Antiviral Research, 2 (1982) 69–79
Elsevier Biomedical Press

ANTIVIRAL EFFECTS OF RIBAVIRIN AND 6-MERCAPTO-9-TETRAHYDRO-2-FURYLPURINE AGAINST DENGUE VIRUSES IN VITRO

WAYNE C. KOFF, JOE L. ELM, Jr. and SCOTT B. HALSTEAD

Department of Tropical Medicine and Medical Microbiology, John A. Burns School of Medicine, University of Hawaii, 3675 Kilauea Avenue, Honolulu, HI 96816, U.S.A.

(Received 19 May 1981; accepted 3 December 1981)

The antiviral effects of ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) and 6-mercapto-9-tetrahydro-2-furylpurine (6-MPTF) against dengue viruses were examined in vitro. Ribavirin significantly reduced the growth of dengue virus types 1–4 in LLC-MK2 cells at concentrations well below cytotoxic levels (cell viability was determined by trypan blue dye exclusion). Addition of guanosine to ribavirin-treated dengue virus-infected cell cultures completely reversed the antiviral effect of the drug. In contrast, ribavirin had no effect on dengue virus replication in human peripheral blood leukocytes (PBL). 6-MPTF, a specific inhibitor of hypoxanthine-guanine phosphoribosyltransferase, did not significantly reduce the growth of dengue viruses in either LLC-MK2 cells or human PBL. However, synergistic effects of 6-MPTF and ribavirin were observed, as combined treatment of the two drugs markedly suppressed the replication of dengue viruses in human PBL. The successful demonstration that dengue virus replication in mononuclear leukocytes is markedly suppressed by the combined treatment of ribavirin and 6-MPTF signals a need to evaluate the efficacy of this treatment against dengue virus infections in vivo.

combination chemotherapy dengue virus 6-mercapto-9-tetrahydro-2-furylpurine ribavirin

INTRODUCTION

Dengue viruses are a major cause of morbidity and mortality among children in tropical Asia [10]. The clinical spectrum of dengue virus disease ranges from relatively benign dengue fever to the more severe dengue hemorrhagic fever (DHF) and life-threatening shock syndrome (DSS) [14]. These viruses are known to occur in nature as four distinct antigenic types [19], and several studies have demonstrated a significant correlation between the severity of disease caused by dengue and the immune status of the host prior to infection [11,15]. At the present time, efforts to control the spread of dengue have not been entirely successful. Insect control measures are faced with the increasing resistance of mosquito vectors to chemical pesticides [29]. In addition, vaccine development must preclude the possibility of homotypic vaccines sensitizing individuals towards the more severe secondary heterologous dengue infections [32]. As a result, antiviral chemotherapeutic agents are presently being evaluated as a means for prophylactic and therapeutic control of dengue virus disease.

0166-3542/82/0000–0000/$02.75

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been reported to exhibit antiviral activity against a broad spectrum of DNA and RNA viruses [35]. The molecular conformation of ribavirin closely resembles that of guanosine [30], and phosphorylated derivatives of the drug have been reported to interfere with the biosynthesis of guanosine nucleotides [27]. In particular, ribavirin-5′-monophosphate has been shown to competitively inhibit inosine-5′-monophosphate (IMP) dehydrogenase, a cellular enzyme required for guanosine-5′-monophosphate biosynthesis [38]. Although the exact mode of action of ribavirin against viral replication is unknown, the reduction of guanosine nucleotide pools and inhibition of 5′ messenger RNA cap formation which occurs in the presence of the drug are thought to play an important role in the decreased production of virus particles [37].

Dengue viruses are known to infect mononuclear leukocytes in vitro [17], and many reports suggest that these cells are directly involved with the pathogenesis of dengue virus infections in vivo [2,26,28]. Since peripheral blood mononuclear leukocytes exhibit a limited capacity for de novo purine biosynthesis [33], the inhibitory effect of ribavirin against dengue virus infections in these cells might be expected to be minimal, and preliminary studies in this laboratory confirmed this prediction (Elm, J.L., Jr. et al.: Abst. A-10, American Society for Microbiology, Annual Meeting, 1981). Analysis of several virus–host–drug relationships has clearly indicated that an important mechanism for improving the efficacy of antiviral agents is through combination chemotherapy. By selecting pairs of drugs which inhibit viral replication at different sites, synergistic effects may result due to concurrent inhibition, sequential blockage or inhibition through complementation [1].

6-Mercapto-9-tetrahydro-2-furylpurine (6-MPTF) is a specific inhibitor of hypoxanthine-guanine phosphoribosyltransferase (HGPRTase), a major salvage pathway enzyme for purine biosynthesis [22]. 6-MPTF has been shown to have antitumor activity [4,31] and is especially effective when administered in combination with inhibitors of de novo purine biosynthesis [22]. In the present study, we have examined the effects of 6-MPTF and ribavirin against dengue virus replication in vitro. Our results indicate that combined treatment of ribavirin and 6-MPTF effectively suppresses dengue virus replication in vitro, and demonstrates the need to investigate the efficacy of combination chemotherapy against dengue virus infections in vivo.

MATERIALS AND METHODS

Virus assays

Dengue virus type 1 (strain 16007), type 2 (strain 16681), type 3 (strain 16562), and type 4 (strain H-241) were recovered in BSC-1 cells or in suckling mice from acute-phase sera from patients with dengue hemorrhagic fever [16,19]. These viruses were propagated in LLC-MK2 cells (rhesus monkey kidney) maintained with Eagle's basal medium (BME) with Earle's salts and 10% calf serum. Stock seed virus was released from cells by freeze-thawing and concentrated by ultracentrifugation as previously described [13].

Assays of virus titers were done by modification of a plaque method in LLC-MK2 cells [12]. Briefly, 3-day-old LLC-MK2 cell monolayers grown in 1 ounce bottles (Brockway Glass Co., Brockway, PA) were rinsed with Hanks' balanced salt solution (HBSS; Microbiological Associates, Bethesda, MD) and inoculated with 0.2 ml of serially diluted virus samples for 90 min at 37°C with continuous gentle rocking to facilitate adsorption. The inoculum was poured off, and cells were overlayed with 4 ml of 1% purified agar (BBL; Cockeysville, MD) in BME with final concentrations of 10% calf serum, 200 units penicillin/ml, 200 μg streptomycin/ml, 2 mM L-glutamine, 0.1% sodium bicarbonate, and neutral red diluted to 1 : 24,000. The bottles were incubated in the dark for 7 days at 37°C before plaques were counted. All samples were assayed in triplicate.

Infection of LLC-MK2 cells

Cells were rinsed with HBSS and inoculated with dengue viruses at a multiplicity of infection (m.o.i.) of 0.01–0.1. After adsorption for 90 min at 37°C, the inoculum was aspirated off and replaced with 5 ml of virus maintenance media (BME supplemented with 2% calf serum). Culture medium was harvested daily in 0.5 ml aliquots and stored at -70°C for future examination of virus titers. In general, peak titers of virus were obtained at 5 days post-infection. Nucleosides and/or ribavirin were added immediately following the viral adsorption period and remained in contact with the cells for the entire 5-day culture period.

Infection of mononuclear leukocytes

Preservative-free heparinized whole blood was collected by venipuncture of normal adult human volunteers. Mononuclear leukocytes were isolated by standard Ficoll–Hypaque (Pharmacia, Uppsala, Sweden; sodium diatrizoate, Winthrop, New York, NY) density gradient centrifugation [5]. Cells were washed three times in phosphate-buffered saline (Ca^{2+}/Mg^{2+}-free, PBS, pH 7.4), once in HBSS, and resuspended in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 20 mM Hepes (Calbiochem, San Diego, CA), 0.2% sodium bicarbonate, 2 mM L-glutamine, 200 units penicillin/ml and 200 μg streptomycin/ml. 6×10^6 mononuclear leukocytes were incubated with dengue virus type 2 (m.o.i. = 0.1–1.0) and anti-dengue 4 serum (1 : 200 final dilution) in a total volume of 1 ml for 90 min at 37°C. This concentration of anti-dengue 4 rhesus monkey serum regularly enhanced dengue virus type 2 replication in both non-immune human leukocytes and non-immune rhesus leukocytes [18]. Cultures were washed in RPMI 1640 medium to remove any unadsorbed virus, and cells were resuspended at 2×10^6/ml in RPMI 1640 medium supplemented with 2% fetal calf serum. One ml cultures were incubated at 37°C in a 5% CO_2 in air atmosphere for 4–5 days, harvested and stored at -70°C. As with LLC-MK2 cells, dengue virus titers reached a maximum level by 4–5 days post-infection. Ribavirin and/or 6-MPTF were included in virus cultures following the removal of virus inoculum, and remained in contact with the cells for the entire culture period. Growth of dengue viruses in mononuclear leukocytes was assayed by the LLC-MK2 plaque method described above.

Cell viability

Viability of mononuclear leukocytes was assayed by trypan blue dye exclusion [24]. Briefly, 50 μl of a 0.1% trypan blue solution were added to 0.2 ml cell cultures for 30 s followed by 0.1 ml of 4% acetic acid to terminate the reaction. Replicate samples of each culture were counted to yield accurate percentages (± 3%) of cell viability.

Statistical analysis

Student's *t* test was used to evaluate the significance of observed differences between experimental and control groups.

Chemicals

Ribavirin was obtained from ICN Pharmaceuticals, Inc. (Covina, CA). Guanosine, xanthosine and adenosine were obtained from Sigma Chemical Co. (St. Louis, MO). 6-MPTF was obtained from the Drug Synthesis and Chemistry Branch (Division of Cancer Treatment, National Cancer Institute, Silver Spring, MD). Ribavirin and 6-MPTF were dissolved in sterile distilled water, filtered, and stored at 4°C until use.

RESULTS

Dose-response studies

Fig. 1 describes the effect of increasing concentrations of ribavirin on the replication of dengue virus type 2 in LLC-MK2 cells. The minimal inhibitory concentration (MIC) of ribavirin is defined as the concentration which causes 50% reduction in the number of plaques. Interpolation from our dose-response data indicates that the MIC for ribavirin against dengue virus type 2 is approximately 2.0 μg/ml. Concentrations of drug less than 100 μg/ml did not produce any microscopically detectable damage to the cells. Higher doses resulted in generalized rounding and destruction of the cell sheet. These viability studies agree with previous findings which showed that ribavirin's cytotoxic effects do not vary significantly among cell types, with the 50% cytotoxic dose of the drug being 200–1000 μg/ml [34].

Treatment of dengue virus type 2-infected LLC-MK2 cells with 6-MPTF at concentrations ranging from 10 μM to 500 μM had no effect on virus replication (Table 1). At concentrations greater than 500 μM, 6-MPTF treatment for 5 days of uninfected LLC-MK2 resulted in observable cytopathic effects. In addition, combined treatment of ribavirin plus 6-MPTF at various doses did not improve the antiviral efficacy of ribavirin alone in dengue-infected LLC-MK2 cells (Table 1).

Effect of ribavirin on the replication of different dengue virus types

Table 2 shows the effect of ribavirin on the growth of each of the four dengue virus types in LLC-MK2 cells. At a concentration of 50 μg/ml, the replication of all four types

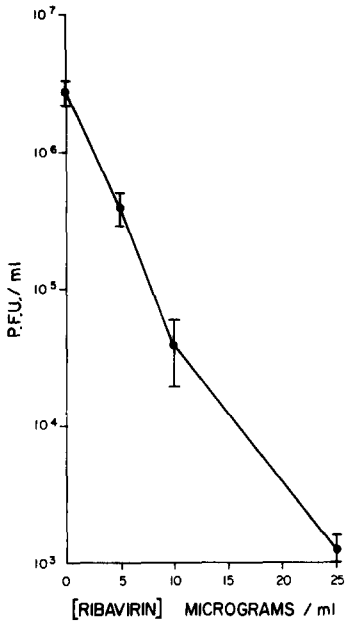

Fig. 1. Dose–response curve of ribavirin on the infection of LLC-MK2 cells with dengue virus type 2. Drug was added immediately following the viral adsorption period. Stock solution of ribavirin was made up in sterile distilled water and filtered prior to use. At 5 days post-infection, aliquots of culture fluids were harvested and assayed in LLC-MK2 cells for determination of virus titers. Values indicate means from four experiments. Bars denote standard error of mean.

TABLE 1

Effect of ribavirin and 6-MPTF on dengue virus replication in LLC-MK2 cells[a]

Ribavirin[b]	6-MPTF[c]	p.f.u./ml[d]
–	–	4.63×10^6
1.0	–	2.03×10^6
10.0	–	1.75×10^5
25.0	–	1.58×10^3
–	10	2.05×10^6
–	100	4.83×10^6
–	500	3.51×10^6
1.0	500	1.33×10^6
10.0	100	5.33×10^5
25.0	100	3.50×10^3
25.0	500	2.50×10^3

[a] Dengue virus type 2 was inoculated into LLC-MK2 cells and culture fluids were harvested 5 days post-infection and assayed for released virus in an LLC-MK2 plaque assay as described in Methods.

[b] Concentration of ribavirin expressed as μg/ml.

[c] Concentration of 6-MTPF expressed as μM.

[d] Values indicate the mean p.f.u./ml from three replicate experiments.

TABLE 2

Inhibition of dengue virus replication by ribavirin[a]

Dengue virus types	Drug[b]	p.f.u./ml[c]	Log_{10} reduction in virus titers[d] ($P < 0.01$)
1	-	7.8×10^5	–
1	+	5.0×10^1	4.19
2	-	9.2×10^6	–
2	+	4.2×10^3	3.34
3	-	2.2×10^5	–
3	+	2.7×10^1	3.91
4	-	4.2×10^5	–
4	+	1.1×10^2	3.58

[a] Dengue viruses were inoculated into LLC-MK2 cells and culture fluids were harvested 5 days post-infection and assayed for released virus in an LLC-MK2 plaque assay as described in Methods.

[b] Ribavirin was added immediately following the viral adsorption period at a concentration of 50 μg/ml.

[c] Values indicate the mean p.f.u./ml from three replicate experiments.

[d] Statistical analysis by the Student's *t* test.

of dengue viruses is suppressed by greater than three logarithms. When comparisons were made of the relative sensitivity of each dengue virus type of ribavirin, no statistical differences were observed. Thus, it may be concluded that all four types of dengue viruses are susceptible to the antiviral effect of ribavirin when cultured in LLC-MK2 cells, and that the degree of sensitivity is similar for the different virus types.

Reversal of the antiviral effect of ribavirin by addition of exogenous nucleosides

Previous studies with other viruses have demonstrated that the antiviral effect of ribavirin in vitro could be significantly reversed by including exogenous nucleosides in the virus–cell cultures [38]. Table 3 describes the effect of guanosine, xanthosine and adenosine on the inhibition of dengue virus type 2 growth in LLC-MK2 cells caused by ribavirin. At 50 μg/ml, the three nucleosides had no effect on dengue virus replication in the absence of ribavirin. When ribavirin alone was added to the dengue-infected cells, titers of virus harvested from supernatant medium 5 days post-infection were reduced by more than three logarithms. Addition of guanosine to ribavirin-treated infected cell cultures completely reversed the antiviral effect of the drug. Of the three nucleosides tested, the ability to reverse the inhibitory effect of ribavirin on dengue virus replication was limited to guanosine. Dose–response studies indicate that concentrations of guanosine as low as 5 μg/ml can significantly reduce the antiviral effect of ribavirin on dengue virus growth in vitro (data not shown).

TABLE 3

Interference with the anti-dengue virus activity of ribavirin by exogenous nucleosides

Sample[a]	Drug[b]	p.f.u./ml[c]
Dengue virus type 2	-	6.33×10^6
plus guanosine	-	4.16×10^6
plus adenosine	-	3.00×10^6
plus xanthosine	-	1.03×10^7
Dengue virus type 2	+	1.67×10^3
plus guanosine	+	3.66×10^6
plus adenosine	+	5.33×10^2
plus xanthosine	+	5.80×10^3

[a] Dengue virus type 2 was infected into LLC-MK2 cells at an m.o.i. = 0.1. Culture fluids were harvested 5 days post-infection and assayed for released virus in an LLC-MK2 plaque assay described in Methods. Nucleosides were added at a final concentration of 50 μg/ml immediately following the viral adsorption period and remained in contact with the cells for the entire 5-day incubation period.

[b] Ribavirin was added immediately following the viral adsorption period at a concentration of 25 μg/ml.

[c] Values indicate the mean p.f.u./ml from three replicate experiments.

Effect of ribavirin and 6-MPTF on the growth of dengue viruses in human PBL

Since dengue virus infection of mononuclear leukocytes is thought to play an important role in the pathogenesis of severe dengue virus disease [2,26,28], we examined the antiviral efficacy of ribavirin and/or 6-MPTF against dengue virus infection of human PBL. The results of these experiments are described in Table 4. At 50 μg/ml, a concentration of the drug that suppressed the growth of dengue viruses in LLC-MK2 cells, ribavirin, had no significant effect on the replication of dengue virus type 2 in human PBL. Concentrations of ribavirin greater than 100 μg/ml were found to be cytotoxic for human PBL. In addition, when cultures of dengue-infected PBL were treated with 6-MPTF, viral titers were only minimally suppressed. However, combined treatment of ribavirin and 6-MPTF significantly ($P < 0.01$) reduced the growth of dengue virus type 2 in human PBL. The concentration of 6-MPTF which, when combined with ribavirin (33 μg/ml), caused a 50% reduction in the number of plaques was interpolated from our data to be 40 μM. The observed suppression of dengue virus replication by ribavirin and 6-MPTF was not due to cytotoxic effects of the drugs, since viability studies demonstrated no significant differences between control and drug-treated cultures.

DISCUSSION

This investigation has focused on the effects of ribavirin and 6-MPTF alone and, in combination, on the replication of dengue viruses in vitro. Ribavirin, at concentrations

TABLE 4

Effects of ribavirin and 6-MPTF on the growth of dengue virus type 2 in human peripheral blood leukocytes

Sample[a]	[Ribavirin] (μg/ml)	[6-MPTF] (μM)	p.f.u./ml[b]	Viability[c]
Control	–	–	4.66×10^3	93
Ribavirin alone	33	–	4.84×10^3	NT[d]
Ribavirin alone	50	–	2.82×10^3	92
6-MPTF alone	–	10	3.33×10^3	90
6-MPTF alone	–	100	4.01×10^3	NT
6-MPTF alone	–	500	1.10×10^3	90
Ribavirin + 6-MPTF	33	10	3.30×10^3	NT
Ribavirin + 6-MPTF	33	100	3.00×10^2	91
Ribavirin + 6-MPTF	33	500	$< 5.0 \times 10^1$	87

[a] Peripheral blood leukocytes were infected with dengue virus type 2 as described in Methods. Ribavirin and/or 6-MPTF were added immediately following the viral adsorption period. Cultures were harvested after 4 days incubation and virus titers were determined by an LLC-MK2 plaque assay.

[b] Values indicate the means of three replicate experiments.

[c] Viability studies by trypan blue dye exclusion.

[d] Not tested.

well below cytotoxic levels, markedly reduced the growth of all four types of dengue virus in LLC-MK2 cells. In contrast, concentrations of ribavirin up to 50 μg/ml did not reduce the replication of dengue viruses in human PBL. These findings support the suggestion of previous studies with DNA viruses that the antiviral action of ribavirin is dependent on the host cell type [21]. Previous studies with measles virus demonstrated a reversal of the antiviral effect of ribavirin when guanosine or xanthosine was added to measles virus-infected cell cultures [6,38]. In our dengue virus LLC-MK2 cell system, guanosine alone could reverse the antiviral effect of ribavirin, while xanthosine or adenosine had no effect. These observations support the theory that the mode of action of ribavirin involves alterations in the biochemical pathways leading to or in association with guanosine nucleotides [36].

Combination chemotherapy has proven to be an effective method for antimicrobial and cancer chemotherapy [3,7,20,23], but has received minimal attention in antiviral chemotherapy [1,8,25]. Recently, studies by Galegov et al. [9] demonstrated that a combination of rimantadine and ribavirin was more effective in tissue culture infected with influenza A virus than the sum of the effects of the drugs given individually. In addition, Wilson et al. [39] showed that combined aerosol treatment of ribavirin and amantadine to influenza-infected mice was considerably more effective than single drug therapy. These studies with influenza provide evidence that other drugs with specific mechanisms of action differing from those postulated for ribavirin may be used in combination with ribavirin to potentiate the antiviral response.

Jadhav et al. [22] showed that 6-MPTF potentiates the effect of azaserine, an inhibitor of de novo purine biosynthesis, on the survival time of mice bearing L5178Y ascites tumors. Of particular interest in these studies was the observation that neither azaserine or 6-MPTF alone exhibited marked activity against this murine tumor, while administration of the drugs in combination significantly increased the survival time. Similarly, our studies showed that neither ribavirin or 6-MPTF significantly reduced the production of dengue viruses in human PBL. Using the azaserine–6-MPTF system as a model, we postulated that combined treatment of ribavirin and 6-MPTF might potentiate the antiviral effect against dengue virus replication. Our data clearly demonstrated that, indeed, combination of ribavirin and 6-MPTF markedly reduced the growth of dengue viruses in human PBL without any significant effect on cell viability. It is interesting to note that 6-MPTF did not potentiate the antiviral effect of ribavirin in LLC-MK2 cells, while combined treatment of the drugs in PBL significantly inhibited dengue replication. The lack of effect of combined therapy in LLC-MK2 cells may be due to the minimal use of salvage pathway enzymes in the production of dengue viruses in these cells. Since mononuclear leukocytes infected with dengue have been strongly implicated in the pathogenesis of severe dengue virus disease [2,26,28], these findings signal a need to determine the efficacy of ribavirin and 6-MPTF against dengue virus infections in vivo.

ACKNOWLEDGEMENT

This work was supported by USPHS NIH grant No. 2-R01-HD08693.

REFERENCES

1 Ayisi, N.K., Gupta, V.S., Meldrum, J.B., Taneka, A.K. and Babiuk, A. (1980) Combination chemotherapy: interaction of 5-methoxymethyldeoxyuridine with adenine arabinoside, 5-ethyldeoxyuridine, 5-iododeoxyuridine, and phosphoacetic acid against herpes simplex virus types 1 and 2. Antimicrob. Agents Chemother. 17, 558–566.

2 Bhamarapravati, N., Toochinda, P. and Boonyapaknavik, V. (1967) Pathology of Thailand hemorrhagic fever: a study of 100 autopsy cases. Ann. Trop. Med. Parasitol. 61, 500–510.

3 Bourque, M., Quintiliani, R. and Tilton, R.C. (1976) Synergism of cefazotin-gentamycin against Enterococci. Antimicrob. Agents Chemother. 10, 157–163.

4 Bowles, W.A., Schneider, F.H., Lewis, L.R. and Robins, R.K. (1963) Synthesis and antitumor activity of 9-(tetrahydro-2-furyl)purine analogs of biologically important deoxynucleosides. J. Med. Chem. 6, 471–478.

5 Boyum, A. (1974) Separation of blood leukocytes, granulocytes and lymphocytes. Tissue Antigens 4, 269–274.

6 Browne, M.J. (1978) Mechanism and specificity of action of ribavirin. Antimicrob. Agents Chemother. 15, 747–753.

7 Devita, V.T., Young, R.C. and Cannellos, G.P. (1975) Combination versus single agent chemotherapy – a review of the basis for selection of drug treatment of cancer. Cancer 35, 98–110.

8 Fiala, M., Chow, A.W., Miusaki, K. and Guze, L.B. (1974) Susceptibility of herpes virus to three nucleoside analogs and their combinations and enhancement of the antiviral effect at acid pH. J. Infect. Dis. 129, 82–85.

9 Galegov, G.A., Pushkarskaya, N.L., Obrosova-Serova, N.P. and Ahdanov, V.M. (1977) Combined action of ribavirin and rimantadine in experimental myxovirus infection. Experientia 33, 905–907.

10 Halstead, S.B. (1966) Mosquito borne hemorrhagic fevers of South and Southeast Asia. Bull W.H.O. 35, 3–15.

11 Halstead, S.B. (1970) Observations related to the pathogenesis of dengue hermorrhagic fever. VI. Hypothesis and discussion. Yale J. Biol. Med. 42, 350–363.

12 Halstead, S.B. and Simasthien, P. (1970) Observations related to the pathogenesis of dengue hemorrhagic fever. II. Antigenic and biologic properties of dengue viruses and their association with disease response in the host. Yale J. Biol. Med. 42, 276–292.

13 Halstead, S.B. and O'Rourke, E.J. (1977) Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. J. Exp. Med. 146, 201–217.

14 Halstead, S.B., Nimmannitya, S., Yamarat, C. and Russell, P.K. (1967) Hemorrhagic fever in Thailand: recent knowledge regarding etiology. Jpn. J. Med. Sci. Biol. 20, 96–103.

15 Halstead, S.B., Nimmannitya, S. and Cohen, S. (1970) Observations related to the pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. Yale J. Biol. Med. 42, 311–328.

16 Halstead, S.B., Udomsakdi, S., Simasthien, P., Singharaj, P., Sukhavachana, P. and Nisalak, A. (1970) Observations related to the pathogenesis of dengue hemorrhagic fever. I. Experience with classification of dengue viruses. Yale J. Biol. Med. 42, 261–275.

17 Halstead, S.B., Marchette, N.J., Chow, J.S. and Lolekha, S. (1976) Dengue virus replication enhancement in peripheral blood leukocytes from immune human beings. Proc. Soc. Exp. Biol. Med. 151, 136–139.

18 Halstead, S.B., O'Rourke, E.J. and Allison, A.C. (1977) Dengue viruses and mononuclear phagocytes. II. Identity of blood and tissue leukocytes supporting in vitro infection. J. Exp. Med. 146, 218–229.

19 Hammon, W. McD., Rudnick, A. and Sather, G.E. (1960) Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. Science 131, 1102–1103.

20 Henderson, E.S. and Sanaka, R.J. (1969) Evidence that drugs in multiple combinations have materially advanced the treatment of human malignancies. Cancer Res. 29, 2272–2280.

21 Huffman, J.H., Sidwell, R.W., Khare, G.P., Witkowski, J.T., Allen, L.B. and Robins, R.K. (1973) In vitro effect of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) on deoxyribonucleic acid and ribonucleic acid viruses. Antimicrob. Agents Chemother. 3, 235–241.

22 Jadhav, A.L., Townsend, L.B. and Nelson, A.L. (1979) Inhibition of hypoxanthine-guanine phosphoribosyl transferase. Biochem. Pharmacol. 28, 1057–1062.

23 Jawetz, E. (1968) The use of combinations of antimicrobial drugs. Ann. Rev. Pharmacol. 8, 151–170.

24 Koff, W.C., Peavy, D.L. and Knight, V. (1979) Inhibition of in vitro proliferative responses of human lymphocytes by rimantadine hydrochloride. Infect. Immun. 23, 665–669.

25 Lerner, A.M. and Bailey, E.J. (1974) Synergy of 9-β-D-arabinofuranosyladenine and human interferon against herpes simplex virus type I. J. Infect. Dis. 130, 549–552.

26 Marchette, N.J., Halstead, S.B., Falkler, W.A., Stenhouse, A. and Nash, D. (1973) Studies on the pathogenesis of dengue infection in monkeys. III. Sequential distribution of virus in primary and heterologous infections. J. Infect. Dis. 128, 23–30.

27 Miller, J.P., Kigwana, L.J., Streeter, D.G., Robins, R.K., Simon, L.N. and Roboz, J. (1976) The relationship between the metabolism of ribavirin and its proposed mechanism of action. Ann. N.Y. Acad. Sci. 284, 211–229.

28 Nisalak, A., Halstead, S.B., Singharaj, P., Udomsakdi, S., Nye, S.W. and Vinichaikul, K. (1970) Observations related to the pathogenesis of dengue hemorrhagic fever. III. Virologic studies of fatal disease. Yale J. Biol. Med. 42, 293–310.

29 Pal, R. and Brown, A.W.A. (1974) Problems of insecticide resistance. Z. Parasitenk. 45, 211–219.
30 Prusiner, P. and Sundaralingham, M. (1973) A new class of synthetic nucleoside analogs with broad spectrum antiviral properties. Nature New Biol. 244, 116–117.
31 Robins, R.K. (1964) Antitumor activity and structural relationships of purine derivatives and related compounds against neoplasms in experimental animals. J. Med. Chem. 7, 186–199.
32 Schlesinger, R.W. (1977) Dengue Viruses. Virology Monographs Volume 16 (Springer Verlag, New York) pp. 1–132.
33 Scholar, E.M. and Calabresi, P. (1973) Identification of the enzymatic pathways of nucleotide metabolism in human lymphocytes and leukemia cells. Cancer Res. 33, 94–103.
34 Sidwell, R.W. (1980) Ribavirin: in vitro antiviral activity. In: Ribavirin: a Broad Spectrum Antiviral Agent. Eds.: Smith, R.A. and Kirkpatrick, W. (Academic Press, New York) pp. 23–42.
35 Sidwell, R.W., Huffman, J.H., Khare, G.P., Allen, L.B., Witkowski, J.T. and Robins, R.K. (1972) Broad spectrum antiviral activity of Virazole: 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. Science 177, 705–706.
36 Sidwell, R.W., Robins, R.K. and Hillyard, I.W. (1979) Ribavirin: an antiviral agent. Pharmacol. Ther. 6, 123–146.
37 Smith, R.A. (1980) Mechanisms of action of ribavirin. In: Ribavirin: a Broad Spectrum Antiviral Agent. Eds.: Smith, R.A. and Kirkpatrick, W. (Academic Press, New York) pp. 99–118.
38 Streeter, D.G., Witkowski, J.T., Khare, G.P., Sidwell, R.W., Bauer, R.J., Robins, R.K. and Simon, L.N. (1973) Mechanism of action of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole), a new broad spectrum antiviral agent. Proc. Natl. Acad. Sci. U.S.A. 70, 1174–1178.
39 Wilson, S.Z., Knight, V., Wyde, P.R., Drake, S. and Couch, R.B. (1980) Amantadine and ribavirin aerosol treatment of influenza A and B infection in mice. Antimicrob. Agents Chemother. 17, 642–648.