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Asplatin, a fusion of aspirin and cisplatin, exhibits significant cytotoxicity to tumor cells and almost fully overcomes the drug resistance of cisplatin resistant cells. Asplatin is highly accumulated in cancer cells and is activated upon the reduction by ascorbic acid.

Aspirin, also known as acetylsalicylic acid, is one of the most widely used medicines in the world. It possesses antipyretic, analgesic and anti-inflammatory activities that are largely associated with its inactivation of COX-1 and COX-2, the rate-limiting enzymes responsible for the biosynthesis of prostaglandins. Recently, aspirin has attracted great interest for its antineoplastic effects. It has been proven that aspirin can prevent the initiation and progression of colorectal and other cancers. Further studies showed that aspirin also inhibits the accumulation, decreased intracellular inactivation with anticancer effect of platinum through increased cellular uptake pathways of both platinum and aspirin. Hence, asplatin could improve the anticancer effect of platinum through increased cellular accumulation, decreased intracellular inactivation with alternated cellular response in comparison with cisplatin.

Asplatin was prepared by the reaction of oxoplatin with acetylsalicylic anhydride in dimethylsulfoxide (DMSO). Two folds of acetylsalicylic anhydride was added to the oxoplatin solution in DMSO and the mixture was stirred at room temperature. After 24 hours reaction, the solution was lyophilized and the product was washed with acetone and diethyl ether, and dried in vacuum. The purity was verified with HPLC (> 95%) and the product was characterized using 1H-NMR and ESI-MS (Fig. S1-S3). 1H NMR (D2O, 25°C): δ=7.90 (d, 1 H); 7.67 (t, 2 H), 7.45 (t, 3 H), 7.23 (d, 4 H), 7.07 (d, 2 H), 6.92 (s, 1 H); 2.47 (s, 3 H); 1.27 (s, 3 H); 2.28 (d, 4 H). ESI-MS: m/z=496.86 (cald. 497.01).
resistant A549R cells. Cell viability measurements showed that asplatin demonstrated significant cytotoxicity to all cell lines analyzed in this work (Table 1). The cytotoxicity of asplatin exceeds that of cisplatin with much lower IC<sub>50</sub> (up to 10 fold), which is more effective than that of the most Pt(IV) prodrugs of cisplatin been investigated. In addition, asplatin almost fully overcomes the drug resistance of A549R cells by reducing the drug resistant factor (RF) from 5.38 to 1.11. Control experiments showed that aspirin alone had negligible cytotoxicity up to 200 μM (Fig. S4). This value is in accordance with the literature that aspirin needs millimolar doses to elicit cytotoxicity. Without the aspirin ligand, the Pt(IV) complex oxoplatin showed moderate cytotoxicity with IC<sub>50</sub> 20-80 μM. These results indicate that the ligation of aspirin to oxoplatin exhibits superior synergistic inhibitory effect on the viability of cancer cells. In contrast, the mixture with equimolar aspirin did not improve the cytotoxicity of oxoplatin.

### Table 1. Inhibitory effect (IC<sub>50</sub> in μM) of asplatin on cancer cells.

<table>
<thead>
<tr>
<th></th>
<th>HeLa</th>
<th>MCF-7</th>
<th>HepG2</th>
<th>A549</th>
<th>A549R</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>4.51 ± 1.70</td>
<td>10.10</td>
<td>8.25 ± 1.91</td>
<td>9.65 ± 0.7</td>
<td>51.92</td>
<td>5.38</td>
</tr>
<tr>
<td>Oxoplatin</td>
<td>21.45 ± 3.08</td>
<td>78.64 ± 14.89</td>
<td>36.03 ± 6.34</td>
<td>51.16 ± 4.99</td>
<td>203.8 ± 32.5</td>
<td>3.98</td>
</tr>
<tr>
<td>Oxoplatin + Aspirin</td>
<td>23.01 ± 5.12</td>
<td>78.19 ± 12.02</td>
<td>36.78 ± 6.61</td>
<td>52.00 ± 7.02</td>
<td>169.5 ± 26.0</td>
<td>3.26</td>
</tr>
<tr>
<td>Asplatin</td>
<td>0.45 ± 0.16</td>
<td>3.10 ± 0.3</td>
<td>1.49 ± 0.24</td>
<td>8.53 ± 0.42</td>
<td>9.48 ± 1.31</td>
<td>1.11</td>
</tr>
<tr>
<td>Fold increase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.02 ± 3.26</td>
<td>5.56</td>
<td>1.13</td>
<td>5.48</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> IC<sub>50</sub> ratio of cisplatin/asplatin

The reductions of asplatin by AsA and GSH were investigated using analytical RP-HPLC. Results showed that asplatin could be fully reduced by equimolar ascorbic acid, with high concentration (100 molar ratio) and long reaction time (30 h) (Fig. S5A). On the contrary, GSH led to only minor reduction of asplatin even with high concentration (100 molar ratio) and long reaction time (30 h) (Fig. S5B). This result indicates that the reduction of asplatin by GSH is much less efficient than by AsA, which is consistent with the reduction of oxoplatin. The activation of asplatin has been analyzed by the platination of DNA upon the reduction. (A) The platination of DNA with different ratio of Pt/nucleotide ratio (r<sub>i</sub>). (B) Time dependent platination of DNA with the r<sub>i</sub> ratio of 0.2. All reactions were performed on 0.01 μM platinum complex. Cells were double stained with annexinV-FITC and propidium iodide (PI). Results showed that asplatin induced apoptosis much more effectively than cisplatin and oxoplatin (Table 2). The mixture of equimolar aspirin did not influence the activity of oxoplatin. These data indicate that the high cytotoxicity of asplatin is associated with the enhanced cell apoptosis of asplatin.

### Table 2. Quantification of apoptosis in HeLa cells using an annexinV/PI assay.

<table>
<thead>
<tr>
<th></th>
<th>Early apoptosis, %</th>
<th>Late apoptosis, %</th>
<th>Necrosis, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>3.37</td>
<td>1.17</td>
<td>0.39</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>7.98</td>
<td>4.38</td>
<td>1.34</td>
</tr>
<tr>
<td>Oxoplatin</td>
<td>5.87</td>
<td>1.63</td>
<td>0.63</td>
</tr>
<tr>
<td>Oxoplatin + Aspirin</td>
<td>6.24</td>
<td>1.86</td>
<td>0.26</td>
</tr>
<tr>
<td>Asplatin</td>
<td>25.8</td>
<td>13.6</td>
<td>2.36</td>
</tr>
</tbody>
</table>

<sup>a</sup> All compounds are in 1 μM and the incubation time is 30 h.
The in vivo antitumor activity of asplatin was analyzed on HepG2 tumor xenograft model. The mice were inoculated with HepG2 cancer cells and the tumor grew to a size of 80-150 mm³ after 12 days. The mice were randomly divided into five groups with 8 mice in each group. Cisplatin and asplatin were given 5 times via tail iv injection in 3-day intervals at doses of 0.5 or 2 mg Pt per kg. The tumor volumes were measured in every two days (Fig. 3A). The result showed that both treatments of cisplatin and asplatin led to significant dose-dependent inhibition of the tumor growth in comparison with the control, while asplatin exerted the antitumor effect relatively higher than cisplatin (P < 0.05 at 2 mg Pt/kg). The tumor weight and size at the end of treatments showed the effect of asplatin treatment (Fig. 3B, 3C). This result indicates that the pronounced cytotoxicity of asplatin also leads to the high antitumor effect in vivo.

The toxicity of the platinum drugs was also evaluated with the mean body weight of mice. The result showed that both drugs caused the reduction of body weight at the dose of 2 mg Pt/kg (Fig. 3D). It is worth noting that less body weight reduction was observed in the treatment of asplatin, suggesting less toxicity of this compound. At the dose of 0.5 mg Pt/kg, cisplatin still reduced the body weight relative to the control, whereas asplatin showed nearly no influence at this dose. Combined with the tumor growth inhibition result, these data indicated that asplatin possesses a higher anti-tumor activity with lower systemic toxicity than cisplatin.

Aspirin is recently found to be effective in the cancer prevention and remission. However, high dose of aspirin (~ mM) is required to achieve the effect. Here in this work, we found that the ligation of aspirin to Pt complex demonstrated greatly synergistic effect in low μM range. The mixture of aspirin showed no detectable effect on oxoplatin. This result indicates that the coordination of aspirin is crucial for the synergistic effect.

In summary, asplatin demonstrates significant inhibitory effect on the growth of cancer cells. Asplatin is highly accumulated in tumor cells and binds to DNA efficiently upon reduction by ascorbic acid. The aspirin coordination significantly enhances the anti-tumor activity in low micromolar concentration, indicating great synergistic effect of the ligation of two drugs. This result suggests that aspirin could modulate the cellular response to the platinum drug and sensitizes the tumor cells to cisplatin converted from the prodrug. Interestingly, asplatin nearly completely overcomes the drug resistance of the cisplatin-resistant cell, probably due to the different cellular responses of asplatin relative to cisplatin. In vivo assay demonstrated that asplatin possesses higher antitumor efficacy with lower toxicity in comparison with cisplatin. This result offers a novel strategy to enhance sensitivity and reduce toxicity of platinum drugs by incorporating the anti-inflammation drug aspirin for the potential use in the clinic.

This work was supported by the National Basic Research Program of China (973 Program, 2012CB932502) and the National Science Foundation of China (U1332210, 21171156, 513040482, 51125012).

Notes and references

Electronic Supplementary Information (ESI) available: [Experimental details, characterization of asplatin by HPLC, ESI-MS, NMR and cytotoxicity assay]. See DOI: 10.1039/c000000x/