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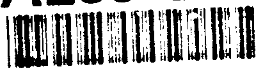

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12a. DISTRIBUTION/AVAILABILITY STATEMENT

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This project is concerned with the synthesis of suicide inhibitors of reverse transcriptase. This is a key enzyme in replication of HIV, the causative agent of AIDs. A suicide substrate contains a latent reactive group which becomes activated only as a consequence of the normal catalytic activity of the target enzyme and irreversibly inactivates it. Suicide substrates are thus inherently selective and have less side effects when used as drugs. We have synthesized over 40 compounds with these potentialities and screened them for antiviral activity in tissue cultur A number of active compounds, including a new class, the nucleoside spiroxiranes, have been identified which inhibit viral replication in cell culture. These compounds do not inhibit normal cell growth as shown by their favorable therapeutic index on lymphocyte cytotoxicity assays and because of their novel properties, show considerable promise as a new generation of antivirals.

RAI; Antiviral; Virology; HTLV-III; Biochemistry; HIV

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SUMMARY

This project is concerned with the synthesis of suicide inhibitors of reverse transcriptase. This is a key enzyme in replication of HIV, the causative agent of AIDS. A suicide substrate contains a latent reactive group which becomes activated only as a consequence of the normal catalytic activity of the target enzyme and irreversibly inactivates it. Suicide substrates are thus inherently selective and have less side effects when used as drugs. We have synthesized over 40 compounds with these potentialities and screened them for antiviral activity in tissue culture. A number of active compounds, including a new class, the nucleoside spiroxiranes, have been identified which inhibit viral replication in cell culture. These compounds do not inhibit normal cell growth as shown by their favorable therapeutic index on lymphocyte cytotoxicity assays and because of their novel properties, show considerable promise as a new generation of antivirals.

The drug, Foscarnet (PFA, Phosphonoformate) is an excellent inhibitor of reverse transcriptase *in vitro*, but is much less active against the AIDS virus growing in tissue culture. This is related to the highly charged nature and consequent low cellular bioavailability of the compound. We have developed synthetic routes to new PFA analogs which are readily taken up by cells and which are 20-30 times more active than the parent compound PFA against virus replication in tissue culture.

One of the problems with AZT has been the short blood half life necessitating administration every 4-6 hours. We have developed prodrug forms of AZT which overcome this problem.

The rationale underlying this diversification of approaches and the three types of viral chemotherapeutic agents which have been developed, is as follows:

It is estimated that there are currently between 1 and 2 million asymptomatic HIV-infected individuals in the U.S. today. In the near-term unavailability of a vaccine, chemotherapy is the only available antiviral treatment. Administration of AZT to asymptomatic HIV carriers shows promise in slowing progression of the disease. By reducing infectious virus load, it may also provide some of the immediate functions of a vaccine. However, there are problems associated with the widespread use of chemotherapy in HIV infection:

1. The incidence of serious side effects in many patients taking AZT;
2. The development of drug resistant variants of HIV;
3. The high cost (≈ $7,500 per patient annually);
4. Low compliance in asymptomatics because of high dosage frequency and cost.

These developments have established a clear need for new drugs to allow alternative therapies when drug resistance develops or side effects occur. Combination therapy with drugs based on different mechanisms of actions will reduce the emergence of resistant mutants and increase the opportunity for additive or synergistic effects. Combinations of prodrugs which increase cellular bioavailability and decrease blood clearance will reduce both drug dose and frequency.

The nucleoside spiroxirane and suicide/affinity inhibitor compounds we have synthesized, have a different mechanism of action than chain terminators such as AZT, DDC and DDI. This will reduce the possibility of developing co-resistance.

The sterol Phosphonoformate diesters by increasing the bioavailability of PFA (foscarnet), reduce drug dosage and frequency and because of their different site of action are excellent candidates for combination therapy with nucleoside analogs.

The AZT-sterol Dicarboxylates increase the blood half life of AZT from 30 minutes to over 72 hours and show good accumulation in tissues, including brain. Their observed antiviral activity in tissue culture is due presumably to release of free AZT following uptake and hydrolysis (this hypothesis will be tested in the upcoming final year of the grant by using the ^{3}H-labeled AZT derivatives). By sparing AZT and allowing weekly administration, our new derivatives have the potential for reducing the costs of AZT therapy for all HIV-positives in the U.S. population (if this becomes desirable) from a prohibitive $10 billion to less then $300 million annually.

A. INTRODUCTION

Retroviruses are characterized by the anomalous storage of their genetic information in th form of RNA. They are a diverse group of organisms which have been shown to be causative agent in a number of mammalian and avian diseases states (1-3). Lentiviruses are a subfamily o retroviruses which have been linked to the induction of arthritis, encephalitis, and slow neurologic diseases in certain species. Many of these viruses are characterized by their ability to develop nove antigenic variants that can escape temporarily from host immune surveillance (5-6). Interest i retroviruses in human disease states has increased with the isolation of HTLV I and II as the causativ agents of adult human T cell leukemias and of HIV I and II as the cause of Acquired Immun Deficiency Syndrome (AIDS) (7-8).

The most recent evaluation of the AIDS epidemic in the U.S. (4) indicates that the currer total of approximately 70,000 AIDS cases is accompanied by a further 1 to 1.5 million infecte individuals, the majority of whom will progress to the clinically overt disease state in the next 5- years. In the near-term absence of an effective vaccine an increase in this infected/symptomle category is likely to continue for a number of years.

In addition to an antiviral therapy for acute cases of AIDS therefore, there is clearly pressin need for an effective antiviral drug regimen suitable for administration to large numbers of otherwis healthy individuals in order to arrest progression of the disease. By preventing viral replication, suc therapy may also reduce dissemination of the virus and thus indirectly fulfill some of the immedia functions of a vaccine. The current drug of choice, AZT can have undesirable side effects when use at maximum therapeutic dosages over prolonged periods. In addition HIV-variants with increase resistance to AZT have recently begun to appear. Combination therapy with drugs having a differe mechanism of action can reduce the likelyhood that resistant variants will appear. The drugs beir developed in this project are designed with the above potential for use in long-term combinatic therapy low of toxicity as a primary goal.

Replication of retroviruses is critically dependent upon a single enzyme called rever transcriptase (RT). This is an RNA-dependent DNA polymerase first found in the purified virio of Rous sarcoma virus by Temin (10) and murine leukemia virus by Baltimore (11). The enzym transcribes the viral RNA into DNA in the first step of viral replication. Since host cells do n contain reverse transcriptase, several molecules of the enzyme are packaged in each virion and ent the cell together with the viral RNA. It is at this stage that viral replication is most sensitive inhibitors of the reverse transcriptase, before amplification of the viral genome and pol gene directe synthesis of endogenous RT has taken place.

The principle that inhibitors of reverse transcriptase will inhibit replication of retroviruse is well established. For example, 3'-azido-3' deoxythymidine, the triphosphate of which inhibits th RT activity of HIV, is a potent inhibitor of virus replication in cultured H-9 cells in the rage of 1 10 micromolar (14) and is being used successfully in patients with AIDS. Prolonged administratio may cause serious side effects. Another agent which has shown promise is phosphonoformate (PFA Foscarnet) which inhibits the RT activity of with an I_{50} of only 0.1 micromolar. However, muc higher concentrations of up to 340 micromolar are required for complete inhibition of HI replication in H-9 cells (15). Other drugs including the dideoxycytidines which are also based upo inhibition of RT by chain termination of the viral template are in clinical trial. Since none of thes drugs permanently inactivate the reverse transcriptase and since they do not accumulat intracellularly in significant amounts, virus replication will resume when blood levels of the drug decrease.

Since there is no complete animal model for HIV which reproduces the immunodeficiency, as an adjunct to the tissue culture studies thisproject will also utilize the Rauscher murine leukemia virus which has a reverse transcriptase with close homology to that of HIV, for in vivo testing of new antiviral drugs. This will be supplemented by testing drug toxicity in human T lymphocytes growing in tissue culture.

Suicide Inhibitors of Reverse Transcriptase

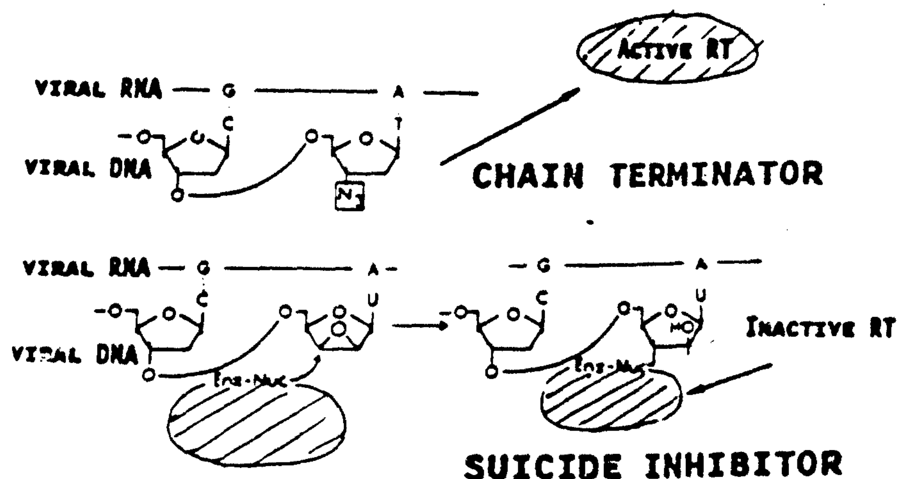
Suicide inhibitors, also known as Kcat inhibitors, are active site directed substrate analogs which contain a latent, reactive group. Following cleavage of the analog by the target enzyme the reactive group is released at the active site and inactivates the enzyme (12,13). Inhibitors of this type have several potential therapeutic advantages int that they can be designed to be highly specific for

the target enzyme. Since most normal cells do not contain active or functional reverse transcriptases (29), this reduces the possibility of side effects. Furthermore, by their nature, inhibitors of this type remain metabolically inert unless specifically cleaved by the target enzyme. Finally, the inhibition is permanent and irreversible, so that viral replication will not resume when the blood levels of the drug decrease. This feature could be particularly useful in long-term therapy of chronic human retrovirus infection.

Inhibition of retroviral replication by 3'-Nucleoside Spiroxirane Derivatives:

An apparent suicide inhibitor of E.Coli DNA polymerase was described by Abboud et al. (48). It was shown that adenosine 2'3'-riboepoxide 5'-triphosphate irreversibly inactivated the enzyme by a covalent interaction. Because of the intrinsic reactivity of the epoxides however they are not suited for use under physiological conditions. We will therefore synthesize a series of nucleoside 3'-spiroxirane analogs. These are designed to inhibit the enzyme by an analogous suicide mechanism and are based upon the observation that the uridine 3'-ribospiroxirane derivative is an inhibitor of retroviral replication in cultured cells and selectively suppresses reverse transcriptase production by the VCF 21 (Moss) vaccinia HIV-RT recombinant. Furthermore at therapeutic levels it has no significant effects on ^{3}H-thymidine incorporation in cultured T-lymphocytes.

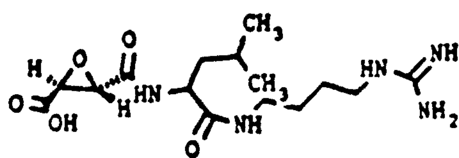
In the first step of replication of viral DNA by reverse transcriptase, activation of the primer 3'-OH by the enzyme results in incorporation of the suicide nucleotide by a normal 3',5'-phosphodiester linkage. At this point the inhibition resembles a simple chain termination similar to that produced by other 3'-blocked nucleoside analogs such as 3'-azidothymidine (AZT) and dideoxycytidine. However when the next step in the elongation reaction is attempted, the enzyme by activating the 3'-oxygen of the spiroxirane functionality becomes alkylated by the substrate and effectively commits suicide. A primary objective of these studies will be to characterize the kinetics of inhibition of the purified HIV-reverse transcriptase by the nucleoside spiroxirane triphosphates to determine if this second suicide step occurs and contributes to the observed antiviral activity of these compounds. Initial indications that the analogs can accomplish irreversible inactivation of intracellular RT support this possibility, and further efforts will be made to incorporate features into the molecule which favor the suicide pathway.
Because of the greater stability of the oxirane ring under physiological conditions these analogs offer several advantages over the simple epoxide types: A schematic representation of the consequences of an interaction with a suicide inhibitor versus a chain terminator is given below.



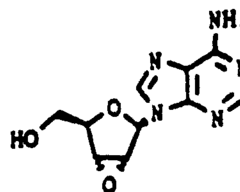
B. WORK ACCOMPLISHED

1. Epoxide Suicide Inhibitors:

Epoxide-containing irreversible (suicide) inhibitors have been reported for a number enzymes, the structures of two such compounds are shown below. One of the first, [N-(L-3-*tran* carboxyxiran-2-carbonyl)-L-leucyl]-amido (4-guanido) butane was isolated from *Aspergill japonicus* and was found to irreversibly inhibit cysteine proteases (49) by alkylation of the sulfydr group at the active site.

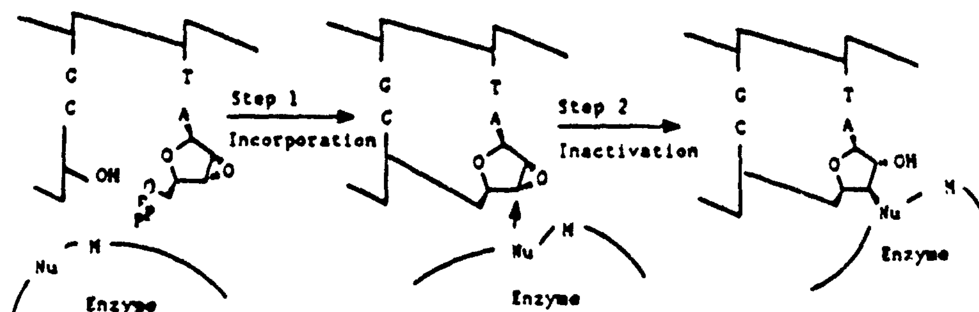


[N-(L-3-*trans*-carpoxioxiran-2-carbonyl) -L-leucyl]-amido(4-guanido)butane



9-(2,3-Anhydro-β-D-ribofuranosyl)adenine

A 2',3'-riboepoxynucleoside, 9-(2,3-anhydro-β-D-ribofuranosyl)adenine, was shown, as the 5' triphosphate, to irreversibly inhibit avian myeloblastosis virus DNA polymerase (a revers transcriptase) (48). Its mechanism of inactivation, shown below, is believed to involve alkylation c the enzyme in the active site upon activation of the epoxide ring after incorporation of the nucleosid to the end of the growing DNA strand (46).



A proposed mechanism for RT inactivation by 2',3'-riboepoxyadenosine.

A similar compound, 1-(2,3-anhydro-β-D-lyxofuranosyl)cytidine, was recently synthesized by Broder et. al. and was found to inhibit HIV *in vitro* (50). Although the mechanism of inactivation was not elucidated, irreversible inactivation was considered a possibility. We have also synthesized the corresponding 1-(2,3-anhydro-β-D-lyxofuranosyl) uridine and found it to have antiviral activity. However these epoxide derivatives unlike the spiroxiranes (see below) are also cytotoxic at higher concentrations probably owing to the greater reactivity of the epoxide ring (50).

2. Preliminary Synthetic and Antiviral Screening Studies:

The reverse transcriptase of HIV is a highly error prone enzyme, a factor which probably contributes to the high mutation rate of the HIV genome. We have shown that reverse transcriptase readily incorporates dUTP in place of dTTP when transcribing from a poly rAdT template, and this incorporation, like that of dTTP, is particularly sensitive to inhibition by PFA (figure).

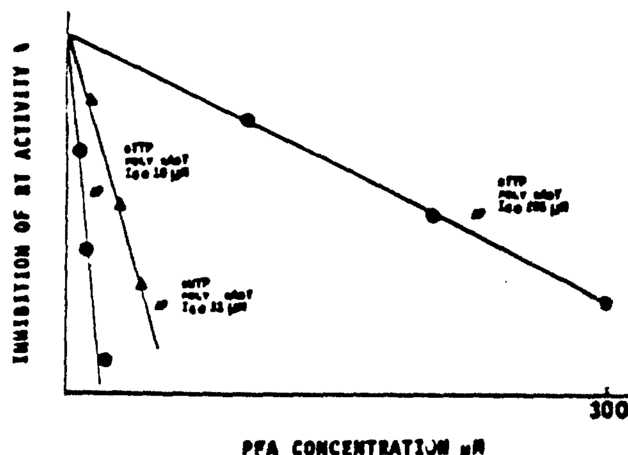


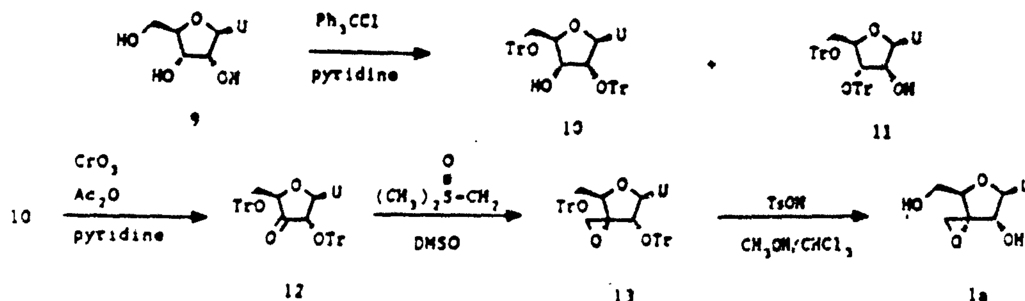
Figure 1: Reverse Transcriptase catalyzed Incorporation of d-UTP: Differential Inhibition of First-strand Synthesis by Foscarnet.

Since uridine is an unnatural base for the cellular DNA polymerases, initial studies were carried out using uridine nucleosides to enhance the antiviral selectivity.

3. Synthesis of Uridine 2' and 3'-Ribospiroxiranes:

3'-uridine spiroxirane was synthesized from uridine by the sequence of reactions shown below. The procedure begins with the selective tritylation of uridine by the procedure of Cook and Moffat (38). Chromatographic separation of the isomers yielded the 2'5'and 3'5'-di-O-trityluridines in good yield. The 2',5'isomer was oxidized with chromium trioxide in pyridine/acetic anhydride to 3',5'-di-O-trityl 3'-ketouridine. Treatment of this compound with two equivalents of dimethyloxosulfonium methylide yielded the 2',5'-di-O-trityl spiroxirane derivative which upon mild acid catalyzed hydrolysis yielded the deprotected 3'-uridine spiroxirane.

The corresponding 2'-spiroxirane derivative was also prepared from the 2',3'-di-O-trityl isomer by analagous procedures and characterized by IR and NMR spectroscopy.



4. Antiviral Activity of Uridine Spiroxiranes and Cytotoxicity Screening in Human T-Lymphocytes:

A series of approximately 30 nucleoside analogs, suggested by the considerations outlined in section B, were synthesized. These were screened for ability to inhibit replication of equine infectious anemia virus growing in equine dermal fibroblasts. This is a lentivirus having a close homology with HIV (30) and is non pathogenic to humans. It is used as a preliminary screen for antiviral drugs before testing against HIV grown in A 3.01 human T-lymphocytes as described in section D. Cytotoxicity was evaluated at concentrations from 25-500μM in IL-2 supplemented CTLL T-Lymphocyte cultures pulsed with ^{3}H thymidine at 24 and 48 hours.

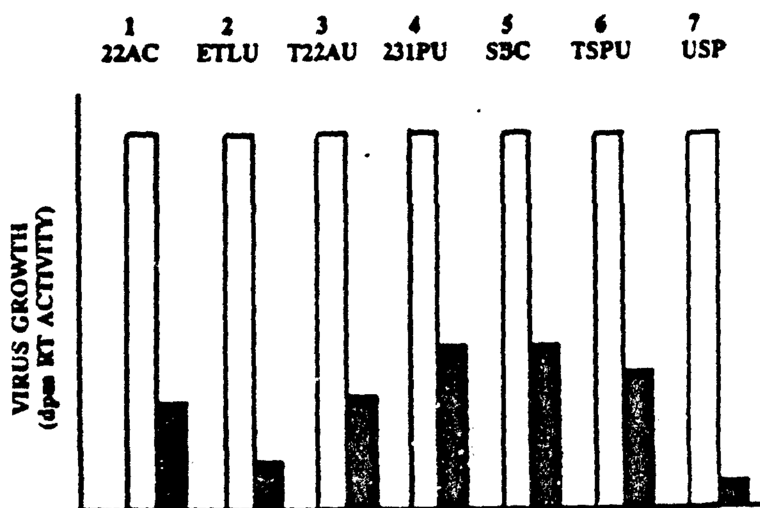


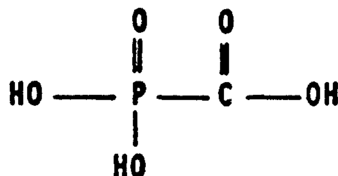
Figure 2: Antiviral Activity of Some Epoxy- and Oxirane Nucleosides:

Viral replication was monitored by RT assay on the pelletized virions from the culture medium. As illustrated in the figure above, 7 of these compounds inhibited viral replication by greater than 50% when present in the culture medium at a concentration of 100μM. The two most effective analogs were the uridine 2', 3'-lyxo epoxide (compound 2) and the uridine 3'-ribospiroxirane (compound 7). Both compounds were also tested for cytotoxicity by measuring their effects on ^{3}H-thymidine incorporation in the IL-2 dependent CTLL T-lymphocyte cell line in tissue culture.

The 3'-uridine spiroxirane derivative was added to control and EIAV infected cultures over a range of concentrations from 5 to 100μM and reverse transcriptase activity was assayed on the pelletized virions after 8 days. Virus replication was almost completely inhibited by 100μM concentrations of the 3' uridine spiroxirane the I_{50} being about 25μM. The corresponding 2'-spiroxirane used as a control had no effect at similar concentrations. The 3'-spiroxirane was non-toxic to T-lymphocytes,the I_{50} for inhibiting ^{3}H-thymidine incorporation being greater than 500μM. Since the epoxide derivative was somewhat inhibitory to T-cell proliferation at concentrations above 100μM, whereas the spiroxirane analog was not, the nucleoside spiroxirane family has been selected for further in depth characterization in this project.

5. Inhibition of Reverse Transcriptase by Phosphonoformic Acid (PFA)

Previous studies have shown that the pyrophosphate analog phosphonoformic acid (PFA) is : excellent inhibitor of reverse transcriptase (14,32).



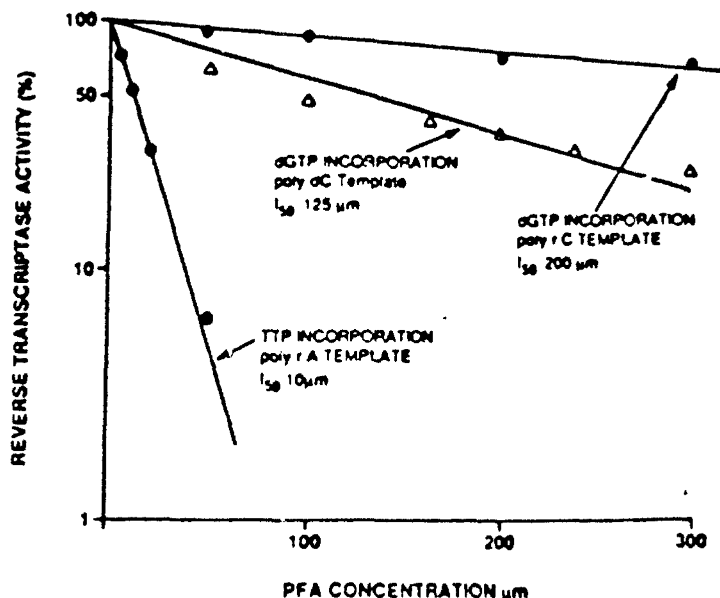
(PFA, FOSCARNET)

Because of reports that PFA differentially inhibited incorporation only of certain nucleoti triphosphates into the viral DNA (35) and in view of the substantial effort to be invested in the ster phosphonoformates (see below) we have carried out an extensive kinetic characterization of the interacti between reverse transcriptase and the inhibitor PFA.

The HIV-RT, was prepared from the vaccinia virus HIV pol-gene construct VCF-21 (Moss). T reverse transcriptase from Moloney Murine Leukemia Virus (MMuLV-RT) and Avian Myeloblastosis Vir (AMV-RT), two retroviruses having homologies with the HIV-RT (15-16), were also used in these init studies.

By use of homologous template-primer combinations with the appropriate ^{3}H labelled nucleoti triphosphate substrate, it was confirmed that RT is remarkably sensitive to inhibition by phosphonoforma but only when incorporating thymidine during first strand synthesis. As shown in Figure 1, PFA inhibit reverse transcriptase incorporation of dTTP using a poly rAdT template. But dGTP incorporation frc either a poly rCdG template or a poly dCdG template (corresponding to second strand virus DNA synthesi was relatively insensitive.

Figure 1: Differential Sensitivity of dTTP and dGTP Incorporation to Inhibition by Phosphonoformate (PF/



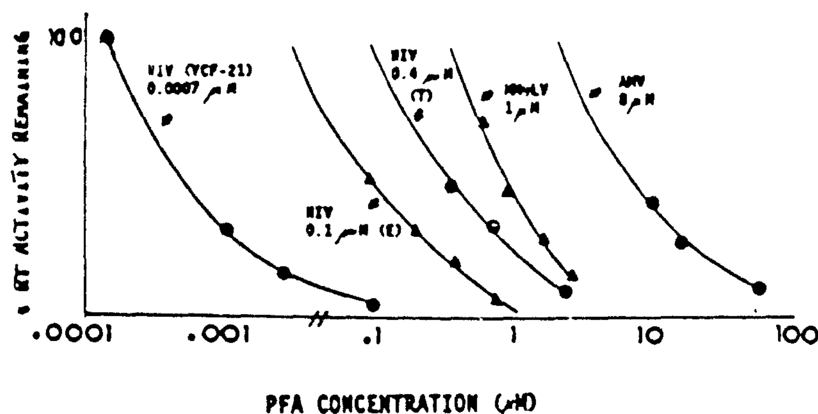
A similar insensitivity to PFA was demonstrated for incorporation of both dCTP and dATP. T kinetics of TTP inhibition by PFA were investigated in depth for the HIV-RT and the AMV and MMuL reverse transcriptases. Despite the close similarity in the reaction mechanism for these three enzymes, t kinetics of inhibition were quite different. This could have important therapeutic consequences f combination drug therapy of HIV with AZT or other kinase inhibitors which lower cellular TTP since it w determine whether inhibition will be additive or synergistic. Whereas the sensitivity of MMuLV-RT to PF was markedly increased by lowering the substrate TTP, the AMV reverse transcriptase, while displayi lower rates of thymidine incorporation at the lower TTP concentrations, was not proportionately mc sensitive to PFA.

At low TTP concentrations, the sensitivity of MMuLV-RT to PFA is increased about five fold frc an I_{50} of 8 μM down to only 1.5 μM PFA. Thus the effectiveness of PFA as a retrovirus inhibitor is likt to be synergistic at low intracellular TTP concentrations only if the viral reverse transcriptase is of t MMuLV type. However, the HIV-RT shows the same sensitivity to PFA over a range of TTP concentratic from 0.5 to 20 μM. Thus the combination of AZT and PFA would be predicted to have additive effe based upon the TTP lowering properties of AZT in certain cell types.

These experiments also demonstrated another potential advantage of PFA if the cellular permeabil problems of the drug can be overcome. The HIV-Reverse transcriptase is the most sensitive to PFA of the retroviral RT's tested the I_{50} ranging from only 0.1 μM to 0.4 μM depending upon the particular templ: primer system used (Figure 2). Also shown in this figure is the enhanced sensitivity of the vaccii recombinant HIV-RT to Foscarnet when expressed in monkey kidney cells. The I_{50} of this enzyme is oı 0.0007 μM. The reasons for this remarkable difference are currently the subject of further investigatic (17) which are however outside the focus of the present project.

PROPERTIES OF WILD TYPE AND RECOMBINANT HIV-REVERSE TRANSCRIPTASES

SENSITIVITY OF VIRAL REVERSE TRANSCRIPTASES TO INHIBITION BY PHOSPHONOFORMATE



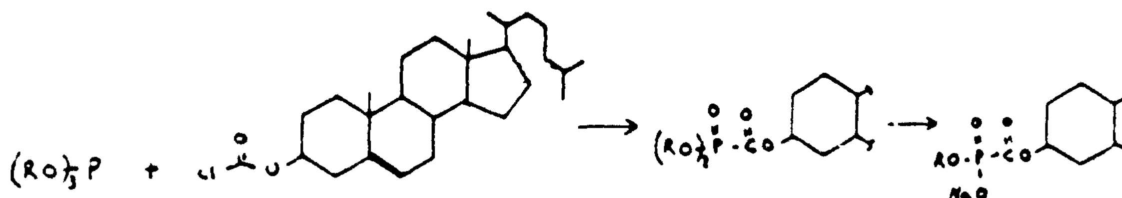
5. Antiviral Properties of the Sterol Phosphonoformates

Despite the sensitivity of the HIV-RT to inhibition by PFA, replication of the virus in tissue cult is relatively insensitive, the I_{50} being of the order of 50 μM.

We have shown in previous studies, by use of sterol ester analogs such as the sterically hindered a hydrolysis resistant cholesterol αα-methyl ethyl caproate (CMEC), that intact cholesterol esters enter ce via a specific endocytotic transport process (18). To enhance deliverability of the active PFA moiety to endosomal sites of viral replication, cholesteryl phosphonoformate ester analogs of PFA have be synthesized and characterized.

I. Cholesteryloxycarbonylphosphonoformates:

A series of mono- and di-alkyl cholesteryloxycarbonylphosphonoformates have been successfu prepared via an Arbozov reaction (19) between trialkylphosphites and cholesterylchloroformate.



Triesters gave acceptable spectroscopic characteristics and were selectively hydrolyzed (19) to the mon and di-sodium salts (20).

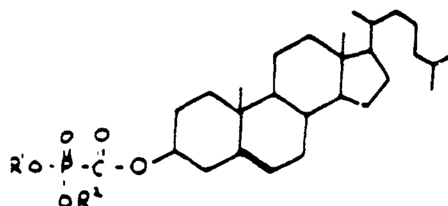


Table 1: Properties of Some Sterol Phosphonoformates.

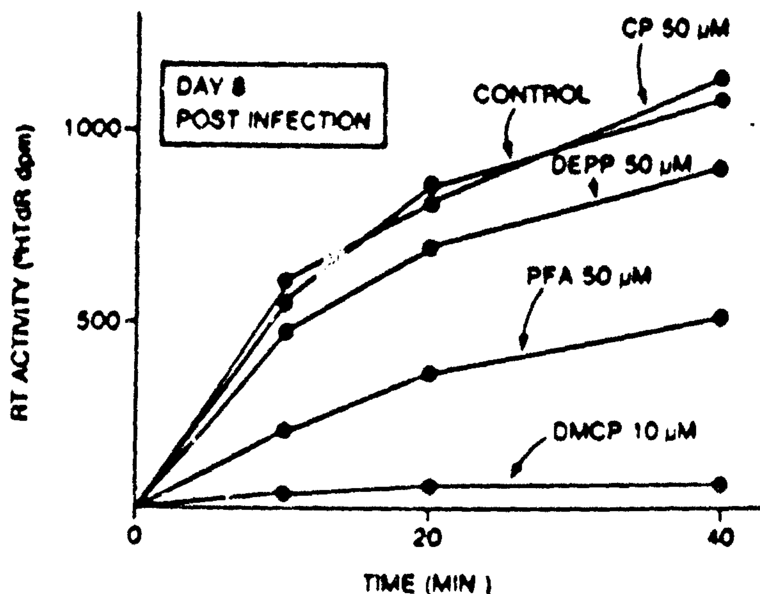
Cmpd.5	R^1	R^2	MP (C°)	R_f
a	C_2H_5	C_2H_5	110-111	0.60 (A)°
b	$CH_2CH_2CH(CH_3)_2$	$CH_2CH_2CH(CH_3)_2$	67-69	0.51 (B)
c	$CH_2CHO(CH_3)_2OCH_3$	$CH_2CHO(CH_3)OCH_3$	106-108	0.30 (A)
d	$CH_2CH_2CH_2CO_2C_2H_5$	$CH_2CH_2CH_2CO_2C_2H_5$	oil	0.33 (A)
e	C_2H_5	Na	≥220	0.29 (C)
f	Na	Na	≥300	-

° Solvent systems: A, 50% ethyl acetate/hexanes, B, 20% ethyl acetate/hexanes, C, 52/14/1/1 $CHCl_3/CH_3OH$/con NH_4OH/H_2O.

A number of compounds of this type having both aromatic and aliphatic substituents have been for antiviral activity in vitro, as detailed below. Incorporation of the sterol moiety in an appropriate t environment can result in compounds having 20-30 times the antiviral potency of the parent compoun Table I lists the effect of increasing the number and size of the R groups on the melting poi chromatographic mobilities of some of these derivatives.

Pending development of the low biohazard CPE assay for HIV infectivity described belo antiviral potency of the drugs was evaluated against Equine Infectious Anemia Virus (EIAV) growing i equine fibroblasts. This is a lentivirus with a reverse transcriptase having the highest degree of hon with HIV-1 RT. The assay consists in measurements of RT activity on pelletized supernatants of in cultures 8 days following infection. These sterol esters of phosphonoformate show an interesting patt antiviral activity in this system (Figure 3).

Figure 3:



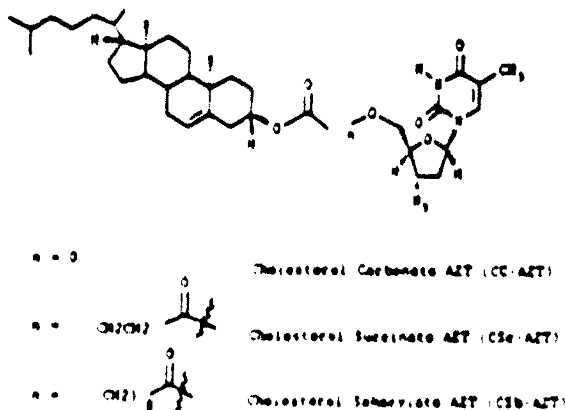
The approximate I_{50} for inhibition of EIAV replication by PFA is between 40 and 50 µM. 1 liethyl-phenyl-phosphonoformate ester (DEPP) is only about 20% as active as PFA and the doubly char lisodium cholesterol phosphonoformate (CP) is essentially inactive. Substitution of the phenyl group w cholesterol group, however, results in a major enhancement of activity. For example, the closely rel DMCP (lower curve, Figure 3) in which cholesterol is esterified to the formate moiety, has 10-20 times m nti-viral activity than the parent compound PFA. Some of the more active analogs display activities cl o the theoretical maximum predicted by the enzyme kinetic studies.

The sterol phosphonoformates display another useful property since the antiviral effects persist fc a considerable time (up to 8 days) after removal of the drug from the culture medium. It seems likely th: this prolonged antiviral protection may be due to intracellular accumulation of the analog followed by slo hydrolysis and sustained release of the active PFA moiety. One of the objectives in this Project is to explo this observation in more depth by measuring rates of intracellular accumulation and hydrolysis of ster phosphonoformates using ^{14}C-labelled compounds synthesized by analagous procedures. In this way ligan which enhance uptake can be distinguished from those which enhance (or reduce) hydrolysis. Th information will be used to enhance pharmacokinetic properties which may prove clinically useful.

SYNTHESIS AND ANTIVIRAL ACTIVITY OF AZT-STEROL DICARBOXYLATES:

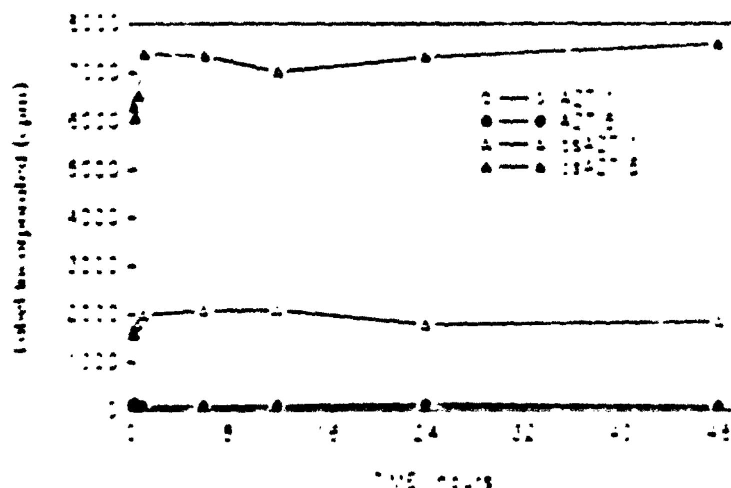
^{3}H-labelled AZT was prepared by $NaB^{3}H_4$ reduction of the 5'aldehyde derivative and coupled cholesterol by a C_{10} (Sebacate) linker as described in section D, to yield cholesteryl sebacyl ^{3}H-AZT (CSI AZT). Unlabelled cholesteryl succinyl AZT (CS-AZT) and cholesteryl carbonate AZT (CCAZT) having tl structures illustrated below, were also synthesized (21).

STRUCTURES OF SOME SYNTHETIC
AZT STEROL DIESTERS



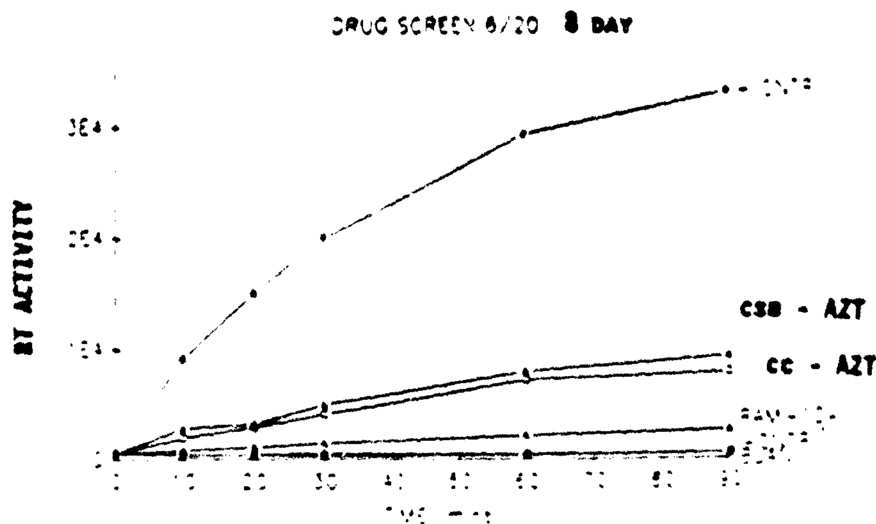
The uptake and accumulation of the ^{3}H-labelled AZT sterol sebacylate was compared with that c ^{3}H-AZT in the human T-lymphocyte cell line over a 48 hr period at two concentrations (1 μM and 5 μM in the medium. Cells were harvested at intervals and washed using a Mash harvester before countin Maximum accumulation and rentention (following the wash procedure) occured within 1 hour and w: approximately 15 and 80 fold greater for the sterol derivatives than for free AZT at the two concentratior (1μM and 5μM) respectively (Figure 4).

ENHANCED ACCUMULATION OF RADIOLABELLED CSB - AZT IN T LYMPHOCYTES



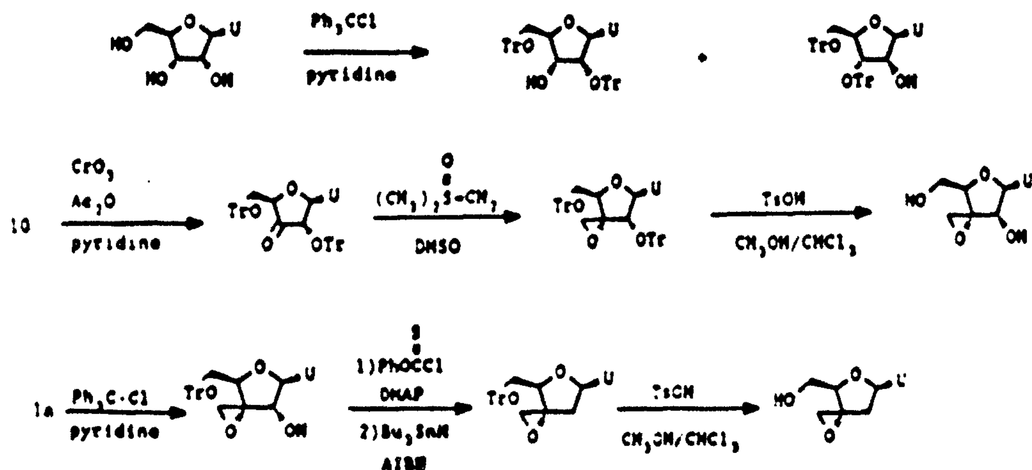
The unlabelled AZT-sterol dicarboxylates showed good antiviral activity at similar concentratio as measured by suppression of viral RT levels in culture supernatants (Figure 5) suggesting that the prodru were being hydrolyzed intracellularly to the free form. Use of the radiolabelled analogs to explore facto regulating this intracellular accumulation and release will be a major objective of the further studies propos in this project.

ANTIVIRAL ACTIVITY OF SOME STEROL AZT DIESTERS



E. Synthesis of Spiroxirane Nucleosides:

This will be carried out by the general procedures used for synthesis of the uridine 3'-ibosproxirane described in section C above. The further extension of this to the synthesis of the 2'-piroxiranes from the 3'5'-di-O-trityl isomers and the 3'-dideoxy spiroxiranes from the 5'-mono-O-trityl derivative is summarized in the scheme below.



This route to the deoxynucleoside spiroxiranes is preferred since direct conversion of the orresponding deoxynucleosides by procedures analagous to steps 1-3 in the above scheme may lead o base elimination following conversion to the 3'-keto derivative (55). Since trityl ethers of primary lcohols are more readily hydrolyzed than those of secondary alcohols it is not possible to obtain the '-mono-trityl uridine spiroxirane directly from the 3',5' derivative, which must first be completely eprotected and converted by the procedure of Michelson and Todd (47). Free radical deoxygenation f C-2 by the procedure of Acton et al (32) via the 2'-O-phenylthiocarbonate followed by eprotection of the 5'-hydroxyl group yields the desired 2'-deoxy-3'-spiroxirane. The absolute onfigurations of the 3' and 2' uridine spiroxiranes obtained by this synthesis have not yet been ssigned. However it has been reported by Corey and Chaykousky (33) that dimethyloxo-sulfonium nethylide affords spiroepoxides with an equatorial methylene in reaction with 4-t-butyl yclohexanone, which would lead to spiroxiranes having the ribo-configurations illustrated. Spiroxiranes in the alternative lyxo configuration however may also be obtained by this general procedure by reaction of the 3'-ketoderivatives with dimethyl sulfonium methylide, as illustrated n the scheme below. In contrast to dimethyloxosulfonium methylide, this reagent yields piroexpoxides with an axially oriented methylene (33).

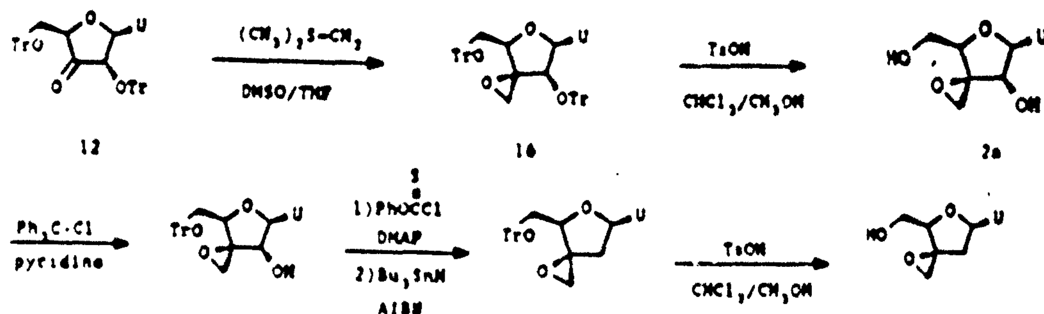


Fig. 6

9. EXPERIMENTAL PROTOCOLS:

1. Synthesis and Metabolism of ^{14}C Sterol Phosphonoformates:

The objective is to synthesize a series of α-^{14}C labelled sterol phosphonoformate mono and dieste containing different ester ligands and to study their uptake and hydrolysis in primary human T-lymphocyt and T-cell lines. The nature of the ester ligands will be varied to define those which increase the rate uptake and those which enhance (or decrease) intracellular hydrolysis to PFA. A number of compounds this type have already been synthesized in non-radioactive form by the procedures described in part II section D, and several have been shown to posess good antiviral activity in tissue culture. The α-^{14}C labell counterparts will be made using similar procedures by the Arbuzov reaction between cholesteryl chloroformate and the appropriate phosphite triesters. Cholesteryl ^{14}C choloroformate will be synthesiz by the reaction of ^{14}C phosphogene (commercially available, New England Nuclear) with cholesterol. T labelled sterol phosphonoformates will be incubated with T-lymphocyte suspensions and aliquots remov by centrifugation, washed and portions counted. The remaining cells will be extracted and the radiolabell hydrolysis products identified by TLC and autoradiography as described in section II.

2. Synthesis and Uptake and Hydrolysis of ^{3}H labelled Sterol Dicarbocylates of AZT, DDC a Nucleoside Spiroxiranes (NSO):

^{3}H labelled AZT was synthesized by conversion of the 5' CH_2OH to the aldehyde and reduction ba to the alcohol using NaB^3H_4. ^{3}H labelled dideoxy-cytidine is commercially available and uridine spiroxirane will be made from ^{3}H-uridine by the procedures developed for the unlabelled compounds (secti C). The labelled nucleosides will be coupled to cholesterol using the diacyl chloride of the appropriate link dicarboxylic acid as described in section II. The series of labelled sterol dicarboxylates so obtained will used to evaluate factors which influence uptake accumulation and release of the active antiviral componen The labelled analogs will be incubated with T-lymphocyte cell suspensions and aliquots removed at interva washed and counted. The radiolabelled hydrolysis products will be extracted and analyzed by TLC described in section II.

3. Cytotoxicity Assays Of Synthetic Analogs and Combination Drugs in Cultured T-Lymphocyt

These studies will be carried out by measuring ^{3}H-thymidine incorporation into PHA stimulat primary T-lymphocytes and in the IL-2 dependent CTLL T-lymphocyte cell line. The analogs will be test over a range of concentrations up to 100 times their effective antiviral concentration. Cultures will be puls (for 8 hours) with ^{3}H TdR at 24 hrs and 48 hrs after addition of either PHA or IL-2 to primary or CTL T-lymphocyte cultures respectively. The ratio of the concentrations of drug analog required to give 5(inhibition of cell and viral replication respectively will be derived as an indication of the therapeutic ind for each drug or drug combination. In this way a measure of the probable side effects to be anticipated fr a given effective antiviral dosage can be compared.

4. Tissue Distribution and Half Life of Radiolabelled Sterol Phosphonoformates and Nucleosi Sterol Dicarboxylates:

In order to reduce the amounts of radioactive drugs required for these studies small Wistar str white rats weighing 125-150g will be used. Groups of 14 rats will be injected via the tail vein with t radiolabelled drugs in dosages determined from the 100% inhibitory concentration for viral replication tissue culture. Pairs animals will be killed at intervals up to 72 hours and tissue samples of brain, sple kidney, heart, lung, blood and skeletal muscle will be taken and the concentration of radioactive drug a metabolites measured by scintillation counting. At 72 hours samples of the same tissues will be homogeniz extracted and the concentrations of prodrug, free drug and radiolabelled metabolites will be analyzed by Tl and scintillation counting.

5. Blood Half life Following Intravenous, Intramuscular and Transdermal Administration:

These studies will be carried out in pairs of New Zealand white rabbits in order to facilit comparison of the invasive administration procedures with transdermal administration via a rabbit ear pat Blood samples will be taken at intervals and analyzed by three separate procedures.

a. Distribution of the lipid soluble prodrugs will be analyzed following separation into t LDL, VLDL, HDL, chylomicron and supernatant fractions by utracentrifugation.

b. Blood samples will be separated by Hypaque-Ficoll density gradient centrifugation a distribution of labelled drugs in the lymphocyte, platelet, PMN and red blood cell fracions will determined.

c. Blood samples will be extracted by the Folch procedure and distribution of radioactiv between the sterol ester and free forms of the drug measured by TLC as described in section II.

6. Steady-State Levels and Antiviral Therapeutic Potency During Weekly Biweekly and Mont Intramuscular Regimens Ex vivo:

These studies will be carried out in new Zealand white rabbits in order to obtain sufficient serum f the ex vivo anti-viral evaluations for each prodrug to be tested. Groups of 3 rabbits will be giv intramuscular injections of the radio-labelled prodrug at weekly, biweekly or monthly intervals as indicat by the clearance studies described above. Blood samples will be taken at intervals to measure the steady st blood levels. On a weekly basis and immediately before a new drug injection, a blood sample will be tak and the serum prepared from it will be tested for ability to prevent HIV replication in cultured A 3.01 lymphocytes as described in section II.

Assay Procedure for Reverse Transcriptase.

The cloned HIV-reverse transcriptase from VCF 21 vaccinia Pol gene construct is assayed b modification of the procedure of Goodman and Spiegelman (27). The reaction mixture (200μL) is incuba in small plastic incubation vials containing tris-HCl buffer (10 μL 1M, pH 8.3), $MgCl_2$ (10 μL 100 mM), K (10 μL, 1M), dithiothreitol (DDT, 2 μL 10 mg/mL), ^{3}H dTTP substrate (10 μL 10 mM) incubated at 30°C a reaction is initiated by addition of enzyme solution (2 μL 4 units transcriptase. At intervals of 5, 10, 20 a 30 minutes, aliquots (25 μL) are withdrawn and the radioactive synthetic DNA is precipitated by additi of EDTA (100 μL, 20 mM) containing salmon sperm DNA carrier, 50 μg) and trichloroacetic acid (2 mL 2 w/v). Tubes are cooled on ice for 5 minutes and the precipitated DNA filtered on a Millipore glass fib filter. Filters are washed 5x with 8% TCA, 5x with 95% ethanol, dried and radioactivity measured scintillation counting using Aquasol (NEN) scintillation fluid. Inhibitors, when present, will be added to t assay dissolved in Tris buffer (50 mM, pH 8.3) to give final inhibitor concentrations in the range of 1 to 1 μM.

Since considerable differences in sensitivity have been noted for different viral reverse transcripta inhibitors showing activities against the recombinant HIV enzyme will be confirmed by testing against t endogenous enzyme prepared from intact virions by the following procedure. Virus containing culture flu harvested from A301 cells infected with HIV will be inactivated by heating at 50°C for 1 hour. T inactivated virus will be precipitated with polyethylene glycol. This procedure does not inactive the reve transcriptase. Specifically to each 5 mL of culture fluid will be added NaCl (0.2 mL, 4M) and polyethyle glycol (PEG, carbowax 6000, 2mL of 30% w/v) and virus allowed to precipitate at 4°C overnight. Followi centrifugation at 4000xg for 30 minutes at 4°C, the precipitate will be resuspended in glycerol. (3mL of 5 v/v containing tris-HCl (25 mM pH 7.5) buffer, DTT, (5mM), KCl (150mM) and Triton X-100 (0.025%) a the virions will be disrupted and reverse transcriptase and viral RNA released by addition of Triton X-1 (1 mL 0.9 % in 150 mM KCl). The supernatant fluid will be used for assay of reverse transcript sensitivity to inhibitors transcribing the viral template as described above. For these endogenous templ assays, incubations will be supplemented with oligo dT (10 μg) to enhance transcription of the viral R (23).

Sterol Phosphonoformate and Nucleoside Cholesteryl Dicarboxylate Metabolism In Human T Lymphocytes.

a. Testing Hydrolysis of Sterol Phosphonoformates and Sterol Dicarboxylates by Lymphocy Acid Hydrolases.

Following delivery of the sterol-derivatized inhibitors to the lysosomes via endosomal sterol est transport it is necessary that the acid sterol hydrolases are able to degrade the molecule thus liberating t active inhibitor. In order to test the enzyme susceptibility of synthetic sterol phosphonoformates ai nucleosides, the following procedure will be adopted. Human peripheral blood lymphocytes will be disrupt by brief sonication in phosphate buffer (0.1 M pH 7). The lysed cell suspensions (1×10^7 cells/mL) will incubated with ^{14}C labelled sterol phosphonoformates at 37°C. Aliquot portions will be removed at interv and extracted with chloroform/methanol by a modification of the Folch procedure. The extracts will separated by TLC and hydrolysis of ^{14}C labelled substrate to free ^{14}C ligands will be assayed by measuri the plates in a Vanguard TLC scanner or autoradiography, followed by scraping of the radioactive bands ai quantitation by liquid scintillation counting. This procedure is currently in routine use for measuri hydrolysis of sterol ester analogs

b. Measuring Uptake of ^{14}C labelled Sterol Phosphonoformates and ^{3}H Labelled Nucleosi Cholesteryl Carboxylates by Human Peripheral Blood Lymphocytes:

Sterol phosphonoformates labelled with ^{14}C in the sterol moiety (and sterol sebacyl ^{3}H-AZT or NS synthesized by the general procedures described below, will be used to measure the intracellular uptake the labelled analogs by human PBLs. Briefly 1×10^6 human PBLs will be incubated in 5 mL of HRP medium with the addition of the test compound added as a tincture in ethanol such that the fir concentration of ^{14}C labelled sterol phosphonoformates is approximately 10 μM. The cultures will incubated at 37°C and aliquot portions of cells removed at intervals of 1 hour up to 24 hours. Cells will removed by centrifugation and washed 2 x with albumin saline and the uptake of labelled sterol derivativ assayed by liquid scintillation counting. This procedure has previously been used successfully to measu uptake of sterol ester analogs by cultured mouse lymphoid cells

Differential Cell Toxicity Studies:

a. Differential Activity of RT Inhibitors Against DNA and RNA Synthesis in IL-2 Depende Cytotoxic TC Cells:

The basic catalytic mechanism of reverse transcriptase, i.e transfer of mononucleotide residues fro a nucleotide triphosphate substrate to the 3' OH of a growing primer chain is one which is shared by all the cellular DNA and RNA polymerases. The first objective in developing inhibitors of RT is to design ar test activatable substrate nucleotide analogs directed against the displacement reactions occurring at tl nucleotide phosphate and the 3' OH groups respectively. The second objective is to improve the selectivi of these analogs against reverse transcriptase (vis a vis the cellular DNA and RNA polymerases) and the thi objective is to maximize the effectiveness of the inhibitors against HIV replication without significant impairing the normal replication and transcription mechanisms of the lymphocyte. This latter can effective be monitored by testing the influence of the various RT inhibitors on normal replication and mitogen responses by lymphocytes in tissue culture.

The cytotoxic T-cell line CTLL is an excellent model for the terminal events in antigen activated cell proliferation, since it responds readily to added interleukin-2 (IL-2) but will not grow in its absen

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PROJECT: DAMD 17-87-C-7171

TITLE: SUICIDE INHIBITOR OF REVERSE TRANSCRIPTASE IN THERAPY OF AIDS AND OTHER RETROVIRUSES.

PRINCIPAL INVESTIGATOR: Dr. J. M. Bailey, Ph.D., D.Sc.
Professor of Biochemistry.

PRODUCTIVITY REPORT

Publications:

1. Nucleotide and template selectivity for inhibition of reverse transcriptase by PFA: Inplications for retroviral therapy. J. M. Bailey and M. M. Lightfoote. *Proc.IV*[th] *Int. AIDS. conf:* 4, 3223 (1988)

2. Differential sensitivity of wildtype and recombinant HIV-Reverse transcriptase to inhibition by foscarnet. M.M. Lightfoote and J.M. Bailey. *Proc. V*[th] *Int. AIDS Conf. Montreal* (1989) in press.

3. Antiviral activities of some sterol phosphonoformate diesters. J. M. Bailey, K. Nelson, M. Lightfoote. *J. Clin. Exp. Ther.* in preparation.

4. Nucleoside spiroxiranes: A new class of retroviral inhibitor. J. M. Bailey, K. Nelson, M. Lightfoote. *J. Virol.* in preparation.

5. Synthesis and antiviral activities of some sterol dicarboxylate esters of 3'Azido thymidine (AZT). J. M. Bailey, R. M. Mook, M. Lightfoote. *J. Clin. Exp. Ther.*, in preparation

6. Synthesis of mono and di-substituted cholesterol phosphonoformates by the Arbuzov reaction. J. M. Bailey and Keith Nelson. *Tetrahedron Letters*, in preparation.

COMPOUNDS SYNTHESIZED:

Compounds synthesized and prepared for shipment to USAMRIID for antiviral testing.

1. 2',O²-Anhydrouridine
2. 2',O²-Anhydrocytidine hydrochloride
3. 3',5'-Di-O-Benzoyl-2'-O²-anhydrouridine
4. 5'-O-t-Butyldimethylsilyl-3'-O-benzoyl-2',O'-anahydrouridine
5. 2',3'-Anhydro-5'-O-trityluridine
6. 3'-Deoxy-2'-thymidinene
7. N³-Benzyl-2',5'-di-O-trityluridine

8. 5'-O-t-Butyldimethylsilylanhydrouridine

9. N^4-Benzoylcytidine

10. 2',3'-Di-O-mesyl-5'-O-trityluridine

11. 5'-O-t-Butyldimethylsilyl-2',3'-isopropylideneuridine

12. 2',3'-Isopropylideneuridine

13. 2',3'-O-Sulfinyluridine

14. 2',3'-Benzylideneuridine

15. N^4-Benzoyl-2',3'-O-sulfinylcytidine

16. 2',3'-O-Sulfinylcytidine

17. 3',5'-Di-O-trityl-2'deoxy-2'-oxouridine

18. 3',5'-Di-O-t-butyldimethylsilyl-2'-deoxy-2'-oxouridine

19. 2',5'-Di-O-t-butyldimethylsilyl-2'-deoxy-3'-oxouridine

20. Diethyl (cholesteryloxycarbonyl)phosphonate

21. Disodium (cholesteryloxycarbonyl)phosphonate

22. Di-[1-(3-carboepoxypropyl)] cholesteryloxycarbonyl phosphonate

23. Di-(2,3-isopropylideneglyceryl) cholesteryloxycarbonyl phosphonate

24. Di-[1-(3-methylbutyl)] cholesteryloxycarbonyl phosphonate
Di-[1-(lithium 3-carboxypropyl)] cholesteryloxycarbonyl phosphonate

25. Sodium ethyl (cholesteryloxycarbonyl) phosphonate

26. Sodium 1-(3-carboxypropyl) 1-(30carboethoxypropyl)[cholesteryloxycarbonyl]phosphonate

27. Adenosine 2',3'-Riboepoxide

28. Thymidine 5'-(1,3,2-dioxaphosphorin-2-oxide)

29. Thymidinene 5'-(1,3,2-dioxaphosphorin-2-oxide)

30. Thymidinene

31. 2-Ethoxy-5-chloro-6-methyl-1,3,2-dioxaphosphorin-5-ene-2-oxide

32. 2-Ethoxy-5-chloro-1,2-oxaphosphol-4-ene-2-oxide

33. 2,4-dichloro-5-methyl-1,3,2-dioxaphosphole-2-oxide

34. 2-methoxy-4,5-dimethyl-1,3,2-dioxaphole-2-oxide

35. Thymidine 3',5'-oxetane