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New acridone-4-carboxylic acid derivatives as potential inhibitors of *Hepatitis C virus* infection

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ABSTRACT

A new class of compounds—acridone derivatives—was tested using the direct fluorometric helicase activity assay to determine the inhibitory properties of the derivatives towards the NS3 helicase of *Hepatitis C virus* (HCV). The compounds were also tested as putative transcription inhibitors of in vitro transcription based on the DNA-dependent T7 RNA polymerase. Most of the acridone derivatives tested were transcription inhibitors; however, only four of them inhibited the NS3 helicase at low concentrations (IC_{50} from 3 μM to 20 μM) and were therefore selected for further studies on the mechanism of inhibition. The acridone derivatives probably act via intercalation into double-stranded nucleic acids but they may also interact directly with viral enzymes. Selected carboxamides were tested in the subgenomic HCV replicon system. Two of the compounds: *N*-(pyridin-4-yl)-amide and *N*-(pyridin-2-yl)-amide of acridone-4-carboxylic acid are efficient RNA replication inhibitors with selectivity indexes of 19.4 and 40.5, respectively, proving that the acridone derivatives may be regarded as potential antiviral agents.

1. Introduction

About 180 million people worldwide are affected with chronic hepatitis caused by *Hepatitis C virus* (HCV; WHO, 2007). As the current therapy is long and ineffective in about 50% of individuals affected with genotype 1b,[1–3] 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 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 protease and RNA helicase/NTP-ase activities, or the NS5B protein, an RNA-dependent RNA polymerase.[4–6] One advantage of this approach is that these proteins have no close homologs among cellular enzymes.[6,7] Our studies focused on the potential inhibitors of the activity of the HCV RNA helicase—the C-terminal portion of the NS3 protein indispensable for viral replication—an enzyme that unwinds double-stranded (ds) forms of RNA and thus allows viral replication and translation to occur.[5,8,9]

As the three-dimensional structure of the HCV NS5B protein is similar to that of the T7 phage RNA polymerase, and since both polymerases contain the same structural domains, as well as the conserved GDD sequence motif,[10,11] an in vitro transcription assay based on the T7 RNA polymerase was used to test possible transcription inhibition capacities of selected compounds. This assay is routinely applied to examine potential inhibitors of RNA synthesis that may interact with DNA or RNA, such as acridine or acridone derivatives,[12,13] and therefore seemed a good tool to perform a parallel pre-screening of acridone-4-carboxylic acid derivatives to identify compounds with anti-polymerase activities.

Acridone derivatives have been known for many years as antimicrobial agents.[14] Recently their anticancer[14,15] and antiviral properties have been revealed. An acridone derivative, cycloferon, is known to act against *Influenza virus* and adenoviruses,[16] whereas other acridone derivatives were shown to inhibit *Human immunodeficiency virus*,[17] *West Nile virus* [18] *and Herpes simplex virus* replication.[19–21]

Since acridones are not nucleoside analogs, their specificity towards viral enzymes may be higher[6] as was demonstrated by recent studies in cell cultures on the replication of viruses of the *Flaviviridae* family. The studies of Tabarrini et al. led to the identification of acridone compounds that inhibit both *Bovine viral diarrhea virus* and HCV replication, and show selective antiviral activities.[22]

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Here, we demonstrate that a different approach—to study the anti-helicase activity of a large group of acridone derivatives so as to select potential specific inhibitors of HCV replication—may also ultimately yield effective anti-HCV drugs. Their antiviral activity may be further enforced by inhibition of the RNA-dependent RNA polymerase, NS5B.[4,6]

2. Results

2.1. Chemistry

For an extensive structure-activity relationship study of acridone-4-carboxylic acid amides, we first synthesized *N*-(carboxyphenyl)anthranilic acid (**1**) on a preparative scale, using the method of Jourdan–Ulmann copper-catalysed condensation of anthranilic acid with *ortho*-bromobenzoic acid. Cyclization to the acridone-4-carboxylic acid (ACA) (**2**) was achieved by heating compound **1** with excess polyphosphoric acid at 120 °C.[23,24] The synthesis of ACA (**2**) and its amides is shown in Scheme 1.

N-Substituted acridone-4-carboxamides were synthesized by amide bond formation between an activated form of ACA and an appropriate amine. A convenient technique for carboxylic group activation was developed that allowed us to obtain high yields of the products. Following this methodology, we prepared an essentially complete set of acridone-4-carboxamides whose amide fragments were formed by the alkyl groups or aryl-, heteryl-ring systems bearing exocyclic groups at different ring positions. The structures of the compounds studied are presented in Scheme 1.

2.2. Helicase inhibition assay

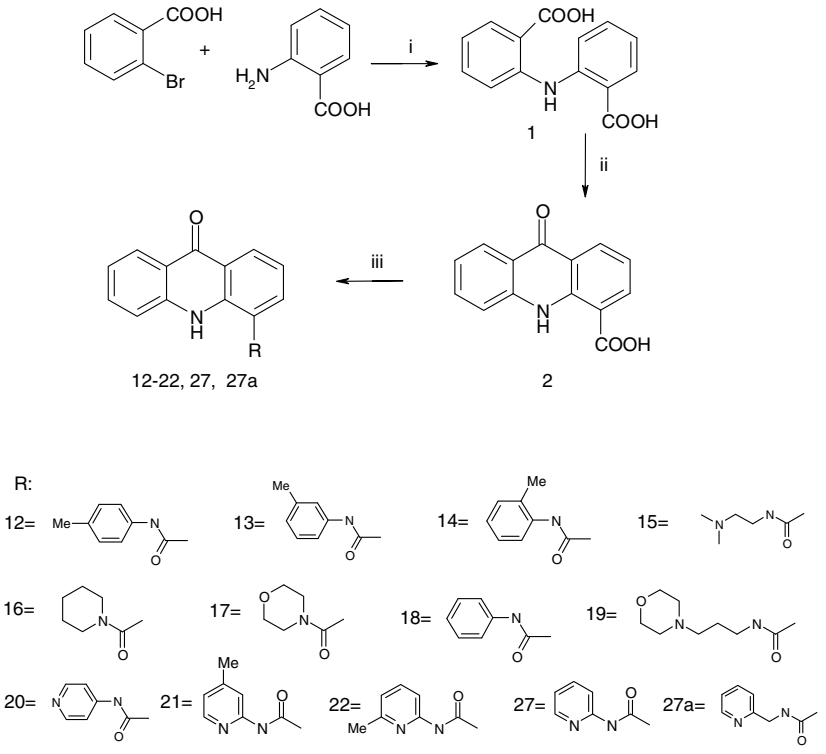
To test the library of new acridone derivatives, the fluorometric helicase activity assay was applied.[25,26] The compounds were initially tested at 10–100 μM and those showing anti-helicase activity at concentrations lower than 100 μM were subsequently tested in smaller increments near the presumed 50% inhibitory concentrations (IC_{50}).

Potent inhibitory activity towards the NS3 helicase was exhibited only by derivatives with pyridine heterocyclic tailpieces (**20, 21, 22** and **27**; Table 1). Compound **27** appeared to be the best inhibitor of helicase activity with an IC_{50} of 3.82 μM, while compound **20** had slightly weaker inhibitory properties with an IC_{50} of 8.9 μM. Addition of CH_3 substituents to the pyridine ring of **27** also slightly decreased the anti-helicase activity of **21** and **22**, resulting in IC_{50} values of 6.2 μM and 20.2 μM, respectively. The length of the linker at the pyridine-bearing amide fragment appears crucial for anti-helicase activity since addition of -CH_2–

Table 1
Inhibitory activities of acridone-4-carboxylic acid derivatives

Compound	M_W	NS3 helicase inhibition IC_{50}[a] ± SD[c] (μM)	Transcription inhibition IC_{99}[b] (μM)
12	328.37	>100	10
13	328.37	>100	40
14	328.37	>100	80
15	309.37	>100	80
16	306.36	>100	>100
17	308.34	>100	>100
18	314.34	>100	40
19	365.43	>100	>100
20	315.3	8.9 ± 0.4	20
21	329.3	6.2 ± 1.3	40
22	329.3	20.2 ± 2.3	80
27	315.3	3.8 ± 0.3	40
27a	329.3	>100	>100

[a] Concentration of inhibitor necessary to reduce the NS3 helicase activity by 50%.
[b] Inhibitor concentration necessary to reduce the T7 RNA polymerase activity by 99%.
[c] Standard deviation.



Scheme 1. Synthesis of acridone-4-carboxylic acid derivatives. Reagents and conditions: (i) Cu, K_2CO_3/DMF, 60–120 °C, 2 h; (ii) PPA, 120 °C, 1 h; (iii) $SOCl_2$/Py, toluene, amine, Et_3N, room temperature, 6–7 h.

in compound **27a** led to complete loss of its inhibitory properties (Table 1).

2.3. Mechanism of anti-helicase action

A series of experiments were designed to establish the mechanism of action of ACA derivatives on the helicase activity. Interaction/competition of helicase inhibitors (**20**, **21**, **22** and **27**) with the enzyme or a ds DNA substrate was studied in helicase activity assays performed at increasing enzyme or substrate concentrations and constant inhibitor concentrations, close to their IC_{50} values (10, 5, 33 and 5 μM, respectively). Inhibition was independent of enzyme concentration, suggesting lack of competition and interaction (Fig. 1A), while in the presence of increasing substrate concentrations a decrease of inhibition was observed (Fig. 1B), clearly indicating interaction of the compounds with the substrate.

To check if inhibition of helicase activity by acridone-4-carboxamides depends on the ATP concentration, the IC_{50} of **21** was measured at three ATP concentrations (Fig. 2). The three inhibition curves and the IC_{50} values did not differ, proving that the ATPase activity is not inhibited. This was further confirmed for all four inhibitors in the ATPase radioactive assay (data not shown).

2.4. Inhibition of T7 polymerase transcription

Taking into account the topological and functional similarity of polymerases, and the fact that the T7 RNA polymerase does not require a radioactive technique to evaluate and visualize transcription products,[12] we used it as an in vitro model to study polymerase inhibition by the set of ACA derivatives.

The phenylamide **18** and tolylamides bearing exocyclic methyl groups at positions *meta-*, *ortho-* and *para-* (**13**, **14** and **12**) exhibited significant, position-dependent activity on cell-free RNA transcription (Table 1). Compound **12** appeared to be the best transcription inhibitor, almost completely suppressing RNA synthesis at 10 μM (Fig. 3). The derivatives with pyridine heterocyclic tailpieces (**20**, **21**, **22** and **27**) exhibited comparable inhibition of RNA synthesis that was also substituent-dependent, compound **20** being the best inhibitor of this group of compounds (concentration of the inhibitor necessary to reduce the enzyme activity by 99%, IC_{99}, equals 20 μM). The carboxamide **27a** with the short CH_2 linker at the pyridine-bearing amide fragment completely lost the capacity to inhibit RNA synthesis (Table 1).

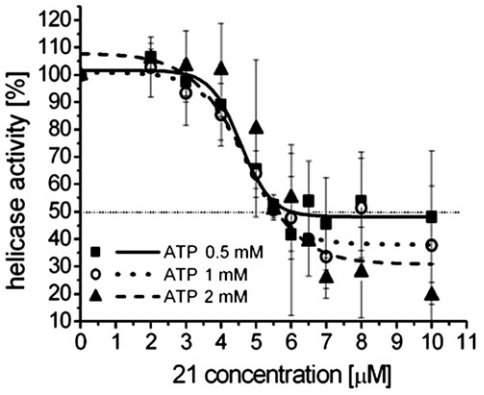


Figure 2. Inhibition of the NS3 helicase activity by compound **21** at three ATP concentrations. The results are presented as the percent activity of the helicase tested in the same conditions but with DMSO instead of inhibitor.

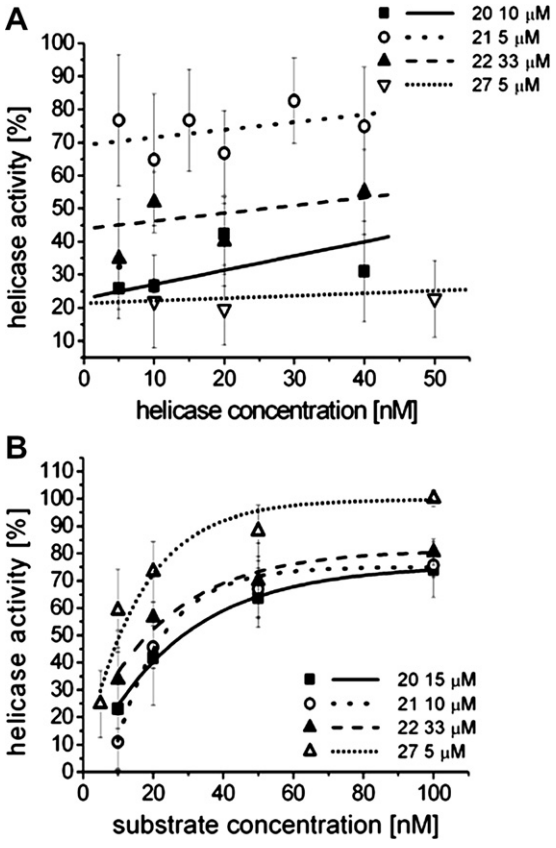


Figure 1. Inhibition of NS3 helicase activity by compounds **20**, **21**, **22** and **27**, (A) at increasing enzyme concentrations. (B) At increasing dsDNA substrate concentrations. The results are presented as the percent activity of the helicase tested in the same conditions but with DMSO instead of inhibitor.

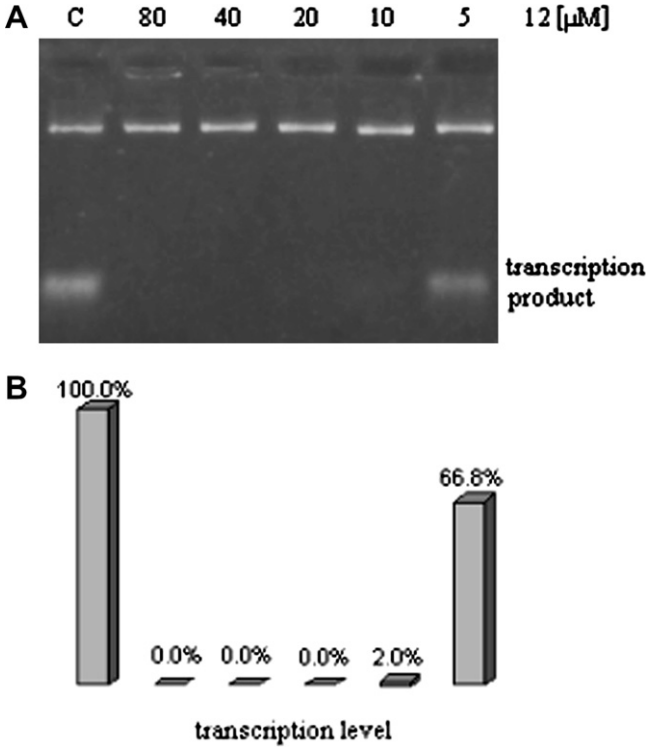


Figure 3. T7 polymerase transcription assay for compound **12**. Inhibition of transcription is reflected by the disappearance of the transcription product in an agarose gel (A), presented as percent of the positive control C (B).

2.5. Intercalation studies

ACA derivatives exerting anti-helicase activity (**20**, **21**, **22** and **27**) and structurally most similar compound (**27a**) as well as the most potent transcription inhibitors (**12**, **13**, **14** and **18**) were tested for their intercalatory properties. Initially, a dsDNA gel migration retardation approach was applied with a large excess of inhibitors.[27] In this assay the compounds that intercalate into dsDNA inhibit ethidium bromide (EtBr) intercalation and the dsDNA band disappears partially or totally. This approach revealed weak dsDNA intercalation properties of the compounds at 100 μM or 500 μM (Fig. 4A), much weaker than that of epidoxorubicin used as control. Compounds **20**, **22** and **27** seem to be the strongest dsDNA intercalators of all the acridone derivatives analysed; a slightly lower effect was observed for **18**.

The intercalatory properties were further studied using dsRNA, the cellular target of the HCV helicase. The results (Fig. 4B) demonstrate that of the nine compounds compared, **20**, **22** and **27** have the strongest intercalatory properties, visible at 20 μM; **14**, **18** and **21** are slightly weaker intercalators, while the other compounds do not intercalate into dsRNA. Partial reappearance of the ds nucleic acid (NA) band at 500 μM **20** is probably due to precipitation of the compound.

2.6. HCV replicon studies

Selected carboxamides were tested at concentrations ranging from 1 μM to 200 μM both in the replicon RNA amplification and cytotoxicity studies. Of nine compounds tested, eight inhibited RNA replication with EC_{50} (50% effective concentration defined as the inhibitor concentration that reduced luminescence by 50%) ranging from 5 μM to 20 μM, but most of them were cytotoxic at similar concentrations (Table 2). Nevertheless, two compounds, **20** and **27**, exhibited antiviral activity together with low cytotoxicity (50% cytotoxic concentration defined as the concentration of compound that inhibited cell growth by 50%, CC_{50} above 150 μM), reducing the HCV RNA level in a dose-dependent manner, with a selectivity index ($SI = CC_{50}/EC_{50}$) of 19.4 and 40.5, respectively.

Table 2
Inhibition of HCV replication in Huh-7 cells carrying the subgenomic replicon[a]

Compound	EC_{50}[b] (μM)	CC_{50}[c] (μM)	SI[d] = CC_{50}/EC_{50}
12	>100	>100	n/a[e]
13	5.6	5.5	1.0
14	19.0	99.3	5.2
18	12.9	90.9	7.1
20	9.0	174.8	19.4
21	6.5	7.0	1.1
22	11.1	39.2	3.5
27	10.2	411.9	40.5
27a	4.3	10.2	2.4
4'-Azidocytidine	1.4	>200	n/a

[a] Each experiment was performed at least three times independently.
[b] Inhibitor concentration needed to reduce viral replication to 50%.
[c] Inhibitor concentration that inhibits cell growth by 50%.
[d] Selectivity index.
[e] Not applicable.

3. Discussion

Natural and synthetic acridone drugs are known to share a common property of NA intercalation with low selectivity towards NA sequence. The specific activity of the compound is most often due to side-chain substitutions protruding into the major or minor grooves of the NA.[13,19,28] As the structure of the DNA duplex plays an important role in the unwinding process conducted by the NS3 helicase,[29] the inhibition of enzyme functions may be due to interaction of acridone-4-carboxamides with the DNA and subsequent disruption of the helical structure of the DNA that leads to inhibition of protein translocation along the DNA. It was also proposed that the biological activities of acridone drugs are not only due to

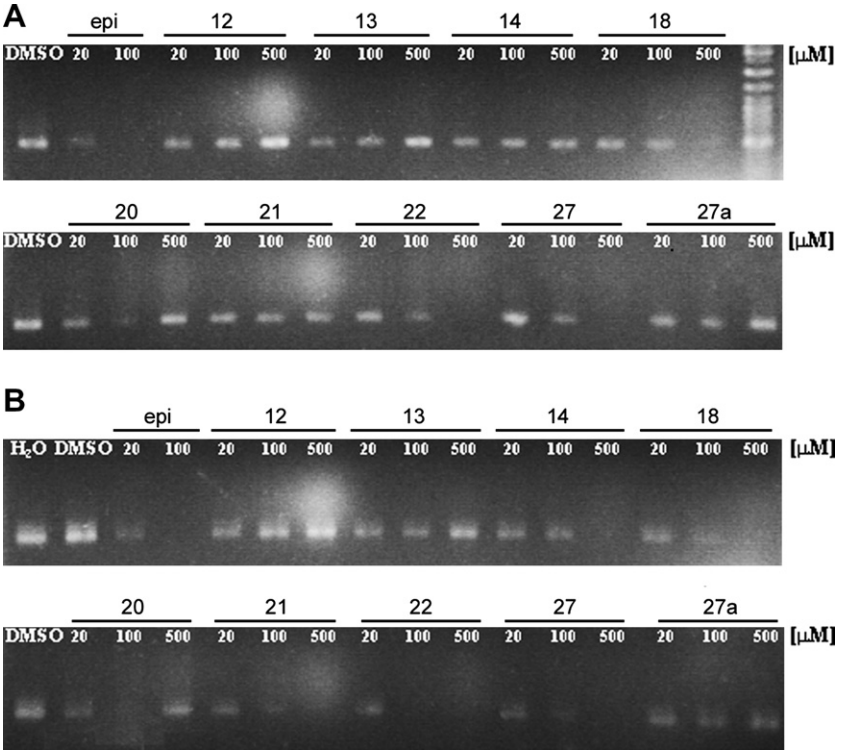


Figure 4. Intercalation assay for selected acridone-4-carboxylic acid derivatives. Their intercalatory properties are reflected by the disappearance of dsDNA (A) or dsRNA (B) bands. DMSO: dsNA incubated with DMSO, Epi: dsNA incubated with epidoxorubicin; **12–27a**: dsNA incubated with selected compounds at given concentrations.

their binding to DNA but also to their direct interaction with topoisomerases and telomerases,[14,19,30] and thus interaction with other enzymes (e.g. helicase or polymerase) cannot be excluded. The position and nature of the substituent plays an important role in the antiviral activity of acridones[15,21] and our data point to the pyridine heterocyclic tailpieces as key pharmacophores for the anti-helicase activity of the compounds, while the tolyl and pyridine amide fragments determine the anti-polymerase activity.

Pyridylamides **20**, **22** and **27** appeared to be the most potent dsDNA intercalators and this could explain their anti-helicase and anti-polymerase activities. On the other hand phenylamide **18** exhibited no anti-helicase activity in spite of its intercalatory properties, while the tolylamide **12**, the most potent polymerase inhibitor did not show any intercalatory properties up to 500 μM (Table 1 and Fig. 4). Thus we conclude that intercalation is not a sufficient mechanism for potent inhibition of either helicase or polymerase activities.

All the intercalating compounds showed a preference for dsRNA, an advantageous feature for inhibitors of amplification of RNA viruses since such molecules could modify secondary structures of viral untranslated regions (UTRs) and of dsRNA intermediates formed during genome replication without affecting cellular DNA.

All four helicase inhibitors (**20**, **21**, **22** and **27**, Table 1) are replication inhibitors in the HCV replicon system based on their EC_{50} values but the cell viability assay revealed significant cytotoxicity of **21** and **22**, which may be the main reasons of the antiviral effect observed (Table 2). Cytotoxicity may be due to the presence of the $-CH_3$ substituent in the pyridyl ring that could favour a specific conformation of the compound and thus cause strong interaction of acridone derivatives with dsDNA thereby interfering with cellular processes. Intercalation of acridone derivatives into dsRNA seems to be the most important mechanism of the inhibition of HCV RNA replication in the cell as all dsRNA intercalators studied had EC_{50} values below 20 μM (Table 2). A high specificity for dsRNA structures as well as lack of methyl group in the pyridyl ring may be the main reasons for the very low cytoxicities of **20** and **27** (Table 2).

We presume, however, that the mechanism of inhibition of viral replication cannot be based solely on the dsRNA intercalatory properties of the acridone derivatives. As both carboxamides **20** and **27** are good inhibitors of T7 polymerase transcription and helicase-mediated NA unwinding (Table 1), their inhibition of HCV RNA replication in the replicon system may be due to a double effect of inhibition of two HCV enzymes, the NS3 helicase and the NS5B polymerase (Table 2). This possibility is further supported by the fact that the full-length NS3 activity is dependent on its interaction with NS5B.[31,32] These possibilities will be verified by NS5B polymerase inhibition studies that are currently in progress.

Preliminary computer modeling analyses revealed that the position of the nitrogen atom in the pyridyl ring considerably affects the spatial location of the ligand in the hydrophobic pocket of the T7 polymerase catalytic site. The ligand-receptor complex is stabilized by the formation of relatively short (2–2.5 Å) and effective hydrogen bonds between the ligand and amino acid residues, NA or NTPs. These studies, together with computer modeling of ligand-NS3 helicase interactions, will be the subject of a future publication.

4. Conclusions

A series of acridone derivatives were synthesized and the anti-HCV activity of the compounds was evaluated. The anti-helicase activity of the compounds studied is determined by the pyridine heterocyclic tailpieces while the tolyl and pyridine amide fragments are key pharmacophores for the anti-polymerase activity. The ability of the compounds to intercalate into dsNA is not sufficient to explain the inhibition of either transcription, NA unwinding by the helicase, or the cytotoxicity of acridone derivatives in Huh-7 cells. However, intercalation of the acridone derivatives into dsRNA may play an important role in inhibition of HCV replication in the replicon system. Pyridylamides **20** and **27** are efficient HCV replication inhibitors with SI values of 19.4 and 40.5, respectively.

Both compounds are the best helicase inhibitors (**27** has the lowest IC_{50} value) and are moderate transcription inhibitors. Therefore in the replicon system they can act via inhibition of both NS3 and NS5B activities.

Compound **27** was designed on the basis of first results of helicase inhibition by pyridyl derivatives. Demonstration of its efficiency as the helicase and RNA replication inhibitor proves that the approach adopted in search for antiviral agents was appropriate. The molecules selected could be further modified to improve their biological activity and obtain specific anti-HCV drugs.

5. Experimental

5.1. Chemistry

^{1}H NMR spectra were measured with a Mercury 400 (400 MHz) spectrometer; chemical shifts are expressed in d units using tetramethylsilane as standard (NMR peak description: s, singlet; d, doublet; t, triplet; m, multiplet; and br, broad peak). Mass spectra were recorded with an Agilent 1100 (HPLC APCI MS) spectrometer in a positive mode. Reactions were monitored by TLC on Merck silica gel plates ($60F_{254}$) and visualised by UV light. Melting points of compounds were determined with a Boetius apparatus. All commercial reagents and organic solvents were used with further purification.

5.1.1. 9-Oxo-9,10-dihydro-acridine-4-carboxylic acid *p*-tolylamide (12)

Thionyl chloride (81.3 μL, 1.13 mmol) and freshly dried pyridine (91.3 μL, 1.13 mmol) were added to the suspension of compound **2** (193.5 mg, 0.81 mmol) in 10 ml of dry toluene. The reaction mixture was stirred at room temperature for 3–4 h, combined with excess (267 mg, 2.5 mmol) *p*-toluidine, triethylamine (337 μL, 2.4 mmol) and stirred for another 3 h. The reaction was monitored by TLC. The following day the solvent was removed under vacuum and water was added to the solid residue. The precipitate formed was filtered, washed with water and dried. The crude product was crystallized several times from *n*-butanol–DMF (19:1) yielding 193 mg of analytically pure compound **12**. Yield: 73%; mp 338–342 °C. ^{1}H NMR (DMSO-d_6) δ 2.36 (3 H, CH_3, s), 7.16 (2H, d, J = 8.0 Hz), 7.27 (1 H, t, J = 6.8), 7.34 (1H, t, J = 7.8), 7.65–7.69 (4H, m), 8.24 (1H, d, J = 7.6 Hz), 8.37 (1H, d, J = 7.2 Hz), 8.48 (1H, d, J = 7.6 Hz), 10.47 (1H, s, CONH), 12.04 (1H, s, NH); LC–MS m/z $[M+H]^+$ 329.

5.1.2. 9-Oxo-9,10-dihydro-acridine-4-carboxylic acid *m*-tolylamide (13)

Compound **13** was prepared from ACA (**2**) and *m*-toluidine as a yellow solid, using an approach similar to that described for compound **12**. Yield: 54%; mp 276–277 °C (toluene–DMF); H NMR (DMSO-d_6) δ 2.39 (3H, s, CH_3), 6.95 (1H, d, J = 7.6 Hz), 7.22–7.29 (2H, m), 7.35 (1H, t, J = 7.6 Hz), 7.54 (1H, d, J = 8.0 Hz), 7.65–7.71 (3H, m), 8.24 (1H, d, J = 8.0 Hz), 8.36 (1H, d, J = 7.2), 8.59 (1H, d, J = 7.6 Hz), 10.46 (1H, s, CONH), 12.02 (1H, s, NH); LC–MS m/z [M+H] 329.

5.1.3. 9-Oxo-9,10-dihydro-acridine-4-carboxylic acid *o*-tolylamide (14)

Compound **14** was prepared from ACA (**2**) and *o*-toluidine as a yellow solid, using an approach similar to that described for com-

pound **12**. Yield: 45%; mp 256–258 °C (*n*-butanol); ^{1}H NMR (DMSO-d_6) δ 2.33 (3H, s, CH_3), 7.21–7.30 (4H, m), 7.36 (2H, m), 7.62 (1H, d, J = 8.0 Hz), 7.69 (1H, t, J = 6.8), 8.34 (1H, d, J = 8.0 Hz), 8.48 (2H, dd, J = 7.6), 10.29 (1H, s, CONH), 12.32 (1H, s, NH); LC–MS m/z $[M+H]^+$ 329.

5.1.4. 9-Oxo-9,10-dihydro-acridine-4-carboxylic acid (15)

Compound **15** was prepared from ACA (**2**) and *N*,*N*-(dimethylethylene)diamine as a yellow solid, using an approach similar to that described for compound **12**. Yield: 42%; mp 171–173 °C (toluene); ^{1}H NMR (DMSO-d_6) δ 2.27 (6H, s, $N(CH_3)_2$); 2.58 (2H, t, $NHCH_2$); 3.44–3.49 (2H, m, CH_2CH_2); 7.23–7.29 (2H, m); 7.58 (1H, d, J = 8.4 Hz); 7.69 (1H, t, J = 8.4 Hz); 8.23 (2H, d, J = 8.4 Hz); 8.42 (1H, d, J = 8.0 Hz), 8.77 (1H, br s, CONH); 12.62 (1H, br s, NH); LC-MS *m/z* $[M+H]^+$ 310.

5.1.5. 4-(Piperidine-1-carbonyl)-10H-acridin-9-one (16)

Compound **16** was prepared from ACA (**2**) and piperidine as a pale yellow solid, using an approach similar to that described for compound **12**. Yield: 46%; mp 234–236 °C (ethanol); ^{1}H NMR (DMSO-d_6) δ 1.67–1.68 (6H, CH_2, m), 3.24 (2H, CH2, m), 3.74 (2H, CH_2, m), 7.21–7.26 (2H, m), 7.56 (1H, d, J = 8.4 Hz), 7.66 (1H, t, J = 7.2 Hz), 7.83 (1H, d, J = 8.4), 8.21 (1H, d, J = 8.0), 8.32 (1H, d, J = 7.2), 10.67 (1H, s, NH); LC–MS m/z $[M+H]^+$ 307.

5.1.6. 9-Oxo-9,10-dihydro-acridine-4-carboxylic acid morpholylamide (17)

Compound **16** was prepared from ACA (**2**) and morpholine as a yellow solid, using an approach similar to that described for compound **12**. Yield: 46%; mp 196–198 °C (ethanol); ^{1}H NMR (DMSO-d_6) δ 3.21–3.40 (2H, CH_2, m), 3.55–3.80 (6H, CH_2, m), 7.22–7.28 (2H, m), 7.61 (1H, d, J = 7.2 Hz), 7.66 (1H, t, J = 7.2 Hz), 7.83 (1H, d, J = 8.4 Hz), 8.22 (1H, d, J = 8.0 Hz), 8.34 (1H, d, J = 7.6 Hz), 10.76 (1H, s, NH); LC–MS m/z $[M+H]^+$ 309.

5.1.7. 9-Oxo-9,10-dihydro-acridine-4-carboxylic acid phenylamide (18)

Compound 18 was prepared from ACA (**2**) and phenylamine as a yellow solid, using an approach similar to that described for compound **12**. Yield: 78%; mp 255–258 °C (*n*-butanol); ^{1}H NMR (DMSO-d_6) δ 7.14 (1H, t, J = 7.2 Hz), 7.28 (1H, t, J = 7.6 Hz), 7.38 (3H, m), 7.69 (2H, m), 7.79 (1H, d, J = 8.0 Hz), 8.24 (1H, d, J = 8.0 Hz), 8.37 (1H, d, J = 7.6 Hz), 8.49 (1H, d, J = 7.6 Hz), 10.55 (1H, s, CONH), 11.98 (1H, s, NH); LC-MS *m/z* $[M+H]^+$ 315.

5.1.8. 9-Oxo-9,10-dihydro-acridine-4-carboxylic acid N-[(morphol-4-yl)propyl]amide (19)

Compound **19** was prepared from ACA (**2**) and 4-(aminopropyl)morpholine as a yellow solid, using an approach similar to that described for compound **12**. Yield: 69%; mp 161–162 °C (n-propanol); ^{1}H NMR (DMSO-d_6) δ 1.78–2.05 (2H, m, NCH_2), 2.42–2.49 (6H, CH_2, m), 3.48–3.52 (2H, CH_2, m), 3.61–3.68 (4H, CH_2, m), 7.23–7.30 (2H, m), 7.59 (1H, d, J = 8.4 Hz), 7.68–7.73 (1H, m), 8.23 (2H, d, J = 8.4 Hz), 8.43 (1H, d, J = 8.4), 8.92 (1H, t, CONH), 12.66 (1H, s, NH); LC-MS *m/z* $[M+H]^+$ 366.

5.1.9. 9-Oxo-9,10-dihydro-acridine-4-carboxylic acid pyridin-4-ylamide (20)

Compound **20** was prepared from ACA (**2**) and 4-aminopyridine as a pale yellow solid, using an approach similar to that described for compound **12**. Yield: 75%; mp 373–376 °C (DMF); ^{1}H NMR (DMSO-d_6) δ 7.30 (1H, t, J = 7.0 Hz), 7.36 (1H, t, J = 7.6 Hz), 7.70–7.72 (2H, m), 7.81–7.83 (2H, m), 8.25 (1H, d, J = 8.0 Hz), 8.36 (1H, d, J = 6.8 Hz), 8.47–8.54 (3H, m), 10.86 (1H, s, CONH), 11.75 (1H, s, NH); LC-MS *m/z* $[M+H]^+$ 316.

5.1.10. 9-Oxo-9,10-dihydro-acridine-4-carboxylic acid (4-methyl-pyridin-2-yl)-amide (21)

Compound **21** was prepared from ACA (**2**) and 4-methyl-2-aminopyridine as a pale yellow solid, using an approach similar to that described for compound **12**. Yield: 76%; mp 207–210 °C (*n*-butanol); ^{1}H NMR (DMSO-d_6) δ 2.45 (3H, s, CH_3), 7.00 (1H, d, J = 4.8 Hz), 7.25–7.33 (2H, m), 7.64 (1H, d, J = 8.0 Hz), 7.72 (1H, t, J = 7.2 Hz), 8.12 (1H, s), 8.25 (2H, d, J = 8.0 Hz), 8.50 (2H, d, J = 7.6 Hz), 11.00 (1H, s, CONH), 12.00 (1H, s, NH); LC–MS m/z $[M+H]^+$ 330.

5.1.11. 9-Oxo-9,10-dihydro-acridine-4-carboxylic acid (3-methyl-pyridin-2-yl)-amide (22)

Compound **22** was prepared from ACA (**2**) and 6-methyl-2-aminopyridine as a yellow solid, using an approach similar to that described for compound **12**. Yield 19%; mp 233–235 °C (*n*-butanol); ^{1}H NMR (DMSO-d_6) δ 2.50 (3H, s, CH_3), 7.01(1H, d, J = 7.6 Hz), 7.29–7.32 (2H, m), 7.63–7.73 (3H, m), 8.05 (1H, d, J = 8.4 Hz), 8.24 (1H, d, J = 7.6 Hz), 8.48 (2H, d, J = 7.6 Hz), 10.97 (s, 1H, CONH), 12.00 (1H, s, NH); LC–MS m/z $[M+H]^+$ 330.

5.1.12. 9-Oxo-9,10-dihydro-acridine-4-carboxylic acid pyridin-2-ylamide (27)

Compound **27** was prepared from ACA (**2**) and 2-aminopyridine as a yellow solid, using an approach similar to that described for compound **12**. Yield 42%; mp 285–287 °C (*n*-butanol–DMF); ^{1}H NMR (DMSO-d_6) δ 7.17 (1 h, t, J = 6.8 Hz), 7.27–7.33 (2H, m), 7.66–7.70 (2H, m), 7.85 (1H, t, J = 7.6 Hz), 8.25 (2H, t, J = 6.8 Hz), 8.40 (2H, d, J = 8.40, 8.47–8.50 (3H, m), 11.11 (1H, s, CONH), 11.91 (1H, s, NH); LC–MS m/z $[M+H]^+$ 316.

5.1.13. 9-Oxo-9,10-dihydro-acridine-4-carboxylic acid (pyridin-2-yl-methyl)-amide (27a)

Compound **27a** was prepared from ACA (**2**) and 2-(aminomethyl)pyridine as a pale yellow solid, using an approach similar to that described for compound **12**. Yield 31%; mp 211–214 °C (*n*-butanol–DMF); ^{1}H NMR (DMSO-d_6) δ 4.68 (2H, d, J = 4.8 Hz, CH_2), 7.23–7.32 (3H, m), 7.41 (1H, d, J = 8.0 Hz), 7.59 (1H, d, J = 8.0 Hz), 7.67–7.75 (3H, m), 8.23 (1H, d, J = 7.6 Hz), 8.38 (1H, d, J = 7.2), 8.46 (1H, d, J = 8.0 Hz), 8.52 (1H, d, J = 8.0 Hz), 9.52 (1H, t, J = 4.8 Hz, NH); LC–MS m/z $[M+H]^+$ 330.

5.2. Biology

5.2.1. Helicase assay

The fluorometric helicase activity assay was performed as described in Boguszewska-Chachulska et al. with minor modifications concerning the reaction temperature and volume (37 °C and 60 μl).[25,26]

5.2.2. In vitro transcription assay

The in vitro transcription reactions of plasmid DNAs were performed as described in Melton et al.[33] Nucleoside triphosphates, T7 RNA polymerase and ribonuclease inhibitor (RiboLock™) were from Fermentas. Each reaction mixture contained 0.5 μg of EcoRI-restricted plasmid (pTZ19R with a 341 bp insert, cloned into the Ecl136II site), 2 mM of each NTP, 1 U/μl of RNase inhibitor and 12 U of T7 RNA polymerase in reaction buffer (40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl and 10 mM DTT).

The compounds dissolved in DMSO were added at four concentrations (3.125, 6.25, 12.5 and 25 μg/ml) prior to the addition of T7 RNA polymerase. The final reaction mixture (20 μl) was incubated at 37 °C for 1 h to obtain a concentration-dependent response. The reactions were stopped by cooling to −20 °C. The reaction products were visualized by 1 h electrophoresis in a 1.2% agarose gel, in TBE

buffer supplemented with EtBr. The concentration of inhibitor necessary to reduce the enzyme activity by 99% (IC_{99}) was determined by densitometric evaluation of the intensity of the electrophoresis bands using the Scion Image for Windows, Release Beta 4.0.3.

5.2.3. Nucleic acid intercalation

The intercalatory properties of selected compounds were studied by the dsNA migration retardation assay.[27] dsDNA was prepared by PCR amplification of a cDNA fragment of the HCV con1 isolate coding for the 3′ UTR,[34] and dsRNA by annealing products of in vitro transcription reactions performed on the PCR product of the con1 3′ UTR (Krawczyk et al., in preparation). dsDNA and dsRNA (both 15 nM, composed of 490 bp) were incubated for 45 min at room temperature with 20, 100 and 500 μM compounds in 20 mM Tris pH 7.5, and then submitted to electrophoresis in 1% agarose gel in 1× TAE. The gel was subsequently stained with EtBr.

5.2.4. HCV replicon and cells

The human hepatoma cell line Huh-7, carrying the subgenomic HCV genotype 1 replicon with the luc-ubi-neo (reporter/selective) fusion gene,[35] was kindly provided by Dr. Ralf Bartenschlager (University of Heidelberg, Heidelberg). The cells were grown as described in Gozdek et al.[36]

5.2.5. Anti-HCV replicon studies

The conditions of the assay used to test the antiviral activity of the compounds were described in Paeshuyse et al. and Gozdek et al.[36,37] The experiments were carried out at least three times with three replicates for each inhibitor concentration; for comparison 4′-azidocytidine[38] (kindly provided by J. Neyts, Katolik Universität, Leuven) was tested at concentrations from 0.1 μM to 200 μM. The data were calculated as the percentage of the luminescence of control samples containing 1–2% DMSO. The 50% effective concentration (EC_{50}) is defined as the inhibitor concentration that reduced luminescence by 50%.

5.2.6. Cytotoxicity assay

Huh-7 cells carrying the HCV replicon were seeded and grown with serial dilutions of the compounds as described for the replicon assay.[36] After 3 days of incubation at 37 °C, the medium was removed and 100 μl of Dulbecco modified Eagle's medium (DMEM; Invitrogen) without phenol red supplemented with 0.2 mg/ml XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-(phenylamino)-carbonyl-2 H-tetrazolium hydroxide; Serva) and 25 μM phenazine methosulfate (PMS; Serva) was added. Wells without cells containing 100 μl of culture medium with XTT-PMS staining solution ('blank') were also prepared. The plates were incubated for 3–4 h at 37 °C. The absorbance was measured with Synergy HT (Biotek) at 450 nm and at 690 nm as a reference; the 'blank' absorbance values were subtracted from the sample values. Data were calculated as the percentage of cell growth in the control samples containing 1–2% DMSO. The 50% cytotoxic concentration (CC_{50}) is the concentration of compound that inhibited cell growth by 50%.

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