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Synthesis of sulfonamide-containing *N*-hydroxyindole-2-carboxylates as inhibitors of human lactate dehydrogenase-isoform 5

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ABSTRACT

N-Hydroxyindole-2-carboxylates possessing sulfonamide-substituents at either position 5 or 6 were designed and synthesized. The inhibitory activities of these compounds against isoforms 1 and 5 of human lactate dehydrogenase were analysed, and K_i values of the most efficient inhibitors were determined by standard enzyme kinetic studies. Some of these compounds displayed state-of-the-art inhibitory potencies against isoform 5 (K_i values as low as 5.6 μM) and behaved as competitive inhibitors versus both the substrate and the cofactor.

Tumor cells require a high production of energy (ATP) and anabolites, even under hypoxic conditions, to ensure their proliferation and survival. For this purpose, glucose metabolism in cancer cells is shifted from normal oxidative phosphorylation (OXPHOS) to glycolysis, leading to the final production of lactate. This metabolic switch, also known as the Warburg effect,[1] is very often associated with invasive tumor phenotypes,[2] as well as with stem cells.[3,4] The peculiar metabolism of carbohydrates occurring in tumors is currently being considered as a novel target for non-toxic and selective therapeutic interventions against cancer.[5,6] In fact, therapies resulting from this approach should be directed specifically against cancer cells and, therefore, devoid of important undesirable side effects.[7]

Some of the enzymes involved in the glycolytic process are currently being considered as anti-cancer targets, and there are several small molecules at the preclinical stage which are reported to act as metabolic modulators in cancer cells,[8] although there are only a few examples in clinical trials. Lonidamine, an inhibitor of hexokinase (HK), the enzyme that catalyzes the phosphorylation of the 6-position of glucose, thus starting the glycolytic process, has completed a phase 3 trial, but its efficacy was mined by pancreatic and hepatic toxicity.[9] Among HK inhibitors, it is worthwhile to mention 2-deoxyglucose (2-DG),[10] and 3-bromopyruvate,[11] although their clinical results still need to be definitely assessed. Finally, dichloroacetate (DCA) is a promising pyruvate dehydrogenase kinase (PDK) inhibitor under phase 2 clinical study, whose action restores the normal oxidative demolition of pyruvate by reactivating PDH and, as a consequence, indirectly diverting anaerobic glycolysis.[12] As of yet, there are no clinical trials involving anti-cancer agents that inhibit lactate dehydrogenase (LDH). LDH catalyzes the reduction of pyruvate into lactate and constitutes a major checkpoint in the glycolytic pathway for the switch from OXPHOS to anaerobic glycolysis. Human isoforms of LDH are tetrameric, and may be composed of three types of subunits: LDH-A (or LDH-M, muscle), LDH-B (or LDH-H, heart) and LDH-C.[13] Subunit C has been found only in a homotetrameric isoform LDH-C_4 (or *h*LDHX), which is involved in male fertility.[14] On the other hand, subunits A and B form five isoforms deriving from their various possible combinations in the following way: *h*LDH1 = LDH-B_4, *h*LDH2 = LDH-AB_3, *h*LDH3 = LDH-A_2B_2, *h*LDH4 = LDH-A_3B, *h*LDH5 = LDH-A_4. Whereas *h*LDH1 is mostly represented in the heart, *h*LDH5 is prevalent in liver and skeletal muscle. Isoform 5, containing only the A-subunit, was found to be overexpressed in highly invasive and hypoxic carcinomas,[15,16] and it was clearly associated with hypoxia inducible factor 1α (HIF-1α).[17] Moreover, an augmented expression of the LDH-A subunit was found in several tumor lines, together with the overproduction of glucose transporter GLUT1, after exposure to oxygen deprivation,[18] due to the increased glucose consumption required by anaerobic glycolysis.

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The reduced oxygen supply often found in invasive hypoxic tumors increases the vulnerability of their cells to *h*LDH5-inhibition because of the glycolysis upregulation and the inability to switch back to oxidative phosphorylation in oxygen-deprived conditions. In fact, *h*LDH5 has been acknowledged as one of the most promising targets for anti-cancer therapy, because repression of its expression cuts the main energy production process in hypoxic tumors, as shown by a reduced invasiveness in metastatic cell lines.[19,20] Most importantly, *h*LDH5-inhibition should not give rise to important side-effects in humans, since hereditary LDH-A deficiency causes myopathy only after intense anaerobic exercise, but does not provoke any symptoms under ordinary circumstances.[21] Unfortunately, only a few *h*LDH5-inhibitors are now discussed in the scientific literature, mainly because there had been no evidence for health benefits deriving from this action until recent times.[22] About a year ago, compound FX-11, an efficient inhibitor of *h*LDH5 (K_i = 8 μM), was reported to block tumor progression both in vitro and in vivo.[23] Furthermore, a phenylbutyric acid derivative linked to an adenosine-like fragment was shown to inhibit *h*LDH5 with an IC_{50} value of 14.8 μM,[24] although no data relative to cancer cell proliferation assays were reported.

We have recently developed a new class of *N*-hydroxyindole-2-carboxylates (NHIs), and found that those containing a phenyl ring at either position 5 or 6 (**1**, Fig. 1) possess efficient inhibitory properties of *h*LDH5, with K_i values reaching the low micromolar range (as low as 4.7 μM).[25] These compounds displayed a marked selectivity for isoform *h*LDH5, since their inhibition of the other homotetrameric isoform, *h*LDH1, was either undetectable or very low (⩽11% at 125 μM). The introduction of triazole linkers between the aryl substituent and the NHI scaffold did not produce significant improvements of the inhibition potency.[26] The generally lower activity found with these triazole-substituted NHIs (**2**, Fig. 1) was presumed to be due to the desolvation energy associated with the presence of these additional polar heterocycles,[27] which is not sufficiently counterbalanced by the nice fit in the enzyme active site that these compounds show by computational methods.[26]

For the purpose of expanding the chemical classes of *h*LDH5-inhibitors, and in an attempt to find new potent and isoform -selective derivatives, which are still scarcely represented in the literature in spite of the growing interest in this emerging target, we decided to extend this NHI series by introducing sulfonamide-based substituents (**3**–**5**, Fig. 2). Specifically, we have designed and synthesized a series of 22 sulfonamide-bearing NHIs in which the aminosulfonyl group is either directly bound to the indole scaffold in position 6 or 5 (compounds **3a**–**q** or **4**, respectively) or linked to the central scaffold through a phenyl spacer (compounds **5a**–**d**). The substituents present on the sulfonamide nitrogen atom (R^1 and R^2) are either independent groups (**3a–c,e–m**, **4**, **5a**–**c**) or part of the same cycle (**3d,n–q**, **5d**).

The synthesis of 5-sulfonamido-substituted NHIs **3a**–**q** is displayed in Scheme 1. Condensation of 4-methyl-3-nitrobenzene-1-sulfonyl chloride (**6**) with the appropriate amine was carried out under various reaction conditions in the presence of different bases (see Supplementary data for details). The resulting sulfonamides (**7a**–**q**) were treated with sodium hydride/dimethyl oxalate to generate α-ketoester intermediates, which were then directly submitted to a reductive cyclization step, leading to NHI-esters **8a**–**q**.

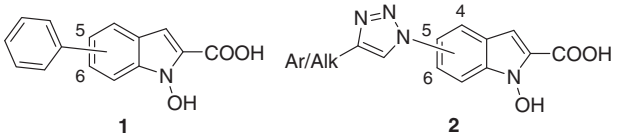
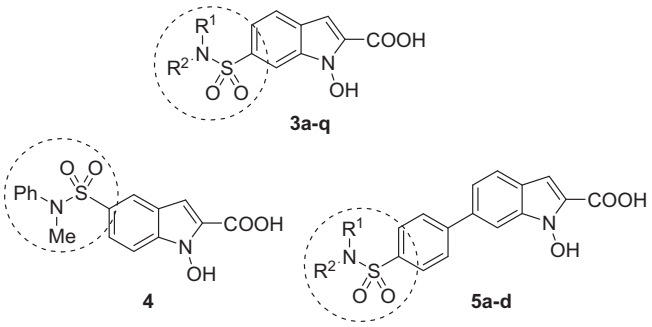


Figure 1. Structures of reference phenyl- (**1**) and triazole-substituted (**2**) NHIs.



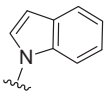
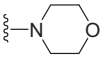
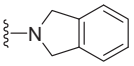
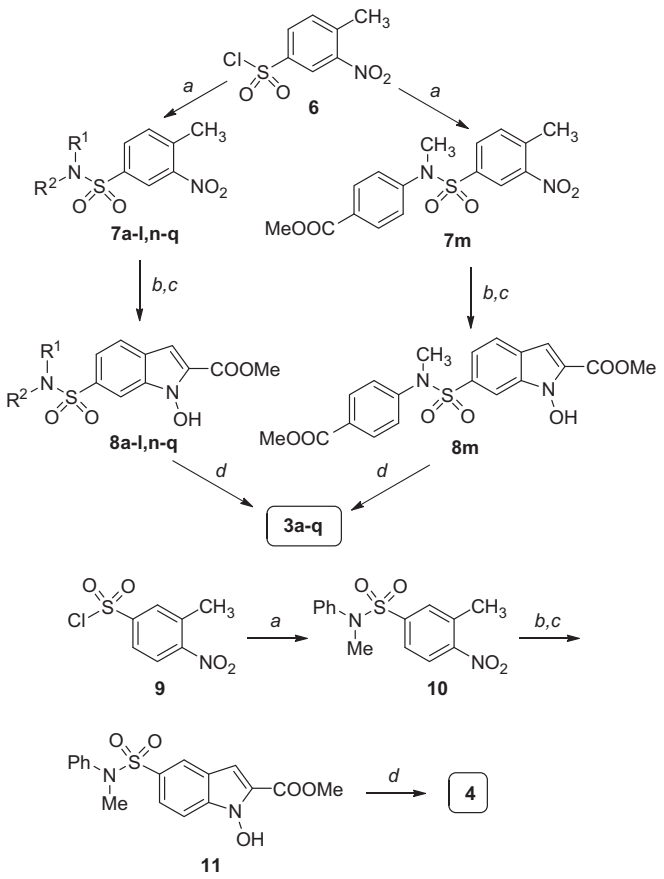
	R^1	R^2		R^1	R^2
a	CH_3	CH_3	**i**	CH_3	3-Cl-Ph
b	CH_3	Ph	**j**	CH_3	2-Cl-Ph
c	*n*-Bu	Ph	**k**	CH_3	2,4-Cl_2-Ph
d			**l**	CH_3	3,4-Cl_2-Ph
			m	CH_3	4-COOH-Ph
e	CH_3	4-CH_3-Ph	**n**		
f	CH_3	4-CF_3-Ph	**o**		
g	CH_3	4-F-Ph	**p**		
h	CH_3	4-Cl-Ph	**q**		

Figure 2. Structures of newly developed sulfonamide-containing NHIs **3**–**5**.

This last step required a fine optimization of the reaction conditions, since various amounts of over-reduced indole side-products were also obtained. The simplest member of this class (**8a**) was prepared by reaction with $SnCl_2$ in DME.[28,29] In the synthesis of **8b**, the same conditions caused the formation of considerable amounts of indole side-product; therefore, this compound was instead obtained by using milder reducing conditions (sodium hypophosphite in the presence of palladium over charcoal).[30] A combination of $SnCl_2$ and $PhSH/Et_3N$[31,32] proved to be the optimal reagent for the preparation of compounds **8c**,**d**,**i**–**q**. The remaining NHI-esters (**8e**–**h**) were instead obtained by a reduction of the appropriate precursors in the presence of lead and triethylformate in hot methanol.[33] Final hydrolysis of the COOMe group was obtained upon treatment with an aqueous 2 N solution of LiOH in a 1:1 mixture of THF and methanol.

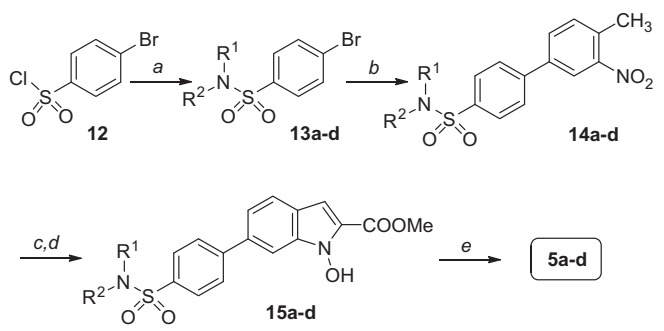
An analogous synthetic pathway was followed for the preparation of compound **4**, bearing the *N*-methyl-*N*-phenylsulfonamide group in position 5 (Scheme 1), starting from 3-methyl-4-nitrobenzene-1-sulfonyl chloride (**9**).[34] It is worth mentioning that, in this case, the most efficient reductive cyclization step was promoted by the $SnCl_2/PhSH/Et_3N$ system.[31,32]

A slightly modified reaction sequence was applied to the preparation of the 'phenyl-spaced' series of sulphonamido-NHIs (**5a**–**d**, Scheme 2). Sulfonamide **13a** was commercially available, whereas **13b,c** were prepared by direct condensation of an excess of the



Scheme 1. Reagents and conditions: (a) R^1R^2NH (see Supplementary data); (b) $(COOMe)_2$, NaH 60%, dry DMF, −15 °C to rt; (c) **8a**: $SnCl_2{\cdot}2H_2O$, dry DME, 4 Å MS, 0 °C to rt; **8b**: $H_2PO_2Na{\cdot}H_2O$, Pd-C 10%, H_2O/THF (1:1), rt; **8c**,**d**,**i**–**q**, **11**: $SnCl_2{\cdot}2H_2O$, C_6H_5SH, Et_3N, CH_3CN, rt; **8e**–**h**: Pb, TEAF, MeOH, 55 °C; (d) aqueous 2 N LiOH, THF/MeOH (1:1), rt.

appropriate aniline with 4-bromobenzene-1-sulfonyl chloride (**12**). Indole-containing sulfonamide **13d** required different conditions for preparation, such as the use of finely powdered NaOH and tetrabutylammonium hydrogensulfate in CH_2Cl_2. These aryl bromides were then subjected to Pd-catalyzed cross-coupling reactions to obtain nitro-toluene intermediates **14a**–**d**. As seen in the previous scheme, the last steps of this synthesis also involved sodium hydride-promoted condensation with dimethyl oxalate, reductive



Scheme 2. Reagents and conditions: (a) R^1R^2NH, DCM, rt (**13b**,**c**) or NaOH, TBAHS, DCM, 0 °C to rt (**13d**); (b) 4-methyl-3-nitrobenezeneboronic acid, $Pd(OAc)_2$, TBAB, Na_2CO_3, H_2O, μW, 5 min (**14a**) or $Pd(OAc)_2$, PPh_3, aqueous 2 M Na_2CO_3, toluene, EtOH, 100 °C, 24 h (**14b**–**d**); (c) $(COOMe)_2$, NaH 60%, dry DMF, −15 °C to rt; (d) $SnCl_2{\cdot}2H_2O$, C_6H_5SH, Et_3N, CH_3CN, rt; (e) aqueous 2 N LiOH, THF/MeOH (1:1), rt.

cyclization operated by the $SnCl_2$/PhSH/Et_3N system,[31,32] and final ester hydrolysis, to produce NHIs **5a**–**d**.

The inhibitory activities of compounds **3a**–**q**, **4** and **5a**–**d** on the two purified human enzyme isoforms *h*LDH5 and *h*LDH1 were measured by standard enzyme kinetics experiments. Initially, we evaluated the percent inhibition of *h*LDH5 relative to control at a compound concentration of 125 μM in the presence of 25 μM NADH. Enzyme activity was determined by measuring the absorbance decrease at 340 nm, due to the consumption of NADH. Compounds displaying an inhibition level higher than 50% at 125 μM were then submitted to a full enzyme kinetics characterization, to establish their type of inhibition versus both NADH and pyruvate, as well as their isoform selectivity (*h*LDH5 versus *h*LDH1). Therefore, we initially measured the Michaelis–Menten constants (K_m) relative to the cofactor (NADH) and the substrate (pyruvate) for *h*LDH5, derived from Lineweaver–Burk plots. Next, we evaluated the K'_m values in the presence of the selected compounds (concentration range = 25 ÷ 100 μM). Final K_i values (Table 1) were then determined by double-reciprocal Lineweaver–Burk plots obtained from the values of K'_m, obtained as described above for each compound.

The smallest member of the 6-sulfonamido-substituted series, bearing only two methyl groups (**3a**), did not show any appreciable activity, whereas the introduction of one phenyl ring in the sulfonamide nitrogen atom produced a potent inhibitor (**3b**) displaying K_i values of 17 μM versus NADH and 9.2 μM versus pyruvate. When the same *N*-methyl-*N*-phenylsulfamoyl group was moved from position 6 of **3b** to position 5, the resulting compound (**4**) completely lost the *h*LDH5-inhibitory activity. Other inactive compounds were produced when the sulfonamide nitrogen atom was part of cyclic systems, such as indole (**3d**), piperidine (**3n**), pyrrolidine (**3o**), morpholine (**3p**), or isoindoline (**3q**). Replacement of the *N*-methyl group with larger alkyl substituents, such as a *n*-butyl (**3c**), did not modify the competitive inhibition potencies versus the cofactor (K_i = 18 μM), whereas it substantially reduced the efficiency of its competition with the substrate (K_i = 90 μM). An explorative series of *N*-aryl-substituted analogs of **3b** were then analyzed (**3e**–**l**). Among these compounds, 4-trifluoromethylphenyl-substituted derivative **3f** showed a certain improvement of the pyruvate-competitive *h*LDH5-inhibition, whereas its competition versus NADH was less efficient than that of its non-substituted counterpart **3b**. The introduction of a *para*-chloro atom generated the most potent compound of this series (**3h**), which displayed very efficient inhibition levels versus both the cofactor (K_i = 6.6 μM) and the substrate (K_i = 5.6 μM). The shift of the chlorine atom from the *para*-position

Table 1
Results of enzyme (*h*LDH5) inhibition assay for selected compounds

Entry	Compound	K_i[a] (μM) (versus NADH)[b]	K_i[a] (μM) (versus Pyr)[c]
1	**3b**	17	9.2
2	**3c**	18	90
3	**3e**	20	20
4	**3f**	26	6.0
5	**3g**	125	28
6	**3h**	6.6	5.6
7	**3i**	48	43
8	**3j**	14	25
9	**3k**	30	40
10	**3l**	18	36
11	**5b**	16	22
12	**5d**	7.7	8.8

[a] Values are reported as the average of three or more measurements; the error in these values is within ±30% of the average.
[b] Saturating concentration (2.0 mM) of sodium pyruvate and competitive increasing concentrations (12.5 ÷ 150 μM) of NADH.
[c] Saturating concentration (200 μM) of NADH and competitive increasing concentrations (25 μM ÷ 1.0 mM) of sodium pyruvate.

of **3h** to the *meta*- (**3i**) and *ortho*- (**3j**) positions generally produced reductions of the inhibition potencies. The same negative trend was observed when an additional chloro-group was inserted either in the *ortho*- (**3k**) or *meta*- (**3l**) positions of compound **3h**. The insertion of other types of *para*-substituents caused either a reduction (**3e**: 4-CH_3; **3g**: 4-F) or a complete loss (**3m**: 4-COOH) of the inhibitory activity. The restricted series of 'phenyl-spaced' derivatives **5a**–**d** generally afforded weaker inhibitors. In fact, compounds **5a** and **5c** were completely inactive. Compound *N*-methyl-*N*-phenyl-substituted **5b** proved to be a slightly poorer inhibitor than its non-spaced counterpart **3b**. However, the most potent inhibitor of this 'phenyl-spaced' subclass was the indole-sulfonamide **5d**, in sharp contrast to its inactive close analog **3d** belonging to the 'non-phenyl-spaced' family. In fact, **5d** displayed K_i values (7.7 and 8.8 μM) approaching those of the best sulfonamide inhibitor so far, compound **3h**.

As for isoform selectivity, it is important to notice that none of these active *h*LDH5-inhibitors displayed any detectable inhibition of *h*LDH1 (<3%) at 125 μM, thus confirming the generally high specificity of NHI-based inhibitors for *h*LDH5.

All the reported compounds were analyzed by means of docking studies. Active inhibitors **3b**,**c**,**e**–**l** are characterized by a common interaction scheme. As shown in Figure 3, and in agreement with our previously published results,[25,26] the carboxylic group of the most potent inhibitor of this series, compound **3h**, shows strong polar interactions with R169 and T248; moreover, its *N*-hydroxy group displays an H-bond with the backbone nitrogen atom of T248, as well as with a water molecule that mediates the interaction with H193. The indole central scaffold of **3h** is placed in a cleft mainly delimited by N138, A238, V241, I242, T248, and I252. The sulfonamide group does not seem to participate in specific polar interactions; rather, it places the aromatic substituent of **3h** in a lipophilic cleft mainly constituted by V31, A98, and Y247. Similar binding poses were found for all the other active inhibitors. On the other hand, the low activity of carboxy-substituted NHI **3m** could be explained by the fact that the additional COOH group of this compound does not participate in any significant interaction with the enzyme (Fig. S1, Supplementary data).

The insertion of the sulfonamide nitrogen atom into a cycle such as indole (**3d**), piperdine (**3n**), pyrrolidine (**3o**), morpholine (**3p**), or isoindoline (**3q**) causes a marked decrease of activity. The docking of these derivatives highlights the overturning of the indole central scaffold, when compared to active inhibitors (see above) with the loss of the H-bonds involving the *N*-hydroxy group, and a consequent distortion of the interactions between the carboxylic substituent and R169, as shown by representative example **3n** in Figure 4A. In order to further clarify why these compounds behaved so differently from their 'non-cyclic' counterparts, a manual docking of representative 'cyclic' sulfonamide **3n** was carried out, in which this derivative was forced to assume the binding pose previously obtained with active inhibitors, such as **3h** (cf. Fig. 3). The results obtained by this procedure with **3n** are shown in Figure 4B: the resulting complex shows that the piperidine cyclic substituent of **3n** is oriented towards the alkyl chain of V31 and displays an intense steric clash (red arrow, Fig. 4B) with this residue that prevents this type of compound from assuming an efficient interaction with the enzyme active site.

The addition of a phenyl spacer between the indole central scaffold and the sulfonamide portion of the ligands is tolerated in two examples (**5b** and **5d**). Our docking results confirm the good putative binding of indole-substituted 'spaced' derivative **5d** (Fig. 5). In fact, **5d** participates in an additional H-bond with the backbone of R99 by means of one of its sulfonamide oxygen atoms. Furthermore, the indole portion connected to the sulfonamide group shows a strong aromatic interaction with the aryl side-chain of Y247.

The loss of the inhibitory activity found with compound **4**, which is caused by the shift of the sulfonamide group from position 6 to 5, can be explained only partially by a simple docking analysis of its putative binding pose. In fact, as shown in Figure 6, the indole central scaffold of **4** is sensibly shifted from the posi-

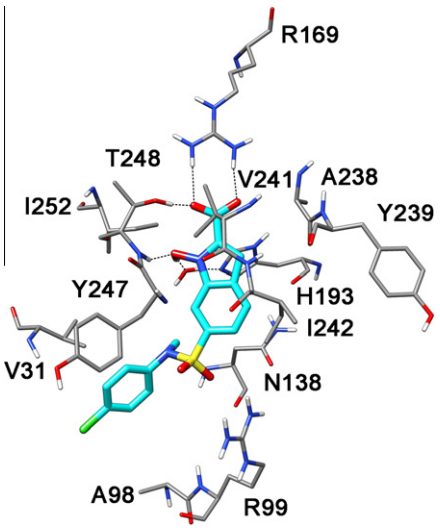


Figure 3. Docking analysis of **3h** into the LDH-A subunit.

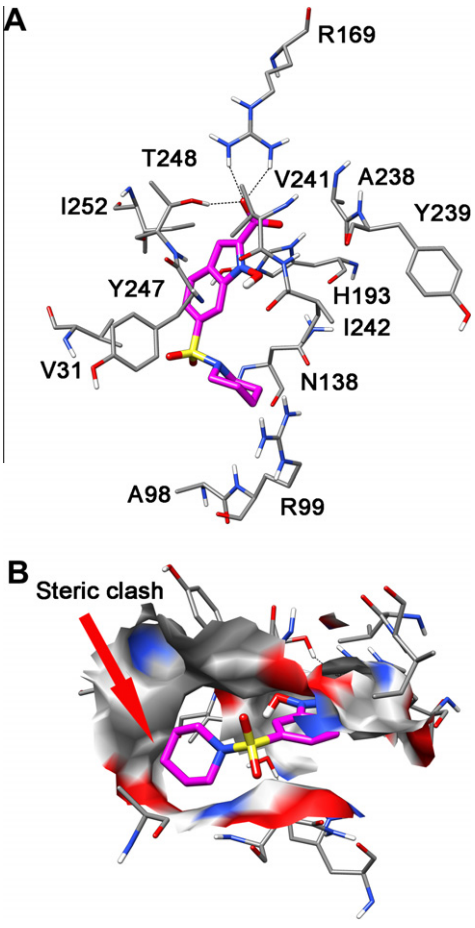


Figure 4. Docking analysis of **3n** into the LDH-A subunit (A) and manual docking of **3n** using the results obtained for compound **3h** as a reference disposition (B), displaying the steric clash occurring in this case.

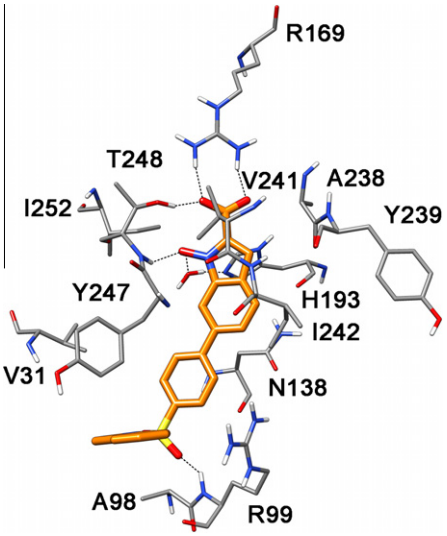


Figure 5. Docking analysis of **5d** into LDH-A subunit.

tion occupied by the same portion of its 6-substituted analogs of type **3** (see Fig. 3). This shift causes a weakening of the interaction between the carboxylic group of **4** and R169, and the concomitant loss of its interaction with the nitrogen backbone of T248 by means of the N–OH group, although the polar link between its COOH and the hydroxy group of T248 is preserved. The phenyl ring bound to the sulfonamide group of **4** still shows lipophilic interactions with Y239. Overall, this binding pose would not be sufficient to justify the dramatic loss of activity displayed by compound **4**. Therefore, in order to further understand the different biological outcomes obtained within this class of compounds and the presumed modes of interaction with the enzyme active site, representative derivatives **3h**, **4**, and **5d** were submitted to 10 ns of molecular dynamic (MD) simulations. An analysis of the root-mean-square deviation (rmsd) from the initial model of the α carbons of the enzyme, showed that after an initial increase, the rmsd remained approximately constant around the value of 1.0–1.6 Å during the last 7 ns for all three complexes (Fig. S2, Supplementary data).

In agreement with the docking analysis, MD simulations confirmed that compounds **3h** and **5d** maintain their strong H-bond interactions with R169, T248, and the structural water molecule. The strength of the π–π interaction occurring between residue Y247 and either the phenyl group of **3h** or the indole substituent of **5d** is further confirmed by this MD analysis. Furthermore, the H-bond between the sulfonamide oxygen atom of **5d** and the nitrogen backbone of R99 is also maintained (Fig. S3, Supplementary data).

On the other hand, the analysis of the MD trajectory for the complex obtained by molecular docking of **4** into the LDH-A active site clearly supports the low activity of this 5-substituted derivative. In fact, as shown in Figure 7, during MD simulation compound **4** completely loses its crucial interaction with R169 of the enzyme and the structural water molecule, whereas it maintained the H-bond interaction only between its carboxylic group and the OH group of the side chain of T248. This behavior would indicate a much weaker interaction of compound **4** and the LDH-A active site and, therefore, would justify the experimentally-found poor inhibitory activity of **4**.

In conclusion, the results presented here give the following SAR indications concerning the *h*LDH5-inhibitory efficiency of this class of sulfonamide-containing NHIs: (1) the sulfonamide substituent must be placed in position 6 of the NHI scaffold, rather than in position 5 (compare compounds **3b** and **4**); (2) the sulfonamide-nitrogen atom should bear one aryl substituent (R^2) in addition to the methyl group (R^1) (in fact compound **3a** is inactive); (3) large R^1-alkyl groups, such as *n*-butyl, partially (**3c**) or completely (**5c**) compromise the ability of the resulting compounds to compete with the small natural substrate pyruvate in the deepest cavity of the enzyme active site; (4) cyclic 'non-spaced' sulfonamides are not suitable for this purpose (**3d**,**n**–**q**); (5) an extra COOH group in the phenyl substituent causes a loss of activity (**3m**), whereas the introduction of a *para*-chlorine atom in the same position affords the most potent inhibitor of this series (**3h**); (6) sulfonamide groups linked to position 6 of the NHI scaffold through a phenylene spacer maintain a good inhibition level and, in this case, indole-substituted derivative **5d** proves to be the most potent inhibitor, in contrast to its 'non-spaced' counterpart (**3d**). Molecular modeling studies nicely confirm the experimental inhibition results obtained by enzyme kinetics assays. Overall, this new chemical class produced some of the most potent and selective *h*LDH5-inhibitors, displaying K_i values in the low micromolar range. In the near future, a full pharmacological evaluation will be carried out on selected compounds from this series, in order

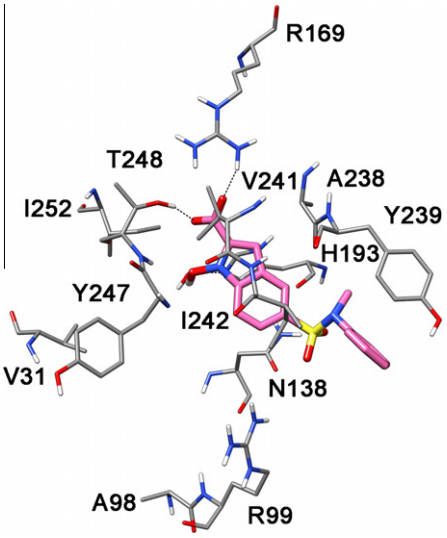


Figure 6. Docking analysis of **4** into LDH-A subunit.

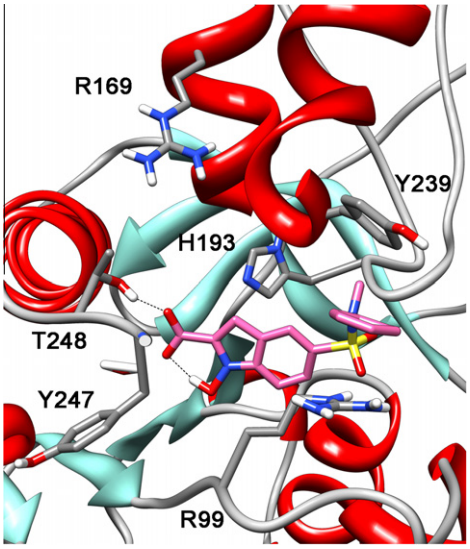


Figure 7. MD simulation results for the complex of LDH-A with **4**.

to assess their therapeutic potential against cancer cells. Meanwhile, the enzyme-inhibition results presented here open the way to the development of a novel chemical class of *h*LDH5-inhibitors, whose synthesis can be easily implemented by combining various amine and sulfonyl precursors, thus generating large collections of new potentially active sulfonamide-containing derivatives.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.10.031.

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