Effect of ST2825 on the proliferation and apoptosis of human hepatocellular carcinoma cells

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ABSTRACT. The purpose of this study was to investigate the effect of ST2825, an inhibitor of myeloid differentiation factor 88 (MyD88), on the proliferation and apoptosis of human hepatocellular carcinoma (HCC) cells as well as the potential mechanism and clinical significance of ST2825 in the treatment of HCC. Immunohistochemical staining with an MyD88 antibody was performed on tissues from 80 human HCC patients and adjacent normal tissues. In the in vitro experiment, human HCC HepG-2 cells cultured in vitro were divided into the following groups: blank, control (1% DMSO), low-dose (2 µM), medium-dose (10 µM), and high-dose ST2825 (20 µM). Cell apoptosis was detected by the Annexin V-FITC assay, and HepG-2 cell proliferation was detected by the MTT assay. The expression of IκB, p65, cyclin D1, caspase-3, and bcl-2 in the cells after a 48-h treatment was assayed by western blot analysis. MyD88 expression in the HCC tissue was significantly higher than that in the adjacent normal tissue (P < 0.05). The proliferation and apoptosis rates of control HCC cells displayed no significant differences compared with those of the blank group (P > 0.05). Compared with the control, ST2825 significantly inhibited the proliferation of and promoted the apoptosis of HCC cells. Moreover,
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ST2825 significantly decreased bcl-2 expression, increased cleaved caspase-3 expression (P < 0.05), and reduced p65 nuclear expression (P < 0.05) in a dose-dependent manner. ST2825 inhibits the proliferation and promotes the apoptosis of HCC cells, thereby suggesting that ST2825 may be a new drug for HCC treatment.

**Key words:** Hepatocellular carcinoma; ST2825; Apoptosis; Proliferation

**INTRODUCTION**

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide, with a high rate of mortality and poor prognosis. Currently, combined therapy with surgery, chemotherapy, radiation therapy, and biological therapy is used for the clinical treatment of HCC (Rasool et al., 2014). However, the majority of HCC patients are diagnosed with middle-advanced cancer, a stage in which the best opportunity for surgical treatment has passed. Additionally, owing to its insensitivity to conventional radiotherapy and chemotherapy regimens, HCC recurs easily after surgery (Bruix et al., 2014). Thus, the development of new chemotherapeutic drugs with high efficiency and few side effects is of great importance in the treatment of HCC. Myeloid differentiation factor 88 (MyD88) plays a key role in the development, progression, and invasion of HCC (Liang et al., 2013; Jia et al., 2014), and ST2825 is a specific inhibitor of MyD88 (Van Tassell et al., 2011). In this study, we investigated the effect of ST2825 on the proliferation and apoptosis of HCC cells to provide a new approach for the clinical treatment of HCC.

**MATERIAL AND METHODS**

**General information**

We recruited 80 patients with HCC who received surgical treatment in our hospital from October 2010 to May 2014. Postoperative diagnosis was jointly made by three experienced pathologists. The study was approved by our hospital Ethics Committee, and all patients signed a written informed consent form prior to surgery.

**Immunohistochemical staining**

Tissue specimens were immersed in neutral formalin, embedded in paraffin, and cut into 4-µm sections for immunohistochemical staining. The tissue sections were dried in an oven at 70°C for 30 min followed by overnight incubation at 56°C. The dried sections were dewaxed in xylene twice (5 min each time) and dehydrated with a gradient of ethanol (75, 85, 95, and 100%). After being rinsed with tap water, the sections were washed with phosphate-buffered saline (PBS) on a shaker twice (10 min each time). Antigen retrieval was performed using antigen retrieval buffer, and the sections were blocked with normal goat serum blocking solution for 1 h at room temperature. After the blocking solution was discarded, MyD88 antibody dilution solution was immediately added, and the sections were incubated in a 37°C incubator for 2 h. The sections were then washed thoroughly with PBS and incubated with a secondary antibody for 1 h. The sections were washed again with PBS and incubated with horseradish peroxidase-conjugated streptavidin for 15 min. After washing with PBS, a color reaction was performed by adding dinitrobenzidine, and hematoxylin
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was used to double-stain the nucleus for 1 min. The sections were subsequently dehydrated with an ethanol gradient, clarified with xylene, mounted with neutral resin, and then observed by microscopy. The negative control was prepared with PBS substituting for the primary antibodies.

Interpretation of the immunohistochemical staining results and the scoring criteria were performed as previously described (Liang et al., 2013; Bruix et al., 2014): MyD88-positive cells were recognized upon the observation of brown-yellowish precipitate, and the results were independently interpreted by two experienced pathologists. Each section was examined in 15 fields of view at high magnification (400X) using a double-blind method. The average number of MyD88-positive cells in 100 cells was obtained as the percentage of positive cells. The positive immunohistochemical staining results (appearance of brown-yellowish particles in the nucleus or cytoplasm) were interpreted according to the literature (Jia et al., 2014) based on the following two aspects: 1) percentage of positive cells characterized as <1% for 0 points, 1-10% for 1 point, 10-30% for 2 points, 30-60% for 3 points, and >60% for 4 points; and 2) staining intensity of positive cells characterized as negative for 0 points, yellowish for 1 point, yellow for 2 points, and brown-yellowish for 3 points. The results were determined as follows: 0-2 points for negative and 3-7 points for positive.

Cells and reagents

The human HCC cell line (HepG-2) was provided by the Shanghai Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences. RPMI 1640 medium was purchased from Gibco (USA). MTT (M2128) was purchased from Sigma (USA). The Annexin V-FITC Apoptosis Detection Kit was purchased from BD Biosciences (USA). Total cellular protein (P0013), nuclear/plasma protein extraction reagent (P0028), and ECL reagent (P0018) were purchased from Beyotime (Shanghai, China). The bcl-2, H3, cyclin D1, p65 (sc-109), H3 (sc-8654) and β-actin (sc-7210) primary antibodies were purchased from Santa Cruz (USA). The cleaved caspase-3 antibody was purchased from Cell Signaling (USA). The horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (sc-2020) was purchased from Bioworld. ST2825 was purchased from MedChem Express.

HCC HepG-2 cell culture

The HepG-2 HCC cell line was restored and seeded in culture flasks containing RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were incubated at 37°C in a humidified 5% CO2 incubator. Cells were passaged once every 2 to 3 days, and logarithmic growth phase cells were collected for drug intervention.

Drug treatment and grouping

Cells were seeded on 96-well plates at 1 x 10^4 cells per well with 29 replicate wells for each group. Culture medium (100 µL) was added to each well. The experiment was performed with the following five groups: blank (no intervention), control (1% DMSO), low-dose ST2825 (2 µM), medium-dose ST2825 (10 µM), and high-dose ST2825 (20 µM). The final concentration of ST2825 was controlled at 1% for the three treatment groups.

Annexin V-FITC apoptosis assay

Cells were collected after 24, 48, and 72 h of treatment. HepG-2 cell apoptosis was assayed by flow cytometry following the manufacturer protocol.
MTT colorimetric assay of cell proliferation

Plates added with drug were placed in a humidified 37°C, 5% CO₂ cell culture incubator for 24, 48, or 72 h of continuous culture. To each well, 20 µL MTT solution was added 4 h before termination of culture. After termination of culture, the supernatant was completely removed from each well by suction followed by the addition of 150 µL DMSO per well and oscillation for 10 min. The absorbance of the reaction solution in each well at 570 nm was measured using an ELISA reader. The results are reported as means of six replicate wells. The inhibition rate of cell proliferation in each group was calculated as follows: inhibition rate = (1 - OD<sub>treatment</sub> / OD<sub>blank</sub>) × 100%; where OD is the absorbance at 570 nm.

Western blot assay of p65, cyclin D1, bcl-2, and cleaved caspase-3 expression

After 48 h of treatment, the HepG-2 cells in the ST2825 and control groups were washed twice with PBS and centrifuged at 12,000 g for 15 min. Total and nuclear proteins were extracted from cells using the corresponding extraction reagent in accordance with manufacturer protocol. Nuclear protein was used for the p65 assay with H3 as the internal control, and total protein was used for the cyclin D1, caspase-3, and bcl-2 expression assays. Protein quantification was performed using the bicinchoninic acid method. Protein samples (30 µg each) were electrophoresed, transferred to a membrane, and blocked with 5% nonfat milk for 1 h. H3, p65, cyclin D1, β-actin, cleaved caspase-3, and bcl-2 antibodies were diluted with 5% milk (1:200) and incubated with the membrane at 4°C overnight. After being washed with TBST, the membrane was incubated with horseradish peroxidase-conjugated rabbit anti-goat secondary antibody diluted in 5% milk. A 1:1 chemiluminescent working solution was prepared according to the ECL instructions. Gel images were acquired using a gel imaging system, and densitometry was performed using the Image J software.

Statistical analysis

Statistical analysis was performed using SPSS Version 13.0. Quantitative data are reported as means ± SD. Comparison of the means between groups was performed using one-way ANOVA. The difference was considered significant at P < 0.05.

RESULTS

MyD88 expression in HCC and adjacent normal tissues

The number of MyD88-positive cells was higher in HCC tissues than that in adjacent normal tissues, showing a statistically significant difference between groups (P < 0.05; Figure 1 and Table 1).

Effect of ST2825 on HepG-2 cell proliferation

The inhibition rate of HepG-2 cell proliferation did not significantly differ between the control and blank groups (P > 0.05; Table 2). Compared with the control, ST2825 significantly inhibited HepG2 cell proliferation (P < 0.05; Table 2) in a time- and dose-dependent manner. The inhibition rate remained significantly higher in the high-dose ST2825 group than that in the low-dose group (P < 0.05; Table 2).
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**Figure 1.** MyD88 expression in hepatocellular carcinoma and adjacent normal tissues.

![Image](adjacent_normal_liver_tissues_Hepatocellular_carcinoma_tissues.jpg)

**Table 1.** MyD88 expression in hepatocellular carcinoma and adjacent normal tissues

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>MyD88 expression</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive cases (%)</td>
<td>Negative cases (%)</td>
<td></td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>80</td>
<td>50 (62.5)</td>
<td>30 (37.5)</td>
<td>13.33</td>
</tr>
<tr>
<td>Adjacent normal tissues</td>
<td>80</td>
<td>10 (12.5)</td>
<td>70 (87.5)</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05, compared with the blank group; #P < 0.05, compared with the low- and medium-dose groups.

**Table 2.** Effect of ST2825 on HepG-2 cell proliferation (means ± SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>0.1 ± 0.02</td>
<td>0.1 ± 0.09</td>
<td>0.09 ± 0.01</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Low-dose ST2825</td>
<td>10</td>
<td>0.2 ± 0.01</td>
<td>0.9 ± 3.2**</td>
<td>10.1 ± 2.7**</td>
<td>24.8 ± 2.6**</td>
</tr>
<tr>
<td>Medium-dose ST2825</td>
<td>10</td>
<td>0.03 ± 0.09</td>
<td>15.6 ± 3.9**</td>
<td>27.2 ± 3.1**</td>
<td>32.5 ± 3.5**</td>
</tr>
<tr>
<td>High-dose ST2825</td>
<td>10</td>
<td>0.01 ± 0.08</td>
<td>51.3 ± 5.6**</td>
<td>58.3 ± 7.2**</td>
<td>63.2 ± 8.6**</td>
</tr>
</tbody>
</table>

*P < 0.05, compared with the blank group; #P < 0.05, compared with the low- and medium-dose groups.

**Effect of ST2825 on HepG-2 cell apoptosis**

The apoptotic rate of HepG-2 cells did not change significantly between the blank and control groups (P > 0.05). Compared with the control, 24 and 48 h of ST2825 treatment significantly increased the rate of HepG-2 cell apoptosis (P < 0.05) in a dose-dependent manner. The apoptotic rate of HepG-2 cells in the high-dose ST2825 group was significantly higher than that in the low-dose group (P < 0.05; Table 3).

**Table 3.** Effect of ST2825 on HepG-2 cell apoptotic rate (%).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>10</td>
<td>0.01 ± 0.001</td>
<td>2.3 ± 0.09</td>
<td>3.2 ± 0.13</td>
<td>4.2 ± 0.15</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>0.02 ± 0.001</td>
<td>2.5 ± 0.08</td>
<td>3.3 ± 0.14</td>
<td>4.1 ± 0.16</td>
</tr>
<tr>
<td>Low-dose ST2825</td>
<td>10</td>
<td>0.02 ± 0.001</td>
<td>3.6 ± 0.15</td>
<td>4.8 ± 0.96</td>
<td>8.2 ± 1.23**</td>
</tr>
<tr>
<td>Medium-dose ST2825</td>
<td>10</td>
<td>0.01 ± 0.002</td>
<td>6.9 ± 1.01**</td>
<td>8.9 ± 1.35**</td>
<td>10.2 ± 1.79**</td>
</tr>
<tr>
<td>High-dose ST2825</td>
<td>10</td>
<td>0.03 ± 0.001**</td>
<td>10.6 ± 1.42**</td>
<td>13.36 ± 2.31**</td>
<td>16.9 ± 2.91**</td>
</tr>
</tbody>
</table>

*P < 0.05, compared with the blank group; #P < 0.05, compared with the low- and medium-dose groups.

**Effect of ST2825 on IκB expression and p65 nuclear expression in HepG-2 cells**

Compared with the control, 24 h of ST2825 intervention significantly increased IκB (Figure 2) expression in HepG-2 cells in a dose-dependent manner (P > 0.05). Moreover, ST2825 signifi-
cantly inhibited p65 (Figure 3) expression in the nucleus in a dose-dependent manner (P < 0.05; Figures 2, 3 and Table 4).

![Western blot analysis of IκB, cyclin D1, cleaved caspase-3, and Bcl-2](image1.png)

**Figure 2.** Effect of ST2825 on IκB, cyclin D1, cleaved caspase-3, and Bcl-2 expression in HepG-2 cells as determined by western blot analysis (lane 1, blank; lane 2, control; and lane 3, high-dose ST2825).

![Western blot analysis of p65](image2.png)

**Figure 3.** Effect of ST2825 on p65 expression in HepG-2 cells as determined by western blot analysis (lane 1, blank; lane 2, control; and lane 3, high-dose ST2825).

![Table 4](table.png)

**Table 4.** Densitometry of the western blot of IκB, p65, cyclin D1, Bcl-2, and cleaved caspase-3 expression in HepG-2 cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>IκB</th>
<th>p65</th>
<th>Cyclin D1</th>
<th>Bcl-2</th>
<th>Cleaved caspase-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.92 ± 0.32</td>
<td>1.01 ± 0.34</td>
<td>0.56 ± 0.31</td>
<td>0.68 ± 0.21</td>
<td>0.13 ± 0.04</td>
</tr>
<tr>
<td>Control</td>
<td>0.09 ± 0.06</td>
<td>1.03 ± 0.42</td>
<td>0.52 ± 0.25</td>
<td>0.64 ± 0.19</td>
<td>0.24 ± 0.12</td>
</tr>
<tr>
<td>High-dose ST2825</td>
<td>0.88 ± 0.29**</td>
<td>0.34 ± 0.11**</td>
<td>0.19 ± 0.09**</td>
<td>0.21 ± 0.09**</td>
<td>0.96 ± 0.29**</td>
</tr>
</tbody>
</table>

*P < 0.05, compared with the blank group; **P < 0.05, compared with the control group.

**Effect of ST2825 on IκB, cyclin D1, cleaved caspase-3, and Bcl-2 expression in HepG-2 cells**

Compared with the control, 24 h of ST2825 treatment significantly inhibited cyclin D1 and Bcl-2 expression (P > 0.05; Figure 2 and Table 4), but 24-h treatment with ST2825 significantly increased cleaved caspase-3 expression (P < 0.05; Figure 2 and Table 4) in the high-dose group.
The solvent did not affect cyclin D1, cleaved caspase-3, and bcl-2 expression in control HepG-2 cells compared with that observed for the blank group (P < 0.05; Figure 2 and Table 4).

**DISCUSSION**

MyD88 is a member of the Toll/IL-1R family and death domain family, which has a pivotal role in regulating the transduction of the NF-κB signaling pathway (Wang et al., 2014). MyD88 is essentially a soluble cytoplasmic protein containing three functional domains: an N-terminal death domain of 90-amino acid residues that mainly mediates the interaction between death sequence-containing proteins; an intermediate region; and a C-terminal Toll region of 130-amino acid residues that mainly transfers signals by recruiting connexin. The death domain of MyD88 can bind to the death domain of interleukin-1 receptor-associated kinase, causing the autophosphorylation of the kinase. Ultimately, this binding will induce κB activation through a series of activation reactions leading to NF-κB activation and translocation. Previous studies have found that the NF-κB signaling pathway has a role in the development and progression of HCC. NF-κB activity is significantly increased in human HCC tissue (Qiao et al., 2006), and the inhibition of NF-κB expression substantially suppresses the proliferation and promotes the apoptosis of HCC cells (Wu et al., 2009). Moreover, many studies have indicated that MyD88 has an important role in the development, progression, invasion, and metastasis of a variety of tumors (Kfoury et al., 2014). The results obtained from the present study showed that the MyD88 expression level in HCC tissue was significantly higher than in adjacent normal tissue, which is consistent with the results of previous studies. Additionally, MyD88 is closely related to the clinical stage of HCC patients. The downregulation of MyD88 expression significantly inhibits the proliferation and promotes the apoptosis of HCC cells (Liang et al., 2013). Together, these results suggest that MyD88 is an important component in the proliferation and apoptosis of HCC cells, and may therefore be an attractive new treatment target in HCC.

ST2825 is a small-molecule compound consisting of a halogenated heptapeptide (Hinz et al., 1999; Loiarro et al., 2007), and it competitively binds to the Toll/IL-1 receptor domain of MyD88 to inhibit MyD88 dimerization. This mechanism prevents MyD88 from activation and TLR signal transduction, thereby blocking the transduction of the TLR signaling pathway. The present study found that ST2825, a selective inhibitor of MyD88, significantly inhibited cell proliferation and promoted apoptosis in the human HCC cell line HepG-2 in a dose-dependent manner. Thus, we used high-dose ST2825 to probe the underlying mechanism. The results showed that ST2825 significantly inhibited the translocation of p65 to the nucleus, thus inhibiting the transcription, translation, and expression of the NF-κB-mediated downstream factor cyclin D1. Cyclin D1 is an important component in HCC cell proliferation (Deane et al., 2001), which may be the main reason why ST2825 can inhibit HCC cell proliferation. Additionally, ST2825 significantly increased cleaved caspase-3 protein expression but decreased the protein expression of bcl-2, an anti-apoptotic protein, in HCC cells, which may be one of the reasons why ST2825 can promote apoptosis in HCC cells.

In summary, ST2825, a selective inhibitor of MyD88, significantly inhibits the proliferation and promotes the apoptosis of HCC cells. The possible mechanism is related to the inhibition of the NF-κB signaling pathway activity. The effect of ST2825 needs to be tested in animal models to provide evidence for its application in the clinical treatment of HCC.

**Conflicts of interest**

The authors declare no conflict of interest.
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