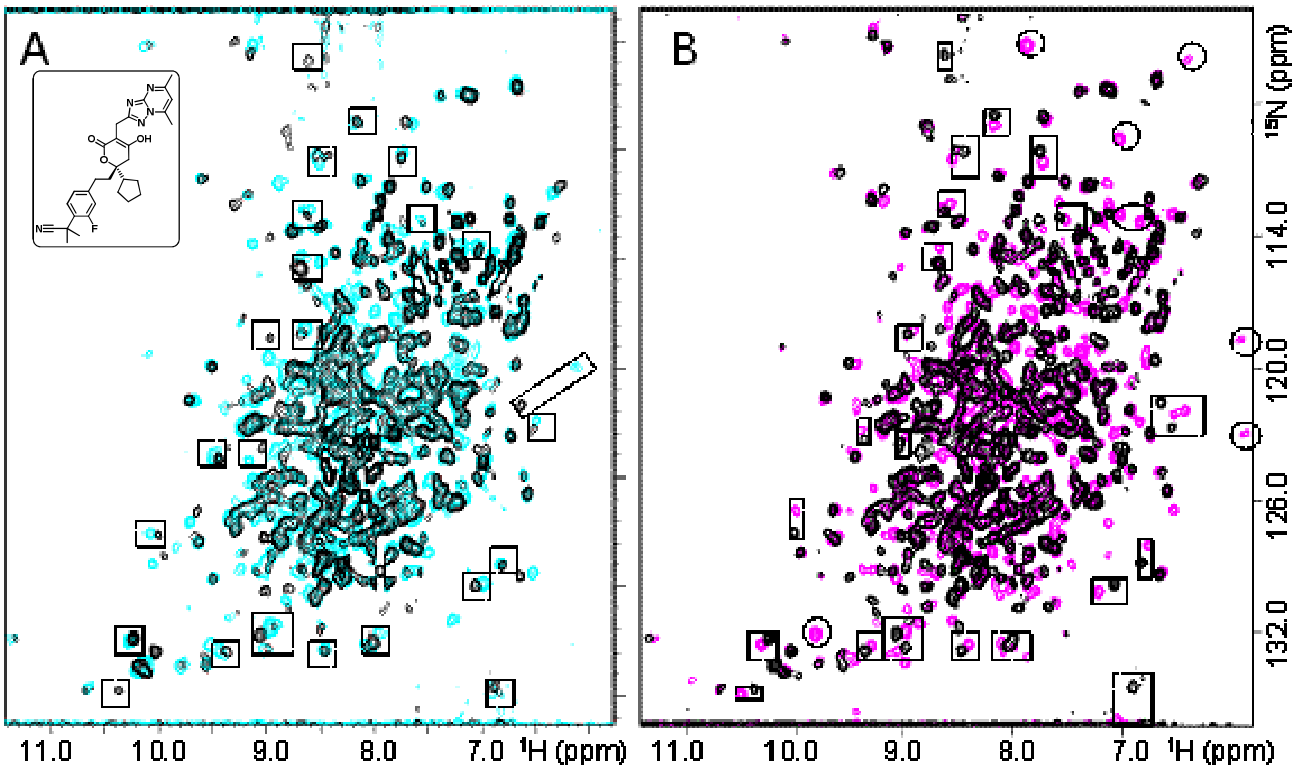
2D ^{15}N-HSQC NMR NMR experiments were performed at 25 ^{o}C on a Bruker Avance 600 MHz NMR spectrometer equipped with a 5 mm triple resonance probe head. The fast ^{15}N-^{15}N-HSQC experiment[25] was used to improve sensitivity and to avoid water saturation. All HSQC experiments were acquired with 304 scans per t1 increment, and a recycle delay of 0.7 s. The ^{1}H and ^{15}N spectral widths were 8,000 Hz with 1024 (t2) complex points and 2000 Hz with 48 indirect complex points, respectively. Linear prediction to 96 complex points was performed in the indirect dimension before Fourier transformation. NMR data were processed and analyzed with Felix program (FELIX Corporate Headquarters, CA). Figure 1A shows the superposition of the ^{15}N-HSQC spectra of NS5B in the absence (black) and in the presence (cyan) of HCV-796. Significant chemical shift changes were observed for many protein residues. Chemical shift changes of selected peaks indicated site specific binding to the palm domain of NS5B. Figure 1B shows the superposition of the ^{15}N-HSQC spectra of NS5B in the absence (black) and in the presence (magenta) of the thienopyrimidine inhibitor (**73**). Again, the chemical shift changes of selected peaks induced by the binding of the inhibitor indicated site specific binding. A detailed comparison of the chemical shift changes induced by the two compounds revealed that many of the same peaks were shifted in both NMR spectra in Figure 1A and 1B (depicted by boxes in Figure 1). This observation suggested that the thienopyrimidine inhibitor (**73**) has an overlapping binding site with HCV-796 and, hence, binds to the palm domain. There are, however, distinct differences in the chemical shift perturbation patterns between the two inhibitors. For many protein peaks both the extents and the directions of peak shifts are different. In addition, some peaks are shifted by compound (**73**), but not HCV-796, indicating possible differences in the binding mode for the two compounds. Finally, several new protein peaks emerged upon binding the thienopyrimidine inhibitor (**73**) (displayed by circles in Figure 1B). The appearance of new peaks suggests protein conformational changes induced by binding of compound (**73**), which may explain, at least in part, the difficulty in obtaining crystal structures of pyridine carboxamide inhibitors in complex with HCV NS5B

polymerase (personal communication, Dr. Charles A. Lesburg at Merck Research Laboratories, Kenilworth, NJ). A detailed comparison of the induced chemical shift changes between HCV-796 and the thienopyrimidine inhibitor (**73**) thus suggests that the pyridine carboxamide inhibitors bind in the palm domain of HCV NS5B polymerase and may induce protein conformational changes upon binding.

We also titrated a known thumb site inhibitor of HCV NS5B into a ^{15}N-labeled polymerase sample. Figure 1C shows the superposition of the ^{15}N-HSQC NMR spectrum of NS5B in the absence (black) and presence (green) of the dihydropyranone derivative (compound TS2) which was previously shown to bind at Thumb Site 2.[18,19] Highlighted with red boxes are those peaks that underwent chemical shift changes upon the binding of this compound but were unchanged when binding the palm site inhibitors above (compare Figure 1C with 1A and 1B). Moreover, many peaks that were shifted in the presence of the palm site inhibitors were unchanged in the presence of the thumb site inhibitor (indicated by diamond boxes in Figure 1C). Taken together, the NMR chemical shift perturbation data clearly show that the thumb site binder exhibits a distinct binding mode from that of the palm site binders above, reinforcing our conclusions that the thienopyrimidine inhibitor (**73**) binds to the palm site of HCV NS5B polymerase.



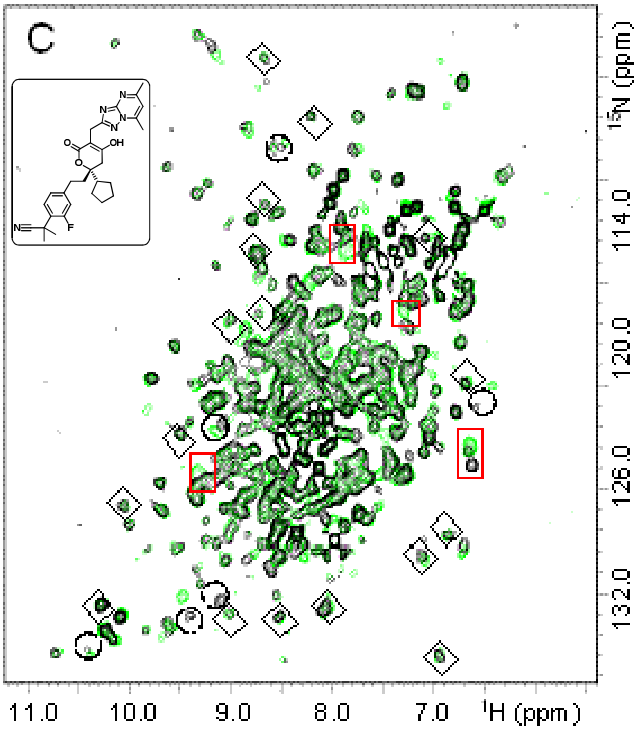


Figure 1. (A) Superposition of 2D ^{15}N-HSQC spectra of ^{15}N-labeled NS5B (150 μM) in the absence (black contours) and presence of HCV-796 (300 μM) (cyan contours). (B) Superposition of 2D ^{15}N-HSQC spectra of ^{15}N-labeled NS5B (200 μM) in the absence (black contours) and presence of the thienopyrimidine inhibitor (**73**) (400 μM) (magenta contours). The boxes depict peaks that were shifted in both (A) and (B). The circles in (B) display new peaks that emerged upon the binding of the thienopyrimidine inhibitor (**73**) to NS5B. (C) Superposition of 2D ^{15}N-HSQC spectra of ^{15}N-labeled NS5B (115 μM) in the absence (black contours) and presence of the dihydropyranone compound (TS2) (394 μM) (green contours). Peaks that are unchanged in the presence of the palm site binders HCV-796 and (**73**) but shifted as a result of binding TS2 are highlighted in red. Peaks that are unchanged in the presence of TS2 but shifted in the presence of HCV-796 or (**73**) are depicted by black diamond boxes. Only a few peaks (circled) are shifted by both HCV-796 and TS2.

Assay Protocols HCV NS5B polymerase activity was measured in a radiolabeled nucleotide incorporation assay as described.[23] Briefly, 50 μL reactions containing 20 mM HEPES (pH 7.3), 7.5 mM DTT, 20 units/mL RNasIN, 1 μM GTP, ATP and UTP, 60 μCi/ml [33P]-CTP supplemented to 20 nM CTP, 10 mM $MgCl_2$, 60 mM NaCl, 100 μg/ml BSA, 100 nM heteropolymer RNA template, 0.25

mM trinucleotide initiator and 30 nM NS5B (Δ21) enzyme were incubated at room temperature for 2 h during which the rate of incorporation was linear. Assay was terminated by the addition of 50 μL 500 mM EDTA. The reaction mixture was transferred to Millipore DE81 filter plate and the incorporation of labeled CTP was determined by Packard TopCount. Compound IC_{50} values were calculated from experiments with 10 serial twofold dilutions of the inhibitor in duplicate.

To measure cell-based anti-HCV activity, replicon cells (1b-Con1) were seeded at 5000 cells/well in 96-well plates in the presence of the pyridine carboxamide derivatives. Various concentrations of an inhibitor were added to the assay mixture, the starting concentration of the compound ranging from 250 μM to 1 μM. The final concentration of DMSO was 0.5%; fetal bovine serum was 5%, in the assay media. Cells were harvested on day 3 post dosing. The replicon RNA level was measured using real time PCR (Taqman assay) as described.[24] EC_{50} values were calculated from experiments with 10 serial twofold dilutions of the inhibitor in duplicate. Cellular toxicity was determined in replicon cells seeded and dosed identically as in replicon activity assays, by MTS read-out 3 days post dosing according to the manufacturer's protocol (Promega, Cat #G3580).

Mechanism of Action Study NS5B primer-extension assay, as detailed in Reference 23, measured multiple rounds of initiation and primer extension, similar to the enzyme activity assay used to generate IC_{50} data of compounds described. Briefly, the trinucleotide initiator was replaced by 100 nM of an 11-mer RNA primer complementary to the 3'-end of the RNA template. The single-round elongation assay measured the burst phase activity (reaction carried out for 15 minutes at room temperature) consisting of single-round extension of pre-formed enzyme-primer-template after 22 hour preincubation at room temperature. Compound **73** behaved as an inhibitor of initiation, as it was inactive against single-round elongation.

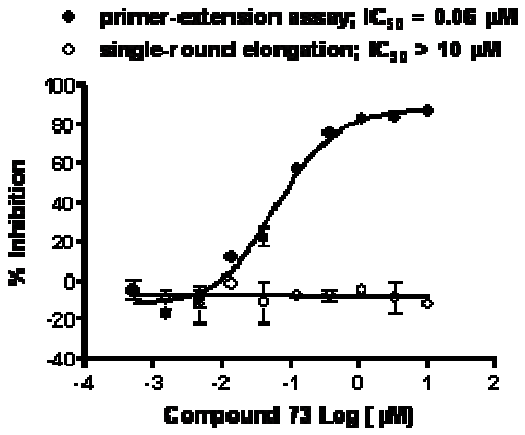


Figure 2. Mechanism of action of compound **73**.

2-Bromo-4-isopropyl-1-nitrobenzene (**6**) To the solution of compound **5** (4.28 g, 20 mmol) in toluene (150 mL) was slowly added *m*-chloroperoxybenzoic acid (25 g) portionwise (Caution: exothermic!). The reaction mixture was brought to reflux and stirred for overnight. The mixture was cooled to room temperature and filtered. The filtrate was diluted with ether, and washed with aqueous NaOH solution (10%) and brine. The organic layer was concentrated. The residue was purified by column chromatography. The product, compound **6**, was eluted with a mixture of Hexane:DCM (4:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (d, *J* = 8.8 Hz, 1H), 7.58 (d, *J* = 1.6 Hz, 1H), 7.29 (dd, *J* = 8.4, 2.0 Hz, 1H), 2.96 (septet, *J* = 7.2 Hz, 1H), 1.27 (d, *J* = 7.2 Hz, 6H).

***t*-Butyl 4-(5-isopropyl-2-nitrophenyl)piperazine-1-carboxylate** (**46**) To the solution of compound **6** (1.22 g, 5 mmol) in DMF was added *tert*-butyl piperazine-1-carboxylate (1.2 g, 6 mmol) and DIEA (1.3 mL, 7.5 mmol). The reaction mixture was stirred in microwave at 200 °C for 20 minutes. The mixture was cooled to room temperature and concentrated. The residue was purified by column chromatography to give compound **46**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, *J* = 8.8 Hz, 1H), 6.96-6.91 (m, 2H), 3.64-3.54 (m, 4H), 3.09-2.98 (m, 4H), 2.92 (septet, *J* = 6.8 Hz, 1H), 1.48 (s, 9H), 1.25 (d, *J* = 6.8 Hz, 6H).

***t*-Butyl 4-(2-amino-5-isopropylphenyl)piperazine-1-carboxylate** (**47**) To the solution of compound **46** (1 g) in methanol (30 mL) was added palladium on carbon (10%, 0.5 g). The reaction mixture was

stirred under an atmosphere of hydrogen at room temperature for overnight. The mixture was filtered through celite. The filtrate was concentrated. Compound **47** was obtained in the amount of 711 mg.

***t*-Butyl 4-(5-isopropyl-2-(2-methylnicotinamido)phenyl)piperazine-1-carboxylate** (**67**) To the solution of 2-methylnicotinic acid (68.6 mg, 0.5 mmol) in DMF was added HATU (190mg, 0.5 mmol), followed by the addition of compound **47** (160 mg, 0.5 mmol) and DIEA (260 μL, 1.5 mmol). The reaction mixture was stirred at 80 ºC for overnight. The mixture was cooled to room temperature and concentrated. The product, compound **11**, was purified by column chromatography using a mixture of EtOAc:Et_3N:MeOH (96:2:2) as the eluent.

***N*-(4-isopropyl-2-(piperazin-1-yl)phenyl)-2-methylnicotinamide** (**68**) Compound **67** was treated with TFA (neat). The mixture was stirred at room temperature for 30 minutes, and then concentrated. Compound **68** was obtained as an HCl salt. ^{1}H NMR (500MHz, DMSO-d_6) δ 9.7 (s, 1H, amide NH), 9.2 (br, 1H, amine NH), 8.8 (d, J = 5 Hz, 1H), 8.3 (d, J = 8 Hz, 1H), 7.9 (d, J = 8 Hz, 1H), 7.8 (t, J = 6, 1H), 7.1 (d, J = 8, 1H), 7.0 (s, 1H), 3.2 (br, 4H), 3.1 (br, 4H), 2.9 (septet, J = 7 Hz, 1H), 2.7 (s, 3H), 1.2 (d, J = 7 Hz, 6H). Mass calculated for formula $C_{20}H_{26}N_4O$ 339.21792 (M+H); observed HRMS 339.21756 $(M+H)^+$.

***N*-(2-(4-(7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperazin-1-yl)-4-isopropylphenyl)-2-methylnicotinamide** (**71**) Compound (**68**) (28.7 mg, 0.084 mmol) was dissolved in DMA (2 mL) and then 6-chloro-7-deazapurine (32.3 mg, 0.210 mmol), and DIEA (73.2 uL, 0.420 mmol) were added and microwaved at 200 °C for 20 min. At the end of the reaction the solution solvent was evaporated in vacuo, then dissolved in DMSO/acetonitrile (3:1), filtered through 0.45 μM filter cartridge and purified using reverse phase HPLC to provide desired product **71**. HPLC (TFA, 10 min) RT = 2.17 min. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.9 (s, 1H, amide NH), 10.0 (s, 1H), 8.82 (d, J = 8 Hz, 2H), 8.6 (d, J = 8 Hz, 2H), 8.4 (s, 1H), 7.8-7.6 (d, 1H, and t, 1H), 7.1 (s, 1H, broad), 7.0-7.4 (s, 1H and s, 1H, broad), 4.2 (br, 4H), 3.2 (br, 4H), 2.9-2.8 (s and septet overlap, 4H), 1.1 (m, 6H). Mass calculated for formula $C_{26}H_{29}N_7O$ 456.25062 (M+H); observed HRMS 456.24976 $(M+H)^+$.

***N*-(4-isopropyl-2-(4-(thieno[3,2-*d*]pyrimidin-4-yl)piperazin-1-yl)phenyl)-2-methylnicotinamide**

(**73**) Compound (**68**) (45mg, 0.135 mmol) was dissolved in DMA (2 mL) and then 4-chlorothieno[3,2-*d*]pyrimidine (57.6 mg, 0.337 mmol), and DIEA (117 uL, 0.675 mmol) were added and microwaved at 200 °C for 20 min. At the end of the reaction the solution solvent was evaporated in vacuo, then dissolved in DMSO/acetonitrile (3:1), filtered through 0.45 μM filter cartridge and purified using reverse phase HPLC to provide desired product **73**. HPLC (TFA, 10 min) RT = 2.23 min. ^{1}H NMR (400MHz, DMSO-d_6) δ 10.0 (s, 1H, amide NH), 8.9 (s, 1H), 8.80 (d, *J* = 6 Hz, 1H), 8.65 (d, *J* = 6 Hz, 1H), 8.55 (d, *J* = 7 Hz, 1H), 7.9 (t, 2H), 7.62 (s, 1H), 7.06 (s, 1H), 7.02-7.04 (d, *J* = 9 Hz, 1H), 4.25 (br, 4H), 3.08 (br, 4H), 2.82-2.78 (s and septet overlap, 4H), 1.08 (m, 6H). Mass calculated for formula $C_{26}H_{28}N_6OS$ 473.21179 (M+H); observed HRMS m/z 473.21135 $(M+H)^+$.