Therapeutic effects of sorafenib on the A549/DDP human lung adenocarcinoma cell line in vitro

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Abstract. The aim of the present study was to observe the effects of sorafenib on the proliferation, apoptosis and invasion of A549/DDP cisplatin-resistant lung adenocarcinoma cells cultured in vitro. The A549/DDP cisplatin-resistant lung adenocarcinoma cell strain was cultured in vitro, the cell culture group incubated in culture medium only was set as the control group (Group S0) and the four concentration gradients of sorafenib were added to the culture groups as the experimental groups: S1, 2 µmol/l; S2, 4 µmol/l; S3, 8 µmol/l; and S4, 16 µmol/l. The MTT assay was used to determine the growth inhibition rate of the cells, which were respectively subjected to sorafenib treatment for 24, 48 and 72 h. Flow cytometry was used to determine the rate of apoptosis in each group following sorafenib treatment for 72 h. Furthermore, the Transwell invasion experiment was used to determine the effect on A549/DDP cell invasion following sorafenib treatment for 24 h. Based on the MTT assay, it was found that the inhibition rates of A549/DDP cisplatin-resistant lung adenocarcinoma cells in groups S1-4 following sorafenib treatment for 24 h were 4.58±2.82, 14.93±2.62, 37.58±7.13 and 58.39±8.15%, respectively. For 48 h, inhibition rates in S1-4 were 14.98±2.93, 26.28±7.31, 63.00±3.05 and 78.84±3.96%, respectively, and for 72 h, inhibition rates were 18.80±2.82, 32.71±2.55, 58.2±2.3, and 82.7±2.3, which was significantly lower compared with the control group. The number of cells permeating the septum in groups S1-4 following treatment with sorafenib for 24 h was 58.2±2.3, 41.3±1.3, 22.6±2.1 and 14.7±1.1/HPS, which was significantly lower compared with the control group. The number of cells permeating the septum in each experimental group decreased with the enhancement of the concentration gradient. The differences were statistically significant (P<0.05). In conclusion, sorafenib inhibits the proliferation of A549/DDP cisplatin-resistant lung adenocarcinoma cells in a time- and concentration-dependent manner. In addition, sorafenib induces apoptosis in A549/DDP cisplatin-resistant lung adenocarcinoma cells, thus reducing their invasiveness.

Introduction

At present, lung cancer is a malignant tumor with the globally highest morbidity and fatality rates. ~80-85% of lung cancers are non-small-cell lung carcinoma (NSCLC). Surgical resection is considered the main treatment for NSCLC, with surgery being indicated for ~20-30% of patients and the postoperative recurrence and the metastasis rate being ≥50% (1). Therefore, for the lung cancer patients who are unable to undergo surgery and those with postoperative recurrence or metastasis, combinations of platinum-containing agents are the primary chemical treatment to extend the lifetime. However, to date, no effective measures have been established for patients who are subject to such regimen control, and who present with new cancer progression. Sorafenib is a small molecule with activity against neoplasms via multiple biological targets. Sorafenib has a dual function of inhibiting tumor cell proliferation and angiogenesis. At present, the Food and Drug Administration (FDA, Silver Spring, MD, USA) has approved sorafenib for the use in the clinical treatment of kidney and liver cancers (2,3). To date, basic research and clinical trials using sorafenib for the prevention and treatment of lung cancer have made positive
progress globally (4,5). The present study used the A549/DDP cisplatin-resistant lung adenocarcinoma cell strain to study the biological effects of sorafenib. Effects on proliferation, apoptosis and invasion of the A549/DDP cisplatin-resistant lung adenocarcinoma cell strain was observed, which provided scientific experimental data and a theoretical basis for the application of sorafenib for the subsequent treatment of cisplatin-resistant lung cancer.

Materials and methods

Cell strains. The A549 lung adenocarcinoma cell strain and the A549/DDP cisplatin-resistant lung adenocarcinoma cell strain were purchased from the tumor cell bank of the Chinese Academy of Sciences (Shanghai, China), with a resistance index of 13 times.

Main reagents. Sorafenib was purchased from Bayer (Leverkusen, Germany), RPMI-1640 culture medium and fetal bovine serum (FBS) were from HyClone (Logan, UT, USA), cisplatin (DDP) from Haosen Pharmacy, Ltd. (Ganzhou, China), MTT and dimethylsulfoxide (DMSO) from Sigma-Aldrich (St. Louis, MO, USA), Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit from BioVision (Mountain View, CA, USA) and Matrigel® from BD Biosciences (Franklin Lakes, NJ, USA).

Cell culture. The A549 and A549/DDP cells were cultured in RPMI-1640 medium at 37°C with 5% CO₂ and 95% saturated humidity. To maintain the stable drug-resistance of A549/DDP cells, 2 μmol/l DPP was added into the RPMI-1640 with 10% FBS for joint culture and the cells were cultured in the medium without DDP for one week prior to beginning the trial.

Experimental groups. The experiment involved five groups: S0 and experimental groups S1, S2, S3 and S4. The control group S0 was incubated with the culture medium RPMI-1640 only; S1-4 were incubated with 2, 4, 8 and 16 μmol/l sorafenib, respectively.

MTT assay. Cells were collected at the exponential growth phase, the cell density was adjusted to 3x10³ ml⁻¹ and 100 μl was inoculated into each well on 6 culture plates and 7 repeated wells set for each 96-well plate. One culture plate was removed each day at the same time, MTT reagent (20 μl) was added, placed in a CO₂ incubator and incubated for 4 h. Following removal of the medium, DMSO (100 μl) was added to each well and the plate was placed on an agitator for 10 min to totally dissolve the crystals. The optical density (OD) of each well was measured at 490 nm using an ELISA reader (R&D Systems, Minneapolis, MN, USA). The blank controls (containing medium, MTT and DMSO) and control wells (containing cells, medium, MTT and DMSO) were measured. The formula adopted for the calculation of the inhibition rate of cell proliferation is: Inhibition rate = 100% x [(OD value of the control well - OD value of the blank control well)-(OD value of the experimental well - OD value of the blank control well)]/(OD value of control well - OD value of the blank control well). Each experiment was repeated three times.

Flow cytometric analysis of apoptosis. The inoculated cells were cultured in a 5% CO₂ incubator for 24 h and the original culture medium was discarded. Different concentrations of sorafenib (2, 4, 8 and 16 μmol/l) were added to the experimental groups at 2 ml/well in RPMI medium. The control group was added with the same amount of culture medium without sorafenib. Three complexes were prepared for each group. The culture plates were incubated for a further 72 h. The cells of each group were digested with EDTA-free pancreatin (Gibco, Grand Island, NY, USA) and collected. Binding buffer (500 μl; KeyGen Biotech, Nanjing, China) was added to prepare a cell suspension. Under the exclusion of light, 5 μl Annexin V-FITC and 5 μl PI was added to each group of cell suspension. The groups were allowed to react for 15 min at room temperature in the dark. Flow cytometric analysis was performed within one hour and the percentage of apoptotic cells was calculated using CellQuest software, version 3.0 (BD Biosciences).

Transwell assay. A549/DDP cells were collected during the exponential growth phase, the concentration was adjusted to 5x10⁵ ml⁻¹ and the cells were inoculated into the upper chamber of a Transwell plate (6.5 mm; 24-well plate, Costar; Corning, NY, USA) at 200 μl/well and three repeated wells were set for each group. Sorafenib was added to the upper chamber with a final concentration of 2, 4, 8 and 16 μmol/l for the experimental groups, and no reagent was added for the control group. At the same time, RPMI-1640 (500 μl) culture medium containing 10% FBS was added into the lower chamber and the plate was cultured for a further 24 h. The cells were removed from the Transwell chamber, washed twice with phosphate-buffered saline and the filter membrane was fixed with 4% paraformaldehyde for 30 min. The filter membrane was subjected to crystal violet staining (Beyotime Institute of Biotechnology, Shanghai, China) for 20 min following air drying and was then rinsed with double distilled water. Five fields of view at upper, lower, left, right and central locations were captured under a light microscope (x400). To assess the number of migrated cells the membrane to the lower chamber surface, the average values were analyzed. The number of migrated cells reflected the level of tumor cell invasiveness. Accordingly, a reduced number of cells permeating the septum indicated marked inhibition of invasion.

Statistical analysis. Values are presented as the mean ± standard deviation and were determined by the statistical software SPSS 11.0 (SPSS Inc., Chicago, IL, USA). Groups were compared using one-way analysis of variance. Homogeneity of variance was tested by the least significant difference method, abd heterogeneity of variance was tested using Dunnett’s T method. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison between the growth curves of A549/DDP cisplatin-resistant lung adenocarcinoma cells and A549 parental cells. Microscopic observation indicated that the adherent growth of cisplatin-resistant lung adenocarcinoma cells A549/DDP was marked and vigorous, indicating a satisfying light refraction of
cytoplasm. Compared with A549 cells, the A549/DDP cells were slightly larger in volume, growing in fusiform or polygonal structures, with a large cell nucleus. Compared with parental A549 cells, the A549/DDP cisplatin-resistant cells exhibited a higher cell proliferation rate and reached the exponential growth phase 2-4 days following passage, while the A549 cells required 3-5 days to reach the exponential growth phase (Fig. 1).

Sorafenib inhibits the proliferation of A549/DDP cisplatin-resistant lung adenocarcinoma cells. The MTT assay was used to determine the inhibition rate of sorafenib on the proliferation of A549/DDP cisplatin-resistant lung adenocarcinoma cells at different concentrations and time-points. The results showed that, compared with the control group, sorafenib inhibited the proliferation of A549/DDP cisplatin-resistant lung adenocarcinoma cells within the concentration range of 2, 4, 8 and 16 µmol/l. With the extension of the action time and increase of the concentration, the inhibition rate of sorafenib on A549/DDP cisplatin-resistant lung adenocarcinoma cells gradually increased (P<0.05). In addition, the differences in the inhibition rates of sorafenib on A549/DDP cisplatin-resistant lung adenocarcinoma cells at different times and concentrations were statistically significant (P<0.05), as shown in Table I and Figs. 2 and 3.

Sorafenib induces apoptosis of A549/DDP cisplatin-resistant lung adenocarcinoma cells. The rate of apoptosis was determined using Annexin V-FITC/PI staining and flow cytometric analysis. It was observed that the rate of apoptosis of the control group S0 was 8.89±0.81% and the rates of apoptosis in the experimental groups S1-4 were 12.84±0.24, 17.27±0.78, 21.98±0.75 and 49.67±1.38%, respectively. Compared with the control group, the differences were statistically significant (P<0.01); the differences of further pairwise comparison in the same group were also statistically significant (P<0.01), as shown in Fig. 4.

Sorafenib inhibits the invasion of A549/DDP cisplatin-resistant lung adenocarcinoma cells. The Transwell assay was used to determine the number of cells permeating the

Table I. The inhibition rate of sorafenib on A549/DDP cisplatin-resistant lung adenocarcinoma cells at different incubation times (mean ± standard deviation).

<table>
<thead>
<tr>
<th>Group</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>4.58±2.81</td>
<td>14.98±2.93 (^a)</td>
<td>18.80±2.82 (^b)</td>
</tr>
<tr>
<td>S2</td>
<td>14.93±2.62</td>
<td>26.28±7.31 (^a)</td>
<td>32.71±2.55 (^b)</td>
</tr>
<tr>
<td>S3</td>
<td>37.58±7.13</td>
<td>63.00±3.05 (^a)</td>
<td>75.51±4.73 (^c)</td>
</tr>
<tr>
<td>S4</td>
<td>58.39±8.15</td>
<td>78.84±3.96 (^a)</td>
<td>87.50±3.36 (^c)</td>
</tr>
</tbody>
</table>

\(^a\) P<0.01 vs. 24 h; \(^b\) P<0.05 vs. 48 h; \(^c\) P<0.01 vs. 48 h. S1-4, incubation with sorafenib at 2, 4, 8 and 16 µmol/l.
Transwell membrane in each group. The results showed that, following 24 h of incubation, the average number of cells permeating the septum in the normal control group S0 reached 82.7±2.3/high power lens (HP), while the average number of cells permeating the septum in S1-4 in the presence of sorafenib at concentrations of 2, 4, 8 and 16 µmol/l, were decreased to 58.2±2.5, 41.3±1.3, 22.6±2.1 and 14.7±1.1/HP, respectively. Compared with the control group, the difference in the number of cells permeating the Matrigel® of each group was statistically significant (P<0.01). When the concentration increased and the cell permeation of the septum gradually decreased, the differences obtained by pairwise comparisons among the groups also became statistically significant (P<0.01). In addition, observation by optical microscopy following crystal violet staining indicated that the cells in the normal control group which had not been treated with sorafenib were of fusiform, polygonal or oval shaped, with a larger cell volume a dense distribution at the lower surface.
of the Transwell chamber, while the cells of the experimental
groups, which had been treated with sorafenib, gradually
became smaller, pseudopodia decreased and the shape of the
cells became round and sparsely scattered at the lower surface
of the Transwell chamber (Fig. 5).

Discussion

Sorafenib is an antineoplastic agent with multiple biological
targets, which inhibits tumor growth, invasion and metastasis
by preventing the proliferation of tumor cells and angiogenesis
in tumor tissue. In 2005 and 2007, the FDA approved the use
of sorafenib for the clinical treatment of advanced kidney
and liver cancer (6,7). At present, basic research and clinical
trials using sorafenib for the prevention and treatment of lung
cancer are progressing with positive preliminary results (4,5).

Dy et al (8) have tested sorafenib in a Phase II clinical trial as a
first-line treatment of advanced NSCLC and the results showed
that among the 25 patients selected for the study, three went
into partial remission and six achieved a stable condition, with
progression-free survival (PFS) for 2.8 months, and a certain
clinical efficacy was obtained. Spigel et al (9) performed a
clinical trial of combining sorafenib with erlotinib, or using
only erlotinib as the second-line treatment of patients with
advanced NSCLC. A total of 168 patients were involved. The
results showed that the disease control rates of the combina-
tion group and the group with only erlotinib were 54 and
38%, respectively, and PFSs were 3.38 and 1.94, respectively,
indicating that sorafenib has potential as a second-line treat-
ment. In the present study, in order to provide a novel method
for the second-line treatment of NSCLC, the A549/DDP
cisplatin-resistant lung adenocarcinoma cell strain was chosen
to assess the effect of sorafenib on the proliferation, apoptosis
and invasion in vitro.

In the present study, the MTT assay was used to determine
the activity of sorafenib on the proliferation of A549/DDP
cisplatin-resistant lung adenocarcinoma cells and to observe
its inhibition effects. The results showed that compared with
the parental A549 cells, the A549/DDP cisplatin-resistant lung
adenocarcinoma cells were slightly larger in volume, growing
in fusiform or polygonal structures, with a large intracellular
nucleus. The cell proliferation was faster, reaching the exponen-
tial phase within 2-4 days following passage, while A549
cells reached the exponential phase within 3-5 days following
passage, indicating that the metabolic activity of A549/DDP
cisplatin-resistant lung adenocarcinoma cells was significantly
enhanced and marked mitosis was observed following acquisi-
tion of drug-resistance characteristics; thus, they were able
to enter the logarithmic phase faster than the parental A549
cells. This is consistent with the observations on the growth
characteristics of A549/DDP cisplatin-resistant lung adenocar-
cinoma cells reported by Giard et al (10). A further analysis
of the inhibitory effect of sorafenib on the proliferation of
A549/DDP cisplatin-resistant lung adenocarcinoma cells was
conducted, showing that sorafenib is capable of inhibiting the
proliferation of the A549/DDP cisplatin-resistant lung adenocar-
cinoma cells within the concentration range of 2-16 µmol/l.
Furthermore, following treatment of A549/DDP cells with
varying concentrations of sorafenib, the inhibition rate of cells
was gradually increased with increasing time, while at the same
time, the inhibition rate was gradually increased as well with
increasing drug concentration. The results of this trial showed
that sorafenib was capable of inhibiting the proliferation of
A549/DDP cisplatin-resistant lung adenocarcinoma cells and
this inhibitory effect was time- and concentration-dependent.
The Annexin V-FITC/PI assay was used to determine the rate
of apoptosis of A549/DDP cisplatin-resistant lung adenocarcin-
oma cells and to observe the induction effect of sorafenib on
the apoptosis of A549/DDP cells. The results showed that marked
apoptosis appeared in the cells in each experimental group
following treatment with sorafenib at different concentrations
for 72 h. In addition, with the increase of the drug concentra-
tion, the rate of apoptosis also increased. The difference from
the control group was statistically significant (P<0.05), indi-
cating that sorafenib is capable of promoting the apoptosis of
A549/DDP cisplatin-resistant lung adenocarcinoma cells, and
this effect was dependent on the concentration. The difference
obtained by pairwise comparison among experimental groups
was also statistically significant (P<0.05). The results of the
present study were similar to those investigating the effects of
sorafenib on the proliferation and apoptosis of parental A549
cells reported by Yu et al (11) and Mao et al (12). In the present
study, to further determine the influence of sorafenib on the
invasiveness of A549/DDP cisplatin-resistant lung adenocarcin-
oma cells, the Transwell assay was used for each experimental
group in the presence of sorafenib for 24 h. The results showed
that with increasing drug concentration, the number of cells
permeating the basement membrane in each experimental
group significantly decreased. The difference between each
experimental and the control group was statistically significant
(P<0.05), indicating that sorafenib had an in vitro inhibitory
effect on the invasion of A549/DDP cisplatin-resistant lung
adenocarcinoma cells. At the same time, it was noted that the
cells permeating the septum in each experimental group
markedly changed their shape, the occurrence of pseudopodia
decreased and the cell volume decreased.

In conclusion, sorafenib inhibited the proliferation of
A549/DDP cisplatin-resistant lung adenocarcinoma cells in a
time- and concentration-dependent manner. Sorafenib also
induces apoptosis and reduces the invasiveness of A549/DDP
cells, which provided scientific experimental evidence for the
theoretical basis of the application of sorafenib in the subse-
cquent treatment of cisplatin-resistant lung cancer and offers a
novel and effective strategy for further treatment of advanced

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latin and paclitaxel in patients with advanced non-small cell