Biochemical Pharmacology, 1962, Vol. 11, pp. 761–768. Pergamon Press Ltd., Printed in Great Britain.

STUDIES ON THE BIOCHEMICAL PHARMACOLOGY OF 5-IODO-2′-DEOXYCYTIDINE *IN VITRO* AND *IN VIVO*

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(*Received* 26 *March* 1962; *accepted* 26 *April* 1962)

Abstract—Some of the biological and biochemical effects of 5-iodo-2′-deoxycytidine (ICDR*) and 5-iodo-2′-deoxyuridine (IUDR) have been compared. ICDR is less effective in inhibiting the growth and decreasing the viability of P815Y mastocytoma cells grown in culture. In these cells ICDR is incorporated into DNA as IUDR 5′-phosphate, in place of equivalent amounts of thymidine 5-phosphate; this correlates with the finding that ICDR increases markedly the sensitivity of P815Y cells to injury by X-irradiation.

The utilization of ^{14}C-formate and ^{3}H-thymidine for the biosynthesis of DNA-thymine of L5178Y and Ehrlich ascites tumor cells *in vitro* is inhibited markedly by IUDR and to a lesser extent by ICDR. Whereas IUDR inhibits the growth of *Streptococcus faecalis* (ATCC 89043) in media supplemented with pteroylglutamic acid, thymine, or thymidine, ICDR in similar molar concentrations exerts little or no effect.

The toxicity produced by ICDR in mice is significantly less than that observed with IUDR. After injection of mice with ^{3}H-ICDR, chromatography of the urine showed that the drug is extensively metabolized. There is no marked difference between ICDR and IUDR in their ability to inhibit the growth of L1210 and L5178Y neoplasms in mice.

THE BIOLOGICAL activities of various halogenated derivatives of 2′-deoxyuridine (UDR) and 2′-deoxycytidine (CDR) have been investigated in several laboratories. The recent synthesis by Chang and Welch[1] of 5-iodo-2′-deoxycytidine (ICDR), a compound that cannot be made by procedures employed successfully for 5-iodo-2′-deoxyuridine (IUDR) or the corresponding bromine-containing derivatives of UDR and CDR, has permitted the extension of these studies to include this new compound. A discussion of the theoretical advantages of derivatives of CDR, as potential chemotherapeutic agents, because of the possibility of greater metabolic stability, has been presented elsewhere.[2] In essence, it was hoped that the halogenated derivatives of CDR, like the parent compound, would be poor donors of deoxyribose, but would be converted intracellularly into the halogenated derivative of 2′-deoxycytidine 5′-phosphate (CDR 5′-P), and then deaminated by deoxycytidylate deaminase to form the halogenated derivative of 2′-deoxyuridine 5′-phosphate (UDR 5′-P), which can be used by the cell as a precursor of DNA.[2] Although 5-bromo-2′-deoxycytidine (BCDR) exhibited greater metabolic stability than did the corresponding derivative

*Although a form of abbreviation that would be more in keeping with that of accepted nomenclature of deoxyribonucleosides might by IdCR or ICdR, the widespread usage of FUDR for 5-fluoro-2′-deoxyuridine (5-fluorouracil deoxyribonucleoside) has made preferable the choice of terms such as ICDR and IUDR for the 5-iodo-derivatives of 2′-deoxycytidine and 2′-deoxyuridine, respectively.

of uracil in the rat *in vivo*,[3] a similar degree of resistance to catabolic influences was not observed in the mouse.[4] The present report describes a study of ICDR with respect to its metabolism by, toxicity for, and mechanism of inhibitory action in (a) mammalian cells (P815Y murine neoplastic mast cells) in culture, (b) *Streptococcus faecalis* (ATCC 8043), and (c) mice (*in vivo*).

Studies in cell culture. Both the growth and the viability of neoplastic mast cells in culture are decreased significantly by ICDR and to a greater extent by IUDR (Table 1); this difference, which is uniform over a wide range of concentrations of these

TABLE 1. COMPARISON OF THE EFFECT OF IUDR AND ICDR ON GROWTH INHIBITION AND DECREASE IN CELL VIABILITY OF MURINE NEOPLASTIC CELLS IN CULTURE*

	Concentration of analog in culture medium (μmoles/liter)	
Deoxyribonucleoside	50% Growth inhibition†	50% Cell viability‡
IUDR	2·7	0·8
ICDR	14	2·0
BUDR	56	
BCDR	450	
FUDR	5×10^{-4}	

* The culture medium, cell line, and techniques (Tables 1–3) have been described previously.[5]

† 48-hr assay.

‡ Cells were incubated at 37 °C for 36 hr with graded levels of the drugs, harvested by centrifugation, washed once to remove most of the residual drug, resuspended in a large volume of fresh medium, and reincubated (zero-time). Aliquots were counted at suitable time intervals and the number of viable cells for each drug level was determined (essentially as has been described by Alexander[21] from the intercept obtained by extrapolation of the outgrowth curves to zero-time.

agents in the medium, may reflect, as cited previously with BCDR,[5] a metabolic limitation of the cell either to transport ICDR through the cell wall or to phosphorylate and deaminate it, or both. ICDR is much more active as an inhibitor of the reproduction of the mast cells than is BCDR (the concentrations required in the medium for 50 per cent inhibition of growth are 14 μmolar for ICDR and 450 μmolar for BCDR). With maximally toxic doses of ICDR, cellular reproduction is completely inhibited after one cell-division, whereas in the presence of a maximally toxic dose of BCDR the cells reproduce twice before lysing.[5]

In an earlier report,[5] cross-resistance to BCDR of a cloned subline of P815Y mast cells (P815Y/FUDR) selected for resistance to FUDR suggested that conversion of the drug to phosphorylated derivatives of BUDR in the parent line occurred *after* deamination. Since the resistant subline is also cross-resistant to ICDR, it follows that the pathway of ICDR metabolism in P815Y cells may be the same.

Chromatography of nucleotides obtained by enzymatic degradation of the DNA of P815Y cells grown for two generations with ^{3}H-ICDR (0.1 μc/μmole; prepared by the method of Chang and Welch,[1] shows that this compound is incorporated into DNA in the form of 5-iodo-2′-deoxyuridine 5′-monophosphate (IUDR 5′-P) in an amount that replaces an equivalent quantity (34 per cent) of the thymidylic acid (dTMP), calculated on the basis of newly synthesized DNA (see Table 2). As shown in Table 2. deoxyadenylic acid (dAMP) (1·69 μmoles) and dTMP plus IUDR 5′-P (1·48 μmoles)

are found in nearly equivalent amounts in the DNA digest, in accordance with the Watson-Crick[6] formulation for base-pairing. Incorporation of ICDR or BCDR[5] into the cellular DNA as the 5′-monophosphate of the corresponding halogenated derivative of 2′-deoxyuridine shows that the ultimate anabolic fate of these compounds within the cells is similar.

TABLE 2. INCORPORATION OF ICDR AS 5-IODO-2′-DEOXYURIDINE 5′-PHOSPHATE INTO THE DNA OF MURINE NEOPLASTIC CELLS (P815Y) IN CULTURE*

Deoxyribonucleotide	Nucleotide in DNA (μmoles)		Replacement of thymidylic acid by IUDR5′-P (%)
dAMP		1·69	
dTMP	1·01		
IUDR 5′-P	0·47	1·48	34

* The cells were grown for slightly more than two cell divisions in the presence of 2×10^{-5}M ^{3}H-ICDR (0·1 μc/μmole). The methods for isolation and determination of nucleotides have been reported previously.[5]

TABLE 3. COMPARISON OF THE EFFECT OF X-IRRADIATION ON THE VIABILITY OF MAST CELL NEOPLASMS (P815Y) GROWN IN CULTURE WITH IUDR OR ICDR*

Drug	Drug conc. (μmoles/ml)	X-ray dose (roentgens)	Viable cells (%)	Irradiated / Nonirradiated
None		0	100	67
		150	67	
ICDR	1×10^{-3}	0	100	49
		150	49	
IUDR	1×10^{-3}	0	57	39
		150	22	

* Cells were incubated for 36 hr at 37 °C with the respective agents in the medium at the concentrations indicated, X-irradiated in the glass culture tubes (physical factors were: 250 kv; 15 ma; ½ mm Cu; 1 mm Al; distance of target to culture tubes, 56 cm; time, 90 sec), harvested by centrifugation, washed once to remove most of the residual drug, resuspended in a large volume of fresh medium, and reincubated. The viability determinations were made as described in Table 1.

As anticipated from the work of others on the effect of irradiation with either ultraviolet or X-rays of mammalian cells grown in the presence of IUDR,[7,8] ICDR increases the sensitivity of the P815Y cell to X-irradiation; furthermore, ICDR does not appear to differ significantly from IUDR in this respect (Table 3). No *marked* differences are found between IUDR- and ICDR-treated cells exposed to X-irradiation in dosages other than those reported here; a similar finding also has been made recently by Szybalski, who compared the effect of X-irradiation on human cells (D98S) grown in culture in the presence of either IUDR or ICDR.[9]

Studies with S. faecalis. In a range of concentrations (0·2-2·0 mM) that, with IUDR, leads to marked inhibition of the growth of this microorganism (Table 4), ICDR does not inhibit significantly the growth of *S. faecalis*. The reasons for this inactivity of ICDR are now under investigation; however, it appears likely that in this organism ICDR either is not deaminated to IUDR, or is not phosphorylated to form ICDR 5′-P, or the latter is not deaminated to IUDR 5′-P.

Studies in mice. To estimate the metabolic stability of ICDR, each of 10 mice was injected with a single intraperitoneal dose of 2 μmoles of ^{3}H-ICDR (1·25 μc/μmole), and the animals were placed in a metabolism cage. The combined urine was collected for a 24-hr period in a tube suspended in a dry ice-alcohol bath, diluted fourfold with water, adjusted to pH 10·8, and chromatographed on a Dowex-1 formate column (200–400 mesh, 30 × 2·5 cm) by gradient elution with formic acid, as reported previously in similar studies with IUDR.[10] At least six major and seven minor radioactive fractions were obtained. Although the compounds excreted have not yet been completely identified or the relative quantities determined, the results clearly indicate that extensive metabolic alteration of ICDR occurs in the mouse, a circumstance previously encountered with IUDR.[10]

TABLE 4. COMPARISON OF THE EFFECT OF IUDR AND ICDR ON GROWTH INHIBITION OF *S. faecalis* IN MEDIA SUPPLEMENTED WITH THYMINE, THYMIDINE, OR PTEROYLGLUTAMIC ACID*

	Per cent growth					
	5-Iodo-2′deoxyuridine			5-Iodo-2′-deoxycytidine		
Concentration of analog (μmole/ml)	Pteroyl-glutamic acid	Thymine	Thymidine	Pteroyl-glutamic acid	Thymine	Thymidine
none	100	100	100	100	100	100
0·05	52	85	88	87	100	100
0·1	48	56	85	80	100	100
0·2	38	32	80	80	100	100
0·5	33	24	34	80	100	100
1·0	18	20	14	80	100	88
2·0	18	19	7		100	74

* IUDR and ICDR in the amounts indicated were added to culture tubes that contained basal medium[22] and either thymine, thymidine (both at 5 mμmoles/ml), or pterolyglutamic acid (0·01 mμmole/ml) in a final volume of 10 ml. After inoculation the tubes were incubated at 37 °C for 18 hr, and growth was measured turbidimetrically in a Klett-Summerson photoelectric colorimeter (filter no. 66).

IUDR caused the death of 50 per cent of Ha/ICR Swiss mice within 24 hr after a single intraperitoneal dose of 2·5 g/kg;[10] on the other hand, in BDF_1 mice the same dose of IUDR caused the death (within 96 hr) of 80 per cent of the animals, and all mice died after a single dose of 3·5 g/kg. In contrast, ICDR was not lethal for BDF_1 mice in single doses ranging from 1·25 to 3·5 g/kg; since the supply of this costly compound has been very limited, larger quantities have not yet been administered. No marked differences in weight loss among the groups given ICDR were observed; the maximal losses (− 2·1 to − 2·6 g) were seen within 2 to 3 days after injection. Even after the repeated daily intraperitoneal administration of ICDR to BDF_1 mice, during periods of 10 days, a significant lethality was not observed, except with the maximal daily dosage of 500 mg/kg (Table 5). In contrast, in earlier studies with IUDR in Ha/ICR Swiss mice all animals, succumbed after the administration of ten daily intraperitoneal doses of 300 mg/kg.[10] From these data it will be evident that ICDR is appreciably less toxic for the mouse than is IUDR.

The comparative effects of the iodinated pyrimidines on neoplastic growth were measured, and the results obtained are presented in Table 6. With molar-equivalent doses, ICDR and IUDR appeared to be equally effective in inhibiting the growth *in vivo* of L1210 and L5178Y lymphomas.

TABLE 5. LETHALITY OF REPEATED DOSES OF ICDR IN BDF_1 MICE*

Daily dose (mg/kg)	No. of deaths	Av. Δ wt. (g)*†
500	6/10	− 4·9
450	0/10	− 4·4
400	0/10	− 4·5
350	1/10	− 4·0
300	0/5	− 3·9
250	0/5	− 3·7
200	0/5	− 4·2
150	0/5	− 1·9

* BDF_1 mice of either sex were used and the compounds were administered once daily by intraperitoneal injection for 10 consecutive days.

† Average weight change from initiation to termination of treatment with ICDR.

TABLE 6. COMPARATIVE EFFECT OF ICDR AND IUDR ON THE GROWTH OF SUBCUTANEOUS IMPLANTS OF THE L1210 AND L5178Y LYMPHOMAS

Neoplasm	Treatment	Daily dose* (mg/kg)	Av. tumor† wt. (mg)	Av. Δ wt.‡ (g)
L1210	Control		450	+ 1·8
	IUDR	150	136	− 1·3
	ICDR	150	149	− 0·5
	ICDR	300	109	− 2·4
L5178Y	Control		226	+ 0·6
	IUDR	150	50	− 2·0
	ICDR	150	91	− 1·6
	ICDR	300	31	− 2·4

* Beginning 24 hr after tumor implantation, the compounds were administered once daily for 6 consecutive days to groups of 10 mice.

† The animals were weighed and then sacrificed; the tumors were excised and weighed on day 8 after tumor implantation.

‡ Average weight change from initiation to termination of treatment with compound.

Effect of ICDR on the biosynthesis of DNA by murine neoplastic cells in vitro. A comparison of the effects of IUDR and ICDR on the alternate metabolic routes available for the formation of DNA-thymidylic acid, *in vitro*, in ascites cell forms of L5178Y lymphoma and Ehrlich carcinoma, indicated that IUDR causes a marked inhibition of the incorporation of both ^{14}C-formate and ^{3}H-thymidine into DNA-thymine of both cell lines. Under these circumstances, ICDR produced significant inhibition in the Ehrlich ascites cells, but was less effective than IUDR, whereas it was essentially inert in the other cell line (Table 7). The lesser degree of inhibition by ICDR, as compared with IUDR, is probably a reflection of the low enzymatic capacities of these cells to convert ICDR to the active form, presumably phosphorylated

derivatives of 5-iodo-2′-deoxyuridine. Which host tissues are capable of converting ICDR to IUDR and its derivatives is a problem now under investigation.

The marked increase in the utilization of ^{14}C-formate in the presence of CDR is in agreement with previously reported studies.[11] Accordingly, the failure of ICDR in the present study to potentiate the utilization of ^{14}C-formate for the biosynthesis of DNA-thymine provides additional evidence that no significant direct dehalogenation of the compound occurred. The available evidence indicates that, in the mouse, 5-iodouracil

TABLE 7. COMPARISON OF THE INHIBITION BY IUDR AND ICDR OF THE UTILIZATION OF RADIOACTIVE FORMATE OR THYMIDINE FOR THE BIOSYNTHESIS OF DNA-THYMINE BY NEOPLASTIC CELLS *in vitro**

Deoxyribonucleoside incubated	Relative specific activity of DNA-thymine†		
	L5178Y lymphoma cells		Ehrlich ascites carcinoma cells
	^{14}C-formate	^{3}H-thymidine	^{3}H-thymidine
None (control)	1·00	1·00	1·00
IUDR	0·26	0·03	0·13
ICDR	0·82	0·92	0·45
CDR	2·5		

* The reaction mixture consisted of packed cells (0.25 ml), horse serum (0·2 ml), ^{3}H-thymidine (0·1 μmole, 5 μc) or ^{14}C-formate (3 μc), the indicated 2′-deoxyribonucleoside (5 μmoles), and Krebs III buffer to give a final volume of 2·3 ml. The incubations were conducted in triplicate in 20-ml beakers in a Dubnoff metabolic shaker at 37 °C (air); agitation was at 90 cpm for 2 hr. The preparation of the cells, the methods of isolation, and the determination of the specific activity of the DNA-thymine have been described previously.[16]

† The specific activities of the control values were equated to 1·00.

is cleaved from IUDR before dehalogenation occurs, with the formation of uracil and free iodide.[10] It has been shown in man that IUDR is rapidly dehalogenated[12, 13], but this probably occurs after cleavage to 5-iodouracil; Pohl *et al.*[14] have demonstrated the extensive debromination of 5-bromouracil in man, and it has been proposed that loss of halogen is preceded by a metabolic reduction of the 5,6-double bond.[15]

DISCUSSION

The incorporation of ICDR into the DNA of murine neoplastic mast cells in culture, via deamination and phosphorylation, in the form of 5-iodo-2′-deoxyuridine 5′-phosphate, in place of equivalent amounts of thymidylic acid, probably accounts in part for its action in inhibiting the reproduction of cells, decreasing their viability, and increasing their sensitivity to X-irradiation; however, under these circumstances the compound is not superior to IUDR. Evidence obtained *in vitro* with other murine neoplastic cells (L5178Y lymphoblasts; Ehrlich ascites carcinoma) demonstrates that ICDR, or more probably its derivative (IUDR 5′-P),[16] inhibits at another possible site—namely, the utilization of thymidine for the biosynthesis of DNA-thymine at either the nucleoside or nucleotide level. Although it has not been established unequivocally that either ICDR or BCDR[5] may be deaminated directly by neoplastic cells before phosphorylation, the studies with mast cells resistant to FUDR and

cross-resistant to BCDR, IUDR, and ICDR, but not to FCDR, strongly indicate that primary deamination of ICDR to form IUDR does occur with the parent cells, and studies are in progress with cell-free fractions of such cells with a view to the identification of the responsible enzyme. Pertinent also are preliminary studies by our colleague, Dr. W. A. Creasey, who has shown that ICDR and BCDR are rapidly deaminated, in the absence of added ATP, by fractions obtained from particle-free, dialyzed portions of homogenates of mouse kidney. In view of reports that deoxycytidylate deaminase activity, present in rapidly growing mammalian tissues (e.g., neoplasms, embryonic tissues, regenerating liver, thymus, and bone marrow) is absent or is suppressed[17–19] in many normal tissues, it will be of much interest to study the distribution of the deaminase for which ICDR and BCDR appear to be direct substrates.

It should be emphasized that the currently available findings do not permit the conclusion that, *in vivo*, ICDR serves only as a source of IUDR and that an intermediary formation of ICDR 5′-P does not occur. As has been indicated elsewhere,[11] CDR increased the utilization of ^{14}C-formate for the biosynthesis of DNA-thymine in Ehrlich ascites cells to a greater extent than did UDR; this finding has been attributed to the fact that CDR provides a larger supply of deoxyuridine 5-phosphate than does UDR *per se*.[20] In Ehrlich ascites cells in mice, ^{82}Br-BCDR was a better source of intracellular radioactivity than was ^{82}Br-BUDR;[3] furthermore, such uptake did not occur with K^{82}Br, and the stability of the label (greater than that after the administration of ^{82}Br-BUDR) during subsequent cell replications indicated that incorporation into DNA had occurred. Accordingly, it may be anticipated that, *in vivo*, ICDR may provide higher intracellular levels of IUDR 5′-P in certain types of cells than does IUDR *per se*. In addition, the lower toxicity of ICDR, its greater solubility at neutral pH, and its higher resistance to thermal decomposition in solution suggest that it may have several practical, as well as metabolic, advantages over IUDR as a chemotherapeutic agent.

Acknowledgements—These studies, which were reported in preliminary form,[23] were supported by grants CY-2817 and CY-5262 from the National Cancer Institute, and by supplies of 5-iodo-2′-deoxyuridine, obtained by the Cancer Chemotherapy National Service Center, each of the United States Public Health Service; also grant support was provided by the American Cancer Society (T-17 and T-23). In addition, we are particularly grateful to the Connecticut Division of the American Cancer Society for special funds for the procurement of 5-iodo-2′-deoxyuridine, and, more recently, together with the national organization, for an emergency grant that will provide supplies of 5-iodo-2′-deoxycytidine for clinical investigation.

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