Inhibitors of histone deacetylase 6 based on a novel 3-hydroxy-isoxazole zinc binding group

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ABSTRACT
Histone deacetylase 6 (HDAC6) is an established drug target for cancer treatment. Inhibitors of HDAC6 based on a hydroxamic acid zinc binding group (ZBG) are often associated with undesirable side effects. Herein, we describe the identification of HDAC6 inhibitors based on a completely new 3-hydroxy-isoxazole ZBG. A series of derivatives decorated with different aromatic or heteroaromatic linkers, and various cap groups were synthesised and biologically tested. In vitro tests demonstrated that some compounds are able to inhibit HDAC6 with good potency, the best candidate reaching an IC₅₀ of 700 nM. Such good potency obtained with a completely new ZBG make these compounds particularly attractive. The effect of the most active inhibitors on the acetylation levels of histone H3 and α-tubulin and their anti-proliferative activity of DU145 cells were also investigated. Docking studies were performed to evaluate the binding mode of these new derivatives and discuss structure-activity relationships.

1. Introduction
Histone deacetylases (HDACs) are post-translational enzymes that remove acetyl groups from lysine residues, thereby regulating key processes, such as gene expression. Eleven HDAC isoforms, clustered into four classes, are currently described. HDAC6 is a histone deacetylase isoform belonging to class IIa that contains two catalytic domains and, unlike other HDACs with nuclear localisation, is found mainly in the cytoplasm. Importantly, HDAC6 deacetylates non-histone proteins, such as tubulin, Hsp90 and cortactin.

Several HDAC inhibitors (HDACi) have been developed so far, mainly active against cancer and neurodegenerative diseases, resulting in five approved drugs. The vast majority of the developed inhibitors contain a hydroxamic acid zinc binding group (ZBG). However, several studies have shown that hydroxamic acids are genotoxic due to the Lossen rearrangement of the O-ester, resulting in five approved drugs. The vast majority of the developed inhibitors contain a hydroxamic acid zinc binding group (ZBG). However, several studies have shown that hydroxamic acids are genotoxic due to the Lossen rearrangement of the O-ester, which yields a reactive isocyanate able to covalently modify various cellular components. This undesirable effect motivates the search for HDAC inhibitors with alternative ZBGs.

In a recent study, we described the effect of different linker chemotypes on the potency and selectivity of a series of HDAC6 inhibitors carrying a hydroxamime ZBG. Interestingly, during the synthesis of N-hydroxy-3-phenyl-propiolamide, we observed the formation of 5-aryl-3-hydroxy-isoxazole, presumably formed through intramolecular cyclisation by 1,4 addition of the hydroxyl group to the conjugated alkyne bond. Compound 12 converted into 13 over time, even when stored at solid state both at room temperature and at −20°C, demonstrating that compound 13 is stable. Being constituted by a small heteroaromatic ring with hydrogen bond acceptor and donor atoms, we hypothesised that 3-hydroxy-isoxazole moiety could satisfy the pharmacophore requirements necessary for the coordination of the catalytic zinc ion of HDAC6. Therefore, in this work we investigated a series of 3-hydroxy-isoxazole derivatives bearing different aromatic or heteroaromatic linkers and various cap groups.

2. Results
2.1. Ligand-based analyses
We firstly investigated the tautomeric preference of compound 13 in water solvent by means of quantum-chemical calculations, performed with Jaguar of the Schrödinger suite 2020−1, with default settings. Indeed, five-membered heteroaromatic rings, such as 3-hydroxy-isoxazole, could undergo keto-enol tautomerism in water. Results of the quantum-chemical calculations indicated that the enol form is 0.5 kcal/mol more stable than the keto tautomer, which is likely to be favoured by aromaticity. This result is in line with previous in silico and experimental studies on the tautomeric equilibria of 3-hydroxy-substituted isoxazoles, suggesting that the 3-hydroxy tautomer is preferentially present in solution. Therefore, all in silico calculations were conducted with the enol form of the ligands.

To evaluate whether compound 13 could provide a valuable biosimilar replacement of previously reported HDAC6 ZBGs, additional studies were performed to investigate the in vitro activity of compound 13 on DU145 cells. Compound 13 inhibits HDAC6 with an IC₅₀ of 700 nM, demonstrating that the compound is able to inhibit HDAC6 with good potency. The effect of the most active inhibitors on the acetylation levels of histone H3 and α-tubulin and their anti-proliferative activity of DU145 cells were also investigated. Docking studies were performed to evaluate the binding mode of these new derivatives and discuss structure-activity relationships.
The synthesised compounds are reported in Figure 2. With the heteroaromatic) and the introduction of selected cap groups. Modifications included different linker portions (aromatic and compounds based on the 3-hydroxy-isoxazole scaffold. Bioisosteric replacement of the hydroxamic acid moiety. suggest that the 3-hydroxy-isoxazole could represent a valuable bind to the Zn\(^{2+}\) ion with a bidentate coordination in crystallo-

### 2.2. Chemical synthesis

We therefore synthesised and *in vitro* tested a small series of compounds based on the 3-hydroxy-isoxazole scaffold. Modifications included different linker portions (aromatic and heteroaromatic) and the introduction of selected cap groups. The synthesised compounds are reported in Figure 2. With the exception of 13 and 29, all the other 3-hydroxy-5-aryl/heteroaryl-isoxazole derivatives were synthesised according to the retrosynthetic strategies depicted in Scheme 2. All the experimental synthetic procedures and the discussion of each synthetic pathway are reported in the Supplementary material, together with the synthetic Schemes S1–S6.

As a first attempt, the 3-hydroxy-5-arylisoxazole scaffold was directly obtained by cyclisation from hydroxylamine and the 2,3-dibromo-3-arylpropanoate ester following the procedure of Panda et al.\(^\text{15}\), as reported in the Pathway A. The 2,3-dibromo-3-arylpropanoate ester was readily prepared by brominating the appropriate aryl cinnamate ester, which was previously synthesised by esterification of the corresponding aryl cinnamic acid. This synthetic pathway provided easily the ester intermediate with high yield. On the contrary, the last step gave low yields, probably due to the harsh reaction conditions, which produced several and unidentified by-products, making the subsequent purification procedure particularly hardworking. Pathway B resulted in cyclisation of the appropriate 3-arylpropiolate ester with hydroxylamine using milder reaction conditions.\(^\text{16}\) The 3-arylpropiolate ester derives from the corresponding acid, which is obtained from the 2,3-dibromo-3-arylpropanoate ester by a dehydrohalogenation reaction. The dibromo-derivative was in turn obtained from bromination of an aryl cinnamate ester, which was produced by Wittig reaction between the appropriate aryl aldehyde and the triphenylphosphonium salt. By using this synthetic pathway, the final desired products were obtained with high yield and purity. However, the main limitations of this procedure are the low commercial availability of various 3-arylpropiolate esters and their limited synthetic accessibility. In this regard, besides the Sonogashira’s reaction followed in the past, a second strategy for the synthesis of the 3-arylpropiolate esters was adopted. It requires a dehydrohalogenation of the corresponding 2,3-dibromo-derivative, using alcoholic KOH, then a re-esterification of the obtained 3-arylpropanoic acid. However, although this retrosynthetic pathway adds two synthetic steps and the strong alkaline conditions of dehydrohalogenation lead to the formation of by-products, the overall yield was higher than that obtained following the previously described Pathway A. Moreover, during the bromination reaction of the methyl 3–(5-methylthiophen-2-yl)acrylate, in addition to the desired dibromo-derivative, an α-bromoaryl-acrylate (methyl 2-bromo-3–(5-methylthiophen-2-yl)acrylate) was isolated and identified. This compound gave us the opportunity to follow another approach for the synthesis of the 3-arylpropiolate esters (Pathway C). According to Kim et al.\(^\text{17}\) α-bromoacrylate can be converted into the corresponding 3-arylpropiolate ester using a mixture of sodium amide (Na\(_{2}\)NH\(_{2}\)) and potassium tert-butoxide (t-BuOK) in a non-protic solvent (i.e. THF). This procedure, tested on the methyl 2-bromo-3–(5-methylthiophen-2-yl)acrylate, allowed us to obtain the 3–(5-methylthiophen-2-yl)propionic acid with quantitative yield and without by-products. Thus, the next 3-arylpropiolate esters were prepared accordingly to the improvements achieved following Pathway C. The appropriate 2-bromo-3-

![Scheme 1](https://example.com/scheme1)

**Scheme 1.** Conversion of the N-hydroxy-3-phenyl-propiolamide 12 into the 3-hydroxy-5-phenyl-isoxazole 13.

![Figure 1](https://example.com/figure1)

**Figure 1.** 3D ligand-based alignment of 13 with respect to the known HDAC6 inhibitor ChEMBL16300. 13 and ChEMBL16300 are represented as raspberry and deep teal sticks, respectively.

### 3. Experimental Section

**Figure 2:** The synthesised compounds are reported in Figure 2. With the exception of 13 and 29, all the other 3-hydroxy-5-aryl/heteroaryl-isoxazole derivatives were synthesised according to the retrosynthetic strategies depicted in Scheme 2. All the experimental synthetic procedures and the discussion of each synthetic pathway are reported in the Supplementary material, together with the synthetic Schemes S1–S6.

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Figure 2. Chemical structures of the investigated 3-hydroxy-5-arylisoxazole compounds.

Scheme 2. Retrosynthetic strategies adopted for the synthesis of the 3-hydroxy-5-arylisoxazoles.
S. Synthesis of 29.

arylacrylate esters were easily synthesised by reacting the arylaldehyde with 2-bromoacetate esters in the presence of titanium tetrachloride (TiCl₄) and triethylamine (TEA), as reported by Augustine et al.18. This procedure was successfully applied for the synthesis of diverse aryl and heteroaryl α-bromo-acrylates and gave high yields. Unfortunately, starting from pyridyl carboxaldehyde this synthetic procedure was not effective. Therefore, for the synthesis of the methyl 2-bromo-3-(5-bromopyridin-2-yl)acrylate a variant of the Wittig’s reaction was adopted (Pathway D)19. The 5-bromopyridin-2-yl carboxaldehyde was condensed with the brominated ylide to give directly the α-bromopyridyl acrylate. The brominated ylide was prepared by reacting the triphenylphosphonium salt with NaOH to obtain the ylide, which was successively mono-brominated with bromine. A last and different synthetic approach was used to obtain the 5-phenylisoxazole-3-carboxylic acid 29. The ethyl 2,4-dioxo-4-phenylbutanoate was cyclized with hydroxylamine hydrochloride (a) and the obtained ethyl ester 29.1 was then hydrolysed with NaOH 1 M (b, Scheme 3).

Since the synthesised compounds have not be subjected to in vivo assays, their purity was evaluated by NMR analysis (>90%). In this regard and as an example, the 1H NMR spectra images of the most interesting derivatives (i.e. 17, 23, 25 and 27) have been reported, together with that of the first compound (13) of this series (see Supplementary material).

2.3. Biological evaluation

The obtained compounds were tested in vitro for the inhibition of recombinant human HDAC6, through dose-response assays performed in the 300 μM to 135 nM concentration range. The results are reported in Table 1, and dose-response curves are reported in Figure S1. While 14–16, 19, 20 and 29 were inactive, displaying more than 55% HDAC6 activity at the highest tested concentration (300 μM), compounds 13, 22, 26 and 28 were scarcely active showing IC₅₀ values of 98.1, 73.3, >100, and 82.3 μM, respectively. We identified three active molecules with IC₅₀ values ranging from 10 to 50 μM (18, 21, and 24), three compounds (17, 25, 27) with IC₅₀ values below 10 μM and one compound (23) with IC₅₀ below 1 μM. The most active compound (23) reached an IC₅₀ of 700 nM. The most active compounds were further tested on the acetylated p53 peptide -residues 379–382 (RHKKAcAMC) as substrate for HDAC6. The results corroborate the inhibitory activity of the compounds lower than 10 μM. The most active compounds 17, 23, 25 and 27 were tested in vitro in DU145, an androgen insensitive prostate cancer cell line (PCa) with very low or undetectable AR levels, which represents the castration-resistant PCa (CRPC) model. HDAC6 contribution to CRPC is known20 and the possibility to arrest CRPC proliferation by acting on HDAC targets is expanding in the scientific literature21,22. After administration of the selected compounds for 24 h at 10 μM concentration, the effect on the acetylation levels of histone H3 and α-tubulin (respectively, HDAC1 and HDAC6 targets) was investigated. While no effect was observed on the acetylation levels of histone H3 (Figure S3), the acetylation of α-tubulin increased after treatment with 23 and 25, suggesting a potential HDAC6 selectivity for both compounds. Lastly, in the same cell line, the anti-proliferative effect was measured in dose and time dependent manner (Figure S4). As shown in Figure S4, the compounds reduced the proliferation rate of DU145 cells at a 50 μM concentration. In particular, 23, 25 and 27 exerted anti-proliferative activity in late phases at the lowest concentrations as well.

2.4. Molecular docking

To rationalise the structure-activity relationships (SAR) of the reported compounds, docking calculations were performed into a representative HDAC6 crystal structure (PDB code: 5WGI23). The experimental details are reported in the Supplementary material. Docking calculations were performed with the Glide software of the Schrödinger suite 2020–124. According to the predicted binding modes, the 3-hydroxy-isoxazole moiety establishes a bidentate coordination with the catalytic Zn²⁺ of the HDAC6 active site, similarly to other previously reported ligands with different ZBGs23,25–27. In particular, the compounds under investigation were predicted to coordinate the catalytic zinc ion with the 3-hydroxy group, and the nitrogen and oxygen atoms of the isoxazole ring. Moreover, the 3-hydroxyl group established a hydrogen bond interaction with the His573 in the protonated form, similarly to Trichostatin A (TSN) in the 5WGI crystal complex23,28. Interestingly, docking calculations performed in the 5WGI structure with a neutral His573 provided similar results, the Root Mean Square Deviations (RMSD) values observed for the respective predicted poses being below 2.0 Å (Table S2). This result, which is due to the ability of the 3-hydroxyl group to establish both H-bond acceptor and donor interactions, suggests that the His573

| Compound ID | HDAC6 IC₅₀ (μM) |
|-------------|-----------------|
| Trichostatin A | 0.026 |
| 13 | 98.1 |
| 14 | n.a. |
| 15 | n.a. |
| 16 | n.a. |
| 17 | 1.3 |
| 18 | 16.4 |
| 19 | n.a. |
| 20 | n.a. |
| 21 | 29.6 |
| 22 | 73.3 |
| 23 | 0.7 |
| 24 | 21.9 |
| 25 | 1.5 |
| 26 | >100 |
| 27 | 8.2 |
| 28 | 82.3 |
| 29 | n.a. |

n.a.: not active. The compound displayed more than 55% enzyme activity at the highest tested concentration (300 μM).
protonation state does not significantly affect the binding mode of the investigated ligands. Likewise, docking results obtained with negatively charged ligands potentially originating from the dissociation of the 3-hydroxyl group were fully comparable, the RMSD obtained between the poses being always below 1.5 Å (Table S2). According to the predicted binding poses, 29, which presents a carboxylic acid group at position 3 of the isoxazole ring and turned out to be inactive in our in vitro assays (Table 1), did not establish interactions with the zinc ion of HDAC6. Moreover, compounds with sterically hindered linkers, such as 19 and 20 (Figure 3, panel a), could not be accommodated in the catalytic tunnel of the histone deacetylase, due to steric clashes with the Phe643, Phe583 and His614 side chains. Similar considerations apply also for 15, which is substituted with an ortho chlorine substituent. Compounds with a 4-substituted phenyl (e.g. 18) (Figure 3, panel b) or 5-methyl-thiophene group (i.e. 24) (Figure 3, panel c) are able to establish favourable \( \pi-\pi \) stacking interactions with the side chains of the residues lining the catalytic tunnel. Ligands with polar substituents in meta position, such as 14, could not establish a favourable coordination of the HDAC6 catalytic zinc, thus resulting inactive or significantly less active.

In compound 25, one of the most active ligands of the series (Table 1 and Figure 3, panel d), the 1,3-benzodioxolane moiety establishes favourable \( \pi-\pi \) stacking interactions with Phe643, Phe583 and His614, and a hydrogen bond interaction with the 1095 water molecule. The latter interaction, observed also in other crystallographic complexes, appears to contribute significantly to the binding of ligands to HDAC6. The furan derivative 26 was almost as active as the phenyl derivative 13. However, substitution with a bromine, such as in 21 and 22, provided slightly better activities. Compounds bearing flexible aromatic cap groups, such as 17 (Figure 3, panel e) and 27, were among the most active of the series (Table 1). According to the predicted binding modes, the distal aromatic portion of these molecules establishes favourable hydrophobic interactions with the side chains of Phe583 and Pro464, similarly to the crystallographic ligand in the complex. Indeed, superimposition of the docking poses of 17 and 27 with HDAC6 crystallographic ligands revealed structural details important for the activity. In particular, compounds 17 and 27 present a methyl-ether linker, which is often replaced, in potent HDAC6 inhibitors, by chemical moieties potentially able to interact with Ser531 and/or water molecules in the proximity of the cap region. Notably, the substitution of the methyl-ether linker in 17 with methylene (i.e. 23 – Figure 3, panel f) resulted in a slight improvement of HDAC6 inhibitory activity (Table 1). Altogether, the performed in silico analyses helped to rationalise the inhibitory activity of the investigated compounds.

3. Conclusions

In conclusion, we have reported a series of HDAC6 inhibitors bearing a completely new 3-hydroxy-isoxazole zinc binding group. Some of the compounds had good potency, the most active one having an IC\(_{50}\) of 700 nM, and showed also anti-proliferative activity in prostate cancer cells. In particular, the results of the in vitro assays on recombinant proteins allowed us to identify 17, 23 and 25 as the most interesting candidates of the series. Molecular docking and ligand similarity calculations allowed us to predict their binding mode and discuss the SARs of the reported ligands. Interestingly, the compounds present good activity and low
molecular weight. Most importantly, they are based on an entirely new zinc binding group, thus representing an interesting alternative to classical hydroxamic acids. Therefore, they constitute valuable candidates for further preclinical optimisation of HDAC6 inhibitors based on a novel zinc binding group.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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