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ANTIVIRAL MECHANISMS OF ACTION

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INTRODUCTION

Extensive efforts have been made in recent years to develop drugs for the safe and effective treatment of viral diseases. Such efforts have begun to yield promising results, with over ten compounds now utilized as antiviral drugs in clinics around the world (1). Despite such tentative successes, it is generally agreed that better antiviral agents are needed and should be designed, since toxicity of varying degrees, development of resistant viruses, inadequate spectrum of viral inhibition, and insufficient effect on established viral diseases are generally associated with the antiviral substances now in clinical use.

Research in recent years has begun to define details of biochemical differences between the normal host cell, the virus-infected cell, and the virus itself. Thus we should be in a position to take advantage of these differences in improving our success rate in antiviral drug development. Assistance in this drug design approach is provided by information now known of the

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general types of chemical structures which have antiviral activity and by exploiting mechanisms whereby that antiviral activity is exerted.

It is the purpose of this review to consider the major sites of attack of antiviral substances and to illustrate them with known compounds. Disadvantages or shortcomings of the example compounds, as well as the antiviral targets, are pointed out, where known. It is hoped that such a review will stimulate well-designed studies leading to the development of significant and acceptable new antiviral drugs.

THE PROBLEM OF MULTIFACETED MECHANISMS OF ACTION

In beginning this review, it is important to recognize that many antiviral agents, including some now with degrees of clinical utility, have more than one apparent mechanism of action. Thus the issue of defining exactly which mechanism is antiviral and which may result in a toxic reaction is often clouded.

An example of this problem is seen with the drug 9-β-D-arabinofuranosyladenine (ara-A, Vidarabine®), now being marketed in the United States for treatment of herpes eye infections and for parenteral therapy of herpes encephalitis. Ara-A is readily converted into its corresponding mono-, di-, and triphosphates in the cell (2), although available evidence indicates the compound also is rapidly degraded to hypoxanthine (ara-Hx) (3, 4) which may in turn be converted to its corresponding mono-, di-, or triphosphates (5) or may be further degraded to xanthine and uric acid. It is generally accepted that the antiviral effect of ara-A is the preferential inhibition of DNA-dependent DNA polymerase of herpes virus by ara-ATP (6). Ara-ATP is also a competitive inhibitor, at higher concentrations, of cellular DNA polymerases α and β which is one reason for the drug's inhibition of cellular proliferation (6–9). However, ara-A is incorporated into the DNA of cells and of herpes virus (10, 11). In cells, it is found in the internucleotide linkage (12, 13), and in the virus it appears in terminal positions resulting in incomplete viral DNA chains (10). This incorporation into herpes viral DNA was not seen by other investigators under different conditions (14), further confusing the situation. Ribonucleotide phosphate reductase, considered by many to be a key enzyme in DNA synthesis, is also inhibited by ara-A di- and triphosphate (15, 16). Ara-ATP also inhibits terminal deoxynucleotidyl transferase (7, 17). Finally, ara-A itself has been shown to be an apparent suicide inactivator of human lymphoblast *S*-adenosylhomocysteine hydrolase, an action thought to be one reason for the cytotoxicity exerted by ara-A (18). Thus at least six enzymes are known to be inhibited by this drug or one of its metabolites. At this point we can only

make qualified postulates concerning which action results in antiviral activity and which causes cytotoxicity.

Another example of a clinically active compound having a multifaceted mechanism of action is the triazole nucleoside, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin). Ribavirin has a broad spectrum of antiviral effects, inhibiting DNA and RNA viruses in vitro and in vivo, and, in limited human trials reported to date, is efficacious against several viral diseases in man, including influenza, hepatitis type A, herpes zoster and progenitalis, and measles (for review see 19). The metabolites of ribavirin include its 5'-mono-, di-, and triphosphates, the deribosylated base, and the carboxylic acids of the nucleoside and of the free base (20). Ribavirin-5'-monophosphate (RMP) is a competitive inhibitor of inosine monophosphate (IMP) dehydrogenase (21), an action now thought to be related to the cytotoxicity exerted by the compound, particularly the immunosuppression seen at high prolonged dosages (31). It also inhibits GMP synthesis (23). Ribavirin itself appears to be a strong inhibitor of cellular thymidine phosphorylation (22). Ribavirin-5'-triphosphate (RTP) has been shown to be a selective inhibitor of influenza virus RNA polymerase (24), although it does not inhibit various cellular RNA polymerases (24, 25). The influenza viral RNA polymerase inhibition is particularly important, since influenza virus is one of the most sensitive to the effects of the compound. Further, it has also been shown (24) that the influenza virus RNA polymerase is stimulated by guanosine-containing nucleotides which are incorporated into the 5' termini (26). Indeed, evidence exists that ribavirin is incorporated into the 5' "cap" of messenger RNA in place of guanosine (27). Ribavirin assumes a conformation strikingly similar to guanosine, as determined by X-ray crystallography (28). In studies with vaccinia virus, ribavirin was found to prevent the polypeptide coating of viral DNA, resulting in formation of incomplete viral particles (30), and it also appears to inhibit selectively the synthesis of the influenza viral polypeptides (29).

An important characteristic shared by both ara-A and ribavirin is the absence of development of resistance by viruses to these agents. This is a major attribute for an antiviral drug, which probably results from its multifaceted mechanism of action.

VIRUS-INACTIVATING AGENTS

Several chemical agents are known which exert rather intriguing antiviral activity by directly inactivating the virus. Calcium elenolate, a monoterpine obtained from acid-hydrolyzed aqueous extracts of various parts of the olive plant, exerts virucidal action in vitro against a variety of RNA and DNA viruses, apparently by interaction with the protein coat of the virus particle

(32). In an animal study, intranasal application reduced parainfluenza virus yields without significant toxicity (33, 34). Human trials with this compound indicated efficacy only if treatment began very early after virus exposure (H. E. Renis, personal communication).

3-Ethoxy-2-oxobutyraldehyde hydrate (kethoxal) has an in vitro antiviral effect against DNA and RNA viruses (36–38), and topical application affects upper-respiratory parainfluenza virus infections in hamsters (38) and cutaneous herpes virus infections in mice (39). The compound is a potent inactivator of extracellular virus, although it also inhibits intracellular virus multiplication (38). A double-blind trial against type 1 herpes virus–induced lip lesions showed efficacy only against very early infections (40).

Certain dihydroisoquinolines have exhibited an inactivating effect on influenza A and B viruses and on parainfluenza virus; a strong antiviral effect was seen against these viruses in cell culture studies, and moderate activity was then seen in animal experiments. The compounds failed to achieve the required antiviral effect in humans, however (41–46).

Several light-sensitive dyes also inactivate viruses; these include proflavine, neutral red, and acridine orange. During replication of the virus, these dyes become incorporated within the virus structure, apparently by intercalation between the stacked bases of the viral DNA. The virus is inactivated when the dye-DNA complex absorbs sufficient light of defined wavelength to produce a degradation reaction. Such degradations result in loss of guanine, gaps in the base sequence, and subsequent strand breaks in the viral DNA (for review see 47). Proflavine is the dye currently recommended for human studies (47). Clinical studies using such light-sensitive dye treatment have been reported for herpes virus infections of the genitals, eye, oral cavity, lip, and skin, with varying degrees of success reported (47). Careful scrutiny of the results indicates that success depends on careful attention to methods, such as treatment of new lesions, removal of the tops of the vesicles, adequate exposure of the vesicle to the dye, use of light of the proper wavelength and for sufficient time.

Concern has been expressed that photoinactivation of herpes viruses may be clinically hazardous because the inactivated virus is capable of transforming normal nonhuman cells to a malignant state (48); the dyes, which possess a strong affinity for cell nuclei, also have the capability of damaging their genetic makeup (49, 50). To date, however, proflavine itself has not been shown to be carcinogenic (47, 51). Since herpes viruses also may play an etiologic role in human cancer, especially in those cases where the infection induced by the virus persists throughout the life of the patient, as occurs with herpes genitalis, a dilemma ensues. Should one tolerate a continuous infection which is potentially carcinogenic or attempt to cure the infection with light-sensitive dyes involving the as yet unconfirmed risk of cancer due to the treatment itself?

The overall concept of a virus-inactivating agent would appear meritorious if the substance used is sufficiently selective for the virus. Disadvantages to such agents include the need for topical application of the inactivating agent, which limits to a degree the viral infections which may be treated, and the apparent need for very early treatment before the viral infection is well established.

INHIBITION OF VIRAL ATTACHMENT, PENETRATION, AND UNCOATING

As the virus initially infects a eucaryotic cell, several general stages in the infection process occur which are potential sites of attack by antiviral drugs. In these stages, the infecting virion attaches to receptors of the cell membrane, penetrates the cell membrane, and, once in the cytoplasm of the cell, the virion's protein coat is removed, releasing the viral nucleic acid.

Viral attachment or adsorption has been the least effective site for attack by antivirals, with no substances yet found sufficiently active to warrant clinical testing. A sulfated polysaccharide has been reported to interact with virus particles, causing a decrease in their rate of attachment to cells in vitro (52). Viruses affected include encephalomyocarditis, echo, influenza, dengue, and rabies. Moderate in vivo activity was also seen against dengue virus in mice. Heparin, a negatively charged mucopolysaccharide, apparently forms a noninfectious complex with herpes virus which prevents it from attaching to the host cell (53, 54). Activity has been seen against the herpes virus both in vitro and in animal experiments, the latter utilizing the injection of heparin into rabbit skin prior to or simultaneously with infection. Because of the ionic nature of the interaction, it is likely that heparin would have a considerable degree of nonspecificity.

Inhibition of viral penetration and/or viral uncoating has proven to be an acceptable mechanism of antiviral action, since 1-adamantanamine HCl (amantadine), a relatively nontoxic, clinically effective antiinfluenza virus drug, apparently acts at both sites (55–61). Recent work by Oxford, Patterson & Dourmashkin (62) indicates, however, that amantadine may act instead by inhibition of viral polypeptide synthesis. A number of compounds possessing structures based on the *trans*-decalin nucleus also appear to act by blocking viral penetration into the cell (63–65). Significant in vitro anti-influenza virus activity with little associated cytotoxicity has been seen using a number of these decalin derivatives, but no effect was seen in vivo, possibly due to rapid metabolic breakdown in the animal (64). 3,4-Dihydro-1-isoquinolineacetamide HCl similarly appears to act by delaying viral penetration into cells (66); this compound has exhibited in vivo activity against influenza A, echo, Columbia SK, and herpes viruses (67, 68). Chlo-

roquine, a relatively ineffective antiviral agent, has been reported to inhibit the uncoating of Newcastle disease virus (69), although this uncoating may be related to effects of the compound on cellular membranes (70).

Thus with the possible exception of amantadine, these sites of attack by antiviral compounds have not proven of practical use. In a very interesting study (35), the combination of amantadine and ribavirin showed considerable synergy against influenza both in vitro and in vivo.

INHIBITORS OF VIRION-ASSOCIATED ENZYMES

DNA Polymerases

A large number of substances are believed to have antiviral activity as a result of inhibition of virion-associated DNA polymerases. Antiviral agents of this type can be broadly grouped into analogues of pyrophosphate and analogues of natural nucleoside polyphosphates. The latter group is often modified only in the sugar moiety or in the purine or pyrimidine portion of the molecule, but rarely in both. There are two interesting compounds in the first category: trisodium phosphonoformate (PFA) and trisodium phosphonoacetate (PA). PFA inhibits herpes simplex virus type I DNA polymerase 50% at 3.5 μM (71). Its effect on eucaryotic DNA polymerase α can be overcome by increasing the amount of enzyme. Cell proliferation (HeLa cells) required greater than 100 μM PFA in the medium to achieve 50% inhibition (71). PFA is most active in vitro against the DNA-containing viruses herpes simplex virus 1 and 2 and pseudorabies virus (71). Like PFA, PA appears to be a potent inhibitor of herpes simplex virus–induced DNA polymerase (72, 75), but it has no effect on the host cell (WI-38) polymerase (73, 75). In a very detailed kinetic study of the polymerizing and the pyrophosphate nucleoside triphosphate exchange reactions using the turkey herpes virus–induced DNA polymerase, Leinbach et al (74) showed that PA interacts with DNA polymerase at the pyrophosphate binding site. Overby et al (75) demonstrated that resistance of virus to PA was directly correlated with the same relative resistance of the corresponding cell-free DNA polymerase.

In an interesting report, Öberg & Helgstrand (76) have shown that ribavirin triphosphate (RTP) is an inhibitor of herpes virus DNA polymerase, showing 35% inhibition at 100 μM. More recently, Y. C. Cheng (personal communication) has reinvestigated this inhibition, and using 0.5 mM RTP was unable to show inhibition of the DNA polymerase of either herpes virus types 1 or 2. While ribavirin has been reported as a potent inhibitor of DNA synthesis in a number of systems, this inhibition may be indirect (22) (see below) and is possibly related to its effects on the cellular total deoxynucleotide pool.

Arabinosyl nucleotides, particularly ara-A and arabinosyl cytosine (ara-C), as their triphosphates, are very strong inhibitors of a number of deoxynucleotide polymerizing enzymes (7). Earlier studies (13) have clearly established that ara-A is phosphorylated intracellularly and primarily exists as the triphosphate, although it is rapidly deaminated to inosine as well (86). A number of studies recently reviewed and carefully analyzed by Müller (6) reveal that herpes simplex virus–induced DNA polymerase is at least 40-fold more sensitive to ara-ATP than are the host cell DNA polymerases α and β. In a very careful study, DiCioccio & Srivastava (7) showed that ara-ATP and ara-CTP inhibit terminal deoxynucleotidyl transferase competitively with respect to any deoxynucleotide substrate and inhibit DNA polymerases α and β from a number of human cells competitively with respect to their analogue substrates (dATP for ara-ATP and dCTP for ara-CTP); noncompetitive inhibition is seen with respect to nonanalogous deoxynucleoside triphosphates. Neither compound is inhibitory to DNA polymerase γ. In a very interesting study, Le Page (77) has shown that an ara-A–resistant human leukemia shows no inhibition of DNA synthesis in cell extracts by ara-ATP, and in two lines of Gardner lymphosarcoma, which showed very different sensitivity to ara-A, DNA synthesis in cell extracts of the more sensitive lines showed very much greater inhibition by ara-ATP.

Trifluorothymidine (5-trifluoromethyl-2'-deoxyuridine dF_3T) is readily phosphorylated by thymidine kinase in intact eucaryotic systems and further phosphorylated to both the 5'-di- and 5'-triphosphate (78). dF_3TTP is a competitive inhibitor (versus dTTP) of both the α and β DNA polymerases of HeLa cells and a very much more powerful inhibitor of the vaccinia virus–induced DNA-dependent DNA polymerase (79). Müller (6) also states that the herpes simplex virus–induced DNA polymerase is competitively inhibited by dF_3TTP with respect to dTTP.

9-(2,2-hydroxyethoxymethyl)guanine(A-cycloguanosine, acyclo-G) is a guanosine analogue which possesses marked antiviral activity in animal models of herpes virus infections (80). It is phosphorylated in extracts of viral cells infected with herpes simplex virus at 30 to 120 times the rate that the same phosphorylation takes place in uninfected cells (81). This rapid phosphorylation was attributed to the viral-induced thymidine kinase, since herpes mutants lacking this enzyme did not produce the same effect (81). Chen & Prusoff (82) have shown that thymidylate kinase and pyrimidine deoxynucleoside kinase induced by herpes simplex virus are associated with the same protein moiety, and therefore one would expect monophosphates produced by thymidine kinase to be readily phosphorylated to the triphosphate level. Indeed, Elion et al (83) have shown that acyclo-GTP does accumulate in herpes simplex type 1–infected vero cells to more than 30-

fold the level found in uninfected cells, and further that it is a competitive inhibitor to dGTP with DNA polymerases. The authors report (83) that the K_i of acyclo-GTP for the herpes simplex virus type 1 DNA polymerase is only 0.08 μM, whereas that for the HeLa cell DNA polymerase α was 2.1 μM. Thus with the viral selectivity observed, it would be expected that viral DNA chains would terminate upon incorporation of acyclo-GMP.

The inhibitory effect of 5-iodo-2'-deoxyuridine triphosphate (IdUTP) on DNA polymerase has been demonstrated (84), but the main antiviral effect of 5-iodo-2'-deoxyuridine (IU) is believed to be exerted after its incorporation into and formation of nonfunctional DNA (85).

A number of derivatives of rifamycin are known to inhibit RNA-directed DNA polymerases in cell-free systems (87), but their overall antiviral activity may be ascribed to other intracellular interactions. Since several significant antiviral substances, particularly PFA, ara-A, and acyclo-G, appear to act against DNA viruses primarily by inhibition of virion-associated DNA polymerases, this enzyme would appear to be a key target for design of new chemotherapeutic agents.

RNA Polymerases

A number of substances are known to inhibit DNA- and RNA-directed RNA polymerases in vitro, and, in some cases, this activity is thought to account in part for antiviral action. For example, in a very careful study, Eriksson et al (24) have shown that ribavirin triphosphate (RTP), a quantitatively important intracellular form of ribavirin (88), is a strong inhibitor of the influenza virus–induced RNA polymerase. The inhibition of the viral polymerase is competitive with respect to ATP and GTP, but not with UTP or CTP. The influenza viral RNA polymerase is known to be remarkably stimulated by dinucleotides containing guanine, and Plotch & Krug (89) showed that ApG or GpC were incorporated into the 5' end of the influenza A cRNA. Eriksson et al (24) found that RTP abolished the enhancement of the viral polymerase by ApG and GpC. It is possible that this observation may account for the very great sensitivity of influenza A viruses to ribavirin (90). Eriksson et al (24) made the further important observation that RTP inhibition of influenza A virus RNA polymerase is selective since a variety of other bacterial and eucaryotic RNA polymerases were essentially insensitive to effects by this substance. Müller et al (25) also showed that RTP does not inhibit eucaryotic RNA polymerases I and II, and is without effect on eucaryotic poly(A) polymerase.

Phosphonoformate (PFA) is also known to inhibit the influenza A RNA polymerase (71). This study reveals 50% inhibition of the enzyme at 20 μM PFA, and it appears to be quite selective since calf thymus RNA polymerases I and II and *E. coli* RNA polymerase are relatively insensitive

to the substance, showing 50% inhibition at greater than 500 μM. It is disappointing that PFA was only inhibitory to influenza virus at a high concentration (500 μM).

An interesting compound, gliotoxin, first isolated in 1936 [see (91) for recent review], was shown to inhibit completely the synthesis of polio virus RNA (92), presumably by formation of a disulfide bond (93) with an essential sulfhydryl group in the viral RNA-dependent RNA polymerase.

A recent study by Smith et al (86) employing herpes simplex virus type 1 in KB cells has clearly shown that deoxyadenosine, but not adenosine, reverses the antiviral effect of ara-A, but requires the presence of coformycin, a potent adenosine deaminase inhibitor. Reversal by deoxyadenosine strongly implicates ara-A in processes involving DNA rather than RNA. Nonetheless, in other systems, for example ara-A inhibition of DNA synthesis in TA3 cells (94), reversal is seen with adenosine but not deoxyadenosine. Thus, ara-A or its triphosphate may also be expected to interfere in RNA synthesis or processing. In a very interesting report, Rose & Jacob (95) have found that ara-ATP inhibits poly(A) synthesis in rat liver chromatin, but shows no effect at the same concentration on RNA synthesis in chromatin. The K_i for ara-ATP for the chromatin enzyme was 4 μM, and for the cell sap enzyme was 60 μM. Thus, Rose & Jacob (95) conclude that ara-ATP inhibits the initiation of polyadenylylation, but elongation of poly(A) chains is not so potently inhibited. These authors also point out that DNA viruses must depend on nuclear poly(a) polymerase for the polyadenylylation of viral-specific mRNA produced after infection, and thus inhibition of poly(A) synthesis could well block processing of viral-specific mRNA and subsequently the synthesis of viral-specific proteins. As a consequence, viral replication is arrested.

Cordycepin (3'-deoxyadenosine, 3'-dA) is an inhibitor of oncornavirus induction and of herpes, Semliki forest, western equine encephalitis, Newcastle disease, and reoviruses (91). 3'-dA is an adenosine analogue which is readily phosphorylated to its mono-, di-, and triphosphate intracellularly (96), and which is thought to be a highly specific inhibitor of synthesis of those RNA species which contain poly(A) (97). Müller and his associates (98) have studied the effect of 3'-dATP on the DNA polymerases α and β found in L5178 Y cells and report no inhibition. They do report a moderate inhibition of RNA polymerases I, II, and III from mouse liver with the K_i ranging from 40 to 75 μM. Much more significantly, the K_i for 3'-dATP with the oviduct poly(A) polymerase was 7.3 μM, and for the calf thymus terminal riboadenylate transferase, it was 16 μM. These K_i values are sufficiently below the K_m value for ATP in these systems that they appear quite significant in terms of the mode of action of the drug. Legraverend & Glazer (99) have suggested that since phosphorylation of

nonhistone chromosomal proteins is inhibited by 3'-dATP this inhibition may affect transcription. Alternatively, Beach & Ross (100) have shown that 3'-dA inhibits newly synthesized mRNA in mouse fetal erythroid cells, and they further hypothesize that there may be a fourth type of RNA polymerase resistant to 3'-dATP but insensitive to α-amanitin.

Deoxypyrimidine Nucleoside Kinase and Thymidine Kinase

There are at least two very different ways by which deoxypyrimidine kinases of viral origin could be inhibited; the first, of course, is direct competition with the natural substrate, and the second is inhibition of the catalysis by allosteric modulators. Jamieson et al (101) demonstrated that herpes simplex virus types 1 and 2 induce a kinase able to phosphorylate both thymidine and deoxycytidine, while pseudorabies virus and vaccinia virus induce a kinase only able to phosphorylate thymidine. Kit et al (102, 103) have carefully characterized the herpes virus kinase, and in some properties, particularly phosphoryl donor specificity, liken it to the human and mouse mitochondrial enzyme; however, the viral thymidine kinases are not inhibited by dCTP. All the thymidine kinases investigated (103) were feedback inhibited by dTTP. Cheng et al (104) have shown that a number of thymidine analogues possess antiviral activity, but only if the herpes simplex virus strain can induce thymidine kinase, and De Clercq et al (105) have shown that some of the same thymidine analogues are remarkably selective for herpes viruses. In a very careful study, Cheng (106, 107) has shown that a number of 5-substituted deoxyuridine derivatives are excellent inhibitors of herpes simplex virus types 1 and 2 thymidine kinase. 5-IdU, 5-BrdU, 5-vinyl-dU, 5-allyl-dU, 5-ethyl-dU, and 5-propyl-dU are excellent inhibitors of both thymidine kinases, while 5-IdC and 5-BrdC are more potent competitors only with the herpes simplex type 1 thymidine kinase. A number of the above compounds were active inhibitors of either herpes simplex type 1 or herpes simplex type 2 replication, but not of a mutant herpes simplex virus type 1 lacking the ability to induce thymidine kinase. These compounds are presumably phosphorylated and because of the reported association of the herpes- induced deoxypyrimidine nucleoside kinase and thymidylate kinase (82) are probably present in the cell as di- and triphosphates, in which form they might be expected to exert other influences on nucleotide processing systems and may be powerful modulators of thymidine kinase. Oddly enough, acyclo-G is phosphorylated by thymidine kinase (81), but it is a poorly competing substrate with thymidine and probably exerts its antiviral effect when phosphorylated at the DNA synthesis level.

In an interesting series of papers, Lin et al (108) have achieved the synthesis of 5-iodo-5-amino-2',5'-dideoxyuridine (IaddU) and shown it

(109) to be a potent inhibitor of herpes simplex virus type 1 replication, but also exhibiting little, if any, cellular toxicity. This group also showed (110) that IaddU became incorporated into the nucleotide pools and into DNA only when cells were infected with herpes simplex virus type 1. IaddUTP was shown (111) to be a 60-fold more potent feedback inhibitor of *E. coli* thymidine kinase than is dTTP at pH 7.8. These studies would presumably extrapolate to the viral enzymes, since all the isozymes of thymidine kinase so far examined have shown dTTP regulation (102).

Gentry & Aswell (112) have shown that 1-β-D-arabinosylthymine (ara-T) is a selective inhibitor of herpes simplex virus types 1 and 2 replication in hamster cell cultures. Miller et al (113), employing only compounds that are activated in virus-infected cells as a means to increase antiviral selectivity, have shown that ara-T is phosphorylated by herpes simplex virus type 1–infected, but not–uninfected, cells, and further, ara-T was not effective with herpes simplex virus strains lacking thymidine kinase. Similar results were shown with varicella zoster virus replication (113) and again attributed to ara-T phosphorylation by deoxypyrimidine kinase which has both directly (115) and indirectly (116) been shown to be induced by this virus. Having demonstrated that ara-T depends on virally induced thymidine kinase to show its antiviral effects, Aswell et al (117, 118) have also shown that 5-methylarabinosylcytosine (4-amino-arabinosylthymine, aminoara-T) is a very effective antiherpetic agent in cells that contain deoxycytidine deaminase, which would allow its conversion to ara-T.

Like pyrazofurin (119), ribavirin is phosphorylated intracellularly by adenosine kinase (120) rather than thymidine kinase. However, although both show broad-spectrum antiviral activities (121), their modes of antiviral action are very different. De Clercq & Torrence (123) have recently discussed the data showing that pyrazofurin-5'-phosphate inhibits host cell orotidine-5'-phosphate decarboxylase (122). Pyrazofurin is extremely toxic in animals (123). These authors (123) have also summarized the inhibitors of host cell thymidylate synthetase. Nonetheless, while ribavirin is phosphorylated intracellularly, it clearly has a marked inhibitory effect on the phosphorylation of thymidine (22). In subsequent work (J. C. Drach, personal communication; Y. C. Cheng, personal communication), it has been shown that neither RMP nor RTP nor ribavirin alone has an inhibitory effect on thymidine kinase. It may be possible thus to account for this marked decrease in thymidine phosphorylation by another form of inhibition of thymidine kinase thus far unknown. While the experiments of Drach et al (22) clearly show the inhibition of [^{3}H]thymidine phosphorylation in KB cells treated with ribavirin, it was simultaneously shown that these cells were synthesizing DNA by independent incorporation of inorganic $^{32}P_i$. Thus, earlier conclusions indicating that ribavirin inhibited DNA synthesis

(124, 125) which depended on the incorporation into DNA of [^{3}H]thymidine via its phosphorylated form may be very much in error and must now be reevaluated.

Inhibition of viral-associated deoxypyrimidine nucleoside kinases has thus been a target for antiviral agents which is both popular and of considerable merit since relatively selective effects are seen. A problem which appears, however, is the ease with which affected viruses can develop resistance to such agents (114).

Viral Neuraminidase

While different points of view exist concerning the role of virion-associated neuraminidase, whether in penetration or assembly, it is clear that the severity of influenza symptoms increases in volunteers with decreasing concentration of serum antineuraminidase antibody (126). 2-Deoxy-2,3-dehydro-*N*-trifluoroacetyl neuraminic acid is an influenza virus inhibitor (127). It apparently prevents the enzymatic removal of neuraminic acid from the virus envelope leading to extensive aggregation of virus particles and ultimately to inhibition of viral replication (128, 129).

mRNA Guanylyltransferase and mRNA Methyltransferase

It was recently clearly established that a number of viral and eucaryotic mRNAs contain a 5' terminal "cap" structure consisting of a 7-methylguanosine residue linked from its 5' position via a triphosphate bridge to a 2'-*O*-methylribonucleoside (130), and enzymes capable of synthesizing "cap" structures are found in vaccinia and reovirus cores (131, 132). Recent reviews of this area (130–132) show that a number of RNA and DNA-containing viruses possess "cap" structures, but notably polio virus does not (130), and since ribavirin is not active against polio virus, its effect on the capping process was investigated. Goswami et al (27) showed that RTP is an excellent competitive inhibitor of the vaccinia virus mRNA guanylyltransferase ($K_i = 32\ \mu$M and K_m for GTP $= 22\ \mu$M). It was further shown that in the absence of GTP, RTP at 1 mM inhibited the methylation of vaccinia mRNA, although sinefungin (133), an antifungal agent, is much more effective. Studies by Oxford (29) have shown that influenza virus–coded peptides are less readily synthesized in the presence of ribavirin, while the host kidney cell peptide synthesis is not altered. This observation may be ascribed to altered viral RNA synthesis or "cap" formation. More recently, however, Bouloy et al (134) have shown that influenza virus replication in reticulocytes scavenges the 5' "cap" plus about 15 terminal nucleotides from preformed globin mRNA (135). It is also clear that influenza virus replication requires simultaneous synthesis of host cell mRNA and thus depends on host capping mechanisms.

INHIBITORS OF THE TRANSLATIONAL PROCESSES OF VIRAL mRNA

mRNA Translation

Translation of a number of mRNAs in the wheat germ system has been shown to be inhibited by 7-methylguanosine-5'-monophosphate (m^7-GMP) (136–139), but guanosine nucleotides lacking the 7-methyl group, or bearing more methyl groups (139), are ineffective. Interestingly (138), m^7-GMP inhibits even noncapped satellite tobacco necrosis virus RNA translation in the wheat germ system, suggesting that the "cap" binding site may be part of another recognition site. In structural studies using reovirus mRNA in the wheat germ system, Adams et al (140) showed that 7-methyl, 7 ethyl, or 7-benzyl groups were essential, and further, that the amino group in position 2 of guanine was important in the binding process. In the vesicular stomatitis virus mRNA translation, Lodish & Rose (141) have shown that m^7-GMP and 7-methyl-guanosine-5'-triphosphate-5'-nucleoside-phosphate yield 90% inhibition in the wheat germ system, but only 20% in the reticulocyte system. In a later study, Bergmann & Lodish (142) determined that at low K^+ concentrations wheat germ ribosomes bound and translated appreciable quantities of vesicular stomatitis virus mRNA. They also conclude that under any reaction conditions translation of the mRNAs in the reticulocyte system is less dependent on the 5' "cap."

Nascent Viral Polypeptide Chains

Parafluorophenylalanine (pFPhe) was first used as an amino acid analogue in 1951 (143) and subsequently shown to have antiviral activity against a wide range of RNA and DNA viruses (91). Its mode of action appears to be replacement of phenylalanine in proteins leading to defective viral peptides, and thus to prevention of viral maturation (144).

In a recent, very novel approach, Carrasco (145) has shown that virally infected cells become leaky, and thus β-γ-methylene-GTP enters those cells (but not normal cells) thus inhibiting the protein synthesis of encephalomyocarditis virus in 3T6 cells. In further work (169), it has also been shown that other translation inhibitors are similarly taken up by a number of virally infected cells, but not by normal cells.

Two thiosemicarbazones [1-methylisatin-3-thiosemicarbazone(methisazone) and isatin-β-thiosemicarbazone(isatin)] are effective therapeutic agents in man against vaccinia infection (146) and as a prophylactic agent against smallpox (147). A study of these agents has shown that late mRNA (synthesized 3 hr post vaccinia virus infection) is not normal and that the proteins programmed from it are not expressed (148). However, Katz et al (149) believe that it is the cleavage of late poststructural proteins that is

inhibited by isatin. More recently, Fox et al (150) and Levinson et al (151) have shown that thiosemicarbazones, as their Cu^{2+} chelates, bind to double-stranded and single-stranded DNA and RNA, thus presumably perturbing transcriptive and translational processes.

INHIBITORS OF THE SYNTHESIS OF VIRAL DNA

Many of the compounds that inhibit viral DNA synthesis may do so by direct inhibition of polymerases (and have been covered in a prior section), or they may act by interference in the synthesis of precursors, or by incorporation into DNA leading to faulty material, or finally by interaction with the templates so as to block DNA replication.

Compounds Incorporated into DNA

The incorporation of 5-IdU into viral DNA in place of thymine and the subsequent fragility and mispairing of this DNA recently has been reviewed (6), and even more extensively covering a wider range of halogenated deoxypyrimidine nucleosides (152). Incorporation of these substances may lead to nonfunctional DNA, thus disturbing the flow of genetic information. In addition, there are other deoxythymine analogues incorporated into DNA which have been carefully reviewed by De Clercq & Torrence (123). It is very interesting that 5-AIddU is incorporated into herpes simplex DNA (111), and the authors point out the well-known acid lability of the P–N bond thus formed. But the biological effect of this incorporation may be much more complex, since DNA is not normally exposed to acid in nature. Ara-AMP is incorporated into DNA of herpes simplex virus (16) and also into the DNA of L5178 Y cells (12) and mouse fibroblasts (13). Generally the arabinosyl nucleotide is found in internucleotide linkage in cellular DNA (12, 13) and in terminal positions in herpes virus DNA (10). Thus it acts as a chain terminator in newly synthesized herpes chains. Ara-CMP is also incorporated into DNA (153, 154), but not into RNA. It unfortunately inhibits DNA synthesis to a greater extent in uninfected cells than in herpes simplex–infected cells (125). Acyclo-G also appears to be incorporated into DNA where it presumably acts as a chain terminator (83).

Inhibitors of Nonviral Enzymic Processes Involving DNA Synthesis

Among the processes that could alter the rate or extent of DNA synthesis, antiviral agents chiefly affect thymidylate synthetase and deoxynucleoside triphosphate pool sizes either directly or indirectly. A large number of deoxyuridine derivatives show a powerful inhibition of TMP synthetase; for example, the 5'-monophosphates of 5-fluorodeoxyuridine, 5-trifluoromethyldeoxyuridine (155), as well as 5-iodoacetamidomethyldeoxyuridine

(156) and 5-ethyldeoxyuridine (157). But a very significant assay which permits detection of TMP synthetase inhibitors in vivo has been developed by De Clercq et al (158) in which nucleoside analogues are screened for their ability to block incorporation of deoxyuridine, but not deoxythymidine, into DNA. 5-Fluorodeoxyuridine, 5-trifluoromethyldeoxyuridine, 5-thiocyanodeoxyuridine, 5-cyanodeoxyuridine, and 5-nitrodeoxyuridine were among the compounds so classified, and their antiviral effects were readily reversed with thymidine (158).

Another point of attack is at the level of perturbation of nucleotide pools. For example, ribavirin, which is readily phosphorylated intracellularly (159, 160), is, as its monophosphate, a powerful inhibitor of IMP dehydrogenase, having a competitive K_i of 2.7×10^{-7} M, while that of GMP, the natural regulator of that enzyme, is 7.7×10^{-5} M (21). Clearly, ribavirin causes perturbation of the nucleotide pools, not only decreasing the dGTP pool size in L5178 Y cells by more than 50%, but also oddly increasing the dTTP pool size by greater than 100% (161) and showing no real effect on dATP and dCTP pools. Similarly, the ribonucleoside triphosphate pools, as shown by Zimmerman & Deeprose (88), are highly altered with GTP declining by 50%, and UTP and CTP increasing significantly. It is difficult to imagine that these perturbations in the nucleotide pool levels as a result of administration of ribavirin could totally account for its antiviral activities, since, if that were the case, one would expect it to show no viral selectivity or specificity.

Interactions with Template

There are a number of substances that interact with DNA and, as a result of intercalation, effectively block DNA replication. While many of these substances have been shown to be active antiviral agents, most also affect host-cell DNA replication as well. Müller (6) has recently reviewed these substances with respect to their antiviral activity, and Kersten & Kersten (162) have recently completed an excellent review of these agents. Daunomycin and adriamycin are very similar in action and inhibit only the DNA viruses, particularly herpes simplex virus and vaccinia virus (163), and oncogenic RNA viruses, which replicate via a DNA intermediate (6). Both of these substances are considered intercalating agents (164), and both inhibit nucleic acid synthesis involving DNA templates and, as a result, are quite generally toxic.

Actinomycin also appears to be an intercalating agent preferring dG-C-rich regions of the DNA. It is active against DNA, but not RNA, viruses with the exception of the oncogenic RNA viruses (165).

Distamycin is a powerful antiviral against DNA-containing viruses (166), and possibly the oncogenic RNA viruses (6). It is not selective, since it also severely blocks cell growth. Distamycin strongly interacts with dA-T-rich

regions of helical DNA (167), and only weakly with single-stranded DNA or RNA. It does not inhibit RNA-dependent polymerase reactions (168).

INHIBITORS OF THE BIOSYNTHESIS OF VIRAL GLYCOPROTEINS AND ASSEMBLY

A variety of both DNA and RNA viruses contain envelopes into which virus-coded glycopeptides are incorporated, suggesting another possible target for antiviral drugs. Influenza virus contains hemagglutinin 'spikes," which are the major glycoprotein component of the virus envelope, and which are responsible for the attachment of the virus particle to host cell receptor sites (reviewed in 170, 171). Another important membrane component in influenza virus is the enzyme neuraminidase (N-acetylneuraminate glycohydrolase), located on the surface of the virus, and, like hemagglutinin, it appears to attach to the lipid membrane of the virus (reviewed in 1972). The effects of compounds on neuraminidase are discussed in an earlier section.

2-Deoxy-D-glucose and D-glucosamine inhibit the formation of viral hemagglutinin and neuraminidase (173), and therefore prevent the formation of mature virions. In cell culture, 2-deoxy-D-glucose also inhibits Newcastle disease virus (174), herpes virus (175), respiratory syncytial virus (177, 178), Semliki forest virus (176), sinbis virus (176), parainfluenza type 3 virus (177), and measles virus (178). In vivo activity has been seen against herpes and influenza infections (179, 180, 204). D-Glucosamine also reversibly inhibits respiratory syncytial virus and parainfluenza type 3 virus in vitro (177). 2-Deoxy-D-glucose has been successfully used in human trials against herpes genitalis (204). The presence of D-glucose or D-mannose in the culture medium reversed the action of 2-deoxy-D-glucose (174, 177). 2-Deoxy-D-glucose produced virions whose surface projections were greatly reduced. Both compounds inhibit the glycosylation of viral glycoproteins in the infected cells (181), and it has been postulated that fusion of the virus with the host cell membrane subsequently cannot take place. With Semliki forest virus, guanosine diphosphate-2'-deoxy-D-glucose (GDP2dGl) has been found to be the agent responsible for inhibition of glycosylation of viral glycoproteins when 2-deoxy-D-glucose is added to the growth medium, causing a block in the synthesis of infective particles by preventing membrane glycosylation (182). GDP2dGl prevents the natural GDP-mannose which accumulates under these conditions from participating in mannosylation of the viral glycoprotein. Mannose has been shown to be the largest carbohydrate component of viral glycoprotein of sindbis virus (183). In glucose-containing medium, D-glucosamine continues to inhibit the synthesis of glycoproteins of fowl plague virus and Semliki forest virus even

though multiplication of the virus does not cease (184). It has been suggested that D-glucosamine might interfere with the activation of other sugars involved in the synthesis of viral glycoprotein via depletion of the UTP pool. In fructose-containing media, D-glucosamine depletes the cells of UTP to such an extent that it becomes rate limiting for viral RNA synthesis (184).

2-Deoxy-2-fluoro-D-glucose (2d2FGl) and 2-deoxy-2-fluoro-D-mannose (2d2FM) have been studied and compared to 2-deoxy-D-glucose as inhibitors of Semliki forest and fowl plague viruses in cell culture; 2d2FGl was found most effective (185). In chick embryo cells, both fluoro derivatives were activated by UTP and GTP to give the corresponding UDP and GDP fluoro sugars (186). A smaller amount of the corresponding 2-fluorosugar was incorporated into glycoproteins than with 2-deoxy-D-glucose. This was explained (186) by the fact that although GDP2dG1 functions as a good donor in in vitro glycosylation reactions, the corresponding GDP-2-deoxy-2-fluorosugar does not, but most likely exerts its significant antiviral effect by specific enzymatic inhibition.

A combination of 2-deoxy-D-glucose and the pyrimidine nucleoside, 3-deazauridine, synthesized by Robins et al (187), was synergistic in the inhibition of Japanese encephalitis virus production (188). It was postulated that 3-deazauridine diphosphate-2'-deoxy-D-glucose is a possible inhibitor of the glycosylation of viral glycoprotein. 3-Deazauridine is an in vitro inhibitor of several RNA viruses (189, 190); the activity of the compound can be reversed by addition of uridine (189).

In 1971, a new antibiotic, tunicamycin, was isolated and found active against certain plant viruses and Newcastle disease virus (191), especially when grown in embryonated eggs (192). The antibiotic was originally shown (193) to contain two molecules of D-glucosamine, and the antiviral effect was partially reversed by addition of D-glucosamine to the cellular growth medium. It has been suggested (193) that tunicamycin inhibits glycosylation of the viral glycoprotein, since it greatly inhibited the incorporation of D-glucosamine and D-glucose in Newcastle disease viral–infected chick embryo fibroblasts (194). Tunicamycin has been shown to arrest the formation of infective virions of Semliki forest and fowl plague virus by the inhibition of glycosylation of the glycoprotein (195). It also has recently been proposed as an inhibitor of N-acetylglucosamine-lipid biosynthesis, thereby preventing the glycosylation of newly synthesized viral glycoproteins (196). Tunicamycin did not affect the nonenveloped encephalomyocarditis virus in BHK cells, indicating that it is not a general inhibitor of protein synthesis. The structure of tunicamycin has recently been assigned as that of a uracil nucleoside trisaccharide (197) containing acetyl-D-glucosamine and D-galactosamine. Its structural resemblance to

natural uridine diphosphate-N-acetyl-D-glucosamine is quite apparent. Recently Schultz & Oroszlan (198) have shown that the molecular weight of the precursor polyprotein to the envelope glycoprotein of Rauscher murine leukemia virus is reduced from 85,000 to 68,000 by the presence of tunicamycin, which prevents glycosylation of the asparagine residues. Morrison et al (199) have recently shown that not only does tunicamycin give rise to an unglycosylated form of vesicular stomatitis virus glycoprotein, but also the migration of this glycoprotein from the rough endoplasmic reticulum to smooth intracellular membranes is prevented, which is a significant inhibition of viral assembly. Although tunicamycin has an interesting broad antiviral spectrum, it also inhibits cellular polysaccharide biosynthesis (200–202) as well as glycosylation of interferon (203), and would appear to be too toxic for practical use.

CONCLUSION

This review focuses on the major sites of attack by antiviral substances, illustrating activity at these sites using known compounds. Other sites of antiviral attack exist; these include inhibition of late viral mRNA and late viral protein (e.g. methisazone, rifampin), inhibitors of viral assembly (e.g. guanidine, rifampin), and interferon inducers. It was felt that compounds such as methisazone, guanidine, and rifampin, as well as other pioneer antiviral substances, are well known and widely discussed in previous reviews; the number of the types of interferon inducers is so large as to require a full separate discussion. It is hoped, however, that despite such obvious omissions, this review will be of assistance to those dedicated to the development of more effective antiviral substances.

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