



Research paper

In silico-guided design, synthesis and in vitro evaluation of thiazolo-pyridines targeting SARS-CoV-2 Nsp13

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ARTICLE INFO

Keywords:

Thiazolo-pyridines

Nsp13

GRID-Guided optimization

Integrated CADD workflow

SARS-CoV-2

P450 metabolism prediction

Ligand designer

ABSTRACT

In the search for novel antiviral agents targeting SARS-CoV-2 non-structural protein 13 (Nsp13), we applied an integrated *in silico* drug design workflow combining ADME profiling, molecular interaction field analysis, molecular docking and thermodynamic evaluations to identify thiazolo-pyridine derivatives as promising scaffolds. This computational strategy enabled the rational prioritization of compounds and guided the design and synthesis of a novel series of [1,3]thiazolo[5,4-*b*]pyridine-6-carboxamides (**19** and **20**) and [1,3]thiazolo[5,4-*h*] [1,6]naphthyridines (**21** and **22**). Among the synthesized compounds, **19h** and **19n** emerged as the most potent candidates, demonstrating dual inhibitory activity with IC₅₀ values for unwinding and ATPase activities in the low micromolar range. Kinetic analyses confirmed a competitive inhibition mechanism, as these compounds successfully compete with the natural substrate ATP for the enzyme's active site. Overall, our findings highlight the effectiveness of integrated computational approaches for accelerating antiviral lead optimization and establish the thiazolo-pyridine core as viable chemotype for further Structure-Activity Relationship (SAR) studies and preclinical development.

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), still represents a global concern, due also to the ongoing spread of this virus and its variants. Although multiple COVID-19 vaccines have been developed in the last few years [1], there is still a lack of effective therapies for the treatment of infections caused by SARS-CoV-2. Therefore, the identification of alternative strategies to fight COVID-19 is needed.

SARS-CoV-2 is an enveloped, positive-sense single-stranded RNA *Betacoronavirus* belonging to the family of *Coronaviridae* [2,3]. The SARS-CoV-2 genome contains approximately 30 kbs and encodes two open reading frames (ORF1a and ORF1b) which are translated in two

polypeptides (pp1a and pp1ab). After virus entry, these polyproteins are processed by viral proteases 3CLpro and PLpro into 4 structural proteins (Spike, Nucleocapsid, Matrix and Envelope), 16 non-structural proteins (Nsp1-16) and 9 accessory proteins. Non-structural proteins including primase (Nsp8), RNA-dependent RNA polymerase RdRp (Nsp12) and helicase (Nsp13) are involved in the formation of the replication-transcription complex (RTC), playing an essential role in viral gene replication [4]. Among non-structural proteins, Nsp13 has been identified as one of the most attractive targets for the development of new antiviral agents, due both to its high degree of conservation and its essential role in SARS-CoV-2 replication [5,6]. It belongs to the helicase superfamily 1B (SF1B) and acts by catalysing the unwinding of dsDNA or RNA in a 5' to 3' direction in a nucleoside triphosphate (NTP)

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dependent manner. Additionally, it has been shown that Nsp13 is a bifunctional enzyme which also possesses an RNA 5' triphosphatase activity within the same NTPase active site: by removing the γ -phosphate group of the 5'-terminal RNA nucleotide, Nsp13 could play a further essential role during the formation of the 5' mRNA cap, thus contributing to the human immune system evasion and viral diffusion [7]. Nsp13 helicase is one of the most well-conserved non-structural proteins in the SARS-CoV-2 genome, sharing a 99.8% sequence identity compared to SARS-CoV, from which it differs for only a single amino acid residue (V570 in SARS-CoV-2 Nsp13 instead of I570 in SARS-CoV) [8]. With a triangular pyramidal shape, SARS-CoV-2 Nsp13 contains five conserved domains essential for helicase activity: the N-terminal zinc-binding domain (ZBD) coordinating three zinc ions, the stalk domain and the 1B domain which are responsible of protein–protein and/or nucleic acid interactions within the RTC; two “RecA-like” domains 1A and 2A containing the active site for NTP binding and hydrolysis [7]. Structural analysis and experimental fragment screening of SARS-CoV-2 Nsp13 revealed several druggable conserved pockets that could be relevant for structure-based drug design, with particular attention to the nucleotide binding site [9]. All these features make Nsp13 an ideal target for the development of broad-spectrum inhibitors acting with new mechanisms of action, in order to allow combined therapies that would reduce the risk of selection of drug-resistant viral strains.

Thiazoles have been found in many bioactive molecules endowed with anti-coronavirus properties at micromolar level [10–13], also appearing as a recurrent moiety in heterocyclic compounds exhibiting a demonstrated or predicted helicase inhibition [9,14–16].

In recent years, advances in computer-aided drug design (CADD) have enabled the development of integrated workflows capable of translating structural and energetic information from target proteins into rational chemical modifications, thereby accelerating lead optimization and improving hit-to-lead efficiency [17]. Accordingly, in our efforts to identify novel SARS-CoV-2 antiviral agents, we applied an integrated computational workflow combining GRID-based interaction mapping, ligand design tools, molecular docking, MM/GBSA analyses, and *in silico* metabolic prediction to guide the optimization of a new series of [1,3]thiazolo[5,4-*b*]pyridines and [1,3]thiazolo[5,4-*h*][1,6]naphthyridines (Chart 1) targeting the ATPase pocket of SARS-CoV-2 Nsp13 helicase. Although computational predictions cannot fully

reproduce the complexity of biological systems, these approaches provide a powerful and cost-effective strategy for prioritizing compounds and guiding experimental efforts in a hypothesis-driven manner. Based on these computational insights, the selected derivatives were rationally designed and synthesized in order to evaluate their ability to inhibit SARS-CoV-2 Nsp13 unwinding and ATPase functions.

2. Results and discussion

2.1. Drug design of SARS-CoV-2 Nsp13 inhibitors

The starting point for our computational approach was the crystal structure of the wild-type SARS-CoV-2 Nsp13 helicase complexed with the 1-(2-methylphenyl)-1,2,3-triazole-4-carboxylic acid fragment **Z2327226104** (Fig. 1a), within the nucleotide binding site (PDB ID: 7NNG). With the aim of improving the binding affinity against Nsp13 we applied the GRID program, a widely used computational tool for rational drug design in medicinal chemistry. To evaluate the chemical features of the binding site, after removing the ligand, we focused our attention on 4 pivotal probes: O and N1 which simulated the carbonyl oxygen atom and the nitrogen of the amide, respectively, C1 = and CRY, which corresponded to the aromatic and lipophilic regions. A comparative analysis of the GRID experiments was carried out by assessing the energies and geometric positions of the molecular interaction fields (MIFs) around the target. The MIFs revealed an extended surface for C1 = between the triazole and benzene rings, indicating that the introduction of fused aromatic heterocycles would improve their interactions with the binding pocket (Fig. 1b). In light of these insights, we applied a scaffold hopping strategy, replacing the triazole ring with a set of six condensed heteroaromatic cores (1–12, Table S1), which are significant in medicinal chemistry due to their broad spectrum of biological activities [18–20]. By analyzing the root mean square deviation values (RMSD) between the X-ray co-crystallized fragment and the generated docking poses, we evaluated the capability of our protocol to reproduce the binding mode of the experimental bioactive pose (RMSD value equal to 0.64 Å). Thus, we obtained a Glide Gscore cut-off value of −4.38 kcal/mol to be applied as a filter. Therefore, docking results revealed that all designed compounds were able to accommodate into the nucleotide binding pocket. MM/GBSA calculations validated this scaffold hopping approach, pointing out that the compound containing

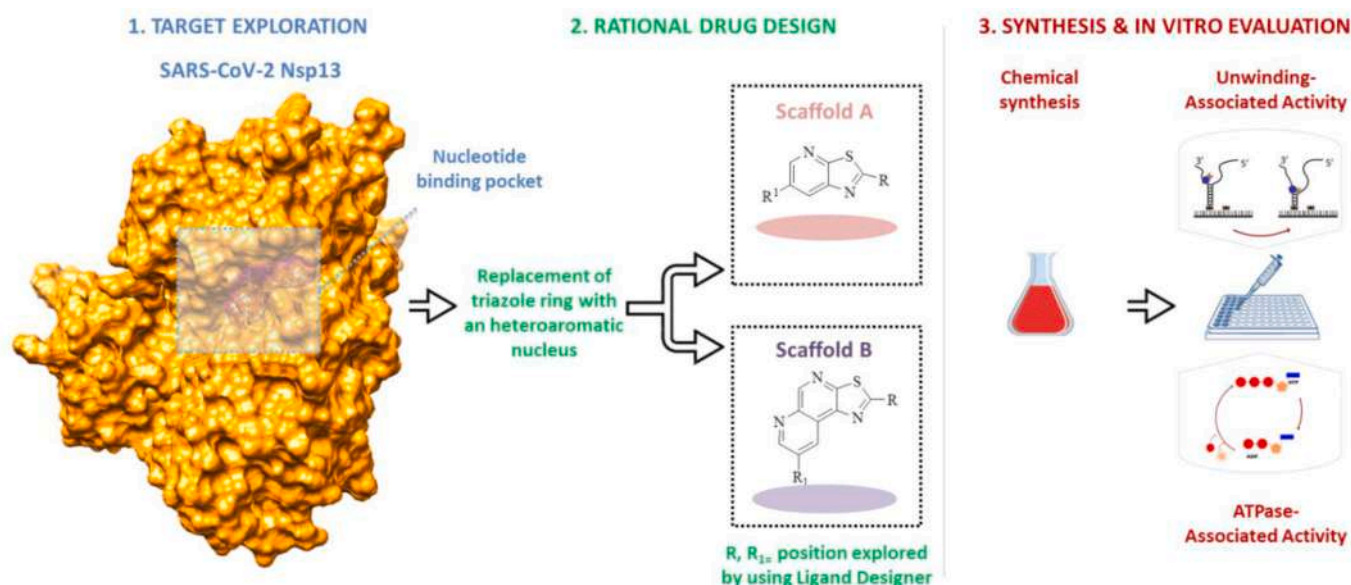


Chart 1. Workflow for the identification and optimization of [1,3]thiazolo[5,4-*b*]pyridine and [1,3]thiazolo[5,4-*h*][1,6]naphthyridine scaffolds targeting the ATP-binding site of SARS-CoV-2 Nsp13.

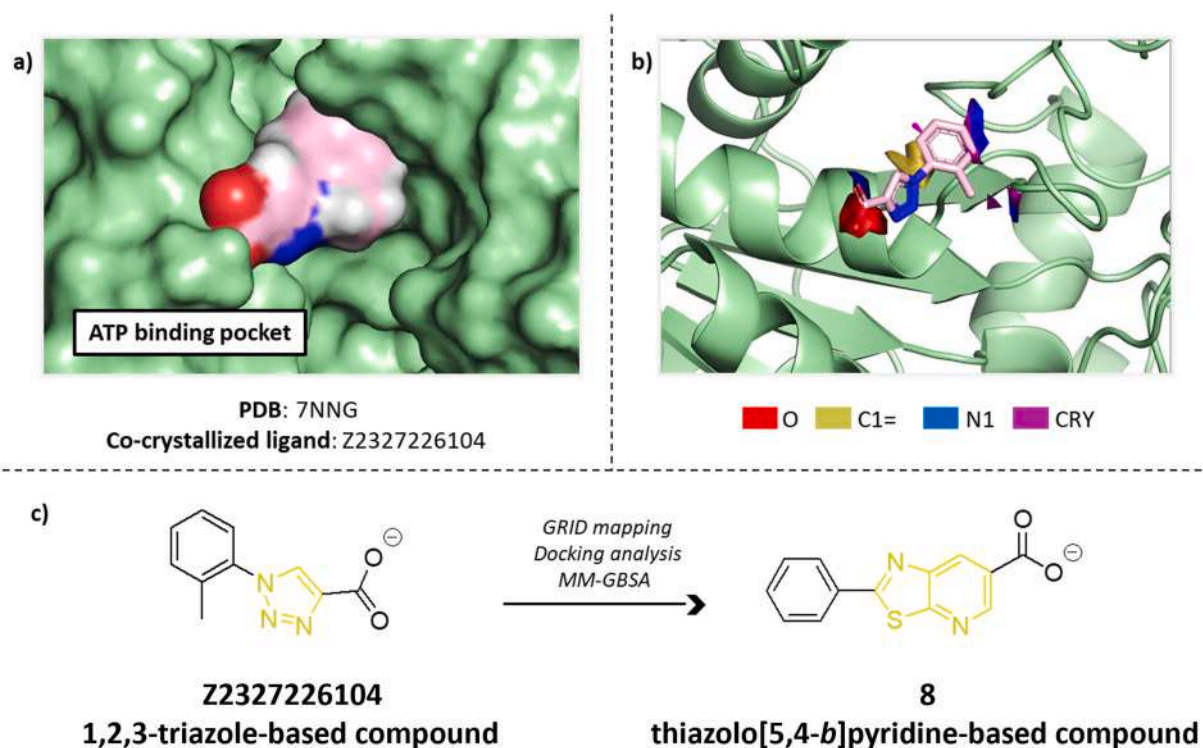


Fig. 1. a) Zoom-in view of the binding pocket of the co-crystallized structure 1-(2-methylphenyl)-1,2,3-triazole-4-carboxylic acid (**Z2327226104**) into the SARS-CoV-2 Nsp13. The receptor and the inhibitor are represented as green and rose surface, respectively; b) GRID generated pocket with the accommodated ligand. The protein and the ligand are reported as a green cartoon and rose carbon sticks, respectively, while O, C1 =, N1, and CRY molecular interaction fields (MIFs) are represented as red, yellow, blue and purple solid surfaces, respectively; c) Chemical features of **Z2327226104** and thiazolo[5,4-*b*]pyridine-based compound **8**.

the thiazolo[5,4-*b*]pyridine nucleus (**8**) exhibited a comparable binding affinity (ΔG_{bind} equal to -31.09 kcal/mol, see Supporting Information, Table S1) to the original reference fragment **Z2327226104** ($\Delta G_{bind} = -30.29$ kcal/mol) (Fig. 1c).

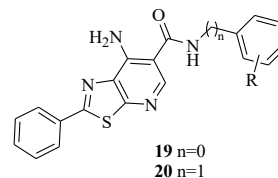
This favorable binding energy is consistent with the interest that both thiazole and pyridine scaffolds have attracted in the field of antiviral research [21], encouraging us to consider the thiazolo[5,4-*b*]pyridine core as a focal point for the development of new antiviral candidates.

Following GRID analysis, the binding pocket was further investigated to identify potential modifications aimed at enhancing the theoretical affinity toward Nsp13. It was highlighted the presence of the isocontour maps of O and N1 probes close to the aromatic region, thus indicating the need to add a donor/acceptor group at position 6. For this reason, analyzing the attractive growth space around the pocket, the Ligand Designer tool, implemented in Schrödinger Suite, was used to generate virtual ligands matching the shape and chemical features of the binding pocket. A virtual library was generated by the combinatorial enumeration of 43 R-groups at position 6 of compound **8**. Only 10 enumerated molecules (**9-18**, Table S2) were obtained and subjected to QikProp properties, molecular recognition and MM/GBSA analysis. Among the resulted compounds, the calculations suggested that the presence of the anilide group incorporated into the thiazolo[5,4-*b*]pyridine scaffold ensured a favoured thermodynamic profile ($\Delta G_{bind} = -33.15$ kcal/mol) within the Nsp13 nucleotide binding pocket (Supporting Information, Table S2).

To improve solubility and reduce lipophilicity while preserving theoretical binding affinity, an amino group was introduced at position

Table 1

Theoretical free energies of binding of designed 7-amino-*N*,2-diphenyl[1,3]thiazolo[5,4-*b*]pyridine-6-carboxamides **19a-m** and 7-amino-*N*-benzyl-2-phenyl[1,3]thiazolo[5,4-*b*]pyridine-6-carboxamides derivatives **20a-m**.



Cpd	n	R	MM/GBSA (kcal/mol)	Cpd	n	R	MM/GBSA (kcal/mol)
19a	0	H	-36.45	20a	1	H	-38.26
19b	0	2-OH	-38.76	20b	1	2-OH	-40.80
19c	0	2-NH ₂	-39.13	20c	1	2-NH ₂	-43.16
19d	0	2-NO ₂	-36.94	20d	1	2-NO ₂	-46.12
19e	0	2-OCH ₃	-34.37	20e	1	2-OCH ₃	-39.10
19f	0	3-OH	-33.11	20f	1	3-OH	-31.80
19g	0	3-NH ₂	-33.84	20g	1	3-NH ₂	-30.39
19h	0	3-NO ₂	-39.58	20h	1	3-NO ₂	-47.08
19i	0	3-OCH ₃	-33.69	20i	1	3-OCH ₃	-48.45
19j	0	4-OH	-38.44	20j	1	4-OH	-37.20
19k	0	4-NH ₂	-36.90	20k	1	4-NH ₂	-48.76
19l	0	4-NO ₂	-35.62	20l	1	4-NO ₂	-36.62
19m	0	4-OCH ₃	-36.59	20m	1	4-OCH ₃	-43.90

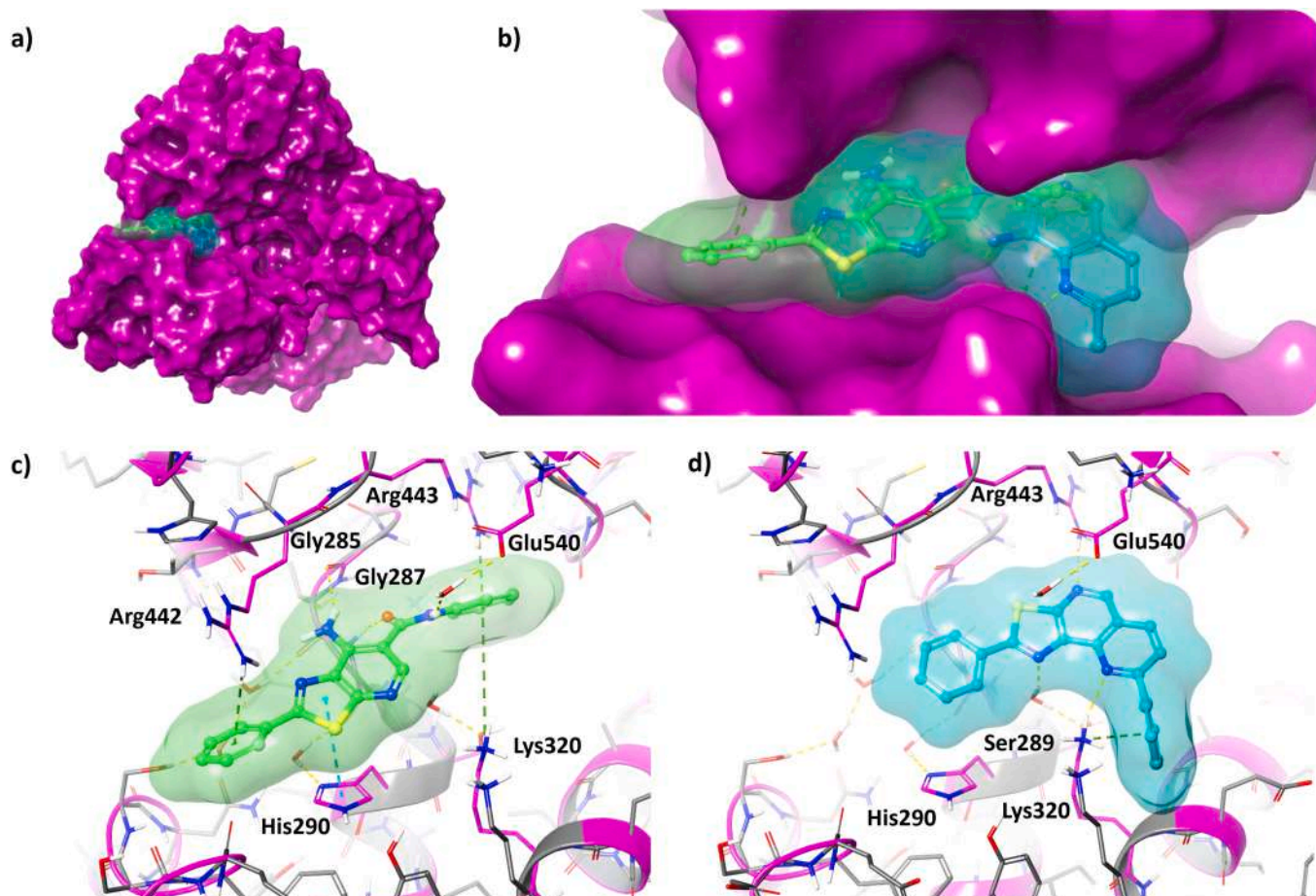
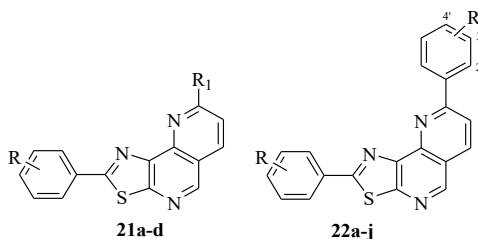


Fig. 2. a) Superposition of the binding modes of compounds **19a** (green ball-and-stick) and **21c** (cyan ball-and-stick); b) zoomed-in view of the catalytic site, with Nsp13 depicted as a fuchsia surface. Three-dimensional representation of the binding modes of c) **19a** and d) **21c** within the binding pocket. The protein is depicted as a fuchsia cartoon, while the ligands are shown as transparent surfaces in green and cyan for **19a** and **21c**, respectively. Hydrogen bonds and cation- π interactions are depicted as dashed lines in yellow and green, respectively.

Table 2

Theoretical free energies of binding of designed [1,3]thiazolo[5,4-*h*][1,6]naphthyridines **21a-d** and **22a-j**.



MM/GBSA (kcal/mol)				MM/GBSA (kcal/mol)					
Cpd	R	R ₁		Cpd	R	R ₁	2'	3'	4'
21a	H	Me	−38.01	22a	H	F	−38.48	−38.36	−36.70
21b	4-OCH ₃	Me	−42.44	22b	4-OCH ₃	F	−43.79	−43.14	−42.86
21c	H	Ph	−39.98	22c	H	NO ₂	−42.65	−39.61	−37.27
21d	4-OCH ₃	Ph	−45.53	22d	4-OCH ₃	NO ₂	−48.27	−45.15	−43.31
				22e	H	CF ₃	−39.24	−37.95	−34.50
				22f	4-OCH ₃	CF ₃	−44.96	−43.81	−40.72
				22g	H	Cl	−44.58	−39.85	−39.51
				22h	4-OCH ₃	Cl	−50.09	−45.35	−45.61
				22i	H	OCH ₃	−41.17	−40.25	−39.83
				22j	4-OCH ₃	OCH ₃	−46.95	−46.03	−45.62

7 of the thiazolo[5,4-*b*]pyridine ring (**19a**, Table 1). Thermodynamic analysis and visual inspection indicated that this modification enhanced the binding affinity toward Nsp13, as evidenced by a ΔG_{bind} value of -36.45 kcal/mol. Notably, compound **19a** established an additional cation- π interaction involving the benzene ring and Arg442, as well as a π - π stacking interaction between the thiazole moiety and His290. Furthermore, the amino group formed a hydrogen bond with the backbone carbonyl of Gly285, providing an additional anchoring point that may contribute to the improved binding profile (Fig. 2c). The portion containing the amide is positioned toward the pocket that accommodates RNA. Given the available space within the Nsp13 binding pocket, we explored the replacement of the phenyl ring with a benzyl group to better occupy the cavity and enhance molecular interactions. To further optimize this region, we functionalized both benzyl and phenyl amide moieties with electron-donating and electron-withdrawing groups at the *ortho*, *meta*, and *para* positions, yielding a total of 26 compounds and aiming to fine-tune binding affinity and physicochemical properties (Table 1).

After docking calculations, MM/GBSA analysis was carried out on the designed compounds; 18 of these exhibited more favorable binding free energies than compound **19a**. Consequently, these molecules were prioritized for synthetic accessibility assessment.

We also explored the effect of the increased molecular rigidity on target engagement. To achieve this, we adopted a rigidification strategy by expanding the [1,3]thiazolo[5,4-*b*]pyridine-6-carboxamides into a fused polycyclic aromatic system. Specifically, the resulting rigid tricyclic [1,3]thiazolo[5,4-*h*][1,6]naphthyridine scaffold was assessed.

As an initial step, we investigated the binding mode of the naphthyridine scaffold bearing a methyl (**21a**) and phenyl group (**21c**) at position 8 (R_1). Phenyl substitution was found to be more energetically favorable than methyl substitution, with the phenyl derivative exhibiting a 1.97 kcal/mol advantage (Table 2).

By comparing the binding modes of compounds **19a** and **21c**, we observed that the naphthyridine core in **21c** appears to orient the

molecule more toward the inner region of the target protein. In contrast, **19a** remains oriented toward the more solvent-exposed region (Fig. 2c and d). This difference in binding orientation resulted in a more favorable binding free energy for **21c**, as indicated by its MM/GBSA score of -39.98 kcal/mol, compared to that of **19a**. Notably, the positioning of the phenyl ring of **21c** in proximity to Lys320 suggested an opportunity for further optimization through ring decoration, aimed at enhancing key protein-ligand interactions. Indeed, the introduction of a methoxy group in the *para* position of the 2-phenyl moiety further enhanced the interaction in terms of energy (**21b,d**).

Taking this into account, a focused library of thiazolo-naphthyridine derivatives **22a-j** was generated and prepared using LigPrep, introducing a variety of substituents (R_1 =F, Cl, CF_3 , OCH_3 , NO_2) at the 2', 3' and 4' positions of the phenyl ring in **21c**. All derivatives were docked into the SARS-CoV-2 Nsp13 binding site, and the top-scoring poses were subjected to further thermodynamic evaluation using the MM/GBSA method, following our established computational protocol. The energy analysis revealed that substitutions at the 2' position were the most favorable in terms of binding affinity (Table 2), orienting the synthetic route towards 2'-substituted derivatives. Additionally, the introduction of a *para* - OCH_3 group at the 2-phenyl moiety consistently improved the binding affinity.

Chart 2 outlines the systematic computational strategy used to prioritize both the [1,3]thiazolo[5,4-*b*]pyridine and [1,3]thiazolo[5,4-*h*][1,6]naphthyridine derivatives as potential Nsp13 inhibitors.

The combined use of GRID-based interaction mapping and ligand design tools represents a powerful strategy for lead optimization, as it enables the direct translation of spatial and energetic information from the binding site into rational chemical modifications. GRID methods, originally developed to compute MIF, provide a detailed three-dimensional description of favorable interaction regions within the receptor pocket, highlighting hydrophobic areas, hydrogen bond donor/acceptor sites, and charged regions. This allows the identification of previously unexploited “hotspots” and offers a predictive framework

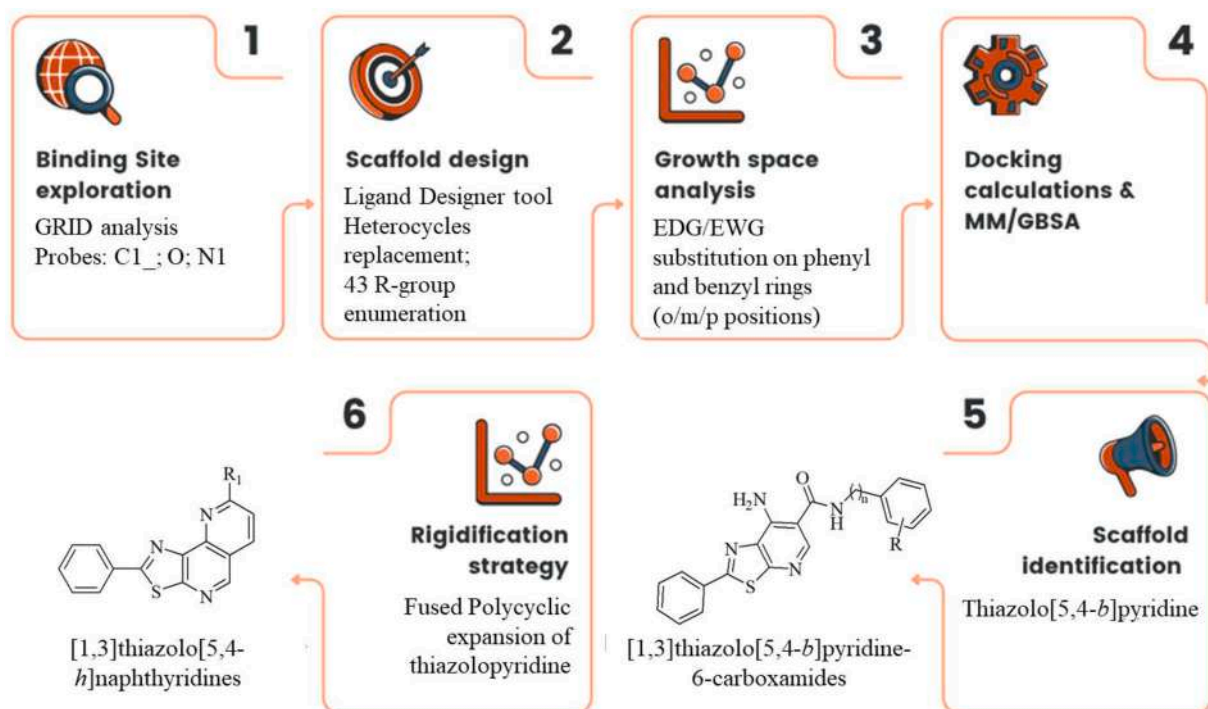


Chart 2. Computational workflow for the identification of [1,3]thiazolo[5,4-*b*]pyridine-6-carboxamide and [1,3]thiazolo[5,4-*h*][1,6]naphthyridine scaffolds against Nsp13.

indicating where specific functional groups can be advantageously introduced. Here, this computational protocol proved particularly valuable for identifying a favorable aromatic interaction region within the ATPase binding pocket. Exploitation of this aromatic hotspot guided the introduction of aromatic moieties into the ligand scaffold, ultimately leading to the development of the thiazolo-pyridine derivatives, as the promising chemotype.

In parallel, ligand design platforms, whether based on fragment growing, bioisosteric replacement, or scaffold optimization, leverage this information to generate chemically meaningful modifications. Importantly, these tools are not inherently tied to GRID methodologies, but when used in combination, they enable a workflow in which structural insights guide molecular generation in a hypothesis-driven manner. Rather than exploring chemical space randomly, the design process is constrained by the physicochemical features of the binding site, thereby increasing the likelihood of improving binding affinity and selectivity. As already mentioned, the generated compounds were prioritized through the combined evaluation of molecular docking and MM/GBSA binding free-energy analyses. Docking studies enabled the rapid identification of favorable binding poses and key interaction patterns within the ATPase pocket, whereas MM/GBSA calculations provided additional energetic support for estimating the relative stability of the protein–ligand complexes. The integration of these complementary computational approaches enabled a more reliable selection of compounds for synthesis. Although both methodologies rely on approximations and cannot fully account for long-timescale conformational dynamics, entropic contributions, or the complexity of the cellular environment, their combined application represents a powerful and cost-effective strategy for reducing the number of experimentally tested candidates and enriching the selection in structurally and energetically consistent hits.

2.2. Chemistry

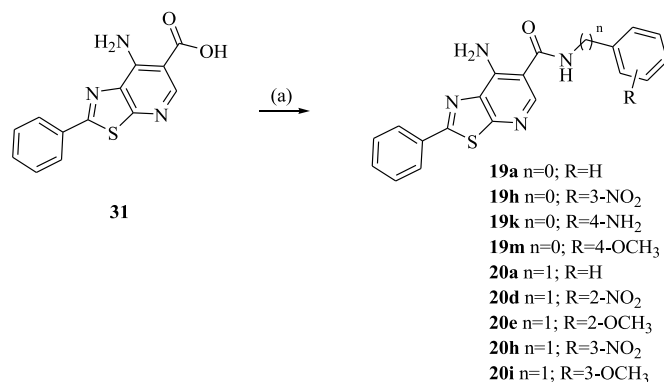
Leveraging our expertise in the synthesis of bioactive heterocyclic systems [22–25], we optimized a synthetic route to [1,3]thiazolo[5,4-*b*]pyridines starting from *N*-(2-amino-2-oxoethyl)benzamide derivatives **24a,b**. These precursors were prepared by reacting the appropriate benzoyl chlorides (**23a,b**) with glycine hydrochloride in the presence of triethylamine (Scheme 1). The obtained *N*-(2-amino-2-oxoethyl)benzamides **24a,b** have been converted in the corresponding thioamide derivatives **25a,b** using phosphorus pentasulfide in anhydrous pyridine.

This synthetic approach allowed us not only to afford the desired thioamides **25a,b** but also to isolate small amounts of 2-phenyl-1,3-thiazol-5-amino derivatives **26a,b**. The latter were obtained from moderate to good yields (30–60%) by cyclization of **25a,b** with phosphorous tribromide in anhydrous ethyl acetate, at room temperature (Scheme 1).

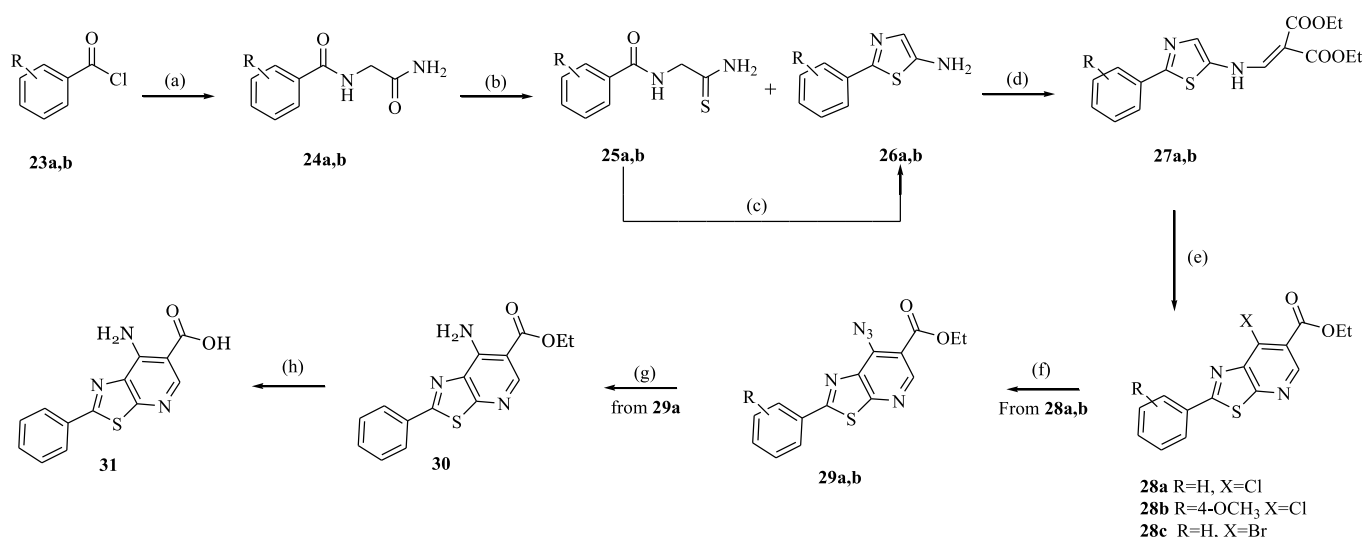
Condensation with diethyl ethoxymethylenemalonate (EMME) in anhydrous ethanol led to the diethyl {[2-phenyl-1,3-thiazol-5-yl]amino}methylidene}propanedioate intermediates **27a,b**, in turn subjected to an oxidative ring closure with phosphoryl chloride or bromide, resulting in the 7-chloro intermediates **28a,b** and 7-bromo intermediate **28c**, respectively (Scheme 1).

Nucleophilic substitution of **28a,b** with sodium azide in *N,N*-dimethylformamide (DMF) allowed to obtain the ethyl 7-azido-2-phenyl[1,3]thiazolo[5,4-*b*]pyridine-6-carboxylate derivatives **29a,b**. Furthermore, derivative **29a** has been subjected to selective reduction of the azido group with sodium dithionite to afford the desired product **30** in excellent yield (98%). Compound **30** was subsequently heated under reflux with sodium hydroxide 2 N in ethanol, allowing the isolation of the corresponding 7-amino-2-phenyl[1,3]thiazolo[5,4-*b*]pyridine-6-carboxylic acid **31** (Scheme 1).

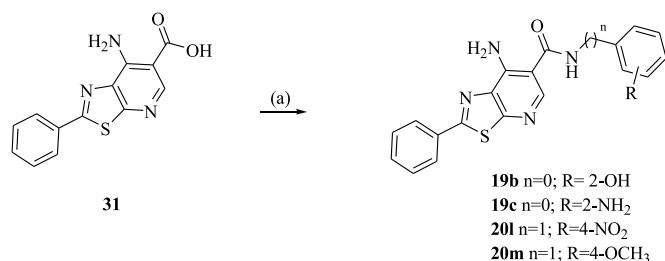
7-Amino-*N*,2-diphenyl[1,3]thiazolo[5,4-*b*]pyridine-6-carboxamides **19** and 7-amino-*N*-benzyl-2-phenyl[1,3]thiazolo[5,4-*b*]pyridine-6-carboxamides **20** were obtained by coupling the 7-amino-2-phenyl[1,3]thiazolo[5,4-*b*]pyridine-6-carboxylic acid **31** with the proper



Scheme 2. Reagents and conditions: (a) aniline or benzylamine, ACN, TCFH, NMI, r.t.



Scheme 1. Reagents and conditions: (a) glycine hydrochloride, TEA, DCM, r.t.; (b) P₂S₅, pyridine, reflux; (c) PBr₃, EtOAc, r.t.; (d) diethyl ethoxymethylenemalonate, EtOH, reflux; (e) POCl₃ or POBr₃, reflux; (f) NaN₃, DMF, r.t.; (g) Na₂O₄S₂, CH₃OH, reflux; (h) EtOH, NaOH 2 N, reflux.



Scheme 3. Reagents and conditions: (a) aniline or benzylamine, ACN, HOBT, EDC·HCl, DMAP, microwave irradiation (50 °C, 60 W, 40').

substituted anilines (for **19a,h,k,m,n**) or benzylamines (for **20a,d,e,h,i,n**) in acetonitrile and in the presence of *N,N,N',N'*-tetramethylchloroformamidinium hexafluorophosphate (TCFH) and *N*-methylimidazole (NMI). Unfortunately, when the reaction was performed with 2-nitroaniline, it was not possible to isolate the desired compound (**Scheme 2**).

Different experimental conditions were applied to afford the 7-amino-*N*,2-diphenyl[1,3]thiazolo[5,4-*b*]pyridine-6-carboxamides **19b,c** and 7-amino-*N*-benzyl-2-phenyl[1,3]thiazolo[5,4-*b*]pyridine-6-carboxamides **20l,m** obtained by reaction of **31** with 2-aminophenol (**19b**), *o*-phenylenediamine (**19c**), 4-nitrobenzylamine hydrochloride (**20l**) or 4-methoxybenzylamine (**20m**) respectively, in acetonitrile and in the presence of hydroxybenzotriazole hydrate (HOBT), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl) and 4-dimethylaminopyridine (DMAP), through a Microwave-Assisted Organic Synthesis (**Scheme 3**).

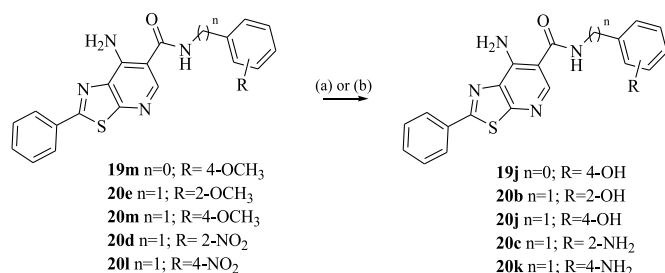
Demethylation reaction of **19m**, **20e** and **20m** with boron tribromide allowed us to obtain derivatives **19j**, **20b** and **20j** respectively, in excellent yields (100%) (**Scheme 4**).

Finally, reduction of the amino group of compounds **20d** and **20l** with tin(II) chloride in anhydrous methanol led to derivatives **20c** and **20k**, respectively (**Scheme 4**).

Furthermore, intermediates **29a,b** were subjected to a simultaneous reduction of both azido and ethoxycarbonyl groups with lithium aluminium hydride in anhydrous tetrahydrofuran (THF), at room temperature, affording the (7-amino-2-phenyl[1,3]thiazolo[5,4-*b*]pyridin-6-yl)methanol derivatives **32a,b**, which serve as versatile synthons for the synthesis of the tricyclic [1,3]thiazolo[5,4-*h*][1,6]naphthyridine system. Indeed, they were subjected to oxidation with Dess-Martin periodinane, thus leading to the corresponding key intermediate *o*-aminoaldehydes **33a,b**. [1,3]Thiazolo[5,4-*h*][1,6]naphthyridine derivatives **21** and **22** were achieved through Friedländer condensation of derivatives **33a,b** with acetone or properly substituted acetophenones, in basic media (**Scheme 5**).

2.3. Evaluation of the Nsp13 enzymatic profile

All the synthesized compounds were tested for their ability to block the catalytic activities of the SARS-CoV-2 Nsp13. The SARS-CoV-2



Scheme 4. Reagents and conditions: for **19m**, **20e** and **20m** (a) BBr₃, DCM, r.t.; for **20d** and **20l** (b) SnCl₂, CH₃OH, reflux.

Nsp13 unwinding-associated activity was performed by a fluorescence-based method in black 384-well plates with 1 nM purified Nsp13, 0.5 mM ATP and 500 nM annealed-Cy3/BHQ-2 labelled DNA substrate. The SARS-CoV-2 Nsp13 ATPase-Associated Activity was measured in a transparent 96-well plate with 25 nM purified Nsp13 and 250 μM ATP by quantifying the inorganic phosphate released with the Biomol Green Reagent. The half-maximal inhibitory concentration (IC₅₀) was determined for each compound (**Tables 3 and 4**), using compound **1b** from Corona et al. [26] as positive control of inhibition.

2.4. Structure activity relationship studies

Out of 32 tested compounds, 17 derivatives were identified as dual Nsp13 inhibitors (**Tables 3 and 4**). These include 15 derivatives from the [1,3]thiazolo[5,4-*b*]pyridine-6-carboxamide series **19** (**19a,c,h,j,k,m**) and **20** (**20a,b,d,e,h,i,j,l,m**) as well as the [1,3]thiazolo[5,4-*h*][1,6]naphthyridines **21d** and **22a**. Comparing the two classes of compounds (**19,20** vs **21,22**) clearly revealed that structural rigidification is detrimental to the activity. Specifically, the [1,3]thiazolo[5,4-*h*][1,6]naphthyridines displayed dual inhibitory activity at higher IC₅₀ values than derivatives **19** and **20**. In particular, **21d** and **22a** inhibited unwinding activity with IC₅₀ values of 16.90 and 26.13 μM, respectively, while their ATPase inhibition (IC₅₀ = 15.50 and 10.15 μM, respectively) was nearly comparable to the reference compound **1b** (7.2 μM). Moreover, three [1,3]thiazolo[5,4-*h*][1,6]naphthyridines (**22e**, **22h** and **22i**) were found to affect exclusively the Nsp13 unwinding activity at micromolar level (**Table 4**). Unfortunately compounds **21c** and **22g** were not soluble at the tested concentration.

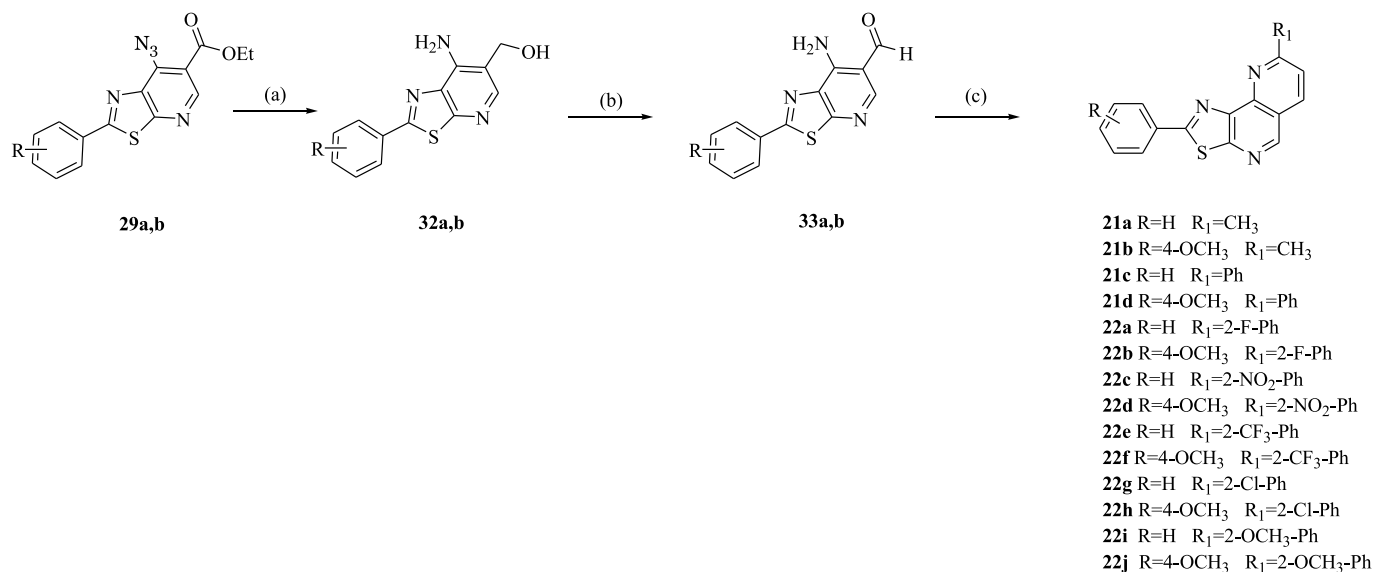
The most interesting results were obtained among the 7-amino-*N*-phenyl-[1,3]thiazolo[5,4-*b*]pyridine-6-carboxamides **19**, which demonstrated enhanced unwinding (IC₅₀ 3.30 – 23.13 μM) and ATPase activity (IC₅₀ 2.66 – 21.19 μM) compared to the *N*-benzyl-analogues **20** (unwinding: IC₅₀ 6.77 – 26.30 μM; ATPase: IC₅₀ 6.48 – 24.00 μM).

The most active compound proved to be **19h**, which bears a 3-nitro group on the benzamido moiety. With an unwinding activity of 3.30 μM and an ATPase inhibition of 2.87 μM, it exhibited dual inhibitory activity at lower IC₅₀ values than the reference compound **1b**. The second best activity was obtained with **19m**, which bears a 4-methoxy group on the benzamido moiety (unwinding: 6.83 μM; ATPase: 2.66 μM). Replacing the 4-methoxy group with a 4-amino substituent (**19k**) led to a further decrease in inhibitory potency, as evidenced by the higher IC₅₀ values observed for both ATPase and unwinding activities compared to **19m**; nevertheless, both **19m** and **19k** retained higher ATPase inhibitory potency than the reference compound **1b**. While the unsubstituted derivative **19a** retained micromolar unwinding activity (IC₅₀ = 7.62 μM), ATPase activity was negatively affected. Finally, moving the amino group from *para* position (**19k**) to the *ortho* position (**19c**) or replacing the 4-methoxy group with a hydroxyl group (**19j**), proved detrimental to both inhibitory activities (**Table 3**).

Elongation of the carbon chain from the carboxamide series **19** to the *N*-benzyl-analogues **20** did not yield an increase in dual inhibitory potency; with ATPase inhibitory activity generally exceeding the unwinding potency. Among these, the most active compound was **20i**, bearing a 3-methoxy group, with an unwinding activity of 9.14 μM and an ATPase inhibition of 6.48 μM, while the 3-nitro derivative **20h** (analogue of **19h**), displayed ATPase activity comparable to the reference compound **1b**. With the exception of the 2-methoxy derivative **20e**, which retained dual inhibitory activity at micromolar level (unwinding: IC₅₀ 9.69 μM; ATPase: IC₅₀ 9.38 μM), the introduction of 2-nitro or 2-hydroxyl groups resulted in reduced potency. Finally, moving the methoxy group to the position 4 (**20m**) or replacing it with a nitro (**20l**) or a hydroxyl group (**20j**), proved detrimental to the biological activity.

2.5. Kinetics of Nsp13 inhibition

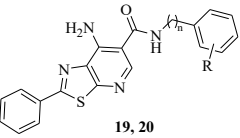
Among the most potent compounds, the methoxy substituted



Scheme 5. Reagents and conditions: (a) LiAlH₄, THF, 0–5 °C; (b) Dess-Martin periodinane, DCM, r.t.; (c) R₁COCH₃, KOH, EtOH, reflux.

Table 3

[1,3]Thiazolo[5,4-*b*]pyridine carboxamides **19** and **20**: structures and inhibition of SARS-CoV-2 Nsp13-associated enzymatic activities.

Series	Cpd	n	R	Unwinding IC ₅₀ (μM) ^a	ATPase IC ₅₀ (μM) ^b
	19a	0	H	7.62 ± 0.54	11.44 ± 1.49
	19b	0	2-OH	> 30 (54%) ^c	> 30 (70%) ^c
	19c	0	2-NH ₂	21.50 ± 1.6	16.40 ± 0.4
	19h	0	3-NO ₂	3.30 ± 0.65	2.87 ± 0.93
	19j	0	4-OH	23.13 ± 1.58	21.19 ± 5.13
	19k	0	4-NH ₂	7.97 ± 0.15	4.88 ± 0.01
	19m	0	4-OCH ₃	6.83 ± 1.50	2.66 ± 0.54
	20a	1	H	18.61 ± 3.30	12.22 ± 1.30
	20b	1	2-OH	14.23 ± 1.14	11.74 ± 1.58
	20c	1	2-NH ₂	> 30 (100%) ^c	> 30 (100%) ^c
	20d	1	2-NO ₂	23.99 ± 3.46	12.23 ± 0.63
	20e	1	2-OCH ₃	9.69 ± 0.88	9.38 ± 2.72
	20h	1	3-NO ₂	6.77 ± 1.40	9.28 ± 1.36
	20i	1	3-OCH ₃	9.14 ± 0.19	6.48 ± 0.59
	20j	1	4-OH	19.79 ± 0.19	10.94 ± 2.01
	20k	1	4-NH ₂	> 30 (63%) ^c	> 30 (52%) ^c
	20l	1	4-NO ₂	17.70 ± 0.1	14.10 ± 0.8
	20m	1	4-OCH ₃	26.30 ± 4.8	24.00 ± 3.5
	1b	---	---	5.2 ± 1.6	7.2 ± 1.4

^a Compound concentration required to inhibit the SARS-CoV-2 Nsp13-associated unwinding activity by 50%.

^b Compound concentration required to inhibit the SARS-CoV-2 Nsp13-associated ATPase activity by 50%.

^c Percentage of residual enzymatic activity measured in the presence of the highest compound concentration.

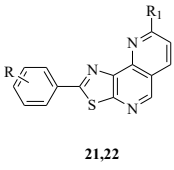
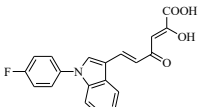
derivatives **19m** and **20i** and the nitro substituted derivative **19h**, were chosen to better characterize the mode-of-action. In order to confirm the predicted binding mode within the ATP binding pocket, we investigated the effect of the compound on the enzyme kinetics towards the ATP substrate on the ATPase activity by titrating the ATP in the reaction mixture in the absence or presence of an increasing amount of the selected compounds. The results, analyzed by a Lineweaver-Burk plot and reported in Fig. 3, showed an increase value for K_m along with a constant V_{max}, confirming that **19h**, **19m** and **20i** inhibited the enzyme by competing with the natural substrate ATP for its binding in the active site.

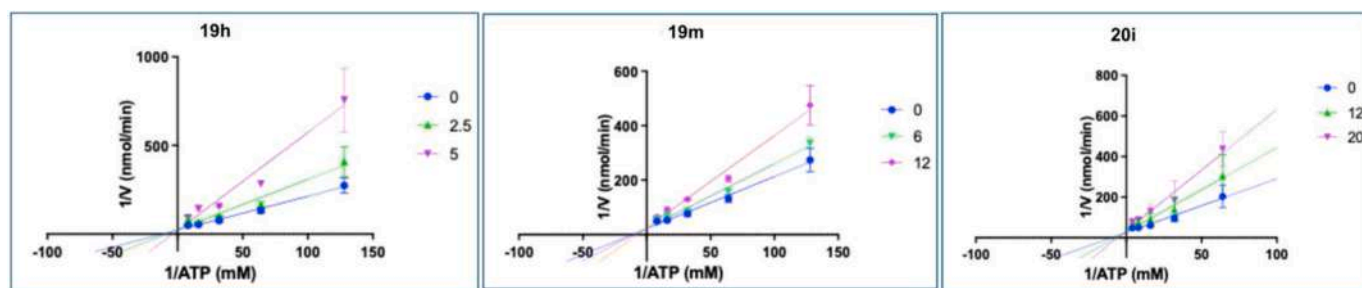
2.6. In silico metabolic profiling toward rational design of compounds **19n** and **20n**

In silico metabolic investigations of the most active compounds **19h**, **19m**, and **20i** were performed, as predicting sites of metabolism (SOM) provides insight into metabolic stability enabling a deeper evaluation of the pharmacokinetic properties.

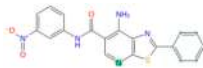
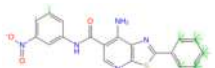
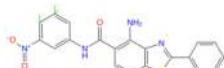
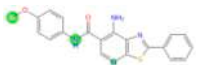
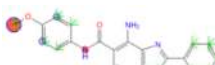
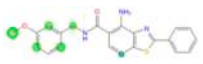
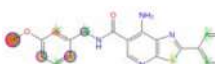
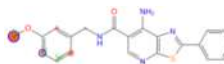
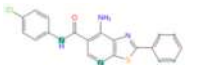
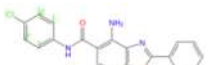
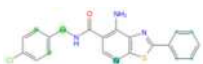
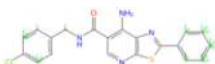
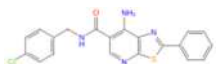
Since cytochrome P450 (CYP) enzymes play a central role in drug metabolism, the P450 SOM tool (Schrödinger Suite 2024-2) was employed to evaluate CYP3A4-, CYP2C9-, and CYP2D6-mediated metabolism for these compounds. For CYP2C9 and CYP2D6, predictions combined induced-fit docking, to assess accessibility to the enzyme reactive center, with a rule-based evaluation of intrinsic reactivity. For CYP3A4, which has a highly flexible binding site, only intrinsic reactivity was considered.

Table 4[1,3]Thiazolo[5,4-*h*][1,6]naphthyridines **21** and **22**: structures and inhibition of SARS-CoV-2 Nsp13-associated enzymatic activities.

Series	Cpd	R	R ₁	Unwinding IC ₅₀ (μM) ^a	ATPase IC ₅₀ (μM) ^b
 21,22	21a	H	CH ₃	> 30 (82%) ^c	> 30 (66%) ^c
	21b	4-OCH ₃	CH ₃	> 30 (91%) ^c	> 30 (66%) ^c
	21c	H	Ph	NS ^d	NS ^d
	21d	4-OCH ₃	Ph	16.90 ± 0.1	15.50 ± 3.8
	22a	H	2-F-Ph	26.13 ± 0.76	10.15 ± 0.71
	22b	4-OCH ₃	2-F-Ph	> 30 (78%) ^c	> 30 (53%) ^c
	22c	H	2-NO ₂ -Ph	> 30 (100%) ^c	> 30 (90%) ^c
	22d	4-OCH ₃	2-NO ₂ -Ph	> 30 (66%) ^c	> 30 (76%) ^c
	22e	H	2-CF ₃ -Ph	16.70 ± 1.2	> 30 (61%) ^c
	22f	4-OCH ₃	2-CF ₃ -Ph	> 30 (100%) ^c	> 30 (100%) ^c
	22g	H	2-Cl-Ph	NS ^d	NS ^d
	22h	4-OCH ₃	2-Cl-Ph	11.42 ± 2.69	> 30 (58%) ^c
	22i	H	2-OCH ₃ -Ph	19.30 ± 1.5	> 30 (52%) ^c
	22j	4-OCH ₃	2-OCH ₃ -Ph	> 30 (60%) ^c	> 30 (79%) ^c
 1b	1b	---	---	5.2 ± 1.6	7.2 ± 1.4

^a Compound concentration required to inhibit the SARS-CoV-2 Nsp13-associated unwinding activity by 50%.^b Compound concentration required to inhibit the SARS-CoV-2 Nsp13-associated ATPase activity by 50%.^c Percentage of residual enzymatic activity measured in the presence of the highest compound concentration.^d NS: Not soluble.**Fig. 3.** Lineweaver Burk analysis of the kinetics of the Nsp13-associated ATPase activity in the absence (blue line, DMSO) or presence of compounds **19h**, **19m**, **20i**.**Table 5**

Predicted P450 metabolism sites of the studied compounds using Schrödinger Suite 2024-2.

Cpd	CYP3A4 ^a	CYP2C9 ^b	CYP2D6 ^b
19h			
19m			
20i			
19n			
20n			

^a In each compound, atoms accessible and intrinsically reactive for CYP3A4 are marked with green circles, with radius proportional to the score (larger = higher reactivity).^b For CYP2C9 and CYP2D6, Fe-accessibility is shown as green rays from the atom. Each full ray = 1 unit, and partial rays indicate the decimal part of the score. Circles indicate overall score and intrinsic reactivity (radius = score; red intensity = reactivity; dark red = highest). A blue ring shows the atom passed CYP filtering; no ring = failed.

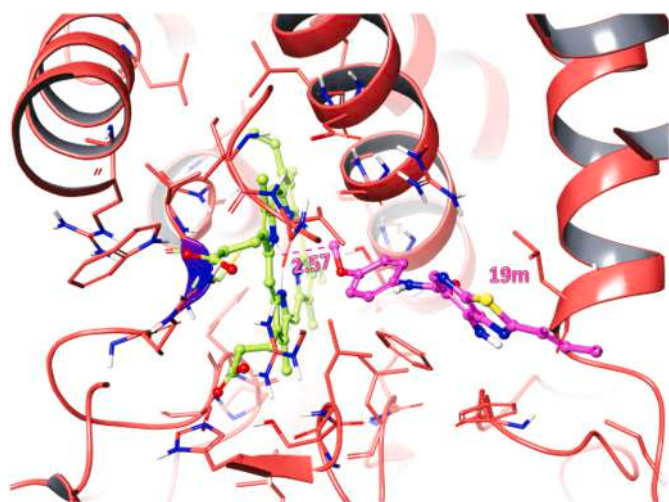


Fig. 4. P450SOM docked pose of compound **19m** (pink sticks) in the CYP2C9 binding site (salmon cartoon). Binding site residues are shown as lines, and the heme group is depicted as light green sticks. The distance between the metabolically susceptible atom on the phenyl ring and the iron center is indicated by a pink dashed line, expressed in Å.

The predicted metabolic profiles for the selected compounds are detailed in Table 5. Based on the calculations, compounds **19m** and **20i** are accommodated in the CYP2C9 and CYP2D6 binding site, with the methoxy substituent projecting toward the heme iron. The methoxy groups in both **19m** and **20i**, were found to lie at approximately 2.57 (Fig. 4) and 2.50 Å (Fig. 5a) from the heme iron, respectively, identifying these regions as the main metabolic soft spots. As shown in Fig. 5, compound **20i** is oriented toward the heme iron not only via the methoxy substituent but also through the *ortho* (Fig. 5c) and *para* (Fig. 5b) carbon atoms and the benzylic methylene ($-\text{CH}_2-$) (Fig. 5d). This suggests multiple possible sites of metabolic transformation mediated by the heme-containing enzyme.

In contrast, compound **19h** is not able to access the receptor binding sites nor establish interactions with the heme group.

Analysis of the predicted metabolic profiles of the most active compounds **19h**, **19m**, and **20i** revealed that the impact of substitution on metabolic stability depends on both the nature and the position of the substituent. Among the methoxylated derivatives, *meta* substitution (**20i**) showed a higher number of predicted soft spots compared to the *para* isomer **19m** (Table 5). In contrast, the *meta* nitro substitution in compound **19h** provided favorable metabolic stability, effectively deactivating the ring and thereby eliminating the need for further optimization. For the methoxylated derivatives, since the *para* substituted compound (**19m**) already exhibited fewer metabolic liabilities than its *meta* counterpart (**20i**), it was selected as the most strategic

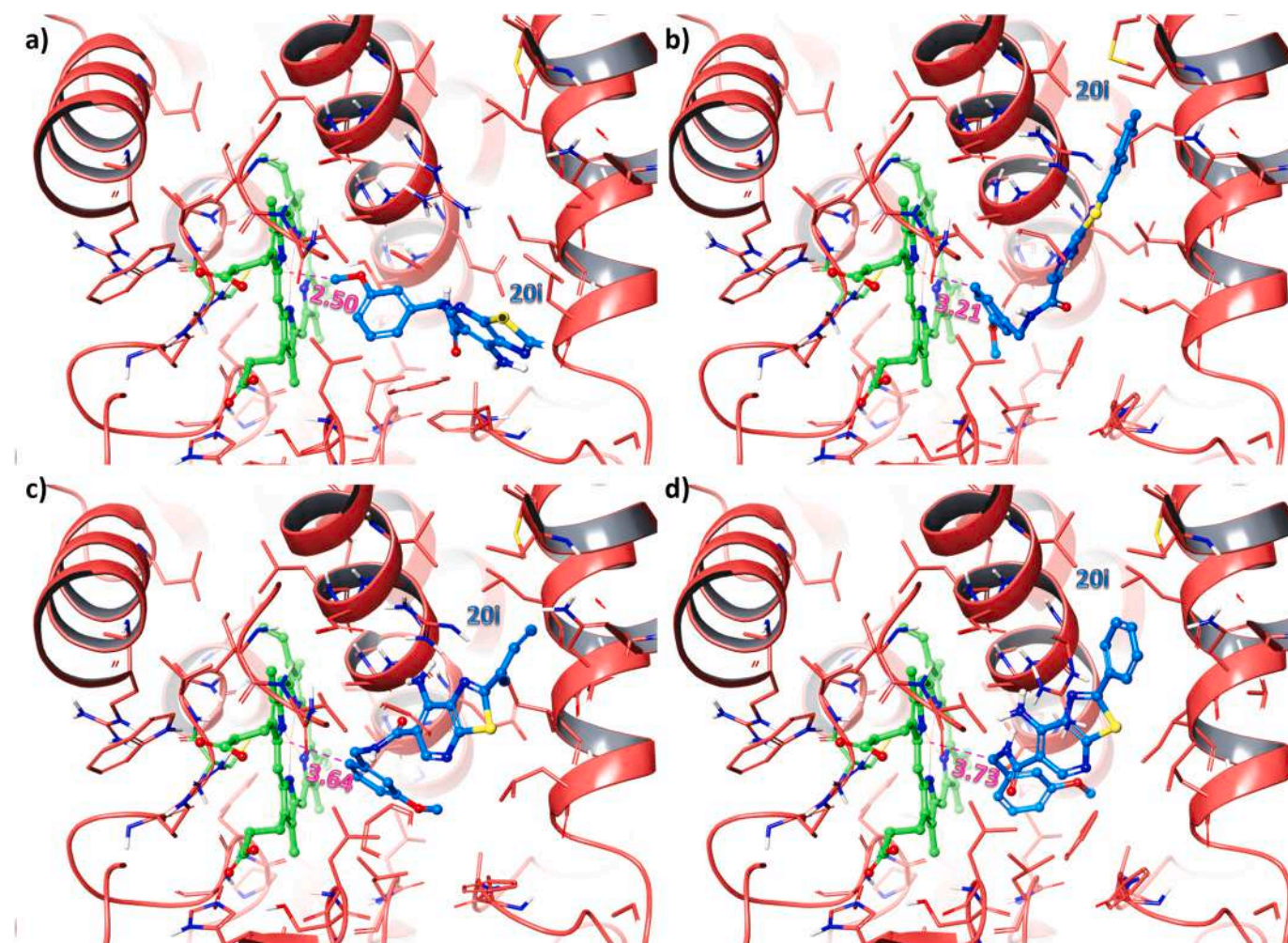


Fig. 5. P450SOM docked poses of compound **20i** (blue sticks) in the CYP2C9 binding site (salmon cartoon). Binding site residues are shown as lines, and the heme group is depicted as light green sticks. The distance between the metabolically susceptible atom on the phenyl ring and the iron center is indicated by a pink dashed line, expressed in Å.

starting point for further optimization. This choice was also supported by *in vitro* data, which indicated that replacing the methoxy group with a hydroxyl group in both the phenyl (**19**) and benzyl (**20**) series reduced ATPase activity. Crucially, the methoxy group is susceptible to metabolic O-demethylation, which would yield less active hydroxyl derivatives.

Given that the introduction of a chlorine atom is a well-established strategy for enhancing metabolic resistance in bioactive molecules [27], the *para* chloro substituted derivatives **19n** and **20n** were designed. *In silico* metabolic prediction confirmed a loss of potential soft spots and, consequently, a reduction of potential sites of metabolism compared to the methoxylated compounds (Table 5).

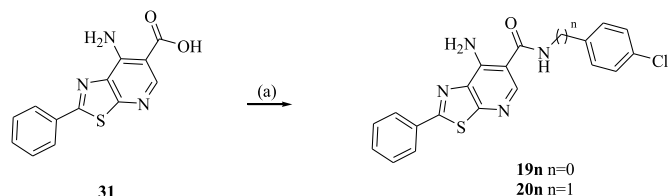
Subsequently, compounds **19n** and **20n** were docked into the binding site and their binding affinities were estimated using MM/GBSA calculations. Both compounds showed more favorable ΔG_{bind} values than **19h** and **19m**, with calculated values of -41.70 and -42.21 kcal/mol, respectively.

Based on the favorable prediction, compounds **19n** and **20n** were synthesized according to the procedure previously described (Scheme 6).

We subsequently evaluated both compounds for their ability to inhibit Nsp13-associated activities (Table 6). The introduction of a chlorine atom at the *para* position proved an effective strategy, as both designed compounds **19n** and **20n** exhibited enhanced inhibition of Nsp13-associated activity. Specifically, **19n** showed higher ATPase inhibitory activity ($IC_{50} = 2.39 \mu\text{M}$) than the reference compound **1b**, while its unwinding activity remained comparable to that of the most active derivative in the series, **19h**. Within the *N*-benzyl-derivatives, **20n** showed a slight increase in both the inhibition of Nsp13-mediated unwinding ($IC_{50} = 6.45 \mu\text{M}$) and ATPase activities ($IC_{50} = 4.78 \mu\text{M}$) with respect to derivative **20i**.

The binding modes of the most active compounds were comparatively analyzed by visualizing their key interactions within the enzyme active site. Previous structural studies on the SARS-CoV-2 Nsp13 ATPase pocket highlighted the critical role of residues His290, Arg442, Lys320/Lys323, and Glu261 in nucleotide recognition and catalytic stabilization, with His290 and Arg442 forming the conserved aromatic/cationic network involved in ATP binding [28]. Consistent with these observations, the most active compounds identified in the present study preserved key interactions within this catalytic region. Compound **19m** (Fig. 6b) displayed a well-defined binding pattern, establishing a π - π interaction with Arg442 and a cation- π interaction with His290. The carbonyl group formed a hydrogen bond with Lys323, while the thiazole-pyridine moiety engaged in an additional cation- π interaction with Lys320, contributing to the stabilization of the ligand within the binding site. Compounds **19h** and **19n** adopted a similar binding orientation (Fig. 6d), with the chlorine and nitro substituents directed toward Arg443. In particular, **19h** (Fig. 6a) showed a π - π interaction with Arg442 and a water-mediated bridge with Glu540. The nitro group was involved in salt bridge interactions with Lys288 and Arg443, while aromatic hydrogen bonds were observed with Gly538 and Glu540. Furthermore, the amide carbonyl formed a H-bond with Gly287.

Compound **19n** (Fig. 6c) exhibited a partially overlapping interaction profile, forming an aromatic hydrogen bond with Asp260, a residue located within the stalk/tower region implicated in helicase



Scheme 6. Reagents and conditions: (a) 4-Chloroaniline or 4-chlorobenzylamine, ACN, TCFH, NMI, r.t.

Table 6

Inhibition of SARS-CoV-2 Nsp13-associated enzymatic activities by compounds **19n** and **20n**.

Cpd	n	R	Unwinding IC_{50} (μM) ^a	ATPase IC_{50} (μM) ^b
19h	0	3-NO ₂	3.30 ± 0.65	2.87 ± 0.93
19n	0	4-Cl	3.33 ± 0.61	2.39 ± 0.35
19m	0	4-OCH ₃	6.83 ± 1.50	2.66 ± 0.54
20i	1	3-OCH ₃	9.14 ± 0.19	6.48 ± 0.59
20n	1	4-Cl	6.45 ± 0.28	4.78 ± 0.69
1b			5.2 ± 1.6	7.2 ± 1.4

^a Compound concentration required to inhibit the SARS-CoV-2 Nsp13-associated unwinding activity by 50%.

^b Compound concentration required to inhibit the SARS-CoV-2 Nsp13-associated ATPase activity by 50%.

conformational dynamics [29]. The thiazole-pyridine system interacted with Arg442 through both hydrogen bonding and cation- π interactions, whereas the carbonyl group engaged Lys320. Additionally, the chlorine substituent established a halogen bond with Arg443.

2.7. Cell-based assays

The cellular antiviral activity and cytotoxicity of **19h**, **19n**, **19m**, **20i** and **20n** were evaluated in cell-based assays (Table 7). The compounds were assessed against SARS-CoV-2 Omicron Strain BA.4 in VERO-E6 cells. The compounds proved non-toxic behavior in VERO-E6 cells, but only compound **20i** showed a detectable reduction of the viral cytopathic effect (CPE) with an EC_{50} of $61 \mu\text{M}$. Moreover, the compounds were tested for their cytopathic effect in HUH-7 cells confirming a non-toxic behavior, with the sole exception of compound **20i** that had a CC_{50} of $42.5 \mu\text{M}$.

3. Conclusions

Overall, by combining GRID-based interaction mapping and ligand design tools, we identified the thiazolo-pyridine derivatives as a promising scaffold for our *de novo* rational drug design in the antiviral field. A key advantage of this approach lies in its iterative nature: interaction field analysis informs ligand modification, which is subsequently evaluated through docking and rescoring methods, and further refined in successive cycles. This facilitates improved shape complementarity, optimization of directional interactions such as hydrogen bonds, and reduction of steric clashes or desolvation penalties. Compared to traditional medicinal chemistry strategies, this integrated computational framework significantly enhances the efficiency of local chemical space exploration, focusing efforts on high-value modifications.

Nevertheless, certain limitations must be considered. GRID calculations typically rely on a static representation of the protein and may not fully capture conformational flexibility, while ligand design tools can occasionally propose structures with limited synthetic feasibility. Despite these constraints, their complementary use provides a robust and rational basis for guiding lead optimization, particularly when integrated with downstream validation techniques such as molecular docking and MM/GBSA analysis. Indeed, our *in silico* protocol led to the synthesis of novel [1,3]thiazolo[5,4-*b*]pyridine-6-carboxamides, among which compounds **19h** and **19n** emerged as the most active compounds, exhibiting dual inhibitory activity against ATPase and unwinding functions of SARS-CoV-2 Nsp13, with IC_{50} values in the low micromolar range.

Kinetic studies further corroborated our computational model, confirming a competitive inhibition mechanism against ATP. Furthermore, *in silico* metabolic investigations provided deeper insights into the scaffold metabolic stability, guiding the refinement of the thiazolo-pyridine core. In particular, the application of advanced computational P450 metabolism site prediction tools enabled a rapid and high-throughput evaluation of the ligand series, allowing the identification

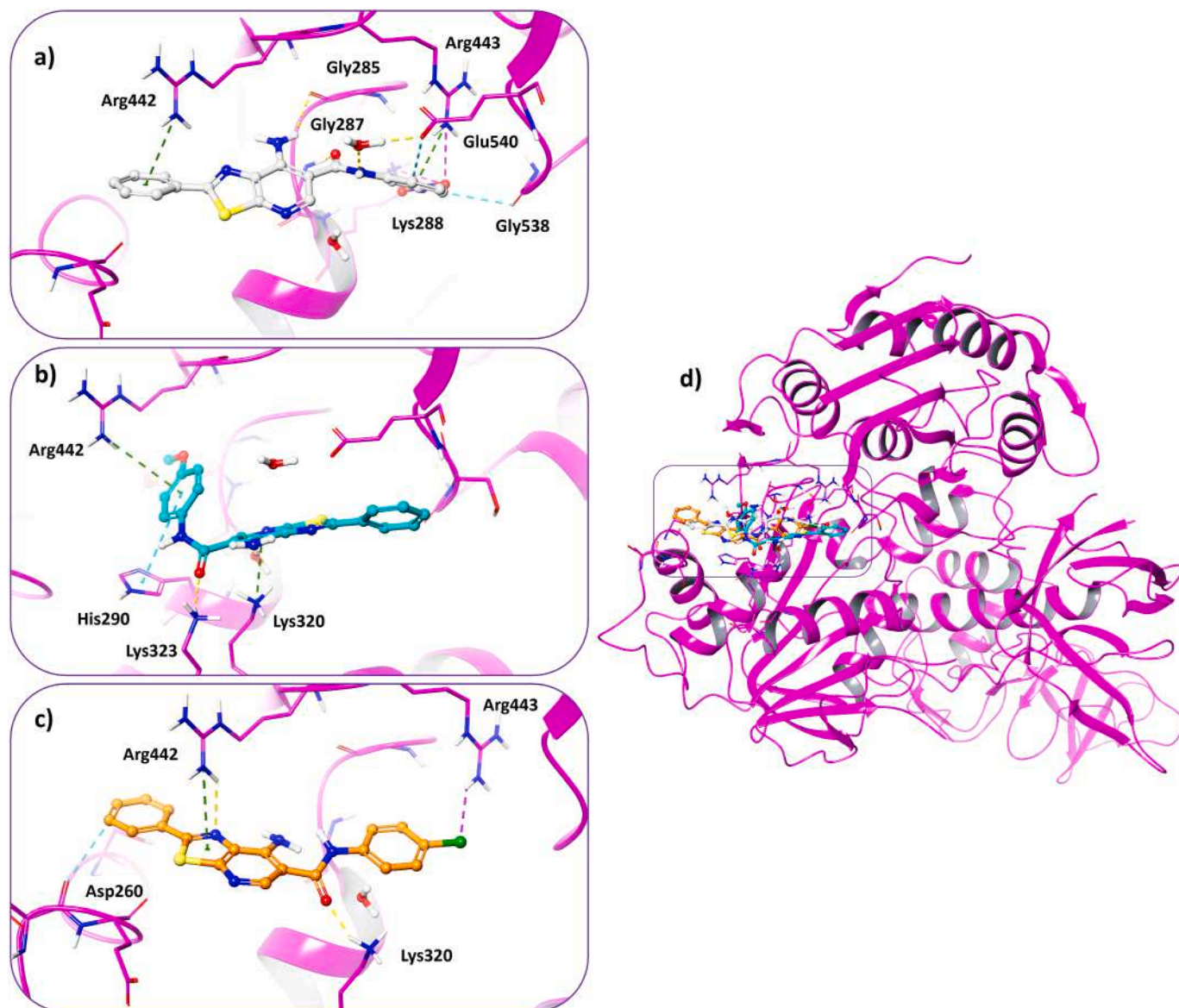


Fig. 6. Three-dimensional representation of the binding modes of **a) 19h**, **b) 19m**, and **c) 19n** within the Nsp13 binding pocket. The protein is depicted as a fuchsia cartoon, while the ligands are shown as grey, cyan, and orange sticks, respectively. Hydrogen bonds, cation- π interactions, and π - π interactions are represented as yellow, green, and blue dashed lines, respectively. Aromatic hydrogen bonds and halogen bonds are depicted as cyan and violet dashed lines, respectively. **d)** Superposition of the binding modes of compounds **19h**, **19m**, and **19n** within Nsp13, shown as a fuchsia cartoon.

of potential metabolic soft spots and liabilities at an early stage of the optimization process. By integrating structural, electronic, and steric determinants associated with CYP-mediated biotransformation, these computational approaches provide a powerful framework for anticipating metabolic behavior prior to experimental testing, substantially accelerating lead optimization while reducing time and resource consumption. Although the majority of compounds did not exhibit strong cellular antiviral activity, compound **20i** showed a moderate reduction in virus-induced CPE. At present, while these results support helicase inhibition as a promising strategy, future efforts focused on SAR insights will be essential to further optimize the thiazolo-pyridine class of compounds to identify the most promising chemical modifications to achieve optimal cellular potency and selectivity.

4. Experimental section

4.1. Computational methods

4.1.1. Target preparation

All calculations were performed using the Schrödinger Suite 2024-2. To run the molecular modelling, we used the crystal structure of the SARS-CoV-2 helicase in complex with 1-(2-methylphenyl)-1,2,3-triazole-4-carboxylic acid (Z2327226104), deposited in the Protein Data Bank (PDB) with the PDB code 7NNG. This structure is part of a series of deposited PDB entries derived from a crystallographic fragment screening performed by Newman et al., aiming at encouraging the rational drug design and structure-based optimization of novel Nsp13 inhibitors [9]. The Protein Preparation Wizard tool implemented in Maestro was used to prepare the receptor structure with OPLS_2005 as the force field [30,31]. Residual crystallographic buffer components, water molecules and phosphate ion were removed; hydrogens atoms were then added, side chain protonation states were calculated at pH

Table 7
Inhibition of SARS-CoV-2 induced cytopathic effect in Vero-E6 cells.

Cpd	n	R	SARS-CoV-2 EC ₅₀ (μM) ^a	Vero-E6 72 h CC ₅₀ (μM) ^b	HUH-7 72 h CC ₅₀ (μM) ^b
19h	0	3- NO ₂	>100	>100 (71%)	>100 (81%)
19n	0	4-Cl	>100	>100 (98%)	>100 (80%)
19m	0	4- OCH ₃	>100	>100 (73%)	>100 (90%)
20i	1	3- OCH ₃	61.04 ± 13.82	>100 (69%)	42.5 ± 12.0
20n	1	4-Cl	>100	>100 (86%)	>100 (76%)
GC376	---	---	0.28 ± 0.03	>100 (100%)	ND
Camptotecin	---	---	---	ND	43.4 ± 15.1

^a Compound concentration required to inhibit the SARS-CoV-2 viral induced CPE by 50%.

^b Compound concentration required to reduce cell viability by 50%.

7.4, and an energy minimization was carried out.

4.1.2. GRID

Starting from the prepared crystal structure of Nsp13, we applied the GRID software [32], a widely used computational method in the medicinal chemistry field. GRID program was able to run a mapping of the three-dimensional (3D) space surroundings the ligand into the nucleotide binding site by using the so called “probes”. The probes reproduced the chemical features of most common atom types and small moieties expected to find a good binder of that target. The selected probes were the following: CRY for lipophilic regions; C1 = for a sp² carbon atom of aromatics and olefins; N1 and O for a hydrogen bond donor and acceptor, respectively; CL, BR, and F that mimic organic chlorine, bromine, and fluorine atoms. Software settings were left by default, and the GRID Graphical User Interface (GUI) was used to display the computed isocontour maps.

4.1.3. Rational library design

All designed ligands were generated by means of the Ligand Designer tool [33] implemented in Maestro to enhance the binding affinity *versus* Nsp13 nucleotide and RNA accommodation sites. Firstly, we employed the experimental co-crystal fragment (1-(2-methylphenyl)-1,2,3-triazole-4-carboxylic acid) as starting point. The “Fill growth space” feature implemented in the Ligand Designer Panel provided us the possibility to identify empty spaces around the ligand to be filled with specific functional groups in order to enhance the ligand affinity and maximized the interaction with key residues into Nsp13 binding pocket.

ADME properties such as logP, polar surface area (PSA), hydrogen bond donor (HBD), hydrogen bond acceptor (HBA) and molecular weight (MW) were calculated, and Multi-Parameter Optimization (MPO) score was useful to select the best candidates for further modifications. MPO score provided an overall score between 0 (bad) and 1 (good) by taking the weighted geometric mean of the normalized scores of each considered property values. Through the Custom R-Group Enumeration tool [33], we generate different substitutions on the identified scaffold from a library of R-group fragments based on their synthetic feasibility.

All generated new ideas were submitted to molecular recognition calculations to evaluate their theoretical binding affinity towards Nsp13 by using Glide software v7.8. The grid box was generated by Receptor Grid Generation using OPLS_2005 as force field; it was centred onto the co-crystallized fragment and its size was 10 × 10 × 10 Å. The standard precision (SP) algorithm was employed, generating 10 poses per ligand. In order to assess the accuracy and reliability of the docking protocol, we performed re-docking calculations of the co-crystallized fragment by

means of the Glide software version [34]. Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) calculations were performed on the best docking poses of each compound obtained from the docking procedure to compute also the binding free energy (ΔG_{bind}) of the designed ligand complexed to Nsp13 nucleotide binding site [35,36].

4.1.4. P450 site of metabolism

Compounds **19h**, **19m**, **19n**, **20m**, **20i**, **20n** were evaluated for their potential sites of metabolism by P450 site of metabolism (P450 SOM) workflow (P450Site of Metabolism Prediction, version 1.1, Schrödinger, LLC, New York, NY, 2011) implemented in Schrödinger suite 2024-2. As reported in other studies [27], for each molecule, P450 SOM performed a calculation of intrinsic reactivity coupled to induced-fit docking (IFD) for the selected isoform of cytochrome. The reactivity rules have been parameterized in P450 SOM to predict atomic reactivity profiles for promiscuous P450 enzymes that are thought to be mostly independent of structural restrictions on the binding poses. The reactivity is predicted with a linear free energy approach based on the Hammett and Taft scheme, where the reactivity of a given atom is the sum of a baseline reactivity rate and a series of perturbations determined by the connectivity [37]. The induced-fit docking approach is a variation on the normal protocol. The initial sampling is enhanced by generating multiple starting conformations, so that a wider range of poses is found in the initial docking stage. The initial docking includes van der Waals scaling of the receptor and alanine mutation of the most flexible residues.

In the Prime refinement step, any residue with an atom within 5 Å of any ligand pose is selected for side-chain prediction. The subsequent minimization includes the ligand, side chains, and backbones of the flexible residues. The ligand is then redocked into each of the low energy protein conformations, determined by a 40 kcal/mol cut-off. There is no final scoring stage, because all poses are considered in determining which atoms are sufficiently accessible to the reactive heme iron. Any atom within the cut-off distance of 5 Å from the heme iron is considered as a potential site of metabolism [38].

4.1.5. Pan assay interference compounds evaluation

The pan assay interference compounds (PAINS) properties of the 19 active compounds were theoretically investigated using the ZINC PAINS Pattern Identifier web server. The analysis did not highlight any PAINS associated with the molecules investigated.

4.2. Chemistry

All melting points were taken on a Büchi melting point M – 560 apparatus. IR spectra were determined in bromoform with a Shimadzu FT/IR 8400S spectrophotometer. ¹H and ¹³C NMR spectra were recorded on a Bruker Avance II 200 (¹H: 200 MHz, ¹³C: 50.0 MHz), Bruker AC 300 (¹H: 300 MHz, ¹³C: 75 MHz), a Bruker Avance II 400 (¹H: 400 MHz, ¹³C: 100 MHz), in DMSO-*d*₆ or CDCl₃ solution using tetramethylsilane (TMS) as internal standard.

Column chromatography was performed with Merck silica gel (230–400 mesh ASTM) or a Büchi Pure FlashPrep C-850 Chromatography System. Elemental analyses (C, H, N) were within ±0.4% of theoretical values and were performed with a VARIO EL III elemental analyzer. The purity of all the tested compounds was >95%, determined by HPLC (Agilent 1100 series).

4.2.1. General procedure for the synthesis of N-(2-amino-2-oxoethyl) benzamides (**24a,b**)

To a stirred solution of glycine hydrochloride (7.96 mmol) in anhydrous dichloromethane (30 mL), triethylamine (4.4 mL, 31.50 mmol) was added dropwise at 0 °C. The reaction mixture was stirred for 15 min and then a solution of the proper benzoyl chloride **23a,b** in anhydrous dichloromethane (10 mL) was added at room temperature. After 16 h, the reaction mixture was washed with NaHCO₃

saturated aqueous solution and extracted with ethyl acetate. The organic layer was dried over sodium sulphate and the solvent was evaporated under reduced pressure to afford a solid which was recrystallized from dichloromethane.

4.2.1.1. *N*-(2-amino-2-oxoethyl)benzamide **24a.** This compound was obtained by reaction with benzoyl chloride **23a**. Mp: 185.1–186.3 °C; Yield: 88%; IR cm^{-1} : 4144 (NH), 4021 (NH), 3745 (OH), 1680 (CO); ^1H NMR DMSO- d_6 (ppm): 3.84 (2H, d, $J = 6.0$ Hz, CH_2), 7.10 (1H, s, NH), 7.39 (1H, s, OH), 7.42–7.56 (3H, m, H_3 , H_4 , H_5), 7.90 (2H, dd, $J = 8.3$ Hz, 1.4 Hz, H_2 , H_6), 8.68 (1H, t, $J = 6.0$ Hz, NH); ^{13}C NMR CDCl_3 (ppm): 42.4, 127.3 (2 x C), 128.2 (2 x C), 131.2, 134.1, 166.4, 171.0. Anal. Calcd for $\text{C}_9\text{H}_{10}\text{N}_2\text{O}_2$ (178.19): C, 60.66; H, 5.66; N, 15.72. Found: C, 60.91; H, 5.84; N, 15.57.

4.2.1.2. *N*-(2-amino-2-oxoethyl)-4-methoxybenzamide **24b.** This compound was obtained by reaction with 4-methoxy-benzoyl chloride **23b**. Mp: 205.3–206.0 °C; Yield: 82%; IR cm^{-1} : 3310 (NH), 3156 (NH), 3041 (OH), 1639 (CO); ^1H NMR DMSO- d_6 (ppm): 3.80 (2H, d, $J = 6.0$ Hz, CH_2), 3.81 (3H, s, CH_3O), 6.98–7.03 (3H, m, H_3 , H_5 , NH), 7.36 (1H, s, OH), 7.88 (2H, d, $J = 8.8$ Hz, H_2 , H_6), 8.53 (1H, t, $J = 6.0$ Hz, NH); ^{13}C NMR CDCl_3 (ppm): 45.2, 55.2, 113.4 (2 x C), 126.3, 129.3 (2 x C), 161.7, 165.9, 171.3. Anal. Calcd for $\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_3$ (208.21): C, 57.68; H, 5.81; N, 13.45. Found: C, 57.38; H, 5.93; N, 13.58.

4.2.2. General procedure for the synthesis of *N*-(2-amino-2-thioxoethyl) benzamides (**25a,b**)

To a solution of *N*-(2-amino-2-oxoethyl)benzamide derivatives **24a, b** (2.90 mmol) in anhydrous pyridine (10 mL) phosphorus pentasulfide (2.90 mmol) was added under nitrogen atmosphere at room temperature. The reaction mixture was heated under reflux for 6 h. After cooling, NaHCO_3 saturated aqueous solution was added and the mixture was extracted with ethyl acetate. The organic layer was dried over sodium sulphate and the solvent was removed under reduced pressure. The residue was purified by silica gel chromatography using dichloromethane/ethyl acetate (7:3) as eluent, allowing the isolation of the desired *N*-(2-amino-2-thioxoethyl)benzamide derivatives **25a,b**. From the same reaction mixture, it was possible to isolate also the 2-phenyl-1,3-thiazol-5-amine derivatives **26a,b** (yield: 30–38%).

4.2.2.1. *N*-(2-amino-2-thioxoethyl)benzamide **25a.** This compound was obtained by reaction of **24a**. Mp: 112.6–113.0 °C; Yield: 41%; IR cm^{-1} : 3463–3438 (NH_2), 3255 (NH), 1675 (CO); ^1H NMR CDCl_3 (ppm): 4.49 (2H, d, $J = 5.2$ Hz, CH_2), 7.39–7.63 (5H, m, H_3 , H_4 , H_5 , NH_2), 7.85 (2H, dd, $J = 8.0$ Hz, 1.8 Hz, H_2 , H_6), 8.38 (1H, bs, NH); ^{13}C NMR CDCl_3 (ppm): 49.7, 127.2 (2 x C), 129.0 (2 x C), 132.2, 136.5, 167.9, 204.1. Anal. Calcd for $\text{C}_9\text{H}_{10}\text{N}_2\text{OS}$ (194.25): C, 55.65; H, 5.19; N, 14.42. Found: C, 55.87; H, 5.30; N, 14.19.

4.2.2.2. *N*-(2-amino-2-thioxoethyl)-4-methoxybenzamide **25b.** This compound was obtained by reaction of **24b**. Mp: 167.8–168.1 °C; Yield: 45%; IR cm^{-1} : 3265 (2 x NH), 2557 (SH), 1646 (CO); ^1H NMR CDCl_3 (ppm): 3.75 (2H, d, $J = 6.0$ Hz, CH_2), 3.86 (3H, s, CH_3O), 6.95 (2H, d, $J = 8.0$ Hz, H_3 , H_5), 7.17 (1H, s, NH), 7.71 (1H, s, SH), 7.82 (2H, d, $J = 8.0$ Hz, H_2 , H_6), 8.25 (1H, s, NH). The poor solubility of compound **25b** prevented ^{13}C NMR spectra from being recorded. Anal. Calcd for $\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_2\text{S}$ (224.28): C, 53.55; H, 5.39; N, 12.49. Found: C, 53.61; H, 5.51; N, 12.38.

4.2.3. General procedure for the synthesis of 2-phenyl-1,3-thiazol-5-amines (**26a,b**)

To a solution of *N*-(2-amino-2-thioxoethyl)benzamide derivatives **25a,b** (2.47 mmol) in anhydrous ethyl acetate (12 mL), phosphorous tribromide (1.97 mmol) was added under nitrogen atmosphere and the reaction mixture was stirred at room temperature for 20 min.

Phosphorous tribromide (0.05 mL) has been further added and the reaction mixture was stirred at room temperature for 48 h. Ethyl acetate (50 mL) was added, and the mixture was washed with NaHCO_3 saturated aqueous solution (25 mL) and extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulphate and the solvent removed under reduced pressure. The solid obtained was purified by silica gel chromatography using dichloromethane/ethyl acetate (9:1) as eluent.

4.2.3.1. 2-Phenyl-1,3-thiazol-5-amines **26a.** This compound was obtained by reaction of **25a**. Yield: 67%; IR cm^{-1} : 3352–3298 (NH_2); ^1H NMR CDCl_3 (ppm): 3.79 (2H, s, NH_2), 7.09 (1H, s, H_4), 7.30–7.45 (3H, m, H_3 , H_4 , H_5), 7.78 (2H, dd, $J = 7.8$ Hz, 1.6 Hz, H_2 , H_6); ^{13}C NMR CDCl_3 (ppm): 125.5 (2 x C), 126.7, 128.8 (2 x C), 128.9, 134.1, 146.6, 156.3. Anal. Calcd for $\text{C}_9\text{H}_8\text{N}_2\text{S}$ (176.24): C, 61.34; H, 4.58; N, 15.90. Found: C, 61.44; H, 4.67; N, 15.97.

4.2.3.2. 2-(4-Methoxyphenyl)-1,3-thiazol-5-amine **26b.** This compound was obtained by reaction of **25b**. Mp: 89.5–89.8 °C; Yield: 55%; IR cm^{-1} : 3346–3301 (NH_2); ^1H NMR CDCl_3 (ppm): 3.76 (2H, s, NH_2), 3.83 (3H, s, CH_3O), 6.89 (2H, d, $J = 8.8$ Hz, H_3 , H_5), 7.04 (1H, s, H_4), 7.71 (2H, d, $J = 8.8$ Hz, H_2 , H_6); ^{13}C NMR CDCl_3 (ppm): 55.4, 114.2 (2 x C), 126.7, 127.0, 127.2 (2 x C), 145.7, 156.7, 160.4. Anal. Calcd for $\text{C}_{10}\text{H}_{10}\text{N}_2\text{OS}$ (206.26): C, 58.23; H, 4.89; N, 13.58. Found: C, 58.10; H, 5.06; N, 13.30.

4.2.4. General procedure for the synthesis of diethyl {[2-phenyl-1,3-thiazol-5-yl]amino} methylidene} propanedioates (**27a,b**)

To a solution of the suitable 2-phenylthiazol-5-amine **26a,b** (1.12 mmol) in anhydrous ethanol (10 mL), diethyl ethoxymethylenemalonate (1.12 mmol) was added and the reaction mixture was refluxed for 12 h. After cooling, the solvent was removed under reduced pressure and the residue was recrystallized from ethanol.

4.2.4.1. Diethyl {[2-phenyl-1,3-thiazol-5-yl]amino}methylidene} propanedioate **27a.** This compound was obtained by reaction of **26a**. Mp: 122.7–123.4 °C; Yield: 50%; IR cm^{-1} : 3390 (NH), 1720 (CO), 1707 (CO); ^1H NMR CDCl_3 (ppm): 1.33 (3H, t, $J = 7.2$ Hz, CH_3), 1.38 (3H, t, $J = 7.2$ Hz, CH_3), 4.21–4.33 (4H, m, 2 x CH_2), 7.39–7.48 (3H, m, H_3 , H_4 , H_5), 7.49 (1H, s, H_4), 7.80–7.87 (2H, m, H_2 , H_6), 8.19 (1H, d, $J = 12.8$ Hz, CH), 11.1 (1H, d, $J = 12.8$ Hz, NH); ^{13}C NMR CDCl_3 (ppm): 14.2, 14.4, 60.4, 60.8, 95.4, 126.0 (2 x C), 129.1 (2 x C), 130.2, 130.9, 133.2, 140.2, 154.5, 161.1, 164.9, 168.9. Anal. Calcd for $\text{C}_{17}\text{H}_{18}\text{N}_2\text{O}_4\text{S}$ (346.40): C, 58.94; H, 5.24; N, 8.09. Found: C, 58.85; H, 5.15; N, 8.25.

4.2.4.2. Diethyl {[2-(4-methoxyphenyl)-1,3-thiazol-5-yl]amino}methylidene} propanedioate **27b.** This compound was obtained by reaction of **26b**. Mp: 101.3–101.8 °C; Yield: 59%; IR cm^{-1} : 3392 (NH), 1721 (CO), 1710 (CO); ^1H NMR CDCl_3 (ppm): 1.32 (3H, t, $J = 7.0$ Hz, CH_3), 1.38 (3H, t, $J = 7.0$ Hz, CH_3), 3.85 (3H, s, CH_3O), 4.18–4.39 (4H, m, 2 x CH_2), 6.91 (2H, d, $J = 8.8$ Hz, H_3 , H_5), 7.43 (1H, s, H_4), 7.78 (2H, d, $J = 8.8$ Hz, H_2 , H_6), 8.15 (1H, d, $J = 12.8$ Hz, CH), 11.07 (1H, d, $J = 12.8$ Hz, NH); ^{13}C NMR CDCl_3 (ppm): 14.3, 14.4, 55.4, 60.3, 60.8, 95.2, 114.4 (2 x C), 126.1, 127.6 (2 x C), 130.8 (2 x C), 139.2, 154.7, 161.3, 164.9, 169.1. Anal. Calcd for $\text{C}_{18}\text{H}_{20}\text{N}_2\text{O}_5\text{S}$ (376.43): C, 57.43; H, 5.36; N, 7.44. Found: C, 57.15; H, 5.23; N, 7.19.

4.2.5. General procedure for the synthesis of ethyl 7-chloro-2-phenyl[1,3]thiazolo[5,4-*b*]pyridine-6-carboxylates (**28a,b**)

The proper diethyl {[2-phenyl-1,3-thiazol-5-yl]amino}methylidene} propanedioate **27a,b** (0.89 mmol) was dissolved in phosphorous oxychloride (48 mmol) and the reaction mixture was refluxed for 12 h. After cooling, the excess of phosphoryl chloride was removed under reduced pressure. The residue was poured onto crushed ice and neutralized with NaHCO_3 saturated aqueous solution. The formed solid was filtered off and dried.

4.2.5.1. Ethyl 7-chloro-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxylate **28a.** This compound was obtained by reaction of **27a**. Mp: 131.2–131.6 °C; Yield: 55%; IR cm^{-1} : 1727 (CO); ^1H NMR CDCl_3 (ppm): 1.47 (3H, t, $J = 7.3$ Hz, CH_3), 4.50 (2H, q, $J = 7.3$ Hz, CH_2), 7.48–7.61 (3H, m, H_3 , H_4 , H_5), 8.15 (2H, bs, H_2 , H_6), 8.98 (1H, s, H_5); ^{13}C NMR CDCl_3 (ppm): 14.3, 62.2, 124.0, 127.9 (2 x C), 129.3 (2 x C), 132.3, 132.8, 137.6, 145.5, 148.7, 161.2, 164.1, 170.2. Anal. Calcd for $\text{C}_{15}\text{H}_{11}\text{ClN}_2\text{O}_2\text{S}$ (318.78): C, 56.52; H, 3.48; N, 8.79. Found: C, 56.73; H, 3.59; N, 8.68.

4.2.5.2. Ethyl 7-chloro-2-(4-methoxyphenyl)[1,3]thiazolo[5,4-b]pyridine-6-carboxylate **28b.** This compound was obtained by reaction of **27b**. Mp: 138.7–138.9 °C; Yield: 77%; IR cm^{-1} : 1726 (CO); ^1H NMR CDCl_3 (ppm): 1.46 (3H, t, $J = 7.2$ Hz, CH_3), 3.90 (3H, s, CH_3O), 4.49 (2H, q, $J = 7.2$ Hz, CH_2), 7.00 (2H, d, $J = 8.8$ Hz, H_3 , H_5), 8.08 (2H, d, $J = 8.8$, H_2 , H_6), 8.93 (1H, s, H_5); ^{13}C NMR CDCl_3 (ppm): 14.3, 55.6, 62.1, 114.6 (2 x C), 123.9, 125.6, 129.7 (2 x C), 130.9, 145.6, 148.2, 161.3, 163.0, 164.2, 169.9. Anal. Calcd for $\text{C}_{16}\text{H}_{13}\text{ClN}_2\text{O}_3\text{S}$ (348.80): C, 55.09; H, 3.76; N, 8.03. Found: C, 55.21; H, 3.64; N, 8.24.

4.2.6. Procedure for the synthesis of ethyl 7-bromo-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxylate (**28c**)

A mixture of diethyl {[2-phenyl-1,3-thiazol-5-yl]amino}methylidene)propanedioate **27a** (0.3 mmol) and phosphorous oxybromide (0.9 mmol) was heated at 100 °C for 2 h. After cooling, the excess of phosphoric tribromide was removed under reduced pressure, the residue was neutralized with a 25% aqueous ammonium hydroxide and extracted with dichloromethane. The organic phase was dried over sodium sulphate and the solvent was removed under reduced pressure. The solid collected was purified by silica gel chromatography using dichloromethane as eluent. Mp: 136.4–137.2 °C; Yield: 31%; IR cm^{-1} : 1724 (CO); ^1H NMR CDCl_3 (ppm): 1.48 (3H, t, $J = 7.1$ Hz, CH_3), 4.51 (2H, q, $J = 7.1$ Hz, CH_2), 7.50–7.62 (3H, m, H_3 , H_4 , H_5), 8.11–8.18 (2H, m, H_2 , H_6), 8.88 (1H, s, H_5); ^{13}C NMR CDCl_3 (ppm): 14.2, 62.2, 126.5, 127.3, 127.9 (2 x C), 129.2 (2 x C), 132.2, 132.8, 147.0, 148.2, 148.8, 164.6, 170.0. Anal. Calcd for $\text{C}_{15}\text{H}_{11}\text{BrN}_2\text{O}_2\text{S}$ (363.23): C, 49.60; H, 3.05; N, 7.71. Found: C, 49.48; H, 3.22; N, 7.89.

4.2.7. General procedure for the synthesis of ethyl 7-azido-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxylates (**29a,b**)

To a suspension of the suitable ethyl 7-chloro-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxylate **28a,b** (0.69 mmol) in *N,N*-dimethylformamide (1.5 mL), sodium azide (0.69 mmol) was added at room temperature under nitrogen atmosphere. The reaction mixture was stirred overnight and then it was poured onto crushed ice. The formed solid was filtered off and dried to afford the desired compounds.

4.2.7.1. Ethyl 7-azido-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxylate **29a.** This compound was obtained by reaction of **28a**. Mp: 159.3–160.5 °C; Yield: 52%; IR cm^{-1} : 2120 (N_3), 1630 (CO); ^1H NMR CDCl_3 (ppm): 1.44 (3H, t, $J = 7.2$ Hz, CH_3), 4.44 (2H, q, $J = 7.2$ Hz, CH_2), 7.42–7.58 (3H, m, H_3 , H_4 , H_5), 8.03–8.07 (2H, m, H_2 , H_6), 8.90 (1H, s, H_5); ^{13}C NMR CDCl_3 (ppm): 14.3, 61.7, 115.0, 127.5 (2 x C), 129.3 (2 x C), 132.0, 132.7, 140.6, 141.4, 149.7, 161.9, 164.0, 167.5. Anal. Calcd for $\text{C}_{15}\text{H}_{11}\text{N}_5\text{O}_2\text{S}$ (325.35): C, 55.38; H, 3.41; N, 21.53. Found: C, 55.42; H, 3.55; N, 21.40.

4.2.7.2. Ethyl 7-azido-2-(4-methoxyphenyl)[1,3]thiazolo[5,4-b]pyridine-6-carboxylate **29b.** This compound was obtained by reaction of **28b**. Mp: 111.8–112.7 °C; Yield: 63%; IR cm^{-1} : 2117 (N_3), 1604 (CO); ^1H NMR CDCl_3 (ppm): 1.44 (3H, t, $J = 7.2$ Hz, CH_3), 3.90 (3H, s, CH_3O), 4.43 (2H, q, $J = 7.2$ Hz, CH_2), 7.03 (2H, d, $J = 8.8$ Hz, H_3 , H_5), 7.99 (2H, d, $J = 8.8$ Hz, H_2 , H_6), 8.86 (1H, s, H_5); ^{13}C NMR CDCl_3 (ppm): 14.3, 55.6, 66.7, 114.6 (2 x C), 115.1, 125.6, 129.3 (2 x C), 140.7, 140.8, 149.1, 161.9, 162.8, 164.1, 167.3. Anal. Calcd for $\text{C}_{16}\text{H}_{13}\text{N}_5\text{O}_3\text{S}$

(355.37): C, 54.08; H, 3.69; N, 19.71. Found: C, 54.37; H, 3.53; N, 20.02.

4.2.8. Procedure for the synthesis of ethyl 7-amino-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxylate (**30**)

To a solution of ethyl 7-azido-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxylate **29a** (0.3 mmol) in anhydrous methanol (10 mL), sodium dithionite (0.3 mmol) was added at room temperature and the reaction mixture was refluxed at 80 °C for an hour and 30 min. Sodium dithionite (0.3 mmol) was further added and the reaction mixture was heated at reflux for an hour and 30 min. After cooling, the mixture was poured onto crushed ice and the solid was filtered off, dried and recrystallized from ethanol [39]. Mp: 183.2–184.0 °C; Yield: 98%; IR cm^{-1} : 3332–3247 (NH_2), 1625 (CO); ^1H NMR CDCl_3 (ppm): 1.43 (3H, t, $J = 7.1$ Hz, CH_3), 4.40 (2H, q, $J = 7.1$ Hz, CH_2), 6.34 (1H, s, NH_2), 7.40–7.56 (3H, m, H_3 , H_4 , H_5), 8.01–8.03 (2H, m, H_2 , H_6), 8.85 (1H, s, H_5); ^{13}C NMR CDCl_3 (ppm): 14.3, 60.8, 104.5, 127.1 (2 x C), 129.0 (2 x C), 131.1, 133.4, 135.3, 149.4, 150.6, 160.0, 165.0, 167.7. Anal. Calcd for $\text{C}_{15}\text{H}_{13}\text{N}_3\text{O}_2\text{S}$ (299.35): C, 60.18; H, 4.38; N, 14.04. Found: C, 60.29; H, 4.22; N, 13.89.

4.2.9. Procedure for the synthesis of 7-amino-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxylic acid (**31**)

To a solution of ethyl 7-amino-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxylate **30** (0.18 mmol) in ethanol (10 mL), sodium hydroxide 2N (0.24 mL) was added and the reaction mixture was heated at reflux for 2 h. After cooling, the solvent was removed under reduced pressure, cold water was added and the crude was acidified to pH = 5 with acetic acid. The solid was filtered off and dried allowing the isolation of the desired pure product. Mp: 272.8–273.6 °C; Yield: 92%; IR cm^{-1} : 3331–3250 (NH_2), 3127 (OH), 1631 (CO); ^1H NMR $\text{DMSO}-d_6$ (ppm): 4.09 (1H, s, OH), 6.39 (2H, s, NH_2), 7.56–7.57 (3H, m, H_3 , H_4 , H_5), 8.07–8.09 (2H, m, H_2 , H_6), 8.70 (1H, s, H_5); ^{13}C NMR $\text{DMSO}-d_6$ (ppm): 104.3, 127.3 (2 x C), 129.8 (2 x C), 131.7, 133.4, 135.2, 149.9, 151.4, 159.4, 163.0, 169.8. Anal. Calcd for $\text{C}_{13}\text{H}_9\text{N}_3\text{O}_2\text{S}$ (271.29): C, 57.55; H, 3.34; N, 15.49. Found: C, 57.38; H, 3.51; N, 15.67.

4.2.10. General procedure for the synthesis of 7-amino-*N*,2-diphenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamides (**19a,h,k,m,n**) and 7-amino-*N*-benzyl-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamides (**20a,d,e,h,i,n**)

To a solution of 7-amino-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxylic acid **31** (0.37 mmol) in anhydrous acetonitrile (4 mL), the proper aniline (for **19a,h,k,m,n**) (0.48 mmol) or benzylamine (for **20a,d,e,h,i,n**) (0.44 mmol), *N,N,N',N'*-tetramethylchloroformamidinium hexafluorophosphate (TCFH, 0.44 mmol for **19a,h,k,m,n** and 0.41 mmol for **20a,d,e,h,i,n**) and *N*-methylimidazole (NMI, 1.30 mmol for **19a,h,k,m,n** and 0.78 mmol for **20a,d,e,h,i,n**) were added. The reaction mixture was stirred at room temperature until the complete depletion of the starting material (TLC). Then the reaction mixture was poured onto crushed ice and the solid was filtered off, dried and crystallized or purified by silica gel chromatography.

4.2.10.1. 7-amino-*N*,2-diphenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide **19a.** This compound was obtained by reaction of **31** with aniline after 12 h and it was crystallized from diethyl ether. Mp: 307.8–308.6 °C; Yield: 77%; IR cm^{-1} : 3398 (NH), 3387–3284 (NH_2), 1641 (CO); ^1H NMR $\text{DMSO}-d_6$ (ppm): 7.11 (1H, t, $J = 7.3$ Hz, ArH), 7.36 (2H, t, $J = 7.9$ Hz, ArH), 7.57–7.59 (3H, m, ArH), 7.74 (4H, d, $J = 7.6$ Hz, ArH, NH_2), 8.10–8.12 (2H, m, ArH), 8.75 (1H, s, H_5), 10.28 (1H, s, NH). ^{13}C NMR $\text{DMSO}-d_6$ (ppm): 109.1, 121.3 (2 x C), 121.8, 124.3, 127.4 (2 x C), 129.1 (2 x C), 129.9 (2 x C), 131.8, 133.4, 135.3, 139.3, 148.5, 149.1, 163.5, 166.7. Anal. Calcd for $\text{C}_{19}\text{H}_{14}\text{N}_4\text{OS}$ (346.41): C, 65.88; H, 4.07; N, 16.17. Found: C, 69.03; H, 3.95; N, 16.31.

4.2.10.2. 7-amino-*N*-(3-nitrophenyl)-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide **19h.** This compound was obtained by reaction of **31**

with 3-nitroaniline after 12 h and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (9:1) as eluent. Mp: 329.0–329.8 °C; Yield: 66%; IR cm^{-1} : 3412 (NH), 3374–3292 (NH_2), 1654 (CO), 1475 (NO_2); ^1H NMR DMSO- d_6 (ppm): 7.58–7.60 (3H, m, ArH), 7.67 (1H, t, $J = 8.2$ Hz, ArH), 7.81 (2H, s, NH_2), 7.98 (1H, d, $J = 8.3$ Hz, ArH), 8.11–8.14 (3H, m, ArH), 8.78–8.82 (2H, m, H_5 , ArH), 10.73 (1H, s, NH). ^{13}C NMR DMSO- d_6 (ppm): 108.5, 115.1, 118.7, 127.0, 127.4, 129.8, 130.5, 131.9, 133.2, 135.2, 140.5, 148.3, 148.6, 149.2, 159.2, 163.6, 167.2. Anal. Calcd for $\text{C}_{19}\text{H}_{13}\text{N}_5\text{O}_3\text{S}$ (391.40): C, 58.30; H, 3.35; N, 17.89. Found: C, 58.40; H, 3.22; N, 18.02.

4.2.10.3. 7-amino-N-(4-aminophenyl)-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 19k. This compound was obtained by reaction of **31** with *p*-phenylenediamine after 36 h and it was crystallized from diethyl ether. Mp: 299.0–299.8 °C; Yield: 64%; IR cm^{-1} : 3433 (NH), 3337–3255 (2 x NH_2), 1638 (CO); ^1H NMR DMSO- d_6 (ppm): 6.55 (2H, d, $J = 8.7$ Hz, ArH), 7.33 (2H, d, $J = 8.7$ Hz, ArH), 7.57–7.59 (4H, m, ArH, NH_2), 7.71–7.73 (3H, m, ArH, NH_2), 8.09–8.11 (2H, m, ArH), 8.70 (1H, s, H_5), 9.93 (1H, s, NH). ^{13}C NMR DMSO- d_6 (ppm): 109.4, 114.4, 123.2, 127.4, 128.3, 129.8, 131.8, 133.3, 135.2, 145.3, 148.2, 149.0, 158.7, 163.4, 166.0. Anal. Calcd for $\text{C}_{19}\text{H}_{15}\text{N}_5\text{O}_3\text{S}$ (361.42): C, 63.14; H, 4.18; N, 19.38. Found: C, 63.22; H, 4.02; N, 19.55.

4.2.10.4. 7-amino-N-(4-methoxyphenyl)-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 19m. This compound was obtained by reaction of **31** with *p*-anisidine after 12 h and it was crystallized from diethyl ether. Mp: 285.2–285.8 °C; Yield: 84%; IR cm^{-1} : 3389 (NH), 3307–3241 (NH_2), 1635 (CO); ^1H NMR DMSO- d_6 (ppm): 3.75 (3H, s, CH_3O), 6.94 (2H, d, $J = 8.9$ Hz, ArH), 7.57–7.59 (3H, m, ArH), 7.63 (2H, d, $J = 8.9$ Hz, ArH), 7.73 (2H, s, NH_2), 8.10 (2H, d, $J = 3.7$ Hz, ArH), 8.74 (1H, s, H_5), 10.17 (1H, s, NH). ^{13}C NMR DMSO- d_6 (ppm): 55.7, 109.1, 114.2 (2 x C), 123.0 (2 x C), 127.4 (2 x C), 129.8 (2 x C), 131.8, 132.2, 133.4, 135.3, 148.4, 149.1, 156.2, 158.9, 163.4, 166.4. Anal. Calcd for $\text{C}_{20}\text{H}_{16}\text{N}_4\text{O}_2\text{S}$ (376.43): C, 63.81; H, 4.28; N, 14.88. Found: C, 63.93; H, 4.15; N, 14.99.

4.2.10.5. 7-amino-N-(4-chlorophenyl)-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 19n. This compound was obtained by reaction of **31** with 4-chloroaniline after 4 h and it was crystallized from diethyl ether. Mp: 323.8–324.6 °C; Yield: 70%; IR cm^{-1} : 3402 (NH), 3323–3268 (NH_2), 1644 (CO); ^1H NMR DMSO- d_6 (ppm): 7.42–7.77 (9H, m, ArH, NH_2), 8.10 (2H, s, ArH), 8.74 (1H, s, H_5), 10.39 (1H, s, NH). ^{13}C NMR DMSO- d_6 (ppm): 108.9, 122.7 (2 x C), 127.4 (2 x C), 127.8, 129.0 (2 x C), 129.8 (2 x C), 131.9, 133.3, 135.2, 138.3, 148.5, 149.1, 159.0, 163.5, 166.8. Anal. Calcd for $\text{C}_{19}\text{H}_{13}\text{ClN}_4\text{O}_3\text{S}$ (380.85): C, 59.92; H, 3.44; N, 14.71. Found: C, 60.11; H, 3.31; N, 14.87.

4.2.10.6. 7-amino-N-benzyl-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 20a. This compound was obtained by reaction of **31** with benzylamine after 12 h and it was crystallized from diethyl ether. Mp: 277.2–278.0 °C; Yield: 88%; IR cm^{-1} : 3434 (NH), 3391–3302 (NH_2), 1653 (CO); ^1H NMR DMSO- d_6 (ppm): 4.50 (2H, s, CH_2), 7.26–7.36 (5H, m, ArH), 7.54–7.63 (3H, m, ArH), 7.85 (2H, s, NH_2), 8.09 (2H, s, ArH), 8.69 (1H, s, H_5), 9.13 (1H, s, NH). ^{13}C NMR DMSO- d_6 (ppm): 42.8, 108.2, 127.3, 127.4 (2 x C), 127.8 (2 x C), 128.8 (2 x C), 129.8 (2 x C), 131.8, 133.4, 135.4, 140.0, 148.0, 149.2, 153.2, 163.3, 167.9. Anal. Calcd for $\text{C}_{20}\text{H}_{16}\text{N}_4\text{O}_3\text{S}$ (360.43): C, 66.65; H, 4.47; N, 15.54. Found: C, 66.76; H, 4.32; N, 15.67.

4.2.10.7. 7-amino-N-[(2-nitrophenyl)methyl]-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 20d. This compound was obtained by reaction of **31** with (2-nitrophenyl)methanamine after 3 h and it was crystallized from diethyl ether. Mp: 268.0–268.8 °C; Yield: 83%; IR cm^{-1} : 3434 (NH), 3353–3264 (NH_2), 1641 (CO), 1498 (NO_2); ^1H NMR DMSO- d_6 (ppm): 4.77 (2H, s, CH_2), 7.57–7.81 (8H, m, ArH and NH_2), 8.09 (3H, s,

ArH), 8.71 (1H, s, H_5), 9.19 (1H, s, NH). The poor solubility of compound **20d** prevented ^{13}C NMR spectra from being recorded. Anal. Calcd for $\text{C}_{20}\text{H}_{15}\text{N}_5\text{O}_3\text{S}$ (405.43): C, 59.25; H, 3.73; N, 17.27. Found: C, 59.38; H, 3.61; N, 17.39.

4.2.10.8. 7-amino-N-[(2-methoxyphenyl)methyl]-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 20e. This compound was obtained by reaction of **31** with 2-methoxybenzylamine after 36 h and it was crystallized from diethyl ether. Mp: 265.0–265.7 °C; Yield: 86%; IR cm^{-1} : 3392 (NH), 3299–3234 (NH_2), 1639 (CO); ^1H NMR DMSO- d_6 (ppm): 3.83 (3H, s, CH_3O), 4.46 (2H, d, $J = 5.8$ Hz, CH_2), 6.92 (1H, t, $J = 7.4$ Hz, ArH), 7.0 (1H, d, $J = 7.9$ Hz, ArH), 7.22–7.26 (2H, m, ArH), 7.56–7.59 (3H, m, ArH), 7.82 (2H, s, NH_2), 8.07–8.10 (2H, m, ArH), 8.71 (1H, s, H_5), 8.94 (1H, d, $J = 5.8$ Hz, NH). ^{13}C NMR DMSO- d_6 (ppm): 37.8, 55.8, 108.3, 110.9, 120.6, 127.1, 127.3 (2 x C), 127.8, 128.4, 129.8 (2 x C), 131.8, 133.3, 135.3, 148.1, 149.2, 157.0, 158.8, 163.3, 168.0. Anal. Calcd for $\text{C}_{21}\text{H}_{18}\text{N}_4\text{O}_2\text{S}$ (390.46): C, 64.60; H, 4.65; N, 14.35. Found: C, 64.73; H, 4.52; N, 14.48.

4.2.10.9. 7-amino-N-[(3-nitrophenyl)methyl]-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 20h. This compound was obtained by reaction of **31** with (3-nitrophenyl)methanamine after 48 h and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (9:1) as eluent. Mp: 269.0–269.7 °C; Yield: 37%; IR cm^{-1} : 3418 (NH), 3364–3287 (NH_2), 1646 (CO), 1504 (NO_2); ^1H NMR DMSO- d_6 (ppm): 4.61 (2H, d, $J = 5.8$ Hz, CH_2), 7.56–7.59 (3H, m, ArH), 7.65 (1H, t, $J = 7.9$ Hz, ArH), 7.82–7.84 (3H, bd, NH_2 , ArH), 8.07–8.14 (3H, m, ArH), 8.22 (1H, t, $J = 1.8$ Hz, ArH), 8.70 (1H, s, H_5), 9.26 (1H, t, $J = 5.8$ Hz, NH). ^{13}C NMR DMSO- d_6 (ppm): 42.3, 107.9, 122.3 (2 x C), 127.3 (2 x C), 129.8 (2 x C), 130.4, 131.8, 133.3, 134.6, 135.3, 142.4, 148.0, 148.3, 149.2, 158.9, 163.4, 168.1. Anal. Calcd for $\text{C}_{20}\text{H}_{15}\text{N}_5\text{O}_3\text{S}$ (405.43): C, 59.25; H, 3.73; N, 17.27. Found: C, 59.39; H, 3.60; N, 17.42.

4.2.10.10. 7-amino-N-[(3-methoxyphenyl)methyl]-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 20i. This compound was obtained by reaction of **31** with 3-methoxybenzylamine after 12 h and it was crystallized from diethyl ether. Mp: 288.8–289.4 °C; Yield: 71%; IR cm^{-1} : 3443 (NH), 3367–3288 (NH_2), 1633 (CO); ^1H NMR DMSO- d_6 (ppm): 3.74 (3H, s, CH_3O), 4.46 (2H, d, $J = 4.7$ Hz, CH_2), 6.82 (1H, d, $J = 7.7$ Hz, ArH), 6.91 (2H, bs, ArH), 7.26 (1H, t, $J = 7.4$ Hz, ArH), 7.57 (3H, bs, ArH), 7.86 (2H, s, NH_2), 8.09 (2H, s, ArH), 8.68 (1H, s, H_5), 9.12 (1H, s, NH). ^{13}C NMR DMSO- d_6 (ppm): 42.7, 55.4, 108.2, 112.5, 113.4, 119.8, 127.3 (2 x C), 129.8 (2 x C), 129.9, 131.8, 133.3, 135.3, 141.6, 147.9, 149.2, 158.7, 159.7, 163.4, 167.9. Anal. Calcd for $\text{C}_{21}\text{H}_{18}\text{N}_4\text{O}_2\text{S}$ (390.46): C, 64.60; H, 4.65; N, 14.35. Found: C, 64.73; H, 4.51; N, 14.47.

4.2.10.11. 7-amino-N-[(4-chlorophenyl)methyl]-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 20n. This compound was obtained by reaction of **31** with 4-chlorobenzylamine after 18 h and it was crystallized from diethyl ether. Mp: 270.2–270.8 °C; Yield: 70%; IR cm^{-1} : 3419 (NH), 3374–3263 (NH_2), 1642 (CO); ^1H NMR DMSO- d_6 (ppm): 4.47 (2H, d, $J = 5.3$ Hz, CH_2), 7.39 (4H, bs, ArH), 7.58 (3H, bs, ArH), 7.84 (2H, s, NH_2), 8.09 (2H, s, ArH), 8.68 (1H, s, H_5), 9.14 (1H, s, NH). ^{13}C NMR DMSO- d_6 (ppm): 42.1, 108.0, 127.3 (2 x C), 128.7 (2 x C), 129.6 (2 x C), 129.8 (2 x C), 131.7, 131.8, 133.3, 135.3, 139.0, 148.0, 149.2, 158.8, 163.4, 167.9. Anal. Calcd for $\text{C}_{20}\text{H}_{15}\text{ClN}_4\text{O}_3\text{S}$ (394.88): C, 60.83; H, 3.83; N, 14.19. Found: C, 60.94; H, 3.69; N, 14.32.

4.2.11. General procedure for the synthesis of 7-amino-N,2-diphenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamides (19b,c) and 7-amino-N-benzyl-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamides (20l,m)

To a solution of 7-amino-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxylic acid **31** (0.37 mmol) in anhydrous acetonitrile (4 mL), the proper aniline (for **19b,c**) (0.56 mmol) or benzylamine (for **20l,m**), hydroxybenzotriazole hydrate (HOBt, 0.074 mmol), *N*-(3-

Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC-HCl, 0.74 mmol) and 4-dimethylaminopyridine (DMAP, 0.74 mmol) were added. The desired product was obtained through a Microwave-Assisted Organic Synthesis (MAOS) technique, irradiating the reaction for 40 min (2 cycles of 20 min each) with a power of 60 W and a temperature of 50 °C. After cooling, the reaction mixture was poured onto crushed ice and the formed solid was filtered off. The solid was dried and crystallized or purified by silica gel chromatography.

4.2.11.1. 7-Amino-N-(2-hydroxyphenyl)-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 19b. This compound was obtained by reaction of **31** with 2-aminophenol and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (95:5) as eluent. Mp: 311.2–312.0 °C; Yield: 15%; IR cm^{-1} : 3390 (NH), 3331–3250 (NH_2), 3127 (OH), 1631 (CO); ^1H NMR CDCl_3 (ppm): 3.77 (2H, s, NH_2), 6.49 (1H, s, OH), 6.83–6.90 (2H, m, ArH), 7.11–7.14 (2H, m, ArH), 7.52 (3H, bs, ArH), 7.92 (1H, s, ArH), 8.06–8.07 (2H, m, ArH), 9.13 (1H, s, NH). The poor solubility of compound **19b** prevented ^{13}C NMR spectra from being recorded. Anal. Calcd for $\text{C}_{19}\text{H}_{14}\text{N}_4\text{O}_2\text{S}$ (362.41): C, 62.97; H, 3.89; N, 15.46. Found: C, 62.79; H, 4.07; N, 15.31.

4.2.11.2. 7-Amino-N-(2-aminophenyl)-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 19c. This compound was obtained by reaction of **31** with *O*-phenylenediamine and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (95:5) as eluent. Mp: 319.6–320.4 °C; Yield: 40%; IR cm^{-1} : 3387 (NH), 3334–3253 (2 x NH_2), 1642 (CO); ^1H NMR DMSO- d_6 (ppm): 6.59 (1H, t, $J = 7.0$ Hz, ArH), 6.78 (1H, d, $J = 8.0$ Hz, ArH), 6.96–7.00 (1H, m, ArH), 7.16 (1H, d, $J = 7.7$ Hz, ArH), 7.58–7.59 (4H, m, ArH, NH_2), 7.76 (2H, s, NH_2), 8.09–8.12 (3H, m, ArH), 8.82 (1H, s, H_5), 9.67 (1H, s, NH). The poor solubility of compound **19c** prevented ^{13}C NMR spectra from being recorded. Anal. Calcd for $\text{C}_{19}\text{H}_{15}\text{N}_5\text{OS}$ (361.42): C, 63.14; H, 4.18; N, 19.38. Found: C, 63.33; H, 3.98; N, 19.26.

4.2.11.3. 7-Amino-N-[(4-nitrophenyl)methyl]-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 20l. This compound was obtained by reaction of **31** with 4-nitrobenzylamine hydrochloride and it was crystallized from dichloromethane. Mp: 259.2–260.0 °C; Yield: 71%; IR cm^{-1} : 3466–3398 (NH_2), 3344 (NH), 1664 (CO), 1482 (NO_2); ^1H NMR DMSO- d_6 (ppm): 4.61 (2H, d, $J = 5.8$ Hz, CH_2), 7.56–7.58 (3H, m, ArH), 7.62 (2H, d, $J = 8.7$ Hz, ArH), 7.84 (2H, s, NH_2), 8.04–8.10 (2H, m, ArH), 8.21 (2H, d, $J = 8.7$ Hz, ArH), 8.71 (1H, s, H_5), 9.27 (1H, t, $J = 5.8$ Hz, NH). ^{13}C NMR DMSO- d_6 (ppm): 42.5, 107.9, 124.0 (2 x C), 127.4 (2 x C), 128.7 (2 x C), 129.8 (2 x C), 131.8, 133.3, 135.4, 146.9, 148.1, 149.3, 158.9, 159.8, 163.4, 168.2. Anal. Calcd for $\text{C}_{20}\text{H}_{15}\text{N}_5\text{O}_3\text{S}$ (405.43): C, 59.25; H, 3.73; N, 17.27. Found: C, 59.40; H, 3.58; N, 17.13.

4.2.11.4. 7-Amino-N-[(4-methoxyphenyl)methyl]-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 20m. This compound was obtained by reaction of **31** with 4-methoxybenzylamine and it was crystallized from dichloromethane. Mp: 243.0–243.8 °C; Yield: 75%; IR cm^{-1} : 3460–3392 (NH_2), 3348 (NH), 1659 (CO); ^1H NMR DMSO- d_6 (ppm): 3.72 (3H, s, CH_3O), 4.42 (2H, d, $J = 5.0$ Hz, CH_2), 6.90 (2H, d, $J = 8.3$ Hz, ArH), 7.28 (2H, d, $J = 8.3$ Hz, ArH), 7.56 (3H, s, ArH), 7.84 (2H, s, NH_2), 8.08 (2H, s, ArH), 8.65 (1H, s, H_5), 9.06 (1H, t, $J = 5.0$ Hz, NH). ^{13}C NMR DMSO- d_6 (ppm): 42.3, 55.5, 108.3, 114.2 (2 x C), 127.3 (2 x C), 129.2 (2 x C), 129.8 (2 x C), 131.8, 131.9, 133.4, 135.4, 148.0, 149.2, 158.7, 158.8, 163.3, 167.8. Anal. Calcd for $\text{C}_{21}\text{H}_{18}\text{N}_4\text{O}_2\text{S}$ (390.46): C, 64.60; H, 4.65; N, 14.35. Found: C, 64.47; H, 4.81; N, 14.52.

4.2.12. General procedure for the synthesis of 7-amino-N,2-diphenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide (19j) and 7-amino-N-benzyl-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamides (20b,j)

To a suspension of the suitable 7-amino-N,2-diphenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide (for **19j**) or 7-amino-N-benzyl-2-phenyl

[1,3]thiazolo[5,4-b]pyridine-6-carboxamide (for **20b,j**) (0.13 mmol) in anhydrous dichloromethane (5 mL), BBr_3 (0.64 mmol) was added dropwise at 0 °C. The reaction mixture was stirred overnight at room temperature. Then the reaction mixture was poured onto crushed ice and the formed solid was filtered off, dried and crystallized from diethyl ether or dichloromethane.

4.2.12.1. 7-amino-N-(4-hydroxyphenyl)-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 19j. This compound was obtained by reaction of **19m** and it was crystallized from diethyl ether. Mp: 349.6–350.0 °C; Yield: 100%; IR cm^{-1} : 3411 (NH), 3387–3304 (NH_2), 3109 (OH), 1638 (CO); ^1H NMR DMSO- d_6 (ppm): 5.06 (2H, s, NH_2), 6.75–6.78 (2H, m, ArH), 7.47–7.51 (2H, m, ArH), 7.59–7.63 (3H, m, ArH), 8.12–8.17 (2H, m, ArH), 8.39 (1H, s, OH), 8.87 (1H, s, H_5), 10.24 (1H, s, NH). ^{13}C NMR DMSO- d_6 (ppm): 109.6, 115.5, 123.1, 127.6, 129.9, 130.4, 132.3, 132.7, 135.8, 145.2, 150.3, 154.5, 164.3, 165.0, 165.2. Anal. Calcd for $\text{C}_{19}\text{H}_{14}\text{N}_4\text{O}_2\text{S}$ (362.41): C, 62.97; H, 3.89; N, 15.46. Found: C, 62.79; H, 4.07; N, 15.31.

4.2.12.2. 7-amino-N-[(2-hydroxyphenyl)methyl]-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 20b. This compound was obtained by reaction of **20e** and it was crystallized from dichloromethane. Mp: 298.0–298.6 °C; Yield: 100%; IR cm^{-1} : 3432 (NH), 3378–3321 (NH_2), 3138 (OH), 1647 (CO); ^1H NMR DMSO- d_6 (ppm): 4.45 (2H, d, $J = 5.5$ Hz, CH_2), 6.44 (3H, s, OH, NH_2), 6.77 (1H, t, $J = 7.4$ Hz, ArH), 6.84 (1H, d, $J = 7.4$ Hz, ArH), 7.07–7.11 (1H, m, ArH), 7.21 (1H, d, $J = 7.6$ Hz, ArH), 7.58–7.65 (3H, m, ArH), 8.14–8.17 (2H, m, ArH), 8.91 (1H, s, H_5), 9.15 (1H, t, $J = 5.5$ Hz, NH). ^{13}C NMR DMSO- d_6 (ppm): 37.9, 108.4, 110.4, 115.4, 117.5, 119.3, 127.6 (2 x C), 127.9, 129.9 (2 x C), 130.9, 132.4, 132.5, 136.2, 143.7, 151.1, 154.7, 164.7, 166.6. Anal. Calcd for $\text{C}_{20}\text{H}_{16}\text{N}_4\text{O}_2\text{S}$ (376.43): C, 63.81; H, 4.28; N, 14.88. Found: C, 63.92; H, 4.15; N, 15.09.

4.2.12.3. 7-amino-N-[(4-hydroxyphenyl)methyl]-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 20j. This compound was obtained by reaction of **20m** and it was crystallized from diethyl ether. Mp: 276.2–276.8 °C; Yield: 100%; IR cm^{-1} : 3425 (NH), 3327–3279 (NH_2), 3097 (OH), 1634 (CO); ^1H NMR DMSO- d_6 (ppm): 4.38 (2H, d, $J = 5.7$ Hz, CH_2), 5.08 (2H, s, NH_2), 6.71–6.75 (2H, m, ArH), 7.15–7.19 (2H, m, ArH), 7.57–7.64 (3H, m, ArH), 8.11–8.16 (2H, m, ArH), 8.66 (1H, s, OH), 8.80 (1H, s, H_5), 9.17 (1H, t, $J = 5.7$ Hz, NH). ^{13}C NMR DMSO- d_6 (ppm): 42.5, 108.6, 115.5 (2 x C), 127.6 (2 x C), 129.3 (2 x C), 129.6 (2 x C), 129.9, 132.3, 132.6, 136.1, 144.2, 150.8, 152.5, 156.8, 164.5, 166.4. Anal. Calcd for $\text{C}_{20}\text{H}_{16}\text{N}_4\text{O}_2\text{S}$ (376.43): C, 63.81; H, 4.28; N, 14.88. Found: C, 63.67; H, 4.09; N, 15.12.

4.2.13. General procedure for the synthesis of 7-amino-N-[(2-aminophenyl)methyl]-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 20c and 7-amino-N-[(4-aminophenyl)methyl]-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 20k

To a solution of the suitable 7-amino-N-benzyl-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide **20d** or **20l** (0.12 mmol) in anhydrous methanol (5 mL), SnCl_2 (1.2 mmol) was added and the reaction mixture was refluxed for 4 h. After cooling, the solvent was removed under reduced pressure and the residue was poured onto crushed ice and neutralized to pH = 9 with Na_2CO_3 saturated aqueous solution. The formed solid was filtered off, dried and crystallized from diethyl ether or purified by silica gel chromatography.

4.2.13.1. 7-amino-N-[(2-aminophenyl)methyl]-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 20c. This compound was obtained by reaction of **20d** and it was crystallized from diethyl ether. Mp: >350 °C; Yield: 100%; IR cm^{-1} : 3383 (NH), 3302–3257 (2 x NH_2), 1646 (CO); ^1H NMR DMSO- d_6 (ppm): 4.32 (2H, t, $J = 5.9$ Hz, CH_2), 5.13 (2H, s, NH_2), 6.52 (1H, td, $J = 7.4, 1.2$ Hz, ArH), 6.63 (1H, dd, $J = 8.0, 1.1$ Hz, ArH),

6.94-6.98 (1H, m, ArH), 7.06 (1H, dd, $J = 7.4$, 1.2 Hz, ArH), 7.52-7.59 (3H, m, ArH), 7.82 (2H, s, NH_2), 8.07-8.11 (2H, m, ArH), 8.64 (1H, s, H_5), 8.98 (1H, t, $J = 5.9$ Hz, NH). ^{13}C NMR DMSO- d_6 (ppm): 42.1, 108.2, 115.1, 116.3, 122.4, 127.3 (2 x C), 128.3, 129.5, 129.8 (2 x C), 131.6, 131.8, 133.3, 138.1, 146.6, 147.9, 149.1, 158.7, 168.0. Anal. Calcd for $\text{C}_{20}\text{H}_{17}\text{N}_5\text{OS}$ (375.45): C, 63.98; H, 4.56; N, 18.65. Found: C, 64.14; H, 4.39; N, 18.79.

4.2.13.2. 7-amino-N-[(4-aminophenyl)methyl]-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxamide 20k. This compound was obtained by reaction of **20l** and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (9:1) as eluent. Mp: 265.0-265.5 °C; Yield: 51%; IR cm^{-1} : 3405 (NH), 3351-3278 (2 x NH_2), 1642 (CO); ^1H NMR DMSO- d_6 (ppm): 4.30 (2H, d, $J = 5.7$ Hz, CH_2), 4.95 (2H, s, NH_2), 6.52 (2H, d, $J = 8.3$ Hz, ArH), 7.01 (2H, d, $J = 8.3$ Hz, ArH), 7.56-7.58 (3H, m, ArH), 7.83 (2H, s, NH_2), 8.07-8.10 (2H, m, ArH), 8.62 (1H, s, H_5), 8.93 (1H, t, $J = 5.7$ Hz, NH). ^{13}C NMR DMSO- d_6 (ppm): 42.5, 108.4, 114.2 (2 x C), 126.8, 127.3 (2 x C), 128.8 (2 x C), 129.8 (2 x C), 131.8, 133.3, 135.3, 147.9, 148.0, 149.1, 158.6, 163.3, 167.6. Anal. Calcd for $\text{C}_{20}\text{H}_{17}\text{N}_5\text{OS}$ (375.45): C, 63.98; H, 4.56; N, 18.65. Found: C, 64.79; H, 4.69; N, 18.57.

4.2.14. General procedure for the synthesis of (7-amino-2-phenyl[1,3]thiazolo[5,4-b]pyridin-6-yl)methanols 32a,b

To a suspension of lithium aluminium hydride (2.11 mmol) in tetrahydrofuran (10 mL), the suitable ethyl 7-azido-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carboxylate **29a,b** (0.64 mmol) was added at 0 °C and the suspension was stirred for 16 h at room temperature. Then the reaction mixture was poured onto crushed ice and the formed solid was filtered off, dried and recrystallized from diethyl ether.

4.2.14.1. [7-amino-2-phenyl[1,3]thiazolo[5,4-b]pyridin-6-yl]methanol 32a. This compound was obtained by reaction of **29a**. Mp: 180.8-180.9 °C; Yield: 52%; IR cm^{-1} : 3590 (OH), 3450-3358 (NH_2); ^1H NMR DMSO- d_6 (ppm): 4.56 (2H, d, $J = 5.2$ Hz, CH_2), 5.17 (1H, t, $J = 5.2$ Hz, OH), 6.58 (2H, bs, NH_2), 7.53-7.63 (3H, m, H_3 , H_4 , H_5), 8.04-8.15 (3H, m, H_5 , H_2 , H_6); ^{13}C NMR CDCl_3 (ppm): 61.5, 104.5, 127.1 (2 x C), 129.1 (2 x C), 130.9, 132.2, 132.4, 146.9, 149.5, 158.0, 167.8. Anal. Calcd for $\text{C}_{13}\text{H}_{11}\text{N}_3\text{OS}$ (257.31): C, 60.68; H, 4.31; N, 16.33. Found: C, 60.84; H, 4.47; N, 16.45.

4.2.14.2. [7-amino-2-(4-methoxyphenyl)[1,3]thiazolo[5,4-b]pyridin-6-yl]methanol 32b. This compound was obtained by reaction of **29b**. Mp: 179.9-180.1 °C; Yield: 63%; IR cm^{-1} : 3480 (OH), 3320-3240 (NH_2); ^1H NMR DMSO- d_6 (ppm): 3.85 (CH_3O), 4.55 (2H, bs, CH_2), 5.14 (1H, bs, OH), 6.46 (2H, bs, NH_2), 7.12 (2H, d, $J = 8.0$ Hz, H_3 , H_5), 8.03 (3H, s, H_2 , H_6 , H_5); ^{13}C NMR DMSO- d_6 (ppm): 55.5, 58.3, 114.6 (2 x C), 117.0, 126.0, 128.4 (2 x C), 134.4, 146.0, 146.8, 156.4, 161.5, 161.6. Anal. Calcd for $\text{C}_{14}\text{H}_{13}\text{N}_3\text{O}_2\text{S}$ (287.34): C, 58.52; H, 4.56; N, 14.62. Found: C, 58.32; H, 4.70; N, 14.50.

4.2.15. General procedure for the synthesis of 7-amino-2-phenyl[1,3]thiazolo[5,4-b]pyridine-6-carbaldehydes (33a,b)

To a solution of (7-amino-2-phenyl[1,3]thiazolo[5,4-b]pyridin-6-yl)methanol derivative **32a,b** (1.74 mmol) in anhydrous dichloromethane (20 mL), Dess-Martin periodinane (2.44 mmol) was added under nitrogen atmosphere and at room temperature. The reaction mixture was stirred for 48 h and then diethyl ether (20 mL) was added. The organic phase was washed with a 5% solution of sodium thiosulfate (20 mL) and a NaHCO_3 saturated aqueous solution (20 mL) and subsequently extracted with ethyl acetate. The organic phase was dried over sodium sulphate and the solvent was evaporated under reduced pressure. The obtained solid was used in the next step without further purifications.

4.2.16. General procedure for the synthesis of 2-phenyl[1,3]thiazolo[5,4-h][1,6]naphthyridines 21a-d, 22a-j

To the suspension of 7-amino-2-phenylthiazolo[5,4-b]pyridine-6-carbaldehyde derivative **33a,b** (0.5 mmol) and potassium hydroxide (0.6 mmol) in anhydrous ethanol (20 mL), the proper substituted acetophenone (0.5 mmol) was added. To obtain derivatives **21a,b**, o-aminoaldehydes **33a,b** (0.5 mmol) were added to a solution of potassium hydroxide (0.6 mmol) in acetone (20 mL). The reaction mixture was refluxed under nitrogen atmosphere for 12 h. After cooling, the solvent was removed under reduced pressure and the residue was purified by silica gel chromatography.

4.2.16.1. 8-Methyl-2-phenyl[1,3]thiazolo[5,4-h][1,6]naphthyridine 21a.

The product was obtained by reaction of **33a** with acetone and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (95:5) as eluent. Mp: 181.3-182.5 °C; Yield: 22%; ^1H NMR DMSO- d_6 (ppm): 2.82 (3H, s, CH_3), 7.59-7.63 (3H, m, ArH), 7.70 (1H, d, $J = 8.4$ Hz, ArH), 8.16-8.21 (2H, m, ArH), 8.61 (1H, d, $J = 8.4$ Hz, ArH), 9.30 (1H, s, H_5); ^{13}C NMR DMSO- d_6 (ppm): 26.0, 121.2, 124.1, 127.7 (2 x C), 129.9 (2 x C), 132.1, 133.5, 137.3, 151.5, 155.3, 158.9, 161.1, 165.2, 166.6. Anal. Calcd for $\text{C}_{16}\text{H}_{11}\text{N}_3\text{S}$ (277.34): C, 69.29; H, 4.00; N, 15.15. Found: C, 69.40; H, 4.15; N, 14.83.

4.2.16.2. 2-(4-Methoxyphenyl)-8-methyl[1,3]thiazolo[5,4-h][1,6]naphthyridine 21b. The product was obtained by reaction of **33b** with acetone and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (95:5) as eluent. Mp: 191.0-191.7 °C; Yield: 25%; ^1H NMR CDCl_3 (ppm): 2.97 (3H, s, CH_3), 3.91 (3H, s, CH_3O), 7.04 (2H, d, $J = 8.7$ Hz, ArH), 7.53 (1H, d, $J = 8.4$ Hz, H_7), 8.22-8.33 (3H, m, ArH), 9.07 (1H, s, H_5); ^{13}C NMR CDCl_3 (ppm): 26.3, 55.5, 114.2 (2 x C), 120.6, 123.2, 126.3, 129.5 (2 x C), 136.2, 142.4, 144.5, 149.4, 155.4, 162.2, 165.0, 168.1. Anal. Calcd for $\text{C}_{17}\text{H}_{13}\text{N}_3\text{OS}$ (307.37): C, 66.43; H, 4.26; N, 13.67. Found: C, 66.57; H, 4.12; N, 16.79.

4.2.16.3. 2,8-Diphenyl[1,3]thiazolo[5,4-h][1,6]naphthyridine 21c. The product was obtained by reaction of **33a** with acetophenone and was crystallized from ethanol and diethyl ether. Mp: 194.8-195.1 °C; Yield: 44%; ^1H NMR CDCl_3 (ppm): 7.52-7.59 (6H, m, ArH), 8.11 (1H, d, $J = 8.6$ Hz, ArH), 8.27-8.40 (4H, m, ArH), 8.47 (1H, d, $J = 8.7$ Hz, ArH), 9.15 (1H, s, H_5); ^{13}C NMR CDCl_3 (ppm): 120.0, 121.2, 127.8 (2 x C), 128.3 (2 x C), 128.9 (2 x C), 129.0 (2 x C), 130.6, 131.3, 133.6, 137.1, 138.5, 142.7, 145.1, 149.9, 155.9, 161.9, 168.1. Anal. Calcd for $\text{C}_{21}\text{H}_{13}\text{N}_3\text{S}$ (339.41): C, 74.31; H, 3.86; N, 12.38. Found: C, 74.01; H, 4.00; N, 12.65.

4.2.16.4. 2-(4-Methoxyphenyl)-8-phenyl[1,3]thiazolo[5,4-h][1,6]naphthyridine 21d. The product was obtained by reaction of **33b** with acetophenone and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (95:5) as eluent. Mp: 185.5-186.3 °C; Yield: 21%; ^1H NMR CDCl_3 (ppm): 3.90 (3H, s, CH_3O), 7.00-7.07 (2H, m, ArH), 7.52-7.63 (3H, m, ArH), 8.10 (1H, d, $J = 8.6$ Hz, H_7), 8.22-8.26 (2H, m, ArH), 8.35-8.40 (2H, m, ArH), 8.46 (1H, d, $J = 8.6$ Hz, H_6), 9.11 (1H, s, H_5); ^{13}C NMR CDCl_3 (ppm): 55.5, 114.3 (2 x C), 119.9, 121.2, 126.4, 128.3 (2 x C), 128.9 (2 x C), 129.4 (2 x C), 130.5, 137.0, 138.5, 142.8, 144.8, 149.4, 155.7, 161.7, 162.2, 168.0. Anal. Calcd for $\text{C}_{22}\text{H}_{15}\text{N}_3\text{OS}$ (369.44): C, 71.52; H, 4.09; N, 11.37. Found: C, 71.34; H, 4.28; N, 11.21.

4.2.16.5. 8-(2-Fluorophenyl)-2-phenyl[1,3]thiazolo[5,4-h][1,6]naphthyridine 22a. The product was obtained by reaction of **33a** with 2-fluoroacetophenone and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (95:5) as eluent. Mp: 200.2-201.0 °C; Yield: 43%; ^1H NMR CDCl_3 (ppm): 7.20-7.27 (1H, m, ArH), 7.39 (1H, t, $J = 7.5$ Hz, ArH), 7.48-7.53 (4H, m, ArH), 8.16 (1H, dd, $J = 8.6$, 2.1 Hz, ArH), 8.27-8.29 (2H, m, ArH), 8.42-8.48 (2H, m, ArH), 9.18 (1H, s, H_5);

^{13}C NMR CDCl_3 (ppm): 116.3 (d, $J_{\text{C}^3-\text{F}} = 22.5$ Hz), 121.2, 123.8 (d, $J_{\text{C}^6-\text{F}} = 10.1$ Hz), 124.9 (d, $J_{\text{C}^5-\text{F}} = 2.8$ Hz), 127.0 (d, $J_{\text{C}^4-\text{F}} = 11.5$ Hz), 127.8 (2 x C), 129.0 (2 x C), 131.3, 131.9, 132.0, 132.5, 133.6, 136.5, 142.7, 145.0, 150.0, 155.8, 158.7, (d, $J_{\text{C}^2-\text{F}} = 251.5$ Hz), 168.3. Anal. Calcd for $\text{C}_{21}\text{H}_{12}\text{FN}_3\text{S}$ (357.40): C, 70.57; H, 3.38; N, 11.76. Found: C, 70.45; H, 3.52; N, 11.91.

4.2.16.6. 8-(2-Fluorophenyl)-2-(4-methoxyphenyl)[1,3]thiazolo[5,4-*h*][1,6]naphthyridine 22b. The product was obtained by reaction of **33b** with 2-fluoro acetophenone and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (95:5) as eluent. Mp: 198.2–199.0 °C; Yield: 28%; ^1H NMR CDCl_3 (ppm): 3.92 (3H, s, CH_3O), 7.05 (2H, d, $J = 8.4$ Hz, ArH), 7.22–7.26 (1H, m, ArH), 7.41 (1H, t, $J = 7.5$ Hz, ArH), 7.49–7.56 (1H, m, ArH), 8.18 (1H, d, $J = 8.6$ Hz, ArH), 8.24 (2H, d, $J = 8.4$ Hz, ArH), 8.44 (1H, dd, $J = 7.7, 1.7$ Hz, ArH), 8.50 (1H, d, $J = 8.8$ Hz, ArH), 9.19 (1H, s, H_5). ^{13}C NMR CDCl_3 (ppm): 55.5, 114.3 (2 x C), 116.2 (d, $J_{\text{C}^3-\text{F}} = 22.5$ Hz), 121.2, 123.7 (d, $J_{\text{C}^6-\text{F}} = 9.8$ Hz), 124.9 (d, $J_{\text{C}^5-\text{F}} = 3.0$ Hz), 126.5, 129.5 (2 x C), 131.9 (d, $J_{\text{C}^4-\text{F}} = 8.2$ Hz), 132.5, 136.4, 142.8, 144.9, 149.4, 155.6, 158.6, 161.4 (d, $J_{\text{C}^2-\text{F}} = 205.5$ Hz), 160.6, 162.3, 168.2. Anal. Calcd for $\text{C}_{22}\text{H}_{14}\text{FN}_3\text{OS}$ (387.43): C, 68.20; H, 3.64; N, 10.85. Found: C, 68.32; H, 3.46; N, 10.70.

4.2.16.7. 8-(2-Nitrophenyl)-2-phenyl[1,3]thiazolo[5,4-*h*][1,6]naphthyridine 22c. The product was obtained by reaction of **33a** with 2-nitro acetophenone and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (9:1) as eluent. Mp: 269.0–270.2 °C; Yield: 62%; IR cm^{-1} : 1529 (NO_2); ^1H NMR $\text{DMSO}-d_6$ (ppm): 7.62 (3H, bs, ArH), 7.84 (1H, bs, ArH), 7.95 (2H, bs, ArH), 8.10–8.19 (4H, m, ArH), 8.92 (1H, d, $J = 8.0$ Hz, ArH), 9.47 (1H, s, H_5). The poor solubility of compound **22c** prevented ^{13}C NMR spectra from being recorded. Anal. Calcd for $\text{C}_{21}\text{H}_{12}\text{N}_4\text{O}_2\text{S}$ (384.41): C, 65.61; H, 3.15; N, 14.57. Found: C, 65.78; H, 3.01; N, 14.74.

4.2.16.8. 2-(4-methoxyphenyl)-8-(2-nitrophenyl)[1,3]thiazolo[5,4-*h*][1,6]naphthyridine 22d. The product was obtained by reaction of **33b** with 2-nitro acetophenone and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (9:1) as eluent. Mp: 330.2–330.7 °C; Yield: 15%; IR cm^{-1} : 1531 (NO_2); ^1H NMR CDCl_3 (ppm): 3.91 (3H, s, CH_3O), 7.03 (2H, d, $J = 7.4$ Hz, ArH), 7.68–7.79 (3H, m), 7.90 (1H, d, $J = 7.4$ Hz, ArH), 8.11 (1H, d, $J = 8.5$ Hz, ArH), 8.21 (2H, d, $J = 8.5$ Hz, ArH), 8.51 (1H, d, $J = 8.5$ Hz, ArH), 9.20 (1H, s, H_5). The poor solubility of compound **22d** prevented ^{13}C NMR spectra from being recorded. Anal. Calcd for $\text{C}_{22}\text{H}_{14}\text{N}_4\text{O}_3\text{S}$ (414.44): C, 63.76; H, 3.40; N, 13.52. Found: C, 63.87; H, 3.23; N, 13.67.

4.2.16.9. 2-Phenyl-8-[2-(trifluoromethyl)phenyl][1,3]thiazolo[5,4-*h*][1,6]naphthyridine 22e. The product was obtained by reaction of **33a** with 2-trifluoromethyl acetophenone and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (9:1) as eluent. Mp: 213.2–214.4 °C; Yield: 38%; ^1H NMR CDCl_3 (ppm): 7.48–7.53 (3H, m, ArH), 7.64 (1H, t, $J = 7.7$ Hz, ArH), 7.72 (1H, t, $J = 7.7$ Hz, ArH), 7.80–7.86 (3H, m, ArH), 8.24–8.27 (2H, m, ArH), 8.50 (1H, d, $J = 8.5$ Hz, ArH), 9.24 (1H, s, H_5). The poor solubility of compound **22e** prevented ^{13}C NMR spectra from being recorded. Anal. Calcd for $\text{C}_{22}\text{H}_{12}\text{F}_3\text{N}_3\text{S}$ (407.41): C, 64.86; H, 2.97; N, 10.31. Found: C, 64.99; H, 2.73; N, 10.19.

4.2.16.10. 2-(4-Methoxyphenyl)-8-[2-(trifluoromethyl)phenyl][1,3]thiazolo[5,4-*h*][1,6]naphthyridine 22f. The product was obtained by reaction of **33b** with 4-methoxy acetophenone and it was purified by silica gel chromatography using dichloromethane as eluent. Mp: 241.8–242.4 °C; Yield: 35%; ^1H NMR CDCl_3 (ppm): 3.90 (3H, s, CH_3O), 7.01 (2H, d, $J = 9.0$ Hz, ArH), 7.64 (1H, t, $J = 7.5$ Hz, ArH), 7.72 (1H, t, $J = 7.5$ Hz, ArH), 7.80–7.86 (3H, m, ArH), 8.22 (2H, d, $J = 9.0$ Hz, ArH), 8.49 (1H, d, $J = 8.5$ Hz, ArH), 9.20 (1H, s, H_5). The poor solubility of

compound **22f** prevented ^{13}C NMR spectra from being recorded. Anal. Calcd for $\text{C}_{23}\text{H}_{14}\text{F}_3\text{N}_3\text{OS}$ (437.44): C, 63.15; H, 3.23; N, 9.61. Found: C, 63.26; H, 3.11; N, 9.79.

4.2.16.11. 8-(2-Chlorophenyl)-2-phenyl[1,3]thiazolo[5,4-*h*][1,6]naphthyridine 22g. The product was obtained by reaction of **33a** with 2-chloro acetophenone and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (93:7) as eluent. Mp: 219.4–220.2 °C; Yield: 51%; ^1H NMR CDCl_3 (ppm): 7.43–7.48 (2H, m, ArH), 7.50–7.55 (4H, m, ArH), 7.95–7.97 (1H, m, ArH), 8.06 (1H, d, $J = 8.5$ Hz, ArH), 8.26–8.30 (2H, m, ArH), 8.49 (1H, d, $J = 8.5$ Hz, ArH), 9.23 (1H, s, H_5). ^{13}C NMR CDCl_3 (ppm): 115.1, 121.1, 124.4, 127.3, 127.9 (2 x C), 129.0 (2 x C), 130.1, 130.6, 131.4, 132.0, 132.4, 132.6, 133.5, 135.7, 139.0, 150.0 (2 x C), 160.9, 162.2. Anal. Calcd for $\text{C}_{21}\text{H}_{12}\text{ClN}_3\text{S}$ (373.86): C, 67.47; H, 3.24; N, 11.24. Found: C, 67.66; H, 3.07; N, 11.51.

4.2.16.12. 8-(2-Chlorophenyl)-2-(4-methoxyphenyl)[1,3]thiazolo[5,4-*h*][1,6]naphthyridine 22h. The product was obtained by reaction of **33b** with 2-chloro acetophenone and it was purified by silica gel chromatography using dichloromethane as eluent. Mp: 226.6–227.4 °C; Yield: 38%. ^1H NMR CDCl_3 (ppm): 3.90 (3H, s, CH_3O), 7.02 (2H, d, $J = 8.9$ Hz, ArH), 7.42–7.55 (3H, m, ArH), 7.96 (1H, dd, $J = 6.9, 2.4$ Hz, ArH), 8.04 (1H, d, $J = 8.5$ Hz, ArH), 8.22 (2H, d, $J = 8.9$ Hz, ArH), 8.47 (1H, d, $J = 8.5$ Hz, ArH), 9.19 (1H, s, H_5). ^{13}C NMR CDCl_3 (ppm): 55.5, 113.2, 114.3 (2 x C), 121.1, 124.3, 126.4, 127.2, 129.6 (2 x C), 130.1, 130.6, 132.4, 132.6, 135.6, 139.0, 142.8, 149.4, 155.7, 162.0, 162.3, 168.3. Anal. Calcd for $\text{C}_{22}\text{H}_{14}\text{ClN}_3\text{OS}$ (403.88): C, 65.42; H, 3.49; N, 10.40. Found: C, 65.56; H, 3.61; N, 10.26.

4.2.16.13. 8-(2-Methoxyphenyl)-2-phenyl[1,3]thiazolo[5,4-*h*][1,6]naphthyridine 22i. The product was obtained by reaction of **33a** with 2-methoxy acetophenone and was crystallized from diethyl ether. Mp: 158.3–159.6 °C; Yield: 20%; ^1H NMR CDCl_3 (ppm): 3.91 (3H, s, CH_3O), 7.05 (1H, d, $J = 8.2$ Hz, ArH), 7.16–7.23 (1H, m, ArH), 7.45–7.52 (4H, m, ArH), 8.20–8.27 (4H, m, ArH), 8.39 (1H, d, $J = 8.7$ Hz, H_6), 9.16 (1H, s, H_5); ^{13}C NMR CDCl_3 (ppm): 55.4, 111.4, 114.5, 120.6, 121.0, 121.4, 124.9, 127.8 (2 x C), 128.7, 128.9 (2 x C), 130.1, 131.4, 132.5, 133.2, 133.6, 135.2, 149.9, 157.6, 161.8, 167.9. Anal. Calcd for $\text{C}_{22}\text{H}_{15}\text{N}_3\text{OS}$ (369.44): C, 71.52; H, 4.09; N, 11.37. Found: C, 71.37; H, 3.96; N, 11.52.

4.2.16.14. 8-(2-Methoxyphenyl)-2-(4-methoxyphenyl)[1,3]thiazolo[5,4-*h*][1,6]naphthyridine 22j. The product was obtained by reaction of **33b** with 2-methoxy acetophenone and it was purified by silica gel chromatography using dichloromethane:ethyl acetate (95:5) as eluent. Mp: 161.5–162.3 °C; Yield: 33%; ^1H NMR CDCl_3 (ppm): 3.90 (3H, s, CH_3O), 3.91 (3H, s, CH_3O), 7.00–7.08 (3H, m, ArH), 7.16–7.24 (1H, m, ArH), 7.45–7.53 (1H, m, ArH), 8.20 (2H, d, $J = 8.0$ Hz, ArH), 8.22 (2H, d, $J = 8.0$ Hz, ArH), 8.38 (1H, d, $J = 8.7$ Hz, H_6), 9.13 (1H, s, H_5); ^{13}C NMR CDCl_3 (ppm): 55.5, 55.7, 111.4, 114.3 (2 x C), 121.0, 121.4, 124.8, 126.5, 128.8, 129.4 (2 x C), 131.4, 132.5, 135.1, 142.8, 144.9, 149.3, 155.3, 157.6, 161.7, 162.1, 167.8. Anal. Calcd for $\text{C}_{23}\text{H}_{17}\text{N}_3\text{O}_2\text{S}$ (399.46): C, 69.15; H, 4.29; N, 10.52. Found: C, 68.98; H, 4.13; N, 10.65.

4.3. Biology

The plasmid for N-terminally His-ZB tagged Nsp13 pNIC-ZB vector was acquired from Addgene (159614). The protein was obtained as described [9]. Briefly the expression was carried out in *E. coli* BL21 Rosetta2 cells. The protein was purified combining IMAC, Hi-salt and Size Exclusion chromatography. For the first step of purification, a HisTrap column was used, and eluted fractions were further purified on a HiTrap SP HP column. After overnight digestion with TEV protease, sample was loaded onto a HiLoad 16/600 Superdex pg column. The protocol was optimized for high-yield and high-purity protein production. The final yield of purification was around 3 mg of pure protein from

4 L of culture. Quality control analyses (SDS-PAGE, enzymatic validation assays) were performed.

The Enzymatic activities were measured as reported [26]. Briefly SARS-CoV-2 Nsp13 unwinding-associated activity was measured in black 384 well plate, in 20 μ L reaction volume containing 20 mM Tris-HCl pH 7.2, 50 mM NaCl, 2 μ M Hel Capture oligo (5'- TGG TGC TCG AAC AGT GAC -3') 5 mM $MgCl_2$, and, to avoid aspecific binding 10 μ g/mL of BSA and 180 μ M TCEP, were added. Compounds were resuspended in DMSO to a final concentration of 5%, 1 nM of purified Nsp13 was added. The reaction mixture containing the enzyme was pre-incubated for 10 min with inhibitor at room temperature. The reaction was started adding 0.5 mM ATP and 500 nM dsDNA substrate (5'- AGT CTT CTC CTG GTGCTC GAA CAG TGA C-Cy3-3', 5'- BHQ-2-GTC ACT GTT CGA GCA CCACCT CTT CTG A-3'). After 15 min of incubation at 37 °C, the reaction products were measured with Victor Nivo (Perkin) at 530/580 nm.

The SARS-CoV-2 Nsp13 ATPase-associated activity was measured in a transparent 96 well plate (PerkinElmer). Briefly, in 25 μ L reaction volume containing 20 mM Tris-HCl pH 7.2, 50 mM NaCl, 2 mM $MgCl_2$, and, to avoid a specific binding 10 μ g/mL of BSA and 180 μ M TCEP were added and Nsp13 was tested at 25 nM. The reaction mixture containing the enzyme was pre-incubated for 10 min with inhibitor, at room temperature. The reaction was started adding 250 μ M ATP. After 30 min of incubation at 37 °C, 50 μ L of Biomol® Green Reagent (Prod. No. BML-AK111, Enzo Lifescience) were added and the reaction was incubated for 10 min at room temperature, protected from the light. Products were measured with Victor Nivo (Perkin) at 650 nm.

The kinetic of inhibition of Nsp13 ATPase activity was performed in a transparent 96 well plate (PerkinElmer), in 25 μ L of reaction mixture, as reported for Nsp13 ATPase-associated activity assay, titrating the ATP, with final concentrations of 0.25 mM, 0.12 mM, 0.6 mM, 0.3 mM and 0.1 mM, in absence and presence of increasing concentrations of inhibitors. Reaction was started with the addition of 25 nM Nsp13. On the same plate a calibration curve of inorganic phosphate (Phosphate Standard, Prod. No. BML-KI102, Enzo Lifescience) was added. After 30 min of incubation at 37 °C, 50 μ L of Biomol® Green Reagent (Prod. No. BML-AK111, Enzo Lifescience) were added and reaction was incubated for 10 min at RT, protected from the light. Products were measured with Victor Nivo (Perkin) at 650 nm. The pmols of inorganic phosphate produced during the reaction were interpolated from the calibration curve. Results were plotted in a Lineweaver-Burk graph as the reciprocal of pmols produced (1/Vmax) vs the reciprocal of ATP concentrations.

Data analysis of assay development results was performed using GraphPad Prism Version 9.1.2. Test compound results were normalized relative to respective controls. Dose response curves were fitted to a nonlinear regression of (log10) dose vs normalized response-variable slope. Assay quality was assessed using the Z'-factor calculation with $Z' > 0.5$ as threshold for acceptance.

Determination of cytotoxicity and viral replication inhibition in Vero-E6-GFP: The SARS-CoV-2 strain Omicron Strain BA.4 was purchased from NCPV 2209291v. The African green monkey kidney cell line (ATCC:CRL-1586™), previously engineered to constitutively express GFP (Vero-E6-GFP), was kindly provided by Janssen Pharmaceutical. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 2% v/v fetal bovine serum (FBS; Gibco), 0.075% sodium bicarbonate (7.5% solution, Gibco) and 1 $\times$ Pen-strep (Euroclone) and maintained under 5% CO₂ at 37 °C. Cells were seeded at 10,000 cells/well in 96-well plates. The following day, cells were incubated with compounds and virus at different MOI 0.01, in the presence of 2 μ M P-gp inhibitor CP-100356. The compound GC376 was used as a positive control. Medium was removed 72 h post-infection and total GFP well fluorescence was measured with a Cytation5 with excitation wavelength of 485/535 nm.

Determination of cytotoxicity and viral replication inhibition in GFP Expressing Huh-7 Stable Cell Line (purchased from Applied Biological

Materials). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% v/v fetal bovine serum (FBS; Gibco), 1 $\times$ Pen-strep (Euroclone), 1.4 μ g/mL Puromycin and maintained under 5% CO₂ at 37 °C. Cells were seeded at 10,000 cells/well in 96-well plates. The following day, cells were incubated with compounds. The compound Camptotecin was used as a positive control for cytotoxicity. Medium was removed 72 h post-infection and total GFP well fluorescence was measured with a Cytation5 with excitation wavelength of 485/535 nm.

The experiment are results of two independent biological replicates in triplicate. Data analysis of assay development results was performed using GraphPad Prism Version 9.1.2. Test compound results were normalized relative to respective controls. The compound cytotoxicity was determined as the % of reduction of the constitutive GFP expression in uninfected samples and the inhibition of viral-induced CPE was calculated as a percentage of the cytopathic effect induced by the virus on untreated infected controls. Dose response curves were fitted to a nonlinear regression of (log10) dose vs normalized response-variable slope.

CRediT authorship contribution statement

Roberta Bivacqua: Methodology, Formal analysis, Data curation. **Roberta Emmolo:** Methodology, Investigation, Formal analysis. **Gianmarco Gualtieri:** Methodology, Formal analysis. **Simona Di Martino:** Formal analysis. **Marilia Barreca:** Methodology, Formal analysis, Data curation. **Virginia Spanò:** Methodology, Formal analysis. **Maria Valeria Raimondi:** Methodology, Data curation. **Angela Corona:** Methodology, Investigation, Formal analysis, Data curation. **Francesco Ortuso:** Supervision, Software. **Enzo Tramontano:** Supervision. **Isabella Romeo:** Writing – original draft, Methodology, Conceptualization. **Alessandra Montalbano:** Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Paola Barraja:** Writing – review & editing, Supervision, Funding acquisition. **Stefano Alcaro:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by EU funding within the NextGenerationEU-MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT) and the “1° Memorial Vincenzo&Vincenzo”. COVID-SARS2 NSP13 was a gift from Opher Gileadi (Addgene plasmid # 159614; <http://n2t.net/addgene:159614>; RRID: Addgene_159614).

Abbreviations

ORF = open reading frame
 3CLpro = 3-chymotrypsin-like protease
 PLpro = papain-like protease
 Nsp = non-structural protein
 RdRP = RNA-dependent RNA-polymerase
 RTC = replication-transcription complex
 dsDNA = double-stranded DNA
 NTP = nucleoside triphosphate
 ZBD = zinc-binding domain
 MIFs = molecular interaction fields
 RMSD = root mean square deviation
 MM/GBSA = Molecular Mechanics/Generalized Born Surface Area
 HSV = herpes simplex virus

TEA = triethylamine
 DCM = dichloromethane
 EtOAc = ethyl acetate
 EtOH = ethanol
 DMF = *N,N*-Dimethylformamide
 THF = Tetrahydrofuran
 EMME = diethyl ethoxymethylenemalonate
 TCFH = *N,N,N,N*-tetramethylchloroformamidinium hexafluorophosphate
 NMI = *N*-methylimidazole
 ACN = acetonitrile
 HOBt = 1-hydroxybenzotriazole
 EDC HCl = *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride
 DMAP = 4-dimethylaminopyridine
 SOM = sites of metabolism
 CYP = cytochrome P450
 ADME = Absorption, Distribution, Metabolism, Excretion
 SAR = structure-activity relationship
 PDB = Protein Data Bank
 GUI = Graphical User Interface
 PSA = polar surface area
 HBD = hydrogen bond donor
 HBA = hydrogen bond acceptor
 MW = molecular weight
 MPO = Multi-Parameter Optimization
 SP = standard precision
 IFD = induced-fit docking
 PAINS = pan assay interference compounds
 DMSO = dimethyl sulfoxide
 MAOS = Microwave-Assisted Organic Synthesis
 IMAC = Immobilized Metal Affinity Chromatography
 TEV = Tobacco Etch Virus
 BSA = Bovine Serum Albumin
 TCEP = Tris(2-carboxyethyl)phosphine
 r.t. = room temperature

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmech.2026.119338>.

Data availability

Data will be made available on request.

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