Journal of Medicinal Chemistry Article

J. Med. Chem. **2010,** *53,* 3117–3126 **3117**
DOI: 10.1021/jm901741p

Synthesis of New Acridone Derivatives, Inhibitors of NS3 Helicase, Which Efficiently and Specifically Inhibit Subgenomic HCV Replication

Anna Stankiewicz-Drogoń,[‡,†] Bernd Dörner,[§,†] Thomas Erker,*[,§] and Anna M. Boguszewska-Chachulska*[,∥,⊥]

[‡]*Institute of Biochemistry and Biophysics, PAS, Pawinskiego 5a, 02-106 Warsaw, Poland,* [§]*University of Vienna, Department of Medicinal Chemistry, Althanstrasse 14, 1090 Vienna, Austria,* [∥]*Genomed, Ponczowa 12, 02-971 Warsaw, Poland, and* [⊥]*Warsaw University of Technology, Faculty of Chemistry, Noakowskiego 3, 00-664 Warsaw, Poland.* [†]*These authors contributed equally to this work.*

Received November 24, 2009

A new goup of acridone derivatives, obtained by reaction of acridone-4-carboxylic acid derivatives with aromatic amines, was tested to determine the inhibitory properties toward the NS3 helicase of hepatitis C virus (HCV). Six compounds inhibited the NS3 helicase at low concentrations (IC_{50} from 1.5 to 20 μM). The acridone derivatives probably act via intercalation into double-stranded nucleic acids with a strong specificity for double-stranded RNA, although an interaction with the enzyme cannot be excluded. Testing in the subgenomic HCV replicon system revealed that compounds **10** and **13** are efficient RNA replication inhibitors, with EC_{50} of 3.5 and 1 μM and therapeutic indexes of > 28 and 20, respectively. Compound **16**, with $EC_{50} < 1$ μM and TI > 1000, is extremely specific and practically noncytotoxic at the concentrations tested, proving that the acridone derivatives may be regarded as potential antiviral agents. Although the mechanism of action of **16** in the replicon system remains unclear, it is the key lead compound for further development of anti-HCV drugs.

Introduction

About 180 million people worldwide are chronically infected with hepatitis C virus (HCV[a]), an agent responsible for 50−76% of all cases of liver cancer and for two-thirds of all liver transplants in the developed world (World Health Organization (WHO), 2007). As the current combination therapy of pegylated interferon and ribavirin is long and ineffective in about 60% of patients chronically infected with HCV genotype 1 and in about 20% of those with HCV genotypes 2 and 3,[1] there is an urgent need for more effective, better tolerated, and less expensive treatments.

The development of antiviral agents directly targeting the viral life cycle seems to be the most promising therapeutic strategy, as it should block HCV replication and thus spread of infection. This goal could be achieved by direct inhibition of viral enzymes involved in the replication process, such as the NS3 protein, exerting both serine protease and RNA helicase/NTPase activities, or the NS5B protein—the RNA-dependent RNA polymerase. A few drugs targeting the NS3/4A protease,[2−4] or the NS5B polymerase,[5] are already in clinical trials, but no NS3 helicase inhibitor has reached this level of development, probably because its mechanism of action is not yet entirely understood, thus hampering the rational design of compounds targeting the helicase.[6]

Still, as an enzyme indispensable for HCV replication that unwinds double-stranded (ds) forms of RNA and allows viral replication and translation to occur,[7] the NS3 helicase/NTPase represents a tempting target for specific anti-HCV drug design. Another advantage is that NS3 helicase does not possess close homologues among human cellular enzymes.[6,8,9] Its inhibitors could be used together with inhibitors of other viral proteins in a cocktail, preventing HCV from escaping the treatment pressure by the emergence of drug-resistant mutants. Identification of several helicase inhibitors, belonging to various chemical groups, capable of inhibiting viral replication, was reported by our laboratory[10,11] but there is still a need for compounds with lower effective concentrations and/or higher therapeutic indices.

Recently, we presented results of our studies on a series of acridone derivatives that inhibited HCV RNA synthesis in the subgenomic replicon system in a dose-dependent manner with an activity/cytotoxicity window of 40,[12] supporting the possibility that acridone derivatives could feature as lead compounds in the development of anti-HCV drugs. This finding was further confirmed by Manfroni and co-workers, who identified a group of acridone derivatives that inhibited HCV RNA replication in the subgenomic replicon system and for one compound demonstrated a weak inhibition of NS3 helicase activity.[13] One main difference between our lead structure and the acridone derivatives used by Manfroni et al. was the carboxylic function. The amide bonding formed after the derivatization with amines seemed to increase affinity and selectivity for NS3 helicase as we could prove in our paper.[12]

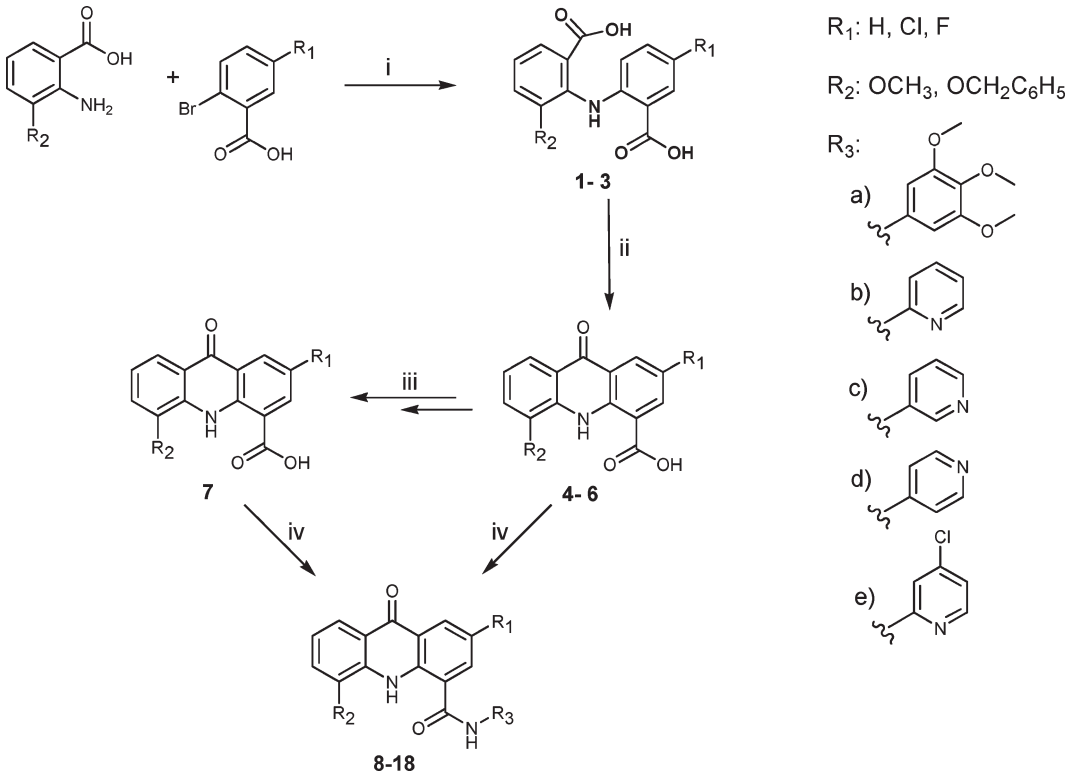
In this study, we report the synthesis of new derivatives of acridone, namely of 5-methoxyacridone-4-carboxylic acid (MACA) and selection of compounds that not only efficiently inhibit the NS3 helicase in the in vitro assay but are also potent

*To whom correspondence should be addressed. For T.E., chemistry: phone, +43 1 4277 55003; fax, +43 1 4277 9551; E-mail: thomas.erker@univie.ac.at. For A.M.B-C., biology: phone, +48 22 498 2948; fax, +48 22 644 60 25; E-mail: annab-ch@genomed.pl.

[a] Abbreviations: HCV, Hepatitis C virus; MACA, 5-methoxyacridone-4-carboxylic acid; CC_{50}, concentration of compound that inhibited cell growth by 50%; EC_{50}, 50% effective concentration; IC_{50}, 50% inhibitory concentration; TI, therapeutic index; IFN, interferon; NTP, nucleoside triphosphate.

Published on Web 03/25/2010

Scheme 1. Synthesis of Compounds **8**−**18**[a]



[a] Reagents and conditions: (i) Cu^0/K_2CO_3, EtOH, reflux; (ii) $POCl_3$, CH_3CN, reflux; (iii) (1) HBr, Ac_2O, reflux, (2) benzylchloride, K_2CO_3, DMF, 50 °C, (3) aq LiOH, CH_3OH, reflux; (iv) $SOCl_2$/pyridine, toluol, amine, Et_3N, room temperature.

inhibitors of HCV replication with low cytotoxicity for human hepatoma cells.

Results

Chemistry. The acridone derivatives **8**−**18** were synthesized as outlined in Scheme 1. Therefore 2-amino-3-methoxybenzoic acid was coupled with either 2-bromobenzoic acid, 2-bromo-4-chlorobenzoic acid, or 2-bromo-4-fluorobenzoic acid in a copper-catalyzed reaction to obtain compounds **1**,[14] **2**, and **3**. Cyclization with phosphorus(V)oxychloride yielded the acridonic acid derivatives **4**,[14] **5**, and **6**. Compound **7** was synthesized in a three-step reaction as described in literature[15] outgoing from **4**. The acridonic acid derivatives **4**, **5**, **6**, and **7** were activated with thionyl chloride and then reacted with aromatic amines[12] to achieve the acridone-4-carboxamide derivatives **8**−**18** (Table 1).

Biology. Helicase Expression and Activity. The NS3 helicase of genotype 1a was obtained as previously described.[16] New variants of the NS3 helicase (genotype 1b and 3a) were isolated and cloned from infected blood samples from Polish HCV-infected patients and their nucleotide sequences were established and deposited in the GeneBank database under the following accession numbers: 1255063 and 1255068 (Bernatowicz-Najda et al., submitted). Sequence comparisons with the helicase of genotype 1a 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 NTP-ase and helicase motifs was changed. New helicase variants of genotypes 1b and 3a were expressed in a Bac-to-Bac expression system and purified from insect cells.

Table 1. List of Substituents of Compounds **1**−**18**

compd	R_1	R_2	R_3
1, **4**	H	OCH_3	
2, **5**	Cl	OCH_3	
3, **6**	F	OCH_3	
7	H	$OCH_2C_6H_5$	
8	H	OCH_3	a
9	H	OCH_3	b
10	H	OCH_3	c
11	H	OCH_3	e
12	Cl	OCH_3	b
13	Cl	OCH_3	c
14	Cl	OCH_3	e
15	F	OCH_3	b
16	F	OCH_3	c
17	F	OCH_3	e
18	H	$OCH_2C_6H_5$	d

About 15 mg/mL were obtained for each of the new helicase variants.

In the fluorometric helicase activity assay using a dsDNA substrate,[12,16] 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 4-fold higher than that of helicase 1b.

Inhibition of HCV Helicase Activity. To test the potential antihelicase activity of acridone derivatives, the fluorometric helicase activity assay with the genotype 1a helicase was applied as previously described.[12] The compounds dissolved in dimethyl sulfoxide (DMSO) were initially tested at 10−100 μM or 10−500 μM concentrations, depending on the solubility of the inhibitor. The compounds showing antihelicase activity were subsequently tested in smaller increments

around the approximated IC_{50} values (concentration of inhibitor that reduces helicase activity by 50%).

All the compounds studied exerted an inhibitory effect on unwinding of dsDNA by the helicase, but the strength of this interaction differed significantly, with IC_{50} values ranging from 1.5 μM for compound **14** to 751 μM for compound **8** (Table 2). The position of the nitrogen atom in the pyridyl ring as well as the presence of chloride or fluoride substitutions at the C2 position of the acridone structure or at the C4 position of the pyridyl ring modified the inhibitory potential of acridone derivatives. On the other hand, addition of a 3,4,5-trimethoxyphenyl group instead of the pyridyl ring led to the weakest inhibitor, compound **8**.

The mechanism of antihelicase activity of the most potent helicase inhibitors ($IC_{50} \leq 20\ \mu$M) with respect to the enzyme or dsDNA was studied as described previously, in the helicase assay with increasing enzyme or substrate concentration and constant inhibitor concentration.[10−12] Inhibition appeared independent of the enzyme concentration (Figure 1), suggesting lack of competition and interaction, while in the presence of increasing substrate concentrations, a decrease of inhibition was observed (Figure 2), clearly indicating interaction of the compounds with the substrate. Additionally, IC_{50} values of 8.6 ± 2.7 μM and 8.4 ± 0.4 μM established for compound **16** in the assay with 20 nM or 50 nM enzyme, respectively, confirmed the hypothesis that the inhibitory properties of the compounds tested are not influenced by helicase concentration, at least at the enzyme concentrations studied.

Table 2. Inhibitory Activities of MACA Derivatives[a]

compound	IC_{50}[b] ± SD[c] (μM)
8	751.0 ± 33.7
9	6.2 ± 2.1
10	20.6 ± 6.3
11	35.8 ± 10.2
12	64.7 ± 44.6
13	54.6 ± 4.0
14	1.5 ± 0.9
15	4.7 ± 1.3
16	8.6 ± 2.7
17	46.3 ± 17.9
18	8.0 ± 2.2

[a] All the data represent mean values for three independent experiments. [b] Concentration of inhibitor necessary to reduce the NS3 helicase activity by 50%. [c] Standard deviation.

To further examine the possibility that the compounds interact with the helicase, the IC_{50} values for compound **16** were established using 50 nM enzymes from different HCV genotypes: 1a, 1b, and 3a. Inhibition of helicases 1a and 3a appeared comparable (IC_{50} of 8.4 μM and 7.3 μM, respectively), while helicase 1b was 2.5−3-fold more resistant to the inhibitory activity of **16** (IC_{50} of 19.3 μM), which may imply a specific interaction of **16** with the helicase (Figure 3).

To check if the inhibition of the helicase activity by acridone derivatives depends on the ATP concentration, the IC_{50} values of **14**, **15**, and **16** were measured at three ATP concentrations, with a constant Mn^{2+} to ATP molar ratio of 4:1. The three inhibition curves (data not shown) and the IC_{50} values (Table 3) did not differ, proving that the ATPase activity is not inhibited.

Compounds inhibiting the HCV helicase activity with $IC_{50} \leq 20\ \mu$M were tested for their intercalatory properties on both dsDNA and dsRNA substrates in a dsNA gel migration retardation assay as described previously.[11,12] In this assay, the compounds that intercalate into dsNA inhibit ethidium bromide intercalation and the dsNA band disappears partially or totally. This approach revealed weak dsDNA intercalation properties of the compounds at 100 μM or 500 μM, much lower than that of epidoxorubicin, a potent intercalator, used as a positive control (Figure 4A). Compound **9** seems to be the strongest dsDNA intercalator of all the compounds analyzed; a slightly lower effect was observed for **10**. For dsRNA, the cellular target of the HCV helicase, the intercalatory properties of the compounds, were stronger, visible already at 100 μM compound **10**. Compounds **13** and **16** were slightly weaker intercalators because the RNA band disappeared only at 500 μM compound, whereas the other compounds did not intercalate into dsRNA (Figure 4B).

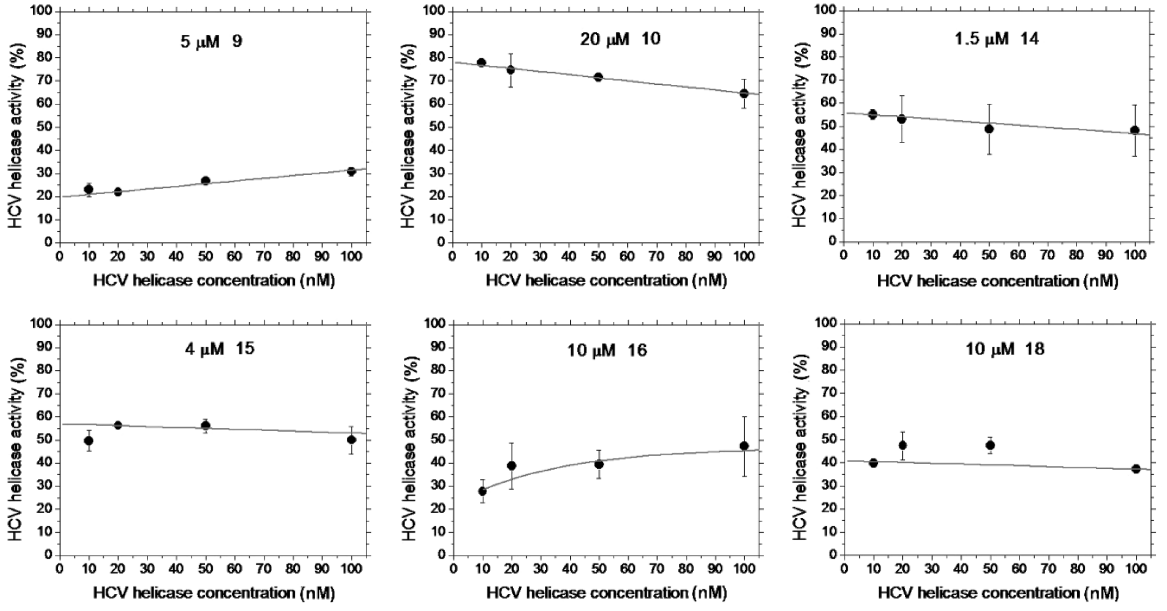


Figure 1. Inhibition of the NS3 helicase activity by MACA derivatives at increasing enzyme concentrations. The results are presented as the percent activity of the helicase tested in the same conditions but with DMSO instead of inhibitor.

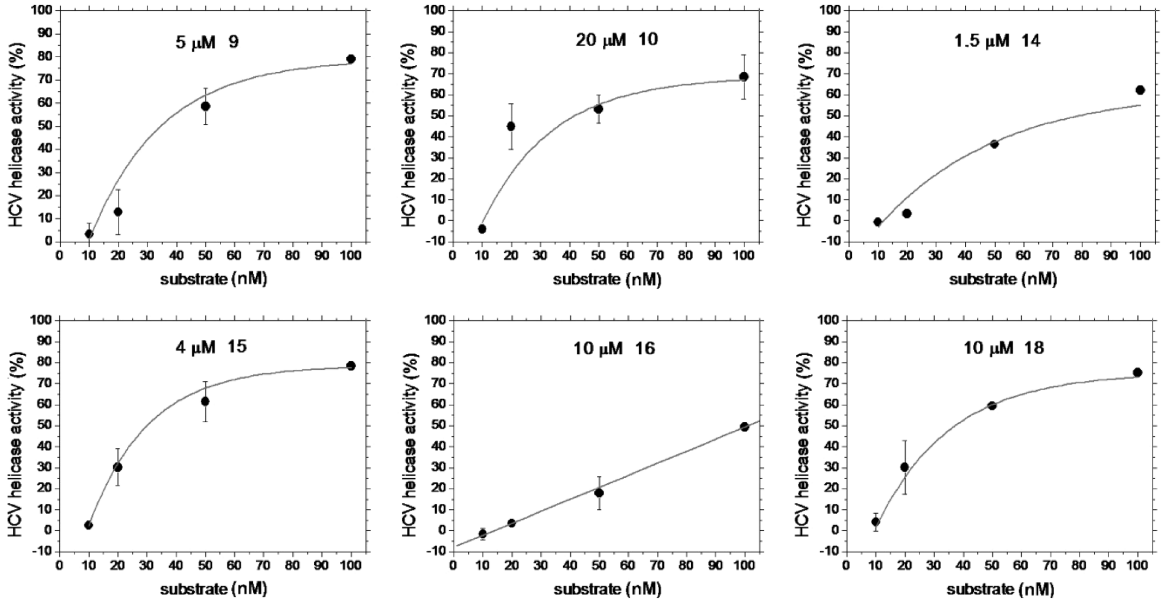


Figure 2. Inhibition of the NS3 helicase activity by MACA derivatives at increasing dsDNA substrate concentrations. The results are presented as the percent activity of the helicase tested in the same conditions but with DMSO instead of inhibitor.

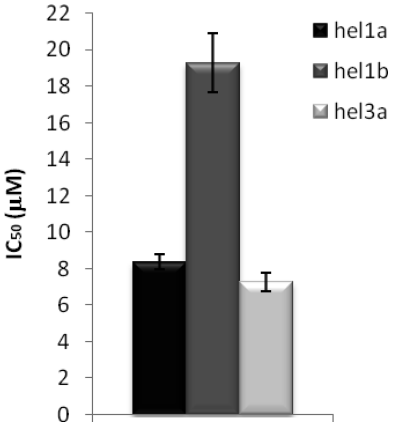


Figure 3. Inhibition of the helicase activity of helicases derived from various HCV genotypes: 1a (hel1a), 1b (hel1b), and 3a (hel3a) by compound **16**. IC_{50}, concentration of inhibitor necessary to reduce the NS3 helicase activity by 50%.

Antiviral Activity in the HCV Replicon System. Selected acridone derivatives were tested at concentrations ranging from 1 to 200 μM or from 1 μM to 1 mM, depending on the compound solubility in DMSO, both in the replicon RNA amplification assay[10−12] and in the cytotoxicity studies using the XTT method.[12] Of 11 compounds tested, five inhibited RNA replication, with EC_{50} (50% effective concentration defined as the inhibitor concentration that reduces luminescence by 50%) values ranging from 1 to 20 μM (Table 4). However, the strong inhibition of HCV replication observed for compounds **9** and **12** was rather due to the cytotoxicity of the compounds than to the actual inhibition of RNA synthesis. Derivatives synthesized on the basis of **9** had either weak potency, decreasing HCV replication only by 30% without greater decrease in RNA levels upon further addition of the compound (**11** or **15**), or had no effect on RNA replication at the concentrations examined. Nevertheless, three compounds, **10**, **13**, and **16**, exhibited antiviral activity together with low cytotoxicity (50% cytotoxic concentration defined as the concentration of the compound that inhibits cell growth by 50%, CC_{50}), reducing the HCV RNA level in a dose-dependent manner with a therapeutic index ($TI = CC_{50}/EC_{50}$) of >28, 20.1, and >1000, respectively. In our studies, **16** was a stronger inhibitor of RNA synthesis than ribavirin (EC_{50} of 0.98 μM and 17.6 μM, respectively) and comparable to 4′-azidocytidine (EC_{50} of 1.4 μM in our assay (Table 4) and 1.28 μM according to previous reports[17]).

Table 3. Inhibition of the NS3 Helicase Activity by Compounds **14**, **15**, and **16** at Three ATP Concentrations[a]

	IC_{50}[b] ± SD[c] [μM]		
compd	0.5 mM ATP	1.5 mM ATP	2.5 mM ATP
14	1.1 ± 0.3	1.5 ± 0.9	1.1 ± 0.2
15	6.5 ± 0.4	4.7 ± 1.3	5.1 ± 1.0
16	10.9 ± 1.9	8.6 ± 2.7	9.9 ± 2.8

[a] The results are presented as the percent activity of the helicase tested in the same conditions but with DMSO instead of inhibitor. [b] Concentration of inhibitor necessary to reduce the NS3 helicase activity by 50%. [c] Standard deviation.

The inhibition of HCV RNA replication by the best compound, **16**, was additionally measured using reverse transcription and real-time PCR. For this purpose, Huh-7 cells carrying the genotype 1b subgenomic replicon were grown for 7 days with **16** (1, 10, and 100 μM) or without inhibitor. For each sample, total RNA was isolated and 5 μg of RNA were used to obtain cDNA in the reverse transcription reaction. The same amount of cDNA from each sample was used as template for real-time PCR. EC_{50} estimated by this method was 4.5 ± 2.4 μM, which gives a TI above 220. At 10 μM, compound **16** replication was inhibited up to 85% and this level of replication was maintained at 100 μM compound.

To test a possible synergy/antagonism of acridone derivative **16**, the best inhibitor of HCV RNA replication, with other anti-HCV compounds, the antiviral activity of **16** was investigated in combination with interferon-γ (IFN-γ) or ribavirin. The Prichard and Shipman MacSynergy II software,[18] which examines drug interaction using either the Bliss Independence or the Loewe Additivity as null reference model for additivity, was used. In this algorithm, if the

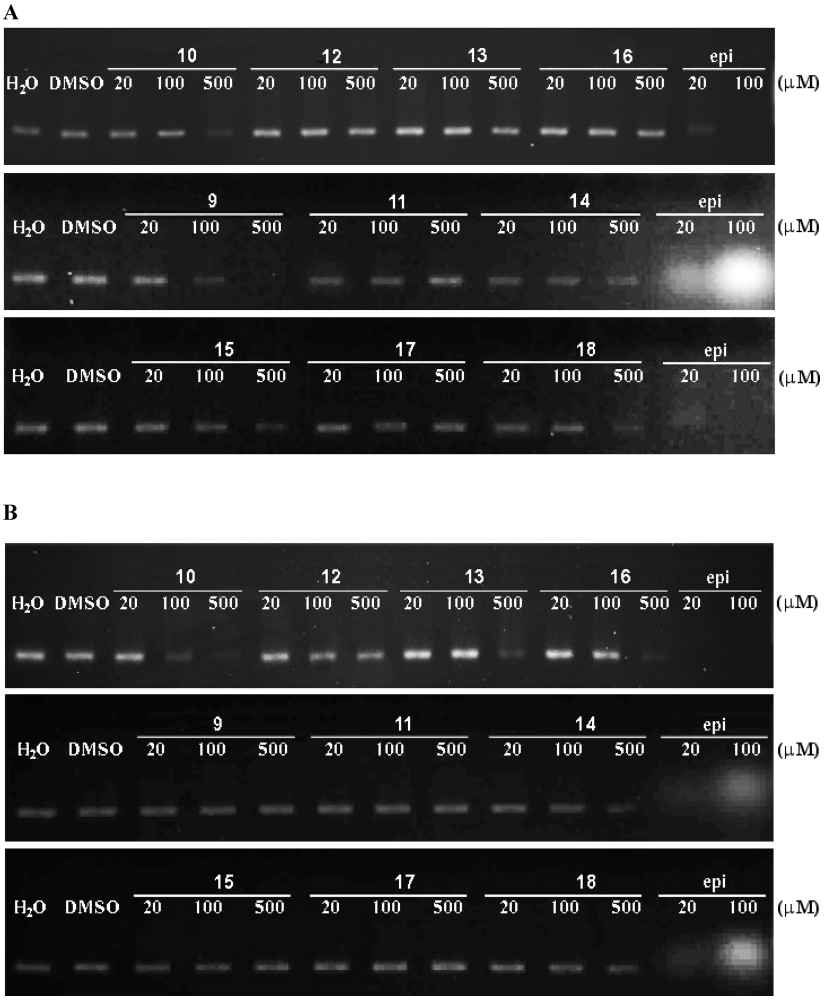


Figure 4. Intercalation assay for the MACA derivatives. Their intercalatory properties are reflected by the disappearance of dsDNA (A) or dsRNA (B) bands. DMSO: dsNA incubated with DMSO; Epi: dsNA incubated with epidoxorubicin; **8**−**18**: dsNA incubated with selected compounds at given concentrations (μM).

Table 4. Inhibition of HCV Replication in Huh-7 Cells Carrying the Subgenomic Replicon[a]

compd	EC_{50}[b] (μM)	CC_{50}[c] (μM)	TI[d]
8	202.7 ± 26.2	> 500	> 2.5
9	2.3 ± 1.0	2.5 ± 0.4	1.1
10	3.5 ± 1.6	> 100	> 28
11	*f*	171.3 ± 26.3	n/a[e]
12	5.0 ± 0.9	15.8 ± 1.9	3.2
13	0.96 ± 0.36	19.3 ± 3.2	20.1
14	> 100	> 100	n/a
15	*f*	> 100	n/a
16	0.98 ± 0.37	> 1000	> 1000
17	> 100	> 100	n/a
18	> 100	> 100	n/a
ribavirin	17.6 ± 2.2	1060 ± 321	60.2
4′azidocytidine	1.4 ± 0.7	> 200	> 143

[a] Each experiment was performed independently at least three times. [b] Inhibitor concentration needed to reduce viral replication to 50%. [c] Inhibitor concentration that inhibits cell growth by 50%. [d] Therapeutic index. [e] Not applicable. [f] Inhibition of HCV replication of 30% was achieved at 5.8 μM **11** and 2.7 μM **15** and maintained at this level even when the compound concentration was increased to 100 μM.

inhibitory effect of the compounds studied is additive, 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 of inhibitors, while a surface below the zero plane indicates an antagonistic effect. The synergy areas were displayed graphically after subtracting the theoretical additive surface from the one obtained experimentally. The data presented are the result of three independent experiments. The combinations of **16** with either IFN-γ or ribavirin resulted in an additive activity (Figure 5).

To obtain replicon mutants resistant to compound **16**, Huh-7 cells bearing the subgenomic replicon were grown in the presence of 10 μM **16** and under the selective pressure of G418. In these conditions, cells lacking the replicon or containing the replicon susceptible to **16** are killed and those that acquire mutations conferring resistance to **16** survive. Derivative **16** inhibited HCV RNA replication with EC_{50} = 0.98 μM in naïve cells but with EC_{50} as high as 251 μM in mutant cells, i.e., more than 2 orders of magnitude less efficiently. RNA from compound **16**-resistant colonies was isolated, reverse transcribed, and sequenced to search for mutations responsible for the resistance to the compound. No nucleotide (and thus no amino acid) substitution was found in the NS3-NS5B region of the compound **16**-resistant cells.

Discussion

All MACA derivatives tested appeared efficient inhibitors of the HCV helicase activity in the in vitro assay. The position and nature of the substituent is important for the biological

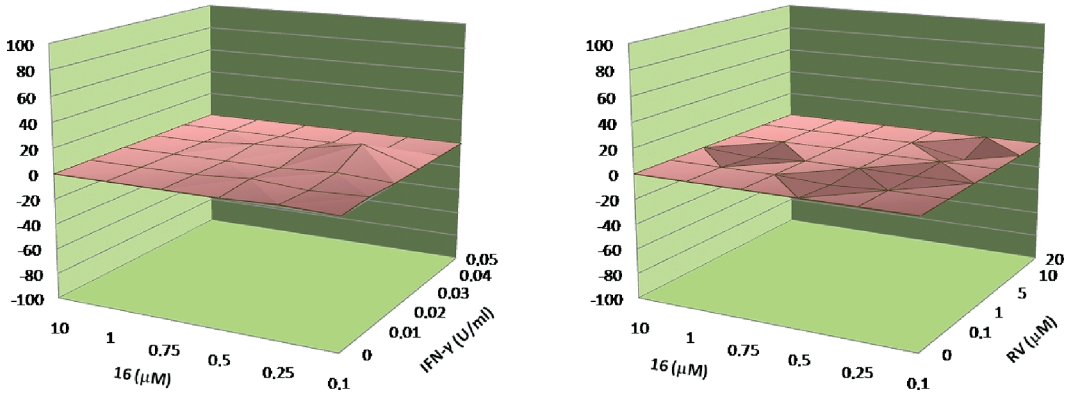


Figure 5. Inhibition of HCV RNA replication by (A) a combination of **16** and IFN- γ and (B) **16** and ribavirin (RV). Values under the zero plane indicate antagonistic activity, values in the zero plane indicate additive activity, and the values above the zero plane indicate synergistic activity.

activity of acridines and acridones,[19,20] and our data strongly support this assumption.

Presence of the pyridyl ring and the position of the nitrogen atom at the ring determined the strength of the antihelicase activity as demonstrated by virtual lack of activity of compound **8** and a 3-fold lower IC_{50} value reached for **9** in comparison with **10**. Moreover, the addition of a chloride (**12**) or a fluoride (**15**) atom at the C2 position of the acridone structure of **9** led to two compounds that differed more than 10-fold in their inhibitory potential, with IC_{50} of 5.6 μM for **15** and 64.7 μM for **12**. Halogenation of **10** at the same position gave similar differences in activity of **16** (IC_{50} = 8.6 μM) and **13** (IC_{50} = 55.6 μM), and in both situations the substitution of a hydrogen by a fluoride atom produced more potent helicase inhibitors than prototype compounds, while the substitution by a chloride atom decreased the anti-HCV activity of MACA derivatives. The addition of a chloride or fluoride moiety to C4 at the pyridyl ring also influenced the antihelicase activity of the compounds, and the combination of chloride substitution at this position with chloride at the C2 position of the acridone structure led to the most potent helicase inhibitor, **14** (IC_{50} = 1.5 μM).

Our studies on the mechanism of action of acridone derivatives as helicase inhibitors suggest that these compounds interact with the dsDNA substrate rather than with the enzyme. Because natural and synthetic acridine and acridone drugs are known to share the common property of nucleic acid (NA) intercalation,[21] dsDNA intercalation seems a possible way of inhibiting the helicase in our in vitro assay. Translocation of the enzyme along the NA strand is inhibited both by the increased demand for the energy due to stabilization of dsNA structure by intercalators[22] and by its modification as the NS3 helicase seems sensitive to the NA duplex structure.[23] Still, if dsDNA intercalation were the only mechanism of helicase inhibition by the acridone derivatives, all of them would inhibit NS3 helicase-mediated dsDNA unwinding with the potency reflected by their intercalatory potential. However, this is not the case because the most potent helicase inhibitor **14** did not reveal any intercalatory properties.

It was shown that side chain substitutions in the acridone derivatives, protruding into the major or minor grooves of the NA, are important for compound−NA interactions,[21,24,25] and that the biological activities of acridone drugs are not only due to their binding to DNA but also to their direct interaction with cellular topoisomerases and telomerases.[19,26] Thus interaction of acridone derivatives with other enzymes (e.g., the HCV helicase) should be considered. Although direct interaction of acridone derivatives with the viral helicase was not detected in our enzymatic assay (Figure 1), creation of a ternary complex enzyme:DNA:intercalator cannot be excluded.[22] A specific compound **16**-NS3 interaction is suggested by the differences in the inhibition of helicases from genotypes 1a, 1b, and 3a exerted by **16** (Figure 3).

Further studies conducted in the HCV subgenomic replicon system showed that antihelicase properties of the compounds do not necessarily imply their inhibitory effect on RNA synthesis. Although the MACA derivative **9** was a stronger helicase inhibitor than **10**, the latter and its derivatives exerted more potent inhibition of HCV RNA synthesis in the replicon system without cytotoxic effect on Huh-7 cells. Interestingly, a double chloride substitution of **9** that led to the most potent helicase inhibitor **14** resulted in complete loss of anti-HCV properties, probably due to a decrease in the permeability of the cell membrane for **14**. These data suggest that an additional mechanism must be involved in the inhibition of viral RNA synthesis in the replicon system, probably based on the intercalating properties of acridone derivatives as revealed by a strong affinity of **10**, **13**, and **16** for dsRNA. This preference for dsRNA is an advantageous feature for inhibitors of amplification of RNA viruses because such molecules could modify the secondary structures of viral untranslated regions (UTRs) and of dsRNA intermediates formed during genome replication without affecting cellular DNA.

Compound **16** was also tested in the replicon system for its synergy/antagonism in combination with ribavirin or IFN-γ; the effect of both drug combinations was additive, which suggests that **16** does not interfere with the metabolism of either IFN-γ or ribavirin and probably has a different mode of action. Both synergistic and antagonistic effects of anti-HCV drug combinations have already been reported, e.g., telaprevir and boceprevir, both NS3/4A inhibitors, with nitazoxanide[27] or ribavirin with a nucleoside analog 2′-C-methylcytidine, an NS5B polymerase inhibitor.[28] Thus the additive effect of the **16** combination with IFN-γ or ribavirin is a good prognostic if it is considered as a lead compound for development of a new generation of anti-HCV drugs.

Moreover, colonies resistant to compound **16** were obtained, although no nucleotide substitution in the region coding for the nonstructural proteins (NS3-NS5) was detected. The possibility of a mutation in the reporter gene,

luciferase, was excluded by real-time PCR experiments that clearly showed a dose-dependent decrease in viral RNA levels upon addition of **16**. Thus, the action of **16** could consist in its binding to the 5′UTR and blocking RNA replication at the very beginning of the process by preventing the NS5B polymerase from binding to the viral RNA. Another hypothesis presumes compound **16**-induced mutations of host cell factors, e.g., inosine monophosphate dehydrogenase (IMPDH). Since acridone-based inhibitors of IMPDH have been reported recently[29] and since one of the putative mechanisms of ribavirin action in anti-HCV therapy is IMPDH inhibition,[30] inhibition of this enzyme by **16** could contribute to its antiviral effect. Inhibition of topoisomerase II and protein kinase C (PKC) should also be considered, since inhibition of these proteins was reported for other antiviral acridone derivatives.[19,31] Yet, interaction of compound **16** with the NS3 helicase cannot be excluded since a marginal antiviral effect exerted by **16** in mutant cells was observed. Mechanism of action of compound **16**, whose understanding is crucial for development of highly effective anti-HCV drugs, is a subject of further investigations.

Conclusion

Although the mechanism of action of compound **16** in the replicon system remains unclear and may involve inhibition of various viral and host factors, compound **16** constitutes the best inhibitor of HCV replication among all the helicase inhibitors published until now, with a TI above 1000 and EC_{50} below 1 μM, similar to values obtained for 4′-azidocytidine, a lead compound in the development of balapiravir, a drug already submitted to clinical trials,[17] proving that compound **16** is an excellent lead for further rational design of drugs against HCV.

Experimental Section

Chemistry. Unless otherwise stated, all chemicals were of analytical grade and obtained from Sigma-Aldrich Chemie GmBH (Schnelldorf, Germany), TCI Europe (Zwijndrecht, Belgium), or Apollo Scientific Limited (Bredbury, UK) and used without further purification. ^{1}H- and ^{13}C NMR spectra were recorded on a Bruker Advance DPx200 (200 and 50 MHz). Chemical shifts are reported in δ units (ppm) relative to Me_4Si as internal standard for DMSO-d_6 spectras or alternatively the solvent signal for *d*-trifluoroaceticacid (*d*-TFA) spectras (s, bs, d, m, Cq for singlet, broad singlet, doublet, multiplet, and quartenary carbon, respectively) and *J* values are reported in hertz. Mass spectra (MS) were obtained with a Shimadzu (GC-17A; MS-QP5050A) spectrometer. The purity of the tested compounds and intermediates is larger than 95% and was established by combustion analysis with a Perkin-Elmer 2400 CHN elemental analyzer.

General Synthesis Procedure for Compounds 8−18. To a suspension of **4**, **5**, **6**, or **7** (1 mmol) in 10 mL of dry toluol, thionyl chloride (110 μL, 1.5 mmol), and pyridine (120 μL, 1.5 mmol) were added. The mixture was stirred at room temperature for 4 h, then the amine (2 mmol) and triethylamine (250 μL, 2 mmol) were added and stirred overnight. The solvent was removed under vacuum, and water was added. The precipitate was filtered, washed with water, and dried. The solid was purified by crystallization in DMF/EtOH/H_2O.

5-Methoxy-*N*-(3,4,5-trimethoxyphenyl)-acridone-4-carboxamide (8). The title compound was synthesized according to the general procedure with **4** (0.269 g, 1 mmol) as acridonic acid derivative and 3,4,5-trimethoxyaniline (0.366 g, 2 mmol) as amine. Yield: 0.169 g (39%) yellow solid; mp: 263−267 °C. ^{1}H NMR (DMSO-d_6): δ 3.69 (s, 3H, OCH_3), 3.82 (s, 6H, 2× OCH_3), 4.06 (s, 3H, OCH_3), 7.16 (s, 2H), 7.21−7.32 (m, 1H), 7.36−7.49 (m, 2H), 7.77−7.85 (m, 1H), 8.41−8.55 (m, 2H), 10.58 (s, 1H, NH), 12.25 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 55.8 (2× OCH_3), 56.4 (OCH_3), 60.1 (OCH_3), 99.5 (2× CH), 112.8 (CH), 116.9 (CH), 118.7 (Cq), 120.1 (CH), 120.9 (Cq), 121.5 (Cq), 121.6 (CH), 130.7 (CH), 130.9 (Cq), 133.7 (CH), 134.2 (Cq), 134.4 (Cq), 139.6 (Cq), 147.6 (Cq), 152.6 (Cq), 166.6, (CON), 176.3 (CO). MS *m*/*z*: 434 (41%, M^+), 252 (40%), 209 (24%), 183 (100%), 168 (65%).

5-Methoxy-*N*-(pyridin-2-yl)-acridone-4-carboxamide (9). The compound was synthesized according to the general procedure with **4** (0.269 g, 1 mmol) as acridonic acid derivative and 2-aminopyridine (0.188 g, 2 mmol) as amine. Yield: 0.176 g (51%) yellow solid; mp: 272−276 °C. ^{1}H NMR (DMSO-d_6): δ 7.21−7.33 (m, 2H), 7.33−7.45 (m, 2H), 7.77−7.85 (m, 1H), 7.88−8.00 (m, 1H), 8.10−8.19 (m, 1H), 8.43−8.64 (m, 3H), 11.17 (s, 1H, NH), 12.26 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 56.5 (OCH_3), 112.9 (CH), 115.9 (CH), 116.9 (CH), 117.7 (Cq), 120.2 (CH), 120.4 (CH), 120.9 (Cq), 121.6 (Cq), 121.7 (CH), 130.9 (Cq), 131.2 (CH), 134.7 (CH), 138.2 (CH), 139.9 (Cq), 147.6 (Cq), 148.1 (CH), 151.5 (Cq), 167.5 (CON), 176.3 (CO). MS *m*/*z*: 345 (98%, M^+), 251 (100%), 236 (32%), 223 (86%).

5-Methoxy-*N*-(pyridin-3-yl)-acridone-4-carboxamide (10). The compound was synthesized according to the general procedure with **4** (0.269 g, 1 mmol) as acridonic acid derivative and 3-aminopyridine (0.188 g, 2 mmol) as amine. Yield: 0.136 g (39%) yellow solid; mp: 330−335 °C. ^{1}H NMR (DMSO-d_6): δ 4.06 (s, 3H, OCH_3), 7.21−7.33 (m, 1H), 7.36−7.51 (m, 3H), 7.77−7.85 (m, 1H), 8.14−8.24 (m, 1H), 8.41 (bs, 1H), 8.48−8.57 (m, 2H), 8.94 (bs, 1H), 10.85 (s, 1H, NH), 12.24 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 56.5 (OCH_3), 112.9 (CH), 116.9 (CH), 118.0 (Cq), 120.2 (CH), 120.9 (Cq), 121.6 (Cq), 121.7 (CH), 123.6 (CH), 128.7 (CH), 130.9 (Cq), 131.1 (CH), 134.1 (CH), 134.9 (Cq), 139.7 (Cq), 142.9 (CH), 145.3 (CH), 147.6 (Cq), 167.2 (CON), 176.3 (CO). MS *m*/*z*: 345 (47%, M^+), 251 (64%), 223 (69%), 209 (62%), 153 (67%), 94 (100%).

***N*-(4-Chloropyridin-2-yl)-5-methoxyacridone-4-carboxamide (11).** The compound was synthesized using the general procedure with **4** (0.269 g, 1 mmol) as acridonic acid derivative and 2-amino-4-chloropyridine (0.237 g, 2 mmol) as amine. Yield: 0.143 g (38%) yellow solid. mp: 321−327 °C. ^{1}H NMR (DMSO-d_6): δ 4.08 (s, 3H, OCH_3), 7.20−7.46 (m, 4H), 7.78−7.86 (m, 1H), 8.27 (s, 1H), 8.42−8.62 (m, 3H), 11.46 (s, 1H, NH), 12.17 (s, 1H, NH). ^{13}C NMR (*d*-TFA): δ 58.4 (OCH3), 116.3 (CH), 117.6 (CH), 118.0 (Cq), 118.9 (Cq), 119.1 (Cq), 119.5 (CH), 125.4 (CH), 127.2 (CH), 130.1 (CH), 135.0 (CH), 135.6 (Cq), 140.3 (CH), 140.6 (Cq), 140.9 (CH), 150.2 (Cq), 150.7 (Cq), 171.5 (Cq), 172.3 (Cq). MS m/*z*: 379 (32%, M^+), 251 (87%), 223 (100%), 152 (52%), 75 (63%).

2-Chloro-5-methoxy-*N*-(pyridin-2-yl)-acridone-4-carboxamide (12). The compound was synthesized according to the general approach with **5** (0.304 g, 1 mmol) as acridonic acid derivative and 2-aminopyridine (0.188 g, 2 mmol) as amine. Yield: 0.197 g (52%) dark-yellow solid; mp: 244−251 °C. ^{1}H NMR (DMSO-d_6): δ 4.06 (s, 3H, OCH_3), 7.21−7,35 (m, 2H), 7.36−7.47 (m, 1H), 7.75−7,83 (m, 1H), 7.88−8.00 (m, 1H), 8.08−8.17 (m, 1H), 8.28−8.50 (m, 2H), 8.64−8.70 (m, 1H), 11.38 (s, 1H, NH), 12.20 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 56.5 (OCH_3), 113.2 (CH), 116.0 (CH), 116.8 (CH), 116.9 (CH), 120.5 (CH), 120.8 (Cq), 122.1 (CH), 122.5 (Cq), 124.7 (Cq), 129.6 (CH), 130.7 (Cq), 134.4 (CH), 138.3 (CH), 138.4 (Cq), 147.6 (Cq), 151.4 (Cq), 166.3 (CON), 175.2 (CO). MS *m*/*z*: 381 (19%, M^+, ^{37}Cl) 379 (56%, M^+, ^{35}Cl), 285 (62%), 257 (63%), 151 (54%), 78 (100%).

2-Chloro-5-methoxy-*N*-(pyridin-3-yl)-acridone-4-carboxamide (13). The compound was synthesized according to the general procedure with **5** (0.304 g, 1 mmol) as acridonic acid derivative and 3-aminopyridine (0.188 g, 2 mmol) as amine. Yield: 0.102 g (27%) yellow solid; mp: 280−283 °C. ^{1}H NMR (DMSO-d_6): δ 4.04 (s, 3H, OCH_3), 7.20−7.54 (m, 3H), 7.72−7.80 (m, 1H), 8.13−8.21 (m, 1H), 8.37−8.46 (m, 2H), 8.56 (s, 1H), 8.92 (s, 1H), 10.88 (s, 1H, NH), 12.17 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ

56.5 (OCH_3), 113.1 (CH), 116.9 (CH), 120.00 (Cq), 120.1 (Cq), 120.8 (Cq), 122.0 (CH), 122.6 (Cq), 123.6 (CH), 124.6 (Cq), 128.6 (CH), 129.5 (CH), 133.6 (CH), 138.3 (Cq), 142.9 (CH), 145.4 (CH), 147.5 (Cq), 147.6 (Cq), 165.8 (CON), 175.2 (CO). MS *m*/*z*: 381 (36%, M^+, ^{37}Cl), 379 (100%, M^+, ^{35}Cl), 285 (98%), 256 (65%).

2-Chloro-*N*-(4-chloropyridin-2-yl)-5-methoxyacridone-4-carboxamide (14). The compound was synthesized according to the general procedure with **5** (0.304 g, 1 mmol) as acridonic acid derivative and 2-amino-4-chloropyridine (0.237 g, 2 mmol) as amine. Yield: 0.176 g (43%) orange solid; mp: 298−304 °C. ^1H NMR (DMSO-d_6): δ 4.07 (s, 3H, OCH_3), 7.21−7.44 (m, 3H), 7.74−7.83 (m, 1H), 8.21−8.25 (m, 1H), 8.37−8.50 (m, 2H), 8.60−8.65 (m, 1H), 11.60 (s, 1H, NH), 12.08 (bs, 1H, NH). ^{13}C NMR (*d*-TFA): δ 58.5 (OCH_3), 116.4 (CH), 117.7 (CH), 118.5 (Cq), 119.6 (CH), 120.0 (Cq), 120.7 (Cq), 125.6 (CH), 130.3 (CH), 133.3 (CH), 134.0 (Cq), 135.6 (Cq), 138.8 (Cq), 140.5 (CH), 141.3 (CH), 149.9 (Cq), 150.8 (Cq), 160.1 (Cq), 170.5 (Cq), 172.0 (Cq). MS *m*/*z*: 415 (65%, M^+, $^{37}Cl^{35}Cl$), 413 (80%, M^+, $^{35}Cl_2$), 285 (100%), 270 (32%), 257 (87%).

2-Fluoro-5-methoxy-*N*-(pyridine-2-yl)-acridone-4-carboxamide (15). The compound was synthesized using the general procedure with **6** (0.287 g, 1 mmol) as acridonic acid derivative and 2-aminopyridine (0.188 g, 2 mmol) as amine. Yield: 0.196 g (54%) yellow solid; mp: 299−305 °C. ^1H NMR (DMSO-d_6): δ 4.07 (s, 3H, OCH_3), 7.22−7.46 (m, 3H), 7.75−7.84 (m, 1H), 7.89−8.01 (m, 1H), 8.11−8.24 (m, 2H), 8.46−8.62 (m, 2H), 11.30 (s, 1H, NH), 12.20 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 58.5 (OCH_3), 116.0 (CH), 117.4 (CH), 117.9 (Cq), 118.4 (d, CH, J = 24.2 Hz), 119.8 (CH), 120.2 (d, Cq, J = 8.3 Hz), 121.6 (d, Cq, J = 6.5 Hz), 124.8 (CH), 130.4 (CH), 130.9 (d, CH, J = 28.8 Hz), 135.7 (Cq), 137.6 (Cq), 139.8 (CH), 149.4 (Cq), 150.6 (CH), 150.7 (Cq), 160.2 (d, CF, J = 253.3 Hz), 170.5 (d, Cq, J = 1.1 Hz), 171.8 (d, Cq, J = 4.6 Hz). MS *m*/*z*: 363 (100%, M^+), 269 (74%), 254 (27%), 241 (58%).

2-Fluoro-5-methoxy-*N*-(pyridine-3-yl)-acridone-4-carboxamide (16). The compound was synthesized according to the general procedure with **6** (0.287 g, 1 mmol) as acridonic acid derivative and 3-aminopyridine (0.188 g, 2 mmol) as amine. Yield: 0.182 g (50%) yellow solid; mp: 289−295 °C. ^1H NMR (DMSO-d_6-): δ 4.04 (s, 3H, OCH_3), 7.20−7.44 (m, 3H), 7.73−7.80 (m, 1H), 8.14−8.22 (m, 2H), 8.42−8.51 (m, 2H), 8.94 (bs, 1H), 10.84 (s, 1H, NH), 12.15 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 56.5 (OCH_3), 112.9 (CH), 115.2 (d, CH, J = 21.9 Hz), 116.7 (2× CH), 119.9 (Cq), 120.0 (Cq), 120.1 (Cq), 121.8 (CH), 122.1 (CH), 122.4 (d, CH, J = 26.9), 122.5 (Cq), 128.6 (CH), 130.8 (Cq), 136.6 (Cq), 142.9 (CH), 145.5 (CH), 147.5 (Cq), 155.4 (d, CF, J = 238.6 Hz), 165.9 (CON), 175.5 (CO). MS *m*/*z*: 363 (100%, M^+), 269 (92%), 241 (59%), 213 (20%), 185 (18%).

***N*-(4-Chloropyridin-2-yl)-2-fluoro-5-methoxyacridone-4-carboxamide (17).** The compound was synthesized according to the general approach with **6** (0.287 g, 1 mmol) as acridonic acid derivative and 2-amino-4-chloropyridine (0.237 g, 2 mmol) as amine. Yield: 0.254 g (64%) yellow solid; mp: >350 °C. ^1H NMR (*d*-TFA): δ 4.27 (s, 3H, OCH_3), 7.59−7.88 (m, 3H), 8.03 (s, 1H), 8.16−8.27 (m, 1H), 8.50−8.58 (m, 1H), 8.69−8.77 (m, 2H). ^{13}C NMR (*d*-TFA): δ 58.5 (OCH_3), 116.0 (CH), 117.4 (CH), 118.0 (Cq), 118.4 (d, CH, J = 23.6 Hz), 119.6 (CH), 120.2 (d, Cq, J = 8.4 Hz), 121.4 (d, Cq, J = 6.7 Hz), 125.6 (CH), 130.4 (CH), 131.0 (d, CH, J = 28.7 Hz), 135.7 (Cq), 137.6 (Cq), 140.5 (CH), 149.9 (Cq), 150.7 (Cq), 160.1 (Cq), 160.2 (d, Cq, J = 253.2 Hz), 170.4 (d, Cq, J = 1.5 Hz), 171.8 (d, Cq, J = 5.2 Hz). MS *m*/*z*: 399 (29%, M^+, ^{37}Cl), 397 (81%, M^+, ^{35}Cl), 269 (100%), 254 (31%), 241 (73%).

5-Benzyloxy-*N*-(pyridine-4-yl)-acridone-4-carboxamide (18). The compound was synthesized according to the general procedure with **7** (0.345 g, 1 mmol) as acridonic acid derivative and 4-aminopyridine (0.188 g, 2 mmol) as amine. Yield: 0.081 g (19%) yellow solid; mp: 283−285 °C. ^1H NMR (DMSO-d_6): δ 5.42 (s, 2H, OCH_2), 7.22−7.34 (m, 1H), 7.39−7.56 (m, 5H), 7.67−7.90 (m, 5H), 8.49−8.62 (m, 4H), 10.97 (s, 1H, NH), 12.41 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 70.0 (OCH_2), 114.4 (CH), 114.5 (CH), 117.3 (CH), 118.0 (Cq), 120.1 (CH), 121.1 (Cq), 121.6 (Cq), 121.7 (CH), 126.9 (2× CH), 127.9 (CH), 128.4 (2× CH), 131.2 (Cq), 131.4 (CH), 134.1 (CH), 136.5 (Cq), 139.8 (Cq), 145.4 (Cq), 146.4 (Cq), 150.3 (2× CH), 167.8 (CON), 176.3 (CO). MS *m*/*z*: 421 (30%, M^+), 330 (9%), 236 (42%), 185 (11%), 91 (100%).

Biology. 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.[16] 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 (Bernatowicz-Najda et al., submitted), protein expression in the baculovirus system and purification from insect cells performed as previously described.[16]

Helicase Inhibition Assay. The fluorometric helicase activity assay and inhibitor screening were performed as described in Boguszewska-Chachulska et al.,[32] with minor modifications concerning the reaction temperature and volume that were 37 °C and 60 μL, respectively.

Nucleic Acid Intercalation. Intercalatory properties of selected compounds were studied by the dsNA migration retardation assay as described previously.[12] dsRNA was prepared as described in Krawczyk et al.[11]

RNA Isolation and Reverse Transcription. RNA spin Mini kit (GE Healthcare, Little Chalfont, UK) was used to isolate total RNA from Huh-7 cells bearing the subgenomic replicon, according to the manufacturer's instructions. To obtain cDNA for sequencing of the NS3-NS5B fragment from naïve and mutant Huh-7 cells, 8 pmoles of a specific primer (3UTR: 5′ACWTGATCTGCAGAGAGRCC3′ or NS3Rev1: 5′GMRCAYTCYTCCATYTCRTC3′) and 1−2 μg of RNA were used. To obtain cDNA for the real-time PCR reactions, 5 μg of RNA and a mixture of primers were used: 0.25 μM 3UTR, 2.5 μM oligo(dT)$_{15}$, and 2.5 ng/μL random hexamers. To facilitate annealing, the primer was incubated with RNA for 15 min at 65 °C followed by 2−3 min incubation on ice. After addition of Master Mix (1× First Strand buffer, 10 mM DTT, 200 U Super Script III reverse transcriptase (Invitrogen, Carlsbad, CA), 1 mM dNTPs (metabion, Munich, Germany), and 40 U RiboLock RNase inhibitor (Fermentas, Vilnius, Lithuania), the reverse transcription reaction was carried for 1 h at 50 °C and stopped by placing at 75 °C for 15 min.

Real-Time PCR. For each reaction, 2 μL of cDNA diluted 1:10 was used with 8 μL of the reaction mixture (JumpStart Taq ReadyMix, Sigma, Poznań, Poland) and 0.2 μM forward and reverse primer: RT1Up: 5′CTGCGGAGGAAACCAAG3′ and RT1Low: 5′GAATGTGGGGGCGTCAG3′ for the amplification of the viral target gene (a fragment of NS5B) and TubF: 5′CTTCAAGCGCATCTCGGAGC3′ and TubR: 5′TGCGGTGGCATCCTGGTACT 3′ for the amplification of the cellular reference gene (a tubulin fragment). Real-time PCR was carried out in the Light Cyler 480 (Roche Diagnostics, Warsaw, Poland) apparatus using the following program: 10 min initial denaturation at 95 °C followed by 45 cycles of 10 s denaturation at 95 °C, 20 s annealing at 56 °C and 10 s elongation at 72 °C. To calculate the efficiency of reactions for each pair of primers the absolute quantification method, a part of the program supplied with Light Cyler 480, was applied. To verify the inhibitory potential of **16** on viral RNA replication, the basic relative quantification method was used.

HCV and Cell Toxicity Replicon Studies. The human hepatoma cell line Huh-7, carrying the subgenomic HCV genotype 1 replicon with the luc-ubi-neo (reporter/selective) fusion gene,[33] was kindly provided by Dr. Ralf Bartenschlager (University of Heidelberg, Heidelberg, Germany). The cells were grown as described.[10,33] The conditions of the luminescence-based assay used to test the antiviral activity as well as

cytotoxicity of the compounds were previously described.[10−12] 4′azidocytidine was kindly provided by Dr. Johan Neyts (Katolik Universitat, Leuven, Belgium); ribavirin was purchased from ICN Biochemicals (ICN Biochemicals, Cleveland, OH).

For the real time-PCR experiments, cells were seeded at a density of 4×10^5 on a 100 mm culture dish in DMEM medium (Invitrogen) and grown in the presence of 0, 1, 10, or 100 μM **16** for 7 days.

To evaluate the combinatorial effect of **16** with ribavirin or IFN-γ (Institute of Biotechnology and Antibiotics, Poland) on HCV replication, increasing concentrations of the compounds were added: 0, 0.1, 0.25, 0.5, 0.75, 1, 10, 20 μM compound **16**; 0, 0.01, 0.02, 0.03, 0.04, and 0.05 U/mL IFN-γ, and 0, 0.1, 1, 5, 10, and 20 μM ribavirin. The final concentration of DMSO was always 1%. After 3 days, luminescence was measured and Prichard and Shipman's MacSynergy II software was used to calculate the data.[18]

To obtain mutants resistant to **16**, Huh-7 cells bearing the subgenomic replicon were seeded at a density of 2×10^6 cells in a 100 mm diameter culture dish (Sarstedt) and grown at 37 °C and 5% CO_2 in complete DMEM medium (Invitrogen) supplemented with 250 μg/mL G418 and 10 μM **16**. The cells were passaged upon reaching confluence (every 3−4 days). After 4 weeks, the colonies of cells resistant to G418, and thus to the compound, were obtained, and total RNA from the mutant cells isolated, reverse transcribed and the NS3-5B fragment sequenced.

Acknowledgment. This work was supported by the Ministry of Science and Higher Education grant N N401 2329 3. We thank Dr. Anne-Lise Haenni for careful reading of the manuscript and helpful remarks.

Supporting Information Available: Synthesis and spectroscopic data for the nonkey compounds **1**−**6**. Elemental analyses for compounds **8**−**11**, **13**−**18** and high resolution mass for compound **12**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hayashi, N.; Takehara, T. Antiviral therapy for chronic hepatitis C: past, present, and future. *J. Gastroenterol.* **2006**, *41*, 17–27.
(2) Reesink, H. W.; Zeuzem, S.; Weegink, C. J.; Forestier, N.; Van Vliet, A.; Van De Wetering De Rooij, J.; McNair, L.; Purdy, S.; Kauffman, R.; Alam, J.; Jansen, P. L. M. Rapid decline of viral RNA in hepatitis C patients treated with VX-950: a phase Ib, placebo-controlled, randomized study. *Gastroenterology* **2006**, *131*, 997–1002.
(3) Manns, M.; Muir, A.; Adda, N.; et al. Telaprevir in hepatitis C genotype-1-infected patients with prior non-response, viral breakthrough or relapse to peginterferon-alfa-2a/b and ribavirin therapy: SVR results of the PROVE3 study. In 44th Annual Meeting of the European Association for the Study of the Liver (EASL 2009), Copenhagen, 2009.
(4) Kwo, P.; Lawitz, E.; McCone, J.; Schiff, E.; Vierling, J.; Pound, D.; Davis, M.; Galati, J.; Gordon, S.; Ravendhran, N.; Rossaro, L.; Anderson, F.; Jacobson, I.; Rubin, R.; Koury, K.; Brass, C.; Chaudhri, E.; Albrecht, J. HCV SPRINT-1 final results: SVR 24 from a phase 2 study of boceprevir plus pegintron (peginterferon alfa-2b)/ribavirin in treatment-naïve subjects with genotype-1 chronic hepatitis C. In 44th Annual Meeting of the European Association for the Study of the Liver (EASL), Copenhagen, 2009.
(5) Thompson, A.; Patel, K.; Tillman, H.; McHutchison, J. G. Directly acting antivirals for the treatment of patients with hepatitis C infection: a clinical development update addressing key future challenges. *J. Hepatol.* **2009**, *50*, 184–194.
(6) Frick, D. N. The hepatitis C virus NS3 protein: a model RNA helicase and potential drug target. *Curr. Issues Mol. Biol.* **2007**, *9*, 1–20.
(7) Lam, A. M.; Frick, D. N. Hepatitis C virus subgenomic replicon requires an active NS3 RNA helicase. *J. Virol.* **2006**, *80*, 404–411.
(8) Kim, J. L.; Morgenstern, K. A.; Griffith, J. P.; Dwyer, M. D.; Thomson, J. A.; Murcko, M. A.; Lin, C.; Caron, P. R. Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. *Structure* **1998**, *6*, 89–100.
(9) Lam, A. M.; Rypma, R. S.; Frick, D. N. Enhanced nucleic acid binding to ATP-bound hepatitis C virus NS3 helicase at low pH activates RNA unwinding. *Nucleic Acids Res.* **2004**, *32*, 4060–4070.
(10) Gozdek, A.; Zhukov, I.; Polkowska, A.; Poznanski, J.; Stankiewicz-Drogon, A.; Pawlowicz, J. M.; Zagorski-Ostoja, W.; Borowski, P.; Boguszewska-Chachulska, A. NS3 Peptide, a novel potent hepatitis C virus NS3 helicase inhibitor: its mechanism of action and antiviral activity in the replicon system. M. *Antimicrob. Agents Chemother.* **2008**, *52*, 393–401.
(11) Krawczyk, M.; Wasowska-Lukawska, M.; Oszczapowicz, I.; Boguszewska-Chachulska, A. M. Amidinoanthracyclines—a new group of potential anti-hepatitis C virus compounds. *Biol. Chem.* **2009**, *390*, 351–360.
(12) Stankiewicz-Drogon, A.; Palchykovska, L. G.; Kostina, V. G.; Alexeeva, I. V.; Shved, A. D.; Boguszewska-Chachulska, A. M. New acridone-4-carboxylic acid derivatives as potential inhibitors of Hepatitis C virus infection. *Bioorg. Med. Chem.* **2008**, *16*, 8846–8852.
(13) Manfroni, G.; Paeshuyse, J.; Massari, S.; Zanoli, S.; Gatto, B.; Maga, G.; Tabarrini, O.; Cecchetti, V.; Fravolini, A.; Neyts, J. Inhibition of subgenomic hepatitis C virus RNA replication by acridone derivatives: identification of an NS3 helicase inhibitor. *J. Med. Chem.* **2009**, *52*, 3354–3365.
(14) Sharp, M. J.; Mader, C. J.; Strachan, C.; Synthesis of acridine derivative as multidrug-resistant inhibitors. (Glaxo Group Ltd., UK). PCT Int. Appl., 1998.
(15) Dörner, B.; Kuntner, C.; Bankstahl, J. P.; Bankstahl, M.; Stanek, J.; Wanek, T.; Stundner, G.; Mairinger, S.; Löscher, W.; Müller, M.; Langer, O.; Erker, T. Synthesis and Small-Animal Positron Emission Tomography Evaluation of [11C]-Elacridar As a Radiotracer to Assess the Distribution of P-Glycoprotein at the Blood−Brain Barrier. *J. Med. Chem.* **2009**, *52*, 6073–6082.
(16) Boguszewska-Chachulska, A. M.; Krawczyk, M.; Stankiewicz, A.; Gozdek, A.; Haenni, A. L.; Strokovskaya, L. Direct fluorometric measurement of hepatitis C virus helicase activity. *FEBS Lett.* **2004**, *567*, 253–258.
(17) Klumpp, K.; Leveque, V.; Le Pogam, S.; Ma, H.; Jiang, W. R.; Kang, H.; Granycome, C.; Singer, M.; Laxton, C.; Hang, J. Q.; Sarma, K.; Smith, D. B.; Heindl, D.; Hobbs, C. J.; Merrett, J. H.; Symons, J.; Cammack, N.; Martin, J. A.; Devos, R.; Najera, I. The novel nucleoside analog R1479 (4′-azidocytidine) is a potent inhibitor of NS5B-dependent RNA synthesis and hepatitis C virus replication in cell culture. *J. Biol. Chem.* **2006**, *281*, 3793–3799.
(18) Prichard, M. N.; Shipman, C., Jr. A three-dimensional model to analyze drug−drug interactions. *Antiviral Res.* **1990**, *14*, 181–205.
(19) Bastow, K. F. New acridone inhibitors of human herpes virus replication. *Curr. Drug Targets Infect. Disord.* **2004**, *4*, 323–330.
(20) Bernardino, A. M.; Castro, H. C.; Frugulhetti, I. C.; Loureiro, N. I.; Azevedo, A. R.; Pinheiro, L. C.; Souza, T. M.; Giongo, V.; Passamani, F.; Magalhaes, U. O.; Albuquerque, M. G.; Cabral, L. M.; Rodrigues, C. R. SAR of a series of anti-HSV-1 acridone derivatives, and a rational acridone-based design of a new anti-HSV-1 3H-benzo[b]pyrazolo[3,4-*h*]-1,6-naphthyridine series. *Bioorg. Med. Chem.* **2008**, *16*, 313–321.
(21) Adams, A. Crystal structures of acridines complexed with nucleic acids. *Curr. Med. Chem.* **2002**, *9*, 1667–1675.
(22) Tuteja, N.; Phan, T. N.; Tuteja, R.; Ochem, A.; Falaschi, A. Inhibition of DNA unwinding and ATPase activities of human DNA helicase II by chemotherapeutic agents. *Biochem. Biophys. Res. Commun.* **1997**, *236*, 636–640.
(23) Tackett, A. J.; Wei, L.; Cameron, C. E.; Raney, K. D. Unwinding of nucleic acids by HCV NS3 helicase is sensitive to the structure of the duplex. *Nucleic Acids Res.* **2001**, *29*, 565–572.
(24) Goodell, J. R.; Madhok, A. A.; Hiasa, H.; Ferguson, D. M. Synthesis and evaluation of acridine- and acridone-based anti-herpes agents with topoisomerase activity. *Bioorg. Med. Chem.* **2006**, *14*, 5467–5480.
(25) Piestrzeniewicz, M. K.; Wilmanska, D.; Studzian, K.; Szemraj, J.; Czyz, M.; Denny, W. A.; Gniazdowski, M. Inhibition of RNA synthesis in vitro by acridines—relation between structure and activity. *Z. Naturforsch., C: J. Biosci.* **1998**, *53*, 359–368.
(26) Dziegielewski, J.; Slusarski, B.; Konitz, A.; Skladanowski, A.; Konopa, J. Intercalation of imidazoacridinones to DNA and its relevance to cytotoxic and antitumor activity. *Biochem. Pharmacol.* **2002**, *63*, 1653–1662.
(27) Glenn, J. S. Small molecule approaches to NS4B and other novel non-structural protein targets. In HCV Drug Discovery, San Diego, CA, **2009**.
(28) Coelmont, L.; Paeshuyse, J.; Windisch, M. P.; De Clercq, E.; Bartenschlager, R.; Neyts, J. Ribavirin antagonizes the in vitro anti-hepatitis C virus activity of 2′-C-methylcytidine, the active component of valopicitabine. *Antimicrob. Agents Chemother.* **2006**, *50*, 3444–3446.

(29) Watterson, S. H.; Chen, P.; Zhao, Y.; Gu, H. H.; Dhar, T. G.; Xiao, Z.; Ballentine, S. K.; Shen, Z.; Fleener, C. A.; Rouleau, K. A.; Obermeier, M.; Yang, Z.; McIntyre, K. W.; Shuster, D. J.; Witmer, M.; Dambach, D.; Chao, S.; Mathur, A.; Chen, B. C.; Barrish, J. C.; Robl, J. A.; Townsend, R.; Iwanowicz, E. J. Acridone-based inhibitors of inosine 5′-monophosphate dehydrogenase: discovery and SAR leading to the identification of *N*-(2-(6-(4-ethylpiperazin-1-yl)pyridin-3-yl)propan-2-yl)-2-fluoro-9-oxo-9,10-dihydroacridine-3-carboxamide (BMS-566419). *J. Med. Chem.* **2007**, *50*, 3730–3742.

(30) Zhou, S.; Liu, R.; Baroudy, B. M.; Malcolm, B. A.; Reyes, G. R. The effect of ribavirin and IMPDH inhibitors on hepatitis C virus subgenomic replicon RNA. *Virology* **2003**, *310*, 333–342.

(31) Vance, J.; Bastow, K. F. Inhibition of DNA topoisomerase II catalytic activity by the antiviral agents 7-chloro-1,3-dihydroxyacridone and 1,3,7-trihydroxyacridone. *Biochem. Pharmacol.* **1999**, *58*, 703–708.

(32) Boguszewska-Chachulska, A. M.; Krawczyk, M.; Najda, A.; Kopanska, K.; Stankiewicz-Drogon, A.; Zagorski-Ostoja, W.; Bretner, M. Searching for a new anti-HCV therapy: synthesis and properties of tropolone derivatives. *Biochem. Biophys. Res. Commun.* **2006**, *341*, 641–647.

(33) Vrolijk, J. M.; Kaul, A.; Hansen, B. E.; Lohmann, V.; Haagmans, B. L.; Schalm, S. W.; Bartenschlager, R. A replicon-based bioassay for the measurement of interferons in patients with chronic hepatitis C. *J. Virol. Methods* **2003**, *110*, 201–209.