Cytotoxic Nucleotide Analogs as Potential Anticancer Agents.[1] 5-Bromodeoxyuridine 5′-Methylphosphonate

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The synthesis and the cytotoxicity to Chinese hamster (V-79) cells of ammonium 5-bromo-2′-deoxyuridine 5′-methylphosphonate (**3**) are described; this compound is a structural analog of 2′-deoxythymidine 5′-phosphate. When the cells were grown on dishes for 1 day with initial concentrations of 10–1000 μM **3** in the medium partial cytotoxicity was observed; however, a 5-day exposure to 10 μM **3** produced total cytotoxicity. If a cell culture was treated simultaneously with thymidine and **3**, the cells were protected from **3**. Culture cells with sublethal damage by **3** were found to be extremely sensitive to light. The stability of **3** in the culture medium and the interaction of **3** with the hamster cells were demonstrated. The treatment of hamster cells with **3** for 1–8 days, the subsequent removal of **3**, and the addition of thymidine to the medium for the remainder of a 13-day time period produced no reversal of the cytotoxic activity of **3**. Thus, **3** may be the first known nonphosphate nucleotide analog that can penetrate the membrane of living cells to produce irreversible cytotoxicity.

DNA synthesis in cancer cells may be prevented by the irreversible inhibition of one of the enzymes required for the biosynthesis of the deoxyribonucleoside phosphates that are incorporated into DNA.[2] Another possibility would be to administer a deoxyribonucleotide with a halogen or a sulfur atom on the pyrimidine moiety for incorporation into DNA; the incorporated analog could render the DNA metabolically inactive.[2] This latter approach has been ineffectual because nucleotides do not readily penetrate the membrane of a living cell.[3]

A cytotoxic nucleoside, 5-bromodeoxyuridine (BrdUrd), readily enters living cells; the phosphate group may be added to BrdUrd by an intracellular kinase.[4] Since the halogenated nucleotide formed inside the cell is an effective inhibitor of DNA synthesis, BrdUrd may be utilized as an antimetabolite.[5] On the other hand, certain cells may not contain the kinase that is required to convert BrdUrd into a metabolically active nucleotide.[6] Furthermore, the cells may contain a nucleoside phosphorylase that can separate the pyrimidine moiety from the pentose portion of a halogenated nucleoside before the phosphate group is added.[7]

Since it appeared likely that it is the diionized phosphate group which prevents the penetration of a nucleotide through the cell membrane, 5-fluoro-2′-deoxyuridine 5′-sulfate was prepared as a monoionized nucleotide analog and potential antimetabolite.[8] Price[9] observed that the ribofuranosyl 5′-sulfate penetrates the membrane and produces a disorientation of DNA synthesis; however, the compound is not cytotoxic to L-1210 leukemia cells. In the present investigation, a methylphosphonate group was utilized as a substitute for the phosphate group; a monoionized nucleotide analog prepared in this way might penetrate the cell membrane to mimic the activity of a nucleotide with respect to the enzymes of DNA synthesis.

The biological experiments reported here suggest that ammonium 5-bromo-2′-deoxyuridine 5′-methylphosphonate (**3**) penetrates the membrane of Chinese hamster cells intact and kills all the cells after a time interval equivalent to approximately 8 generation cycles. The delay in the cytotoxicity may be related to the amount of **3** that must be incorporated into DNA to inhibit the synthesis of new DNA or the transcription of RNA. Further studies on the effect of **3** on human cancer cells will be reported in a subsequent publication.

Experimental Section

Ammonium 5-Bromo-2′-deoxyuridine 5′-Methylphosphonate (3). A solution of 5.0 g of 5-bromo-2′-deoxyuridine (16.3 mmoles, Schwarz Bio-Research, Inc.) and 8.0 g of triphenylchloromethane in 100 ml of dry pyridine was stored at 25° for 7 days. The solution was poured into 1000 ml of cold water, 100 ml of $CHCl_3$ was added, and the mixture was warmed and stirred for 2 hr at 25°. The mixture was extracted 3 times with 200-ml portions of $CHCl_3$, and the water fraction was discarded. The $CHCl_3$ solution was pooled and dried with Na_2SO_4. The $CHCl_3$ was removed under vacuum, and the residue was recrystallized from Me_2CO and C_6H_6 to yield 7.0 g (12.8 mmoles) of 5-bromo-2′-deoxy-5′-*O*-trityluridine[10] [tlc on Mallinckrodt ChromAR Sheet 1000 with 9:1 $CHCl_3$–EtOAc (solvent I), R_f 0.13].

A solution of 6.8 g (12.4 mmoles) of 5′-*O*-tritylbromodeoxyuridine and 5.0 ml of Ac_2O in 80 ml of dry pyridine was stirred at 28° for 24 hr. The solution was poured into 60 ml of cold water; the suspension was warmed and stirred for 1 hr at 25°. The solvent was removed under vacuum, and the residue was exhaustively dried under vacuum [tlc with solvent I, R_f 0.38; and with 95:5 $CHCl_3$–MeOH (solvent II), R_f 0.79].

The powder was dissolved in 180 ml of warm 8:2 AcOH–H_2O, and the solution was heated at 70° for 4 hr. The solution was stored at 4° for 20 hr to permit crystallization of triphenylcarbinol, the suspension was filtered, and the crystals were washed with aqueous AcOH. The AcOH solution was pooled, the solvent was removed under vacuum, and the residue was pulverized and dried. The powder was washed 3 times with 5-ml portions of C_6H_6 to yield 3.4 g (9.8 mmoles) of compound **1**.[10] Recrystallization was accomplished from EtOAc; tlc with solvent II, R_f 0.55.

Compound **1** (660 mg, 1.9 mmoles) and methylphosphonic acid[11] (590 mg, 6.1 mmoles) were dissolved in 30 ml of dry pyridine, and the solvent was removed under vacuum. This procedure was repeated to provide a dry reaction mixture. The residue was dissolved in 60 ml of dry pyridine and 5.5 g (26.7 mmoles) of dicyclohexylcarbodiimide was added.[11] The solution was stored at 25° for 4 days in a desiccator. The solution was poured into 120 ml of cold water; the suspension was warmed and stirred at 25° for 2 hr. The mixture was filtered, the precipitate was discarded, and the filtrate was extracted 3 times with 100-ml portions of petroleum ether (bp 30–60°). The organic fraction was discarded, and the solvent was removed from the aqueous fraction under vacuum. The residue was dissolved in 25 ml of MeOH, 25 ml of 1.5 *M* aqueous NH_4OH was added, and the mixture was shaken at 25° for 16 hr. The solution was filtered, and the solvent was removed from the filtrate under vacuum. The residue was dissolved in 5.4 ml of H_2O, and the solution was applied to a 2.5 × 100 cm column of Sephadex G-15. The column was developed with 340 ml of H_2O in upward flow before the product appeared in the eluent. The product was eluted in 110 ml of H_2O, and the fractions that contained the product were pooled and lyophilized to a powder. The residue was recrystallized from MeOH and Et_2O to yield 530 mg (70%) of hygroscopic white crystals of the ammonium salt of **3**, mp 148–150°. Chromatography on Whatman No. 3MM paper in descent showed

one zone with 75:5:20 *i*-PrOH–concd NH_4OH–H_2O, R_f 0.19, and 5:2:3 *n*-BuOH–AcOH–H_2O (solvent III), R_f 0.39. *Anal.* ($C_{10}H_{17}N_3O_7PBr \cdot 0.2H_2O$) C, H, N: calcd, 29.60, 4.32, 10.35; found, 29.63, 4.45, 10.09.

Cell Culture Assay. Compound **3** was tested with cell cultures of a clone of the V-79 line of Chinese hamster cells,[12] originally derived from lung tissue (this cell line was kindly supplied to us by Dr. E. Y. Chu, Oak Ridge National Laboratory). The clone used here was selected by plating single cells and the isolation of a colony grown from an individual cell. This clone has a plating efficiency (ratio of the number of colonies formed divided by the number of cells inoculated) of 70%. The cells have a generation cycle of 10 hr; there is a DNA synthesis period (S) of 6 hr, a pre-DNA synthesis period (G_1) of 1.5 hr, a post-DNA synthesis period (G_2) of 1.5–2 hr, and a mitotic period (M) of 0.5–1 hr (as determined by the labeled metaphase technique[13]).

The survival fraction of cells exposed to **3** was measured by the formation of colonies; this method has been used to study the response of mammalian cells to X-rays,[12] hydroxyurea,[12] fluorodeoxyuridine,[14] and ribofuranosylthiopyrimidines.[15] The survival fraction was calculated from the ratio of the number of colonies counted on dishes that contained **3**, to the number of colonies on control dishes with no **3**.

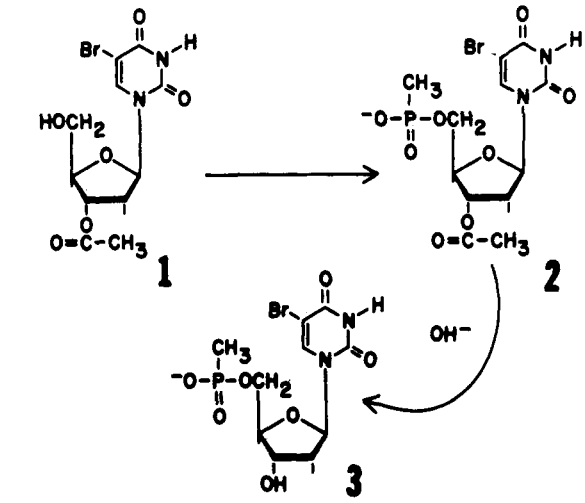
For each experiment, monolayer cultures were trypsinized and the cells were diluted with Eagle Minimum Essential Medium (Cat. No. F-12, Grand Island Biological Corp., Grand Island, N. Y.) supplemented with 15% fetal bovine serum (Microbiological Associates, Bethesda, Md.), penicillin (100 units/ml), streptomycin (100 μg/ml), neomycin sulfate (100 μg/ml), and Phenol Red (20 μg/ml). Final concentrations of 100, 500, or 2000 cells in 15 ml of medium were prepared for plating in a 60-mm Falcon plastic dish. Three replicate dishes were prepared for each cell concentration and each concentration of **3**. Solutions of **3** in sterile water were prepared immediately before use and sterilized by passage through a Swinny millipore filter holder containing one DN 0.65 μ pore size filter. The concentration of **3** in water on each dilution was determined after filtration by the uv absorbance of **3** at 279 mμ with a Gilford 240 spectrophotometer. These solutions were diluted with sterile Eagle medium and 1.0 ml of each solution was added to each dish to provide initial concentrations of **3** of 10, 100, and 1000 μ*M*. Five replicate dishes for each cell concentration and zero **3** served as the controls; control dishes received 1.0 ml of medium without **3**.

Immediately after addition of **3** the dishes were incubated at 37° and pH 7.0 in a humid atmosphere of 5% CO_2 in air. The viable cells attach to the surface of the plastic and the medium may be decanted and replaced with no effect on the viability of the cells. This procedure was performed in several groups of dishes to remove **3** from the dishes and to collect the medium containing **3** which had been exposed to living cells. A quantitative analysis for **3**, the 5-bromouracil (BrUra), and the BrdUrd in the medium was performed. The dishes were rinsed twice with balanced salt solution at different time intervals from 1 to 9 days and refilled with fresh medium. The dishes were reincubated at 37° under controlled pH and humidity for the remainder of the 13-day time period. For example, the dishes that were rinsed after 1 day were reincubated for 12 days, and the dishes that were rinsed after 4 days were reincubated for 9 days. At the end of the 13-day period of incubation, the cells were fixed, the colonies were stained with Giemsa, and the number of colonies per dish was counted.

The cytotoxic effect of an exposure to **3** and "visible" light was determined in selected experiments. Monolayers of hamster cells on plastic dishes were illuminated for 1 hr at 37° with the light from four fluorescent lamps (20-W, Champion "Cool White" tubes, 60-cm long); the lamps were mounted in a fixture with a reflector, 11 cm apart, and 33 cm above the dishes. The light treatments were performed inside the incubator, and the lamps were turned on 1 hr in advance to allow sufficient time for stabilization of the light and the temperature. The thermostat was readjusted to provide a constant temperature of 37°. Control dishes with no **3** were given the same light treatment; the viability of these cells was not influenced by light.

Synthetic Results. The synthesis of 3′-*O*-acetyl-5-bromo-2′-deoxyuridine (**1**)[10] and 3′-*O*-acetyl-5-iodo-2′-deoxyuridine[16] have been reported; however, the yield of **1** was improved. Compound **1** was found to be more stable in acid than BrdUrd.[17] The esterification of **1** with pyridinium methylphosphonate (Scheme I) was performed by the procedure of Myers, *et al.*,[11] to provide 3′-*O*-acetyl-5-bromo-2′-deoxyuridine-5′-methylphosphonate (**2**). The removal of the acetyl group from compound **2** is difficult to accomplish because the 5-bromouracil moiety of **2** and **3** is unstable under alkaline conditions.[18] Therefore, the hydrolysis of **2** to yield the ammonium salt of compound **3** was performed with dilute aqueous alkali at a relatively low temperature (Scheme I). The preparation of the 5′-methylphosphonate derivatives of adenosine[11] and 6-azauridine[19] from the appropriate isopropylidine intermediates has been reported.

Scheme I



Biological Results. The hamster cell bioassay of the nucleotide analog (**3**) demonstrates the effect of different concentrations of **3** and different exposure time intervals on the capacity of mammalian cells to undergo cell division and multiplication. The effect of **3** on the formation of colonies from viable V-79 hamster cells was determined at different initial **3** concentrations and cell concentrations (Figure 1). The colonies were counted 13 days after the administration of **3**. Since each colony represents a viable cell, the data in Figure 1 show that approximately half the cells are killed by a 1-day treatment with 1000 μ*M* **3**. All of the cells are killed by a 4-day exposure to 100 μ*M* **3**; therefore, an increase in the concentration of **3** is less cytotoxic than an increase in the exposure time interval.

The competitive effect of thymidine was investigated (Figure 2).

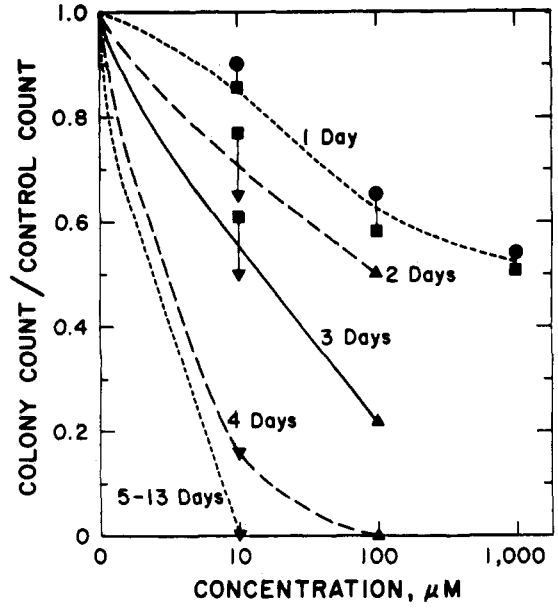


Figure 1. The effect of different initial concentrations of **3** on the survival fraction (calculated from the average values of the colony count per dish, for 3 dishes of Chinese hamster cells treated with **3**, *divided* by the average count per dish with no added **3**) for different durations of treatment with **3**. Since no significant difference was observed in the survival fraction (determined at 13 days) between dishes with initial cell concentrations of 100 cells per dish and 500 cells per dish, the mean values for the survival fraction were calculated. The different symbols indicate mean values for different experiments.

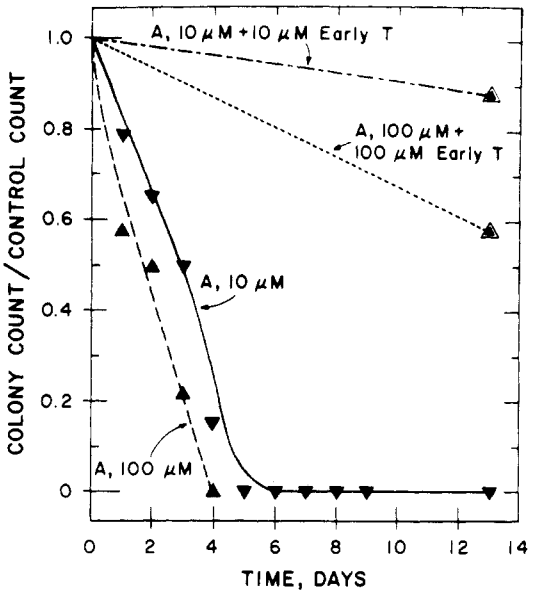


Figure 2. The hamster cells were treated with initial concentrations of zero, 10 μM, and 100 μM **3** for 1-13 days. The antimetabolite was removed, and the cells were reincubated for the remainder of the 13-day period (12-0 days) with fresh growth medium. The cells were also incubated with **3** and equimolar concentrations of thymidine for 13 days. The triangles represent the mean of the survival fraction for initial cell concentrations of 100 and 500 cells per dish, and compound **3** is represented by the symbol "A". Thymidine that is added immediately after inoculation is designated "Early T."

The hamster cells were inoculated into medium with an initial **3** concentration of 10 μM plus equimolar thymidine (10 μM) and were permitted to grow in this mixture for 13 days. Almost 90% of the cells survived the 13-day treatment with equimolar **3** and thymidine, as shown in Figure 2; therefore, thymidine protects the cells against the antimetabolite activity of **3.** For comparative purposes, the cells were treated with 10 μM **3** for 1-9 days, the medium with **3** was decanted, and the cells were reincubated for the remainder of the 13-day period. The results of Figure 2 show the time course of the cytotoxicity of **3.** None of the cells survives a 5-day treatment with 10 μM **3** alone, whereas virtually all the cells survive a 13-day treatment with **3** if an equimolar amount of thymidine is added at the same time as the **3.**

It may be seen from Figure 2 that at concentrations of 100 μM **3** plus 100 μM thymidine, the protective action of the latter compound may be less effective; only 60% of the cells survive a 13-day treatment with the mixture. On the other hand, all the cells are killed by a 4-day treatment with 100 μM **3** when thymidine is not added to the dishes.

From the results of the foregoing experiments it appears possible that **3** may be cleaved at the cell membrane to produce free methylphosphonate, BrdUrd, or BrUra. Thus, the observed cytotoxicity could be due to the breakdown products formed from **3.** To test this possibility, the cells were incubated with 1000 μM ammonium methylphosphonate under the same conditions; however, this salt did not have any effect on the survival of the cells. In other experiments, the cells (at initial concentrations of 100 and 500 cells per dish) were treated with 100 μM BrdUrd for 1 day. The mean value of the survival fraction determined in these experiments was 0.49; thus, the clone of V-79 cells used in these studies is sensitive to BrdUrd.

Several experiments were performed in an attempt to detect BrdUrd in the cytoplasm of cells that were incubated with a high concentration of compound **3.** Monolayer cell cultures were grown in bottles in the presence of 1000 μM **3**; 1.0×10^6 cells were incubated for 1 day and 2.0×10^5 cells were incubated for 9 days. The medium was removed, and the cells were washed and trypsinized to detach them from the glass surface. The cell membrane was broken by sonication; the particulate fraction was separated by centrifugation, the cytoplasmic fraction of the cells was pooled and lyophilized, and the powder was taken up in a minimal quantity of water. The solution was chromatographed on paper (solvent III), and the chromatograms were examined with a uv lamp. No detectable **3,** BrdUrd, or BrUra was found in the cell cytoplasmic fraction with this technique. Although **3** may be bound to the particulate fraction, we have delayed an examination of the particulate fraction until an isotopically labeled derivative of **3** is available.

Table I. Recovery of Compound **3** from Growth Medium[a]

Time, days	Compound **3**, μM	
	Cells, none	Cells, 33/ml[b]
0	100	100
1	94	31
2	91	26
4	103	10
9	110	10

[a] Eagle's minimal essential medium was used at 37°. [b] The cells were from the V-79 line of Chinese hamster cells.

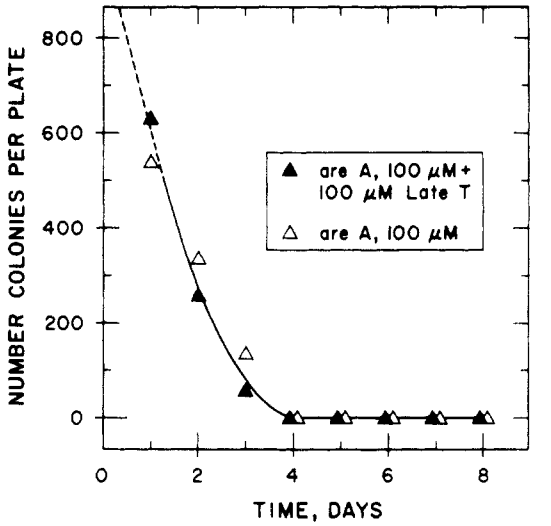


Figure 3. The hamster cells, at an initial concentration of 2000 cells per dish, were treated with 100 μM compound **3** for 1-8 days. The antimetabolite was removed, the cells were reincubated in fresh growth medium containing 100 μM thymidine (▲) and zero thymidine (△), for the remainder of the 13-day period (12-5 days). The symbols represent average values for the colonies on 3 dishes, counted at 13 days. Compound **3** is represented by the symbol "A" and thymidine that is added after **3** is removed is designated "Late T."

To determine the stability of **3,** 100 μM **3** was incubated with Eagle medium at 37° in the absence of cells under sterile conditions. The medium was decanted from several dishes, pooled, and lyophilized. The powder was taken up in water and chromatographed on paper (solvent III) to detect the **3.** The zones on the paper that contained **3** were eluted into an aqueous buffer, and the amount of **3** was determined from the uv absorbance. The concentration of **3** that was found in the medium is given in Table I. The results show that compound **3** is stable to chemical decomposition within the experimental error and does not interact with the components of the growth medium. The effect of hamster cells on **3** in the medium was determined; the cells were incubated with 100 μM **3,** the cells were washed, and the medium was removed. The concentration of **3** that remained in the medium was analyzed by paper chromatography (solvent III) and the uv absorbance (see Table I). The concentration of **3** in the medium decreased to approximately 10 μM; however, the breakdown products of **3,** BrUra and BrdUrd, were not detected in the medium. These findings demonstrate that the antimetabolite **(3)** interacts with hamster cells.

Miura and Wilt[20] demonstrated that BrdUrd inhibits the growth of mitotically active chick embryo cells; however, if BrdUrd (130 μM) was removed and the inhibited cells were transferred to a growth medium containing thymidine, the cytotoxicity of BrdUrd was reversed. To determine whether the cytotoxicity of **3** is reversed by thymidine, the hamster cells were incubated with 100 μM **3** for 1-8 days, and **3** was removed by decantation of the medium from the dishes. The cells were washed and reincubated for the remainder of the 13-day period (12-5 days) with thymidine concentrations of 100 μM or zero in fresh growth medium. It may be seen,

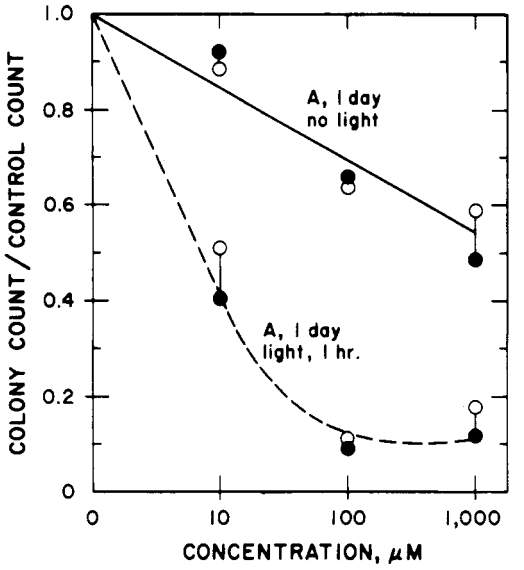


Figure 4. The cytotoxic effect of a 1-day treatment with different initial concentrations of compound 3 (represented by the symbol "A"), in the absence of light, is shown in the upper curve. The effect of light for 1 hr on cells with sublethal damage by 3 is shown in the lower curve. The filled circles represent the average survival fraction for an initial cell concentration of 500 cells per dish, performed in triplicate, and the unfilled circles represent the average for an initial concentration of 100 cells per dish.

from Figure 3 that, in comparison with the cells with no added thymidine, the treatment with thymidine after the removal of 3 does not influence cell viability within the experimental error. Thus, the cytotoxicity of 3 is irreversible if thymidine is added after 3.

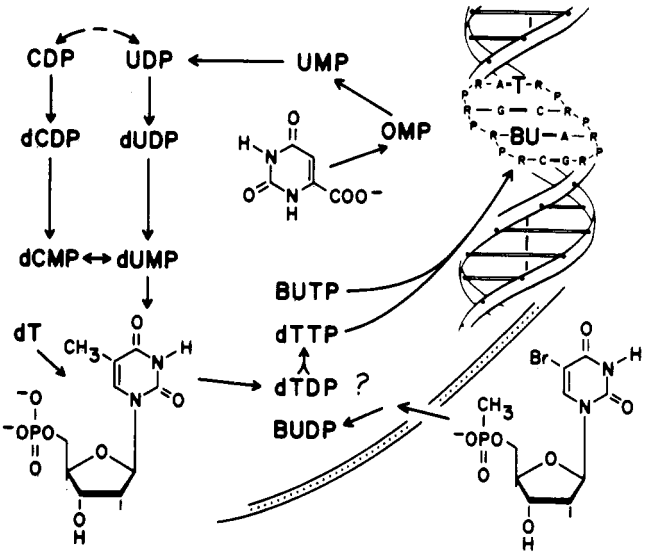
The incorporation of BrUra into DNA produces a polymer that is more sensitive to light than natural DNA.[21] Chinese hamster cells are killed by incubation with BrdUrd followed by a treatment with "visible" light.[22] In the experiments with compound 3 (Figure 4), the cells were incubated on dishes for 1 day with different initial concentrations of 3. The exposure of the dishes to light was similar to that described by Puck and Kao.[22] After the light treatment, the medium was decanted and the dishes were reincubated with fresh medium for 12 days. The results of the average colony counts, performed in triplicate, may be compared with the data obtained with cells that were given the same treatments with 3, but were not exposed to "visible" light. Only 10% of the cells survived the exposure to 100 μM 3 plus light, whereas 60% of the cells survived in darkness.

Discussion

A tentative explanation of the experimental results with compound 3 and Chinese hamster cells is presented in the hypothetical metabolic pathway of Scheme II. It is suggested that compound 3 penetrates the cell membrane intact and competes with thymidine 5′-phosphate (dTMP) as a substrate for thymidylate kinase.[23] The enzymatic phosphorylation of compound 3 would produce a potential substrate for dTDP kinase and the second phosphorylation would produce a potential substrate for DNA polymerase. If the 5′-pyrophosphate derivative of 3 is utilized for an enzymatic esterification at the 3′-terminal hydroxyl group of a DNA strand, compound 3 would become the 3′-terminal unit of the DNA chain with the release of inorganic pyrophosphate. Upon the subsequent addition of a 2′-deoxyribonucleotide to the 3′ position of 3 in the DNA chain, compound 3 would be incorporated intact in an internal position of a DNA strand. Thus, the BrUra moiety and the deoxyribosyl group with an uncharged methylphosphonate group may partially replace the dTMP residues in hamster cell DNA.

The concentration of 3 decreased in the growth medium

Scheme II



in the presence of hamster cells, but no breakdown products were detected in the growth medium or the cell cytoplasm. The resistance of a nucleoside methylphosphonate to alkaline phosphomonoesterases and a 5′-nucleotidase[19] suggests that the monoionized compound 3 penetrates the hamster cell membrane intact. This suggestion is consistent with reports that a monoionized nucleoside phenylphosphate[24] and a nucleoside cyclic phosphate[25] appear to enter cells.

When hamster cells are grown in equimolar mixtures of compound 3 and thymidine, the cells are protected against the cytotoxicity of 3 (Figure 2). Two possible explanations can be suggested to interpret this competitive phenomenon. Since thymidine is converted to dTMP inside the cells by thymidine kinase,[4] compound 3 and dTMP may compete as substrates for the active site of thymidylate kinase and for incorporation into DNA. On the other hand, thymidine and compound 3 may compete for the same membrane sites for penetration from the medium into the cell. The low survival of cells treated with 100 μM thymidine (and 100 μM 3) compared with the survival of cells treated with 10 μM thymidine (and 10 μM 3), reported in Figure 2, may be due to the known cytotoxic action of elevated concentrations of thymidine in the growth medium.[26]

The incorporation of compound 3 into hamster cell DNA, suggested in Scheme II, is consistent with the experimental results with hamster cells. It is apparent from Figure 2 that the removal of 3 (100 μM) from the medium after approximately two cell cycles (1 day) permits approximately 60% of the cells to survive and multiply even though the survivors may sustain sublethal damage as evidenced by their sensitivity to "visible" light (Figure 4). On the other hand, after two generation cycles with an initial concentration of 100 μM 3, colony formation is prevented for 40% of the cells; there is no detectable increase in the viability of these cells if they are subsequently exposed to thymidine for 12 days (Figure 3).

If compound 3, at an initial concentration of 100 μM, is allowed to remain in the medium for 8 generation cycles, all the cells are killed. The delay in the observed cytotoxicity with 3 is consistent with the time that may be required for sufficient incorporation of the BrUra moiety into DNA for a lethal effect.[27] Apparently, cells grown in the dark can survive a submaximal incorporation of 3 into the DNA; but the light treatment of cells with sublethal damage causes a strong enhancement of the cytotoxicity. Studies to com-

pare the cytotoxicity of **3** with BrdUrd and experiments to demonstrate the incorporation of an isotopically labeled compound **3** into the DNA of hamster cells are in progress. The effect of the hypothetical methylphosphonate diester bond (in DNA) upon the biosynthesis, enzymatic repair, and physical properties of DNA remains to be investigated.

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Amidines.† 3.[1] Thioureas Possessing Antihypertensive Activity

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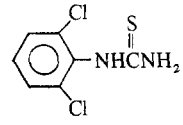
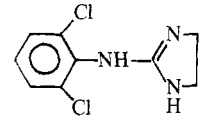
A series of 2- and 2,6-substituted phenylthioureas were found to have potent antihypertensive activity; the 2,6-dimethyl compound was particularly effective and had an unusually high ratio of efficacy to lethality (>1000). These compounds are orally active in the rat but not in the dog. Several potential metabolites were synthesized, one of which was active in both species.

In the course of an investigation into the potential antihypertensive activity of amidines,‡ we observed that the thiourea I was as potent (approx 1 mg/kg) in producing prolonged blood pressure depression as the clinically effective hypotensive agent 2-(2,6-dichloroanilino)-2-imidazoline§ (II) when administered orally to metacorticoid rats. Although II is reported to have a low incidence of side effects in man,[3a,b] in rats it has an efficacy:lethality (E:L)# of only 10, whereas I shows an E:L ratio of 50-200.

Further testing showed that I administered orally did not produce blood pressure lowering in either normotensive or neurogenic hypertensive dogs. The biological data (see Pharmacology Section) suggested that inactivity in the dog might be due to the failure of these thioureas to be converted to an active metabolite, rather than due to metabolic inactivation. The possibility of a species-specific metabolic activation and the unexpectedly large potency and high E:L ratio of I prompted the synthesis of related thioureas and several possible metabolites, and the investigation of their antihypertensive activity.



I



II

Chemistry. Highly hindered and weakly basic amines react poorly with alkali metal thiocyanates under the usual conditions for thiourea synthesis. It had previously been observed that the use of trifluoroacetic acid with NaOCN led to carbamylation in systems which were otherwise refractory.[4,5] When trifluoroacetic acid was used in the reaction of aryl amines with NaSCN, excellent yields of the monosubstituted thioureas were obtained. The 1,3-disubstituted thioureas were prepared by reaction of 2,6-dichlorophenyl isothiocyanate[6] with the appropriate amines.

The acetylthiourea **15** was more conveniently obtained

†We include in the term "amidines," those compounds containing the moiety $N-C=X$ (X = C, N, O, or S).

‡A preliminary communication concerning active amidines is given by Loev, *et al.*[2]

§Catapres®.

#The term efficacy:lethality (E:L) is used in this paper to mean the ratio of the minimal oral dose producing lethality in the normal rat to that producing significant blood pressure depression in the metacorticoid hypertensive rat.