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THE MECHANISMS OF ACTION OF THREE FLUORINE SUBSTITUTED CYTOSINE ANALOGS: IMPLICATIONS FOR CANCER CHEMOTHERAPY

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Abstract—The mechanisms of action in DNA synthesis of three fluorine substituted cytosine analogs have been studied in human phytohaemagglutinin-stimulated lymphocytes. 5-Fluorocytidine (over a wide range of concentrations) and 5-fluorocytosine (at high concentration) inhibited ^{3}H-deoxyuridine incorporation into DNA without inhibiting ^{3}H-thymidine incorporation. 5-Fluorocytidine caused reduction in the free cell concentration of TTP. In contrast, ara-fluorocytosine markedly inhibited both ^{3}H-deoxyuridine and ^{3}H-thymidine incorporation into DNA but did not cause a consistent change in thymidine triphosphate concentration. These results indicate that 5-fluorocytidine, and to a lesser extent, 5-fluorocytosine block *de novo* TMP synthesis in human cells. It seems likely that they are both metabolized to 5-fluorodeoxyuridine monophosphate which inhibits the enzyme TMP synthetase. On the other hand, the results suggest that ara-fluorocytosine has an action identical to that of cytosine arabinoside, inhibition of DNA polymerase. It is postulated that cells resistant to cytosine arabinoside which have high cytidine deaminase levels might be selectively sensitive to 5-fluorocytidine and to 5-fluorocytosine.

A NUMBER of fluorine substituted pyrimidines have been synthesized and tested for toxicity in experimental tumour systems,[1–6] but only 5-fluorouracil and 5-fluorodeoxyuridine have been widely used to treat human malignant diseases. 5-Fluorocytosine (5-FC) was found to be essentially non-toxic but 5-fluorocytidine (5-FCR) and ara-fluorocytosine (ara-FC)* are both toxic to mammalian cells.[7] Studies *in vivo* have shown that 5-fluorocytosine is not metabolized in man, but some fungi have been found to deaminate this drug to 5-fluorouracil, and 5-fluorocytosine is, therefore, a useful drug for treating certain fungal infections.[8,9] 5-Fluorocytidine and ara-fluorocytosine, unlike 5-fluorocytosine, are substrates for mammalian cytidine deaminase,[7] and theoretically they might, therefore, have a dual mechanism of action, acting not only as cytosine antimetabolites, but also as inhibitors of thymidylate synthetase after they have been metabolized to dUMP analogs. We have now carried out experiments to delineate the mechanisms of inhibition of DNA synthesis in human cells by these three fluorinated compounds, 5-fluorocytosine, 5-fluorocytidine and ara-fluorocytosine.

The method used for collecting human lymphocytes and the culture conditions were those of Das and Hoffbrand.[10] Separated lymphocytes were incubated at 37°

* 1-β-D-Arabinofuranosyl 5-fluorocytidine.

in 3 ml volume at a cell concentration of 10^6/ml in medium TC 199 containing 30% autologous serum and 0·1 ml phytohaemagglutinin (PHA, Wellcome). After 72 hr incubation, cultures were pooled, reallocated into 3 ml volumes each containing 3×10^6 cells and, where appropriate, drugs added in 0·1 ml volumes of physiological saline. Equivalent volumes of saline were added to controls. One μCi of ^{3}H-thymidine (^{3}H-methyl, 5 Ci/m-mole) or 1 μCi ^{3}H-deoxyuridine (^{3}H-6, 24 Ci/m-mole) was added in 0·1 ml volume of saline. After incubation for 1 hr at 37° the amount of ^{3}H-nucleoside incorporated into DNA in control and drug-treated cells was estimated by extracting DNA as described by Hryniuk and Bertino.[11] For deoxyribonucleotide triphosphate estimation, drug-treated and control cells were also incubated in 13 ml for 1 hr but without addition of labelled nucleoside. These cells were collected by centrifugation, washed once in ice-cold phosphate-buffered saline, pH 7·4, and 1 ml 60% methanol added to each cell pellet for nucleotide extraction. The assay for deoxyribonucleoside triphosphates (dNTP) has been described elsewhere.[12,13]

Table 1 shows the effect of 5-FC (5-fluorocytosine), 5-FCR (5-fluorocytidine) and ara-FC (ara-fluorocytosine) on the dNTP levels and on the incorporation of labelled deoxyuridine and labelled thymidine into DNA. At a concentration of 10^{-2} M and in two of three experiments at 10^{-3} M, 5-FC caused slight inhibition of ^{3}H-deoxyuridine incorporation without consistently influencing ^{3}H-thymidine incorporation into DNA, but even at these high concentrations the drug had no consistent effect on the cell concentration of dNTP. 5-FCR, at concentrations ranging from 10^{-3} to 10^{-7} M, caused inhibition of ^{3}H-deoxyuridine incorporation into DNA but this drug also raised ^{3}H-thymidine incorporation into DNA and reduced the free cell thymidine triphosphate (dTTP) concentration. On the other hand, ara-5FC (10^{-3}–10^{-7} M) markedly inhibited both ^{3}H-thymidine and ^{3}H-deoxyuridine incorporation into DNA but produced no consistent changes in the level of any of the dNTP.

These results suggest that both 5-FCR (over a wide range of concentration) and 5-FC (at high concentrations only) inhibit TMP synthetase in human lymphocytes. The effects of 5-FCR on ^{3}H-deoxyuridine and ^{3}H-thymidine uptake and on cellular dTTP concentrations closely resemble those produced by two known inhibitors of *de novo* thymidylate synthesis, methotrexate and 5-fluorouracil.[14,15] The inhibition by methotrexate of ^{3}H-deoxyuridine incorporation into DNA is associated with an accumulation of intracellular dUMP, and this observation supports the belief that dUMP methylation is inhibited by methotrexate.[16] 5-FdUMP inhibits TMP synthetase directly, and thus may inhibit ^{3}H-deoxyuridine incorporation into DNA in a similar manner. The rise in ^{3}H-thymidine incorporation into DNA with these drugs is thought to be due to lowering in the free cell pool of dTTP with consequent reduced feed-back inhibition of thymidine kinase but also to alteration in specific activity of the ^{3}H-thymidine in the dTTP pool caused by the depletion of cell dTTP levels. Under these conditions enchanced ^{3}H-thymidine uptake does not indicate enhanced DNA synthesis, and the results may mask an actual reduction in the rate of DNA synthesis. Thus, at least in lymphocytes, it would seem that 5-FCR is readily deaminated to give 5-FUR, and this compound is presumably phosphorylated to the active TMP synthetase inhibitor, 5-FdUMP. Ara-FC, on the other hand, had effects on ^{3}H-thymidine and ^{3}H-deoxyuridine incorporation into DNA and on the dNTP pools similar to those caused by ara-C and, therefore, like ara-C, probably inhibits DNA polymerase.[17] The suggestion that the major effect of ara-FC is inhibition on

TABLE 1.

Expt. No.	Drug	Concn (M)	^{3}H-TdR/DNA 10^6 cells (cpm)	(% of control)	^{3}H-UdR/DNA 10^6 cells (cpm)	(% of control)	dATP pmoles/10^6 cells	dTTP pmoles/10^6 cells	dGTP pmoles/10^6 cells	dCTP pmoles/10^6 cells
I	Control		19,400	—	3154	—	4·7	13·5	4·3	—
	5-FCR	10^{-4}	20,000	103	611	19	3·1	6·2	2·5	—
		10^{-5}	22,000	113	630	20	4·5	7·0	3·7	—
		10^{-6}	18,300	94	730	23	5·0	7·0	3·5	—
II	Control		23,470	—	4 5 2 0	—	1·6	3·8	1·4	0·6
	5-FC	10^{-3}	21,100	90	3580	79	1·4	3·5	1·4	0·6
	5-FCR	10^{-5}	25,405	108	110	2	2·4	2·5	1·3	0·8
	Ara FC	10^{-5}	1375	6	260	6	1·5	4·0	1·4	0·6
III	Control		43,807	—	15,500	—	4·3	8·8	1·6	1·3
	5-FC	10^{-3}	45,006	103	12,860	83	4·5	8·5	0·8	—
	5-FCR	10^{-5}	53,823	123	665	4	—	3·8	0·6	0·8
	Ara FC	10^{-5}	2770	6	685	4	4·4	9·5	0·6	1·3
IV	Control		52.931	—	8560	—				
	5-FC	10^{-2}	54,039	102	6837	80				
		10^{-3}	55,830	105	8901	104				
		10^{-4}	51,165	97	9393	110				
	5-FCR	10^{-5}	65,079	123	1884	22				
		10^{-6}	58,611	111	3945	46				
		10^{-7}	51,348	97	7075	83				
	Ara FC	10^{-5}	3645	7	660	8				
		10^{-6}	8 1 5 4	15	1578	18				
		10^{-7}	23.184	45	4 3 1 1	50				

The effect of 5-fluorocytidine (5-FCR), 5-fluorocytosine (5-FC) and ara 5-fluorocytosine (ara FC) on the incorporation of radioactive thymidine (^{3}H-TdR) and radioactive deoxyuridine (^{3}H-UdR) into DNA in PHA-stimulated lymphocytes and on the free cellular concentrations of deoxyadenosine triphosphate (dATP), thymidine triphosphate (dTTP), deoxytidine triphosphate (dCTP) and deoxyguanosine triphosphate (dGTP). Each result is the mean of triplicate cultures.

DNA polymerase, presumably by competition with the natural substrate, deoxycytidine triphosphate, is consistent with the report of Burchenal *et al.*[18] that deoxycytidine will reverse the toxicity of ara-FC (as it will that of ara-C). This predominant effect of ara-FC of inhibition of DNA polymerase may possibly mask an additional effect of ara-FC on TMP synthetase. Alternatively, it may be that ara-FC is much less readily deaminated than 5-FC and 5-FCR and thus has little or no effect on *de novo* TMP synthesis.

It has been postulated previously that ara-C resistant tumours with raised cytidine deaminase activity might be particularly susceptible to 5-fluorocytosine.[19] The present study suggests that 5-fluorocytidine would be a better selective antimetabolite for use in ara-C resistant solid tumours and in ara-C resistant leukaemias.

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REFERENCES

1. C. HEIDELBERGER, N. K. CHANDHUIR, P. DANNENBERG, D. MOOREN and L. GRIESBACH, *Nature, Lond.* **179,** 663 (1957).
2. R. DUSCHINSKY, E. PLEVEN, J. MALBICA and C. HEIDELBERGER, *Abstr. Am. Chem. Soc. Meeting.* 8–13 Sept. 19C–20C (1957).
3. N. C. YUNG, J. H. BURCHENAL, R. FECHER and R. DUSCHINSKY, *J. Am. Chem. Soc.* **83,** 4060 (1961).
4. J. J. FOX, N. MILLER and I. WEMPEN, *J. Med. Chem.* **9,** 101 (1966).
5. I. WEMPEN, R. DUSCHINSKY, L. KAPLAN and J. J. FOX, *J. Am. Chem. Soc.* **83,** 4755 (1961).
6. K. KIKUGAWA and M. ICHINO, *J. Org. Chem.* **37,** 284 (1972).
7. M. R. DOLLINGER, J. H. BURCHENAL, W. KREIS and J. J. FOX, *Biochem. Pharmac.* **16,** 681 (1967).
8. B. A. KOECHLIN, F. RUBIO, S. PALMER, T. GALVID and R. DUSCHINSKY, *Biochem. Pharmac.* **15,** 435 (1966).
9. R. J. FASS and R. L. PERKINS, *Ann. Int. Med.* **74,** 535 (1971).
10. K. C. DAS and A. V. HOFFBRAND, *Br. J. Haemat.* **19,** 459 (1970).
11. W. M. HRYNIUK and J. R. BERTINO, *J. clin. Invest.* **48,** 2140 (1969).
12. A. W. SOLTER and R. E. HANDSCHUMACHER, *Biochem. biophys. Acta* **174,** 585 (1969).
13. U. LINDBERG and L. SKOOG, *Anal. Biochem.* **34,** 152 (1970).
14. A. V. HOFFBRAND, E. TRIPP, D. CATOVSKY and K. C. DAS, *Br. J. Haemat.* **25,** 497 (1973).
15. M. H. N. TATTERSALL, A. LAVOIE, K. GANESHAGURU, E. TRIPP and A. V. HOFFBRAND, manuscript in preparation.
16. M. H. N. TATTERSALL, R. C. JACKSON, T. A. CONNORS and K. R. HARRAP, *Eur. J. Cancer* (in press).
17. J. J. FURTH and S. S. COHEN, *Cancer Res.* **28,** 2061 (1968).
18. J. H. BURCHENAL, H. H. ADAMS, N. S. NAVELL and J. J. FOX, *Cancer Res.* **26,** 370 (1966).
19. M. H. N. TATTERSALL, *Br. J. Cancer* **27,** 406 (1973).