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Mini-review

The interplay between antiviral activity, oligonucleotide hybridisation and nucleic acids incorporation studies

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Dedicated to Prof. Erik De Clercq on the occasion of reaching the status of Emeritus-Professor at the Katholieke Universiteit Leuven in September 2006.

Abstract

Nucleoside analogues have been the most successful antiviral compounds. Likewise, they are the most intriguing antiviral compounds, because of their structural relationship to natural nucleosides. This is also the reason why the design process of a potential selective antiviral nucleoside is so difficult. Too many natural processes (from cellular uptake to DNA incorporation) and too many enzymes are involved in their biological effect (activity/toxicity/catabolism/anabolism) to make the design process readily predictable. The relationship between the physicochemical and biochemical properties of nucleoside analogues and their antiviral activity is very complex and could only be understood on a very long term basis. Here we try to explain some of the reasoning that was made during the design process leading to new potent antivirals with a phosphonate functionality.

Keywords: HSV; HIV oligonucleotides; Polymerase; Reverse transcriptase; Hexitol nucleoside; Cyclohexenyl nucleoside; Threosyl nucleosides

Contents

1. Introduction

Nucleoside and nucleotide analogues are small molecules and are considered as privileged leads in antiviral and antitumoral drug design. Oligonucleotides, on the other hand, are being developed to control gene expression (antisense, ribozymes, siRNA) and have a potentially much wider applicability. The first report on the antiviral activity of natural and modified oligo(poly)nucleotides dated from 1969 (De Clercq and Merigan, 1969a; De Clercq et al., 1969b). It was recognized that this biological activity was indirect (interferon induction) and that it was dependent upon the formation of stable secondary (helical) structures, which could be thermally induced (De Clercq and Merigan, 1969a; De Clercq et al., 1970). The biochemical link between nucleosides and oligonucleotides is obvious, as natural nucleosides are converted intracellularly to their corresponding triphosphate, which function as a building block for nucleic acid biosynthesis. This biochemical event, which occurs in every living organism, is controlled by kinases and polymerases. These enzymes are, obviously, also involved in the mode of action of antiviral nucleosides. Two common examples are HSV and HIV infections. Anti-HSV nucleosides reach a high level of safety (in vivo) because two viral specific, enzymatic systems (kinase, polymerase) are involved in their

Abbreviations: HIV, human immunodeficiency virus; HSV, herpes simplex virus; CeNA, cyclohexenyl nucleic acids; TNA, threose nucleic acids; HNA, hexitol nucleic acids; RT, reverse transcriptase

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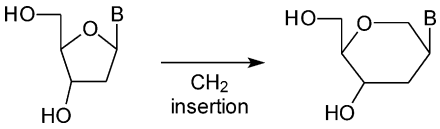
Table 1
Anti-HSV activity of base modified hexitol nucleosides and kinetic parameters for incorporation of hexitol nucleosides in DNA using the M184V mutant of HIV-RT (a) and Vent DNA polymerase (b)



	B	HSV-1 (μg/ml)	HSV-2 (μg/ml)
	5-Iodouracil	0.02	0.4
	Cytosine	0.7	0.04
	Guanine	0.2	0.02
	5-Ethyluracil	0.02	0.4
	Diaminopurine	0.2	0.7
	5-Trifluoromethyluracil	0.02	0.4

Substrate	V_{Max} (% min^{-1})	K_m (μM)
(a) dATP	2.80 (±0.10)	0.23 (±0.04)
(a) hATP	2.17 (±0.12)	2.26 (±0.05)
(b) dATP	2.31 (±0.05)	0.63 (±0.03)
(b) hATP	2.20 (±0.09)	0.44 (±0.03)

mode of action (the kinase having the function as first filter). Most anti-HIV nucleosides are more toxic because selectivity can only be reached at the level of polymerases versus reverse transcriptase.

2. Discussion

The broader substrate specificity of HSV-kinases has been a rewarding property for drug design. This may be exemplified by the anti-HSV activity of many base modified hexitol nucleosides (Verheggen et al., 1993, 1995) (Table 1). Insertion of a methylene group between the sugar oxygen atom of a furanose nucleoside and the anomeric carbon atom renders the molecule more stable against enzymatic degradation without abolishing its substrate properties for HSV kinase.

Structural analysis has demonstrated that these hexitol nucleosides are mimics of furanose nucleosides frozen in the 3′-endo-conformation (Verheggen et al., 1995). Because of this property, oligonucleotides build-up of hexitol nucleosides (HNA) have an A-type geometry, preorganized for hybridization with RNA (Lescrinier et al., 2000) (Fig. 1). HNA forms very stable duplexes with RNA (Hendrix et al., 1997) and can be used to control gene expression, as demonstrated by the inhibition of P-glycoprotein expression in a cellular system (Kang et al., 2004). When considering the triphosphates of hexitol nucleosides, it has been demonstrated that such triphosphates can be efficiently incorporated into DNA, using Vent DNA polymerase, opposite their natural complement and that chain termination is achieved after insertion of two or three hexitol-A nucleotides (Vastmans et al., 2000). The kinetic parameters for incorporation by Vent DNA polymerase are very similar for dATP and hexitol-ATP. HIV-reverse transcriptase and its M184V mutant are able to incorporate three hexitol nucleotides after each other, the M184V mutant being the most efficient (Vastmans et al., 2001). The first two building blocks are incorporated as efficiently as 2′-deoxyadenosine triphosphate. At high nucleoside concentration, HIV-RT prefers hexitol nucleotide incorporation above natural nucleotide incorporation, Vent DNA polymerase prefers natural nucleotide incorporation above hexitol nucleotide incorporation. Kinetic parameters for incorporation into DNA by HIV-RT show similar V_{max} values, but increased K_m values for hATP versus dATP (Vastmans et al., 2001). Modelling experiments show that there is a direct interaction of the amino acid Met184 side chain of RT with the sugar moiety of the nucleoside triphosphate and that hexitol nucleotides have a rigid sugar moiety which hampers conformational changes needed for chain elongation (resulting in chain termination). The active site of HIV-1-RT is more flexible than those of other polymerases, i.e. HIV-1-RT is capable of inserting more hexitol nucleotides under similar conditions.

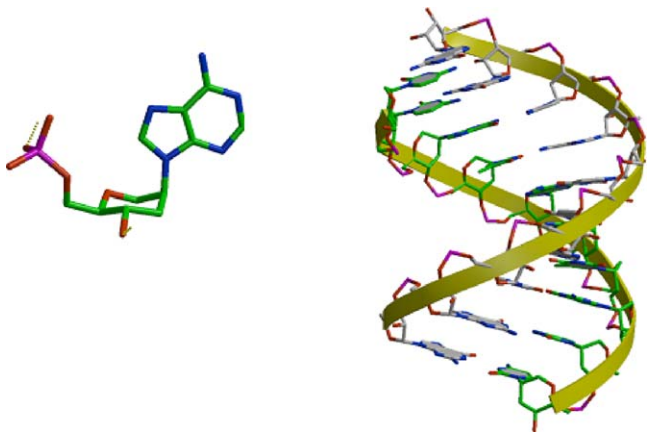


Fig. 1. Structure of a hexitol nucleoside and of a HNA:RNA duplex (Maier et al., 2005).

These studies have led to the conclusion that hexitol nucleosides are selectively phosphorylated by herpes virus kinases (not by human kinases) and that they undergo an induced fit principle for binding to kinases in the high energy state (Champness and Sanderson, 1998), which may be one of the factors hampering phosphorylation by cellular kinases. But even if hexitol nucleosides (or their deoxy congeners) would have been phosphorylated well by cellular kinases, they would stay poor anti-HIV compounds, because of the poor selectivity of their triphosphates at the polymerase level (DNA polymerase versus HIV-1 RT). The physicochemical reason why they are good antisense oligonucleotides (rigid preorganized structure) makes them poor antivirals (Herdewijn, 1999).

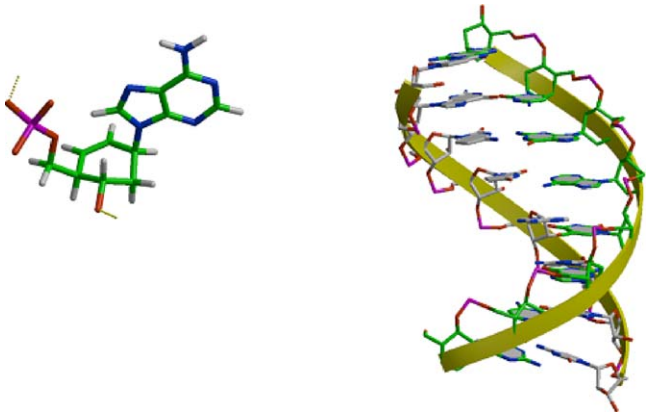


Fig. 2. Structure of a cyclohexenyl nucleoside and of a CeNA:RNA duplex (Nauwelaerts et al., in press).

Therefore, more conformational flexibility was introduced into the six membered ring by synthesizing cyclohexenyl nucleosides (Wang et al., 2000a). In cyclohexenyl nucleosides, the furanose oxygen atom is replaced by a carbon–carbon double bond. These nucleosides exist in several low-energy conformations which are easily interconvertable. The energy difference between the Northern-type and Southern-type conformation is 1.6 kJ/mol, with a preference for the N-conformation in the deoxy series (Nauwelaerts et al., 2005) (Fig. 2). The energy barrier between both conformations is 10 kJ/mol. We have demonstrated that both conformations may occur after incorporation of cyclohexenyl nucleosides into oligonucleotides (Verbeure et al., 2001; Nauwelaerts et al., 2005). The thermal stability of fully modified oligo-(cyclohexenyl-A) with DNA and RNA complement is similar (which is not the case for oligo-HNA and oligo-DNA) (Wang et al., 2000b). Cyclohexenyl nucleosides seems, therefore, a much better mimic of a natural furanose nucleoside than hexitol nucleoside. How do they behave then at the level of the polymerases and at the antiviral level? Kinetic parameters have shown that the K_m value for incorporation of cyclohexenyl triphosphates into DNA using Vent DNA polymerase is somewhat reduced with respect to a deoxynucleoside triphosphate and that the V_{Max} value has doubled (Kempeneers et al., 2005). The triphosphate of a cyclohexenyl nucleoside is a better substrate for Vent DNA polymerase and for reverse transcriptase than the triphosphate of a hexitol nucleoside (Kempeneers et al., 2005). A cyclohexenyl nucleotide is a good substitute for ribonucleotides in a siRNA experiment (Nauwelaerts et al., in press). The better fit of the properties of CeNA (than HNA) with the physicochemical properties of natural nucleic acids makes cyclohexenyl nucleosides also better antivirals. The antiviral activity of cyclohexenyl G against HSV-1 and HSV-2 is increased (when compared with hexitol nucleosides) and some analogues are active against viruses, other than herpes viruses (Wang et al., 2000a; Gu et al., 2003). This activity remains low, most probably, due to non-optimal phosphorylation kinetics by kinases.

That introduction of flexibility may lead to increased antiviral effect (Herdewijn, 1999), is demonstrated with the synthesis of the unsaturated hexitol congeners with adenine and thymine base (Table 2). Both nucleosides show low but significant activity against HIV-1 (Herdewijn, 1997a).

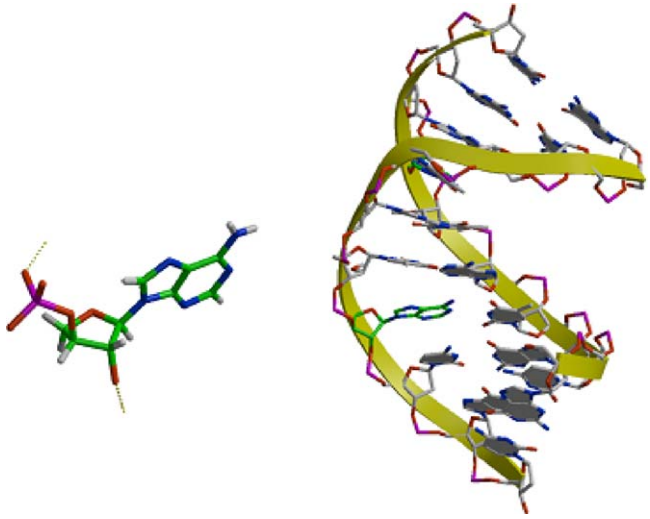
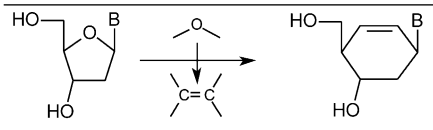
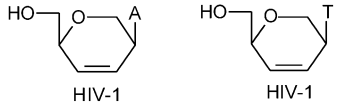


Fig. 3. Structure of a threosyl nucleoside and of a TNA nucleoside incorporated in an A-type DNA duplex (Pallan et al., 2003).

Table 2
Anti-herpes virus activity of cyclohexenyl nucleosides and kinetic parameters for the incorporation of cyclohexenyl nucleosides in DNA using Vent DNA polymerase



	(μg/ml)			
B	HSV-1	HSV-2	VZV	CMV
Guanine	0.002	0.05	0.5	0.6
Cytosine	40	40	0.05	ND

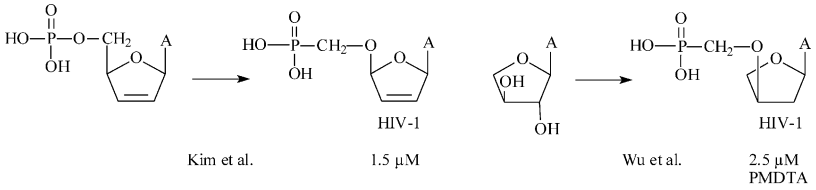


HIV-1 8 μg/ml

HIV-1 3 μg/ml

Substrate	K_m (μM)	K_{cat} (min^{-1})
dATP	1.46 (± 0.19)	16.90 (± 0.65)
CeATP	0.98 (± 0.08)	7.08 (± 0.15)

Table 3
Structure of a threosyl phosphonate nucleoside (PMDTA) and kinetic parameters for the incorporation of threosyl nucleosides in DNA using HIV-RT (a) and Vent DNA polymerase (b) and for the incorporation of PMDTA in DNA using HIV-RT (c)



Substrate	K_m (μM)	K_{cat} (min^{-1})
(a) dTTP	2.63 (±0.43)	0.65 (±0.02)
(a) tTTP	7.3 (±3.3)	0.68 (±0.008)
(b) dTTP	0.53 (±0.13)	9.46 (±0.66)
(b) tTTP	1.16 (±0.31)	1.74 (±0.14)
(c) dATP	0.10 (±0.018)	0.66 (±0.019)
(c) PMDTApp	0.29 (±0.026)	0.79 (±0.016)

In search for nucleoside analogues that may have improved antiviral activity and selectivity, we analyzed several nucleic acids for their physicochemical property to hybridize as well with RNA as with DNA with roughly equally stability. An obvious candidate is α-L-threose nucleic acids (TNA) which were first synthesized by Eschenmoser and co-workers (Schöning et al., 2000) and which are capable of cross-pairing with RNA and DNA (Fig. 3). With respect to hybridization strength, TNA resembles RNA. To analyze the functional properties of TNA, α-L-threose nucleosides were incorporated into a hammerhead ribozyme (Kempeneers et al., 2004). Ribozymes are RNA sequences with enzyme-like activity, i.e. they can catalyze chemical reactions in the absence of protein cofactors. The TNA-modified ribozyme, however, shows reduced cleavage kinetics, due to the shortening of the interphosphate distance and the change of the ground-state structure at the catalytic core of the ribozyme.

In a second functional assay we evaluate threose nucleoside triphosphates to function as substrates for several polymerases (Kempeneers et al., 2003). Vent DNA polymerase is more efficient than HIV reverse transcriptase to incorporate threosyl nucleotides into DNA. Chain termination occurs after incorporation of two threosyl nucleotides. The efficiency of incorporation of hexitol nucleotides by DNA polymerase and HIV-RT is higher than that of the incorporation of threosyl nucleotides. The kinetic parameters for HIV-RT show a highly reduced K_{cat} value and, likewise, for Vent DNA polymerase (although to a lesser extent). The further investigation of threosyl nucleosides as potential antivirals was stopped at this level, because we realized that their triphosphates are bad substrates for reverse transcriptase and they show opposite selectivity than desired for an anti-HIV agent (DNA polymerase versus HIV-RT). The efficiency of phosphorylation (substrate for kinases) as well as the selectivity (as triphosphates) for HIV-RT should be increased.

A way to do this is to synthesize nucleoside phosphonates by an isosteric replacement of the phosphate function. The development of antivirals in this way has its precedent in nucleoside chemistry (Kim et al., 1991) (Table 3). In our case of threosyl nucleosides (PMTA), the 2′-OH group was removed (giving PMDTA) to end up with a potential chain terminator.

The diphosphate of PMDTA shows very similar K_{cat} as dATP for the enzymatic incorporation into DNA by HIV-1 reverse transcriptase (Wu et al., 2005). The kinetic parameter for the incorporation by DNA polymerase α could not be measured because PMDTA was such a poor substrate for the enzyme, which points to the selectivity of the compound. In addition, the compound shows potent anti-HIV-1 activity, similar to the activity of the "golden standard", phosphonate, i.e. PMPA, which suggests that phorphorylation by cellular kinases should be possible. Elongation of the TNA building block with a phosphonate moiety, proved to be the right decision to develop a selective anti-HIV-1 agent (Wu et al., 2005).

3. Conclusion

In conclusion, measuring hybridization properties of modified oligonucleotides and measuring kinetics of incorporation of the triphosphates of modified nucleosides into DNA/RNA by various enzymes may give a good insight into their biochemical behavior. This information is very valuable to the design of antiviral nucleosides (i.e. to decide whether the chemistry of the selected modification should be pursued or not). Ideally, this information should be filled-up with kinetic studies at the level of the different kinases involved in the metabolic activation of the nucleoside (Herdewijn, 1997b). Chemically spoken, however, there are now several tools available to circumvent problems with poor intracellular phosphorylation such as phosphonate synthesis and phosphate prodrug synthesis.

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