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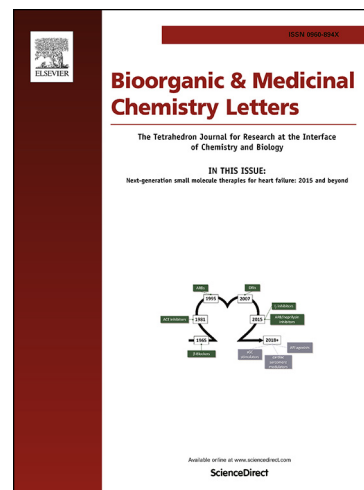
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Synthesis, Biological Evaluation and Mode of Action Studies of Novel Amidinourea Inhibitors of Hepatitis C Virus (HCV)

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ABSTRACT

Novel amidinourea derivatives have been synthesised and evaluated for their antiviral activity against Hepatitis C Virus (HCV). A compound with an amidinourea-spermine chemical structure, different from that of standard anti-HCV drugs, showed micromolar activity against HCV and excellent viability. Studies on the mode of action revealed that the new compound may act against HCV through the inhibition of IRES-mediated translation.

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Hepatitis C virus (HCV) infection represents a disease of significant global impact that afflicts around 170 million people worldwide.¹⁻² A small proportion of infected people clear the virus naturally,³ whilst the majority develop chronic hepatitis C (CHC) which can lead to a spectrum of liver diseases from mild inflammation to extensive liver fibrosis and cirrhosis, conferring significant morbidity and mortality to affected individuals.⁴ The world health organization (WHO) estimates that globally around 200 million people are chronically infected with HCV, with 3-4 million new infections occurring every year.⁵ The standard therapy for HCV infection has been based for more than a decade on the use of pegylated interferon alpha (PEG-IFN α) and the antiviral nucleoside analogue ribavirin (RBV).⁶ Since 2011, a number of directly acting antivirals (DAAs), such as the protease inhibitors (PIs) telaprevir, boceprevir and simeprevir,⁷ and the viral RNA-dependent RNA polymerase inhibitor sofosbuvir,⁸⁻⁹ have been licensed for use as part of combination therapies for HCV and CHC infections. These innovative treatment regimens have revolutionized the field of HCV medicine and provided optimism to find a cure in HCV patients. However, the fight against HCV infections is not fully over yet, because of the high costs associated with the therapies as well as the emergence of mutant strains resistant to DAA drugs.¹⁰⁻¹²

We recently reported the design and discovery of new amidinourea HCV inhibitors with structure **A** (Figure 1), as analogues of the antiviral drug moroxydine.¹³ As a continuation of this work, the synthesis and biological evaluation of a new series of amidinourea¹⁴ derivatives with general structure **B** was planned. Since the natural polyamines¹⁵ spermine, spermidine and

putrescine were recently reported to possess inhibitory activity against HCV,¹⁶ we became intrigued by the possibility to merge both the amidinourea and the polyamine moieties into a hybrid structure. This resulted led to the design of a series of amidinourea-polyamine hybrids with general structure **B** (Figure 1).

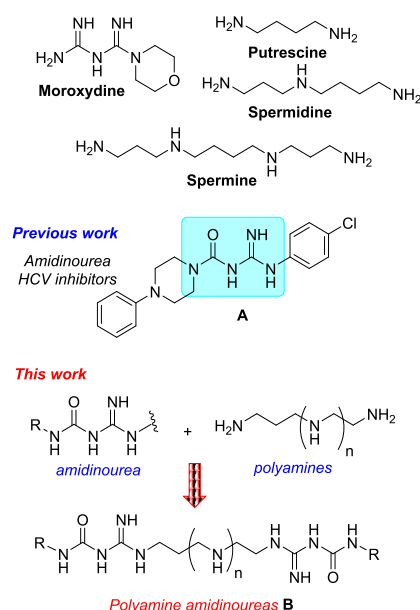
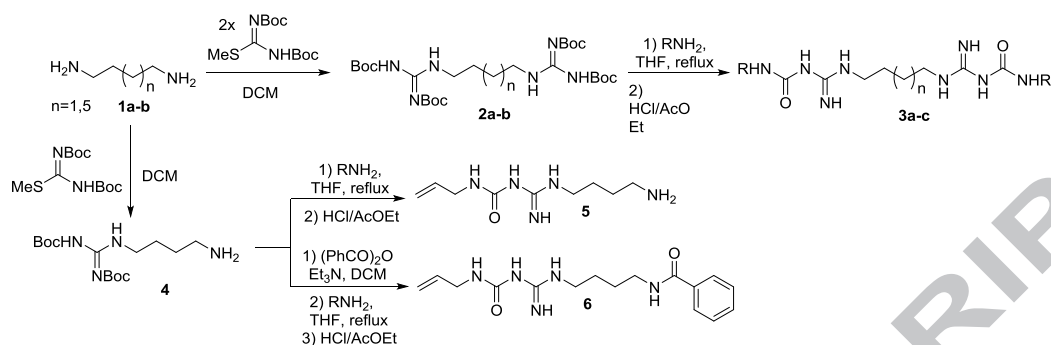
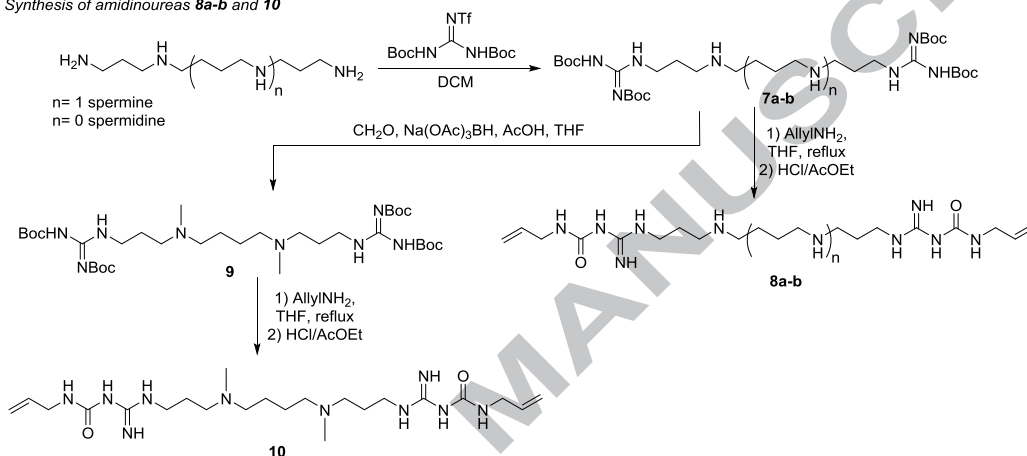


Figure 1. Structure of polyamines and anti-HCV amidinoureas

a) Synthesis of amidinoureas **3a-c**, **5** and **6**b) Synthesis of amidinoureas **8a-b** and **10**Scheme 1. Synthesis of amidinoureas derivatives **3a-c**, **5**, **6**, **8a-b** and **10**

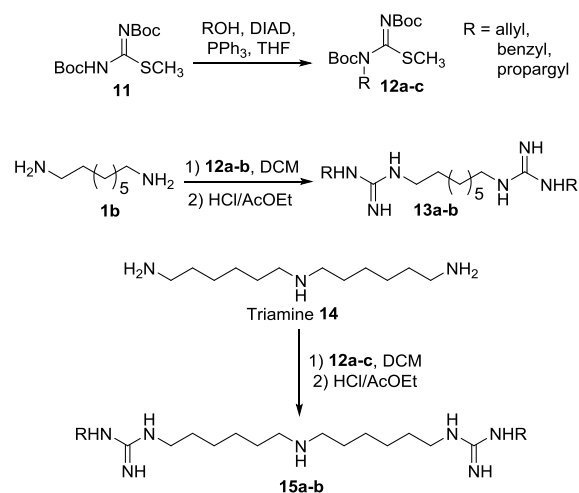
In this work, the synthesis, biological evaluation and mode of action studies of new polyamine amidinourea **B** as inhibitors of HCV is described.

The synthesis of a set of amidinourea compounds derived from the diamines putrescine **1a** and 1,8-diaminooctane **1b** was first planned. The diamines **1a-b** were reacted with *N,N'*-di-Boc-S-methylisothiurea in DCM at room temperature affording the Boc-guanidines **2a-b**.¹⁷ The latter were reacted with an appropriate amine (allylamine, benzylamine and *p*-Cl-aniline) and then treated with HCl/AcOEt solution to afford the corresponding amidinoureas **3a-c**. Scheme 1. Putrescine was also converted into the monoguanidine derivative **4** which in turn led the amidinoureas **5** and **6** through reaction with allylamine, benzoyl chloride and Boc deprotection as shown in Scheme 1. The choice of an allyl substituent on the amidinourea moiety arose from preliminary data and previous work on similar amidinourea derivatives endowed with antimicrobial activity.¹⁸⁻¹⁹

Amidinoureas **8a-b** bearing a spermine and spermidine backbone respectively were synthesised in a similar way as reported in Scheme 1. The guanylated intermediates **7a-b**, obtained via guanylation of the appropriate polyamine, were reacted with allylamine in refluxing THF affording, after Boc deprotection, the derivatives **8a-b**. The secondary amine groups in **7a** were also converted into tertiary amines via reductive amination reaction leading to **9**, in order to explore the importance of an NH moiety for the antiviral activity. The reaction of **9** with allyl amine and Boc-deprotection led to amidinourea **10**.

Finally, a set of guanidine derivatives **13a-b** and **15a-b** were synthesised as described in Scheme 2 with the aim to evaluate a

correlation between the presence of an amidinourea moiety on a polyamine backbone with the antiviral activity. The alkylated S-methylisothiurea derivatives **12a-c** were synthesised via Mitsunobu reaction according to previously reported procedures.²⁴ Reaction of **12a-c** with 1,8-diaminooctane **1b** and the triamine **14**, followed by Boc cleavage with HCl/AcOEt led to guanidine derivatives **13a-b** and **15a-b**.

Scheme 2. Synthesis of guanylated polyamines **13a-b** and **15a-b**

All the compounds **3a-c**, **5**, **6**, **8a-b**, **10**, **13a-b** and **15a-b** were assayed for their potential cytotoxicity at a single dose of 10 μ M on Huh7 cells. Interestingly, all compounds proved to be not toxic, with the exception of **3a** and **3b** which exhibited high cellular toxicity and therefore were excluded from further

experiments (Figure 2a). The non-cytotoxic compounds were then assayed as potential HCV antiviral agents, specifically as potential HCV entry or replication inhibitors.

Initially, the antiviral activity of amidinourea and guanidine derivatives on HCV entry using HCV pseudo-particles (HCVpp) at a single dose of 10 μM was evaluated. All the compounds showed no entry effect, in contrast with the great inhibition detected from the positive control bafilomycin (Figure 2b). The same compounds were then tested on HCV transient replication using a sub-genomic HCV replicon. Accordingly, Huh7 cells were electroporated with HCV RNA and seeded in the presence of the potential inhibitors at the concentration of 10 μM for 24h. As shown in Figure 2c, the spermine-amidinourea derivative **8a** was the sole active molecule, exhibiting a strong antiviral activity (90%). Since only one compound showed antiviral activity, a Structure Activity Relationship (SAR) study was not possible. However, it looks clear that the anti-HCV activity must be associated to the presence of a spermine backbone mainly. Moreover, the amine groups in the spermine chain must be secondary, as no activity was observed for the tertiary amine derivative **10**.

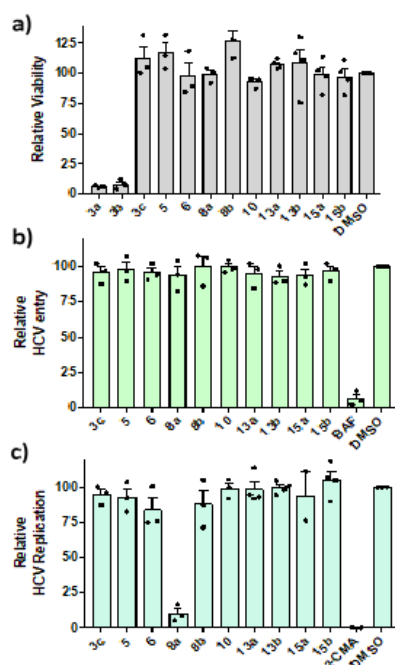


Figure 2. Antiviral activity and cytotoxicity of amidinourea and guanidine compounds. a) Huh7 cells were treated with 10 μM of each compound and incubated for 24h before evaluating cell viability. b) Huh7 cells were treated with each compound and the concentration of 10 μM for 1h and then infected with HCVpp for 3h in the presence of the compounds. Viral entry was detected 72h post infection. c) Huh7 cells, electroporated with HCV subgenomic RNA, were seeded and treated with 10 μM of each compound for 24h before measuring luciferase.

However, despite the disappointing screening results, the amidinourea **8a** showed an excellent antiviral profile and thus it was further characterised performing a full dose response scale on the transient replication using a starting concentration of 30 μM , following 1:3 dilutions (Figure 3a). A significant dose dependency was observed, with an IC_{50} of 2.65 μM . Again, no effects on the cellular viability were detected, except a modest toxic effect at 30 μM .

Based on the results of the initial screening which showed a good inhibitory activity and low toxicity for **8a**, and intrigued by its innovative and uncommon chemical structure when compared to standard anti-HCV agents, we decided to further investigate this compound try to elucidate its mode of action.

We initially validated our findings by evaluation of compound **8a** on the fully infectious HCV cell-cultured, using the JFH-1 clone.²⁰ Following viral inoculation for 3h, Huh7-J20 infected cells were exposed to **8a** treatment for a period of 72h with the same dose-response concentrations tested for transient replicons. A good cellular viability was observed, with a moderate cytotoxicity detected only at the concentration of 30 μM and a predicted CC_{50} value of 81.1 μM . The compound also showed a good antiviral activity on fully infectious HCV cell-cultured, with an IC_{50} value of 12.3 μM (Figure 3b), in line with the antiviral effect observed on the HCV replicon. In order to understand whether the slightly reduced antiviral activity of **8a** was due to a structure-related issue affecting the drug entry or drug stability or to a different mechanism of action, the amidinourea **8a** was re-tested against the transient replicon at the concentration of 10 μM , allowing the replication to establish for 24 h before treating with **8a** for an additional 24h. Interestingly, a reduced antiviral activity (~45%, Figure 4a) compared to immediate exposure to the compounds (~90%) was observed, suggesting a potential role of **8a** in the early stage of HCV replication. With the aim to explore this hypothesis, Huh7 cells transfected with wild-type replicon RNA (WT) were seeded in the presence of the compound **8a** and monitored at 2, 4, 8 and 24h. Interestingly, a strong antiviral phenotype was observed after only 2 h and maintained throughout the experiment (Figure 4b). Thus, we decided to evaluate the efficacy of **8a** against a replication-defective replicon (GND), which, once transfected, is only able to be translated to generate mature proteins, resulting in a loss of signal in 24h. As it is evident from Figure 4c, **8a** exhibited a consistent antiviral activity against GND, showing a constant reduction from 2 to 8 hours post-transfection. Comparing inhibition levels between WT and GND showed same antiviral efficacy at every time point considered (Figure 4d), suggesting that the antiviral effect of **8a** is exerted through inhibition of viral translation. We finally evaluated the effect of **8a** on a stable replicon cell line, persistently expressing HCV subgenomic genome (Huh7-J17).²¹ A moderate effect was detected on viral replication, with the maximal antiviral effect exerted in the first 24h (45%) (Figure 4e).

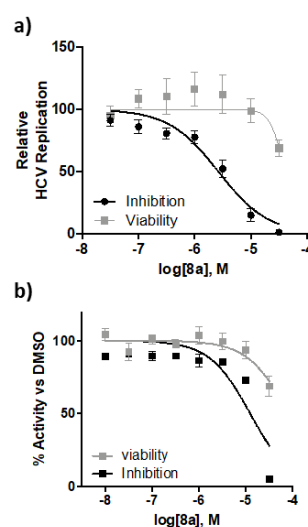


Figure 3. Dose response scale curves for amidinourea **8a** on HCV replicon or HCVcc. a) Huh7 cells were electroporated with HCV replicon RNA and exposed to **8a** for 24h. b) Huh7-J20 cells

were infected with fully infectious HCV (JFH1), treated for 72h and then harvested to evaluate viral replication. Grey dots and

line: cellular viability; black dots and line: viral replication.

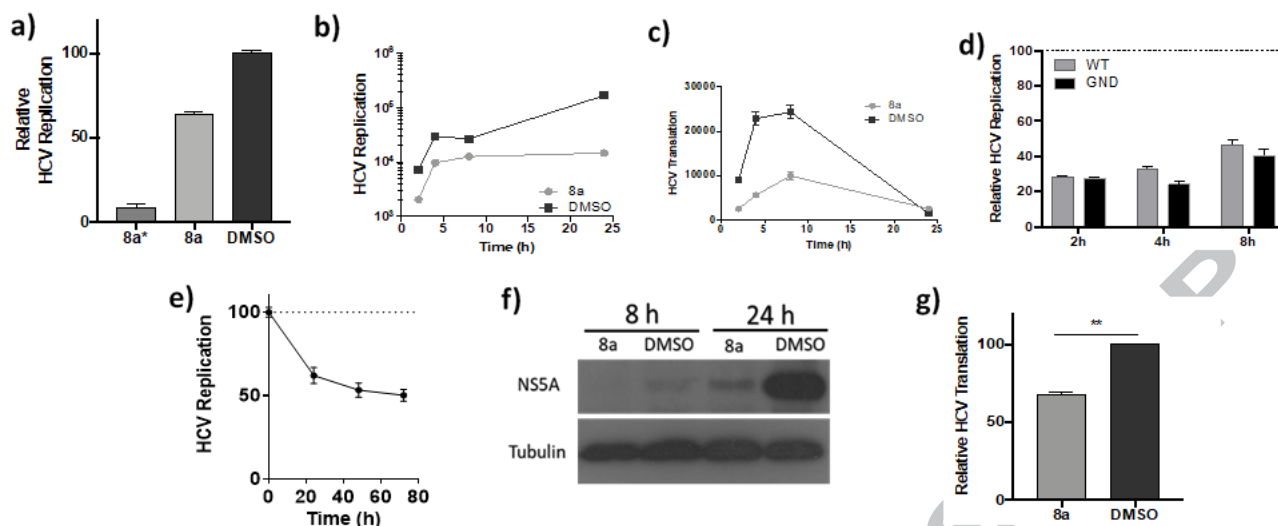


Figure 4. Effect of amidinorea **8a** on early stage of viral replication and on initial processing and IRES-mediated translation. a) Huh7 cells were electroporated with HCV subgenomic RNA and immediately treated with **8a** (left bar) or incubated for 24h before being treated with **8a** for 24h (centre bar). b-c) Time course of **8a** on HCV replicon (b) or replication defective GND (c). Huh7 cells were transfected with HCV RNA and seeded in the presence of **8a**. Viral replication/translation was evaluated at 2, 4, 8 and 24h post transfection. d) Antiviral effect of **8a** at each time point evaluated compared to untreated cells. e) Huh7 cells, stably expressing HCV replicon RNA, were treated with **8a** and the viral replicon was evaluated at 24, 48 and 72h. f) Huh7 cells were transfected with HCV replicon RNA and immediately treated with **8a** for 8 or 24h. g) Huh7 were transfected with a plasmid encoding for firefly luciferase under the control of HCV IRES and co-transfected with a plasmid encoding for the Renilla luciferase to normalise for transfection efficiency

It was speculated that **8a** could affect the protein translation or processing in HCV replication cycle. The viral protein processing was thus investigated, to establish whether the HCV inhibition was determining an increased amount of unprocessed polyprotein, due to a block in the processing, or a reduced amount of mature protein, indicating in this case an impaired translation.

For this purpose, Huh7 cells, transfected with HCV replicon RNA, were treated with **8a** for 8 or 24h and then assayed by western blot to evaluate the viral protein NS5A. As reported in Figure 4f, we detected a significantly reduced amount of NS5A, while no accumulation of unprocessed polyprotein was detected, suggesting a role for **8a** in affecting viral translation. To confirm our hypothesis, the effect of **8a** on the HCV IRES-mediated translation was finally evaluated. To this scope, Huh7 cells were co-transfected with two plasmids: 1) one expressing firefly luciferase under the control of HCV IRES and 2) another expressing renilla firefly under the control of TK promoter to normalise data for transfection efficiency. Cells were exposed to **8a** for 24h before assaying for dual luciferase. Results showed a moderate, although significant, inhibition (40%, Figure 4g) of firefly translation, supporting the hypothesis that **8a** is controlling HCV replication through a modulation of its IRES-mediated translation. It is noteworthy, that some of the known HCV IRES inhibitors described in the literature to date bear a guanidine/bis-guanidine moiety,²²⁻²⁴ clearly indicating a key role of this, and similar groups like amidinoureas, for antiviral activity.

In conclusion, a novel inhibitor **8a** of HCV, with a novel amidinourea-spermine structure, has been identified. The new compound **8a** disclosed with this work showed a good antiviral profile and excellent viability. Preliminary studies on the mode of action suggest that compounds **8a** could inhibit HCV by modulating the RNA IRES translation. Further studies are in

progress in our labs to fully confirm the mode of action and to design analogues of **8a** bearing a spermine backbone to be tested against wild-type and resistant HCV.

Acknowledgments

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Supplementary Material

Experimental procedures and full characterization for new compounds and intermediates are reported in the Supporting Information.

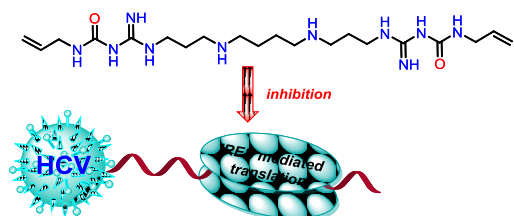
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