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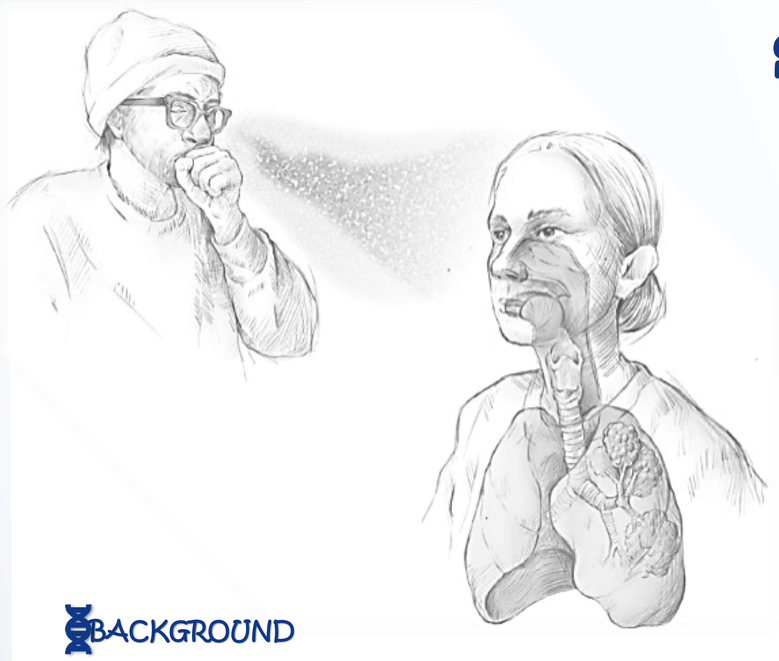
SMALL MOLECULE DERIVATIVES AS DUAL INHIBITORS OF SARS-CoV-2 NSP13 BLOCKING VIRAL REPLICATION

Madia, V. N.;[a] Ialongo, D.;[a] Patacchini, E.;[a] Arpacioglu, M.;[a] Messore, A.;[a] Tramontano, E.;[b] De Santis, R.;[c] Lista, F.;[c] Costi, R.;[a] and Di Santo, R.[a]

[a] *Istituto Pasteur-Fondazione Cenci Bolognetti, Dipartimento di Chimica e Tecnologie del Farmaco, "Sapienza" Università di Roma, p.le Aldo Moro 5, I-00185 Rome, Italy;* [b] *Department of Life and Environmental Sciences, University of Cagliari, Cittadella Universitaria di Monserrato, SS554 -09042 Monserrato (CA) Italy;* [c] *Army Medical Center, Scientific Department, p.za Celimontana 50, Rome 00184, Italy.*



SARS-CoV-2, responsible for the COVID-19 pandemic, has infected over 500 million people and caused more than 6 million deaths worldwide as of June 2022. While antigen-based vaccines have demonstrated significant success at mitigating severe disease and spread, the need to treat infected patients as well as the evolution of potentially vaccine-resistant mutants make the development of new potent antivirals a pressing concern. A promising target for antiviral drug development against SARS-CoV-2 is nonstructural protein 13 (**NSP13**), because it plays a critical role in virus life-cycle and its inhibition in SARS-CoV-1 has demonstrated to lead to the blocking of viral replication.

BACKGROUND

SARS-CoV-2 NSP13 is a 67 kDa protein that belongs to the helicase superfamily SF1B. It utilizes the energy of nucleotide triphosphate hydrolysis to catalyze the unwinding of double-stranded DNA or RNA in a 5′ to 3′ direction. NSP13 has been shown to interact with the viral RNA-dependent RNA polymerase NSP12 and acts in concert with the replication-transcription complex (NSP7/NSP8/NSP12). In addition to its helicase activity, NSP13 also possesses RNA 5′ triphosphatase activity within the same active site, suggesting a further essential role for NSP13 in the formation of the viral 5′ mRNA cap. The enzyme contains 5 domains: a N-terminal Zinc binding domain (ZBD) that coordinates 3 structural Zinc ions, a helical "stalk" domain, a β-barrel 1B domain and two "RecA like" helicase subdomains 1A and 2A that contain the residues responsible for nucleotide binding and hydrolysis (**Figure 1**). NSP13 is also the most conserved among the non-structural proteins in the SARS-CoV-2 genome.[1]

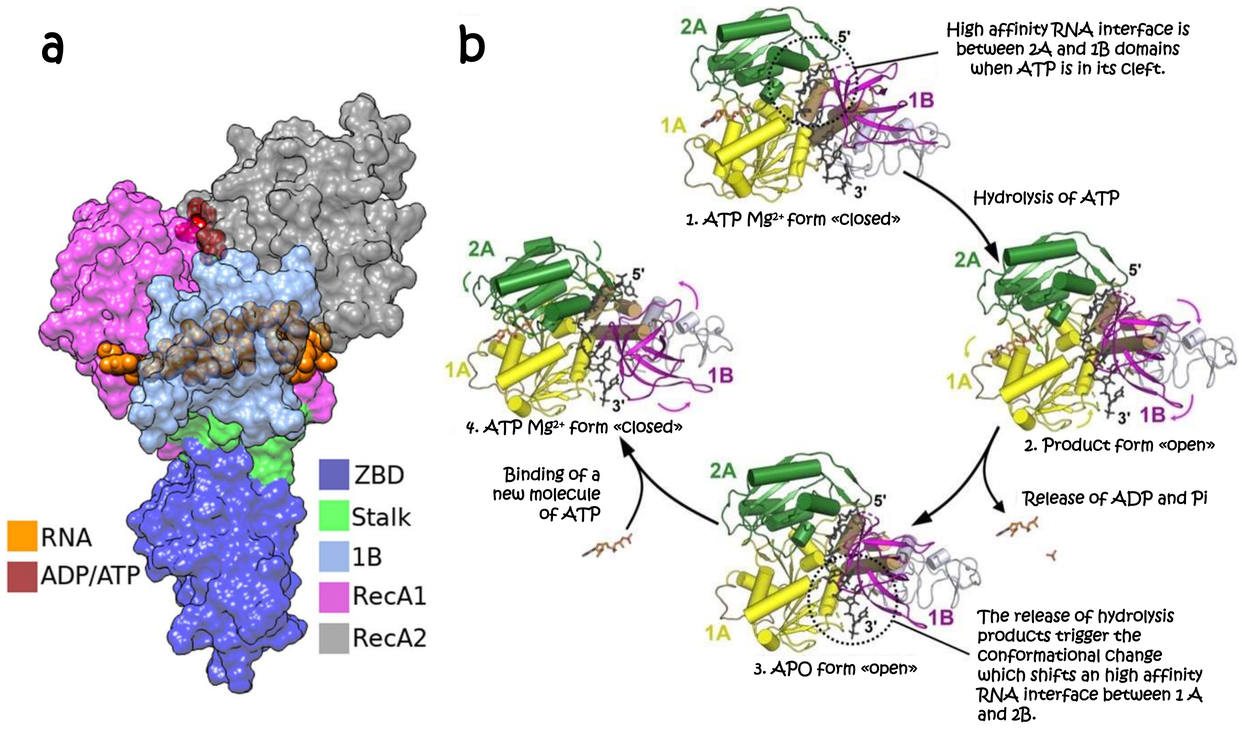


Figure 1. (a) Surface representation of the NSP13 model, with the principal domains highlighted with color. (b) Proposed catalytic cycle for NSP13 based on the transition from the closed to the open form initiated by the binding, hydrolysis and release of ATP, which triggers the conformational changes and remodels the RNA interface. Hydrolysis as subsequent charge repulsion could trigger the opening of the cleft between the two domains with conserved motifs on the 2A domain primarily contacting the product phosphate whilst the ADP product interacts with the 1A domain. Image remodeled from Newman, et al., Nat. Commun. 12, 4848 (2021).

HIT IDENTIFICATION

As the heterocyclic compounds have been rigorously involved in the ailments including infections, cancer and other diseases, there exists a profound scope of exploring these multiple nuclei to curb also SARS-CoV-2. Previously, aryl diketo acids (**DKAs**) have been described as inhibitors of SARS-CoV-1 NSP13 helicase activity. This is probably due to the possibility of this scaffold to chelate functional metal ions, like magnesium ions which are present in the catalytic pocket of the enzyme.[2] Hence, basing on the 99.8% amino acid sequence identity between SARS-CoV-1 and SARS-CoV-2 NSP13 and our longstanding expertise in the design and synthesis of DKA compounds endowed with antiviral activity,[3] we decided to carry out a **virtual structure-based semi-random screening** on our in-house library. This *in silico* evaluation allowed us to identify some hit compounds as putative NSP13 inhibitors characterized by an aromatic core endowed with a diketo hexenoic and diketo butanoic chain, agreeing with literature data. In order to confirm these first predictions, we decided to evaluate the antiviral activity of these molecules with biological assays. As a results, a class of indole-based compounds emerged as potent new SARS-CoV-2 NSP13-helicase inhibitors. In particular, the newly identified hits were characterized by an indole skeleton possessing a diketo hexenoic chain in 3-position (**Figure 2**).

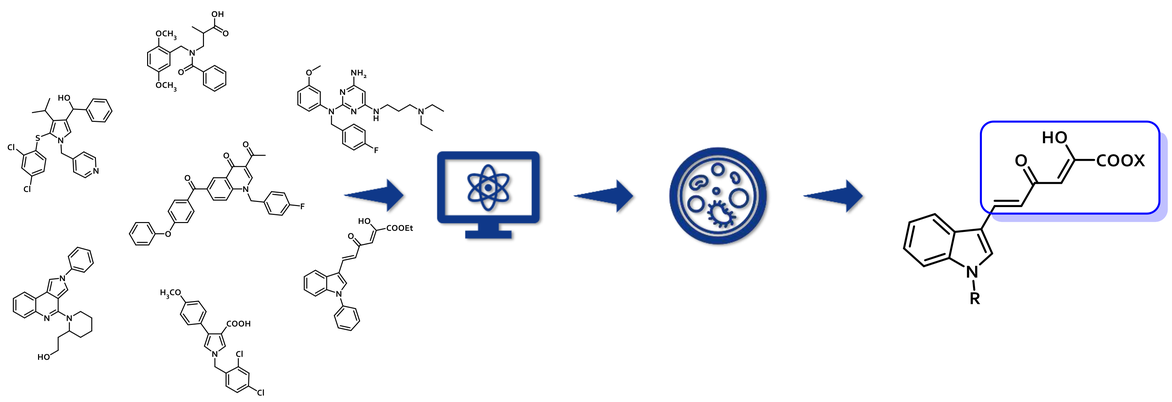
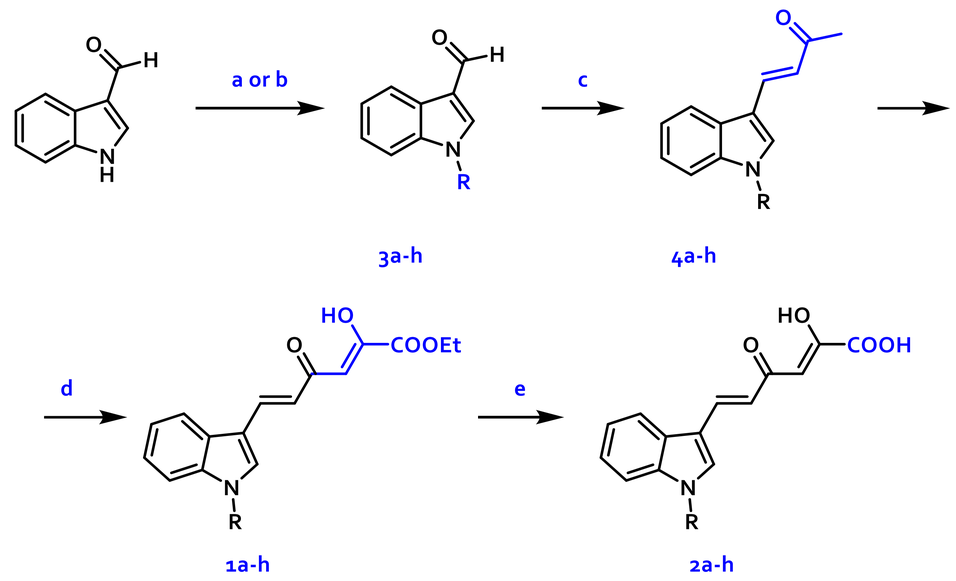


Figure 2. Over the years, our laboratory has worked extensively on the design and synthesis of biologically active compounds, resulting in an in-house library that mainly consists of structurally diverse, small heterocyclic molecules endowed with various biological activity[3]. In a first round of screening, we selected some compounds considering literature date, and among them some DKA emerged as potent SARS-CoV-2 NSP13 inhibitors. To confirm these results, the most promising compounds were then evaluated for their inhibitory activity in vitro *assays. Indolyl compounds with a 3-diketo hexenoic long chain emerged as a new scaffold for a hit-to-lead optimization process.*

SYNTHESIS

We decide to design and synthesize new derivatives structurally related to the new identified hits. Maintaining the indolyl DKA core, alkyl, phenyl, and benzyl N-substituents were introduced in order to investigate structure-activity relationships. The general synthesis is reported in **Scheme 1**.

Scheme 1. Synthesis of new indolyl DKA compounds.



Reagents and conditions. (a) proper halide, DMF dry, 1-24 h, 90 °C, 50-70%; (b) arylboronic acid, cupric acetate, NMP/pyridine 1:1, microwave (60 W, 120 °C, 50", open vessel), 57%; (c) 5 N NaOH, acetone, 2 days, 50 °C, 100%; (d) diethyl oxalate, NaOEt, THF, 1,5 h, r.t., 60-80%; (e) 1 N NaOH, THF:MeOH 1:1, 30 min, r.t., 100%.

Indole-3-carboxaldehyde was arylated with the appropriate arylboronic acid following a microwave-assisted Suzuki coupling procedure to afford aryl derivatives **3a-c** or alkylated with the appropriate alkyl halide using NaH as a base to give alkyl derivatives **3d-h**. Then, aldehydes **3a-h** were condensed with acetone in the presence of 5 N NaOH. The enones **4a-h** that formed were reacted with diethyl oxalate in basic conditions (NaOEt) to give diketo esters **1a-h** that were finally hydrolyzed with 1 N NaOH to afford the corresponding acids **2a-h**.

BIOLOGICAL RESULTS AND DISCUSSIONS

The newly synthetized esters **1a-h** and the corresponding diketo acids **2a-h** were tested against SARS-CoV-2 NSP13, and the results are shown in **Table 1**. Some molecules showed promising IC_{50} values in the low micromolar range. However, it is worth to underline that among the active compounds, all of them are more active in inhibiting the SARS-CoV-2 NSP13 unwinding activity than the NTPase activity, confirming some literature data on the ability of DKAs to have helicase inhibitory activity. In general, acidic compounds are more active than esters, although compound **1d** emerged as the most potent compound in *in vitro* enzymatic assays and compounds **1c** and **1d** are more active than the corresponding acids **2c** and **2d**. At a later stage, we decided to perform also *in cellulo* assays using **VERO E6 GFP infected cells**. Some compounds proved to be active in the micromolar range, showing a percentage of viral inhibition of about 100% at 25 or 50 μM. In addition, none of the synthetized compounds exerted cytotoxicity at the tested concentrations. Considering all data, compounds **2g** is the most potent and effective SARS-CoV-2 NSP13 helicase inhibitory compounds.

Table 1. Activity of newly synthetized compounds.

Cmpd	R	SARS-CoV-2 NSP13 Helicase IC_{50} (μM)	SARS-CoV-2 NSP13 NTPase IC_{50} (μM)	Viral inactivation *in cellulo*	CC_{50}
1a	4-OH-Ph[a]	>30	>30	0	>30
1b	4-CN-Ph	>30	>30	0	>30
1c	4-F-Ph	1.2±0.48	10.5±0.3	96.0% at 50 μM	>30
1d	Bn[b]	0.44±0.19	21.7±1.9	0	>30
1e	4-OH-Bn	>30	>30	0	>30
1f	$CH_2CH(CH_3)$=CH	>30	>30	0	>30
1g	CH_2CH=$(CH_3)_2$	>30	>30	0	>30
1h	$CH_2(C$=$O)_2$Morph[c]	>30	>30	0	>30
2a	4-OH-Ph	6.2±2.7	>30	0	>30
2b	4-CN-Ph	2.67±0.19	>30	0	>30
2c	4-F-Ph	5.62±1.4	>30	99.9% at 25 μM	>30
2d	Bn	7.17±3.7	>30	99.4% at 25 μM	>30
2e	4-OH-Bn	1.14±0.40	18.9±1.3	0	>30
2f	$CH_2CH(CH_3)$=CH	>30	>30	99.9% at 50 μM	>30
2g	CH_2CH=$(CH_3)_2$	0.77±0.41	>30	99.6% at 25 μM	>30
2h	$CH_2(C$=$O)_2$Morph[c]	>30	>30	0	>30

[a]Ph: Phenyl. [b]Bn: Benzyl. [c]Morph: Morpholine.

TAKE HOME MESSAGE

- The development of vaccines is undoubtedly essential to contain the diffusion of the SARS-CoV-2, and a joint effort never seen before led to a worldwide vaccination campaign In less than 1 year after the COVID-19 outbreak. However, vaccines may be less effective or even ineffective against emerging variants of SARS-CoV-2 and it is still to be determined how long this vaccine-induced immunity will last.
- The development of antiviral drugs targeting SARS-CoV-2, so, remain undoubtly necessary.
- A promising target for antiviral drug development against SARS-CoV-2 is nonstructural protein 13 (NSP13), because it plays a critical role in viral replication and its sequence is conserved across all coronavirus species.
- In this work, we presented a novel class of indolyl 3-diketo hexenoic derivatives endowed with SARS-CoV-2 NSP13 helicase inhibitory activity.
- Some of the newly synthesized compounds showed promising activity in enzymatic and cellular assays. In particular, compound **2g** emerged as a new hit for further hit-to-lead optimization.

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SAPIENZA UNIVERSITÀ DI ROMA

ISTITUTO PASTEUR ITALIA FONDAZIONE CENCI BOLOGNETTI

valentinanoemi.madia@uniroma1.it