Nucleic Acids. II. Cytotoxicity Studies with Nucleotides and Dinucleoside Phosphates Containing *ara*-Cytidine

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Thirty-one derivatives and analogs of 1-β-D-arabinofuranosylcytosine (*ara*-cytidine), including simple substituted nucleosides, nucleotides, and dinucleoside phosphates, were tested for cytotoxicity to KB carcinoma cells in culture. The simple nucleotides showed good activity in this system which was prevented by deoxycytidine in the cases tested. The 3′→5′-dinucleoside phosphates of *ara*-cytidine and various other nucleosides showed significant cytotoxic activity *vs.* KB cells and were usually more active than their corresponding 2′→5′ analogs. The 5′→5′-dinucleoside phosphates of *ara*-cytidine also showed good activity. No conclusions can yet be drawn regarding the possible penetration and/or action of the intact dinucleoside phosphates. Twenty of the compounds were tested for cytotoxicity *vs.* L5178Y mouse leukemia cells in culture and its *ara*-cytidine-resistant mutant ("kinaseless"). For the most part, the compounds investigated, including the simple nucleotides, were highly cytotoxic to the parent cells but completely cross-resistant with *ara*-cytidine against the mutant. Of particular interest was the finding that certain adenosine-containing dinucleoside phosphates and adenosine *per se* were cytotoxic to both the parent and *ara*-cytidine-resistant lines. Although hydrolysis to liberate adenosine in the mutant cell is the likely explanation for sensitivity to several dinucleoside phosphates, additional studies are needed to establish this point unequivocally. Unlike the situation with KB cells, the 2′→5′-dinucleoside phosphates tested were as active as the 3′→5′ congeners against L5178Y mouse leukemia. Structure–activity relationships which can be drawn from this study are discussed.

1-β-D-Arabinofuranosylcytosine (cytarabine, cytosine arabinoside, *ara*-cytidine, CA, *ara*-C) is a synthetic abnormal nucleoside which shows marked inhibitory activity against mammalian cells and DNA viruses *in vitro* and *in vivo*.[1-7] This agent has shown unequivocal activity against leukemias and lymphomas and herpes simplex keratitis in man.[8-14] The experiments described in this paper result from a determined effort to prepare a wide variety of nucleotides and dinucleoside phosphates containing *ara*-C in the hope of uncovering unique activity in mammalian cells or whole animals. The results of cytotoxicity studies with a variety of such compounds in three mammalian cell lines and, in selected cases, the effects of deoxycytidine upon such activity are presented here. A companion paper describes the synthesis of the new compounds.[15] Preliminary reports of these experiments and the antiviral properties of the substances have appeared.[16-18]

Materials and Methods.—Cytotoxicity against KB human epidermoid carcinoma cells was determined in the 3-day test described previously.[19] The compounds were added at zero time and protein content determined on day 3. The sensitive parent strain of L5178Y cells and its *ara*-C-resistant mutant (Ca55) were maintained according to the procedure of Chu and Fischer[20] with the compounds added at zero time and cell growth determined by counting cell numbers on day 2 or 4. Abbreviations for dinucleoside phosphates are explained in Chart II of the accompanying paper.[15] Deoxycytidine and adenosine were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. A_p and pA were purchased from Schwartz Bioresearch, Inc., Orangeburg, N. Y., and A_pU, U_pCA, and U^pCA were purchased from Zellstoffabrik Waldhof, Mannheim, Germany (U. S. representative, H. L. Jensen, Inc., Montclair, N. J.).

Results

Cytotoxicity Studies with KB cells. In the standard 3-day cytotoxicity test,[19] the following highly substituted nucleosides tested had low activity ($ID_{50} > 20$ μg/ml) against KB cells: TrCA(AcAcNAc), CA(AcAcNAc), CA(NAc), MTrCA(NAc), CA(BzBzNBz), TrCA, TrCA(NBz). (See footnote 6 of ref 15 for an explanation of the abbreviations used here and in the tables.) Since the ID_{50} of *ara*-C under these test conditions is 0.05–0.1 μg/ml, no detailed studies were undertaken with these relatively inactive compounds.

Cytotoxicity data on compounds which showed significant and reproducible inhibition of KB cells are present in Table I. It is obvious that all of the simple nucleotides of *ara*-C (2′-, 3′-, and 5′-phosphates) were at least as active as *ara*-C on a molar basis. In five of the seven cases in which dinucleoside phosphates were compared, the 3′→5′ phosphate was more active than was the 2′→5′ phosphate (CA_pA *vs.* CA^pA; CA_pCA *vs.* CA^pCA; CA_pdA *vs.* CA^pdA; CA_pT *vs.* CA^pT and CA_pU *vs.* CA^pU). On the contrary, U^pCA and A^pCA were as cytotoxic to KB cells as were the corresponding 3′→5′-dinucleoside phosphates U_pCA and A_pCA. In most of these cases, the activity of the 3′→

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TABLE I
CYTOTOXICITY OF *ara*-CYTIDINE DERIVATIVES TO KB CELLS

Compd	Median ID_{50} μg/ml	Median ID_{50} m$M \times 10^4$	Range of ID_{50}, μg/ml	No. of expts
CA	0.05	2	0.03–0.11	22
CA^p	0.05	1.5	0.03–0.05	5
CA$_p$	0.06	1.85	0.03–0.07	5
pCA	0.06	1.75	0.04–0.15	10
MepCA	0.4	11	0.3–0.55	6
PhpCA	0.07	1.7	0.05–0.08	5
CA^{p}A	0.60	10	0.48–0.67	6
CA$_p$A	0.12	2	0.08–0.16	6
CA^{p}dA	2.3	40	1.7 and 2.8	2
CA$_p$dA	0.12	2.1	0.12–0.22	3
CA^{p}CA	0.66	12	>0.4 and 0.66	2
CA$_p$CA	0.06	1	0.05 and 0.07	2
CA^{p}U	0.47	8.3	0.44 and 0.50	2
CA$_p$U	0.15	2.65	0.09–0.24	3
CA^{p}T	1.7	30	1.7–2.0	3
CA$_p$T	0.19	3.4	0.19–1.9	3
A^{p}CA	0.05	0.9	0.03–0.07	3
A$_p$CA	0.08	1.4	0.05–0.1	3
U^{p}CA	0.13	2.3	0.12–0.15	3
U$_p$CA	0.13	2.3	0.12–0.15	3
dU$_p$CA	0.16	3	0.05–0.27	4
pCAdC	0.11	2.1	0.06–0.14	8
pCACA	0.20	3.6	0.19–0.25	6
pCAC	0.1	2	>0.1–0.14	3
pCAdU	0.1	1.6	0.07 and 0.12	2

5′-dinucleoside phosphate equalled that of *ara*-C *per se* on a molar basis.

5′→5′-Dinucleoside phosphates were approximately as active against KB as was *ara*-C. It is interesting that pCAdC showed striking activity, particularly when one considers that dC prevents the activity of *ara*-C when added to the assay medium in excess. It should be noted that PhpCA is several times more cytotoxic to KB cells than is MepCA, both on a weight and on a molar basis.

Under various test conditions, deoxycytidine prevents or delays the cytotoxic action of *ara*-C both *in vitro vs.* mammalian cells and DNA viruses and in whole animals.[1,3,21] The cytotoxicity of selected dinucleoside phosphates was determined in the presence and absence of 10 μg of deoxycytidine/ml to determine whether direct evidence could be obtained that these compounds were acting by a mechanism not involving cleavage to *ara*-C. The results of these experiments are presented in Table II. These data show significant prevention of the cytotoxic action of pCA and the three dinucleoside phosphates tested in addition to the free nucleoside. Further studies in which the concentration of deoxycytidine was varied from 0.08 to 10 μg/ml confirmed the marked reduction of activity at the highest level of deoxycytidine. Significant reversal was observed at the lower concentrations of deoxycytidine but not in a direct ratio to the amount of this nucleoside present. Thus, no evidence was obtained with the nucleosides investigated that their action at the molecular level was significantly different from *ara*-C *per se* by this test.

Cytotoxicity Studies with L5178Y and Its Resistant Mutant.—In a recent publication,[20] Chu and Fischer reported the isolation of a mutant cell line of L5178Y mouse leukemia which was resistant to *ara*-C, presumably

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TABLE II
EFFECT OF DEOXYCYTIDINE ON CYTOTOXICITY OF SELECTED COMPOUNDS IN KB CELLS

Agent	ID_{50}, μg/ml −dC	ID_{50}, μg/ml +dC (10 μg/ml)
CA	0.06	>1
pCA	0.1	>1
pCAdC	0.1	>1
CA$_p$U	0.09	>1
CA$_p$dA	0.12	>1
CA$_p$A	0.13	>0.4
CA^{p}A	0.67	>2
MepCA	0.55	>2

lacking a kinase for phosphorylating this nucleoside. These cultures were obtained through the courtesy of Dr. Fischer and a variety of *ara*-C derivatives and dinucleoside phosphates were tested for cytotoxic activity therein. The results of these investigations are shown in Table III. In some of these cases, the

TABLE III
CROSS-RESISTANCE STUDIES IN L1578Y CELL LINES

Agent	Median ID_{50}, μg/ml Parent	Kinaseless mutant[a]	No. of expts
CA	0.02	35	7
CA^{p}A	0.09	3.2[b]	6
CA$_p$A	0.07	1.6[b]	7
A^{p}CA	0.05	1.3	2
A$_p$CA	0.05	1.5	2
pCAA	0.05	1.7	3
CA^{p}dA	0.06	85	3
CA$_p$dA	0.05	>50	3
pCA	0.03	>10	1
pCA(NBz)	0.5	>10	2
CA$_p$	0.03	>50	2
CA^p	0.03	>10	2
T$_p$CA	0.03	>10	1
CA$_p$T	0.03	19	2
CA^{p}T	0.07	>50	2
CA$_p$dU	0.04	>10	2
CA^{p}dU	0.02	>10	2
pCACA	0.04	25	2
pCAdU	0.03	>10	2
pCAC	0.04	50	2
PhpCA	0.03	>25	3
MepCA	0.07	25	3
A	0.3	0.4	7
pA	0.5	0.44	1
A$_p$	0.3	0.3	1
A$_p$A	0.6	0.5	1
U$_p$A	11	>40	3
A$_p$U	>4[c]	>4	3
U$_p$CA	0.03	20	2
U^{p}CA	0.04	20	2

[a] Clone Ca55 isolated from a subline of L5178Y. [b] $p < 0.01$. [c] Values for three assays were 3.2, >4, and >4 μg/ml.

absolute end point of activity was not determined since clear-cut cross-resistance was demonstrated, which was the primary purpose of the experiment.

The unique cytotoxicity of adenosine (A) and A-containing dinucleoside phosphates should be noted. Although the partial cross-resistance of all the compounds which inhibited the Ca55 line can be explained by hydrolysis of the dinucleoside phosphate to liberate adenosine, reversal studies with deoxycytidine are not wholly consistent with this explanation. As shown

TABLE IV
REVERSAL STUDIES IN L5178Y AND Ca55 CELLS[a]

Compd	ID_{50} μg/ml: Parent	Mutant
CA	0.02	40
CA + dC	0.08	55
CA + U	0.01	30
A	0.3	0.4
A + dC	7	0.98
A + U	7	17
CA_pA	0.07	1.6
CA_pA + dC	0.5	7
CA_pA + U	>0.01	>20
CA^pA	0.09	4
CA^pA + dC	0.23	30
U	>40	...

[a] Reversing agents added at 10 μg/ml.

in Table IV, dC reversed the cytotoxic activity of A in the parent L5178Y cells to a much greater extent than was observed with the Ca55 mutant. With the A-containing compounds CA_pA and CA^pA, however, the reversal of activity in the mutant cell line with dC was greater than observed with either A or *ara*-C alone.

If the compounds which were only partially cross-resistant in the Ca55 mutant are active by virtue of hydrolysis to liberate the cytotoxic component A, U_pA and A_pU show a lower order of activity than anticipated. When combinations of A and U were tested together, the cytotoxicity of A was reversed by U, as shown in Table IV. These data were confirmed on repeat experiments and suggest that the low order of activity of dinucleoside phosphates containing A and U is due to reversal of the cytotoxic activity of A by U rather than by slower hydrolysis of these compounds.

Discussion

Based on the studies reported here, certain structure–activity relationships can be deduced. The simple nucleotides of *ara*-C show marked cytotoxic activity against both KB cells and the mouse L5178Y cell in culture. Converting the 5′-phosphate of *ara*-C to the phenyl ester allows retention of considerable activity, whereas conversion of this nucleotide to the methyl ester results in a marked loss of activity in KB. Almost all of the *ara*-C-containing compounds examined were highly cytotoxic to L5178Y cells in culture.

In general, the 3′→5′-dinucleoside phosphate esters containing *ara*-C are more active against KB cells than the corresponding 2′→5′ derivatives (A^pCA and U^pCA are notable exceptions). On the whole, the 5′→5′-dinucleoside phosphates containing *ara*-C showed good cytotoxicity equalling, in some cases, that of *ara*-C *per se* on a molecular basis. In all cases investigated thus far, including a small number of nucleotides and dinucleoside phosphates, cytotoxicity to KB or L5178Y cells was decreased by the addition of deoxycytidine at zero time, as is the case for *ara*-C. Although this observation suggests that the substituted compounds ultimately act in the cell at the same locus as does *ara*-C, it is based on an indirect measure. This conclusion cannot be firmly drawn until further biochemical studies with radioactive substrates are completed. The partial sensitivity demonstrated for certain A-containing dinucleoside phosphates in L5178Y cells and their "kinaseless" mutant may be due to hydrolysis liberating A or pA, both of which are highly cytotoxic to the mutant cell. Aronow[22] reported that adenine, but not adenosine, was cytotoxic to mammalian cells in culture at concentrations of 10^{-3} *M* (280 μg/ml) and that the inhibition was partially reversed by uridine. With the exception of certain reversal data, the partial cross-resistance of A-containing compounds can be explained by hydrolysis of the compound to liberate A which is the cytotoxic component in the L5178Y lines. Added dC may reverse the cytotoxicity of CA_pA and CA^pA to Ca55 cells more than would be expected from its effects on A or *ara*-C alone. Although the 5′→5′-dinucleoside phosphate of mercaptopurine riboside was not cross-resistant with the free nucleoside against a mercaptopurine-resistant cell line in culture,[23] other investigations with substituted nucleotides of 5-fluorodeoxyuridine failed to demonstrate any unique properties which could be attributed to biological activities of the unhydrolyzed compounds.[24–26] In the present work, the 5′→5′-dinucleoside phosphate of *ara*-C *was* cross-resistant with *ara*-C itself in the mutant cell line (see Table III).

In spite of the fact the deoxycytidine is able to prevent the activity of the derivatives tested and no unique biological effects were observed in the systems studied, this does not mean *a priori* that the same biological effects will be observed in a whole animal as are seen with *ara*-C alone. Organ penetration of dinucleoside phosphates or nucleotides may well differ from that of *ara*-C resulting in high concentrations of *ara*-C, its phosphate, or congeners in a particular site in the body in which the free nucleoside does not localize. As an example, pdC was shown to be metabolized differently from dC itself in a uracil-requiring strain of *E. coli*, while MepdC did not serve as a pyrimidine source.[27] In addition, the deamination of *ara*-C, known to occur in mammalian cells and whole animals,[2,9,28] may not proceed with the dinucleoside phosphates or may proceed at such a low rate that this reaction will not limit the effective concentration of *ara*-C delivered to a target organ. For these reasons, additional studies in whole animals are warranted with selected dinucleoside phosphates containing *ara*-C.

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