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POTENT AND SELECTIVE ACTIVITY OF A NEW CARBOCYCLIC NUCLEOSIDE ANALOG (CARBOVIR: NSC 614846) AGAINST HUMAN IMMUNODEFICIENCY VIRUS IN VITRO

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Carbocyclic 2',3'-didehydro-2',3'-dideoxyguanosine (Carbovir: NSC 614846), a novel nucleoside analog, emerged as a potent and selective anti-HIV agent from a large screening program conducted by the National Cancer Institute and its contractors. Its hydrolytic stability and its ability to inhibit the infectivity and replication of HIV in T-cells at concentrations of approximately 200- to 400-fold below toxic concentrations make carbovir a top-priority candidate for development as a potential antiretroviral agent in the treatment of AIDS patients.

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Abbreviations: Human Immunodeficiency Virus (HIV); Acquired Immunodeficiency Syndrome (AIDS); 3'-azido-2',3'-dideoxythymidine (AZT); dideoxycytidine (ddC); dideoxyadenosine (ddA); dideoxyinosine (ddI); National Cancer Institute (NCI); fetal calf serum (FCS); interleukin-2 (IL-2); multiplicity of infection (MOI); microculture tetrazolium assay (MTA); and phenazine methosulfate (PMS); 2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide, inner salt, sodium salt (XTT); fetal bovine serum (FBS).

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Following the identification of a human retrovirus (HIV) as the etiologic agent of AIDS (1-3), an intense effort has been made to identify drugs for treatment of this debilitating, lethal disease. Using human host-cell-based screening systems, it was possible to identify AZT as a candidate for development and, with unprecedented speed, enter it into clinical testing (4). In randomized placebo-controlled trials, AZT was rapidly shown to have therapeutic value and has subsequently become the "drug of choice" for treatment of AIDS (5). Other nucleoside analogs, ddC, ddA, and ddI, have also been shown to have an in vitro anti-HIV activity commensurate with development to clinical trials (6). Early clinical results with ddC have indicated therapeutic activity, but also some undesirable side effects (e.g., peripheral neuropathy) (7). It should also be noted that AZT, while clinically useful in many settings, also is associated with substantial toxicity, especially myelosuppression (8).

As part of a very large-scale anti-HIV drug screening and AIDS drug development program sponsored by the NCI (9), we have tested a series of carbocyclic nucleoside analogs. These compounds were given priority for screening because of design features compatible with action as DNA chain terminators and because of previous evidence for antiviral activities of some carbocyclic nucleoside analogs in other viral systems. These include carbocyclic analogs of arabinofuranosylpurine nucleosides (10,11), aminonucleosides (12), lyxofuranosyladenine (13), and xylofuranosylpurine nucleosides (14,15). Carbocyclic nucleosides are structurally analogous to natural and synthetic nucleosides, the only difference being a methylene group which replaces the oxygen atom of the carbohydrate ring. Earlier studies showed that these analogs, which lack the labile glycosidic bond, are stable to cleavage by phosphorylases and hydrolases while retaining the potential for therapeutically useful interaction with other enzymes involved in nucleotide metabolism (16). The chemical and biological properties of carbocyclic nucleosides has recently been reviewed (17).

We describe here the identification of a novel carbocyclic nucleoside that is a potent and selective inhibitor of HIV in vitro. Carbovir (Fig. 1)

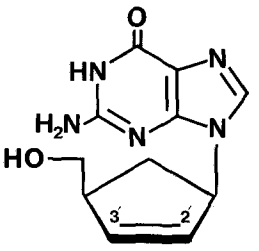


Figure 1. Chemical structure of carbocyclic 2',3'-didehydro-2'-3'-dideoxyguanosine (carbovir: NSC 614846).

emerged and was rapidly confirmed as the most potent and selective anti-HIV compound in primary screening of 75 carbocyclic nucleoside analogs.

MATERIALS AND METHODS

Compounds. All carbocyclic nucleosides were from the common intermediate, cis-4-acetamidocyclopent-2-enemethyl acetate, previously described (12). Spectra (infrared, ultraviolet, and proton magnetic resonance), and elemental analysis (C, H, N) are consistent with the structures present in Fig. 1. Details of the synthetic methods will be presented elsewhere.

Infection and Distribution of Cells to Microtiter Trays. Cells are treated in 50-ml conical centrifuge tubes for 30 minutes with 1-2 µg/ml of polybrene at 37°C, and pelleted (8 min., 1200 rpm). Virus is added [in RPMI-1640, 10% FCS, with IL-2 (for ATH8 cells), and antibiotics] to provide an MOI of approximately 0.01. An MOI of 0.01 is obtained by adding 10^3 infectious units of virus to 10^5 cells. Medium alone is added to virus-free control cells. The treated or control cells are incubated for 1 hour at 37°C in 5% CO_2 in air. Infected or uninfected cells are diluted to give 1 x 10^4 cells/100 µl (2 x 10^4 cells/100 µl for ATH8 cells). Infected or uninfected cells (100 µl) are distributed to appropriate wells of a 96-well, U-bottom, microtiter plate. Each compound dilution is tested in duplicate with infected cells. Uninfected cells are examined for drug cytotoxicity in a single well for each dilution of compound. Drug-free control cells, infected and uninfected, are run in triplicate. Drug blanks receiving medium alone are also included. The plates are then incubated at 37°C in 5% CO_2 until ready for drug addition.

Drug Dilution and Addition. The first dilution of each drug is made in a test tube according to the dilution specified. The remaining dilutions are made in 96-well plates. All wells of each plate are filled with 225 µl of medium using a Cetus liquid handling system. Twenty-five microliters (25 µl) of 2 diluted compounds are manually added to a filled dilution plate in the same order in which the drugs will appear on the test plate. The two compounds are then serially diluted 10-fold using the Cetus liquid handling system. Using a multi-channel pipettor with 6 microtips, 100 µl of each drug dilution is transferred to the test plate. Test plates are incubated at 37°C in 5% CO_2 in air for 7 days or until the virus-infected control cells are lysed as determined microscopically.

Quantitation of Viral Cytopathogenicity and Drug Activity. Data are derived from the MTA as previously described (18). An XTT-PMS solution is prepared immediately prior to its addition to the wells of the culture dish

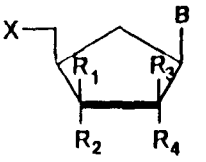
[1 mg/ml XTT (19,20) solution in media without FCS]. The stock PMS (15.3 mg PMS/ml FBS) solution is diluted 1:100 (0.153 mg/ml). The diluted PMS (40 µl) was added to every ml of XTT required (this will give a final PMS concentration of .02 mM after addition to the plate). The XTT-PMS mixture (50 µl) is added to each of the appropriate wells. The plate is incubated for 4 hours at 37°C. The plate lids are removed and replaced with adhesive plate sealers (Dynatech). The sealed plate is inverted, placed in the V-MAX plate reader (Molecular Devices, Inc.), and read at 450 nm to quantitate the formazan production. Antiviral activity is indicated by a dose-related increase in formazan production, as a result of increased cellular survival, with increasing concentrations of active antiviral drugs. This increase is followed by a decrease in formazan production at the highest concentrations as a result of drug toxicity (Fig. 2).

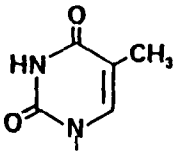
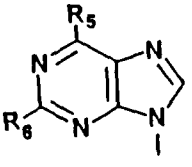
RESULTS AND DISCUSSION

Table 1 summarizes the chemical structures and anti-HIV activities of several carbocyclic nucleoside analogs. The corresponding nucleosides in which the C-8 of the purine ring is replaced by N are not included in the table. Five analogs of carbovir were also confirmed to have reproducible *in vitro* anti-HIV activity (defined as 50% or greater reduction of cytopathic effect in two or more independent experiments). Structure-activity relationship studies (data not shown) indicated that replacement of the guanosine heterocycle by adenine (NSC 614841) decreased selectivity 10-fold. Also, saturation of the carbocyclic sugar moiety either decreased activity (NSC 614844) or abolished (NSC 615813) activity. In addition, substitution of the purine moiety with a pyrimidine or replacement of the C-8 carbon of the purine with nitrogen abolished activity. None of the analogs with substituents at either the 2'- or 3'-position exhibited activity. Even an azido function at these positions did not confer activity. Thus, either a 2',3'-dideoxy or a 2',3'-unsaturated sugar moiety is an essential structural requirement for anti-HIV effect.

In addition to AZT, ddC ranks among the most potent and selective inhibitors of the AIDS virus (6,21). Thus, the active compounds from the primary screen (see Table 1) were further tested in direct comparison to ddC using a variety of host cell lines. The 2',3'-unsaturated guanosine analog, carbovir, was the most selective anti-HIV compound. Its therapeutic index (ratio of 50% cytotoxic dose, IC_{50}, to 50% antiviral effective dose, EC_{50})

Table 1. Structures and Anti-HIV Activities of Carbocyclic Nucleoside Analogs



B	NSC#	R_1	R_2	R_3	R_4	R_5	R_6	X	Activity
	615823	H	H	H	H			OH	-
	615824	H	--	H	--			OH	-
	613805	H	OH	OH	H	Cl	H	OH	-
	614842	H	H	H	H	Cl	H	OH	-
	613824	H	--	H	--	Cl	H	OH	-
	613807	OH	H	OH	H	NH_2	H	OH	-
	615828	H	OH	OH	H	NH_2	H	OH	-
	613822	H	OH	OH	H	NH_2	H	Cl	-
	613820	H	OH	OH	H	NH_2	H	NH_2	-
	613803	H	OH	OH	H	NH_2	H	CH_2OH	-
	613806	OH	H	H	OH	NH_2	H	OH	-
	613809	H	N_3	OH	H	NH_2	H	OH	-
	614835	H	N_3	H	OH	NH_2	H	OH	-
	613812	H	H	H	N_3	NH_2	H	OH	-
	614834	H	NH_2	OH	H	NH_2	H	OH	-
	614836	H	NH_2	H	OH	NH_2	H	OH	-
	614844	H	H	H	H	NH_2	H	OH	+
	614841	H	--	H	--	NH_2	H	OH	+
	614845	H	H	H	H	OH	H	OH	-
	614848	H	--	H	--	OH	H	OH	-
	613804	H	OH	OH	H	SH	H	OH	-
	615826	OH	H	H	OH	SH	H	OH	-
	614843	H	H	H	H	SH	H	OH	-
	613826	H	--	H	--	SH	H	OH	-
	613816	H	OH	OH	H	Cl	NH_2	OH	-
	615812	H	H	H	H	Cl	NH_2	OH	-
	613825	H	--	H	--	Cl	NH_2	OH	+
	613819	H	OH	OH	H	NH_2	NH_2	OH	-
	615814	H	H	H	H	NH_2	NH_2	OH	+
	614850	H	--	H	--	NH_2	NH_2	OH	+
	615827	OH	H	H	OH	OH	NH_2	OH	-
	615813	H	H	H	H	OH	NH_2	OH	-
(carbovir)	614846	H	--	H	--	OH	NH_2	OH	+

Primary anti-HIV screening was performed using the MT-2 cell line and the HTLV-IIIB virus isolate. Details of the testing protocol are described in the methods section. Activity (+) is defined as a reduction of virus-induced viral cytopathic effects by 50% or more in two independent experiments.

ranged from 184 to 403 depending on the cell line used to measure the anti-HIV effect (Table 2). Figure 2 compares the inhibitory effects of carbovir on the cytopathogenicity of HIV in MT-2 cells with ddC (left panel), and in ATH8 cells with AZT (right panel). Although carbovir is less potent than AZT, the in vitro data reveal that it is consistently more selective than ddC.

Table 2. Inhibition of Human Immunodeficiency Virus (HIV)-Induced Cytopathic Effects in Different Host Cells by the Carbocyclic Analog of 2',3'-Dideoxy-2',3'-Didehydroguanosine (Carbovir: NSC-614846)*

	ATH8			MT-2			CEM		
Compound	EC_{50} µg/ml	IC_{50} µg/ml	TI	EC_{50} µg/ml	IC_{50} µg/ml	TI	EC_{50} µg/ml	IC_{50} µg/ml	TI
Carbovir	0.19	35.0	184	0.19	41.8	220	0.15	60.5	403
ddC	0.24	18.0	75	0.29	44.0	152	0.11	12.3	112
AZT	0.10	30.1	301	0.09	64.0	711	0.05	184	3680

*All data represent the means of several experiments.

The effective concentration, 50% (EC_{50}) represents the concentration of test agent that increases (protects) formazan production in infected cultures to 50% of untreated, uninfected cell controls. The inhibitory concentration, 50% (IC_{50}) represents the toxic concentration of drug that reduces formazan production in uninfected cultures to 50% of untreated, cell controls. Microcomputer-calculated EC_{50} is determined by simple linear interpolation from the data as illustrated in Figure 2. The therapeutic index is determined by dividing the IC_{50} by the EC_{50}.

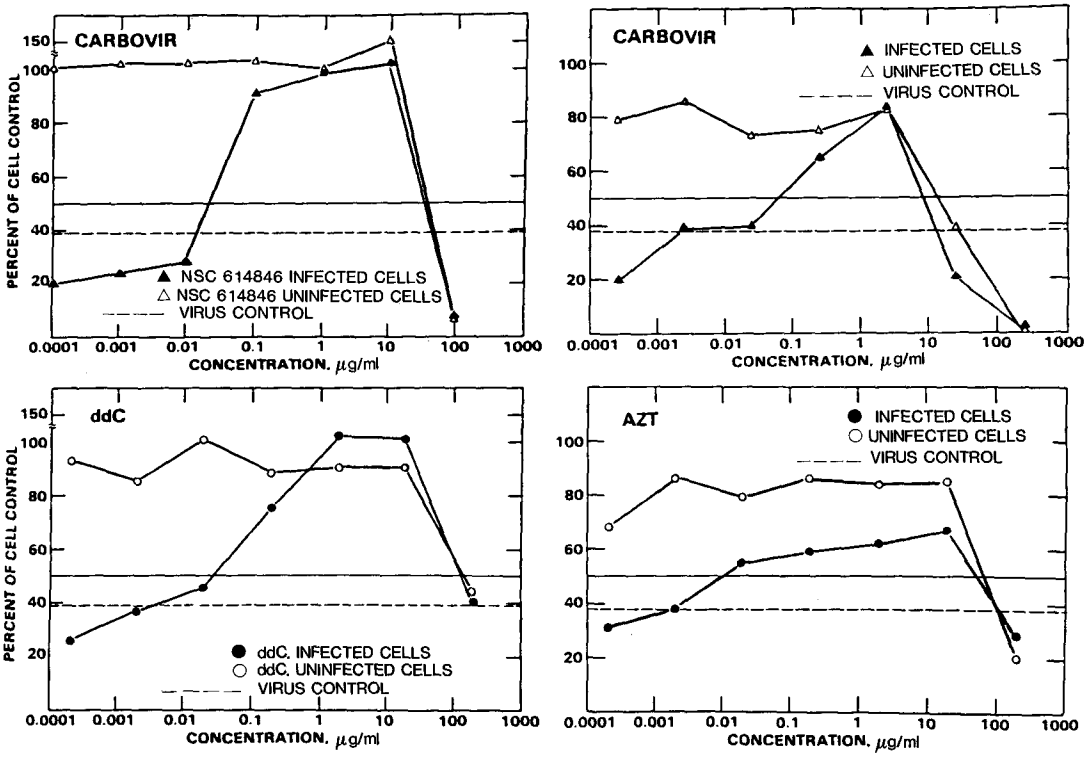


Figure 2. HIV antiviral assays of carbovir and ddC in MT-2 cells (left panel), and of carbovir and AZT in ATH8 cells (right panel). Infected (bottom curve with closed symbols in each panel) or uninfected (top curve with open symbols in each panel) target cells (10^4/well) were treated with 10-fold serial dilutions of drugs. Data were derived from the microculture tetrazolium assay as described in the methods section. The solid horizontal line in each panel indicates 50% level and is used for determination of EC_{50} and IC_{50}.

Table 3. Effect of Carbovir (NSC-614846) on HIV Replication When Measured by p24 gag Protein Expression in CEM Cells

Sample	p24 Synthesis (ng/ml)	Reduction in p24 Expression (%)
Virus Controls	>800	--
Carbovir (NSC-614846)	28	>99.97
AZT	32	>99.96
ddA	38	>99.95

Suppression of p24 internal core antigen synthesis by test drugs. Data obtained by antigen capture ELISA (duPont) of supernatants from treated or untreated (virus control) microcultures prior to the addition of tetrazolium salt. For each drug, p24 determinations were performed at the drug concentration exhibiting maximum therapeutic effect.

Carbovir was also evaluated for its inhibitory effects on the expression of viral antigen in HIV-infected CEM cells (Table 3). Production of viral p24 core antigen at optimal inhibitory concentrations of the antiviral agents indicated comparable activities for AZT, ddA, and carbovir.

Carbovir represents the most promising compound from this new class of potent and selective anti-HIV agents. Its hydrolytic stability and its ability to inhibit the infectivity and replication of HIV in T-cells at concentrations of approximately 200- to 400-fold below toxic concentrations make carbovir an excellent candidate for development as a potential antiretroviral agent in the treatment of AIDS patients. Because of these features, the NCI's Decision Network Committee has selected carbovir for initial preclinical development studies. As a result, animal pharmacokinetic studies, toxicity evaluations, and mechanism of action studies are currently underway in several laboratories.

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