

Novel 2,4-Dichloro-5-sulfamoylbenzoic Acid Oxime Esters: First Studies as Potential Human Carbonic Anhydrase Inhibitors

Jaydeo T. Kilbile, Suryakant B. Sapkal, Gioele Renzi, Ilaria D'Agostino, Luigi Cutarella, Mattia Mori, Barbara De Filippis, Imadul Islam, Maria Luisa Massardi, Elena Somenza, Roberto Ronca, Yasinalli Tamboli,* Fabrizio Carta,* and Claudiu T. Supuran



Cite This: *ACS Med. Chem. Lett.* 2024, 15, 972–978



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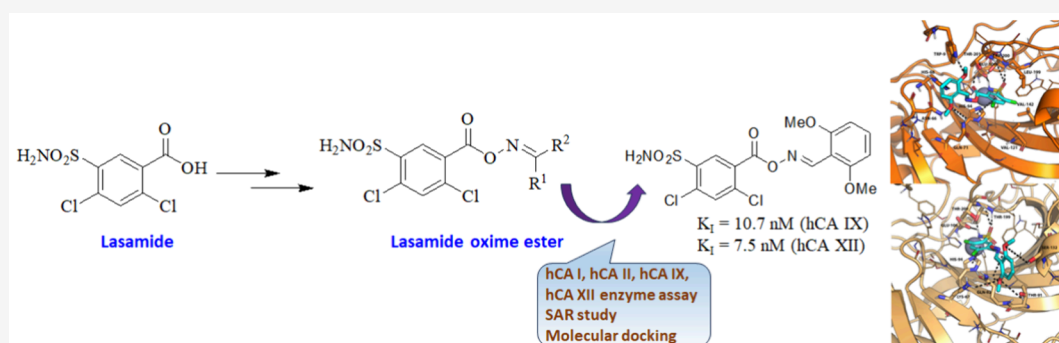
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ABSTRACT: In this study, a focused library of oxime ester derivatives of 2,4-dichloro-5-sulfamoylbenzoic acid (lasamide) containing Schiff bases was synthesized and tested *in vitro* for their ability to inhibit the cytosolic human carbonic anhydrases (hCAs) I and II, as well as the transmembrane and tumor-associated IX and XII isoforms. As a result, we obtained a first line of knowledge on lasamide derivatives potentially useful for development as CA inhibitors (CAIs). In particular, we focused our attention on the derivative **11**, which was selective toward hCAs IX and XII over the cytosolic isoenzymes. An *in silico* study was conducted to assess the binding mode of **11** within hCAs IX and XII. Also, antiproliferative assays highlighted promising derivatives. The data obtained in this study are currently in use for the development of better-performing compounds on the tumor-associated isoforms.

KEYWORDS: Cancer, Carbonic Anhydrase, Benzenesulfonamide, Lasamide, O-Benzoyl Oximes, Schiff Base

Cancers are pervasive global health issues that significantly impact the quality of life for millions of people worldwide.¹ They are a leading cause of morbidity and fatal events that profoundly affect individuals and the entire society in the long term. To address the challenges posed by the disease, a multifaceted approach is required that includes prevention, early and accurate diagnoses, and improved treatments associated with supportive care. In such a context, the validation of new druggable targets is highly beneficial for the sustainability of the currently used therapeutical protocols. Among the most promising experimental targets, a place of honor is held by the metalloenzymes carbonic anhydrases (CAs; EC 4.2.1.1), which are proven to be crucial for fundamental cancer-related cellular processes.^{2,3} CAs are involved in regulating pH levels, transporting ions, and maintaining the CO₂ homeostasis. The α -class of CAs expressed in humans (hCAs) accounts for 16 distinct isoforms and, among them, the transmembrane isoforms IX and XII are directly involved in carcinogenesis, whereas the cytosolic isoforms hCA I and hCA II are widely expressed in organs, such as eyes, erythrocytes, osteoclasts, the gastrointestinal

system, and kidney cells, and are cooperative with the tumor-associated isoenzymes.^{4–9}

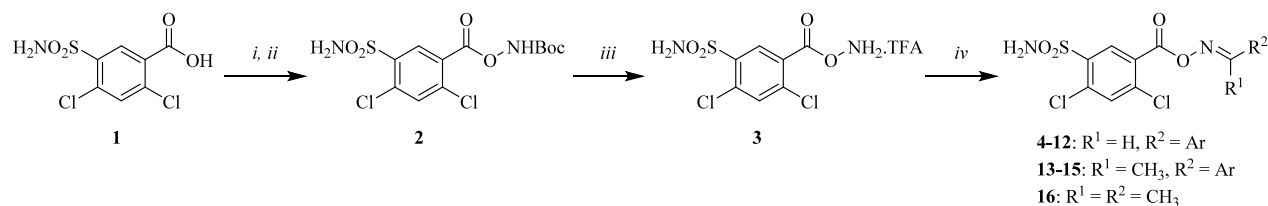
The primary sulfonamides (–SO₂NH₂) are the prototypical CA inhibitor (CAI) moieties and have a long-standing history in clinical practice spanning over 70 years to date. Such a group is widely recognized for its capability to directly inhibit the catalytic action of CAs¹⁰ through coordination of the zinc ion.^{11,12}

2,4-Dichloro-5-sulfamoylbenzoic acid (lasamide, **1**) is the key intermediate for the synthesis of the diuretic furosemide and is a key precursor for experimental compounds with potential pharmaceutical applications,¹³ thus including those acting as CAIs.^{14,15} Two lasamide salts are reported in

Received: May 1, 2024
Revised: May 18, 2024
Accepted: May 21, 2024
Published: May 23, 2024



Scheme 1. Synthetic Approach for Oxime Ester Series (4–16) of Lasamide 1 Reported in This Work



^aReagents and conditions: (i) SOCl₂, toluene, 45 °C, 6 h; (ii) *N*-Boc-hydroxylamine, TEA, THF, 5 °C to rt, 10 h; (iii) TFA, DCM, 5 °C, 5 h; (iv) aldehyde or ketone, AcONa, EtOH, 75 °C, 5 h.

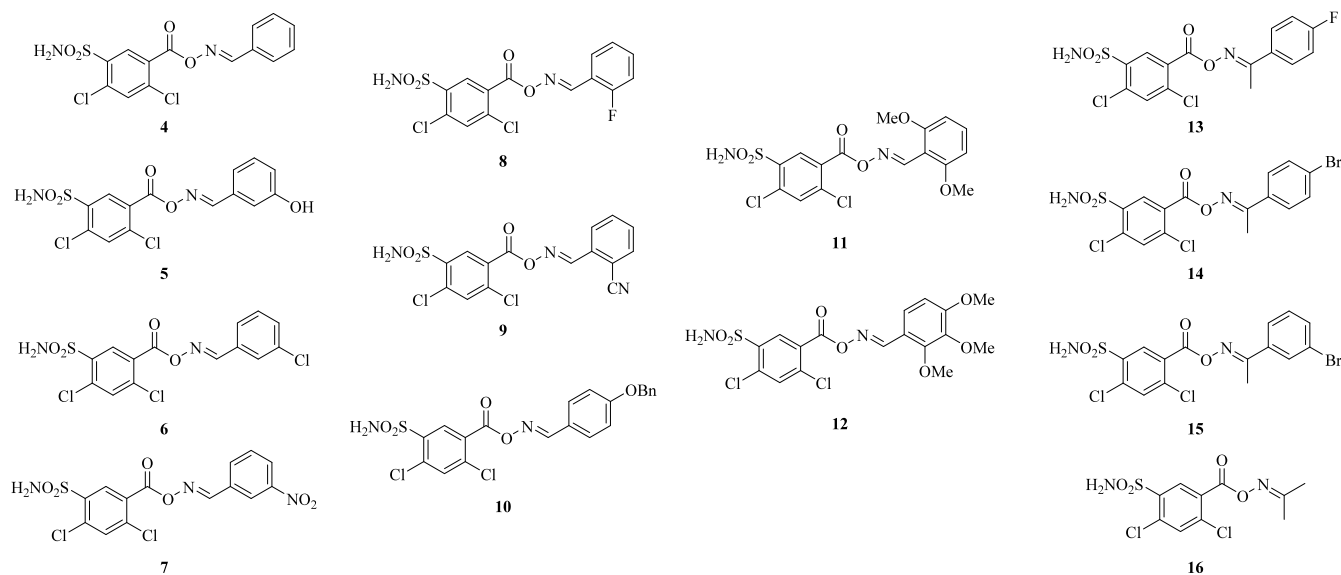


Figure 1. Chemical structures of *O*-benzoyl oximes 4–16.

complexes with hCA I and hCA II and found to possess a potent inhibitory activity.¹⁶ The Schiff base derivatives possess a broad spectrum of bioactivities,¹⁷ and various oxime derivatives containing the coumarin/sulfonamide moiety are also described as hCA IX and XII inhibitors.^{18–21}

Hence, we applied a divergent synthetic approach to obtain a series of primary sulfonamide compounds having the oxime ester–imine backbone in order to evaluate their CA inhibitory potential against two cytosolic isozymes (hCAs I and II) and tumor-associated isozymes (hCAs IX and XII).

In contrast with conventional approaches to preparing oxime esters (Supporting Information), late-stage modification has emerged as a convenient and efficient strategy to achieve the rapid generation of a diverse library of products through linear synthesis. Hence, we prepared intermediate 3 from commercial lasamide 1 by oxime esterification with *N*-Boc-hydroxylamine followed by Boc deprotection, as depicted in Scheme 1. Further, compound 3 was subsequently transformed to the corresponding novel oxime ester derivatives by Schiff's base reaction with enhanced atom economy.

After unsuccessful attempts (Supporting Information),^{22,23} we resorted to a more efficient and economically advantageous one-pot, two-step protocol, which involved *in situ* acyl chloride formation followed by reaction with *N*-Boc-hydroxylamine. Combinations of solvents and temperatures were screened, and the addition of thionyl chloride (SOCl₂) in toluene at 45 °C for 6 h resulted in optimal conditions in order to reduce the formation of several byproducts due to the generation of *N*-sulfonylsulfonamides. The excess SOCl₂ and toluene were

distilled off, and the crude reaction mass was directly used in the next step with *N*-Boc-hydroxylamine under the basic condition of triethylamine (TEA) at room temperature to afford compound 2. Boc cleavage of the latter was achieved using trifluoroacetic acid (TFA) in dichloromethane (DCM) to afford 3 as a trifluoroacetate salt, which was directly reacted with different aldehydes and ketones (Scheme 1). The last step involved Schiff's base formation using sodium acetate (NaOAc) and the appropriate aldehyde/ketone in ethanol at 75 °C. The obtained products were easily purified by recrystallization from either ethanol or a mixture of ethanol and isopropyl ether. The structures of final compounds 4–16 are depicted in Figure 1.

The inhibition profiles for intermediate 2 and *O*-benzoyl oximes 4–16 on the physiologically relevant cytosolic hCAs I and II and the tumor-associated hCAs IX and XII were determined through the stopped-flow CO₂ hydrase assay.^{24–29} Inhibition constants (K_i)^{28,30} were reported in Table 1 with lasamide 1 and acetazolamide (AAZ) as reference compounds.

Overall, lasamide 1 was found to strongly inhibit all the tested hCA isoforms within subnanomolar (i.e., I and II) and low nanomolar (i.e., IX and XII) ranges. Similarly to the parent lasamide 1, intermediate 2 proved to be highly potent in inhibiting the hCA I with a K_i value slightly under the nanomolar concentration ($K_i = 0.93$ nM), whereas the *in vitro* kinetic values for the remaining isoforms were increased up to 4.8-fold for the hCA XII isoform (Table 1). Interesting structure–activity relationships (SARs) were obtained on the

Table 1. Inhibition Data of Lasamide 1, Intermediate 2, Derivatives 4–16, and AAZ on hCAs I, II, IX, and XII^a

compound	K_i (nM)					
	hCA I	hCA II	hCA IX	SI _{IX/II}	hCA XII	SI _{XII/II}
1	0.52	0.33	2.6	7.9	7.5	22.7
2	0.93	2.9	3.3	1.1	36.3	12.5
4	84.4	69.7	41.3	0.59	7.9	0.1
5	6.7	36.3	41.3	1.1	27.4	0.75
6	21.3	81.5	35.2	0.4	7.0	0.09
7	8.2	279	8.8	0.03	382	1.4
8	44.6	91.6	3.2	0.03	59.8	0.65
9	3.7	52.7	39.4	0.74	39.9	0.8
10	263	78.3	35.4	0.45	199	2.5
11	255	44.1	10.7	0.24	7.5	0.17
12	445	220	38.3	0.17	57.8	0.26
13	36.5	33.9	43.3	1.3	8.9	0.26
14	69.5	81.8	42.6	0.52	263	3.2
15	29.5	59.9	32.8	0.54	49.7	0.83
16	5.3	0.04	3.4	85.0	47.5	1188
AAZ	250	12.1	25.8		5.7	

^aMean from three different assays by a stopped-flow technique. Errors were in the range of ± 5 –10% of the reported values.

O-benzoyl oxime series and were reported per each CA isoform considered in this study.

- All compounds profiled for the hCA I isoform showed quite varied K_i values, and all were higher when compared with that of lasamide 1 precursor (Table 1). Specifically, the introduction within the phenyl moiety of 4 ($K_i = 84.4$ nM) of single groups potentially able to establish hydrogen bonds, such as the –OH in 5, the NO₂ in 7, and the –CN in 9, determined a sensible reduction of the K_i values down to the low nanomolar range ($K_i = 6.7, 8.2,$ and 3.7 nM for derivatives 5, 7, and 9, respectively). On the contrary, the –Cl and –F halogens at the same position (i.e., 6 and 8 in Table 1) were slightly beneficial for the inhibition potency when compared with 1 ($K_i = 21.3$ and 44.6 nM for 6 and 8, respectively). The bulky *O*-benzyl moiety in *para*-position, as in 10, greatly increased the K_i value to 263 nM, thus making it comparable with the bi- and trimethoxyphenyl-substituted derivatives 11 and 12 ($K_i = 255$ and 445 nM, respectively). Better results were obtained for the methyl oxime series 13–16. The halogen effect was clearly observed for 13 and 14 with the former 1.9-fold more potent than the bromine derivative ($K_i = 36.5$ and 69.5 nM for 13 and 14, respectively). Interestingly, the introduction of the bromo atom in *meta*-position of the phenyl ring, as in 15, allowed it to regain inhibition potency up to 29.5 nM. Reduction of the tail bulkiness with the isopropyl oxime ester, as in 16, was highly beneficial for the hCA I inhibition ($K_i = 5.3$ nM).
- As for the hCA II, substitution of the phenyl in 4 with the phenol to afford 5 halved the K_i value (K_i of 69.7 and 36.3 nM for 4 and 5, respectively). A slight improvement of the inhibition potency was obtained with the cyanophenyl derivative 9 (K_i of 52.7 nM). The chloro (6) and fluoro (8) phenyl derivatives were 1.2- and 1.3-fold, respectively, less effective than the unsubstituted phenyl 4 (Table 1). As expected, the bulky *O*-benzyl derivative 10 was a medium nanomolar

inhibitor with a $K_i = 78.3$ nM. However, the nitrophenyl 7 resulted to be a high nanomolar inhibitor of hCA II ($K_i = 279$ nM). Quite interestingly, the di- (11) and tri- (12) methoxy derivatives were highly discriminative for the hCA II with the former being a 5.0-fold more effective inhibitor when compared with the latter ($K_i = 44.1$ and 220 nM for 11 and 12, respectively). The kinetic trend for 13–16 toward the hCA II was partially similar to that for hCA I being the *para*-bromo derivative 14 was 2.4-fold less effective than its fluoro-substituted counterpart 13 ($K_i = 33.9$ and 81.8 nM for 13 and 14, respectively). Again, the switch of the halogen from *para*- to *meta*-position (i.e., from 14 to 15) allowed the compound to regain hCA II inhibition potency up to 59.9 nM. The introduction of the isopropyl oxime ester, as in 16, resulted in a drastic increase in the inhibition potency with subnanomolar K_i ($= 0.04$ nM).

- Overall, compounds 4–16 showed a flatter kinetic profile for the hCA IX when compared with the cytosolic isoforms I and II. For instance, the phenyl 4, the phenolic 5, and the cyanophenyl derivative 9 resulted in being equipotent hCA IX inhibitors (i.e., $K_i = 41.3, 41.3,$ and 39.4 nM for 4, 5, and 9, respectively). Similarly, the chlorophenyl (6) and the *O*-benzyl (10) derivatives were both medium nanomolar inhibitors ($K_i = 35.2$ and 35.4 nM for 6 and 10, respectively). Quite interestingly, the introduction of fluoro (i.e., 8) instead of the chloro atom in compound 6 strongly reduced the K_i value up to 11.0-fold ($K_i = 35.2$ and 3.2 nM for 6 and 8, respectively). Also, the nitro derivative 7 was quite effective in inhibiting hCA IX ($K_i = 8.8$ nM). The halogen effect for 13 and 14 was almost undetectable ($K_i = 43.3$ and 42.6 nM for 13 and 14, respectively), whereas the kinetics was slightly affected by the regioselectivity since the *meta*-bromo 15 was 1.3-fold more effective than its *para*-substituted counterpart 14 ($K_i = 42.6$ and 32.8 nM for 14 and 15, respectively). The polymethoxy derivatives 11 and 12 elicited K_i values of 10.7 and 38.3 nM, respectively, against hCA IX. Finally, and in analogy to hCAs I and II, the isopropyl derivative 16 was the most potent inhibitor within the compound series ($K_i = 3.4$ nM).
- As for the other tumor-associated isoform XII, the chloro-containing derivative 6 was a slightly more potent inhibitor than its progenitor phenyl 4 ($K_i = 7.9$ and 7.0 nM for 4 and 6, respectively). Quite interestingly, such values were almost superimposable to the dimethoxy 11 ($K_i = 7.5$ nM). As for the remaining compounds, higher K_i values were obtained. For instance, the substitution of the phenyl moiety in 4 with the phenolic ring, as in 5, increased the inhibition value up to 3.5 fold ($K_i = 7.9$ and 27.4 nM for 4 and 5, respectively). Significant reduction of the inhibition potency was observed for the nitrophenyl derivative 7 ($K_i = 382$ nM) and for the *O*-benzyl 10 ($K_i = 199$ nM). Medium nanomolar K_i values were obtained for the *ortho*-fluoro 8 and the *ortho*-cyano 9 ($K_i = 59.8$ and 39.9 nM for 8 and 9, respectively). Interestingly, 8 showed an inhibitory profile against hCA XII almost similar to that of the trimethoxyphenyl derivative 12 ($K_i = 57.8$ nM). Among the oxime esters 13–16, the fluoro derivative 13 was highly effective in inhibiting hCA XII ($K_i = 8.9$ nM), whereas its bromo counterpart 14 was found to be 29.6-fold less potent (K_i

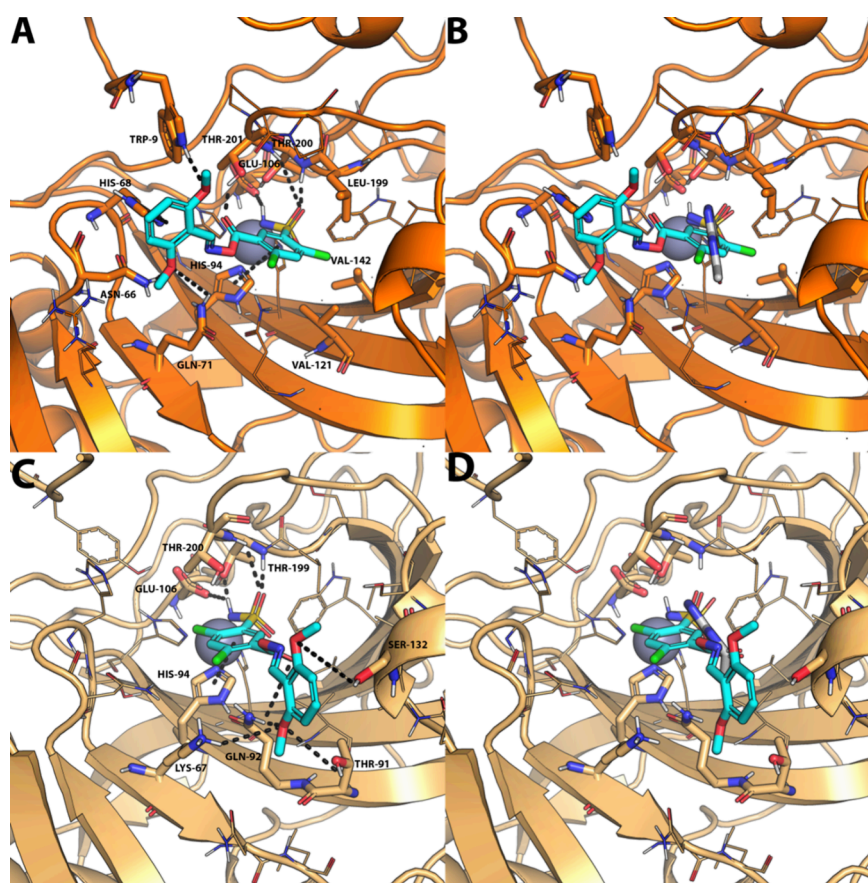


Figure 2. Predicted binding mode of compound **11** against the crystallographic structure of (A) hCA IX (orange cartoons and lines) and (C) hCA XII (gold cartoons and lines). Compound **11** is shown as cyan sticks, and the catalytic Zn (II) ion is shown as a gray sphere. Polar interactions in panels (A) and (C) are highlighted by black dashed lines, and residues contacted by the ligand are shown as sticks. In panels (B) and (D), the overlay between the docking pose of compound **11** and the cocrystallized AAZ (colored gray) is shown.

= 263 nM). A strong regioselective effect was reported for the *meta*-bromophenyl derivative **15**, which resulted in a 5.3-fold more potent inhibitor ($K_i = 49.7$ nM). Conversely to the previously considered hCA isoforms, derivative **16** was a medium nanomolar hCA XII inhibitor with a $K_i = 47.5$ nM.

The selectivity indexes (SIs) of compounds **1–16** for the tumor-associated isoforms IX and XII in comparison with the ubiquitous and cooperative hCA II were reported in Table 1. Since the experimental inhibitory activities in Table 1 accounted for compound **11** being highly selective inhibitors for the tumor-associated isoforms, we consider investigating its binding modes and affinity scores on such enzymes (i.e., IX/XII) by molecular docking simulations.

X-ray crystallography structures of hCA IX and hCA XII cocrystallized with the primary sulfonamide inhibitor AAZ were selected for docking simulation purposes.³¹ The reliability of the docking protocol^{11,32} was preliminarily assessed by redocking the cocrystallized inhibitor to both isoforms to obtain a docking pose that overlaps with the crystallographic pose (root mean square deviation, RMSD < 1.0 Å, data not shown).

Docking results on the hCA IX are reported in Figure 2A and show the primary sulfonamide moiety of **11** to interact with the catalytic zinc ion to establish H-bond interactions with GLU-106, as well as with the backbone of THR-201 and THR-200, thus in a manner that is superimposable to the

cocrystallized AAZ in Figure 2B. The dichlorophenyl moiety is T-shaped with the side chain of HIS-94, while the two chlorine atoms occupy a hydrophobic region of the enzyme bounded by LEU-199, VAL-142, and VAL-121. The aromatic ring of the dimethoxyphenyl portion establishes a T-shaped stacking interaction with HIS-68. Other interactions between the compound and hCA IX include H-bonds with GLN-71, ASN-66, and TRP-9 (Figure 2A). The GOLDSCORE Fitness score of the docking pose of **11** to hCA IX is 64.86.

As for the hCA XII, the interaction of **11** within the catalytic cleft accounted for the primary sulfonamide moiety of **11** to coordinate the zinc ion in a conformation superimposable to the cocrystallized ligand AAZ (Figure 2C,D) and to establish an H-bond interaction with GLU-106. Additionally, H-bonds are established with the backbones of THR-199 and THR-200. Similarly to hCA IX, the aromatic ring of the dichlorophenyl moiety in **11** establishes a T-shaped stacking interaction with HIS-94. Other valuable interactions between **11** and hCA XII include the H-bonds between the dimethoxyphenyl portion with LYS-67, THR-91, GLN-92, and SER-132 (Figure 2C). The GOLDSCORE Fitness score of the docking pose of **11** to hCA IX is 64.73.

Overall, molecular docking simulations provide a structural hypothesis of the interaction of **11** within the catalytic site of the two tumor-associated hCAs, i.e., hCA IX and hCA XII, that suggests the sulfonamide group is responsible for zinc coordination, while the tail of the molecule interacts with

key residues within the catalytic site, thus corroborating the strong inhibitory activity observed by experiments.

Representative compounds particularly selective *in vitro* for the tumor-associated hCAs (i.e., **4**, **11**, and **12**) were selected to evaluate their antiproliferative effect *in vitro* on the human triple-negative breast cancer cell line MDA-MB-231,³³ and SLC-0111 was used as reference. Cancer cells were treated with the compounds at different concentrations, and their efficacy to inhibit the proliferation was assessed after 72 h of treatment under hypoxia by flow cytometry-based cell counting. As shown in Figure 3, SLC-0111 was found to be

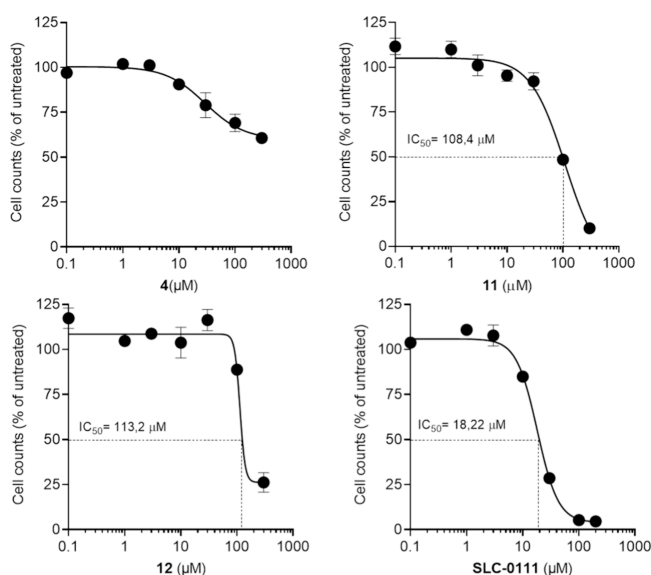


Figure 3. Cells proliferation of triple-negative breast cancer MDA-MB-231 cells treated for 72 h with **4**, **11**, **12**, and SLC-0111. Cell count refers to the untreated/control as 100%.

effective in reducing the proliferation of MDA-MB-231 with an IC_{50} of 18.22 μ M. Interestingly, compounds **11** and **12** showed a promising antiproliferative effect with IC_{50} values of 108.4 and 113.2 μ M, respectively. Indeed, no significant activity was observed for compound **4** at the higher concentration evaluated.

In vitro cellular assays of **4**, **11**, and **12** under normoxia (not shown) reported very similar results, thus confirming the efficacy of the hCAs and indicating a wider set of additional targets to be implicated as expected for such small molecular entities.

In summary, 14 novel Schiff bases derived from lasamide **1** were synthesized by a convergent synthesis approach as effective CAIs. All compounds were assessed for their inhibitory activities against the cytosolic hCAs I and II along with transmembrane tumor-associated isoforms IX and XII. hCA I was effectively inhibited by derivative **9** bearing an *ortho*-cyano phenyl ring, whereas the isoform II was most effectively inhibited by the acetone-derived analogue **16**. All compounds (**1**–**16**) elicited inhibitory activity against cancer-related hCA IX isoform with K_I values in the range of 3.4–43.3 nM. SAR analysis revealed that inhibitory potential against hCA XII was more vulnerable to the position and size of the halogen substituent (–F, –Br) on the aromatic ring. Among the compound series considered in this study, the dimethoxyphenyl derivative **11** proved to be a highly effective inhibitor and selective for both the tumor-associated isoforms IX and

XII, and therefore, an *in silico* investigation was conducted in order to establish their binding modes. Selected compounds **4**, **11**, and **12** showed interesting antiproliferative effects on human triple-negative breast cancer cell line MDA-MB-231. Hence, the findings of this work suggested that the oxime ester derivatives of lasamide **1** could lead to potentially anticancer agents by targeting hCAs IX and XII. In this context, current studies are in progress to develop derivatives with better-oriented *in vitro* selectivity toward the targeted hCAs and advanced in-cell-based experiments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmmedchemlett.4c00206>.

Previous synthetic attempts for oxime ester derivatives of lasamide **1**, experimental procedures, general chemistry, synthetic procedures, carbonic anhydrase inhibition assay, *in silico* studies, cell proliferation assays, characterization of the compounds, NMR and ESMS spectra, and references (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Yasinalli Tamboli – King Abdullah International Medical Research Center (KAIMRC), King Saud Bin Abdulaziz University for Health Sciences, Ministry of National Guard-Health Affairs, Riyadh 14811, Saudi Arabia; orcid.org/0000-0002-5161-0170; Email: yasinmedchem@gmail.com

Fabrizio Carta – NEUROFARBA Department, Sezione di Scienze Farmaceutiche e Nutraceutiche, University of Florence, 50019 Florence, Italy; orcid.org/0000-0002-1141-6146; Email: fabrizio.cartat@unifi.it

Authors

Jaydeo T. Kilbile – Department of Chemistry, School of Basic and Applied Sciences, MGM University, Chhatrapati Sambhajnagar 431003 Maharashtra, India

Suryakant B. Sapkal – Department of Chemistry, School of Basic and Applied Sciences, MGM University, Chhatrapati Sambhajnagar 431003 Maharashtra, India

Gioele Renzi – NEUROFARBA Department, Sezione di Scienze Farmaceutiche e Nutraceutiche, University of Florence, 50019 Florence, Italy; orcid.org/0009-0008-9109-030X

Ilaria D'Agostino – NEUROFARBA Department, Sezione di Scienze Farmaceutiche e Nutraceutiche, University of Florence, 50019 Florence, Italy; Department of Pharmacy, University of Pisa, 56126 Pisa, Italy; orcid.org/0000-0002-4870-7326

Luigi Cutarella – Department of Biotechnology, Chemistry and Pharmacy, University of Siena, 53100 Siena, Italy

Mattia Mori – Department of Biotechnology, Chemistry and Pharmacy, University of Siena, 53100 Siena, Italy; orcid.org/0000-0003-2398-1254

Barbara De Filippis – Department of Pharmacy “G. d’Annunzio”, University of Chieti-Pescara, 66100 Chieti, Italy

Imadul Islam – King Abdullah International Medical Research Center (KAIMRC), King Saud Bin Abdulaziz University for Health Sciences, Ministry of National Guard-Health Affairs, Riyadh 14811, Saudi Arabia

Maria Luisa Massardi – Department of Molecular and Translational Medicine, University of Brescia, 25123 Brescia, Italy

Elena Somenza – Department of Molecular and Translational Medicine, University of Brescia, 25123 Brescia, Italy

Roberto Ronca – Department of Molecular and Translational Medicine, University of Brescia, 25123 Brescia, Italy

Claudiu T. Supuran – NEUROFARBA Department, Sezione di Scienze Farmaceutiche e Nutraceutiche, University of Florence, 50019 Florence, Italy; orcid.org/0000-0003-4262-0323

Complete contact information is available at:
<https://pubs.acs.org/10.1021/acsmchemlett.4c00206>

Author Contributions

The manuscript was written through the contributions of all authors. All authors approved the final version of the manuscript.

Funding

Funding no. NRC23R/680/10 by King Abdullah International Medical Research Center (KAIMRC) for Y.T. Associazione Italiana per la Ricerca sul Cancro (AIRC) grant IG 2019 – ID.23151 and CIB (Consorzio Interuniversitario per le Biotecnologie) for R.R. National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 - Call for tender No. 3277 of 30 December 2021 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU, Project code ECS_00000017, Concession Decree No. 1055 of 23 June 2022 adopted by the Italian Ministry of University and Research, CUP B83C22003930001, project title “Tuscany Health Ecosystem – THE” for F.C.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Y.T. gratefully acknowledges King Abdullah International Medical Research Center (KAIMRC), Riyadh, Saudi Arabia for necessary facilities to carry out this research work.

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