

Curcumin Affects Components of the Chromosomal Passenger Complex and Induces Mitotic Catastrophe in Apoptosis-Resistant Bcr-Abl-Expressing Cells

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Abstract

The Bcr-Abl oncoprotein plays a major role in the development and progression of chronic myeloid leukemia and is a determinant of chemotherapy resistance occurring during the blast crisis phase of the disease. The aim of this article was to investigate the possibility of combating the resistance to apoptosis caused by Bcr-Abl by inducing an alternative cell death process. As a model of chronic myeloid leukemia, we employed Bcr-Abl-transfected mouse progenitor 32D cells with low and high Bcr-Abl expression levels corresponding to drug-sensitive and drug-resistant cells, respectively. The drug curcumin (diferuloylmethane), a known potent inducer of cell death in many cancer cells, was investigated for efficacy with Bcr-Abl-expressing cells. Curcumin strongly inhibited cell proliferation and affected cell viability by inducing apoptotic symptoms in all tested cells; however, apoptosis was a relatively late event. G₂-M cell cycle arrest, together with increased mitotic index and cellular and nuclear morphology resembling those described for mitotic catastrophe, was observed and preceded caspase-3 activation and DNA fragmentation. Mitosis-arrested cells displayed abnormal chromatin organization, multipolar chromosome segregation, aberrant cytokinesis, and multinucleated cells—morphologic changes typical of mitotic catastrophe. We found that the mitotic cell death symptoms correlated with attenuated expression of survivin, a member of the chromosomal passenger complex, and mislocalization of Aurora B, the partner of survivin in the chromosomal passenger complex. Inhibition of survivin expression with small interfering RNA exhibited similar mitotic disturbances, thus implicating survivin as a major, albeit not the only, target for curcumin action.

This study shows that curcumin can overcome the broad resistance to cell death caused by expression of Bcr-Abl and suggests that curcumin may be a promising agent for new combination regimens for drug-resistant chronic myeloid leukemia. (Mol Cancer Res 2006;4(7):457–69)

Introduction

The elimination of tumor cells by commonly used anticancer drugs is predominantly mediated by triggering apoptosis. However, tumor cells often evade apoptosis by affecting apoptosis regulatory proteins and pathways, thereby enhancing their survival potential. The failure to undergo apoptosis in response to anticancer therapy can result in drug resistance. Consequently, new approaches are needed that enhance the elimination of resistant cells. Cancer cells can still be effectively eliminated by nonapoptotic mechanisms, such as mitotic catastrophe or senescence (reviewed in refs. 1, 2). Combined strategies have been proposed which aim to overcome drug resistance by causing permanent growth arrest and nonapoptotic cell death to prevent the progression of drug-resistant clones.

Mitotic catastrophe is defined as a mode of cell death caused by aberrant mitosis (for a review, see ref. 3). Typical features of this process are block in mitosis, mitotic spindle disorganization, failed chromosome segregation, and formation of multinucleated cells. Mitotic catastrophe is considered to be fundamentally different from apoptosis (2); however, others claim that mitotic catastrophe is followed by apoptotic symptoms, such as caspase activation (4). One of the mechanisms leading to mitotic catastrophe results from a combination of deficient cell cycle checkpoints and cellular damage. Failure to arrest the cell cycle before or at mitosis triggers an attempt of aberrant chromosome segregation, which culminates in the activation of the apoptotic pathway. Another way of inducing mitotic catastrophe is to interfere directly with mitosis (e.g., by microtubule-damaging agents) leading to mitotic spindle and chromosome segregation abnormalities (5-7). Finally, the inactivation or dysfunction of the chromosomal passenger complex (CPC), which is necessary for the coordination of all chromosomal and cytoskeletal events in mitosis, often leads to mitotic cell death (reviewed in ref. 8).

Recently, the use of phytochemicals as anticancer agents with potential to overcome cellular resistance to apoptosis has gained considerable attention. Curcumin, a naturally occurring

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phytochemical has shown chemopreventive properties against various malignancies. As a pharmacologically safe agent, curcumin could be used alone to prevent cancer and in combination with chemotherapy to treat cancer. The main cell death mode induced by curcumin is apoptosis (reviewed in ref. 9); however, there is evidence showing that curcumin disrupts the mitotic spindle structure and induces micronucleation in MCF-7 cells (10), which suggests mitotic catastrophe. Several researchers, including ourselves, have shown the ability of curcumin to induce cell death in cancer cells resistant to other treatments. We showed that curcumin was able to overcome apoptosis resistance in cancer cells overexpressing P-glycoprotein, multidrug resistance-associated protein 1, and heat shock protein 70 (11, 12). Recently, curcumin was described as a tumor suppressive agent against adult T-cell leukemia (13). These observations indicated that curcumin may have potential in inducing cell death in apoptosis-resistant Bcr-Abl-expressing cells. Bcr-Abl is a consequence of the Philadelphia chromosome generation. The chimeric protein exhibits constitutive tyrosine kinase activity and is believed to be the critical determinant of chronic myeloid leukemia. The disease initially presents as an indolent chronic phase and progresses to an aggressive and drug-resistant stage called blast crisis. Cells in blast crisis display a broad drug-resistant phenotype, resulting in insensitivity to treatment (14-16).

We used Bcr-Abl-transfected mouse progenitor 32D cells, expressing different levels of Bcr-Abl and exhibiting differences in their sensitivity to drug treatment (17, 18). The C2 clone expresses a low level of Bcr-Abl and is sensitive to drug treatment, thus mimicking the chronic phase of the disease, whereas the C4 clone expresses high amounts of the oncogene and is highly drug resistant, corresponding to the blast crisis. The parental cell line 32D and two progeny Bcr-Abl-expressing lines (C2 and C4) were treated with curcumin, which in all three lines led to cell death. We show here that curcumin arrested the cell cycle in G₂-M and induced mitotic catastrophe in the parental and the Bcr-Abl-expressing cells. This type of cell death overcame the apoptosis resistance that is typical of high Bcr-Abl-expressing cells. Moreover, we offer the first evidence that curcumin down-regulates the level of survivin in Bcr-Abl-expressing cells. This was associated with mislocalization of Aurora B (the partner of survivin in the CPC), defective spindle assembly, chromosome segregation, and cytokinesis. The ability of survivin to mediate these effects was indicated by RNA interference.

Results

Curcumin Decreases the Viability and Proliferation of Bcr-Abl-Expressing Cells

Hematopoietic parental 32D cells and two Bcr-Abl-expressing cell lines (C2 and C4) were treated with 5, 10, or 20 μmol/L curcumin. C4 cells were highly resistant to apoptosis induced by etoposide (18) and displayed resistance to other agents, such as Ara-C and H₂O₂ (Fig. 1A).

Curcumin is a potent inhibitor of cell proliferation. Accordingly, a dose-dependent inhibition of proliferation was observed after 18 hours of curcumin treatment as measured by

the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 1B). Interestingly, there was no difference between the three cell lines, including the drug-resistant C4 cells. Cells treated with 20 μmol/L curcumin displayed ~40% of the proliferation capacity of the control (untreated) cells. This concentration of curcumin was subsequently employed in further experiments. The MTT assay done in curcumin-treated cells preincubated for 1 hour with 50 μmol/L of the pan-caspase inhibitor zVAD.fmk showed that the growth arrest induced by curcumin was caspase independent at least up to 18 hours of treatment. Cells treated with 50 μmol/L zVAD.fmk alone had a MTT value ~85% of the control level in 32D and C2 cells and ~100% of control level in C4 cells.

Cell proliferation measurement by using a more direct method, such as bromodeoxyuridine (BrdUrd) incorporation assay, confirmed these observations (Fig. 1C). Significant inhibition of proliferation was evident after 6 hours of curcumin treatment; at that time, BrdUrd incorporation was reduced to 40% to 50% of the control level.

To determine the cytotoxicity of curcumin, we assessed propidium iodide (PI) uptake by flow cytometry (Fig. 1D). After 18 hours of curcumin treatment, <10% of cells were nonviable. The percentage increased with time and averaged 15% to 20% after 24 hours in all three cell lines. At 36 and 48 hours, the percentage of dead cells was 2-fold higher in the 32D compared with the C2 and C4 cells, indicating a greater susceptibility to death following the initial cell cycle arrest. The apoptosis-resistant C4 cells showed the same susceptibility to death induced by curcumin as C2 cells.

Curcumin Arrests Cells in G₂-M Phase of the Cell Cycle and Affects Cell Cycle Regulators

We did cell cycle analysis by flow cytometry. Histograms of DNA content are shown in Fig. 2A. Control cells showed a typical pattern with 10% to 15% of cells in the G₂-M phase. It had been noted previously that these Bcr-Abl-expressing 32D cells have a slightly reduced G₂-M component (18) and this was also reflected here. A significant increase in the percentage of cells in the G₂-M phase was visible after 6 hours of curcumin treatment. The G₂-M fraction at 18 hours increased in comparison with control cells and was 2.6-, 3.4-, and 3.1-fold higher in treated 32D, C2, and C4 cells, respectively (Fig. 2B). Notably, the sub-G₁ fraction did not exceed 10% after 18 hours and 15% after 24 hours of curcumin treatment in all three cell lines (Figs. 2A and 3).

The levels of the cell cycle regulatory proteins p21^{Cip1/WAF1}, p27^{Kip1}, and cyclin D2 were examined by Western blotting (Fig. 2C and D). Cyclin D2 is a critical factor promoting cell cycle progression and is overexpressed in Bcr-Abl-expressing cells (19). As expected, C2 and C4 cells displayed a high basal level of cyclin D2. After 18 hours of curcumin treatment, cyclin D2 was down-regulated to a nearly undetectable level in all three cell lines. Curcumin strongly induced p21 expression in 32D cells, which was detected after 6 hours of curcumin treatment and persisted until 18 hours. In Bcr-Abl-expressing cells, an increased p21 level was particularly evident at 18 hours. The protein level of p27 was significantly lower in the Bcr-Abl-expressing cells than in parental 32D. This was in

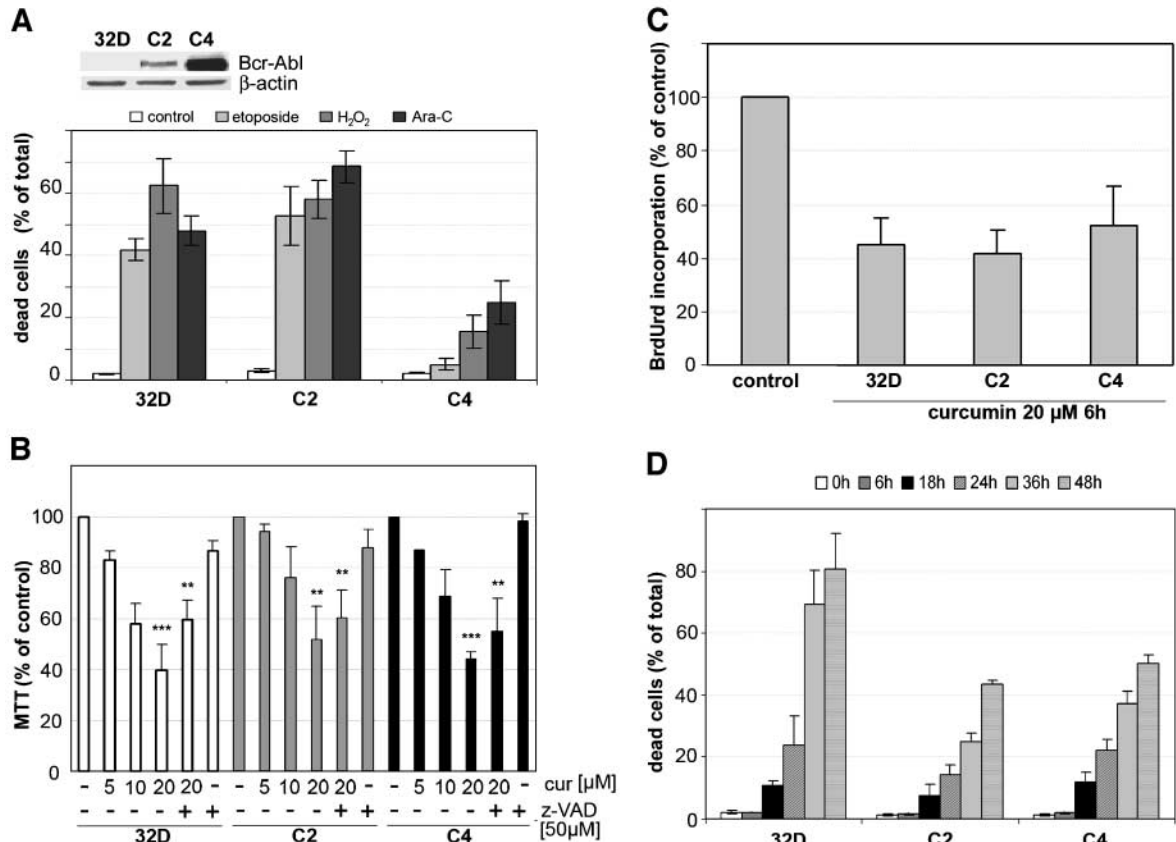


FIGURE 1. Curcumin decreases viability and proliferation of Bcr-Abl-expressing cells. **A.** Susceptibility of 32D, C2, and C4 cells to death induced by 18 hours of treatment with 7.5 $\mu\text{g/mL}$ (12.7 $\mu\text{mol/L}$) etoposide, 100 $\mu\text{mol/L}$ H_2O_2 , or 25 $\mu\text{mol/L}$ Ara-C as measured by PI uptake and flow cytometry. **B** and **C.** Proliferation of 32D, C2, and C4 cells incubated with curcumin. **B.** MTT assay. Cells were treated with 5, 10, or 20 $\mu\text{mol/L}$ curcumin for 18 hours or pretreated for 1 hour with 50 $\mu\text{mol/L}$ zVAD.fmk alone or followed by 20 $\mu\text{mol/L}$ curcumin. Proliferation was estimated by the MTT assay and calculated as the percentage of control. **, $P < 0.001$; ***, $P < 0.0001$ versus untreated (Student's t test). **C.** BrdUrd incorporation. Cells were incubated with 20 $\mu\text{mol/L}$ curcumin for 6 hours and BrdUrd was added for 15 minutes. Cells were fixed, immunostained, and analyzed by flow cytometry. **D.** Viability of curcumin-treated cells measured by PI uptake and flow cytometry. Cells were treated with 20 $\mu\text{mol/L}$ curcumin and analysis was done at indicated time points. Columns, mean ($n = 3$); bars, SD.

agreement with the published data showing that the Bcr-Abl oncogene suppresses p27 gene expression (20). Incubation with curcumin for 18 hours led to the up-regulation of p27 in all three cell lines.

Curcumin Increases Mitotic Index and Induces Abnormalities of Mitosis and Cytokinesis

As curcumin arrested cells in G_2 -M phase of the cell cycle, which subsequently induced cell death, we considered that curcumin may be inducing mitotic catastrophe. To verify this hypothesis, we studied the changes in nuclear morphology on curcumin treatment (Fig. 4A). We found that the cells exhibited pronounced changes in nuclear morphology and chromatin organization, however, without the formation of apoptotic bodies. Cells with multipolar chromosome segregation and multinucleation were visible. 32D cells with the abnormal mitotic nuclei accounted for 10% and 30% at 6 and 18 hours of treatment, respectively. C2 and C4 cells showed mitosis perturbances more frequently and the percentage of changed nuclei increased from 20% to 30% after 6 hours to 40% to 45% after 18 hours of treatment (Fig. 4B). Interestingly, the cells were

arrested not only in metaphase but also in later stages of mitosis, indicating that the mitotic spindle assembly checkpoint was not activated. Observation of cell morphology under phase contrast showed the appearance of giant cells with an unusual shape typical of cells with failed cytokinesis (Fig. 4C). These data indicated that mitotic catastrophe was the primary mediator of cell death activated by curcumin.

Mitosis entry is correlated with dephosphorylation of Cdk1 (p34) protein leading to its activation. To investigate whether curcumin-treated cells undergo mitotic cell death, we analyzed the phosphorylation status of Cdk1 protein. We found phosphorylated protein in control samples, but after curcumin treatment its level decreased, indicating mitosis entry followed by massive mitotic arrest (Fig. 4D). The Cdk1 kinase together with cyclin B1 forms an active heterodimer called "mitosis-promoting factor." Its activation is a necessary step for the progression from G_2 to M phase and must be sustained from prophase to metaphase, whereas in anaphase cyclin B1 is degraded. Up-regulation of cyclin B1 and prolonged activation of cyclin B1/Cdk1 complex are typical markers of mitotic catastrophe proceeding with arrest at the mitotic spindle assembly

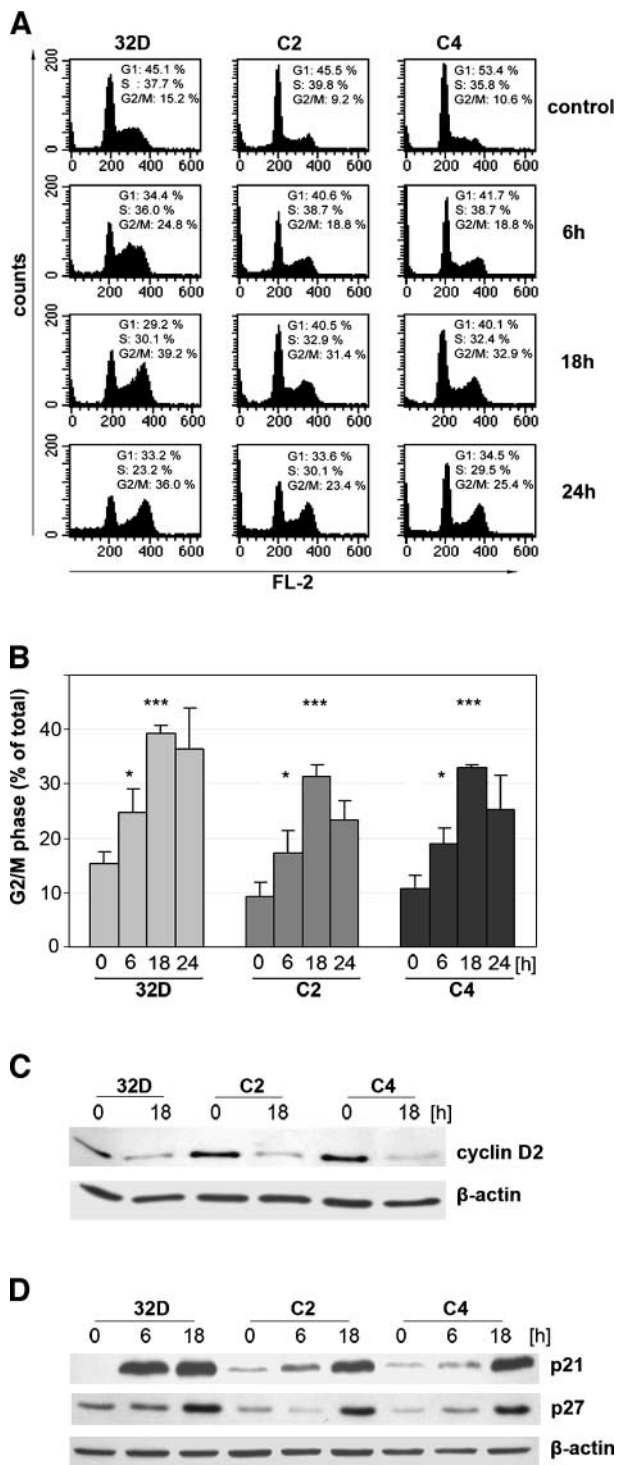


FIGURE 2. Curcumin arrests cells in the G₂-M phase and affects cell cycle regulatory proteins. **A.** DNA content analyzed by flow cytometry after 6, 18, or 24 hours of curcumin treatment (20 μmol/L). Ten thousand cells were analyzed for each time point. Typical histograms for three separate experiments. Percentage of cells in each phase of the cell cycle: G₁, S, and G₂-M was estimated after analysis using ModFit software. **B.** Percentage of cells arrested in the G₂-M phase of the cell cycle. Columns, mean of three experiments; bars, SD. **C.** Level of cyclin D2 protein. **D.** Levels of p21 and p27 proteins estimated by Western blotting. Whole-cell extracts were prepared at indicated time points of treatment with 20 μmol/L curcumin. β-Actin was used as a loading control.

checkpoint, often observed in cells incubated with microtubule poisons. Conversely, CPC disturbances or spindle assembly checkpoint deregulations, which usually lead to pre-early anaphase entry, are associated with cyclin B1 degradation (21). This was a feature of all curcumin-treated cells, which exhibited slow down-regulation of cyclin B1, correlating with morphologic features of aberrant mitosis, without metaphase arrest, but progression into later stages with many abnormalities (Fig. 3A and D).

To distinguish cells that are in the G₂ phase from those in the M phase of the cell cycle and to confirm that cells are arrested in mitosis, we measured the mitotic index by laser scanning cytometry (iCys) using a MPM-2 antibody, which specifically recognizes phosphoproteins abundant in mitotic cells from early prophase to anaphase. It is important to note that MPM-2 positivity is characteristic for cells in early stages of mitosis only and is not sustained. Double staining with MPM-2 and 4',6-diamidino-2-phenylindole (DAPI) allowed for observation of nuclear morphology in MPM-2-positive and MPM-2-negative subpopulations. As expected, only a few percentage of control cells were in mitosis, whereas at 6 hours of curcumin treatment the fraction of C2 and C4 cells in M phase reached 10% to 15% and further increased with time up to 15% to 20% (Fig. 4E). The level of MPM-2-positive 32D cells was lower compared with the Bcr-Abl-expressing cells. A single-cell gallery presenting nuclear morphology of cells gated from the MPM-2-positive and MPM-2-negative subpopulations is shown as part of Fig. 4E. It clearly indicates that mitosis-arrested MPM-2-positive cells show morphologic changes of nuclei with condensed chromatin, typical of mitotic catastrophe and comparable with those presented in Fig. 4A. Unlike the MPM-2-positive cells, most of the negative ones had interphase nuclear morphology; however, some of MPM-2-negative cells with 4N DNA content (G₂-M phase of the cell cycle) displayed abnormal mitotic morphology with multinuclei but without condensation. This phenotype was observed predominantly after 18-hour incubation with curcumin. As cells were not only arrested in the early stages of mitosis but also progressed into later stages, some cells would be already MPM-2 negative as the antibody recognizes phosphoproteins in cells in early prophase to anaphase. This would explain the underestimate compared with the morphologic study presented in Fig. 4B, showing ~30% of parental and 40% of Bcr-Abl-expressing cells displaying aberrant mitotic morphology.

Mitotic Catastrophe of Bcr-Abl-Expressing Cells Is followed by Caspase Activation and Apoptotic DNA Fragmentation

The sub-G₁ fraction measured after 24 hours of incubation with curcumin did not exceed 15% in all tested cells (Fig. 3). An increased number of cells in the sub-G₁ subpopulation was detected after 48 and 72 hours, reaching ~40% to 45% in 32D cells and 20% to 30% in C2 and C4 cells. Figure 4A shows a time course of the percentage of the sub-G₁ fraction detected after curcumin treatment. It is noteworthy that the sub-G₁ fraction followed the G₂-M block. After 24 hours of treatment, ~40% of all cells had active caspase-3. However, it is notable that in 32D cells activation of caspase-3 was earlier, with 35% of cells showing positivity at 18 hours, whereas in C2 and C4 cells the percentage of cells with active caspase-3 at that time did

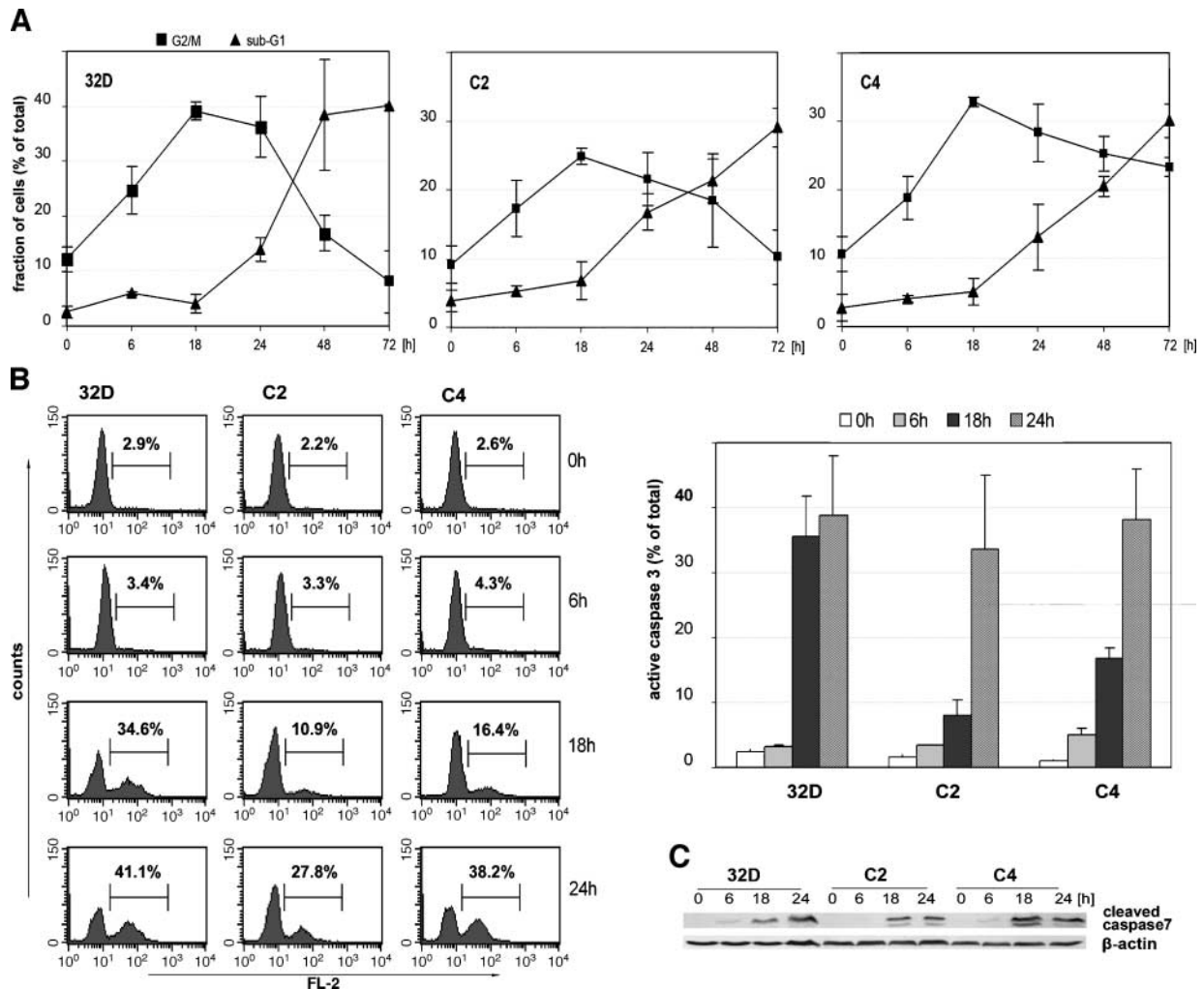


FIGURE 3. Mitotic catastrophe symptoms caused by curcumin are followed by apoptosis. **A.** Time course of the percentage of cells arrested in the G₂-M phase of the cell cycle (■) and apoptotic cells estimated as the sub-G₁ population (▲) based on DNA content analysis by flow cytometry. Cells were treated with 20 μmol/L curcumin. Points, mean ($n = 4$); bars, SD. **B.** Caspase-3 activity on curcumin treatment. Cells were treated for 6, 18, or 24 hours with curcumin, fixed, stained with anti-active caspase-3 antibody, and analyzed by flow cytometry. Representative histograms and statistical analysis. Columns, mean ($n = 3$); bars, SD. **C.** Caspase-7 cleavage in curcumin-treated cells shown by Western blotting in whole-cell extracts was prepared at indicated time points.

not exceed 10% to 15% (Fig. 3B). Caspase-7 cleavage products were also examined by Western blotting and indicated prominent activation at 18 hours. These results confirmed the data from the previous cytotoxicity assay (Fig. 1D), indicating caspase-dependent death as a relatively late event but also suggesting that 32D cells are more susceptible to the initiation of apoptosis following the disturbances at G₂-M. C4 cells activated caspase-3 and DNA fragmentation on curcumin treatment with the same efficiency as the apoptosis-sensitive C2 cells. This suggests that their resistance to specific drugs may be a result of a block in the initiation of apoptotic signaling in response to certain types of damage rather than disturbances of the executory machinery.

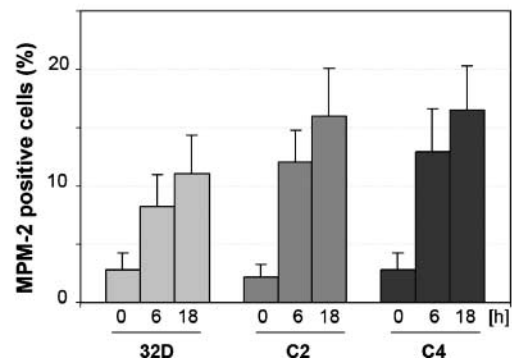
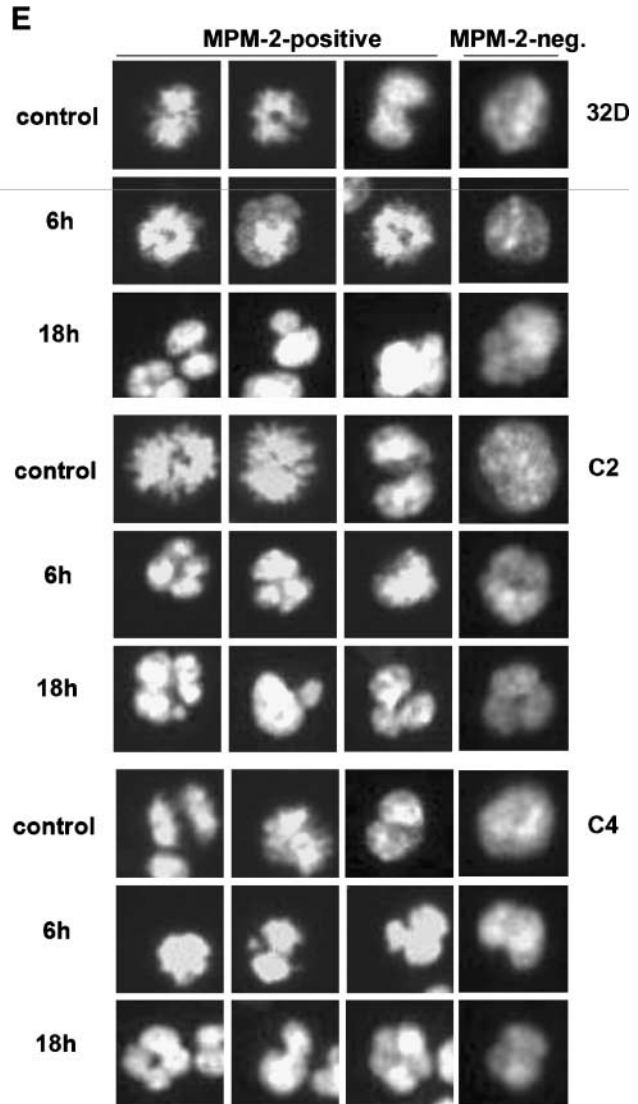
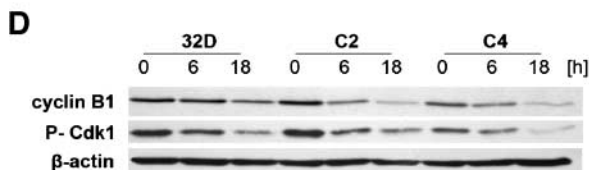
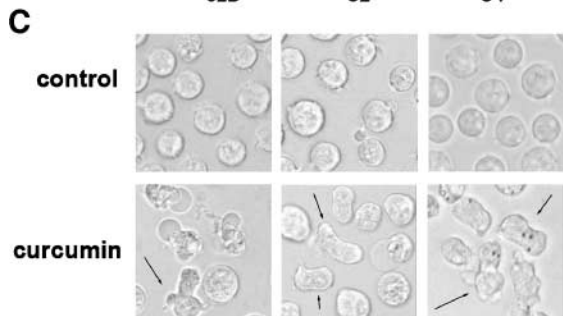
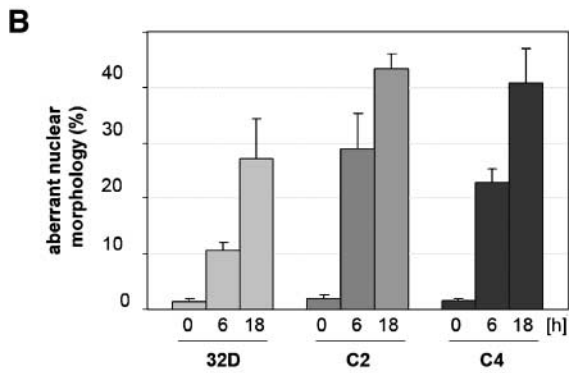
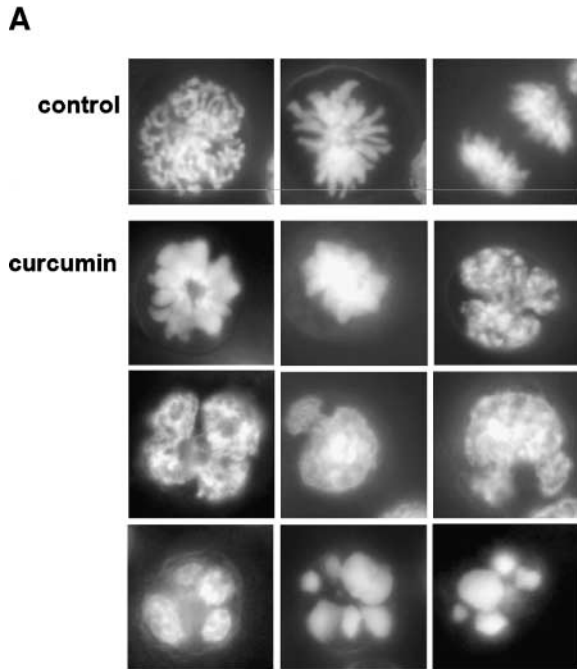
Curcumin Down-Regulates Survivin Expression and Causes Aurora B Mislocalization in Mitotic Cells

It has been shown that survivin, a member of the inhibitors of apoptosis family, not only inhibits caspases but also is a

CPC protein, controlling mitotic events. We investigated survivin expression and protein levels after 6 and 18 hours of curcumin treatment (Fig. 5A and B). Curcumin decreased survivin expression, however very slightly in 32D cells, which correlated with the changes observed on the protein level. The most significant decrease of survivin mRNA level was detected in C4 cells and amounted 0.79 of control level after 6 hours and 0.4 of control after 18 hours of curcumin treatment. We observed a significant decrease in the survivin protein level in C2 and C4 cells after 6 hours of curcumin treatment, and it was barely detectable after 18 hours (Fig. 5B). The survivin level was slightly decreased on curcumin treatment in 32D cells as well but not as dramatically as in the C2 and C4 cell lines. Because the decrease of survivin occurred in cells treated with curcumin for 6 and 18 hours, when mitotic perturbations were evident, the effect of curcumin on survivin expression might be connected with mitotic abnormalities and finally mitotic catastrophe

induction. Survivin displays a characteristic localization pattern in mitotic cells as it is present at metaphase chromosomes, central spindle midzone, and midbody of control cells (Fig. 5C, *a-d*). After curcumin treatment, survivin staining indicated a

much lower level of the protein with a more diffuse localization (Fig. 5C, *e-h*). Nevertheless, cells regularly reached anaphase and telophase, albeit with many abnormalities in chromosome segregation.



To further examine the consequences of survivin depletion, we assessed the level and localization of the Aurora B protein kinase (known previously as AIM-1), which is a CPC subunit and a survivin-binding partner regulating mitotic events. In mammals, this kinase plays an essential role in chromosome segregation, including kinetochore attachment, spindle assembly and disassembly, control of spindle checkpoints, chromosome segregation, and cytokinesis. Vader et al. recently showed that survivin cooperates with Aurora B and mediates targeting of the CPC to the centromere and midbody (22). Moreover, survivin enhances the Aurora B kinase activity involved in the regulation of the mitotic events.

As expected, in untreated mitotic cells, Aurora B was found in the centromeres of chromosomes, central spindle midzone, and midbody, corresponding to the localization of survivin (Fig. 5D, *a-d*). We did not detect any changes in the level of Aurora B protein in curcumin-treated cells (Fig. 5B), whereas immunostaining showed mislocalization of Aurora B (Fig. 5D). Aurora B protein staining in most of the cells showed diffused localization without any pattern characteristic for mitotic cells. Some aggregates of Aurora B were detected only in the disorganized metaphase cells. In other mitotic cells with multipolar spindle, improperly segregated sister chromatids, or multinuclei, typical staining was not visible. This suggests that the down-regulation of survivin may have resulted in the disruption of Aurora B localization.

Survivin Depletion Causes Polyploid Formation, Defects of Chromosome Segregation, and Aurora B Mislocalization

To investigate whether the absence of survivin can lead to mitotic cell death in Bcr-Abl-expressing cells, we did RNA interference to down-regulate survivin. C4 cells were employed for this study as they are resistant to classic apoptosis but sensitive to curcumin-induced mitotic catastrophe. We used small interfering RNA (siRNA) designed against the mouse survivin Birc5 gene. Splice variants of survivin have been described, but they play no role in mitosis regulation, as previous siRNA studies have shown (23). C4 cells were electroporated to introduce survivin siRNA as well as a negative control siRNA and the level of survivin was determined after 24, 48, and 72 hours after transfection (Fig. 6A). Survivin siRNA, but not the negative siRNA, greatly diminished survivin expression to a nearly undetectable level. Contrary to curcumin-treated cells, p21 and p27 was not up-regulated in survivin siRNA-transfected cells until 72 hours after transfection. At that time, slight increase of both p21 and p27 was observed. A viability study showed a moderate increase in cell death, which affected 15% of cells after

24 hours and increased to 36% at 72 hours (Fig. 6B). The cell cycle analysis presented in Fig. 6C shows strong G₂-M arrest followed by polyploid formation in survivin-depleted cells. At 24 hours, 40% of cells were found in the G₂-M phase, which decreased to 10% to 15% at 48 and 72 hours. More than 15% of cells had a DNA content greater than 4N at 48 hours after transfection and this fraction increased to 25% after 72 hours. Observation of nuclear morphology indicated mitotic abnormalities after survivin RNA interference (Fig. 6D, DAPI staining). The changes resembled those observed in curcumin-treated cells with exclusion of giant nuclei, which probably correspond to polyploid cells. These abnormalities indicated defective sister chromatid separation and disturbances in cytokinesis. The diffuse pattern of Aurora B found in survivin-depleted cells was also very similar to that in curcumin-treated cells (Figs. 5 and 6D). These data indicate that cells without survivin display similar features to cells treated with curcumin.

Discussion

Curcumin, a natural chemopreventive and anticancer agent, interferes with many signaling pathways, bringing about cessation of proliferation, inhibiting cytokine production, and inducing cell death. There is evidence that curcumin treatment leads to the inhibition of tumor promotion (24-26). The ability of curcumin to induce cell death in various cancer cells has been reported by many groups, including ourselves (11, 12, 27-31). Interestingly, curcumin was able to suppress cell growth of human T-cell leukemia virus type I-infected T-cell lines as well as primary adult T-cell leukemia cells but not normal peripheral blood mononuclear cells (13). Although the mechanism of curcumin-induced cell death seems to be cell specific and dependent on curcumin concentration, the main reported method of cell death induced by curcumin is apoptosis. Recently, however, an ability of curcumin to induce alternative death pathways in cancer cells that are resistant to other treatments has gained attention.

Here, we show that curcumin induced mitotic catastrophe in Bcr-Abl-expressing cells. Moreover, the level of Bcr-Abl expression did not affect the susceptibility to curcumin. Cells were arrested in G₂-M phase of the cell cycle and displayed increased mitotic index, mitotic spindle disorganization, failed chromosome segregation, and formation of MPM-2-positive multinucleated cells. The mitotic catastrophe was followed by activation of caspase-3 and cells with sub-G₁ content, thus implying apoptosis as the death process finalizing the mitotic catastrophe. Our data indicate that Bcr-Abl-expressing cells were equally susceptible to cell cycle arrest compared with parental cells; however, a lower percentage of 32D cells displayed mitotic abnormalities. Caspase-3 activation and loss of viability occurred

FIGURE 4. Curcumin induces mitotic catastrophe in 32D and Bcr-Abl-expressing cells. **A.** Mitotic nuclear morphology after Hoechst staining of untreated (control) and curcumin-treated cells. Typical pictures presenting mitosis abnormalities found on curcumin treatment. Magnification, $\times 600$. **B.** Percentage of nuclei with abnormal mitotic morphology after 6 or 18 hours of curcumin treatment. **C.** Cell morphology of untreated cells or cells after 18 hours curcumin treatment. Arrows, large, elongated cells. Magnification, $\times 200$. **D.** Cyclin B1 and phosphorylated Cdk1 protein levels after 6 or 18 hours of curcumin treatment. **E.** Nuclear morphology of MPM-2-positive and MPM-2-negative cells. Mitotic cells were determined using iCys scanning cytometry by quantifying cells with elevated MPM-2 signal. The MPM-2-positive or MPM-2-negative subpopulation of untreated or curcumin-treated cells was gated and the single-cell gallery presents nuclear morphology after DAPI staining. Magnification, $\times 400$. Representative pictures for three separate experiments. Data are percentage of MPM-2-positive cells measured in three separate experiments. Columns, mean; bars, SD.

earlier in 32D cells. The 32D cells appear therefore to exit cell cycle arrest and enter apoptosis faster. We also showed that curcumin was able to negate the broad resistance to apoptosis of Bcr-Abl-expressing cells, which have constitutively active pro-survival and antiapoptotic signals (as reviewed in refs. 32, 33).

Curcumin strongly inhibited proliferation of all cell lines and affected expression of cell cycle regulatory proteins, cyclin D2, p27, and p21 (Fig. 2C and D). Bcr-Abl-expressing cells displayed high basal expression of cyclin D2, which has been described as a critical determinant of chronic myeloid leukemia

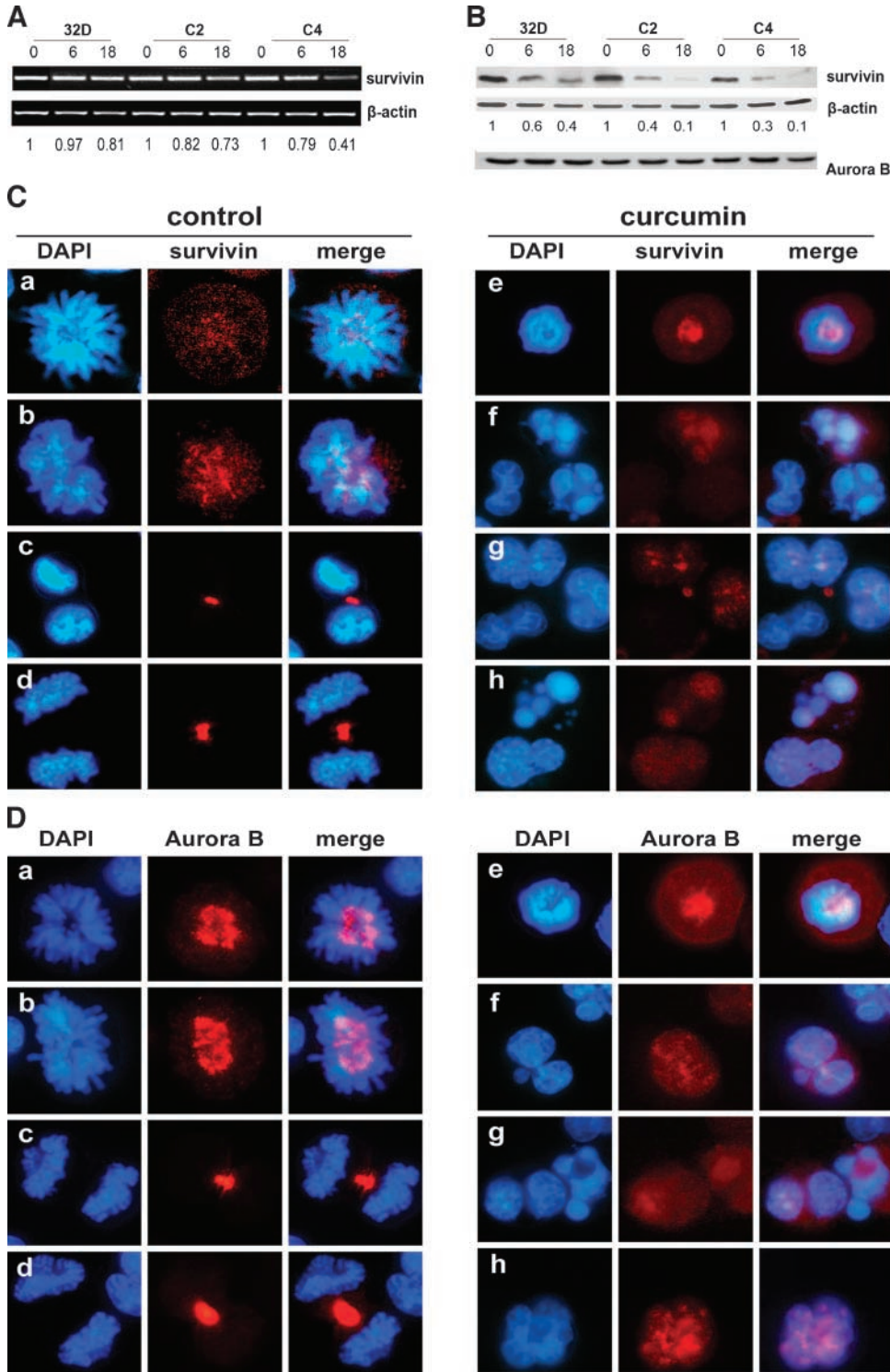


FIGURE 5. Curcumin down-regulates survivin expression and causes Aurora B mislocalization. **A.** Survivin expression level estimated by reverse transcription-PCR in untreated cells and curcumin-treated 32D, C2, and C4 cells for 6 or 18 hours. Densitometric analysis was done to calculate the survivin/actin ratio. **B.** Survivin and Aurora B protein levels estimated by Western blotting after 6 or 18 hours of curcumin treatment. β -Actin was used as a loading control. Densitometric analysis was done to calculate the survivin/actin ratio, which was set at 1 for each control sample. **C** and **D.** Localization of CPC proteins. Survivin (**C**) and Aurora B (**D**) immunostaining of untreated cells and cells treated for 18 hours with curcumin. Cells were stained with anti-survivin or anti-Aurora B antibody followed by DNA staining by DAPI. Typical pictures presenting control or curcumin-treated mitotic cells stained with DAPI, specific antibody, and both (*merge*).

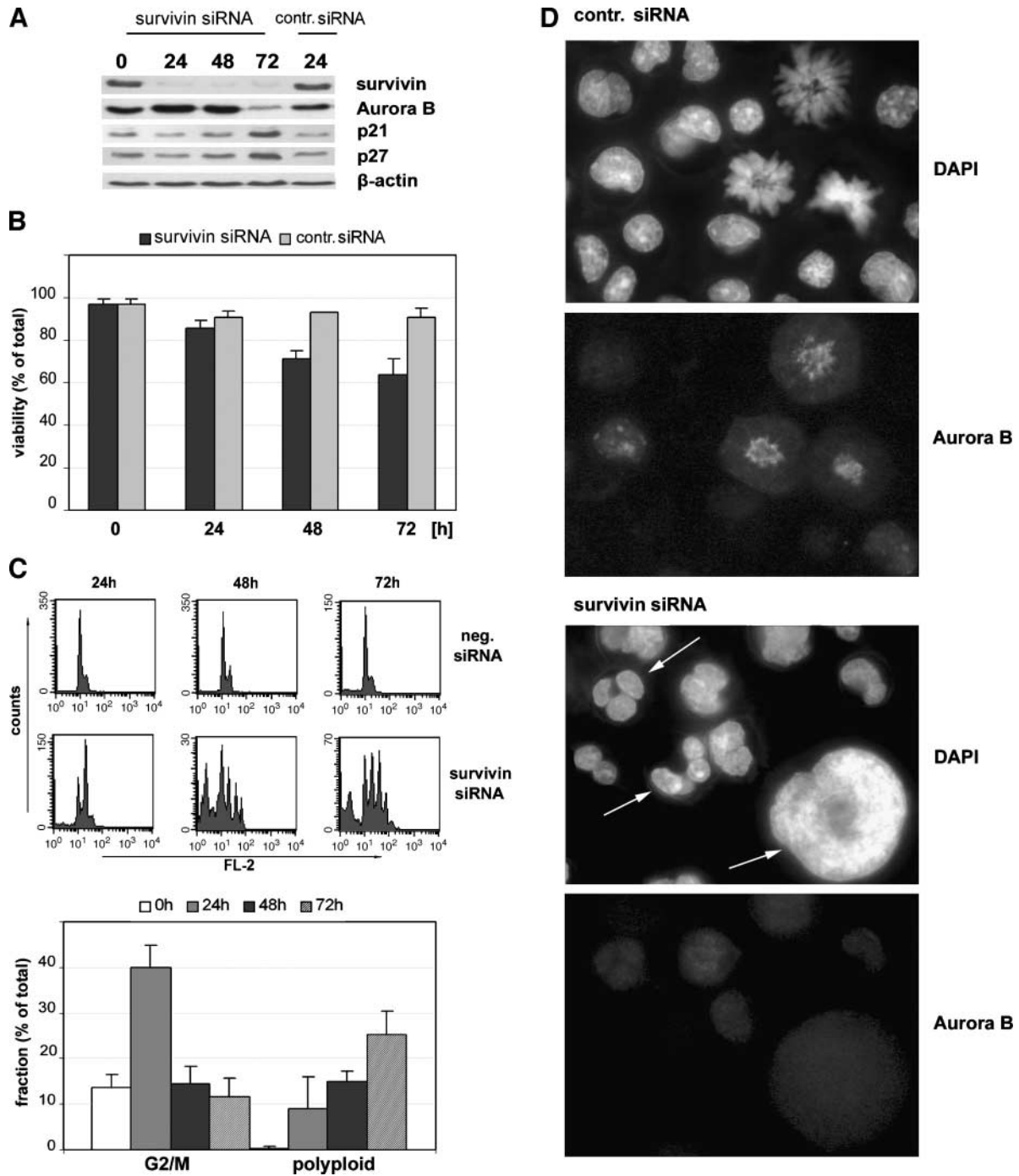


FIGURE 6. Survivin down-regulation causes decreased viability and mitosis abnormalities. **A.** Survivin, Aurora B, p21, and p27 levels after transfection with control or survivin siRNA estimated by Western blot. Whole-cell extracts were prepared 24, 48, and 72 hours after electroporation. β -Actin was used as a loading control. **B.** Viability of cells transfected with control (*gray columns*) or survivin (*black columns*) siRNA estimated by PI uptake and flow cytometry at indicated time points. mean; bars, SD. ($n = 4$). **C.** Cell cycle analysis done by flow cytometry. Typical histograms presenting cell cycle at different time points after transfection. Data are percentage of cells found in G₂-M phase and cells with DNA content higher than 4N (polyploid) from four independent experiments. Columns, mean; bars, SD. **D.** Nuclear morphology (DAPI) and Aurora B staining after transfection with control siRNA or survivin siRNA. Cells were collected after 48 hours, fixed, and stained. Typical pictures. Arrows, cells with abnormal mitotic morphology.

cell proliferation. Bcr-Abl induces cyclin D2 expression (19, 34) and STI-571, a Bcr-Abl tyrosine kinase inhibitor, down-regulates cyclin D2 (20). On curcumin treatment, cyclin D2 was decreased to a barely detectable level. This is in

agreement with results of others showing cyclin D1 and D2 down-regulation in various curcumin-treated cell lines (35). STAT5 is proposed to be the main determinant of Bcr-Abl-dependent cyclin D2 overexpression, and it could be a

curcumin target (36, 37). Moreover, the cyclin D2 gene promoter contains binding sites for the activator protein-1 and nuclear factor- κ B transcription factors, and curcumin inhibits their DNA-binding activity (36, 38).

We found p21^{WAF1} to be strongly up-regulated in curcumin-treated 32D cells at 6 hours, whereas in Bcr-Abl-expressing cells this was more prominent at 18 hours. Zheng et al. also reported that curcumin induced a G₂-M block followed by apoptosis in melanoma cells, and this was associated with p53 and p21 up-regulation (39). p27 was also more prominent in Bcr-Abl-expressing cells after 18 hours of curcumin treatment. The slight delay in the elevation of those proteins compared with 32D may be a reflection of the fact that there are fewer S-G₂-M cells in the Bcr-Abl-expressing cells initially, or there may be direct effect of Bcr-Abl on these regulators. Bcr-Abl causes low constitutive expression of p21, which is predominantly cytosolic (40). Cytosolic p21 has been reported to have a pro-survival role in other transformed cells (41). It was shown by others that p27 expression was suppressed in Bcr-Abl-expressing cells via the phosphoinositol-3-kinase/Akt pathway (20), and inhibition of Bcr-Abl tyrosine kinase activity with STI-571 rapidly increased p27 protein level (42). There are only a few articles showing p27 up-regulation after curcumin treatment (39, 43), and our data are in line with those.

Mitotic catastrophe can be a result of DNA damage in cells with abrogated checkpoints; other causes include compromised mitotic spindle or microtubules, survivin down-regulation, or dysfunction of other CPC proteins. In our system, we did not observe symptoms of mitotic assembly checkpoint arrest, such as elevation of cyclin B1 level and metaphase arrest, which are usually connected with disorganization of microtubules and/or failed formation of the mitotic spindle. We investigated whether curcumin influenced mitosis by affecting survivin expression, as it has been shown very recently that 50 μ mol/L curcumin causes G₂-M cell cycle arrest and down-regulates survivin in adult T-cell leukemia cells (13). Survivin is a member of the inhibitors of apoptosis family and is overexpressed in various human cancers. It is also a member of the CPC, which is essential for mitotic progression. Studies using survivin antisense oligonucleotides or siRNA showed that down-regulation of survivin in dividing cells is associated with dysfunction of the spindle checkpoint mechanism, G₂-M arrest, failed cytokinesis, and multinucleation (44-46). High expression of survivin has been found in chronic myeloid leukemia patients, particularly in blast crisis (47), and its down-regulation sensitized Bcr-Abl-positive blast crisis cells to STI-571-induced apoptosis (48). All these findings are extremely important, as they suggest that anti-survivin strategies may have therapeutic potential in patients with chronic myeloid leukemia.

We detected a significant decrease of survivin protein level after treatment with curcumin. Bcr-Abl-expressing cells displayed a stronger down-regulation, which temporally correlated with a block in mitosis. Survivin mRNA determination using reverse transcription-PCR method together with protein level analysis shows that curcumin much greater decreases the protein than mRNA levels in C2 and C4 cells. This suggests that curcumin affects both translation and protein stability of survivin.

In untreated cells, a dynamic association of survivin with the mitotic apparatus was observed. Multiple defects in cell division were, however, evident (aberrant spindle formation, chromosome disorganization, and cytokinesis abnormalities) following survivin ablation by curcumin (Fig. 5).

The siRNA experiments were done to directly study the effects of survivin depletion on the respective cell lines. Survivin down-regulation caused extensive G₂-M arrest followed by polyploid formation (Fig. 6). Our finding was in agreement with previous reports describing pleiotropic cell division defects on survivin down-regulation (45, 49); however, it was in contrast with others who found no cells with DNA content higher than 4N (46). It has been suggested that the cell cycle arrest caused by survivin depletion depends on the p53-p21-mediated checkpoint response. Beltrami et al. showed that loss of survivin resulted in up-regulation of a p53 downstream target, p21, which also participated in the p53-dependent G₂-M arrest (50). Moreover, they found that the mitotic block induced by survivin ablation was lost in p53^{-/-} HCT-116 cells and in HeLa cells with functionally inactivated p53, whereas wild-type p53 limited the expansion of polyploid cells on survivin depletion. These findings are in line with our observations, as in contrary to curcumin-treated cells we did not observe p21 and p27 up-regulation at 24 and 48 hours after siRNA transfection. Slight up-regulation was observed only after 72 hours of survivin depletion (Fig. 6A). It suggests the possible role of p21 and p27 in the regulation of G₂-M arrest in curcumin-treated cells but is not the case after survivin depletion and allows polyploid formation. The nuclear changes occurring in survivin-depleted mitotic cells were similar to the abnormalities observed in curcumin-treated cells; however, in survivin siRNA-treated cells, large polyploid nuclei were visible together with multinuclei.

These data suggest that survivin down-regulation could play a role in the curcumin-induced mechanism of mitotic catastrophe, but it is unlikely to be the sole target of the drug, as cell death was activated earlier in curcumin-treated cells than in the cells with survivin depletion caused by siRNA. Curcumin also inhibits the activator protein-1 and nuclear factor- κ B transcription factors, and nuclear factor- κ B has been indicated as a very potent pro-survival agent in Bcr-Abl-expressing cells. Moreover, curcumin is a potent inhibitor of STAT activity, which is activated by the Bcr-Abl tyrosine kinase (19, 51, 52). It has also been reported recently that curcumin treatment of hepatoma cells leads to a significant decrease of histone acetylation by inhibition of histone acetyltransferase activity (53).

The enzymatic core of the CPC complex is Aurora B, which is complexed with survivin and inner centromeric protein. A functional CPC is involved in coordinating the chromosomal and cytoskeletal events of mitosis, regulating kinetochore formation, spindle assembly checkpoint, assembly of a stable bipolar spindle, and cytokinesis completion. CPC formation is necessary for the binding of the BubR1 and MAD2 proteins and mitotic assembly checkpoint activation inhibiting anaphase entry. The Bub and Aurora B proteins cooperate to maintain BubR1-mediated inhibition of anaphase-promoting complex (54). Survivin plays a crucial role in the CPC complex as it is a partner of Aurora B protein. They physically associate at

kinetochores; moreover, binding of survivin is required for full Aurora B activity and stability of binding (55). We examined whether survivin down-regulation affected Aurora B localization in mitotic cells, resulting in pre-early anaphase entry. Immunostaining experiments indicated mislocalization of Aurora B in curcumin-treated cells (Fig. 5) although without a decrease in total Aurora B protein. In cells depleted of survivin using RNA interference, Aurora B failed to concentrate at kinetochores, mitotic spindle midzone, and midbody, which corresponded to the abnormalities observed in curcumin-treated cells (Fig. 6). The down-regulation of survivin and dislocalization of Aurora B were sufficient to deregulate mitotic spindle formation and mitotic assembly checkpoint leading to premature anaphase entry and failed cytokinesis.

These data suggest a connection between the survivin down-regulation caused by curcumin and mitosis abnormalities that precede cell death in these cells. To our knowledge, this is the first report showing that curcumin affects the CPC function in Bcr-Abl-expressing cells. We have shown that mitotic catastrophe may be induced by curcumin treatment of Bcr-Abl-expressing cells that are resistant to conventional chemotherapy. Further study using different dose regimens are needed to determine whether effective concentrations are achievable and curcumin may represent a useful agent for future combination regimens, which may also include synergistically acting compounds.

Materials and Methods

Cell Culture and Treatment

The 32D mouse progenitor cells and Bcr-Abl-expressing C2 and C4 clones were kindly provided by Dr. S. McKenna (17, 18, 41). 32D cells were maintained in RPMI 1640 containing 10% FCS, 2 mmol/L L-glutamine, 1% penicillin/streptomycin (Life Technologies, Warsaw, Poland), and 10% WEHI conditioned medium as a source of IL-3. Clones expressing Bcr-Abl were cultured in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 1% penicillin/streptomycin, and 0.2 µg/mL puromycin. To induce cell death, cells were treated with either curcumin ($\geq 95\%$ purity, Cayman Chemical Co., Ann Arbor, MI) dissolved in DMSO (Sigma-Aldrich, Poznam, Poland) and added to the medium to a final concentration of 5, 10, or 20 µmol/L for MTT study or 20 µmol/L for other experiments or etoposide (Calbiochem, Warsaw, Poland) dissolved in DMSO and used at a 7.5 µg/mL (12.7 µmol/L) final concentration. PI was obtained from Sigma-Aldrich; the cell-permeable pancaspase inhibitor Z-Val-Ala-DL-Asp-fluoromethylketone (Bachem, St. Helens, Merseyside, United Kingdom) was added to 50 µmol/L concentration 1 hour prior curcumin treatment. Protease inhibitor cocktail was from Roche Diagnostics Co. (Warsaw, Poland).

Proliferation Measurement

For MTT assay, after appropriate time of incubation with curcumin, 50 µL MTT (Sigma-Aldrich) in PBS (5 mg/mL) was added to 0.5×10^5 cells in 100 µL in quintuplicate in 96-well plates. The cells were incubated for 2 hours at 37°C in humidified atmosphere (5% CO₂ in air). Plates were centrifuged at 1,300 rpm, and supernatants were discarded. DMSO (150 µL) was added to solubilize the purple formazan product

formed by the action of mitochondrial enzymes in living cells. The absorbance of each cell suspension was measured at 570 nm using a microplate reader (Reader 400 SFC, LabInstruments, Hamburg, Germany). Cell proliferation was calculated according to the following formula: (mean of tested sample / mean of control) $\times 100\%$.

For BrdUrd assay, after appropriate time of incubation with curcumin, BrdUrd (Sigma-Aldrich) was added to the culture medium to a final concentration of 10 µmol/L for 20 minutes. Then, 1×10^6 cells were collected and washed with PBS containing 0.1% Tween 20 followed by 30-minute incubation with 2 mol/L HCl at room temperature. After washing with 0.1 mol/L sodium tetraborate (Sigma-Aldrich) and twice with PBS-0.1% Tween 20, samples were incubated for 1 hour at room temperature with primary antibody against BrdUrd (Becton Dickinson, Warsaw, Poland), washed in PBS-0.1% Tween 20, and incubated with a secondary, phycoerythrin-conjugated antibody (Becton Dickinson). After washing, cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

Assessment of Cell Viability

Cell viability was assessed by PI exclusion assay on a FACSCalibur flow cytometer. Cells (0.5×10^6) were resuspended in PBS with 50 µg/mL PI before analysis. The criteria for cell death were based on changes in the permeability to PI (measured on the FL-2 channel).

Caspase-3 Activity Measurement

Cells were collected, washed with PBS, and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 15 minutes at 4°C. Then, cells were washed in PBS twice and fixed in cold 70% ethanol overnight at -20°C. After washing twice in PBS containing 0.1% Tween 20, the cells were incubated for 1 hour with phycoerythrin-conjugated antibody against active form of caspase-3 (Becton Dickinson). Cells were washed to remove unbound antibody, suspended in PBS-0.1% Tween 20, and analyzed on a FACSCalibur.

Cell Lysis and Western Blotting

The antibodies used were survivin (D-8; sc-17779), cyclin B1 (H-433; sc-752), p21 (C-19; sc-397), cyclin D2 (M-20; sc-593), p-Cdc2 p34 (Thr¹⁴/Tyr¹⁵; sc-12340), and Cdc2 p34 (B-6; sc-8395; all obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Anti-active caspase-3 antibody was obtained from BD PharMingen (Warsaw, Poland) and caspase-7 antibody was from Cell Signaling (Danvers, MA). Anti-Aurora B (AIM-1) and anti-p27^{Kip1} were from BD Transduction Laboratories (Warsaw, Poland). Anti-β-actin (Ab-1) antibody was obtained from Calbiochem. Cells were lysed in modified radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 2 mmol/L AEBSF, protease inhibitor cocktail, 1 mmol/L Na₃VO₃, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L NaF] on ice for 20 minutes. Equal amounts of protein (50-60 µg) were resolved using SDS-PAGE and electrotransferred to nitrocellulose. All secondary antibodies were horseradish peroxidase conjugated (DAKO, Gdymia, Poland) and detection was done using enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom).

DNA Content Analysis

Cell cycle as well as apoptotic DNA fragmentation measured by hypodiploid DNA content (sub-G₁ fraction) were analyzed by flow cytometry. One million cells were collected, washed, and suspended in Nicoletti buffer [0.1% sodium citrate (pH 7.4), 0.1% Triton X-100, 50 µg/mL PI]. DNA content was determined on a flow cytometer; 10,000 events were counted for each sample (FACSCalibur). The sub-G₁ fraction represents apoptotic cells; cellular debris was excluded from the analysis. Cell cycle analysis was done using the ModFit program (Becton Dickinson).

Semiquantitative Reverse Transcription-PCR

Total RNA was prepared using a QIAshredder homogenizer and RNeasy Mini kit (Qiagen, Düsseldorf, Germany). Total RNA (3 µg) was used in reverse transcription reaction with 1 mmol/L deoxynucleotide triphosphates (Roche Diagnostics), 0.5 µg anchored oligo(dT) primer (Sigma-Aldrich), 10 units RNase inhibitor (Sigma-Aldrich), and 1 µL reverse transcription polymerase (Sigma-Aldrich). Diluted cDNA was used as a template for PCR done in 25 µL total volume using Taq PCR Core kit (Qiagen). The annealing temperature was 59°C (for 60 seconds), amplification was at 72°C (for 60 seconds), and 25 cycles of PCR were done. Primers for survivin and β-actin were designed using GeneFisher (<http://bibiserv.techfak.uni-bielefeld.de/genefisher/>). Primer used were survivin forward 5'-AGGAATTGGAAGGCTGGGAA and reverse 5'-CCAGCTGCTCAATTGACTGA and β-actin forward 5'-GACCCAGATCATGTTTGA and reverse 5'-CTTCATGAGGTAGTCTGTCA. The amplified fragments were separated on 3% agarose gel and visualized by ethidium bromide staining. For each reverse transcription-PCR, a negative control without template was done (data not shown).

Mitotic Index

Mitotic index was estimated by the MPM-2 assay. The anti-phosphorylated Ser/Thr-Pro MPM-2 antibody (Upstate Cell Signaling Solutions, Warsaw, Poland) recognizes a phosphorylated epitope in proteins that are phosphorylated at the onset of mitosis. Staining was done according to producer's recommendations, except that as a secondary antibody, Alexa Fluor 633 goat anti-mouse IgG (H + L; Molecular Probes, Warsaw, Poland), was used at a concentration of 4 µg/mL. Additionally, after MPM-2 cells were stained for nuclear morphology with Hoechst 33258 (Sigma-Aldrich) in PBS at 2 µg/mL. Samples were analyzed on a scanning cytometer iCys Research Imaging Cytometer (CompuCyte, Cambridge, MA).

Morphology Observations

Cell morphology was observed under transmitted light or fluorescence microscopy. Whole-cell morphology was observed under inverse light microscope Olympus (Hamburg, Germany) IX70. Observation of the nuclei under fluorescence microscopy was done after Hoechst 33258 staining. Cells (0.5×10^5 – 0.75×10^5) were centrifuged onto cytospin coverslips and fixed with cold 70% ethanol at 4°C overnight. Fixed cells were washed in PBS and stained for 20 minutes in 1 µmol/L

Hoechst 33258 dye. The coverslips with stained cells were mounted on slides in the gel mount mounting medium (Sigma-Aldrich) and analyzed in a fluorescence microscope (Nikon, Warsaw, Poland) with a ×60 immersion objective.

Immunostaining

For immunostaining, cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature and permeabilized with 0.2% Triton X-100/PBS. After blocking in 2% bovine serum albumin/PBS, cells were incubated for 1.5 hours with a primary antibody against Aurora B (1:200) or survivin (1:200) and washed thrice in 0.05% saponin/PBS to remove unbound antibody. Then, the cells were incubated for 1 hour with Alexa Fluor 544–conjugated secondary antibody (1:200; Molecular Probes). For observation of nuclear morphology, cells were stained with DAPI (Sigma-Aldrich). The coverslips with stained cells were mounted on slides in the gel mount mounting medium and analyzed in a fluorescence microscope with a ×60 immersion objective.

RNA Interference

Survivin siRNA oligonucleotide corresponding to nucleotide sequence 5'-CCGUCAGUGAAUUCUAGAA-3' (Silencer Pre-designed siRNA: ID 1160715) was obtained from Ambion (Huntingdon, Cambridge, United Kingdom). siRNA was resuspended in RNase-free water and stored at –200°C. Transfection of siRNA was carried out using electroporation (Bio-Rad Gene Pulser Xcell Total System, Warsaw, Poland). Three million cells in 600 µL RPMI 1640 were incubated with siRNA (final concentration, 100 nmol/L) in a 0.4 cm cuvette for 5 minutes on ice before electroporation (260 V, 950 µF). After additional 5-minute incubation on ice, cells were resuspended in 3 mL RPMI 1640 supplemented with glutamine and 10% FSC without antibiotics. Antibiotics (1% penicillin/streptomycin) were added at 6 hours after electroporation. All measurements were done 24 to 72 hours after transfection.

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