Antiviral Research 86 (2010) 144–153

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

A novel Hepatitis C virus p7 ion channel inhibitor, BIT225, inhibits bovine viral diarrhea virus *in vitro* and shows synergism with recombinant interferon-α-2b and nucleoside analogues

Carolyn A. Luscombe[a], Zhuhui Huang[b], Michael G. Murray[b], Michelle Miller[a], John Wilkinson[a], Gary D. Ewart[a,*]

[a] *Biotron Limited, Sydney, NSW, Australia*
[b] *Hepatitis Research Program, Southern Research Institute, Frederick, MD 21701, USA*

ARTICLE INFO

Article history:
Received 7 August 2009
Received in revised form
21 December 2009
Accepted 4 February 2010

Keywords:
Novel antiviral compound
Flavivirus inhibitor
BVDV
HCV
p7

ABSTRACT

The novel small molecule, BIT225 (*N*-[5-(1-methyl-1*H*-pyrazol-4-yl)-napthalene-2-carbonyl]-guanidine: CAS No. 917909-71-8), was initially identified using a screening strategy designed to detect inhibitors of Hepatitis C virus (HCV) p7 ion channel activity. Here we report that BIT225 has potent stand-alone antiviral activity against the HCV model pestivirus bovine viral diarrhea virus (BVDV) with an IC_{50} of 314 nM. Combinations of BIT225 with recombinant interferon alpha-2b (rIFNα-2b) show synergistic antiviral action against BVDV and the synergy is further enhanced by addition of ribavirin. Synergy was also observed between BIT225 and two nucleoside analogues known to inhibit the HCV RNA-dependent RNA polymerase.

BIT225 has successfully completed a phase Ia dose escalating, single dose safety trial in healthy volunteers and a phase Ib/IIa trial to evaluate the safety and pharmacokinetics of repeated dosing for selected doses of BIT225 in HCV-infected persons. A modest, but statistically significant drop in patient viral load was detected over the 7 days of dosing (ref. www.biotron.com.au). Given the critical role of the p7 protein in the HCV life cycle and pathogenicity, our data indicate that molecules like BIT225, representing a new class of antiviral compounds, may be developable for therapeutic use against HCV infection, either as monotherapy, or in combination with other HCV drugs.

1. Introduction

The *Flaviviridae* are a family of enveloped viruses with nucleocapsids containing one molecule of a single stranded, positive sense, RNA genome of around 9500–12500 nucleotides (Dimmrock et al., 2001). The family is comprised of three genera—Pestiviruses (e.g. bovine viral diarrhea virus—BVDV), Flaviviruses (e.g. dengue virus), and Hepaciviruses (e.g. Hepatitis C virus—HCV). Members of the latter two genera cause significant diseases in humans.

From a medical research perspective, HCV is perhaps the most notorious (reviews Bartenschlager et al., 2004; Penin et al., 2004), although mosquito-borne flavirus diseases such as dengue fever and yellow fever also cause significant disease burden in humans. HCV infects ∼170 million people, or approximately 3% of the world's population and can lead to life-threatening liver disease including hepatocellular carcinoma. No vaccines are available to prevent HCV infection and the current standard of care therapy relies on treatment with pegylated interferon α (Peg-IFN) and ribavirin. Peg-IFN and ribavirin therapy requires a long duration of therapy (48 weeks) and is not universally effective at reducing viral burden or preventing disease progression. For treatment of genotype 1 HCV infections the sustained viral response rate (SVR) is approximately 50%. It is also expensive and frequently associated with side effects that limit eligibility for and compliance to therapy (Manns et al., 2006). Hence, there is still an urgent need for new anti-HCV agents that target different aspects of virus replication to work, either in concert with Peg-IFN and ribavirin, or perhaps to replace those drugs with combinations of multiple new specifically targeted anti-HCV (STAT-C) compounds. Such combinations are likely to result in improved rates of control and elimination of virus from HCV-infected individuals.

There are several new classes of direct acting anti-HCV agents under clinical development that may potentially be used in the next decade. These compounds are primarily agents that have activity against the HCV polymerase or protease (Kwong et al., 2008; Thompson et al., 2009). Both drug classes are potent inhibitors of HCV replication and in clinical trials show enhanced response rates

* Corresponding author at: Biotron Limited, Innovations Bld. #124, Eggleston Rd., Canberra, ACT 2602, Australia.
E-mail address: gewart@biotron.com.au (G.D. Ewart).

0166-3542/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2010.02.312

compared to current standard of care therapy (Kwong et al., 2008; Thompson et al., 2009).

Another target for new STAT-C compounds is p7. The p7 protein is a small membrane protein encoded between the E2 and NS2 proteins. It is known to be required for HCV replication in chimpanzees (Sakai et al., 2003), and is crucial for virus replication *in vitro*, acting at a late stage involving virus assembly and release (Steinmann et al., 2007a). Similarly, the p7 polypeptides of BVDV (Harada et al., 2000) and HCV's closest relative, the *hepacivirus* GBV-B (Takikawa et al., 2006), are also known to be crucial for virus replication. Hence, p7 is a valid target for antiviral drug development.

p7 oligomerizes in phospholipid membranes to form a cation-selective ion channel (Griffin et al., 2003; Pavlovic et al., 2003; Premkumar et al., 2004; Clarke et al., 2006), which is a drug targetable molecular activity of the protein; the only one so far characterized. Consequently, p7 belongs to the same family of virus-encoded ion-channel-forming proteins as the influenza A protein M2 and HIV-1 Vpu. This family of proteins are known collectively as viroporins (Gonzalez and Carrasco, 2003). Viroporin inhibitors were first approved for use in humans over 40 years ago in the form of the anti-influenza A drugs amantadine and rimantadine, establishing a clear precedent for successful pharmaceutical development for this class of antiviral compounds (Couch, 2000; Oxford et al., 2003). Amantadine and rimantadine inhibit influenza A by blocking H^+-conduction through the M2 ion channel, an activity affecting conformational changes in virus proteins which are essential for virus replication (Hay et al., 1985; Pinto and Lamb, 2006).

In contrast to the influenza story, the sequence of molecular mechanisms by which *Flaviviridae* p7 facilitates virus assembly and release have not yet been elucidated. However, the simplest starting hypothesis is that a mechanism similar to that of M2, involving the p7 ion channel activity, may be operating. Further, whatever the actual mechanism, it would seem likely that inhibitors of p7 ion channels – as long as they are able to reach the p7 target in infected cells – will show antiviral activity. Indeed, to date, a number of compounds have been identified that inhibit HCV p7 ion channels (Griffin et al., 2003; Pavlovic et al., 2003, 2005; Premkumar et al., 2004), and Griffin et al. (2008) have recently demonstrated that such compounds are capable of inhibiting HCV replication in the JFH1 *in vitro* HCV cell culture system.

Here we report initial identification of BIT225, a novel small molecule that is the first representative of a new class of HCV p7 ion channel inhibitors.

As part of early characterization of the antiviral properties of BIT225, we tested the compound against the well-established HCV model virus, BVDV (Buckwold et al., 2003a), and found that BIT225 inhibits replication of BVDV at a sub-micromolar IC_{50}. The BVDV system was also used to examine drug interactions between BIT225 and other known antiviral compounds and revealed synergistic antiviral activity between BIT225 in combination with recombinant interferon α-2b (rIFNα-2b) and ribavirin, as well as between BIT225 and two nucleoside analogues known to inhibit the HCV RNA-dependent-RNA polymerase.

2. Materials and methods

2.1. BIT225

BIT225 (Fig. 1) was synthesized for Biotron Limited at Epichem Pty. Ltd. (Murdoch, WA, Australia). For long-term storage, the compound was stored desiccated at −80 °C. Stock solutions at 500 mM were made in DMSO and were stored at −20 °C protected from light for up to 1 week. For inhibition of p7 ion channels in planar lipid bilayer experiments, a working solution of 100 mM BIT225 in DMSO was prepared and 1 μl aliquots were added to the *cis* and *trans* chambers with stirring (the final concentration of BIT225 in each chamber was 100 μM). DMSO was also used as the compound carrier in virological assays and carrier-only controls were included in all experiments.

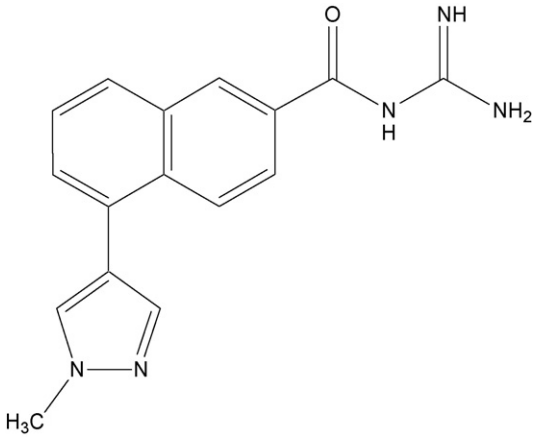

Fig. 1. Structure of BIT225 (*N*-[5-(1-Methyl-1*H*-pyrazol-4-yl)-napthalene-2-carbonyl]-guanidine.) [CAS No. 917909-71-8].

2.2. P7 ion channel recording in planar lipid bilayers

The synthesis and characterization of a peptide corresponding to the p7 protein of HCV H77 (genotype 1a) was described previously (Premkumar et al., 2004). The peptide sequence is ALENLVILNAASLAGTHGLVSFLVFFCFAWYLKGRWVPGAVYAFYGMWPLLLLLLALPQRAYA; it shows a strong peak at *m*/*z* ratio 7018.4 by matrix-assisted laser desorption/ionization time of flight mass spectroscopy and appears as a single protein band on Coomassie stained polyacrylamide gels (4–20% gel run in Tris–Glycine–EDTA buffer with 8 μg of peptide loaded per lane).

Lipid bilayer experiments were also performed essentially as described previously (Premkumar et al., 2004), except that a lipid mixture of palymitoyl-oleoly-phospatidylethanolamine, palymitoyl-oleoly-phospatidylcholine and palymitoyl-oleoly-phospatidylserine (6:3:1) in *n*-decane was used, and unless specified otherwise, buffers in the *cis* and *trans* chambers were symmetrical; 50 mM KCl in 10 mM MES buffer (pH 7.2). The p7 peptide was dissolved in 2,2,2-trifluoroethanol (TFE), initially at 7.5 mg/ml, then diluted to 0.15 mg/ml in TFE, and 2 μl (0.3 μg) was added to the *cis* and *trans* chambers, for a final peptide concentration of approximately 40 nM bathing both sides of the bilayer. Both chambers were stirred to facilitate spontaneous incorporation of the peptide into the bilayer and channel activity was typically detected within 5–10 min at a holding potential of −60 mV (*trans* relative to *cis*). Currents were filtered at 1 kHz, and digitized at 2 kHz. For subsequent analysis and presentation, data traces were filtered at 200 Hz and mean currents were determined over a period of 10–30 s.

2.3. BVDV antiviral assays

The antiviral efficacy against BVDV (strain NADL; American Type Culture Collection [ATCC]) was evaluated as inhibition of virus-induced cytopathic effect (CPE) in infected Madin–Darby bovine kidney (MDBK) cells [ATCC], essentially as described by Buckwold et al. (2003b). Antiviral assays were designed to test 6 half-log concentrations of each compound in triplicate against the challenge virus. Cell viability was measured 7 days post-drug addition by staining the cells with Cell Titer 96 (Promega, Madison, WI). Averaged replicate data were converted to % inhibition of CPE, based

on 100% being the average cell control value (uninfected cells, no drug) and 0% being the average virus control value (infected cells, no drug). Assessment of compound cytotoxicity was run in parallel; with % of cell viability expressed within the range between 100% = average cell control O.D. and 0% = average background O.D. (wells with no cells).

2.4. Antiviral combination assays

For characterization of the efficacy of combinations of two compounds against BVDV-induced CPE, a checkerboard dilution matrix of all permutations of 2-fold serial dilutions of each compound was prepared, and the MacSynergy II software (Prichard and Shipman, 1996) was used – as per (Buckwold et al., 2003b) – to evaluate whether there was evidence of synergy or antagonism for antiviral activity and/or cytotoxicity. For BIT225, eight 2-fold dilutions were used (max. conc. 4 μM) and combined with five 2-fold dilutions of either rIFNα-2b (max. conc. 80 IU/ml); ribavirin (max. conc. 20 μg/ml); 2′-C-methyladenosine (CMA; max. conc. 10 μM); or, 2′-C-methylcytidine (CMC; max. conc. 10 μM). For testing the effect of triple combinations of BIT225, rIFNα-2b and ribavirin, experiments were set up with a fixed concentration of rIFNα-2b (either 5 or 10 IU/ml) in all wells and a checkerboard of BIT225 and ribavirin concentrations, as above.

Using the MacSynergy II software on the basis of the Bliss independence mathematical definition, the expected additive antiviral protection was determined from the dose–response curves of the individual drugs and subtracted from the experimental antiviral activity for each combination concentration (Prichard and Shipman, 1990). The resultant data matrix was plotted as a three-dimensional surface and two values – corresponding to the total volumes of the surfaces above and below the zero plane (plane of additivity) – were calculated. Volumes of positive value reflect synergism and negative volumes reflect antagonism. Synergy or antagonism volumes were statistically calculated at 95% confidence level as $Synergy_{95}$, and $Antagonism_{95}$. $Synergy_{95}$ values of between 50 and 100 U (in units of concentration times concentration times percent; e.g. IU/ml μM%; μM^2%, μg/mlμM%) were defined to be slightly synergistic and values of greater than 100 U to be highly synergistic; with corresponding interpretation for the negative values of $Antagonism_{95}$. $Synergy_{95}$ or $Antagonism_{95}$ values in the range ±50 U are considered to fall within the range of additivity.

3. Results

3.1. Identification of BIT225 as a potential p7 ion channel inhibitor

BIT225 (Fig. 1) was identified as an inhibitor of HCV p7 after screening Biotron's library of antiviral compounds. The initial screen employed an indirect, semi-quantitative, moderate throughput p7 bacterial bioassay. The method is described fully elsewhere (Ewart and Best, 2006; Cox et al., 2007; Gage et al., 2007): essentially, a synthetic cDNA fragment encoding the HCV p7 protein (strain H77 genotype 1a), with codons optimized for expression in *Escherichia coli* (GenScript, Piscataway, NJ), was cloned into the expression vector pPL451(Love et al., 1996), creating the plasmid pPLp7, in which expression of p7 is downstream of the tandem λ p_R and p_L promoter pair and under control of the temperature sensitive $cI^{ts}857$ repressor. Expression of p7 is tightly repressed at 30 °C and increasing levels of protein can be induced by raising the culture temperature; with a maximal expression level induced at 42 °C. As shown previously, for growth on minimal nutrient media, expression of a Na^+/K^+ conductive viroporin, like p7, in the *E. coli* plasma membrane causes a de-energized state leading to the leak of essential metabolites (e.g. adenine, proline) from the cell that are required for growth (Ewart et al., 1996). In the presence of inhibitors of the p7 ion channel, normal ionic gradients are maintained in the *E. coli* cell, the metabolites are retained, and the cells are able to grow despite expression of p7. Thus, partial rescue of the growth of *E. coli* cells expressing HCV p7 provided the first evidence that BIT225 is able to inhibit the HCV p7 ion channel. This method is used as the first screening step for all compounds of the Biotron compound library. In the format used, small volumes (~1 μl) of compound containing solutions were applied in discrete locations on minimal medium agar plates that had been inoculated at 30 °C with a background lawn of *E. coli* cells containing the p7 expression vector. After allowing the compounds to absorb into the agar, the plates were incubated overnight at 35–37 °C to induce expression of p7 (which inhibits growth of the cells in the background lawn). The plates were examined by eye and compounds were scored as positive if a halo of *E. coli* cell growth (indicating inhibition of expression of the p7 channel activity) was clearly visible around the site of application of the compound. BIT225 consistently scored positive in this assay.

3.2. BIT225 inhibits HCV p7 ion channel activity in planar lipid bilayers

As reported previously by ourselves and others, HCV p7 forms ion channels in artificial planar lipid bilayers that are weakly cation selective and conductive to K^+, Na^+, and Ca^{2+} ions (Griffin et al., 2003; Pavlovic et al., 2003; Premkumar et al., 2004). Chloride ions are also permeable, though to a lesser extent (Premkumar et al., 2004). We also reported that p7 channels are blocked by 5-(*N*-*N*-hexamethlyene)-amiloride (Premkumar et al., 2004). Here, as illustrated in Fig. 2, we show that BIT225 inhibits p7 ion channel activity. The traces in Fig. 2A–C were obtained at a transmembrane holding potential of −60 mV: the *cis* and *trans* chambers both contained 50 mM KCl. The bilayer was painted and gave a stable capacitance of 130 pF in tests using an applied linear voltage ramp (1 V/s), and Fig. 2A shows the baseline current data for the bilayer prior to addition of a peptide corresponding to p7 from HCV genotype 1a, strain H77. Peptide was added to the *cis* and *trans* chambers, at a final concentration of approximately 40 nM, and after stirring both chambers for approximately 8 min, ion channel activity was detected: a 10 s sample trace is shown in Fig. 2B. Over a period of about 7 min, current flowing through the bilayer fluctuated between 0 pA (channel closed state; as indicated by the dashed line in Fig. 2B) and a maximal open state current of around 3 pA, with an average current over the period of 0.65 pA (SD 0.27; Fig. 2D). Upon addition of 2 μl of 50 mM BIT225 in DMSO to the *cis* and *trans* chambers (final concentration; 100 μM), channel activity ceased after a brief period of stirring (Fig. 2C) and average bilayer current returned to baseline over the subsequent 2 min, after which time the experiment was terminated.

Complete p7 channel inhibition by 100 μM BIT225 (as per Fig. 2D) was seen in 3 out of 3 replicate experiments. Inhibition was strictly dependent on the presence of BIT225: addition of control volumes of DMSO carrier alone did not effect the ion currents (data not shown).

It should be noted that these experiments were not designed to fully characterize the interaction of BIT225 with p7. Undoubtedly, much time and effort could be devoted to such experimentation. However such experiments were not our priority, which was rather to establish whether BIT225 could inhibit the p7 ion channel at a reasonable concentration. We (arbitrarily) set 100 μM as an upper limit, by which we should expect to see significant inhibition of the p7 channel in bilayers if physiologically relevant inhibition were likely to be seen at sub-toxic levels in cell culture experiments and we were both pleasantly surprised and satisfied to see that

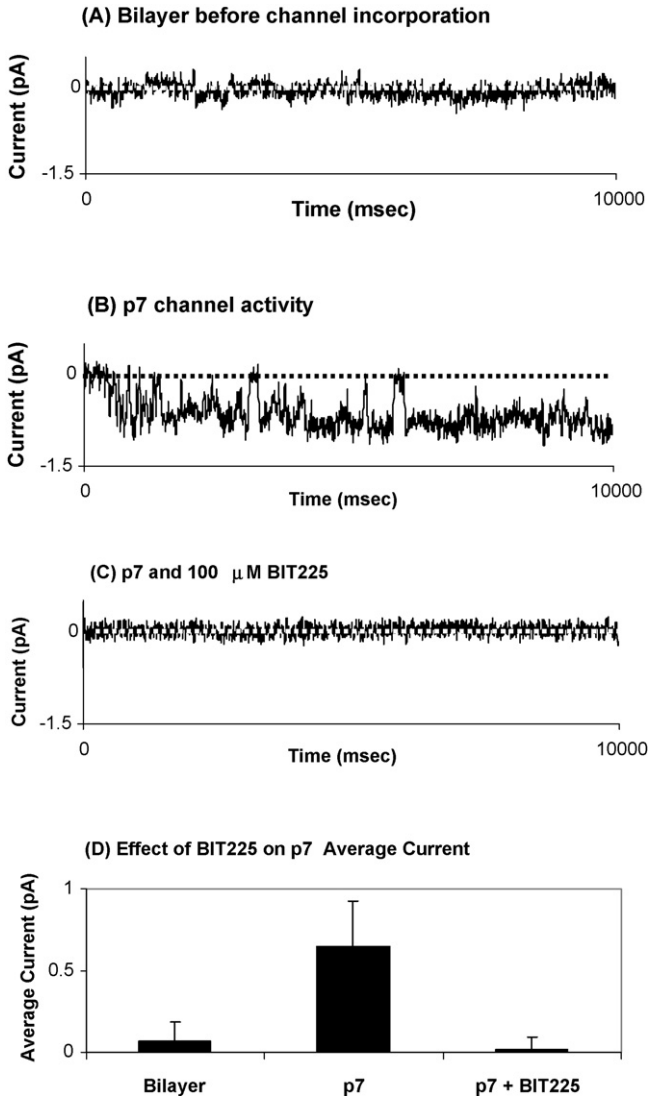

Fig. 2. BIT225 inhibits HCV p7 ion channels in planar lipid bilayers. Bilayer experiments were performed as described in Section 2. Representative 10 s traces of bilayer currents, filtered at 200 Hz: (A) baseline bilayer current; (B) a period of maximal p7 channel activity; (C) addition of BIT225 inhibits the p7 channel activity. (D) Left to right: average bilayer current over periods of 2–5 min corresponding to conditions in A–C.

BIT225 showed complete channel inhibition at that concentration in artificial bilayers.

In summary, results from the planar lipid bilayer experiments, in conjunction with the p7 bacterial bioassay, showed that BIT225 is able to inhibit the ion channel activity of the HCV p7 protein.

3.3. BIT225 inhibits BVDV in vitro and shows synergy with rIFNα-2b

In vitro replication of BVDV is a well-established, readily accessible and user-friendly experimental system that, before the advent of JFH1-based cell culture replicative HCV systems, was widely accepted as a surrogate system for initial testing of potential anti-HCV compounds (Buckwold et al., 2003a). It is still a valid choice for certain aspects of drug development, such as assessing synergy or antagonism of combinations of antiviral drugs. Such experiments are not yet standard in the JFH1 system.

BIT225 is highly active against BVDV: the dose–response curve is shown in Fig. 3. In 6 experiments BIT225 gave an average IC_{50} of 314 nM (95% confidence interval 295–335 nM). In these experiments, no significant cytotoxicity was seen in uninfected MDBK cells up to 2 μM, with approximately 16% cell death seen at 4 μM (Fig. 3A). In a single earlier screening experiment the 50% cytotoxicity concentration (CC_{50}) of BIT225 for MDBK cells was measured at 11.6 μM, giving an antiviral index (IC_{50}/CC_{50}) in these cells of around 40. Clearly, BIT225 inhibits replication of BVDV at concentrations lower than those causing cytotoxicity. We were therefore able to use this system to evaluate potential interactions between BIT225 and other compounds with known anti-HCV activity.

We tested the effects of (a) double compound combinations of BIT225 with rIFNα-2b or ribavirin; as well as, (b) triple combinations of BIT225, rIFNα-2b and ribavirin. As described in Section 2, culture plates of MDBK cells were set up in a checkerboard fashion to test combinations of concentration over the effective ranges of each compound and the resultant cytotoxicity or CPE data were analyzed using the MacSynergy II program (Prichard and Shipman, 1996). The experiments testing triple compound combinations were set up at two fixed concentrations of interferon (5 IU/ml or 10 IU/ml) in each culture well, with varying concentrations of BIT225 and ribavirin. The results for all combination studies are summarized in Table 1 and MacSynergy plots for representative experiments are shown in Fig. 4.

Antiviral synergy was observed between BIT225 and rIFNα-2b (Fig. 4A) with an average $Synergy_{95}$ volume (see Section 2 for definition) of 87 μM IU/ml% over 3 experiments (Table 1).

On the other hand, antagonism was observed between the BIT225 and ribavirin (Fig. 4B). This effect occurs primarily at the higher concentrations of both compounds. As reported in the literature and confirmed in these studies, ribavirin on its own did not show significant antiviral activity at concentrations up to 20 μg/ml (<5% inhibition of CPE) (Fig. 3C). Therefore, the results may suggest that, at higher concentrations, ribavirin interferes with the strong antiviral activity of BIT225.

Despite the observation of a degree of antagonism between BIT225 and high concentrations of ribavirin, the presence of low concentrations of ribavirin enhanced the $Synergy_{95}$ volumes derived from the triple compound experiments as compared to combinations of BIT225 and interferon only (Table 1). Further, this effect was seen to primarily occur at the lower concentrations of BIT225 and at the higher concentrations of BIT225 the net effect of the presence of interferon was to cancel the antagonism between BIT225 and ribavirin (Fig. 4C and D). Ribavirin is well known to enhance the activity of IFN against BVDV, as well as improve the success rate of Peg-IFN treatment for HCV patients (Buckwold et al., 2003b; Buckwold, 2004), and the results presented here show that the ribavirin is also able to increase the antiviral synergy of combinations of BIT225 and rIFNα-2b.

Antagonistic effects in cytotoxicity are favorable interactions, suggesting that addition of one drug could offset partial cytotoxicity of another drug. The cytotoxicity arms of the experiments indicate some antagonism in the case of the triple compound combinations (Fig. 4 G and H) that was not seen in the double compound experiments (Fig. 4 E and F). Again, this effect was predominantly at the higher concentrations of ribavirin. Our data indicated that addition of BIT225 to rIFNα-2b and ribavirin achieved reduced combined cytotoxicity.

The effect of rIFNα-2b and ribavirin on BIT225 dose–response for BVDV-induced CPE was further analyzed by fitting sigmoidal curves (using GraphPad Prism® software) to individual data sets. In general, we found that ribavirin alone did not significantly affect the IC_{50} of BIT225 against BVDV. In contrast, increasing concentrations of rIFNα-2b caused a reduction of BIT225 IC_{50} values: using consistent curve fitting parameters (variable Hill slope; TOP and BTM constants constrained to 93% and 8%, respectively), the IC_{50} values (95% confidence intervals in brackets) for BIT225 in the

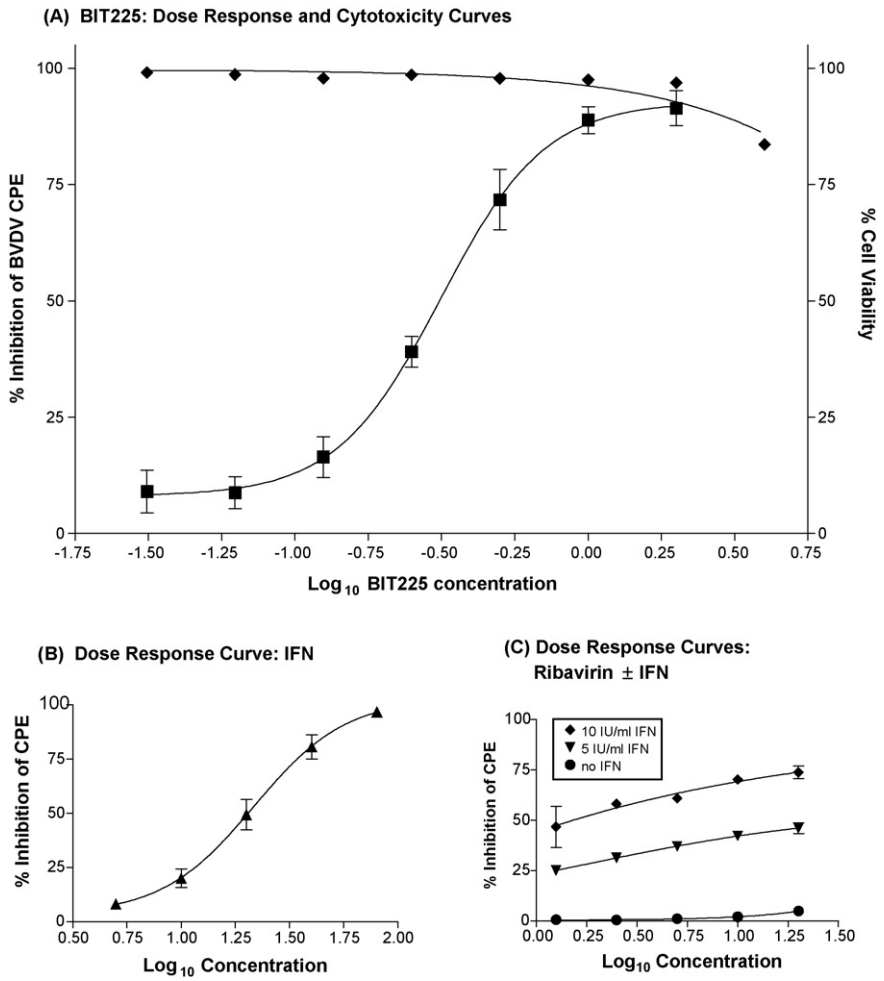

Fig. 3. Dose–response curves for single compounds against BVDV in MDBK cells. Sigmoidal curves were fit to the experimental data for % inhibition of BVDV CPE and % cell viability after exposure to BIT225, and IC_{50} values were determined using Graphpad Prism® software. Curve fitting parameters allowed variable Hill slope and unrestrained TOP and BTM constants. (A) Combined dose–response (squares) and cytotoxicity (diamonds) curves for BIT225 as a stand-alone compound against BVDV: for dose–response, IC_{50} = 314 nM (95% CI: 295–335), error bars are SEM for n = 6 experiments, goodness of curve fit; R^2 = 0.999. (B) Dose–response curve for rIFNα-2b: IC_{50} = 21.7 IU/ml (95% CI: 17.8–26.5), error bars are SEM for n = 3 experiments, goodness of curve fit; R^2 = 1.00. (C) Dose–response curve for ribavirin alone (circle symbols): IC_{50} = not applicable, error bars are SEM for n = 3 experiments. Also shown in C are the dose–response curves for ribavirin in the presence of 5 and 10 IU/ml rIFNα-2b (triangle and diamonds, respectively).

presence of 0, 5 and 10 IU/ml of rIFNα-2b, respectively, were 314 nM [295–335]; 127 nM [90–179]; and 55 nM [35–86]. Although the numbers are slightly different when the curves are fit with unrestrained TOP and BTM parameters, the same trend of reducing BIT225 IC_{50} with increasing interferon is seen.

Addition of low concentrations of ribavirin to combinations of BIT225 plus rIFNα-2b greatly increased the antiviral activity, as illustrated in Fig. 5A. Fig. 5B highlights the synergy of triple compound combinations at the lowest concentration of BIT225 tested (31 nM). For example, it can be seen that the combination of 5 IU/ml rIFNα-2b plus 31 nM BIT225 plus the lowest concentration of ribavirin tested (1.25 μg/ml), yielded >70% inhibition of BVDV CPE. The same low concentrations of BIT225 and ribavirin, in the presence of 10 IU/ml IFNα yielded >90% virus inhibition. For comparison: 5 IU/ml IFNα alone gave ~8% inhibition; 31 nM BIT225 alone gave ~5% inhibition; and 1.25 μg/ml ribavirin alone showed no antiviral activity. Clearly the triple combination is highly efficacious against BVDV.

Table 1
$Synergy_{95}$/$Antagonism_{95}$ volumes for drug combinations containing BIT225.

Combination (BIT225+)	Antiviral activity		Cytotoxicity	
	$Synergy_{95}$/$Antagonism_{95}$ Volumes	Interpretation	$Synergy_{95}$/$Antagonism_{95}$ Volumes	Interpretation
rIFNα-2b[a]	87/−1	Slight synergy	1/−7	Additive
Ribavirin[b]	1/−72	Slight antagonism	2/−4	Additive
Ribavirin[b] + rIFNα-2b (5 IU/ml)	138/0	High synergy	0/−59	Slight antagonism
Ribavirin[b] + rIFNα-2b (10 IU/ml)	127/0	High synergy	0/−67	Slight antagonism
2′-C-methyl-adenosine[c]	107/−3	High synergy	2/−53	Slight antagonism
2′-C-methyl-cytidine[c]	71/0	Slight synergy	0/−18	Additive

[a] Units for $Synergy_{95}$/$Antagonism_{95}$ volumes: IU/ml μM%.
[b] Units for $Synergy_{95}$/$Antagonism_{95}$ volumes: μg/ml μM%.
[c] Units for $Synergy_{95}$/$Antagonism_{95}$ volumes: μM^2%.

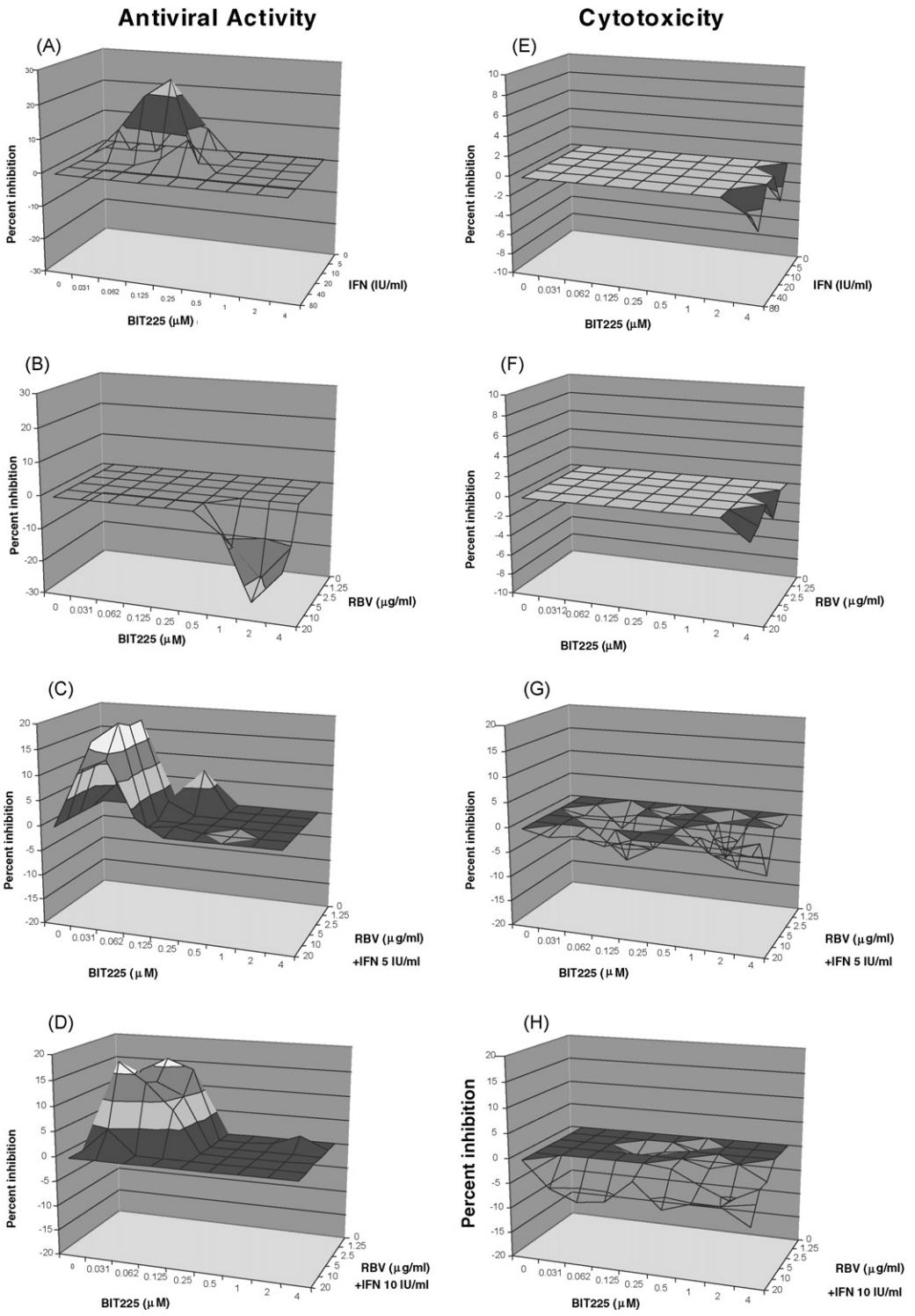

Fig. 4. MacSynergy II plots for antiviral activity against BVDV (A–D) and cytotoxicity (E–H) data for representative compound combination experiments. As described in Section 2, these plots represent the surfaces generated by the $Synergy_{95}$ and $Antagonism_{95}$ values calculated from the data. Mountains above the plane of additivity at zero percent inhibition represent regions of significant ($p = 0.05$) synergy and valleys represent antagonism. All plots show compounds in combination with BIT225 (*x*-axis): rIFNα-2b (A and E); ribavirin (B and F); ribavirin with fixed concentration of 5 IU/ml rIFNα-2b (C and G); ribavirin with fixed concentration of 10 IU/ml rIFNα-2b (D and H).

3.4. BIT225 shows synergy with nucleoside analogues

No other therapeutic compounds, apart from IFNs and ribavirin, have yet been approved for use in HCV patients. However, of those under development, the two main classes are the nucleoside analogues and the protease inhibitors (Tomei et al., 2005; Sarrazin et al., 2007; Liu-Young and Kozal, 2008). Of these, some nucleoside analogues have been shown to be effective in the BVDV model. We further investigated the antiviral interaction between BIT225 and two nucleoside analogues active against BVDV: 2′-C-methyladenosine (CMA), and 2′-C-methylcytidine (CMC). Their anti-HCV activities have previously been reported (Carroll et al., 2003). Our data suggested that both of these nucleoside analogues as stand-alone

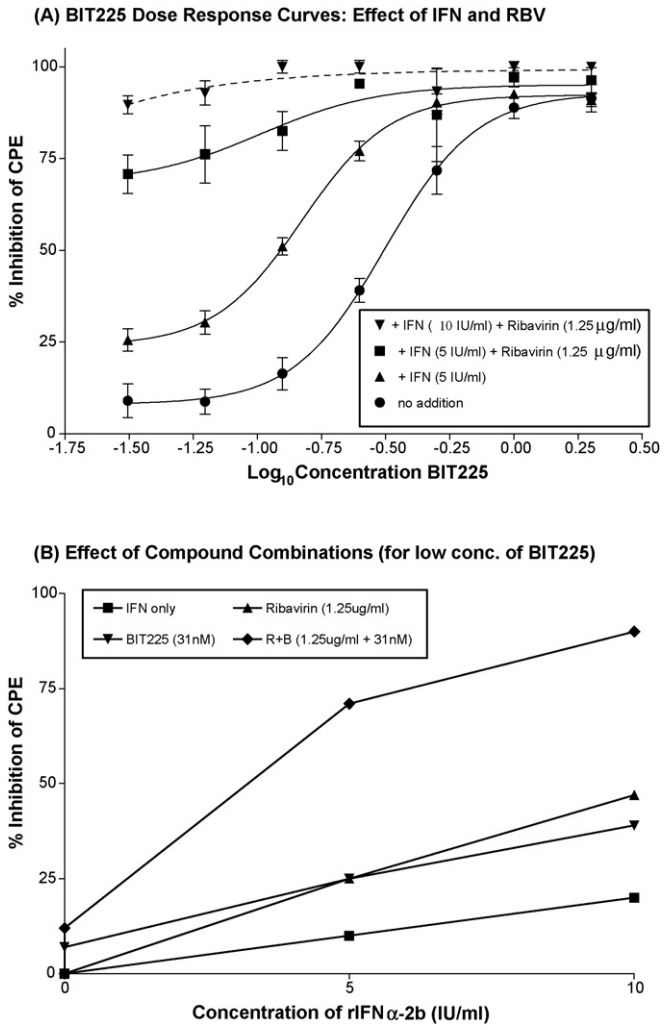

Fig. 5. The antiviral effect of representative combinations of BIT225 and rIFNα-2b ± ribavirin against BVDV. (A) The effect on BIT225 dose–response curves of: BIT225 alone (circles); BIT225 plus 5 IU/ml rIFNα-2b (triangles); BIT225 plus 5 IU/ml rIFNα-2b plus 1.25 μg/ml ribavirin (squares); BIT225 plus 10 IU/ml rIFNα-2b plus 1.25 μg/ml ribavirin (inverted triangles with dashed line). (B) Comparative antiviral effects of particular compound concentrations, either stand-alone or in the indicated combinations.

compounds were active against BVDV (Fig. 6A). Combination experiments revealed that increasing concentrations of either CMA or CMC caused a leftward shift of BIT225 dose–response curves, indicative of decreased IC_{50} values for BIT225 (Fig. 6B and C). MacSynergy II analysis confirmed that there was significant synergy between BIT225 and both CMA and CMC (Fig. 6D and E; Table 1). The combination of 31 nM BIT225 plus 2.5 μM CMA caused approximately 75% inhibition of BVDV CPE (Fig. 6B).

4. Discussion

BIT225 – a novel small molecule – was identified as an inhibitor of the ion channel activity of HCV p7 after screening Biotron's antiviral compound library. Against the HCV-related pestivirus, BVDV, BIT225 has potent stand-alone antiviral activity with an IC_{50} of 314 nM (Fig. 3A). Also in the BVDV system, there is strong synergism between the antiviral actions of BIT225 and rIFNα-2b (Fig. 4A). This synergy was enhanced in the presence of low concentrations of ribavirin (Fig. 4C and D), such that almost complete inhibition of virus replication was achieved with a triple-drug combination containing only 31 nM BIT225 (one-tenth of its stand-alone IC_{50} against BVDV) plus concentrations of IFN and ribavirin well below their stand-alone IC_{50} values.

Synergistic antiviral activity was also observed for combinations of BIT225 with the nucleoside analogues 2′-C-methyl-adenosine and 2′-C-methyl-cytidine (Fig. 6), both of which are known to inhibit the HCV RNA-dependent RNA polymerase.

Over the years, BVDV system has proven to be a very good predictor of anti-HCV activity for compounds and anti-flaviviral drug combinations. For example, imminosugars (Durantel et al., 2001) and amantadine (Griffin et al., 2004) inhibited both BVDV and HCV *in vitro*, and IFN/ribavirin combinations showed similar synergistic activity against BVDV, as they did for HCV. Consequently, BIT225 not only has potential application as a tool for basic research or veterinary use in the bovine industry, but also, investigation of the potential utility of BIT225 (or BIT225-like molecules) in anti-HCV therapeutic cocktails is a high priority in our future drug development strategy.

To date, BIT225 has completed two phase I human trials (see www.biotron.com.au): a phase Ia escalating single dose safety and pharmacokinetics study in healthy volunteers; and a phase Ib 7-day multi-dosing study in HCV-infected subjects at two selected doses (also primarily a safety and pharmacokinetics study). In the latter trial a modest but statistically significant drop in viral load was associated with BIT225 treatment at the higher dose, establishing proof of concept and providing impetus for further development of this new class of anti-HCV compounds.

The obvious direction for future research lies in characterization of BIT225 and its mechanism of action in HCV cell culture systems. So far, difficulties of access to the JFH1 system (in part associated with our commercial goals) have limited our ability to characterize BIT225 in this system. However, preliminary experiments with JFH1 – performed by T. Suzuki and T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan) – revealed evidence of anti-HCV activity of BIT225 (manuscript in preparation). In addition, experiments with subgenomic HCV replicons – which do not express p7 – show a lack of inhibition by BIT225, consistent with inhibition of p7 being integral to the antiviral mechanism of action.

The major reason for development of therapeutic antiviral drug combinations is that stand-alone small molecule compounds rapidly lead to development of viral resistance. For HCV, this has been documented for NS3 protease and NS5B polymerase inhibitors in clinical trials (Tomei et al., 2005; Sarrazin et al., 2007; Liu-Young and Kozal, 2008) as well as for amantadine (Chan et al., 2002; Maynard et al., 2006). As shown by current highly active antiretroviral therapy (HAART) strategies used for management of HIV-1 infection, the rate of generation of resistance is greatly reduced by simultaneously treating viral infections with multiple drugs, targeting different viral and cellular proteins. The same approach is likely to be useful in HCV treatments, with the added benefit that it is possible to cure viral infection from individual patients after relatively short-term treatments. Indeed, a recent small scale phase II study reported sustained virological response in 8 out of 12 genotype 1 patients after treatment with triple combinations of the protease inhibitor telepravir, Peg-IFN α-2a and ribavirin (Lawitz et al., 2008) and these results are supported by data from the larger scale phase II trials PROVE 1 and PROVE 2 (Zeuzem, 2008).

The strong synergy between BIT225, rIFNα-2b and ribavirin (as well as anti-HCV nucleoside analogues) that we report here, argues well for the possibility of future development of BIT225, or related compounds, for use in combination therapies for HCV treatment.

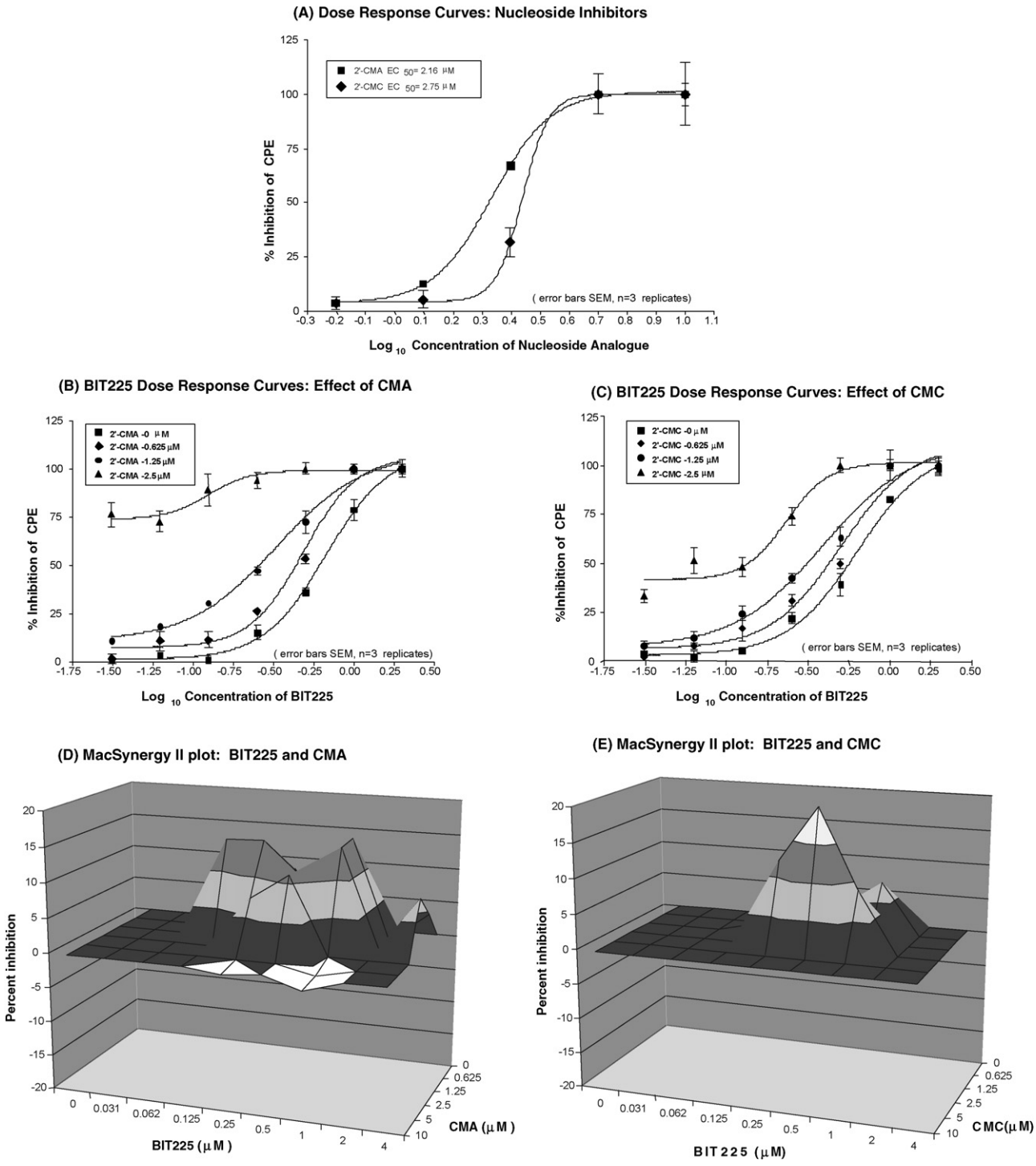

Fig. 6. Effect of nucleoside analogues and combinations of BIT225 plus nucleoside analogues on BVDV CPE: (A) shows the dose–response curves for CMA (squares) and CMC (diamonds); (B and C) respectively, show the effect of various concentrations (as indicated in the inset legends) of CMA and CMC on the dose–response curves for BIT225. Error bars are SEM for $n = 3$ experiments. (D and E) show MacSynergy II plots for combinations of BIT225 with CMA and CMC, respectively.

What is the mechanism of action of BIT225? In terms of standalone BIT225, there are two clear observations concerning the compound reported in this manuscript: (a) BIT225 inhibits BVDV replication and (b) it inhibits the ion channel activity of HCV p7. Clearly many details remain to be elucidated to establish all the links in chain of the mechanism of antiviral activity of BIT225. Nevertheless, based on current knowledge of viroporins and their roles in virus replication, we propose that the simplest broad hypothesis remains that inhibition of p7 channel activity in infected host cells is involved in the mechanism of action of BIT225: as opposed to the alternate hypothesis that the compound acts on BVDV by some completely identified pathway not involving the p7 protein.

To reiterate the evidence that BIT225 inhibits p7 channel activity: as a consequence of screening Biotron's compound library, BIT225 was identified using two separate assays of p7 function. The bacterial bioassay was used as the initial high-throughput screen

to identify compounds able to reverse growth inhibition caused by expression of p7 channels in the bacterial cell membrane. This cell-based method is a relatively indirect assay, however, the compounds selected by this screen are then further characterized in a very direct assay of p7 channel function: in the planar lipid bilayer system, a highly purified synthetic peptide with sequence corresponding to the p7 protein of HCV strain H77 (genotype 1a) was reconstituted into synthetic phospholipid bilayers and ion currents across the membrane, due to spontaneous p7 channel formation, were measured. Although the bilayer experiments are relatively intricate and time consuming, the advantage of the system is that we can confidently attribute the ion currents to channels formed by the p7 peptide. Consequently, the complete and reproducible cessation of the ion currents upon addition of BIT225 (Fig. 2) allows us to confidently conclude that BIT225 is able to inhibit HCV p7 ion channels in planar lipid bilayers. This conclusion is valid even though, in the experiments reported here, only a single chosen concentration (100 μM) was tested. It is, possible that BIT225 inhibits BVDV by targeting an activity of a protein other than the p7 ion channel. We believe this is unlikely.

Formation of cation-selective ion channels is the only functional activity of p7 so far identified (Griffin et al., 2003; Pavlovic et al., 2003; Premkumar et al., 2004), and from a viral replication perspective, HCV p7 protein is known to be involved in virion assembly and release (Steinmann et al., 2007a). Thus, a circumstantial mechanistic link between these two activities is implied. Mutagenesis experiments have firmed up the link between the ion channel activity of p7 and its roles in virus replication: site-directed mutations in the loop region of p7 – a short hydrophilic segment between the two trans-membrane helices – abolish both p7 ion channel activity (Griffin et al., 2004) and infectivity of HCV in chimpanzees (Sakai et al., 2003). The same correlation has been demonstrated with BVDV p7 mutants (Harada et al., 2000).

BIT225 may represent a new tool for probing the role of ion channel function in the replication HCV and related viruses: for example, future attempts to generate BIT225 resistant mutants and to correlate resistance to changes in the p7 gene and ion channel function may prove fruitful. Such resistance mutants would provide another piece in the puzzle; but still the precise mechanism by which ion channel activity assists virus replication would remain to be elucidated. In the case of the analogous viral ion channel encoded by influenza A, the M2 protein, the H^+-conductive ion channel activity is known to critically affect conformation of hemagluttinin during intra-cellular trafficking. It seems likely that a similar general process may apply in HCV, whereby p7 channel activity manipulates the ion gradients across membranes of intracellular vesicles at the site of HCV assembly, affecting local protein conformations and influencing HCV assembly and release.

Strengthening the argument for analogous actions of M2 and p7 is the remarkable observation that the influenza A/M2 inhibitors amantadine and rimantadine also turn out to inhibit HCV p7 ion channel activity and *in vitro* HCV replication (Griffin et al., 2008). Influenza and HCV are widely divergent viruses, just as M2 and p7 are widely divergent proteins sharing very little primary amino acid sequence homology. Similarly, amantadine inhibits the p7 proteins from BVDV and HCV, where again only very low conservation of the primary amino acid sequences is observed. The common mechanism of inhibition by amantadine is not known, but, there is a high degree of similarity in the predicted secondary structures of the proteins (Griffin et al., 2004), which presumably contribute features that are involved in the binding of channel blocking molecules. In the light of this, and given that we have shown that BIT225 can bind to HCV p7 (required for channel blocking activity), as per our working hypothesis, the most likely explanation for the mode of action of BIT225 against BVDV is via inhibition of BVDV p7 ion channel. This remains to be tested.

In apparent contrast to amantadine's "broad-spectrum" ability to inhibit ion channels of diverse primary sequence such as M2 and p7, it is also well known that specific, single amino acid substitutions in the M2 protein can cause drug resistance in influenza A. Similarly, the amino acid sequence of p7 affects the amantadine sensitivity of HCV (Griffin et al., 2008). Clearly, further research is required to clarify the subtleties of interactions between p7 and its inhibitors.

In addition to amantadine and rimantadine (Griffin et al., 2003), other compounds have been reported that inhibit the HCV p7 ion channel, namely, long alkylchain imminosugars (Pavlovic et al., 2003, 2005), and hexamethylene amiloride (HMA) (Premkumar et al., 2004; StGelais et al., 2007). Imminosugar derivates were found to inhibit replication of three chimeric JFH-1 reporter viruses *in vitro* with representative p7 sequences from genotypes 2a, 1a and 1b (Steinmann et al., 2007b); and HMA is able to inhibit p7 channels from genotypes 1a (Premkumar et al., 2004) and 1b (StGelais et al., 2007), although no data has been reported related to the anti-HCV activity of HMA.

No p7 inhibitor has yet been definitively proven to be effective against HCV *in vivo*. So far, clinical data is available for amantadine where results have been variable, some trials showing a sustained virological response in previous interferon non-responders, although the net effect was not statistically significant (Deltenre et al., 2004). In this regard, it is noteworthy that the ability of amantadine to inhibit HCV p7 ion channels and HCV replication *in vitro* appears to be highly dependent on the viral genotype and sequence of the p7 isolate (Haqshenas et al., 2007; Steinmann et al., 2007b; Griffin et al., 2008). A phase II trial of the imminosugar UT-231B failed to demonstrate any antiviral efficacy in genotype 1, interferon non-responder HCV-infected subjects (United Therapeutics company website, clinical trial identifier NCT00069511).

In summary, BIT225 represents the first molecule of a new class of compounds with antiviral activity against HCV and related viruses: substituted napthoyl guanidines. The probable mechanism of action of BIT225 is via inhibition of p7 ion channel activity, though this remains to be definitively proven. The strong synergy between BIT225, rIFNα and ribavirin bodes well for the future development of BIT225, or related analogues, in combination therapies for HCV treatment.

Acknowledgements

We are grateful to Mr. Leigh Harty for excellent technical assistance and perseverance with the bilayer experiments. We also thank Dr. Wayne Best of Epichem Pty. Ltd. for advice related to the design and synthesis of BIT225.

References

Bartenschlager, R., Frese, M., Pietschmann, T., 2004. Novel insights into hepatitis C virus replication and persistence. Adv. Virus Res. 63, 71–180.

Buckwold, V.E., 2004. Implications of finding synergic in vitro drug–drug interactions between interferon-alpha and ribavirin for the treatment of hepatitis C virus. J. Antimicrob. Chemother. 53, 413–414.

Buckwold, V.E., Beer, B.E., Donis, R.O., 2003a. Bovine viral diarrhea virus as a surrogate model of hepatitis C virus for the evaluation of antiviral agents. Antiviral Res. 60, 1–15.

Buckwold, V.E., Wei, J., Wenzel-Mathers, M., Russell, J., 2003b. Synergistic in vitro interactions between alpha interferon and ribavirin against bovine viral diarrhea virus and yellow fever virus as surrogate models of hepatitis C virus replication. Antimicrob. Agents Chemother. 47, 2293–2298.

Carroll, S.S., Tomassini, J.E., Bosserman, M., Getty, K., Stahlhut, M.W., Eldrup, A.B., Bhat, B., Hall, D., Simcoe, A.L., LaFemina, R., Rutkowski, C.A., Wolanski, B., Yang, Z., Migliaccio, G., De Francesco, R., Kuo, L.C., MacCoss, M., Olsen, D.B., 2003. Inhibition of hepatitis C virus RNA replication by 2′-modified nucleoside analogs. J. Biol. Chem. 278, 11979–11984.

Chan, J., O'Riordan, K., Wiley, T.E., 2002. Amantadine's viral kinetics in chronic hepatitis C infection. Dig. Dis. Sci. 47, 438–442.

Clarke, D., Griffin, S., Beales, L., Gelais, C.S., Burgess, S., Harris, M., Rowlands, D., 2006. Evidence for the formation of a heptameric ion channel complex by the hepatitis C virus p7 protein in vitro. J. Biol. Chem. 281, 37057–37068.
Couch, R.B., 2000. Prevention and treatment of influenza. N. Engl. J. Med. 343, 1778–1787.
Cox, G., Ewart, G., Gage, P., 2007. Method of Modulating Ion Channel Functional Activity. US Patent 7,179,803, 20 February 2007.
Deltenre, P., Henrion, J., Canva, V., Dharancy, S., Texier, F., Louvet, A., De Maeght, S., Paris, J.C., Mathurin, P., 2004. Evaluation of amantadine in chronic hepatitis C: a meta-analysis. J. Hepatol. 41, 462–473.
Dimmrock, N., Easton, A., Leppard, K., 2001. Introduction to Modern Virology, Fifth edition. Blackwell Science.
Durantel, D., Branza-Nichita, N., Carrouée-Durantel, S., Butters, T.D., Dwek, R.A., Zitzmann, N., 2001. Study of the mechanism of antiviral action of iminosugar derivatives against bovine viral diarrhea virus. J. Virol. 75, 8987–8998.
Ewart, G., Best, W., 2006. Antiviral Compounds and Methods. World Intellectual Property Organisation, Publication number WO 2006/135978 A1 (28 December).
Ewart, G.D., Sutherland, T., Gage, P.W., Cox, G.B., 1996. The Vpu protein of human immunodeficiency virus type 1 forms cation-selective ion channels. J. Virol. 70, 7108–7115.
Gage, P., Cox, G., Ewart, G., 2007. Method for Determining Ion Channel Activity of a Substance. US Patent RE39815, 4 September 2007.
Gonzalez, M.E., Carrasco, L., 2003. Viroporins. FEBS Lett. 552, 28–34.
Griffin, S., Stgelais, C., Owsianka, A.M., Patel, A.H., Rowlands, D., Harris, M., 2008. Genotype-dependent sensitivity of hepatitis C virus to inhibitors of the p7 ion channel. Hepatology 48, 1779–1790.
Griffin, S.D., Beales, L.P., Clarke, D.S., Worsfold, O., Evans, S.D., Jaeger, J., Harris, M.P., Rowlands, D.J., 2003. The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. FEBS Lett. 535, 34–38.
Griffin, S.D., Harvey, R., Clarke, D.S., Barclay, W.S., Harris, M., Rowlands, D.J., 2004. A conserved basic loop in hepatitis C virus p7 protein is required for amantadine-sensitive ion channel activity in mammalian cells but is dispensable for localization to mitochondria. J. Gen. Virol. 85, 451–461.
Haqshenas, G., Dong, X., Ewart, G., Bowden, S., Gowans, E.J., 2007. A 2a/1b full-length p7 inter-genotypic chimeric genome of hepatitis C virus is infectious in vitro. Virology 360, 17–26.
Harada, T., Tautz, N., Thiel, H.J., 2000. E2-p7 region of the bovine viral diarrhea virus polyprotein: processing and functional studies. J. Virol. 74, 9498–9506.
Hay, A.J., Wolstenholme, A.J., Skehel, J.J., Smith, M.H., 1985. The molecular basis of the specific anti-influenza action of amantadine. EMBO J. 4, 3021–3024.
Kwong, A.D., McNair, L., Jacobson, I., George, S., 2008. Recent progress in the development of selected hepatitis C virus NS3.4A protease and NS5B polymerase inhibitors. Curr. Opin. Pharmacol. 8, 522–531.
Lawitz, E., Rodriguez-Torres, M., Muir, A.J., Kieffer, T.L., McNair, L., Khunvichai, A., McHutchison, J.G., 2008. Antiviral effects and safety of telaprevir, peginterferon alfa-2a, and ribavirin for 28 days in hepatitis C patients. J. Hepatol. 49, 163–169.
Liu-Young, G., Kozal, M.J., 2008. Hepatitis C Protease and Polymerase Inhibitors in Development, vol. 22. AIDS Patient Care STDS, pp. 449–457.
Love, C.A., Lilley, P.E., Dixon, N.E., 1996. Stable high-copy-number bacteriophage lambda promoter vectors for overproduction of proteins in *Escherichia coli*. Gene 176, 49–53.
Manns, M.P., Wedemeyer, H., Cornberg, M., 2006. Treating viral hepatitis C: efficacy, side effects, and complications. Gut 55, 1350–1359.
Maynard, M., Pradat, P., Bailly, F., Rozier, F., Nemoz, C., Si Ahmed, S.N., Adeleine, P., Trepo, C., 2006. Amantadine triple therapy for non-responder hepatitis C patients. Clues for controversies (ANRS HC 03 BITRI). J. Hepatol. 44, 484–490.
Oxford, J.S., Bossuyt, S., Balasingam, S., Mann, A., Novelli, P., Lambkin, R., 2003. Treatment of epidemic and pandemic influenza with neuraminidase and M2 proton channel inhibitors. Clin. Microbiol. Infect. 9, 1–14.
Pavlovic, D., Fischer, W., Hussey, M., Durantel, D., Durantel, S., Branza-Nichita, N., Woodhouse, S., Dwek, R.A., Zitzmann, N., 2005. Long alkylchain iminosugars block the HCV p7 ion channel. Adv. Exp. Med. Biol. 564, 3–4.
Pavlovic, D., Neville, D.C., Argaud, O., Blumberg, B., Dwek, R.A., Fischer, W.B., Zitzmann, N., 2003. The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. Proc. Natl. Acad. Sci. U.S.A. 100, 6104–6108.
Penin, F., Dubuisson, J., Rey, F.A., Moradpour, D., Pawlotsky, J.M., 2004. Structural biology of hepatitis C virus. Hepatology 39, 5–19.
Pinto, L.H., Lamb, R.A., 2006. The M2 proton channels of influenza A and B viruses. J. Biol. Chem. 281, 8997–9000.
Premkumar, A., Wilson, L., Ewart, G.D., Gage, P.W., 2004. Cation-selective ion channels formed by p7 of hepatitis C virus are blocked by hexamethylene amiloride. FEBS Lett. 557, 99–103.
Prichard, M.N., Shipman Jr., C., 1990. A three-dimensional model to analyze drug–drug interactions. Antiviral Res. 14, 181–205.
Prichard, M.N., Shipman Jr., C., 1996. Analysis of combinations of antiviral drugs and design of effective multidrug therapies. Antivir. Ther. 1, 9–20.
Sakai, A., Claire, M.S., Faulk, K., Govindarajan, S., Emerson, S.U., Purcell, R.H., Bukh, J., 2003. The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. Proc. Natl. Acad. Sci. U.S.A. 100, 11646–11651.
Sarrazin, C., Kieffer, T.L., Bartels, D., Hanzelka, B., Muh, U., Welker, M., Wincheringer, D., Zhou, Y., Chu, H.M., Lin, C., Weegink, C., Reesink, H., Zeuzem, S., Kwong, A.D., 2007. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. Gastroenterology 132, 1767–1777.
Steinmann, E., Penin, F., Kallis, S., Patel, A.H., Bartenschlager, R., Pietschmann, T., 2007a. Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions. PLoS Pathog. 3, e103.
Steinmann, E., Whitfield, T., Kallis, S., Dwek, R.A., Zitzmann, N., Pietschmann, T., Bartenschlager, R., 2007b. Antiviral effects of amantadine and iminosugar derivatives against hepatitis C virus. Hepatology 46, 330–338.
StGelais, C., Tuthill, T.J., Clarke, D.S., Rowlands, D.J., Harris, M., Griffin, S., 2007. Inhibition of hepatitis C virus p7 membrane channels in a liposome-based assay system. Antiviral Res. 76, 48–58.
Takikawa, S., Engle, R.E., Emerson, S.U., Purcell, R.H., St Claire, M., Bukh, J., 2006. Functional analyses of GB virus B p13 protein: development of a recombinant GB virus B hepatitis virus with a p7 protein. Proc. Natl. Acad. Sci. U.S.A. 103, 3345–3350.
Thompson, A., Patel, K., Tillman, H., McHutchison, J.G., 2009. Directly acting antivirals for the treatment of patients with hepatitis C infection: a clinical development update addressing key future challenges. J. Hepatol. 50, 184–194.
Tomei, L., Altamura, S., Paonessa, G., De Francesco, R., Migliaccio, G., 2005. HCV antiviral resistance: the impact of in vitro studies on the development of antiviral agents targeting the viral NS5B polymerase. Antivir. Chem. Chemother. 16, 225–245.
Zeuzem, S., 2008. Telaprevir, peginterferon alfa-2a, and ribavirin for 28 days in chronic hepatitis C patients. J. Hepatol. 49, 157–159.