PROGRESS WITH ANTIVIRAL AGENTS

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Introduction

It is perhaps pretentious to employ the current popular term 'antiviral chemotherapy' when only three classes of compounds are officially approved for *limited* clinical application: *N*-methylisatin-3-thiosemicarbazone as a prophylactic agent in smallpox [1]; adamantamine, for prophylaxis in humans subject to influenza A-2 virus infections [2,3]; and 5-iodo-2′-deoxyuridine (IUdR) for therapy of herpes simplex corneal keratitis [4–7], a major source of blindness in man.

Differences of opinion still exist as to whether the future lies with antiviral agents of vaccines [2], the latter of which exploit natural defence mechanisms [8,9]. However specific immunization is feasible only in prophylaxis of viral infections which possess a limited number of serotypes. The feasability of designing broad-spectrum antiviral agents (see below) will provide the decisive answer to this question.

The vast majority of new antiviral agents reported during the past 10 years do not pass the stage of tests on cell cultures or small animals. But the intense research activity of the past 5 years has provided a number of drugs approved for investigational purposes, many of which are promising. This brief outline concentrates on only a limited range of compounds, mainly nucleoside and poly(nucleotide) analogues, principally with a view to illustrating methods of approach, applicable also to other compounds. An extensive compilation of antiviral (and antitumour) substances of natural origin has appeared [10].

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Antiviral and antitumour agents

The search for new or improved antiviral agents has now become inextricably linked with similar endeavours in the field of antitumour agents, stimulated enormously by the discovery of reverse transcriptase which clarified the molecular mechanism of tumour induction by oncornaviruses [11], in at least apparent agreement with the known fact that some compounds are therapeutically effective both as antiviral and antitumour agents.

Of the three types of DNA viruses known to induce tumours, viz. the adenoviruses, papova viruses and herpes viruses, the latter are very widespread and persistent in humans, who display increased susceptibility on immunosuppression treatment. Herpes viruses cause cancer in animals and there is extensive evidence for and intimate association between these viruses and malignancies in man; the interest in this subject is testified to by the number of symposia [12–14] and reviews [15–17] during the past 2 years. It is of special interest that no vaccines have been developed against herpes.

Some viruses are also known to be responsible for severe deformities both in animals and man; but the role of viruses in congenital malformations, although considered established, is less well known. The subject of viral teratogens has been recently reviewed [18].

Mode of action of antiviral agents

The most promising aspect of present-day research is the increasing attention being devoted to the mode of action of potential antiviral agents. Most of these act at steps subsequent to uncoating, and will be discussed below. One exception is adamantamine and some of its derivatives, the activity of which is directed against some early step in penetration or against uncoating following penetration [19]. Another specific inhibitor of the uncoating process in the case of ECHO virus [20] is 2-thio-4-oxothiazolidine, which reacts reversibly with the coat protein. Eggers (Sept. 1973 Congress on Viral Chemotherapy) reports that this compound at 100 μg/ml gives a 4-log decrease in viral replication, while 150 μg/ml is not toxic to the cell, nor affects cellular RNA or protein synthesis. The review by Dales [21] on early events in cell–animal virus interactions is very timely with respect to the activity of such agents. Meanwhile Welsh et al. [22] have found that adamantadine·HCl effectively inhibits lymphocytic choriomeningitis virus synthesis even when added after all the cells were infected; virion penetration was also delayed, so that the agent is apparently effective both in the early and late stages of infection.

Estimation of viral susceptibility

General experimental procedures for determination of antiviral activities in vitro and in vivo, together with associated toxicities and the resulting therapeutic indices, are described in the volume edited by Bauer [1]. A more recent and interesting innovation is the development of laboratories for the rapid clinical diagnosis of common viruses, as well as their susceptibilities in tissue culture to various antivital agents available to the clinician (e.g. [23,24]).

Purine and pyrimidine derivatives

The literature literally teems with reports of purine and pyrimidine analogues exhibiting antiviral activity [25–27], but many should be treated with reserve because of a paucity of adequate data. A notable exception is 5-fluorouracil, probably the most intensively investigated pyrimidine antimetabolite; it is readily converted to the riboside in both bacterial and mammalian systems, followed by phosphorylation and incorporation into RNA in the case of mammalian cells. But, while active against DNA viruses in cell cultures, its in vivo efficacy is limited [28,29].

In a recent exemplary study [30] on the synthesis of Rous sarcoma virus on monolayer cultures of chick embryo fibroblasts, it was shown that 5-fluorouridine inhibits cellular RNA synthesis by more than 80%, largely by irreversible blockage of formation of rRNA. Although normal quantities of virus are produced, the infectivity was lowered; correlating with a profound inhibition of viral glycoprotein synthesis. Under the same conditions 5-fluorouracil did not affect the yield of infectious virus. By contrast, another nucleoside analogue, 5-bromotubercidin, reversibly blocked synthesis of heterogeneous nuclear, as well as ribosomal, RNA; it also blocked synthesis of RSV-RNA and, after a lag period, synthesis of viral proteins and mature virions.

Both 6-thioguanine and its riboside exhibit high in vitro activity against cytomegalovirus, a highly infective virus found in patients on immunosuppressive treatment, and responsible for severe congenital effects in newborn infants. The therapeutic index for both of these is exceptionally high, about 100, and several additional analogues were almost as active [31]. It is of interest that 6-thioguanine is very effective in combination antitumour therapy with araC [32–34].

A remarkable example of a pyrimidine analogue with antiviral activity is 5-(3,4-dichlorophenyl)-5-ethyl-hexahydropyrimidine-2,4,6-trione [35]. This is more easily recognized as a barbital derivative, 5-ethyl-5′-(3,4-dichlorphenyl)-barbital. It was the only member of a large number of clinically useful barbiturates, with weak activity against Coxsackie A21 virus on AV2 cells by the plaque reduction test. But, when administered in the diet to mice and gerbil at 10–70 mg/kg daily, it gave a high protective effect with a therapeutic index in excess of 12. Since this compound is a member of a class regarded as clinically safe, and is effective orally, trials in man appear to be indicated. Worthy of note is the fact that, on the basis of in vitro tests alone, this analogue would have been rejected.

One further example is 5-methyl-6-carbethoxy-2-thiomethyl-pyrimidine, which has been reported highly active in vitro against polio [36].

Nucleoside analogues

Nucleoside analogues, both synthetic and of natural origin, have provided some of the most potent antiviral, and antitumour agents. Interest in this class of compounds stems in large part from the progress achieved in recent years on the metabolism, and the replication mechanisms, of nucleic acids, facilitating studies on the mechanism of action of these analogues, as well as the 'rational' design of new ones. A nucleoside analogue, 5-iodo-2′-deoxyuridine (IUdR) was the first purely synthetic antiviral agent, with effective in vitro activity against herpes simplex, vaccinia and some other virulent viruses, and with beneficial effects in clinical treatment of corneal herpes and, to a lesser extent, cutaneous herpes, herpes zoster, herpes genitalis and herpes encephalitis [4–7].

A number of additional nucleoside analogues subsequently proved effective against herpes, including araC, 5-fluorodeoxyuridine (FUdR), 5-bromodeoxyuridine (BUdR), 5-methylamino-2′-deoxyuridine, araA, 5-trifluoromethyl-2′-deoxyuridine, 5-ethyl-2′-deoxyuridine. The relative clinical merits of some of these are reviewed by Kaufman [6], e.g. trifluorothymidine is superior to IUdR for treatment of dendritic keratitis. The mode of action of these various analogues clearly cannot be identical; this is reflected in their range of action, e.g. 5-methylaminodeoxyuridine is inactive against other viral systems; IUdR is more effective that FUdR against herpes type 1, whereas the reverse is true for type 2. By contrast, araC inhibited both types equally, whereas all three were inactive against adenoviruses. In turn, araA is active against IUdR-resistant herpes.

The mode of action of IUdR and BUdR is still the object of intensive study [5,7]. Although results vary somewhat with the test system employed, they apparently undergo conversion to the triphosphates, followed by incorporation into viral DNA, with possibly resultant inhibition of mRNA transcription [37].

The biochemical effects of araA have been thoroughly reviewed by Suhadolnik [38]. Like most nucleoside analogue antiviral agents, it is converted intracellularly to the triphosphate, which inhibits ribonucleotide reductase (but less efficiently than dATP) and DNA polymerase [39]. It is apparently not incorporated into DNA or RNA [40]. Prior to phosphorylation it is slowly deaminated in vivo; the resultant araI is equally effective, an advantage not shared by other analogues, such as araC (see below).

AraC biochemistry is exhaustively reviewed by Cohen [41], Roy-Burman [29] and Goz and Prusoff [4]. Earlier proposals implicating araCDP as an inhibitor of CDP reductase no longer appear valid. By contrast araCTP *selectively* inhibits some polymerases (see below) and is incorporated into DNA; but a difference of opinion still exists as to whether it acts solely as a chain terminator [42] or is also incorporated within the DNA strands [43]. Particularly noteworthy is the fact that araC is active against oncogenic viruses, and is widely employed singly, or in combination tumour therapy [32–34].

The mode of action of trifluorothymidine involves phosphorylation to the triphosphate, which then replaces 2–8% of the thymidine in the DNA of vaccinia virus. This, together with the observed morphological changes in the virus particles, is believed to account for the antiviral effect. However the 5′-monophosphate is a powerful inhibitor of thymidylate synthetase, a fact perhaps not without significance [44].

5-Alkyl nucleosides

The antiviral activity of some nucleoside analogues is due, at least in part, to their incorporation into DNA (see above). For such analogues as IUdR and BUdR, this may entail accompanying mutagenic effects, as well as activation of oncogenic genomes. Hence the potential utility of an analogue which might exhibit base-pairing properties identical with the parent base, thus minimizing or eliminating possible mutagenic effects.

5-Ethyluracil is such an analogue of thymine; the pK for dissociation of the ring N_3 hydrogen (the donor in Watson–Crick base pairing) in 5-ethyluracil and its pentosides is identical with those for thymine and thymidine. Furthermore 5-ethyluracil undergoes moderate incorporation into bacterial DNA and 5-ethyldeoxyuridine (EtUdR) extensive incorporation into phage DNA. In reversions of T_4 rII mutants to r^+, where BUdR gives the expected frequencies of revertants, EtUdR was without effect [27]. EtUdR restores cell proliferation in human phytohemagglutinin-stimulated lymphocytes grown under condi-

tions of DNA synthesis block due to amethopterine induced thymidine deficiency, with incorporation into nuclei of cells entering the S phase; the analogue induced no chromatid aberrations in metaphase under conditions where BUdR did so [45].

EtUdR was found to be almost as effective as IUdR and BUdR against vaccinia virus on HeLa cells [46], and was somewhat less effective than IUdR against herpes simplex on chick embryo fibroblast monolayers (H. Kaufman, personal communication). Other reports of activities against these viruses are even more promising ([47] and references therein), and further trials are now under way with Dr. E. DeClercq. Meanwhile 5-ethyl-2′-deoxycytidine has been synthesized [48]; in tests conducted by DeClercq against herpes, vaccinia and VSV viruses on primary rabbit kidney cells, it exhibited weak activity only against herpes, probably via cellular deamination to the active EtUdR [48].

The mode of action of EtUdR is under study. The low incorporation of 5-ethyluracil into *E. coli* DNA (about 15% replacement of thymine) is due in part to its poor properties as a substrate of thymidine phosphorylase. EtUdR is a moderate inhibitor of thymidine kinase and is itself a poorer substrate than thymidine [47].

Muraoka and Ueda [49] have synthesized 5-ethyluracil-1-β-D-galactopyranoside and the corresponding xylopyranoside, both of which were claimed to exhibit appreciable activity against the Mahoney strain of polio virus. However the earlier claims of these authors for antiviral activities of several other 5-alkyluracil ribonucleosides, in particular, 5-butyluridine, have not been confirmed [50]; undoubtedly the 2′-deoxyuridine would be of more interest.

Nucleoside anomers and enantiomers

Chemical syntheses of 2′-deoxynucleosides yield mixtures of the α- and β-anomers, the separation of which is usually tedious (but see ref. [51]). It is frequent practise to discard the 'biologically inactive' α-anomer. Two recent reports point to the need for a revision of this concept.

Christensen et al. [52] found the α-anomers of 2-chloro- and 2-hydrazino-2′-deoxyadenosine to exhibit appreciable activity against several bacterial systems and, albeit to a lesser extent than the β-anomer, against leukemia L1210. Activity was not due to cleavage of the glycosidic bond.

The foregoing recalls the earlier report by Peery and LePage [53] that both the α- and β-anomers of 2′-deoxy-6-thioguanine inhibit some mouse tumours. The α-anomer was less active, but also less toxic to the host because it was converted to the active nucleotide by tumours (including human) but not by normal bone marrow cells. The lower toxicity made possible higher and multiple doses with concomitant enhanced activity.

No tests of antiviral or antitumour activities appear to have been made with the α-anomers of ribo- and arabinonucleosides. But it should be noted that traces of α-cytidylic acid have been isolated from yeast RNA [54]; while two cytosine nucleosides possessing the α-L configuration, pentopyranine A and C, are elaborated in the culture medium by the Blasticidin S-producing microorganism *Streptomyces griseochromogenous* [55].

The L-enantiomer of araC, although resistant to enzymatic deamination, proved inactive against lymphoid leukemia L1210 and Ridgeway osteogenic sarcoma [56].

Nucleotide analogues

Nucleoside antiviral and antitumour agents are usually converted intracellularly to the active phosphorylated forms. But some cells may not contain the necessary nucleoside kinase(s); others, rich in nucleoside phosphorylase, may cleave the glycosidic linkage before phosphorylation occurs.

Nucleotides are usually incapable of penetrating the cellular membrane because of the diionized phosphate group at physiological pH. Wigler and Lozzio [57] overcame this difficulty by preparing the methyl ester of BUdR-5′-phosphate, which readily penetrated Chinese hamster cells intact and killed all the cells over a time interval equivalent to 8 generation cycles. It was postulated that BUdR-5′-methylphosphate is transformed by thymidylate and dTDP kinases to the triphosphate, followed by incorporation into DNA with resultant lethality. But another possibility not envisaged is the intracellular hydrolysis of this analogue by phosphodiesterase I to BUdR-5′-

phosphate [58], followed by phosphorylation to the normal triphosphate.

An interesting variant of the foregoing [59] profited from the observation [60] that cAMP is resistant to muscle AMP deaminase and crosses cell membranes intact. This suggested 3′,5′-cyclic-araCMP, which proved as active in vitro as araC against several DNA viruses. In infected mice it proved more active than araC against herpes and vaccinia, presumably because of resistance to deamination by deoxycytidylate deaminase. It was not established whether the enhanced antiviral activity was a property of the cyclic phosphate derivative, or was preceded by cleavage of the cyclic phosphate ring by cyclic phosphodiesterase. The same authors had earlier shown that some 3′,5′-cyclic phosphates of adenosine analogues resistant to cAMP phosphodiesterase, exhibit antiviral activity. It should be noted that the replacement of araC by its 3′,5′-cyclic phosphate also obviates the deleterious effects on araC activity of intracellular cytidine deaminase (see below).

Both the foregoing procedures are clearly promising and applicable on a wider scale. It should be recalled that 2′-acylated cAMP is without effect on glycogen phosphorylation in vitro, but is more active than cAMP when tested on liver slices, the obvious interpretation being that the acylated derivative more readily crosses the cell membrane and is then hydrolyzed by cellular esterase(s) to the active cAMP [61].

Hazards associated with nucleoside antiviral agents

Apart from potential toxicity to the host associated with the in vivo use of any drug, the properties of several antiviral nucleosides call for special comment, e.g. IUdR has been employed on a number of patients with herpes simplex encephalitis, and FUdR and IUdR against congenital cytomegalovirus infections [5]. Both these analogues are incorporated into DNA and are mutagenic. The ability of another analogue, BUdR, to produce chromosomal aberrations (see above) is well known. Clinical use of such agents for other than topical applications are consequently limited to cases of potentially lethal infections.

An additional hazard now attaches to the foregoing. It has long been known that murine leukemia virus (MLV) may be 'activated' in mice by aging, carcinogens (shown to be also mutagens, see ref. [62]), X-rays, specific antigens, etc., in accord with both the 'provirus' and 'oncogene' hypotheses. It has now been established that IUdR and BUdR are potent activators of MLV in AKR cells, the extent of activation being as high as 10^6-fold the spontaneous rate [63]. Klement (see ref. [63]) also found BUdR to activate synthesis of murine sarcoma virus in a nonproducer line of rat cells transformed by Kirsten sarcoma virus. According to the authors, "these drugs may be extremely useful agents for activating a leukemia virus genome in cells from mice and other species, including man", but this is no comfort to those interested in development of antiviral agents. Meanwhile Vesely and Cihak [64] found that 5-azacytidine is also an activator, and Gerber [65] that BUdR activates Epstein–Barr virus in human lymphoid cells. Fortunately several other nucleoside antiviral agents, such as araC, 6-azauridine, 6-mercaptopurine are reported to be inactive. In a comparative study of IUdR and EtUdR, I.B. Weinstein (personal communication) found the latter inactive.

Intracellular potentiation of activity

The intracellular metabolism of drugs is by no means a new problem (e.g. [66]) and antiviral and antitumour agents are no exception. Occasionally intracellular enzymes play a key role in formation of the active agent, e.g. phosphorylation of nucleoside analogues, discussed above. Following are several illustrations of procedures developed to circumvent the deleterious effects of intracellular enzymes on antiviral and antitumour agents.

The use of araC as a therapeutic agent requires complex dose schedules, largely due to its rapid intracellualr deamination to inactive araU. In patients treated for acute myeloblastic leukemia the half-life of araC in the plasma varied from 3–9 min [67], and its efficacy has been linked directly with the level of cytidine deaminase in the bone marrow of leukemic patients [68]. The distribution of deaminases (and kinases) of araC in tissues of the mouse and man have been investigated by Ho [69].

The activity of araC in L1210 leukemic mice is enhanced upon simultaneous administration of 3,4,5,6-tetrahydrouridine, a known potent inhibitor

of cytidine deaminase [70]. Additional deaminase inhibitors have been developed, such as N^4-hydroxy-5-methyl-2′-deoxycytidine [71]. It has been proposed [72] that such inhibitors be employed with 5-azacytidine, a promising agent in treatment of acute myelocytic leukemia, the activity of which is reduced as a result of its deamination by peripheral leukemic leucocytes. But more efficient methods have now been developed to circumvent the deleterious effects of tissue deaminases.

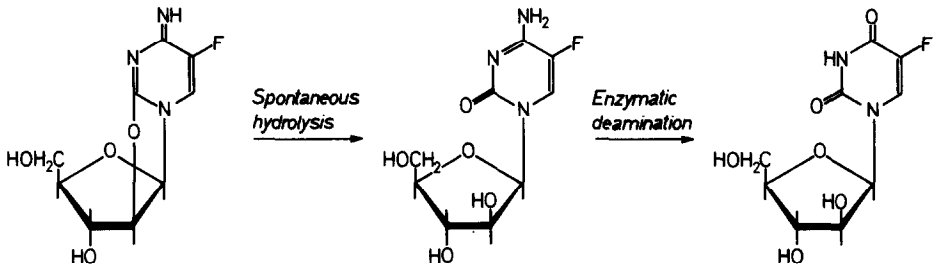
It was initially found by Hoshi et al. [73] that 2,2′-anhydro-araC is markedly active at high dose levels against mouse leukemia L1210, with a therapeutic index of 50 as compared to 10 for araC; the increased activity was ascribed to the resistance of araC to tissue deaminases. Simultaneously Nagyvary (cited in ref. [38]) reported that 2,2′-anhydro-araC-3′phosphate was active against Rauscher leukemia virus and leukemia due to this virus in mice, and suggested that this was due to hydrolysis of the compound to active araC in the blood stream, results confirmed and extended by numerous observers (e.g. ref. [74]). Anhydro-araC is also inherently unstable and is converted slowly to araC in neutral medium, with a half-life at pH 7.4 and 37°C of 32 hr; it does not undergo phosphorylation in vivo, nor is it taken up by Ehrlich ascites cells prior to conversion to araC; furthermore an araC resistant 2′-deoxycytidine kinase-deficient subline of L1210 in mice was cross-resistant to 2,2′-anhydro-araC [75]. The cumulative toxicity of the cyclocytidine derivative is surprisingly low relative to araC [76]. It is clear that 2,2′-anhydro-araC is a depot form of araC, the effectivity of which is now under intensive investigation (see also below).

Another approach to the development of sustained action derivatives of araC arose out of the synthesis of the 5′-ester of araC with adamantane-1-carboxylic acid. The resulting araC-5′-adamantoate proved to be a much more effective antitumour agent than araC, with single-dose therapy as efficient as therapy with araC on an optimum schedule of courses of multiple closely spaced doses with appropriate intervals for host recovery [77]. Further investigation demonstrated that the drug is hydrolyzed by mammalian blood plasma (probably by non-specific esterases) to active araC [78]. This principle had been applied much earlier by Nishizawa et al. [79] who used esters of 5-fluoro-2′-deoxyuridine to reduce hydrolysis by cellular nucleoside phosphorylase. Subsequently a variety of other 5′-esters of araC were shown to be equally effective as antitumour agents in mice, in protecting mice from the lethal effects of intracranial herpes simplex infection, in inhibition of DNA synthesis in phytohemagglutinin-stimulated human lymphocytes, etc. [80]. The corresponding 2′-*O*- and 3′-*O*-esters proved less effective [81,82].

There is little doubt but that the foregoing esters are protected from in vivo deamination prior to hydrolysis of the ester substituents. This is supported by the direct synthesis of all the possible *O*′-methyl and *O*′-ethyl derivatives of araC, which proved highly or totally resistant to kidney cytidine deaminase [83–85], and which exhibit low, if any, activity against several viruses in cell culture (DeClercq and Shugar, in preparation), in accordance with the expected resistance of such derivatives to esterases and other tissue enzymes. It is of interest that 2′-*O*-methyl-araC, which is theoretically capable of being converted to the 5′-triphosphate, is inactive, pointing to the importance of the unsubstituted arabinosyl ring for its activity.

An ingenious application of two of the foregoing principles involved the synthesis of 2,2′-anhydro-ara-5-fluorocytosine [86]. This was expected to undergo hydrolysis to ara-5-fluorocytosine (ara-FC), followed by deamination to ara-5-fluorouracil (ara-FU), as in scheme 1. Each of these is chemotherapeutically active by inhibition of DNA polymerase and thymidylate synthetase, respectively. Experiments demonstrated, in fact, significant prolongation of survival times in mice innoculated with leukemia L1210 and its mercaptopurine-resistant variant L1210/6-MP, when administered as a single daily dose, or even orally and as much as 48 hr after infection, so that this 'double-barreled' agent proved more effective than araC, ara-FC, anhydro-araC or ara-FU.

An additional illustration of the use of intracellular enzymes for enhancing drug efficiency was provided by LePage et al. [87]. When a nucleotide, such as the antiviral araAMP, was administered to mice, it was rapidly excreted at the same rate and in the same form as if the nucleoside were administered, due to the high kidney phosphatase level. But human kidney samples were found to have much lower phosphatase levels, and administration of the nucleotide to human



2,2'-anhydro-1-(β-D-arabinosyl)-5-fluorocytosine (2,2'-anhydro-ara-FC)

1-β-D-arabinofuranosyl-5-fluorocytosine (ara-FC)

1-β-D-arabinofuranosyl-5-fluorouracil (ara-FU)

SCHEME 1

patients led to a slow conversion to nucleoside, resulting in a sustained blood plasma level of the latter. This procedure possesses the additional advantage that nucleotides are more soluble and may be given intravenously in small volume. It should be noted that araA is readily deaminated to araI by intracellular adenosine deaminase, but in this instance the antiviral activity of the latter against herpes simplex is equal to that of araA [6].

Broad-spectrum antiviral agents

Practically all antiviral agents hitherto reported possess a limited spectrum of activity, with the exception of interferons (which exhibit species specificity) and interferon inducers, whose value is mainly prophylactic.

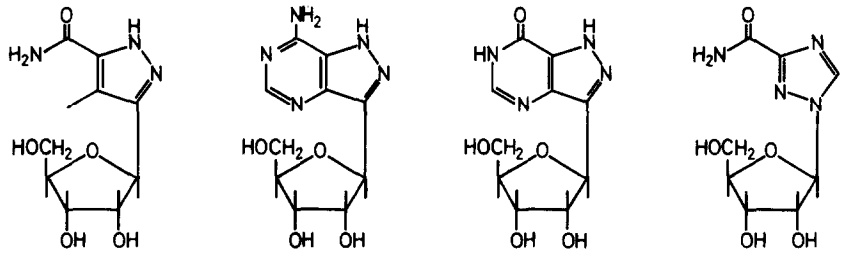
The possibility of developing broad-spectrum antiviral agents was demonstrated by Bauer et al. [88]. It had been commonly accepted that methisazone (*N*-methyl-isatin-3-thiosemicarbazone), an effective agent against smallpox, is active only against some DNA viruses. The foregoing authors found that this agent, and some of its analogues, are active against a variety of RNA viruses in tissue culture, including foot-and-mouth, polio, some rhinoviruses, arboviruses and influenza A and B. No in vivo results were reported. The activity against RNA viruses was considered in accord with the mode of action of this agent which, in the case of vaccinia, induces dissolution of the late mRNA–ribosome complex, with concomitant loss of ability to synthesize proteins required for encapsidation of newly formed viral DNA [89].

Swallow [3] and, indirectly Dales [21], have drawn attention to the broad antiviral spectra of the adamantamines. For example α-methyl-1-adamantane methylamine hydrochloride not only provides significant protection against influenza A-2 in mice even 4 days after infection, but shows in vitro activity against rubella, rubeola and Rous and Esh sarcoma viruses; while adamantamine itself is active in vitro against lymphocytic choriomeningitis [22].

The ribofuranosyl nucleoside antibiotic, pyrazomycin, isolated in 1969 by R.H. Williams, of the Lilly Research Laboratories from a strain of *Streptococcus candidus*, was found to prevent proliferation of rhinovirus, measles, herpes simplex and vaccinia in cultured cell monolayers, with a minimum inhibitory concentration for vaccinia of less than 2 μg/ml. Vaccinia virus lesions in mice were reduced even on oral administration.

Pyrazomycin is structurally related to the nucleoside antibiotics formycins A and B (analogues of adenosine and inosine, Scheme 2), both with antiviral activity [90]. The biologically active form of pyrazomycin has been identified as its 5'-phosphate [91], which is an antagonist of uridine metabolism, being 20-fold more potent than 6-aza-UMP as an inhibitor of orotidylic acid decarboxylase. Antitumour activity of pyrazomycin has been investigated by Sweeney et al. [92].

The relatively broad antiviral spectrum of pyrazomycin prompted the synthesis of a number of ribofuranosides of 5-membered heterocyclic rings, culmi-



SCHEME 2

nating in 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, denoted as Virazole [93,94], and exhibiting highly significant in vitro activity against 7 DNA and 10 RNA viruses tested. In vivo experiments were equally promising: topical application inhibited herpes keratitis in rabbits, and tail lesions induced by herpes, vaccinia and vesicular stomatitis viruses in mice. Intraperitoneally in mice it inhibited Friend leukemia virus-induced splenomegaly and hepatomegaly, as well as respiratory infections by influenza A_0, A_2, B and parainfluenza 1 viruses. Intracerebral viral infections were unresponsive, perhaps because the drug does not effectively penetrate the blood–brain barrier [95–97]. The oral activity in IV/A_2-infected mice was independently confirmed in three other laboratories [96] and is especially significant since only adamantanamine • HCl had hitherto proven effective against influenza A and A_2 virus infections, and only prophylactically; Virazole exhibited significant therapeutic value even when administered 24 hr after infection.

It is clear that, apart from its potential therapeutic significance, the availability of an agent with such a broad antiviral spectrum should prove a useful tool in studies on the biochemical mechanisms of viral replication and inhibition. Virazole is not an interferon inducer in cell cultures or mice [95]. Its action against measles virus in Vero cell cultures is reversed by xanthosine, guanosine, less so by inosine. In vitro studies with isolated enzyme systems showed that the 5′-phosphate is a potent competitive inhibitor of 5′-IMP dehydrogenase, with resultant blocking of 5′-GMP synthesis in infected cells on the pathway from 5′-IMP to 5′-XMP [98]. Although demonstrated that virazole undergoes phosphorylation in vivo, further studies are necessary to clarify its mode of action. Meanwhile some additional analogues have been prepared, but none was more active than the parent compound, nor did any exhibit broad-spectrum activity [99].

It is to be anticipated that this important development will stimulate further efforts in this direction, in the same way as the first synthetic antiviral agent, IUdR, initiated the era leading to the present state of progress.

Inhibitors of viral polymerases

The discovery of RNA-directed DNA polymerases in oncornaviruses provided a new impetus in the search for selective inhibitors of polymerases not only for tumour therapy, but against other viral infections as well.

Earlier observations had already pointed to the possibility of selective inhibition of polymerase activity. Cleaver [100] found that hydroxyurea effectively blocks semi-conservative DNA replication in HeLa cells without affecting repair replication. It was subsequently noted by Brown [101] that 6-(*p*-hydroxyphenylazo)-uracil completely inhibited semi-conservative DNA replication in Gram-positive organisms, while allowing normal repair replication of UV-damaged DNA to proceed in *B. subtilis* 168 thy^- ind^-; in agreement with this was the observation that this agent did not inhibit DNA pol I in vitro. Simultaneously Gamma Reddy et al. [102] showed that, in in vitro systems, araCTP inhibits *E. coli* DNA pol III, to a much lesser extent pol II and T_4 DNA polymerase, and is without effect on DNA pol I, pointing

to the utility of araCTP for studies of repair replication in the absence of semi-conservative replication. But no less significant is the fact that such selective inhibition is exhibited by the active form of an agent with potent antiviral and antitumour properties. It was, in fact, shortly afterwards demonstrated [103] that araCTP is a potent inhibitor in vitro of partially purified Rauscher leukemia virus (RLV)-DNA polymerase, using either RNA or DNA templates, in accord with the known activity of araC against oncornaviruses, and the generally accepted belief that its activity depends on its intracellular transformation to the triphosphate, which then interferes with DNA replication.

Screening of 1 000 synthetic compounds for selective inhibition of Qβ replicase yielded one, 4-(2-propinyloxy-β-nitrostyrene), which at 10 μg/ml inhibited phage multiplication in *E. coli* by 99% with only 50% inhibition of bacterial growth and no inhibition of DNA polymerase. The replicases from poliovirus-infected cells, and Friend leukemia virus-infected tissues, were strongly inhibited by this agent at 100 μg/ml, but no in vivo trials were reported [104].

Various classes of molecules have been found which block reverse transcriptases in vitro, including the ansamacrolides (rifamycins, streptovaricins), anthracyclines (daunorubicin, adriamycin, etc.), cactinomycins, polypeptides (distamycins, bleomycins), etc. All of these, and what is known of their mode of action, have been admirably reviewed by Chandra et al. [105] and Apple [106].

The most popular of the foregoing is the antibiotic rifamycin-SV and some of its semi-synthetic analogues, which specifically bind to, and block, DNA-directed bacterial RNA polymerases, but do not inhibit mammalian polymerases. The potential clinical applications and antiviral activities of the rifamycins are reviewed by Wehrli and Staehlin [107], who refer to this class of compounds as the 'wonder drug', but more useful as a biochemical 'tool' because of the high toxicity of therapeutic doses. Of 180 rifamycin derivatives screened by Green et al. [108] and Gurgo et al. [109] for activity against RNA- and DNA-directed activities of various RNA tumour viruses, a number showed promise, but no toxicity data were presented.

Another potential complication in the search for specific inhibitors of RNA-directed DNA polymerase is the demonstration of such enzymes in HeLa cells derived from a cervical carcinoma, and in WI-38 cells, a normal diploid strain from human embryonic lung tissue [110]. The existence of such enzymes of apparently cellular origin may complicate the design of specific inhibitors for reverse transcriptases. Furthermore, can one expect reverse transcriptase inhibitors to be *therapeutically* effective if the function of this enzyme is necessary only to *initiate* the transformed state, but not to maintain it? It is true that therapeutic effects have been demonstrated both in animals and man [106], but the data presently available are too meagre to provide an unequivocal answer. Clearly more extensive studies on the role of reverse transcriptase inhibitors as function of template requirements are called for.

Polynucleotide inhibitors of viral polymerases

An additional, and newer, approach to selective inhibition of viral replicases makes use of synthetic polynucleotides which to some extent mimic part of the viral genome. For example, following the discovery that the initial portion of RNA transcribed from Qβ RNA is rich in adenosine, it was shown that in vitro replication was appreciably inhibited by poly (rU) or the copolymer poly (rA,rU), and less so by poly (rA) [111].

With increasing knowledge of viral genomes and of the template requirements of viral polymerases, it may prove feasible to inhibit a given polymerase activity with the aid of polynucleotide analogues which interact with the template, or polymerase, or both. One striking feature of RNA tumour viruses is the presence in their genomes of poly (A) tracts 25–50-fold more extensive than in non-oncogenic viruses [112]. It had earlier been shown by Tuominen and Kenney [113] that single-stranded polyribonucleotides competitively bind to RLV-DNA polymerase and that poly (rU) almost abolished the reverse transcriptase activity in vitro, independently of the nature of the template; under the same conditions DNA polymerases from *E. coli* and mouse embryo cells were unaffected. Poly (dU) is also an effective in vitro inhibitor of avian myeloblastosis virus reverse transcriptase [114], suggesting the utility of trials with poly (dT), which complexes even more effectively with poly (A).

Can such inhibitors act in vivo, where the situation is much more complex? Tennant et al. [115] showed that poly (rA) gave 50% inhibition of replication of the Mahoney strain of RLV on mouse embryo cells at a concentration without effect on the population rate growth of the cells. In contrast to the situation in vitro, poly (rU) was less effective; while poly (rG) was too cytotoxic. It was concluded that poly (rA) suppresses polymerization of virus-specific DNA by interfering with the function of viral DNA polymerase in the cells. Furthermore poly (2′-*O*-methyl-A) at a concentration of 10–50 μg/ml inhibited viral replication by as much as 90% with no observable toxic effects on the cells [116]. The activity in this system of poly (2′-*O*-methyl-A) excludes possible interferon induction, such polymers being inactive. The authors do not comment on the lower in vivo activity of poly (rU) relative to poly (rA), the reverse of that found in vitro; this could be due to susceptibility of poly (rU) to intracellular nucleases and suggests trials with poly (2′-*O*-methyl-U) which is completely resistant to RNase and highly resistant to other nucleases [117].

Relatively little has been reported on specific polynucleotide inhibitors for lytic RNA viruses, such as poxvirus, the virions of which contain RNA replicases. However, by analogy with the situation cited above for Qβ replicase, the development of such inhibitors should be feasible (see [118]). It may also prove advantageous to concentrate on the use of deoxypolymers or 2′-*O*-methyl polynucleotides, which do not normally exhibit messenger activity [119], and are also apparently less toxic to the host cells.

Two additional developments merit comment. Pitha et al. [120] have employed vinyl analogues of polynucleotides, poly (1-vinyluracil) and poly (1-vinyladenine), with molecular weights in excess of 10^5, as inhibitors of leukemia virus replication. These analogues are electrically neutral, resistant to enzymic hydrolysis, complex specifically with polynucleotides with the expected complementarity, and are apparently taken up by mammalian cells. Both of these significantly inhibited the early stage of acute murine leukemia virus infections in mouse embryo cells, but did not affect replication of Sindbis and Vesicular Stomatitis viruses. Chandra and Bardos [121] and Srivasta and Bardos [122] found that synthetic and natural polynucleotides, in which some of the uracil and/or cytosine residues have been thiolated at the 5-position, selectively inhibit polymerase activities from various sources, including RNA tumour viruses, e.g. 'thiolated' poly (rC) with 9% of the cytosine residues converted to 5-mercaptocytosine equally effectively inhibited the DNA-directed DNA polymerases of Friend leukemia virus and murine sarcoma virus, but was more effective against the RNA-directed DNA polymerase of MSV. Kinetic studies indicated that thiolated poly (rC) blocks binding of the enzyme to the template. One possible mechanism of action is covalent binding of inhibitor to enzyme via mixed disulfide linkages [123]. While premature to predict the possible therapeutic value of such inhibitors, the ability to introduce thiolated residues in both model and natural polynucleotides with tailored or selected sequences argues in favour of further studies in this direction.

Photodynamic therapy in topical viral infections

It was shown by Thiele and Wacker [124] that the efficacy of IUdR therapy of herpes simplex keratitis in man was enhanced by simultaneous exposure to light. Although not further explored, this effect is readily interpretable if viral DNA incorporation of IdUrd occurs, since the incorporated IUdR residues absorb at longer wavelengths than thymine residues and are susceptible to photochemical reactions involving dehalogenation and accompanying strand breaks in DNA [125]. This is in accord with the known increased susceptibility to inactivation of nucleic acids, in which thymine residues are replaced by 5-halogenouracils, by day-light lamps which emit traces of radiation down to 300 nm [126].

A more direct application of light therapy applicable, as in the foregoing case, to topical viral infections, was the use by Moore et al. [127] of proflavine, followed by irradiation with visible light, to treat herpes keratitis in the rabbit. An extension of this procedure to recurrent herpes simplex infection of the skin and mucous membrane in man involved the surface application of Neutral Red, followed by exposure to visible light. This procedure is based on sound theoretical considerations, for the photodynamic inactivation of viral infectivity has been known for many years and its mechanism reasonably well

understood [126,128,129]. The sensitivity of viruses to visible light in the presence of a dye is dependent on the nature of the dye; with Toluidine Blue, e.g., the relative rate constants for inactivation of vaccinia, adenoviruses and polioviruses are 1 : 0.17 : 0.001, and it has been proposed that the relatively high resistance to photosensitized inactivation of polioviruses be profited from to eliminate potentially harmful organisms from live polio vaccines [126].

A more extensive clinical trial of the foregoing procedure was conducted by Felber et al. [130] on more than 100 patients, with particular emphasis on 32 with an average of at least four recurrences of herpes per year for a minimum of two years. Neutral Red was the dye employed, with controls involving the use of phenol sulfonphthalein (a red dye with no photoreactive or virucidal activity). The results reported were impressive, the more so in view of the simplicity of the technique. Particularly significant was the fact that herpes type 2 virus was even more sensitive than type 1, in agreement with the observation that both types 1 and 2 were photoinactivated equally effectively in tissue culture, this procedure being thus effective against type 2 herpes genitalis, difficult to treat by other methods.

Polynucleotide interferon inducers

Interferon, produced endogenously by cells during virus infections, is a potent antiviral agent with a very broad spectrum, relatively stable and generally believed to be non-toxic. However, because of its species specificity, it was until recently considered that only interferon derived from human cells could be applied in human diseases, and the prospects, based on production from human leucocyte cultures, do indeed show promise [6]. More recent data suggest that certain interferons are active in cells from heterologous species [131]. Nonetheless production of adequate amounts of interferon for prophylactic or therapeutic purposes poses formidable technical problems. Hence the widespread interest in interferon inducers and attempts to purify and characterize the structure and conformation of interferon molecules [132].

Most interferon inducers are polyanions. A few are low molecular weight substances, the most remarkable being 2,7-bis(2-diethylaminoethoxy)-fluoren-9-one dihydrochloride (tilorone hydrochloride), the only inducer active orally and, in mice, effective against a number of viruses, but least so against the influenza group [3,133,134]. Unfortunately this inducer has proven less effective in rabbits and had little or no measureable effect in man [6].

It was first shown by Hilleman's group [135] that the most potent synthetic interferon inducers, as potent as viruses, are double-stranded polynucleotides such as poly (rI)·poly (rC). Since it is technically feasible to produce substantial quantities of synthetic polynucleotides by polymerization of ribonucleoside-5′-pyrophosphates with polynucleotide phosphorylase or the 5′-triphosphates with polymerases, extensive trials have been conducted to examine the structural requirements of these polynucleotides for inducing activity, as well as increasing activity with concomitant decrease in toxicity. Relevant data are summarized by DeClercq and Merigan [136], Colby [137] and Finter [131].

The results of a typical study [138] are shown in table 1, from which the following conclusions emerge: (a) an increase in stability (or melting temperature T_m) of the double-stranded inducer above a given value is of no special significance; (b) blocking of the 2′-hydroxyls of the poly (rC) strand dramatically decreases or abolishes activity. More recent studies with other polynucleotide analogues have been reported by Torrence et al. [139] and DeClercq and Janik [140]. Surprisingly the effects of a 2′-*O*-methyl purine polynucleotide have not yet been examined (but see below). It is perhaps not without relevance that the 2′-*O*-methyl polynucleotides are also inactive as messengers in in vitro systems [119]. However, W.A. Carter has apparently found that replacement of poly (rI) by a copolymer of poly (rI) and poly (2′-*O*-MeI) does not affect activity, while simultaneously decreasing toxicity (personal communication from E. DeClercq). In a paper in press [141], it has been shown by means of competition experiments that single-stranded polynucleotides do not bind to cellular receptor sites for interferon induction, whereas triple-stranded complexes do bind but fail to trigger the necessary message for induction.

The structural requirements of poly (rI)·poly (rC) for interferon induction have been extensively investi-

Table 1

Activity against vesicular stomatitis virus (VSV) in primary rabbit kidney cell cultures of various polynucleotide 1:1 helical complexes, administered as such or by sequential administration of the two components. The T_m values for the helical complexes are at pH 7.8 in 0.15 M NaCl

Polynucleotide no. 1	Polynucleotide no. 2	T_m (°C)	Minimum inhibitory concentration* (μg/ml)		
			Poly no. 1 complexed to poly no. 2	Poly no. 1, then poly no. 2 in 1 hr	Poly no. 2, then poly no. 1 in 1 hr
Poly (rA)	Poly (rU)	59	0.04	>10	>10
Poly (rA)	Poly (2′-*O*-MeU)	70.5	>10	>10	>10
Poly (rI)	Poly (rC)	64	0.0004	0.00004	0.0004
Poly (rI)	Poly (5-MerC)	80.5	0.001	0.0004	1
Poly (rI)	Poly (2′-*O*-MeC)	61.5	4	>10	4–10
Poly (rI)	Poly (dC)	55	⩾10	⩾10	>10
Poly (rI)	Poly (5-MedC)	65	>10	>10	10

* Concentration of polymer (either the polynucleotide complex, or polynucleotide no. 2, if polynucleotide no. 2 is added second, or polynucleotide no. 1, if polynucleotide no. 1 is added second) required to reduce VSV plaque formation by 50%.

gated by Carter et al. [142]. These authors had previously observed that the initial step in induction, adsorption of the double-stranded helix, is very rapid in human cells in vitro, suggesting that an intact primary structure of the inducer complex is required only as a triggering event, and that subsequent presence of the complex is largely detrimental to the cell, including toxicity. They therefore prepared double-stranded helices of poly (rI), which has been shown to be the component of poly (rI)·poly (rC) more effectively adsorbed to the cell receptor site [143], with copolymers of C and U, and C and G. These exhibited in vitro antiviral activities comparable to poly (rI)·poly (rC), but were hydrolyzed by nucleases 5- to 8-fold more rapidly. It remains to evaluate the efficacy of these new models in vivo, but this approach is one of many illustrating the possibilities of manipulating the polynucleotide inducing system.

The mode of action of interferon, administered as such or formed by inducers, is still far from clarified. A recent paper [144], summarizing earlier results, reported that vaccinia virion DNA-directed RNA polymerase is inhibited after infection if the cells were pretreated with interferon or interferon-inducer, leading to marked inhibition of vaccinia mRNA formation.

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