In silico identification, design and synthesis of novel piperazine-based antiviral agents targeting the hepatitis C virus helicase.

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Abstract

A structure-based virtual screening of commercial compounds was carried out on the HCV NS3 helicase structure, with the aim to identify novel inhibitors of HCV replication. Among a selection of 13 commercial structures, one compound was found to inhibit the subgenomic HCV replicon in the low micromolar range. Different series of new piperazine-based analogues were designed and synthesized, and among them, several novel structures exhibited antiviral activity in the HCV replicon assay. Some of the new compounds were also found to inhibit HCV NS3 helicase function in vitro, and one directly bound NS3 with a dissociation constant of 570 ± 270 nM.

Highlights

- Virtual screening studies on the HCV NS3 helicase.
- Identification of a substituted piperazine as new anti-HCV scaffold.
- Synthesis of different series of novel piperazine-based structures.
- New inhibitors of the subgenomic HCV replicon 1b genotype identified.
- Novel inhibitors of the HCV NS3 helicase discovered.

Key words
Structure-based virtual screening; HCV NS3-helicase; piperazine derivatives; anti-HCV activity; NS3 helicase inhibitors.

1. Introduction

The hepatitis C virus (HCV) is a major cause of chronic liver disease because it infects approximately 3\% of the global population [1]. HCV infection becomes chronic in 60-85\% of patients, who are at high risk of developing hepatic steatosis, fibrosis, cirrhosis and hepatocellular carcinoma [2, 3]. An HCV vaccine is currently not available, while the standard of care for many years was a combination of pegylated interferon (pegIFN) and ribavirin, a therapy that was not specific for HCV, was effective in

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only 50% of HCV patients, and was associated with many side effects [4]. However, new interferon-free combinations of direct-acting antivirals (DAAs) have revolutionized HCV prognosis and treatment.

HCV has a ~9,000 nt long single-stranded, positive-sense RNA genome, which encodes six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) essential for virus replication [5]. The new FDA approved DAAs are highly potent inhibitors of the NS3 protease [6, 7], the NS5B polymerase [8], and the NS5A protein [10]. Drugs still in development target other HCV proteins, like NS4B [9]. All-oral combination therapies with NS5B polymerase, NS3 protease and/or NS5A inhibitors are now the standard of HCV care, but they are associated with high costs [11, 12], and resistant mutants have been reported for each approved DAA [13-17]. Due to these limitations, new therapeutics that will reduce the costs of treatment and avoid the development of resistance are still needed.

In the search for novel inhibitors of HCV replication, a still underexploited target remains the HCV NS3 helicase, for which relatively few specific inhibitors have been reported so far, none reaching the clinical evaluation stage [18]. The HCV helicase is essential for HCV replication [19, 20], and is most likely needed for ATP-dependent unwinding of double-stranded RNA sequences formed during HCV replication [21, 22]. An HCV helicase inhibitor was also recently shown to enhance the efficacy of the latest generation of HCV protease inhibitors [23].

Due to its essential role and relative lack of selective inhibitors under development, the HCV NS3 helicase was chosen as a target for the in silico identification and synthesis of antivirals, through their potential interference with the known RNA binding cleft.

2 Results and discussion

2.1 Molecular modelling

The HCV NS3 helicase occupies the C-terminal portion of the NS3 protein and is formed by three domains, which define an ATP binding pocket in the cleft separating domain 1 from domain 2, and a single-stranded nucleic acid binding site at the interface of the three domains (Figure 1) [24].
As revealed in several crystal structures available for the enzyme in complex with ssDNA or ssRNA oligonucleotides [25, 26], the nucleic acid is bound within the closed conformation of the enzyme in a narrow central space defined by the three main domains (Figure 1). Due to the essential role demonstrated for Thr269, Arg393, Thr411 and Trp501 [27], computer-based studies were directed to identify compounds binding the region defined by these residues. In particular, the sub-site defined by Trp501 and Arg393 in the 3KQN crystal structure [25] was used for the virtual screening of the SPECS library of commercial compounds [28]. The approximately 450,000 structures available were analysed with MOE 2014.10 conformational search tool [29]; 500 low-energy conformations were kept for each input molecule. The main interactions between the target residues and the co-crystallised substrate were considered to build a pharmacophoric query, and the selection was restricted to five features (Figure 2).
The 3,500 molecules matching the search criteria were further analysed with a molecular docking procedure, using Glide in the standard precision SP mode [30]. The output poses were re-scored with the Glide extra precision scoring function XP [31], FlexX [32] and Plants ChemPLP [33] scoring functions. The rescoring results were combined with a consensus scoring procedure, in which for each scoring function a pose is considered a hit if ranked in the top 25% of the score value range for all the poses of the database. As a further analysis, the molecules matching this criterion for all three scoring functions were re-docked with Glide SP [30] in the RNA binding pocket of the 3KQH crystal structure, which corresponds to the high-affinity open conformation of the enzyme. A final selection of 13 derivatives was made after visual inspection of the docking results. These compounds were purchased and tested in the HCV replicon assay (Table S1). Among them, 1a (Figure 3) showed an interesting antiviral profile against HCV replication, with an EC$_{50}$ value in the low micromolar range (Table 1).
Figure 3: Structure and predicted binding mode of 1a in the 3KQH crystal structure.

The predicted binding mode found for 1a indicates good spatial occupation of the target site, with one aromatic ring in close proximity to Trp501, the piperazine linker filling the central space surrounding Asn556, and the opportunity of hydrogen-bond formation between one sulfonamido group and Arg393 and Thr411 backbone (Figure 3). Given its potential to inhibit HCV replication, a series of derivatives of 1a was designed and prepared for further investigations.

2.2 Chemistry

Compound 1a is characterised by a symmetrical structure, with a central piperazine ring and two aliphatic linkers connected by a sulfonamide group to two equal aromatic systems. The main modifications designed included different aromatic substitutions, linker chain variations and disruption of the molecule symmetry, as summarised in Figure 4.

Figure 4: Proposed modifications on the scaffold of 1a.

A first series of new derivatives was designed to explore the effect of the aromatic substituent on the
original structure, by varying the substitutions on the phenyl rings while keeping the central piperazine nucleus, maintaining the original three-carbon saturated linker or symmetrically shortening it to a two-carbon chain. Different commercially available aromatic sulfonyl chlorides (2a-s) and sulphonamide 5 were used to obtain piperazine derivatives 1a-u and 8a-h according to an optimised two-step synthetic pathway (Scheme 1). A further modification was designed to rigidify the three-carbon linker and study the importance of the positive charge/H-bond acceptors of the piperazine amine groups, inserting an amide group in correspondence of piperazine nitrogen atoms, using the scaffold of β-alanine (8) as new linker for a third series of symmetrical compounds 11a, d, f-h (Scheme 1).

Scheme 1: Reagents and conditions: a) i. Et$_3$N, an. DCM, 0 °C, 10 min; ii. NaH, DMF, rt, 1h, followed by 1,3-dibromopropane, 0 °C, 10 min; iii. piperazine, NaHCO$_3$, EtOH, reflux, 24h; iv. LiOH, THF, H$_2$O, 80 °C, on. b) i. NaOH, H$_2$O, 35 °C, 2h; ii. TBTU, HOBt, DiPEA, an. THF, rt, 4h.

The strategy to obtain amine compounds 1a-s and 8a-h began with the preparation of sulfonamides 6a-s and 7a-h, all obtained with a nucleophilic displacement of amino alkylbromides 3-4 and the different sulfonylchlorides. In order to avoid a self-reaction between two molecules of the nucleophile, the amine group of the alkyl bromides was slowly released by the dropwise addition of triethylamine at 0 °C. 6u was obtained by treating sulfonamide 5 with sodium hydride, followed by the addition of 1,3-dibromopropane. Final symmetrical products 1a-s, 1u and 8a-h were obtained through the displacement of the intermediate bromide leaving groups by piperazine, using NaHCO$_3$/ethanol as base/solvent system and refluxing the reaction mixture for 24 h. Compound 1t, with a carboxylic function on the aromatic rings, was obtained after basic hydrolysis of ester 1s. Symmetrical amide products 11a, d, f-h were obtained reacting the different sulfonyl chlorides with β-alanine 9, with the formation of carboxylic acid intermediates 10a-d, f-h, l, n, which were subsequently used for a coupling reaction with piperazine, using TBTU as coupling agent. With the purpose to further explore the role of the aliphatic linker, its elongation was also envisaged: compound 17 with a four-carbon linker was obtained after optimisation of a four-step synthetic pathway (Scheme 2).
Scheme 2: Reagents and conditions: (i) Et₃N, an. DCM, rt, 7h; (ii) Mesyl chloride, Et₃N, an. DCM, rt, 2h; (iii) Piperazine, NaHCO₃, EtOH, reflux, 24h; (iv) TFA, H₂O, rt, 30min, followed by 2a, Et₃N, an. DCM, 0 °C to rt, 1h.

The free amine in 4-aminobutanol 12 was first protected with BOC to give 14, and then the hydroxy function was converted to mesylate ester in 15. Once displaced the mesylate groups with piperazine (16), the two terminal amine groups were deprotected by hydrolysis of the carbamate ester with trifluoroacetic acid, thus obtaining an intermediate salt, which was precipitated from the reaction mixture and directly treated with an excess of 4-chloro-benzenesulfonyl chloride (2a) in basic conditions to give 17.

The role of the sulfonamide groups was also explored by replacement with amide functions in 20 (Scheme 3a), obtained by reacting acyl chloride 18 with alkyl bromide 3, and then treating intermediate 19 with piperazine at r.t. in THF. Moreover, an attempt to investigate the importance of the piperazine central nucleus was made by replacing it with a para-phenylenediamine group in 21, which maintains the overall length of the original scaffold (Scheme 3b).

Scheme 3: Reagents and conditions: a) i. Et₃N, an. DCM, 0 °C, 10 min; ii. Piperazine, Et₃N, an. THF, rt, 48 h; b) i. p-Phenylenediamine, Et₃N, an. THF, rt, 72 h.

Finally, the role of molecular symmetry for antiviral activity was also taken into consideration, and three small series of unsymmetrical compounds were designed and synthesised. All the compounds prepared
followed a rational approach guided by the biological results obtained for the previous series of structures. In particular, the presence of two aromatic sulfonamide groups was to be maintained, along with a \textit{para} hydrophobic substituent in at least one phenyl ring, the central piperazine nucleus and at least one three-carbon linker (\textbf{Scheme 4}). As a further confirmation of the importance of the central disubstituted piperazine, derivative \textbf{39}, in which only half of the original molecule is present, was prepared by reacting intermediate \textbf{6a} with piperidine \textbf{38}, as shown in \textbf{Scheme 4}.

\textbf{Scheme 4: Reagents and conditions:} a) i. Piperazine, NaHCO$_3$, EtOH, reflux, 24h; ii. 6b-c, l-n, NaHCO$_3$, EtOH, reflux, 24h. b) i. NaHCO$_3$, EtOH, reflux, 24h. c) i. TBTU, HOBt, DiPEA, an. THF, rt, 4h. d) i. NaHCO$_3$, EtOH, reflux, 24h.

A first series of unsymmetrical compounds \textbf{23-35} was designed to maintain the original scaffold while introducing different \textit{para} hydrophobic substituents in the two aromatic rings, combining together the most successful substitutions previously found (4-chloro, 4-methyl, 4-\textit{tert}-butyl and 4-trifluoromethyl), along with the unsubstituted phenyl moiety and the biphenyl one. To achieve this result, the two amine groups of piperazine had to be functionalised with two different alkyl bromides: first the pure monosubstitution products were isolated, and then these intermediates were reacted with the second alkyl bromide. A second small series of unsymmetrical derivatives \textbf{36a-b, l} was designed to maintain the same \textit{para} hydrophobic substituent in the two aromatic rings, while reducing the length of one linker from three to two methylene groups. Final products \textbf{36a-b, l} were obtained by reacting mono-substituted intermediates \textbf{22} with ethyl bromides \textbf{7a-b, l}. A third and final series of unsymmetrical structures \textbf{37a-c, l, n} was planned to functionalise one piperazine amine group to amide, thus partially rigidifying the scaffold, while keeping the overall length of the molecule and the same hydrophobic substituent in the \textit{para} position of the two aromatic rings. Mono-substituted intermediates \textbf{22a-c, l, n} were reacted with carboxylic acids \textbf{10a-c, l, n}, following a TBTU-assisted coupling reaction. Finally, half-molecule
derivative 39 was obtained by refluxing intermediate 6a with piperidine 38 in EtOH for 24 hours, using NaHCO₃ as base.

2.3 Biological activity

2.3.1 HCV replicon and cytostatic assay

All the newly synthesised compounds were evaluated for their potential antiviral activity in the Huh5-2 replicon system (Table 1) [34]. The HCV protease inhibitor telaprevir (VX-950) was included as positive control.

Table 1: Antiviral effect of the test compounds on hepatitis C virus replication in the Huh5-2 replicon system and inhibition of NS3 helicase unwinding activity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC₅₀(µM)a,d</th>
<th>EC₉₀(µM)b,d</th>
<th>CC₅₀(µM)c,d</th>
<th>SIe</th>
<th>Unwinding IC₅₀(µM)f</th>
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<td>&gt;92.5</td>
<td>&gt;92.5</td>
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<td>&gt;83.2</td>
<td>&gt;83.2</td>
<td>-</td>
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<td>-</td>
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<td>&gt;104</td>
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<td>&gt;97.5</td>
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<td>(VX-950)</td>
<td>0.8±0.2</td>
<td>-</td>
<td>47</td>
<td>58.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>Primuline</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10±2</td>
</tr>
<tr>
<td>Aurintricarboxylic Acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.3±0.1</td>
</tr>
</tbody>
</table>

*EC₅₀ = 50% effective concentration (concentration at which 50% inhibition of virus replication is observed).*

*EC₉₀ = 90% effective concentration (concentration at which 90% inhibition of virus replication is observed).*

*CC₅₀ = 50% cytostatic/cytotoxic concentration (concentration at which 50% adverse effect is observed on the host cell).*

*The EC₅₀, EC₉₀ and CC₅₀ values are the mean of at least 2 independent experiments, with standard deviations of ±10% of the value quoted unless otherwise stated (mean value ± standard deviations).*

*SI = the ratio of CC₅₀ to EC₅₀.*

*Concentration needed to reduce rates of helicase-catalysed DNA unwinding by 50%.*

n.d. Not determined

Considering the results obtained for 1a-t, most of the antiviral potential is associated with a hydrophobic substituent in the para position of the two aromatic rings, as can be observed for 1a, 1c, 1l and 1m. Moreover, moving the original 4-chloro group to position 3 (1i) or 2 (1u) is associated with activity reduction. The lowest EC₅₀ value is reached when a second phenyl group is placed in the position 4 of the two aromatics (1n), although this modification also shows an increased cytotoxic effect. Compounds 1e and 1h, with a para-nitrophenyl and 2-naphthyl aromatic moieties respectively, are associated with an increased toxic effect in comparison with the other analogues in this series, while the removal of the para-substituent or its replacement with methoxy groups leads to loss of activity (1b, d, f, g). Replacement of the original phenyl rings with heteroaromatic moieties in 1p-r leads as well to loss of activity, and the same effect can be observed for the substitution of the 2-naphthyl group (1h) with a 1-
naphthyl in 1o: even though cytotoxicity is reduced, any activity against the viral replication is lost. Trying to mimic the effect of the 4-nitro group of 1e, a 4-carboxylate function was introduced as aromatic substituent in 1t: with this last modification, both cytotoxicity and antiviral activity are abolished. A mild inhibition of the viral replication is observed for ethyl ester 1s, but its antiviral potential is reduced in comparison with 1a. Symmetrical shortening of the aliphatic linker in 8a-h is associated with loss of activity, possibly indicating an important role of the linker for the viral replication inhibition. An exception to this trend is represented by 8h and 8e, for which the presence of a 4-nitrophenyl and a 2-naphthyl rings, respectively, results in activity retention in comparison with their three-carbon counterparts, while the shortening of the linker appears to reduce the toxic effect found for 1h and 1e. Antiviral results for symmetrical amides 11a, d, f-h indicate that either the presence of the positive charge on the piperazine amine groups or the flexibility of the two linkers are important for the viral replication inhibition, since this modification is correlated with loss of activity for all seven derivatives prepared. A longer aliphatic linker of four methylene groups (17) is also associated with loss of activity, confirming that the length of the molecule plays an important role in HCV replication inhibition. Another feature that appears important is the presence of the two sulfonamide groups: their replacement with amide groups in the symmetrical scaffold of 20 is associated with a dramatic loss of activity. Biological results found for 21, where the 4-phenylendiamine central ring both rigidifies the structure and removes the positive charge of the linker compared to 1a, suggest an increased cytotoxicity, even if a low micromolar EC\textsubscript{50} value is retained. The loss of antiviral activity found for 39 indicates the importance of the overall length of the molecule, and taken together with the results found for 21 this evidence suggests that the presence of a di-substituted piperazine nucleus is essential for the antiviral activity of the novel scaffold. Antiviral data found for compounds 23-35 suggest that symmetry is not essential for antiviral activity, since the insertion of different \textit{para}-hydrophobic substituents in the two aromatic rings is tolerated: EC\textsubscript{50} values for most of these compounds are in the range of 1-10 \textmu M, suggesting activity retention as a general trend. Nevertheless, in some cases the small modification carried out seems to strongly and unexpectedly affect cytotoxicity, as revealed for 25 and 28-30. In the case of biphenyl products 27, 31, 33 and 34 this cytotoxic effect seems to be at least partially in line with the results obtained for 1n. In the case of unsymmetrical compounds 36a-b, 1, in which one aliphatic linker is shortened to two methylene groups, the small change in the molecular structure is associated with a retained antiviral effect, further confirming that symmetry is not required for antiviral activity. Nevertheless, with the exception of 36b, also in this second series of unsymmetrical derivatives the small modification is correlated with an increased cytotoxic effect. Finally, biological results for unsymmetrical compounds 37a-c, 1 and n, in which one piperazine nitrogen is functionalised to amide, suggest activity retention, with 37a, 37l and 37n showing EC\textsubscript{50} values in the low micromolar range, even though the toxic effect seems to be enhanced, particularly for 37l. Data found for 37b, which, as expected, does not show any antiviral activity, confirm the essential role played by a \textit{para}-hydrophobic aromatic group for the viral replication inhibition.

\textbf{2.3.2 HCV NS3 helicase enzymatic assays}
About half of the newly synthesised compounds were tested for their ability to inhibit HCV NS3 helicase-catalysed nucleic acid unwinding. Somewhat surprisingly, most compounds tested did not effect observed rates of DNA strand separation, even when supplied at concentrations as high as 1 mM (Table 1). Primuline [18] and aurintricarboxylic acid [35] were included as positive controls.

The most active compound, 31 (IC\textsubscript{50}=90±30 µM), was selected for additional analysis along with a compound with intermediate activity, 1l (IC\textsubscript{50}=310±190), and two compounds that did not inhibit helicase-catalysed DNA unwinding, 1a and 11a (Figure 5A). Each compound was tested for its ability to either dislodge HCV helicase from a bound oligonucleotide (Figure 5B) or prevent HCV helicase from hydrolysing ATP (Figure 5C). Only 31 was able to fully displace NS3h from an oligonucleotide at concentrations below 1 mM (Figure 5B). Both 31 and 11 inhibited ATP hydrolysis, but again 31 was a far more potent inhibitor in this assay (Figure 5C).

Interestingly, about 10 times less 31 was needed to inhibit NS3h-catalysed ATP hydrolysis than was needed to inhibit nucleic acid binding to the same extent. Since small molecules can inhibit helicase-catalysed ATP hydrolysis either by preventing ATP from binding or by preventing nucleic acids from stimulating ATP hydrolysis, we next tested the ability of 31 to inhibit ATP hydrolysis in the absence and presence of various concentrations of poly(U) RNA. In the absence of RNA, 31 did not inhibit NS3h-catalysed ATP hydrolysis even at concentrations as high as 1 mM (data not shown). However, 31 inhibited ATP hydrolysis in the presence of RNA in a manner in which 31 appeared to compete with RNA for a binding site on the enzyme (Figure 5D). Data best fit a kinetic model that assumes that 31 affects the apparent dissociation constant for RNA (K\textsubscript{RNA}), but does not influence the V\textsubscript{max}. However, unlike what is seen with classic competitive inhibitors, the observed K\textsubscript{RNA} values were not linearly dependent on the concentration of the inhibitor (i.e. 31). Instead, a plot of K\textsubscript{RNA} vs. 31 concentration reveals a sigmoidal relationship, indicative of a cooperative effect (Figure 5E).

To rule out the possibility that 31 might be acting by binding helicase substrates instead of NS3, we also performed direct binding assays by monitoring the effect of 31 on intrinsic protein fluorescence. Both NS3h and 31 fluoresce at 340 nm when excited at 280 nm. When each is alone, NS3h fluoresces about 70 times more brightly than the same amount of 31 at these wavelengths. However, when a solution of NS3h is titrated with 31, a new species forms that fluoresces twice as brightly as free NS3h, and 140 times brighter than free 31. Titration data fit a model in which a 1:1 complex forms with a dissociation constant of 0.57 µM. Importantly, this high affinity seems to best reflect the ability of 31 to inhibit replication of HCV replicons where the average observed EC\textsubscript{50} value was 1.3 µM.
Figure 5: Inhibition of HCV NS3 helicase activity for the newly synthesised piperazine derivatives. (A) Compounds selected for analysis. (B) The ability of each compound to displace NS3h from a DNA oligonucleotide. Numbers in parenthesis are IC$_{50}$ values with errors representing 95% confidence intervals seen in non-linear regression analysis of 2 independent titrations. (C) Ability of each compound to inhibit NS3 helicase-catalysed ATP hydrolysis. Numbers in parenthesis are as in (B). (C) Rates of ATP hydrolysis (nM ATP cleaved/s/nM NS3h) observed in the presence of various concentrations of poly(U) RNA and 31. Data are globally fit to Eq. 1 (Methods) assuming 31 does not affect V$_{max}$. (E) Amount of RNA needed to stimulate ATP hydrolysis to 50% maximum (K$_{RNA}$) observed at various concentrations of 31. (F) Fluorescence observed with various concentrations of 31 in the absence of NS3h (circles) or in the presence of 100 nM NS3h (squares). Data are globally fit to Eq. 2 (Methods) with the noted dissociation constant and an F$_{c}$ of 308, F$_{s}$ of 4.5. and F$_{c}$ of 641.

These results suggest that enzymatic in vitro reactions might not be the best assays to detect antivirals that target a helicase. The above observation that a compound that clearly binds directly to NS3h fails to inhibit the enzyme at concentrations where it should be fully bound suggests that assay conditions might prevent the enzyme-inhibitor interaction. For example, the helicase might form oligomers or higher ordered structures when bound to nucleic acids or ATP such that ligand binding is blocked. Alternatively, the rapid conformational changes that occur while the helicase acts as a molecular motor in these assays might dislodge a bound inhibitor. Based on our results with 31, it is likely that other compounds also bind NS3h with high affinity (examples: 1h, 1l-o, 1s-t, 8a, 8h, 17, 30-31, 34, 36, 37a, 37l, and 37n). However, testing for direct interaction between these other compounds and NS3h has been challenging because they do not possess the same optical properties as 31. We are therefore still exploring other possible assays to determine the affinity of other compounds generated in this project for the HCV helicase, many of which are associated with less cytotoxicity in the replicon assay than 31 (Table 1).

Based on the results obtained so far, some inhibition of the enzyme unwinding activity can be observed with a naphthyl aromatic ring in the symmetrical structure (1h, 1o, 8h), both with a three or two-carbon
linker, with a 4-carboxylic or 4-ethoxylate groups (1s-t), with a 4-chlorophenyl system and a two-carbon or four-carbon linker (8a and 17), and with the original three-carbon symmetrical scaffold and a 4-tert-butylphenyl, 4-CF₃-phenyl or a 4-biphenyl ring (11-n). The trend for the enzymatic activity does not seem to parallel the HCV replicon assay, since in this set of data the length of the linker does not play an essential role, even if the presence of a bulky hydrophobic substituent in the para position of the aromatic ring seems relevant for activity. Some ability to inhibit helicase-catalysed unwinding can also be observed in all three series of unsymmetrical structures, in particular with a 4-tert-butyl group in at least one aromatic (30-31, 36l, 37l), with at least one biphenyl moiety (31, 34, 37n), or with two equal 4-chlorophenyl substituents in the unsymmetrical amide scaffold (37a).

This data would suggest that the antiviral effect of 31, should be at least in part due to its interference with the NS3 helicase function, and that several commonly used helicase assays grossly underestimate the potency of this, and likely related, compounds. The antiviral effect of the newly prepared structures might alternatively result from the interference with an additional target, viral or cellular. Additional studies aiming to improve the antiviral activity and understand the mechanism of action of these compounds are ongoing and will be reported in due course.

3 Conclusions

The application of computer-aided techniques to the study of the HCV NS3 helicase led to the identification of one commercial piperazine-based analogue with an antiviral effect against HCV replication in the low micromolar range. Starting from its structure, different modifications were designed and carried out to explore the biological activity associated with the new scaffold. Several analogues of 1a inhibited the HCV subgenomic replicon replication. Different structural modifications were explored to understand the role of aromatic substituents, sulfonamide groups, linker chains, piperazine central nucleus and symmetry of the original molecule. Two equal phenyl rings with a hydrophobic substituent in the para position are essential for antiviral activity, along with the presence of the two sulfonamide groups and the central piperazine nucleus. The length and nature of the two linker chains is also important for activity retention: the presence of at least one three-carbon aliphatic linker is essential, while shortening or elongating the two linkers at the same time is associated with loss of activity. The same effect can be observed with the symmetrical functionalization of piperazine amine groups to amide, by inserting a carbonyl group in the terminal methylene of the two linkers. Replacement of the piperazine central ring with para-phenylenediamine is associated with loss of activity, and activity is lost as well when piperazine central nucleus is replaced with piperidine, maintaining only half of the original scaffold. The overall symmetry of the structure can tolerate small perturbations such as two different para hydrophobic aromatic substituents, or different linker chains. Unsymmetrical derivatives are in general associated with increased cytotoxicity in comparison with their symmetrical counterparts.

In vitro evaluations of some of the newly synthesised compounds show that different inhibit the NS3 helicase activity. In particular, compound 31 bound free helicase with a sub-micromolar dissociation constant, and it influenced the protein’s ability to bind nucleic acid substrates needed to stimulate ATP hydrolysis. Even if the antiviral effect of the new structures could be at least partially correlated with inhibition of the NS3 helicase, other viral or cellular targets could still be involved, and the toxicity of
suggests it might also act against related cellular motor proteins. Further exploration of both antiviral activity and biological targets of these compounds is the current focus of ongoing investigations.

4 Experimental

4.1 Materials and methods

All solvents used for chromatography were HPLC grade from Fisher Scientific (UK). $^1$H and $^{13}$C NMR spectra were recorded with a Bruker Avance DPX500 spectrometer operating at 500 and 125 MHz, with Me$_4$Si as internal standard. Mass spectra were determined with a Bruker microTOF spectrometer using electrospray ionization (ESI source). For mass spectra, solutions were made in HPLC grade methanol. Flash column chromatography was performed with silica gel 60 (230–400 mesh) (Merck) and TLC was carried out on precoated silica plates (kiesel gel 60 F$_{254}$, BDH). Compounds were visualised by illumination under UV light (254 nm). Melting points were determined on an electrothermal instrument and are uncorrected. All solvents were dried prior to use and stored over 4 Å molecular sieves, under nitrogen. All compounds were more than 95% pure.

Intermediates 6-7, 10, 13, 14, 19, 22 were prepared according to literature procedures, described in detail along with compound characterisation in the Supporting Information. Preparation and characterisation details on the new target compounds 1a-s, u, 8a-h, 11a, d, f-h, 17, 20, 21, 23-35, 37a-c, l, n are given below. $^1$H-NMR spectra of all final compounds are reported in the Supporting Information.

4.1.1 General method for the preparation of $N,N'$-(piperazine-1,4-diyl)bis(alkyl)dialkysulfonamides 1, 8

Piperazine (0.05 g, 0.6 mmol) and NaHCO$_3$ (0.11 g, 1.3 mmol) were suspended in absolute ethanol (9 mL). The different alkyl bromide 6 or 7 (1.3 mmol) was then added portionwise to the suspension and the reaction mixture was stirred under reflux for 24 h. The solvent was evaporated under reduced pressure and the crude residue was purified by flash column chromatography.

4.1.1.1 $N,N'$-(3,3'-(Piperazine-1,4-diyl)bis(propane-3,1-diyl))bis(4-chlorobenzene-sulfonamide) (1a)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 64% yield as a pale yellow solid. M.p. 168-170°C. TLC (9:1 DCM-MeOH, Rf: 0.61). $^1$H-NMR (DMSO-d$_6$), $\delta$: 1.48-1.52 (m, 4H), 2.18-2.26 (m, 12H), 2.73-2.78 (m, 4H), 7.69 (d, $J$ = 8.6 Hz, 4H), 7.71 (bs, 2H), 7.81 (d, $J$ = 8.6 Hz, 4H). $^{13}$C-NMR (DMSO-d$_6$), $\delta$: 26.0, 40.8, 52.5, 54.7, 128.4, 129.3, 137.1, 139.3. Anal. Caled for C$_{22}$H$_{30}$Cl$_2$N$_4$O$_4$S$_2$: C, 48.08; H, 5.50; N, 10.20. Found: C, 47.95; H, 5.11; N, 10.07. MS [ESI, m/z]: 549.0, 551.0 [M+H].

4.1.1.2 $N,N'$-(3,3'-(Piperazine-1,4-diyl)bis(propane-3,1-diyl))dibenzene-sulfonamide (1b)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 64% yield as a white solid. TLC (9:1 DCM-MeOH, Rf: 0.46). M.p. 136-138°C. $^1$H-NMR (CDCl$_3$), $\delta$: 1.60 (m, 4H), 2.38 (m, 12H), 3.04 (t, $J$ = 5.8 Hz, 4H), 7.10 (bs, 2H), 7.50
(m, 4H), 7.56 (m, 2H), 7.83 (m, 4H). $^{13}$C-NMR (CDCl$_3$), $\delta$: 24.0, 44.0, 53.0, 57.9, 126.8, 129.0, 132.6, 140.1. Anal. Calcd for C$_{22}$H$_{32}$N$_4$O$_4$S$_2$: C, 54.98; H, 6.71; N, 11.66. Found: C, 54.85; H, 6.99; N, 11.48. MS [ESI, m/z]: 481.1 [M+H].

4.1.1.3 $N,N'$-(3,3'-Piperazine-1,4-diyl)bis(propane-3,1-diyl)bis(4-methylbenzene-sulfonamide) (1c) [36]

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 95:5 v/v. Obtained in 68% yield as a white solid. M.p. 176-178°C (lit. 181-183°C) [33]. TLC (9:1 DCM-MeOH, Rf: 0.67). $^1$H-NMR (CDCl$_3$), $\delta$: 1.65 (m, 4H), 2.46 (bs, 18H), 3.07 (t, $J=5.6$ Hz, 4H), 7.12 (bs, 2H), 7.32 (d, $J=8.2$ Hz, 4H), 7.74 (d, $J=8.2$ Hz, 4H). $^{13}$C-NMR (DMSO-d$_6$), $\delta$: 20.9, 26.0, 40.9, 52.6, 54.8, 126.5, 129.5, 137.8, 142.4. Anal. Calcd for C$_{24}$H$_{36}$N$_4$O$_4$S$_2$: C, 56.67; H, 7.13; N, 11.01. Found: C, 56.89; H, 6.92; N, 11.14. MS [ESI, m/z]: 509.1 [M+H].

4.1.1.4 $N,N'$-(3,3'-Piperazine-1,4-diyl)bis(propane-3,1-diyl)bis(4-methoxybenzene-sulfonamide) (1d)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 91:9 v/v. Obtained in 60% yield as a white solid. M.p. 172-174°C. TLC (9:1 DCM-MeOH, Rf: 0.72). $^1$H-NMR (DMSO-d$_6$), $\delta$: 1.47 (m, 4H), 2.18 (bs, 12H), 2.72 (m, 4H), 3.83 (s, 6H), 7.11 (d, $J=8.3$ Hz, 4H), 7.41 (t, $J=5.6$ Hz, 2H), 7.70 (d, $J=8.3$ Hz, 4H). $^{13}$C-NMR (DMSO-d$_6$), $\delta$: 25.6, 39.7, 51.9, 54.3, 56.0, 114.4, 126.5, 131.8, 165.2. Anal. Calcd for C$_{24}$H$_{36}$N$_4$O$_6$S$_2$: C, 53.31; H, 6.71; N, 10.36. Found: C, 53.44; H, 6.92; N, 10.40. MS [ESI, m/z]: 541.1 [M+H].

4.1.1.5 $N,N'$-(3,3'-Piperazine-1,4-diyl)bis(propane-3,1-diyl)bis(4-nitrobenzene-sulfonamide) (1e)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 91:9 v/v. Obtained in 43% yield as a pale yellow solid. M.p. 226-228°C. TLC (9:1 DCM-MeOH, Rf: 0.59). $^1$H-NMR (DMSO-d$_6$), $\delta$: 1.49 (m, 4H), 2.18 (m, 12H), 2.82 (t, $J=6.8$ Hz, 4H), 7.98 (bs, 2H), 8.03 (d, $J=8.7$ Hz, 4H), 8.42 (d, $J=8.7$ Hz, 4H). $^{13}$C-NMR (DMSO-d$_6$), $\delta$: 26.1, 40.8, 52.5, 54.6, 124.5, 128.0, 146.1, 149.5. Anal. Calcd for C$_{22}$H$_{20}$N$_6$O$_8$: C, 46.31; H, 5.30; N, 14.73. Found: C, 45.99; H, 5.63; N, 14.66. MS [ESI, m/z]: 571.1 [M+H].

4.1.1.6 $N,N'$-(3,3'-Piperazine-1,4-diyl)bis(propane-3,1-diyl)bis(3,4-dimethoxybenzene-sulfonamide) (1f)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 95:5 v/v. Obtained in 65% yield as a yellow solid. M.p. 152-154 °C. TLC (9:1 DCM-MeOH, Rf: 0.70). $^1$H-NMR (CDCl$_3$), $\delta$: 1.66 (m, 4H), 2.47 (m, 12H), 3.06 (t, $J=5.7$ Hz, 4H), 3.93 (s, 6H), 3.96 (s, 6H), 6.53 (bs, 2H), 6.95 (d, $J=8.4$ Hz, 2H), 7.33 (d, $J=2.1$ Hz, 2H), 7.46 (dd, $J_1=8.4$ Hz, $J_2=2.1$ Hz, 2H). $^{13}$C-NMR (CDCl$_3$), $\delta$: 24.1, 44.0, 53.0, 56.1, 56.2, 57.7, 109.7, 110.5, 120.7, 131.9, 149.0, 152.3. Anal. Calcd for C$_{26}$H$_{40}$N$_4$O$_8$: C, 51.98; H, 6.71; N, 9.33. Found: C, 51.96; H, 6.94; N, 9.30. MS [ESI, m/z]: 601.1 [M+H].

4.1.1.7 $N,N'$-(3,3'-Piperazine-1,4-diyl)bis(propane-3,1-diyl)bis(2,5-dimethoxybenzene-sulfonamide)
Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 95:5 v/v. Obtained in 71% yield as a white solid. M.p. 170-172 °C. TLC (9:1 DCM-MeOH, Rf: 0.73). \(^1\)H-NMR (CDCl\(_3\)), \(\delta\): 1.71 (m, 4H), 2.55 (bs, 12H), 3.00 (t, \(J = 6.2\) Hz, 4H), 3.83 (s, 6H), 3.95 (s, 6H), 7.00 (d, \(J = 8.9\) Hz, 2H), 7.08 (dd, \(J = 8.9\) Hz, \(J = 3.1\) Hz, 2H), 7.47 (d, \(J = 3.1\), 2H). \(^1^3\)C-NMR (CDCl\(_3\)), \(\delta\): 25.3, 44.4, 52.5, 55.4, 56.0, 57.2, 114.1, 114.8, 121.0, 132.0, 150.4, 153.3. Anal. Calcd for C\(_{26}\)H\(_{40}\)N\(_4\)O\(_8\)S\(_2\): C, 51.98; H, 6.71; N, 9.33. Found: C, 51.80; H, 7.02; N, 9.29. MS [ESI, m/z]: 601.1 [M+H].

4.1.1.8 \(N,N'-(3,3'-(Piperazine-1,4-diyl))bis(propane-3,1-diyl))dinaphthalene-2-sulfonamide (1h)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 79% yield as a white solid. M.p. 186-188 °C. TLC (9:1 DCM-MeOH, Rf: 0.62). \(^1\)H-NMR (CDCl\(_3\)), \(\delta\): 1.66 (m, 4H), 2.46 (bs, 12H), 3.13 (t, \(J = 5.5\) Hz, 4H), 7.32 (bs, 2H), 7.66 (m, 4H), 7.83 (dd, \(J = 8.7\) Hz, \(J = 1.8\) Hz, 2H), 7.95 (d, \(J = 7.9\), 2H), 7.99 (m, 4H), 8.44 (s, 2H). \(^1^3\)C-NMR (DMSO-d\(_6\)), \(\delta\): 25.9, 40.8, 52.4, 54.7, 122.2, 127.3, 127.5, 127.7, 128.6, 129.1, 129.3, 131.7, 134.0, 137.4. Anal. Calcd for C\(_{30}\)H\(_{36}\)N\(_4\)O\(_4\)S\(_2\): C, 62.04; H, 6.25; N, 9.65. Found: C, 61.89; H, 5.97; N, 9.48. MS [ESI, m/z]: 581.1 [M+H].

4.1.1.9 \(N,N'-(3,3'-(Piperazine-1,4-diyl))bis(propane-3,1-diyl))bis(3-chlorobenzene-sulfonamide) \(1i\)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 79% yield as a white solid. M.p. 156-158 °C. TLC (9:1 DCM-MeOH, Rf: 0.66). \(^1\)H-NMR (DMSO-d\(_6\)), \(\delta\): 1.48 (m, 4H), 2.20 (m, 12H), 2.79 (m, 4H), 7.64 (t, \(J = 7.8\) Hz, 2H), 7.74 (m, 6H), 7.78 (t, \(J = 1.8\) Hz, 2H). \(^1^3\)C-NMR (DMSO-d\(_6\)), \(\delta\): 26.0, 40.8, 52.5, 54.7, 125.1, 126.0, 131.3, 132.2, 133.8, 142.4. Anal. Calcd for C\(_{22}\)H\(_{30}\)Cl\(_2\)N\(_4\)O\(_4\)S\(_2\): C, 48.08; H, 5.50; N, 10.20. Found: C, 47.90; H, 5.76; N, 10.08. MS [ESI, m/z]: 549.0, 551.0 [M+H].

4.1.1.10 \(N,N'-(3,3'-(Piperazine-1,4-diyl))bis(propane-3,1-diyl))bis(4-(tert-butylbenzene-sulfonamide) \(1l\)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 79% yield as a white solid. M.p. 188-190 °C. TLC (9:1 DCM-MeOH, Rf: 0.59). \(^1\)H-NMR (DMSO-d\(_6\)), \(\delta\): 1.31 (s, 18H), 1.46 (m, 4H), 2.16 (m, 12H), 2.75 (m, 4H), 7.49 (t, \(J = 5.8\) Hz, 2H), 7.60 (d, \(J = 8.4\) Hz, 4H), 7.70 (d, \(J = 8.4\) Hz, 4H). \(^1^3\)C-NMR (DMSO-d\(_6\)), \(\delta\): 26.0, 30.7, 34.7, 40.8, 52.5, 54.8, 125.9, 126.3, 137.6, 155.1. Anal. Calcd for C\(_{30}\)H\(_{48}\)N\(_4\)O\(_4\)S\(_2\): C, 60.78; H, 8.16; N, 9.45. Found: C, 60.96; H, 8.00; N, 9.51. MS [ESI, m/z]: 593.3 [M+H].

4.1.1.11 \(N,N'-(3,3'-(Piperazine-1,4-diyl))bis(propane-3,1-diyl))bis(4-(trifluoromethyl)-benzene sulfonamide) \(1m\)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 61% yield as a white solid. M.p. 184-186 °C. TLC (9:1 DCM-MeOH, Rf: 0.65). \(^1\)H-NMR (DMSO-d\(_6\)), \(\delta\): 1.48 (m, 4H), 2.16 (bm, 12H), 2.81 (m, 4H), 7.49 (bs, 2H), 7.99 (m, 8H). \(^1^3\)C-NMR (DMSO-d\(_6\)), \(\delta\): 26.0, 40.8, 52.5, 54.6, 123.6 (q, \(J = 272.7\) Hz), 126.4 (q, \(J = 3.7\) Hz), 127.4,
132.2 (q, J = 32.2 Hz), 144.5. Anal. Calcd for C_{24}H_{30}F_{6}N_{4}O_{4}S_{2}: C, 46.75; H, 4.90; N, 9.09. Found: C, 46.91; H, 5.11; N, 9.18. MS [ESI, m/z]: 617.1 [M+H].

4.1.1.12 N,N‘-(3,3’-(Piperazine-1,4-diyl))bis(propane-3,1-diyl))bis(biphenyl-sulfonamide) (I

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 87% yield as a white solid. M.p. 183-185 °C. TLC (9:1 DCM-MeOH, Rf: 0.59). \(^1\)H-NMR (DMSO-d_{6}), \(\delta\): 1.46-1.50 (m, 4H), 2.12-2.22 (m, 12H), 3.51-3.56 (m, 4H), 7.41-7.44 (m, 2H), 7.49-7.53 (m, 4H), 7.62 (bs, 2H), 7.71-7.75 (m, 4H), 7.84-7.89 (m, 8H). \(^{13}\)C-NMR (DMSO-d_{6}), \(\delta\): 26.0, 40.9, 52.5, 54.8, 127.0, 127.1, 127.3, 128.4, 129.0, 138.5, 139.2, 143.8. Anal. Calcd for C_{34}H_{40}N_{4}O_{4}S_{2}: C, 64.53; H, 6.37; N, 8.85. Found: C, 64.24; H, 6.51; N, 8.78. MS [ESI, m/z]: 633.2 [M+H].

4.1.1.13 N,N‘-(3,3’-(Piperazine-1,4-diyl))bis(propane-3,1-diyl))dinaphthalene-2-sulfonamide (I

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 91% yield as a white solid. M.p. 216-218 °C. TLC (9:1 DCM-MeOH, Rf: 0.67). \(^1\)H-NMR (DMSO-d_{6}), \(\delta\): 1.36 (m, 4H), 1.93 (bs, 8H), 1.99 (t, J = 6.8 Hz, 4H), 2.79 (m, 4H), 7.67 (m, 6H), 7.91 (bs, 2H), 8.10 (d, J = 8.2 Hz, 2H), 8.64 (d, J = 8.4 Hz, 2H). \(^{13}\)C-NMR (DMSO-d_{6}), \(\delta\): 25.8, 40.7, 52.3, 54.6, 124.4, 124.6, 126.7, 127.5, 127.7, 128.5, 128.9, 133.6, 133.8, 135.4. Anal. Calcd for C_{30}H_{36}N_{4}O_{4}S_{2}: C, 62.04; H, 6.25; N, 9.65. Found: C, 61.91; H, 6.33; N, 9.63. MS [ESI, m/z]: 581.2 [M+H].

4.1.1.14 N,N‘-(3,3’-(Piperazine-1,4-diyl))bis(propane-3,1-diyl))diquinoline-8-sulfonamide (I

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 76% yield as a white solid. M.p. 200-202 °C. TLC (CDCl_{3}), \(\delta\): 1.67 (m, 4H), 2.37 (bs, 12H), 2.95 (m, 4H), 6.79 (bs, 2H), 7.59 (dd, J = 8.4, J = 4.2, 2H), 7.68 (t, J = 7.6 Hz, 2H), 8.08 (d, J = 8.0 Hz, 2H), 8.31 (d, J = 8.0 Hz, 2H), 8.46 (d, J = 7.1 Hz, 2H), 9.04 (m, 2H). \(^{13}\)C-NMR (CDCl_{3}), \(\delta\): 26.1, 42.4, 52.9, 56.0, 122.2, 125.7, 128.8, 131.4, 133.1, 136.0, 136.9, 143.4, 151.1. Anal. Calcd for C_{28}H_{34}N_{6}O_{4}S_{2}: C, 57.71; H, 5.88; N, 14.42. Found: C, 57.55; H, 6.04; N, 14.34. MS [ESI, m/z]: 583.2 [M+H].

4.1.1.15 N,N‘-(3,3’-(Piperazine-1,4-diyl))bis(propane-3,1-diyl))dithiophene-2-sulfonamide (I

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 83% yield as a white solid. M.p. 138-140 °C. \(^1\)H-NMR (DMSO-d_{6}), \(\delta\): 1.52 (m, 4H), 2.24 (m, 12H), 2.86 (m, 4H), 7.18 (dd, J = 5.1, J = 3.7, 2H), 7.57 (dd, J = 3.7, J = 1.3, 2H), 7.79 (bs, 2H), 7.92 (dd, J = 5.1, J = 1.3, 2H). \(^{13}\)C-NMR (DMSO-d_{6}), \(\delta\): 25.8, 41.2, 52.6, 54.9, 127.6, 131.3, 132.2, 141.4. Anal. Calcd for C_{5}H_{8}N_{4}O_{4}S: C, 43.88; H, 5.73; N, 11.37. Found: C, 43.59; H, 5.96; N, 11.31. MS [ESI, m/z]: 493.0 [M+H].

4.1.1.16 N,N‘-(3,3’-(Piperazine-1,4-diyl))bis(propane-3,1-diyl))dipyridine-3-sulfonamide (I

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-
MeOH 91:9 v/v. Obtained in 63% yield as a pale yellow solid. M.p. 140-142 °C. TLC (9:1 DCM-MeOH, Rf: 0.52). \(^1^H\)-NMR (DMSO-\(d_6\), \(\delta\): 1.50 (m, 4H), 2.20 (bs, 12H), 2.82 (bs, 4H), 7.65 (dd, \(J_1 = 8.0\) Hz, \(J_2 = 4.9\) Hz, 2H), 7.85 (bs, 2H), 8.16 (dt, \(J_1 = 8.0\) Hz, \(J_2 = 1.9\) Hz, 2H), 8.82 (dd, \(J_1 = 4.9\) Hz, \(J_2 = 1.9\) Hz, 2H), 8.94 (d, \(J = 2.2\) Hz, 2H). \(^1^C\)-NMR (DMSO-\(d_6\), \(\delta\): 26.0, 40.8, 52.5, 54.6, 124.2, 134.4, 136.8, 146.9, 152.9. Anal. Calcd for C\(_{20}\)H\(_{30}\)N\(_6\)O\( _4\)S\( _2\): C, 49.77; H, 6.27; N, 17.41. Found: C, 49.55; H, 6.07; N, 17.23. MS [ESI, m/z]: 483.1 [M+H].

4.1.1.17 Ethyl 3-(N-(3-(4-(3-(4-carboxyphenylensulfonamido)propyl) piperazin-1-yl)propyl) sulfamoyl)benzoate (1s)
Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 41% yield as a white solid. M.p. 170-172 °C. TLC (9:1 DCM-MeOH, Rf: 0.56). \(^1^H\)-NMR (CDCl\( _3\), \(\delta\): 1.44 (t, \(J = 7.1\) Hz, 6H), 1.67 (m, 4H), 2.47 (m, 12H), 3.11 (t, \(J = 5.6\) Hz, 4H), 4.44 (q, \(J = 7.1\) Hz, 4H), 7.45 (bs, 2H), 7.92 (d, \(J = 8.5\) Hz, 4H), 8.19 (d, \(J = 8.5\) Hz, 4H). \(^1^C\)-NMR (CDCl\( _3\), \(\delta\): 14.2, 23.8, 44.3, 53.0, 57.9, 61.6, 126.8, 130.2, 133.9, 144.1, 165.2. Anal. Calcd for C\(_{28}\)H\(_{40}\)N\( _4\)O\( _8\)S\( _2\): C, 53.83; H, 6.45; N, 8.97. Found: C, 53.69; H, 6.71; N, 8.76. MS [ESI, m/z]: 625.2 [M+H].

4.1.1.18 N,N’-(3,3’-(Piperazine-1,4-diyl)bis(propane-3,1-diyl))bis(2-chlorobenzene-sulfonamide) (1u)
Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 98:2 v/v. Obtained in 80% yield as a white solid. M.p. 162-164 °C. TLC (9:1 DCM-MeOH, Rf: 0.79). \(^1^H\)-NMR (DMSO-\(d_6\), \(\delta\): 1.49 (m, 4H), 2.17 (m, 12H), 2.85 (m, 4H), 7.54 (td, \(J_1 = 7.4\) Hz, \(J_2 = 1.5\) Hz, 2H), 7.65 (m, 4H), 7.88 (t, \(J = 5.1\) Hz, 2H), 7.96 (dd, \(J_1 = 7.9\) Hz, \(J_2 = 1.3\) Hz, 2H). \(^1^C\)-NMR (DMSO-\(d_6\), \(\delta\): 25.7, 41.0, 52.5, 54.9, 127.6, 130.5, 130.5, 131.7, 133.9, 137.8. Anal. Calcd for C\(_{22}\)H\(_{30}\)Cl\( _2\)N\( _4\)O\( _4\)S\( _2\): C, 48.08; H, 5.50; N, 10.20. Found: C, 48.27; H, 5.34; N, 10.32. MS [ESI, m/z]: 549.1, 551.1 [M+H].

4.1.1.19 N,N’-(2,2’-(Piperazine-1,4-diyl)bis(ethane-2,1-diyl))bis(4-chlorobenzene sulfonamide) (8a)
Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 95:5 v/v. Obtained in 45% yield as a white solid. M.p. 218-220 °C. TLC (9:1 DCM-MeOH, Rf: 0.66). \(^1^H\)-NMR (CDCl\( _3\), \(\delta\): 2.17, (bs, 8H), 2.43 (t, \(J = 5.5\) Hz, 4H), 3.08 (m, 4H), 5.14 (bs, 2H), 7.51 (d, \(J = 8.5\) Hz, 4H), 7.82 (d, \(J = 8.5\) Hz, 4H). \(^1^C\)-NMR (DMSO-\(d_6\), \(\delta\): 40.9, 52.2, 54.9, 128.6, 129.7, 137.9, 139.8. Anal. Calcd for C\(_{20}\)H\(_{26}\)Cl\( _2\)N\( _4\)O\( _4\)S\( _2\): C, 53.83; H, 6.45; N, 10.20. Found: C, 54.6; H, 6.71; N, 10.87. MS [ESI, m/z]: 521.0, 523.0 [M+H].

4.1.1.20 N,N’-(2,2’-(Piperazine-1,4-diyl)bis(ethane-2,1-diyl))dibenzenesulfonamide (8b)
Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 53% yield as a white solid. M.p. 158-160 °C. TLC (9:1 DCM-MeOH, Rf: 0.40). \(^1^H\)-NMR (CDCl\( _3\), \(\delta\): 2.17, (s, 8H, H-3’), 2.39 (t, \(J = 5.8\) Hz, H-2’), 3.00 (m, 4H, H-1’), 5.13 (bs, 2H, NH), 7.53 (m, 4H, H-3), 7.60 (tt, \(J_1 = 7.3\) Hz, \(J_2 = 2.1\) Hz, 2H, H-4), 7.88 (m, 4H, H-2). \(^1^C\)-NMR (CDCl\( _3\), \(\delta\): 39.1, 52.2, 55.4, 127.0, 129.0, 132.6, 139.6. Anal. Calcd for C\(_{20}\)H\(_{28}\)N\( _4\)O\( _2\): C, 53.08; H, 6.24; N, 12.38. Found: C, 52.89; H, 6.53; N, 12.32. MS [ESI, m/z]: 453.1 [M+H].
4.1.1.21 \(N,N'(2,2'-(Piperazine-1,4-diyl)bis(ethane-2,1-diyl))bis(4-methylbenzene-sulfonamide)\) (8c) [37]

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 98:2 v/v. Obtained in 89% yield as a white solid. M.p. 182-184°C (lit. 185-187°C) [34]. TLC (9:1 DCM-MeOH, Rf: 0.59). \(^1\)H-NMR (CDCl\(_3\)), \(\delta\): 2.24, (bs, 8H), 2.39 (t, \(J = 5.7\), 4H), 2.45 (s, 6H), 2.98 (m, 4H), 5.10 (bs, 2H), 7.32 (d, \(J = 8.2\) Hz, 4H), 7.57 (d, \(J = 8.2\) Hz, 4H). \(^{13}\)C-NMR (DMSO-d\(_6\)), \(\delta\): 20.9, 40.0, 52.4, 56.7, 126.4, 129.5, 137.6, 142.4. Anal. Calcd for C\(_{22}\)H\(_{32}\)N\(_4\)O\(_4\)S\(_2\): C, 54.98; H, 6.71; N, 11.66. Found: C, 54.93; H, 6.95; N, 11.64. MS [ESI, m/z]: 481.1 [M+H].

4.1.1.22 \(N,N'(2,2'-(Piperazine-1,4-diyl)bis(ethane-2,1-diyl))bis(4-methoxy-benzenesulfonamide)\) (8d)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 87% yield as a white solid. M.p. 170-172 °C. TLC (9:1 DCM-MeOH, Rf: 0.45). \(^1\)H-NMR (DMSO-d\(_6\)), \(\delta\): 1.45 (t, \(J = 5.4\), 4H), 2.24, (bm, 8H), 2.71-2.75 (m, 4H), 3.89 (s, 6H), 7.09 (d, \(J = 8.8\) Hz, 4H), 7.41 (t, \(J = 5.5\) Hz, 2H), 7.81 (d, \(J = 8.8\) Hz, 4H). \(^{13}\)C-NMR (CDCl\(_3\)), \(\delta\): 39.1, 52.3, 55.4, 55.6, 114.1, 129.2, 131.1, 162.8. Anal. Calcd for C\(_{22}\)H\(_{32}\)N\(_4\)O\(_6\)S\(_2\): C, 51.54; H, 6.29; N, 10.93. Found: C, 51.39; H, 6.47; N, 10.84. MS [ESI, m/z]: 513.1 [M+H].

4.1.1.23 \(N,N'(2,2'-(Piperazine-1,4-diyl)bis(ethane-2,1-diyl))bis(4-nitrobenzene-sulfonamide)\) (8e)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 63% yield as a pale yellow solid. M.p. 212-214 °C. TLC (9:1 DCM-MeOH, Rf: 0.68). \(^1\)H-NMR (DMSO-d\(_6\)), \(\delta\): 2.18, (bs, 8H), 2.55 (t, \(J = 6.6\), 4H), 2.98 (t, \(J = 6.6\) Hz, 4H), 7.92 (bs, 2H), 8.05 (d, \(J = 8.8\) Hz, 4H), 8.40 (d, \(J = 8.8\) Hz, 4H). \(^{13}\)C-NMR (DMSO-d\(_6\)), \(\delta\): 40.0, 52.3, 56.8, 124.4, 127.9, 146.4, 149.4. Anal. Calcd for C\(_{20}\)H\(_{26}\)N\(_6\)O\(_8\)S\(_2\): C, 44.27; H, 4.83; N, 15.49. Found: C, 44.17; H, 4.66; N, 15.27. MS [ESI, m/z]: 543.0 [M+H].

4.1.1.24 \(N,N'(2,2'-(Piperazine-1,4-diyl)bis(ethane-2,1-diyl))bis(3,4-dimethoxy-benzenesulfonamide)\) (8f)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 74% yield as a white solid. M.p. 152-154 °C. TLC (9:1 DCM-MeOH, Rf: 0.68). \(^1\)H-NMR (CDCl\(_3\)), \(\delta\): 2.20, (bs, 8H), 2.39 (t, \(J = 5.7\), 4H), 2.45 (s, 6H), 2.98 (m, 4H), 5.10 (bs, 2H), 5.26 (t, \(J = 4.9\) Hz, 2H), 6.94 (m, 2H), 7.34 (m, 2H), 7.49 (m, 2H). \(^{13}\)C-NMR (CDCl\(_3\)), \(\delta\): 39.1, 52.3, 56.8, 124.4, 127.9, 146.4, 149.4. Anal. Calcd for C\(_{24}\)H\(_{36}\)N\(_4\)O\(_8\)S\(_2\): C, 50.33; H, 6.34; N, 9.78. Found: C, 50.19; H, 6.50; N, 9.62. MS [ESI, m/z]: 573.1 [M+H].

4.1.1.25 \(N,N'(2,2'-(Piperazine-1,4-diyl)bis(ethane-2,1-diyl))bis(2,5-dimethoxy-benzenesulfonamide)\) (8g)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 95:5 v/v. Obtained in 77% yield as a white solid. M.p. 176-178 °C. TLC (9:1 DCM-MeOH, Rf: 0.47). \(^1\)H-NMR (CDCl\(_3\)), \(\delta\): 2.31, (bs, 8H), 2.42 (t, \(J = 5.6\), 4H), 2.98 (m, 4H), 3.83 (s, 6H), 3.92 (s, 6H), 5.58 (t, \(J = 4.9\) Hz, 2H), 6.96 (d, \(J = 9.1\) Hz, 2H), 7.08 (dd, \(J_1=9.1\) Hz, \(J_2= 3.1\) Hz, 2H), 7.47 (d, \(J = 3.1\) Hz,
2H. $^1$H-NMR (CDCl$_3$), $\delta$: 39.8, 52.6, 56.0, 56.1, 57.1, 113.7, 114.8, 120.3, 127.8, 150.2, 153.4. Anal. Calcd for C$_{24}$H$_{36}$N$_4$O$_8$S$_2$: C, 50.33; H, 6.34; N, 9.78. Found: C, 50.26; H, 6.65; N, 9.76. MS [ESI, m/z]: 573.1 [M+H].

4.1.1.26 N,N'-(2,2'-(Piperazine-1,4-diyl)bis(ethane-2,1-diyl))dinaphthalene-2-sulfonamide (8h)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 82% yield as a white solid. M.p. 176-178 °C. TLC (9:1 DCM-MeOH, Rf: 0.56). $^1$H-NMR (CDCl$_3$), $\delta$: 2.18, (bs, 8H), 2.37 (t, $J$ = 5.8, 4H), 3.01 (m, 4H), 5.22 (bs, 2H), 7.66 (m, 4H), 7.82 (dd, $J_1$ = 8.8 Hz, $J_2$ = 1.8 Hz, 2H), 7.92 (d, $J$ = 8.1 Hz, 2H), 7.97 (m, 4H), 8.45 (s, 2H).

$^{13}$C-NMR (CDCl$_3$), $\delta$: 39.2, 52.3, 55.5, 122.2, 127.7, 127.9, 128.6, 128.9, 129.2, 129.4, 131.9, 134.7, 137.3. Anal. Calcd for C$_{28}$H$_{32}$N$_4$O$_4$S$_2$: C, 60.85; H, 5.84; N, 10.14. Found: C, 60.71; H, 6.03; N, 10.06. MS [ESI, m/z]: 553.1 [M+H].

4.1.2 Synthesis of 3-(N-(3-(4-(3-(4-Carboxyphenylensulfonamido)propyl)piperazin-1-yl)-propyl)sulfamoyl) benzoic acid (1t)

Compound 1s (0.15 g, 0.3 mmol) was dissolved 3 mL of THF. LiOH monohydrate (0.07 g, 1.7 mmol) was dissolved in 4 mL of distilled water and added to the THF solution. The reaction was stirred o.n. at 80 °C. The organic solvent was then removed at reduced pressure and the water residue was acidified to pH 5 with 1M HCl solution. The resulting precipitate was filtered, washed with water and dried under vacuum to afford a pure white solid in 88% yield. M.p. > 300 °C. $^1$H-NMR (D$_2$O), $\delta$: 1.48 (m, 4H), 2.15 (t, $J$ = 7.8 Hz, 4H), 2.31 (bm, 8H), 2.69 (t, $J$ = 7.1 Hz, 4H), 7.72 (d, $J$ = 8.1 Hz, 4H), 7.88 (d, $J$ = 8.1 Hz, 4H). $^{13}$C-NMR (D$_2$O), $\delta$: 27.8, 43.5, 51.5, 55.6, 126.2, 129.1, 138.7, 145.2, 174.5. Anal. Calcd for C$_{24}$H$_{32}$N$_4$O$_8$: C, 50.69; H, 5.67; N, 9.85. Found: C, 50.53; H, 5.45; N, 9.71. MS [ESI, m/z]: 591.1 [M+Na].

4.1.3 General method for the preparation of N,N'-(piperazine-1,4-diyl)bis(3-oxopropane-)diaryl sulfonamides 11

The different 3-arylsulfonylamino propionic acid 10 (1.3 mmol), TBTU (0.45 g, 1.4 mmol) and HOBt (0.19 g, 1.4 mmol) were suspended in anhydrous THF (9 mL) at r.t. DiPEA (0.7 mL, 4.2 mmol) was then added to the reaction mixture, followed by piperazine (41) (0.05 g, 0.6 mmol). The reaction mixture was left stirring at r.t. for 4 h. The organic solvent was then removed at reduced pressure and the residue was suspended in EtOAc (100 mL). The organic layer was washed with saturated NaHCO$_3$ solution (2 x 70 mL), then with saturated NH$_4$Cl solution (2 x 70 mL) and finally with brine (70 mL). The organic solvent was removed under vacuum after drying over MgSO$_4$. The crude residue was purified by flash column chromatography.

4.1.3.1 N,N'-(3,3'-(Piperazine-1,4-diyl)bis(3-oxopropane-3,1-diyl))bis(4-chlorobenzene-sulfonamide) (11a)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 98:2 v/v. Obtained in 41% yield as a white solid. M.p. 206-208 °C. TLC (9:1 DCM-MeOH, Rf:
0.76). $^1$H-NMR (CDCl$_3$), $\delta$: 2.62 (t, $J = 5.2$ Hz, 4H), 3.33 (m, 4H), 3.43 (m, 4H), 3.56 (m, 4H), 7.1 (bs, 2H), 7.52 (d, $J = 8.5$ Hz, 4H), 7.83 (d, $J = 8.5$ Hz, 4H). $^{13}$C-NMR (DMSO-d$_6$), $\delta$: 32.5, 38.8, 40.6, 40.9, 44.3, 44.6, 128.4, 129.3, 137.2, 139.2, 168.6. Anal. Calcd for C$_{22}$H$_{26}$Cl$_2$N$_4$O$_6$S$_2$: C, 45.75; H, 4.54; N, 9.70. Found: C, 45.61; H, 4.81; N, 9.52. MS [ESI, m/z]: 598.9, 600.9 [M+Na].

4.1.3.2 $N,N'-(3,3'-(Piperazine-1,4-diyl))bis(3-oxopropane-3,1-diyl))bis(4-methoxybenzene-sulfonamide) (11d)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 98:2 v/v. Obtained in 46% yield as a white solid. M.p. 178-180 °C. TLC (9:1 DCM-MeOH, Rf: 0.65). $^1$H-NMR (CDCl$_3$), $\delta$: 2.59 (t, $J = 5.6$ Hz, 4H), 3.23 (m, 4H), 3.42 (m, 4H), 3.60 (m, 4H), 3.89 (s, 6H), 5.42 (bs, 2H), 7.00 (d, $J = 8.8$ Hz, 4H), 7.82 (d, $J = 8.8$ Hz, 4H). $^{13}$C-NMR (CDCl$_3$), $\delta$: 33.0, 33.1, 39.0, 39.1, 41.2, 44.7, 44.9, 55.6, 114.3, 129.1, 131.7, 162.8, 168.8. Anal. Calcd for C$_{24}$H$_{32}$N$_4$O$_8$S$_2$: C, 50.69; H, 5.67; N, 9.85. Found: C, 50.67; H, 5.92; N, 9.76. MS [ESI, m/z]: 591.1 [M+Na].

4.1.3.3 $N,N'-(3,3'-(Piperazine-1,4-diyl))bis(3-oxopropane-3,1-diyl))bis(3,4-dimethoxy-benzene-sulfonamide) (11f)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 98:2 v/v. Obtained in 47% yield as a white solid. M.p. 102-104 °C. TLC (9:1 DCM-MeOH, Rf: 0.65). $^1$H-NMR (CDCl$_3$), $\delta$: 2.80 (bs, 4H), 3.21 (m, 4H), 3.55 (m, 4H), 3.95 (m, 12H), 4.04 (bs, 4H), 5.25 (bs, 1H), 5.54 (bs, 1H), 6.97 (m, 2H), 7.33 (m, 2H), 7.49 (m, 2H). $^{13}$C-NMR (CDCl$_3$), $\delta$: 33.0, 33.2, 38.8, 39.0, 41.1, 45.1, 56.2, 56.3, 56.4, 56.5, 109.6, 109.7, 110.6, 110.7, 120.8, 120.9, 129.9, 131.6, 149.2, 149.5, 152.0, 152.7, 169.8, 171.8. Anal. Calcd for C$_{26}$H$_{36}$N$_4$O$_{10}$S$_2$: C, 49.51; H, 6.03; N, 8.89. Found: C, 49.51; H, 6.03; N, 8.89. MS [ESI, m/z]: 651.1 [M+Na].

4.1.3.4 $N,N'-(3,3'-(Piperazine-1,4-diyl))bis(3-oxopropane-3,1-diyl))bis(2,5-dimethoxy-benzene-sulfonamide) (11g)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 52% yield as a white solid. M.p. 90-92 °C. TLC (9:1 DCM-MeOH, Rf: 0.63). $^1$H-NMR (CDCl$_3$), $\delta$: 2.55 (t, $J = 6.0$ Hz, 4H), 3.22 (m, 4H), 3.37 (m, 4H), 3.60 (m, 4H), 3.84 (s, 6H), 3.97 (s, 6H), 5.92 (t, $J = 6.1$ Hz, 2H), 7.00 (d, $J = 9.0$ Hz, 2H), 7.09 (dd, $J_1 = 9.0$ Hz, $J_2 = 3.0$ Hz, 2H), 7.46 (d, $J = 3.0$ Hz, 2H). $^{13}$C-NMR (DMSO-d$_6$), $\delta$: 32.5, 38.9, 40.5, 40.8, 44.2, 44.4, 55.7, 56.5, 114.2, 114.3, 119.5, 128.2, 150.1, 152.3, 168.9. Anal. Calcd for C$_{26}$H$_{36}$N$_4$O$_{10}$S$_2$: C, 49.61; H, 5.93; N, 8.89. Found: C, 49.61; H, 5.93; N, 8.89. MS [ESI, m/z]: 651.1 [M+Na].

4.1.3.5 $N,N'-(3,3'-(Piperazine-1,4-diyl))bis(3-oxopropane-3,1-diyl))dinaphthalene-2-sulfonamide (11h)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 54% yield as a white solid. M.p. 228-230 °C. TLC (9:1 DCM-MeOH, Rf: 0.79). $^1$H-NMR (CDCl$_3$), $\delta$: 2.55 (m, 4H), 3.28 (m, 8H), 3.52 (m, 4H), 5.61 (bs, 2H), 7.66 (m, 4H), 7.86 (dd, $J_1 = 8.7$ Hz, $J_2 = 1.7$ Hz, 2H), 7.93 (d, $J = 7.9$ Hz, 2H), 7.99 (m, 4H), 8.45 (s, 2H). $^{13}$C-NMR (DMSO-d$_6$), $\delta$: 32.5, 38.9, 40.5, 40.8, 44.2, 44.4, 122.2, 127.3, 127.5, 127.7, 128.6, 129.1, 129.3, 131.6, 134.11,
137.3, 168.5. Anal. Calcd for C$_{30}$H$_{32}$N$_4$O$_6$S$_2$: C, 59.19; H, 5.30; N, 9.20. Found: C, 58.84; H, 5.61; N, 9.96. MS [ESI, m/z]: 631.0 [M+Na].

4.1.4 Synthesis of [4-[(4-tert-Butoxycarbonylamino-butyl)-piperazin-1-yl]-butyl]-carbamic acid tert-butyl ester (16)
Piperazine (0.17 g, 2.1 mmol) and NaHCO$_3$ (0.38, 4.5 mmol) were suspended in 3 mL of absolute ethanol and added dropwise of 79 (1.21 g, 4.5 mmol) diluted in 5 mL of ethanol. The reaction mixture was stirred under reflux for 24 h. The solvent was evaporated under vacuum, and the residue was suspended in DCM (30 mL) and washed with saturated NaHCO$_3$ solution (2 x 30 mL) and brine (30 mL). The organic phase was evaporated at reduced pressure after drying over MgSO$_4$ to give a yellow solid in 70% yield. $^1$H-NMR (CDCl$_3$), $\delta$: 1.37 (m, 18 H), 1.63 (m, 8H), 2.32 (bs, 4H), 2.56 (bs, 8H), 3.33 (t, $J$ = 5.3 Hz, 4H), 5.56 (bs, 2H). $^{13}$C-NMR (CDCl$_3$), $\delta$: 24.4, 25.7, 28.4, 28.5, 40.5, 53.0, 58.0, 78.8, 156.0. MS [ESI, m/z]: 397.3 [M+H].

4.1.5 N,N’-(4,4’-((Piperazine-1,4-diyl)bis(butane-4,1-diyl))bis(4-chlorobenzene-sulfonamide) (17)
Compound 16 (0.63 g, 1.5 mmol) was dissolved at 0 °C in a mixture of 2 mL of TFA and 0.2 mL of distilled water. The reaction mixture was left stirring at r.t for 30 min., then the volume was reduced under vacuum and the remaining suspension was poured dropwise into 5 mL of ice-cooled diethyl ether. The resulting white precipitate was left in the fridge o.n. before being filtered and washed with cold diethyl ether. The white solid was suspended with 4-chlorobenzensulfonyl chloride (2a) (0.63 g, 3.0 mmol) in 7 mL of anhydrous DCM; the resulting mixture was treated dropwise with triethylamine (1.3 mL, 9.5 mmol) under ice-cooling and stirred for 1 h at r.t. The reaction mixture was then diluted with DCM and washed with water (2 x 30 mL) and brine (30 mL). The organic solvent was removed under vacuum after drying over MgSO$_4$ and the crude residue was purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 91:9 v/v to give a white solid in 15% yield. M.p. 182-184 °C. TLC (9:1 DCM-MeOH, Rf: 0.64). $^1$H-NMR (CDCl$_3$), $\delta$: 1.63 (m, 8H), 2.50 (bm, 4H), 2.77 (bm, 8H), 2.96 (t, $J$ = 5.3 Hz, 4H), 7.49 (d, $J$ = 8.6 Hz, 4H), 7.81 (d, $J$ = 8.6 Hz, 4H). $^{13}$C-NMR (CDCl$_3$), $\delta$: 23.9, 28.2, 42.8, 51.8, 57.7, 128.4, 129.2, 138.7, 139.2. Anal. Calcd for C$_{24}$H$_{34}$Cl$_2$N$_4$O$_4$S$_2$: C, 49.91; H, 5.93; N, 9.70. Found: C, 49.67; H, 6.11; N, 9.61. MS [ESI, m/z]: 577.1, 579.1 [M+H].

4.1.6 Synthesis of N,N’-(3,3’-(piperazine-1,4-diyl))bis(propane-3,1-diyl))bis(4-chlorobenzamide) (20) [38]
A mixture of 19 (0.5 g, 1.8 mmol), piperazine (0.07 g, 0.8 mmol) and triethylamine (0.24 mL, 1.7 mmol) in 7 mL of anhydrous THF was stirred under nitrogen atmosphere for 48 h at r.t. The reaction mixture was then diluted with DCM (20 mL), washed with saturated NaHCO$_3$ solution (2 x 30 mL) and brine (30 mL) and dried over MgSO$_4$. The organic solvent was removed under vacuum and the crude residue was purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 91:9 v/v to give a white solid in 36% yield. M.p. 180-182 °C. TLC (9:1 DCM-MeOH, Rf: 0.63). $^1$H-NMR (CDCl$_3$), $\delta$: 1.82 (m, 4H), 2.56 (m, 12H), 3.59 (m, 4H), 7.42 (d, $J$ = 8.5 Hz, 4H), 7.78 (d, $J$ = 8.5 Hz, 4H), 8.05 (bs, 2H). $^{13}$C-NMR (DMSO-d$_6$), $\delta$: 26.1, 37.9, 52.7, 55.6, 128.2, 129.0, 133.3, 135.7, 164.9.
Anal. Calcd for C_{24}H_{30}Cl_{2}N_{4}O_{2}: C, 60.38; H, 6.33; N, 11.74. Found: C, 60.58; H, 6.51; N, 11.90. MS [ESI, m/z]: 477.1, 479.1 [M+H].

4.1.7 Synthesis of \(N,N'-(2,2'-(1,4-phenylenebis(azanediyl))bis(ethane-2,1-diyl))bis(4-chlorobenzensulfonamide)\) (21)

A mixture of 7a (1.05 g, 3.5 mmol), 4-phenylendiamine (0.16 g, 1.5 mmol) and triethylamine (0.47 mL, 3.4 mmol) in 13 mL of anhydrous THF was stirred under nitrogen atmosphere for 72 h at r.t. The reaction mixture was then diluted with DCM (35 mL), washed with saturated NaHCO\(_3\) solution (2 x 30 mL) and brine (30 mL) and dried over MgSO\(_4\). The organic solvent was removed under vacuum and the crude residue was purified by flash column chromatography eluting with \(n\)-hexane-EtOAc 100:0 v/v increasing to \(n\)-hexane-EtOAc 20:80 v/v to afford a light brown solid in 17% yield. M. p. 162-164 °C. TLC (8:2 EtOAc-\(n\)-hexane, Rf: 0.46).

1\(^{H}\)-NMR (DMSO-d\(_6\)), \(\delta\): 2.87 (t, \(J = 6.5\) Hz, 4H), 2.97 (m, 4H), 4.70 (t, \(J = 6.1\) Hz, 2H), 6.32 (s, 4H), 7.65 (d, \(J = 8.6\) Hz, 4H), 7.79 (d, \(J = 8.6\) Hz, 4H), 7.81 (bs, 2H). 13\(^{C}\)-NMR (DMSO-d\(_6\)), \(\delta\): 41.8, 43.6, 113.7, 128.3, 129.3, 137.2, 139.3, 139.6. Anal. Calcd for C\(_{22}\)H\(_{24}\)Cl\(_2\)N\(_4\)O\(_4\)S\(_2\): C, 48.62; H, 4.45; N, 10.31. Found: C, 48.81; H, 4.39; N, 10.38. MS [ESI, m/z]: 543.0, 545.0 [M+H].

4.1.8 General method for the preparation of unsymmetrical sulfonamides 23-36

A mixture of the different \(N\)-(3-piperazin-1-yl-propyl)-arylsulfonamide 22 (1.0 mmol), \(N\)-(3-bromopropyl)arylsulfonamide 3 or \(N\)-(2-bromoethyl)arylsulfonamide 2 (1.5 mmol) and NaHCO\(_3\) (0.16 g, 2 mmol) in absolute ethanol (7 mL) was stirred under reflux for 24 h. The solvent was evaporated under reduced pressure and the crude residue was purified by flash column chromatography.

4.1.8.1 \(N\)-{3-[4-(3-Benzensulfonylamino-propyl)-piperazin-1-yl]-propyl}-4-chloro-benzene sulfonamide (23)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 58% yield as a white solid. M. p. 128-130 °C. TLC (9:1 DCM-MeOH, Rf: 0.59). 1\(^{H}\)-NMR (DMSO-d\(_6\)), \(\delta\): 1.44-1.51 (m, 4H), 2.11-2.26 (m, 12H), 2.74-2.79 (m, 4H), 7.56-7.62 (m, 3H), 7.61-7.64 (m, 1H), 7.66-7.70 (m, 3H), 7.78 (d, \(J = 8.3\) Hz, 2H). 13\(^{C}\)-NMR (DMSO-d\(_6\)), \(\delta\): 26.0, 26.1, 40.8, 40.9, 52.5, 54.7, 54.8, 126.4, 128.4, 129.1, 129.3, 137.2, 137.3, 139.6. Anal. Calcd for C\(_{22}\)H\(_{31}\)ClN\(_4\)O\(_4\)S\(_2\): C, 51.30; H, 6.07; N, 10.88. Found: C, 51.19; H, 6.35; N, 10.67. MS [ESI, m/z]: 515.1, 517.1 [M+H].

4.1.8.2 4-Chloro-\(N\)-(3-(4-(4-methylbenzenesulfonylamido)propyl)-piperazin-1-yl)-propylbenzene sulfonamide (24)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 86% yield as a white solid. M. p. 172-174 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.57). 1\(^{H}\)-NMR (DMSO-d\(_6\)), \(\delta\): 1.43-1.51 (m, 4H), 2.10-2.28 (m, 12H), 2.38 (s, 3H), 2.69-2.81 (m, 4H), 7.39 (d, \(J = 8.1\) Hz, 2H), 7.48 (t, \(J = 5.6\) Hz, 1H), 7.64-7.71 (m, 5H), 7.79 (d, \(J = 8.7\) Hz, 2H). 13\(^{C}\)-NMR (DMSO-d\(_6\)), \(\delta\): 20.9, 26.0, 40.8, 40.9, 52.5, 54.7, 54.8, 126.5, 128.4, 129.3, 129.5, 137.1, 137.6, 139.4, 142.4. Anal. Calcd for C\(_{23}\)H\(_{35}\)ClN\(_4\)O\(_2\): C, 52.21; H, 6.29; N, 10.59. Found: C, 52.09; H, 5.94;
4.1.8.3 4-tert-Butyl-N-(3-(4-(3-(4-chlorophenylsulfonylamido)propyl)-piperazin-1-yl)-propyl)benzene sulfonamide (25)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 60% yield as a white solid. M.p. 130-132 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.44). 1H-NMR (DMSO-d6), δ: 1.30 (s, 9H), 1.41-1.53 (m, 4H), 2.11-2.35 (m, 12H), 2.72-2.80 (m, 4H), 7.49 (t, J = 5.5 Hz, 1H), 7.60 (d, J = 8.5 Hz, 2H), 7.65-7.72 (m, 5H), 7.78 (d, J = 8.5 Hz, 2H). 13C-NMR (DMSO-d6), δ: 26.0, 30.7, 34.7, 40.8, 40.9, 52.5, 52.6, 54.7, 54.8, 125.9, 126.3, 128.4, 129.3, 137.1, 137.7, 139.4, 155.1. Anal. Calcd for C26H39ClN4O4S2: C, 54.67; H, 6.88; N, 9.80. Found: C, 54.51; H, 6.49; N, 9.61. MS [ESI, m/z]: 571.2, 573.2 [M+H].

4.1.8.4 4-Chloro-N-(3-(4-(3-(4-(trifluoromethyl)phenylsulfonylamido)propyl)-piperazin-1-yl)-propyl)benzenesulfonamide (26)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 85% yield as a white solid. M.p. 158-160 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.61). 1H-NMR (DMSO-d6), δ: 1.44-1.51 (m, 4H), 2.11-2.27 (m, 12H), 2.74-2.83 (m, 4H), 7.67 (d, J = 8.5 Hz, 2H), 7.69 (bs, 1H), 7.78 (d, J = 8.5 Hz, 2H), 7.86 (bs, 1H), 7.94-8.03 (m, 4H). 13C-NMR (DMSO-d6), δ: 26.0, 40.7, 40.8, 52.5, 54.6, 54.7, 126.4 (q, J = 3.7 Hz), 127.4, 128.4, 129.2, 131.8, 131.9, 137.1, 139.4, 144.5. Anal. Calcd for C23H30ClF3N4O4S2: C, 47.38; H, 5.19; N, 9.61. Found: C, 47.32; H, 5.17; N, 9.48. MS [ESI, m/z]: 583.1, 585.1 [M+H].

4.1.8.5 N-(3-(4-(3-(4-Chlorophenylsulfonylamido)propyl)-piperazin-1-yl)-propyl)biphenyl-4-sulfonamide (27)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 71% yield as a white solid. M.p. 138-140 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.63). 1H-NMR (DMSO-d6), δ: 1.42-1.54 (m, 4H), 2.15-2.31 (m, 12H), 2.72-2.81 (m, 4H), 7.44 (t, J = 5.7 Hz, 1H), 7.50-7.54 (m, 2H), 7.62 (t, J = 5.7 Hz, 1H), 7.65-7.79 (m, 3H), 7.74 (d, J = 7.1 Hz, 2H), 7.78 (d, J = 8.6 Hz, 2H), 7.85 (d, J = 8.6 Hz, 2H), 7.89 (d, J = 8.6 Hz, 2H). 13C-NMR (DMSO-d6), δ: 26.0, 26.1, 40.8, 40.9, 52.5, 54.7, 54.8, 127.0, 127.1, 127.3, 128.4, 129.0, 129.3, 137.1, 138.5, 139.2, 139.3, 143.8. Anal. Calcd for C25H35ClN4O4S2: C, 56.89; H, 5.97; N, 9.48. Found: C, 56.73; H, 6.08; N, 9.30. MS [ESI, m/z]: 591.1, 593.1 [M+H].

4.1.8.6 4-tert-Butyl-N-(3-(4-(3-(phenylsulfonylamido)propyl)-piperazin-1-yl)-propyl)benzene sulfonamide (28)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 54% yield as a white solid. M.p. 134-136 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.55). 1H-NMR (DMSO-d6), δ: 1.30 (s, 9H), 1.43-1.49 (m, 4H), 2.11-2.26 (m, 12H), 2.72-2.78 (m, 4H), 7.49 (t, J = 5.6 Hz, 1H), 7.58-7.62 (m, 5H), 7.66-7.69 (m, 1H), 7.70 (d, J = 8.5 Hz, 2H), 7.78 (d, J = 7.8 Hz, 2H). 13C-NMR (DMSO-d6), δ: 26.0, 26.1, 30.7, 34.7, 40.8, 40.9, 52.5, 52.6, 54.8, 54.9, 125.9,
126.3, 126.4, 129.1, 132.2, 137.6, 140.5, 155.2. Anal. Calcd for C_{26}H_{40}N_{4}O_{4}S_{2}: C, 58.18; H, 7.51; N, 10.44. Found: C, 58.35; H, 7.46; N, 10.47. MS [ESI, m/z]: 537.2 [M+H].

4.1.8.7 4-tert-Butyl-N-(3-(4-(3-(4-methylphenylsulfonylamido)propyl)-piperazin-1-yl)-propyl)benzene sulfonamide (29)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 83% yield as a white solid. M.p. 155-157 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.64). \(^1\)H-NMR (DMSO-d\(_6\)), \(\delta\): 1.30 (s, 9H), 1.43-1.49 (m, 4H), 2.09-2.24 (m, 12H), 2.38 (s, 3H), 2.70-2.78 (m, 4H), 7.39 (d, \(J = 8.1\) Hz, 2H), 7.49 (bs, 2H), 7.60 (d, \(J = 8.5\) Hz, 2H), 7.66 (d, \(J = 8.1\) Hz, 2H), 7.70 (d, \(J = 8.5\) Hz, 2H). \(^{13}\)C-NMR (DMSO-d\(_6\)), \(\delta\): 20.9, 26.0, 30.7, 34.7, 40.8, 40.9, 52.5, 52.6, 54.8, 54.9, 125.9, 126.3, 126.5, 129.5, 137.6, 137.7, 142.4, 155.1. Anal. Calcd for C_{27}H_{42}N_{4}O_{4}S_{2}: C, 58.88; H, 7.69; N, 10.17. Found: C, 59.07; H, 7.92; N, 10.23. MS [ESI, m/z]: 573.2 [M+Na].

4.1.8.8 4-tert-Butyl-N-(3-(4-(3-(4-(trifluoromethyl)phenylsulfonylamido)propyl)-piperazin-1-yl)-propyl)benzenesulfonamide (30)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 95:5 v/v. Obtained in 70% yield as a white solid. M.p. 156-158 °C. TL C (DCM-MeOH 9:1 v/v, Rf: 0.76). \(^1\)H-NMR (DMSO-d\(_6\)), \(\delta\): 1.30 (s, 9H), 1.42-1.51 (m, 4H), 2.09-2.26 (m, 12H), 2.73-2.77 (m, 2H), 2.78-2.82 (m, 2H), 7.49 (t, \(J = 5.5\) Hz, 1H), 7.60 (d, \(J = 8.4\) Hz, 2H), 7.70 (d, \(J = 8.4\) Hz, 2H), 7.86 (bs, 1H), 7.97-9.01 (m, 4H). \(^{13}\)C-NMR (DMSO-d\(_6\)), \(\delta\): 26.0, 30.7, 34.7, 40.8, 40.9, 52.5, 52.6, 54.6, 54.8, 125.9, 126.3, 126.4, 126.5, 127.4, 132.2, 137.7, 144.5, 155.1. Anal. Calcd for C_{27}H_{39}F_{3}N_{4}O_{4}S_{2}: C, 53.62; H, 6.50; N, 9.26. Found: C, 53.46; H, 6.71; N, 9.25. MS [ESI, m/z]: 605.2 [M+H].

4.1.8.9  \(N-(3-(4-(3-(4-tert-Butylphenylsulfonylamido)propyl)-piperazin-1-yl)-propyl)\) biphenyl-4-sulfonamide (31)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 58% yield as a white solid. M.p. 146-148 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.62). \(^1\)H-NMR (DMSO-d\(_6\)), \(\delta\): 1.29 (s, 9H), 1.41-1.53 (m, 4H), 2.07-2.25 (m, 12H), 2.73-2.77 (m, 2H), 2.78-2.82 (m, 2H), 7.44 (t, \(J = 7.5\) Hz, 1H), 7.47-7.53 (m, 3H), 7.58-7.64 (m, 3H), 7.70 (d, \(J = 8.5\) Hz, 2H), 7.74 (d, \(J = 7.2\) Hz, 2H), 7.85 (d, \(J = 8.5\) Hz, 2H), 7.89 (d, \(J = 8.5\) Hz, 2H). \(^{13}\)C-NMR (DMSO-d\(_6\)), \(\delta\): 26.0, 26.1, 30.7, 34.7, 40.8, 40.9, 52.5, 52.6, 54.8, 125.9, 126.3, 126.4, 126.5, 127.4, 132.2, 137.7, 144.5, 155.1. Anal. Calcd for C_{32}H_{44}N_{4}O_{4}S_{2}: C, 62.71; H, 6.71; N, 9.14. Found: C, 62.69; H, 7.32; N, 9.09. MS [ESI, m/z]: 613.2 [M+H].

4.1.8.10 4-Methyl-\(N-(3-(4-(3-(phenylsulfonylamido)propyl)-piperazin-1-yl)-propyl)\)benzene sulfonamide (32)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 95:5 v/v. Obtained in 52% yield as a white solid. M.p. 137-139 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.65). \(^1\)H-NMR (DMSO-d\(_6\)), \(\delta\): 1.43-1.51 (m, 4H), 2.13-2.26 (m, 12H), 2.38 (s, 3H), 2.70-2.79 (m, 4H), 7.39 (d, \(J = 8.1\) Hz, 2H), 7.48 (t, \(J = 5.4\) Hz, 1H), 7.56-7.62 (m, 3H), 7.66 (d, \(J = 8.1\) Hz, 2H), 7.61-
7.64 (m, 1H), 7.78 (d, J= 7.5 Hz, 2H). $^{13}$C-NMR (DMSO-$d_6$), $\delta$ 20.9, 26.0, 26.1, 40.9, 52.6, 54.8, 126.4, 126.5, 129.2, 129.5, 132.2, 137.6, 140.5, 142.4. Anal. Calcd for $C_{23}H_{34}N_4O_4S_2$: C, 55.84; H, 6.93; N, 11.33. Found: C, 55.73; H, 7.18; N, 11.20. MS [ESI, m/z]: 495.2 [M+H].

4.1.8.11 $N$-(3-(4-(3-(4-Methylphenylsulfonylamido)propyl)-piperazin-1-yl)-propyl)biphenyl-4-sulfonamide (33)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 67% yield as a white solid. M.p. 134-136 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.58). $^1$H-NMR (DMSO-$d_6$), $\delta$ 1.42-1.54 (m, 4H), 2.13-2.25 (m, 12H), 2.37 (s, 3H), 2.69-2.74 (m, 2H), 2.78-2.83 (m, 2H), 7.38 (d, J= 8.1 Hz, 2H), 7.44 (t, J= 7.3 Hz, 1H), 7.47 (t, J= 5.5 Hz, 1H), 7.49-7.53 (m, 2H), 7.63 (t, J= 5.5 Hz, 1H), 7.66 (d, J= 8.2 Hz, 2H), 7.72-7.75 (m, 2H), 7.86 (d, J= 8.5 Hz, 2H), 7.89 (d, J= 8.5 Hz, 2H). $^{13}$C-NMR (DMSO-$d_6$), $\delta$ 20.9, 26.0, 26.1, 40.9, 52.6, 54.8, 54.9, 126.5, 127.0, 127.2, 127.3, 128.4, 129.1, 129.6, 137.6, 138.5, 139.3, 142.5, 143.8. Anal. Calcd for $C_{29}H_{38}N_4O_4S_2$: C, 61.03; H, 6.71; N, 9.82. Found: C, 60.86; H, 6.52; N, 9.74. MS [ESI, m/z]: 571.2 [M+H].

4.1.8.12 $N$-(3-(4-(3-(Phenylsulfonamido)propyl)-piperazin-1-yl)-propyl)biphenyl-4-sulfonamide (34)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 71% yield as a white solid. M.p. 124-126 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.47). $^1$H-NMR (DMSO-$d_6$), $\delta$ 1.42-1.54 (m, 4H), 2.11-2.25 (m, 12H), 2.73-2.77 (m, 2H), 2.78-2.82 (m, 2H), 7.44 (t, J= 7.3 Hz, 1H), 7.50-7.53 (m, 2H), 7.56-7.61 (m, 3H), 7.61-7.66 (m, 2H), 7.70 (d, J= 7.4 Hz, 2H), 7.77-7.79 (m, 2H), 7.85 (d, J= 8.5 Hz, 2H), 7.89 (d, J= 8.5 Hz, 2H). $^{13}$C-NMR (DMSO-$d_6$), $\delta$ 26.0, 40.9, 52.5, 54.8, 54.9, 126.4, 127.0, 127.2, 127.4, 128.4, 129.0, 129.1, 132.3, 138.5, 139.3, 142.5, 143.8. Anal. Calcd for $C_{28}H_{36}N_4O_4S_2$: C, 60.41; H, 6.52; N, 10.06. Found: C, 60.27; H, 6.81; N, 9.87. MS [ESI, m/z]: 557.2 [M+H].

4.1.8.13 $N$-(3-(4-(3-(Phenylsulfonamido)propyl)-piperazin-1-yl)-4-(trifluoromethyl) benzene sulfonamide (35)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 71% yield as a white solid. M.p. 119-121 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.72). $^1$H-NMR (DMSO-$d_6$), $\delta$ 1.43-1.51 (m, 4H), 2.11-2.23 (m, 12H), 2.73-2.77 (m, 2H), 2.78-2.82 (m, 2H), 7.58-7.61 (m, 4H), 7.62-7.66 (m, 2H), 7.77-7.79 (m, 2H), 7.88 (bs, 1H), 8.00 (s, 4H). $^{13}$C-NMR (DMSO-$d_6$), $\delta$ 26.0, 40.8, 40.9, 52.5, 54.6, 54.8, 126.4, 126.5, 127.4, 128.4, 129.0, 129.1, 132.2, 132.3, 137.6, 144.5. Anal. Calcd for $C_{23}H_{16}F_3N_4O_4S_2$: C, 50.35; H, 5.70; N, 10.21. Found: C, 50.51; H, 5.53; N, 10.34. MS [ESI, m/z]: 549.2 [M+H].

4.1.8.14 4-Chloro-$N$-(3-(4-(2-(4-chlorophenylsulfonylamido)ethyl)-piperazin-1-yl)-propyl) benzene sulfonamide (36a)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 72% yield as a white solid. M.p. 133-135 °C. TLC (DCM-MeOH 9:1 v/v,
4.1.8.15  N-(3-(4-(2-(Phenylsulfonamido)ethyl)-piperazin-1-yl)-propyl) benzenesulfonamide (36b)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 47% yield as a white solid. M.p. 135-137 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.69). 1H-NMR (DMSO-d6), δ: 1.43-1.49 (m, 2H), 2.14-2.28 (m, 12H), 2.73-2.78 (m, 2H), 2.81-2.86 (m, 2H), 7.49 (bs, 1H), 7.55-7.62 (m, 5H), 7.61-7.66 (m, 2H), 7.76-7.82 (m, 4H). 13C-NMR (DMSO-d6), δ: 26.0, 30.7, 39.0, 40.0, 40.8, 52.4, 52.5, 54.8, 56.7, 125.8, 125.9, 126.0, 126.3, 137.6, 137.7, 155.1. Anal. Calcd for C29H46N4O4S2: C, 60.17; H, 8.01; N, 9.68. Found: C, 60.11; H, 8.22; N, 9.69. MS [ESI, m/z]: 579.2 [M+H].

4.1.8.16 4-tert-Butyl-N-(3-(4-(2-(4-tert-butylphenylsulfonamido)ethyl)-piperazin-1-yl)-propyl) benzene sulfonamide (36l)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 96:4 v/v. Obtained in 53% yield as a pale yellow solid. M.p. 86-88 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.55). 1H-NMR (CDCl3), δ: 1.64-1.70 (m, 2H), 2.37-2.34 (m, 6H), 2.54 (t, J= 5.2 Hz, 2H), 3.07 (t, J= 5.7 Hz, 2H), 3.18-3.24 (m, 2H), 3.37-3.41 (m, 2H), 3.54-3.60 (m, 2H), 5.84 (bs, 1H), 6.63 (bs, 1H), 7.47-7.51 (m, 4H), 7.78-7.82 (m, 4H). 13C-NMR (CDCl3), δ: 24.7, 32.8, 39.2, 41.5, 43.3, 45.0, 52.6, 52.7,
57.0, 128.4, 128.5, 129.3, 129.4, 138.7, 138.8, 138.9, 139.0, 169.4. Anal. Calcd for C_{22}H_{33}Cl_{2}N_{4}O_{5}S_{2}: C, 46.89; H, 5.01; N, 9.94. Found: C, 46.92; H, 4.76; N, 9.95. MS [ESI, m/z]: 563.1, 565.1 [M+H].

4.1.9.2 N-(3-(4-(3-(Phenylsulfonamido)propanoyl)piperazin-1-yl)-3-oxopropyl)benzene sulfonamide (37b)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 37% yield as a colourless waxy solid. M.p. 30-32 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.54). \(^1\)H-NMR (CDCl\(_3\)), \(\delta\): 1.59-1.64 (m, 2H), 2.29 (t, \(J= 4.7\) Hz, 2H), 2.32 (t, \(J= 4.7\) Hz, 2H), 2.37 (t, \(J= 6.0\) Hz, 2H), 2.49 (t, \(J= 5.6\) Hz, 2H), 3.04 (t, \(J= 6.0\) Hz, 2H), 3.17-3.21 (m, 2H), 3.33 (t, \(J= 4.7\) Hz, 2H), 3.50-3.54 (m, 2H), 5.80 (t, \(J= 6.2\) Hz, 1H), 6.66 (bs, 1H), 7.48-7.52 (m, 4H), 7.54-7.58 (m, 2H), 7.82-7.86 (m, 4H). \(^{13}\)C-NMR (CDCl\(_3\)), \(\delta\): 24.8, 32.7, 39.2, 41.4, 43.1, 45.0, 52.5, 52.7, 56.8, 126.8, 126.9, 129.11, 129.16, 132.55, 132.59 (CH, C-aromatic), 140.11, 140.21 (C, C-aromatic), 169.43 (C, C-6'). Anal. Calcd for C_{22}H_{30}N_{4}O_{5}S_{2}: C, 53.42; H, 6.11; N, 11.33. Found: C, 53.55; H, 5.89; N, 11.40. MS [ESI, m/z]: 495.1 [M+H].

4.1.9.3 4-Methyl-N-(3-(4-(3-(4-methylphenylsulfonamido)propanoyl)piperazin-1-yl)-3-oxopropyl)benzenesulfonamide (37c)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 34% yield as a white solid. M.p. 149-151 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.64). \(^1\)H-NMR (CDCl\(_3\)), \(\delta\): 1.65-1.70 (m, 2H), 2.36-2.42 (m, 6H), 2.44 (s, 3H), 2.45 (s, 3H), 2.54 (t, \(J= 5.5\) Hz, 2H), 3.10 (t, \(J= 5.8\) Hz, 2H), 3.20-3.24 (m, 2H), 3.40 (t, \(J= 4.7\) Hz, 2H), 5.52 (t, \(J= 6.7\) Hz, 1H), 6.40 (bs, 1H), 7.31-7.35 (m, 4H), 7.75 (d, \(J= 8.3\) Hz, 2H), 7.77 (d, \(J= 8.3\) Hz, 2H). \(^{13}\)C-NMR (CDCl\(_3\)), \(\delta\): 21.2, 21.4, 24.6, 32.8, 39.1, 41.4, 43.5, 45.0, 52.7, 52.8, 57.4, 126.9, 126.9, 129.6, 129.7, 137.1, 137.12, 143.2, 143.3, 169.4. Anal. Calcd for C_{24}H_{34}N_{4}O_{5}S_{2}: C, 55.15; H, 6.56; N, 10.72. Found: C, 54.96; H, 6.34; N, 10.56. MS [ESI, m/z]: 523.2 [M+H].

4.1.9.4 4-tert-Butyl-N-(3-(4-(3-(4-tert-butylphenylsulfonamido)propanoyl)piperazin-1-yl)-3-oxopropyl)benzenesulfonamide (37I)

Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 47% yield as a white solid. M.p. 189-191 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.61). \(^1\)H-NMR (CDCl\(_3\)), \(\delta\): 1.36 (s, 9H), 1.37 (s, 9H), 1.66-1.71 (m, 2H), 2.37 (t, \(J= 5.0\) Hz, 2H), 2.42 (t, \(J= 5.0\) Hz, 2H), 2.46 (t, \(J= 5.9\) Hz, 2H), 2.57 (t, \(J= 5.6\) Hz, 2H), 3.11 (t, \(J= 5.9\) Hz, 2H), 3.21-3.25 (m, 2H), 3.42 (t, \(J= 5.0\) Hz, 2H), 3.60 (t, \(J= 5.0\) Hz, 2H), 5.54 (t, \(J= 6.6\) Hz, 1H), 6.28 (bs, 1H), 7.51-7.55 (m, 4H), 7.78 (d, \(J= 8.5\) Hz, 2H), 7.80 (d, \(J= 8.5\) Hz, 2H). \(^{13}\)C-NMR (CDCl\(_3\)), \(\delta\): 24.7, 31.1, 32.9, 35.1, 39.1, 41.4, 43.4, 45.0, 52.7, 52.8, 57.2, 126.0, 126.1, 126.8, 137.0, 137.1, 156.2, 156.3, 169.4. Anal. Calcd for C_{30}H_{46}N_{4}O_{5}S_{2}: C, 59.38; H, 7.64; N, 9.23. Found: C, 59.22; H, 8.01; N, 9.04. MS [ESI, m/z]: 607.3 [M+H].

4.1.9.5 N-(3-(4-(3-(Biphenyl-4-ylsulfonamido)propanoyl)piperazin-1-yl)-3-oxopropyl) biphenyl-4-sulfonamide (37n)
Purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 97:3 v/v. Obtained in 49% yield as a white solid. M.p. 176-178 °C. TLC (DCM-MeOH 9:1 v/v, Rf: 0.54). 1H-NMR (CDCl₃), δ: 1.65-1.71 (m, 2H), 2.35-2.39 (m, 4H), 2.42 (t, J= 5.8 Hz, 2H), 2.56 (t, J= 5.5 Hz, 2H), 3.13 (t, J= 5.8 Hz, 2H), 3.25-3.29 (m, 2H), 3.40 (t, J= 4.6 Hz, 2H), 3.59-3.61 (m, 2H), 5.75 (t, J= 6.3 Hz, 1H), 6.53 (bs, 1H), 7.40- 7.46 (m, 2H), 7.47-7.51 (m, 4H), 7.60-7.64 (m, 4H), 7.71-7.75 (m, 4H), 7.91-7.96 (m, 4H). 13C-NMR (CDCl₃), δ: 24.7, 32.8, 39.2, 41.5, 43.4, 45.1, 52.7, 52.8, 57.1, 127.2, 127.3, 127.4, 127.5, 127.6, 128.0, 128.4, 128.5, 129.0, 129.1, 138.7, 138.8, 139.2, 139.3, 145.4, 145.5, 169.4. Anal. Calcd for C₃₄H₃₈N₄O₅S₂: C, 63.13; H, 5.92; N, 8.66. Found: C, 63.18; H, 5.87; N, 8.79. MS [ESI, m/z]: 647.2 [M+H].

4.1.10 Synthesis of 4-chloro-N-(3-(piperidin-1-yl)propyl)benzenesulfonamide (39)
Piperidine 38 (0.1 mL, 1.2 mmol) and NaHCO₃ (0.11 g, 1.3 mmol) were suspended in 9 mL of absolute EtOH. N-(3-Bromopropyl)-4-chlorobenzenesulfonamide 6a was then added portionwise to the suspension and the mixture was stirred under reflux for 24 h. The reaction mixture was then concentrated under reduced pressure. The residue was diluted with EtOAc (30 mL), washed with water (3 x 30 mL) and the organic phase was concentrated under vacuum after drying over MgSO₄. The residue was purified by flash column chromatography eluting with DCM-MeOH 100:0 v/v increasing to DCM-MeOH 93:7 v/v to give the title compound as a pale yellow oil in 85% yield. TLC (DCM-MeOH 95:5 v/v, Rf: 0.30). 1H-NMR (CDCl₃), δ: 1.50 (m, 2H), 1.67 (m, 6H), 2.43 (m, 6H), 3.09 (t, J= 5.3 Hz, 2H), 7.50 (d, J= 8.5 Hz, 2H), 7.82 (d, J= 8.5 Hz, 2H). 13C-NMR (CDCl₃), δ: 23.60, 24.00, 25.70, 26.00, 25.70, 44.32, 54.39, 58.71, 128.42, 129.21, 138.61, 138.98. Anal. Calcd for C₁₄H₂₁ClN₂O₂S: C, 53.07; H, 6.68; N, 8.84. Found: C, 53.05; H, 6.64; N, 8.87. MS [ESI, m/z]: 317.1, 319.1 [M+H].

4.2 Molecular Modelling
Molecular modelling simulations were performed on a MAC pro 2.66 GHz Quad-Core Intel Xeon, running Ubuntu. Molecular Operating Environment (MOE) 2014.10 [29] and Maestro (Schrodinger version 9.0) [30] were used as molecular modelling softwares. All minimisations were performed with MOE 2014.10 until RMSD gradient of 0.001 Kcal mol⁻¹ Å⁻¹ with the AMBER99 force field. Partial charges were automatically calculated. Conformational analyses were performed with MOE 2010.10; conformers with a strain energy >4 kcal/mol were discarded, and the maximum number of conformations per ligand was set to 500. In every step MOE default settings were applied. Pharmacophoric filters were created within MOE 2010.10 choosing the PCH (polar-charged-hydrophobic) scheme. Docking experiments were carried out using GlideSP module in Maestro [30] with the default options. A 12 Å docking grid was generated using as centroid defined by Asp296, Arg393 and Trp501 in the 3KQN and 3KQH crystal structures. Docking results were rescored using FlexX [32], Plants ChemPLP [33] and Glide XP [30] scoring functions.

4.3 HCV replicon assay
The compounds were dissolved in dimethyl sulfoxide, stored at -20 °C protected from light and further diluted in culture medium prior to use. The Huh 5-2 HCV subgenomic replicon-containing cells were
provided by Prof. R. Bartenschlager (University of Heidelberg, Heidelberg, Germany). Huh 5.2 cells, containing the hepatitis C virus genotype 1b I389Luc-ubi-neo/NS3-3'5.1 replicon were sub-cultured in DMEM supplemented with 10% FCS, 1% non-essential amino acids, 1% penicillin/streptomycin and 2% Genetin at a ratio of 1:3 to 1:4, and grown for 3-4 days in 75 cm² tissue culture flasks. One day before addition of the compound, cells were harvested and seeded in assay medium (DMEM, 10% FCS, 1% non-essential amino acids, 1% penicillin/streptomycin) at a density of 6,500 cells/well (100 μL/well) in 96-well tissue culture microtiter plates for evaluation of antiviral effect and CulturPlate (Perkin Elmer) for evaluation of the antiviral effect. The microtiter plates were incubated overnight (37 °C, 5% CO₂, 95-99% relative humidity), yielding a nonconfluent cell monolayer. The evaluation of the antimetabolic as well as antiviral effect of each compound was performed in parallel.

Four-step, 1-to-5 compound dilution series were prepared for the first screen, to collect data for a more detailed dose-response curve, an eight-step, 1-to-2 dilution series was used. Following assay setup, the microtiter plates were incubated for 72 hours (37 °C, 5% CO₂, 95-99% relative humidity). For the evaluation of anti-metabolic effects, the assay medium was aspirated, replaced with 75 μL of a 5% MTS solution in phenol red-free medium and incubated for 1.5 hours (37 °C, 5% CO₂, 95-99% relative humidity). Absorbance was measured at a wavelength of 498 nm (Safire2, Tecan), and optical densities (OD values) were converted to percentage of untreated controls. For the evaluation of antiviral effects, assay medium was aspirated and the cell monolayers were washed with PBS. The wash buffer was aspirated, and 25 μL of Glo Lysis Buffer (Promega) was added allowing for cell lysis to proceed for 5 min at room temperature. Subsequently, 50 μL of Luciferase Assay System (Promega) was added, and the luciferase luminescence signal was quantified immediately (1000 ms integration time/well, Safire, Tecan). Relative luminescence units were converted into percentage of untreated controls.

The EC₅₀ and EC₉₀ (values calculated from the dose-response curve) represent the concentrations at which 50% and 90% inhibition, respectively, of viral replication is achieved. The CC₅₀ (value calculated from the dose-response curve) represents the concentration at which the metabolic activity of the cells is reduced by 50% as compared to untreated cells.

### 4.4 HCV NS3 helicase enzymatic assays

The ability of compounds to inhibit HCV helicase-catalysed nucleic acid unwinding was determined using molecular beacon-based NS3 helicase assays as described by Hanson et al. [39] Reactions contained 25 mM MOPS pH 6.5, 1.25 mM MgCl₂, 5% DMSO, 5 μg/ml BSA, 0.01% (v/v) Tween20, 0.05 mM DTT, 5 nM florescent DNA substrate, 12.5 nM NS3h, and 1 mM ATP.

The ability of compounds to displace NS3 from a DNA oligonucleotide was monitored as described by Mukherjee et al. [40]. Each assay contained 15 nM NS3h, 25 mM MOPS, pH 7.5, 1.25 mM MgCl₂, 0.0025 mg/ml BSA, 0.005% (v/v) Tween20, 0.025 mM DTT and 12.5 nM NS3h.

The ability of NS3 to hydrolyse ATP was monitored as described by Sweeney et al. [41]. Reactions performed in the presence of RNA contained 25 mM MOPS pH 6.5, 1.25 mM MgCl₂, 15 μM poly(U) RNA (Sigma), 6 nM NS3h in 5 μg/mL BSA, 0.001% Tween 20.

To determine the compound concentration needed to reduce activity by 50% (IC₅₀) in each of the above assays, reactions were performed in duplicate two-fold dilution series such that final compound
concentrations ranged from 1 mM to 0.78 µM. Data obtained from all reactions within the linear range of the assays were normalized to controls lacking inhibitor (100%) and controls lacking enzyme (0%), and fit to a normalized dose response equation with a variable Hill slope using GraphPad Prism (v. 6). Average IC₅₀ values ± standard deviations are reported.

To determine the amount of RNA needed for half maximal stimulation of ATP hydrolysis ($K_{RNA}$), steady state rates were fit to Eq. 1:

$$V = \left( \frac{V_{\text{max}} \times R}{K_{RNA} + R} \right) + V_b$$  \hspace{1cm} (Eq. 1)

In equation 1, $V_{\text{max}}$ is maximum ATP hydrolysis rate, $R$ is the concentration of poly(U) RNA, and $V_b$ is the basal rate of ATP hydrolysis in the absence of RNA.

Direct binding assays using intrinsic protein fluorescence were performed as described by Ndjomou (2012) [42] using truncated NS3 lacking the protease domain (NS3h) isolated from the JFH1 strain of HCV genotype 2a. After correcting for dilution and inner filter effects, fluorescence intensities (excitation: 280 nm, emission: 340 nm) were fit to Eq. 2.

$$F = F_e \times \left( \frac{E}{EL} \right) + F_s \times \left( \frac{L-EL}{E} \right) + F_c \times \left( \frac{EL}{E} \right)$$  \hspace{1cm} (Eq. 2)

where $EL = \frac{((K_d + L+E)-\sqrt{(K_d + L+E)^2-4*(L*E))}}{2}$

In eq. 1, $L$ is the total concentration of compound 13, $E$ is the concentration of NS3h, $K_d$ is the dissociation constant describing complex formation, $F_e$ is coefficient describing free protein fluorescence, $F_s$ is a coefficient describing free compound 13 fluorescence, and $F_c$ is a coefficient describing the fluorescence of the protein-ligand complex.

**List of abbreviations**

pegIFN= pegylated-Interferon

DAAs= Direct-Acting-Antivirals

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