Bioorganic & Medicinal Chemistry Letters 20 (2010) 7054–7058

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Mycoepoxydiene, a fungal polyketide, induces cell cycle arrest at the G2/M phase and apoptosis in HeLa cells

Jifeng Wang [a], Baobing Zhao [a], Wei Zhang [a], Xuan Wu [a], Ruoyu Wang [a], Yaojian Huang [a], Dong Chen [b,c], Kum Park [c], Bart C. Weimer [b,c,*,†], Yuemao Shen [a,b,*]

[a] Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, School of Life Sciences, Xiamen University, Xiamen, Fujian 361005, China
[b] Joint Center for Systems Biology of Xiamen University and Utah State University, Xiamen University, Xiamen, Fujian 361005, China
[c] Center for Integrated BioSystems, Utah State University, 4700 Old Main Hill, Logan, UT 84322, USA

ARTICLE INFO

Article history:
Received 14 July 2010
Revised 2 September 2010
Accepted 21 September 2010
Available online 25 September 2010

Keywords:
Mycoepoxydiene
Polyketide
G2/M arrest
MAPK
Apoptosis

ABSTRACT

Mycoepoxydiene (MED) is a polyketide isolated from a marine fungus associated with mangrove forests. It contains an oxygen-bridged cyclooctadiene core and an α,β-unsaturated δ-lactone moiety. MED induced the reorganization of cytoskeleton in actively growing HeLa cells by promoting formation of actin stress fiber and inhibiting polymerization of tubulin. MED could induce cell cycle arrest at G2/M in HeLa cells. MED-associated apoptosis was characterized by the formation of fragmented nuclei, PARP cleavage, cytochrome *c* release, activation of caspase-3, and an increased proportion of sub-G1 cells. Additionally, MED activated MAPK pathways. Interestingly, the time of JNK, p38, and Bcl-2 activation did not correlate with the release of cytochrome *c*. This study is the first report demonstrating the action mechanism of MED against tumor cell growth. These results provide the potential of MED as a novel low toxic antitumor agent.

Cancer is one of the most prevalent and important life-threatening diseases worldwide. Despite the discoveries of new biological molecules and immune-mediated therapies, chemotherapy remains the major clinical approach to treating cancer.[1,2] However, the current chemotherapies are far from optimal, and there is evidence that resistance to the available drugs is increasing. Since natural products have played a central role in the development of cancer chemotherapies[3–6] the search for new therapeutics from natural sources is in critical need of expansion.

Mycoepoxydiene (MED) (Fig. 1A), a novel polyketide containing an oxygen-bridged cyclooctadiene core and an α,β-unsaturated δ-lactone moiety, was isolated from the marine fungus *Diaporthe* sp. HLY-1 found in submerged rotten leaves of *Kandelia candel* in a mangrove forest in Fujian Province, China.[7] MTT assays were used to measure the inhibitory activity of MED, exhibited cytotoxic activity against KB cell line with an IC_{50} less than 21.5 μM.[7] The same compound was found in the fungal strain OS-F66617 isolated from twig litter,[8] and the endophytic *Phomopsis* sp. HANT25 from the Thai medicinal plant *Hydnocarpus anthelminthicus*.[9] Due to interest in its unique structural features, the total chemical synthesis of MED was achieved in 2002.[10] The natural and synthetic forms of MED showed similar cytotoxicity to human tumor cell lines with an IC_{50} ranging from 2.4 to 21.5 μM.[8,9,11] However, the inhibitory mechanism of MED against the growth of tumor cells remains unknown. This study is the first report demonstrating the mechanism of tumor cell growth inhibition by MED. MED induces a cascade of cellular events that includes cytoskeletal rearrangement, cell cycle arrest at G2/M, p38 MAPK/JNK signaling, cytochrome *c* release, and caspase-3-mediated apoptosis.

To begin this work, MTT assays were done in 12 different human tumor cell lines. MED inhibited growth of cells various cancer types—AGS (gastric), HT-29 (gastric), DU 145 (prostate), MDA-MB-435 (breast), MDA-MB-231 (breast), MCF-7 (breast), U2OS (bone), HeLa (cervical), OVCAR3 (ovarian) and A549 (lung) (Supplementary Fig. 1). With such broad activity across tissue types, we selected HeLa cells to examine the effects and mechanisms of cell death induced by MED. Additional in vitro assays demonstrated that the MED IC_{50} was 16.8 μM (Fig. 1B) using HeLa cells in vitro using propidium iodide (PI), a fluorescent probe to measure membrane integrity. Using flow cytometry to observe PI staining, we found that the ratio of dead cells increased as the MED concentration increased (Fig. 1C). As expected, the ratio of dead cells also increased with increasing exposure time (Fig. 1D).

MED induced morphological changes in cells within 9 h of exposure (Fig. 2A). The cells shrank, became rounded, and contained fragmented nuclei, all of which are morphological features (i.e.,

* Corresponding authors. Tel.: +592 2184180; fax: +592 2181722 (Y.S.).
E-mail addresses: bcweimer@ucdavis.edu (B.C. Weimer), yshen@xmu.edu.cn (Y. Shen).
† Present address: University of California—Davis, Veterinary Medicine, Population Health and Reproduction, One Shields Ave, 2055 Haring Hall, Davis, CA 95616, USA.

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.09.105

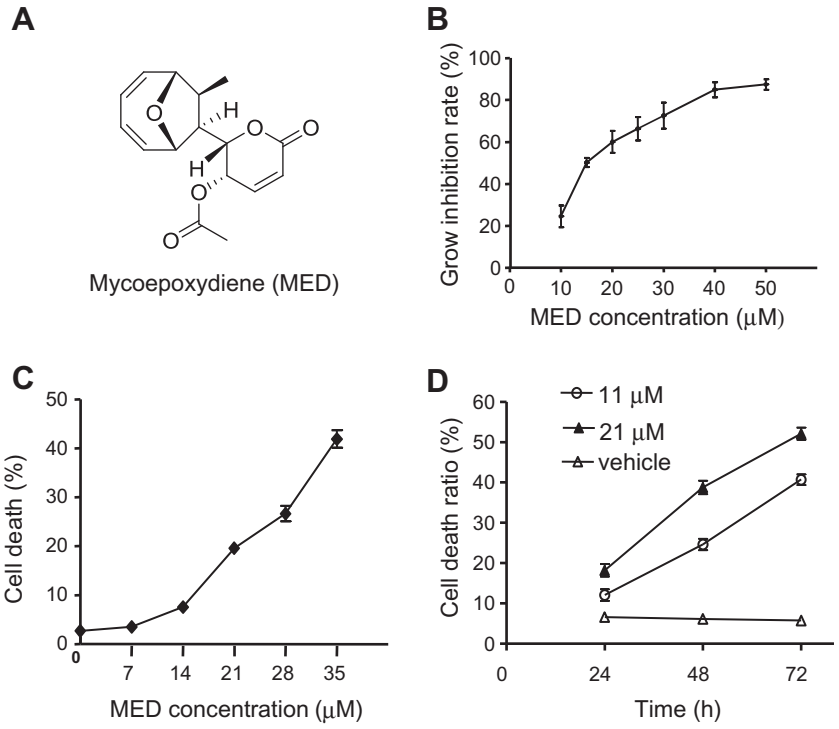

Figure 1. MED inhibits HeLa cell growth. (A) Structure of MED. (B) Viability of HeLa cells following 72 h exposure to various concentrations of MED. The cell survival rate was calculated by MTT assay as described in Supplementary data. Each value represents the mean ± SD of three independent experiments. Flow cytometric analysis of MED-induced cell death in HeLa cells. Cells (5×10^5 cells/well in 6-well plates) were treated with various MED concentrations for 24 h (C) or treated for various incubation times at 11 and 21 μM MED (D) and then stained with PI. Cell numbers were determined by flow cytometry.

condensed nuclei) typical of stressed cells moving into apoptosis.[12,13] These observations led us to hypothesize that MED exposure resulted in cytoskeletal changes reflected in molecular changes that led to apoptosis.

Based on the morphological changes induced by MED addition to growing HeLa cells, we looked for alterations in the cytoskeleton. MED induced a concentration- and time-dependent increase in actin stress fiber formation across the cell body beyond that observed for control cells (Fig. 2B). Interestingly, MED caused a time-dependent disruption of the tubulin cytoskeleton characterized by microtubule rearrangement within 9 h of treatment (Fig. 2C). Based on these observations, we concluded that MED dramatically disrupted the cytoskeleton of HeLa cells, producing a diffuse microtubule network and an increase in actin stress fibers.

Consistent with the cytoskeletal changes, we hypothesized that additional changes in the cell cycle likely led to signs of apoptosis (i.e., condensed nuclei). Treatment of cells with MED caused inhibition of cell cycle progression after 12 h that resulted in an increase in the percentage of cells at G2/M phase compared to the control (Fig. 3A, top panel). Western blot analysis revealed proteins associated with cell cycle-associated changes due to MED addition. Additionally, apoptotic cells recognized by flow cytometry showed diminished uptake of PI. The mean apoptotic population was 1.5% (sub-G1 phase) in the control, while in MED-treated cells it increased to 23.3% after 36 h (Fig. 3A, bottom panel).

MED treatment resulted in a time- and concentration-dependent reduction in the expression of Cdc2 p34, GADD45α, Wee1, Myt1, Chk1, and Chk2 proteins. Increased production of p21 and cyclin B1 accompanied these changes (Fig. 3B). Taken together, these findings indicate that MED induced cycle arrest at G2/M phase after 24–36 h via protein expression regulation.[14] Since only a portion of the cells entered cycle arrest and nuclear fragment bodies were found even with higher concentrations of MED. This suggests that MED has weak activity on the inducing of G/M arrest as a mechanism to induce apoptosis.

Observation of widespread cell membrane disruption, cytoskeletal changes, cell cycle arrest, and nuclear condensation led us to hypothesize that MED directly induces apoptosis via established cell death signaling pathways, all of which have an intersection via mitochondrial disruption. To test this hypothesis, we first monitored release of cytochrome *c*, which is an important mitochondrial inner membrane-associated protein that plays a role in caspase-induced apoptosis when released into the cytoplasm.[15,16] After 20 h treatment with 21 μM MED, cytochrome *c* was released into the cytoplasm, As show in (Fig. 4A), the cytosolic fraction confirmed that MED-treated cells contained increasing amounts of cytochrome *c* with increasing MED exposure. Tubulin (cytosol) and Hsp60 (mitochondria) served as controls to verify localization. Taken together, these results confirm that MED exposure induces cytochrome *c* release into the cytosol.

Subsequently, we determined expression of a number of proteins involved in activation of the mitochondrial dysfunction to initiate apoptosis. Initially, we examined Bcl-2, Bak, and Bad; in concert, these proteins regulate mitochondrial integrity and retention of cytochrome *c* inside mitochondria.[17] The expression of Bak and Bad were inversely regulated as compared to Bcl-2 (Fig. 4B). This change led to a change in the pro/anti-apoptotic balance modulated by the Bcl-2 ratio leading to disruption of mitochondrial integrity that in turn results in the release of cytochrome *c* into the cytoplasm and initiation of apoptosis via at least three signal transduction routes.[18]

Mitogen-activated protein kinases (MAPK) superfamily, consists of three family members: the extracellular signal-regulated kinases (ERK), c-jun N-terminal kinase (JNK) and p38 MAPK. JNK and p38 MAPK pathway are implicated primarily in the induction of apoptosis and inflammation after exposure to different

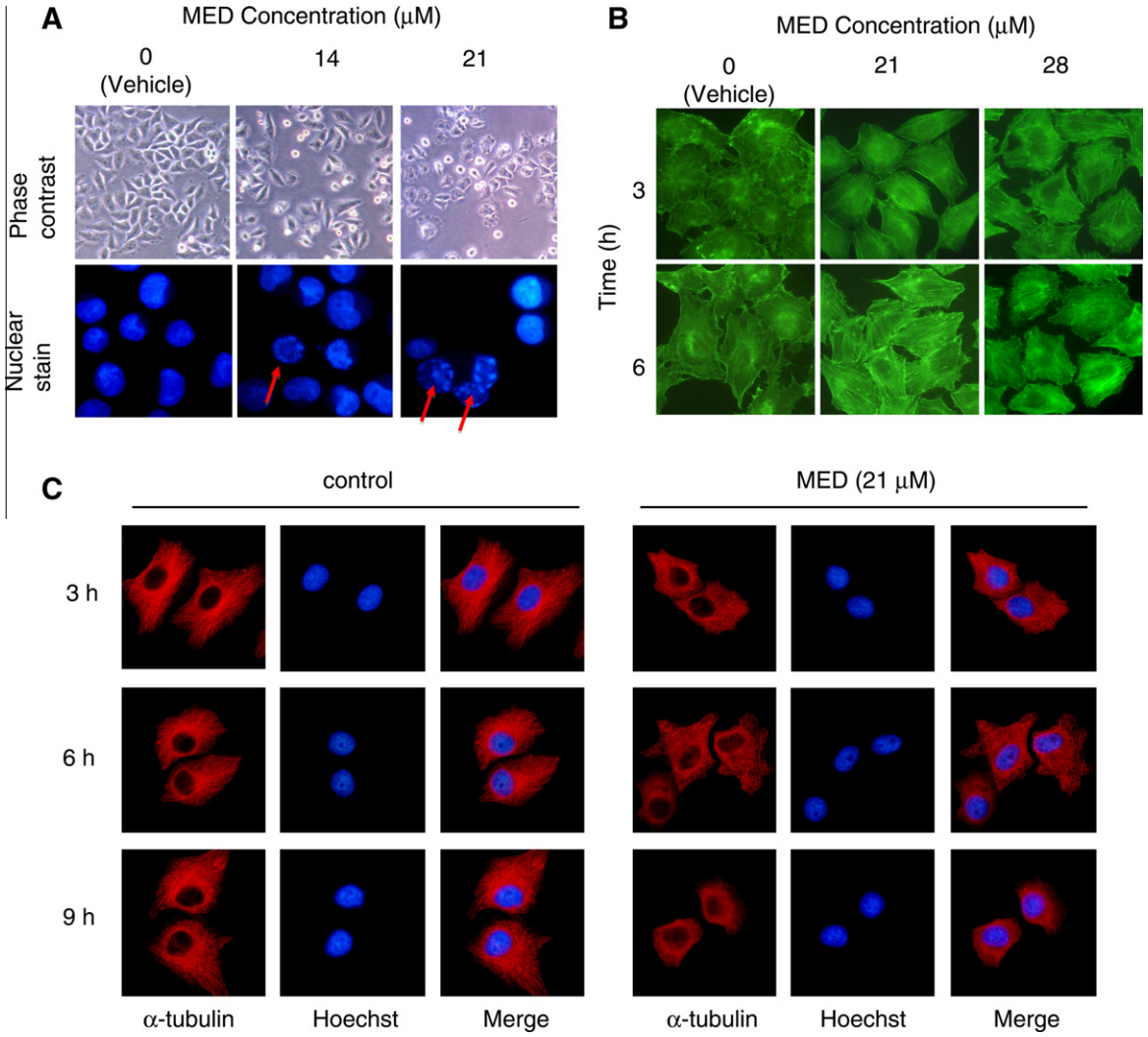

Figure 2. (A) Microscopy of HeLa cells treated with increasing concentrations of MED. HeLa cells were treated for 12 h with 0, 14 or 21 μM MED. The images were captured using phase contrast (12 h at 400×) or fluorescence (24 h at 1000×) for nuclear staining with 0 (vehicle), 14 or 21 μM MED. Apoptotic cells with condensed nuclei were observed using nuclear staining as indicated with red arrows. To observe the effect of MED on the HeLa cytoskeleton, HeLa cells were seeded onto glass coverslips that were precoated with 0.2 mg/ml gelatin. The cells were allowed to adhere overnight before treatment for different times with or without MED. Cells were fixed and stained for observation of the cytoskeleton. (B) Effect of MED on the F-actin cytoskeleton; MED enhances the polymerization of F-actin. (C) Effect of MED on the tubulin cytoskeleton. MED disrupts the polymerization of α-tubulin.

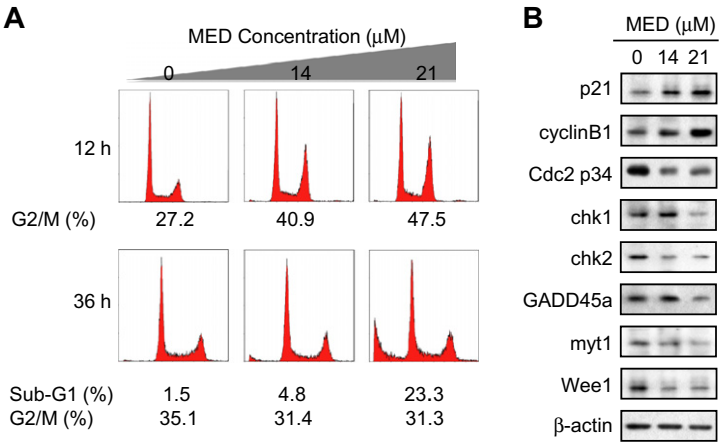

Figure 3. The effects of MED on the cell cycle distribution of HeLa cells. Representative histogram plots of the total PI-labeled populations are shown. (A) MED caused cell cycle arrest at the G2-M phase (12 h) and the appearance of a sub-G1 peak (36 h). Cells were treated with vehicle or MED for 12 or 36 h, and cell cycle distribution was assessed by flow cytometry. Cells appearing in the sub-G1 region of the DNA content histogram represent apoptotic cells. Percentage values were calculated from the number of gated events in the G2/M or sub-G1 region out of the total events (20,000). (B) The effect of MED on cell cycle-related proteins. The levels of various proteins in HeLa cells treated with 21 μM MED for 12 h were determined by immunoblot assay.

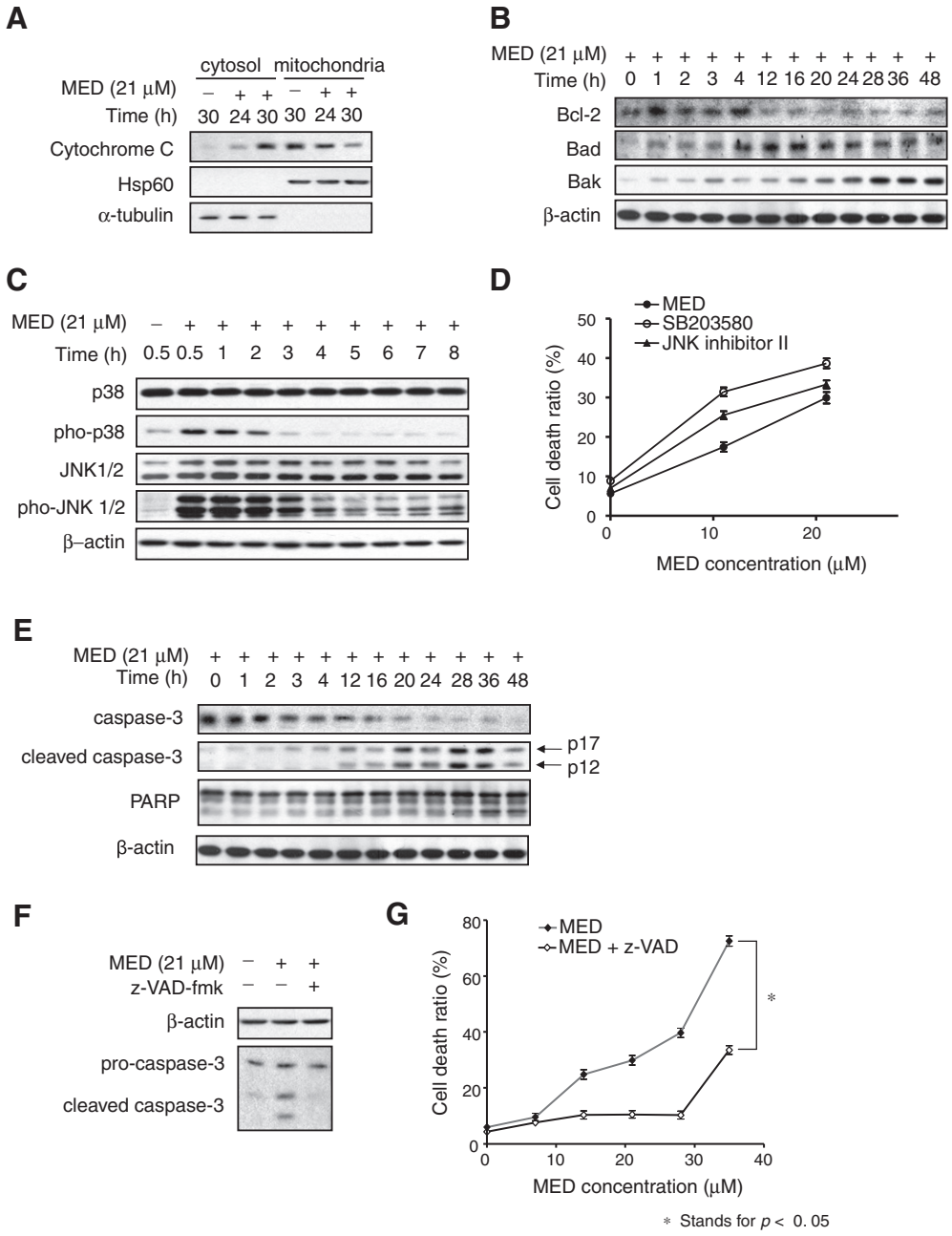

Figure 4. Analysis of apoptosis induced by MED. (A) Western blot analysis revealed a greater amount of cytochrome *c* in the cytoplasm of MED-treated cells compared to controls. The absence of Hsp60 in the cytoplasm indicated that mitochondrial contamination of the cytoplasm was negligible. The presence of tubulin was used as a control to assess the purity of each fraction, as it should only appear in the cytosol. (B) Western blot analysis for the expression of apoptosis-related proteins. (C) MED increased the activation of MAPK. (D) Effects of MAPK inhibitors on MED-induced cell death. Cells were pre-treated with MAP kinase inhibitors 1 h prior to the addition of MED (14 or 21 μM). The number of live cells (PI-negative) was analyzed by fluorescence flow cytometry 48 h post treatment. (E) Western blot analysis of MED-induced caspase-3 activation and the cleavage of PARP. (F) Western blot analysis of MED-induced caspase-3 activation in HeLa cells treated with MED (21 μM) for 24 h. The cleavage of pro-caspase-3 was blocked by pre-treatment with z-VAD-fmk. (G) Effect of the pan-caspase inhibitor z-VAD-fmk on MED-induced apoptosis. Exponentially growing cells were treated for 48 h with z-VAD-fmk (25 μM) in addition to MED. The number of live cells (PI negative cells) was analyzed by flow cytometry. *Stands for p <0.05.

agents.[19,20] To understand the decline in Bcl-2, we investigated p38 MAPK/JNK levels and phosphorylation as a mechanism to activate Bcl-2-related cytochrome *c* release.[21] Treatment with MED increased JNK phosphorylation (Thr183/Tyr185) and p38 phosphorylation (Thr180/Tyr182) transiently within 30 min of exposure, but the expression of unphosphorylated p38 and JNK remained constant (Fig. 4C). To examine the functional consequence of the p38 MAPK and JNK phosphorylation changes, cells were pre-treated with MAP kinase inhibitors 1 h prior to MED exposure. Pre-treatment with inhibitors of JNK (JNK inhibitor II) or p38 MAPK (SB203580) did not relieve the cytotoxic effect of MED-induced cell apoptosis (Fig. 4D).

To uncover additional mechanisms of apoptosis that are independent of JNK and p38, we investigated cytochrome *c*-mediated activation of caspase-3 (Casp-3), which is an established mechanism of apoptosis.[22] Treatment with MED (21 μM) decreased pro-Casp-3 over time with a concomitant increase in the cleavage fragments p17 and p12 (Fig. 4E). Notable increases in cleavage fragments appeared after 12 and 28 h of incubation. We also observed cleavage of PARP (Fig. 4E) by Casp-3 during the onset of

apoptosis. Pre-treatment of the cells with the caspase inhibitor z-VAD-fmk, which broadly inhibits caspase cleavage,[23] inhibited MED-induced Casp-3 cleavage (Fig. 4F) and significantly ($p \leqslant 0.05$) reduced MED-induced cell death (Fig. 4G).

Antitumor natural products can be classified into cytotoxic and mechanism-based agents. Cytotoxic agents act on ubiquitous targets such as Hsp90, topoisomerases, mTOR, DNA or tubulin.[3] Mechanism-based agents were developed on the basis of significant advances in cancer biology, which act on cancer-specific molecular targets.[24] However, those targets need to be strictly validated before moving forward to drug discovery,[25] and mechanism-based antitumor agents have the tendency of facing resistance in therapeutics.[26] As the major types of solid human tumors (breast, lung, prostate, and colon) are multi-causal in nature (in contrast to, e.g., chronic myeloid leukemia, which is mostly driven by a specific genetic abnormality), therefore, the search for improved cytotoxic agents continues to be an important line of modern anticancer drug discovery that will be critical for future advances in cancer therapy.

Mycoepoxydiene (MED) is a fungal polyketide possessing a novel backbone. It showed evident cytotoxicity in the previous research.[7–9] Here, for the first time, we discovered MED inhibited the growth of HeLa cells by inducing apoptosis accompanied with cytoskeletal rearrangement and cell cycle arrest at G2/M phase. MED induced G2/M arrest through increasing p21 and cyclin B1 expression and decreasing the expression of cdc2, GADD45, wee1, myt1, chk1 and chk2. Apoptosis in HeLa cells was induced by decrease of the Bcl-2 level, and increase of the Bad and Bak levels resulting in release of cytochrome *c* from mitochondria that subsequently activated caspases and the cleavage of PARP. Additionally, MED activated p38, MAPK, and JNK; but the function of this activation needs further exploration to determine the biological significance. These unique mechanisms induced by MED treatment indicate that this compound may be useful as an anti-cancer treatment.

Acknowledgments

This work was supported by NSFC (90913024), 863 Program (2007AA091503 and 2006AA09Z410), 973 Program (2010CB83 3802), the National Science Fund for Distinguished Young Scholars to Y.-M.S. (30325044), and the United States Department of Agriculture (2008-34526-19199) to B.C.W.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.105.

References and notes

1. Neidle, S.; Thurston, D. E. *Nat. Rev. Cancer* **2005**, *5*, 285.
2. Rajamanickam, S.; Agarwal, R. *Drug Dev. Res.* **2008**, *69*, 460.
3. Butler, M. S. *Nat. Prod. Rep.* **2005**, *22*, 162.
4. Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2007**, *70*, 461.
5. Paterson, I.; Anderson, E. A. *Science* **2005**, *310*, 451.
6. McAlpine, J.; Romano, A.; Ecker, D. *Curr. Opin. Drug Disc. Dev.* **2009**, *12*, 186.
7. Lin, X.; Huang, Y.; Fang, M.; Wang, J.; Zheng, Z.; Su, W. *FEMS Microbiol. Lett.* **2005**, *251*, 53.
8. Cai, P.; McPhail, A. T.; Krainer, E.; Katz, B.; Pearce, C.; Boros, C.; Caceres, B.; Smith, D.; Houck, D. R. *Tetrahedron Lett.* **1999**, *40*, 1479.
9. Prachya, S.; Wiyakrutta, S.; Sriubolmas, N.; Ngamrojanavanich, N.; Mahidol, C.; Ruchirawat, S.; Kittakoop, P. *Planta Med.* **2007**, *73*, 1418.
10. Takao, K.; Watanabe, G.; Yasui, H.; Tadano, K. *Org. Lett.* **2002**, *4*, 2941.
11. Takao, K.; Yasui, H.; Yamamoto, S.; Sasaki, D.; Kawasaki, S.; Watanabe, G.; Tadano, K. *J. Org. Chem.* **2004**, *69*, 8789.
12. Habold, C.; Poehlmann, A.; Bajbouj, K.; Hartig, R.; Korkmaz, K. S.; Roessner, A.; Schneider-Stock, R. *J. Cell. Mol. Med.* **2008**, *12*, 607.
13. Hsu, H. Y.; Wu, H. L.; Tan, S. K.; Li, V. P.; Wang, W. T.; Hsu, J.; Cheng, C. H. *Mol. Pharmacol.* **2007**, *71*, 344.
14. Yang, H.; Chung, D. H.; Kim, Y. B.; Choi, Y. H.; Moon, Y. *Toxicology* **2008**, *243*, 145.
15. Kroemer, G.; Zamzami, N.; Susin, S. A. *Immunol. Today* **1997**, *18*, 44.
16. Zou, H.; Li, Y.; Liu, X.; Wang, X. *J. Biol. Chem.* **1999**, *274*, 11549.
17. Reed, J. C. *Nature* **1997**, *387*, 773.
18. Dejean, L. M.; Martinez-Caballero, S.; Guo, L.; Hughes, C.; Teijido, O.; Ducret, T.; Ichas, F.; Korsmeyer, S. J.; Antonsson, B.; Jonas, E. A.; Kinnally, K. W. *Mol. Biol. Cell* **2005**, *16*, 2424.
19. Lewis, T. S.; Shapiro, P. S.; Ahn, N. G. *Adv. Cancer Res.* **1998**, *74*, 49.
20. Obata, T.; Brown, G. E.; Yaffe, M. B. *Crit. Care Med.* **2000**, *28*, N67.
21. Ohtsuka, T.; Zhou, T. *J. Biol. Chem.* **2002**, *277*, 29294.
22. Cohen, G. M. *Biochem. J.* **1997**, *326*, 1.
23. Misaghi, S.; Korbel, G. A.; Kessler, B.; Spooner, E.; Ploegh, H. L. *Cell Death Differ.* **2006**, *13*, 163.
24. McLaughlin, F.; Finn, P.; La Thangue, N. B. *Drug Discovery Today* **2003**, *8*, 793.
25. Benson, J. D.; Chen, Y. N.; Cornell-Kennon, S. A.; Dorsch, M.; Kim, S.; Leszczyniecka, M.; Sellers, W. R.; Lengauer, C. *Nature* **2006**, *441*, 451.
26. Chabner, B. A.; Roberts, T. G., Jr. *Nat. Rev. Cancer* **2005**, *5*, 65.