Biochemical Pharmacology, Vol. 43, No. 7, pp. 1579–1585, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00
© 1992. Pergamon Press plc

DECREASE IN TTP POOLS MEDIATED BY 5-BROMO-2′-DEOXYURIDINE EXPOSURE IN A HUMAN GLIOBLASTOMA CELL LINE

DONNA S. SHEWACH,*† JOANN ELLERO,* WILLIAM R. MANCINI* and WILLIAM D. ENSMINGER*‡

Departments of *Pharmacology and ‡Internal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109-0504, U.S.A.

(*Received* 4 *September* 1991; *accepted* 4 *December* 1991)

Abstract—The antitumor and radiosensitizing properties of 5-bromo-2′-deoxyuridine (BUdR) appear to be due, in part, to its incorporation into cellular DNA. To optimize conditions for incorporation of 5-bromo-2′-deoxyuridine-5′-monophosphate (BrdUMP) into DNA, we investigated the metabolism of BUdR to its DNA precursor form, the 5′-triphosphate BrdUTP, in the U251 human glioblastoma cell line. The results demonstrated that BrdUTP accumulated rapidly in this cell line, achieving steady-state values within 2 hr of drug addition. The level of BrdUTP accumulation was proportional to the amount of exogenous BUdR up to a concentration of 100 μM, without apparent saturation. Exposure of glioblastoma cells to BUdR was associated with substantial selective decreases in both the cellular dCTP and TTP pools, the extent of which was dependent on the exogenous BUdR concentration. In the absence of exogenous BUdR, BrdUTP was eliminated rapidly from cells with an initial half-life of approximately 15 min. As the cellular BrdUTP level declined, the dCTP and TTP levels increased to control values. Incorporation of BrdUMP into DNA appeared linear with time as long as the cellular BrdUTP level remained constant. This incorporation was not enhanced by the addition of 5-fluoro-2′-deoxyuridine (FUdR), a potent inhibitor of thymidylate synthetase, which at a concentration of 10 nM had no effect on TTP pools in this cell line. Thus, the decrease in cellular TTP pools mediated by BrdUTP allows the halogenated pyrimidine to enhance its own incorporation into DNA.

5-Bromo-2′-deoxyuridine (BUdR§) is a thymidine analog which is readily incorporated into the DNA of cells, bacteria and viruses as the 5′-monophosphate derivative (BrdUMP) [1, 2]. DNA incorporation of this fraudulent nucleotide is associated with diverse biologic activities, including antitumor and antiviral properties, mutagenic effects and the ability to increase the sensitivity of cells to radiation damage [1–3]. The radiosensitization effect has been exploited clinically by administering BUdR in conjunction with radiation therapy to patients with malignancies [4]. Glioblastomas are well-suited to treatment with BUdR since the rapidly dividing tumor cells should readily incorporate the halogenated analog whereas the slower dividing normal brain cells would be less vulnerable to the toxic effects of this agent. Initial attempts to administer BUdR during a prolonged infusion to patients were hampered by vascular and infectious complications, and myelosuppression limited the amount of BUdR that could be administered [5, 6]. The use of an implantable infusion pump to administer BUdR intraarterially has eliminated the vascular and infectious complications associated with prolonged intravenous infusion of this drug [7, 8]. Furthermore, the development of a rapid and sensitive assay system has permitted adjustment of the drug dose to maintain plasma concentrations of BUdR which are therapeutic but not myelosuppressive [8, 9]. Preliminary clinical results with the combination of BUdR and radiation therapy are encouraging [8, 10].

Since BUdR must be incorporated into cellular DNA in order to act as a radiosensitizer, recent reports have examined the relationship between BUdR concentration and incorporation of its 5′-monophosphate into glioblastoma DNA both *in vitro* and *in vivo* [8, 11]. The *in vitro* studies have demonstrated that the incorporation of BrdUMP into cellular DNA can vary significantly between cultured cell lines. However, the reason for this variation has not been elucidated. To be incorporated into DNA, BUdR must be metabolized intracellularly to 5-bromo-2′-deoxyuridine-5′-triphosphate (BrdUTP), the immediate DNA precursor. There are three distinct kinases responsible for this phosphorylation [2, 12], and in addition BUdR and its phosphorylated metabolites can be degraded by other enzymes. Heterogeneity between cell lines at any one of these metabolic steps can lead to variable BrdUTP levels, resulting in differential DNA incorporation of this analog. Alternatively, the relative concentration of the competing endogenous TTP pool may contribute to the heterogeneity of BrdUMP incorporation between cell lines. Despite the fact that the extent of BrdUMP incorporation is

† Corresponding author: Donna S. Shewach, Ph.D., Department of Pharmacology, 3608 Upjohn Center for Clinical Pharmacology, University of Michigan Medical Center, Ann Arbor, MI 48109-0504. Tel. (313) 763-5810; FAX (313) 763-3438.

§ Abbreviations: BrdUMP, 5-bromo-2′-deoxyuridine-5′-monophosphate; BrdUTP, 5-bromo-2′-deoxyuridine-5′-triphosphate; BUdR, 5-bromo-2′-deoxyuridine; dNTP, deoxyribonucleoside 5′-triphosphate; FUdR, 5-fluoro-2′-deoxyuridine; and PBS, phosphate-buffered saline.

dependent on the ability of the cell to accumulate BrdUTP, the rate and extent of accumulation of this metabolite have not been well studied. Evaluation of the cellular metabolism of BUdR may identify cell-specific differences which result in variable DNA incorporation of this analog and, furthermore, such studies may suggest new methods for increasing the amount of BrdUMP incorporated into DNA to produce greater cytotoxicity and radiosensitization. Thus, the data presented here describe the metabolism of BUdR in glioblastoma cells in culture. We also report the effects of 5-fluoro-2′-deoxyuridine (FUdR), a potent inhibitor of thymidylate synthetase, on this metabolism. A preliminary account of these studies has been presented [13].

MATERIALS AND METHODS

Chemicals. RPMI 1640 cell culture medium and horse serum were obtained from GIBCO BRL (Grand Island, NY). Bovine calf serum was purchased from Hyclone (Logan, UT). Nucleosides and nucleotide standards were obtained from the Sigma Chemical Co. (St. Louis, MO). Ammonium phosphate buffer was purchased from J. T. Baker Inc. (Phillipsburg, NJ). All other chemicals were reagent grade.

Cells. U251 glioblastoma cells were a gift from Dr. Darell Bigner, Duke University Medical Center [14]. The cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with 10% bovine calf serum. It was determined that less than 0.1 μM thymidine was present in the serum. Cells used in the experiments described here were passaged at least 3 days prior to the beginning of the study to ensure that the population was in exponential growth at the time of drug addition.

To harvest the cells for nucleotide analysis, medium from the cultures was poured off rapidly and the plate was washed once with phosphate-buffered saline (PBS). Trypsin was added (0.25%) to detach the cells from the plate, at which point ice-cold PBS with 10% horse serum was added rapidly to stop the trypsinization process. An aliquot of the well-dispersed cell suspension was removed for estimation of cell number using an electronic particle counter (Coulter Electronics, Hialeah, FL), and the remainder was centrifuged at −4°. The cell pellet was then extracted with 0.4 N perchloric acid and neutralized with potassium hydroxide to isolate the acid-soluble nucleotides. The cell extract was stored at −20° until analysis.

Nucleotide analysis. For analysis of cellular deoxyribonucleotides, the cell extract was first separated on a boronate affinity column to remove the majority of the ribonucleotides.* Deoxyribonucleotides were then analyzed using a Waters high pressure liquid chromatograph equipped with a U6K injector, two model 501 pumps and a model 490 four-channel absorbance detector. The system was controlled by means of Maxima 820 software operated on a NEC Powermate 2 computer. The deoxyribonucleotides from the extract were loaded onto a strong anion exchange column (Whatman, NJ) and eluted with a linear gradient of ammonium phosphate buffer ranging in concentration from 0.15 to 0.6 M. The pH of the buffer was varied from 3.5 to 4.1. Nucleotides were identified based on their UV absorbance at 254, 281 and 292 nm and comigration with authentic standards. Nucleotides in cell extracts were quantitated by comparison of their peak areas with that of a known amount of the appropriate standard.

Incorporation of BrdUMP into DNA. Cells were incubated with BUdR and trypsinized rapidly as described above. Between 2×10^6 and 5×10^6 cells were aliquotted routinely for estimation of BrdUMP incorporation and processed by a previously reported procedure [15, 16]. Briefly, cells were lysed and treated with proteinase K. DNA was then isolated by phenyl/chloroform-isoamyl alcohol extraction followed by ethanol precipitation. The DNA was hydrolyzed to individual bases by treatment with DNAse I, snake venom phosphodiesterase, alkaline phosphatase and thymidine phosphorylase. The bases were extracted into ethyl acetate, derivatized with bis-trimethylsilyl trifluoroacetamide. Resulting products were then detected and quantitated using a Hewlett–Packard 5987A gas chromatograph/mass spectrometer in the selected ion monitoring mode.

Analysis of BUdR in the cell medium. Immediately prior to harvesting cells exposed to BUdR, an aliquot of the cell medium was removed and frozen at −20° for quantitation of the amount of BUdR remaining at that time. Thawed medium samples were diluted and then analyzed without further processing for their BUdR content by a standard HPLC procedure [9].

RESULTS

Accumulation of BrdUTP. The time- and concentration-dependence of BrdUTP accumulation was assessed in U251 glioblastoma cells. Initial studies demonstrated that, with BUdR concentrations ranging from 2 to 20 μM, BrdUTP accumulated rapidly, achieving steady-state values within 1 hr which were maintained for at least 8 hr after drug addition (data not shown). Furthermore, the exogenous concentration of BUdR remained unchanged for at least 6 hr following drug addition at concentrations in excess of 2 μM. Hence, the relationship between BUdR concentration and BrdUTP accumulation was assessed in U251 cells during a 2-hr incubation period. When the exogenous BUdR concentration ranged from 1 to 100 μM, BrdUTP accumulated intracellularly in a linear fashion without apparent saturation (data not shown).

Effects of endogenous deoxyribonucleotide pools. Since it has been reported that BUdR can result in inhibition of CDP reduction by ribonucleotide reductase [17], it was of interest to analyze the effects of BUdR on the deoxyribonucleoside triphosphate pools in the glioblastoma cells. For this study, U251 cells were incubated with 2 μM BUdR for 25 hr to assess potential time-dependent effects on nucleotide pools. Within 2 hr of drug addition, BrdUTP had accumulated intracellularly to a value of 72 pmol/10^7 cells, a level which was maintained

* Manuscript in preparation.

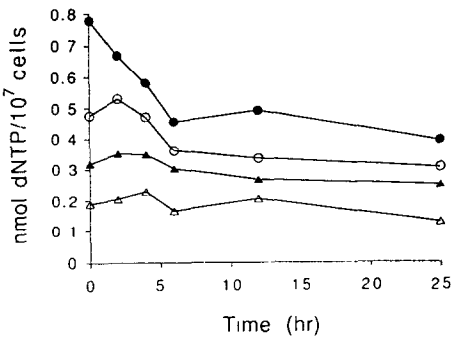

Fig. 1. Effect of BUdR on deoxyribonucleoside 5'-triphosphate (dNTP) pools in glioma cells. U251 cells were incubated with 2 μM BUdR for 25 hr. Deoxyribonucleotide pools were measured periodically by HPLC techniques as described in Materials and Methods. Values at 0 hr represent control deoxyribonucleotide levels. Key: (●) TTP; (○) dCTP; (▲) dATP; and (△) dGTP. Results are single determinations.

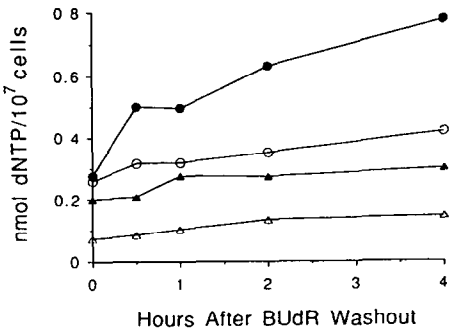

Fig. 3. Recovery of TTP and dCTP pools in U251 cells after BUdR exposure. Cells were incubated with 10 μM BUdR for 2 hr followed by replacement of the medium with drug-free medium. Deoxyribonucleotide pools were measured by HPLC techniques as described in Materials and Methods. Key: (●) TTP; (○) dCTP; (▲) dATP; and (△) dGTP. Values represent single determinations from a typical experiment.

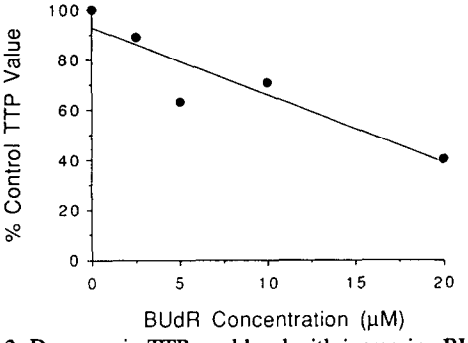

Fig. 2. Decrease in TTP pool level with increasing BUdR concentrations. U251 cells were incubated with the indicated concentrations of BUdR for 2 hr. TTP levels were measured by HPLC techniques as described in Materials and Methods. The control TTP value was 0.329 nmol/10^7 cells. Values represent single determinations from a typical experiment.

for at least 12 hr. Figure 1 illustrates the effects of this drug exposure on the endogenous deoxyribonucleoside triphosphate pools. The TTP and dCTP pools exhibited the greatest alterations, decreasing to 60 and 70% of their initial values, respectively. In contrast, the levels of dATP and dGTP remained relatively constant during this incubation. The decline in the TTP and dCTP values was most evident during the first 6 hr after BUdR addition, whereas between 6 and 25 hr these values decreased only slightly.

Other similar experiments have demonstrated consistently that exposure of U251 cells to BUdR resulted in a decline in TTP and dCTP pools, with little or no effect on the purine deoxyribonucleotide pools. This effect on pyrimidine deoxyribonucleotide pools was exaggerated at higher BUdR concentrations, and TTP pools were depressed consistently to a greater extent than dCTP pools. Figure 2 illustrates the relationship between increasing BUdR concentrations and the endogenous TTP levels. Exposure to 20 μM BUdR for 2 hr reduced TTP to approximately 40% of the untreated control value (Fig. 2), whereas higher BUdR concentrations (up to 100 μM) resulted in a decrease in TTP to approximately 10% of the control value (data not shown). At concentrations of BUdR as high as 100 μM, no significant perturbations in dATP or dGTP levels were observed (data not shown).

These studies demonstrate that BrdUTP can achieve an intracellular concentration that exceeds that of the endogenous deoxyribonucleoside triphosphates. Indeed, at 100 μM BUdR, BrdUTP can achieve a level more than five times higher than the TTP value. However, at the clinically achievable plasma concentration of 2 μM, BrdUTP is typically 5- to 10-fold lower than the endogenous TTP level.

Metabolic effects after removal of BUdR from medium. To determine whether or not the observed decreases in dCTP and TTP pools were reversible, U251 cells were incubated with 20 μM BUdR for 2 hr followed by replacement of the medium with fresh drug-free medium. Cells were harvested periodically and analyzed for nucleotide content. As illustrated in Fig. 3, the levels of the dCTP and TTP pools increased following washout of the BUdR, attaining the initial untreated control value within 4 hr. Only minor changes in the dATP and dGTP pools were observed during this interval. Thus, the changes in the pyrimidine deoxyribonucleotide pools induced by BUdR were readily reversible following removal of the exogenous BUdR.

In the same experiment described in Fig. 3, the rate of decay of BrdUTP was assessed. As indicated in Fig. 4, BrdUTP was eliminated rapidly from the cells following removal of exogenous BUdR. The rate of BrdUTP elimination was multiexponential, with the initial half-life calculated as approximately 15 min. One hour after removal of BUdR, the BrdUTP half-life increased to more than 2 hr. These

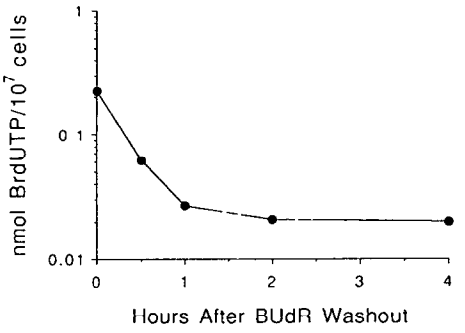

Fig. 4. Rate of elimination of BrdUTP in glioma cells. U251 cells were incubated with 10 μM BUdR for 2 hr, at which point the medium in each culture was replaced with drug-free medium. BrdUTP was measured periodically by HPLC techniques as described in Materials and Methods. Results are the means of duplicate determinations.

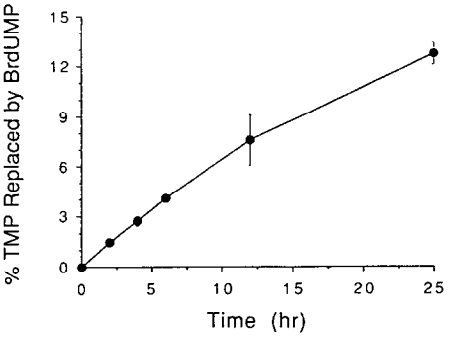

Fig. 5. Incorporation of BrdUMP into U251 DNA. Glioma cells were incubated with 2 μM BUdR for 25 hr. The amount of BrdUMP incorporated into DNA was determined at the indicated intervals as described in Materials and Methods. Values are the means ± SEM of triplicate determinations.

results demonstrate that, in the absence of exogenous BUdR, the majority of the intracellular BrdUTP (88%) was eliminated within 1 hr. Hence, the greatest cellular exposure to BrdUTP occurs during the BUdR incubation period.

Incorporation of BrdUMP into DNA. While the incorporation of BrdUMP into DNA has been documented in many cell lines, the relationship between the metabolism of BUdR and DNA incorporation has not been studied. Previous results with the U251 cell line demonstrated that BrdUMP can replace a maximum of 48% of the thymidylate residues in DNA [11]. We studied the incorporation of BrdUMP in cells incubated with 2 μM BUdR for 25 hr, as illustrated in Fig. 5. Incorporation of BrdUMP into cellular DNA increased in an apparent linear fashion during the first 12 hr. The data suggest that the rate of incorporation decreased at some point between 12 and 25 hr. Examination of the nucleotide pools during this experiment demonstrated that BrdUTP accumulated to a value of 72 pmol/10^7 cells by 2 hr after drug addition and remained at approximately that level for the next 10 hr. However, by 25 hr after drug addition, the intracellular BrdUTP concentration had decreased to 18 pmol/10^7 cells. Analysis of the amount of BUdR remaining in the medium during the course of the experiment indicated that the drug remained at a constant level for at least the first 6 hr, but by 24 hr it had decreased to 0.18 μM. These data suggest that, in the presence of a constant exogenous BUdR concentration, U251 cells maintain a constant BrdUTP level which is associated with a continual increase of BrdUMP incorporation into DNA. However, degradation of the exogenous BUdR results in a decrease in the cellular BrdUTP concentration and a slower rate of incorporation of the analog into DNA.

Table 1. Effect of FUdR on BrdUMP incorporation into U251 DNA

[BUdR] (μM)	% TMP replaced by BrdUMP in DNA	
	No FUdR	+ 10 nM FUdR
1.0	5.70 ± 0.04	5.65*
2.5	7.03 ± 0.57	6.99 ± 0.015
5.0	7.64 ± 0.125	8.67 ± 0.09

Cells were incubated with BUdR alone or in the presence of 10 nM FUdR for 6 hr. BrdUMP incorporation into DNA was analyzed by a gas chromatography/mass spectrometry assay [15, 16]. Values are means ± SEM; N = 3 except where indicated.

* Single determination.

Attempts to increase the DNA incorporation of BrdUMP have centered around adding agents which decrease endogenous TTP pools such as FUdR or FUra [2, 3, 11, 18]. However, Mancini *et al.* [11] have reported recently that the incorporation of BrdUMP into U251 cellular DNA during a 24-hr incubation with BUdR was not augmented by simultaneous exposure to FUdR. In an effort to elucidate the mechanism responsible for this lack of modulation by FUdR, we examined the effect of FUdR on the cellular levels of BrdUTP and TTP and, in the same cell population, measured the incorporation of BrdUMP into cellular DNA. We employed a 6-hr incubation period for these studies to expose the cells to a constant level of BUdR. Table 1 illustrates that, over a 6-hr time period, 10 nM FUdR did not increase substantially the incorporation of BrdUMP into the DNA of U251 glioblastoma cells, in accord with previous results employing longer incubation periods [11]. Table 2 demonstrates that FUdR did not decrease the cellular TTP pool level in these cells, nor did it affect the amount of BrdUTP that accumulated in these cells. Other experiments have demonstrated consistently that a 6-hr incubation of U251 cells with 10 nM FUdR does not depress TTP pools (data not shown). Thus, since the levels of TTP and BrdUTP were not altered by FUdR, this analog should have no effect on the incorporation of BrdUMP into DNA. It will

Table 2. Effect of FUdR on TTP and BrdUTP pools

[BUdR] (μM)	[FUdR] (nM)	TTP (nmol/10^7 cells)	BrdUTP (nmol/10^7 cells)
0	0	0.316	
1.0	0	0.212	0.011
2.5	0	0.240	0.038
5.0	0	0.224	0.052
0	10	0.286*	
1.0	10	0.274	0.011
2.5	10	0.200	0.016
5.0	10	0.218	0.044

U251 cells were incubated with BUdR alone or in the presence of 10 nM FUdR for 6 hr. Nucleotide pool measurements were performed as described in Materials and Methods. Values are averages of duplicate determinations, except where indicated.

* Single determination.

be important to determine whether longer incubation periods or higher concentrations of FUdR are able to depress TTP pools in these cells and, if not, the mechanism by which U251 cells are insensitive to the nucleotide effects of FUdR must be elucidated.

DISCUSSION

While the exact mechanisms by which BUdR exerts its biologic effects are not known, it appears that several of these effects are due, in part, to its incorporation into cellular DNA [1, 2]. Although this incorporation is dependent upon the intracellular conversion of BUdR to BrdUTP, there are few reports describing the rate and extent of accumulation of this important metabolite. Despite current interest in BUdR as adjunct therapy in patients with brain tumors, the data on the metabolism of BUdR in gliomas are sparse. Thus, we have investigated the metabolism of BUdR in a cultured glioblastoma cell line as a first step in the elucidation of the relevant biochemical parameters which determine the level of BrdUMP incorporation into DNA.

Thymidine kinase is the first enzyme in the metabolic pathway that converts BUdR to BrdUTP. BUdR is reported to be a good substrate for this enzyme, with projected K_m values in the range of 2 to 3 μM, similar to the K_m value for the native substrate thymidine [19–21]. In the data presented here, BrdUTP rapidly accumulated to a steady-state level, which may reflect a high affinity of thymidine kinase in the U251 cells for BUdR. These data also demonstrated that BrdUTP accumulation was not saturated at concentrations of BUdR as much as 50-fold above its putative K_m value for thymidine kinase. This finding may be explained by the observation that the endogenous TTP level decreased substantially in response to BUdR exposure. Since TTP can act as a feedback inhibitor on thymidine kinase [22–24], it is possible that reduction in the TTP level allows the U251 cells to phosphorylate BUdR faster by relieving the innate inhibition of the enzyme. Other researchers have demonstrated that BrdUTP can act as a feedback inhibitor of partially purified mammalian thymidine kinase [19]. Thus, it may have been expected that accumulation of high levels of BrdUTP would inhibit its further phosphorylation. However, our data indicate that BrdUTP at levels up to 544 pmol/10^7 cells does not inhibit the phosphorylation of BUdR in the U251 line. It is likely that the interaction between the decreasing TTP levels and perhaps the presence of other cellular nucleotides prevent BrdUTP from significantly inhibiting thymidine kinase activity in the intact glioblastoma cells.

The results in Fig. 1 illustrate that exposure to BUdR results in a significant decrease in both the endogenous dCTP and TTP pool levels. Other investigators have also demonstrated that the cellular dCTP pools decrease during exposure to BUdR, apparently due to feedback inhibition of ribonucleotide reductase by BrdUTP [17, 25]. BrdUTP may be acting in a manner similar to dUTP which, according to the current models of regulation of ribonucleotide reductase, results in decreased production of both dCTP and TTP [26, 27]. Indeed, adding deoxyuridine to lymphoid cells in culture can produce reductions in dCTP pools [28]. The observation that TTP pool levels were decreased to a greater extent than dCTP levels may be explained by the fact that decreases in TTP may also be effected through inhibition of thymidine kinase activity, as described in the preceding paragraph. In addition, cellular accumulation of BrdUMP may result in inhibition of thymidylate synthetase activity, as reported for bacterial sources of this enzyme [29, 30]. Thus, there are potentially three mechanisms by which TTP pool levels can be decreased in glioblastoma cells, whereas dCTP pools appear to be decreased through only one mechanism.

To our knowledge, there has been only one previous report describing a reduction in the cellular TTP pool in response to BUdR [31]. Using Friend leukemia cells, it was demonstrated that 4 μM BUdR (in the presence of 1 mM butyric acid) reduced the TTP pools to approximately 50% of control within 30 min and sustained that reduced nucleotide level for at least 6 hr. The results presented here illustrate that this reduction in TTP occurs with BUdR treatment in the U251 glioblastoma cells (in the absence of other additives). In addition, the data in Figs. 2 and 3 extend the previous results by demonstrating that the reduction in the TTP pool was dependent upon the BUdR concentration and, furthermore, that this effect was readily reversible upon washout of BUdR. In contrast to the results presented here, the data from the Friend leukemia cells demonstrated that there was little, if any, change in the dCTP pool in response to BUdR, while a 2-fold increase in dATP was noted. Thus, these data indicate that nucleotide pool changes mediated by BUdR are cell line specific and emphasize the necessity to define these alterations for each cell line under study.

Although the reduction in TTP level in response to BUdR has important implications for the antitumor and radiosensitizing properties of the thymidine analog (as discussed below), little information exists on this effect, which may be explained by a couple of observations. First, many

investigators employ the DNA polymerase assay to measure the levels of cellular deoxyribonucleotides, and it has been shown that this assay cannot distinguish between BrdUTP and TTP [32]. Thus, TTP levels were not measured in many studies of BUdR effects on nucleotide pools. Second, it was recognized more than 30 years ago that BrdUMP incorporation into DNA could be enhanced by decreasing the endogenous level of the competing TTP pool [3]. Thus, many studies examining the biologic effects of BUdR also employ inhibitors of TTP synthesis, such as FUdR. In such studies, depression of TTP pools is expected and attributed to the effects of the inhibitor of TTP synthesis. The data presented in this paper demonstrate that, in addition to the depression of cellular dCTP pools, BUdR induces an even more prominent reduction in the endogenous TTP level. This effect should be taken into consideration when adding BUdR and FUdR in combination since, at high levels of BUdR, the reduction in TTP pools may not be enhanced by FUdR. Indeed, Mancini *et al.* [11] demonstrated that, in the D-54 glioblastoma cell line, BrdUMP incorporation into DNA could be enhanced by FUdR at concentrations of BUdR less than 10 μM, but at higher BUdR concentrations this effect was abolished. Experiments are underway to determine whether FUdR reduced the TTP pool levels at low but not high BUdR concentrations.

The BUdR-induced decrease in TTP pools has a dual beneficial effect on the incorporation of BrdUMP into DNA. As discussed above, depression of the endogenous TTP pool may increase the phosphorylation of BUdR, thus increasing the amount of precursor available for DNA incorporation. Furthermore, a reduction in cellular TTP decreases the concentration of the natural competitor of BrdUTP for the replicative DNA polymerases which should enhance the incorporation of BrdUMP into DNA. Considering that the reduction in TTP pools became more profound as the BUdR concentration was increased, it may be possible to administer BUdR at a concentration that would suppress the TTP pool to an undetectable level. However, at least 10% of the control level of TTP remained in the cell in the presence of 100 μM BUdR, a highly cytotoxic concentration that is impractical to achieve clinically [1, 2, 8, 11]. Thus, it would be most desirable to employ a second agent to effect an efficient decrease in endogenous TTP pools with the goal of enhancing BrdUMP incorporation into DNA at less toxic BUdR concentrations. The thymidylate synthetase inhibitor FUdR is a logical choice of agents to use since it can decrease cellular TTP pools at concentrations which do not appear to be toxic [30, 33]. However, data presented in this paper demonstrated that 10 nM FUdR was unable to effect a decrease in the endogenous TTP pools in the U251 cell line either by itself or in the presence of BUdR. Consequently, BrdUMP incorporation into cellular DNA was not enhanced by the simultaneous administration of FUdR. A previous report indicated that concentrations of FUdR up to 25 nM were unable to enhance BrdUMP incorporation into U251 cell DNA [11]. The failure of 10 nM FUdR to decrease TTP pools is most likely due to a lack of inhibition of thymidylate synthetase, for which there may be several explanations. FUdR requires phosphorylation to its 5'-monophosphate, FdUMP, which must then combine with thymidylate synthetase and a reduced folate cofactor in order to inhibit the enzyme [30, 33]. Hence, inadequate accumulation of FdUMP (possibly due to high cellular thymidine levels) or too little endogenous folate could prevent formation of the inhibitory complex. Alternatively, these cells may possess high levels of thymidylate synthetase or an altered form of the enzyme which is insensitive to inhibition by FdUMP. These hypotheses are presently under investigation.

While incorporation of BrdUMP into cellular DNA appears to be necessary for the antitumor and radiation sensitizing effects of BUdR, it is not known whether the overall level of incorporation of BrdUMP or perhaps its misincorporation for a nucleotide other than thymidylate results in its biologic effects. These two types of incorporation have been considered in detail in studies of the mutagenic activity of BUdR [25, 34–36]. Although some investigators believe that mutagenesis is proportional to the total amount of BrdUMP incorporated into DNA [34], other data indicate that the less frequently occurring substitution of BrdUMP for dCMP is the critical mutagenic event [25, 35]. If the antitumor and/or radiosensitizing effects are due to the overall level of BrdUMP incorporation into DNA, then reducing the cellular concentration of TTP will be crucial for enhancing BrdUMP incorporation. However, if misincorporation of BrdUMP for dCMP is critical for its biologic effects, then it will be more important to reduce the endogenous dCTP pools to increase the likelihood that this type of misincorporation will occur. Hence it is necessary to distinguish which of these biochemical events is responsible for the antitumor and radiosensitizing effects of BUdR so that this information can be exploited in designing optimal chemotherapeutic protocols for this agent.

Acknowledgement—This work was supported in part by NIH Grant PO1CA42761 from the National Cancer Institute.

REFERENCES

1. Prusoff WH and Goz B, Halogenated pyrimidine deoxyribonucleosides. In: *Handbook of Experimental Pharmacology* (Eds. Sartorelli AC and Johns DG), Vol. 38, Part 2, pp. 272–347. Springer, New York, 1975.
2. Goz B, The effects of incorporation of 5-halogenated deoxyuridines into the DNA of eukaryotic cells. *Pharmacol Rev* **29**: 249–272, 1978.
3. Djordjevic B and Szybalski W, Genetics of human cell lines: III. Incorporation of 5-bromo- and 5-iododeoxyuridine into the deoxyribonucleic acid of human cells and its effect on radiation sensitivity. *J Exp Med* **112**: 509–531, 1960.
4. Szybalski W, X-ray sensitization by halopyrimidines. *Cancer Chemother Rep* **58**: 539–557, 1974.
5. Hoshino T, Radiosensitization of brain tumors. In: *Modern Radiotherapy and Oncology—Central Nervous System Tumors* (Ed. Deely TJ), pp. 170–183. Butterworths, London, 1974.

6. Kinsella TJ, Mitchell JB, Russo A, Aiken M, Morstyn G, Hsu SM, Rowland J and Glatstein E, Continuous intravenous infusions of bromodeoxyuridine as a clinical radiosensitizer. *J Clin Oncol* **10**: 1144–1150, 1984.
7. Phillips TW, Chandler WF, Kindt GW, Ensminger WD, Greenberg HS, Seeger JF, Doan KM and Gyves JW, New implantable continuous administration and bolus dose intracarotid drug delivery system for the treatment of malignant gliomas. *Neurosurgery* **11**: 213–218, 1982.
8. Hegarty TJ, Thornton AF, Diaz RF, Chandler WF, Ensminger WD, Junck L, Page MA, Gebarski SS, Hood TW, Stetson PL, Tankanow RM, McKeever PE, Lichter AS and Greenberg HS, Intra-arterial bromodeoxyuridine radiosensitization of malignant gliomas. *Int J Radiat Oncol Biol Phys* **19**: 421–428, 1990.
9. Stetson PL, Shukla UA, Amin PR and Ensminger WD, High performance liquid chromatographic method for the determination of bromodeoxyuridine and its major metabolite, bromouracil, in biological fluids. *J Chromatogr* **341**: 217–222, 1985.
10. Kinsella TJ, Mitchell JB, Russo A, Morstyn G and Glatstein E, The use of halogenated thymidine analogs as clinical radiosensitizers: Rationale, current status, and future prospects: non-hypoxic cell sensitizers. *Int J Radiat Oncol Biol Phys* **10**: 1399–1406, 1984.
11. Mancini WR, Stetson PL, Lawrence TS, Wagner JG, Greenberg HS and Ensminger WD, Variability of 5-bromo-2′-deoxyuridine incorporation into DNA of human glioma cell lines and modulation with fluoropyrimidines. *Cancer Res* **51**: 870–874, 1991.
12. Kit S, Dubbs DR, Piekarski LJ and Hsu TC, Deletion of thymidine kinase activity from L cells resistant to bromodeoxyuridine. *Exp Cell Res* **31**: 297–312, 1963.
13. Ellero J, Ensminger WD and Shewach DS, Metabolism of BUdR in a human glioblastoma cell line. *Proc Am Assoc Cancer Res* **32**: 416, 1991.
14. Bigner DD, Bigner SH, Pontent J, Westermark B, Mahaley MS Jr, Ruoslahti H, Herschman H, Eng LF and Wikstrand CJ, Heterogeneity of genotypic and phenotypic characteristics of fifteen permanent cell lines derived from human gliomas. *J Neuropathol Exp Neurol* **40**: 201–229, 1981.
15. Maybaum J, Kott MG, Johnson NJ, Ensminger WD and Stetson PL, Analysis of bromodeoxyuridine incorporation into DNA: Comparison of gas chromatographic/mass spectrometric, CsCl gradient sedimentation, and specific radioactivity methods. *Anal Biochem* **161**: 164–171, 1987.
16. Stetson PL, Maybaum J, Shukla UA and Ensminger WD, Simultaneous determination of thymine and bromouracil in DNA hydrolysates using gas chromatography-mass spectrometry with selected-ion monitoring. *J Chromatogr* **375**: 1–9, 1986.
17. Meuth M and Green H, Induction of a deoxycytidineless state in cultured mammalian cells by bromodeoxyuridine. *Cell* **2**: 109–112, 1974.
18. Kaufman ER and Davidson RL, Bromodeoxyuridine mutagenesis in mammalian cells: Mutagenesis is independent of the amount of bromouracil in DNA. *Proc Natl Acad Sci USA* **75**: 4982–4986, 1978.
19. Lee L-S and Cheng Y-c, Human deoxythymidine kinase II: Substrate specificity and kinetic behavior of the cytoplasmic and mitochondrial isozymes derived from blast cells of acute myelocytic leukemia. *Biochemistry* **15**: 3686–3690, 1976.
20. Bresnick E and Thompson UB, Properties of deoxythymidine kinase partially purified from animal tumors. *J Biol Chem* **240**: 3967–3974, 1965.
21. Kizer DE and Holman L, Purification and properties of thymidine kinase from regenerating rat liver. *Biochim Biophys Acta* **350**: 193–200, 1973.
22. Ives DH, Morse PA Jr and Potter VR, Feedback inhibition of thymidine kinase by thymidine triphosphate. *J Biol Chem* **238**: 1467–1474, 1963.
23. Prusoff WH and Chang PK, Regulation of thymidine kinase activity by 5-iodo-2′-deoxyuridine 5′-triphosphate and deoxythymidine 5′-triphosphate. *Chem Biol Interact* **1**: 285–299, 1969/1970.
24. Fischer PH and Baxter D, Enzyme regulatory site-directed drugs: Modulation of thymidine triphosphate inhibition of thymidine kinase by 5′-amino-5′-deoxythymidine. *Mol Pharmacol* **22**: 231–234, 1982.
25. Ashman CR and Davidson RL, Bromodeoxyuridine mutagenesis in mammalian cells is related to deoxyribonucleotide pool imbalance. *Mol Cell Biol* **1**: 254–260, 1981.
26. Moore EC and Hurlbert RB, Regulation of mammalian deoxyribonucleotide biosynthesis by nucleotides as activators and inhibitors. *J Biol Chem* **241**: 4802–4809, 1966.
27. Thelander L and Reichard P, Reduction of ribonucleotides. *Annu Rev Biochem* **48**: 133–158, 1979.
28. Perez DJ and Tattersall MHN, The metabolic basis of deoxyuridine cytotoxicity: Studies of cultured human lymphoblasts. *Mol Pharmacol* **22**: 166–174, 1982.
29. Flaks JG and Cohen SS, Virus-induced acquisition of metabolic function. III. Formation and some properties of thymidylate synthetase of bacteriophage-infected *Escherichia coli*. *J Biol Chem* **234**: 2981–2986, 1959.
30. Wataya Y, Santi DV and Hansch C, Inhibition of *Lactobacillus casei* thymidylate synthetase by 5-substituted 2′-deoxyuridylates. Preliminary quantitative structure–activity relationship. *J Med Chem* **20**: 1469–1473, 1977.
31. Bick MD, Bromodeoxyuridine inhibition of Friend leukemia cell induction. Mechanism of reversal by deoxycytidine. *Biochim Biophys Acta* **476**: 279–286, 1977.
32. Bick MF, A quantitative method for distinguishing BrdUTP and dTTP in soluble pools. *Anal Biochem* **78**: 582–585, 1977.
33. Heidelberger C, Fluorinated pyrimidines and their nucleosides. In: *Handbook of Experimental Pharmacology* (Eds. Sartorelli AC and Johns DG), Vol. 38, Part 2, pp. 193–231. Springer, New York, 1975.
34. Hopkins RL and Goodman MF, Deoxyribonucleotide pools, base pairing, and sequence configuration affecting bromodeoxyuridine- and 2-aminopurine-induced mutagenesis. *Proc Natl Acad Sci USA* **77**: 1801–1805, 1980.
35. Davidson RL, Broeker P and Ashman CR, DNA base sequence changes and sequence specificity of bromodeoxyuridine-induced mutations in mammalian cells. *Proc Natl Acad Sci USA* **85**: 4406–4410, 1988.
36. Sargent RG, Ji J, Mun B and Mathews CK, Ribonucleotide reductase: A determinant of 5-bromodeoxyuridine mutagenesis in phage T4. *Mol Gen Genet* **217**: 13–19, 1989.