Fluorinated Pyrimidines. XXXI. Mechanisms of Inhibition of Vaccinia Virus Replication in HeLa Cells by Pyrimidine Nucleosides* (33480)

MAKOTO UMEDA[1] AND CHARLES HEIDELBERGER[2]
(Introduced by H. P. Rusch)
McArdle Laboratory for Cancer Research, The Medical School, University of Wisconsin, Madison, Wisconsin 53706

It is known that local application to rabbit or human eyes afflicted with *herpes simplex keratitis* of 5-iodo-2′-deoxyuridine (IUDR),[3] 5-bromo-2′-deoxyuridine (BUDR), and cytosine arabinoside (CA) results in therapeutic antiviral activity (1–3). However, 5-fluoro-2′-deoxyuridine (FUDR), a potent inhibitor of DNA synthesis (4), and of vaccinia virus replication in tissue culture (5), had no effect in the *in vivo* herpes system (6), although there is one clinical report of its activity by frequent instillation into the eye at high concentrations (7). The 5-trifluoromethyl-2′-deoxyuridine (trifluorothymidine, F_3TDR), which was first synthesized in this laboratory (8), also inhibits DNA synthesis as a consequence of inhibition of thymidylate synthetase (9) (the same enzyme inhibited by the nucleotide of FUDR), is incorporated into the DNA of bacteriophage T4 (10) and mammalian cells (11), is a powerful tumor-inhibitory compound (12) that is now undergoing clinical trial in advanced cancer patients, and has a potent antiviral effect against herpes and vaccinia infections of the rabbit's eye (13). In fact, F_3TDR was found in that system to be considerably more active on a molar basis than BUDR, IUDR, and CA (14), and was active against an IUDR-resistant strain of the virus (13).

In the present report, a comparative study of the antiviral activity of these pyrimidine nucleoside analogs has been carried out, using vaccinia virus grown in HeLa cell cultures, in order to obtain further information on their mode of action.

Materials and Methods. Cells, virus, and media. HeLa S3 cells (mycoplasm-free), grown in shaker culture, were used both for experiments on the growth inhibition tests of host cells and for the virus experiments. The W.R. strain of vaccinia virus, a neurotropic strain, had been passaged through mouse brain, and a suspension of infected mouse brain was kindly supplied to us by Dr. D. L. Walker of this University. The virus stock used in these experiments was grown in HeLa cells for more than 5 passages before use.

The medium used for cell growth in shaker culture was MEM for suspension culture supplemented with 10% calf serum, 0.1% Pluronic F18 (14), and antibiotics (S-medium). For the medium in the colony formation experiments, MEM plus 10% calf serum and antibiotics was employed. For the maintenance medium for virus growth, the calf serum was replaced in the S-medium by 0.1% bovine serum albumin fraction V (MS-medium). All media and sera were obtained from the Grand Island Biological Co., Grand Island, N. Y.

Chemicals. The FUDR was generously supplied by Hoffmann-LaRoche, Inc., and CA by the Upjohn Co. The F_3TDR was supplied by the Cancer Chemotherapy National Service Center, Bethesda, Md. The

* This work was supported in part by Grant CA-7175 from the National Cancer Institute, National Institutes of Health, US Public Health Service. A preliminary abstract of part of this work appeared in Federation Proc. 26, 450 (1967).

[1] Holder of National Institutes of Health International Research Fellowship 2 F05 TW-950-02. Present address: Tissue Culture Laboratory, School of Medicine, Yokohama City University, Yokohama, Japan.

[2] American Cancer Society Professor of Oncology.

[3] Abbreviations used in this work: IUDR, 5-iodo-2′-deoxyuridine; FUDR, 5-fluoro-2′-deoxyuridine; F_3TDR, 5-trifluoromethyl-2′-deoxyuridine; CA, cytosine arabinoside; TDR, thymidine; CDR, deoxycytidine; BUDR, 5-bromo-2′-deoxyuridine; UDR, 2′-deoxyuridine; MEM, minimum essential medium.

IUDR was purchased from the Aldridge Co., Milwaukee, Wis.

Colony cell counts. After treatment of cells with the drugs for 1 or 2 days in MS-medium, viable cells were determined by colony formation. The cell suspension was diluted 10 × serially, and 0.1 ml of several dilutions was plated in petri dishes containing 5 ml. of prewarmed S-medium. After cultivation for 1 week at 37°, the dishes were washed with saline, fixed with methanol, and stained with Giemsa. The colonies visible to the naked eye were counted, and the plating efficiency was determined and compared to that of untreated controls (85–90%).

Conditions of virus infection. HeLa cells, after being washed with MS-medium, were suspended in the vaccinia virus stock solution diluted with MS-medium at a cell density of 1–2 × 10^6 cells/ml and with a virus multiplicity of 10–15. The cell–virus mixture was incubated for 1 hr at 37° in a shaking incubator, washed with medium to remove unadsorbed virus, and was suspended in the experimental medium, consisting of MS-medium supplemented with various concentrations or combinations of the drugs and metabolites at a cell density of 2 × 10^5 cells/ml. They were then incubated with shaking at 37° until the samples were removed for plaque assays.

Plaque assay. The virus titers were measured by the plaque formation technique using a starch overlay on HeLa cell monolayers (15). Serial 10-fold dilutions of sonicated infected cell samples were frozen at —20° until the assay, and prepared in MEM plus 0.1% bovine serum albumin fraction V. Complete monolayers of HeLa cells in petri dishes were inoculated with 0.2 ml of the appropriate virus dilutions and incubated for 1 hr at 37° in a humidified 5% CO_2 incubator with occasional shaking. Two plates were used for each dilution. Then the dishes received an overlay medium consisting of 85% MEM and 15% tryptose phosphate broth (Difco), to which was added 0.8% starch (starch hydrolyzed for gel-electrophoresis, Lot 245-1, Connaught Medical Research Laboratories, Toronto, Canada). A second overlay to which 0.01% neutral red was added was performed on the second day, and the plaques were counted the following day. Virus titers were expressed as plaque forming units per ml (pfu/ml).

Results. The effects under identical conditions of various concentrations of the four pyrimidine nucleoside analogs on the replication of vaccinia virus in HeLa cell cultures are shown in Fig. 1. All compounds produced an inhibition of vaccinia viral replication at 10^{-6} *M*, with the highest activity shown by F_3TDR which inhibited at 10^{-7} *M*. In contrast to the other compounds, FUDR exerted its inhibition primarily during the first day, after which the rate of virus production increased.

The toxicity of the compounds to HeLa cells cultured in the MS-medium under the conditions for viral infection (the cell number of the controls doubled in 2 days) is shown in Fig. 2. The cell viability was determined after 1-/and 2-days treatment with the drugs by colony counting as described in "Materials and Methods" and is expressed as plating efficiencies as percentage of the untreated controls. Comparison of the data in Figs. 1 and 2 reveals, particularly at 2 days, that IUDR and F_3TDR are more toxic to virus replication than to the cells, FUDR is equally toxic, and CA more toxic to the cells than to viral replication. Thus, at least in this system, IUDR and F_3TDR have a considerable therapeutic advantage over FUDR and CA.

Since IUDR, FUDR, and F_3TDR are analogs of thymidine, and it is known that deoxycytidine reverses the effects of CA in various systems, experiments were carried out to see whether simultaneous administration of the normal metabolite and analog would reverse the inhibitory effect of these drugs on vaccinia viral replication. As shown in Fig. 3, in all cases reversal did occur, and viral replication was observed. Equimolar concentrations of TDR reversed the inhibitory effects of IUDR, CA, and F_3TDR. In the case of FUDR, lower concentrations of TDR also reversed the inhibition.

It is known that FUDR blocks DNA synthesis as a consequence of the inhibition by its nucleotide (FUDRP) of thymidylate synthetase, but it is not incorporated into DNA

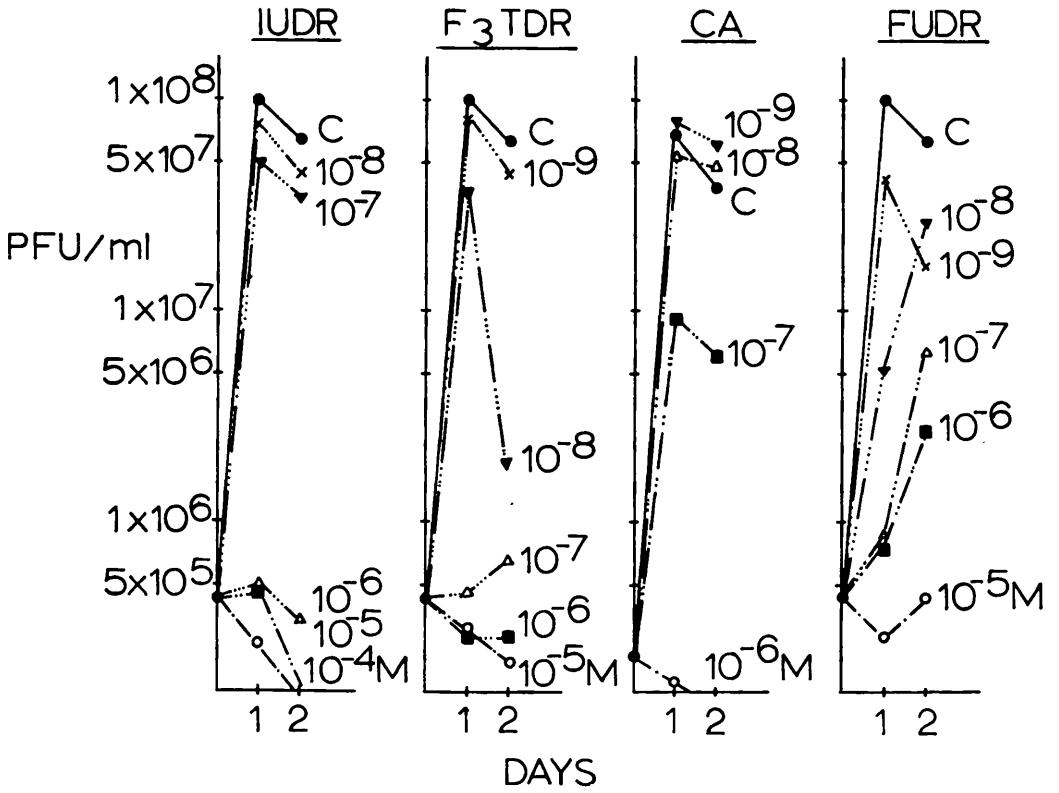

FIG. 1. The effects of pyrimidine nucleoside analogs added at the time of infection of vaccinia viral replication in HeLa cells. For the exact conditions, see "Materials and Methods."

(4). On the other hand, the nucleotide of F_3TDR also inhibits thymidylate synthetase (9), but the analog is incorporated into DNA (10). It has been demonstrated that IUDR is incorporated into the DNA of vaccinia virus (16). Therefore, it seemed likely that the greater selectivity of the inhibition of vaccinia viral replication produced by IUDR and F_3TDR as compared to FUDR and CA might be a consequence of the incorporation of the former two compounds into the DNA of vaccinia virus. The situation with respect to the incorporation of CA into DNA is at present unclear (see "Discussion"). With these considerations in mind, the experiments shown in Fig. 4 were designed. The virus-infected cells were exposed to inhibitory doses of the drugs for 1 day, and were then washed and treated with equimolar concentrations of TDR or CDR known (from Fig. 3) to reverse completely the inhibition produced by the drugs when given simultaneously. In Fig. 4 it is demonstrated that at equimolar concentrations there was almost complete rescue of virus production by TDR from FUDR inhibition. In the case of IUDR and F_3TDR treatment there was no rescue by TDR above the level of the input multiplicity of the virus. However, when CDR was added 1 day after treatment with CA some rescue of virus production was achieved, amounting to a fourfold increase over the input multiplicity. These results clearly show that during the first day of infection in the presence of IUDR and F_3TDR some irreversible event occurs, which cannot be remedied by subsequent TDR addition. It seems most reasonable to suppose that this event is the incorporation of IUDR and F_3TDR into viral DNA, which is known for IUDR (16) and has recently been demonstrated also for F_3TDR (17).

Discussion. It is clear from the experiments presented here that the inhibition by

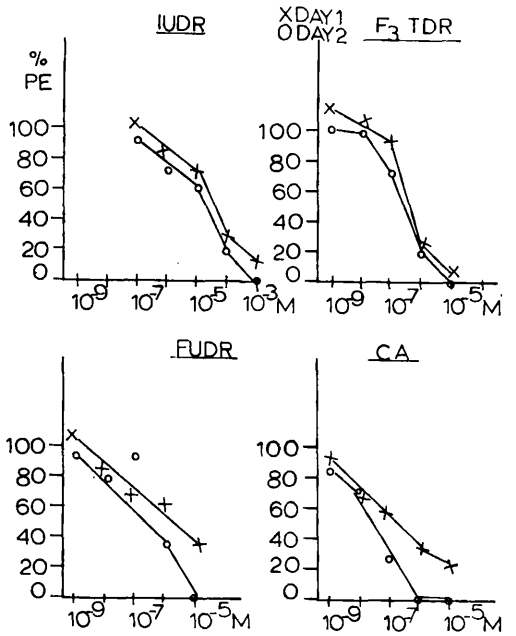

FIG. 2. The plating efficiencies of HeLa cells as compared with the untreated controls, 1 and 2 days after incubation with various concentrations of the pyrimidine nucleoside analogs in MS-medium under the conditions used for viral infection.

FUDR of the replication of vaccinia virus in HeLa cell cultures results from an inhibition of viral DNA synthesis, since the viral replication in the presence of FUDR could be reversed by thymidine given simultaneously, and rescued after delayed thymidine addition. With IUDR and F_3TDR, although the inhibition of viral growth was reversed when TDR was given at the same time, the replication could not be rescued when TDR was supplied to the cells 1 day after infection and drug addition. The concentration of IUDR required for irreversibility was 10^{-4} *M* (demonstrated in other experiments), whereas only 10^{-6} *M* F_3TDR was needed, which confirms in this system the greater activity of F_3TDR than of IUDR previously reported for the inhibition of *herpes simplex* keratitis in the rabbit's eye (14). The case of CA is more complicated. Although its inhibition of viral replication was reversed by CDR, in the delayed experiment the rescue of virus production was only partial. This analog is converted to the mono- and triphosphates, and it has been claimed that its primary effect is to inhibit the enzymes catalyzing the conversion of cytidine diphosphate to deoxycytidine diphosphate (18), but the conclusion that this effect is biologically significant has been disputed (19). The incorporation of CA into the DNA of L cells (20) and leukemic cells (21) in culture has been reported, but no evidence for the incorporation of CA triphosphate into DNA in a purified DNA polymerase system could be detected (22). Therefore, the mode of action of this interesting analog is not clear. It appears from the results of our reversal and rescue experiments, that the major cause of the inhibition of vaccinia viral replication produced by CA in this system is the inhibition of DNA synthesis. However, the fact that virus production could not be rescued completely, suggest that there may be some incorporation of the analog into viral DNA. Since we are primarily interested in the mechanism of the antiviral activity of F_3TDR, studies of this analog are continuing (17).

Summary. A comparative study of the effects of four pyrimidine nucleoside analogs on the replication of vaccinia virus in HeLa cell cultures was carried out. It was found that 5-iodo-2′-deoxyuridine and 5-trifluoromethyl-2′-deoxyuridine were potent irreversible inhibitors, with selective toxicity to the virus as compared with the cells. Cytosine arabinoside and 5-fluoro-2′-deoxyuridine had no selective toxicity against the virus, and the inhibition produced by the latter compound was transient. When given simultaneously, thymidine reversed the inhibitory effects of 5-fluoro-2′-deoxyuridine, 5-trifluoromethyl-2′-deoxyuridine, and 5-iodo-2′-deoxyuridine; and deoxycytidine reversed the inhibition produced by cytosine arabinoside. If the infected cells were treated with the drugs for 1 day, washed, and normal metabolites added, complete virus production was rescued by thymidine in the case of 5-fluoro-2′-deoxyuridine, partial rescue by deoxycytidine from cytosine arabinoside, but no rescue above the level of the input multiplicity was achieved by thymidine from the inhibition produced by 5-iodo-2′-deoxyuridine and 5-trifluoromethyl-2′-deoxyuridine. These results

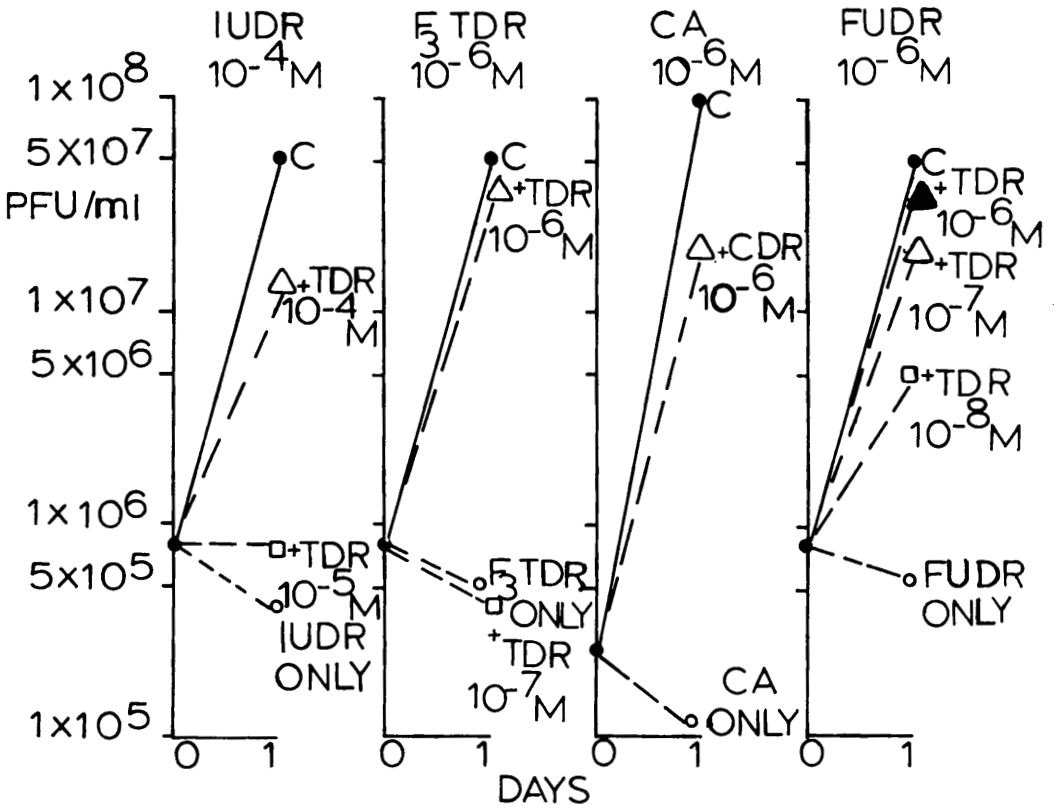

FIG. 3. The reversal by normal metabolites, given simultaneously with the pyrimidine nucleoside analogs, of the inhibition of vaccinia viral replication in HeLa cells.

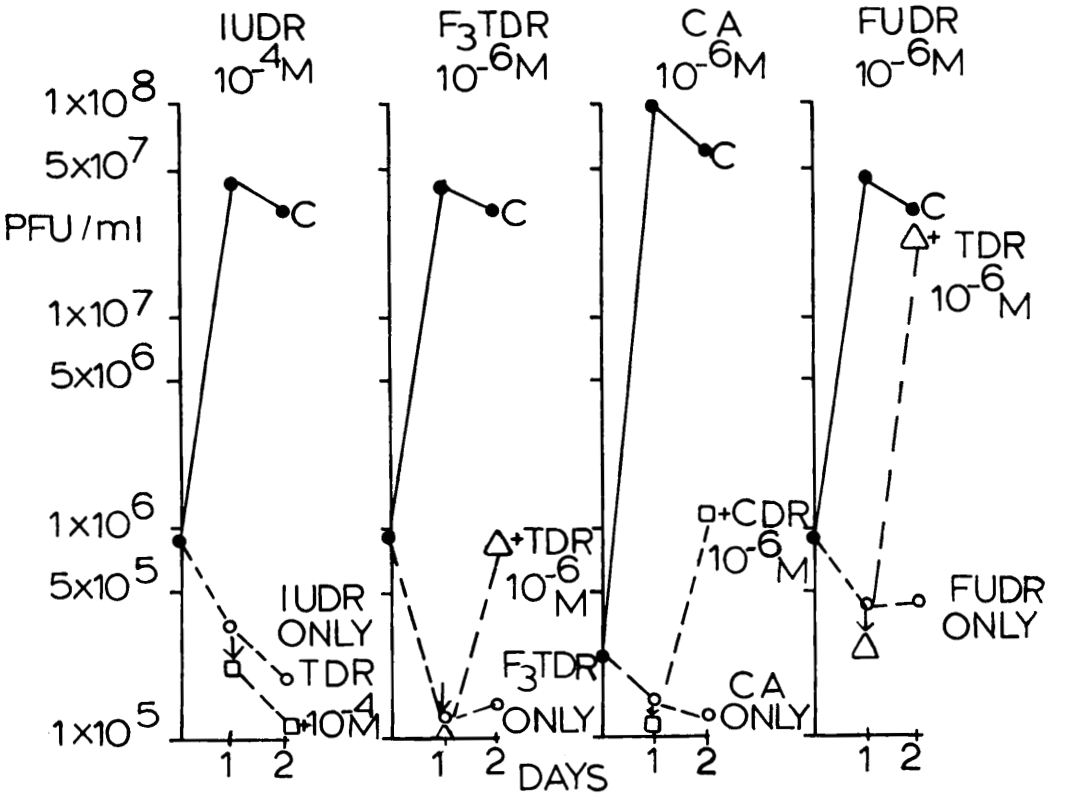

FIG. 4. The possible rescue of vaccinia viral replication in HeLa cells by normal metabolites added 1 day after infection and treatment with the pyrimidine nucleoside analogs, which were washed out prior to addition of the normal metabolites. The time of addition of the normal metabolite is indicated by an arrow.

are discussed in terms of the mechanisms of action of these analogs, and it is concluded that the major (but perhaps not the only) antiviral activity of the latter two compounds is probably a consequence of their incorporation into the viral DNA.

1. Kaufman, H. E., Proc. Soc. Exptl. Biol. Med. **109**, 251 (1962).

2. Kaufman, H. E. and Maloney, E. D., Arch. Ophthalmol. **69**, 626 (1963).

3. Underwood, G. E., Proc. Soc. Exptl. Biol. Med. **111**, 660 (1962).

4. Heidelberger, C., Progr. Nucleic Acid Res. Mol. Biol. **4**, 1 (1965).

5. Salzman, N. P., Virology **10**, 150 (1960).

6. Kaufman, H. E., Maloney, E. D., and Nesburn, A. B., Invest. Ophthalmol. **1**, 686 (1962).

7. Lerman, S. and Eisenschmid, J., Am. J. Ophthalmol. **56**, 725 (1963).

8. Heidelberger, C., Parsons, D. G., and Remy, D. C., J. Med. Chem. **7**, 1 (1964).

9. Reyes, P. and Heidelberger, C., Mol. Pharmacol. **1**, 14 (1965).

10. Gottschling, H. and Heidelberger, C., J. Mol. Biol. **7**, 541 (1963).

11. Szybalski, W., Cohn, N. K., and Heidelberger, C., Federation Proc. **22**, 532 (1963).

12. Heidelberger, C. and Anderson, S. W., Cancer Res. **24**, 1979 (1964).

13. Kaufman, H. E. and Heidelberger, C., Science **145**, 585 (1964).

14. Kaufman, H. E., Ann. N. Y. Acad. Sci. **130**, 168 (1965).

15. DeMaeyer, E. and Schonne, E., Virology **24**, 13 (1964).

16. Prusoff, W. H., Bakhle, Y. S., and McCrea, J. F., Nature **199**, 1310 (1963).

17. Heidelberger, C., Umeda, M., and Khwaja, T. A., Proc. 5th Intern. Congr. Chemotherapy, Vienna, **1967**, 225.

18. Chu, M. Y. and Fisher, G. A., Biochem. Pharmacol. **11**, 423 (1962).

19. Moore, E. C. and Cohen, S. S., J. Biol. Chem. **242**, 2116 (1967).

20. Silagi, S., Cancer Res. **25**, 1446 (1965).

21. Chu, M. Y. and Fisher, G. A., Biochem. Pharmacol. **14**, 333 (1965).

22. Cardeilhac, P. T. and Cohen, S. S., Cancer Res. **24**, 1595 (1964).

Received July 3, 1968. P.S.E.B.M., 1969, Vol. 130.