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Review article

Virus drug-resistance: mechanisms and consequences

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antiviral agents; mechanisms of resistance; properties of drug-resistant HSV mutants

Introduction

The discovery and subsequent widespread use of effective antibiotics marked an enormous advance in the ability to treat bacterial infections. Although these drugs have little effect on the replication of viruses, it was anticipated that similarly useful antiviral agents would soon be found. Ideally, such compounds should exert maximum inhibitory action on viral replication with minimal disruptive effects on normal cellular processes. Indeed, over the years a large number of agents with antiviral activity have been described, but unfortunately many of these also showed considerable toxicity to the host cell. Thus, it has proved quite difficult to find inhibitors that act selectively on viral replication, largely because of the intimate relationship between viral and host cell synthetic activities. Significant advances, however, have been made recently in the development of highly selective and potent inhibitors of herpes simplex virus (HSV).

As a result, a number of compounds are licenced for clinical use and we are now entering a new era in the treatment of herpes virus infections, which will see for the first time widespread use of effective, relatively non-toxic antiviral drugs.

It has been demonstrated that resistant mutants can be isolated following exposure of sensitive virus strains to virtually any of the selective inhibitors of viral replication so far discovered. In fact, it has been proposed that development of drug-resistance is a good indication that a compound has specific antiviral activity [127]. In view of the rapidly increasing clinical use of antiherpes compounds it is obviously extremely important that the potential problems of drug-resistance are addressed. Study of resistant mutants in the laboratory has provided valuable information about the mechanisms of drug-resistance, and hopefully it will help to identify classes of mutants that are likely to cause clinical problems. Furthermore, the investigation of cross-re-

sistance of mutants to other inhibitors might establish patterns which would facilitate the choice of alternative chemotherapy, should this become necessary.

In this review after a brief consideration of the development of resistance to a number of inhibitors whose antiviral activity has been long established, we shall focus attention on the mode of action of inhibitors of HSV, discussing mechanisms of resistance and pathogenic properties of drug-resistant mutants. The in vivo effects of antiherpes drugs both in animals and man, and the important role of latency in the chemotherapy of herpesvirus infections have been recently reviewed elsewhere [74]. Finally, the likely consequences of extensive clinical use of the new inhibitors are discussed in the light of recent reports of the isolation of drug-resistant herpesviruses from humans undergoing drug therapy.

Early antiviral compounds

Early extensive efforts in the search for selective antiviral drugs resulted in the discovery of only a small number of compounds showing sufficient promise to warrant further development for treatment of virus infections. The most notable of these include; thiosemicarbazone derivatives (isatin-β-thiosemicarbazone (IBT) and *N*-methylisatin-β-thiosemicarbazone (methisazone) guanidine, 5-iodo-2′-deoxyuridine (IUdR), 5-trifluoromethyl-2′-deoxyuridine (trifluorothymidine, TFT), and adamantane derivatives (1-adamantanamide hydrochloride, amantadine) and α-methyl-1-adamantanemethylamine hydrochloride (rimantadine). Of these drugs, IUdR and TFT are used clinically to treat herpes simplex virus infections and therefore their modes of action and mechanisms by which the virus acquires resistance to them will be discussed later.

The thiosemicarbazones which are selective inhibitors of pox viruses [13,258] have been used clinically in the treatment of vaccinia virus infections [184] and in the prophylaxis of smallpox [14,225]. These agents have little effect upon early viral protein synthesis and DNA replication, but prevent the formation of mature, infectious virions by inhibiting late protein synthesis [15,95,214,280]. This might occur as a result of direct interaction between the drug and viral structural proteins [214]. Mutants resistant to these compounds have been readily isolated by successive passage of a sensitive virus strain in tissue culture in the presence of increasing concentrations of IBT, or by serial passage of virus intranasally in mice receiving IBT therapy [7]. The resistant strains were stable, as they retained the resistant phenotype after passage in the absence of IBT [7]. A thiosemicarbazone-dependent mutant also has been isolated following a single passage of parental virus in tissue culture, in the presence of IUdR plus a low concentration of IBT [141].

Guanidine is an inhibitor of human enteroviruses, and was extensively studied as a potential agent for the treatment of poliovirus infection [69,173]. This compound selectively inhibits the replication of poliovirus at concentrations below those which cause adverse effects to host cells [69,254]. Viral RNA replication is inhibited probably as a result of the interaction of guanidine with viral structural proteins [66,69,157,197]. The protective effect of guanidine treatment on experimentally infected monkeys is, however, minimal and is thought to be at least partly due to the

emergence of drug-resistant mutants in vivo [185]. Guanidine-resistant strains have been obtained following treatment of sensitive virus in tissueculture, and they have also been isolated from animals receiving guanidine therapy [174,185]. In addition, guanidine-dependent mutants have been isolated following prolonged passage of sensitive virus in the presence of the inhibitor [174,195]. It is interesting that highly guanidine-dependent mutants fail to cause paralysis in monkeys [175,176], probably through failure to replicate normally in vivo.

The adamantane derivatives, amantadine and rimantadine are active against certain strains of influenza virus, both in vitro and in vivo [80,236,263]. In general, influenza A strains tend to be sensitive to these inhibitors, whilst influenza B strains are relatively resistant [80,236,263]. However, it is apparent that considerable variation occurs in the degree of sensitivity of different influenza A strains [236], and some influenza B strains are inhibited in tissue culture by amantadine [244]. Both inhibitors are useful therapeutic agents when given during the early period of an influenza A infection [276]. Amantadine and rimantadine are thought to act in a similar way, although their mode of action is not clearly understood. Different effects on viral replication are seen depending on the dose of amantadine that is used [239]. Initial virus RNA transcription and protein synthesis are inhibited by high doses of amantadine and evidence suggests that it is virus uncoating which is disrupted at this early stage [31,140,156,244]. Influenza virus enters the cell by endocytosis, and low pH (around pH 5) is required for membrane fusion and release of viral ribonucleocapsid from vesicles [133,167,168]. A high concentration of amantadine raises the pH of the vesicles, thereby causing inhibition of membrane fusion and virus uncoating [168,187,200].

Genetic evidence from the use of recombinant virus strains has indicated that sensitivity to amantadine is influenced by RNA segment 7, the gene which codes for matrix protein [121,179]. It is not clear whether matrix protein is involved directly in mediating amantadine sensitivity either early in infection, or at a later stage such as virion maturation. In addition, it has been claimed that rimantadine may prevent the release of matrix protein from ribonucleoprotein complexes, such that they are unable to penetrate the nucleus [31]. Thus, it is probable that amantadine and rimantadine act at a number of stages in the replication of influenza virus, but the disruptive mechanisms await further clarification.

Amantadine- and rimantadine-resistant mutants have been isolated by passaging sensitive influenza A strains in tissue culture in the presence of the inhibitors [6,32,57,179,239]. Some work suggests that selection of such mutants is difficult [207], although in other studies, amantadine-resistant strains were isolated in tissue culture following only a single exposure to the compound [57, J. McCauley, pers. commun.]. Amantadine resistance has also been observed in vivo, as passage of an influenza A strain in mice undergoing amantadine therapy resulted in the isolation of resistant mutants [206]. The mechanism of resistance of these mutants is not clear and it is hoped that further study of such variants will help to elucidate the mode(s) of action of these inhibitors.

Recent antiviral compounds

Of the antiviral compounds described more recently, a number show great promise as potentially useful antiherpesvirus agents. These inhibitors are generally most active against HSV types 1 and 2, and against varicella-zoster virus (VZV), but usually show little activity against Epstein–Barr virus (EBV) or human cytomegalovirus (HCMV). The highly selective mode of action of these compounds results in considerable antiviral activity combined with significantly lower cytotoxicity than earlier antiherpes agents. However, mutant strains resistant to most of these compounds have been isolated following growth of sensitive virus in tissue culture in the presence of the drugs.

Earlier examples of selective inhibitors of herpesviruses are 9-β-D-arabinofuranosyladenine (Ara A) [234], phosphonoacetic acid (PAA) [204,241] and the thymidine analogues, 1-β-D-arabinofuranosylthymine (Ara T) [9,116] and 5-iodo-5′-amino-2′,5′-dideoxyuridine (IADDU) [45,51] whose modes of action will be described in the following section. At the forefront of the 'new generation' inhibitors of herpesviruses is the acyclic guanosine anlogue, acyclovir (9-(2-hydroxyethoxymethyl)guanine, ACV) [96,235]. This analogue is a potent inhibitor of HSV types 1 and 2 in tissue culture, HSV-2 strains generally being slightly less sensitive than HSV-1 strains [26,64,70,104,247]. VZV is less sensitive than HSV [19,70,235] and EBV and HCMV are both considerably less sensitive [70,77,264], although EBV DNA replication is inhibited by relatively high concentrations of ACV [61]. Murine cytomegalovirus strains are significantly more sensitive to ACV than HCMV strains [33,70,264] although the reason for this is as yet unclear. ACV has proved effective when used to treat HSV infections in animal models [103,147,208,209,235], and some evidence demonstrates that the establishment of latent infection can be prevented providing treatment is initiated soon after virus inoculation [103,152,208]. Furthermore, the bulk of experimental evidence suggests that ACV treatment has no effect upon latent infection once established [20,103,152,208]. Controlled clinical trials with ACV have proved it to be useful in the treatment of many herpes infections [11,26,30,67,150,190, 251,281,282], and formulations of the drug are now licenced for clinical use.

A number of highly active inhibitors of herpes viruses in addition to acyclovir have recently been described and include: (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdU) [84], 1-(2′-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) [178], 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU) [269] and 9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]guanine (alternatively abbreviated to: BIOLF-62, 2′-NDG, BW 759 or DHPG) [8,56,245,248]. The spectrum of activity of each of these nucleoside analogues differs slightly from that of ACV. The thymidine analogue, BVdU, is highly active against HSV-1 and VZV, but perhaps surprisingly is relatively ineffective against strains of HSV-2 [84–86]. FIAC and FMAU are also extremely effecitive against HSV-1 and HSV-2 [178,262,269], but in addition, FIAC is also active against VZV [178]. DHPG, which is structurally related to ACV is active against both HSV types 1 and 2 [8,56,245,248] and additionally has some activity against VZV and EBV [56,248]. It is claimed that this compound is a good inhibitor of HCMV [56] with ED_{50} values ranging from 1.0–4.8 μM, although one report does not show such high activity of DHPG towards this virus [248]. The mode of action of the

inhibitors discussed above and mechanisms by which resistance to them can develop will be described in the following sections.

Mode of action of herpes simplex virus inhibitors

The most successful inhibitors of herpes simplex virus replication so far discovered are nucleoside or pyrophosphate analogues. The nucleoside analogues require activation in virus-infected cells by phosphorylation to the triphosphate form, and the resulting analogue triphosphates may interrupt virus replication in one of two ways. Firstly, by direct inhibitory action on the virus-specified DNA polymerase, thus causing suppression of viral DNA synthesis. Secondly, by disrupting replicative events as a result of incorporation of analogue into newly synthesized virus DNA. By contrast, pyrophosphate analogues do not require prior activation but act directly and selectively as inhibitors of the virus-specified DNA polymerase.

A more detailed discussion of the interactions between these inhibitors and enzymes is presented below. Consideration of such interactions is pertinent to a discussion of mechanisms of drug-resistance. In fact, it has frequently been the case that the mode of action of an anti-herpes compound has been confirmed, or elucidated through study of mutant virus strains (see, e.g., [85,96,97,114,122,221]). It should be borne in mind that nucleoside analogues may affect enzyme functions other than those recognized as having major roles in their modes of action. A potential candidate is the ribonucleotide reductase activity induced during HSV-infection [60,134,159,217] which is probably virus-coded [94] and has been investigated as a possible target for a number of nucleoside analogue triphosphates [196].

Nucleoside analogues

Many nucleoside analogues have been described which exhibit activity against HSV, but these vary considerably in therapeutic index (see reviews [81,82,249]). All have fundamentally similar modes of action in that they require conversion to an active triphosphate form, and it is this which inhibits virus replication. The majority of these compounds are thymidine analogues and so initial phosphorylation is generally performed by a thymidine kinase (TK). The nucleoside analogue triphosphates are then utilized by DNA polymerase in place of natural nucleotides. The therapeutic index of each inhibitor is partly determined by the differential specificities of virus-specified and host enzymes.

It has been shown that the thymidine analogues IUdR and TFT which were recognized some time ago to have anti-HSV activity [143–146] can be phosphorylated equally well by both cellular and virus-specified TKs [24,25,114,149,220,274] and the monophosphates are phosphorylated by cellular enzymes to the triphosphate forms which are used either by cellular or virus-specified DNA polymerases in place of dTTP [10,108,123,202,259]. The analogues are incorporated into DNA [106,108,142,233] and this appears to be the major factor contributing to the disruption of virus and cellular replication. Reports suggest that incorporation of IUdR into DNA might destabilize it and result in faulty transcription [106,203], and incorporation of TFT probably results in a decreased rate of DNA synthesis [233]. In addition, a direct

relationship has been established between incorporation of IUdR into virus DNA and loss of infectivity of the virus [106]. However, there is evidence which shows that IUdR and its nucleotide derivatives also exert inhibitory effects on cellular enzymes (e.g. [219]). Similarly it has been well documented that the monophosphate derivative of TFT is a potent inhibitor of cellular thymidylate synthetase [224]. Thus, as there is little enzymatic selectivity in the action of these analogues, they are relatively toxic and cannot be given systematically [5,23]. A degree of antiviral selectivity does exist however, probably attributable to differences in the levels of TK in HSV-infected and normal cells. Virus-specified TK is synthesized in large amounts during HSV infection, whereas there are far lower concentrations of TK in normal or non-proliferating cells [21,119,148,154]. Therefore, differences between the amount of analogue phosphorylated in uninfected and virus-infected cells may confer some of the selectivity of action of IUdR and TFT which allows topical use of these drugs [139,271].

Some of the more recently described nucleoside analogues are extremely potent and selective inhibitors of HSV replication in tissue culture, animals and man [26,81,82,178,248]. The basis of their selectivity lies primarily in the preferential phosphorylation of the compounds by HSV-specified TK [49,50,144,148,154], an enzyme which appears to possess a broader spectrum of substrate-specificity than its cellular counterparts. The broad spectrum of substrate specificity of HSV TK is reflected in its potential to phosphorylate the natural substrates deoxycytidine and dTMP, in addition to thymidine, when tested by in vitro assay [44,47,135,136]. Enzyme kinetic studies have demonstrated that the majority of the 'new generation' nucleoside analogue inhibitors are extremely good substrates for the virus-induced TK but poor substrates for cellular kinases [9,45,49,50,53,54]. The apparent binding affinity of virus TK for the analogues and the phosphorylation rates are similar to those of the natural substrate, thymidine [49,50,53,54]. Some of these compounds, which are generally 5-substituted deoxyuridine derivatives, are even better substrates than thymidine. For example, HSV-1 TK has higher affinity for the analogue (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdU) than for thymidine [53,161]. In contrast, some analogues which are still selectively phosphorylated by HSV TK are markedly poorer substrates for the enzyme. One such compound is the guanosine analogue acyclovir as the virus-induced TK gives a km value of around 200 μM for ACV, 500- to 1000-fold higher than the thymidine km, suggesting significantly lower binding affinity [114,160]. However, this compound is an extremely poor substrate for cellular enzymes since it is not phosphorylated to significant levels in uninfected cells [96], thus accounting in part for its high selectivity.

Following initial phosphorylation by virus TK, the analogues are converted to the di- and triphosphate forms either by cellular kinases alone, or in combination with virus-specified enzyme activities. For example, it has been shown that ACV is converted from the mono- to the diphosphate forms exclusively by cellular GMP kinase [188], and to the triphosphate by a variety of cellular nucleoside diphosphate kinases [189], apparently with no involvement of virus-specified enzymes. However, there is some evidence that the dTMP kinase activity associated with HSV TK might be responsible for converting certain nucleoside analogue monophosphates to their respective diphosphates. This has been shown for the compound IADDU and is

probably also the case for BVdU with the type 1 TK [45,113]. Presumably, cellular nucleoside diphosphate kinases are responsible for the conversion of these nucleoside analogue diphosphates to the triphosphates. It should be noted that where tested, it has generally been found that the phosphorylated nucleoside analogues are poorer substrates for cellular kinases than the natural nucleotides (see, e.g. [16,188]).

Nucleoside analogues such as ACV are unlikely to be phosphorylated exclusively by HSV TK, as several lines of evidence suggest that a host cell enzymatic activity (or activities) might be responsible for phosphorylating very small amounts of this compound. Firstly, different tissue culture cell lines show considerable variation in sensitivity to inhibition by ACV [104], and particular virus strains also show variation in sensitivity to the inhibitor when tested in a variety of cell lines [81,104]. Secondly, murine cytomegalovirus strains do not induce a virus-specified TK during infection and so the particularly high sensitivity of these strains to ACV cannot be attributed to phosphorylation of the analogue by such an enzyme [33,192]. Furthermore, ACV is phosphorylated in EBV genome-activated cells, although it is unclear whether EBV encodes a TK capable of performing this function [48,62,77,201]. Thus, it is possible that phosphorylation of ACV in these cells is due to a host enzymatic activity which might be induced during virus infection [48,201]. It is clear that the low level of phosphorylated derivatives generated by cell enzymes is insufficient to inhibit the growth of all herpes viruses. This can be seen particularly with pseudorabies virus which, although it induces TK activity, is resistant to ACV. However, if the virus is treated with ACV in 'TK-transformed' cells which express HSV TK constitutively, it appears as sensitive to ACV as HSV-1 strains [76]. The interpretation of this result is that the level of ACV phosphorylation in 'TK-transformed' cells is considerably higher than in normal cells and so the system reveals viruses which are sensitive to phosphorylated derivatives of ACV.

It was stated earlier that the 'active' form of these inhibitors is thought to be the nucleoside analogue triphosphate which causes inhibition of virus multiplication by blocking the action of HSV-specified DNA polymerase and in some cases by causing termination of DNA chain elongation. Multiplication may also be inhibited as a result of incorporation of analogue into virus DNA. The precise mechanisms by which virus multiplication is inhibited following incorporation have not been fully elucidated. There is, however, better understanding of the action of inhibitors which cause direct inhibition of HSV DNA polymerase and chain termination. For example, it has been demonstrated that the triphosphate of ACV (Acyclo-GTP) is a competitive inhibitor of the incorporation of dGMP into DNA catalyzed either by HSV-specified or host cell DNA polymerases [89,109]. However, this inhibitor shows considerable selectivity, as the virus enzyme has a much greater affinity for Acyclo-GTP than have cellular polymerases [89] and studies using radio-labelled Acyclo-GTP have shown that it is incorporated into DNA in vitro at a greater rate by virus DNA polymerase than by host cell polymerase [89]. Treatment of HSV 'TK-transformed' cells with [^{14}C]ACV results in the accumulation of very short DNA fragments which cannot be 'chased' into higher molecular weight DNA [110]. This supports the hypothesis that incorporation into DNA of Acyclo-GMP residues lacking extendable 3'-hydroxyl moieties causes chain termination.

Kinetic studies have demonstrated that HSV DNA polymerase has extremely high affinity for Acyclo-GTP (Ki value $\simeq$ 0.003 μM), in fact considerably higher than for the natural substrate dGTP (Ki = 0.2 μM) [89]. However, Acyclo-GTP will not support normal DNA synthesis when it replaces dGTP in an in vitro DNA polymerase assay [89]. In addition, Acyclo-GMP terminated DNA is itself a potent and selective inhibitor of HSV DNA synthesis and the effect cannot be reversed by the 3',5'-exonuclease activity associated with HSV DNA polymerase since it is unable to remove 3'-terminal Acyclo-GMP residues from DNA [89]. Thus the current view of the inhibition of virus DNA synthesis by Acyclo-GTP is as follows: The analogue triphosphate initially acts as an extremely good alternative substrate for HSV DNA polymerase but not host cell polymerases, and the virus enzyme readily incorporates Acyclo-GMP residues into DNA. Tight association between the enzyme and 3'-Acyclo-GMP terminated DNA, results in retardation in cycling time of the polymerase, in addition to chain termination. It is likely that this tight association results in the DNA polymerase being irreversibly bound to the DNA template causing total cessation of DNA synthesis (P.A. Furman, pers. commun.).

Another nucleoside anlogue which inhibits herpes virus replication is Ara A [234,273]. This compound is currently used to treat clinical herpes infections, however, the drug is not ideal as some toxicity is associated with its use [164,228,272]. Like other nucleoside analogues, it is the triphosphate form of Ara A (Ara-ATP) which appears to be the active metabolite. Phosphorylated derivatives of Ara A are found in similar concentrations both in uninfected and HSV-infected cells, and so there is little or no selectivity in phosphorylation [17,240,242]. Evidence suggests that initial phosphorylation can be performed by a variety of cellular kinases including; adenosine kinase, deoxycytidine kinase and deoxyguanosine kinase but not by virus-specified kinases such as TK [41,114,118,158,170,267]. It is also clear that Ara A is extensively deaminated in vivo by adenosine deaminase [27,28]. This is significant, as although the derived metabolite, 9-β-D-arabinofuranosylhypoxanthine (AraHx), has antiviral activity, it is less potent than Ara A itself [12,29]. It is interesting that the antiviral activity of Ara A can be reversed by deoxyadenosine only when an inhibitor of adenosine deaminase is present [250].

Ara A causes inhibition of both cellular and viral DNA synthesis, although viral DNA synthesis is more sensitive [211,240,242]. However, the mechanism by which the active metabolite, Ara-ATP, brings about this inhibition is still unclear, as is the basis of the antiviral selectivity of the drug. Some evidence shows that Ara-ATP is a relatively good inhibitor of cellular ribonucleotide reductase [40,191], that it can prevent the initial polyadenylation of mRNA precursors [227] and in addition, that Ara A itself inactivates cellular *S*-adenosylhomocystine hydrolase [128]. The major inhibitory action of Ara-ATP is however, probably directed towards DNA polymerase and DNA synthesis. Enzyme kinetic studies have shown that Ara-ATP competitively inhibits the incorporation of dAMP residues into DNA [91,112,182,193,194,202]. A number of reports have shown that HSV-specified DNA polymerase has higher affinity than cellular polymerases for Ara-ATP, suggesting that this might account for the antiviral selectivity of the drug ([193,194,202] H.S. Allaudeen, pers. commun.). Further evidence that the major site of action of Ara-ATP, at least in uninfected cells,

is at DNA polymerase came from study of an Ara A-resistant tumour cell line which makes altered DNA polymerase with reduced ability to bind Ara-ATP [169]. The precise mechanism of inhibition of DNA synthesis remains controversial. Studies have shown that Ara-AMP residues are internally incorporated into DNA of uninfected cells [215,216], although Müller et al. [193] showed that following Ara A-treatment of HSV-infected cells, many Ara-AMP residues are located at 3′-terminal positions in viral DNA. This implies that termination of HSV DNA chain elongation might occur, and that different mechanisms may operate in the inhibition of cellular and viral DNA synthesis. This hypothesis is supported in part by the work of Pelling et al. [213], who found that although Ara-AMP residues were internally incorporated into HSV DNA, there was significantly more incorporation of the analogue into cellular DNA. They concluded that the selective inhibition of viral DNA synthesis was unlikely to be due to such internal incorporation of Ara-AMP residues. The possibility that the 3′,5′-exonuclease activity associated with HSV DNA polymerase is able to excise 3′-terminal Ara-AMP residues, was investigated by Derse and Cheng [88]. They demonstrated that this enzyme activity will hydrolyze these fraudulent residues from DNA. In addition, Ara-ATP would not replace dATP in supporting DNA synthesis in vitro, and DNA primer-templates containing 3′-terminal Ara-AMP residues were poorly extended by HSV DNA polymerase [88]. Thus, it seems that few Ara-AMP residues are actually internally incorporated into viral DNA because the 3′,5′-exonuclease activity of the viral DNA polymerase actively removes most 3′-terminal Ara-AMP residues before further primer elongation occurs.

In summary, the basis for the antiviral selectivity of Ara A is still not fully understood. Recent work has demonstrated that HSV-induced ribonucleotide reductase is selectively inhibited by Ara-ATP to a considerably higher degree (20-fold) than the cellular enzyme (W.H. Shannon, pers. commun.). However, the most significant effect of the nucleoside analogue triphosphate is probably on the HSV DNA polymerase. This enzyme has slightly higher affinity than cellular polymerase, both for Ara-ATP and natural triphosphates, attributes which might make it more susceptible to the action of Ara-ATP. This compound is not an absolute chain terminator like Acyclo-GTP, but its incorporation into DNA results in a significant decrease in the rate of viral DNA synthesis, which has deleterious effects on transcription [130] and translation [211] of viral genes.

Data now accumulating provide some explanations for the observed reduction in viral DNA synthesis and inhibition of virus replication by the class of nucleoside analogues which are selectively phosphorylated by HSV TK, and readily incorporated into viral DNA [4,46,52,163,229,230]. The triphosphates of these compounds, preferentially formed in HSV-infected cells, appear good substrates both for virus and host cell DNA polymerases [3,229]. For example, studies using isolated enzymes have shown that BVdU-triphosphate competitively inhibits incorporation of dTMP into DNA catalyzed by HSV-specified DNA polymerase [3,229]. Enzyme kinetic data indicated that viral polymerase has relatively high affinity for BVdU-triphosphate ($K_i \simeq 0.1$ μM) [3,162,229] an affinity considerably lower however, than that shown for Acyclo-GTP ($K_i \simeq 0.003$ μM) [89,162]. Thus, the relatively strong affinity of the viral DNA polymerase for BVdU-triphosphate again probably reflects the apparent high

affinity this enzyme shows for natural substrates when compared to host cell polymerases, and it is unlikely that there is specific selectivity [163].

As previously stated, there is ample evidence which shows that BVdU-triphosphate, and other similar nucleoside analogue triphosphates, are utilized by HSV DNA polymerase and readily incorporated in viral DNA [4,46,163,180,229,230,232]. Therefore, it was correctly pointed out be Sagi et al. [232] and Larsson and Öberg [163] that these analogues are unlikely to be selective inhibitors of the virus-specified DNA polymerase, but probably exert their antiviral effect(s) following incorporation into DNA. Again, it should be appreciated that the triphosphate forms of these nucleoside analogues might exert an inhibitory effect upon the virus-induced ribonucleotide reductase. In fact, it has been demonstrated that BVdU-triphosphate and 5-propenyl-2'-deoxyuridine-triphosphate, but not Acyclo-GTP, are relatively good inhibitors of this enzyme activity [196]. It is possible that such inhibition might contribute to the antiviral activities of a number of compounds. However, the major antiviral effects are once again probably mediated through analogue incorporation into viral DNA, although the consequences of this have been the subject of much speculation. Unfortunately, there is relatively sparse experimental data available to clarify these mechanisms of action. It has been demonstrated however, that incorporation of IADDU into viral DNA results in an increase in the number of single and double-stranded breaks in the DNA [106]. In addition, recent work [180] has shown that incorporation of BVdU might facilitate viral DNA degradation. Therefore, incorporation of these analogous into viral DNA might simply cause destabilization and increased degradation of the DNA, rendering it nonfunctional and unavailable for replication or transcription. Furthermore, detailed analysis of the effect of IADDU and BVdU on HSV replication showed specific alterations in expression of virus-induced β and γ proteins ([203], M.J. Otto, pers. commun.). The authors suggested that this effect was probably due to faulty transcription of viral DNA containing nucleoside analogue residues. BVdU also caused impaired glycosylation of viral glycoproteins. Furthermore, virions from IADDU-treated cells had altered virion proteins and showed decreased ability to induce synthesis of all viral proteins in infected cells [203]. Therefore, incorporation of these analogues into viral DNA might generally result in faulty transcription of virus-specified mRNAs with subsequent effects on protein synthesis. However, it is likely that different nucleoside analogous exert inhibitory effects by several different mechanisms. The mechanisms of action of Ara A, BVdU and ACV are outlined in Fig. 1.

Pyrophosphate analogues

A number of years ago it was discovered that certain pyrophosphate analogues showed anti-herpes activity and the most active and selective of these, PAA, was chosen for further investigation [204,241]. Early studies demonstrated that PAA inhibited virus growth in tissue culture at doses which did not affect cell growth [204], and it was also effective when used to treat experimental herpes infections in animals [117,151,186]. However, it became apparent that PAA caused significant skin irritation when applied topically in high concentrations [2,120], and on systemic administration the compound accumulated in the bone of experimental animals [22]. More

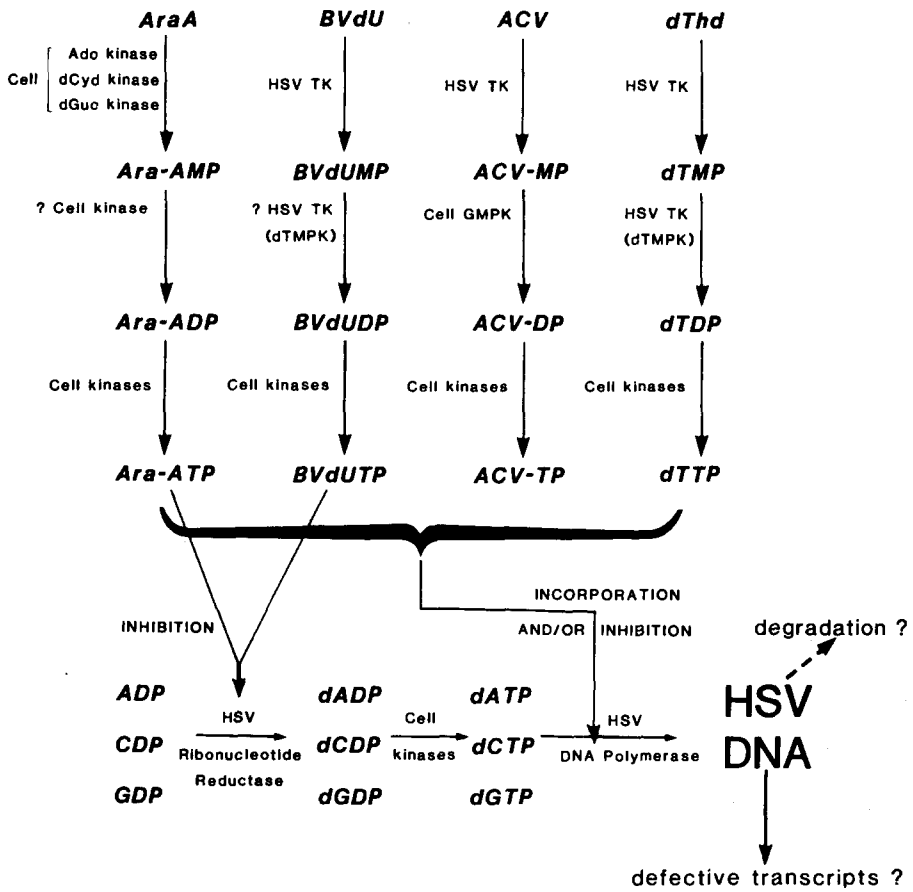

Fig. 1. Enzymes involved in the biosynthesis of Ara A, BVdU and ACV to their respective triphosphate forms, and the sites of action of these inhibitors. The cellular and virus-specified enzymes which phosphorylate these analogues in HSV-infected cells are detailed at each step. The phosphorylation pathway of thymidine and its nucleotides in infected cells (right-hand column) is shown for comparison. Targets of the nucleoside analogue triphosphates are indicated. (Ado = adenosine, dCyd = deoxycytidine, dGuo = deoxyguanosine).

recently, another pyrophosphate analogue, phosphonoformate (PFA), has been described which is an equally active anti-herpes agent but seems less toxic [2,98,126,198]. For this reason, PFA has now been selected for further development.

Evidence to date suggests that PAA and PFA share the same, or a similar, mode of action [97,98]. Initial work established that treatment of HSV-infected cells with PAA resulted in a marked reduction of viral DNA synthesis [204]. In vitro studies by Mao et al. [181] showed that PAA inhibited the activity of HSV-specified DNA polymerase, not by binding directly with template DNA like an intercalating agent, but by specific interaction with the polymerase. Thus, this virus-induced enzyme was identified as a target for the antiviral activity of the drug. Detailed biochemical analysis of the DNA

polymerase induced by herpes virus of turkeys revealed the enzyme to be inhibited by PAA in a non-competitive manner with respect to deoxynucleoside triphosphates and competitively with respect to pyrophosphate [166]. It was concluded from this investigation that PAA interacts with the polymerase at the pyrophosphate binding site, a conclusion which was subsequently confirmed for HSV-specified DNA polymerase by Overby et al. [205], who speculated that by binding to the enzyme, PAA inhibits the addition of deoxynucleotides to the elongating DNA chain. Recent investigations have shown that PFA inhibits HSV DNA polymerase in the same way, and to a similar degree [55,90,98,202]. Thus, PFA is also a non-competitive inhibitor of HSV DNA polymerase with respect to deoxynucleoside triphosphates, and a competitive inhibitor of the pyrophosphate exchange reaction [55,90,98,202].

Genetic data has accumulated which supports the view that the viral DNA polymerase is the major target for PAA and PFA. Hay and Subak-Sharpe [122], showed that HSV mutants selected for resistance to PAA induced altered DNA polymerase activities which were less sensitive to inhibition in vitro by the analogue. This finding was later substantiated by Honess and Watson [131] for PAA, and Derse et al. [90] for PFA. Specific genetic evidence for the antiviral target of PAA came from work by Jofre et al. [138] and Purifoy and Powell [222] who used temperature sensitive (*ts*) mutants of HSV. They found that certain *ts* mutants with lesions in the DNA polymerase gene, were also resistant to PAA, and the DNA polymerases induced by these mutants were less sensitive in vitro than wild-type enzyme to inhibition by the compound [222]. In addition, revertants of these mutants to ts^+ phenotype resulted in the viruses regaining sensitivity to PAA [222] strong evidence that HSV DNA polymerase is the site of PAA, and presumably PFA sensitivity. Genetic mapping studies using intertypic recombinants have subsequently defined more precisely the position of the PAA-resistant lesion with respect to the DNA polymerase gene [42,43].

The selective basis for the antiviral activity of PAA and PFA appears to rely on host cell DNA polymerases being less sensitive to inhibition by these analogues than the viral enzyme. It has been determined that host cell polymerase α is inhibited by both PAA and PFA to a similar degree, and the mechanism of this inhibition is similar to that by which virus enzyme is inhibited [231]. However, the cellular enzyme is around 15 to 30 times less sensitive to both compounds than the virus polymerase. Furthermore, host cell polymerases β and γ are relatively insensitive to inhibition by PAA and PFA [231].

Mechanisms of resistance to anti-herpes drugs

It has been frequently shown that drug-resistant variants of HSV can be isolated relatively easily in tissue culture by exposing sensitive wild-type (*wt*) virus strains to inhibitor. In some cases this has been accomplished following only a single passage of *wt* virus in the presence of a drug (see e.g. [93,104,223]). However, it is apparent that the frequencies with which resistant variants arise, and the relative degrees of resistance are not constant for all inhibitors. The basis of such resistance appears to be mutational change in the viral genome, resulting in synthesis of altered products of viral genes. These genetic changes are heritable, and tend to be stable, even when variants are

extensively passaged in the absence of inhibitor [35,93]. It is believed that the best explanation for the origin of these resistant variants is that mutants arise randomly at very low frequency, and are always present in *wt* virus populations [205,210]. Therefore, drug-treatment probably results in selection of pre-existing mutants which have arisen spontaneously and are better able to replicate in the presence of the inhibitor than the *wt* virus. These mutant strains are unlikely to be induced by contact with an inhibitor unless the compound is itself a mutagen, as exemplified by 5-bromo-2'-deoxyuridine (BUdR) [18,107,165,171,172].

Viral drug-resistance is rarely absolute, especially when cellular functions are involved in the metabolism of an antiviral compound to an active form, and so resistance is always an arbitrarily defined, relative decrease in drug sensitivity. In addition, it is important to appreciate that the degree of resistance of a mutant to an inhibitor will probably vary according to the cell-type used to evaluate such resistance [83,104,252] and the metabolic state of the cells used. It is worthwhile to consider mechanisms of resistance observed in the study of antibiotics and anticancer agents where the following general mechanisms have been recognized, since these mechanisms may be relevant to antiviral drug-resistance.

1. Decreased permeability of the cell membrane to inhibitor.
2. Reduction or loss of synthesis of an enzyme responsible for activating an inhibitor.
3. Compensatory increase in synthesis of the target enzyme of an inhibitor.
4. Specific change in affinity between a target or activating enzyme and inhibitor.
5. Increased degradation of an inhibitor or its metabolites.

The most significant and widespread mechanism of antibiotic resistance discovered is induction of enzymes which specifically degrade the drugs, for example, β-lactamases which break down penicillins [1]. Genes coding for these enzymes are often on plasmids and can be readily transferred by conjugation between bacterial cells allowing rapid and multiple spread of antibiotic resistance [78,79]. Fortunately, this type of acquisition of extra genetic information cannot occur with herpesviruses. It is theoretically possible that all mechanisms outlined above could operate with HSV. However, in practice only two of them have so far been described.

The loss of synthesis of an enzyme involved in activation of an inhibitor is the most common mechanism by which HSV acquires resistance to nucleoside analogues. It has been shown that mutation can result in loss of the virus-specified TK activity [93], a function which is not essential for viral replication in tissue culture [93]. These mutants are cross-resistant to all nucleoside analogues which require initial phosphorylation by TK for production of the active triphosphate. The second resistance mechanism recognized is reduction in affinity between an enzyme and inhibitor. This was first described for the virus-specified DNA polymerase [122], and more recently this mechanism has been demonstrated for HSV TK [75]. There are likely to be quite rigid constraints on the types of mutation that can occur in genes coding for enzymes with essential functions like the viral DNA polymerase [221], ensuring that the normal enzymatic activity is retained. Any dramatic alteration resulting in loss of the enzyme function obviously would be lethal to the virus. One prediction based on this assumption is that these types of mutant, which induce altered enzymes, should arise at quite

low frequency. A further mechanism by which HSV could theoretically acquire resistance to nucleoside analogues is possibly by a change in the 3',5'-exonuclease activity associated with the viral DNA polymerase. For example, synthesis of viral DNA might be inhibited to a lesser degree by Acyclo-GTP if an altered 3',5'-exonuclease activity was able to remove 3'-terminal Acyclo-GMP residues incorporated into DNA [88,89].

It is recognized that viral infection alters the host cell membrane [124,125] but specific mutations resulting in decreased drug sensitivity by an effect on the entry of analogues into cells have not so far been described. Similarly, no drug-resistant HSV mutants have been described with increased ability to degrade inhibitors, or which make increased levels of the target enzyme for an inhibitor. One could imagine that a mutant which induces a specifically altered nucleoside triphosphatase might be resistant through enhanced degradation of a nucleoside analogue triphosphate [279]. Discovery of such a mutant would lend support to the hypothesis that this enzyme activity is virus-coded [38,277,278]. Mutation that leads to overproduction of a viral enzyme which is the target of an inhibitor might result in decreased sensitivity to that inhibitor. For example, a mutation resulting in increased synthesis of the virus-specified DNA polymerase might afford decreased sensitivity to DNA polymerase inhibitors like PAA and PFA. In fact, it has been shown that host-cell DNA polymerase α is only inhibited by these compounds at low enzyme concentrations [126], but this mechanism of resistance has not so far been described for HSV.

Loss of TK activity

HSV mutants which fail to induce TK activity (TK^-) were first isolated by passaging *wt* virus in tissue culture, in the presence of BUdR [93]. As mentioned above, selection of these mutants demonstrated that induction of TK activity was not essential for viral replication in tissue culture, and it also provided strong evidence that HSV encodes a TK gene. Reports of resistance to IUdR in tissue culture were published about the same time as the discovery of HSV TK^- mutants [35,246]. These studies showed that IUdR-resistant mutants were easily selected by passaging *wt* virus in the presence of the drug [223], but as these mutants were not extensively characterized their ability to induce TK activity was not established. In retrospect, it seems likely that the majority of these mutants were unable to induce TK activity, especially as Dubbs and Kit [93] showed that BUdR-selected TK^- mutants were cross-resistant to IUdR. Smith [246] demonstrated that *wt* virus populations contained IUdR-resistant mutants at frequencies between 0.5 to 4% suggesting a relatively high mutation rate. These frequencies were later substantiated for other nucleoside analogue inhibitors [210,247], and so it is possible that *wt* virus populations contain quite high proportions of naturally occurring TK^- variants. The development of resistance to TFT in tissue culture has also been demonstrated, but again these mutants were not tested for induction of TK activity [115]. It is possible that they were also TK^-, as they showed cross-resistance to IUdR [115]. More recent studies with independently derived HSV TK^- mutants, demonstrated that they were cross-resistant to TFT when tested in cells lacking cytosol TK activity, but were less resistant in normal TK^+ cells [105]. It is thus important to emphasize the use of TK^- cells when testing the sensitivity of mutants to nucleoside analogues which are phosphorylated by cellular TK. In fact, Dubbs and

Kit [93] found that BUdR-resistant TK^- mutants showed greater sensitivity to BUdR when tested in normal TK^+ LM cells, than in TK^- cells.

There seems to be little comprehensive data available on the properties of mutants resistant to IUdR and TFT. This might be because interest in these drugs waned after the extent of their toxicity became apparent. In contrast, the advent of highly selective anti-herpes inhibitors saw a resurgence of interest in the study of drug-resistant mutants. Initially, they served as useful tools in order to facilitate understanding of the modes of action of these compounds. Thus, part of the evidence which established that ACV is selectively phosphorylated by HSV TK came from studies with TK^- mutants [58,96,104,114,238]. It was first found by Elion et al. [96] that a TK^- mutant was resistant to ACV, strongly suggesting that this guanosine analogue is specifically recognized as a substrate by the viral TK, and phosphorylated in *wt* virus-infected cells. It soon became evident that other independently selected TK^- mutants were also resistant to ACV [104,247]. Furthermore, it was shown that the sensitivity to ACV could be restored to TK^- viruses if the tests were performed in 'TK-transformed' cells where the constitutively expressed HSV TK was able to complement the defects in the mutants [104]. Attempts were made to isolate ACV-resistant mutants in tissue-culture, by exposing *wt*-sensitive virus to high concentrations of the drug. It was concluded from a number of studies that ACV-resistant variants could be easily isolated at relatively high frequency [58,104,238,247]. Biochemical characterization of the mutants showed that the majority had lost ability to induce TK activity [58,104,235]. In fact, Schnipper and Crumpacker [238] found that selection of ACV-resistant mutants in tissue-culture paralleled reduction in expression of viral TK activity. Investigations performed with BVdU, FIAC and DHPG showed that TK^- mutants of HSV-1 were resistant to these compounds, again suggesting that viral TK is responsible for their initial phosphorylation [56,85,178]. Furthermore, in a study designed to obtain BVdU-resistant mutants in tissue-culture, the majority of resistant isolates tested were found to be TK^- [101]. Variants resistant to DHPG and FIAC have also been isolated in tissue-culture [248,261], although biochemical properties of these mutants were not established. It is interesting to note that the degrees of resistance of different, independently isolated TK^- mutants to a particular nucleoside analogue, are very similar when tested in the same tissue culture system [104]. In summary, the studies using 'new generation' nucleoside analogue inhibitors show that resistance arising in tissue-culture is most commonly due to TK^- mutants. The level of this resistance is high and seems to be relatively uniform for each nucleoside analogue inhibitor.

Alteration in DNA polymerase

It was discussed in the previous section that the pyrophosphate analogues, PAA and PFA have a similar or identical mode of action, which involves direct inhibition of the virus-specified DNA polymerase. Not surprisingly, it has been found that resistance to these compounds can occur through alteration in the properties of DNA polymerase. Isolation of PAA-resistant mutants, by exposing *wt* virus to the analogue in tissue-culture, was first reported by Klein and Friedman-Kien [151]. They found that these mutants could be selected with relative ease, an observation which has been substantiated in a number of subsequent studies where PAA- or PFA-resistant

mutants were isolated [90,97,131,151,205]. Some evidence indicates that *wt* virus stocks contain low but significant levels of PAA-resistant variants [205] and thus, it has been suggested that exposure of *wt* virus to PAA or PFA results in selection of pre-existing resistant variants from the virus population. It is apparent that cloned, pyrophosphate analogue resistant mutants display variable levels of resistance to these analogues when tested in the same cell-type, suggesting that a variety of mutants may be generated in a single *wt* virus population. Analysis of the virus-specified DNA polymerases induced by these mutants revealed decreased affinities for PAA and PFA, presumably accounting for the resistance to the analogues [90,97,122,131]. Furthermore, Chartrand and his associates [42,43] have used marker rescue and analysis of intertypic recombinants to demonstrate that PAA-resistance markers (*paa*^{r}) in both type 1 and type 2 are clustered in a 2.9 kb region of the genome between map units 40 and 41.8. All other mutations associated with DNA polymerase function which were examined in this study mapped in a slightly larger region (map units 38.6–41.8) which encompassed the PAA-resistance loci, thus providing further strong evidence that mutations affecting PAA-resistance are located in the DNA polymerase gene.

Cross-resistance studies with this class of mutant have proved extremely interesting, and have been useful in elucidating mechanisms of resistance to certain other herpesvirus inhibitors. It has been established that most, if not all PAA-resistant mutants show cross-resistance to PFA [97], and the reverse relationship with PFA-resistant mutants is also true [12,90,96]. Furthermore, it has been demonstrated that some PAA-resistant mutants show reduced sensitivity to Ara A [59,97], suggesting that the viral DNA polymerase is a target for Ara-ATP. Recently, a proportion of both PAA- and PFA-resistant variants were found to display increased sensitivity to aphidicolin relative to *wt* viruses [12,132,199], an inhibitor of DNA polymerase [92, 212]. On the basis of these obervations, it was suggested that aphidicolin could be considered for use in combinaton therapy to reduce the likelihood of the emergence of resistant variants with mutations in the DNA polymerase gene [12]. It should be noted that PAA- and PFA-resistant mutants seem only to have alterations in the viral-induced DNA polymerase, whilst other viral-specified functions, such as TK, remain unchanged [12,111]. An early study by Honess and Watson [131] described PAA-resistant mutants which expressed altered DNA polymerase and were also TK$^-$, although these were only isolated following extensive passage of virus in the presence of PAA and acquisition of the TK$^-$ phenotype may have been fortuitous.

Obviously, nucleoside analogues such as ACV will be phosphorylated in cells infected with PAA- or PFA-resistant mutants if they express normal TK levels. The acquisition of ACV-resistance by loss of TK activity has been discussed earlier, however, it was predicted from the mechanism of action of ACV that mutation of the viral DNA polymerase gene might also confer resistance to ACV. PAA-resistant variants with mutations in the DNA polymerase gene were found to be relatively cross-resistant to ACV [58,238], observations which provided evidence for a second locus of resistance to ACV. Genetic recombination experiments between PAA-resistant and TK$^-$ mutants demonstrated that these resistance loci were distinct on the herpesvirus genome [58]. Recent analysis of the DNA polymerases induced by a group of

PFA-resistant mutants showed that these enzymes had decreased affinity for Acyclo-GTP, in addition to PFA and PAA [90] and, not surprisingly, these variants were found to be cross-resistant to ACV in tissue culture [12,56], although they were all sensitive to the ACV-derivative, DHPG [56]. Crumpacker and his colleagues [71] have analysed a set of intertypic recombinants generated by rescue of a *ts* lesion in the DNA polymerase gene of an HSV-2 strain using fragments of DNA derived from a PAA-resistant HSV-1 strain. These were the recombinants used in the earlier study to map the location of the *paa*^{r}*-1* locus [43] and in this study they were used to map the location of an ACV-resistance locus (acg^r-1) making use of the observation that the parent PAA-resistant HSV-1 strain was co-resistant to ACV. The *acg*^{r}*-1* locus segregated with the *paa*^{r}*-1* locus and was mapped between map units 40.2 and 41.8. It is likely therefore that the lesion in DNA polymerase conferring PAA-resistance also conferred ACV-resistance, a result entirely consistent with previous observations [58,238].

In a later study the same series of recombinants was used yet again to map the loci for resistance to Ara A and BVdU since the parent HSV-1 mutant was also resistant to these analogues [72]. The *ara-A*^{r}*-1* locus segregated in the recombinants along with the *paa*^{r}*-1* and *acg*^{r}*-1* loci. These data should not be overinterpreted. They define the approximate location of a lesion (or series of lesions) in the polymerase gene of a single PAA-resistant HSV-1 strain which can confer resistance to at least three structurally distinct analogues. The mutation may be in those sequences encoding the 'active centre' of the enzyme as was proposed [155] but it would seem equally likely that mutation at a remote site could affect substrate-binding by altering the overall three-dimensional structure of the enzyme.

An interesting observation was that the *bvdu*^{r}*-1* locus apparently segregated independently of the other three but mapped close to them. These experiments provided some suggestive evidence that it is possible for mutations in DNA polymerase to confer cross-resistance to PAA, ACV and Ara A without generating BVdU-resistance and also that it is possible to generate BVdU-resistance by mutation in DNA polymerase.

It must be stressed that the observations discussed above relating to the mapping of ACV, Ara A and BVdU-resistance loci have all been made using the single parent mutant HSV-1 strain 17 (*ts*$^+$, *paa*^{r}*-1*) and recombinants derived from it. Far more information will be required before we can assess with any degree of confidence the relative importance of different regions of the polymerase gene with respect to resistance to different analogues. In fact, recent studies by Honess and his colleagues [132] have made it clear that any simplistic view that lesions conferring resistance to particular analogues will map to localized well-defined regions of the polymerase gene (see refs. [72,155]) must be regarded with considerable scepticism.

Although it was established using PAA-resistant mutants that mutation of the HSV DNA polymerase gene could confer resistance to ACV, it has proved extremely difficult to isolate in tissue culture TK$^+$, ACV-resistant mutants which induce altered DNA polymerase using ACV as the selective agent [58,104,111]. The reason for this is unclear, especially as mutants resistant to PAA and PFA can be isolated with relative ease, and indeed, all of those so far tested have shown varying degrees of cross-resis-

tance to ACV. In fact, probably only two TK^+ mutants with altered DNA polymerases have been isolated to date using ACV. These are BW^r, which induces an altered polymerase with decreased affinity for Acyclo-GTP [111] and C1 [101] P_2C_5, which also appears to be ACV-resistant due to an altered polymerase [104, unpubl. results]. Both mutants are sensitive to PAA and PFA, and show different patterns of cross-resistance from TK^- mutants to nucleoside analogue inhibitors [105,111]. A number of TK^- and PAA-resistant double mutants have been isolated using ACV, providing additional evidence for the existence of two loci conferring resistance to ACV [58,104]. These mutants, which are substantially more resistant to ACV than TK^- mutants, were in two cases isolated following multiple passage in the presence of a high ACV concentration [104]. Enzyme kinetic studies have demonstrated that the DNA polymerases induced by two such double mutants, ACG^r4, and SC16 R_9C_2, have reduced binding affinity for both Acyclo-GTP and PAA [111,283]. Thus, all of the PAA- and PFA-resistant mutants tested are cross-resistant to ACV, although not all ACV-resistant mutants selected using ACV and expressing altered DNA polymerase, are cross-resistant to PAA and PFA.

Attempts have been made to isolate highly resistant mutants in tissue culture using Ara A, however, so far this has proved relatively difficult [12]. This might be due to Ara A and its derivatives having multiple sites of action which could require virus to acquire multiple mutations to achieve high level resistance. There is some indication from study of PAA-resistant strains that HSV can acquire resistance to Ara A by mutation of the DNA polymerase gene, although the level of resistance observed was not very high [59,97]. It is claimed in a recent study by Coen et al. [59] that Ara A-resistant mutants can be isolated relatively easily following a single passage of wt virus in the presence of the inhibitor, and Ara A-resistant mutants have been isolated by others [237,260]. However, an adenosine deaminase inhibitor was not used in these experiments, and so these investigators might really have been isolating AraHx-resistant mutants. It is also claimed that Ara A-resistant variants induced DNA polymerases with decreased ability to bind Ara-ATP, as was demonstrated for the polymerase induced by a PAA-resistant strain, PAA^r5 [59], and for PFA-resistant mutants [12]. However, similar enzyme kinetic studies were not undertaken with the polymerases induced by the mutant strains selected using Ara A [59]. It is interesting that the majority of these mutants also showed some cross-resistance to PAA, again implicating viral DNA polymerase as the target for Ara-ATP or at least for AraHx. Further support for this notion has come from recent work which demonstrated conclusively that the purified DNA polymerase induced by an HSV-1 mutant selected for resistance to Ara A, showed decreased affinity for Ara-ATP (H.S. Allaudeen, pers. commun.).

There have been no other reports to date of the isolation of nucleoside analogue-resistant mutants expressing altered DNA polymerase, although it might be expected that these mutants will arise because of the involvement of viral DNA polymerase in the mode of action of these inhibitors. The use of PAA-resistant mutants has again been useful in identifying the viral DNA polymerase gene as a locus able to confer resistance to another nucleoside analogue. Crumpacker et al. [72] showed that an HSV-1 PAA-resistant mutant was cross-resistant to BVdU. The properties of the

DNA polymerase induced by this strain have been investigated and confirm that the enzyme has altered recognition for BVdU-triphosphate (H.S. Allaudeen, pers. commun.). Furthermore, reduction in binding affinity of DNA polymerases induced by PAA-resistant mutants for FIAC-triphosphate and FMAU-triphosphate, was also seen (H.S. Allaudeen, pers. commun.).

Alteration in TK substrate specificity

Until recently, the only mechanism known by which HSV could acquire resistance to nucleoside analogues and retain the TK^+ phenotype, was by mutation of the DNA polymerase gene [58,104,238]. Another mechanism now recognized is by mutation of the viral TK gene resulting in the induction of an enzyme with altered substrate specificity, such that its ability to phosphorylate thymidine is retained, whilst phosphorylation of the nucleoside analogue is severely impaired [75]. The first mutant in this class to be described was isolated by passaging *wt* virus in the presence of ACV [75]. These passages were performed in serum-starved baby hamster kidney (BHK) cells, a tissue culture system designed to favour the replication of TK^+ viruses [136]. It was anticipated that the TK^+, ACV-resistant mutant isolated from this selection system would induce an altered DNA polymerase. Initial evidence however, suggested that resistance was due to mutation of the TK gene [75]. More detailed investigations subsequently confirmed these observations, showing that the TK induced by this mutant strain differed from that of the *wt* in a number of physical and kinetic characteristics, including loss of ability to phosphorylate ACV and reduced thermal stability [160,161]. Two additional mutants which induce TK activity, one isolated in the presence of BVdU [101] and the other with ACV [162] have been characterized [161,162] and both induce altered TKs with reduced ability to phosphorylate the inhibitor used in their selection. Furthermore, analysis of the DNA polymerases induced by these resistant strains showed them to be indistinguishable from the polymerase induced by *wt* virus [162] indicating that induction of an altered DNA polymerase was unlikely.

The pattern of cross-resistance of these TK substrate specificity mutants is complex and relatively unpredictable, although not surprisingly, they are all sensitive to the DNA polymerase inhibitors, PAA and Ara A, whose action is independent of HSV TK ([75], our unpubl. results). The pattern of cross-resistance to other nucleoside analogue inhibitors however, is less predictable and this will probably be found to be the case for other mutants in this class. Interestingly, it was shown that the BVdU-resistant mutant (SC16 B3) was completely sensitive to ACV when tested in tissue-culture [101]. The implications of changes in the TKs induced by the ACV-resistant mutants, SC16 S1 and SC16 Tr7, for the behaviour of the variants towards other inhibitors was investigated in two ways. Firstly, the apparent affinities of the TKs for a range of nucleoside analogues were tested by determining K_i values. Secondly, the resistance of these mutants to the analogues was directly tested by plaque reduction assay (see Table 1).

In each case, decreased affinity of the enzyme for ACV was associated with decreased affinity for other nucleoside analogues. Furthermore, in some cases but not all, decreased affinity for an analogue correlated with increased resistance of the

TABLE 1

Cross-resistance of ACV-resistant variants which induce TK with altered substrate specify

Nucleoside analogue	SC16 (*wt*)		SC16 S1		SC16 Tr7	
	Ki (μM)[a]	ED_{50} (μM)[b]	Ki (μM)	ED_{50} (μM)	Ki (μM)	ED_{50} (μM)
ACV	200	0.10	2200	10	2500	22
BVdU	0.10	0.03	5	0.54	1.5	0.37
IUdR	0.14	0.56	6	1.30	0.72	0.70
TFT	0.28	1	6.8	20.3	0.80	2.03
Ara T	3.6	0.80	420	8.10	60	2.44
FMAU	0.24	0.01	50	2.10	1.6	0.03
FIAC	0.50	0.10	138	5.70	29	0.43

[a] Ki values were determined using purified TK as described [161].

[b] ED_{50} values were determined using BHK cells, or BU-BHK (TK^-) cells (for IUdR and TFT assays) in plaque reduction assays as described [104].

mutant virus to that inhibitor. A study by Cheng et al. [56] showed that these TK substrate specificity mutants also differed in sensitivity to DHPG, SC16 S1 and SC16 B3 being sensitive, and SC16 Tr7 being relatively resistant to this inhibitor.

It is interesting to note that the kinetic properties of the TK induced by SC16 B3 are similar to those of HSV-2-induced TK with respect to interaction with BVdU. HSV-2 is significantly less sensitive to BVdU in tissue culture than HSV-1, although both virus types induce TK activity [53,85]. It has been suggested that this difference might be due to the ability of the dTMP kinase activity associated with HSV-1 TK to phosphorylate BVdU-monophosphate (BVdUMP) to BVdU-diphosphate (BVdUDP) more readily than the HSV-2 TK activity [113]. However, when BVdU and not BVdUMP is used as substrate, HSV-1 TK will not convert this nucleoside to BVdUDP in vitro [113], which casts some doubt on the importance of the dTMP kinase activity in these reactions in vivo. Analysis of the TKs purified from SC16 B3, *wt* HSV-1 and HSV-2 infected cells has shown that both HSV-2 and SC16 B3 TKs have much lower binding affinities for this analogue than the *wt* HSV-1 enzyme [53,161]. Phosphorylation of BVdU by HSV-2 and SC16 B3 TKs can be detected in vitro when the analogue is present at high concentrations [53,162] which implies that consideration of relative binding affinities of TK for analogues rather than maximum phosphorylation rates, might give a better indication of the sensitivity of a virus to a particular nucleoside analogue inhibitor.

The group of altered TK mutants described above are not unique as another nucleoside analogue-resistant mutant expressing altered TK has been reported recently [265,266]. This was isolated in tissue culture using 5-methoxymethyl-2'-deoxyuridine (MMdU) and induced nearly normal levels of TK activity (50–70% those of the *wt*) but was resistant to MMdU, ACV and Ara T [265]. A recent report has confirmed that this mutant ($MMdU^r$-20) induces TK with altered substrate specificity with reduced ability to recognize MMdU, Ara T and BVdU [266]. Interestingly, it was demonstrated that the mutant TK in 'crude'-infected cell extract, showed increased thermostability compared with the *wt* enzyme activity. Other possible candidates are

two mutants isolated following exposure of virus to IUdR in tissue culture [39] and in people suffering from herpes infections [129], and the BUdR-resistant mutant C1(101)TK5 isolated by Summers et al. [253] in tissue culture. This latter mutant induced only 25% TK activity compared with the *wt* virus, a result similar to that found with SC16 S1 TK [74,160]. Detailed kinetic analyses of the TKs and DNA polymerases induced by these mutants have not been performed, and so it is not possible to say whether they have mutations in the TK or DNA polymerase genes, or both.

Induction of low TK activity

The general properties and patterns of cross-resistance of TK^- mutants have been described above. It is likely that the TK gene of a 'true' TK^- mutant contains a frameshift mutation [e.g. 270] or a base-pair substitution such that an early termination codon is generated [253]. Such a nonsense mutation would give rise to premature termination of translation, resulting in the induction of no TK polypeptide, or a truncated polypeptide with no detectable TK activity [253]. Alternatively, mutation affecting a regulatory function might result in failure to make TK polypeptide, although no mutant in this class has yet been found. It is apparent that many so-called 'TK^-' mutants induce low but significant levels of TK activity [253]. Mutants isolated with ACV have been described which induce around 5% [104] and 14% and 16% [58] TK activity compared with *wt* virus. A mutant isolated using BVdU induced TK activity which was 5% that of *wt* virus [101] and Veerisetty and Gentry [265] used MMdU to isolate resistant mutants, most of which induced low levels of TK activity. It should be emphasised that the use of TK^- cells is important in order to obtain an accurate assessment of such low levels of viral TK activity. There are a number of probable explanations why these low levels are induced in mutant-infected cells. A trivial, but probably frequent explanation, is that the virus stock generated consists of a mixture of a large amount of true TK^- mutant and a small proportion of contaminating *wt* TK^+ virus [99], and therefore, only plaque-purified clones of mutant virus should be used. A second possible explanation is that there is occasional read-through of a termination codon in a transcript from a TK gene containing a nonsense mutation. Infection of cells with this class of mutant will result in synthesis of low levels of *wt* TK polypeptide. This explanation was proposed by Cremer et al. [68] for the mutant C1(101)TK4 which induces no TK polypeptide [253] but consistently gave 1–2% TK activity. The mutant was suppressable in vitro, and it was suggested that the low TK activity observed in tissue culture might be due to read-through by low activity suppressor tRNAs. A third class of mutation, possibly the most common, would be the missense type, which would lead to an amino acid substitution in a critical region within the TK polypeptide, resulting in expression of normal amounts of an altered polypeptide having low residual TK activity. These mutants might represent the lower end of a wide spectrum of variants which all induce altered TKs and exhibit different levels of TK activity. Recent work has shown that the ACV-resistant double mutant, SC16 R_9C_2 [104], induces TK activity of this type. This mutant makes a similar amount of full length TK polypeptide in infected cells compared to the *wt* virus, but the TK activity is only about 5% [283]. Study of the SC16 R_9C_2-induced TK has shown

that it has altered properties, including significantly reduced binding affinity for ACV, indicating it has a changed substrate specificity [283]. Thus, 'TK$^-$' variants which induce low levels of TK activity might consists of a variety of genetically distinct mutants. The possible significance of such residual TK activity will be discussed later.

The different classes of ACV-resistant HSV mutants that have been isolated in tissue culture are summarized in Table 2.

Pathogenicity of drug-resistant variants of HSV

HSV has evolved over many years during which it has become extremely well-adapted to its human host, so much so that the majority of individuals in the population are infected but few suffer any serious clinical problems. During the primary acute disease latent infections are established in the peripheral nervous system and these may reactivate from time to time providing virus with an opportunity to spread horizontally in the population. This pattern of disease means that an individual once infected is a potential source of future infections for many years to come (for review see [275]). This relationship between virus and host has developed unchallenged until recent times. However, the treatment of infected patients with effective anti-herpes drugs introduces a new factor into the equation and can lead to the appearance of virus strains better adapted than their predecessors to survive in the presence of the drug [34,73,243,268].

In the previous section it was shown that HSV can acquire resistance to inhibitors by several mechanisms each of which involves alteration in virus phenotype. The demonstration that a particular phenotype is drug-resistant in tissue culture tells us little however, about the ability of such a phenotype to establish itself in the population, or about its 'pathogenic potential' (potential to cause disease) in the individual human host. Studies on the effects and implications of drug-resistant variants arising during treatment of humans are currently in their infancy (see page 27). Animal model systems can however, shed considerable light on the in vivo potential of particular drug-resistant phenotypes and it is these studies which will now be discussed.

TABLE 2

Classes of ACV-resistant mutants isolated in tissue culture

Mutant	TK activity (%wt)	DNA polymerase activity	Refs.
TK$^-$	<1	normal	[75,93,104,253]
'Low Producer' TK^r	1–15	normal	[58,101,104,265]
TK^r (altered substrate specifity)	15–100	normal	[75,160,161,162,265]
DNA Pol^r	100	altered	[104,111]
TK$^-$ DNA Pol^r	<1	altered	[58,104]
'Low producer' TK^r DNA Pol^r	1–15	altered	[104,283]

If our initial premise, that HSV is well-adapted for survival in the human population, is correct then we would expect drug-resistant variants which compete successfully with *wt* strains to possess similar in vivo properties. Specifically, we would expect similar patterns of growth in host tissues, establishment of latent infections in neurological tissues and subsequent reactivations. Widespread use of a particular drug would of course give a resistant variant considerable selective advantage and this might counterbalance some degree of attenuation in other in vivo characteristics.

When considering the consequences of the appearance of a resistant variant we must look both at the implications for the population and also at the implications for the individual concerned. It may be possible, for example, for a resistant mutant to have extremely poor survival potential in the human population where it must compete with *wt* strains, but at the same time it might exhibit an altered and damaging pattern of pathogenicity in the individual host. It is important to separate these concepts since the particular properties which ensure success of the virus in one sphere may not ensure success in the other.

What then can we learn about pathogenicity and survival potential of different drug-resistant phenotypes using animal model systems?

Loss of TK activity

Viruses which have lost the ability to express TK activity (TK^- phenotype) have been investigated in several different animal systems. The results have been somewhat variable but there is general agreement that these mutants perform less well than their wild type antecedents in most systems.

We will consider firstly growth at the periphery, at the site of inoculation. It has been shown that TK^- viruses reach significantly lower titres than parental TK^+ strains when inoculated at similar doses into the tissues of the ear pinna of the mouse [75,100,102]. Similar results were obtained by Price and Khan [218] using a mouse ear model. In contrast, Tenser and his colleagues [255–257], in their studies of TK^- viruses using eye inoculation in both mice and guinea pigs, saw no significant differences between TK^+ and TK^- viruses in their patterns of growth.

Although the results concerning growth at peripheral sites have been somewhat contradictory, a much clearer picture emerges when we consider the ability of these strains to invade and replicate in local sensory ganglia. There is now general agreement that TK^- viruses replicate poorly in ganglial tissues [102, 218, 255–257]. Furthermore, it is difficult to demonstrate the presence of latent virus infection by culture of ganglia after the acute infection has subsided. The failure to recover TK^- virus from cultured ganglia can of course be interpreted in one of two ways. It either means that latent infection was not established during the acute infection, or it means that latent infections established by TK^- viruses cannot be reactivated by convential techniques. Again there are differences in results from different laboratories. For example, Price and Khan [218] could not demonstrate latent infections of superior cervical ganglia following ocular infection with TK^- variants in the mouse, whereas Tenser and his associates [256] recovered TK^- viruses from about 20% of trigeminal ganglia examined using a similar system. There are even differences between TK^- strains when examined in the same system. Using two variants of the type 1 strain C1(101) we were

able to show relatively efficient establishment of latent infections using the TK^- ACV-resistant variant, P_2C_6, but no latent infection using the Dubbs and Kit mutant, B2006 [100].

It is pertinent at this point to introduce several notes of caution concerning the interpretation of results obtained in animal systems using TK^- viruses.

i. Some of the apparent differences observed may be due to differences between systems employed such as the animals used, the routes of inoculation, the doses of virus employed or the particular ganglia examined.

ii. Some so-called TK^- variants may be 'low producers' inducing some enzyme activity which might alter the pattern of pathogenicity. A virus is usually classified as 'TK^-' on the basis of an in vitro enzyme assay and the results of such an assay are dependent on the conditions used both for the infection and the assay. Large variations can be seen with the same virus under different conditions [257].

iii. As discussed earlier, HSV TK is a multifunctional enzyme able to recognise in addition to thymidine itself, both deoxycytidine and dTMP as substrates. In view of this, the phenotype of the virus should be described not only in terms of TK function but also in terms of deoxycytidine kinase (dCK) and dTMP kinase (dTMPK). It may be possible, for example, for a virus to possess a TK^-, dCK^-, $dTMPK^+$ phenotype and such a virus might exhibit a different pattern of pathogenicity from one with a TK^-, dCK^-, $dTMPK^-$ phenotype. This is speculation since there is no current information on the roles of these additional activities in vivo, or whether they possess a functional role at all. However, such information might be forthcoming if the phenotypes of TK^- strains are described in more detail.

iv. Some differences between TK^- variants might be due to inapparent lesions in other functions. If the difference in pathogenicity between a TK^- variant and its parent is due solely to the lesion in TK then *wt* TK^+ revertants [37] would behave in vivo like the parent virus. Tests such as this might remove some of the apparent anomalies in these systems.

v. TK^- viruses might revert to TK^+ in vivo and some of the apparent properties could be attributed to activities of the revertants. For example, TK^+ viruses have been recovered from the ganglia of animals originally inoculated with TK^- variants (H.J. Field, pers. commun.) although it was not clearly established whether these were revertants. Differences in reversion frequencies would result in apparent differences in properties.

Another important characteristic of HSV is its ability to invade the central nervous system [36,65,226] with the occasional development of herpes encephalitis [177]. It is therefore important to characterize drug-resistant viruses with respect to their ability to replicate in the CNS since any improvement compared to *wt* might have serious consequences. A convenient way of doing this is to assess the lethality of mutants following intracerebral inoculation. Experiments such as this in the mouse have fortunately shown marked attenuation in 'neurovirulence' of TK^- variants [75,100,102].

It would appear therefore from the data available on the pathogenicity of TK^- variants that these viruses replicate less well than *wt* in vivo with the biggest differences in growth being seen in tissue of the nervous system. There are also defects in the

establishment of latent infections or in reactivation. These factors taken together imply that the consequences following the appearance of TK^- variants in normal individuals undergoing drug therapy would not be serious either for the individual or for the community.

TK mutants with altered substrate-specificity

Another class of TK mutant is that in which subtle changes in the enzyme result in failure to phosphorylate particular nucleoside analogues, with little change in their ability to phosphorylate thymidine. Few mutants in this class have been described but the animal data available clearly show these viruses to be more pathogenic than TK^- variants. The three mutants investigated so far in our laboratory, SC16 S1 and SC16 Tr7, both isolated using ACV [75,162] and SC16 B3, isolated using BVdU [101] grow as well as *wt* virus in the tissues of the ear pinna of the mouse. They can be recovered from latently infected ganglia with an efficiency similar to that of *wt* virus although they do show slight (10-fold) attenuation of neurovirulence when assessed by intracerebral inoculation [75,101,283].

The in vivo properties of these viruses suggest that they might compete favourably with *wt* strains in natural situations. However, apart from their resistance to particular drugs they do not appear at present to have properties which would pose a more serious threat to the individual than that of normal *wt* strains.

'Low TK producers'

As discussed above (page 21), when drug-resistant mutants are isolated, many of the variants obtained express low but significant levels of TK activity (e.g. [58,104,253]). Such mutants are similar to the substrate-specificity mutants described above in that they have impaired or reduced function of the enzyme rather than complete loss of activity, and for this reason divisions between the two groups are necessarily artificial and arbitrary. It may be most convenient to define a 'low producer' as a virus inducing $< 15\%$ of the activity seen with *wt* virus. This definition would include the 'moderate TK producers' (5–15% TK) and undoubtedly some of the TK^- viruses ($<5\%$ TK) described by Tenser and his colleagues [257]. In this range of activity the actual level of enzyme induced appears to have a marked effect on the ability of the virus to replicate in the trigeminal ganglion [257]. However, it is not always easy to apply a seemingly simple definition such as this. The mutant Pat_5^-, for example [257] could be defined either as a substrate-specificity mutant expressing 60% *wt* TK activity, or a low producer expressing only 10% depending on the conditions of virus growth and the assay system used. Nevertheless, providing it is remembered that the two groups of mutant are not distinct but simply represent two ends of a continuous spectrum, and providing the grounds for placing a mutant in one group rather than the other are clearly stated, it is probably worthwhile to make the distinction.

It appears that the ability of a mutant to express a low level of TK activity is beneficial to the virus when confronted with the prospect of replicating in tissues of the

nervous system. Tenser and his associates [257] showed a relationship between the extent of replication in the trigeminal ganglion following ocular infection, and the level of TK expressed by the virus. Our experience with mutants from the type 1 strain SC16 have been similar. For example, the 'neurovirulence' of the mutant SC16 R_1C_1, which expresses 1–2% of the activity observed with *wt* virus, is approximately 10-fold higher (LD_{50} about 3×10^3 PFU) than that of the TK^- strain SC16 R_5C_1 (LD_{50}, 5×10^4 PFU) [100]. We have recently investigated in some detail the properties of recombinants made by crossing the double mutant SC16 R_9C_2 with *wt* virus. One of these recombinants, RSC-11, has the TK gene of SC16 R_9C_2 in an otherwise *wt* genetic background [283]. Like the parent double mutant, this recombinant induces about 5% of the TK activity induced by the *wt* virus. The LD_{50} dose in mouse brain for this mutant is approximately 200 PFU, placing it in an intermediate position between the substrate-specificity mutants (LD_{50} doses in the range, 50–70 PFU) and SC16 R_1C_1 (LD_{50} dose, 3×10^3 PFU), and much more 'neurovirulent' than TK^- strains (LD_{50} doses in the range 10^4–10^6 PFU). This recombinant has also been recovered from latently infected cervical ganglia following ear inoculation [283].

It is clear from the results discussed above that retention of residual TK activity can enhance the neurovirulence and in vivo growth potential of a virus when compared to strictly TK^- variants. The properties of the 'low producers' studied to date suggest that they are intermediate in virulence, between the TK^- variants at one end of the spectrum and substrate-specificity mutants at the other.

DNA polymerase mutants

Mutations in the DNA polymerase gene of HSV can confer resistance to a range of antiherpes drugs and naturally such a mutation does not affect TK function. However, DNA polymerase is an essential virus function and so we might expect changes to alter the behaviour of the virus in vivo. Unfortunately, very little data is available on the pathogenicity of DNA polymerase mutants. What is available already suggests that it will be difficult, if not impossible, to make generalizations about these mutants.

Klein and his colleagues [153] have investigated the properties of a single PAA-resistant mutant using orofacial inoculation in the mouse. They observed slight attenuation in growth at the periphery but they were able to recover latent virus from local sensory ganglia.

We have looked at the pathogenicity of two ACV-resistant DNA polymerase mutants, C1(101)P_2C_5 and RSC-26, and rather different results emerged [100,283].

C1(101)P_2C_5 grew as well as *wt* virus in the mouse ear, was recovered from latently infected ganglia with a similar efficiency to *wt*, but did show some attenuation of neurovirulence when inoculated into mouse brain (LD_{50} dose 8 PFU compared with 1 PFU for C1(101), the *wt*). However, the LD_{50} dose was in the range observed with other type 1 strains (e.g. LD_{50} dose for SC16 is 7 PFU). On the basis of the pathogenicity studies carried out on this virus, it could not be distinguished from other authentic type 1 strains [100] and there is no reason to suppose that its behaviour would be in any way significantly different in vivo.

A somewhat different picture has emerged with the mutant RSC-26. This virus is another recombinant isolated from the cross of SC16 R_9C_2 with *wt*. Unlike RSC-11,

this recombinant expresses *wt* TK activity but it retains a lesion in DNA polymerase identical to the polymerase lesion in SC16 R_9C_2. Its polymerase has a marked reduction in sensitivity to inhibition by ACV-triphosphate and the virus itself is approximately 10-fold less sensitive than *wt* to ACV [283]. This virus replicated poorly in the mouse ear pinna and it was considerably attenuated with respect to neurovirulence ($LD_{50} > 10^3$ PFU). In contrast to the in vivo results, growth in tissue culture appeared normal. Furthermore, the lack of virulence could not be explained by the virus having a *ts* phenotype. We have not excluded the possibility that there is a further unidentified lesion in both SC16 R_9C_2 and RSC-26 which contributes to the attenuation of in vivo growth. However, if such a lesion exists, it must be close to the DNA polymerase gene locus since other recombinants with the in vitro phenotype of RSC-26 are significantly attenuated [283].

Obviously many questions remain unanswered with regard to DNA polymerase mutants and they will remain so until many more mutants have been investigated. However, the data obtained with C1(101)P_2C_5 strongly imply that lesions in DNA polymerase need not be associated with decreased virulence and mutants containing such lesions are of potential clinical importance.

The properties of a series of ACV-resistant mutants isolated from the type 1 strains, SC16 and C1(101) illustrating many of the points discussed above are shown in Table 3.

Resistant isolates from humans

Although studies of the mechanisms which can confer drug-resistance, and investigation of the pathogenic potential of drug-resistant mutants are of value in attempting to assess the likely clinical importance of drug-resistance, ultimately, these only provide a background for the careful study of human isolates. It is only by looking at clinical isolates and monitoring the appearance and effects of resistant strains in individuals and in the population that we can properly assess the importance of the phenomenon.

IUdR has been in use for many years for the treatment of ocular infections and in that time there have been numerous reports of 'treatment failures' (see [183]) although few of these could be directly attributed to the appearance of resistant strains [63,129,137]. In the past year or so there have been several reports documenting the isolation of ACV-resistant mutants from patients undergoing therapy. In many, but not all cases so far reported, the patients had severely compromised immune systems, either resulting from innate immune deficiencies [34,73,243] or as a result of bone marrow transplantation [34,268], and this may have influenced the nature of the resistant isolates selected. Furthermore, there was no clear evidence that the resistant strains were contributing to the disease although this was certainly a possibility in some cases [73,87,243]. The results of a large scale study of isolates from patients receiving ACV therapy have been recently reported by the Burroughs Wellcome group, NC, U.S.A. [87]. They have examined multiple isolates from about 300 immunocompetent and immunocompromised patients, and found evidence for ACV-resistant viruses obtained from patients receiving either ACV or placebo. None of the

TABLE 3

Properties of ACV-resistant mutants of HSV

Virus	Phenotype	ACV-resistance (ED_{50} in BHK cells – μM)	TK induction (% *wt*)	LD_{50} in *mouse brain* (PFU)	Latency (% ganglia + ve)
SC16	*wt*	0.09	100	6	70–85
SC16 S6	TK^-	13	0[a]	$>10^6$	0
SC16 R_5C_1	TK^-	31	0	5×10^4	0
SC16 R_1C_1	'Low producer' TK	13	1.3	3×10^3	10
RSC-11	'Low producer' TK	13	5	2×10^2	R
SC16 Tr7	Substrate specificity TK	18	>100	50	35
SC16 S1	Substrate specificity TK	13	30–40	60	40
RSC-26	DNA Pol^r	1.3	100	1.3×10^3	R
SC16 R_9C_2	'Low Producer' TK, DNA Pol^r	160	5	$>10^5$	0
C1(101)	*wt*	1.3	100	1	100
C1(101)P_2C_5	DNA Pol^r	110	>100	8	80
C1(101)TK^-P_7	TK^-, DNA Pol^r	110	0	2×10^4	ND

[a] Values for uninfected BU-BHK (TK^-) cells subtracted.

R = virus recovered from latent ganglia but precise figures not available.

ND = Not determined.

Data from refs. [75,100,104,283].

patients from which ACV-resistant virus was isolated following therapy had recurrences with resistant strains, although two such patients had recurrences which gave ACV-sensitive virus. Four patients who gave relatively resistant pre-therapy isolates, however, had recurrences with similarly resistant viruses. This indicates that latent virus infection and subsequent reactivations in humans can occur with relatively resistant virus strains. It is interesting that they also found that some of the single isolates consisted of mixtures of *wt* and resistant viruses, observations which are similar to those made by Field [99], using a mouse infection model. The importance of these reports is that they are a clear demonstration that resistant strains can appear in humans undergoing ACV-therapy, and they are a warning that we can expect to see many more in the near future.

The extent of characterization of the resistant variants isolated to date has been far from adequate, leaving many important questions unanswered. Many isolates appear not to have been plaque purified and so some or all may be mixtures. Little can be said about the lesions conferring resistance except that all the viruses appear to have defects in TK function. Few attempts have been made to exclude the possibility of further defects in DNA polymerase. One of the isolates was shown to be PAA-sensitive [73] but since not all ACV-resistant DNA polymerase mutants are cross-resistant to PAA ([105,111], unpubl. results) this result is inconclusive. In three out of four studies it appeared that the method chosen to demonstrate a lesion in TK was to show that the isolates failed to induce an ACV-phosphorylating activity [34,243,268]. Unfortunately, this assay (using [^{14}C]ACV) does not distinguish between TK$^-$, 'low producer' and substrate-specificity mutants, any of which may be defective in ACV kinase activity [75,160,162]. The isolate described by Crumpacker et al. [73] is even more intriguing in that it expresses a low level [10%] of thymidine phosphorylating activity. The possibility exists that this is a 'low producer' mutant, however, since it does not appear to have been plaque-purified, it is equally likely to comprise a mixture of TK$^+$ and TK$^-$ viruses. Clearly, there is a need for more precise biochemical and genetic characterization of isolates from humans if we are to relate experience gained in tissue culture and in animal model systems to observations made in humans.

Conclusions

Experiments in vitro have revealed that herpes simplex virus can acquire resistance to antiviral drugs by two distinct strategies, either: i. reduction or loss of synthesis of the enzyme responsible for activating the inhibitor (i.e. TK) or ii. a specific change in affinity between the target (i.e. DNA polymerase) or activating enzyme (i.e. TK), and the inhibitor.

In the case of HSV, none of the other mechanisms of drug-resistance described for antibiotics or anti-cancer drugs have been so far recognised (see page 13). The first strategy for acquiring resistance (outlined above) results in a considerable decrease in the pathogenicity of the virus and is unlikely to yield variants able to compete successfully with wild type virus in the normal environment. Such variants may appear in individuals undergoing therapy and may influence the course of disease,

particularly if the individuals concerned are immunosuppressed, but they are unlikely to become established in the population. The second mechanism in contrast, can lead to variants showing little attenuation of in vivo growth potential or pathogenicity and thus variants derived by this mechanism may pose a more serious threat both to the individual and to the population. We may derive some consolation from the observations that in tissue culture systems mutants appear to arise by the first mechanism considerably more frequently than by the second [58,104,238,247]. It is to be hoped that the constraints operating in vitro to reduce the frequency of variants showing specific changes in enzyme affinities are equally strong or stronger in vivo. Even if this is the case we can only hope for a delay in their ultimate appearance. Finally, the way in which drugs are used could influence the appearance of resistant strains. The protracted use of low doses of a single drug might, for example, be more likely to result in the emergence of drug-resistance, than the use of combination therapy with drugs having independent modes of action.

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