

Antiproliferative effect of ME3738, a derivative of soyasapogenol, on hepatocellular carcinoma cell lines *in vitro* and *in vivo*

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Received June 14, 2016; Accepted October 17, 2016

DOI: 10.3892/br.2016.792

Abstract. Soyasapogenol, an aglycon of soyasaponin, ameliorates liver injury induced by concanavalin A in mice. A derivative of soyasapogenol, 22 β -methoxyolean-12-ene-3 β , 24(4 β)-diol (ME3738), was reported to induce the gene expression of interferon (IFN)- β in hepatitis C virus replicon cells. The effect of ME3738 on hepatocellular carcinoma (HCC) cell lines was investigated in the present study. A total of 11 HCC cell lines were cultured in medium containing 0–10 μ M ME3738, and after 24, 48, or 72 h of culture, morphological observation and MTT cell growth assays were performed. Furthermore, the effects of ME3738 with or without PEG-IFN- α -2b on cell lines were investigated. Induction of apoptosis was examined on cells treated with 1 μ M of ME3738 using an Annexin V assay. The effect of ME3738 (0.63 and 2.5 μ M) on cell cycle progression was analyzed on two cell lines. The mice with subcutaneous tumors were divided into four groups: i) Control; ii) ME3738 alone; iii) PEG-IFN- α -2b alone and iv) ME3738+PEG-IFN- α -2b (combination). ME3738 was mixed with food (1.5 mg/g) and was taken orally for 15 days. PEG-IFN- α -2b (1,920 IU/mouse) was subcutaneously injected twice a week for two consecutive weeks. On day 15, the mice were sacrificed and the tumors were resected. A dose-dependent anti-proliferative effect was observed to various degrees in all the HCC cell lines *in vitro*. This inhibitory effect reached its maximal level 24 h after the treatment and the 50% inhibitory dose was between 0.8 and 2.4 μ M. The combination treatment did not show a synergistic effect. Induction of apoptosis was not observed. Cell cycle arrest at S-phase was observed in two of the examined cell lines. On day 15, the tumor volume of mice receiving ME3738, PEG-IFN- α -2b, and ME3738+PEG-IFN- α -2b was 69, 30, and 33%, respectively,

of the control tumor volume. ME3738 induced antiproliferative effects on the HCC cells *in vitro* and *in vivo*. The data suggested potential clinical application of ME3738.

Introduction

Soyasaponin is classified among the sugar conjugates of triterpenes, and can take various chemical and sugar-chain structures, such as soyasapogenol. Numerous saponins have demonstrated antimutagenic, anticarcinogenic and anti-metastatic effects against multiple cell lines (1). Triterpenoid saponins are natural sugar conjugates of triterpenes that possess various biological effects. Soyasaponin exerts antiviral effects on herpes simplex virus, human cytomegalovirus, influenza virus, and human immunodeficiency virus through its suppression of gene and viral protein synthesis (2,3). Soyasaponin was reported to suppress cancer cell growth in the colon cancer cell lines (4–6), and reduced colon cancer rates were identified in individuals who consume large amounts of soy legumes (7), suggesting that soyasaponins may have a protective effect against colon cancer. In addition, soyasaponin showed hypotensive action in spontaneous hypertension (8).

Soyasapogenol, an aglycon of soyasaponin, extracted from Glycine max Merr, 22 β -methoxyolean-12-ene-3 β , 24(4 β)-diol (ME3738) is a derivative of soyasapogenol B. ME3738 has been reported to ameliorate liver damage in several experimental models of acute and chronic liver injury induced by concanavalin A (Con A), ethanol, lithocholate, and bile duct ligation (9–13).

ME3738 induces interleukin-6 (IL-6) expression, and serum amyloid A and α 1 acid glycoprotein as downstream targets of the IL-6 signal protecting against Con A-induced liver injury.

Hepatocellular carcinoma (HCC) is one of the most common cancer types worldwide and a leading cause of cancer mortality, especially in countries with a high prevalence of chronic infections with hepatitis B virus and hepatitis C virus (HCV). ME3738 suppresses HCV replication by inhibiting mRNA in HCV replicon cells, along with NS3 and core proteins (14). Saibara *et al* treated chronic hepatitis C patients for 48 weeks with a combination of PEG interferon (IFN)- α -2b and ME3738 (15). Authors of that study reported that the

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Key words: HCC, HCV, ME3738, IFN, soyasapogenol

subjects became HCV RNA-negative during the administration, and that combination treatment was very safe with no side effects other than those seen with PEG IFN- α -2b alone. ME3738 is effective in treating chronic hepatitis C, however, to the best of our knowledge, there are no reports available on the effect of ME3738 on HCC. In the present study, we investigated the antiproliferative effects of ME3738 on HCC cell lines.

Materials and methods

Cell lines and cell culture. The present study used 11 HCC cell lines (KIM-1, KYN-1, KYN-2, KYN-3, HAK-1A, HAK-1B, HAK-2, HAK-3, HAK-4, HAK-5 and HAK-6). The HCC cell lines were originally established in our laboratory, and each cell line retained the morphological and functional features of the original tumor as described elsewhere (16-22). The cells were grown in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) and supplemented with 2.5% heat-inactivated (56°C, 30 min) fetal bovine serum (Bioserum, Victoria, Australia), 100 U/ml penicillin, 100 μ g/ml streptomycin (Gibco BRL, Gaithersburg, MD, USA) and 12 mmol/l sodium bicarbonate, in a humidified atmosphere of 5% CO₂ in air at 37°C.

Effects of ME3738 on the proliferation of HCC cell lines *in vitro*. The cells (1.5–6.5 \times 10³ cells/well) were seeded 96-well plates (Thermo Fisher Scientific, Roskilde, Denmark), cultured for 24 h and the medium was replaced with ME3738 (Meiji Seika Pharm Co., Ltd., Tokyo, Japan; 0, 0.08, 0.16, 0.32, 0.63, 1.25, 2.5, 5 and 10 μ M). After culturing for 24, 48 or 72 h, the number of viable cells was examined using MTT cell growth assay kits (Chemicon International, Inc., Temecula, CA, USA). The 50% inhibitory concentration (IC₅₀) of each cell line was estimated at 24 h of culture with ME3738.

Quantitative analysis of ME3738-induced apoptosis *in vitro*. Cells cultured with or without ME3738 (1 μ M) for 72 h were stained with the Annexin V-enhanced green fluorescent protein (EGFP) Apoptosis Detection kits (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) according to the manufacturer's protocol. After staining, the cells were analyzed using a FACScan (BD Biosciences, San Jose, CA, USA), and the Annexin V-EGFP-positive apoptotic cell rate was determined.

Effects of ME3738 on cell cycle. HAK-1B and HAK-4 were cultured with ME3738 (0.63 or 2.5 μ M) for 12, 24 or 48 h, labeled with 10 μ M BrdU for 30 min, fixed in 70% cold ethanol at 4°C overnight, stained with anti-BrdU and propidium iodide, and then analyzed using a FACScan. Staining was performed using the modified technique described elsewhere (23).

Effects of ME3738 with or without PEG-IFN- α -2b on the proliferation of HCC cell lines *in vitro*. The cells (1.5–6.5 \times 10³ cells/well) were seeded in 96-well plates (Thermo Fisher Scientific), cultured for 24 h and the medium was replaced with ME3738 (0, 0.1 or 0.5 μ M) with or without PEG-IFN- α -2b (PEGIntron®; MSD K.K., Tokyo, Japan; 0 or 1,000 IU/ml). After culturing for 72 h, the number of

viable cells was examined using MTT cell growth assay kits (Chemicon International, Inc.).

Effects of ME3738 with or without PEG-IFN- α -2b on the proliferation of HCC cell lines in BALB/c mice. HAK-1B cells (1 \times 10⁷ cells/mouse) were transplanted subcutaneously into the backs of 4-week-old female BALB/c mice. After tumor formation was confirmed, the mice were divided into four groups (n=7 in each group), i.e., control group, ME3738 alone group, PEG-IFN- α -2b alone group and ME3738+PEG-IFN- α -2b (combination) group. ME3738 was mixed with food (1.5 mg/g) and was taken orally for 15 days. PEG-IFN- α -2b (1,920 IU/mouse) was subcutaneously injected twice a week for two consecutive weeks. The clinical dose of PEG-IFN- α -2b in chronic hepatitis C treatment was 9.6 \times 10⁴ IU/kg and was 3-fold that of the lowest dose (3.2 \times 10⁴ IU/kg) in the experiment. The tumor size was measured in two directions using calipers (until day 15), and the tumor volume (mm³) was estimated using the equation 'length x (width)² x 0.5'. On day 15, the mice were sacrificed by cervical dislocation and the resected tumor was fixed in formalin and prepared into paraffin sections.

In addition, immunostaining was performed with anti-mouse endothelial cell antibody (anti-mouse CD34 monoclonal antibody, clone MEC14.7, final dilution, 1/100, cat. no. MCA1825; AbD Serotec, Raleigh, NC, USA) and anti-human Ki-67 monoclonal antibody (clone MIB-1, final dilution, 1/50, cat. no. M724029; Dako, Carpinteria, CA, USA).

The number of blood vessels and MIB-1 index in a unit area (mm²) of every section was calculated and its mean was obtained.

The present study was approved by the Ethics Committee of Kurume University (approval no. 10248). Animal experiments for the current study were approved by the Ethics Review Committee for Animal Experimentation of Kurume University School of Medicine.

Statistical analysis. Data are expressed as the mean \pm standard deviation. Comparisons between groups were performed using Student's t-test and two-factor factorial ANOVA. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of ME3738 on the proliferation of HCC cell lines *in vitro*. In all the cell lines, a dose-dependent antiproliferative effect was observed at various degrees in 24, 48 and 72 h cultures with ME3738. The cell number was suppressed in 10 cell lines to <50% of the control at 24 h (Fig. 1) and the suppression was statistically significant in all the cell lines with a dose range of 0.08–10 μ M (P<0.001). The IC₅₀ of ME3738 cultured for 24 h was 0.8–2.4 μ M, with the exception of KYN-2 (Table I).

Quantitative analysis of ME3738-induced apoptosis *in vitro*. Quantitative analysis of apoptosis revealed ME3738 did not induce a significant increase in the amount of apoptosis (Table II).

Effects of ME3738 on cell cycle. In HAK-1B and HAK-4, the ratio of the cells in the S-phase increased, and ratio of the

Table I. IC₅₀ of ME3738 in the cell lines.

Cell line	IC ₅₀ (μM)
KIM-1	1.8
KYN-1	2.0
KYN-2	ND
KYN-3	2.0
HAK-1A	2.0
HAK-1B	0.8
HAK-2	1.9
HAK-3	1.2
HAK-4	1.4
HAK-5	1.1
HAK-6	2.4

ND, not determined.

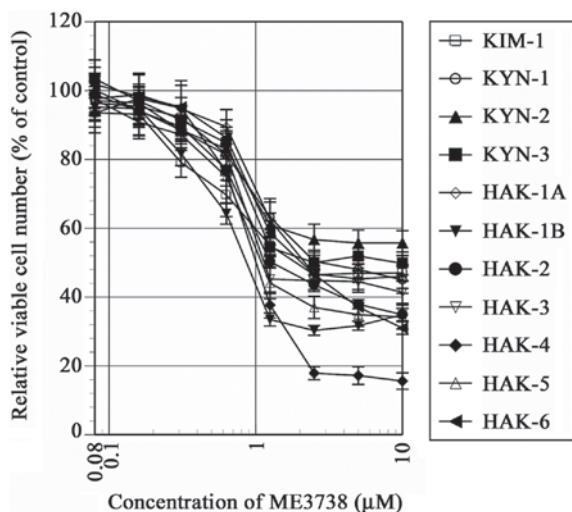


Figure 1. Antiproliferative effect of ME3738. In all the cell lines, a dose-dependent antiproliferative effect was observed at various degrees in 24 h cultures with ME3738. The suppression was statistically significant in all the cell lines at a dose range of 0.08-10 μM ($P<0.001$). ME3738, 22β-methoxyolean-12-ene-3β, 24(4β)-diol.

cells in the G2/M phase slightly decreased or did not change, compared with those in the control cells at any time-point 12-48 h after the addition of ME3738 (Fig. 2).

Effects of ME3738 with or without PEG-IFN-α-2b on the proliferation of HCC cell lines in vitro. A total of 72 h after the addition of PEG-IFN-α-2b alone, the relative viable cell number was suppressed in all the cell lines. The combination treatment of ME3738 and PEG-IFN-α-2b did not exert synergistic antiproliferative effects in any cell lines (Fig. 3).

Effects of ME3738 with or without PEG-IFN-α-2b on the proliferation of HCC cell lines in nude mice. On day 15, the tumor volume of mice receiving ME3738 ($P<0.05$), PEG-IFN-α-2b ($P<0.001$), and ME3738+PEG-IFN-α-2b ($P<0.001$) was 69.3, 30.9, and 33.5%, respectively, of the

Table II. Quantitative analysis of apoptosis induced by ME3738 in 11 liver cancer cell lines.

Cell line	Annexin V-EGFP-positive apoptotic cells (%)	
	Control	ME3738
KIM-1	6.4±1.3	9.1±2.1
KYN-1	9.6±1.1	10.7±0.8
KYN-2	13.6±3.2	16.8±1.5
KYN-3	6.9±1.3	6.9±1.3
HAK-1A	7.9±1.6	7.3±3.5
HAK-1B	12.9±1.8	12.8±2.1
HAK-2	8.6±2.4	7.9±2.0
HAK-3	9.2±1.9	9.9±0.8
HAK-4	6.3±2.1	5.3±1.8
HAK-5	7.9±1.2	7.1±0.8
HAK-6	5.4±3.4	5.6±2.9

Cells were cultured with medium alone (control) or medium with 1 μM of ME3738. Apoptosis was measured by Annexin V-EGFP staining. EGFP, enhanced green fluorescent protein.

control tumor volume (Fig. 4). The MIB-1 index showed >70% positive cells in all the groups, and there were no significant differences in the mean number of blood vessels per unit area among the groups (data not shown).

Discussion

We have previously reported that PEG-IFN-α-2b and IFN-β, which are widely used in the treatment of HCV, suppress cell proliferation by inducing apoptosis and/or cell cycle arrest in many HCC cell lines (23,24). However, some cell lines showed low sensitivity for PEG-IFN-α-2b and IFN-β on proliferation. By contrast, ME3738 was able to suppress cell proliferation at various concentrations in all the cell lines we studied, including those with low sensitivity to IFN. Even KYN-2, which had the lowest degree of cell suppression, showed a 40% inhibition of cell proliferation at doses of ≥1.25 μM.

The findings of the present study suggest that the suppression of HCC cell proliferation by ME3738 was due, not to apoptosis, but rather to the inhibition of cell cycle progression. ME3738 upregulates the production of IFN-β in non-neoplastic liver cells (25). We examined the effect of ME3738 on IFN-β expression in HAK-1B cells by microarray, but no change in IFN-β expression was observed (data not shown). Our previous studies have reported that the suppression of cell proliferation by IFN-β in HCC cell lines was primarily due to apoptosis (24). However, the present study was unable to confirm the induction of apoptosis by ME3738, suggesting that the mechanism of induction of IFN-β by ME3738 may differ between non-neoplastic and neoplastic cells.

ME3738 was reported to increase the expression of IL-6 in the liver (26). From the point of view of host defense, IL-6

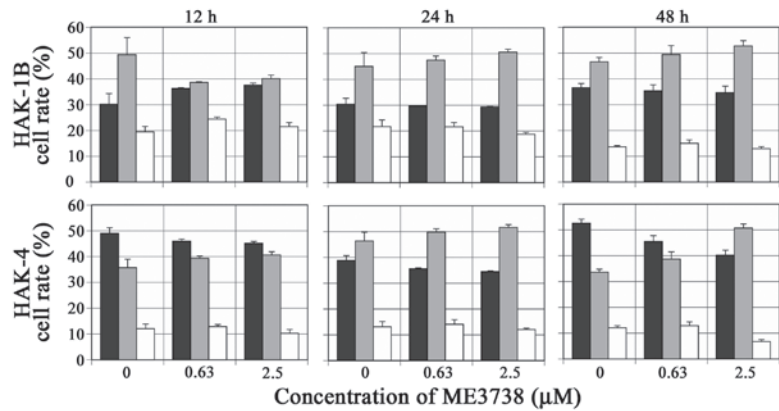


Figure 2. Cell cycle analysis [G1 phase (black bar), S-phase (grey bar), G2/M phase (white bar)]. In HAK-1B and HAK-4, the ratio of cells in the S-phase increased, whereas the ratio of cells in the G2/M phase slightly decreased or did not change, compared with those in the control at any time-point 12-48 h after the addition of ME3738. ME3738, 22β-methoxyolean-12-ene-3β, 24(4β)-diol.

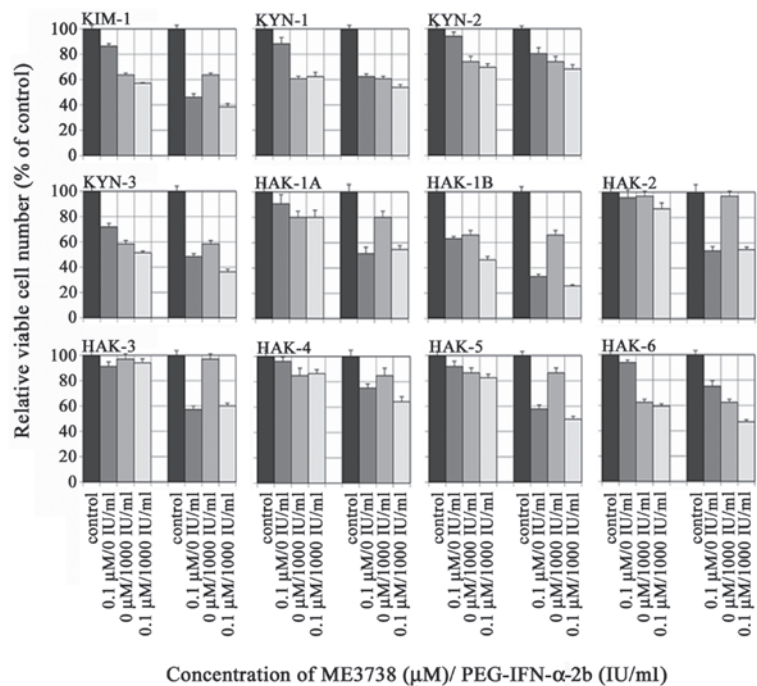


Figure 3. Antiproliferative effect of ME3738 in combination with PEG-IFN-α-2b. Cells were incubated with ME3738 in the presence of PEG-IFN-α-2b, and the relative viable cell number (% of control) was determined after 72 h of culture. ME3738, 22β-methoxyolean-12-ene-3β, 24(4β)-diol.

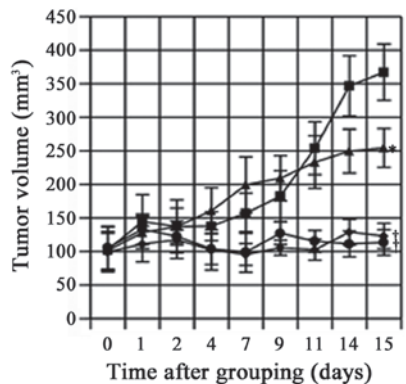


Figure 4. Time course change in estimated tumor volume of s.c. transplanted HAK-1B in nude mice. The mice received ME3738 alone (▲), PEG-IFN-α-2b alone (●), ME3738 and PEG-IFN-α-2b (◆) or control (■). All the mice were sacrificed on day 15. *P<0.05 vs. control; †P<0.001 vs. control. ME3738, 22β-methoxyolean-12-ene-3β, 24(4β)-diol.

is a multifunctional cytokine that, not only plays an important role in the immune reaction, but is also involved in acute phase response and cell growth and differentiation. ME3738 is known to induce IL-6 by increasing the expression of Bcl-2 and Bcl-xL, which have anti-apoptotic effects, thereby protecting the cells from Fas-mediated death. Furthermore, the anti-apoptotic effects of IL-6 are reported to be mediated by downstream STAT3 signaling (27). Therefore, although it is not direct, ME3738 has an anti-apoptotic effect. We did not observe anti-proliferative effects of HCC cells mediated by apoptosis in the present study. Thus studies should be conducted. On the other hand, S-phase cell cycle arrest was observed in two of the HCC cell lines we studied. Ellington *et al* similarly reported that soyasaponin B inhibited the activation of cyclin-dependent kinase-2 and induced S-phase arrest in a colon cancer cell line (28). However, a different study found

that saponins such as ginsenoside induced G₀/G₁ arrest in multiple cell lines (1), suggesting that cell-cycle regulation by saponin differs according to cell type or saponin source.

With regard to the suppression of tumor formation in nude mice, ME3738 alone significantly inhibited tumorigenicity compared with the control, but we found with PEG-IFN- α -2b alone that tumor diameters at day 15 of administration were approximately the same as that at the start of the experiment, and combination treatment with ME3738 and PEG-IFN- α -2b produced no additive or synergistic effects on tumor suppression, similar to the *in vitro* results. CD34 immunostaining revealed no differences in the number of blood vessels among the groups, indicating that ME3738 had no antiangiogenic effect. There was no significant difference on MIB-1, which is known to be a marker of cell proliferation in many tumors, among the groups. Tumor volume decreased significantly in the treatment groups with ME3738 as compared with the control. MIB-1 is detected in all the phases except G₀/G₁, and one of the reasons that no differences in MIB-1 expression were seen in the mouse tissue may be that, as ME3738 showed S-phase arrest *in vitro*, ME3738 treatment in HAK-1B-transplanted mice may have induced S-phase arrest, with the result that similar levels of MIB-1-positive cells were observed in the ME3738-treated mice and in the controls. In addition, S-phase arrest *in vitro*, while not remarkable, may help account for the fact that MIB-1 expression showed no change after ME3738 administration *in vivo*, regardless of the number of doses or length of observation period.

IFN has been widely used for therapies for chronic hepatitis C. ME3738 promotes the secretion of INF- β and phase II trials of the combination treatment with ME3738 and PEG-IFN- α -2a were conducted to determine whether the combination treatment would increase the rate of patients achieving a sustained virological response (SVR). However, a clear additional effect on SVR was not confirmed in phase II trials (15). Furthermore, direct acting antivirals (DAA) radically altered the treatment of hepatitis C (29). Oral administration of DAAs, which acts directly to suppress HCV replication, is able by itself to achieve high rates of SVR, and as a result the use of IFN to treat chronic hepatitis C no longer constituted an issue.

The introduction of DAAs was expected to further improve SVR rates and reduce the incidence of HCC associated with HCV (30-36). Nevertheless, it has been reported that even among patients who achieved SVR, some still develop HCC, and risk factors of HCC are known to affect the elderly, male gender, advanced liver fibrosis, and high AFP levels (30-36). In a rat bile duct ligation model, ME3738 was shown to inhibit hepatic stellate cell activation and collagen synthesis, resulting in the suppression of liver fibrosis (13). Furthermore, no significant adverse effects on the combination treatment of ME3738 and Peg-IFN- α 2a in the clinical trials have been reported thus far, and the safety of ME3738 treatment in chronic hepatitis patients has been confirmed (15). ME3738 may exert a protective effect against hepatocarcinogenesis through the inhibition of hepatic fibrosis. Our current study shows that, ME3738 suppressed cell proliferation to varying degrees in all the HCC cell lines we tested. In conclusion, our findings suggest that ME3738 may exert antitumorigenic

effects in a wide range of HCC patients, and that when used in combination with current therapeutic medications it may function as a modulator to increase the antitumorigenic effect in HCC.

Acknowledgements

We would like to thank Ms. Akemi Fujiyoshi for her assistance in our experiment.

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