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Differences Between 5-Fluoro-2′-deoxyuridine and 5-Fluorouridine in Their Cytotoxic Effect on Growth of Murine Lymphoma L5178Y Cells in *in Vivo* and *in Vitro* Systems*

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Abstract—*The present studies were performed to determine the difference* in vivo *and* in vitro *conditions on the cytotoxic effects of FUdR and FUR by using the same cell line, L5178Y, in both systems. The prolonged activity of FUdR and FUR on survival time of tumor-bearing mice was examined. FUR increased the survival time to* 58% over control at 3 mg/kg *daily for* 5 *days; on the other hand, FUdR at* 300 mg/kg (*this being its optimal dose*), *did not show even the minimum effect. Namely, FUdR was less than one-hundredth of FUR in* in vivo *chemotherapeutic activity. The* in vitro *antiproliferating effects of FUdR and FUR varied under different experimental conditions as follows. In the cytotoxicity by continuous exposure to drugs on the proliferation of cells in culture, FUdR was approximately* 8*-fold higher than FUR. On the other hand, in the cytotoxicity by brief* (60 min) *exposure to drugs on the subsequent growth of cells cultured in a drug free medium, FUdR is about one-sixteenth of FUR. These observations indicate that the result of cytotoxicity by brief exposure but not continuous exposure, is reflected* in vivo *chemotherapeutic activity.*

INTRODUCTION

THE ANTIMETABOLITE, 5-fluorouracil (FU), is one of the most prominent clinical antitumor agents [1]. It has been thought to exert its effects through metabolic conversion to nucleotides, which are formed via the intermediate nucleosides, 5-fluorouridine (FUR) and 5-fluoro-2′-deoxyuridine (FUdR) [2–6]. The cytotoxic effect of FUR and FUdR, which were synthesized by Duschinsky *et al.* [7], has been examined in various laboratories. The latter deoxynucleoside, hoping to take advantage of the powerful inhibitor of the thymidylate synthetase and hence of DNA synthesis [8, 9], was markedly effective in inhibiting the proliferation ability of a number of mouse cell lines *in vitro*. Its activity was 10–100-fold higher than that of FUR on a molar basis [10–12]. On *in vivo* studies, however, FUR has been reported to be much more effective than FUdR in increasing the lifespan of tumor bearing mice [13–16]. Thus the contradictory results between *in vivo* and *in vitro* conditions on the cytotoxic effect of these two analogs should be noted. The studies reported here were, therefore, undertaken to examine the cytotoxic effects of FUR and FUdR by using the same cell line, L5178Y, on both *in vivo* and *in vitro* systems, and analyze the causes which produce the difference between *in vivo* and *in vitro* systems on their cytotoxic effects.

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MATERIALS AND METHODS

Chemicals

FUR and FUdR were generously supplied from Mitsui Pharmaceuticals, Inc. (Tokyo, Japan).

Evaluation of antitumor activity in in vivo *system*

Male BDF_1 (C57BL/6 × DBA/2) mice weighing 19–22 g were purchased from Shizuoka Agr. Co-op. Assoc. Lab. Animals (Hamamatsu, Japan) and a pellet diet (CA-1. CLEA Japan Inc.) and water were offered *ad libitum*.

Six animals in each group were inoculated with 10^7 cells of the murine lymphoma L5178Y which were maintained in male BDF_1 mice by i.p. transplantation every 10 days, and i.p. injected with the drug once daily for 5 days, starting 24 hr after transplantation. Thereafter the survival times of the mice were recorded. Antitumor activity of the drugs was evaluated by the increase in lifespan over controls (ILS) as reported previously [17]. ILS_{30}, optimal dose and maximum ILS were calculated.

Evaluation of antitumor activity in in vitro *systems*

Stock cultures of the murine lymphoma L5178Y cells were maintained in an exponential growth by subculture three times weekly as suspension cultures in Fischer's medium (Grand Island Biological Co., Berkeley, Ca., U.S.A.). supplemented with 10% horse serum, 100 μg/ml streptomycin and 100 U/ml penicillin in a CO_2 incubator with a humid atmosphere of 5% CO_2 and 95% air as described by Fischer and Sartorelli [18]. Stock cultures were restarted from frozen stocks every 6 weeks.

Two different methods were applied to study the cytotoxicity of drugs against the cell proliferation of L5178Y *in vitro*. First, cytotoxic effect by continuous exposure to drugs on the cell proliferation was evaluated as follows; duplicate 10 ml cultures, initially containing 2.5×10^4 cells/ml, were incubated in a drug containing medium at various concentrations in capped 50 ml bottles for 48 hr at 37°C. After incubation, cell numbers were counted by TOA Microcellcounter CC-108 (TOA Medical Electronics Co., Kobe, Japan), and the cell proliferation ratio of treated vs control cultures calculated. The antiproliferating activities were evaluated by the IC_{50} values, which was a concentration required for 50% inhibition against the cell proliferation of the control cultures during a 2-day growth period.

The second method was applied, in order to determine the effect by brief exposure to drugs on the subsequent growth of cells cultured in a drug-free medium, as follows; duplicate 10 ml cultures containing 2×10^5 cells/ml were exposed to drugs at various concentrations. After specified periods, drug exposures were terminated by changing the culture medium, the cells were collected by centrifugation (500 *g*, 3 min), washed once with a warm drug-free medium and then resuspended in the fresh medium and incubated for 4 days at 37°C with sampling at regular intervals to determine cell density. After a 4-day culture period, cumulative cell numbers were calculated by multiplying the cell densities by the dilution factors involved in each culture subdivision, and the cytotoxicity of the drugs was evaluated by the IC_{99} value, which was a concentration required for 99% inhibition against the cell proliferation of the control cultures during a 4-day growth period.

RESULTS

Cytotoxic effects in in vivo *system*

As shown in Fig. 1, FUR, at 1–3 mg/kg daily for 5 days, was found to be much more effective than any dose of FUdR in increasing the survival of tumor-bearing mice. Its ILS_{30}, optimal dose and maximum ILS were 0.3, 3 mg/kg and 58%, respectively, and its therapeutic ratio (optimal dose/ILS_{30}) was 10. On the other hand, in the case of FUdR, it was shown that the maximum lifespan was achieved when FUdR was presented in about 100-fold molar excess over FUR. However its maximum ILS was only 29%, being less than minimum effect (ILS_{30}), therefore, it was impossible to calculate the value of the therapeutic ratio.

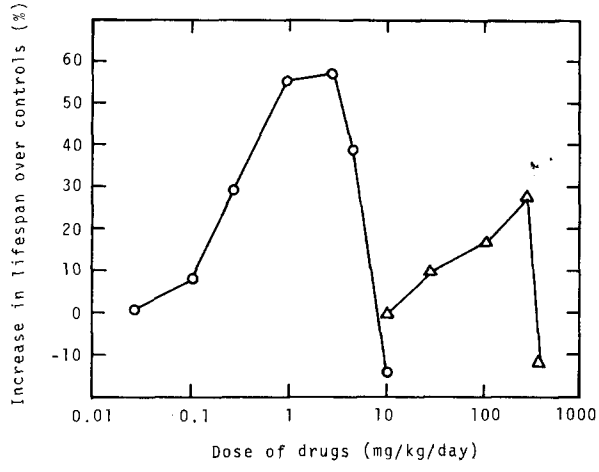

Fig. 1. The effect of FUR or FUdR on the survival of mice bearing L5178Y cells in ascites form. Starting 24 hr after implantation with L5178Y cells (10^7), the mice (BDF_1) received an i.p. injection of FUR (—○—) or FUdR (—△—) once daily for 5 days. Control mice were treated with 0.9% NaCl solution at the same times. Thereafter the survival times of the mice were recorded, and the increase in lifespan (ILS) was calculated on the basis of the mean survival time of treated, vs the control animals.

Antiproliferating effects by continuous drug exposure in in vitro *system*

The comparative cytotoxicity by continuous exposure of FUR and FUdR on the proliferation of L5178Y cells in culture are shown in Fig. 2. FUdR inhibited the cell proliferation by 10, 49 and 88% at 0.25, 0.5 and

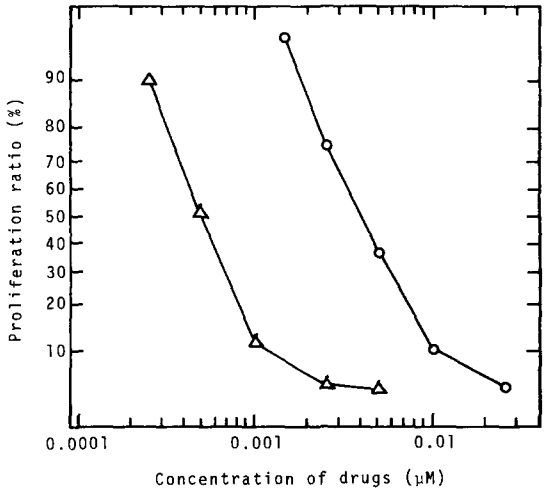

Fig. 2. The effect of contiuous exposure of FUR or FUdR on the proliferation of cultured L5178Y cells. Cells were cultured in a medium containing FUR (—○—) or FUdR (—△—) at the indicated concentrations. After a 2-day *culture period, the proliferation ratio was calculated on the basis of the increased cell numbers of treated, vs the control cultures.*

1 nM respectively, and its IC_{50} was 0.5 nM. On the other hand, at 1 nM of FUR, L5178Y cells proliferated continuously in culture and its IC_{50} was 4 nM which is 8-times that of FUdR. Thus it could be seen that FUR, although of much greater cytotoxicity than FUdR in the therapy of tumor-bearing mice, was less than FUdR in inhibiting cell proliferation by continuous exposure.

Antiproliferating effects by brief drug exposure in in vitro *system*

To clarify the difference between *in vivo* and *in vitro* with respect to cytotoxicity of drugs, effects of brief exposure to drugs on the subsequent growth of cells cultured in a drug free medium, were examined. The opposite phenomena can be observed between a brief and continuous exposure; FUR, which showed 99% inhibition against cell proliferation at 0.16 μM, was approximately 16-fold more toxic than FUdR, as shown in Fig. 3. It was also found that FUdR inhibited cell proliferation by 99% at 2.5 μM, which was about 5000-fold higher than that required to achieve

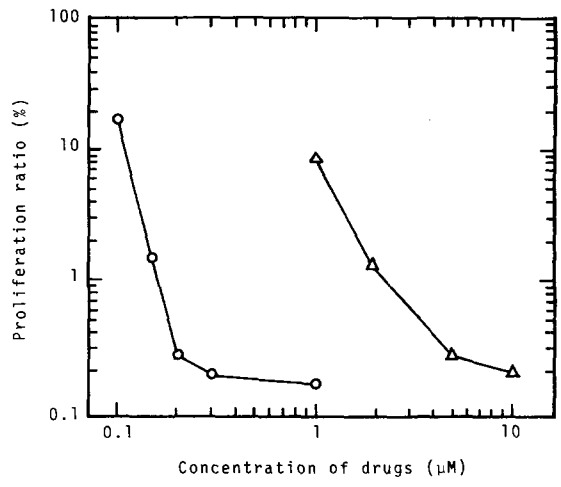

Fig. 3. The effect of a brief exposure of FUR or FUdR on the subsequent growth of L5178Y cells cultured in a drug-free medium. Cultures were treated for 1 hr *with the indicated concentration of FUR (—○—) or FUdR (—△—), drug exposure was terminated by transferring cells to a drug-free medium and incubating at* 37°C. *After a* 4-day *culture period, the proliferation ratio was calculated on the basis of the cumulative cell numbers of treated, vs control cultures.*

the same degree of inhibition by continuous exposure to FUdR. Such a decrease in cytotoxic effect by brief exposure was also observed in FUR, however, IC_{99} was only 7-fold of that by continuous exposure.

Changes in cytotoxicity with drug exposure time was next examined.

Table 1 shows the proliferation ratio of L5178Y cells treated with FUR and FUdR at various concentrations during 1, 3, 6, 12, 18 and 24 hr. From a 24-hr exposure, the L5178Y cells grew continuously at 5 nM or below that of FUR. But at a concentration of 10, 15, 20 and 30 nM, FUR inhibited the cell proliferation by 23.0, 63.8, 99.6 and 99.9%, respectively, and the IC_{99} of FUR was 23 nM. On the other hand, 10 nM of FUdR was sufficient to produce an inhibition ratio exceeding 99% and its IC_{99} was 3.9 nM, as shown in Table 1. The order in cytotoxic activity of drugs from a 24 hr exposure was similar to that from continuous exposure.

The relationship between cytotoxicity and exposure time is more clearly shown in Fig. 4. FUdR was 6-fold and 2-fold more toxic than FUR after a 24 and 18 hr exposure, respectively. After a 12-hr exposure, on the contrary, FUR was 2-fold more toxic than FUdR. This superiority of FUR to FUdR increased by shortening the exposure time. Cytotoxicity of FUdR is superior to that of FUR only when the exposure time is greater than 15 hr.

Table 1. Effects of duration of exposure time on the antiproliferating activities of FUR and FUdR against L5178Y cells in culture

Drugs	(μM)	Exposure time (hr) 1	3	6	12	18	24
FUR	0.3	0.21*					
	0.2	0.28					
	0.15	1.71	0.12				
	0.1	17.2	0.24				
	0.07		3.52	0.20			
	0.05		8.20	0.42			
	0.03			10.7	0.5	0.17	0.091
	0.02			60.6	26.9	14.6	0.32
	0.01				63.1	82.8	77.0
	0.005						100.0
FUdR	10	0.22					
	5	0.29					
	2	1.57	0.4				
	1	9.0	1.73	0.20			
	0.5		7.2				
	0.3			2.7	0.22		
	0.25		26.3				
	0.2			7.4			
	0.1			32.3	0.64		0.21
	0.03				2.1	0.64	
	0.01					2.0	0.62
	0.003					12.0	1.19
	0.001						4.68

*The value indicates the proliferating ratio by percentage. Cultures were treated for the indicated periods in a medium containing FUR or FUdR at the indicated concentrations. After a 4-day culture period, the proliferating ratio was calculated on the basis of the cumulative cell number of treated vs a control culture. The 99% inhibition concentration (IC_{99}) was determind by dose-response curve and is presented in Fig. 4.

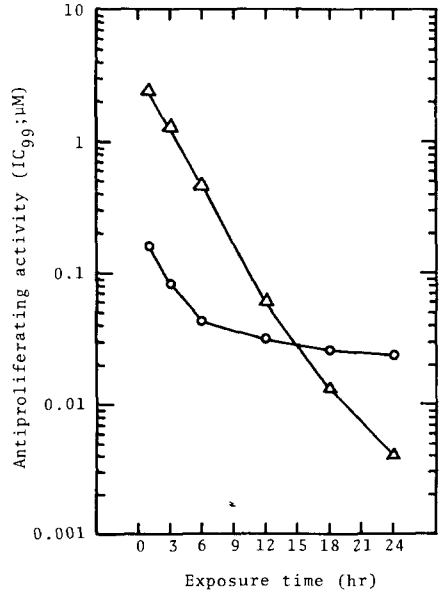

Fig. 4. The effect of exposure time to FUR or FUdR on drug concentration, inhibiting cell proliferation by 99%. This figure presents analytical data from the experiment described in Table 1. FUR; (—○—), FUdR; (—△—).

DISCUSSION

Activity of FUR against several transplanted tumors has been reported to compare with that of FUdR. In both Ehrlich ascites carcinoma and Novikoff hepatoma, Heidelberger *et al.* [14, 15] reported that FUR was more active in prolonging life than FUdR. Similarly, Burchenal [13] found FUR at 1.25–2.5 mg/kg daily for 5 days to be more effective in increasing survival time than any dose of FUdR against lines of L1210 made resistant to methotrexate. Sugiura [16] reported that a 5 mg/kg dose of FUR was sufficient to inhibit the growth of ascites sarcoma 180 and Ehrlich solid carcinoma, whereas, FUdR was necessary to 40 mg/kg.

In *in vitro* studies, Burchenal [13] reported that growth inhibitory IC_{50} values of FUR and FUdR, against L1210 cells in culture, were 2 and 0.2 nM respectively. This result is similar to our study with L5178Y cells and to those of Roosa *et al.* with P-388 [12], of Laskin *et al.*, with Sarcoma 180 [11]. From these data of studies with many kinds of tumor, it can be generalized that the order of FUR and FUdR in an *in vivo* cytotoxicity is inverted in an *in vitro* system, in which cells are exposed to drugs continuously. Comparing the conditions of *in vivo* and *in vitro* experiments with each other, the length of exposure time to drugs differed greatly between both experiments. In contrast to the conditions of continuous exposure to fixed concentrations of drugs for a long period *in vitro* systems [10–12], plasma levels of drugs declined rapidly after dosing and drugs were essentially cleared from plasma in 1 hr in animals [19] and humans [20]. In the similitude of *in vivo* conditions, we attempted to examine the cytotoxicity of drugs by a brief drug exposure in an *in vitro* system. As expected, it can be demonstrated that FUR was also superior to FUdR in an *in vitro* system, similar to the results in mice. Further work will be necessary to determine whether these findings constitute a special case or are generally applicable. However, Tidd and Paterson [21] have reported the observation, being similar to our results, in cytotoxicity of 6-mercaptopurine analog *in vitro* system. That is, 6-methylthioinosine, being less effective *in vivo*, markedly inhibited the proliferation of L5178Y cells by continuous exposure but this cytotoxicity was reversed when the cells were resuspended in a drug-free medium after a brief drug exposure. These findings suggest that the *in vitro* assay after brief exposure is an accurate method corresponding more closely to the *in vivo* assay. On the other hand, an *in*

vitro assay by continuous exposure might risk overestimating the therapeutic effect of drugs whose cytotoxic action is dependent upon exposure time.

From this experiment, the relationship between cytotoxicity and drug exposure time has been determined, it was demonstrated that the cytotoxic action of FUR is rapid and completed within 6 hr, whereas the cytotoxicity of FUdR depended markedly upon the drug exposure time. This high time-dependency of FUdR may contribute in part to the low *in vivo* chemotherapeutic effect of FUdR.

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REFERENCES

1. C. Heidelberger and F. J. Ansfield, Experimental and clinical use of fluorinated pyrimidines in cancer chemotherapy. *Cancer Res.* **23,** 1226 (1963).
2. L. Bosch, E. Harbers and C. Heidelberger, Studies on fluorinated pyrimidines. V. Effects on nucleic acid metabolism *in vitro*. *Cancer Res.* **18,** 335 (1958).
3. E. Bresnick and U. B. Thompson, Properties of deoxythymidine kinase partially purified from animal tumors. *J. biol. Chem.* **240,** 3967 (1965).
4. N. K. Chaudhuri, B. J. Motag and C. Heidelberger, Studies on fluorinated pyrimidines. III. The metabolism of 5-fluorouracil-2-C^{14} and 5-fluoro-orotic-2-C^{14} acid *in vivo*. *Cancer Res.* **18,** 318 (1958).
5. P. Reyes, The synthesis of 5-fluorouridine 5′-phosphate by pyrimidine phosphoribosyltransferase of mammalian origin. I. Some properties of the enzyme from P1534J mouse leukemia cells. *Biochemistry* **8,** 2057 (1969).
6. O. Sköld, Nucleoside derivatives of 5-fluorouracil. Formation with enzymes from Ehrlich ascites tumor. *Arkiv. Kemi.* **17,** 59 (1960).
7. R. Duschinsky, E. Pleven and C. Heidelberger, The synthesis of 5-fluoropyrimidines. *J. Amer. Chem. Soc.* **79,** 4559 (1957).
8. S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb and J. Lichtenstein, The mode of action of 5-fluorouracil and its derivatives. *Proc. nat. Acad. Sci. (Wash.).* **44,** 1004 (1958).
9. K.-U. Hartmann and C. Heidelberger, Studies of fluorinated pyrimidines. XIII. Inhibition of thymidylate synthetase. *J. biol. Chem.* **236,** 3006 (1961).
10. J. H. Burchenal, From wild fowl to stalking horses: alchemy in chemotherapy. Fifth Annual David. A. Karnofsky Memorial Lecture. *Cancer (Philad.)* **35,** 1121 (1975).
11. J. D. Laskin, E. F. Jordan, L. N. Kenny, D. Sugg, A. Y. Divekar and M. T. Hakala, Differences in the sensitivity to 5-fluoropyrimidines of cells in culture. *Proc. Amer. Ass. Cancer Res.* 71 (1976).
12. R. A. Roosa, T. D. Bradley, L. W. Law and L. A. Herzenberg, Characterization of resistance to amethopterin, 8-azaguanine and several fluorinated pyrimidines in the murine lymphocytic neoplasm, P 388. *J. cell. comp. Physiol.* **60,** 109 (1962).
13. J. H. Burchenal, E. A. D. Holmberg, J. J. Fox, S. C. Hemphill and J. A. Reppert, The effects of 5-fluorodeoxycytidine, 5-fluorodeoxyuridine, and related compounds on transplanted mouse leukemias. *Cancer Res.* **19,** 494 (1959).
14. C. Heidelberger, L. Greisbach, O. Cruz, R. J. Schnitzer and E. Grunberg, Fluorinated pyrimidines. VI. Effects of 5-fluorouridine and 5-flouro-2′-deoxyuridine on transplanted tumors. *Proc. Soc. exp. Biol. N.Y.* **97,** 470 (1958).
15. C. Heidelberger, A. V. Sunthankar, L. Griesbach and S. Randerson, Fluorinated pyrimidines. XII. Effects of simple nucleotides on transplanted tumors. *Proc. Soc. exp. Biol. N.Y.* **104,** 127 (1960).
16. K. Sugiura, Relative sensitivity of the solid and ascites forms of sarcoma 180 and Ehrlich carcinoma to inhibitory compounds. *Ann. N. Y. Acad. Sci.* **76,** 575 (1958).
17. A. Hoshi, F. Kanzawa and K. Kuretani, Antitumor activity of cyclocytidine in a variety of tumors. *Gann* **63,** 353 (1972).
18. G. A. Fischer and A. C. Sartorelli, Development, maintenance and assay of drug resistance. *Methods med. Res.* **10,** 247 (1964).

19. M. Chadwick and W. I. Rogers, The physiological disposition of 5-fluorouracil in mice bearing solid L-1210 lymphocytic leukemia. *Cancer Res.* **32,** 1045 (1972).
20. W. E. MacMillan, W. H. Wolberg and P. G. Welling, Pharmacokinetics of fluorouracil in humans. *Cancer Res.* **38,** 3479 (1978).
21. D. M. Tidd and A. R. P. Paterson, Distinction between inhibition of purine nucleotide synthesis and the delayed cytotoxic reaction of 6-mercaptopurine. *Cancer Res.* **34,** 733 (1974).