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# Biochemical and biological properties of 5-bromotubercidin: Differential effects on cellular DNA-directed and viral RNA-directed RNA synthesis

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**Abstract**—We have studied the biochemical and biological properties of 5-bromotubercidin (4-amino-5-bromo-7-β-D-ribofuranosyl-pyrrolo [2,3-*d*]pyrimidine) (BrTu), a synthetic analogue of the highly cytotoxic pyrrolo[2,3-*d*]pyrimidine ribonucleoside antibiotic tubercidin (Tu) that interferes with numerous cellular processes, and has been shown to possess biological specificity and selectivity. Thus, BrTu entered the mammalian cell nucleotide pool by phosphorylation, was incorporated into RNA in an unmodified form and, as a consequence, reversibly inhibited (15 μM) mammalian cell growth and the synthesis of high-molecular-weight cellular RNA species (i.e., mRNA and rRNA). However, BrTu (300 μM) did not inhibit picornavirus RNA synthesis or multiplication, and thus discriminated between virus RNA-dependent and all forms of DNA-dependent RNA synthesis whether of cellular or viral origin; because of this BrTu should prove valuable as a metabolic probe for studying the cell–virus relationship. Furthermore, BrTu is a substrate for adenosine kinase ($K_m$ = 24 μM), and is also its potent inhibitor ($K_i$ = 0.93 μM); thus, low concentrations of BrTu (1.5 μM), which did not inhibit cell growth, blocked phosphorylation and the cellular uptake of other, highly cytotoxic pyrrolo-pyrimidine nucleoside analogues (e.g., tubercidin). This block in cellular uptake and incorporation of toxic analogues was associated with the protective effect of BrTu against cell killing by the analogues, providing a mechanism by which BrTu and these analogues can, as we reported elsewhere [*J. Virol.* **1999**, *73*, 6444], be used for the selective inactivation of replicating picornaviruses.


## 1. Introduction

Previous studies of pyrrolo[2,3-*d*]pyrimidine nucleosides and nucleotides have demonstrated that these purine analogues are incorporated into nucleic acids by mammalian cells in culture[1–4] and by enzyme preparations in vitro.[5,6] Furthermore, these compounds can substitute for the normal adenosine counterparts in a wide variety of enzyme reactions. In comparison with adenine, the pyrrolo[2,3-*d*]pyrimidine structure provides an extra valence which permits the introduction of diverse functional groups, thereby making possible the preparation of a wide variety of ring-substituted derivatives. The position of the extra valence is significant, since it corresponds to the location of the N-7 of adenine; and the presence at this position of even bulky space filling groups does not obstruct the H-bonding sites involved in base-pairing, or otherwise interfere with the formation of polynucleotide helices.[5–8] Thus, pyrrolo-[2,3-*d*]pyrimidine nucleotides are efficient substrates for polymerizing enzymes and may form polymers with unusual physical and biochemical properties. Finally, the large number of derivatives, substituted with a variety of functional groups, may provide metabolic probes which possess biological selectivity and specificity with respect to nucleotide and polynucleotide function. For these reasons we have studied some of the biochemical and biological properties of 5-bromotubercidin (4-amino-5-bromo-7-β-D-ribofuranosyl-pyrrolo[2,3-*d*]pyrimidine) (BrTu) (Fig. 1), a synthetic derivative of pyrrolo[2,3-*d*]pyrimidine ribonucleoside antibiotic tubercidin[1] (Tu); the aglycone of BrTu (4-amino-5-bromo-pyrrolo[2,3-*d*] pyrimidine) is the natural product isolated from a marine sponge.[9] Tubercidin is a highly cytotoxic adenosine analogue that interferes with numerous cellular pro-

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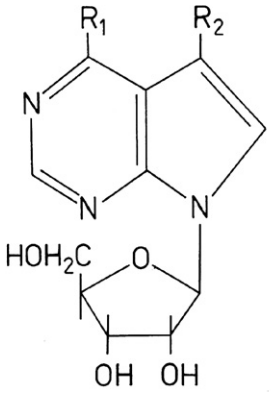


| | $R_1$ | $R_2$ |
|---|---|---|
| Tubercidin | $NH_2$ | H |
| 5-Bromotubercidin | $NH_2$ | Br |
| Deazanebularin | H | H |

**Figure 1.** Structures of 5-bromotubercidin and the related analogues, tubercidin and deazanebularin, used in the present work.

cesses including mitochondrial respiration, de novo purine synthesis, rRNA processing, methylation of tRNA and nucleic acid and protein synthesis.[1,4] The results presented here reveal that in contrast to the highly cytotoxic nucleoside tubercidin, the C-5 substituted analogue BrTu is more selective in its biological activity involving a variety of cellular and enzymatic reactions. For instance, BrTu reversibly inhibits the synthesis of cellular RNA, but does not inhibit either picornavirus RNA synthesis or multiplication, providing a means for discriminating between viral and host replicating systems. Furthermore, by inhibiting adenosine kinase, BrTu strongly inhibits the phosphorylation and cellular uptake of other, highly cytotoxic pyrrolo-pyrimidine ribonucleosides, such as Tu and deazanebularin (DN) (Fig. 1). This block in cellular uptake of Tu or DN was associated with the protective effect of BrTu against cell killing by the analogues, providing the mechanism by which BrTu and these analogues can be used for the selective inactivation of replicating picornaviruses.[10]

## 2. Results and discussion

### 2.1. 5-Bromotubercidin inhibits cell growth and macromolecule synthesis reversibly

Our previous studies of antiviral properties of a series of pyrrolo-pyrimidine nucleoside analogues of adenosine, including tubercidin,[1] deazanebularin,[2] toyocamycine,[11] sangivamycin, formycin and 5-bromotubercidin,[10] have shown that only 5-bromotubercidin (BrTu) possesses unusual selectivity with respect to its effects on some RNA and DNA containing viruses in cultured cells. Hence, BrTu was totally inert with regard to picornavirus (e.g., mengovirus) RNA synthesis or multiplication, whereas it strongly suppressed the synthesis of RNA in host cells just as it did the growth of DNA viruses (e.g., vaccinia virus). This differential effect of BrTu on the cellular DNA-directed and picornavirus RNA-directed RNA synthesis was of interest for two reasons. First, it suggested that BrTu can be used as an experimental model system for studying the cell–virus relationship in the background of cellular RNA synthesis inhibition, and second that it may provide a metabolic probe which possesses biological selectivity and specificity with respect to nucleotide and polynucleotide function. We therefore investigated the substrate specificity of BrTu and its phosphorylated derivatives for a variety of cellular, viral and enzymatic reactions. Several aspects of BrTu action were important: (1) BrTu inhibited the growth of mouse fibroblasts in culture (L2-cells) at relatively low concentrations (Fig. 2); complete growth stopage required 15 μM BrTu. At a BrTu concentration of 3 μM, a consistent rate of cell multiplication was maintained, although it was not quite as rapid as in untreated control cultures. When cells which had been exposed to BrTu (15–300 μM) were washed and incubated in fresh medium free of inhibitor, growth resumed relatively rapidly. The lag period which intervened between the removal of BrTu and resumption of growth increased with increasing time of exposure to a single drug concentration (30 μM) (Fig. 3a), but it did not change with increasing drug concentrations (15–300 μM) at a fixed time (8 h) of exposure (Fig. 3b). Continued microscopic examination of the cultures revealed that the increase in cell number which took place after BrTu removal was not due to excessively rapid growth of a minority of the cell population: all the cells seen in many fields under microscopic observation divided mitotically in a normal manner. In contrast to BrTu, whose effects on cell growth are reversibly cytostatic, its parent compound Tu is both more potent and, at concentrations above 37 nM, rapidly and irreversibly cytotoxic (data not shown and Ref. 10). (2) The data in Figure 4 show that

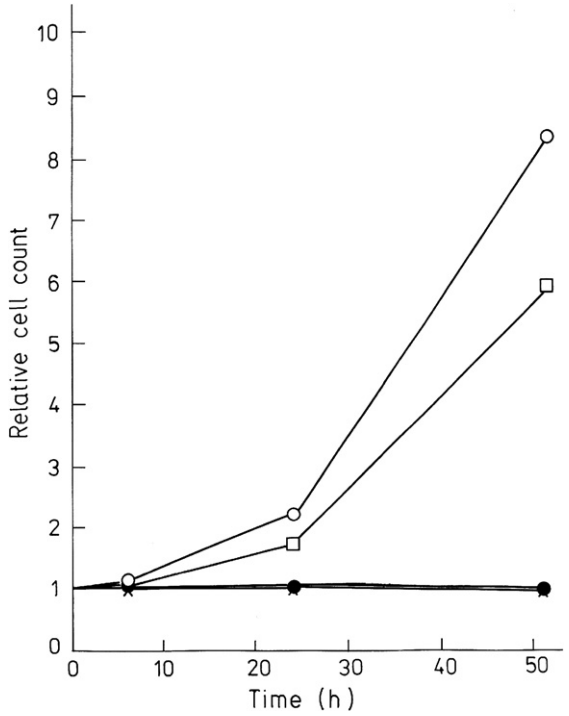


**Figure 2.** Effect of 5-bromotubercidin on the growth of L-cells. Cells were plated at $1 \times 10^5$ per 60 mm Petri dish and incubated overnight. BrTu was then added and maintained at the indicated concentrations throughout the period of observation. Cell counts were performed at the times shown on defined areas of duplicate plates. (○), control; (□), BrTu (3 μM); (×), BrTu (15 μM); (●), BrTu (30 μM).

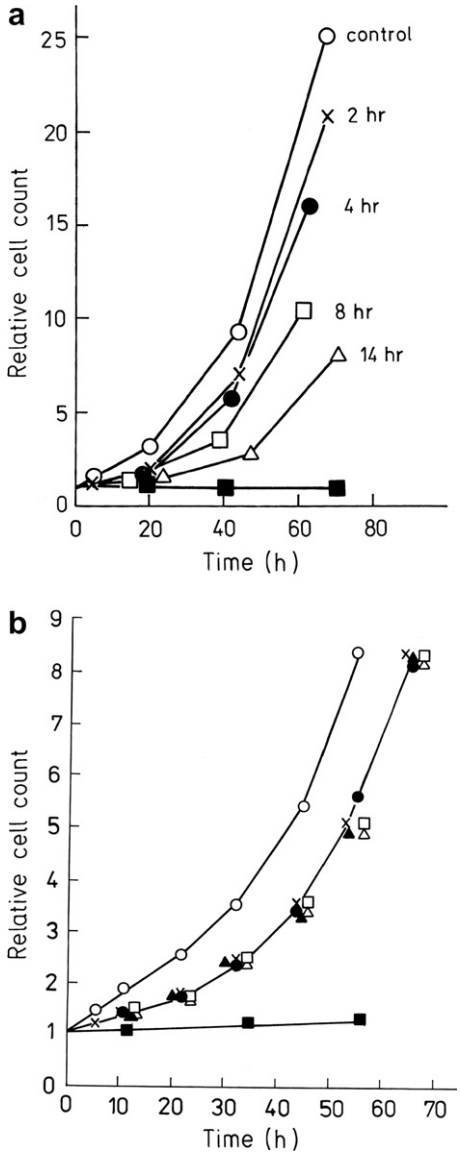


**Figure 3.** 5-Bromotubercidin inhibits cell growth reversibly. The conditions for cell growth were as in Figure 2. Cell cultures were incubated either for various lengths of time with a single drug concentration (30 μM) (a), or for 8 h with indicated concentrations of BrTu (b). At the indicated times thereafter the cultures were washed and incubation continued in drug-free medium. Cell counts were performed at the indicated times on defined areas of duplicate plates. (a) Control (○); BrTu (30 μM): (×), 2 h; (●), 4 h; (□), 8 h; (△), 14 h; (■), continuous. (b) Control (○); BrTu: (×), 15 μM; (●), 30 μM; (□), 60 μM; (△), 150 μM; (▲), 300 μM; (■), 15 μM continuous.

of the classes of macromolecules examined, BrTu preferentially inhibited the synthesis of both RNA (4A) and DNA (4B); the synthesis of protein was relatively unimpaired for some hours (data not shown). DNA synthesis was gradually slowed with increasing concentrations of BrTu (from 15 to 300 μM) and was ultimately completely arrested. RNA synthesis was also slowed by BrTu (4A). The degree of inhibition rose rapidly over the concentration range of 3–15 μM and reached a maximum at 15–30 μM. At these concentrations RNA synthesis was reduced to 15–20% of the normal and may proceed at this rate for up to 9 h. Further increases in concentration of BrTu (to 300 μM) did not produce any additional inhibition of RNA synthesis. These effects on RNA and DNA synthesis, like those on growth, were reversed on removal of BrTu (data not shown). The time required for restoration of the normal rate of RNA and DNA synthesis was inversely related to period of exposure to BrTu. (3) The results of several experiments demonstrated that the inhibition of cellular RNA synthesis by BrTu mainly involved the species of ribosomal RNA, and that the synthesis of both rapidly labelled nuclear 45 S RNA and tRNA was more resistant to the drug (Fig. 5); the synthesis of heterogeneous nuclear RNA was, as determined previously,[12] also inhibited by BrTu (data not shown). Cultures treated by BrTu synthesized as compared with untreated controls approximately 5-fold less amount of rapidly labelled 45 S RNA (Fig. 6), whose conversion to rRNA species was apparently impaired in the presence of the drug (Fig. 7); the rapidly labelled 45 S RNA, which was synthesized in the presence of BrTu, was following drug removal not normally processed into ribosomal RNA species in the presence of actinomycin D (AMD) (Fig. 8, Chart II + AMD), as compared with untreated controls (Chart I + AMD; Chart III − AMD). (4) Several lines of experiments have been performed to test whether the inhibition of RNA synthesis by BrTu reflects some interference with polymerization or is it due to changes in the metabolism of nucleotide monomers. To test for a possible effect of BrTu on purine metabolism, experiments were performed with [$^{14}$C]formate as radioactive precursor for polynucleotide synthesis. If BrTu inhibited nucleic acid synthesis by blocking the formation of purine nucleotides, the incorporation of formate into RNA and DNA should be more sensitive than that of nucleosides and aglycons. However, this was not the case: a given concentration of BrTu inhibited the incorporation of formate into RNA to exactly the same degree as that of nucleoside precursors (data not shown). One corollary of this result is that the incorporation of formate into acid-soluble nucleotide pool was also unaffected by BrTu. This suggested that unlike many purine and purine nucleoside analogues[13] including tubercidin,[4] BrTu did not inhibit purine biosynthesis de novo. Therefore, its inhibitory effects on cell growth and macromolecule synthesis are probably the result of a primary action of BrTu on polynucleotide synthesis or metabolism.

### 2.2. Metabolism of 5-bromotubercidin: characterization of acid-soluble nucleotides and incorporation into nucleic acid

In view of the above observations it is desirable to obtain information concerning the metabolism of BrTu and the incorporation, if any, into polynucleotides. As seen in Table 1 the fraction of radioactive BrTu in the culture medium which appeared in the cellular nucleotide pool was very small; the maximum concentration was reached within 10–25 min and did not change measurably during several hours. The distribution of acid-soluble radioactivity resembled that found for other metabolizable nucleoside analogues in that most of the residues (>80%) were in the form of the 5′-triphos-

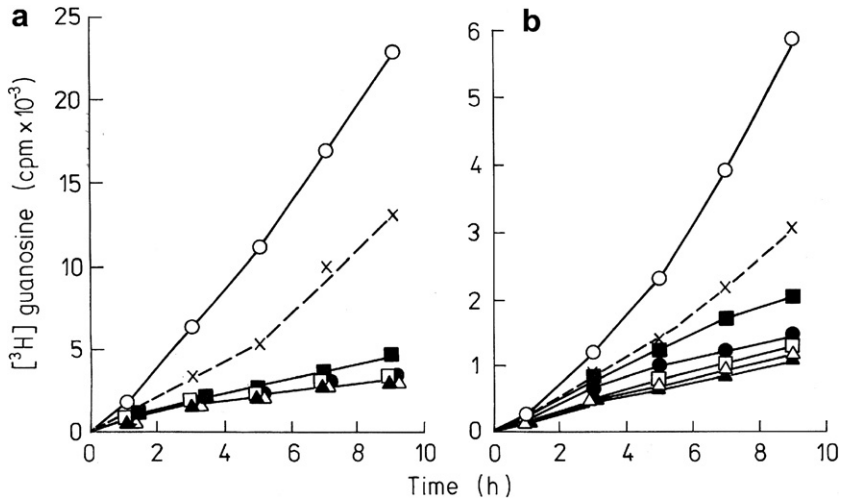


**Figure 4.** Effects of 5-bromotubercidin on macromolecule synthesis in L-cells. Cell monolayers ($3 \times 10^5$ per 60 mm Petri dish) were exposed to indicated concentrations of BrTu followed 20 min later by [$^3$H]guanosine (2 μCi/ml) for DNA and RNA synthesis. At the indicated times the cells were washed, and the radioactivity incorporated into RNA (a) and DNA (b) was measured as described in Section 3. (○), control; BrTu: (×), 3 μM; (■), 15 μM; (●), 30 μM; (□), 60 μM; (△), 150 μM; (▲), 300 μM.

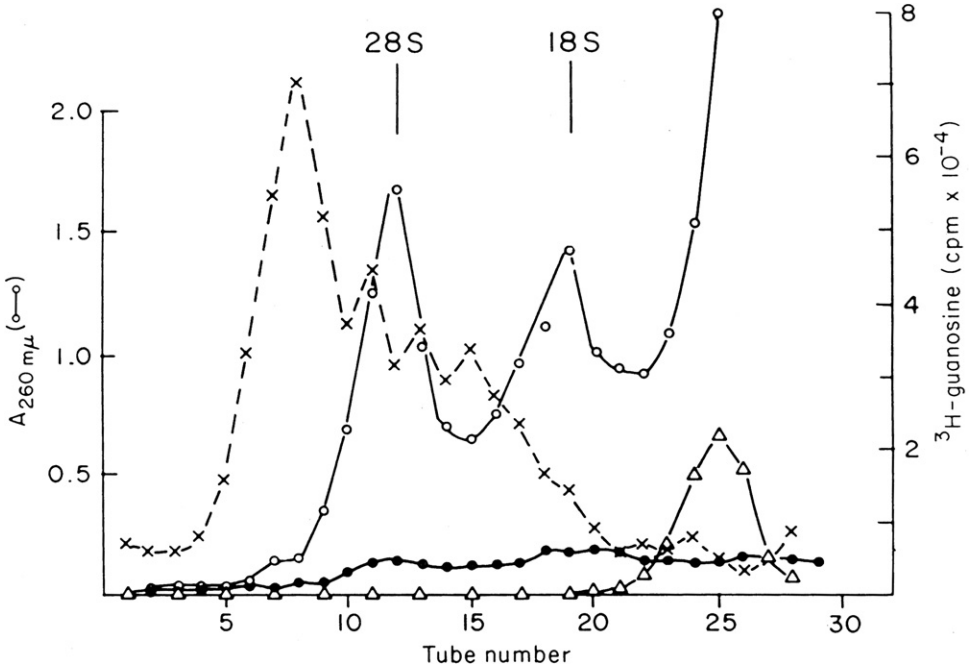


**Figure 5.** Synthesis of RNA in nuclear, ribosomal and postribosomal fractions of L-cells treated with 5-bromotubercidin. Cell monolayers ($3 \times 10^6$ per 100 mm Petri dish, 4 dishes) were exposed to BrTu (37.5 μM; 12.5 μg/ml), followed 20 min later by [$^3$H]guanosine (2 μCi/ml), and incubation was continued for 2 h. Cells were then collected, disrupted in a homogenizer, and RNA was analysed by sucrose density gradient (20–50%) centrifugation, after being extracted from nuclear, ribosomal and postribosomal fractions (Section 3). (○), $A_{260\ nm}$; (×), nuclear RNA; (●), ribosomal RNA; (△), soluble RNA.

phates. The flow of [$^3$H]BrTu into acid-insoluble cellular fractions shows that at 24 μM BrTu the radioactivity incorporated into RNA reached the maximal value within one hour after addition of the drug (Fig. 9). When the cells were incubated in the presence of 2.4 μM BrTu the incorporation of radioactive compound into RNA was not completely interrupted and proceeds beyond the level which was observed in the culture treated with 24 μM BrTu. Incorporation of BrTu into cellular RNA occurred through DNA-dependent reaction, since it was sensitive to inhibition by actinomycin D (data not shown). BrTu was also incorporated into DNA, but the total amount of radioactivity found in this macromolecule was also quite low; we did not analyse whether BrTu was transformed to a 2′-deoxy derivative intracellularly and incorporated in this form into DNA. A low but measurable amount of radioactive BrTu, which was incorporated into cellular RNA, was found to be in an unmodified form. Thus, the radioactive material released on alkaline hydrolysis of RNA,

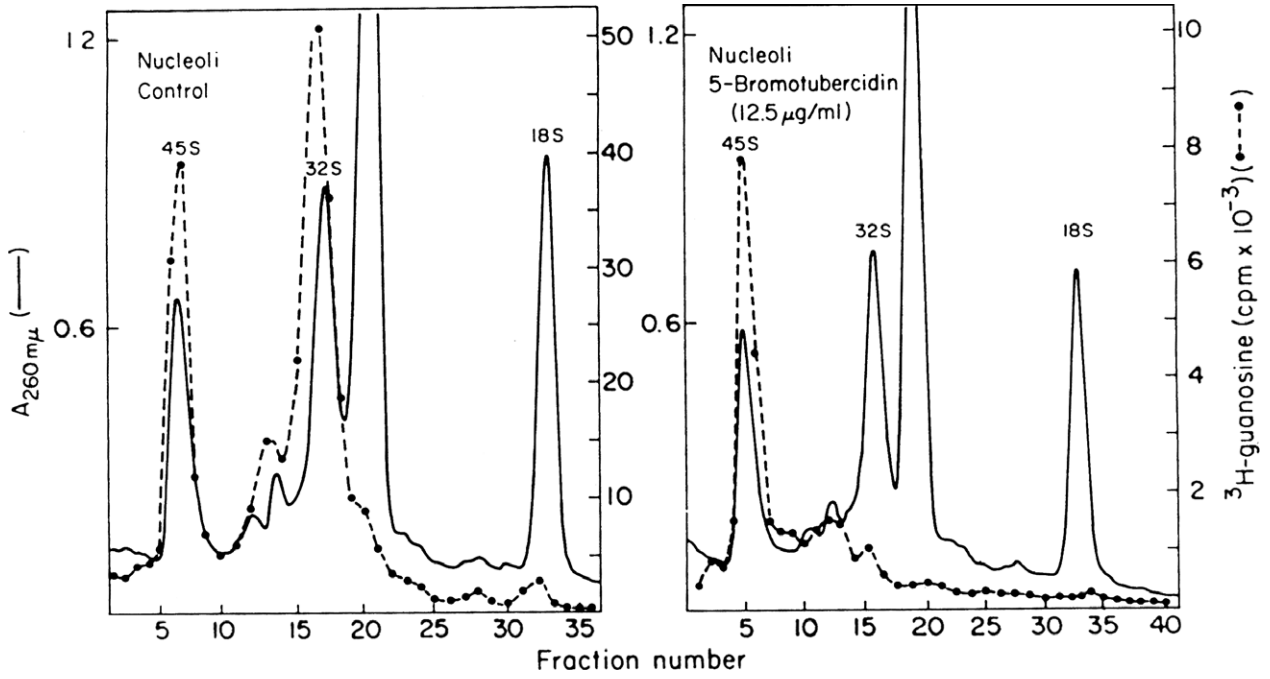


**Figure 6.** Effect of 5-bromotubercidin on the synthesis of RNA species in nucleolar fraction. Experimental conditions were as in Figure 5, except that the cells were incubated with radioactive guanosine for 30 min. After this, the cells were collected and separated into nucleolar and nucleoplasmic fractions, and the RNA was extracted and analysed on polyacrylamide gel electrophoresis (Section 3).

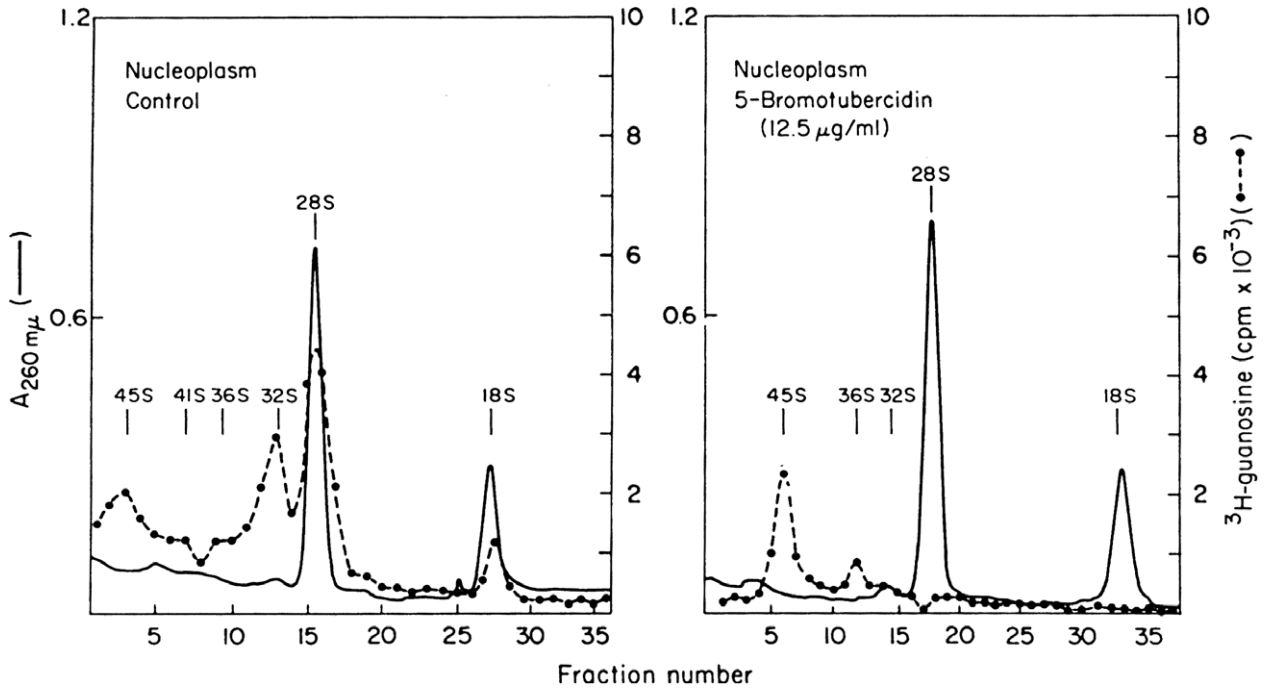


**Figure 7.** Effect of 5-bromotubercidin on the synthesis of RNA species in nucleoplasmic fraction. Experimental conditions were as in Figure 6.

which was isolated from tRNA fraction, was chromatographically and electrophoretically indistinguishable from 2′(3′)BrTuMP. It was converted by *Escherichia coli* alkaline phosphatase to a compound which chromatographically resembled authentic BrTu (data not shown). The radioactivity incorporated into tRNA was found to be in the nucleotide (81%) and nucleoside (19%) form. This suggests that, apart from phosphorylation, BrTu does not undergo metabolic modification in fibroblast cultures.

Several of the above observations are unexpected. In particular, the fact that the uptake of BrTu reaches a plateau quickly, corresponding to a very low concentration in the nucleotide pool, is in marked contrast to the phosphorylation of adenosine and all other adenosine

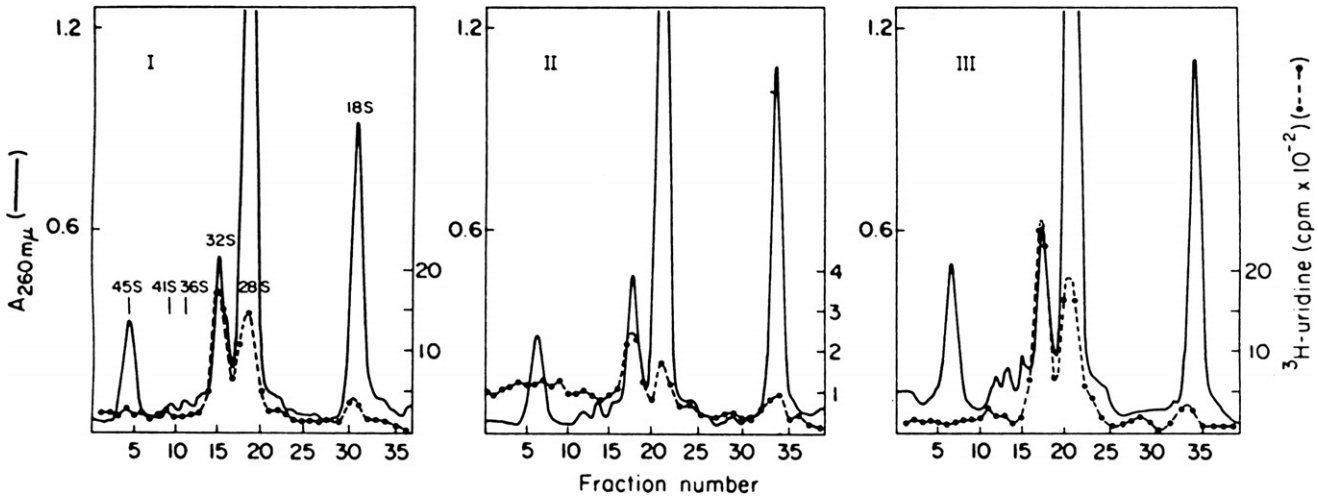


**Figure 8.** 5-Bromotubercidin affects the maturation of ribosomal 45 S RNA precursor. Three sets of cultures were established ($3 \times 10^6$ cells per 100 mm Petri dish, 4 dishes), one-set receiving BrTu (37.5 μM; 12.5 μg/ml) (II), and two sets serving as untreated controls (I and III). After 20 min all sets were labelled by a 20 min pulse of [$^3$H]uridine (2 μCi/ml; spec. act. 5 Ci/mmol), washed and incubated for additional 4 h in fresh drug-free medium (III) containing 0.1 μg/ml of actinomycin D (I and II). At the end of the period of incubation the nucleoli were isolated, RNA extracted and analysed as in Figure 6. I, control (+AMD); II, BrTu (+AMD); III, control (−AMD).

**Table 1.** Cellular uptake of [$^3$H]5-bromotubercidin into acid-soluble pool

| [$^3$H]5-Bromotubercidin (μM) | Incubation (min) | Radioactivity (cpm) |
|---|---|---|
| 2.4 | 25 | 481 |
| 2.4 | 120 | 459 |
| 2.4 | 240 | 509 |
| 24 | 25 | 825 |
| 24 | 120 | 767 |
| 24 | 240 | 794 |

Cell cultures ($2 \times 10^5$ cells per 60 mm Petri dish) were incubated for various lengths of time with indicated concentrations of [$^3$H]BrTu (spec. act. 4.8 μCi/μmol). At the indicated times, the cells were thoroughly washed with phosphate-buffered saline, the nucleotide pool extracted with 0.25 M perchloric acid for 45 min in an ice bath and the radioactivity determined (Section 3).

analogues examined to date.[1,2,10,12] This low but measurable incorporation of BrTu nucleotides into RNA coupled to BrTu mediated block in the synthesis and maturation of high-molecular-weight cellular RNA (Figs. 7 and 8), probably accounts for the excellent recovery of cells from the treatment with BrTu. Further, the low cytotoxicity of BrTu, its ready reversibility and inertness to purine synthesis de novo, all suggested that the uptake of BrTu is not limited by generalized breakdown of cellular metabolism or any significant change in nucleotide metabolism. We presumed that the cellular uptake of BrTu might be impaired at the level of the cytosolic nucleoside or nucleotide phosphorylation and/or membrane nucleoside transportation. We have performed a series of experiments to identify the reaction which restricts BrTu uptake. For this purpose we have explored the phosphorylation of BrTu and of some

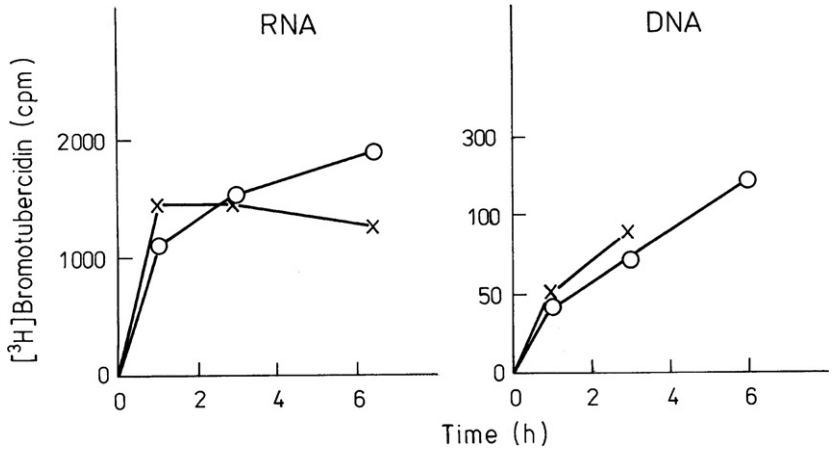


**Figure 9.** Incorporation of [$^3$H]5-bromotubercidin into DNA and RNA. The conditions for cell growth ($8 \times 10^5$ cells per 60 mm Petri dish) and labelling with radioactive BrTu were as in Table 1. The cells remaining after extraction with 0.25 M perchloric acid were resuspended in alkali, and the radioactivity in RNA and DNA was determined (Section 3). BrTu: 24 μM, (×); 2.4 μM, (○).

**Table 2.** Nucleoside and nucleotide kinase activity in L-cell homogenate or $S_{100}$ fraction

| Compound | Fraction of nucleoside converted to phosphates[a] (cpm) | | | |
|---|---|---|---|---|
| | Crude cell homogenate[b] | | $S_{100}$ fraction[b] | |
| | Monophosphate | Triphosphate | Monophosphate | Triphosphate |
| Bromotubercidin | 0.084 ± 0.011 | 0.010 ± 0.002 | 0.078 ± 0.023 | 0 |
| Deazanebularin | 0.53 ± 0.22 | 0.050 ± 0.002 | 0.640 ± 0.170 | 0.012 ± 0.003 |
| Adenosine | 0.35 ± 0.095 | 0.135 ± 0.041 | 0.142 ± 0.053 | 0.470 ± 0.154 |

[a] Reaction products after electrophoretic separation of radioactive nucleotides (Section 3).
[b] Average ± SD for three separate experiments.

**Table 3.** Kinetic constants for nucleoside analogues with rabbit liver adenosine kinase

| Compound rel. $V_{max}/K_m$ | $K_m$[a] (μM) | $K_i$[b] (μM) | $V_{max}$ (μM $min^{-1}$) | Relative[c] $V_{max}$ | Substrate efficiency |
|---|---|---|---|---|---|
| Adenosine | 5.5 | | 3.13 | 100 | 16.95 |
| BrTu | 24 | 0.93 (Adn); 0.27 (DN); 0.5 (Tu) | 2.94 | 94 | 3.77 |
| DN | 12.5 | | 3.0 | 96 | 7.7 |
| Tu | 9.5 | | 3.57 | 114 | 12.0 |

[a] $K_m$ and $V_{max}$ values were derived from five velocity measurements over a 10-fold nucleoside concentration which spanned the $K_m$ value.
[b] The $K$ values for inhibition of the enzyme by BrTu for adenosine, DN and Tu were 0.93, 0.27 and 0.5 μM, respectively.
[c] $V_{max}$ values are relative to the $V_{max}$ of adenosine which was arbitrarily set at 100.

other adenosine analogues, such as tubercidin (Tu) and deazanebularin (DN), in crude and in partially fractionated homogenates of L-cells. From the data presented in Tables 2 and 3, the following facts may be noted: (1) All the nucleosides tested (BrTu, DN, adenosine) and their corresponding 5′-monophosphates were converted to their 5′-triphosphates in crude homogenates of L-cells (Table 2). BrTu was, however, in this reaction less effective substrate than adenosine or DN; this matches our observation (Fig. 10) that BrTu was a substrate for partially purified rabbit liver adenosine kinase ($K_m$ = 24 μM), although less effective than adenosine ($K_m$ = 5.5 μM). (2) Only adenosine and its 5′-monophosphate, and to a lesser extent DN and its 5′-monophosphate, were phosphorylated to their triphosphates

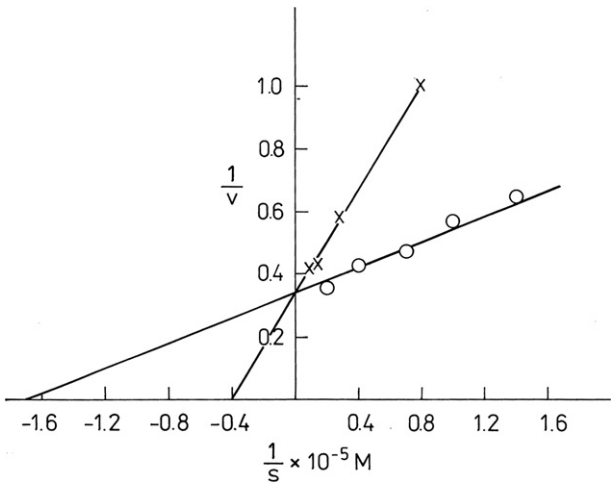


**Figure 10.** Lineweaver-Burk plots of rabbit liver adenosine kinase activity at four concentrations of BrTu (×) in the range 10–120 μM, and at five concentrations of adenosine (○) in the range 5–50 μM. The plotted data were from a single experiment in triplicate.

in the 100,000*g* supernatant from such homogenates (Table 1). BrTu and its 5′-monophosphate were therefore converted to the triphosphate only in fractions which contain some of the particulate fractions of L-cells. (3) The presence of BrTu strongly inhibited the phosphorylation of DN and Tu both in cell homogenate and rabbit liver adenosine kinase preparations; the phosphorylation of adenosine in the respective enzyme preparations was moderately inhibited by BrTu. Thus, the $K$ values for inhibition of the rabbit liver adenosine kinase by BrTu for adenosine, DN and Tu were 0.93, 0.27 and 0.5 μM, respectively (Table 3). Replacement of N-7 in adenosine by C–Br, as occurs in BrTu, did not appreciably affect the value of $V_{max}$ while causing a 5-fold increase in the $K_m$ value, thus having a marked effect upon the substrate efficiency of BrTu. A comparison of BrTu with tubercidin and deazanebularin exhibited a slight increase in the maximal velocity but showed a 2- to 2.5-fold decrease in the $K_m$ values, thus making BrTu inferior to these compounds in terms of substrate efficiency. (4) Bromotubercidin 5′-triphosphate (BrTuTP) was shown to be an effective energy source for the phosphorylation of adenosine and nucleoside analogues or of BrTu itself (data not shown). In addition, BrTuTP was a substrate for *E. coli* RNA polymerase with the synthetic polydeoxyribonucleotides as templates (data not shown). Efficiency of BrTuTP to polymerize in these reactions is comparable to that of rATP, indicating that the bulky C–Br group does not interfere with the formation of BrTu containing polynucleotides. These observations are of some interest for two reasons. First, they show that BrTu is a substrate for adenosine kinase, and a second result of significance is that BrTu inhibits the phosphorylation of other adenosine analogues, such as deazanebularin and tubercidin. Since these analogues are rapidly and irreversibly cytotoxic, whereas BrTu is not, it was possible to test whether the observations at the level of cell-free homog-

**Table 4.** BrTu blocks cellular uptake of tubercidin

| Incubation with [$^3$H] tubercidin (h) | Radioactivity (% inhibition) | | |
|---|---|---|---|
| | Acid-soluble pool | RNA | DNA |
| 1 | 94.3 | 94.4 | 89.3 |
| 2 | 96.9 | 95.6 | 94.2 |
| 3 | 97.6 | 95.4 | 94.8 |

Two parallel sets of cultures were established ($6 \times 10^5$ cells per 60 mm Petri dish), one-set receiving BrTu (30 μM). After 30 min both sets were supplemented with [$^3$H]tubercidin (3.7 μM; spec. act. 155 mCi/mmol). Dishes were withdrawn from each set of cultures at the indicated times, washed and analysed for radioactivity in the acid-soluble pool, RNA and DNA (Section 3).

enates could be reproduced with the intact cells. Thus, the incorporation of [$^3$H]Tu (Table 4) or [$^3$H]DN (data not shown) into acid-soluble and acid-insoluble material of L-cells was reduced to almost undetectable levels in the presence of BrTu. Electrophoretic analysis of acid-soluble pool revealed that Tu was not phosphorylated in the presence of BrTu (data not shown), whereas in control cultures most of the radioactivity was found in the triphosphate region.[1,2] Interestingly, the BrTu mediated block in cellular uptake and incorporation of toxic Tu and/or DN was associated with the protective effect of BrTu against cell killing by the toxic analogues. Thus, the growth of BrTu-treated cell cultures, even those simultaneously exposed to DN, was following drug removal the same as that of the untreated control, whereas cell multiplication did not resume in the culture that had been transiently incubated with DN alone (Table 5). Identical results were obtained with Tu in comparable experiments.[10] In short, the presence of BrTu simply nullified the cytotoxic action of other adenosine analogues. This suggested that the extent of cellular uptake and incorporation of a given toxic analogue could be modulated by BrTu. The possibility of controlling the rate of analogue metabolism in this way encourages attempts to differentiate between viral and host replicating systems[10] and possibly between different cell and tissue types for the rational design of pharmacologically active nucleoside analogues.

The rationale for achieving differential effects of analogues could be as follows. Adenosine analogues (BrTu, Tu, DN) can enter the nucleoside pool either via the nucleoside transport system[14,15] driven by rapid intracellular phosphorylation by cytosolic nucleoside and nucleotide kinases,[16] or by the pathways independent of adenosine kinase involving the intermediate formation of analogue base.[17] On the other hand, multiple pathways are available for the corresponding adenine nucleotides including the de novo purine nucleotide biosynthetic pathway, which is not affected by BrTu. Since the nucleoside uptake is a result of both membrane transport and subsequent intracellular metabolism, it is likely that BrTu inhibits nucleoside analogue (e.g., Tu or DN) uptake at the level of the membrane nucleoside transporter coupled to inhibition of adenosine kinase; the degree of inhibition of analogue uptake by BrTu will therefore be determined in part by the analogue's ability to cross the membrane as a substrate for the nucleoside transporter. Several lines of evidence indicate the existence of two major transporter families that mediate nucleoside permeation of mammalian cells: the concentrative nucleoside transporters are $Na^+$-dependent, and the equilibrative nucleoside transporters are $Na^+$-independent.[15,18] It was recently reported[19] that 5-iodotubercidin, a potent adenosine kinase inhibitor just like BrTu, showed a significant inhibition of adenosine uptake and adenine nucleotide formation at concentrations above 100 nM in rat glioma cells expressing an equilibrative transporter, while concentrations above 3 μM were required for cells expressing concentrative transporter. This study demonstrates that the effects of 5-iodotubercidin in whole cell assays are dependent upon nucleoside transporter expression independently of its inhibition of adenosine kinase. Thus, cellular and tissue differences in expression of nucleoside transporter subtypes may affect the pharmacological action of adenosine kinase inhibitors including BrTu. It may therefore be possible to promote selectively, in the presence of BrTu, the incorporation of the toxic analogue (e.g., Tu or DN) in particular target tissues, while protecting specific tissues.

### 2.3. Effects of 5-bromotubercidin on growth of some animal viruses

The growth of viruses in cultured cells provides an excellent system for detecting the differential susceptibility to inhibitors of the various nucleic acid replication reactions. We have studied the effects of BrTu on the life cycles of several RNA viruses (mengovirus, poliovirus, reovirus, vesicular stomatitis virus, Newcastle disease virus, sindbis virus, influenza virus, Rous sarcoma virus), and three DNA viruses (vaccinia virus, SV40, herpes simplex virus). The results of these experiments were described in detail elsewhere.[10,12,20,21] The observa-

**Table 5.** BrTu protects against the cytotoxic action of DN

| Incubation with nucleoside analogue (h) | Relative cell count | | | | | |
|---|---|---|---|---|---|---|
| | Control | BrTu (60 μM) alone | BrTu + DN (8 μM) | BrTu + DN (20 μM) | BrTu + DN (40 μM) | DN (40 μM) alone |
| 18 | 1.96 | 1.72 | 1.66 | 1.93 | 1.72 | 0.75 |
| 28 | 3.53 | 3.50 | 3.32 | 3.30 | 3.32 | 0.48 |
| 45 | 8.20 | 6.80 | 7.07 | 8.00 | 7.35 | 0 |

L-cell monolayers ($8 \times 10^4$ per 60 mm Petri dish) were incubated for 5 h with the indicated concentrations of each nucleoside, where BrTu was present 60 μM. After 5 h, the cultures were washed and incubation was continued in drug-free medium. Cells were counted at the indicated times in defined areas of each plate. The presence of BrTu protected the cells against the toxicity resulting from the transient exposure to DN.

**Table 6.** Effects of 5-bromotubercidin on virus multiplication

| Virus | 5-Bromotubercidin (μM) | Incubation (h) | No. of PFU/cell |
|---|---|---|---|
| Mengovirus | None | 0 | 2 |
| | None | 24 | 560 |
| | 15 | 24 | 868 |
| | 30 | 24 | 875 |
| | 75 | 24 | 806 |
| | 150 | 24 | 953 |
| | 300 | 24 | 706 |
| Vaccinia virus | None | 0 | 0.10 |
| | None | 24 | 79 |
| | 15 | 24 | 18 |
| | 30 | 24 | 11.5 |
| | 75 | 24 | 7.4 |
| | 150 | 24 | 2.0 |
| | 300 | 24 | 0.55 |

Monolayers of L2-cells ($2 \times 10^5$ cells per 60 mm Petri dish) were incubated for 20 min with indicated concentrations of BrTu after which the cells, in separate plates, were infected with mengovirus and vaccinia virus at a multiplicity of 10 plaque-forming units (PFU)/cell, each. At the end of the period of adsorption (1 h at 37 °C) the plates were washed three times with culture medium and one plate for each virus type was frozen as zero-time control. Other dishes received 3 ml of complete medium with BrTu as indicated. After 24 h of incubation all cultures were frozen and thawed three times and virus titrations were performed as in Ref. 10.

tions which are relevant for the present discussion are given in Table 6 and Figure 11. It can be seen that both the growth and the synthesis of mengoviral RNA were

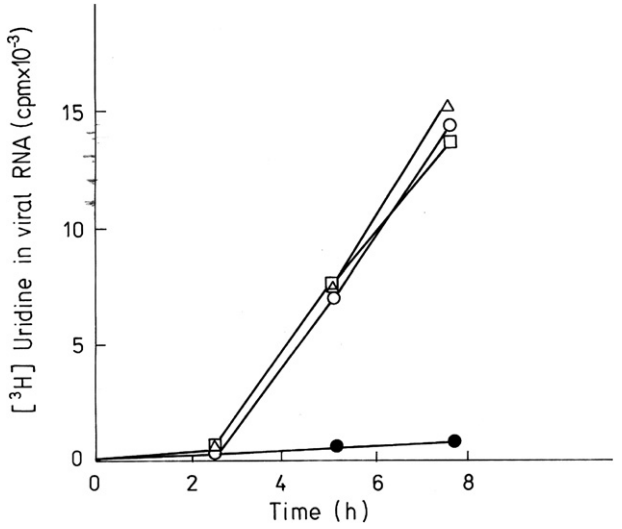


**Figure 11.** 5-Bromotubercidin does not block mengovirus RNA synthesis. L-cells were plated at $3 \times 10^5$ per 60 mm Petri dish and incubated overnight. Actinomycin D (2 μg/ml) was added to all cultures and maintained thereafter throughout the experiment. Twenty minutes later mengovirus (10 PFU/cell) was added in a small volume (0.3 ml) and adsorbed to the monolayer during 1 h at 37 °C; one set of cultures was mock infected. Two sets of cultures then received medium containing BrTu as indicated, the other set serving as control, and incubation was continued for 20 min, at which time [$^3$H]uridine (2 μCi/ml) was added to all plates. At the indicated times thereafter the radioactivity incorporated into material insoluble in 5% trichloroacetic acid was measured as described in Section 3. Control (○); BrTu: 15 μM (△); 60 μM (□); mock (●).

completely resistant to concentrations of BrTu (300 μM) much higher than those (15 μM) which strongly inhibited the growth of the host cell (Fig. 2), of vaccinia virus (Table 6), and of the DNA-dependent RNA synthesis of uninfected cells (Fig. 4a). These findings are of interest for several reasons: (1) It is apparent that BrTu inhibits cellular DNA-directed RNA synthesis, and, by implication, also that of the DNA virus vaccinia; in contrast the growth and RNA synthesis of mengovirus are unaffected. This distinction by BrTu between DNA-dependent and RNA-dependent RNA synthesis is not the result of differences in cytological localization of the two processes, since the entire life cycle of vaccinia virus like that of mengovirus runs its course in the cytoplasm; BrTu also inhibits the growth of SV40[21] or herpes simplex virus both of which replicate in the nucleus of the respective host cells. The differential susceptibility of the two replication reactions more likely reflects differences in the interaction of BrTuTP with the respective polymerizing systems. Indeed, experiments using [$^3$H]BrTu showed that BrTu is not incorporated into mengovirus RNA or virus particles (data not shown), which could be the reason for the inertness of BrTu in both the growth and RNA synthesis of mengovirus. Since BrTuTP is an in vitro substrate for both mengovirus RNA polymerase[22] and *E. coli* RNA polymerase (data not shown), and thus substitutes completely for ATP in pairing with uracil, it is possible that substrate selection by mengovirus polymerase is not determined solely by the constraints of suitable base-pairing. Additional determinants are therefore involved in substrate selection: (i) Given the fact that the fraction of radioactive BrTu in the culture medium of both uninfected and mengovirus-infected cells, which appeared in cellular nucleotide pool (Table 1) and cellular RNA (Fig. 9), was very small but undetectable in mengovirus RNA (data not shown), it is possible that low levels of BrTu nucleotide pool coupled to the previously reported[22–24] low substrate affinity ($K_m$) of mengovirus RNA polymerase for nucleoside analogue triphosphates might account for the lack of BrTu incorporation into viral RNA. Namely, replacement of the natural rATP by BrTuTP (as shown in Ref. 22) uniquely alters the kinetics of the mengovirus polymerase reaction by sharply reducing initial rates of synthesis in non-linear fashion. (ii) Since mengovirus plus-stranded RNA (which serves as messenger RNA) replicates through a minus-stranded RNA intermediate, these observations suggest that mengovirus replicase has a unique ability to respond to its plus and minus strands. Thus, the initial product of plus strand-primed replicase reaction in the presence of substrate analogue (BrTuTP) would be minus strands containing analogue base which is not properly recognized by the mengovirus replicase as a biologically significant template. (iii) Additional reason for this differential incorporation of BrTu might be that viral and cellular polymerases draw on different intracellular nucleotide pools.[25]

(2) Another noteworthy conclusion can be drawn from the complete resistance to BrTu of mengovirus growth. Since the normal growth of the virus requires the maintenance of the integrity of cellular systems for protein

synthesis and energy metabolism, it follows that BrTu does not interfere with these complex processes. Taken together, our results suggest that BrTu produces a single pattern of effects on picornavirus RNA and all forms of DNA transcription, whether of viral or cellular origin. This distinction by BrTu between cellular DNA-dependent and picornavirus RNA-dependent RNA synthesis could be used for designing specifically antiviral nucleosides, which we reported elsewhere.[10] Briefly, being a potent adenosine kinase inhibitor BrTu can be used to prevent cellular uptake of other, more cytotoxic adenosine analogues, such as Tu (Table 4) and DN. This block in cellular uptake of Tu is associated with the protective effect of BrTu against cell killing by the toxic analogue. However, in mengovirus-infected cells BrTu does not prevent Tu from incorporating into mengovirus RNA, giving rise to functionally defective polynucleotides that abort the virus growth cycle under conditions that do no affect the viability of normal uninfected cells.

In conclusion, three aspects of BrTu action are important: (1) BrTu reversibly inhibited cellular RNA synthesis, but it did not inhibit either the picornavirus RNA synthesis or multiplication. This distinction by BrTu between cellular DNA-directed and picornavirus RNA-directed RNA synthesis should prove valuable for studying the virus intracellular life cycle in the background of cellular RNA synthesis inhibition. (2) BrTu, an adenosine kinase inhibitor, limited the amount of highly cytotoxic Tu or DN that entered the cellular nucleotide pools; the resulting intracellular concentration of toxic adenosine analogues was too low to inhibit cell growth. (3) Since BrTu inhibited the synthesis of high-molecular-weight cellular RNA, it protected the cell against damage secondary to Tu or DN incorporation. This facet of 2-fold protection by BrTu probably accounts for the excellent recovery of cells from the combined treatment with BrTu and either of the two cytotoxic analogues; the presence of BrTu simply nullified the cytotoxic action of the other nucleoside analogues. Given the toxicity that accompanied the incorporation of even small amounts of Tu into cellular or viral nucleic acids, it was possible to achieve antiviral selectivity[10] by promoting Tu incorporation into picornavirus infected cells while preventing uptake into uninfected cells. Furthermore, it could also be possible to exploit nucleoside transport and incorporation differences between different cell and tissue types for the rational application of these pharmacologically active nucleoside analogues.

## 3. Experimental

### 3.1. Chemicals

All pyrrolo-pyrimidine nucleosides were synthesized in the laboratory of Dr. R.K. Robins, University of Utah, Salt Lake City, and were generously provided by Drs. H. Wood and R. Engle, National Cancer Institute, Bethesda; tubercidin was obtained through the generosity of Dr. G. Hitchings, the Welcome Research Laboratory, Tuckahoe, NY. The normal nucleosides and nucleotides, amino acids and other metabolites, radioactive and non-radioactive, were obtained from regular commercial sources, and were the best grade available.

Radioactive 5-bromotubercidin, tubercidin and deazanebularin were prepared by New England Nuclear Corporation, Boston, Mass, through catalytic tritium exchange in dimethylformamide. The crude radioactive product was purified to a single chromatographically homogeneous material of constant specific radioactivity by paper and column chromatography as previously described.[2,6]

Mono-, di- and triphosphates were chemically synthesized as described by Ward et al.[6]

### 3.2. Synthesis of poly r(BrTu-U) and poly r(BrTu)

The conditions for synthesizing BrTu riboheteropolymers and ribohomopolymers were previously described.[6] Briefly, one millilitre reaction mixture contained 40 mM Tris–HCl, pH 7.9, 1 mM $MnCl_2$, 4 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 0.04 mM poly d(A-T) or 0.09 mM poly (dT), 0.4 mM [$^3$H]rATP or [$^3$H]BrTuTP at specific activities of $10^5$–$10^6$ cpm/μmol, 0.4 mM UTP and 5–15 U of *E. coli* RNA polymerase. The reaction mixtures were incubated at 37 °C and the extent of synthesis determined by measuring the trichloroacetic acid-precipitable radioactivity in 50 μl aliquots on millipore filters. For the large scale synthesis of poly r(BrTu-U) or poly r(BrTu) the reaction mixtures (5–10 ml) were incubated at 37 °C until the rate of polymerization started to plateau. DNase I (10 μg/ml) was then added and incubation continued for a further 30 min. The reaction mixture was deproteinized with phenol. The aqueous layer was extensively dialysed against 500–100 mM KCl, 10 mM Tris–HCl, pH 7.9, and was used for subsequent experiments.

### 3.3. Enzymes

*Escherichia coli* RNA polymerase was prepared and assayed according to Chamberlain and Berg.[26] *E. coli* alkaline phosphatase, pancreatic DNase, RNase, snake venom phosphodiesterase and hexokinase were obtained from Worthington Corp., Freehold, NJ, USA; myokinase, phosphoenol pyruvate kinase were purchased from Calbiochem, La Jolla, CA, USA. Rabbit liver adenosine kinase was prepared and assayed as described by Lindberg et al.[27] L-cells particulate and soluble fractions were prepared in the following way: washed cells were homogenized in a Dounce homogenizer in 10 mM Tris, pH 7.2, containing 10 mM NaCl, 1.5 mM $MgCl_2$ and 1 mM EDTA. The homogenate was centrifuged at 1000*g* for 10 min, the supernatant collected, and an aliquot saved as particulate enzyme preparation. The remaining portion of the supernatant was then centrifuged at 100,000*g* for 1 h and the supernatant saved. The conditions used for assaying adenosine kinase in both preparations with various nucleosides as substrates were as previously described[27]: the assay mixture (1 ml) contained 20 mM $KPO_4$, pH 5.8, 0.7 mM ATP, 0.25 mM phosphoenol pyruvate, 0.5 mM $MgCl_2$,

50 mM KCl, 20 μg of pyruvate kinase, 7–70 μM of radioactive nucleoside substrate and 25–100 μg of adenosine kinase preparation. The incubation was at 25 °C for various time intervals, the reaction was stopped by adding 0.25 M perchloric acid, cooled in ice and electrophoresed on 3 MM Watman paper at 1500 V for 60–90 min in 0.05 M citrate buffer, pH 3.5. The spots containing nucleotides were cut out and the radioactivity was measured in a liquid scintillation spectrometer.

### 3.4. Cells and viruses

Mouse fibroblasts (strain L2) were maintained and propagated as monolayer cultures in minimal essential medium (MEM) supplemented with 5% foetal bovine serum (Gibco/BRL Life Technologies, Gaithersburg, MD). Vaccinia virus and mengovirus were grown and assayed as previously reported.[2,10]

### 3.5. Macromolecule synthesis

The incorporation of radioactive precursors into cellular macromolecules was followed by the procedures described previously.[2,12] In brief, cells from monolayer cultures exposed to the radioactive precursors for varying periods were harvested and washed with ice-cold saline. They were then extracted with 0.25 M perchloric acid for 45 min with occasional shaking. After centrifugation the acid-soluble material was adsorbed on Norit for 1 h at 4 °C, the Norit-adsorbable nucleotides were then eluted in 50% ethanol containing 2% ammonia, and the aliquots were measured for $OD_{260nm}$ as well as radioactivity in scintillation fluid. The acid-insoluble fraction was dissolved and incubated in KOH (0.5 M, 37 °C, 18 h); the precipitate which formed on acidification of the alkaline solution contained the cellular DNA, whereas the material which remained soluble represented the degraded cellular RNA. The DNA fraction, which was degraded by perchloric acid (0.5 M, 90 °C, 30 min), and the soluble RNA fraction were measured for radioactivity in scintillation fluid.

The procedure used for monitoring viral RNA of mengovirus-infected L-cells was previously reported.[2,10] In brief, monolayers of L-cells were incubated with actinomycin D (2 μg/ml) for 20–30 min. After this, the medium was removed and the cells were infected with mengovirus at a multiplicity of infection of 10–100. Following the period of virus adsorption (60 min, 37 °C) the cells were incubated in fresh medium containing actinomycin D and radioactive RNA precursor. At the end of periods of incubation the cells were washed, and the radioactivity in RNA alkali labile fraction or trichloroacetic acid-precipitable material was determined in a scintillation counter.

### 3.6. Cell fractionation and RNA extraction

Nucleolar, nucleoplasmic and cytoplasmic fractions were isolated according to a modified procedure reported earlier.[12] In brief, washed cells were resuspended in homogenizing media (HM) (10 mM Tris, pH 7.2, 150 mM NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100), stirred gently with a glass rod and spun down by a low speed centrifugation (1000*g*, 3 min). Resulting pellet was resuspended in HM containing 1% Triton X-100 and 0.5% deoxycholate, vortexed very shortly to get homogeneous suspension and spun down as above. This step was repeated once more with the pellet, which was finally examined under phase microscope for the purity of nuclei. All supernatants were combined and saved as the cytoplasmic fraction. The clean nuclei were lysed in high salt buffer containing 10 mM Tris, pH 7.4, 500 mM NaCl, 10 mM $MgCl_2$ (2 ml for $30 \times 10^6$ cells), vortexed shortly and treated with DNase I (50 μg/ml), which caused the clearing of the mixture. Following centrifugation at 15,000*g* for 5 min the pellet was saved as nucleolar fraction, and the supernatant represented nucleoplasmic fraction.

RNA was extracted from nucleolar, nucleoplasmic and cytoplasmic fraction with phenol–sodium dodecyl sulfate as follows: nucleoplasmic fraction was precipitated with 2.2 vol of ethanol in cold and spun down at 10,000*g* for 10 min. The pellet was mixed with 2 ml of TNE–SDS buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% SDS). The clean nucleoli were resuspended in 2 ml of the same buffer, and the cytoplasmic fraction was made 0.5% in SDS. From that point on, all three fractions were treated equally to extract RNA with phenol–SDS and were analysed by electrophoresis on 2.5% acrylamide gels as previously described.[12]

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