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Graphical Abstract

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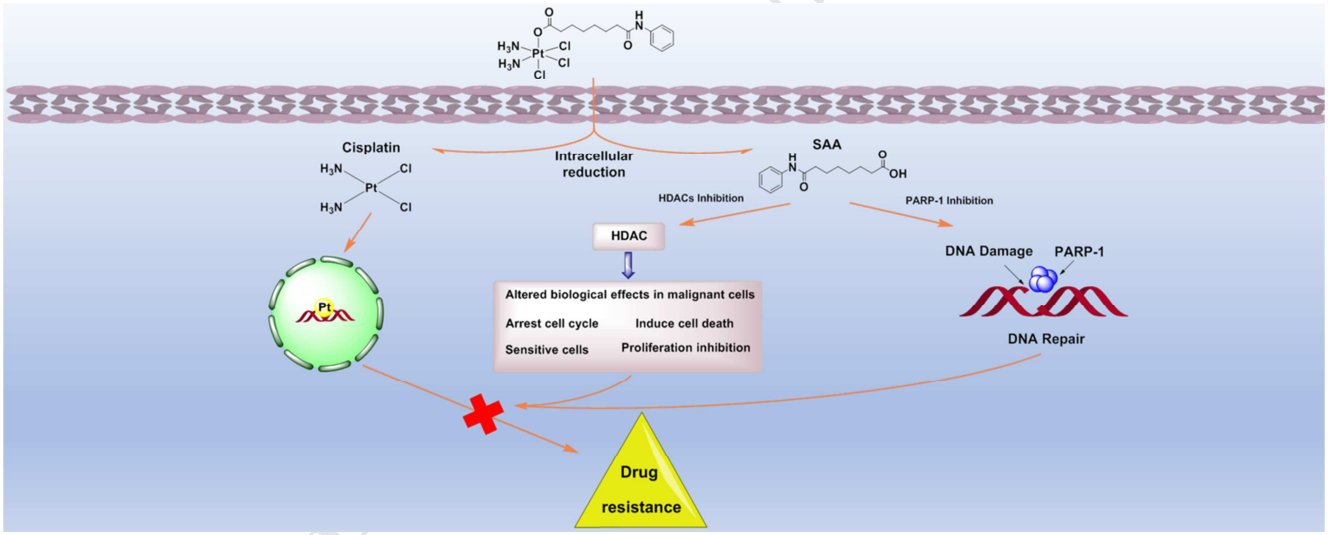

Platinum(IV) prodrugs multiply targeting genomic DNA, histone deacetylases and PARP-1

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ABSTRACT: Several Pt(IV) prodrugs containing SAA, a histone deacetylases inhibitor, were designed and prepared for multiply targeting genomic DNA, histone deacetylases and PARP-1. The resulting Pt(IV) prodrug had significantly strong antiproliferative activity against the tested cancer cell lines, especially SAA1, derived from the conjugation of cisplatin and SAA, had potent ability to overcome cisplatin resistance. Under the combined action of DNA platination and inhibition of HDACs and PARP-1 activity, the cytotoxic activity of SAA1 was 174-fold higher than cisplatin against cisplatin-resistant SGC7901/CDDP cancer cells. The mechanism of action of SAA1 was preliminarily investigated, in which cellular uptake, cell apoptosis and cell cycle arrest as well as western blot analysis were made by treating SAA1 with SGC7901/CDDP cells. Besides, HDACs inhibition activity and PARP-1 enzyme inhibition of SAA1 were also studied.

Keywords: multi-target, Pt(IV) prodrug, HDAC inhibitor, PARP-1 inhibitor, overcoming drug-resistance.

1. Introduction

Histone deacetylases (HDACs), a class of zinc metalloenzymes that deacetylate core histone lysine residues, have attracted increased attention for the critical role in the regulation of gene expression owing to governing the acetylation state of lysine residues [1, 2]. The finding that overexpression of HDACs transforms the function and expression of tumor-related proteins, chiefly involved in cell proliferation, migration, metastasis and angiogenesis, has promoted histone deacetylase inhibitors (HDACIs) as a new class of anticancer agents [3-5].

Generally HDAC inhibitors consist of three domains: a chelator refers to the zinc binding group (ZBG); a cap component fits into the active site pocket, and a linker domain connects the ZBG and the cap group [6]. So far, a series of diverse HDACIs have been found to be effective antitumor agents via multiple mechanisms, such as autophagic cell death, cell-cycle arrest, mitotic cell death and so on [7, 8]. Moreover, HDACIs have been found to have few adverse effects on normal cells. In the nucleus, DNA is noncovalently related to histone to form the nucleosomes which constitute chromatin subunits. HDACIs, inducing hyperacetylation of histone proteins, could enhance the accessibility of DNA within chromatin [9, 10] and consequently reinforce the antitumor activities of platinum and other DNA-damaging drugs [11,

12]. These facts indicate that cancer cell death caused by simultaneous HDAC inhibition and DNA damage has emerged as a highly promising alternative approach in cancer therapy.

SAA, a derivative of the drug SAHA, has been reported to exert HDAC inhibitory activity [13] by carboxylic acid groups partially chelating with zinc ions and the aromatic groups occupying the tubular pocket. In addition, it is also found to possess PARP inhibitory activity with benzamide, a major pharmacophoric group in many PARP inhibitors [14], to form multiple hydrogen bonds with two critical amino acid residues in the PARP active site, Gly-863 and Ser-904 [15-17]. PARP-1 is regarded as a valuable target in the prospecting cancer treatment due to its key role in the repair of DNA strand breaks [18, 19]. As a DNA repair enzyme [20], PARP has been observed overexpressed in a number of hematological and solid tumors as compared to normal cells [14]. Besides, it has the catalytic function to transfer ADP-ribose units from nicotinamide adenine dinucleotide (NAD+) to a variety of receptor proteins [21, 22], and is also responsible for the formation of ADP-ribose polymers, which is crucial in the repair of damaged DNA caused by chemotherapeutic agents [23]. Thus, PARP-1 contributes to the diminished cancer therapies effectiveness and the resistance that often develops after cancer therapy [24-26]. Moreover, PARP-1 is playing an important role in maintaining the integrity of the structure of chromosomes [27] and participating in DNA replication, transcription [28], and cellular death processes [29]. Therefore, inhibition of PARP-1 activity can enhance the effect of chemotherapy drugs [30] and overcome drug resistance to improve the result of the treatment of tumor [31-38].

Cisplatin (CDDP) has been widely used in cancer therapy as a classic cytotoxic drug for its brilliant antitumor effects on numerous cancers. However, drug resistance is one of the drawbacks to limit its chemotherapeutic efficacy in tumors, which is partly due to DNA damage repair in addition to the other effects [39-41]. Recently, a strategy of applying Pt(IV) complexes with two axial bioactive groups has been adopted to promote the efficacy of cisplatin and overcome its resistance [11, 12, 42-45]. According to the literature reports, divalent platinum chemotherapy drugs have shortcomings such as serious side effects, low bioavailability and so on.[46, 47] Compared to Pt(II), Pt(IV) prodrugs are more kinetically inert, resulting in less toxicity. In addition, Pt(IV) prodrugs are easy to be reduced to liberate axially connected bimolecules and divalent platinum species, which may play a joint action to overcome drug resistance and improve the pharmacological activity of platinum-based anticancer drugs. In our

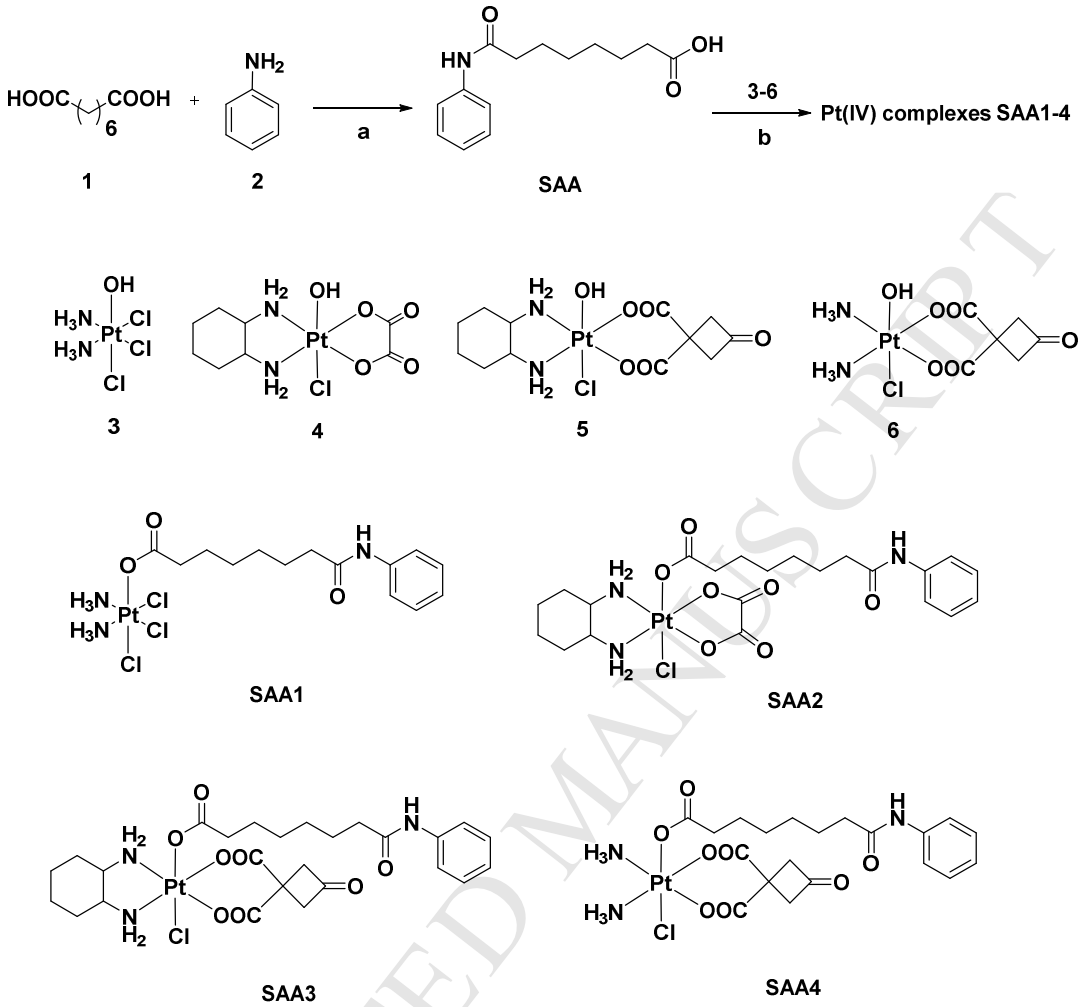
recent research on prodrugs derived from the conjugation of Pt(IV) complexes with different biological units, Pt(IV) complexes with only one bioactive ligand in an axial position and a chlorine atom or hydroxyl group in the opposite axial position were involved, because they were found more likely to be reduced than those with two bioactive species in two axial positions and subsequently to release the pharmacophore [48-52].

Based on the above, SAA was chosen as a bioactive ligand (HDACs and PARP-1 dual inhibitor) to conjugate with four Pt(IV) complexes derived from cisplatin, oxaliplatin, DN603 and DN604 [53], respectively (Fig. 1). The resulting compounds were expected to simultaneously target genomic DNA, HDACs and PARP-1, hypothesizing that the incorporation of SAA into platinum drug might produce new multifunctional Pt(IV) prodrugs by maintaining the effective Pt(II) species to bind nuclear DNA and getting ability to overcome drug resistance (Fig. 2).

2. Results and discussion

2.1. Chemistry.

The synthetic procedure is listed in Scheme 1. SAA was obtained according to a literature procedure [54]. Meanwhile, Pt(IV) precursors 3-6 were synthesized through the oxidative chlorination of the corresponding Pt(II) complexes (cisplatin, oxaliplatin, DN603 and DN604) with N-chlorosuccinimide (NCS) in water [52, 55]. Finally, four Pt(IV) prodrugs, SAA1-4, were obtained via SAA coupling with the corresponding Pt(IV) intermediate in the presence of O-(benzotriazol-1-yl)-N,N,N’,N’- tetramethyluronium tetrafluoroborate (TBTU) as the coupling agent [48]. The products were purified by column chromatography, whose structures were confirmed by ^{1}H, ^{13}C, ^{195}Pt NMR and IR spectra together with ESI-MS spectroscopy.



Scheme 1. Preparation of Pt(IV) prodrugs SAA1-4. Reagents and conditions: (a) 180 °C; (b) TBTU, Et_3N, DMF, room temperature.

2.2. HPLC analysis on the stability and reduction of complex SAA1.

The stability of SAA1 in a solution of PBS/DMF was examined by HPLC technique at different time. As shown in Fig. S1 (see Supporting Information), SAA1 was stable in a period of 48 h under the physiological condition. In order to verify whether SAA1 could be reduced to its Pt(II) equivalent and release SAA in accordance with our design, SAA1 was studied by HPLC in a solution of acetonitrile/water (25:75, v:v) in the presence of ascorbic acid. As shown in Fig. S2 (see Supporting Information), SAA1 was gradually reduced to release SAA as time passed, accompanied by the decreasing peak of SAA1 and the rising peak of SAA. It was noted that

cisplatin was not observed owing to its weak chromophore under the ultraviolet detecting condition.

2.3. In vitro antiproliferative activity.

SAA1-4 and their parent divalent Pt(II) complexes were screened against four different human cancer cell lines including breast (MCF-7 and MDA-MB-231), colon (HCT-116) and gastric carcinoma (SGC7901) along with normal liver cells LO2 using MTT assay. Parent Pt(II) complexes as well as SAHA and SAA were used as positive controls. The corresponding cytotoxic data are given in Table 1.

It was noted that SAHA presented potent antitumor activities comparable to cisplatin and oxaliplatin, while SAA displayed negligible cytotoxicity against all the tested tumor cell lines, with IC_{50} values greater than 200 μM. In addition to HCT-116, cytotoxicity of cisplatin is generally stronger than that of oxaliplatin, and the two clinical drugs were more effective than DN603 and DN604. It is pleasing to find that by introducing SAA, complexes SAA1-4, the Pt(IV) derivatives of cisplatin, oxaliplatin, DN603 and DN604, exhibited significant ameliorative antitumor activity, especially against breast (MCF-7) and gastric (SGC7901) cancer cell lines, in which SAA1 and SAA2, as the trend with their parent compounds, had particularly better cytotoxic activity. Compared to oxaliplatin, SAA2 has a slight increased antitumor activity for MCF-7, but for SGC7901 cancer cells, it was 1.5 times more effective than oxaliplatin. Regrettably, SAA2 showed higher toxicity to normal cells LO2 compare to oxaliplatin at the same time. Encouragingly, SAA1 exhibited 13.4 times more effective than cisplatin against gastric carcinoma (SGC7901) cell lines, with IC_{50} value of 0.95 μM in comparison to 12.69 μM of cisplatin, but showed lower cytotoxicity against normal liver cells LO2 compared with the positive drugs cisplatin and SAHA, prompting it to be a good drug candidate.

2.4. Antitumor activity against drug-resistant cancer cell lines.

Cisplatin has been used as a first-line chemotherapy agent for human gastric cancer, while drug resistance is a key factor that restricts the efficacy of cisplatin in treatment. Upon the cytotoxic data in Table 1, we further tested the typical complex, SAA1, against cisplatin-resistant SGC7901/CDDP cells. The corresponding data for cisplatin were also determined for

comparative purposes. The cytotoxic effect of cisplatin was significantly influenced in cisplatin-sensitive SGC7901. As shown in Table 2, the IC_{50} value of cisplatin was increased to 67.88 μM with the resistance factor (RF) value of 5.35. In contrast, the cytotoxicity of SAA1 was markedly promoted against SGC7901/CDDP while compared with that of SGC7901. SAA1 was nearly 174 times as effective as cisplatin against cisplatin-resistant gastric (SGC7901/CDDP) cell lines. The RF value of SAA1 was surprisingly 0.41, which was 13-fold superior to cisplatin, demonstrating its strong sensitivity to cisplatin resistance cancer cells. It was noted that SAA1 showed much potent cytotoxicity toward both cisplatin sensitive and resistant cancer cell lines. As far as we know, only a limited number of Pt(IV) complexes targeting both genomic DNA and histone deacetylases have been reported to achieve favorable activity in the past a few years [42, 56], in which resistance factors against cisplatin-resistant cancer cell lines were approximately 1. As postulated, the mechanism of action of SAA1 was not only involved in the process associated with platination of DNA like cisplatin, but also involved in other processes not affected by the mechanisms of resistance developed by cells towards DNA damaging cisplatin. In addition, we conducted two critical control tests. The results indicated that, obviously, mechanically equimolar mixed CDDP and SAA (1:1) could not overcome the resistance of cisplatin. The Pt(IV) drug molecule used in SAA1 was not as potent as SAA1 against SGC7901 cells and showed a strong drug resistance in SGC7901/CDDP cells. Based on the above two control tests, the rationality of using tetravalent platinum strategy is also proved

2.5.Cellular uptake.

Since SAA1 showed better cytotoxic activity, it was selected for cell uptake assays in gastric cancer cells using inductively coupled plasma mass spectrometry (ICP-MS). As displayed in Fig. 3 and Table S1, SGC7901/CDDP cells were treated with the complex (12.0 μM) for 12 hours, resulting in the higher uptake of SAA1 than that of cisplatin and ctc-$[Pt(NH_3)_2(OH)Cl_3]$. In particular, the intracellular platinum concentration of SAA1-treated cells reached 269 ng/10^6 cells, which was 2.36-fold and 1.93-fold higher than that of cisplatin (114 ng/10^6 cells) and ctc-$[Pt(NH_3)_2(OH)Cl_3]$ (139 ng/10^6 cells), respectively. In addition, we had run the blank test with cells washed immediately after treatment with platinum complexes without incubation. The level of Pt in cells was almost negligible compared with that of incubation group. According to the

results of cell uptake tests and cytotoxic assay, they appeared to have a positive correlation, implying enhanced cellular uptake can lead to increased cytotoxicity.

2.6.HDACs inhibition activity.

HDACs have received increasing attention in recent years because of their close association with cancer. HDACIs as a class of antineoplastic drugs can alter gene transcription and anticancer, such as cell growth inhibition, differentiation, apoptosis and tumor angiogenesis. And several studies suggested that HDACIs have a synergistic effect on DNA damage agent cisplatin. Thus, our complexes were designed to release SAA inside the cells so as to exert HDACs inhibition. In the test, Hela cell nuclear extract, a rich source of HDACs, was used to efficiently evaluate the most active Pt(IV) prodrug SAA1. As expected, cisplatin lacked the activity to inhibit HDACs with IC_{50} value exceeding 100 μM. As shown in Fig.4 and Table S2, SAA1 had potent HDACs inhibitory activity with IC_{50} value of 26.83 μM in the nuclear extract, but showed weaker HDACs inhibitory ability than the positive drug SAHA (IC_{50}= 0.13 μM) and SAA (IC_{50}= 16.77 μM). This observation is basically in agreement with the well-known HDACs inhibitory effect of SAA [13], indicating that the addition of SAA to the axial position of a Pt(IV) atom is a useful way to introduce HDAC activity into the Pt(IV) prodrug.

2.7.PARP-1 enzyme inhibition.

According to the previous reports [42, 56], the axial ligands concerned only had a single HDAC inhibitory effect. While SAA, as an axial ligand in our complexes, was reported to not only have a HDAC inhibitory activity, but also have a PARP-1 inhibition effect. It has been indicated that PARP-1 plays an important role in DNA single strand breaks repair (SSB) by implementing basic excision repair (BER) pathway. And DNA damage caused by agents like cisplatin is usually repaired by BER pathway. Thus inhibition of PARP-1 may interfere with BER pathway thereby enhancing the cytotoxicity of these agents and sensitizing cancer cells to them. Based on the above, SAA1 as well as SAA and cisplatin was evaluated in vitro for their PARP-1 enzyme inhibition activity (Fig.5 and Table S3). As expected, SAA1 indicated activity with IC_{50} value of 38.26 μM against PARP-1 comparable to SAA, revealing that addition of SAA into a Pt(IV) moiety resulted in a significant increase in enzyme potency, while cisplatin had hardly inhibitory activity with IC_{50} beyond 100 μM. Consistent with the HDAC result above,

SAA1 had a strong PARP-1 inhibition activity, which may explain the better cytotoxic activity of SAA1 against gastric cancer cells and the ability to overcome cisplatin resistance.

2.8.Effect on cell cycle arrest.

In order to investigate the effect of our Pt (IV) complexes on cell cycle arrest, the cycle distribution of SGC7901/CDDP cells treated with different concentrations of SAA1 after 24 hours was analyzed by flow cytometry with untreated cells as negative control and cisplatin-treated cells as positive control. As shown in Fig.6, SAA1 was as effective as cisplatin in blocking the cell cycle in G2/M phase. For the untreated cells, the percentage of cells in G0/G1 phase was 67.47% and in G2/M phase was only 6.66%. It was noted that SAA did not have much effect on the cell cycle, the percentage of cells in G0/G1 and G2/M phase was comparable to that of the untreated group. (Fig. S25) After treatment with 2 μM of SAA1, the percentage of cells in G2/M phase increased to 18.74%, while the percentage in S phase did not change significantly. At 4 μM of SAA1, 42.61% of cells were arrested in G2/M phase, which was higher than the percentage of cells treated with high-dose of cisplatin (24.90%). These results showed that SAA1 evidently arrested the G2/M phase of the cell cycle in a dose-dependent manner.

2.9.Induced apoptotic cell death and western blot analysis.

To confirm whether the inhibitory effect of SAA1 on cancer cell proliferation was accompanied by enhanced apoptosis, we performed a FITC-Annexin V/propidium iodide (PI) staining and flow cytometry assay. The compounds tested were co-cultured with SGC7901/CDDP cells at increasing concentrations for 24 hours with cisplatin as a positive control. As illustrated in Fig.7 and Fig. S26, only a few apoptotic cells were present in the control group (6.68%) and SAA essentially had little effect on apoptosis. Gratifyingly, a dose-dependent increase in the percentage of apoptotic cells was observed after being treated with SAA1. The population rose to 15.44% after treatment with 2 μM of SAA1 for 24 h and further increased to 37.42% after treatment with 4 μM of SAA1, which was significantly higher than that after treatment with 4 μM of cisplatin (15.79% apoptotic cells at the same concentration). Overall, the results above evidently verified that complex SAA1 effectively induced apoptosis in SGC7901/CDDP cells.

In addition, the promotion of apoptosis induced by platinum complexes was closely related to induction of tumor suppressor p53 [57-59]. Since p53 plays such a pro-apoptotic role in apoptosis that we studied its pathway involved in the mechanism of action of SAA1 in SGC7901/CDDP cells. As depicted in Fig.8, treatment of SGC7901/CDDP cells with SAA1 resulted in an increase in p53 activation, which was consistent with cisplatin. These results indicated that activation of the p53 pathway was involved in SAA1-induced cancer cell death.

3. Conclusion

In summary, we successfully developed several multifunctional Pt(IV) prodrugs containing a HDACs and PARP-1 dual inhibitor for the first time. Notably, SAA1 is the most prominent one, whose cytotoxicity against MDA-MB-231 breast cancer and SGC7901 gastric cancer cells is superior to its parent complex, cisplatin. Noteworthy, the markedly improved ability of SAA1 to overcome cisplatin resistance was found in cisplatin-resistant SGC7901 cells. The resistance factor was 0.41 for SAA1, much lower than that for cisplatin. This suggests that the mechanism underlying the biological action of SAA1 is different from that of cisplatin, allowing SAA1 to successfully overcome the resistance mechanisms operating in the case of cisplatin. The biological results also indicates that the mechanism also comprises inhibitory effect towards HDACs and PARP-1, leading to increasing the accessibility of DNA in chromatin and DNA damage caused by the platinum moiety and hereafter hindering the DNA repair process to sensitize cancer cells. In addition, through the combined action, the cell cycle of cancer cells was successfully blocked in the G2/M phase and the apoptosis was elevated in cisplatin-resistant cancer cells compared to the same dose of cisplatin. Consequently, our study provided a new and promising platform for constructing multi-target platinum drugs as well as a broad implication for multi-target chemotherapy in cancer treatment.

4. Experimental Section

4.1. Materials and measurements.

All chemicals and solvents were obtained from commercial sources and used directly without further purification, except where noted. Compounds 3, 4, 5 and 6 were prepared according to literature reports [55]. The purity of all compounds used in the biological studies was ≥95%. β-

acitn and p53 antibodies were purchased from Imgenex, USA. All tumor cell lines were purchased from Nanjing KeyGEN BioTech company (China). ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 on a Bruker 300 MHz spectrometer. ^{195}Pt NMR spectra were measured in DMSO-d_6 with a Bruker 400 MHz spectrometer using a solution of 10 mM potassium hexachloroplatinate in 95% H_2O/5% D_2O as an external standard. IR spectra of the complexes were detected by Thermo fisher Nicolet iS10 spectrometer. Decomposition points were measured by SGW X-4 Microscopic melting point meter (Shanghai Precision Scientific Instrument Co. Ltd). Platinum contents were determined by inductively coupled plasma-mass spectrometer (ICP-MS, Optima 5300DV, PerkinElmer, USA). High solution mass spectra were measured by an Agilent 6224 ESI/TOF MS instrument. The stability and released ability of complex was measured by Waters 1525 binary HPLC instrument.

4.2. Chemistry

4.2.1. Synthesis of compound SAA.

A mixture of suberic acid (5.73 g, 55.0mmol) and aniline (5.12 g, 55.0 mmol) was stirred at 180 °C for 1 h. After cooling, the mixture was diluted with AcOEt–THF and filtered. The filtrate was washed with saturated aqueous $NaHCO_3$, and the aqueous layer was acidified with concentrated HCl. The precipitated crystals were collected by filtration to give SAA as a white solid.

4.2.2. Synthesis of compounds SAA1-4.

To a solution of SAA (0.30 g, 1.2 mmol), TBTU (0.39 g, 1.2 mmol), and Et_3N (0.12 g, 1.2 mmol) in dry DMF (15 mL), and complex 4, 5, 6 or 7 was added, respectively. The mixture was stirred at room temperature overnight. After completion of reaction, the whole mixture was added to CH_2Cl_2 (120 mL), and then extracted twice with water (100 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified on silica gel column eluted DCM/MeOH (50:1) to give the desired product as a yellow solid.

4.2.3. Compound SAA.

Yield, 66.7%; ^{1}H NMR (300 MHz, methanol-d_4) δ 7.53 (d, J = 8.2 Hz, 2H), 7.29 (t, J = 7.8 Hz, 2H), 7.07 (t, J = 7.4 Hz, 1H), 2.33 (dt, J = 21.8, 7.4 Hz, 4H), 1.67 (dt, J = 23.8, 7.1 Hz, 4H), 1.49 – 1.30 (m, 4H). ESI-MS (m/z): calcd for $C_{14}H_{19}NO_3$ $[M-H]^-$: 249.13649, found: 248.12759.

4.2.4. Compound SAA1.

Yield, 56.9%; IR (KBr, cm^{-1}): 3261, 3173, 2933, 2854, 1659, 1597, 1498, 1464, 831, 793, 722, 690. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.81 (s, 1H), 7.58 (d, J = 7.8 Hz, 2H), 7.28 (t, J = 7.6 Hz, 2H), 7.02 (d, J = 8.1 Hz, 1H), 6.18 (s, 6H), 2.35 – 2.18 (m, 4H), 1.53 (d, J = 28.7 Hz, 4H), 1.31 (s, 4H). ^{13}C NMR (75 MHz, DMSO) δ 181.20, 171.73, 139.83, 129.08, 123.36, 119.52, 36.91, 36.78, 29.02, 28.89, 25.84, 25.53. ^{195}Pt NMR (129 MHz, DMSO-d_6) δ 552.41 ppm. ESI-MS (m/z): calcd for $C_{14}H_{24}Cl_3N_3O_3Pt$ $[M-H]^-$: 583.79800, found: 582.05684.

4.2.5. Compound SAA2.

Yield, 62.5%; IR (KBr, cm^{-1}): 3182, 2936, 2857, 1705, 1666, 1625, 1597, 1532, 1498, 1440, 753, 693. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.84 (s, 1H), 7.58 (d, *J* = 7.7 Hz, 2H), 7.33 – 7.23 (m, 2H), 7.01 (t, *J* = 7.4 Hz, 1H), 2.54 (s, 2H), 2.29 (t, *J* = 7.5 Hz, 2H), 2.20 (t, *J* = 7.4 Hz, 2H), 2.01 (dd, *J* = 35.2, 12.3 Hz, 2H), 1.61 – 1.39 (m, 8H), 1.30 (p, *J* = 3.7 Hz, 4H), 1.07 (d, *J* = 8.5 Hz, 2H).^{13}C NMR (75 MHz, DMSO) δ 174.94, 171.67, 164.37, 164.35, 139.79, 129.09, 123.37, 119.48, 61.94, 60.97, 36.82, 34.09, 31.18, 30.72, 28.86, 28.79, 25.45, 24.85, 24.23, 24.10. ^{195}Pt NMR (129 MHz, DMSO-d_6) δ 1221.83 ppm. ESI-MS (m/z): calcd for $C_{22}H_{32}ClN_3O_7Pt$ $[M-H]^-$: 680.15765, found: 680.06525.

4.2.6. Compound SAA3.

Yield, 65.9%; IR (KBr, cm^{-1}): 3136, 3055, 2933, 2857, 1793, 1659, 1598, 1535, 1498, 1441, 857, 756, 693. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.79 (s, 1H), 7.57 (d, *J* = 7.9 Hz, 2H), 7.26 (t, *J* = 7.8 Hz, 2H), 7.00 (t, *J* = 7.3 Hz, 1H), 3.53 (s, 2H), 3.39 (s, 2H), 2.63 (dd, *J* = 22.2, 11.0 Hz, 2H), 2.27 (t, *J* = 7.2 Hz, 4H), 2.16 – 1.93 (m, 2H), 1.62 – 1.01 (m, 14H). ^{13}C NMR (75 MHz, DMSO) δ 203.34, 180.35, 176.01, 175.93, 171.17, 139.31, 128.57, 122.86, 119.02, 45.74, 37.68, 36.56, 36.35, 35.21, 35.12, 30.88, 30.48, 28.44, 28.36, 25.17, 24.98, 23.56, 23.42. ^{195}Pt NMR (129 MHz, DMSO-d_6) δ 1195.47 ppm. ESI-MS (m/z): calcd for $C_{26}H_{36}ClN_3O_8Pt$ $[M-H]^-$: 749.12100, found: 748.17727.

4.2.7. Compound SAA4.

Yield, 61.7%; IR (KBr, cm^{-1}): 3198, 2934, 2854, 1791, 1624, 1597, 1541, 1498, 1442, 857, 755, 693. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.82 (s, 1H), 7.58 (d, *J* = 7.5 Hz, 2H), 7.27 (t, *J* = 7.3 Hz, 2H), 7.02 (d, *J* = 6.8 Hz, 1H), 6.30 (s, 6H), 3.50 (s, 4H), 2.27 (d, *J* = 7.4 Hz, 4H), 1.52 (d, *J* = 28.7 Hz, 4H), 1.26 (d, *J* = 13.6 Hz, 4H). ^{13}C NMR (75 MHz, DMSO) δ 203.48, 179.21, 175.86, 175.65, 171.20, 139.32, 128.57, 122.85, 119.02, 45.48, 37.57, 36.36, 35.51, 28.49, 28.38, 25.16, 24.98. ^{195}Pt NMR (129 MHz, DMSO-d_6) δ 1337.74 ppm. ESI-MS (m/z): calcd for $C_{20}H_{28}ClN_3O_8Pt$ $[M-H]^-$: 668.99100, found: 668.11416.

4.3. The stability of complex SAA1 in PBS/DMF buffer.

The stability of Pt(IV) complexes in a PBS/DMF (99:1, v:v) was investigated by HPLC. The incubation was generated by adding SAA1 to PBS/DMF buffer, which was performed at 25 °C for 0, 6, 12, 24 and 48 h, separately. Reversed phase HPLC was implemented on a 250 × 4.5 mm ODS column and the HPLC profiles were recorded on UV detection at 210 nm. Mobile phase consisted of acetonitrile/water (25:75, v/v), and flow rate was 1.0 mL/min. The samples were taken for HPLC analysis after filtration by 0.45 μm filter.

4.4. Released ability of complex SAA1 under reduction with ascorbic Acid.

The released ability of SAA1 in a solvent comprising acetonitrile/water (25:75, v:v) was investigated by HPLC. The standard compounds were made by adding ascorbic acid, SAA, and SAA1, respectively, to a solvent containing 25% acetonitrile and 75% water. The incubation was generated by adding test compounds (10 mM) to a solvent containing 25% acetonitrile and 75% water in the presence of 15 mM ascorbic acid, which was performed at 25 °C for 0, 1, and 2 h, separately. Reversed phase HPLC was implemented on a 250 × 4.5 mm ODS column and the HPLC profiles were recorded on UV detection at 210 nm. Mobile phase consisted of acetonitrile/water (25:75, v/v), and flow rate was 1.0 mL/min. The samples were taken for HPLC analysis after filtration by 0.45 μm filter.

4.5. Cell culture.

Breast (MCF-7 and MDA-MB-231), colon (HCT-116), gastric (SGC7901 and cisplatin resistant SGC7901) carcinoma cells along with normal liver cells LO2 were cultured at 37 °C in a 5% CO_2 atmosphere using the following monolayer culture media containing 10% fetal bovine serum (FBS), 100 mg/mL of penicillin and 100 mg/mL of streptomycin.

4.6. Cytotoxicity analysis.

The growth inhibitory effect towards human cell lines was evaluated by means of MTT assay. Briefly, 1×10^5 cells/well, MCF-7, MDA-MB-231, HCT-116, SGC7901, SGC7901/CDDP and LO2 cell lines, were separately seeded in 96-well microplates in DMEM medium with 10% FBS and then incubated 24 h at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The medium was removed and the cells were then exposed to a fresh one containing the compounds to be studied at the appropriate concentration. Triplicate cultures were established for each treatment. After incubated for 72 h, each well was treated with 10 μL of a 5 $mg{\cdot}mL^{-1}$ MTT. And after 5 h additional incubation, the medium was replaced by 100 μL DMSO. The inhibition of cell growth induced by the tested complexes was detected by measuring the absorbance of each well at 570/630 nm using enzyme labeling instrument.

4.7. HDACs inhibition activity assay.

HDACs inhibition activity assays were conducted as reported previously [60, 61]. At first, various concentrations of tested compound (50 μL) was mixed with 10 μL of HeLa nuclear extract solution and incubated at 37 °C for 10 min. Then 40 μL of fluorogenic substrate Boc-Lys (acetyl)-AMC was added. After incubation at 37 °C for 30 min, 100 μL of developer, containing trypsin and Trichostatin A (TSA), was added to stop the reaction. After 20 min incubation, fluorescence intensity was measured using a microplate reader at excitation and emission wavelengths of 390 and 460 nm, respectively. The inhibition ratios were calculated by the fluorescence intensity readings of tested wells deducting those of control wells, and the IC_{50} values were calculated using the software GraphPad Prism (version 5.0).

4.8. PARP-1 inhibition assay.

The inhibition of the target compounds on PARP-1 enzymatic assay was determined by ELISA in 96-well plates as previously reported [62]. Histone (20 μg/mL) was diluted in 100 μL

PBS buffer prepared by 10 mM Na_2HPO_4, 10 mM NaH_2PO_4 and 150 mM NaCl. The mixture was precoated in each well and incubated at 4 °C overnight. 100 μM NAD^+, 200 nM slDNA and 25 μM biotinylated NAD^+ diluted in 30 μL of reaction buffer (2 mM $MgCl_2$, 50 mM Tris) were added into each well, and then 5 μL of solvent control or various concentrations of test compound was added. 20 μL PARP-1 (50 ng/well) was added to initiate the reaction at 30 °C. After 1 h, 50 μL streptavidin conjugated HRP was added and maintain temperature at 30 °C for 30 min. At last, 100 μL mixture of luminol and H_2O_2 in citrate buffer (0.1 M) was added. Luminescent signal was measured by spectrophotometer (Molecular Devices SpectraMax M5 microplate reader) and the concentration required for 50% inhibition of PARP-1 enzymatic activity (IC_{50}) was calculated using the software GraphPad Prism (version 5.0).

4.9. Cellular uptake test.

SGC7901/CDDP cells were seeded in 6-well plates in grow medium. When the cells achieved about 80% confluence, SGC7901/CDDP cells were treated with 12 μM of ctc-$[Pt(NH_3)_2(OH)Cl_3]$, cisplatin and SAA1 for 12 h, respectively. Cells were collected and washed three times with cold PBS, harvested and counted, then centrifuged at 1000 × g for 8 min and resuspended in 1 mL PBS. A volume of 100 μL was taken out to determine the cell density. Finally, the cells were digested in 200 μL 65% HNO_3 for 10 h at 65 °C. The Pt level in cells was measured by ICP-MS.

4.10. Cell cycle analysis.

SGC7901/CDDP cells were treated with suitable concentration of SAA, SAA1 and cisplatin, separately. After 24 h of incubation, cells were collected by trypsinization, washed three times with ice-cold PBS, fixed, permeabilized with ice-cold 70% ethanol and stored at -20 °C overnight. After washed with ice-cold PBS, the cells were incubated with 100 μg/mL RNase A for 30 min at 37 °C, stained with 1 mg/mL propidium iodide (PI) for 30 min in the dark at 4 °C. Finally, analysis was performed by flow cytometry using a fluorescence-activated cell sorting (FACS) Calibur (Becton Dickinson, Palo Alto, CA).

4.11. Apoptosis analysis.

SGC7901/CDDP cells were seeded at the density of 2×10^6 cells/mL of the DMEM medium with 10% FBS on 6-well plates to the final volume of 2 mL. Cisplatin was positive control at a concentration of 4 μM. The plates were incubated for overnight and then treated with different concentrations compound SAA, SAA1 and cisplatin for 24 h. Briefly, after incubation 24 h, cells were collected and washed with PBS twice, and then resuspend cells in 1 × Binding Buffer (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) at a concentration of 1×10^6 cells/mL. The cells were stained with 5 μL of FITC Annexin V (BD, Pharmingen) and 5 μL propidium iodide (PI) staining using annexin-V FITC apoptosis kit followed; 100 μL of the solution was transferred to a 5 mL culture tube and incubated for 30 min at room temperature (25 °C) in the dark. The apoptosis ratio was quantified by system software (Cell Quest; BD Biosciences).

4.12. Western blot analysis.

Cisplatin-resistant SGC7901 cells were treated with tested compounds at the indicated concentrations for 24 h. Afterwards, cells were harvested and lysed in cell in RIPA buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% NP40), and were centrifuged at 13000 rpm for 20 min at 4 °C. Proteins from cell lysates was electrophoresed on a 12% SDS-PAGE and blotted to a nitrocellulose membrane. The membrane was blocked with PBST containing 5% non-fat dry milk for 1 h and another 1 h incubation with primary antibodies at 37 °C. The membrane was further stained with the corresponding secondary antibodies for 1 h at room temperature (25 °C). Protein blots were detected by ECL according to the manufacturer's protocol (GE). β-actin was used as a loading control.

Notes

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Compounds' characterization by ^{1}H, ^{13}C and ^{195}Pt-NMR spectra together with ESI mass spectroscopy and HPLC chromatograms. Cellular uptake of measured samples on the tested cancer cell line and biological assays such as HDAC and PARP-1 inhibitory activity. Supplementary data related to this article can be found at ****.

Abbreviations List

HDAC	Histone deacetylase
HDACIs	Histone deacetylase inhibitors
PARP-1	Poly (ADP-ribose) polymerase 1
ZBG	Zinc binding group
NAD+	Nicotinamide adenine dinucleotide
CDDP	Cisplatin
NCS	N-chlorosuccinimide
TBTU	O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate
RF	Resistance factor
ICP-MS	Inductively coupled plasma mass spectrometry
SSB	Single strand breaks
BER	Basic excision repair
PI	Propidium iodide
DCM	Dichloromethane
UV	Ultraviolet
HPLC	High performance liquid chromatography

FBS	Fetal bovine serum
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
TSA	Trichostatin A
HRP	Horseradish peroxidase
FACS	Fluorescence-activated cell sorting
RIPA	Radio immunoprecipitation assay
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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TABLES

Platinum(IV) prodrugs multiply targeting genomic DNA, histone deacetylases and PARP-1

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Table 1.

Effect of target compounds against cell viability of different cell lines[a]

Complex	IC_{50} values (μM)				
	HCT-116	MCF-7	MDA-MB-231	SGC7901	LO2
Cisplatin	7.44 ± 0.72	3.75 ± 0.29	7.96 ± 0.64	12.69 ± 0.82	8.68 ± 0.35
Oxaliplatin	8.67 ± 0.33	18.35 ± 1.18	14.33 ± 0.55	26.82 ± 2.16	16.95 ± 1.17
DN603	43.15 ± 3.36	30.45 ± 2.17	14.25 ±1.28	8.14 ± 0.64	14.11 ± 1.27
DN604	32.50 ± 2.59	9.36 ± 0.82	40.99 ± 3.23	8.20 ± 0.57	35.84 ± 3.12
SAHA	6.98 ± 0.52	3.11 ± 0.16	3.18 ± 0.25	7.43 ± 0.54	2.48 ± 0.18
SAA	>200	>200	>200	>200	>200
SAA1	16.27 ± 1.21	6.70 ± 0.58	5.72 ± 0.36	0.95 ± 0.07	16.87 ± 0.35
SAA2	15.43 ± 1.08	15.07 ± 1.10	18.17± 0.29	17.39 ± 0.16	6.93 ± 0.32
SAA3	45.96 ± 4.35	15.68 ± 1.23	17.57 ± 1.69	14.75 ± 1.12	6.71 ± 0.54
SAA4	32.90 ± 3.08	10.25 ± 0.95	46.46 ± 2.58	6.84 ± 0.49	82.88 ± 6.25

[a] Results are expressed as the mean ± SD from three independent experiments.

Table 2.

In vitro growth inhibitory effect of SAA1 against cisplatin-resistant cell lines SGC7901[a]

Complex	IC_{50} values (μM)			
	SGC7901	SGC7901/CDDP	LO2	RF
Cisplatin	12.69 ± 0.82	67.88 ± 2.16	8.68 ± 0.35	5.35
SAHA	7.43 ± 0.54	7.18 ± 0.48	2.48 ± 0.18	0.96
SAA	>200	>200	>200	
SAA1	0.95 ± 0.07	0.39 ± 0.01	16.87 ± 0.35	0.41
SAA : CDDP =1:1	1.37 ± 0.09	13.82 ± 1.12	nd*	10.08
ctc-$[Pt(NH_3)_2(OH)Cl_3]$	3.44 ± 0.25	13.46 ± 0.34	nd*	3.9

[a] Results are expressed as the mean ± SD from three independent experiments.

nd* means not detected

Figures

Platinum(IV) prodrugs multiply targeting genomic DNA, histone deacetylases and PARP-1

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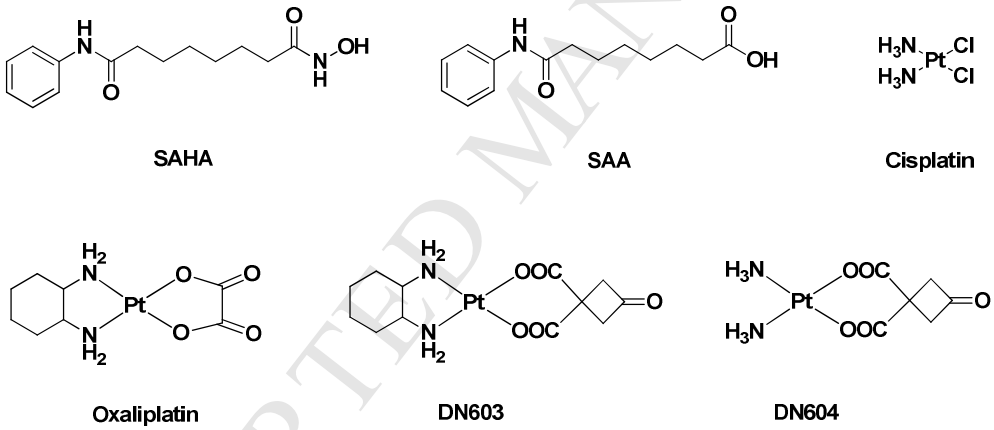

Fig. 1. Chemical structure of SAHA, SAA and platinum(II) complexes.

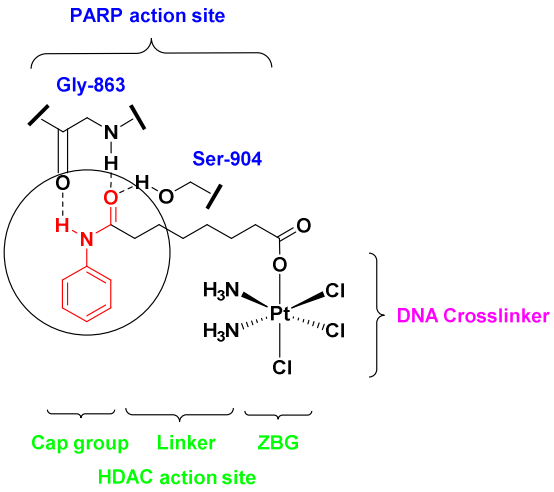

Fig. 2. Design of a novel platinum(IV) prodrug derived from cisplatin, for example, as a potential HDAC inhibitor, PARP inhibitor and antitumor agent.

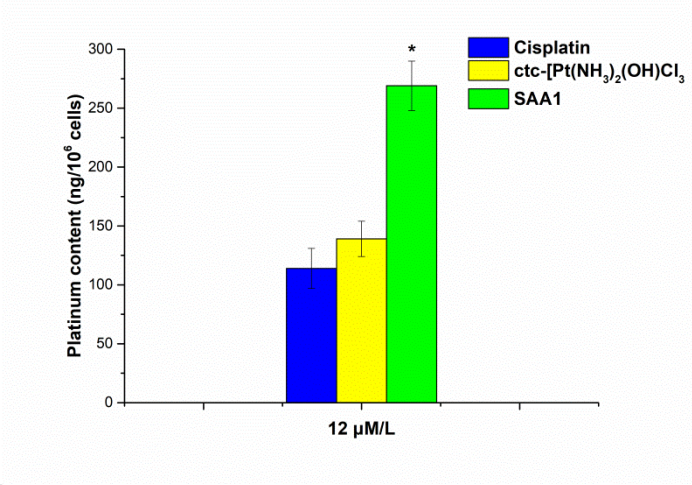

Fig. 3. Intracellular accumulation of ctc-$[Pt(NH_3)_2(OH)Cl_3]$, cisplatin and SAA1 (12 μM) in SGC7901/CDDP cells after 12 h. Each value shown in the table is in nanograms of platinum per 10^6 cells. Results are expressed as the mean ± SD for three independent experiments. *$P < 0.05$ was calculated relative to cisplatin.

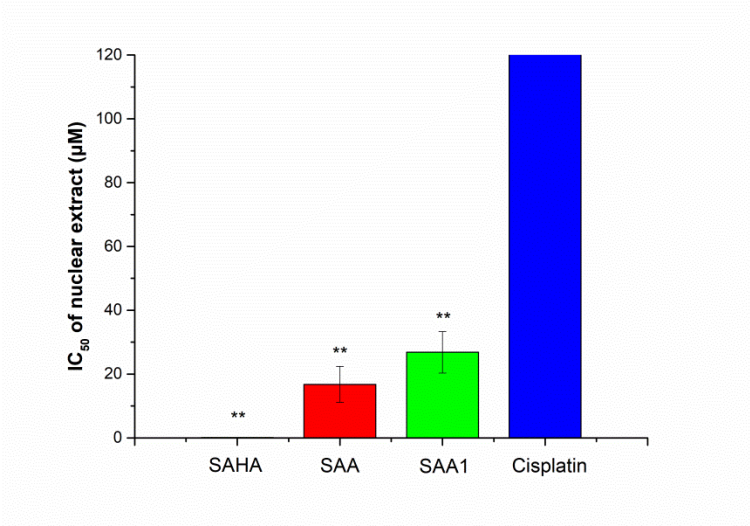

Fig. 4. HeLa cell nuclear extract inhibitory activity of cisplatin, SAHA, SAA and SAA1. Results are expressed as the mean ± SD for three independent experiments. **P < 0.01 was calculated relative to cisplatin.

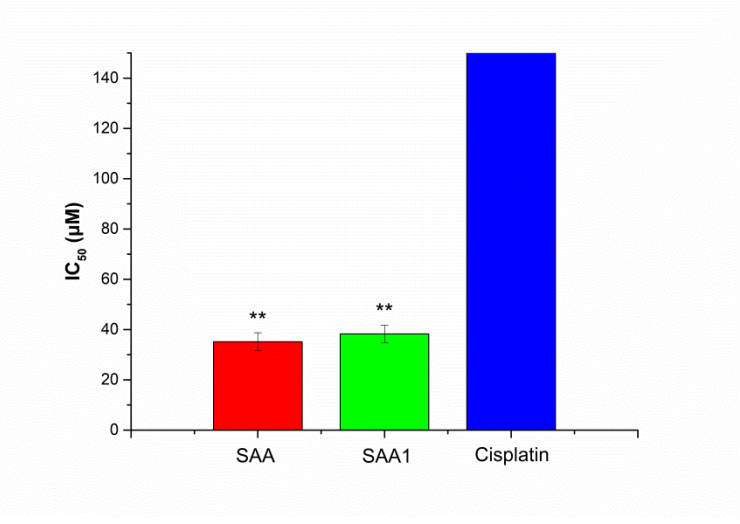

Fig. 5. Inhibitory activities against PARP-1 of SAA-1, SAA and cisplatin. Results are expressed as the mean ± SD for three independent experiments. **P < 0.01 was calculated relative to cisplatin.

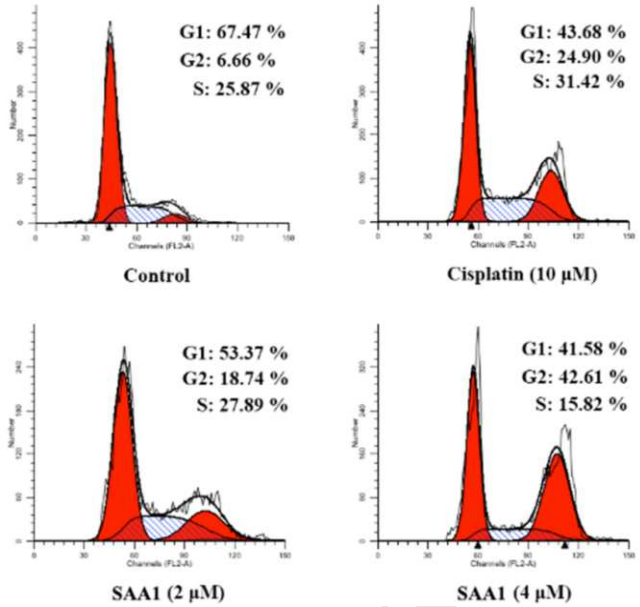

Fig. 6. Effects of SAA1 on cell cycle arrest in SGC7901/CDDP cells. Cells were treated with 2 and 4 μM of SAA1 for 24 h. Then the cells were fixed and stained with PI to analyze DNA content by flow cytometry.

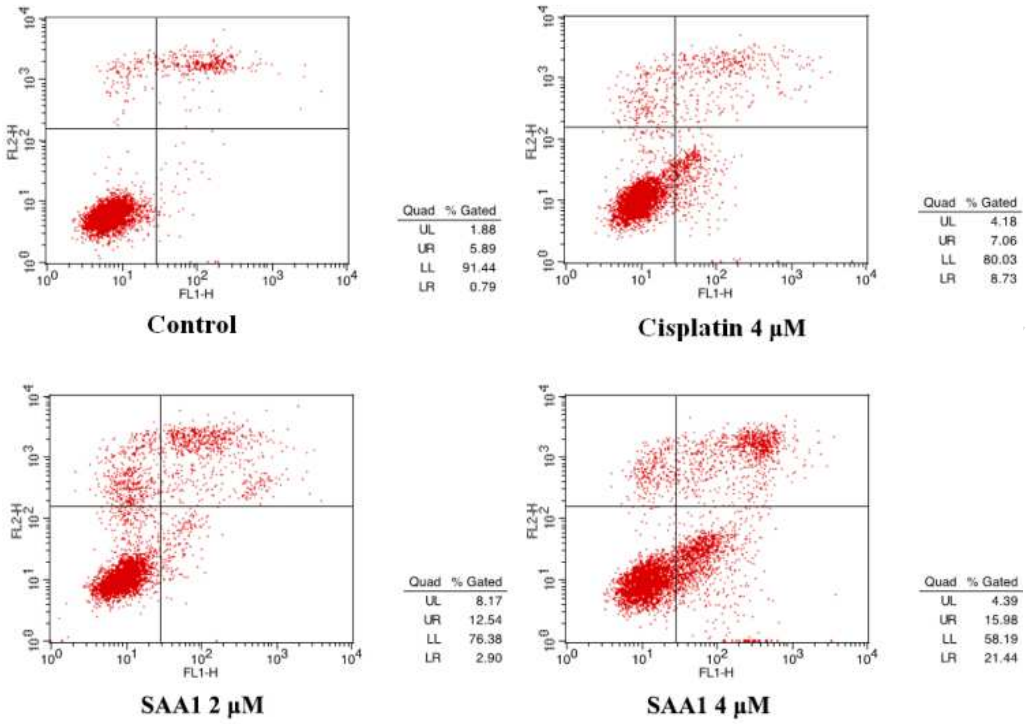

Fig. 7. Induction of apoptosis at 24 h by cisplatin and SAA1 in SGC7901/CDDP cells. The cells were harvested and labeled with annexin-V-FITC and PI, and analyzed by flow cytometry. Data are expressed as the mean ± SEM for three independent experiments.

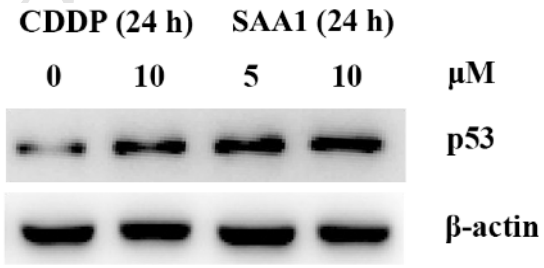

Fig. 8. Western blot analysis of p53 after treatment of SGC7901/CDDP cells with cisplatin (10 μM) and SAA1 (5 μM and 10 μM) for 24 h, respectively. β-Actin antibody was used as reference control.

Highlights

Platinum(IV) prodrugs multiply targeting genomic DNA, histone deacetylases and PARP-1

Pt(IV) prodrugs were designed to exert multifunctional anticancer effects.

SAA1 effectively inhibited HDAC and PARP-1 in addition to damage DNA.

By taking a joint action, SAA1 could overcome cisplatin resistance significantly.