through an understanding of enantiostructure multiactivity relationships of significant pharmaceuticals, we should improve therapeutic indices and promote enhanced benefit to risk ratios. In this specific case, since hyperlipidemia and platelet hyperactivity are both implicated in atherosclerosis, *R*-(+) desmethyl isomers of clofibric acid may provide leads for the development of more efficacious and safer drugs for the treatment of coronary artery disease.

(18) Thus, it would be desirable to assess the enantiomers for serum triglyceride lowering activity in other animal models.

Acknowledgment. We gratefully acknowledge support for this work through U.S. Public Health Service Grant HL 12740 from the National Heart, Lung and Blood Institute.

Stereospecificity of the Chloride Ion Channel: The Action of Chiral Clofibric Acid Analogues

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2-(*p*-Chlorophenoxy)isobutyric acid (clofibric acid (1) or CPIB) is a drug known to block chloride membrane conductance (G_{Cl}) in rat striated muscle. In the present study chiral analogues of CPIB (2-(*p*-chlorophenoxy)propionic acid (2) and 2-(*p*-chlorophenoxy)butyric acid (3)) have been tested to evaluate the influence of chirality on Cl ion flux in the channel. The results showed that the chloride channel conductance strongly depends on the absolute configuration: in fact, the *S*-(−) isomers of the tested compounds strongly decreased the G_{Cl} of skeletal muscle membrane, whereas the *R*-(+) isomers were virtually ineffective. These data allow the hypothesis that, like other ion channels present in various biological systems, the chloride channel of skeletal muscle membrane could also have a stereospecific binding site (or receptor) regulating chloride ion flux.

In skeletal muscle 65–85% of the resting membrane conductance is due to chloride ions.[1,2] This large chloride conductance (G_{Cl}) stabilizes the resting membrane potential of mammalian muscle. In fact, muscles with abnormally low G_{Cl} can become hyperexcitable and produce trains of action potentials as observed in some forms of hereditary myotonia of goats[3] or humans and in myotonia produced by certain drugs.[4-7]

In spite of the important role of resting G_{Cl} in mammalian muscle excitability, relatively few studies are available on the molecular mechanism underlying this function.

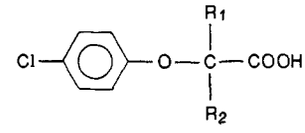
Recently we showed that 2-(*p*-chlorophenoxy)isobutyric acid (1) (CPIB), an in vivo metabolite of clofibrate,[8] specifically decreases membrane G_{Cl} of rat skeletal muscle.[9,10] There are no reports in the literature on stereospecificity of the chloride channel in skeletal muscle. Experiments were performed to evaluate the capability of chiral analogues of CPIB to interfere with chloride channel conductance and to produce myotonia.

Results and Discussion

In a preceding paper we presented evidence that 2-(*p*-chlorophenoxy)isobutyric acid (1) (clofibric acid or CPIB) is capable of blocking in vivo and in vitro G_{Cl} in rat extensor digitorum longus (EDL) muscle fibers.[9] Recently, it was found[10] that the CPIB concentration required for 50% block ($IC_{50} \pm SD$) of G_{Cl} was 205 ± 0.8 μM.

In order to interfere more specifically with chloride channel conductance, a series of CPIB chiral derivatives with an asymmetric carbon atom directly linked to the carboxy group has been synthesized, resolved, and tested.

(*RS*)-2-(*p*-Chlorophenoxy)propionic acid[11] (2) and (*RS*)-2-(*p*-chlorophenoxy)butyric acid[12] (3) were synthesized by means of known methods and they were resolved into the corresponding enantiomers by fractional crystallization of their brucine salts. Optically pure (*S*)-(−)-2 and (*S*)-(−)-3 were obtained by crystallizing the diastereomeric mixture from ethanol. (*R*)-(+)-2 and (*R*)-(+)-3 were isolated from the mother liquors of the preceding crystallization.



1: $R_1 = R_2 = CH_3$
2: $R_1 = CH_3$, $R_2 = H$
3: $R_1 = C_2H_5$, $R_2 = H$

The absolute configuration of 2 was known[11] whereas the configuration of the compound 3 was established by

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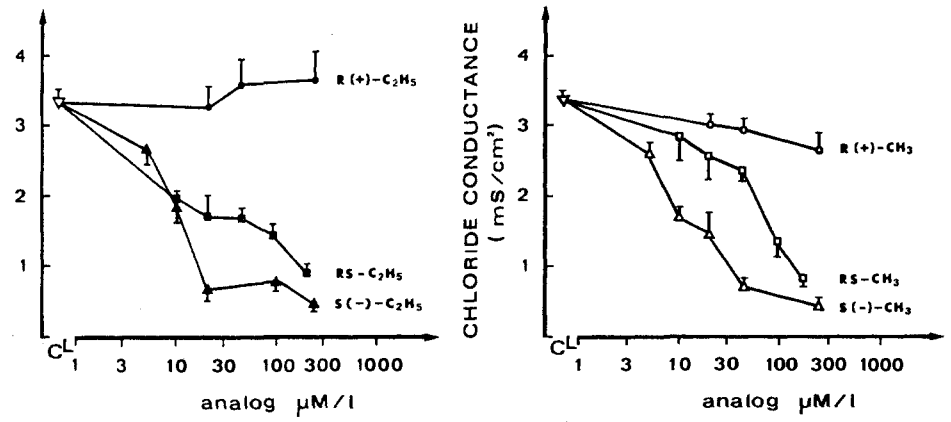

Figure 1. Effect of (*RS*)-, (*S*)-, and (*R*)-2-(*p*-chlorophenoxy)propionic and -butyric acids on chloride conductance (G_{Cl}) of rat EDL muscle. Each point represents the mean ± SEM of 7–79 fibers from 2–16 muscles. The average G_{Cl} before the addition of drug was taken as the control (Δ) ($p < 0.01$ for each concentration).

Table I. Effect of CPIB Analogues on Resting Membrane Potential and Excitability of Rat EDL Muscle Fibers[a]

	control	(*RS*)-**2**	(*S*)-(−)-**2**	(*R*)-(+)-**2**	(*RS*)-**3**	(*S*)-(−)-**3**	(*R*)-(+)-**3**
concentration, μM		92	20	230	92	20	230
resting potential, −mV	78 ± 2	74 ± 5	70 ± 2	73 ± 2	65 ± 5*	68 ± 3	70 ± 8
latency, ms	6.1 ± 0.8	24.3 ± 8.1	45.5 ± 2.5*	6.2 ± 2.1	18.3 ± 2.9*	30.2 ± 3.5*	5.2 ± 0.3
threshold current, nA	110 ± 11	79.1 ± 4.4	65 ± 5*	90 ± 15	67.3 ± 10.7	65 ± 11*	121 ± 16
no. of spikes	6.8 ± 0.6	12.5 ± 0.6	18.0 ± 1.4*	6.5 ± 1.2	15.6 ± 1.6*	15.0 ± 2.0*	6.5 ± 0.8
fibers/preparations	48/16	18/2	15/3	8/3	12/3	8/2	13/2

[a] Values (mean ± SEM) are as follows. Concentration: for racemates and *S*-(−) enantiomers, the minimal concentration at which nearly all the parameters studied are significantly different from the control. The *R*-(+) enantiomers were ineffective at the maximal concentration tested. Resting membrane potential. Latency: maximum latency to start of action potential from start of stimulus. Threshold current: minimal current intensity for a long pulse that would elicit a single action potential. Number of spikes: maximum elicited by strong depolarizing pulses. Number of fibers over number of preparation sampled (*): significantly different from mean of control group ($p < 0.01$ or less).

comparison of its ORD and CD curves with those obtained from α-substituted phenoxyacetic acids of known absolute configuration.[11,13]

Addition of (*RS*)-**2** and (*RS*)-**3** to rat EDL muscle in vitro in increasing concentrations produced a dose-dependent decrease of G_{Cl} (Figure 1).

Moreover, both of these racemates were more potent than **1** in their blocking activity of membrane G_{Cl}: in fact, the IC_{50} ± SD of the two products for the chloride channel block were 81 ± 0.8 and 44 ± 1.2 μM, respectively. These values indicate also that, of the two tested racemates, the butyric acid derivative **3** appears to be the more potent.

When *R*-(+) and *S*-(−) isomers, either of **2** or **3**, were tested for their activity on chloride channel conductance, it was found that the *S*-(−) enantiomer of both analogues demonstrated a much greater blocking activity with respect to the optical antipode. In fact, the *S*-(−) isomers of **2** and **3** tested in vitro (5–230 μM) on rat EDL muscle produced a dose-dependent block of G_{Cl} (Figure 1) with an IC_{50} ± SD of 12.0 ± 0.9 and 10.3 ± 0.9 μM, respectively. In contrast, the *R*-(+) isomers, tested at the same concentrations, were practically ineffective (Figure 1).

The time for the effect on chloride channel to develop was dose related for both the racemates and the *S*-(−) enantiomers. In particular, it was almost immediate after the addition of the *S*-(−) isomers (10–15 μM) and it was fully reversible after 30 min of washing. On the other hand, all the tested compounds (racemates and enantiomers) did not show any significant effect on the residual membrane conductances, which are attributable largely to potassium (G_K). In addition, the resting membrane potential of the tested fibers changed little after exposure to any of the compounds (Table I). The small observed decrement is probably due to the inherent "rundown" of the EDL fibers at 30 °C during the 2-h experiment following dissection.

The compounds synthesized were also examined for their effect on the excitability characteristics of EDL fibers. It is known that an abnormally low G_{Cl} is the cause of the altered excitability and the repetitive firing seen in some forms of human hereditary myotonia and in myotonia produced by monocarboxylic aromatic acids.[2,4,5] In particular it has been shown that the degree of myotonia produced by those compounds is directly related to their ability to reduce chloride channel conductance.[3-6] The same correlation was found for the chiral compounds examined in this study and the effects on excitability characteristics are reported in Table I. In muscle fibers exposed to increasing concentrations of (*RS*)-**2** and (*RS*)-**3**, the current pulses required to reach threshold current were much smaller than those of the control, as can be expected[3,7] from the reduction of G_{Cl}.

Also the delay (latency) between onset of current pulse and initiation of action potential was significantly and dose dependently increased by **2** and **3** just as reported for the monocarboxylic aromatic acids.

The firing capability of the treated fibers was increased by the compounds, and self-sustaining repetitive action potentials were observed.

In conclusion, the racemates were able to produce a pharmacological model of myotonia specifically due to the block of G_{Cl}. Also, with respect to the excitability of EDL fibers, the antipodes of **2** and **3** showed different effects: the *S*-(−) enantiomers strongly (Table I) altered the excitability characteristics of EDL fibers, producing in vitro a model of myotonia similar to that of monocarboxylic aromatic acids. In contrast, the *R*-(+) isomers were again ineffective. The other characteristics of the action potential (magnitude of AP overshoot) were not influenced by the tested drugs.

Finally, the derivatives synthesized here were also tested for their ability to block chloride channel conductance and

to induce myotonia in vivo. EMG recordings consistent with the "low chloride" myotonic syndrome[6] have been registered in rats acutely treated with the racemic and levo forms of **2** and **3**; no effects were observed with the (+) isomers. Precisely characteristic myotonic discharges were observed in more than 50% of the rats treated with 200 mg/kg (in a single dose) of the racemates and in all the rats treated with the same amount of the (−) isomers.

Conclusions

In this study in which chiral compounds have been used, we have demonstrated that the Cl ion flux in the channel of rat skeletal muscle membranes is strongly influenced by the absolute configuration of the inducing molecule. In fact, in our experiments, only the *S* enantiomers of the tested compounds were capable of decreasing chloride channel conductance in a concentration-dependent manner and concomitantly increasing excitability leading to a myotonic state, whereas optically pure *R* enantiomers, at the highest tested concentrations, were ineffective. The substituent on the asymmetric carbon atom also influences the interaction. In fact, compounds **2** and **3** tested either in their racemic or in their optically active forms gave different quantitative results in blocking the Cl ion flux in the channel. The comparison of IC_{50} values indicates that the ethyl derivative **3** is more potent than its methyl analogue **2**.

These data lead us to hypothesize that, like other channels present in various biological systems,[14] the chloride channel of skeletal muscle membrane has a stereospecific binding site or receptor that may regulate chloride ion flux. Moreover, the activity of racemic derivatives **2** and **3** seems to be lower than expected, taking into account the quantity of the *S* isomer present in the racemic mixture. Further experiments are in progress with various substituted derivatives of *p*-chlorophenoxyacetic acid in order to determine if the *R* isomer exhibits any antagonist activity.

Experimental Section

Chemistry. All melting points were taken on a Tottoli apparatus and are uncorrected. Mass and IR spectra were determined with a Kratos MS 80 spectrometer and a Perkin-Elmer 283 spectrophotometer, respectively. ^1H NMR spectra were measured on a Varian EM390 spectrometer using Me_4Si as the internal standard (chemical shifts are expressed in δ). Optical rotations at different wavelengths were measured with a Perkin-Elmer 241 MC polarimeter. CD curves were measured in MeOH with a Cary 61 dichograph using a 10-mm cell. Microanalysis were conducted with a HP Model 185 CHN analyzer (the analytical results are within ±0.4% of the theoretical values).

(*RS*)-2-(*p*-Chlorophenoxy)propionic Acid [(*RS*)-2]. To a solution of Na (17.0 g, 0.74 mol) in 500 mL of absolute EtOH was added with stirring *p*-chlorophenol (95 g, 0.74 mol) in 200 mL of absolute EtOH during 20 min. After the addition, ethyl 2-bromopropionate (135.5 g, 0.74 mol), dissolved in 150 mL of absolute EtOH, was added dropwise within 20 min and the mixture refluxed for 4 h. The solvent was removed under reduced pressure and the residue poured into H_2O and extracted with Et_2O. The ether layer was washed with H_2O and dried over Na_2SO_4. Evaporation of the solvent gave an oil (165 g), which was refluxed for 45 min with 10% alcoholic KOH (600 mL). The solvent was removed under reduced pressure and the residue was poured into H_2O and washed with Et_2O. The aqueous layer was acidified with dilute HCl and then extracted with $CHCl_3$. The organic layer was washed with H_2O and dried over Na_2SO_4. Evaporation of the solvent gave a solid, which was crystallized from $CHCl_3$–petroleum ether: yield 95 g; mp 112–114 °C (lit.[11] mp 113–114 °C); NMR ($CDCl_3$) 1.62 (d, 3, CH_3), 4.71 (q, 1, CH), 6.70–7.35 (m, 4, aromatic), 10.11 (s, 1, COOH); IR (KBr) 1710 (C=O) cm^{-1}; MS, m/z (relative abundance) 200 (M^+, 36), 155 (39), 128 (100). Anal. ($C_9H_9ClO_3$) C, H.

Resolution of (*RS*)-2. Compound (*RS*)-**2** (45 g, 0.22 mol) and brucine dihydrate (97 g, 0.22 mol) were mixed and crystallized several times from EtOH.

After six crystallizations the salt had $[\alpha]^{20}_D$ −25.8° (*c* 2.1, MeOH). This salt was treated with 5% H_2SO_4 and then extracted with $CHCl_3$, affording (*S*)-(−)-**2**, which was crystallized from $CHCl_3$–petroleum ether: mp 103–104 °C; $[\alpha]^{20}_D$ −34.9° (*c* 1.9, MeOH) (lit.[11] mp 104–105 °C, $[\alpha]^{25}_D$ −34.95°); ORD (*c* 0.0301, MeOH) $[\Phi]_{366}$ −130 °, $[\Phi]_{334}$ −300°, $[\Phi]_{313}$ −570°, $[\Phi]_{302}$ −965°, $[\Phi]_{296}$ −1430°; CD (*c* 0.0233) $[\Theta]_{300}$ −200°, $[\Theta]_{287}$ −1660°, $[\Theta]_{285}$ −1250°, $[\Theta]_{278}$ −2540°, $[\Theta]_{275}$ −1900°, $[\Theta]_{272}$ −2200°, $[\Theta]_{258}$ −340°, $[\Theta]_{247}$ −1520°, $[\Theta]_{245}$ −1220°, $[\Theta]_{230}$ −5420°, $[\Theta]_{210}$ −1690°.

The mother liquors from the first crystallization of the brucine salt were concentrated and crystallized several times. After six crystallizations the mother liquors were evaporated to dryness, and the obtained salt was treated with 5% H_2SO_4 and extracted with $CHCl_3$. The organic layer was extracted with $NaHCO_3$-saturated solution. The aqueous layer was acidified with dilute HCl and extracted with $CHCl_3$. Evaporation of the solvent gave (*R*)-(+)-**2**, which was crystallized from $CHCl_3$–petroleum ether: mp 103–104 °C; $[\alpha]^{20}_D$ +35° (*c* 5.0, MeOH) (lit.[11] mp 104–105 °C; $[\alpha]^{25}_D$ +34.1°).

(*RS*)-2-(*p*-Chlorophenoxy)butyric Acid [(*RS*)-3]. It was obtained with the procedure described for (*RS*)-**2**, starting from ethyl 2-bromobutyrate (130 g, 0.66 mol): yield 80 g; mp 76–78 °C (from $CHCl_3$–petroleum ether) (lit.[12] mp 77–78 °C); NMR ($CDCl_3$) 1.07 (t, 3, CH_3), 1.83–2.23 (m, 2, CH_2), 4.54 (t, 1, CH), 6.68–7.40 (m, 4, aromatic), 10.43 (s, 1, COOH); IR (KBr) 1710 (C=O) cm^{-1}; MS m/z (relative abundance) 214 (M^+, 22), 169 (11), 128 (100). Anal. ($C_{10}H_{11}ClO_3$) C, H.

Resolution of (*RS*)-3. Compound (*RS*)-**3** (40 g, 0.19 mol) and brucine dihydrate (82 g, 0.19 mol) were mixed and crystallized several times from EtOH. After four crystallizations the salt had $[\alpha]^{20}_D$ −43.2° (*c* 4.6, MeOH), which remained constant after two more crystallizations. This salt was treated with 5% H_2SO_4 and then extracted with $CHCl_3$, affording (*S*)-(−)-**3**, which was crystallized from petroleum ether: mp 88–90 °C; $[\alpha]^{20}_D$ −43° (*c* 4.7, MeOH); ORD (*c* 0.0314, MeOH) $[\Phi]_{366}$ −310°, $[\Phi]_{334}$ −510°, $[\Phi]_{313}$ −820°, $[\Phi]_{302}$ −1230°, $[\Phi]_{297}$ −1710°; CD (*c* 0.096), $[\Theta]_{295}$ −120°, $[\Theta]_{287}$ −2300°, $[\Theta]_{285}$ −2070°, $[\Theta]_{280}$ −3060°, $[\Theta]_{273}$ −2320°, $[\Theta]_{272}$ −2430°, $[\Theta]_{255}$ −100°, $[\Theta]_{232}$ −7910°, $[\Theta]_{210}$ +2110°.

The mother liquors from the first crystallization of the brucine salt were concentrated and crystallized three more times. At this point the mother liquors were evaporated to dryness, and the solid was treated with 5% H_2SO_4 and extracted with $CHCl_3$. The organic layer was extracted with $NaHCO_3$-saturated solution. The aqueous layer was acidified with dilute HCl and extracted with $CHCl_3$. Evaporation of the solvent gave (*R*)-(+)-**3**, which was crystallized from petroleum ether: mp 88–90 °C; $[\alpha]^{20}_D$ +41.5° (*c* 5.0, MeOH).

Pharmacology. Male Wistar rats of 180–220 g were used for all experiments. For in vitro studies, extensor digitorum longus muscles, removed as previous described,[15] were placed in a muscle bath maintained at 30 ± 1 °C and were perfused with Ringer's solution with or without the tested compounds. The tested derivatives were only slightly soluble at pH 7, but reasonably stable stock solutions of 10^{-3} g/mL were prepared in 1% sodium bicarbonate aqueous solution. Final concentrations were obtained for further dilution in normal or Cl^--free Ringer's solution and were buffered at pH 7.3–7.4. Composition of the normal and Cl^--free methyl sulfate Ringer's solution and microelectrode techniques for intracellular recordings were all as detailed in earlier papers.[15,16] The measurements included membrane resting potentials, cable parameters, component conductances, and intracellular excitability.

The resting membrane component conductances were calculated from the cable parameters measured in chloride-containing and in chloride-free Ringer's solution,[15,16] before and after the

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addition of the tested analogue. The reciprocal of membrane resistance in chloride-containing solution was assumed to be total membrane conductance (G_m) and the same parameter measured in chloride-free solution was considered largely potassium conductance (G_K). The mean chloride conductance G_{Cl} of each experimental group of fibers was estimated as the mean G_m minus the mean G_K. Sodium and other small conductances were neglected. These determinations were made in several fibers of different preparations at three or more concentrations of each examined analogue in order to construct the dose-response curves for G_{Cl}. Moreover, for each compound the resultant conductances vs. concentration curves were fit to a single site binding equation with a nonlinear least-squares method from which the $IC_{50} \pm SD$ (concentration required for half-maximal G_{Cl} block ± standard deviation) was determined. The excitability characteristics of the sampled fibers were determined intracellularly[15] at a different concentration of each compound by observing the intracellular membrane potential response recorded from one microelectrode to a square-wave constant-current delivered by a second microelectrode inserted within 100 μm from the voltage electrode. In each fiber the membrane potential was set by a steady holding current to −80 mV, before passing the depolarizing pulses.

For in vivo studies some of the tested compounds, thoroughly mixed in a bolus, were administrated in a single dose to different groups of rats. Electromyographic recordings were performed at different times, allowing sufficient time for the substance to distribute and for the effect to develop fully. Data are expressed as means ± SEM. Significance of differences between group means was calculated by the Student's t test. The estimates for SEM of G_{Cl} were obtained from the variances of G_m and G_K, assuming no covariance, by standard methods.[17] Standard deviations for the IC_{50} values were calculated from the variance-covariance matrix obtained during nonlinear fitting procedures.

Acknowledgment. This research was supported by Italian CNR Grant 85426 and MPI 1985 and 1986 grants.

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3′-Substituted 2′,3′-Dideoxynucleoside Analogues as Potential Anti-HIV (HTLV-III/LAV) Agents†

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A series of 2′,3′-unsaturated and 3′-substituted 2′,3′-dideoxynucleoside analogues of purines and pyrimidines have been synthesized and evaluated for their inhibitory activity against human immunodeficiency virus (HIV). The 2′,3′-unsaturated analogues of 2′,3′-dideoxycytidine (ddeCyd) and 2′,3′-dideoxythymidine (ddeThd), 3′-azido-2′,3′-dideoxythymidine (AzddThd), 3′-fluoro-2′,3′-dideoxythymidine, 2′,3′-dideoxycytidine (ddCyd), and 2′,3′-dideoxyadenosine (ddAdo) emerged as the most potent inhibitors of HIV-induced cytopathogenicity in the human T lymphocyte cell lines ATH8 and MT4. In ATH8 cells ddCyd, ddeCyd, and ddAdo had the highest therapeutic index whereas in MT4 cells AzddThd, ddThd, ddCyd, and ddAdo were the most selective. Derivatives from ddThd in which the substituent group was linked to the 3′-carbon atom via a thio, sulfonyl, or oxygen bridge were far less inhibitory to HIV than was AzddThd.

Acquired immunodeficiency syndrome (AIDS) is an immunosuppressive disease characterized by an immune impairment associated with life-threatening opportunistic infections and a high susceptibility to unusual forms of certain neoplasms (i.e., Kaposi's sarcoma).[1-3] Human T-cell lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV), recently designated as human immunodeficiency virus (HIV),[4] has been recognized as the etiologic agent of AIDS.[5,6] Several compounds with different chemical structures (i.e., Suramin,[7-9] Evans Blue,[9] aurintricarboxylic acid,[10] HPA-23,[11,12] phosphonoformic acid,[9,13] ribavirin,[14] interferon-α,[15] AL-721,[16] and 2′,3′-dideoxyribonucleosides[17-20]) have been reported as having significant inhibitory effect against HIV in vitro. Of these compounds, 2′,3′-dideoxyribonucleosides have thus far proven to be the most potent antiretroviral agents in vitro.[17-20] Indeed, Mitsuya and co-workers reported that 3′-azido-2′,3′-dideoxythymidine (AzddThd) protected human T cells (clone ATH8) against the cytopathogenic effect of HIV at 1–5 μM,[17] and 2′,3′-dideoxycytidine (ddCyd) completely suppressed HIV-induced cytopathogenicity in vitro at 0.5 μM.[18] The 2′,3′-unsaturated analogue of ddCyd, ddeCyd, proved equally effective as ddCyd in protecting ATH8 cells against HIV,[19] while ddeThd, the 2′,3′-unsaturated analogue of 2′,3′-dideoxythymidine (ddThd), was

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† Dedicated to Prof. Wolfgang Pfleiderer on the occasion of his 60th birthday.
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§ National Institutes of Health.

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