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Studies on the anti-hepatitis C virus activity of newly synthesized tropolone derivatives: Identification of NS3 helicase inhibitors that specifically inhibit subgenomic HCV replication

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ABSTRACT

We synthesized new tropolone derivatives substituted with cyclic amines: piperidine, piperazine or pyrrolidine. The most active anti-helicase compound (IC_{50} = 3.4 μM), 3,5,7-tri[(4′-methylpiperazin-1′-yl)methyl]tropolone (**2**), inhibited RNA replication by 50% at 46.9 μM (EC_{50}) and exhibited the lowest cytotoxicity (CC_{50}) >1 mM resulting in a selectivity index (SI = CC_{50}/EC_{50}) >21. The most efficient replication inhibitor, 3,5,7-tri[(4′-methylpiperidin-1′-yl)methyl]tropolone (**6**), inhibited RNA replication with an EC_{50} of 32.0 μM and a SI value of 17.4, whereas 3,5,7-tri[(3′-methylpiperidin-1′-yl)methyl]tropolone (**7**) exhibited a slightly lower activity with an EC_{50} of 35.6 μM and a SI of 9.8.

1. Introduction

Hepatitis C virus (HCV) remains one of the most important viral threats to human health in spite of advanced studies on the development of new drugs.[1] The present treatment for HCV infection is still limited to the pegylated interferon-α (IFN-α) in combination with ribavirin, with response rates (sustained virological response) between 40% and 50% for genotype 1, the most prevalent genotype in Europe and the United States and up to 80% for genotypes 2 and 3.[2] Even the introduction of new directly acting antivirals targeted against the viral protease and polymerase will not solve one of the major problems encountered when treating HCV infections—that of viral breakthroughs due to the emergence of HCV mutants resistant to the treatment applied, observed also during the clinical trials with telaprevir, a protease inhibitor.[1]

Searching for an alternative, effective and well tolerated therapy against HCV we continued our studies on derivatives of tropolone. Tropolone is an aromatic but non-benzenoid compound consisting of a seven carbon ring. Tropolones naturally occur in the wood of members of the *Cupressaceae* family and have already been demonstrated to possess antimicrobial,[3,4] antifungal and insecticidal properties.[5,6] The tropolone derivative β-thujaplicinol is a selective inhibitor of the ribonuclease H activity of the HIV reverse transcriptase.[7] Furthermore, copper chelates of α-, β- and γ-thujaplicins inhibited apoptosis induced by *Human influenza virus* and prevented viral release and spread of the infection, and α-thujaplicin showed a strong cytotoxic effect in vitro in the cell line of murine lymphocytic leukaemia, while its 50%-lethal dose value in mice was quite low (256 mg/kg).[8–10] Hinokitiol (β-thujaplicin) and tropolone exhibit strong cytotoxic effects on selected murine and human tumour cell lines.[11]

Our previous screening for potential HCV NS3 helicase inhibitors revealed the inhibitory activity of tropolone derivatives, especially 3,7-dibromo-5-(morpholin-4′-ylmethyl)tropolone (**11**).[12] On the basis of these initial results, we synthesized new tropolone derivatives substituted with cyclic amines: piperidine, piperazine or pyrrolidine and tested their activity against the HCV NS3 helicase. To examine the anti-HCV activity of tropolone derivatives selected using the helicase assay, studies on inhibition of RNA replication in the HCV subgenomic replicon system developed by Bartenschlager and co-workers were carried out.[13,14] Our results confirm that the helicase assay allows the selection of compounds with significant antiviral activity that may be considered as lead compounds for the development of anti-HCV agents.

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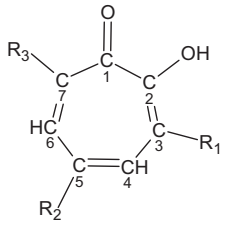
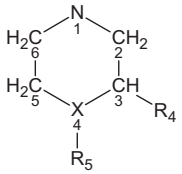
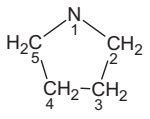




Cyclic amine (A_1)



Cyclic amine (A_2)

Compound	R_1	R_2	R_3	Cyclic amine (A_1)		
				X	R_4	R_5
1	CH_2-A_1	CH_2-A_1	CH_2-A_1	C	H	H
2	CH_2-A_1	CH_2-A_1	CH_2-A_1	N	H	CH_3
3	CH_2-A_2	CH_2-A_2	CH_2-A_2	-	-	-
4	CH_2-A_1	CH_2-A_1	CH_2-A_1	C	H	A_2
5	Cl	H	Cl	-	-	-
6	CH_2-A_1	CH_2-A_1	CH_2-A_1	C	H	CH_3
7	CH_2-A_1	CH_2-A_1	CH_2-A_1	C	CH_3	H
8	CH_2-A_1	CH_2-A_1	CH_2-A_1	C	H	OH
9	Br	H	Br	-	-	-
10	Br	H	H	-	-	-
11	Br	CH_2-A_1	Br	O	H	-

Scheme 1. Structures of tropolone derivatives.

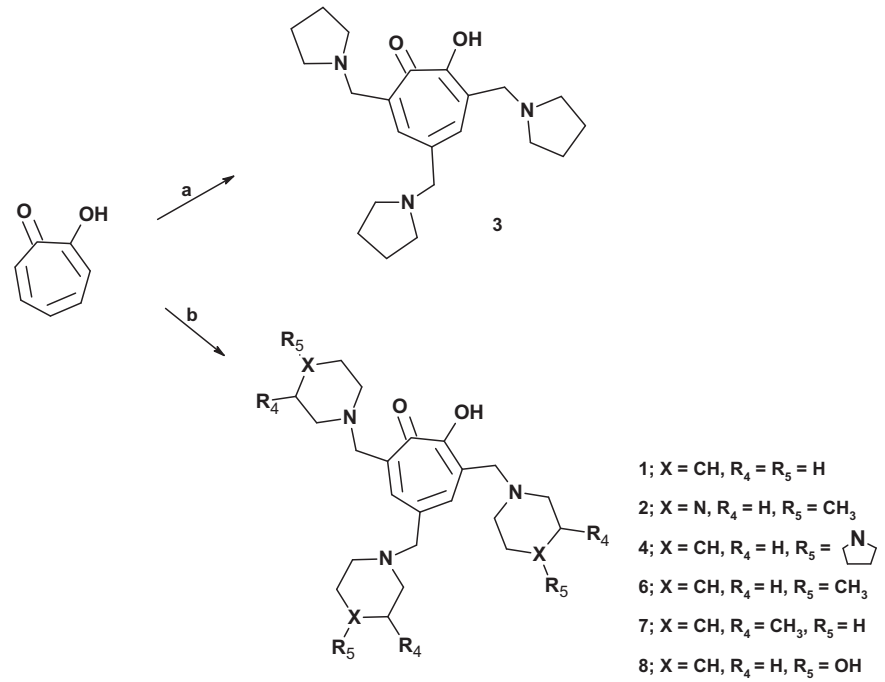
2. Results and discussion

2.1. Synthesis and characterization of tropolone derivatives

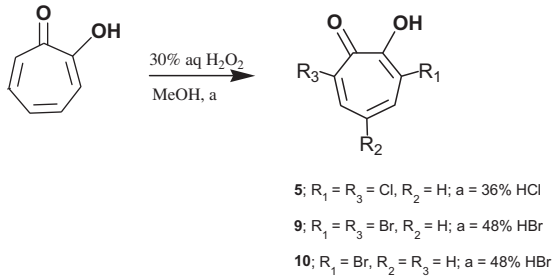
The tropolone derivatives employed in this study and their abbreviations are presented in Scheme 1. 3,5,7-Tri(piperidin-1′-ylmethyl)tropolone (**1**) was synthesized by a modification of the published method.[15] The mixture of tropolone and 37% aqueous formaldehyde was treated with 1-methylpiperazine. After adding the amine the temperature rose, the suspension dissolved and the resulting dark-orange solution was stirred at 70 °C for 7 min. After cooling, the product precipitated and was purified by crystallization. The method mentioned above was slightly modified to obtain the other analogs of tropolone, 3,5,7-tri(pyrrolidin-1′-ylmethyl)tropolone (**3**), 3,5,7-tri[(4′-pyrrolidin-1″-ylpiperidin-1′-yl)methyl]tropolone (**4**) and 3,5,7-tri [(4′-hydroxypiperidin-1′-yl)methyl]tropolone (**8**) (Scheme 2).

When the mixture of tropolone and 1-methylpiperazine was treated with formaldehyde and stirred at 65 °C for 5 min, 3,5,7-tri[(4′-methylpiperazin-1′-yl)methyl]tropolone (**2**, Scheme 2) was obtained and purified by crystallization. This method was also used for the synthesis of 3,5,7-tri [(4′-methylpiperidin-1′-yl)methyl]tropolone (**6**) and 3,5,7-tri [(3′-methylpiperidin-1′-yl)methyl]tropolone (**7**) (Scheme 2).

The halogeno derivatives of tropolone were synthesized using the procedure reported by Barhate et al. for the halogenation of aliphatic or aromatic hydrocarbons, aromatic amines and naphthalenes.[16–18] This method relies on the oxidation of hydrochloric or hydrobromic acid by hydrogen peroxide to generate positive halogen species in situ, leading to chloro or bromo derivatives of aromatic compounds. For the synthesis of 3,7-dichlorotropolone (**5**, Scheme 3) about 6 equiv of 36% aqueous HCl with 30% aqueous H_2O_2 were used. The 3,7-dibromotropolone (**9**) was obtained together with 3-bromotropolone (**10**) (Scheme 3) using 2 equiv of 48% aqueous HBr and 30% aqueous H_2O_2 in a methanolic solution at a low temperature (4 °C). The compounds were separated by crystallization from methanol.



Scheme 2. Synthesis of compounds **1**, **2**, **3**, **4**, **6**, **7** and **8**. (a) CH_2O, pyrrolidine; (b) **1**: CH_2O, piperidine; **2**: CH_2O, 1-methylpiperazine; **4**: CH_2O, 4-pyrrolidin-1-yl-piperidine, EtOH; **6**: CH_2O, 4-methylpiperidine; **7**: CH_2O, 3-methylpiperidine; **8**: CH_2O, 4-hydroxypiperidine.



Scheme 3. Synthesis of compounds **5**, **9** and **10**.

All the tropolone analogues were purified to homogeneity and characterized by NMR spectroscopy and mass spectrometry. In the ^{1}H NMR spectrum of **5** the signal of 3-H and 7-H is absent, while the signals of 4-H and 6-H form a doublet coupled to the 5-H triplet with ^3J = 10.7 Hz. These spectral features indicate substitution with chlorine at positions 3 and 7. In compound **9** the same positions are substituted by bromine. This results in minor changes in resonance positions, but does not affect the coupling pattern in comparison to **5**. In compound **10** the symmetry of the ^{1}H spin system is broken by bromine substitution at position 3. This is manifested by the appearance of four well resolved and mutually coupled resonances assigned to 4-H, 5-H, 6-H and 7-H. In the remaining compounds the 4-H and 6-H nuclei are magnetically equivalent and their resonance appears as a singlet. This indicates 3,5,7 substitutions. An additional singlet at δ = 3.43–3.62 (two protons) is assigned to the 5-CH_2 linkage, while a singlet at δ = 3.67–3.85 (four protons) comes from 3- and 7-CH_2 chemically equivalent linkages. The resonances coming from protons of cyclic amines which are connected to the CH_2 linkages mentioned are observed as unresolved multiplets. Due to extensive overlap of these signals, 2D NMR techniques were exploited to assign the multiplets and to confirm the structures of the newly synthesized tropolone derivatives.

Various solvents can be used to dissolve tropolone derivatives due to the different solubility of these compounds. Halogeno derivatives of tropolone are soluble in dimethyl sulfoxide (DMSO), but poorly soluble in chloroform and in methanol or EtOH; in turn cyclic amine-substituted tropolones are insoluble in DMSO, but soluble in chloroform (except **8**), EtOH and in water (with concentration limitations).

2.2. New helicase variants and helicase inhibition assay

New variants of the NS3 helicase (genotypes 1b and 3a) were isolated and cloned from infected blood samples from Polish HCV-infected patients. The nucleotide sequences of the NS3 helicase of the new variants were established and deposited in the GeneBank database under the following accession numbers: GU056838 and GU056839. Sequence comparisons with the helicase of genotype 1a used in our previous studies[19] revealed a significant divergence of nucleotide and amino acid sequences, typical of HCV isolates (79% and 91% identity for the isolates of genotype 1, 70% and 81–83% identity for both isolates of genotypes 1 and 3, respectively). None of the highly conserved NTPase and helicase motifs was changed.

The helicases of genotypes 1a, 1b and 3a were expressed in the baculovirus system and purified from insect cells following the protocol developed by Boguszewska-Chachulska et al.[19] About 15 mg/mL were obtained for each of the new helicase variants. The purity of the proteins reached 95% as estimated by SDS–PAGE and Coomassie staining.

To test the helicase activity of the new helicase variants, a fluorometric helicase activity assay was applied,[19] with 6 mM $MnCl_2$, 10 nM dsDNA substrate, 20 nM enzyme, 125 nM capture strand and 1.5 mM ATP. The proteins appeared to have different initial reaction velocities (data not shown), helicase 3a being the most potent enzyme that at 20 nM could unwind dsDNA with an initial velocity fourfold higher than that of helicase 1b. This phenomenon has already been reported for the enzymes derived from HCV genotypes 1a, 1b and 2a, and various mechanisms underlying differences in the enzyme activities were proposed, such as differences in the rate of ATP hydrolysis, nucleoside triphosphate (NTP) preference and strength of DNA binding.[20]

The helicase assay was applied to test new tropolone derivatives in the optimal buffer and ATP conditions selected on the basis of results obtained previously.[21] The tropolone derivatives were tested at 1–300 μM concentrations, depending on their solubility and the inhibition effect obtained. Compounds **5**, **9**, **10** and **11** were dissolved in DMSO, **3** in methanol, while **1, 2, 4, 6, 7** and **8** were dissolved in 70% EtOH. All the compounds tested were preincubated for 15 min at room temperature with the dsDNA substrate and the enzyme to facilitate formation of active complexes and interaction of inhibitors with their targets. The unwinding reaction was initiated by addition of ATP. All the 50% inhibitory concentrations (IC_{50}) values obtained as well as the IC_{50} value previously determined for 3,7-dibromo-5-(morpholin-4′-ylmethyl)tropolone (**11**) are presented in Table 1.[12] In several cases no IC_{50} values could be determined due to the low solubility of the compounds and/or lack of anti-helicase activity.

Compound **2**, the most efficient helicase inhibitor, was tested against three different variants of the NS3 helicase belonging to two main genotypes, 1 and 3, to determine its specificity (Table 2). No significant differences in the IC_{50} values were observed, indicating not only that there are no significant structural differences between various HCV isolates but also that this new inhibitor is not genotype-specific, which is an advantage in the design of anti-HCV drugs.[22]

2.3. Inhibition of replication of HCV subgenomic replicon

To test the effect of the compounds studied on RNA amplification as well as their cytotoxicity, Huh-7 cells harbouring the replicating subgenomic HCV were grown in the presence of the tropolone derivatives at concentrations ranging from 1 μM to 1 mM. In each experiment a constant volume of inhibitor was added, resulting in a final constant 1% concentration of solvent. As positive control, Huh-7 cells were grown with 1% solvent. The remaining level of HCV RNA replication in the cells was determined by measuring the luminescence signal.

The 50% effective concentration defined as the inhibitor concentration that reduced luminescence by 50% (EC_{50}), the 50% cytotoxic concentration defined as the compound concentration that inhib-

Table 1
IC_{50} values obtained for tropolone derivatives in the helicase assay

Compound	IC_{50}[a] (μM)
1	26.5 ± 6.8
2	3.4 ± 2.1
3	183.6 ± 25.5
4	7.0 ± 2.6
5	>150
6	10.5 ± 1.1
7	17.8 ± 2.1
8	22.7 ± 4.3
9	>150
10	>150
11	17.6 ± 6.8

[a] All the data represent mean values for at least three independent experiments ± standard deviation.

Table 2
IC_{50} values for compound **2** obtained with helicases of various genotypes

Genotype	IC_{50}[a] (μM)
hel1a	3.4 ± 2.1
hel1b	3.5 ± 1.9
hel3a	4.5 ± 2.9

[a] The data represent mean values for three independent experiments ± standard deviation.

ited cell growth by 50% (CC_{50}) and the selectivity index (SI = CC_{50}/EC_{50}) values obtained for the compounds tested, are presented in Table 3.

The tropolone derivatives tested proved to be weaker HCV replication inhibitors than amidinoanthracyclines or even acridone derivatives,[21,23] with EC_{50} values in the micromolar range. Unexpectedly, in the context of the results obtained in the helicase assay, two strong inhibitors of helicase activity (compounds **4** and **11**) as well as most of the remaining compounds were unable to inhibit replication of the HCV subgenomic replicon. They either showed no effect (**3, 4, 8**) or their CC_{50} values were similar to the EC_{50} values, resulting in SI values close to 1 (**5, 9, 10, 11**). Two isomers of methyl-substituted piperidine derivatives of tropolone, **6** and **7**, were the strongest inhibitors of replication among the tropolone derivatives tested, with EC_{50} values of 32 and 35.6 μM, respectively, while the methylpiperazine derivative of tropolone, compound **2**, with an EC_{50} value of 46.9 μM was the least cytotoxic compound tested (CC_{50} >1 mM) resulting in the highest SI value (>21) (Fig. 1). Surprisingly, another derivative of tropolone with unsubstituted piperidine (compound **1**) was a weak inhibitor with an EC_{50} value of 137 μM and an SI value of 3.6. The CH_3 group possibly plays an important role in the antiviral activity of the six-membered cyclic amine derivatives of tropolone evaluated in this study, especially considering the lack of antiviral activity of two differently substituted piperidine derivatives of tropolone: the pyrrolidine-substituted (**4**) and hydroxyl-substituted derivatives (**8**). However, this is the methylpiperazine substitution at positions 3, 5 and 7 of the tropolone ring that is the key pharmacophore element identified by these studies, as it seems responsible for the highest anti-helicase activity of compound **2** as well as its high antiviral activity, only slightly lower than that of compounds **6** and **7**.

Table 3
EC_{50}, CC_{50} and SI values of the tropolone derivatives

Compound	EC_{50}[a] (μM)	CC_{50}[a] (μM)	SI
1	137.4 ± 48.9	>500	>3.6
2	46.9 ± 11.7	>1000	>21.3
3	>500	>500	
4	>200	>200	
5	334.3 ± 6.0	253.8 ± 17.9	0.8
6	32.0 ± 1.1	556.0 ± 77.7	17.4
7	35.6 ± 2.5	348.0 ± 53.9	9.8
8	>250	>250	
9	89.8 ± 4.9	92.7 ± 9.0	1.0
10	121.3 ± 16.4	85.2 ± 3.0	0.7
11	176.5 ± 50.5	227.3 ± 37.5	1.3
4′-Azidocytidine	10.54 ± 2.50	>100	n.a.
Ribavirin	120.00 ± 37.11	177.94 ± 49.39	1.5

[a] All the data represent mean values for at least three independent experiments ± standard deviation.

In comparison with ribavirin, whose SI value in this assay was 1.5, compounds **2**, **6** and **7** had good SI values (>21.3, 17.4 and 9.8, respectively), indicating that there is a reasonable cytotoxicity/activity window. When compared to 4′-azidocytidine (kindly provided by Dr. Johan Neyts, Katolik Universität, Leuven, Belgium), compounds **2**, **6** and **7** showed higher EC_{50} values with similar or lower cytotoxicity. It should be emphasized that the most cytotoxic compounds are halogeno derivatives of tropolone.

2.4. Derivative 2 and 7-resistant mutants

To select resistant mutants, Huh-7 cells harbouring the subgenomic HCV replicon were grown in the presence of 100 μM derivatives **2** or **7** under the selective pressure provided by Geneticin (G418). In these conditions cells either lacking the replicon or containing a susceptible replicon are killed and the remaining cells form colonies within 3–4 weeks. After the selection the susceptibility of resistant colonies was tested. The results showed that the EC_{50} value for compound **2** tested in compound **2**-resistant colonies was threefold higher than the EC_{50} tested in naïve cells (131.35 ± 15.34 μM vs 46.9 ± 11.6 μM). It was similar to the EC_{50} of compound **7**-resistant mutants that was almost threefold higher than that of naïve cells (90.06 ± 3.90 μM vs 35.6 ± 2.5 μM). Moreover, the mutants selected against compound **2** were also resistant to **7** and vice versa, as shown in Figure 2. RNA of the selected mutant cells was harvested, reverse-transcribed to cDNA and the NS3-coding region was amplified by PCR. The PCR products were sequenced and only one mutation in the NS3 helicase sequence was detected (A to G), the same mutation for both types of mutants, which was not present in the HCV RNA of naïve cells. The mutation leads to one amino acid substitution in the NS3 helicase protein, threonine at position 477 is changed to alanine (Fig. 3). This mutation (T477A) is localised in the region of the NS3 helicase domain 2 that is not part of any conserved structure. Therefore inhibition is probably not competitive, but rather allosteric and this is consistent with the results of dependency experiments performed for the first tropolone inhibitor of helicase identified.[12] However, the exact mechanism of action remains unknown. Interestingly, this mutation is located on the NS3 protease-facing

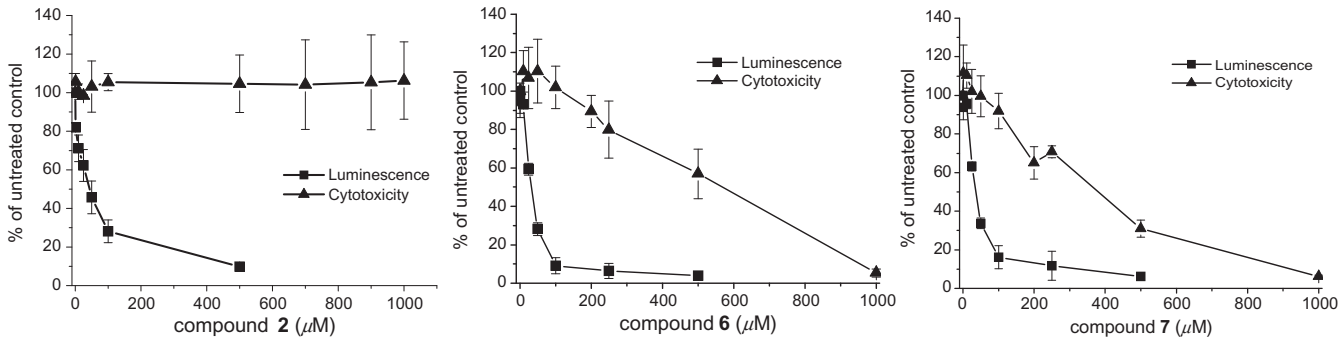


Figure 1. Inhibition of replication of the HCV subgenomic replicon by compounds **2**, **6** and **7**. Each point is the mean of three independent assays, with three replicates.

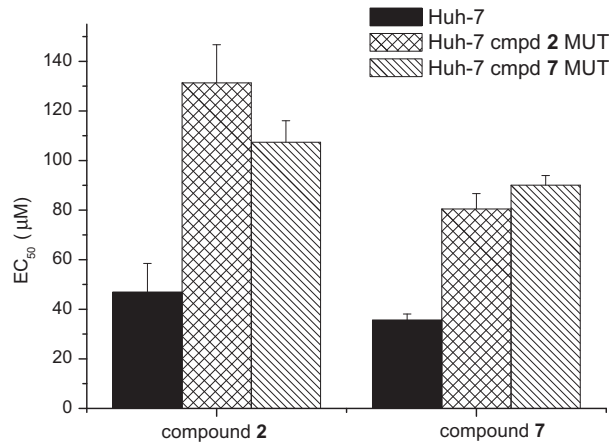


Figure 2. EC_{50} values of the Huh-7 cells resistant to compounds **2** and **7** as well as of naïve Huh-7 cells tested against either compound.

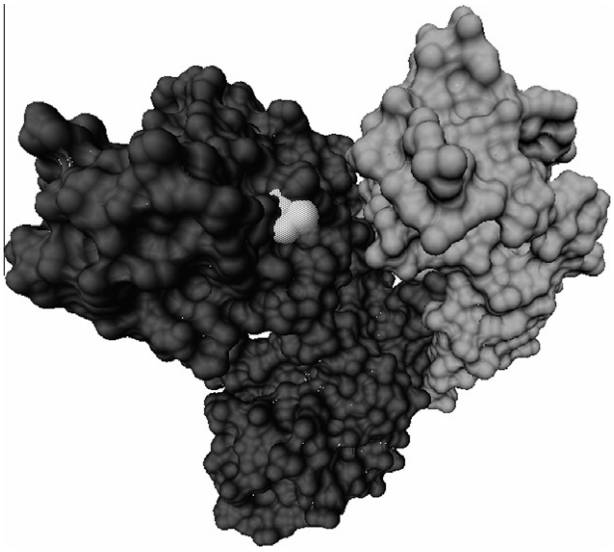


Figure 3. Structure of the NS3 protein (1CU1) with the mutation conferring resistance to compounds **2** and **7** marked in white. The helicase domain is highlighted in black and the protease domain in grey. The mutation is located on the NS3 protease-facing surface.

surface and tropolone binding may influence helicase-protease interactions (Fig. 3). Computer modelling of ligand-NS3 helicase interactions based on this experimental result is currently in progress but it must be supported by new interaction studies.

2.5. Synergy studies

It is important to test drug candidates in combination with other drugs or compounds that might be used in combinatorial therapies commonly employed in the treatment of viral infections. These experiments may prevent the use of combinations of drugs that antagonize each other, rendering the therapy ineffective. The effects of the compound **2**/IFN-γ and **2**/ribavirin combinations in cell culture were therefore evaluated. The results were analysed as described previously[24] and are presented in Figure 4A and B, respectively. When the effect of drug combination is additive, the data points form a horizontal surface that equals the zero plane. A surface that lies above the zero plane indicates a synergistic effect of the combination, and a surface below the zero plane indicates antagonism. In our study both combinations result in an additive effect with a very slight tendency to synergy, which might be expected of two drugs that have different modes of action and that do not interfere with their respective metabolism.

While cases of compounds that antagonize IFN are rare, there are examples of antagonism between ribavirin and a number of nucleoside analogs. In 1987 Vogt et al. discovered that ribavirin antagonizes the anti-*Human immunodeficiency virus* (anti-HIV) activity of the 3′-azido-3′-deoxythymidine (AZT) and more recently it was similarly demonstrated that ribavirin antagonizes the inhibitory activity of 2′-C-methylcytidine, an active component of the experimental anti-HCV drug valopicitabine.[25,26]

3. Conclusions

Three tropolone derivatives (**2, 6** and **7**) inhibit replication of the HCV subgenomic replicon in cell cultures, a property that has been shown only for a handful of HCV helicase inhibitors: a peptide inhibitor,[27] TBBT, DRBT[28] as well as acridone, anthracycline[21,23] and triphenylmethane derivatives.[29] Two of these tropolone derivatives, **2** and **7**, are the first anti-helicase compounds that inhibit HCV replication with the ability to cause the appearance of resistant mutants, which further proves that inhibition of replication is the result of inhibition of the helicase activity. The mutation conferring resistance to compound **2** was the same as for compound **7**,

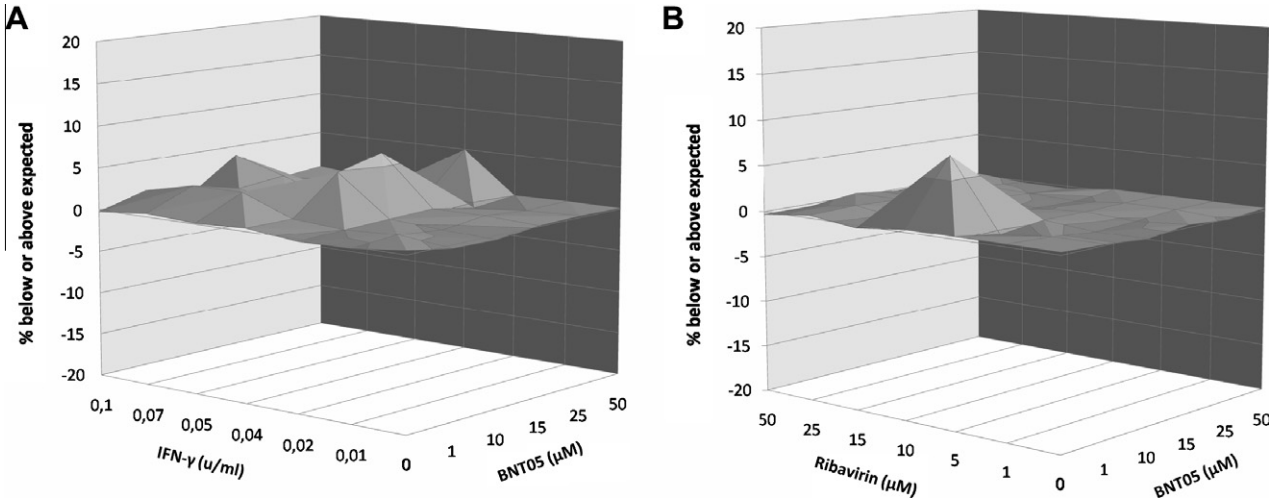


Figure 4. Combined anti-HCV activity of IFN-γ and compound **2** (A) and of ribavirin and compound **2** (B). Values under the zero plane indicate antagonistic activity, values in the zero plane indicate additive activity and the values above the zero plane indicate synergistic activity.

suggesting the same mechanism of action, possibly an allosteric inhibition. Localisation of this mutation on the NS3 protease-facing surface (Fig. 3) suggests that tropolone binding may influence helicase-protease interactions.

Despite its relatively high EC_{50} value of 46.9 μM, 3,5,7-tri[(4′-methylpiperazin-1′-yl)methyl]tropolone (**2**) is the most promising tropolone derivative identified here as it is non-cytotoxic at the highest concentrations studied (1 mM) that precluded the exact determination of its SI. Consequently, compound **2**, containing the methylpiperazine moiety as the key pharmacophore element, may be regarded as a lead to develop new compounds that can be used in future multidrug therapy.

4. Experimental

4.1. Chemistry

^{1}H, ^{1}H–^{1}H COSY, ^{1}H–^{13}C GHMBC and ^{1}H–^{13}C GHSQC NMR experiments were recorded on a Bruker Avance II 300 MHz spectrometer. The data of chemical shifts are reported with reference to Me_4Si (internal standard). They are reported in the following format: chemical shift (multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet), integration, coupling constants and assignment). NMR spectra were obtained in the Laboratory for Solid State NMR, Institute of Physical Chemistry, Polish Academy of Sciences (PAS). Mass spectra were recorded on a Micro-mass ESI Q-TOF spectrometer at the Mass Spectrometry Laboratory, Institute of Biochemistry and Biophysics (IBB), PAS. Starting materials were purchased from Sigma–Aldrich (Poznań, Poland). The derivatives of tropolone: 3,7-dibromotropolone (**9**), 3-bromotropolone (**10**) and 3,7-dibromo-5-(morpholin-4′-ylmethyl)tropolone (**11**) were synthesized by adapting the methods developed in our laboratory.[12] All solvents were of reagent grade. All yields reported are for dry compounds that require no further purification.

4.1.1. 3,5,7-Tri(piperidin-1′-ylmethyl)tropolone (1)

Piperidine (690 μL, 6.97 mmol) was slowly added to the mixture of tropolone (227 mg, 1.86 mmol) and 37% aqueous formaldehyde (720 μL, 9.61 mmol) cooled in an ice bath. While adding the amine the suspension dissolved and the resulting dark-orange solution was stirred at 70 °C for 7 min. After cooling the solution to room temperature, a precipitate was obtained and crystallized from acetone to give 400 mg (0.97 mmol, 52% yield) of compound **1**. MS (TOF) calcd for $C_{25}H_{40}N_3O_2^+$ (MH^+) *m/z* 414.3121, found 414.2466. ^{1}H NMR ($CDCl_3$, 298 K) δ: 1.33–1.71 [m, 18H; resolved in 2D spectra: 1.47 (3,5,7-tri-4′-CH_2), 1.62 (3,5,7-tri-3′-CH_2 and 3,5,7-tri-5′-CH_2)], 2.30–2.59 [m, 12H; resolved in 2D spectra: 2.40 (5-2′-CH_2 and 5-6′-CH_2), 2.47 (3,7-di-2′-CH_2 and 3,7-di-6′-CH_2)], 3.45 (s, 2H, 5-CH_2), 3.67 (s, 4H, 3-CH_2 and 7-CH_2), 7.83 (s, 2H, 4-H and 6-H); ^{13}C NMR ($CDCl_3$, 298 K) δ: 24.2 (3,5,7-tri-4′-CH_2), 26.1 (3,5,7-tri-3′-CH_2 and 3,5,7-tri-5′-CH_2), 54.2 (5-2′-CH_2 and 5-6′-CH_2), 54.7 (3,7-di-2′-CH_2 and 3,7-di-6′-CH_2), 60.1 (3-CH_2 and 7-CH_2), 67.6 (5-CH_2), 133.8 (C3 and C7), 136.8 (C5), 138.6 (C4 and C6), 168.4 (C1 and C2).

4.1.2. 3,5,7-Tri[(4′-methylpiperazin-1′-yl)methyl]tropolone (2)

A mixture of tropolone (227 mg, 1.86 mmol) and 1-methylpiperazine (750 μL, 6.76 mmol) was treated with 37% aqueous formaldehyde (720 μL, 9.61 mmol) added in two steps. After adding the first portion of aqueous formaldehyde, the mixture turned into a clear solution and started to boil. It was stirred at 65 °C for 5 min. After cooling the solution to room temperature, a precipitate was obtained and crystallized twice from ethyl acetate and petroleum ether to give 617 mg (1.34 mmol, 72% yield) of compound **2** as yellow needles. MS (TOF) calcd for $C_{25}H_{43}N_6O_2^+$ (MH^+) *m/z* 459.3447, found 459.2846. ^{1}H NMR ($CDCl_3$, 298 K) δ: 2.23–2.69 [m, 33H; resolved in 2D spectra: 2.31 (3 × CH_3), 2.49 (3,5,7-tri-3′-CH_2 and 3,5,7-tri-5′-CH_2), 2.51 (5-2′-CH_2 and 5-6′-CH_2), 2.58 (3,7-di-2′-CH_2 and 3,7-di-6′-CH_2)], 3.49 (s, 2H, 5-CH_2), 3.72 (s, 4H, 3-CH_2 and 7-CH_2), 7.81 (s, 2H, 4-H and 6-H); ^{13}C NMR ($CDCl_3$, 298 K) δ: 46.1 (3 × CH_3), 52.8 (5-2′-CH_2 and 5-6′-CH_2), 53.2 (3,7-di-2′-CH_2 and 3,7-di-6′-CH_2), 55.3 (3,5,7-tri-3′-CH_2 and 3,5,7-tri-5′-CH_2), 59.2 (3-CH_2 and 7-CH_2), 66.7 (5-CH_2), 133.5 (C3 and C7), 136.2 (C5), 138.7 (C4 and C6), 168.5 (C1 and C2).

4.1.3. 3,5,7-Tri(pyrrolidin-1′-ylmethyl)tropolone (3)

Pyrrolidine (600 μL, 7.20 mmol) was slowly added to a mixture of tropolone (244 mg, 2.00 mmol) and 37% aqueous formaldehyde (774 μL, 10.33 mmol). While adding the amine the temperature rose, the suspension dissolved and the resulting yellow solution was stirred at 60 °C for 10 min. After cooling to room temperature and stirring for 12 h, no precipitate was obtained. The solution was extracted with chloroform (10 mL) and water (3 × 10 mL), the organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to produce oil. The oil was co-evaporated twice with methanol producing a light yellow precipitate. The precipitate was crystallized twice from acetone providing 329 mg (0.89 mmol, 44% yield) of compound **3** as a yellow powder. MS (TOF) calcd for $C_{22}H_{34}N_3O_2^+$ (MH^+) *m/z* 372.2651, found 372.2523. ^{1}H NMR ($CDCl_3$, 298 K) δ: 1.71–1.93 [m, 12H; resolved in 2D spectra: 1.78 (5-3′-CH_2, and 5-4′-CH_2), 1.82 (3,7-di-3′-CH_2, and 3,7-di-4′-CH_2)], 2.44–2.73 [m, 12H; resolved in 2D spectra: 2.53 (5-2′-CH_2 and 5-5′-CH_2), 2.62 (3,7-di-2′-CH_2 and 3,7-di-5′-CH_2)], 3.62 (s, 2H, 5-CH_2), 3.85 (s, 4H, 3-CH_2 and 7-CH_2), 7.79 (s, 2H, 4-H and 6-H); ^{13}C NMR ($CDCl_3$, 298 K) δ: 23.6 (5-3′-CH_2 and 5-4′-CH_2), 23.7 (3,7-di-3′-CH_2 and 3,7-di-4′-CH_2), 53.9 (5-2′-CH_2 and 5-5′-CH_2), 54.3 (3,7-di-2′-CH_2 and 3,7-di-5′-CH_2), 57.6 (3-CH_2 and 7-CH_2), 64.4 (5-CH_2), 134.6 (C3 and C7), 137.3 (C5), 138.7 (C4 and C6), 168.4 (C1 and C2).

4.1.4. 3,5,7-Tri[(4′-pyrrolidin-1″-ylpiperidin-1′-yl)methyl] tropolone (4)

A solution of 4-pyrrolidin-1-ylpiperidine (1.112 g, 7.21 mmol) in 20 mL of ethanol was added to a mixture of tropolone (242 mg, 1.98 mmol) and 37% aqueous formaldehyde (774 μL, 10.33 mmol). After adding the amine the mixture turned into a clear yellow solution that was stirred at 65 °C. The course of the reaction was monitored by MS spectrometry. Additional portions of 4-pyrrolidin-1-ylpiperidine were added over a period of 3 days (in total 482 mg, 3.13 mmol) and the reaction mixture was cooled to room temperature and stirred for 12 h to complete the reaction. The solvent was evaporated and the residue was co-evaporated with toluene, dissolved in chloroform (20 mL) and washed with water (3 × 20 mL). The organic extract was dried over anhydrous Na_2SO_4, filtered and co-evaporated with acetone. The precipitate was crystallized twice from acetone to produce 736 mg (1.18 mmol, 60% yield) of **4** as a yellow powder. MS (TOF) calcd for $C_{37}H_{61}N_6O_2^+$ (MH^+) *m/z* 621.4856, found 621.5017. ^{1}H NMR ($CDCl_3$, 303 K) δ: 1.44–2.29 [m, 31H; resolved in 2D spectra: 1.53 (5-3′-CH axial and 5-5′-CH axial), 1.63 (3,7-di-3′-CH axial and 3,7-di-5′-CH axial), 1.77–1.79 (3,5,7-tri-3″-CH_2 and 3,5,7-tri-4″-CH_2), 1.84 (5-3′-CH equatorial and 5-5′-CH equatorial), 1.90 (3,7-di-3′-CH equatorial and 3,7-di-5′-CH equatorial), 1.95 (5-4′-CH), 2.03 (5-2′-CH axial and 5-6′-CH axial), 2.17 (3,7-di-2′-CH axial and 3,7-di-6′-CH axial)], 2.45–2.68 (m, 12H, 3,5,7-tri-2″-CH_2 and 3,5,7-tri-5″-CH_2), 2.74–3.06 [m, 8H; resolved in 2D spectra: 2.83 (5-2′-CH equatorial and 5-6′-CH equatorial), 2.87 (3,7-di-2′-CH equatorial and 3,7-di-6′-CH equatorial), 2.97 (3,7-di-4′-CH)], 3.43 (s, 2H, 5-CH_2), 3.68 (s, 4H, 3-CH_2 and 7-CH_2), 7.85 (s, 2H, 4-H and 6-H); ^{13}C NMR ($CDCl_3$, 303 K) δ: 23.2 (3,5,7-tri-3″-CH_2 and 3,5,7-tri-4″-CH_2), 31.4 (5-3′-CH_2 and 5-5′-CH_2), 31.8 (3,7-di-3′-CH_2 and

3,7-di-5′-CH_2), 50.9 (3,5,7-tri-4′-CH), 51.5 (3,5,7-tri-2″-CH_2 and 3,5,7-tri-5″-CH_2), 52.4 (5-2′-CH_2 and 5-6′-CH_2), 52.9 (3,7-di-2′-CH_2 and 3,7-di-6′-CH_2), 59.0 (3-CH_2 and 7-CH_2), 67.1 (5-CH_2), 134.0 (C3 and C7), 137.0 (C5), 138.6 (C4 and C6), 168.0 (C1 and C2).

4.1.5. 3,7-Dichlorotropolone (5)

To the cold (ice + acetone bath) solution of tropolone (500 mg, 4.09 mmol) in 5 mL of methanol, aqueous HCl (1.66 mL, 36%) was added, followed by dropwise addition of 30% aqueous H_2O_2 (1.67 mL). The reaction mixture was stirred at room temperature for 5 days. Additional aqueous HCl (0.8 mL, 36%) and 30% aqueous H_2O_2 (0.8 mL) were added at room temperature and the mixture was stirred for an additional 48 h. The yellow precipitate formed was filtered and washed with diethyl ether to give 31 mg (0.16 mmol, 4% yield) of compound **5** as a white powder. MS (TOF) calcd for $C_7H_5Cl_2O_2^+$ (MH^+) *m/z* 190.9667, found 190.9651. ^{1}H NMR (DMSO-d_6, 303 K) δ: 6.86 (t, 1H, ^3J = 10.7 Hz, 5-H), 7.90 (d, 2H, ^3J = 10.7 Hz, 4-H and 6-H); ^{13}C NMR (DMSO-d_6, 303 K) δ: 122.4 (C5), 133.4 (C3 and C7), 137.7 (C4 and C6), 164.9 (C1 and C2).

4.1.6. 3,5,7-Tri [(4′-methylpiperidin-1′-yl)methyl]tropolone (6)

A mixture of tropolone (227 mg, 1.86 mmol) and 4-methylpiperidine (800 μL, 6.76 mmol) was treated with 37% aqueous formaldehyde (720 μL, 9.61 mmol). After adding formaldehyde the mixture turned into a clear solution and started to boil. It was stirred at 60 °C for 5 min and then at room temperature for 12 h. The precipitate obtained was crystallized four times from ethyl acetate or acetone and washed with diethyl ether or acetone to give 643 mg (1.41 mmol, 76% yield) of compound **6**. MS (TOF) calcd for $C_{28}H_{46}N_3O_2^+$ (MH^+) *m/z* 456.3590, found 456.2868. ^{1}H NMR ($CDCl_3$, 298 K) δ: 0.89–0.97 [m, 9H; resolved in 2D spectra: 0.92 (5-CH_3), 0.94 (3,7-di-CH_3)], 1.11–1.50 [m, 7H; resolved in 2D spectra: 1.28–1.36 (5-3′-CH_2 and 5-5′-CH_2), 1.36 (3,5,7-tri-4′-CH)], 1.50–1.73 (m, 8H, 3,7-di-3′-CH_2 and 3,7-di-5′-CH_2), 1.90–2.21 [m, 6H; resolved in 2D spectra: 2.00 (5-2′-CH and 5-6′-CH), 2.12 (3,7-di-2′-CH and 3,7-di-6′-CH)], 2.76–2.97 [m, 6H; resolved in 2D spectra: 2.82 (5-2′-CH and 5-6′-CH), 2.85 (3,7-di-2′-CH and 3,7-di-6′-CH)], 3.47 (s, 2H, 5-CH_2), 3.68 (s, 4H, 3-CH_2 and 7-CH_2), 7.82 (s, 2H, 4-H and 6-H); ^{13}C NMR ($CDCl_3$, 298 K) δ: 21.8 (3 × CH_3), 30.6 (3,5,7-tri-4′-CH), 34.5 (3,5,7-tri-3′-CH_2 and 3,5,7-tri-5′-CH_2), 53.7 (5-2′-CH_2 and 5-6′-CH_2), 54.2 (3,7-di-2′-CH_2 and 3,7-di-6′-CH_2), 59.8 (3-CH_2 and 7-CH_2), 67.2 (5-CH_2), 133.9 (C3 and C7), 136.8 (C5), 138.4 (C4 and C6), 168.3 (C1 and C2).

4.1.7. 3,5,7-Tri [(3′-methylpiperidin-1′-yl)methyl]tropolone (7)

A mixture of tropolone (244 mg, 2.00 mmol) and 3-methylpiperidine (845 μL, 7.20 mmol) was treated with 37% aqueous formaldehyde (774 μL, 10.33 mmol). After adding formaldehyde the mixture turned into a clear solution and started to boil. It was stirred at 65 °C for 5 min, then cooled to room temperature and stirred for 12 h. The precipitate formed was crystallized three times from acetone to give 543 mg (1.19 mmol, 60% yield) of compound **7**. MS (TOF) calcd for $C_{28}H_{46}N_3O_2^+$ (MH^+) *m/z* 456.3590, found 456.2708. ^{1}H NMR ($CDCl_3$, 298 K) δ: 0.80–0.97 [m, 12H; resolved in 2D spectra: 0.86 (3 × CH_3), 0.89 (3,5,7-tri-4′-CH axial)], 1.52–1.81 [m, 15H; resolved in 2D spectra: 1.63 (5-6′-CH axial), 1.64 (3,5,7-tri-3′-CH), 1.65 (3,5,7-tri-5′-CH axial), 1.70 (3,5,7-tri-4′-CH equatorial), 1.75 (3,5,7-tri-5′-CH equatorial), 1.77 (3,7-di-6′-CH axial)], 1.87–2.10 [m, 3H; resolved in 2D spectra: 1.94 (5-2′-CH axial), 2.04 (3,7-di-2′-CH axial)], 2.71–2.85 [m, 6H; resolved in 2D spectra: 2.74 (5-6′-CH equatorial), 2.77 (5-2′-CH equatorial and 3,7-di-6′-CH equatorial), 2.80 (3,7-di-2′-CH equatorial)], 3.44 (s, 2H, 5-CH_2), 3.67 (s, 4H, 3-CH_2 and 7-CH_2), 7.81 (s, 2H, 4-H and 6-H); ^{13}C NMR ($CDCl_3$, 298 K) δ: 19.5 (3 × CH_3), 25.5 (3,5,7-tri-3′-CH_2), 31.0 (3,5,7-tri-5′-CH_2), 32.7 (3,5,7-tri-4′-CH_2), 53.5 (5-2′-CH_2), 54.1 (3,7-di-2′-CH_2), 59.7 (3-CH_2 and 7-CH_2), 61.6 (5-6′-CH_2), 62.0 (3,7-di-6′-CH_2), 67.3 (5-CH_2), 133.8 (C3 and C7), 136.3 (C5), 138.6 (C4 and C6), 168.1 (C1 and C2).

4.1.8. 3,5,7-Tri [(4′-hydroxypiperidin-1′-yl)methyl]tropolone (8)

To a solution of tropolone (248 mg, 2.03 mmol) in ethanol (1 mL), aqueous (37%) formaldehyde (780 μL, 10.41 mmol) and 4-hydroxypiperidine (732 mg, 7.24 mmol) were added and the mixture was stirred and heated to 65 °C for 5 min. It was then cooled to room temperature, stirred for 12 h and evaporated to produce oil. After adding acetone (3 mL) a precipitate formed that was filtered, and crystallized from methanol with acetone giving 799 mg (1.73 mmol, 85% yield) of compound **8**. MS (TOF) calcd for $C_{25}H_{40}N_3O_5^+$ (MH^+) *m/z* 462.2968, found 462.1859. ^{1}H NMR (CD_3OD, 298 K) δ: 1.43–1.73 [m, 6H; resolved in 2D spectra: 1.57 (5-3′-CH and 5-5′-CH), 1.68 (3,7-di-3′-CH and 3,7-di-5′-CH)], 1.73–1.99 [m, 6H; resolved in 2D spectra: 1.86 (5-3′-CH and 5-5′-CH), 1.89 (3,7-di-3′-CH and 3,7-di-5′-CH)], 2.19 (t, 2H, 5-2′-CH and 5-6′-CH), 2.52 (t, 4H, 3,7-di-2′-CH and 3,7-di-6′-CH), 2.72–2.83 (m, 2H, 5-2′-CH and 5-6′-CH), 2.91–3.05 (m, 4H, 3,7-di-2′-CH and 3,7-di-6′-CH), 3.43 (s, 2H, 5-CH_2), 3.54–3.77 [m, 3H; resolved in 2D spectra: 3.62 (5-4′-CH), 3.72 (3,7-di-4′-CH], 3.84 (s, 4H, 3-CH_2 and 7-CH_2), 7.59 (s, 2H, 4-H and 6-H); ^{13}C NMR (CD_3OD, 298 K) δ: 34.4 (3,7-di-3′-CH_2 and 3,7-di-5′-CH_2), 35.4 (5-3′-CH_2 and 5-5′-CH_2), 51.8 (3,7-di-2′-CH_2 and 3,7-di-6′-CH_2), 52.2 (5-2′-CH_2 and 5-6′-CH_2), 62.4 (3-CH_2 and 7-CH_2), 67.2 (3,7-di-4′-CH), 67.9 (5-CH_2), 68.6 (5-4′-CH), 130.6 (C3 and C7), 131.9 (C5), 141.5 (C4 and C6), 176.1 (C1 and C2).

4.1.9. 3,7-Dibromotropolone (9)

The compound was synthesized as described by Boguszewska-Chachulska et al.[12] MS (TOF) calcd for $C_7H_5Br_2O_2^+$ (MH^+) *m/z* 280.8636, found 280.8701. ^{1}H NMR (DMSO-d_6, 298 K) δ: 6.68 (t, 1H, ^3J = 10.7 Hz, 5-H), 8.12 (d, 2H, ^3J = 10.7 Hz, 4-H and 6-H); ^{13}C NMR (DMSO-d_6, 298 K) δ: 123.1 (C5), 125.3 (C3 and C7), 140.8 (C4 and C6), 164.7 (C1 and C2).

4.1.10. 3-Bromotropolone (10)

The compound was synthesized as previously described.[12] MS (TOF) calcd for $C_7H_6BrO_2^+$ (MH^+) *m/z* 200.9551, found 200.9507. ^{1}H NMR (DMSO-d_6, 298 K) δ: 6.91 (dt, 1H, ^3J = 10.2 Hz, ^3J = 10.2 Hz, ^4J = 0.8 Hz, 5-H), 7.29 (dd, 1H, ^3J = 10.4 Hz, ^4J = 0.8 Hz, ^5J = 0.2 Hz, 7-H), 7.46 (dt, 1H, ^3J = 10.2 Hz, ^3J = 10.4 Hz, ^4J = 1.0 Hz, 6-H), 8.29 (dd, 1H, ^3J = 10.2 Hz, ^4J = 1.0 Hz, ^5J = 0.2 Hz,, 4-H); ^{13}C NMR (DMSO-d_6, 298 K) δ: 119.3 (C7), 125.5 (C5), 130.4 (C3), 136.8 (C6), 141.1 (C4), 164.7 (C1), 170.2 (C2).

4.2. Biology

4.2.1. Protein cloning, expression and purification

The NS3 helicase domain of genotype 1a was expressed in a baculovirus system and purified from insect cells as described.[19] The helicase domains of genotypes 1b and 3a were obtained by reverse transcription and PCR amplification using RNA extracted from the blood of Polish HCV-infected patients as templates; this was followed by cloning, protein expression and purification performed as previously described[19] (see Supplementary data: Experimental protocols on cloning and expression of new HCV helicase variants).

4.2.2. Enzymatic assay

Helicase assays were performed in 60 μL reaction buffer with 10 nM dsDNA substrate and 125 nM capture strand as described.[21] The enzyme was preincubated with the inhibitors at various concentrations in the reaction mixture without ATP for 15 min at room temperature. The unwinding reaction was started by addition of 1.5 mM ATP and was carried out at 30 °C for 60 min in a Synergy

HTi Fluorescence Reader (BioTek Instruments, Inc., Winooski, VT). The fluorescent signal was registered every 2 min. The enzyme activity was calculated as the initial reaction velocity as previously described.[21] Inhibition was calculated as the percent activity of the helicase incubated without inhibitor. The ORIGIN 6.1 program (OriginLab) and EXCEL (Microsoft) were used to calculate the data.

4.2.3. HCV replicon and cell toxicity studies

The human hepatoma cell line Huh-7 and the plasmid pFK-luc-ubi-neo/NS3-3′/Con1/5.1 carrying the subgenomic (NS3—3′nontranslated region) HCV con1 replicon with the luc-ubi-neo (reporter/selective) fusion gene were kindly provided by Dr. Ralf Bartenschlager (Department of Molecular Virology, University of Heidelberg, Heidelberg, Germany).[14] Stable Huh-7 clones carrying persistently replicating subgenomic HCV replicons were obtained using the protocol described by Lohmann et al. with minor modifications.[30] Huh-7 cells were grown and passaged as previously described.[14,21,27] The conditions of the assay applied to test antiviral activities and cell toxicity of tropolone derivatives were as described.[27,28]

4.2.4. Selection and analysis of mutants resistant to 2 and 7

Huh-7 cells were seeded at a density of 4×10^5 cells per 10 cm diameter culture dish in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 250 μg/mL G418 and with 100 μM compounds **2** or **7**. They were grown at 37 °C and 5% CO_2 and were passaged upon reaching confluency for a total time of 1 month. Finally the cells were collected by centrifugation and frozen at −80 °C. Total RNA was extracted using the RNAspin RNA isolation kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. The RNA was eluted in 40 μL of RNase-free water and the NS3-coding region was reverse-transcribed using the SuperScript III First-Strand synthesis system (Invitrogen) according to the manufacturer's instructions and a mixture of random primers and the specific primer, NS3rev1 (see Supplementary data). The NS3 region was amplified using the following primers: AACGTCTAGGCCCCCCGAACC and AATACGCGGCCAGAGCTGCTAGG (Laboratory of DNA Sequencing and Oligonucleotide Synthesis, IBB PAS) and sequenced at IBB PAS.

4.2.5. Combined inhibition of replication by tropolone derivatives, ribavirin and IFN-γ

To study the effect of the combined inhibition of replication by tropolone derivatives and IFN-γ (Institute of Biotechnology and Antibiotics, Warsaw, Poland) or ribavirin (ICN Biochemicals, Cleveland, USA), Huh-7 cells bearing the subgenomic replicon were seeded in a 96-well microtiter plate and grown with increasing concentrations of compound **2** (1, 10, 15, 25 and 50 μM) together with increasing concentrations of IFN-γ (0.01, 0.02, 0.04, 0.05, 0.07 and 0.1 μ/mL), or in another experiment with ribavirin (1, 5, 10, 15, 25 and 50 μM).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.05.066.

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