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Discussion Letter

Factors contributing to the inhibition of HIV reverse transcriptase by chain-terminating nucleotides in vitro and in vivo

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Arguments are presented leading to the conclusion that two major factors contribute to the potency of inhibition of DNA-polymerase activity by chain-terminating nucleotides. The relative significance of these factors varies with the reaction conditions, particularly with the length of the template and the concentration ratio of enzyme (reverse transcriptase or other DNA polymerase) to primer. It is concluded that potent inhibition of HIV-reverse transcriptase activity under typical in vitro and in vivo conditions arises from different features of the interaction of chain terminators with the enzyme. A new method of testing for the parameter important under in vivo conditions is suggested.

Chain termination; Azidothymidine; HIV; AIDS; Reverse transcriptase

1. INTRODUCTION

The only currently available chemotherapy of HIV infections is that provided by the use of a nucleoside analog, 3′-azidothymidine (review: [1]). While there are serious and limiting side-effects caused by the use of this agent, there is also good evidence that it has a positive influence on the course of the disease. Other dideoxy nucleoside analogs, in particular dideoxy cytidine and dideoxy inosine, appear to have similarly beneficial effects, with different side-effects. The experience with these drugs may be taken as encouragement to proceed with the search for alternative and better potential drugs from this class of compounds, and to help achieve this aim, a detailed knowledge of their mode of action is needed.

Most of the nucleoside derivatives which have been identified as having potent anti-HIV activity belong to the family of 2′,3′-dideoxynucleosides, i.e. they are analogs of the naturally occurring 2′-deoxynucleosides in which the 3′-hydroxyl group has been replaced by another moiety, in the simplest case by a hydrogen. In their triphosphorylated form, they are able to inhibit DNA synthesis by DNA-polymerases by incorporation of the analog monophosphate at the 3′-end of the growing chain, which leads to chain termination. This, together with a more potent effect on reverse transcriptase than on cellular polymerases, appears to be the basis of their inhibitory effect on HIV replication. Thus, the derivatives must, after entering the cell in their non-phosphorylated form, first be phosphorylated by cellular kinases to give the active form of the drug. This process is obviously of great importance, and since several kinases of varying specificity are involved, it occurs with varying efficiency with the different agents available. The problems occurring during pro-drug activation will not be discussed here, but it is clear that a more rational approach to optimising substrate properties towards cellular kinases is desirable.

Once the nucleoside analog is present in cells in its triphosphorylated form, its interaction with the viral reverse transcriptase after invasion by HIV appears to be the crucial factor involved in inhibition of viral replication. An understanding of the mechanism of this inhibition, and of the reasons for the specificity of the effect for the viral enzyme when compared with cellular DNA polymerases is of great importance for the further search and development of better nucleoside-based drugs. We believe that there may be some misunderstanding of the relative importance of different aspects of the interactions involved, and also that currently used in vitro reverse transcriptase inhibition assays are not necessarily appropriate for screening and comparison of this class of inhibitors.

2. THEORY

Two main factors appear to contribute to inhibition of DNA polymerases by chain terminators. The first of these is competition between the dideoxynucleotide and

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the natural deoxynucleotide in binding to the enzyme and incorporation into the DNA chain. To examine the results of this type of competition in a quantitative manner, we consider a system consisting of a template molecule (RNA or DNA), a primer (tRNA or an oligonucleotide) and an excess of reverse transcriptase (RT) over primer/template complex (P/T). This means that all potential sites of primer extension are occupied by polymerase molecules, which in turn means that steps involving dissociation of the enzyme from completed or terminated duplexes need not be considered in this analysis. The situation after addition of a nucleoside triphosphate complementary to the template can be described formally by the following scheme:

$$\mathrm{RT + P/T} \underset{K_{p/t}}{\rightleftharpoons} \mathrm{RT{\cdot}P/T} \overset{+\ \mathrm{dNTP}}{\underset{K_s}{\rightleftharpoons}} \mathrm{RT{\cdot}P/T{\cdot}dNTP} \xrightarrow{k} \mathrm{RT{\cdot}P\text{-}dNMP/T}$$

Scheme 1. RT is reverse transcriptase, P/T is the primer/template complex, dNTP is a deoxynucleotide triphosphate and P-dNMP is the primer which has been extended by addition of one deoxynucleotide monophosphate residue. $K_{p/t}$ is the dissociation constant of the RT·P/T complex, K_s is the dissociation constant for dNTP from its complex with RT·P/T and k is the rate constant for the transfer of dNMP to the 3′-end of the primer.

A simiiar scheme applies for a chain-terminator, and here we use the constants K_i and k_i to correspond to K_s and k. We now assume that the template is a homopolymer, e.g. polyrA, which means that for the substrate (dTTP), steps 2 and 3 of Scheme 1 occur in a cyclical manner until the primer is extended to the length of the template. Whether the polymerase dissociates during the course of this filling up reaction or not depends on the processivity of the enzyme, which is in turn dependent on the relative rates of the polymerisation reaction and the rate of dissociation of the enzyme from extended primer/template complexes which still have an overhanging 5′-template end. The probability of dissociation obviously also increases with the length of the template, but under the conditions used for this analysis such events are of no consequence, since free primer ends will be rapidly occupied by other reverse transcriptase molecules.

Under the conditions defined here, the effective second order rate constant defining the rate of incorporation of substrate into the growing chain is k/K_s. This expression is analogous to the well-known k_{cat}/K_m value in classical enzyme kinetics. The corresponding expression for the chain terminator is k_i/K_i, and the relative rate of incorporation of substrate and chain terminator at each position of the template (i.e. for each mononucleotide added) is given by the expression:

$$k_{app}/k_{app,i} = k[S]K_i/k_i[I]\,K_s \qquad (1)$$

where k_{app} and $k_{app,i}$ are the respective pseudo-first-order rate constants for incorporation of substrate and chain-terminator, respectively, into the growing chain. The probability, p, that substrate, and not chain terminator, is incorporated at each step is given by

$$p = k_{app}/(k_{app} + k_{app,i}) \qquad (2)$$

where p lies between 0 and 1. Considering the first position to be filled at the 3′-end of the primer, and using C_0 as the concentration of primer bound to template, the concentration of substrate incorporated (C_1) is given by

$$C_1 = C_0 p \qquad (3)$$

and at position 2 by

$$C_2 = C_0 p^2 \qquad (4)$$

and in general for the i^{th} position by

$$C_i = C_0 p^i \qquad (5)$$

The sum of the concentrations of substrate incorporated at each step gives the amount of substrate incorporated (C_{inc}) if the reaction is allowed to go to completion in the presence of a chain terminator. This is given by

$$C_{inc}/C_o = \sum_{i=1}^{i=n} p^i = \sum_{i=1}^{i=n}(p + p^2 + p^3 + \ldots\ldots p^n) \qquad (6)$$

where n is the number of bases wich can be added to each primer molecule as determined by the length of the template. This finite geometric series can be converted to the standard form

$$C_{inc}/C_o = \sum_{i=1}^{i=n} p(1 + p + p^2 + \ldots\ldots p^{n-1}) \qquad (7)$$

whose sum is known to be equal to

$$p((p^n - 1)/(p - 1)) \qquad (8)$$

and thus

$$C_{inc} = C_o p((p^n - 1)/(p - 1)) \qquad (9)$$

We can then define the relative amounts of substrate incorporated in the presence and absence of chain terminator (I_f, the fractional incorporation) by dividing this equation by nC_o, which is the concentration of substrate which can be incorporated in the absence of chain terminator, by

$$I_f = p((p^n - 1)/n(p - 1)) \qquad (10)$$

Expressing this as a percentage ($I_\%$)

$$l_{\%} = 100p((p^n - 1)/n(p - 1)) \quad (11)$$

A more useful form of the equation uses r, the ratio of the effective rate constants for incorporation of chain terminator and substrate ($k_{app,i}/k_{app}$). From Eqn. 2, it is easy to show that

$$p = 1/(r + 1) \quad (12)$$

Substituting this in Eqn. 11, we obtain

$$l_{\%} = 100(1 - (1/(r + 1))^n)/nr \quad (13)$$

A practical form of the equation is obtained by substituting for r in Eqn. 13 using the relationship

$$r = k_i K_s i/k K_i s \quad (14)$$

where s and i are the substrate and inhibitor concentrations, respectively. If we also substitute $k_i K_s/k K_i$ by the parameter k_r, which is the relative size of the effective second-order rate constants for chain terminator and substrate, we obtain the equation

$$l_{\%} = 100s(1 - (1/(k_r i/s + 1))^n)/nk_r i \quad (15)$$

3. EXAMPLES

We can now begin to examine the expected degree of chain termination and of inhibition of the extent of substrate incorporation in the presence of chain terminators in different situations with respect to the length of the template and the relative concentrations of primer/template and enzyme.

3.1. *Short templates*

In the first example, the nature of the template/primer complex is such that only 10 nucleotides can be added to the primer before the double-stranded region is complete. If we assume that k_r is equal to 1, which appears to be the case for AZTTP compared with TTP [2], and assume that substrate is present at 1000 times the concentration of chain terminator (AZTTP shows significant inhibition at such relative concentrations in standard in vitro assays), p is 0.999, and from Eqn. 5 the concentration of chains which reach position 10 on the template is $0.999^{10}C_0$ or $0.99C_0$. Thus, 99% of the chains are completed, which means that only 1% chain termination will have occurred, and if inhibition were defined to be the degree of chain termination, it would be 1%. This is the parameter which is of potential interest in judging the potency of the inhibitor in vivo, since chain termination at any position (except the last) will lead to lack of completion of the new DNA strand. However, standard in vitro tests measure the amount of nucleotide incorporation, and using Eqn. 15 it can be calculated that the incorporation of substrate in the presence of chain terminator, $l_{\%}$, is 99.45%. Thus, there will be 0.55% inhibition of the extent of incorporation under these conditions, which would be barely measurable. At a ratio of 100:1 of substrate to chain terminator, this would become 5.3%. 50% inhibition of incorporation would be seen at relative concentrations of terminator and substrate of ca. 0.2, corresponding to 4 μM if the substrate concentration is 20 μM, which is a commonly used value. This is 2–3 orders of magnitude higher than that seen with AZTTP/TTP, indicating that chain termination per se together with subsequent removal of the terminated chain from the pool of growing chains cannot be responsible for the potent inhibition seen in in vitro tests by AZTTP and other dideoxynucleotides. Thus, another factor must be responsible for the potent inhibition seen, and this arises from the fact that in the in vitro assays, catalytic amounts of enzyme with respect to primer are used, i.e. there is a large excess of primer over enzyme. Under these conditions, the probable mode of inhibition is a consequence of the processivity of the enzyme. The processivity is determined by the relative rates of chain propagation and dissociation of the polymerase from its complex with template/primer. High processivity (i.e. a large number of nucleotides added in a processive manner without the enzyme leaving the RNA–DNA or DNA–DNA complex) is ensured by a low rate of spontaneous dissociation. We [2] have measured this rate to be ca. 0.04 s^{-1} at 25°C for a chain-terminated complex in the case of HIV-1 reverse transcriptase, or a factor of ca. 10 lower from a complex analogous to the 'dead end' complex described for other polymerases and which is a complex formed between template, chain-terminated primer, polymerase and the deoxynucleotide triphosphate which would have been added next in a template-directed manner if chain termination had not occurred. The affinity of the reverse transcriptase-template/primer interaction is ca. 10^{10} M^{-1} in such complexes [2], or a factor of ca. 10 lower without deoxynucleotide triphosphate. The affinity for non-terminated template/primer complexes appears to be similar, at least in the absence of dNTP, but under polymerisation conditions, its effective affinity is given by its K_m value, which will be much higher than the true dissociation constant (at least with short templates), since polymerisation and (presumably) dissociation from filled up complexes (i.e. blunt ends) gives the enzyme a faster way out of this stable complex. Thus, an irreversibly terminated template/primer complex is a very effective inhibitor of the normal enzyme-template/primer interaction.

Such a mode of inhibition has been described in detail for the case of herpes simplex DNA-polymerase and the antiviral agent acyclovir, which functions in its phosphorylated form as a chain terminator [3]. The antiviral action of this analog is thought to arise from this type of inhibition. It is thus of interest to consider whether

the anti-HIV action of AZT and other nucleoside analogs might arise from a similar mechanism.

3.2. *Long templates*

At this point, it is important to realize that there is a fundamental difference in the concentration relationships in the in vitro tests and in cells which have just been penetrated by HIV. This arises because each molecule of viral RNA has its 'own' reverse transcriptase molecule, whereas in the in vitro tests, each polymerase molecule must move between a large number of potential polymerisation sites. If it cannot do this, or only very slowly, because of chain termination by nucleotide analogs and formation of a dead end complex, inhibition of polymerisation will result because many primer molecules will never 'see' an enzyme molecule. Although the strategic details of reverse transcription in retroviruses are not yet fully understood, it seems unlikely that removal of reverse transcriptase molecules from the pool available for polymerisation will contribute to the inhibition observed, for the simple reason that there are at least as many polymerase molecules as viral RNA molecules. However, this does not, of course, alter the fact that chain termination at any stage of transcription will lead to incomplete and presumably non-functional DNA chains. Nevertheless, it seems likely that potent inhibition can occur in this situation because of the much greater length of the template than in the in vitro experiments. If we take the same probability, p, of incorporation of the natural nucleotide as was used in the first example, and then choose a chain length of 3000 (approximately the number of thymidines which are incorporated on reverse transcription of the HIV RNA) for the template instead of 10, the probability that termination has not occurred after 1000 bases is $(0.999)^{3000}$ or ca. 0.05, meaning that ca. 95% chain termination will have occurred, compared to 1% after 10 added nucleotides. The degree of inhibition of nucleotide incorporation can be calculated to be ca. 68.4%. The figure which is relevant for judging the potency in terms of antiviral activity is the degree of chain termination. Thus, if an AZTTP concentration which is 1000th of the TTP concentration can be achieved in cells prior to invasion by HIV, which seems likely [4], potent inhibition should occur. We therefore conclude that inhibition of completion of transcription by low concentrations of AZTTP is a quantitatively realistic mechanism for the antiviral effect of AZT, but that the origin of the potent inhibition is not the same as that in the standard in vitro tests.

4. CONCLUSIONS

In pragmatic terms, it is initially not of great importance which of the two mechanisms for potent inhibition is responsible for the antiviral effect of AZT. However, in searching for alternative agents, it is possible that some promising candidates might be eliminated on the basis of the in vitro test using short templates. It is conceivable that a substance which has very good incorporation kinetics, which is the important parameter in vivo, and which is also well metabolized by cellular kinases, might be missed at an early stage in screening because it does not form a particularly stable dead end complex, which is the decisive factor for in vitro inhibition. The different possible mechanisms of inhibition are also of relevance to studies of the selectivity of AZTTP and similar reagents for reverse transcriptase as compared to cellular polymerases. Such studies have also been done using short templates, and might thus give results which do not apply to the in vivo situation with long templates.

A convenient assay which removes these uncertainties would be valuable. The most rigorous method, the examination of single step kinetics, is not a realistic approach for screening large numbers of substances. The use of long single-stranded templates, although experimentally less convenient than standard template/primer complexes, gives a better approximation to the in vivo situation, since the significance of formation of dead end complexes is reduced, although still not removed if catalytic amounts of enzyme are used, which will normally be the case if the time dependence of the incorporation is to be followed conveniently. However, it is conceivable to obtain an estimate of the relative rates of incorporation of a chain terminator and a substrate using Eqn. 15 without the need to follow the polymerisation reaction at high temporal resolution. The experimental design would be similar to that used for the theoretical examples discussed above, i.e. an excess of reverse transcriptase over primer/template would be used, and the amount of substrate incorporated after allowing the polymerisation reaction to go to completion in the presence and absence of chain terminator would be compared. If the $l_{\%}$ is determined at various inhibitor concentrations and constant substrate concentration, Eqn. 15 can be used in conjunction with one of the commercially available computer programs which allow non-linear regression fits to equations entered by the user (e.g. Grafit) to obtain a value for k_r. This could be done with long or short templates, as long as n, the number of sites at which the chain terminator can potentially be added, is known.

There are several advantages of the suggested method of evaluating the properties of chain terminating nucleotides. Firstly, a parameter is obtained which is an objective measure of the efficiency of addition of the nucleotide monophosphate onto the end of the growing DNA chain. This is in strong contrast to the parameter which is called K_i or alternatively IC_{50} obtained from the standard steady-state assays. This value is purely empirical and applies only to the exact conditions used for the determination. In particular, it will be clear from the above analysis that this parameter will vary with the

nature of the primer template complex, in particular with the length of the template, and with the concentration ratio of enzyme/primer. In general, there will be contributions of both types of inhibition mechanism (i.e. chain termination per se and dead end inhibition), but the relative significance of these will vary according to the exact concentration relationships and the length of the template. Secondly, if an RNA (or DNA) template containing all four bases is used, the same template will serve for testing chain terminators having any of these as their base component, with the only modification that n, the number of sites at which the chain terminator could potentially be added, must be changed in the fitting equation. Similar assays could be set up for testing the inhibitory properties against cellular polymerases, allowing a more objective comparison of the inhibitory power of the substances against the viral and host cell polymerases.

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