Evaluation and SAR analysis of the cytotoxicity of tanshinones in colon cancer cells

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[ABSTRACT]
AIM: This study was designed to evaluate the anti-cancer actions of tanshinone I and tanshinone IIA, and six derivatives of tanshinone IIA on normal and cancerous colon cells. Structure activity relationship (SAR) analysis was conducted to delineate the significance of the structural modifications of tanshinones for improved anti-cancer action.

METHOD: Tanshinone derivatives were designed and synthesized according to the literature. The cytotoxicity of different compounds on colon cancer cells was determined by the MTT assay. Apoptotic activity of the tanshinones was measured by flow cytometry (FCM).

RESULTS: Tanshinone I and tanshinone IIA both exhibited significant cytotoxicity on colon cancer cells. They are more effective in p53+/+ colon cancer cell line. It was also noted that the anti-cancer activity of tanshinone I was more potent and selective. Two of the derivatives of tanshinone IIA (N1 and N2) also exhibited cytotoxicity on colon cancer cells.

CONCLUSIONS: The anti-colon cancer activity of tanshinone I was more potent and selective than tanshinone IIA, and is p53 dependent. The derivatives obtained by structural modifications of tanshinone IIA exhibited lower cytotoxicity on both normal and colon cancer cells. From steric and electronic characteristics point of view, it was concluded that structural modifications of ring A and furan or dihydrofuran ring D on the basic structure of tanshinones influences the activity. An increase of the delocalization of the A and B rings could enhance the cytotoxicity of such compounds, while a non-planar and small sized D ring region would provide improved anti-cancer activity.

[KEY WORDS] Tanshinones; Synthetic derivatives; Colon cancer; Cytotoxicity; SAR analysis

[Introduction]
Colorectal cancer (CRC) is the third most common cancer globally and the fourth most frequent cause of cancer-related deaths. Both metastases and drug resistance to chemotherapy are the major concerns in colon cancer therapy [¹-³]. Plants are available source for discovery of new anticancer agents. Successful cases, such as camptothecin and taxol, have been reported in recent years [⁴]. Salvia miltiorrhiza Bunge, also known as “Danshen”, has been used to treat various diseases including heart diseases, hepatitis and cancer in China for period of time with minimal side effects [⁵]. To this end, tanshinones have been reported to have potent anti-cancer activity in some cancer cells, such as liver and prostate cancer cells [⁶-⁷]. Recently, the anti-colon cancer activity of tanshinone I and tanshinone IIA was also reported [⁸-⁹]. As a key regulator of cell growth and cell death, p53 is activated by DNA-damaging agents. Activation of p53 protects genome by inducing growth arrest to allow cells to repair the damage or apoptosis. Mutation or deficiency of such a tumor suppressor protein p53 occurs in more than 50% of all human cancers and causes drug resistance to a number of chemotherapeutic
agents, including 5-fluorouracil (5-FU) and oxaliplatin\textsuperscript{[10-11]}. Whether the cytotoxicity of tanshinone I and tanshinone IIA in colon cancer cells was p53 dependent was evaluated in this study by comparing their cytotoxicity and apoptotic activity between HCT116 p53\textsuperscript{+/+} and HT29 cell lines (p53 mutant, G-> A mutation in codon 273).

It is known that biological activity is closely related to structure. The differences among the structures of tanshinones are in ring A, ring C, and ring D (Fig. 1). On that basis, tanshinone IIA, a major and relative cheap constituent from Danshen, was selected as a starting structure to make structural modifications in order to have more functional derivatives and seek improved compounds for cancer treatment. Taking the advantage of the nucleophilicity of the furan $\alpha$-C of tanshinone IIA, derivatives of tanshinone IIA (N1$^to$N5) (Fig. 2) were synthesized by reaction of tanshinone IIA with aromatic aldehydes in the presence of $p$-TsOH. N6 was obtained through radical bromination of tanshinone IIA\textsuperscript{[12-13]}. The cytotoxicity of these derivatives was evaluated on human normal colonic fibroblasts and colon cancer cells in order to assess their therapeutic safety. Information from these structural modifications on tanshinone IIA would assist in the synthesis of better therapeutic agents for colon cancer in the future.

**Fig. 1** Structures of tanshinones I and IIA

<table>
<thead>
<tr>
<th>Compounds</th>
<th>N1</th>
<th>N2</th>
<th>N3</th>
<th>N4</th>
<th>N5</th>
</tr>
</thead>
<tbody>
<tr>
<td>-R</td>
<td>H</td>
<td>OH</td>
<td>F</td>
<td>NO$_2$</td>
<td>CF$_3$</td>
</tr>
</tbody>
</table>

**Materials and Methods**

**Compounds and reagents**

$^1$H- and $^{13}$C-NMR spectra were measured on a Varian Mercury-400 NMR spectrometer (Palo Alto, CA) (using tetramethylsilane as the internal standard). All reagents and solvents were of commercial grade and used as such unless specified. The correct molecular weights were determined on an automatic ThermoFinnigan LCQ-Advantage MS/MS analysis system (San Jose, CA), equipped with a Gilson 322 pump, Gilson UV/vis-152 detector, Gilson 215 liquid handler (Lewis Center), and a fluent splitter with a 5-cm Phenimax C$_{18}$ column (5 $\mu$m). HPLC gradient was buffer A: 0.05% TFA/H$_2$O; buffer B: 0.05% TFA/acetonitrile. Buffer B was linear from 0% to 100% in 5 min at a flow rate of 1.0 mL·min$^{-1}$. HPLC grade authentic standards of Tanshinone I and tanshinone IIA were purchased from Chengdu Congcon Bio-tech Co., Ltd. (Sichuan, China). Doxorubicin hydrochloride was purchased from Enzo (NY). 5-Fluorouracil was from Sigma (St. Louis, MO, USA). Oxaliplatin was purchased from TOCRIS Biosciences (Bristol, United Kingdom). All the test compounds were dissolved with DMSO to make a stock solution at a concentration of 10mM, which were then diluted to appropriate concentrations with appropriate solution before each experiment. FITC-conjugated annexin V was purchased from BD Biosciences (Bedford, MA, USA). EMEM medium, McCoy 5A medium and RPMI1640 medium, fetal bovine serum (FBS), penicillin and streptomycin, and trypsin were purchased from Invitrogen (Carlsbad, CA, USA). Unless otherwise specified, all chemicals used in this study were purchased from Sigma. The structures of tanshinone I ($\geq$ 97%) and tanshinone IIA ($\geq$ 97%) were verified by $^1$H-NMR and the HPLC-UV analysis.

**Cell cultures**

Human colon cancer HCT116 cell line (wild type, p53\textsuperscript{+/+}), HT29 cell line (p53\textsuperscript{mut/mut}), and CCD18-co cell line (normal human colon fibroblast) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). HCT116 and HT29 cell lines were routinely cultured in RPMI 1640 medium. CCD18-co cells were routinely cultured in EMEM medium. The medium was supplemented with 10% FBS (GIBCO/BRL, NY, USA) and 100 mg·mL$^{-1}$ streptomycin sulfate and 100 U·mL$^{-1}$ penicillin G. The cells were cultured at 37 °C in a 5% CO$_2$ atmosphere.

**Cytotoxicity assay**

Cells were incubated with serial dilutions of tanshinones, 5-FU, doxorubicin hydrochloride, or oxaliplatin (0–100 $\mu$mol·L$^{-1}$) in 96-well culture plates for 48 h. MTT solution (100 $\mu$L, 0.5 mg·mL$^{-1}$) was added to each well and the plate was incubated at 37°C for 4 h, followed by removal of the supernatant and replacement with DMSO (100 $\mu$L). Cells without treatment served as blank control. DMSO 1% ($V/\text{}V$) served as vehicle control. The product was quantified by measuring absorbance at 570 nm using a Biorad Microplate Reader (Bio-Rad, Philadelphia, United States).
### Apoptosis analysis

Cells were seeded in 24-well culture plate and cultured overnight. Then the test compounds at the appropriate concentrations were added into the medium, except for the control and vehicle control groups. Cells without treatment served as the blank control. Cells with 0.1% (V/V) DMSO served as the vehicle control. After treatment for 48 h, cells were digested with 0.25% trypsin, and washed twice with cold PBS. The cell precipitation was re-suspended in a binding buffer and FITC Annexin V (5 µL) and proidium iodide (10 µL, 50 µg·mL⁻¹) were added into the cell suspension. They were then gently vortexed and incubated for 15 min at room temperature (25 °C) in the dark. Samples were analyzed by flow cytometry (BD LSRFortessa Cell Analyzer) within 2 h.

### Statistical analysis

Data were analyzed by the one-way or two-way analysis of variance, followed by Dunnett test or Bonferroni post-tests to detect differences intra group with GraphPad Prism software 5.0 (USA). Significant difference was considered when \( P < 0.05 \).

### Results

#### General procedure for the synthesis of tanshinone derivatives

According to the literature [12-13], in the presence of \( p \)-TsOH, tanshinone IIA reacted with some aromatic aldehydes in chloroform to give 2, 2′-(substituted methylene) bis-(1, 6, 6-trimethyl-6, 7, 8, 9-tetrahydrophenanthro[1, 2-b]furan-10,11-dione) derivatives (N1–N5). In addition, under the initiation of benzoyl peroxide (BPO), transhinone IIA reacted with some aromatic aldehydes for 2 h to give 2-bromo-1, 6, 6-trimethyl-6, 7, 8, 9-tetrahydrophenanthro[1, 2-b]furan-10,11-dione (N6) in 70% yield. Among these derivatives, N3 and N5 are reported as new compounds (Fig. 2). The spectroscopic details for the N1–N6 derivatives are presented.

2. 2′-((4-Hydroxyphenyl)methylene)bis(1, 6, 6-trimethyl-6, 7, 8, 9-tetrahydrophenanthro[1, 2-b]furan-10, 11-dione) (N1) \(^1\)H NMR (400 MHz, CDCl₃) \( \delta \): 7.60 (d, \( J = 8.4 \) Hz, 2H), 7.45 (d, \( J = 8.0 \) Hz, 2H), 7.46 (d, \( J = 8.4 \) Hz, 2H), 7.24–7.37 (m, 5H), 5.62 (s, 1H), 3.16 (t, \( J = 6.4 \) Hz, 4H), 2.18 (s, 6H), 1.75–1.77 (m, 4H), 1.64–1.75 (m, 4H), 1.27 (s, 12H); ESI-MS m/z 677.3 [M + H]+.

2. 2′-((4-Hydroxyphenyl)methylene)bis(1, 6, 6-trimethyl-6, 7, 8, 9-tetrahydrophenanthro[1, 2-b]furan-10, 11-dione) (N2) \(^1\)H NMR (400 MHz, CDCl₃) \( \delta \): 7.51 (d, \( J = 8.4 \) Hz, 2H), 7.14 (d, \( J = 4.4 \) Hz, 2H), 7.04 (d, \( J = 8.0 \) Hz, 2H), 7.12 (d, \( J = 4.4 \) Hz, 2H), 6.86 (d, \( J = 8.4 \) Hz, 2H), 5.55 (s, 1H), 3.16 (t, \( J = 6.0 \) Hz, 4H), 2.16 (s, 6H), 1.74–1.75 (m, 5H), 1.62–1.63 (m, 4H), 1.26 (s,12H); ESI-MS m/z 693.8 [M + H]+.

2. 2′-(4-Fluorophenyl)methylene)bis(1, 6, 6-trimethyl-6, 7, 8, 9-tetrahydrophenanthro[1, 2-b]furan-10,11-dione) (N3) \(^1\)H NMR (400 MHz, CDCl₃) \( \delta \): 7.60 (d, \( J = 8.4 \) Hz, 2H), 7.45 (d, \( J = 8.0 \) Hz, 2H), 7.02–7.06 (m, 2H), 5.59 (s, 1H), 3.16 (t, \( J = 6.4 \) Hz, 4H), 2.180 (s, 6H), 1.75–1.77 (m, 6H), 1.62–1.64 (m, 6H), 1.27(s, 12H); ESI-MS m/z 695.8 [M + H]+.

2. 2′-(4-Nitrophenyl)methylene)bis(1, 6, 6-trimethyl-6, 7, 8, 9-tetrahydrophenanthro[1, 2-b]furan-10,11-dione) (N4) \(^1\)H NMR (400 MHz, CDCl₃) \( \delta \): 8.24 (d, \( J = 8.8 \) Hz, 2H), 7.60 (d, \( J = 8.0 \) Hz, 2H), 7.41–7.46 (m, 4H), 5.70 (s, 1H), 3.17 (t, \( J = 6.0 \) Hz, 4H), 2.22 (s, 6H), 1.76–2.02 (m, 4H), 1.27 (s, 12H); ESI-MS m/z 722.9 [M + H]+.

2. 2′-((4-Trifluoromethyl)phenyl)methylene)bis(1, 6, 6-trimethyl-6, 7, 8, 9-tetrahydrophenanthro[1, 2-b]furan-10, 11-dione (N6) \(^1\)H NMR (400 MHz, CDCl₃) \( \delta \): 7.62 (d, \( J = 8.0 \) Hz, 1H), 7.514 (d, \( J = 8.0 \) Hz, 1H), 7.15 (t, \( J = 6.4 \) Hz, 4H), 2.23 (s, 3H), 1.76–1.79 (m, 3H), 1.62–1.64 (m, 4H), 1.33 (s, 6H); ESI-MS m/z 737.04 [M + H]+ (100%), 375.03 [M + 2 + H]+ (97%).

### Cytotoxicity of newly synthesized tanshinones against colon cancer cells with various p53 genotypes and normal colon fibroblasts

The cytotoxicity of tanshinone I and tanshinone IIA was verified in normal and colon cancer cells. The cytotoxicity of the positive drugs (doxorubicin, 5-FU and oxaliplatin) was also evaluated for comparison (Table 1). HCT116 cells were more sensitive to tanshinones than HT29 cells, and similar observations were seen for doxorubicin and oxaliplatin.

In addition, tanshinone I exhibited no cytotoxicity in normal human fibroblast CCD18-co cells at its respective half inhibitory concentration (IC₅₀) (Fig. 3), while this selectivity was not observed for tanshinone IIA. As for the other derivatives, there was no detectable cytotoxicity on CCD18-co cells (data not shown). In Fig. 3, HCT116 and CCD18-co cells were treated with the tanshinones at the concentration close to their IC₅₀ for 48 h, and cell viability was detected with the MTT assay.

#### Table 1  Cytotoxicity of tanshinones I and IIA against colon cancer cells (Mean ± SEM, \( n = 3 \))

<table>
<thead>
<tr>
<th>Compounds</th>
<th>HCT116 p53&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>IC₅₀ (µmol·L&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>HT29 p53&lt;sup&gt;mut/mut&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tanshinone I</td>
<td>3.16 ± 1.07</td>
<td>21.11 ± 3.41</td>
<td></td>
</tr>
<tr>
<td>Tanshinone IIA</td>
<td>7.08 ± 1.51</td>
<td>27.94 ± 3.35</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>2.82 ± 0.16</td>
<td>9.38 ± 1.62</td>
<td></td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>3.69 ± 1.68</td>
<td>21.13 ± 6.82</td>
<td></td>
</tr>
<tr>
<td>5-Fluourouracil</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

ND: Non detectable
Apoptosis induced by tanshinones in colon cancer cells

The cytotoxicity exhibited by the tanshinones could be due to the induction of apoptosis. Indeed apoptosis could be induced by tanshinone I and tanshinone IIA (6.25 µmol·L\(^{-1}\)) and this could be detected at 24 h after incubation in HCT116 p53\(^{+/+}\) cells. Tanshinone I produced higher potency. However, the apoptotic activity of these tanshinones at the same concentration was much weaker in the p53 mutant cells, and there was no significant apoptotic activity by both tanshinones in HT29 cells. (Fig. 4) In Fig. 4, the cells without tanshinone treatment served as blank control. Cells with 0.1% (V/V) DMSO served as the vehicle control. Cells in experimental groups were treated with the tanshinones (6.25 µmol·L\(^{-1}\)) for 24 h. At the end of the incubation, cells were collected and stained with FITC Annexin V and PI for 15 min at RT (25 °C) in the dark. The samples were analyzed by flow cytometry (BD FACS). The histogram expresses the percentage of apoptotic cells.

Cytotoxicity of synthetic compounds derived from tanshinone IIA in HCT116 p53\(^{+/+}\) cells

Among the six compounds synthesized from tanshinone IIA, only N1 [IC\(_{50}\) (51.8 ± 1.7) µmol·L\(^{-1}\)] and N2 [IC\(_{50}\) (35.9 ± 1.2) µmol·L\(^{-1}\)] showed significant cytotoxicity in HCT116 p53\(^{+/+}\) cells. However, this was weaker than that of tanshinone IIA [IC\(_{50}\) (7.08 ± 1.51) µmol·L\(^{-1}\)]. The structures of the new compounds are presented in Fig. 2.

Discussion

Tanshinone IIA and tanshinone I are reported to be the most abundant tanshinones in Danshen. These results showed that a p53-dependent pathway was involved in the cytotoxicity and apoptotic activity of tanshinone I and tanshinone IIA. This was demonstrated by the lack of apoptotic activity of the tanshinones in HT-29 cells, a p53 mutant cell type. However, other mechanisms led to this drug resistant effect, such as overexpression of manganese superoxide dismutase or Bcl-2 cannot be excluded\(^{[14-16]}\). It was also noted that selective cytotoxicity of tanshinone IIA was not as good as tanshinone I. Therefore, modification of the structure of tanshinone IIA is meaningful for the discovery of more potent compounds in the treatment of colon cancer.

Comparing the structures of tanshinones, it was noted that the naphthalene or tetrahydronaphthalene rings A and B, and the ortho-quinone moiety in ring C should be essential structures for the activity of tanshinones, while structural modifications of ring A and furan or dihydrofuran in ring D would influence the activity. It was reported that the introduction of polar substituent groups into A ring or D ring enhanced the cytotoxicity of tanshinones\(^{[17]}\). Therefore, the new compounds were designed as the following strategy: keeping effective quinone (ketene) group and aromatic rings as first consideration, and then concentrating on modification of the furan ring. Among the analogues synthesized, all showed no cytotoxicity on normal colon cells, and only N1 and N2 showed weak cytotoxicity on colon cancer cells when compared with tanshinone IIA (Table 2). The SAR showed that the presence of polar and electron-withdrawing groups such as -F, -NO\(_2\) or -CF\(_3\) in the para position of the aromatic aldehydes greatly affected the activity. It was unexpected that the activity was vanished when a bromo group was substituted in the furan ring of tanshinone IIA. The hydroxyl-substituting group in the para position of aromatic aldehyde presented better pharmacological properties than those of other derivatives.

In conclusion, the possibility of a p53-dependent pathway was shown to occur in the anti-colon cancer activity of tanshinone IIA and tanshinone I. Derivatives of tanshinone IIA were synthetized and their cytotoxicity against normal and colon cancer cells was evaluated. Furthermore, from the steric and electronic characteristic point of view, some preliminary conclusions on SAR of tanshinones could be drawn: increasing the delocalization of the A and B rings could enhance the cytotoxicity, while a non-planar and small sized D ring region are a better choice.

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Author contributions

WANG Lin, LI Xu-Qin and CHO Chi-Hin designed the experimental protocols; WANG Lin and ZHANG Fei-Long...
performed research; LIU An contributed the HPLC purifying and identification tools; WANG Lin and LIU An analyzed the data; WANG Lin and LI Xu-Qin wrote the paper.

References
