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Original Article

Molecular docking and inhibition of matrix metalloproteinase-2 by novel difluorinatedbenzylidene curcumin analog

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Abstract: We recently described the synthesis and characterization of a novel difluorinatedbenzylidene analog of curcumin, commonly referred as CDF, which demonstrated significantly enhanced bioavailability and *in vivo* anticancer activity. CDF targets many factors similar to curcumin, albeit with more potency, as reported previously. To further highlight this differential behavior of CDF, we chose matrix metalloproteinase protein MMP-2 which is involved in the processes of invasion and metastasis of human tumors. Both curcumin and CDF were characterized for their binding characteristics using *in silico* docking studies; they were also evaluated via biological assays involving gelatin zymography, miRNA analysis, invasion assays and ELISA. CDF was found to inhibit MMP-2 expression and activity in A549 and H1299 NSCLC cells much more effectively than curcumin, validating molecular modeling results. miR-874, an MMP-2-targeting miRNA, was up-regulated by CDF. Thus, it appears that CDF can inhibit MMP-2 through multiple mechanisms. Our results are suggestive of a more potent inhibition of invasion and metastasis by CDF, compared to curcumin, thus warranting its further evaluation as an effective anticancer agent.

Keywords: MMP-2, CDF, curcumin, miR-874, *in silico* molecular docking

Introduction

Curcumin (diferuloylmethane) is a nutraceutical obtained from plant *Curcuma longa*, which is commonly used as a spice in tropical Southeast Asia. It has been extensively investigated for its putative chemo-preventive and therapeutic uses. Despite numerous pre-clinical studies detailing its beneficial effects against various human cancers, its entry into the clinical practice has been severely limited due to poor bioavailability and rapid metabolism [1]. In an attempt to overcome such limitations, investigations in our laboratory led to the synthesis of its novel analog, viz. 3, 4-difluorobenzylidene curcumin, which is commonly referred to as CDF. This analog exhibited enhanced bioavailability in pancreatic and other tissues [2, 3]. Subsequent investigations from our laboratory have also reported on potent regulation of multiple molecular targets such as NF-κB, Akt, COX-2, IL-6, VEGF, PTEN and miRNAs by CDF [4-8]. Such pleiotropic action of nutraceuticals against multiple therapeutic targets is well-documented [9], and such a multi-targeting effect could be a good attribute of CDF for the prevention of tumor progression and/or treatment of human malignancies.

In the present study, we tested whether CDF can inhibit matrix metalloproteinase-2 (MMP-2), a member of the family of metalloproteinases. Members of this family are involved in the degradation of extracellular matrix facilitating the process of metastasis, and thus MMP-2 is an important target to inhibit invasion and metastasis. An increased expression of MMP-2 has been reported in various tumors including ovarian, breast and prostate tumors as well as melanoma [10]. Studies in recent years have suggested a role of MMP-2 as cancer prognostic marker [11] which is associated with poor prog-

nosis [12]. In order to validate MMP-2 as a target of CDF, docking studies were performed to assess the activity of CDF versus curcumin through MMP-2 crystal structure. CDF activity was compared to that of curcumin, its parent compound. Subsequently, biological assays were performed and compared for CDF and curcumin using gelatin zymography, invasion and ELISA techniques. We observed a positive correlation between docking studies and biological assays validating the use of our integrated approach to find new molecular target of CDF as a novel anticancer agent.

Materials and methods

Cell lines and reagents

The human lung adenocarcinoma cell lines A549 and H1299 were purchased from the American Type Culture Collection (Manassas, VA) and maintained according to the American Type Culture Collection's instructions. Cells were cultured in 5% CO_2-humidified atmosphere at 37°C. The cell lines have been tested and authenticated through the core facility (Applied Genomics Technology Center at Wayne State University) by short tandem repeat profiling using the PowerPlex 16 System from Promega.

Docking studies

The NMR structure of MMP-2 protein PDB id: 1HOV available at Protein databank [13] was used for docking of CDF and curcumin. Molecular docking was carried out using Auto Dock 4.2 software program [14]. This protein contains 11 models of macromolecules and previous studies have reported no differences in the docking studies using all 11 models. Hence, the catalytic domain containing S1', S1, S2', S3' of the model 1 of PDB id 1HOV was selected for docking study [15]. The docking parameters were optimized through self-docking exercise carried out using the co-crystallized ligand. Polar hydrogen atoms were added followed by assignment of Kollman united atom charge to MMP-2. All rotatable bonds within the ligand were allowed to rotate freely and the molecule was considered rigid. For the zinc ion, the parameter set reported by Stote and group was used (r=1.1 A, e=0.25 kcal mol^{-1} and a formal charge of +2e) [16]. AutoGrid program was used to generate 3D affinity grid fields with grid map of 40 × 40 × 40 points with spacing of 0.375 Å. AutoDock tools utility was used to generate both grid and docking parameter files (i.e., gpf and dpf). Docking parameters were set to 25 automated runs for a 150 population size with a 2,500,000 maximum number of energy evaluations for each docking experiment with Lamarckian genetic algorithm as the search method. The Lamarckian Genetic Algorithm (LGA) was used as the search engine keeping other parameters to default value [17]. About 25 conformations for curcumin and CDF were generated. The interactions of docked complex MMP-2 protein-ligand conformations, including hydrogen bonds and the bond lengths were analyzed using PyMOL v0.99rc6 [18]. The docking studies were performed at Tata Consultation Services, Pune.

miRNA transfections

Transfections of pre-miR-874 in A549 and H1299 cells were done using methodology previously described [19]. Briefly, cells were seeded (2.5×10^5 cells per well) in six well plates and transfected with pre-miR-874 or non-specific (NS) pre-miRNA controls (Life Technologies) at a final concentration of 200 nM, using DharmaFECT transfection reagent (Dharmacon). After 48 hours of transfection, cells were passaged and transfected once again before evaluating the levels of target (MMP-2) by real-time RT-PCR.

MMP-2 zymography

Activity of MMP-2 was assessed in the culture supernatants by gelatin zymography. A549 and H1299 cells were first exposed to vehicle control or curcumin/CDF (10 μM) for 72 hours. At the end of incubation, 50 μl of culture supernatant was mixed with sample buffer and resolved on a 12% SDS-PAGE under non-reducing conditions. The gel was co-polymerized containing 0.5 mg/ml of gelatin (Sigma, St. Louis, USA). Gel was washed twice for 30 minutes with renaturation buffer (2.5% Triton X-100) at room temperature before incubation in the incubation buffer (50 mM Tris-HCl pH7.5, 200 mM NaCl, 10 mM CaCl2, 1 μM ZnCl2) at 37°C for 16 hours. Thereafter, gel was stained for 2 hours in 0.25% coomassie brilliant blue R-250 and then de-stained. White bands were observed against a blue background after de-staining, indicating MMP-2's gelatinolytic activity.

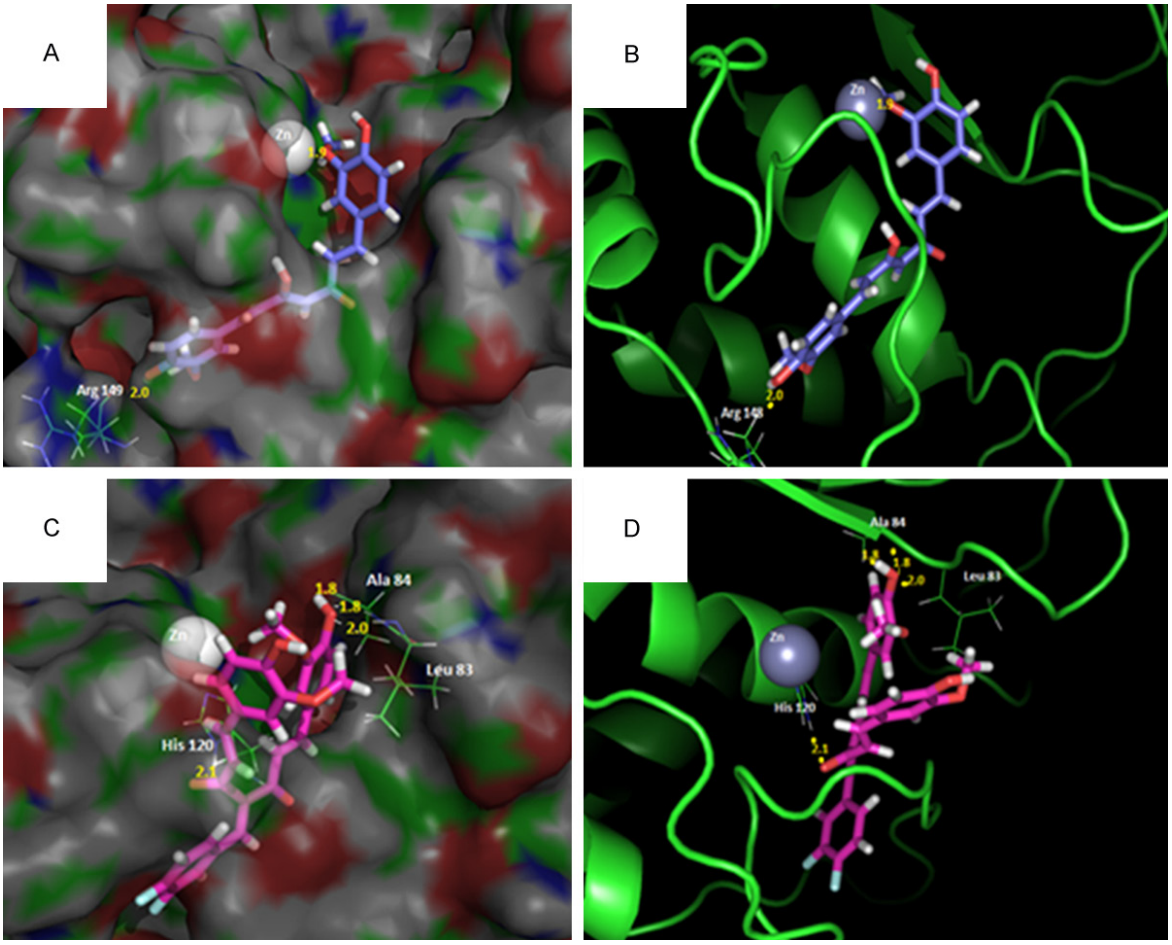

Figure 1. Binding of curcumin and CDF in the active site of MMP-2 protein cavity as assessed by molecular docking. A. The surface view of docking of curcumin (blue) in the catalytic cavity of MMP-2; it interacts with Zn seen as ball and Arg 149 forming hydrogen bonds. It can be seen that curcumin extends in the S1' pocket. B. The docking of curcumin (blue) in MMP-2 protein shown in cartoon form with the secondary structure of protein visible. The interaction with Zn and Arg 149 can be seen. Arg 149 is shown as lines. C. The surface view of docking of CDF (magenta) in the catalytic cavity of MMP-2; the interaction of CDF with residues Leu 83, Ala 84 and His 120 forming hydrogen bonds can be seen. Zn atom is seen as ball. It can be seen that CDF extends in the S1' pocket. D. The docking of CDF (magenta) in MMP-2 protein shown in cartoon form with the secondary structure of protein visible. The interaction with residues Leu 83, Ala 84 and His 120 can be seen. The interacting residues are shown as lines.

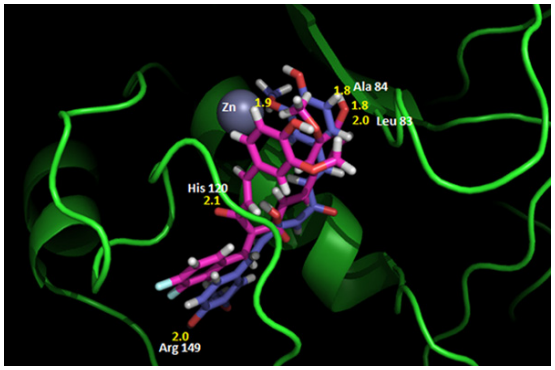

Figure 2. The cartoon view of superimposed image curcumin (yellow) and CDF (cyan) in the catalytic domain of MMP-2 protein.

Real-Time RT-PCR

Real-Time RT-PCR analyses were done as described previously [20]. Total RNA was isolated using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Real-time PCR was used to quantify mRNA expression and the amount of RNA was normalized to GAPDH expression. For miRNA analysis, total RNA was isolated using the mirVana miRNA isolation kit (Life Technologies). The levels of miRNAs were determined using miRNA-specific Taqman probes from the Taqman MicroRNA Assay (Life Technologies). The relative amounts of miRNA were normalized to RNU48.

Table 1. Docking analysis and consensus scores of Curcumin and CDF in the active site of MMP-2 protein assessed by computer modeling studies

Compound	Minimum Binding energy (Kcal/mol)	No of hydrogen bonds	Amino acid interacting	Hydrogen bond Distance (Å)
Curcumin	-7.35	2	Arg 149 (H)	2.5
			Zn	1.9
CDF	-6.39	4	Leu 83 (HN)	2.0
			Ala 84 (HN)	1.8
			Ala 84 (O)	1.8
			His 120 (HN)	2.1

Cell invasion assay

Cell invasion assay was performed using 24 well transwell permeable supports with 8 μM pores (Corning) [20]. After treatment of cells with curcumin/CDF or the DMSO vehicle (control) for indicated times, cells were suspended in serum free medium and seeded into the transwell inserts coated with growth factor reduced Matrigel (BD Biosciences). Bottom wells were filled with complete media. After 24 hours, cells were stained with 4 μg/ml calcein AM (Life Technologies) in PBS at 37°C for 1 h. Cells were detached from inserts by trypsinization and fluorescence of the invaded cells was read in ULTRA Multifunctional Microplate Reader (TECAN, San Jose, CA).

MMP-2 activity assay

The activity of MMP-2 was further assayed using human MMP-2 ELISA kit (Invitrogen, USA) following the vendor's protocol. The assay is based on solid phase sandwich ELISA wherein a highly purified antibody specific for MMP-2 is coated onto the wells of the microtiter strips purchased from the vendor. During the first incubation, standards of known MMP-2 content, controls, and assay samples were pipetted into the coated wells. A biotinylated secondary detection antibody was added after washing followed by further washing and addition of streptavidin-peroxidase enzyme bound to the biotinylated antibody to complete four-member sandwich. After a third incubation and washing to remove all unbound enzyme, the substrate solution was added. The bound enzyme acted upon the substrate to produce color which was read at 450 nM using ULTRA Multifunctional Microplate Reader (TECAN, San Jose, CA). The intensity of the color was directly proportional to the concentration of MMP-2 present.

Results

MMP-2 as a molecular target

To evaluate binding stabilities of curcumin and CDF in the protein cavity of MMP-2, we first assessed the binding of a standard compound in the pre-defined active site of MMP-2 protein by using Autodock software program. The active site of MMP-2 protein consists of Zn and consensus (HEBGHXLGLXHS) of amino acids for Zn binding motif which are conserved throughout the MMPs and contain three histidine residues which are bound to Zn [21]. Previous reports have suggested that amino acids in the catalytic cavity play an important role in binding of inhibitor in the protein cavity [15, 22, 23]. The protein cavity containing Zn and important amino acids was selected for docking of curcumin and CDF. Both compounds were found to dock into the active site of MMP-2 protein cavity with a good fit (**Figures 1**, **2** and **Table 1**). The aryl hydroxyl group of CDF was found to be involved in two hydrogen bonding interactions with Ala 84 (both 1.8 Å) and one with Leu 83 (2.0 Å), while one of the central diketo group interacted with His 120 (2.1 Å). These residues are found to be similar with those involved in the binding of known inhibitors of MMP-2 [15, 22] and hence CDF is anticipated to exhibit pronounced inhibition of MMP-2. On the other hand the binding interactions of curcumin are substantially different wherein one of the hydroxyl groups on the side aryl ring is found to be involved in H-bonding with Arg 149 (2.5 Å) while the methoxy group on the other aryl ring is found to interact with Zn (1.9 Å) atom. Although these interactions yield stronger binding energy for curcumin (minimum binding energy: -7.35 Kcal/mol) than CDF (minimum binding energy: -6.39 Kcal/mol), such binding is not expected to influence MMP-2 inhibition, confirmation of which is provided by the proceeding assays.

Inhibition of MMP-2 activity

In order to explore the inhibition of MMP-2 activity, we chose A549 and H1299 lung can-

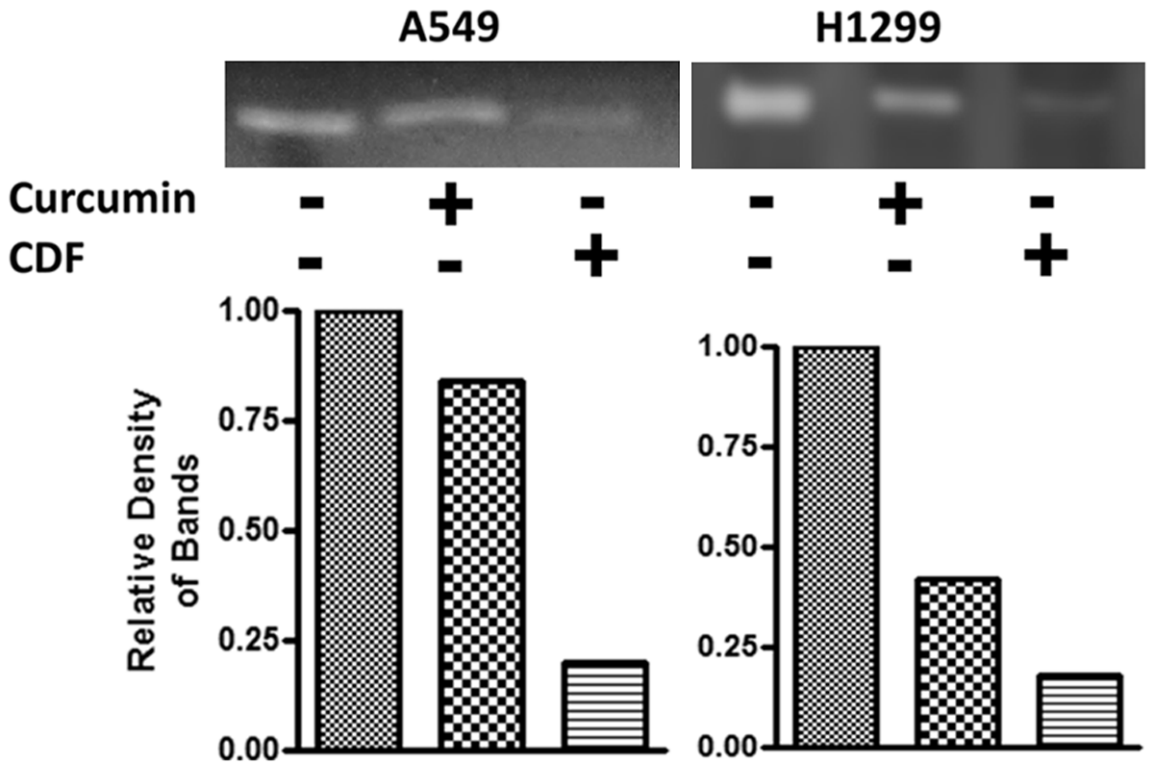

Figure 3. MMP-2 gelatinolytic activity (zymography) comparing the effects of curcumin and its derivative CDF. A549 and H1299 lung cancer cells were exposed to vehicle control (DMSO) or 10 μM curcumin/CDF for 72 hours. The collected conditioned medium was subjected to zymography (upper panel). Densitometric analysis was performed on the bands from zymography gel (lower panel) where the band from control DMSO-treated sample was assigned a value of 1.0 and the intensity of curcumin- and CDF-treated samples' bands was calculated relative to control band.

cer cells as our models because these cells are known to express MMP-2 [24, 25]. We first performed gelatin zymography to directly test the ability of curcumin and CDF to modulate the expression of MMP-2. Cells were treated with either vehicle control or 10 μM solutions of the two compounds. Cultures from these treated cells were then collected and subjected to zymography. We found that treatment with CDF more effectively inhibited the activity of MMP-2 (**Figure 3**). A densitometric analysis of the bands revealed 16% inhibition of MMP-2 activity by curcumin and 80% inhibition by CDF in A549 cells (**Figure 3**). CDF was observed to be a potent inhibitor of MMP-2 activity in H1299 cells, as well, with 82% inhibition, compared to 58% inhibition by curcumin (**Figure 3**).

Transcriptional regulation of MMP-2 activity

To evaluate whether curcumin/CDF could transcriptionally regulate MMP-2, we quantitated MMP-2 mRNA by real-time RT-PCR after treating A549 and H1299 cells with these compounds for 72 hours. While curcumin inhibited the MMP-2 mRNA by ~23% in A549 and 30% in H1299 cells, the inhibition by CDF was significantly more ($p < 0.01$) in both the cell lines tested- ~40% in A549 cells and 51% in H1299 cells (**Figure 4A**). Regulatory effect of mRNA transcripts often involves miRNAs. miR-874 has been reported to regulate MMP-2 levels in A549 and H1299 cells [25], therefore, we evaluated the levels of this tumor-suppressive miRNA in curcumin/CDF-treated cells. Both, curcumin and CDF were found to up-regulate miR-874 (**Figure 4B**) but the up-regulation by CDF was significantly more. As a proof of concept, we transfected A549 and H1299 cells with pre-miR-874 and observed down-regulation in the expression of its target MMP-2 (**Figure 4C**), thus validating the regulation of MMP-2 by miR-874 in our model system. Biological significance of such MMP-2 inhibition was evaluated by invasion assays, and while both curcumin and CDF inhibited the invasion of A549 and H1299 cells (**Figure 4D**), CDF again showed as a more potent compound.

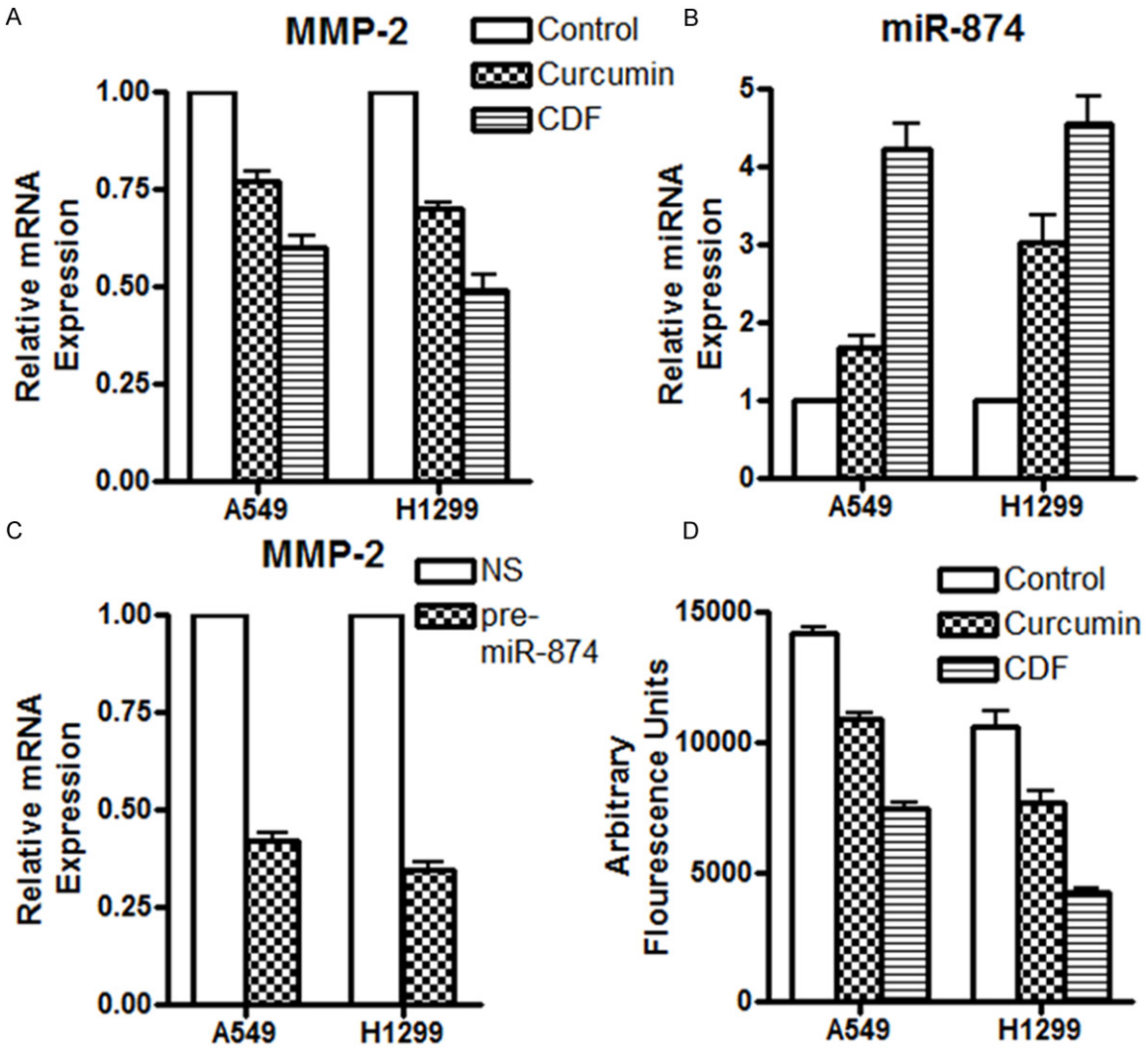

Figure 4. Transcriptional regulation of MMP-2. A549 and H1299 cells were treated with 10 μM curcumin/CDF for 72 hours and the MMP-2 mRNA transcripts (A) or the miR-874 levels (B) were quantitated by Real-Time RT-PCR. (C) MMP-2 mRNA levels were also measured after the cells were transfected with pre-miR-874 (*NS, non-specific pre-miRNA control*). Finally, A549 and H1299 cells treated with curcumin/CDF were subjected to invasion assays by plating cells in matrigel-coated inserts. The invading cells were collected by trypsinization and their fluorescence was quantitated using plate reader.

Thus, MMP-2 inhibition was correlated with the inhibition of invasive potential of both the cell lines.

Quantitative analysis of MMP-2 activity inhibition

We assayed the secreted MMP-2 using quantitative ELISA assay. A549 and H1299 cells were first exposed to increasing concentrations of curcumin and CDF for 72 hours and then MMP-2 levels were assayed in the culture supernatants. As seen in **Figure 5**, CDF inhibited the secretion of MMP-2 to a greater extent than curcumin, at all the concentrations tested. At the highest tested concentration of 10 μM, inhibition by curcumin was ~25% and ~85% by CDF, in A549 cells. In H1299 cells, inhibitions were ~39% and ~83%, respectively. Using this dose of 10 μM, we also tested a time-dependent inhibition of MMP-2 by curcumin and CDF. Aliquots of conditioned media were collected at the indicated time periods and subjected to ELISA. CDF was found to be much more effective in down-regulating MMP-2 secretion, compared to curcumin, at all the time periods and in both the cell lines (**Figure 6**).

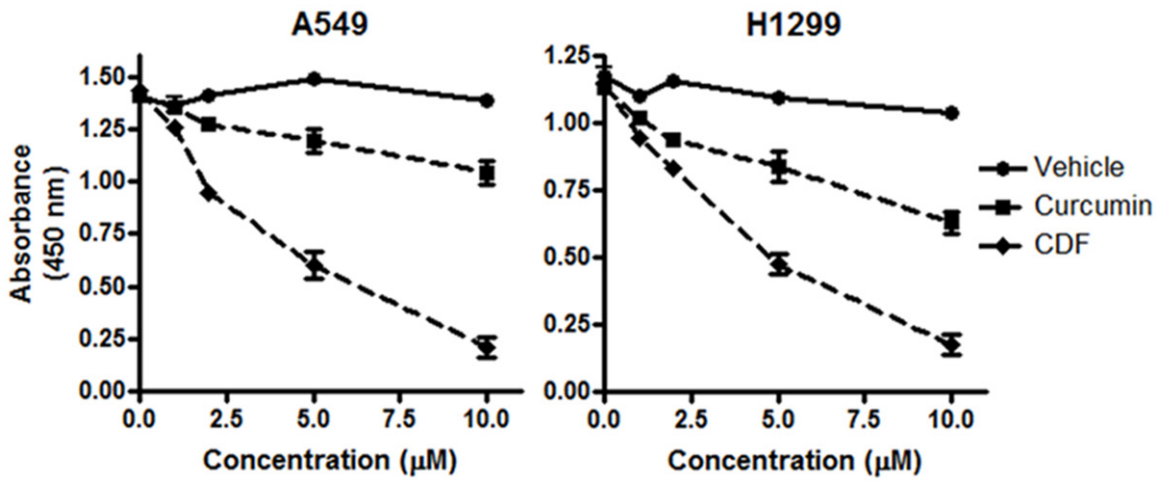

Figure 5. Concentration-dependent inhibition of MMP-2 secretion. A549 cells and H1299 were treated with indicated doses of curcumin/CDF or the equivalent quantities of DMSO (vehicle control). Treatment was carried out for 72 hours and then the samples were subjected to MMP-2 ELISA.

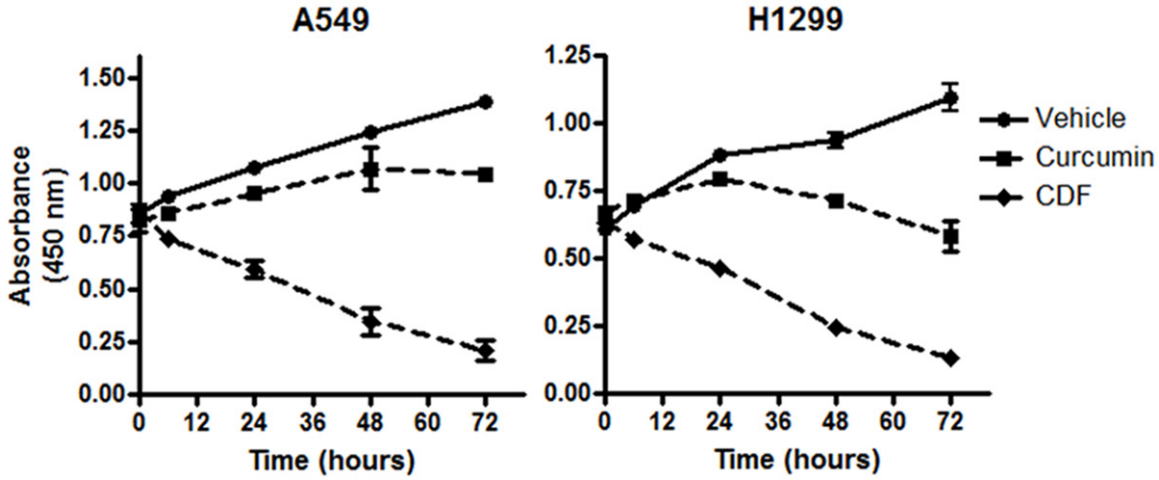

Figure 6. Time-course inhibition of MMP-2 secretion. A549 cells and H1299 were treated with 10 μM doses of curcumin/CDF or the equivalent quantities of DMSO (vehicle control) for indicated time periods. Conditioned media was collected at the end of different time-points and stored at -80°C until ready to be assayed. Once all the samples were collected, they were subjected to MMP-2 ELISA.

Discussion

Whenever a novel anticancer compound is synthesized that exhibits promising cytotoxic effects against cancer cell lines, the first and foremost challenge is to elucidate its molecular targets that are associated with its biological activity. This is true even for the compounds that are analogs of established and extensively studied phytochemicals. In the present study, we tested MMP-2 as a protein target of CDF, which is a novel and potent analog of curcumin. MMP-2 is an enzyme that belongs to the family of matrix metalloproteinases which are calcium-dependent, zinc-containing endopeptidases [26, 27]. The family of human MMPs consists of 23 different forms that are divided into six groups but are structurally and functionally related [27]. They are secreted in an active/inactive zymogen or a pro-MMP form. MMP-2 belongs to the gelatinase group of MMPs, which also comprises of MMP-9. This group is so named because these two MMPs primarily digest gelatin, the denatured form of collagen. In the present study, activity of MMP-2 was analyzed by a technique called substrate

zymography. This technique identifies MMP-2 through the degradation of its preferential substrate gelatin on a polyacrylamide gel, and is particularly sensitive since it can detect levels of MMP-2 as low as 10 pg [28]. While zymography is a reliable technique to study functional MMP-2s, it is not necessarily very quantitative, and hence we also employed ELISA technique for its quantitation. Inhibition of MMP-2 activity was determined to be 16% by zymography and 25% by ELISA in case of curcumin while it was found to be 80% by zymography and 85% by ELISA for CDF.

The compounds used for MMP inhibition usually contain zinc binding or chelating groups like hydroxymate, carboxylate, phosphinate and thiolate, respectively [29]. It has been observed that the hydroxymate group has unfavorable pharmacokinetics and is associated with toxicities [29, 30]. Thus, new group of compounds need to be evolved as MMPs inhibitors. Based on the present work we should like to propose that phytochemical curcumin and its difluobenzylidene analog CDF are the novel MMP-2 inhibitors. Although Zn chelation is generally considered as a requirement for MMP inhibitors, the interactions with key amino acid residues in the catalytic site are also of foremost importance for influencing the inhibitory activity [31]. In fact, there are reports documenting that MMP-13 inhibitors do not interact with Zn center, suggesting that Zn chelation is not the only criteria for inhibition of MMP's [30, 32]. The interactions with the hydrophobic pocket S1' is also found to be crucial for inhibiting MMP-2 [30, 33].

Analyzing the present docking results reveals that both CDF and curcumin bind to the MMP-2 in similar way as the co-crystal compound i52 going through the S1' pocket containing hydrophobic residues Phe 148, Phe 115, Thr145 and Leu150. However, CDF showed interactions with Leu 83 (S3'pocket) and Ala 84(S1 pocket) similar to the interactions in the co-crystal structure of hydroxamic acid inhibitor i52. The Ala 84 is a highly conserved residue in the active site of MMP protein, and thus interaction with this residue is considered crucial [15]. The active site also contains three histidine residues (His 120, His124 and His 130) bound to the Zn atom which are highly conserved in MMPs. In the present case, CDF was found to form hydrogen bond with His 120 which is helpful in anchoring the compound in the active site near Zn atom while extending itself into the S1' hydrophobic pocket analogous to the known co-crystal inhibitor. These interactions similar to other known MMP-2 inhibitors along with anchorage in the S1' pocket may help explain the specificity of CDF for MMP-2 protein [15, 29, 34]. On the contrary, curcumin did not exhibit any interactions with the important residues although it interacted with Zn through its enolic hydroxyl group, resulting in high binding energy which alone may not be important for the MMP-2 inhibitory activity. Additionally, curcumin did show H-bonding interaction with Arg149 but in the neighboring pocket [15]. Such differential interactions with the important amino acid residues seem to provide the basis for the potent MMP-2 inhibitory activity of CDF compared to curcumin despite having lower binding energy in the docking site.

Molecular docking studies are indicative of a post-translation regulation of target protein by anticancer agents. The inhibition of MMP-2 activity by curcumin and CDF seems to stem from their ability to interact with the specific regions on the protein surface, as described above. Interestingly, we also observed a transcriptional regulation of MMP-2 by curcumin and CDF which suggests the regulation of MMP-2 at multiple steps. As a mechanism, an up-regulation of miR-874 was observed. miR-874 is a tumor suppressive miRNA [35] which has been shown to target MMP-2 in exactly the same NSCLC cell lines [25] as used in the present study, namely A549 and H1299. Similar to our current observations, this study [25] also reported an inhibition of MMP-2 in pre-miR-874-transfected cells. Further, this study also evaluated an *in vivo* effect of miR-874 up-regulation wherein pre-miR-874 treated A549 cells showed much reduced orthotopic tumor burden when implanted intra-thoracic in athymic nude mice. Combined with the invasion assays described by us, there is a clear biological relevance of MMP-2 regulation in the NSCLC cells.

Previous work from our laboratory has shown an action of CDF against membrane type 1-metalloproteinase (MT1-MMP), also known as MMP-14 [8]. CDF was observed to down-regulate MT1-MMP through a mechanism that involved up-regulation of microRNA-200s (miR-200s) and PTEN. It is interesting to note that MT1-MMP is involved in the activation of MMP-2

[36-38] and, thus, CDF influences multiple steps in MMP-2 activation cascade by interacting with MT1-MMP as well as MMP-2. The miR-200s are markers of epithelial phenotype. Another epithelial marker E-cadherin has recently been connected to MMP-2 and invasion of A549 NSCLC cells, especially because the loss of E-cadherin was shown to promote invasion in an MMP-2-dependent manner [39]. For that reason, we initially used A549 cells in the current study as a model for the inhibition of MMP-2 activity. In order to rule out cell line specific effects, and also to study the transcriptional regulation involving miR-874, as reported earlier [25], we corroborated our results in another NSCLC cell line, H1299. The available literature indicate a nexus of MT-MMP, MMP-2, miR-200s and EMT driving the processes of invasion and metastasis, and our current study indicates attenuation of such nexus by CDF, along with a novel regulation involving miR-874, a revelation that needs to be tested further in future studies.

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Disclosure of conflict of interest

None of the authors report any conflict of interest or financial disclosure.

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