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The inhibition of human immunodeficiency virus type 1 in vitro by a non-nucleoside reverse transcriptase inhibitor MKC-442, alone and in combination with other anti-HIV compounds

Tara M. Brennan [a], Debra L. Taylor [a,*], C. Gordon Bridges [a], James P. Leyda [b], A. Stanley Tyms [a]

[a] *MRC Collaborative Centre, 1–3 Burtonhole Lane, Mill Hill, London NW7 1AD, UK*
[b] *Marion Merrell Dow Research Institute, Cincinnati, OH, USA*

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Abstract

MKC-442, a derivative of the non-nucleoside reverse transcriptase (RT) inhibitor 1-[(2-hydroxyethoxy)methyl)-6-(phenylthio)thymidine (HEPT), showed potent and selective inhibition of human immunodeficiency virus type 1 (HIV-1) replication in vitro, using a range of host-cell/virus systems including human peripheral blood mononuclear cells infected with primary clinical isolates. MKC-442 was evaluated in combination with the nucleoside analogues AZT, ddI and ddC, the non-nucleoside RT inhibitor nevirapine, the HIV-1 proteinase inhibitor Ro-31-8959, and the α-glucosidase 1 inhibitor, MDL-28,574, using a cell viability assay. Drug interactions were evaluated by the isobologram technique and by calculating combination indices. Notable synergistic inhibition of HIV-1 replication was observed when MKC-442 was combined with AZT and MDL-28,574 and moderate synergy with ddI. In combination with ddC, nevirapine or Ro-31-8959, only a slightly better than additive effect was observed. Impressive synergy was seen using the three-drug combinations of MKC-442, AZT and MDL-28,574 or MKC-442, AZT and Ro-31-8959. No additional cytotoxicity was observed as measured by [^{3}H]thymidine incorporation by concanavalin A-stimulated peripheral blood mononuclear cells, when MKC-442 was combined with any of the above-mentioned compounds. The use of MKC-442 in a two- or three-drug combina-

* Corresponding author. Fax: +44 (81) 906 1395.

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tion regimen with other RT inhibitors, a proteinase inhibitor or an α-glucosidase 1 inhibitor should be considered for HIV-1-related chemotherapy.

Keywords: Human immunodeficiency virus; Drug combination, Non-nucleoside RT inhibitor

1. Introduction

The non-nucleoside reverse transcriptase (RT) inhibitors (NNRTIs) such as 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)-thymine (HEPT) and its derivatives (Baba et al. 1989, 1991; Miyasaka et al., 1989; Tanaka et al., 1991), tetrahydroimidazol[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-thiones (TIBO, e.g. R-82913 and R-82150) (Pauwels et al., 1990), nevirapine (BI-RG-587) (Merluzzi et al., 1990), pyridinones (e.g. L-697,661) (Goldman et al., 1991), bis(heteroaryl)piperazines (BHAPS) (e.g. U-87201E) (Romero et al., 1991), [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide (TSAO) (Balzarini et al., 1992) and α-anilinophenylacetamide (Pauwels et al., 1993), are antiretroviral agents with potent and selective activity in vitro against the human immunodeficiency virus type 1 (HIV-1). These compounds are not inhibitory to any DNA or RNA viruses other than HIV-1 and are targeted at a non-substrate binding site of the HIV-1 RT (Cohen et al., 1991; Balzarini et al., 1992; Dueweke et al., 1992). MKC-442 (6-benzyl-1-ethoxymethyl-5-isopropuluracil) (see Fig. 1) a novel HEPT derivative was recently described as a potent inhibitor of HIV-1 in vitro, with a favourable toxicity and pharmacokinetic profile in vivo (Yuasa et al., 1993; Baba et al., 1994). We have confirmed its antiviral activity in vitro using different host-cell/virus systems including human peripheral blood mononuclear cells (PBMCs) infected with primary clinical isolates of HIV-1, in comparison with other inhibitors of the HIV-1 RT.

As with the clinically investigated nucleoside analogues zidovudine (AZT, Retrovir, 3′-azido-2′,3′-dideoxythymidine), didanosine (ddI, Videx, 2′,3′-dideoxyinosine) and zalcitabine (ddC, Hivid, 2′,3′-dideoxycytidine), the major drawback of the use of NNRTIs for the treatment of patients with the acquired immune deficiency syndrome (AIDS) is the rapid emergence of drug-resistant strains of HIV-1 (Larder et al., 1989; St Clair et al., 1991; Richman et al., 1992; Saag et al., 1993). However, this type of compound has been demonstrated to show clinical efficacy in the short term and may have a role in

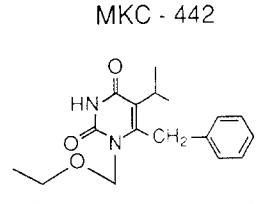

Fig. 1. Structural formulae of MKC-442 (6-benzyl-1-ethoxymethyl-5-isopropuluracil), a HEPT derivative.

drug combination regimens where the concurrent use of different antiretroviral agents may inhibit or delay the development of viral resistance (Larder et al., 1992; Balzarini et al., 1993; Chow et al., 1993). With the aim of identifying synergistic or antagonistic drug interactions, we have evaluated the in vitro anti-HIV activity of MKC-442, as a representative NNRTI, in combination with other RT inhibitors (convergent combination approach). These were AZT, ddI, and ddC, as well as a second NNRTI, nevirapine. In addition, we investigated MKC-442 in combination with two compounds which are targeted at different stages in the HIV replication cycle (divergent combination approach). Ro-31-8959 (Saquinavir, Invirase) is a peptidomimetic inhibitor of HIV-1-encoded proteinase (Roberts et al., 1990), and MDL-28,574 the 6-*O*-butanoyl derivative of castanospermine, an inhibitor of α-glucosidase 1 of the glycoprotein processing enzymes (Taylor et al., 1991, 1994a). Furthermore, we have investigated the three-drug combination regimens of MKC-442 with AZT and either Ro-31-8959 or MDL-28,574. To ensure that there was no additional cytotoxicity when MKC-442 was used in combination with the other compounds, the uptake of [^{3}H]thymidine by PBMCs in response to mitogen was determined.

2. Materials and methods

2.1. Compounds

MKC-442, prepared as previously described (Tanaka et al., 1994), was provided by Mitsubishi Kasei Corp. AZT and ddI were purchased from the Sigma Chemical Co. (Poole, Dorset, UK), Ro-31-8959 and ddC were provided by Roche Products Ltd. (Welwyn Garden City, UK) and nevirapine (BI-RG-587) provided by Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT USA). MDL-28,574 was synthesized from castanospermine as previously described (Liu et al., 1990).

2.2. Virus and cells

The HIV-1 strains RF and GB8, and the T-cell lines C8166, MT-4 and JM were obtained from the MRC AIDS Directed Programme Reagent Project (Dr. Harvey Holmes, NIBSC, UK). Human PBMCs were isolated by Lymphoprep™ (Nycomed Pharma As, Oslo, Norway) density gradient centrifugation from acid citrate dextrose (ACD)-treated buffy coat (North London Blood Transfusion Centre, Colindale, London). The different cell lines were cultured in the suspension medium RPMI 1640 (Gibco BRL, Middlesex, UK) supplemented with 10% (v/v) fetal calf serum, 290 μg/ml glutamine, 100 U/ml penicillin and 50 μg/ml streptomycin. In addition, PBMCs used for antiviral assays were depleted of CD8 + cells using T cytotoxic/suppressor dyna beads according to the manufacturer's instructions (Dyna, Oslo, Norway) and then stimulated for 3 days with 10 μg/ml phytohaemoglutinin (PHA) and 10 U/ml interleukin 2 (IL-2) (Boehringer Mannheim, Germany). PBMCs infected with the clinical isolates HIV-1_{CC2}, HIV-1_{CC3} and HIV-1_{CC4} were separated from heparinized

blood samples obtained from patients with AIDS (provided by Dr. J. Parkin, St Bartholomews Hospital Medical School, London).

2.3. Antiviral assays

C8166 and JM cells were infected with HIV-1_{RF} and HIV-1_{GB8} respectively, for 1 h at room temperature. The cells were then washed three times and cultured in the presence of varying concentrations of test compound for 3 days at 37°C. The cell-free supernatant fluid was then assayed for levels of p24 viral core antigen using the ELISA method of Moore et al. (1990). Antiviral activity of the compounds in MT-4 cells infected with HIV-1_{RF} were determined using the tetrazolium reduction (MTT) cell viability assay as previously described (Pauwels et al., 1988). For the assessment of antiviral activity against clinical isolates of HIV-1, PBMCs isolated from HIV-1-antibody-positive patients were co-cultured with PHA-stimulated uninfected PBMCs in a 1 : 10 ratio, and cultured in the presence of varying concentrations of drug. After 7 days, the cell-free supernatant fluid was assayed for p24 viral antigen using a commercially available p24 antigen ELISA (Coulter, UK).

2.4. Drug combination assays

The MTT cell viability assay originally published by Pauwels et al. (1988) was adapted for analyzing the anti-HIV-1 effects of drug combinations. For each combination assay, the inside wells of six 96-well flat-bottomed plates were used, with quadruplicate wells for each combination of drugs. The various drug combinations were achieved by creating a chequerboard, with one compound being titrated horizontally and the other being titrated vertically across the plates. The correct final concentrations were achieved by adding 50 μl of the first drug ($\times 4$ concentrated) and 50 μl of the second drug ($\times 4$ concentrated) followed by 100 μl of the infected cell suspension. For three drug combination assays, chequerboards of two drugs were created on a background of fixed concentrations of the third compound. These assays used only duplicate wells for each drug combination, but still required the use of six 96-well cell culture plates. As previously described, MT-4 cells were infected with 100 $TCID_{50}$ of HIV-1_{RF} per 5×10^4 cells and distributed into wells at a concentration of 5×10^4 cells per well. The cell culture plates were incubated at 37°C for 5 days and cell viability was assessed by adding 10 μl of MTT (7.5 mg/ml) in PBS to each well, and incubating the plates for an additional hour. The formazan crystals which formed were solubilized by adding 100 μl of acidified isopropanol to each well and mixed. The absorbance was read at 540 nm. Raw ASCII data from the plate reader were captured by Ultroterm and further processed by Microsoft Excel with minimal user intervention. A series of linked Excel macros were written to parse, calculate the means and standard deviations of OD values (the replicate error on each point was always $< 10\%$), and then plot dose–response graphs.

2.5. Analysis of results

From the series of dose–response curves which were generated, the 50% inhibitory concentrations (IC_{50}s) of each drug alone and at a fixed concentration of the other were

determined. These IC_{50} values were used to construct isobolograms (Berenbaum, 1989; Suhnel, 1990) and to calculate a combination index (CI) using the following formula:

$$CI = \frac{(D)_A}{(Dx)_A} + \frac{(D)_B}{(Dx)_B} + \alpha\frac{(D)_A(D)_B}{(Dx)_A(Dx)_B}$$

as previously described (Chou and Talalay, 1984; Suhnel, 1990). In this formulae $(Dx)_A$ = the IC_{50} of drug A alone, $(Dx)_B$ = the IC_{50} of drug B alone, and $(D)_A$ or $(D)_B$ = the concentrations of drugs A and B in combination giving 50% protection of the cells. The constant $\alpha = 1$ for mutually non-exclusive agents and $\alpha = 0$ for mutually exclusive agents. In all cases, the formula for mutually non-exclusive agents was used, with the exception of the combination of the two NNRTIs, MKC-442 and nevirapine, where CI values were calculated using the formulae for both mutually exclusive and non-exclusive agents. Confidence limits for the CI indicating zero interaction were obtained from mixing proportions of an identical compound in the combination assay ($CI = 0.87 \pm 0.07$) (verified additive combination) and in our laboratory a CI value of less than 0.8 indicated synergy (95% confidence). Comparisons of different combinations were achieved by performing the two sample *t*-test on CI values and synergistic interactions were established by comparison with CI values for the verified additive combination.

For three-drug combinations CI values were calculated using the general isobole equation:

$$CI = \frac{(D)_A}{(Dx)_A} + \frac{(D)_B}{(Dx)_B} + \frac{(D)_C}{(Dx)_C}$$

as described by Berenbaum (1989). Where $(Dx)_A$, $(Dx)_B$ and $(Dx)_C$ = the IC_{50}s of drugs A, B and C alone, and $(D)_A$, $(D)_B$ or $(D)_C$ = the concentrations of drugs A, B and C in combination giving 50% protection of the cells.

2.6. Human lymphocyte proliferation

Freshly isolated human PBMCs were distributed into 96-well round-bottomed cell culture plates at a concentration of 2×10^5 cells per well and stimulated with concanavalin A (2.5 μg/ml), in the presence or absence of different concentrations of drugs alone or in combination, for 48 h at 37°C. The cells were then labelled with [^{3}H]thymidine (1 μCi/well) for 24 h and radiolabel incorporation assessed by cell harvest onto glass fibre paper followed by measurement in a β-spectrometer. For assays involving the use of AZT which would potentially antagonize the uptake of thymidine, deoxy-[5-^{3}H]cytidine incorporation and cell counts were assessed in parallel cultures.

3. Results

3.1. Antiviral effect and cytotoxicity of MKC-442 tested alone

MKC-442 had potent antiviral activity against both laboratory-adapted strains of HIV-1 in established T-cell lines as well as primary clinical isolates in human PBMCs

Table 1
Anti-HIV activity of MKC-442 in comparison with other RT inhibitors

RT inhibitor	IC_{50} (μM)					
	MT-4/HIV-1_{RF} [a]	C8166/HIV-1_{RF} [b]	JM/HIV-1_{GB8} [b]	PBMC/HIV-1_{CC2} [b]	PBMC/HIV-1_{CC3} [b]	PBMC/HIV-1_{CC4} [b]
MKC-442	0.018 ± 0.0093	0.0024 ± 0.002	0.0016	0.01	0.04	0.0022
Nevirapine	0.26 ± 0.06	0.21 ± 0.07	0.14	0.022	0.07	0.015
AZT	0.082 ± 0.08	0.05 ± 0.05	> 10	0.004	0.0019	0.018
ddI	26 ± 4.6	4.4 ± 0.56	6	0.52	0.035	0.028
ddC	0.46 ± 0.25	0.16 ± 0.07	0.098	0.018	0.015	0.032

[a] MTT cell viability assay.
[b] p24 reduction assay.

(Table 1). The IC_{50} values were in the range 0.0016–0.018 μM and compared favourably with other RT inhibitors tested in parallel. No cytotoxicity was seen in the T-cell lines used at concentrations of up to 100 μM as determined by MTT cell viability assay. The concentration of MKC-442 required to inhibit by 50% the uptake of [^{3}H]thymidine by PBMCs in response to the mitogen concanavalin A was 52.5 ± 20 μM ($n = 4$). This provided a selectivity index in excess of 2000.

3.2. Two-way antiviral combinations

The MTT cell viability assay was used to assess the antiviral effects of MKC-442 in combination with other compounds. Using this assay MKC-442 had a mean IC_{50} value of 0.038 ± 0.02 μM ($n = 11$). When this drug was studied in combination with AZT or MDL-28,574, it was clear that the IC_{50} value could be significantly reduced by the addition of low concentrations of the second compound (Table 2). Similarly, the addition of low concentrations of MKC-442 reduced the concentrations of the other drug required to restrict the growth of HIV-1. When the data generated was used to calculate CI values, these ranged from 0.40–0.56 for the combination of MKC-442 and AZT, to 0.51–0.67 for MKC-442 in combination with MDL-28,574, which indicated a notable synergistic antiviral effect in both cases (Table 2). This was illustrated by the construction of isobolograms (Fig. 2A, B). In contrast, the CI values (Table 2) and isobolograms (Fig. 2C–F) showed that MKC-442 produced only moderate synergy with ddI, with little more than an additive effect with ddC, Ro-31-8959 or nevirapine. These results were consistently reproducible in a number of experiments.

3.3. Three-way antiviral combinations

Further to the results obtained in the two-way combination assays, MKC-442 was studied in two different three-way combinations. These were MKC-442, AZT and MDL-28,574 (Table 3) and MKC-442, AZT and Ro-31-8959 (Table 4). Such three-way combination assays when carried out using a chequerboard format generate a large

Table 2
Combination indices for MKC-442 in two-way drug combination assays

Combination	Concentrations (μM) giving 50% inhibition of cell death [a]		Molar ratio	CI [b]	P [c]
	MKC-442	2nd compound			
MKC-442/AZT	*0*	0.11	–		
	0.08	*0*	–		
	0.003	0.056	1 : 19	0.56	
	0.01	*0.03*	1 : 3	0.43	0.0001 (S)
	0.023	*0.01*	1 : 0.4	0.40	
	0.03	0.0068	1 : 0.2	0.46	
MKC-442/MDL-28,574	*0*	29	–		
	0.023	*0*	–		
	0.0003	17	1 : 56666	0.60	
	0.001	13	1 : 13000	0.51	0.001 (S)
	0.003	*10*	1 : 3333	0.51	
	0.01	5	1 : 500	0.67	
MKC-442/ddI	*0*	16.4	–		
	0.08	*0*	–		
	0.0064	*10*	1 : 1562	0.74	
	0.01	7.2	1 : 720	0.62	0.036 (S)
	0.03	3.6	1 : 120	0.68	
	0.035	*3*	1 : 86	0.70	
MKC-442/ddC	*0*	0.7	–		
	0.033	*0*	–		
	0.028	*0.03*	1 : 1.1	0.83	
	0.02	*0.1*	1 : 5	0.83	0.30 (A)
	0.01	0.29	1 : 29	0.84	
	0.0094	*0.3*	1 : 32	0.83	
MKC-442/nevirapine	*0*	0.088	–		
	0.038	*0*	–		
	0.03	*0.01*	1 : 0.3	0.99 (0.90)	
	0.015	*0.03*	1 : 2	0.86 (0.84)	0.48 (A)
	0.01	0.044	1 : 4.4	0.89 (0.76)	
	0.003	0.065	1 : 22	0.87 (0.82)	
MKC-442/Ro-31-8959	*0*	0.0088	–		
	0.035	*0*	–		
	0.03	0.0002	1 : 0.007	0.90	
	0.026	*0.001*	1 : 0.04	0.93	0.54 (A)
	0.014	*0.003*	1 : 0.2	0.88	
	0.01	0.004	1 : 0.4	0.86	

[a] Only a selection of results from each combination assay is shown. The fixed drug concentration in each combination is shown in italics.
[b] CI values have been calculated using the formula for mutually non-exclusive agents. The CI values calculated using the formula for mutually exclusive agents are shown in parentheses.
[c] P = probability value (95% confidence level) for a two sample t-test, using the verified additive effect. S, synergism; A, additivity.

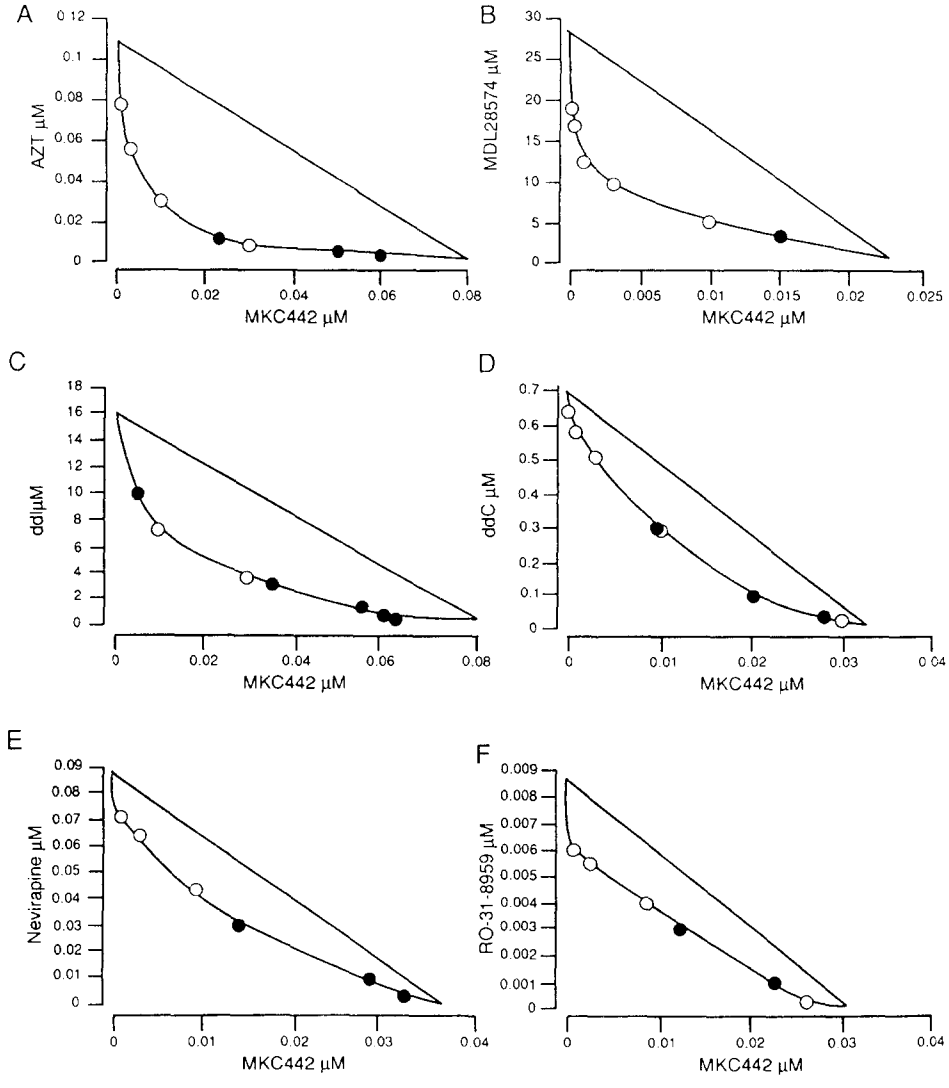

Fig. 2. Isoboles for the combination of MKC-442 with the nucleoside analogues AZT, ddI, ddC, the non-nucleoside RT inhibitor nevirapine, the HIV-1 proteinase inhibitor Ro-31-8959 and the α-glucosidase 1 inhibitor MDL-28,574. The MTT cell viability assay was used to calculate the IC_{50} values for each compound alone and at a fixed concentration of the other. These values were used to plot isobolograms, the concave shape of which is indicative of synergy. ○, fixed concentration of MKC-442: ●, fixed concentration of second compound. (A) Isoboles for the combination of MKC-442 and AZT; (B) MKC-442 and MDL-28,574; (C) MKC-442 and ddI; (D) MKC-442 and ddC; (E) MKC-442 and nevirapine; (F) MKC-442 and Ro-31-8959.

amount of data, that is: (1) dose–response curves for all drugs used individually; (2) dose–response curves for each drug at different fixed concentrations of each of the other two drugs; and (3) dose–response curves for each drug with fixed concentrations of the

Table 3
Combination indices for two- and three-drug combinations of MKC-442, MDL-28,574 and AZT

Compounds and concentrations (μM) giving 50% inhibition of cell death [a]			Molar ratio	CI	P [b]
MKC-442	MDL-28,574	AZT			
0.04	*0*	*0*			
0	90	*0*			
0	*0*	0.04			
0.01 (4)	*0*	0.0043 (9)	1 : 0.4	0.38	
0.003 (13)	*0*	0.018 (2)	1 : 6	0.56	0.038 (S)
0.003 (13)	*0*	0.0064 (6)	1 : 2.1	0.25	
0.03 (1.3)	45 (2)	*0*	1 : 15000	0.61	
0.01 (4)	17 (5)	*0*	1 : 1700	0.56	0.0091 (S)
0.004 (10)	*30* (3)	*0*	1 : 7500	0.46	
0	25 (3.6)	*0.003* (13)	1 : 0.0001	0.24	
0	*10* (9)	0.004 (10)	1 : 0004	0.23	< 0.0001 (S)
0	*3* (30)	0.0075 (5)	1 : 0025	0.22	
0.01 (4)	*3* (30)	0.0032 (12.5)	1 : 300 : 0.32	0.36	
0.01 (4)	3.6 (25)	*0.003* (13)	1 : 360 : 0.3	0.37	< 0.0001 (S)
0.003 (13)	*3* (30)	0.0046 (8)	1 : 1000 : 1.5	0.22	
0.003 (13)	7 (13)	*0.003* (13)	1 : 2333 : 1	0.23	

[a] Only a selection of the data generated is shown. The fixed drug concentrations in each combination are shown in italics. Numbers in parentheses represent the fold reduction compared to the IC_{50} of the drugs alone.
[b] P = probability value (95% confidence level) for a two-sample t-test, using the verified additive effect. S, synergism; A, additivity.

other two drugs in combination. For simplicity we have only shown a selection of the results in Tables 3 and 4, but similar results were obtained for specific drug combinations irrespective of which compounds were used at fixed concentrations. Each three-way combination experiment was repeated at least twice and similar results were obtained. Interestingly, a number of the three-drug combinations, for example: MKC-442 0.01 μM, AZT 0.01 μM and MDL-28,574 3 μM; or MKC-442 0.001 μM, AZT 0.01 μM and Ro-31-8959 0.01 μM, showed complete protection of the cells which did not allow further analysis and calculation of a combination index.

In these three-way combination assays the results obtained for MKC-442 in two-way combinations confirmed those reported in Table 2, for example MKC-442 was synergistic with AZT and MDL-28,574, but only additive with Ro-31-8959. Consistent with previous reports AZT and MDL-28,574 in combination were synergistic, but AZT and Ro-31-8959 were only additive (Taylor et al., 1994b). Overall it was apparent from the CI values that by using three different types of anti-HIV compound in combination, impressive synergy could be obtained and the concentrations of each drug significantly reduced. Greater synergy was obtained when MKC-442 and AZT were used in a three-way combination with MDL-28,574 (CI values in the range 0.22–0.37) than when they were used in combination with Ro-31-8959 (CI values in the range 0.44–0.78)

Table 4
Combination indices for two- and three-drug combinations of MKC-442, Ro-31-8959 and AZT

Compounds and concentrations (μM) giving 50% inhibition of cell death [a]			Molar ratio	CI	P [b]
MKC-442	Ro-31-8959	AZT			
0.06	*0*	*0*			
0	0.012	*0*			
0	*0*	0.023			
0.03 (2)	0.0038 (3)	*0*	1 : 0.1	0.97	
0.01 (6)	0.0064 (1.8)	*0*	1 : 0.6	0.89	0.15 (A)
0.004 (15)	*0.01* (1.2)	*0*	1 : 2.5	0.95	
0.024 (2.5)	*0*	*0.003* (7.6)	1 : 0.1	0.58	
0.01 (6)	*0*	0.008 (2.9)	1 : 0.8	0.57	0.0009 (S)
0.007 (8.6)	*0*	*0.01* (2.3)	1 : 1.4	0.60	
0	0.004 (3)	*0.01* (2.3)	1 : 2.5	0.91	
0	0.0065 (1.8)	*0.003* (7.7)	1 : 0.5	0.74	0.81 (A)
0	*0.0003* (40)	0.02 (1.15)	1 : 67	0.91	
0.017 (3.5)	*0.0003* (40)	*0.003* (7.6)	1 : 0.02 : 0.20	0.44	
0.001 (60)	0.005 (2.4)	*0.003* (7.6)	1 : 5 : 3	0.56	0.023 (S)
0.0049 (12)	*0.001* (12)	*0.01* (2.3)	1 : 0.2 : 2	0.60	
0.001 (60)	*0.0003* (40)	0.017 (1.3)	1 : 0.3 : 17	0.78	

[a] Only a selection of the data generated is shown. The fixed drug concentrations in each combination are shown in italics. Numbers in parentheses represent the fold reduction compared to the IC_{50} of the drugs alone.
[b] P = probability value (95% confidence level) for a two-sample t-test, using the verified additive effect. S, synergism; A, additivity.

($P = 0.021$). This is most likely related to the fact that MKC-442, AZT and MDL-28,574 all interact synergistically with each other in two-way combinations.

3.4. Cytotoxicity

The ability of PBMCs to proliferate in response to mitogen whilst cultured in the presence of MKC-442, Ro-31-8959, AZT or MDL-28,574 alone or in two- or three-way combinations, was investigated. The concentrations of drug required to inhibit by 50% the incorporation of [^{3}H]thymidine into cellular DNA in response to concanavalin A (CC_{50}) was 52.5 ± 20 μM ($n = 4$) for MKC-442, 4.36 ± 1.9 μM ($n = 5$) for AZT, 780 ± 136 μM ($n = 8$) for MDL-28,574 and 13.3 ± 6.6 μM ($n = 5$) for Ro-31-8959 when used as single agents. With respect to AZT, similar results were obtained when deoxy-[^{3}H]cytidine incorporation was assessed and cell numbers were significantly reduced at concentrations above the CC_{50}. The endpoints for AZT, MDL-28,574 and Ro-31-8959 were not altered by the addition of MKC-442 at concentrations up to 10 μM. Similarly, the CC_{50} for MKC-442 was not altered by the addition of concentrations of AZT up to 1 μM, MDL-28,574 up to 100 μM and Ro-31-8959 up to 10 μM. For studying the effects of three drugs together, we combined 1 μM MKC-442 with 1 μM AZT and either 100 μM MDL-28,574 or 1 μM Ro-31-8959 and noted no reduction in

the incorporation of [^{3}H]thymidine, compared with untreated cells. These results indicated that at the low concentrations of drugs required for synergistic antiviral activity, no cytotoxicity or detrimental effects on mitogen-induced cell division within the immune system should occur.

4. Discussion

Despite the fact that the NNRTIs, nevirapine and L-697,661 have shown clinical efficacy in HIV-1-infected patients, as determined by a profound decrease in p24 antigen levels and viral load and a corresponding increase in CD4 cell counts, the rapidity with which drug-resistant variants are selected for, means that the antiviral effect is short-lived (Richman et al., 1992; Saag et al., 1993; Havlir et al., 1993). This finding was not unexpected as HIV-1 strains resistant to NNRTIs are rapidly generated in cell culture experiments (Nunberg et al., 1991; Richman et al., 1991) and the mutations selected for by specific classes of NNRTIs are well characterized and documented (Richman et al., 1991; Mellors et al., 1992; Schinazi et al., 1994). We have shown that the HEPT derivative, MKC-442, is a potent and selective anti-HIV-1 agent in vitro, but its use as a monotherapy for the treatment of patients with AIDS will undoubtedly be thwarted by the same resistance problem. This assumption is supported by the fact that drug-resistant variants to MKC-442 have recently been selected for in vitro (Seki et al., 1994). Analysis of the nucleotide sequences within the RT encoding region of these drug-resistant mutants revealed amino acid substitutions at positions 181 (Tyr → Cys), in common with other NNRTIs (Richman et al., 1991; Mellors et al., 1992; Schinazi et al., 1994), and additionally at positions 103 (Lys → Arg) and 108 (Val → Ile) (Seki et al., 1994). Interestingly, resistance was slower to emerge in response to MKC-422 compared with nevirapine, and it has been suggested that this may be related to the requirement for a greater number of mutations (Seki et al., 1994).

The problem of drug resistance was first encountered with the use of the nucleoside analogue AZT (Larder et al., 1989). It is now apparent that strains of HIV-1 resistant to other nucleoside RT inhibitors including ddI, ddC and 3TC can be selected for both in vitro and in vivo (St Clair et al., 1991; Gu et al., 1992; Tisdale et al., 1993; Gao et al., 1993), in addition to the NNRTIs. Recently, the isolation of mutants resistant to HIV proteinase inhibitors, after selection in cell culture (Dianzani et al., 1993; Craig et al., 1993; Otto et al., 1993) and in the clinic (Emini et al., 1994; Jacobsen et al., 1994), has also been reported. Although the importance of drug-resistant viruses in pathogenesis has not yet been proven, it is likely that the emergence of the resistant phenotype may make the drug less effective. The apparent role for most of these drugs in the future for the treatment of HIV-1-infected patients appears to be in combination regimens which could potentially delay or prevent the development of drug resistance. There is now a wealth of information available on the specific mutations selected for by particular classes of drugs (Schinazi et al., 1994) and it is also apparent that certain amino acid substitutions can suppress the effects of mutations responsible for AZT resistance (Larder, 1992). Similarly, combination treatment can result in a different resistance pattern compared with single-drug therapy (Balzarini et al., 1993). Careful consideration

of the specific mutations selected for by particular compounds may reveal the most appropriate drug combinations to prevent multidrug resistance.

The use of combination therapy could produce enhanced antiviral effects thereby enabling dosage reductions and lessening drug side effects. In the current study, using a convergent approach to drug combinations, MKC-442 showed an impressive synergistic antiviral effect with AZT, and moderate synergy with ddI. This was consistent with the findings of Baba et al. (1994) and Yuasa et al. (1993). In contrast, and not unexpectedly considering the allosteric nature of the inhibition, MKC-442 in combination with the other NNRTI, nevirapine, showed little better than an additive effect.

The more conventional approach to drug combination therapy aims at targeting different stages in the HIV replication cycle. This divergent combination approach was investigated using MKC-442 in combination with the HIV-1 proteinase inhibitor, Ro-31-8959, or the glycoprotein processing inhibitor, MDL-28,574. Ro-31-8959 inhibits the processing of viral *gag* and *gag–pol* polyproteins, resulting in the production of immature non-infectious virions. In combination with this HIV-1 proteinase inhibitor, MKC-442 showed only an additive or marginally synergistic effect, despite the fact that the two drugs act at different stages of the HIV-1 life cycle and against two distinct viral enzymes. Similarly, Ro-31-8959 was previously shown to have only an additive effect with another RT inhibitor, AZT using the same cell viability assay (Taylor et al., 1994b). In contrast, a notable synergistic effect was observed when MKC-442 was combined with MDL-28,574, the 6-*O*-butanoyl derivative of castanospermine. MDL-28,574 is a potent inhibitor of cellular α-glucosidase 1, of the glycoprotein processing enzymes, and treatment of HIV-1-infected cells results in the production of virions which are non-infectious due to the presence of abnormally processed, glucosylated envelope glycoproteins (Taylor et al., 1994a). The reasons for the synergistic interaction of this compound are not clear, but consistent with the results presented here MDL-28,574 also showed synergy with other nucleoside (AZT, ddI and ddC) and non-nucleoside (nevirapine) RT inhibitors (Taylor et al., 1994b).

Overall the results showed that MKC-422 did not have any antagonistic effects on the antiviral activity of a series of HIV-1 inhibitors and in a number of instances showed synergy. No additional cytotoxicity was seen when MKC-442 was used in combination with any of the compounds investigated. Furthermore, the three-drug combination regimens investigated here: MKC-442, AZT and Ro-31-8959 in combination; or MKC-442, AZT and MDL-28,574 in combination, allowed a remarkable reduction in the concentration of each drug required to inhibit HIV-1 growth, compared to when each drug was used alone. These results suggest that clinical benefit may be afforded by the use of MKC-442 or other NNRTIs in the two- or three-drug combination regimens described. Whether the rate of emergence of drug-resistant viruses is delayed or abrogated by the use of such drug combination regimens, compared to monotherapy, remains to be addressed.

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