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Discovery of potent nucleotide-mimicking competitive inhibitors of hepatitis C virus NS3 helicase

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ABSTRACT

Among the enzymes involved in the life cycle of HCV, the non-structural protein NS3, with its double function of protease and NTPase/helicase, is essential for the virus replication. Exploiting our previous knowledge in the development of nucleotide-mimicking NS3 helicase (NS3h) inhibitors endowed with key structural and electronic features necessary for an optimal ligand–enzyme interaction, we developed the tetrahydroacridinyl derivative **3a** as the most potent NS3h competitive inhibitor reported to date (HCV NS3h K_i = 20 nM).

Hepatitis C virus (HCV) is a positive-stranded RNA virus of the *Flaviviridae* family.[1] More than 170 million people are chronically infected with HCV worldwide.[2] At the moment, the only drugs approved for treatment of chronic HCV infection are interferon-alpha (pegylated and conventional) and ribavirin, administered alone or in combination therapy.[3] However, the current treatments for HCV infection are far from being ideal, mainly due to efficacy and safety issues.[3] Consequently, the development of specifically targeted antiviral therapies for HCV is urgently needed.[4,5] Among the enzymes involved in the HCV life cycle, the non-structural protein NS3 is a multifunctional enzyme essential for virus replication.[6,7] The N-terminal domain of NS3 is a serine protease whereas the C-terminal domain has NTPase and helicase activities and unwinds both DNA and RNA.[8,9]

Inhibition of NS3 helicase (NS3h) is a challenging approach for the development of innovative anti-HCV drugs.[10] Drugs that target unwinding activity could act in different ways: by inhibiting ATPase activity by interfering with ATP binding or hydrolysis, by preventing nucleic acid (NA) binding and thus blocking the unwinding process, or by intercalating into the NA chains.[11–14] Few NS3h inhibitors competing for the NA binding site have been so far reported,[12,15–18] but most of them suffer from modest potency, inappropriate pharmacokinetic properties or toxicity.

We previously reported the discovery of the first NS3h nucleotido-mimetic inhibitor (**1**, QU663, Fig. 1), which inhibits the helicase activity by competitively binding the DNA recognition site of the enzyme (K_i = 0.75 μM).[19] In our studies the NS3h inhibitory activity of **1** was explained by considering its peculiar electronic and conformational features resembling those calculated for RNA–DNA nucleotides.[19] Indeed, the heterocyclic system of **1** presents a partially negative charged area around the hydrazide moiety positioned at 5.92–6.23 Å away from the basic quinoline nitrogen, thus reproducing the electronic distribution of purine and pyrimidine nucleotides, which have a positively charged area ~5.8 and ~7.3 Å from the negatively charged phosphate moiety, respec-

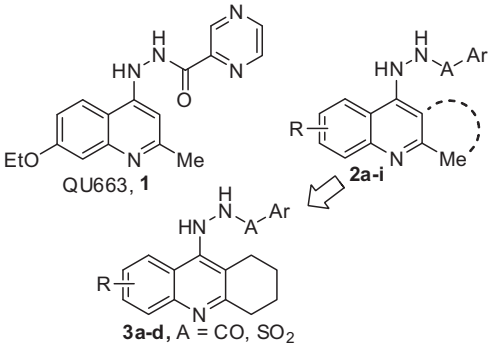

Figure 1. Reference and title compounds.

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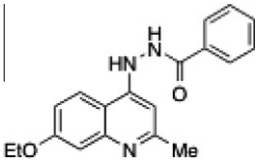
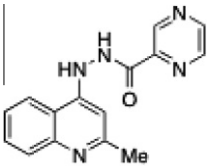
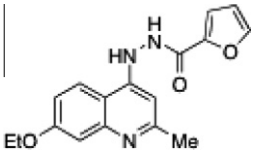
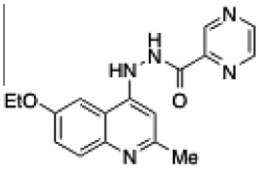
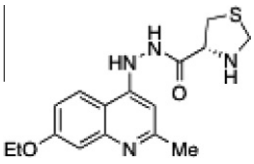
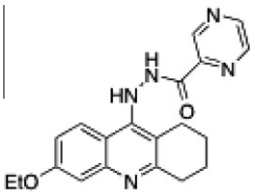
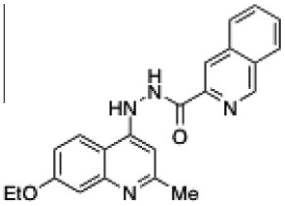
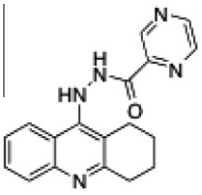
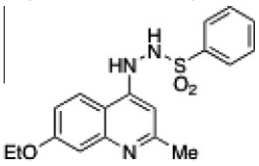
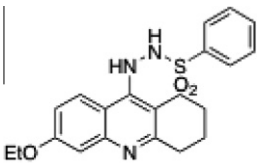
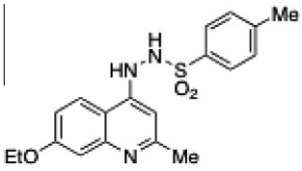
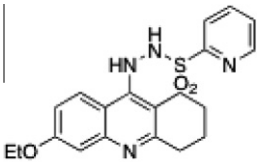
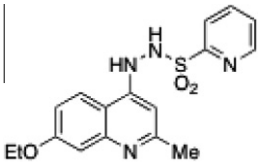
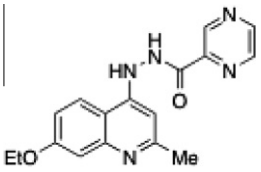
Table 1
Inhibition of HCV NS3h by inhibitors **1**, **2a–i** and **3a–d**

Compds	Structure	NS3h K_i[a] (μM)	Compds	Structure	NS3h K_i[a] (μM)
2a		125	**2h**		>300
2b		>300	**2i**		>300
2c		>300	**3a**		0.020
2d		250	**3b**		>300
2e		5.0	**3c**		0.16
2f		75	**3d**		0.10
2g		0.30	**1**[b]		0.75

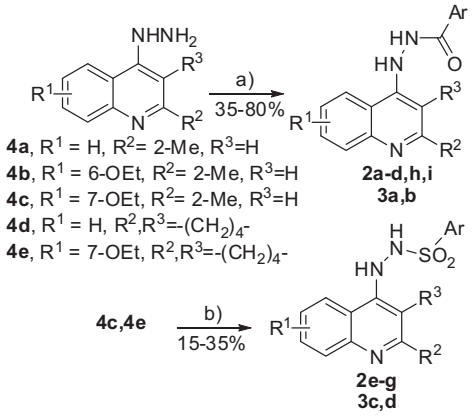
[a] Values are means of three experiments, standard deviation is within 10% of the mean.
[b] See Ref. 19.

tively.[19] Moreover, docking studies revealed that compound **1** was able to engage several interactions with key amino acids (e.g., Trp501) important for NA binding and for helicase activity.[19] Based on this rational, and with the aim to further investigate this class of compounds for their potential role as anti-HCV drugs, we synthesized and biochemically characterized quinolylhydrazides **2a–i**, and 1,2,3,4-tetrahydroacridinylhydrazides **3a–d** (Fig. 1 and Table 1) as potent NS3h inhibitors.

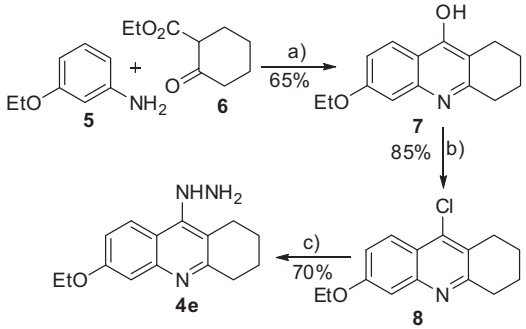
The synthesis of target inhibitors **2a–i** and **3a–d** is reported in Scheme 1. Hydrazines **4a–e**, prepared following reported procedures,[19,20] were coupled with the appropriate carboxylic acid using aldrithiol and triphenylphosphine as activating reagents in dichlorometane,[19] to afford the desired compounds in 35–80% overall yields after chromatographic purification and re-crystallization. Sulfonic hydrazides **2e–g** and **3c**,**d** were obtained in reasonable yield by reacting hydrazines **4c** or **4e** with the suitable arylsulfonyl chloride in pyridine at 25 °C. Hydrazine **4e**, necessary for the synthesis of compounds **3a**,**c**,**d**, was prepared as described in Scheme 2, following classic literature protocols, starting from 3-ethoxyaniline **5** and 2-ethoxycarboxylcyclohexanone **6**.[20]

Inhibition of HCV NS3h by the synthesized inhibitors was measured by using the helicase assay previously described[19] and results are reported in Table 1.

We first investigated the role of the aromatic hydrazide moiety for enzyme affinity. The pyrazine system of **1** proved to have a key role for the interaction with the enzyme. Indeed, substitution of the pyrazine system by a phenyl ring (**2a**) led to a 170-fold drop in inhibitory activity with respect to **1**. Also introduction of



Scheme 1. Synthesis of inhibitors **2a–i** and **3a–d**. Reagents and conditions: (a) (i) $ArCO_2H$, PPh_3, aldrithiol, DCM, 25 °C, 2–3 h, (ii) **4a–e**, DCM, 25 °C, 18 h; (b) $ArSO_2Cl$, pyridine, 25 °C, 12 h. Structures of compounds **2a–i** and **3a–d** as displayed in Table 1.



Scheme 2. Synthesis of hydrazine intermediate **4e**. Reagents and conditions: (a) Ph_2O, reflux, 3 h; (b) $POCl_3$, 25 °C, 1 h; (c) $NH_2NH_2{\cdot}H_2O$, 5 min, 150 W, 150 °C.

five-membered heterocycles such as a 2-furyl (**2b**) or a (*R*)-4-thiazolidinyl (**2c**) was detrimental for affinity, as well as the bulkier 2-isoquinoline system of **2d**. More encouraging results were obtained when a sulfonyl group replaced the carbonyl group of **2a**. Accordingly, **2e**, bearing a benzenesulfonyl moiety, was 25 times more potent than **2a**, although 7 times less potent than **1**. While the *p*-toluensulfonyl derivative **2f** was less potent than **2e**, probably due to steric constraints, a significant increase of inhibitory activity was achieved with the 2-pyridylsulfonyl derivative **2g**, this latter showing a 2.5-fold potency increase with respect to our prototype compound **1**.

Subsequently, we investigated substitutions at the quinoline system. The 7-OEt substituent of **1** revealed to be an important determinant for inhibitory activity. Accordingly, a marked decrease of potency was observed when it was removed (**2h**), or shifted at C6 (**2i**). Contrastingly, modification at C2–C3 of the quinoline moiety, and in particular replacing the quinoline system of **1** by a substituted 1,2,3,4-tetrahydroacridine ring resulted in the synthesis of **3a**, which displayed a K_i of 0.020 μM, thus being 37.5 times more potent than our prototype compound **1**, and representing the most potent NS3h inhibitor known to date. Compounds that lack a 6-OEt substituent displayed a marked decrease in inhibitory activity (**3b** vs **3a**). With compound **3a** in our hand, the synthesis of sulfonyl derivatives **3c** and **3d** was a logical choice in an attempt to further improve the inhibitory potency of **3a**. As expected, sulfonyl derivatives **3c** and **3d** resulted more potent than their quinoline counterparts (**2e** and **2g**, respectively). However, **3d** proved to be less potent than **3a**, probably due to the lack of synergism among the key pharmacophoric elements for enzyme interaction, namely the sulfonyl and the 1,2,3,4-tetrahydroacridine moieties.

Due to its exceedingly high inhibitory potency, compound **3a** was selected for further studies aimed at confirming its mechanism of inhibitory activity. As shown in Figure 2A, titrating increasing amounts of compound **3a** resulted in a dose-dependent reduction of the reaction products, visualized as displaced single

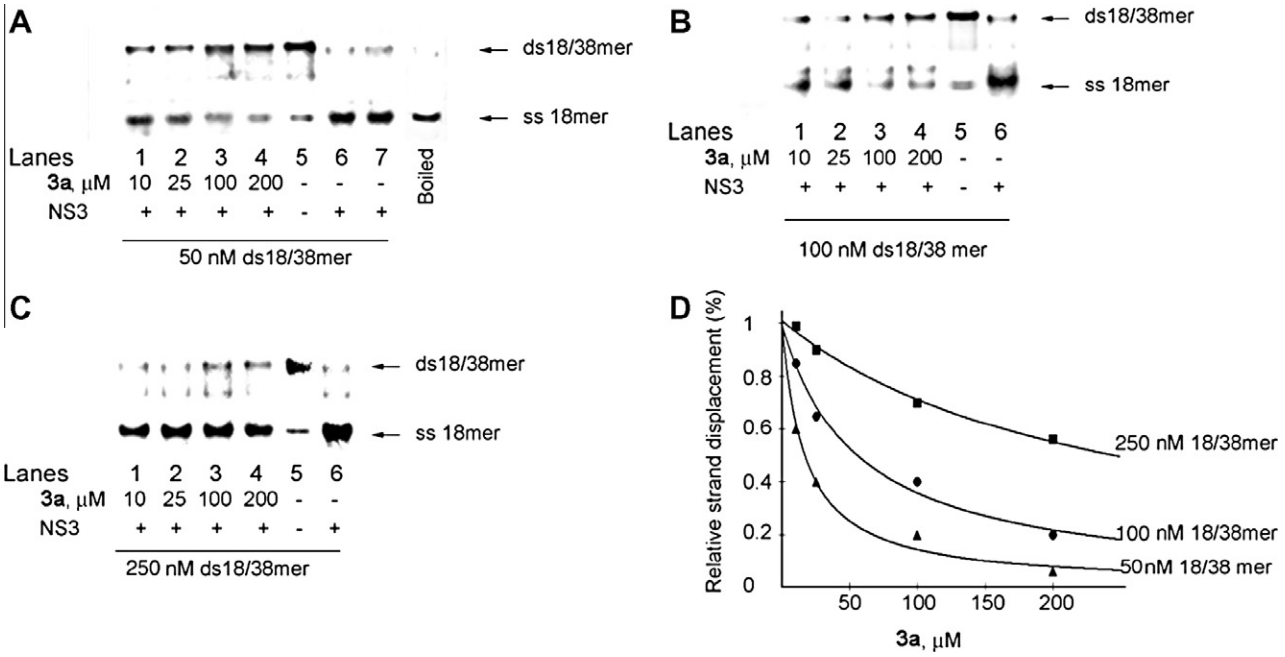


Figure 2. Compound **3a** is a competitive inhibitor of the NS3h activity. Experiments were performed as described in Ref. 19. (A) Native PAGE analysis of the reaction products of the NS3h activity in the presence of 5 nM NA template 5′-FAM-18/66-mer and in the absence (lane 6) or in the presence of increasing amounts of **3a** (lanes 1–4). Lane 5, control reaction in the absence of the enzyme. Lane 7, boiled substrate. (B) As in panel A, but in the presence of 10 nM NA template. (C) As in panel A, but in the presence of 25 nM of **3a**. (D) Dose–response curves for the inhibition of the NS3h activity by **3a** in the presence of increasing fixed NA concentrations. Values are the means of three independent experiments. Error bars are ±SD.

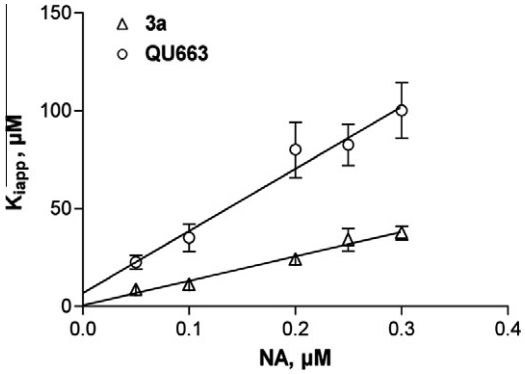


Figure 3. Variation of the apparent inhibitory constants (K_{iapp}) of **1** (QU663) and **3a** as a function of the NA concentrations. Data were fitted to the linear relationship: $K_{iapp} = K_i + (K_i/K_m^{NA}) \cdot [NA]$. Values are the means of three independent experiments. Error bars are ±SD.

strand (ss) (compare lanes 1–4 with lanes 6, 7). When the same experiment was repeated in the presence of increasing amounts of the NA substrate (Fig. 2B and C), the NS3h inhibitory effect of increasing amount of **3a** was reduced as the NA substrate concentration increased (compare panel B, lanes 1–4 with panel C, lanes 1–4). The dose–response curves shown in panel D clearly show that the potency of inhibition was inversely correlated to the concentration of the NA substrate, indicating a fully competitive mechanism of action of **3a**. The inhibition constant (K_i) for **3a** to the NS3h was 0.02 ± 0.002 μM. As shown in Figure 3, the apparent inhibitory constant (K_{iapp}) values for both **3a** and **1** increased as the NA substrate concentration increased, clearly showing an identical competitive mechanism of action. As expected, compound **3a** was much more potent than compound **1**, as indicated by the different slopes ($\approx K_i/K_m^{NA}$) of the curves. Similarly to **1**, compound **3a** did not show inhibition towards the ATPase activity of NS3 up to 100 μM, nor revealed any significant NA intercalating ability (data not shown).

In conclusion, exploiting our previous goal in the field and our understanding of the key structural and electron distribution features necessary to reproduce in a small organic molecule the electron distribution of purine and pyrimidine nucleotides,[19] we discovered **3a** as the most potent NS3h competitive inhibitor reported to date. As its simplified analog **1**, **3a** represents the prototypic compound of the class of nucleotide-mimicking competitive inhibitors of NS3h. This work could lead to a novel class of promising anti-HCV agents, typified by **3a**, potentially useful in the fight against this viral infection, although further biological assays to characterize these selective inhibitors are necessary.

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References and notes

1. Choo, Q. L.; Kuo, G.; Weiner, A. J.; Overby, L. R.; Bradley, D. W.; Houghton, M. *Science* **1989**, *244*, 359.
2. Ascione, A.; Tartaglione, T.; Di Costanzo, G. G. *Dig. Liver Dis.* **2007**, *39*, S4.
3. Jain, M. K.; Zoellner, C. *Expert. Opin. Pharmacother.* **2010**, *11*, 673.
4. Webster, D. P.; Klenerman, P.; Collier, J.; Jeffery, K. J. *Lancet Infect. Dis.* **2009**, *9*, 108.
5. Opar, A. *Nat. Rev. Drug Discov.* **2010**, *9*, 501.
6. Kolykhalov, A. A.; Mihalik, K.; Feinstone, S. M.; Rice, C. M. *J. Virol.* **2000**, *74*, 2046.
7. Borowski, P.; Schalinski, S.; Schmitz, H. *Antiviral Res.* **2002**, *55*, 397.
8. Tai, C. L.; Chi, W. K.; Chen, D. S.; Hwang, L. H. *J. Virol.* **1996**, *70*, 8477.
9. Gwack, Y.; Kim, D. W.; Han, J. H.; Choe, J. *Eur. J. Biochem.* **1997**, *250*, 47.
10. Sampath, A.; Padmanabhan, R. *Antiviral Res.* **2009**, *81*, 6.
11. Gordon, C. P.; Keller, P. A. *J. Med. Chem.* **2005**, *48*, 1.
12. Chen, C. S.; Chiou, C. T.; Chen, G. S.; Chen, S. C.; Hu, C. Y.; Chi, W. K.; Chu, Y. D.; Hwang, L. H.; Chen, P. J.; Chen, D. S.; Liaw, S. H.; Chern, J. W. *J. Med. Chem.* **2009**, *52*, 2716.
13. Borowski, P.; Deinert, J.; Schalinski, S.; Bretner, M.; Ginalski, K.; Kulikowski, T.; Shugar, D. *Eur. J. Biochem.* **2003**, *270*, 1645.
14. Krawczyk, M.; Wasowska-Lukawska, M.; Oszczapowicz, I.; Boguszewska-Chachulska, A. M. *Biol. Chem.* **2009**, *390*, 351.
15. Diana, G. D.; Bailey, T. R. U.S. 5633,388, 1997.
16. Janetka, J. W. L.; Mullican, M. D. WO 00/24725, 2000.
17. Phoon, C. W.; Ng, P. Y.; Ting, A. E.; Yeo, S. L.; Sim, M. M. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1647.
18. Kandil, S.; Biondaro, S.; Vlachakis, D.; Cummins, A. C.; Coluccia, A.; Berry, C.; Leyssen, P.; Neyts, J.; Brancale, A. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2935.
19. (a) Maga, G.; Gemma, S.; Fattorusso, C.; Locatelli, G. A.; Butini, S.; Persico, M.; Kukreja, G.; Romano, M. P.; Chiasserini, L.; Savini, L.; Novellino, E.; Nacci, V.; Spadari, S.; Campiani, G. *Biochemistry* **2005**, *44*, 9637; (b) Campiani, G.; Aiello, F.; Fabbrini, M.; Morelli, E.; Ramunno, A.; Armaroli, S.; Nacci, V.; Garofalo, A.; Greco, G.; Novellino, E.; Maga, G.; Spadari, S.; Bergamini, A.; Ventura, L.; Bongiovanni, B.; Capozzi, M.; Bolacchi, F.; Marini, S.; Coletta, M.; Guiso, G.; Caccia, G. *J. Med. Chem.* **2001**, *44*, 305.
20. (a) Gemma, S.; Kukreja, G.; Tripaldi, P.; Altarelli, M.; Bernetti, M.; Franceschini, S.; Savini, L.; Campiani, G.; Fattorusso, C.; Butini, S. *Tetrahedron Lett.* **2008**, *49*, 2074; (b) Campiani, G.; Fattorusso, C.; Butini, S.; Gaeta, A.; Agnusdei, M.; Gemma, S.; Persico, M.; Catalanotti, B.; Savini, L.; Nacci, V.; Novellino, E.; Holloway, H. W.; Greig, N. H.; Belinskaya, T.; Fedorko, J. M.; Saxena, A. *J. Med. Chem.* **2005**, *48*, 1919.