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Dual inhibitors of LSD1 and spermine oxidase†

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We have previously described the synthesis and evaluation of 3,5-diamino-1,2,4-triazole analogues as inhibitors of the flavin-dependent histone demethylase LSD1. These compounds are potent inhibitors of LSD1 without activity against monoamine oxidases A and B, and promote the elevation of H3K4me2 levels in tumor cells *in vitro*. We now report that the cytotoxicity of these analogues in pancreatic tumor cells correlates with the overexpression of LSD1 in each tumor type. In addition, we show that a subset of these 3,5-diamino-1,2,4-triazole analogues inhibit a related flavin-dependent oxidase, the polyamine catabolic enzyme spermine oxidase (SMOX) *in vitro*.

Introduction

Lysine and arginine residues on nucleosomal histone protein tails undergo reversible mono-, di- and, in the case of lysine, trimethylation that serves to regulate gene expression. Unlike histone acetylation, which activates gene transcription, histone methylation can either activate or silence gene expression, depending on the specific chromatin mark involved. The primary function of the flavin-dependent amine oxidase lysine-specific demethylase 1, (LSD1, also known as KDM1A), is to remove methyl groups from the activating chromatin marks monomethyl histone 3 lysine 4 (H3K4me) and dimethyl histone 3 lysine 4 (H3K4me2). LSD1 is also known to demethylate lysine 370 of the tumor suppressor p53, and has been shown to play a regulatory role in a number of cancer and non-cancer disease states.[1] Over-expression of LSD1 has been observed in a variety of tumor cell lines, and promotes the aberrant silencing of tumor suppressor genes. For these reasons, LSD1 is regarded as an attractive target for therapeutic intervention. A number of LSD1 inhibitors have been described (Fig. 1), including oligoamines such as verlindamycin **1** (ref. 2–5) and related isosteric ureas and thioureas,[5,6] tranylcypromine-based irreversible inhibitors such as GSK2879552 (**2**)[7] and ORY-1001 (**3**),[8–10] reversible benzohydrazide inhibitors such as SP-2509 (**4**)[10] and triazole-based reversible inhibitors such as **5**.[11] In addition, dithiocarbamate-urea hybrid LSD1 inactivators such as **6**,[12] and linear[13–16] and cyclic[17,18] peptide inhibitors have been reported. The most potent peptide inhibitor to date is **7** (LSD1 IC_{50} = 107 nM). Compounds **2**, **3** and **4** are currently being evaluated in human clinical trials.

Our group previously described a series of 3,5-diamino-1,2,4-triazoles related to **5** that are effective reversible inhibitors of LSD1. Triazole **5**, the most potent analogue in the initial series, was shown to inhibit LSD1 with an IC_{50} of 1.2 μM, and a K_i of 2.2 μM.[11] Compound **5** produced a cell type-specific cytotoxicity in a panel of 5 tumor cell lines, and effectively increased cellular levels of H3K4me2. Importantly, **5** did not inhibit monoamine oxidases A and B at concentrations up to 100 μM.

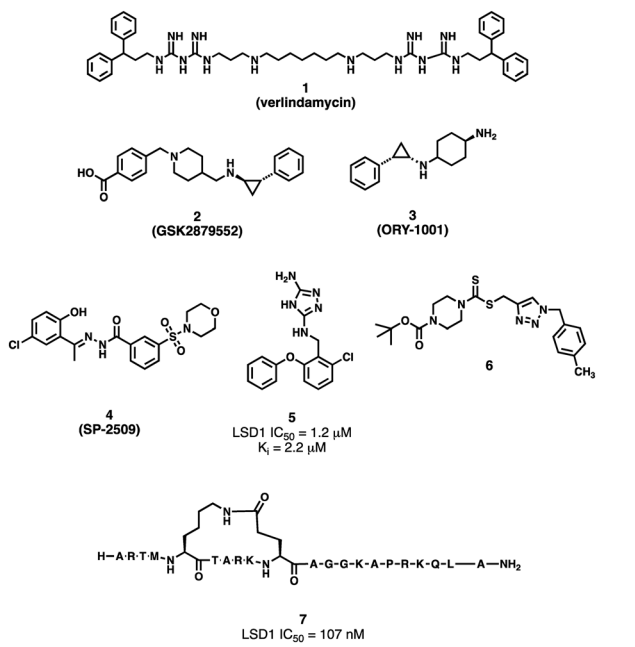

Fig. 1 Structures of known LSD1 inhibitors **1**–**7**.

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The closely related flavin-dependent amine oxidase known as spermine oxidase (SMOX) was discovered and first characterized by our group in 2001.[19,20] SMOX and its peroxisomal homologue polyamine oxidase (PAOX) are key components of the polyamine back-conversion pathway.[21] SMOX has recently been implicated as a causative factor in gastric cancer initiated by infection with *Helicobacter pylori*.[22–24] *H. pylori* infection has been shown to induce SMOX in gastric epithelial cells, which generates hydrogen peroxide leading to DNA damage and possibly apoptosis.[23] Inhibition of SMOX by the pan polyamine oxidase inhibitor MDL 72527 (ref. 25) is capable of reducing these effects. However, MDL 72527 is an irreversible inhibitor of SMOX with unacceptably low potency. To date, few inhibitors of SMOX have been identified,[26,27] and no specific inhibitors of the enzyme have been discovered.[11]

In the present study, we initiated hit-to-lead optimization studies intended to reveal new 3,5-diamino-1,2,4-triazole-based LSD1 inhibitors with improved potency and selectivity compared to the parent compound **5**. Our initial goal was to identify inhibitors selective for LSD1, and with little or no activity against MAO A and B, SMOX or PAOX. During the course of our enzyme specificity determinations, we discovered that a subset of these compounds had significant activity against SMOX that was superior to the currently used agent MDL 72527, and that have potential for use as chemopreventive agents. The synthesis of these molecules and the corresponding biological evaluation studies are described below.

Results

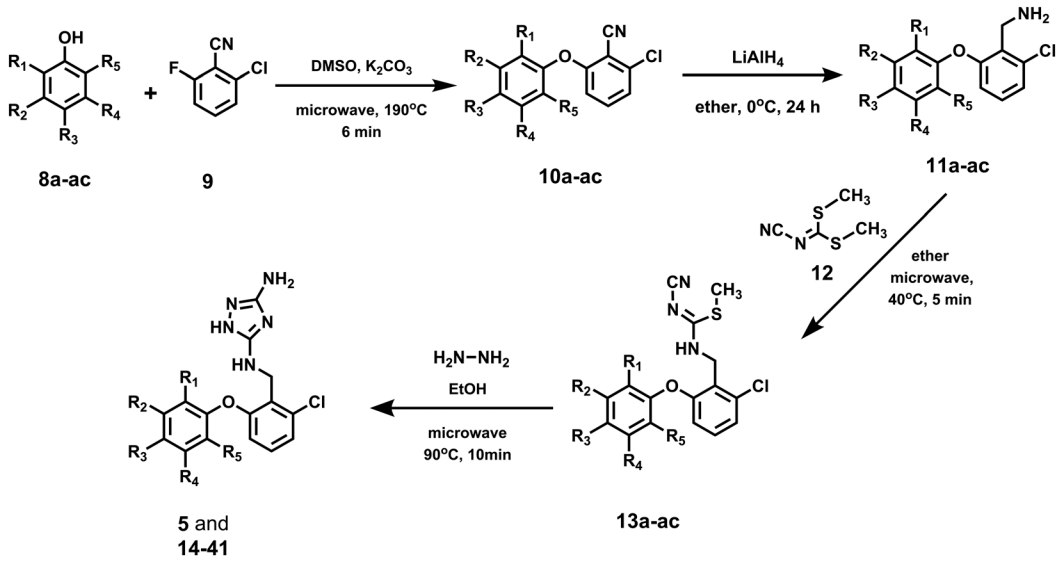
Synthetic procedures

To generate a library of analogues related to the parent 3,5-diamino-1,2,4-triazole **5**, we used our previously published synthetic route,[11] as shown in Scheme 1. Condensation of phenols **8a–ac** and 2-cyano-3-chlorofluorobenzene **9** (K_2CO_3, microwave, 190 °C, 6 min) resulted in the phenoxyphenyl intermediates **10a–ac**. The cyano group in compounds **10a–ac** was then reduced ($LiAlH_4$) to afford primary amine **11a–ac**, which was reacted with **12** (microwave, 40 °C, 5 min) to yield **13a–ac**. Intermediates **13a–ac** were then treated with hydrazine (microwave, 90 °C, 10 min) to produce the desired 3,5-diaminotriazole **5** and the previously unreported 3,5-diaminotriazole analogues **14–41** (Table 1).

To determine whether the chlorine substituent was required for inhibitory activity, a novel synthetic route was developed (Scheme 2) that allowed formation of compounds featuring the dechlorinated 3,5-diamino-1,2,4-triazole scaffold, and that supported the rapid synthesis of analogues. In this way, we were able to produce the penultimate intermediate in bulk, and rapidly synthesize new analogues in the final step. Thus, the 1,3-dihalogenated-2-cyanobenzene **9** was reduced in the presence of $LiAlH_4$ to yield the corresponding primary amine **42**. Compound **42** was then appended to **12** (see Scheme 1) to afford intermediate **44**, which was cyclized in the presence of hydrazine to form **43**. At this step, intermediate **43** was derivatized in small portions to complete the addition of a variety of phenols **8a–p**, resulting in the rapid formation of a library of analogues related to **45**. Unexpectedly, conversion of **44** to the final product **45** yielded the corresponding dechlorinated product. We postulate that this effect is due to stabilization of the aromatic ring *via* anchimeric assistance that promotes the elimination of the chlorine prior to attack of the phenol on fluorine. Coupling of **43** with phenols **8a–s** then afforded the dechlorinated analogue **45** and the previously unreported 3,5-diaminotriazoles analogues **46–60**, shown in Table 1.

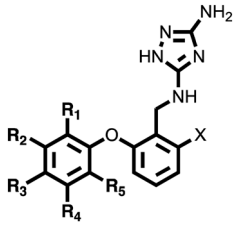
Enzyme inhibition studies

Compounds **5**, **14–41**, **45–60** were evaluated for the ability to inhibit recombinant LSD1/CoREST using a commercially



Scheme 1

Table 1 Inhibition of LSD1/CoREST at 10 μM by compounds **5**, **14**–**41** and **45**–**60**. Tranylcypromine (TCP) and the known spermine oxidase inhibitor MDL 72527 were used as positive and negative controls, respectively. Where IC_{50} values are reported, they are derived from a curve with data points collected in triplicate, and which varied by 10% or less in each case

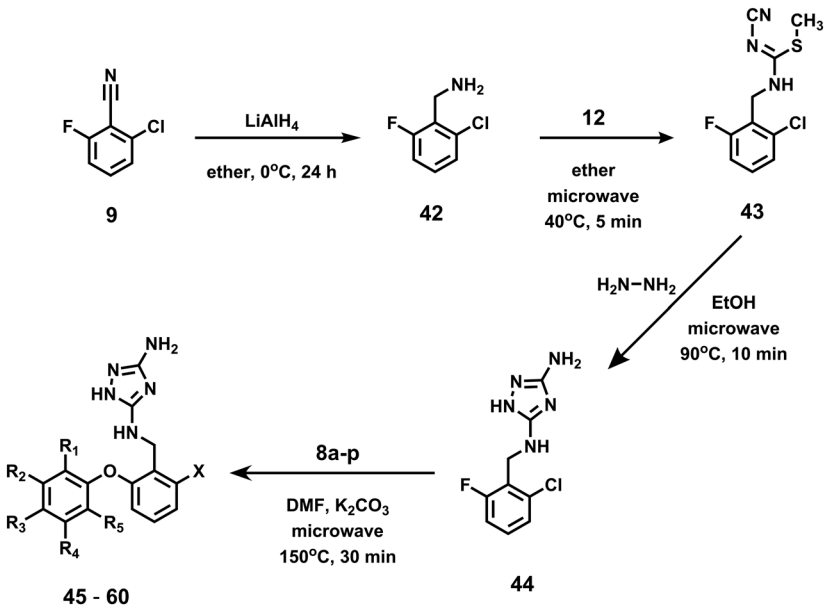


Cmpd.	R_1	R_2	R_3	R_4	R_5	X	% inhibition of LSD1 (10 μM)
TCP	—	—	—	—	—	—	68.1 ± 1.9
MDL 72527	—	—	—	—	—	—	0.0 (IC_{50} > 100 μM)
5	H	H	H	H	H	Cl	84.9 ± 4.7 (IC_{50} 1.19 μM)
14	H	H	CF_3O	H	H	Cl	38.6 ± 5.6
15	H	H	CH_3	H	H	Cl	59.3 ± 6.1
16	Isopropyl	H	H	CH_3	H	Cl	56.3 ± 3.2
17	H	CH_3O	H	H	H	Cl	44.6 ± 5.1
18	H	H	*t*-Butyl	H	H	Cl	58.2 ± 8.8
19	H	CH_3	H	CH_3	H	Cl	67.9 ± 6.5
20	H	CF_3	H	CF_3	H	Cl	44.9 ± 4.6
21	H	H	CF_3S	H	H	Cl	Not determined (insoluble)
22	Br	H	Br	H	H	Cl	Not determined (insoluble)
23	CF_3	H	Br	H	H	Cl	67.8 ± 5.4
24	H	H	CH_3S	H	H	Cl	Not determined (insoluble)
25	CH_3O	H	CH_3	H	H	Cl	84.7 ± 3.2
26	H	CH_3O	H	CH_3O	H	Cl	63.3 ± 8.6
27	CH_3	CH_3	H	H	H	Cl	80.6 ± 1.9
28	CH_3	H	H	H	H	Cl	74.2 ± 5.2
29	H	–O–CH_2–O–		H	H	Cl	65.8 ± 6.4
30	–CH=CH–CH=CH–		H	H	H	Cl	95.3 ± 8.8 (IC_{50} 0.23 μM)
31	H	–CH=CH–CH=CH–		H	H	Cl	92.9 ± 12.8 (IC_{50} 0.19 μM)
32	H	H	Phenyl	H	H	Cl	66.8 ± 9.3
33	Ethoxy	H	CH_3	H	H	Cl	77.7 ± 7.0
34	–NH–CH=CH–		H	H	H	Cl	67.6 ± 4.6
35	H	H	F	H	H	Cl	76.4 ± 4.7
36	H	CH_3O	CH_3O	CH_3O	H	Cl	67.5 ± 3.4
37	–CH_2–CH_2–CH_2–		H	H	H	Cl	68.3 ± 2.6
38	H	$(Et)_2N$	H	H	H	Cl	64.4 ± 6.4
39	F	H	F	H	H	Cl	55.5 ± 3.8
40	3-Methoxy-phenethyl	H	H	H	H	Cl	82.2 ± 7.8
41	Cl	H	Cl	H	H	Cl	Not determined (insoluble)
45	H	H	H	H	H	H	51.3 ± 3.17
46	H	CH_3O	CH_3O	CH_3O	H	H	56.0 ± 3.6
47	–CH=CH–CH=CH–		H	H	H	H	80.2 ± 2.6
48	Ethoxy	H	CH_3	H	H	H	73.8 ± 3.5
49	F	H	F	H	H	H	58.4 ± 0.23
50	H	$(Et)_2N$	H	H	H	H	61.1 ± 3.56
51	Isopropyl	H	H	CH_3	H	H	56.4 ± 2.85
52	H	H	*t*-Butyl	H	H	H	57.9 ± 9.13
53	H	CH_3	H	CH_3	H	H	54.8 ± 3.39
54	CF_3	H	Br	H	H	H	58.3 ± 2.28
55	H	H	CH_3S	H	H	H	80.2 ± 0.11
56	H	CH_3O	H	CH_3O	H	H	57.0 ± 3.22
57	CH_3	CH_3	H	H	H	H	52.2 ± 8.17
58	H	–CH=CH–CH=CH–		H	H	H	52.1 ± 3.5
59	H	H	Phenyl	H	H	H	52.1 ± 8.17
60	H	H	F	H	H	H	54.6 ± 1.63

available assay kit (#700120, Cayman Chemical, Ann Arbor, MI). The results of this assay are summarized in Table 1. The most potent analogues, **30** and **31**, were found to have IC_{50} values of 0.23 and 0.19 μM, respectively (Fig. 2).

LSD1 QSAR

Using an integrated feature of Molecular Operating Environment (MOE. Chemical Computing Group, Montreal), computational methods were employed to generate a traditional QSAR



Scheme 2

model to predict changes in substituents that would increase compound efficacy. New and previously reported small molecules known to be reversible inhibitors of LSD1 were used to build a QSAR model based on positive and negative descriptors within a defined chemical space. This model was developed using chemical descriptors such as size, weight, clog *P*, aromaticity, hydrogen donors/acceptors, molar refractivity, number of rigid bonds and radius of substituents *versus* potency towards LSD1. Some specific computational positive descriptors that were used were SlogP_VSAO, opr_brigid, weinerPol and zagrab. Negative descriptors were vsa_acc, opr_nrot, SMR_VSA4 and radius. Computational analysis was then performed using compounds developed in our laboratories as well as compounds described in the literature, as long as they fit our selection criteria and had defined IC_{50} values toward LSD1. Results were plotted as a linear *XY* plot having experimentally derived *in vitro* pIC_{50} values along the *x*-axis *versus* computationally predicted pIC_{50} values on the *y*-axis (Fig. 3). The resulting graph revealed an R^2 value of 0.71 and a cross-validated predictive strength (XPRED) of 0.798. Efforts were made to increase the R^2 value to greater than 0.80, but there was insufficient inhibition data among the small molecules that fall within the parameters for specific inhibition of LSD1. This model will be used for the design of second-generation 3,5-diamino-1,2,4-triazoles related to **5**.

Inhibition of related flavin-dependent oxidase enzymes

In order to assess the specificity/selectivity of **5**, **14–41** and **45–60** for LSD1, selected analogues were evaluated for the ability to inhibit monoamine oxidases (MAO) A and B using a commercial assay kit (#V1401, Promega Biosciences, Madison, WI). The results of this assay are displayed in Table 2. TCP inhibited both isoforms of MAO within the range of previously published IC_{50} values.[28]

As a further monitor of specificity for LSD1, selected compounds were evaluated for the ability to inhibit two enzymes from the polyamine metabolic pathway, SMOX and N^1-acetylpolyamine oxidase (PAOX), as previously described.[29] The most effective SMOX inhibitors were compounds **30**, **31**, **54** and **59** (Table 2), with IC_{50} values of 147.9, 162.7, 120.96 and 25.71 μM, respectively. By contrast, the parent analogue **5** possessed

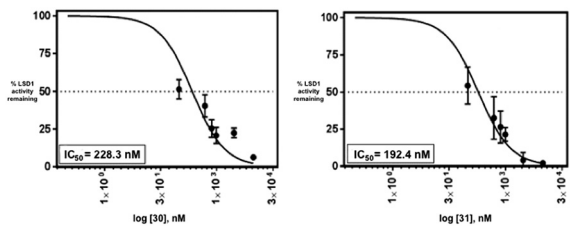


Fig. 2 Determination of the IC_{50} values for inhibition of LSD1/CoREST by compounds **30** and **31**. Each data point is the average of three determinations ± SEM.

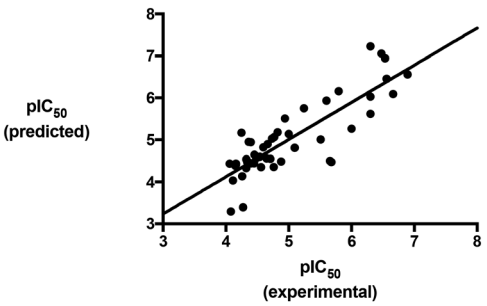
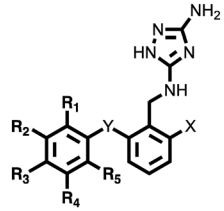


Fig. 3 Predictive QSAR model for LSD1 inhibitor library. New and previously reported small molecule competitive inhibitor IC_{50} values were entered into MOE to develop a predictive QSAR model. The model used various chemical descriptors *vs.* IC_{50} values to allow the prediction of future inhibitors.

Table 2 Inhibition of LSD1/CoREST, MAO-A, MAO-B, SMOX and PAOX at 10 μM by compounds **5**, **30**, **31**, **45** and **51**–**60**. Tranylcypromine (TCP) and the known spermine oxidase inhibitor MDL 72527 were used as controls. Where IC_{50} values are reported, they are derived from a curve with data points collected in triplicate, and which varied by 10% or less in each case



Cmpd.	R_1	R_2	R_3	R_4	R_5	Y	X	% inhibition of LSD1 (10 μM)	% inhibition of MAO-A (10 μM)	% inhibition of MAO-B (10 μM)	% inhibition of SMOX (10 μM)	% inhibition of PAOX (10 μM)
TCP	—	—	—	—	—	—	—	68.1 ± 2.0	(IC_{50} = 4.2 μM)	(IC_{50} = 5.8 μM)	0	0
MDL 72527	—	—	—	—	—	—	—	0	0.0 (IC_{50} > 200 μM)	0.0 (IC_{50} > 200 μM)	4.8 ± 9.6 (IC_{50} 89.7 μM)	32.1 ± 13.2
5	H	H	H	H	H	O	Cl	84.9 ± 4.7 (IC_{50} 1.19 μM)	0.0 (IC_{50} > 200 μM)	0.0 (IC_{50} > 200 μM)	28.0 ± 14.2 (IC_{50} 177.9 μM)	0
30	–CH=CH–CH=CH–		H	H	H	O	Cl	95.3 ± 8.8 (IC_{50} 0.19 μM)	0.0 (IC_{50} > 200 μM)	7.12 (IC_{50} > 200 μM)	19.0 ± 10.9 (IC_{50} 147.9 μM)	ND
31	H	–CH=CH–CH=CH–		H	H	O	Cl	92.9 ± 12.8 (IC_{50} 0.25 μM)	0.0 (IC_{50} > 200 μM)	0.0 (IC_{50} > 200 μM)	23.6 ± 3.9 (IC_{50} 162.7 μM)	ND
45	H	H	H	H	H	O	H	51.3 ± 3.2	IC_{50} = 50.2 ± 1.0 μM	18.77 ± 4.77	0 (IC_{50} > 200 μM)	0 (IC_{50} > 200 μM)
51	Isopropyl	H	H	CH_3	H	O	H	56.4 ± 2.9	(IC_{50} = 55.5 ± 11.3)	49.73 ± 9.78	13.9 ± 3.7 (IC_{50} > 200 μM)	0 (IC_{50} > 200 μM)
52	H	H	*t*-Butyl	H	H	O	H	57.9 ± 9.139	(IC_{50} = 61.5 ± 1.1)	32.34 ± 7.46	0 (IC_{50} > 200 μM)	0 (IC_{50} > 200 μM)
53	H	CH_3	H	CH_3	H	O	H	54.8 ± 3.4	(IC_{50} = 91.4 ± 11.8 μM)	83.41 ± 3.15	0 (IC_{50} > 200 μM)	0 (IC_{50} > 200 μM)
54	CF_3	H	Br	H	H	O	H	58.3 ± 2.3	(IC_{50} = 3.0 ± 1.2 μM)	90.08 ± 2.34	0 ± 13.49 (IC_{50} 120.96 μM)	0 (IC_{50} > 200 μM)
55	H	H	CH_3S	H	H	O	H	80.2 ± 0.1	IC_{50} = 38.4 ± 5.6	31.66 ± 7.51	0 (IC_{50} > 200 μM)	0 (IC_{50} > 200 μM)
56	H	CH_3O	H	CH_3O	H	O	H	57.0 ± 3.2	0.0 (IC_{50} > 200 μM)	17.06 ± 27.61	0 (IC_{50} > 200 μM)	0 (IC_{50} > 200 μM)
57	CH_3	CH_3	H	H	H	O	H	52.2 ± 8.2	0 (IC_{50} > 200 μM)	68.61 ± 5.43	0 (IC_{50} > 200 μM)	0 (IC_{50} > 200 μM)
58	H	–CH=CH–CH=CH–		H	H	O	H	52.1 ± 3.5	(IC_{50} = 79.2 ± 1.1 μM)	81.58 ± 0.96	0 (IC_{50} > 200 μM)	0 (IC_{50} > 200 μM)
59	H	H	Phenyl	H	H	O	H	52.11 ± 8.2	(IC_{50} = 50.0 ± 4.2 μM)	26.35 ± 9.80	25.7 ± 11.4 (IC_{50} 25.7 μM)	0 (IC_{50} > 200 μM)
60	H	H	F	H	H	O	H	54.58 ± 1.6	(IC_{50} = 89.33 ± 1.16 μM)	40.7 ± 6.14	0 (IC_{50} > 200 μM)	0 (IC_{50} > 200 μM)

an IC_{50} value against SMOX of 177.9 μM. Importantly, compound **59** is significantly more potent against SMOX than the known polyamine oxidase inhibitor MDL 72527 (IC_{50} = 89.7 μM, Table 2), retains good activity against LSD1 (Table 1), and is thus the most potent SMOX inhibitor reported to date. It is noteworthy that the naphthalene-containing compounds **30**, **31** and **58** are significantly less potent against SMOX, suggesting that the positioning of the aromatic rings and their ability to

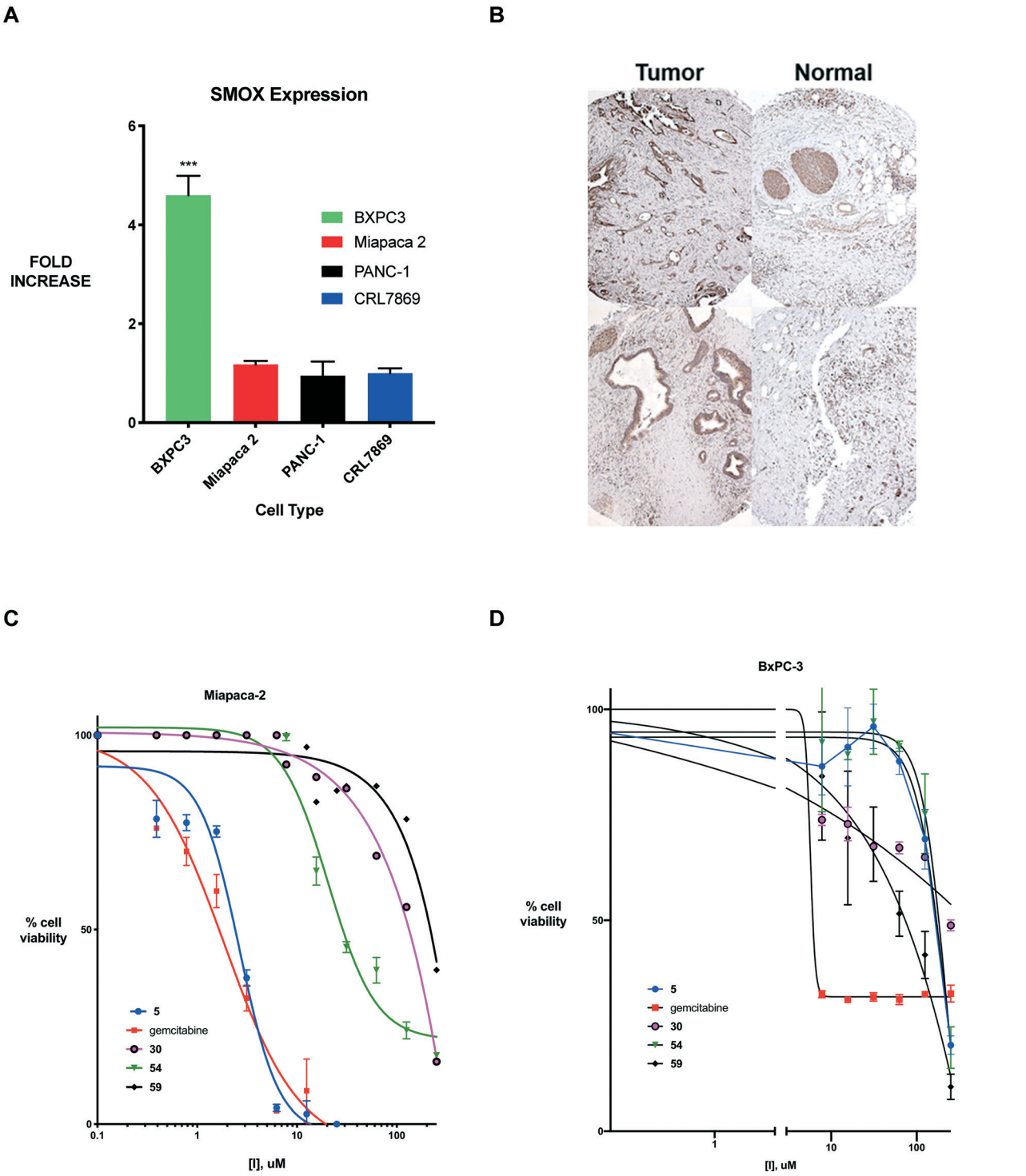


Fig. 4 Growth inhibition by SMOX in the Miapaca2 and BxPC-3 pancreatic tumor cell lines is related to the degree of overexpression of SMOX. Panel A: expression of SMOX in 3 pancreatic tumor cell lines and in CRL7869 intestinal epithelial cells as determined by western blot. Each data point is the average of 3 determinations ± SEM; panel B: immunohistochemical staining of human pancreatic tissue slices from cancerous (left) and non-cancerous patients; panel C: response of Miapaca2 pancreatic tumor cells to **5**, gemcitabine, **30**, **55** and **61**; panel C: response of BxPC-3 pancreatic tumor cells to **5**, gemcitabine, **30**, **55** and **61**. For panels C and D, each data point is the average of three determinations ± SEM.

rotate freely are important in terms of binding to the SMOX active site. Additional experimentation will be required to test this hypothesis, and to develop structure/activity parameters for SMOX inhibition. None of the analogues tested had inhibitory activity against PAOX.

LSD1 and SMOX expression in pancreatic tumor cell lines and response to inhibition

In order to determine whether there was a relationship between LSD1 or SMOX expression levels in pancreatic tumor cell lines and cytotoxicity, we determined the expression level of these enzymes in the BxPc-3, Miapaca2 and PANC-1 pancreatic tumor cell lines, and in the CRL7869 human intestinal cell line as a reference standard, by western blotting (Fig. 4). All cell lines were purchased from ATCC (Manassas, VA). There were no significant differences in LSD1 expression among the three tumor cell lines tested (data not shown). However, the levels of SMOX in the BxPc-3 pancreatic tumor cell line were 4.6-fold higher than in the MiaPaca-2, PANC-1 or CRL7869 cell lines. (Fig. 4A). We next obtained unidentified pancreatic tissue slices from malignant pancreatic and non-cancerous patients from our in-house tissue bank, and measured SMOX levels by immunohistochemistry (Fig. 4B). The levels of SMOX in the pancreatic tumor tissue (left) was significantly higher than that in the normal pancreatic tissue (right). Using gemcitabine (a nucleoside-based chain terminator that is a first-line treatment for pancreatic cancer), the potent LSD1 inhibitors **5** and **30**, and the two most potent SMOX inhibitors **54** and **59**, we then determined the IC_{50} values for growth inhibition in the Miapaca2 and BxPc-3 cell lines. In the Miapaca cell line, **5** and gemcitabine were essentially equipotent, exhibiting IC_{50} values of 2.66 and 1.86 μM, respectively, while compounds **30**, **54** and **59** had IC_{50} values between 30 and 200 μM (Fig. 4C). By contrast, in the BxPc-3 cell line, gemcitabine exhibited an IC_{50} of 5.71 μM, but growth inhibition plateaued at 30% inhibition (Fig. 4D). Our most potent SMOX inhibitor, **59**, inhibited cell growth with an IC_{50} of 61.5 μM. The remaining compounds, including the potent LSD1 inhibitors **5** and **30** and the weak SMOX inhibitor **54**, had IC_{50} values well above 100 μM. (Fig. 4D). These data suggest that cytotoxicity of these 3,5-diamino-1,2,4-triazoles in the MiaPaCa2 cell line may be mediated by an as yet unknown mechanism, and may correspond with the expression level of SMOX in the BxPc-3 cell line. Additional compounds and *in vitro* assays will be performed to verify this hypothesis, and reported in a subsequent publication.

Discussion

Based on data gathered for the compounds described in this paper, it is possible to postulate a number of structure/function relationships within the 3,5-diamino-1,2,4-triazole series of LSD1 inhibitors. There was a decrease in LSD1 inhibitory activity when single substituents were appended to the phenoxy ring, as in compounds **14–41**, with the exception of an *ortho* methoxy group or an *ortho* ethoxy *para* methyl substitution (compounds **25** and **33**, respectively), where activity was retained. Conversion of the phenoxy substituent to a naphthyloxy moiety resulted in an increase in inhibitory activity, which was slightly larger for the 1-naphthyloxy analogue **30** (IC_{50} 0.19 μM) as compared to the 2-naphthyloxy compound **31** (IC_{50} 0.25 μM). Compounds **30** and **31** are thus roughly 5 times more active against LSD1 than the parent compound **5a**. This indicates that the active site pocket surrounding the bound FAD cofactor can accommodate additional bulk, and that the orientation of the second aromatic moiety is also important. This may also explain why activity is retained in compound **40**, which bears a 3-methoxyphenethyl substituent on the phenoxy ring that could assume the same position as the naphthylene ring system.

Removal of the chlorine substituent from the molecule resulted in a reduction in LSD1 inhibitory activity across the board (compare compounds **5** and **45**, compounds **30** and **47**, compounds **33** and **48** and compounds **31** and **58**). The most effective compounds in the dechloro series were **47** (dechloro **30**) and **55**. In addition, many of the dechloro analogues were found to have modest activity against MAO-A and MAO-B. Thus, inclusion of the chlorine in this scaffold is required for optimal activity. Additional analogues in this and other series are now being made to refine our structure/activity model.

It was recently reported that the increased incidence of gastric cancer in patients infected with *Helicobacter pylori* is mediated by reactive oxygen species (ROS) produced from increased expression of spermine oxidase (SMOX). In addition, the polyamine oxidase inhibitor MDL 72527 was able to protect against ROS-mediated damage, potentially leading to a reduced risk of gastric cancer.[22,23] It has now been demonstrated that pretreatment with compound **5** can effectively prevent the formation of ROS in RAW 264.7 macrophages that have been infected with *H. pylori* (unpublished observations). In these studies, and as previously reported,[11] **5** is not cytotoxic in a number of cell lines. These data suggest that **5** may be an effective inhibitor of SMOX *in vitro*, and led us to test whether analogues of **5** were inhibitors of purified SMOX. As shown in Table 2 above, compounds **5**, **30**, **31**, **54** and **59** were all able to inhibit SMOX with IC_{50} values between 25.7 and 177.9 μM. While these inhibitory constants are modest, they compare favorably to the known inhibitor MDL 72527 (IC_{50} = 89.7 μM), and compound **59** is 3.5-fold more potent against the purified enzyme than MDL 72527. As such, **59** is the most potent SMOX inhibitor reported to date. In addition, the SMOX inhibitors described in Table 2 are the only known SMOX inhibitors that do not also inhibit PAOX. Thus, these compounds can serve as chemical tools to study the effects of differential inhibition of SMOX. We postulate that dual inhibitors of LSD1 and SMOX could be useful, since they produce both antitumor and chemopreventive activities. The synthesis and evaluation of analogues in this class are an ongoing concern in our laboratories.

Experimental

Synthesis

All reagents and dry solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI), Sigma Chemical Co. (St.

Louis, MO), VWR (Radnor, PA) or Fisher Scientific (Chicago, IL) and were used without further purification except as noted below. Triethylamine was distilled from potassium hydroxide and stored in a nitrogen atmosphere. Dry methanol, ethyl acetate, tetrahydrofuran, dimethyl formamide and hexane were prepared using a Glass Contour solvent purification system (Pure Process Technology, LLC, Nashua, NH). Preparative scale chromatographic procedures were carried out using a CombiFlash Rf200 chromatography system (Teledyne-Isco, Lincoln, NE) fitted with silica gel 60 cartridges (230–440 mesh). Thin layer chromatography was conducted on Merck precoated silica gel 60 F-254. Ion exchange chromatography was conducted on Dowex1X8-200 anion exchange resin.

All ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer, and all chemical shifts are reported as δ values referenced to TMS or DSS. Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad peak. In all cases, ^{1}H-NMR, ^{13}C-NMR and MS spectra were consistent with assigned structures. Mass spectra were recorded by LC/MS on a Waters autopurification liquid chromatography with a model 3100 mass spectrometer detector. Prior to biological testing procedures, all compounds were determined to be >95% pure by UPLC chromatography (95% H_2O/5% acetonitrile to 20% H_2O/80% acetonitrile over 10 minutes) using a Waters Acquity H-series ultrahigh-performance liquid chromatograph fitted with a C18 reversed-phase column (Acquity UPLC BEH C18 1.7 M, 2.1 × 50 mm). Compounds **5** and **14–41** were synthesized according to the general procedures[11] described below. Compounds **5** and **14–29** have been previously reported.[11]

General procedure for the synthesis of substituted 2-chloro-6-aryloxybenzonitriles

Synthesis of 2-chloro-6-phenyloxybenzonitrile, 10a. To a 20 mL microwave vial containing a magnetic stir bar was added 0.78 g (5.0 mmol) of 2-fluoro-6-chlorobenzonitrile **9**, (1.04 g, 7.5 mmol) of K_2CO_3, 0.52 g (5.5 mmol) of phenol **8a** and 12.0 mL of anhydrous DMSO. The vial was then sealed and stirred to distribute the contents evenly. The mixture was then microwaved at 190 °C for 6 min at high absorption to insure even heating. The reaction mixture was poured into a beaker containing 100 mL of crushed ice to precipitate the product. The aqueous layer was extracted with three 50 mL portions of diethyl ether, and the ether layer was washed with 25 mL of saturated NaCl, dried over anhydrous Na_2SO_4, filtered, and the ether was removed *in vacuo* to yield 1.11 g of the desired diaryl ether **10a** (97% yield). The crude product was pure enough to be used in the next reaction without further purification.

General procedure for the synthesis of substituted 2-chloro-6-aryloxybenzylamines

Synthesis of 2-chloro-6-phenyloxybenzylamine, 11a. A 1.11 g portion of **10a** (4.8 mmol) was dissolved in 50 mL of anhydrous diethyl ether, cooled to 0 °C in an ice bath and stirred while bubbling dry argon into the reaction mixture for 10 min. A 14.49 mL portion of 1.0 M $LiAlH_4$ in THF (14.49 mmol) was then added dropwise with stirring over 20 min. The resulting reaction mixture was allowed to stir for 2 h at 0 °C, and then warmed to room temperature and allowed to stir overnight. The mixture was cooled to 0 °C, and the reaction was quenched by the slow addition of $Na_2SO_4{\cdot}10H_2O$. When the evolution of gas subsided, the reaction was stirred for 30 min at room temperature, and the mixture was filtered through a Celite pad. The filtrate was concentrated to dryness to yield crude 2-chloro-6-phenyloxybenzylamine **11a**. The crude product was pure enough to be used in the next reaction without further purification.

General procedure for the synthesis of substituted N^5-(2-chloro-6-aryloxybenzyl)-1*H*-1,2,4-triazole-3,5-diamines

Synthesis of N^5-(2-chloro-6-phenyloxybenzyl)-1*H*-1,2,4-triazole-3,5-diamine, 5. A 0.935 g portion of benzylamine **11a** (4.0 mmol) was dissolved in 12 mL of diethyl ether and added to a 20 mL microwave vial equipped with a magnetic stir bar. A 0.702 g portion of dimethyl cyanodithioiminocarbonate **12** (4.8 mmol) was added and the vial was sealed. The contents were microwaved at 45 °C for 5 minutes, cooled to room temperature, and the ether was removed *in vacuo* to yield the intermediate **13a** as a white to pale yellow solid. A 0.192 g portion of hydrazine hydrate (6.0 mmol) in 12 mL of dry ethanol was then injected, the vial was stirred to break up the solid intermediate, and the resulting mixture was microwaved at 90 °C for 10 min at high absorption. The ethanol was removed *in vacuo*, and the residue was purified by silica gel chromatography (9% MeOH in CH_2Cl_2) to afford 1.07 g of pure **5** (85%) as an off-white, amorphous solid. ^{1}H NMR (400 MHz, CD_3OD) δ 4.21 (s, 2H), 6.77–6.80 (dd, 4H), 6.99–7.01 (d, 2H), 7.11–7.15 (t, 4H), 7.20–7.27 (m, 2H), 7.33–7.38 (t, 2H). UPLC retention time: 12.1 min. MS calculated 359.08, found 316.33 ($[M + 1]^+$).

Synthesis of N^5-(2-chloro-6-(naphthalen-1-yloxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 30. Compound **30** was prepared from **11r** exactly as described above for compound **5** in 85% yield as a white, amorphous solid. ^{1}H NMR (400 MHz, DMSO) δ 4.49 (s, 2H), 6.67–6.80 (dd, 1H), 6.99–7.01 (d, 1H), 7.25 (d, 2H), 7.54–7.59 (m, 3H), 7.77–7.79 (dd, 1H), 7.99–8.01 (dd, 1H), 8.12–8.14 (dd, 1H). UPLC retention time: 14.34 min. MS calculated 365.10, found 366.21 ($[M + 1]^+$).

Synthesis of N^5-(2-chloro-6-(naphthalen-2-yloxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 31. Compound **31** was prepared from **11s** exactly as described above for compound **5** in 80% yield as a white powder. ^{1}H NMR (400 MHz, CD_3OD) δ 2.14 (s, 2H), 6.72 (dd, 1H), 6.81–6.86 (dd, 1H), 7.07 (d, 1H), 7.08 (s, 1H), 7.17 (d, 1H), 7.32 (m, 3H), 7.51 (dd, 1H), 8.19 (dd, 1H). UPLC retention time: 14.53 min. MS calculated 365.82, found 366.21 ($[M + 1]^+$).

Synthesis of N^5-(2-([1,1′-biphenyl]-4-yloxy)-6-chlorobenzyl)-1*H*-1,2,4-triazole-3,5-diamine, 32. Compound **32** was prepared

from **11t** exactly as described above for compound **5** in 81% yield as an off-white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.59 (s, 2H), 6.77–6.79 (d, 1H), 7.14 (m, 3H), 7.24 (d, 2H), 7.35 (d, 2H), 7.42 (d, 2H), 7.54 (d, 2H), 7.59 (d, 2H). UPLC retention time: 14.55 min. MS calculated 391.1, found 392.26 ($[M + 1]^+$).

Synthesis of N^5-(2-chloro-6-(2-ethoxy-4-methylphenoxy)-benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 33. Compound **33** was prepared from **11u** exactly as described above for compound **5** in 40% yield as a white, amorphous solid. ^{1}H NMR (400 MHz, CD_3OD) δ 2.40 (s, 3H), 3.75 (s, 3H), 6.54–6.57 (m, 3H), 6.83–6.88 (d, 1H), 7.06–7.18 (m, 2H). UPLC retention time 14.7 min. MS calculated 359.11, found 360.29 ($[M + 1]^+$).

Synthesis of N^5-(2-((1*H*-indol-7-yl)oxy)-6-chlorobenzyl)-1*H*-1,2,4-triazole-3,5-diamine, 34. Compound **34** was prepared from **11v** exactly as described above for compound **5** in 72% yield as a pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.11 (s, 2H), 6.46 (s, 1H), 6.85–6.87 (d, 1H), 6.86–6.88 (d, 1H), 7.01–7.07 (m, 2H), 7.24 (d, 21H), 7.31–7.33 (d, 1H). UPLC retention time: 14.79 min. MS calculated 354.80, found 355.98 ($[M + 1]^+$).

Synthesis of N^5-(2-chloro-6-(4-fluorophenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 35. Compound **35** was prepared from **11w** exactly as described above for compound **5** in 68% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.97 (s, 2H), 6.52–6.54 (d, 1H), 6.66–6.68 (d, 1H), 6.87–6.88 (d, 1H), 6.90–6.91 (d, 1H), 6.94–6.96 (d, 1H), 6.98–7.00 (d, 1H), 7.08–7.09 (d, 1H). UPLC retention time: 12.65 min. MS calculated 333.80, found 334.92 ($[M + 1]^+$).

Synthesis of N^5-(2-chloro-6-(3,4,5-trimethoxyphenoxy)-benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 36. Compound **36** was prepared from **11x** exactly as described above for compound **5** in 89% yield. ^{1}H NMR (400 MHz, CD_3OD) δ 3.74 (s, 3H), 3.78 (s, 3H), 3.84 (s, 2H), 6.19 (s, 3H), 6.83–6.85 (d, 1H), 7.04–7.08 (m, 1H), 7.16–7.19 (d, 1H), 7.3–7.33 (d, 2H). UPLC retention time: 21.45 min. MS calculated 405.12, found 406.20 ($[M + 1]^+$).

Synthesis of N^5-(2-chloro-6-((2,3-dihydro-1*H*-inden-4-yl)-oxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 37. Compound **37** was prepared from **11y** exactly as described above for compound **5** in 65% yield as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.71 (s, 2H), 2.84–2.87 (M, 2H), 2.97–3.0 (m, 4H), 6.83–6.85 (d, 1H), 6.92–6.95 (d, 1H), 6.99–7.03 (M, 1H), 7.08 (s, 1H), 7.12–7.14 (d, 1H), 7.40–7.42 (d, 1H). UPLC retention time: 20.25 min. MS calculated 355.83, found 357.23 ($[M + 1]^+$).

Synthesis of N^5-(2-chloro-6-(3-(diethylamino)phenoxy)-benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 38. Compound **38** was prepared from **11z** exactly as described above for compound **5** in 68% yield. ^{1}H NMR (400 MHz, CD_3OD) δ 1.15–1.18 (t, 6H), 2.98 (s, 2H), 3.31–3.36 (m, 4H), 6.12–6.14 (dd, 1H), 6.19–6.20 (m, 1H), 6.27–6.29 (dd, 1H), 7.04–7.08 (m, 1H), 7.24–7.26 (d, 1H), 7.57–7.59 (d, 1H), 8.01 (s, 1H). UPLC retention time: 0.46 min. MS calculated 386.16, found 387.55 ($[M + 1]^+$).

Synthesis of N^5-(2-chloro-6-(2,4-difluorophenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 39. Compound **39** was prepared from **11aa** exactly as described above for compound **5** in 57% yield as an off-white, amorphous solid. ^{1}H NMR (400 MHz, CD_3OD) δ 4.07 (s, 2H), 6.63–6.65 (d, 1H), 7.02 (s, 1H), 7.32–7.33 (d, 1H), 7.58–7.59 (d, 1H), 7.74–7.78 (m, 1H), 8.15–8.17 (d, 1H). UPLC retention time: 0.56 min. MS calculated 351.07, found 352.42 ($[M + 1]^+$).

Synthesis of N^5-(2-chloro-6-(2-(3-methoxyphenethyl)-phenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 40. Compound **40** was prepared from **11ab** exactly as described above for compound **5** in 75% yield as an off-white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 4.43 (s, 2H), 4.84 (s, 2H), 5.02 (s, 2H), 6.55–6.58 (d, 1H), 6.94–6.95 (d, 1H), 6.98–7.01 (d, 1H), 7.04 (s, 1H), 7.06 (d, 2H), 7.09 (s, 1H), 7.10 (d, 1H), 7.11 (s, 1H), 7.13 (d, 1H), 7.18–7.19 (m, 3H), 7.37–7.38 (d, 1H). UPLC retention time: 20.47 min. MS calculated 449.94, found 450.23 ($[M + 1]^+$).

Synthesis of N^5-(2-chloro-6-(2,4-dichlorophenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 41. Compound **41** was prepared from **11ac** exactly as described above for compound **5** in 75% yield. ^{1}H NMR (400 MHz, CD_3OD) δ 3.75 (s, 2H), 6.52 (s, 1H), 6.56–6.57 (m, 1H), 6.68–6.71 (d, 1H), 6.81–6.84 (d, 1H), 7.23–7.26 (dd, 2H). UPLC retention time: 21.45 min. MS calculated 384.65, found 385.73 ($[M + 1]^+$).

Synthesis of 2-chloro-6-fluorobenzylamine, 42. 2-Chloro-6-fluorobenzonitrile **9** (1.0 g, 6.43 mmol) was dissolved in 50 mL of diethyl ether and stirred at 0 °C while bubbling argon through the solution for 10 minutes, followed by the dropwise addition of 15 mL (3.0 equivalents) of 1.0 M $LiAlH_4$ in THF over 20 min. Following addition of the reducing agent, the reaction was stirred for 2 hours at 0 °C, warmed to room temperature and allowed to stir overnight. The reaction was cooled to 0 °C and quenched by slow addition of $Na_2SO_4 \cdot 10H_2O$. When the evolution of gas was complete, the reaction was warmed to room temperature and allowed to stir for an additional 30 minutes, and then filtered through a Celite pad. The filtrate was then concentrated to dryness *in vacuo*. The crude material was purified on silica (0–5% MeOH in dichloromethane), to produce the desired 2-chloro-6-fluorobenzylamine **42** (0.913 g, 89% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.88 (s, 2H), 7.09 (m, 1H), 7.28 (m, 2H).

Synthesis of N^5-(2-chloro-6-fluorobenzyl)-4*H*-1,2,4-triazole-3,5-diamine, 44. A 0.913 g (5.72 mmol) of 2-chloro-6-fluorobenzylamine **43** was dissolved in 12 mL of diethyl ether and added to a 20 mL microwave vial equipped with a magnetic stirring bar. Dimethyl cyanodithioiminocarbonate **12** (1.0 g, 6.43 mmol) was then added and the vial was sealed. The contents were microwaved at 45 °C for 5 minutes, then allowed to cool to room temperature, and the ether was removed *in vacuo* to yield the intermediate **44** as a white solid. Hydrazine hydrate (0.275 g, 8.58 mmol) in 12 mL of ethanol was then injected into the vial, and the reaction was stirred to break up the intermediate solid. The dispersed reaction mixture was microwaved at 90 °C for 10 min on high absorption. The mixture was allowed to cool to room temperature, and the ethanol was removed *in vacuo* to yield the crude product. The crude material was purified on silica (10% MeOH in dichloromethane), to afford 1.27 g of pure **44** as a white, amorphous powder (92% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.32 (s, 2H), 7.20–7.22 (t, 4H), 7.31–7.36 (m, 2H).

UPLC retention time: 0.46 min. MS calculated 241.05, found 242.30 ([M + 1]$^+$).

General procedure for the synthesis of substituted N^5-(6-phenyloxybenzyl)-4*H*-1,2,4-triazole-3,5-diamines

Synthesis of N^5-(2-phenyloxybenzyl)-1*H*-1,2,4-triazole-3,5-diamine 45. To a 20 mL microwave vial equipped with a magnetic stir bar was added N^3-(2-chloro-6-fluorobenzyl)-1*H*-1,2,4-triazole-3,5-diamine (1.0 g, 4.14 mmol) K_2CO_3, (0.429 g, 4.55 mmol) phenol **8a**, and 12 mL of anhydrous dimethylsulfoxide. The vial was then sealed and stirred to distribute contents evenly. The reaction was microwaved at 190 °C for 6 min on high absorption, and the contents were poured into a beaker containing 100 mL crushed ice. The product was extracted with three 50 mL portions of diethyl ether, and the ether layer was washed with 25 mL of saturated NaCl. The organic layer was dried over Na_2SO_4, filtered, and the filtrate was removed *in vacuo* to provide crude **45**. The crude material was purified on silica (10% MeOH in dichloromethane), to afford pure1.14 g of pure **45** (98% yield) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 4.61 (s, 2H), 6.80–6.82 (dd, 1H), 7.25–7.33 (d, 2H), 7.27–7.3 (m, 1H), 7.50–7.52 (m, 2H), 7.68–7.77 (d, 1H), 7.95–7.00 (m, 1H), 8.1–8.2 (d, 1H). UPLC retention time: 0.47 min. MS calculated 278.5, found 279.65 ([M + 1]$^+$).

Synthesis of N^5-(2-(3,4,5-trimethoxyphenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 46. Compound **46** was prepared from **8b** exactly as described above for compound **45** in 58% yield as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.86 (s, 6H), 3.90 (s, 3H), 4.89 (s, 2H), 6.44 (s, 2H), 6.80–6.82 (dd, 1H), 7.22–7.25 (m, 1H), 7.76–7.81 (m, 1H), 7.83–7.85 (d, 1H). UPLC retention time: 0.42 min. MS calculated 371.16, found 372.70 ([M + 1]$^+$).

Synthesis of N^5-(2-(naphthalen-1-yloxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine 47. Compound **47** was prepared from **8c** exactly as described above for compound **45** in 56% yield as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.66 (s, 2H), 7.36–7.38 (d, 2H), 7.53–7.57 (d, 1H), 7.63–7.64 (m, 1H), 7.68–7.70 (d, 1H), 7.84–7.85 (d, 1H), 7.91–7.93 (d, 2H), 8.15–8.16 (m, 2H), 8.17–8.19 (m, 1H). UPLC retention time: 0.44 min. MS calculated 331.14, found 332.43 ([M + 1]$^+$).

Synthesis of N^5-(2-(2-ethoxy-4-methylphenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 48. Compound **48** was prepared from **8d** exactly as described above for compound **45** in 68% yield as a white solid. ^{1}H NMR (400 MHz, CDCl) δ 1.15–1.18 (t, 6H), 2.98 (s, 2H), 3.31–3.36 (m, 4H), 6.12–6.14 (dd, 1H), 6.19–6.20 (m, 1H), 6.27–6.29 (dd, 1H), 7.04–7.08 (m, 1H), 7.24–7.26 (d, 1H), 7.57–7.59 (d, 1H), 7.75–7.83 (m, 1H), 8.01 (s, 1H). UPLC retention time: 0.46 min. MS calculated 339.17, found 340.65 ([M + 1]$^+$).

Synthesis of N^5-(2-(2,4-difluorophenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 49. Compound **49** was prepared from **8e** exactly as described above for compound **45** in 42% yield as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.07 (s, 2H), 6.63–6.65 (d, 1H), 7.02 (s, 1H), 7.32–7.33 (d, 1H), 7.58–7.59 (d, 1H), 7.74–7.78 (m, 1H), 7.86–7.88 (m, 1H), 8.15–8.17 (d, 1H). UPLC retention time: 0.56 min. MS calculated 315.11, found 316.61 ([M + 1]$^+$).

Synthesis of N^5-(2-(3-(diethylamino)phenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 50. Compound **50** was prepared from **8f** exactly as described above for compound **45** in 68% yield as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.15–1.18 (t, 6H), 2.98 (s, 2H), 3.31–3.36 (m, 4H), 6.12–6.14 (dd, 1H), 6.19–6.20 (m, 1H), 6.27–6.29 (dd, 1H), 7.04–7.08 (m, 1H), 7.24–7.26 (d, 1H), 7.57–7.59 (d, 1H), 7.75–7.83 (m, 1H), 8.01 (s, 1H). UPLC retention time: 0.46 min. MS calculated 352.20, found 353.65 ([M + 1]$^+$).

Synthesis of N^5-(2-(2-isopropyl-5-methylphenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 51. Compound **51** was prepared from **8g** exactly as described above for compound **45** in 55% yield as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.21 (s, 3H), 1.23 (s, 3H), 2.35 (s, 3H), 4.69 (s, 2H), 6.64 (d, 1H), 6.80 (s, 1H), 7.10–7.12 (d, 1H), 7.32–7.34 (d, 1H), 7.54–7.57 (m, 1H), 7.73–7.75 (d, 1H), 7.79–7.87 (m, 1H). UPLC retention time: 1.99 min. MS calculated 337.43, found 338.66 ([M + 1]$^+$).

Synthesis of N^5-(2-(4-(*tert*-butyl)phenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 52. Compound **52** was prepared from **8h** exactly as described above for compound **45** in 66% yield as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.27 (s, 9H), 4.70 (s, 2H), 6.77–6.79 (d, 1H), 7.11–7.13 (d, 2H), 7.54–7.59 (d, 2H), 7.57–7.59 (d, 1H), 7.73–7.77 (m 1H), 7.82–7.84 (m, 1H). UPLC retention time: 0.71 min. MS calculated 337.19, found 338.36 ([M + 1]$^+$).

Synthesis of N^5-(2-(3,5-dimethylphenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 53. Compound **53** was prepared from **8i** exactly as described above for compound **45** in 48% yield as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 1.27 (s, 3H), 2.36 (s, 3H), 4.68 (s, 2H), 6.78 (s, 1H), 6.80 (s, 1H), 6.93 (s, 1H), 7.74–7.78 (m, 1H), 7.82–7.84 (m, 1H), 8.16–8.17 (d, 1H), 8.22–8.24 (d, 1H). UPLC retention time: 0.45 min. MS calculated 309.16, found 310.57 ([M + 1]$^+$).

Synthesis of N^5-(2-(4-bromo-2-(trifluoromethyl)phenoxy)-benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 54. Compound **54** was prepared from **8j** exactly as described above for compound **45** in 48% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.18 (s, 2H), 6.81–6.83 (d, 1H), 6.91–6.93 (d, 1H), 7.41–7.42 (d, 1H), 7.44 (s, 1H), 7.54–7.56 (dd, 1H), 7.71–7.78 (m, 1H), 8.15–8.17 (m, 1H). UPLC retention time: 0.47 min. MS calculated 427.03, found 428.55 ([M + 1]$^+$).

Synthesis of N^5-(2-(4-(methylthio)phenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 55. Compound **55** was prepared from **8k** exactly as described above for compound **45** in 48% yield as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.53 (s, 3H), 4.60 (s, 2H), 6.81–6.84 (d, 1H), 7.21–7.22 (d, 2H), 7.42–7.44 (d, 2H), 7.69–7.71 (d, 1H), 7.87–7.89 (m, 1H), 7.91–8.00 (m, 1H). UPLC retention time: 0.89 min. MS calculated 327.12, found 328.26 ([M + 1]$^+$).

Synthesis of N^5-(2-(3,5-dimethoxyphenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 56. Compound **56** was prepared from **8l** exactly as described above for compound **5** in 68% yield as a brown solid. ^{1}H NMR (400 MHz, CD_3OD) δ 2.90 (s, 3H), 2.98

(s, 3H), 4.74 (s, 2H), 6.33–6.34 (m, 1H), 6.38–6.39 (m, 1H), 6.88–6.90 (d, 1H), 7.10–7.12 (d, 1H), 7.77–7.81 (s, 1H), 8.07 (s, 2H). UPLC retention time: 0.46 min. MS calculated 341.15, found 342.63 ($[M + 1]^+$).

Synthesis of N^5-(2-(2,3-dimethylphenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 57. Compound **57** was prepared from **8m** exactly as described above for compound **5** in 48% yield as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 2.14 (s, 3H), 2.38 (s, 3H), 4.71 (s, 2H), 6.54–6.56 (d, 1H), 6.95–6.97 (d, 1H), 7.13–7.15 (d, 1H), 7.18–7.22 (m, 1H), 7.57–7.61 (d, 1H), 7.69–7.73 (m, 1H), 7.71–7.81 (m, 1H). UPLC retention time: 0.55 min. MS calculated 309.16, found 310.38 ($[M + 1]^+$).

Synthesis of N^5-(2-(naphthalen-2-yloxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine 58. Compound **58** was prepared from **8n** exactly as described above for compound **5** in 64% yield as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.70 (s, 2H), 6.81–6.83 (d, 1H), 7.35–7.38 (dd, 1H), 7.52–7.55 (m, 1H), 7.56–7.59 (m, 2H), 7.75–7.77 (d, 1H), 7.79–7.83 (d, 1H), 7.87 (s, 1H), 7.91–7.93 (m, 1H), 7.90–7.99 (d, 1H), 8.15–8.17 (d, 1H). UPLC retention time: 0.95 min. MS calculated 331.14, found 332.60 ($[M + 1]^+$).

Synthesis of N^5-(2-([1,1′-biphenyl]-4-yloxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, 59. Compound **59** was prepared from **8o** exactly as described above for compound **5** in 64% yield. ^{1}H NMR (400 MHz, CD_3OD) δ 4.74 (s, 2H), 6.85–6.87 (d, 1H), 7.26–7.28 (d, 2H), 7.38–7.42 (m, 1H), 7.47–7.51 (m, 2H), 7.62–7.64 (d, 2H), 7.68–7.71 (d, 2H), 7.77–7.81 (m, 1H), 7.85–7.88 (m, 1H), 8.15–8.17 (d, 1H). UPLC retention time: 0.43 min. MS calculated 357.16, found 358.37 ($[M + 1]^+$).

N^5-(2-(4-Fluorophenoxy)benzyl)-1*H*-1,2,4-triazole-3,5-diamine, synthesis of 60. Compound **60** was prepared from **8p** exactly as described above for compound **5** in 44% yield as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.17 (s, 2H), 6.52–6.54 (d, 1H), 6.70–6.72 (d, 2H), 7.23–7.25 (d, 1H), 7.57–7.59 (m, 1H), 7.84–7.86 (d, 2H), 7.94–7.99 (m, 1H). UPLC retention time: 0.46 min. MS calculated 299.12, found 300.50 ($[M + 1]^+$).

Determination of LSD1 activity

Compounds **5**, **14–41** and **45–60** were evaluated for the ability to inhibit recombinant LSD1/CoREST using a commercially available assay kit (#700120, Cayman Chemical, Ann Arbor, MI). The substrate and all compounds were incubated in assay buffer from 30 min up to 4 h at 37 °C as described in the commercial protocol. The volume of each reaction well was 50 μl, containing 5 μl of a 200 μM solution of substrate peptide and 20 μl of a 15 ng μl^{-1} enzyme solution. All compounds were diluted in 1% DMSO with assay buffer to a final volume of 50 μM. Fluorescence was measured at the recommended wavelengths of k_{ex} = 530 nm, k_{em} = 590 nm. IC_{50} determinations were performed using serial dilutions at 250, 125, 62.5, 31.25, 6.25, 3.125, 1.563, 0.781 and 0.390 μM. Tranylcypromine (TCP) and the known spermine oxidase inhibitor MDL 72527 were used as positive and negative controls, respectively. All blanks contained 1% DMSO to determine any solvent effects.

Measurement of MAO-A and MAO-B activity

In order to assess the specificity/selectivity of the target compounds for LSD1, selected analogues were evaluated for the ability to inhibit monoamine oxidases (MAO) A and B using a commercial assay kit (#V1401, Promega Biosciences, Madison, WI), as previously described.[11] Compounds were dissolved in DMSO and diluted to the desired concentration with PBS, using TCP as a positive control and 1% DMSO as a negative control. Compounds were added to a mixture of substrate and enzyme, and the plate was allowed to incubate for 1 h at RT. Data were generated using a luminescent plate reader.

Measurement of SMOX and PAOX activity[29]

For the measurement of oxidase activity, the enzyme was diluted to a concentration of 15 ng μL^{-1}. Each reaction contained 62.2 μL of 0.5 M glycine buffer, 20 μL of the enzyme solution, 17.8 μL of the inhibitor or vehicle, and 20 μL of 1.5 mM spermine or N^1-acetylspermine for SMOX or PAOX activity, respectively (final concentration = 250 μM) in a 0.5 mL reaction tube. As a control, one tube contained enzyme that had been boiled for 5 minutes prior to addition. Each reaction was incubated at 37 °C for one hour in a thermal cycler, then immediately extracted with acid and labeled with dansyl chloride. The production of spermidine from spermine (or acetylated spermine) was then determined by HPLC. Where indicated, inhibitors were added at the specified concentrations 2 min prior to the addition of substrate. Per cent inhibition of SMOX and PAOX were calculated using the following equation:

$$[\text{Spermidine produced (with inhibitor)}/\text{spermine produced (no inhibitor)}] \times 100.$$

Western blotting

Western blot analysis was performed according to BioRad technical bulletin 6376. Cells were plated at 350 000 cells per well in a 6 well plate in a total volume of 2.0 mL per well. Cells were incubated at 37 °C in a humidified environment containing 5% CO_2. For protein collection, wells were washed twice with cold PBS and lysed with 200 μL of cold RIPA buffer containing 1% Halt protease & phosphatase inhibitor (Thermo Fisher Scientific) for 30 min on ice. Individual cell lysates were centrifuged at 16 000 × *g* for 20 min in a 4 °C pre-cooled centrifuge. The supernatant fractions were collected, and proteins were measured using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific). SDS gel electrophoresis and immunoblot analysis were performed using the methodology previously described.[30] Anti-LSD1 and anti-GAPDH monoclonal primary antibodies were used with HRP-conjugated secondary antibodies (Abcam). Bands were detected using a c600 gel imager (Azure Biosystems, Dublin, CA). The band size and intensity for each signal were quantified using Image Studio Lite ver5.2 (LI-COR Biosciences) and

normalized to the loading control GAPDH. The relative density value of the MCF-10A control lysate was set as 1.

Cell viability assay

All cell lines (BxPc-3, Miapaca2 and PANC-1 pancreatic tumor cell lines, and the CRL7869 normal human intestinal cell line) were purchased from ATCC (Manassas, VA). For the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (MTS) reduction assay, 2000 cells per well were seeded in 50 μL of complete medium per well of a 96-well plate and the cells were allowed to attach overnight at 37 °C in a 5% CO_2 atmosphere. The medium was aspirated and cells were treated with 100 mL of fresh medium containing appropriate concentrations of each inhibitor to be tested. The cells were incubated for 72 h at 37 °C in 5% CO_2, after which 20 μL of the MTS reagent solution (Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay) was added to the medium. The cells were incubated for another 2 h at 37 °C, and absorbance was measured at 490 nm on a SpectraMax M5 instrument (Molecular Devices) equipped with SOFTmax PRO 4.0 software to determine cell viability. A reference wavelength of 690 nm was used to subtract the background. Percent cell death was calculated by the following equation: % Cell Death = (Abs Control − Abs sample)/Abs Control × 100. A dose response curve was constructed for each inhibitor, and each data point was the average of 3 determinations obtained during a single experiment ± S.E.M. IC_{50} values were calculated using the GraphPad Prism 5 software package (Graph-Pad, San Diego, California).

Conclusions

We have synthesized and evaluated **44** new LSD1 inhibitors based on the 3,5-diamino-1,2,4-triazole scaffold. Synthetic routes were developed leading to 2 separate series, one featuring a chlorine in the 2-position of the aromatic ring appended to the triazole (**5** and **14–41**) and one dechloro series (**45**, and **46–60**). The most potent of these analogues, compounds **30** and **31**, inhibit purified LSD1/CoREST with IC_{50} values of 190 and 250 nM, respectively. None of the analogues in the chloro series had significant activity against MAO-A and MAO-B, while some of the dechloro analogues were modest inhibitors of MAO (see Table 2). Thus, removal of the chlorine substituent resulted in compounds that were less potent against LSD1, and less enzyme specific. Compound **5** was essentially equipotent with gemcitabine against the Miapaca-2 pancreatic tumor cell line, which exhibited a 3.3-fold increase in cellular LSD1 content. However, in the PANC-1 cell line, which was found to have a 1.57-fold elevation of LSD1, compound 5 and gemcitabine were ineffective, supporting the theory that cellular LSD1 levels correlate positively with the response of cells to LSD1 inhibition. Finally, a subset of 3,5-diamino-1,2,4-triazole analogues (**30**, **31**, **54** and **59**) proved to be inhibitors of SMOX, albeit with modest IC_{50} values. However, compound **59** is 3.5-fold more potent against SMOX *in vitro* than the known inhibitor, making it the most potent SMOX inhibitor discovered to date. Importantly, the SMOX inhibitors we have identified do not inhibit PAOX, and thus will be useful as chemical tools to study differential inhibition of SMOX on polyamine metabolism. Further SAR studies are required to determine the structural requirements for optimal binding of analogues in this series to either SMOX or LSD1.

Conflicts of interest

There are no conflicts to declare.

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