

BINDING-SITE CHARACTERIZATION AND RESISTANCE TO A CLASS OF NON-NUCLEOSIDE INHIBITORS OF THE HCV NS5B POLYMERASE*.

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Running Title: HCV NS5B Inhibitor Binding Site

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The virally-encoded NS5B RNA-dependent RNA polymerase has emerged as a prime target in the search for specific HCV anti-virals. A series of benzimidazole 5-carboxamide compounds inhibit the cellular RNA replication of a HCV sub-genomic replicon and we have advanced our understanding of this class of inhibitors through a combination of complementary approaches that include biochemical cross-linking experiments with a photo-reactive analogue followed by MS-analysis of the enzyme. A novel binding site has been localized for these inhibitors at the junction of the thumb-domain and the N-terminal finger loop. Furthermore, the isolation and characterization of resistant replicon mutants that co-localize to this region distinguished this class of compounds from other non-nucleoside NS5B inhibitors that bind to distinct allosteric sites. Resistant mutations that emerged with the benzimidazole 5-carboxamide and related compounds were found at three amino acid positions in the thumb domain: P495 with substitutions to S, L, A or T; P496 substitutions to S or A; and a V499A substitution. Mutations at each of these positions conferred different levels of resistance to this drug class: the P495 changes provided the greatest shifts in compound potency, followed by moderate changes in potency with the P496 substitutions, and finally only minor shifts in potency with V499A. Combinations that include the benzimidazole 5-carboxamide polymerase inhibitors and compounds that bind other sites or other HCV targets, including HCV protease inhibitors, are complementary in cell culture models of HCV RNA replication at suppressing the emergence of resistant variants.

This novel class of compounds and unique binding site expand the diversity of HCV anti-virals currently under development and offer the potential to improve the treatment of chronic HCV infection.

More than two percent of the world population are chronically infected with hepatitis C virus (HCV), a flavivirus that is the etiological agent of non-A non-B hepatitis (1,2). A large proportion of patients fail to achieve a sustained response to current therapies consisting of a combination of pegylated-interferon and ribavirin. The discovery and development of specific anti-HCV chemotherapies aims to address this unmet clinical need and has focused on inhibitors of virally encoded functions. HCV encodes a linear polyprotein of approximately 3010 amino acids that is cleaved at multiple sites by cellular and viral proteases to produce structural and non-structural (NS) proteins [for review, see (3)]. One of the non-structural proteins, NS5B, catalyzes the RNA-dependent RNA polymerization of a negative strand intermediate and the subsequent generation of multiple copies of the plus strand viral genome; this enzyme has emerged as a principal target for chemotherapeutic inhibition of HCV replication (4).

The three dimensional structure of the NS5B polymerase reveals an organization comparable to other nucleic acid polymerases with the familiar features of fingers, palm and thumb domains that are organized in a "right-hand" motif (5-7). A distinct feature of the HCV polymerase (and closely related RdRp) active site cavity is the protrusion of a unique beta-hairpin from the thumb subdomain that apparently plays a role in the initiation of de novo RNA synthesis as

demonstrated by both structural and biochemical studies (8-11). Another additional feature of the HCV polymerase is two loops that bridge the fingers and thumb sub-domain and result in an encircled active site. This feature is now known to be shared by other RdRp from rhinovirus, bacteriophage phi6, rabbit hemorrhagic disease virus, bovine viral diarrhea virus, Norwalk virus and poliovirus (12-19). Interestingly, the interface between the HCV polymerase N-terminal λ 1 loop and the thumb subdomain is the location of a GTP binding site (8) although its precise biological role is unsolved.

A number of different HCV polymerase inhibitors have emerged that can be broadly divided into three categories: (i) nucleoside analogs (ii) pyrophosphate mimics and (iii) non-nucleoside inhibitors reviewed in (20). Notably, many nucleoside analogues with the potential to inhibit HCV RNA replication have 2'-methylribose structures; mechanism of action studies have demonstrated that these nucleosides act as chain-terminators (21). The pyrophosphate mimics are exemplified by a series of diketo acids and hydroxypyrimidine carboxylic acids that selectively bind the NS5B active site divalent cations and act as product-like inhibitors of the polymerase reaction (22).

The non-nucleoside inhibitors (NNI) are thus far the most diverse class of inhibitors and represent compounds that bind to distinct pockets on the HCV polymerase. One promiscuous pocket on the thumb domain binds at least three different classes of NNI: the thiophene 2-carboxylic acids, phenylalanine derivatives and cyclopentyl dihydropyran-2-ones (23,24) classes of compounds all bind to a pocket with a central M423 residue. Another class of compounds are exemplified by benzothiadiazines that give rise to resistant replicon mutants at position M414 (25,26) which is located at the base of the palm and thumb domain in the active site cleft.

Benzimidazole 5-carboxamide inhibitors have also been extensively pursued as HCV NS5B specific inhibitors (25,27-31,35). These compounds inhibit an initiation phase of the reaction, and a modified NS5B polymerase with lower RNA-substrate affinity was used to identify these compounds in a screening campaign (32). These compounds are non-competitive with respect to NTP incorporation, but inhibit

productive binding of RNA template/primer substrate. The compounds have a unique resistance profile and preliminary resistance studies with HCV replicons identified substitutions in one residue, P495 in the NS5B thumb domain, as conferring resistance to this class of inhibitor (33).

In an effort to further characterize the benzimidazole class of NS5B inhibitors and map the location of inhibitor binding, we describe herein cross-linking experiments with a photo-reactive analogue followed by MS-analysis of the enzyme, that localize a putative binding-site for these inhibitors at the junction of the thumb-domain and the NS5B N-terminal λ 1 loop. Furthermore, the isolation and characterization of replicon resistant mutants that co-localize to this region distinguished this class of compounds from other non-nucleoside NS5B inhibitors that bind to other allosteric sites. Combinations that include the benzimidazole 5-carboxamide polymerase inhibitors and compounds that bind other sites, as well as other classes of compounds such as HCV protease inhibitors, are complementary in cell culture models of HCV RNA replication at suppressing the emergence of resistant variants. The mounting number and diversity of HCV antivirals currently in discovery, expand the potential for improving the treatment of chronic HCV infection.

Material and Methods:

HCV inhibitor compounds

The HCV NS5B polymerase inhibitors (Fig. 1) were prepared according to published procedures (29,31,34-36). BILN 2061 was synthesized by the medicinal chemistry group of Boehringer Ingelheim (Canada) Ltd. R&D division (37). Interferon- α (IFN- α) isolated from human leucocytes was purchased from Sigma.

Photoaffinity labeling experiments

The purified HT-NS5B Δ 21 native protein in buffer (30 μ M in 25 mM Tris pH 7.5/ 1 mM TCEP/ 10% glycerol/ 300 mM NaCl) was pre-incubated in the dark with a 1:1.2 molar ratio of inhibitor compound B (or DMSO control; final DMSO concentration was 2% in both samples) at 4°C for 15 minutes. The sample was distributed in

aliquots in a white Microfluoar 96-well plate that was then photolyzed for up to 40 min at room temperature using a Spectroline ENF-280C (Spectronics Corp.) lamp rated at 500 $\mu\text{W}/\text{cm}^2$ light intensity at 365 nm and at a 6.5 cm distance from the sample. Aliquots were withdrawn at different times to assess enzymatic activity, using the HCV polymerase enzymatic assay described below.

An additional set of irradiated samples were pooled together after irradiation for proteolysis and mass spectrometry analysis. Three volumes of a mixture of 8 M urea and 0.4 M ammonium bicarbonate (pH 8.0) was added to one volume of the cross-linked protein solution. After an incubation period of 15 minutes at 60°C, reduction was performed with DTT at 50°C for 15 minutes, followed by alkylation with 10 mM iodoacetamide at room temperature for 15 minutes. Samples were then digested at 37°C for 24 hours with agitation to completion with immobilized TPCK trypsin (Pierce) that was previously equilibrated in the protein solution buffer. After digestion, samples were centrifuged to pellet the TPCK trypsin beads and the supernatants were recovered for LC-MS analysis of digests.

Mass spectrometry

Identification of the labeled tryptic peptide was performed using an Apex II 7-T Fourier transform mass spectrometer (Bruker) and an analytical HPLC system (Shimadzu) with the flow split to 3 $\mu\text{L}/\text{min}$ through a 0.30 x 150 mm Pepmap C18 column (LC Packings, San Francisco, CA) (38). During gradient chromatography with water/acetonitrile (0-90%+ 0.1% formic acid) 128 digitized spectra were acquired at ~20 000 resolution. The data were converted from Bruker Xmass to Waters MassLynx format and searched for m/z values corresponding to peptides covalently cross-linked to compound B. The control was treated identically to the labeled sample except for the absence of inhibitor.

Cell culture

Human hepatoma Huh-7 and all HCV replicon containing Huh-7 cell lines (32,39) used for the experiments were maintained in DMEM

supplemented with 10% fetal bovine serum at 37 °C in an atmosphere of 5% CO₂. Replicon cells were maintained in medium supplemented with 0.5 mg/ml G418, unless indicated otherwise.

Cell-based assay for replicon inhibition

Prior to the assay, the cells were plated in medium without G418 at a density of 10,000 cells per well in a 96 well plate and incubated at 37 °C. The following day, medium was removed and replaced by the same volume of DMEM containing 10% FBS, 0.5% DMSO and serial dilutions of the compound to be tested. The cells were incubated in the presence of compound for 72 hr after which replicon RNA levels were determined by Taqman quantitative real time RT-PCR. Briefly, total RNA was extracted from the replicon cells using the RNeasy 96 kit (Qiagen) and quantified using RiboGreen (Molecular Probe). The Real-Time RT-PCR was performed on an ABI Prism 7700 Sequence Detection System using the TaqMan EZ RT-PCR (Applied Biosystems) (39). The RNA copy number was normalized (by RiboGreen RNA quantification of the RNA extracted from the cell culture well) and expressed as genome equivalents / μg of total RNA.

Selection of replicons resistant to inhibitor

The bicistronic replicon system described by Lohmann et al. (1999) is dependent on the function of HCV non-structural proteins and enzymes. The S22.3 cell line, derived from the Con-1 sequence as previously described (39) and a highly adapted derivative clone, termed R3 (40) that contains additional adaptive mutations, were used in this study.

Cells were trypsinized and resuspended in fresh medium (DMEM, 10% fetal calf serum) containing 1 mg/ml G418 (Invitrogen). 150,000 cells were plated into one well of a 6-well plate. The following day ($t = \text{day } 0$), fresh medium containing the NS5B polymerase inhibitor at a pre-determined concentration and 1 mg/ml G418 was added to the well. On day 3, when the cells approached confluency, they were trypsinized and transferred into a 10 cm plate. Medium (containing fresh inhibitor and 1 mg/ml G418) was changed on day 6 and day 10. By day 11, cell death was evident, as the majority of cells were susceptible to G418. At day 14, the medium was changed,

with fresh medium harboring the same concentration of inhibitor and only 0.5 mg/ml G418. Fresh medium with inhibitor and 0.5 mg/ml G418 was replaced at day 17 and day 20. Cells that formed visible colonies by day 21 were picked for expansion into 48-well plates; the colonies were also counted by fixing and staining the cells with crystal violet. Picked colonies were grown in 48-well plates in the presence of G418 to obtain sufficient cells for further characterization.

The total cellular RNA, containing the HCV subgenomic replicon RNA, was isolated from 1×10^6 cells by Qiagen RNeasy protocol. HCV sequences were amplified by RT-PCR and the DNA product sequenced with HCV specific primers.

Plasmid construction

The reconstruction of isogenic replicons with the P495A, P495L or P496A single amino acid substitution in the NS5B gene was done as follows: two restriction sites, *EcoRV* and *NotI*, flanking the NS5B gene were engineered into the R3 cDNA clone by site-directed mutagenesis; the *EcoRV* site spans the NS5A-5B cleavage site and encodes a V258I substitution in the Con-1 replicon sequence that is found in other genotype 1b isolates; the *NotI* site is located in the variable region of the 3'UTR. A subclone harboring only the *EcoRV*-*NotI* NS5B cassette was used to introduce the specific substitutions generated by single base change with the Quick-Change TMII site-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions. The resulting plasmids were digested with *NotI* and *EcoRV*, and the fragment was purified and ligated into the R3 replicon cDNA with *NotI*/*EcoRV*. Alternatively, the other substitutions were introduced into R3 cDNA by standard subcloning techniques. The modifications were verified by DNA sequencing on an ABI3100 capillary electrophoresis sequence analyzer.

Bacterial expression plasmids pET28 (Invitrogen) harboring the coding region of genotype 1b NS5B were also constructed and modified to encode the single amino acid substitutions as described above; these clones express the His-NS5B Δ 21.

Expression and purification of recombinant proteins from E. coli

The recombinant HCV NS5B polymerase can be produced in soluble form by expression of a variant that lacks the C-terminal 21 amino acids (41-43). The N-terminal hexa-histidine NS5B polymerase (termed HT-NS5B Δ 21) was expressed from a pET vector in *E. coli* strain JM109 (DE3) and induced with 0.4 mM IPTG for 3 hours at 24°C. Cells were harvested and lysed in a microfluidizer. The lysate, after centrifugation, was purified by sequential chromatographic steps on Ni-NTA, DEAE-Sepharose and heparin-Sepharose columns (32). The protein was thereafter concentrated on a Resource S column, and applied to a Superdex 200 column where peak fractions containing highly pure HT-NS5B Δ 21 were pooled.

NS5B Polymerase assays

The assay was performed using 10 nM of enzyme, 0.5 μ Ci of [³H]-UTP, 1 μ M UTP, 250 nM 5'-biotinylated oligo(rU₁₂), 10 μ g/mL poly(rA) in 20 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 0.2 U/ μ L of RNasin, 5% DMSO, 3% glycerol, 30 mM NaCl, 0.33% dodecyl- β -D-maltoside, 0.01% IGEPAL. Aliquots of 8 μ L were removed at specific times, spotted onto DE81 filter paper discs, washed in phosphate buffer to remove unincorporated UTP, rinsed and counted to quantify the amount of UMP incorporated into bound product RNA; alternatively, reactions were terminated after 90 minutes at 22°C by the addition of stop solution containing streptavidin-coated beads (8 mg/mL in 0.5 M EDTA) for Scintillation Proximity Assay (Perkin Elmer- Amersham). After 30 min at room temperature, 75 μ L of 5 M cesium chloride were added to the wells and the plate was left at room temperature for one hour before quantifying the radioactive UMP incorporated onto the biotinylated primer by counting for 60 seconds on a TopCount (Packard).

In vitro transcription, transfection of synthetic RNA and selection of replicon cell lines

HCV subgenomic replicon RNA transcripts harboring specific substitutions in the NS5B segment were synthesized using a T7 Ribomax kit (Promega) according to the manufacturer's protocol. RNA was purified using RNeasy columns (Qiagen), and DNA was

removed using an RNase-free DNase kit (Qiagen). Huh-7 cells were electroporated with 0.01-10 μg of purified *in vitro* transcripts and seeded into two 15 cm dishes with fresh medium; 24 hr later, medium supplemented with G418 (0.25 mg /ml) initiated the selection process. Colonies that were visible after 3 weeks were isolated and expanded into cell lines for further analysis.

Inhibitor combination studies

Long-term resistant replicon selection with a combination of BILN 2061 and compound C was performed as described above for compound C alone, with the exception that sustained levels of both inhibitors (at the indicated concentrations) were maintained during the selection; resistant colonies on the plates were fixed, stained with crystal violet and counted.

Results

Irreversible photo-inactivation of the HCV NS5B

Compound A (IC_{50} 0.15 μM) is a representative benzimidazole 5-carboxamide inhibitor and under a variety of assay conditions has consistently shown potent inhibition of the NS5B enzyme (29,32). This series of compounds are being actively pursued as potential therapies for the treatment of chronic HCV infection and although progress has been made in characterizing their mechanism of action, little is known about their physical binding site or pocket (33). Based on the accumulated structure-activity relationship with this class of inhibitors, we have shown that the compounds tolerate extensive substitutions off the left-hand side imidazole portion without significantly affecting their inhibition of NS5B (27). The tolerability for substitutions at the C-2 position was exploited to prepare compound B, a potent (IC_{50} 0.63 μM) derivative that incorporated a tethered photoreactive benzophenone moiety. This appendage was used for photo-affinity labeling studies with the HCV NS5B enzyme to physically map the binding region for this class of compounds. Benzophenone derivatives have been used to specifically label the targets of other viral inhibitors (38). Upon exposure to ultraviolet light, the benzophenone carbonyl oxygen atom forms a radical intermediate that can cross-link to a proximal protein segment via abstraction then

coupling to form a stable covalent bond. NS5B was mixed with a slight excess of the photo-reactive compound B and irradiated with UV light. The samples were assayed for enzyme activity after a 2000-fold dilution, which dissociated non-covalently bound compound B from the enzyme. Fig. 2 shows the results of a typical photolabeling experiment. Enzyme activity remained unchanged with time when enzyme alone (DMSO control) was irradiated. Moreover, the enzyme retained activity when mixed with compound B in the dark, or when irradiated with a benzophenone derivative that was a poor inhibitor of NS5B (not shown). NS5B activity, however, decreased in a time-dependent irreversible manner as enzyme plus compound B was irradiated. The typical nonlinear curve (Fig. 2) of the photolabeling provided an inactivation rate constant of 0.133 min^{-1} .

Mapping the site of NS5B photolabeling

In a preparative scale photolabeling experiment, NS5B protein was incubated with compound B and irradiated at 365nm for 25 min, followed by tryptic digestion to identify the photolabeled tryptic peptide of NS5B. A signal corresponding to the mass of a photoaffinity labeled peptide was clearly evident upon $\mu\text{LC-FTMS}$ analysis; Fig. 3 A shows the presence of a component eluting at 21.2 min that was consistent with the +2 ion of the labeled tryptic peptide NMVYSTTSR, which was absent from a control digest analyzed under similar conditions (Fig. 3B). In the corresponding mass spectrum (Fig. 3a inset), the observed monoisotopic peak had a m/z of 981.9216, consistent with the value predicted (981.9220, ± 0.4 ppm with external calibration) for the +2 ion labeled with compound B. An additional analysis was performed to induce fragmentation, which was limited and attributable to the sequence of hydroxylated residues, but was suggestive of an attachment at one of the first two residues (NM). The tryptic peptide NMVYSTTSR spans amino acids 35 to 43 of the genotype 1b NS5B polymerase used in our studies (Fig. 4). This region constitutes a segment of the N-terminal $\lambda 1$ loop that bridges the finger domain with the thumb; amino acids 35 and 36 are part of a short helix that contacts the thumb domain.

Inhibition of HCV replicons and resistance to benzimidazole 5-carboxamide inhibitors

Though compound A is a potent inhibitor of NS5B *in vitro*, the effect on HCV RNA replication in cell culture as measured with the HCV replicon system (44), was marginal (63% inhibition at 50 μM) and attributable to the poor cell permeability accorded by the acid functionalities and net negative charge of the compound (28). In an effort to identify C-5 carboxamide analogues that retained *in vitro* potency but demonstrated superior cell culture activity, a combinatorial chemistry approach [M.A. Poupart unpublished result; (31)] led to the discovery of compound C with an EC_{50} in a 72 hour replicon assay of 1.1 μM . Other benzimidazoles that have demonstrated cell-based inhibition of HCV replicons have a free C-5 carboxylic acid (33) whereas compound C features a distinct C-5 carboxamide extension. A benzimidazole C-5 carboxylic acid inhibitor was previously used in cell culture to select for resistant replicons (33) that had a substitution at only one NS5B amino acid position (P495). In an effort to gain insight into the potential differences between benzimidazole inhibitors with a C-5 carboxylic acid or an extended carboxamide appendage, we performed a replicon selection experiment with compound C. Huh-7 cells harboring HCV subgenomic replicons were cultured in the presence of a sustained amount of G418 and compound C. The concentration of compound C ranged from 1 μM to 20 μM (which is significantly below the cytotoxic concentration of the compound) and G418 was initially used at 1 mg/ml; after 10 days of selection, constant levels of compound C were maintained, but the concentration of G418 was decreased to 0.5 mg/ml. Following the three week selection, colonies were observed on all plates. Notably, the frequency of colony formation was inversely proportional to the quantity of compound C used in selection (fig. 5A). Colonies from multiple experiments were picked and individual clones expanded (in the presence of G418 and the respective concentrations of inhibitor) for further analysis. Total RNA was isolated from the individual clones and the HCV replicon was amplified by RT-PCR and sequenced. Amino acid substitutions in the NS5B polymerase domain identified in at least 20 individual clones that were

selected in the presence of 6X, 10X or 20X the EC_{50} of compound C [or two other closely related carboxamide analogues (36)] are shown in Table 1. Each of these substitutions was the result of a single nucleotide change. Substitutions in P495 and P496 were selected with both low and high inhibitor concentrations; though the P495 substitutions were predominant at the high inhibitor concentrations. V499 substitution was only seen with low inhibitor concentration. The selection of a substitution only at P495 with a 5-carboxylic acid benzimidazole was previously described (33) and is included in an extensive list of potential resistant mutants in Table 1. Notably, with the 5-carboxamide benzimidazoles, we also selected P495S and P495T mutants in addition to the previously noted P495A and P495L substitutions. Furthermore, the P496S, P496A and V499A that emerged in selection with the C5-carboxamides represent previously uncharacterized changes at proximal residues (fig 5B).

Characterization of modified NS5B enzymes

In order to examine the effect of a representative set of mutations on the activity of the NS5B and its inhibition, the HT-NS5B Δ 21 gene products that encode single amino acid substitutions of P495S, P495L, P495A, P496A, P496S, and V499A were over-expressed in *E. coli*, purified and characterized. All of the mutant enzymes maintained comparable polymerase activity relative to the wild-type control, however, the IC_{50} of compounds A and C were affected to varying extents by the different mutants (Table 2). On the other hand, the IC_{50} of an unrelated 2-thiophene carboxylic acid non-nucleoside inhibitor (24) that binds to a well characterized pocket in the lower thumb portion, was similar for all of the mutant enzymes and indistinguishable from wild-type (not shown). As shown in Table 2, the IC_{50} values for compound A relative to the wild type NS5B, was greater than 100-fold higher for the P495 substituted enzymes, and only 2.7 to 3.5-fold higher for the P496 and V499A substituted enzymes. The IC_{50} values for compound C were greater than 100-fold higher with the P495S and P495L enzymes, 39-fold higher with the P495A mutant enzyme, 10-13-fold higher with the P496A or S mutant NS5B, and only 2.3-fold higher with the V499A modification.

Characterization of single-mutant replicons

In order to examine the effect of changes at each of the NS5B 495, 496 and 499 residues in a cell-based replicon context, representative mutations (P495S, or P495A, or P496S, or V499A) were introduced into the Con-1 adapted, R3 replicon plasmid. *In vitro* transcribed RNA (0.5-10 ng) was used to transfect Huh-7 cells and stable clones were selected with G418. Colony formation efficiency with the replicon RNA harboring each of these mutations was slightly lower, but within an order of magnitude of our adapted R3 clone (1×10^6 cfu/ μ g); the sequence of the mutant replicons from the cell lines described in Table 3 was determined to verify that each of the clones in this representative set had no other NS5B changes. In contrast, replicons encoding a P495L or P496A mutation had significantly reduced colony formation efficiency ($< 1 \times 10^3$ cfu/ μ g) which raised concern that the resulting colonies encoded additional changes (33). In order to eliminate the possibility of a potential second locus effect, we therefore restricted our analysis to the mutant replicons listed in Table 3.

The EC_{50} for BILN 2061 (3.7 ± 0.6 nM) with the wild type replicon was reproduced with all of the NS5B mutant replicon cell lines. However, the P495S mutant NS5B replicon was resistant to inhibition by compound C with an EC_{50} value $>86 \mu$ M that represented more than an 80-fold shift in sensitivity. The P495A and P496S mutant replicons both shifted the EC_{50} of compound C by about 25-fold and did not have as profound an affect as the P495S mutant. Lastly, only a minimal 2-fold shift in EC_{50} was seen with the V499A mutant.

Inhibition by a combination of BILN 2061 and compound C

Compound C resistant replicons did not confer cross-resistance to a potent NS3 protease inhibitor BILN2061 (Table 3). We followed-up this observation with a combination study in short-term replicon inhibition, in addition to examining their combined effect on the emergence of resistant replicons. The activity of the 5-carboxamide class of compounds was investigated in a two-drug combination experiment with BILN 2061 for possible synergy in inhibiting HCV RNA

replication. All fixed-ratio combinations of the two inhibitors exhibited a predominantly additive effect (not shown). A combination of the two drugs, at various ratios that spanned a 1-20 μ M concentration range of compound C and 2-40 nM of BILN 2061 was also maintained in a three week selection with replicon cells cultured in the presence of G418. Twenty-four distinct two-drug combinations were evaluated as illustrated in the matrix of Fig. 6. As noted above, the frequency of colony formation was inversely proportional to the quantity of either compound C or BILN 2061 used in selection. In excess of 400 colonies emerged with relatively low levels (i.e. less than 3X the EC_{50}) of single drug exposure (Fig. 6). However, a marked reduction in colony formation was seen on all plates that had combinations of compound C and BILN 2061 at greater than 1 μ M and 6 nM, respectively; only one colony formed in the presence of 12 nM BILN 2061 and 3 μ M compound C. In addition to providing insight regarding the specificity of genetically mapped binding pockets, the lack of cross-resistance between these compounds revealed that a combination of the two could efficiently suppress the emergence of resistant replicons.

Discussion

The non-nucleoside inhibitors of the HCV polymerase can be broadly categorized on the basis of their binding site location. Different chemical classes of compounds such as the thiophene 2-carboxylic acids and phenylalanine derivatives (24), as well as the cyclopentyl dihydropyran-2-one inhibitor (23), bind to a distinct allosteric site in the thumb domain centered on M423 which is distally removed from the central active site. Another distinct class of inhibitors are exemplified by benzothiadiazines that give rise to resistant mutants at position M414 locating another binding site for non-nucleoside inhibitors at the base of the palm and thumb domain in the active site cleft (25,26).

An initial analysis of the benzimidazole class of inhibitors indicated that these compounds were distinct from other non-nucleosides and bound an alternative allosteric site by virtue of a single resistant mutation at codon 495 in the thumb domain of the NS5B (33). The use of a benzimidazole 5-carboxylic acid is an important

feature distinguishing the earlier study from our current analysis. Compounds A and C have extensions from the 5-carboxamide portion that were lacking from the benzimidazole analogues used in the earlier study. Based on our previously published SAR with this class of inhibitors, we hypothesize that this extension provides for additional interactions with the binding pocket. The substantial improvement in cell culture inhibition of HCV RNA replication conferred by compound C allowed us to investigate the potential for additional interactions mediated by the carboxamide class that may not have been evident with the free acid version. The P495 changes previously found with the benzimidazole carboxylates and now with our carboxamide analogues suggests that a structural feature, shared by both series of compounds, interacts with P495. The benzimidazole-cyclohexyl core is common to these inhibitors and tolerates few changes without significant loss in inhibitor potency (27). Hence, the P495 residue may represent a critical component of the NS5B binding pocket that interacts with the core of this class of inhibitors. In support of this proposal was our finding that the changes at P495 had the most profound effect on inhibitor potency.

Compound C was also used to select replicons with unique NS5B substitutions at P496 and V499 that were not observed in resistance studies performed with the 5-carboxylate derivative (33). One possible interpretation is that only the right-hand side carboxamide appendage interacts with these residues of the binding site. Notably, the right hand side extension of these inhibitors tolerates a higher degree of chemical diversity than the core (27,29); consequently, changes in the amino acids of the binding pocket that constitute the recognition elements for this appendage may have less of an effect on inhibitor potency than substitutions that affect core recognition. The observation that the various P496 and V499 mutations only shifted compound C potency 10 to 25-fold and 2 to 3-fold, respectively (and less than 4-fold with compound A) is consistent with this interpretation. Furthermore, we have also noted that the potency of benzimidazole 5-carboxylic acids that lack the right hand side appendage did not significantly shift with these NS5B mutants (data not shown).

Another striking feature of this class of HCV inhibitors is the extremely broad chemical diversity demonstrated by a variety of substitutions at the C2-benzimidazole “left-hand side” (27). We exploited this site of promiscuity by tethering a benzophenone and cross-linked the inhibitor to a short helix that forms an important contact between the N-terminal finger loop and thumb domain. In a three-dimensional spatial model of the HCV NS5B polymerase these residues are in close proximity to amino acids P495, P496, V499 that confer resistance to this series of inhibitors (Fig. 7).

The localization of a binding site for the benzimidazole class of non-nucleoside inhibitors (by both genetic and biophysical methods) to a region that presumably encompasses the interface between the N-terminal λ 1 loop and the thumb domain, adds to a growing body of experimental data that this region can allosterically regulate polymerase activity. Mutations in a highly conserved leucine at position 30 in the λ 1 loop that anchors the loop to a pocket in the thumb, alter the conformation of the NS5B polymerase and inactivate the enzyme (45). Furthermore, the λ 1 loop-thumb interface also binds a GTP that is proposed to allosterically regulate NS5B activity; notably, the GTP also contacts amino acid P495 in the thumb as well as amino acids in the λ 1 loop (8,33). Additional evidence for an important regulatory role for this region was suggested by the recent crystallization of the thiophene 2-carboxylic acid class of inhibitors with the genotype 2a NS5B polymerase (46). Although these compounds do not directly bind the λ 1 loop-thumb interface, as suggested with benzimidazole inhibitors, the proximal location of their pocket within the thumb apparently elicits subtle conformational changes at the loop-thumb interface upon binding. We have attempted to soak a variety of benzimidazole inhibitors into NS5B protein crystals and have thus far failed to identify electron density for a bound inhibitor; although we consistently observe the loss of electron density for a portion of the λ 1 loop that contacts the thumb (R. Coulombe and G. Kukulj, unpublished observation). Recently derived structures from X-ray crystallography indicate that closely related inhibitors may bind the thumb and displace the λ 1 loop (53,54).

The N-terminal loop that bridges the fingers and thumb domain is a structural feature shared by most RNA-dependent RNA polymerases from a variety of viruses (12-19). The observation that small molecules such as the benzimidazole compounds may specifically bind this interface and inhibit polymerase activity, suggests that the design of alternative compounds that target the specific loop-thumb interface from other viruses may be feasible.

RNA viruses replicate with poor fidelity and this characteristic has been exploited with the HCV sub-genomic replicon system to select for a number of variants that confer resistance to a variety of inhibitors that target not only different pockets on the NS5B polymerase, but also different targets in the HCV non-structural region (47-50). These studies have proven useful in the genetic mapping of potential sites of action for various compounds. Additionally, they may provide insight into the potential emergence of drug resistant variants during chemotherapeutic treatment with these inhibitors. Although the benzimidazole NS5B inhibitors are particularly sensitive to changes in the P495 and P496 residues of NS5B, these mutations do not confer cross resistance to a separate class of inhibitor such as BILN2061 that targets the NS3 protease (51). Given that HCV replicates at extremely high rates in humans (52), there is an increased potential for the selection of drug resistant viral variants during chemotherapy. Multi-drug combinations that inhibit multiple targets may be required to achieve a sustained viral response, even with small molecules that are specific for HCV enzymes. Our evaluation of the frequency of resistant colony formation with either BILN 2061 or compound C demonstrated that these inhibitors individually suppressed the emergence of resistant HCV replicons to a moderate extent. Remarkably, the combination of BILN 2061 and compound C was extremely effective at abrogating the emergence of resistant replicons and may be predictive of a beneficial co-therapy in the treatment of chronic HCV infection.

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FOOTNOTES

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The abbreviations used are: μ LC-FTMS, capillary scale liquid chromatography-Fourier Transform mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; MS, mass spectrometry; RdRp, RNA dependent RNA polymerase; NNI, non-nucleoside inhibitor; DMSO, dimethyl sulfoxide; TCEP, Tris [2-carboxyethyl] phosphine; EDTA, ethylenediamine tetra-acetic acid, FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; RT-PCR, reverse-transcription polymerase chain reaction; DTT, dithiothreitol; IPTG, iso-propyl β -D-thiogalactopyranoside; cfu, colony forming units.

FIGURE LEGENDS

Fig. 1. Chemical structure of HCV inhibitors used in this study. Compound A was previously described as an optimized benzimidazole inhibitor of the NS5B polymerase (29). Compound B is a derivative of A, with a tethered benzophenone substitution on the C2 benzimidazole left hand side that was used for the photo-affinity labeling experiment in this study. Compound C is a derivate of A with an alternative C5 carboxamide substitution that provides for improved inhibition cell culture HCV RNA replication. IC_{50} (\pm standard deviation) of the individual compounds in an NS5B enzyme assay are depicted.

Fig. 2. Photo-inactivation of NS5B by compound B. NS5B enzyme was pre-incubated in the dark with a 1:1.2 molar ratio of inhibitor compound B (closed triangles) or DMSO control (closed boxes) at 4°C for 15 minutes. Samples were then irradiated at room temperature with low intensity light at 365 nm. The progress of the photo-inactivation was monitored by assaying NS5B polymerase activity at indicated illumination times after a 2000-fold dilution. Individual points represent the mean NS5B polymerase activity (with error bars) determined from three independent experiments.

Fig. 3. LC/MS of the trypsin digest of HCV NS5B polymerase photoaffinity labeled with compound B. Panel A, the extracted ion chromatogram for $m/z = 981.92$ (+2 charge state for mass 1961.83) from the photoaffinity-labeled sample is compared with the chromatogram from the control sample without inhibitor. Panel B, The peak eluting at 21.2 min is not present in the DMSO treated control. The inset in upper panel A shows the theoretical isotope distribution calculated for the +2 ion formed from residues NMVYSTTSR with label (top) and the partial mass spectrum obtained for this peak (bottom).

Fig. 4. Linear representation of the structural features of HCV NS5B polymerase. Red highlights the palm subdomain; purple and green delineate the fingers and thumb subdomains, respectively; yellow, represents the segments that comprise two loops interconnecting the fingers and thumb subdomains. The location of tryptic peptide $^{35}\text{NMVYSTTSR}^{43}$ in the $\lambda 1$ loop is indicated.

Fig. 5. Selection of replicons resistant to different concentrations of compound C.

Panel A. Huh-7 cells maintaining the HCV subgenomic replicon were exposed to a broad range of compound C concentrations in the presence of G418 as described in the Material and methods section. The concentrations of compound C are indicated along the top part of the figure. Colonies were picked from a variety of plates for expansion and subsequent analysis; remaining colonies were stained with crystal violet and quantified as indicated by the inset numbers. Panel B: Summary of all of the amino acid substitutions identified from sequence analysis of the resistant clones.

Fig. 6. The effect of combinations of BILN2061 and compound C on the frequency of resistant colony formation. Replicon cells were exposed to various concentrations of BILN2061 and/or compound C for 3 weeks in the presence of G418. The top row depicts colonies selected with the indicated concentrations of compound C alone; the left-most column depicts the colonies selected with the indicated concentration of BILN2061; the remaining plates depict colonies that were selected with a dual combination of BILN2061 and compound C at the indicated concentrations derived from the matrix. The value indicated in each square represents the number of colonies; if the colony count was too high to be determined accurately, a qualitative evaluation was expressed as a number of (+) which was proportional to the intensity of the staining.

Fig. 7. Three-dimensional structure of NS5B. The structure of the NS5B polymerase highlights the three amino acids: P495, P496, V499 (in space filling mode) identified as compound C resistant mutants. The striped bar highlights the tryptic peptide $^{35}\text{NMVYSTTSR}^{43}$ in the $\lambda 1$ loop that cross-linked to compound B.

Table 1: Sequence Changes in Resistant Clones

Selection	Codon	Change	New Codon	AA Change
20X	CCG	1484c>T	CTG	P495L
20X	CCG	1483c>T	TCG	P495S
20X	CCG	1483c>T	TCG	P495S
20X	CCG	1483c>A	ACG	P495T
20X	CCG	1483c>A	ACG	P495T
20X	CCG	1483c>T	TCG	P495S
20X	CCC	1483c>T	TCC	P495S
20X	CCC	1483c>T	TCC	P495S
20X	CCG	1483c>A	ACG	P495T
20X	CCG	1483c>G	GCG	P495A
20X	CCG	1483c>G	GCG	P495A
20X	CCC	1486c>T	TCC	P496S
20X	CCG	1483c>G	GCG	P495A
20X	CCG	1483c>G	GCG	P495A
20X	CCG	1484c>T	CTG	P495L
10X	CCC	1486c>T	TCC	P496S
10X	CCG	1483c>G	GCG	P495A
10X	CCC	1486c>T	TCC	P496S
10X	CCC	1486c>T	TCC	P496S
10X	CCC	1486c>G	GCC	P496A
10X	CCG	1483c>G	GCG	P495A
10X	CCG	1483c>T	TCG	P495S
10X	CCC	1486c>T	TCC	P496S
10X	CCC	1486c>T	TCC	P496S
6X	CCC	1486c>T	TCC	P496S
6X	CCC	1486c>T	TCC	P496S
6X	CCC	1486c>T	TCC	P496S
6X	GTC	1496t>C	GCC	V499A
6X	CCC	1486c>T	TCC	P496S
6X	GTC	1496t>C	GCC	V499A
6X	CCC	1486c>T	TCC	P496S
6X	CCC	1486c>T	TCC	P496S
6X	CCC	1486c>T	TCC	P496S
6X	CCC	1486c>T	TCC	P496S

Table 2: Inhibition [IC₅₀ (±S.D.) μM]* of recombinant HT-NS5BΔ21 containing resistance mutations

	WT 1b	P495S	P495L	P495A	P496A	P496S	V499A
Compound A	0.15(±0.042)	>25	>25	>25	0.52(±0.16)	0.53(±0.22)	0.41(±0.12)
Compound C	0.27(±0.05)	>32	>25	10.6(±3.2)	2.6(±0.4)	3.8(±2.1)	0.63(±0.15)

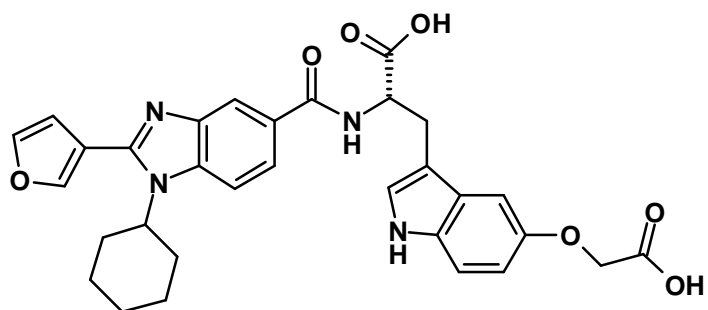
* Mean determined from at least 4 measurements

Table 3: Potency of inhibitors [EC₅₀ (±S.D.) μM]* in stable replicon cell lines with NS5B resistance mutations

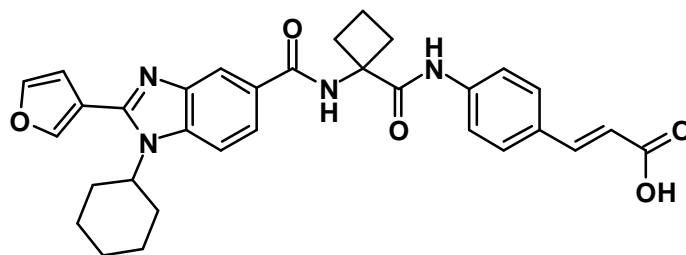
	WT 1b	P495S	P495A	P496S	V499A
Compound C	1.1(±0.2)	>86	28 (±6)	28 (±8)	2.3 (±1.2)
BILN 2061	0.0037 (±0.0006)	0.0036 (±0.0005)	0.0024 (±0.0005)	0.0035 (±0.001)	0.0027 (±0.0006)

* Mean determined from at least 3 independent measurements

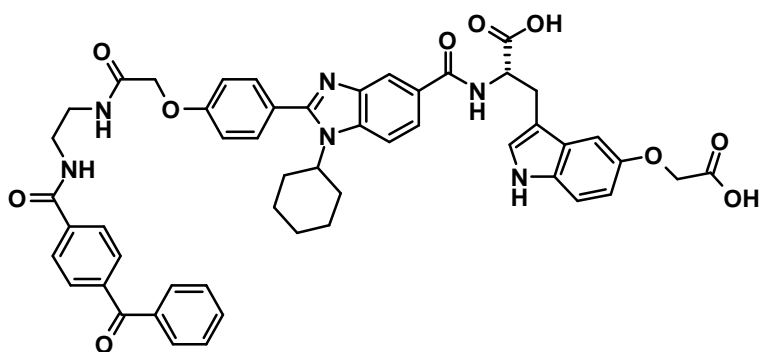
Figure 1



Compound A $IC_{50} = 0.15 (\pm 0.042) \mu M$

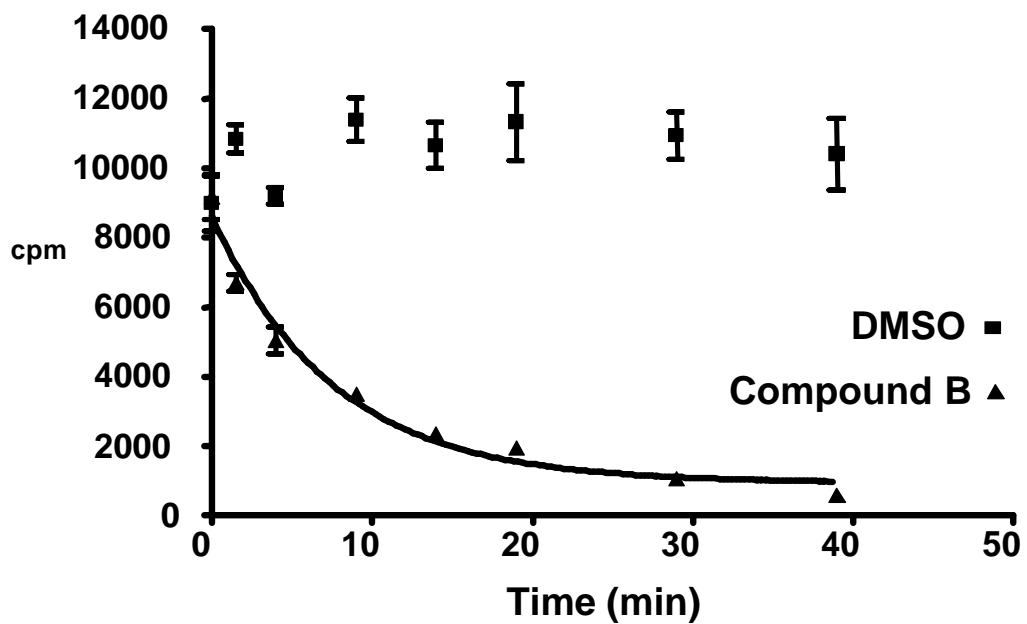


Compound C $IC_{50} = 0.27 (\pm 0.05) \mu M$



Compound B $IC_{50} = 0.63 (\pm 0.02) \mu M$

Figure 2



Rate constant = $0.13 \pm 0.02 \text{ min}^{-1}$
 $R^2 = 0.95$

Figure 3

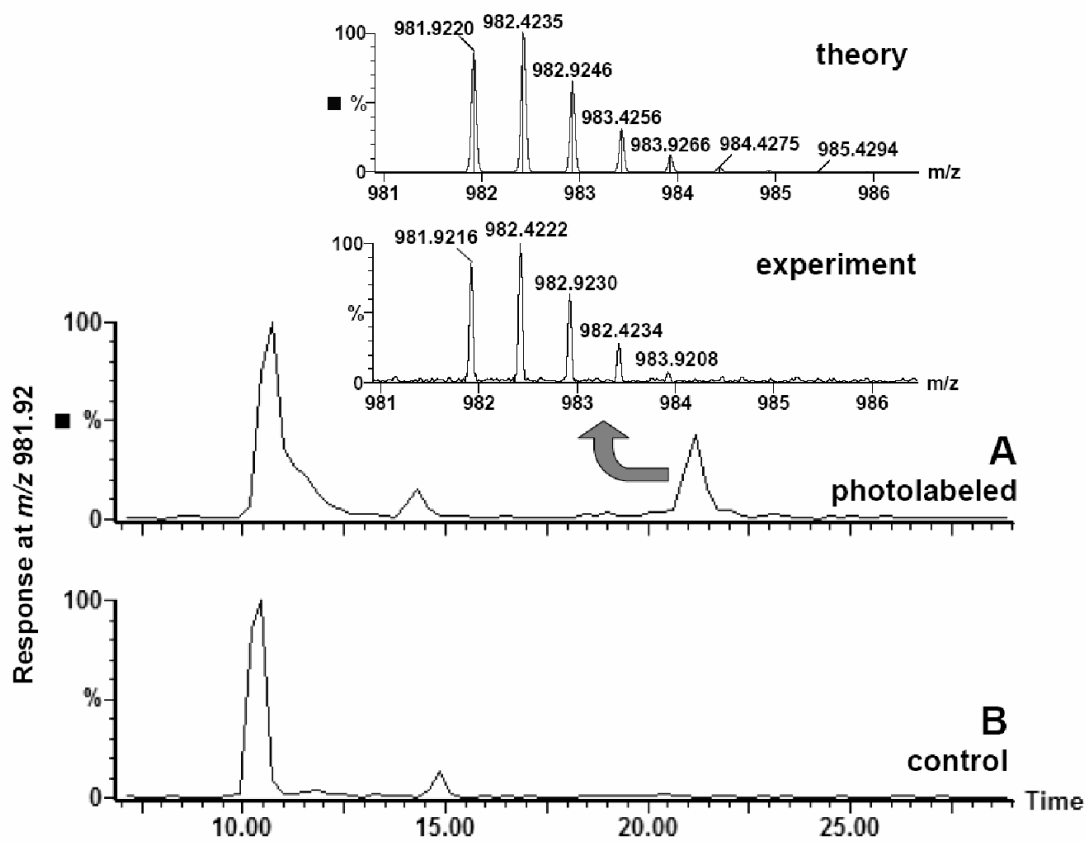


Figure 4

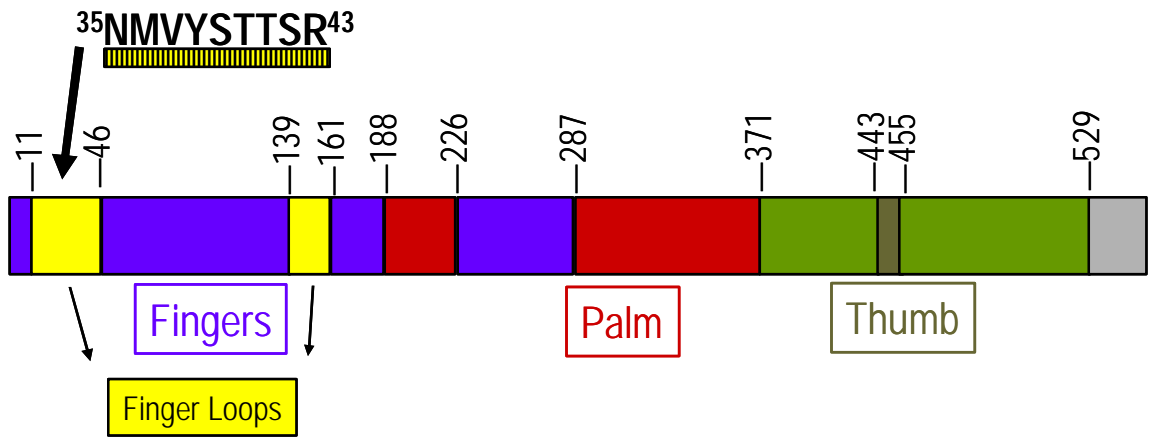


Figure 5

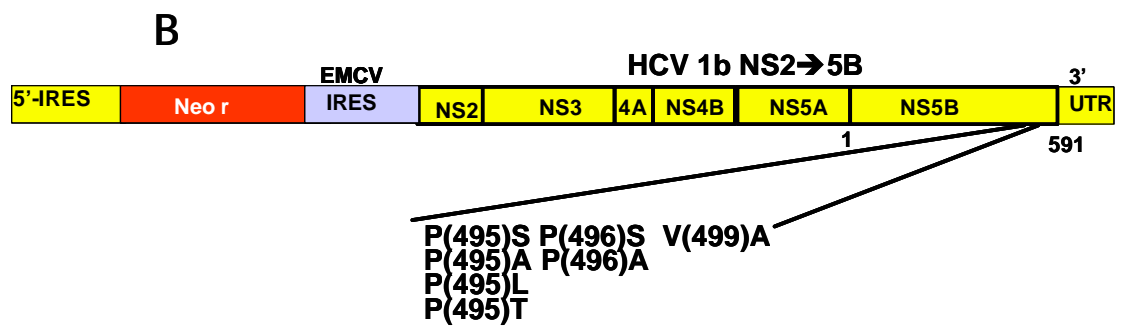
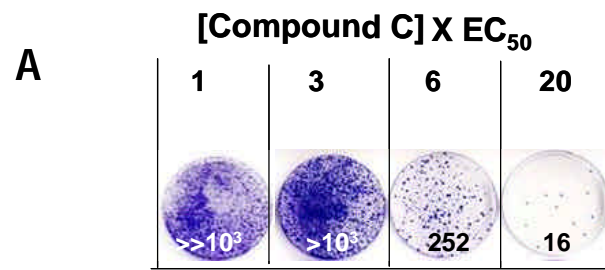


Figure 6

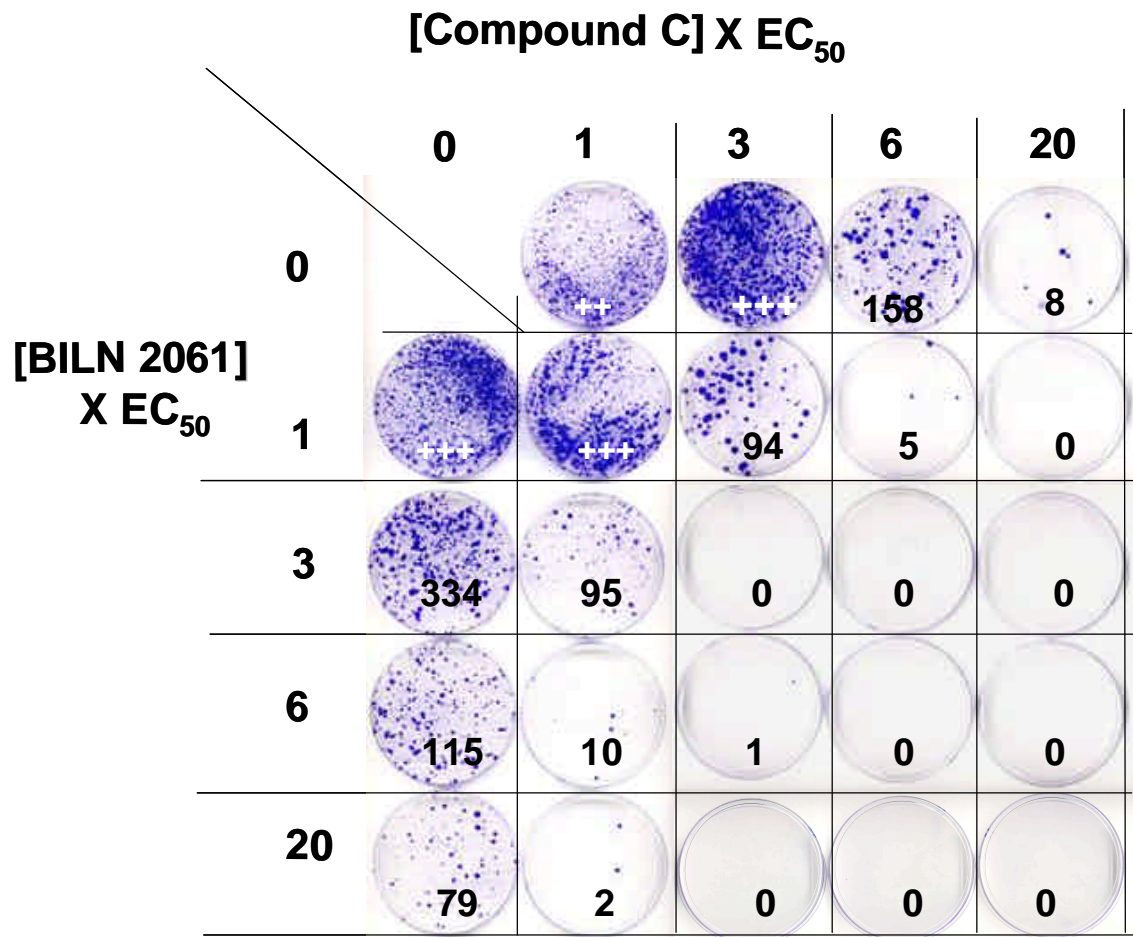


Figure 7

