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POTENTIAL RADIOSENSITIZING ANTIVIRAL AND ANTICANCER PYRIMIDINE NUCLEOSIDES

Technical Progress Report

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Content:

A. Scope and Purpose

B. Abstract Progress Report

C. Technical Progress Report

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A. Scope and Purpose

The scope and purpose is the synthesis of specific nucleoside analogs that will not only sensitize neoplastic tissues and appropriate topical viral infections to the lethal effects of radiations, but also will augment the latter by a concomitant specific antiviral effect. Biologically active compounds will be investigated in regard to site as well as mechanism of action. The photochemical events involved will be elucidated.

Although the yield of 6-aza nucleoside analogs were not markedly improved adequate amounts are being prepared for radiation studies. If needed more effort will be made to increase yield. Additional novel nucleosides, for example 5-halomethylene analogs of deoxyuridine, will be synthesized as potential radiation sensitizers.

Additional 5'-amino analogs of various nucleosides will also be synthesized because in general this class of compounds have proven to be either non-toxic or to possess an improved therapeutic index relative to the parent compound. Our development of a novel synthesis for this type of compound will afford large scale synthesis for animal studies. The 5'-amino analog of 5-iodo-2'-deoxycytidine (AIC) is more potent than 5-iodo-2'-deoxyuridine (AIU), and may have a broader spectrum of antiviral activity. Exploration of the potential of these and other 5'-amino nucleoside analogs is a major objective. Thus this program continues to afford a novel approach to antiviral and cancer chemotherapy.

B. Abstract Progress Report

A newly developed method of synthesis now affords the large scale production of 5'-amino analogs of a variety of nucleosides. This procedure has been used to synthesize the 5'-amino analog of 5-iodo-, 5-bromo-, 5-chloro-, 5-fluoro-, 5-trifluoromethyl-2',5'-dideoxyuridine. In addition to the previously established

antiviral activity of 5-iodo-5'-amino-2',5'-dideoxyuridine (AIU) we have found 5-bromo- (ABrU) and 5-iodo-5'-amino-2',5'-dideoxycytidine (AIC) to be even more potent antiviral agents than AIU both in vitro and of AIC against experimental herpatic keratitis in rabbits. Initial studies indicate a non-toxicity in vitro. There is sound reason to predict AIC and ABrC to each have a broader spectrum of antiviral activity than AIU and to have a similar lack of toxicity. 5-Fluoro- as well as 5-trifluoromethyl-5'-amino-2',5'-dideoxyuridine, and 5'-amino arabinosyl cytosine (A-ara C) have antiviral activity which is less potent than the parant compound, but each has a vastly improved therapeutic index. Additional experiments have been performed with experimental herpetic keratitis which confirm that AIU has similar efficacy but less potency than iodoxuridine (odeo), however the favorable therapeutic index of AIU should make it a preferable agent.

Metabolic studies show AIU to be incorporated into the DNA of only cells infected with herpes simplex virus (HSV) type 1, and to be phosphorylated by a cell-free extract from only HSV infected cells. These findings explain the complete lack of toxicity to the uninfected cell whether in vitro or in vivo. The triphosphate of AIU which is a phosphoramidate has been synthesized and its unique biological and physico-chemical properties are being studied.

The UV radiation sensitizing effects of various halogenated nucleoside triphosphates on thymidine kinase and dTMP kinase have been investigated as well as their photochemistry. The kinetics of UV-inactivation by AIU and 6-aza-IdUrd have been studied.

C. Technical Progress Report

The synthesis of 5-iodo-5'-amino-2',5'-dideoxyuridine (AIU) has been improved by use of a novel procedure that solves the problem of preparing large amounts. Up to now 2'-deoxyuridine was tosylated in the 5' position, converted to the 5'-azido analog, reduced to the 5'-amino, and then converted to a mercurial complex in the 5-position prior to iodination. Elaborate purification using column technique was required to ensure removal of any contaminating mercury salt. We now avoid this problem by starting with 5-iodo-2'-deoxyuridine and after converting it into the 5'-azido analog via the 5'-tosyl derivative, we can by use of triphenylphosphine effect a direct reduction to the 5'-amino without removal of the iodine in the 5-position of the pyrimidine moiety. This new procedure which we developed enables us to prepare gram quantities, or larger if need be, with no difficulty since the use of columns has been eliminated. This new procedure has increased the yield markedly.

Studies on the biological and chemical properties of AIU is a major direction. In addition to our previous finding of no toxicity to a variety of murine, avian, simian and human cells in culture, no toxicity, as evidence grossly or histologically, was found when AIU was injected into new born or 8-day old mice in vivo. At even lower amounts, 5-iodo-2'-deoxyuridine (IUdR, IDU, IdUrd, Idoxuridine) was very toxic to there mice. Studies of potential mutagenicity of AIU in L5178Y cells in culture were performed by Dr. Capizzi and found to be negative. Additional experiments with AIU and IdUrd in rabbits with experimental herpetic keratitis confirm that AIU has similar efficacy but less potency than idoxuridine, however the very favorable therapeutic index of AIU should make AIU a preferable agent either alone or in combination with other agents.

Another compound we developed recently, 5-iodo-5'-amino-2',5'-dideoxycytidine, has been found to be even more potent than AIU against herpes simplex

virus type 1 not only in cell culture but more importantly in the therapy of herpetic keratitis in rabbits. These exciting new findings will obviously be rapidly expanded to include the effect on other viruses as well as study of its mechanism of action.

Metabolic studies show AIU labelled with ^{125}I is transported into Vero cells in culture only when infected with herpes simplex virus, and is phosphorylated by a cell-free extract from only herpes simplex virus infected cells. AIU labelled with ^{125}I has been shown to be incorporated into the DNA of herpes simplex virus and of Vero cells, but only when infected with this virus. Upon treatment of the radioactive DNA derived from the virus infected cells with beef pancreatic DNAase the ^{125}I becomes acid soluble. Further treatment with Crotolus adamantus venom phosphodiesterase produces radioactive AIU.

The presence of AIU in DNA results in a marked instability of the DNA to low pH. It should be noted that phosphoramidates are notoriously acid labile. Whereas AIU labelled DNA is resistant to the action of microccal nuclease, 5-iodo-2'-deoxyuridine (IdUrd) labeled DNA is readily solubilized. Although DNAase II hydrolyzes AIU labelled DNA, it is resistant to subsequent treatment with spleen phosphodiesterase. These data indicate that AIU is incorporated into the DNA polymer.

Bouyant density centrifugation analysis indicates a 72% and 30% substitution of IdUrd for thymidine in viral and host cell DNA respectively when derived from virus infected cells, however although ^{125}I-AIU is incorporated into viral DNA the buoyant density of the DNA is not affected which means a very low degree of incorporation. Thus these experiments show that the incorporation of this novel analog into DNA is induced by herpes simplex virus. What this unique virus process is which is responsible for the anabolism of AIU uniquely in only the virus infected cell is being actively pursued.

Having established the incorporation of this 5'-amino nucleoside into DNA poses the question of how this is accomplished. To this end we synthesized chemically the 5'-triphosphate of AIU (5-iodo-5'-amino-2',5'-dideoxyuridine-5'-N-triphosphate; AIdUTP) which is the phosphoramidate analog of 5-iodo-2',5'-dideoxyuridine-5'-triphosphate (IdUTP). We have investigated some of the chemical and biological properties of AIdUTP.

Although AIdUTP is stable in alkaline solution, below pH 8 the rate of hydrolysis increased markedly following first order reaction kinetics and plateaued between pH 3 to 4. Below pH 3 a very marked increase in the rate of hydrolysis again occurred. The inclusion of magnesium ion in the reaction mixture decreases the rate of hydrolysis. Protonation of a group on AIdUTP which has a pKa of 6.18 (and presumably is the ionized oxygen on the gamma phosphate) must precede the hydrolysis. The effect of magnesium is probably expressed by a chelation which results in a decrease of the pKa of this moiety from 6.18 to a pKa of 5.15. The hydrolytic products found include the nucleoside, AIU, and trimetaphosphate, but neither the mono- nor the diphosphate of AIU.

Initial biological studies have examined the role of AIdUTP as an allosteric regulator of E. coli thymidine kinase. AIdUTP like dTTP and IdUTP converted this enzyme into an inactive dimer with a sedimentation coefficient of 5.85. AIdUTP is about 60-fold more potent an allosteric inhibitor than is dTTP. 5-Iodo-2'-deoxycytidine-5'-triphosphate (IdCTP), a potent activator of thymidine kinase can only prevent 85% of the AIdUTP inhibition. Whereas at high pH IdUTP became a potent activator of thymidine kinase, the inhibitory effect of dTTP but not of AIdUTP was markedly reduced.

The ultraviolet radiation sensitizing effects of various halogenated nucleoside triphosphates are under investigation with the objective to determine whether an allosteric enzyme can be locked into either the active or inactive form. If

achieved than this may be another approach to control the activity of a particular metabolic pathway. Whereas dTTP, dCTP, BrdUTP and BrdCTP decreased the rate of inactivation of thymidine kinase, IdUTP and IdCTP enhanced the rate of UV inactivation. The enhancement of IdCTP can be prevented by the inclusion of dTTP but not with thymidine.

The IdCTP effect follows saturation kinetics and is very similar to the activation property: $k_{1/2} \doteq k_m$. Similarly thymidine could not prevent the IdUTP enhancement of the rate of UV inactivation.

Photochemical studies of IdCTP indicated extensive dehalogenation to form dCTP, and similar studies with IdUTP showed a pattern similar to that found previously with IdUrd. The corresponding bromo-derivative of dCTP, BrdCTP, when irradiated with UV showed at best a very small extent of debromination.

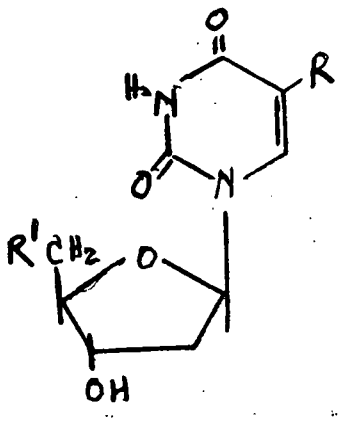
AIU although not a substrate for E. coli thymidine kinase was previously found to be a poor inhibitor of the phosphorylation of thymidine with a K_I of 240 uM. AIU enhanced the rate of UV inactivation of this enzyme with saturation kinetics.

Of the various compounds studied to date, only AIU and 6 Aza-IdUrd enhanced the rate of UV inactivation of thymidylate (dTMP)-kinase. The K_I of 6 Aza-IdUrd is 0.109 mM whereas the K_m for dTMP is 0.062. Saturation kinetics were not found and dTMP did not affect the rate of UV inactivation of 6 Aza-IdUrd, hence it would appear that the interaction of 6 Aza-IdUrd with dTMP kinase is non-specific in nature.

Whereas we earlier found the 5'-azido analogs of IdUrd, BrdUrd and CldUrd enhanced the rate of UV inactivation of E. coli thymidine kinase relative to the respective 5'-hydroxy analogs, no such enhancement has been found with dTMP-kinase.

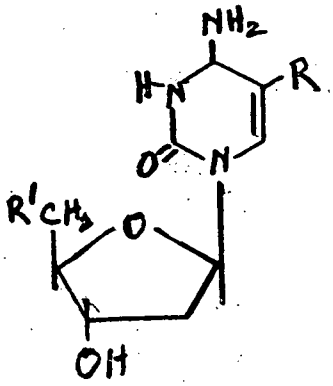
The following new compounds have been synthesized this past year:

I. Deoxyuridine Analogs



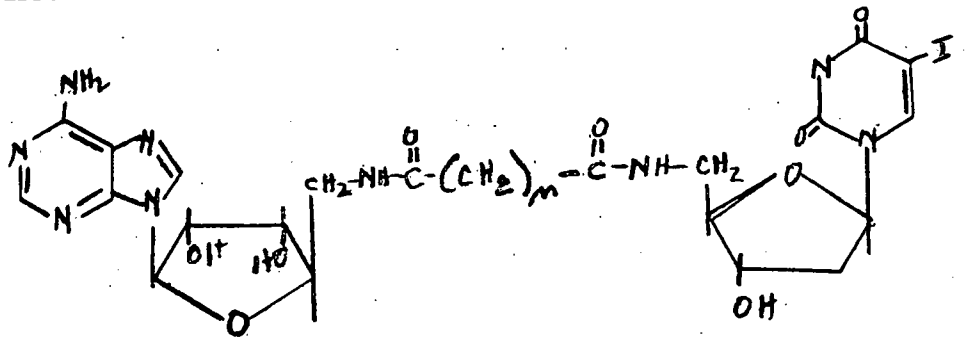
	R'	R	
1	N_3	F	
2	N_3	CF_3	
3	N_3	H	
4	NH_2	I	(AIU) via new method
5	NH_2	Br	(ABrU) via new method
6	NH_2	Cl	(AClU) via new method
7	NH_2	F	(AFU) via new method
8	NH_2	CF_3	(ACF_3U) via new method
9	NH_2	H	

II. Deoxycytidine Analogs:



	R^1	R	
10	N_3	I	
11	N_3	Br	
12	N_3	H	
13	NH_2	I	(AIC)
14	NH_2	Br	(ABrC)
15	NH_2	H	

III. Multisubstrate Analog of ATP and 5-Iodo-2'-deoxyuridine



This and related compounds were not prepared because with the replacement of Dr. J. P. Neenan with Dr. T. S. Lin we have not been able to reproduce the original biological activity with new preparations of the bisubstrate analog of ATP and thymidine (ATA). Although elemental analysis, UV spectra, relative migration of the new and old preparation of ATA in several solvent systems are identical, biological activity is lacking. We have contacted Dr. Neenan and are making arrangements for his repeating the synthesis with Dr. Lin or me in order to sort out the problem. Because the development of this type of compound is a major development in its own right, the decision was made to seek separate funding from the NIH for this specific aspect. Only those bisubstrate analogs with potential radiation sensitizing properties will be attempted, as for example, the structure depicted above, but only if we first sort out the problem of lack of biological activity. To date Dr. Lin and Dr. Shiau, both very critical and meticulous chemists, have carefully documented each step in the synthesis of ATA by elemental analysis, NMR and IR, but the product is not biologically active.

In summary, we feel that we have a very strong and viable program particularly in the development of potent antiviral agents with unique lack of toxicity and great virus sensitization potential.