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Combination chemotherapy: interaction of 5-methoxymethyldeoxyuridine with trifluorothymidine, phosphonoformate and acycloguanosine against herpes simplex viruses

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Summary

Methoxymethyldeoxyuridine (MMUdR) when used in combination with either trifluorothymidine (F_3TdR) or phosphonoformate (PFA) showed synergistic activity against herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) in vitro, whereas MMUdR and acycloguanosine (ACG) combination was antagonistic against herpes viruses. HSV-1 mutants resistant to ACG, arabinofuranosyladenine (Ara-A), MMUdR or PFA were isolated. Drug-resistant HSV-1 virus mutants were analyzed for cross sensitivity to ACG, Ara-A, F_3TdR, MMUdR, MMUdR-5'-monophosphate (MMUdR-MP) and PFA. The Ara-A-resistant (Ara-A^R) virus exhibited 3-fold resistance to MMUdR-MP (ID_{50} = 105 μM). The ACG-resistant (ACG^R) mutant was 160-fold less sensitive to MMUdR ($ID_{50} > 1138$ μM). The MMUdR-resistant ($MMUdR^R$) mutant remained sensitive to all other antiviral drugs in vitro. Ara-A provided protection against HSV-1 encephalitis in immunosuppressed mice inoculated with a low dose (200 PFU/mouse) of $MMUdR^R$ virus or wild-type HSV-1. F_3TdR decreased incorporation of tritiated deoxyuridine ([^{3}H]UdR) in RK-13 cells by 50% at 0.068 μM. Under similar conditions, MMUdR (up to 600 μM) and PFA (up to 208 μM) were without effect on incorporation of [^{3}H]UdR into DNA. In combination chemotherapy experiments, MMUdR (up to 300 μM) used along with F_3TdR (up to 1.08 μM) neither decreased nor enhanced cytotoxicity of F_3TdR as measured by incorporation of [^{3}H]UdR into cellular DNA. Similarly, MMUdR (up to 300 μM) in combination with PFA (up to 166 μM) was nontoxic to host cells.

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methoxymethyldeoxyuridine; trifluorothymidine; phosphonoformate; acycloguanosine; chemotherapy; herpes simplex virus

Introduction

Nucleoside analogues have received considerable attention for the treatment of herpes simplex infections [11,13,19,29,31,32,34]. However, their use is usually accompanied by adverse effects on host cells, which limit their usefulness in therapeutics [24,28,31,35]. The best possible way to overcome host cell toxicity is to develop drugs which inhibit only unique viral functions. Unfortunately, no such drug has been developed to date. Therefore, drugs which elicit antiviral activity by selectively utilizing or inhibiting virus specified functions to a greater degree than host cells are presently of major interest for the treatment of viral diseases [10,11,13,16,19,29]. In this laboratory, we have been studying the antiviral activity and biological properties of a nucleoside analogue, 5-methoxymethyldeoxyuridine (MMUdR [1–5,19]).

In addition to the problem of toxicity with antiviral drugs, another limitation is the emergence of drug-resistant mutants [8,9,26]. Combination chemotherapy has proven to be an effective means of dealing with drug toxicity and drug resistance problems in antimicrobial and cancer chemotherapy [15,23,25,27]. Therefore, it was reasoned that these limitations of antiviral drugs could also be overcome by the use of a combination of antiviral drugs. For such an approach to be therapeutically beneficial, the use of two drugs in combination should fulfill the following criteria: (a) they must interact to produce, preferably, a synergistic effect or at least an additive effect; (b) they should produce no increased toxicity relative to the agents alone; and (c) they should inhibit emergence of mutants resistant to either drug or their combination. In order to test this hypothesis, the interaction of MMUdR with other antiviral drugs was investigated in cell culture [1,3]. These studies have shown that synergistic activity can be accomplished by combination of drugs whose mechanism of action is directed at different viral synthetic pathways [3]. Furthermore, synergistic activity was achieved without concurrent increase in toxicity. Studies on the efficacy of MMUdR in combination with arabinofuranosyladenine (Ara-A) have shown that the simultaneous application of drug combinations gave a better therapeutic response than individual drugs for controlling viral keratitis and arresting the progression of herpes vaginitis. They also reduced the general problem of development of drug-resistant mutants, and prevented latency if the treatment was initiated at an early stage [2,4,30]. In view of these positive findings, further studies on the interaction of MMUdR with other antiviral drugs were undertaken.

Materials and Methods

Drugs and chemicals

Ara-A, trifluorothymidine (F_3TdR) and phosphonoformate (PFA) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. MMUdR and MMUdR-monophos-

phate (MMUdR-MP) were synthesized [19,35]. Molecular weights of drugs used in these studies are: ACG, 225; Ara-A, 267; F_3TdR, 296; MMUdR, 273; MMUdR-MP, 353; and PFA, 192. Cyclophosphamide (procytox) and acycloguanosine (ACG) were obtained from Horner Co., Montreal, P.Q., Canada and Burroughs Wellcome, Research Triangle Park, Durham, NC, U.S.A., respectively. Since Ara-A is poorly soluble in aqueous solution, a stock solution for antiviral assays was prepared as follows: the drug (1024 μg/ml) was dissolved in phosphate-buffered saline (0.05 M, pH 7.0) at 50°C, filter-sterilized and diluted with assay media (MEM containing 4% FCS) to give a final concentration of 512 μg/ml. All other drugs were dissolved at the appropriate concentration in the assay media. For in vivo studies, Ara-A was used as a suspension in sterile phosphate-buffered saline (0.1 M, pH 7.5). [*methyl*-^{3}H]Thymidine (specific activity 20 Ci/mmol) and [6-^{3}H]deoxyuridine (specific activity 17.5 Ci/mmol), NCS solubilizer and chemicals for radioactivity counting were obtained from Amersham Searle, Oakville, Ontario, Canada. Stock solutions of radioactive compounds were prepared in MEM.

Cell culture and viruses

Rabbit kidney (RK-13) cells were cultured as previously described [3,5]. Herpes simplex virus type 1 (HSV-1) strains MAC, NAH and 76 and type 2 (HSV-2) strains X-265 and MS were used in these studies. The general conditions for the preparation of virus stocks have been described previously [3,5]. Antibodies specific to each herpes virus were prepared using the procedures described earlier [3].

The MMUdR-resistant (MMUdR^R) mutant of HSV-1 strain 76 stocks were prepared by infecting confluent cultures of Hep-2 cells at a multiplicity of infection (m.o.i.) of 0.001 in the presence of 32 μg/ml of MMUdR. After approximately 7–10 days, few minute plaques were visible. The cells present in the plaque were isolated and passaged (cocultivated) with fresh uninfected Hep-2 cells. The virus released was once again cultured in the presence of 32 μg/ml of MMUdR. Numerous plaques were produced upon secondary passage in the presence of 32 μg/ml of MMUdR. The virus was subsequently passaged in progressively higher concentrations (2-fold) of MMUdR until the virus was resistant to 2000 μg/ml of MMUdR. ACG-resistant (ACG^R), Ara-A-resistant (Ara-A^R) and PFA-resistant (PFA^R) mutants of HSV-1 strain 76 were isolated in essentially the same manner as the MMUdR^R mutant, except that initial passage began at concentrations of 10 μg/ml ACG, 30 μg/ml Ara-A and 50 μg/ml PFA, respectively. The resistant strains were finally selected by growth in the presence of 100 μg/ml ACG, 64 μg/ml Ara-A and 750 μg/ml PFA. Each resistant virus strain was grown twice in drug-free medium prior to use in antiviral assays. These mutants remained resistant to their respective drugs for at least 10 passages (maximum tested) in the absence of the drugs.

Drug inhibition assays

Antiviral assays were carried out using the procedure described previously [3,5]. Briefly, confluent cell monolayers were infected with either 10 or 50 plaque forming units (PFU) of virus per well in a microtitre plate. Each antiviral drug at the appropriate concentration containing 1 or 2 neutralizing units of a specific antibody for each

herpes virus type was added to minimize the production of secondary plaques. The tissue culture plates were incubated at 37°C for 72 h prior to fixation, staining and enumeration [3,5]. In each experiment, toxicity controls (containing test compound and medium only), cell controls (containing medium only) and virus controls (containing virus and medium only) were run simultaneously. The percentage of inhibition (calculated from reduction in the number of plaques) at each concentration of the compound was determined. These data were used to draw dose–response curves for each antiviral compound. From these graphs, the concentration of each drug needed to reduce the number of plaques by 50% was determined. In combination chemotherapy experiments, each drug was dissolved at the appropriate concentration and both compounds were added together. Dose isobolograms were used to study the type of interaction between drugs [3,4]. Dose–response curves for single drugs and for each level of the first drug (e.g. MMUdR) in combination with various concentrations of the second drug (e.g. PFA) were drawn for each pair of antiviral drugs. The concentrations of various drugs in combination were multiples (0.25, 0.50, 1, 2, 4 times) of amounts required to cause 50% inhibition of PFU when used alone. From these curves, the amount of the second drug in combination required to give 50% reduction in PFU was determined. Fractional inhibitory concentrations (FIC) were then calculated for each drug by dividing the concentration of each drug in the combination by the amount of drug that would be required to give the same degree of inhibition by itself. FIC of each pair of drugs were plotted to construct isobolograms and the nature of the interaction between drugs was deduced from these figures. When the effects of two compounds are additive, the points fall on a straight line, connecting unity on the ordinate axis with unity on the abscissa axis. Deviations to the right of this theoretical line suggest interference or antagonism; deviations to the left indicate synergism between two drugs.

Virus yield studies

The effect of antiviral drugs alone and in combination on virus titre was investigated using RK-13 cultures infected with HSV-2, MS strain according to the procedure described earlier [4]. Virus control (virus and medium) and drug control (drug and medium) were run simultaneously. Antiserum was not included in the overlay. The drugs were combined in ratios on the basis of the concentrations shown in Table 2. These concentrations were chosen because each drug at this concentration, when used in combination with the other drug, resulted in maximum synergistic activity. Media from samples which had received similar treatment were pooled and titrated to determine the amount of virus present. The assay for each drug was carried out in quadruplicate.

Deoxyribonucleoside uptake studies

Incorporation of [*methyl*-^{3}H]thymidine ([^{3}H]TdR) and [6-^{3}H]deoxyuridine ([^{3}H]UdR) into DNA was studied using RK-13 cells. The cells were seeded in petri dishes (10 × 35 mm) and allowed to grow to confluency (approximately 10^6 cells/plate). The growth medium was aspirated and the appropriate concentration of each compound (2 ml) dissolved in the assay media was added to each plate. The cultures

were incubated at 37°C in a 5% CO_2 atmosphere for 24 h. Following drug exposure, the monolayers were washed twice with 1.0 ml of Hank's balanced salt solution and then incubated for 1 h with 1 ml of MEM containing 1.0 μCi of the appropriate labelled compound. The monolayers were washed twice with 2.0 ml of PBS (0.05 M, pH 7.0) and treated with trypsin–EDTA solution to dislodge cells. The cells were suspended in 2.0 ml of 10 mM Tris, pH 7.8 containing 1 mM NaCl and 1 mM EDTA and precipitated with 5% trichloroacetic acid. The precipitate was collected by filtration and washed successively with 5% trichloroacetic acid, 6% sodium pyrophosphate and 90% ethanol. After drying at 55°C for 30 min, filter discs were transferred into scintillation fluid and the amount of radioactivity incorporated into DNA was determined using a liquid scintillation counter (Beckman Model LS 8000). The composition of the scintillation fluid was: 5 g PPO; 75 mg POPOP; 1 l toluene. Control cells (without exposure to drug) were processed in an identical manner for each experiment. The results are expressed as the percentage ratio of radioactivity incorporated by cells exposed to antiviral drugs over that of concurrently processed control cells. The effect of combination of drugs on [^{3}H]deoxyribonucleoside incorporation into DNA was also investigated. In these experiments, each compound was dissolved at the appropriate concentration and both drugs were added simultaneously. The concentrations of various drugs in combination were multiples (1, 2, 4 times) of the amount needed for maximum synergistic activity against HSV-2, strain MS. The concentrations of MMUdR and F_3TdR used were (μg/ml): 5 : 0.02, 10 : 0.04, 20 : 0.08, 40 : 0.16 and 80 : 1.32; whereas the concentrations of MMUdR and PFA used were (μg/ml): 40 : 4, 80 : 8, 160 : 16 and 320 : 32.

Efficacy of Ara-A against HSV-1 encephalitis

Animals White Swiss mice were obtained from the Animal Resources Centre, University of Saskatchewan. Male mice, weighing 18–20 g, were used in all experiments to ensure uniform susceptibility to infection. The animals were housed in special quarters with ventilation and isolation techniques designed for infectious disease studies. Food (mouse pellets) and water were available ad libitum at all times. The temperature was maintained at 21°C.

Lethal effects of HSV-1 180 mice were injected intraperitoneally (i.p.) with a single dose (250 mg/kg) of cyclophosphamide to suppress their immune response. After 30 min, the mice were inoculated intravenously with 0.05 ml of various dilutions of MMUdR^R virus or HSV-1, strain 76. The dosages of virus used were 200, 2000 or 20 000 PFU/mouse. Virus dilutions were prepared in sterile MEM.

Experimental design Infected mice were randomly separated into groups of 10. Treatment was initiated 6 h following virus inoculation. Ara-A was administered i.p. as a single daily injection at dosages of 80 mg/kg per day and 400 mg/kg per day for 5 days. Infected control animals (virus controls) received a volume of vehicle equal to the volume of injected drug. The mice were observed for 21 days following inoculation. The criteria used to determine efficacy in these studies were number of survivors at 21 days and increased length of survival time. Mice were inspected twice daily, at 10 a.m. and 10 p.m. Deaths were recorded at the beginning of each period.

Groups of 5 mice (uninfected) treated with cyclophosphamide alone or cyclophos-

phamide and Ara-A for each treatment level were run simultaneously to ensure deaths were not due to drug alone.

Results

Relative antiviral activity of ACG, F_3TdR, MMUdR, and PFA against HSV-1 and HSV-2

F_3TdR and ACG were found to be potent inhibitors of both HSV-1 and HSV-2 and their 50% inhibitory levels (ID_{50}) ranged from 0.1 to 4 μM. These ID_{50} values correspond closely to previously reported ID_{50} values [14,16,21]. The antiviral potency of PFA against HSV-2 was greater than HSV-1 with the ID_{50} values for PFA ranging from 5 to 40 μM against HSV-2 strains and 16 to 110 μM against HSV-1 strains. These results are in general agreement with values reported earlier [14,22].

Collateral sensitivity of drug-resistant mutants

In order to determine whether resistance to one drug would also alter susceptibility to the other drugs, drug-resistant viruses were analyzed for cross-resistance to six antiviral agents (MMUdR, MMUdR-MP, ACG, F_3TdR, Ara-A and PFA) by determining the concentration of drug that inhibited 50% of virus plaque formation (Table 1). The HSV-1 (strain 76) wild type virus was sensitive to all antiherpes drugs tested. The ID_{50} values for drug-resistant mutants of HSV-1 were 27, 105, 573 and 1176 μM for ACG, Ara-A, PFA and MMUdR, respectively. The $MMUdR^R$ mutant remained sensitive to ACG, Ara-A and PFA, but became approximately 2-fold more resistant to its corresponding 5'-monophosphate, MMUdR-MP. The $Ara\text{-}A^R$ virus exhibited a 3-fold resistance to MMUdR-MP ($ID_{50} = 145$ μM) but remained sensitive to MMUdR, F_3TdR, PFA and ACG. The susceptibility of the ACG^R mutant to MMUdR ($ID_{50} > 1138$ μM) was approximately 160-fold less than the wild type virus. No change

TABLE 1

Relative in vitro sensitivities to antiherpes drugs of drug-resistant mutants and of parental strain of herpes simplex virus

Compound	ID_{50} concentration[a] (μM)				
	Wild type (HSV-1, strain 76)	$MMUdR^R$	ACG^R	$Ara\text{-}A^R$	PFA^R
MMUdR	7.0	1176.0	>1138.0	7.0	9.0
MMUdR-MP	51.0	91.0	65.0	145.0	51.0
ACG	0.9	0.7	27.0	0.3	1.1
F_3TdR	0.5	0.07	0.01	0.4	0.5
Ara-A	30.0	19.0	19.0	105.0	30.0
PFA	31.0	31.0	31.0	57.0	573.0

Antiviral assays were carried out using RK-13 cells. All drugs were added immediately after virus infection.
[a] Concentration required to cause 50% reduction in plaque numbers. Amount of virus used was 50 PFU.

in susceptibility of this mutant to MMUdR-MP, Ara-A and PFA was observed. Interestingly, the ACG^R virus and $MMUdR^R$ virus were considerably more susceptible to F_3TdR than the wild type strain. The PFA^R virus remained sensitive to MMUdR, MMUdR-MP, Ara-A and ACG.

Inhibition of HSV-1 and HSV-2 plaque formation by simultaneous treatment with combination of two antiviral drugs

MMUdR in combination with F_3TdR or PFA resulted in a greater degree of inhibition of plaque formation than either drug alone (Fig. 1A, B). In contrast, the combination of MMUdR with ACG was less effective than for the individual drugs (Fig. 1C). Analysis of this data by dose isobolograms revealed that MMUdR in combination with either F_3TdR or PFA showed synergistic activity against both HSV-1 and HSV-2, whereas its interaction with ACG was antagonistic (Fig. 2). The amounts of each drug required to inhibit viral plaques by 50% when used alone and in combination are presented in Table 2.

Effect of antiviral drugs on HSV-2 replication (virus yield)

The concentration of each compound needed to inhibit in vitro production after

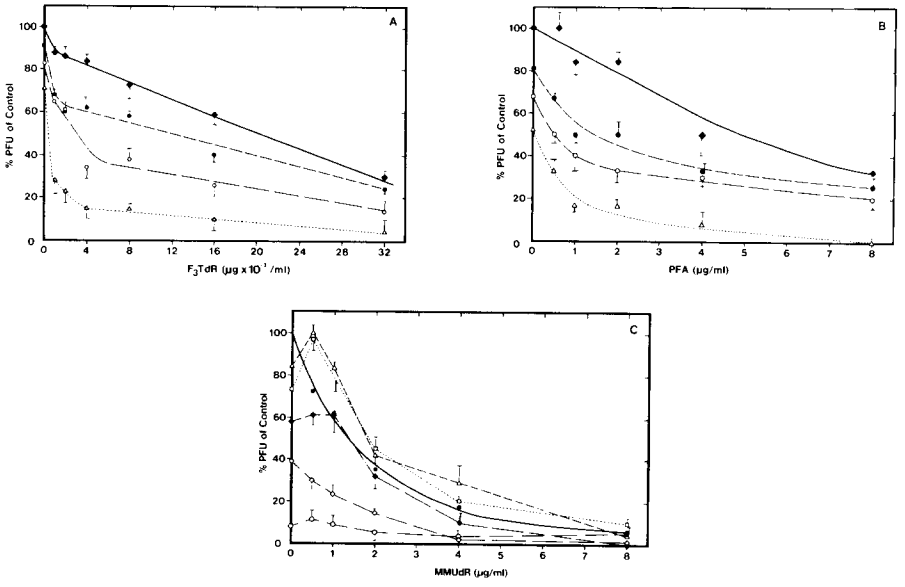

Fig. 1. Dose–response curves. (A) MMUdR and F_3TdR combination against HSV-2, strain MS. MMUdR concentrations in combination with F_3TdR were: zero (◆—◆), 4 μg/ml (●---●), 8 μg/ml (○-.-○) and 16 μg/ml (△...△). (B) MMUdR and PFA combination against HSV-2, strain MS. MMUdR concentrations in combination with PFA were: zero (◆—◆), 8 μg/ml (●---●), 16 μg/ml (○---○) and 32 μg/ml (△...△). (C) MMUdR and ACG combination against HSV-1, strain 76. ACG concentrations in combination with MMUdR were: zero (●—●), 0.03 μg/ml (△...△), 0.06 μg/ml (□...□), 0.12 μg/ml (◆-.-◆), 0.25 μg/ml (□-...-□), and 0.5 μg/ml (○-...-○). The points lying on the response axis (—) represent % PFU of control produced by these MMUdR (A and B) or ACG (C) concentrations in single-drug experiments performed concurrently with the combination experiments. Antiviral assays were carried out using RK-13 cells.

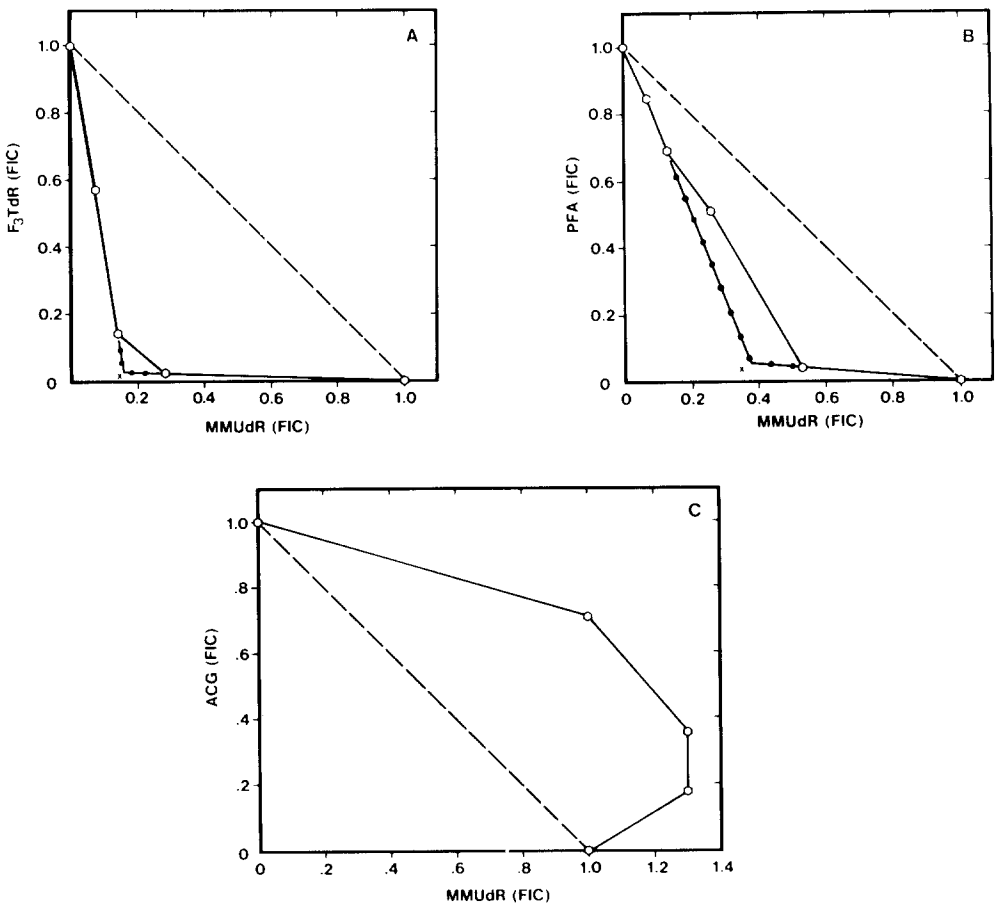

Fig. 2. Isobolograms for combinations of antiviral drugs: (A) MMUdR and F_3TdR against HSV-2, strain MS; (B) MMUdR and PFA against HSV-2, strain MS; and (C) MMUdR and ACG against HSV-1, strain 76. When the effects of two compounds are additive, the points fall on a straight line, connecting unity on the ordinate axis with unity on the abscissa axis (—). Deviations to the left of this theoretical line indicate synergism between two drugs (A and B, ●—●. x is the point of maximum synergy); deviations to the right suggest interference or antagonism (C, ○——○). Antiviral assays were performed using RK-13 cells.

incubation for 72 h was: MMUdR (256 μg/ml), F_3TdR (>0.16 μg/ml) and PFA (8 μg/ml). In combination, MMUdR (18 μg/ml) plus F_3TdR (0.0008 μg/ml) and MMUdR (18 μg/ml) plus PFA (1.4 μg/ml) prevented virus production. After washing of drugs and incubation of cells with fresh maintenance medium for 3 more days, the amounts of each compound required to prevent virus production were: 256 μg/ml MMUdR, 0.16 μg/ml F_3TdR and >32 μg/ml PFA. In combination, 36 μg/ml MMUdR plus 0.0016 μg/ml F_3TdR and 72 μg/ml MMUdR plus 5.6 μg/ml PFA prevented production of infectious virus particles. These results show that a considerably enhanced antiviral effect resulted when MMUdR was used in combination with

TABLE 2

Effectiveness of the combination of MMUdR with F_3TdR and PFA against HSV-1 and HSV-2 in vitro

Virus[b]	Drug combination	Amount of each drug required[a]	
		In combination (μg/ml)	Alone (μg/ml)
HSV-1	(A) MMUdR	0.03	0.95
	+		
	F_3TdR	0.015	0.06
HSV-2	(B) MMUdR	10.0	36
	+		
	PFA	0.7	5
HSV-1	(C) MMUdR	0.7	1.9
	+		
	PFA	0.5	8.4
HSV-2	(D) MMUdR	9.3	58
	+		
	F_3TdR	0.0004	0.02

[a] The concentration required to inhibit viral plaque formation by 50%. The ID_{50} concentration for each drug when used in combination was determined by multiplying the fractional inhibitory concentration (FIC) at maximum synergy (e.g., point x in Fig. 2A, B) by the amount of single drug that will cause 50% inhibition of PFU.

[b] Antiviral assays were carried out using RK-13 cells. HSV-1, strain 76 and HSV-2, strain MS were used for infection. Amount of virus used was either 10 PFU (A and B) or 50 PFU (C and D). The virus titre was varied to determine the effect of MMUdR on virus inoculum.

either F_3TdR or PFA. Furthermore, when these compounds were used in combination, a substantially lower concentration of each compound was required to effect the same degree of virus yield reduction over the activity of these compounds when used individually. The relationship between the dose of drugs and virus yield is shown in Fig. 3.

Inhibitory effects of F_3TdR and PFA on normal cell morphology and cellular DNA synthesis

For any drug to be useful in vivo, the concentrations required to inhibit viral replication should be considerably lower than those that cause alterations of cellular functions. The minimum concentrations required to produce definite evidence of microscopic toxicity (granulation, vacuolation or cytomegaly) in confluent monolayers of RK-13 cells were: 0.4 μM F_3TdR; 1330 μM PFA; >2276 μM ACG and 3760 μM MMUdR. Cytotoxicity was also determined by studying the incorporation of [^{3}H]UdR or [^{3}H]TdR into DNA. The relationship between the dose of antiviral drugs and the incorporation of deoxyribonucleosides into the DNA of RK-13 cells is shown in Fig. 4. The addition of F_3TdR to RK-13 cells resulted in potent inhibition of [^{3}H]UdR incorporation into cellular DNA. At a concentration of 0.5 μg/ml of F_3TdR, [^{3}H]UdR incorporation was <20% of control. Under similar conditions, 160 μg/ml of MMUdR (highest concentration tested) had little effect on the incorporation of

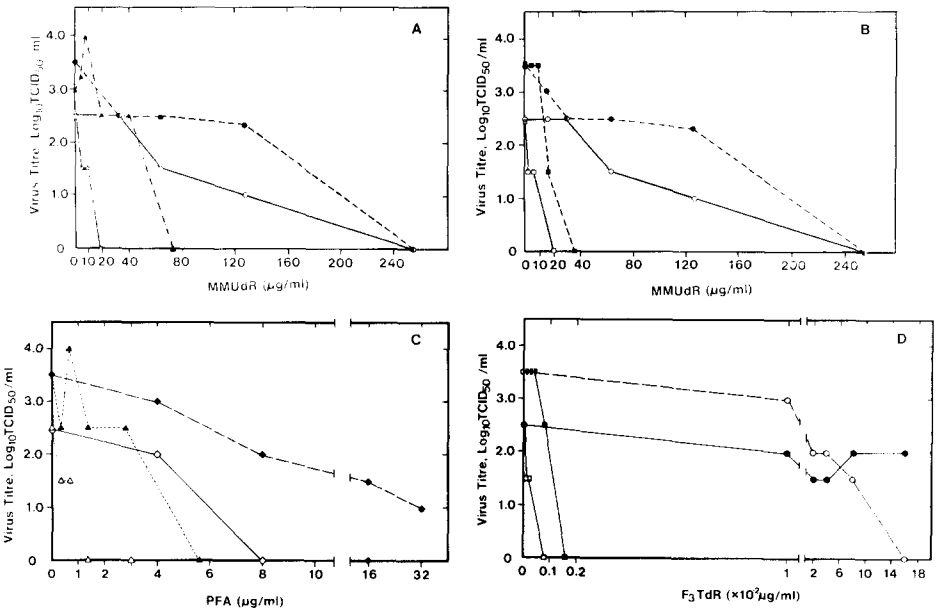

Fig. 3. Effect of antiviral drugs singly, and in combination, on infectious virus particles yield: (1) after incubation of infected cells in the presence of each compound for 72 h: MMUdR (○---○), PFA (◇—◇) and F_3TdR (○---○); (2) after washing of drugs and incubation of cells with fresh maintenance medium for 72 h more: MMUdR (●---●), PFA (◆---◆) and F_3TdR (●---●). Concentrations (μg/ml) of drugs when used alone were: MMUdR, 16, 32, 64, 128 and 256; F_3TdR, 0.01, 0.02, 0.04, 0.08 and 0.16; and PFA, 4, 8, 16 and 32. For combination chemotherapy experiments, MMUdR and PFA were used in the ratios (μg/ml) 4.5:0.35, 9:0.7, 18:1.4, 36:2.8 and 72:5.6, whereas MMUdR and F_3TdR were combined in the ratios 2.25:0.0001, 4.5:0.0002, 9:0.0004, 18:0.0008 and 36:0.0016. After incubation of infected cells for 72 h with combination of drugs: (A) MMUdR + PFA (△—△); (B) MMUdR + F_3TdR (□—□); (C) PFA + MMUdR (△...△); and (D) F_3TdR + MMUdR (□—□). After washing of drugs and incubation of cells with fresh maintenance medium for 72 h more: (A) MMUdR + PFA (△-.-△); (B) MMUdR + F_3TdR (■---■); (C) PFA + MMUdR (▲---▲); and (D) F_3TdR + MMUdR (■—■). The media from samples which had received similar treatment were pooled and titrated to determine the amount of virus present. The antiviral assays were carried out using HSV-2 strain MS in RK-13 cells. Amount of virus used was 20 PFU.

[^{3}H]UdR [35], whereas 100 μg/ml of PFA reduced incorporation of [^{3}H]UdR by 27%. Both F_3TdR and PFA stimulated the uptake of [^{3}H]TdR. In this respect the behaviour of F_3TdR and PFA is similar to EtUdR [10] and other fluorodeoxyuridine derivatives [35,37]. In contrast, the incorporation of [^{3}H]TdR was not affected on exposure to 160 μg/ml of MMUdR [35]. In order to determine whether MMUdR would enhance cytotoxicity of F_3TdR, the effect of MMUdR in combination with F_3TdR on [^{3}H]UdR incorporation was also studied. In combination, MMUdR (up to 80 μg/ml) neither decreased nor enhanced cytotoxicity of F_3TdR. Similarly, MMUdR (up to 320 μg/ml) plus PFA (up to 32 μg/ml) was nontoxic to host cells as measured by incorporation of [^{3}H]UdR into cellular DNA (Fig. 4).

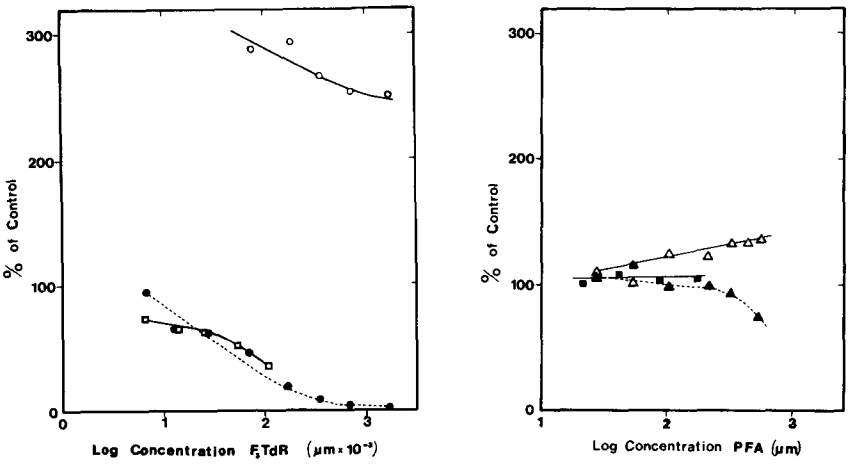

Fig. 4. Effect of antiviral drugs on [^{3}H]UdR and [^{3}H]TdR incorporation into DNA of RK-13 cells. [^{3}H]UdR: F_3TdR (●---●); PFA (▲---▲); F_3TdR + MMUdR (□—□); PFA + MMUdR (■---■). [^{3}H]TdR: F_3TdR (○—○) and PFA (△—△). In combination, MMUdR and F_3TdR were used in the ratios (μg/ml) 5:0.02, 10:0.04, 20:0.08, 40:0.16, and 80:1.32. Similarly MMUdR and PFA were combined in the ratios (μg/ml) 40:4, 80:8, 160:16 and 320:32.

Efficacy of Ara-A against HSV-1 encephalitis

Animals inoculated with MMUdR^R virus or wild type virus developed characteristic neurological symptoms between 4 and 8 days post-inoculation. The average survival time for untreated mice was 5, 6.5 and 9 days after inoculation of 2×10^2, 2×10^3 and 2×10^4 PFU respectively of either wild type or MMUdR^R virus. Ara-A provided complete protection against HSV-1 encephalitis to mice inoculated with MMUdR^R virus or wild type HSV-1, provided the virus dose used for infection was low (200 PFU/mouse). In contrast, Ara-A was only partially effective (30–50% mortality) if mice were inoculated with a higher dose (2×10^3 PFU/mouse) of virus. Again, no difference in the levels of protection was observed whether MMUdR^R virus or wild type virus was used for inoculation. However, if the virus inoculation dose was increased to 2×10^4 PFU/mouse, Ara-A was not efficacious even after administration of 400 mg/kg per day.

Discussion

The major goal of chemotherapy is selective inhibition of the infective organism with a minimal effect on host cells. For F_3TdR, an inhibitory effect on HSV-1 replication was obtained at a concentration only marginally lower than the concentration at which normal cell metabolism was impaired (as monitored by cellular DNA synthesis, Fig. 4). Thus, F_3TdR by itself has a very poor margin of safety. These results are in agreement with earlier reports [14,21]. Since F_3TdR is very efficiently phosphorylated by cellular kinases and F_3TdR-5′-monophosphate is a potent inhibitor of

thymidylate synthetase [21], this could presumably explain the cellular toxicity of F_3TdR. PFA has a considerably better margin of safety than F_3TdR, but it has a tendency to accumulate in bones, albeit to a lesser degree than its congener phosphonoacetic acid [22,33]. The synergistic interaction observed in cell culture when MMUdR was used in combination with either F_3TdR or PFA is interesting and possibly of therapeutic importance because the amount of each compound required for antiviral activity was far less than when each was used alone. These results warrant in vivo trials; studies on the efficacy of the combination of MMUdR and F_3TdR against keratitis in rabbits, and MMUdR in combination with PFA for the treatment of herpes genitalis using a guinea pig model are in progress.

Another very important finding of these studies was that MMUdR in combination with F_3TdR or PFA was far more effective in reducing the yield of infectious virus particles in comparison to individual drugs. Complete inhibition of viral replication (virucidal effect) was achieved at concentrations considerably lower than when virus was exposed to the individual drugs. The observation that MMUdR, when used in combination, did not enhance cytotoxicity of F_3TdR or PFA (measured by inhibition of cellular DNA synthesis) is also of importance because these results indicate that synergistic inhibition of herpes simplex virus replication was most likely due to a direct effect on the metabolic functions of the virus and not due to interference with host cell metabolism. The results of virus yield experiments and antimetabolic activity reported in this communication complement our earlier findings on the interaction of MMUdR with other antiviral drugs [2,4,30,35]. In addition, these studies further support the hypothesis suggested by us earlier [2,4] that by the judicious choice of antiviral drug combinations it should be possible to achieve maximum antiviral effects and simultaneously minimize toxicity.

Although the reasons for synergy at the biochemical level are not known at this time, a number of theoretical mechanisms have been proposed [18,20]. Our rationale for drug combinations was based on the hypothesis that by selecting pairs of drugs which interfere with viral replication at different enzymatic steps, enhancement or synergistic activity may result due to a sequential blockade, concurrent inhibition or complementary inhibition. The selective antiviral activity of MMUdR results from its phosphorylation by the virus-induced deoxypyrimidine kinase [38] and MMUdR-5′-triphosphate is a potent competitive inhibitor of viral DNA-dependent DNA polymerase (Gupta et al., unpublished results). F_3TdR inhibits competitively both viral-induced and cellular dT-kinases [21]. The 5′-triphosphate of F_3TdR has been shown to inhibit viral-induced DNA polymerase to a greater degree than the cellular enzymes and also produces defective virions by incorporating into virion DNA [21,37]. PFA has been shown to interfere with the pyrophosphate binding sites on DNA polymerase and is a noncompetitive inhibitor of virus-induced DNA polymerase [17,33]. This is generally believed to be responsible for its antiviral activity. Thus F_3TdR, MMUdR and PFA inhibit viral replication by interfering at different biochemical steps in the pathways of DNA synthesis. Therefore synergistic interactions observed between MMUdR–F_3TdR and MMUdR–PFA seem to be consistent with the predictions made on the basis of kinetic models for inhibitor or substrate–enzyme interactions [18,20]. However, we do recognize that these standard explanations are

too simplistic for understanding the overall mechanism of action of inhibitors of nucleic acid synthesis, because of a complex system of reactions which tightly regulate the metabolic machinery of the cell through a network of feedback and activation controls. The alternative hypothesis put forward by Cheng and colleagues that cytotoxic drugs may have enhanced activity by the mechanisms of 'self potentiation' may also apply to antiviral drugs [6,7]. Metabolic studies using labelled compounds are necessary to understand the biochemical basis of interaction between multiple agents. Further work on these aspects will be undertaken after synthesis of radioactive compounds.

Development of drug resistance to antiviral drugs is a phenomenon that occurs naturally [8,9] and studies reported in this paper substantiate earlier findings. However, the results of the cross sensitivity of virus mutants ACG^R, Ara-A^R, $MMUdR^R$ and PFA^R to other antiviral drugs suggest that alternative drug therapy should be possible for the treatment of herpes simplex infections which may fail to respond because of the development of drug-resistant mutants during therapy. The observation that Ara-A was equally effective in protecting mice inoculated with $MMUdR^R$ virus or wild type virus gives credence to this suggestion. Simultaneous application of MMUdR and Ara-A for the treatment of herpes simplex vaginitis in experimental models indicates that development of drug-resistant mutants was largely prevented [2]. Thus, it is possible that the use of MMUdR with either F_3TdR or PFA may also decrease the problem of development of drug-resistant mutants to these antiviral drugs.

The results of antiviral activity experiments indicate that a considerable degree of variation in susceptibility occurs between herpes simplex virus types and isolates within these types. Thus, if a specific isolate is susceptible to one drug, it does not mean that similar susceptibility to other drugs exists. Therefore, further success of drug treatments could be based on determining drug sensitivity prior to initiation of treatment, or during treatment, so as to optimize the therapeutic regimen with antiviral drugs.

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