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SYNTHESIS OF 5-CHLORODEOXYURIDINE AND A COMPARATIVE STUDY OF 5-HALODEOXYURIDINES IN *E. COLI**

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Abstract—5-Chlorodeoxyuridine (ClUDR) was synthesized for the purpose of comparing its inhibitory properties with 5-fluorodeoxyuridine (FUDR), 5-bromodeoxyuridine (BrUDR) and 5-iododeoxyuridine (IUDR). With *Escherichia coli* K 12 as the test organism, it was found that FUDR was about 4000 times more effective than ClUDR which, in turn, was nine times more effective than BrUDR for inhibiting growth of the bacteria to 50 per cent of a control. IUDR was not inhibitory to growth of *E. coli* K 12. Low concentrations of ClUDR, BrUDR or IUDR strongly potentiated the inhibitory effect of FUDR in *E. coli* over a 200- to 300-fold concentration range by limiting the utilization of thymidylate for DNA synthesis. Stimulation of growth with higher concentrations of ClUDR, BrUDR or IUDR in the presence of FUDR indicates that these halogen derivatives serve as thymidine substitutes for DNA synthesis.

INTRODUCTION

AN INTERESTING feature of the inhibitory effects of 5-bromodeoxyuridine (BrUDR),§ 5-fluorodeoxyuridine (FUDR) and 5-iododeoxyuridine (IUDR) on nucleic acid metabolism is that the nature of the halogen in the 5-position of the pyrimidine influences markedly the effectiveness and type of inhibition produced by the deoxynucleoside derivatives. FUDR inhibits DNA synthesis by blocking the methylation of deoxyuridylate to thymidylate,[1, 2] whereas BrUDR[3, 4] and IUDR[5] inhibit incorporation of thymine compounds into DNA. In addition, 5-bromouracil,[6–8] 5-chlorouracil[8] and 5-iodouracil[6, 8] replace thymine of DNA, while 5-fluorouracil,[1, 9, 10] and to a lesser extent 5-chlorouracil,[11] are incorporated in place of uracil into RNA.

5-Chlorodeoxyuridine (ClUDR) was prepared to make possible additional comparisons of the inhibitory effects of 5-halodeoxyuridine derivatives. ClUDR has been identified previously as a constituent of DNA from bacteria grown in the presence of 5-chlorouracil.[12, 13]

Escherichia coli K 12 was chosen as a test organism for the comparative studies because FUDR, ClUDR or BrUDR inhibit growth of this strain which does not require exogenous pyrimidines for growth. Therefore inhibitory effects of the halodeoxyuridine compounds may be assumed to be the result of interference with

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§ The abbreviations used are: BrUDR, 5-bromo-2′-deoxyuridine; ClUDR, 5-chloro-2′-deoxyuridine; FUDR, 5-fluoro-2′-deoxyuridine; IUDR, 5-iodo-2′-deoxyuridine; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

intermediates arising *de novo* rather than with intermediates of "salvage" pathways of synthesis, such as the conversion of pyridimidine nucleosides to the corresponding 5′-phosphates.

MATERIALS AND METHODS

Materials

Deoxyuridine, deoxycytidine, uridine, cytidine, orotic acid, thymidine, thymidine-5′-phosphate and IUDR were purchased from the California Corporation for Biochemical Research, Los Angeles. FUDR was obtained through the courtesy of the Cancer Chemotherapy National Service Center, Bethesda, Maryland. BrUDR was synthesized by methods previously described.[14] ClUDR was prepared as described below.

5-Chlorodeoxyuridine

Powdered deoxyuridine (5·25 g, 0·023 mole) was dissolved in 300 ml of glacial acetic acid at room temperature. A 10% excess of dry chlorine in 200 ml of cold anhydrous carbon tetrachloride was added and the solution allowed to stand overnight. After removal of the solvents by lyophilization, the residue was deacetylated in 400 ml of anhydrous methanol containing from 10 to 15% anhydrous ammonia. Hydrolysis was complete upon standing at room temperature for 3 days. The volume was reduced to 30 ml and crystallization was induced by addition of ethyl acetate until the solution became cloudy. The crystalline product which appeared upon standing was recrystallized from absolute ethanol. The white crystals, 1·95 g (30 per cent yield), melted at 178–179·5 °C (uncorrected). The λ of maximum and minimum absorption was 278 mμ and 239 mμ, respectively, at pH 7 (a_M max., 10·06 $\times$ 10^3).

Anal. Calcd. for $C_9H_{11}O_5N_2Cl$: C,41·15; H, 4·22; N, 10·67. Found: C, 41·05; H, 4·06; N, 10·91.

Assay Methods

The halodeoxyuridine compounds were assayed with *E. coli* K 12 in a glucose salts medium using methods previously described.[15] The tubes were incubated at 37 °C for 18 hr, at which time growth of the controls was maximal. Growth was measured as turbidity in a Klett–Summerson photoelectric colorimeter (filter no. 66). All determinations were run in duplicate or triplicate.

RESULTS AND DISCUSSION

The growth response of *E. coli* K 12 to increasing concentrations of FUDR, ClUDR, BrUDR and IUDR is summarized in Fig. 1. Comparison of the concentrations of the analogs which limit growth to 50 per cent of a control reveals that FUDR is about 4000 times more inhibitory than ClUDR which, in turn, is about nine times more active than BrUDR. IUDR was not inhibitory at concentrations up to 2000 μM. Complete repression of growth for an 18-hr period was produced by 0·08 μM FUDR or 1600 μM ClUDR, but 2600 μM BrUDR caused only partial inhibition. Growth was completely suppressed for an indefinite period by 3 μM FUDR, whereas 2600 μM ClUDR did not inhibit growth during longer incubation periods. 5-Chlorouracil, 5-bromouracil or 5-iodouracil were not inhibitory.

Several pyrimidine compounds known to participate in the biosynthesis of nucleic acids were tested for their ability to reverse the growth inhibition of *E. coli* K 12 by ClUDR or FUDR (Figs. 2–4). Such inhibition reversal studies are often not informative in determining the nature of inhibitory action. However, since FUDR, IUDR and BrUDR have been studied extensively and known to have inhibitory actions at specific

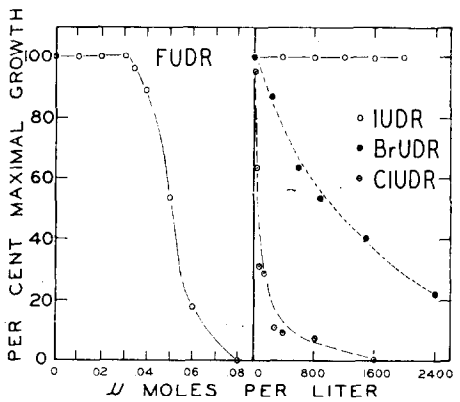

FIG. 1. Comparison of the inhibitory effect of 5-fluorodeoxyuridine (FUDR), 5-iododeoxyuridine (IUDR), 5-bromodeoxyuridine (BrUDR) and 5-chlorodeoxyuridine (ClUDR) on the growth of *E. coli* K 12. Incubated at 37 °C for 18 hr in a glucose-salts medium.

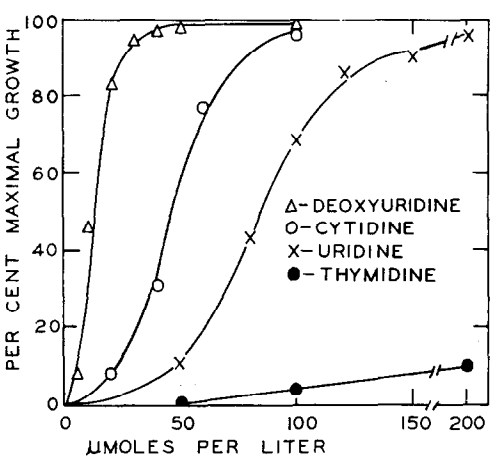

FIG. 2. Comparison of the effectiveness of various pyrimidine compounds in reversing the growth of *E. coli* K 12 in the presence of 0·5 μM 5-fluorodeoxyuridine. Incubated at 37 °C for 18 hr in a glucose-salts medium.

metabolic sites, it was expected that interpretation of reversal data in the presence of ClUDR would be more specific than is usually possible.

It is apparent from the data of Figs. 2–4 that cytosine and uracil ribonucleosides or deoxyribonucleosides are effective to a variable extent in reversing the inhibitory effects of ClUDR or FUDR. No valid interpretations can be made from the fact that differences were observed in the effectiveness of these nucleosides in reversing inhibition because facile interconversions are possible. For example, cytosine compounds

may be converted to uracil compounds by deaminases, and deoxynucleosides may be precursors of ribonucleotides by a prior cleavage to uracil with deoxynucleoside phosphorylase. The relative rates of these reactions *in vivo* are not known. In addition, it is probable that the halodeoxyuridine compounds inhibit to a variable extent the

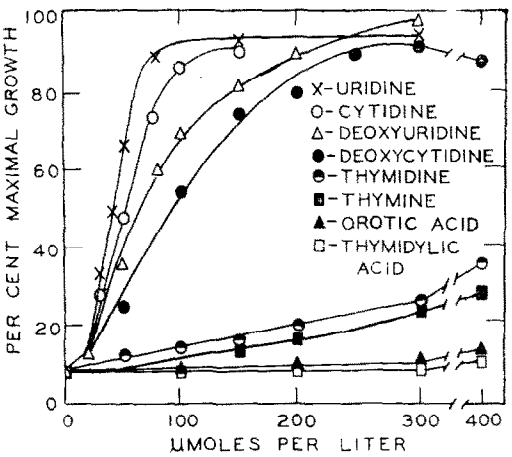

FIG. 3. Comparison of the effectiveness of pyrimidine compounds for reversing the growth of *E. coli* K 12 in the presence of 800 μM 5-chlorodeoxyuridine. Incubated at 37 °C for 18 hr in a glucose–salts medium.

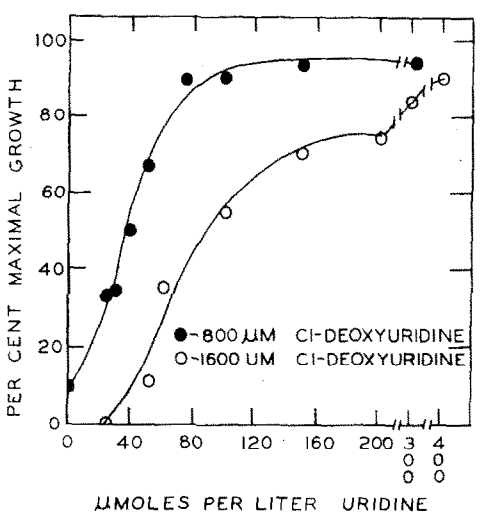

FIG. 4. Reversal of *E. coli* K 12 growth by uridine at two concentrations of 5-chlorodeoxyuridine. Incubated at 37 °C for 18 hr in glucose–salts medium.

conversion of the reversing agents to compounds of the nucleotide pool. At a concentration of FUDR which is about six times the minimum concentration for complete inhibition, thymidine is a relatively ineffective reversing agent (Fig. 2). It is apparent from Fig. 3 that thymine, thymidine and thymidylic acid are ineffective reversing agents at a concentration of ClUDR which does not produce complete inhibition. Deoxyuridine, uridine, deoxycytidine and cytidine reverse the inhibition of

ClUDR to approximately that of control growth (Fig. 3), but as shown in Fig. 4 reversal with uridine is not competitive.

Thymine compounds do not undergo the facile interconversions mentioned above. For this reason, and since FUDR,[1, 2] BrUDR[3, 4] and IUDR[5] are known to inhibit metabolism of thymine compounds, the effectiveness of thymidine for reversing inhibitions produced by halodeoxyuridine compounds was of interest. In the presence of 0·061 μM FUDR, which allows 2 per cent of control growth, thymidine is an effective reversing agent, but at higher concentrations of FUDR (0·5 μM) thymidine produced only a slight reversal effect (Fig. 5). These results support previous data[1, 2]

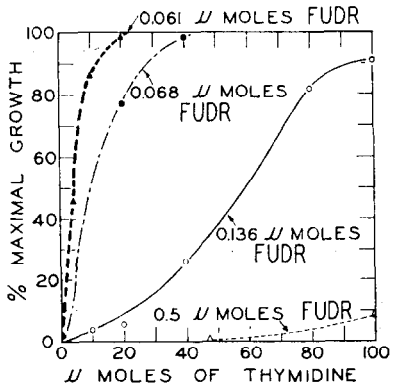

FIG. 5. Reversal of *E. coli* K 12 growth by thymidine in the presence of various concentrations of 5-fluorodeoxyuridine (FUDR). Incubated at 37 °C for 18 hr in glucose–salts medium.

indicating that the primary effect of FUDR on *E. coli* growth is the inhibition of thymidylate synthetase. The additional inhibitory effects, which probably are the result of interference with RNA metabolism by prior cleavage to fluorouracil,[2] can be overcome with either uracil or cytosine nucleosides (Fig. 2).

Fig. 6 summarizes the effectiveness of thymidine as a reversal agent in the presence of ClUDR. At 800 μM ClUDR, thymidine had no effect. Only partial reversal was produced by thymidine in the presence of 200 μM ClUDR which allowed about 30 per cent of control growth. From this it may be concluded that, if ClUDR inhibits thymidylate synthetase or thymidylic acid utilization, there are additional sites of inhibition at this concentration. The lack of a good growth response to thymidine in the presence of uridine and 800 μM ClUDR (Fig. 6) substantiates the above conclusion.

Since bromouracil, chlorouracil and iodouracil are incorporated into DNA in place of thymine under conditions of thymine deficiency,[12] it was of interest to determine the effect of the corresponding nucleoside derivatives on growth of *E. coli* K 12 in the presence of a low concentration of FUDR which specifically limits thymidylate synthesis (Fig. 5). At these conditions it is reasonable to expect that BrUDR, ClUDR or IUDR would be incorporated into DNA to a greater extent than in the presence of an uninhibited supply of endogenous thymidine phosphates. Furthermore, since FUDR limits thymidylate synthesis,[1, 2] and BrUDR[3, 4] or IUDR[5] inhibits thymidylate utilization, it seemed logical to predict that a combination of FUDR with BrUDR or IUDR would act synergistically as growth inhibitors. It was therefore of interest to compare the effects of various concentrations of ClUDR, IUDR and BrUDR on

growth and viability of *E. coli* in the presence of a low concentration of FUDR which limits thymidylate formation selectively[1, 2] (Figs. 7 and 8).

Fig. 7 summarizes the data obtained from the use of a combination of ClUDR and FUDR. Almost identical results were obtained by substituting BrUDR or IUDR for ClUDR (Fig. 8). At a concentration of FUDR (0·034 μM), which allows 97 per cent maximal growth, 0·03 μM to 10 μM ClUDR, IUDR or BrUDR inhibited

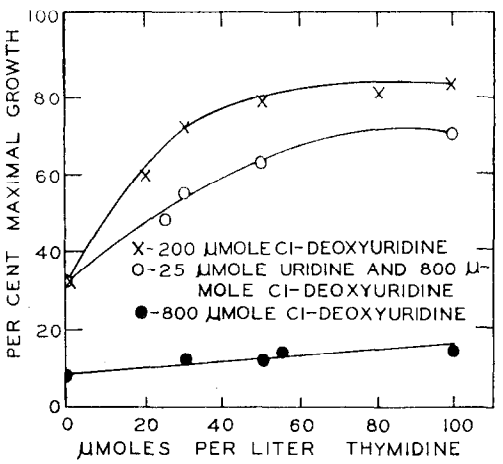

FIG. 6. Reversal of *E. coli* K 12 growth by thymidine in the presence of 5-chlorodeoxyuridine. Incubated at 37 °C for 18 hr in glucose-salts medium.

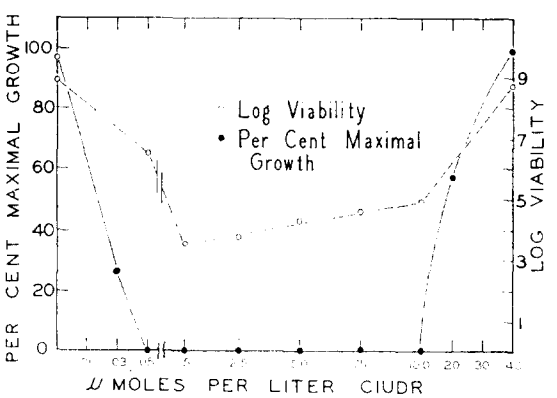

FIG. 7. Effect of various concentrations of 5-chlorodeoxyuridine (ClUDR) in the presence of 0·034 μM 5-fluorodeoxyuridine (FUDR) on growth and viability of *E. Coli* K 12. Incubated at 37 °C for 18 hr in glucose-salts medium.

growth completely and cell viability was reduced to about 1 per cent of the inoculum. Thus, although IUDR alone does not inhibit growth of *E. coli* K 12 at concentrations up to 2000 μM, it is a very potent inhibitor in the presence of FUDR which allows 97 per cent control growth. Similarly, ClUDR or BrUDR completely inhibit growth over a 200- to 300-fold concentration range (Figs. 7 and 8).

One μM thymidine or 10 μM thymine completely reversed the inhibitory effect of the combined halonucleosides. Viability and turbidity were also restored to

control growth by concentrations of ClUDR, BrUDR or IUDR above 10 μM. Thus, ClUDR, BrUDR or IUDR at low concentrations in combination with FUDR are potent inhibitors of growth and viability over a 200- to 300-fold concentration range, whereas higher concentrations of the same analogs reverse this effect (Figs. 7 and 8).

The synergistic effect on growth (Fig. 8) is consistent with activities of FUDR,[1, 2] BrUDR[3, 4] and IUDR,[5] which block sequential reactions of thymine nucleotide metabolism. BrUDR or IUDR, which are known to inhibit thymidylate utilization, are relatively poor inhibitors of *E. coli* K 12 growth when they are not used in combination with FUDR (Fig. 1). This ineffectiveness may be due to a rapid *de novo* synthesis

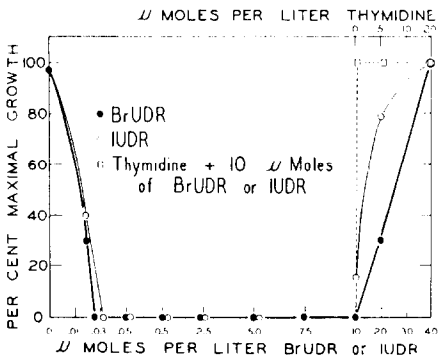

FIG. 8. Effect of various concentrations of 5-bromodeoxyuridine (BrUDR) or 5-iododeoxyuridine (IUDR) in the presence of 0·034 μM 5-fluorodeoxyuridine (FUDR) on growth of *E. coli* K 12. Incubated at 37 °C for 18 hr in glucose–salts medium.

of thymidylate. However, when thymidylate is limited by a concentration of FUDR which allows 97 per cent control growth, BrUDR or IUDR are potent inhibitors. It may be concluded that the combination of BrUDR and FUDR or IUDR and FUDR specifically limits the availability of thymine nucleotides for DNA synthesis since thymine or thymidine reverse the inhibitory effect completely.

The effects of ClUDR (Fig. 7) are almost identical to those produced by BrUDR or IUDR (Fig. 8). By analogous reasoning it may be concluded that ClUDR also inhibits utilization of thymidylate. From Fig. 3 it is apparent that ClUDR causes additional inhibitory effects which are reversed with uracil or cytosine nucleosides but not thymidine. These effects are not produced by a prior cleavage of ClUDR to 5-chlorouracil, since the latter compound, in contrast to 5-fluorouracil, does not inhibit *E. coli* K 12.

The observation that higher concentrations of ClUDR, BrUDR or IUDR (Fig. 7 and 8) circumvent both inhibitory effects and produce growth, suggests that these 5-halopyrimidine compounds substitute for thymine nucleotides in DNA synthesis. If this interpretation is correct, it may be concluded that substitution of the 5-halopyrimidines for thymine in DNA does not have a permanent lethal effect on the viability of a substantial population of the cells. This conclusion is consistent with the data of Zamenhof and Griboff[7] who found that from 17 to 19 per cent incorporation of 5-bromouracil into *E. coli* can occur without affecting the speed of cell division. A variable tolerance of tumour cells to replacement of thymine by 5-bromouracil has been demonstrated by Hakala[16] who found that the replacement limit in HeLa

cells is about 50 per cent and in mouse fibroblasts greater than 80 per cent. The similar effectiveness of ClUDR, BrUDR and IUDR in replacing the thymine requirement for growth suggests that chlorouracil, iodouracil and bromouracil are comparable in serving as non-lethal substitutes for DNA thymine.

It is unlikely that conditions (Figs. 7 and 8) are ideal for continued incorporation of unnatural bases into DNA during the entire 18-hr incubation period. As the number of cells increase, the conversion rate of nucleoside derivatives to free bases by nucleoside phosphorylase increases. Thus, as growth proceeds, it would be expected that the concentration of FUDR would drop[2] to a level which does not inhibit thymidylate formation. As endogenous thymidylate becomes available, incorporation of ClUDR, BrUDR and IUDR would be expected to decrease. This does not detract from the conclusion that growth is probably stimulated (Figs. 7 and 8) by incorporation of the halonucleosides into DNA in place of thymidylate during the first few hours of incubation.

It is probable[1, 2] that FUDR owes its anti-tumor activity to the fact that it produces cell death because of its primary action on thymidylate synthesis, thus producing a chemically-induced "thymidineless death" of rapidly dividing cells.[17] The present data show that in *E. coli* K 12 a relatively narrow concentration range of FUDR is effective for limiting thymidylate synthesis, without at the same time inducing other inhibitory mechanisms (Fig. 5). A thymidine-less condition is produced by a low concentration of FUDR in combination with ClUDR, BrUDR or BrUDR over a wide range of the latter compounds. This combination of inhibitors completely inhibits *E. coli* multiplication over 200- to 300-fold concentration range without inhibiting reactions other than those involving thymidylate formation or utilization. Under these conditions, DNA synthesis is limited specifically by an inadequate supply of thymine compounds and cell death as well as growth inhibition occurs (Fig. 7). At higher levels of ClUDR, BrUDR or IUDR in combination with FUDR, an entirely different effect may occur; namely cell death by incorporation of ClUDR, IUDR, or BrUDR into DNA.[16] These conditions offer interesting possibilities for chemotherapy investigations.

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