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Cytological and Subcellular Response of Cells Exposed to the Type-1 RIP Curcin and its Hemocompatibility Analysis

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Curcin, a type 1 ribosome inactivating protein (RIP) is investigated here for its cellular competence on six mammalian cell lines. Cells exposed to curcin (100 µg/ml) for 72 h exhibited significant cellular metabolic arrest, with the cancer cell lines being more sensitive. The viability assessment of the cancer cells in a 3D cell culture based assay revealed highly restricted sprouting and proliferation with near to complete dead cell population. Prominent mitochondrial dysfunction, elevated reactive oxygen species levels, nuclear degeneration, structural/mechanical destabilization and suppression of defense mechanisms were imminent with the RIP treated cells. Expression levels of nuclear factor κB (NF-κB), cytoskeletal focal adhesion kinases (FAK) and vinculin were significantly diminished. Vital cellular organelles as nucleus, mitochondria and actin were severely incapacitated on RIP exposure resulting in multimodal apoptosis and necrosis. The ability of curcin to impart comprehensive shutdown of the cells, especially cancer cells, complemented with its hemocompatibility, opens up possibilities of utilizing this ribotoxin as a prospective therapeutic candidate against cancers of diverse origins.

Phytomolecules have long served a pivotal role as potent active components towards strengthening and widening the pharmaceutical options against various ailments. Ribosome inactivating proteins (RIPs) are one such highly cogent group of proteins, mainly of plant, bacterial and fungal origin, which have conventionally hosted the ability to irreversibly depurinate the ribosomal RNA and terminate protein synthesis^{1–3}. Traditionally, RIPs are recognized to deadenylate the large ribosomal RNA with high site specificity and inactivate the ribosomes owing to their RNA N-glycosidase activity^{4–6}. The potential of some of the RIPs to depurinate the rRNA at multiple sites⁷ along with several other polynucleotide substrates⁸ has been addressed. Broad and diverse pharmacological attributes have been associated with RIPs, as protein synthesis-inhibitory, immunosuppressive, anti-tumor, antiviral, anti-HIV and abortifacient traits to mention a few⁹.

The fascinating multimodal activities of RIPs have since long time generated interest towards developing antitumor drugs that selectively target tumor cells¹⁰. A stern resolve in this regard, particularly in the case of type 1 RIPs, are the immunotoxins^{11–15}, RIPs conjugated to specific antibodies, which may present promising options for treating various diseases and interestingly against HIV-infected cells¹⁶. The action mechanism of RIPs have been extremely imperceptible and numerous accounts have been put forth to decipher the same. It was earlier believed that the mortality of cells treated with RIPs or RIP-immunotoxins was a result of protein synthesis inhibition alone with subsequent cellular necrosis, however, a stark similarity between RIP treated cells and cells undergoing apoptosis or programmed cell death (PCD) has been observed^{17–21}.

Jatropha curcas is a well-known and mass cultivated multipurpose plant with immense medicinal value^{22–24}. Felke²⁵ isolated the toxic principle curcin, from the seeds of this plant which is a type 1 RIP possessing strong inhibitory activity on protein synthesis¹ along with N-glycosidase and antitumor activities^{4,26–28}. Recent works have demonstrated the cancer directed therapeutic potential of this RIP in conjunction with peptides and nanoparticles^{29,30}, additionally emphasizing the compatibility of curcin to collaborate with various delivery strategies without compromising on its activity.

In this study, we explore the superior toxicity of curcin against normal and cancer cell lines. Morphological events related to organelle degeneration and membrane protein disruptions during apoptosis have also been

described with an emphasis on the inherent ability of curcumin to affect Nuclear factor κ B (NF- κ B), Focal adhesion kinases (FAK), Poly (ADP-ribose) polymerase (PARP) and reactive oxygen species (ROS). Further, the toxic effects of curcumin on 3D in vitro cancer mass was assayed and recorded, disrupting and destroying the cancer colonies with intense proficiency. In addition, suppression of the anti-apoptotic survivin and the extra cellular matrix binding vinculin proteins, have been evidenced correlating the multiple modes of action of this potent phytochemical to induce apoptotic stimuli and consequent cytotoxic reactions.

Results

Cytotoxic effects of curcumin. The level of cellular metabolic activity greatly depends on proper functioning of organelles and any dysfunction leads to impaired cyto-metabolism. With respect to the cell lines under study, cytotoxic rather than cytostatic effects of curcumin were observed. Dose dependent toxicity was recorded, with the severity increasing with increase in curcumin concentration (Fig. 1A). In the case of normal cell lines, L929, HCN-1A and HuVEC at the highest concentration of curcumin (100 μ g/ml), approximately 70%, 35% and 60% of cells, respectively, were viable. The fibroblast L929 has a higher mitotic index when compared to the other two normal cell lines, which may be a factor in the relatively increased viability. Endothelial cells, though with lower mitotic index, showed only moderate response to curcumin treatment. This could be due to the efflux transport mechanism these cells exhibit

as part of being in vascular system, exporting molecules across tissues and blood stream.

The effect of curcumin on cancer cells was profound with glioma registering below 20% viability at 100 μ g/ml and about 65% at 1 μ g/ml of the RIP. The mortality rate of MCF-7 was nearly 70% at 100 μ g/ml of curcumin and around 45% at 10 μ g/ml. Though susceptible, yet the most resistant among the cancer cell lines was the multi-drug resistant MDA-MB-453 presenting around 45% viability at the highest and nearly 55% at the mid concentration of curcumin. As these cell lines possess more or less similar mitotic index, the variance in sensitivity to curcumin may be linked to their drug resistance, which is unique to each cell population. We speculate that less sensitive MDA-MB-453, which is a triple negative multi-drug resistant variant, exhibited better drug efflux when compared to the other cancer cell lines. Glioma's increased sensitivity to curcumin shows that it exhibits nil or minimal drug resistance.

Curcumin treated cells predominantly exhibited apoptotic and necrotic characteristic events at hypodiploid regions, in many cases both signals recorded from same cells, invoking the complexity of toxicity rendered by the toxin (Fig. 1B). These observations were emulated in the flow cytometry (Supplementary Fig. S2) analysis, with curcumin treated populations exhibiting prominent apoptosis and necrosis when compared to the controls.

To visually ascertain the metabolic arrest of cells treated with curcumin, a new and simple approach based on the alamar blue cytotoxicity assay was attempted. The alamarBlue® Assay incorporates a fluorometric growth indicator based on detection of metabolic

Figure 1 | (A) Cytotoxicity profile of curcumin on normal (L929, HCN-1A, HuVEC) and cancer cells (Glioma, MCF-7, MDA-MB-453). Control group of cells (devoid of curcumin) showed 100% viability whereas the curcumin treated cells presented dose dependent toxicity. Glioma cells were the most susceptible at all concentrations of curcumin with the lowest viability at 100 μ g/ml of curcumin (17% viability). The other cell lines presented a maximum viability of 70% for L929, 35% for HCN-1A, 60% for HuVEC, 30% for MCF-7 and 45% for MDA-MB-453 at 100 μ g/ml curcumin. Columns, means of three independent experiments; bars $\pm$ SD. * Significantly different from corresponding control group by a one-way ANOVA (* $p < 0.05$). (B) Early apoptotic cells stained with annexin V and necrotic cells exhibiting PPI intake were dominant in curcumin treated population. Majority of cells expressed both cell death signals, proposing the induction of both cytotoxic mechanisms simultaneously by curcumin. (C) Alamar blue assisted confocal laser microscopy detection of cellular metabolism. Images g–l represents the fluorescent intensity signals from control and s–x of curcumin treated cells. The presence of a bright hue in control cells signifies the reduction of non-fluorescent resazurin to the fluorescent resorufin dye, indicating the active metabolic state of the cells. On the contrary, the curcumin treated cells were devoid of any significant levels of fluorescence, evidence of their imminent metabolic arrest.

activity. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth. The underlying principle involves the reduction of non-fluorescent resazurin to a fluorescent dye, resorufin by metabolically active cells, which is not possible with cells whose metabolic activities have been terminated. Correspondingly, control cells showed highly intense fluorescent signals (Fig. 1Cg–l) which is directly proportional to cell's viability and its metabolic activity. In the case of curcumin treated cells, nil or extremely weak intensity of fluorescence was recorded (Fig. 1Cs–x), translating directly to the diminished metabolic potential of the cells. The arrested metabolic state of the cells provides insight into the intrinsic stress that cells undergo due to exposure to toxins as curcumin. To emphasize on the potential significance of this method, specifically dead cell population or cells undergoing apoptosis was concentrated upon in the case of curcumin treated cells, which enables the enhanced discrimination potential of the metabolically active and dead cells.

In addition, corresponding morphological analysis revealed that curcumin treated cells showed specific similarities to those undergoing a suicidal apoptotic fate. Visual indications included cellular content shrinkage, shriveling, membrane blebbing, plasma membrane integrity loss and clumping of cells, loss of organelle recognition and appearance of apoptotic bodies, among others.

In vitro 3D cancer colony regression and extirpation. The role of curcumin, as a cancer repressor was analyzed with 3D colony assay. Cancer cells grow as 3D colonies, resembling tumor mass, on gel matrices. Accordingly, glioma developed into a 3D spheroid with the main colony being surrounded by smaller ones (Fig. 2a1), whereas MCF-7 and MDA-MB-453 grew as multi layered cellular mats (Fig. 2b1,c1). 96 h curcumin treated groups showed decreased colony area (Fig. 2a4) and formation of elongated tubes projecting outwards of the cancer mass (Fig. 2d1,d2,e1,e2). Glioma spheroid remained as a dead mass of cells (Fig. 2a4) which kept on shrinking in due progression to curcumin exposure and finally disintegrated. MCF-7 and MDA-MB-453 cancer masses were highly disrupted and reduced to tiny patches (Fig. 2b4,c4) associated with each other by thick tubular protrusions (in the case of MCF-7, Fig. 2e1,e2) which eventually disintegrated (Fig. 2b4). Also, majority of the treated cells had undergone an apoptotic or necrotic fate (Fig. 2a6,b6,c6). Calcein-AM, acetoxymethyl ester of calcein, is highly lipophilic and cell membrane permeable. Though Calcein-AM itself is not a fluorescent molecule, the calcein generated from Calcein-AM by esterase in a viable cell emits strong green fluorescence differentiating live and dead cells. On the other hand, PPI, a nuclei staining dye, cannot pass through a viable cell membrane. It reaches the nucleus by passing through disordered areas of dead cell membrane, and intercalates with the DNA double helix of the cell to emit red fluorescence. The observations based on the fluorometric assay were in line with this principle, correlative of the cell membrane disruptions and related apoptotic/necrotic events on curcumin exposure. The cancer colonies failed to propagate in culture even after regular media supplementation, evidencing the loss of sustenance and proliferation potential of the cells. These results reemphasize the profound detrimental effects administered by curcumin to monolayer as well as multi-layered 3D carcinomas.

ROS generation and mitochondrial response. ROS triggering ability of curcumin was complimentary to the cell viability data, reiterating the dose dependent activity of curcumin. In normal and cancer cells alike, ROS was significantly more than that of control group. L929 cells showed an ROS generation of nearly 50% more than the control cells whereas HCN-1A and HuVEC cells produced a little more than 10 and 15% of the amount of ROS of control group, respectively. The cancer cells in particular exhibited enhanced ROS generation tendencies at all the concentrations of curcumin tested,

glioma and MDA-MB-453 being more responsive. Glioma cells presented an ROS capacity of nearly double that of the untreated cells. Meanwhile MCF-7 and MDA-MB-453 cells showed an increase in ROS of 40% and 75% respectively (Fig. 3A).

To analyze the secondary effects of ROS, immediate mediator of ROS-induced cell death mediated signaling, NF- κ B activation was profiled. Increased absorbance intensity of NF- κ B was observed in test groups (Fig. 3B). In the case of normal cell lines, L929, HCN-1A and HuVEC, the amount of activated NF- κ B was slightly higher than controls whereas, glioma showed nearly twice the intensity of NF- κ B compared to control. MCF-7 and MDA-MB-453 also presented noticeable increase in NF- κ B levels.

Also, it could be clearly observed from Fig. 3C that the curcumin treated cells showed diminished or any calcein fluorescence (Fig. 3Ca5,b5,c5,d5,e5,f5), depicting the leaching out of dye from mitochondrial pores, induced as a result of the increased ROS load in the mitochondria. In addition, loss of prominent fluorescence and patched signals in the cytosol portrayed the severe damage to mitochondrial membrane (Fig. 3Ca6,b6,c6,d6,e6,f6), induced as a result of toxin exposure. Additional prominent loss of mitochondrial integrity (Supplementary Fig. S3) has also been provided as concrete proof of the imminent toxicity of curcumin on the cell's energy hub.

Nuclear deterioration and PARP inhibition. Significant nuclear deterioration was observed with cells exposed to curcumin. The effects were prominent in 72 h curcumin incubated cells with the formation of micronucleus, nuclear shrinkage, condensation of nuclear material (Fig. 4Ab4,d4,f4,h4,j4,l4) and total loss of morphological integrity (Fig. 4Aa4,c4,e4,g4,i4,k4). Time dependent progression of nuclear deformities with increase in curcumin incubation was evident from the observations. The poly (ADP-ribose) polymerase (PARP) family of enzymes plays a critical role in the maintenance of DNA integrity as part of the base excision pathway of DNA repair. The results clearly indicated that curcumin inhibited PARP by nearly 45% (Fig. 4B) at highest concentration and the inhibition rate was concentration dependent. PARP inhibition leads to cells being prone to DNA damage consequently switching on the DNA damage mediated apoptotic pathway, correlative of the observations with nuclear distortions on curcumin exposure.

Cytoskeletal protein degeneration. Visual observations of curcumin treated cells revealed a major structural and morphological transition from the normal (untreated) cells, with cytoplasmic shrinkage (Fig. 5m–r) and microtubule like outgrowths (Fig. 4k3,i2), highly arrested neuritic and dendritic structures (Fig. 6o) and eventual complete cellular collapse. An analysis of the cytoskeletal protein actin revealed that control groups devoid of curcumin presented well-organized and elaborate actin network scaffolding throughout the cytoplasm (Fig. 5Ag–l) whereas cells treated with curcumin showed prominent actin filament degeneration and distortion (Fig. 5As–x). The fine network of actin filaments were reduced to circumferential lining (Fig. 5Av) or to highly condensed matter (Fig. 5At, w, x). These results directly correlate to the morphological changes observed with curcumin treated cell lines due to structural integrity loss. The expression levels of FAK, one of the main signaling molecules involved in variety of actin-mediated signaling pathways, in test group of cells decreased when compared to the controls (Fig. 5B). Curcumin treated HCN-1A and MCF-7 showed nearly 50% decrease in FAK expression when compared to control groups. Glioma and HuVEC showed nearly 40% reduced expression of FAK, whereas, L929 and MDA-MB-453 showed very slight decrease in FAK expression. The data clearly implied a significant decrease of FAK levels on curcumin treatment, which could have led to the steric hindrance and physical deformation of actin network.

Vinculin. Another important observation was made with vinculin, which is one of the membrane proteins that play a major role in cell-cell and cell-matrix interaction. The expression of vinculin is

Figure 2 | In vitro 3D cancer mass extirpation and live/dead analysis. (A) Post 96 h *in vitro* tumor induction, curcumin at 100 $\mu\text{g/ml}$ was added to Glioma, MCF-7 and MDA-MB-453. Treated groups presented marked difference from the controls in all aspects. Cancer mass regression with respect to size (a4) in case of glioma and complete disruption of the 3D cellular mat in the case of MCF and MDA-MB were evident. Sprouting of new independent cancer colonies (a1, arrows) were totally arrested in the curcumin treated samples. The live/dead results were overwhelming as nearly 99% of the treated population was positive to PPI staining (a6,b6,c6) with minimum to nil calcein staining (a5,b5,c5) as compared to the controls (a2,b2,c2). (B) Stress-tube formation in the case of Glioma (d1,d2 arrows) and MCF-7 (e1,e2 arrows) was observed post 48 h curcumin exposure. The elongated structures were observed to originate from the periphery or within the colonies, evidently reaching out for substratum, nutrition or cell-cell contact signaling. The tubes appeared from 48 h curcumin treatment as thick cellular projections, which eventually shrunk and disintegrated by 96 h. The appearances of such cellular adaptations for survival are proof of the stress induced by curcumin to the cells, which could be beneficial in arresting the tumor growth and proliferation.

essential for the cell to establish contact with its microenvironment. Vinculin expression was completely down-regulated after curcumin administration and the cells exhibited loss of structural and mechanical integrity (Fig. 6A). Control group presented vinculin expression throughout the cellular span (Fig. 6b, f, j, n, r, v) whereas the curcumin treated cells exhibited diminished expression with lack of discrete signals (Fig. 6d, h, l, p, t, x) indicating the loss of cell's structural integrity, culminating in reduced inter-cellular and cell-substratum contact signaling.

Survivin. Because survivin has a recognized role as an inhibitor of apoptosis, we next investigated whether and how the observed down-regulation of survivin by the toxin would relate to the known ability

of curcumin to induce apoptosis. Control group of cells presented orderly distributed survivin expression (Fig. 7b, f, j, n, r, v) whereas in the case of curcumin treated cells, the expression was significantly arrested (Fig. 7d, h, l, p, t, x) indicating the loss of cell's survival cues and increasing their susceptibility to apoptosis. Thus, the magnitude of survivin down-regulation caused by curcumin closely correlated with the extent of apoptosis and with the degree of short-term growth and survival (as determined by alamar blue assay), as well as long-term survival (as determined by 3D cancer colony assay) of these cells.

Lipid peroxidation assay. Mitochondria are known as the main source for the generation of ROS. These ROS are quenched by

Figure 3 | (A) Reactive oxygen species (ROS) production, assayed using the fluorogenic dye 2',7'-dichlorofluorescein diacetate (DCFDA) on control and cells treated with curcumin at 100, 10 and 1 µg/ml. The ROS production of control cells were considered as 100% and the ROS of curcumin treated cells represented correspondingly. The results were complimentary to the cytotoxicity data with all the curcumin treated cells showing increased ROS generation compared to control cells. Among the normal cells, the fibroblast L929 exhibited around 50% increase in ROS levels when compared to controls, while HCN-1A and HuVEC presented slight increase. Cancer cells, presented considerably elevated ROS values with MCF-7 being moderate. (B) the normal cells presented slightly higher levels of activated NF-κB than their controls. Glioma cells showed maximum NF-κB activity, nearly twice of the control while MCF-7 and MDA-MB-453 had noticeable increased expressions. Columns, means of three independent experiments; bars $\pm$ SD. * Significantly different from corresponding control group by a one-way ANOVA (* $p < 0.05$). (C) Change of mitochondrial membrane potential transition by curcumin. Effect of curcumin on cellular calcein fluorescence. Transient openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. Curcumin caused a rapid release of calcein from cells, as shown by the disappearance of the green calcein fluorescence post treatment. The discrete appearance of calcein in mitochondria in the controls was absent to a major extent in the curcumin treated cells. The highly diminished calcein fluorescence (a5,b5,c5,d5,e5,f5), was indicative of the leaching out of dye from mitochondrial pores induced by curcumin. The mitochondrial toxicity of curcumin was evident from the lack of definite signals and presence of bright fluorescence throughout the cytosol (a6,b6,c6,d6,e6,f6).

certain classes of enzymes as superoxide dismutases (SOD) and catalases in the cell. Erythrocytes on the other hand, are incapable of producing SOD and catalases, hence are vulnerable to the extraneous toxicants and the cell membrane may be easily damaged during lipid peroxidation. Lipid peroxides, reactive products of lipid peroxidation, have deleterious effects on the cell membrane. Curcumin exposure increased the LPO rate nearly 2 folds when compared to control sample (Fig. 8C). This clearly indicated the ability of curcumin to oxidize lipids of cell membrane causing a permanent damage. The cellular ROS generation induced as a response to curcumin exposure (Fig. 4A) has already been established, therefore the current observations may be a linked phenomenon.

Hemocompatibility. With the cytotoxic behavior of curcumin, one can expect that it exhibits lesser compatibility to blood cells. However, curcumin was found to be comparatively lesser toxic towards blood cells. Erythrocyte sedimentation level (Fig. 8A) and hemolysis (Fig. 8B) analysis depicted slight change in the values compared to the control group. The platelet adhesion quantification assay by LDH (Fig. 8D) showed that the adhesion onto curcumin-coated plates was extremely lower than on glass or polystyrene substrates proving the hemocompatible nature of curcumin. This clearly depicted that curcumin is incapable

of inducing platelet adhesion and subsequent activation. As platelet activation can lead to complications as primary hemostasis, the inability of curcumin to do so could favor in its consideration as a blood-compatible molecule. The thrombus (Fig. 8E) and plasma recalcification profile (Fig. 8F) showed that there is no significant variation of coagulation levels from control samples, though a slight increase in plasma recalcification was found in curcumin treated samples post 30 mins, when compared to control samples. The initial slope of the linear portion of each profile was calculated as a measure of the rate of clot formation. No difference was observed between the slopes of control (0.0061) and curcumin (0.0055) treated PPP that confirms that curcumin is comparatively less coagulative. Though the basic study of hemocompatibility depicted curcumin as safer, it is necessary for much detailed study as this knowledge could suggest significant clinical interest.

Discussion

More than 30% of the pharmaceutical agents currently available are of natural origins or their derivatives which form the major source of therapeutic options against a wide range of diseases^{31,32}, signifying not only the advantages of such compounds, but urging the need for newer and more potent versions. In this study, we reported on the

Figure 4 | (A) Curcumin induced the nuclear morphological change in cell lines under study. Representative DAPI staining showing homogeneous staining of the nucleus in viable cells and irregularly stained micro-bodies as a result of chromatin condensation and nuclear fragmentation of the curcumin treated groups were visible (b4,d4,g4,j3,j4). Curcumin treatment induced significant cytoplasmic shrinkage with the nucleus appearing as the sole organelle with higher volume ratio when compared to the whole cytoplasm. As the nucleus suffers lack of a buffer environment, the effects of the RIP seem to be more pronounced resulting in severe nuclear toxicity. (B) The ability of curcumin on inhibiting PARP enzyme activity was analyzed. The dose dependent inhibition of PARP was observed with nearly 50% inhibition at 100 $\mu\text{g/ml}$ of curcumin. PARP inhibition is highly significant since it sensitizes cells to DNA damaging agents. The combined PARP inhibition and observed nuclear toxicity could be a linked phenomenon of curcumin exposure. Columns, means of three independent experiments; bars $\pm$ SD. * Significantly different from corresponding control group by a one-way ANOVA (* $p < 0.05$).

multi-dimensional cytotoxic effects of curcumin on different cell lineages. Previous studies on curcumin have shown that like other RIPs, curcumin induces cell death^{33,34} predominantly by inhibiting cell-free translation and protein synthesis in reticulocyte lysate systems^{35,36}. Type 1 RIPs have been portrayed as non-toxic or compara-

tively lesser toxic agents with respect to the type 2 RIPs as the former are devoid of cell binding moieties which eventually render them incapable of cellular entry on their own, though with exceptions of some specialized cells presenting themselves as vulnerable. Though earlier works narrate the anti-tumor ability of curcumin, detailed study

Figure 5 | The dynamics of actin microfilaments, comprising polymerization and cytoskeletal arrangement during curcumin-induced cell death was imaged with rhodamine phalloidin. (A) Actin was present in its intact and defined structural form in the control group of cells (g–l). The structural rigidity and integrity of actin was observed to be completely lost in curcumin treated cells (s–x). Correlative to the bright field images of the treated cells (m–r), which present visual evidence of loss of structural integrity, the actin filaments were also disorganized and in many cases confined to discrete spots, signifying the role of curcumin in depolymerization of the actin filaments along with disruption of the cellular cytoskeleton. (B) Corresponding to the visual observations of cellular skeletal breakdown, the FAK expression in curcumin treated cells was observed to be lower than the controls. Nearly 50% decrease in FAK with HCN-1A and MCF-7 was seen as compared to the control groups. A 40% reduction in FAK expression was observed in glioma. L929 and MDA-MB-453 presented slight decrease in FAK expression when compared to their controls. Columns, means of three independent experiments; bars $\pm$ SD. * Significantly different from corresponding control group by a one-way ANOVA (* $p < 0.05$).

on the factors influencing apoptosis have not been reported. We demonstrate the toxic potential of such a type 1 RIP on normal and cancer cell lines alike, which depicts the varying degree of toxicity to the cell types. Curcumin significantly inhibited cell proliferation that arrested or degenerated various essential organelles involved in major metabolic pathways. All the cell types, irrespective of normal or cancer origin were susceptible to curcumin.

Programmed cell death (PCD), is a genetically regulated process and is pivotal to the homeostasis of all cells. An apoptotic event leading to monitored cellular disorganization culminates from the disturbance of nuclear dynamics complemented by the distortions in the vital cytoplasmic and skeletal organelle structure, function and distribution. It is characterized by cytoplasmic retraction, cytoskeletal disorganization, chromatin condensation, chromosomal DNA

Figure 6 | Fluorescence microscopy after double immunocytochemical detection of the cytoskeletal component, vinculin in cell lines under study revealed the marked difference in expression patterns of control and curcumin treated cells. The discrete signals of vinculin throughout the cellular body in the control (b, f, j, n, r, v) was highly diminished in the curcumin treated cells with weak or nil fluorescence (d, h, l, p, t, x) signifying the action of curcumin on vinculin expression and disruption of the structural integrity of cells. The bright field images coincided with cells experiencing severe structural breakdown, with cellular collapse and loss of inter-cellular contacts, symptoms relative to the lack of vital structural and contact signaling cues as vinculin.

fragmentation, mitochondrial swelling with alterations in the membrane potential and permeability, decrease in the anti- and increase in pro-apoptotic stimulus, loss of plasma membrane integrity, decreased metabolic activity, and the packaging of cellular constituents into apoptotic vesicles^{37,38}.

Here, we demonstrated that curcumin-treated cell lines exhibited several morphological alterations similar to autophagy and apopto-

sis, predominantly. The cells exhibited enhanced ROS production and mitochondrial damage, with ROS playing additional role in the lipid peroxidation of erythrocytes. Loss of membrane integrity, consequentially the mitochondrial potential, elevated the NF- κ B and decreased the anti-apoptotic survivin expression. Survivin's function is to preserve the mitotic apparatus and to allow normal mitotic progression, whereas its anti-apoptotic function is executed via its

Figure 7 | Expression of survivin in control and curcumin treated cell populations was analyzed to determine the competence of cellular defensive apparatus against the invading toxin. Survivin, one of the major defense molecules of cells, especially cancer cells was significantly downregulated in curcumin exposed cells. The clear and discrete immunocytochemical signals of survivin in the cytoplasm of control cells (b, f, j, n, r, v) was natural of the cells displaying ordered and active defense machinery. On the other hand, the RIP exposed cells presented relatively diminished, if present, survivin signals (d, h, l, p, t, x) signifying the action of curcumin on the expression of the anti-apoptotic survivin.

Figure 8 | Blood compatibility of curcumin. (A) Erythrocyte sedimentation rate post curcumin exposure was analyzed visually with Triton X and PEG as positive and negative controls, respectively. The photograph of erythrocyte sedimentation reveals that there was no significant deviation of curcumin incubated samples from the negative control whereas the Triton X treated blood exhibited highly elevated levels of ESR. (B) The ESR results were emulated in the RBC hemolysis analysis as well where compared to negative control (PEG), curcumin induced hemolysis by a meager 5%, in contrast to the significantly increased levels with Triton X (C) Lipid peroxidation analysis revealed that the LPO was nearly double in the case of curcumin treated sample when compared to the control. This may be a phenomenon which correlates to the ROS generation tendency of curcumin in cells. As blood cells do not have a proper mechanism to curb the elevated ROS, curcumin was able to induce higher LPO levels. (D) The number of adhered platelets on polystyrene plates and curcumin coated polystyrene plates was determined by colorimetric quantification of LDH activity post cell lysis. The results depict that platelet adhesion was nearly half on curcumin-coated plates when compared to polystyrene plates suggesting the inability of curcumin to induce significant platelet adhesion and possible subsequent activation. (E) Whole blood clotting time analysis. Curcumin exposed and negative control blood samples were incubated at 37°C. At equal intervals, samples were collected, diluted with distilled water and the absorbance of hemoglobin released was measured. Curcumin exposed as well as negative control showed similar blood clotting patterns. (F) Clotting kinetic profiles of the absorbance at 405 nm as a function of time for PPP incubated with curcumin. Citrated PPP (without the addition of calcium) serves as a negative control. The slope of the linear portion of each curve was examined as a measure of clotting rate. Though there was a slight increase in the plasma recalcification, the slope values were comparatively equal. Columns, means of three independent experiments; bars $\pm$ SD. * Significantly different from corresponding control group by a one-way ANOVA (* $p < 0.05$).

ability to prevent caspase activation. It has been shown in several other experimental systems that the down-regulation of survivin expression, for example by antisense or siRNA approaches results in elevated “basal level” apoptosis^{39–41}. With curcumin exposure, mitochondrial membrane potential is lost, leading to the possible release of caspases. Evidently, with the downregulation of survivin, caspase

activation is unhindered, leading to the initiation of the apoptotic protocol.

The mitochondrial pore transition is a resultant of increased ROS load in the cytosol, which acts as pro-apoptotic factor influencing the mitochondrial permeability. Mitochondria are crucial in the control of apoptosis, being involved not only in the intrinsic but also the

extrinsic pathways. Mitochondrial membrane potential loss is linked to release of apoptotic factors such as cytochrome c and caspases, which in turn activate the downstream apoptotic signaling, guiding the cells to PCD^{42,43}. This mitochondrial pore transition occurs as the secondary effects of ROS elevation in cytosol, attributed here, to curcumin exposure. Furthermore, the condensation and shrinkage of nuclear material among treated cell lines, along with PARP inhibition ability of curcumin reinforced the notion of an autophagic cell death phenotype. NF- κ B is considered an important protein in regulating cellular responses as it belongs to the category of “rapid-acting” primary transcription factors present in cells in an inactive state which do not require new protein synthesis for activation, allowing for a rapid response to harmful cellular stimuli. Interestingly, NF- κ B has been described to be activated upon oxidative stress induced by cytotoxic or foreign substances. Upon ROS induction, I κ B kinases phosphorylate and trigger degradation of inhibitory I κ B proteins. This releases NF- κ B resulting in its activation and translocation into nucleus and act as transcription factor for apoptosis^{44–46}. The clear correlation of NF- κ B activation with ROS levels suggests that NF- κ B activation occurs under oxidative stress. We attribute the observed nuclear toxicity by curcumin to be from the translocation of NF- κ B from cytoplasm to nucleus, triggering the apoptosis-signaling pathway. In addition, PARP1 is known to be overexpressed in variety of cancers, and its expression has been associated with overall prognosis in cancer, especially breast cancer⁴⁷. A series of new therapeutic agents that are potent inhibitors of the PARP1 and PARP2 isoforms have demonstrated important clinical activity in patients with cancers⁴⁸. The loss of base excision repair capacity produced on PARP inhibition by curcumin can prompt the evaluation of this RIP as potential enhancer of DNA damaging cytotoxic chemotherapeutic molecules such as alkylating agents (for example, platinum, cyclophosphamide) and topoisomerase 1 inhibitors.

The morphological changes observed in curcumin treated cells were in accord with previous observations and presented ultrastructural alterations of organelles. It is widely known that apoptotic cells reorganize their cytoskeleton, with fragmentation of the microfilament bundles conferring plasticity to the cell and promote volume decrease as well as cell shrinkage⁴⁹. On the other hand, cytoskeletons are also important in the execution phase, when they form a cortical network under the plasmalemma, thus preserving cell morphology and plasma membrane integrity. The maintenance of these structures depends on high cellular ATP levels, so that they undergo disorganization in late apoptosis, when mitochondria suffer extensive depolarization. Using drugs that influence actin stabilization/depolymerization, it has been shown that stimulating alterations in the microfilaments status is sufficient to induce apoptosis⁵⁰. Curcumin treated cell lines also exhibited a considerable retraction of the cellular mass with detrimental effects on the cytoskeletal proteins, vinculin and actins. Earlier reports show that vinculin promotes traction force at FA, mediating conversion of forces generated in the cytoskeleton that drive retrograde flow into traction force on the ECM during FA maturation. The tail domain of Vinculin associates with F-actin acting as an effective component of the molecular clutch in determining the architecture of cell to matrix attachment. FAK is a nonreceptor protein tyrosine kinase that predominantly localizes in focal adhesion complexes (FAC). The actin remodeling or disruptions break up the FACs, thereby drastically affecting any activation of FAK, which is functionally linked to mature and stable FACs. The reduced cytoskeletal remodeling is thus likely to enlarge the initial cytoskeletal aberrations induced directly by curcumin. As FACs are multiprotein complexes and form direct contact between actin and extracellular environment, they have a role in actin mediated signaling^{51,52}. Thus, the loss of mature FACs on curcumin treatment implies significant aberrations in intracellular signaling pathways, which could culminate in a diverse variety of malfunctions in cellular homeostasis and should be considered as a highly warranted and extremely

significant toxicological effect of the RIP. It is also worth noting that the different organelles have reciprocal interactions with each other, both in viable and apoptotic cells. The actin cytoskeleton and microtubules can also interact with mitochondria, which are considered as the central processing organelles in most apoptotic pathways. Apoptotic signals (either from outside or inside the cell) converge to mitochondria that can initiate the execution phase of apoptosis.

We also observed lower metabolic activity and 3D tumor suppression post curcumin exposure. Apart from the prominent apoptotic indications, cyto-necrosis due to curcumin was observed which is thought to occur via binding of the protein to cell membrane, causing membrane destabilization, facilitating easy entry of propidium iodide.

Drugs intended for long-term contact with blood must be subjected to thorough scrutiny, since hemocompatibility is the most important aspect during any drug administration. Prospective drug candidates in contact with blood must not induce any pernicious effects as thrombosis, thromboembolisms, antigenic responses, destruction of blood constituents and plasma proteins, etc. In this study, the hemocompatibility of curcumin was investigated by analyzing the hemolytic index, erythrocyte sedimentation rate, plasma recalcification and platelet adhesion. Hemolysis was less than 5% for test samples and was well within the permissible limit set for biomaterials. ESR rate also supports the claim that curcumin exposure did not cause significant deleterious effects. In addition, the platelet adhesion study as well as plasma recalcification analysis assures the blood compatible nature of curcumin. Being an RIP, the observations made were interesting in lieu of considering curcumin for further in vivo therapeutic analysis.

In conclusion, we project curcumin as a highly cogent toxic RIP capable of multiple modules of action on shutting down the cellular machinery. The multitudes of activities encompassing ROS generation, loss of cellular organelle function and integrity, decline of mitochondrial membrane potential, induction of NF- κ B, disruption of the cytoskeletal scaffold leading to cellular collapse, suppression of FAK, and more interestingly the downregulation of survivin and membrane adhesion proteins, in all culminating into PCD. The 3D cancer mass regression and complete disruption by curcumin provides concrete supplementary proof for its inevitable activity on malignant solid tumors, eliminating the core of the cancer mass and with it, the chance of future proliferation and recurrence. As many RIPs have been proposed and tested as potent immunotoxins and few in nano-drug delivery for specific tumor targeting, and on the basis of the comprehensive destruction of cancer cells in nearly every possible aspect, we present curcumin as a potent option not only as a robust cytotoxic phytomolecule, but also as a puissant means to curb the progression of cancer-metastasis.

Methods

Cell culture. L929 (mouse fibroblast), MCF-7 and MDA-MB-453 (breast cancer), Glioma (brain cancer) were from Riken Bioresources, Japan and HCN-1A (neural) was from ATCC. HuVEC was from Gibco. L929, HCN-1A, MCF-7 and Glioma were maintained in T25 flasks using DMEM medium supplemented with FBS, while HuVEC was grown in Med 200 supplemented with LSGS and antibiotics (100 U/ml penicillin and streptomycin) and incubated in a 5% CO₂ incubator at 37°C. MDA-MB-453 were maintained in L-15 media supplemented with 10% FBS in an incubator at 37°C. The cells were sub-cultured every two-three days. For all confocal experiments, cells were cultured in 35 mm glass base dishes (Iwaki) 24 h.

Cytotoxicity assay. Approximately 5000 cells were seeded into each well of 96 well plates. After attaining visual confluency, curcumin was added at different concentrations (100 μ g/ml, 10 μ g/ml and 1 μ g/ml). Control group was devoid of curcumin. After 72 h of incubation with curcumin, cells were washed with PBS and 0.2 ml of respective medium was added. Cell viability was assayed with alamar blue in a 96 well plate reader (Multidetector microplate scanner, Dainippon Sumitomo Pharma).

Alamar blue assisted visual fluorescence detection of cytotoxicity. Curcumin (100 μ g/ml) was added to the test cells and incubated for 72 h. Cells were washed thoroughly with PBS and observed in PBS at an excitation wavelength of 561 nm in a confocal laser scanning microscope (CLSM, Olympus IX 81 under DU897 mode).

Analysis of apoptosis and necrosis. Apoptotic and necrotic dead cell staining was performed employing annexin-V and PPI respectively. The test cells were treated with curcun at 100 µg/ml for 72 h. Cells were washed post treatment, stained and imaged using CLSM under 488 and 561 nm excitation.

ROS generation. Cells were cultured in 96 well plates, 5000 cells/well and treated with 100 µg/ml, 10 µg/ml and 1 µg/ml of curcun. Control groups and blanks were maintained. After 72 h of treatment, 20 µl of DCFH-DA dye was added to each well at a concentration of 10 mM. The plate was incubated for 30 min and the fluorescence in each well was quantified at 480 nm excitation using a microplate reader. The fluorescence of the test and control cells was directly proportional to the amount of ROS produced.

NF-κB and FAK estimation. Approximately 5000 cells were seeded onto 96 well plates. Cells were grown under ambient condition for 24 h. 100 µg/ml of curcun was added to test wells and the plates were maintained at culture conditions for 72 h. The NF-κB estimation was carried out as per manufacturer's instruction and resultant absorbance was read spectrophotometrically at 490 nm. FAK estimation was carried out as per manufacturer's instruction with cell maintenance and curcun treatment conditions similar to NF-κB estimation.

Mitochondrial membrane transition pore assay. Approximately 25,000 cells were seeded onto 35 mm glass base dishes. Test group of cells were treated with 100 µg/ml of curcun. After 72 h, the plates were stained for pore transition as per manufacturer's instruction and analyzed.

PARP inhibition assay. 100, 10 or 1 µg/ml of curcun was incubated along with PARP enzyme and PARP cocktail mix, followed by assessing the inhibition rate of PARP according to the manufacturer's instructions. Negative control was maintained without curcun exposure.

Organelle disruption study. Curcun (100 µg/ml) was added to the test cells and incubated for 72 h with observations being made every 24 h till 72 h. DAPI and Mitotracker were freshly prepared and added to the cells. After incubation of 10 min (DAPI) and 45 min (Mitotracker) cells were washed with PBS (pH 7.4) and fresh media added prior to observation. Actin cytoskeletal staining was performed with phalloidin rhodamine on the 3rd day of curcun treatment. Cells were visualized at an excitation wavelength of 405 nm (DAPI), 488 nm (Mitotracker) and at 561 nm (Phalloidin Rhodamine).

Immunocytochemical assays for vinculin and survivin. Cells were treated with 100 µg/ml concentration of curcun for 72 h. After the incubation period, cells were washed and fixed using formaldehyde and acetone and treated with antibodies to either survivin or vinculin. After 1 h, the secondary fluorescent antibodies (Goat polyclonal Secondary Antibody to Mouse IgG - H&L (FITC) abcam) were added and the plates were incubated for 1 h. Plates were then washed and observed under CLSM.

3D cancer colony assay. Glioma, MCF-7 and MDA-MB-453 were loaded onto thick (3 mm) Geltrex® (Invitrogen) basement membrane matrix coated 96 well plates. The well-established cancer colonies (post 96 h) were supplemented with 100 µg/ml of curcun and effects monitored periodically. Live dead staining was performed using Calcein/PPI according to manufacturer's instructions, post 96 h curcun treatment.

Lipid peroxidation (LPO) assay. The reaction of MDA with TBA has been widely adopted as a sensitive assay method for lipid peroxidation⁵³. LPO assay characterizes the degradation of the TBA and the concentration of melondialdehyde reacted *in vitro* to give a fluorescent product that can be measured at 532 nm. Lipid peroxidation in RBCs due to curcun exposure was analyzed accordingly. 0.2 ml of healthy human blood (collected from volunteer) was incubated with 1.5 mL thiobarbituric acid, 0.2 mL of SDS and 1.5 mL of 20% acetic acid solution at 100°C for 1 h. Post incubation, the solution was centrifuged at 3500 rpm for 10 min and the supernatant was collected to measure the OD value.

Hemolysis. 100 µl of blood was incubated with 100 µl (100 µg/ml) of curcun solution for 3 h at 37°C. Positive and negative controls were maintained employing the use of either 100 µl of Triton-X and 40% PEG respectively. The mixtures were centrifuged at 800 g for 15 min. The degree of hemolysis was determined by measuring the absorbance of the supernatant at 540 nm, as previously reported⁵⁴. The absorbance value accounted for the amount of hemoglobin release induced by curcun.

Erythrocyte sedimentation. 100 µl of erythrocyte suspension (1×10^6 cells/ml) was incubated with 100 µl (100 µg/ml) of curcun or 40% PEG (negative control) or with Triton-X (positive control) in eppendorf for 1 h at 37°C. The erythrocyte sedimentation was investigated and recorded using digital camera.

Whole blood clotting time (thrombus formation). The thrombogenicity of curcun was evaluated using a whole blood kinetic clotting time method. Briefly, the clotting reaction was activated with the addition of 100 µl of (100 µg/ml) curcun to 100 µl of blood sample and incubated at 37°C for 15 min. Control samples are maintained without curcun exposure. Post incubation, to 50 µl of the test mixture, 25 µl of CaCl₂ was added. All samples were incubated at room temperature for 5, 20, 40 and 60 min. At the end of each time point, the samples were incubated with 3 ml of distilled water

for 5 min. Each well was sampled in triplicate (200 µl each) and transferred to a 96-well plate. The red blood cells that were not trapped in a thrombus were lysed with the addition of distilled water, thereby releasing hemoglobin into the water for subsequent measurement. The concentration of hemoglobin in solution was assessed by measuring the absorbance at 540 nm using a 96 well plate reader. The size of the clot is inversely proportional to the absorbance value.

Platelet adhesion. Platelet rich plasma (PRP) were isolated as previously described⁵⁵. PRP was dropped onto bare polystyrene culture plates or curcun coated culture plates and was incubated for 1 h at 37°C under static conditions. The suspension was aspirated and each well was rinsed carefully three times with PBS. The number of adherent platelets was determined by detecting the amount of lactate dehydrogenase (LDH) present after cell lysis. Briefly for LDH analysis, adherent platelets were lysed by incubation with 2% Triton-PBS buffer for 30 min at 37°C. A colorimetric substrate for LDH was added and incubated for 20 min at 37°C. The reaction was stopped and the optical density was measured at 490 nm with reference wavelength of 650 nm.

Plasma recalcification profile. Platelet poor plasma (PPP) was utilized for this study and was prepared as previously described^{56,57}. 100 µl of test sample were incubated with 100 µl of PPP in 96 well plates. Controls consisted of tissue culture-treated plastic (TCP) exposed to PPP with and without CaCl₂. Following the addition of PPP, 100 µl of 0.025 M CaCl₂ was added to each well (except without Ca⁺², negative control). The plate was then immediately placed in a 96 well plate reader, where the kinetics of the clotting process due to recalcification were monitored by measuring the absorbance at 405 nm (every 3 min for 45 min) at 37°C. In calculating the mean absorbance at each time point, three wells were averaged per sample. The slope of the linear portion of each profile were calculated and analyzed.

Statistical analysis. All quantitative experiments were conducted with at least three independent experiments. Statistical evaluation was performed using Origin Pro Software. Data were analyzed by a one-way ANOVA for multiple data set analyses and were expressed as mean $\pm$ SD. A difference was considered statistically significant when $p < 0.05$.

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Author contributions

M.S.M., S.V. and D.S.K. conceived and designed the experiments and drafted the final manuscript. H.M., Y.S.¹ and A.E. assisted in chromatography and electrophoresis. Y.N. assisted in CLSM studies. A.A. assisted in flow cytometry. Y.S.² contributed in HPLC and blood compatibility analysis. A.C.P. assisted in statistical analysis. Y.Y., T.M. and D.S.K. analyzed the data and contributed reagents/materials/analysis tools. All authors read and approved the manuscript.

Additional information

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