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Identification of Weak Points of Hepatitis C Virus NS3 Protease Inhibitors Using Surface Plasmon Resonance Biosensor-Based Interaction Kinetic Analysis and Genetic Variants

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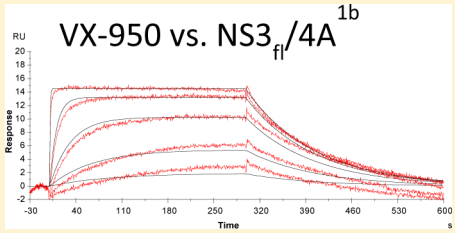
ABSTRACT: To aid the design of next generation hepatitis C virus (HCV) drugs, the kinetics of the interactions between NS3 protease inhibitors and enzyme from genotypes 1a, 1b, and 3a have been characterized. The linear mechanism-based inhibitors VX-950 (telaprevir) and SCH 503034 (boceprevir) benefited from covalent adduct formation. However, the apparent affinities were rather weak (VX-950, K_D* of 340, 8.5, and 1000 nM for genotypes 1a, 1b and 3a, respectively; SCH 503034, K_D* of 90 and 3.9 nM for 1b and 3a, respectively). The non-mechanism-based macrocyclic inhibitors BILN-2016 (ciluprevir) and ITMN-191 (danoprevir) had faster association and slower dissociation kinetics, indicating that rigidification is kinetically favorable. ITMN-191 had nanomolar affinities for all genotypes (K_D* of 0.13, 1.6, and 0.52 nM), suggesting that a broad spectrum drug is conceivable. The data show that macrocyclic scaffolds and mechanism-based inhibition are advantageous but that there is considerable room for improvement of the kinetics of HCV protease targeted drugs.



INTRODUCTION

Hepatitis C virus (HCV) is a global health problem, with around 170 million people chronically infected worldwide and the cause of more than 360 000 deaths each year.[1,2] HCV is an RNA virus belonging to the Flaviviridae family and that exists in a very large number of genotypes and subpopulations. The global distribution of the six major genotypes (1−6) and subclasses (1a, 1b, and so forth) is widespread.[3,4] Genotype 1 is the most common genotype in the U.S. and Europe and also the most difficult to treat. Among patients infected with genotype 1, 50−60% fail to achieve sustained virological response (SVR) using standard INF-based therapy, whereas for other genotypes (2 and 3) SVR is achieved in up to 90% of the cases using the same therapy.[5,6]

A major problem is that new subclasses emerge rapidly because of viral evolution. This aggravates the already widespread prevalence of the infection, since one patient can be infected with more than one subclass. It is consequently essential to develop drugs that are not sensitive to genetic variations, although combination therapies will most likely be needed in order to overcome this challenge. The standard treatment has until recently been a combination of pegylated interferon α (IFNα) and ribavirin, an indirect, costly, time-consuming, and very harsh treatment with serious side effects and limited success rate, especially for some genotypes. Direct acting viral drugs targeting the viral protease have now become available in the clinic, but none are suitable for treatment without IFNα and ribavirin.

The targeted viral protease resides in the nonstructural protein 3 (NS3) of HCV. It is, together with host cellular proteases and the viral NS2-3 protease, responsible for processing of the single polyprotein produced upon translation of the 9.6 kb HCV genome, resulting in 10 mature viral proteins. The NS3 protease is a chymotrypsin type serine protease that constitutes the N-terminal domain of NS3, a multifunctional protein that has a helicase/NTPase in the C-terminal domain. Although having completely different functions, these domains are known to be interdependent, enhancing each other's activities.[7−9] NS3 is also involved in the reduced innate immunological response associated with HCV infection.[10,11] Direct acting antivirals targeting NS3 are therefore believed to have multiple roles. The approval of two NS3 protease inhibitors telaprevir (VX-950, Incivek) and boceprevir (SCH 503034, Victrelis) (Figure 1) by the FDA for clinical use in 2011 has therefore been an important proof-of-concept and a major step forward. Although these drugs have considerably improved the standard treatment of HCV, especially for patients infected with genotype 1, they have to be used in combination therapy with IFN and ribavirin and resistance has already been demonstrated.[12−14] The latest addition to the clinically available drugs, simeprevir (TMC435, Olysio),[15,16] appears to have a higher genetic barrier to resistance and better pharmacokinetic properties, illustrating that new drugs will most likely supplement the first generation of compounds, but it is not yet clear what the additional

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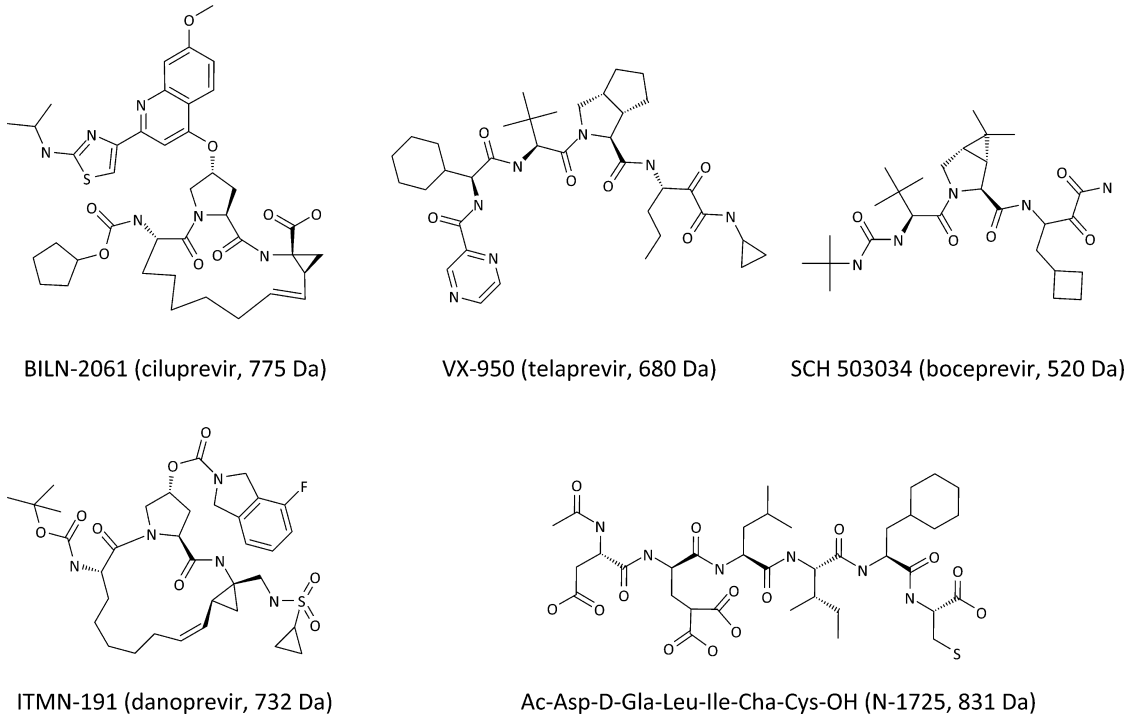

Figure 1. Structures, names, and molecular weights of the studied inhibitors.

benefits will be or how they will complement the already available drugs.

As for HIV, there will be a continued pressure for development of additional HCV drugs in order to battle resistance development, especially inhibitors that can be used without interferon and ribavirin.[17] Such follow-up drugs should preferably not only have novel structural features influencing their interaction with the active site, thereby improving their kinetics and affinities for a broad spectrum of targets and avoiding development of resistance. They should ideally also have novel modes of action involving alternative mechanistic features or interactions with allosteric sites, e.g., as a recently described inhibitor identified by a fragment-based approach.[18] The development of compounds targeting the viral polymerase and NS5A protein is also an important complement to the development of protease inhibitors, since combination therapies will most likely be needed, as in the case of HIV.[19]

To better understand how efficient and safe drugs can be designed, our research is focused on analyzing the kinetic and mechanistic details of interactions between lead compounds and drugs with their targets. As part of our HCV drug discovery program, we therefore established a surface plasmon resonance (SPR) biosensor-based assay for analysis of interactions between NS3 protease inhibitors and full-length NS3 protease from genotype 1a.[20] In the current study we extended the assay also for studies of NS3 protease from genotypes 1b and 3a. In addition, it has comprised analysis of recombinant variants including heterodimeric NS3/4A complexes and truncated NS3 protein encompassing the protease domain alone in order to clarify the consequences of using different types of model systems.

The possibility of obtaining detailed mechanistic and kinetic information from the biosensor assay makes it an important complement to conventional activity-based inhibition assays, which only indirectly monitor the interaction and are typically limited to equilibrium-based data (e.g., K_i). An often disregarded fact is that differences in K_m values and preferred experimental conditions make it difficult to compare inhibition data for different enzyme variants. In fact, it may not even be possible to identify a common set of conditions for all enzyme variants, as we have recently demonstrated for HCV NS3 protease.[21] The study not only showed that the catalytic properties and thus the inhibition of the target protein variants clearly differed in different assay buffers (as expected) but also demonstrated that a standard assay condition suitable for inhibition analysis of all target variants could not be defined. The study clearly demonstrates that it is rather futile to compare K_i values for inhibitors of HCV NS3 inhibitors obtained with different assays and with different genotypes. The biosensor-based approach does not suffer from the same limitation, since it does not have to consider differences in K_m and catalytic rates for different enzyme variants. It has therefore been found to be useful for obtaining data on the interaction mechanism and kinetics rather than the inhibitory effect. The possibility of determining the interaction kinetics of inhibitor–target interactions is important for better understanding the interaction as such, as well as for understanding the pharmacokinetics of drugs and their effects on viral kinetics, thus complementing the mathematical modeling that is used to understand these complex phenomena.[22−24]

The compounds in this study represent different generations of inhibitors and with different structures and characteristics (Figure 1).[17] Ac-*Asp-D-Gla-Leu-Ile-β-Cha-Cys*-OH (compound **14** in ref 25, here denoted N-1725) is a hexapeptide analogue of the N-terminal cleavage product with low nanomolar affinity.[25] Ciluprevir (BILN-2061) and danoprevir (ITMN-191) are both second generation inhibitors in which the N-terminal region of the parent compound was modified by incorporation of a macrocycle and a large bulky P_2 substituent, reducing the length and the flexibility of the compound.[26−29] BILN-2061 was the first HCV protease inhibitor evaluated in clinical trials. Although it was shown to be a highly effective

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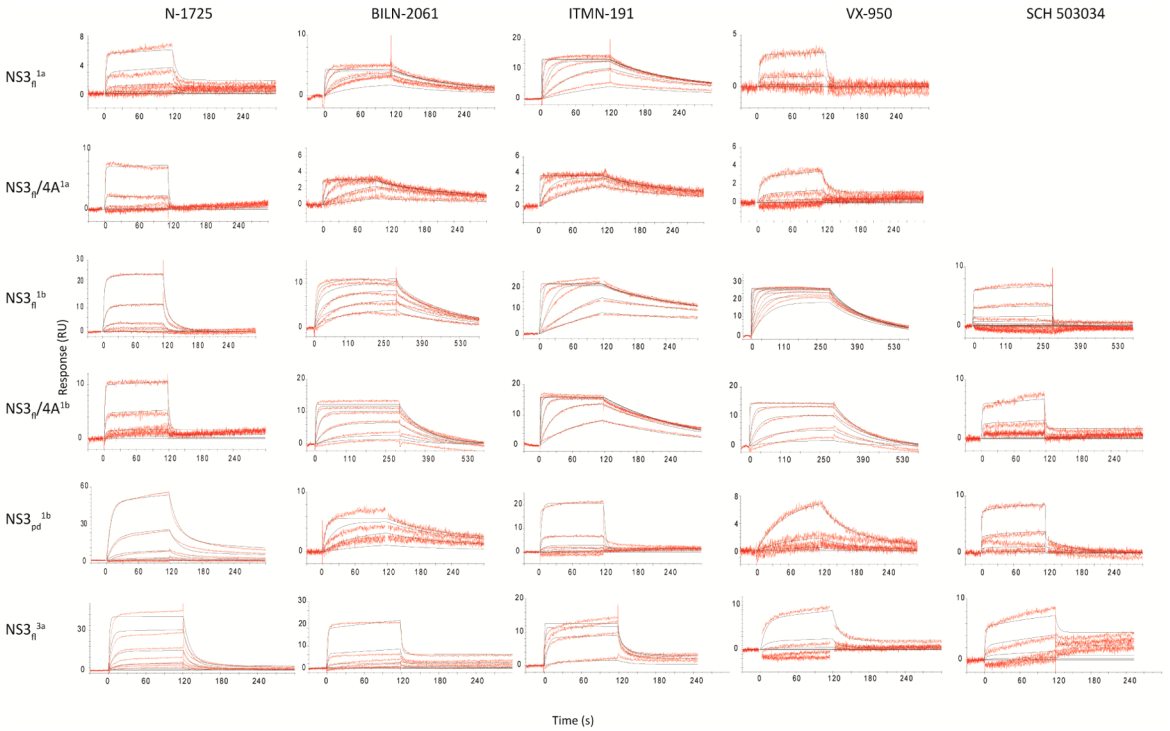

Figure 2. Sensorgrams showing the interaction profiles for the inhibitors injected in concentration series over the immobilized protein variants. Best fit theoretical curves (black) for global nonlinear regression analysis using a two-step interaction model overlay the experimental sensorgrams (red). The sensorgrams in the figure are truncated and scaled in a way that facilitates a visual comparison of the different data sets. Data analysis was performed with longer dissociation phases. The corresponding kinetic data and R_{max} values are presented in Table 1.

inhibitor, illustrating an important proof-of-concept, it was withdrawn from clinical trials because of toxic effects in animals.[30] ITMN-191 is today in phase II clinical trials. An alternative design was used for optimization of VX-950 and SCH 503034, two first generation inhibitors and the only compounds yet approved for clinical use.[17] These drugs are both linear analogues of the original N-terminal product peptide but with a shorter chain and a reactive electrophilic C-terminal group. They are significantly smaller than the macrocyclic inhibitors and gain their efficiency from a mechanism that involves the formation of a covalent, reversible bond with the catalytic serine.

By elucidation of the interaction kinetic properties of these inhibitors and their interactions with a set of target variants, it was demonstrated that the compounds are all kinetically suboptimal, explaining why they are biochemically rather poor inhibitors,[21] at least from the perspective of what is expected from an effective drug. There are several basic structural features that may be further exploited in the design of future generations of HCV NS3 inhibitors that can serve as more efficient drugs and with a potential to target a broader range of genotypes and subtypes.

RESULTS

Preparation of Sensor Surfaces. Seven different variants of NS3 were produced for the study. The full length NS3 protein for each of the three genotypes ($NS3_{fl}{}^{1a}$, $NS3_{fl}{}^{1b}$, $NS3_{fl}{}^{3a}$) was produced, being the physiologically most relevant protein variant. To assess how the kinetics were influenced by the helicase domain, the protease domain alone from genotypes 1a and 1b were produced ($NS3_{pd}{}^{1a}$, $NS3_{pd}{}^{1b}$). These variants were thought to also be useful as references since much published work has been based on these truncated forms of NS3. Similarly, to discern if the NS4A cofactor had a significant influence on the interaction between NS3 and inhibitors, full length variants with NS4A coexpressed were also produced for genotypes 1a and 1b ($NS3_{fl}/4A^{1a}$, $NS3_{fl}/4A^{1b}$).

Six of the seven proteins were successfully immobilized to the sensor chip surface by amine coupling. The level of immobilization was normally in the range of 4000−10 000 resonance units (RU), a range acceptable for the current inhibitors considering that the signals from SPR biosensor experiments depend on both the amount of immobilized protein and the molecular weights of the analytes. Variants of the 1b and 3a genotypes were more easily immobilized because of higher concentrations obtained upon production and higher protein stabilities, while the immobilization of genotype 1a protein was more difficult. The amount of genotype 1a protein immobilized to the surface was doubled by using a CM 7 sensor chip. This was necessary to get adequate signal levels in the interaction studies for this protein variant but still not sufficient for analysis of the compound with the lowest molecular weight (SCH 503034) and only just adequate for analysis of the next smallest compound (VX-950) (see Figure 2). Truncated proteins were more difficult to attach to the surface than full-length proteins. This was most evident for the truncated genotype 1a protein, for which no suitable conditions for immobilization by amine coupling were identified.

Interaction Kinetic Analysis. Time series data (sensorgrams) for a series of compound concentrations were collected and analyzed for all inhibitor−protein interactions for which significant signals could be obtained (Figure 2). The low level of $NS3_{fl}{}^{1a}$ and $NS3_{fl}/4A^{1a}$ immobilization was not sufficient for the detection of interactions with SCH 503034. The signals

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were also rather low for the interactions between VX-950 and the genotype 1a protein variants, but data could be collected.

To identify the interaction mechanism and estimate the rate constants and affinities for the interactions, different models were fitted to the sensorgrams using global nonlinear regression. Models for a 1:1 interaction and a heterogeneous ligand were both excluded because they did not generally result in a good fit of the data, although they were adequate for some interactions (data not shown). A two-state model was chosen, since it gave satisfactory fits for all inhibitor interactions. This model (Scheme 1) can be interpreted as an induced fit

Scheme 1

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E^*I$$

mechanism, where the binding of inhibitor is followed by a conformational change of the complex, either locally in the binding site or in a more extensive rearrangement of the overall protein structure. It also applies to the reversible mechanism-based inhibition expected for compounds with a reactive electrophilic C-terminal group.

The kinetic parameters estimated by nonlinear regression of the type of data shown in Figure 2 are presented in Table 1. The K_D^* value is the overall/observed affinity, i.e., the combined equilibrium dissociation constant for both interaction steps (eq 1).

$$K_D^* = \frac{k_{-1}}{k_1}\frac{k_{-2}}{k_{-2} + k_2} \quad (1)$$

In order to visualize the results, the rate constants for the first (k_1 vs k_{-1}) and second (k_2 vs k_{-2}) steps were plotted in interaction kinetic plots (Figure 3). The graphs were helpful when trying to find trends among the interactions and when assessing the relative contribution of the second step to the overall interaction. In the k_1 vs k_{-1} plot the compounds with high affinity are positioned in the top left-hand corner. Similarly, the compounds in this position in the k_2 vs k_{-2} plot have a second step that contributes significantly to the interaction. This is also evident from a comparison of the magnitudes of K_D and K_D^* (Table 1). Interestingly, this step appeared to be rate limiting not only for the two mechanism-based inhibitors but also for the other inhibitors and certain protein variants. However, there was no simple correlation between either the enzyme variants or compounds. The following analysis is primarily based on the data for the first step (k_1, k_{-1}, and K_D); the second step (k_2 and k_{-2}) was considered when it had a significant effect (defined as $K_D/K_D^* \geq 5$) on the overall interaction. Table 1 and Figure 3 are both useful for the analysis.

Interactions with Full-Length NS3 and Genotype Comparisons. The prototypic N-terminal product peptide analogue, N-1725, had weak affinities for all genotypes (K_D^* = 0.2−5.6 μM) as a result of slow association and dissociation rates (all points were positioned to the right of the diagonal in the k_1 vs k_{-1} plot). There were relatively small differences in the kinetics for the interactions between N-1725 and the different genotypes. It was most efficient on $NS3_{fl}^{1a}$ for which the second step appeared to contribute significantly (K_D/K_D^* = 20).

BILN-2061 had relatively high affinity for all three genotypes, but the interactions were relatively genotype dependent. The overall affinity was highest for genotype 1a (1 nM) and lowest for genotype 3a (160 nM), the latter having a rate limiting second step (K_D/K_D^* = 21). The higher affinity for BILN-2061 compared to N-1725 can be attributed to up to 100-fold faster association rates and 10-fold slower dissociation rates.

ITMN-191 had the highest affinities of all compounds for the enzyme from all three genotypes and was less genotype dependent, with K_D^* = 0.13 nM for genotype 1a, K_D^* = 1.6 nM for genotype 1b, and K_D^* = 0.52 nM for genotype 3. The high affinities could be attributed to relatively fast association and slow dissociation rates (points shifted to the left of the diagonal in the k_1 vs k_{-1} plot). The second step played an important role for the interactions with the genotypes 1a and 3a proteins (K_D/K_D^* of 19 and 40, respectively) but not for the genotype 1b interaction.

As expected, the target interactions with VX-950 and SCH 503034 were for some of the enzyme variants significantly influenced by the second step (K_D/K_D^* = 6−436), in accordance with their mechanism-based inhibition. However, their affinities were still not very high for any of the genotypes, at least compared to BILN-2061 and ITMN-191. For SCH 503034 this can clearly be attributed to slow association and dissociation kinetics (all points were positioned to the right of the diagonal in the k_1 vs k_{-1} plot), with the covalent step being critical for efficacy. Interestingly, SCH 503034 had a preference for genotype 3a NS3, with K_D^* = 3.9 nM.

NS4A Cofactor and Helicase Effects. The two types of constructs used as references to the full length proteins showed that both the NS4A cofactor and the helicase domain influenced the interactions with the inhibitors. The cofactor had an effect that was different for genotypes 1a and 1b and that was apparently influenced by the inhibition mechanism.

More pronounced differences were seen between the interactions with truncated and full length genotype 1b NS3 protein. The second step did not contribute significantly to the stability of the complex, as illustrated in the k_2 vs k_{-2} plot by the position of point for the truncated protein generally being located to the right. This was supported by the fact that a 1:1 interaction model was adequate for the analysis of the interactions with this protein variant. ITMN-191, VX-950, and SCH 503034 interacted considerably more efficiently with the full length protein than the truncated protein, as judged from the K_D^* values, while N-1725 and BILN-2061 had higher affinity for the truncated enzyme.

DISCUSSION

The biosensor-based approach previously developed for analysis of inhibitor interactions with HCV NS3 from genotype 1a[20] was here exploited to develop interaction kinetic assays for NS3 from two additional genotypes (1b and 3a). Assays enabling assessment of the role of NS4A and the helicase domain on the mechanism and kinetics of the interactions were also developed. The possibility to establish sensitive and stable sensor surfaces differed for the different protein variants, with only one variant not being possible to immobilize in sufficient amounts. This may be a consequence of the different content of lysine residues, critical for the immobilization chemistry used. The protease domain of genotype 1a only has four lysine residues while the 1b protein has five, both significantly less than the full length protein since the helicase domain has approximately 15 additional lysine residues, depending on genotype.[21] The sensitivities of the surfaces that could be created were sufficient for detection of interactions with all

Table 1. Kinetic Data for Interactions between Studied Inhibitors and NS3 Protease Variants, Assuming a Two-Step Model (Scheme 1)[a]

	$NS3_{fl}^{1a}$	$NS3_{fl}/4A^{1a}$	$NS3_{fl}^{1b}$	$NS3_{fl}/4A^{1b}$	$NS3_{pd}^{1b}$	$NS3_{fl}^{3a}$
			N-1725 NMB			
k_1 (10^6 M^{-1} s^{-1})	0.038	0.022	0.026	0.056	0.0075	0.060
k_{-1} (10^{-2} s^{-1})	15	79	19	32	5.4	8.3
k_2 (10^{-3} s^{-1})	3.2	0.48	10	1.5	3.2	0.96
k_{-2} (10^{-3} s^{-1})	0.18	0.41	3.9	0.36	2.7	1.7
K_D (nM)	3900	36000	7200	5800	7200	1400
K_D* (nM)	200	17000	5600	2000	3300	870
K_D/K_D*	20	2	1	3	2	2
R_{max} (RU)	10	35	35	18	70	45
			BILN-2061 NMB			
k_1 (10^6 M^{-1} s^{-1})	3.2	3.9	0.18	0.16	1.1	0.15
k_{-1} (10^{-2} s^{-1})	0.95	0.80	3.9	1.1	0.81	51
k_2 (10^{-3} s^{-1})	1.2	0.01	16	0.0017	2.5	3.3
k_{-2} (10^{-3} s^{-1})	0.94	6.5	7.1	2.6	1.4	0.17
K_D (nM)	3.0	2.1	220	71	7.3	3300
K_D* (nM)	1.3	2.1	67	71	2.5	160
K_D/K_D*	2	1	3	1	3	21
R_{max} (RU)	4	3	12	16	2	5
			ITMN-191 NMB			
k_1 (10^6 M^{-1} s^{-1})	3.3	2.1	3.3	0.95	0.060	24
k_{-1} (10^{-2} s^{-1})	0.83	0.85	0.53	0.62	110	52
k_2 (10^{-3} s^{-1})	0.40	2.4	0.00016	0.024	2.1	2.3
k_{-2} (10^{-3} s^{-1})	0.022	1.4	0.0082	6.0	2.3	0.057
K_D (nM)	2.5	4.0	1.6	6.5	19000	21
K_D* (nM)	0.13	1.6	1.6	6.5	10000	0.52
K_D/K_D*	19	3	1	1	2	40
R_{max} (RU)	13	5	20	16	68	13
			VX-950 MB			
k_1 (10^6 M^{-1} s^{-1})	0.12	0.0068	0.91	0.15	0.0045	0.0015
k_{-1} (10^{-2} s^{-1})	25	9.4	0.78	0.97	1.8	9.6
k_2 (10^{-3} s^{-1})	1.0	3.8	0.0051	0.0014	2.3	2.6
k_{-2} (10^{-3} s^{-1})	0.20	0.0050	5.8	2.3	6.3	0.041
K_D (nM)	2000	14000	8.6	67	4100	66000
K_D* (nM)	340	17	8.5	66	3000	1000
K_D/K_D*	6	824	1	1	1	66
R_{max} (RU)	1	4	23	20	11	11
			SCH 503034 MB			
k_1 (10^6 M^{-1} s^{-1})	nd	nd	0.056	0.062	0.13	0.12
k_{-1} (10^{-2} s^{-1})	nd	nd	88	48	210	20
k_2 (10^{-3} s^{-1})	nd	nd	0.41	2.9	8.4	9.3
k_{-2} (10^{-3} s^{-1})	nd	nd	0.56	0.013	21	0.022
K_D (nM)	nd	nd	16000	7700	16000	1700
K_D* (nM)	nd	nd	90	38	3200	3.9
K_D/K_D*			178	203	5	436
R_{max} (RU)			29	12	4	9

[a] K_D* is the overall/observed affinity (eq 1) and $K_D = k_{-1}/k_1$. For consistency, this model was used also for $NS3_{pd}^{1b}$, although a simple model was sufficient. R_{max} is the theoretical maximal response at steady state, obtained as a parameter in the regression analysis. NMB indicates a non-mechanism-based mode of inhibition, and MB indicates a mechanism-based mode of inhibition.

compounds except SCH 503034, for which the immobilization levels with $NS3_{fl}^{1a}$ and $NS3_{fl}/4A^{1a}$ were not satisfactory for the detection of interactions with this small inhibitor. Although the protein variants had slightly different tags (for details, see ref 21), introduced to improve expression, purification, and stability, they were not structurally near the inhibitor binding site and were assumed not to influence the interactions studied.

The possibility to use SPR biosensor-based analysis of interactions for detection of conformational changes and mechanistic complexities is a great advantage but requires carefully designed experiments and cautious interpretations.[31] Only interactions with the truncated protein, i.e., lacking the helicase domain, were adequately described by a simple one-step model. This confirmed that the helicase domain plays an important role in the NS3 interactions with SCH 503034 but not as clearly for the other inhibitors.[9]

It was more unexpected that all inhibitors had complex interaction mechanisms with the full-length proteins. Although it is logical that the mechanism-based inhibitors follow a two-step mechanism, it is not obvious that the non-mechanism-

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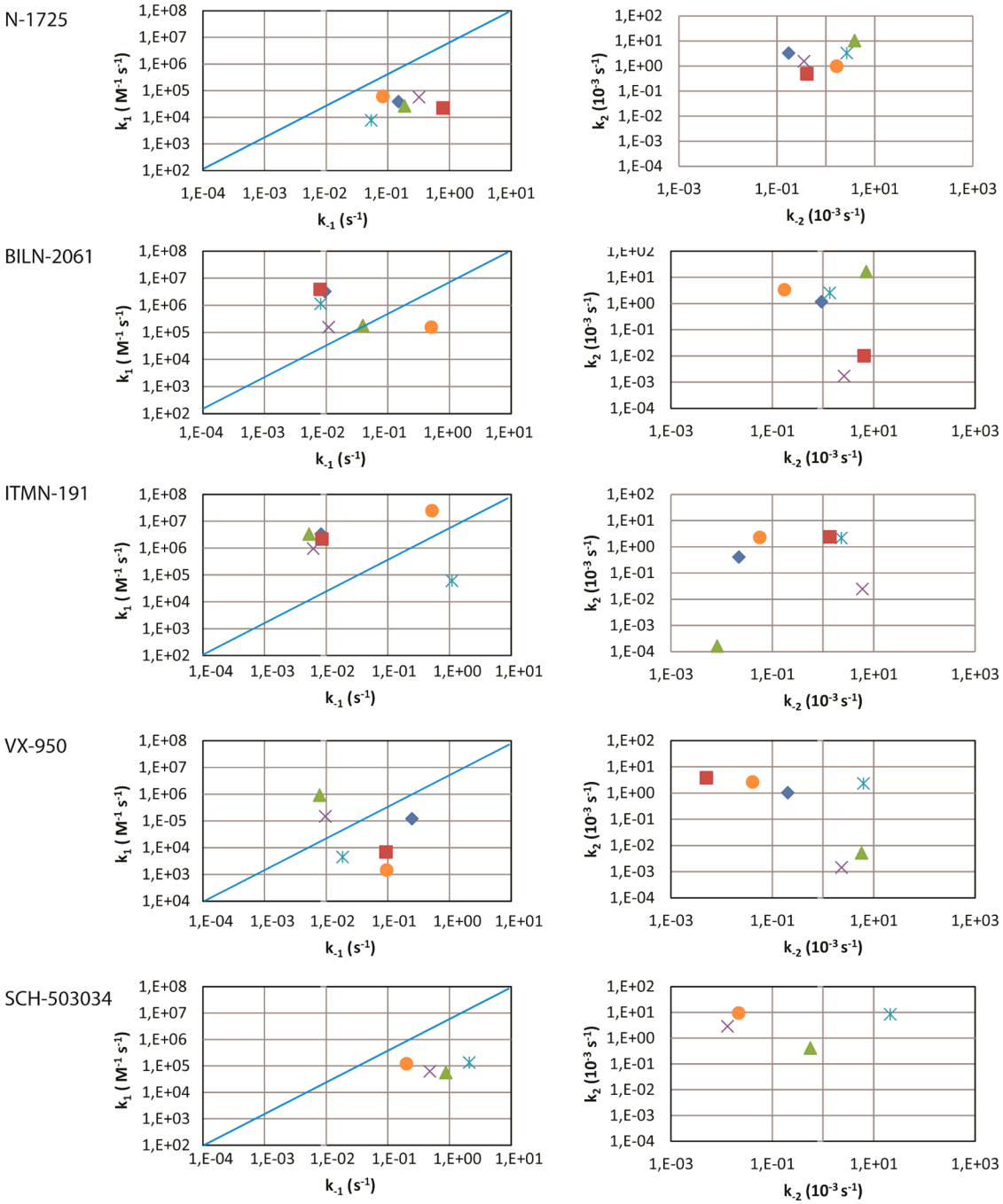

Figure 3. Interaction kinetic plots for (left) k_1 vs k_{-1}, and (right) k_2 vs k_{-2}, for the interactions between the inhibitors and HCV NS3 protease variants, assuming a two-step mechanism: ◆ $NS3_{fl}^{1a}$; ■ $NS3_{fl}/4A^{1a}$; ▲ $NS3_{fl}^{1b}$; × $NS3_{fl}/4A^{1b}$; ∗ $NS3_{pd}^{1b}$; ● $NS3_{fl}^{3a}$. The diagonal in the k_1 vs k_{-1} plot is a visual aid, facilitating the graphical analysis.

based inhibitors should also be better described by this model. However, dynamic changes of the protein are common and have been hypothesized to improve the specificity of the interaction, since the associated inhibitor−protease complex is allowed to rearrange into a more stable form.[32] The complexity can therefore probably be attributed to conformational changes induced by inhibitor binding or the stabilization of the protein in a conformation not energetically favorable in the absence of inhibitor. This may be related to the observation that some inhibitors have an ability to activate NS3 at low concentrations, indicating that they stabilize an active form of the enzyme.[33] It can be speculated that it involves different conformations of the protein with respect to the orientation of the protease and helicase domains.

The current data were consistently analyzed with a two-step model, although a heterogeneous model was selected for the analysis in the original publication of the method.[20] The new data were more extensive, and a careful comparison of the suitability of the two mechanistic models was again performed. Although the heterogeneous model could also be used for some of the data sets, it was not perfect for others. Since the heterogeneous model is based on the assumption that there are at least two populations of the target with different interaction characteristics for the analyte, it is unlikely that the model

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would not be generally suitable for all of the inhibitors, considering that they have the same binding site. However, although it cannot be excluded that free NS3 occurs in different conformations that have different interaction characteristics, there was no evidence for this in the current data. The data for the two non-mechanism-based inhibitors in common in the two studies (ITMN-191 and BILN-2061) matched well, despite the different models used. This indicates that the differences between the models did not influence the estimation of the critical parameters.

In addition to the analysis of the effect of the helicase domain on the interaction with inhibitors, the effect of coexpressed full-length NS4A was explored in this study. However, there were no clear trends in the current data, making it difficult to interpret how the effect of NS4A can be rationalized. It may be attributable to the more nonspecific structural role of NS4A, indirectly influencing the geometry of the active site. The presence of tightly bound NS4A is thus expected to have a much smaller effect than the larger and more dynamic rearrangements expected for the helicase domain.

Since the aim of this study was to improve our understanding of suitable characteristics of inhibitors and aid the design of next generation HCV drugs, we focused on the mechanistic and kinetic details of the interaction between the inhibitors and the full-length NS3 protein from different genotypes. By correlation of this with the structural and mechanistic features of the inhibitors, certain interpretations could be made. The compounds selected for the study included NS3 protease inhibitors in the clinic or that have reached clinical trials. N-1725 was included as a reference, since it represents an analogue of the prototypic linear N-terminal cleavage product. It is large and has a long extended interaction interface with the enzyme, while the other two linear compounds, VX-950 and SCH 503034, are rather small tetrapeptides and gain their potency from the formation of a covalent bond (see below).

The affinities for the interaction between N-1725 and the protease from all genotypes were weak. This might be associated with the size of this inhibitor and also from the fact that it is very substrate-like and therefore not optimized for high affinity binding. There are little data in the literature for this inhibitor, but we have recently performed extensive analysis of its inhibition characteristics under different conditions.[21] The inhibition data correlate well with the interaction kinetic data except for the inhibition of NS3 from genotype 3a, for which the inhibition was lower than for the other genotypes. The same pattern was not seen in the interaction data.

The tripeptides BILN-2061 and ITMN-191 have a shorter peptidic chain than the linear compounds, and they all contain a large macrocyclic structure, which is thought to rigidify the compound and stabilize the inhibitor−protease complex. BILN-2061 also has a large P_2 substituent, which has been found to be important for its inhibitory potency. Although BILN-2061, ITMN-191, VX-950, and SCH 503034 are structurally different and exhibit different mechanisms of action, they share a similar core, corresponding to the P_1 and P_2 side chains. The data showed that the macrocyclic compounds had faster association and slower dissociation kinetics than the linear compounds, indicating that rigidifying the compounds may be advantageous from an interaction kinetic perspective. Although ITMN-191 is not mechanism-based, it was also stabilized by a second step, apparently because of an induced fit mechanism.

BILN-2061 is a potent inhibitor in both enzyme activity-based and replicon-based assays.[26] It has also been found to be equally potent against the 1a and 1b genotypes, although with reduced effect against genotype 3.[34] This correlates with the current interaction data that showed that the interactions were more efficient with the genotypes 1a and 1b protease, although the compound had relatively high affinity for protease from all three genotypes. A critical difference was a relatively fast dissociation (k_{-1}) for the 3a variant, suggesting that the compound is unable to form a stable complex, although it has a capacity to interact with the active site. This may be associated with the rigid macrocyclic structure and the loss of flexibility and may be structurally rationalized by a comparison of crystal structures of BILN-2061 in complex with NS3 from genotypes 1 and 3, which has previously indicated that different protein residues are involved in the interactions.[35]

The current data suggest that ITMN-191 is more flexible in its structure in comparison to BILN-2061, since it interacted with equal affinities to all three genotypes. In fact, it had the highest affinities of all compounds for the enzyme from all three genotypes. Although the interaction with $NS3_{fl}^{3a}$ exhibited different kinetics, it still resulted in a high affinity interaction. This was unexpected, since we have recently shown that this inhibitor has a significantly reduced inhibition of genotype 3a NS3.[21] ITMN-191 appears to be rather resilient to changes in the active site of the target protein, since it was also unaffected by the absence/presence of the NS4A cofactor. ITMN-191 interacted with low affinity with the truncated protein, which is not very surprising because the helicase has proven to be of importance for inhibitor binding. The lowered affinity, which is a result from a reduced association rate and an increased dissociation rate, might be due to structural arrangements that are usually provided by the helicase domain.

VX-950 and SCH 503034 represent a new generation of mechanism-based HCV NS3 protease inhibitors, as they carry ketoamide groups expanding into the prime side of the protease. These groups are highly reactive and bind covalently but reversibly to the protease catalytic serine. However, the kinetic advantage of this mechanism has not been fully established and may vary with the structure of the inhibitor scaffold, the hydration state of the ketoamide, and the genotype, since they are very dependent on the detailed architecture of the active site. We have earlier explored mechanism-based inhibitors of HCV NS3 protease and found that the electrophilic group provided less of an advantage than expected, possibly because the reactive group may not be properly positioned in the active site and binding of these inhibitors is more a result of noncovalent interactions rather than the expected covalent interaction.[36]

VX-950 and SCH 503034 did not have very high affinities for any of the genotypes, as we and others have found previously.[21,37] This is rather surprising for compounds in the clinic and despite the expected advantage of a mechanism-based inhibition. However, it is in accordance with previous studies that suggest that the mechanism for both inhibitors involves a formation of a noncovalent complex followed by an isomerization where the biggest contribution to their potency is their long half-life.[28,38] The poor kinetics may be attributed to a smaller and more flexible structural scaffold than the macrocyclic inhibitors.

The analysis indicates that VX-950 is kinetically suboptimal and does not benefit as much as SCH 503034 from the supposed formation of a covalent bond. For SCH 503034 the covalent mechanism was essential, as it had very slow association and fast dissociation kinetics, and the stabilization

Table 2. Description of the Constructs and Genotypes of the Studied NS3 Protease Variants

protein	description
$NS3_{fl}^{1a}$	full length NS3, genotype 1a[39]
$NS3_{fl}/NS4A^{1a}$	full-length NS3 coexpressed with full length NS4A, genotype 1a[21]
$NS3_{pd}^{1a}$	protease domain of NS3, genotype 1a[21]
$NS3_{fl}^{1b}$	full length NS3, genotype 1b[40]
$NS3_{fl}/NS4A^{1b}$	full-length NS3 coexpressed with full length NS4A, genotype 1b[41]
$NS3_{pd}^{1b}$	protease domain of NS3, genotype 1b[21]
$NS3_{fl}^{3a}$	full length NS3, genotype 3a[21]

of the complex via a covalent bond was both significant and critical. Optimization of SCH 503034 would thus benefit more on the design of analogues with faster association and slower dissociation rates.

CONCLUSIONS

The current study has highlighted the importance of resolving inhibitor interaction kinetics in the evaluation of NS3 protease inhibitors and that the choice of model system (e.g., constructs with/without the helicase domain or NS4A) can have a large effect when assessing the characteristics and potency of a compound. Comparative studies of inhibitors clearly need to be performed with model systems that are well matched. The use of NS3 from genotypes 1a, 1b, and 3a for the analysis was important, since it showed that these inhibitors varied in their potential to interfere with the replication of HCV from different genotypes.

Although quantifying functional inhibitory effects of compounds by conventional enzyme activity analysis is useful, the indirect determination of K_i values makes them more uncertain than directly determined K_D values, especially for complex interactions and mechanism-based inhibitors. They may therefore be misleading for structure−activity relationship analysis and potency ranking. The current kinetic data have therefore been important for exposing characteristics of NS3 protease inhibitors that are of relevance for the design of lead compounds that are more efficient and less sensitive to genetic variations in NS3. In particular, features such as compound flexibility and mechanism-based inhibition have been exposed. The two drugs in the clinic that target HCV NS3 are clearly suboptimal in the sense that their association rates are relatively slow and the dissociation rates fast. Their affinity is to a large extent dependent on their mechanism-based mode-of-action. It is reasonable to assume that a new generation of more potent inhibitors that combine the favorable characteristics of the macrocyclic and the mechanism-based inhibitors could be designed.

EXPERIMENTAL SECTION

Enzymes and Inhibitors. Expression and purification of the different genotypes and variants of NS3 were performed as described elsewhere.[21] The paper also provides the sequences and the exact constructs used, as well as their kinetic properties and the effects of the inhibitors studied here. The different variants of NS3 are listed in Table 2.

BILN-2061 was obtained from Boehringer-Ingelheim, VX-950 from the VIRGIL DrugPharm Team and SCH 503034 from Medivir (Huddinge, Sweden). ITMN-191was synthesized according to published procedures (International Publication Number WO 2005/037214 A2, compound AR00334191), and N-1725 was purchased from Bachem (Bubendorf, Switzerland). The inhibitors are illustrated in Figure 1.

Immobilization of NS3 Proteins. Interaction experiments were performed using a Biacore S51 biosensor (GE Healthcare, Uppsala, Sweden) which is based on SPR technology. Proteins were immobilized to CM5 or CM7 (for $NS3_{fl}^{1a}$ and $NS3_{fl}/NS4A^{1a}$) series S sensor chips (GE Healthcare, Uppsala, Sweden) using standard amine coupling, as previously described.[20] In short, the sensor surface was first activated with 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Sigma-Aldrich Sweden AB, Stockholm, Sweden) and 0.1 M *N*-hydroxysuccinimide (NHS) (Sigma, Stockholm, Sweden) followed by a 10 min injection (2 μL/min) of protein. Unreacted dextran was then deactivated with injections of 20 mM Tris-HCl (Sigma-Aldrich Sweden AB, Stockholm, Sweden), pH 7.4. Just prior to immobilization the proteins were subjected to buffer change (50 mM MES (Sigma-Aldrich Sweden AB, Stockholm, Sweden), pH 6.0, 0.1% *n*-octyl-β-D-glucopyranoside (OGP) (Anatrace, Maumee, OH, USA) or 0.1% Triton X-100 (Merck KgaA, Darmstadt, Germany) (used for $NS3_{fl}/NS4A^{1a}$ and $NS3_{fl}/NS4A^{1b}$, respectively) and 4 mM ditiothreitol (DTT) (Sigma-Aldrich Sweden AB, Stockholm, Sweden)) using Pierce columns (Fischer Scientific Inc., Rockford, IL, USA). Protein concentrations used for immobilization were in the range of 30−150 μg/mL. As running buffer 50 mM HEPES (Sigma-Aldrich Sweden AB, Stockholm, Sweden), pH 7.4, 0.1% OGP or 0.1% Triton X-100 (used for $NS3_{fl}/NS4A^{1a}$ and $NS3_{fl}/NS4A^{1b}$) and 2 mM DTT were used.

Interaction Kinetic Analysis. All interaction experiments were performed at 25 °C in the same buffer as for immobilization but with the addition of 5% (v/v) dimethyl sulfoxide (DMSO). The flow rate was 30 μL/min in all experiments. All inhibitors were in stock solutions of 10 mM in 100% DMSO except for N-1725 that was kept in a 2 mM stock solution. Inhibitors were prepared in 4-fold concentration series ranging from 10 μM to 2.5 nM. These were further injected over the immobilized surface for 120 s (300 s for $NS3_{fl}^{1b}$ and $NS3_{fl}/4A^{1b}$) followed by a dissociation of 500 s. A single concentration of ITMN-191 was used as positive control. No regeneration was used because of the poor stability of the protein under nonideal conditions. All experiments were done in triplicates.

Data analysis was performed using the Biacore T100 evaluation software 2.0, the Biacore S51 evaluation software 2.1, and the BIAevaluation Software (all three from GE Healthcare). All interactions were reference-subtracted using unmodified dextran as a reference surface. Signals were also blank-subtracted using blank injections of running buffer, and a DMSO correction curve was used to correct for solvent disturbances.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HCV, hepatitis C virus; IFN, interferon; OGP, *n*-octyl β-D-glucopyranoside; NHS, *N*-hydroxysuccinimide; RU, resonance units; SPR, surface plasmon resonance; SVR, sustained virological response

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