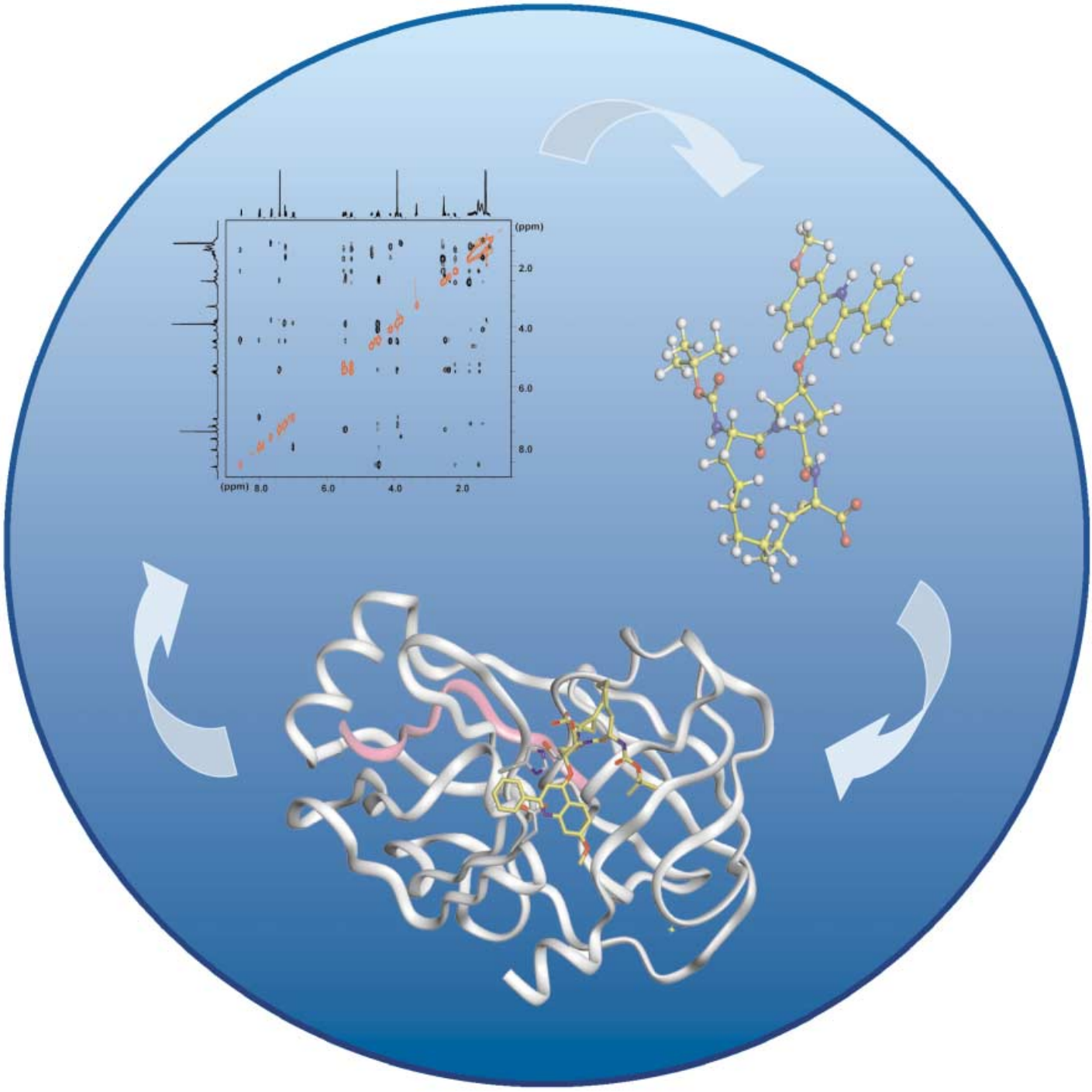
Communications



Potent and selective macrocyclic inhibitors of the hepatitis C virus NS3 serine protease based on the conformation of a enzyme-bound substratelike hexapeptide demonstrate many of the desirable properties of a druglike archetype, which could lead to an antiviral agent for the treatment of hepatitis C in man. For more details see the following communication by Tsantrizos et al.

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Protease Inhibitors

Macrocyclic Inhibitors of the NS3 Protease as Potential Therapeutic Agents of Hepatitis C Virus Infection**

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The hepatitis C virus (HCV) is the etiologic agent of chronic hepatitis C infection, an important health problem that can lead to cirrhosis of the liver and hepatocellular carcinoma in humans.[1] The number of infected individuals is estimated to exceed 170 million people worldwide and the current available treatments (interferon α alone or in combination with ribavirin) are of limited efficacy.[2] In spite of intensive efforts, many aspects of the disease and the biology of the hepatitis C virus remain unclear, making drug discovery an extremely challenging endeavor. We present herein the design of a novel class of selective inhibitors of the HCV NS3 protease, an enzyme which is essential for viral replication in vivo.[3] These compounds exhibit many of the desirable biopharmaceutical properties of antiviral agents and represent a significant advancement towards the discovery of a therapeutic agent for the treatment of hepatitis C in humans.

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

The HCV RNA genome contains a long open reading frame, encoding a polyprotein of about 3011 amino acids which must be proteolytically processed into four structural and six nonstructural (NS) polypeptides.[1] The structural polypeptides are cleaved from the polyprotein by host enzymes, whereas the nonstructural proteins are processed by two virally encoded enzymes, the NS2/NS3 and the NS3 proteases;[2] the latter enzyme is responsible for processing four out of the five cleavage sites of the nonstructural region, thus it represents a particularly important target for drug discovery.

Initial investigations revealed that the N-terminal peptide products derived from sequences of the HCV NS3 protease substrates are competitive inhibitors of this enzyme, and these peptides became leads for the design of peptidomimetic inhibitors.[4,5] Similar peptides were used for exploring the interactions between substrate-based ligands and the NS3 protease by NMR spectroscopy,[6–9] molecular modeling, and crystallography.[10] However, the inherent conformational flexibility of these peptides, in addition to the solvent exposure of the shallow S1–S3 binding region of the NS3 protease,[11] posed a significant challenge to the design of relatively small peptidomimetic compounds that could penetrate cell membranes and block replication of the HCV RNA genome in cell culture.

To design of a novel class of inhibitors which could potentially provide a therapeutic agent for the treatment of hepatitis C in humans, the bimolecular recognition elements involved in an NS3–ligand complex were evaluated extensively by NMR spectroscopy and molecular modeling. The HCV NS3 enzyme is fairly unique amongst serine proteases in that it is activated by its structure-modifying cofactor NS4A[12] and by its ligands.[13] Interactions between NS3 and its NS4A cofactor induce conformational changes that significantly reduce (but not entirely eliminate) the plasticity of the enzyme.[13] In addition, stabilization of the NS3 active site upon binding of a substrate-based ligand has been proposed.[8] We explored the interactions of the NS3 protease domain with the substrate-based inhibitor **1** (Figure 1a) by NMR spectroscopy.[6,9,14]

Transferred nuclear Overhauser effects and transferred ^{13}C spin–lattice relaxation NMR experiments indicate that peptide **1** binds to the protease in an extended conformation and undergoes extensive rigidification upon binding. Studies on protease-induced differential line broadening suggested that the P5 and P6 residues are solvent-exposed and interact only weakly with the protease,[6] consistent with reports by other investigators.[7,15] In the inhibitor–protein complex, the P3 side chain lies on the solvent-exposed surface of the protein and close to the P1 norvaline side chain. This latter group is folded, placing the δ CH_3 group close to α H of P1 (Figure 1b).

On the basis of this data and literature precedence,[16] we speculated that intramolecular linking of the P1 side chain to the P3 side chain with a hydrocarbon bridge would lead to a macrocyclic inhibitor which would, in the free state, preferentially adopt the conformation of NS3-bound peptide **1**. A rigid macrocyclic scaffold would also assure that the P2–P3 amide bond would adopt exclusively the *trans* geometry

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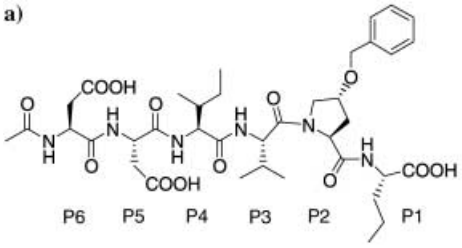

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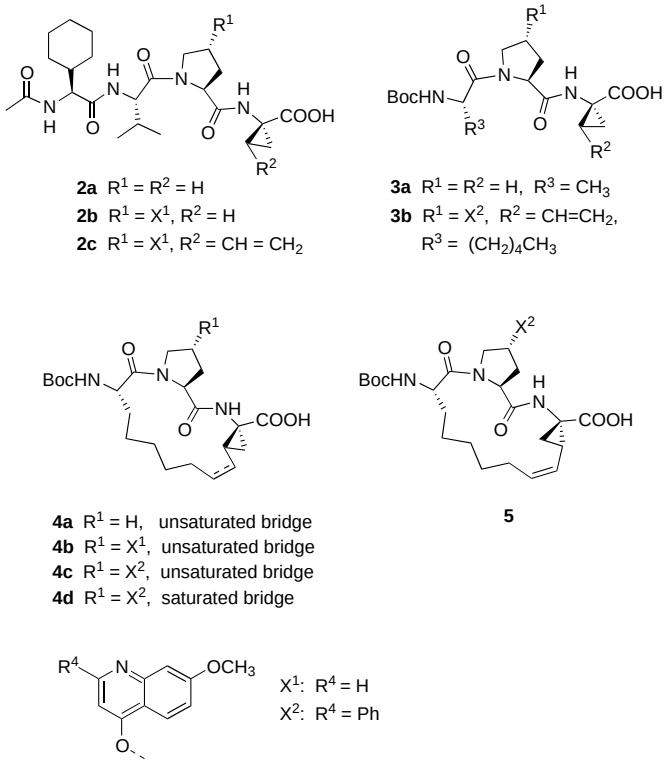
Figure 1. a) Structure of hexapeptide **1**; b) model of P1–P4 moieties of **1** docked in the active site of the apo form of the NS3 protease.[17] Differential line broadening (DLB) NMR data (blue = negative DLB, red = positive DLB) of NS3-bound **1**.

observed in the bound conformation, unlike linear peptides which exist as mixtures of *cis* and *trans* rotamers. The anticipated reduction of the conformational entropic penalty, paid upon binding of this inhibitor, could lead to an increase in the overall binding energy. A model of an inhibitor–enzyme complex was created by simply inserting the NMR-derived structure of NS3-bound **1** into the active site of the apoenzyme (Figure 1 b).[17] This model supported our hypothesis and suggested that additional interactions could be gained between the hydrocarbon bridge of the cyclic inhibitor and the S1–S3 binding pocket of the enzyme. Furthermore, as a consequence of the net reduction in the peptidic nature of the macrocyclic inhibitors, we speculated that these molecules may exhibit more desirable biopharmaceutical properties than their corresponding linear peptides.

The design and synthesis of the rigid macrocyclic inhibitors began with an evaluation of the interactions between the NS3 protease and the basic peptidic scaffold of previously developed tetrapeptide inhibitors, typified by compound **2** (Scheme 1).[18] During these studies, we observed that the tetrapeptide scaffold **2a** and its truncated analogue **3a** are poor inhibitors of the NS3 enzyme in vitro (Table 1, $<20\,\%$ inhibition at 1000 μM was observed). However, we discovered that the 15-membered macrocyclic peptide **4a**, where the double bond is *Z* configured, is more potent than either of the two linear peptides (Table 1, $IC_{50} = 400$ μM).[19] Optimization of **4a** by substitution of the proline with a 4-hydroxy-7-methoxyquinoline moiety (X^1), as previously reported,[18] led to inhibitor **4b** with an approximate 16600-fold increase in potency over the "naked" scaffold **4a**, a close to 50-fold increase in potency over the linear tetrapeptide **2b**, and a 2-fold increase in potency over **2c** which has a vinyl substituent at the cyclopropyl ring (Table 1).[20] Addition of a phenyl substituent to X^1 ($\rightarrow X^2$) led to a doubling of the potency in the in vitro assay (Table 1, **4b** vs **4c**).[21] However, a more than 36-fold drop in potency was observed upon cleaving the bridge of inhibitor **4c** to produce the corresponding open-chain analogue **3b** (Table 1), which confirms the importance of macrocyclization. Predictably, the binding affinity and potency of these inhibitors is highly dependent on the ring size and the stereochemistry of each chiral center.[19] For example, peptide **4c** is 180-fold more potent than its epimer **5**, which further validates the specificity of these inhibitors for their intended molecular target.

Steady-state kinetic analysis of the mode of inhibition by compound **4c** demonstrated competitive inhibition of the NS3–NS4A protease heterodimer of genotype 1b with a K_i of 1.0 nM. For the



Scheme 1. Structures of tested inhibitors for the NS3 protease.

Table 1: Potency and specificity of the HCV NS3 protease inhibitors **2**–**5** in enzymatic (IC_{50}[a]) and cell-based assays (EC_{50}[b]); n.d. not determined.[29]

Compound	IC_{50}	EC_{50}	IC_{50}(HLE[c])	IC_{50}(HLCatB[d])	IC_{50}(BPChym[e])
2a	>1000				
2b	1.4	>20.0			
2c	0.047	>5.0			
3a	>1000				
3b	0.400	n.d.	>30	n.d.	>30
4a	400.0				
4b	0.024	1.20	>30	n.d.	>30
4c	0.011	0.077	>30	>30	>30
4d	0.028	0.12	>75	>75	>75
5	2.00				

[a] In vitro IC_{50} [μM] determination in enzymatic assays using an NS3–NS4A heterodimer protein of genotype 1b. [b] EC_{50} [μM] determination in Huh-7 cells harboring the bicistronic subgenomic HCV RNA replicon. [c]–[e] In vitro specificity assays used: human leukocyte elastase, human liver cathepsin B, bovine pancreatic chymotrypsin.

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heterodimer of genotype 1a a K_i of 1.4 nM was obtained. The compounds **4** were also highly selective in inhibiting the HCV NS3 protease without inhibiting mammalian proteases, even at concentrations exceeding 30 μM (Table 1).[29]

Inhibition of the HCV NS3 protease by the macrocyclic inhibitors **4** was subsequently confirmed in a cell-based assay,[22] using human hepatoma (Huh-7) cells transfected with a selectable subgenomic HCV RNA.[23] In this assay, RNA replication is dependent on the key virally encoded enzymes and is sensitive to compounds which specifically inhibit these enzymes.[24] Negligible reduction in the HCV RNA levels was observed with tetrapeptides **2b** and **2c** even at their solubility limits. In contrast, a median effective concentration (EC_{50}) of 1.2 μM and 0.077 μM was measured for the macrocyclic inhibitors **4b** and **4c**, respectively (Table 1). Saturation of the P1–P3 olefinic bridge of inhibitor **4c** resulted in compound **4d**, which was almost equipotent to its precursor in both the enzymatic and the cell-based assay (Table 1, $IC_{50} = 0.028$ μM, $EC_{50} = 0.120$ μM). Thus, the electronic properties (π character) and rigidification elements of the olefin moiety did not significantly impact the overall stability of the NS3 protease–inhibitor complex.

Binding of the macrocyclic inhibitors in the active site of the NS3 protease was also confirmed by X-ray crystallography. Previously reported crystallographic data revealed the typical chymotrypsin-like fold of the NS3 protease, complexed with the key central segment of its NS4A cofactor (a peptide composed of residues 21–34 of the NS4A and known as $NS4A_{peptide}$).[25,26] The $NS4A_{peptide}$ was shown to intercalate within the N-terminal domain of the protease, forming a β sheet with the enzyme. More recently, X-ray and NMR data of α-keto acid inhibitors covalently bound to the NS3 protease were reported.[8,10] In addition, a crystal structure analysis of an engineered single polypeptide, containing the C-terminal helicase domain and the N-terminal protease covalently linked to the NS4A cofactor, revealed the binding of the C-terminal carboxylic acid moiety of the helicase to the active site of the protease.[27] This is the only earlier report of a C-terminal carboxylate bound to the active site of the NS3–NS4A complex and the system represents a model of product inhibition for this enzyme. However, with the **4c**–NS3–$NS4A_{peptide}$ complex (Figure 2) we present the first X-ray structure analysis of a small-molecule carboxylic acid bound to the active site of this enzyme.

We were successful in obtaining small needlelike co-crystals of the wild-type BK* NS3 protease–$NS4A_{peptide}$ complexed with inhibitor **4c** that diffracted X-rays to 3.0 Å resolution (Figure 2). The macrocyclic compound **4c** was found at the NS3 active site and was clearly defined in the difference electron density. This structure revealed how the carboxylate group interacts with the active site and provided additional insight into the mechanism of product inhibition observed with natural substrates of the HCV NS3 protease. The heteroatom-to-heteroatom distances suggest that one of the carboxylate oxygen atoms of the inhibitor binds in the oxyanion hole of NS3 (NH of G137 and S139), while the other forms a hydrogen bond with the H57 (δ N) residue (Figure 2b). In addition, the side chain (γ O) of S139 appears to form weak asymmetric bifurcated hydrogen bonds with both carboxylate oxygen atoms of the inhibitor. The binding affinities of **4c** toward a wild-type 1b HCV NS3 protease and its corresponding S139A and H57A (catalytically inactive) single amino acid mutants were determined by using a fluorescence polarization assay. We obtained K_D values of 0.04 μM with wild-type NS3 and of 0.007 μM with the S139A mutant,[28,29] whereas a dramatically lower affinity ($K_D >$ 10 μM) was estimated for the H57A mutant. These results suggest that the H57 side chain plays a crucial role in stabilizing the enzyme–inhibitor complex, whereas the net contributions of S139 (γ O) are modulated by many other factors, including perhaps the energy penalty associated with dehydration and disruption of the hydrogen bond with H57.

Formation of canonical hydrogen bonds between the amide moieties of the inhibitor and the NS3 main chain hydrogen bond donor/acceptor residues R155 and A157 was also observed. The *tert*-butyl group was well defined and

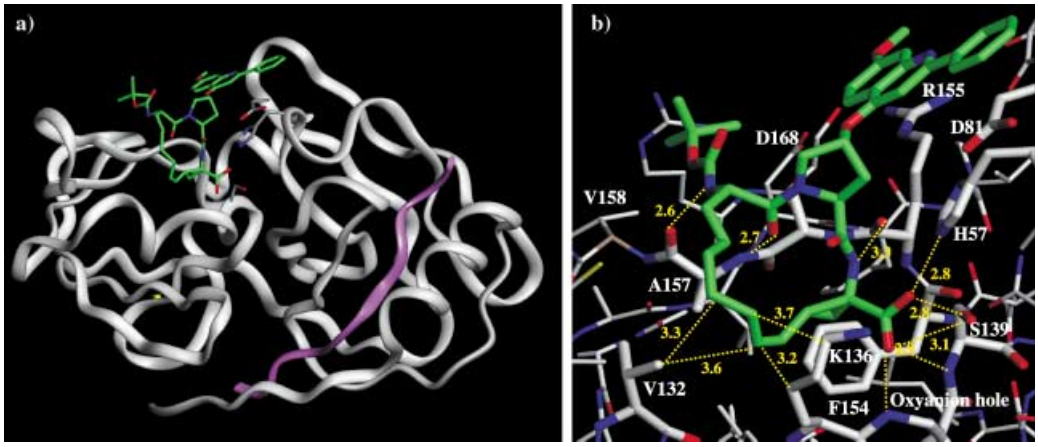

Figure 2. Structure of **4c** bound to the HCV NS3–$NS4A_{peptide}$ complex as determined by X-ray structure analysis. a) **4c** bound in the active site of the complex of BK* NS3 protease (white ribbon with Zn^{2+} ion shown as a yellow ball) with $NS4A_{peptide}$ (purple). b) Key interactions of **4c** with the active site; figures at dashed yellow lines are distances in Å, measured from heteroatom to heteroatom that may be implicated in hydrogen-bond or van der Waals interactions. NS3 residues distal from the binding pocket are shown in thinner lines. Carbon atoms of the inhibitor are colored in green, those of NS3 in white. All nitrogen and oxygen atoms are colored in blue and red, respectively.

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positioned above V158 of the protein surface. The aliphatic bridge between P1 and P3 of the inhibitor was clearly visible in the electron density, which indicates that the bridge is conformationally fixed within the S1–S3 pocket. The NS3 residue V132 appears to be within van der Waals distance of this bridge and may contribute to the overall binding energy in the inhibitor–enzyme complex (Figure 2b). Furthermore, inhibitor-induced conformational changes of the R155 and D168 side chains leads to the formation of a salt bridge between these residues and facilitates an interaction of the guanidine group of R155 with the methoxy quinoline unit (Figure 2b).

A comparison of the X-ray data of our inhibitor–enzyme complex with those of the previously reported complex between the apo form of the NS3 protease and $NS4A_{peptide}$ of the BK-strain hepatitis C virus reveals a similar overall structure with only modest differences at the active site.[25] However, our structure provides strong evidence for the molecular basis of the interactions between a potent and selective druglike inhibitor and the NS3 protease, which lead to the distortion of the catalytic triad and the disruption of the hydrogen bond between S139 and H57. Furthermore, the hydrogen bond between H57 and one of the carboxylate oxygen atoms of the inhibitor appears to be forcing a side chain rotation of S139, which places γ O deeper into the core of the protein.

Data from preliminary pharmacokinetic studies on the macrocyclic inhibitors **4c** and **4d** were also encouraging.[29] Both inhibitors were found to be relatively stable to in vitro metabolism in human and rat liver microsomes. After i.v. (intra venous) administration (5.0 $mg\,kg^{-1}$) in rat, both inhibitors were excreted in the bile, which suggests that the liver is the main clearance organ. However, compound **4c** was cleared more rapidly than **4d** and gave a lower volume of distribution at steady state (V_{dss}) (this indicates lower general tissue distribution of **4c**). Oral administration of the inhibitors at a dose of 25 $mg\,kg^{-1}$ to conscious male rats resulted in a peak plasma concentration (c_{max}) of about 0.2 (**4c**) and 1.3 μM (**4d**) and a bioavailability (*F*, calculated by comparison between the i.v. and oral profile) of 2 (**4c**) and 22% (**4d**). Based on the Caco-2 model, the cell membrane permeability for inhibitor **4c** was also significantly lower (~10-fold lower) than that for **4d,** a fact that might contribute to the overall differences in oral bioavailability between the two compounds.[29]

In summary, we have elucidated the subtle details governing the structure–activity relationship between a substrate-based peptidomimetic ligand and the HCV NS3 serine protease. We have used this knowledge to design a novel class of highly specific and potent inhibitors of the HCV NS3 protease for the most prevalent HCV genotypes 1a and 1b, which are also the most resistant to chemotherapy with interferon α. Compound **4c** and its saturated analogue **4d** are the first inhibitors of the NS3 protease which inhibit HCV RNA replication in the cell-based replicon assay. In addition, they are orally absorbed and stable to metabolic breakdown. Thus, these compounds show many of the desirable properties of a druglike archetype and could lead to a clinically useful antiviral agent for the treatment of hepatitis C viral infections in humans.

Experimental Section

Confirmation of the structure and purity of all inhibitors and details on the biological assays and pharmacokinetic studies are provided in the Supporting Information.

The BK* NS3 protease was derived from a construct which encoded a modified BK strain sequence that spanned amino acids 1–180 and contained the C-terminal solubilization motif ASKKKK as previously reported.[9] The protease was complexed with $NS4A_{peptide}$ (KK-^{21}GSVVIVGRIILSGR^{34}-K) and exposed to the inhibitor **4c**. Crystal data: Space group: $P3_221$, cell constants: 76.5, 76.5, 169.2 Å. Data to 3.0 Å resolution were collected at the MPI wiggler beamline BW6B at DESY Hamburg using monochromatic X-radiation with a wavelength of 1.05 Å. Data were integrated, scaled, and merged with the HKL package.[30] Structure solution was done by molecular replacement using 1JXP coordinates from PDB as the search model.[25] Two crystallographically independent molecules were found in each asymmetric unit. An initial difference electron density map unambiguously showed the entire structure of the bound inhibitor. The model was refined with CNS,[31] using data between 20.0 and 3.0 Å resolution and applying noncrystallographic symmetry restraints. (Data collection statistics: resolution limits 100.0–3.0 Å, total observations 41980, unique reflections 11050, completeness 92.7%, *R* merge 0.065; refinement statistics: number of atoms in model: protein 2774, inhibitors 102, zinc ions 2, number of reflections (free set) 10493 (527), rms deviation from ideal geometry: bond lengths 0.01 Å, bond angles 1.59°, temperature factors [$Å^2$]: all protein atoms 60.9 $Å^2$, rms bonded 1.2 $Å^2$, *R* 0.271, R_{free} 0.327.)

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