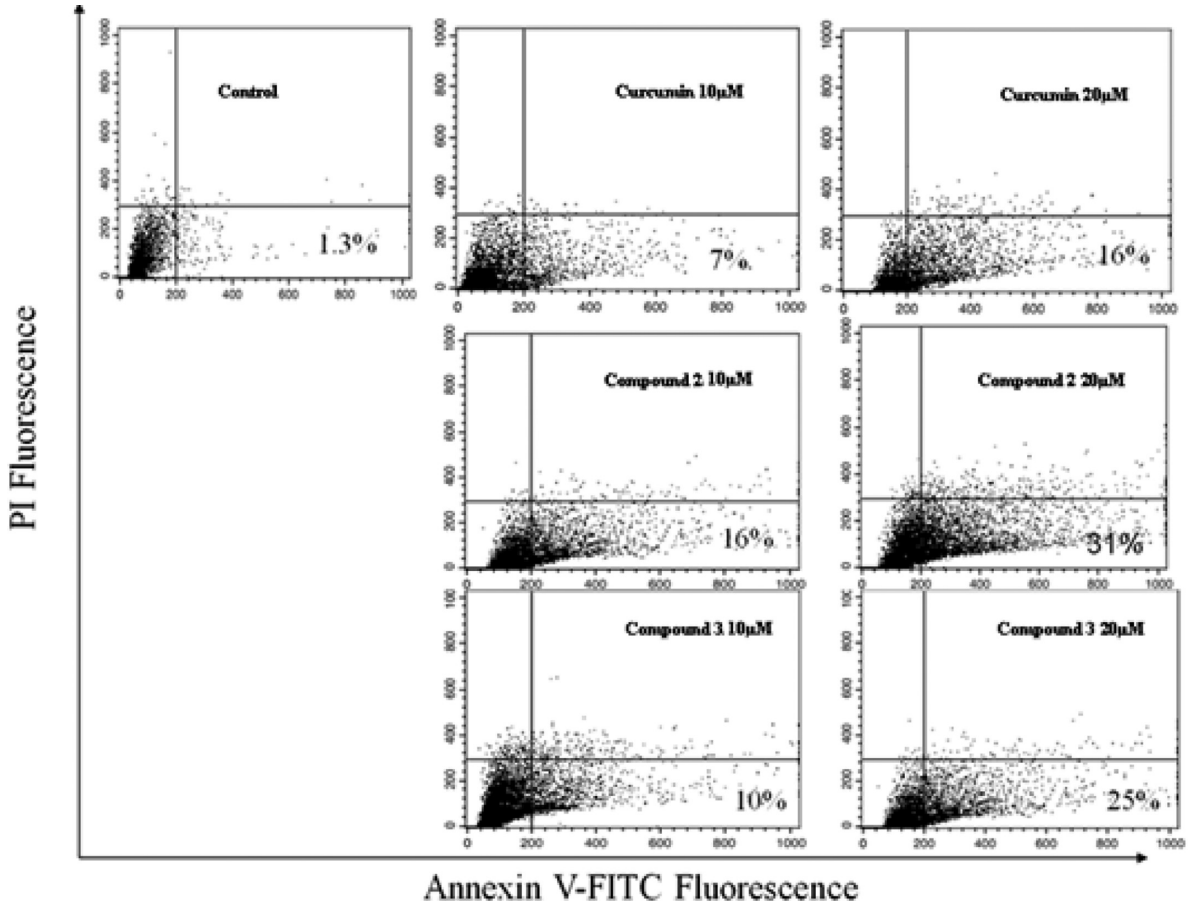

Figure 8. Induction of apoptosis in the cultured A549 cells upon treatment with curcumin, compound **2**, and compound **3**, under serum-free conditions. A dot plot representation of the annexin V-FITC fluorescence (x-axis) vs PI fluorescence (y-axis) of the apoptotic A549 (annexin V-positive) cells, treated with curcumin, compound **2**, and compound **3** (10−20 μM) for 48 h. Data are represented as the best of three independent experiments (n = 3) with similar results.

is probably attributed to reductive unfolding of the protein in the presence of DTT; as a result, curcumin is released from the hydrophobic pocket of the protein (BSA). The released curcumin is very much susceptible to degradation. David et al. have reported the denaturation (unfolding) of BSA in the presence of DTT.[44] The explanation also justifies the result obtained by Aggarwal et al. and others.[33,34] The degraded byproducts of curcumin formed in the presence of TCEP and DTT were also isolated and identified (data not shown), and the products were found to be different from vanillin or ferulic acid, which made us speculate that in the presence of a reducing agent the degradation of curcumin follows a different mechanism. Therefore, it can be concluded that the enol form of curcumin is highly susceptible to degradation, and the protection of the enol form through hydrophobic encapsulation may be a promising way of enhancing stability. In the quest for making more stable curcumin analogues, a series of derivatives were prepared with substitution in the diketone region of curcumin to avoid the keto−enol transition. Among differently substituted curcumin, compounds **2** and **3** have exhibited a dramatic improvement in curcumin stability and have antiproliferative activity comparable to that of curcumin. Even in the presence of a strong reducing agent, such as TCEP, compounds **2** and **3** remain stable (Figure 4). The pyrazole (compound **3**) and isoxazole (compound **2**) derivatives of curcumin are more stable because of the absence of tautomerism, which makes the active methylene carbon less nucleophilic, reducing the availability of acidic protons. Of these, the pyrazole analogue is more stable than its isoxazole counterpart possibly because of the disparity in size and chemical nature between oxygen and nitrogen atoms, making the isoxazole system less stable.

As the isoxazole and pyrazole substitutions improved curcumin stability, we next addressed if these substitutions caused any alteration in the binding affinity of these molecules for tubulin. No significant changes in the affinity constants of these compounds as compared to curcumin were observed. These results further led us to check if they bind to a site similar to or different from that of curcumin. Curcumin binds at the interdimer interface, between the α- and β-subunits of two α,β-dimers, close to the vinblastine binding site. Our docking result indicates that the binding pocket of compounds **2** and **3** closely resembles that of curcumin (Figure 6). Another interesting feature of this investigation is that despite changing the dicarbonyl group we did not observe any significant change in the binding affinity constant, indicating that the dicarbonyl group of curcumin is not essential for tubulin recognition in contrast to acetylation of the phenolic groups at the two termini of curcumin that generates a less effective curcumin analogue.[22]

We further checked the anticancer potential of the compounds in cell culture medium devoid of serum. We found that the IC_{50} value of compounds **2** and **3** against A549 cells remains unaltered over time; in contrast, the IC_{50} value of

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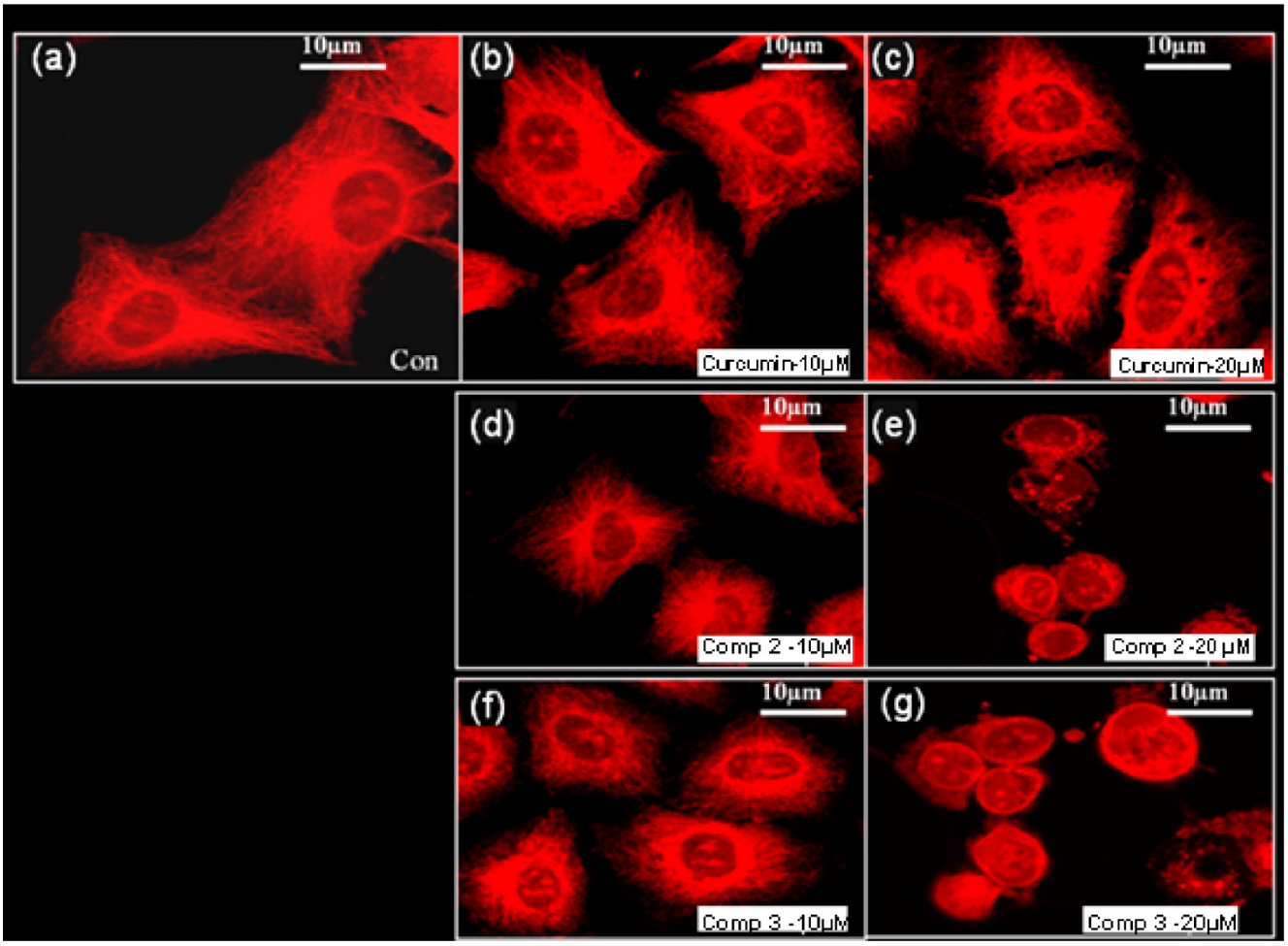

Figure 9. Disruption of the microtubule network in the cultured A549 cells upon treatment with curcumin, compound **2**, and compound **3**, under serum-free conditions. Cultured A549 cells were incubated in the presence of varying concentrations of curcumin, compound **2**, and compound **3** (10−20 μM) for 24 h, under serum-free conditions. The samples were stained for immunofluorescence using a mouse monoclonal anti-α-tubulin antibody and the corresponding rhodamine-conjugated (red) secondary antibody. Microtubule images of untreated cells (a) and cells treated with curcumin (b and c), compound **2** (d and e), and compound **3** (f and g) were taken under a Zeiss confocal microscope (LSM 510 Meta). The results represent the best of data collected from three identical experiments (n = 3).

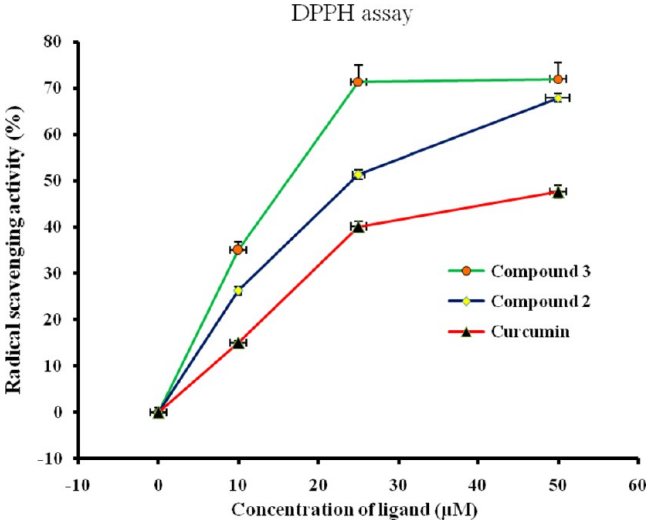

Figure 10. Free radical scavenging activity of different curcumin analogues measured by a DPPH-based free radical scavenging assay.

curcumin increases gradually, which further indicates that compounds **2** and **3** are stable even in the absence of serum (Figure 7), and other cell culture-based experiments also support this finding (Figure 9). In our previous work, we have convincingly established that compounds **2** and **3** mediate their anticancer action through targeting tubulin and their IC_{50} value also closely resembles that of curcumin. These new curcumin analogues also have enhanced stability and better free radical scavenging activity (Figure 10), which can be effectively utilized in anticancer therapy. Together, this investigation provides valuable information regarding the structure, stability, and activity of several curcumin analogues that may be useful in the design of an anticancer agent.

■ ASSOCIATED CONTENT

Supporting Information

Chemical structures of TCEP and DTT (Figure S1) and normalized fluorescence intensity (excitation at 295 nm and emission at 335 nm) of a BSA/curcumin mixture (2 μM BSA, 50 mM PIPES buffer (pH 7), 25 °C), monitored as a function of the concentration of curcumin (normalized to the fluorescence of ligand-free BSA), where the solid line indicates

dx.doi.org/10.1021/bi400734e | *Biochemistry* 2013, 52, 7449−7460

the best fit to eq 1 (the best fit was obtained with $n = 4$, and the resulting K_d value is shown) (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Authors

*MBU, Indian Institute of Science, Bangalore 560012, India. Telephone: +91-80-22932714. E-mail: surolia@mbu.iisc.ernet.in.

*Department of Biochemistry, Bose Institute, Kolkata 700054, India. Telephone: +9133-2355-9544. E-mail: bablu@jcbose.ac.in.

Author Contributions

S.C. and G.D. contributed equally to this work.

Funding

This work was supported by a Raja Ramanna Fellowship from the Department of Atomic Energy (DAE) to B.B. and the Centre of Molecular Medicine by the Department of Biotechnology to A.S. P.C. and A.S. are recipients of the J. C. Bose National Fellowships from the Department of Science and Technology (DST). A.S. holds a Bhatnagar Fellowship of the Council of Scientific and Industrial Research (CSIR), India. S.C. and G.D. acknowledge fellowships from CSIR.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

PIPES, piperazine *N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; $MgCl_2$, magnesium chloride; GTP, guanosine 5′-triphosphate; PDB, Protein Data Bank.

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