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Cancer Cell-Selective Targeting by Arylcyclopropylamine-Vorinostat Conjugates

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ABSTRACT: Anticancer drug delivery by small molecules offers a number of advantages over conventional macromolecular drug delivery systems. We previously developed phenylcyclopropylamine (PCPA)-drug conjugates (PDCs) as small-molecule-based drug delivery vehicles for targeting lysine-specific demethylase 1 (LSD1)-overexpressing cancers. In this study, we applied this PDC strategy to the HDAC-inhibitory anticancer agent vorinostat. Among three synthesized PCPA or arylcyclopropylamine (ACPA)-vorinostat conjugates **1**, **9**, and **32**, conjugate **32** with a 4-oxybenzyl linker showed sufficient stability in buffer solutions, potent LSD1 inhibition, efficient LSD1-dependent vorinostat release, and potent and selective antiproliferative activity towards LSD1-expressing human breast cancer and small-cell lung cancer cell lines. These results indicate that the conjugate selectively releases vorinostat in cancer cells. A similar strategy may be applicable to other anticancer drugs.

KEYWORDS: anticancer drug, vorinostat, prodrug, LSD1, targeted therapy

Chemotherapy, including targeted therapy with anticancer agents, is one of the most important treatment methods for cancer. However, cancer chemotherapy is frequently associated with severe side effects, because many anticancer agents show poor selectivity for cancer cells over normal cells. To reduce the side effects of cancer chemotherapy, several drug delivery and prodrug strategies, such as antibody-drug conjugates (ADCs) and small molecule-drug conjugates (SMDCs), have been developed.[1–3] However, ADCs suffer from several limitations, such as poor tissue penetration, immunogenicity, and high cost, due to their macromolecular structure,[4] while SMDCs also have several disadvantages, such as severe side effects and low bioavailability.[5] Moreover, ADCs and SMDCs release anticancer drugs in an uncontrolled fashion, due to the *in vivo* instability of their thiosuccinimide and disulfide linkers.[6,7] Therefore, there remains a need to develop new strategies for the targeted delivery and release of anticancer agents.

In this context, we previously proposed phenylcyclopropylamine (PCPA)-drug conjugates (PDCs) as small-molecule-based drug delivery vehicles.[8-10] PCPA is a well-known inhibitor of lysine-specific demethylase 1 (LSD1); it forms an adduct with FAD, a coenzyme of LSD1, through one-electron oxidation by FAD, followed by radical-radical reaction with release of an ammonia molecule by hydrolysis of the imine intermediate (Figure 1a).[11-18] We utilized this reaction mechanism to develop PDCs targeting LSD1, which is overexpressed in various cancer cells. The PCPA-FAD adduct is formed with concomitant LSD1-dependent generation of the amine intermediate, followed by intramolecular cyclization and release of the drug in the cells (Figure 1b). Although PDCs still have concerns about bioavailability or stability, they are useful as molecules which selectively release a drug in cancer cells. Here, we applied this PDC strategy to a histone deacetylase (HDAC) inhibitor. We focused on vorinostat (Figure 2) as a representative HDAC inhibitor that is clinically used for the treatment of cutaneous T-cell lymphoma.[19,20] Vorinostat has several undesirable side effects, including thrombocytopenia, hyperglycemia, fatigue, diarrhea, and nausea.[21] Although clinical trials of vorinostat to treat hematological and solid tumors have been reported, its efficacy was only moderate due to dose-limiting toxicity.[22-25] We considered that this issue might be overcome by developing conjugates that release vorinostat selectively in cancer cells, thereby reducing the toxicity of vorinostat to normal cells. Herein, we report the rational design and synthesis of conjugates of vorinostat and arylcyclopropylamine (ACPA), and their biological evaluation as cancer cell-selective targeting agents.

With the aim of reducing the toxicity of vorinostat, we initially designed PCPA-vorinostat conjugate **1** which is composed of PCPA, an ethylaminocarbonyl linker, and vorinostat

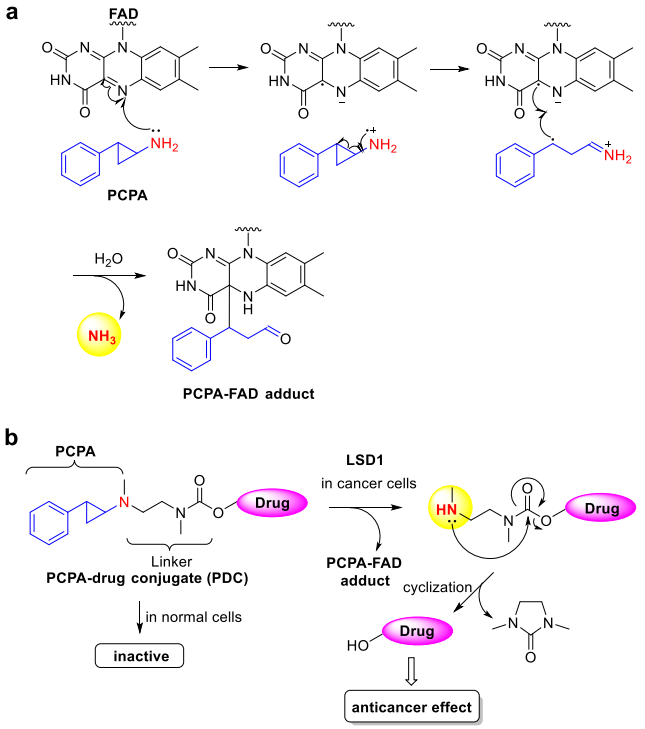

Figure 1. (a) Mechanism of LSD1 inhibition by PCPA. (b) Design concept of PCPA-drug conjugates (PDCs) as small-molecule-based drug delivery vehicles.

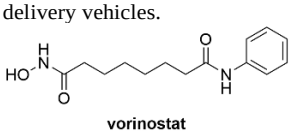

Figure 2. Structure of vorinostat.

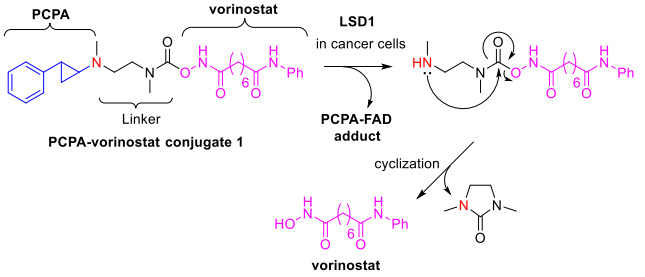

Figure 3. Expected mechanism of release of vorinostat from PCPA-vorinostat conjugate **1**.

(Figure 3). Conjugate **1** was expected to show cytotoxicity toward LSD1-overexpressing cancer cells by releasing vorinostat, while it should be less cytotoxic to normal cells, which express lower levels of LSD1. Before experimentally testing conjugate **1**, we simulated its binding mode to LSD1 by using Glide software. We tried to dock two enantiomers of the *trans*-cyclopropylamine conjugate into the pocket where it can react with FAD, because the *trans*-form is generally used for LSD1 inhibitors. However, docking poses were obtained for the (1*R*, 2*S*)-form, but not for the (1*S*, 2*R*)-form. The most stable pose and its g-score are shown in Figures S1, S2a,b, and Table S1. The benzene ring of PCPA was located at a pocket formed by Thr 335, Ala 539, Tyr 761, and Ala 809 of LSD1. The NH group of the anilide in vorinostat formed a hydrogen bond with the carboxylate of Glu 559. These results suggest that at least one enantiomer of conjugate **1** could bind to LSD1 and release vorinostat through the mechanism shown in Figure 3. Therefore, for convenience, we decided to synthesize and evaluate conjugate **1** as a racemic mixture (Scheme S1). However, we found that synthesized compound **1** is unstable in HEPES buffer solutions (pH 7.4) at 37°C (Figure S3), probably due to hydrolysis of the carbamate, so we could not investigate its biological activity.

To improve the stability of **1**, we designed PCPA-vorinostat conjugate **9**, in which vorinostat is coupled with PCPA through a linker with a 4-oxybenzyl group (Figure 4 and Scheme S2). This was inspired by the work of Jung's group on nitroreductase-mediated release of LSD1 inhibitors from prodrugs.[26] We expected that the carbamate of compound **9** would be more resistant to hydrolysis than that of **1**, because the leaving-group ability of the phenol in **9** is weaker than that of the hydroxamic acid in **1**. As shown in Figure 4, conjugate **9** was predicted to be recognized by LSD1 through the formation of a PCPA-FAD adduct, thereby releasing an amine. Vorinostat should be released by intramolecular cyclization and subsequent 1,6-elimination (Figure 4). Next, we confirmed that one enantiomer of **9** could bind to LSD1 by means of docking simulation. The most stable conformation and its g-scores are shown in Figures S1, S2c,d, or Table S1. Similarly to the case of **1**, the benzene ring of PCPA was located at the pocket formed by Thr 335, Ala 539, Tyr 761, and Ala 809, and the anilide in vorinostat was positioned in a region where its NH group can form a hydrogen bond with Glu 379. Thus, conjugate **9** was expected to act as a prodrug of vorinostat, targeting cancers in which LSD1 is overexpressed. This compound was prepared as a racemic mixture, and its stability in buffer solution (pH 7.4) was investigated. As expected, it was stable in the buffer solution (data not shown). We then evaluated its LSD1-inhibitory activity. Indeed, PCPA-vorinostat conjugate **9** potently inhibited LSD1 with an IC_{50} of 1.29 μM, being 19 times more potent than PCPA (Table 1). Next, we performed HPLC analysis to confirm the release of vorinostat mediated by LSD1 inhibition by compound **9** (Figure 5). A mixture of compound **9** and LSD1 or FAD in assay buffer was incubated for 24 h and then analyzed by HPLC. While the peak corresponding to vorinostat was observed in the mixture of **9** and LSD1 (Figure 5a,c), the peak intensity was very low in the mixture of **9** and FAD (Figure 5a,b). These results strongly

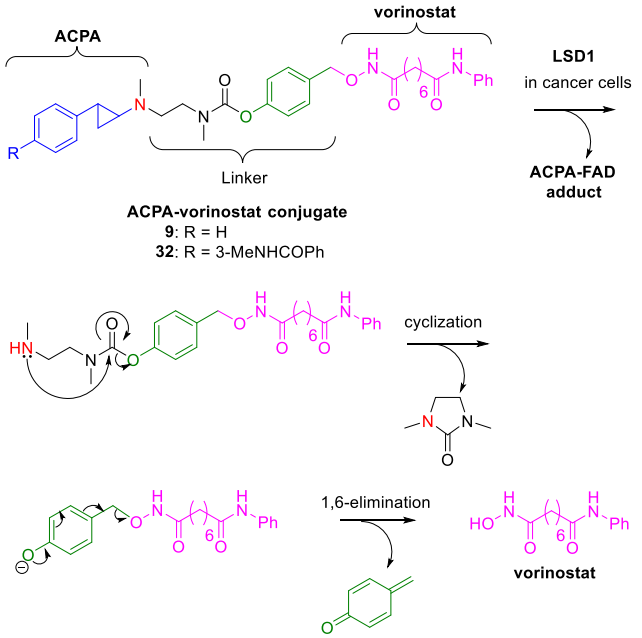

Figure 4. Expected mechanism of the release of vorinostat from arylcyclopropylamine (ACPA)-vorinostat conjugates **9** and **32**.

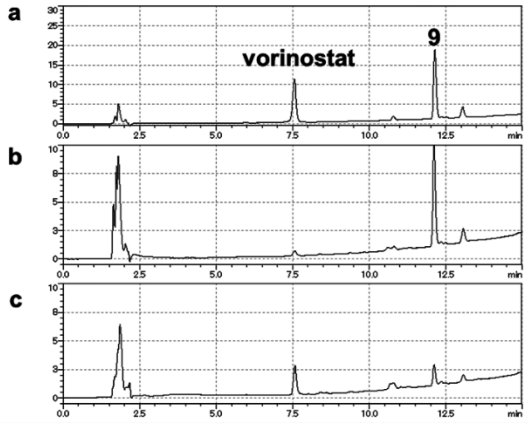

Figure. 5 Detection of vorinostat generated from PCPA-vorinostat conjugate **9** in the presence of LSD1; (a) mixture of authentic vorinostat (7.5 min) and **9** (12.2 min); (b) mixture of **9** and FAD; (c) mixture of **9** and LSD1.

suggest the occurrence of LSD1-dependent release of vorinostat from conjugate **9**. Next, we evaluated the antiproliferative activity of **9** toward LSD1-overexpressing MDA-MB-231 breast cancer cells[27] and human mammary epithelial cells (HMEC), which are normal, non-cancerous cells with a low level of LSD1 expression.[8] Vorinostat inhibited the growth of MDA-MB-231 cells with a GI_{50} of 2.73 μM (Table 1). It also impaired the viability of HMEC cells with an IC_{50} of 10.7 μM, and its selectivity index (SI, IC_{50} for HMEC cells/GI_{50} for MDA-MB-231 cells) was only 3.92 (Table 1). Although PCPA-vorinostat conjugate **9** did not affect the viability of HMEC cells (IC_{50} > 100 μM), it showed weak activity against MDA-MB-231 cells (GI_{50} = 34.3 μM). We considered that the reason for the weak antiproliferative activity might be that the LSD1-inhibitory activity of **9** is not sufficient to release vorinostat efficiently in MDA-MB-231 cells.

To improve the LSD1-inhibitory activity and antiproliferative activity toward MDA-MB-231 cells, we next designed compound **32** in which the phenyl group of **9** is replaced with a 3-MeNHCOPh group (Figure 4 and Scheme S3). Compound **32** is expected to show more potent LSD1 inhibition, because we previously reported that a PCPA-based LSD1 inhibitor containing a 3-MeNHCOPh group is more potent than one bearing a phenyl group.[28] As with conjugates **1** and **9**, we performed a docking study of **32** to LSD1. The docking simulation indicated that both enantiomers of **32** can bind to the catalytic site of LSD1 (Figures S1, S2e–h, and Table S1). Though the conformations of the isomers are different, their biphenyl structures are located in the region surrounded by Thr 335, Ala 539, Tyr 761, and Ala 809, and the *N*-methyl amide groups form hydrogen bonds with the carboxylate of Asp 555; the conformation of the biphenyl moiety in (1*R*, 2*S*)-**32** was similar to and that of the parent LSD1 inhibitor which has the same stereochemical configuration (Figures S2ef, S4ab, and Table S1), whereas that of (1*S*, 2*R*)-**32** was different from that of the parent one (Figures S2gh, S4cd, and Table S1). The anilide in vorinostat of each isomer can also form a hydrogen bond with Glu 559. These results suggest that both enantiomers can be expected to inhibit LSD1, followed by the release of vorinostat. Thus, we prepared racemic and enantiomeric conjugates **32** as shown in Schemes S3 and S4 and examined its LSD1-inhibitory activity. As expected, the inhibitory activity of racemic **32** toward LSD1 was much higher (IC_{50} = 0.126 μM) than that of **9** (IC_{50} = 1.29 μM) and was similar to that of its optically active compounds (1*R*, 2*S*)-**32** and (1*S*, 2*R*)-**32** (Table 1). Therefore, we tested racemic **32** for further studies. The HPLC data shown in Figure 6 revealed that 42% of compound **32** released vorinostat when a mixture of **32** and LSD1 was incubated in assay buffer for 24 h, while incubation with FAD was ineffective (Figure 6). Encouraged by these data, we next investigated the cancer-cell-selective antiproliferative activity. As shown in Table 1, conjugate **32** displayed potent antiproliferative activity toward human breast cancer MDA-MB-231 cells and small cell lung cancer NCI-H526 cells, which both overexpress LSD1, with GI_{50}s of 4.49 μM and 1.00 μM, respectively. Conjugate **32** was eight times more potent than conjugate **9** for inhibition of MDA-MB-231 cell growth (Table 1). Furthermore, conjugate **32** was inactive towards HMEC cells (IC_{50} > 100 μM). Thus, the selectivity of **32** for cancer cells was much higher than that of **9** or vorinostat (SI values: vorinostat 3.92; **9** > 2.92; **32** > 22.3 for MDA-MB-231 cells, vorinostat 22.2; **32** > 100 for NCI-H526 cells) (Table 1). In addition, we tested the cancer cell growth inhibitory activities of 4-(chloromethyl)phenyl acetate (4-CMA) which releases para-quinone methide in cells.[29] Its GI_{50} values were 2.05 μM for MDA-MB-231 cells and 2.02 μM for NCI-H526 cells (Table 1). The effect of conjugate **32** on the accumulation of methylated and acetylated histone H3 was also evaluated using Western blot analysis. As expected, compound **32** promoted both methylation of Lys 4 of histone H3 (H3K4) and acetylation of H3K9 (Figure 7). Because HDAC1-inhibitory activity of **32** is approximately 40 times lower than that of vorinostat (Table S2), conjugate **32** should not directly inhibit HDAC in cells. We also confirmed that the combined treatment with vorinostat and NCD38, an LSD1 inhibitor,[13] showed a synergistic effect and the further combined treatment with 4-CMA provided an additive effect (Figure S5). These results indicate that **32** strongly inhibited the cancer cell growth presumably via LSD1 inhibition by the ACPA moiety of **32**, HDAC inhibition by the released vorinostat, and cytotoxicity of para-quinone methide released from **32** (Figure 4).

In conclusion, we designed and synthesized three PCPA/ACPA-vorinostat conjugates as small-molecule-based drug delivery vehicles. Among them, ACPA-vorinostat conjugate **32** potently inhibited LSD1 and efficiently released vorinostat upon binding to LSD1. Moreover, conjugate **32** selectively inhibited the growth of breast cancer cells and small cell lung cancer cells without exhibiting cytotoxicity toward normal cells. Further studies are currently in progress in our laboratory to assess the generality of our PDC strategy.

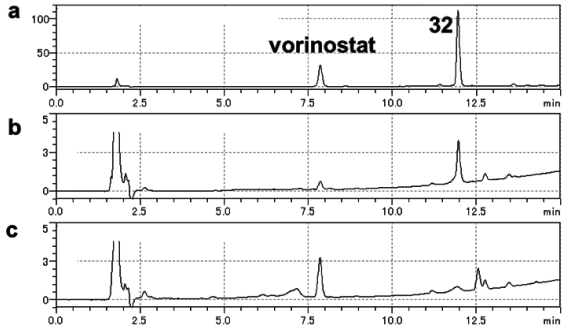

Figure 6. Detection of vorinostat generated from ACPA-vorinostat conjugate **32** in the presence of LSD1; (a) mixture of authentic vorinostat (7.5 min) and **32** (12.2 min); (b) mixture of **32** and FAD; (c) mixture of **32** and LSD1. The release yield of vorinostat was determined to be 42% by quantitative analysis.

Table 1. LSD1-inhibitory activity and antiproliferative activity of PCPA, vorinostat, and PCPA/ACPA-vorinostat conjugates 9 and 32.[a]

Compound	IC_{50} (μM)	GI_{50} (μM)		IC_{50} (μM)	Selectivity Index (SI)	
	LSD1	MDA-MB-231 (breast cancer cells)	NCI-H526 (lung cancer cells)	HMEC (normal cells)	IC_{50} (HMEC)/ GI_{50} (MDA-MB-231)	IC_{50} (HMEC)/ GI_{50} (NCI-H526)
PCPA	24.8±2.8	798±75	N.D.	1420 ±67.9	1.78	N.D.
vorinostat	N.D.	2.73±0.13	0.481±0.18	10.7 ±0.61	3.92	22.2
9	1.29±0.21	34.3±1.30	N.D.[b]	>100	>2.92	N.D.
(*rac*)-**32**	0.126±0.011	4.49±0.10	1.00±0.46	>100	>22.3	>100
(1*R*, 2*S*)-**32**	0.444±0.119	N.D.	N.D.	N.D.	N.D.	N.D.
(1*S*, 2*R*)-**32**	0.457±0.030	N.D.	N.D.	N.D.	N.D.	N.D.
4-CMA	N.D.	2.05±0.18	2.02±0.20	N.D.	N.D.	N.D.

[a]Values are the mean ± S.D. of at least three experiments. [b]N.D. = not determined.

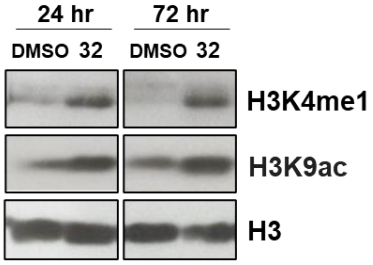

Figure 7. Western blot analysis of histone acetylation and methylation in NCI-H526 cells treated with 16 μM ACPA-vorinostat conjugate **32**.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.XXXXXXX. Synthetic experimental details, characterization data, and biological assay protocols (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

4-CMA, 4-(chloromethyl)phenyl acetate; ADC, antibody-drug conjugates; PCPA, phenylcyclopropylamine; PDC, PCPA-drug conjugate; LSD1, lysine-specific demethylase 1; ACPA, arylcyclopropylamine; SMBC, small molecule-drug conjugates; HDAC, histone deacetylase; HEPES, 4-(2-hydroxyethyl)-1-

piperazineethanesulfonic acid; HMEC, human mammary epithelial cells; GI_{50}, half-maximum growth-inhibitory concentration.

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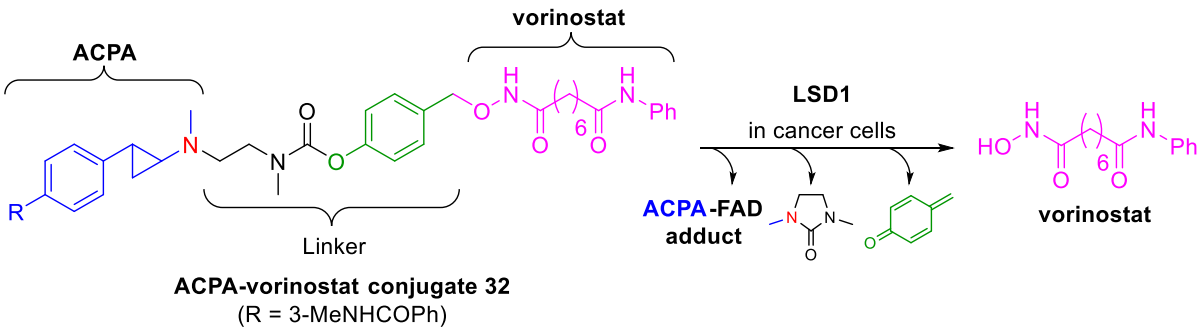
Table of Contents Graphic



ACPA-vorinostat conjugate 32
(R = 3-MeNHCOPh)

vorinostat: cancer cell selectivity = 3.92 (MDA-MB-231); 22.2 (NCI-H526)
32: **cancer cell selectivity > 22.3** (MDA-MB-231); **> 100** (NCI-H526)