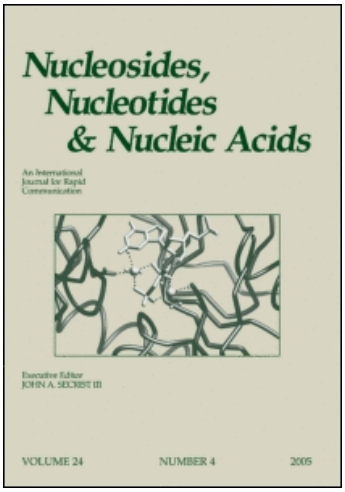

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Synthesis and Antitumor Activity of 5-Bromo-1-Mesyluracil

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SYNTHESIS AND ANTITUMOR ACTIVITY OF 5-BROMO-1-MESYLURACIL

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▫ *Large-scale preparation of 5-bromo-1-mesyluracil (BMsU) 4 has been optimized. BMsU was synthesized by condensation of silylated 5-bromouracil and MsCl in acetonitrile or by the reaction of 5-bromouracil with MsCl in pyridine. The same product was obtained by bromination of 1-mesyluracil. The purpose of this study was to elucidate the effects of BMsU on the biosynthetic activity of tumor cell enzymes involved in DNA, RNA and protein syntheses, and in de novo and salvage pyrimidine and purine syntheses. Investigations were performed in vitro on human cervix carcinoma cells (HeLa). BMsU displayed inhibitory effects on DNA and RNA syntheses in HeLa cells after 24 h of treatment. De nova biosynthesis of pyrimidine and purine was also affected. Antitumor activity of BMsU is closely associated with its inhibitory activity on the enzymes that play an important role in the metabolism of tumor cells. In vivo antitumor activity of BMsU was also investigated. The model used in investigations was a mouse anaplastic mammary carcinoma transplanted into the thigh of the right leg of CBA mice. Significant reduction in tumor growth time was achieved with BmsU administered at a dose of 50 mg/kg.*

Keywords *N*-1-Sulfonylpyrimidine Derivatives, Human Cervix Carcinoma Cell (HeLa), Enzyme Activity, Anaplastic Mammary Carcinoma

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INTRODUCTION

A number of nucleoside analogues have shown promise as anticancer agents.[1,2] The sulfonamide group $R'SO_2$-NHR^2 is a common pharmacophore found in various biologically active molecules, enzyme inhibitors, and receptor antagonists.[3–5] In the last few years, we have been involved in the synthesis and biological evaluation of novel pyrimidine nucleic base derivatives possessing a sulfonamide pharmacophore as potential antitumor agents. We have prepared the sulfonylcyclourea derivatives by attaching the sulfonyl fragment onto N-1 of pyrimidine bases.[6–8] The compounds showed potent growth inhibitory activity against human tumor cell lines in vitro at concentrations of 10^{-5}–10^{-8} M.[9] In comparison with 5-FU, some of *N*-1-sulfonylpyrimidine derivatives showed 10 times stronger inhibitory effects while the effects on normal human cell lines were much lower. Our additional studies showed that *N*-1-sulfonylpyrimidine derivatives have a strong antiproliferative activity and ability to induce apoptosis in treated tumor cells.[9]

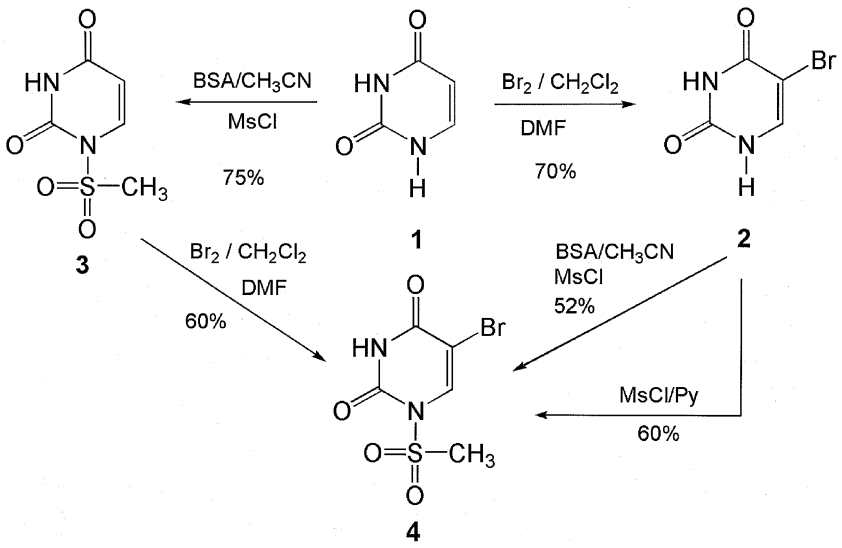
Here, we report a large scale synthesis of 5-bromo-1-(methanesulfonyl)uracil, BMsU, (**4**) as a new candidate with antitumor activity. The purpose of this study was to elucidate the effects of BMsU **4** on the biosynthetic activity of tumor cell enzymes involved in DNA, RNA and protein syntheses, and in de novo and salvage pyrimidine and purine syntheses. Investigations were performed in vitro on human cervix carcinoma cells (HeLa). In vivo antitumor activity of BMsU was also investigated. The model used in the investigations was a mouse anaplastic mammary carcinoma transplanted into the thigh of the right leg of CBA mice.

RESULTS

The synthesis involved condensation of silylated uracil (**1**) or 5-bromouracil (**2**) with methanesulfonyl chloride (MsCl) or the condensation reaction of 5-bromouracil (**2**) with MsCl in pyridine (Scheme 1).

Silylation of uracil (**1**) was accomplished with bis(trimethylsilyl)acetamide (BSA) in acetonitrile at 80°C. The silylated derivative was condensed with MsCl, giving I-(methanesulfonyl)uracil (**3**) in 75% yield. Bromination of **3** by bromine in CH_2Cl_2/DMF at room temperature afforded the corresponding 5-bromo-1-(methanesulfonyl)uracil, BMsU (**4**), in 60% yield (45% from uracil). Surprisingly, attempts to brominate the C-5 position of MsU with N-bromosusuccinimide (NBS) failed.[10]

In the condensation reaction of 5-bromouracil (**2**) with MsCl in pyridine, product **4** was obtained in 60% yield after several recrystallizations from methanol. On the other hand, the silylated 5-bromouracil **2** in the reaction with MsCl in acetonitrile gave **4**, which crystallized from the reaction mixture. Recrystallization horn methanol gave an analytically pure 5-bromo derivative **4** in 52% yield.



SCHEME 1

Antiproliferative Activity In Vitro

The 5-bromo-1-(methanesulfonyl)uracil (BMsU) (**4**) displayed antimetabolic activity on the cervix carcinoma cell line (HeLa). DNA synthesis was significantly inhibited (by 48%) in treated HeLa cells in the first hour of incubation. During the same period of incubation time, RNA synthesis was slightly inhibited (13%) and protein synthesis was unaffected. De novo pyrimidine and purine syntheses were partially blocked as well (Table 1).

TABLE 1 Antiproliferative-Activity of BMsU **4** in HeLa Cells

	% of Control (X ± SD)
DNA synthesis[a]	42 ± 5
RNA synthesis[b]	87 ± 2
Protein synthesis[c]	101 ± 8
De novo pyrimidine synthesis[d]	78 ± 5
De novo puke synthesis[e]	87 ± 1

Cells were incubated for 60 min, at 37°C in medium containing 1×10^{-6} M of investigated compound, or in the absence of inhibitor (control cells). At the indicated intervals the appropriate isotope was added as described under methods. Incorporation of radioactivity was measured in count per minute (c.p.m.) and expressed in percent relative to controls. Results are shown as mean values ± standard deviation. In controls incorporation was: [a]1716 c.p.m., [b]23,172 c.p.m., [c]2594 c.p.m., [d]1678 c.p.m., and [e]4032 c.p.m.

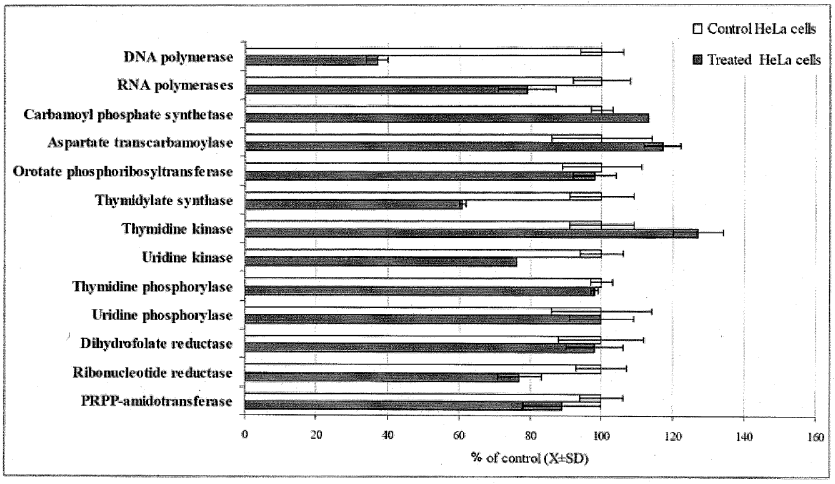


FIGURE 1 Effects of 5-bromo-1-mesyluracil **4** on the biosynthetic activity of enzymes involved in de novo and salvage pathways of nucleotide and nucleic acids syntheses.

Effects on the Biosynthetic Activity of Enzymes

Activity of specific enzymes involved in nucleic acids syntheses, such as DNA polymerase α, was reduced by BMsU 4 by 63%. The RNA polymerase biosynthetic activity was inhibited by 20%. The activity of PPRP amidotransferase, the regulatory enzyme of purine biosynthetic pathway, was reduced by 14%, while the activity of carbamoyl phosphate synthetase, the regulatory enzyme of pyrimidine synthesis, was increased by 24% in BMsU-treated cells. Key enzymes in the de novo synthesis of pyrimidine, aspartate transcarbamoyl synthetase, and orotate phosphoribosyl-transferase, were not inhibited in treated cells compared to control cells. Synthetic activity of aspartate transcarbamylase and thymidine kinase was increased by 17% and 24%, respectively. Thymidylate synthase (38%), uidine kinase (24%), and ribonucleotide reductase (33%) were also inhibited in BMsU-treated HeLa cells. Effects on the activities of other investigated enzymes were not appreciable (Figure 1).

Antitumor Effects In Vivo

Investigation results for the antitumor effects of BMsU 4 are shown in Table 2. The BMsU applied in a single dose of 50 mg/kg, one day after tumor cells implantation, had good antitumor activity against anaplastic mammary carcinoma ($p < 0.01$). Tumor growth time in this group of animals was prolonged by 30% (2.5 days) compared to tumor growth time in the control group. However, the antitumor effect of this compound applied according to different time schedules, *i.e.*, on day 1, 3, 5, 7, and 9 or 6, 8, 10, 12, and 14, and at different doses was not statistically significant compared to the controls.

TABLE 2 Antitumor Activity of BMsU **4** on Tumor Growth Time (TGT)

Treatment (mg/kg/day)	TGT (X ± SD/days)	T/C[a] (%)
Control group	7.5 ± 0.9	100
50/day 1	10.0 ± 0.7*	130
50/day 6	8.5 ± 1.2	110
30/day 1, 3, 5, 7, 9	8.1 ± 1.3	110
30/day 6, 8, 10, 12, 14	8.1 ± 1.3	110

[a]T represents arithmetic mean tumor growth time of treated animals and C represents arithmetic mean tumor growth time of control animals.

*$p < 0.01$.

DISCUSSION

Only a few reports on *N*-1-sufonylpyrimidine derivatives could be found in the literature. Martirosyan et al.[11] isolated I-(*p*-toluenesulfonyl)uracil as an unwanted product in the transformation of C-4 keto group of uracil, and Kaldrikyn et al.[12] examined the synthesis of 1-*p*-alkoxybenzenesulfonyl-5-bromouracil derivatives possessing antibacterial activity. According to Tada,[13] benzoyl and arenylsulfonyl-5-fluorouracil derivatives are more active and less toxic than 1-(2-tetrahydrofuryl)uracil in the Leukemia L/1210 system. However, the in vitro anticancer activity of *N*-1-sulfonylcytosine derivatives was described for the first time in our patent.[8] The best method for the synthesis of 5-bromo-1-(methanesulfonyl)uracil, BMsU (**4**), was the condensation reaction of silylated 5-bromouracil (**2**) with MsCl in acetonitrile. The simplicity of the synthesis and product isolation made this method attractive and useful for a large scale synthesis of BMsU.

N-1-Sulfonylpyrimidine analogue, 5-bromo-1-mesyluracil (BMsU), showed strong cytotoxic activity and the ability to induce apoptosis in different human carcinoma (cervix, colon, pancreatic, lymph node metastasis) cell lines.[9] The investigated compound, at a concentration of 1×10^{-6} M, inhibited DNA and RNA syntheses in human cervix carcinoma cells (HeLa) after 60 min of treatment. Inhibition of DNA synthesis could be due to partial DNA degradation in tumor cells treated by BMsU, presumably as a result of endonuclease activity and significant inhibition of DNA polymerase (63%) and thymidylate synthase activity (Figure 1).

Similar results were obtained in investigations of the 5-fluorouracil and 2′-deoxy-5-fluorouridine antiproliferative activity, where the DNA lesions induced in treated tumor cells were not repaired due to insufficiency of dTTP.[14,15]

Key enzymes in de novo syntheses of pyrimidine, aspartate transcarbamylase, and orotate phosphoribosyltransferase were not inhibited in treated cells compared to control cells. Catalytic activity of carbamoyl phosphate synthetase, the first regulatory enzyme involved in de novo pyrimidine synthesis, was increased by 24% in BMsU-treated cells.

The activity of PPRP-amidotransferase, the regulatory enzyme of puke biosynthetic pathway, was negligibly reduced (14%). Inhibition of thymidylate

synthase catalytic activity was observed in tumor cells exposed to BMsU. Thymidylate synthase is the rate-limiting enzyme in de novo pyrimidine biosynthesis, and the consequence of its inhibition is inadequate thymidylate synthesis, which then leads to inhibition of DNA synthesis.[16]

Ribonucleotide reductase, which consists of two non-identical protein subunits, catalyzes the rate-limiting step in the de novo synthesis of deoxyribonucleoside-5′-triphosphates required for DNA replication.[17] Its catalytic activity is regulated by pyrimidine and puke nucleotides concentrations, which are, depending on the cell's requirement, inhibitors or activators of ribonucleotide reductase. Reduced ribonucleotide reductase.activity was observed in tumor cells exposed to the investigated substance, which could lead to decreased deoxyribonucleotide levels in the cells and to lower cell proliferative capacity. Similar results were obtained with a number of ribonucleotide analogues designed to reduce ribonucleotide reductase activity.[18–20] Due to disequilibrium in pyrimidine synthesis, purine synthesis was also decreased, and hence a drop in the de novo purine synthesis was observed in our experiments.

Sources of the cell pyrimidine and purine nucleotides are de novo synthesis and *salvage* metabolic pathways, both of which exist in tumor cells. Since de novo synthesis is very expensive, generation of pyrimidine and purine pools has an important role in preserving metabolic energy, especially in highly proliferative tissues.[21] In almost all tumor cells, activities of *salvage* pathway enzymes involved in purine and pyrimidine syntheses are more pronounced than the activities of enzymes involved in de novo synthesis. The role of a *salvage* pathway is usually to keep the *steady state* in pyrimidine and purine nucleotide levels. Lai et a1.[22] found that thymidine kinase activity was markedly elevated in cancer cells, including human colon carcinoma and rat hepatoma and sarcoma, due to an increased amount of this enzyme, which is attributable to higher mRNA production, Thymidine kinase activity was significantly higher than thymidylate synthase activity and activities of other enzymes involved in purine and pyrimidine nucleotide metabolisms.[23] In BMsU-treated cells, the activity of uridine kinase was diminished and that of thymidine kinase was significantly increased compared to untreated cells. The investigated compound **4** was able to reduce de novo purine synthesis within 60 min, which could further account for the observed suppression of DNA and RNA syntheses. Uridine phosphorylase and thymidine phosphorylase, present at higher levels in a wide variety of solid tumors than in the adjacent non-neoplastic tissues, were not inhibited by the investigated substance.[24,25]

The present results allow the conclusion that antitumor activity of 5-bromo-1-mesyluracil (BMsU) **4** is closely associated with its inhibitory effect on the enzymes that play an important role in the metabolism of tumor cells.

For the in vivo experiments, doses of the tested derivative were chosen on the basis of data previously obtained in LD_{50} experiments (LD_{50} = 150 mg/kg) (unpublished results). Investigation results for the BMsU antitumor effects are given in Table 2. It can be seen from this data that BMsU, applied in a single dose of 50 mg/kg on day 1 after tumor cells implantation, had good antitumor activity against

anaplastic mammary carcinoma ($p < 0.01$). If the drug was administrated in multiple doses or when the tumor was larger, the antineoplastic effect failed to appear. A possible explanation could be that these doses were not sufficiently high for cytotoxic effects or that the tumor mass was too large. Another explanation may be the development of resistance, as in the case of many other antineoplastic drugs.[26–29]

Preliminary results of the antitumor activity of BMsU show reasonably good antitumor activity against anaplastic mammary carcinoma grown into CBA mice. The antitumor activity of BMsU **4** depends on the dose applied, and on the treatment schedule.

EXPERIMENTAL

Chemistry

Solvents were distilled horn appropriate drying agents shortly before use. TLC was carried out on DC-plastikfolien Kieselgel 60 F254. Melting points were determined on a Kofler hot-stage apparatus and were uncorrected. UV spectra (λ_{max}/nm, log ε/$dm^3 \cdot mol^{-1} \cdot cm^{-1}$) were taken on a Philips PU8700 UV/VIS spectrophotometer. lR spectra (ν_{max}/cm^{-1}) were obtained for KBr pellets on a Perkin-Elmer 297 spectrophotometer. The ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 spectrometer, operating at 75.46 MHz for the ^{13}C nucleus. The samples were dissolved in DMSO-d_6 and measured at 20°C in 5-mm NMR tubes. Chemical shifts (δ/ppm) were referred to TMS.

1-(Methanesulfonyl)Uracil (MSU) (3)

A mixture of uracil (**1**) (6 g, 53.5 mmol) and *N,O*-bis(trimethylsilyl)acetamide (26.4 mL, 107 mmol) was heated under reflux in dry acetonitrile (80 mL) for 30 min. The colorless solution was cooled to 0°C and.methanesulfonyl chloride (8.3 mL, 107 mmol) was added. The reaction mixture was heated under reflux for 20 h. The resulting suspension was cooled and the solid was separated by filtration. Recrystallization from hot methanol gave the analytically pure product **3**, as white crystals: 7.6 g (75%); R_f=0.81 (CH_2C1_2/MeOH 3:l); m.p. 228–231°C; UV (MeOH) λ_{max}: 210.8 and 245.1 (log ε: 3.86 and 3.93); IR (KBr) ν_{max},: 3200 (m), 3080 (m), 2950 (w), 2850 (w), 1725–1695 (s), 1640 (m), 1440 (s), 1360 (s), 1275 (s), 1180 (s), 1170 (s); ^1H NMR (DMSO-d_6) δ: 11.87 (s, 1H, NH), 7.87 (d, 1H,J=8.3 Hz, H-6), 5.80 (d, 1H, *J*=8.3 Hz, H-5), 3.70 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ: 163.28 (s, C-4), 148.76 (s, C-2), 138.23 (d, C-6), 103.41 (d, C-5), 41.62 (q, CH_3). *Anal.* Calcd. for $C_5H_6N_2O_4S$ (M_r=190.18): C 31.58, H 3.18, N 14.73%; Found: C 31.81, H 3.06, N 14.89%.

5-Bromo-1-(Methanesulfonyl)Uracil (BMSU) (4)

A) 1-Mesyluracil **3** (5 g, 26.3 mmol) was dissolved in dry DMF (200 mL), and the solution of bromine in dichloromethane (75 mL; 1 mL Br_2/25 mL CH_2Cl_2) was added dropwise. The red solution was stirred at room temperature for 4 h, and the

solvent was evaporated under pressure. Ethanol was added into the remaining oil, and the obtained solid was filtered off and recrystallized from hot water, yielding product **4**, as white crystals: 3.67 g (52%); R_f=0.78 (CH_2C1_2/MeOH 20:1); m.p. 235°C; UV (MeOH) λ_{max}=264.1 (log ε=3.86); IR (KBr) ν_{max}: 3150 (m), 3100 (s), 3050–3010 (br, s), 2940 (m), 2860 (m), 1730 (s), 1680 (br, s), 1610 (s), 1420 (s), 1370 (s), 1325 (s), 1255 (s), 1180 (s); ^{1}H-NMR (DMSO-d_6) δ: 12.35 (s, 1H, NH), 8.10 (s, 1H, H-6), 3.71 (s, 3H, CH_3); ^{13}C-NMR (DMSO-d_6) δ: 159.19 (s, C-4), 147.94 (s, C-2), 137.02 (d, C-6), 98.10 (d, C-5), 41.55 (q, CH_3). *Anal* Calcd. for $C_5H_5N_20_4SBr$ M_r=269.08): C 22.32, H 1.87, N 10.41%; Found: C 22.37, H 1.77, N 10.23%.

B) A mixture of 5-bromouracil (**2**) (4 g, 21 mmol) and *N*,*O*-bis(trimethylsilyl)acetamide (10.4 mL, 42 mmol) was heated under reflux in dry acetonitrile (32 mL) for 30 min. The colorless solution was cooled to 0°C and methanesulfonyl chloride (3.3 mL, 42 mmol) was added. The reaction mixture was heated under reflux for 20 h. The resulting suspension was cooled, and the solid was separated by filtration. Recrystallization from hot methanol gave 2.9 g (52%) of product **4**.

C) A solution of 5-bromouracil (**2**) (2 g, 10.5 mmol) in dry pyridine (40 mL) was cooled to 0°C and methanesulfonyl chloride (1.63 mL, 21 mmol) was added dropwise. After stirring at room temperature overnight, the resulting dark suspension was evaporated under pressure. Methanol was added and the obtained brown solid was filtered off. The crude product was treated with dichloromethane, filtered, and, after a few recrystallizations from hot methanol, product **4** was obtained (1.68 g, 60%).

MATERIALS

In Vitro Study

For the in vitro study, BMsU was dissolved in a phosphate buffered saline (PBS). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL, Life Technologies (Parsley, UK). Radioisotopes: [methyl-^{3}H]-thymidine (25 Ci/mmol), [2-^{3}H]-adenine (16 Ci/mmol), [5-^{3}H]-uridine (26 Ci/mmol), deoxy[5-^{3}H]-uridine-5′-monophosphate (15 Ci/mmol), [5-^{3}H]-uridine-5′-triphosphate (14.5 Ci/mmol), [5-^{3}H]-cytidine-5′-triphosphate (19 Ci/mmol), [8-^{14}C]-adenozin-5′-triphosphate (52 mCi/mmol), [1-^{14}C]-glycine (53 mCi/mmol), [2-^{14}C]-thymidine (57 mCi/mmol), and [^{14}C] formic acid sodium salt (56 mCi/mmol) were purchased from Amersham Pharmacia Biotech (Buckinghamshire, GB). Radioactivity was determined in Wallac scintillation fluid in a Pharmacia β-counter. The substrate and cofactors were obtained from Sigma Biochemicals (St. Louis, MO).

Cell Lines and Culture

Cervix carcinoma cells (HeLa) were kindly provided by Professor Kresimir Pavelic, M.D. (Division of Molecular Medicine, Ruder Bošković Institute, Zagreb,

Croatia). The cell line was grown as a monolayer and cultivated in Dulbecco's modified Eagle's medium (DMEM), which was supplemented with 10% *v/v* fetal bovine serum, 2 mM glutamine, 100 U penicillin, and 100 μg/mL streptomycin. HeLa cells were incubated at 37°C in a humidified atmosphere with 5% (*v/v*) CO_2.

Incorporation Studies

Incorporation of labeled precursors into DNA, RNA, and protein for 10^6 CaCo2 cells was determined by the method of Liao et al.[30] Incorporation of the [^{14}C]-fotmic acid sodium salt (53.0 mCi/mmo1) into pyrimidines was determined by the method of Christopherson et al.[31] Incorporation of [1-^{14}C]-glycine (15.0 Ciim-mol) into purines was determined by the methods described by Cadman et al.[32] and Agarwal et al.[33]

Enzyme Assays

Inhibition of several enzyme activities was carried out by first preparing an appropriate HeLa cells homogenate or subcellular fraction, and then adding the drug to be tested during the enzyme assay. Protein content was determined by the method of Lowry et al.[34] Inhibition of enzyme activity was determined at 1 μM of drugs for 60 min of incubation. DNA polymerase (Y activity was determined in cytoplasmic extracts by the methods of Sedwick et al.[35] and Eichler et al.[36] RNA polymerase activities were assayed using the method of Anderson et al.[37] The following enzyme activities were determined using cell homogenates. Ribonucle-otide reductase activity was analyzed by the method of Moore et al.[38] and Matsmoto et al.[39] Carbamoyl phosphate synthetase activity was assessed by the method of Kalman et al.[40] Aspartate trsnscarbamylase activity was determined by the method of Koritz et al.[41] The method of Houghton et al.[14] was used for determination of the orotate phosphoribosyltransferase (OPRTase) activity. Thymidylate synthase activity was obtained by the method of Kampf et al.[42] and Peters et al.[43] Thymidine-5′-diphosphate kinase activities were measured by the method of Maley et al.[44] and Weber[45] and separated by thin-layer chromatography. The method of Ho et al.[46] was used for determination of the dihydrofolate reductase activity. The activity of 5-phosphoribosyl-2-pyrophosphate amidotransferase (PRPP-amidotransferase) was assessed by Martin's method.[47] Results are expressed as the mean of counts per minute (c.p.m.) in replicate wells.

Pilot Study In Vivo

For the in vivo study, BMsU **4** was dissolved in an isotonic physiological saline immediately before being injected into animals.

Animals

Weight to ten 10–14-week-old male and female CBA mice were used in each group per experiment. The animals were treated with different doses of the

examined compound and each experiment was repeated twice. Mice were obtained from the Ruder Boškoviæ Institute breeding colony. During the experiment, three to four animals were kept in a cage. Food and water were supplied ad libitum. All procedures performed in this study complied with the European document entitled "Directive for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes" (86/609/EEC) as well as the Croatian Act of Animal Protection in Experimental Work.

Tumor

Anaplastic mouse mammary carcinoma (AMC) induced into female CBA mice and maintained by serial transplantation in syngenic recipients (CBA mice) was used in the study. AMC was transplanted by injecting 10^6 tumor cells (0.02 mL) into the thigh of the right hind leg. Tumor volume was measured by caliper and calculated according to the formula $A \times B \times C \times \pi/6$, where A, B, and C represent three orthogonal diameters. The end point of tumor response was the tumor growth time (TGT), *i.e.*, the time needed for an individual tumor to increase its volume 5 times over the volume at the beginning of treatment. The effect of the therapy was evaluated by comparing the tumor growth time in treated groups of animals with the tumor growth time in the control/untreated group of animals.

Treatment

Animals were divided into several groups: Control/untreated animals received physiological saline only while the treated animals received the new agent dissolved in physiological saline. BMsU **4** was given in different doses intraperitoneally (i.p.) according to the body weight of mice, *i.e.*, 0.02 mL of solution was given per 10 g of body weight, in different treatment schedules.

Treatment Protocols

Group 1: Animals received the compound in a single dose of 50 mg/kg/day one day (24 h) after tumor cells implantation, Group 2: Animals received the compound in a single dose of 50 mg/kg/day on the 6th day after tumor cells implantation, Group 3: Animals received the compound in repeated doses of 30 mg/kg/day on day l, 3, 5, 7, and 9 after tumor cells implantation, Group 4: Animals received the compound in repeated doses of 30 mg/kg/day on day 6, 8, 10, 12, and 14 after tumor cells implantation.

Statistical Analysis

In vitro experiments: In all experiments, the mean values of 4 independent experiments i standard deviation (S.D.) were calculated. In vivo experiments: All results were presented as arithmetic mean ± standard deviation (X ± SD) of tumor growth time (TGT). The ratio T/C, in which T represents the arithmetic mean of tumor growth time of treated animals and C represents the arithmetic mean of

tumor growth time of control animals, was calculated for all treated groups and was shown as percentage. The results were tested by the ANOVA test for independent samples. The level of statistical significance between control and treated groups was set up at the level of $p < 0.05$.

REFERENCES

1. MacCoss, M.; Robins, M.J. *Chemistry of Antitumor Agents*; Wilman, D.E.V., Ed.; Blackie and Son: UK, 1990.
2. Robins, M.J.; Kinin, G.D. *Chemistry of Antitumor Agents*; Wilman, D.E.V., Ed.; Blackie and Son: UK, 1990.
3. Lendnicer, D.; Mitscher, L.D. *The Organic Chemistry of Drug Synthesis*; John Wiley: New York, 1977; Vol. 1, 120–132.
4. Mohamadi, F.; Spels, M.M.; Grindey, G.B. Sulfonylureas: a new class of cancer chemotherapeutic agents. J. Med. Chem. **1992**, *35*, 3012–3016.
5. Howbert, J.J.; Grossman, C.S.; Crowell, T.A.; Rieder, B.J.; Harper, R.W.; Kramer, K.E.; Tao, E.V.; Aikins, I.; Poore, G.A.; Rinzel, S.M.; Grindey, G.B.; Shaw, W.N.; Todd, G.C. Novel agents effective against solid tumor. The Diarylsulfonylureas. Synthesis, activities and analysis of quantitative structure-activity relationships. J. Med. Chem. **1990**, *3*, 2393–2407.
6. Kašnar, B.; Krizmanić, I.; Žinić, M. Synthesis of the sulfonylpyrimidine derivatives as a new type of sulfonylcycloureas. Nucleosides Nucleotides **1997**, *16*, 1067–1071.
7. Žinić, B.; Krizmanić, I.; Vikić-Topić, D.; Žinić, M. 5-Bromo- and 5-iodo-*N*-1-sulfonylated cytosine derivatives. Exclusive formation of keto-imino tautomers. Croat. Chem. Acta **1999**, *72*, 957–966.
8. Žinić, B.; Žinić, M.; Krizmanić, I. *Sulfonylpyrimidine Derivatives with Anticancer Activity*; Ruder Bošković Institute, 2003.
9. Glavaš-Obrovac, L.J.; Karner, I.; Žinić, B.; Pavelić, K. Antineoplastic activity of novel *N*-1-sulfonylpyrimidine derivatives. Anticancer Res. **2001**, *21*, 1979–1986.
10. Kumar, R.; Wiebe, L.I.; Knaus, E.E. A mild and efficient methodology for the synthesis of 5- halogenouracil nucleosides that occurs via a 5-halogeno-6-azido-5,6-dihydro intermediate. Can. J. Chem. **1994**, *72*(9), 2005–2010.
11. Martirosyan, Z.A.; Gunar, V.I.; Zav'yalov, S.I. Tosylation of nucleobases. Izv. Akad. Nauk SSSR, Ser. Him. **1970**, (8), 1841–1844.
12. Kaldrikyn, M.A.; Geboyan, V.A.; Ter-Yakharyn, Yu.Z.; Paronikyan, R.V.; Garibdzhanyan, B.T.; Stepanyan, GM.; Paronikyan, G.M. Synthesis and biological activity of *N*′-4-alkoxybenzenesulfonyl-5-halouracis. Khim. Farm. Zh. **1986**, *20*, 928–932.
13. Tada, M. Antineoplastic agents. Synthesis of some 1-substituted 5-fluorouracil derivatives. Chem. Lett. **1975**, *2*, 129–130.
14. Houghton, J.A.; Houghton, P.J. Elucidation of pathways of 5-fluorouracil metabolism in xenografts of human colorectal adenocarcinoma. Eur. J. Clin. Oncol. **1983**, *19*, 807–815.
15. Tazawa, K.; Sakamoto, T.; Kuroki, Y.; Ysmashita, I.; Okamoto, M.; Katuyama, S.; Fujimaki, M. Inhibitory effects of fluorinated pyrimidines, 5′-DFUR, UFT and T-506, in a model of hepatic metastasis of mouse colon 26 adenocarcinoma assessment of inhibitory activity and adverse reactions at the maximum tolerated dose. Clin. Exp. Metastasis **1997**, *15*, 266–271.
16. Fukushima, M.; Fujioka, A.; Uchida, I.; Nakagawa, F.; Takechi, T. Thymidylate synthase (TS) and ribonucleotide reductase (RNR) may be involved in acquired resistence to 5-fluorouracil(5-FU) in human cancer xenografts in vivo. Eur. J. Cancer **2001**, *137*, 1681–1687.
17. Kolberg, M.; Strand, K.R.; Graff, P.; Anderson, K.K. Structure, function, and mechanism of ribonucleotide reductase. Biochem. Biophys. Acta **2004**, *1699*, 1–34.
18. Takahaski, T.; Nakashima, A.; Kanazawa, J.; Yamaguchi, K.; Akinaga, S.; Tamaoki, T.; Okabe, M. Metabolism and ribonucleotide reductase inhibition of (E)-2′ deoxy-2′-(fluormethylene)cytidine MDL 101.731 in human cervical carcinoma HeLa S3 cells. Commun. Chemother. Pharmacol. **1998**, *41*, 268–274.
19. Hurta, R.A.; Wright, J.A. Alterations in the activity and regulation of mammalian ribonucleotide reductase by chlorambucil, a DNA damaging agent. J. Biol. Chem. **1992**, *267*, 7066–7071.

20. Smith, B.D.; Karp, J.E. Ribonucleotide reductase: an old target with new potential. Leuk. Res. **2003**, *27*, 1075–1076.
21. Weber, G. Significance of enzyme degradation rate and enzyme inhibition in targeted chemotherapy. Advan. Enzyme Regul. **2004**, *in press*.
22. Lai, M.H.T.; Weber, G. Increased concentration of thymidine kinase in rat hepatoma. Biochem. Biophys. Res. Commun. **1983**, *111*, 280–287.
23. Niedzwicki, J.G.; Ilzsch, M.H.; ElKouni, M.H.; Cha, S. Structure activity relationship of ligands of the pyrimidine nucleoside phosphorylases. Biochem. Pharmacol. **1984**, *33*, 2383–2395.
24. Pizzomo, G.; Cao, D.L.; Leffert, J.J.; Russel, R.L.; Zhang, D.K.; Handschumacher, R.E. Homeostatic control of uridine and the role of uridine phosphorylase: a biological and clinical update. Biochem. Biophys. Acta **2002**, *1587*, 133–144.
25. Liu, M.; Cao, D.; Russell, R.; Handschumacher, R.E.; Pizzomo, G. Expression, characterization, and detection of human uridine phosphorylase and identification of variant phosphate activity in selected human tumors. Cancer Res. **1998**, *58*, 5418–5424.
26. Matsui, H.; Suzuka, K.; Iitsuka, Y.; Ya,azawa, K.; Tanaka, N.; Mitsuhashi, A.; Seki, K.; Sekiya, S. Salvage combination chemotherapy with S-fluorouracil and actinomycin D for patients eighth refractory, high-risk gestational trophoblastic tumors. Cancer **2002**, *95*, 1051–1054.
27. Ardalan, B.M.; Buscaglia, D.; Schein, P.S. Tumor 5-fluorodeoxyuridylate concentration as a determinant of 5-fluorouracil response. Biochem. Pharmacol. **1978**, *27*, 2009–2013.
28. Guo, Y.P.; Kotova, E.; Chen, Z.S.; Lee, K.; Hopper-Borge, E.; Belinsky, M.G.; Kruh, G.D. MRP8, ATP-binding cassette C11 (ABCC11), is a cyclic nucleotide effux pump and a resistance factor for fluoropyrimidine 2′,3′-dideoxycytidine and 9′-(2′-phosphonylmethoxyethyl)adenine. J. Biol. Chem. **2003**, *278*, 29509–29514.
29. Anton, A.; Aranda, E.; Carrato, A.; Marcuello, E.; Massuti, B.; Vervantes, A.; Abad, A.; Sastre, J.; Fenandez-Martos, C.; Gallen, M.; Diaz-Rubio, E.; Huarte, L.; Ba lcells, M. Irinotecan (CPT-11) in metastatic colorectal cancer patiens resistant to 5-fluorouracil(5-FU): a phase II study. Methods Find. Exp. Clin. Pharmacol. **2003**, *25*, 639–643.
30. Liao, L.; Kupchan, S.M.; Horowitz, S.B. Mode of action of the antitumor compound bruceantin an inhibitor of protein synthesis. Mol. Pharmacol. **1976**, *12*, 167–176.
31. Christopherson, R.I.; Yu, M.L.; Jones, M. An overall radioassay for the first three reactions of de novo pyrimidine synthesis. Anal. Biochem. **1981**, *111*, 240–249.
32. Cadman, E.; Heimer, R.; Benz, C. The influence of methotrexate pretreatment on S-flaxouracil metabolism in L1210 cells. J. Biol. Chem. **1981**, *256*, 1695–1704.
33. Agarwal, R.P.; He, J.; Bansal, M.; Gupta, V. Affect of long-term zidovudine exposure on salvage and de novo purine and pyrimidine nucleotide syntheses. Biochim. Biophys. Acta **1995**, *1266*, 223–228.
34. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randal, R. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. **1951**, *193*, 265–275.
35. Sedwick, W.D.; Wang, T.S.; Kom, D. Purificaton and properties of nuclear and cytoplasmic deoxyribonucleic acid polymerases from human Kb cells. J. Biol. Chem. **1972**, *247*, 5026–5033.
36. Eichler, D.C.; Fisher, P.A. Effect of calcium on the recovery distribution of DNA polymerase α form cultured human cells. J. Biol. Chem. **1977**, *252*, 4011–4014.
37. Anderson, K.M.; Mendelson, I.S.; Guizik, G. Solubilized DNA-dependent nuclear RNA polymerases from the mammary glands of late-pregnant rats. Biochem. Biophys. Acta **1975**, *383*, 56–66.
38. Moore, E.C.; Hulbert, R.B. Regulation of mammalian deoxyribonucleotide biosynthesis by nucleotides as activators and inhibitors. J. Biol. Chem. **1966**, *24*(20), 4802–4809.
39. Matsmoto, M.; Tihan, T.; Cory, J.G. Effects of ribonucleotide reductase inhibitors on the growth of human colon carcinoma HT-29 cells in culture. Cancer Chemother. Pharmacol. **1990**, *26*, 323–329.
40. Kalman, S.M.; Duflield, P.H.; Brzozwki, T.J. Purification and properties of a bacterial carbamyl phosphate synthetase. J. Biol. Chem. **1966**, *241*(8), 1871–1877.
41. Koritz, S.B.; Cohen, P.P. Calorimetric determination of carbamyl amino acid and related compounds. J. Biol. Chem. **1968**, *243*, 3924.
42. Kampf, A.; Bariknecht, R.; Schaffer, P.; Osaki, S.; Mertes, M.P. Synthetic inhibitors of *E. coli*, calf thymus and Ehrlich ascites tumor thymidylate synthetase. J. Med. Chem. **1976**, *19*(7), 903–908.
43. Peters, G.J.; Van der Wilt, C.L.; Van Groeningen, C.J.; Smid, K.; Meijer, S.; Pinedo, H.M. Thymidilate synthase inhibition after administration of fluorouracil with or without leucovorin in colon cancer patients: implications for treatment with fluorouracil. J. Clin. Oncol. **1994**, *12*, 2035–2042.

44. Maley, F.; Ochoa, S. Enzymatic phosphorylation of deoxycytidylic acid. J. Biol. Chem. **1958**, *233*, 1538–1543.
45. Weber, G.; Shiotani, T.; Kizaki, H.; Tzeng, D.; Williams, J.C.; Gladstone, N. Biochemical strategy of the genome as expressed in regulation of pyrimidine metabolism. Adv. Enzyme Regul. **1978**, *16*, 3–19.
46. Ho, Y.K.; Hakala, T.; Zakrzewski, S. 5-(1-Adamantyl)pyrimidines as inhibitors of folate metabolism. Cancer Res. **1972**, *32*, 1023–1028.
47. Martin, D.W. Radioassay for enzimatic production of glutamate from glutamine. Anal. Biochem. **1972**, *46*, 239–242.