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Uptake of L-Glutamate into Synaptic Vesicles: Competitive Inhibition by Dyes with Biphenyl and Amino- and Sulphonic Acid-substituted Naphthyl Groups

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ABSTRACT. The specificity of the vesicular L-glutamate carrier was characterized using dyes with biphenyl and amino- and sulphonic acid substituted naphthyl groups, structurally similar to the specific vesicular L-glutamate inhibitor Evans Blue. The dye Trypan Blue was the most potent inhibitor; the IC_{50} value was determined to be 49 nM. Naphthol Blue Black, Reactive Blue 2, Benzopurpurin 4B, Ponceau SS, Direct Blue 71 and Acid red 114 were also highly potent inhibitors with IC_{50} values from 330 to 1670 nM (series 1). The dyes were competitive inhibitors of vesicular glutamate uptake, and acted therefore on the glutamate transporter. Their IC_{50} values for the vesicular uptake of γ-aminobutyric acid (GABA) were all higher than 20 μM. They had no effect on synaptosomal uptake of glutamate. Furthermore, we have also found several other dyes with IC_{50} values for the vesicular uptake of glutamate ranging between 1 and 30 μM and for γ-aminobutyric acid higher than 50 μM (series 2). The most potent inhibitor Trypan Blue contains a biphenyl group, linked by azo groups to side chains containing sulphonic, amino and/or hydroxyl groups coupled to a naphthalene ring system. Trypan Blue and Evans Blue are by molecular mechanics, shown to have planar structures with conjugated double bonds throughout the structure. The other dyes, which were less effective, had phenyl and/or naphthalene groups linked by an azo group. We have also tested a series of amino and/or hydroxyl naphthalene di-/sulphonic acids that correspond to the side chains of the most potent dyes, but they had no effect on glutamate nor on γ-aminobutyric acid uptake. We conclude that the inhibitory action of these compounds is strictly dependent of the complete molecule. BIOCHEM PHARMACOL **56**;9:1243–1249, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. vesicular uptake; glutamate; GABA; dyes; Trypan Blue; glutamate transporter

During the last years, several papers have described the uptake of amino acids into synaptic vesicles from mammalian brain [1–5]. Glutamate is quantitatively the most important excitatory amino acid neurotransmitters of the vertebrate CNS, whereas GABA† is the most important inhibitory one [6–8]. The vesicular uptakes of the amino acid neurotransmitters are dependent upon an electrochemical proton gradient, generated by a MgATPase located in the vesicle membrane [9–11]. Vesicular uptake of glutamate is driven mainly by the membrane potential, whereas GABA uptake is driven by both the membrane potential and the pH gradient of the electrochemical gradient [12–14].

Glutamate and GABA are taken up into separate populations of synaptic vesicles with K_m values determined to be about 1 and 5 mM, respectively [2–4]. Low concentrations of Cl^- or Br^- are essential for the uptake of glutamate into synaptic vesicles, but not for the uptake of GABA [2, 15]. The vesicular amino acid transporters have so far not been cloned.

In a previous paper Roseth *et al.* [16] reported that a biphenyl derivative of 1,3-naphthalene disulphonic acid, known as Evans Blue and originally used diagnostically in blood volume determination, was a very potent inhibitor of vesicular glutamate uptake with IC_{50} estimated to 87 nM. Evans Blue is also a known blocker of a subset of α-amino-3-hydroxy-5-methyl-isoxazole/kainate receptors (IC_{50} = 355 nM) for the subunit combination GluR1,2 [17]. The closely related compound Chicago Skye Blue 6B (CSB) was 35-fold less potent in inhibiting the vesicular glutamate uptake. Evans Blue and CSB contain sulphonic acids with two centres of negative charge at physiological pH. Roseth *et al.* [16] have also shown that the disulphonic acid 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulphonic acid (SITS), which is an inhibitor of the chloride transport in other organs, was a competitive inhibitor of the vesicular glutamate uptake and non-competitive with respect to chloride ions.

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† *Abbreviations:* CSB, Chicago Skye Blue 6B; GABA, γ-aminobutyric acid; and SITS, 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulphonic acid.

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In this paper, we have continued our previous search for specific inhibitors of the vesicular glutamate uptake and studied several new compounds having structural similarities with Evans Blue. In addition, the effect of the various related compounds have been studied to identify the molecular regions responsible for the inhibitory effect. This information may be helpful in characterizing the glutamate binding site on the vesicular glutamate transporter and may be of help in isolating the transporter in the future. An abstract of part of the work has been presented [18].

MATERIALS AND METHODS

Materials

ATP (disodium salt), GABA, L-glutamate (dipotassium salt) and Trypan Blue were purchased from Sigma Chemical Co. [2,3-^{3}H]GABA (25–40 Ci/mmol) and L-[2,3-^{3}H]glutamate (17–25 Ci/mmol) were obtained from New England Nuclear. 1,5-Naphthalenedisulphonic acid, 1-naphthol-3,6-disulphonic acid, 2-naphthol-3,6-disulphonic acid, 1-naphthylamine-4-sulphonic acid, 2-Naphthylamine-1,5-disulphonic acid, sulphanilic acid azochromotrop and sulphonazo 111 were from Fluka. 1-Amino-8-naphthol-2,4-disulphonic acid, 1-amino-8-naphthol-3,6-disulphonic acid, 1-naphthylamine-4,8-disulphonic acid, 2-naphthylamine-3,6-disulphonic acid, 2-naphthylamine-4,8-disulphonic acid, 2-naphthylamine-5,7-disulphonic acid, 2-naphthylamine-5,7-disulphonic acid and 2-naphthylamine-6,8-disulphonic acid were from Tokyo Kasei Organic Chemicals. 3-Amino-4-hydroxybenzoic acid, 4,5-dihydroxy-1,3-benzendisulphate, 4,5-dihydroxynapthalene-2,7-disulphonic acid, 4-amino-3-hydroxy-1-naphthalene sulphonic acid, 4-amino-5-hydroxy-2,7-naphthalene disulphonic acid, Acid Blue 161, Acid Red 114, Azocarmine B, Azomethine-H, benzene-1,3-disulphonic acid, Benzopurpurin 4B, Brilliant Crocein MOO, Chromotrop 2R, Direct Blue 71, Direct Red 75, Eriochrome Blue Black, Hydroxy Naphthol Blue, Naphthalene-1,3,6-trisulphonic acid, Naphthol Blue Black, Nitrazine Yellow, Nitroso R-salt, Orange G, Ponceau S, Ponceau SS and Reactive Blue 2 were from Aldrich. Male Wistar rats (150–200 g) were obtained from Møllegaard, Denmark.

Preparation of Synaptic Vesicles

Synaptic vesicles were isolated in principle as described by Whittaker *et al.* [19] and in detail by Fykse and Fonnum [3]. Homogenates (10%) from brains of male Wistar rats (150–200 g) were prepared in 0.32 M of sucrose, 10 mM Tris-maleate (pH 7.4) and 1.0 mM EGTA and centrifuged, first for 10 min at 800 *g*. Pellets were removed and supernatants centrifuged for 30 min at 20,000 *g* to get crude synaptosomal fractions (P_2). The crude synaptosomal fractions were osmotically shocked by resuspension in 10 mM Tris-maleate (pH 7.4) and 0.1 mM EGTA and centrifuged for 30 min at 17,000 *g*. The remaining supernatants containing vesicles, were subjected to 0.4 and 0.6 M of sucrose density gradient centrifugation in a Contron TST 28.38 rotor at 65,000 *g* for 2 hr. The vesicle fractions were isolated from the 0.4 M of sucrose band and stored in liquid nitrogen without measurable loss of activity.

Assay for Vesicular Uptake

Vesicular L-glutamate and GABA uptakes were determined as described by Fykse and Fonnum [3]. The uptake assay mixture contained 110 mM potassium tartrate (if not otherwise stated), 10 mM Tris-maleate (pH 7.4) and 4 mM $MgCl_2$. Synaptic vesicles (0.07–0.1 mg of protein) were preincubated at 30° for 15 min in absence or presence of inhibitor followed by adding the substrate containing 1 mM L-[^{3}H]glutamate or [^{3}H]GABA and 2 mM ATP (disodium salt neutralized with Tris base). The mixture was further incubated for 3 min and the reaction was stopped by adding 7.0 mL of ice-cold 0.15 M KCl, immediately followed by filtration through Millipore HAWP filters (diameters 25 mm and pore size 0.45 μM). The filters were washed twice with 0.15 M KCl before adding 10 mL of Filter Count scintillation fluid (Packard). Filters were counted for retained radioactivity in a Packard Tri-Carb 2200 liquid scintillation spectrophotometer. All values were corrected for non-specific binding (blanks). Blanks were treated in the same way, but incubated on ice (2–3°) to inactivate the MgATPase. The blank values for the uptake of GABA and glutamate accounted for 15–20% and 5–10% of the total uptake, respectively. Assays were carried out in duplicate. Protein measurements were performed according to Lowry *et al.* [20]. Test agents were prepared in 1 mM stock solutions in 10 mM Tris-maleate, pH 7.4.

Molecular Mechanics

For the calculations of Trypan Blue, Evans Blue and Chicago Skye Blue 6B, software from Molecular Simulations Inc. of San Diego, CAc was used. The Insight II 95.0 modelling system was used for model building and visualization, and the Discover 95.0/3.0.0 program was used for molecular mechanics calculations, using the cuff forcefield without cross terms and with a harmonic potential of the bond stretching term. The calculations were performed at SINTEF Unimed MR Center, Trondheim, Norway. As starting point for Insight, the HEWBUG structure (dicalcium tris(3,3′-(1,1′-biphenyl)-4,4′-diazo-4,4′-diyl-bis(4-amino-1-naphthalenesulfonate)) octakis(ethyleneglycol) hydrate) from the Cambridge Structural Database from Cambridge Crystallographic Data Centre of Cambridge, UK, was used for the modeled molecules. A total of 2^7 (128) different conformations of each compound were energy minimized. The conformations were generated by starting with a model built from the HEWBUG crystal structure, and generating all possible combinations of 0 or 180° rotations of the 7 torsion angles that can be defined for the bonds connecting the ring systems. It was thus assumed that the relative orientation of the ring systems

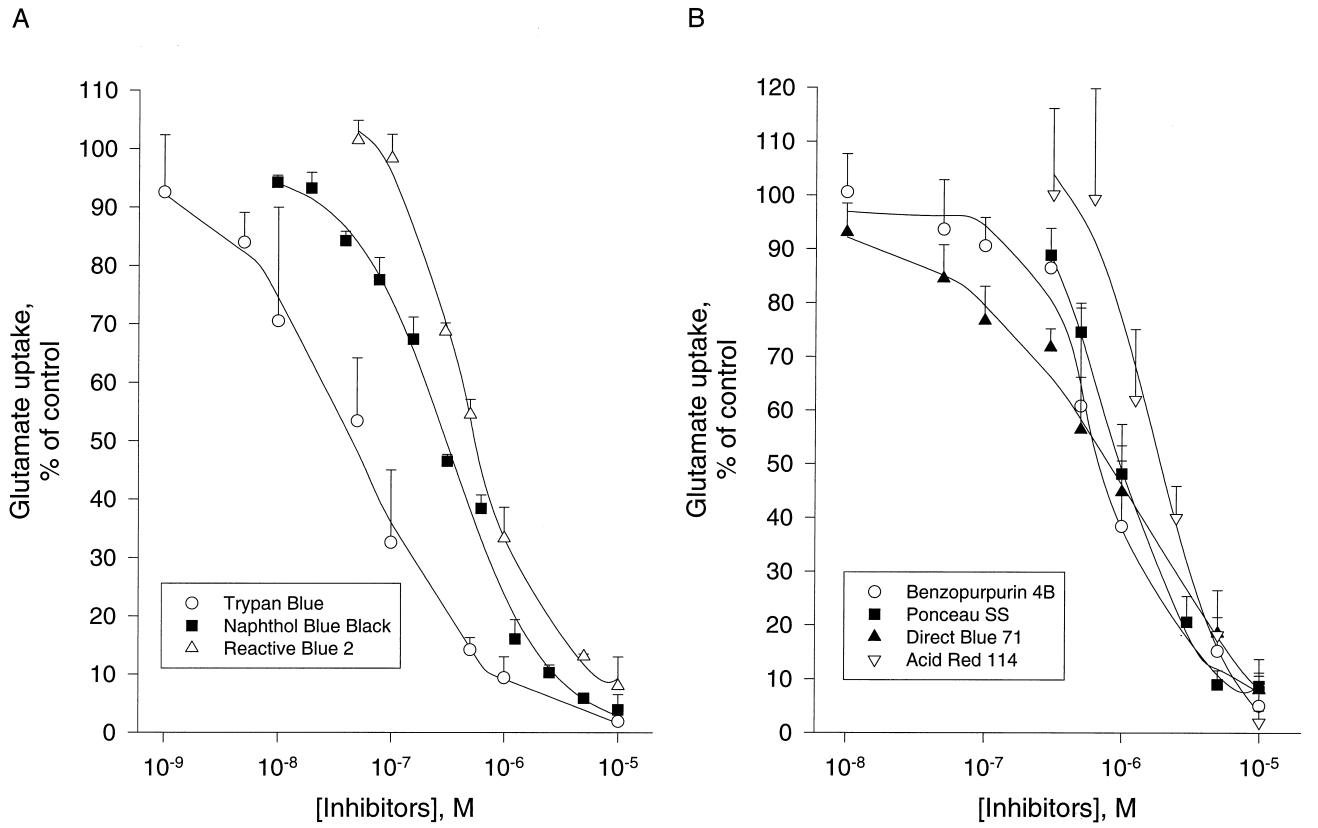

FIG. 1. Effects of various concentrations of the test agents Trypan Blue, Naphthol Blue Black, Reactive Blue 2 (A) and Benzopurpurin 4B, Ponceau SS, Direct Blue 71 and Acid Red 114 (B) on the ATP-dependent uptake of L-glutamate into synaptic vesicles. The uptake activity of glutamate is expressed as percentage of the activity in the absence of test agent (control). The inhibitors were preincubated with the vesicles for 15 min. The uptake reaction was started by the addition of the substrate. The results are expressed as mean of at least three different experiments (relative uptake, percentage of control). The uptake of glutamate in the control was 2787 ± 371 (N = 21) pmol/min/mg of protein.

found in these compounds is similar to the HEWBUG structure.

Statistical Analysis

The results are expressed as mean ± SD values of absolute uptake or as relative uptakes (percentage of controls). All figures show mean values from three separate experiments. Regression analysis (linear and non-linear) was performed.

RESULTS

In this study we have found that several dyes (Fig. 1) are highly potent inhibitors of the vesicular uptake of glutamate. The most potent one was Trypan Blue (Fig. 1A) with an estimated IC_{50} value of 49 nM. Also, Naphthol Blue Black (Fig. 1A), Reactive Blue 2 (Fig. A), Benzopurpurin 4B (Fig. 1B), Ponceau SS (Fig. 1B) and Direct Blue 71 (Fig. 1B) were very potent glutamate-uptake inhibitors (series 1). The estimated IC_{50} values are summarized in Table 1. The IC_{50} values for the vesicular uptake of GABA were 100–1000-fold higher than for the uptake of glutamate, except for Acid Red 114, the weakest of the inhibitors. Trypan Blue (0.5 μM) had no effect on the high affinity uptake of

TABLE 1. IC_{50} values of the most potent inhibitors of the vesicular uptake of glutamate and GABA

	IC_{50} values (μM)	
Series 1	**L-glutamate**	**GABA**
Trypan Blue	0.049	50
Evans Blue	0.087*	23*
Naphthol Blue Black	0.33	>50
Reactive Blue 2	0.44	44
Benzopurpurin 4B	0.68	50
Ponceau SS	0.80	>50
Direct Blue 71	1.36	>50
Acid Red 114	1.67	20
Series 2		
Chicago Skye Blue 6B	3*	>50*
Nitrazine Yellow	4	18
Eriochrome Blue Black	6	>50
Brilliant Crocein MOO	7	>50
Acid Blue 161	10	>50
Chromotrop 2R	20	>50
Direct Red 75	30	>50
Nitrozo R-sale	>50	23
2-amino-3,6-naphthalenedisulphonic acid	>50	>50

The vesicular uptake was performed as described in Materials and Methods. The IC_{50} values from Series 1 were estimated by regression analysis from at least 3 different experiments. The IC_{50} values from series 2 (the less active) were estimated graphically from at least 3 experiments.

*Taken from Roseth *et al.* (1995).

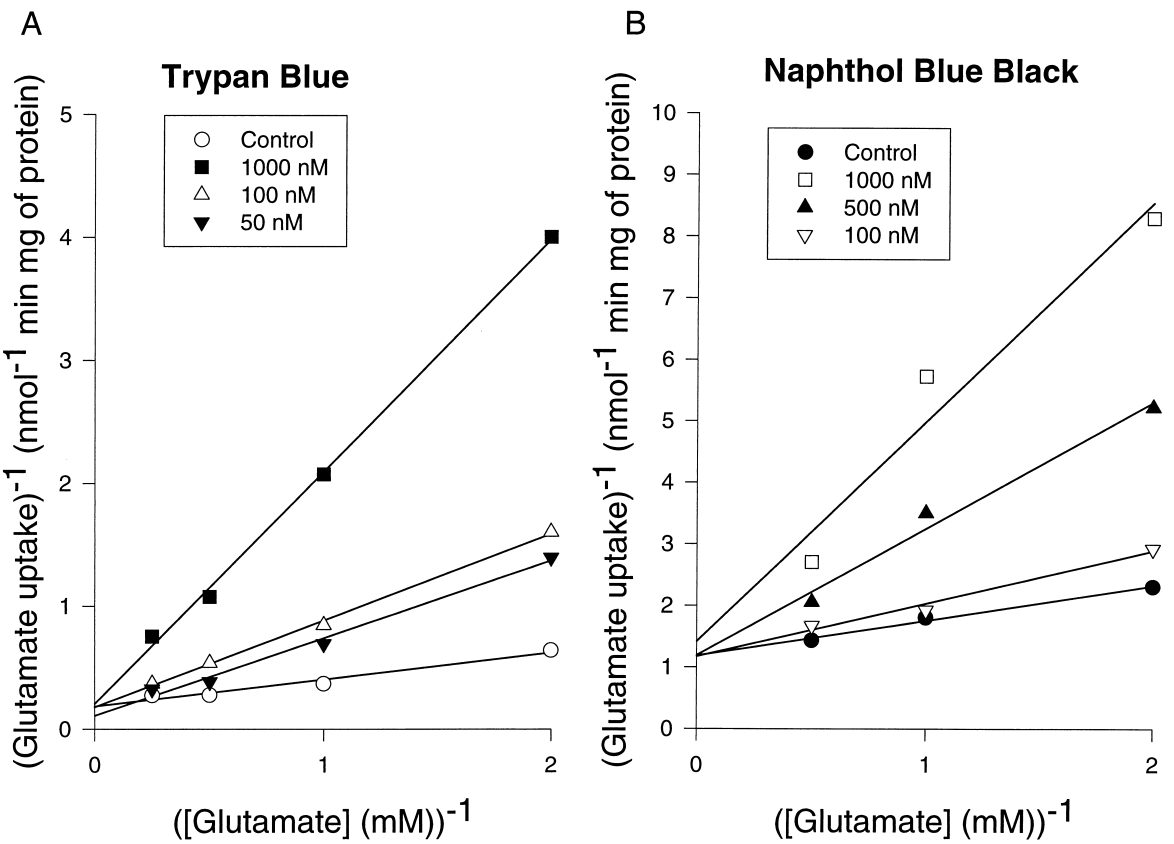

FIG. 2. Double reciprocal plots (Lineweaver–Burk plots) of the vesicular uptake of L-glutamate in absence or presence of different concentrations of Trypan Blue (A) and Naphthol Blue Black (B). The concentration of glutamate was varied between 0.5 and 4 mM. The apparent K_i values were determined by a secondary replot of the slopes of the Lineweaver–Burk plots and estimated to 50 nM and 200 nM for Trypan Blue and Naphthol Blue Black, respectively.

glutamate in plasma membranes. Another series (series 2) of related dyes, where the naphthyl and/or phenyl groups are connected by an azo group, had IC_{50} values for vesicular glutamate uptake between 1 and 30 μM and for GABA more than 50 μM (Table 1). These dyes have conjugated double bonds, but lack the dimethyl biphenyl group in the middle of the molecule. We have also studied the inhibitory effect of a series of amino and hydroxyl naphthalene di-/sulphonic acids which correspond to the different side chains of the biphenyl dye molecules. These are 4-amino-3-hydroxy-1-naphthalene sulphonic acid, 4-amino-5-hydroxy-2,7-naphthalene disulphonic acid, 1-amino-8-naphthol-2,4-disulphonic acid, 1-amino-8-naphthol-3,6-disulphonic acid, benzene-1,3-disulphonic acid, 4,5-dihydroxy-1,3 benzendisulphate, 4,5-dihydroxynaphthalene-2,7-disulphonic acid, 1,5-naphthalenedisulphonic acid, naphthalene-1,3,6-trisulphonic acid, 1-naphthol-3,6-disulphonic acid, 1-naphthylamine-4,8-disulphonic acid, 1-naphthylamine-4-sulphonic acid, 2-naphthol-3,6-disulphonic acid, 2-naphthylamine-1,5-disulphonic acid, 2-naphthylamine-3,6-disulphonic acid, 2-naphthylamine-4,8-disulphonic acid, 2-naphthylamine-5,7-disulphonic acid, 2-naphthylamine-6,8-disulphonic acid and 3-amino-4-hydroxybenzoic acid. At 10 μM these naphthalene derivatives gave no inhibition of either vesicular glutamate or GABA uptake, except of 4-amino-3-hydroxy-1-naphthalene sulphonic acid which inhibited the uptake of glutamate by 30%. The 2-naphthylamine-3,6-disulphonic acid which is the derivative of the most potent inhibitor, Trypan Blue, was also tested at 50 μM, but gave no inhibition of glutamate or GABA uptake.

Kinetic studies showed that the two mostly potent dyes, Trypan Blue and Naphthol Blue Black, were competitive inhibitors of the vesicular uptake of glutamate when the concentration of glutamate was varied and the concentration of Cl^- was kept at 4 mM (Fig. 2A and B). The K_i values were determined from a secondary replot of the slopes of the Lineweaver–Burk plots shown in Fig. 2 and were calculated to ≈50 nM and ≈200 nM for Trypan Blue and Naphthol Blue Black, respectively.

The modelling analysis of Trypan Blue, Evans Blue and CSB showed that the conjugated double bonds give these molecules an almost planar conformation (Fig. 4).

DISCUSSION

The most effective inhibitors of the vesicular uptake of glutamate were Trypan Blue, Evans Blue, Naphthol Blue Black and Benzopurpurin 4B (series 1). These are planar structures with conjugated double bonds throughout the structure (dyes) and with amino and sulphonic acid substituted naphthyl groups at the ends (Fig. 3). The effect on the vesicular uptake of GABA was 100-1000-fold less than

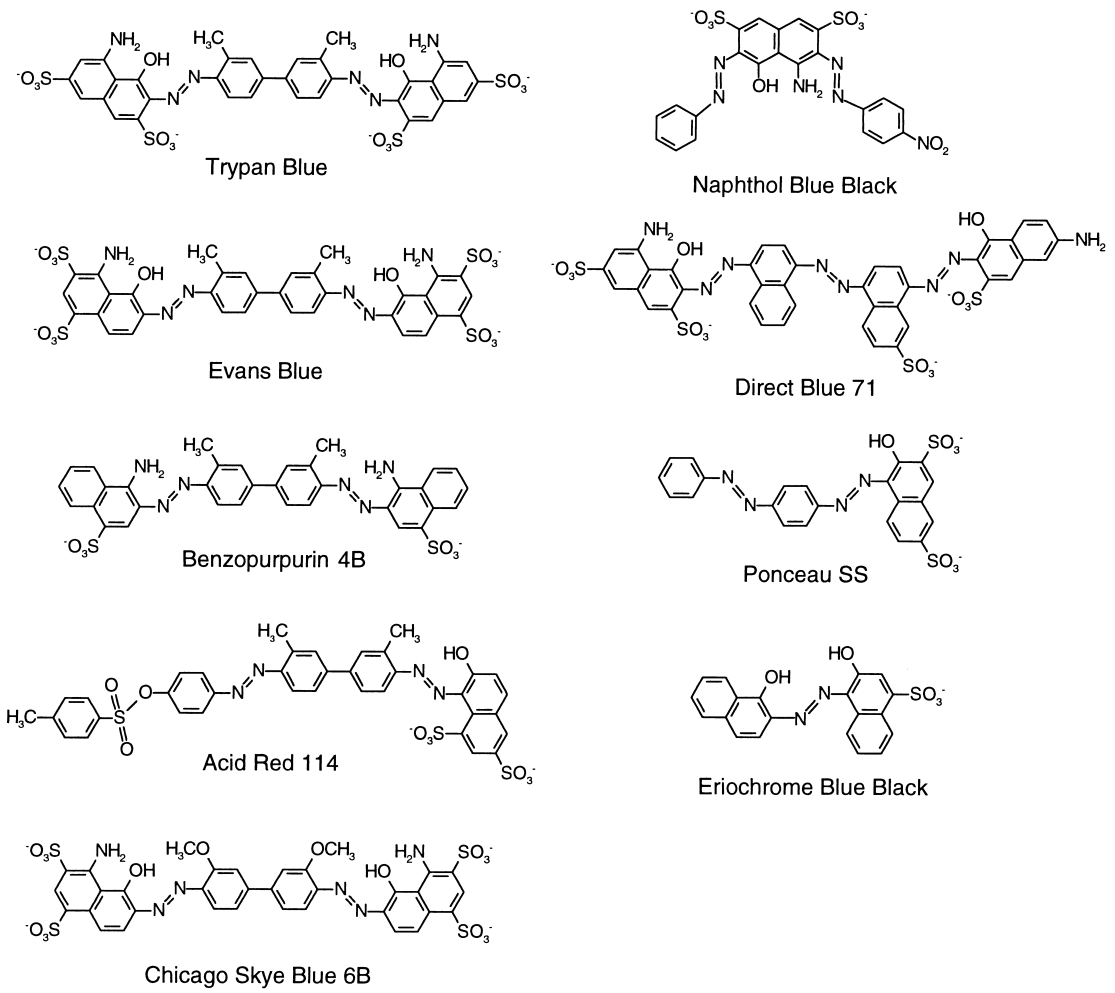

FIG. 3. Chemical structures of the most specific inhibitors of the vesicular uptake of glutamate; Trypan Blue, Evans Blue, Benzopurpurin 4B, Acid Red 114, Ponceau SS, Naphthol Blue Black, Direct Blue 71, Eriochrome Blue Black and Chicago Skye Blue 6B.

on glutamate which clearly demonstrates their specificity for the glutamate transporter.

In our previous paper [16] it was suggested that the amino and sulphonic acid substituted naphthyl groups of Evans Blue would resemble a glutamate like structure and therefore inhibit the glutamate site. The present investigation shows that this can not be the case because the naphthylamine sulphonic acids did not inhibit the vesicular uptake of glutamate, even at concentrations 1000-fold higher than used of the dyes.

We have also used molecular mechanics for visualization of the presumed most stable conformations of the best inhibitors. The calculations were performed on Trypan Blue, Evans Blue and CSB in their neutral form in vacuum simulation. Our data modelling analysis showed that conjugated double bonds give these molecules an almost planar conformation, except for a 31° torsion around the central bond on these compounds. The hydrogen bonds between the amino- and sulphonic groups also tend to favor a planar structure. None of the calculated conformations with planar geometry were substantial lower in energy than the others, indicating flexibility and possibilities for changes in conformation on interaction with the surroundings. Among the conformations lowest in energy were the almost planar structures given in Fig. 4. We observe that the conformations of the three structures are in principal identical except for the functional groups. However, Trypan Blue might distinguish itself from the other two because of the different positions of the charged groups. In Trypan Blue they are located at C3 and C6, whereas in Evans Blue they are located at C5 and C7 (Fig. 3). The glutamate binding site of the glutamate transporter has not been identified, making a calculation of the interaction between the glutamate binding site and the inhibitors impossible.

The highly potent action of Trypan Blue and Evans Blue [16] may be due to a hydrophobic attraction through the dimethyl-biphenyl group, located in the middle of the molecule, and the transporter molecule. Keller *et al.* [17] also proposed that hydrophobic or steric interaction between the binding site and the central biphenyl region of Evans Blue, is fundamental for the blocking of α-amino-3-

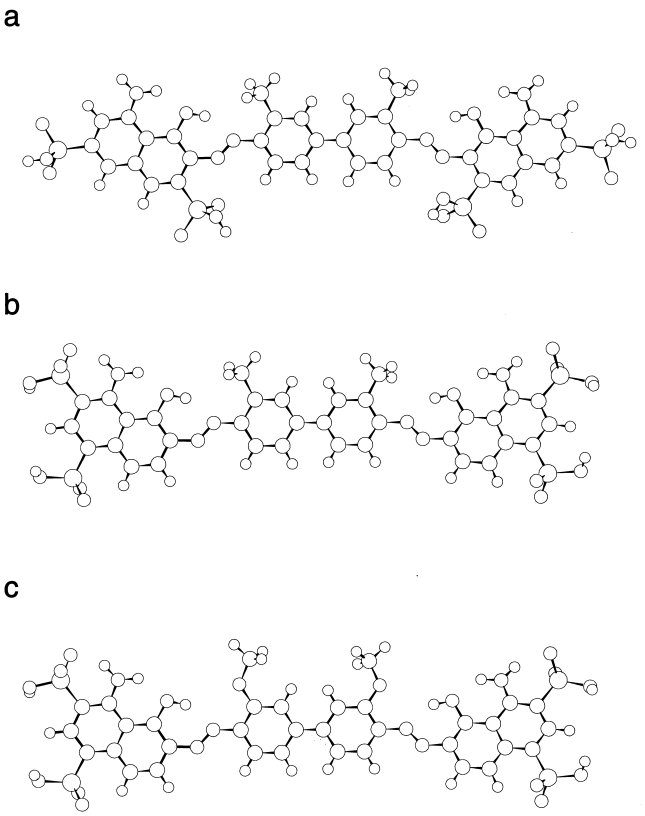

FIG. 4. Structures of the presumed most stable conformations of Trypan Blue (a), Evans Blue (b) and Chicago Skye Blue 6B (c) visualized by the Insight II 95.0 modelling system.

hydroxy-5-methyl-isoxazole and kainate glutamate receptors. In this respect it is interesting that we recently found in our lab that some PCBs (2,2-dichlorodiphenyl) are excellent inhibitors of vesicular uptake (J. Andersen, Norwegian Defense Research Establishment, personal communication).

In the case of Benzopurpurin 4B, which has such a central dimethyl-biphenyl group, the reduced inhibitory action may be explained by a decreased charge contribution in the side chains compared to Trypan Blue/Evans Blue. Acid Red 114 has, in contrast to Trypan Blue, Evans Blue and Benzopurpurin 4B, a slightly twisted conformation, shown by molecular mechanics in the vacuum phase. This conformation change is due to the -O-SO_2-linkage which bends the terminal toluene group out of the plane because both the -O-linkage and -SO_2-linkage are 120° (Dr. L. M. Bjerkeseth, Norwegian Defense Research Establishment, personal communication). In addition substitution of the two terminal groups are different from the other members of series 1. All the other azo dyes tested (Fig. 1 and 2), lack such a central hydrophobic biphenyl region, which may explain their reduced inhibitory action (Fig. 3).

The great difference in the inhibition between the almost identical Trypan Blue/Evans Blue and CSB is difficult to explain. In CSB the methyl groups are replaced by a methoxy group, and data modelling show that the methyl groups in Trypan Blue/Evans Blue are oriented outwards, whereas in CSB the methoxy groups have to be bent inwards due to steric hindrance (Fig. 4). A possible explanation is that the orientations of the methyl groups in Trypan Blue and Evans Blue do fit more precisely into the glutamate binding site of the transporter than the methoxy groups of CSB. Another explanation may be that the substitution of methyl groups with methoxy groups reduces the hydrophobicity of the molecule, which may reduce the binding to the vesicular glutamate transporter [17].

The kinetic studies show competitive kinetics between the most active inhibitors and the substrate glutamate. This is in agreement with the findings for Evans Blue [16]. These competitive kinetics probably indicate that the inhibitors, in some way, act with the glutamate binding site. This could be achieved both by binding directly to the glutamate binding site or a binding interference with the nearby area. However, no similarities between the structure of these molecules and glutamate have been seen.

It is an interesting observation that within the series of inhibitors, which can easily be compared, λ_{max} in the visible specter which reflect the chromophore (light absorbing group, i.e. the conjugated double bonds), changes from blue with Trypan Blue (607 nm), toward red (Acid Red 114 has a λ_{max} of 514 nm) with a decrease in inhibitory power.

In conclusion, the present study shows that large, planar and conjugated organic sulphonic acids, such as Trypan Blue, specifically inhibit the vesicular uptake of glutamate, and the inhibitory action is strictly dependent on the entire molecule. The most powerful inhibitors are Trypan Blue and Evans Blue, which contain a central hydrophobic dimethyl biphenyl part and amino- and sulphonic acid substituted naphthyl groups at the ends. However, the specific target of these dyes at the glutamate transporter is not known because the structure of the transporter and its glutamate binding site has not been characterized.

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