same enzyme that catalyzes the conversion of U to UMP(5′), but a different enzyme from orotidylic acid pyrophosphorylase. In the resistant subline, the mechanism of resistance to AzU is attributed to a reduction in the level of this enzyme, uridylic acid pyrophosphorylase; consequently, growing cultures are unable to form AzUMP from AzU, and inhibition of the synthesis *de novo* of pyrimidine is not achieved.

Department of Pharmacology,
Yale University School of Medicine,
New Haven, Conn., U.S.A.

LEWIS J. MARKOFF
ROBERT E. HANDSCHUMACHER

REFERENCES

1. E. S. CANELLAKIS, *J. biol. Chem.* **227**, 329 (1957).
2. R. J. RUBIN, J. J. JAFFE and R. E. HANDSCHUMACHER, *Biochem. Pharmac.* **11**, 563 (1962).
3. R. E. HANDSCHUMACHER, *J. biol. Chem.* **235**, 2917 (1960).
4. R. E. HANDSCHUMACHER, *Biochim. biophys. Acta* **23**, 428 (1957).
5. R. W. BROCKMAN, J. M. DAVIS and P. STUTTS, *Biochim. biophys. Acta* **40**, 22 (1960).

Biochemical Pharmacology, 1966, Vol. 15, pp. 763–768. Pergamon Press Ltd., Printed in Great Britain.

Carcinostatic agents—XVI. Inhibition of purine biosynthesis and of tetrahydrofolic acid formylase by β-4-methoxybenzoyl-β-bromoacrylic acid

(*Received* 7 *October* 1965; *accepted* 22 *December* 1965)

Cis-β-4-methoxybenzoyl-β-bromoacrylic acid (MBBA) has been prepared as a potential anticancer agent.[1] It proved effective against mammary adenocarcinoma, Crookers' sarcoma and SAK sarcoma in mice, and Yoshida sarcoma in rats.[2, 3] Administration of the compound to a number of patients with advanced carcinoma of the uterus resulted in a marked subjective and sometimes objective improvement.[4] Studies of the mode of action of MBBA indicate that this drug interferes with purine biosynthesis. The details of this investigation are presented in this paper. MBBA and its parent compound β-4-methoxybenzoylacrylic acid were used in the form of their sodium salts. Tetrahydrofolic acid was prepared from folic acid (Lepetit) by catalytic hydrogenation of platinum in glacid acetic acid[5] and was dissolved in a 0·76% solution of cysteine, pH 8, a few drops of 1% $NaHCO_3$ being added to accelerate solution.

METHODS

Enzyme preparation. The enzyme preparation used in all experiments was obtained by extraction of the acetone powder of pigeon liver with 10 vol. of 0·05 M barbiturate buffer, pH 7·6, at 0°. After standing for 10 min at 0° the residue was removed by centrifugation at 2000 *g* and the supernatant used for the experiments.

Purine biosynthesis. The biosynthesis of purines was followed by measuring the incorporation of formate-^{14}C into inosinic acid.[6]

Determination of the effect of MBBA on formimino transferase + cyclodeaminase activity. To 3 μmoles tetrahydrofolate, 13·8 μmoles sodium formiminoglutamate, 19·0 μmoles cysteine, and 3·2 ml 0·1 M triethanolamine–HCl buffer (pH 7·0) was added 0·3 ml enzyme extract to give a total volume of 4·4 ml. Incubation was carried out at 37°. Immediately after addition of the enzyme and after 60-min incubation, 1·2-ml samples were withdrawn and deproteinized with 2·4 ml of 8% perchloric acid. The amount of methenyltetrahydrofolate formed was determined spectrophotometrically at 355 mμ.[7] A nonincubated sample was used as a blank.

Determination of the effect of MBBA on serine aldolase and methylenetetrahydrofolate dehydrogenase activity.[8] Tetrahydrofolate, 1·37 μmoles, 10·0 μmoles cysteine, 1·2 μmoles NADP, 9·5 μmoles serine, 2·0 ml 0·1 M phosphate buffer (pH 7), and 0·2 ml enzyme extract were incubated in a total volume of 3 ml at 37°. Immediately after addition of the enzyme and after 30-min incubation, 1·2-ml samples were withdrawn, deproteinized by 2·4 ml of 8% perchloric acid, and the amount of methylenetetrahydrofolate formed was determined spectrophotometrically at 355 mμ.

RESULTS

As demonstrated by Fig. 1, MBBA inhibits the incorporation of formate-^{14}C into the purine ring of inosinic acid. In order to determine which of the enzymes involved in the synthesis of inosinic

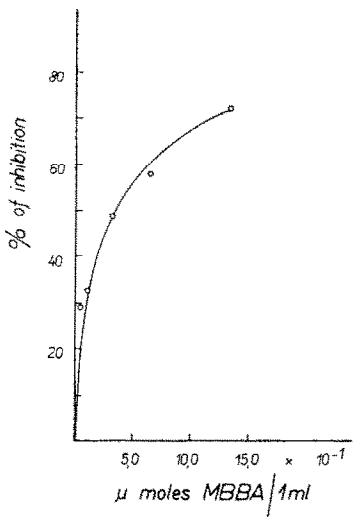

FIG. 1. Inhibition of purine biosynthesis by MBBA. The incubation mixture contained: 19·1 μmoles ATP, 19·2 μmoles homocysteine, 27·3 μmoles glutamine, 19·0 μmoles glycine, 10·0 μmoles riboso-5-phosphate, 7·3 μmoles Na phosphoglycerate, 47·0 μmoles $MgSO_4.7H_2O$, 1·3 μmoles $H^{14}COONa$ (2·0 μc), 2·0 μmoles tetrahydrofolic acid, 0·44–6·96 μmoles MBBA, and 1·2 ml of 0·05 M veronal buffer, pH 7·6. To this mixture 2·0 ml enzyme extract was added to give a total volume of 4·4 ml. The samples were incubated at 37°. Portions of 1·8 ml were withdrawn from each reaction vessel immediately after the addition of the enzyme and after incubation for 45 min. The samples were deproteinized with 1·0 ml 10% trichloroacetic acid. To each sample, 2·0 mg hypoxanthine was added as a carrier. Trichloroacetic acid and veronal were removed by 3-fold extraction with 5·0-ml portions of ether. For hydrolysis of the nucleotides and destruction of radioactive coenzymes, 0·5 ml 2% $FeCl_3$ in 2 N HCl was added, and the samples were heated at 100° for 30 min and evaporated under an infrared lamp to dryness. The residues were dissolved in 0·5 ml water and applied to Whatman paper no. 3. The chromatograms were developed with water-saturated *n*-butanol, and the hypoxanthine bands were cut out and eluted directly onto aluminum planchettes. The eluates were counted on a thin-window tube with the apparatus of Friesecke-Hoepfner, and corrections for background were made.

acid is primarily inhibited by MBBA, the concentration of the metabolites participating in the reaction sequence was varied. Table 1 demonstrates that it was not possible to affect the inhibition in this manner. It was therefore thought that the drug might inhibit enzymes involved in the transformation of tetrahydrofolate coenzymes. As a result, the effect of MBBA on tetrahydrofolate formylase action

TABLE 1. THE EFFECT OF TWO DIFFERENT SUBSTRATE CONCENTRATIONS ON THE INHIBITORY EFFECT OF MBBA ON THE PURINE BIOSYNTHESIS

Substrate	Substrate (μmoles)	Inhibition (%)
ATP	19·1	35·5
ATP	38·2	34·4
Homocysteine	19·2	33·0
Homocysteine	38·4	36·4
Glutamine	27·3	35·5
Glutamine	54·6	36·7
Glycine	19·0	50·8
Glycine	38·0	47·5
Ribose-5-phosphate	10·0	50·8
Ribose-5-phosphate	20·0	42·2
3-Phosphoglyceric acid	7·3	35·5
3-Phosphoglyceric acid	14·6	37·0
$H^{14}COONa$	1·3	50·8
$H^{14}COONa$	2·6	53·1
Aspartic acid	7·1	35·9
Aspartic acid	14·2	34·6

The concentration of MBBA was 0·168 μmole/1 ml incubation mixture.

was examined. Figure 2 shows that the compound interferes with the activity of this enzyme. It is interesting that MBBA inhibits formylase activity at a lower concentration than it does the bio-

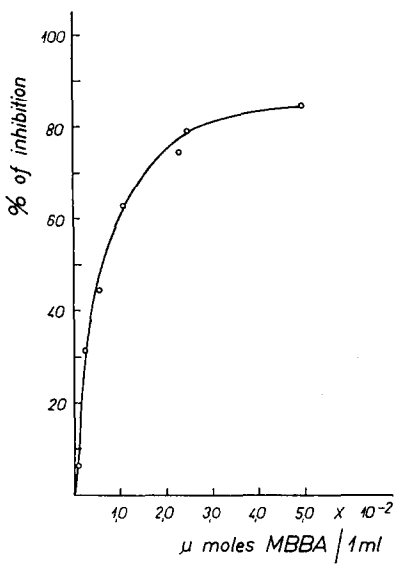

FIG. 2. Inhibition of tetrahydrofolic acid formylase activity by MBBA. The incubation mixture contained: 1·96 μmoles ATP, 2·33 μmoles FH_4, 12·5 μmoles cysteine, 100·0 μmoles NaF, 100 μmoles KCl, 20·0 μmoles $MgCl_2$, 89·7 μmoles Na formate, 3·0–150 mμmoles MBBA, and 1·2 ml 0·1 N veronal buffer, pH 7. The reaction was started by addition of 0·2 ml enzyme extract to give a total volume of 3·0 ml. Immediately after addition of the enzyme and after 1-hr incubation at 37°, samples of 1·2 ml were deproteinized by addition of 2·4 ml of 8% $HClO_4$, and the amount of $N^{5,10}$ methenyltetrahydrofolate formed was determined spectrophotometrically at 355 mμ,[10] N^{10} formyltetrahydrofolic acid formed originally by the enzymatic reaction from tetrahydrofolic acid and formate is converted to $N^{5,10}$ methenyltetrahydrofolic acid by action of perchloric acid.[11]

synthesis of purines. Inhibition of the tetrahydrofolate formylase is competitive (Fig. 3) with regard to ATP, but uncompetitive with tetrahydrofolate and formate (Figs. 4 and 5). In addition to tetrahydrofolate formylase which uses formate as the one-carbon source, other enzyme systems that can catalyze the formation of formyl derivatives of tetrahydrofolic acid from serine and formimino

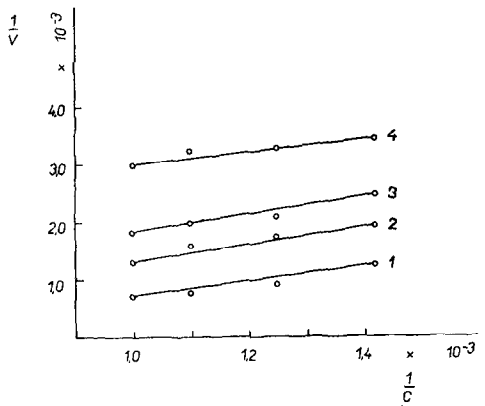

FIG. 3. Lineweaver-Burk plot demonstrating the type of inhibition of tetrahydrofolate formylase by MBBA at various tetrahydrofolate concentrations. The experimental conditions were the same as shown in Fig. 2. The concentrations of tetrahydrofolate used were 1·0–6·95 × 10^{-3} M. The MBBA concentrations were: (1) 0; (2) 2·3 × 10^{-6} M; (3) 3·5 × 10^{-6} M; (4) 5·8 × 10^{-6} M.

glutamic acid were examined for MBBA inhibition. Neither the serine aldolase + methylenetetrahydrofolate dehydrogenase nor the formino transferase + cyclodeaminase system was inhibited by a concentration of MBBA varying between 0·5 and 3·5 × 10^{-3} M. Under the same experimental conditions no inhibitory effect was exerted by the drug on folate reductase.

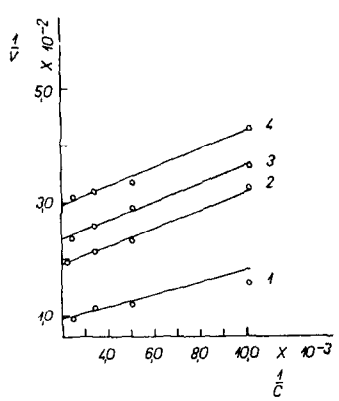

FIG. 4. Lineweaver-Burk plot demonstrating the type of inhibition of tetrahydrofolate formylase by MBBA at various formate concentrations. The experimental conditions were the same as shown in Fig. 2. The concentrations of formate used were 1·00–10·00 × 10^{-3} M. The MBBA concentrations were: (1) 0; (2) 2·3 × 10^{-6} M; (3) 3·5 × 10^{-6} M; (4) 5·8 × 10^{-6} M.

In addition to MBBA, the parent compound β-4-methoxybenzoylacrylic acid was tested as a possible inhibitor of the inosinic acid-synthesizing system and of tetrahydrolfolic acid formylase. None of these systems was inhibited by this compound at concentrations 0·1 to 2·0 × 10^{-3} M. This observation shows that the presence of the halogen in the β position of the acrylic acid moiety is necessary for the inhibitory effect of MBBA.

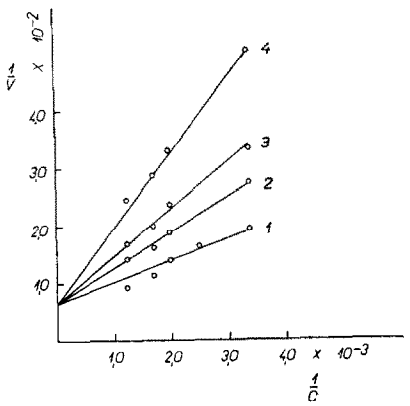

FIG. 5. Lineweaver-Burk plot demonstrating the type of inhibition of tetrahydrofolate formylase by MBBA at various ATP concentrations. The experimental conditions were the same as shown in Fig. 2. The concentrations of ATP used were 0·77–2·85 × 10^{-3} M. The MBBA concentrations were: (1) 0; (2) 2·3 × 10^{-6} M; (3) 3·5 × 10^{-6} M; (4) 5·8 × 10^{-6} M.

DISCUSSION

The observed inhibition of tetrahydrofolic acid formylase by MBBA can be explained in a number of ways. The marked chemical reactivity of MBBA may lead to its chemical interaction with tetrahydrofolic acid or, alternatively, it may result in its binding to the active enzyme site. The incubation of MBBA with tetrahydrofolic acid in the absence of the enzyme did not, however, lead to the formation of any stable complex detectable by paper chromatography. It is likely, therefore, that the compound exerts its activity by attaching itself to the enzyme. If this attachment were formed by means of a covalent bond, the inhibition would be uncompetitive against all substrates. The established competitive relationship with ATP excludes this possibility. It appears that MBBA attaches itself to the ATP site of the enzyme.[12, 13] The nucleophilic residue of MBBA, which is the halogen atom, most likely attaches itself to the electrophilic groups to which the phosphate moiety of ATP normally attaches. The observed competition between the drug and ATP may be for these groups.

The observation that the inhibition of inosinic acid biosynthesis is not amenable to reversal by ATP indicates that the inhibition of the formylase is not the only site of activity of MBBA. Such other sites will have to be elucidated by further investigations.

Institute of Hematology and Blood Transfusion,
Prague, Czechoslovakia

V. SLAVÍKOVÁ
M. SEMONSKÝ*
K. SLAVÍK†
J. VOLEJNÍKOVÁ

* Pharmaceutical and Biochemical Research Institute, Prague.
† Laboratory for Protein Metabolism, Charles University, Prague.
Present address: Dept. of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, N.Y., 14203.

REFERENCES

1. M. Semonský, E. Ročková, V. Zikán, B. Kakáč and V. Jelínek, *Coll. Czech. chem. Commun.* **28**, 377 (1963).
2. V. Jelínek and M. Semonský, *Čas. Lék. čes.* **102**, 183 (1963).
3. V. Francová, K. Ráž, Z. Franc, V. Zikán, V. Jelínek and M. Semonský, *Čs. farm.* In press.
4. O. Dvorák, J. Venta and M. Semonský, *Neoplasma.* **12**, 93 (1965).
5. B. L. O'Dell, J. M. Vandenbelt, E. S. Bloom and F. F. Pfiffner, *J. Am. chem. Soc.* **69**, 250 (1947).
6. V. Slavíková and K. Slavík, *Biochim. biophys. Acta* **71**, 604 (1963).
7. H. Tabor and L. Wyngaarden, *J. biol. Chem.* **234**, 1830.
8. V. Slavíková and K. Slavík, *Experientia* **17**, 113 (1961).
9. M. J. Osborn and F. M. Huennekens, *Biochim. biophys. Acta* **26**, 646 (1957).
10. G. R. Greenberg, L. Jaenicke and M. Silverman, *Biochim. biophys. Acta* **17**, 589 (1965).
11. D. B. Cosulich, B. Roth, J. M. Smith, M. E. Hultquist and R. P. Parker, *J. Am. chem. Soc.* **73**, 5006 (1957).
12. H. R. Whiteley, M. J. Osborn and F. M. Huennekens, *J. biol. Chem.* **239**, 1538 (1959).
13. H. R. Whiteley and F. M. Huennekens, *J. biol. Chem.* **237**, 1290 (1962).