Biochemical, Structural, and Biological Evaluation of Tranylcypromine Derivatives as Inhibitors of Histone Demethylases LSD1 and LSD2

ARTICLE *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · APRIL 2010

Impact Factor: 12.11 · DOI: 10.1021/ja101557k · Source: PubMed

J|A|C|S ARTICLES

Published on Web 04/23/2010

Biochemical, Structural, and Biological Evaluation of Tranylcypromine Derivatives as Inhibitors of Histone Demethylases LSD1 and LSD2

Claudia Binda,[†,#] Sergio Valente,[‡,#] Mauro Romanenghi,[§] Simona Pilotto,[†,‡] Roberto Cirilli,[||] Aristotele Karytinos,[†] Giuseppe Ciossani,[†] Oronza A. Botrugno,[§] Federico Forneris,[†,¶] Maria Tardugno,[‡] Dale E. Edmondson,[⊥] Saverio Minucci,[*,§,▽] Andrea Mattevi,[*,†] and Antonello Mai[*,‡]

Department of Genetics and Microbiology, University of Pavia, Via Ferrata 1, 27100 Pavia, Italy, Department of Drug Chemistry and Technologies, University "La Sapienza", P.le A. Moro 5, 00185 Roma, Italy, European Institute of Oncology, Via Adamello 16, 20139 Milan, Italy, Italian National Institute of Health, Department of Therapeutic Research and Medicines Evaluation, Via Regina Elena 299, 00161 Roma, Italy, Department of Biochemistry, Emory University, 1510 Clifton Road, Atlanta, Georgia 30322, and Department of Biomolecular Sciences and Biotechnologies, University of Milan, Via Celoria, 26, 20133 Milan, Italy

Received February 23, 2010; E-mail: saverio.minucci@ifom-ieo-campus.it; antonello.mai@uniroma1.it; andrea.mattevi@unipv.it

Abstract: LSD1 and LSD2 histone demethylases are implicated in a number of physiological and pathological processes, ranging from tumorigenesis to herpes virus infection. A comprehensive structural, biochemical, and cellular study is presented here to probe the potential of these enzymes for epigenetic therapies. This approach employs tranylcypromine as a chemical scaffold for the design of novel demethylase inhibitors. This drug is a clinically validated antidepressant known to target monoamine oxidases A and B. These two flavoenzymes are structurally related to LSD1 and LSD2. Mechanistic and crystallographic studies of tranylcypromine inhibition reveal a lack of selectivity and differing covalent modifications of the FAD cofactor depending on the enantiomeric form. These findings are pharmacologically relevant, since tranylcypromine is currently administered as a racemic mixture. A large set of tranylcypromine analogues were synthesized and screened for inhibitory activities. We found that the common evolutionary origin of LSD and MAO enzymes, despite their unrelated functions and substrate specificities, is reflected in related ligand-binding properties. A few compounds with partial enzyme selectivity were identified. The biological activity of one of these new inhibitors was evaluated with a cellular model of acute promyelocytic leukemia chosen since its pathogenesis includes aberrant activities of several chromatin modifiers. Marked effects on cell differentiation and an unprecedented synergistic activity with antileukemia drugs were observed. These data demonstrate that these LSD1/2 inhibitors are of potential relevance for the treatment of promyelocytic leukemia and, more generally, as tools to alter chromatin state with promise of a block of tumor progression.

Introduction

Alterations in the structural and functional states of chromatin are involved in the pathogenesis of a variety of diseases.[1] Histone lysine demethylases represent very attractive targets for epigenetic drugs and are gaining increasing attention. A lysine can be mono-, di-, and trimethylated. Each modification on the same amino acid can exert different biological effects.[2] Consistently, the recent discovery of histone lysine demethylases has revealed two types of enzymatic mechanisms.[3] The iron-dependent enzymes can demethylate lysine side chains in all three methylation states. Conversely, the oxidative chemistry that underlies the function of flavin-dependent histone demethylases makes it impossible for these enzymes to act on trimethylated lysine and restricts their activity to mono- and dimethylated substrates.[4]

Mammals contain two flavoenzyme demethylases: LSD1 and LSD2 known also as KDM1A and KDM1B.[5,6] LSD1 is typically associated with the corepressor protein CoREST,[7] and removes methyl groups from mono- and dimethyl Lys4 of histone H3, a gene activation mark. The enzyme is an interesting target for

[†] University of Pavia.
[#] These authors contributed equally.
[‡] University "La Sapienza".
[§] European Institute of Oncology.
[||] Italian National Institute of Health.
[¶] Present address: Crystal and Structural Chemistry, Bijvoet Center for Biomolecular Research, Utrecht University, The Netherlands.
[⊥] Emory University.
[▽] University of Milan.

(1) Best, J. D.; Carey, N. *Drug Discovery Today* **2009**, *15*, 65.
(2) Bhaumik, S. R.; Smith, E.; Shilatifard, A. *Nat. Struct. Mol. Biol.* **2007**, *14*, 1008.
(3) Anand, R.; Marmorstein, R. *J. Biol. Chem.* **2007**, *282*, 35425.
(4) Forneris, F.; Binda, C.; Battaglioli, E.; Mattevi, A. *Trends Biochem. Sci.* **2008**, *33*, 181.

epigenetic drugs as suggested by its overexpression in solid tumors,[8] its role in various differentiation processes,[9] its involvement in herpes virus infection,[10] and its association to histone deacetylase 1, a validated drug-target. LSD2, like LSD1, displays a strict specificity for mono- and dimethylated Lys4 of H3. However, the biology of LSD2 is proposed to differ from that of LSD1 since LSD2 does not bind CoREST and has not been found so-far in any LSD1-containing protein complex.[6,11,12]

LSD1 and LSD2 share a similar catalytic domain (45% sequence identity) that is structurally homologous with the amine oxidases, a class of flavin-dependent enzymes that act on biogenic amines.[4,5] Among these proteins, human monoamine oxidases (MAOs) A and B have been the subject of more than 50 years of research that has led to the development of a multitude of inhibitors including antidepressive and antiparkinson drugs.[13] Their similarity in the catalytic and structural properties prompted the investigation of antiMAO drugs as potential LSD1 inhibitors.[14] It was found that tranylcypromine (Table 1), a MAO inhibitor used as antidepressive drug, is able to inhibit LSD1.[15−18] On this basis, we sought to design compounds that would be more selective for demethylases using tranylcypromine as the lead scaffold. Here, we report the synthesis of a series (more than 40) of new tranylcypromine analogues and a biochemical and biological evaluation of their inhibitory properties with human LSD1, mouse LSD2, human MAO A, and human MAO B (see Supporting Information). The results demonstrate that many of these compounds are effective LSD1 and LSD2 inhibitors, and most importantly, the prototype **14e** (Table 1) exhibits synergistic activities with antileukemia drugs.

Materials and Methods

Syntheses of all tested compounds are described in the Supporting Information. Human recombinant MAO A and MAO B were expressed in *Pichia pastoris* and purified as published.[19] Inhibition assays and K_i values were measured using kynuramine (MAO A) and benzylamine (MAO B) substrates at pH 7.5 according to published procedures (Table 1).[19] Mouse recombinant LSD2 was expressed in *Escherichia coli* and purified as described.[6] Human recombinant LSD1/CoREST were expressed in *E. coli* as separate proteins and co-purified following previously reported procedures.[20] Enzymatic activities and inhibition assays with both demethylases were carried out at pH 7.5−8.0 using methylated H3 peptides.[6,20] Crystals of LSD1/CoREST inhibitor complexes were obtained by cocrystallization under conditions identical to those previously reported for other structural studies on these proteins.[14,20] Diffraction data were measured at the European Synchrotron Radiation Facility and the Swiss Light Source. Data processing and structure refinement were carried out using standard procedures.[20,21] Crystallographic and refinement statistics together with the PDB accession codes are reported in Table S5. Figures were prepared with Pymol (www.pymol.org).

NB4 cells were treated at different concentrations of **14e** (Table 1). Whole cell lysates were subjected to SDS-PAGE, and then immunoblotted using antibodies against different histone modifications (from Abcam: H3K4me2, H3K9me2, H3; antiacetylated H4, T25[22]). Analysis of NB4 cells and murine APL blasts growth and differentiation was performed as described previously.[23,24]

Data Deposition. Coordinates have been deposited with the Protein Data Bank. Accession codes are 2XAF, 2XAG, 2XAH, 2XAJ, 2XAQ, 2XAS (Table S5).

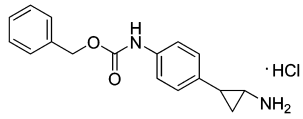
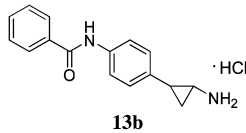
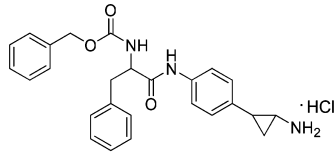
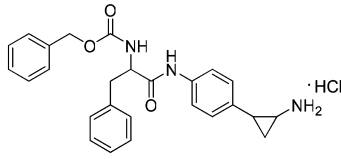
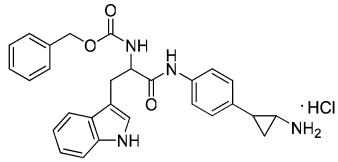
Results

Enantioselectivity of the Inhibition. Tranylcypromine is a racemic mixture of (±)-*trans*-2-phenylcyclopropyl-1-amine·HCl (tPCPA) that covalently inhibits the LSD and MAO enzymes. The first question we addressed in our study was the difference, if any, between the two tPCPA enantiomers with respect to their inhibition activities. (+)- and (−)-tPCPA were synthesized and their absolute configuration determined (see Supporting Information). Biochemical analysis showed that the inversion of the configuration has a marginal effect on inhibition of the two LSD enzymes, whereas it is significant for inhibition of MAO B.[25] In addition, the crystallographic analysis of LSD1/CoREST inhibitor complexes highlighted a surprising feature: the two enantiomers differ both in the binding orientation and nature of the covalent adduct with the flavin (Figure 1−3; Scheme 1). This feature was confirmed using the *para*-brominated derivatives whose electron-rich substituent provided enhanced clarity of the electron density maps (Figure 1). (−)-tPCPA engages its carbonyl carbon in a covalent bond with the flavin N5 atom and positions its phenyl ring in the core of the substrate-binding pocket, stacking above the flavin ring. In contrast, the phenyl ring of the (+)-tPCPA binds in a lateral niche of the substrate-binding pocket and points away from the flavin ring (Figure 1−3). Furthermore, the covalent bond with the flavin N5 atom involves the phenyl-substituted carbon of the inhibitor rather than the carbonyl carbon as observed for the (−)-enantiomer (Scheme 1).

To further analyze these different binding modes, we compared our crystal structures with those previously determined

(5) Shi, Y.; Lan, F.; Matson, C.; Mulligan, P.; Whetstine, J. R.; Cole, P. A.; Casero, R. A.; Shi, Y. *Cell* **2004**, *119*, 941.
(6) Karytinos, A.; Forneris, F.; Profumo, A.; Ciossani, G.; Battaglioli, E.; Binda, C.; Mattevi, A. *J. Biol. Chem.* **2009**, *284*, 17775.
(7) Lee, M. G.; Wynder, C.; Cooch, N.; Shiekhattar, R. *Nature* **2005**, *437*, 432.
(8) Schulte, J. H.; Lim, S.; Schramm, A.; Friedrichs, N.; Koster, J.; Versteeg, R.; Ora, I.; Pajtler, K.; Klein-Hitpass, L.; Kuhfittig-Kulle, S.; Metzger, E.; Schüle, R.; Eggert, A.; Buettner, R.; Kirfel, J. *Cancer Res.* **2009**, *69*, 2065.
(9) Hu, X.; Li, X.; Valverde, K.; Fu, X.; Noguchi, C.; Qiu, Y.; Huang, S. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 10141.
(10) Gu, H.; Roizman, B. *J. Virol.* **2009**, *83*, 4376.
(11) Ciccone, D. N.; Su, H.; Hevi, S.; Gay, F.; Lei, H.; Bajko, J.; Xu, G.; Li, E.; Chen, T. *Nature* **2009**, *461*, 415.
(12) Yang, Z.; Jiang, J.; Stewart, D. M.; Qi, S.; Yamane, K.; Li, J.; Zhang, Y.; Wong, J. *Cell. Res.* **2010**, *20*, 276.
(13) Edmondson, D. E.; Binda, C.; Wang, J.; Upadhyay, A. K.; Mattevi, A. *Biochemistry* **2009**, *48*, 4220.
(14) Yang, M.; Culhane, J. C.; Szewczuk, L. M.; Gocke, C. B.; Brautigam, C. A.; Tomchick, D. R.; Machius, M.; Cole, P. A.; Yu, H. *Nat. Struct. Mol. Biol.* **2007**, *14*, 535.
(15) Lee, M. G.; Wynder, C.; Schmidt, D, M.; McCafferty, D. G.; Shiekhattar, R. *Chem. Biol.* **2006**, *13*, 563.
(16) Schmidt, D. M.; McCafferty, D. G. *Biochemistry* **2007**, *46*, 4408.
(17) Yang, M.; Culhane, J. C.; Szewczuk, L. M.; Jalili, P.; Ball, H. L.; Machius, M.; Cole, P. A.; Yu, H. *Biochemistry* **2007**, *46*, 8058.
(18) Mimasu, S.; Sengoku, T.; Fukuzawa, S.; Umehara, T.; Yokoyama, S. *Biochem. Biophys. Res. Commun.* **2008**, *366*, 15.
(19) Binda, C.; Li, M.; Hubalek, F.; Restelli, N.; Edmondson, D. E.; Mattevi, A. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 9750.
(20) Forneris, F.; Binda, C.; Adamo, A.; Battaglioli, E.; Mattevi, A. *J. Biol. Chem.* **2007**, *282*, 20070.
(21) Collaborative Computational Project, Number 4. *Acta Crystallogr.* **1994**, *D50*, 760.
(22) Ronzoni, S.; Faretta, M.; Ballarini, M.; Pelicci, P.; Minucci, S. *Cytometry, Part A* **2005**, *66*, 52.
(23) Minucci, S.; et al. *Blood* **2002**, *100*, 2989.
(24) Di Croce, L.; Raker, V. A.; Corsaro, M.; Fazi, F.; Fanelli, M.; Faretta, M.; Fuks, F.; Lo Coco, F.; Kouzarides, T.; Nervi, C.; Minucci, S.; Pelicci, P. G. *Science* **2002**, *295*, 1079.
(25) Zirkle, C. L.; Kaiser, C.; Tedeschi, D. H.; Tedeschi, R. E.; Burger, A. *J. Med. Pharm. Chem.* **1962**, *91*, 1265.

Table 1. Inhibition of Selected Tranylcypromine Derivatives against LSD1, LSD2, and Monoamine Oxidases

Compound	LSD1 [a,b] K_i (μM)	LSD2 [a,b] K_i (μM)	MAO A [a,c] K_i (μM)	MAO B [a,c] K_i (μM)
(±)-tPCPA·HCl	271	186	19	16
(+)-tPCPA·HCl [d]	284	137	nd	4.4
(-)-tPCPA·HCl [d]	168	127	nd	89
(+)-Br-tPCPA·HCl [d]	58	66	nd	0.4
(-)-Br-tPCPA·HCl [d]	28	82	nd	2.3
(+)-cPCPA·HCl [e]	364	131	nd	39
(-)-cPCPA·HCl [e]	506	68	nd	50
(+)-Br-cPCPA·HCl [e]	23	61	nd	0.7
(-)-Br-cPCPA·HCl [e]	44	21	nd	1.9
 **13a**	1.9	20	0.5	7.4 no spectral changes
 **13b**	1.1	61	2.3	3.5 no spectral changes
 **14e**	1.3	38	1.2	no inhibition [f]
 **15**	2.1	20	4.0	no inhibition [f]
 **14l**	40	12	49	no inhibition [f]

[a] Enzymatic activities were measured at 25 °C using the peroxidase-coupled assay (see Materials and Methods). Errors in the determination of K_i are within 30% of their values; nd, not determined. The K_i values were determined by steady-state competition experiments. The slow rate of irreversible inhibition allowed these experiments to be performed by normal steady-state approaches. [b] LSD1 activities were assayed in 50 mM Hepes/NaOH pH 7.5 using a histone H3 peptide monomethylated at Lys4 as substrate. LSD2 activities were measured in 50 mM Hepes/NaOH pH 8.0 with the substrate histone H3 peptide dimethylated at Lys4. [c] MAO A and MAO B assays were performed in 50 mM Hepes/NaOH pH 7.5, 0.5% (v/v) reduced Triton X-100 by using kynuramine and benzylamine as substrates, respectively. [d] (+)-tPCPA·HCl and (+)-Br-tPCPA·HCl have 1*S*,2*R* absolute configuration, and (−)-tPCPA·HCl and (−)-Br-tPCPA·HCl have 1*R*,2*S* absolute configuration. [e] (+)-cPCPA·HCl and (+)-Br-cPCPA·HCl have 1*S*,2*S* absolute configuration, and (−)-cPCPA·HCl and (−)-Br-cPCPA·HCl have 1*R*,2*R* absolute configuration. [f] No detectable inhibition effect at the maximum tested concentrations 133 μM, 267 μM, and 67 μM for **14e**, **15**, and **14l**, respectively. Higher concentrations produced turbidity.

of LSD1 in complex with racemic tranylcypromine.[17,18] In these complexes, the inhibitor was found to bind exactly with the same binding mode as observed with (−)-tPCPA indicating that, when probed with a racemic mixture, LSD1 selectively binds the (−)-enantiomer. This is consistent with (−)-tPCPA being a slightly stronger LSD1 inhibitor than the opposite enantiomer (Table 1). The differences in the binding modes of the two stereoisomers become even more remarkable when compared with the case of human MAO B. In this enzyme, (+)- and (−)-tPCPAs adopt identical binding modes: they both form covalent adducts with the flavin C4a (rather than N5 as found in LSD1) atom and their phenyl moieties bind in positions that closely resemble that observed for (+)-tPCPA in LSD1[19] (Figures 1 and 2B; to be published elsewhere). In summary, tPCPAs are confirmed to be nonselective inhibitors, but key aspects of binding differ among enzymes and inhibitor enantiomers: (i) the cyclopropyl atom forming the covalent linkage, (ii) the flavin ring position that is involved in the covalent bond with the inhibitor, and (iii) the position of the inhibitor aromatic ring with respect to the flavin.

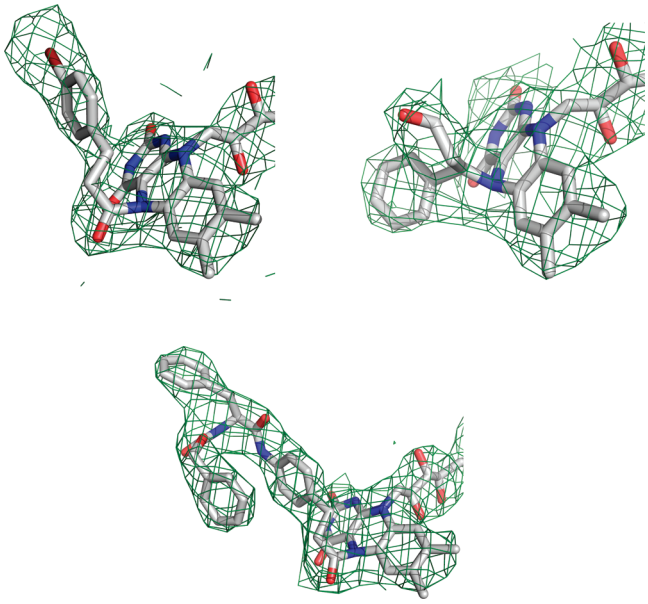


Figure 1. Crystallographic data on inhibitor binding to human LSD1/CoREST. The pictures show the weighted $2F_o - F_c$ maps (1.2 σ contour level, 3.0 Å resolution) for the FAD cofactor and *p*-bromo-(−)-tPCPA (upper left panel), (+)-tPCPA (upper right panel), and **14e** (bottom). The electron densities clearly indicate the presence of a flavin-inhibitor covalent adduct. The orientation of the two phenyl rings of **14e** was further confirmed by the structural analysis of a brominated derivative. Carbons are in gray, nitrogens in blue, oxygens in red, and bromines in dark red.

***Cis* versus *Trans* Diastereoselectivity of Inhibition.** The above-described findings raised the question of the effect of changing the configuration of only one chiral center, that is, the inhibitory efficacy of the *cis* isomers of 2-phenylcyclopropyl-1-amine·HCl (cPCPA). Both the (+) and (−)-enantiomers of cPCPA are covalent inhibitors of LSD1, LSD2, and MAO B, although they exhibit weaker affinities than the corresponding trans diastereomers (Table 1). The crystal structure of LSD1/CoREST with (+)-Br-cPCPA shows an inhibitor binding mode which is identical to that of the (−)-enantiomer of tPCPA in terms of both orientation of the phenyl ring and covalent linkage with the flavin N5 atom (Figure 1). These findings fully support the notion that 2-phenylcyclopropyl-1-amines are nonspecific inhibitors and predict that they are likely to inhibit other flavin-dependent amine oxidases.

Toward Selective Inhibitors. We sought to design and evaluate new molecules with improved inhibitory properties against LSD1/2. Our strategy was to exploit the vastly different architectures of the substrate binding sites in the amine oxidase enzymes.[17,19,20,26] LSD1 has an open cleft that hosts the H3 N-terminal tail residues through a network of very specific interactions (Figures S8 and S9). MAO A and MAO B feature internal cavities that are gated by surface loops. On the basis of these structural observations, we synthesized tPCPA derivatives with increasing larger substituents that contained a mix of hydrophobic and hydrophilic groups.

Virtually all synthesized compounds inhibit either LSD1 and LSD2 though with different efficacies. The most potent compounds were **13a**, **13b**, and **14e** with K_i values of 1−2 μM for LSD1/CoREST and of 20−60 μM for LSD2 which are at least 100-fold better than tPCPA (Table 1). Structural analysis of their

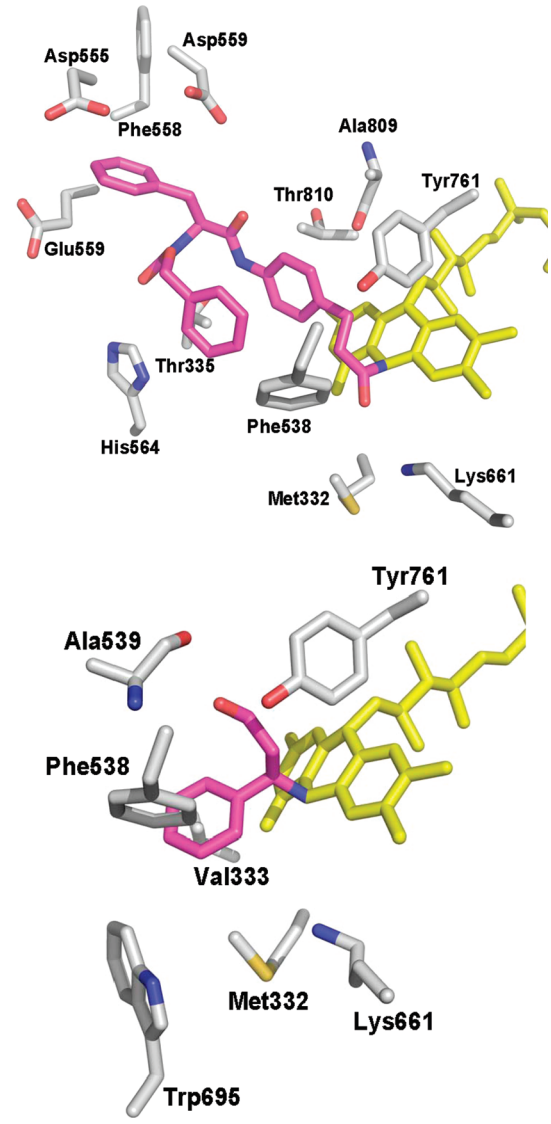


Figure 2. Three-dimensional structure of (A) **14e** and (B) (+)-tPCPA binding. The pictures show the FAD (yellow), the inhibitor (pink carbons), and the surrounding side chains (gray carbons). For Ala809, also the backbone atoms are shown. Nitrogens are in blue, oxygens in red, and bromines in dark red.

complexes with LSD1/CoREST confirmed that they form a covalent bond with the flavin N5 atom (Figures 1 and 3; Scheme 1). In all cases, the structure analysis revealed that the phenylpropyl group is in a conformation identical to that observed for (−)-tPCPA (Figure 1 and Figure S10). This binding mode enables the bulky inhibitor chain to extend from the flavin in an orientation that is roughly orthogonal to the cofactor ring. In particular, one of the aromatic rings of the "branched" **14e** partly protrudes out of the binding cleft (Figure S9). In essence, these inhibitors act as a sort of plug that fills the substrate-binding cleft of LSD1 and is "anchored" to the flavin ring through a covalent linkage.

Having identified relatively strong LSD1/CoREST inhibitors, we probed them for their inhibitory power against human MAO A and MAO B. First of all, the branched **14e** and **14l** inhibitors did not inhibit MAO B. This is consistent with the MAO B three-dimensional structure that shows two adjacent flat cavities that would be too small to accommodate these bulky molecules.[19] Furthermore, smaller linear compounds such as **13a**

(26) Son, S. Y.; Ma, J.; Kondou, Y.; Yoshimura, M.; Yamashita, E.; Tsukihara, T. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 5739.

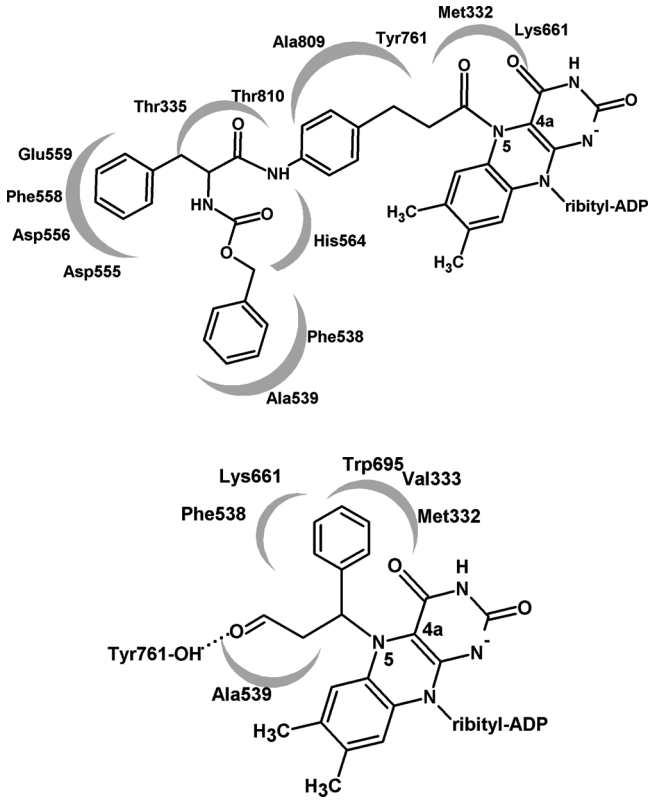
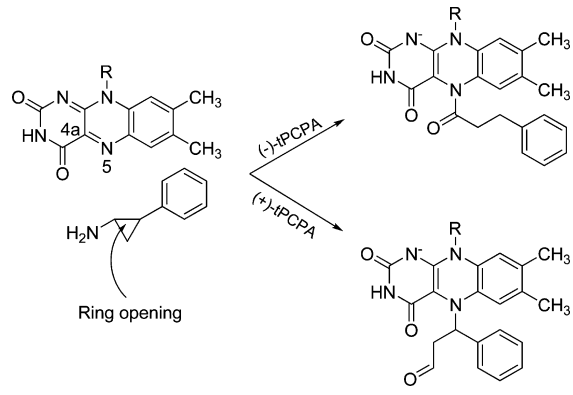


Figure 3. Schematic diagram of the inhibitor binding sites; (A) **14e**, and (B) (+)-tPCPA. H-bonds are shown with dashed lines. Consistent with the data of Yang et al.[17] on racemic tPCPA, the electron densities for **14e** (Figure 1), (−)-tPCPA, and **13b** do not rule out the possibility that at least part of the bound inhibitor molecules forms a cyclic adduct that in addition to the depicted bond between flavin N5 and the inhibitor carbonyl carbon contains also a covalent linkage between the flavin C4a and the phenyl-substituted carbon of the inhibitor.

Scheme 1



and **13b** displayed good K_i values against MAO B but did not induce spectral perturbations of the flavin, indicating that they are noncovalent inhibitors. This may reflect a binding mode that does not position the cyclopropyl unit in the proper orientation with respect to the flavin to promote formation of a covalent linkage.[27] The data on MAO B inhibition contrast with those measured on MAO A. All tested compounds were effective covalent inhibitors of this enzyme with measured K_i values in the 1−50 μM range. MAO A has a large 650 Å^3 active site cavity which is gated by a flexible loop.[26,28] This property apparently enables the enzyme to bind relatively large molecules such as **14e** and its *cis* diastereomer **15** (Table 1). These findings emphasize the adaptability of the MAO A active site as compared to that of MAO B, which is consistent with chemical labeling studies.[29]

Biological Evaluation. The good selectivity (and water solubility) of **14e** identified this compound as a logical choice for an initial evaluation of the biological activities of the synthesized molecules. We chose a cellular model of acute promyelocytic leukemia (APL) since the pathogenesis of this disease involves the aberrant activities of several chromatin modifiers (histone deacetylases and methyltransferases). These abnormalities are associated with a block of the normal hematopoietic differentiation at the promyelocytic stage. Pharmacological concentrations of retinoic acid, acting on the fusion protein PML-RAR (originated by the t15;17 chromosomal translocation that is the trigger of the disease), release the differentiation block and lead to clinical remission of the patients.[30]

Initially, we checked whether **14e** modulated its target in the APL-derived NB4 cell line. We treated the cells with various concentrations of the compound for various times. After a short (6 h) treatment, we observed a selective, dose-dependent increase in H3-Lys4 dimethylation (Figure 4A). At later time points (12 h), the increase in Lys4 dimethylation showed a less evident dose-dependence that could be explained by the irreversible mechanism of action of the inhibitor. We could not measure significant changes in the levels of H3-dimethylLys9, whereas after 24 h, we observed an increase in histone H4 acetylation (Figure S11), in line with the postulated cross-talks between LSD1 and deacetylases.[4] Next, we measured the effect of **14e** on cell growth. Singly, the compound did not affect the growth of NB4 cells up to 7 days of treatment, even at a concentration of 2 μM, which was sufficient to achieve the highest target modulation. However, LSD1 inhibition exhibited a striking synergism with retinoic acid growth inhibitory effect: at low retinoic acid concentrations (10 nM) that affect only mildly cell growth, co-treatment with **14e** led to a >10-fold stronger effect, with a reduction in cell number even stronger than that observed with high retinoic acid concentrations given alone (1 μM, Figure 4B). In part, this phenomenon is due to an enhanced apoptosis rate (Figure S12). The effect on cell growth led us to hypothesize that **14e** could also affect differentiation of NB4 cells. We therefore measured differentiation by appearance of surface markers (such as CD11b), and by morphological features. **14e** alone led to a significant enhancement of CD11b expression (from 10% to 40% of CD11b positive cells) with limited morphological changes. It also potentiated the differentiating effect of retinoic acid at all concentrations (Figure S13), greatly enhancing CD11b expression at low retinoic acid concentrations (Figure 4C). To verify that the effect was not limited to a cell line, we took advantage of the availability of primary murine APL blasts derived from an animal model.[23] **14e** exhibited a strong effect on the growth of leukemic blasts in a semisolid medium, even when given alone (Figure 4D). This correlated with enhanced differentiation as gathered from

(27) Culhane, J. C.; Wang, D.; Yen, P. M.; Cole, P. A. *J. Am. Chem. Soc.* **2010**, *132* (9), 3164.
(28) De Colibus, L.; Li, M.; Binda, C.; Lustig, A.; Edmondson, D. E.; Mattevi, A. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 12684.
(29) Hubalek, F.; Pohl, J.; Edmondson, D. E. *J. Biol. Chem.* **2003**, *278*, 28612.
(30) Botrugno, O. A.; Santoro, F.; Minucci, S. *Cancer. Lett.* **2009**, *280*, 134.

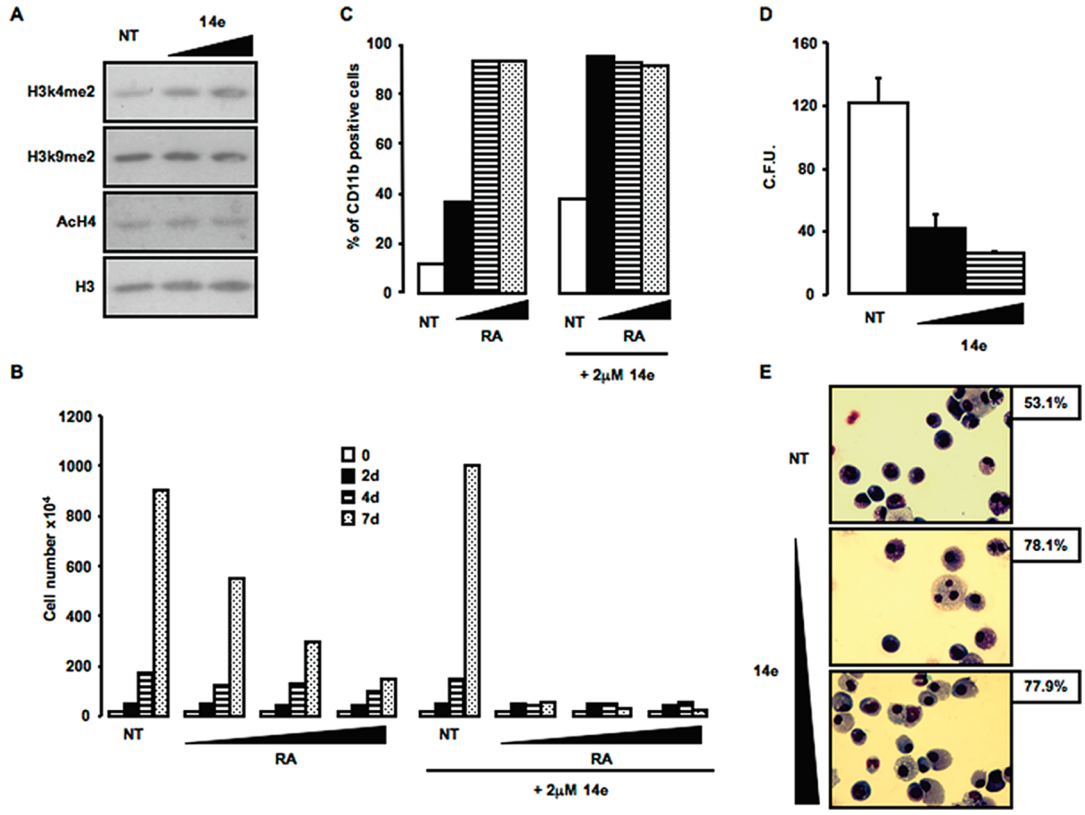


Figure 4. Biological evaluation of **14e**. (A) **14e** modulates histone methylation in whole cells. NB4 cells were treated for 6 h with increasing concentrations of **14e** (0.25−2 μM) or vehicle (NT), and then whole cell lysates were analyzed by Western blot against the indicated histone post-translational modifications (H3K4me2, dimethylation level of Lys4 of histone H3; H3K9me2, dimethylation level of Lys9 of histone H3; AcH4, lysine acetylation levels of histone H4). Total H3 served as loading control. (B) **14e** synergizes with retinoic acid (RA) in inhibiting cell growth. NB4 cells were treated with increasing concentrations of retinoic acid (10 nM, 100 nM, and 1 μM), in the absence or in the presence of **14e** (2 μM). At the indicated time points, cells were counted by Trypan blue exclusion. NT, untreated cells (vehicle only). A representative experiment is shown. (C) **14e** enhances expression of the differentiation marker CD11b in NB4 cells. Cells were treated as in panel B. After 48 h, the CD11b marker was analyzed by FACS. (D) **14e** inhibits cells growth of murine APL blasts. Primary leukemic cells (20 000 cells) from spleen were seeded in triplicate in semisolid medium (MethoCult 3434) in the presence of increasing concentrations of **14e** (0.25 and 2 μM) or vehicle (NT). After 7 days, colonies (C.F.U.) were counted. (E) **14e** induces differentiation of murine APL blasts. APL blasts were seeded in semisolid medium as in panel D. At the end of the assay, colonies were pooled, and cells, cyto-spun on glass slides, were stained (May Grunwald-Giemsa). Percentage of differentiated cells is indicated.

morphological features of the single cells (Figure 4E) and colonies (that show a spread pattern as opposed to the compact structure of colonies derived from APL blasts; Figure S14) as well as induction of genes associated with differentiation (Figure S15).

Discussion

Our investigation of 2-phenylcyclopropylamines has led to the discovery of novel compounds with improved inhibitory activities against LSD1/2, unraveling several key features about their inhibition mechanism. The prevailing view in the current literature is that tPCPA reacts by transferring one electron to the flavin, followed by recombination of the radicals resulting from homolytic cyclopropane ring cleavage to form the covalent adduct (Scheme 1).[31] In the course of our studies, we have observed that inhibition of both MAO B and LSD1 generates hydrogen peroxide with a H_2O_2/protein molar ratio of about 0.3−0.7. Thus, the reaction is partly uncoupled: occasionally, the flavin is reoxidized and/or the oxidized tPCPA is released before formation of the covalent linkage can occur. This lends support to the idea that 2-phenylcyclopropylamines behave like canonical amine oxidase substrates that are oxidized by the enzyme, resulting in H_2O_2 generation. The difference between cyclopropylamines and normal substrates is that the former can react to generate stable covalent adducts.

Independently from the exact mechanism, it is clear that inhibition occurs with cyclopropyl ring-opening. An insightful finding is that (+)- and (−)-tPCPA generate positionally distinct inhibitor−flavin linkages, possibly via differing mechanistic pathways (Scheme 1). This feature contrasts with MAO B, in which the two enantiomers exhibit the same orientation and identical C4a adduct.[19] A tentative explanation for the N5 versus C4a difference between LSD1 and MAO B is that the MAO B adduct initially occurs as an N5 adduct that undergoes a migration to the C4a atom as found in model flavin reactions.[32] Taken together, these data indicate that, although the ± and *cis*/*trans* stereoisomers are unspecific LSD and MAO inhibitors, the effect of chirality must not be neglected.

Another crucial aspect revealed by these studies is that most of the investigated molecules inhibit both MAO and LSD enzymes. It appears that the common evolutionary origin and folding topology in these otherwise functionally divergent enzymes manifest themselves in similar inhibition and binding properties. However, the "branched" set of compounds does show some selectivity. **14e** and **14l** are unable to inhibit MAO

(31) Silverman, R. B. *J. Biol. Chem.* **1983**, *258*, 14766.

(32) Walker, W. H.; Hemmerich, P.; Massey, V. *Helv. Chim. Acta* **1967**, *50*, 2269.

B, representing *per se* a valuable property because there is only a handful of known inhibitors that are so effective in the MAO A versus MAO B selectivity.[13] A similarly bulky tPCPA derivative recently described exhibits similar properties.[33] The inability of the "branched" compounds to discriminate between LSD1 and MAO A likely reflects the absence of specific directional inhibitor−protein interactions (i.e., H-bonds) as indicated by the crystal structure of **14e** bound to LSD1/CoREST. Nevertheless, molecules such as **14e** represent a step forward the development of selective inhibitors. **14e** is not only an effective inhibitor of LSD1 *in vitro*, but it also exhibits relevant biological activities in cellular models. The compound strongly enhances the efficacy of retinoic acid on growth and differentiation of acute promyelocytic leukemia cells, including primary murine APL blasts. This result demonstrates that LSD1 inhibitors have potential to both alter differentiation via alterations of the chromatin functional state and act synergistically with drugs that block and/or reduce tumor progression.

Abbreviations: LSD, lysine-specific histone demethylase; MAO, monoamine oxidase; tPCPA, *trans*-2-phenylcyclopropyl-1-amine as hydrochloride salt; cPCPA, *cis*-2-phenylcyclopropyl-1-amine as hydrochloride salt; APL, acute promyelocytic leukemia.

Acknowledgment. The support by the Italian Ministry of Science (PRIN08 and FIRB), Ministry of Health, Fondazione Roma, Fondazione Cariplo, EU (Epitron), and the Italian Association for Cancer Research is gratefully acknowledged.

Supporting Information Available: Statistics about crystallographic procedures, experimental details about the biological evaluation of the inhibitors, detailed description of the inhibitor syntheses, and complete reference 23. This material is available free of charge via the Internet at http://pubs.acs.org.

JA101557K

(33) Ueda, R.; Suzuki, T.; Mino, K.; Tsumoto, H.; Nakagawa, H.; Hasegawa, M.; Sasaki, R.; Mizukami, T.; Miyata, N. *J. Am. Chem. Soc.* **2009**, *131*, 17536.