Prodrugs

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Targeting Cancer with PCPA-Drug Conjugates: LSD1 Inhibition-Triggered Release of 4-Hydroxytamoxifen

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Abstract: *Targeting cancer with small molecule prodrugs should help overcome problems associated with conventional cancer-targeting methods. Herein, we focused on lysine-specific demethylase 1 (LSD1) to trigger the controlled release of anticancer drugs in cancer cells, where LSD1 is highly expressed. Conjugates of the LSD1 inhibitor trans-2-phenylcyclopropylamine (PCPA) were used as novel prodrugs to selectively release anticancer drugs by LSD1 inhibition. As PCPA-drug conjugate (PDC) prototypes, we designed PCPA-tamoxifen conjugates* ***1a*** *and* ***1b****, which released 4-hydroxytamoxifen in the presence of LSD1 in vitro. Furthermore,* ***1a*** *and* ***1b*** *inhibited the growth of breast cancer cells by the simultaneous inhibition of LSD1 and the estrogen receptor without exhibiting cytotoxicity toward normal cells. These results demonstrate that PDCs provide a useful prodrug method that may facilitate the selective release of drugs in cancer cells.*

The chemotherapy of cancer usually provides a certain level of beneficial therapeutic effect, while simultaneously causing serious side effects because of the cytotoxicity of the employed drugs toward normal cells.[1] To reduce the adverse effects of anticancer drugs, several methodologies including drug delivery systems for anticancer drugs have been developed to date.[2] Such examples include antibody-drug conjugates (ADCs)[2b] and small molecule-drug conjugates (SMDCs),[2c] which show both potent and selective cytotoxicity toward cancer cells expressing a specific protein (Supporting Information, Figure S1 a,b). However, owing to their large structure, ADCs and SMDCs suffer from several limitations, such as immunogenicity and high costs.[2b,c] However, poor absorption, insolubility, and toxicity can be circumvented by using prodrug strategies (Supporting Information, Figure S1 c).[2d] Prodrugs enzymatically or spontaneously undergo a chemical reaction at a target site and release a drug to show an actual drug action. This controlled release of anticancer drugs in cancer cells can provide good clinical results in cancer therapy. Herein, we report a new method for prodrugs based on small molecules.

With the aim of designing novel prodrugs that target cancer cells, we focused on the FAD-dependent lysine-specific demethylase 1 (LSD1),[3] since it has already attracted much attention as a biomarker and as a molecular target for cancer therapy. LSD1 demethylates predominantly mono- and dimethylated lysine 4 of histone 3 (H3K4me1/me2).[3a] Furthermore, LSD1 is not only highly expressed in various cancer cell lines (Supporting Information, Figure S2) such as neuroblastoma,[3b] glioma,[3c] and breast cancer cells[3d] but is also involved in the cell growth of cancer. It has been reported that relative to unaffected tissue, the expression of LSD1 is significantly increased in the tumor tissue of patients with various forms of cancer, including breast cancer.[3e,f] In addition, targeted recruitment of LSD1 in promoter regions is associated with the proliferation of cancer cells.[3g,h] To date, a number of LSD1 inhibitors have been identified,[4] and most of them are based on *trans*-2-phenylcyclopropylamine (PCPA) (Supporting Information, Figure S3). As shown in Figure 1 a, PCPA inhibits LSD1 through a reaction sequence

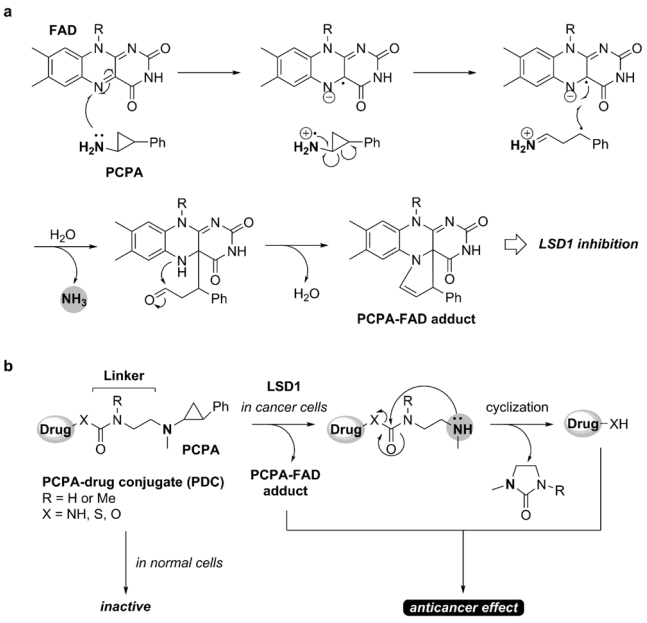

Figure 1. a) The chemical structure of PCPA and its inhibition mechanism for LSD1. b) Concept for a prodrug strategy based on PCPA-drug conjugates (PDCs).

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including a single-electron transfer, radical opening of the cyclopropyl ring, and formation of a covalent bond with FAD.[5] During the inactivation of LSD1, the imine intermediate is hydrolyzed, which leads to the extrusion of the nitrogen atom of PCPA in the form of ammonia. Based on the mechanism of the PCPA-induced inhibition of LSD1, we propose that PCPA-drug conjugates (PDCs) should be able to target cancer cells, in which LSD1 is highly expressed (Figure 1b). As shown in Figure 1b, PDCs should be recognized by LSD1 and deactivate it in a similar manner to PCPA itself, that is, through a single-electron transfer mechanism. Subsequently, the drug should be released together with the linker moiety of the PDCs through hydrolysis of the imine intermediate, and an ensuing intramolecular cyclization should eventually separate the linker from the drug. Thus, PDCs could serve as prodrugs that selectively release an anticancer drug upon binding to LSD1. This method would induce significantly lower levels of side effects, as such molecules are inactive against normal cells, where the expression of LSD1 is lower. Furthermore, PDCs can expect the combined effect of LSD1 inhibition and the release of an anticancer drug to further increase their anticancer activity.

As a proof-of-concept study, we designed PCPA-tamoxifen conjugates **1a** and **1b** (Figure 2) as PDC prototypes. These consist of PCPA, a linker, and 4-hydroxytamoxifen (4OHT),[6] that is, an anti-estrogen agent for breast cancer treatment (Figure 2a). According to the mechanism shown in Figure 2b, we expected **1a** and **1b** to exhibit anticancer

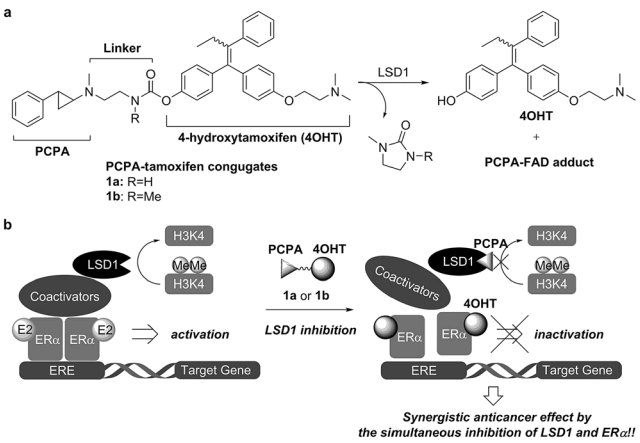

Figure 2. a) Design of PCPA-tamoxifen conjugates **1a** and **1b**. b) Scheme for the expected mechanism of action for **1a** and **1b** in LSD1/ERα-positive breast cancer cells.

activity, and theoretical simulations suggested that the recognition of **1a** and **1b** by LSD1 should be effective, as isomers of the conjugates are likely to fit well within the active pocket of LSD1 (Supporting Information, Figure S4). Subsequently, 4OHT should be released in LSD1-expressing breast cancer cells to antagonize the estrogen receptor α (ERα). Conjugates **1a** and **1b** should furthermore induce a synergistic anticancer effect by the simultaneous inhibition of LSD1 and ERα, as the interaction of LSD1 and ERα in breast cancer cells promotes cell growth.[3g,h]

Scheme S1 in the Supporting Information shows the synthetic route to **1a** and **1b**, which were obtained as diastereomers, as one isomer of 4OHT easily isomerizes under physiological conditions.[7]

Initially, we examined the recognition of **1a** and **1b** by LSD1 and their ability to inhibit the catalytic activity of the enzyme. As expected, the inhibitory activity toward LSD1 was much higher for **1a** and **1b** relative to PCPA (IC_{50} values: PCPA, 24.8 μM; **1a**, 0.339 μM; **1b**, 0.155 μM; Supporting Information, Table S1). In addition, **1a** and **1b** exhibited weak activity toward other FAD-dependent monoamine oxidases such as MAOA and MAOB (Supporting Information, Table S1). These results suggest that **1a** and **1b** are recognized selectively by LSD1, thereby effectively achieving its inhibition. Moreover, a kinetic analysis suggested that this inhibition is irreversible (Figure 2a).[5] The kinetic analysis, performed using two different substrate concentrations, exhibited nonlinear progress curves, which ultimately reach a plateau, thus indicating that **1a** and **1b** inhibit LSD1 in a time-dependent manner (Supporting Information, Figures S5 and S6). Furthermore, significantly higher k_{inact}/K_i values were observed for **1a** and **1b** relative to those of PCPA, which demonstrates the highly selective recognition and inhibition of LSD1 by **1a** and **1b** (Supporting Information, Table S2). Subsequently, we carried out a MALDI-TOF-MS analysis to examine the potential inhibition of LSD1 by **1a** and **1b** through the formation of PCPA-FAD adducts (Figure 2a). Peaks at $m/z = 918$ and 900, corresponding to the PCPA-FAD adduct and its dehydrated form, respectively (Figure S7b,d), were observed in the presence of LSD1 but not in its absence (Supporting Information, Figure S7a,c). The results obtained from the kinetic and MALDI-TOF-MS analyses are thus consistent with the irreversible inhibition of LSD1 by **1a** and **1b** through the formation of PCPA-FAD adducts as shown in Figure 2a.

To confirm that the release of 4OHT is triggered through the inhibition of LSD1 by **1a** and **1b**, we also carried out an ESI-MS analysis. If **1a** and **1b** engage with FAD on the active site of LSD1, 4OHT should be released upon formation of the PCPA-FAD adduct (Figure 2a). As expected, the release of 4OHT was detected by ESI-MS in the presence of LSD1 (Supporting Information, Figure S8b,d) and not observed in its absence (Supporting Information, Figure S8a,c). Moreover, the release was found to be time-dependent (Figure 3a,b and the Supporting Information, Figure S8) and significantly suppressed by known LSD1 inhibitors such as PCPA and NCD38[4b] (Figure 3c and the Supporting Information, Figure S9), which provides further support for our hypothesis that the release of 4OHT depends on the enzyme activity of LSD1.

Because of the simultaneous inhibition of LSD1 and ERα (Figure 2b), **1a** and **1b** were expected to inhibit the growth of breast cancer cells. To evaluate the in-cell activity of **1a** and **1b**, we selected ERα-positive breast cancer MCF7 cells, in which LSD1 is overexpressed (Supporting Information, Figure S2a).

The level of the LSD1 substrate H3K4me2 present in MCF7 cells upon treatment with **1a** and **1b** was examined by western blotting analysis. As shown in Figure 4a, a dose-

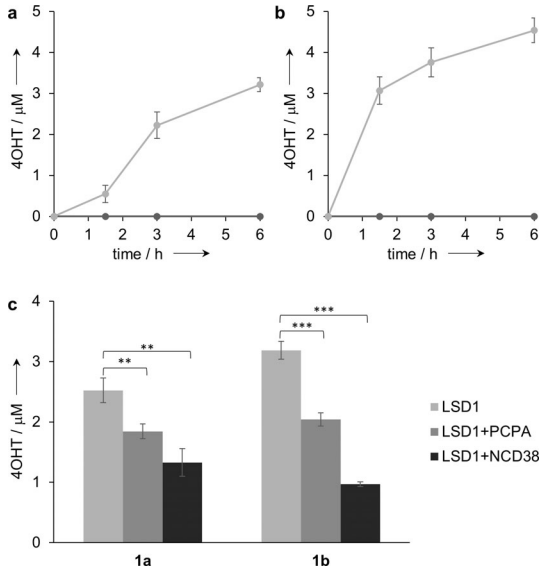

Figure 3. Release of 4OHT from **1a** and **1b**. a) Mixture of **1a** and LSD1 (light circles), mixture of **1a** and FAD in the absence of LSD1 (dark circles); b) mixture of **1b** and LSD1 (light circles), mixture of **1b** and FAD in the absence of LSD1 (dark circles); and c) mixture of **1a**-LSD1 or **1b**-LSD1 in the presence of LSD1 inhibitors PCPA (300 μM) or NCD38 (10 μM). Values represent mean ± SD of at least three experiments, $p < 0.01$ (**) and $p < 0.0001$ (***) (Student's t-test).

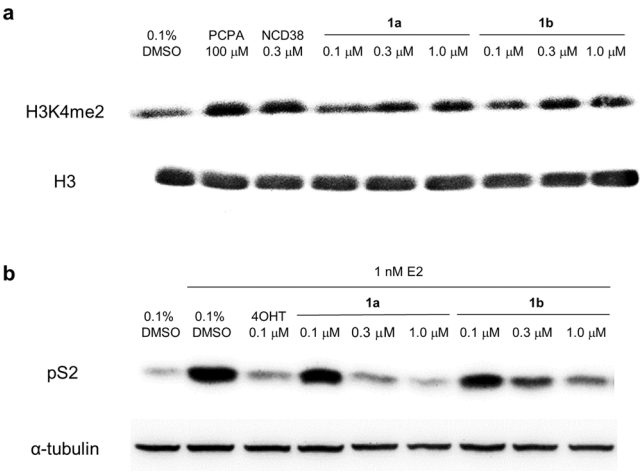

Figure 4. a) Western blot detection of H3K4me2 levels in MCF7 cells treated for 8 h with PCPA, NCD38, **1a**, or **1b**. b) Western blot detection of pS2 levels in MCF7 cells treated for 24 h with 4OHT, **1a**, or **1b** in the presence of E2.

dependent increase of the level of H3K4me2 was observed, which corroborates the active role of **1a** and **1b** in the inhibition of LSD1 in MCF7 cells.

We then evaluated the expression level of pS2, which is a representative ERα-target gene,[8] in MCF7 cells in the presence of the endogenous ER agonist β-17-estradiol (E2). While the expression of pS2 was induced by E2, it was blocked by **1a** and **1b** in a dose-dependent manner (Figure 4b), which suggests that **1a** and **1b** inhibit the function of ERα in MCF7 cells. To dismiss the possibility of a direct interaction of **1a** and **1b** with ERα, which may result in a decreased expression level of pS2, we investigated the affinity of **1a** and **1b** in vitro and found that **1a** and **1b** exhibited significantly lower activity in the ER-binding assays than E2 and 4OHT (Supporting Information, Figure S10). To further dismiss this possibility in cells, we used compounds **S1** and **S2** as negative controls (Supporting Information, Figure S11 and Schemes S2 and S3). As shown in the Supporting Information, Figure S12a, negative controls **S1** without a PCPA moiety exerted little influence on the expression of pS2 induced by E2 in MCF7 cells as compared to **1b**. However, negative controls **S2a** and **S2b**, which contain 3′-hydroxytamoxifen (3′OHT) with a much lower affinity for ERα than 4OHT (Supporting Information, Figure S10),[6b] did not inhibit the expression of pS2 induced by E2 in MCF7 cells (Supporting Information, Figure S12b). Although the ERα-binding affinity of **1a** and **1b** is weaker than 3′OHT (Supporting Information, Figure S10), which exerted little influence on the expression of pS2 induced by E2 in MCF7 cells at 1 μM (Supporting Information, Figure S12c), **1a** and **1b** completely inhibited the ERα function in cells at the same concentration (Figure 4b and the Supporting Information, Figure S12c). To further investigate the inhibition of ERα, we examined the change of pS2 expression levels by a combined treatment of 4OHT with LSD1 inhibitors PCPA and NCD38 and observed a significant decrease of the level of pS2 expression in MCF7 cells (Supporting Information, Figure S13). The results shown in the Supporting Information Figures S10, S12, and S13 rule out the possibility of a direct involvement of **1a** and **1b** with ERα-mediated pS2 expression. Instead, it seems more likely that the interaction between 4OHT released from **1a** and **1b** and ERα is responsible for the decreased level of pS2 expression.

Prior to testing the antiproliferative activity of **1a** and **1b**, we evaluated the effect of the combined treatment of 4OHT with PCPA and NCD38 in MCF7 cells. In the presence of E2, this combination resulted in a higher antiproliferative activity relative to a treatment with each individual agent. In particular, the combined treatment with 4OHT and NCD38, a more selective LSD1 inhibitor than PCPA, showed a synergistic effect (Supporting Information, Figure S14). During our studies on their antiproliferative activity, we observed that 0.1 μM **1a** and **1b** significantly reduced the growth of MCF7 cells stimulated by E2 (Supporting Information, Figure S14). Moreover, for concentrations of 0.01–0.1 μM, **1a** and **1b** showed a dose-dependent antiproliferative activity in the presence of E2 (Figure 5a). The results obtained on the cytotoxicity of **1a** and **1b** toward human mammary epithelial cells (HMEC), that is, non-cancerous cells with a lower expression of LSD1 are also noteworthy (Figure S2a). For concentrations up to 2.5 μM, **1a** and **1b** did not affect the viability of HMEC cells (Figure 5b), which supports the selective inhibition of the growth of ERα-positive breast cancer cells in preference to normal cells.

In conclusion, we propose PDCs, represented by the prototypical PCPA-tamoxifen conjugates **1a** and **1b**, as novel prodrugs based on small molecules. In vitro, **1a** and **1b** irreversibly inactivate LSD1 by forming PCPA-FAD adducts,

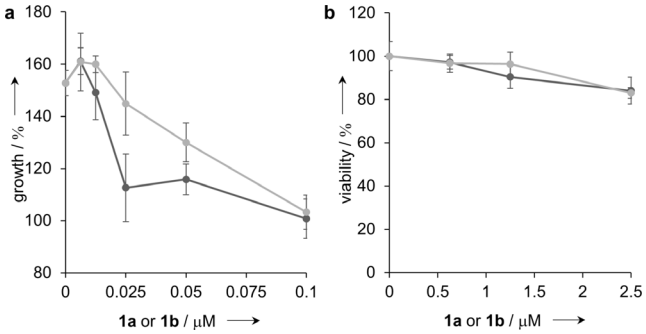

Figure 5. Growth-inhibition activity and cytotoxicity of **1a** (dark circles) and **1b** (light circles). a) MCF7 cells and b) HMEC cells. Values represent mean ± SD of at least four experiments.

under concomitant LSD1-enzyme activity-dependent release of 4OHT. Moreover, **1a** and **1b** inhibit the growth of breast cancer cells by the simultaneous inhibition of LSD1 and ERα without exhibiting cytotoxicity toward normal cells. A tuning of the activity of PDCs by optimizing the structure of the aromatic ring of PCPA and/or the linker appears feasible according to the activity of the drugs.[9] Irreversible LSD1 inhibition may be a concern in terms of efficient targeting through protein turnover. However, the in situ-generated PCPA-FAD adduct can be replaced by endogenous FAD in cancer cells,[10] which reactivates LSD1. In addition to their role as ERα antagonists, PDCs could also be applied to other anticancer drugs such as epigenetic modulators, nuclear receptor ligands, and various cytotoxic drugs.[3g,h] To assess the scope of this concept, further studies on various PCPA-drug conjugates are currently in progress in our laboratory.

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Keywords: drug design · epigenetics · prodrugs · small molecules · targeted cancer therapy

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Communications

Prodrugs

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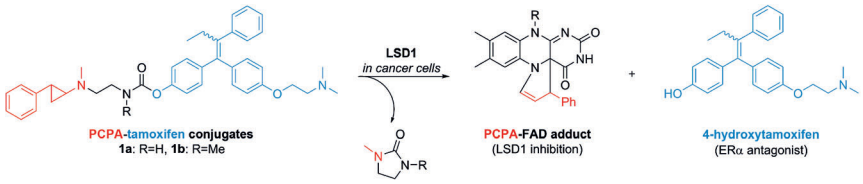
Targeting Cancer with PCPA-Drug Conjugates: LSD1 Inhibition-Triggered Release of 4-Hydroxytamoxifen



I want a new drug: PCPA-drug conjugates (PDCs) were designed as the basis of a new prodrug strategy. The prototype PCPA-tamoxifen conjugates **1a** and **1b** released 4-hydroxytamoxifen upon inhibition of LSD1. While **1a** and **1b** exhibited antiproliferative activity in breast cancer cells by the simultaneous inhibition of LSD1 and ERα, no cytotoxicity was observed in normal cells.