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Mismatched double-stranded RNA (polyI-polyC$_{12}$U) is synergistic with multiple anti-HIV drugs and is active against drug-sensitive and drug-resistant HIV-1 in vitro☆

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Abstract

Although highly active anti-retroviral therapy (HAART) is successful in the treatment of HIV infection, problems with toxicity, drug-resistant variants, and therapeutic failures have compromised the long-term utility of existing combination regimens. Mismatched double-stranded RNA (polyI-polyC$_{12}$U) is an immune modulator with inherent anti-HIV activity. Cell toxicities and anti-HIV activities of fourteen anti-HIV agents were determined alone and in combination with polyI-polyC$_{12}$U. Combination analyses for anti-HIV activity were performed at three drug ratios. Using Mixed Dose Effect analyses and the CalcuSyn for Windows software package, combination indeces were determined for all drug combinations. In general, polyI-polyC$_{12}$U was synergistic in combination with abacavir, zidovudine, zalcitabine, didanosine, stavudine, efavirenz, indinavir, ritonavir, nelfinavir, and amprenavir. It was synergistic to antagonistic with lamivudine, delavirdine, nevirapine, and saquinavir. Thus, polyI-polyC$_{12}$U is synergistic with most anti-HIV agents at most drug ratios and across most effective concentrations in vitro, although, certain members of each class were exceptions. PolyI-polyC$_{12}$U alone was equally active against wild-type HIV and HIV resistant to nevirapine, protease inhibitors, or nucleoside analogue reverse transcriptase inhibitors. These results suggest that polyI-polyC$_{12}$U should be re-evaluated as a potential adjunct therapy in patients who have failed current anti-retroviral therapeutic regimens.

Keywords: Experimental therapies; Reverse transcriptase; Protease; Acquired immune deficiency syndrome

1. Introduction

Mismatched double-stranded RNA (polyI-polyC$_{12}$U, Ampligen®) is an immune modulator. It was shown earlier to be a potent inhibitor in vitro against the human immunodeficiency virus type 1 (HIV-1) in a variety of cell types (Montefiori and Mitchell, 1987; Montefiori et al., 1988a,

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1989a). Furthermore, it was synergistic with zidovudine and a number of experimental anti-HIV agents including castanospermine, interferon, and amphotericin B (Montefiori et al., 1989c). More recently, polyI-polyC_{12}U was shown to be synergistic with zidovudine against zidovudine-resistant HIV without synergistic toxicity to bone marrow cells (Gillespie et al., 1994). Based in part upon these in vitro analyses, phase I and II/III clinical trials were initiated. The original phase I clinical trial demonstrated that patients on polyI-polyC_{12}U had augmentation of delayed hypersensitivity skin reactions with only limited toxicity. Furthermore, nine of ten patients had viral RNA levels that decreased to undetectable levels and six of seven showed a decrease in viral load in co-culture assays. Finally, patients showed maintenance of $CD4^+$ lymphocyte levels, as well as improvements in HIV-related symptoms and a rise in neutralizing antibody titers (Carter et al., 1987). Based on this study, a phase II/III placebo-controlled trial was initiated. In 36 HIV infected patients receiving zidovudine and either polyI-polyC_{12}U or placebo, polyI-polyC_{12}U was well-tolerated and patients showed clinical improvement on polyI-polyC_{12}U plus zidovudine compared with placebo plus zidovudine (Thompson et al., 1996). The subsequent identification of new and improved anti-retroviral agents, coupled with the profound effects of highly active anti-retroviral therapy (HAART) (Collier et al., 1996; Hammer et al., 1994, 1997) slowed development of polyI-polyC_{12}U as an anti-HIV agent.

Current multi-drug regimens, i.e. HAART, have proven highly effective at decreasing the levels of viral replication as measured by plasma viral RNA (Collier et al., 1996; Hammer et al., 1994, 1997). HAART has also significantly delayed the progression of HIV infection to AIDS in most patients (Collier et al., 1996; Hammer et al., 1994, 1997). Although early work suggested that HAART could significantly reduce levels of HIV in the lymph nodes (Cavert et al., 1997), as well as in the circulation, recent studies have demonstrated persistence of HIV-1 proviral genomes within certain compartments in patients on HAART (Furtado et al., 1999; Hockett et al., 1999; Zhang et al., 1998, 1999). In addition, replication competent virus is maintained within resting $CD4^+$ lymphocytes (Finzi et al., 1997; Wong et al., 1997). Furthermore, therapy is complicated by the emergence of single- and multiple-drug-resistant HIV (Condra et al., 1995) and its subsequent transmission to uninfected individuals (Hecht et al., 1998). Thus, the long-term benefits of HAART without the development of new anti-HIV agents is in jeopardy. The sum of these studies has again raised interest in immune modulator therapy as an adjunct to conventional HAART.

It is clear that any new anti-HIV agent, regardless of mechanism of action, will need to be active in the presence of existing anti-HIV drugs. Furthermore, new anti-HIV agents will likely need to be active against variants of HIV that are resistant to currently available anti-HIV drugs. Since the mechanism of action of polyI-polyC_{12}U is different from other anti-HIV agents, we hypothesized that polyI-polyC_{12}U would be synergistic with at least some anti-HIV agents and that it would be effective against drug-resistant variants of HIV. To test this hypothesis, the activities of polyI-polyC_{12}U in combination with fourteen anti-HIV agents currently approved by US Food and Drug Administration were tested. These compounds included the nucleoside analogue reverse transcriptase inhibitors (NRTI), abacavir (Daluge et al., 1997), zidovudine (Mitsuya et al., 1985), zalcitabine (Chu et al., 1988; Mitsuya and Broder, 1986), didanosine (De Clercq et al., 1989; Mitsuya and Broder, 1986), lamivudine (Coates et al., 1992) and stavudine (Chu et al., 1988; Lin et al., 1987), the non-nucleoside analogue reverse transcriptase inhibitors (NNRTI), efavirenz (Young et al., 1995), delavirdine (Romero et al., 1993), and nevirapine (Merluzzi et al., 1990), and the protease inhibitors (PI), indinavir (Vacca et al., 1994), ritonavir (Kempf et al., 1995; Markowitz et al., 1995), saquinavir (Craig et al., 1991; Roberts et al., 1990), nelfinavir (Patick et al., 1996), and amprenavir (Baba et al., 1997; Partaledis et al., 1995; St. Clair et al., 1996). Although the fourteen anti-HIV agents have been studied earlier, their toxicities and anti-HIV activities in one laboratory using standardized reagents at contemporary time points have never been reported. Thus, as a preliminary study, all fifteen drugs were studied alone for toxicity and anti-HIV activity using a

$CD4^+$ lymphoblastoid cell line and HIV_{LAI}. Once baseline activities were established, the anti-HIV activities of each drug alone and in combination with polyI-polyC_{12}U were determined. In addition, the anti-HIV activities of polyI-polyC_{12}U against several reverse transcriptase inhibitor- and protease inhibitor-resistant HIV molecular clones were assessed.

2. Methods

2.1. Compounds and drugs

PolyI-polyC_{12}U was a gift from Hemispherx Biopharma, Inc. It was provided as reconstituted, quality-controlled, RNAse free solution at 2.44 mg/ml and was stored at 4°C. Since not all anti-HIV agents are readily available for purchase or from the manufacturers in pure form, we chose to study each agent in its prescription formulation. William M. Mitchell (Vanderbilt University) provided all drugs in their prescription form. The advantage of this approach is that any investigator can readily obtain the source materials. The disadvantage is that stabilizers, inert ingredients, and preservatives can influence solubility and activity of the active ingredient. The results in Table 1 (Section 3) suggest that additives did not have a major effect on any assays performed in this study. Caplets and tablets were crushed and the powders dissolved, while gelcaps were dissolved whole. All drugs were dissolved and the final stock concentrations were calculated based on concentrations of the pure drug, as indicated in the dosing, without including the weights of any

Table 1
Observed cell toxicity and observed and reported anti-HIV activities of fifteen anti-HIV agents

Drug	Class	C_{max}[a] (μM)	Cell toxicity		Anti-HIV activity		
			Replicate assays	CT_{50}[b] (μM)	Replicate assays	EC_{50}[b] (nM)	Published[c]
Abacavir	NRTI	4.47 (1.33)	5	135.6 (125.5)	5	321 (48)	3700–5800
Lamivudine	NRTI	4.02 (1.35)	3	2667 (842)	9	1534 (1577)	2–15 000
Zidovudine	NRTI	6.78 (1.95)	4	349 (368)	4	42 (14)	11–48
Zalcitabine	NRTI	0.120	4	119 (61)	4	605 (263)	30–500
Didanosine	NRTI	NA	3	985 (384)	4	4549 (784)	2500–10 000
Stavudine	NRTI	NA	3	564 (195)	3	1712 (635)	9 μM–4 mM
Delavirdine	NNRTI	35 (20)	3	1654 (530)	6	14.4 (8.9)	5–30
Efavirenz	NNRTI	12.9 (3.7)	3	50.4 (1)	4	2.8 (1.1)	1.7–25[d]
Nevirapine	NNRTI	7.5 (1.5)	3	743 (120)	4	193 (39)	10–100
Indinavir	PI	12.6 (4.0)	3	198 (18)	4	19.2 (3.0)	25–100[d]
Saquinavir	PI	3.73	3	23 (4)	7	24.2 (7.5)	1–30
Ritonavir	PI	15.53 (4.99)	4	137 (82)	4	29 (5.2)	3.8–153
Nelfinavir	PI	4.52–6.02	4	155 (153)	4	202 (61)	7–196
Amprenavir	PI	10.59	3	9 (1)	4	48.4 (12.7)	12–80
PolyI-polyC_{12}U[g]	dsRNA	NA	3	128 (47)[e]			
(moi > 1)					38	1383 (674)[f]	1000–1600[f]
(moi < 0.1)					18	828 (240)[f]	1000–1600[f]

[a] Physicians' Desk Reference, 2000. Values are the C_{max}. Values in parentheses are S.D. If no S.D., then none was reported.
[b] Mean; values in parentheses are 1 S.D.
[c] References are Physicians' Desk Reference, 2000. Citations for each individual drug are reported in the introduction.
[d] EC_{95}, no EC_{50} published.
[e] For polyI-polyC_{12}U, μg/ml.
[f] For polyI-polyC_{12}U, ng/ml; NA, not available; i.e. not in the literature.
[g] For polyI-polyC_{12}U anti-HIV activity was measured at both an moi < 1 and at an moi > 1; both sets of data are shown.

additives. Zalcitabine, zidovudine, didanosine, abacavir, and lamivudine were dissolved in distilled H_2O. Efavirenz was solubilized in one part ethanol (EtOH), four parts H_2O, and five parts dimethylsulfoxide (DMSO). Delavirdine, nevirapine, nelfinavir, amprenavir, saquinavir, ritonavir, and indinavir were solubilized in three parts EtOH, three parts DMSO, and four parts H_2O. Stavudine was solubilized in 25% EtOH. All stock solutions were diluted into RPMI-1640 containing 2 mM L-glutamine and 11.5% heat-inactivated fetal bovine serum (FBS, growth medium), filtered through 0.45 μm nitrocellulose filters, and stored at −70°C.

2.2. Cells and viruses

H9 and MT-2 cells were cultured in growth medium. Both cell lines were reduced by half and re-fed every other day. HeLa cells were released using trypsin and split 1:4 three times per week. HIV_{LAI} and drug-resistant molecular clones of HIV, unless noted differently, were obtained from the NIH AIDS Research and Reference Reagent Program. All catalog numbers (Cat. #) refer to this program. HIV_{NL4-3} is the parental molecular clone (Cat. #114, kindly donated by Dr Malcolm Martin). M184V is HIV_{NL4-3} containing a single codon change resulting in a change at position 184 of RT from methionine to valine (Gao et al., 1993; Gu et al., 1992) (provided by Dr Paul Krogstaad, UCLA). JF26A7 is HIV_{NL4-3} containing five changes in RT that result in zidovudine resistance (Fitzgibbon et al., 1993) (provided by Dr Paul Krogstaad, UCLA). L10R is HIV_{NL4-3} containing five changes in protease (leucine 10 to arginine, methionine 46 to isoleucine, leucine 63 to proline, valine 82 to threonine, and isoleucine 84 to valine) that are identical to those following 40 weeks of indinavir therapy (Cat. #2840, donated by Dr Emilio Emini and Dr William Schleif) (Condra et al., 1995). M46I is HIV_{NL4-3} containing four changes in protease (methionine 46 to isoleucine, leucine 63 to proline, valine 82 to threonine, and isoleucine 84 to valine) that are identical to those following 40 weeks of indinavir therapy (Cat. #2841, donated by Dr Emilio Emini and Dr William Schleif) (Condra et al., 1995). Nevirapine resistant virus was selected by passage of clinical isolate A018A in the presence of nevirapine and contains a single RT mutation of tyrosine at position 181 to cysteine (Richman et al., 1991b) (Cat. #1392, donated by Dr D. Richman).

Molecular clones were transfected onto HeLa cells using Lipofectin (Gibco/BRL). After 40 h, uninfected H9 cells were added to the culture and 48 h later the non-adherent cells were cultured. HIV was harvested when the culture was 100% positive for HIV antigens by indirect immunofluorescence assay (IFA) as described (Montefiori et al., 1988b; Robinson et al., 1996a,b). Uncloned HIV_{LAI} was grown in H9 cells. All viruses were clarified of cells by low-speed centrifugation followed by filtration through 0.45 μm nitrocellulose filters.

2.3. Cell toxicity and anti-HIV data

2.3.1. Cell toxicity

The 50% cell toxic dose (CT_{50}) was determined as described earlier (Montefiori et al., 1988b; Robinson et al., 1996a,b). Briefly, compounds were diluted in eight, 2-fold serial dilutions from stock solutions in triplicate wells of a 96 well plate. To each compound, approximately 2×10^5 MT-2 cells were added in a final volume of 200 μl. Cells and compound were incubated at 37°C in growth medium for 3 days. Cell viability was determined using Finter's Neutral Red dye as described (Montefiori et al., 1988b). The absorption (A_{540}) was measured on a microcolorimeter and compared with eight cell control replicates (no drug added). Each assay was performed at least three times for a total of at least nine replicate wells. This method detects both cytostatic and cytolytic effects of drugs.

2.3.2. Anti-HIV assay

Anti-HIV activity of each drug, except polyI-polyC$_{12}$U, was determined using a vital dye assay essentially as described (Montefiori et al., 1988b; Robinson et al., 1996a,b). Drugs were diluted in triplicate wells of a 96-well plate in eight, 2-fold serial dilutions. Next, 50 μl of HIV-1_{LAI} was added to a final multiplicity of infection (moi) of

approximately 0.1–1. Virus and drug were incubated for 1 h at 37°C. To each drug and virus mixture, approximately 2×10^5 MT-2 cells were added to a final volume of 200 μl. Cells and virus were incubated for 3 days at 37°C. Cell viability was determined using Finter's Neutral Red dye. The absorption was measured on a microcolorimeter (A_{540}) and compared with eight cell control replicates (no virus or drug added) and eight virus control wells (no drug added). Each assay was performed a minimum of three times. The 50% effective concentration (EC_{50}), or that concentration that inhibits HIV-induced cell death by 50%, was calculated from each dose response curve. Results using this assay have compared favorably to other measures of HIV replication, including reverse transcriptase release, RNA synthesis, protein synthesis by immunofluorescence and most importantly infectious virus release as measured by 50% tissue culture infectious dose (Robinson et al., 1989). Furthermore, it is more cost effective than other dye assays for cell growth and viability. Finally, as virtually all agents fell within their published values (Section 3), the data are consistent with other measures of viral replication. For polyI-polyC_{12}U, the assay was performed identically except that diluted polyI-polyC_{12}U was pre-incubated with cells for 4 h prior to addition of virus. PolyI-polyC_{12}U pre-incubated with virus had no anti-HIV activity (data not shown).

2.3.3. Mixed dose effect analyses

After an EC_{50} was obtained for each drug, the anti-HIV activity of each drug alone and in combination with polyI-polyC_{12}U was assayed in a single 96-well plate. Column 1 was the cell control and column 2 the virus control. Columns 3–5 contained dilutions of the drug to be tested in triplicate. Columns 6–8 contained dilutions of polyI-polyC_{12}U in triplicate. Columns 9–11 were the combination of polyI-polyC_{12}U and the drug to be tested in triplicate. Assays were designed so that the EC_{50} of the drug would occur in the middle of the dilution range. Therefore, dose response curves from an ineffective concentration to a maximally effective concentration could be determined for each drug. Three different ratios were used for these studies as indicated in Tables 3–6. The ratios were based on the 50% effective concentration (EC_{50}) for each compound alone. The first assay was designed so that the ratio of polyI-polyC_{12}U with each drug was approximately the EC_{50}:EC_{50}. In the second experiment, one-third the concentration of polyI-polyC_{12}U was mixed with the other drug at the same concentration as in the first experiment. In the third experiment, polyI-polyC_{12}U at the same concentration as in experiment one was mixed in combination with the appropriate drug at one-third the concentration as in experiment one. The result was that each drug was compared with polyI-polyC_{12}U at three different ratios. After incubation with HIV, the cells were harvested as described in the anti-HIV assay above. PolyI-polyC_{12}U was pre-incubated with cells and the other drugs were pre-incubated with the virus. Following vital dye staining, the percentage of viable cells was calculated as a mean of triplicate infections (triplicate wells). The Fraction affected (Fa) at each dilution was calculated (i.e. Fa of 0.25 would equal 25% viable cells). This was calculated for the drug to be tested, for polyI-polyC_{12}U, and for each ratio of polyI-polyC_{12}U and drug. The combination indeces (CI) were calculated according to the method of Chou and Talalay (Chou, 1991; Chou and Chou, 1985; Chou and Talalay, 1983, 1984) using CalcuSyn for Windows software. For ease of interpretation, the CI for only the calculated EC_{50}, EC_{90}, and EC_{99} are reported herein.

3. Results

For combination analyses to be meaningful, the toxicities and anti-HIV activities of each drug must be established under standardized conditions. Each agent was tested a minimum of three times and each assay was performed in triplicate. Thus, each drug was tested a minimum of nine times to determine its CT_{50} against uninfected MT-2 cells. The results of these studies are illustrated in Table 1. Each drug was next tested for its anti-HIV activities using the same cell line and a tissue-culture established isolate of HIV,

HIV_{LAI}. The EC_{50} of each agent is illustrated in Table 1. The referenced CT_{50} and EC_{50} values are literature values obtained from the Physician's Desk Reference, a compendium of published and unpublished values from multiple laboratories. Since different assays, cell lines, and viral isolates can lead to different CT_{50} and EC_{50} values, the PDR values are useful summaries of observed anti-HIV activity. Specific citations for the antiviral activity of each drug are given in the introduction. Two drugs were more potent anti-HIV agents than published in the PDR (Physicians' Desk Reference, 2000): abacavir and indinavir. Two drugs were less potent than reported in the literature (Physicians' Desk Reference, 2000) — nevirapine and zalcitabine. The remaining agents had EC_{50} within the published range. Only the EC_{50}'s of abacavir and nevirapine were more than two standard deviations from the published values. The EC_{50} of abacavir was, however, almost identical to its published activity of 260 nM against clinical isolates of HIV in peripheral blood mononuclear cells (Daluge et al., 1997). The discrepancies between published EC_{50} and observed EC_{50} may be secondary to different reagents, i.e. different cells, methods, and viruses, different times of harvest, or may be due to the methods used to solubilize the drugs. The critical issue, however, is that all of the drugs were tested using one virus isolate, the same moi, the same target cell line, and the same methodology was utilized to measure protection in this study; therefore, relative potencies can be directly compared. These data should not, however, be used to compare in vivo potencies as differences in bioavailability, serum half-life, metabolism, and tissue distribution can all affect efficacy.

Next, polyI-$polyC_{12}U$ was studied in combination with fourteen anti-HIV agents approved for use in HIV-infected individuals within the US. The CI for each combination at three different drug ratios was calculated using the CalcuSyn for Windows software package. CI of less than 1 indicates synergy, greater than 1 indicates antagonism and equal to 1 indicates additivity. For simplicity, only the CI at the EC_{50}, EC_{90}, and EC_{99} are illustrated. The results for polyI-$polyC_{12}U$ in combination with NRTI are illustrated in Table 2. PolyI-$polyC_{12}U$ was synergistic with abacavir, zidovudine, zalcitabine, didanosine, and stavudine at all three EC's and at all drug ratios tested. However, polyI-$polyC_{12}U$ was synergistic to antagonistic with lamivudine, depending on the drug ratio and EC measured (Table 2). The mean results for polyI-$polyC_{12}U$ and lamivudine suggested additivity to antagonism. The results for polyI-$polyC_{12}U$ in combination with NNRTI are illustrated in Table 3. PolyI-$polyC_{12}U$ was synergistic with efavirenz at virtually all EC and all drug ratios. However, polyI-$polyC_{12}U$ was generally antagonistic with both delavirdine and nevirapine (Table 3). The exception to these results was at the 1:1 experimental ratio where polyI-$polyC_{12}U$ was synergistic with both delavirdine and nevirapine. The raw data for the strongly synergistic combination of efavirenz and polyI-$polyC_{12}U$ are illustrated in Fig. 1. It is clear that the presence of polyI-$polyC_{12}U$ shifts the dose response curve of efavirenz strongly to the left, demonstrating synergy. All CI for all drug combinations were determined from similar dose response curves. Interestingly, the dose response curve for polyI-$polyC_{12}U$ was shifted far less than the efavirenz curve; however, the potency of inhibition of polyI-$polyC_{12}U$ was much stronger in the presence of efavirenz, resulting in a favorable CI. The results for polyI-$polyC_{12}U$ in combination with PI are illustrated in Table 4. PolyI-$polyC_{12}U$ was additive to synergistic with indinavir, ritonavir, nelfinavir, and amprenavir. At the EC_{90} and EC_{95} polyI-$polyC_{12}U$ was potently synergistic with all four of these PI. However, polyI-$polyC_{12}U$ was slightly synergistic to additive with saquinavir (Table 4). Again, the best ratio was the 1:1 experimental ratio for saquinavir and polyI-$polyC_{12}U$.

The software package also calculates the specific concentrations of each inhibitor necessary to achieve the appropriate EC at each ratio. These EC are calculated directly from the dose response curves for each drug in combination. Since the mixed dose effect analyses and the EC for each drug alone were performed on the same plate, any effect of additives on the EC would be present in both the drug alone and drug in combination results. The calculated EC_{99}'s for synergistic com-

Table 2
Mixed dose effect analysis of polyI-polyC$_{12}$U with NRTI

Drug	Experimental drug ratio[a]			Mean[b]	Synergy[c]
Abacavir Sulfate	1:1.63	1:4.9	1:14.6		
EC_{50}	0.799 (0.128)	0.79 (0.065)	1.052 (0.176)	0.883	+
EC_{90}	0.561 (0.097)	0.523 (0.063)	0.88 (0.152)	0.655	+++
EC_{99}	0.385 (0.092)	0.354 (0.073)	0.726 (0.205)	0.488	+++
Lamivudine	1:1.63	1:4.88	1:14.8		
EC_{50}	0.881 (0.058)	1.173 (0.190)	1.479 (0.413)	1.178	−
EC_{90}	0.716 (0.081)	0.744 (0.121)	1.630 (0.489)	1.030	±
EC_{99}	0.578 (0.119)	0.456 (0.118)	1.823 (1.11)	0.952	±
Zidovudine	1:25.42	1:76.25	1:228.75		
EC_{50}	0.777 (0.134)	0.976 (0.232)	0.727 (0.065)	0.827	++
EC_{90}	0.845 (0.252)	0.496 (0.225)	0.399 (0.069)	0.580	+++
EC_{99}	0.959 (0.485)	0.271 (0.252)	0.262 (0.085)	0.497	+++
Zalcitabine	1:5.08	1:15.25	1:45.75		
EC_{50}	0.879 (0.171)	0.735 (0.079)	1.112 (0.185)	0.909	±
EC_{90}	0.818 (0.192)	0.568 (0.096)	0.329 (0.067)	0.572	+++
EC_{99}	0.802 (0.276)	0.431 (0.113)	0.128 (0.038)	0.454	+++
Didanosine	3.94:1	1.3:1	1:2.29		
EC_{50}	0.822 (0.158)	0.710 (0.093)	0.713 (0.075)	0.748	++
EC_{90}	0.629 (0.197)	0.532 (0.110)	0.388 (0.075)	0.516	+++
EC_{99}	0.485 (0.265)	0.394 (0.133)	0.235 (0.07)	0.371	+++
Stavudine	6.15:1	2.05:1	1:1.46		
EC_{50}	0.728 (0.120)	0.876 (0.127)	0.993 (0.154)	0.866	+
EC_{90}	0.509 (0.113)	0.655 (0.112)	0.669 (0.095)	0.611	+++
EC_{99}	0.361 (0.135)	0.487 (0.138)	0.611 (0.124)	0.486	+++

[a] Combination Indeces for three experimental drug ratios at the calculated EC_{50}, EC_{90}, and EC_{99}. Experimental ratio of the drugs to be tested to polyI-polyC$_{12}$U, based on EC_{50}'s. The actual ratio of drug: polyI-polyC$_{12}$U is shown. Values in the table are CI; numbers in parentheses are standard deviations. Experiments were performed as described in Section 2.

[b] Mean CI at the EC for the three different drug ratios.

[c] Synergy was determined by the method of Chou and Talalay. −−−, antagonism; −−, moderate antagonism; −, slight antagonism; ±, nearly additive; +, slight synergism; ++, moderate synergism; +++, synergism; and ++++, strong synergism.

Table 3
Mixed dose effect analysis of polyI-polyC$_{12}$U with NNRTI[a]

Drug	Experimental drug ratio			Mean	Synergy
Delavirdine	1:41	1:119	1:366		
EC_{50}	1.495 (0.482)	0.808 (0.109)	1.256 (0.299)	1.186	−
EC_{90}	1.556 (0.732)	0.725 (0.135)	1.539 (0.578)	1.270	−−
EC_{99}	2.425 (1.893)	0.643 (0.181)	1.921 (1.173)	1.663	−−−
Efavirenz	1:508	1:1525	1:4575		
EC_{50}	1.001 (0.253)	0.921 (0.154)	0.575 (0.169)	0.832	++
EC_{90}	0.45 (0.201)	0.542 (0.148)	0.168 (0.081)	0.387	+++
EC_{99}	0.205 (0.178)	0.338 (0.156)	0.079 (0.065)	0.207	++++
Nevirapine	1:8.13	1:23.8	1:73.2		
EC_{50}	1.035 (0.168)	0.735 (0.125)	1.071 (0.267)	0.947	±
EC_{90}	1.435 (0.387)	0.667 (0.169)	2.074 (0.499)	1.392	−−−
EC_{99}	2.165 (1.019)	0.601 (0.242)	4.31 (1.75)	2.359	−−−

[a] Combination indeces for three experimental drug ratios at the calculated EC_{50}, EC_{90}, and EC_{99}. See legend to Table 2.

binations of polyI-polyC$_{12}$U with NRTI, NNRTI, and PI (Table 5) are well below the toxic concentrations of the agents (Table 1). When these val-ues are compared with the reported maximum plasma concentration (C_{max}) for each agent (Table 1), only zalcitabine would require higher doses in patients than the C_{max}. However, that is also true for zalcitabine alone (Table 1).

If polyI-polyC$_{12}$U is to be used in a clinical setting, it will likely be used in the context of HIV variants already resistant to one or more anti-HIV agents. Therefore, the activities of polyI-polyC$_{12}$U against NRTI-resistant, NNRTI-resistant, and PI-resistant variants of HIV were tested. To minimize the effects of other mutations, replication-defective variants, and poorly defined genotypes of clinically-derived viruses, drug-resistance was studied in a defined viral background. All mutations were in the context of an HIV$_{NL4-3}$ backbone except for the nevirapine-resistant virus. Thus, the only mutations present between different viruses were those known to encode for the appropriate resistance pattern. As illustrated in Table 6, all of the viruses were resistant to the appropriate anti-HIV agent. The lamivudine-resistant, nevirapine-resistant, and zidovudine-resistant viruses were completely

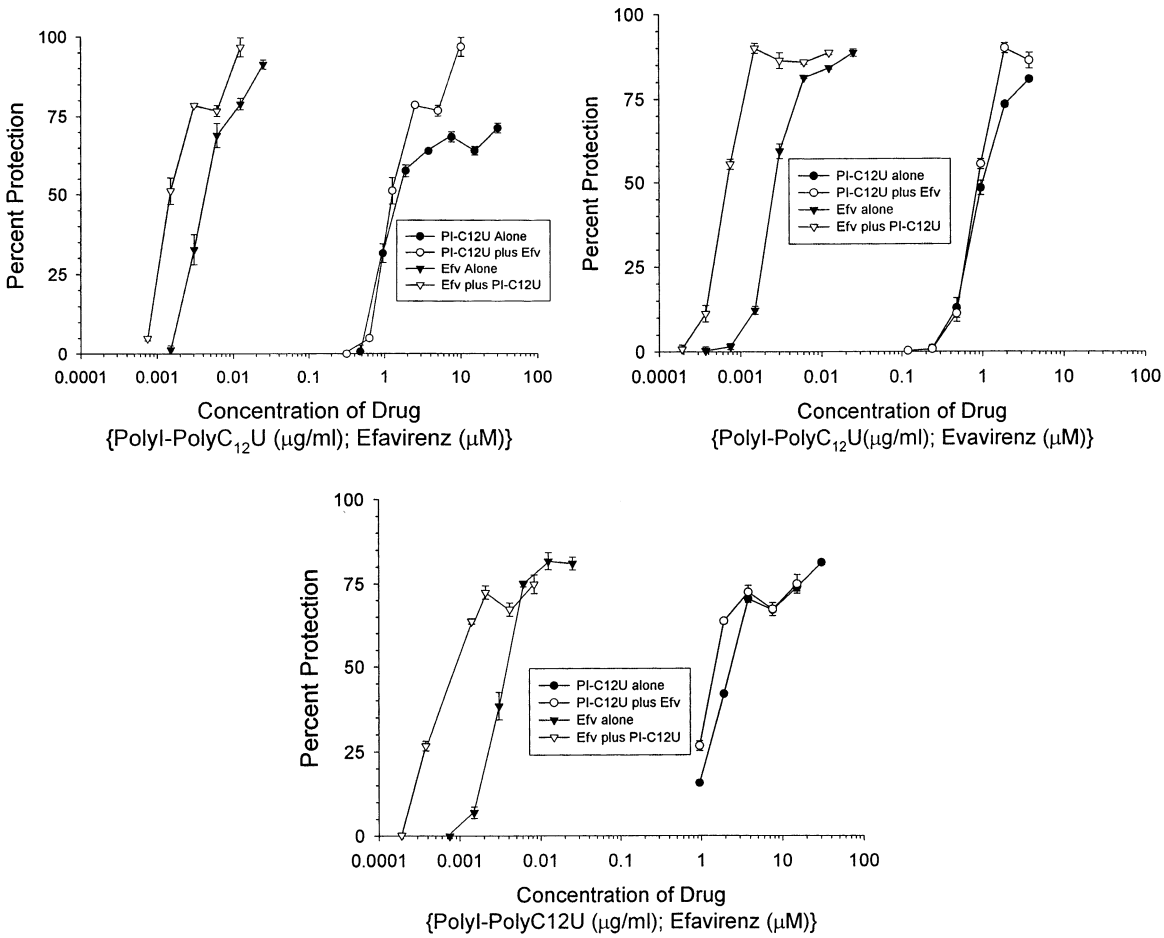

Fig. 1. Representative dose response curves for the strongly synergistic combination of polyI-polyC$_{12}$U and Efavirenz. Panel A, the 1:508 drug ratio; panel B, the 1:1525 drug ratio; and panel C, the 1:4575 drug ratio. For all three panels, the closed circle is the dose response curve for polyI-polyC$_{12}$U alone; the open circle is the dose response curve for polyI-polyC$_{12}$U in combination with Efavirenz; the closed, inverted triangle is the dose response curve for Efavirenz alone; the open, inverted triangle is the dose response curve for evavirenz in combination with polyI-polyC$_{12}$U.

Table 4
Mixed dose effect analysis of polyI-polyC$_{12}$U with PI[a]

Drug	Experimental ratio			Mean	Synergy
Indinavir	1:25.4	1:76.25	1:229		
EC_{50}	0.912 (0.175)	1.132 (0.207)	1.110 (0.275)	1.051	±
EC_{90}	0.769 (0.172)	0.769 (0.147)	0.364 (0.108)	0.634	+++
EC_{99}	0.640 (0.217)	0.523 (0.157)	0.116 (0.073)	0.426	+++
Saquinavir	1:42.4	1:127	1:381.25		
EC_{50}	1.188 (0.329)	1.148 (0.267)	1.098 (0.193)	1.145	−
EC_{90}	1.195 (0.218)	0.784 (0.216)	1.191 (0.215)	1.057	±
EC_{99}	0.830 (0.260)	0.532 (0.217)	1.300 (0.340)	0.887	+
Ritonavir	1:16.9	1:51	1:152		
EC_{50}	0.981 (0.074)	1.295 (0.22)	0.775 (0.18)	1.017	±
EC_{90}	0.569 (0.038)	0.501 (0.087)	0.535 (0.113)	0.535	+++
EC_{99}	0.359 (0.034)	0.232 (0.077)	0.381 (0.108)	0.324	+++
Nelfinavir	1:12.7	1:38	1:114		
EC_{50}	0.829 (0.183)	1.053 (0.105)	0.838 (0.215)	0.907	±
EC_{90}	0.392 (0.076)	0.772 (0.066)	0.701 (0.197)	0.622	+++
EC_{99}	0.199 (0.051)	0.557 (0.076)	0.578 (0.28)	0.445	+++
Amprenavir	1:20	1:60	1:183		
EC_{50}	1.487 (0.262)	1.116 (0.093)	0.939 (0.235)	1.108	−
EC_{90}	0.784 (0.144)	0.965 (0.107)	0.523 (0.116)	0.757	++
EC_{99}	0.399 (0.108)	0.823 (0.147)	0.329 (0.108)	0.517	+++

[a] Combination indeces for three experimental drug ratios at the calculated EC_{50}, EC_{90}, and EC_{99}. See legend to Table 2.

Table 5
EC_{99} doses of synergistic combinations of polyI-polyC$_{12}$U with anti-HIV agents

Drug	Combinations of each drug (nM) + polyI-polyC$_{12}$U (ng/ml)[a]		
Abacavir	580 + 945	413 + 2025	480 + 7033
Zidovudine	195 + 4956	48 + 3667	60 + 13 734
Zalcitabine	901 + 4582	265 + 4044	238 + 10 889
Didanosine	6263 + 1591	4306 + 3312	3314 + 7589
Stavudine	3871 + 631	4152 + 2025	2210 + 3236
Efavirenz	18 + 9079	2.5 + 3735	2.5 + 11 445
Indinavir	80 + 2038	64 + 4846	8.6 + 1968
Ritonavir	46 + 781	77 + 3890	12 + 1897
Nelfinavir	179 + 2271	98 + 3722	35 + 4037
Amprenavir	61 + 1243	62 + 3716	15 + 2677

[a] The first number is the concentration of the drug being tested (in nM) plus the second number (the concentration of polyI-polyC$_{12}$U in ng/ml) necessary to achieve the EC_{99}. Only synergistic combinations are illustrated for three different drug ratios.

resistant to the appropriate drug while the PI-resistant viruses were only partially resistant (Table 6). This partial resistance of three- to five-fold was quite similar to the eight-fold resistance to indinavir reported for these constructs by Condra et al. (Condra et al., 1995). PolyI-polyC$_{12}$U showed equal efficacy against all drug-resistant viruses (Table 7).

4. Discussion

PolyI-polyC$_{12}$U is a relatively non-toxic anti-HIV agent, potent in tissue culture, and promising in earlier clinical trials. Thus, if polyI-polyC$_{12}$U can be shown to be synergistic with existing anti-HIV agents and active against drug-resistant virus, it should be considered for further clinical

evaluation in combination with existing anti-HIV agents. As illustrated, polyI-polyC$_{12}$U was synergistic at some experimental ratios with all existing anti-HIV agents (Tables 2–4). Second, it is equally active against both drug-resistant and drug-sensitive HIV (Table 7). Finally, it shows a therapeutic ratio (EC_{50} to CT_{50}) of approximately 200, similar to that of anti-HIV agents approved for human use (Table 1). Neither synergy nor lack of toxicity in vitro can be directly translated into patient benefits. However, the demonstration of synergy between anti-HIV agents in vitro (Patick et al., 1995; Richman et al., 1991a; Zhu et al., 1996) has been borne out by synergistic effects in HIV-infected individuals. Therefore, the in vitro synergy between polyI-polyC$_{12}$U and most drugs currently in use to treat HIV-infected individuals suggest that polyI-polyC$_{12}$U may prove useful in HAART.

Table 6
Resistance phenotype of drug-resistant variants of HIV

HIV clone[a]	Drug	Replicate assays	EC_{50}[b] (nM)
HIV_{NL4-3}	Zidovudine	1	18.3
	Indinavir	4	6.3 (1.6)
	Nevirapine	3	174 (22)
	Lamivudine	3	120 (54)
M184V	Lamivudine	1	>12 500
JF26A7	Zidovudine	2	>20 000
L10R	Indinavir	3	31.3 (10.5)
M46I	Indinavir	3	17.7 (4.0)
Nev. Res.	Nevirapine	3	>1250

[a] The specific mutations for each clone are given in Section 2. All except the nevirapine-resistant clone are in the NL4-3 background.

[b] Values are mean EC_{50}. The values in parentheses are standard deviations. No standard deviation could be determined for those viruses that were resistant to the drug at all concentrations tested.

Table 7
Antiviral activity of polyI-polyC$_{12}$U against drug-resistant strains of HIV-1[a]

HIV clone	Resistance	Replicates assays	EC_{50} (μg/ml)[b]
HIV_{NL4-3}	None	4	3.53 (2.85)
M184V	Lamivudine	3	4.68 (2.34)
JF26A7	Zidovudine	4	3.18 (0.51)
L10R	Indinavir	4	1.79 (0.94)
M46I	Indinavir	5	1.82 (1.18)
Nev. Res.	Nevirapine	3	2.18 (0.44)

[a] For the genotype of each clone, Section 2.

[b] Values are the means from each replicate assay performed in triplicate. Values in parentheses are standard deviations. Concentrations of polyI-polyC$_{12}$U are in μg/ml as molar concentrations cannot be accurately calculated.

There are several methods to determine synergy. These include commercially available software packages for the methods of Chou and Talalay (CalcuSyn) and MacSynergy, the latter utilizing the method of Prichard et al. for testing antiviral agents in combination (Prichard et al., 1993). The latter method demonstrated that, in some circumstances, the Chou and Talalay methodology was flawed. However, we utilized the method of Chou and Talalay in these studies for three reasons. First, the software is widely available and relatively affordable. Second, this method is by far the most utilized for anti-HIV drug testing and has been reported in numerous studies. Finally, and most importantly, when both methods were used to study anti-HIV agents in combination they generated similar results and similar conclusions, especially if more than one drug ratio was employed (Deminie et al., 1996). Therefore, we chose three drug ratios and used only the method of Chou and Talalay for these studies.

The mechanism of anti-HIV activity of polyI-polyC$_{12}$U appears to be through direct activation of the 2′,5′-oligoadenylate synthetase pathway (Ushuima et al., 1993). The subsequent activation of RNase L leads to delayed release of virus from infected cells (Schroder et al., 1989). An additional mechanism of action may be through the direct inhibition of HIV RT by 2′,5′-oligoadenylate (Montefiori et al., 1989b). In addition to its anti-HIV effects, polyI-polyC$_{12}$U is also an immune modulator. It has been shown to increase the antibody response to HIV in patients and to increase $CD4^+$ lymphocyte counts in treated HIV-infected individuals (Carter et al., 1987; Thompson et al., 1996). Thus, polyI-polyC$_{12}$U has

the added benefit of both innate anti-HIV activity and the potential to stimulate the immune system to rebound from HIV infection.

While the translation from in vitro activity to clinical success is far from guaranteed, the activities of polyI-polyC_{12}U against drug-resistant viruses, coupled with its potent synergy with most existing anti-HIV agents suggest that polyI-polyC_{12}U should be useful in HIV infected individuals, especially in those who have failed HAART or who are infected by drug-resistant viruses. These results, combined with the success of polyI-polyC_{12}U in phase I and phase II/III clinical trials suggest that polyI-polyC_{12}U, in combination with any HIV inhibitor except for lamivudine and delavirdine, should be evaluated for clinical use in HIV-infected individuals.

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