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Resistance towards exonucleases of dinucleotides with stereochemically altered internucleotide phosphate bonds

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Abstract—Kinetic constants for the hydrolytic susceptibility of the internucleotide phosphate bond in normal dinucleotides [e.g., 2′-deoxycytidylyl-(3′ > 5′)-2′-deoxyuridine (dCpdU) and 2′-deoxyadenylyl-(3′→5′)-2′-deoxycytidine (dApdC)] and isomeric dinucleotides [e.g., 2′-deoxycytidylyl-(3′→5′)-1′-deoxy-2′-isouridine (dCpisodU) and 1′-deoxy-2′-isoadenylyl-(3′→5′)-2′-deoxycytidine (isodApdC)], toward 5′- and 3′-exonucleases, phosphodiesterase I (PDE I) and phosphodiesterase II (PDE II) were experimentally determined and remarkable differences emerged. The study is of importance in the discovery of nuclease-stable inhibitors of HIV integrase, but may also have ramifications in the area of anti-sense oligonucleotides of therapeutic interest.

The 5′- and 3′-exonucleases, phosphodiesterases I (PDE I) and phosphodiesterase II (PDE II) degrade the internucleotide phosphodiester bond sequentially from small and large oligonucleotides and nucleic acids. These exonucleases have been utilized as tools for the sequential degradation of nucleic acids. Phosphodiesterase I successively releases 5′-mononucleotides from 3′-hydroxy terminated ribo- and deoxyribo-nucleosides.[1,2] On the other hand, phosphodiesterase II releases 3′-mononucleotides sequentially from the free 5′-hydroxy end of ribo- and deoxyribo-oligo-nucleotides.[3] These exonucleases are relatively non-specific with respect to base or sugar moieties of nucleotides,[4] but may be sensitive to the stereochemistry and conformation of the substrate. Even photomodified oligonucleotides possess sufficient substrate behavior to be sequenced by these exonucleases.[5] The 5′-OH protected ribo- and deoxyribo-oligonucleotides are not substrates of the PDE II enzyme.[6] Degradation of di- and small oligonucleotides by exonucleases may limit their usefulness as potential therapeutic agents. We have synthesized a number of novel isomerically modified dinucleotide 5′-monophosphates that exhibit inhibitory activity against recombinant HIV-1 integrase.[7,8] This communication examines the stability of the internucleotide bond of these HIV integrase inhibitors towards 5′- and 3′-exonucleases. The compounds chosen for study were the dinucleotides (without the terminal 5′-phosphates) so that the hydrolytic cleavage study would focus on the internucleotide phosphodiester linkage without complications from the 5′-phosphate group. Comparisons were made with the behavior of their natural counterparts and with the integrase inhibitors. An explanation of the data in terms of structure, stereochemistry and conformation is provided.

Kinetic parameters (K_m, V_{max}, catalytic efficiency) of the normal and isomeric dinucleotides, for example dApdC, isodApdC, dCpdU and dCpisodU (Fig. 1) and other compounds were determined using the exonucleases, phosphodiesterase I (bovine intestinal mucosa) and phosphodiesterase II (bovine spleen) which were procured from Sigma. The assays using these enzymes are described.[9] It is clear from Table 1 that the dinucleotides, dApdC and isodApdC, were hydrolyzed by PDE I as well as by PDE II enzymes. However, there were major differences in the catalytic efficiencies (V_{max}/K_m) for the hydrolysis of natural compound, dApdC, compared to the non-natural, isomeric dinucleotide, isodApdC. For example, the latter showed only 29% of the catalytic efficiency of the natural compound with PDE I (Table 1). This indicates that there is marked impairment of the hydrolytic susceptibility of the internucleotide phosphate bond of pisodApdC with the 5′-

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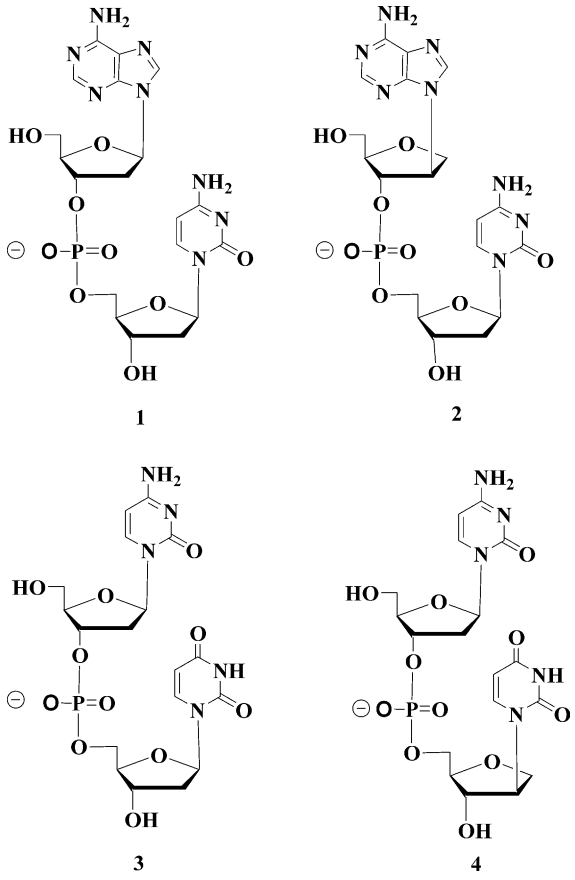

Figure 1. Examples of dinucleotides examined for exonuclease substrate activity.

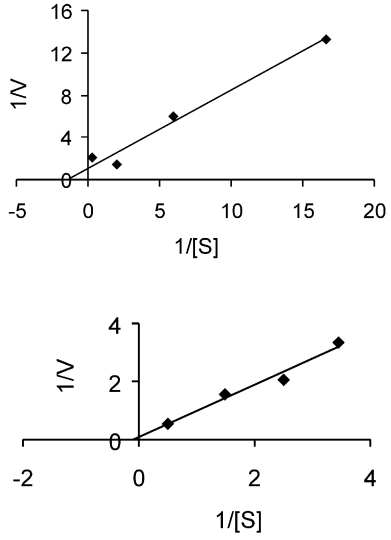

Figure 2. Lineweaver–Burk plots (1/V vs 1/[S], mM^{-1}) of the substrate activities of the natural compound, dCpdU, (top) and the novel non-natural compound, dCpIsodU (bottom) with 5′-exonuclease (PDE I).

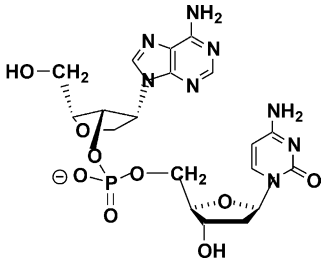

Figure 3. Unusual internucleotide phosphate bond resulting from the combination of an isomeric (L) and a natural (D) nucleoside.

exonuclease. Similarly, in the case of PDE II, the isomeric dinucleotide, isodApdC, with a catalytic efficiency (V_{max}/K_m) of 4.5 μmol/min/mM, is only 21% as active with respect to substrate behavior as the natural dinucleotide, dApdC ($V_{max}/K_m = 21.1$ μmol/min/mM).

In the pyrimidine series examined, the results were even more dramatic (Table 1). For example, the non-natural dinucleotide, dCpisodU, shows 0.3% of the activity of its natural counterpart with PDE I (5 vs 1614 μmol/min/mM). This is exemplified graphically in Figure 2. It is also of interest to note that the non-natural compound is not a substrate at all for PDE II, whereas the natural compound is a good substrate with a V_{max}/K_m of 88 μmol/min/mM.

Finally, it is remarkable that non-natural dinucleotides with terminal 5′-phosphate bonds, such as the anti-HIV integrase inhibitor, pdCpisodU, are not substrates for either 5′- or 3′-exonucleases. In contrast, for example, the corresponding natural compound, pdCpdU, is a good substrate for 5′-exonuclease ($V_{max}/K_m = 225$).

While resistance to exonucleases have been previously observed with chemically modified internucleotide

Table 1. Kinetic constants of the hydrolytic cleavage of dinucleotides with PDE I and PDE II

Substrate	Enzyme	K_m (mM)[a]	V_{max} (μmol/min)	V_{max}/K_m
dApdC **1**	PDE 1	2.7	141.4	52.4
isodApdC **2**	PDE 1	1.25	19.2	15.4
dApdC **1**	PDE II	1.63	34.4	21.1
isodApdC **2**	PDE II	1.35	6.1	4.5
dCpdU **3**	PDE I	0.90	1453	1614.4
dCpisodU **4**	PDE I	12	60	5.0
dCpdU **3**	PDE II	1.52	133.4	87.8
dCpisodU **4**	PDE II		NS	
pisodApdC, 5′-phosphate of **2**	PDE I PDE II		NS	
pdCpisodU, 5′-phosphate of **4**	PDE I PDE II		NS	

NS, non-substrate.

[a] Substrate concentrations chosen were close to the K_m values. Kinetic constants were calculated by linear regression analysis from the average of two duplicate experiments.

phosphate bonds,[10–12] the resistance of these non-natural dinucleotides towards hydrolytic cleavage of the internucleotide phosphate bond must have its basis on other factors. In isomeric nucleosides which are components of the dinucleotides under study, transposition of the glycosidic bond from the natural 1′-position to the isomeric 2′-position leads to isonucleosides which can be viewed structurally as having features of carbocyclic nucleosides with transposed endocyclic oxygens.[11] In addition, these compounds may be viewed stereochemically as belonging to the L-related class and their mirror images as being in the D-related class. The dinucleotides of this study are therefore of mixed L- and D-nucleoside stereochemistry. Thus, this combination of an isomeric deoxynucleoside with a natural deoxynucleoside introduces an unusual 3′,5′-internucleotide phosphate bond into the molecule which is conformationally distinct from the internucleotide linkage found in natural systems as shown in Figure 3. While the binding of both natural and non-natural dinucleotides to the exonucleases appear to occur with approximately the same efficiency on the basis of K_m values, the turnover of substrate as represented by V_{max} values is markedly lower for the non-natural compounds. The most dramatic differences were seen with the potently active, integrase inhibitor, pdCpIsodU, which is totally resistant toward both enzyme systems. Further studies on the molecular design and chemoenzymatic synthesis of other analogues with anti-integrase activity based on these results are in progress.

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9. Phosphodiesterase I assays: The reaction mixtures were prepared using 20 μL (0.32 mM), 40 μL (0.64 mM), 60 μL (0.96 mM), and 80 μL (1.28 mM) of dApdC and isodApdC (and other modified dinucleotides) in 250 μL of 50 mM Tris–Cl buffer (pH 7.6) containing 10 mM $MgCl_2$. Reactions were initiated by the addition of 40 μL of enzyme (5 units/mL) to each reaction mixture at 37 °C for 1 h. The reactions were stopped by addition of 250 μL of 1 M phosphate buffer pH 4.0 containing 5 mM EDTA. The reactions were analyzed by HPLC using a C_{18} column.
Phophodiesterase II assays: The assay mixtures were prepared using 20 μL (0.32 mM), 40 μL (0.64 mM), 60 μL (0.96 mM), and 80 μL (1.28 mM) of dApdC and isodApdC and other substrates in the 250 μL of 0.1 M acetate buffer (pH 6.0) containing 10 mM $MgCl_2$. The reactions were initiated by the addition of 5 μL of enzyme (5 units/mL) to the reaction mixture at 37 °C for 1 h. The reactions were stopped by addition of 250 μL of 0.5 M Tris–Cl buffer (pH 10.0) containing 5 mM EDTA. The reactions were analyzed by HPLC.
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