Diosmetin induces apoptosis by upregulating p53 via the TGF-β signal pathway in HepG2 hepatoma cells

BIN LIU*, YUFENG SHI†, WENDING PENG*, QINGYU ZHANG, JIE LIU, NIANPING CHEN and RUNZHI ZHU

Laboratory of Hepatobiliary Surgery, Guangdong Medical University; Zhanjiang Key Laboratory of Hepatobiliary Diseases, Affiliated Hospital of Guangdong Medical University, Zhanjiang, Guangdong 524001, P.R. China

Received June 18, 2015; Accepted April 25, 2016

DOI: 10.3892/mmr.2016.5258

Abstract. Diosmetin (Dio) is a major active component of flavonoid compounds. A previous study demonstrated that Dio exhibited anticancer activity and induced apoptosis in HepG2 human hepatoma cells via cytochrome P450, family 1-catalyzed metabolism. The present study observed that cell proliferation of HepG2 cells was inhibited by Dio treatment and tumor protein p53 was significantly increased following Dio treatment. Following addition of recombinant transforming growth factor-β (TGF-β) protein to Dio-treated HepG2 cells, cell growth inhibition and cell apoptosis was partially reversed. These findings suggest a novel function for the TGF-β/TGF-β receptor signaling pathway and that it may be a key target of Dio-induced cell apoptosis in HepG2 cells.

Introduction

Natural products, particularly those that bind to microtubules, are important in the development of anticancer therapeutic agents (1). Diosmetin (Dio) is a flavone compound in various dietary sources, including oregano, citrus fruits, and it may also be extracted from certain medicinal herbs, such as Rosa agrestis Savi (Rosaceae), Chrysanthemum morifolium (Asteraceae) and Dianthus versicolor Fisch (Caryophyllaceae) (2,3). Flavonoids are considered be associated with a variety of beneficial effects, including antioxidant activity (4), which protect tissues against oxidative stress and associated pathologies, such as inflammation (5).

Dio is used in traditional Mongolian medicine to treat liver diseases (6). Hepatocellular carcinoma (HCC) is a global health problem (7) and therapeutic strategies for HCC are predominantly focused on chemotherapy, for example, the alkylating agent cisplatin or the topoisomerase inhibitor doxorubicin (8).

Dio exerts synergistic cytostatic effects in HepG2 cells via cytochrome P450, family 1 (CYP1)-catalyzed metabolism, activation of c-Jun N-terminal kinase (JNK)/extracellular signal-regulated kinase (ERK) and tumor protein p53 (p53)

DOI: 10.3892/mmr.2016.5258

Contributed equally

Key words: diosmetin, TGF-β, HepG2, apoptosis
X protein (Bax) expression (17). Thus, the aim of the present study was to investigate the association between TGF-β and Dio-induced cell apoptosis in HepG2 cells.

Materials and methods

Compounds and reagents. Diosmetin (C₁₄H₁₂O₆; Fig. 1A) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The original concentration of Dio stored at -20°C was 10 mg/ml. TGF-β human recombinant was purchased from Prospec-Tany TechnoGene, Ltd. (East Brunswick, NJ, USA) and anti-human antibodies against p53, Bcl-2, Bax, TGF-β, TβRII, Smad3, phosphorylated (p)-Smad2/3 and GADPH were all purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody was obtained from EarthOx Life Sciences, LLC (Millbrae, CA, USA). Cell Counting Kit-8 (CCK8) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from Beyotime Institute of Biotechnology (Haimen, China).

Cell culture and Dio treatment. HepG2 cells were provided by the Affiliated Hospital of Guangdong Medical College (Zhanjiang, China). The cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C, and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., USA) supplemented with 10% (v/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 U/ml streptomycin. HepG2 cells were grown in standard media, and when 60-70% confluent, the cells were treated with different concentrations of Dio (5, 10 and 15 µg/ml) or TGF-β protein/Dio (10 µg/ml) for 24 h. Images were captured by microscopy (magnification, x100).

Annexin V/propidium iodide (PI) double staining. Apoptotic cells (2×10⁴ cells) were quantified with an Annexin V-fluorescein isothiocyanate (FITC)/PI kit (BD Biosciences, Franklin Gardens, NJ, USA) and detected with the FACSCalibur™ flow cytometer (BD Biosciences). Data were analyzed with Modfit 3.2 and BD FACSDiva 6.1.3 software (BD Biosciences). Briefly, cells were pretreated with 5, 10, 15 and 20 µg/ml Dio for 24 h and washed with phosphate-buffered saline (PBS), and then cells were collected and resuspended in binding buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 2.5 mM CaCl₂ and 140 mM NaCl]. Cells were incubated with Annexin V-FITC and PI for 15 min in the dark, prior to flow cytometric analysis. Annexin V-positive cells indicated early stage apoptosis, whereas Annexin V and PI-positive cells indicated late stage apoptosis.

MTT and CCK8 analysis. HepG2 cell density was adjusted to 2×10⁴ cells/100 µl, and the cells were seeded into 96-well plates and placed in an incubator overnight (37°C in 5% CO₂) to allow for attachment and recovery. MTT and CCK8 analyses were performed separately. Briefly, cells were pretreated with 5, 10, 15 and 20 µg/ml Dio for 24 h. A total of 20 µl MTT solution (5 mg/ml in PBS) solution was transferred to each well to yield a final 120 µl/well and to separate wells a total of 10 µl CCK8 (5 mg/ml in PBS) was transferred. The plates were incubated for 4 h at 37°C in 5% CO₂ and the absorbance was recorded using the EnSpire™ 2300 Multilabel Plate Reader (PerkinElmer, Inc., Waltham, MA, USA) at wavelengths of 595 nm and 450 nm, respectively. The half maximal inhibitory concentration (IC₅₀) of Dio was calculated using software (Shmm, 1.0.0.0).

Western blotting. Cells were collected and lysed in lysis buffer [100 mM Tris-HCl (pH 6.8), 4% (m/v) sodium dodecylsulfate (SDS), 20% (v/v/g) glycerol, 200 mM 2-mercaptoethanol, 1 mM phenylmethyl sulfonylfluoride and 1 g/ml aprotinin] for 30 min on ice. The lysates were separated using centrifugation at 4°C for 15 min at 3,913 x g. The total protein concentration in the supernatants was detected using the BCA Protein assay kit (Beyotime Institute of Biotechnology). Proteins (20 µg) were separated using 8-15% SDS-PAGE and subsequently transferred to nitrocellulose membranes. These were saturated with 5% milk in Tris-buffered saline and 1% Tween-20 (TBST) and incubated with the following primary antibodies in a diluent overnight at 4°C: p53 (9282), Bcl-2 (2876), Bax (2772), TGF-β (3711), TGF-βRII (11888), Smad3 (9513), p-Smad2/3 (8828) and GAPDH (2118) (all 1:1,000; Cell Signaling Technology). Membranes were washed three times with TBST and incubated with HRP-conjugated goat anti-rabbit IgG (E030120-01; EarthOx Life Sciences) for 1 h at room temperature, followed by washing four times with TBST. An enhanced chemiluminescence kit (GE Healthcare Life Sciences, Chalfont, UK) was added to the membrane and detection was performed using an Odyssey® CLX Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Immunofluorescence (IF). Cells were seeded into 6-well plates and pretreated with 10 µg/ml Dio for 24 h. All floating and attached cells were harvested and fixed with ice-cold 4% formaldehyde for 10 min and then washed with ice-cold PBS. Cells were permeabilized with 0.3% Triton X-100, washed with ice-cold PBS and stained with the following primary antibodies against: p53 (9282), p-p53(Ser15) (9284), p-p53(Ser46) (2521), p-p53(Ser20) (9287), p-p53(Thr81) (2676), p-p53(Ser37) (9289), p-p53(Thr18) (2529) and p-p53(Ser33) (2526) (all 1:500; Cell Signaling Technology). Cells were subsequently incubated with Alexa Fluor® secondary antibodies (Invitrogen; Thermo Fisher Scientific, Inc.). Following staining with 4,6-diamidino-2-phenylindole (DAPI), cells and nuclei were observed with a fluorescence microscope (Leica TCS SP5II, Leica Microsystems GmbH, Germany) at wavelengths of 520±20 nm and blue fluorescence at 460 nm.

Statistical analysis. Data from the present study were analyzed using GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, CA, USA). Data are presented as the mean ± standard deviation from triplicate experiments unless otherwise stated. Statistical differences were assessed using the Student’s t-test and *P<0.05 was considered to indicate a statistically significant difference.

Results

Dio inhibits cell proliferation and promotes cell apoptosis. It was demonstrated that untreated HepG2 cells grew well and
Figure 1. Dio specifically induces apoptosis in HepG2 cells. (A) Chemical structure of Dio. (B) Cell proliferation in HepG2 cells treated with different concentrations (5, 10, and 15 µg/ml) of Dio for 24 h were visualized by microscopy (magnification, x100). (C) Cell growth inhibition rates with different concentrations (5, 10, 15 and 20 µg/ml) of Dio for 24 h were analyzed by MTT and CCK8 methods. (D) Cell apoptosis treated with different concentrations (5, 10 and 15 µg/ml) of Dio for 6 h were visualized by flow cytometry. Dio, diosmetin; CCK8, Cell Counting Kit-8.

Figure 2. TGF-β partly ameliorated Dio-mediated cell apoptosis. (A) TGF-β (2 ng/ml) reversed Dio (10 µg/ml)-induced cell apoptosis (magnification, x100). (B) Cell were treated with 10 µg/ml Dio and different TGF-β protein concentrations for 24 h, and cell growth inhibition rates were analyzed by MTT assay. (C) Cells were treated with 10 µg/ml Dio and 2 ng/ml TGF-β protein for 24 h, and cell growth inhibition rates were analyzed by MTT assay. Dio, diosmetin; TGF-β, transforming growth factor-β.
were observed to have with normal skeletons, whereas cells treated with Dio were distorted and a number of them became round and floating. The number of normal cells was reduced, and sloughed cells were increased in a concentration-dependent manner (Fig. 1B). MTT and CCK8 analysis was used to evaluate the inhibitory effects of Dio in HepG2 cells. Results from the present study demonstrate that Dio inhibits cell proliferation in HepG2 cells in a concentration-dependent manner (Fig. 1C). Cell apoptosis was detected by flow cytometry with the results indicating that Dio may induce cell apoptosis in a concentration-dependent manner (Fig. 1D). In the early stages of apoptosis, cells were Annexin V-positive (Q4), whereas Annexin V and PI-positive cells were considered to be in the late stage of apoptosis (Q2). The IC_{50} of Dio was determined to be 12.0 µg/ml.

**TGF-β is important in Dio-triggered apoptosis.** To investigate whether cell growth reversed following recombinant TGF-β administration, HepG2 cells were treated with 10 µg/ml Dio and different concentrations of TGF-β and images were captured using microscopy (magnification, x100; Fig. 2A and B). Cells were treated with or without Dio (10 µg/ml) and with or without TGF-β (2 ng/ml) for 24 h. The MTT assay was performed to detect the proliferation inhibition rate of the cells (Fig. 2C), and the inhibition rate of cells treated with Dio (10 µg/ml) and TGF-β (2 ng/ml) was compared with Dio treatment, however, higher than that of TGF-β treatment.

**Dio regulates expression levels of apoptosis-associated proteins and TGF-β signaling pathway protein.** As cell apoptosis is associated with the Bcl-2-associated mitochondria-dependent apoptosis signaling pathway (18), the present study further investigated the link between p53 and Bcl-2 in HepG-2 cells treated with Dio (Fig. 3). Cells were exposed to 0.5, 10, or 15 µg/ml Dio for 24 h and the expression levels of p53 were demonstrated to be upregulated in a dose-dependent
manner (P<0.001), while Bcl-2 was downregulated in a
dose-dependent manner (P<0.01). Protein expression levels
of TGF-β, TβRII, Smad3 and p-Smad2/3 were reduced, also
in a dose-dependent manner (P<0.05; Fig. 3A). Bax protein
expression levels show no change compared with the control.
The results demonstrated that cell growth was partially
reversed following treatment with recombinant TGF-β.
Correspondingly, Bcl-2, TGF-β, TβRII, Smad3 and P-Smad2/3
increased when stimulated by TGF-β compared with the
control. Furthermore, p53 protein expression levels decreased
when stimulated by TGF-β compared with the control and Bax
protein expression levels showed no change when compared
with the control. p53 protein expression levels decreased when
with TGF-β protein and Dio, compared with Dio treatment
alone, while protein expression levels of Bcl-2, TGF-β, TβRII,
Smad3 and p-Smad2/3 (Fig. 3C).

Dio induces apoptosis by enhancing p53 expression and phosphorylation at Ser15, 33 and 37. p53 is important in the
cellular response to DNA damage and other genomic aberra-
tions. Activation of p53 may lead to cell cycle arrest and DNA
repair, or apoptosis (19). Bcl-2 protein expression levels were
reduced and total p53 expression was increased in HepG2
cells treated with Dio for 24 h. Using IF microscopy, nuclear
accumulation of p-p53 (at Ser15, 33 and 37) was observed
(Fig. 4). It was observed that following Dio treatment for 24 h,
p53 phosphorylation increased at Ser15, 33 and 37, however, no
marked increase was detected at Ser20, 46, Try18 or 81.

Discussion

Natural products have been widely used in the development
of anticancer therapeutic agents, although the underlying
mechanisms of cancer cell suppression remain to be eluci-
dated (20). Previous studies into Dio-induced cell apoptosis
predominantly focused on cytochrome P450 (11,21). Dio exerts
cytostatic effects in MDA-MB 468 cells, due to CYP1A1 and
CYP1B1-catalyzed conversion to the flavone luteolin and
CYP1 enzymes increase the antiproliferative activity of dietary
flavonoids in breast cancer cells (9,11). Cyp26 b1 regulates
retinoic acid-dependent signals in T cells and its expression
is inhibited by transforming growth factor-β (22). To increase
understanding of TGF-β signal participation, HepG2 cells
were treated with different concentrations of Dio and cell
growth was observed. Cell growth was inhibited and
the expression levels of apoptosis-associated and TGF-β signaling
pathway-associated proteins was altered. However, following
TGF-β administration, cell apoptosis was partly reversed.
TGF-β signaling pathway-associated proteins were also regulated. Notably, Dio reduced TjRII expression levels, however, addition of exogenous TGF-β protein regulated Dio-induced inhibition of cell proliferation and apoptosis, which indicates that TGF-β signaling pathway is key in Dio-induced cancer cell apoptosis. The present study hypothesized that exogenous TGF-β protein compensates for decrease in receptor expression levels. TGF-β and TGF-β receptor binding initiates a range of cellular responses via binding to and activation of specific cell surface receptors with intrinsic serine/threonine kinase activity. These activated TGF-β receptors stimulate the phosphorylation of R-Smad proteins, which have been identified as important in regulating the expression levels of extracellular matrix proteins via the TGF-β signaling pathway (23,24).

p53 uses various cellular inputs to regulate apoptosis, proliferation and differentiation (25,26). p53 has also been associated with cancer cell metastasis, metabolism and small G protein signal transduction. p53 can induce apoptosis in response to multiple stimuli via cell growth arrest, apoptosis and senescence (16,27). As a transcriptional factor, p53 regulates transcription and expression of a variety of target genes, such as Bcl-2 and Bax, ultimately leading to cell cycle arrest and apoptosis (19,28). The present study demonstrated that Dio significantly upregulates p53 expression and increases the ratio of Bax/Bcl-2 proteins in cells treated with Dio, which suggests p53 and Bax/Bcl-2 proteins are key in Dio-induced cell apoptosis. Furthermore, Dio increases intracellular p53 protein expression level, particularly the p-p53 at Ser15, 33 and 37. p53 may be phosphorylated by ataxia telangiectasia mutated, ataxia telangiectasia and Rad3 related, and DNA-dependent protein kinase. Phosphorylation impairs the ability of MDM2 proto-oncogene to bind p53, promoting accumulation and activation of p53 in response to DNA damage. Exogenous TGF-β partly reverses Dio-induced cell apoptosis in HepG2 cells, which demonstrates that TGF-β/TjRII signaling pathway may be an important target for therapeutic agents based on Dio-induced cell apoptosis in HepG2 cells.

Acknowledgements

The present study was supported in part by grants from the Zhanjiang 2013 Annual Financial Capital Competitive Project Science and Technology Project, Zhanjiang Key Laboratory of Hepatobiliary Diseases (grant no. 2013A402-4), Zhanjiang 2014 Annual Financial Capital Competitive Project Science and Technology Project (grant no. 2014A010929), the Start Project of Doctor Scientific Research Funds, Affiliated Hospital of Guangdong Medical College (grant no. B2012039).

References

1. Yue QX, Liu X and Guo DA: Microtubule-binding natural products for cancer therapy. Planta Med 76: 1037-1043, 2010.
2. Bits L, Kultur S, Melkoglu G, Ozsoy N and Can A: Flavonoids and antioxidant activity of Rosa agrestis leaves. Nat Prod Res 24: 580-589, 2010.
3. Androutsopoulos VP and Spandios DA: The flavonoids diosmetin and luteolin exert synergistic cytostatic effects in human hepatoma HepG2 cells via CYP1A-catalyzed metabolism, activation of JNK and ERK and P53/P21 up-regulation. J Nutr Biochem 24: 496-504, 2013.
4. Rice-Evans CA, Miller NJ and Paganga G: Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 20: 933-956, 1996.
5. Quati L, Bortolozzo S, Stragotto S, Moro S, Pavanetto M, Nassi A, Palatini P and Floreni M: Flavonoids diosmetin and hesperetin are potent inhibitors of cytochrome P450 2C9-mediated drug metabolism in vitro. Drug Metab Pharmacokinet 25: 466-476, 2010.
6. Obmann A, Werner I, Presser A, Zehl M, Sloboda Z, Pursuren S, Narayana Y, Ketterl C and Glasl S: Flavonoid C- and O-glycosides from the Mongolian medicinal plant Dianthus versicolor Fisch. Carbohydr Res 346: 1866-1875, 2011.
7. Yang JD and Roberts LR: Hepatocellular carcinoma: A global view. Nat Rev Gastroenterol Hepatol 7: 448-458, 2010.
8. Poons RT and Borys N: Lyso-thermosensitive liposomal doxorubicin: An adjuvant to increase the cure rate of radiofrequency ablation in liver cancer. Future Oncol 7: 937-945, 2011.
9. Androutsopoulos VP, Mahale S, Arroo RR and Potter G: Anticancer effects of the flavonoid diosmetin on cell cycle progression and proliferation of MDA-MB-468 breast cancer cells due to CYPI activation. Oncol Rep 21: 1525-1528, 2009.
10. Androutsopoulos V, Wilsher N, Arroo RR and Potter GA: Bioactivation of the phytoestrogen diosmetin by CYPI cytochromes P450. Cancer Lett 274: 54-60, 2009.
11. Androutsopoulos VP, Ruparelia K, Arroo RR, Tatsakis AM and Spandios DA: CYPI-mediated antiproliferative activity of dietary flavonoids in MDA-MB-468 breast cancer cells. Toxicology 264: 162-170, 2009.
12. Drabsh Y and ten Dijke P: TGF-β signaling in breast cancer cell invasion and bone metastasis. J Mammary Gland Biol Neoplasia 16: 97-108, 2011.
13. Busse A and Keilholz U: Role of TGF-β in melanoma. Curr Pharm Biotechnol 12: 2165-2175, 2011.
14. HeldinCH, LandstrmM and Moustakas A: Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. Curr Opin Cell Biol 21: 166-176, 2009.
15. Meulmeester E and ten Dijke P: The dynamic roles of TGF-β in cancer. J Pathol 223: 205-218, 2011.
16. Zhang Q, Liu J, Liu B, Xia J, Chen N, Chen X, Cao Y, Zhang C, Lu C, Li M and Zhu R: Dihydromyricetin promotes hepatocellular carcinoma regression via a p53 activation-dependent mechanism. Sci Rep 4: 4628, 2014.
17. Wu S, Liu B, Zhang Q, Liu J, Zhou W, Wang C, Li M, Bao S and Zhu R: Dihydromyricetin reduced Bcl-2 expression via p53 in human hepatoma HepG2 cells. PloS One 8: e67886, 2013.
18. Youle RJ and Strasser A: The BCL-2 protein family: Opposing activities that mediate cell death. Nat Rev Mol Cell Biol 9: 47-59, 2008.
19. Brady CA, Jiang D, Mello SS, Johnson TM, Jarvis LA, Kozak MM, Kenzelmann Broz D, Basak S, Park EJ, McLaughlin ME, et al: Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. Cell 145: 571-583, 2011.
20. Harvey AL: Natural products in drug discovery. Drug Discov Today 13: 894-901, 2008.
21. Ciolino HP, Wang TT and Yeh GC: Diosmin and diosmetin are agonists of the aryl hydrocarbon receptor that differentially affect cytochrome P450 1A1 activity. Cancer Res 58: 2754-2760, 1998.
22. Takeuchi H, Yokota A, Ohoka Y and Iwata M: Cyp26b1 regulates retinoic acid-dependent signals in T cells and its expression is inhibited by transforming growth factor-β. PloS One 6: e16089, 2011.
23. Nagaraj NS and Datta PK: Targeting the transforming growth factor-beta signaling pathway in human cancer. Expert Opin Investig Drugs 19: 77-91, 2010.
24. Perrot CY, Javelaud D and Mauriel A: Insights into the transforming growth factor-β signaling pathway in cutaneous melanoma. Ann Dermatol 25: 135-144, 2013.
25. Speidel D: Transcription-independent p53 apoptosis: An alternative route to death. Trends Cell Biol 20: 14-24, 2010.
26. Javelaud D, Alexaki VI, Dennler S, Mohammed KS, Guise TA and Mauriel A: TGF-β/SMAD/GLI2 signaling axis in cancer progression and metastasis. Cancer Res 71: 5060-5061, 2011.
27. Lee IH, Gaddamereth SJ, Gurtuk N, Ye R and Presser A: DNA damage-specific control of cell death by cryptochrome in p53-mutant ras-transformed cells. Cancer Res 73: 785-791, 2013.
28. Burman M, Shi Y, Hedström E, Kogner P and Selivjanova G: Dual targeting of wild-type and mutant p53 by small molecule inhibitors results in the induction of N-Myc and key survival oncogenes and kills neuroblastoma cells in vivo and in vitro. Clin Cancer Res 19: 5092-5103, 2013.