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Curcumin homing to the nucleolus: mechanism for initiation of an apoptotic program

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Abstract

Curcumin is a plant-derived polyphenol that displays anti-tumor properties. Incubation of cultured SF-767 glioma cells with curcumin gave rise to intense intra-nuclear foci of curcumin fluorescence. *In vitro* studies revealed that nuclear homing by curcumin is not a result of DNA/chromatin binding. On the other hand, curcumin fluorescence co-localized with nucleophosmin, a nucleolus marker protein. To determine the temporal relationship between curcumin-induced apoptosis and nucleolar homing, confocal live cell imaging was performed. The data show that curcumin localization to the nucleolus occurs prior to cell surface exposure of phosphatidylserine. In studies of the mechanism of curcumin-induced apoptosis in SF-767 cells, its effect on the subcellular location of p14^{ARF} was determined. Whereas p14^{ARF} was confined to the nucleolus in untreated cells, 2 h following incubation with curcumin, it displayed a diffuse nuclear distribution. Given the role of nuclear p14^{ARF} in binding the E3 ubiquitin ligase, MDM2 (Mouse Double Minute 2 Homolog), the effect of curcumin treatment on cellular levels of the tumor suppressor protein, p53, was examined. Between 2 and 4 h following curcumin treatment, p53 levels increased with maximum levels reached by 8 h. Thus, curcumin homing to the nucleolus induces redistribution of p14^{ARF} to the nucleoplasm where interaction with MDM2 leads to stabilization of p53, with subsequent initiation of apoptosis.

Keywords

Curcumin; nucleolus; apoptosis; p14^{ARF}; p53; confocal fluorescence microscopy

Introduction

Curcumin is a yellow colored polyphenol abundant in rhizomes of the plant, *Curcuma longa*. As an ingredient of the Indian spice, turmeric, health benefits of curcumin have been described for centuries. More recently, research efforts have provided compelling evidence that curcumin possesses intrinsic anti-cancer properties and can induce apoptosis both *in*

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vitro and *in vivo* [1–6]. Curcumin exerts pleiotropic effects by modulating the activity of transcription factors, growth factors and cytokines [7]. Intriguingly, although curcumin has been shown to induce cytotoxic effects in multiple tumor cell lines, normal cells appear to be largely spared from cytotoxicity [7 – 9].

In an effort to overcome problems associated with water insolubility and poor bioavailability, Ghosh et al. [10] described a tripartite, nanoscale delivery vehicle for curcumin, termed nanodisks [ND; 11, 12]. In studies with cultured lymphoma cells, curcumin-ND displayed an enhanced ability to induce apoptosis compared to free curcumin [13]. Subsequent studies of the effect of curcumin-ND on cultured SF-767 glioblastoma cells included examination of its intracellular itinerary following uptake [14]. Confocal fluorescence microscopy analysis of intrinsic curcumin fluorescence after 4 h incubation with cultured SF-767 cells revealed the presence of bright intra-nuclear foci of curcumin fluorescence. Insofar as the nuclear space is comprised of distinct sub-compartments that harbor proteins involved in cell proliferation, cell cycle arrest and apoptosis [15, 16], curcumin homing to discrete sub-nuclear sites is of great interest. To characterize this homing phenomenon, the intra-nuclear site of curcumin fluorescence intensity has been determined and the temporal relationship between nucleolar homing and apoptosis examined. The results reveal that curcumin-induced apoptosis of SF-767 cells correlates with redistribution of p14^{ARF} from the nucleolus to the nucleoplasm, an event that leads to stabilization of the tumor suppressor protein, p53 [17]. Knowledge of the biochemical and cell biological basis underlying curcumin-induced apoptosis should increase opportunities for therapeutic exploitation of its anti-cancer properties.

Materials and methods

Reagents

Curcumin was obtained from Cayman Chemical and used without further purification. Calf thymus DNA was from Sigma Aldrich (St. Louis, MO). Mouse monoclonal antinucleophosmin, Alexa Fluor 647 goat anti-mouse IgG and Hoechst 33342 were from Life Technologies Corp. (Carlsbad, CA). Monoclonal anti-p53 (clone DO-1) was from Sigma Aldrich. Rabbit polyclonal anti-p14^{ARF}, mouse monoclonal β -actin and DyLight 405-conjugated goat anti-rabbit IgG were from Novus Biologicals (Littleton, CO). Western Lightning Plus-ECL, obtained from PerkinElmer, Inc. (Waltham, MA) was used for immunoblot analysis. Biotin-conjugated annexin V, Alexa Fluor 350-labeled streptavidin and propidium iodide (PI) were components of the Vybrant Apoptosis Assay Kit obtained from Life Technologies Corp. Goat anti-mouse HRP-conjugated secondary, NE-PER nuclear and cytoplasmic extraction reagents, Halt protease inhibitor cocktail and Restore western blot stripping buffer were from Thermo Scientific (Rockford, IL). HRP-conjugated goat anti-rabbit IgG secondary antibody was from Vector laboratories (Burlingame, CA).

Curcumin incubations with calf thymus DNA

A stock solution of 4.5 mg/ml calf thymus DNA was prepared in water by gently inverting the sample overnight at 4 °C. The DNA concentration was determined using a NanoDrop instrument. For spectrophotometric studies, a 25 mM curcumin stock solution, prepared in

absolute ethanol, was used. Prior to fluorescence or absorbance measurements, a 2.5 mM curcumin working solution was prepared from the stock using 20 mM sodium phosphate, pH 7.0, 50 mM NaCl [18]. The absorbance and fluorescence properties of curcumin were determined in the absence and presence of increasing concentrations of calf thymus DNA. Ultraviolet-visible (UV/Vis) absorbance spectra were obtained on a Perkin-Elmer Lambda 20 spectrophotometer (Perkin-Elmer, Norwalk, CT). Samples were scanned from 350 to 500 nm. Fluorescence spectra were obtained on a Perkin-Elmer LS 50B luminescence spectrometer. Samples were excited at 420 nm and emission monitored from 480 to 600 nm (2.5 nm slit width).

Cell culture studies

SF-767 glioblastoma cells were obtained from Dr. Trudy M. Forte (Children's Hospital Oakland Research Institute) and maintained in high glucose DMEM (Thermo Scientific Hyclone) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. HepG2 cells were cultured in DMEM supplemented with 10% FBS. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. For cell incubation studies, a 20 mg/ml curcumin stock solution (in DMSO) was diluted 100 fold with 20 mM sodium phosphate, pH 7.4, 150 mM NaCl (PBS) immediately before use.

Co-localization study of curcumin and propidium iodide (PI)

To investigate curcumin interaction with cellular nucleic acid, SF-767 cells were incubated with 20 µM curcumin (as curcumin-ND [7]) for 24 h, washed with PBS, fixed with methanol/acetone (1:1 v/v) for 5 min and stained with 1 µg/ml PI for 15 min. Coverslips were mounted on a glass slide using Vectashield Mounting Medium (Vector Laboratories) and sealed. Confocal images were acquired on a Zeiss LSM710 microscope with a 63 ×, 1.4NA oil objective with a pinhole of 90 µm. Z-stack recordings of 0.566 µm thick overlapping images were obtained. Image processing, deconvolution, 3D reconstruction and co-localization analysis were performed with Huygens Essential and Bitplane Imaris Suite package of Scientific Volume Imaging. The ImarisColoc module was used for co-localization analysis. This module uses an iterative procedure to determine an intensity threshold (0–255 pixel intensity scale) for the green (curcumin) and red (PI) fluorescent labels. The region of interest was defined using red as the mask channel. In conjunction with the automatic threshold, the region of interest was used to obtain co-localization data represented as a 2-D histogram.

Nuclear protein extraction

HepG2 cells were harvested with trypsin-EDTA, centrifuged at 500 × g for 5 min and the cell pellet washed with PBS. Nuclear proteins were extracted using the NE-PER reagent according to the manufacturer's instructions. Total protein was quantified using the BCA Protein Assay (Thermo Scientific) using bovine serum albumin (BSA) as standard.

Live imaging confocal fluorescence microscopy

SF-767 cells were seeded onto 35 mm glass bottom microwell dishes (MatTek Corporation; Ashland, MA) and incubated with 20 µM curcumin. After 4 h or 24 h, cells were rinsed with

annexin V binding buffer (Vybrant Apoptosis Assay kit component) and incubated with biotin-conjugated annexin V followed by Alexa Fluor 350 streptavidin solution. Cells were co-stained with PI and images acquired on a Zeiss LSM710 microscope with a C-Apochromat 63 $\times$, 1.20 W Korr M27 water objective. Some photobleaching occurred since no anti-fade reagent was used.

Immunoblot analysis

SF-767 cells, seeded onto 12 well plates at a density of 5×10^5 cells/ml, were left untreated or incubated with 20 μ M curcumin. At indicated time points, cells were lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100 and 1 $\times$ Halt protease inhibitor cocktail). Ten μ g total cell protein from each lysate were separated on a 4% to 20% acrylamide SDS gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% (w/v) non-fat milk. Target proteins were visualized using the ECL detection system after incubation with anti-p53 (1:5000), anti- p14^{ARF} (1:1000), anti- MDM2 (1:200) and anti- β -actin (1:4000) antibodies. Goat anti-mouse and anti-rabbit HRP-conjugated secondary antibody was used at 1:10000 and 1:2000 dilutions, respectively. The experiment was performed three times.

Confocal fluorescence microscopy

Cells were plated overnight on coverslips in 12 well plates at a density of 1.5×10^5 cells/ml in a final volume of 1 ml. Cells were incubated with 20 μ M curcumin for specified times at 37 $^{\circ}$ C. Subsequently, the coverslips were washed, the cells fixed with 4 % paraformaldehyde for 15 min on ice and washed with PBS containing 1% BSA. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature followed by three washes with PBS plus 1% BSA. Nucleophosmin and p14^{ARF} were detected after incubation with anti-nucleophosmin or anti- p14^{ARF} (both diluted 1:50) for 2 h at room temperature. Cells were washed and incubated with either Alexa Fluor 647-conjugated anti-mouse IgG (1:150 dilution for nucleophosmin) or DyLight 405-conjugated anti-rabbit IgG (1:200 for p14^{ARF}) for 1 h. In some cases samples were stained with Hoechst 33342 before mounting with Vectashield for image acquisition on a Zeiss LSM710 confocal microscope.

Results

Recently, Ghosh and Ryan [14], studied curcumin uptake by cultured glioblastoma cells using curcumin-ND as the delivery vehicle. Investigation of curcumin's intracellular itinerary revealed a time-dependent accumulation of this polyphenol in the nucleus. To assess whether this phenomenon occurs in other cell types and if it may be specific to the mode of curcumin delivery, SF-767 and HepG2 cells were incubated with free curcumin. As seen in Figure 1, after 24 h incubation, both cell types displayed distinct intra-nuclear foci of curcumin fluorescence. Given that curcumin fluorescence is concentrated in these discrete intra-nuclear sites, as opposed to a generalized dispersion, experiments were conducted to characterize the apparent homing process, identify the intra-nuclear site(s) of accumulation and examine the biological significance of this phenomenon.

In vitro DNA binding studies

Others have reported that curcumin binds the minor groove of DNA, albeit weakly [18, 19]. Thus, it is conceivable that nuclear homing by curcumin is driven by a specific interaction with nucleic acid. Although this would also be consistent with an ability of curcumin to adopt a planar molecular structure, incubations with calf thymus DNA had little effect on either the UV/Vis absorbance spectrum or the fluorescence emission properties of curcumin (Figure 2). On the basis of these data, it may be concluded that curcumin does not bind DNA *in vitro*.

Co-localization studies with PI

A possible explanation for results obtained upon incubation of curcumin with calf thymus DNA is that interaction with nucleic acid requires other components or features present only in intact chromatin. To explore this, SF-767 cells were incubated with curcumin for 24 h, fixed and stained with the fluorescent DNA intercalating dye, PI. Merged confocal fluorescence microscopy images provided no evidence of fluorescence co-localization between curcumin and PI (Figure 3). The Z-stack recording of overlapping images spanning the entire cell volume clearly shows that the intra-nuclear foci of curcumin fluorescence (green) are distinct from sites of PI fluorescence (red). A two-dimensional intensity histogram encompassing the entire Z stack indicates that, at no place within the cell, do curcumin and PI co-localize.

Curcumin homing to the nucleolus

The apparent lack of curcumin binding to DNA/chromatin prompted further probing of the intra-nuclear site(s) of intense curcumin fluorescence. Considering the size, shape and appearance of the fluorescent foci, it was hypothesized that curcumin homes to the nucleolus. To examine this, curcumin treated SF-767 and HepG2 cells were fixed and probed with an antibody directed against nucleophosmin, a nucleolus marker protein [20]. Confocal microscopy images (Figure 4) revealed that the intra-nuclear foci of curcumin fluorescence co-localize with nucleophosmin. The merged image shows complete fluorescence overlap, confirming a nucleolar localization of curcumin.

Curcumin interaction with nuclear protein

Given the apparent lack of curcumin binding to nucleic acid/chromatin, it was hypothesized that curcumin localization to the nucleolus is driven by protein interaction. Previous studies have shown that curcumin associates with myriad proteins *in vitro* [21] and these interactions may be related to its biological effects. When curcumin was incubated with a nuclear protein extract from HepG2 cells (Figure 5), a 40 nm blue shift (from 540 nm to 500 nm) was observed in the wavelength of maximum curcumin fluorescence emission that was accompanied by a protein concentration dependent enhancement in curcumin fluorescence intensity.

Nucleolar localization of curcumin precedes initiation of apoptosis

To investigate the potential physiological significance of nucleolar homing, curcumin localization to this site was correlated with the onset of apoptosis in cultured SF-767 cells.

Live cell imaging showed that, whereas foci of curcumin fluorescence were present 4 h after incubation with 20 μ M curcumin, the cells had not yet initiated an apoptotic program, as judged by a lack of cell surface exposure of phosphatidylserine, exclusion of PI and maintenance of cell morphology (Figure 6). On the other hand, at 24 h, cells were positive for annexin V and PI and adopted a rounded morphology. Thus, curcumin homing to the nucleolus precedes the onset of apoptosis and, therefore, may be a cause rather than an effect of programmed cell death.

Effect of curcumin on p14^{ARF} localization

The cell cycle regulatory protein p14^{ARF} is normally confined to the nucleolus, where it interacts with other proteins, including nucleophosmin [22]. Upon mitogenic signaling, however, p14^{ARF} is released to the nucleoplasm where it interacts with MDM2, thereby inhibiting its ability to ubiquitinate the tumor suppressor protein, p53. Thus, p14^{ARF} interaction with MDM2 leads to stabilization of p53, permitting it to induce cell cycle arrest or apoptosis [23, 24]. Using confocal fluorescence microscopy, the effect of curcumin on the subcellular localization of p14^{ARF} was examined in SF-767 cells (Figure 7). As expected, no curcumin fluorescence was detected at time zero. By 2 h curcumin fluorescence was apparent, displaying a perinuclear and nucleolar localization pattern. At time zero, p14^{ARF} was confined to the nucleolus and a merged image shows complete co-localization with nucleophosmin. At 2 h following incubation with curcumin, however, evidence of p14^{ARF} migration from the nucleolus to the nucleoplasm (where it displayed a diffuse distribution pattern) was present. No such redistribution occurred in the case of nucleophosmin, which retained its nucleolar localization. Thus, curcumin-mediated effects on p14^{ARF} redistribution do not appear to be the result of generalized nucleolar dissolution.

Effect of curcumin on cellular levels of p14^{ARF}, MDM2 and p53

Given the established role of p14^{ARF} in p53 mediated apoptosis [23–25], we hypothesized that curcumin's effect on p14^{ARF} redistribution from the nucleolus to the nucleoplasm may be mechanistically linked to curcumin-induced apoptosis. To explore this, SF-767 cells were incubated with curcumin and the cellular levels of p14^{ARF}, MDM2 and p53 were determined as a function of time (Figure 8). Compared to changes in p53, only minor differences in p14^{ARF} and MDM2 protein levels were detected over an 8 h time course. At 16 h the 97 kDa MDM2 band intensity was decreased, concomitant with an increase in its corresponding 60 kDa caspase cleavage product [26]. The lack of a p53 signal in untreated cells is fully consistent with its rapid degradation as a consequence of MDM2-mediated ubiquitination and proteasomal degradation. By contrast, between 2 and 4 h following curcumin treatment, an increase in p53 signal was observed that reached a maximum level at 8 h and persisted at least until 16 h. Upon stabilization, p53 can function as a transcription factor, inducing cell cycle arrest and apoptosis [17]. Thus, early events associated with curcumin-induced cell death, including p14^{ARF} migration from the nucleolus to the nucleoplasm followed by p53 stabilization, occur subsequent to nucleolar homing of this polyphenol.

Discussion

Curcumin is a remarkable, plant-derived polyphenol that has been used medicinally for centuries. Research on curcumin indicates its biological actions are pleiotropic, manifesting both anti-cancer and anti-neurodegenerative properties [7, 27]. Studies indicate curcumin-dependent inhibition of uncontrolled cell proliferation involves an ability to induce apoptosis. Furthermore, curcumin has been documented to inhibit formation of oligomers and fibrils composed of the amyloid β peptide [28]. In studies of curcumin solubilized in ND complexes, a strong pro-apoptotic effect was observed in cultured lymphoma and glioblastoma cells [13, 14]. In the latter study confocal fluorescence microscopy analysis of intracellular curcumin provided evidence that it localizes to discrete intra-nuclear sites, and this curious finding was explored further in the present study. The observation that incubation of either HepG2 or SF-767 cells with curcumin leads to intense intra-nuclear foci of fluorescence, suggests this is a general phenomenon. The data indicate that, upon entering cells, curcumin migrates to the nucleus where it interacts with one or more resident components, resulting in its accumulation/sequestration. Given the discrete nature of the intra nuclear site(s) of curcumin fluorescence intensity, studies were undertaken to identify its interaction partner(s). Whereas little evidence of DNA/chromatin binding was obtained, the foci of curcumin fluorescence intensity co-localized with the nucleolus marker protein, nucleophosmin. Subsequently, spectroscopic studies provided evidence of curcumin interaction with a nuclear protein extract.

A question that arises, however, is whether interaction with nuclear proteins affects the fluorescence properties of curcumin or if the fluorescence intensity of nucleolar localized curcumin is due to concentrated polyphenol accumulation in this compartment. Others have examined the effect of protein on the spectral properties of curcumin with the finding that interaction with amyloid or fibrillar proteins induces a large enhancement in fluorescence emission quantum yield that is accompanied by a ~40 nm blue shift in the wavelength of maximum fluorescence emission. For example, Singh et al. [29] reported that curcumin binds preferentially to oligomers of α -synuclein, as compared to monomeric α -synuclein, and this binding is accompanied by characteristic changes in curcumin fluorescence emission. Likewise, McCrate et al. [30] reported that curcumin binds specifically to curli, extracellular amyloid fibers present on the surface of *E. coli*. Again, interaction with curli was accompanied by a strong enhancement in curcumin fluorescence emission intensity. Thus, it seems that curcumin preferentially interacts with fibrillar proteins and such interactions induce characteristic changes in curcumin fluorescence emission properties.

The nucleolus was identified over 200 years ago and is the most prominent subnuclear structure. Histological staining methods have led to the identification of intra nucleolar sites termed “nucleolar organizing regions” that contain protein fibrils. Distinct nucleolar sub-regions include fibrillar centers, dense fibrillar centers and granular component [31, 32]. Whereas the nucleolus is primarily involved in ribosome biogenesis, the number of nucleoli present, their size and activity increases in rapidly dividing cells [33]. Moreover, changes in nucleolar phenotype correlate with execution of a cell death program. Thus, it is conceivable that the effects of curcumin on cell proliferation and apoptosis could be manifest through its interactions with the nucleolus. Any connection between curcumin, the nucleolus and

apoptosis, however, is likely to involve protein interaction and proteomic studies of isolated nucleoli indicate the presence of >700 unique proteins. Annotation of the nucleolar proteome indicates approximately one third of the proteins function in production of ribosome subunits while others play a role in pre-mRNA processing, cell-cycle control and DNA replication and repair. Additionally, an interesting aspect of the nucleolar proteome relates to dynamic changes that occur under different metabolic conditions (e.g. cellular stress). An example of how the nucleolus functions to regulate aspects of the stress response relevant to the present discussion involves the tumor-suppressor protein, p53 [34]. Under normal conditions, p53 is continuously ubiquitinated by the E3 ubiquitin-protein ligase, MDM2, resulting in proteasomal degradation of this key tumor suppressor protein. As such, p53 is normally present in cells at very low levels. Upon exposure to exogenous stress (e.g. DNA damage, heat shock, hypoxia), however, p53 becomes stabilized, activating a cascade of events that lead to cell-cycle arrest or apoptosis [17]. Accumulation of p53 induces p21-mediated inhibition of cyclin D1 and cyclin E1, resulting in cell-cycle arrest in G1 phase. In human cells, p53 stabilization is conferred by MDM2 binding to p14^{ARF} [23, 35, 36]. Various stimuli are capable of “activating” p14^{ARF} leading to its redistribution from the nucleolus to the nucleoplasm. p14^{ARF} then associates with MDM2, inhibiting its ability to ubiquitinate p53. The present results indicate that, in SF-767 glioma cells, curcumin manifests its biological effects through this pathway. The data presented indicate curcumin homing to the nucleolus precedes redistribution of p14^{ARF} from the nucleolus to the nucleoplasm. Once in the nucleoplasm, p14^{ARF} physically sequesters MDM2 abrogating its ubiquitin ligase activity [23–25], resulting in stabilization of p53, which drives the initiation of apoptosis. Further study of the process of curcumin homing and p14^{ARF} mobilization will likely provide novel insight into the mechanism whereby this potent, yet nontoxic, polyphenol elicits its biological effects. Because numerous activities have been ascribed to curcumin that can lead to apoptosis, future studies need to focus on whether individual or multiple activities may be at play. For example, it has been reported that curcumin interacts directly with p53 [37], disrupting its conformation and gene transactivation properties. Hence, curcumin-mediated effects on p53 could involve both direct and indirect (via p14^{ARF}/MDM2) processes. By understanding these mechanistic processes in detail, new strategies to employ curcumin as a therapeutic agent will emerge.

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References

1. Lontas A, Yeger H. Curcumin and resveratrol induce apoptosis and nuclear translocation and activation of p53 in human neuroblastoma. *Anticancer Res.* 2004; 24:987–998. [PubMed: 15161054]
2. Anand P, Sundaram C, Jhurani S, Kunnumakkara AB, Aggarwal BB. Curcumin and cancer: an “old-age” disease with an “age-old” solution. *Cancer Lett.* 2008; 267:133–164. [PubMed: 18462866]
3. Goel A, Kunnumakkara AB, Aggarwal BB. Curcumin as “Curecumin”: from kitchen to clinic. *Biochem Pharmacol.* 2008; 75:787–809. [PubMed: 17900536]

4. Zanotto-Filho A, Braganhol E, Edelweiss MI, Behr GA, Zanin R, Schroder R, et al. The curry spice curcumin selectively inhibits cancer cells growth in vitro and in preclinical model of glioblastoma. *J Nutr Biochem.* 2012; 23:591–601. [PubMed: 21775121]
5. Park W, Amin AR, Chen ZG, Shin DM. New perspectives of curcumin in cancer prevention. *Cancer Prev Res (Phila).* 2013; 6:387–400. [PubMed: 23466484]
6. Guo LD, Chen XJ, Hu YH, Yu ZJ, Wang D, Liu JZ. Curcumin inhibits proliferation and induces apoptosis of human colorectal cancer cells by activating the mitochondria apoptotic pathway. *Phytother Res.* 2013; 27:422–430. [PubMed: 22628241]
7. Ravindran J, Prasad S, Aggarwal BB. Curcumin and cancer cells: how many ways can curry kill tumor cells selectively? *AAPS J.* 2009; 11:495–510. [PubMed: 19590964]
8. Kunwar A, Barik A, Mishra B, Rathinasamy K, Pandey R, Priyadarsini KI. Quantitative cellular uptake, localization and cytotoxicity of curcumin in normal and tumor cells. *Biochim Biophys Acta.* 2008; 1780:673–679. [PubMed: 18178166]
9. Syng-Ai C, Kumari AL, Khar A. Effect of curcumin on normal and tumor cells: role of glutathione and bcl-2. *Mol Cancer Ther.* 2004; 3:1101–1108. [PubMed: 15367704]
10. Ghosh M, Singh AT, Xu W, Sulchek T, Gordon LI, Ryan RO. Curcumin nanodisks: formulation and characterization. *Nanomedicine.* 2011; 7:162–167. [PubMed: 20817125]
11. Ryan RO. Nanodisks: hydrophobic drug delivery vehicles. *Expert Opin Drug Deliv.* 2008; 5:343–351. [PubMed: 18318655]
12. Ryan RO. Nanobiotechnology applications of reconstituted high density lipoprotein. *J Nanobiotechnology.* 2010; 8:28. [PubMed: 21122135]
13. Singh AT, Ghosh M, Forte TM, Ryan RO, Gordon LI. Curcumin nanodisk-induced apoptosis in mantle cell lymphoma. *Leuk Lymphoma.* 2011; 52:1537–1543. [PubMed: 21699455]
14. Ghosh M, Ryan RO. ApoE enhances nanodisk-mediated curcumin delivery to glioblastoma multiforme cells. *Nanomedicine (Lond).* 2013 Epub ahead of print, July 24.
15. Zimmer A, Nguyen QD, Gespach C. Nuclear bodies and compartments: functional roles and cellular signalling in health and disease. *Cell Signal.* 2004; 16:1085–1104. [PubMed: 15240004]
16. Gallagher SJ, Kefford RF, Rizos H. The ARF tumour suppressor. *Int J Biochem Cell Biol.* 2006; 38:1637–1641. [PubMed: 16600663]
17. Ljungman M. Dial 9-1-1 for p53: mechanisms of p53 activation by cellular stress. *Neoplasia.* 2000; 2:208–225. [PubMed: 10935507]
18. Nafisi S, Adelzadeh M, Norouzi Z, Sarbolouki MN. Curcumin binding to DNA and RNA. *DNA Cell Biol.* 2009; 28:201–208. [PubMed: 19364279]
19. Zsila F, Bikadi Z, Simonyi M. Circular dichroism spectroscopic studies reveal pH dependent binding of curcumin in the minor groove of natural and synthetic nucleic acids. *Org Biomol Chem.* 2004; 2:2902–2910. [PubMed: 15480453]
20. Colombo E, Alcalay M, Pelicci PG. Nucleophosmin and its complex network: a possible therapeutic target in hematological diseases. *Oncogene.* 2011; 30:2595–2609. [PubMed: 21278791]
21. Gupta SC, Prasad S, Kim JH, Patchva S, Webb LJ, Priyadarsini IK, et al. Multitargeting by curcumin as revealed by molecular interaction studies. *Nat Prod Rep.* 2011; 28:1937–1955. [PubMed: 21979811]
22. Sherr CJ. Divorcing ARF and p53: an unsettled case. *Nat Rev Cancer.* 2006; 6:663–673. [PubMed: 16915296]
23. Kruse JP, Gu W. Modes of p53 regulation. *Cell.* 2009; 137:609–622. [PubMed: 19450511]
24. Ozenne P, Eymin B, Brambilla E, Gazzeri S. The ARF tumor suppressor: structure, functions and status in cancer. *Int J Cancer.* 2010; 127:2239–2247. [PubMed: 20549699]
25. Llanos S, Clark PA, Rowe J, Peters G. Stabilization of p53 by p14ARF without relocation of MDM2 to the nucleolus. *Nat Cell Biol.* 2001; 3:445–452. [PubMed: 11331871]
26. Pochampally R, Fodera B, Chen L, Shao W, Levine EA, Chen J. A 60 kd MDM2 isoform is produced by caspase cleavage in non-apoptotic tumor cells. *Oncogene.* 1998; 17:2629–2636. [PubMed: 9840926]

27. Lee WH, Loo CY, Bebawy M, Luk F, Mason RS, Rohanizadeh R. Curcumin and its derivatives: their application in neuropharmacology and neuroscience in the 21st century. *Curr Neuropharmacol*. 2013; 11:338–378. [PubMed: 24381528]
28. Yang F, Lim GP, Begum AN, Ubeda OJ, Simmons MR, Ambegaokar SS, et al. Curcumin inhibits formation of amyloid beta oligomers and fibrils, binds plaques, and reduces amyloid in vivo. *J Biol Chem*. 2005; 280:5892–5901. [PubMed: 15590663]
29. Singh PK, Kotia V, Ghosh D, Mohite GM, Kumar A, Maji SK. Curcumin modulates alpha-synuclein aggregation and toxicity. *ACS Chem Neurosci*. 2013; 4:393–407. [PubMed: 23509976]
30. McCrate OA, Zhou X, Cegelski L. Curcumin as an amyloid-indicator dye in *E. coli*. *Chem Commun (Camb)*. 2013; 49:4193–4195. [PubMed: 23287899]
31. Hadjiolov, AA. The nucleolus and ribosome biogenesis. Springer-Verlag: 1985.
32. Boisvert FM, van Koningsbruggen S, Navascues J, Lamond AI. The multifunctional nucleolus. *Nat Rev Mol Cell Biol*. 2007; 8:574–585. [PubMed: 17519961]
33. Horky M, Kotala V, Anton M, Wesierska-Gadek J. Nucleolus and apoptosis. *Ann N Y Acad Sci*. 2002; 973:258–264. [PubMed: 12485873]
34. Vlatkovic N, Boyd MT, Rubbi CP. Nucleolar control of p53: a cellular Achilles' heel and a target for cancer therapy. *Cell Mol Life Sci*. 2013
35. Lohrum MA, Ludwig RL, Kubbutat MH, Hanlon M, Vousden KH. Regulation of HDM2 activity by the ribosomal protein L11. *Cancer Cell*. 2003; 3:577–587. [PubMed: 12842086]
36. Suzuki A, Kogo R, Kawahara K, Sasaki M, Nishio M, Maehama T, et al. A new PICTURE of nucleolar stress. *Cancer Sci*. 2012; 103:632–637. [PubMed: 22320853]
37. Moos PJ, Edes K, Mullally JE, Fitzpatrick FA. Curcumin impairs tumor suppressor p53 function in colon cancer cells. *Carcinogenesis*. 2004; 25:1611–1617. [PubMed: 15090465]

Highlights

- Curcumin forms fluorescent foci within the nucleus of glioblastoma cells
- Co-localization studies reveal curcumin homes to the nucleolus
- Nucleolar homing precedes induction of apoptosis and correlates with p53 stabilization

Figure 1. Curcumin fluorescence in cultured glioblastoma and hepatoma cells
Cells were incubated with 20 μ M curcumin for 24 h, fixed, stained with Hoechst and visualized by confocal fluorescence microscopy. Scale bar: 10 μ m.

Figure 2. Effect of calf thymus DNA on the spectral properties of curcumin

UV/Vis absorbance (A) and fluorescence (B) spectra of a 50 μ M curcumin solution were obtained in the absence (a) or presence of 14 μ g (b), 49 μ g (c) 79 μ g (d) calf thymus DNA. Curve (e) depicts spectra of 79 μ g calf thymus DNA alone. Absorbance spectra were collected from 350 to 500 nm while fluorescence emission spectra were obtained from 480 to 600 nm (excitation 420 nm).

Figure 3. Cellular localization of curcumin and PI

SF-767 cells were incubated with 20 μM curcumin for 24 h and stained with PI. Images were obtained with a Zeiss LSM710 confocal microscope at $63\times$ magnification. The upper panel shows Z-stack images (0.56 μm thick slices) from a representative cell. The lower panel is a two-dimensional intensity histogram for the entire Z stack. Data represents pairwise distribution of pixel intensities obtained from the green (curcumin) and red (PI) channels on a 0 – 255 scale.

A. SF-767 **B. HepG2**

Figure 4. Intracellular localization of curcumin and nucleophosmin

SF-767 (A) and HepG2 (B) cells were incubated with 20 μ M curcumin at 37 °C. After 24 h the cells were fixed and curcumin detected by confocal fluorescence microscopy. Anti-nucleophosmin IgG was added to the fixed cells and Alexa Fluor 647 labeled secondary antibody was used for detection. Cell nuclei were stained with Hoechst 33342. Scale bar: 10 μ m.

Figure 5. Effect of nuclear protein extract on curcumin fluorescence emission

Nuclear protein was extracted from HepG2 cells as described in Section 2.5. Samples were excited at 420 nm and fluorescence emission monitored from 450 to 600 nm. Curve a) 40 µg nuclear protein extract; b) 20 µM curcumin; c) curcumin plus 20 µg nuclear protein extract and d) curcumin plus 40 µg nuclear protein extract.

Figure 6. Temporal aspects of curcumin nucleolar homing and initiation of apoptosis

SF-767 cells were exposed to 20 μ M curcumin for 4 and 24 h. At the indicated times, live cells were incubated with a biotinylated annexin V followed by Alexa fluor 350 labeled streptavidin. Cells were co-stained with PI. Images were obtained by confocal microscopy. Far left) DIC image of cellular morphology. Far right) merged image of all channels. Scale bar: 10 μ m.

Figure 7. Effect of curcumin on the subcellular localization of p14^{ARF}

SF-767 cells were incubated with 20 μ M curcumin for 0 h and 2 h, fixed and stained for p14^{ARF} and nucleophosmin and examined by confocal fluorescence microscopy. Treatment conditions are indicated on each panel.

Figure 8. Effect of curcumin on the cellular content of p14^{ARF}, MDM2 and p53

SF-767 cells were incubated with 20 μ M curcumin for the indicated times. Cells were lysed and 10 μ g total protein separated by SDS-PAGE. Proteins were transferred to PVDF membrane and probed for p14^{ARF}, MDM2, p53 and β -actin as described in Section 2.7. The image depicted is representative of an experiment that was performed on three separate occasions with similar results.