Synthesis, biological evaluation and mode of action studies of novel amidinourea inhibitors of hepatitis C virus (HCV)

Andrea Magri, Omar Mokrane, Kate Lauder, Arvind H. Patel, Daniele Castagnolo

A R T I C L E  I N F O

Keywords:
HCV
Amidinourea
Polyamine
HCV translation
RNA IRES

A B S T R A C T

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.

Hepatitis C Virus (HCV) infection represents a disease of significant global impact that affects around 170 million people worldwide. A small proportion of infected people clear the virus naturally, 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. 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. The standard therapy for HCV infection has been based for more than a decade on the use of pegylated interferon alpha (PEG-IFNα) and the antiviral nucleoside analogue ribavirin (RBV). Since 2011, a number of directly acting antivirals (DAAs), such as the protease inhibitors (PIs) telaprevir, boceprevir and simeprevir, and the viral RNA-dependent RNA polymerase inhibitor sofosbuvir, 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.

We recently reported the design and discovery of new amidinourea HCV inhibitors with structure A (Fig. 1), as analogues of the antiviral drug moroxydine. As a continuation of this work, the synthesis and biological evaluation of a new series of amidinourea derivatives with general structure B was planned. Since the natural polyamines spermine, spermidine and putrescine were recently reported to possess inhibitory activity against HCV, we became intrigued by the possibility to merge both the amidinourea and the polyamine moieties into a hybrid structure. This resulted in the design of a series of amidinourea-polyamine hybrids with general structure B (Fig. 1).

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-methylisothiourea in DCM at room temperature affording the Boc-guanidines 2a–b. The latters 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.

Amidinoureas 8a–b bearing a spermine and spermidine backbone...
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 (Fig. 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 24 h. As shown in Fig. 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.

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 (Fig. 3a). A significant dose dependency was observed, with an IC50 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 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. Following viral inoculation for 3 h, Huh7-J20 infected cells were exposed to 8a treatment for a period of 72 h 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 CC50 value of 81.1 µM. The compound also showed a good antiviral activity on fully infectious HCV cell-cultured, with an IC50 value of 12.3 µM (Fig. 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 24 h. Interestingly, a reduced antiviral activity (~45%, Fig. 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 24 h. Interestingly, a strong antiviral phenotype was observed after only 2 h and maintained throughout the experiment (Fig. 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 24 h. As it is evident from Fig. 4c, 8a exhibited a consistent antiviral activity against GND, showing a constant reduction from 2 to 8 h post-transfection. Comparing inhibition levels between WT and GND showed the same antiviral efficacy at
every time point considered (Fig. 4d), suggesting that the antiviral effect of 8a is exerted through the 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 24 h (45%) (Fig. 4e).

It was speculated that 8a could affect the protein translation or processing in the 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 24 h and then assayed by western blot to evaluate the viral protein NS5A. As reported in Fig. 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 24 h before assaying for dual luciferase. Results showed a moderate, although significant, inhibition (40%, Fig. 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

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

Fig. 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 24 h. b) Huh7-J20 cells were infected with fully infectious HCV (JFH1), treated for 72 h and then harvested to evaluate viral replication. Grey dots and line: cellular viability; black dots and line: viral replication.
action and to design analogues of 8a bearing a spermine backbone to be tested against wild-type and resistant HCV.

Acknowledgments

We gratefully acknowledge the EPSRC UK National Mass Spectrometry Facility for providing the mass spectrometry data. Royal Society (RG160870) is acknowledged for financial support. MRC (grant MC_UU12014/2) is acknowledged for financial support.

Appendix A. Supplementary data

Supplementary data (Experimental procedures and full characterisation for new compounds and intermediates) to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.01.008.

References

1. Mohamed AA, Elbedewy TA, El-Serafy M, El-Toukhy N, Ahmed W, El Din ZA. Hepatitis C virus: A global view. World J Hepatol. 2015;7:2676–2680.
2. http://www.who.int/news-room/fact-sheets/detail/hepatitis-c.
3. Grebely J, Prins M, Hellard M, et al. Hepatitis C virus clearance, reinfection, and persistence, with insights from studies of injecting drug users: towards a vaccine. Lancet Infect Dis. 2012;12:408–414.
4. Chen SL, Morgan TR. the natural history of hepatitis C virus (HCV) infection. Int J Med Sci. 2010;7:47–52.
5. Shepard CW, Finelli L, Ahn M, et al. Hepatitis C virus clearance, reinfection, and persistence, with insights from studies of injecting drug users: towards a vaccine. Lancet Infect Dis. 2012;12:408–414.
6. Santantonio T, Pausano M, Sinisi E, et al. Efficacy of a 24-week course of PEG-interferon alpha-2b monotherapy in patients with acute hepatitis C after failure of spontaneous clearance. J Hepatol. 2005;42:329–333.
7. Lin TL, Lent O, Faiming G, et al. In vitro activity and preclinical profile of TMC435350, a potent hepatitis C virus protease inhibitor. Antimicrob Agents Chemother. 2009;53:1377–1385.
8. Bhatta HK, Singh H, Grewal N, Natt NK. Sofosbuvir: a novel treatment option for chronic hepatitis C infection. J Pharm Anal. 2014;5:278–284.
9. Smith K. Viral hepatitis: Sofosbuvir: a new milestone in HCV treatment? Nat Rev Gastroenterol Hepatol. 2013;10:258–259.
10. Issur M, Götte M. Resistance patterns associated with HCV NS5A inhibitors provide limited insight into drug binding. Viruses. 2014;6:4227–4241.
11. Gane EJ, Metivier S, Nahass R, et al. The emergence of NS5B resistance associated substitution S282T after sofosbuvir-based treatment. Hepato Commun. 2017;1:536–549.
12. Walker A, Filipe S, Lúbbe N, et al. Detection of a genetic footprint of the sofosbuvir resistance-associated substitution S282T after HCV treatment failure. Viral J. 2017;14:106.
13. Magri A, Reilly R, Scalacci N, et al. Rethinking the old antiviral drug moroxydine: discovery of novel analogues as potent anti-hepatitis C virus (HCV) agents. Bioorg Med Chem Lett. 2015;25:5372–5376.
14. Castagnolo D. Chapter 5 New Strategies in Chemical Synthesis and Catalysis. Wiley-VCH; 2012:97.
15. Castagnolo D, Scheneose S, Botta M. Guanylated diamines, triamines, and polyanilines: chemistry and biological properties. Chem Rev. 2011;111:5247.
16. Kourtisina AN, Tumikiai VK, Khomutov MA, et al. Biogenic polyamines spermine and spermidine activate RNA polymerase and inhibit RNA helicase of hepatitis C virus. Biochemistry (Mosc). 2012;77:1172–1180.
17. Bass R, Jenkinson S, Wright J, Smulders-Srinivasan T, Marshall JC, Castagnolo D. Synthesis and biological evaluation of amidinourea and triazine congeners as inhibitors of MDA-MB-231 human breast cancer cell proliferation. ChemMedChem. 2017;12:1–5.
18. Sanguinetti M, Sanfilippo S, Castagnolo D, et al. Novel macrocyclic amidinourea: potent non-azole antifungals active against wild-type and resistant candida species. ACS Med Chem Lett. 2013;4:852–857.
19. Manetti F, Castagnolo D, Raffi F, et al. Synthesis of new linear guanidines and macrocyclic amidinourea derivatives endowed with high antifungal activity against Candida spp. and Aspergillus spp. J Med Chem. 2009;52:7376–7379.
20. Wakioka T, Pirsigmeier M, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. J Gen Virol. 2005;86:739–745.
21. Magri A, Bellire R, Renda S, et al. Hexokinase I N-terminal based peptide prevents the helicase activity of hepatitis C virus. J Hepatol. 2004;50:382–388.
22. Davis DR, Seth PP, Robinson DE, et al. Biaryl guanidine inhibitors of in vitro HCV-IRES. Bioorg Med Chem Lett. 2004;14:5139–5143.
23. Davis DR, Seth PP. Therapeutic targeting of HCV internal ribosomal entry site RNA. Annu Rev Chem. 2010;21:117–128.
24. Dhroor SM, Parsons J, Carnevali M, et al. Hepatitis C virus translation inhibitors targeting the internal ribosomal entry site. J Med Chem. 2014;57:1694–1707.