# INHIBITION OF ACETYLCHOLINE ESTERASE AND BUTYRYLCHOLINE ESTERASE WITH ISOQUINOLINE ALKALOIDS

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The inhibition of the mentioned enzymes with 16 natural and modified isoquinoline alkaloids was investigated. Protoberberines are very strong inhibitors of both enzymes, quaternary tetrahydroprotoberberines and benzylisoquinolines are comparably strong in butyrylcholine esterase and slightly weaker in the case of acetylcholine esterase. Representatives of the pavinane and protopine groups are the weakest inhibitors of both enzymes in the series tested. The results confirm that these substances are bound competitively to butyrylcholine esterase and non-competitively to acetylcholine esterase, and they also show that the charge has a decisive effect on the force of the interaction. On the other hand the effect of the hydrophobicity of the inhibitor molecule on the bond strength is relatively small. Among the substances tested allylberberrubine is the strongest inhibitor of butyrylcholine esterase, its inhibition constant is $1\cdot3 \cdot 10^{-7}$.

Acetylcholine esterase (EC.3.1.1.7) and butyrylcholine esterase (EC.3.1.1.8) catalyse the hydrolysis of physiologically important choline esters. Compounds with quaternary nitrogen atom are frequent reversible inhibitors of these enzymes[1,2]. Relatively very strong interactions of some isoquinoline alkaloids (protoberberines, benzophenanthridines, and aporphines) with these enzymes[3,4] have been described. It followed from an analysis of the inhibition that in the case of acetylcholine esterase these substances are bound outside the active centre of the enzyme, probably into the region of the so-called $\gamma$-anionic site[3], while in the case of butyrylcholine esterase they directly penetrate the active centre of the enzyme and they complete with by the substrate[4]. The presence of the positive charge in the molecule of the inhibitor is a prerequisite of the binding of these substances to both enzymes, and hydrophobic forces also seem important for the bond strength[3,4].

The aim of this study is the completion of the series of the isoquinoline alkaloids tested so far by representatives of further groups of these alkaloids (*i.e.* quaternary tetrahydroprotoberberines, benzylisoquinolines, pavinanes, and protopines), the checking of the effect of the charge on the bond strength (using substances with a dissociable hydroxyl group and substances which form uncharged pseudobases), and also a more detailed testing of the effect of the hydrophobicity of the molecule

on the bond strength (using two series of semi-synthetic protoberberines with variously long alkyl groups in the molecule).

## EXPERIMENTAL

### Material and Methods

Compounds *II—VIII* (chlorides) were prepared from berberine sulphate (*I*) (E. Gurr, Great Britain) using a procedure described in ref.[5]. Their purity was checked by melting point measurement, TLC, and spectroscopically, as given in ref.[5]. Natural alkaloids from the *Papaveraceae* family (compounds *IX—XVI*) were isolated at the Department of Medical Chemistry, Medical Faculty, Purkyně University, Brno. Acetylcholine esterase was purchased from Sigma Co. (USA). Before use the enzymatic preparation was dialysed against 0·1 mol/l sodium phosphate buffer of pH 7·8. Human blood serum was used as the source of butyrylcholine esterase activity.

The activities of the enzymes were measured photometrically using a kinetic automat Vitatron--AKES (Holland) or a spectrophotometer Cary 118 (Varian, USA). The measurements were carried out in 0·1 mol/l sodium phosphate buffer of pH 7·8 in the presence of 0·25 mmol/l of 5,5'--dithio-bis(2-nitrobenzoate) and 0·5 mmol/l of acetylthiocholine at 25°C (substantially according to ref.[6]). The reactions were started by addition of enzymes (usually 10 μl) and the starting reaction rate ($v_0$) was determined from the absorbance increase at 405 nm. The testing of the inhibitor activity was carried out under analogous conditions in the presence of certain concentrations of alkaloids (in the case of acetylcholine esterase the resulting activity of the enzyme added into a 1 ml cell with an optical path of 1 cm was equal to 100 nkat/l, in the case of butyrylcholine esterase 30 nkat/l). The inhibition forces of the substances are given in percent of the inhibition of the initial reaction rate at the above given conditions, as $K_{0.5}$ values (*i.e.* the concentration of the inhibitor at which the initial rate decreases to one half under the given conditions) or as inhibition constants $K_{is}$. The character of the inhibition was tested at variable concentrations of the acetylthiocholine substrate (0·2—1 mol/l) and the inhibitor (I) (0—40 μmol/l). From the data obtained (a total of 30 values $v_0$ at various [S] and [I]) the values of parameters $K_{is}$ (inhibition constant of the slope) and $K_{ii}$ (inhibition constant of the intercept) were obtained by non-linear regression[7] according to equation (*1*), using a computer. The values of the Michaelis constant ($K_m$) and of the maximum rate ($V_m$) were determined from data obtained in the absence of the inhibitor.

$$1/v_0 = 1/V_m(1 + [I]/K_{ii}) + K_m/V_m \cdot 1/[S] (1 + [I]/K_{is}) \qquad (1)$$

## RESULTS AND DISCUSSION

The basic results of the inhibition measurements with both enzymes are given in Table I. The values of the inhibition of acetylcholine esterase with berberine and sanguinarine (tested earlier) are slightly higher than given[3]. This is probably due to the fact that in our case a pure enzyme preparation was used, while in ref.[3] only partly purified material. (This result is in agreement with the conclusion made in ref.[8], where the affinities of some isoquinoline alkaloids to acetylcholine esterase were tested by means of equilibrium methods). Some substances were selected from Table I (especially those which may be indicated as very strong inhibitors at least with respect

to one of the enzymes tested) and their inhibitory effect was investigated in greater detail. Berberine ($I$) is a non-competitive inhibitor of acetylcholine esterase (the values of the inhibition constants $K_{is}$ and $K_{ii}$ were practically identical and they did not differ from the $K_{0.5}$ value), but with respect to butyrylcholine esterase this compound acts practically as a competitive inhibitor ($K_{is} = 0.6$ µmol/l, $K_{ii}$ is more than an order of magnitude higher). An analogous behaviour was also assumed for other substances tested (this assumption should be fulfilled with respect to the similarity of the structures of the alkaloids – cf. similar results in refs[3,4]). The inhibitory effect of the alkaloids selected was expressed in the case of acetylcholine esterase simply by means of constant $K_{0.5}$ (which in the case of non-conpetitive inhibition is identical with the inhibition constant[9]), while in the case of butyrylcholine esterase the inhibition constants of the slope $K_{is}$ are given for the substances tested (Table II).

The results in Tables I and II show that the alkaloids of all groups tested are capable of inhibiting both investigated enzymes. The strongest inhibitors of this

TABLE I

Inhibition of acetylcholine esterase (A) and butyrylcholine esterase (B) with the tested isoquinoline alkaloids. The conditions of the measurements are given in Methods, the values given represent the average of at least 3 measurements, the number in brackets indicate the concentration of the alkaloids (µmol/l) at which the indicated percents of inhibition were achieved

| Compound | Class | Inhibition, % | |
| --- | --- | --- | --- |
| | | A | B |
| $I$ berberine[a] | protoberberines | 76(0·5) | 25(15) |
| $II$ 13-methylberberine | protoberberines | 64(0·5) | 18(15) |
| $III$ 13-ethylberberine | protoberberines | 56(0·5) | 12(15) |
| $IV$ 13-propylberberine | protoberberines | 35(0·5) | 10(15) |
| $V$ 13-butylberberine | protoberberines | 45(0·5) | 10(15) |
| $VI$ 13-ethoxyberberine | protoberberines | 30(0·5) | 8(15) |
| $VII$ butylberberrubine | protoberberines | 40(0·5) | 13(15) |
| $VIII$ allylberberrubine | protoberberines | 45(0·5) | 92(15) |
| $IX$ cis-N-methylstylopinium iodide | tetrahydroprotoberberines | 69(10) | 15(15) |
| $X$ escholidine | tetrahydroprotoberberines | 85(10) | 59(15) |
| $XI$ cyclanoline | tetrahydroprotoberberines | 44(10) | 18(15) |
| $XII$ sanguinarine[a] | benzophenanthridines | 49(10) | 50(15) |
| $XIII$ escholamine | benzylisoquinolines | 80(10) | 52(15) |
| $XIV$ papaverine | benzylisoquinolines | 56(10) | 8(15) |
| $XV$ californidine | pavinanes | 61(10) | 9(50) |
| $XVI$ protopine | protopines | 38(10) | 4(50) |

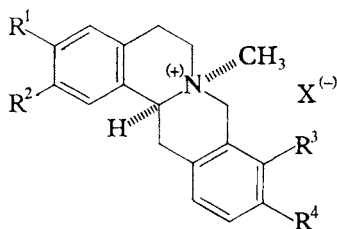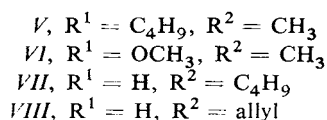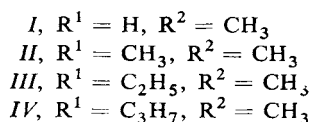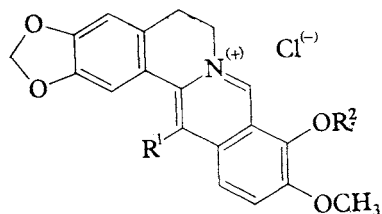[a] These compounds were tested earlier[3,4].

series of alkaloids have inhibition constants of the order $10^{-6} - 10^{-7}$ mol/l with respect to both enzymes. The data indicated for acetylcholine esterase differ only little from those in ref.[3] (*cf.* above), but the data for butyrylcholine esterase show that the affinity of this enzyme for the substances tested is higher than given[4]. This difference is caused by the fact that in ref.[4] the inhibitory effect of these substances to butyrylcholine esterase was expressed by means of constants $K_{0.5}$ which in the case of competitive inhibitors, and when relatively high substrate concentrations are used, may be higher even by several orders of magnitude than the inhibition constants $(K_{is})$. The experimental conditions in this study differ from those mentioned[4] even in the use of substrate (acetylthiocholine instead of butyrylthiocholine). However, in view of the very broad substrate specifity of this enzyme[1,2] this plays only a minor role. Since non-purified serum was used as the source of the butyrylcholine esterase activity, it may be expected that the true affinity of this enzyme to the tested substances is a little higher than the values given in Table II indicate. From Table I and II it follows that the bond strength of individual groups of isoquinoline alkaloids differs slightly in the two enzymes. It is clear that so far untested benzylisoquinoline alkaloids (especially escholamine $(XIII)$, but also the pharmacologically important papaverine $(XIV)$) as well as pavinanes $(XV)$ and protopines $(XVI)$ interact with these enzymes (even though the link of the substances from the last two groups especially to butyrylcholine esterase is somewhat weaker than in the reference substance $I$ — Table I). In the quaternary tetrahydroprotoberberines $IX - XI$ a distinct inhibitory activity was demonstrated (in the case of butyrylcholine esterase the inhibition constants of these alkaloids are comparable with the values obtained for very active protoberberines, while in the case of acetylcholine esterase these substances are somewhat weaker inhibitors in comparison with protoberberines).

TABLE II

Inhibition constants characterizing the binding of some alkaloids to acetylcholine esterase (A) and butyrylcholine esterase (B). The values of $K_{0.5}$ and $K_{is}$ were determined as indicated in Methods (the $K_{0.5}$ values are burdened by an approximately 10% error, the $K_{is}$ values by an approximately 15—20% error)

| Compound | A<br>$K_{0.5}$, μmol/l | B<br>$K_{is}$, μmol/l |
|---|---|---|
| *I* berberine | 0·28 | 0·6 |
| *V* 13-butylberberine | 0·57 | 2·0 |
| *VIII* allylberberrubine | 0·60 | 0·13 |
| *IX* *cis*-N-methylstylopinium iodide | 1·4 | 5·0 |
| *X* escholidine | 1·1 | 0·35 |
| *XIII* escholamine | 1·3 | 0·40 |

The data in Table I and II also permit a relatively good estimation of the effect of hydrophobicity in the strongest of the tested inhibitors, *i.e.* in protoberberine. None of the substances tested, having an alkyl group (or alkoxy group) bound to the protoberberine skeleton (series *II — VI* and *VII*, with respect to *I*) was a stronger



*I*,  $R^1 = H$,  $R^2 = CH_3$

*II*,  $R^1 = CH_3$,  $R^2 = CH_3$

*III*,  $R^1 = C_2H_5$,  $R^2 = CH_3$

*IV*,  $R^1 = C_3H_7$,  $R^2 = CH_3$

*V*,  $R^1 = C_4H_9$,  $R^2 = CH_3$

*VI*,  $R^1 = OCH_3$,  $R^2 = CH_3$

*VII*,  $R^1 = H$,  $R^2 = C_4H_9$

*VIII*,  $R^1 = H$,  $R^2 = $ allyl



*IX*,  $R^1 + R^2 = R^3 + R^4 = OCH_2O$,  $X^{(-)} = I^{(-)}$

*X*,  $R^1 + R^2 = OCH_2O$,  $R^3 = OCH_3$,  $R^4 = OH$,  $X^{(-)} = ClO_4^{(-)}$

*XI*,  $R^1 = OCH_3$,  $R^2 = OH$,  $R^3 = OH$,  $R^4 = OCH_3$,  $X^{(-)} = I^{(-)}$
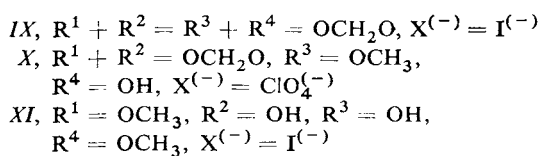
inhibitor than the basic substance, *i.e.* berberine (*I*). Hence, it is probable that in the binding of the tested substances to both enzymes hydrophobic interactions are not of basic importance. A weak decrease of the affinity with the alkyl length can evidently be attributed to the negatively acting interactions of these groups with amino acid residues of the binding sites of the enzymes for these substances (which may be connected with the steric restriction of the binding sites). These negative effects will probably prevail over a possible weakly positive effect of the increasing hydrophobicity of the molecule. The behaviour of compound *VI* is in agreement with this conclusion. This substance is the weakest inhibitor from the series of modified protoberberines (*I — VII*) with respect to both enzymes. In addition to the unfavourably bulky ethoxyl substituent (comparable with the propyl group) its low hydrophobicity is manifested negatively (according to the parameter $\pi$, which is a measure of hydrophobicity[10], it should be similar as in compound *I*). Among the semi-synthetic protoberberines tested only allylberberrubine (*VIII*) displayed an increased affinity (to butylrylcholine esterase) in comparison with the basic substance *I*. (Table II).

The very low inhibition constant ranges this substance among very strongly bound inhibitors. However, it is a substance bound reversibly, because dialysis of a mixture of serum with butyrylcholine esterase activity and compound *VIII* (10 μmol/l) led to a recovery of the enzymatic activity from the original 17% to 92%. At present relatively very few very strong reversible inhibitors of butyrylcholine esterase are known. Allylberberrubine *(VIII)* could be classified among substances which act on this enzyme specifically even in a complex biological medium, such as blood serum.
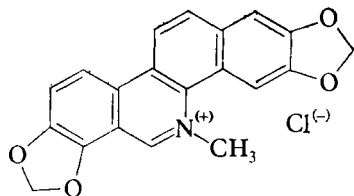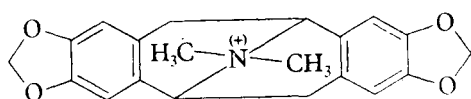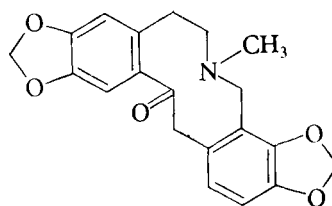


*XII*
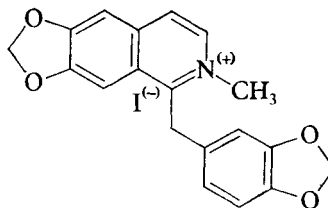


*XIII*
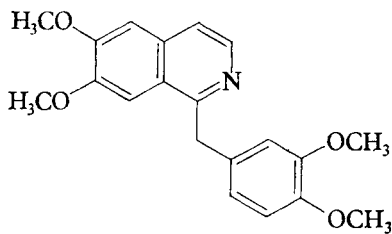


*XIV*



*XV*



*XVI*

Compound *VIII* is bound practically equally strongly as the recently described inhibitor EGYT-2347 (2-chloro-12-(2-piperidinoethyl)-dibenzo[*d,g*][1,3,6]-dioxazocin hydrochloride) the inhibition constant of which is 0·15 μmol/l, but which acts by a non-competitive mechanism[11]. When *VIII* is bound to butyrylcholine esterase

the dipole–dipole interaction of the double bond of the substituent with some suitably oriented polar group of the binding site of the enzyme will probably manifest itself favourably. The connection of the $\pi$-electrons of the allyl group double bond into the system of mobile electrons in the molecule of this substance may have a positive effect on the bond of *VIII*. The behaviour of allylberberrubine (*VIII*) with respect to butyrylcholine esterase is a further argument against the assumption of a distinctly hydrophobic site for alkaloids (and thus of the active centre as well) in this enzyme.

Hydroxyl groups are also very important in the interaction of butyrylcholine esterase with isoquinoline alkaloids, since their presence in certain positions in the molecule of the alkaloid increases the inhibitory effect substantially[4]. This effect was also confirmed in the tested tetrahydroprotoberberine alkaloids (Table I and II). In the case of acetylcholine esterase the inhibitory force of escholidine (*X*), which has an —OH group, is comparable with the effect of compound *IX*. In contrast to this, in the case of butyrylcholine esterase compound *IX* is a relatively weak inhibitor, while *X* is one of the strongest inhibitors of the substances tested. It is interesting that an introduction of a further hydroxyl into the alkaloid molecule (*cf. XI vs X* in Table I) decreases the inhibitory effect on both enzymes substantially. In the case of butyrylcholine esterase this may depend on the combination of two contradictory effects — a distinctly positive effect of the —OH group presence at a certain site of the inhibitor molecule (for example with a suitable orientation for the formation of a hydrogen bond with some group of the binding site) and a weak negative effect of the decreased hydrophobicity in consequence of the introduction of another hydroxyl.

Although the distinct and specific effect of the hydroxyl groups in the molecules of isoquinoline alkaloids becomes manifest only when they interact with butyryl-



FIG. 1

Inhibitory effect of some alkaloids on acetylcholine esterase in dependence on the pH value of the medium. The conditions of the measurements are mentioned in the Methods, the buffers used were 0·1 mol/l sodium phosphate (up to pH 8·4) and 0·1 mol/l of NaOH-glycine (above pH 9·0) ones, % means percent of inhibition. *1* berberine (*I*) (0·5 µmol/l); *2* escholidine (*X*) (5 µmol/l); *3* sanguinarine (*XII*) (10 µmol/l)
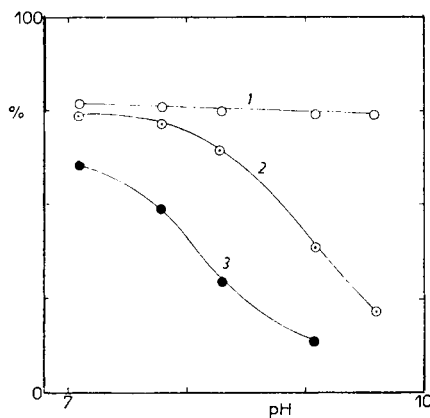
choline esterase, a substantial decrease in inhibition was observed in both enzymes when these groups were ionized. This effect was tested in media with different pH values and in the case of acetylcholine esterase it is documented in Fig. 1 (butyryl-choline esterase behaves analogously). However, this fact depends rather on the loss of the cationic character of the inhibitor molecule, because sanguinarine (*XII*), which does not contain —OH groups, but which is converted in weakly alkaline medium to an electroneutral pseudobase[12,13], also inhibits substantially less at higher pH values (Fig. 1). In contrast to this berberine (*I*), which is a cation in the pH range tested (it forms a pseudobase only in strongly alkaline medium[14]), displays practically the same inhibitory effect at various pH values (Fig. 1). The character of the curves for inhibitors *X* and *XII* in Fig. 1 is in agreement with the p$K$ values of these compounds (p$K$ ionization of the —OH group of *X* is about 8·5, the p$K$ value of *XII* is equal to about 8·0, *cf.* refs[12,13]). The data obtained confirm the assumed[3,4] decisive effect of the positive charge of the isoquinoline alkaloids during their interaction with acetylcholine esterase and buryrylcholine esterase. Also in agreement with this is the fact that the compounds with a quaternary nitrogen are (in the series tested) better inhibitors than the compounds with a tertiary nitrogen (*cf.* for example *XIII* and *XIV* and also the relatively weak activity of *XVI* on both enzymes, Table I). Both enzymes tested differ from other enzymes (the interactions of which with the alkaloid of this group have already been described in detail in refs[15−18]) in this strict requirement of a distinctly cationic character of the alkaloid and also in a relatively low selectivity with respect to various structural types of quinoline alkaloids.

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