Identification of Lactate Dehydrogenase 5 Inhibitors using Pharmacophore-Driven Consensus Docking

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Abstract: Background: Human lactate dehydrogenase 5 (*h*LDH5) represents a promising anticancer target, particularly for the treatment of hypoxic tumors, where it is often hyperexpressed. In fact, by catalyzing the reduction of pyruvate to lactate, *h*LDH5 allows the survival of tumor cells under hypoxic conditions by means of glycolysis. Despite the efforts dedicated to the identification and development of *h*LDH5 inhibitors, only few compounds showing promising activity in cancer cell lines have been reported.

Objective: In the present study, we developed a virtual screening (VS) protocol aimed at identifying new small molecule inhibitors of *h*LDH5.

Method: The VS strategy consisted in a pharmacophore-driven consensus docking (CD) approach, combining a structure-based pharmacophore screening and CD protocol employing three different docking methods.

Results: The VS protocol was applied to filter the Enamine commercial database and allowed the selection of three candidate ligands to be subjected to *h*LDH5 inhibition assays. One of the selected compounds showed a promising activity, compared to its low molecular weight, with an IC_{50} of 180.7 ± 16.5 μM.

Conclusion: We identified a new small-molecule inhibitor of *h*LDH5 that can be considered as a new lead for the development of potent *h*LDH5 inhibitors. Moreover, these results demonstrate the reliability of the VS protocol developed.

1. INTRODUCTION

Human lactate dehydrogenase (*h*LDH) is the enzyme responsible for the catalysis of the last step of glycolysis, which is the reversible reduction of pyruvate to lactate, with simultaneous oxidation of cofactor NADH to NAD^+. Five functional tetrameric human isoforms (*h*LDH1-5) are distinguished on the basis of the possible combinations of the two monomeric subunits: LDH-A and LDH-B. Among them *h*LDH5 (*h*LDH-A_4) has attracted great attention initially as an anti-malarial target and, more recently, as a potential anticancer target, since it plays a key role in the metabolism of invasive tumours.[1] The *h*LDH5-catalyzed reduction of pyruvate to lactate allows a continued ATP production even under hypoxic conditions by means of glycolysis without the oxygen-dependent oxidative phosphorylation. *h*LDH5 is over-expressed in many human tumor tissues and it was found to correlate with tumor size and poor prognosis. Silencing this enzyme by genetic techniques or by pharmacological inhibition proved to decrease cell proliferation and migration.[2] Therefore, inhibition of *h*LDH5 may be considered as a challenging and promising anticancer strategy, since it should cause a starvation of cancer cells by reducing their conversion of glucose to lactate.[3,4] *h*LDH5 inhibition for the treatment of tumours is a potentially safe strategy, considering that individuals homozygous for LDH-A deficiency do not show any particular clinical symptoms except for myoglobinuria upon intense physical exertion.[5] In the last years, great efforts have been dedicated to the development of new *h*LDH5 inhibitors by both academic and industrial groups. A diacid malonate-based bifunctional inhibitor (compound **1** or AZ-33, Fig. **1**) was developed by NMR fragment-based approaches at AstraZeneca UK. AZ-33 is very potent on the isolated enzyme ($IC_{50} = 0.5$ μM), but unfortunately it failed to reach a good activity on cancer cells, probably because of the diacid functionality that hinders membrane permeability.[6] Screening of the Genentech/Roche corporate compound collection and subsequent lead optimization led to the development of other *h*LDH5 inhibitors, such as 2-thio-6-oxo-1,6-dihydropyrimidine **2** (Fig. **1**), which showed an inhibition potency in the low micromolar range ($IC_{50} = 0.48$ μM), but it was scarcely effective in reducing lactate production in cancer cells.[7] The most recent inhibitor discovered by Genentech Inc. is the trisubstituted hydroxylactam **3** (Fig. **1**), which displayed a nanomolar affinity for the enzyme ($IC_{50} = 3$ nM), and it also reached an excellent potency in the reduction of lactate

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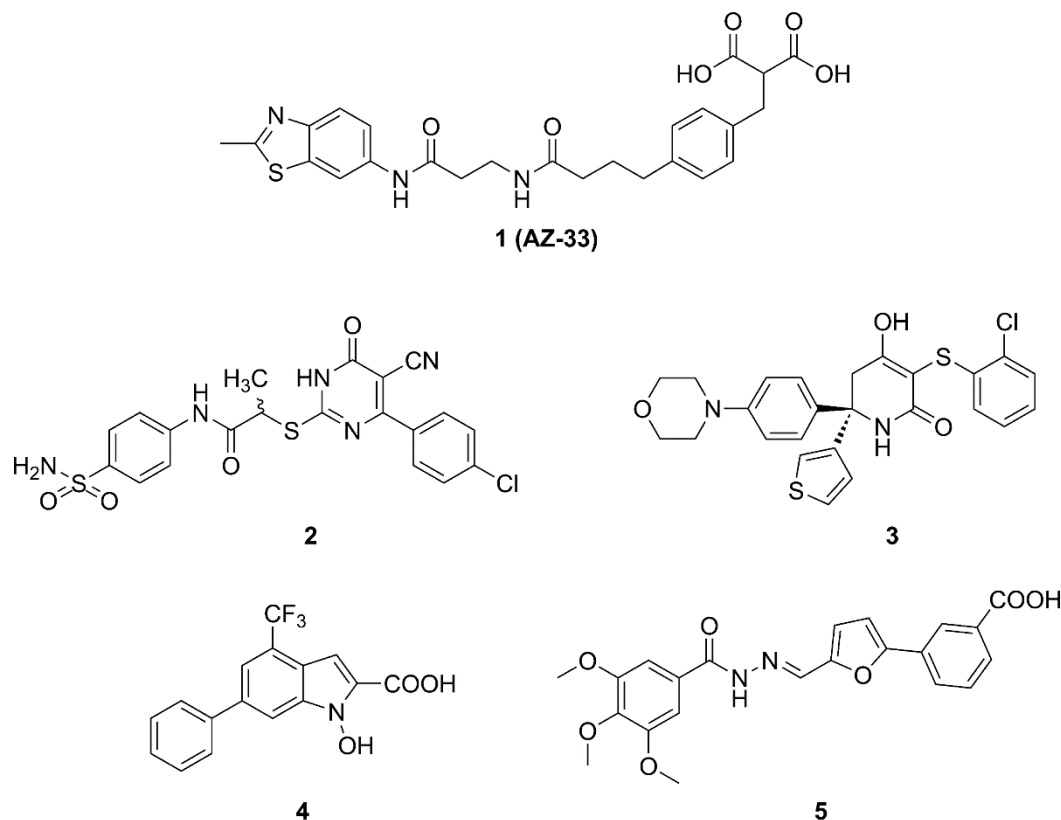
Fig. (1). Structures of some *h*LDH5 inhibitors.

production in pancreatic MiaPaca2 cancer cell line (IC$_{50}$ = 670 nM), showing very promising pharmacokinetic properties to be orally administered in mice.[8] Our group discovered a new class of *N*-hydroxyindole-based inhibitors of *h*LDH5, and one of the most active compound is reported in Fig. **1** (compound **4**),[9-13] which inhibits the enzyme with IC$_{50}$ values in the low micromolar range, and reduces lactate production and cell viability in various cancer cell cultures. As a further step in the development of this class, we functionalized *N*-hydroxyindole-based compounds with sugar portions in order to enhance their uptake by cancer cells, thus exploiting a dual targeting of the Warburg effect.[14] Among other chemical classes of LDH-inhibitors, *N*-acylhydrazone derivative **5** (Fig. **1**), developed at the University of Bologna, inhibits *h*LDH5 at the micromolar level and reduces lactate production, NAD$^+$/NADH ratio and induces apoptosis in cancer cells.[15] In the last few years, a certain number of new *h*LDH inhibitors, such as galloflavin, have been identified through receptor-based virtual screening (VS) studies [16] relying on docking into different crystal structures of the enzyme.[15,17] Recently, we developed and optimized a consensus docking (CD) protocol combining ten different docking methods, which was found to predict ligand binding poses better than the single docking procedures and showed to be a promising strategy for improving performance and hit rates of VS campaigns.[18,19] Our CD protocol has already been

successfully employed for the identification of new non-covalent fatty acid amide hydrolase (FAAH) inhibitors,[20] while a mixed ensemble/CD approach using only four docking procedures allowed the identification of mild *h*LDH inhibitors.[21] On the other hand, we recently demonstrated that receptor-based pharmacophore screening may represent a reliable strategy to identify new small molecule inhibitors of *h*LDH.[22] For this reason, we decided to develop a pharmacophore-driven CD approach combining pharmacophore-based VS and a CD protocol employing a reduced number of docking procedures, in order to identify novel compounds endowed with *h*LDH-A inhibitory activity (Fig. **2**).

2. MATERIALS AND METHODS

2.1 Receptor-Based Pharmacophore Generation. The receptor-based pharmacophore hypothesis was developed based on the X-ray structure of human lactate dehydrogenase A (*h*LDH-A) in complex with the 2-amino-5-aryl-pyrazine inhibitor (compound **18**) reported by Fauber et al. and the coenzyme NADH (PDB code 4M49).[23] The receptor-based pharmacophore model was built by using the software PHASE.[24] After generating an exhaustive model including all the possible pharmacophore features identified by the software for compound **18**, only the five features representing the main ligand-protein and ligand-cofactor interactions observed in the crystal structure were selected

and retained in the final pharmacophore model. The other features were thus discarded. The *create_xvolReceptor* utility of PHASE was used to generate the receptor excluded volume, representing the region of space surrounding the pharmacophore model that cannot be occupied by the database compounds when aligned to the model in a pharmacophore screening. The spheres defining the excluded volume shell were generated taking into account all the receptor and coenzyme heavy atoms placed at a distance ranging from 2 to 5 Å from the ligand surface. An additional excluded volume sphere representing the structural water molecule interacting with the ligand, R169 and the coenzyme was also considered. All other settings were left as their default.
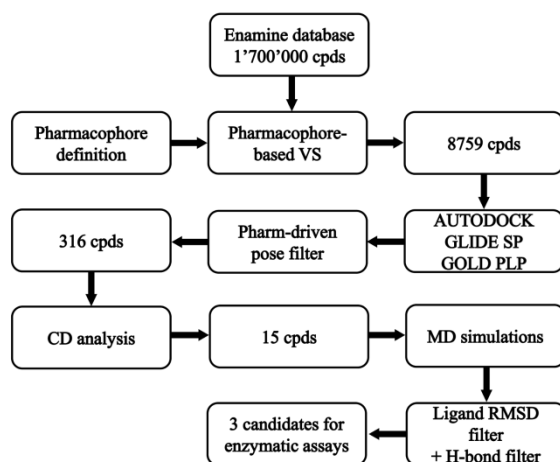

Fig. (2). Workflow of the pharmacophore-driven CD approach.

2.2 Database Generation and Pharmacophore Screening. The Enamine database, including more than 1'700'000 commercial compounds, was used as the screening database. The database was processed through the *phasedb_manage* and *phasedb_confsites* utilities of PHASE, in order to generate a PHASE 3D database in which multiple conformers, together with their corresponding pharmacophore sites, were created and stored for each molecule. The "thorough" sampling method was chosen, thus generating complete conformational ensembles for both the core and the peripheral groups of the compounds, while all other settings where left as their defaults. The PHASE 3D database was screened by using the pharmacophore model and the corresponding excluded volume previously generated. The pharmacophore search was carried out by setting the negative ionizable feature of the model as mandatory and the other ones as optional. Then, it was imposed that only the compounds matching at least three out of the five total features of the model and respecting the volume constraints were retrieved.

2.3 Docking Procedures. The screened compounds were docked into the crystal structure of *h*LDH-A in complex with compound **18** and NADH (PDB code 4M49).[23] The coenzyme and the water molecule interacting with R169, the ligand and NADH was kept within the receptor pocket used for all the different docking calculations. Only the top-scored

poses of the docked compounds were considered as a result of each docking procedure.

GOLD 5.1. The docking site was defined as the region of space including all residues which stayed within 10 Å from the bound ligand in the *h*LDH-A X-ray structure. The compounds were subjected to 30 genetic algorithm runs employing ChemPLP as fitness function. The "allow early termination" option was disabled, while the possibility for the ligand to flip ring corners was enabled. The GOLD defaults were left for all other settings.[25]

GLIDE 5.0. The docking grid was defined considering the bound ligand as the center of a cubic box of 10 Å in the x, y and z directions. The option allowing only the docking of ligands containing a defined range of atoms was disabled, so that all the ligands were docked independently from their number of atoms. The standard precision (SP) method was used for the study and all other settings were left as GLIDE defaults.[26]

AUTODOCK 4.2.3. Receptor and ligands were processed by using AUTODOCK Tools to define the torsion angles in the compounds, to add the solvent model and to assign partial atomic charges to the ligands and the protein. The receptor site in which the compounds were docked was delimited by a grid box of 10 Å in the x, y and z directions centered on the bound ligand. Energetic map calculations were carried out by employing a grid spacing of 0.375 Å and a distance dependent function of the dielectric constant. Each docked compound was subjected to 20 Lamarckian genetic algorithm runs using 2'500'000 steps of energy evaluation and default values for all other parameters.[27]

2.4 Pharmacophore-Driven Pose Filtering. Applying the three docking methods, three different binding dispositions were obtained for each compound docked into the *h*LDH-A binding site; these docking results were filtered through a rigid PHASE pharmacophore search, carried out by using the "ScoreInPlace" option. By applying this procedure, the top-scored poses of the docked compounds were superimposed to the pharmacophore model without applying any change to their coordinates, and only the poses matching at least three out of the five total features of the model and respecting the volume constraints were retrieved. As in the pharmacophore screening, the matching of the negative ionizable feature was set as mandatory for pose retrieval. Only the compounds for which all three docking poses passed the filter were taken into account for the consensus docking analysis, while the other ones were discarded.

2.5 Consensus Docking Evaluation. The RMSD of each of the three docking poses obtained for each compound against the remaining two was calculated by using the rms_analysis software of the GOLD suite.[28] On this basis, a 3x3 matrix was generated for each analyzed compound reporting the RMSD results. By using an in-house program these results were processed, so that the similar docking poses among the three results were clustered together. The complete-linkage method was used as clustering algorithm, which is an agglomerative type of hierarchical clustering. This method starts considering each element in a cluster of its own. The clusters are then sequentially combined into larger ones,

until all elements are in the same cluster. At each step, the two clusters separated by the shortest distance are combined. We selected an RMSD threshold of 1.5 Å, therefore the so obtained clusters contained the group of poses which were less than 1.5 Å away from all others poses belonging to the same cluster.

2.6 Molecular Dynamic Simulations. All simulations were performed using AMBER 14.[29] The crystal structure of *h*LDH-A in complex with compound **18** and cofactor NADH (PDB code 4M49) was used as a template to set up the simulations protocol. The complexes were placed in a parallelepiped water-box (TIP3P explicit solvent model) and solvated with a 10 Å water cap; the system was then neutralized through the addition of chloride ions. General amber force field (GAFF) parameters were assigned to the ligands and NADH, while the AM1-BCC method was used to calculate their partial charges. Two different minimization steps were performed prior to the molecular dynamic (MD) simulations. The first step was carried out for the minimization of the solvent, by applying a position restraint of 100 kcal/(mol•Å^2) to the complex. In the second step, only the protein α carbons were restrained with a harmonic potential of 10 kcal/(mol•Å^2), thus the whole system was minimized through 10'000 steps of steepest descent followed by conjugate gradient, until a convergence of 0.05 kcal/(mol•Å2) was reached. The minimized complexes were used as input structures for the MD simulations which were run by using particle mesh Ewald electrostatics, a cutoff of 10 Å for the non-bonded interactions and periodic boundary conditions. SHAKE algorithm was employed to keep all bonds involving hydrogen fixed, thus a simulation time step of 2.0 fs was used. Constant-volume periodic boundary conditions were used for the first 300 ps of simulation, during which the temperature was raised from 0 to 300 K. Constant-pressure conditions were employed for the remaining 4.7 ns of MD simulation, while the system temperature was kept constant at 300 K by using the Langevin thermostat. A total of 5 ns of MD simulation was thus performed for each ligand-protein-cofactor complex studied. The analysis of the MD trajectories to calculate H-bonds and water-bridged interactions occupancies, as well as the RMSD of the ligands with respect to their input poses, were performed by using the Cpptraj suite of AMBER 14.

2.7 Enzymatic Essays. The inhibition properties of the compounds were evaluated against purified human lactate dehydrogenase isoform 5 (Lee Biosolution, Inc.). The "forward" direction (pyruvate → lactate) of the lactate dehydrogenase reaction was conducted, and the kinetic parameters were measured by fluorescence (emission wavelength at 460 nm, excitation wavelength at 340 nm) to monitor the rate of conversion of NADH to NAD$^+$. Compounds were tested in the presence of scalar concentrations of NADH. They were added in scalar amounts (concentration range = 10–200 μM) to a reaction mixture containing phosphate buffer, 1.4 mM pyruvate and scalar concentrations of NADH (12.5 μM-150 μM). Finally, LDH solution was added (0.015 U•mL^{-1}). The LDH activity was measured by recording the decrease in NADH fluorescence using a Victor X3 Microplates Reader (PerkinElmer®). The experimental data were analyzed by

non-linear regression analysis with GraphPad Prism software, using a second order polynomial regression analysis, and by applying the mixed-model inhibition fit.[30]

3. RESULTS AND DISCUSSION.

With the aim of identifying small molecule inhibitors of *h*LDH5 belonging to novel chemical classes, we developed a pharmacophore-driven consensus docking (CD) strategy combining a receptor-based pharmacophore search with a consensus docking approach. The pharmacophore model was derived from the X-ray structure of *h*LDH-A complexed with the cofactor NADH and the 2-amino-5-aryl-pyrazine inhibitor compound **18** reported by Fauber et al. (PDB code 4M49).[23] As shown in Fig. **3**, this inhibitor is anchored to the active site of the enzyme thanks to a series of hydrogen bonds established with both protein residues and NADH. The carboxylic group of the ligand shows ionic interactions with the positively charged H193, which is a key residue for the enzyme catalytic activity, while four different hydrogen bonds are formed by its 2-aminopyrazine moiety: one with the backbone oxygen of T248, another one with the hydroxyl group of the same residue and two different H-bonds with a pyrophosphate oxygen of NADH. Moreover, a structural water molecule is supposed to mediate an H-bond network among the ligand carboxylic group, the amide carbonyl of the cofactor and the guanidine group of R169, which is another fundamental catalytic residue of the enzyme. In fact, these water bridged interactions (Fig. **3**) are clearly shown in chain A of the crystal structure 4M49. In the other two chains were the ligand is bound to the enzyme (chains C and D), such a water molecule is either absent or 4.6 Å away from the inhibitor carboxylic group, being involved in a different water bridged H-bond between R169 and T248. In chain B, the inhibitor is replaced by a lactate molecule, which is the substrate of the enzyme.
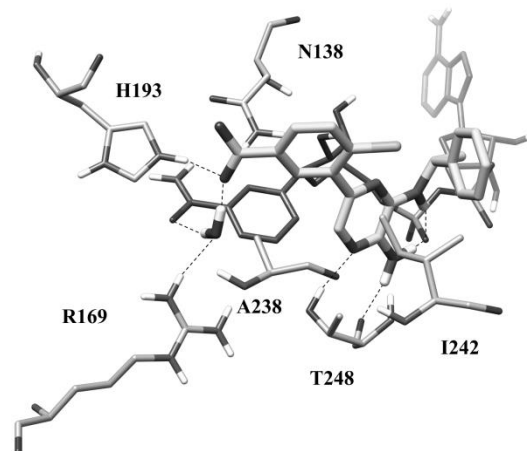

Fig. (3). Main ligand-protein interactions identified in the X-ray structure of *h*LDH, chain A, in complex with compound **18** (stick) and NADH. Hydrogen bonds are shown as black dashed lines.

In order to estimate the importance of these possible water mediated H-bonds and the strength of the direct interactions between ligand and protein/cofactor, we performed a 5 ns long MD simulation and analyzed the stability of the whole complex. After only 400 ps of MD simulation the system

reached an equilibrium, since during the following 4.6 ns of simulation its total energy was found to be constant, while the protein heavy atoms showed an average root-mean-square deviation (RMSD) with respect to the input structure of 1.0 Å (Fig. S1 in Supporting Information). The ligand and the coenzyme NADH perfectly maintained their binding disposition within the protein catalytic site, since their average RMSD with respect to the corresponding crystallographic pose were found to be 0.96 and 0.75 Å, respectively. Moreover, all the interactions identified in the X-ray complex were maintained for most of the MD simulation. In particular, the ligand H-bonds with H193, T248 and NADH showed more than 95% of occupancy, while the water-bridged interaction among R169, the ligand and the cofactor was detected for about 70% of the whole simulation. Given the results obtained with the MD simulation analysis, we decided to build a receptor-based pharmacophore model taking into account all the ligand moieties involved in these interactions to be used in a VS study. Therefore, the generated pharmacophore model (Fig. **4**) included a negative ionizable feature representing the inhibitor carboxylic group interacting with H193 and the structural water molecule, an H-bond acceptor feature representing its aromatic nitrogen interacting with T248 hydroxyl group and three H-bond donor features representing the three NH groups of the ligand interacting with NADH and T248 backbone oxygen. The pharmacophore model was completed with an excluded volume shell composed by spheres mimicking the steric hindrance of the receptor, which were generated by taking into account the heavy atoms of the cofactor and protein residues in proximity of the ligand, as well as the structural water molecule.
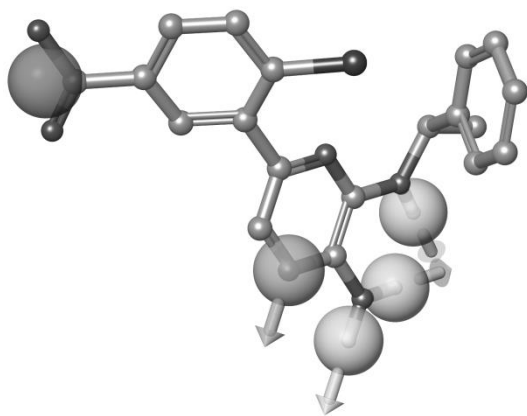

Fig. (4). Receptor-based pharmacophore model used for the VS study; the reference inhibitor together with the negative (dark gray), H-bond acceptor (gray) and H-bond donor (pale gray) features are shown; the excluded volume spheres are hidden for clarity.

The receptor-based pharmacophore model was used to screen the Enamine database, collecting about 1'700'000 commercially available compounds. Since the carboxylic group of compound **18** was responsible of the interactions with the catalytic residues H193 and R169, the pharmacophore screening was performed by setting the negative ionizable feature of the model as obligatory, while the other features were set as optional. The compounds

matching at least three out of the five pharmacophore features of the model and satisfying the steric constraints of the receptor were retrieved during the screening. By applying this filter, only 8759 compounds were selected and taken into account for docking studies. As mentioned above, we have recently developed a CD protocol combining different docking procedures, whose reliability in VS studies has been extensively tested using enriched databases and experimentally confirmed through the identification of new FAAH and LDH inhibitors. In the present VS study we decided to use a pharmacophore-driven CD protocol, i.e. a simplified CD approach employing three docking methods to identify only those compounds that were most likely to bind LDH catalytic site by assuming a disposition fitting the receptor-based pharmacophore. The 8759 compounds selected through the pharmacophore screening were thus docked into the crystal structure of *h*LDH-A in presence of NADH and the structural water molecule (PDB code 4M49), by using three procedures whose reliability in docking LDH inhibitors was previously assessed through enriched database analysis (i.e. Autodock, GLIDE with the SP method and GOLD with ChemPLP fitness function).[21] Then, before performing pose clustering, we first superimposed each docking result to the receptor-based pharmacophore model. Only the compounds for which all the three docking poses (obtained using the three different procedures) matched at least three out of the five features of the model were considered for the CD analysis. Moreover, the matching of the negative ionizable feature was set as mandatory in this post-docking filter, as imposed in the pharmacophore screening. As a result, only 316 compounds were retained and subjected to pose clustering, in order to search for common binding modes. In our latest evaluation and optimization of the CD procedure with enriched databases, we studied the effect of the RMSD tolerance used for pose clustering on the VS performance and we evidenced that the best results in terms of enrichment factors were obtained by using an RMSD cutoff of 1.5 Å. For this reason, we used the same RMSD threshold for the CD analysis of the 316 compounds passing the pharmacophore-driven pose filter. As shown in Table 1, only 15 compounds reached a consensus level of 3, which was also obtained for the reference inhibitor, compound **18**.

Table 1. Consensus docking results for the filtered commercial compounds.

Consensus Level	Number of Compounds
3	15
2	85
1	216

These ligands were thus subjected to MD simulation studies to verify the stability of their predicted binding mode by using the same MD protocol reported above that was tested on the reference X-ray complex. The RMSD of the position of each ligand during the whole 5 ns of simulation, with respect to the corresponding docking pose, was calculated and the 6 compounds showing an average RMSD higher

than 1.5 Å were discarded. The remaining 9 ligands were further analyzed to evaluate the stability of their interactions with the cofactor and the protein residues represented in the receptor-based pharmacophore model. The three compounds maintaining the interaction with H193 for more than 90% of the whole simulation and at least two other interactions with NADH, T248 and R169 (considering the water-bridged H-bond), for at least 70% of the simulation were selected to be purchased and tested for their inhibitory activity against *h*LDH5. Table 2 reports the biological results obtained for the selected ligands, together with the known inhibitor galloflavin that was used as a reference: compound **VS3** showed a promising activity, with an IC_{50} of 180.7 ± 16.5 μM. In fact, due to its very low molecular weight and number of heavy atoms, compound **VS3** demonstrated a higher ligand-efficiency (LE = 0.40)[31] compared to that calculated for compound **18** (LE = 0.31) and galloflavin (LE = 0.28), although showing a lower activity (compound **18** IC_{50} = 2 μM). [23] Hence, compound **VS3** could represent a valuable starting point for a lead optimization campaign.

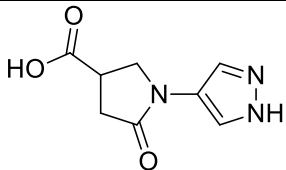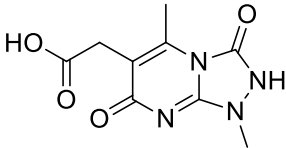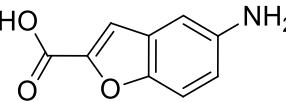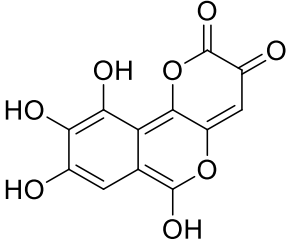**Table 2.** Structure and *h*LDH5 inhibitory activity of the tested compounds.

	Structure	IC50 (µM)
VS1		>>500
VS2		494.1 ± 42.2
VS3		180.7 ± 16.5
Galloflavin		91.7 ± 10.7

Fig. **5** shows the predicted binding mode of compound **VS3**. The ligand is placed with its bicyclic core between A238 and I242 from one side, and NADH from the other side, thus forming van der Waals interactions with both these residues and the cofactor. The amino group of the ligand forms two

different hydrogen bonds that were maintained for more than 80% of the MD simulation: one with the backbone oxygen of T248 and another one with NADH pyrophosphate. The carboxylic group of compound **VS3** is placed in close proximity of both the catalytic residue H193, with which it interacts during the whole MD simulation, and the structural water molecule; in fact, the water bridged H-bond network involving the ligand, NADH and R169 was detected for more than 90% of the simulation. Finally, a hydroxyl group of NADH forms an additional hydrogen bond with the ligand endocyclic oxygen.
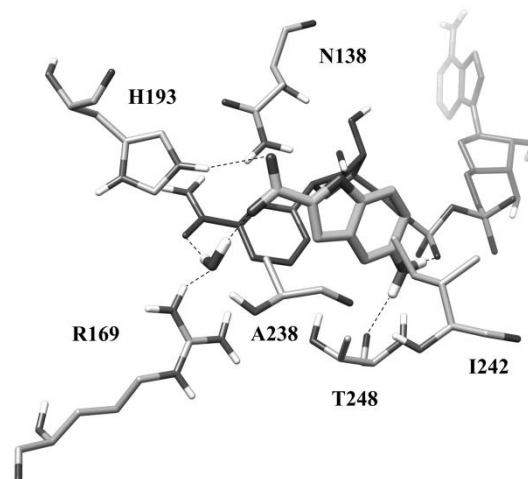

Fig. (5). Minimized average structure of compound **VS3** docked into *h*LDH-A catalytic site.

4. CONCLUSIONS.

In the present study, we developed a pharmacophore-driven CD protocol focused on the identification of new *h*LDH5 inhibitors. For this purpose, we built a receptor-based pharmacophore model based on the X-ray structure of *h*LDH-A in complex with the cofactor NADH and a 2-amino-5-aryl-pyrazine inhibitor. The generated pharmacophore model, representing the key ligand-protein and ligand-cofactor interactions identified in the crystallographic complex, was used to filter a database including about 1'700'000 compounds. The ligands selected through the screening were subjected to a CD approach employing three docking methods and aimed at identifying the compounds that were most likely to bind LDH catalytic site assuming a disposition fitting the pharmacophore model. The predicted binding mode of the top-scored compounds was then analyzed through MD simulations studies, which allowed the selection of three candidate ligands that were tested for *h*LDH5 inhibitory activity. Enzymatic assays revealed a promising activity for one of the selected compound, which showed an IC_{50} of 180.7 ± 16.5 μM, and thus validated the reliability of the VS protocol herein described. Moreover, the identified ligand can already be considered as a new lead compound for the development of potent *h*LDH5 inhibitors, especially considering its simple chemical scaffold and very low molecular weight (compared to the reference inhibitors galloflavin and compound **18**), which can allow a wide range of possible ligand functionalization in future lead optimization studies. A series

of derivatives of compound **VS3** will be thus synthesized in order to develop more potent analogues, and with the aim of identifying highly active ligands to be subjected to cell proliferation and migration assays.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

SUPPLEMENTARY MATERIAL

Figure S1. Analysis of the MD simulation of *h*LDH-A in complex with the cofactor NADH and the 2-amino-5-aryl-pyrazine ligand.

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