Enhanced Antiproliferative and Pro-apoptotic Activities of a Novel Curcumin-related Compound in Jurkat Leukemia T-Cells

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Abstract. *Background/Aim: Inhibition of arachidonic acid metabolism by curcumin has been suggested to be a key mechanism for its anti-carcinogenic action. Recently, we reported on the synthesis of curcumin analogues and their evaluation as selective COX1 inhibitors. Two compounds (HP109/HP102) were selected for evaluation of their anti-proliferative and pro-apoptotic potential in Jurkat T-cells. Materials and Methods: Jurkat T-cells were stimulated with phorbol 12-myristate 13-acetate/ phytohemagglutinin (PMA/PHA) in the absence and presence of different concentrations of curcumin or HP109/HP102. Interleukin 2 (IL2) production and IL2 promoter activity were analyzed by enzyme-linked immunosorbent assay and a luciferase reporter assay, respectively. Proliferation and cell viability were monitored by 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide assay, annexin -V/7-amino-actinomycin D staining and western blotting. Results: HP102 was about 10-times more effective in blocking IL2 synthesis compared to curcumin. Enhanced effects of HP102 were also observed in reducing the proliferation rate and cell viability. In contrast to HP102, HP109 did not exhibit enhanced effects compared to curcumin. Conclusion: The curcumin analog HP102 had strongly improved the anti-proliferative and pro-apoptotic potential in Jurkat T-cells compared to curcumin.*

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The polyphenol curcumin (diferuloylmethane) is produced in the rhizome of Curcuma longa and has been shown to have a number of pharmacological and therapeutic activities, including antioxidant, anti-inflammatory, and anti-carcinogenic properties. Various molecular targets modulated by this agent include transcription factors, growth factors and their receptors, cytokines, enzymes, and genes related to cell proliferation and apoptosis (1). Curcumin has been shown to modulate production of many pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), Interleukin 1 (IL1) and IL6, as well as chemokines (2-4). The anti-inflammatory potential of curcumin promotes modulation in activation of different transcription factors, *e.g.* nuclear factor-kappa B, activator protein 1 and nuclear factor of activated T-cells (5). Curcumin has been also shown to inhibit the proliferation and survival of almost all types of tumor cells. Due to these activities, many researchers have suggested that curcumin-induced cell death is mediated both by the activation of cell death pathways and by the inhibition of growth and proliferative pathways (6-8). Curcumin causes DNA damage and endoplasmic reticulum stress and apoptosis through activation of the caspase signaling pathway (6).

In addition, inhibition of arachidonic acid metabolism by curcumin has been suggested to be a key mechanism for its anti-carcinogenic activity. Cyclo-oxygenase-1 and -2 (COX1/2) have been detected in various tumors and are considered to be promising therapeutic targets for cancer (9, 10). Handler *et al.* reported on the synthesis of novel curcumin analogs and their evaluation as selective COX1 inhibitors (11). All described curcumin analogs were selective and potent COX1 inhibitors with sub-micromolar to micromolar IC_{50} values. In the present study, we compared the effects of curcumin with the curcumin analogs HP109 and HP102 (compounds 2 and 3 in 11) on Jurkat T-cells, focusing on the impact of these compounds on cell activation, proliferation and apoptotic cell death.

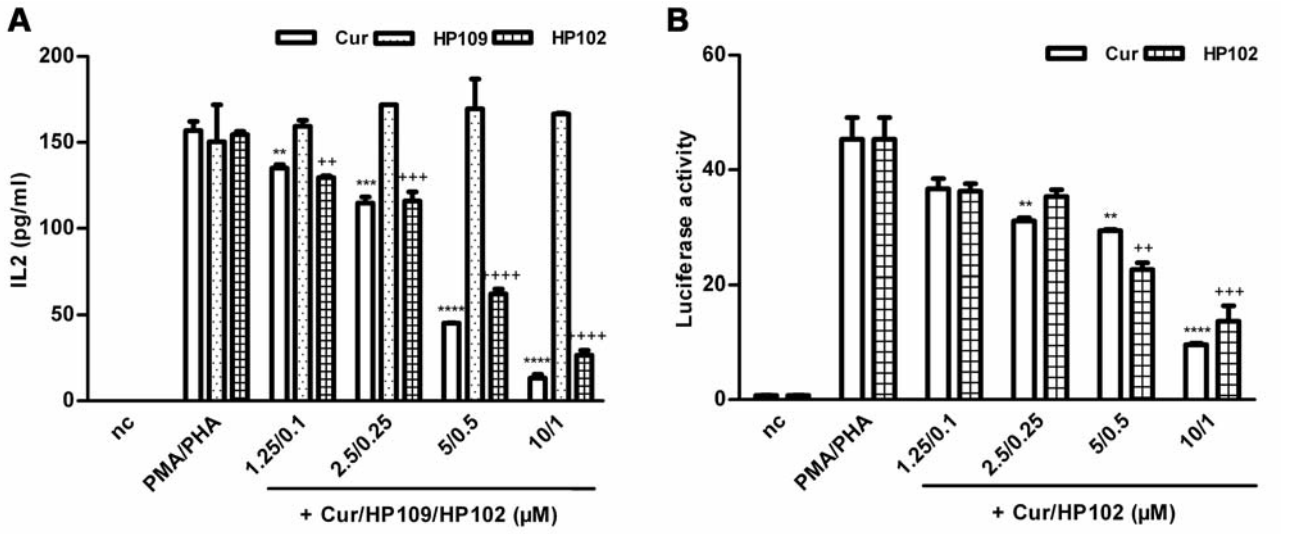

Figure 1. *HP102 attenuates Interleukin 2 (IL2) production and IL2 promoter activation in Jurkat T-cells. A: Jurkat T-cells were stimulated for 24 h with phorbol 12-myristate 13-acetate/phytohemagglutinin (PMA/PHA) in the absence or presence of different concentrations of curcumin or HP109 or HP102. IL2 levels in cell culture supernatants were detected by enzyme-linked immunosorbent assay. B: Jurkat T-cells were stimulated for 6 h with PMA/PHA in the absence or presence of different concentrations of curcumin or HP102 and luciferase activity was measured by a luminometer. Columns represent the mean±SEM. Experiments were performed in triplicates and repeated two times. nc, Negative control; Cur, Curcumin ($^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$), HP102 ($^{++}p<0.01$; $^{+++}p<0.001$; $^{++++}p<0.0001$), compared to the untreated control.*

Materials and Methods

Cells and cell culture. Jurkat T-cells containing a luciferase reporter gene under the control of the *IL2* promoter were kindly provided by K Schmetterer (Medical University Vienna, Austria). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (Life Technologies, Thermo Fisher Scientific, Inc. Waltham, MA, USA) at 37˚C and 5% CO_2. To induce IL2 expression, cells were treated with phorbol 12-myristate 13 acetate (PMA; 100 ng/ml) in combination with phytohemagglutinin (PHA; 1 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA) for 6 or 24 h. Curcumin was from Curcuma longa (Turmeric, purity ≥65%; Sigma-Aldrich), the curcumin analogs HP102 (1E,6E)-1,7-di[4-(methylsulfonyl)phenyl]-1,6-heptadien-3,5-dion and HP109 (1E,6E)-1,7-di[4-(methylmercapto)phenyl]-1,6-heptadien-3,5-dion were kindly provided by the Department of Pharmaceutical Chemistry (University Vienna, Austria) and synthesized as previously described (11). All compounds were dissolved in dimethylsulfoxide (DMSO) (purity ≥99.5%) and diluted into cell culture medium. In all samples, the final DMSO concentration was 0.5% (v/v).

Cytokine measurement. The concentration of IL2 in cell culture supernatants was quantified by enzyme-linked immunosorbent assay (ELISA) 24 h after activation with PMA/PHA in the absence or presence of different concentrations of the compounds. A commercially available kit was used according to the manufacturer´s instructions (eBioscience, San Diego, CA, USA).

Proliferation assay. Jurkat T-cells (1×10^4 cells/well) were grown for 24 h in the absence or presence of different concencentrations of curcumin or HP109 or HP102. After the addition of (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) reagent (Roche Diagnostics GmbH, Vienna, Austria), cell proliferation was estimated at 490/655 nm using a 96-well microplate reader (iMark; Bio-Rad Laboratories, Inc. Hercules, CA, USA).

Luciferase assay. Jurkat T-cells (1×10^5 cells/well) were stimulated for 6 h with PMA/PHA in the absence or presence of different concentrations of the compounds. Luciferase activity was measured in a luminometer following the instructions given in a luciferase assay kit (Promega, Madison, WI, USA). Data are represented in relative luciferase units (RLU).

Annexin-V/7-amino-actinomycin D (7-AAD) staining. Jurkat T-cells (1×10^5 cells/well) were grown for 24 h in the absence or presence of different concentrations of curcumin or HP102 and stained with Annexin-V/7-AAD reagent (Merck Millipore, Darmstadt, Germany) following the manufacturer's instructions. Cells were then analyzed with the Muse Cell Analyzer (Merck Millipore), as previously described (12).

Western blot analysis. Jurkat T-cells (2×10^6 cells/dish) were treated for 24 h with different concentrations of curcumin or HP102 and disrupted in sodium dodecyl sulfate (SDS) sample buffer (Roti-Load1; Carl Roth GmbH, Karlsruhe, Germany). Cell lysates were boiled for 5 min at 95˚C and treated with a short impulse of ultrasound (10 s) to minimize viscosity. Solubilized extracts were separated by SDS polyacrylamide gel electrophoresis (PAGE) on 12% polyacrylamide mini-gels, and electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc. Hercules, CA, USA). After blocking for 1 h at room temperature with 5% nonfat dried milk in PBST (1×PBS containing 0.1% Tween

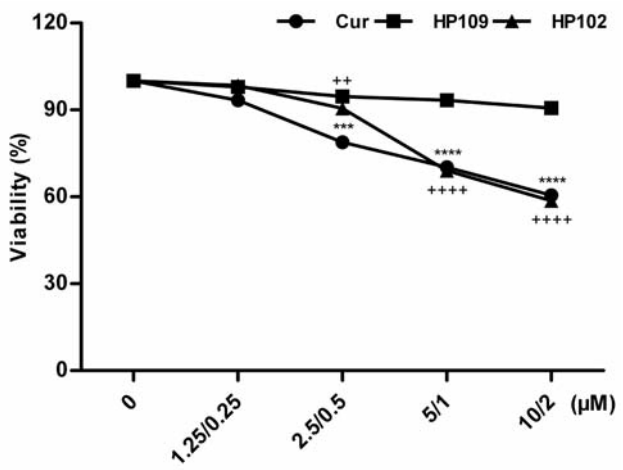

Figure 2. *HP102 induces cell growth arrest in Jurkat T-cells. Jurkat T-cells were grown for 24 h with different concentrations of curcumin or HP109 or HP102. After the addition of the XTT reagent, cells were incubated for an additional 21 h. Proliferation was estimated by measuring the absorbance at 490/655 nm. Columns represent the mean±SEM. Experiments were performed in triplicates and repeated once. Treatment with curcumin (Cur) from 2.5 μM or HP102 from 0.5 μM significantly reduced cell proliferation (*** $p<0.001$; **** $p<0.0001$; ++ $p<0.01$; ++++ $p<0.0001$).*

20), the membranes were probed overnight at 4˚C with specific antibodies to caspase-3/-7/-9, B-cell lymphoma2 (BCL2), BCLxL and Myeloid cell leukemia1 (MCL1) (Cell Signaling, New England Biolabs, Frankfurt am Main, Germany); antibody against tubulin was from Sigma-Aldrich. The membranes were washed with PBST and incubated for 2 h with horseradish peroxidase coupled anti-rabbit secondary antibody in PBST containing 1% non-fat dried milk. Finally, the stained bands were visualized by the Roti-Lumin plus substrate (Roth) and chemoluminescence signals were detected with the GeneGnome device (Syngene, Cambridge, UK).

Statistical analysis. Statistical analysis was performed with GraphPad Prism 5.0 (La Jolla, CA, USA) and a *p*-value of less than 0.05 was determined as the acceptable level of significance. The data are presented as means±SEM and analyzed with one-way ANOVA.

Results

HP102 suppresses IL2 expression and IL2 promoter activation in Jurkat T-cells. To study the effects of curcumin and the curcumin analogs HP109 and HP102 on T-cell activation, we used the Jurkat leukemia T-cell line as a model system. Jurkat T-cells were stimulated for 24 h with PMA/PHA in the absence or presence of different concentrations of curcumin (1.25-10 μM) or HP109 or HP102 (0.1-1 μM). Data show that treatment of Jurkat T-cells with PMA/PHA resulted in a strong increase in IL2 production (Figure 1A). In the presence of curcumin or HP102, IL2 expression was reduced in a dose-dependent manner (Figure 1A). We observed that 1 μM HP102 reduced IL2 expression to a level comparable to that when cells were treated with 10 μM of curcumin, clearly indicating the stronger efficacy of HP102 against Jurkat T-cells at lower doses (1-2 μM). Interestingly, HP109 did not have inhibitory any effects on IL2 expression (Figure 1A).

Additionally, we studied the effects of curcumin and HP102 on *IL2* promoter activation. Jurkat T-cells containing a luciferase gene under the control of the *IL2* promoter were stimulated for 6 h with PMA/PHA in the absence or presence of curcumin (1.25-10 μM) or HP102 (0.1-1 μM). Compared to PMA/PHA-stimulated cells, luciferase activity decreased in the presence of 1 μM HP102 at 70 and 10 μM curcumin at 80%, confirming the enhanced effects of HP102 at lower concentrations than curcumin (Figure 1B).

HP102 inhibits proliferation of Jurkat T-cells. Curcumin has been shown to inhibit cell growth and induces apoptosis of many types of cancer cells. Herein, we analyzed the impact of HP109 and HP102 on cell proliferation in comparison to that of curcumin. Jurkat T-cells were treated for 24 h with different concentrations of curcumin (1.25-10 μM), HP109 or HP102 (0.25-2 μM). XTT, a substrate to monitor mitochondrial activity, was added and the cells were incubated for another 21 h. Data show that both curcumin and HP102 reduced proliferation of Jurkat T-cells in a dose-dependent manner (Figure 2). At 10 μM curcumin, proliferation rate was reduced at about 40%. More importantly, treatment of the cells with 2 μM HP102 led to similar inhibitory effects on cell proliferation as observed with 10 μM curcumin (Figure 2). HP109 however, did not affect proliferation of Jurkat T-cells (Figure 2).

HP102 induces apoptotic cell death in Jurkat T-cells via the caspase signaling cascade. To determine whether HP102 reduced cell viability *via* activation of apoptotic processes, the cells were treated for 24 h with increasing concentrations of curcumin (1.25-20 μM) or HP102 (0.1-2 μM) and stained with Annexin-V/7-AAD. Data show that treatment of Jurkat T-cells with up to 5 μM curcumin had no significant effect on cell viability (Figure 3A). At 10 μM curcumin, 35% of the cell population was in the early apoptotic stage and 25% in the late apoptotic/dead stage. HP102 up to 0.5 μM did not negatively influence cell viability (Figure 3B). At 1 μM HP102, 25% of the cells were in the early apoptotic stage and 30% in the late apoptotic/dead stage (Figure 3B), highlighting the major sensitivity of Jurkat T-cells to HP102.

In addition, we investigated whether HP102 induced apoptosis *via* the caspase signaling pathway. Jurkat T-cells were treated with curcumin or HP102 at the same concentrations as described above and activation of the caspase signaling cascade was analyzed by western blots. As

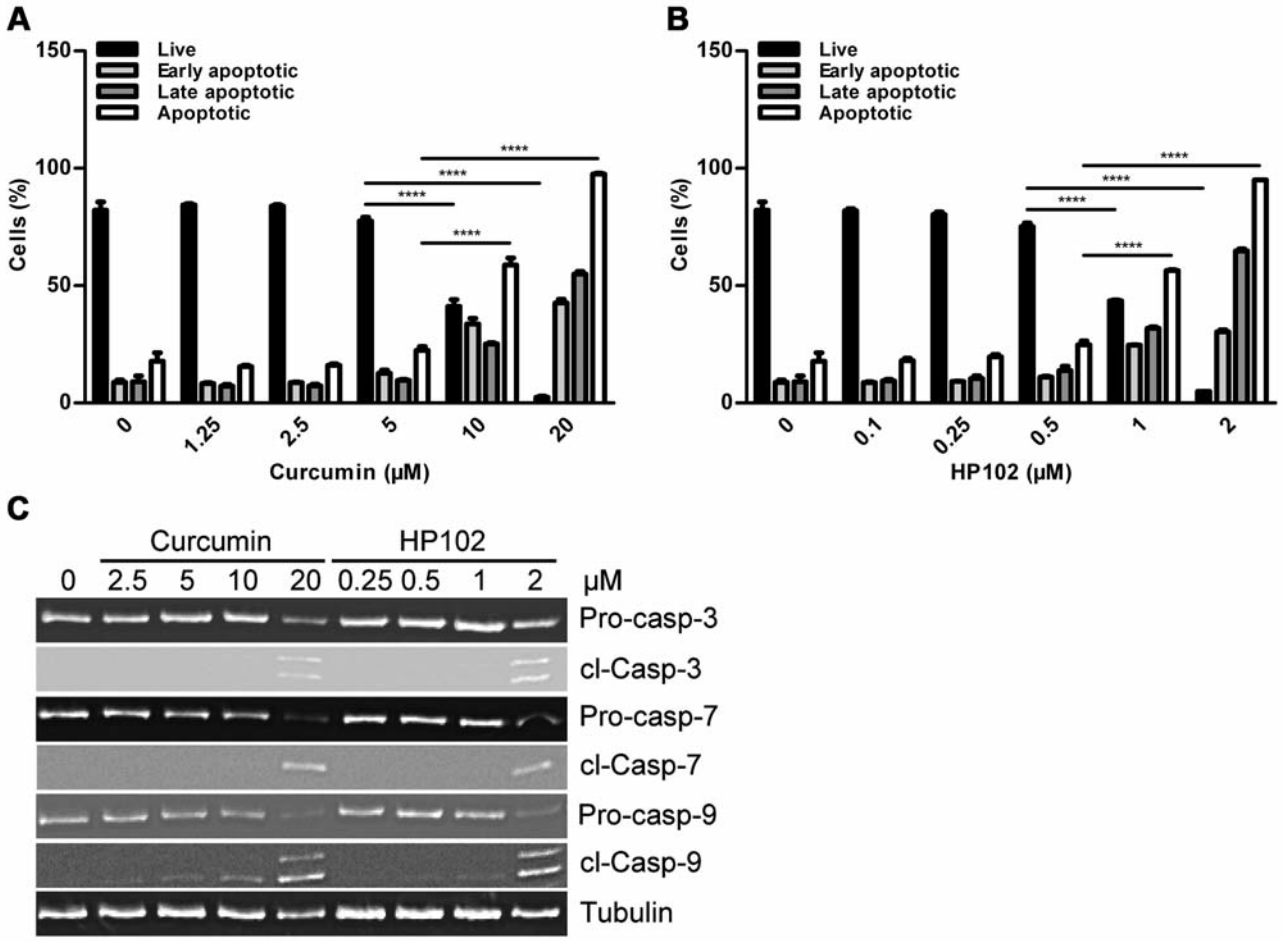

Figure 3. *HP102 induces apoptotic cell death in Jurkat T-cells via activation of caspases. Jurkat T-cells were grown for 24 h with indicated concentrations of curcumin (A) or HP102 (B) and labeled with Annexin-V/7-AAD. Columns represent the mean±SEM. Experiments were performed in duplicates and repeated once. Treatment with 10 μM curcumin or 1 μM HP102 dramatically reduced cell viability and led to apoptotic cell death (****p<0.0001). C: Jurkat T-cells were treated for 24 h with the indicated concentrations of curcumin or HP102 and cleavage of pro-Caspase-3/-7/-9 was analyzed by western blot. cl: Cleaved form.*

shown in Figure 3C, pro-caspase-9 and the effector caspases pro-caspase-3 and -7 were cleaved at 20 μM curcumin. Similar results were obtained when the cells were treated with 2 μM HP102 (Figure 3C).

HP102 induces cleavage of MCL1 protein. The BCL2 family members BCL2, BCLxL and MCL1 are known for their pro-survival and anti-apoptotic activities. Therefore, in the next experiment, we investigated whether curcumin and HP102 affected BCL2, BCLxL and MCL1 protein expression. Jurkat T-cells were treated for 24 h with different concentrations of curcumin (2.5-20 μM) or HP102 (0.25-2 μM) and cellular levels of BCL2, BCLxL and MCL1 proteins were analyzed by western blots (Figure 4). Data show that BCL2, BCLxL and MCL1 expression was constant up to treatment with 10 μM of curcumin or 1 μM HP102, but decreased at 20 μM curcumin and 2 μM HP102 (Figure 4). The decrease in BCL2, BCLxL and MCL1 protein levels were also accompanied by a drop of the "housekeeping" protein tubulin, indicating the cytotoxic effects of curcumin and HP102 at higher concentrations. MCL1 is a highly regulated anti-apoptotic protein which can be degraded by the proteasome pathway upon genotoxic stress (13), or cleaved by caspase-3 (14). Interestingly, cleavage products of MCL1 were detected at 20 μM curcumin and 2 μM HP102 (Figure 4), suggesting that MCL1 cleavage could be a downstream target of caspase-3.

Discussion

Curcumin, a naturally-occurring polyphenolic compound, has been shown to prevent tumor formation and metastasis (15). Many studies have implicated COX2 and COX1 in the

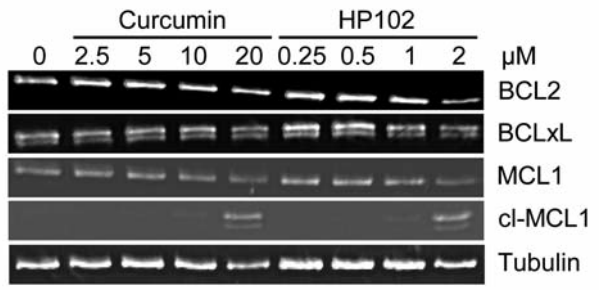

Figure 4. *HP102 induces cleavage of Myeloid cell leukemia1 (MCL1) in Jurkat T-cells. Jurkat T-cells were treated for 24 h with indicated concentrations of curcumin or HP102 and expression/cleavage of B-cell lymphoma2 (BCL2), B-cell lymphoma- extra large (BCLxL) and MCL1 proteins was analyzed by western blot. cl: Cleaved form.*

development and progression of various human cancer types (16, 17). In the present study, we evaluated the possible antiproliferative and pro-apoptotic activities of two compounds (HP109 and HP102) in Jurkat T-cells. Data demonstrate that HP102 has promising anticancer properties being much more effective than curcumin in inhibiting leukemia T-cell growth. HP102 blocked IL2 expression and proliferation of Jurkat T-cells at relatively low concentrations (1-2 μM), whereas curcumin reached the same effects at concentrations 5- to 10-fold higher. Whether the enhanced arrest in cell proliferation through HP102 was due to its high affinity to COX1, which in turn may lead to a drop in prostaglandin synthesis, has to be investigated in further experiments. Interestingly, the curcumin analog HP109 neither blocked IL2 synthesis nor affected cell proliferation at low concentrations, suggesting that the methylsulfonyl group in HP102 may be responsible for its enhanced activity.

Several studies considered curcumin as a pro-apoptotic drug. In this context, we investigated whether HP102-driven proliferation arrest was due to induction of apoptosis. Our data show that HP102 induced apoptosis of Jurkat T-cells at concentrations which correlated well with those effective in inhibition of proliferation. HP102 induced activation of the caspase signaling pathway by initiating cleavage of pro-caspase-9 followed by the effector caspases 3 and 7, apparently being 10-fold more efficient than treatment with curcumin. Other proteins that are inhibited by curcumin include the anti-apoptotic proteins BCL2, BCLxL and MCL1. Curcumin and HP102 reduced expression of BCL2, BCLxL and MCL1 at concentrations which were already toxic to the cells. We observed that MCL1 was cleaved both by curcumin and HP102 in a dose-dependent manner, which was in accordance with the cleavage of pro-caspase-3, -7, and -9, suggesting a direct link between caspase activation and MCL1 cleavage. Recent data indicate that MCL1 may play more profound roles than BCL2 and BCLxL in response to cell death (18). Weng *et al*. reported that apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand of Jurkat T-cells required the specific cleavage of MCL1 by caspase-3, while other anti-apoptotic proteins such as BCL2 and BCLxL seemed not to be affected (14). TRAIL belongs to the tumor necrosis factor (TNF) superfamily and initiates apoptosis through engagement of death receptors DR4 and DR5 (19). Jung *et al*. reported that curcumin sensitizes TRAIL-induced apoptosis through reactive oxygen species (ROS)-mediated upregulation of DR5 in human renal cancer cells (20). In this respect, we will investigate whether this process could also be relevant in curcumin-treated Jurkat leukemia T-cells.

To conclude, the novel curcumin analog HP102 had enhanced antiproliferative and pro-apoptotic activities in Jurkat T-cells compared to curcumin. In further studies, we will investigate the pro-apoptotic potential of HP102 in other types of cancer cells (*e.g*. colon cancer, breast cancer).

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