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Synthesis and biological evaluation of a novel artesunate–podophyllotoxin conjugate as anticancer agent

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ABSTRACT

A novel conjugate of artesunate–podophyllotoxin was prepared and evaluated for its cytotoxicity against diverse normal and multidrug resistance human cancer cell lines by CCK-8 assay. The conjugate exhibited good cytotoxicity on all the cell lines with IC_{50} values of 0.453 ± 0.156–3.011 ± 0.272 μM and reduced the resistant factor. The conjugate was further found to disrupt the microtubule network and induce G2/M cell cycle arrest in multidrug resistance K562/ADR cells. Meanwhile, Hoechst staining analysis suggested that conjugate induced cell death by apoptosis. Furthermore, conjugate could downregulate the levels of P-glycoprotein (P-gp) in P-gp overexpressing K562/ADR cells.

Artemisinin,[1] a natural sesquiterpene lactone, isolated from Chinese herb *Artemisia annua*,[2] has been used in the treatment of malaria widely.[3] After many years of research, artemisinin and its derivatives were found to possess several other pharmacological properties like anticancer,[4–6] antimalarial,[7,8] antiviral,[9] and antitubercular[10] effects. Artesunate (ART, **1**, Fig. 1), one of the derivatives of artemisinin, was approved as an antimalarial agent against multidrug-resistant malaria.[11] Increasing studies have also demonstrated that ART exhibits anti-proliferative activity towards various human cancer cell lines in vitro.[12,13] The anticancer activity of ART is mediated by several mechanisms inducing apoptosis,[14] G2/M cell cycle arrest[15] and DNA damage,[16] inhibiting angiogenesis[17] and the expression of HIF-1α[18] and NF-κB,[19] and radiosensitizing effect.[20] Meanwhile, ART showed antitumor activity against cancer xenografts in vivo.[21,22] Furthermore, ART had been proven to have synergistic antitumor effect with other drugs, such as doxorubicin[23] and oxaliplatin.[24]

On the other hand, podophyllotoxin[25] (PPT, **2**, Fig. 1), a well-known arylnaphthalene lignan lactone, isolated mainly from a variety of natural plants including *Podophyllum peltatum* and *Podophyllum hexandrum*, holds extensive antineoplastic properties against various cancer cells through several antiproliferative mechanisms including inhibition of tubulin[26] and topoisomerase II.[27] However, due to side effects, poor water solubility and drug-resistance, the clinical application of PPT has been deserted as an anticancer agent. Therefore, PPT becomes an attractive lead candidate for chemical modifications in the field of antitumor medicine.[28] So far, to overcome the limitations, numerous derivatives of PPT have been synthesized, and several chemical modifications, including etoposide and teniposide, have been used against various cancers in clinical, such as testicular carcinoma, small cell lung cancer and lymphoma.[29]

Nowadays, to develop more effective drug candidates in medicinal chemistry field, hybridization of natural products, natural product-drug or natural product-heterocycle represents one of the most promising approaches for the design of new lead structures.[30–32] Horwedel et al.[33] reported the synthesis of homodimers of artesunic acid molecules and heterohybrids of artesunic acid and betulin, which inhibited the proliferation of sensitive and multidrug-resistant CCRF-CEM leukemia cells and induced G0/G1 cell cycle arrest, apoptosis, and formation of reactive oxygen species. More recently, Li and coworkers[34] confirmed that camptothecin–artesunate conjugate revealed better inhibitory activity against MCF7 breast cancer cells and SMMC-7721 liver cancer cells than camptothecin or artesunate. Meanwhile, the conjugates of PPT and 5-FU exhibited more potent anticancer activity than etoposide and 5-FU.[35] It was reported that lignopurines, prepared by the junction of the non-lactonic podophyllic aldehyde and purines, demonstrated increased cytotoxic activity by disruption of the microtubule network and inducing G2/M cell cycle arrest.[36]

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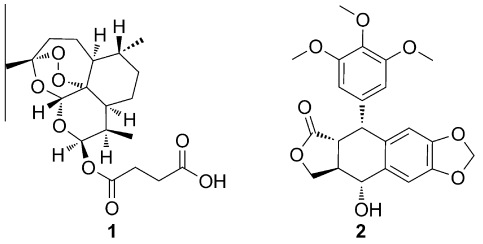


Figure 1. Structures of artesunate and podophyllotoxin.

Recently, some researchers found that dichloroplatinum (II) complexes of PPT displayed potent cytotoxicity against sensitive (K562) and resistant (K562/ADR) cancer cell lines, and induced cell cycle arrest in the G2/M phase.[37]

To our best knowledge, only one Letter had reported the synthesis of the hybrid molecule of ART and GL331 (a PPT derivative) without any further biological activity study.[38] Herein, with the aim to find new natural product-derived antineoplastic agent,[39] in this Letter, we prepared a novel conjugate of artesunate and PPT, and evaluated its antiproliferative effects against a panel of cancer cell lines in vitro. Furthermore, the conjugate was investigated for its effects on cell cycle progression, apoptosis and microtubule network, and induction to P-gp.

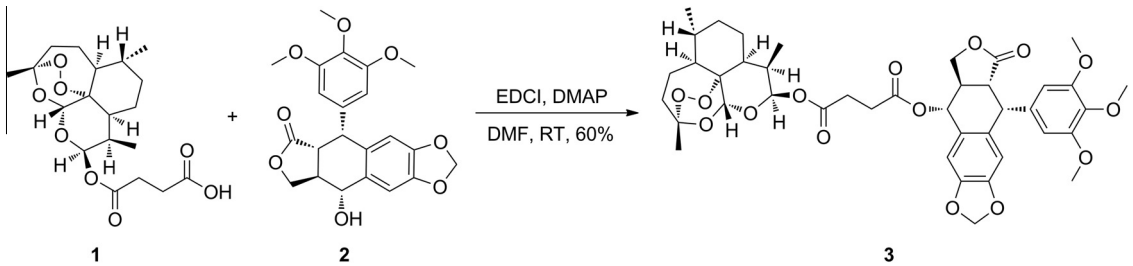
Synthesis of the conjugate **3** is illustrated in Scheme 1. The ART was reacted with PPT catalyzed by l-(3-dimethylaminopropyl)3-ethylcarbo-diimide hydrochloride (EDCI) and 4-dimethylaminopyridine (DMAP) at room temperature to generate compound **3**. Its structure was characterized by IR, ^{1}H NMR, ^{13}C NMR and high-resolution mass spectrum (HR-MS).

The in vitro cytotoxic activity of the test compounds (**3**, PPT and ART) was evaluated against sensitive human cancer cells (human hepatoma HepG2 cells, human lung adenocarcinoma A549 cells, human cervical carcinoma HeLa cells and human leukemic K562 cells), multidrug-resistant K562/ADR leukemia cells and human umbilical vein endothelial cells (HUVEC) by CCK-8 assay, and the results are summarized in Table 1. Etoposide was taken as positive control. The results showed that the compound **3** exhibited effective anti-proliferative activities against all the six cell lines with IC_{50} values from the low micromolar to nanomolar range. Interestingly, **3** exhibited much better inhibitory effects than ADR, and also showed inhibition comparable to those of etoposide. However, it was found that the anticancer activities of **3** were observably lower than those of PPT. Additionally, **3** showed more potent cytotoxicities against four cell lines (HepG2, HeLa, HUVEC and K562) than A549 and K562/ADR cell lines. Particularly, **3** not only exhibited significant cytotoxicity against K562 cells with IC_{50} value of 0.453 ± 0.156 μM, but also showed a potent inhibitory effect on drug-resistant Leukemia K562/ADR cells with IC_{50} value of 2.018 ± 1.201 μM. The resistance factor (4.454) of **3** was obviously lower than that of PPT (14.166), comparable with etoposide (4.903) and higher than ART (1.042). It was suggested from the data that compound **3** had the potential to overcome the multidrug resistance of K562/ADR cells.

Recently, CIP-36, a novel derivative of PPT, was reported to have the potential to induce apoptosis and cell cycle arrest in K562/ADR cells.[41] To determine whether conjugate **3** has similar effects on tumor cells, the effects of **3**, PPT and ART on cell cycle progression were determined by FACS analysis in K562/ADR cells. Treatment with 2.5 μM **3**, 0.1 μM PPT and 6.5 μM ART led to an accumulation of cells in the G2/M phase with a concomitant decrease in the population of G1 phase cells, as seen in Figure 2. G2/M phase arrest was tested after 48 h of treatment: 21.39 ± 1.94%, 33.17 ± 3.35% and 16.93 ± 1.7% of the cells were in G2/M phase after tested compounds (**3**, PPT and ART), respectively, compared with 8.32 ± 0.81% in untreated cultures. These results revealed that **3** could interfere with cell proliferation by arresting the cell cycle, and induce G2/M arrest in K562/ADR cells just like PPT and ART.

To understand whether the conjugate can induce K562/ADR cell apoptosis, an Annexin V-APC/7-AAD binding assay was performed and the percentages of apoptotic cells were determined by flow cytometry. K562/ADR cells were treated with vehicle alone as control and with 2.5 μM **3**, 0.1 μM PPT and 6.5 μM ART for 48 h. As shown in Figure 3, in the untreated group, the frequency of cancer cell apoptosis was 3.18 ± 0.09%. There was low frequency (5.50 ± 0.39%) of K562/ADR cell apoptosis under 2.5 μM **3**, which was slightly higher than control. However, following treatment with 0.1 μM PPT and 6.5 μM ART, 35.35 ± 1.75% and 15.28 ± 1.23% cell apoptosis on cancer cells were observed, respectively, which were significantly stronger than control. These results indicated that **3** could induce apoptosis in K562/ADR cells; however, the apoptosis-inducing effect was unobvious. To confirm the effect of the conjugate on induction of apoptosis, K562/ADR cells were examined using Hoechst 33342 staining. Control cells exhibited excellent growth characteristic after 48 h incubation shown in Figure 4. Whereas, K562/ADR cells treated with 2.5 μM **3** exhibited the characteristics of apoptosis, such as cell shrinkage, nuclear fragmentation and condensation. Similar effects were also observed when cell were exposed to 0.1 μM PPT or 6.5 μM ART. These results demonstrated that **3** could prevent the proliferation of cancer cells by arresting the cell cycle and induce G2/M arrest accompanied by apoptosis in K562/ADR cells.

As shown in Figure 3, it can be seen that the apoptosis inducing effect of **3** was much less than PPT and even less than ART. So, it seems that the aspect of apoptosis inducing may contribute less to the cytotoxicity of the conjugate. It was clearer that the G2/M cell cycle arrest could be related with the inhibitory activity. Previous studies showed that PPT and its derivatives could inhibit tubulin polymerization and disturb the microtubule skeleton.[42–44] Because compound **3** is a derivative of PPT, it could be feasible that the G2/M cell cycle arrest might be due to the disruption of the microtubule network. To determine whether **3** was able to depolymerize cellular microtubules like PPT, we further investigated the effects of **3**, PPT and ART on microtubule skeleton using indirect immunofluorescence. K562/ADR cells were treated with vehicle,



Scheme 1. Synthesis of artesunate–podophyllotoxin conjugate.