Structural requirements for antiviral activity in nucleosides

Piet Herdewijn

The discovery of several new series of nucleoside analogues with antiviral activity has altered the classical way of thinking about nucleoside analogues as antiviral agents. Modelling studies and conformational analysis have entered the arena and contributed to our knowledge of the mode of action. New lead structures have uncovered previously unknown targets for antiviral drug design, which may be exploited in the future to discover more potent and less toxic antiviral nucleosides with, eventually, a broad spectrum of activity. This review summarizes some of the new directions that have been taken by industrial and academic researchers in their effort to find the ideal antiviral nucleoside.

There has been and probably always will be much scepticism in the pharmaceutical industry about the development of nucleosides as antiviral agents. Indeed, a nucleoside having a free primary hydroxyl group or a phosphonate function in the 'sugar' moiety is always a candidate for enzymatic phosphorylation, potentially leading to incorporation of the modified nucleotide into DNA. This incorporation may lead to mutagenesis, subsequently followed by uncontrolled cell proliferation, although it has been demonstrated with acyclovir (**1**, Figure 1) that this does not always happen. The disadvantage of acyclovir, however, is its very narrow spectrum of activity[1].

The enzymes used by virus and host to activate nucleosides and incorporate them into DNA are very similar. Because the reaction mechanism used by these enzymes is the same, the binding site for the nucleoside must be well conserved. A nucleoside accepted as a substrate for a viral enzyme is therefore also a potential substrate for host enzymes. Moreover, the enzymatic machinery used to activate a nucleoside so that it becomes an antiviral agent and/or a toxic compound is often the same. This can be demonstrated using anti-HIV nucleosides as models: for example, zidovudine (**5**), stavudine (**6**), zalcitabine (**7**), didanosine (**8**), lamivudine (**9**) (Figure 2). These nucleosides have to be phosphorylated to their mono-, di- and triphosphate before interacting with their final target:

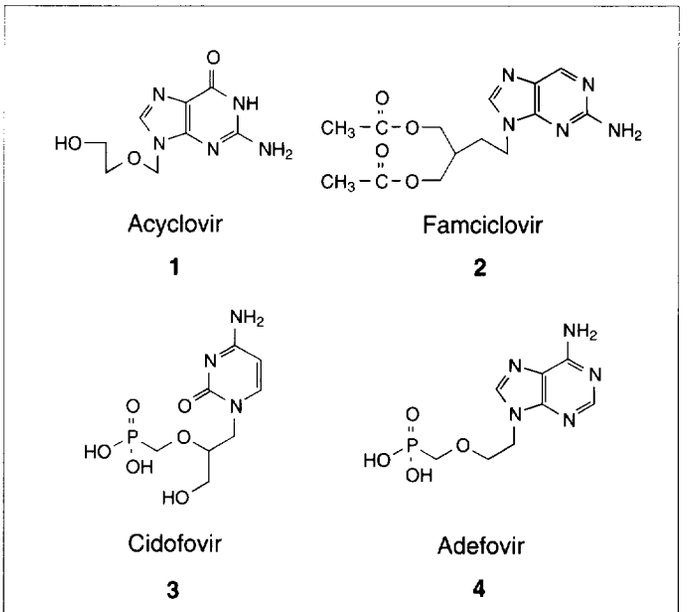

Figure 1. *Structure of acyclic nucleosides acyclovir (**1**) and famciclovir (**2**) and some analogues with a phosphonate functionality (**3**, **4**).*

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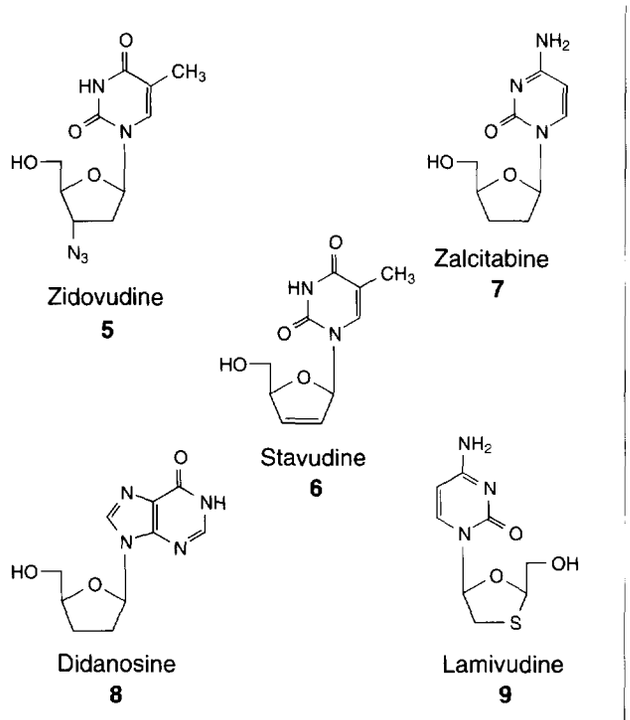


Figure 2. *Structure of anti-HIV nucleosides.*

reverse transcriptase. These phosphorylations are carried out by host enzymes, which means that the triphosphates of the 'dideoxynucleosides' are also formed in noninfected cells and presented to the host DNA polymerases. The selectivity filter between activity and DNA toxicity is therefore only dependent on the substrate specificity of the triphosphates for two related enzymes.

The situation is somewhat safer for most anti-HSV-2 (antiherpes simplex virus type 2) nucleosides because two viral enzymes are involved in their mode of action: thymidine kinase and DNA polymerase. On the other hand, HSV-2 infections are less harmful than HIV infections and, in the past, less toxicity was accepted for an antiherpes agent than for an anti-HIV compound. This situation is slowly changing. Given the potential for lifelong exposure, the standards of nontoxicity for an anti-HIV agent are increasing. However, generally, a nucleoside is only considered safe if it is not incorporated into DNA.

The antiviral nucleosides that are currently on the market were discovered through the intuitive thinking of chemists as to which structural analogues of natural substrates might confound the viruses, or they were developed as structural analogues of already existing antiviral compounds. Recent examples of such antivirals are cidofovir[2] (**3**, Figure 1) of the first series and famciclovir[3] (**2**, Figure 1) of the second series. Lead structures were never really designed as antiviral agents on an 'intellectual' basis – i.e. starting from a structural analysis of the target and/or a conformational analysis of the substrates of the target enzymes. The question is: should we proceed in that direction or will it be possible in the future to rationalize antiviral activity on a structural basis? Is there any development in the antiviral nucleoside field that might question the general (but perhaps unjust) belief that a potent antiviral nucleoside has to be toxic *per se*? Is it necessary that a nucleoside passes through the phosphorylation cascade in order to be active? That this may not be the case can be demonstrated by the discovery of *S*-adenosylhomocysteine hydrolase inhibitors, represented here by 3-deazaneplanocin A (**10**) and 5′-noraristeromycin (**11**) (Figure 3). Both compounds show activity against several viruses, including human cytomegalovirus (CMV)[4]. However, as the mode of action is based on interference of cellular enzymes, a very high selectivity index can not be expected with these compounds.

In this short review, I will give some examples of recent developments in the antiviral nucleoside field and try to demonstrate in which directions research in the antiviral field may evolve, based on a structural analysis of recently discovered active antiviral nucleosides. The article does not aim to give a comprehensive overview of all the antiviral nucleosides in the pipeline.

Stereoselective action

At the molecular level, the living world on earth is a completely asymmetric environment. This is reflected in the SARs of the drugs we use. Mostly, the two enantiomers of a chiral compound possess qualitative and/or quantitative differences in activity. In most cases, one enantiomer is active,

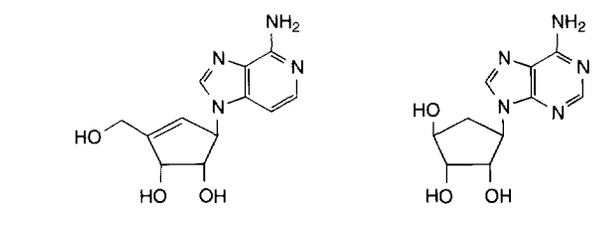


Figure 3. *Nucleoside analogues that inhibit S-adenosylhomocysteine hydrolase.*

the other not. Classical drugs are often made by total synthesis, and separation of the enantiomers is necessary to evaluate the difference in activity between them. This is followed by the synthesis and further development of only one of the two isomers. The inactive enantiomer in the racemic mixture is, at best, only supplementary and unnecessary ballast for the body, which has to eliminate it after metabolism. In the discovery of drugs from natural origins, however, the process is the reverse.

The compounds presented by nature are mostly optically active and only one enantiomer is biologically evaluated. This is also the case when new compounds are synthesized using natural products as starting materials. Because of the complexity of most natural compounds, the mirror image compound is often never synthesized and evaluated for its potential biological activity. This is the first possible reason why it took so long for L-nucleosides to be evaluated. Natural nucleosides have the D-configuration, and only recently have L-nucleosides been found to possess antiviral activity. For example, it was rather surprising that L-3TC (**9**) is more active and less toxic than D-3TC (Refs 5,6). The first L-nucleosides were synthesized back in the 1960s, but the great interest in the L-series of nucleosides is very recent. Research into L-nucleosides as antivirals started in the field of carbocyclic nucleosides (e.g. C-BVDU). These molecules were first synthesized as racemic mixtures[7] and separation was necessary to analyse the biological activity of each enantiomer[8].

Impact of AIDS

There is a second and more important reason why the discovery of potent antiviral L-nucleosides has been long in coming. L-Nucleosides were discovered as antiviral compounds because of the setting up of large screening systems against HIV during the last decade. Without the appearance of AIDS, their discovery might perhaps have taken another decade. This has to do with the structure of the discovered compounds. A normal ribonucleoside has four chiral centres and a deoxynucleoside has three stereogenic centres. However, the substituents on only two of these chiral centres are absolutely required for the activity of the classical nucleosides. The biological activity of a nucleoside is dependent on the nature of the base moiety; thus the aglycon moiety at position C1 is considered as absolutely necessary. As the nucleosides have to be phosphorylated to become active, the presence of a primary hydroxyl group at position C4 is likewise a prerequisite for biological activity. Thus, the configuration of the C1 and C4 atoms of a nucleoside is of crucial importance. The difference between a D-nucleoside and an L-nucleoside in the dideoxy series is in fact not very pronounced. When the base moiety and the hydroxymethyl group are considered as reference points, the ring oxygen function is situated on the backside in D-nucleosides and on the frontside in L-nucleosides, opposite to the C2–C3 bond (Figure 4). D- and L-dideoxynucleosides overlap very well[9]. Therefore it is not surprising that antiviral activity has been found mainly in the series of L-dideoxynucleosides [e.g. L-dideoxycytosine (**12**), L-fluorodideoxycytosine (**13**) and L-dideoxydidehydroadenosine (**14**)[10–12]] and in the related L-oxathiolane[13] series, with the cytosine (**9**) and 5-fluorocytosine (**15**) analogues as important examples (Figure 5). These are mainly cytosine nucleosides, which may be a reflection of the kinases involved not being very selective. Indeed, similar antiviral activity of D- and L-dideoxynucleosides is not a general rule, as demonstrated by carbovir (**23**, Figure 10)[9]. The stereoselectivity of antiviral activity between the enantiomers of carbovir is a reflection of the different phosphorylation by cellular enzymes (5′-nucleotidase, GMP kinase, nucleoside-diphosphate kinase)[9].

Stereogenic centres

Nucleosides with two stereogenic centres may be phosphorylated by cellular kinases, and the triphosphates of these nucleosides are able to interact with polymerases. Once a third asymmetric centre is introduced into the nucleoside, the interaction between substrate and enzyme becomes more complex, and it is less likely that the D- and L-forms of the nucleoside analogue will both be recognized by the different enzymes involved in nucleoside metabolism. Briefly, the discrimination by the nucleoside metabolic enzymes increases with the number of stereogenic centres present.

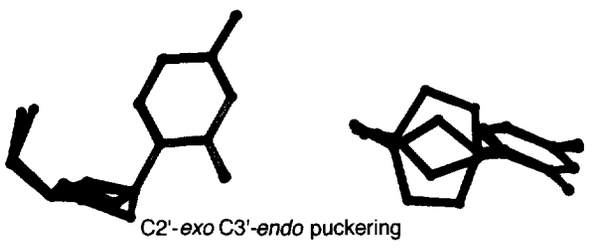


Figure 4. *Molecular overlap of* D- *and* L-*dideoxynucleosides.*

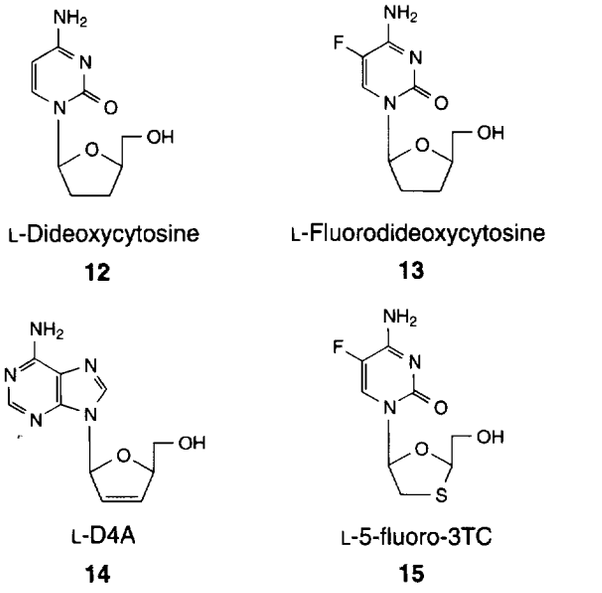


Figure 5. *L-Dideoxynucleoside analogues showing anti-HIV activity (lamivudine is mentioned in Figure 2).*

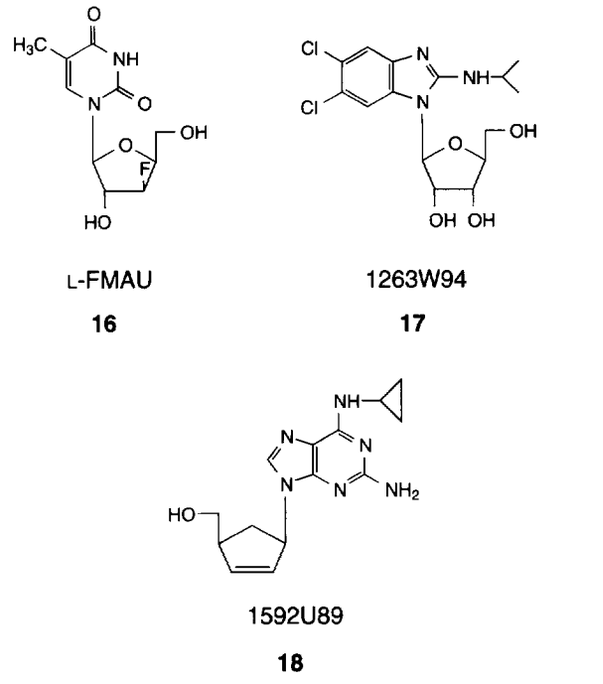


Figure 6. *Lead structure of some new antivirals whose mode of action differs from that of the classical antiviral nucleosides.*

The L-analogue of the potent anti-HIV compound zidovudine (**5**), for example, is not active[14], although it is considered to be a dideoxy analogue. This compound indeed has three chiral centres. The secondary hydroxyl group is replaced by an azido group and structurally it belongs to the 2′-deoxynucleosides and not the dideoxynucleosides. It is not known if the inactivity of the L-analogue is due to insufficient phosphorylation and/or the fact that its triphosphate is not recognized by reverse transcriptase.

Looking at the structures of other L-nucleosides with antiviral activity, there seem to be some data that contradict the above observation. Two compounds, L-FMAU (**16**)[15] and 1263W94 (**17**)[16], have four chiral centres and are active in the β-L-configuration (Figure 6). L-FMAU is a selective inhibitor of Epstein–Barr virus replication, and 1263W94 is an anti-CMV compound significantly more active than ganciclovir. However, the latter compound (1263W94) is a viral DNA synthesis inhibitor and its mechanism of activity is not mediated through inhibition of CMV DNA polymerase. The virus-specific gene product that functions as a target for 1263W94 is still under investigation. L-FMAU, on the other hand, could be converted to its mono-, di- and triphosphate metabolites. However, the triphosphate of L-FMAU is not a substrate for viral or human DNA polymerase. The mode of action of this compound is also different from that of other antiherpes nucleosides. Thus, both apparent exceptions in fact open up new fields in antiviral chemotherapy. They uncover new modes of action with probably less danger of these compounds being incorporated into human DNA (one of the mechanisms that may lead to mutagenesis).

New antiviral nucleoside development

From a structural point of view, the question may be asked in which directions can new antiviral nucleosides be developed. This is, of course, difficult to predict, but two recent findings put forward some new starting points for thinking. Antiviral nucleosides consist of a base moiety and a carbohydrate or a carbohydrate mimic. It was generally believed that conformational flexibility of the carbohydrate part of a nucleoside is important for the metabolic activation of the antiviral nucleoside and for interaction with the target enzyme. On the level of each phosphorylation step and the polymerase reaction, the conformation of the sugar part of the active nucleoside may be different, and these conformations may even change during the reaction. Therefore, new antivirals were developed that predominantly belonged to the group of acyclic nucleosides and the group of nucleosides with a five-membered ring sugar.

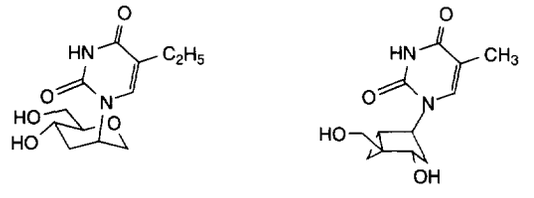
Six-membered ring carbohydrates

Some years ago, however, a new series of nucleosides was discovered with a six-membered ring carbohydrate moiety. These nucleosides have a 1,5-anhydrohexitol structure (**19**, Figure 7) with the heterocyclic base situated in the 2-position[17]. The solution- and solid-phase conformation of these compounds indicate the presence of an axially oriented heterocyclic moiety. Interestingly, the antiviral activity disappears when the oxygen atom is removed and the base moiety adopts an equatorial position.

The conformational preference of these nucleosides may be explained by the influence of steric factors and bond lengths. This means that the molecule can be forced into its biologically active conformation by creating more steric hindrance around the hydroxymethyl group than in the neighbourhood of the base moiety. Figure 8 shows an overlap between the antiviral anhydrohexitol nucleoside and natural thymidine in its frozen 2′-*endo*/3′-*exo* and 3′-*endo*/2′-*exo* conformations. These are the two extreme conformations of a natural nucleoside and the energy difference between them is around 4 kcal/mol. From this overlap it is clear that the anhydrohexitol nucleosides geometrically fit to a furanose nucleoside in the 3′-*endo*/2′-*exo* conformation, and no decent overlap is possible between the carbocyclic analogues and thymidine in these two conformations. These data suggest a similar conformational preference of furanose nucleosides during one of the crucial enzymatic steps leading to antiviral activity. This finding is surprising given the observation that the most usual conformation of thymidine itself is the 3′-*exo*/2′-*endo* form.

Activity of (N)-methanocarba T

A second interesting observation is the described antiherpes activity of (*N*)-methanocarba T (Ref. 18) (**20**, Figure 7).



Anhydrohexitol nucleoside **19** (*N*)-Methanocarba T **20**

Figure 7. *Example of conformationally constrained nucleoside analogues with antiherpes activity.*

Although we do not know the exact mode of action of this bicyclo[3.1.0]hexane nucleoside, the discovery of its biological activity fits nicely with the observations mentioned above. This compound can be considered as an analogue of carbocyclic thymidine with a 4′,6′-methylene bridge. It is a conformationally rigid analogue of thymidine in its 2′-*exo* conformation. The other analogue with a cyclopropane ring between C1′ and C5′ does not show antiherpes activity. This compound can be considered as a thymidine analogue frozen in its 3′-*exo* conformation. The reason for its inactivity may be steric hindrance. These observations indicate that at least one of the crucial enzymes involved in nucleoside activation accepts the 2′-*exo* conformation. Studies of both the anhydrohexitol nucleosides and the methanocarbocyclic nucleosides demonstrate that a more rational approach to new antivirals may be possible in the future.

Finding the right combination of a carbohydrate mimic and a heterocyclic ring, based on configurational and conformational analysis of enzymes and substrates, is certainly one of the right ways to proceed, but, in this respect (with the exception of HIV research), it is amazing how little is known about the structure of the viral enzymes that are considered as targets for antiviral chemotherapy. A step in the right direction is the study of a quantitative explanation for

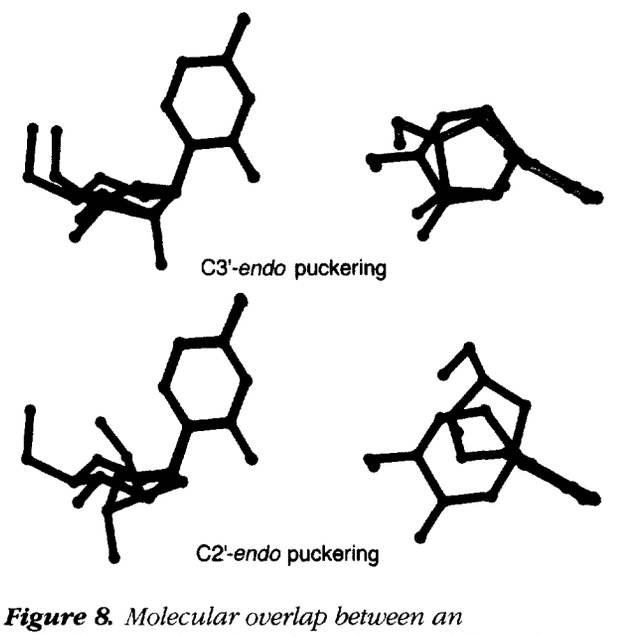


Figure 8. *Molecular overlap between an anhydrohexitol nucleoside and natural thymidine in its frozen C3′-*endo *and C2′-*endo *puckering conformation.*

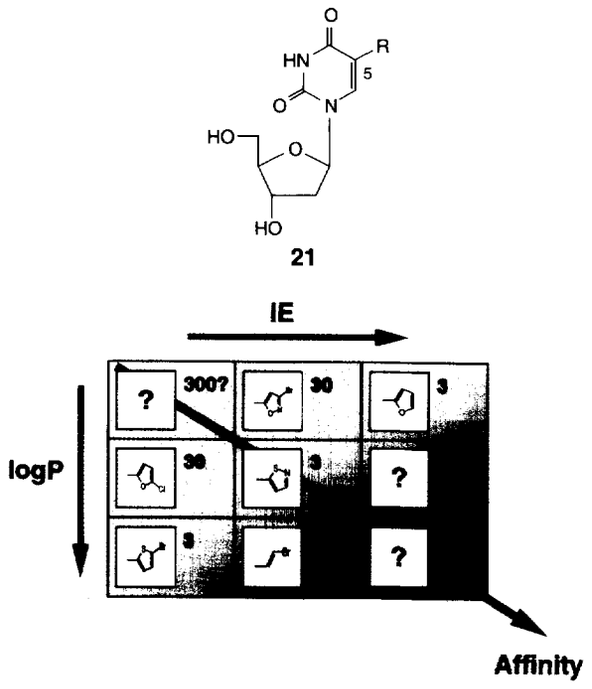


Figure 9. *Schematic representation depicting the counterbalance between hydrophobicity (log P) and interaction energy (IE) of the 5-substituted side chain on the affinity of the compound for HSV-1 thymidine kinase. Affinities are in μM and are only given as an indication.*

the HSV-1 thymidine kinase affinity of 5-substituted 2′-deoxyuridines (**21**)[19]. 5-Substituted 2′-deoxyuridine derivatives were the first antiviral nucleosides to be discovered. These compounds have to be enzymatically phosphorylated before they can exert antiviral activity. The phosphorylation in HSV-infected cells is carried out by a virus-specific thymidine kinase. This selective phosphorylation in virus-infected cells partly explains the selectivity of these compounds. Based on crystallization experiments, a study has been undertaken to analyse the structural characteristics of a heterocyclic ring substituted in the 5-position of 2′-deoxyuridine in view of its affinity for the activating enzyme[19]. The interaction energy between substrate and enzyme is often counterbalanced by the hydrophobicity of the substituent; this is demonstrated in Figure 9. Improved binding affinity for the enzyme can be obtained by ameliorating the hydrophobicity of the compound by introduction of halogen or methyl groups. The introduction of large groups into the heterocyclic system, however, often leads to a decrease in interaction energy (e.g. due to steric clashes) with the enzyme. The net result is that the final affinity remains largely unaffected. Figure 9 also demonstrates that there is still room for improvement.

Of course, enzymatic phosphorylation is not the only factor that determines antiviral activity. Some nucleosides are very efficiently phosphorylated but are worthless as antivirals; the opposite is also true. A nice example of the first series is 5-chloro-3′-fluoro-2′,3′-dideoxyuridine[20,21] (935U83). This compound (**22**, Figure 10) shows excellent anti-HIV activity *in vitro*, a high selectivity index and it is efficiently phosphorylated in cells[21]. But, it is not useful in the clinic. In three clinical studies involving more than 140 HIV-infected persons [monotherapy and in combination with DDI (didanosine) or AZT/3TC], the compound has demonstrated a lack of *in vivo* antiviral effects (W. Spreen, pers. commun.). Prediction of *in vivo* behaviour of an antiviral nucleoside is still not possible and will, perhaps, never be. However, there is indeed an urgent need for more rigorous SAR studies of substrates and enzymes involved in antiviral nucleoside metabolism. This area of fundamental research is just beginning and there is no doubt that it will lead to new, safer antivirals.

Pharmacokinetic and pharmacodynamic factors

The above considerations lead us to some other problems associated with antiviral drug development; this may best be demonstrated using 1592U89 as a model[22] (**18**, Figure 6). This compound is a carbocyclic nucleoside analogue, active *in vivo* against HIV-1 infections and well tolerated. It can be considered as a prodrug of the phosphates of (–)-carbovir (**23**)[23], and a correlation has been demonstrated between carbovir triphosphate formation and anti-HIV activity.

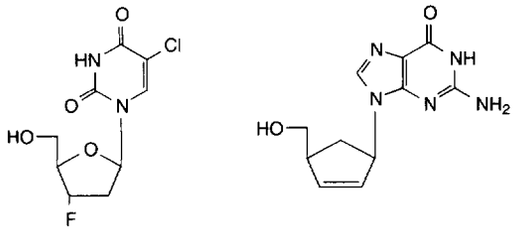


Figure 10. *Structure of some potent anti-HIV compounds that never reached the market because of insufficient* in vivo *activity (**22**) or toxicity (**23**).*

1592U89, however, does not show the same toxicity as carbovir. It allows the nucleoside carbovir, which has caused problems in animals (e.g. very insoluble, precipitates in the kidney, poor penetration of the CNS, poor oral absorption)[22], to be bypassed. The pharmacokinetic, distribution and toxicity profile of 1592U89 is distinct from and improved over that of (–)-carbovir (**23**, Figure 10)[22]. Moreover, *in vitro* it is as active against HIV-1 as zidovudine, but *in vivo* it is more potent than zidovudine, giving a more pronounced reduced viral load over a longer time than zidovudine itself. This means that our ideas about nucleoside behaviour *in vivo* are too simplistic and there is no *in vitro* model that is predictive of *in vivo* activity. The influence of pharmacokinetic and pharmacodynamic factors on the final activity of a compound is very large. Research into the development of all kinds of prodrug forms of nucleosides should be further encouraged, but these molecules should be considered as separate entities rather than as masked nucleoside analogues. They should be evaluated for their efficacy *in vivo*, before studies of their *in vitro* properties by extensive biochemical and serum stability research are begun.

Structurally, 1592U89 (**18**, Figure 6) belongs to the carbocyclic nucleosides, which means that the ring oxygen atom is replaced by a methylene group. When looking at the general structure of such a nucleoside analogue, the difference in structure with that of a natural nucleoside seems very small. This is because we have learned to look at structures in an oversimplified way and thereby make wrong conclusions. The difference between a carbocyclic nucleoside and a nucleoside with a pentofuranose ring is very large. The anomeric centre is removed, as is the stereoelectronic influence of the ring oxygen atom which creates the characteristics of a 'nucleoside'. This means that the SAR of carbocyclic nucleosides is different to that of normal nucleosides[24].

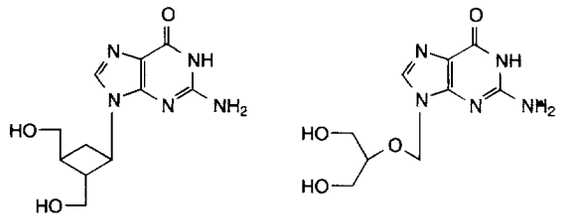
Staying with the above example, the furanose analogue of carbovir is of low potency and has only a very small safety margin. Despite this, we keep going by extrapolating SAR studies and synthesizing the carbocyclic analogues of those furanose nucleosides that have been demonstrated to posses antiviral activity. However, compounds considered structurally as nucleoside analogues are not always functional nucleoside analogues, and in this way we will miss a lot of potentially important compounds. An in-depth structural analysis of every separate class of nucleoside analogues based on steric and stereoelectronic considerations is needed to increase the chance that we do indeed combine the right heterocycle with the right carbohydrate mimic in order to obtain antiviral activity.

Desirable mode of action and spectrum of activity

A last problem that I would like to discuss briefly is what kind of antivirals are needed. A historical example of drug development in the field of anti-infectious diseases is the antibiotics, with the structural beauty of penicillin as an example. These compounds, with a broad spectrum of antibacterial potency, exert their activity by forming and breaking covalent bonds. To target bacteria and viruses is different from targeting cellular receptors with equilibrium binding compounds. The latter compounds are mostly too gentle for the infectious agent, which quickly learns to escape the action of these substances by mutating. A potent antiviral agent should interfere with a specific viral target by formating and breaking covalent bonds, which is a less reversible process and increases the possibility of destroying the infectious agent. This concept, in fact, fits the mode of action of most nucleoside analogues. Once they are incorporated into DNA, for example, they may cause serious damage and impede normal viral replicative cycles. This is one of the reasons why most antivirals that are on the market are nucleoside analogues. Their activity could be improved by using combination chemotherapy. A drug mixture may be used in which the individual compounds are aimed at interfering with different viral targets. From what we know about antiviral compounds, however, we can predict that a nucleoside will always have its place in such an antiviral cocktail.

The second lesson that we may learn from antibiotics is that it would be advantageous to have at our disposal antivirals with a broad spectrum of activity. Although antiviral nucleosides with a broader activity spectrum, such as lobucavir (**24**, Figure 11)[25], have been discovered in the past, the breakthrough really came with the discovery of the acyclic nucleoside phosphonates.

Antiviral compounds often show activity against a series of related viruses. However, this does not mean that the mode of action is the same in the different viruses. For example, compounds active against HSV are often active against varicella-zoster virus or even CMV (e.g. ganciclovir; **25**, Figure 11)[26]. However, only recently a gene (UL97) product was found to be involved in the phosphorylation of ganciclovir in human cytomegalovirus (HCMV) infected cells[27], opening up opportunities for the design of new anti-HCMV compounds.



Lobucavir **24** Ganciclovir **25**

Figure 11. *Examples of second generation antiviral nucleosides whose activity spectrum is broader than that of the parent lead compound.*

Compounds active against HIV are sometimes also active against hepatitis B virus (e.g. adefovir; **4**, Figure 1)[28]. This is understandable because the replicative machinery of these viruses has some common mechanisms. The first example of a really broad-spectrum antiviral compound is cidofovir (**3**, Figure 1)[28]. This compound is active against herpesviruses, hepadnaviruses, papillomaviruses, adenoviruses and poxviruses. Cidofovir and adefovir are nucleoside phosphonates; this means that the enzymatically labile phosphate function of the nucleotide is replaced by a more stable phosphorus–carbon functionality. In this way the first step in the metabolic activation of the nucleoside (i.e. phosphorylation) can be overcome, although this mechanism may not be the only one explaining its biological activity.

The mode of action of cidofovir may be different from one virus to another. Similarly, cidofovir has side effects, and there seems to be a relationship between broad-spectrum antiviral potency and increase in toxicity. Here also there is room for improvement, and there is no reason to believe that cidofovir will remain the only broad-spectrum antiviral. Research on nucleotide mimics, say at the level of the mono-, di- or triphosphate, still has to commence and should lead to the discovery of new interesting structures with antiviral activity.

Paradoxical findings

Perhaps the attentive reader has discovered that some of the data mentioned in this review are contradictory. For example, I mentioned that safe nucleosides should best not be phosphorylated but, on the other hand, I gave examples of new directions in nucleoside research where phosphorylation of the compound is necessary for antiviral activity. This, however, reflects the versatility of antiviral nucleoside research. A nucleoside is more than the sum of carbohydrate + heterocycle, and interaction with DNA is not the only mechanism for antiviral activity. A nucleoside may be an ideal transport system for a heterocycle, selectivity with nucleosides may be obtained just by making use of natural uptake systems, or a nucleoside analogue may have a beneficial effect on the immune system, which contributes to its biological activity. There is still so much we do not understand about nucleosides, and many pathways of antiviral nucleoside metabolism and targets for antiviral nucleosides are still to be discovered.

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