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Discovery of substituted *N*-phenylbenzenesulphonamides as a novel class of non-nucleoside hepatitis C virus polymerase inhibitors

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ABSTRACT

The RNA-dependent RNA polymerase NS5B of the hepatitis C virus (HCV) has emerged as one of the key targets for antiviral drug discovery. Here we describe a novel non-nucleoside inhibitor (NNI) chemotype identified by screening: The substituted *N*-phenylbenzenesulphonamides (SPBS) which showed reversible inhibition of NS5B from HCV genotype 1b with IC_{50} values up to 40 nM. Based on the decreased inhibitory activity against a recombinant NS5B protein carrying the mutation L419M or M423T we assumed that the SPBS inhibitors bind to the thumb site II which has already been described as the allosteric binding site for the NNI carboxy thiophene. The postulated binding site was consequently confirmed by solving two co-crystal structures of NS5B in complex with SPBS analogues at 2.3 and 2.2 Å resolutions. The inhibitors are hydrogen-bonded to the main chain Ser476 and Tyr477 and to the side chain of Arg501. In addition, the inhibitors displayed van der Waals interactions with several residues of the hydrophobic binding pocket Leu419, Ile482, Leu497, Met423 and Trp528. Notably, the two SPBS analogues reported here revealed significant differences in addressing the NH-group of the main chain Tyr477 by hydrogen-bonds, water-mediated or directly, which provoked a shift of the carboxyphenyl group of the inhibitors towards the His475 position for the water-mediated binding mode. Interestingly, the differences observed in the binding mode led to a different cross resistance profile at positions M423 and I482. Using a panel of 38 individual NS5B proteins derived from different HCV genotypes, we could demonstrate inhibitory activity of the SPBS against polymerases from HCV genotypes 1a and 1b whereas the inhibitor class failed to inhibit any of the non-genotype 1 polymerases efficiently. Furthermore we demonstrated initial antiviral activity for SPBS against the subgenomic replicons of HCV genotypes 1a and 1b, respectively, and no considerable cytotoxic potential against a panel of ten different cell types.

1. Introduction

Three percent of the world's population is infected with the hepatitis C virus (HCV), an enveloped 9.6 kb positive-sense single-stranded RNA virus belonging to the *Flaviviridae* family. In 70% of cases the infection becomes chronic with the risk of developing liver cirrhosis and hepatocellular carcinoma (Hoofnagle, 2002). The standard treatment of HCV infection, a combination of pegylated interferon alpha (pegIFN-α) and ribavirin (RBV), is associated with severe side effects and the response rate is unfortunately low for individuals infected with genotype 1, the most prevalent strain in North America, Europe and Asia. Therefore a high unmet medical need exists for the development of safer and more effective drugs. Direct-acting antivirals (DAA), currently investigated in clinical trials for the so called specifically targeted antiviral therapy of HCV (STAT-C), mainly focus on three molecular targets: the NS3/4A serine protease, the NS5A phosphoprotein, and the NS5B polymerase. Fortunately, very recently the first two DAAs (Telaprevir and Boceprevir) have been approved by the US Food and Drug Administration for the treatment of patients infected with HCV. NS5B, an RNA-dependent RNA polymerase, is structurally organised in a typical 'right hand' polymerase shape with finger, palm, and thumb subdomains surrounding a completely encircled active site (Bressanelli et al., 1999; Lesburg et al., 1999). The RNA-dependent RNA polymerase activity with no counterpart activity in humans is strictly essential for viral replication (Behrens et al., 1996; Kolykhalov et al., 2000) which makes NS5B an attractive antiviral drug target. HCV polymerase inhibitors can be classified into two major categories, the nucleoside analogue

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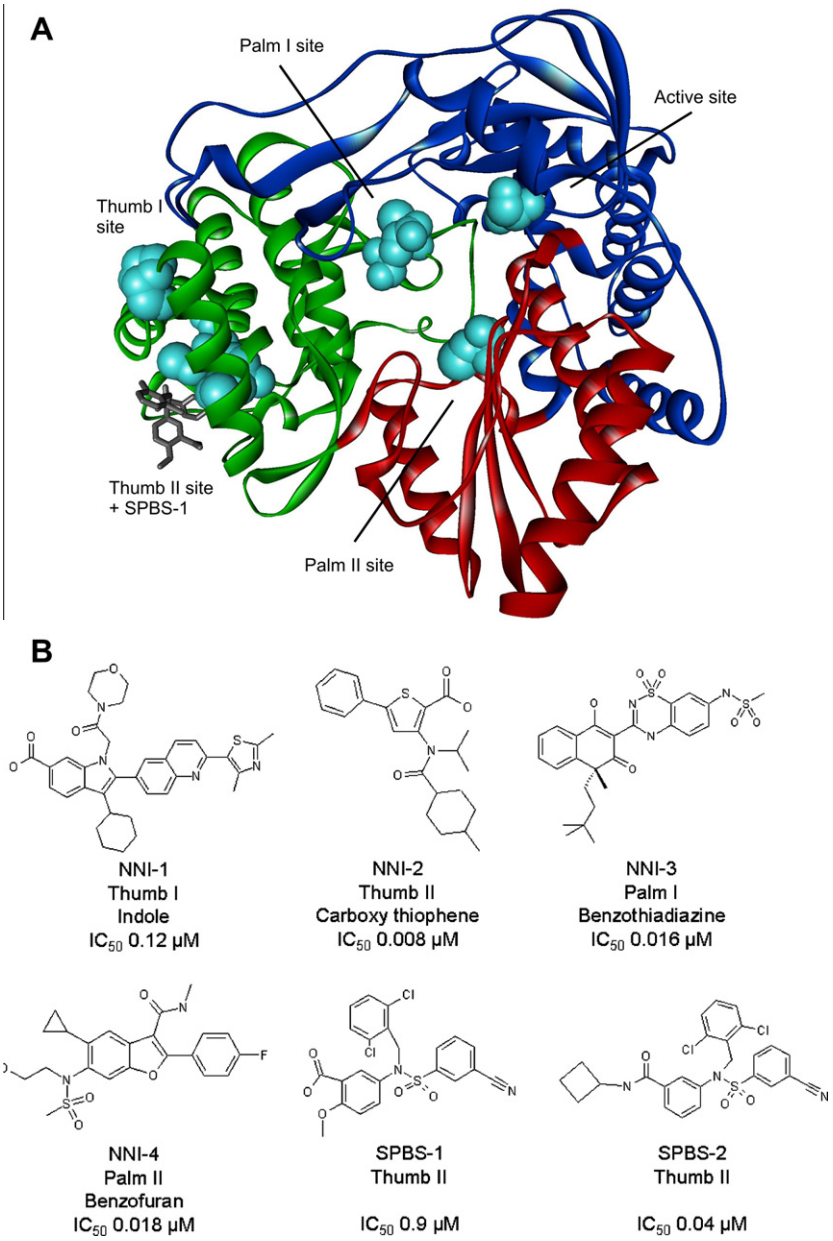

Fig. 1. 3D structure of HCV polymerase NS5B and structural formula of NNIs used in this study. (A) Palm, finger and thumb subdomain of NS5B are coloured in red, blue and green, respectively. Residues P495 (thumb site I), L419 and M423 (thumb site II), Q446 and C451 (palm site I), C316 (palm site I) and S282 (active site) associated in NNI or NI resistance are shown as light blue spheres. SPBS-1 bound to thumb site II is shown in grey. (B) For the NNI reference inhibitors (Bosse et al., 2008; Botyanszki et al., 2006; Chan et al., 2004b; Kneteman et al., 2009) and SPBS analogues designated as SPBS-1 and SPBS-2 the respective binding site is given below the structure. IC_{50} values have been determined in biochemical primer-dependent transcription assay on HCV genotype 1b polymerase (GeneBank CAB10747).

inhibitors (NIs) which act as chain terminators and bind to the active site and the non-nucleoside inhibitors (NNIs) which bind to one of at least four different allosteric binding sites outside the active site. In the last years several NNI scaffolds have been described targeting one of these binding pockets, namely the indoles which bind to the thumb site I also known as thumb-finger site or NNI-1 site (Botyanszki et al., 2006), the thiophenes which bind to the thumb site II (NNI-2 site) Wang et al., 2003, the benzothiadiazines which bind to the palm site I (NNI-3 site) (Bosse et al., 2008) and finally the benzofurans which bind to the palm site II (NNI-4) Howe et al., 2008 (Fig. 1).

It is anticipated that the emergence of viral resistance is likely to limit the use of NNIs (and all other DAAs) as monotherapy and that the future HCV therapy must consist of the combination of two or more DAAs which inhibit different viral targets or have different mode of actions (Sarrazin and Zeuzem, 2010). Following this logic, NS5B has the great advantage of multiple inhibitor binding sites, and due to the non-overlapping resistance profiles of NS5B inhibitors targeting different binding sites, it might be possible to add more than one of them in the combination regime. It is thus still important to discover new NNIs with different binding modes and different resistance profiles even though some promising NS5B inhibitors are currently undergoing clinical investigations (Franciscus, 2011).

In this report, we describe the SPBS as novel class of HCV polymerase NNIs. Biochemical studies using the primer-dependent transcription assay, crystallisation of NS5B-inhibitor complexes, and the subgenomic HCV replicon system were used in this study

to investigate the kinetic behaviour, the binding mode, the cross resistance profile to known NNI reference inhibitors, the genotype coverage, and the antiviral activity of the novel NNI scaffold.

2. Material and methods

2.1. Non-nucleoside inhibitors

The non-nucleoside polymerase inhibitors NNI-1 (Indole Botyanszki et al., 2006), NNI-3 (Benzothiadiazine Bosse et al., 2008) and NNI-4 (Benzofurane Kneteman et al., 2009), which were used as reference compounds, were synthesized at Shanghai Medicion Inc. (Shanghai, China) according to published procedures. The SPBS (Urban et al., 2007) as well as the reference inhibitor NNI-2 (Carboxy thiophene Chan et al., 2004b), were synthesized at the Chemistry department at Bayer Pharma AG (Wuppertal) according to published procedures.

2.2. NS5B sequences and site-directed mutagenesis

As described elsewhere, all NS5B sequences used in this study were cloned into a modified pET21b(+) plasmid (Novagen, Madison, US) to obtain NS5B proteins with C-terminal 21 amino acid deletions and His6-tags (May et al., 2011). High-throughput screening and protein–ligand co-crystallisations were performed using NS5B sequence CAB10747 (GeneBank) Lohmann et al., 1997. Mutations for binding site identification or cross-resistance analysis were introduced into the NS5B sequence CAB46913 (GeneBank) Lohmann et al., 1999 using the QuickChange® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and the respective oligonucleotide primers listed in the supplementary material (Table A.1). To improve the NS5B expression yield, all sequences derived from CAB46913 (GeneBank) have been additionally modified by an insertion of a small DNA fragment upstream the NS5B sequence according to (Ivanov et al., 2006). To increase the expression yield of NS5B proteins derived from non-CAB46913 sequences, the sequences were optimised using prediction software and changed to the preferred codon usage for *Escherichia coli* before they were synthesized at GENEART (Regensburg, Germany) and cloned into the modified pET21b(+) plasmid.

2.3. NS5B expression and purification

E. coli Rosetta 2 (DE3) (Novagen, Madison, US) were transformed with expression vectors containing the respective NS5B sequence. To perform the RdRp assay cell growth, protein expression and purification via nickel-affinity chromatography was performed as described before (May et al., 2011). To obtain NS5B in the highest purity suitable for protein–ligand co-crystallisations, transformed Rosetta 2 (DE3) cells were grown as described elsewhere (May et al., 2011), spun down, frozen-thawed using liquid nitrogen, and resuspended in lysis buffer (50 mM K_2HPO_4/ KH_2PO_4 pH 7.2 (71.7% dibasic and 28.3% monobasic), 500 mM NaCl, 5 mM 2-mercaptoethanol, 20 mM imidazole, 10% glycerol, one tablet per 50 mL Complete EDTA-free protease inhibitor (Roche, Mannheim, Germany), 1 mg/mL lysozyme). After incubation on ice for 45 min, cells were disrupted by sonication. Cell debris was subsequently removed by high speed centrifugation. Cleared cell lysate was incubated with 5 mL Ni–NTA beads QIAGEN (Hilden, Germany) for 90 min, 4 °C, on a rocking-shaker. Beads were transferred in a column and packed by gravity flow. Protein was eluted by linear gradient using an Äkta Explorer chromatography system and elution buffer (50 mM Tris/HCl pH 7.2, 50 mM K_2HPO_4/ KH_2PO_4 (71.7% dibasic and 28.3% monobasic) pH 7.2, 500 mM NaCl, 5 mM 2-mercaptoethanol, 500 mM imidazole, 10% glycerol). Fractions containing NS5B were pooled and dialysed overnight against 1 M glycine pH 7.0, 250 mM NaCl, 2 mM DTT). The dialysate was subjected to a ResourceS column (GE Healthcare Technologies, Uppsala, Sweden) and eluted by linear gradient using buffer A (1 M glycine pH 7.0, 2 mM EDTA, 2 mM DTT) and buffer B (1 M glycine pH 7.0, 1 M NaCl, 2 mM EDTA, 2 mM DTT). Protein containing fractions were pooled and concentrated before gel filtration was performed with a Superdex200 column (GE Healthcare Technologies, Uppsala, Sweden) and a buffer containing 10 mM Tris pH 7.5, 300 mM NaCl and 2 mM DTT to separate aggregates and exchange the buffer.

Table 1
Atomic coordinates data collection, crystallographic, and refinement statistics.

	SPBS-1	SPBS-2
Wavelength (Å)	1.5418	1.5418
Resolution (Å)	77.15–2.3	77.62–2.22
Completeness (%)[a]	94.8 (94.8)	99.7 (96.4)
I/σ[a]	10.4 (2.1)	18.0 (4.6)
R_{sym} (%)	9.2 (49.6)	8.6 (33.9)
Space group	$P3_221$	$P3_221$
Unit cell parameters (Å) *a*	89.02	89.72
B	89.02	89.72
C	186.17	186.84
Monomers per ASU[b]	1	1
R_{cryst} (%)	19.9	18.8
R_{free} (%)	23.8	21.9
Wilson B factor (Å^2)	32.0	17.9
r.m.s.d. bond length (Å)	0.008	0.010
r.m.s.d. bond angles (°)	1.063	1.303

[a] (highest resolution bin).
[b] asymmetric unit.

2.4. Co-crystallisation of protein ligand complexes

Crystals were obtained by vapour diffusion using the hanging drop technique. Usually 2 μL mother liquor was mixed with 2 μL protein–inhibitor mixture (1 mM final inhibitor concentration, 2% DMSO). The protein–inhibitor mix was incubated on ice for 15 min and centrifuged at 13000*g*, 4 °C for 10 min. Clear supernatant was solely used for crystallisation. Crystals appeared after 2 days and continued growing for weeks. Crystals of NS5B-SPBS-1 and NS5B-SPBS-2 grew under the following conditions: 2 M Na/K tartrate, 100 mM Tris pH 7, 200 mM Li_2SO_4; 2 M Na/K phosphate, 100 mM Na-Acetate pH 4.5; 2 M Na/K phosphate, 100 mM imidazole pH 8, 200 mM NaCl. Crystals of space group $P3_221$ were measured with a Rigaku MicroMax-007 equipped with a Marresearch mar345 image plate detector. Data were indexed and integrated with MOSFLM (Leslie and Powell, 2007) and scaled with SCALA (Evans, 2006). Molecular replacement using PDB structure 1YVF as search model was performed with AMoRe (Trapani and Navaza., 2008) and PHASER (McCoy et al., 2007). The inhibitors were placed into the F_{obs}–F_{calc} electron density maps followed by real space refinement using the computer graphics program COOT (Emsley et al., 2010). Iterative rounds of model building using COOT and maximum-likelihood refinement using REFMAC5.2 (Murshudov et al., 1997) completed the model. Data collection and refinement statistics are summarised in Table 1. Ligand interactions were analysed in Schrödinger Suite 2011 (Maestro 9.2) (see Supplementary Material, Fig. A1).

2.5. RNA-dependent RNA polymerase assay

RNA polymerase activity was determined using a primer-dependent transcription assay as described elsewhere (Adachi et al., 2002; May et al., 2011) and were performed in 384 well microtiter plates. For NS5B polymerases derived from HCV geno-

type 1b the enzymatic reaction mixtures contained 80 nM of purified protein, 20 mM Tris–HCl pH 7.5, 75 mM potassium glutamate, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 0.01% bovine serum albumin, 100 U/mL RNasin, 6 μg/mL poly(A) template, 90 nM oligo U_{12} primer, 5 μM UTP and 16 μCi/mL [^{3}H]-UTP in a final reaction volume of 60 μL. Reactions were initiated by the addition of enzyme and incubated for 2 h at 30 °C and stopped by the addition of 0.1 M EDTA and 100 μg of streptavidin coated beads per reaction well. Plates were incubated for 60 min at room temperature and analysed by Microbeta TriLux 1450 LSC & Luminiscence Counter (Perkin Elmer, Rodgau, Germany). For genotypic profiling experiments a panel of 38 individual NS5B sequences from genotypes 1a (14 sequences), 1b (9 sequences), 2a (3 sequences), 3a (5 sequences), 4a (1 sequence), 5a (1 sequence), and 6a (5 sequences) were used. As published recently, the assay conditions were adjusted regarding the $MgCl_2$ concentration, pH and reaction temperature to non-genotype 1b polymerases (May et al., 2011). All experiments were performed at least three times in duplicates and the IC_{50}s were determined by sigmoidal dose–response curves with variable hill slope using non-linear regression in GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA). The fold change (FC) in susceptibility was calculated by dividing the IC_{50} of the tested NS5B variant by the IC_{50} of the respective wild type NS5B.

2.6. Testing for reversible NS5B inhibition

To distinguish between a reversible or irreversible inhibition, the recovery of polymerase activity after a rapid 100-fold dilution of the NS5B-inhibitor complex was measured (Copeland, 2005). After a 30 min pre-incubation of 8 mM NS5B with 0.39 μM SPBS-2 (10-fold the IC_{50}) at 30 °C, the mixture was diluted 100-fold in reaction buffer containing the template, the primer and the substrate to start the reaction as described before. The enzyme incubated without inhibitor or incubated with SPBS-2 in 10-fold, 1-fold and 0.1-fold IC_{50} concentrations were used as controls without any pre-incubation and without any pre-dilution.

2.7. Anti-HCV assay

To determine the antiviral activity of the inhibitors, stable HCV replicon cell lines were used. Huh5–2 cells (Lohmann et al., 1999), SG-Neo (L + I) cells Blight et al., 2003, and pSGR-JFH1 cells (Kato et al., 2003) were seeded at a density of 6×10^3 cells per well in a 96-well plate in completed DMEM (Invitrogen) supplemented with 10% heat-inactivated foetal calf serum (Biochrom), 1× MEM non-essential amino acids solution (Gibco) and 250 μg/ml G418 at 37 °C and 8.5% CO_2. Compounds were tested at serial dilutions with appropriate compound-free DMSO controls. After 3 d total RNA was isolated via "RNeasy Mini Kit" (QIAGEN, Hilden, Germany) to determine the HCV RNA copy numbers by real-time PCR detection using LightCycler® 480, Roche Applied Science, Mannheim, Germany. Used primers for all cell types: Forward primer 5′-AATGCCTGGAGATTTGGGC-3′; Reverse primer 5′-TTTCGCGACCCAACACTACTC-3′. Probe (2–5′UTR-p): 6-Carboxyfluorescin-TGCCCCCGCGAGACTGCTAGC-*N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine. All experiments were performed at least in quadruplicates and the EC_{50}s were determined by sigmoidal dose–response curves with variable hill slope using non-linear regression in GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

2.8. Cytotoxicity

In vitro cytotoxic activity of SPBS was tested on a panel of 10 cell lines from different species and different organs namely the J774A.1 (ATCC No. TIB-67), HepG2 (ATCC No. HB-8065), BALB/3T3 clone A31 (ATCC No. CCL-163), N18TG2 (Department of Toxicology, Bayer Schering Pharma AG, Wuppertal, Germany), H9c2 (ATCC No. CRL-1446), NRK-52E (ATCC No. CRL-1571), THP-1 (ATCC No. TIB-202), H9 (ATCC No. HTB-176), NHDF(Clonetics San Diego, CA, USA) and Huh-7 (Reblikon GmbH, Schriesheim, Germany). Cells were cultivated under conditions described on the ATCC product sheets or in DMEM (PAN), 10% FCS (Biochrom), 1% Na-pyruvate (Invitrogen), 1% L-glutamine (PAN), 1% Pen/Strep (Invitrogen) for N18TG2 cells, and in DMEM (PAN), 10% FCS (Biochrom), 1% L-glutamine (PAN), 1% Pen/Strep (Invitrogen), 1% NEAA (Invitrogen) for Huh-7 cells, and MEM (Invitrogen), 10% FCS (Biochrom), 1% L-glutamine (PAN), 1% Pen/Strep (Invitrogen) for NHDF cells, respectively. Serial dilutions with appropriate compound-free DMSO and cell-free medium controls were performed as mentioned above for the replicon assay in 96-well transparent microtiter plates. After 72 h of incubation at 37 °C and 5% CO_2 a final concentration of 10% alamarBlue (BioSource International, Camarillo, CA,) was added per well. Ensuing an additional incubation period of 4–8 h the fluorescence was measured with a Tecan SpectraFlour Plus with an excitation wavelength of 550 nm, emitting a wavelength of 595 nm. CC_{50} values of viability were calculated by the use of Workflow 3.0 (Quattro Research GmbH, Martinsried, Germany). After transformation and normalisation of data points, a dose response curve with variable slope was calculated. CC_{50} values are expressed as arithmetic mean of a double determination.

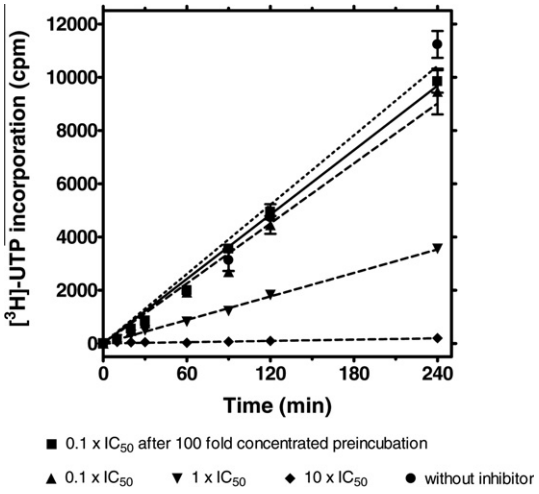

Fig. 2. Reversible inhibition mode of SPBS inhibitors. After 30 min pre-incubation of NS5B with SPBS-2 at a concentration of 10-fold IC_{50} the primer dependent transcription assay was started with a 100-fold dilution while adding the substrate (filled squares and solid line). A control reaction with SPBS in the same final concentration of 0.1 × IC_{50} was performed without any pre-incubation (triangles and dashed line). The control without inhibitor is represented by filled circles and a dotted line. Additional controls are SPBS-2 in a concentration of 10 × IC_{50} (diamonds and dashed line) and 1 × IC_{50} (downward triangles and dashed line).

3. Results

3.1. Testing for reversible NS5B inhibition

By screening a library of more than one million compounds in a biochemical target assay, several scaffolds came up as potential NS5B inhibitors. One of the most promising chemotypes, the substituted *N*-benzyl-*N*-phenylbenzene-sulphonamides (SPBS), has been investigated in this study. The screening hit revealed an IC_{50} of 900 nM in the biochemical target assay which could be improved to 40 nM for one of the most potent analogues (Fig. 1 B). The reversibility of NS5B inhibition was analysed by measuring

Table 2
Binding site identification via comparison of the SPBS resistance profile to those of four published reference compounds on selected mutations. The change in the IC_{50} is given as fold change compared to the IC_{50} of the wild type enzyme (GeneBank CAB46913). A decrease in inhibitory activity by a factor of 20 or higher is highlighted in bold writing.

	NNI-1	NNI-2		NNI-3		NNI-4	
	P495L	M423T	L419 M	C451R	Q446E	C316Y	C316F
SPBS-1	6	10	**31**	0.1	1	4	12
SPBS-2	1	**37**	6	3	3	1	1
Indole (NNI-1)	**82**	1	1	1	1	1	2
Thiophene (NNI-2)	7	**42**	**48**	12	13	1	2
Benzothiadiazine (NNI-3)	2	1	1	**47**	**29**	14	**20**
Benozofuran (NNI-4)	2	1	1	7	11	**86**	**>100**

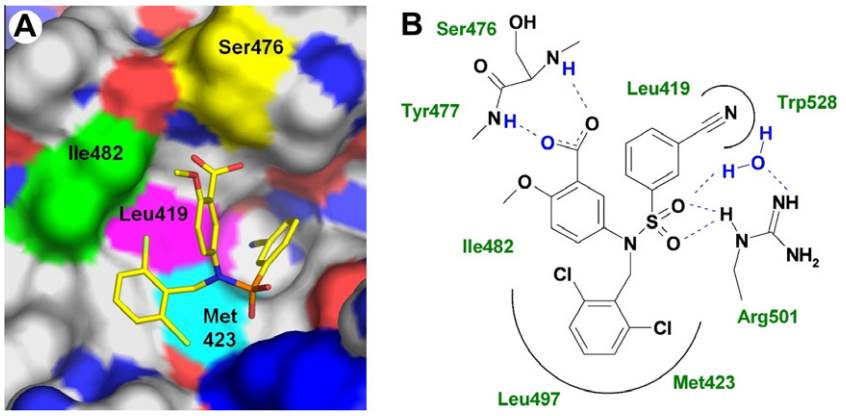

Fig. 3. X-ray structure (A) and binding mode (B) of the SPBS-1 within the thumb II pocket of the HCV polymerase NS5B. Acidic and basic amino acid residues are coloured in red and blue, respectively. (A) Amino acid positions associated with NNI-2 resistance are highlighted in different colours. (B) Amino acids forming the binding pocket are highlighted in green while hydrogen bonds between inhibitor and participating amino acids are highlighted in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the recovery of polymerase activity after rapid 100-fold dilution of NS5B-inhibitor complexes according to the protocol of (Copeland, 2005). As illustrated in Fig. 2 SPBS-2 revealed similar linear progress curves, either after pre-incubation at a concentration of 10-fold IC_{50} and rapid dilution to the assay concentration of 0.1-fold IC_{50} or under standard conditions without any pre-treatment. So the SPBS analogue could be classified as a rapidly reversible inhibitor. This was also true for all tested reference compounds from Fig. 1 B (data not shown).

3.2. Binding site identification of SPBS inhibitors

To elucidate the binding site of the SPBS inhibitors by resistance profiling, at least one published mutation for each NNI binding site, known to confer resistance to the respective reference compound (Fig. 1), was introduced into the wild type NS5B gene via site-directed mutagenesis. The mutant proteins were expressed and purified as described in material and methods. The change in the IC_{50} value of the reference compounds seen on these modified polymerases was compared to the changes in IC_{50} values of the SPBS. The observed increase in IC_{50} values for the reference compounds occurred on the expected mutation as shown in Table 2 (given as fold change). The IC_{50} value of the indole inhibitor binding to NNI-1 site increased on the NNI-1 mutation P495L, whereas the carboxy thiophene as binding site NNI-2 representative showed increased IC_{50} values on the NNI-2 mutations M423T and L419M. The benzothiadiazine inhibitor demonstrated increased IC_{50} values on the NNI-3 mutations Q446E and C451R as expected. Using the benzofuran, the respective NNI-4 marker mutations C316F and C316Y could be confirmed by high fold changes. Interestingly, the NNI-3 inhibitor also showed a 20-fold increase in the IC_{50} value on the mutation C316F.

As the SPBS inhibitors revealed cross resistance only to the carboxy thiophene with a fold change of 31 for SPBS-1 using NS5B with mutation L419M and a fold change of 37 for SPBS-2 using NS5B with mutation M423T, respectively, we hypothesised that the SPBS target the binding site NNI-2 in the thumb domain.

3.3. Binding mode of the SPBS inhibitors

To confirm the predicted inhibitor binding site, X-ray crystal structures of SPBS-1 and SPBS-2 (Fig. 1 B) bound to NS5B were determined at 2.3 and 2.2 Å resolutions (Table 1). The binding pocket of the SPBS inhibitors is situated 35 Å from the active site near to the allosteric GTP site (Bressanelli et al., 2002) (Fig. 1 A) and was previously described as NNI-2 binding site (Biswal et al., 2006; Wang et al., 2003). The hydrophobic part of the pocket is formed mainly by the side chains of Arg422, Trp528, Met423, Leu419, Leu497 and Tyr477 and the main chain atoms of His475 and Leu474. The pocket is occupied by the cyanophenyl groups of SPBS-1 and SPBS-2 with extensive van der Waals interactions with residues Leu419, Arg422, His475 and Trp528 (Fig. 3). The carboxyl oxygens of SPBS-1 are hydrogen-bonded to the main chain amide nitrogen atoms of Ser476 and Tyr477. Other significant interactions via hydrogen-bonds are formed between the central sulfon oxygen atoms of the SPBS inhibitors and the guanidinium moiety of Arg501 (Fig. 3). Finally the 2,6-dichlorophenyl group is located above the hydrophobic protein surface with van der Waals interactions with residues Met423, Ile482 and Leu497. Notably, the two SPBS analogues reported here, revealed significant differences in the binding mode regarding the interaction with the main chain of Ser476 and Tyr477. While as mentioned above for SPBS-1 both oxygen atoms of the carboxyl group targeted the amid groups of Ser476 and Tyr477 directly via hydrogen bonds, only the Ser476

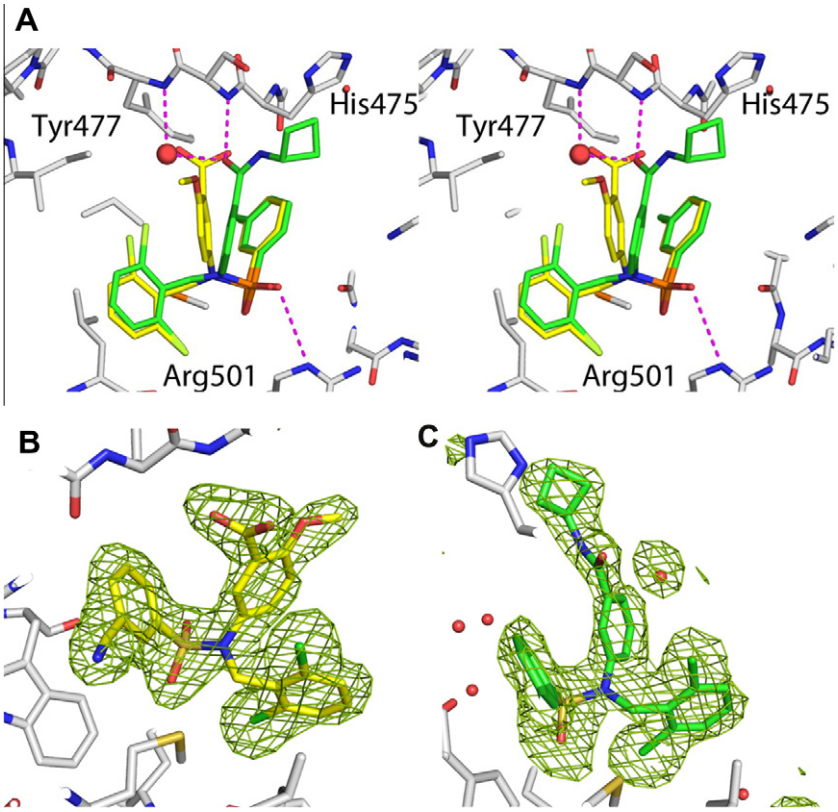

Fig. 4. Stereo view ball-and-stick diagram showing the different binding mode by superimposition of SPBS-1 (yellow) and SPBS-2 (green) within the thumb II pocket of the HCV polymerase (A). The backbone binding of SPBS-2 is mediated by a water molecule (red ball). Hydrogen bridge linkages are represented by the magenta broken lines. F_{obs}–F_{calc} omit maps (contour level 3 I/σ) of SPBS-1 (B) and SPBS-2 (C). Binding modes are unambiguously defined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Inhibitor cross-resistance on binding site 2 mutations – a comparison of the SPBS-1 and SPBS-2 with two reference NNI-2 inhibitors. Changes in the IC_{50} values were given as fold changes compared to those of the wild type enzyme (GeneBank CAB46913). A decrease in the inhibitory activity by a factor of 20 or higher is highlighted in bold writing.

Mutation	Reference	SPBS-1	SPBS-2	Carboxy thiophene	Hydroxydihydropyranone (Li et al., 2009)
L419I	Herlihy et al., 2008	7	2	7	0.5
L419M	De Francesco and Carfi, 2007; Koch and Narjes, 2007; Le Pogam et al., 2006	**23**	8	**59**	3
L419M + M423T	Le Pogam et al., 2006	**21**	**>100**	**>100**	**>100**
M423I	Le Pogam et al., 2006; Shi et al., 2008	3	**67**	16	**>100**
M423L	De Francesco and Carfi, 2007; Koch and Narjes, 2007	4	**>100**	0.4	1
M423V	De Francesco and Carfi, 2007; Koch and Narjes, 2007; Shi et al., 2008	1	**65**	**31**	**>100**
M423K	Malancona et al., 2010	8	**50**	**>100**	**>100**
M423T	Le Pogam et al., 2006; Shi et al., 2008	8	**50**	**52**	**>100**
M426T	Shi et al., 2008	3	3	2	9
S476A	Koch and Narjes, 2007	18	**>100**	7	1
I482L	Herlihy et al., 2008; Le Pogam et al., 2006	16	2	**56**	2
I482S	Shi et al., 2008	**25**	9	5	**34**
I482T	Shi et al., 2008	**22**	4	1	7
V494A	Shi et al., 2008	0.1	1	**21**	3

is hydrogen-bonded directly by the carbonyl group of SPBS-2. However, the Tyr477 is addressed water-mediated by the same carbonyl oxygen (Fig. 4) thus the unique water molecule involved in the binding mode of SPBS-2 mimics the missing second oxygen of the carboxyl group present in SPBS-1. In addition the water-mediated binding mode of SPBS-2 provokes a shift of the benzamide residue oxygen towards the His475 residue which main chain is now extra targeted by the cyclobutyl group. Further details about the binding mode of SPBS-1 and SPBS-2 are shown in ligand interaction diagrams in the supplementary material (Fig. A1).

3.4. Cross resistance within the NNI-2 binding site

To reveal inhibitor cross resistance within the NNI-2 binding site, the resistance profiles of the two SPBS inhibitors were compared to two published NNI-2 inhibitors, a carboxy thiophene and a hydroxydihydropyranone (Chan et al., 2004b; Li et al., 2009). For this purpose a panel of published resistance mutations, found in clinical trials or in the HCV replicon studies, was created via site-directed mutagenesis. The IC_{50}s were determined on each mutation in the primer-dependent transcription assay and listed

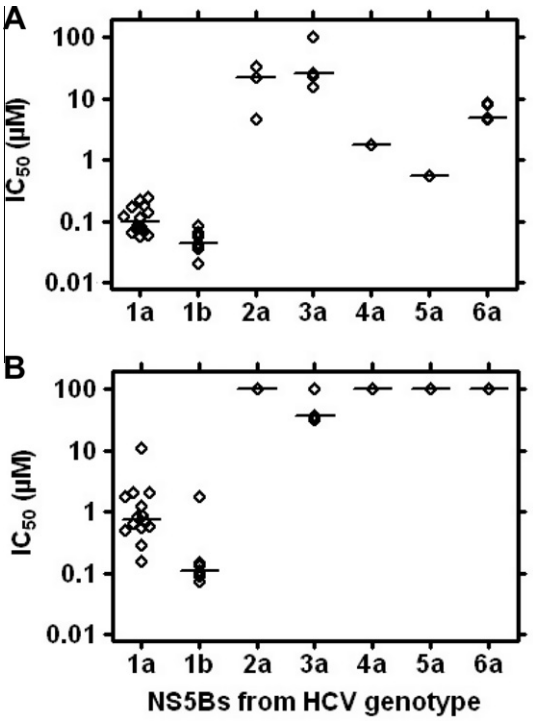

Fig. 5. Genotypic profiling of non-nucleoside NS5B inhibitors binding to the thumb II site, (A) carboxy thiophene, and (B) SPBS-2. Each diamond represents an IC_{50} value of the respective inhibitor against one individual NS5B sequence of the HCV genotype 1a (14), 1b (9), 2a (3), 3a (5), 4a (1), 5a (1) or 6a (5) (numbers of individual NS5B sequences for each genotype panel are given in brackets). Median IC_{50}s against each panel are highlighted as bars.

in Table 3 as fold change compared to the IC_{50}s against the wild type enzyme. We considered a compound with fold change above 20-fold to be inactive or weakly active. No cross resistance of the two SPBS analogues to the carboxy thiophene was observed on mutations in position V494. In position 419 SPBS-1 showed cross resistance to the carboxy thiophene at an exchange of Leu with Met, whereas SPBS-2 notably revealed only a moderate increase in fold change provoked by these mutation. Surprisingly, further differences in the resistance profile between the two SPBS analogues could be found for at least two additional positions. A substitution of Met423 resulted in a considerable fold change increase for SPBS-2 comparable to the carboxy thiophene and to the hydroxihydropyranone. In contrast, for SPBS-1 the inhibitory activity was maintained to a degree by any of the Met423 substitutions. On the other hand any Ile482 substitution was tolerated by SPBS-2, but provoked a considerable increase in fold change for SPBS-1. The impact of the position 482 on the inhibitory activity of the reference inhibitors strongly depended on the substituent. A M482L mutation decreased the inhibitory activity of the carboxy thiophene, whereas a Ser at this position led to an increase in fold change for the hydroxydihydropyranone. Finally SPBS-2 demonstrated only poor, SPBS-1 only moderate inhibitory activity on mutation S476A which was not observed for any of the reference inhibitors.

Table 5
Cytotoxic activity of SPBS inhibitors on a panel of ten different cell lines expressed as CC_{50}.

Cell line	Species, organ and cell type	SPBS-1 (μM)	SPBS-2 (μM)
THP1	Human monocytic leukemia	>33	>33
H9	Human cutaneous T lymphocyte	>33	>33
NHDF	Human dermal fibroblast	>33	29
HepG2	Human liver epithelial	28	33
Huh-7	Human hepatoma	25	26
J774A.1	Mouse monocyte macrophage	>33	>33
BALB/3T3.A31	Mouse embryo fibroblast	31	>33
N18TG2	Mouse neuroblastoma	>33	>33
H9C2	Rat heart muscle	>33	>33
NRK52E	Rat kidney epithelial	>33	>33

3.5. Genotypic profiling

To profile the cross-genotype efficacy of SPBS-2 in comparison to the carboxy thiophene inhibitor, IC_{50} values were determined using the primer-dependent transcription assay on a panel of 38 individual NS5B proteins derived from HCV sequences from the genotypes 1–6 (May et al., 2011). Good activities against genotype 1a and 1b were observed for both inhibitors (Fig. 5) with median IC_{50}s of 0.1 and 0.05 μM for the carboxy thiophene and 0.7 and 0.1 μM for SPBS-2 against the 1a and 1b polymerase panel, respectively. In contrast, both NNIs demonstrated only impaired activity on polymerases derived from non-1 genotypes, whereas SPBS-2 completely failed to inhibit polymerases from genotypes 2a, 4a, 5a, and 6a and the carboxy thiophene showed some minor activities on HCV 4a, 5a and 6a.

3.6. Antiviral activity and cytotoxicity of the SPBS in cell culture

To determine the antiviral activity of the SPBS inhibitors in cell culture the subgenomic replicon system from HCV genotype 1a and 1b was used. In these assays SPBS-1 showed initial activity on HCV 1a replicon cells with an EC_{50} of 0.97 μM and a selectivity index (SI) greater than 34 whereas for SPBS-2 an EC_{50} of 4.8 μM and a SI of 6.3 was measured. In contrast, on HCV 1b replicon cells SPBS-1 demonstrated equal antiviral activity as on genotype 1a with an EC_{50} of 1.3 μM (SI > 33) whereas SPBS-2 despite being highly active in the biochemical assay demonstrated very poor cellular activity with an EC_{50} of 28 μM (SI > 1.2) near to the detection limit of the replicon assay (Table 4). No cytotoxic effects were observed on the majority of the tested cell cultures as displayed in Table 5. Effects were observed for SPBS-1 on human liver epithelial cells (HepG2), human hepatoma cells (Huh7), and mouse embryo fibroblasts (BALB/3T3.A31) with CC_{50} values of 28, 25, and 31 μM, respectively. SPBS-2 displayed also CC_{50} values on HepG2 and Huh7 cells of 33 μM and 26 μM. An additional effect was observed for this compound on human dermal fibroblasts (NHDF) with a CC_{50} value of 29 μM.

4. Discussion

In the early days of DAA drug discovery the very first NS5B inhibitors have shown disappointing results in clinical trials and

Table 4
Antiviral EC_{50} data, cytotoxic CC_{50} data and selectivity index (SI) for SPBS-1 and SPBS-2 in replicon cells of HCV genotype (GT) 1a and 1b.

HCV replicon	GT	SPBS-1			SPBS-2		
Cell line		EC_{50} (μM)	CC_{50} (μM)	SI	EC_{50} (μM)	CC_{50} (μM)	SI
SG-Neo (L + I)	1a	0.97 ± 0.17	>33	>34	4.8 ± 0.75	30 ± 2.5	6.3
Huh5-2	1b	1.3 ± 0.23	>33	>25	28 ± 2.1	>33	>1.2

although there has been put a lot of pioneer work into this drug candidates they had to be discontinued (Meanwell and Koszalka, 2008). Nonetheless, today promising follow up candidates of the discontinued front runners or completely new encouraging NNI scaffolds are dominant among protease inhibitors and NIs in the HCV pipeline. It is very likely that the most perfect NNI compounds will pass through the trials and will become available in the coming years for patients suffering from chronic HCV infection (Franciscus, 2011). However, multiple options of NNI drugs targeting different NS5B binding sites and/or showing different resistance profiles might be desirable for the clinicians to find the best combination regime to treat HCV infection in the future. Therefore, there is still a medical need to discover novel NNIs. In this work the SPBS, identified by high-throughput screening in a biochemical NS5B assay, was reported as novel NNI-2 scaffold.

In a first cross-resistance analysis using a small panel of NS5B proteins with marker mutations of each NNI-binding site the SPBS inhibitors showed a similar resistance pattern to a NNI-2 reference inhibitor. But whereas the carboxy thiophene demonstrated increased IC_{50}s on both resistance mutations M423T and L419M, the SPBS-1 and SPBS-2 showed a drop in efficacy either on M423T or L419M. Except for a slight reduction in sensitivity to mutation C316F which is located in the palm II site we did not find significant cross resistance to NNI-1, NNI-3 or NNI-4 inhibitors. This fact led us to the hypothesis that SPBS analogues, just like carboxy thiophenes, bind in a reversible manner to the allosteric inhibitor binding site thumb II. However, there might be some differences in the binding mode of carboxy thiophenes and SPBS. To verify this hypothesis, a co-crystallisation of the HCV polymerase with the SPBS-1 compound was performed.

Due to the nature of the thumb site II a high lipophilicity and the presence of a carboxylic acid moiety are typical features for NNI-2 inhibitors namely the carboxy thiophenes (Biswal et al., 2005), the phenylalanine derivatives (Wang et al., 2003), and the pyranoindoles (Howe et al., 2006). In preference to the carboxylic acid group the dihydropyrones contain an enol/ketone oxygen functionality (Love et al., 2003). SPBS-1 also contains a carboxylic acid residue, which is directly hydrogen bonded to main chain Ser476 and Tyr477. This binding behaviour revealed by the co-crystallisation associates a resemblance to the carboxy thiophenes. In contrast, the cyclobutyl group of SPBS-2 targets His475 while the amide oxygen interacts with the main chain Ser476 via a direct H-bond and Tyr477 via a water molecule. The overall NS5B architecture undergoes minimal changes upon inhibitor binding, as it was previously described for the binding of thumb site II inhibitors phenylalanine derivatives and dihydropyrones, respectively (Love et al., 2003; Wang et al., 2003). However, due to side chain movements e.g. in positions Leu497 and Arg501, the architecture of the pocket itself varies among the bound ligands. By binding a dihydropyrone the pocket looks like a deep narrow cleft and Leu497 is part of the escarpment with a distance between the side chains of Ile482 and Leu497 of 8.2 Å at the narrowest point of the cleft (PDB ID: (2HAI Li et al., 2006)). A similar architecture with an Ile482 and Leu497 side chain distance of 9.5 Å was observed when a carboxy thiophene was bound to the pocket (PDB ID: 2D3Z (Biswal et al., 2006)). By contrast, the binding of SPBS-1 produces a flat wide pocket, since the side chain of Leu497 together with the Met423 side chain forms the bottom of the cleft under the 2,6-dichlorophenyl group of SPBS-1 and the side chain of Arg501 stands on the opposite side of the cleft with a distance to the Ile482 side chain of 12.7 Å. A similar shallow architecture of the thumb site II was found in the co-crystal structure of NS5B and a phenylalanine derivative with a distance of 12.4 Å between the side chains of Ile482 and Arg501 (PDB ID: 1NHU (Wang et al., 2003)).

Due to the fact that SPBS-1 showed consistent activity on all mutations in position M423 whereas SPBS-2 as well as the carboxy thiophene and the hydroxydihydropyranone demonstrated reduced activity on these mutations, the distinct binding modes of SPBS-1 and SPBS-2 observed in the co-crystal structures, seem to lead to distinct tolerances for mutations in position M423. The increase in IC_{50} values of the reference compounds on mutations in position M423 was previously described (Le Pogam et al., 2006; Shi et al., 2008). Interestingly, SPBS-2 decreased in activity by factor 50 or higher on all amino acid exchanges tested in position M423. Therefore the methionine in position 423 seems to play a crucial role in the formation of the binding pocket, as the co-crystallisation revealed no direct interaction between M423 and the inhibitor. The binding mode of SPBS-2 differs to the carboxy thiophenes and the hydroxydihydropyranones concerning the amino acid position S476. An exchange of serine with alanine results in a significant decrease in activity of SPBS-2 whereas SPBS-1 as well as both reference compounds remains active. This observation underlines the different behaviour of SPBS-1 and SPBS-2 among each other and compared to the reference compounds. In addition, resistance mutations in position V494 were reported to be associated with viral breakthrough during treatment with carboxy thiophenes (Cooper et al., 2009). In contrast to this observation which is in line with a high fold change in IC_{50} for the carboxy thiophene used in this study, the potency of both SPBS inhibitors has been maintained against a NS5B protein with a V494A mutation.

Due to incomplete sequence conservation within each of the four NNI binding sites among the HCV genotypes, several studies emphasise, that NNIs normally suffer from a narrow genotype coverage contrary to NIs which bind to the highly conserved active site and as a consequence they show sustainable activities over all genotypes (Gray et al., 2007; Kneteman et al., 2009; Le Pogam et al., 2008; Ludmerer et al., 2005; Pauwels et al., 2007; Shi et al., 2009). The benzofuran, a NNI-4 inhibitor class, is an exception of this rule by showing a high cross-genotype efficacy (Kneteman et al., 2009). For the SPBS-2 and also for the carboxy thiophene we observed stable inhibitory activities on genotype 1a and 1b polymerases. This was not surprising as screening for novel NNIs and respective hit-to-lead optimisation is normally done with genotype 1 polymerases. Interestingly, SPBS-2 showed both a higher diversity in the inhibitory activity and a higher IC_{50} median against genotype HCV 1a polymerases compared to HCV 1b polymerases. We found that differences in the amino acid sequence between 1a and 1b NS5B proteins used in this study (see supplementary material of reference (May et al., 2011) for sequence information) were located outside or in the rim of the thumb site II. Even though the mechanism of NNI-2 inhibition is not well understood there are hints that NNI-2 inhibitors may cause similar to NNI-1 inhibitors a perturbation of interdomain communication between the finger and the thumb domain (Biswal et al., 2005; Di Marco et al., 2005). It might be possible that even mutations or combinations of mutations away from the effective thumb site II could interfere with the inhibition process. By analysing the amino acid sequences of the 15 non-genotype 1 NS5B proteins, several well-known NNI-2 resistance mutations could be identified as possible reasons for the loss of SPBS-2 activity against non-genotype 1 polymerases. This may include that in all HCV 2a sequences, mutations in positions 419, 476 and 482 were recognised. We have shown in Table 3 that although Ser in position 476 is addressed by backbone binding a substitution with Ala for instance leads to a complete loss of SPBS-2 activity. In HCV 3a polymerases we found the same mutations L419I and I482L as in the HCV 2a polymerases. Albeit the cross-resistance analysis brought out that different from SPBS-1 and carboxy thiophene the single mutations alone had only a minor effect on the inhibitory activity of SPBS-1, it may well be that in combination these two mutations are more

effective in preventing inhibitor binding. In addition, the mutation A421V was found in HCV 3a polymerases in the deep part of the hydrophobic pocket close to the position L419. Further in terms of the thumb II binding site the same pre-existing drug resistance mutations as in polymerases of genotype 2a were detected for HCV 4a and HCV 6a polymerases. Finally the HCV 5a polymerases used in this study had an Ile instead of a Met in position 423 and may have in this way a reduced affinity to SPBS-2.

To our disappointment, most of the SPBS analogues showed poor cellular activity and unfortunately, even though the target potency of SPBS-2 was significantly improved compared to the screening hit, this progress did not provide any improvements in cellular activity. On the contrary, we observed a reduction in antiviral activity for SPBS-2. A possible general low passive cell permeability of the SPBS class may result in rather average antiviral activities which might be clarified by future caco-2 or PAMPA permeability studies. Differences in the physchem properties of SPBS-1 and SPBS-2 may influence their permeability and thereby influence their antiviral activities to varying degrees. In addition, similar discrepancies between the NNIs potency in target assays compared to cell-based replicon assays have been described before and possible reasons for this phenomenon have been discussed: The inhibitor binding affinities may be influenced to varying degrees by conformational differences existing between the NS5B in complex with a homopolymeric template/primer RNA in the biochemical assay and the NS5B in complex with the viral RNA and associated with several viral and cellular protein cofactors in the replicon assay (Chan et al., 2004a,b). Secondly, non-physiological enzyme concentration may general exceed the potency of some inhibitors in biochemical assays (Nyanguile et al., 2010). A sequence variation within the thumb site II between the two different NS5B sequences used in the biochemical assay and in the replicon cell could not be the reason for the unexpected low cellular activity of SPBS-2, since both NS5B sequences were derived from the same HCV con1 sequence.

In summary, we identified the SPBSs as novel class of thumb site II inhibitors of the HCV polymerase NS5B. Cross-resistance studies revealed both an overlapping resistance to known NNI-2 inhibitors and distinct differences for some positions. SPBS-1 and SPBS-2 by itself revealed differences in their susceptibility to mutations at position M423, which might be caused by the structural differences observed in their binding modes. SPBSs seem to inhibit only genotype 1 sequences sufficiently and the rather average cellular activity leaves room for improvement to the future work.

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Appendix A. Supplementary data

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