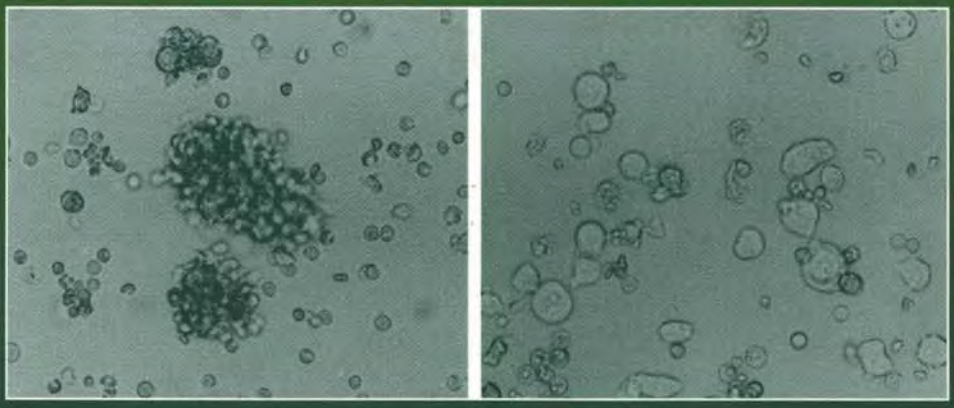
METHODS IN MOLECULAR MEDICINE™

Antiviral Methods and Protocols

Edited by

Derek Kinchington
Raymond F. Schinazi



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Changing Methods for Discovering Antiviral Drugs

Philip S. Jones

1. Introduction

Viral diseases were largely untreatable 40 yr ago. Now effective and safe therapies are available. This has led to significant improvements in the quality of life for large numbers of patients. New viral diseases are, however, continuing to emerge and established viruses have been shown to develop resistance to available therapies making this a fertile area for continued drug discovery. The processes used to discover drugs have also changed enormously over the past 40 yr. Nowhere have these changes been more apparent than in the field of antiviral therapy. Therefore, the development of antiviral drugs makes an excellent example for documenting the changes in approaches used to discover active agents. This brief chapter describes some of these changes—from the broad screening in animals and tissue culture first used to the mechanism–based approaches using computer assisted techniques and biostructural information.

2. Beginnings

The origins of antiviral therapies can be traced to the early 1950s, when sulfonamide antibiotics were tested for activity against poxviruses using mice infected with vaccinia ***(1)***. A decade of work at the Wellcome laboratories culminated in the development of methisazone, which was introduced in 1960 for the prophylaxis of smallpox (*see* **Scheme 1**). Notable success in the smallpox epidemic in Madras in 1963 demonstrated the value of this compound, but vaccines introduced soon after led to eradication of the disease and made the compound redundant. However, the principle that chemotherapy was effective for treating antiviral diseases had been demonstrated.

Influenza was another viral disease where chemotherapeutics were available in this early period. Once again this can be ascribed in part to appropriate

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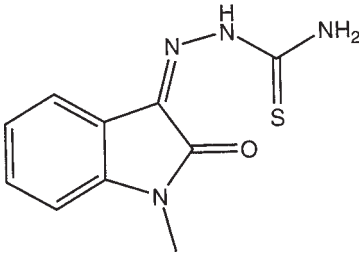
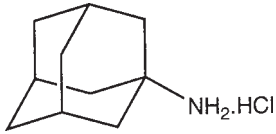


Scheme 1. Methisazone.

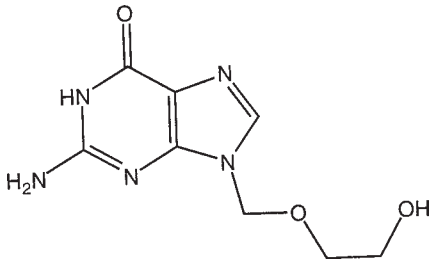


Scheme 2. Amantidine.

animal models being available for the testing of compounds. Antiviral activity against influenza A was observed for amantidine (licensed in 1966) and the related rimantidine (*see* **Scheme 2**). The mechanism of action of these compounds was elucidated much later. Analysis of nucleotide sequences of drug-resistant mutants revealed that the proton channel M2 protein had changed. Blockade of this channel leads to interference of virus uncoating ***(2)***.

3. Nucleoside Analogs

In the mid-1960s, screening of natural product nucleosides, isolated for anticancer programs, revealed activity vs some DNA viruses. However, the development of acyclovir for the treatment of herpes infections in the late 1970s marked the "coming of age" of antiviral therapy as it was the first example of a highly selective, efficacious antiviral drug. The discovery of acyclovir also preceded a detailed knowledge of its mechanism of action. Studies subsequently showed that the active entity is acyclovir triphosphate, which can be incorporated into a nascent DNA chain preventing further extension (*see* **Scheme 3**) ***(3)***. The triphosphate also inhibits the DNA polymerase directly. The administered drug is initially converted to its monophosphate by a viral enzyme and then to triphosphate by host kinases. The viral enzyme carries out the initial phosphorylation approx 200 times faster than host cell enzymes. This leads to higher concentrations of the active inhibitor in infected cells than in healthy ones and, therefore, to a high degree of selectivity ***(4)***. A second facet of selectivity is derived from the fact that the viral polymerase incorporates acyclovir triphosphate more readily than natural nucleoside phosphates.



Scheme 3. Acyclovir.

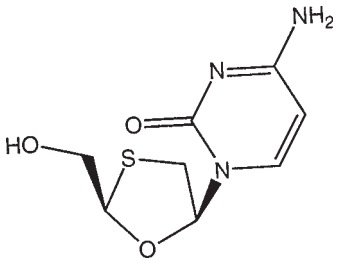
The success of acyclovir demonstrated that useful selectivity for viral enzymes over human host enzymes was a realizable target.

There are now many other DNA synthesis inhibitors in use, e.g., cidofir, idoxuridine, famciclovir, ganciclovir, and trifluorothymidine, and this class of compound makes up the majority of antiviral agents currently available or in clinical trials. However poor selectivity (and the associated toxicity) is still a key problem in this area owing to the similarities of viral and cellular metabolism ***(5)***.

By the end of the 1970s, large numbers of compounds had been tested against a variety of viruses, but many had failed to have the required selectivity profile. It became clear that a greater knowledge of virus life cycles would be required to enable the identification of critical functions unique to the virus. More accurate methods of assaying activity and of testing for subtle differences between host and viral processes were also needed. The timely coincidence of the development of molecular biological techniques (allowing a more detailed understanding of the life cycle of viruses and the preparation of useful quantities of viral proteins) and the appearance of a "high-profile" viral disease in AIDS provided the basis for one of the most intensive (and public) scientific endeavors of this century. A worldwide effort to understand the life cycle of HIV immediately followed the discovery of the virus. These studies suggested several processes in which intervention could be expected to lead to therapeutic benefit. Two will be focused on here: HIV reverse transcriptase (RT) and proteinase.

4. HIV RT Inhibitors

An unusual feature of the retrovirus family, of which HIV is a member, is the RT enzyme. This enzyme is essential for replication and has the capacity to generate DNA from RNA. The RNA-dependent DNA polymerase function of the RT provides at least one target for drug discovery. Nucleoside analogs once again proved to be effective inhibitors. Wellcome's experience in the nucleo-



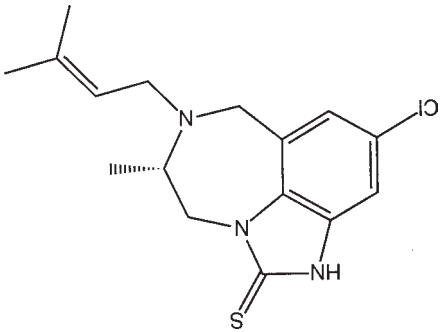
Scheme 4. 3TC.

side field enabled it to rapidly respond to the opportunity provided by HIV and led to the launch of AZT—the first licensed therapy for AIDS ***(6)***. However, as with many nucleoside analogs, toxicity was a problem. Several other related compounds followed (e.g., DDC and DDI) but their use was still restricted by dose limiting toxicity. This led to using the compounds in combination, which has proved effective in keeping the virus in check while providing an acceptable side effect profile. A novel approach to the selectivity issue was taken with the nucleoside inhibitor 3TC (*see* **Scheme 4**). In this case, the unnatural enantiomer of the sugar is a more potent inhibitor of RT and is less cytotoxic than the natural enantiomer. This property has been exploited with the development of this compound. The understanding of the mechanistic basis of the action of RT inhibitors and the recognition of a similar function in hepatitis B virus (HBV) (genetically distinct from HIV) has led to active compounds vs HIV RT being tested against HBV to good effect.

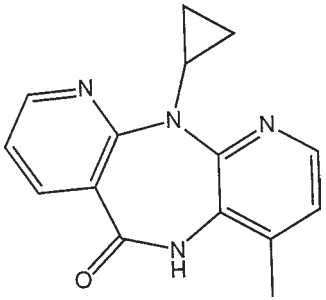
Advances in the screening of chemical libraries has led to the discovery of nonnucleoside inhibitors of RT (NNRTIs). Two approaches can be followed. When an appropriate antiviral assay is available compounds can be screened in whole cells. These assays are frequently labor intensive and therefore generally have a lower throughput than isolated enzyme assays. These assays have the advantage that chemical leads generated by this approach have already overcome the barriers of cell penetration and stability.

The discovery of the TIBO class of NNRTIs represents a fine example of this first approach. The problems of lower throughput were reduced by screening smaller focused libraries of compounds. The lead structure for the TIBO class of compound was identified and subsequently optimized (*see* **Scheme 5**) ***(7)***.

The discovery of another NNRTI, nevirapine, illustrates the second approach (*see* **Scheme 6**). Here it is no longer necessary to have access to an antiviral assay to discover active entities. Compounds are screened against an isolated enzyme. High throughput of compounds in the screen is a key advantage of this method enabling the testing of thousands of compounds; however, com-



Scheme 5. TIBO R82913.



Scheme 6. Nevirapine.

pounds active against the enzyme may lose all their activity in an infected cell or animal. A variety of reasons may be responsible for these failings, e.g., uptake, metabolism, or cellular penetration. In the case of nevirapine, a large library of thousands of compounds was screened against recombinant RT. Having identified an initial hit, medicinal chemists tackled the problem of optimizing the properties and demonstrated that high activity vs the isolated enzyme could lead to good antiviral activity. Screening of a series of related compounds also led to an excellent correlation of anti-HIV activity with inhibition of isolated RT, leading to the conclusion that these compounds were indeed exerting their antiviral effect through RT inhibition ***(8)***.

Further NNRTIs have now been discovered ***(9)***. None of these compounds require metabolic activation, but they are only active vs HIV-1 RT. Biochemical studies showed that these NNRTIs were noncompetitive inhibitors of RT. That is, in contrast to the nucleoside derivatives, which act as competitive substrates or inhibitors, these compounds do not bind at the active site, but exert their influence on the enzyme via an allosteric mechanism. The techniques of biostructure-based drug design enabled rationalization of much of the work on

RT inhibitors. RT is a heterodimeric enzyme made up of 51 and 61 kDa polypeptides. An X-ray crystallographic structure of the enzyme complexed with nevirapine confirmed the biochemical analyses by indicating that the binding site was close, but distinct from, the polymerase active site. However, conformational changes at the polymerase active site did occur on binding of these inhibitors ***(10)***.

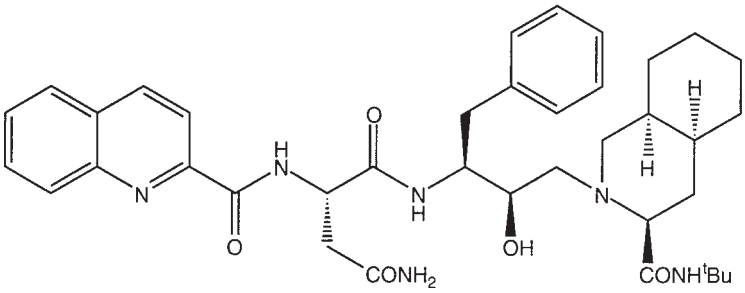
Clinical evaluation of nevirapine indicated that resistance emerges rapidly. The nature of the resistance was characterized and specific mutations in the RT were identified. It can be rationalized that as these compounds bind away from the active site, there is less mechanistic pressure for these residues to remain unmutated. Some amino acid residues in the vicinity of the binding site of the NNRTIs are conserved in other retrovirus RTs perhaps implying that they are required for functional protein. This has led to the strategy of using the X-ray structure of the enzyme and inhibitors to design compounds that interact with these residues and, hence, avoid resistance issues ***(11)***.

Other NNRTIs complexed with enzyme have now been crystallized and the binding modes compared. Although these molecules are structurally very different, they bind in a similar region to nevirapine ***(12)***.

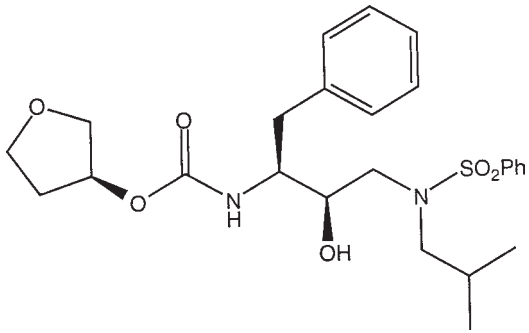
5. HIV Proteinase Inhibitors

The discovery in the mid-1980s that HIV encoded a unique aspartyl proteinase responsible for posttranslational cleavage of the viral polyprotein led to a number of drug research programs in search of inhibitors. These programs represent a paradigm for modern drug discovery—with substrate-based, biostructure-based, and high-throughput screening approaches all successfully employed. In the substrate based approach, knowledge of the cleavage sequence of the natural substrate and the mechanism of enzyme function suggests transition state-based compounds, which traditionally have proven effective for enzyme inhibition. The work in the HIV field has built on studies used to design inhibitors of renin, another aspartyl proteinase. The use of this approach rapidly led to efficacious drugs, e.g., saquinavir (*see* **Scheme 7**) ***(13)***. Several proteinase inhibitors designed using this approach have recently received regulatory approval.

While this approach rapidly led to a new treatment for HIV infection, the availability of an X-ray structure of the enzyme complexed to inhibitors allowed theories for improving interactions between inhibitor and enzyme to be developed and tested. Improved interactions allowed other moieties in the inhibitors to be modified, generating compounds with improved pharmacokinetic profiles. This work has culminated in second generation inhibitors exemplified by VX478, in which a structure-based approach using X-ray data has resulted in a compound that retains high affinity for the enzyme but with



Scheme 7. Saquinavir.

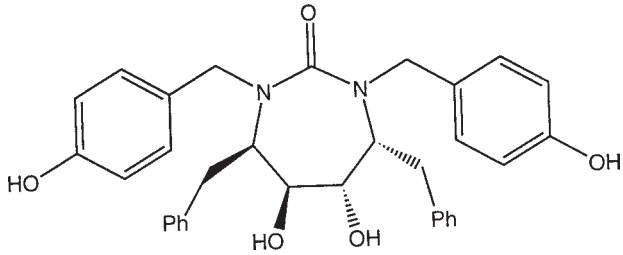


Scheme 8. VX478.

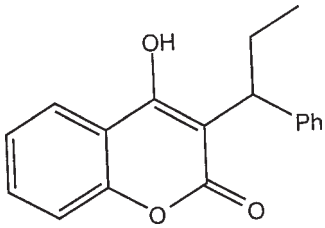
reduced molecular weight (an important feature in hepatic clearance) (*see* **Scheme 8**) ***(14)***. Some of the amide bonds, typically present in first-generation compounds and representing another pharmacokinetic liability, have also been removed.

As had been predicted prior to the determination of the X-ray structure, the proteinase assembles its catalytic machinery using a C2 symmetric homodimer (as opposed to a monomer in renin). The symmetrical nature of the enzyme suggested that C2 symmetric inhibitors might be effective. The X-ray structure also revealed the presence of an "ordered" water molecule bound to two of the carbonyl groups of the inhibitor. Displacement of such a water molecule with an inhibitor should, theoretically, lead to greatly enhanced activity. This theory was exploited with a class of cyclic inhibitors that mimicked the interactions of the water molecule with functionality within the inhibitor leading to highly potent and "compact inhibitors," e.g., XM-323 (*see* **Scheme 9**) ***(15)***.

Finally, the ability to screen large numbers of compounds has also been used in this area leading to the discovery of structurally novel leads. A notable example was the discovery of the pyran shown in **Scheme 10**. The X-ray crys-



Scheme 9. XM-323.



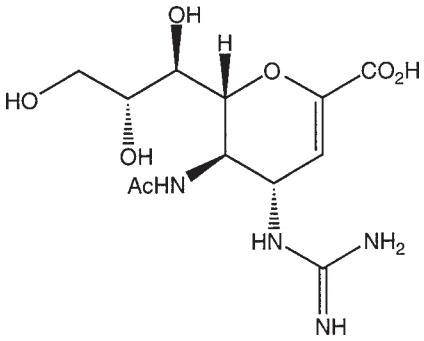
Scheme 10. Pyran.

tal structure of this compound complexed with the enzyme assisted further optimization ***(15)***.

Another intriguing application of structural information in the development of better therapies has been in the characterization of resistance in both the HIV proteinase and HIV RT areas. The generation of resistance in HIV is thought to be owing to the poor fidelity of the RTleading to point mutations. Research on the use of different HIV proteinase inhibitors led to the identification of mutations in characteristic but differing positions ***(16)***. These mutant proteins have, in some cases, been crystallized, leading to a better understanding of resistance at the molecular level. Frequently the mutation results in loss or gain of only a single methylene in the side chain of one of the amino acids in the proteinase, yet this can have a significant effect on the affinity of the inhibitor for the modified enzyme. These studies have suggested possibilities for the design of subsequent generations of inhibitors that might circumvent the effects of mutation.

6. Influenza Neuraminidase Inhibitors

A fine example of structure-based drug design has been reported for the design of an influenza virus neuraminidase inhibitor (*see* **Scheme 11**). Neuraminidase is an enzyme expressed on the surface of influenza virions and it is thought to be important for the successful release of progeny. Inhibition of



Scheme 11. Neuraminidase inhibitor.

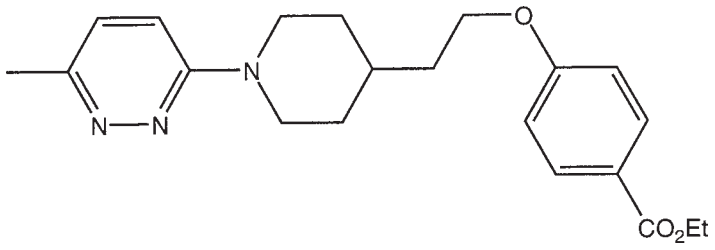
this enzyme should lead to effective therapy against both A and B strains, but selectivity for the viral neuraminidase over human enzymes was an issue. High-resolution X-ray data on the enzyme bound to a weak inhibitor were used to predict areas where extra binding interactions could be added. Synthesis of inhibitors based on this analysis revealed compounds with activity improved by four orders of magnitude and also with very high selectivity ***(17)***. One compound, GG167, is now used in clinical trials.

7. Rhinovirus Canyon Blockers

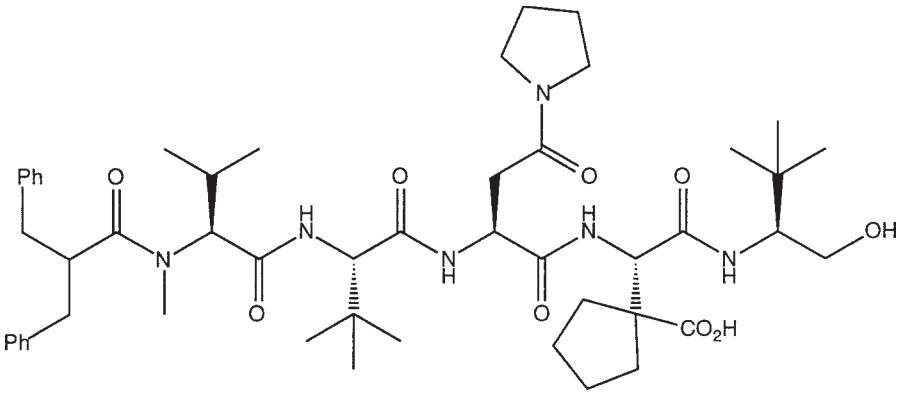
An early step in any viral life cycle is the attachment of the virus particle to the target cell. Inhibition of this process potentially offers an attractive target for therapeutic intervention. To date, despite several programs based on this approach, none has been successful in producing effective therapies. Attempts at intervening in the rhinovirus life cycle did, however, lead to promising early results ***(18)***. Here, screening provided a lead compound with activity in a virus infectivity assay. Assay data indicated that the likely target was the capsid. Published results using X-ray crystallographic data of whole virions indicated that a second class of compound bound to a "canyon" beneath the surface of the capsid. Combining features from both molecules led to pirodavir—an antiviral with activity against many rhinovirus strains that is also believed to bind in this "canyon" (*see* **Scheme 12**).

8. HSV Ribonucleotide Reductase Inhibitors

As described above, acyclovir is an effective therapy for the treatment of herpes infections. Working on the principle that emerging resistance may be countered by combination therapy, targeting a second enzyme may well provide a useful additional treatment. HSV encodes its own ribonucleotide reductase. A novel approach might be to inhibit this enzyme, which is responsible



Scheme 12. Pirodavir.



Scheme 13. HSV RR inhibitor.

for the conversion of ribonucleotides to deoxyribonucleotides, the essential building blocks of DNA (*see* **Scheme 13**). HSV ribonucleotide reductase is a tetramer made up of two of each of two types of subunit. It was discovered that a nonapeptide inhibited the function of the enzyme in a reversible but noncompetitive manner, and it was postulated that this molecule competed with the small subunit for the binding site on the large subunit and hence prevented association of the functional complex. A nonapeptide would often be regarded as too large to represent a useful lead for the discovery of compounds active in vivo. However, exceptional increases of activity vs the enzyme have been observed by modification of the side chains of the amino acids in the lead compound demonstrating that large improvements can be attained even without exploiting the benefits of transition state analogs ***(19)***.

9. Antisense

A totally different approach to the design of antiviral therapeutics is the use of antisense oligonucleotides. Here, the mechanistic target for intervention is

the messenger RNA, rather than the protein itself. The rules for preparing highly specific agents are those described by Watson and Crick, i.e., the base pairing propensities of the nucleoside bases. In theory the design of highly specific agents relies only on knowing the target gene sequence. Chemical modifications to the sequences are required to resist the destructive actions of host nucleases. Delivery and cellular uptake of compounds, which are frequently highly charged, are also significant issues. Animal studies have, however, demonstrated clear effects and a range of clinical trials are ongoing, which, if successful, will demonstrate a new type of approach to antiviral therapy ***(20)***.

10. Conclusions

Antiviral therapy has made considerable advances over the past four decades. Programs targeting HSV and HIV, in particular, have made excellent contributions and have begun to meet the challenges posed by these viruses. The methods used to produce antiviral drugs have been innovative and varied. A knowledge of the substrate provided an entry for the early polymerase inhibitors. Substrate-based approaches also provided early success in the preparation of the first generation of HIV proteinase inhibitors, but biostructural information, as it became available, has played a key role in the discovery of later classes of compounds. It is interesting to note that for human cytomegalovirus proteinase the X-ray structure had been reported prior to the clinical development of any compound, and for the hepatitis C virus NS3 proteinase, the X-ray structure had been reported prior to the patenting of any inhibitors. Here, it is likely that this information will play a significant role in the first generation of inhibitors. High-throughput screening has been particularly successful for HIV RT inhibitors, generating a range of structurally diverse leads. The methods used to discover drugs, however, are continually evolving, and it will be interesting to see what role high-throughput chemistry using parallel synthesis and robots to make, as well as test, compounds will have.

Viruses have been shown to be particularly adept at developing resistance to drugs, and the effective management of viral diseases may well rely on combination therapy. This may take the form of either targeting a single virus function with multiple agents or using several agents to attack several targets in the life cycle. Many viral diseases still require new treatments. These facts ensure that there will be many new challenges for antiviral drug therapy in the future. The evidence from the past suggests that the challenges will be met.

Acknowledgments

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2

Laboratory Safety Considerations

Paul M. Feorino, John D. Williamson, and Raymond F. Schinazi

1. Introduction

In the laboratory it is important that potentially pathogenic agents be controlled to protect the laboratory worker from infection and the experiment from contamination. The operation of a safe laboratory depends on many factors: the training and judgement of laboratory personnel; the implementation of protocols, the selection and use of equipment and reagents; and the location and design of the laboratory. These should be integrated in order to provide maximum safety for the personnel without impeding operation of the laboratory.

2. Laboratory-Associated Virus Infections

A survey conducted 20 years ago and based on a total of 3921 cases showed 59% of laboratory-acquired infections had occurred in research laboratories *(1)*. The majority of infections were of laboratory personnel but, in some cases, staff working outside the laboratory were also affected. Other events have emphasized the need for effective biosafety measures. These include: two "escapes" of smallpox virus from laboratories that resulted in members of the public becoming fatally infected; the emergence of new viral diseases with high case-fatality rates; and the recognition that laboratory-based investigations would need to be made on viruses for which no prophylactic or therapeutic measures were available. **Table 1** lists some viruses that have been identified as causal agents of laboratory-acquired infections *(2–4)*.

3. Microbiological Risk Assessment

Factors that can influence the risk of laboratory-acquired infection include: the virus under investigation, laboratory practices being used and the host. The consequences of release of the virus into the environment must also be considered.

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Table 1
Laboratory-Acquired Viral Infections

Ebola virus
Hantaviruses
Hepatitis B virus
Hepatitis C virus
Human herpesviruses
Herpesvirus simiae (B virus)
Human immunodeficiency virus
Lassa virus
Lymphocytic choriomeningitis virus
Marburg virus
Parvovirus B19
Rabies virus
Rift Valley fever virus
Simian immunodeficiency virus
Venezuelan equine encephalitis virus
Vesicular stomatitis virus

Table 2
Infectious Doses for Some Virus Infections (25–50% of Human Volunteers)

Disease or agent	Inoculation route	Dose[a]
Measles	Intranasal spray	0.2
Rhinovirus	Nasal drops	1
Venezuelan equine encephalitis	Subcutaneous	1
West Nile fever	Intramuscular	1
Poliovirus 1	Ingestion	2
Rubella	Pharyngeal spray	10
Influenza A2	Nasopharyngeal	790

[a]Numbers of cell culture infectious doses or animal infectious units.

3.1. Virus

1. Virulence may be defined as the ability of a virus to invade host tissues and cause disease. **Table 2** shows the infectious doses of some viruses are very low (data taken from **ref. *3***).
2. Transmissibility: The risk of spread of infection will be determined by the probability of secondary and tertiary cases. This can be assessed by the case-morbidity or case-mortality rates found by epidemiological studies of naturally acquired infection.
3. Latency: Delayed onset of disease with few or no clinical signs during the latent phase may delay recognition that infection has already occurred.

Table 3
Routes of Infection Associated with Laboratory Activities

Route	Laboratory activity
Ingestion (through the mouth)	Mouth pipeting
	Contaminated articles or fingers placed in mouth
	Eating, drinking, or smoking
Inoculation (through the skin)	Needle-stick accidents or cuts from sharp objects, such as syringes and glass slides ("sharps accidents")
Contamination (of skin and mucous membranes)	Splashes into mouth, eyes, or nose
	Splashes on damaged skin
	Transfer from contaminated fingers to eyes or mouth
Inhalation (through the lungs)	Exposure to aerosols generated by various laboratory procedures

4. Persistence: Continued excretion after infection increases the risk of transmission and may even result in "carriers" of the infection.

3.2. Laboratory Practices

1. Experience of laboratory workers: Training in good microbiological practice is the foundation of safe laboratory procedures; its importance cannot be overemphasized.
2. Type of laboratory: Industrial and research laboratories are more likely to work with large quantities of concentrated viral preparations, which increases the risk of infection following a spillage. Exposure to high virus concentrations may also result in infection by other routes than the natural route of infection.
3. Procedures: The most commonly reported types of activities associated with laboratory-acquired infections are listed in **Table 3**.

3.3. Host

1. Exposure route: Outcome may be affected by route of infection; for example, blood-borne viruses do not readily cause infection by the respiratory route.
2. Individual host characteristics: Compromising factors that can influence the particular consequences of exposure to infection include immune status and pregnancy.
3. Presence in body fluids: An awareness should be maintained of both viral and other, nonviral pathogens that may be present in body fluids (blood, saliva, sputum, semen, and breast milk).
4. Broken biological barriers: Skin is a particularly good barrier against infection, but it is breached by cuts or abrasions.

5. Prophylaxis and chemotherapy: Vaccines and/or drugs, if available, should be used to protect against infection or for treatment if infection is suspected or known to have occurred.

3.4. Environment

1. Vectors: Insects or other animals may become infected following the release of viruses from the laboratory and spread the infection to other animals and/or humans.
2. Environmental factors: The survival of virus particles in a potentially pathogenic state is influenced by ambient temperature and humidity.
3. Population characteristics: Herd immunity resulting from vaccination programs or from infections endemic in the local community, together with other factors, such as social behavior, can affect the spread of infection in the community.

4. Hazard, Risk, and Containment

"Hazard" is the potential danger associated with a particular virus, and "risk" is the probability that the hazard will be expressed as an exposure with the possibility of infection. "Containment" refers to the control measures used to reduce the possibility of exposure. Some authorities categorize viruses according to hazard *(4,5)*, whereas others have drawn up a classification based on risk, which includes such factors as pathogenicity, transmissibility, prophylaxis, and therapeutic measures *(6)*.

4.1. Classification of Viruses by Hazard/Risk Group (see Table 4)

- Hazard/Risk Group 1: A virus that is unlikely to cause human disease and offers no or minimal hazard to laboratory workers.
- Hazard/Risk Group 2: A virus that can cause human disease but is unlikely to be a serious hazard to laboratory workers. Accidental laboratory infection may cause serious infection but effective treatment and preventive measures are available. The risk of spread of infection is limited.
- Hazard/Risk Group 3: A virus that may cause serious human disease and offers a special hazard to laboratory workers. It may present a risk of spread in the community but effective treatment and preventive measures are usually available.
- Hazard/Risk Group 4: A virus that usually causes serious human disease and is extremely hazardous to laboratory workers. It may be readily transmitted from one individual to another causing serious epidemic disease. Effective treatment and preventive measures are not usually available.

5. Biosafety/Laboratory Containment Level Criteria

The hazard of a virus and/or the risks associated with its use in particular laboratory procedures determine the appropriate level of containment. Most classifications are designated in ascending order and include four kinds of laboratories: US Department of Health and Human Services (USDHHS) and the

Table 4
Classification of Viruses on the Basis of Hazard or Risk as Adopted in the European Community (EC), the United Kingdom (UK) and the United States (US)

Virus	EC	UK	US
Dengue viruses types 1–4	3	3	2
Ebola virus	4	4	4
Hantaan (Korean hemorrhagic fever) virus	3	3	3
Hepatitis A virus	2	2	2
Hepatitis B virus	3	3	2
Hepatitis C virus	3	3	2
Hepatitis D virus	3	3	2
Hepatitis E virus	3	3	2
Herpesvirus simiae	3	3	3
Human herpes viruses 1–8	2	2	2
Human immunodeficiency viruses	3	3	2
Human papilloma viruses	2	2	2
Human T-cell lymphotropic viruses I and II	3	3	2
Influenza types A, B, and C	2	2	2
Japanese encephalitis virus	3	3	3
Lassa virus	4	4	4
Marburg virus	4	4	4
Measles virus	2	2	2
Mumps virus	2	2	2
Monkeypox virus	3	3	2
Rabies virus	3	3	2
Vaccinia virus	2	2	2
Variola (smallpox) virus	4	4	4
Venezuelan equine encephalitis virus	3	3	3

World Health Organization (WHO) identify Biosafety Levels 1, 2, 3, and 4 and in the United Kingdom the Advisory Committee on Dangerous Pathogens (ACDP) categorizes Laboratory Containment Levels 1, 2, 3, and 4 ***(4–6)***. All share the same objective: to identify biosafety or laboratory containment levels that minimize the risk to the laboratory worker, to the outside community, and to the environment. At Biosafety/Laboratory Containment Level 2, exposure risks to the laboratory worker arise mainly from contact through a contaminated work environment. As the risk of airborne infection increases, Biosafety/Laboratory Containment Level 3 provides facilities to prevent aerosol transmission. Additional safeguards to protect the outside community and the environment are found at Biosafety/Laboratory Containment Level 4, which is

designed to provide both a safe and a secure laboratory for work with the most dangerous human viruses.

Although the Biosafety/Laboratory Containment Level can be determined directly by the Hazard/Risk Group, a strict relationship may not always be required. Some discretion applies to assessment of the infection risks associated with airborne transmission of particular viruses. For example, the USDHHS recommends that a Biosafety Level 2 facility but with Biosafety Level 3 practices and equipment be used for activities with human retroviruses and hepatitis viruses if these viruses are produced in research quantities or are manipulated as concentrated preparations, or if procedures are used that generate droplets or aerosols. In the United Kingdom, the Control of Substances Hazardous to Health Regulations (COSHH) 1994 requires that the minimum Laboratory Containment Level match the Hazard Group. However, work with specified viruses in the ACDP Hazard Group 3 (human hepatitis viruses and human retroviruses), which does not involve their propagation or concentration, can be carried out at a reduced level of containment if the risk of airborne transmission is low. The most important safety precautions applicable to these blood-borne viruses are to minimize contamination of surfaces and avoid the use of instruments or equipment that may accidentally cause cuts, for example, syringes or glass slides. *See* **Table 5** for a summary of biosafety containment requirements.

6. Laboratory Biosafety

The principal element of containment is strict adherence to standard microbiological practices and procedures. They are fundamental to laboratory biosafety at all levels of containment and are designed primarily to protect the laboratory worker by avoiding any activities that are potential sources of infection (*see* **Table 3**). Attention must also be paid to the additional protection provided by safety equipment (primary barriers) and facility design (secondary barriers), particularly with regard to work at Biosafety/Laboratory Containment Levels 3 and 4.

6.1. Standard Microbiological Practices

The following summary is based on the USDHHS publication "Biosafety in microbiological and biomedical laboratories" ***(4)***, the UK guidelines "Categorization of biological agents according to hazard and categories of containment" prepared by the ACDP ***(5)*** and the WHO "Laboratory Biosafety Manual" ***(6)***.

1. A biosafety manual is prepared or adopted. Laboratory personnel receive training on potential hazards associated with the work involved, precautions to prevent exposures, and exposure evaluation procedures. Appropriate immunizations

Table 5
Biosafety/Laboratory Containment Level Facility Requirements

Facility requirement	Biosafety/laboratory containment level			
	1	2	3	4
Separated from other activities in the same building	No	No	Yes	Yes
Access restricted to authorized persons	No	Yes	Yes	Yes
Double door entry	No	No	Yes	Yes
Airlock and shower	No	No	No	Yes
Ventilation				
Inward air flow (negative pressure)	No	Yes[a]	Yes	Yes
Filtered air exhaust	No	No	Yes	Yes
Biological safety cabinets				
Classes I and II	No	Yes	Yes	Yes[a]
Class III	No	No	No	Yes
Contains its own equipment	No	No	Yes[a]	Yes
Specified disinfection procedures	Yes	Yes	Yes	Yes
Autoclave				
On site	Yes	Yes	Yes	Yes
In the laboratory	No	No	Yes	Yes
Double-ended	No	No	Yes[a]	Yes
Dunk tank	No	No	No	Yes
Sealable for decontamination	No	No	Yes[a]	Yes

[a]May be required/permitted by particular regulatory authorities

or tests are carried out for infectious agents handled or potentially present in the laboratory. Annual updates or additional training are provided as necessary.

2. Access to the laboratory is restricted to authorized persons. Hazard warning signs on access doors identify the names of personnel authorized to enter and the name(s) and telephone number(s) of person(s) responsible for the laboratory.
3. Laboratory coats or gowns and gloves are worn to prevent contamination or soiling of street clothing and hands. Protective clothing must be removed and left in the laboratory before leaving; if contaminated, it must be decontaminated and cleaned or, if necessary, destroyed.
4. Eating, drinking, smoking, handling contact lenses, and applying cosmetics are not permitted in the laboratory work area. Persons who wear contact lenses in the laboratory also wear goggles or a face shield. Food is stored outside the work area in cabinets or refrigerators designated and used for this purpose only.
5. Mouth pipeting is prohibited; mechanical pipeting devices are used.
6. All procedures are performed carefully to minimize the creation of splashes or aerosols. Biological (microbiological) safety cabinets are used for procedures that may generate aerosols.