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

TUNG-WU CHANG1,2*, CHUEH-YU LIN3*, YIN-JEH TZENG4 and HUU-SHENG LUR1

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

Abstract. Aim: To determine the combative 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-G1 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 combinatorial synergism.

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).
Industries, Kibbutz Beit Haemek, Israel), in a humidified incubator
Israel), 100 U/ml penicillin and 100 μg/ml streptomycin (Biological
bovine serum (FBS, Biological Industries, Kibbutz Beit Haemek,
(DMEM; Gibco, New York, USA) supplemented with 10% fetal
maintained in Dulbecco’s modified Eagle’s medium with phenol red
kindly provided by Dr. SY Lo at Tzu-Chi University. The cells were
5474
The human hepatoma cell line HepG2 was
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% CO₂ conditions.

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% CO₂ 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 sulfoxide) at a
concentration of 10 mg/ml as stock.

Cell viability assay. The WST-1 (4-[3-[4-(iodophenyl)-2-(4-
itrrophenyl)-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, 1×10⁴ 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 group) ×100. The semi-inhibitory concentration (IC₅₀) 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 and IC₅₀ (CI₅₀) values were based on cell viability (WST-1
assay) and calculated using the following equation CI₅₀= CI₅₀/IC₅₀
+ Cr₅₀/ICr₅₀, where CI₅₀ and IC₅₀ are the concentrations of Tan IIA
and Resv, respectively, used in combination to achieve 50% of
cytotoxic effect. IC₅₀ and IC₅₀ are the individual agents Tan IIA
and Resv used to achieve the same effect (i.e., the same as IC₅₀). A
CI₅₀ value of less than, equal to and more than 1, respectively
indicates the synergism, additiveness and antagonism of the
combinatorial effects. CI values, in addition to CI₀, were calculated
and plotted over a range of fractional doses 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
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 (G0-G1, S, and G2-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.

**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 (IC50 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 IC50 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 two compounds at ratios of 1/2:1/2 and 1/3:2/3 by w/v in parallel experiments and examined their inhibitive effects. IC50 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 (CI50). Tan IIA combined with Resv showed synergistic effects (CI50<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 I. Cytotoxicity of tanshinone IIA, trans-resveratrol, mixture of the two compounds and cisplatin against HepG2 cells**

<table>
<thead>
<tr>
<th>Treatment time</th>
<th>TanIIA</th>
<th>Resv</th>
<th>Cisplatin</th>
<th>1/2TanIIA 1/2Resv</th>
<th>1/3TanIIA + 2/3Resv</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (μg/mL)</td>
<td>P</td>
<td>P</td>
<td>IC50 (μg/mL)</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>24 h</td>
<td>&gt;100</td>
<td>0.0044</td>
<td>0.0001</td>
<td>74.59±1.62</td>
<td>0.0104</td>
</tr>
<tr>
<td>36 h</td>
<td>91.63±8.93</td>
<td>0.0103</td>
<td>0.0156</td>
<td>57.53±3.73</td>
<td>0.0035</td>
</tr>
<tr>
<td>48 h</td>
<td>40.28±11.63</td>
<td>0.0468</td>
<td>0.0458</td>
<td>41.89±11.99</td>
<td>0.0442</td>
</tr>
</tbody>
</table>

IC50 Values were statistically compared for single- and dual-compound treatments. P, p-value, compared to the mixture at a ratio of 1/2:1/2; P, p-value, compared to the mixture at a ratio of 1/3:2/3.

**Table II. Synergistic combination of tanshinone IIA and trans-resveratrol demonstrated by combination index at IC50 (CI50<1).**

<table>
<thead>
<tr>
<th>Treatment time</th>
<th>1/2TanIIA+1/2Resv</th>
<th>1/3TanIIA + 2/3Resv</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI50</td>
<td>CI50</td>
<td>CI50</td>
</tr>
<tr>
<td>24-h</td>
<td>0.62</td>
<td>0.39</td>
</tr>
<tr>
<td>36 h</td>
<td>0.50</td>
<td>0.47</td>
</tr>
<tr>
<td>48 h</td>
<td>0.31</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Combinative synergism of Tan IIA and trans-Resv toward cisplatin-comparable cytotoxicity. We compared the combinative effects of the two mixture ratios with that of cisplatin. The results showed that comparable...
IC50 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-G1 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-G1 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 G2/M and S populations at 12 hours following treatment, with normalized fractions of 26.43% (G2/M) and 20.08% (S) as compared with 17.85% (G2/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-G1 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.
The late stage apoptosis and necrosis support the combinatorial 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-G1 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 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).
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-G1 stage, while Resv evoked S- and G2/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-G1 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 phyto compounds 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 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 IC50 values (from several to dozens of μg/ml) (12, 31) still exceed the Cmax value. In the present study Resv reduced the IC50 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 sulfonate and acetyltanshinone II A, 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

This work was supported by grants from Agriculture and Food Agency, Council of Agriculture, Executive Yuan (992101010503-030103Z1, 1002101010504-030103Z1) and Tzu-Chi University (TCMRC-C95002-04). We also thank Jonathan Brody for reviewing and editing the manuscript and Dr. Chung-Lin Tsai for the preparation of the graphs.
Conflicts of Interest

The Authors declare no conflicts of interest.

References


Received June 3, 2014
Revised July 11, 2014
Accepted July 14, 2014