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Mini-review

Is HIV drug resistance a limiting factor in the development of anti-HIV NNRTI and NRTI-based vaginal microbicide strategies?

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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

Antiviral drugs that act at specific sites within the HIV life cycle have important rationale for development as anti-HIV microbicides. However, to be effective, such drugs must act by directly interfering with viral enzymatic function and eliminate the ability of HIV to mediate infection. Compounds that are developed as microbicides must have high potency, and should ideally not be well absorbed from the vaginal cavity in order to minimize any potential problems of drug resistance. Such compounds should also be active over long periods of time and should be able to be combined with other active agents, in order to promote the concept of synergy, such as that which has been demonstrated in HIV therapeutic studies.

Keywords: HIV drug resistance; Microbicides; NNRTIs; NRTIs

Contents

1. Introduction

The worldwide HIV epidemic has become an enormous burden for women in the developing world (UNAIDS). Women are mainly infected by heterosexual transmission of HIV and often have no control over condom use by sexual partners. Estimates on the potential of vaginal microbicides for the prevention of HIV infection is promising (Foss et al., 2003; Smith et al., 2005), its use is thus clearly justified. Among the various compounds under investigation as potential microbicides specific anti-HIV drugs have a major role to play. Yet, an obvious concern is the potential of such compounds to select for drug resistance or the possible loss of activity of such substances against transmission of viruses from drug-resistant HIV carriers.

It is known that approximately ten percent of all new HIV infections in North America and Western Europe are now attributable to viruses that contain at least one drug resistance-associated mutation in either the reverse transcriptase (RT) or protease (PR) genes (Little et al., 2002; Salomon et al., 2000; Tamalet et al., 2003). In some cases, patients may be unlucky enough to become infected with viruses that contain multiple mutations that may remarkably confer resistance against all

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three families of antiviral drugs, i.e. protease inhibitors, non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside inhibitors (Brenner et al., 2002). The purpose of this review is to shed light on how and whether HIV drug resistance might affect the possible use of nucleoside/tide reverse transcriptase inhibitors (NRTIs) and NNRTIs as vaginal microbicides and to propose a framework for research on this topic.

Resistance may be an issue for several reasons. One is the potential for HIV transmission of resistant strains that may overcome a microbicide that is based on the use of specific antiviral drugs (ARVs). A second is the potential for selection of drug resistance by a woman who uses an ARV-based microbicide, without knowing that she is HIV-infected. The potential for relevant compounds to then be systemically absorbed and select for resistance will be key. It is also possible that the concentrations of drug that are ultimately present in the blood and lymphoid tissue will be inadequate to select for resistance in this circumstance.

A related subject is the use of a ARV-based microbicide by a woman who knows that she is HIV-positive but uses it to protect her sexual partner. Might she in any way be at risk of transmitting a resistant virus? Each of these topics is important, as is the subject of whether the vaginal microenvironment is one in which selection of resistant virus might take place. This might depend on the extent to which viral replication occurs in this environment as well as the propensity of an ARV to carry drug selection if formulated as a gel or foam. The answers to these important questions are not known.

2. Considerations of drug resistance

This problem assumes relevance in the context of developing country scenarios in which antiretroviral drugs have not generally been available, in view of the decision by the World Health Organization to scale up antiviral therapy through use of the co-formulation of stavudine/(d4T), lamivudine/(3TC), nevirapine (NVP). This combination of two nucleoside inhibitors plus a NNRTI has the potential to save millions of lives in the shortest possible period of time and hence should be supported. At the same time, however, the potential for development of drug resistance against any of the agents in this combination is very real and, accordingly, the World Health Organization (WHO) has instituted policies that will monitor the development of resistance in settings in which this combination is administered (Zewdie et al., 2004). This notwithstanding, it should be pointed out that two of the drugs in the combination, i.e. 3TC and NVP, possess a low genetic barrier for resistance. A fuller discussion of this problem can be found elsewhere (Wainberg, 2005).

Indeed, data from prevalence studies of HIV-1 drug resistance have revealed a wide range of results. In general, populations that have never been exposed to antiretroviral drugs (ARVs) may be expected to harbor low rates of resistance mutations (Gittens et al., 2003; Laurent et al., 2002; Petch et al., 2005; Vergne et al., 2003), since prevalence of drug resistance is closely coupled to access to therapy as shown in industrialized countries (Grant et al., 2002; Wensing et al., 2005). Under current circumstances in most developing countries, it may be impossible to discern individuals who have drug access from those that do not in regard to likelihood of harbouring susceptible viruses. A practical assumption may be to consider every patient as a potential carrier of drug-resistant viruses.

Consequently, a relevant issue that arises is whether microbicides might be equally able to protect against transmission of both wild-type and drug-resistant viruses in the developing countries. Several reports have revealed development of high rates of drug resistance when national HIV treatment programs were poorly implemented (Adje et al., 2001; Harries et al., 2001; Vergne et al., 2002). Drug resistance may become an issue in developing-country settings. To some extent, a danger exists for women who have received single dose nevirapine for prevention of mother to child transmission (MTCT), given that even limited exposure to this drug can result in selection of drug resistance (Abrams, 2004; Eshleman and Jackson, 2002; Eshleman et al., 2001; Jourdain et al., 2004; Martinson et al., 2004; Morris et al., 2004; Wainberg, 2005), since they may be compromised with regard to future therapeutic options. This subject also has relevance for NNRTI-based microbicide development, because women harbouring resistant viruses might conceivably transmit them to male sexual partners even if a microbicide were used. At the same time, vulnerable seronegative women could still potentially be protected by microbicides since their male sexual partners would in all likelihood not harbour resistant viruses if they had themselves never received treatment.

However, the WHO 3 × 5 initiative will likely transform the context of microbicide use so that we will ultimately need to be concerned about drug-resistant viruses harboured by the male sexual partners of women at risk and the nature of the mutations that are present in such population. A relevant question is whether or not all HIV drug-resistant viruses are likely to be transmitted with equal frequency or whether some viruses, that possess mutations associated with diminished replicative fitness, may be found less frequently in new infections than either wild-type viruses or viruses containing mutations that do not impact on fitness. Several studies, including one from our group, have reported that resistant viruses may be transmitted with a lower frequency than expected (de Mendoza et al., 2004; Leigh Brown et al., 2003; Yerly et al., 2004). In addition, estimates are that transmitted HIV-1 resistance will most likely remain low despite increased access to ARVs (Blower et al., 2001). For instance, viruses containing the M184V mutation in reverse transcriptase, associated with resistance to 3TC, are less likely to be found in new cases of HIV infection, i.e. primary HIV infection, than are either wild-type viruses or viruses containing mutations associated with resistance to other nucleosides and/or non-nucleoside reverse transcriptase inhibitors (de Mendoza et al., 2004; Quinn et al., 2000; Turner et al., 2004). This is interesting, but there are two important caveats in the interpretation of results. First, the 184V mutation may result in lower levels of viral load than those associated with wild-type viruses (Machouf et al., 2006). Therefore, the diminished frequency of transmission of M184V viruses may be attributable to the fact that individuals with low viral loads are less infectious in general than are people with higher viral loads (Turner et al., 2004; Wensing et al., 2005). Second, as pointed out in our paper, viruses carrying the M184V

mutation might be less easily detectable by genotype due to deselection and/or diminished replicative capacity compared to wild-type (Turner et al., 2004). Of course, it is also possible that viruses containing M184V are less frequently transmitted for reasons related to ability to cross-mucosal barriers and/or other considerations. These notions are consistent with factors known to be associated with the diminished replicative capacity of M184V, including diminished reversed transcriptase processivity or efficiency, diminished ability to participate in initiation of reverse transcription, and diminished capacity to participate in reverse transcriptase template switching reactions (Back et al., 1996; Devereux et al., 2001; Diallo et al., 2003a,b; Feng and Anderson, 1999; Gotte et al., 2000). Similar observations have been made for the L74V mutation in RT, principally associated with resistance to ddI (Diallo et al., 2003b; Frankel et al., 2005; Miranda et al., 2005). Detailed assessments of the impact of individual reverse transcriptase mutations on RT biochemistry have not been undertaken in regard to most other nucleoside mutations or mutations associated with the NNRTI family of drugs.

Another issue is that much of the relevant work carried out to date, with regard to both diminished transmission of M184V viruses and the impact of M184V on reverse transcriptase biochemistry, has been performed solely using subtype B viruses. Clearly, a broader study involving a variety of different subtypes is necessary in order to assess the risk in most developing country settings in which subtype C viruses are prevalent along with various recombinant viruses e.g. A/G.

Therefore, cohorts should be reassessed in an attempt to determine likelihood of transmission of viruses containing distinct mutations associated with HIV drug resistance. It is important to note that work to date suggests that viruses containing thymidine associated mutations (TAMs) and mutations associated with resistance to NNRTIs are probably transmitted at similar frequency to wild-type, whereas viruses associated with resistance to 3TC, containing the M184V mutation and PI mutations are transmitted with much lower efficiency (de Mendoza et al., 2004; Turner et al., 2004). Studies are needed to validate these findings with larger number of patient samples that include data from our own and other cohorts and results of international collaborations. Therefore, analyses of transmission of viruses associated with drug resistance should be extended toward a broader assessment of non-subtype B viruses as well as distinct analyses of individual mutational profiles in this regard. One hypothesis is that viruses associated with resistance to NNRTIs are probably transmitted at higher frequency than are viruses associated with certain nucleoside or nucleotide compounds. This may be related to the possibility that NNRTI-related mutations do not impact on replicative or transmission fitness to the same extent as other mutations, although studies on this subject are required. Among the latter is the drug tenofovir (TDF) that can select for the K65R mutation in RT that is also associated with diminished rates of transmission and diminished replicative capacity. Interestingly, rates of K65R presence have risen in recent years, largely due to the increasing use of this compound in clinical trials and in HIV therapeutics. This topic is also relevant, as TDF continues to be mentioned as a compound with important potential as a microbicide and in pre-exposure prophylaxis. Conceivably, this potential might not be compromised, if it could be demonstrated that viruses containing the K65R mutation are significantly less likely to be sexually transmitted than are either wild-type viruses or viruses associated with resistance to NNRTIs and thymidine analogs (ZDV and d4T). However, recent results establish concern. Notably, the selection of the K65R with tenofovir (TDF) in tissue culture can occur quite quickly, i.e. within 12 weeks, when subtype C viruses are studied, whereas such selections of resistance are far more difficult to achieve with subtype B viruses, (Brenner et al., in press). These findings clearly need to be validated in clinical studies.

An important topic for immediate discussion, is that related to the possibility that viruses associated with resistance to NNRTIs may be transmitted as well as are viruses of wild-type origin. This is due to the compelling rationale for use of such compounds as TMC-120 and UC-781 as microbicidal agents. In this context, the following points should be made:

1. Data available to date reflect the fact that such non-nucleoside mutations as L100I, K103N, V106A, Y181C, and Y181I are seemingly not impacted in regard to likelihood of transmission in primary cohort investigational studies performed to date (de Mendoza et al., 2004; Turner et al., 2004). This fact might compromise the potential utility of TMC-120, UC-781 and other tight-binding NNRTIs to be employed as microbicidal agents, since the male transmitter of HIV might conceivably harbor an NNRTI-resistant virus. (It should be noted that neither TMC-120 nor UC-781 are being developed for oral treatment, but both agents have been licensed for microbicide use). Although this point is undoubtedly more valid in wealthy as opposed to developing country situations at this time, work in this field must anticipate the more widespread use of antivirals in the context of the WHO 3×5 initiative.
2. Relatively little is known about whether novel tight-binding NNRTIs might be compromised as microbicides to the same extent as viruses that contain mutational profiles associated with the current therapeutic use of NNRTIs in wealthy countries. Accordingly, such agents as TMC-120 and UC-781 might not necessarily be impacted to full extent, even if one or several mutations associated with resistance to currently approved NNRTIs are present in the transmitting viral population. More information on this topic is required.
3. It is possible that certain NNRTI mutations may act differentially in viruses of different subtypes. As an example, research by our group showed that a V106M mutation was more likely to develop in viruses of subtype C origin as opposed to a V106A substitution which occurs more commonly in subtype B viruses. These differences are attributable to differences in codon usage at amino acid position 106 in RT among viruses of subtype B versus subtype C (Brenner et al., 2003). In all likelihood, further differences will emerge in regard to different subtypes and their resistance to select drugs. It is possible that we may only learn much more about this topic following the widespread introduction of antivirals into developing country settings.

Studies on cohorts of recent and primary HIV infection are key to facilitate an analysis of "transmission fitness" of viruses containing mutations associated with HIV drug resistance. This should be distinguished from the more commonly referred to characteristic of "replication capacity" (RC), which refers to a situation whereby mutations associated with drug resistance may impact on the ability of HIV to replicate. Preliminary findings have shown that the M184V mutation in HIV reverse transcriptase (RT) may impact significantly on the ability of HIV to be transmitted, although the precise reasons for this deficit are unclear (Petrella and Wainberg, 2002).

3. Differential transmission of drug-resistant viruses

The concept of "transmission fitness" will likely prove useful from the public health perspective since it could provide an estimation of how efficiently different HIV types and strains can establish infection in new hosts. Since it is impossible to simulate all factors influencing HIV transmission in a laboratory setting judicious observation of this phenomenon as it occurs in real life will be required. Transmission of HIV doubtless results from a complex interplay of factors (magnitude and frequency of exposure, mechanisms of exposure, patterns of sexual practices, use of ARVs, inherent capabilities of the transmitted virus, etc.) that ultimately lead to entry of infectious virions into a susceptible host cell and the incorporation of viral DNA into host cell DNA. In order to estimate "transmission fitness" of viruses circulating in a given population appropriate recording of these multiple important factors in HIV transmission will be necessary.

Studies should assess the potential for differential transmission of HIV-1 containing select mutations associated with resistance against various compounds. Results to date indicate a less efficient transmission of viruses containing the M184V mutation in contrast to those associated with resistance to NNRTIs and thymidine analogs (de Mendoza et al., 2004; Turner et al., 2004). In addition, viruses containing mutations associated with protease inhibitors may also be less prevalent in the context of primary HIV infection than of wild-type, although not to the extent of compromise observed with the M184V substitution (Turner et al., 2004). It is now necessary to extend these studies by relying on data from multiple cohorts through published results and websites. Establishment of new primary HIV infection cohorts, e.g. in Botswana, will also derive relevant information pertaining not only to likelihood of transmission of viruses associated with drug resistance in a context of viruses of subtypes other than B. The predominant subtype in Botswana is subtype C. As stated above, the case with which selection of the K65R mutation, conferring resistance to tenofovir (TDF), can be accomplished in tissue culture establishes concern for the use of tenofovir (TDF) in such settings as Botswana in both pre-exposure prophylaxis (PREP) and microbicide use.

An important topic as well is that of transmission of resistant forms of HIV from females to males. At present, this subject is relevant in the case of many women who have received NNRTIs for prevention of MTCT. An additional danger is that a woman who is already HIV seropositive might be exposed to drug in the form of a microbicide. Obviously, the resistant virus might not then be impeded by the microbicide, even though it might be argued that an effective microbicide might reduce female to male transmission of wild-type HIV even more effectively than male to female transmission. Some assessment of this issue might be possible by asking about the transmission of resistance-related mutations in acute HIV infection to women as opposed to men in the context of primary HIV infection cohorts.

The fact that single-dose nevirapine is associated with the development of drug resistance in the context of prevention of mother-to-child transmission of HIV is of obvious concern. It is well known from clinical experience that the only way of preventing HIV drug resistance is to use appropriate combinations of drugs rather than any single compound as monotherapy. In all likelihood, combinations of drugs may also be required to prevent HIV drug resistance in the context of microbicides. Are multiple drug-resistance-associated mutations associated with diminished transmission efficacy? As stated, the M184V mutation is impacted in this regard (Turner et al., 2004). Multiple data sets should be evaluated on transmission of viruses containing drug-resistance (DR) mutations from multiple centers in order to obtain greater statistical significance and to generalize conclusions. RT mutations that are known to impact on viral RC, i.e. M184V, K65R, L74V, will also be those that are less likely to be transmitted relative to mutations that confer resistance to NNRTIs and the thymidine-associated mutations (TAMs) that are associated with resistance to zidovudine (ZDV) and stavudine (d4T). Interestingly, the current widespread use of tenofovir (TDF) in western countries may lead to accelerated occurrence of TAMs more than K65R as mutations associated with resistance. Patients newly identified as HIV+ have been mostly diagnosed by PCR prior to seroconversion. This means that the overgrowth of an initially unfit viral population by more replication competent viral revertants is unlikely to have occurred in such settings.

4. Are there differences among drug-resistant viral subtypes in regard to HIV-1 transmission?

When compared with subtype B, subtype C has lower replicative fitness in peripheral blood mononuclear cells (PBMC) but a similar one in skin-derived Langerhans cells (Ball et al., 2003). Although these observations suggest equivalent transmission efficiency of these two subtypes, subtype C HIV-1 has become predominant in Asia and Africa despite the presence of concurrent (Essex, 1999) or even preexisting subtypes (Kuiken et al., 2002). The apparent advantage for heterosexual transmission of subtype C thus remains unexplained. Prevalent host factors that promote inflammation, cause epithelial trauma and transitions in the cervico-vaginal microenvironment may contribute to this advantage (Myer et al., 2004, 2005; Rottingen et al., 2001). The fact is, however, that both wild type and drug-resistant HIV-1 are transmitted and microbicides will have to be efficient as well as harmless within this milieu.

Most work to date on transmission of drug-resistant HIV-1 have dealt with viruses of subgroup B origin, since the latter are those most predominant in western countries in which access to ARVs has been broadly available. Yet, atleast several important

differences among mutations that confer resistance to different drugs have been reported in regard to viral subtypes. Some of the time, these differences are attributable to variations in codon usage and redundancy of the genetic code. Examples are:

1. The preferential occurrence of the L90M mutation as a cornerstone of the development of resistance to the protease inhibitor (PI) nelfinavir in subtype C as opposed to subtype B viruses, in which a D30N substitution is commonly observed.
2. The occurrence of a V106M substitution in viruses of subtype C as a key determinant of resistance to NNRTIs, whereas this mutation is only rarely seen in viruses of subtype B.

Studies should now ask whether the K65R mutation, associated with resistance to TDF, may be more common in viruses of subtype C than subtype B. This topic is important for the following reasons:

a. K65R is relatively rare in comparison with TAMs as a determinant of resistance to TDF in heavily treated populations.
b. K65R is seen relatively infrequently in patients treated with first-line TDF containing regimens. This is true even in cases of the failure of such regimens (e.g. TDF, 3TC, EFV) to achieve durable suppression of viral load.
c. TDF is now the most commonly prescribed NRTI/NtRTI in first-line regimens in western countries and is now being studied in pre-exposure prophylaxis protocols (PREP) to prevent the horizontal transmission of HIV.

Resistance against TDF should be selected in further comparative tissue culture studies that employ wild-type clinical isolates and clonal derivatives of subtype B versus subtype C viruses, and the mechanisms responsible for more rapid selection of K65R, is subtype C discerned. Previous studies did identify K65R as a signature mutation for TDF in subtype B viruses, although this substitution was observed in only 4 of 12 independent selections in cell culture (Wainberg et al., 1999). The latter findings are consistent with the clinical observation that K65R is seen relatively rarely in patients with subtype B viruses who fail TDF-based therapies. Unfortunately, TDF has not been available as a therapeutic option in countries in which subtype C is predominant. Hence, data on the propensity of TDF to select for K65R in the clinic in such settings are not available.

5. Clustering of HIV infection

Research by a number of groups, has shown that a high proportion of new cases of HIV infection occur in clusters. Recently, Wawer et al. showed in the Rekai region of Uganda that 43% of new HIV infections were apparently caused by people who were themselves only recently infected by the virus (Martinson et al., 2004). A different group recently showed that such clusters may represent 50% of new infections in the Quebec primary infection cohort (Brenner et al., 2005). These studies involve the complete sequencing of the RT and PR genes and the demonstration of extensive sequence homology among viruses obtained from recently infected individuals and/or seroconverters. These data have important public health implications as they suggest that high proportions of recent HIV infections are being transmitted by relatively small numbers of individuals.

In this context, the possibility that viral drug resistance mutations might impact on transmission of HIV-1 acquires special significance. It is important to ascertain whether the diminished "transmission fitness" associated with the M184V mutation, cited above, is relevant in the case of patients who acquired drug resistance (DR) mutations while on therapy versus recently infected individuals with high viral loads who may be hyper-transmitters of HIV, as suggested by the cluster analyses performed by Wawer and co-workers (Brenner et al., 2005; Wawer et al., 2005).

Accordingly, transmission data in regard to DR mutations should be reanalyzed based on what is known about the time since infection and viral loads among potential transmitters. New data may show that mutations such as M184V, K65R, and L74V are not more likely to impact transmission from individuals who are themselves undergoing primary HIV infection (and who have never been treated with ARVs) than from individuals who acquired such mutations as a consequence of treatment failure in association with an accumulation of relevant DR mutations.

In the context of drug resistance, this discussion is also pertinent to the development of compounds that interfere with the CCR5 co-receptor. At least three such compounds have now entered phase 3 clinical trials (Schering, Pfizer, GSK), although work on one of these compounds (GSK) was stopped because of problems of hepatotoxicity. Conceivably, such entry inhibitors may work best against HIV transmitted by individuals, who were themselves infected as a result of sexual contact with recently infected untreated individuals as opposed to individuals with more advanced disease. The reason is that the former may be expected to transmit a higher proportion of viruses that employ the CCR5 co-receptor as opposed to CXCR4, which mostly predominates in later stage disease. Although biological filters, e.g. dendritic cells, clearly exist that result in the vast majority of new infections being of CCR5 as opposed to CXCR4 preference, new data may show that individuals who are infected with some CXCR4-using variants are more prone to develop resistance to CCR5 inhibitors, as a result of alternative co-receptor usage, than patients who harbour more homogenous populations of CCR5-using viruses at baseline. This subject is beyond the context of the current review, but may have greater relevance in years to come, particularly if co-receptor antagonists are someday developed as antiviral microbicides (Kuhmann and Moore, 2005; Moyle and Lalezari, 2005).

6. Can a NNRTI enter virions?

One important biological issue in regard to the potential use of NNRTIs as anti-HIV vaginal microbicides is whether such agents might inactivate the infectivity of virions or whether their activity might be restricted to HIV-infected cells. Both of these possibilities are relevant, in view of the fact that human ejaculate contains both cell-free viruses as well as infected cells, each of which is thought to play an important role in regard

to mediation of new infection (Ho et al., 1984; Stewart et al., 1985; Van Voorhis et al., 1991; Xu et al., 1997). The basis upon which NNRTIs achieve antiviral effect is through binding as non-competitive inhibitors to the active site of HIV RT. This effect can easily be achieved within cells, as NNRTIs have been shown to penetrate into HIV-infected cells where they can maintain a presence over atleast 24 h. In addition, both of the NNRTIs that are approved for clinical treatment, i.e. nevirapine and efavirenz, have been shown to maintain very long half lives in plasma, i.e. >36 h. The NNRTIs are also known to be able to directly inactivate purified recombinant RT molecules by directly acting on the active site of the enzyme. However, no group has convincingly demonstrated that these NNRTIs can penetrate inside of virions to destroy viral infectious capacity.

This subject should be addressed using radio-labelled preparations of the two tight-binding NNRTIs that have been licensed for microbicide development, i.e. UC-781 and TMC-120. The ability of radio-labelled NNRTIs to penetrate within virions and to inactivate HIV-1 infectivity can be easily addressed by exposure of radio-labelled NNRTIs, such as TMC-20 and UC-781.

Alternatively, purified HIV particles can be exposed to select NNRTIs, following which virus particles can be pelleted and resuspended in fresh solution in the absence of any NNRTI. Thus, non-tight-binding NNRTI should be washed away by the centrifugation step while tight-binding NNRTIs should remain attached to the virus particles which can then be assessed for infectivity on fresh cell populations.

Finally, studies should assess the ability of purified recombinant RT to both initiate reverse transcription reactions and to participate in strand transfer events in regard to synthesis of early stage products of reverse transcription, in the presence of these same NNRTIs.

7. Conclusions

Studies of the type described here will be essential to advance the rationale for the use of tight-binding NNRTIs, perhaps in co-administration with other ARVs, as an anti-HIV microbicide. This type of work might next lead to animal studies in which such agents as TMC-120 are studied for ability to protect macaques against infection by SHIVs (hybrid HIV/SIV viruses) in which the RT is derived from HIV. We hope as well that further studies with agents such as TDF will validate the use of NRTIs as microbicides. Ultimately, of course, clinical trials to test these concepts must take place.

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