Biochemical Pharmacology, Vol. 25, pp. 355 357. Pergamon Press, 1976. Printed in Great Britain.

Effects of 5-fluorodeoxyuridine on cell viability and uptake of deoxycytidine and [^{3}H]cytosine arabinoside in L5178Y cells*

(*Received* 31 *July* 1974; *accepted* 30 *June* 1975)

1-β-D-Arabinofuranosyl cytosine (cytosine arabinoside, Ara-C) has been demonstrated to be a potent inhibitor of a wide variety of organisms, such as bacteria [1, 2], mammalian cells in culture [3, 4], DNA viruses [5, 6], infectious virus [7], and transplantable tumors in mice and rats [8]. In humans, Ara-C has been effective in the treatment of neoplasms [9, 10] and for viral infections of the eye [11, 12].

The possible mechanisms whereby Ara-C causes cell death are: (1) inhibition of the formation of deoxycytidine diphosphate from cytidine diphosphate [3, 13, 14]; (2) incorporation of [^{3}H]Ara-C into DNA fractions of mammalian cells [13–20]; (3) inhibition of DNA polymerase [19–22]; and (4) incorporation of [^{3}H]Ara-C into RNA fractions [13, 16–18]. This report is concerned with the modification of Ara-C induced acute cell death by 5-fluorodeoxyuridine (FUDR) and the biochemical effects of the sequential treatment of FUDR and Ara-C on L5178Y murine leukemic cells.

The methods of propagation of murine lymphoblast leukemic cells (L5178Y) have been described [23]. Cells used in all experiments were in the exponential phase of growth and were maintained at 37° in either Fischer's medium (FM) or in Fischer's medium containing 10% horse serum (FMS) during the experiment. Ara-C was obtained from Dr. J. H. Hunter of the Research Laboratories of the Upjohn Co. [^{3}H]Ara-C, purified by chromatography as described previously [13–16], [^{3}H]deoxycytidine ([^{3}H]CdR), deoxycytidine hydrochloride and deoxythymidine (TdR) were purchased from Schwartz Bio Research, Inc. 5-Fluorodeoxyuridine (FUDR) was obtained from CalBiochem Co.

Kinetics of cell death. L5178Y murine leukemic cells ($2{\cdot}0 \times 10^5$/ml) were exposed to FUDR ($1{\cdot}0 \times 10^{-8}$ M) or Ara-C ($3{\cdot}3 \times 10^{-6}$ M) singly and sequentially and incubated for periods ranging from 1 to 4 hr. Cell viability was determined by the dilute agar-colony method. Cloning efficiency of untreated cells was 75 per cent [17]. Deoxythymidine ($2{\cdot}0 \times 10^{-6}$ M) and/or deoxycytidine ($5{\cdot}0 \times 10^{-5}$ M) were added to the cloning medium.

Uptake of [^{3}H]CdR or [^{3}H]Ara-C in cells pretreated with FUDR. Leukemic cells (5×10^7/20 ml) were pretreated with FUDR (1×10^{-8} M) for 2 hr in FM or FMS. The drug was removed by washing and the cells were incubated with [^{3}H]CdR ($1{\cdot}6 \times 10^{-6}$ M, 373 μCi/mole) or [^{3}H]Ara-C ($3{\cdot}3 \times 10^{-6}$ M, 121·7 μCi/mole) for 1 hr; the cell fractions were prepared [13, 17] and counted in a liquid scintillometer.

We have reported the sensitization of L5178Y cells by pretreatment with FUDR (1×10^{-8} M) 2 hr prior to Ara-C ($3{\cdot}3 \times 10^{-6}$ M) 1 through 4 hr [24], as shown in Fig. 1. The rate of cell death with sequential FUDR and Ara-C was 60 per cent/hr and with Ara-C alone was 32 per cent/hr. Deoxythymidine, when added to the cloning medium, did not alter the cell survival after FUDR treatment. It is known that FUDR inhibits thymidylate synthetase, thereby inhibiting *de novo* thymidylate synthesis [25, 26]. The effect of FUDR on the incorporation of [^{3}H]CdR into DNA was studied. L5178Y cells were pretreated with FUDR (1×10^{-8} M) for 2 hr in FMS, washed to remove the drug and incubated with [^{3}H]CdR ($1{\cdot}6 \times 10^{-6}$ M, 373 μCi/mole) for 1 hr (Table 1). An approximate 3-fold increase in DNA incorporation in the FUDR-pretreated cells was found when compared with the non-FUDR-treated cells. This increase could be due to: (1) partial synchronization or accumulation of cells in S phase by FUDR; (2) the presence of TdR in the horse serum; and (3) conversion of [^{3}H]CdR to [^{3}H]deoxythymidine monophosphate after the removal of FUDR. When TdR ($2{\cdot}0 \times 10^{-6}$ M) was added simultaneously with [^{3}H]CdR after FUDR treatment, there was a sharply increased incorporation of [^{3}H]CdR into DNA. This may be due to the fact that in the presence of deoxythymidine-5′-monophosphate, presumed a product of the inhibiton by FUDR, there was enhanced [^{3}H]CdR incorporation and restoration of DNA synthesis. However, cell survival was unaltered even in the presence of TdR. This work suggests that FUDR may have other sites of action in addition to the inhibition of thymidylate synthetase. In order to study the relationship between FUDR and TdR, the effect of FUDR on incorporation of [^{3}H]CdR was undertaken as described previously, except that horse serum was omitted from the incubation medium (Table 1). The incorporation of [^{3}H]CdR into DNA was greatly inhibited in cells pretreated with FUDR to about 25 per cent of the incorporation in non-FUDR-treated cells. However, the incorporation was increased by 46 per cent in cells pretreated with FUDR in the presence of TdR, although TdR did not rescue the cells from FUDR toxicity.

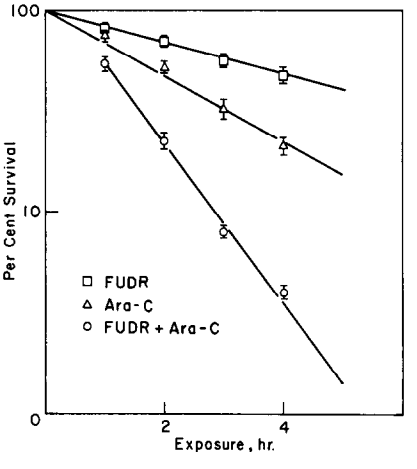

Fig. 1. L5178Y cells in the exponential phase of growth were pretreated with FUDR ($1{\cdot}0 \times 10^{-8}$ M) for 2 hr. Ara-C ($3{\cdot}3 \times 10^{-6}$ M) was added and incubated at 37° for different intervals of time. Cell viability was determined by the dilute agar-colony method, and all data were normalized to 100 per cent of the control. Points represent mean values of three experiments with four replicates per experiment. A minimum of 200 colonies was counted for each point. Vertical lines ± S.E. for FUDR, Ara-C, FUDR pretreated for 2 hr and followed by Ara-C.

* This study was supported by ACS Grant 88-HI, Cancer Center Grant (1-PO2 CA 13943 CAP) and CA 15909.

Table 1. Effect of FUDR on the incorporation of [^{3}H]deoxycytidine*

Fraction	No FUDR pretreatment −TdR	No FUDR pretreatment +TdR	FUDR pretreatment −TdR	FUDR pretreatment +TdR
Cold acid-soluble (cpm/10^6 cells)				
+Horse serum	79 ± 38		62 ± 26	132 ± 33
−Horse serum	20 ± 2·1	15 ± 2·8	16 ± 1·5	20 ± 3·3
DNA (cpm/mg)				
+Horse serum	3150 ± 211		9320 ± 1134	50,000 ± 5183
−Horse serum	2400 ± 547	7020 ± 1338	610 ± 86	10,300 ± 1725
% Survival				
+Horse serum	100		69	71
−Horse serum	100	100	70	79

* L5178Y murine leukemic cells (5×10^7 cells/20 ml) in the exponential phase of growth were pretreated with FUDR (1×10^{-8} M) for 2 hr in FMS or FM. The drug was removed by washing, and the cells were incubated with [^{3}H]CdR ($1{\cdot}6 \times 10^{-6}$ M, 373 μCi/μmole) for 1 hr in FMS or FM; the cell fractions were prepared and counted in a liquid scintillometer. Cell survival was determined by the soft agar-colony method, and all data were normalized to 100 per cent of the control. The procedure was repeated replacing FMS with FM.

Table 2. Effect of FUDR on the incorporation of [^{3}H]cytosine arabinoside*

Fraction	No FUDR pretreatment −TdR	No FUDR pretreatment +TdR	FUDR pretreatment −TdR	FUDR pretreatment +TdR
Cold acid-soluble (cmp/10^6 cells)	274 ± 1·2	421 ± 43	353 ± 7	482 ± 16
RNA (cpm/mg)	739 ± 27	627 ± 46	512 ± 40	865 ± 82
DNA (cpm/mg)	1809 ± 79	2002 ± 105	1168 ± 90	2070 ± 247

* L5178Y cells (5×10^7 cells/20 ml) in the exponential phase of growth were treated with FUDR (1×10^{-8} M) for 2 hr in FM. The drug was removed by washing, and the cells were then incubated with [^{3}H]Ara-C ($3{\cdot}3 \times 10^{-6}$ M, 121·7 μCi/mole) for 1 hr in FM; the cell fractions were prepared and counted in a liquid scintillometer.

Thus, the results obtained from [^{3}H]CdR incorporation in serum-free medium indicate that FUDR did inhibit [^{3}H]CdR incorporation into DNA. This inhibition was markedly diminished by TdR, although cell survival was unaltered. It is presumed that the 17-fold increase in [^{3}H]CdR incorporation results from thymidine-dependent DNA synthesis by DNA replication or DNA repair. The presence of significant amounts of TdR in serum is indicated, since the incorporation of [^{3}H]CdR into DNA was reduced in serum-free medium while an opposite result was obtained in medium containing serum (Table 1). This possible source of TdR is not sufficient to protect cells from FUDR or methotrexate [17] toxicity.

Since [^{3}H]Ara-C was found to be incorporated into lower molecular weight RNA and the incorporation was correlated with the cell kill [18], the effect of FUDR on [^{3}H]Ara-C incorporation was studied. The results shown in Table 2 demonstrated that, in L5178Y cells pretreated with FUDR (1×10^{-8} M) for 2 hr in serum-free medium, the incorporation of [^{3}H]Ara-C ($3{\cdot}3 \times 10^{-6}$ M, 121·7 μCi/mole) was decreased by 37 and 32 per cent into RNA and DNA fractions respectively. Although FUDR pretreatment decreased the incorporation of [^{3}H]Ara-C into RNA and DNA, the cells were not protected from Ara-C toxicity. A similar result was found with methotrexate [27]. In this study, the effect of FUDR on Ara-C did not appear to be related to the incorporation of [^{3}H]Ara-C into RNA or DNA, since this incorporation was decreased in cells pretreated with FUDR. Furthermore, pretreatment with FUDR sensitized the cells to acute cell death by Ara-C and produced a snyergistic effect. However, when TdR was added, the [^{3}H]Ara-C incorporation was increased, although TdR did not rescue the Ara-C-treated cells from FUDR toxicity. FUDR had no effect on [^{3}H]Ara-C incorporation when horse serum was added to the incubation medium.

Acknowledgements—The proficient technical assistance of Mrs. Tara Kasturi, Mrs. Elaine Cheng and Mrs. Grace Shiue is greatly appreciated.

Division of Biological and Medical Sciences, Section of Biochemical Pharmacology and Section of Medicine,
MING Y. CHU
MARVIN L. HOOVIS
GLENN A. FISCHER
Brown University and Roger Williams General Hospital, Providence, R.I. 02908, U.S.A.

REFERENCES

1. L. I Pizer and S. S. Cohen, *J. biol. Chem.* **25,** 2387 (1960).
2. L. Slechta, *Fedn Proc.* **20,** 357 (1961).
3. M. Y. Chu and G. A. Fischer, *Biochem. Pharmac.* **11,** 423 (1962).
4. J. H. Kim and M. L. Eidinoff, *Cancer Res.* **25,** 698 (1964).
5. D. A. Buthala, *Proc. Soc. exp. Biol. Med.* **115,** 69 (1964).
6. H. E. Renis and H. G. Johnson, *Bact. Proc.* **45,** 140 (1962).
7. J. S. Butel and F. Rapp, *Virology* **27,** 490 (1965).
8. J. S. Evans, E. A. Musser, G. D. Mengel, K. R. Forsbald and J. H. Hunter, *Proc. Soc. exp. Biol. Med.* **106,** 350 (1961).
9. R. J. Papac, P. Calabresi, J. W. Hollingsworth and A. D. Welch, *Cancer Res.* **25,** 1459 (1965).

10. R. W. Talley and V. K. Vaitkevicious, *Blood* **21,** 352 (1963).
11. G. E. Underwood, *Proc. Soc. exp. Biol. Med.* **129,** 235 (1968).
12. H. E. Kaufman and E. D. Maloney, *Archs Ophthal., N.Y.* **69,** 626 (1963).
13. M. Y. Chu and G. A. Fischer, *Biochem. Pharmac.* **14,** 333 (1965).
14. R. I. Momparler, M. Y. Chu and G. A. Fischer, *Biochim. biophys. Acta* **161,** 481 (1968).
15. S. Silagi, *Cancer Res.* **25,** 1446 (1965).
16. W. A. Creasey, R. J. Papac, M. D. Markiw, P. Calabresi and A. D. Welch, *Biochem. Pharmac.* **15,** 1417 (1966).
17. M. Y. Chu and G. A. Fischer, *Biochem. Pharmac.* **17,** 753 (1968).
18. M. Y. Chu, *Biochem. Pharmac.* **20,** 2057 (1971).
19. R. I. Momparler, *Biochem. biophys. Res. Commun.* **34,** 465 (1969).
20. F. L. Graham and G. F. Whitmore, *Cancer Res.* **30,** 2636 (1970).
21. A. P. Kimball and M. J. Wilson, *Proc. Soc. exp. Biol. Med.* **127,** 429 (1968).
22. J. J. Furth and S. S. Cohen, *Cancer Res.* **28,** 2061 (1968).
23. G. A. Fischer and A. C. Sartorelli, *Meth. med. res.* **10,** 247 (1964).
24. M. Y. Chu, M. L. Hoovis, P. Calabresi and G. A. Fischer, *Pharmacologist* **13,** 209 (1971).
25. S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb and J. Lichtenstein, *Proc. natn. Acad. Sci. U.S.A.* **44,** 1004 (1958).
26. K. U. Hartman and C. Heidelberger, *J. biol. Chem.* **236,** 3006 (1961).
27. M. Y. Chu and G. A. Fischer, *Biochem. Pharmac.* **17,** 741 (1968).

Biochemical Pharmacology, Vol. 25, pp. 357–358. Pergamon Press, 1976. Printed in Great Britain.

Effects of chronic administration of morphine on pentobarbital responses in the mouse

(*Received* 14 *February* 1975; *accepted* 23 *May* 1975)

The effect of morphine on hepatic microsomal activity has been reported to be species and sex dependent. In the rat, numerous studies have shown that chronic administration of morphine to rats depresses microsomal metabolism of many drugs [1 7]. Several investigators have further shown that the effect of morphine on hepatic drug metabolism could be demonstrated only in sexually mature male rats [7, 8–11]. However, the rat seems to be unique for demonstrating a sex dependency in the metabolism of drugs, since sex differences are not seen in the mouse, guinea pig, cat or dog [7, 12, 13]. Since a sex dependency in drug metabolism could not be demonstrated in the mouse, it was postulated that morphine should exert no depressant effect on the ability of male and female mice to metabolize drugs [11]. In support of the postulate, it was reported that liver microsomes obtained from mice of both sexes receiving morphine sulfate, 20 mg/kg i.p., once daily for 4 days, exhibited no significant differences on several drug-metabolizing parameters including the ability to metabolize ethylmorphine [11]. In the present communication, we present evidence that morphine can inhibit drug microsomal activity in both male and female mice, using as measurements *N*-demethylation of ethylmorphine and pentobarbital sleeping time.

Both male and female ICR mice (Simonsen Labs., Gilroy, Calif.), weighing 23–25 g, were rendered tolerant to and physically dependent on morphine by the subcutaneous implantation of a specially formulated morphine pellet [14]. Control mice were implanted with placebo pellets for the same period of time. To assess the effect of this treatment on the effect of Na-pentobarbital, both sleeping time and lethality were determined after 72 hr of pellet implantation. The sleeping time of each group was measured after a single dose of Na-pentobarbital, 60 mg/kg i.p., with twelve to fourteen mice in each group. The duration of sleeping time was taken as the time between the loss of righting reflex of the animals and the time they righted themselves. In the lethality experiments, the 24 hr LD_{50} and 95% confidence limits of Na-pentobarbital were estimated, using at least three doses of Na-pentobarbital and eight mice per dose [15]. The activity of hepatic drug-metabolizing enzymes was determined by the *N*-demethylation assay technique [16] which involves the isolation of microsomes by centrifugation and the incubation of the microsomes fortified with an NADPH-generating system in the presence of ethylmorphine. The degree of enzyme activity was indicated by the amount of formaldehyde formed from the *N*-demethylation of ethylmorphine. The number of assays per group was between six and eight.

The chronic administration of morphine by 3 days of pellet implantation potentiated the effects of pentobarbital on sleeping time and on lethality in both male and female mice. As summarized in Table 1, mice of both sexes receiving a morphine pellet implant for 72 hr exhibited a sleeping

Table 1. Enhancement of pentobarbital responses after morphine pellet implantation in the mouse

Treatment	Sleeping time (min)		LD_{50} (50% confidence limits)	
	Male	Female	Male	Female
Placebo pellet	55·8 ± 11·8	52·6 ± 5·2	120 (102·7–140·3)	115 (110·4–119·8)
Morphine pellet	141·9 ± 15·8*	236·8 ± 25·4*	86 (79·2–93·4)	86 (81·1–91·2)

* $P < 0.0005$.