Effect of (E)-5-(2-Bromovinyl)uracil on the Catabolism and Antitumor Activity of 5-Fluorouracil in Rats and Leukemic Mice

Claude Desgranges, Gabriel Razaka, Erik De Clercq, Piet Herdewijn, Jan Balzarini, F. Drouillet, and Henri Bricaud

ABSTRACT

In contrast to thymine and 5-fluorouracil (FUra) which were cleared from the bloodstream within 2–4 h after their i.p. administration (200 μmol/kg) to rats, (E)-5-(2-bromovinyl)uracil (BVUra) maintained a concentration of 50–70 μM for at least 6 h and was still present in the plasma 24 h after its administration. In vitro experiments with rat liver extracts indicated that BVUra was not a substrate but an inhibitor for the reductive step in pyrimidine degradation catalyzed by dihydrothymine dehydrogenase. Kinetic and dialysis experiments suggested that BVUra was an irreversible inhibitor of this enzyme. The binding of BVUra to the enzyme depended on the presence of reduced nicotinamide adenine dinucleotide phosphate in the reaction mixture. Dihydrothymine dehydrogenase activity was also inhibited in the dialysed 105,000 × g supernatant fraction of livers from rats that had previously been treated with BVUra. Such inhibitory effects also occurred in vivo; previous administration of BVUra increased the plasma half-lives of thymine and FUra by 10- and 5-fold and their area under the curve by 9- and 8-fold, respectively.

The effect of BVUra on the antitumor activity of FUra was evaluated in DBA/2 mice inoculated with 10⁶ P388 leukemia cells. The mean survival times for the control and FUra-treated mice (5 mg/kg at 1, 3, 5, and 7 days after tumor cell inoculation) were 9.7 and 12.4 days, respectively. When BVUra (200 μmol/kg) was administered 1 h before each injection of FUra, the mean survival time was extended to 17.1 days. BVUra alone did not affect the mean survival time. When the dose of FUra was increased to 20 mg/kg, the mean survival time was 15.3 days; upon a preceding injection of BVUra the mean survival time decreased to 9.2 days. The latter effect probably resulted from an increased toxicity of FUra. Similar results were obtained if FUra was replaced by 5-fluoro-2'-deoxyuridine and BVUra by (E)-5-(2-bromovinyl)-2'-deoxyuridine. The enhancement of both the antitumor and toxic effects of FUra by BVUra were most probably due to an inhibition of FUra degradation, since, like in rats, BVUra increased the plasma half-life of FUra in DBA/2 mice. Hence BVUra appears to be an interesting compound, increasing the potency of FUra by decreasing its degradation.

INTRODUCTION

Thy and Ura are both subject to the same catabolic pathway which is, in contrast to that of the purine bases, a reductive pathway (see Ref. 1 for review). The first step of the catabolism of Thy and Ura is the reduction of the 5:6 double bond of the pyrimidine ring, followed by the cleavage of the 3:4 bond to give β-ureidosobutyric acid and β-ureidopropionic acid from which β-amino acids are formed with the release of ammonia and carbon dioxide (2–5). The first enzyme of this catabolic pathway, H₂Thy dehydrogenase (EC 1.3.1.2), was identified as the rate-limiting enzyme (5–8). The liver appears to be the major site of pyrimidine catabolism (7–9); the activity of H₂Thy dehydrogenase in the liver is 20-fold higher than in lung, bone marrow, and colon (10).

5-Substituted Ura analogues and in particular 5-halogenated Ura are also substrates for this enzyme (11, 12), and it is obvious that the degradation of FUra via H₂Thy dehydrogenase may affect the therapeutic efficacy of FUra in the treatment of cancer. The use of H₂Thy dehydrogenase inhibitors may be helpful not only for the elucidation of the mechanism of action of FUra and its analogues but also with respect to an enhancement of their therapeutic activity. Some inhibitors have been described previously; among the Ura analogues (9, 13–15), 5-cyanouracil (11, 16, 17) and DUra (10, 15, 16, 18–20) are the most potent inhibitors of pyrimidine degradation in vitro or in vivo. Most of these compounds act in a competitive manner, since they are also substrates of H₂Thy dehydrogenase (11–14). Some of them act in vivo, both by decreasing the degradation of Thy and FUra and by increasing their incorporation into DNA and RNA (18, 19).

As a consequence, they not only enhance the antitumoral activity of FUra but also its toxicity (17, 18). While we were studying the catabolism of the potent and selective antiviral agent, BVdUrd (21) in the rat, we found that its degradation product BVUra (Fig. 1), in contrast to other pyrimidine bases, had a relatively long half-life in plasma (22). We have now pursued these observations, and here we demonstrate that BVUra, being itself not active as a substrate, reduces the degradation of other pyrimidine bases, such as FUra, by inhibition of the H₂Thy dehydrogenase activity in vivo and in vitro. Moreover, BVUra enhances the antitumor activity of FUra in the P388 leukemia model in DBA/2 mice.
MATERIALS AND METHODS

Chemicals. Thy, FURA, DURa, other Ura analogues, their corresponding deoxynucleosides and NADPH were obtained from Sigma Chemical Co., St. Louis, MO. EThy was kindly provided by Robugen GmbH (Esslingen, West Germany). BVdUrd was synthesized according to Jones et al. (23). BVUra was prepared as previously described (24). 6-AThr was obtained by alkaline cyclization of α-methylcyanoacetyleurea according to the procedure of Bergmann and Johnson (25). [methyl-\textsuperscript{3}H]Thy (60 mCi/mmol) and 5-fluoro[\textsuperscript{14}C]Ura (55 mCi/mmol) were purchased from Amersham (Buckinghamshire, United Kingdom).

In Vitro Pyrimidine Degradation. A 105,000 x g rat liver extract was prepared according to the procedure of Shiotani and Weber (12). This extract was dialyzed overnight against 0.25 M sucrose in 35 mM potassium phosphate buffer (pH 7.4), 5 mM 2-mercaptoethanol, and 2.5 mM MgCl\textsubscript{2}. Thy and FURA degradation was assayed by a radiochemical method involving production of radioactive catabolites from \textsuperscript{[14}C]Thy and \textsuperscript{[14}C]FURA. The assay mixture contained 20 μM of radioactive Thy or FURA, 250 μM NADPH, 35 mM potassium phosphate, and liver extract (0.5 mg protein/ml of reaction mixture). After incubation at 37°C, the reaction was stopped by 30% perchloric acid; precipitated proteins were eliminated by centrifugation. After neutralization, 10 μl of the supernatant were spotted onto Merck cellulose (for Thy) or silica gel (for FURA)-coated plates. Ascending chromatography was carried out with eluents of tert-butyl alcohol:methylethylketone:water:ammonium hydroxide (40:30:30:10) (26) for cellulose plates or a mixture of chloroform:methanol:acetic acid (30:10:5) (27) for silica gel plates. The plates were scanned with a thin layer radiochromatogram scanner to locate the radioactivity. The plates were then developed and radioactivity was quantitated by HPLC have been described previously (22).

The schedule or administration is described in the legends to the charts.

In Vivo Pyrimidine Degradation. The inhibition of pyrimidine degradation in vivo of BVUra was found to be completely and specifically dependent on the presence of NADPH (Table 1A). Indeed, when liver extract was preincubated with NADPH, the degradation of both Thy and FURA was significantly reduced; the rate of degradation of both Thy and FURA decreased as a function of the NADPH concentration in the incubation medium (Fig. 2). A 50% inhibition of the degradation of 20 μM Thy or FURA was obtained at a BVUra concentration of 2–3 μM. An identical 50% inhibitory dose was obtained for BVUra or BVdUrd if partially purified H\textsubscript{2}Thy dehydrogenase was used instead of crude liver extract.

Pyrimidine Catabolism by Liver Extracts. The in vitro catabolism of BVUra was compared with that of Thy and FURA. For these experiments, liver extracts were used, since the liver appears to be the major site for pyrimidine catabolism in vivo (7–9). When Thy was incubated with crude liver extract in the presence of NADPH (under the conditions described in "Materials and Methods"), it was rapidly transformed to β-ureidobutyric acid and β-aminoobutyric acid with an initial velocity of 35 nmol/h/mg protein. N\textsubscript{2}Thy was detected, which suggests that the reductive conversion of Thy to H\textsubscript{2}Thy is the limiting step in the catabolism of Thy. When assayed under the same conditions, FURA was degraded twice as fast as Thy (70 nmol/h/mg protein).

Pyrimidine Catabolism by Liver Extracts. The in vitro catabolism of BVUra was compared with that of Thy and FURA. For these experiments, liver extracts were used, since the liver appears to be the major site for pyrimidine catabolism in vivo (7–9). When Thy was incubated with crude liver extract in the presence of NADPH (under the conditions described in "Materials and Methods"), it was rapidly transformed to β-ureidobutyric acid and β-aminoobutyric acid with an initial velocity of 35 nmol/h/mg protein. N\textsubscript{2}Thy was detected, which suggests that the reductive conversion of Thy to H\textsubscript{2}Thy is the limiting step in the catabolism of Thy. When assayed under the same conditions, FURA was degraded twice as fast as Thy (70 nmol/h/mg protein). α-fluoro-β-ureidopropionic acid and α-fluoro-β-alanine were detected in the incubation medium while 5-fluorodihydrouracil was not. When Thy or FURA was incubated with H\textsubscript{2}Thy dehydrogenase separated from dihydropyrimidine cyclohydrase and N-carbamoyl-β-alanine aminohydrolase by DEAE-cellulose chromatography, only the formation of H\textsubscript{2}Thy or 5-fluorodihydrouracil could be demonstrated. In contrast with Thy or FURA, BVUra was not degraded when incubated with either crude liver extract or partially purified H\textsubscript{2}Thy dehydrogenase.

In Vivo Inhibition of Pyrimidine Degradation by BVUra. BVUra not only was not a substrate of H\textsubscript{2}Thy dehydrogenase but acted as an inhibitor of this enzyme. Indeed, when liver extract was preincubated at 37°C with BVUra, the degradation of both Thy and FURA was significantly reduced; the rate of degradation of both Thy and FURA decreased as a function of the BVUra concentration in the incubation medium (Fig. 2). A 50% inhibition of the degradation of 20 μM Thy or FURA was obtained at a BVUra concentration of 2–3 μM. An identical 50% inhibitory dose was obtained for BVUra if partially purified H\textsubscript{2}Thy-dehydrogenase was used instead of crude liver extract.

In Vivo Inhibition of Pyrimidine Degradation by BVUra. BVUra not only was not a substrate of H\textsubscript{2}Thy dehydrogenase but acted as an inhibitor of this enzyme. Indeed, when liver extract was preincubated at 37°C with BVUra, the degradation of both Thy and FURA was significantly reduced; the rate of degradation of both Thy and FURA decreased as a function of the BVUra concentration in the incubation medium (Fig. 2). A 50% inhibition of the degradation of 20 μM Thy or FURA was obtained at a BVUra concentration of 2–3 μM. An identical 50% inhibitory dose was obtained for BVUra if partially purified H\textsubscript{2}Thy-dehydrogenase was used instead of crude liver extract.

In Vivo Inhibition of Pyrimidine Degradation by BVUra. BVUra not only was not a substrate of H\textsubscript{2}Thy dehydrogenase but acted as an inhibitor of this enzyme. Indeed, when liver extract was preincubated at 37°C with BVUra, the degradation of both Thy and FURA was significantly reduced; the rate of degradation of both Thy and FURA decreased as a function of the BVUra concentration in the incubation medium (Fig. 2). A 50% inhibition of the degradation of 20 μM Thy or FURA was obtained at a BVUra concentration of 2–3 μM. An identical 50% inhibitory dose was obtained for BVUra if partially purified H\textsubscript{2}Thy-dehydrogenase was used instead of crude liver extract.

In Vivo Inhibition of Pyrimidine Degradation by BVUra. BVUra not only was not a substrate of H\textsubscript{2}Thy dehydrogenase but acted as an inhibitor of this enzyme. Indeed, when liver extract was preincubated at 37°C with BVUra, the degradation of both Thy and FURA was significantly reduced; the rate of degradation of both Thy and FURA decreased as a function of the BVUra concentration in the incubation medium (Fig. 2). A 50% inhibition of the degradation of 20 μM Thy or FURA was obtained at a BVUra concentration of 2–3 μM. An identical 50% inhibitory dose was obtained for BVUra if partially purified H\textsubscript{2}Thy-dehydrogenase was used instead of crude liver extract.

In Vivo Inhibition of Pyrimidine Degradation by BVUra. BVUra not only was not a substrate of H\textsubscript{2}Thy dehydrogenase but acted as an inhibitor of this enzyme. Indeed, when liver extract was preincubated at 37°C with BVUra, the degradation of both Thy and FURA was significantly reduced; the rate of degradation of both Thy and FURA decreased as a function of the BVUra concentration in the incubation medium (Fig. 2). A 50% inhibition of the degradation of 20 μM Thy or FURA was obtained at a BVUra concentration of 2–3 μM. An identical 50% inhibitory dose was obtained for BVUra if partially purified H\textsubscript{2}Thy-dehydrogenase was used instead of crude liver extract.
INHIBITION OF PYRIMIDINE DEGRADATION BY BROMOVINYLURACIL

was observed. If NADPH was present in the preincubation of NADPH and then extensively dialyzed, no inhibitory activity was preincubated at 37°C with BVUra in the absence dialyzing the enzyme extract after preincubation with 20 μM that obtained with preincubation at 37°C. In B, the enzyme extract was preincubated for 10 min at 37°C with BVUra at different concentrations and then assayed for Thy and FUra degradation assay. Activity of the control sample was 47 nmol/h/mg protein.

Table 1
Effect of preincubation of the enzyme preparation with BVUra on the inhibition of FUra degradation

In A, the enzyme extract was preincubated for 10 min at 37°C with or without BVUra and/or NADPH. At the end of this period FUra (20 μM), and in some cases NADPH and BVUra, were added to the reaction mixture. The enzyme activity was determined as previously described and the percentage of inhibition was determined in comparison to the control sample. In B, the enzyme extract was preincubated for 10 min at 37°C with or without BVUra and/or NADPH; the mixture was then dialyzed extensively against 35 mM phosphate buffer (pH 7.4) and used as enzyme source in the FUra degradation assay. Activity of the control sample was 47 nmol/h/mg protein.

<table>
<thead>
<tr>
<th>Preincubation mixture</th>
<th>BVUra (4 μM)</th>
<th>NADPH (250 μM)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>17 ± 6</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>16 ± 6</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>76 ± 6</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Preincubation mixture</th>
<th>BVUra (20 μM)</th>
<th>NADPH (250 μM)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>86 ± 7</td>
<td></td>
</tr>
</tbody>
</table>

Inhibition obtained with preincubation at 4°C was only 31% of that obtained with preincubation at 37°C.

These experiments indicated that BVUra might be irreversibly linked to H₂Thy-dehydrogenase. This hypothesis was verified by dialyzing the enzyme extract after preincubation with 20 μM BVUra with or without NADPH or FUra, and assaying the remaining activity with FUra as substrate (Table 1B). When the enzyme was preincubated at 37°C with BVUra in the absence of NADPH and then extensively dialyzed, no inhibitory activity was observed. If NADPH was present in the preincubation medium before dialysis, the degradation of FUra was inhibited by 86%. If the concentration of FUra in the preincubation mixture was increased to 100 μM, it interfered with the binding of BVUra to the enzyme, since, under these conditions, the inhibition was only 7%. Thus, inhibition of H₂Thy dehydrogenase occurred only when BVUra was preincubated with the enzyme extract in the presence of NADPH and was not reversed by extensive dialysis. These results are consistent with an irreversible inhibition of H₂Thy dehydrogenase by BVUra, whereby BVUra competes with FUra for a common binding site. Kinetics analyses are in progress to confirm this hypothesis. BVdUrd did not show any inhibitory activity under conditions where it could not be degraded to BVUra, i.e., by pyrimidine nucleoside phosphorylases that may occasionally be present in H₂Thy dehydrogenase preparations.

In Vivo Clearance of Thy, FUra, and BVUra. When Thy or FUra were administered i.p. to rats at 200 μmol/kg, Thy or FUra appeared in the plasma within a few minutes but were rapidly cleared from the bloodstream according to a first order process (Fig. 3) with a half-time of 22 and 14 min, respectively (Table 2). Similarly, when the pyrimidine deoxynucleosides dThd orFdUrd were administered i.p., they also appeared in the blood within a few minutes and were then rapidly eliminated from the circulation.

Table 2
Pharmacokinetics of Thy and FUra in the plasma of rats

Each compound was administered i.p. at 200 μmol/kg according to the schedules described in Figs. 3 and 4. Mean ± SD for t1/2 and AUC were calculated from the individual values obtained for each rat. The increases in t1/2 or AUC are indicated in parentheses.

<table>
<thead>
<tr>
<th>Administration</th>
<th>No. of rats</th>
<th>t1/2 (min)</th>
<th>AUC (μM x h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thy alone</td>
<td>6</td>
<td>22 ± 5 (x1)</td>
<td>171 ± 26 (x1)</td>
</tr>
<tr>
<td>BVUra + Thy</td>
<td>3</td>
<td>232 ± 47 (x10.5)</td>
<td>1569 ± 558 (x9.2)</td>
</tr>
<tr>
<td>dThd alone</td>
<td>4</td>
<td>14 ± 3 (x1)</td>
<td>50 ± 15 (x1)</td>
</tr>
<tr>
<td>BVUra + dThd</td>
<td>5</td>
<td>206 ± 47 (x14.7)</td>
<td>1063 ± 321 (x21.3)</td>
</tr>
<tr>
<td>BVdUrd + dThd</td>
<td>3</td>
<td>199 ± 23 (x14.2)</td>
<td>861 ± 135 (x17.2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasma FUra</th>
<th>t1/2 (min)</th>
<th>AUC (μM x h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUra alone</td>
<td>7</td>
<td>14 ± 5 (x1)</td>
</tr>
<tr>
<td>BVUra + FUra</td>
<td>4</td>
<td>74 ± 12 (x5.3)</td>
</tr>
<tr>
<td>FdUrd alone</td>
<td>3</td>
<td>21 ± 12 (x1)</td>
</tr>
<tr>
<td>BVUra + FdUrd</td>
<td>3</td>
<td>82 ± 8 (x3.9)</td>
</tr>
<tr>
<td>BVdUrd + FdUrd</td>
<td>3</td>
<td>77 ± 7 (x3.7)</td>
</tr>
</tbody>
</table>

CANCER RESEARCH VOL. 46 MARCH 1986

1096
The corresponding bases resulting from the phosphorolysis of the deoxynucleosides appeared in the plasma and were eliminated within 2–3 h (Fig. 4). Ura, 5-iodouracil, and 5-trifluoromethyluracil followed the same clearance pattern as Thy and FUra (data not shown).

On the contrary, after i.p. administration at 200 µmol/kg, BVUra was eliminated from the plasma very slowly, so that it maintained a concentration of 50–70 µM for at least 6 h. BVUra was then cleared from the plasma according to an apparent first order reaction with a mean half-life of about 8 h. Consequently, BVUra was still present in the plasma at a concentration of 5–20 µM 24 h after its administration. The mean AUC for BVUra in the plasma was about 1000 µM×h. Following i.v. administration at 200 µmol/kg, BVUra reached a plasma concentration of 100 µM at 20 min; the first order elimination gave a half-life of 7.5 h and an AUC of 1175 µM×h. When BVdUrd was administered i.p. to rats it was rapidly transformed to BVUra upon the action of pyrimidine nucleoside phosphorylases (22). Consequently, BVUra reached a plasma concentration of 70 µM 2–4 h after BVdUrd administration and was then eliminated with a half-life of 7.5 h; its mean AUC was 1075 µM×h. After i.v. administration of BVdUrd, a maximal concentration of 60 µM BVUra was reached within 2–4 h; the half-life of BVUra was about 7.1 h and its plasma AUC was 782 µM×h.

The peculiar behavior of BVUra most probably resulted from its resistance to the action of H2Thy dehydrogenase. Elimination of BVUra from the circulation by the kidney was low. The amount of BVUra eliminated in the urine during the first 48 h following administration of BVUra accounted for only 1.5–10% of the amount of BVUra administered. The maximum renal excretion of BVUra occurred between 4 and 24 h after its administration and reached peak urinary concentration of 700 µM. After 48 h, at a time when BVUra could not longer be detected in the plasma, low concentrations of BVUra were still present in the urine.

HPLC analyses demonstrated the presence of BVdUrd and (E)-5-(2-bromovinyl)uridine in the urine as identified by their retention time and by the action of pyrimidine nucleoside phosphorylases. Since these two products were not present in the plasma at the time they were detected in the urine, it could be assumed that they were formed by a pentosyl exchange reaction at the kidney level.

In Vivo Inhibition of Pyrimidine Degradation by BVUra.

The inhibitory effect of BVUra on pyrimidine degradation in vivo was first demonstrated by comparing the H2Thy dehydrogenase activity in the dialyzed 105,000 × g fractions of livers removed from rats pretreated with BVUra (200 µmol/kg) to the livers from control rats. The degradation of FUra was inhibited by 97% in liver extracts from rats pretreated with BVUra as compared to liver extracts from untreated rats.

In vivo inhibition of the catabolism of Thy and FUra was further demonstrated by monitoring the effect of BVUra on the plasma concentrations and pharmacokinetic parameters of Thy and FUra. When BVUra was administered at 200 µmol/kg 1 h before the i.p. injection of Thy or FUra at 200 µmol/kg, plasma concentrations, t½, and AUC were increased (Fig. 3; Table 2). Similarly, plasma concentrations, t½, and AUC of Thy and FUra, generated by the phosphorolytic cleavage of dThd and FdUrd, were increased when these nucleosides were administered 3 h after BVUra (Fig. 4; Table 2). When BVdUrd instead of BVUra was administered, the plasma concentrations, t½, and AUC of Thy and FUra, generated from dThd and FdUrd, respectively, again were markedly increased (Fig. 4; Table 2). Other pyrimidine bases, which are substrates of H2Thy dehydrogenase such as Ura, 5-iodouracil, and 5-trifluoromethyluracil also showed a marked increase in plasma, t½, and AUC following administration of BVUra or BVdUrd (data not presented).

Inhibition of pyrimidine degradation was noted at 8, 18, 24, and even 48 h after a single BVUra (200 µmol/kg) administration, although at 48 h BVUra was undetectable in the plasma. A dose of BVUra, 25 µmol/kg, was as efficient as a dose of 200 µmol/kg, and BVUra, even at 3 µmol/kg, reached 80% of its maximum inhibitory effect. At lower doses the inhibitory effect rapidly declined.

In Vivo Enhancement of the Antileukemic Effects of FUra by BVUra. Since BVUra protected FUra from degradation not only in vitro but also in vivo, BVUra might be expected to enhance the antitumor activity of FUra. This turned out to be the case as illustrated by the experiments shown in Fig. 5. When DBA/2 mice were inoculated i.p. with 1 million P388 leukemia cells, they died within 10 days after tumor cell inoculation. When FUra was administered at either 2.5 or 5 mg/kg at days 1, 3, 5, and 7 after...
tumor cell inoculation, it increased the survival of mice to 118 and 128%, respectively, as compared to the control group (Table 3). However, when BVUra (200 μmol/kg) was administered 1 h before each FUra injection, the survival was further increased to 150 and 176%, respectively. With these FUra doses, survivals of BVUra-treated mice were significantly increased in comparison to those of mice receiving only FUra (Table 4). At 20 mg/kg, FUra alone increased the mean survival time of leukemic mice to 158%, and if this dose of FUra was preceded by BVUra at a dose of 200 μmol/kg, the life span decreased to that of the control mice. When compared to that of mice receiving FUra at 20 mg/kg alone, the survival of BVUra-pretreated mice receiving FUra at 20 mg/kg was considerably decreased (Table 4). Clearly, this treatment regimen was toxic for the mice, as particularly demonstrated by a marked loss of weight. BVUra administered alone at 200 μmol/kg had neither antitumor nor apparent toxic effects (Table 3).

FUra at 10 mg/kg had a very slight antileukemic effect which was considerably increased (mean survival time, 159%) when FUra administration was preceded by BVUra; FUra alone at 200 mg/kg was well tolerated and increased the mean survival time of mice to 164% in comparison to the control group but became toxic (as demonstrated by a marked loss of weight) if BVUra had been injected previously; in this case the survival time was lower than for the control group (Fig. 5). The survival time of mice pretreated with BVUra (as compared to that of mice receiving FUra only) was considerably increased at low doses of FUra while it was significantly decreased at high doses of FUra (Table 4).

Almost identical effects were obtained if BVdUrd (200 μmol/kg) instead of BVUra was injected 90 min before the administration of FUra or FdUrd (Fig. 6; Table 4). The time interval of 90 min was chosen, since after 90 min BVdUrd was almost completely cleared from the circulation and replaced by BVUra.

The enhancing effect of BVUra and BVdUrd on the antitumor activity and toxicity of FUra in DBA/2 mice could be attributed to an inhibition of the degradation of FUra and hence an increase in the therapeutically active concentrations of FUra. Indeed, the plasma concentrations of FUra in mice, as in rats, were considerably increased if the mice had received BVUra or BVdUrd before FUra (Table 5).

In Vivo Effects of Some Other Inhibitors. We have also examined the effects of in vivo pyrimidine degradation of some other inhibitors of H2Thy-dehydrogenase, i.e., DUra (16, 18, 19),

---

Table 3
Effects of FUra, FdUrd, and inhibitors of pyrimidine degradation on the survival of mice bearing P388 leukemic cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Survival (days)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.7 ± 0.8 (48)</td>
<td>100</td>
</tr>
<tr>
<td>FUra (2.5 mg/kg)</td>
<td>11.4 ± 0.4 (6)</td>
<td>118*</td>
</tr>
<tr>
<td>FUra (5 mg/kg)</td>
<td>12.4 ± 3.3 (24)</td>
<td>128*</td>
</tr>
<tr>
<td>FUra (20 mg/kg)</td>
<td>15.3 ± 1.5 (24)</td>
<td>158*</td>
</tr>
<tr>
<td>FdUrd (10 mg/kg)</td>
<td>10.4 ± 1.0 (12)</td>
<td>107*</td>
</tr>
<tr>
<td>FdUrd (50 mg/kg)</td>
<td>11.7 ± 1.3 (12)</td>
<td>121*</td>
</tr>
<tr>
<td>FdUrd (200 mg/kg)</td>
<td>15.9 ± 1.4 (12)</td>
<td>164*</td>
</tr>
<tr>
<td>BVUra (200 μmol/kg)</td>
<td>9.2 ± 0.9 (6)</td>
<td>94*</td>
</tr>
<tr>
<td>BVdUrd (200 μmol/kg)</td>
<td>10.2 ± 0.8 (6)</td>
<td>105*</td>
</tr>
<tr>
<td>DUra (200 μmol/kg)</td>
<td>12.5 ± 2.9 (6)</td>
<td>129*</td>
</tr>
<tr>
<td>DUra (50 μmol/kg)</td>
<td>13.1 ± 0.8 (6)</td>
<td>135*</td>
</tr>
<tr>
<td>ETUra (200 μmol/kg)</td>
<td>9.8 ± 0.8 (6)</td>
<td>101*</td>
</tr>
<tr>
<td>6-ATHy (70 μmol/kg)</td>
<td>9.4 ± 0.8 (6)</td>
<td>97*</td>
</tr>
</tbody>
</table>

* Significantly different (P < 0.001) from control.

Table 4
Influence of inhibitors of pyrimidine degradation on the effects of FUra and FdUrd on the survival of leukemic mice

<table>
<thead>
<tr>
<th>Inhibitors of pyrimidine degradation (μmol/kg)</th>
<th>FUra (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>BVUra (200)</td>
<td>150.2*</td>
</tr>
<tr>
<td>BVdUrd (200)</td>
<td>151.1*</td>
</tr>
<tr>
<td>DUra (200)</td>
<td>80*</td>
</tr>
<tr>
<td>DUra (50)</td>
<td>141.1*</td>
</tr>
<tr>
<td>ETUra (200)</td>
<td>98.6*</td>
</tr>
<tr>
<td>6-ATHy (70)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5
Influence of BVUra and BVdUrd on plasma concentrations of FUra in mice

<p>| Plasma concentrations of FUra (μL) upon injection of |
|-----------------------------------------------|-------------|</p>
<table>
<thead>
<tr>
<th>FUra (mg/kg)</th>
<th>Plasma concentrations of FUra (μL)</th>
<th>FUra alone</th>
<th>BVUra + FdUrd</th>
<th>BVUra + FdUrd</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1 (13.4)*</td>
<td>21</td>
<td>26 (61.5)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>38.5</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>2.5 (59)</td>
<td>79</td>
<td>159 (189)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>4</td>
<td>308</td>
<td>273</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses, rat plasma values.

---

CANCER RESEARCH VOL. 46 MARCH 1986

1098
EtUra (14), and 6-AThy (28). When injected i.p. in rats at 200 
µmol/kg, DUra increased by 15- and 23-fold, respectively, the 
plasma half-life and AUC of Fura. DUra, EtUra, and 6-AThy were also investigated for their enhancing effects on the 
antitle{antileukemic activity of Fura in mice (Table 4). Only DUra at 50 
µmol/kg caused a marked increase in the survival time of leu-
}

Kemic mice over that achieved by Fura alone. Unlike BVUra, 
DUra had itself some antileukemic activity (Table 3), and when 
used at 200 µmol/kg in conjunction with Fura (20 mg/kg) it 
effected a dramatic decrease in the mean life span of the mice 
(Table 4).

**DISCUSSION**

The present results demonstrate that BVUra is more slowly 
cleared from the bloodstream than the other pyrimidine bases 
probably because it is not a substrate for the first enzyme of 
the reductive pathway for pyrimidine degradation, i.e., H₂Thy dehy-
dergenase. Indeed, BVUra is not degraded by liver extract 
which are rich in H₂Thy dehydrogenase (7–9) or by partially 
purified enzyme. In vivo, it takes 48 h before BVUra is entirely 
cleared from the bloodstream after it has been administered to 
rats i.p. at 200 µmol/kg. The plasma AUC values for BVUra 
obtained after i.p. or i.v. administration of BVUra (1000 and 1175 
µMxh, respectively) are clearly higher than those obtained for 
Thy (171 and 222 µMxh, respectively) or Fura (66 and 144 
µMxh, respectively), indicating that BVUra is more slowly cleared 
from the bloodstream than Thy and Fura.

BVUra is only partially eliminated by renal clearance. Although 
high concentrations (up to 700 µM) of BVUra were found in the 
urine within the first 24 h following administration of BVUra, 
urinary BVUra did not represent more than 10% of the adminis-
tered BVUra. Possibly, BVUra could be transformed in vivo to 
BvdUrd by pyrimidine nucleoside phosphorylases (22) then phos-
phorylated by cellular kinases and incorporated into nucleic acids. 
However, BvdUrd is not a substrate for cellular cytosol dThd 
kinese; it only is a good substrate both for the mitochondrial 
dThd kinase and herpes simplex and varicella-zoster virus-en-
coded enzymes (29). Thus, in uninfected cells or tissues BvdUrd 
can be phosphorylated only by mitochondrial dThd kinase. To 
what extent any this phosphorylation contributes to the clear-
ance of BVUra from the circulation is not clear, however. There 
are various other metabolic pathways or other elimination routes 
(e.g., biliary, fecal) by which BVUra may be eliminated, and in 
addition, BVUra may be bound to and retained by plasma or 
tissue proteins.

The present results also demonstrate that BVUra acts as a 
powerful inhibitor of H₂Thy dehydrogenase and thereby inhibits 
the degradation of other pyrimidines that are substrates for this 
enzyme both in vitro and in vivo. In vitro, BVUra reduces the 
degradation of Thy and Fura (Fig. 2). The findings that (a) 
preincubation with liver extract is required for the inhibitory effect 
of BVUra on H₂Thy dehydrogenase activity, (b) this inhibitory 
effect is not reversed by extensive dialysis, and (c) in vivo 
inhibition of pyrimidine degradation is maintained after BVUra 
has been completely cleared from the circulation, suggest that 
like DUra (16), BVUra may be an irreversible inhibitor of H₂Thy 
dehydrogenase. This enzyme is a flavoprotein (12). It has been 
demonstrated (see Ref. 30 for review) that several flavin-depend-
ten enzymes can be inhibited irreversibly by substrate analogues 
which contain a function which is converted to a highly reactive 
group within the active site. This highly reactive group may then 
interact with an essential protein residue or prosthetic group 
before dissociation occurs, thereby causing irreversible inhibition. 
Although the bromovinyl is per se less reactive than the diazo 
group (31, 32), its reactivity may be enhanced considerably in 
the presence of NADPH, since this cofactor is necessary during 
the preincubation period to ensure irreversible binding of BVUra 
to the enzyme.

BVUra inhibits the degradation of Thy and Fura in vivo. When 
rats are pretreated with BVUra before the administration of Thy 
or Fura, plasma AUC of Thy and Fura (Table 2) is considerably 
increased (9.2- and 8.1-fold, respectively). Under these condi-
tions, BVUra probably acts by blocking the H₂Thy dehydrogen-
ase since the activity of this enzyme is markedly suppressed in 
(Please note that the text is not fully visible due to the cropping of the image. The full text is not fully visible due to the cropping of the image.)
may in part be attributed to its own metabolism by the P388 cells, which are, in contrast to other tumor cells, rather poor in thymidine kinase activity (33). This would limit the phosphorylation of FdUrd to 5-fluoro-2'-deoxyuridine-5'-monophosphate in these cells and consequently the inhibitory action of FdUrd on thymidylate synthetase (34) and its incorporation into DNA (35). P388 cells also have low pyrimidine nucleoside phosphorylase activity (33, 35) which means that they are virtually unable to convert FUra to FdUrd or 5-fluorouridine. However, FUMP may be formed directly from FUra upon the action of pyrimidine phosphoribosyl transferase which appears to be quite active in P388 cells (33, 36). Moreover, Mulkins and Heidelberger (33) have demonstrated that P388 cells resistant to FUra have a reduced pyrimidine phosphoribosyl transferase activity in comparison to the wild-type cells. Thus, the inhibitory effects of FUra on P388 cells may be related to the conversion of FUra to FUMP and the eventual incorporation of FUMP into RNA (37). According to our observations BUUra could act by blocking the degradation of FUra, thereby making available greater amounts of FUra to be processed via pyrimidine phosphoribosyl transferase to FUMP, which may then give rise to an increased incorporation of FUra into RNA of P388 cells.

The inhibition of Thy and FUra degradation achieved in vivo when Thy and FUra are administered 3 h after the injection of BVdUrd, i.e., a time when BVdUrd has been completely converted to BVUra in the plasma (22), is apparently mediated by BUUra, although theoretically, it could also be attributed to BVdUrd itself. In vitro, BVdUrd partially inhibits the degradation of Thy and FUra by crude liver extracts. As demonstrated by HPLC analysis, however, BVdUrd, which is an excellent substrate for pyrimidine nucleoside phosphorylases (38, 39), is almost entirely transformed to BVUra by liver extracts. Liver is indeed a rich source of these enzymes (40). When the process of phosphorylation is eliminated by suppression of phosphate in the incubation medium or by the use of a partially purified enzyme devoid of pyrimidine nucleoside phosphorylase activity, inhibition of pyrimidine degradation does not occur. These observations confirm previous findings that pyrimidine deoxynucleosides of which the corresponding base may be either substrate or inhibitor of H2Thy dehydrogenase do not exert any effect on the enzyme activity (14).

In vitro, DUra is a better inhibitor of FUra and Thy degradation than is BVUra (inhibitory dose for DUra, 46–60 nM, as compared to 2–3 µM for BVUra). Among the inhibitors of pyrimidine degradation examined in this study (Table 4), only DUra potenitated the antineoplastic activity of FUra in vivo to a similar degree as BVUra. However, DUra, at 200 µmol/kg was clearly toxic for the mice during the period it was administered, although this toxicity did not appear to affect the mean survival time. At 200 µmol/kg, DUra also increased the toxicity of FUra. At 50 µmol/kg (approximately, 6.9 mg/kg), a concentration close to the 50% lethal dose (10 mg/kg/day for 4 days) for Fischer rats (18), DUra was slightly better tolerated but still showed some signs of toxicity at the beginning of the treatment period. As previously described (41), DUra has by itself some antileukemic activity (increase of mean survival time to 135%), which makes it more difficult to correctly interpret its potentiating effect on the antitumor activity of FUra. In contrast with DUra, BVUra has no antileukemic activity of its own (Table 3). No toxicity studies have been conducted with BVUra. However, its predecessor, BVdUrd has been the subject of toxicity studies, and these have shown that BVdUrd does not lead to untoward effects when administered i.p. to mice at 750–1500 µmol/kg daily for 4 consecutive wk (42). Since BVdUrd is rapidly transformed to BVUra in vivo, it can be surmised that BVUra has no marked toxicity at doses up to 1500 µmol/kg/day.

In conclusion, in addition to its ability to serve as a precursor for the generation of the potent and selective antiviral drug BVdUrd in vivo (22), BUUra also appears to be a powerful inhibitor of the degradation of pyrimidines (such as FUra) via the H2Thy dehydrogenase pathway, allowing the use of 4- to 8-fold lower doses of FUra to achieve an equivalent therapeutic effect in the P388 leukemia mouse model.

ACKNOWLEDGMENTS

The authors thank I. Belloc, F. Dupuch, M. Verstreken for their expert technical assistance and C. Callebaut, N. Grillon and L. Palmaerts for their valuable help in the preparation of the manuscript.

REFERENCES

22. Desgranges, C., Razaka, G., Drouillet, F., Bricaud, H., Herdevin, P., and De Clercq, E. Regeneration of the antiviral drug (E)-5-(2-bromovinyl)-2'-deoxyuridi-
INHIBITION OF PYRIMIDINE DEGRADATION BY BROMOVINYLURACIL

23. Jones, A. S., Verhelst, G., and Walker, R. T. The synthesis of the potent anti-
herpes agent, E-(2-bromovinyl)-2'-deoxyuridine and related compounds. Tet-

24. Barr, P. J., Jones, A. S., Verhelst, G., and Walker, R. T. Synthesis of some 5-
halogenovinyl derivatives of uracil and their conversion into 2'-deoxyribo-nucle-

25. Bergmann, W., and Johnson, T. B. Researches on pyrimidines. CXXXII. A new

26. Fink, K., Cline, R. E., Henderson, R. B., and Fink, R. M. Metabolism of thymine

27. Igo, M., Kuretani, K., and Hoshi, A. Relationship between antitumor effect and
metabolites of 5-fluorouracil in combination treatment with 5-fluorouracil and
guanosine in ascites Sarcoma 180 tumor system. Cancer Res., 43: 5687-
5694, 1983.

28. Matthies, E., Bärwolf, D., and Langen, P. Inhibition by 6-aminothymine of the
degradation of nucleosides (5-iododeoxyuridine, thymidine) and pyrimidine

29. Cheng, Y-C., Dutschman, G., De Clercq, E., Jones, A. S., Rahim, S. G.,
Verhelst, G., and Walker, R. T. Differential affinities of 5-(2-halogenovinyl)-2'-
deoxyuridines for deoxythymidine kinases of various origins. Mol. Pharmacol.,

30. Ghisla, S., Wenz, A., and Thorpe, C. Suicide substrates as irreversible inhibitors
Verlag Chemie, 1980.

31. Previc, E., and Fister, G. Incorporation of 5-diazouracil-[2-14C] into ribonucleic
acid of Escherichia coli during division inhibition. J. Bacteriol., 191: 198-195,
1970.

32. Rando, R. R. On the mechanism of action of antibiotics which act as irreversible

33. Multins, M. A., and Heidelberger, C. Biochemical characterization of fluoro-
pyrimidine-resistant murine leukemic cell lines. Cancer Res., 42: 965-973,
1982.

34. Ardalan, B., Cooney, D. A., Jayaram, H. N., Carrico, C. K., Glazer, R. I.,
Macdonald, J., and Schein, P. S. Mechanisms of sensitivity and resistance of

35. Schuett, J. D., Wallace, H. J., and Diasio, R. B. 5-Fluorouracil incorporation
into DNA of CF-1 mouse bone marrow cells as a possible mechanism of

36. Kessel, D., Hall, T. C., and Reyes, P. Metabolism of uracil and 5-fluorouracil in

37. Wilkinson, D. S., and Pittot, H. C. Inhibition of ribosomal ribonucleic acid
maturation in Novikoff hepatoma cells by 5-fluorouracil and 5-fluorouridine. J.

38. Desgranges, C., Razaka, G., Rabaud, M., Bricaud, H., Baizarni, J. and De
Clercq, E. Phosphorolysis of (E)-5-(2-bromovinyl)-2'-deoxyuridine (BV) and
other 5-substituted 2'-deoxyuridines by purified human thymidine phosphoryl-

39. Liemann, B., and Herrmann, G. (E)-5-(2-Bromovinyl)-2'-deoxyuridine: A good
substrate for mammalian pyrimidine nucleoside phosphorylases. Biomed.

40. Yamada, E. W. Pyrimidine nucleoside phosphorylases of rat liver. Separation
by ion exchange chromatography and studies of the effect of cytidine or uridine

41. Sassenrath, E. N., Kells, A. M., and Greenberg, D. M. Characterization studies

42. Walker, R. T., Jones, A. S., De Clercq, E., Descamps, J., Allaudeen, H. S.,
and Koza, J. W. The synthesis and properties of some 5-substituted uracil
Effect of (E)-5-(2-Bromovinyl)uracil on the Catabolism and Antitumor Activity of 5-Fluorouracil in Rats and Leukemic Mice

Claude Desgranges, Gabriel Razaka, Erik De Clercq, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/3/1094

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.