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The effect of combinations of ampligen and zidovudine or dideoxyinosine against human immunodeficiency viruses in vitro

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Summary

The combinations of ampligen and zidovudine at ratios of 100:1, 25:1, 10:1, and 1:50 acted synergistically to reduce cytopathology caused by HIV in MT-2 cell cultures. Combination indices were less than 1 at all of these ratios representing different combinations of concentrations and at 3 effective doses (ED_{30}, ED_{50}, ED_{70}). Combination of drugs which show synergism at a wide range of ratios of combinations suggest that they may be useful clinically, and that the antiviral efficacy of ZDV may be increased in combination with ampligen. Synergism was also found between ampligen and zidovudine by reduction of HIV-produced plaques in a HeLa cell line expressing CD-4 receptors. However the combination of ampligen and dideoxyinosine against HIV in MT-2 cells was only additive and not synergistic.

Human immunodeficiency virus; AIDS; Antiviral; Drug resistance; Azidothymidine; Zidovudine; Dideoxyinosine

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Introduction

Human immunodeficiency virus (HIV) infection, and AIDS and other disease syndromes attributable to it, present a formidable challenge to the clinician and virologist. Until recently, the only approved antiviral agent against HIV was azidothymidine (ZDV) (Fischl et al., 1987), although in October, 1991, approval for restricted use was given for dideoxyinosine (ddI). More recent studies suggest that besides reducing mortality due to ARC and AIDS, ZDV will also prevent or prolong the interval between the onset of HIV infection and opportunistic infections in HIV-infected subjects with mild degrees of immunodeficiency (Volberding et al., 1990).

Therapy with ZDV is limited by adverse reactions, cost, and by development of resistant strains during treatment (Richman et al., 1987; Larder et al., 1989). The HIV infection is suppressed but not cured, and HIV disease progresses inexorably, even if it is delayed. Besides searching for more effective or less toxic nucleosides, other types of antivirals or measures to strengthen the immune system are being diligently investigated. The eventual strategy may be to use different agents singly or in various combinations.

In this regard, Ampligen® (HEM Research Inc., Philadelphia, PA), a double-stranded RNA, polyriboinosinic: polycytidylic (12:1) uridylic acid (poly (I)n: poly ($C_{12}U$)n), is an interesting agent because potentially, it has both an anti-viral and immuno-modulating action (Carter et al., 1972; Ts'o et al., 1976). It is a less toxic form of the double-stranded RNA, polyinosinic: polycytidylic acid copolymer (poly I: poly C), an antiviral of great potency in some cell culture systems and some animals where it appeared to act mainly as an interferon inducer (Field et al., 1967). In humans, poly I: poly C proved to be toxic to use and did not induce high levels of interferon (Hill et al., 1971). The insertion of uridylate residues in the polycytidylate strand was intended to increase sensitivity to ribonuclease and hence reduce toxicity. Ampligen seems to be less toxic while retaining antiviral potency in cell culture (Carter et al., 1972; Ts'o et al., 1976). In addition to antiviral activity (Montefiori and Mitchell, 1987; Lee et al., 1983) the double-stranded RNA activates 2′-5′ oligoadenylate synthetase (2-5A) (Nilsen et al., 1981), the RNase L pathway, stimulates natural killer (NK) cell activity (Zarling et al., 1980; Nolibe and Thang, 1988), and restored delayed hypersensitivity following thermal injury (Karney-Jones et al., 1990).

Ampligen is thought to exert its anti-HIV effect by activating an intracellular enzyme (RNase-L) against HIV RNA transcripts and by inducing interferons. This compound protected the T cell line C3 and the T lymphoblastoid cell line CEM at doses of 10 to 50 μg/ml against HIV. Infections were monitored by indirect immunofluorescence for viral p24 antigen expression, reverse transcriptase activity for virus production, and vital dye uptake for cytopathic effect (Montefiori and Mitchell, 1987).

Mitchell et al. (1987) first reported that when concentrations of ZDV ranging from 0.025 μM to 0.1 μM were incubated with 25 μg/ml of ampligen, the onset

of cytopathic effect produced by HIV on C3 cells was delayed from 10 and 12 days to 14 and 16 days, respectively. These results suggested a synergistic effect between ZDV and ampligen. Later the same group provided more detailed data on inhibition of HIV cytopathology in MT-2 cells at a combination of ampligen to ZDV of 75:1 (Montefiori et al., 1989). At this ratio concentrations of ampligen from 0.4 to 51.2 μg/ml and of ZDV from 0.02 to 2.56 μM were covered. The effect of other combination ratios was not explored.

In this paper we explore the question of synergism using combination indices for ratios of ampligen to ZDV from 100:1 to 1:100 in MT-2 cells. In addition, the presence or lack of synergism is explored by HIV plaque formation in a transfected HeLa cell line (HT4-6C). Finally we determined whether ampligen acted synergistically with another important anti-HIV nucleoside, dideoxyinosine (ddI).

Materials and Methods

Cell and virus

The H9 cell line infected with HIV-III-B was used as a producer cell line for HIV (Popovic et al., 1984). HIV was pelleted from culture fluid by microcentrifugation in a Beckman type 12 microfuge for 2 h at 4°C and filtered through a 0.45-μm pore membrane. The virus suspension was assayed by reverse transcriptase (RT) assay as described by Gupta et al. (1987).

Anti-HIV drugs

Zidovudine was provided by Burroughs Wellcome, Inc., Research Triangle Park, NC. Dideoxyinosine (ddI) was provided by Bristol Myers, Inc., Wallingford, CT. Mismatched dsRNA (ampligen) was provided as a lyophilized powder in buffered saline by HEM Research, Inc., Rockville, MD. A 2.5 mg/ml stock solution was prepared and stored in glass, at −20°C until used. Ampligen in solution was heated at 50°C in a water bath for 10 min to allow for re-annealing of polynucleotide strands prior to use.

Assays of antiviral activity: MT-2 cell system

The assay was carried out in 96-well plates with 4×10^4 MT-2 cells/200 μl/well in the presence of appropriate concentrations of the inhibitors in triplicate wells. After 4 h incubation, 2.5×10^3 reverse transcriptase units (RTU) of HIV-III-B in 50 μl were added to each well and the plates were incubated 3–5 days until extensive, characteristic cytopathology had developed in virus control wells. The cells were then resuspended and 100 μl was transferred from each well to a poly-L-lysine (PLL)-coated well containing 100 μl of 0.05% neutral red in growth medium. The PLL-coated cells were previously prepared

by incubating a solution of PLL, 100 μg/ml, in 96-well plates for one hour. The plates were then washed twice, dried and stored at 4°C. Cells in the plate containing dye were then permitted to settle, stain, and adhere for one hour at 36°C. The medium was removed carefully, and the dye was eluted with 150 μl of acidified alcohol (50% ethanol in 1% acetic acid). The extracted dye solution was quantitated colorimetrically at 550 nm by a plate reader.

Assay of antiviral activity: HeLa (HT4-6C) cell system

A HeLa cell line (HT4-6C) expressing the human CD4 receptor on its surface was obtained from B.D. Chesebro and K. Wehrly (Harada et al., 1985; Chesebro and Wehrly, 1988). The cell line was used in a plaque reduction assay adapted from the procedure described by Larder et al. (1989). Briefly, 4×10^4 HT4-6C cells in 1 ml were aliquoted into each well of a 24-well plate. After 24 to 48 h, the HT4-6C monolayer was treated for 20 min at 37°C with 8 μg/ml DEAE dextran in RPMI-1640. The cells were washed once with RPMI-1640 and then infected by adding 0.5 ml of HIV (50 000 RTU/ml in RPMI-1640) and incubated for 90 min at 37°C. After the infection, 0.5 ml of inhibitor(s) at 4 times the desired concentration was added to each of the wells and 1 ml of 1% methylcellulose-MEM overlay medium with 3% heat inactivated newborn calf serum was added to each well. The HT4-6C cells were incubated for 7 days at 36°C. The monolayers were then fixed with 10% formaldehyde and stained with 0.25% crystal violet to allow visualization of plaques (individual dense foci of multi-nucleated giant cells).

Effective doses, combination assays and statistics

A. Linear approximation

Average control values and inhibitor treated cytopathology or plaque reduction values for each assay were determined and plotted as a function of log inhibitor concentration. An approximation of the linear portion of the sigmoid curve was obtained by linear regression by using, in the MT-2 assay, the two data points with the greatest log drug concentration having a mean OD approximately equal to that of the virus control, the two data points corresponding to the lowest log drug concentration having a mean average OD approximately equal to cell control, and all of the data points that were in between.

For the HT4-6C assay, a linear approximation of the steepest portion of the curve was performed by linear regression using the two data points with the greatest log drug concentration such that the average PFU's were approximately equal to the average PFU for the virus controls, the two data points with the lowest log antiviral concentration such that the average PFU's were approximately zero, and all of the points in between.

The accuracy of each approximation was determined by the correlation coefficient of the line(s) generated. When a combination of two inhibitors was

used, the concentration of both inhibitors was used to similarly generate a response curve. The abscissa was plotted as the log concentration of one of the components. The concentration of the other component was determined by using the known concentration ratio of the mixture.

B. Calculation of effect of combined drugs

The method of Chou and Talalay (1984) for the calculation of effect of drug combination was followed. From the lines generated in the linear approximations described above, and utilizing 30%, 50%, and 70% of the difference in OD between cell control and virus control for the MT-2 assay (or the difference in pfu between virus control and zero for the HT4-6C assay), we calculated a theoretical log (effective dose (ED)) for a given percentage protection of the cell monolayer by solving for the x-value. Taking the antilog yielded the ED_x for each inhibitor or mixture of inhibitors. We then compared the respective ED_x for each drug alone with the respective ED_x for each component drug in the mixture and used these values to determine the combination index (CI) as described by the multiple drug analysis method of Chou and Talalay (1984). This is described by the equation:

$$CI = (D_1/Dx_1) + (D_2/Dx_2) + [\alpha D_1 D_2)/(Dx_1 Dx_2)]$$

where CI is the combination index, Dx_1 is the dose of 1 to produce x percentage effect alone, and D_1 is the dose of drug 1 required to produce the same x percentage effect when this drug is combined with drug 2. The values of Dx_2 and D_2 are derived in a similar manner for drug 2. The value of α was determined from the plot of the dose effect curve using the median effect equation:

$$f_a/f_u = (D/D_m)^b$$

where f_a is the fraction affected by the dose D, f_u is the fraction unaffected by the dose D, D_m is the dose required for median effect and b is the slope of the dose effect curve. For mutually exclusive drugs, $\alpha = 0$, and both drugs alone and their mixture give parallel lines in the median effect plot. If the drugs are mutually nonexclusive, $\alpha = 1$ and the drugs give parallel lines in the median effect plot, but in the mixture give a concave upward curve. $CI < 1$ indicates synergy, $CI > 1$ indicates antagonism, and $CI = 1$ indicates additive effects.

Results

MT-2 cells infected by HIV-III-B demonstrated characteristic cytopathology. There was a clear development of syncytia, loss of viable cells, and ballooning degeneration of the cytoplasm of affected cells when the concentration of inhibitors was decreased and upon comparing cell control to virus control. This cytopathology was quantitated by neutral red uptake as described in Materials and Methods.

The 30, 50 and 70% effective doses (ED_{30}, ED_{50}, ED_{70}) for ZDV and ampligen were determined after plotting a dose response curve for each drug

TABLE 1

Protective effective doses (ED_{50}) of ampligen and ZDV in combinations against HIV in MT-2 cells

Ampligen: ZDV ratio	ED_{50} (ng/ml)		Combination index			Correlation coefficient
	Ampligen	ZDV	ED_{30}	ED_{50}	ED_{70}	
Ampligen only[a]	6830	NA	NA	NA	NA	NA
100:1	244	2.44	0.3	0.3	0.27	0.85
25:1	203	8.12	0.15	0.17	0.13	0.99
10:1	62	6.2	0.8	0.8	0.8	0.98
1:1	16	16	0.29	0.53	0.17	0.96
1:50	0.4	2	0.13	0.23	0.08	0.87
1:100	0.2	20	2.9	3.6	2.4	0.97
ZDV only[a]	NA	35	NA	NA	NA	NA

Combination index < 1 signifies synergism (see Materials and Methods). Correlation coefficients represent that of the linear portion of the log dose response curve from which ED_x values were estimated. NA = not applicable.

[a]Means of 5 determinations.

separately, and determined each time a combination drug dose response assay was done. The mean and standard deviations for ED_{50} of ampligen and ZDV alone for five separate assays were 6830 $\pm$ 9530 ng/ml and 34.9 $\pm$ 31.2 ng/ml (Table 1). We used the MT-2 assay to investigate a range of ratios of ampligen to ZDV varying from 100:1 to 1:100. For each ratio of combination, two dose response titrations were done. The combined data was used to generate a dose response plot using linear regression as described in Materials and Methods. Thus the data for each combination presented in Table 1 represents the composite of two assays. The table lists the ED_{30}, ED_{50} and ED_{70} for ampligen, and ZDV, and the combination indices, as well as the correlation coefficients for the linear regression lines used to generate ED_x.

It is apparent from Table 1 that at all the ampligen to ZDV ratios (100:1 to 1:50) except 1:100, there is a synergistic effect of the combination of the two drugs against the cytopathic effect of HIV ($CI < 1$). At the effective combination ratios, the ED_{50} dose of ZDV, which by itself was 35 ng/ml, was reduced to 2 to 16 ng/ml in the presence of ampligen. The ED_{50} of ampligen was even more significantly reduced from 6830 ng to 0.4 ng. The latter was in the presence of 2 ng/ml ZDV. The combination indices calculated from data corresponding to ED_{30} and ED_{70} agreed with the trends observed for ED_{50}.

We also used the HeLa-CD4 (HT4-6C) cell line to establish a plaque reduction assay that has allowed us to investigate further the interaction between ampligen and ZDV against HIV. We obtained the ED_{30}, ED_{50} and ED_{70}, the CI's, and correlation coefficients listed in Table 2. It is apparent that in this system, the ED values for protective effect against HIV were quite different than in the MT-2 cell system. However, when combined 1:1 with ampligen, the combination indices for 30, 50 and 70% plaque reduction were all less than 1, which indicated synergism, as was the case in the MT-2 cell system.

TABLE 2

Synergistic effect of ampligen and ZDV against HIV in HeLa-CD4 cells

Correlation drug coefficient	ED_{30}	CI	ED_{50}	CI	ED_{70}	CI	Correlation coefficient
Combination (1:1)	1.4[a]	0.31	10	0.5	60	0.5	0.96
ZDV alone (ng/ml)	4.4	NA	20	NA	120	NA	0.95
Ampligen alone (ng/ml)	760	NA	1100	NA	17000	NA	0.74

[a]Dose of either ZDV or ampligen (1:1 combination in ng/ml) which produced 30, 50 or 70% plaque reduction of HIV.

TABLE 3

Lack of synergistic effect of ampligen and ddI against HIV in MT-2 cells

Ampligen: ddI ratio	ED_{30}	CI	ED_{50}	CI	ED_{70}	CI	Correlation coefficient
Ampligen only	3690	NA	19700	NA	106 000	NA	0.97
1:1	59[a]	1.3	206	0.9	729	0.6	0.93
4:1	210[b]	1.2	899	1.0	3 890	0.8	0.95
ddI only	46	NA	233	NA	1 191	NA	0.84

All doses expressed in ng/ml.

[a]Represents ED_x of either ampligen or ddI at 1:1 combination.

[b]Represents ED_x of ampligen, corresponding doses of ddI may be obtained by dividing by 4 in this 4:1 combination.

We next studied the effect of the combination of ampligen and dideoxyinosine (ddI) against HIV-III-B in the MT-2 cell line. ddI is a nucleoside analogue which acts as a reverse transcriptase inhibitor, a mechanism of action analogous to that of ZDV (Yarchoan et al., 1989). The ED_{50} for ddI alone computed from a dose response experiment as described in Materials and Methods was 233 ng/ml. For combinations of ampligen and ddI at ratios 1:1 and 4:1, the ED_x, CI's, and correlation coefficients are listed in Table 3. The combination indices for 70% protection were 0.6 and 0.8 at the two combination ratios. However the CI was approximately 1 (0.9–1.3) for 30% and 50% protection. Hence there was no consistent synergism between these drugs. Very likely the effect of the combination was additive.

Discussion

Carter et al. (1987) reported that 200 mg of ampligen administered twice weekly over a 6-month period in 10 patients with ARC or AIDS produced a significant clinical and laboratory response. A reduction in HIV RNA in peripheral blood mononuclear cells, a reduction of HIV load as measured by co-culture, an increase in or maintenance of the numbers of helper-inducer T-lymphocytes, augmentation of delayed-type hypersensitivity skin reactions, rises in the titer of neutralizing antibody against HIV, restoration of the natural RNase-L pathway, and improvement in HIV-related symptoms were reported.

There was little or no toxicity noted. However these results could not be confirmed in an as yet unpublished controlled trial conducted by DuPont Company. Publication of any significant new knowledge about this compound is desirable to determine definitely whether it is clinically useful.

Recent phase I studies in our laboratory supported by ACTG indicate that ampligen at doses above 40 mg/m^2 given intravenously twice weekly can decelerate the decline of CD4 counts in patients with 200–500 CD4+ cells who were asymptomatic or had early ARC (unpublished results).

Another approach to the use of ampligen is in combination with ZDV if the combination were highly synergistic in a wide range of combination ratios. In the present study we show that ampligen and ZDV act synergistically over 5 combination ratios ranging from 100:1 to 1:50. It shows that minute amounts of ampligen in combination with ZDV may be biologically effective even though it is not a highly effective anti-HIV agent alone. The two drugs were not synergistic at 1:100 ampligen to ZDV, or probably at higher proportions of ZDV. The lack of synergism at this ratio is probably due to the loss of the biological effect of relatively small doses of ampligen in the presence of the overriding effect of large doses of ZDV.Dideoxyinosine (ddI) is a nucleoside which has recently received limited FDA approval for treatment of HIV infection. It may eventually prove to have clinical problems analogous to ZDV. We were surprised that unlike ZDV, ddI did not act synergistically in combination with ampligen. We postulated that this lack of synergism may be related to the fact that both ddI and ampligen contained inosine. Inosine may be freed from the polyinosinic acid present in ampligen in the patient. Preliminary experiments showed that inosine itself did not inhibit the action of ddI in physiologic concentrations (data not presented). While we cannot explain the lack of synergism between ddI and ampligen, this work shows that synergism may vary with individual compounds within a class.

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References

Fischl, M.A., Richman, D.D., Grieco, M.H. et al. (1987) The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo controlled trial. N. Engl. J. Med. 317, 185–191.

Volberding, P.A., Lagakos, S.W., Koch, M.A. and AIDS Clinical Trials Group (1990) Zidovudine in asymptomatic human immunodeficiency virus infection: a controlled trial of persons with fewer than 500 CD4-positive cells per cubic millimeter. N. Engl. J. Med. 322, 941–949.

Richman, D.D., Fischl, M.A., Grieco, M.H. et al. (1987) The toxicity of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind placebo-controlled trial. N. Engl. J. Med. 317, 192–197.

Larder, B.A., Darby, G. and Richman, D.D. (1989) HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science 317, 1731–1734.

Carter, W.A., Pitha, P.M., Marshall, L.W., Tazawa, I., Tazawa, S. and Ts'o, P.O.P. (1972) Structural requirements of the rIn-rCn complex for induction of human interferon. J. Mol. Biol. 70, 567–587.

Ts'o, P.O.P., Alderfer, J.L., Levy, J., Marshall, L.W., O'Malley, J., Horoszewicz, J.S. and Carter W.A. (1976) An integrated and comparative study of the antiviral effects and other biological properties of polyinosinic acid-polycytidylic acid and its mismatched analogues. Mol. Pharmacol. 12, 299–312.

Field, A.K., Tytell, A.A., Lampson, G.P. and Hilleman, M.R. (1967) Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. Proc. Natl. Acad. Sci. 58, 1004–1009.

Hill, D.A., Baron, S., Levy, H.B. et al. (1971) Clinical studies of induction of interferon by polyinosinic-polycytidylic acid. In: M. Pollard (Ed.) Perspective in Virology 7. New York Academic Press.

Montefiori, D.C. and Mitchell, W.M. (1987) Antiviral activity of mismatched double-stranded RNA against human immunodeficiency virus in vitro. Proc. Natl. Acad. Sci. 84, 2985–2989.Lee, M.H., Wilson, S., Fung, L.F., Fisher, M.M. and Levy, G.A. (1983) The effect of an interferon (IF) inducer on mouse hepatitis type 3 (MHV-3) infection in mice. Hepatology 3, 837.

Nilsen, T.W., Maroney, P.A. and Baglioni, C. (1981) Double-stranded RNA causes synthesis of 2′-5′ oligo(A) and degradation of messenger RNA in interferon-treated cells. J. Biol. Chem. 256, 7806–7812.

Zarling, J.M., Schlais, J., Eskra, L., Greene, J.J., Ts'o, P.O.P. and Carter, W.A. (1980) Augmentation of human natural killer cell activity by polyinosinic acid-polycytidylic acid and its nontoxic mismatched analogues. J. Immunol. 124, 1852–1857.

Nolibe, D. and Thang, M.N. (1988) Stimulation of natural killer cytotoxicity by long treatment with double-stranded polynucleotides without induction of hyporesponsiveness. Cancer Immunol. Immunother. 27, 114–120.

Karney-Jones, R., Hinson, D., Henriques, H.F., Einck, L., Fakhry, S.M. and DePalma, R.G. (1990) Restoration of delayed-type hypersensitivity reaction by an immune modulator following thermal injury. Surgical Forum 95–96.

Mitchell, W.M., Montefiori, D.C., Robinson, W.E., Jr., Strayer, D.R. and Carter, W.A. (1987) Mismatched double-stranded RNA (ampligen) reduces concentration of zidovudine (azidothymidine) required for in vitro inhibition of human immunodeficiency virus. Lancet 890–892.

Montefiori, D.C., Robinson, W.E., Jr. and Mitchell, W.M. (1989) In vitro evaluation of mismatched double-stranded RNA (ampligen) for combination therapy in the treatment of acquired immunodeficiency syndrome. AIDS Res. Hum. Retroviruses 5, 193–204.

Popovic, M., Sarngadharan, M.D., Read, E. and Gallo, R.C. (1984) Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224, 497–500.

Gupta, P., Balchandran, R., Grovit, K., Webster, D. and Rinaldo, C.R., Jr. (1987) Detection of human immunodeficiency virus by reverse transcriptase assay, antigen capture assay, and radioimmunoassay. J. Clin. Microbiol. 6, 1122–1125.

Harada, S., Koyanagi, Y. and Yamamoto, N. (1985) Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay. Science 563.

Chesebro, B.D. and Wehrly, K. (1988) Development of a sensitive quantitative focal assay for human immunodeficiency virus infectivity. J. Virol. 62, 3779–3788.

Chou, T.C. and Talalay, P. (1984) Quantitative analysis of dose-effect relationships the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. 22, 27–55.

Yarchoan, R., Mitsuya, H., Thomas, R.V. et al. (1989) In vivo activity against HIV and favorable toxicity profile of 2′,2′-dideoxyinosine. Science 245, 412–415.

Carter, W.A., Brodsky, I., Pellegrino, M.G. et al. (1987) Clinical, immunological, and virological effects of Ampligen, a mismatched double-stranded RNA, in patients with AIDS or AIDS-related complex. Lancet (i), 1286–1292.