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Combinations of reverse transcriptase, protease, and integrase inhibitors can be synergistic in vitro against drug-sensitive and RT inhibitor-resistant molecular clones of HIV-1

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Abstract

Combinations of anti-HIV agents including one or two reverse transcriptase inhibitors with a protease inhibitor are potent and effective. However, toxicities, costs and the emergence of drug-resistant organisms have compromised their long-term efficacy in people. A next, likely, target for anti-HIV therapy is HIV-1 integrase. Viral integration, catalyzed by integrase, is absolutely required for HIV replication. L-chicoric acid is a potent and selective inhibitor of HIV-1 integrase that also inhibits HIV-1 replication in cell culture. As a first step in understanding the potential role for integrase inhibitors in clinical medicine, the activities of L-chicoric acid alone and in combination with 2′,3′-dideoxycytidine, zidovudine, and a protease inhibitor, nelfinavir, were tested in vitro against molecular clones of HIV-1 resistant to reverse transcriptase inhibitors. L-chicoric acid was equally effective against a wild-type clone of HIV-1, HIV_{NL4-3}, or against HIV-1 resistant to either zidovudine or dideoxycytidine. L-chicoric acid was largely synergistic with zidovudine and synergistic with both dideoxycytidine and nelfinavir.

Keywords: Experimental therapeutics; AIDS; Mixed dose effect analyses; Antiviral agents; Chicoric acid

1. Introduction

Over the past several years there have been remarkable breakthroughs in therapy of human immunodeficiency virus (HIV) infection. Most notable is the initiation of combination therapy for the treatment of HIV infection. Early work prior to the widespread availability of protease (PR) inhibitors demonstrated a potent anti-HIV effect if two reverse transcriptase (RT) inhibitors were used in combination (Caliendo and Hirsch, 1994; Hammer et al., 1994). Clinically useful regimens have included a combination of an inhibitor of RT and PR (Johnson et al., 1992; Kageyama et

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al., 1992). Potent combinations such as those that include two RT inhibitors and a PR inhibitor can suppress viral replication to undetectable levels in patients when measured in the peripheral blood (Collier et al., 1996; Autran et al., 1997; Gulick et al., 1997; Hammer et al., 1997). Recent evidence, however, indicates that replication competent virus exists in other sites such as lymph nodes (Hockett et al., 1999) and in resting CD4 + T-cells (Finzi et al., 1997; Wong et al., 1997; Furtado et al., 1999; Zhang et al., 1999). Indeed, after 2 years of combination therapy, low levels of viral RNA can persist (Furtado et al., 1999).

In addition to evidence of continued viral replication in the presence of potent antiretroviral therapy are more practical concerns including difficult adherence to drug combinations, expense, and appearance of viruses resistant to one or more of these agents (Gulick et al., 1997). Furthermore, many HIV-infected individuals have been on single drug therapy in the past and are infected with viruses that are already resistant to one or more inhibitors (Clumeck, 1993; Cooper et al., 1993; Caliendo and Hirsch, 1994; Swanstrom, 1994; Turriziani et al., 1994; Baldwin et al., 1995; Condra et al., 1995; Tisdale et al., 1995). These issues highlight the need for anti-HIV agents targeted at other HIV proteins.

HIV-1 integrase (IN) is a frequently touted potential target for anti-HIV therapy. IN is an attractive target for therapy because it is an enzyme that is absolutely required for stable and productive infection of cells (LaFemina et al., 1992; Sakai et al., 1993; Engelman et al., 1995). However, it has also proved an elusive target for antiviral drug development. Most small molecule inhibitors of HIV-1 integrase are non-selective and/or inactive in cell culture (reviewed in Robinson, 1998a). Recently, a novel class of IN inhibitors was described (Robinson et al., 1996a,b) that are selective (McDougall et al., 1998), potent, and inhibit HIV-1 replication in cell culture at non-toxic concentrations. This class of inhibitors, comprised of the dicaffeoylquinic acids and dicaffeoyltartaric acids, includes six novel anti-HIV compounds, all of which are potent and selective inhibitors of HIV-1 IN (Robinson et al., 1996a,b; McDougall et al., 1998). The site of action of these agents has been confirmed by the isolation of a drug-resistant variant of HIV-1 and the demonstration that a single amino acid change within IN, a glycine to serine mutation at amino acid 140, was sufficient to confer resistance to L-chicoric acid (L-CA) (King and Robinson, 1998), the 'lead' inhibitor in this class.

Any new anti-HIV agent will be required not only to inhibit HIV-1 replication at non-toxic concentrations, but also to work in the presence of viruses resistant to RT and PR inhibitors. Additionally, as combination therapies are now the standard of care in medicine, new inhibitors should work in combination with existing classes of anti-HIV drugs. To determine whether an inhibitor of IN fit these criteria, the activity of L-CA, was tested in vitro alone or in combination with zalcitabine, (2′,3′-dideoxycytidine, DDC) or zidovudine (ZDV) or nelfinavir (NLF, a PR inhibitor) against HIV-1 clones resistant to ZDV or DDC.

2. Methods

2.1. Cells and viruses

All cell lines were propagated in RPMI-1640 containing HEPES and supplemented with L-glutamine and 11.5% fetal bovine serum (Irvine Scientific, Santa Ana, CA). MT-2 cells are a CD4 + lymphoblastoid cell line that is highly susceptible to the lytic effects of HIV-1 (Harada et al., 1985; Montefiori et al., 1988). Molecular clones of HIV included wild-type HIV_{NL4-3}, $HIV_{NL4-3M184V}$ (Gu et al., 1992; Gao et al., 1993), and $HIV_{NL4-3JF26/A7}$ (Fitzgibbon et al., 1993). All three clones were a generous gift from P. Krogstad (UCLA, Los Angeles, CA). The genotype and phenotypes of each virus are summarized in Table 1. The advantage of such clones is: (1) the mutations associated with RT inhibitor-resistance are found in viruses isolated from patients treated with RT inhibitors; and (2) the only difference between each virus is the RT inhibitor resistance pattern. Viruses were initially transfected in adherent HeLa cells using Lipofectin®. After 48 h, H9 cells were added. Following 24 h of co-culture, non-adherent cells

were removed and cultured. Cells were monitored by indirect immunofluorescence and RT release, as described previously (Robinson et al., 1989), until the culture was 100% infected by HIV-1. Supernatant fluids were collected and clarified of cells by low-speed centrifugation followed by filtration through 0.45 μm filters.

Table 1
Genotype and phenotype of HIV-1 molecular clones

Clone	Mutations	Phenotype
HIV_{NL4-3}	None	Drug-sensitive
$HIV_{NL4-3M184V}$	RT: M184V	Partially resistant to DDC and DDI
$HIV_{NL4-3JF26/A7}$	RT: M41L, D67N, K70R, T215Y, K219Q	Highly resistant to ZDV
$HIV_{NL4-3clone7-1}$	IN: silent mutations	Increased susceptibility to DDC

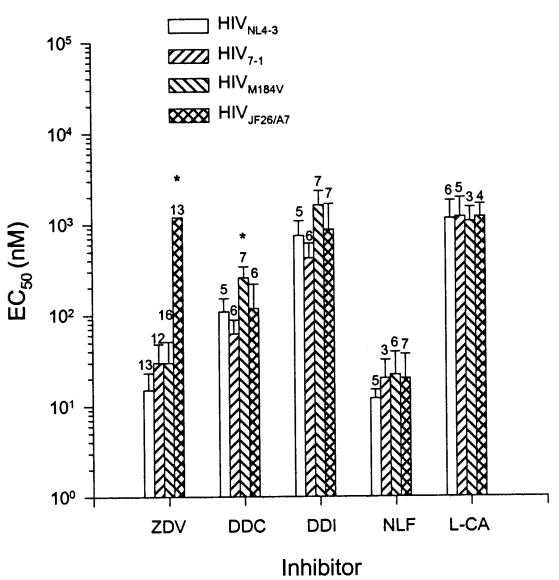

Fig. 1. Anti-HIV activity of RT, PR and IN Inhibitors against HIV-1 molecular clones. The mean EC_{50} was determined for each compound using a cytopathic effect assay as described by Montefiori et al. (1988). The number of experiments performed in triplicate to determine each mean EC_{50} is illustrated above each bar. The error bars are one standard deviation. * indicates $P < 0.001$ by Student's paired t-test. ZDV, zidovudine; DDC, dideoxycytidine; DDI, dideoxyinosine; NLF, nelfinavir; L-CA, L-chicoric acid.

2.2. Compounds

ZDV, DDC, and 2′,3′-dideoxyinosine (DDI) were purchased from Sigma Chemical Co (St. Louis, MO). All three were reconstituted to 1 mM stock solutions in deionized water and stored at −20°C until use. Michael Melnick (Agouron Pharmaceuticals, San Diego, CA) provided NLF. Manfred Reinecke (Texas Christian University, Fort Worth, TX) provided L-CA. Both were reconstituted in deionized water and stored at −70°C until use. All stocks were diluted in growth medium and filter-clarified before being tested for cell toxicity and anti-HIV activity.

2.3. Anti-HIV assays

Anti-HIV activity of compounds was determined both alone and in combination using a cytopathicity based assay as described previously (Montefiori et al., 1988, 1989). This assay utilizes Finter's neutral red dye; protection from HIV-induced cell death is highly correlated with HIV-1 antigen synthesis, RT release, and the formation of infectious progeny virions (Robinson et al., 1989). All drugs were tested at concentrations well below (100–1000-fold) their toxic doses either alone or in combination (data not shown). The 50% effective concentration (EC_{50}) was calculated for triplicate infections. Mean EC_{50} values (shown in Fig. 1) were calculated for each drug against each HIV variant from a minimum of three experiments performed in triplicate.

2.4. Mixed dose effect analyses

Mixed dose effect analyses were performed using the method of Chou and Talalay (Chou, 1974, 1991; Chou and Talalay, 1981, 1983, 1984) and commercially available software: CalcuSyn for Windows (Biosoft, Ferguson, MO, USA). Experiments were designed at a fixed ratio of drugs; the ratio for each drug combination was determined based on the EC_{50} of each drug alone against each HIV variant. Combination indices were calculated on representative experiments performed in triplicate.

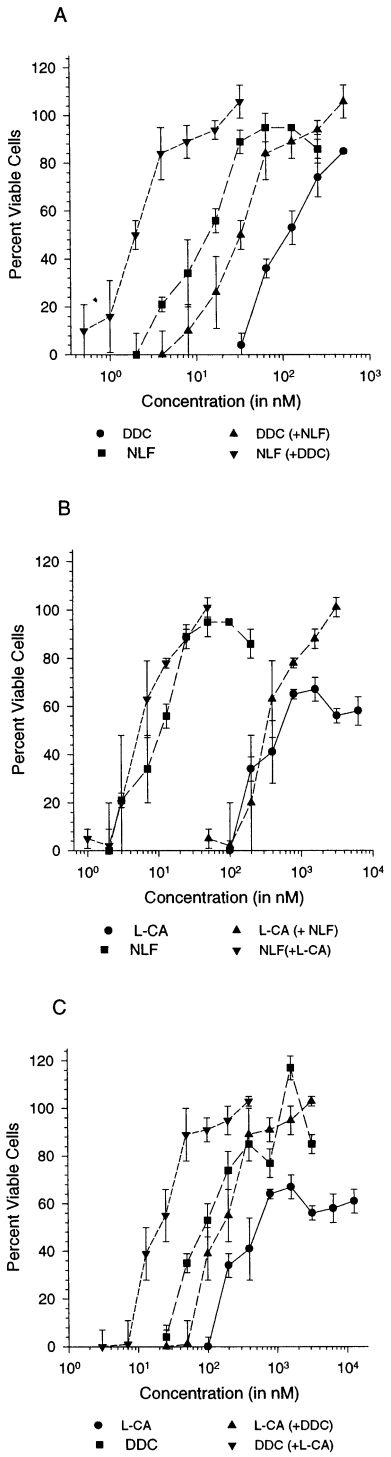

Fig. 2.

3. Results

3.1. Susceptibility of clones to single drug treatment

Although the susceptibilities of several of the clones to each drug have been published previously by other laboratories, their susceptibilities to each drug under our assay conditions and to L-CA were determined as the baseline for this study. These results are illustrated in Fig. 1. The clone containing the methionine to valine change at amino acid 184 of RT ($HIV_{NL4\text{-}3M184V}$) shows slight resistance to both DDI and DDC as previously described (Gu et al., 1992; Gao et al., 1993). The other drug-resistant virus shows a complete resistance pattern: $HIV_{NL4\text{-}3JF26/A7}$ is completely resistant to ZDV as previously described (Fitzgibbon et al., 1993). All viruses showed similar susceptibilities to NLF and L-CA.

3.2. Combinations of agents against drug-resistant virus

To determine whether addition of an IN inhibitor to either RT or PR inhibitors would be synergistic, additive, or antagonistic, combinations of these antiviral agents were tested against wild type and drug-resistant variants of HIV. A representative experiment is shown in Fig. 2. The combination of DDC and NLF or DDC and L-CA was potently synergistic against $HIV_{NL4\text{-}3JF26/A7}$, as the dose-response curves for the combinations are shifted to the left compared to either drug alone (Fig. 2A,C, respectively). NLF with L-CA (Fig. 2B), on the other hand, is closer to additive as each curve in combination can be nearly superimposed on the corresponding curve for each drug alone. At higher drug concentrations, however, more potent synergy is ob-

Fig. 2. Representative dose response curves for anti-HIV agents alone and in combination against $HIV_{NL4\text{-}3JF26/A7}$. The anti-HIV effects of (A) DDC and NLF, (B) NLF and L-CA, or (C) DDC and L-CA alone and in combination are illustrated. Symbols are defined for each figure. Error bars are 1 SD for triplicate samples.

served as the two curves diverge. The EC_{50} of each drug in combination is summarized in Table 2.

To determine whether the observed increase in anti-HIV activity of the combinations was synergy or additivity, the mixed-dose effect plot of Chou and Talalay was used. The combination indices for each drug combination against each virus are shown in Table 3. As illustrated in Table 3A, DDC and L-CA were synergistic against all viruses. For the combination of DDC and NLF, it was synergistic against both of the RT inhibitor resistant viruses and the wild-type clone (Table 3B). The combination of NLF and L-CA was synergistic against all three isolates tested (Table 3C). However, for most of the clones tested this synergy was only strong at high concentrations of both drugs at which 60–90% protection was attained. At lower effective doses the effects were closer to additive (Table 3C). The combination of NLF and ZDV was synergistic against all of the clones tested except $HIV_{NL4\text{-}3cloneJF26/A7}$ (Table 3D). This lack of synergy was due to the complete resistance of clone $HIV_{NL4\text{-}3cloneJF26/A7}$ to ZDV (Fig. 1). Finally, the combination of ZDV and L-CA was synergistic against all of the clones except the wild-type $HIV_{NL4\text{-}3}$ (Table 3E).

Since current anti-HIV agents can suppress viral replication by several logs, the 99% effective concentrations for all of the combinations against all of the clones were predicted using CalcuSyn software. These results are shown in Table 4. Clearly, most of the combinations were synergistic at the EC_{99}. The most potent combinations were those utilizing ZDV, DDC, and NLF. L-CA was synergistic with the other drugs against RT inhibitor resistant virus. The one exception is the combination of ZDV and L-CA, which was not synergistic at the EC_{99} against wild type $HIV_{NL4\text{-}3}$. The reason for this discrepancy when compared to other effective concentrations (Table 3E) is not known.

The results for these studies were highly reproducible. All of the lines in the median dose effect plots were linear with r values greater than 0.93. Most had r values greater than 0.96. Values of greater than 0.9 are recommended for cell culture experiments (CalcuSyn manual). Additionally, the estimated standard deviations shown for the combination indices were appropriately small; the values in Table 3 are 1.96 times the standard deviation (95% CI). Finally, the EC_{50} determinations for each agent alone and in combination as well as the EC_{99} of the combinations were performed on triplicate samples in at least triplicate experiments. Thus, a minimum of nine replicates was used for each of the drugs alone and in combination.

4. Discussion

Recent work has made it clear that any future anti-HIV agent will need to function in the presence of existing anti-HIV agents. Furthermore, the likelihood that any new anti-HIV agent will have to function in the presence of resistance to

Table 2
EC_{50} (nM) of drugs in combination versus drug-sensitive and drug-resistant HIV-1

HIV clone	Drug[a]									
	ZDV {NLF}	ZDV {L-CA}	DDC {PI}	DDC {L-CA}	L-CA {ZDV}	L-CA {DDC}	L-CA {NLF}	PI {ZDV}	PI {DDC}	PI {L-CA}
NL4-3	8[b] (0)	8 (0)	27 (10)	54 (18)	400 (0)	333 (115)	500 (200)	7 (0)	3 (1)	9 (4)
7-1	8 (0)	11 (5)	25 (12)	33 (0)	267 (115)	400 (0)	400 (0)	3 (0)	5 (2.5)	7 (0)
M184V	8 (0)	7 (2)	65 (0)	65 (0)	167 (58)	200 (0)	333 (115)	3 (0)	3 (0)	6 (2)
JF26/A7	521 (180)	371 (39)	44 (18)	54 (18)	500 (200)	333 (115)	333 (115)	23 (8)	5 (2)	6 (2)

[a] All of the EC_{50} were at or below mean maximum achievable serum concentrations (C_{max}) except for L-CA. The C_{max} for L-CA is unknown. C_{max} for ZDV = 6.75 μM; for DDC, 119 nM, and for NLF, 7 μM.

[b] Values are mean EC_{50} from a minimum of three experiments performed in triplicate. They are the concentrations of the indicated drug in the presence of a second inhibitor (indicated in brackets). Values in parentheses are the standard deviations. Thus, 8 nM is the EC_{50} of ZDV in the presence of NLF.

Table 3
Combination indices against drug-sensitive and drug-resistant HIV

Virus (HIV-1)	F_a[a] (protection)	CI[b]	DDC (nM)	L-CA (nM)
(A) DDC plus L-CA				
NL4-3$_{clone7-1}$	0.2	1.144 (0.35)	12.4	148.3
	0.4	0.726 (0.18)	21.1	253.1
	0.6	0.578 (0.14)	32.8	393.7
	0.8	0.493 (0.13)	56.0	672.0
	0.9	0.456 (0.14)	87.1	1046
NL4-3$_{cloneM184V}$	0.2	0.576 (0.27)	21.6	64.8
	0.4	0.358 (0.08)	43.2	129.7
	0.6	0.283 (0.05)	76.8	230.3
	0.8	0.249 (0.05)	153.7	461.2
	0.9	0.242 (0.06)	273	819
NL4-3$_{cloneJF26/A7}$	0.2	2.367 (1.6)	13.0	78.1
	0.4	0.395 (0.1)	23.8	142.8
	0.6	0.214 (0.06)	39.2	235.3
	0.8	0.177 (0.05)	71.8	430.5
	0.9	0.165 (0.09)	118	709
			DDC (nM)	NLF (nM)
(B) DDC plus NLF				
NL4-3$_{clone7-1}$	0.2	0.835 (0.22)	15.2	3
	0.4	0.83 (0.18)	26.4	5.3
	0.6	0.826 (0.17)	41.7	8.3
	0.8	0.823 (0.18)	72.4	14.5
	0.9	0.820 (0.21)	114.2	22.8
NL4-3$_{cloneM184V}$	0.2	0.476 (0.12)	16.6	0.9
	0.4	0.385 (0.07)	34.7	1.8
	0.6	0.336 (0.05)	63.8	3.4
	0.8	0.3 (0.05)	133.0	7.0
	0.9	0.285 (0.06)	244	12.8
NL4-3$_{cloneJF26/A7}$	0.2	0.368 (0.1)	12.6	1.1
	0.4	0.370 (0.08)	23.6	2.1
	0.6	0.371 (0.07)	39.6	3.6
	0.8	0.374 (0.07)	74.0	6.7
	0.9	0.377 (0.08)	124	11
			NLF (nM)	L-CA (nM)
(C) NLF plus L-CA				
NL4-3$_{clone7-1}$	0.2	0.906 (0.29)	2.9	163.6
	0.4	0.472 (0.12)	4.9	279.6
	0.6	0.329 (0.08)	7.6	435.4
	0.8	0.26 (0.08)	13.1	743.9
	0.9	0.239 (0.09)	20.3	1158
NL4-3$_{cloneM184V}$	0.2	1.81 (0.65)	2.5	144
	0.4	0.91 (0.21)	4.7	269.2
	0.6	0.536 (0.12)	7.9	451.5
	0.8	0.293 (0.08)	14.8	844.1
	0.9	0.183 (0.07)	24.8	1416
NL4-3$_{cloneJF26/A7}$	0.2	7.982 (5.3)	4.6	264.5
	0.4	1.138 (0.25)	7.0	401.8
	0.6	0.577 (0.13)	10.0	567.8
	0.8	0.444 (0.11)	15.1	862.5
	0.9	0.388 (0.11)	21.3	1219

Table 3 (Continued)

Virus (HIV-1)	F_a[a] (protection)	CI[b]	DDC (nM)	L-CA (nM)
(D) NLF plus ZDV			NLF (nM)	ZDV (nM)
NL4-3$_{clone7-1}$	0.2	0.304 (0.13)	0.6	1.2
	0.4	0.295 (0.09)	1.3	2.6
	0.6	0.291 (0.07)	2.5	4.9
	0.8	0.294 (0.06)	5.4	10.8
	0.9	0.302 (0.07)	10.4	20.8
NL4-3$_{cloneM184V}$	0.2	0.769 (0.27)	1.3	3.1
	0.4	0.531 (0.14)	2.6	6.1
	0.6	0.394 (0.09)	4.7	10.7
	0.8	0.275 (0.06)	9.3	21.3
	0.9	0.206 (0.05)	16.3	37.5
NL4-3$_{cloneJF26/A7}$	0.2	1.482 (0.36)	9.3	213.4
	0.4	1.286 (0.26)	15.2	349.5
	0.6	1.185 (0.23)	22.8	525.5
	0.8	1.089 (0.23)	37.4	861
	0.9	1.021 (0.25)	56.3	1294
			ZDV (nM)	L-CA (nM)
(E) ZDV plus L-CA				
NL4-3$_{clone7-1}$	0.2	0.898 (0.37)	2.7	67.2
	0.4	1.023 (0.34)	8.7	217
	0.6	1.197 (0.34)	22.9	571.8
	0.8	1.508 (0.44)	73.8	1845
	0.9	1.865 (0.67)	194	4861
NL4-3$_{cloneM184V}$	0.2	0.803 (0.39)	4.9	63.2
	0.4	0.508 (0.17)	10.2	132.7
	0.6	0.373 (0.1)	18.9	245.2
	0.8	0.272 (0.07)	39.6	514.8
	0.9	0.217 (0.07)	73	951
NL4-3$_{cloneJF26/A7}$	0.2	12.6 (8.9)	150.2	450.6
	0.4	1.119 (0.36)	259.8	779.4
	0.6	0.157 (0.08)	409	1226
	0.8	0.017 (0.04)	707	2120
	0.9	0.003 (0.02)	1112	3336

[a] F_a is the fraction affected (i.e. $F_a \times 100$ is the percent protection). Values for 20, 40, 60, 80 and 90% are indicated.

[b] Is the combination index. It was calculated using CalcuSyn for Windows and is one representative experiment from a minimum of three. Each experimental value was determined in triplicate. Values in parenthesis are $1.96 \times$ SD as estimated in the computer program.

Table 4
Predicted EC_{99} for synergistic[a] drug combinations (nM)

Clone	L-CA+ (DCC)	L-CA+ (ZDV[b])	L-CA+ (NLF[b])	DDC+ (NLF[b])	ZDV+ (NLF[b])
HIV$_{NL4-3clone\ 7-1}$	3863+ (322)	NA	4292+ (75)	440+ (88)	143+ (71)
HIV$_{NL4-3M184V}$	4473+ (1491)	5834+ (449)	6536+ (115)	1472+ (77)	201+ (87)
HIV$_{NL4-3JF26/A7}$	3105+ (518)	12 738+ (4246)	3387+ (59)	574+ (52)	NA

[a] Synergism was defined as a CI of <1.

[b] Concentrations of NLF and ZDV above the EC_{99} can be readily achieved in subjects (see footnote to Table 2). NA, not applicable (i.e. no synergy at the EC_{99}).

existing anti-HIV agents such as RT inhibitors and PR inhibitors is high. Therefore, to determine whether inhibitors of HIV IN have a place in combination treatment of HIV, the anti-HIV effects of L-CA, an inhibitor of IN, were evaluated in combination with NLF, ZDV, or DDC.

L-CA is a promising lead compound as an IN inhibitor for several reasons. First, it is a potent and selective inhibitor of HIV-1 IN (Robinson et al., 1996a,b; McDougall et al., 1998; Zhu et al., 1999). Second, it has proven amenable to derivatization, resulting in the synthesis of a large number of analogues, some with increased potency in cell culture compared to other IN inhibitors (King et al., 1999; Lin et al., 1999). Finally, it has been shown to be a non-competitive or irreversible inhibitor of IN that specifically reacts with the IN protein (Zhu et al., 1999). This is an important finding since bis-catechols have been hypothesized to inhibit IN by chelation of absolutely required divalent cations. However, biochemical results from several laboratories indicate that L-CA is not acting as a non-specific metal chelator (McDougall et al., 1998; Zhu et al., 1999).

It is clear from our preliminary results (Fig. 1) that L-CA is as effective an anti-HIV agent as DDI. L-CA is also equally active against both wild-type HIV and variants resistant to either ZDV or DDC. Therefore, L-CA should work in the presence of variants of HIV resistant to existing antiviral agents.

In combination experiments, DDC was synergistic with both NLF and L-CA against all HIV isolates tested (Table 3A,B). ZDV was less synergistic with L-CA except against ZDV-resistant virus (Table 3E). ZDV also showed less synergism in combination with NLF. Indeed, it was only additive against the ZDV-resistant clone $HIV_{NL4\text{-}3JF26/A7}$. These results were consistent with those published previously by Zhu et al. (1996). The strong synergism of ZDV and NLF for wild type HIV is consistent with that described previously (Richman et al., 1991). NLF, as alluded to previously, was synergistic with all three drugs, ZDV, DDC, and L-CA (Table 3B–D). These data were expected from previous studies of NLF with ZDV (Patick et al., 1997). Surprisingly, L-CA was nearly additive with ZDV (Table 3E), although potently synergistic with both NLF and DDC (Table 3A,C). We had demonstrated previously that L-CA was at least additive with a PR inhibitor and ZDV (Robinson, 1998b). Furthermore, this effect was seen with both uncloned tissue-culture adapted HIV and clinical isolates of HIV (Robinson, 1998b). The studies reported herein utilize mathematical analyses to define the nature of the interactions between several PR, RT, and IN inhibitors (in large part synergistic) and their activities against two RT inhibitor resistant viruses.

The most potent combinations were those including the three drugs approved by the United States Food and Drug Administration for use in HIV-infected individuals. In combination, L-CA could, at higher concentrations, substitute for existing anti-HIV agents (Table 4). For example, the concentration of NLF needed to achieve an EC_{99} was the same in the presence of L-CA as in the presence of either ZDV or DDC. Likewise, the concentration of DDC necessary to achieve an EC_{99} in combination was the same whether in the presence of NLF or L-CA. Only ZDV worked better in combination with NLF than L-CA. However, when compared to the EC_{50} value of each drug in combination (Table 2), it was clear that L-CA could substitute effectively for any of the other three drugs, albeit at higher concentrations. For those drugs in clinical usage, the concentrations necessary in combination with L-CA at the EC_{50} are within the known mean maximum serum concentration (C_{max}) (Table 2). In addition, the EC_{99} for both NLF and ZDV in combination with L-CA are well within the known C_{max} (Table 4). Therefore, it would seem that L-CA should prove useful in combination therapy against HIV.

Synergy analysis has been applied to a number of anti-HIV agents in vitro. The clear synergistic effects of RT inhibitors in combination with PR inhibitors in vivo as well as in vitro suggest it is of value in determining clinically-useful drug combinations. The data reported herein support the hypothesis that IN inhibitors such as L-CA may

have a place in the future therapy of HIV infection. Furthermore, the presence of drug-resistant mutations is not likely to hamper such combination treatments. Therefore, development of potent inhibitors of HIV IN should be a high priority. Future studies on triple combinations of anti-HIV agents including DDC, NLF, and L-CA against wild-type viruses as well as viruses resistant to RT, PR or IN inhibitors, will further delineate the potential role of IN inhibitors in clinical therapy of HIV infection.

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