The hepatitis C virus (HCV) non-structural protein 3 (NS3) is a complex multifunctional enzyme. In addition to processing the viral polyprotein, NS3 functions as a viral helicase capable of separating duplex RNA and DNA in reactions fuelled by ATP hydrolysis. A functioning helicase is necessary for HCV replication, showing that the NS3 helicase could be an antiviral drug target. Although early screens for HCV helicase inhibitors yielded few if any antiviral leads, more recent studies have found potent helicase inhibitors that are active against the HCV replicon. Noteworthy HCV helicase inhibitors that are relatively non-toxic to cells and inhibit the HCV replicon include triphenylmethanes, acridones, amidinoanthracyclines, and a rationally designed substituted pyrrole. Also discussed here are assay systems available for discovering and analysing HCV helicase inhibitors, which can broadly be grouped into two categories: those that measure helicase-catalysed hydrolysis of ATP, and those that measure helicase-catalysed separation of double-stranded nucleic acid substrates.

Current HCV therapy is toxic, expensive, and not effective against all HCV genotypes, in part, because interferon and ribavirin were not developed to specifically target HCV. Direct acting antivirals targeting proteins made by HCV and key events in the viral lifecycle might therefore be better than current treatments. Direct acting HCV antivirals, which are sometimes called specifically targeted antiviral therapy for HCV (STAT-C), have been discovered and some have shown marked success in clinical trials. Present STAT-C agents, like the polymerase and protease inhibitors, will need to be supplemented with other compounds if they are to replace current treatment because viruses resistant to most polymerase and protease inhibitors have already been identified. One possible STAT-C target is the hepatitis C virus NS3 helicase, which is needed for HCV replicon replication (Lam and Frick, 2006) and for HCV to replicate in chimpanzees (Kolykhalov et al., 2000).

The HCV helicase was first intensively studied as a drug target starting around 1993, but interest in developing helicase inhibitors as antivirals waned after early attempts failed to yield any promising lead compounds. In the intervening years new assays have been developed for screening, the molecular helicase mechanism has been unveiled and, most excitingly, a few compounds inhibiting HCV NS3 helicase-catalysed reactions have been shown to prevent viral RNA replication in the HCV replicon system. Even if these new inhibitors might not be valuable as lead compounds, they could be used to finally understand why HCV and similar positive sense RNA viruses need to encode a helicase to replicate in cells.

This chapter focuses mainly on assay systems, screening protocols and publicly disclosed HCV helicase inhibitors. We start, however, by briefly reviewing HCV helicase structure and function, highlighting the few studies that were published since we last reviewed the field (Frick, 2007; Belon and Frick, 2009a). If a reader is interested in how the compounds mentioned here might be further developed or used to study HCV biology, there are methods discussed in other
The HCV helicase
HCV non-structural protein 3 (NS3) is a multifunctional protein possessing two major functional domains. The N-terminal one third of the NS3 protein joins with NS4 to produce the mature NS3-NS4 protease, which is responsible for cleaving several locations in the HCV polyprotein to release mature proteins. The C-terminal two thirds of NS3 forms the helicase core, which catalyses the hydrolysis of ATP and uses the free energy to separate double stranded nucleic acids. Recombinant ‘full-length’ NS3 protein containing both the protease and helicase functional domains can be expressed in E. coli and purified. Recombinant NS3 retains its ATP hydrolysis and nucleic acid strand separating helicase activities.

A recombinant protein in which NS3 is truncated to remove the protease portion entirely, termed ‘NS3h’ for NS3-helicase fragment, can be more easily expressed to higher levels in E. coli. NS3h retains an ability to hydrolyse ATP and unwind nucleic acids, although its ability to unwind RNA is somewhat diminished (Frick et al., 2004; Beran et al., 2007). It should be remembered, also, that in most assays not all NS3h proteins are equal. Depending on where the protein is truncated and the location and nature of any attached fusion proteins, various NS3h constructs have remarkably different activities in almost all assays (Frick et al., 2004).

Role of the NS3 helicase in HCV replication
DNA helicases are clearly needed to separate the double helix so that DNA can be copied and transcribed into RNA. The biological roles of RNA helicases are more elusive, but they are probably needed for RNA folding or to modulate secondary structures (Cordin et al., 2006). The NS3 helicase likely separates local regions of double stranded RNA produced during viral replication, but it might also assist in the packaging of viral RNA into virions as has been seen with the closely related Kunjin virus (Liu et al., 2002). Genetic evidence hints at a similar relationship in HCV (Ma et al., 2008), and HCV helicase is most active at a somewhat acidic pH as is typical of the Golgi apparatus, where assembly likely starts (Lam et al., 2004). Other clues to a biological role might come from the fact that in cells, the helicase portion of NS3 protein is methylated (Duong et al., 2005), acetylated, and phosphorylated (Liehebber et al., 2009). The HCV helicase’s promiscuous substrate specificity also hints at possible role in modulating host gene expression. HCV is an RNA virus that is thought to replicate outside the nucleus yet, in addition to being capable of unwinding double stranded RNA, NS3 helicase unwinds double stranded DNA and RNA/DNA hybrids (Tai et al., 1996). The biological significance of HCV helicase action on DNA remains enigmatic, but NS3 has been spotted in the nucleus (Ishido et al., 1997; Muramatsu et al., 1997; Errington et al., 1999) and has the capacity to transform cells (Sakamuro et al., 1995).

Within the HCV replication complex, HCV helicase clearly interacts with itself and other proteins. In such a context, the helicase may function either as a monomer, dimer, or higher order oligomer (Jennings et al., 2009). The NS3 protease/helicase modulates the activity of the NS5B polymerase (Piccininni et al., 2002) and vice versa (Zhang et al., 2005; Jennings et al., 2008). The NS4A protease co-factor enhances the NS3–RNA interaction (Beran et al., 2009) and the presence of an intact NS3 protease domain enhances the ability of NS3 helicase to unwind RNA (Frick et al., 2004; Beran et al., 2007).

HCV helicase: structure and mechanics
In order to understand how compounds might inhibit HCV helicase, it is helpful to understand how HCV helicase is evolutionarily related to other ATP hydrolysing enzymes, its structure, and its functions. This information can be used to design assays, characterize compound specificity, and to rationally design a next generation of derivatives.
Precisely how HCV helicase is related to other viral and cellular helicases has been reviewed in detail previously (Frick and Lam, 2006), but it is important to mention some key points here because if a compound inhibits any particular helicase, it is possible that it will inhibit related helicases as well, sometimes with even greater potency (Zhang et al., 2003; Belon et al., 2010). Helicases are similar to each other and cellular motor proteins, proteasomes, chaperones, kinases, and small GTPases because all these proteins share a common ancestor. As evidence, each retains a ‘Walker-type’ nucleotide binding site formed by two sequence motifs called the Walker A and B sites (Walker et al., 1982). Helicase amino acid sequences also share several additional sequence motifs, which are used to classify helicases into families and these families into superfamilies (Hall and Matson, 1999; Gorbarenaya and Koonin, 2003). HCV helicase is part of the viral DEx/CH box family, which in turn is part of helicase superfamily 2 (SF2). Closely related helicases typically share many structural and functional features; for example HCV NS3 helicase and Dengue virus NS3 helicase share well-conserved sequences and consequently have three-dimensional structures that superimpose nicely.

Structure homology is not limited to closely related helicases. All the members of the helicase class of motors protein possess a similar three-dimensional fold that resembles one first seen in the bacterial recombination protein RecA (Story et al., 1992). The first crystal structure of HCV helicase showed a Y-shaped protein composed of three domains, two of which resemble the RecA (Yao et al., 1997). All helicase structures reported to date possess at least one ‘RecA-like’ motor domain, which function as the core of the helicase molecular motor. HCV helicase, like other SF1 and SF2 helicases has two Rec-A like motor domains linked in tandem on the same polypeptide.

To date, over ten atomic structures of HCV helicase have been deposited in the Protein Data Bank (PDB, www.pdb.org). The ATP and RNA binding sites on NS3 have been resolved (Kim et al., 1998; Mackintosh et al., 2006; Gu and Rice, 2010), and the various structures reveal the precise positions of various amino acid side chains when the protein assumes a variety of conformations. Two PDB files show NS3h from either HCV genotype 1a (PDB file 1HEI) or 1b (PDB file 8OHM) with its motor domains in either a closed or open conformation (Yao et al., 1997; Cho et al., 1998). PDB file 1CU1 shows full-length NS3 with a portion of NS4A tethered to its N-terminus (Yao et al., 1999). There are several views of NS3h bound to DNA oligonucleotides (Kim et al., 1998; Mackintosh et al., 2006; Gu and Rice, 2010). File 1A1V shows a single NS3h with a bound 8-nucleotide long polyU DNA oligonucleotide (Kim et al., 1998) and file 2F55 shows how two NS3h protomers might assemble in tandem on a 13-nucleotide long stretch of DNA (Mackintosh et al., 2006). File 3KQM and file 3KQK show NS3h bound to 6-nt long polymers of (d)A and (d)T, respectively. Precisely how the protein changes conformation upon ATP binding can be visualized by comparing file 3KQK with 3KQN, in which the ground state ATP-mimic ADP(BeF₃) is bound between the motor domains. Changes induced by ATP hydrolysis can be seen by comparing file 3KQK (or 3KQN) with 3KQL, in which ADP(AlF₄) mimics the transition state formed between the motor domains upon ATP hydrolysis. File 3KQU shows another ADP(BeF₃) complex bound to a longer 19-nt long (d)T oligonucleotide (Gu and Rice, 2010). Such a wealth of structural data is a goldmine for rational drug design.

Although all helicases are homologues, they have each evolved to fill a different biological niche. This divergent evolution is reflected in their different enzymatic activities, which vary with regard to substrate specificity, fuel specificity, and direction of movement. Each of these properties affects the design and selection of appropriate assay systems, and how the protein might interact with inhibitors. Because helicases are motor proteins, it is often helpful to classify them based on which direction they move along a nucleic acid strand. Helicase directionality can be probed by creating a partially duplex nucleic acid substrate with a short single-stranded ‘tail’ of about 20 nucleotides on either the 3′ or 5′ end. If the helicase requires a 3′ tail to catalyse strand separation, it likely moves in 3′–5′ direction,
and, inversely, if a S′ tail is required, the helicase likely moves in 5′–3′ direction. Since HCV NS3 helicase will only unwind nucleic acid that has a 3′ single-stranded region, it classified as a 3′–S′ helicase. Proving directionality is difficult, but two commonly used methods are to assess either ability of helicase to displace proteins bound to either the 3′ end or S′ end of oligonucleotides (Morris and Raney, 1999) or a helicase’s ability to quench end-labelled fluorescent oligonucleotides in a time-dependent manner (Fischer et al., 2004). Both of these experiments have been performed with HCV helicase and each suggests that the protein moves in a 3′ to S′ direction (Morris et al., 2002; Matlock et al., 2010).

Exactly how HCV helicase unwinds RNA or DNA is still hotly debated and has been the subject of other reviews (Frick, 2007; Pyle, 2008). Most models assume HCV helicase acts like a molecular inchworm, crawling along nucleic acids by rotating the second motor domain relative to the rest of the protein in a reaction modulated by ATP binding and hydrolysis. Nucleotide modulated Rec-A like motor domain rotation, and how it might move the protein on DNA, was recently visualized in the above series of structures of NS3h bound to nucleotide analogues. In those structures, nucleotide driven conformational changes appear to transfer individual backbone phosphates between two nucleic acid binding sites, located along the nucleic acid binding surface of the two RecA-like motor domains (Gu and Rice, 2010). Other key recent developments include new mathematical models that can be used to analyse single molecule studies (Arunajadai, 2009) like those previous published with NS3 (Dumont et al., 2006; Myong et al., 2007), a plausible explanation for the very different kinetic step sizes that have been reported in various studies (Serebrov et al., 2009), and a clear demonstration that phosphate release limits how rapidly ATP hydrolysis can fuel RNA unwinding (Wang et al., 2010).

Methods to identify and analyse helicase inhibitors

Helicases are enzymes that use the energy derived from NTP hydrolysis to separate regions of double stranded nucleic acid. In this reaction, ATP is considered an activator and the nucleic acid is the substrate. As such, it is possible to measure helicase activity in at least two ways: by measuring the cleavage of ATP or by measuring the conversion of double stranded nucleic acid into single strands. Many methods have been developed to accomplish either of these goals, and each method has advantages and disadvantages, but they all can provide valuable mechanistic insights.

ATPase assays

To fuel its movement on nucleic acids, HCV helicase catalyses water-mediated cleavage of ATP into ADP and inorganic phosphate (Pi). The rate of ATP hydrolysis can be determined by measuring the loss of ATP, the appearance of ADP, or the appearance of Pi.

The most direct method to monitor helicase-catalysed ATP hydrolysis is to measure amounts of ATP and ADP in helicase reactions using high performance liquid chromatography (HPLC) or thin layer chromatography (TLC). In either case, the substrate and product can be detected based on the fact that nucleotides absorb ultraviolet (u.v.) light (Du et al., 2002). More sensitive assays use ATP labelled with a radioisotope. For example, hydrolysis of ATP can be directly measured by combining helicase, buffer, divalent metal, nucleic acid, and [32P]ATP. After stopping the reaction with acid or a metal chelator, products can be separated the using TLC, and quantified by exposing the dried plate to X-ray film (Suzich et al., 1993) or a phosphorimager screen (Howe et al., 1999). Such an assay measures both [a32P] ATP and [a32P]ADP, but not the amount of Pi liberated. By substituting [32P]ATP, the phosphate product can be measured either by using HPLC, TLC, or by simply adding activated charcoal (i.e. Norit; Lam et al., 2003a). Norit absorbs nucleotides and can be rapidly removed by centrifugation or filtration, leaving only phosphate in the supernatant. Assays using radiolabelled nucleotides are remarkably sensitive. Because only the specific activity of ATP limits sensitivity, such assays are routinely performed with sub-nanomolar substrate concentrations in a few microlitres. Although [32P]ATP is typically used for this type of assay, ATP labelled with other
nuclides is commercially available as are other radiolabelled nucleoside triphosphates and some ATP analogues. The disadvantages to this procedure are that use of radioisotopes can be relatively hazardous, and the specialized equipment needed to safely handle and measure radioisotopes is not always available.

Although not as sensitive as the above radioisotope-based assays, there are numerous high-throughput methods available to monitor ATP hydrolysis by measuring the substrate or products using a spectrophotometer (Table 12.1). Phosphate can be measured with one of the many colorimetric assays that use the Fiske-Subbarow ammonium molybdate reagent (Fiske and Subbarow, 1925). For example, an acidic ammonium molybdate solution can be added to a HCV helicase-catalysed reaction after nucleotides are removed using Norit (Lam et al., 2003a). Alternatively, phosphate can be determined without removing ATP using a modified procedure in which colour is amplified with the dye malachite green molybdate. In such an assay, a phosphate complex forms that absorbs light at 630 nm (Lanzetta et al., 1979). Helicase, metal, and ATP are combined in reaction buffer, incubated for a period of time, then quenched with an acidic solution of malachite green and molybdate. A citrate solution is then quickly added, absorbance is measured, and the amount of phosphate generated determined by comparison to a standard curve. For 30 μl helicase reactions performed in microplates, such an assay has a linear range between 10 μM and 200 μM phosphate (Frick et al., 2007; Belon and Frick, 2009b).

Phosphate production can also be measured continuously by coupling its production to other reactions that can be directly monitored. For example, the enzyme purine nucleoside phosphorylase (PNPase), which normally functions without removing ATP using a modified procedure in which colour is amplified with the dye malachite green molybdate. In such an assay, a phosphate complex forms that absorbs light at 630 nm (Lanzetta et al., 1979). Helicase, metal, and ATP are combined in reaction buffer, incubated for a period of time, then quenched with an acidic solution of malachite green and molybdate. A citrate solution is then quickly added, absorbance is measured, and the amount of phosphate generated determined by comparison to a standard curve. For 30 μl helicase reactions performed in microplates, such an assay has a linear range between 10 μM and 200 μM phosphate (Frick et al., 2007; Belon and Frick, 2009b).

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Table 12.1 High-throughput screening assays useful for HCV helicase inhibitor discovery and analysis

<table>
<thead>
<tr>
<th>Assay</th>
<th>Reaction monitored</th>
<th>Equipment needed</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luciferin</td>
<td>ATP hydrolysis</td>
<td>Luminescence spectrophotometer</td>
<td>Koresawa and Okabe (2004)*</td>
</tr>
<tr>
<td>NADH</td>
<td>ADP production</td>
<td>UV–vis spectrophotometer (A&lt;sub&gt;340&lt;/sub&gt;)</td>
<td>Suzich et al. (1993)</td>
</tr>
<tr>
<td>Malachite Green</td>
<td>P&lt;sub&gt;i&lt;/sub&gt; production</td>
<td>UV–vis spectrophotometer (A&lt;sub&gt;630&lt;/sub&gt;)</td>
<td>Frick et al. (2007), Belon and Frick (2009b)</td>
</tr>
<tr>
<td>MESG</td>
<td>P&lt;sub&gt;i&lt;/sub&gt; production</td>
<td>UV–vis spectrophotometer (A&lt;sub&gt;360&lt;/sub&gt;)</td>
<td>Funk et al. (2004)*</td>
</tr>
<tr>
<td>MDCC-PBP</td>
<td>P&lt;sub&gt;i&lt;/sub&gt; production</td>
<td>Fluorescence spectrophotometer</td>
<td>Wang et al. (2010)</td>
</tr>
<tr>
<td>GlassMilk: Filter plates</td>
<td>Oligo:M13 unwinding</td>
<td>Membrane filter plates, plate washer, microplate scintillation counter</td>
<td>Sivaraja et al. (1998)*</td>
</tr>
<tr>
<td>SPA</td>
<td>DNA unwinding</td>
<td>SPA beads, microplate scintillation counter</td>
<td>Kyono et al. (1998)</td>
</tr>
<tr>
<td>FlashPlate</td>
<td>DNA unwinding</td>
<td>FlashPlates, microplate scintillation counter</td>
<td>Hicham Alaoui-Ismaili et al. (2000)</td>
</tr>
<tr>
<td>ECL</td>
<td>DNA unwinding</td>
<td>Magnetic flow cell, ECL instrument</td>
<td>Zhang et al. (2001)*</td>
</tr>
<tr>
<td>ELISA</td>
<td>RNA unwinding</td>
<td>UV–vis spectrophotometer (A&lt;sub&gt;450&lt;/sub&gt;)</td>
<td>Hsu et al. (1998)</td>
</tr>
<tr>
<td>FRET</td>
<td>DNA or RNA unwinding</td>
<td>Fluorescence spectrophotometer</td>
<td>Porter et al. (1998), Boguszewska-Chachulska et al. (2004), Tani et al. (2010)</td>
</tr>
<tr>
<td>TR-RET</td>
<td>DNA unwinding</td>
<td>Fluorescence spectrophotometer</td>
<td>Earnshaw et al. (1999)*</td>
</tr>
<tr>
<td>MBHA</td>
<td>DNA or RNA unwinding</td>
<td>Fluorescence spectrophotometer</td>
<td>Belon and Frick (2008)</td>
</tr>
</tbody>
</table>

*Protocol refers to use with a protein other than HCV helicase.
for purine salvage, also acts on nucleoside analogs whose absorbance spectrums change upon phosphorolysis, such 7-methylthioguanosine (MESG). MESG absorbs light at 330 nm and when phosphate is present, PNPase converts MESG to ribose-1-phosphate and a base (2-amino-6-mercapto-7-methylpurine) that absorbs at a longer wavelength (Webb, 1992). Since the hydrolysis of ATP by the HCV NS3 helicase produces ADP and $P_i$, the activity of the enzyme is linked to the appearance of an absorbance at 360 nm. This assay is sensitive to $\sim 2 \mu M$ phosphate. One concern when using this assay with HCV helicase is that the $k_{cat}$ of the PNPase ($\sim 40 s^{-1}$) is slower than the $k_{cat}$ seen with HN NS3h under optimal conditions (Frick et al., 2007). The PNPase reaction is most useful, however, because it is essentially irreversible, and therefore can be used to remove phosphate contaminates from ATP or other reagents. Such a ‘phosphate mop’ is necessary when one desires to precisely measure very small amounts of phosphate produce when a helicase cleaves ATP.

Helicase-catalysed phosphate release can be most precisely measured using a fluorescently labelled E. coli phosphate binding protein (PBP). This assay uses a site directed mutant of the E. Coli PBP that contains a cysteine to which the fluorescent molecule N-[2-(1-maleimidyl)ethyl]-7-diethylamino)coumarin-3-carboxamide (MDCC) is attached. In the presence of $P_i$, the emission maxima of MDCC-PBP changes from 474 nm to 464 nm (when excited at 425 nm) and fluorescence changes by approximately 5-fold (Brune et al., 1994). To use this very sensitive assay, $P_i$ contaminating ATP or any other reagents must first be removed using MESG and PNPase to mop $P_i$ contamination to less than 0.1 $\mu M$ (Brune et al., 1994). MDCC-PBP is now commercially available (e.g. the ‘phosphate sensor’ from Invitrogen), making this assay relatively easy to set up if a fluorescence spectrophotometer is available (Wang et al., 2010).

Rather than measuring $P_i$, HCV helicase-catalysed ATP hydrolysis can also be monitored by coupling the production of ADP with other enzymes that use ADP as a substrate. For example, when pyruvate kinase (PK) is include in a HCV helicase reaction, along with lactate dehydrogenase (LDH), phosphoenolpyruvate (PEP) and reduced nicotinamide adenine dinucleotide (NADH), ATP hydrolysis will be proportional to NADH oxidation, which can be followed with a spectrophotometer (Suzich et al., 1993). The ADP produced will react with PEP via PK to produce ATP and pyruvate. LDH then reduces pyruvate using NADH to produce lactate and NAD$^+$. Since NADH absorbs at 340 nm while NAD$^+$ does not, this assay can be continuously monitored so that rates can be directly measured. Sensitivity is limited, though, by the extinction coefficient of NADH ($\epsilon_{540} = 6.22 mM/cm$), and any compounds that absorb light at 340 nm or that inhibit PK or LDH will interfere with this assay.

For screening purposes, probably the best method to identify compounds in inhibiting the NS3 ATPase is use a one of the many available luminescent ATP assays (Funk et al., 2004). As is routinely done with kinases (Koresawa and Okabe, 2004) and related proteins, ATP remaining after a helicase reaction is terminated can be measured using the enzyme luciferase to react residual ATP with luciferin, producing a luminescent signal. By comparing the amount of luminescence at the end of a helicase reaction to a control reaction, the amount of ATP consumed can be calculated; the amount of luminescence produced is directly proportional to the amount of ATP remaining in the reaction mixture and thus inversely proportional to ATPase activity. These assays are commercially available (e.g. Promega’s Kinase Glo Kits), and are amenable to screening since only compounds that inhibit luciferase should interfere with the reaction (Koresawa and Okabe, 2004). They are also linear over a wide range of ATP concentrations (1 $\mu M$ to 500 $\mu M$). Such an assay is less useful for precise ‘steady-state’ kinetic analysis because an accurate measurement requires consumption of more than 20% of the ATP substrate.

The above ATPase assays are useful not only for studying HCV NS3 helicase function, but also for analysing the mechanism of action of helicase inhibitors. Any of the above assays could be used for screening, but if they are, hit compounds need to also be tested for their ability to inhibit any number of other similar ATPase enzymes.
Non-specific compounds are more likely to prove toxic in cell-based replicon assays. From the point of view of inhibitor discovery, simply screening for ATPase inhibitors may miss compounds functioning by another mechanism such as interaction at the RNA binding site or a possible allosteric site.

**Unwinding assays**

The ability of a helicase to separate a double helix was first demonstrated using a radiolabelled DNA substrate and S1 nuclease, which only degrades duplex DNA. In such a rudimentary helicase assay, single-stranded DNA products can be estimated by measuring the amount of acid soluble radioactivity produced in the presence of helicase (Abdel-Monem *et al.*, 1976; Hotta and Stern, 1978). Today, the prototype helicase assay measures the conversion of double stranded to single-stranded nucleic acids using isotope-labelled oligonucleotides.

The substrates for these assays are typically made of a labelled shorter strand annealed to a longer complementary strand. Helicase, buffer, divalent metal ions (e.g. Mg$^{2+}$) and ATP are then combined, the reaction allowed to proceed for some time, and metal chelating agents are added to terminate the reaction. Products are then separated using non-denaturing polyacrylamide gel electrophoresis, and detected with autoradiography or a similar method (Matson and Kaiser-Rogers, 1990). Since single-stranded nucleic acids run faster in such a gel, the amount of single-stranded product formed can be used to calculate reaction velocity. With HCV helicase, the shorter oligonucleotide needs to be annealed to a complementary longer strand to leave a 3′ tail necessary for HCV helicase to function. During the reaction (or shortly after the reaction is stopped) a third oligonucleotide must be added that is complementary to one strand to ‘trap’ reaction products and prevent the substrate from re-forming. This process is extremely sensitive, limited only by the specific activity of the labelled DNA probe, and is still the gold standard in the field today. The helicase assays outlined below illustrate how this basic assay can be modified to provide a higher throughput or so that more data points can be obtained to follow reaction kinetics in real time (Table 12.1).

The above assay can be directly used for high throughput screening by employing DNA binding beads (e.g. GeneClean Glassmilk, Qbiogene) and filter plates, as was done to identify an inhibitor of the helicase encoded by herpes simplex virus (HSV; Sivaraja *et al.*, 1998). In this high throughput helicase assay, the substrate is made of a labelled-oligonucleotide annealed to M13 viral DNA. Reactions are performed in well plates with hydrophobic membranes on the bottom, and the beads are added to bind the viral DNA (and any remaining annealed oligonucleotide) along with a detergent. When the plates are then washed, the labelled single-stranded product of the helicase reaction is removed, and only substrate remains in the wells, which can be measured using a scintillation counter.

While the above bead-based assay requires a long DNA substrate, shorter helicase substrates can be monitored in a high throughput format using oligonucleotides and a scintillation proximity assay (SPA). Kyono *et al.* developed a SPA for HCV helicase, which uses a substrate made of a $^3$H-labelled oligonucleotide annealed to an unlabelled RNA oligonucleotide. When the helicase reaction is run, the $^3$H-labelled strand is released, and it is captured with a complementary biotinylated oligonucleotide that is present in high concentration in the reaction mixture. This biotin-labelled $[^3H]$DNA duplex then binds to the scintillant bead via biotin/streptavidin, allowing for detection (Kyono *et al.*, 1998). SPA beads emit photons only when they directly complex radioisotopes. A similar assay using a duplex RNA substrate is now commercially available (GE Healthcare).

A FlashPlate (NEN Lifesciences/Perkin Elmer) assay is a modified scintillation proximity assay where the well plate itself is coated with a scintillant rather than using a bead scintillant. A DNA oligonucleotide directly bound to the plate by a biotin linker is annealed to a complementary $[^33P]$DNA (Earnshaw and Pope, 2001). When a helicase and ATP is added, the radiolabelled DNA is released into solution, away from the scintillant coating of the well, and lower counts are recorded. Such an assay is advantageous in
that the plates can be read at any point without the need to quench the reaction (Earnshaw and Pope, 2001). Such a FlashPlate assay was used by Hicham Alaoui-Ismaili et al. (2000) to measure HCV helicase-catalysed unwinding of DNA in a high-throughput format.

A HCV helicase HTS-capable unwinding assay that eliminates the need for radioisotopes is based on electrochemiluminescence (ECL) and was tested by Zhang et al. (2001) using the DnaB helicase. The ECL substrate is made by attaching a DNA nucleotide to a ruthenium chelate which is in turn annealed to an unlabelled DNA (Zhang et al., 2001). A helicase reaction is run in the presence of a trapping strand that is conjugated to biotin, and the trapped reaction product complex is captured on streptavidin-coated magnetic beads. Application of an electric potential causes the excitation of the ruthenium chelate at the electrode, resulting in the chemiluminescent emission of photons in proportion to the amount of single-stranded DNA produced in the helicase reaction.

HCV helicase-catalysed RNA unwinding can also be measured in a high throughput format using an enzyme linked immunosorbent assay (ELISA; Hsu et al., 1998). The helicase ELISA substrate is composed of a digoxigenin (DIG) labelled RNA annealed to a biotin-labelled RNA. The biotin of the ELISA substrate is bound to a streptavidin coated well plate, then helicase and ATP are added to initiate RNA unwinding (Hsu et al., 1998). After incubating for an hour, plates are washed to remove free DIG-labelled RNA and anti-DIG antibody is added and detected using standard techniques (Hsu et al., 1998). Both biotin- and DIG- labelled oligonucleotides are commercially available.

Most fluorescent helicase assays rely on Förster resonance energy transfer (FRET), which involves two chromophores referred to as the ‘donor’ and the ‘acceptor.’ For FRET to occur, the emission wavelength of the donor must overlap the absorption wavelength of the acceptor. Light of a defined wavelength is used to excite the donor, and either fluorescence emission of the acceptor or the donor is monitored. If the acceptor chromophore is not fluorescent, it will simply act to quench the fluorescence of the donor. In most fluorescent helicase assays the donor and acceptor chromophores are tethered to complementary strands of DNA. As the name FRET implies, a photon is not involved in the transfer of energy from donor to acceptor: it is a dipole–dipole interaction and the efficiency of this transfer falls off rapidly as distance between donor and acceptor increases, as it would if the donor and acceptor are bound to two complementary DNA strands that are separated by a helicase (Lakowicz, 1999).

A wide variety of chromophores have been used in FRET-based unwinding assays. The original helicase FRET assays used coumarin as a donor and fluorescein as an acceptor (Houston and Kodadek, 1994), or a fluorescein/hexachlorofluorescein donor/acceptor pair (Bjornson et al., 1994). The later assay was used by Porter et al. to study HCV NS3h in early mechanistic studies (Porter et al., 1998). Other FRET assays used for HCV helicase high throughput screens have employed a Cy3/Black hole Quencher donor/acceptor pair (Boguszewska-Chachulska et al., 2004), or an Alexa Fluor 448/Black Hole Quencher pair (Tani et al., 2010). Similar fluorescence assays can be used to monitor unwinding with the DNA itself acting as the acceptor chromophore. For example, fluorescence of a DNA oligonucleotide containing the fluorescent base 2-aminopurine in place of adenine decreases when it is annealed to a complementary strand (Raney et al., 1994). Similarly, some fluorescent moieties, such as BODIPY FL, can be quenched through photoinduced electron transfer by nearby guanines in complementary strands (Tani et al., 2009).

The above fluorescent helicase assays are all set up like the prototype radioisotope-based helicase assay except that the trapping oligonucleotide must always be added at the start of the reaction. Otherwise, no reaction will be observed when the acceptor-bearing strand and the donor-bearing strand re-anneal. How additional DNA strands might interact with HCV helicase is not precisely known, although a recent report suggest that HCV helicase might simultaneously interact with three complementary strands of DNA to facilitate strand exchange in a reaction reminiscent of those catalysed by similar helicases needed for DNA recombination (Rypma et al., 2010). Spurred by the idea that HCV helicase might function
to primarily rearrange nucleic acids in such a manner, we recently developed a FRET-based helicase assay that utilizes dual-labelled oligonucleotides, called molecular beacons, which are capable of forming both hetero-duplexes and hairpin structures (Tyagi and Kramer, 1996). Such a molecular beacon-based helicase assay (MBHA) has the advantage over other FRET-based assays in that no trap is required in the reaction (Belon and Frick, 2008). Upon strand separation by HCV NS3, the beacon forms a hairpin, bringing the donor and acceptor next to each other, and fluorescence is quenched. By utilizing a molecular beacon, each strand acts as its own trapping agent, making the reaction essentially irreversible. Also, since the donor and acceptor modifications are located on the same strand, and opposite the strand on which the helicase likely translocates, possible protein–nucleotide interactions are minimized. The MBHA can also be performed in the same vessel as fluorescent NS3 protease assays so that both activates of NS3 can be simultaneously monitored (Frick et al., 2010).

The most common problem seen when fluorescence-based assays are used for high throughput screens is that compounds that absorb or emit light at either the excitation or emission wavelengths will interfere with the assay, either by fluorescing themselves or by quenching the fluorophore being monitored. The two most common methods used to attempt to circumvent compound interference involve monitoring either changes in fluorescence polarization (or anisotropy) or time-resolved fluorescence, instead of fluorescence intensity. Polarization changes when a fluorescence probe-labelled oligonucleotide is displaced from its complement, and such assays have been extensively been used to monitor helicase action (Xu et al., 2003). A time resolved fluorescence energy transfer (TR-FRET) assay, featuring a Eu$^{3+}$/tetramethyl rhodamine donor/acceptor, has also been used in high throughput screens to identify several novel bacterial and viral helicase inhibitors (Earnshaw et al., 1999).

The above list of assays that could be used to screen for inhibitors of HCV helicase-catalysed DNA or RNA unwinding is by no means exhaustive. There are a seemingly endless variety of assays that monitor the action of helicases on DNA or RNA, and numerous HCV helicase assays have been reported that might also be useful for screening and for analysing the effects of the inhibitors listed below. Notable other assays include one that can be used to track NS3 progression along duplex RNA (Serebrov and Pyle, 2004), a procedure that monitors helicase action on an RNA thread held with optical tweezers (Dumont et al., 2006), and a procedure that uses single molecule fluorescence to monitor HCV helicase action on DNA (Myong et al., 2007). More detailed reviews and protocols for these and other helicase can be found in the recent book Helicases: Methods and Protocols (Abdelhaleem, 2010). The selection of a helicase assay depends on experimental goals as well as the time, resources, and instruments available.

**Compounds that inhibit HCV helicase**

Helicases are difficult targets as evidenced by the fact that no helicase inhibitors are approved for clinical use. There has been some preclinical success, however, in developing inhibitors of the herpes simplex virus (HSV) helicase-primase as antiviral drugs (Crute et al., 1995; Crute et al., 2002; Kleymann et al., 2002) and, for this reason alone, all HCV helicase inhibitors should be critically examined for those that might have antiviral potential. Below follows a list of known HCV helicase inhibitors grouped based on where they might interact with NS3, along with summaries of their known potency. For each compound, up to three values are listed that describe its ability to inhibit HCV helicase in helicase assays by 50% (IC$_{50}$), its ability to inhibit the HCV replicon by 50% (EC$_{50}$), and its cellular toxicity reported as the concentration of compound necessary to kill half the cells (CC$_{50}$). When discussing compounds that were tested in replicons, we also report a ‘selectivity index’ (SI), defined here as the simple ratio EC$_{50}$/CC$_{50}$. The SI is a measure of how effective a compound is at inhibiting cellular HCV RNA replication compared with its toxicity in cells. A high SI indicates that the compound is relatively non-toxic. In addition to small molecules, we also briefly discuss small peptide inhibitors, which might be useful in designing peptidomimetic compounds. Other inhibition
strategies have been investigated as well, such as the use of antibodies inhibiting NS3 helicase (Tessmann et al., 2002; Prabhu et al., 2004) and RNA aptamers (Hwang et al., 2004), but these are not discussed here. These alternate approaches have been reviewed previously in detail (Frick and Lam, 2006; Frick, 2007).

Compounds that likely displace ATP
Most nucleoside monophosphates (NMP), nucleoside diphosphates (NDPs), and nucleoside triphosphates (NTPs) bind HCV helicase weakly with dissociation constants that mimic what is seen with ATP. In the presence of RNA, HCV helicase binds ATP with an apparent $K_d$ of 7 mM and in the absence of RNA the protein binds ATP with $K_d$ of 0.9 mM (Wang et al., 2010). In our lab, we have found that all NTPs tested, including ribavirin triphosphate and the non-obligate chain terminators used as NS5B inhibitors, can substitute for ATP and fuel unwinding assays. Guanine nucleoside derivatives are typically hydrolysed more slowly and support the slowest unwinding rates. As such, under certain conditions, high concentrations of GTP can inhibit ATP hydrolysis and ATP-fuelled unwinding (Heck et al., 2008; Belon and Frick, 2009b). Others have also observed that NTPs with inverted sugars such as L-β-dATP, L-β-dTTP, L-β-dCTP and L-β-ddCTP are also very poor fuels for the unwinding reaction (Locatelli et al., 2001). Non-hydrolysable NTP analogues, such as ±/β-methylene-ATP and γ,β-imido-ATP, are poor inhibitors of HCV catalysed DNA unwinding with $IC_{50}$ values of 140 μM and 1.8 mM, respectively (Belon and Frick, 2008).

Some modified nucleosides appear to interact somewhat more tightly with HCV helicase than the above NTPs. For example, some potent compounds were found in screens of ring-expanded, or so-called ‘fat nucleosides’ (Fig. 12.1A). Fat nucleosides were tested for their ability to inhibit the ATPase and helicase action of NS3

Figure 12.1 Nucleoside and base analogue helicase inhibitors presumed to bind to ATP binding site. (A) ‘Fat’ nucleoside analogue 24 (Zhang et al., 2003). This compound inhibits helicase-catalysed DNA unwinding with an $IC_{50}$ of 11 μM, and inhibits RNA unwinding with an $IC_{50}$ of 5.5 μM. This compound also inhibits WNV helicase (DNA $IC_{50}$=5.7 μM, RNA $IC_{50}$=3.3 μM), JEV helicase (DNA $IC_{50}$=150 μM, RNA $IC_{50}$>500 μM) and human Suv3 helicase (DNA $IC_{50}$=5 μM, RNA $IC_{50}$=6 μM). (B) Halogenated nucleoside or base analogues. 5,6-dichloro(ribofuranosyl)benzimidazole (DRBT) inhibits helicase-catalysed DNA unwinding with an $IC_{50}$ of 1.5 μM but the closely related 5,6-dichloro(ribofuranosyl)benzimidazole (DRB) inhibits unwinding with an $IC_{50}$ of 450 μM (Borowski et al., 2003). Tetrabromobenzimidazole (TBBT) inhibits HCV NS3 catalysed DNA unwinding with an $IC_{50}$ of 20 μM (Borowski et al., 2003). Both DRBT and TBBT are active in various different replicons with $EC_{50}$ of ~10–65 μM and no observed toxicity up to 100 μM (Paeshuysje et al., 2008). (C) Compound 4 is an aminotriazine modified analogue of 5-aminoimidazole-4-carboxamide ribotide (AICAR), an intermediate in the purine biosynthesis pathway. It inhibits helicase-catalysed DNA unwinding with an $IC_{50}$ of 37 μM but does not inhibit RNA unwinding (Ujijnamata et al., 2007).
helicases from HCV, West Nile virus (WNV), and Japanese encephalitis virus (JEV; Zhang et al., 2003). Fat nucleoside compound 24 (Fig. 12.1A) inhibits HCV helicase-catalysed DNA and RNA unwinding with an IC\textsubscript{50} of 11 μM and 5.5 μM, respectively. Although this series of analogues would seem likely to compete for the NS3 ATP binding site, none of the ring-expanded nucleosides inhibit the ATPase activity of JEV, HCV or WNV helicases. The efficacy of fat nucleosides is not isolated to flaviviral helicases. The human cellular DEAD box helicase, DDX3, which has been shown to be necessary for HIV replication (Yedavalli et al., 2003), is also inhibited by fat nucleosides, and this effect is sufficient to halt HIV replication in cell culture (Yedavalli et al., 2008).

Other nucleoside analogues that inhibit HCV helicase are built on benzazole base scaffolds. Halogenated benzotriazoles (Fig. 12.1B), such as 5,6-dichloro(ribofuranosyl)benzotriazole (DRBT) have been tested on HCV, WNV and JEV NS3 helicases, and they inhibit DNA unwinding by HCV helicase selectively with an IC\textsubscript{50} of 1.5 μM. They do not inhibit WNV or JEV helicases at concentrations up to 500 μM (Borowski et al., 2003). The importance of the benzotriazole ring was demonstrated by replacing it with a benzimidazole (Fig. 12.1B). The benzimidazole nucleosides are much less potent with an IC\textsubscript{50} of 450 μM (Borowski et al., 2003). A halogenated benzotriazole lacking the ribose moiety, tetrabromobenzotriazole (TBBT; Fig. 12.1B), is also somewhat effective in inhibiting HCV catalysed DNA unwinding with an IC\textsubscript{50} of 20 μM, but it is more potent against WNV NS3 helicase with a 1.7 μM IC\textsubscript{50} (Borowski et al., 2003). Both DRBT and TBBT have been tested in four different replicon cell lines and they both inhibit HCV replication (DRBT EC\textsubscript{50} values of 10–33 μM, TBBT EC\textsubscript{50} values of 40–65 μM). Neither is toxic at concentrations up to 100 μM (Paeshuyse et al., 2008).

Nucleoside analogue 4, an analogue of an intermediate in the purine biosynthesis pathway (Fig. 12.1C), was tested in helicase assays against WNV, HCV, JEV and DV helicases. The compound inhibited DNA unwinding by the HCV helicase with an IC\textsubscript{50} of 37 μM, and was slightly more potent against the WNV helicase with an IC\textsubscript{50} of 23 μM, but was ineffective against JEV and DV helicases. Surprisingly, when the substrate was switched from DNA to RNA, no inhibition of unwinding was observed for any of the helicases (Ujjinamatada et al., 2007).

Only two classes of HCV inhibitors that do not resemble nucleosides are believed to target the HCV helicase ATP binding site. The first class in made of pyrophosphate analogues. The pyrophosphate analogue imidodiphosphate, (Fig. 12.2A) has a K\textsubscript{i} of 12 μM in assays measuring the HCV NS3 catalysed ATP hydrolysis, and pyrophosphate itself appears in such assays to bind HCV NS3 significantly tighter than any nucleoside analogue with an K\textsubscript{i} of 20 μM (Mukovnya et al., 2008). The other class comprises compounds derived for the dye soluble blue HT. Hu et al. used molecular modelling to identify soluble blue HT (Fig. 12.2B) as a potential NS3 helicase inhibitor (Hu et al., 2003). They also solved a co-crystal structure (PDB file 2ZJO) that shows soluble blue HT bound to NS3 with one of its three benzene sulfonate moieties directly adjacent to the catalytic Walker A site (SF2 helicase motif I). Such an arrangement would prevent the enzyme from binding and hydrolysing ATP. Blue HT inhibits HCV NS3 catalysed DNA unwinding with an IC\textsubscript{50} of 40 μM (Chen et al., 2009). Blue HT was later subjected to several rounds of structural refinement by creating derivatives and assaying compounds for efficacy, eventually resulting in the discovery of compound 12 (Fig. 12.2B), which inhibits HCV NS3-catalysed DNA unwinding with an IC\textsubscript{50} of 10.1 μM. Compound 12 inhibits an HCV subgenomic replicon with an EC\textsubscript{50} of only 2.7 μM and a CC\textsubscript{50} of 10.5 μM, a significant increase in selectivity index (i.e. EC\textsubscript{50}/CC\textsubscript{50}) compared with other active compounds in the series that had specificities of approximately 1.4–1.5 (Chen et al., 2009).

Compounds acting at the DNA/RNA binding site

Even though the atomic details of how HCV helicase interacts with nucleic acids have been known for over ten years (Kim et al., 1998), only a few classes of compounds are known to bind this site. Probably the largest class is the set of symmetrical
benzimidazole-phenyl-carboximides that were first reported in a series of patents by Viropharma (Diana and Bailey, 1997) and were later the subject of a detailed structure activity relationship study (Phoon et al., 2001). We have recently studied the mechanism of action of the prototype compound in this series: a head-to-head dimer composed of benzimidazole-phenyl-carboxamide subunits linked to benzene we term (BIP)$_2$B (Belon et al., 2010; Fig. 12.3A). (BIP)$_2$B inhibits DNA and RNA unwinding with IC$_{50}$'s of 0.7 to 5 μM, and binds the HCV NS3h protein in place of nucleic acid, preventing the action of the helicase on its substrate (Belon et al., 2010). The compound also inhibits related helicases from Dengue virus (DV), Japanese encephalitis virus (JEV) as well human DDX3 helicase, and binds DV and JEV helicases substantially tighter than either HCV helicase or DDX3, suggesting (BIP)$_2$B may be an more effective inhibitor of the flaviviral helicases. (BIP)$_2$B does not inhibit the HCV replicon.

Maga et al. describe a series of quinoline and quinoxalines that mimic the electronic characteristic of purines and pyrimidines and compete with nucleic acid but do not affect ATPase activity (Maga et al., 2005). Through progressive rounds of synthesis, molecular modelling and inhibition assays, they created QU663 (Fig. 12.3B), a quinoline-based compound that inhibits DNA unwinding with a K$_i$ of only 750 nM. QU663 and is not toxic to 3T3, NSO, Daudi, or normal human lymphocyte cells (Maga et al., 2005), but was not reportedly tested in a HCV replicon system. Molecular modelling supports the idea that QU663 could bind the RNA binding groove, and was predicted to interact strongly with W501 as well as other key residues located in the RNA binding site including Phe557, Arg393, Thr298, Gln434, and Glu493 (Maga et al., 2005).

Kandil et al. used molecular modelling to de novo design an NS3 helicase inhibitor that binds in place of RNA (Kandil et al., 2009). First, using the crystal structure of NS3h bound to a DNA oligonucleotide (Kim et al., 1998) they sought to target a region surrounding Arg393, a residue that is critical for RNA unwinding (Lam et al., 2003b) because it interacts with the DNA/RNA backbone. Because Arg393 is entirely solvent exposed, the group hypothesized that a molecule interacting with the protein near Arg393 would be significantly stabilized if it formed a covalent bond with an adjacent cysteine, Cys431 (Kandil et al., 2009). The parameters of the initial docking and model building were tailored to produce molecules of comparatively small size with few
chiral centres. The necessity of the covalent bond-forming Michael acceptor was verified by the target compound, compound 4 (Fig. 12.3C), inhibiting HCV NS3 catalysed DNA unwinding with an IC\textsubscript{50} of only 0.26 \( \mu \text{M} \) (Kandil \textit{et al.}, 2009).

In docking simulations, regioisomer 12 (Fig. 12.3C) bound in a conformation where the reactive Michael acceptor was distant to Cys431 and showed no \textit{in vitro} activity at all, up to the 100 \( \mu \text{M} \) tested. Compound 4 was then tested in an HCV subgenomic replicon and demonstrated an EC\textsubscript{50} of 3 \( \mu \text{g/mL} \) (9 \( \mu \text{M} \)) but killed cells with a CC\textsubscript{50} of 10 \( \mu \text{g/mL} \) (30 \( \mu \text{M} \); Kandil \textit{et al.}, 2009).

**Composed of tropolones, thiadiazoles, and substituted pyrimidines.**

The acridone derivatives are particularly intriguing because they were discovered to be helicase inhibitors independently by two different groups using different approaches. The first group to characterize acridone-based helicase inhibitors initially screened a library of acridone derivatives with unwinding assays. Two of the compounds, 20 and 27 (Fig. 12.4A) inhibit DNA unwinding with IC\textsubscript{50}'s of 8.9 and 3.8 \( \mu \text{M} \), respectively (Stankiewicz-Drogon \textit{et al.}, 2008). Acridone derivatives 20 and 27 also inhibit RNA replication in a subgenomic replicon with EC\textsubscript{50}'s of 9.0 and 10.2 \( \mu \text{M} \) while being relatively non toxic with CC\textsubscript{50} values of 174.8 and 411.9 \( \mu \text{M} \), respectively (Stankiewicz-Drogon \textit{et al.}, 2008). Contrary to the methodology used by Stankiewicz-Drogon \textit{et al.}, Manfroni \textit{et al.} first screened drugs for activity against a subgenomic replicon, identified active compounds, and then investigated their mechanism of action. By starting with a drug that showed activity against bovine diarrhoeal virus (BVDV), the group synthesized over 40 derivative compounds and tested them for activity in...
an HCV subgenomic replicon; 5 of these compounds, derivatives 12, 14, 19, 23, and 35 were identified by screening for activity in an HCV subgenomic replicon, and all inhibit RNA replication with an EC₅₀ of 2.3–5.5 μM (5–12 μM; Manfroni et al., 2009). Only compound 23 inhibited helicase-catalysed DNA unwinding, with an IC₅₀ of 50 μg/ml (110 μM; Manfroni et al., 2009).

By employing an initial high throughput enzymatic screen of about 70 compounds, followed by replicon testing, Krawczyk et al. discovered several high potency, low toxicity compounds based on the known anti-cancer drug doxorubicin (Fig. 12.5A). For example, compound 3 had and very low EC₅₀ of 0.057 μM in cell culture, and compound 21 had an EC₅₀ of 0.129 μM, both of which had a selectivity index (i.e. EC₅₀/CC₅₀) of 20 or greater. The anthracyclines are known DNA intercalating agents, and the group demonstrated clear intercalation of compound into DNA with competition studies, direct measurement of substrate–inhibitor complex formation, and ATPase inhibition experiments. These experiments imply a mechanism where helicase is unable to interact productively with the DNA substrate, and may form a stable ternary enzyme–inhibitor DNA complex (Krawczyk et al., 2009).

Tropolones are compounds known to have antiviral, anticancer and other therapeutic uses (Zhao, 2007), and several such compounds are potent HCV helicase inhibitors. For example, dibromo-morpholinomethyltropolone (DBMTr; Fig. 12.5B) inhibits HCV helicase-catalysed DNA unwinding with an IC₅₀ of 18 μM and shows little cytotoxicity in yeast (Boguszewska-Chachulska et al., 2006). Tropolone effects on the HCV NS3 ATPase and HCV helicase-catalysed RNA unwinding, however, are generally small; some even stimulate ATP hydrolysis. For example DMBTr stimulates the NS3 helicase-catalysed ATP hydrolysis rate of by 8% at 100 μM.
Figure 12.5 Helicase inhibits acting at unknown sites. (A) Selected amidinoanthracine helicase inhibitors. These compounds are analogues of the anti-cancer drug doxorubicin. Compounds 3 and 21 are shown due to their high potency in both DNA unwinding assays (IC$_{50}$=80, 500 nM) and subgenomic replicons (EC$_{50}$=57, 129 nM) as well as for their high selectivity index (i.e. EC$_{50}$/CC$_{50}$) of 19 and 33 for compounds 3 and 21, respectively (Krawczyk et al., 2009). (B) Dibromo-morpholinomethytrpopholone (DBMTr) inhibits helicase-catalysed DNA unwinding with an IC$_{50}$ of 18 μM (Boguszewska-Chachulska et al., 2006). (C and D) Thiadiazole (Janetka et al., 2000) and substituted pyrimidine (Hale et al., 2001) helicase inhibitors reported in patents assigned to Vertex Pharmaceuticals, Inc. Compound 13 and IA-19 have IC$_{50}$’s of less than 15 μM in a helicase-catalysed DNA unwinding assay; compound IA-19 also inhibits viral replication in a bovine viral diarrhoea virus (BVDV) assay with an IC$_{50}$ of < 20 μM, although the CC$_{50}$ is 20–40 μM.

(Boguszewska-Chachulska et al., 2006) and does not inhibit helicase-catalysed RNA unwinding (Borowski et al., 2007). The tropolone derivatives do not appear to compete with ATP, DNA or RNA in any assays.

Little has been reported about the final two classes of HCV helicase inhibitors, which have only been described in patents. Vertex Inc. found 56 substituted thiadiazole compounds (Janetka et al., 2000) that all had the ability to inhibit HCV NS3 catalysed ATP hydrolysis with IC$_{50}$ values of less than 15 μM. Of the five most potent compounds in this set that were tested for an ability to inhibit helicase-catalysed DNA unwinding only compound 13 (Fig. 12.5C), had an IC$_{50}$ of less than 25 μM (Janetka et al., 2000). Vertex also reported testing 17 substituted pyrimidine compounds for a their ability to inhibit HCV NS3 catalysed RNA unwinding (Hale et al., 2001).

Nine of the 17 compounds inhibit RNA unwinding with an IC$_{50}$ of less than 15 μM and of those, one compound, IA-19 (Fig. 12.5D), also inhibits replication of bovine viral diarrhoea virus in a cell culture system with an IC$_{50}$ of less than 20 μM. The compound was, however, fairly toxic with a CC$_{50}$ was between 20 and 40 μM (Hale et al., 2001).

Peptide-based inhibitors

Two small peptides have been reported to inhibit HCV helicase, each by a different mechanism, and they each also inhibit the HCV replicon in cells. A 2008 patent by Raney et al. describes a short peptide with the sequence HIDAHLSTQK-GGG-YARAAQRQA that inhibits HCV replicons. The peptide mimics a portion of NS3 that is suspected to be involved in the formation of NS3 dimers and assembly of higher order
oligomers (Raney et al., 2008). The other known small peptide HCV helicase inhibitor appears to bind both ATP and RNA binding sites. This 14 amino acid peptide termed ‘p14’ has the sequence RRGRTGGRGGIYR and was designed to mimic arginine-rich conserved helicase motif VI (Gozdek et al., 2008). In DNA unwinding assays, p14 inhibited with an IC\textsubscript{50} of 725 nM while not affecting ATP hydrolysis at all (Gozdek et al., 2008). In other studies, p14 proved to be slightly more potent than initially described, inhibiting DNA unwinding with an IC\textsubscript{50} of 200 nM (Borowski et al., 2008). NMR and computer docking studies suggest that p14 occupies the nucleic acid binding groove, preventing the enzyme from acting on its nucleic acid substrate, while curving into the region near the P-loop, preventing interaction between helicase domains 1 and 2 (Gozdek et al., 2008). p14 inhibits HCV replicons with an EC\textsubscript{50} of 83 μM (Gozdek et al., 2008).

**Conclusion and future directions**

To date, no inhibitors of any helicase are in clinical use, and much more work must be done if HCV helicase inhibitors are ever to enter the clinic. Recent major progress in the HCV inhibitor field centres on the fact that several relatively non-toxic compounds have been shown to halt HCV RNA replication in cell culture-based replicon assay systems. However, to our knowledge, none of these compounds have been tested in HCV strains capable of replicating in cell culture (e.g. HCV\textsubscript{cc}), and no HCV strains resistant to any of these compounds have been identified. Likewise, only a couple studies have made use of the information in the many NS3 structural or mechanistic studies to target key regions or rationally design compound derivatives. The next steps towards drug development for HCV helicase inhibitors should therefore include validating inhibitors in the HCV\textsubscript{cc} system. Resistant strains will then need to be isolated and sequenced to understand how rapidly resistance develops and if amino acid combinations conferring resistance already exist in nature. Compounds could also be soaked into NS3 crystals or the binding sites could be determined with NMR so that modelling could be used to facilitate the synthesis of more potent analogues.

At this point the utility of compounds that have shown activity in the replicon system is not limited strictly to drug design. Compounds could be used as chemical probes to understand why HCV needs a helicase using one of the subgenomic, genomic, or HCV\textsubscript{cc} culture systems. Similarly, helicase inhibitors can be used to study the HCV helicase itself, a prototype helicase and molecular motor. Inhibitors functioning by various mechanisms of action could be used to elucidate steps in the ATP hydrolysis cycle and the mechanism of helicase-catalysed nucleic acid unwinding. Although HCV NS3 helicase inhibitors may not represent the next generation of anti-HCV drugs, they are currently useful for studying HCV and represent another promising way to fight this potentially serious disease in the future.

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