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Synthesis and evaluation of 3-amino/guanidine substituted phenyl oxazoles as a novel class of LSD1 inhibitors with anti-proliferative properties†

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A series of functionalized phenyl oxazole derivatives was designed, synthesized and screened *in vitro* for their activities against LSD1 and for effects on viability of cervical and breast cancer cells, and *in vivo* for effects using zebrafish embryos. These compounds are likely to act *via* multiple epigenetic mechanisms specific to cancer cells including LSD1 inhibition.

Histones are proteins that bind to DNA and facilitate efficient 'packing' of DNA in eukaryotic cells.[1] They are highly basic in nature due to the presence of positively charged amino acid side chains causing the DNA to fold around them into compact structures (nucleosomes). The ability of histones to regulate gene expression is controlled by post-translational modifications on the N-terminal (and likely C-terminal) "tails" of the core histones which project out of the nucleosome. Modifications including phosphorylation, acetylation, methylation, ubiquitination, sumoylation and biotinylation have been identified on the histone tails.[2] The cellular roles of histone lysine acetylation/deacetylation are the best characterized of the histone modifications – acetylation normally activates transcription whereas deacetylation deactivates this process.[3] Although there are clear links to disease,[4–8] the role of histone methylation is much less understood and appears to be context dependent.

LSD1 (lysine specific demethylase 1) was the first histone demethylase identified. Its discovery significantly advanced the understanding of epigenetic regulation of gene expression, changing the paradigm that methylation is a non-reversible feature of histones.[9] Histone methylation/demethylation has since been found to be an important epigenetic modification linked to activation as well as repression of transcription. Two types of histone demethylases have been discovered. The flavin-dependent demethylase LSD1 acts on lysine 4 and lysine 9 of histone H3 (H3K4 and H3K9). LSD1 selectively catalyzes the oxidation of the methyl group of mono- and dimethylated lysines resulting in an imine intermediate and generation of hydrogen peroxide. The imine product is non-enzymatically hydrolysed to generate a carbinolamine resulting in demethylated lysine and formaldehyde release.[10] The other major class, i.e. Jumonji domain-containing histone demethylases, are Fe(II) and 2-oxoglutarate dependent oxygenases that act on mono-, di- and trimethylated Lys and methylated Arg residues depending on the particular enzymes.[11] Histone demethylase activity is associated with several pathological states. Increased LSD1 expression in prostate tumors correlates significantly with relapse during therapy.[6,7] Suppressed LSD1 expression is associated with vascular smooth muscle cell inflammatory damage in a mouse model of diabetes.[8] Demethylation of p53 (tumor suppressor) by LSD1 prevents p53 interaction with its co-activator 53BP1.[5] Activation of the telomerase reverse transcriptase (hTERT) gene is known to be dependent on LSD1 levels and recruitment to the hTERT promoter.[4]

Studies on LSD1 have identified a few classes of molecules that exhibit inhibitory activity. Several monoamine oxidase inhibitors (MAOIs), more commonly used as antidepressants, inhibit LSD1, indicating one possible direction for small molecule design.[12] Biguanide and bisguanidine polyamine analogs are another class of molecules that was identified for this process. These molecules have been used in cultured colon cancer cells to demonstrate LSD1 inhibition and resultant activation of silenced genes.[13] Peptides containing methionine as the key structural element also showed LSD1 inhibition. However, none of these molecules are likely to be selective for LSD1 or are drugable. Small molecules which can selectively modulate the activity of LSD1 should therefore hold

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†Electronic supplementary information (ESI) available: ^{1}H and ^{13}C NMR, IR and MS/HRMS data of all new compounds. See DOI: 10.1039/c3ob40217g

great promise in two main areas – (1) in laboratory experiments to understand the cellular and physiological effects of LSD1 inhibition and the interaction of histone H3K4 methylation with other epigenetic modifications such as histone acetylation, histone phosphorylation and DNA methylation; (2) in animal studies aimed at defining the potential of clinical therapy as LSD1 inhibitors for the treatment of chronic conditions such as cancer, diabetes and obesity. Herein, we report the synthesis and biological study of a novel class of molecules as probes of LSD1 function, as possible epigenetic modulators or as potential anti-cancer agents.

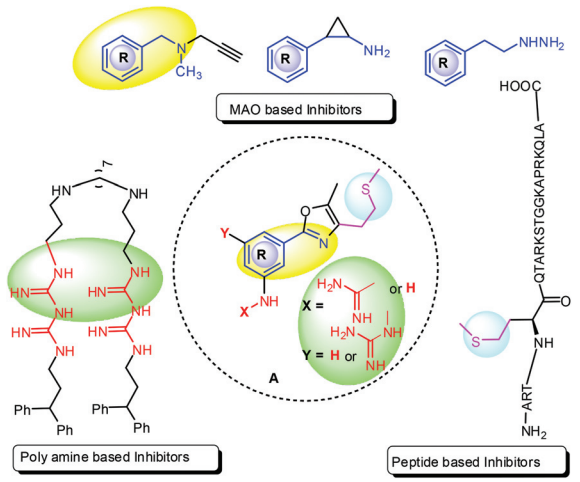
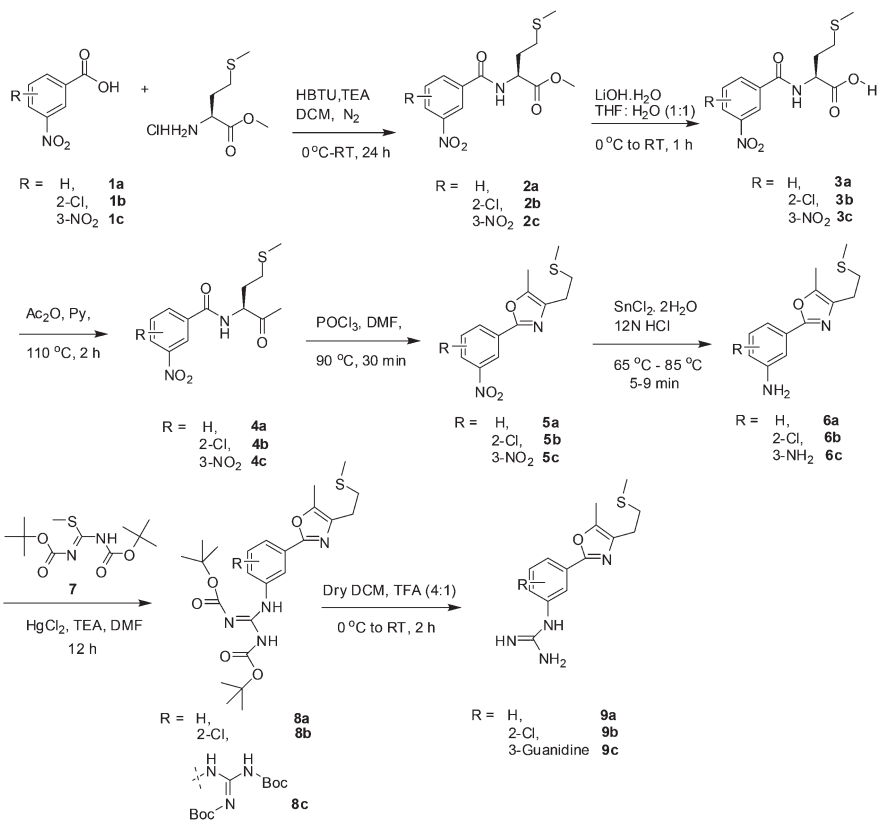


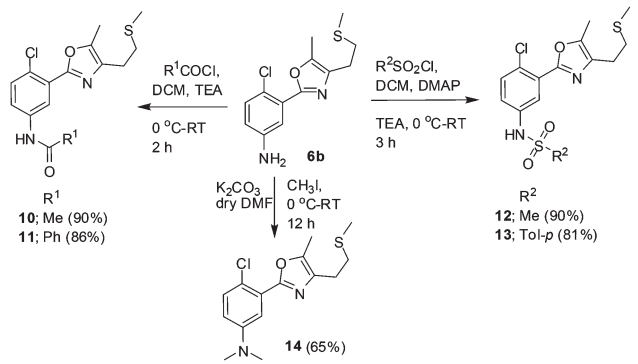
Fig. 1 Design of novel LSD1 inhibitors based on pharmacophore similarity with reported inhibitors.

Using the available X-ray diffraction crystal structure of LSD1 as a starting point,[14] we designed a series of small molecules as potential inhibitors taking the key pharmacophore features identified for the MAO, the polyamine/guanidine and methionine based peptide inhibitors, into account (Fig. 1)[12,13,15–20] being linked through an oxazole moiety. The oxazole linker was chosen as a cyclic bioisostere of an amino acid. The reason for introducing the guanidine or amino group *meta* into the oxazole moiety on the benzene ring was to avoid long conjugation and thereby potential activation of the oxazole moiety towards metabolism. The Schrodinger molecular modelling suite was used to visualize the three-dimensional structure of LSD1 and predict the docking sites, inhibitory interactions and the theoretical effectiveness of small molecules.

The synthesis of target compounds and their derivatives was efficiently performed using the reaction sequence as shown in Scheme 1. 3-Nitrobenzamide derivatives **2**[21,22] were readily obtained by reacting appropriate 3-nitrobenzoic acids **1** with methionine methylester hydrochloride in the presence of HBTU, followed by treatment with $LiOH{\cdot}H_2O$ to give rise to the corresponding carboxylic acids **3**.[23] Those were subsequently converted into keto-amides **4** in the presence of acetic anhydride and pyridine *via* a Dakin–West type reaction.[24] After



Scheme 1 Synthesis of 3-amino/guanidine substituted phenyl oxazoles.



Scheme 2 Synthesis of analogues **10–14** of **6b**.

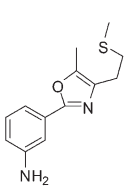
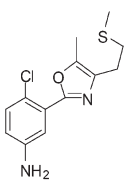
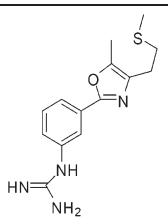
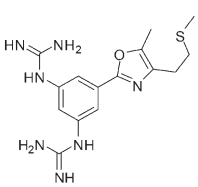
refluxing with $POCl_3$, the keto-amides **4** cyclized spontaneously to produce the 3-nitrophenyl oxazole derivatives **5**[24] which on reduction with $SnCl_2{\cdot}2H_2O$ afforded 3-aminophenyl oxazole derivatives **6**.[25] The installation of a bis-BOC protected 3-guanidine group was achieved by treating **6** with N^1,N^2-bis-(*tert*-butoxycarbonyl)-*S*-methylisothiourea (**7**),[26] to give the corresponding 3-guanidine phenyloxazole derivatives **8**. Finally, on deprotection with trifluoroacetic acid, 3-guanidine phenyl oxazole derivatives **9** were obtained.[26] A number of *N*-substituted derivatives (**10–14**) were also prepared *via N*-acylation, *N*-sulfonation or *N*-alkylation of **6b** (Scheme 2). All synthesized compounds were fully characterized by spectroscopic methods (NMR, IR, MS and HRMS).

Having synthesized the desired target molecules **6** and **9** along with **10–14** we performed their pharmacological evaluation *in vitro* determining the cell viability by a MTT assay. Initially, these compounds were subjected to the cervical cancer cell line HeLa and the breast cancer cell line MDA-MB-231 with concentrations ranging from 0.5 nM to 3.7 nM (log concentrations) for 36 h. Among the compounds tested **6a**, **6b**, **9a** and **9c** showed significant inhibitory effects on these cells (Table 1; for graphs see ESI†). From these data it was evident that a guanidine moiety did not offer any significant advantages over an amino group (*e.g.* **9a** and **9c** *vs*. **6a** and **6b**) with respect to *in vitro* activities. Subsequent dose response studies were performed to calculate the IC_{50} values that were found to be in the range of 10–16 μM.

We focused next on evaluating zebrafish embryo toxicity and apoptosis. Embryos of the zebrafish *Danio rerio* are excellent animal models for studying the effects of small molecules on early development and on toxicity, thus allowing effective *in vivo* evaluations of potential drugs before embarking on expensive studies on mice and humans.[27] The histone demethylase LSD1 is known to be encoded by the zebrafish genome,[28] and the protein sequence has 85% sequence identity with that of human LSD1 protein making the zebrafish embryo an attractive *in vivo* model. Compounds **6b**, **6c** and **9b** showed general toxicity-related effects in zebrafish embryos, resulting in death after 24 h of exposure at 10 μM. In contrast, compounds **6b**, **9a** and **9c** showed increased apoptosis at 10 μM with no general toxicity-related effects even after 72 h of treatment (Table 1, Fig. 2). The data clearly suggest an increased apoptosis as indicated by acridine orange fluorescence, especially in the area of the brain. This observation is consistent with reported brain-related effects of LSD1 inhibition.[29]

Our results indicate that compounds **6a**, **6b**, **9a** and **9c** possess biological activity related to the mechanisms involved in cell viability. The data from *in vitro* LSD1 inhibition are consistent with compounds **6a**, **6b** and **9a** possessing LSD1 inhibitory properties, although the IC_{50} values are higher by an order of 10^4. This suggests that the activity of the compounds on the cells tested is mediated by additional effects apart from LSD1 inhibition. Possible effects include those on epigenetic modifications other than histone methylation, such as histone acetylation and DNA methylation, leading to a significant suppression of cell viability. Such non-specific epigenetic effects are plausible because the compounds were designed based on structural features of the LSD1 protein and that of other known inhibitors of LSD1. The data from studies with zebrafish embryos showed that compounds **6b**, **9a** and **9c** resulted in a significant increase in apoptosis, with no general toxicity-related effects. The treatment was done at a concentration of 10 μM, which was in the range of *in vitro* LSD1 inhibition IC_{50} values. The significant difference in activity shown by these

Table 1 Summary of the pharmacological evaluation of compounds **6a**, **6b**, **9a** and **9c**

Pharmacological activity	 **6a**	 **6b**	 **9a**	 **9c**
HeLa cell viability (IC_{50})	1.29 nM	1.22 nM	1.48 nM	2.14 nM
MDA-MB-231 cell viability (IC_{50})	1.328 nM	1.202 nM	1.328 nM	1.035 nM
In vitro LSD1 inhibition (IC_{50})	16.1 μM	10.1 μM	9.5 μM	>50 μM
Zebrafish embryo apoptosis	Insignificant	Yes (10 μM)	Yes (10 μM)	Yes (10 μM)
Zebrafish embryo toxicity	None	None	Not done	None

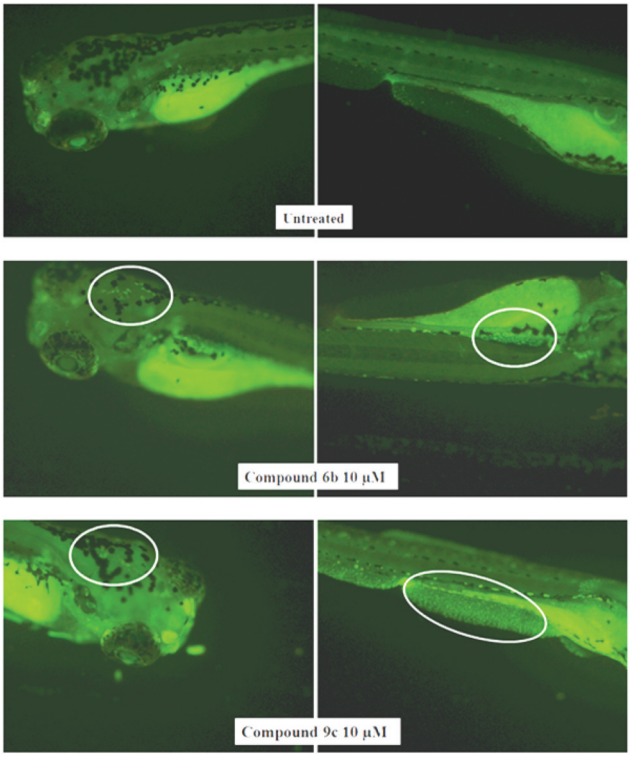


Fig. 2 Zebrafish embryos treated with compounds **6b** and **9c** and stained with acridine orange. Brightly stained spots highlighted with a circle represent apoptotic cells with fragmented nuclei.

compounds in cells and in zebrafish embryos suggests that the cancer cells used may possess features (such as specific mutations or epigenetic targets) that make them more sensitive to the effects of LSD1 inhibition, compared to the developing zebrafish embryos which represent a non-diseased, living organism. This is plausible since cancer cells are known to have several accumulated defects compared to normal cells. Our data thus support the possibility that the compounds are selective for cancer cells over normal cells. Overall, the results of our detailed pharmacological analysis suggest that compounds **6b** and **9a** possess biological activity consistent across *in vitro*, cell culture and *in vivo* systems.

To understand the binding mode and stability of ligand receptor complexes we performed molecular modeling studies (Docking analysis and Molecular dynamics simulations) using **6a**, **6b** and **9a** against LSD1 (Fig. 3, see also ESI†). Docking studies predicted good interactions with the LSD1 enzyme. The guanidine group of **9a** interacted with Glu-308 and Arg-310 *via* H-bonds whereas the phenyl ring participated in a π-cation interaction with Arg-316. In the case of **6b**, (i) the free $-NH_2$ formed a H-bond bond with Ser-760, (ii) the sulfur atom formed a H-bond with Met-332 and Val-333 and (iii) the phenyl ring participated in π–π stacking with Trp-751 and Tyr-761 residues. In the case of **6a**, (i) the free $-NH_2$ and the oxazole nitrogen were involved in H-bonding with Thr-624 and Arg-316 respectively, and (ii) the oxazole ring participated in a π–cation interaction with Arg-316.

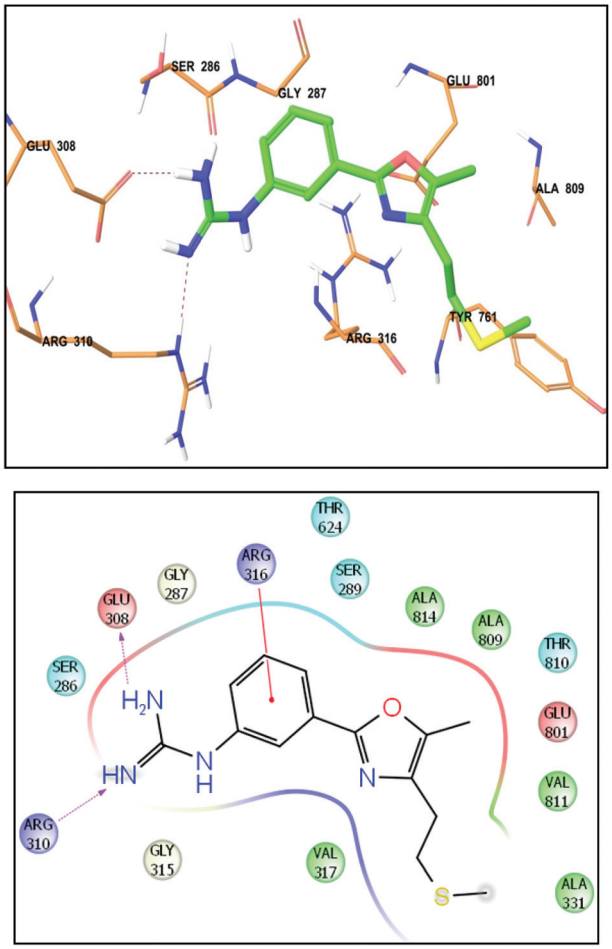


Fig. 3 Binding orientation and interactions of **9a** at the LSD1 inhibitors binding site.

Furthermore, the molecular dynamics (MD) simulations of **9a** at the binding site of protein were also performed using the Desmond package incorporated in Maestro. To evaluate the stability of the MD trajectories and the differences in stability of the MD simulations, the total energy of the ligand–protein complex and RMSD values for the protein backbone atoms relative to the initial minimized structure through the phase of the simulation were calculated. There was no significant change in the total energy of the system observed during the entire simulation period. The RMSD values remained within 6.0 Å for the system after reaching equilibrium, which demonstrated the conformational stabilities of the protein structures. Throughout the MD simulations, the studied compound maintained its binding pose in the expected orientation.

In summary, we have designed, synthesized and performed pharmacological analysis of 3-amino/guanidine substituted phenyl oxazoles as a novel class of LSD1 inhibitors and likely epigenetic modulators. These molecules were conveniently prepared *via* a multistep sequence involving an amide bond formation, construction of an oxazole ring and introduction of a guanidine moiety as key steps. Among the compounds tested, **6a**, **6b** and **9a** showed promising activities against LSD1

(IC_{50} ~ 10–16 μM) and cancer cells (IC_{50} = 1.2–1.4 nM) *in vitro*. Additionally, the compounds **6b** and **9a** were found to increase apoptosis in zebrafish embryos in the brain area when evaluated in a phenotype-based zebrafish assay. All these compounds showed good binding interactions with the LSD1 enzyme *in silico* as indicated by docking studies. Overall, our study provides the basis for future work which can be directed at elucidating the details of mechanisms of action of the compounds presented and the contribution of specific structural features to their activity.

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