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Discovery and Optimization of a Novel Macrocyclic Amidinourea Series Active as Acidic Mammalian Chitinase Inhibitors

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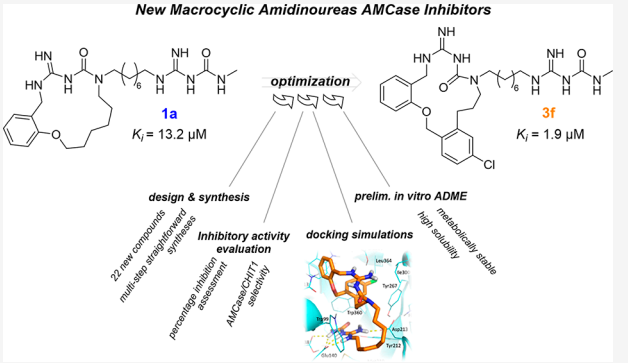
ABSTRACT: Our research group has been involved for a long time in the development of macrocyclic amidinoureas (MCAs) as antifungal agents. The mechanistic investigation drove us to perform an *in silico* target fishing study, which allowed the identification of chitinases as one of their putative targets, with **1a** showing a submicromolar inhibition of *Trichoderma viride* chitinase. In this work, we investigated the possibility to further inhibit the corresponding human enzymes, acidic mammalian chitinase (AMCase) and chitotriosidase (CHIT1), involved in several chronic inflammatory lung diseases. Thus, we first validated the inhibitory activity of **1a** against AMCase and CHIT1 and then designed and synthesized new derivatives aimed at improving the potency and selectivity against AMCase. Among them, compound **3f** emerged for its activity profile along with its promising *in vitro* ADME properties. We also gained a good understanding of the key interactions with the target enzyme through *in silico* studies.



KEYWORDS: *Macrocyclic amidinoureas, human chitinases, AMCase, CHIT1, repurposing*

Chitin is the second most abundant homopolysaccharide after cellulose and is one of the main components of fungi's cell walls and exoskeletons of crustaceans and insects.[1] Chitin is physiologically hydrolyzed by glycosyl hydrolases (GHs), almost ubiquitous enzymes involved in morphogenesis, host defense, and nutrition.[2,3] Although mammals are not able to synthesize chitin, their DNA encodes for several chitinases that contribute to the clearance of inhaled or ingested chitin. In particular, humans produce chitotriosidase (CHIT1)[4] and acidic mammalian chitinase (AMCase),[5] enzymatically active, and the nonhydrolyzing chi-lectins (chitinase-like proteins, CLPs).[6] Recently, human chitinases came under increasing scrutiny, since pieces of evidence[7−9] suggest their implication in inflammation-related diseases. In fact, in addition to the physiological expression of AMCase in the lung, stomach, and gastric mucosa, it was found to be upregulated in animal models of asthma and allergic patients through the interleukin 13 (IL-13) pathway.[7,10] Furthermore, the administration of allosamidin, a potent AMCase inhibitor, and AMCase-neutralizing antibodies significantly decreased both the Th2-induced inflammation and asthma symptoms.[7,10,11] CHIT1, present in activated macrophages and neutrophils along with bronchial epithelial cells, seems to be involved in the regulation of transforming growth factor β (TGFβ) and IL-13 pathways that promote a fibrotic response in the lung and lead to sarcoidosis, chronic obstructive pulmonary diseases, and idiopathic pulmonary fibrosis.[12−14] However, the role of chitinases in these diseases is still debated,[15−17] even if both of these enzymes are known to establish a rich protein−protein association network (see Figure S1 in Supporting Information), confirming their involvement in several biological pathways and, thus, their high value as pharmacological targets. In particular, their implication in the modulation of the exacerbated type-2 immune response, peculiar in chronic inflammatory lung diseases, is widely recognized and drives the scientific community to study modulators of chitinase catalytic activity.

Indeed, a number of chitinase inhibitors have been identified in the last 20 years, including both natural product derivatives, such as the cyclic peptides argifin (Figure 1) and argadin,[18] the pseudotrisaccharides allosamidin and demethylallosamidin,[19] small molecules, such as xanthine derivatives, bisdionins C and

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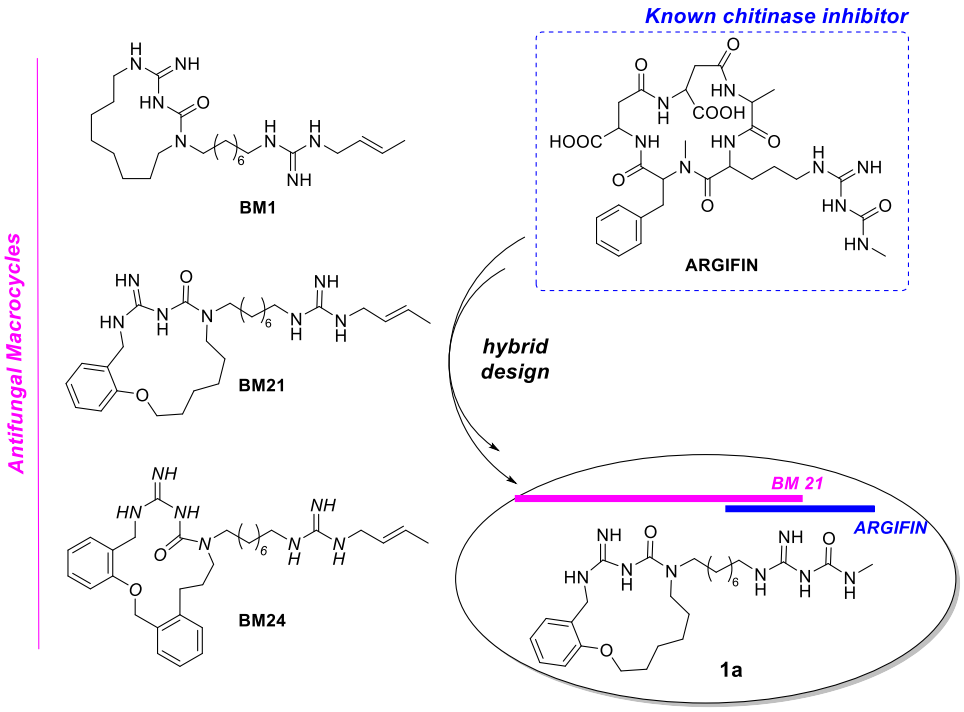

Figure 1. Structure of representative antifungal macrocycles, argifin, and hybrid compound **1a**, previously described as a submicromolar inhibitor of *T. viride* chitinase.

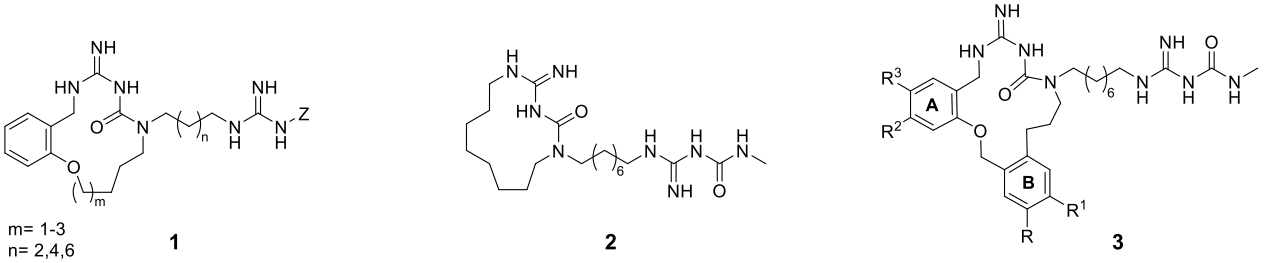

Figure 2. General structure of the three series of derivatives.

F,[20] and many others.[21] However, most of them, even if highly potent, suffer from poor pharmacokinetics,[22] while others resulted in reducing inflammation in an *in vivo* murine model but with low potency and AMCase/CHIT1 selectivity.[23] Still today, only a few compounds library by Molecure SA (formerly Oncoarendi Therapeutics SA) reached the milestone of high potency and selectivity along with good pharmacokinetics properties, and, in particular, OATD-01 was shown to be well tolerated in recent phase I clinical trials for the treatment of pulmonary fibrosis.[24,25]

As part of Prof. Maurizio Botta's research on antimicrobials, a series of macrocyclic amidinoureas (MCAs) (representative compounds **BM1**, **BM21**, and **BM24** are depicted in Figure 1) were developed as potent antifungal agents, especially against *Candida* spp. with minimal inhibitory concentrations (MICs) ranging from 1 to 32 μg/mL.[26−31] Aimed at investigating their mode of action (MoA), we resorted to a computational target fishing approach that pointed attention toward fungal chitinases. Enzymatic assays on *Trichoderma viride* (*T. viride*) chitinase revealed a micromolar inhibition (inhibition constant (K_i) ranging from 14.1 to 50.8 μM), confirming the *in silico* results.[32] Later, a rational design of a hybrid compound, bearing the amidinourea tail of argifin (Figure 1),[18] and the scaffold of compound **BM21**, led to **1a** (Figure 1), showing a submicromolar inhibitory activity against *T. viride* chitinase (K_i = 0.22 μM).[32]

The similarity among *T. viride* and human chitinases and their close phylogenetic relation (see Supporting Information, Figure S2) drove us to investigate the possibility to move toward a different project line targeting human chitinases as a potential therapeutic approach for chronic inflammatory lung diseases, by taking advantage of the knowledge retrieved from *T. viride* chitinase inhibition. Thus, we tested compound **1a** against human chitinases AMCase, resulting in good inhibitory activity, and CHIT1, revealing promising AMCase/CHIT1 selectivity (Table 1). Encouraged by these results and to enlarge our knowledge of the chemical space related to **1a**, we synthesized a small library of more than 20 derivatives by modifying the main structural features of **1a** by performing a rational design.

Starting from the expertise on MCAs retrieved by the long-time development of antifungal agents with similar chemical structures[18] and from the recent finding of the Argifin-MCA hybrid compound **1a** as a potent inhibitor of *T. viride* chitinase,[32] we developed a small class of derivatives endowed with the macrocyclic cores and the argifin tail. The MCAs library was designed into three main series (Figure 2): the first one, including derivatives **1b−l** (Table 1), bearing the

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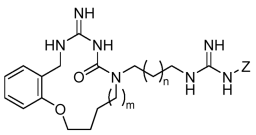
Table 1. Inhibitory Activity Data for MCAs Series on AMCase and CHIT1[d]



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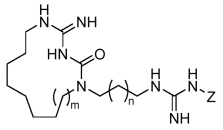

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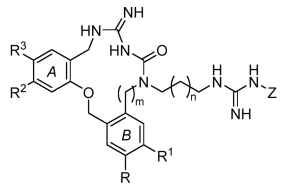

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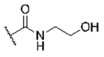
Cpd	m	n	Z	R	R^1	R^2	R^3	% Inh AMCase[a]	K_i AMCase (μM)	% Inh CHIT1[a]	K_i CHIT1 (μM)	SI $\frac{K_i \text{ CHIT1}}{K_i \text{ AMCase}}$
1a	3	6		-	-	-	-	59	13.2 ± 0.1	26	9.9 ± 2.0	0.8
1b	2	6		-	-	-	-	78	5.3 ± 1.1	38	5.7 ± 0.8	1.1
1c	1	6		-	-	-	-	67	10.3 ± 0.8	35	6.5 ± 0.5	0.6
1d	3	4		-	-	-	-	51	12.9 ± 0.5	20	14.0 ± 2.5	1.1
1e	3	2		-	-	-	-	41	27 ± 2	22	12.4 ± 2.4	0.5
1f	3	6		-	-	-	-	8	>100	n.d.[b]	n.d.	-
1g	3	6		-	-	-	-	13	>100	18	15.9 ± 3.0	<0.16
1h	3	6		-	-	-	-	14	>100	<5	>50	-
1i	3	6	H	-	-	-	-	10	>100	n.d.	n.d.	-
2a	1	6		-	-	-	-	50	18.4 ± 0.9	<5	>50	>2.7
3a	3	6		H	H	H	H	84	3.5 ± 0.2	32	7.4 ± 0.6	2.1
3b	3	6		OCF_3	H	H	H	< 5	>50	35	6.5 ± 1.1	<0.12
3c	3	6		F	H	H	H	70	8.2 ± 1.0	46	4.1 ± 0.3	0.5
3d	3	6		Me	H	H	H	79	5.1 ± 0.3	40	5.2 ± 0.9	1.0
3e	3	6		H	F	H	H	83	3.8 ± 0.6	36	6.2 ± 0.2	1.6
3f	3	6		H	Cl	H	H	87	1.9 ± 0.1	22	12.4 ± 1.8	6.5

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Table 1. continued

Cpd	m	n	Z	R	R^1	R^2	R^3	% Inh AMCase[a]	K_i AMCase (μM)	% Inh CHIT1[a]	K_i CHIT1 (μM)	SI $\frac{K_i \text{ CHIT1}}{K_i \text{ AMCase}}$
3g	3	6		H	H	Me	H	70	8.3 ± 0.9	<5	>50	>6.0
3h	3	6		H	H	H	Me	72	6.9 ± 0.3	n.d.	n.d.	-
3i	3	6		H	H	H	OMe	76	5.6 ± 0.6	34	6.8 ± 0.4	1.2
3j	3	6		H	H	Cl	H	61	21 ± 2.2	<5	>50	>2.4
3k	3	6		H	H	F	H	82	2.9 ± 0.2	10	31.4 ± 5.6	10.8
3l	3	6		H	H	H	F	66	18 ± 2.0	20	14 ± 2.8	0.8
			Bisdionin F					59[c]	0.26 ± 0.02	98[c]	0.0014 ± 0.0001	0.0055

[a]At [Inh] = 50 μM. [b]n.d. = not determined. [c]At [Inh] = 1 μM. [d]Data are reported as the mean of at least three independent experiments as a percentage of inhibition at the inhibitor (test compound) concentration of 50 μM and as inhibition constants (K_i) endowed with SD values. Selectivity index (SI) was calculated as the ratio between the percentage of inhibition at 50 μM on AMCase against CHIT1. Hit compound **1a** is highlighted in gray. Bisdionin F is reported as reference control of the experiments. For more information see the experimental section in the Supporting Information.

fingerprinting of the **1a** phenyl-fused core; the second one containing an aliphatic macrocycle, a feature shared with **BM1**, (herein we present only compound **2a** resulting in an amidinourea-tailed **BM1** derivative, Table 1), while the last series included the diphenyl-fused macrocycles (**3a–l**, Table 1).

The design of such derivatives is based on the attempt to explore the chemical space around the endocyclic amidinourea function by decreasing (series 2) or increasing (series 3) the number of phenyl rings fused to the macrocycle with respect to series 1 with the consequent impact on the possible numbers of $\pi-\pi$ or $\pi-$cation stacking interactions they could establish in the enzyme binding site. In series 1, we modified the macrocyclic size in the aliphatic portion (indicated by the letter *m*) by reducing the ring from 15 to 14 and 13 terms (MCAs **1b** and **1c**, respectively, Table 1) in order to make the scaffold more flexible and allow different binding poses in the enzymes pocket. The role of the length of the alkyl tail (indicated by the letter *n*) was also investigated through MCAs **1d** and **1e** (Table 1), bearing 6 and 4 methylene-based chains, respectively.

An insight into the terminal amidinourea (*Z*) and, in particular, into the H-bond network generated by the nitrogen H-bond donor on the enzymatic activity was performed by replacing the *N*-methyl substituent with a more polar *N*-β-hydroxyethyl group (**1f**, Table 1) or by adding another methyl group, resulting in an amidino-*N*,*N*-dimethylurea (**1g**, Table 1). Also, the *N*-methyl urea portion of the terminal function was completely removed, leaving intact the unsubstituted guanidine (**1i**, Table 1) or substituting with its *N*-methyl thiourea isostere (**1h**, Table 1) to actually investigate its role and understand the impact of a bigger and polarizable atom (the sulfur) in the interaction with the target enzyme.

In a second round of the optimization process, in series 2 and 3, we focused our attention on the aromatic portions of the core. In particular, we removed the *O*-phenyl ring (namely, ring A) (**2a**, Table 1) and introduced an additional phenyl group (namely, ring B) (**3a**, Table 1), and we assessed the effect of the electron modulation of these phenyl rings induced by activating (**3d** and **3g–I l**, Table 1) or deactivating (**3a–c**, **3e–f**, and **3j–l**, Table 1) groups.

The inhibitory activity against AMCase and CHIT1 was evaluated via a direct fluorometric assay using 4-methylumbelliferyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose as the enzyme substrate. The percentage of inhibition was initially screened at 50 μM, each compound in DMSO solution. For compounds showing an inhibition ≥70% of the activity, the K_i values were determined using a competitive model of inhibition.[33] Results are reported in Table 1.

The analysis of the *in vitro* enzyme inhibition data for AMCase and CHIT1 enzymes allows a better understanding of the influence of the different structural modifications on the SARs among the first series of compounds (**1a–I**, Table 1). Most importantly, not only did the terminal amidinourea moiety appear to be crucial for the inhibitory activity but also it requires the presence of a single methyl substituent for optimal activity since all its modifications (**1f–i**) or removal (**1i**) resulted in a dramatic loss of enzyme inhibition. Second, the tail length had a less dramatic effect on the inhibitory activity. Reducing the number of carbon atoms from eight (**1a**) to six (**1d**) did not affect potency, while the presence of a significantly shorter side chain (**1e**, 4 carbon atoms) determined a 2-fold increase in the K_i value. Third, variations

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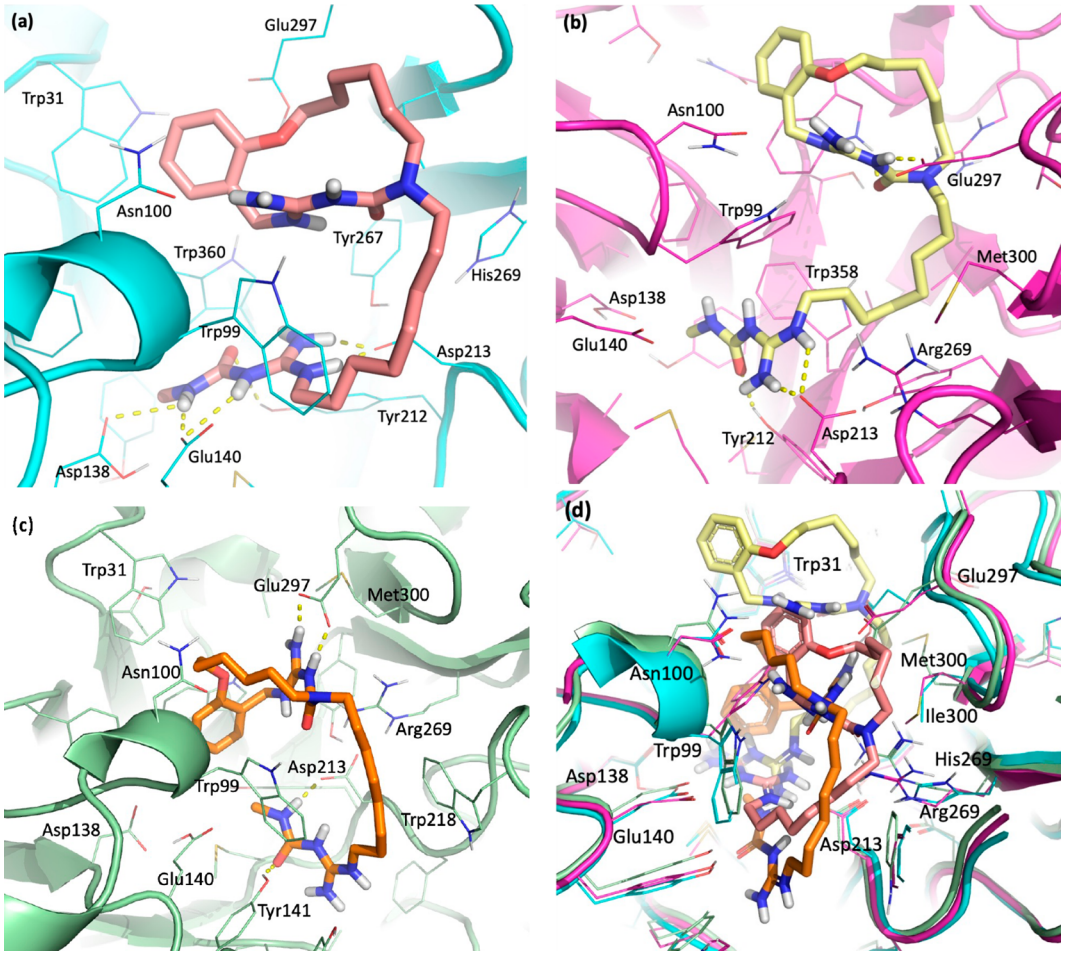

Figure 3. Binding mode of **1a** in AMCase (a, cyan) and in CHIT1 (b, magenta and **c** pale green) binding sites. Protein backbones are presented in cartoon and interacting residues in thin sticks and labeled. Compound **1a** is presented as salmon (a), yellow (b), or orange (c) sticks. Hydrogen bonds are shown as yellow dotted lines. (d) Superimposition of **1a** in AMCase (cyan) and in CHIT1 (magenta, pale green) binding sites. The figure highlights that the previously described differences in the binding sites of AMCase and CHIT1 lead to a strong displacement of **1a** from the binding site with a consequent loss of interactions between the compound and the catalytic residues in CHIT1.

in the ring size of the macrocyclic core (**1b**, 14-membered ring, and **1c**, 13-membered ring) only minimally affect the inhibitory activity, with the 14-membered ring (**1b**) being the best macrocycle dimension.

Furthermore, modifications of the macrocyclic core led to interesting findings. Indeed, removing the *O*-phenyl ring A from **1c** led to **2a** determined a slight decrease in the activity on AMCase (<2-fold increase of the K_i value) and a complete loss of inhibition of CHIT1, whereas the introduction of an additional phenyl group (ring B (as in **3a**)), was associated with a more substantial improvement of the activity on AMCase, lowering the K_i value to 3.5 μM (≈4-fold decrease of the K_i value, compared to that of **1a**). Interestingly, compound **3a** also showed an interesting inhibition profile, with a better activity on AMCase than CHIT1, and represented the first compound in these series to show such a behavior.

Consequently, the functionalization of these phenyl rings with different substituents, i.e., deactivating substituents, such as halogens (**3b, 3c, 3e, 3f, 3j−l**), or activating substituents, such as methyl (**3d**, **3g**, **3h**) or methoxy (**3i**) groups, was also evaluated and displayed a contrasting effect on the inhibitory activity. A general increase in activity was measured in derivatives bearing halogen substituents on ring B (**3e** and **3f**) and in compounds with activating substituents on ring A (**3h** and **3j**). Conversely, the halogen functionalization of ring A resulted in a general decrease of activity, with the sole improvement being for compound **3k**. The most active compound of this series is **3f**, which presents a K_i of 1.9 μM.

Interestingly, the inhibition profile was modulated by the functionalization of both phenyl rings A (R^1) and B (R^2) with halogen or methyl substituents. An enhancement of the preference toward AMCase over CHIT1 was observed with most of such compounds, culminating with derivatives **3f**, **3g**, and **3k** which showed a K_i value up to 11-fold lower for AMCase than for CHIT1.

To rationalize the enzymatic inhibition data, we investigated the pattern of ligand-enzyme interactions by docking the whole library of compounds in both active sites (see Supporting Information for a more detailed discussion).

Observing the binding pose of MCA **1a** in AMCase (Figure 3a), we can notice that the ureic NH group of the terminal amidinourea of **1a** is involved in a hydrogen bonding network with the catalytic residues Asp138 and Glu140, while the amidine group establishes hydrogen bond interactions with the side chains of Tyr212 and Asp213. The binding pose of **1a** in the CHIT1 active site (Figure 3b) is helpful to clarify its decrease in inhibitory activity toward CHIT1 compared to AMCase, highlighting that the inversion of the indole ring of

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Trp99 avoids the interaction of the amidinourea moiety with Asp138 and Glu140. The macrocyclic core of **1a** establishes interactions with the Glu297 side chain, and a salt bridge between the carboxyl group of Asp213 and the amidinourea NH group is formed. We also evaluated the binding mode of **1a** in a CHIT1 crystal structure (PDB ID: 4WKH)[34] characterized by the presence of Trp99 that points out the binding site as occurs in AMCase (Figure 3c).

The steric hindrance due to the longer side chain of Arg269 with respect to His269 in AMCase along with the indole ring of Trp218 are responsible for the lack of interactions between the terminal amidinourea of **1a** and the side chains of Asp138, Glu140, and Asp213 and are in accordance with the lower inhibitory activity toward this enzyme.

A preliminary *in vitro* ADME profile (aqueous solubility, passive permeability, and metabolic stability) was investigated for some of the most promising AMCase inhibitors of the series (**1b**, **3a**, **3c**, **3f**, and **3i**) (Table 2).

Table 2. *In Vitro* Preliminary ADME Evaluation of Compounds 1b, 3a, 3c, 3f, and 3i

	Water Solubility[a]				
Cpd	μg/mL	Log S[b]	Papp[c] (10^{-6} cm/s)	Metabolic Stability (%)[d]	Major Metabolite (%)[e]
1b	1808.1	−2.45	<0.5	80.9	M_1 = 19.1
3a	2014.2	−2.44	<0.5	76.2	M_1 = 23.8
3c	2852.5	−2.34	<0.5	98.5	M_1 = 1.5
3f	23109.1	−2.44	<0.5	91.5	M_1 = 8.5
3i	3075.1	−2.28	<0.5	98.7	M_1 = 1.3

[a]Aqueous solubility was determined through the HPLC-UV/MS method used for the assessment of compound purity (reported in the Supporting Information, Chemical Procedures and Characterizations, section General Chemistry). [b]Logarithm of solubility expressed as mol/L. [c]Apparent permeability. [d]The metabolic stability in the presence of HLMs is expressed as a percentage of the unmodified parent drug. [e]M_1 = M + OH (+ 16).

As expected for a trifluoroacetate salts, all compounds showed a high-water solubility; **3c** and **3i** reached the highest values of 2.8 and 3 mg/mL, respectively. Furthermore, the optimal solubility confirmed by the parallel artificial membrane permeability assay (PAMPA) underlined the very low ability of those compounds to come across phospholipidic bilayers, with Papp values lower than 0.5×10^{-6} cm/s. By combining the solubility and permeability data, we were able to classify these derivatives as BCS Class III compounds endowed with high solubility and low permeability.[35] For a deeper insight, the metabolic stability in the presence of human liver microsomes (HLMs) was studied. **3c**, **3f**, and **3i** were stable with percentages of the unmetabolized compound greater than 90%, while **1b** and **3a** appeared to be less stable to the metabolic action of microsomes (80.95 and 76.2%, respectively) leading to improved percentages of the mono-oxidate metabolite (M_1). All the results are summarized in Table 2, in accordance with those of previously described MCAs.[30] Also, the compounds were found to be noncytotoxic and non-PAINS through predicting tools (see Supporting Information).

In summary, we translated a nonsatisfactory result in the search for antifungal agents into a successful starting point for the development of perspective human chitinase inhibitors. Thus, we synthesized a small library of compounds characterized by chemical modification of the three main structural features of **1a** and evaluated their inhibitory activity on AMCase and CHIT1. The amidinourea moiety and the tail seem to be key structural features of such compounds, being fundamental for the interaction with the enzyme, as also confirmed by docking simulations. The introduction of a second phenyl ring B fused to the macrocyclic core allows us to work on its substitution patterns with activating or deactivating groups. Through the derivatization and derivative design, we were able to develop **3f**, being 7-fold more active than the hit compound **1a** and moderately selective against AMCase (being ≈7-fold more potent on this enzyme than on CHIT-1). Moreover, taking the preliminary *in vitro* ADME results, all tested compounds resulted in BCS Class III derivatives endowed with high-water solubility (Log S between −2.5 and −2) and a consequent low passive permeability (Papp $<0.5 \times 10^{-6}$ cm/s). Many more differences have been appreciated during the metabolic studies in the presence of HLMs, except for **1b** and **3a**, whose stability resulted in lower than 90%, and **3c**, **3f**, and **3i** showed reassuring percentages of stability when in the presence of HLMs. *In silico* studies were performed to rationalize the enzymatic data, leading us to identify the residues that are fundamental to establishing strong interaction with the enzyme, conferring to some of these compounds an inhibition profile showing an improved selectivity toward AMCase (with up to 11-fold lower K_i, when compared to that of CHIT1, see Table 1). In particular, we found key interactions between the compounds and Trp99 and Arg269 residues. Even if the activity data of this small library of derivatives are far from the high potency displayed by the compounds developed by Molecure SA, all the information retrieved from the computational studies and the ADME results constitute a promising scenario in the frame of optimization and development of a new series of selective AMCase inhibitors, repurposing this new scaffold, previously optimized for its antifungal activity, as an inhibitor of human chitinase.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.2c00472.

Protein−protein association network for AMCase and CHIT1; from fungal to human chitinases; synthesis of three series of derivatives; *in silico* study, toxicity prediction, and PAINS assessment; material and methods: enzymatic assays; *in silico* studies; preliminary ADME profile of **1b**, **3a**, **3c**, **3f**, and **3i**; chemical procedures and characterizations; NMR spectra of representative compounds (PDF)

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L.J.I.B., F.O., and G.I.T. synthesized and characterized the compounds. L.J.I.B., F.O., G.I.T., and J.D.D. performed the enzyme inhibition assays. C.I.T. and D.F. performed the *in silico* studies. F.P. performed the *in vitro* ADME experiments. L.J.I.B. and I.D. wrote the first draft of the manuscript. All authors analyzed data and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ DEDICATION

This paper is dedicated to Prof. Maurizio Botta (1950−2019), who drove the first stages of the present study. Prof. Botta was an eminent chemist, an inspiring teacher, and a towering figure in the medicinal chemistry field. His strong devotion to scientific research and his work and life advices will always remain in the hearts and minds of all of us along with all his other colleagues and students.

■ ABBREVIATIONS

AMCase, acidic mammalian chitinase; CHIT1, chitotriosidase; CLP, chitinase-like proteins; MCA, macrocyclic amidinourea; MIC, minimal inhibitory concentrations; MoA, mode of action; GH, glycosyl hydrolase; IL-3, interleukin 13; PAMPA, parallel artificial membrane permeability assay; Papp, apparent permeability; SAR, structure−activity relationship; TGFβ, growth factor beta

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