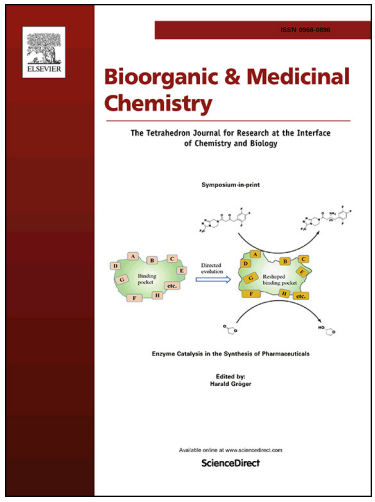


# Synthesis and biological evaluation of paclitaxel and vorinostat co-prodrugs for overcoming drug resistance in cancer therapy in vitro

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## Graphical Abstract

In vitro hydrolysis, co-prodrug **1a** could release PTX and SAHA more efficiently and was further prepared to nanomicelles with $mPEG_{2000}$-$PLA_{1750}$.
**1a** nanomicelles had a comparable or even better cytotoxicity than PTX especially in the drug-resistant MCF-7/ADR cells.

# Synthesis and biological evaluation of paclitaxel and vorinostat co-prodrugs for overcoming drug resistance in cancer therapy in vitro

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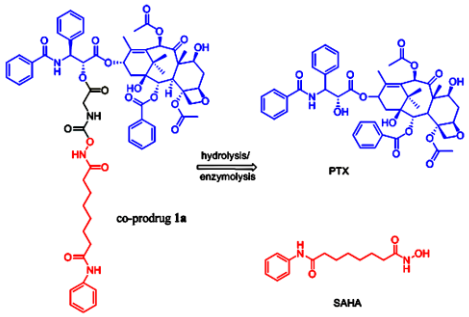

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ABSTRACT

*Paclitaxel (PTX) is the first-line treatment drug for breast cancer. However, drug resistance after a course of treatment and low selectivity restricted its clinical utility sometimes. In this study, we successfully bound PTX and vorinostat (SAHA) to form co-prodrugs based on the synergistic anticancer effects. The PTX-SAHA co-prodrugs were conjugated by glycine (**1a**) and succinic acid (**1b**) respectively and the former have shown better activity in cytotoxicity, cell cycle arrest and western-blot experiments. Therefore, **1a** was further prepared to nanomicelles with $mPEG_{2000}$-$PLA_{1750}$ as the carrier by using thin film method. PTX-SAHA co-prodrug nanomicelles were spherical with a particle size of 20-100 nm. In vitro drug release test showed **1a** nanomicelles had sustained release effect, which could reduce the resistance of PTX. In vitro cytotoxicity was evaluated by SRB assay in HCT-116 cells, MCF-7 and drug-resistant MCF-7/ADR cells. The results showed **1a** nanomicelles had comparable or even better cytotoxicity than PTX especially in the MCF-7/ADR cells. All the results suggested that PTX-SAHA co-prodrug nanomicelles were promising treatment for PTX resistance cancer.*



## 1. Introduction

Nowadays, cancer imposes great threat on people's lives with incidence of various cancer has become higher and lack of eventual cure. The commonly used treatments in the clinical treatment of cancers include surgery, chemotherapy, radiotherapy and biological therapy[1]. Generally speaking, traditional chemotherapy has made a great contribution in cancer therapy but remains limited by inferior biocompatibility, unexpected systemic cytotoxicity and drug resistance of cancer cells.

For all the contemporary problems, drug combination therapies have been widely exploited to decrease these unwanted side-effects and enhance therapeutic effects simultaneously. Combination chemotherapy has improved the efficacy of cancer therapy notably as many instances had exhibited[2]. Paclitaxel (PTX) is one of the most widely used clinical anticancer drugs which has a unique microtubule resistance mechanism and generally used for breast cancer, ovarian cancer and other types of cancer[3]. However, the long-term use of PTX can develop into drug resistance and further limits its application in clinical. The main cause of resistance was that the variation of microtubule structure can alter intracellular drug level and signal transduction, thereby avoiding the apoptosis pathways[4]. Therefore, it's particularly important to increase drug accumulation in tumor tissues and reduce their drug resistance. Histone deacetylase inhibitors (HDACIs) have shown great prospect for a good deal of cancers by using alone or combining with traditional anticancer drugs. Treatment of cancer with HDACIs has multiple-effects such as inhibition of cell proliferation, induction of cell-cycle arrest and apoptosis[5-6]. Suberoylanilide hydroxamic acid (SAHA, Zolinza/ vorinostat) is an oral HDACI which has strong anticancer effects in hematological tumors[7]. Recently, several studies have demonstrated that SAHA could potentiate PTX-induced antitumor effects against some human cancer cells (lung cancer cells, ovarian cancer cells, endometrial cancer cells, etc.) [8-10].

Despite these advantages, sometimes the cancer therapy with combination drugs can be severely restricted by some issues.

PTX is an intravenous preparation and SAHA is an oral preparation which mean they may not reach the target site concurrently. And the physiochemical properties of PTX and SAHA are quite different that may cause the interaction complicated. Besides, SAHA always hydrolyze to some inactive metabolisms like carboxylic acid derivative especially in plasma[11].In that case, restructuring and modifying SAHA is essential for enhancing the stability of SAHA. To date, several strategies have been investigated to improve the stability of SAHA, including clickable pH-responsive prodrug[12], selective enzymatic cleavable prodrug[13], thiol-sensitive prodrug[14], redox-responsive prodrug [15], and so on. Based on the previous researches, we conjugated PTX and SAHA with a nontoxic linker to form a "co-prodrug" for combined administration of PTX and SAHA (**Fig. 1**). In this way, we expected to obtain the synergistic effect of PTX and SAHA by reconverting to the two original anticancer drugs concurrently under particular conditions.

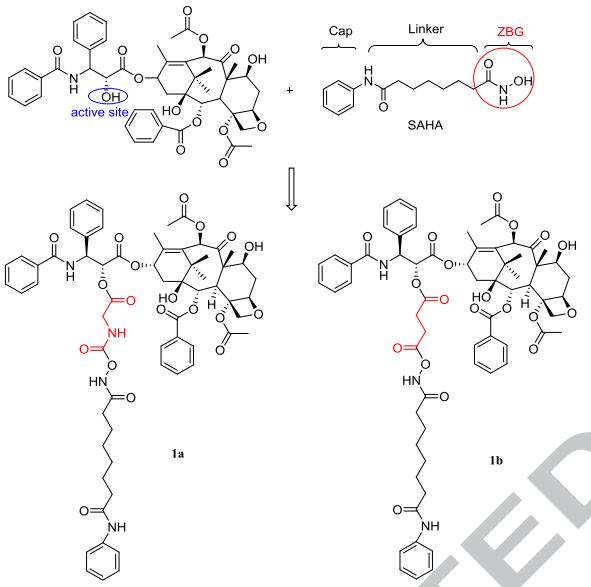



**Fig. 1.** Design of the PTX-SAHA co-prodrugs.

By conjugating the two drugs together in a cleavable way, we enhanced the stability of SAHA and reduced the drug resistance after cellular uptake of the PTX-SAHA co-prodrugs. We connected the hydroxamic acid group of SAHA and the 2′-OH of PTX to form a co-prodrug, and further formed the co-prodrug nanomicelles with $mPEG_{2000}$-$PLA_{1750}$ as the carrier[16]. Glycine (Gly) and succinic acid were used as the linker of PTX-SAHA co-prodrugs. The release mechanism of co-prodrugs was that the carbonic ester connected with SAHA simply hydrolyzed through hydrolysis or enzymolysis firstly. Then, the release rate of PTX mainly depended on the stability of the rest part. It has been reported that PTX-2′-Gly is unsteady when it's exposed to water and the other one is much steadier under same condition[17-18]. This study examined the stability of co-prodrugs and in vitro cytotoxicity in human colorectal cell line HCT-116, breast cancer cell lines MCF-7 and MCF-7/ADR, as well as cell cycle analysis. All these results showed that **1a** released free PTX and SAHA efficiently and had better cytotoxicity than **1b**. Furthermore, PTX-SAHA nanomicelles could enhance the stability of **1a** co-prodrug and prolong the retention time in blood stream through accumulating in tumor site by way of enhanced permeation and retention (EPR) effect. Comparing free PTX and **1a** co-prodrug, PTX-SAHA co-prodrug nanomicelles considerably enhanced the anticancer efficacy and efficiently reversed PTX resistance in cancer therapy.

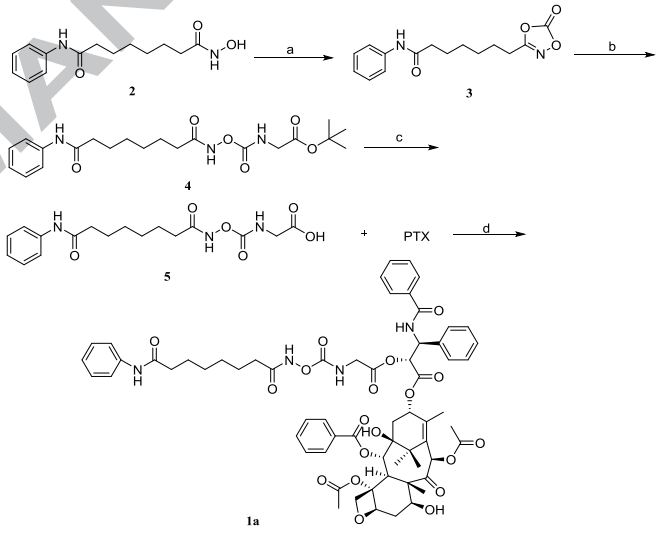
## 2. Results and discussion

### *2.1. Synthesis of the PTX-SAHA co-prodrug*

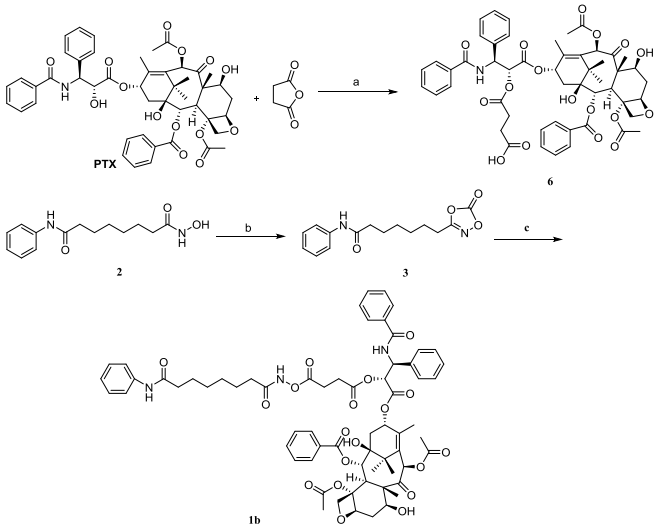
The **1a** were synthesized using a four-step procedure as illustrated in **scheme 1** with $CH_2Cl_2$ as solvent. Firstly, the compound **2** (SAHA) was activated to an active intermediate **3** by using CDI and then reacted with the tert-butyl protected glycine to obtain compound **4**. Subsequently, cleavage of the tert-butyl group from compound 4 was performed using 30% TFA in $CH_2Cl_2$, resulting in compound **5**. Finally, compound **5** was reacted with PTX via an esterification reaction to produce the product **1a**.

The **1b** were synthesized using a different way from **1a** as illustrated in **scheme 2**. PTX was reacted with succinic anhydride to generate compound **6**, which was catalyzed by DMAP and pyridine. Compound **2** (SAHA) was activated to a active intermediate **3** by using CDI and then reacted with the compound **6** to produce the other terminal product **1b**.

The progress of the reaction was monitored by thin layer chromatography (TLC). All reactions processed with good yields and purified to high purity. Each compound was confirmed by $^1$H NMR. The final products were confirmed by $^{13}$C NMR, mass spectrometry and HPLC.




**Scheme 1.** Reagents and conditions: (a) CDI, $CH_2Cl_2$, rt; (b) H-Gly-tBu, rt; (c) 30% TFA in $CH_2Cl_2$, 0°C; (d) EDCI, HOBT, $CH_2Cl_2$, rt.




**Scheme 2.** Reagents and conditions: (a) DMAP, pyridine, $CH_2Cl_2$, rt; (b) CDI, $CH_2Cl_2$, rt; (c) compound **6**, rt.

*2.2. HPLC analysis of PTX-SAHA co-prodrugs*

HPLC with fluorescence detection was used to identify the hydrolysis of co-prodrugs and formation of PTX, SAHA and other intermediates from the co-prodrugs. Formic acid buffer was used in the mobile phase to serve as the ion-pairing reagent, and to serve as the major buffer component. The retention times for SAHA and PTX were 8.42 min and 13.81 min, respectively. In addition, the retention times for compound **6**, **1a** and **1b** were 13.63 min, 14.62 min and 15.06 min, respectively. The others metabolic intermediate of SAHA such as N-phenylotanedi-amide and 8-aniline-8-oxooctanoic acid didn't produced.

*2.3. In vitro degradation and release of PTX-SAHA co-prodrugs in PBS*

The hydrolysis studies of **1a** and **1b** were first performed in PBS at pH 6.0 and 7.4 at 37°C. The disappearance of **1a**/**1b** and the formation of PTX/SAHA were quantified using HPLC. The percentage of remaining **1a**/**1b** and converted PTX/ SAHA was plotted as a function of time (**Fig. 2**). The degradation curves in **Fig. 2A** showed that the two co-prodrugs exhibited greater stability under the slightly acidic physiological environment (pH 6.0). There were over 85% **1a** and **1b** remained 24 hours later in pH 6.0 PBS. Besides, **1b** was much more stable than **1a** so that the reconversion of SAHA and PTX were little. The half life ($t_{1/2}$) of **1a** was 2 h at pH 7.4 and the reconversion of PTX and SAHA reached the highest at 48 h were 50.3% and 56.8%, respectively. These results illustrated in **Fig. 2** indicated that the **1a** can release active anticancer drugs well for synergistic effects.

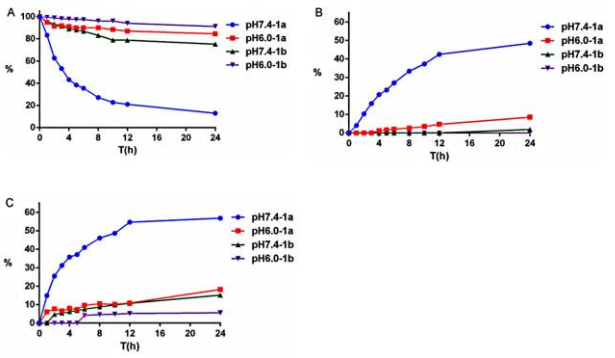



**Fig. 2.** (A) Degradation of the co-prodrugs in PBS (pH7.4) and PBS (pH6.0); (B) PTX released from the co-prodrugs in PBS (pH7.4) and PBS (pH6.0); (C) SAHA released from the co-prodrugs in PBS (pH7.4) and PBS (pH6.0).

*2.4. In vitro degradation and release of PTX-SAHA co-prodrugs in plasma*

The hydrolysis studies of **1a** and **1b** were then performed in human plasma and mouse plasma at 37 °C. The disappearance of **1a**/**1b** and the formation of PTX/SAHA were quantified using HPLC. The percentage of remaining **1a**/**1b** and converted PTX/ SAHA was plotted as a function of time (**Fig. 3**). The two co-prodrugs had a short $t_{1/2}$ nearly about 0.5h in both human and mouse plasma. Besides, the hydrolysis rate of them was faster in mouse plasma due to the difference of carboxylesterase. Similarly, as the PBS stability above, the **1b** was more stable and had less reconversion of PTX than **1a**. The highest reconversion rates of PTX from **1a** were up to 74.1% and 85.8% in human and mouse plasma, respectively. The peak of SAHA was cross with some substance in the plasma so that the conversion rate of SAHA hadn't listed out.

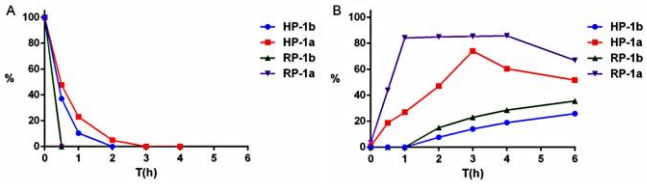



**Fig. 3.** (A) Degradation of the co-prodrugs in human and mouse plasma; (B) PTX released from the co-prodrugs in human and mouse plasma. HP: human plasma; RP: mouse plasma.

*2.5. Cytotoxicity of PTX-SAHA co-prodrugs*

PTX would become nontoxicity after esterification of the hydroxyl group at the C-2' position and SAHA would lose activity after the hydroxamic acid was sealed. The cytotoxicity was principally depending on the reconversion of PTX and SAHA. For the two kinds of co-prodrugs, the rank order of hydrolysis rate and PTX/SAHA reconversion rate was**1a**>**1b**. The cancer cells were incubated with SAHA, PTX, mixture of PTX and SAHA, co-prodrug **1a** or **1b** for 24h to identify if the co-prodrug has the synergistic anticancer effects. Consistent with the stability, **1a** exhibited much more cytotoxic towards all three cells than **1b**. In addition, the $IC_{50}$ of **1a** is a little bit better than PTX alone against MCF-7/ADR which demonstrates that the co-prodrug **1a** may have greater effect against drug-resistant cells (**Table 1**).

**Table 1.** In Vitro Cytotoxicity Assay Data Summary

$IC_{50}$nM (mean± SD)

| Compound | HCT-116 | MCF-7 | MCF-7/ADR |
|---|---|---|---|
| SAHA | 1617.76 ± 201.37 | 939.97 ± 98.02 | 1413.40 ± 110.26 |
| PTX | 2.61 ± 1.67 | <1.5 | >10000 |
| PTX+SAHA | 1.65 ± 0.85 | <1.5 | 369.23 ± 16.63 |
| **1a** | 9.55 ± 3.35 | 1.5 | 1384.45 ± 83.88 |
| **1b** | 144.12 ± 17.09 | 62.35±3.36 | 4019.76 ± 143.35 |

The data are presented as average ± standard error (n=3).

*2.6. Cell cycle analysis*

To investigate the cellular level of the synergistic effects, we have studied the cell cycle variation of HCT-116 cells，MCF-7 and MCF-7/ADR cells treated with PTX/ SAHA mixture, PTX, SAHA, co-prodrug **1a** and **1b**. Cell cycle analysis in these cells indicated that the mixture of PTX and SAHA can induce a marked G2/M cell cycle arrest after 24 h when compared to untreated cells (**Fig. 4**). Both SAHA and PTX induced cell arrests in the G2/M phase. SAHA induced the G2/M cell cycle arrest in a concentration dependent manner in HCT-116 and MCF-7 cells, while there was almost none effect in MCF-7/ADR cells. The **1a** has equivalent effects with PTX in HCT-116 and MCF-7 cells. In addition, there was more induction of G2/M arrest in MCF-7/ADR cells exposed to **1a** than PTX. Hence, the small gap in cell cycle arrest between co-prodrug **1a** and PTX is reasonable and further indicates the effective hydrolyze of **1a** to release PTX and SAHA concurrently for collaborative treatment in tumor cells.

*2.7. Western blot analysis*

On the basis of the cytotoxicity and cell cycle arrest experiments in HCT-116, MCF-7 and MCF-7/ADR cells, we next further determined the HDAC inhibitor activities of the co-prodrugs. Inhibition of HDAC is usually indicated by tubulin acetylation state. The cells were incubated with PTX, SAHA, the mixture of PTX and SAHA, **1a** and **1b** for 10h and 24h. In addition, cells

without any treatment were used as control. Compared to treatment of SAHA or **1b** alone, Western blot analysis showed hyperacetylation of tubulin after treating with **1a** or PTX. The results indicated that co-prodrug **1a** exhibited greater efficacy in inhibiting HDAC towards HCT-116, MCF-7 and MCF-7/ADR cells (**Fig. 5**).

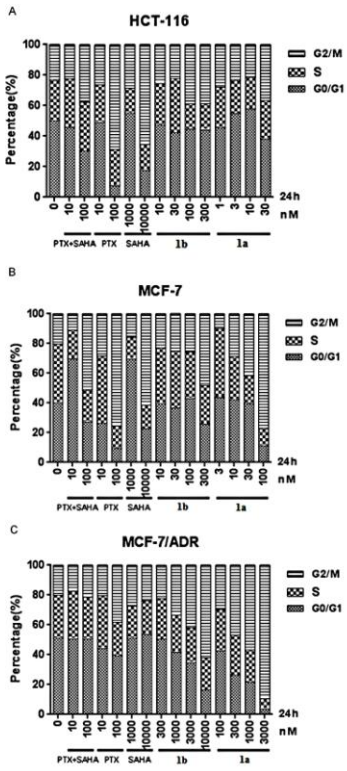



**Fig. 4.** Cell cycle analysis treated with PTX/SAHA mixture, PTX, SAHA, co-prodrug **1b** and **1a**.(A) HCT-116 cells; (B) MCF-7cells; (7) MCF-7/ADR cells.

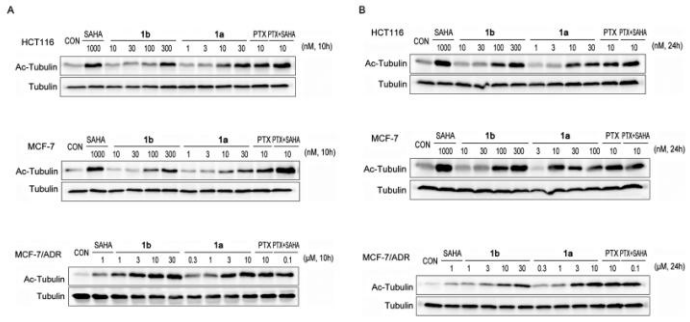



**Fig. 5.** Effect of individual compounds on tubulin depolymerization of HCT-116, MCF-7 and MCF-7/ADR cells.

### *2.8. Characterization of co-prodrug nanomicelles*

The EE (%) and DL (%) of the co-prodrug nanomicelles are 95.6% ± 0.2% and 19.1% ± 0.1%, respectively.

Unlike blood vessels of healthy tissue, tumor blood vessels have enhanced permeability and retention (EPR) effect allowing the size between 20-200 nm particles through. Therefore, Particle size plays a vital role for nanomicelles that affects their in vivo performance and pharmacokinetics. The mean particle size was 72.5 nm, as shown in **Fig. 6A**. And the main hydrodynamic diameter of the nanomicelles was between 20 and 100 nm. These results illustrated that the nanomicelles suffice for passing through the tumor blood vessels. Besides, the zeta potential of nanomicelles showed negative potential at around - 11.5 mV, suggesting the nanomicelles were capable of lengthening the circulation time in tumor because nanomicelles with negative surface charges are inclined to agglomerate due to interaction with protein.

The morphology of the nanomicelles was measured by TEM, as shown in **Fig. 6B**. It can be seen from the figure that the **1a** nanomicelles are spherical structure with a particle size of 20-100nm, which is consistent with the results of the dynamic light scattering (DLS) test.

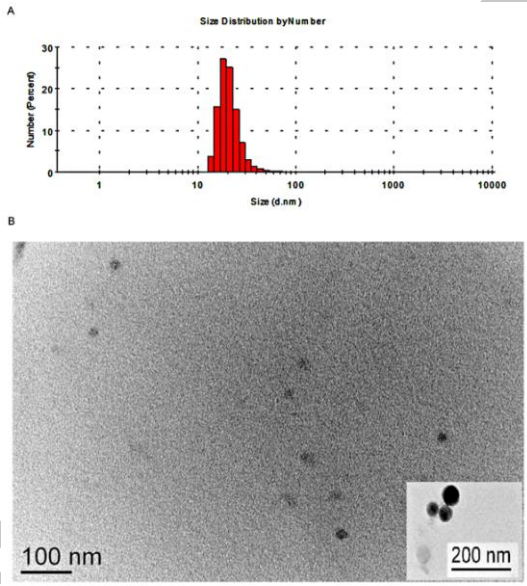



**Fig. 6.** (A) Size and size distribution of **1a** nanomicelles; (B) transmission election microscopy (TEM) of **1a** nanomicelles.

### *2.9. Colloidal stability of nanomicelles*

The colloidal stability of the nanomicelles was investigated by measuring the variation of mean particle size. The nanomicelles were diluted with PBS and placed at 25 °C for 48 h. The results demonstrated that the nanomicelles are stable at this condition with no obvious variation of the mean particle size and PDI was observed as shown in **Fig. 7.**

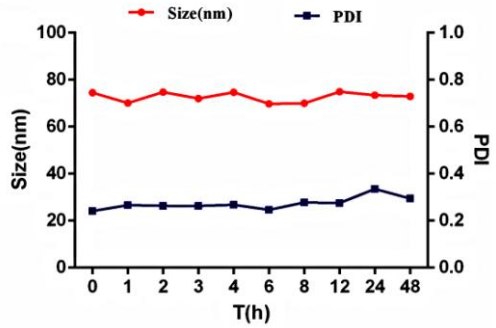



**Fig. 7.** Variation of the mean particle size of **1a** nanomicelles at 25 °C in PBS (means ± SD, n=3).

### *2.10. In vitro drug release from the nanomicelles in PBS*

A long retention time of nanomicelles in the bloodstream is often a prerequisite for successful accumulation in tumor. The **1a** nanomicelles were more stable than the original compound in PBS7.4 as shown in **Fig. 8.** PTX and SAHA released slowly and reached the balance within 48 h. The highest release amount of PTX and SAHA were 12.34% and 40.42%, respectively. The

release amount of compound **1a** was 3%, indicating that it can be rapidly reconverted into the original drug when released from the micelles. In general, the compound nanomicelles have obvious sustained release effect, which can reduce the resistance of PTX and play a better anti-tumor effect.

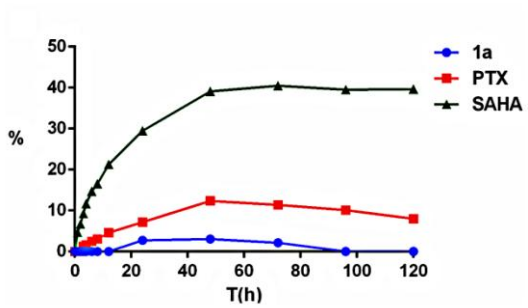



**Fig. 8.** Accumulative drug release of drugs from namomicellers at 37 °C.

*2.11. Cytotoxicity of nanomicelles*

Naturally, your paper should start with a concise and informative title. The inhibitory activity of compounds **1a** nanomicelles, **1a**, SAHA, PTX, the mixture of PTX and SAHA and $mPEG_{2000}$-$PLA_{1750}$ were incubated against the above cells using the SRB assays (**Table 2**). The material $mPEG_{2000}$-$PLA_{1750}$ was found has no cytotoxicity. Compared with the original compound **1a**, the inhibition activity of 1a nanomicelles had increased a lot. In addition, the inhibitory activity of **1a** nanomicelles was equal even better than that of PTX especially in the MCF-7/ADR cells. These results showed that **1a**nanomicelles could reconvert the PTX and SAHA in a slow way and effective concentrations was achieved at the same time.

**Table 2.** In Vitro Cytotoxicity Assay Data Summary

| $IC_{50}$nM(mean± SD) | | | |
|---|---|---|---|
| Compound | HCT-116 | MCF-7 | MCF-7/ADR |
| **1a** | 20.73 ± 5.06 | 19.83 ± 7.94 | 619.96 ± 192.27 |
| **1a** nanomicelles | 3.15 ± 0.59 | 2.88 ± 0.70 | 393.95 ± 193.91 |
| $mPEG_{2000}$-$PLA_{1750}$ | >30000 | >30000 | >30000 |
| SAHA | 1130.15 ± 90.73 | 2030.83 ± 369.92 | 7298.57 ± 963.80 |
| PTX | ≤3.99 | 1.51 ± 0.46 | 1998.28 ± 719.39 |
| SAHA+PTX | 8.89 ± 4.29 | 18.51 ± 10.39 | 1180.62 ± 73.86 |

## 3. Conclusion

We have shown novel PTX-SAHA co-prodrugs which were conjugated by glycine or succinic acid. The biological assessment of **1a** was much better than **1b** and therefore **1a** was further formed the co-prodrug containing nanomicelles with polymer $mPEG_{2000}$-$PLA_{1750}$ as the carrier. We followed up with cellular testing in previous cell lines to determine the effects of anticancer activity. In these follow-up assays, we inferred that the nanomicelles can be used in the treatment of drug-resistant MCF-7/ADR by releasing PTX and SAHA concurrently and maintain effective concentration for a long while in the tumor site. The **1a** co-prodrug nanomicelles possessed several superiorities, including (1) prolonged blood circulation and increased accumulation at the tumor site; (2) sufficiently intracellular drug release, which are contributed to remarkably PTX-resistance reversion efficacy in vitro. The PTX-SAHA co-prodrug and its' nanomicelles with the advantages of anti-drug resistance are potential to be a new generation of synergistic therapy.

## 4. Experimental

*4.1. Materials and methods*

$^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX-400 MHz spectrometer (400 and101 MHz, respectively) using $CDCl_3$, or DMSO-$d_6$ as solvents with TMS as an internal standard. Chemical shifts were reported as d (ppm) and spin-spin coupling constants as J (Hz) values. The mass spectra (MS) were recorded on a Finnigan MAT-95 mass spectrometer. Column chromatography was performed with silica gel (200–300 mesh). All of the starting materials are commercially available and were used without further purification.

Vorinostat (SAHA) was synthesized before. Paclitaxel (PTX) was purchased from Shanghai biocompound medical technology Co., Ltd. BOC-glycine, Succinic anhydride, 4-Dimethyl-aminopyridine (DMAP), 1,1'-carbonyldiimidazole(CDI) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) were all obtained from Energy Chemical Reagent Co. (Shanghai ,China). All other chemical reagents used were of analytical grade quality.

*4.2. Synthesis of the PTX-SAHA co-prodrugs*

*4.2.1. Synthesis of compound **4***

Under the protection of nitrogen, Compound **2** (SAHA) (1g, 3.78mmol) was dispersed in $CH_2Cl_2$ (20 mL), and then CDI (0.61g, 3.78mmol) was added. After stirring for 1 h at room tempetature to form active intermediate 3, BOC-glycine (0.5g, 3.78 mmol) was added and stirred for 10h continually. The reaction mixture was diluted with $CH_2Cl_2$ and washed with 0.2M HCl, dried $Na_2SO_4$, filtered. The filtrate was concentrated under reduced pressure. The residue was purified using silica gel column chromatography with $CH_2Cl_2$ and MeOH to give **4** (1.05 g, 66%) as a white solid. m.p.126-128°C。$^{1}H$ NMR (400 MHz, DMSO) δ 11.41 (s, 1H), 9.84 (s, 1H), 8.01 (t, *J* = 5.8 Hz, 1H), 7.58 (d, *J* = 7.8 Hz, 2H), 7.27 (t, *J* = 7.9 Hz, 2H), 7.01 (t, *J* = 7.3 Hz, 1H), 3.68 (d, *J* = 6.1 Hz, 2H), 2.28 (t, *J* = 7.4 Hz, 2H), 2.06 (t, *J* = 7.2 Hz, 2H), 1.54 (dd, *J* = 16.0, 6.9 Hz, 4H), 1.41 (s, 9H), 1.29 (s, 4H)；MS (ESI): m/z Calcd for $C_{21}H_{31}N_3O_6$ $[M+H]^+$: 422.2, found: 422.1.

*4.2.2. Synthesis of compound **5***

Compound **4** (1.05 g, 2.49 mmol) was dissolved in 30%TFA in $CH_2Cl_2$ (10 mL) at 0 °C for 0.5h. After stirring for 1.5 h at room temperature, the reaction mixture was concentrated directly under reduced pressure. The residue was purified using silica gel column chromatography with $CH_2Cl_2$ and MeOH to give **5** (90%) as a white solid. m.p.143-145°C. $^{1}H$ NMR (400 MHz, DMSO) δ 12.68 (s, 1H), 11.41 (s, 1H), 9.84 (s, 1H), 7.98 (t, *J* = 5.8 Hz, 1H), 7.58 (d, *J* = 7.8 Hz, 2H), 7.28 (t, *J* = 7.9 Hz, 2H), 7.01 (t, *J* = 7.4 Hz, 1H), 3.71 (d, *J* = 6.0 Hz, 2H), 2.29 (t, *J* = 7.4 Hz, 2H), 2.06 (t, *J* = 7.2 Hz, 2H), 1.54 (dd, *J* = 16.6, 6.8 Hz, 4H), 1.29 (s, 4H).$^{13}C$ NMR (101 MHz, DMSO) δ 171.12, 169.95, 155.43, 139.31, 128.58, 122.86, 118.99, 42.29, 36.33, 31.86, 28.28, 24.96, 24.63；MS (ESI): m/z Calcd for $C_{17}H_{23}N_3O_6$ $[M+H]^+$: 366.1, found: 366.2.

*4.2.3. Synthesis of compound **1a***

To a solution of compound **5** (0.213g, 0.586 mol) in $CH_2Cl_2$ (10 mL) were added EDCI (0.112 g, 0.586 mmol) and HOBT (0.079 g, 0.586 mmol). After stirred for 0.5 h at 0 °C, paclitaxel (0.506 g, 0.586 mmol) was added and stirred for 15h at room temperature. The mixture was diluted with $CH_2Cl_2$ and washed

with water, dried over $Na_2SO_4$ and evaporated to dryness to give crude product, which was further purified by silica gel column chromatography with $CH_2Cl_2$ and MeOH to give **1a** (0.132 g, 18%) as a white solid. m.p.147-149°C。$^1$H NMR (400 MHz, $CDCl_3$) δ 11.02 (s, 1H), 9.86 (s, 1H), 8.09 (t, *J* = 15.5 Hz, 4H), 7.76 – 7.64 (m, 3H), 7.55 (dd, *J* = 16.7, 8.0 Hz, 5H), 7.45 (d, *J* = 6.7 Hz, 3H), 7.35 (q, *J* = 7.8 Hz, 4H), 7.28 (s, 1H), 7.24 (s, 1H), 7.13 (t, *J* = 6.8 Hz, 1H), 7.05 (t, *J* = 7.3 Hz, 2H), 6.89 (s, 1H), 6.21 (s, 1H), 5.95 (s, 1H), 5.78 (s, 1H), 5.57 (d, *J* = 6.7 Hz, 1H), 5.35 (d, *J* = 8.0 Hz, 1H), 4.93 (d, *J* = 9.7 Hz, 1H), 4.33 (d, *J* = 16.9 Hz, 1H), 4.24 (d, *J* = 8.5 Hz, 1H), 4.12 (t, *J* = 9.4 Hz, 1H), 3.92 (s, 1H), 3.54 (d, *J* = 5.2 Hz, 1H), 3.14 (d, *J* = 5.8 Hz, 1H), 2.88 (s, 1H), 2.57 (s, 1H), 2.25 (s, 9H), 2.16 (s, 3H), 1.81 (d, *J* = 31.4 Hz, 6H), 1.45 (d, *J* = 14.5 Hz, 3H), 1.38 – 1.18 (m, 8H), 1.10 (d, *J* = 14.0 Hz, 7H).$^{13}$C NMR (101 MHz, $CDCl_3$) δ 202.42, 171.22, 171.03, 170.76, 170.39, 167.96, 167.58, 165.85, 154.72, 141.04, 137.25, 135.31, 133.16, 132.91, 131.31, 130.91, 129.13, 128.18, 127.86, 127.59, 127.49, 126.69, 126.19, 123.06, 118.95, 83.18, 80.20, 77.74, 76.34, 76.22, 76.02, 75.70, 75.17, 74.60, 73.65, 71.24, 69.81, 57.23, 53.96, 45.26, 41.88, 36.03, 34.80, 33.54, 31.59, 28.68, 27.33, 27.00, 25.52, 24.28, 21.47, 20.61, 20.14, 13.53, 8.64；HRMS (ESI): m/z Calcd for $C_{64}H_{72}N_4O_{19}$, $[M+Na]^+$: 1223.4689, found: 1223.4688.

*4.2.4. Synthesis of compound **6***

Under the protection of nitrogen, paclitaxel (0.1 g, 0.117 mmol) was dissolved in $CH_2Cl_2$ (20 mL), and then pyridine (0.044 g, 0.433 mmol), succinic anhydride (0.016 g, 0.163 mmol) and DMAP (0.014 g, 0.117 mmol) were added. After stirred for 13h at room temperature, the mixture was poured into water, adjusted to slightly acidic pH and extracted with $CH_2Cl_2$. The organic layer was washed with brine, dried over $Na_2SO_4$ and filtered. The filtrate was concentrated under reduced pressure. The residue was purified using silica gel column chromatography with $CH_2Cl_2$ and MeOH to give **6** (0.065 g, 58%) as a white solid.m.p.178-180°C。$^1$H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, *J* = 7.6 Hz, 2H), 7.75 (d, *J* = 7.5 Hz, 2H), 7.61 (t, *J* = 7.2 Hz, 1H), 7.52 (d, *J* = 7.3 Hz, 3H), 7.38 (t, *J* = 15.8 Hz, 7H), 7.05 (d, *J* = 9.2 Hz, 1H), 6.25 (dd, *J* = 18.3, 9.3 Hz, 2H), 5.99 (d, *J* = 8.7 Hz, 1H), 5.68 (d, *J* = 6.7 Hz, 1H), 5.53 (s, 1H), 4.97 (d, *J* = 9.3 Hz, 1H), 4.48 – 4.39 (m, 1H), 4.31 (d, *J* = 8.3 Hz, 1H), 4.19 (d, *J* = 8.3 Hz, 1H), 3.80 (d, *J* = 6.7 Hz, 1H), 2.66 (dd, *J* = 11.7, 6.8 Hz, 2H), 2.57 (d, *J* = 6.9 Hz, 3H), 2.44 (s, 3H), 2.36 (dd, *J* = 15.1, 9.3 Hz, 1H), 2.18 (d, *J* = 21.7 Hz, 4H), 1.88 (d, *J* = 24.8 Hz, 5H), 1.67 (s, 3H), 1.20 (t, *J* = 25.3 Hz, 6H)；MS (ESI): m/z Calcd for $C_{51}H_{55}NO_{17}[M+H]^+$: 954.3, found: 954.4.

*4.2.5 Synthesis of compound **1b***

Compound **4** (0.068 mmol) was synthesized as described above. The compound **6**(0.065 g, 0.068 mmol) was added and stirred for 10h at room temperature. The reaction mixture was poured into water and extracted with $CH_2Cl_2$. The organic layer was washed with brine, dried over $Na_2SO_4$ and filtered. The filtrate was concentrated under reduced pressure. The residue was purified using silica gel column chromatography with $CH_2Cl_2$ and MeOH to give **1b** (0.046 g, 57%) as a white solid. m.p.144-146°C. $^1$H NMR (400 MHz, $CDCl_3$) δ 9.28 (s, 1H), 8.13 (d, *J* = 7.4 Hz, 2H), 7.79 (d, *J* = 7.7 Hz, 2H), 7.62 (t, *J* = 7.1 Hz, 1H), 7.58 – 7.34 (m, 12H), 7.29 (s, 3H), 7.08 (t, *J* = 7.2 Hz, 1H), 6.27 (s, 1H), 6.16 (t, *J* = 8.5 Hz, 1H), 5.95 (s, 1H), 5.66 (d, *J* = 6.8 Hz, 1H), 5.49 (d, *J* = 4.2 Hz, 1H), 4.96 (d, *J* = 9.4 Hz, 1H), 4.42 (s, 1H), 4.30 (d, *J* = 8.2 Hz, 1H), 4.18 (d, *J* = 8.3 Hz, 1H), 3.78 (d, *J* = 7.0 Hz, 1H), 2.78 (dd, *J* = 23.6, 7.2 Hz, 4H), 2.58 – 2.48 (m, 2H), 2.40 (s, 3H), 2.30 (t, *J* = 7.0 Hz, 2H), 2.22 (s, 3H), 2.12 (s, 2H), 2.06 – 1.96 (m, 1H), 1.95 – 1.83 (m, 4H), 1.76 (s, 1H), 1.66 (d, *J* = 3.2 Hz, 7H), 1.59 (s, 2H), 1.34 (s, 4H), 1.16 (d, *J* = 35.0 Hz, 6H).$^{13}$C NMR (101 MHz, $CDCl_3$) δ 203.80, 171.68, 171.28, 170.47, 170.35, 169.94, 168.29, 167.42, 167.00, 142.68, 137.96, 136.84, 133.76, 133.63, 132.79, 131.95, 130.23, 129.22, 129.07, 128.99, 128.72, 128.60, 127.34, 126.96, 124.25, 119.81, 84.43, 81.03, 79.06, 77.35, 77.24, 77.03, 76.72, 76.42, 75.60, 75.04, 74.67, 72.10, 71.93, 58.46, 53.24, 45.61, 43.14, 37.06, 35.55, 35.33, 32.38, 29.71, 28.95, 28.09, 27.93, 27.13, 26.76, 25.05, 24.55, 22.66, 22.06, 20.85, 14.83, 9.62；HRMS (ESI): m/z Calcd for $C_{64}H_{72}N_4O_{19}[M+Na]^+$: 1222.4725, found: 1222.4724.

*4.3. HPLC analysis of PTX-SAHA co-prodrugs*

Chromatographic detection was carried out by using Agilent 1200 high performance liquid chromatography (HPLC, Agilent, PaloAlto, CA) with a Diamonsil C18 (250 mm×4.6 mm) column. The detection was conducted at 254 nm (UV detector) with the column temperature at room temperature. The sample volume for each injection was 50 μL. The gradient elution was applied with a mobile phase of acetonitrile (ACN) containing 0.1% formic acid and water containing 0.1% fomic acid with a constant flow rate of 1mL/min: 0–15 min (5% ACN to 95% ACN), 15–25 min (95% ACN). An external standard method was established by HPLC in order to determine the content of each compound.

*4.4. In vitro drug release of PTX-SAHA co-prodrugs in PBS*

PTX-SAHA co-prodrug solutions with concentration of 10 μM/L were diluted to 50 μM/mL by 10 mM PBS buffer (pH 7.4/6.0). After incubation at 37 °C for 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h,12 h and 24 h, the areas of PTX-SAHA co-prodrugs and other species were detected by HPLC as described in Section 4.3. Then, each content was calculated by the standard concentration curve, which was obtained by an external standard method.

*4.5. In vitro drug release of PTX-SAHA co-prodrugs in plasma*

PTX-SAHA co-prodrug solutions with concentration of 100μM/L were diluted to 100μM/mL by plasma (human / mouse). After incubation at 37°C for 0.5h, 1h, 2h, 3h, 4h, 5h and 6h,50μL reaction mixture was quenched by 50μL cold ACN and centrifuged at 30000 rpm×5 min. The supernatant was detected by HPLC as described in Section 4.3. Then, each compound was calculated by the concentration-area standard curve, which was obtained by an external standard method.

*4.6. Cytotoxicity of **1a** and **1b** co-prodrugs*

The in vitro Cytotoxicity assay of **1a** and **1b** was evaluated against human colorectal cell line HCT-116, breast cancer cell lines MCF-7 and drug-resistant breast cancer cell lines MCF-7/ADR using an SRB assay. Briefly, the cells at logarithmic phase were seeded into 96-well plates ($1.0\times10^4$cells per well) in 100μL culture medium. Cells were treated in triplicate with gradient concentrations of test drugs and incubated at 37°C for 72 h. The growth inhibitory effects on the cell lines were measured with SRB assay subsequently. The drug concentration required for 50% growth inhibition ($IC_{50}$) of tumor cells was determined from dose–response curves.

*4.7. Cell cycle assay*

Representative treatment groups were harvested at 24 h. Cells at logarithmic phase were seeded at $2 \times 10^5$ per cells in 6-well plates and treated with compounds at the indicated concentration or with vehicle as a control. After 24 h, these cells were washed three times with cold PBS, treated with trypsin, neutralized with nutrient solution, centrifuged and discarded the supernatent. Following, these cells were washed with cold PBS for once, 300 μl PBS and 700 μl ethanol were added and fixed for overnight at 4 °C. Subsequently, the cells were centrifuged (450 rpm×5 min), discarded ethanol, washed with PBS and discarded supernatent.

At last, the cells were precipitated with 500 µl PBS containing 10 µg/mL RNase and 10 µg/mL PI, stained with propidium iodide (10 µg/mL) for 30 min and then analyzed using Flowjo.

*4.8. Western blot analysis*

Cells at logarithmic phase were seeded in 6-well plates ($3.0 \times 10^5$ cells per well) and cultured overnight. Compounds were added to the wells and cells were incubated at 37 °C for 24 h. Cells without any treatment were used as control. The cells were washed with cold PBS three times, harvested and resuspended in RIPA lysis buffer. The lysates were boiled for 10 min, mixed and collected the protein. Proteins were separated on SDS-polyacrylaminde gel and separated by electrophoresis. The semidry method was used for transferring the proteins to NC membrane. Following, the proteins were stained with ponceaux, sealed for 1 h with confining liquid after marking the proteins. After incubation with corresponding primary antibody at 4 °C overnight, the membrane was eluted with eluent three times. Following, membrane was incubated with secondary antibody which marked with horse radish peroxidase (HRP) for 1 h at room temperature, washed with TBST three times and visualized using ChemiDoc MP detection system (Bio-Rad).

*4.9. Preparation and characterization of **1a** co-prodrug nanomicelles*

Thin-film hydration method was used to prepare nanomicelles. 75 mg compound **1a** and 300 mg $mPEG_{2000}$-$PLA_{1750}$ copolymer were dissolved in 12 mL acetonitrile. The mixture was evaporated slowly under reduced pressure for 2 h and continued to vacuum drying for 24 h to remove organic solvents completely. The mixture skeleton was heated to 50°C until it turned to transparent gel samples completely, then 15 mL ultrapure water was added with vibration adequately. Then the light blue emulsion was filtered with 0.22 µm microfiltration membrane, lyophilized and stored at 4 $^{o}$C.

After the co-prodrug was extracted from the micelles by ACN, the final concentration of co-prodrug was measured by RP-HPLC analysis. The encapsulation efficiency (EE) and drug loading (DL) of the co-prodrug nanomicelles were calculated by the following equations:

$$EE\ (\%) = W_{encapsulated} / W_{input} \times 100\%$$

$$DL\ (\%) = W_{encapsulated} / W_{total} \times 100\%$$

Where, $W_{input}$ is the amount of co-prodrug was added before preparation, $W_{encapsulated}$ is the amount of co-prodrug encapsulated in the nanomicelles after purification and $W_{total}$ is the total amount of co-prodrug nanomicelles after purification.

The mean particle size, size distribution, polydispersity index (PDI) and zeta ( $\zeta$ ) potential were measured by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments,UK). The samples were prepared to a concentration of 1mg/mL with distilled water.

The morphology of the nanomicelles was observed by transmission electron microscopy (TEM) (JM-2100, Japanese). The nanomicelles were diluted with distilled water and dropped onto a 300 mesh copper grid coated with a thin carbon film. The grids were dried at room temperature and observed by TEM subsequently.

*4.10. Colloidal stability of 1a co-prodrug nanomicelles*

The colloidal stability of nanomicelles was investigated by measuring variation of mean particle size and PDI of the nanomicelles. The nanomicelles were diluted with PBS to maintain the final concentration of the **1a** co-prodrug nanomicelles was 1 mg/mL and placed at room temperature for 2 days. The experiment was performed in triplet.

*4.11. In vitro drug release from **1a** co-prodrug nanomicelles in PBS*

Release of PTX, SAHA and **1a** from the nanomicelles in PBS (pH 7.4) was measured by RP-HPLC as described in Section 4.3. The nanomicelles (2 mg/mL) was dissolved in PBS enclosed in dialysis tube (MWCO2000). The dialysis tube was placed at 10 mL PBS solution kept in a THZ-C isothermal shaker at 37°C and 100 rpm. At the predetermined time point, 50µL of the sample solution was withdrawn, and same amount of PBS was added immediately. The accumulative drug release was measured by RP-HPLC.

*4.12. Cytotoxicity of 1a co-prodrug nanomicelles*

The in vitro Cytotoxicity assay of **1a** co-prodrug nanomicelles was evaluated as described in section 4.6.

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## Supplementary Material

Supplementary data related to this article can be found at

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**Highlights**

- Design of PTX and SAHA co-prodrugs are based on the synergistic antitumor effect.
- In this study, we successfully bound PTX and SAHA to form co-prodrugs **1a** and **1b**.
- **1a** was further prepared to nanomicelles with $mPEG_{2000}$-$PLA_{1750}$ as the carrier.
- PTX-SAHA co-prodrug nanomicelles are promising for treatment of drug-fast cancer.