Mini-review

Aspects of successful drug discovery and development

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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

Despite landmark achievements (e.g. >20 new anti-HIV drugs), a number of important therapeutic challenges remain. Although an expanding array of new drug discovery technologies has become available, drug research and development (R&D) productivity in general is still low. The establishment of close functional links between specialists active in early discovery, development and the clinic can thereby contribute to overall efficiency and higher success rates of new drug candidates. One of the more qualitative discovery challenges is to improve the predictability of early stage research models in term of in vivo drug efficacy. A cell-based model using viral replication in human T cells (MT-4) is used as an example from the HIV field to highlight the role of cell-based assays as tools for new target discovery, lead finding and optimization. The development of the next generation HIV non-nucleoside reverse transcriptase inhibitors (NNRTIs) TMC125 and TMC278 and the protease inhibitor (PI) TMC114 (Prezista™), further point to new fundamental strategies to combat and prevent antiviral drug resistance and to the importance of incorporating clinical and pharmaceutical aspects into lead finding and optimization, drug design and drug candidate selection. A more parallel-oriented drug discovery strategy is thus portrayed that harnesses some 'evolutionary' principles in combination with technologies that are currently rationalizing drug discovery.

Keywords: Chemotherapy; NNRTI; Protease inhibitor; HIV; Drug development

Contents

1. Introduction

In 1987, at the occasion of the International Symposium on Frontiers in Microbiology that was dedicated to Prof. P. De Somer who founded the Rega Institute (Belgium), the late Prof.

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D.T. Walker summarized the whole field of antiviral chemotherapy by listing eight licensed antivirals, six of which were nucleoside analogues targeted at herpes simplex virus (HSV) (Walker, 1987). As one of the reasons for this apparent lack of progress, Prof. Walker pointed to the until then held general belief that, because of the close and specific association of a virus with its host, it would be difficult for an antiviral agent to prevent viral replication in the host without harming it. Since those days various strategies in the design of antiviral drugs have been pursued with success and have led to more than 30 licensed antiviral drugs (for comprehensive reviews see De Clercq, 2002, 2004a, 2004b; Litller and Oberg, 2005).

However, the process of drug discovery and development is still a long, complex and multi-stage process where the odds of success, in retrospect, are low. For drugs in general, only 20% of drug discovery projects lead to a clinical candidate and only 10% of the compounds that enter clinical development achieve registration. The industry as a whole thus has been faced in the past with an overall average drug discovery and development cost of at least US$ 800 million (Overby, 2001).

Analysis of the reasons for this apparent low and even declining success rate reveals that projects mainly fail because drug candidates prove inactive in animal models or in patients, display unacceptable toxicity or cause undesirable side-effects upon in vivo administration. Many drug candidates with the right pharmacological properties do not make it because the pharmaceutical hurdles (e.g. large-scale chemical synthesis, pharmaceutical formulation, chemical stability, bioavailability, acceptable and safe metabolism, distribution, excretion, etc.) prove to be insurmountable. Projects can also be halted for quite different reasons such as poor patent protection, unexpected strong competition, lack of differentiation or innovation compared to other internal or external products in development, management decisions to focus on other priorities or (perceived) insufficient market potential. On the other hand, it is well known that health authorities, pressed by past drug-related accidents and the expectation of society for near perfect medicines, have significantly increased the requirements for drug approvals which in turn is leading to larger, more complex clinical trials and trials of longer duration. New monitoring and analysis technology thereby facilitates faster and more sensitive detection of side-effects that are considered to be unacceptable for a new drug that is developed for a particular disease. Another factor that contributes to the increasing difficulty in developing new drugs is that these are targeted at more complex disease processes and aim to cure, rather than to alleviate, disease symptoms. Finally, questions arise in the industry about the financial sustainability of this approach as drug manufacturers are faced with increasingly shorter market exclusivity through fierce competition and the rapid introduction of cheaper generics. The risks and costs are also perceived as an impediment in the pursuit of (antiviral) drugs for diseases with lower economic potential.

In the wake of all these facts there is an urgent need to dramatically improve the overall efficiency, quality and predictability of the drug discovery and development process. One of the questions for the drug hunters of tomorrow is whether the odds of success can be improved by simply quantitative measures such as synthesizing and testing more new compounds in an industrialized setting. Furthermore, with many new technologies each claiming their central role on the drug discovery stage, there is a need for drug discovery organizations to define the optimal niche of each in a streamlined and efficient process. Finally, it is important for basic scientists and technology developers to identify areas that need improvement and true innovation.

In this review, some aspects of the field of antiviral drug discovery and development will be highlighted that relate to the acceleration of the drug discovery and development process, to the improvement of the in vivo predictive value of in vitro discovery models and to the synergies that can be achieved by combining different drug discovery tools such as (ultra) high-throughput screening, target genomics, structural biology, modelling and bio-informatics.

2. Unifying drug discovery and development aspects

The drug discovery and development process is classically regarded as a mainly two-stage endeavour that is executed in quite different organizational structures and in different time frames. Selected drug candidates that emerge from discovery are investigated in a series of fairly well defined clinical development steps to establish whether or not they achieve, in the appropriate pharmaceutical form and dose, the desired therapeutic effects. Drug discovery itself is mostly portrayed as a linear, consecutive process that starts with target and lead discovery, followed by lead optimization and pre-clinical in vitro and in vivo studies that determine if such compounds fulfil a number of pre-set criteria for initiating clinical development.

Case histories of how antiviral concepts are reduced to practice do not reveal a single, one-way path for clinical success or the supremacy of one particular technology platform over the other. However, as compounds evolve into ligands, inhibitors and ultimately into drug candidates, they climb a steep ladder of complexity whereby the ultimate drug reflects a hard fought balance between physicochemical, pharmacological and pharmaceutical requirements (Table 1). Three environments can be identified that capture these differences in complexity, i.e. (i) the chemical space, (ii) the biological space and (iii) the medical space. Table 1 further summarizes a number of elements that characterize each space in terms of physical, technological and strategic assets. The new disciplines and growing number of technologies have led to drug discovery becoming more organized around technology platforms (e.g. genomics, high-throughput screening (HTS), structural biology, bio-informatics, etc.) rather than around particular disease areas. The danger is real that the spaces mentioned earlier, become more and more disconnected and organized as stand-alone entities where information and knowledge sharing is suboptimal. In order to increase the likelihood that drug candidates successfully complete drug development it therefore seems imperative that target discovery, lead selection and optimization, assay development, etc. reflect the clinical reality and pharmaceutical requirements as much as possible and incorporate this knowledge from the start and throughout the program. This should ensure that only those drug candidates are created and advanced that not only perform well at the

Table 1
Unifying view on drug research and development

	Physical assets	Technology assets	Strategic assets
Complexity of drug environment			
Medical space	Patients; patient-derived samples	Pharmacogenomics; computer assisted clinical trial design; biomarker technology; pharmacokinetic and pharmacodynamic modelling	Patient characterization (disease status, pharmacogomics); biomarkers to measure drug effect; clinical trial design; insight in pathogenesis
Biological space	ADME/TOX[a] assays; animal models; cell-based assays; target functional assays; target binding assays	Bio-informatics; high-throughput screening; cell biology; enzymology/protein technology; structural/molecular biology; (functional) genomics	Access to patient-derived samples; rapid data sharing/turnaround; reliable, quantitative, high-content data integrated in data warehouses; in vivo (disease/ADME/TOX) predictive value of in vitro assays
Chemical space	Target structure/model; ligands; compounds; chemical libraries	High-throughput synthesis; drug design/modelling; chemo-informatics; medicinal chemistry	Availability of natural ligands; chemical accessibility of lead; pharmacophore insights; high-quality structural models; experience-medicinal chemists

Critical elements in chemical, biological and medical space that, if used in a coordinated effort, can accelerate the discovery and development of drug candidates with higher degree of success rates.

[a] ADME: absorption, distribution, metabolism, and excretion; TOX: toxicity.

intended disease target level but that are also safe and endowed with acceptable drug-like properties. This can be facilitated by close functional relationships and interactions in and between chemical, biomedical and clinical investigator teams. Table 1 therefore depicts drug discovery and development as essentially a single universe' where the elements of the chemistry, biology and medical space work in concert and towards a common final goal of bringing a drug successfully to the patient. This 'organic' approach should therefore provide all actors with a common, 'drug'-oriented mind set that is beyond the pursuit of only short time, more easy to quantify, objectives falling within the realm of their own technical disciplines (e.g. delivery of drug targets, hits and lead compounds, drug candidates). It also provides a better framework for a learning organization that builds and grows on experience in a particular disease franchise. Finally, a number of antiviral case histories some of which are briefly recapitulated further below, illustrate aspects beyond the scope of this review but which relate to the importance of the 'human chemistry', the passion, commitment and perseverance that has characterized successful discovery teams (for some examples see De Clercq, 2005; De Clercq and Holy, 2005; De Corte, 2005).

3. Success and challenges in anti-HIV drug discovery

The successful development of about 20 new anti-HIV drugs during the last two decades is ample proof that antiviral discovery has truly come of age and that selective antiviral drugs can achieve important clinical benefits. The introduction of these anti-HIV drugs as part of a so-called highly active antiretroviral therapy (HAART) has led to a dramatic reduction of patient mortality and morbidity (Lee et al., 2001). The approved anti-HIV drugs inhibit either (i) the viral envelope gp41-mediated fusion of the viral and host cell membrane (1 drug, for review see Cooper and Lange, 2004), (ii) the HIV reverse transcriptase (RT)-catalyzed transcription of the viral RNA genome to a DNA copy (11 drugs, 2 mechanistic classes differing in binding

sites and mechanism of drug inhibition) or (iii) the HIV protease (PR) mediated cleavage of immature viral proteins into new enzymatic and structural HIV proteins (9 drugs). These well-characterized viral steps in the HIV replication cycle are thus extensively clinically validated since daily and continued administration of certain combinations of these agents (HAART) reduces the HIV load to levels below the limits of detection, prevents ongoing host cell destruction and even allows restoration of CD4 cell numbers and function.

Despite these landmark achievements, a number of important therapeutic challenges remain. Some of them are directly related to the nature of HIV pathogenesis and others are reflecting shortcomings of available therapies. HIV primarily infects CD4+ T lymphocytes whereby continuous viral replication occurs throughout the course of the HIV disease (Coffin, 1996). Most CD4 cells turn over rapidly, but some belong to latent pools with long half-lives (Ho et al., 1995). Because of the viral reservoir function of long-lived target cell populations, antiviral agents are required to be administered over longer periods of time. The retroviral integration of the HIV genome into the host genome and the large and rapid HIV turnover combined with an error-prone replication process (mainly due to the RT step), explains the 'quasi-species' nature of HIV whereby the individual viral strains compete among themselves for survival and propagation (Fisher et al., 1988; Domingo et al., 2001). This capacity makes HIV well equipped to evolve viral variants that display various degrees of resistance to drug inhibition. A number of elements drive and influence this process of drug resistance development such as: (i) the extend of the viral dynamics and the level of ongoing HIV replication, (ii) the viral fitness, (iii) the presence, type, combination and frequency of viral variants that harbour one or more mutations that render the drug target less susceptible to drug inhibition, (iv) the type and use of drugs (e.g. monotherapy, combination therapy) which exert a selection pressure and (v) the peak and through drug levels in the various tissues and compartments where the selection pressure occurs. The first-generation of HIV inhibitors were discovered and developed in

the late 1980s and early 1990s when the full extend of the viral dynamics and drug resistance aspects were poorly known or appreciated. As a result of the important establishment of the correlation between plasma viral load status and disease progression/outcome (Mellors et al., 1996, 1997), HIV viral load measurement became the primary biomarker for measuring drug efficacy in daily clinical management and during clinical trials with investigational agents.

As initial therapeutic regimens did not provide full viral suppression and imposed a low genetic barrier for drug resistance development, HIV variants emerged and spread with significant reduced susceptibilities to single drugs and even whole drug classes (Hertogs et al., 2000; Brown et al., 2003; Richman et al., 2004). Even in this period of HAART, a growing number of patients is cycling through the various remaining therapeutic options and are increasingly becoming dependent on the availability of newly developed anti-HIV agents. This creates a concern for the long-term as HIV-infected patients face many years, if not decades, of chronic antiviral drug treatment. Fortunately, industrial and academic teams have so far continued their search for new potent anti-HIV drugs which inhibit the currently existing drug-resistant viral strains and which provide improved therapeutic options in both drug-naïve and in treatment-experienced clinical settings. In the absence of fully 'resistance proof' anti-HIV drugs, the only practical solution for counteracting drug resistance has been the use of combinations of anti-HIV drugs selected on the basis of phenotypic and/or genotypic resistance testing of patient-derived samples. However, the combined use of many compounds has important limitations: (i) the patient is exposed to an ever increasing number of drugs thereby increasing the likelihood of metabolic interference, pharmacokinetic and pharmacodynamic interactions and more side-effects, (ii) inconvenience for the patient in terms of number and frequency of daily pill intake and (iii) premature exhaustion of particular drug classes that otherwise might serve as back-up in case of therapy failure. Furthermore, the number of the antiviral targets is ultimately likely to be limited. Therefore, it is even more important that antiviral agents are developed that, whether they target an existing or a new drug target, provide sustainable, maximal viral suppression in the blood and, more importantly, in the various tissues and compartments with active HIV replication (e.g. lymphatic system, brain). The new agents individually and combined, must also create a very high genetic barrier for new drug resistance development, i.e. they must be able to tolerate multiple mutations in the drug binding site so that large decreases in drug susceptibility can be avoided. Finally, the chronic nature of anti-HIV therapy requires long-term drug safety and tolerability.

4. Building models for hypothesis testing in the antiviral arena

The early concepts of selective antiviral chemotherapy were reduced to practice only when virus culture models became available so that viral replication mechanisms could be unravelled, viral enzymes identified and the search initiated for compounds that interfered with virus-specific events during the viral replication cycle. Ever since, antiviral target- and cell-based in vitro models have played a pivotal role in testing and validating the antiviral hypothesis that chemical compounds can be identified that: (i) selectively interfere with a critical viral function, (ii) inhibit viral replication at drug concentrations that are achievable and sustainable in the patient and (iii) stop disease progression without causing harm to the host.

In interpreting and utilizing the available models, one should keep in mind that the drug's biological and physiological context in the patient where the drug exerts its therapeutic effects, is vastly more complex, and possibly different, compared to surrogate in vitro models. For instance, cell lines grown under tissue culture conditions and cells in living tissue may have different gene expression patterns and (drug) metabolic activities. Biochemical assay conditions may not reflect those of the cellular environment of the native enzyme and X-ray crystallography provides structural data of a protein and drug binding sites based on measurement of a protein in a particular crystalline and energetically favourable form does not fully represents the native and more dynamic states.

The cell- and target-based assays are nevertheless often the only practical and available tools for drug discovery and can serve a number of purposes: (i) identification of new lead compounds in random or pre-selected chemical libraries, (ii) determination of antiviral selectivity and specificity of newly discovered active compounds, (iii) generation of quantitative affinity, inhibition and/or antiviral activity data during lead optimization and of high quality data sets for structure-activity and pharmacophore studies, (iv) validation of in silico predictions, (v) elucidation of mechanism of drug action, (vi) drug interaction studies, (vii) determination of in vivo target drug levels (e.g. measurements of cell permeability, metabolism, protein binding) and (viii) discovery of new antiviral targets or binding sites.

New insights into the mode of action of existing anti-HIV compounds and the discovery of new targets has opened the way for the design of many new cellular and biochemical models that can be used in the search for new HIV inhibitors (for a comprehensive review of HIV screening assays and methodologies see Westby et al., 2005). Furthermore, the drug discovery process itself has undergone some evolutionary changes in the past decades whereby the original chemistry/animal model/serendipity-based approach gradually took place for a more in vitro, molecular biology and mechanistic, targeted-based strategy. In more recent years, fuelled by the explosion in genomics, structural biology and computer technology, there is even a further shift towards the use of in silico, predictive methods (e.g. virtual screening, docking, etc.). Whereas it is clear that this more industrialized, multidisciplinary approach holds great promise to accelerate drug discovery, it also faces some challenges: (i) bridging the gap between in silico and in vitro discovery findings and clinically effective drugs, i.e. the challenge of in vivo predictability, (ii) massive data generation from which useful knowledge must be extracted and (iii) integration of the various platforms or spaces (see above) into a streamlined process.

5. Example of a cell-based approach in new target discovery, lead finding and drug optimization

The human T lymphoblastoid cell line MT-4 (Koyanagi et al., 1985) is one of the cell types that has been extensively used in anti-HIV drug discovery efforts by scientists at the Rega Institute For Medical Research and at Tibotec and Virco. It will be used here as an illustrative example of the evolving role of a cell-based methodology in different aspects of HIV drug discovery and development (Table 2).

The choice to use a cell-based, rather than a target-based, primary drug discovery system was taken in light of the following strategic considerations: (i) the most important being that measuring drug inhibition of HIV replication in CD4+ human T cells appeared to be the closest in vitro model for the hallmark clinical feature of HIV infection, i.e. the progressive depletion of CD4+ helper T cells, (ii) initially few anti-HIV targets were known, let alone properly characterized and functionally expressed, (iii) a cell-based, infectious assay with several full replication cycles allowed for the discovery of putative antiviral agents that effectively inhibited any of the known or unknown replication steps and provided an unbiased approach for the discovery of new drug targets and/or drug binding sites, (iv) it thus encompassed a certain level of multiplexing as various drug targets were evaluated in parallel, (v) the measurement of drug cytotoxicity in parallel uninfected cells, allowed to identify selective antiviral agents, (vi) many early anti-HIV programs were focussed on the synthesis and evaluation of $2',3'$-dideoxynucleoside analogues and nucleotide analogues which required several intracellular phosphorylation steps into the active $5'$-triphosphate species, and (vii) although it was realized that compounds could be missed with poor cell permeability, limited cell metabolic stability, low antiviral activity or high protein binding, such compounds would have been considered poor lead candidates anyway and lead optimization could only benefit from activity data that also incorporated these pharmaceutically relevant parameters. The MT-4 cell line itself was originally selected among a series of contemporary available CD4+ T cell lines to develop an anti-HIV screening assay for two pragmatic reasons: (i) highly susceptible to HIV-induced cytopathic effects which contributed to a good assay signal to noise ratio, assay robustness and reproducibility and (ii) good in vitro cell growth characteristics in a 96-well plate format which is more amenable to automation (Pauwels et al., 1987, 1988a). The assay was modified and optimized over time in terms of sensitivity, assay duration, viral end-point detection, reproducibility and screening capacity as well as regarding the types of viral challenge (Table 2). Many of the traditional challenges and limitations of cell-based assays, e.g. sterility requirement, longer assay duration, reduced testing capacity, lower reproducibility as a consequence of more variables, as well as the bio-safety considerations, could largely be overcome through the introduction of advanced automated and robotic systems within a dedicated bio-safety infrastructure.

In the course of two decades, this methodology led to the first description of a number of important new anti-HIV agents and/or classes (Table 2), including the acyclic nucleoside phosphonate (ANP) PMEA (adefovir) (Pauwels et al., 1988b) from which the close analogue PMPA (tenofovir, Viread®) is derived (Balzarini et al., 1993) and the first bicyclam JM2763 (De Clercq et al., 1992, 1994) an analogue of JM3100 (AMD3100) which was later shown to act as a CXCR4 antagonist (Schols et al., 1997). The discovery of the first NNRTI, TIBO (Pauwels et al., 1990) also revealed a new mechanism of RT inhibition (allosteric, non-competitive binding) that was later shown by crystallographic studies to involve an asymmetric, hydrophobic cavity, about 10 Å away from the catalytic site of the HIV-1 RT (Kohlstaedt et al., 1992; Das et al., 1996). More recently, a third class of potent and structurally new HIV RT inhibitors was discovered, called nucleotide-competing RT inhibitors (NcRTIs) which do not act via chain termination as do the N(t)RTIs, but through binding to the active site of HIV RT in competition with the next incoming nucleotide (Jochmans et al., 2005).

6. Combining cell-based platforms, patient-derived information and drug design in the search for next generation HIV reverse transcriptase and protease inhibitors

Whereas the original versions of the MT-4 assay included measuring drug susceptibility of different HIV-1, HIV-2 and SIV wild-type and engineered mutant strains, the development of a recombinant methodology for phenotypic drug resistance testing of patient-derived HIV strains (Hertogs et al., 1998) represented a major step towards linking laboratory observations to the clinical reality (Hertogs et al., 2000; Vingerhoets et al., 2003). The rationale for this development originated from the realization in the early 1990s, that target- and cell-based screening strategies as well as drug design efforts had been based on the underlying assumption that potent inhibition of a prototype (wild-type) HIV strain would translate into similar inhibition of clinical isolates in the patient. Phenotypic and genotypic analysis of viral isolates from patients who failed their initial therapies, data of in vitro drug resistance selection experiments, molecular modelling studies as well as the newly obtained insights into HIV dynamics, all revealed why that assumption was not completely valid: the antiviral agents inhibited only HIV strains and enzymes whose drug binding sites were similar, if not identical to the strains that were used to discover, design and optimize these drugs in the first place. Within the HIV quasi-species pre-existed or evolved (in case of incomplete viral suppression) new variants with altered drug binding sites that, in the presence of the selecting drug (combination) would rapidly become the dominant species. Because of cross-resistance among many of the available and experimental agents, there was wide scepticism at that time about the feasibility of finding next generation RT and PR inhibitors that, despite binding to the 'old' site, would fare any better in terms of coping with HIV's polygenetic nature. On the other hand, more detailed phenotypic and genotypic analysis of cross-resistance between hundreds of experimental RTIs and PIs as well as molecular modelling studies, clearly revealed small but quantitative differences in the levels of resistance and binding characteristics and suggested that they could be further modulated and optimized. Although the RT and PR genetic diversity pointed to a

Table 2
Role of MT-4[a] cell culture-based assay systems in various aspects of HIV drug discovery

Generation	Role in drug discovery and development	Assay end-point	Assay duration (days)	Virus inoculum	Assay plate format	Automation and testing capacity	References
First (1987)	Lead finding and optimization. For example first demonstration of anti-HIV activity of 2′,3′-dideoxynucleoside analogue d4T[b] (Stavudine) (Baba et al., 1987)	HIV-induced CPE[c] via trypan blue dye exclusion (cell membrane integrity)	5	Cell-culture adapted, wild-type HIV strain (HXB2)	96-well	Low, <200 assay points/day	Pauwels et al. (1987)
Second (1988)	Lead finding and optimization. Examples. First demonstration of anti-HIV activity of a: nucleotide RT inhibitor (NtRTI): PMEA, adefovir[d] (Pauwels et al., 1988b); *non-nucleoside RT inhibitor (NNRTI)*: TIBO[e] (Pauwels et al., 1990); *bicyclam* JM2763 (De Clercq et al., 1992, 1994): the lead for AMD3100 (CXCR4 antagonist) (Schols et al., 1997); *nucleotide-competing RT inhibitor (NcRTI)*, the first member of a new RT inhibitor class (Jochmans et al., 2005). Lead optimization and clinical drug candidate selection of: NNRTI DAPY[f] compounds TMC125 (Andries et al., 2004) (Fig. 1) and TMC278 (Janssen et al., 2005); protease inhibitor (PI) TMC114 (Surleraux et al., 2005a, De Meyer et al., 2005)	HIV-induced CPE via mitochondrial enzyme mediated tetrazolium-reduction (cell metabolism)	5	Cell culture adapted, wild-type HIV and site-directed mutant strains	96-well	Medium, 5000–50,000 assay points/day	Pauwels et al. (1988a) and Pauwels (1993a)
Third (1998)	Lead finding, lead optimization, phenotypic drug resistance profiling of clinical samples. Examples: phenotypic characterization of protease drug resistance in 6000 patient-derived HIV strains (Hertogs et al., 2000); activity of TMC125 against >1000 (Andries et al., 2004) and >5000 NNRTI-resistant HIV strains (Vingerhoets et al., 2003)	Green fluorescent protein (EGFP) expression under control of HIV-1 long terminal repeat (LTR) and activated by HIV transactivator protein Tat	3	Cell culture-adapted HIV wild-type, site-directed mutant strains; patient-derived (HXB2) recombinant isolates	384-well	High, >100,000 assay points/day	Hertogs et al. (1998)

[a] MT-4: HTLV-1 transformed, human T lymphoblastoid cell line (Koyanagi et al., 1985).
[b] D4T, 2′,3′-dideoxythymidinene (Stavudine).
[c] CPE: cytopathic effecs.
[d] PMEA: 9-(2-phosphonylmethoxyethyl)adenine.
[e] TIBO: tetrahydroimidazobenzodiazepinone.
[f] DAPY: diarylpyrimidine.

Genetic diversity and resistance mutations in HIV PR gene

(subset of several hundred HIV protease amino-acid sequences obtained from clinical samples)
(Legend: black colour: consensus wild type sequence, colours indicate amino-acids that differ from consensus sequence)
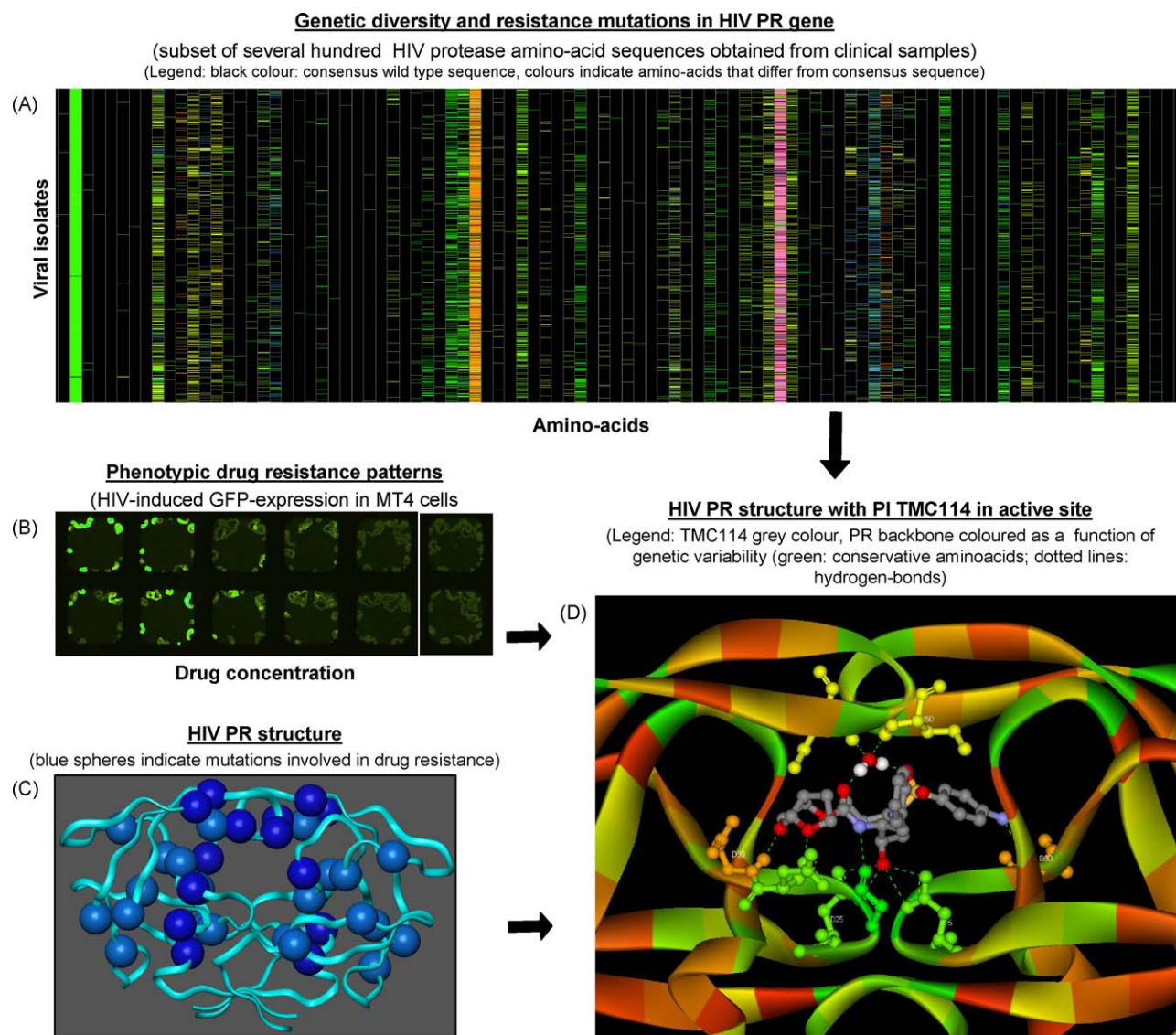

Fig. 1. Genotypic, phenotypic and structural information for the discovery and selection of new HIV inhibitors. (A) Genetic diversity and resistance mutations in HIV PR gene (subset of several hundred HIV protease amino-acid sequences obtained from clinical samples) (legend: black colour: consensus wild-type sequence, colours indicate amino-acids that differ from consensus sequence). (B) Phenotypic drug resistance patterns (HIV-induced GFP-expression in MT4 cells). (C) HIV PR structure (blue spheres indicate mutations involved in drug resistance). (D) HIV PR structure with PI TMC114 in active site (legend: TMC114 grey colour, PR backbone coloured as a function of genetic variability (green: conservative aminoacids; dotted lines: hydrogen-bonds)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

large functional plasticity of these enzymes, large-scale genotyping of clinical samples revealed that certain amino-acids were much more, if not absolutely, conserved and probably critical for maintaining enzyme function. These amino-acids as well as certain atoms of the protein backbone provided attractive theoretical molecular interaction targets for new, broad-spectrum, inhibitors. As a first step towards the discovery of such new generation drugs, a comprehensive RT/PR correlative phenotypic (Hertogs et al., 1998) and genotypic database had to be created as well as a physical sample repository. The resulting 'resistance map' helped to understand the complexity of this challenge whereas new strategic panels of mutant strains could be used in lead selection and optimization. This research would complement structure-based drug design efforts that were based

on wild-type and a limited number of mutant structures. The elements which all contribute to this research approach are illustrated in Fig. 1. An example of a next generation HIV inhibitor that has emerged from such 'evolutionary' process is the PI TMC114 (Prezista™, Darunavir) (Fig. 2). This compound was investigated during an optimization program based on a lead compound (TMC126, UIC-94003) that was structurally related to amprenavir and that contained a bis-tetrahydrofuranyl (bis-THF) moiety (Ghosh et al., 1994, 1998). TMC114 was selected for clinical development from a large series of bis-THF containing PIs based on its: (i) high antiviral potency and selectivity, (ii) its broad-spectrum activity against a large panel of multi-PI-resistant mutant strains, (iii) its higher genetic barrier for new drug resistance development and compared to existing and other
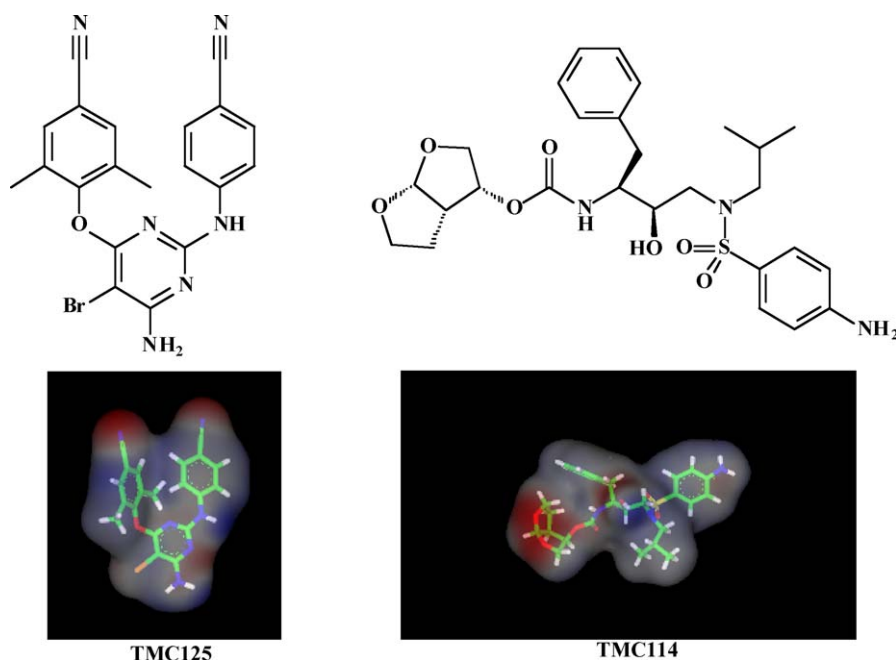
Fig. 2. Chemical structure of the NNRTI TMC125 and the PI TMC114.

bis-THF containing PIs, and (iv) its favourable drug-like properties (King et al., 2004, Surleraux et al., 2005a; De Meyer et al., 2005). TMC114 is currently in late stage phase III clinical development and has demonstrated potent and sustained suppression of HIV replication in treatment-experienced patients with extensive PI resistance (Aresteh et al., 2003; Katlama et al., 2005; Berger et al., 2005). Further modifications of the TMC114 template have yielded new analogues with potent activity against multi-PI-resistant HIV strains (Surleraux et al., 2005b).

7. Non-nucleoside RT inhibitor (NNRTI) research: perseverance and 'flexibility'

One HIV inhibitor series that was discovered in the MT-4 system by screening a library of pharmacologically inactive compounds followed by iterative lead optimization were the TIBO compounds (Pauwels et al., 1990). Their chemical structures were unrelated to that of the then prevailing and widely studied nucleoside analogues. The most active TIBO analogues had single digit nanomolar antiviral activities, high virus/host cell selectivity (more than four orders of magnitude), displayed a unique HIV-1 specificity and inhibited the RT enzyme in a non-competitive fashion (Pauwels et al., 1990). This discovery was soon followed by the description of numerous other, structurally diverse, compounds with a similar antiviral spectrum and mode of action (for review see Balzarini, 2004; De Clercq, 2004a, 2004b). This new class of RT inhibitors is commonly referred to as NNRTIs. Three NNRTIs, nevirapine (Viramune®), delavirdine (Rescriptor®) and efavirenz (Sustiva®, Stocrin®), were approved for the treatment of HIV infection in 1996, 1997 and 1998, respectively and have become one of the three cornerstones of current HAART regiments.

No new NNRTI has since reached the market and clearly illustrates the discovery and development challenges referred to earlier. Despite concerns of rapid drug resistance development, important cross-resistance among the first-generation of NNRTIs and the failure during development of several NNRTIs, some new generation NNRTIs are now advancing to the final stages of clinical development (Pauwels, 2004).

Two of these new anti-HIV drug candidates, TMC125 (etravirine) (Figs. 2 and 3) and TMC278 (rilpivirine) (Fig. 3) are diaminopyrimidine (DAPY) compounds that resulted from a >15 years collaborative research effort that embodied several of the critical success factors mentioned earlier and which was recently reviewed by De Corte (2005). In placebo-controlled clinical trials in drug-naïve and in treatment-experienced patients with NNRTI drug resistance, the treatment with the investigational drugs TM125 (phase III) or TMC278 (phase II) have led to significant viral load reductions, compared to control patients (Gruzdev et al., 2003; Sankatsing et al., 2003; Montaner et al., 2005; Nadler et al., 2005).

The evolution of these clinical candidates has involved the synthesis and evaluation of several thousand compounds in multiple families of NNRTIs, stemming from TIBO and α-APA derivatives (Pauwels et al., 1990, 1993b) which were further optimized to the iminothiourea (ITU) NNRTIs (Ludovici et al., 2001a), the diaryltriazine (DATA) (Ludovici et al., 2001b) NNRTIs and ultimately to the DAPY NNRTIs (Ludovici et al., 2001c; Andries et al., 2004; Janssen et al., 2005) (Fig. 3). Whereas numerous NNRTI families were discovered during this program, the selection of the initial path that led to the ITU/DATA and ultimately DAPY family was triggered by observations of the anti-HIV activity of certain NNRTIs against some mutant, NNRTI-resistant strains (Andries et al., 2004). The viral pharmacogenomic insights obtained from the extensive phenotypic
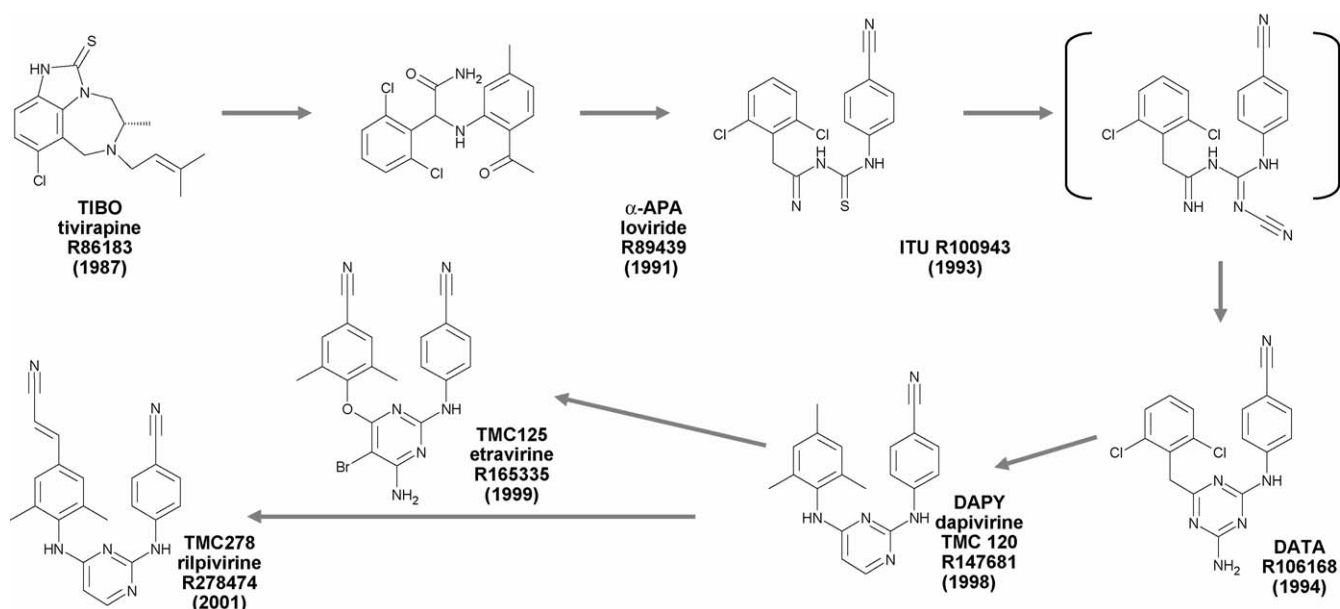
Fig. 3. Chemical evolution from TIBO (R86183) to the DAPY compounds etravirine (TMC125) and rilpivirine (TMC278) (adapted from Janssen et al., 2005).

and genotypic analysis described above, yielded pivotal selection tools to 'evolve' this series towards analogues with potent, broad-spectrum activity against many existing NNRTI-resistant HIV strains (Vingerhoets et al., 2003). Furthermore, systematic drug resistance selection experiments proved critical in selecting new DAPY analogues that provided higher genetic barriers for new resistance development compared to earlier generation NNRTIs or even close analogues (Vingerhoets et al., 2005). In parallel, the medicinal chemistry efforts were directed by new insights obtained from structural and modelling studies (Tantillo et al., 1994; Das et al., 1996, 2004). To increase the drug-like properties, the synthesis of new analogues was also guided in parallel by information of toxicity, metabolism and protein binding of new analogues (Andries et al., 2004). This combined discovery effort thus helped to improve the likelihood that drug candidates were selected with potent, selective and sustained in vivo activity against the HIV quasi-species and even NNRTI-resistant strains.

8. Next generation NNRTIs and PIs with improved drug resistance profiles: molecular determinants of drug inhibition in the context of a variable drug target

The DAPY compounds and TMC114-related PIs are pointing the way to a more fundamental approach to address target variability thereby challenging the long-standing view that antiviral drug resistance is sooner or later inevitable. Drug target variability is illustrated by the different forms of the NNRTI binding pocket depicted in Fig. 4A.

Within the DAPY series, drug potency against wild-type and mutant strains, increased as a result of optimized NNRTI pocket binding via hydrogen bonding to main-chain backbone amino-acids (e.g. Lys101) and interactions with conserved residues (e.g. Trp229) (Janssen et al., 2005). In contrast to first-generation NNRTIs, DAPY compounds can also bind in multiple modes

within the highly flexible pocket as a result of: (i) their access to different possible conformations without paying a significant energetic penalty (Fig. 4B) and (ii) their internal conformational flexibility ("wiggling") and plasticity of their interactions with amino-acids in the binding site ("jiggling") (Fig. 4C) (Das et al., 2004; Janssen et al., 2005; Lewi et al., 2003).

As for TMC114, thermodynamic studies revealed that it is an extreme tight binder of HIV wild-type PR ($K_d = 4.5$ pM) (King et al., 2004) which was approximately two orders of magnitude more than was observed for amprenavir, a first-generation PI and close analogue. Using surface plasmon resonance (SPR) technology with HIV-1 protease immobilized on a sensor chip, Dierynck et al. recently demonstrated that TMC114 shows a fast association but very slow dissociation from wild-type HIV protease compared to commercially available PIs which dissociated 1000-fold faster (Dierynck et al., 2005). These studies again confirmed the tight binding of TMC114 to HIV-PR with an affinity that is three to four orders of magnitude higher than the commercially available PIs (Dierynck et al., 2005). Comparative, structural analysis of these compounds also pointed to the flexibility of TMC114 binding and demonstrated that its increased affinity and potency, is largely due to additional and stronger interactions of the bis-THF moiety with main chain-atoms at the bottom of the active site (King et al., 2004). The multiple and shorter lengths of the H-bonds that characterizes TMC114 binding (Fig. 4D) also led to a mainly enthalpically driven binding in contrast to first-generation PIs where predominantly entropy changes contribute to the free energy of binding (King et al., 2004). Although PI resistance mutations do cause a drop in binding affinity, TMC114 still binds to the mutant enzymes with an affinity that is about 1.5 order of magnitude tighter than the first-generation PIs (King et al., 2004). Finally, Dr. Schiffer and co-workers have also pointed to the fact that TMC114 is fitting within the substrate envelope of HIV PR, in contrast with all first-generation PIs (King et al., 2004).
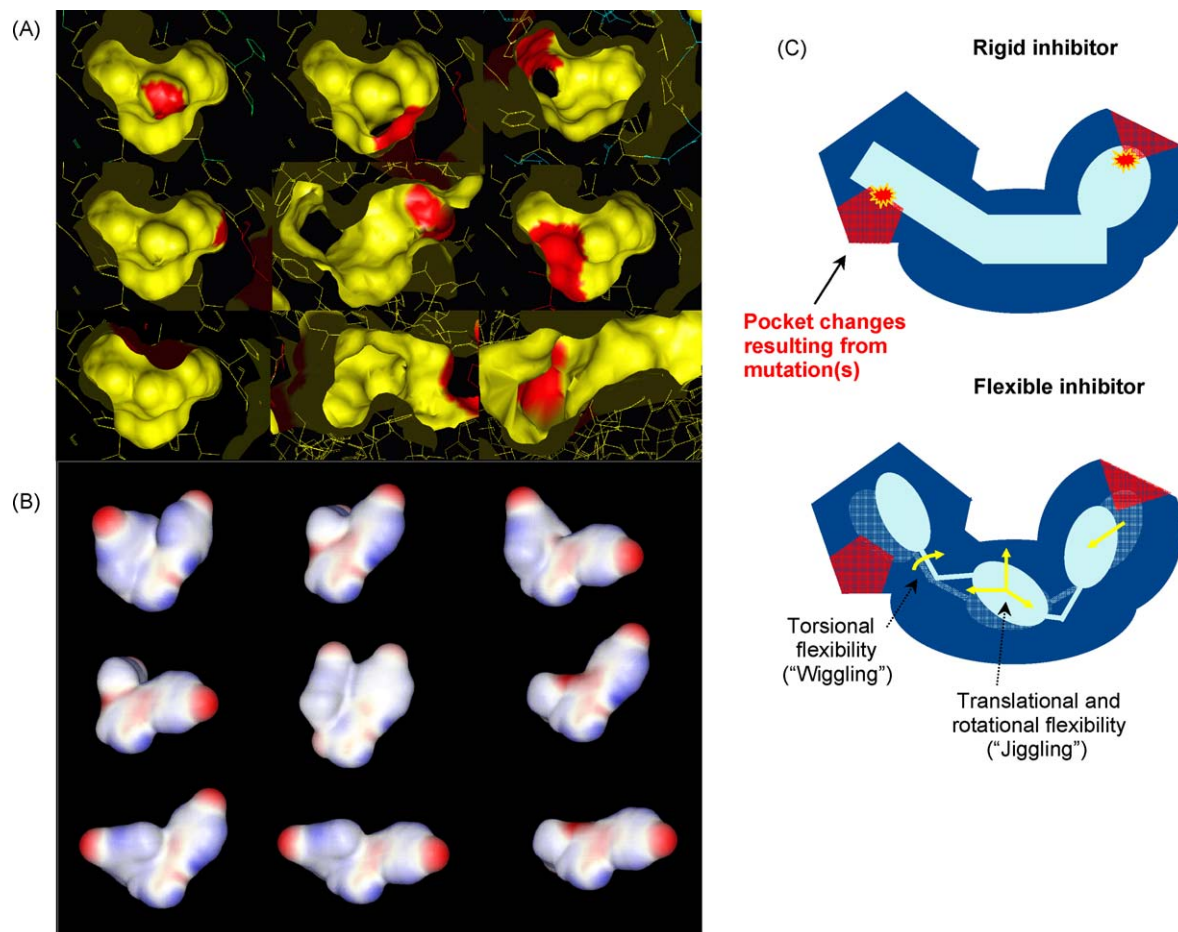
Fig. 4. (A) Different modeled NNRTI binding 'pockets' representing RT genetic diversity and variablity of the drug binding region. (B and C) Two possible molecular mechanisms to overcome the impact of mutations in the drug binding site: different possible TMC125 conformations, mediated by four torsion angles, that can be accessed without significant energetic penalty (from Pauwels, 2004) (B). (C) Schematic diagram of the response of a rigid vs. flexible inhibitor (light blue) in a wild-type (dark blue) or mutated (red) NNRTI binding pocket (adapted from Das et al., 2004). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

9. Conclusion

Drug discovery and development may be one of the few worlds where 'intelligent design' and 'evolutionary' principles, where 'rationality' and 'organized serendipity' can converge. This paradigm, co-inspired on some successful antiviral drug discovery case histories and past pitfalls, can help to bridge the gap between molecular concepts and finally approved antiviral drugs, between research laboratory and the clinic.

The new anti-HIV drug candidates TMC114, TMC125 and TM278 are illustrative of the importance of the in vivo relevance, the quality and the proper alignment and integration of the various discovery tools and platforms. The strategy to combat HIV drug resistance that is discussed in this review points to the importance of in vitro laboratory models that (better) reflect the viral pathogenesis and the complexity of the in vivo biological matrix where the disease processes actually take place. The role of a more parallel, rather than linear, drug discovery approach is emphasized where the drug's capacity for binding and inhibition of the disease-related target is optimized in parallel with those aspects that will impact the drug's ultimate in vivo efficacy, safety and other drug-like properties.

Finally, the DAPY NNRTIs TMC125 and TMC278 and the PI TMC114 exemplify the potential for optimized target binding and inhibition even if that target is polymorphic and prone to evolutionary change as a consequence of various selection pressures. The new molecular insights in the mode of action and the clinical efficacy of these antiviral compounds, even in patients harbouring drug-resistant HIV, argues against the contemporary dogma of 'exhausted' drug classes and provides new avenues of research when approaching new antiviral targets and anti-infective agents in general.

The lessons that have emanated from past antiviral programs and the proper implementation of some aspects that have been discussed in this review, can make future antiviral drug discovery more cost-effective, faster, better and less prone to late stage clinical failures.

breeding ground for many new antiviral scientists. Prof. E. De Clercq and his team at the Rega Institute for Medical Research in Leuven, Belgium, provided during the first decade of the author's career, the ideal environment for learning, experiencing and contributing to the fascinating world of antivirals.

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