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L-Chicoric acid, an inhibitor of human immunodeficiency virus type 1 (HIV-1) integrase, improves on the in vitro anti-HIV-1 effect of Zidovudine plus a protease inhibitor (AG1350)

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Abstract

Combinations of anti-human immunodeficiency virus (HIV) drugs, including reverse transcriptase inhibitors and protease inhibitors, have proven immensely potent in the therapy of acquired immune deficiency syndrome (AIDS). To determine whether HIV integrase is a suitable target for combination therapy, the ability of an HIV integrase inhibitor, L-chicoric acid, to work in combination with a protease inhibitor and Zidovudine was tested in vitro. The addition of L-chicoric acid to either Zidovudine or protease inhibitor improved upon the observed anti-HIV activity of either compound alone. When all three drugs were combined, the anti-HIV activity was substantially better than either of the three compounds alone or any combination of two inhibitors. Doses of both Zidovudine and protease inhibitor could be reduced by more than 33% for an equivalent anti-HIV effect if L-chicoric acid was added. The improved anti-HIV activity was observed with a tissue culture adapted strain of HIV (HIV_{LAI}) and with limited passage clinical isolates of HIV (HIV_{R19} and HIV_{R45}). These data demonstrate that a first generation HIV integrase inhibitor, L-chicoric acid, is at least additive in combination with existing multi-drug regimens and suggest that HIV integrase will be an excellent target for combination therapy of HIV infection.

Keywords: AIDS; Antiviral agents; Experimental therapeutics; Reverse transcriptase

Abbreviations: AIDS, acquired immune deficiency syndrome; L-CCA, L-chicoric acid; DCQA, dicaffeoylquinic acid; DCTA, dicaffeoyltartaric acid; ED_{50}, 50% effective dose; FBS, fetal bovine serum; HIV-1, human immunodeficiency virus type 1; HIV_{LAI}, the LAI isolate of HIV-1; HIV_{R19}, the R19 isolate of HIV-1; HIV_{R45}, the R45 isolate of HIV-1; IC_{50}, 50% inhibitory concentration; IN, integrase; LD_5, 5% lethal dose; MOI, multiplicity of infection; PI, protease inhibitor; RT, reverse transcriptase; ZDV, Zidovudine.

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1. Introduction

Over the past few years, development and Food and Drug Administration (FDA) approval of new classes of anti-retroviral agents has moved at an astounding pace. There are now five nucleoside analogue reverse transcriptase (RT) inhibitors and two non-nucleoside RT inhibitors that have been approved for use in human immunodeficiency virus (HIV) infection as well as four FDA-approved agents that act at HIV protease. The protease inhibitors (PI), unlike the RT inhibitors, are products of rational drug design based upon the structural biology of HIV protease. RT inhibitors, when used in monotherapy, are relatively weak anti-HIV agents whose efficacy is further impaired by toxicity and the emergence of variants of HIV that are resistant to their effects (Larder et al., 1989, Hirsch, 1990, Volberding et al., 1990, Rooke et al., 1991, Aboulker and Swart, 1993, Clumeck, 1993, Cooper et al., 1993, Caliendo and Hirsch, 1994). PIs alone and in combination with RT inhibitors (Brinkworth et al., 1991, Craig et al., 1991, Brinkworth and Fairlie, 1992, Johnson et al., 1992, Kageyama et al., 1992, Alteri et al., 1993, Pollard, 1994) are potent inhibitors of HIV replication. As with the RT inhibitors, however, resistance to PIs can occur (Otto et al., 1993, Culberson et al., 1994, Ho et al., 1994, Swanstrom, 1994, Turriziani et al., 1994, Baldwin et al., 1995, Condra et al., 1995, Gulnik et al., 1995, Maschera et al., 1995, Partaledis et al., 1995, Tisdale et al., 1995).

Because of this resistance to both RT inhibitors and PIs, there is substantial interest in the development of inhibitors targeted at other viral enzymes. One such enzyme is HIV integrase (IN). Recently, we described a group of inhibitors that inhibit HIV IN in biochemical assays and HIV replication at non-toxic concentrations in tissue culture (Robinson et al., 1996a,b). This group of inhibitors, the dicaffeoylquinic acids (DCQAs) and the dicaffeoyltartaric acids (DCTAs), are active in tissue culture in the micromolar range with the most potent compound inhibiting HIV replication by 50% (50% effective dose, ED_{50}) at approximately 3 μM, yet is non-toxic to cells (Robinson et al., 1996a). L-Chicoric acid (L-CCA), a DCTA, is one of the most potent inhibitors of HIV IN yet reported, inhibiting HIV IN activity in biochemical assays by 50% (50% inhibitory concentration, IC_{50}) at a concentration approaching 100 nM. Furthermore, L-CCA demonstrates remarkable selectivity for HIV IN (McDougall et al., 1998).

Due to recent breakthroughs in combination therapy for HIV infection (Larder et al., 1995, Collier et al., 1996, Kline et al., 1996, Autran et al., 1997, Gulick et al., 1997, Hammer et al., 1997), it seems unlikely that any new anti-HIV agent will be introduced into patients unless it has been shown to work in combination with existing anti-HIV therapeutics. To assess the potential of IN inhibitors in combination therapy against HIV, L-CCA was tested for its activity against several HIV isolates alone or in the presence of Zidovudine (ZDV) with or without a PI. The results of these in vitro combination regimens indicate that IN inhibitors can improve upon the observed anti-HIV effect of currently available combination therapy regimens.

2. Materials and methods

2.1. Anti-HIV compounds

ZDV was purchased from Sigma (St. Louis, MO) and was reconstituted with cold deionized water to a final concentration of 1 mM. PI (AG1350) (Munroe et al., 1995) was donated by Michael Melnick (Agouron Pharmaceuticals, La Jolla, CA) and reconstituted in 14% ethanol–37.5% dimethylsulfoxide–48.5% water to a final concentration of 7 mM. AG1350 is slightly (less than 10-fold) less active than the recently FDA-approved PI, Viracept®. L-CCA was synthesized and purified by high-performance liquid chromatography as described (Robinson et al., 1996a,b). The L-CCA was dissolved in water to a final concentration of 2.1 mM.

2.2. Cells and virus

All cell lines were cultivated in RPMI-1640 containing 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mediatech, Herndon, VA] and supplemented with L-glutamine and

11.5% fetal bovine serum (FBS; Irvine Scientific, Irvine, CA) (growth medium). MT-2 cells are a T-lymphoblastoid cell line that is highly susceptible to HIV infection and is lysed completely by HIV (Montefiori et al., 1988). HIV_{LAI} was obtained from the NIH AIDS Reagent Repository and was propagated in H9 cells. The two clinical isolates were obtained from patients visiting the UCI Medical Center AIDS Clinic under an IRB-approved protocol and have been described elsewhere (McDougall et al., 1997). HIV_{R19} and HIV_{R45} were obtained approximately 3 weeks after inoculation with 100 μl of freshly drawn serum onto MT-2 cells (the time required for the MT-2 cells to become infected). Previous work has indicated that such isolates of HIV are predominantly syncytium-inducing, rapid-growing, T-cell-tropic isolates of HIV (Connor et al., 1993). Cell-free supernatants of HIV_{R19} and HIV_{R45} from MT-2 cells were inoculated onto phytohemagglutinin-stimulated peripheral blood mononuclear cells, cultured in growth medium supplemented with 11.5% FBS and 20 units of recombinant human interleukin 2 per milliliter (Boehringer-Mannheim, Indianapolis, IN), and HIV was isolated at peak RT production (7 and 10 days). Both isolates were highly cytopathic and grew to similar titers with similar replication kinetics in tissue culture. The resulting limited passage, cell-free supernatant was directly inoculated onto MT-2 cells for use in antiviral assays.

2.3. Cell toxicity and anti-HIV assays

Cell toxicity and anti-HIV assays were performed as reported previously (Montefiori et al., 1988). Briefly, compounds were diluted 1:1 in growth medium, filter sterilized, and further 2-fold serially diluted from 1:8 to 1:1280 in triplicate wells of a microtiter plate. To each 50 μl of diluted drug, 50 μl of growth medium was added followed by 100 μl of MT-2 cell suspension (2×10^5 cells). Cells were incubated with drug for 48 h at 37°C, then harvested for cell viability in a neutral red dye assay as described previously (Montefiori et al., 1988). Similar toxicities were also seen if the cells were incubated for 72 h prior to harvest (data not shown).

Anti-HIV assays were performed as described (Montefiori et al., 1988). Based upon cell toxicity data, compounds were diluted in growth medium such that a final 1:4 dilution of the sample would result in a concentration of sample that inhibited MT-2 cell growth by 5% (5% lethal dose, LD_5). The LD_5 falls within 1 S.D. of controls treated only with solvent and is, therefore, defined as a non-toxic concentration. The compounds were then 2-fold serially diluted in triplicate. To each 50 μl of diluted compound, 50 μl of HIV_{LAI} was added and the virus–drug mixture was incubated for 1 h at 37°C. Next, 100 μl of MT-2 cell suspension (2×10^5 cells) was added to each well and cells were incubated for 72 h at 37°C. Final multiplicity of infection (MOI) was 1–5. In some assays, virus was diluted 1:25 prior to antiviral assay; this dilution led to a final MOI < 1. Cells were harvested to quantitate cytopathic effect using a neutral red dye assay as described (Montefiori et al., 1988). The antiviral concentration reported is the concentration of drug necessary to protect MT-2 cells from 50% viral-induced cell death; this is referred to as the 50% effective dose (ED_{50}).

2.4. RT assay and immunofluorescence analysis

Each culture supernatant was precipitated with 0.42 ml of 30% polyethylene glycol as described previously (Robinson et al., 1989). Precipitated virus was lysed and incorporation of [^{3}H]thymidine into poly(rA)·oligo(dT) templates was measured according to a modification (Robinson et al., 1989) of the method first described by Poiesz et al. (1980). Trichloroacetic acid precipitable raw counts per minute (cpm) were determined on a Beckman beta-scintillation counter. The mean cpm for the triplicate infections was determined and mean background cpm from three cell control cultures run in parallel to each assay were subtracted. The resultant corrected cpm were multiplied by 8 to convert to cpm/ml of culture supernatant fluid. For immunofluorescence analysis, cells from triplicate wells were combined and spotted onto glass slides. The percentage of cells expressing HIV antigens was quantitated using pooled human anti-HIV serum followed by fluorescein-conjugated goat anti-human IgG and observation under fluores-

cence microscopy as described previously (Robinson et al., 1989).

3. Results

3.1. Cell toxicity and anti-HIV effects of compounds

The anti-HIV effects of L-CCA have been described previously (Robinson et al., 1996a,b). The anti-HIV effects of ZDV have been well documented. The anti-HIV effects of AG1350 have been reported previously (Munroe et al., 1995). Using MT-2 cells as target cells, the cell toxicity and anti-HIV effects of these two compounds and the PI are illustrated in Fig. 1. The MOI in the anti-HIV assay was greater than 1 (except Fig. 1B) and the results shown are protection against the cytopathic effect of HIV given as percent viable cells (Montefiori et al., 1988). As shown in Fig. 1A, PI had no anti-HIV effect at an MOI of greater than 1 as no progeny virions, and thus no subsequent rounds of replication, were required for 100% cytopathic effect. When a lower MOI was employed (<1) the ED_{50} was 1 μM (Fig. 1B). This ED_{50} is significantly higher than the 5.1 nM reported for this compound previously by Munroe et al. (1995). This difference may be related to the assay used, the use of uncloned HIV_{LAI} rather than the HXB2 molecular clone reported by Munroe et al., or a difference in MOI. There was no difference in anti-HIV effect of either L-CCA or ZDV at high versus low MOI. The anti-HIV activity of these two compounds were approximately 2.1 μM and 1 μM, respectively, against HIV_{LAI} at an MOI >1 (Fig. 1C,D) or at an MOI <1 (data not shown).

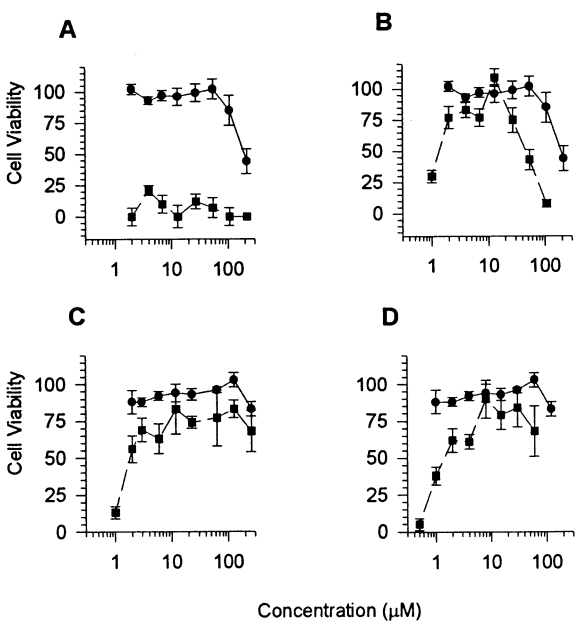

Fig. 1. Anti-HIV and cell toxicity of PI, ZDV, and L-CCA. Cell toxicity (●) and anti-HIV (■) assays were performed as described in Section 2. (A) PI, anti-HIV activity at MOI >1. (B) PI, anti-HIV activity at MOI <1. (C) L-CCA. (D) ZDV. Each point is the mean of triplicate samples; error bars are standard deviations.

3.2. Combination treatments against HIV_{LAI} in vitro

Once individual ED_{50}s against HIV_{LAI} were determined, MT-2 cells were inoculated with HIV_{LAI} in the presence of each drug alone or in combination. For these experiments, HIV infection was monitored by immunofluorescence for antigen synthesis and RT release into the culture supernatant. The results of these infections are illustrated in Fig. 2. A triple combination of L-CCA, ZDV and PI was as effective as double combinations (Fig. 2). However, the dose of each drug was 33% lower in the triple combination regimen than in double combinations and 67% lower than in single drug treatment.

To determine how effective triple combination treatment was compared to single and double drug combinations, MT-2 cells were inoculated with equal concentrations of drugs in single, double and triple combinations. The results of one such experiment are illustrated in Fig. 3. As illustrated, in a single drug treatment, L-CCA was less effective than either ZDV or PI. In dual combinations, L-CCA with PI was the least effective anti-HIV combination but was more potent than any of the solo treatments. Combination of all three anti-viral agents was more potent than any double combination.

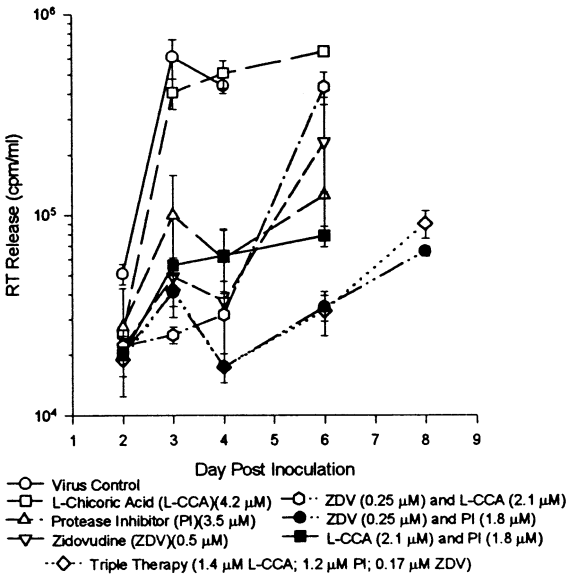

Fig. 2. Addition of L-CCA to combination regimens lowers the dose of PI and ZDV necessary to inhibit HIV_{LAI} infection in vitro. HIV_{LAI} was preincubated with inhibitors, either alone or in combination, then inoculated onto MT-2 cells. Each point is the mean of triplicate infections; error bars indicate standard deviations. Results were confirmed by immunofluorescence assay and were consistent with RT release (data not shown).

3.3. Combination treatment of limited passage clinical isolates of HIV in vitro

One potential concern was that such an effect of L-CCA with ZDV and PI was limited to tissue culture adapted strains of HIV. To test this, MT-2 cells were inoculated with two limited passage isolates of HIV, HIV_{R19} and HIV_{R45}. Both isolates were isolated on MT-2 cells, back-passaged one time on peripheral blood mononuclear cells and then used in these assays. As shown, the triple combination was more potent than the double combination of ZDV and PI against both HIV_{R45} (Fig. 4A) and HIV_{R19} (Fig. 4B). To confirm that this was true not only for triple combination but also for double combinations, MT-2 cells were inoculated with HIV_{R19} and all combinations of drugs. As shown in Fig. 5, results observed with HIV_{R19} were comparable to those seen with HIV_{LAI} (Fig. 3): the triple combination of RT inhibitor, PI and IN inhibitor was more potent than any combination of two drugs.

4. Discussion

Productively infected cells package HIV that is immature and non-infectious; subsequently, the immature gag/pol precursor proteins are proteolytically processed by HIV protease. The mature virion then binds to its receptors and is internalized into the host cell. After entry of the retrovirus into susceptible host cells, the viral enzyme, RT, synthesizes a double-stranded DNA copy of the RNA genome. After reverse transcription, the resulting viral DNA exists as part of a large, stable nucleoprotein complex (Bowerman et al., 1989, Bukrinsky et al., 1993, Heinzinger et al., 1994), which is critical for maintenance of a stable association between viral DNA and the integration machinery, as well as for transport of viral DNA into the nucleus prior to integration (Roe et al., 1993). After nuclear entry, the viral DNA is

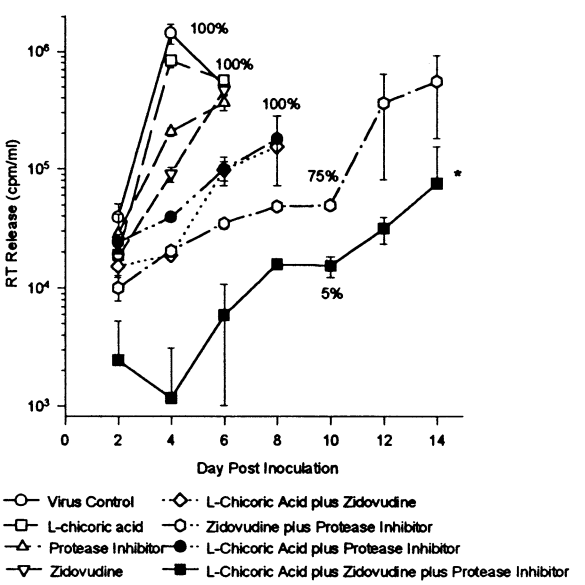

Fig. 3. Triple combination of PI, IN and RT inhibitors is more potent than double combinations of drug against HIV_{LAI} in vitro. L-CCA, PI and ZDV were preincubated with HIV_{LAI} for 1 h prior to addition of the MT-2 target cells. Final inhibitor concentrations tested were: L-CCA, 1.6 μM; PI, 1.3 μM; ZDV, 150 nM. Results are mean RT release from triplicate infections. Error bars are 1 S.D. Percentiles are percentage of cells expressing HIV antigens by immunofluorescence. Asterisk (*) indicates that two of three triplicate infections were positive by immunofluorescence whereas the third was negative.

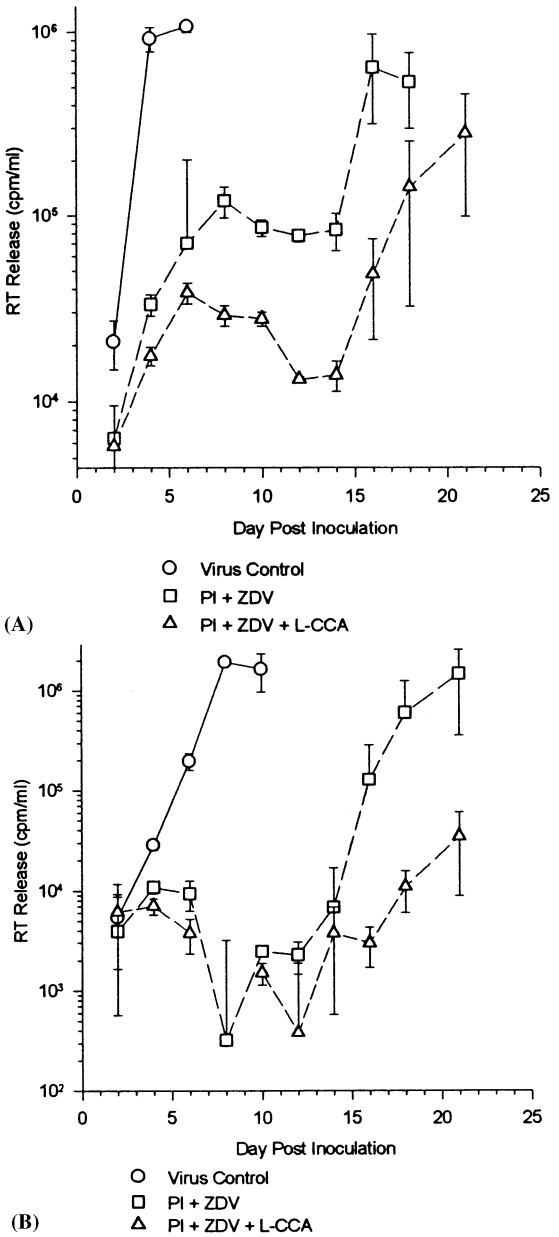

Fig. 4. Triple combination treatment yields improved results for clinical isolates of HIV. HIV_{R45} (A) and HIV_{R19} (B) were pretreated with either PI and ZDV or with PI, ZDV and L-CCA. Final inhibitor concentrations tested were: L-CCA, 1.6 μM; PI, 1.3 μM; ZDV, 150 nM. Results are mean RT release from triplicate infections. Error bars are 1 S.D.. RT data were supported by immunofluorescence assay (data not shown).

covalently joined to the host DNA, forming the provirus. Retroviruses depend upon integration for efficient replication and for maintenance of a stably infected state. Indeed, several reports have demonstrated that mutants of HIV-1 incapable of integration yield viral transcription and synthesis of some viral proteins but do not lead to production of progeny virions (Stevenson et al., 1990, Kulkosky et al., 1992, LaFemina et al., 1992, Cannon et al., 1994, Taddeo et al., 1994). Once integration has occurred, viral RNA and proteins are synthesized. After viral assembly, the HIV specific protease cleaves pol and gag precursor proteins into their mature, processed forms. Such processing is absolutely required for the production of infectious progeny virus. Inhibition of any of these steps in the viral lifecycle has been shown to affect viral growth in tissue culture.

Although both RT inhibitors and PIs are widely accepted as antiviral agents and form important components to the therapy of HIV infection, inhibitors of IN have remained elusive. A

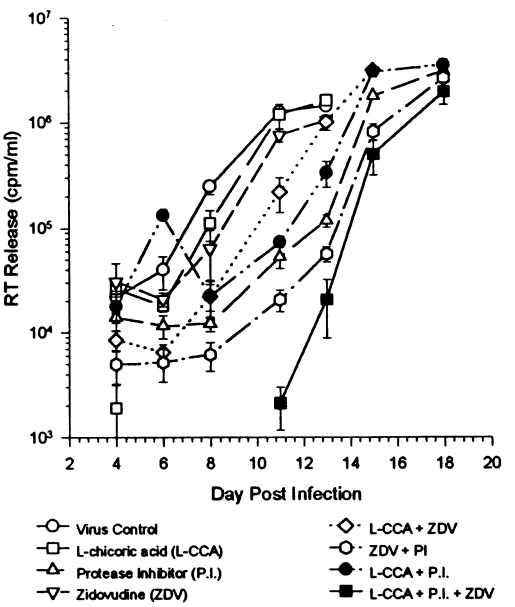

Fig. 5. Triple combination treatment is better than either single or double combination treatment for inhibition of HIV_{R19} infection in vitro. Concentrations for all inhibitors in all combinations were: L-CCA, 1.6 μM; PI, 1.3 μM; ZDV, 150 nM. Values are mean RT release from triplicate infections. Error bars are standard deviations. RT release results were supported by immunofluorescence assay (data not shown).

number of putative IN inhibitors have been reported to date including caffeic acid phenylethyl ester (Fesen et al., 1993, 1994), DNA binding agents (Billich et al., 1992, Fesen et al., 1993, Carteau et al., 1994), topoisomerase inhibitors (Fesen et al., 1993), and bis-catechols (LaFemina et al., 1995) or other hydroxylated aromatic compounds (Burke et al., 1995), aurintricarboxylic acid (Cushman et al., 1995) and cosalane analogues (Cushman and Sherman, 1992). Selectivity of these compounds for IN has been a problem; indeed, even ZDV (Mazumder et al., 1994) and other nucleoside analogues (Mazumder et al., 1996) have been reported to inhibit HIV IN in vitro if the conditions are correct (i.e. high concentrations of drug). Other rather non-specific IN inhibitors include: aurintricarboxylic acid and related compounds (Cushman et al., 1995), DNA binding agents and topoisomerase inhibitors. Importantly, most of the information on IN inhibitors is derived from in vitro experiments using purified IN and a protective effect of small molecule IN inhibitors against HIV infection in tissue culture is either undetectable (LaFemina et al., 1995) or has not been examined. Indeed, several potent, new HIV IN inhibitors were recently described that have not been studied for activity against HIV in tissue culture at non-toxic concentrations (Hong et al., 1997, Neamati et al., 1997, Nicklaus et al., 1997, Zhao et al., 1997a,b,c). Of the reported inhibitors with activity against HIV IN, cosalane analogues had activity in tissue culture, although the data were inconsistent with activity against IN being the mechanism of action. Suramin, another compound reported to have anti-HIV IN activity in vitro has been shown to act through mechanisms other than IN inhibition (Carteau et al., 1993). One recent advance has been the report of an octet oligonucleotide which inhibits IN in vitro and blocks virus replication ex vivo, apparently via inhibition of IN and retroviral integration (Ojwang et al., 1995). This octet oligonucleotide IN inhibitor is currently in phase I clinical trials (Abrams et al., 1996) but its high synthetic cost, low bioavailability and questions concerning its mechanism of action (Cherepanov et al., 1997) may prove problematic for widespread clinical use.

Recently, two promising new classes of HIV IN inhibitors have been reported. The first, the anthraquinones, inhibits HIV preintegration complexes in vitro (Farnet et al., 1996). The lead compound in this class is quinalizarin with an IC_{50} of 1–4 μM using purified HIV IN and 1–6 μM against preintegration complexes (Farnet et al., 1996). This compound has been reported to have anti-HIV activity in tissue culture. The second, the DCQAs and DCTAs, are similar to other bis-catechols except they are more potent inhibitors of HIV IN and are active against HIV in tissue culture at non-toxic concentrations (Robinson et al., 1996a,b).

The results reported herein clearly demonstrate that the addition of a first generation IN inhibitor, L-CCA, to regimens using either a PI or ZDV improves upon the in vitro anti-HIV effect of that regimen. For all of the triple combination studies (Figs. 3–5), all three inhibitors were used at suboptimal concentrations (below the ED_{50}). In addition, if L-CCA is added to ZDV and a PI, one can effectively reduce by at least 33% the amount of ZDV and PI required to provide an equivalent anti-HIV effect. In vitro combination analyses may or may not predict how combinations of antiviral agents will behave in vivo. For HIV, combinations of PIs and RT inhibitors demonstrated additive to synergistic effects in vitro, which were similar to their in vivo effects (Deminie et al., 1996, Patick et al., 1997). With one major limiting factor in anti-HIV combination therapy being the cost of the antiviral agents, especially PIs, such a reduction could substantially increase the number of patients who could be on combination therapy regimens. The results of these in vitro studies, if they were to translate into in vivo results, could have a substantial impact on both the efficacy and costs of newer anti-HIV therapies. Therefore, it is imperative that development of more selective and more potent inhibitors of HIV IN, especially those using L-CCA as a lead compound, should be vigorously pursued.

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