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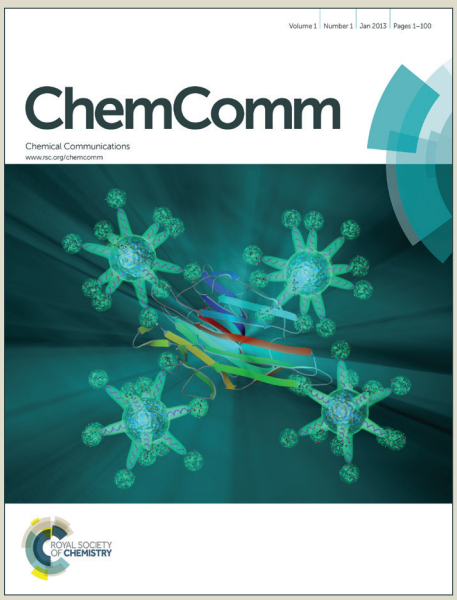

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The Ligation of Aspirin to Cisplatin Demonstrates Significantly Synergistic Effect to Tumor Cells

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Qinqin Cheng,[//a] Hongdong Shi,[//a] Hongxia Wang,[b] Yuanzeng Min,[a] Jun Wang,*[b] and Yangzhong Liu*[a]

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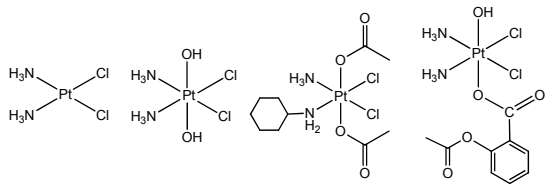
Asplatin, a fusion of aspirin and cisplatin, exhibits significant cytotoxicity to tumor cells and almost fully overcomes the drug resistance of cisplatin resistant cells. Asplatin is highly accumulated in cancer cells and is activated upon the reduction by ascorbic acid.

Aspirin, also known as acetylsalicylic acid, is one of the most widely used medicines in the world.[1] 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.[2] Recently, aspirin has attracted great interest for its antineoplastic effects.[3] It has been proven that aspirin can prevent the initiation and progression of colorectal and other cancers.[4] Further studies showed that aspirin also inhibits the distant metastasis of existing tumors, as well as the incidence of new tumor formation.[5] Although the anti-carcinogenic mechanism of aspirin is not clear, it has been proposed that the aspirin regulates the expression of anti-apoptotic genes, such as COX-2 and BCL-2,[6] and mediates the inhibition of platelet activation.[7] Studies also underscore the combination therapy of aspirin with chemotherapeutic agents, such as cisplatin.[8]

Cisplatin is one of the most widely used antitumor drugs highly effective against a large variety of solid cancers.[9] However, the side-effects and drug resistances limit the application of cisplatin.[10] The drug resistance can arise from different cellular adaptations, including reduced cellular drug uptake, increased drug deactivation, increased DNA repair and/or DNA damage tolerance.[10] Pt(IV) prodrugs offer a promising alternative to overcome the drawback of Pt(II) drugs. Pt(IV) complexes are more inert in coordination chemistry and exhibit less toxic than Pt(II) drugs.[11] Upon entering the cell, the Pt(IV) prodrugs are reduced by intracellular reducing molecules to generate Pt(II) drugs and regain their cytotoxicity.[12, 13] The reductants, such as ascorbic acid (AsA) and glutathione (GSH), are present in much higher concentration in cytoplasm than in extracellular environments. Satraplatin, the first orally administered platinum(IV) complex, demonstrated no cross-resistance with cisplatin in several human cancer cell lines in vitro.[14] Furthermore, the Pt(IV) prodrugs possess two additional axial ligands, which offer an unique approach for the drug design to modify the pharmacokinetic effects of prodrugs, including lipophilicity, redox potential and biological activity.[13]

In this work, we designed an aspirin ligated Pt(IV) complex by tethering aspirin to oxoplatin to generate a satraplatin-like prodrug, asplatin (Scheme 1). We hypothesized that the aspirin ligand could modulate the cellular response to the platinum drug; thus, the metabolite Pt(II) drug could more effectively induce apoptosis and kill cancer cells. In addition, the ligation of aspirin to cisplatin also alters the 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.



Scheme 1. The structure of cisplatin, satraplatin, oxoplatin and asplatin.

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 diethylether, and dried in vacuum. The purity was verified with HPLC (> 95%) and the product was characterized using ^{1}H-NMR and ESI-MS (Fig. S1-S3). ^{1}H NMR (D_2O, 25°C): δ=7.90 (d, 1 H); 7.67 (ter, 2 H), 7.45 (ter, 3 H), 7.23 (d, 4 H), δ 2.47 (s, 3H CH_3). ESI-MS: m/z=496.86 (cald. 497.01).

The *in vitro* antitumor activities of asplatin was evaluated by MTT assay on human cancer cells, including cervical cancer (HeLa), breast carcinoma (MCF-7), hepatocellular carcinoma (HepG2), lung carcinoma (A549) cells and the cisplatin-

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_{50} (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.[15] Without the aspirin ligand, the Pt(IV) complex oxoplatin showed moderate cytotoxicity with IC_{50} 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_{50} in μM) of asplatin on cancer cells.

	HeLa	MCF-7	HepG2	A549	A549R	RF
Cisplatin	4.51 ± 1.70	10.10 ± 1.91	8.25 ± 0.7	9.65 ± 0.66	51.92 ± 6.65	5.38
Oxoplatin	21.45 ± 3.08	78.64± 14.89	36.03 ± 6.34	51.16 ± 4.99	203.8 ± 32.5	3.98
Oxoplatin + Aspirin	23.01 ± 5.12	78.19± 12.02	36.78 ± 6.61	52.00 ± 7.02	169.5 ± 26.0	3.26
Asplatin	0.45 ± 0.16	3.10 ± 0.3	1.49 ± 0.24	8.53 ± 0.42	9.48 ± 1.31	1.11
Fold increase[#]	10.02	3.26	5.56	1.13	5.48	

IC_{50} 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 the release of aspirin within two hours (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.[16]

The activation of asplatin has been analyzed by the platination of DNA, which was detected with fluorescent measurements using EtBr as a probe. The binding of platinum to DNA prevents the intercalation of EtBr, resulting in the stoichiometric loss of fluorescence from the EtBr/DNA complex.[17] The control experiment with cisplatin showed that the platination of DNA led to the fluorescence intensity drop (Fig. 1A). By comparison, asplatin is able to bind to DNA only in the presence of AsA; asplatin alone or with GSH had little effect on DNA even at high concentrations. In addition, HPLC results confirm that the asplatin is activated by AsA in the reaction of oligomeric DNA and the platination adducts are similar to that of cisplatin (Fig. S6). Taken together, these results indicate that asplatin functions as the prodrug of cisplatin upon the activation with the reductant AsA.

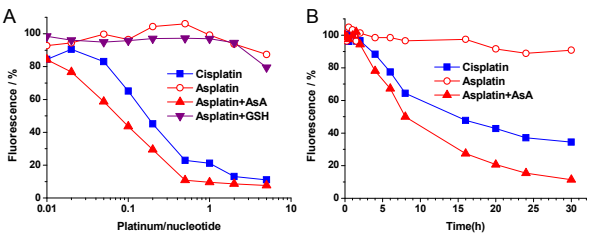


Fig. 1. Reactions of asplatin with DNA upon the reduction. (A) The platination of DNA with different ratio of Pt/nucleotide ratio (r_i). (B) Time dependent platination of DNA with the r_i ratio of 0.2. All reactions were performed on 0.01 mg/ml DNA in 10 mM $NaClO_4$ and 10 mM phosphate buffer (pH=7.4) at 37 °C for 36 h. EtBr (0.04 mg/mL) was added before the fluorescence measurement. The excitation wavelength was 530 nm and the emission was recorded at 592 nm.

To explore the mechanism of enhanced cytotoxicity of asplatin, the cellular uptake of asplatin has been measured. After 3 h incubation with different platinum complexes, the platinum content in HeLa cells was analyzed using ICP-MS. Results showed that the accumulation of asplatin in cells was higher than that of oxoplatin (by 8.4 times) and cisplatin (by 3.2 times) (Fig. 2A). This result indicates that the ligation of aspirin significantly enhanced the drug uptake. In addition, the Pt amount in DNA was also measured after the isolation of DNA from the cells (Fig. 2B). The result showed that much higher level of DNA platination was generated by asplatin than that by cisplatin (2.2 times) and oxoplatin (8.3 times), which is consistent with the Pt content in the cells. These data indicate that the higher cytotoxicity of asplatin could be correlated with its higher DNA targeting efficiency. On the other hand, the aspirin released from the platinum complex could also contribute to the activity of asplatin as more cytotoxicity enhancement (47.7 times) was observed than the DNA platination increasement (8.3 times) in comparison with oxoplatin. Hence, different cellular responses could exist on these Pt complexes.

The apoptosis induced by platinum complexes was quantified using a flow cytometric assay. After the 30 hours treatment of platinum complexes, 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.

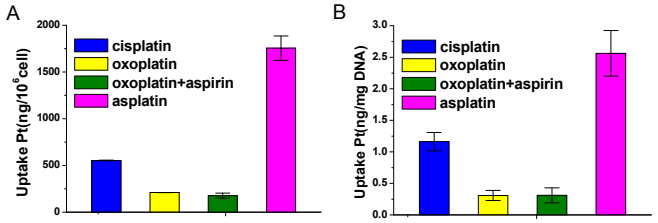


Fig. 2. Cellular Drug Uptake and DNA platination. Platinum in HeLa cells was determined using ICP-MS after the 3 h treatment of 100 μM platinum complex. (A) Platinum in whole cells. (B) Platinum in the DNA.

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

	Early apoptosis, %	Late apoptosis, %	Necrosis, %	Sum, %
Aspirin[#]	3.37	1.17	0.39	4.93
Cisplatin	7.98	4.38	1.34	13.7
Oxoplatin	5.87	1.63	0.63	8.03
Oxoplatin + Aspirin	6.24	1.86	0.26	8.36
Asplatin	27.8	13.6	2.36	43.76

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 mice model. The mice were inoculated with HepG2 cancer cells and the tumor grew to a size of 80-150 mm^3 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 clearly 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 great 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.

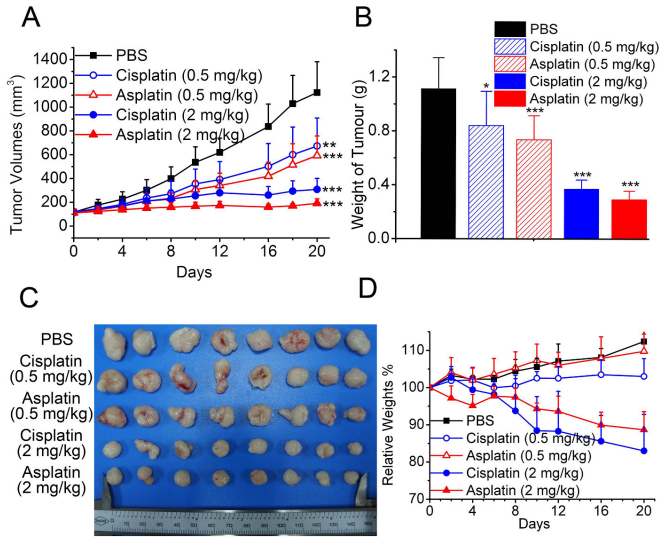


Fig. 3. In vivo antitumor activity of asplatin and cisplatin in HepG2 xenograft tumors. NOD/SCID mice bearing A549 tumors were treated with cisplatin or asplatin (n = 8 at dose 0.5 or 2 mg Pt/kg, q3d × 5). PBS was used as control. (A) Tumor growth as a function of time. (B) The tumor weight in each group at the end of the experiment. (C) The image of tumors at the end of the experiment. (D) The body weight of mice during the treatments. Error bars denote standard deviations and asterisks indicate P-values (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

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 the 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.

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Notes and references

[a] CAS Key Laboratory of Soft Matter Chemistry, CAS High Magnetic Field Laboratory, Department of Chemistry, University of Science and Technology of China, Hefei, Anhui, 230026, China, liuyz@ustc.edu.cn
[b] School of Life Sciences, University of Science and Technology of China, Hefei, Anhui, 230027, China. jwang699@ustc.edu.cn
// These authors contributed equally.

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