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CANCEROSTATIC AGENTS—XXXVII*

THE EFFECT OF β-AROYL-β-HALOGEN ACRYLIC ACIDS ON THE TETRAHYDROFOLIC ACID FORMYLASE

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Abstract—Several derivatives and analogues of β-4-methoxybenzoyl-β-bromoacrylic acid (MBBA) have been tested as inhibitors of tetrahydrofolate formylase from pigeon liver and the effect of structural changes on the inhibitory activity has been studied. The inhibitory effect is dependent on a halogen atom in the β-position of the acrylate moiety adjoining the double bond and further by the free carboxyl of the acrylate moiety. Every change in this area leads to complete loss of the inhibitory activity.

Substitution of the 4 position in the aromatic nucleus by an alkyl or alkoxy group enhances the inhibitory effect, the length of the aliphatic chain being without considerable effect. Substitution by an acetamido group suppresses the effect considerably. The methylation or methoxylation of the aromatic nucleus in the 2, 3 and 6 positions considerably diminishes the inhibitory activity, but it does not eliminate completely the inhibitory effect.

Preincubation of MBBA with tetrahydrofolate formylase does not influence the inhibitory effect; the dialysis of the enzyme-MBBA mixture (either preincubated or not) leads to complete recovery of tetrahydrofolate formylase activity. The inhibition of the enzyme by MBBA and similar substances seems to be completely reversible.

SODIUM salt of *cis*-β-4-methoxybenzoyl-β-bromoacrylic acid, *cis* Br, H (MBBA) proved to have a cytostatic effect in the chemotherapy of several experimental animal tumors[1–3] and it showed also certain chemotherapeutic effect on some types of human tumors.[4, 5]

In our previous communication[6] we have established that MBBA inhibits ^{14}C formate incorporation into inosinate synthesized by pigeon liver enzymes. A more detailed investigation showed that MBBA inhibits tetrahydrofolate formylase isolated from the same source. The inhibition is competitive with ATP and uncompetitive with tetrahydrofolate and formate.

This paper describes the inhibitory effect of some substances related to MBBA on tetrahydrofolate formylase from pigeon liver. The study has been performed to bring some information about the nature of binding of MBBA and related substances to the mentioned enzyme and to get information about the relation between the structure and inhibitory activity.

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MATERIALS AND METHODS

Enzyme preparation. An extract from pigeon liver aceton powder was used as the source of tetrahydrofolate formylase and assayed in the same manner as described previously.[6] The incubation mixture contained: 1·96 μmoles ATP, 2·33 μmoles FH_4, 12·5 μmoles cystein, 100·0 μmoles NaF, 100·0 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; 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.

Derivatives of β-aroyl-β-halogen acrylic acid. The substances tested were chromatographically pure, see references in Table 1. For the inhibition experiments the aqueous solution of sodium salt of the MBBA was employed. The other derivatives were dissolved in 1% sodium bicarbonate.

RESULTS

The following changes in the basic molecule of MBBA performed in order to determine the reactive groups involved in the interaction with tetrahydrofolate formylase and conditioning the cytostatic effect of MBBA:

1. Changes in the aliphatic chain of the β-4-methoxybenzoyl-β-bromoacrylic acid.
2. Replacement of the methoxy group in the 4 position of the aromatic nucleus by other substituents.
3. Additional substitution of the other positions of the aromatic nucleus by methoxy or methyl group or groups.

The comparison of the inhibitory effect of various analogues of MBBA and the concentrations of the analogues causing 50 per cent inhibition are given in Table 1.

From the results presented here several conclusions concerning the inhibitory activities of compounds studied can be made. The substitution of bromine by chlorine does not considerably influence the inhibitory properties of MBBA and of its analogues.

The elimination of the halogen atom leads to a complete loss of the inhibitory activity of MBBA and its chlorine analogues tested. If the halogen atom is present in the α position the inhibitory effect is also completely lost.

The same effect is exhibited by elimination of the double bond in the acrylate moiety even if the halogen atom in the β-position is still present, because β-4-methoxybenzoyl-β-bromopropionic acid proved to be ineffective towards tetrahydrofolate formylase.

The amidation of the acrylate carboxyl by glycine or β-alanine residue as well as by aminoethylamine leads also to a complete loss of the inhibitory activity showing that the free carboxyl of acrylate conditions the inhibitory effect of MBBA.

The substitution of the methoxy group in the 4 position of the aromatic nucleus by other groups effecting the inhibitory activity towards tetrahydrofolate formylase depends to a certain extent on the nature of the substituent. If the alkoxy group was substituted by an alkyl, halogen or hydroxy groups no substantial change in the

biochemical activity could be observed. The prolongation of the carbon chain of the alkoxy group up to the hexyloxy analogue or of the alkyl group up to the butyl analogue does not influence the inhibitory activity.

The elimination of the methoxy group in the 4 position of the aromatic ring diminished the inhibition effect by about 35 per cent, while the substitution of the methoxy group in the position 4 by an acetamido group led to the 50 per cent loss of activity. These findings bring some evidence that a neutral or electrophilic substituent in the position 4 is necessary for the inhibitory activity of the MBBA analogues while a nucleophilic group in this position prevents the binding.

TABLE 1. THE COMPARISON OF THE INHIBITORY EFFECT OF DIFFERENT β-AROYLACRYLATES AND RELATED SUBSTANCES ON TETRAHYDROFOLATE FORMYLASE

Inhibitor	Concentration of inhibitor causing 50% of inhibition (μmoles/ml)
β-Benzoylacrylic acid[12]	no inhibition at concentration $1{\cdot}16 \times 10^{-1}$
β-4-Fluorobenzoylacrylic acid[13]	
β-4-Chlorobenzoylacrylic acid[14]	
β-4-Iodobenzoylacrylic acid[15]	
β-*p*-Toluylacrylic acid[15]	
β-4-Butylbenzoylacrylic acid[16]	
β-4-Pentylbenzoylacrylic acid[16]	
β-4-Hydroxybenzoylacrylic acid[15]	
β-4-Methoxybenzoylacrylic acid[15]	
β-4-Acetamidobenzoylacrylic acid[15]	
β-4-Methoxybenzoyl-β-bromopropionic acid[17]	
β-Benzoyl-β-bromoacrylic acid[18]	$6{\cdot}0 \times 10^{-3}$
β-4-Fluorobenzoyl-β-bromoacrylic acid*	$3{\cdot}5 \times 10^{-3}$
β-4-Chlorobenzoyl-β-bromoacrylic acid[15]	$1{\cdot}16 \times 10^{-3}$
β-4-Iodobenzoyl-β-bromoacrylic acid*	$1{\cdot}87 \times 10^{-3}$
β-*p*-Toluyl-β-bromoacrylic acid[19]	$1{\cdot}16 \times 10^{-3}$
β-4-Ethylbenzoyl-β-bromoacrylic acid*	$1{\cdot}61 \times 10^{-3}$
β-4-*n*-Propylbenzoyl-β-bromoacrylic acid*	$1{\cdot}7 \times 10^{-3}$
β-4-*n*-Butylbenzoyl-β-bromoacrylic acid*	$1{\cdot}16 \times 10^{-3}$
β-4-*n*-Pentylbenzoyl-β-bromoacrylic acid*	$3{\cdot}1 \times 10^{-3}$
β-4-*n*-Decylbenzoyl-β-bromoacrylic acid*	$3{\cdot}4 \times 10^{-3}$
β-4-Hydroxybenzoyl-β-bromoacrylic acid*	$4{\cdot}06 \times 10^{-3}$
β-4-Methoxybenzoyl-β-bromoacrylic acid[1, 19]	$1{\cdot}16 \times 10^{-3}$
β-4-Methoxybenzoyl-β-chloroacrylic acid[1]	$9{\cdot}3 \times 10^{-4}$
β-4-Methoxybenzoyl-α-bromoacrylic acid*	no inhibition at concentration $1{\cdot}16 \times 10^{-1}$
β-4-Ethoxybenzoyl-β-bromoacrylic acid*	$1{\cdot}4 \times 10^{-3}$
β-4-Ethoxybenzoyl-β-chloroacrylic acid[1]	$1{\cdot}52 \times 10^{-3}$
β-4-*n*-Propoxybenzoyl-β-bromoacrylic acid*	$1{\cdot}75 \times 10^{-3}$
β-4-*n*-Butoxybenzoyl-β-bromoacrylic acid*	$1{\cdot}46 \times 10^{-3}$
β-4-*n*-Pentyloxybenzoyl-β-bromoacrylic acid*	$1{\cdot}27 \times 10^{-3}$
β-4-*n*-Pentyloxybenzoyl-β-chloroacrylic acid*	$1{\cdot}4 \times 10^{-3}$
β-4-*n*-Hexyloxybenzoyl-β-bromoacrylic acid*	$1{\cdot}87 \times 10^{-3}$
β-4-Acetamidobenzoyl-β-bromoacrylic acid*	$3{\cdot}83 \times 10^{-2}$
β-4-Methyloxybenzoyl-β-bromoacrylic aminoethylamide[20]	no inhibition at concentration $1{\cdot}16 \times 10^{-1}$
N-(β-4-Methoxybenzoyl-β-bromoacrylyl)glycylglycine ethylester*	
N-(β-4-Methoxybenzoyl-β-bromoacrylyl)-β-alanine[20]	
N-(β-4-*n*-Hexyloxybenzoyl-β-bromoacrylyl)glycine[20]	
β-4-Methoxybenzoyl-β-chloroacrylyl methylamide[8]	
β-4-Methoxybenzoyl-β-chloroacrylyl ethylamide[8]	
β-4-Methoxybenzoyl-β-chloroacrylyl-*n*-propylamide[8]	
N-(β-4-Methoxybenzoyl-β-chloroacrylyl)glycine[20]	

* The synthesis and properties of this compound will be reported in a separate communication.

The substitution of some of the other positions of the aromatic nucleus of MBBA (i.e. positions 2, 3, 6) by a methoxy or methyl groups led to a marked change in the inhibitory activity. In all cases even the 50 per cent of inhibition could not be reached. (See Fig. 1 and Table 2). A marked decrease of the inhibitory activity was observed by the 2,4-dimethoxy analogue and 2,3,4-trimethoxy analogue. 2,4,6-trimethoxy analogue did not show any inhibitory activity at all. The substitution of the 4-methoxybenzoate moiety by the 4 methoxynaphtoate led to the complete loss of the inhibitory activity.

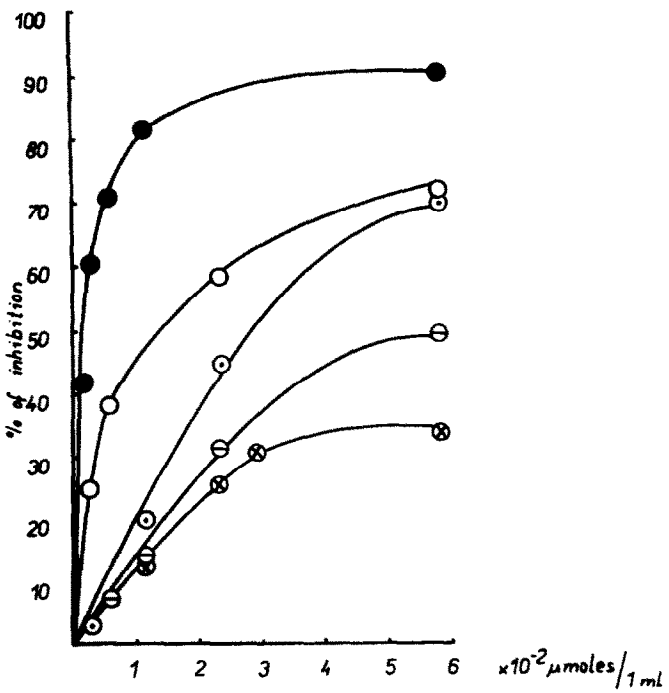

FIG. 1. The effect of concentration of various types of tetrahydrofolate formylase inhibitors. ●—β-4-Methoxybenzoyl-β-bromoacrylic acid (MBBA); ○—β-4-Hydroxybenzoyl-β-bromoacrylic acid; ⊙—β-4-Acetamidobenzoyl-β-bromoacrylic acid; ⊖—β-2,4-Dimethoxybenzoyl-β-bromoacrylic acid; ⊗—β-2,3,4-Trimethoxybenzoyl-β-bromoacrylic acid.

All these findings bring some evidence that the methylation and methoxylation of the positions 2,3,6 (or condensation of the benzoate nucleus with another aromatic ring) affects the inhibitory activity of the MBBA derivatives markedly.

Because of the high chemical reactivity of the halogen in the β-position of the acrylate part of the molecule we supposed that MBBA and its derivatives may bind to the enzyme forming a covalent bond with some nucleophilic site in the enzyme protein. This fact has been demonstrated by Baker[7] on enzyme inhibitors carrying a reactive group in their molecules ("exo" or "endo" alkylating agents of enzymes). If a covalent bond between the inhibitor and enzyme would exist, the preincubation of the enzyme with MBBA should lead either to an increase of the inhibitory activity or at least the inhibited enzyme could not have been reactivated by dialysis.

As demonstrated in Table 3, the preincubation of the MBBA with the enzyme does not considerably influence its inhibitory power on tetrahydrofolate formylase. The dialysis of a preincubated mixture containing enzyme and MBBA leads to the almost complete recovery of its activity. Therefore it seems very probable that MBBA binds to tetrahydrofolate formylase in a reversible manner not inactivating the enzyme by a covalent bond formation. The concentration effect of MBBA and similar substances shown in Fig. 1, eliminates also the possibility that the enzyme is inhibited in a "stoichiometric" manner.

TABLE 2. THE INHIBITORY EFFECT OF DIFFERENT β-4-ALKOXY(ALKYL)AROYLACRYLATES SUBSTITUTED IN THE POSITIONS 2,3 AND 6 OF THE AROMATIC RING (NONE OF THE COMPOUNDS TESTED EXHIBITED THE INHIBITION OF 50%)

Inhibitor	% of inhibition caused by 0·58	1·16	2·32	5·80 × 10^{-2} μmoles
β-2,4,6-Trimethylbenzoyl-β-bromoacrylic acid*	0	0	0	0
β-2,4-Dimethoxybenzoyl-β-bromoacrylic acid*	9	14·0	31·5	49·0
β-2,3,4-Trimethoxybenzoyl-β-bromoacrylic acid*	7·5	13·0	25·5	34·0
β-2,3,4-Trimethoxybenzoyl-β-chloroacrylic acid*	5·0	9·1	23·0	33·0
β-2,4,6-Trimethoxybenzoyl-β-bromoacrylic acid*	0	0	0	0
β-2-Methoxynaftoyl-β-hydroxyacrylic acid*	0	0	0	0
β-4-Methoxynaftoyl-β-bromoacrylic acid*	0	0	0	0

* The synthesis and properties of this compound will be reported in a separate communication.

TABLE 3. EVIDENCE FOR REVERSIBLE BINDING OF MBBA TO TETRAHYDROFOLATE FORMYLASE

Incubation mixture	% of Activity†
Fresh enzyme with MBBA*	47·5
Enzyme preincubated with MBBA for 17 hr	49·3
Enzyme preincubated with MBBA for 17 hr and then dialyzed for 24 hr	92·7
Enzyme + MBBA dialyzed for 24 hr immediately after addition of inhibitor	100·0

* Concentration of MBBA in all experiments was 0·35 × 10^{-2} μmoles/ml.
† In all control experiments (100 per cent of activity) served an enzyme preparation treated in the same manner as the sample but without addition of the inhibitor. During the preincubation (at 4°) or dialysis the enzyme activity varied in the range 2–7 per cent.

DISCUSSION

Although the presented results do not permit to form a clear picture of the nature of binding of MBBA to tetrahydrofolate formylase, several conclusions can be made concerning the groups in the inhibitor molecule involved in the binding with the enzyme.

The main group conditioning the inhibitory activity of MBBA and its analogues is the halogen on the β-carbon atom of the ethylenic double bond, because the elimination of the double bond or the halogen leads to the completely inactive substances. The free carboxyl of acrylate seems to be of similar importance for the inhibitory activity of MBBA because its amidation also completely eliminates the inhibitory effect. Therefore we suppose that the inhibitors belonging to the MBBA group may bind to the enzyme primarily by the β-halogen acrylate moiety containing free carboxyl. It is noteworthy that the bulkiness of the halogen atom does not influence the inhibition. It is perhaps possible that the halogen atom is not directly attached to the binding site of the enzyme for the inhibitor or that there is a relatively large space tolerance in this area. On the other hand, the distance between the carboxyl and halogen atom seems to be very important for the binding because the α-bromo analogue of MBBA is ineffective. Although the halogen atom is reactive and the formation of a covalent bond might be supposed, the dialysis and preincubation experiments bring an evidence that the binding of the inhibitor to the enzyme is reversible.

Concerning the reactivity of carboxylate ion with the enzyme any definite conclusion can be made about the nature of binding. The tested compounds with modified carboxyl group were β-4-methoxybenzoyl-β-bromoacrylylamides[8] with relatively bulky substituents. MBBA esters could not be tested because of their low solubility in aqueous media. Therefore it is hard to decide whether the carboxyl group is bound as a carboxylate ion or in a different way. The ineffectiveness of the compounds in which the carboxyl group was amidated by various substituents could be explained in two ways. Either by the fact that the amidation prevents the ionization of the carboxyl or by the steric hindrance of the binding caused by the spaceful substituent of the amide.

The necessity of the aromatic nucleus for binding to the enzyme is apparent from the influence of its substitution on the inhibitory activity of the compounds tested. The ineffectivity of prolongation of the carbon chain by alkoxy or alkyl groups in the 4 position claims for a relatively large space tolerance in this region, nevertheless the introduction of a nucleophilic substituent (an acetamido group) seems to prevent the binding. The lowering or loss of the inhibitory activity by substitution of positions 2,3,6 may lead to the conclusion that there is a relatively low space tolerance in this area and the mentioned atoms of aromatic ring may be directly involved in the binding to the enzyme. There is no experimental evidence concerning the nature of binding of the aromatic nucleus. But according to Baker's[9] and Plante's[10] experiments with other folate interconverting enzymes a hydrophobic bond between the enzyme and aromatic nucleus could be supposed the atoms 2,3,6 being the sites of attachment to the enzyme molecule.

Tetrahydrofolate formylase is the only enzyme participating in purine biosynthesis inhibited by MBBA but the concentrations of the inhibitor are rather high. Therefore it is not certain whether the inhibition of this enzyme is responsible for the cytostatic

effect of MBBA or if other enzyme systems are the target of this group of cytostatics. A close correlation between the cytostatic properties and tetrahydrofolate formylase inhibition supports an idea about tetrahydrofolate formylase as the target enzyme. Nevertheless, experiments in which MBBA was used as a growth inhibitor of *S. faecalis*,[11] showed that the growth inhibitory effect of MBBA cannot be reversed by either folate or tetrahydrofolate derivatives or purine nucleosides but that is reversed by essential amino acids.

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