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COMMUNICATION

Investigation and Bioorthogonal Anticancer Activity Enhancement of Triphenylphosphine-labile Prodrug of Seleno-Combretastatin-4

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Here, the triphenylphosphine (TPP)-labile prodrug of seleno-Combretastatin-4 (CSeD) has been designed and synthesized. A detail investigation was revealed that CSeD which showed higher safety in the blood circulation could react with TPP to release CA-4 and selenodiazole derivative, accompanying powerful anticancer, antiangiogenesis effects and radiosentization property.

Triarylphosphine (TPP) initiated Staudinger ligation reaction was firstly developed in 2003,[1] which ignited scientist's long-lasting and explosive interest on bioorthogonal chemistry.[2] Several coupling strategies have been well developed over these ten years which allow the covalent linkage of isotope labels, fluorescent probes or affinity tags to different biomolecules.[3-4] Apart from these bioorthogonal ligation reactions, the bond-cleavage type of bioorthogonal reactions were also received tremendous attention[5] and a series of biocompatible reduction, deprotection reactions with various bio-application have emerged after well documented photo-decaging reactions.[6] To extend the application of bioorthogonal cleavage reactions, numerous elegant strategies were developed to use bioorthogonal tools as a trigger for drug release. The key focus is on the development of biocompatible small molecules or reagents as trigger elements. Besides intensively studied metal catalysts, such as Pd, Ru, Cu(I), etc.,[7-9] TPP, both primary and oxidative state of which are nontoxic proved in many known Staudinger ligation reaction, should be a biocompatible key to unlock certain derivatized prodrugs for drug releasing at a controllable manner.[10-11] While the strategies involving TPP as trigger to release drugs with enhanced bioactivities via Staudinger reaction are seldom reported.

Combretastatin A-4 (CA-4), a natural tubulin polymerization inhibitor, can effectively inhibit the aggregation of tubulin, so as to prevent cancer cells from mitosis, block them in G2/M phase, and finally induce apoptosis, exhibiting good prospect of clinical application.[12] Although a large number of studies have carried out on derivatization CA-4 to obtain better drug candidates, its low blood compatibility and obvious toxic side effects for normal tissues still limit its use in clinical research and cancer treatment. Hence, the proper modification of CA-4 is still highly anticipated in order to optimize its clinical behaviour. Selenium (Se), as a necessary trace element for human, plays an important role in many life activities. Organic Se compounds usually possess high bioavailability, strong anticancer activity and high biological safety.[13] In addition, the selenodiazole compounds were proved universal anticancer activity and radiosensitization effect, suggesting high bioavailability, high biological safety and strong medical significance. [13] In view of unique properties Se compouds, we designed a combined prodrug of CA-4 and azide selenodiazole via esterfication and which could react with TPP to release

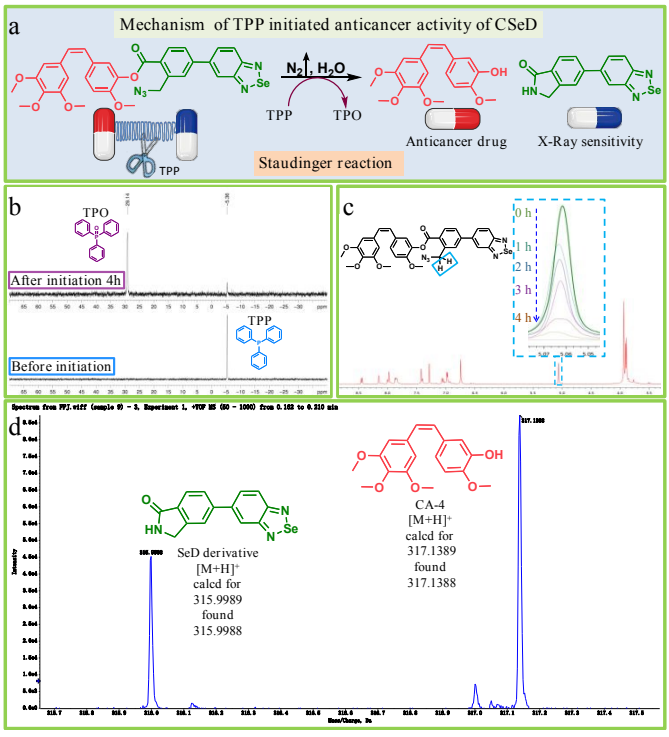

Fig. 1 TPP initiated CSeD monitored by ^{1}H NMR ^{31}P NMR and HMRS. (a) The mechanism of CSeD responding to TPP in the cells. **(b)** A comparison of reactants'

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^{31}P NMR spectrum between before begin and after 4 h. **(c)** The ^{1}H NMR monitoring of characteristic H peaks during 4 h. **(d)** The HMRS has found the mass of CA-4 and SeD derivative.

CA-4 and SeD derivative at certain location, accompanying enhanced anticancer activity and synergy efficiency for cancer treatment **(Fig. 1a)**. With the idea in mind, we firstly synthesized azide seleno diazole (SeD), which was reacted with CA-4 via esterification to gain compound CSeD. All new compounds were well characterized by NMR and HMRS, as shown in **Fig. S11-S26** (see supporting information for detail procedure). To prove CSeD could release CA-4 and SeD derivative by Staudinger reaction, a series transformation were recorded. As presented in **Fig. 1b**, a ^{31}P NMR shows that triphenylphosphine oxide was formed during the mixing of CSeD and TPP in DMSO-d_6 for 4 h. As shown in **Fig. 1c**, a gradual diminishment of the peak of proton next to azide in CSeD was observed during the 4 h as TPP was constantly added to a solution of CSeD. In addition, the HRMS of PBS solution of TPP and CSeD shows the Mass of CA-4 (calcd for $C_{18}H_{21}O_5^+$ ([M+H])$^+$, 317.1389, found, 317.1388) and SeD derivative (calcd for $C_{14}H_{10}N_3OSe^+$ ([M+H])$^+$, 315.9989, found, 315.9988), as presented in **Fig.1d, S27-28**. These results indicated that TPP could serve as a trigger to react with CSeD and achieve bioorthogonal cleavage reactions in the physiological environment.

It's well known that hemocompatibility is an essential requirement for the intravenous use of chemotherapeutic drugs in the clinical situation. Therefore, hemolysis rate of red blood cells (RBCs) treated with different compounds and their morphology were measured and captured. As shown in **Fig. 2a** and **Fig. S1a-b**, hemolysis rate of RBCs treated with CA-4 was notably higher than those of TPP, SeD and CSeD alone. However, co-incubation with TPP and CSeD rapidly enhanced the hemolysis rate and destructed the morphology of RBCs. The results exhibited that free TPP and CSeD showed higher safety in the blood circulation compared with CA-4, but after the combination of TPP and CSeD, it could cause serious damage to the RBCs. Clearly, the Se modified CA-4 demonstrated a positive effect on its physical and chemical properties. Then, MTT assay was performed to assess the antiproliferative effect of compounds against cancer and

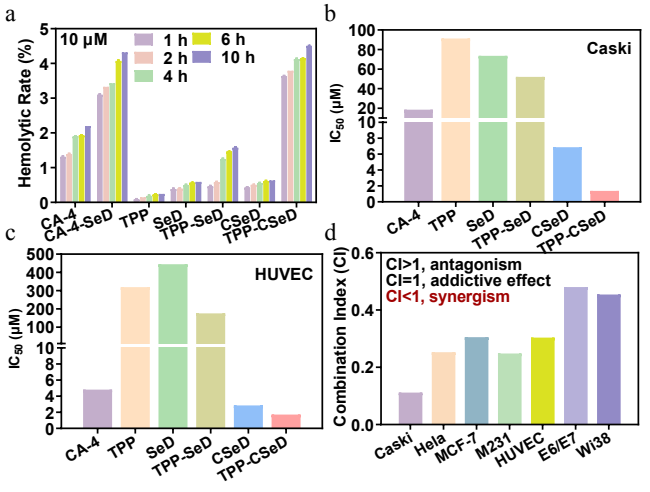

Fig. 2 Anticancer efficiency of different compounds. (a) Effects of different compounds with concentrations of 10 μM on hemolysis of RBCs. **(b)(c)** Cytotoxic effect of various compounds on Caski and HUVEC cells with or without TPP initiating. **(d)** CI values demonstrating the synergistic effect of TPP and CSeD in various cells.

normal cells **(Fig. S2 and Table S1)**. As shown in **Table S1**, TPP have no obvious cytotoxicity toward various types of cancer and normal cells, demonstrating TPP with excellent biological safety can be used as inducer to initiate the reaction with CSeD. Then, in order to find out the time required for TPP to be absorbed by cells, we detected the intracellular uptake of TPP in Caski and HUVEC cells by inductively coupled plasma-mass spectrometry (ICP-MS). The uptake of TPP in Caski and HUVEC cells enhanced in time-dependent manner, while its uptake efficiencies at 4 h were as high as 76.32% and 74.33%, respectively (**Fig. S3**). Hence, according to our design in the study, SeD/CSeD was added to cells after TPP pre-incubation for 4 h in the following biological experiments. Astonishingly, after the conjugation between SeD and CA-4, an obvious improvement in the antiproliferative effect against various cancer cells of CSeD was observed. Taking the Caski cell for example, the IC_{50} value of CSeD against Caski cells was 2.69-fold higher than that of CA-4, demonstrating that the conjugation of SeD increases CA-4's anticancer efficacy. Subsequently, to demonstrate our design, cells were pre-incubated with TPP for 4 h and then co-treated with SeD/CSeD to enable SeD/CSeD react with TPP accumulated in cancer cells. As shown in **Fig. 2b-c**, SeD/CSeD in combination with TPP (TPP-SeD/TPP-CSeD) further suppressed cell proliferation. More strikingly, the IC_{50} value of TPP-CSeD against Caski cells was 13.39-fold higher than that of CA-4. The combination indexes (CI) was used to analysis the relationship of TPP and CSeD, the value of which was lower than 1.0 (**Fig. 2d**), indicating that TPP can be used as a key to react with CSeD and release CA-4 and SeD, achieving synergistic anticancer effect.

Moreover, clonogenic assay demonstrated that treatment with TPP-CSeD compounds effectively inhibit the colony formation of Caski and HUVEC cells (**Fig. S4a-b**). Inhibition effect of TPP-CSeD on Caski tumor spheroids cultured *in vitro* exhibited a dose-dependent manner, further proving antiproliferative effect of TPP-CSeD (**Fig. S5a-b**).

The tumor cells always release a large amount of vascular growth factors (VEGF) to stimulate the directional growth of vascular endothelial cells, resulting in the growth of micro-vessels around and inside the tumor.[15] Previous studies proved that as a common vascular disrupting agent, CA-4 destructs the morphology of endothelial cells by prohibiting tubulin from polymerizing to microtubules and cytoskeletons, finally achieving the purpose of destroying tumour blood vessels. [16] Therefore, we performed the cell migration, transwell and tube formation assays to evaluate the suppression effect of these compounds on angiogenesis activities. As indicated in **Fig. 3**, VEGF stimulates the migration and invasion of HUVEC cells and promotes its tube formation. However, these effects were markedly inhibited by the treatment of TPP-CSeD at non-toxic concentration (1.25 μM). For instance, the migration and invasion of HUVEC cells trigged by VEGF enhanced from 100 % (control group) to 140% and 151%, whereas the treatment of TPP-CSeD significantly decreased the migration and invasion

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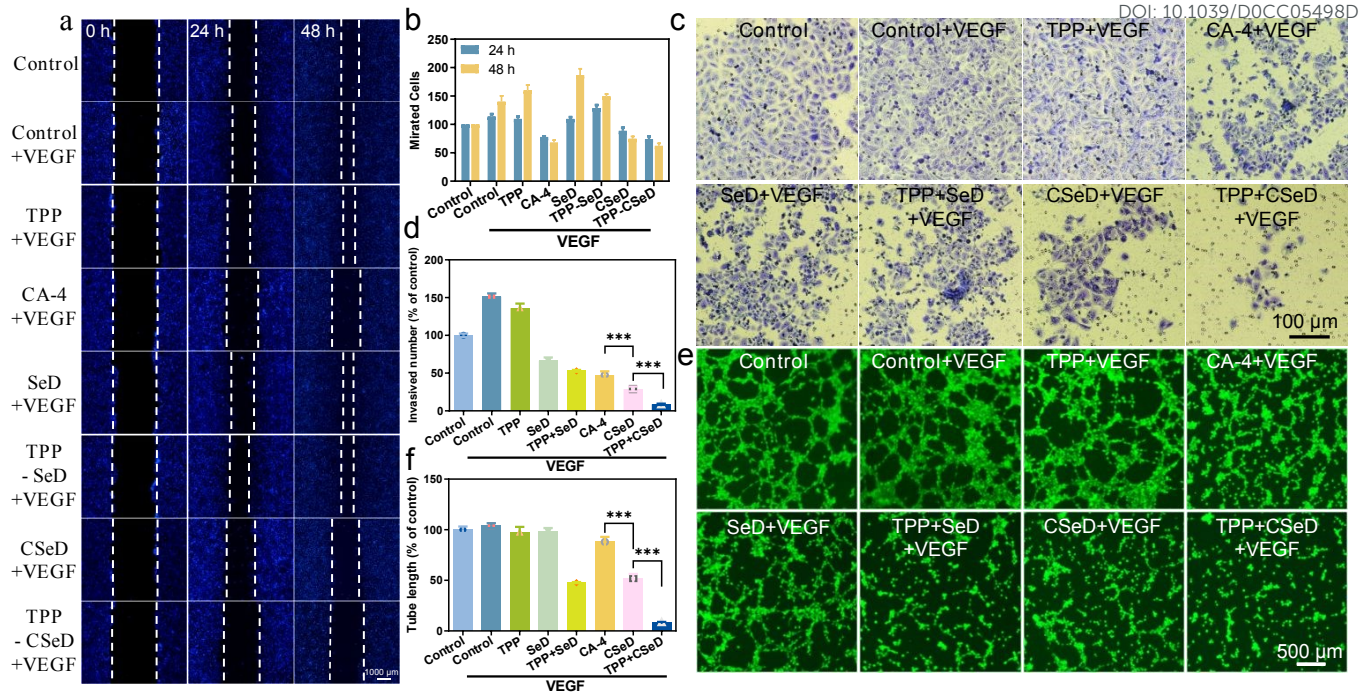

Fig. 3 The comparison of different compounds suppress VEGF-induced HUVEC cell migration, invasion and angiogenesis. Representative images of inhibition of VEGF-induced HUVEC migration **(a)**, invasion **(c)** and tube formation **(e)** by different compounds. Quantitative analysis of migrated cells **(b)**, invasive numbers **(d)** and tube length **(f)**. Significant of difference between groups is indicated at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

rates to 62% and 8.8%, which was much lower than that SeD (186% and 66%) and CA-4 (67% and 47.4%), demonstrating TPP-CSeD enhances inhibition effect for cancer cells migration (**Fig. 3a-b)** and invasion (**Fig. 3c-d**). Similarly, as illustrated in **Fig. 3e-f**, the reduction in tube length in TPP-CSeD group was much greater than that of induced by CSeD or CA-4, indicating a better antiangiogenic activity. Therefore, the efficacy of TPP-CSeD for inhibition of VEGF-induced cancer cell migration, invasion and angiogenesis was further improved versus CA-4 compound.

In addition, TPP-CSeD not only enhances the antiproliferative effect of CSeD, but also provides a possibility to strength the radiotherapeutic effect of X-Ray. Seleno compounds could effectively enhance the radiosensitization of tumor cells for X-ray, resulting in strong anticancer ability according to previous studies.[14] Therefore, the radiosensitization effects of CA-4/SeD/CSeD/TPP-SeD/TPP-CSeD was investigated by irradiating different doses of X-Ray. As shown in **Table S2 and Figure S6a-b**, with the increase of the X-Ray intensity from 0-8 Gy, the IC_{50} values of these compounds obviously decreased. Furthermore, isobologram analysis was used to assess the interaction between X-Ray and compounds, as shown in **Fig. S6c-f**, the data point of in co-treatment group is below the additional effect, revealing that Seleno compounds could synergistically enhanced the radiotherapeutic effect in Caski and HUVEC cells. Overall, TPP-CSeD is not only an efficacy strategy to improve the antitumor effect of CA-4, but also provide a possibility to strength its radiotherapeutic effect of X-Ray.

The death mechanism of Caski and HUVEC cells caused by TPP-CSeD in combination with X-Ray was also explored. After combined with X-Ray irradiation, the population of apoptotic Sub-G1 fraction in Caski cells was further increased (**Figure 4a** and **S7a**), which means TPP-CSeD can also enhance the Caski cell's apoptotic Sub-G1 fraction. Moreover, as shown in **Fig. S8**, CA-4 treatment caused obvious G2/M phase arrest in Caski and HUVEC cells, which was consistent with previous studies.[17]

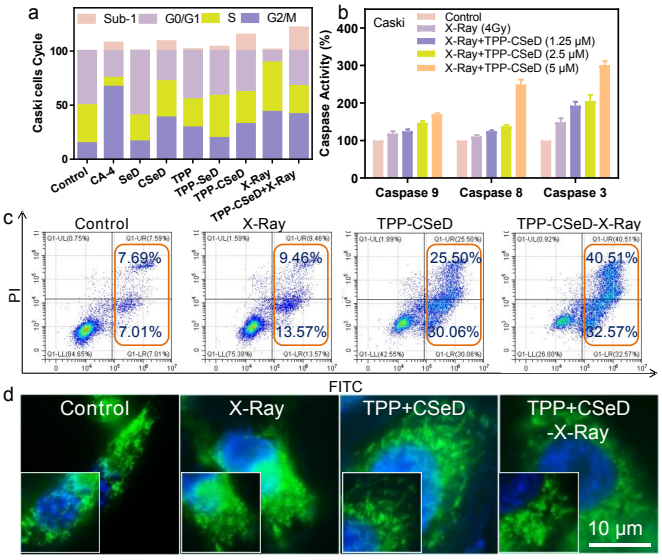

Fig. 4 Action mechanisms of cell apoptosis induced by compounds and X-ray radiotherapy. (a) Cell cycle distribution of Caski cells induced by different treatments for 24 h. **(b)** Combined treatments of X-Ray and compounds activated caspase3/8/9 activity in Caski cells. **(c)** Flow cytometry analysis of apoptosis in Caski and HUVEC cells caused by compounds and X-Ray. **(d)** Effects of TPP-CSeD and X-Ray on mitochondrial dysfunction in Caski cells.

Furthermore, according to the results of Annexin V-FITC apoptosis detection kit, TPP-CSeD combined with X-Ray irradiation effectively enhanced the population of early and late

apoptosis cells in a dose-dependent manner, which was much greater than those of TPP-CSeD or X-Ray alone (**Fig. S9**). These results revealed that under X-Ray irradiation, TPP-CSeD trigged cancer cells death mainly through inducting apoptosis and a small amount of G2/M phase cell arrest. It's well known that mitochondrial dysfunction acts as critical role in inducing apoptotic.[18] Hence, we used mitochondrial probe to dynamically observe the morphological changes of mitochondria in real time. As shown in **Fig. 4d** and **Fig. S7d**, the mitochondria in the control group extended to the whole cytoplasm in a filamentous manner. But in the Caski and HUVEC cells treated with TPP-CSeD and X-Ray, obvious mitochondrial fragmentation and rupture were observed. These results commonly suggested that the combination of TPP-CSeD and X-Ray irradiation plays an important role in inducing mitochondrial dysfunction and triggering cell apoptosis. Many studies reported that caspases, as a family of proteases, plays an important role in initiating and executing apoptosis.[19] Therefore, we next examined the caspase activities including caspase-3/8/9 trigged by different concentrations of TPP-CSeD and X-Ray in combination. A dose-dependent manner increase in caspase 3/8/9 activities were observed after the treatment (**Fig. 4b** and **S7b**). Western blot analysis further showed that treatment of Caski cells with TPP-CSeD and X-Ray also caused dose-dependent increase in activation of caspase-3, caspase-8 and caspase-9 (**Fig. S10**). Taken together, apoptosis mediated by TPP-CSeD and X-Ray is mainly dependent on caspase pathway, mainly through death receptor-mediated and mitochondrial-mediated pathways.

In summary, TPP-labile CSeD prodrug was successfully designed and synthesized by using -OH group from CA-4 and -COOH group from azide SeD via esterification process. TPP could serve as a trigger to react with CSeD for releasing SeD and CA-4 via Staudinger reaction, thus achieving bioorthogonal cleavage reactions. Compound CSeD showed higher safety in the blood circulation compared with CA-4 and the anticancer efficiency of TPP-CSeD was also highly superior to that of CA-4. Moreover, TPP-CSeD displayed excellent radiosentization properties and enhanced the inhibition effect for cancer cells migration, invasion and angiogenesis. The mechanism of cell death suggested TPP-CSeD with X-Ray illumination induced mitochondrial dysfunction and activated of caspase activities, thus triggering cell apoptosis. Taken together, this study provides a new approach for the rational design of late-stage activation multiple chemotherapeutic prodrugs that showed bioorthogonal bioactivity enhancement for simultaneous anticancer and antiangiogenesis therapy.

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Conflicts of interest

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The authors declare that there are no conflicts of interest.

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