

# DNA damage-independent apoptosis induced by curcumin in normal resting human T cells and leukaemic Jurkat cells

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**Curcumin, a phytochemical derived from the rhizome of *Curcuma longa*, is a very potent inducer of cancer cell death. It is believed that cancer cells are more sensitive to curcumin treatment than normal cells. Curcumin has been shown to act as a prooxidant and induce DNA lesions in normal cells. We were interested in whether curcumin induces DNA damage and the DNA damage response (DDR) signalling pathway leading to apoptosis in normal resting human T cells. To this end, we analysed DNA damage after curcumin treatment of resting human T cells (CD3<sup>+</sup>) and of proliferating leukaemic Jurkat cells by the fluorimetric detection of alkaline DNA unwinding (FADU) assay and immunocytochemical detection of  $\gamma$ -H2AX foci. We showed that curcumin-treated Jurkat cells and resting T cells showed neither DNA lesions nor did they activate key proteins in the DDR signalling pathway, such as phospho-ATM and phospho-p53. However, both types of cell were equally sensitive to curcumin-induced apoptosis and displayed activation of caspase-8 but not of DNA damage-dependent caspase-2. Altogether, our results revealed that curcumin can induce apoptosis of normal resting human T cells that is not connected with DNA damage.**

## Introduction

Curcumin, a phytochemical derived from the rhizome of *Curcuma longa* and present in the spice turmeric, has been used in traditional Indian and Chinese medicine for treating a variety of afflictions. Recently, curcumin has attracted the attention of researchers and clinicians as an agent with potential use in therapy for cancer and many other diseases (1). There are several worldwide clinical trials underway and a plethora of studies using animal and cell line models that try to elucidate the molecular mechanisms and biological effects of curcumin (2). Interestingly, curcumin exerts beneficial effects on the organism despite its low bioavailability (3). Curcumin has been shown to inhibit proliferation and to induce cell death in various cancer cells (2). Also our group has shown that curcumin is able to induce cell death in many different cancer cell lines (4–13). It is believed that they are more sensitive to curcumin than normal cells (14). However, we have shown that curcumin-induced cell death also occurs in normal cells (5–7), but the mechanism of cell death in normal resting cells is still elusive.

As there are some reports showing that curcumin causes DNA damage in human peripheral blood mononuclear cells (PBMCs) as detected by the comet assay (15,16), we wanted to know whether DNA damage, which is followed by the activation of the DNA damage response (DDR), could be responsible for the proapoptotic action of curcumin in normal resting T cells. Accordingly, we analysed DNA damage and DDR in normal resting T cells and in proliferating leukaemic Jurkat cells treated with curcumin. Although DDR operates mainly in cycling cells, activation of some key proteins involved in this signalling pathway was recognised also in normal non-proliferating cells, including PBMC (17,18) and resting T cells treated with etoposide (19). The DDR is mediated by DNA damage protein sensors such as the MRN complex that trigger the activation of a signal transduction system including the protein kinases Ataxia telangiectasia mutated (ATM), ATR, Chk1 and Chk2. Ultimately, the DDR activates p53, which contributes to either the apoptotic or the senescence response (20,21). ATM also phosphorylates histone H2AX, and phospho-H2AX, known as  $\gamma$ -H2AX, is a reliable marker of DNA double-strand breaks (DSBs) (22).

Our experiments revealed a high proapoptotic activity of curcumin in normal resting T cells. Moreover, the apoptotic index of curcumin-treated resting T cells was very similar to that found for proliferating leukaemic Jurkat cells. However, we did not detect symptoms of DNA strand breaks in curcumin-treated cells using the antibody against  $\gamma$ -H2AX as well as the fluorimetric detection of alkaline DNA unwinding (FADU) technique. In contrast to the classical DNA-damaging anticancer drug, etoposide (19), curcumin did not induce DDR in Jurkat cells. Our studies indicate the activation of the extrinsic apoptotic pathway, with the engagement of caspase-8, in curcumin-treated lymphocytes.

## Materials and methods

### Cells

Jurkat E6.1 cells obtained from ECACC (European Collection of Cell Culture) were cultured in the RPMI 1640 medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine and antibiotics and kept in a humidified atmosphere (37°C and 5% CO<sub>2</sub> in the air). The cells were seeded 24 h before treatment at a density of 4 × 10<sup>5</sup> cells/ml.

Human T cells were isolated from buffy coats of nine healthy donors (Domestic Blood Center, Warsaw, Poland) using the RosetteSep Human T Cell Isolation Cocktail according to the manufacturer's instruction. The cell purity was usually >95%. For treatment, cells were seeded at a density of 1 × 10<sup>6</sup> cells/ml.

Curcumin (Cayman, Ann Arbor, USA) and etoposide (Sigma-Aldrich, Poznan, Poland) were dissolved in dimethyl sulphoxide (DMSO) and added to the medium to a given final concentration. The DMSO concentration in cell culture did not exceed 0.5% and did not influence cell survival. Nonetheless, control cells were grown in equivalent concentration of DMSO. The project has been approved by the Local Ethic Commission.

### Apoptosis detection

Externalisation of phosphatidylserine to the outer layer of cell membrane was examined using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences, Warsaw, Poland) and FACSCalibur (BD Biosciences) and the CellQuest analysis software (BD Biosciences).

*Immunocytochemistry*

The fixed cells were incubated with primary anti- $\gamma$ -H2AX (Abcam, Cambridge, UK) or anti-53BP1 (Novus Biologicals, Cambridge, UK) monoclonal antibody diluted 1:500 in 1% bovine serum albumin/phosphate-buffered saline (BSA/PBS) (0.5% Tween-20 and 0.1% Triton X-100) for 2 h and with the anti-mouse Alexa 488/anti-rabbit Alexa 555 (Invitrogen, Eugene, USA) secondary antibody diluted 1:500 in 1% BSA/PBS (5% Tween-20 and 0.1% Triton X-100) for 1 h. DNA was stained with DRAQ5 (Biosstatus Limited, Leicestershire, UK) diluted 1:400 in PBS and the cover slips were mounted.  $\gamma$ -H2AX and 53BP1 foci were visualised with a Leica TCS SP2 confocal microscope.

*Western blotting*

Whole cell protein extracts were prepared according to Laemmli (23). Equal amounts of protein were separated electrophoretically in 8 or 12% sodium dodecyl sulphate–polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk dissolved in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature (RT) and incubated overnight at 4°C with one of the following primary antibodies: anti-ATM and anti-phospho-ATM Ser1981 (Millipore, Warsaw, Poland), anti- $\gamma$ -H2AX Ser139 (Abcam), anti-Chk2, anti-phospho-Chk2 Thr68, anti-p53, anti-phospho-p53 Ser15, anti-caspase-2, anti-caspase-8 and anti-caspase-9 (Cell Signaling, Boston, USA) and anti-poly (ADP-ribose) polymerase (PARP) (Enzo Life Sciences, Exeter, UK). Specific proteins were detected after 1 h incubation at RT with one of the horseradish peroxidase–conjugated secondary antibodies (Dako, Gdynia, Poland) using an enhanced chemiluminescent system according to the manufacturer's instructions.

*FADU method*

A modified and automated version of the FADU method was performed to measure the level of DNA damage and repair in cells treated with curcumin or etoposide. The level of DNA strand lesions was analysed 20 min after cell treatment as described previously (24). The measurement of DNA strand breaks by FADU is based on partial denaturation ('unwinding') of double-stranded DNA under controlled alkaline and temperature conditions. DNA strand breaks are sites where unwinding of DNA can start. Briefly, after infliction of DNA damage, cell lysis was performed. Unwinding was terminated by adding a neutralising solution. To quantify the amount of DNA remaining double stranded, a

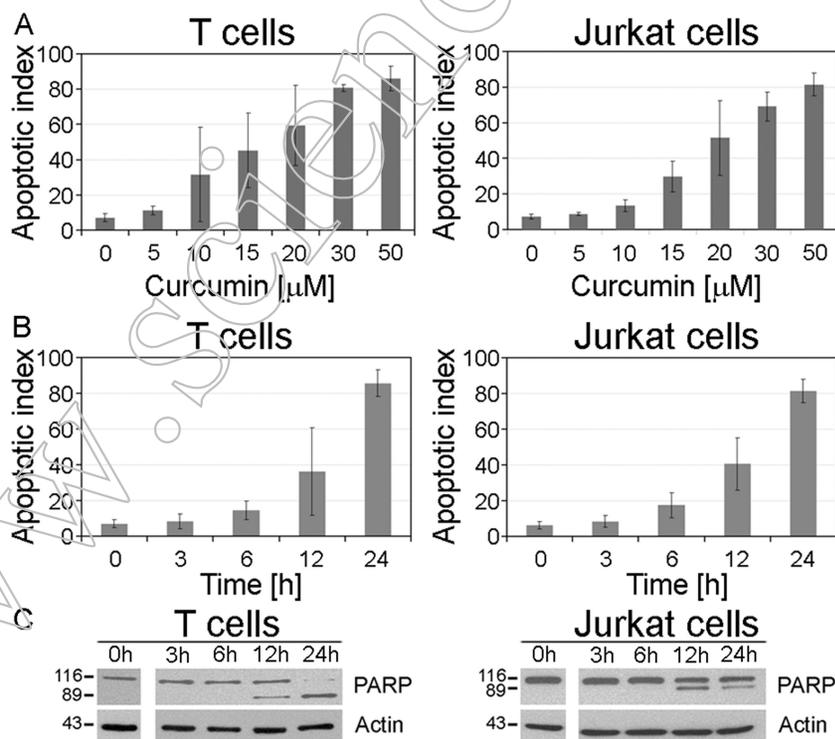
commercially available fluorescence dye (SybrGreen®) was used as a marker for double-stranded DNA.

**Results**

Apoptotic index measured in curcumin-treated cells was defined as the sum of the percentage of cells that were PE Annexin V positive and 7-aminoactinomycin D (7-AAD) negative (early apoptosis, membrane integrity is preserved) and of those that were PE Annexin V and 7-AAD positive (end stage of apoptosis and death).

We showed that curcumin's effect was dose dependent in both types of cell (Figure 1A). Although 15  $\mu$ M curcumin was sufficient to induce apoptosis in a substantial number of cells, for further experiments we chose a higher 50  $\mu$ M concentration that, in our opinion, could result in a more pronounced effect. The time-dependent changes in the percentage of apoptotic cells were very similar in Jurkat and normal T cells after curcumin treatment. Twenty-four hours after treatment, we found 80 and 90% of apoptotic cells in Jurkat and resting T-cell populations, respectively (Figure 1B). These results were confirmed by the detection of the caspase-3 substrate, PARP, cleavage product in both types of cell (Figure 1C).

Recently, it has been shown that curcumin induced DNA damage and DDR in cancer, but not immortalised, pancreatic epithelial cells (25). However, curcumin as an inducer of DNA lesions is still a matter of controversy because its protective action on DNA has also been documented (26). Accordingly, we were interested in whether curcumin-induced apoptosis, by introducing DNA breaks, could lead to DDR in normal resting human T cells and Jurkat cells. First, we checked DNA lesions by using two different methods, namely FADU and immunocytochemical detection of DNA damage foci.



**Fig. 1.** Curcumin-induced apoptosis of resting T cells and Jurkat cells. (A) Dose dependence of apoptotic index in curcumin-treated cells. (B) Time course of apoptotic index in cells treated with 50  $\mu$ M curcumin. Mean  $\pm$  SD values were obtained from four independent experiments in the case of Jurkat cells and from four different donors in the case of T cells. (C) The rate of apoptosis was estimated by the level of PARP proteolysis product. Representative blots of the material derived from three donors (T cells) and three independent experiments (Jurkat cells) are shown. The molecular-mass marker is shown on the left.

The FADU method serves to quantify the formation and repair of DNA single- and DSBs (24,27). Low fluorescence intensities indicate a large number of DNA strand breaks. This method revealed that curcumin did not substantially affect DNA either in leukaemic or in normal cells (Figure 2A). The level of fluorescence was the same for Jurkat control cells and cells treated with increasing concentrations of curcumin. In the case of normal resting T cells, it also seems that curcumin did not affect them substantially as only a slight decrease of fluorescence (<10%) was observed in curcumin-treated cells but without dose dependence. As a positive control, we treated Jurkat cells with etoposide. This DNA-damaging agent induced severe DNA damage in Jurkat cells that was already visible at a concentration <10  $\mu\text{M}$  and acquired plateau (fluorescence about 20%) at 10  $\mu\text{M}$ . Note that measurements using this method were performed in a very short period of time (20 min) when no DNA fragmentation due to apoptosis occurs.

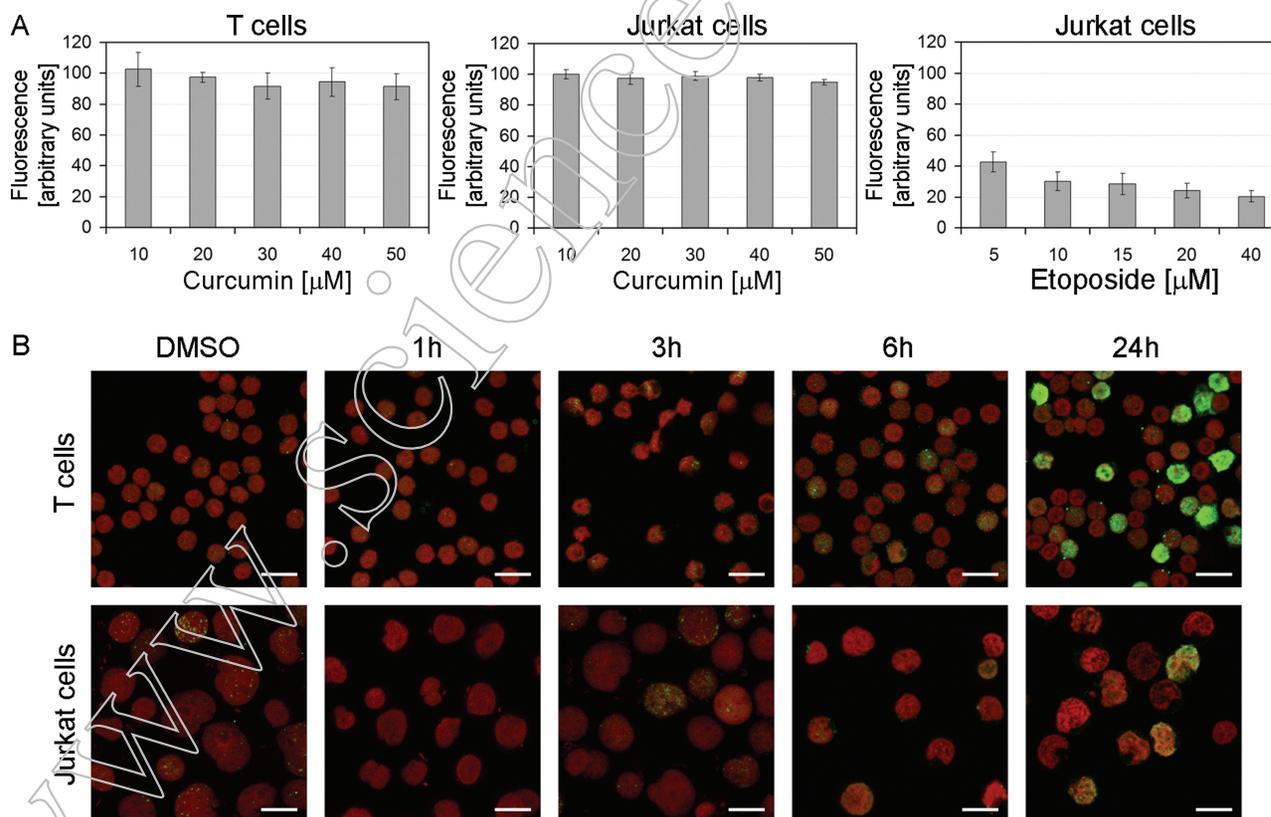
Induction of DSBs triggers phosphorylation of one of the variants of the nucleosome core histone, namely H2AX, on Ser139. This phosphorylation is mediated by ATM, which itself is activated by autophosphorylation on Ser-1981. The presence of phosphorylated H2AX, named  $\gamma$ -H2AX, can be detected immunocytochemically in the form of distinct nuclear foci where each focus is assumed to correspond to a single DSB (22). Concomitant activation of ATM and H2AX phosphorylation is considered to be a reliable hallmark of DSBs (22). Recently, 53BP1 (p53-binding protein 1) has also been recognised as a convenient marker of DSBs, forming nuclear foci together

with  $\gamma$ -H2AX (28). We found that curcumin induced neither  $\gamma$ -H2AX (Figure 2B) nor 53BP1 foci (not shown). However, 24h after treatment with curcumin, many cells stained for  $\gamma$ -H2AX were intensively green (Figure 2B). As was reported previously (29,30), this effect is characteristic of DNA damage in apoptotic cells, which display much stronger phosphorylation of H2AX and more intense fluorescence than observed in the case of primary lesions.

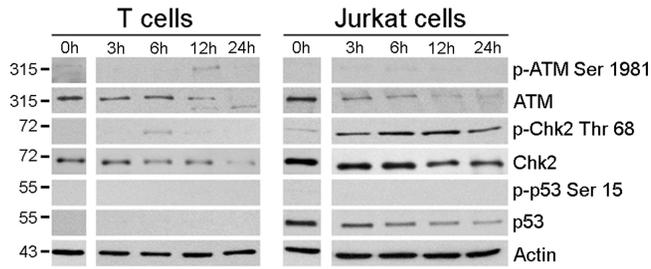
Subsequently, we measured the level of DDR key proteins after curcumin treatment by western blotting. Curcumin did not induce phosphorylation of p53 either in Jurkat or in resting T cells (Figure 3). Only very slight phosphorylation of ATM in resting T cells after 12 h of curcumin treatment was observed.

Interestingly, we observed phosphorylation of Chk2 in curcumin-treated Jurkat cells (3 h and afterwards). A very slight transient increase in phospho-Chk2 was also observed in curcumin-treated T cells (6 h).

On the bases of analysis of PARP proteolysis in both Jurkat and T cells treated with curcumin, we proved that executor caspase-3 was activated (Figure 1C). Therefore, we were interested in activation of upstream caspases—caspase-8, caspase-2 and caspase-9 after curcumin treatment. All these caspases might be activated either in a p53-dependent or p53-independent manner (31). Curcumin induced proteolysis of procaspase-8, but not of procaspase-2 or procaspase-9, in both Jurkat and resting T cells (Figure 4). Although we did not observe the products of procaspase-2 cleavage in curcumin-treated cells, the level of procaspase-2 decreased in time after curcumin treatment in both types



**Fig. 2.** Curcumin does not induce DNA damage. (A) Cells were treated with different concentrations of curcumin as indicated and the analyses were performed at 20 min after treatment. Etoposide-treated Jurkat cells are shown as a positive control. For T cells, mean values  $\pm$  SD from three independent measurements of cells derived from one representative donor are shown. For Jurkat cells, mean values  $\pm$  SD of at least three independent experiments are shown. The fluorescence of control cells is indicated as 100%; low fluorescence intensities indicate a large number of DNA strand breaks. (B) Resting T cells and Jurkat cells were treated with 50  $\mu\text{M}$  curcumin then cytospun, stained with DRAQ5 (red) and anti- $\gamma$ -H2AX conjugated with Alexa 488 (green) and visualised by a confocal microscope. Intensively, green cells stained for  $\gamma$ -H2AX, visible 24h after treatment and represent apoptotic cells.



**Fig. 3.** Curcumin does not induce DDR. The level of proteins involved in the DDR in curcumin-treated T cells and Jurkat cells as indicated. Representative blots of cells derived from three donors and three independent experiments, respectively, are shown. The molecular-mass markers are shown on the left.

of cell. This could be the result of massive cell death, especially cell death that occurred 24 h after treatment.

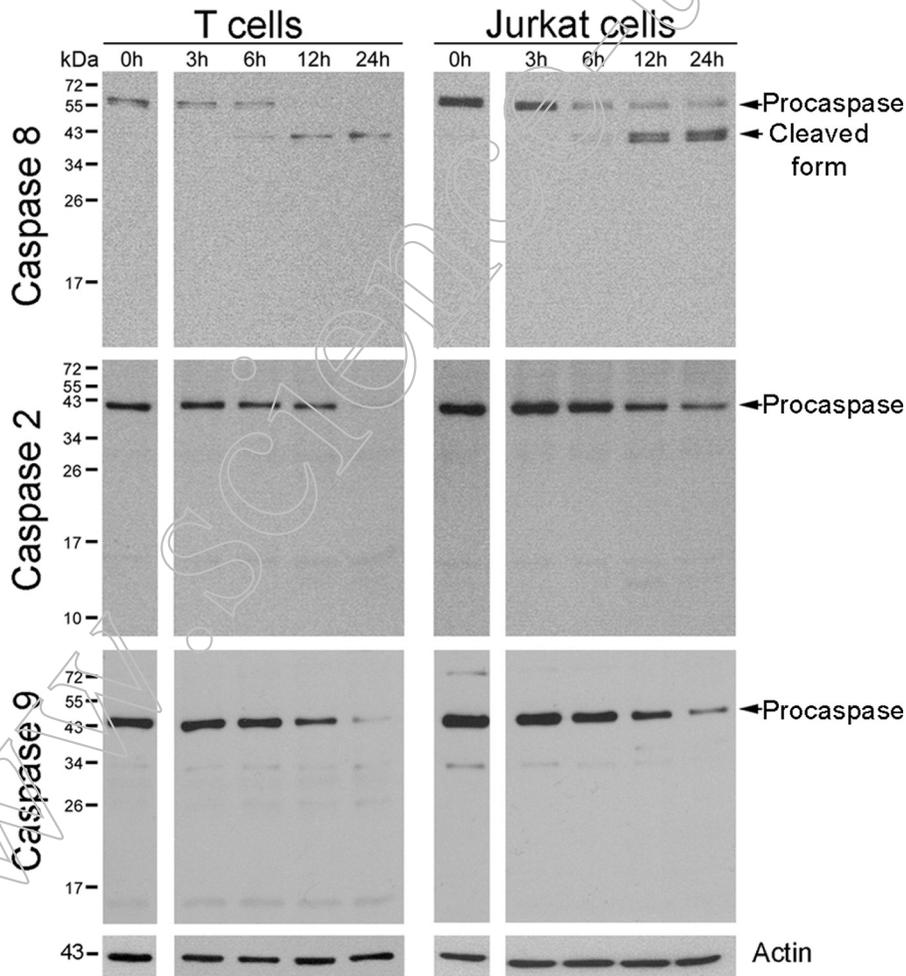
### Discussion

It is believed that normal cells are less prone to curcumin-induced cell death than cancer ones (14). However, low sensitivity to curcumin does not seem to be the case in normal lymphocytes; we (5,7) and others (32) have shown (using different methods and different T-cell subsets than in this study)

that curcumin was very effective as an inducer of apoptosis of normal human lymphocytes.

As several previous articles suggest that human (15,16) and mouse (e.g. 33) lymphocytes are prone to curcumin-induced DNA damage, assessed by the comet assay, we used other methods that measure both single (FADU) and double DNA breaks (FADU, immunocytochemistry of  $\gamma$ -H2AX) to reveal DNA lesions in curcumin-treated resting human T cells. None of them were able to reveal primary DNA damage in these cells. It cannot be excluded that this discrepancy is due to the fact that in studies performed by others, the PBMC fraction was analysed, which consists not only of the T cells (30–50%) but also of B cells, natural killers and monocytes/macrophages. Nonetheless, our experiments performed on proliferating normal mouse germ cells and early embryos (6) as well as on myeloid HL-60 cells (9) also excluded the induction of primary DNA damage by curcumin.

Our results revealed that curcumin did not induce DDR either in proliferating Jurkat cells or in normal resting T cells. Although we observed a slight induction of phospho-ATM at 12 h in curcumin-treated resting T cells, we suppose that this could be due to the recently described role of ATM in the oxidative stress response not connected to DNA damage (34). Interestingly, curcumin caused activation of Chk2 in Jurkat cells. Recently, it has been shown that Chk2 can be activated during mitosis independently of DNA damage and with



**Fig. 4.** Curcumin induces caspase-8 but not caspase-2 and caspase-9 cleavage. Procaspases and their cleavage products are shown. Left panel indicates that only caspase-8 is cleaved in curcumin-treated T cells. Similar results were obtained in Jurkat cells (right panel). Representative blots of cells derived from three donors and three independent experiments, respectively, are shown. Molecular-mass markers are shown on the left.

BRCA1 as the downstream effector (35,36). Actually, we did not observe either substantial DNA damage or ATM phosphorylation in curcumin-treated Jurkat cells. It cannot be excluded that the cells undergo mitotic catastrophe followed by apoptotic death, which might explain the observed increased phosphorylation of Chk2. Mitotic catastrophe finalised with caspase-dependent cell death was observed by us in the HCW-2 cells treated with curcumin (8). However, this explanation cannot be used in the case of non-proliferating T cells, in which we observed a slight activation of Chk2.

Recently, cell cycle arrest in the G<sub>2</sub>/M phase was observed in pancreatic cancer cells after a low (2.5 μM) dose of curcumin (25). Interestingly, activation of the ATM/Chk1 pathway, but not of Chk2, was reported in these cells. ATM predominantly phosphorylates Chk2 at Thr68, whereas ATR (predominantly) or ATM (to a lesser extent) phosphorylate Chk1 at Ser317/345 (37). In our hands, Chk1 phosphorylation on curcumin treatment was undetectable (not shown), so the role of Chk1 and Chk2 in curcumin-induced apoptosis is still awaiting elucidation.

We showed that caspase-8, but not caspase-2 and caspase-9, was cleaved both in curcumin-treated Jurkat and resting T cells. It seems that curcumin induces only the extrinsic p53-independent apoptotic pathway. To our knowledge, this is the first report of such an effect of curcumin on normal non-proliferating human cells. Activation of caspase-8 by curcumin was observed in cancer cells by others (38–40). Moreover, Bush *et al.* (41) showed that in melanoma cell lines, curcumin activated caspase-3 and caspase-8 but not caspase-9 in cells with wild and mutated p53.

Altogether, we show that curcumin induces cell death in an equally efficient manner in resting human T cells and proliferating Jurkat cells. In either case, the primary cause of its proapoptotic activity is not connected with the induction of DNA damage or the DDR. Curcumin induces caspase-8-dependent apoptosis in both types of cell.

Curcumin is considered to be a fully safe compound inducing apoptosis preferentially in cancer cells (14). Our results show that this is not the case for normal resting T cells that are considered relatively resistant to apoptosis. Regarding the future use of curcumin and its derivatives in the clinic and their eventual intravenous application, adverse side-effects could be expected and may include T cells death, and impairment of immune functions such as the suppression of proliferation, IL-2 production and decreased cytotoxicity of T cells.

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Conflict of interest statement: None declared.

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