The hepatitis C virus (HCV) NS3 protease is essential for polyprotein maturation and viral propagation, and it has been proposed as a suitable target for antiviral drug discovery. An N-terminal hexapeptide cleavage product of a dodecapeptide substrate identified as a weak competitive inhibitor of the NS3 protease activity was optimized to a potent and highly specific inhibitor of the enzyme. The effect of this potent NS3 protease inhibitor was evaluated on replication of subgenomic HCV RNA and compared with interferon-α (IFN-α), which is currently used in the treatment of HCV-infected patients. Treatment of replicon-containing cells with the NS3 protease inhibitor or IFN-α showed a dose-dependent decrease in subgenomic HCV RNA that reached undetectable levels following a 14-day treatment. Kinetic studies in the presence of either NS3 protease inhibitor or IFN-α also revealed similar profiles in HCV RNA decay with half-lives of 11 and 14 h, respectively. The finding that an antiviral specifically targeting the NS3 protease activity inhibits HCV RNA replication further validates the NS3 enzyme as a prime target for drug discovery and supports the development of NS3 protease inhibitors as a novel therapeutic approach for HCV infection.

HCV* as a member of the Flaviviridae family is the major etiological agent of non-A, non-B viral hepatitis and an important cause of chronic liver disease leading to cirrhosis and hepatocellular carcinoma in humans (1, 2). An estimated 170 million people worldwide are infected with HCV, and end stage liver disease associated with this virus is now the leading cause of liver transplantation in the western world (3). Many patients treated with IFN-α alone or with a combination of IFN-α plus ribavirin fail to show a sustained virologic response and currently have no other treatment option. Given the high prevalence of the infection, HCV has become the focus of intensive research. Originally cloned in 1989 (1), the viral RNA genome of 9600-nucleotide genome is of positive polarity that encodes a ~3000-amino-acid polyprotein, which is the precursor of at least 10 mature viral proteins: C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B. C is the nucleocapsid protein that binds and encapsulates the viral RNA genome (4), E1 and E2 are the virion glycoproteins, and p7 is of unknown function (5). The NS2 to NS5B proteins inclusively are thought to comprise nonstructural proteins involved in replication and polyprotein processing (6). The individual proteins are processed from the polyprotein by a combination of host and viral proteases. Host signal peptides are responsible for the cleavages between C, E1, E2, p7, and NS2. The cleavage between NS2 and NS3 is performed in an autoproteolytic manner by the metal-dependent protease NS2/3 (7, 8). The proteolytic release of NS4A, NS4B, NS5A, and NS5B is catalyzed by the multifunctional NS3 enzyme, which in conjunction with the mature NS4A cofactor mediates efficient processing (for a review, see Ref. 9). The large polyprotein open reading frame is flanked at the 5'-end by an untranslated region, which functions as an internal ribosome entry site, and at the 3'-end by a highly conserved sequence that is essential for genome replication (10–12). Despite increasing knowledge of the genome structure and the function of individual viral proteins, studies on HCV replication and development of specific HCV antiviral agents have been hampered by the lack of an efficient virus culture system. However, an HCV RNA episomal cell system that preserves the 5'- and 3'-untranslated extremities and expresses the nonstructural region of HCV reconstitutes efficient replication of a subgenomic HCV RNA in the human liver Huh-7 cell line (13).

The activity of the chymotrypsin-like serine protease that is encoded within the amino-terminal 180 amino acids of NS3 is indispensable for HCV infectivity in the chimpanzee model (43). Based on expression, purification, and in vitro enzymatic reconstitution, the NS3 protease is perhaps the most thoroughly characterized HCV enzyme. Efficient processing requires the NS3 protease in combination with the NS4A cofactor and a structural zinc molecule (9). A comparison of the NS3 protease crystal structure, with or without an NS4A segment, shows that the presence of NS4A reorients the position of the catalytic triad within the serine protease (17). The structure of the NS3 protease domain and the full-length protein have been solved by x-ray crystallography (14–17). These studies have been complemented by solution NMR and biochemical studies addressing the structure of target substrate (18) and product analogues (19, 20). The NS3 protease is prone to competitive inhibition by specific penta- or hexapeptides derived from the

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The purified NS3-NS4AFL proteins after Superdex 200 gel filtration chromatography. Lane 1, molecular weight markers; lane 2, NS3-NS4AFL; lane 3, His-NS3-NS4AFL.

Enzymatic Studies—Enzymatic assays and kinetics were performed using a fluorogenic substrate and purified enzyme. Kinetic parameters and inhibition studies were determined with the His-NS3-NS4AFL and the recombinant TEV protease-treated NS3-NS4AFL that removed the histidine tag portion. The inhibition constant \( K_i \) was determined by a steady-state velocity method (26). Briefly, enzyme activity was determined by monitoring the fluorescence change associated with the cleavage of the fluorogenic substrate Ac-Asp-Glu-Asp(EDANS)-Glu-Glu-Ala[O(O)-O]-Ala-Ser-Lys(DABCYL)-NH2 (27) on a PerkinElmer Life Sciences LS50B fluorometer (excitation at 340 nm and emission at 485 nm). Reactions were performed at 23 °C in assay buffer (50 mM Tris, pH 8.0, 250 mM sodium citrate, 0.1% n-dodecyl-β-D-maltoside, 1 mM tris(2-carboxyethyl)phosphate, and 5% dimethyl sulfoxide) containing 1.5 mM enzyme and 4 μM substrate. The onset of inhibition was determined by varying the inhibitor concentration from 0.5 to 6 μM. The reaction was initiated by enzyme addition. The steady-state velocity was estimated by fitting the data to the integrated rate equation describing competitive binding. Then, the apparent \( K_i (K_{i(app)}) \) was obtained by fitting steady-state velocity and inhibitor concentrations to the quadratic equation describing tight binding inhibition (28) using SAS software (Statistical Software System, SAS Institute Inc., Cary, NC). \( K_i \) was obtained by the equation \( K_{i(app)} = K_i (1 + (SV/K_i)^{1/2}) \) for a competitive mode of inhibition. Under similar assay conditions, the kinetic parameters were determined. Calculations were performed by nonlinear regression analysis of initial rates as a function of substrate concentration (29–31). The kinetic parameters were determined by the integrated rate equation describing competitive binding (28) using SAS software (version 3.0; Erthrusco Software Ltd., Staines, United Kingdom).

Selectivity Serine Protease Assays—Bovine pancreatic α-chymotrypsin and human leukocyte elastase (HLE) were obtained from Roche Applied Science and Calbiochem and assayed as described previously (32).

Description of Subgenomic HCV 1b RNA Replicon—The distinguishing details of this construct, selection of neomycin-resistant cell lines, and characterization of adaptive mutants have been described (30). Briefly, a subgenomic HCV 1b replicon was assembled on the basis of the quadric equation describing tight binding inhibition (28) using SAS software (version 3.0; Erthrusco Software Ltd., Staines, United Kingdom).
represents the average of three replicates with quantified by primer- and probe-specific real time RT-PCR. Each point levels were or glyceraldehyde-3-phosphate dehydrogenase mRNA (GAPDH) and human leukocyte elastase (HLE) were administered to replicon-containing cells for 3 days, and HCV RNA (HCV) level by compound A in a replicon cell system. Compound A was administered to replicon-containing cells for 3 days, and HCV RNA recovered relative to an Me2SO control. The percentage of inhibition was then plotted against the compound concentration, and a non-linear curve was fitted (Hill model) to the percentage of inhibition-concentration data. The calculated percentage of inhibition values were then used to determine the median effective concentration EC_{50} slope factor (n), and maximum inhibition (I_{max}) by the nonlinear regression routine procedure of SAS using the following equation.

\[
\text{Percentage of inhibition} = \frac{I_{\text{max}} \times [\text{inhibitor}]^{n}}{\text{inhibitor}^{n} + EC_{50}^{n}}
\]

(Cytotoxicity Assay)—The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide metabolic assay (32) was used to determine the cytotoxicity in Huh-7 replicon-containing cells. EC_{50} corresponds to the concentration of inhibitor that decreased the percentage of formazan production by 50% of that produced by untreated cells after 3 days of incubation. Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was added, and cells were incubated for 3 h. The formazan product was solubilized with 10% Triton X-100 in 0.01 n HCl and quantitated by measuring the optical density at 570 nm.

Inhibition of NS3 Protease-mediated Polyprotein Processing—For Western blot analysis, HCV replicon-containing cells were grown logarithmically in the presence of increasing amounts of inhibitor for 72 h. Cells were lysed with Laemmli buffer, and proteins were analyzed by Western blot analysis.

**Determination of HCV RNA Levels and Cellular GAPDH mRNA Levels**—Huh-7 cells (1 \times 10^6) harboring a bicistronic subgenomic HCV replicon were plated into wells of a 96-well plate and incubated with serial dilutions of inhibitor 24 h after plating. Total cellular RNA was extracted using the RNeasy-96 (Qiagen) cartridge at various times after the compound addition. HCV-specific RNA copy number was quantified (31) by quantitative real-time RT-PCR with the ABI PRISM 7700 sequence detection system and normalized to the total cellular RNA recovered as quantified with RiboGreen (Molecular Probes, Inc., Eugene, OR). Moreover, as a further control for RNA recovery and cell fitness in each assay well, the copy number of cellular glyceraldehyde-3-phosphate dehydrogenase mRNA was also quantified by real-time RT-PCR with specific primers and probe. In the presence of inhibitors, the percentage of inhibition was determined by reduction in HCV RNA levels, which is expressed as genome equivalents per µg of total cellular RNA recovered relative to an Me2SO control. The percentage of inhibition was then plotted against the compound concentration, and a non-linear curve was fitted (Hill model) to the percentage of inhibition-concentration data. The calculated percentage of inhibition values were then used to determine the median effective concentration EC_{50} slope factor (n), and maximum inhibition (I_{max}) by the nonlinear regression routine procedure of SAS using the following equation.
then incubated with methionine-free Dulbecco’s modified Eagle’s medium containing $^{[35]}$S Met/Cys (100 μCi/ml) for 1 h. The inhibitor was present during all incubations. After the labeling period, cells were washed three times with PBS and were lysed with a buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture. Cell lysates were cleared by centrifugation at 13,800 × g for 15 min at 4 °C and processed for immunoprecipitation as described (34). The immunoprecipitation was performed with a rabbit antiserum (K137) specific to NS3 protease and incubated with protein A-conjugated magnetic beads (Dynabeads; Dianova Biosciences). Purification of the NS3-NS4AFL with and without an N-terminal histidine tag resulted in a greater than 90% pure protein as judged by SDS-PAGE (Fig. 1). The protocol yielded about 1.0 mg of purified protein per 10^8 insect cells. Proteolysis at the NS3-NS4A junction was confirmed by SDS-PAGE, Western blot, and N-terminal amino acid sequencing of the NS4A obtained from purified enzyme (data not shown). Kinetic analysis of the cleavage reaction using the internally quenched fluorogenic substrate Ac-Asp-Glu-Asp(EDANS)-Glu-Glu-Abu[C(O)-O]-Ala-Ser-Lys(DABCYL)-NH$_2$ revealed similar kinetic parameters for the NS3-NS4AFL and the His-NS3-NS4AFL proteases: mean $K_m$ = 1.2 and 1.0 μM; mean $k_{cat}$ = 35 and 35 min$^{-1}$, respectively (see Table I). Therefore, the presence of an N-terminal histidine tag followed by a recombinant TEV protease cleavage site did not affect the NS3 serine protease activity and is consistent with previously published results (35).

The N-terminal cleavage products of various peptide substrates of the NS3 protease competitively inhibit the enzyme with micromolar activity (21, 22). That observation led to the design of a series of peptide-based inhibitors (22–24), and efforts aimed at rationally improving inhibitor potency yielded the peptidomimetic compound A (Fig. 2A). The $K_i$ of compound A was determined with the NS3-NS4AFL genotype 1b protease using a steady-state velocity method and by fitting the data to the integrated rate equation describing competitive binding (28). Preliminary experiments demonstrated a competitive mechanism for this class of inhibitors (data not shown). Compound A is a slow, tightly binding inhibitor of the NS3-NS4AFL genotype 1b as shown by the slow onset of inhibition upon enzyme addition (Fig. 2B) with a mean $K_i$ of 74 μM (Fig. 2C). The specificity of compound A was also assessed by evaluating its ability to inhibit a variety of serine proteases. Compound A is highly selective for the HCV NS3 protease and inactive (IC$_{50}$ > 30 μM) against representative serine proteases such as HLE and bovine pancreatic chymotrypsin (Fig. 2C).

**RESULTS AND DISCUSSION**

**Inhibition of NS3 Protease by Compound A and Activity against Other Serine Proteases**—The NS3-NS4AFL was expressed using recombinant baculovirus in Sf21 insect cells. Purification of the NS3-NS4AFL with and without an N-terminal histidine tag resulted in a greater than 90% pure protein as judged by SDS-PAGE (Fig. 1). The protocol yielded about 1.0 mg of purified protein per 10^8 insect cells. Proteolysis at the NS3-NS4A junction was confirmed by SDS-PAGE, Western blot, and N-terminal amino acid sequencing of the NS4A obtained from purified enzyme (data not shown). Kinetic analysis of the cleavage reaction using the internally quenched fluorogenic substrate Ac-Asp-Glu-Asp(EDANS)-Glu-Glu-Abu[C(O)-O]-Ala-Ser-Lys(DABCYL)-NH$_2$ revealed similar kinetic parameters for the NS3-NS4AFL and the His-NS3-NS4AFL proteases: mean $K_m$ = 1.2 and 1.0 μM; mean $k_{cat}$ = 35 and 35 min$^{-1}$, respectively (see Table I). Therefore, the presence of an N-terminal histidine tag followed by a recombinant TEV protease cleavage site did not affect the NS3 serine protease activity and is consistent with previously published results (35).

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**Inhibition of Subgenomic HCV RNA Replication and Cytotoxicity of Compound A**—The HCV replicon cell system that includes the NS2-NS5B NS protein region was developed as previously described by Lohmann et al. (13) and used to evaluate the NS3 protease inhibitory activity of compound A. Although IFN-α is effective in reducing the HCV RNA levels in replicon-containing cells (36–38), the effect of specific NS3
inhibitors on HCV RNA replication has not been thoroughly described. HCV RNA replication in this system is dependent on the various HCV NS proteins (39) and therefore HCV replicon-containing cells are useful for testing specific inhibitors of HCV RNA replication.

The effect of compound A and IFN-α on HCV RNA replication was first determined following a 3-day incubation period with replicon-containing cells. Subgenomic HCV RNA levels were determined by quantitative real time RT-PCR methodology and using total RNA extracted from cells. This detection method spans a broad dynamic range from a low detection limit of $10^6$ to a high of $10^8$ RNA molecules. Treatment of replicon-containing cells with IFN-α (not shown) resulted in a dose-dependent HCV RNA decrease of up to 2 orders of magnitude with an EC$_{50}$ of 0.2 IU/mL. This is consistent with previously reported EC$_{50}$ values for alternative forms of IFN-α (36–38). Following 5 days of incubation with IFN-α, there was a 3-order of magnitude decrease in HCV RNA with no apparent change in EC$_{50}$ determination.

Compound A is a highly hydrophilic and negatively charged molecule of 982 daltons that lacks physico-chemical properties expected to confer a high degree of cell permeability. However, the outstanding potency warranted a determination of its ability to inhibit NS3 protease activity in a cellular setting. The effect of compound A on subgenomic HCV RNA levels was first evaluated using a 3-day assay (Fig. 3). Treatment ($n=3$) of replicon-containing cells with concentrations ranging from 0.15 to 39 μM led to a dose-dependent decrease in HCV RNA of 2 orders of magnitude with an EC$_{50}$ of 1.9 ± 0.2 μM. The effect of compound A was specific to HCV RNA reduction, with no significant reduction in total cellular RNA or control cellular glyceraldehyde-3-phosphate dehydrogenase mRNA. Compound A was not cytotoxic using the same cell setting, and a CC$_{50}$ of >400 μM was observed in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide metabolic assay. These results demonstrate that compound A exclusively targets the NS3 protease and would specifically inhibit cellular HCV RNA replication with low micromolar efficacy and without any evidence for nonspecific inhibitory effects.

Inhibition of NS3 Protease-mediated Polyprotein Processing in HCV Replicon Cell System—In order to confirm the intracellular mode of action of compound A that led to a reduction in subgenomic HCV RNA, the HCV nonstructural protein processing was assessed by Western blot analysis of cell extracts following a 72-h treatment (Fig. 4A). A dose-dependent decrease in mature NS3 protein was observed and resulted in complete disappearance of NS3 protein with 20–100 μM compound A. HCV polyprotein precursor and intermediates were not detected under these conditions, possibly because of a low level and/or a short half-life of the polyprotein. In order to detect HCV nonstructural protein precursors, replicon-containing cells treated with compound A were pulse-labeled with [35S]methionine/cysteine. Following incubation, cell extracts were immunoprecipitated with specific anti-NS3 protein antibody, and products were analyzed by SDS-PAGE followed by phosphorimaging (Fig. 4B). Incubation of cells with 0.3–21 μM compound A resulted in a partial to almost complete disappearance of NS3 protein and the concomitant appearance of a larger molecular weight product consistent with the size of a NS3-5B polyprotein precursor. No other intermediates could be detected. Hence, the effective inhibition of cellular HCV RNA replication by compound A was manifested through efficient blockage of the NS3 protease-mediated polyprotein maturation, a process that is essential for HCV replication in human liver cells.

Half-life of Subgenomic HCV RNA in Cells Treated with Compound A and IFN-α—A detailed kinetic analysis of the decay of subgenomic HCV RNA was performed following a time course incubation of cells with either compound A or with IFN-α, as representatives of two distinct classes of inhibitors (Fig. 5). HCV replicon-containing cells were incubated with maximum nontoxic and nontoxic concentrations of compound A (100 μM) or IFN-α (100 IU/mL), previously determined for a 72-h cell incubation. As noted above, both inhibitors produced a maximal reduction in subgenomic HCV RNA approaching 2 orders of magnitude after a 72-h treatment. The HCV RNA decay rates observed in the presence of compound A and IFN-α were surprisingly similar, with a half-life of 11 ± 1 h with the NS3 inhibitor and 14 ± 1 h with IFN-α. This result is consistent with the half-life reported by Guo et al. (38) for IFN-α as determined by strand-specific Northern blot analysis. The precise mechanism of IFN-α inhibition of HCV RNA replication in infected cells or subgenomic replicon cells remains ill defined. Proposals include cytokine-mediated double-stranded RNA-activated protein kinase R induction and the consequent inhibition of translation and RNase L induction that degrades viral RNA (41, 42). In the replicon-containing cells, both compound A and IFN-α may affect the levels of mature nonstructural proteins produced (compound A through maturation and interferon-α through inhibition of translation) (40). The net result with both agents is a decreased level of mature replication components and parallel drops in HCV RNA levels irrespective of the mechanism. Although a unanimous consensus regarding IFN-α action as an antiviral agent in the HCV replicon system has yet to emerge, these results are very encouraging from a drug discovery standpoint in that they show that HCV protease inhibitors are as efficient as IFN-α. However, as small molecules that target a specific viral enzyme, they would avoid the pleiotropic effects manifested by IFN-α.

Long Term Treatment of HCV Replicon-containing Cells with Compound A and IFN-α—The eradication of HCV replicon RNA was assessed by incubating cells with 100 μM compound A or 100 IU/mL IFN-α for 14 days, either in the presence or absence of the selectable agent G418 (Fig. 6). In untreated control cells, HCV RNA levels were shown to be stable over the 14-day period either in the presence or absence of G418. Blockage of HCV RNA replication, however, rendered the cells sensitive to the selectable agent G418 and resulted in cell death. Therefore, HCV RNA levels were evaluated independently of the number of viable cells by normalization to the yield of total cellular RNA. The detection limit for HCV RNA by RT-PCR is between 10 and 100 genome equivalents/μg of total RNA. These replicon cells contained 8 x 10$^7$ genome equivalents/μg of total RNA. Cells incubated with either compound A (Fig. 6A) or IFN-α (Fig. 6B) for 14 days both showed similar reductions of HCV RNA to undetectable levels that exceeded 4 logs; this drop was independent of the presence of G418.

In order to detect any rebound in HCV RNA, replicon levels were monitored for an additional 7-day period after removal of the respective inhibitor. The experiments in the presence of G418 were terminated after 14 days with both inhibitors because of the small number of surviving viable cells. However, the removal of compound A or IFN-α at day 14 from cells incubated in the absence of G418 still showed that after 7 days subgenomic HCV RNA levels remained undetectable and suggests that these cells were cured of the episomal RNA replicon. HCV RNA levels were still undetectable up to 40 days after the removal of the NS3 protease inhibitor (data not shown). These results demonstrate that both inhibitors can efficiently inhibit HCV RNA replication and reduce HCV RNA levels below the detection limit after 2 weeks of treatment. The observation that HCV RNA did not rebound after cessation of treatment with inhibitors could be explained by (i) the complete elimination of
the HCV replicon from cells; (ii) the lack of selection for the replicon during the rebound period; or (iii) the fact that the HCV subgenomic replicon cell system does not involve a functional virus with cell to cell spread and there is no selective advantage in maintaining the episomal replicon without the appropriate pressure.

We have described a peptidomimetic HCV NS3 protease inhibitor, compound A, that potently inhibits viral protease activity, NS3 protease-mediated polyprotein processing, and HCV RNA replication in cell culture. The mechanism is consistent with the inhibition of NS3-mediated polyprotein cleavage that in turn reduces the production of mature components of the viral replication machinery and results in a reduction in HCV RNA to undetectable levels.

The concept that NS3-mediated processing of the polyprotein is essential for HCV RNA replication is reinforced by the demonstrated effect of a specific inhibitor of the HCV serine protease. The HCV NS3 protease activity was previously shown to

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**Fig. 6.** HCV replicon eradication with compound A and IFN-α. To compare the kinetics of HCV RNA elimination of two different replication inhibitors, cells were incubated with 100 μM NS3 protease inhibitor compound A (A) or with 100 IU/ml IFN-α (B) for 14 days in the presence or absence of 0.5 mg/ml G418. Inhibitor was removed after 14 days, and cells were incubated for another 7 days without compound A or IFN-α. Samples were taken at the indicated time points, and HCV RNA was determined by Taqman real time RT-PCR. The experiments in the presence of G418 were terminated after 14 days, since elimination of the replicon by compound A or IFN-α resulted in cell death. Two independent experiments for each compound were performed, and the average is shown.
be essential to the viral life cycle by introducing an active site mutation in the context of an infectious HCV cDNA that abolishes virus production when injected into the liver of a chimpanzee (43). The reverse genetic and complementary inhibition studies validate the NS3 protease as a target for therapeutic intervention of acute and chronic HCV infection. The peptidomimetic inhibitor described here, in addition to serving as an important lead for the development of more potent and orally available HCV therapeutics, is a useful scientific tool for the biochemical and genetic characterization of HCV RNA replication in cells.

Addendum—While this manuscript was under revision, three reports were published that showed the inhibitory activity of one NS3 protease and two NS5B polymerase inhibitors in the subgenomic replicon system (47, 50, 51).

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An NS3 Serine Protease Inhibitor Abrogates Replication of Subgenomic Hepatitis C Virus RNA

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