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Combinative Interactions of a Human Immunodeficiency Virus (HIV) Tat Antagonist with HIV Reverse Transcriptase Inhibitors and an HIV Protease Inhibitor

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Combinations of the human immunodeficiency virus (HIV) Tat protein antagonist Ro 24-7429 with either the HIV protease inhibitor Ro 31-8959 or the HIV reverse transcriptase inhibitors AZT (3′-azido-3′-deoxythymidine), ddC (2′,3′-dideoxycytidine), ddI (2′,3′-dideoxyinosine), and nevirapine were synergistic or additive in reducing HIV type 1 p24 antigen production in CEM cells or inhibiting HIV type 1-induced syncytium formation in HT4-6C cells.

Chemotherapy of human immunodeficiency virus (HIV) is currently restricted to the use of nucleoside analog prodrugs whose triphosphates are inhibitors of the viral reverse transcriptase (RT). Monotherapy with zidovudine (3′-azido-3′-deoxythymidine [AZT]) has been shown to prolong survival, reduce morbidity, and delay disease progression (14, 15, 38) but is associated with bone marrow toxicity (31), incomplete suppression of viral replication (19), and the selection of drug-resistant mutants (26, 32). Didanosine (2′,3′-dideoxyinosine [ddI]) and zalcitabine (2′,3′-dideoxycytidine [ddC]) have also been licensed for the treatment of HIV infection. The studies to determine whether these two RT inhibitors in combination with AZT will lead to significant benefits in patients are in progress.

A number of studies have shown combinations of anti-HIV compounds to be synergistic in antiviral assays in vitro (2, 22). AZT is synergistic with both nucleoside and nonnucleoside inhibitors of HIV RT (1, 34) and with non-RT inhibitors such as recombinant soluble CD4 (17, 23). AZT and ddC have been shown to synergize with recombinant alpha interferon A (18, 37). Certain three-drug combinations display synergistic interactions as well (23, 25).

Ro 31-8959 (*N-tert*-butyl-decahydro-2-[(*R*)-hydroxy-4-phenyl-3 (*S*)-[[*N*-(2-quinolylcarbonyl)-L-asparaginyl] amino] butyl]-(4aS,8aS)isoquinoline-3(*S*)-carboxamide), a potent viral protease inhibitor which interrupts maturation of infectious virions in both acutely and chronically infected cells (12, 35), is now being assessed in clinical trials. Ro 31-8959 shows additive to synergistic anti-HIV type 1 (HIV-1) activity in combination with AZT, ddC, and recombinant alpha interferon A in vitro (24). Ro 24-7429 [7-chloro-*N*-methyl-5-(1H-pyrrol-2-yl)-3H-1,4-benzodiazepin-2-amine], an antagonist to the viral transcriptional transactivator Tat, inhibits both acute and chronic HIV infections in vitro by suppressing transcription of the viral DNA that becomes integrated in the host cell chromosomes (20, 21). Because combination chemotherapy by agents that disrupt different stages of the replicative cycle of HIV could be more effective in virus suppression, we have evaluated two-drug combinations of Ro 24-7429 with AZT, ddC, ddI, nevirapine (28), and Ro 31-8959 and a three-drug combination of Ro 24-7429, Ro 31-8959, and ddC in antiviral assays in vitro.

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AZT was purchased from Sigma (St. Louis, Mo.), and ddI was purchased from Calbiochem (La Jolla, Calif.). ddC and Ro 24-7429 (20) were synthesized at Hoffmann-LaRoche Inc. (Nutley, N.J.), and Ro 31-8959 was synthesized at Roche Products Ltd. (Welwyn Garden City, United Kingdom) (35). Nevirapine was obtained from Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, Conn.).

CEM and HT4-6C cells were used for the anti-HIV-1 assays. CEM cells, a $CD4^+$ lymphoblastoid cell line, were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 50 μg of gentamicin per ml (10% RPMI). HT4-6C cells, a HeLa cell line expressing CD4, were maintained as previously described (4). Infectious stocks of the N1T strain of HIV-1, the cell-free supernatant of chronically infected CR10(N1T) cells (3), were stored at −135°C, and the numbers of syncytium-forming units (SFU) per milliliter were determined by titration in HT4-6C cells. Virus stocks of the LAV-1_{LAI} (formerly LAV-1_{BRU}) strain of HIV-1 were prepared from cleared lysates of infected CEM cells and stored in aliquots at −70°C (33).

HIV-1 p24 reduction assays were conducted with CEM cells infected at a multiplicity of 1 SFU per cell in 96-well culture plates in 10% RPMI. Following a 2-h adsorption, various concentrations of dimethyl sulfoxide-solubilized compounds were added to duplicate wells so that the final concentration of CEM cells was 10^5/ml and that of dimethyl sulfoxide was 0.5%. After a 4-day incubation at 37°C, cultures were subcultured at a ratio of 1:1 with 10% RPMI. Culture supernatants were harvested on day 7 postinfection and frozen at −135°C until assayed for HIV-1 p24 antigen with an enzyme-linked immunosorbent assay kit (Coulter, Hialeah, Fla.). The cytotoxicities of the compounds and their combinations were determined in parallel assays in mock-infected CEM cells by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction method (29) on day 7 post-drug treatment.

Assays of the reductions in numbers of HIV-1-induced syncytia were performed with monolayers of HT4-6C cells in 24-well plates infected with approximately 200 SFU of LAV-1_{LAI} per well (33). After a 1-h adsorption at 37°C, compounds were added in Dulbecco modified Eagle medium with 5% fetal bovine serum and 0.1% dimethyl sulfoxide, and the cultures were incubated at 37°C for 3 days. The cell monolayers were fixed with 10% formaldehyde and stained with 0.25% crystal

TABLE 1. Effects of Ro 24-7429 alone and in combination with AZT, ddC, and nevirapine on HIV-1-induced syncytium formation in HT4-6C cells

Expt no. and drug combination	Molar ratio of drug concns	Concn (μM)		% Reduction in syncytia from no-drug control[a]	CI[b] at an inhibition of:	
		Ro 24-7429	Combination drug		50%	90%
1. Ro 24-7429 + AZT	None (each drug used alone)	0.1		26		
		0.316		43		
		1.0		68		
		3.16		95		
			0.00316	21		
			0.01	54		
			0.0316	72		
			0.1	86		
	10:1	0.1	0.01	61	0.97	0.51
		0.316	0.0316	80		
		1.0	0.1	97		
	31.6:1	0.1	0.00316	44	0.79	0.44
		0.316	0.01	73		
		1.0	0.0316	93		
		3.16	0.1	99		
	100:1	0.316	0.00316	61	0.97	0.43
		1.0	0.01	92		
		3.16	0.0316	99		
2. Ro 24-7429 + ddC	None (each drug used alone)	0.01		10		
		0.0316		24		
		0.1		26		
		0.316		43		
		1.0		74		
		3.16		91		
		10		94		
			0.001	28		
			0.00316	41		
			0.01	55		
			0.0316	72		
			0.1	88		
	10:1	0.01	0.001	24	0.95	0.45
		0.0316	0.00316	39		
		0.1	0.01	71		
		0.316	0.0316	84		
		1.0	0.1	93		
	31.6:1	0.0316	0.001	16	1.62	0.56
		0.1	0.00316	36		
		0.316	0.01	64		
		1.0	0.0316	90		
		3.16	0.1	94		
	100:1	0.1	0.001	42	0.74	0.59
		0.316	0.00316	69		
		1.0	0.01	85		
		3.16	0.0316	93		
		10	0.1	96		
3. Ro 24-7429 + nevirapine	None (each drug used alone)	0.01		0		
		0.0316		9		
		0.1		23		
		0.316		39		
		1.0		77		
		3.16		96		
		10		99		
			0.01	19		
			0.0316	51		
			0.1	71		
			0.316	84		
			1.0	96		

Continued on following page

TABLE 1—*Continued*

Expt no. and drug combination	Molar ratio of drug concns	Concn (μM)		% Reduction in syncytia from no-drug control[a]	CI[b] at an inhibition of:	
		Ro 24-7429	Combination drug		50%	90%
	1:1	0.01	0.01	30	0.92	0.88
		0.0316	0.0316	42		
		0.1	0.1	72		
		0.316	0.316	90		
		1.0	1.0	97		
	3.16:1	0.0316	0.01	36	0.71	1.31
		0.1	0.0316	60		
		0.316	0.1	79		
		1.0	0.316	89		
		3.16	1.0	96		
	10:1	0.1	0.01	34	0.81	0.98
		0.316	0.0316	62		
		1.0	0.1	93		
		3.16	0.316	99		
		10	1.0	96		

[a] The mean numbers of syncytia per well in no-drug controls were 207, 199, and 71 for experiments 1, 2, and 3, respectively.
[b] For the definition and calculation of CI, see references 5 through 9.

violet to visualize syncytia. Duplicate wells were prepared for each drug dilution and combination.

Two-drug combinations of Ro 24-7429 with AZT, ddC, ddI, and Ro 31-8959 were evaluated by employing multiple-drug-effect analysis by the isobologram method (6, 8) and the median-effect principle (9) under the mutually exclusive assumption. The details of the median-effect principle have been described previously (5, 7), with combination indices of <1, 1, and >1 considered indicative of synergy, additivity, and antagonism, respectively. The three-drug combination of Ro 24-7429, ddC, and Ro 31-8959 was also evaluated by the median-effect principle (25). Isobologram analysis predicts that two compounds interacting additively at a given effect level will produce datum points falling on a straight line connecting inhibitory concentrations on the ordinate and abscissa for each agent alone at that effect level. Datum points falling to the left or right of such a line would indicate synergy or antagonism, respectively. All inhibitory concentrations in this report were determined by dose-effect analysis software (7).

Ro 24-7429, AZT, ddC, and nevirapine displayed concentration-dependent inhibition of HIV-1-induced syncytium formation in HT4-6C cells as single agents (Table 1). Analyzed by the median-effect principle and the isobologram method, the interaction between the HIV Tat antagonist and either nucleoside HIV RT inhibitor showed marked synergy at levels at which more than 50% reduction in HIV-1-induced syncytium formation was achieved, whereas the combination of Ro 24-4279 with the nonnucleoside RT inhibitor nevirapine resulted in an additive effect at levels of inhibition greater than 50% (Table 1).

Ro 24-7429, Ro 31-8959, AZT, ddC, and ddI produced concentration-dependent inhibition of HIV-1 p24 antigen production following acute infection of CEM cells (data not shown). Analysis by isobologram clearly shows that Ro 24-7429 combined with Ro 31-8959 (Fig. 1A), AZT, or ddC (data not shown) produced synergistic inhibition of HIV-1 p24 production. Interestingly, in similar studies, the interaction between Ro 24-7429 and ddI appeared additive (Fig. 1B). The results of combinations in CEM cells were confirmed in repeat experiments. We have found that Ro 24-7429 inhibits the activity of purified recombinant HIV-1 RT (50% inhibitory concentration, 2.1 μM) (11), but such activity does not play an apparent role in anti-HIV activity in chronically infected cells (10). Whether this anti-RT activity of Ro 24-7429 is manifest against

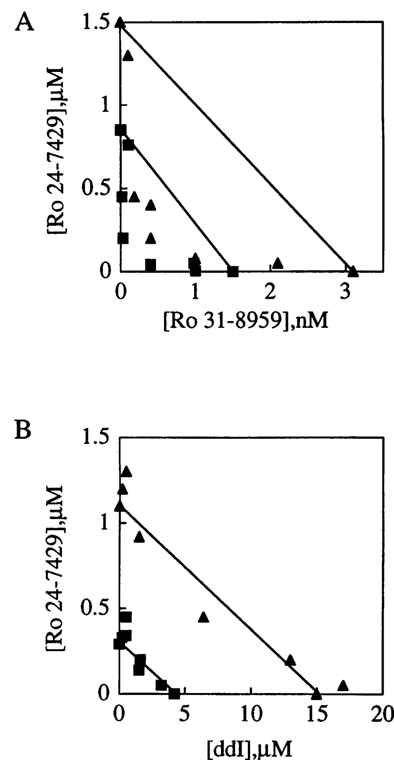

FIG. 1. Isobologram analysis of the two-drug interaction between Ro 24-7429 and Ro 31-8959 (A) or ddI (B) against HIV-1 p24 antigen production in CEM cells at effect levels of 50% (■) and 90% (▲) inhibition.

TABLE 2. CIs of two- and three-drug interactions against HIV p24 antigen production in CEM cells

Drug regimen	Molar ratio of drug concns	Expt no.	CI[a] at an inhibition of:	
			50%	90%
Ro24-7429 + Ro31-8959	400:1	1	0.75	1.03
	400:1	2	0.99	1.07
Ro24-7429 + ddC	400:1	1	0.86	0.84
	400:1	2	0.75	1.01
ddC + Ro31-8959	1:1	1	0.92	0.51
	1:1	2	0.83	1.01
Ro24-7429 + Ro31-8959 + ddC	400:1:1	1	0.95	0.81
	400:1:1	2	1.05	0.84

[a] For the definition and calculation of CI, see references 5 through 9.

acute HIV-1 infection, thus affecting the interaction with the nucleoside triphosphate RT inhibitors, is unclear.

Inhibitors of HIV RT (ddC), HIV Tat (Ro 24-7429), and HIV protease (Ro 31-8959) were combined in two experiments at a 400:1:1 molar ratio against acute HIV-1 infection in CEM cells. This three-drug regimen, combining compounds that target separate points in the HIV replicative cycle, showed increasing synergism at effect levels from 50 to 90% (Table 2). Combination of the agents at concentrations which singly produced little or no effect showed some synergism in a two-drug regimen, but addition of the third agent did not enhance the antiviral effect (data not shown).

It is important to note that none of the two-drug interactions in HT4-6C cells or two- or three-drug interactions in CEM cells was antagonistic over a wide antiviral-effect range. In addition, all single-agent concentrations used in combinations in CEM cells in this study were not cytotoxic as measured by MTT reduction assay (29). No cytotoxicity was observed for the two- or three-drug combinations tested in CEM cells as well.

Chemotherapeutic approaches to the treatment of patients with HIV infection have been suboptimal. As more drugs which target unique sites of HIV replication are identified, the potential for certain combinations to dramatically affect disease progression is increased. In this study we have sought to assess the combinative interactions of three classes of anti-HIV compounds, inhibitors of HIV RT, HIV Tat, and HIV protease. The benefits of certain combinations in vivo may not be discernible in assays of the type conducted in vitro in this study. For example, the benefits of nucleoside combinations like AZT and ddC (27) may exceed those predicted by the synergy observed in vitro (13) and may not be explained by the delayed emergence of resistance (30). It is possible that different nucleosides are phosphorylated to active antiviral compounds differently in separate cell types or at different stages of the cell cycle in a single cell type (16, 36). Similarly, compounds that inhibit HIV replication in chronically infected cells, like Tat and protease inhibitors, may act on populations of cells in infected patients that are unaffected by reverse transcriptase inhibitors which must act prior to integration. Thus, in vitro assays may provide useful guidance to identify potentially synergistic or antagonistic combinations, but such assays cannot reflect the complexity of the infected patient.

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