Synergistic Combinations of Tanshinone IIA and *Trans*-resveratrol Toward Cisplatin-comparable Cytotoxicity in HepG2 Human Hepatocellular Carcinoma Cells

TUNG-WU CHANG[1,2*], CHUEH-YU LIN[3*], YIN-JEH TZENG[4] and HUU-SHENG LUR[1]

[1]*Department of Agronomy, National Taiwan University, Taipei, Taiwan, R.O.C;*
[2]*Division of Crop Improvement, Hualien District Agricultural Research and Extension Station, Council of Agriculture, Hualien County, Taiwan, R.O.C;*
[3]*Institute of Medical Sciences, Tzu-Chi University, Hualien City, Taiwan, R.O.C;*
[4]*Department of Molecular Biology and Human Genetics, Tzu-Chi University, Hualien City, Taiwan, R.O.C*

Abstract. *Aim: To determine the combinative effects of tanshinone IIA (Tan IIA) and trans-resveratrol (Resv) on cytotoxicity, apoptosis, cell-cycle arrest and DNA fragmentation in HepG2 human liver cancer cells. Materials and Methods: Cytotoxicity was detected by the cell proliferation and cytotoxicity WST-1 assay. Cell-cycle arrest and apoptosis were determined using flow cytometry analysis. DNA fragments were separated by gel electrophoresis. Results: Tan IIA and Resv at mixture ratios of 1/2:1/2 and 1/3:2/3 exerted synergistic cytotoxicity comparable to that of cisplatin. Elevated proportions of sub-G_1 and apoptotic cells were respectively found in the combinative treatments in comparison with hypothetic values of additive effects. Moreover, a more intensive pattern of apoptotic DNA fragmentation was visualized in combined treatments than in individual ones. Conclusion: Combining Tan IIA and Resv causes synergistic cisplatin-comparable, cytotoxicity and robustly induces apoptosis, sub-G1 cell cycle arrest and DNA fragmentation. This study provides evidence supporting further pre-clinical investigations of the combinational synergism.*

*These Authors contributed equally to this study.

Correspondence to: Yin-Jeh Tzeng, Department of Molecular Biology and Human Genetics, Tzu-Chi University, No. 701, Sec. 3, Chung-Yang Rd., Hualien City 97004, Taiwan, R.O.C. Tel: +886 38565301 (ext. 2671), Fax: +886 38578386, e-mail: tzeng@mail.tcu.edu.tw or tzeng@gms.tcu.edu.tw, and
Huu-Sheng Lur, Department of Agronomy, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 10, Taiwan, R.O.C.. Tel: +886 33664773, Fax: (02) 23620879, e-mail: lurhs@ntu.edu.tw.

Key Words: Tanshinone IIA, *trans*-resveratrol, synergistic combination, cisplatin, apoptosis, cell cycle arrest.

Liver cancer is the second most common cause of cancer-related death worldwide (1). Although more than 80% of cases of this disease occur in less developed or developing countries of Asia and Africa (1), new cases of liver cancer have increased remarkably in America, Japan and some areas of Europe over the past two decades (2). Epidemiological and clinical statistics indicate the difficulty of managing liver cancer, with fatalities from the disease increasing from 463,000 in 1990 to 598,000 in 2002, to 695,900 in 2008 and 752,100 in 2010 (3-5). Despite increased clinical effectiveness of current and conventional treatments through surgery and chemotherapy, the relative 5-year survival rate is as low as 15% and the chemotherapeutic drugs used in treatment (*e.g.*, cisplatin) are associated with significant negative side effects. Even though the cytotoxic activity of cisplatin is superior to 5-fluorouracil (5-FU) and paclitaxel in the human liver cancer cell line HepG2 (6), its use is limited by side-effects including hemolytic anemia and nephro-, neuro-, oto- and myelotoxicity. Thus, enormous efforts have been invested to develop potent antitumor drugs with fewer side-effects. Among many strategies, compounds isolated from traditional medicinal plants have attracted extensive interest.

Tanshinone IIA (Tan IIA) is a phytoalexin and a major lipophilic ingredient extracted from Danshen (*Salvia miltiorrhiza* Bunge) (Figure 1), a phytomedicine mainly used in traditional Asian folk medicine systems and the clinical treatment of a variety of cardiovascular disorders, such as angina pectoris, myocardial infarction, atherosclerosis or blood clotting abnormalities. Tan IIA has been shown to inhibit the proliferation of cell lines derived from human gastric, colon, breast, ovary, lung and leukocyte carcinomas (7-11). Recently, Tan IIA and its natural analogues have been reported to exert a cytotoxic effect on HepG2 liver cancer cells primarily through apoptosis and cell cycle arrest (12).

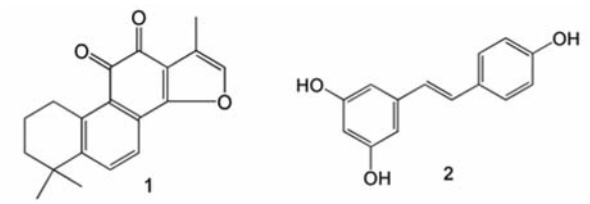

Figure 1. *Chemical structures of tanshinone IIA (1) and trans-resveratrol (2).*

Trans-resveratrol (Resv) (Figure 1) that functions as a phytoalexin is a natural phenolic compound abundant in the skin of grapes, peanuts and *Polygonum cuspidate*. Several studies have shown Resv to have beneficial effects on the cardiovascular system. It exhibits numerous biological properties, including anti-inflammatory and anti-carcinogenesis. It inhibits the proliferation of various cell lines such as those for cancer of the bladder, breast, liver, colon, lung and prostate, along with melanoma and leukemia (13-18), mainly by inducing apoptosis or/and cell-cycle arrest.

More recent attempts to increase anticancer activity have focused on developing synergistic effects by combining two agents. For instance, the antitumor effects of Tan IIA can be combined with cisplatin for the treatment of prostate cancer cells (19), with arsenic trioxide for NB4 cells (20), with imatinib for myeloid leukemia K562 cells (21), and with 5-FU for Colo205 colon cancer cells *in vivo* (22). Resv has been found to enhance the anti-cancer potency of the chemotherapeutic agent clofarabine in human malignant mesothelioma MSTO-211H cells (23), the anti-androgen agent flutamide in prostate cancer cells (24), phytochemicals such as epigallocatechin gallate (EGCG) and gamma-tocotrienol in MCF-7 breast cancer cells (25), and curcumin in Hepa1-6 hepatocellular carcinoma cells (26). Despite its synergistic benefit to anti-tumor compounds, Resv has not been investigated for its combinative synergism with Tan IIA.

The present study aimed at assessing the synergistic cytotoxicity of two phytochemicals Tan IIA and Resv and comparing the cytotoxic activities with that of cisplatin on human liver cancer HepG2 cells. Moreover, the involvement of cell-cycle arrest, apoptosis and DNA fragmentation affected by the two agents and their combinations were studied.

Materials and Methods

Cell line and culture. The human hepatoma cell line HepG2 was kindly provided by Dr. SY Lo at Tzu-Chi University. The cells were maintained in Dulbecco's modified Eagle's medium with phenol red (DMEM; Gibco, New York, USA) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Kibbutz Beit Haemek, Israel), 100 U/ml penicillin and 100 μg/ml streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel), in a humidified incubator under 37˚C, 5% CO_2 conditions.

Chemical compounds. High-performance liquid chromatography (HPLC)-grade Tan IIA and Resv were respectively purchased from Kesure Biotechnology Co. (Kunming, PRC) and Baoji Herbest Bio-Tech Co., (Baoji, PRC). The purity of both agents was 98% or more. Cisplatin (cis-diamineplatinum (II) dichloride) with purity of 99.9% or more was purchased from Sigma-Aldrich Co. (Milwaukee, USA). The agents were dissolved in DMSO (dimethyl sufoxide) at a concentration of 10 mg/ml as stock.

Cell viability assay. The WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]- 1,3-benzene disulfonate) cell proliferation and cytotoxicity assay (Roche Co., South San Francisco, USA) was used to determine cell viability based on the reduction of tetrazolium salt to formazan by mitochondria dehydrogenases. For the cell growth assay, 1×10^4 exponentially- growing cells were plated to each well of a 96-well tissue culture plate and incubated overnight in the same conditions as those cited above to allow cells to attach to the bottom of each well. The cells were then treated with increasing concentrations (0.5 to approximately 100 μg/ml) of Tan IIA, Resv, combined Tan IIA and Resv and cisplatin for 24, 36 and 48 hours. The negative control group was treated with an equal concentration of the solvent vehicle (DMSO). After exposure, the medium was removed and washed with 200 μl PBS. A mixture of 10 μl Cell Proliferation Reagent WST-1 and 190 μl phenol red free DMEM was then added for 30 min under the same incubation conditions. The absorbance was measured in a microplate reader (Thermo Scientific Multiskan Spectrum, New York, USA) at 450 nm. All experiments were carried-out at least thrice and in quadruplicate for each concentration. Cellular survival rate was calculated as follows: Cell survival rate (%)= OD value of experimental group/average OD value of control groups) ×100. The semi-inhibitory concentration (IC_{50}) for each compound as well as combined compounds was obtained from dose-effect curves (not shown) and calculated using the Sigma Plot software (http://www.sigmaplot.com/downloads/download.php). Data were presented as mean±SD of the quadruplicate. Student's two-tailed *t*-test was used to analyze statistical significance between groups.

Statistical analysis of combinatorial effects. Drug synergistic effects were indicated by combination index analysis (27). Combination index at IC_{50} (CI_{50}) values were based on cell viability (WST-1 assay) and calculated using the following equation $CI_{50}= Ct_{50}/ICt_{50} + Cr_{50}/ICr_{50}$, where Ct_{50} and Cr_{50} are the concentrations of Tan IIA and Resv, respectively, used in combination to achieve 50% of cytotoxic effect. ICt_{50} and ICr_{50} are the individual agents Tan IIA and Resv used to achieve the same effect (*i.e.*, the same as IC_{50}). A CI_{50} value of less than, equal to and more than 1, respectively indicates the synergism, additiveness and antagonism of the combinative effects. CI values, in addition to CI_{50}, were calculated and plotted over a range of fraction levels from 0.1 to 0.80 (10-80% of growth inhibition).

Analysis of cell cycle and apoptosis by flow cytometry. For cell-cycle analysis, the exponentially growing HepG2 cells were treated with Tan IIA, Resv and a mixture of both at the desired concentrations, following removal of non-adherent cells by gentle washing. After exposure to the drugs for 6-24 h, the cells were collected and centrifuged at 500 g in a 15 ml tube for 5 min. The cells were then washed with ice cold PBS and fixed with 70% ethanol for 2 hours at –20˚C. The cells were subsequently washed with PBS and treated with 200 μg/ml RNase and 500 μl of

Table I. *Cytotoxicity of tanshinone IIA, trans-resveratrol, mixture of the two compounds and cisplatin against HepG2 cells*

Treatment time	TanIIA			Resv			Cisplatin			1/2TanIIA +1/2Resv	1/3TanIIA +2/3Resv
	IC_{50} (μg/mL)	P_a	P_b	IC_{50} (μg/mL)	P_a	P_b	IC_{50} (μg/mL)	P_a	P_b	IC_{50} (μg/mL)	IC_{50} (μg/mL)
24 h	>100	0.0044	0.0001	74.59±1.62	0.0104	0.0001	35.92±2.14	0.0224	0.3631	53.61±5.34	37.07±1.01
36 h	91.63±8.93	0.0103	0.0156	57.53±3.73	0.0035	0.0019	11.46±0.40	0.0019	0.0039	34.90±2.27	37.82±2.87
48 h	40.28±11.63	0.0468	0.0458	41.89±11.99	0.0442	0.0453	10.14±0.46	0.4185	0.3735	10.51±0.24	10.39±0.17

IC_{50} Values were statistically compared for single- and dual-compound treatments. P_a, *p*-value, compared to the mixture at a ratio of 1/2:1/2; P_b, *p*-value, compared to the mixture at a ratio of 1/3:2/3.

propidium iodide (PI) (20 μg/ml in stock) for 30 min in darkness at room temperature. PI-stained cells were assayed using BD FACSCalibur Flow Cytometry (New Jersey, USA) and cell cycle distributions (G_0-G_1, S, and G_2-M) were analyzed with the CellQuest Pro Software (built-in software).

Apoptosis was detected by staining the cells with an Annexin V-FITC Apoptosis Detection Kit from Strong Biotech Co. (Taipei, Taiwan) following the manufacturer's instructions. Five million HepG2 cells were stained for 15 min with Annexin V- FITC and PI at room temperature in darkness. After staining, the apoptotic cells were counted using BD FACSCalibur Flow Cytometry and the data were processed with the CellQuest Pro software as described above.

Determination of DNA fragmentation. After being treated with various concentrations of Tan IIA, Resv and mixtures of them for 6 h, the cells were harvested and DNA was extracted using the Easy Tissue & Cell Genomic DNA Purification Kit (GeneMark, Taipei, Taiwan) according to the manufacturer's protocol. The DNA solution was separated on 2% agarose gel and stained with ethidium bromide. The experiment was repeated at least three times with similar results.

Results

Synergistic effects of Tan IIA combined with Resv on cytotoxicity. The growth-inhibitory activities of Tan IIA, Resv and cisplatin against HepG2 cancer cells were individually assessed with a WST-1-based colorimetric cell cytotoxicity assay (28) in increasing concentrations from 2 μg/ml to 40 μg/ml and durations from 24 to 48 h with time intervals of 12 h as described in the Materials and Methods section. The WST-1-based assay is relatively more efficient, sensitive and accurate than conventional MTT assays. Results showed that all compounds displayed their anti-proliferative effects in a concentration-dependent manner (data not shown). To measure the anticancer activities of agents *in vitro*, we calculated the corresponding semi-inhibitory concentration (IC_{50} values), with the results listed in Table I. Tan IIA displayed cell growth-inhibiting activity in a time-dependent manner. Similar effects were observed in the cells treated with Resv. Cisplatin was found to be more cytotoxic, exhibiting anticancer activity with 35.92±2.14 μg/ml of IC_{50} value 24 h after treatment, before dropping to 11.46±0.40 μg/ml at 36 h, with little change thereafter. Both of Tan IIA and Resv, respectively, displayed cytotoxicity significantly different but lower to that of cisplatin in all three treatment durations ($p<0.05$). To improve cytotoxic properties of these two phytoalexins, we combined the concentration series of the Tan IIA and Resv at ratios of 1/2:1/2 and 1/3:2/3 by w/v in parallel experiments and examined their inhibitive effects. IC_{50} value data showed that combinative treatments exerted significantly different and more growth-inhibitory effects than each agent alone at all durations ($p<0.05$). We quantified the activity arising from the treatment of the combined agents by calculating combination indices on the basis of the median-effect principle (CI_{50}). Tan IIA combined with Resv showed synergistic effects (CI_{50} <1), respectively, at mixture ratios of 1/2:1/2 and 1/3:2/3 by w/v. In general, the latter exhibited a stronger synergistic effect (Table II). We further examined the CI values with respect to various cell survival fractions after treatment with the two phytochemicals. The plots indicated that synergistic effects (CI<1) were observed at fraction affected less than 0.8 in the two mixture ratios and three lengths of treatment times (Figure 2).

Table II. *Synergistic combination of tanshinone IIA and trans-resveratrol demonstrated by combination index at IC_{50} (CI_{50}<1).*

Treatment time	1/2Tan IIA+1/2Resv CI_{50}	1/3Tan IIA + 2/3Resv CI_{50}
24-h	0.62	0.39
36 h	0.50	0.47
48 h	0.31	0.27

Combinative synergism of Tan IIA and trans-Resv toward cisplatin-comparable cytotoxicity. We compared the combinative effects of the two mixture ratios respectively with that of cisplatin. The results showed that comparable

IC_{50} values ($p>0.05$) were observed at 48 h following treatment with the 1/2:1/2 mixture and at 24, 48 h with the 1/3:2/3 mixture (Table I).

Cell-cycle arrest supports the combinative synergism. Given the remarkable antitumor activity of the combined Tan IIA and Resv treatment, we attempted to gain further insight into the cell biological effects evoked by the two natural compounds underlying the synergistic cytotoxicity. We treated the cells with equal concentrations (5 μg/ml) of each natural compound for 12 and 24 h, respectively, and then investigated the effects on cell cycle progression by flow cytometry analysis after propidium iodide DNA staining. The results (Figure 3) show that the two natural compounds had different effects on cell-cycle progress in two aspects. First, the accumulation of sub-G_1 phase was predominant in the treatment with Tan IIA at normalized fractions of 27.80% and 41.26% as opposed to 2.62% and 14.66% for Resv and as compared with the basal frequencies of 3.79% and 1.76% in the control groups without any treatment using the natural compounds respectively for 12 and 24 h. In dual agent treatment, greater fractions of sub-G_1 phase cells (51.66% and 83.67%) were observed in comparison with those of hypothetical additive values (26.63% and 54.16%). These synergism-like results correspond with the synergistic effect of cytotoxicity carried out using the dual natural compounds as determined by the WST-1 assay. Second, Resv, but not Tan IIA, evoked cell-cycle arrest on the G_2/M and S populations at 12 hours following treatment, with normalized fractions of 26.43% (G_2/M) and 20.08% (S) as compared with 17.85% (G_2/M) and 15.31% (S) in the corresponding control groups. At 24 h following treatment, Resv caused cell arrest at the S phase at a rate of 20.20%, compared with the control value of 11.19%.

The early stage apoptosis supports the combinative synergism. We further studied the non-sub-G_1 phase of the treated cells by means of flow cytometric analysis with FITC Annexin V and PI staining to distinguish early apoptotic (Annexin V positive/PI negative), early necrotic (Annexin V-negative/PI-positive cells) and a mixture of late apoptotic and necrotic cells (positive for both of Annexin V and PI) from viable cells (negative for both Annexin V and PI). The results (Figure 4) showed that at 12 and 24 h following treatment the apoptotic cells were more predominant than the other two kinds of impaired cells described above. Moreover, we examined the populations from the viewpoint of combinative treatments and found that the dual chemical treatment gave an apoptotic rate of 40.12%, which was more than the hypothetic additive fraction (23.19%). This was again in accordance with the synergistic cytotoxicity resulted from dual compounds by WST-1 assay. We also found that the apoptotic fraction at 24 h after treatment (17.59%) was close to the hypothetic additive value (19.26%), possibly because, after longer treatment durations, a number of apoptotic cells undergo undetectable subcellular processes attributed to the synergistic effect.

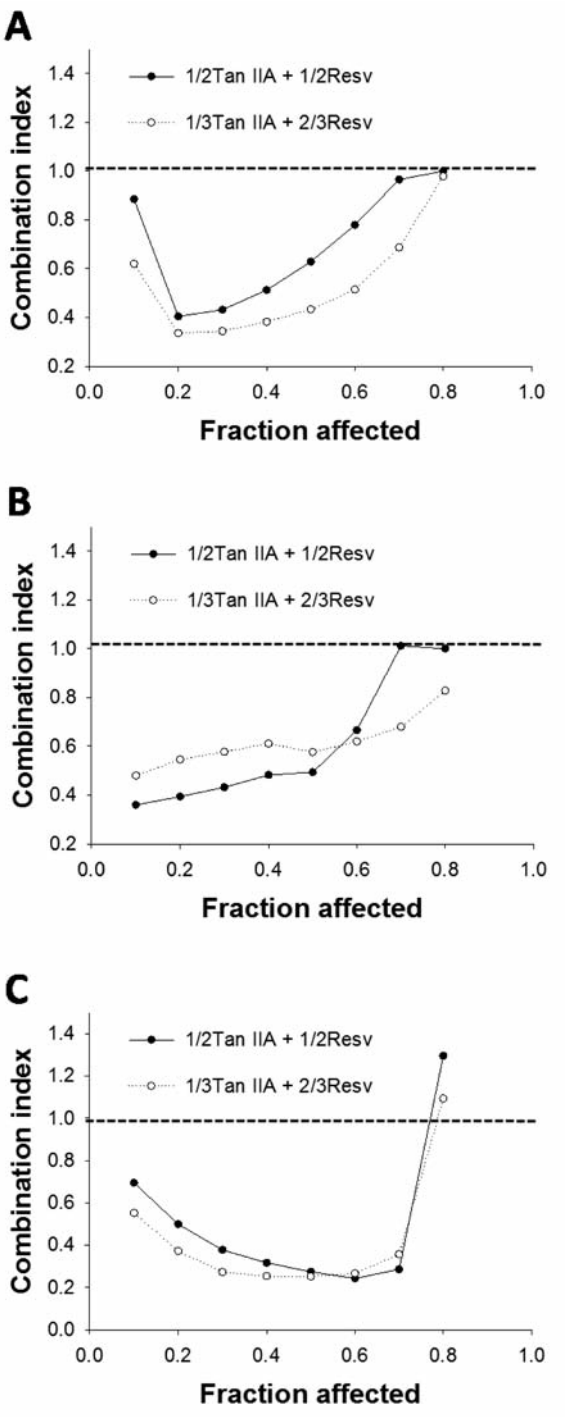

Figure 2. *Synergistic effects of tanshinone IIA and trans-resveratrol with two combination ratios on HepG2 cells.*

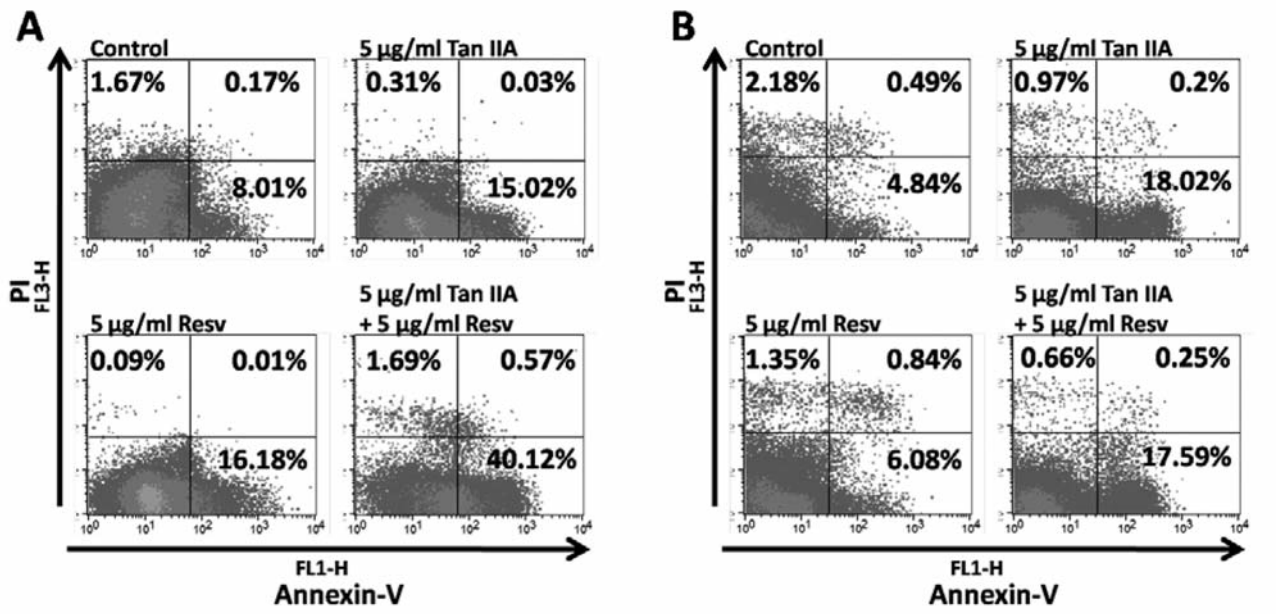

Figure 3. *Effects of tanshinone IIA, trans-resveratrol and mixture of the two compounds on HepG2 cell cycle progression for 12 h (A) and 24 h(B).*

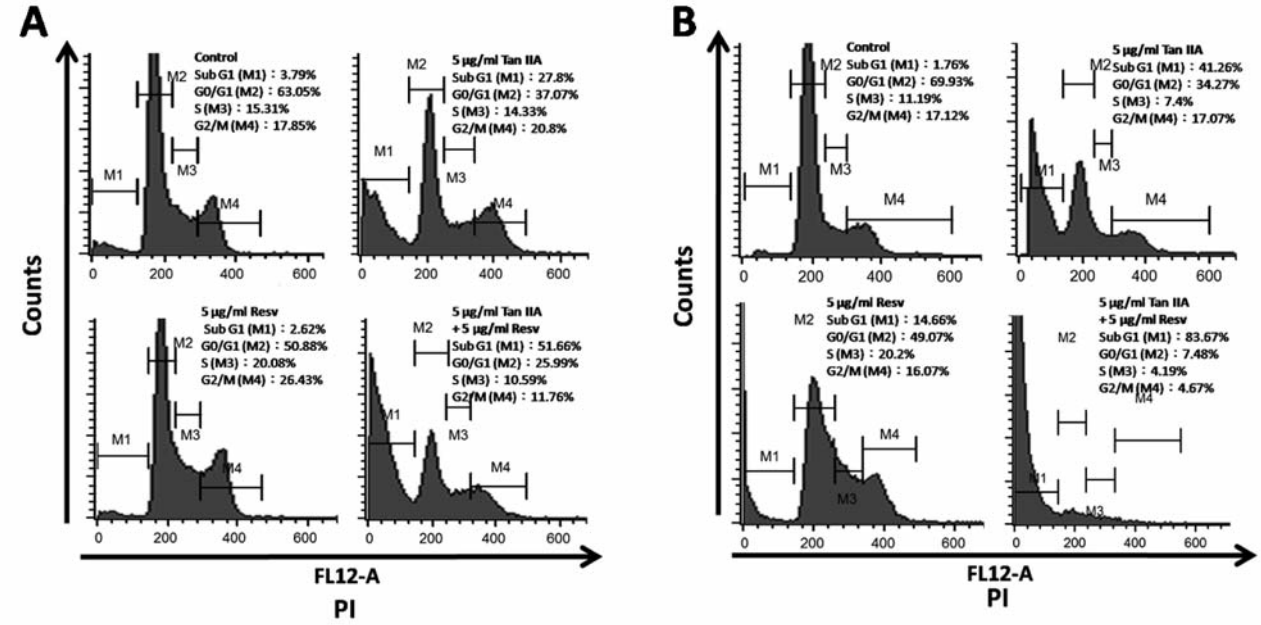

Figure 4. *Apoptosis in HepG2 cells induced by treatment of tanshinone IIA, trans-resveratrol and mixture of the two compounds for 12 h (A) and 24 h (B).*

The late stage apoptosis and necrosis support the combinative synergism. We examined the DNA fragmentation with agarose gel electrophoresis, which is a sign of cell death in later stages (29) and should be included in the sub-G_1 population of the treated cells. As early as 6 h a major pattern of necrosis characterized by smear DNAs was observed in Tan IIA treated cells; while a profound pattern of apoptosis characterized by DNA ladders presented in the Resv exposure. In the combined group both patterns were visualized but the ladder pattern appeared more intensively (Figure 5). The results seemed to further support the synergistic effect determined by the WST-1 assay.

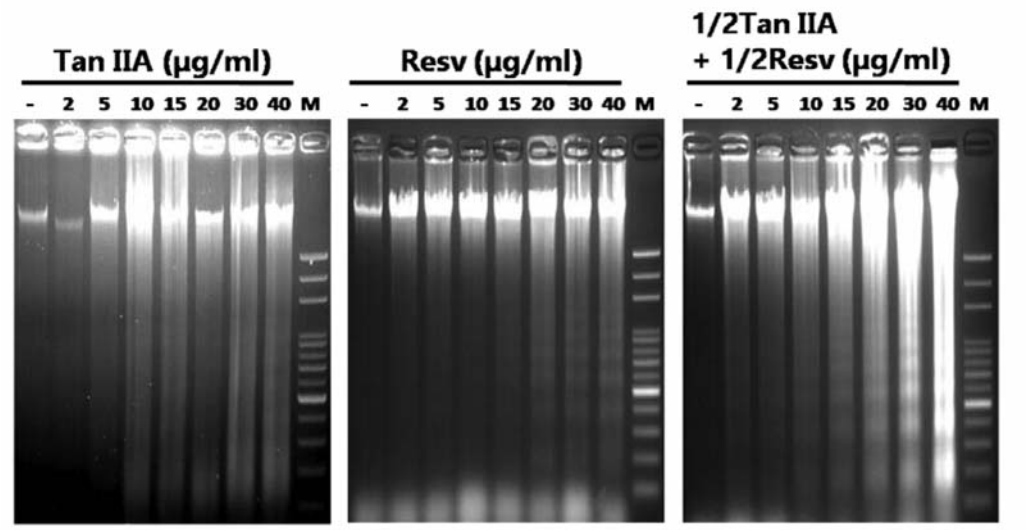

Figure 5. *DNA fragmentation induced by treatment of tanshinone IIA, trans-resveratrol and mixture of the two compounds for 6 h.*

Discussion

In the present study we, for the first time, provide evidence that synergistic combination of Tan IIA and Resv in some way behave as active as cisplatin in the inhibition of HepG2 cell survival. The two natural compounds impaired the cells *via* different ways. Tan IIA blocked the cells at sub-G_1 stage, while Resv evoked S- and G_2/M stage arrest. At early stage, both agents stimulated apoptosis, but Tan IIA exhibited a more pronounced effect. At late stage, Tan IIA provoked a prominent feature of necrosis, while Resv mostly induced apoptosis. After these impairments are merged by combination treatment of Tan IIA and Resv, they can enhance the effects of apoptosis, sub-G_1 cell-cycle arrest, and DNA fragmentation. As noted in the literature (30) the general principles of combination chemotherapy have been attributed to the properties of the component drugs such as nonoverlapping toxicities and different mechanisms of action. On this basis, this model provides a means of assessing the applicability of combination therapy strategies with natural compounds, at least in terms of cytotoxic activity.

While many studies have sought to improve the efficacy of single phytocompounds for preventing cancer cell growth and their corresponding underlying mechanisms of action, few studies have investigated the cytotoxic activities of herb combinations such as Tan IIA and Resv. To our best knowledge the combination of Tan IIA with any natural compound has not been explored. Our study provides evidence for following *in vivo* and *in clinic* investigations and the development of prospective anticancer drug combinations. For instance, Tan IIA is characterized by low bioavailability which may make it difficult to obtain significant results *in vivo* and *in vitro* studies. Recently, pharmacokinetic observations in 12 healthy volunteers revealed that the peak plasma concentration (C_{max}) for orally-administered Tan IIA was 4.00±2.79 ng/ml. In view of the cytotoxicological data for HepG2 cells, all of the IC_{50} values (from several to dozens of μg/ml) (12, 31) still exceed the C_{max} value. In the present study Resv reduced the IC_{50} value of Tan IIA to around 10 μg/ml, which is still far above the Cmax value. Various strategies have been used to increase the hydrophilic solubility of Tan IIA, such as structural modification of sodium Tan IIA sulfonateand acetyltanshinone IIA, constructing novel formulations with solid lipid nanoparticles and cyclic oligosaccharides, and enhancing solid dispersion with chitosan (32). In addition, combinative methods have been used to improve the bioavailability of tanshinones. A mixture of Tan IIA and salvianolic acid B (Sal B) increased the area under the curve (AUC) to a maximum of 14-fold. Moreover, the concentration at 5 minutes after the administration (C5min) of Tan IIA in plasma was significantly increased (up to 28-fold) when combined with polyphenolic extracts (33). The results suggest Sal B and other natural compounds may improve the bioavailability of Tan IIA when combined with Resv. Future *in vivo* anticancer and pharmacokinetic research should pursue the development of such an anticancer formulation.

Acknowledgements

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Conflicts of Interest

The Authors declare no conflicts of interest.

References

1 Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin D, Forman D and Bray F:GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide. In: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer: Available from http://globocan.iarc.fr . Accessed December 12, 2013.

2 Bergsland EK and Venook AP: Hepatocellular carcinoma. Curr Opin Oncol *12*: 357-361, 2000.

3 Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin *61*: 69-90, 2011.

4 Parkin DM, Bray F, Ferlay J, and Pisani P: Global cancer statistics, 2002. CA Cancer J Clin *55*: 74-108, 2005.

5 Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Barker-Collo S, Bartels DH, Bell ML, Benjamin EJ, Bennett D, Bhalla K, Bikbov B, Bin Abdulhak A, Birbeck G, Blyth F, Bolliger I, Boufous S, Bucello C, Burch M, Burney P, Carapetis J, Chen H, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahodwala N, De Leo D, Degenhardt L, Delossantos A, Denenberg J, Des Jarlais DC, Dharmaratne SD, Dorsey ER, Driscoll T, Duber H, Ebel B, Erwin PJ, Espindola P, Ezzati M, Feigin V, Flaxman AD, Forouzanfar MH, Fowkes FG, Franklin R, Fransen M, Freeman MK, Gabriel SE, Gakidou E, Gaspari F, Gillum RF, Gonzalez-Medina D, Halasa YA, Haring D, Harrison JE, Havmoeller R, Hay RJ, Hoen B, Hotez PJ, Hoy D, Jacobsen KH, James SL, Jasrasaria R, Jayaraman S, Johns N, Karthikeyan G, Kassebaum N, Keren A, Khoo JP, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Lipnick M, Lipshultz SE, Ohno SL, Mabweijano J, MacIntyre MF, Mallinger L, March L, Marks GB, Marks R, Matsumori A, Matzopoulos R, Mayosi BM, McAnulty JH, McDermott MM, McGrath J, Mensah GA, Merriman TR, Michaud C, Miller M, Miller TR, Mock C, Mocumbi AO, Mokdad AA, Moran A, Mulholland K, Nair MN, Naldi L, Narayan KM, Nasseri K, Norman P, O'Donnell M, Omer SB, Ortblad K, Osborne R, Ozgediz D, Pahari B, Pandian JD, Rivero AP, Padilla RP, Perez-Ruiz F, Perico N, Phillips D, Pierce K, Pope CA, 3rd, Porrini E, Pourmalek F, Raju M, Ranganathan D, Rehm JT, Rein DB, Remuzzi G, Rivara FP, Roberts T, De Leon FR, Rosenfeld LC, Rushton L, Sacco RL, Salomon JA, Sampson U, Sanman E, Schwebel DC, Segui-Gomez M, Shepard DS, Singh D, Singleton J, Sliwa K, Smith E, Steer A, Taylor JA, Thomas B, Tleyjeh IM, Towbin JA, Truelsen T, Undurraga EA, Venketasubramanian N, Vijayakumar L, Vos T, Wagner GR, Wang M, Wang W, Watt K, Weinstock MA, Weintraub R, Wilkinson JD, Woolf AD, Wulf S, Yeh PH, Yip P, Zabetian A, Zheng ZJ, Lopez AD, Murray CJ, AlMazroa MA and Memish ZA: Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet *380*: 2095-2128, 2012.

6 Brenes O, Arce F, Gatjens-Boniche O and Diaz C: Characterization of cell death events induced by anti-neoplastic drugs cisplatin, paclitaxel and 5-fluorouracil on human hepatoma cell lines: Possible mechanisms of cell resistance. Biomed Pharmacother *61*: 347-355, 2007.

7 Chen J, Shi DY, Liu SL and Zhong L: Tanshinone IIA induces growth inhibition and apoptosis in gastric cancer *in vitro* and *in vivo*. Oncol Rep *27*: 523-528, 2012.

8 Su CC, Chen GW, Kang JC and Chan MH: Growth inhibition and apoptosis induction by tanshinone IIA in human colon adenocarcinoma cells. Planta Med *74*: 1357-1362, 2008.

9 Wang X, Wei Y, Yuan S, Liu G, Lu Y, Zhang J and Wang W: Potential anticancer activity of tanshinone IIA against human breast cancer. Int J Cancer *116*: 799-807, 2005.

10 Jiao JW and Wen F: Tanshinone IIA acts *via* p38 MAPK to induce apoptosis and the down-regulation of ERCC1 and lung-resistance protein in cisplatin-resistant ovarian cancer cells. Oncol Rep *25*: 781-788, 2011.

11 Yun SM, Jeong SJ, Kim JH, Jung JH, Lee HJ, Sohn EJ, Lee MH and Kim SH: Activation of c-Jun N-terminal kinase mediates tanshinone IIA-induced apoptosis in KBM-5 chronic myeloid leukemia cells. Biol Pharm Bull *36*: 208-214, 2013.

12 Lee WY, Chiu LC and Yeung JH: Cytotoxicity of major tanshinones isolated from Danshen (*Salvia miltiorrhiza*) on HepG2 cells in relation to glutathione perturbation. Food Chem Toxicol *46*: 328-338, 2008.

13 Bai Y, Mao QQ, Qin J, Zheng XY, Wang YB, Yang K, Shen HF and Xie LP: Resveratrol induces apoptosis and cell cycle arrest of human T24 bladder cancer cells *in vitro* and inhibits tumor growth *in vivo*. Cancer Sci 101: 488-493, 2010.

14 Osman AM, Bayoumi HM, Al-Harthi SE, Damanhouri ZA and Elshal MF: Modulation of doxorubicin cytotoxicity by resveratrol in a human breast cancer cell line. Cancer Cell Int *12*: 47, 2012.

15 Amiri F, Zarnani AH, Zand H, Koohdani F, Jeddi-Tehrani M and Vafa M: Synergistic anti-proliferative effect of resveratrol and etoposide on human hepatocellular and colon cancer cell lines. Eur J Pharmacol *718*: 34-40, 2013.

16 Yin HT, Tian QZ, Guan L, Zhou Y, Huang XE and Zhang H: In vitro and *in vivo* evaluation of the antitumor efficiency of resveratrol against lung cancer. Asian Pac J Cancer Prev *14*: 1703-1706, 2013.

17 Osmond GW, Masko EM, Tyler DS, Freedland SJ and Pizzo S: In vitro and *in vivo* evaluation of resveratrol and 3,5-dihydroxy-4'-acetoxy-trans-stilbene in the treatment of human prostate carcinoma and melanoma. J Surg Res *179*: e141-148, 2013.

18 Siedlecka-Kroplewska K, Jozwik A, Boguslawski W, Wozniak M, Zauszkiewicz-Pawlak A, Spodnik JH, Rychlowski M and Kmiec Z: Pterostilbene induces accumulation of autophagic vacuoles followed by cell death in HL60 human leukemia cells. J Physiol Pharmacol *64*: 545-556, 2013.

19 Hou LL, Xu QJ, Hu GQ and Xie SQ: Synergistic antitumor effects of tanshinone II A in combination with cisplatin *via* apoptosis in the prostate cancer cells. Yao Xue Xue Bao *48*: 675-679, 2013.

20 Zhang G, Yang YM, Meng WT and Zhou J: Apoptosis of NB4 cells induced by Tanshinone II A combined with arsenic trioxide. Sichuan Da Xue Xue Bao Yi Xue Ban *41*: 57-61, 2010.

21 Jung JH, Kwon TR, Jeong SJ, Kim EO, Sohn EJ, Yun M and Kim SH: Apoptosis Induced by Tanshinone IIA and Cryptotanshinone Is Mediated by Distinct JAK/STAT3/5 and SHP1/2 Signaling in Chronic Myeloid Leukemia K562 Cells. Evid Based Complement Alternat Med *2013*: 805639, 2013.

22 Su CC: Tanshinone IIA potentiates the efficacy of 5-FU in Colo205 colon cancer cells *in vivo* through downregulation of P-gp and LC3-II. Exp Ther Med *3*: 555-559, 2012.

23 Lee YJ, Lee YJ, Im JH, Won SY, Kim YB, Cho MK, Nam HS, Choi YJ and Lee SH: Synergistic anti-cancer effects of resveratrol and chemotherapeutic agent clofarabine against human malignant mesothelioma MSTO-211H cells. Food Chem Toxicol *52*: 61-68, 2013.

24 Kai L and Levenson AS: Combination of resveratrol and antiandrogen flutamide has synergistic effect on androgen receptor inhibition in prostate cancer cells. Anticancer Res *31*: 3323-3330, 2011.

25 Hsieh TC and Wu JM: Suppression of cell proliferation and gene expression by combinatorial synergy of EGCG, resveratrol and gamma-tocotrienol in estrogen receptor-positive MCF-7 breast cancer cells. Int J Oncol *33*: 851-859, 2008.

26 Du Q, Hu B, An HM, Shen KP, Xu L, Deng S and Wei MM: Synergistic anticancer effects of curcumin and resveratrol in Hepa1-6 hepatocellular carcinoma cells. Oncol Rep *29*: 1851-1858, 2013.

27 Chou TC and Talalay P: Generalized equations for the analysis of inhibitions of Michaelis-Menten and higher-order kinetic systems with two or more mutually exclusive and nonexclusive inhibitors. Eur J Biochem *115*: 207-216, 1981.

28 Ngamwongsatit P, Banada PP, Panbangred W and Bhunia AK: WST-1-based cell cytotoxicity assay as a substitute for MTT-based assay for rapid detection of toxigenic Bacillus species using CHO cell line. J Microbiol Methods *73*: 211-215, 2008.

29 Collins JA, Schandi CA, Young KK, Vesely J and Willingham MC: Major DNA fragmentation is a late event in apoptosis. J Histochem Cytochem *45*: 923-934, 1997.

30 Pinto AC, Moreira JN, and Simões S:Current Cancer Treatment - Novel Beyond Conventional Approaches. *In*: Combination Chemotherapy in Cancer:Principles, Evaluation and Drug Delivery Strategies. Ozdemir PO (ed.), pp. 693-715, 2011.

31 Zhong ZH, Chen WG, Liu YH, Li QX, and Qiu Y: [Inhibition of cell growth and induction of apoptosis in human hepatoma cell line HepG2 by tanshione IIA]. Zhong Nan Da Xue Xue Bao Yi Xue Ban *32*: 99-103, 2007.

32 Liu QY, Zhang ZH, Jin X, Jiang YR and Jia XB: Enhanced dissolution and oral bioavailability of tanshinone IIA base by solid dispersion system with low-molecular-weight chitosan. J Pharm Pharmacol 65: 839-846, 2013.

33 Guo ZJ, Zhang Y, Tang X, Li H and Sun QS: Pharmacokinetic interaction between tanshinones and polyphenolic extracts of salvia miltinorrhiza BUNGE after intravenous administration in rats. Biol Pharm Bull *31*: 1469-1474, 2008.

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