



Published in final edited form as:

*Antivir Chem Chemother.* 2008 ; 19(1): 25–31.

## Combinations of 2'-C-methylcytidine analogues with interferon- $\alpha$ 2b and triple combination with ribavirin in the hepatitis C virus replicon system

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### Abstract

**Background**—Hepatitis C virus (HCV) polymerase is an essential enzyme for HCV replication and has multiple inhibitor binding sites making it a major target for antiviral intervention. It is apparent that no single drug can inhibit HCV replication in humans. Hence, combinations of nucleoside analogues  $\beta$ -D-2'-C-methylcytidine (2'-C-MeC; NM-107) or  $\beta$ -D-2'-deoxy-2'-fluoro-2'-C-methylcytidine (2'-F-C-MeC; PSI-6130) with interferon- $\alpha$ 2b (IFN- $\alpha$ 2b) or triple combination with ribavirin (RBV) were evaluated.

**Methods**—Huh-7 cells containing the self-replicating subgenomic HCV replicon (Clone B) were used for drug combination studies. After drug treatment for 5 days, total cellular RNA was then extracted and both ribosomal RNA and HCV replicon RNA were amplified in a single-step multiplex real-time PCR assay. Drug interaction analyses were performed using the CalcuSyn program.

**Results**—Double combinations of 2'-C-MeC or 2'-F-C-MeC with IFN- $\alpha$ 2b at all ratios tested had weighted average combination index ( $CI_{wt}$ ) values  $<1$  indicating synergistic inhibition of HCV replication in the replicon system. For the triple combinations of IFN- $\alpha$ 2b plus RBV with either 2'-C-MeC or 2'-F-C-MeC, the  $CI_{wt}$  values at 1:1:1 ratio tested were 0.5 and 0.8, respectively, indicating synergistic antiviral effects. No apparent cytotoxicity effects were observed with any of the combinations tested.

**Conclusion**—These promising *in vitro* data warrant clinical investigation of the nucleosides analogues such as 2'-C-MeC or 2'-F-C-MeC in their prodrug forms, together with IFN- $\alpha$ 2b and RBV, for successful treatment of HCV infections.

### Introduction

The current standard of care for chronic hepatitis C virus (HCV) infection is a combination of pegylated interferon (IFN)- $\alpha$  and ribavirin (RBV), which results in a sustained virological response in about a half of infected individuals; however, this combined modality is associated with considerable side effects [1,2]. Several new HCV inhibitors have been identified and some are being evaluated in controlled clinical trials [3]. Recently, promising 2'-modified nucleoside analogues with activity against HCV have been reported; for instance, valopicitabine (NM-283), the 3'-valine ester of  $\beta$ -D-2'-C-methylcytidine (2'-C-MeC; NM-107), is a prodrug nucleoside that after intracellular phosphorylation to its 5'-triphosphate metabolite, selectively

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#### Disclosure statement

RFS is a founder and major shareholder of Pharmasset Inc., the developer of PSI-6130. All other authors have no competing interests.

inhibits HCV RNA-dependent RNA polymerase [4,5]. Although valopicitabine was recently evaluated in clinical trials and the combination of valopicitabine with IFN- $\alpha$  (plus RBV) has led to successful viral RNA suppression in a number of HCV-infected individuals, this drug was found to have gastrointestinal toxicity leading to its discontinuation in its present form [6]. Another novel cytidine analogue,  $\beta$ -D-2'-deoxy-2'-fluoro-2'-C-methylcytidine (PSI-6130, 2'-F-C-MeC) has demonstrated potent and specific *in vitro* anti-HCV activity with no apparent cytotoxicity [7,8]. Recently, in a 14 day Phase I monotherapy study, a new prodrug of 2'-F-C-MeC named R7128 was shown to be highly potent at lowering levels of HCV RNA, with a 2.7-log reduction in the viral load of individuals infected with HCV genotype 1 who had failed prior IFN therapy [9,10]. These nucleoside analogues have demonstrated excellent anti-HCV activity *in vitro* in both antireplicon and infectious HCV systems [11]. Furthermore, nucleoside analogue prodrugs can be administered in combination with IFN, with or without RBV, or with other antiviral drugs for the treatment of HCV infections. Previous studies using the HCV replicon have reported that RBV may interfere with pyrimidine nucleoside metabolism [12] and that IFN has shown both cytopathic and non-cytopathic activities [13–14]. Therefore, we determined the *in vitro* anti-HCV activity of these 2'-Me nucleosides with IFN- $\alpha$ 2b to assess the potential drug-drug interactions. Furthermore, the triple combination of 2'-C-MeC or 2'-F-C-MeC with IFN- $\alpha$ 2b and RBV was also evaluated using the replicon system.

## Methods

### Compounds

Recombinant IFN- $\alpha$ 2b (Intron A) was obtained from Schering Corporation, (Kenilworth, NJ, USA). The powder was diluted in sterile water and aliquots were stored at  $-20^{\circ}\text{C}$ . Compounds 2'-C-MeC and RBV were synthesized in our laboratories following published procedures [15,16], whereas 2'-F-C-MeC was synthesized according to the methods of Clark *et al.* [16]. Stocks of 40 mM were prepared in 100% DMSO and stored at  $-20^{\circ}\text{C}$ .

### Cells

Subgenomic HCV replicon RNA-containing Huh7 cell line (Clone B cells; Apath LLC, St Louis, MO, USA) was kindly provided by Dr Charles M Rice [17] through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Bethesda, MD, USA), and was maintained in exponential growth in Dulbecco's modified Eagle's medium supplemented with L-glutamine and 4.5 g/l D-glucose (GIBCO/Invitrogen, Technologies, Carlsbad, CA, USA). The medium was further supplemented with 10% (v/v) heat-inactivated fetal bovine serum,  $1\times$  non-essential amino acids, 100 U/ml of penicillin, 100 ( $\mu\text{g}/\text{ml}$ ) of streptomycin and 500  $\mu\text{g}/\text{ml}$  of G418 (Geneticin, GIBCO/Invitrogen). Cells were maintained at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  and 95% air atmosphere.

### Cell-based antiviral assays

The HCV replicon cells were seeded onto 96-well tissue culture plates (5,000 cells/well) in 50  $\mu\text{l}$  of medium without G418. Immediately after seeding, 50  $\mu\text{l}$  of test compounds, alone and in combination, were added at several concentrations in threefold dilutions. The concentrations of IFN- $\alpha$ 2b, 2'-C-MeC, 2'-F-C-MeC and RBV used ranged from 0.02 to 99 IU/ml, 0.1 to 33  $\mu\text{M}$ , 0.01 to 33  $\mu\text{M}$  and 1.2 to 33  $\mu\text{M}$ , respectively. After 5 days in culture, total cellular RNA was isolated using RNeasy 96 kit (Qiagen, Valencia, CA, USA), and both HCV replicon and endogenous control (TaqMan Ribosomal RNA, Applied Biosystems, Foster City, CA, USA) were amplified in a single-step multiplex real-time PCR assay (7900 HT Sequence Detection System, Applied Biosystems). To express the antiviral effectiveness of the drugs, the average threshold cycle for HCV amplification of the compounds, alone or in combination, was subtracted from the average cycle of the untreated control ( $\Delta\text{Ct}$ ). Determination of the 50% effective concentration ( $\text{EC}_{50}$ ) and the 90% effective concentration ( $\text{EQ}_{90}$ ) in HCV replicon

RNA levels of the compounds were performed as previously described by Stuyver *et al.* [18]. In addition, the 50% cytotoxicity concentration (CC<sub>50</sub>) of the compounds was also determined by calculating the  $\Delta C_t$  for rRNA [18]. Cell proliferation was also determined by 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium salt (MTT) reduction test using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), according to the manufacturer's instructions. Cytotoxicity was considered when the concentration of the test compounds alone or in combination inhibited cell growth by  $\geq 50\%$ .

### Drug combination analyses

To evaluate whether the antiviral effects of two-three-drug combinations of 2'-C-MeC and IFN- $\alpha 2b$  (with or without RBV) or 2'-F-C-MeC and IFN- $\alpha 2b$  (with or without RBV) were synergistic, additive or antagonistic, the drug interactions were analysed using CalcuSyn (Biosoft, Ferguson, MO, USA) computer software. The program allows automated simulation of synergism and antagonism at all dose and effect levels and displays the methods of Chou and Talalay [19], including dose-effect curve, median effect plot, combination index (CI), isobologram, dose-reduction index (DRI) and the Monte Carlo algorithm plot of Belen'kii and Schinazi [20]. We chose the constant ratio drug combination design proposed by Chou and Talalay [19] because it offers several advantages, such as reduction of required data points and generation of maximal useful information on both dual and triple combinations. In addition, it allows the construction of fraction affected (Fa)-CI and Fa-DRI plots with the actual combination data points. Further more, it allows computer simulation of CI and DRI values at all effect levels. As high degree effects are more therapeutically relevant than the low degree of effects, the additional weighted average CI (CI<sub>wt</sub>) of Chou and Talalay [19] was calculated (see review [21]), which uses the formula:  $CI_{wt} = [CI_{50} + 2CI_{75} + 3CI_{90} + 4CI_{95}]/10$ , where CI<sub>50</sub>, CI<sub>75</sub>, CI<sub>90</sub>, and CI<sub>95</sub> are the CI values at 50%, 75%, 90% and 95% inhibition, respectively.

### Results

Combinations of either 2'-C-MeC or 2'-F-C-MeC with IFN- $\alpha 2b$  (and RBV) are synergistic in replicon cells. The *in vitro* effect of the combination of 2'-C-MeC or 2'-F-C-MeC with IFN- $\alpha 2b$  and the triple combination of either 2'-C-MeC or 2'-F-C-MeC with IFN- $\alpha 2b$  and RBV using the replicon system was determined. For each drug pair (or triple combination), three to four independent experiments were performed and all samples were processed in duplicate. HCV replicon (Clone B) cells were incubated with various concentrations of these compounds for 5 days and the anti-HCV activity and cytotoxicity were simultaneously measured. The activity and cytotoxicity effects of 2'-C-MeC, 2'-F-C-MeC, IFN- $\alpha 2b$ , and RBV, alone or in combination, are shown in Tables 1 and 2.

In the present computerized analysis for either one of the four compounds (2'-C-MeC, 2'-F-C-MeC, IFN- $\alpha 2b$  or RBV), the linear correlation coefficient of the median-effect plot (*r* values) ranged from 0.85 to 0.99 and the *r* values for their constant ratio combinations ranged from 0.91 to 0.99 (data not shown). Therefore, the experimental results matched the mass-action law. No cytotoxicity effects of rRNA levels in the replicon cells were observed with 2'-C-MeC, 2'-F-C-MeC and IFN- $\alpha 2b$  (or with the combinations) when tested at the highest antiviral concentrations (Tables 1 and 2). In contrast, RBV alone showed toxicity effects at concentration of  $19 \pm 6.1 \mu M$ . Furthermore, after 5 days of incubation in an MTT assay, there was no apparent cytotoxicity for any of the combinations containing either 2'-C-MeC or 2'-F-C-MeC (up to 33  $\mu M$ ), IFN- $\mu 2b$  (up to 33 IU/ml) and RBV (up to 11  $\mu M$ ; data not shown). A dose-dependent inhibition of HCV replicon replication was observed in cultures treated with each compound alone and their combinations. A 90% reduction of HCV replication was observed when tested alone with 8.8  $\mu M$ , 3.8  $\mu M$  and 17 IU/ml of 2'-C-MeC, 2'-F-C-MeC and

IFN- $\alpha$ 2b, respectively (Table 1). In this system, 2'-F-C-MeC was found to be greater than twofold more potent than 2'-C-MeC.

The EC<sub>90</sub> values of the combined activity of 2'-C-MeC and IFN- $\alpha$ 2b (with or without RBV) at all ratios ranged from 0.8 to 4.9  $\mu$ M and the combined activity of 2'-F-C-MeC and IFN- $\alpha$ 2b (with or without RBV) ranged from 0.4 to 2.6  $\mu$ M (Table 2). These results indicate that both double and triple combinations of either 2'-C-MeC or 2'-F-C-MeC and IFN- $\alpha$  (with or without RBV) were effective against HCV replication in Clone B cells and enhanced potency over the corresponding monotherapies.

In order to determine if the interactions were additive, synergistic or antagonistic, we applied the CalcuSyn approach to the data generated from dual combinations (Table 3 and Figures 1 and 2). Double combination of 2'-C-MeC with IFN- $\alpha$ 2b except for the 1:2 ratio showed favourable dose reduction from EC<sub>50</sub> to EC<sub>95</sub> (Table 3). The combination of 2'-F-C-MeC with IFN- $\alpha$  at ratios of 1:1.5 and 1:1 showed more favourable dose reduction (from EC<sub>50</sub> to EC<sub>95</sub>) than at ratios of 1:10 and 1:5. In addition, all average CI values (see *Methods*) were significantly <1, suggesting synergy when the combination ratios of either 2'-C-MeC or 2'-F-C-MeC and IFN- $\alpha$ 2b were analysed. These results indicate that both combinations have synergistic effects on inhibition of HCV RNA replication in the replicon system. The experimental data were also analysed using Monte Carlo simulation for the estimation of errors in CI and the results are presented in Figures 1A and 1B.

Since the drug combination ratios indicated synergism and there was no overlapping toxicity in the replicon system, the DRI was determined. As shown in Figures 2A and 2B, the three drugs in the two-drug combinations allowed significant dose reductions (DRI >1) for a given effect level. Remarkably, greater DRI values of IFN- $\alpha$ 2b (DRI >10 at EC<sub>90</sub> and EC<sub>95</sub>) were observed, suggesting a greater dose reduction of IFN- $\alpha$ 2b with a potential benefit when administered in combination with either 2'-C-MeC or 2'-F-C-MeC.

Next, the type of interaction obtained with triple combination of either 2'-C-MeC or 2'-F-C-MeC with IFN- $\alpha$ 2b and RBV was determined. The CI values for 2'-C-MeC + IFN- $\alpha$ 2b + RBV at ratio of 2:1:1 at 50% and 95% inhibition levels were 1.4, indicating moderate antagonism; however, at 75% and 90% inhibition levels, CI values were 0.9, indicating slight synergism. Overall, the CI<sub>wt</sub> of 1.1 indicated a nearly additive effect. Similarly, combination of 2'-F-C-MeC + IFN + RBV at the same ratio of 2:1:1 produced CI<sub>wt</sub> value of 1.1, which is considered to be nearly additive (CalcuSyn: Windows software for dose effect analysis program manual). In addition, the combination of 2'-F-C-MeC + IFN + RBV at ratios of 2:1:1 and 1:1:1 were antagonistic at low effect level of 50% inhibition; however, additive to synergistic effects were noted at high effect levels ( $\geq$ 75%) at all ratios tested. Moreover, the triple combination of IFN- $\alpha$ 2b and RBV with either 2'-C-MeC or 2'-F-C-MeC reduced the HCV replicon replication at ratios of 1:1:1. The comparison of the weighted CI values between the two sets of triple combinations indicated that the combination of 2'-C-MeC with IFN- $\alpha$ 2b and RBV at ratio of 1:1:1 yielded greater synergism than 2'-F-C-MeC, IFN- $\alpha$ 2b and RBV at the same ratio, but the difference was not significant (Table 3).

## Discussion

Using a robust drug interactions methodology, we determined that 2'-C-MeC or 2'-F-C-MeC demonstrated synergistic anti-HCV activities when combined with IFN- $\alpha$ 2b alone or combined with IFN- $\alpha$ 2b and RBV in the replicon system without any apparent cytotoxicity. The combination of RBV to the IFN treatment of chronic hepatitis C infection greatly improved responses [22], but unfortunately half of the infected individuals still do not achieve sustained

clearance of HCV [12]. For this reason, newer potent antiviral agents are likely to be used in combination with these agents.

RBV is a guanosine analogue that has broad antiviral activity against several viruses, but its direct antiviral mechanisms on HCV are not completely elucidated. *In vitro* anti-HCV studies have demonstrated that it could have several modes of action: modulating the immune system when favouring the T helper 1 response; increasing the antiviral activity of endogenous IFN, as demonstrated in the respiratory syncytial virus system [23]; increasing the mutation frequency of HCV and error catastrophe or lethal mutagenesis, thus reducing HCV infectivity; inhibiting inosine monophosphate dehydrogenase leading to guanosine 5'-triphosphate depletion; or having a small and unlikely direct effect on HCV RNA replication through polymerase inhibition (see review [24]). We demonstrated that RBV alone at a non-toxic concentration has a very weak inhibitory effect ( $EC_{90} > 19 \pm 6.1 \mu\text{M}$ ) on intracellular HCV replicon replication, which is consistent with results of other studies [25,26]. However, the combinations of IFN- $\alpha$ 2b and either 2'-C-MeC or 2'-F-C-MeC, at several concentration ratios, were synergistic and the addition of RBV also resulted in additive/synergistic activity. None of the combinations presented cytotoxicity (see Table 2, where  $CC_{50}$  of combinations containing RBV were either  $>3.6$  or  $>11 \mu\text{M}$ ). Our findings are in agreement with earlier *in vivo* studies when triple combination of NM283 plus pegylated IFN- $\alpha$  plus RBV were used in individuals with chronic HCV infection and resulted in a significant decline ( $>70\%$ ) of viral load levels [27]. Unfortunately, clinical trials of NM283 were put on hold by the Food and Drug Administration as gastrointestinal side effects were demonstrated [28]. Although the weighted average values in our study increased when comparing double with triple therapies, mainly at the ratio of 2:1:1 for 2'-C-MeC combination, which might be indicative of a negative effect of RBV on the previously observed synergism, it did show synergistic effects at ratio of 1:1:1 (see Table 3). Based on  $CI_{wt}$  values, no significant antagonism was noted for all the combinations. In contrast to the results of the present study, a previous study that used human hepatoblastoma Huh-6 cells containing the HCV replicon showed an antagonistic effect of RBV on the anti-HCV activity of the NM-107 [12]. As the HCV replicon in the Huh-6 cell line replicates independently from ongoing cell proliferation, the toxic effects of RBV may have been masqueraded. Cell toxicity of certain compounds like RBV might be obscured at high concentrations in the HuH6 replicon cells. In fact, we tested the cytotoxicity activity of RBV using HuH6-naïve cells by means of real-time PCR (effect on ribosomal RNA levels) and observed a  $CC_{50}$  value of  $29 \mu\text{M}$  (data not shown), whereas Coelmont *et al.* [12] did not show toxicity of RBV at the highest concentration reported ( $135 \mu\text{M}$ ). Therefore, the system may not solely reflect HCV replication, but the secondary effects of the drug. Moreover, in this study the addition of purine nucleoside analogues to the RBV treatment did not have any major effect on antiviral activity. However, the triple combination presented in our study with pyrimidine nucleoside analogues did have additive/synergistic effects when using equal concentrations of triple (1:1:1) combinations:  $CI_{wt}$  values of 0.5 for 2'-C-MeC and 0.8 for 2'-F-C-MeC (Table 3). Our group [29] reported preliminary data that RBV at a higher ratio (1:5) had a  $CI_{wt}$  value of 1.5, indicating antagonistic effects in the *in vitro* anti-HCV activity of 2'-C-MeC; however, the CI value at 90% inhibition ranged from 0.9 to 2.1, suggesting that the double combination could be either slight synergistic or antagonistic. Nevertheless, and most importantly, we demonstrated that RBV did not interfere with the *in vitro* anti-HCV activity of 2'-C-MeC metabolism (and vice versa) in Huh-7 cells and primary hepatocytes, supporting the results found in the current antiviral studies.

The combination of small-molecule inhibitors of the HCV protease or polymerase with IFN- $\alpha$  and RBV might act synergistically by both decreasing HCV replication and interfering with the ability of HCV to evade the mediators of IFN action. These combinations hold the promise of greatly improved rates of response to therapy of hepatitis C, and perhaps will help to decrease the doses of IFN- $\alpha$  and/or RBV, which are associated with frequent side effects and

contraindicated in many chronically infected individuals. Furthermore, in the present study, the inhibitory effect of HCV RNA replication was increased when 2'-C-MeC was used in combination with IFN- $\alpha$ 2b, which is in agreement with a recent Phase IIb human trial of the anti-HCV activity of a prodrug of 2'-C-MeC (NM-283) shown to produce profound synergistic effects when combined with pegylated IFN [6,30,31]. As mentioned previously, combinations of pegylated IFN- $\alpha$  and RBV have a relatively low sustained viral response especially in individuals infected with HCV genotype 1 [2]. Because of the high genetic heterogeneity, increased efficiency of replication of HCV and expected emergence of resistance mutations, it is likely that the future therapy for HCV will include orally bioavailable drug combinations in addition to RBV and IFN. Furthermore, it is essential to evaluate *in vitro* combinations beforehand to determine their potential effects on antiviral activity as well as cytotoxicity.

In summary, we demonstrate that double or triple combinations of either 2'-C-MeC or 2'-F-C-MeC with IFN- $\alpha$ 2b alone or combined with IFN- $\alpha$ 2b and RBV interacted synergistically in inhibiting HCV in a replicon system. Such studies provide a strong virological rationale for using 2'-C-MeC nucleoside analogues together with the current standard of care treatment for HCV infections.

## Acknowledgments

This work was supported in part by NIH Grant 5P30-AI-50409 (CFAR), 5R37-AI-041980 and by the Department of Veterans Affairs. Part of this work was presented at the *42nd Annual Meeting of the European Association for the Study of the Liver* 11–15 April 2007 Barcelona, Spain, and was rated in the top 10 percentile.

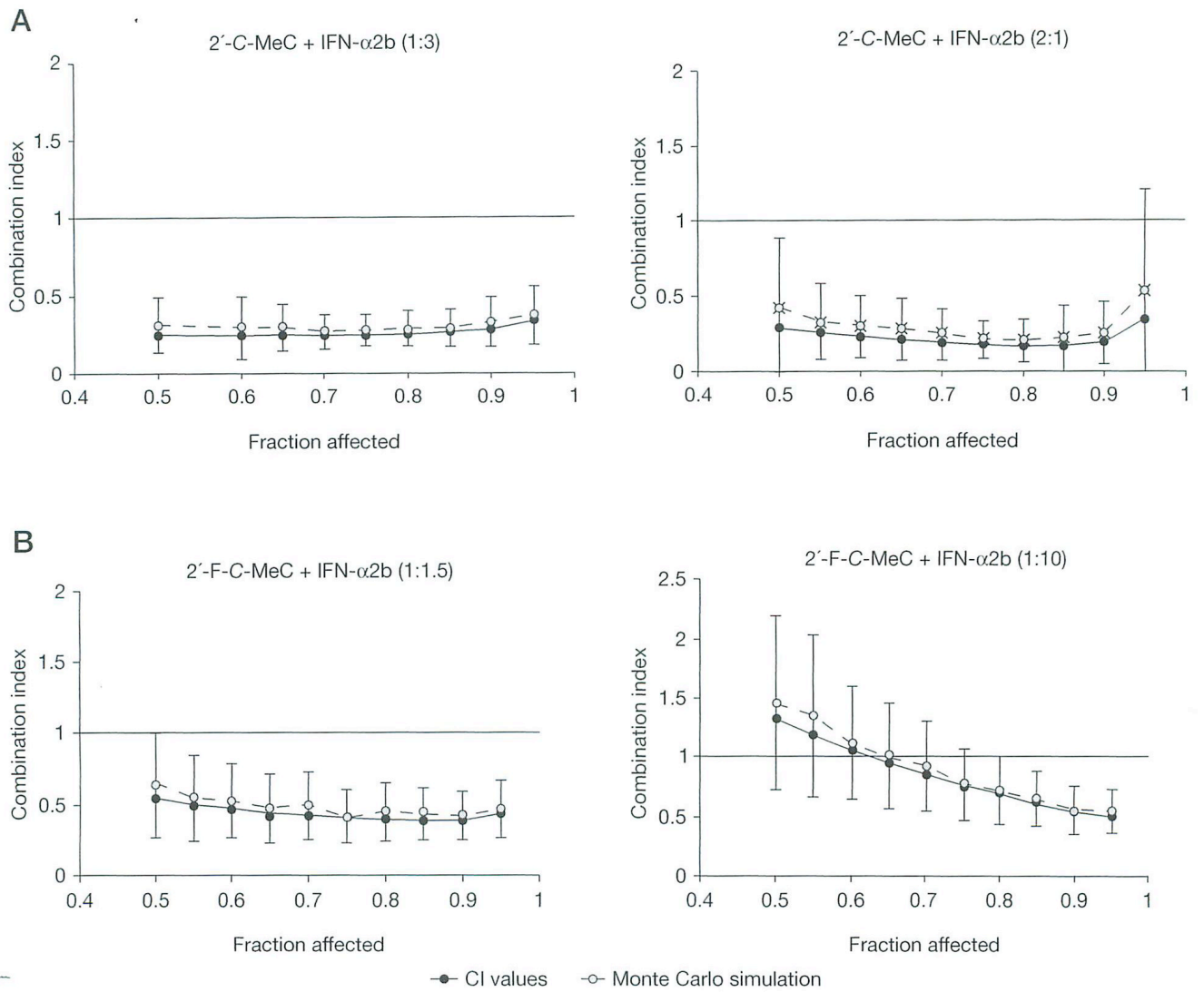
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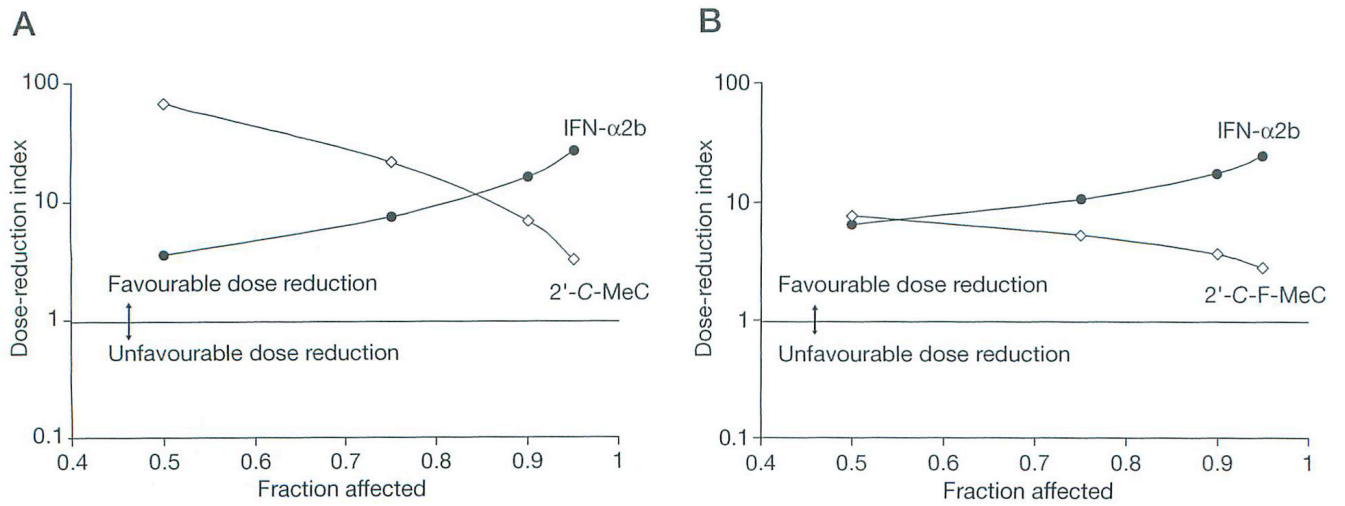
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**Figure 1.**  
Examples of fraction affected–combination index plots and Monte Carlo simulations



**Figure 2.**  
Simulation of the fraction affected-dose-reduction index plot obtained using CalcuSyn computer program

**Table 1**

Anti-HCV activity and cytotoxicity of the four compounds used singly in HCV-replicon-containing Huh-7 cells (Clone B)

Compound	EC <sub>50</sub> <sup>*</sup>	EC <sub>90</sub> <sup>*</sup>	CC <sub>50</sub> <sup>†</sup>
2'-C-MeC, μM	2.4±0.8	8.8±1.9	>33
2'-F-C-MeC, μM	1.2±0.8	3.8±1.7	>33
IFN-α2b, IU/ml	0.5±0.6	17±12	>99
RBV, μM	8.0±3.8	NM <sup>‡</sup>	19±6.1

All values represent the average of four to six experiments and samples were performed in duplicate ± SD.

\* EC<sub>50</sub> and EC<sub>90</sub> effective concentrations required for reducing hepatitis C virus (HCV) levels by 50% and 90%, respectively, on day 5;

† CC<sub>50</sub>, cytotoxicity concentration required for reducing the rRNA levels by 50% on day 5.

‡ Not measurable (NM) because activity at this level was similar to its toxicity. IFN, interferon; RBV, ribavirin.

**Table 2**

Antiviral and cytotoxicity effects of two- to three-drug combination therapy in HCV-replicon-containing Huh-7 cells (Clone B)

Treatment	Ratio	EC <sub>50</sub> $\mu$ M	EC <sub>90</sub> $\mu$ M	CC <sub>50</sub> $\mu$ M*
2'-C-MeC + IFN- $\alpha$ 2b	1:3	0.1	1.3	>33
2'-C-MeC + IFN- $\alpha$ 2b	1:2	0.4	1.8	>33
2'-C-MeC + IFN- $\alpha$ 2b	1:1	0.3	1.6	>33
2'-C-MeC + IFN- $\alpha$ 2b	2:1	0.02	0.8	>33
2'-C-MeC + IFN- $\alpha$ 2b + RBV	2:1:1	0.1	4.9	>11
2'-C-MeC + IFN- $\alpha$ 2b + RBV	1:1:1	0.2	2.2	>3.6
2'-F-C-MeC + IFN- $\alpha$ 2b	1:10	0.09	0.6	>33
2'-F-C-MeC + IFN- $\alpha$ 2b	1:5	0.1	0.8	>33
2'-F-C-MeC + IFN- $\alpha$ 2b	1:1.5	0.2	1.2	>33
2'-F-C-MeC + IFN- $\alpha$ 2b	1:1	0.02	0.4	>33
2'-F-C-MeC + IFN- $\alpha$ 2b + RBV	2:1:1	0.2	2.2	>11
2'-F-C-MeC + IFN- $\alpha$ 2b + RBV	1:1:1	0.09	1.2	>11
2'-F-C-MeC + IFN- $\alpha$ 2b + RBV	1:1.5:1.5	0.2	2.6	>3.6

Values represent the apparent potency of the first compound (2'-C-MeC or 2'-F-C-MeC) listed in the combination.

\* Cytotoxicity concentration (CC<sub>50</sub>) represents only the first compound listed in the combination, and it was tested by calculating the  $\Delta$  threshold cycle of rRNA values. EC<sub>50</sub>, 50% effective concentration; EC<sub>90</sub> 90% effective concentration; IFN, interferon; RBV, ribavirin.

**Table 3**  
Computer-simulated combination index of two- to three-drug combinations at 50%, 75%, 90% and 95% inhibition of HCV replicon replication

Treatment	Ratio	CI values at inhibition of				CI <sub>wt</sub>
		50%	75%	90%	95%	
2'-C-MeC + IFN- $\alpha$ 2b	1:3	0.2	0.3	0.3	0.3	0.3
2'-C-MeC + IFN- $\alpha$ 2b	1:2	1.7	0.6	0.3	0.3	0.5
2'-C-MeC + IFN- $\alpha$ 2b	1:1	0.8	0.5	0.3	0.3	0.4
2'-C-MeC + IFN- $\alpha$ 2b	2:1	0.3	0.2	0.2	0.4	0.3
2'-C-MeC + IFN- $\alpha$ 2b + RBV	2:1:1	1.4	0.9	0.9	1.4	1.1
2'-C-MeC + IFN- $\alpha$ 2b + RBV	1:1:1	0.6	0.5	0.5	0.6	0.5
2'-F-C-MeC + IFN- $\alpha$ 2b	1:10	1.7	0.9	0.6	0.5	0.7
2'-F-C-MeC + IFN- $\alpha$ 2b	1:5	1.3	0.8	0.5	0.5	0.7
2'-F-C-MeC + IFN- $\alpha$ 2b	1:1.5	0.3	0.3	0.3	0.4	0.3
2'-F-C-MeC + IFN- $\alpha$ 2b	1:1	0.7	0.3	0.3	0.3	0.3
2'-F-C-MeC + IFN- $\alpha$ 2b + RBV	2:1:1	2.3	1.0	0.8	1.0	1.1
2'-F-C-MeC + IFN- $\alpha$ 2b + RBV	1:1:1	2.2	0.8	0.5	0.6	0.8
2'-F-C-MeC + IFN- $\alpha$ 2b + RBV	1:1.5:1.5	1.1	0.9	0.9	1.1	1.0

Combination index (CI) values were determined for a mutually exclusive interaction using CalcuSyn program (Biosoft, Ferguson, MO, USA). CI <1, =1 or >1 indicates synergism, additive effect and antagonism, respectively. CI<sub>wt</sub> weighted average CI value was assigned as = [CI<sub>50</sub> + 2CI<sub>75</sub> + 3CI<sub>90</sub> + 4CI<sub>95</sub>]/10 (see review [21]). IFN, interferon; RBV, ribavirin.