Drug Design

DOI: 10.1002/ange.201303999

Lysine-Specific Demethylase 1-Selective Inactivators: Protein-Targeted Drug Delivery Mechanism**

Daisuke Ogasawara, Yukihiro Itoh, Hiroki Tsumoto, Taeko Kakizawa, Koshiki Mino, Kiyoshi Fukuhara, Hidehiko Nakagawa, Makoto Hasegawa, Ryuzo Sasaki, Tamio Mizukami, Naoki Miyata,* and Takayoshi Suzuki**

Reversible histone methylation, a process controlled by two counteracting enzyme families, the histone methyltransferases and the histone demethylases, plays a pivotal role in the regulation of epigenetic gene expression.[1] Lysine-specific demethylase 1 (LSD1) removes methyl groups from mono- and dimethylated Lys4 of histone H3 (H3K4me1/2) through flavin adenine dinucleotide (FAD) dependent enzymatic oxidation.[2] LSD1 also demethylates H3K9me1/2 in prostate cell lines and in cells infected with herpesviruses.[3] Furthermore, histone demethylation by LSD1 is suggested to be associated with certain disease states, including cancer and herpes simplex infection.[1a,3b,4]

trans-2-Phenylcyclopropylamine (PCPA/Tranylcypromine),[5] which was originally found as an inhibitor of monoamine oxidases (MAOs; also FAD-dependent enzymes), is the best-studied LSD1 inhibitor, and biological studies using PCPA have uncovered important roles of LSD1 in several diseases.[3b,4a,c,d] In addition, several groups, including ours, have reported PCPA derivatives with LSD1 inhibitory activity.[6] While many of these LSD1 inhibitors have been suggested to be potential lead compounds for anticancer agents, most of them have various disadvantages, including poor intracellular activity, insufficient inhibitory potency, or inadequate selectivity for LSD1 over MAO A and MAO B. To overcome these issues, we hypothesized that LSD1 could be potently and selectively inactivated by delivering PCPA directly to the LSD1 active site. Herein we describe the design and synthesis of a series of LSD1 inactivators based upon this concept.

PCPA inhibits LSD1 by a single-electron-transfer mechanism (Figures 1 and 2 A).[5a] In the active site of LSD1, FAD first extracts one electron from the nitrogen atom of PCPA to form a cation radical. Then, opening of the cyclopropyl ring occurs and subsequent covalent bond formation with FAD. In the course of LSD1 inactivation, the nitrogen atom of PCPA is released as an ammonia molecule through hydrolysis of the imine intermediate. Taking this mechanism into account, together with our idea of delivering PCPA directly to the

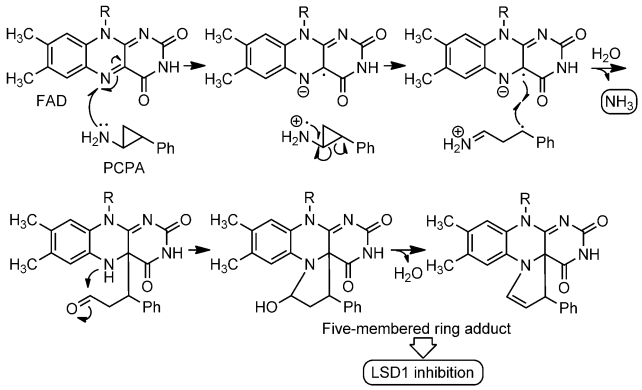

Figure 1. Mechanism of LSD1 inhibition by PCPA.

[*] D. Ogasawara, Dr. Y. Itoh, Prof. T. Suzuki
Graduate School of Medical Science
Kyoto Prefectural University of Medicine, 13 Nishitakatsukasa-cho
Taishogun Kita-ku, Kyoto 603-8334 (Japan)
E-mail: suzukit@koto.kpu-m.ac.jp
D. Ogasawara, Prof. H. Nakagawa, Prof. N. Miyata
Graduate School of Pharmaceutical Sciences
Nagoya City University
3-1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467-8603 (Japan)
E-mail: miyata-n@phar.nagoya-cu.ac.jp
Dr. H. Tsumoto
World-Leading Drug Discovery Research Center, Kyoto University
Kyoto 606-8501 (Japan)
Dr. T. Kakizawa, Dr. K. Fukuhara
Organic Chemistry National Institute of Health Sciences
Tokyo 158-8501 (Japan)
Dr. K. Mino, Dr. M. Hasegawa, Prof. T. Mizukami
Graduate School of Bio-Science
Nagahama Institute of Bio-Science Technology
1226 Tamura-cho, Nagahama, Shiga 526-0829 (Japan)
E-mail: mizukami@nagahama-i-bio.ac.jp
Dr. R. Sasaki
Frontier Pharma Inc., Shiga 526-0829 (Japan)
Prof. T. Suzuki
PRESTO (Japan) Science and Technology Agency (JST)
Saitama 332-0012 (Japan)

[**] We thank Mie Tsuchida, Dr. Yasunao Hattori, and Prof. Kenichi Akaji for their technical support. This work was supported in part by JST PRESTO program (T.S.), a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (T.S. and Y.I.), Hoansha Foundation (T.S), Takeda Science Foundation (T.S.), Naito Foundation (T.S.), and NOVARTIS Foundation (Japan) for the promotion of science (T.S.).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201303999.

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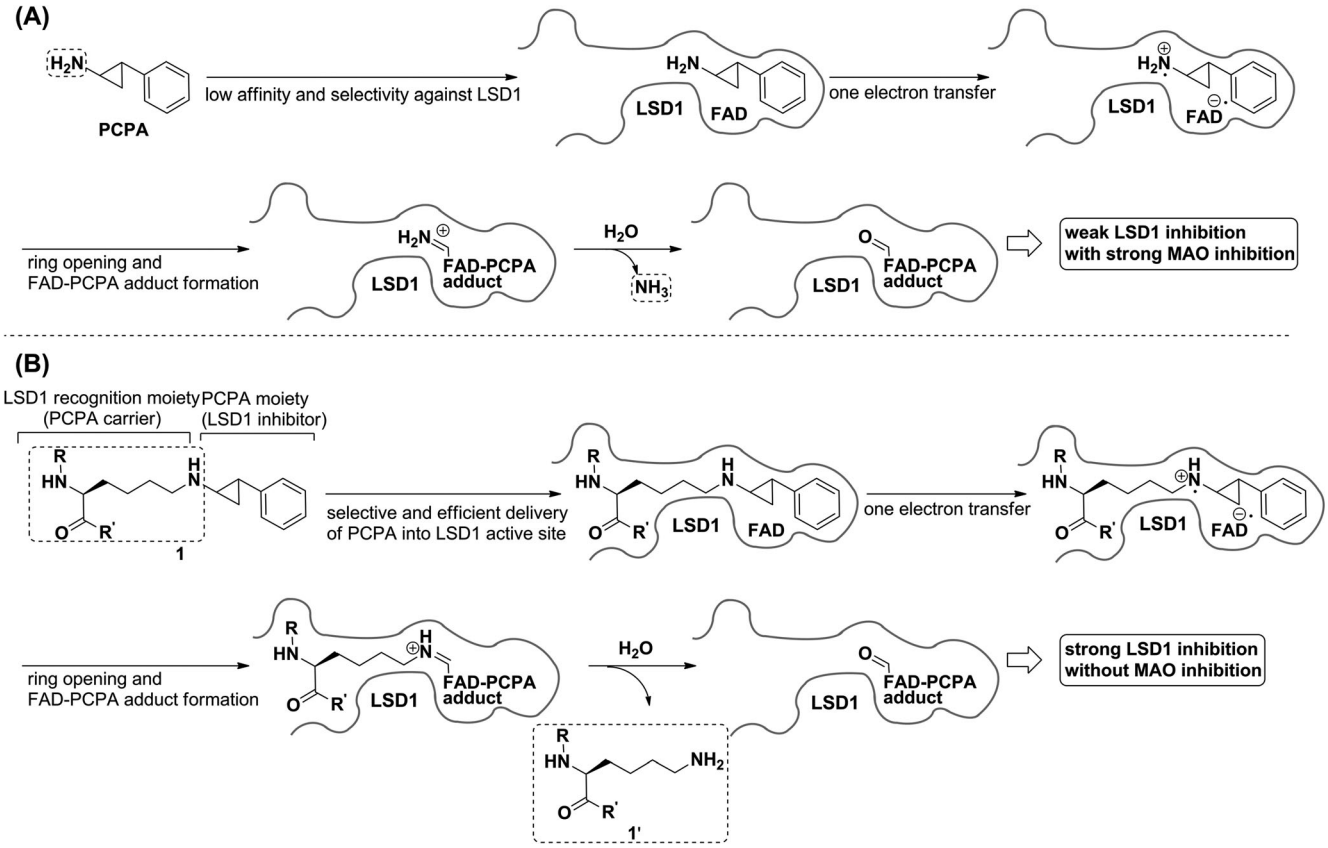

Figure 2. A) Mechanism of LSD1 inhibition by PCPA. B) Putative mechanism of LSD1-targeted delivery of PCPA by **1**.

LSD1 active site, we designed LSD1 inactivators **1** (Figure 2B), in which PCPA is coupled to a lysine carrier moiety at the nitrogen atom. Since methylated lysine is the substrate of LSD1, we expected that the lysine moiety of **1** would be efficiently recognized by LSD1, thus affording high selectivity over MAO A and MAO B. After the PCPA moiety of **1** is carried to the active site of LSD1, we expected that the PCPA moiety would inactivate LSD1 in a similar manner to PCPA itself, through single-electron transfer, radical opening of the cyclopropyl ring, and covalent bond formation with FAD (Figures 1 and 2). Then, the lysine moiety **1'** is expected to be released through hydrolysis of the imine intermediate (Figure 2B). Thus, the lysine moiety of **1** serves as a carrier that delivers PCPA into the active site of LSD1 selectively and efficiently. This approach can be regarded as an LSD1-targeted drug delivery system.

As a proof of concept study, we designed PCPA-Lys-4 H3-21 (**1a**; Figure 3), which bears a PCPA moiety at Lys-4 of a 21-amino-acid LSD1 substrate peptide (H3-21), and evaluated its LSD1-inhibitory activity. We chose the H3-21 peptide as a carrier of PCPA because it is known to be a substrate of LSD1,[2a,7] and several H3-21-peptide-based LSD1 inhibitors were reported.[8] The peptide **1a** was synthesized by displacement of the mesyl group of MesylK4 H3-21 (**2**)[8] with PCPA (see Scheme S1 in the Supporting Information). In a horseradish peroxidase coupled assay,[7] **1a** strongly inhibited LSD1 ($IC_{50} = 0.16\ \mu M$) in a time- and concentration-dependent manner, but did not inhibit MAO A or MAO B ($IC_{50} >$

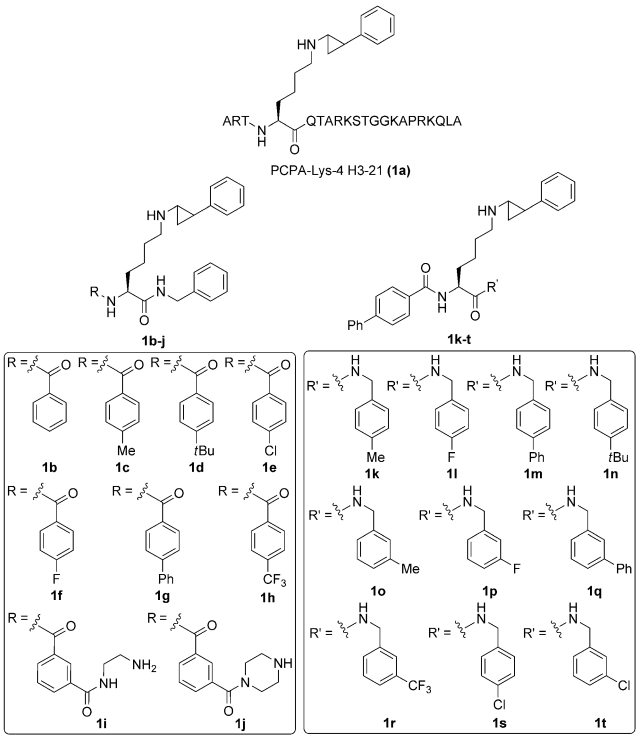

Figure 3. Compounds designed, synthesized, and evaluated in this study.

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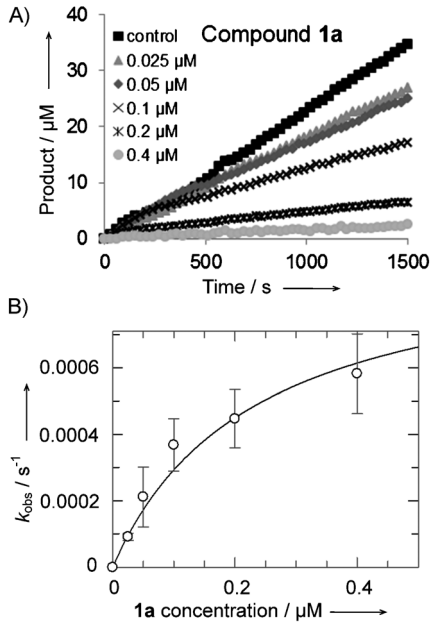

Figure 4. Time- and concentration-dependent inhibition of LSD1 by **1a**. A) Steady-state progress curves obtained for the inactivation of LSD1 by 0 (■), 0.025 (▲), 0.05 (◆), 0.1 (×), 0.2 (*), 0.4 (●) μM **1a**. B) Rate constants (k_{obs}) for the time-dependent inactivation of LSD1 by **1a** were extracted from steady-state progress curve in (A) by single exponential fitting.

100 μM; see Table S1 in the Supporting Information and Figure 4). These results indicate that **1a** is an irreversible LSD1 inactivator, as we had hoped.[9] As expected, the K_i value of **1a** was much smaller than that of PCPA, which indicates that **1a** has a greater binding affinity for LSD1 than does PCPA (Table S1). Next, we performed MALDI MS analysis of the LSD1/**1a** mixture. According to the putative mechanism of LSD1 inhibition by peptide **1a**, the FAD–PCPA adduct and **1′a** should be generated (Figure 2B).[5a] As shown in Figure 5, peaks with *m/z* 918 and 900, corresponding

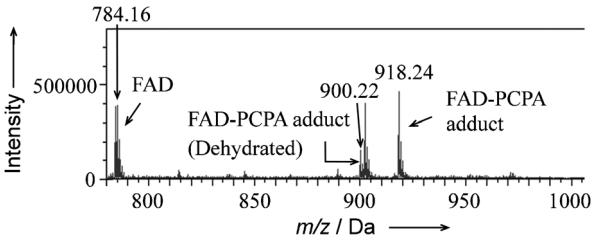

Figure 5. Mass spectrometric detection of FAD PCPA adducts. Mass spectra were obtained from LSD1/**1a** mixture (negative mode).

to the FAD–PCPA adduct and dehydrated adduct, respectively, were observed in the mixture of LSD1 and **1a**. In contrast, no peak corresponding to the FAD–PCPA adduct was observed in control experiments without LSD1 (see Figure S1A in the Supporting Information). Furthermore, the peak corresponding to the released peptide **1′a** (Figure 2B) was also detected in the same mixture (Figure S1B). These mechanistic data strongly support the proposed LSD1-targeted PCPA delivery mechanism (Figure 2B). Next, we evaluated its activity at the cellular level against cervical HeLa and neuroblastoma SH-SY5Y cancer cell lines. We chose these cell lines because LSD1 is overexpressed in both of them, and LSD1 inhibitors have been reported to suppress their cell growth.[4a,6c,d] However, the cellular activity of **1a** was weak (Table S1). We surmise that **1a** has poor cell-membrane permeability, likely a result of the high polarity of its peptide structure. Thus, based on this proof of concept of our LSD1-targeted PCPA delivery strategy, we next sought to apply this strategy to design nonpeptide, small-molecule LSD1 inactivators which would show activity in cell-based assays.

We designed small-molecule, drug-delivery-type LSD1 inactivator candidates, guided by the X-ray crystal structure of LSD1.[10] We designed **1b**, bearing a benzoyl group (R) and benzylamino group (R′; Figure 3), because these groups were expected to fit in the hydrophobic pockets near the entrance of the LSD1 active-site cavity, and should bear the binding affinity to LSD1. We also expected that these small hydrophobic groups would enhance cell membrane permeability. A binding simulation of **1b** with LSD1 using Molegro Virtual Docker 5 confirmed that the benzoyl group of **1b** is located in the hydrophobic pocket delineated by Phe 538, Ala 539, and Phe 692, and the benzylamino group is located in the hydrophobic pocket delineated by Ile 356, Leu 677, and Cys 360 (see Figure S2A in the Supporting Information). Moreover, the PCPA moiety of compound **1b** appears to be located in almost the same place as the FAD–PCPA adduct in the reported X-ray crystal structure (see Figure S3 in the Supporting Information).[5b] This is consistent with the idea that the PCPA moiety of **1b** would be selectively recognized by LSD1 and oxidized by FAD, which would lead to selective inactivation of LSD1 (Figure 2B).

We synthesized **1b** from commercially available Boc-Lys(Cbz)-OH (**3**) as shown in Scheme S2 in the Supporting Information, and the inhibitory activity of **1b** towards LSD1 was evaluated. The compound **1b** exerted potent LSD1 inhibitory activity ($IC_{50} = 0.30$ μM), being at least 100-fold more potent than PCPA itself ($IC_{50} = 31$ μM, which is comparable to that of **1a**; Table S2 in the Supporting Information) without inhibiting MAO A ($IC_{50} > 25$ μM). We also evaluated its cellular activity against HeLa and SH-SY5Y cancer cell lines. As expected, **1b** showed relatively high cell growth inhibitory activity against these cancer cell lines (GI_{50} for HeLa = 35 μM, GI_{50} for SH-SY5Y = 17 μM; Table S2). Encouraged by this finding, we next sought to optimize the lysine moiety of **1b** to improve the activity.

We first set out to optimize the benzoyl group of **1b**. We designed and synthesized **1c–j** which bear substituents at either the *meta* or *para* position of the benzoyl group of **1b** (Figure 3; Figures S2B and S2C, and Schemes S3 and S4 in the Supporting Information). As shown in Table S2 in the Supporting Information, compounds with hydrophobic substituents at the benzoyl group (**1c–h**) showed strong LSD1 inhibitory activity as well as improved growth inhibitory activity towards HeLa and SH-SY5Y cell lines.

Next, we set out to further optimize the benzylamino group of **1g**, which was the most promising compound among

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1c–**h** in terms of potency in the cancer cell growth inhibition assays. We designed and synthesized **1k**–**t** with hydrophobic substituents at either the *meta* or *para* position of the benzylamino group of **1g** (Figure 3; Figure S2D and Schemes S5 and S6 in the Supporting Information). As shown in Table S2 in the Supporting Information, **1k**–**t** showed LSD1 inhibitory activity and further improved cancer cell growth inhibitory activity. Among them, **1s** was the most potent (GI_{50} for HeLa = 3.7 μM, GI_{50} for SH-SY5Y = 1.7 μM).

To confirm the selectivity of these inactivators, we tested the inhibitory activity of selected compounds against MAO A and MAO B. As we expected, while PCPA itself potently inhibited MAO A and MAO B (IC_{50} for MAO A = 2.5 μM, IC_{50} for MAO B = 2.4 μM), **1g**, **1r**, **1s**, and **1t** did not (see Table S3 in the Supporting Information). These results suggest that the lysine moiety of these LSD1 inactivators does not deliver PCPA to the active site of MAO A and MAO B, but rather delivers PCPA specifically to the LSD1 active site with high efficiency.

Having optimized the structure of the lysine moiety of **1b** as a PCPA carrier targeting LSD1, we next investigated the LSD1 inactivation mechanism to confirm that the resulting inactivators indeed inhibit LSD1 by delivering PCPA to the LSD1 active site, as peptide **1a** does. We used **1g** and **1s** in the following mechanistic studies because they showed potent activity in both enzyme assays and cell-based assays. First, we examined whether **1g** and **1s** inhibit LSD1 in a time-dependent manner. As shown in Figure S4 in the Supporting Information, **1g** and **1s** were found to be time-dependent LSD1 inactivators, in accordance with the irreversible mechanism we proposed (Figure 2B).[9] The kinetic parameters of these compounds are shown in Table S4 in the Supporting Information. The k_{inact}/K_i values of **1g** and **1s** were much larger than that of PCPA and comparable to that of **1a**, thus confirming that **1g** and **1s**, as well as **1a**, are much more potent LSD1 inactivators than PCPA. As in the case of peptide **1a**, the K_i values of **1g** and **1s** were greatly improved (more than 120-fold and 48-fold smaller, respectively) compared to that of PCPA. Significantly, the k_{inact} values of **1g** and **1s** remained almost the same as that of PCPA, and the k_{inact} value of **1a** was five-fold less than that of PCPA (Tables S1 and S4). These kinetic parameters indicate that the lysine moiety of **1g** and **1s** contributes to the enhanced LSD1 inhibitory activity of these inactivators by increasing the binding affinity for LSD1 without decreasing the reaction rate of the PCPA moiety with FAD. We surmise that the conformational flexibility of these small-molecule LSD1 inactivators allowed their PCPA moiety to react with FAD in a similar manner to PCPA itself. Next, we performed MALDI MS analysis of the inactivated mixture of LSD1 with **1g** and **1s**. It is expected that inhibition of LSD1 by these inactivators will yield the FAD–PCPA adduct (Figure 2B),[5a] regardless of the structure of the lysine moiety. As shown in Figure S5 in the Supporting Information, peaks with *m*/*z* 918 and 900, corresponding to the FAD-PCPA adduct and the dehydrated adduct, respectively, were observed in the cases of both LSD1/**1g** and LSD1/**1s**. We also successfully detected **1′g** and **1′s**, the lysine moieties released from LSD1/**1g** and LSD1/**1s**, respectively (Figure S6 in the Supporting Information). These mechanistic data strongly support the idea that these small-molecule LSD1 inactivators inhibit LSD1 through efficient and selective delivery of PCPA to the active site of LSD1 with the assistance of their lysine moiety.

In conclusion, we have designed and synthesized a series of novel LSD1 inactivators based on our new concept of LSD1-targeted PCPA delivery. We tested this concept with peptide **1a** and confirmed that **1a** does indeed inhibit LSD1 selectively and efficiently by delivering PCPA directly to the LSD1 active site. The enzyme is then inactivated by FAD-PCPA adduct formation, as in the case of PCPA itself, with release of the carrier peptide **1′a**. We also demonstrated that this strategy could be applied to the design of nonpeptide, small-molecule LSD1 inactivators. Mechanistic studies confirmed that the small-molecule inactivators also inactivate LSD1 by LSD1-targeted PCPA delivery, similar to peptide **1a**. Biological evaluations revealed that these small-molecule compounds are potent LSD1-selective inactivators (comparable to peptide **1a**) in enzyme assays, and they also exhibit potent cell growth inhibitory activities against cancer cell lines. We believe this is the first time that a drug-carrier-type system has been employed to target a specific molecule. These novel LSD1-selective inactivators are considered to be candidates for anticancer agents, as well as bioprobes.

Received: May 10, 2013
Published online: July 3, 2013

Keywords: biological activity · drug delivery · drug design · kinetics · proteins

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