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Impairment of prostate cancer cell growth by a selective and reversible lysine-specific demethylase 1 inhibitor

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Post-translational modifications of histones by chromatin modifying enzymes regulate chromatin structure and gene expression. As deregulation of histone modifications contributes to cancer progression, inhibition of chromatin modifying enzymes such as histone demethylases is an attractive therapeutic strategy to impair cancer growth. Lysine-specific demethylase 1 (LSD1) removes mono- and dimethyl marks from lysine 4 or 9 of histone H3. LSD1 in association with the androgen receptor (AR) controls androgen-dependent gene expression and prostate tumor cell proliferation, thus highlighting LSD1 as a drug target. By combining protein structure similarity clustering and *in vitro* screening, we identified Namoline, a γ-pyrone, as a novel, selective and reversible LSD1 inhibitor. Namoline blocks LSD1 demethylase activity *in vitro* and *in vivo*. Inhibition of LSD1 by Namoline leads to silencing of AR-regulated gene expression and severely impairs androgen-dependent proliferation *in vitro* and *in vivo*. Thus, Namoline is a novel promising starting compound for the development of therapeutics to treat androgen-dependent prostate cancer.

Prostate cancer is the second leading cause of cancer deaths in Western countries. As long as tumors are prostate confined, they can be efficiently treated by surgery and/or radiation therapy in a curative intent. In cases, however, where the tumor has already disseminated an androgen ablation therapy has to be applied.[1] Patients initially respond to androgen ablation, but tumors become androgen resistant within a period of 12–18 months,[2] after which no curative treatment exists. Thus, the urgent need to identify novel therapeutic targets for the treatment of androgen-resistant prostate cancer is evident.

We recently identified lysine-specific demethylase 1 (LSD1), an amine oxidase, as a novel target for prostate cancer therapy.[3] Expression of LSD1 positively correlates with the malignancy of prostate tumors.[3,4] LSD1 functions as a histone demethylase that removes mono- and dimethyl, but not trimethyl marks from either lysine 4 or lysine 9 of histone H3 (H3K4 and H3K9, respectively).[3,5] As a component of corepressor complexes, LSD1 demethylates active methyl marks at H3K4.[5,6] In comparison, when associated with the androgen receptor (AR), the enzyme removes repressive methyl marks from H3K9, thereby enhancing AR-dependent gene expression and prostate tumor cell proliferation.[3] Thus, we hypothesized that selective LSD1 inhibitors are useful precursors for the development of novel drugs for prostate cancer therapy.

Previous studies showed that inhibitors of other members of the amine oxidase family also impair the activity of LSD1.[7–13] However, these amine oxidase inhibitors including clorgyline, pargyline, tranylcypromine, polyamines and derivatives thereof, many of them do not selectively target LSD1 and therefore, limits their use as therapeutics owing to potential side effects. In this study, we found a novel and selective LSD1 inhibitor called Namoline by combining protein structure similarity clustering and *in vitro* screening. Namoline impairs LSD1 demethylase activity and blocks cell proliferation and xenograft tumor growth which provides a promising

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starting compound for the development of new cancer therapeutics.

Material and Methods

Cells and mice

LNCaP cell line was purchased from Cell Lines Service (Heidelberg) on July 2009. The 5-week-old male nude mice were purchased from (Charles River Laboratories, Sulzfeld, Germany). All experiments were performed according to the German animal protection law with permission from the responsible local authorities.

Protein structure similarity clustering

For protein structure similarity clustering (PSSC)[14] the ligand-sensing core of LSD1 was extracted *in silico* from the reported crystal structure.[15] The ligand-sensing core was then submitted to a similarity search against all protein structures in the protein data bank (PDB) using the "protein structure database searching by DaliLite v. 3" website.[16] Superimposed ligand-sensing core structures were inspected and verified visually.

Determination of enzymatic activity

Monoamine oxidase (MAO)-A (Sigma-Aldrich, Schnelldorf, Germany) and MAO-B (Sigma-Aldrich, Schnelldorf, Germany) enzymatic activities were determined using the Amplex® Red Monoamine Oxidase Assay Kit (Molecular Probes, Darmstadt, Germany). For the determination of LSD1 enzymatic activity, 2 μg of baculovirus-expressed and purified GST-LSD1 (pFastBac-HT-GST-A-LSD1 aa2-852) protein were mixed with 9 μg peptide of histone H3 (residues 1–20) carrying two methyl groups at lysine 4 (H3K4me2). The reaction mixture was incubated in demethylase buffer (50 mM Tris, pH 8.5, 50 mM KCl, 5 mM $MgCl_2$) containing 4 μg Amplex Red, and 0.1 units horseradish peroxidase (HRP) in the presence or absence of the indicated concentrations of Namoline. Production of H_2O_2 was analyzed in 96-well black plates by measuring fluorescence (544/590 nm) in a Molecular Devices Spectra Max Gemini plate reader. Chemicals were obtained as indicated: Namoline (Hansa Fine Chemicals, Bremen, Germany), tranylcypromine (Biomol), clorgyline (Molecular Probes, Darmstadt, Germany) and pargyline (Molecular Probes, Darmstadt, Germany).

Inhibitor dilution assay

Two milligram of GST-LSD1 was incubated with either 250 μM Namoline, 100 μM tranylcypromine or DMSO. After 1 hr, 2.5 μL aliquots were removed from all samples and diluted into HRP-assay solution containing substrate and coupling reagents to a final volume of 100 μL. This represents a 40-fold dilution of the inhibitor concentration, which is expected to yield 90% activity of LSD1 for a reversible enzyme inhibitor.

Demethylase assay

Demethylation assays were performed essentially as described earlier.[17] One milligram peptide of histone H3 (residues 1–20) carrying one or two methyl groups at lysine 4 were incubated with 2 μg of GST-LSD1 in the absence or presence of Namoline. The reaction mixture was incubated in demethylation buffer for 4 hr at 37°C and analyzed by mass spectroscopy.

Cell proliferation assays

Experiments were performed as described earlier.[3] LNCaP cells were cultured for 72 hr in the presence or absence of 1 nM R1881 (Sigma-Aldrich, Schnelldorf, Germany) and 50 μM Namoline. The cell proliferation ELISA BrdU colorimetric assay (Roche, Mannheim, Germany) was performed according to the manufacturer's instructions.

Quantitative RT-PCR (qRT-PCR)

qRT-PCR was performed as described previously.[17]

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed as described earlier.[3,17] LNCaP cells were cultivated for 210 min in the presence or absence of 1 nM R1881 and 50 μM Namoline as indicated. Immunoprecipitation was performed with specific antibodies: (anti-AR [06-680], anti-H3K9me1 [07-450], anti-H3K9me2 [07-441] [Millipore, Schwalbach, Germany]), anti-H3K9me2 (39753) (Active Motif), anti-H3 (ab1791) (Abcam) and anti-LSD1.[3]

Growth of xenograft tumors in nude mice

For tumor inoculation, 1×10^7 LNCaP cells were resuspended in matrigel (BD Biosciences, Heidelberg, Germany, Schwalbach, Germany) on ice and administered subcutaneously in nude mice. Intraperitoneal daily injection of vehicle or 0.02 mg Namoline per animal was started 30 days after tumor inoculation. Tumor size was determined by calliper measurements.[17]

Statistics

Statistical analysis was performed using a two-tailed Student's *t*-test. Bars represent mean and + SEM ($n \geq 3$). *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.01$.

Results

The γ-pyrone Namoline selectively inhibits the enzymatic activity of LSD1

To identify novel LSD1 inhibitors, we performed PSSC,[14] an unbiased bioinformatics approach that detects structural similarities between the substrate binding site of different proteins. The ligand-sensing core of LSD1, a spherical cutout of the three-dimensional structure of the substrate-binding site, was extracted *in silico* and subjected to a search against about 69,000 protein structures deposited in the PDB. Although, in principle, the PSSC approach could detect structural similarities between the ligand-sensing cores of LSD1 and distantly related proteins, we exclusively identified members of the amine oxidase family including MAO-A and MAO-B (Supporting Information Table 1).

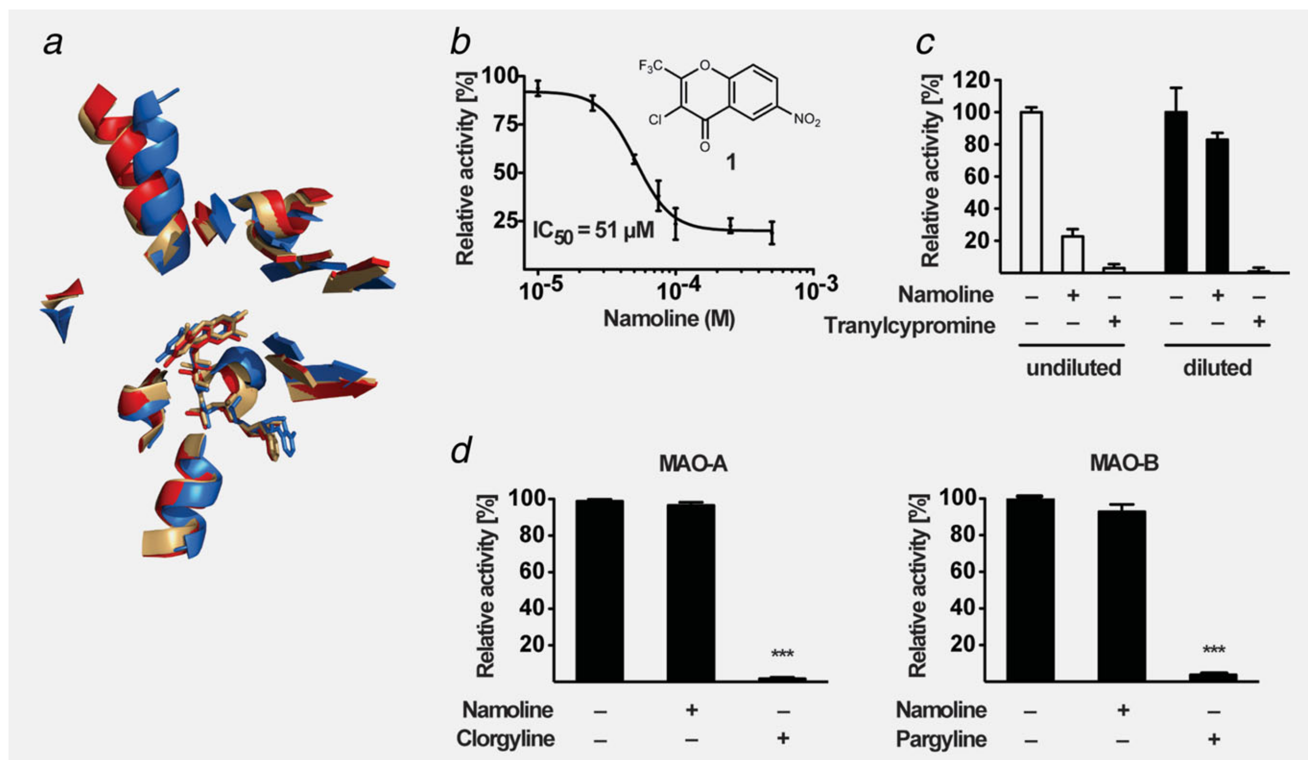

Figure 1. The γ-pyrone Namoline selectively inhibits the enzymatic activity of LSD1. (*a*) Superimposition of the ligand-sensing cores of LSD1 (blue; PDB: 2ejr), MAO-A (brown; PDB: 2bxr) and MAO-B (red; PDB: 1gos). The overall subfold of the ligand-sensing cores of the three enzymes is conserved. (*b*) Namoline inhibits the enzymatic activity of LSD1 with an IC_{50} of 51 μM. IC_{50} values were determined in a HRP-coupled enzymatic assay using recombinant LSD1 and H3K4me2 peptide as substrate. The chemical structure of Namoline is indicated. (*c*) Namoline reversibly inhibits the activity of LSD1. Dilution of Namoline but not of the covalently binding inhibitor tranylcypromine results in recovery of LSD1 activity. (*d*) Namoline does not affect the enzymatic activity of MAO-A or MAO-B at a concentration of 50 μM. The control substances clorgyline (50 μM) and pargyline (50 μM) effectively inhibit MAO-A and MAO-B, respectively.

Recently, we reported γ-pyrones as a novel class of reversible MAO-A/B inhibitors from a focused, natural product-inspired library.[18] The similar three-dimensional subfold of the ligand-sensing cores of LSD1 and MAO-A/B (Fig. 1*a* and Supporting Information Fig. 1) thus suggested that γ-pyrones are candidate inhibitors of LSD1. Furthermore, we hypothesized that the γ-pyrone compound library might also contain selective LSD1 inhibitors, as the amino acid sequence identity between the ligand-sensing cores of LSD1 and the MAO-A/B is only 19% (Supporting Information Fig. 1), thus permitting specific interactions between an inhibitor and individual amino acid side chains of LSD1.

Following these hypotheses, we screened a library comprising 705 compounds for inhibition of LSD1 demethylase activity in a HRP-coupled assay using recombinant LSD1 and a dimethyl H3K4 (H3K4me2) peptide as substrate.[7] In this screen, we identified the γ-pyrone 3-chloro-6-nitro-2-(trifluoromethyl)-4H-chromen-4-one (**1**) as a novel LSD1 inhibitor, which we termed Namoline. Namoline inhibits the demethylase activity of LSD1 with a half-maximal inhibitory concentration (IC_{50}) of 51 μM (Fig. 1*b*). Dilution of the LSD1/Namoline reaction results in recovery of LSD1 activity showing reversibility of the LSD1 inhibition, while, in the presence of the covalently binding inhibitor tranylcypromine, LSD1 activity cannot be recovered (Fig. 1*c*). In contrast to the known inhibitors clorgyline and pargyline for MAO-A and MAO-B, respectively, Namoline does not affect the enzymatic activities of MAOs under these conditions (Fig. 1*d*).

To further validate the inhibitory effect of Namoline on LSD1 demethylase activity, we incubated mono- or dimethyl H3K4 peptides (H3K4me1/me2) with recombinant LSD1 in the presence or absence of Namoline and assayed demethylation by mass spectrometry. The robust demethylation of H3K4me2 (Fig. 2*a*) and H3K4me1 (Fig. 2*b*) was observed in the presence of LSD1, converting K4me2 into mono- or unmethylated lysine. Importantly, demethylation is completely blocked only in the presence of Namoline (Supporting Information Figs. 3a–c).

Namoline impairs androgen-induced proliferation, demethylation and xenograft tumor growth

Next, we addressed the inhibitory potential of Namoline on LSD1 in cell-based assays. Global cellular changes in the histone methylation levels were observed at concentrations more

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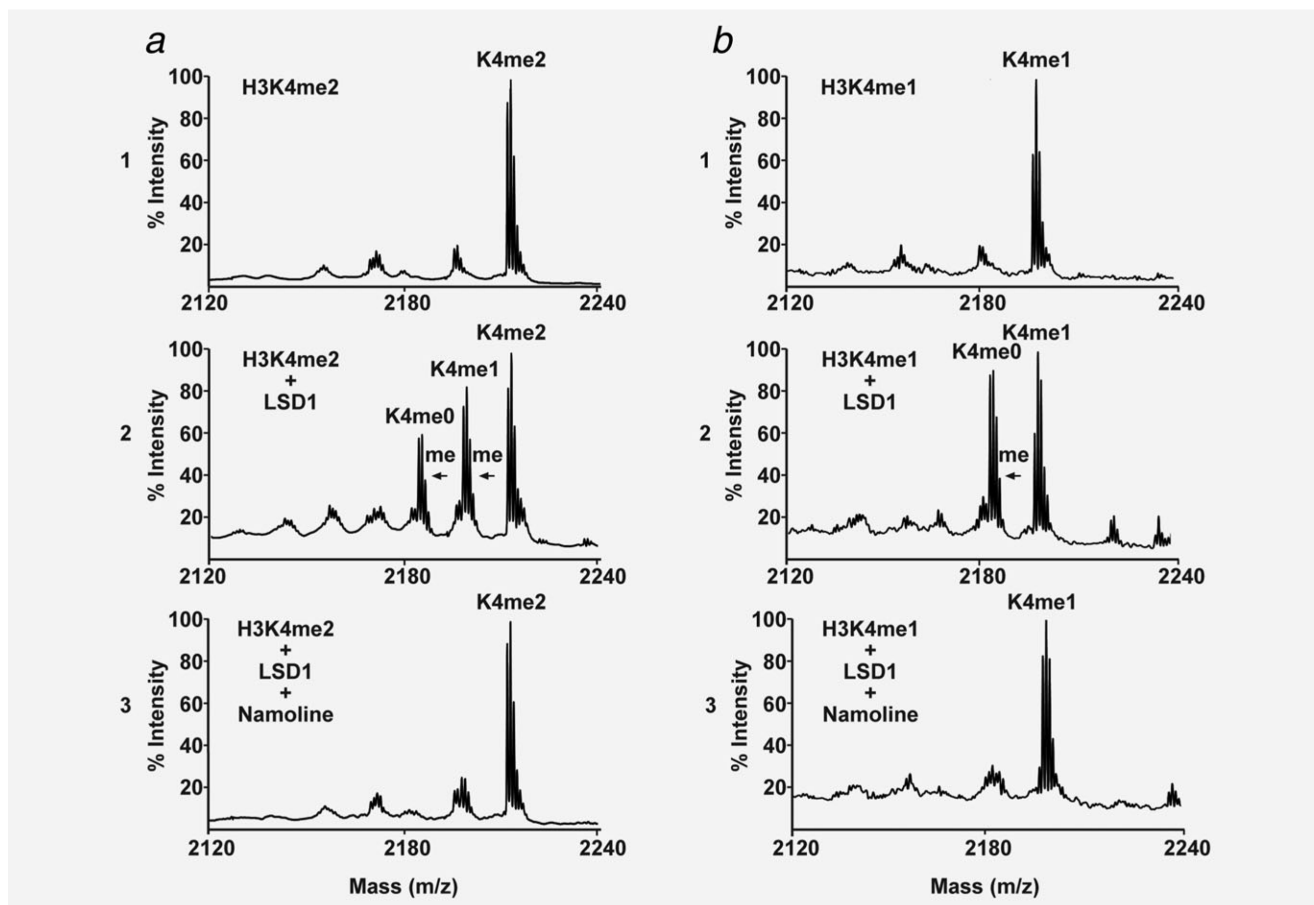

Figure 2. Namoline inhibits demethylation of peptides by LSD1. H3K4me2 (*a*) or H3K4me1 (*b*) peptides were treated with vehicle or incubated with recombinant LSD1 in the absence or presence of 50 μM Namoline. Demethylation reaction were analyzed by mass spectrometry. A mass shift corresponding to the loss of one methyl group is indicated as "me."

than 20 μM of Namoline in dose-dependent manner, indicating its membrane permeability and affinity for cellular LSD1 (Supporting Information Fig. 3d). Treatment of LNCaP cells with more than 100 μM induced a severe reduction in cell viability (data not shown). Based on those cellular effects, we have chosen the concentration of 50 μM for further experiments. As knockdown of LSD1 blocks AR-dependent prostate tumor cell proliferation, we investigated the anti-proliferative properties of Namoline. As shown in Figure 3*a*, androgen-induced proliferation of LNCaP cells is severely reduced in the presence of Namoline. Next, we investigated the effect of Namoline on the expression of endogenous AR target genes shown to be involved in prostate cancer.[17] qRT-PCR analysis of LNCaP cells demonstrates that Namoline severely impairs androgen-induced expression of genes such as *FKBP5*, *TMPRSS2*, *ELK4*, *MAK*, *NKX3.1*, *IGF1R*, *MAF*, *GREB1*, *KLK2* and *PSA* (Fig. 3*b* and Supporting Information Fig. 4).

As previously shown, ligand-dependent expression of AR target genes requires removal of repressive methyl marks from H3K9 by LSD1.[3] ChIP analysis shows that Namoline specifically impairs AR agonist R1881-induced demethylation of H3K9me1 and H3K9me2, but not of H3K9me3 at androgen-response element (ARE)-containing promoters of *FKBP5*,[19] *MAK*, *TMPRSS2* or *ELK4* (Fig. 3*c* and Supporting Information Fig. 5). Neither ligand-induced recruitment of AR nor the presence of LSD1 at the promoter of these androgen-regulated genes is affected by Namoline (Fig. 3*c*).

LSD1 was recently shown to contribute to cell proliferation through control of cell-cycle genes such as *MYBL2* and *CDK1*.[20] Hence, we investigated whether Namoline also impairs the level of histone methylation and expression of those genes in AR-negative PC-3 cells. Indeed, expression of *MYBL2* and *CDK1* and demethylation of H3K9me1 and H3K9me2 on the promoters of those genes are significantly impaired in the presence of Namoline (Supporting Information Fig. 6).

Having established the anti-proliferative property of Namoline in cell lines, we analyzed the effect of Namoline on tumor cell proliferation *in vivo*. Xenograft tumors were generated by subcutaneous implantation of LNCaP cells into nude mice. Upon treatment with Namoline, xenograft tumor growth is severely blunted (Fig. 3*d*). Namoline treatment showed some adverse effects in mice such as a slight weight

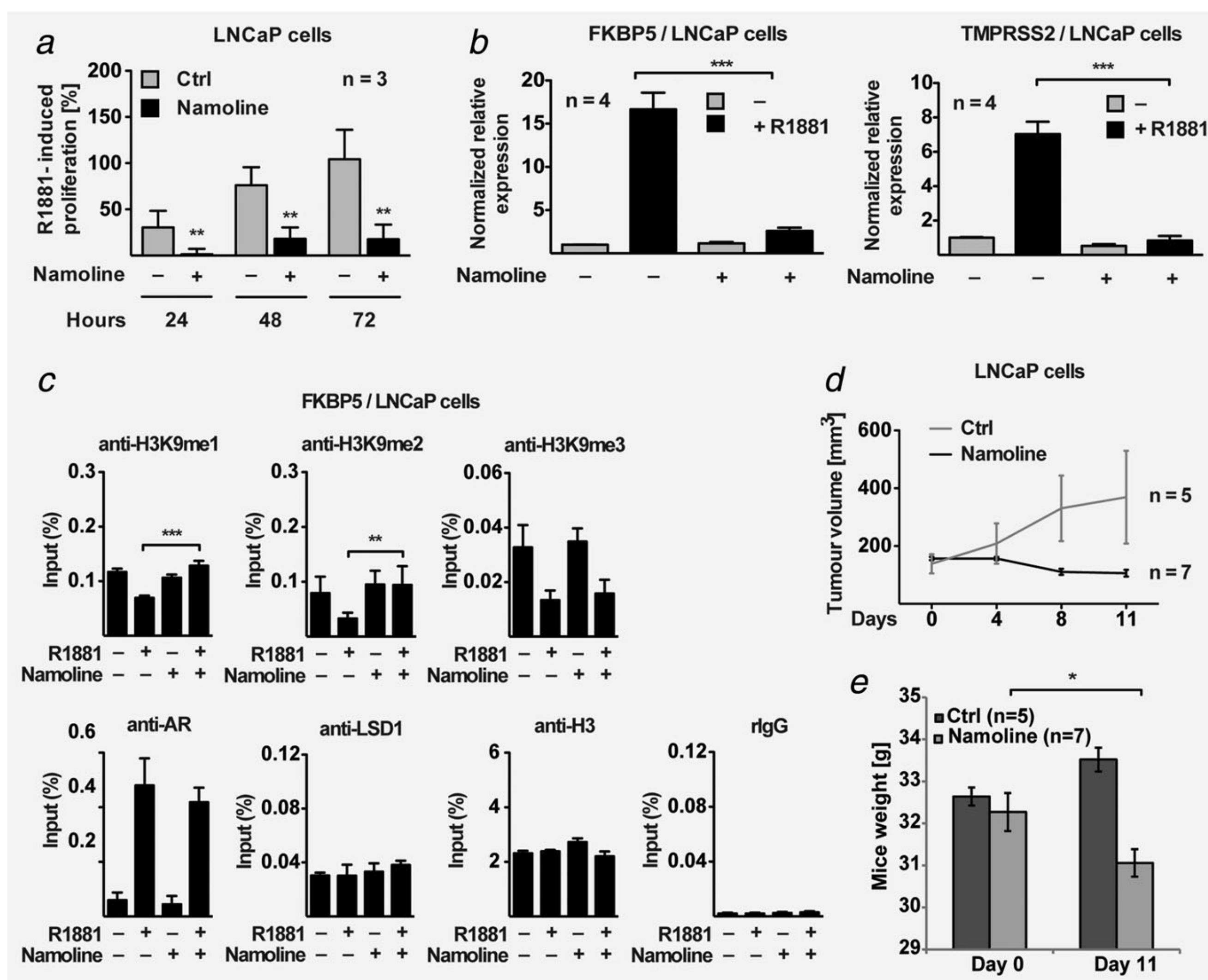

Figure 3. Namoline inhibits LNCaP cell proliferation (*a*) and expression of the AR target genes *FKBP5* and *TMPRSS2* (*b*). (*c*) In LNCaP cells Namoline specifically blocks demethylation of H3K9me2 and H3K9me1 at the promoter of the AR target gene *FKBP5*. (*d*) Namoline inhibits xenograft tumor growth of LNCaP cells in nude mice. LNCaP cells were cultivated in the presence or absence of the AR agonist R1881 and Namoline, as indicated (*a–c*). ChIP analysis (*c*) was performed with the indicated antibodies. The precipitated chromatin was quantified by qPCR analysis using primers flanking the ARE in the promoter of *FKBP2*. (*d*) Namoline treatment showed slight adverse effect in mice as shown by weight loss upon Namoline treatment.

loss (Fig. 3*e*) or minor liver toxicity as determined by microscopic investigation.

Discussion

Based on the similar structural similarities between the ligand-sensing cores of LSD1 and MAO-A/B, we hypothesized that γ-pyrones, a novel class of reversible MAO-A/B inhibitors might also inhibit LSD1. In the screening of the γ-pyrone compound library, we indeed identified a novel LSD1 inhibitor with a moderately potent inhibitory effect which does not inhibit closely related MAO-A/B or spermine oxidase or polyamine oxidase (Supporting Information Fig. 3e/f). Identification of this novel type of inhibitor for LSD1 prompted us to move ahead and profile Namoline in biochemical and cellular tests as a proof-of-concept for LSD1 as a novel target for an antiprostate cancer therapeutic modality.

Hormone refractory prostate cancers overexpressed AR and are hypersensitive to androgens. The development of LSD1 inhibitory compounds represents a new strategy to block the activity of AR. Moreover, expression of LSD1 positively correlates with the malignancy of prostate tumors and the elevated levels of LSD1 may render prostate cancer cells more sensitive toward the loss of LSD1. In this study, γ-pyrone-type LSD1 inhibitor impairs AR target gene expression, androgen-dependent tumor cell proliferation and xenograft tumor growth, showing the possible use of LSD1-based therapy. Thus, Namoline is regarded as a

starting point for the development of a truly novel inhibitor type for LSD1. Some lead optimization toward higher potency while retaining selectivity will allow to select better inhibitors and to carefully determine adverse on-target effects of long-term LSD1 inhibition on noncancerous cells and tissues. Namoline-related compounds from our γ-pyrone compound library did not show any structure–activity relationship possibly owing to the low potency range (Supporting Information Fig. 2). However, other derivatives still carrying an electron-withdrawing substituent at the 6-position would be worth trying.

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