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SYNTHESIS AND CHARACTERIZATION OF AN ARYL-AZIDOPAROXETINE

A NOVEL PHOTO-AFFINITY PROBE FOR SEROTONIN-TRANSPORTER

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Abstract—Paroxetine is an effective antidepressant drug and potent serotonin (5-HT) uptake inhibitor. It selectively labels 5-HT transporter on platelets and neurons. We report here the synthesis of an aryl-azido derivative of paroxetine, which is a novel photoactive and irreversible ligand for the [^{3}H]paroxetine binding site on the platelet 5-HT transporter. The compound inhibited [^{3}H]paroxetine binding (IC_{50}, 55 nM) and 5-HT uptake (IC_{50}, 12 nM) at equilibrium conditions and inactivated 10–20% of [^{3}H]paroxetine binding sites upon irradiation at 320 nm. SDS-PAGE of platelet protein extract labelled with the radioactive analogue of the synthesized probe revealed the presence of four radioactive bands of which the 71-kDa one was the most prominent

Key words: platelet serotonin transporter; aryl-azidoparoxetine; photo-labelling

Several lines of evidence indicate that affective disorders are associated with abnormalities in neuronal 5-HT function [1]. In particular, the inhibition of 5-HT uptake by most antidepressant drugs is one of the elements of the indoleamine hypothesis of depression [2], and the potentiation of serotonergic transmission by blockade of the 5-HT re-uptake system is considered a possible mechanism of antidepressant action.

5-HT transporter protein from human brain and platelets was cloned recently, and its amino acid composition was deduced from the complementary cDNA sequence [3, 4]. Very little is known, however, about the properties of this protein such as the carbohydrate content, the molecular arrangement in the membrane, and the structure of its antidepressant binding site(s). Various purification attempts failed to yield homogenous protein [5, 6] positively identified as a 5-HT transporter.

We report here the synthesis of an aryl-azido derivative of paroxetine, which is a photo-active ligand for covalent labelling of 5-HT transporter. A radioactive analogue of the synthesized probe may facilitate purification of the native platelet or brain 5-HT transporter protein and subsequent structural and functional studies of the 5-HT transport in the reconstituted systems.

MATERIALS AND METHODS

Materials

Paroxetine hydrochloride was a gift from SKBeecham (UK). *N*-Hydroxysuccinimidyl-4-azido-salicylic acid was from the Pierce Chemical Co. (Rockford, IL), and all other chemicals were obtained from Sigma (St. Louis, MO). Radioactive [^{3}H]paroxetine (23.1 Ci/mmol)and [^{3}H]5-HT (23.7 Ci/mmol) were purchased from NEN (DuPont, Canada). Human platelet concentrates were donated by the Ottawa Red Cross Laboratories.

Synthesis of ASP

We have adapted the method described by Henderson *et al.* [7] for linking paroxetine to succinylated albumin and used an activated succinyl-azido derivative of salicylic acid for the synthesis of ASP. The reaction mixture contained 200 μmol paroxetine, 0.5 μCi[^{3}H]paroxetine as a tracer, 100μmol *N*-hydroxy-succinimidyl-4-azido-salicylic acid dissolved in 5 mL dimethylformamide and 5 mL 0.2 M $NaHCO_3$. After a 1-hr incubation at 25°C in the dark, the product was extracted three times with 5-mL aliquots of ethyl acetate. The combined extracts were dried over KCl *in substantia*, evaporated to dryness under nitrogen, and dissolved in 0.1 mM HCl in ethanol. The crude product was purified by TLC on silica gel using ethanol-ethyl acetate conc. ammonium hydroxide (5:1:0.05, by vol.) as a solvent system. The same solvent was used for the extraction of purified compound from silica gel. The final product was dissolved in ethanolic HCl (0.1 mM) and stored in the dark at −20°.

For the synthesis of radioactive ASP, 50 μCi [^{3}H]paroxetine (1.83 nmol) was incubated with 1 nmol of *N*-hydroxy-succinimidyl-4-azido-salicylic acid, and the procedure described for the synthesis of non-radioactive label was followed.

Photoinactivation and UV spectroscopy

A 25 μM solution of ASP in 0.1 mM ethanolic HCl was irradiated for 10 min using GE UV lamp, $\lambda_{max} = 320$ nm, at 24 W and a distance of 15 cm. The sample was then placed in a 1-cm quartz cuvette, and the scanning absorption spectrum was determined using a Varian DMS 90 spectrophotometer. The control spectrum was determined with a sample (25 μM) that was not irradiated.

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‖ Abbreviations: ASP, azidosalicylylparoxetine; and 5-HT, serotonin.

5-HT uptake and [^{3}H]paroxetine binding

These experiments were carried out as described by Hrdina *et al.* [8] and Mellerup *et al.* [9], respectively. Kinetic parameters of 5-HT uptake (K_m and V_{max}) were determined in saturation experiments at 5-HT concentrations of 0.1 to 1.0 μM after Lineweaver-Burk transformation of saturation isotherms. K_d and B_{max} of [^{3}H]-paroxetine binding were determined from Scatchard plots of the equilibrium binding data at [^{3}H]paroxetine concentrations of 0.05 to 1.6 nM and 10 μM fluoxetine to define the nonspecific binding.

The data presented are the results of at least three experiments carried out in triplicate.

Labelling of platelet membrane proteins

Human platelet membranes were obtained as described by Mellerup *et al.* [9]. The incubation mixture contained: 0.1 mL platelet membrane suspension (100 to 150 μg protein), 0.2 to 0.4 μCi [^{3}H]ASP in a total volume of 1 mL buffer (50 mM Tris-130 mM NaCl-5 mM KCl, pH 7.4). The control samples contained 1 mM fluoxetine to define the nonspecific binding. Incubation was carried out for 2 hr in the dark at 25°. The membranes were then spun down in an Eppendorf centrifuge (18,000 *g*, 5 min), washed with 1 mL of ice-cold incubation buffer, resuspended in 0.5 mL of the same buffer containing 0.05 mg bovine serum albumin, and irradiated for 30 min at 0° with 2 GE UV lamp (24 W, distance 15 cm). The membranes were isolated by centrifugation (as above), washed three times with 1-mL aliquots of incubation buffer, resuspended, and used for electrophoresis. An identical procedure was used for the inactivation experiment except that the [^{3}H]ASP was replaced by the non-radioactive compound.

SDS–PAGE

Labelled platelet membranes (100 to 150 μg protein) were suspended in 100 μL of 2% SDS in 1 mM Tris-HCl buffer, pH 7.4, containing protease inhibitors (1 μM phenylmethylsulfonyl fluoride, 0.01% bacitracin and aprotinin), homogenized by multiple pipetting, incubated at room temperature for 30 min, and then centrifuged for 5 min at 18,000 *g*. The supernatant was used for SDS–PAGE according to a standard procedure [10]. The gel was then sliced into 8 × 3 mm fragments that were dissolved by incubation (40°, 24 hr) in 1-mL aliquots of 30% hydrogen peroxide-conc. ammonium hydroxide (19:1, v/v). The resulting samples were mixed with 10 mL scintillation fluid (Ready Safe, Beckman) and counted for radioactivity.

RESULTS

The synthesis reaction of ASP yields only one product, which is easily identifiable by its distinct chromatographic properties in TLC. Since radioactive [^{3}H]paroxetine was added to the reaction mixture as a tracer, we were able not only to identify ASP on TLC plates (*Rf*: paroxetine, 0.17; ASP, 0.87; solvent system, see Materials and Methods), but also to determine its purity; it was over 98% for the final product.

Figure 1 shows the UV spectrum of ASP before and after 10 min of irradiation. Photo-inactivation is indicated by decreased absorbance at 265 nm. It is difficult

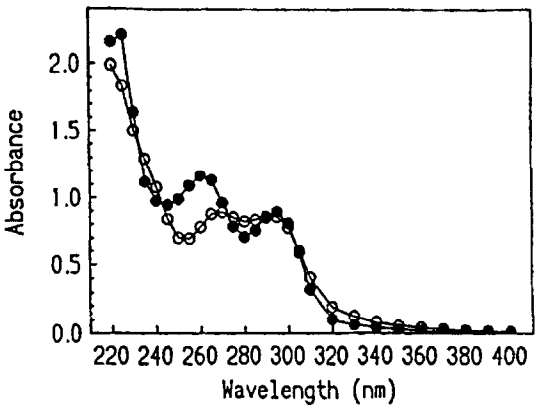


Fig. 1. UV spectrum of ASP before (●) and after (○) irradiation. Experimental conditions: ASP, 25 μM in 0.1 mM HCl in ethanol; irradiation for 10 min at 24 W and 15 cm distance (GE UV lamp).

to provide any quantitative data on the relationship between the extent of irradiation and the change of UV absorbance since inactivation of ASP is very rapid. In fact, the spectrum changes during scanning of the sample. The 10-min irradiation for spectroscopic evaluation was a convenient period of time to carry out the described experiment.

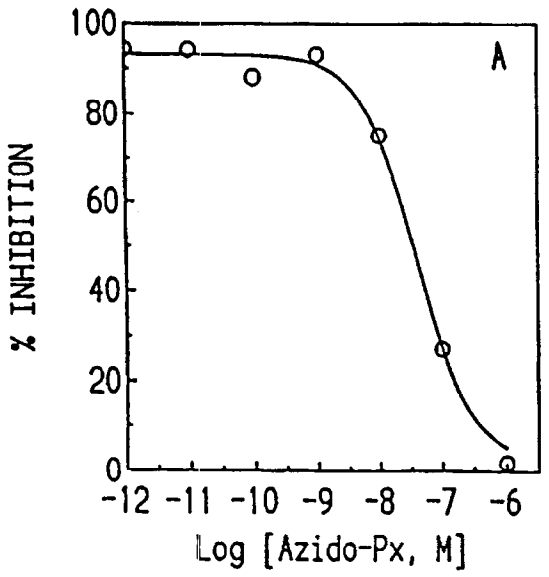
To characterize the pharmacological profile and specificity of the synthesized ASP, we determined its inhibitory activity on 5-HT uptake and [^{3}H]paroxetine binding in human platelets. The platelet 5-HT uptake was inhibited potently by ASP (IC_{50} = 12 nM, Fig. 2A). Analysis of the 5-HT uptake kinetics indicates that this inhibition was competitive as shown by the Lineweaver-Burk transformation of the saturation isotherms (Fig. 2B). ASP also inhibited [^{3}H]paroxetine binding to platelet membranes (IC_{50} = 55 nM; Fig. 3A), and this inhibition was competitive as well (Fig. 3B).

To determine whether ASP could irreversibly inactivate the [^{3}H]paroxetine binding site, the platelet membranes were incubated with an excess of ASP (100 nM), isolated by centrifugation to remove unbound ASP, and then irradiated. Since such a sample was barely optically transparent due to absorption and scattering of light, the extended (30 min) irradiation time was necessary to complete the reaction. Saturation experiments revealed that [^{3}H]paroxetine binding was reduced in ASP-treated membranes due to inactivation of 15–20% of the binding sites and a 50% decrease in affinity (Fig. 4).

The electrophoresis on SDS polyacrylamide gel indicated that [^{3}H]ASP labelled four protein fractions, designated 1–4, on Fig. 5 and corresponding to 120, 100, 71 and 25 kDa, respectively. The radioactivity recovered at the start (Slice No. 1) likely represents precipitated or non-solubilized material. The large peaks of radioactivity at the gel front (Slices Nos. 23–28) correspond to the unbound probe.

DISCUSSION

Photo-affinity labelling of 5-HT transporter from platelet membranes and brain synaptosomes was first described by Rotman and Pribluda [11]. The authors described the synthesis of radioactive 2-azidoimi-



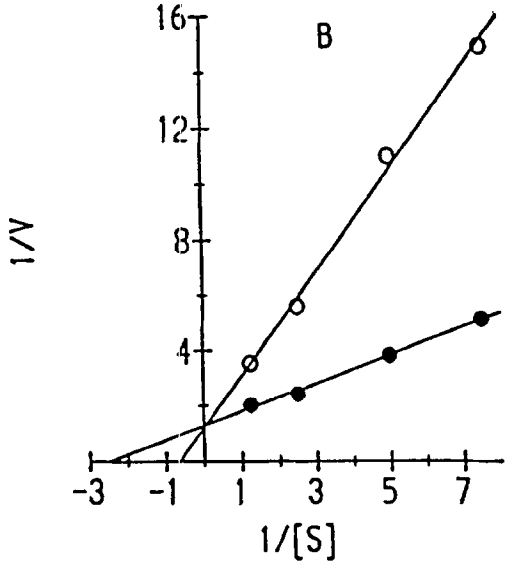
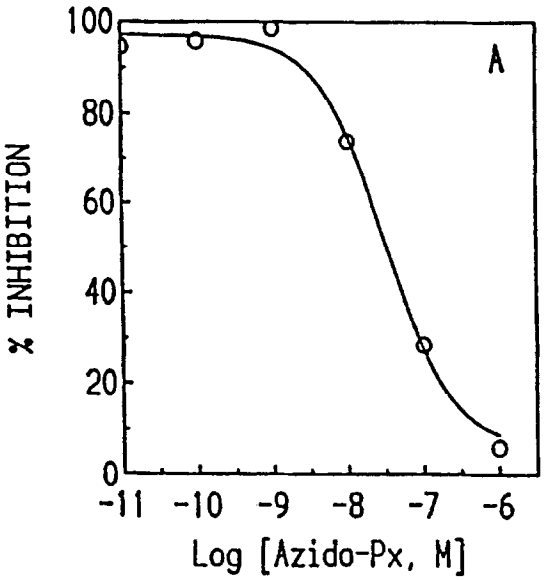


Fig. 2. (A) Inhibition of platelet 5-HT uptake by ASP. (B) Kinetics of 5-HT uptake. Key: (●) control: V_{max}, 0.770 pmol/min × 10^6 platelets; K_m, 0.391 μM; and (○) ASP, 2.33 nM: V_{max}, 0.830 pmol/min × 10^6 platelets; K_m, 1.540 μM.



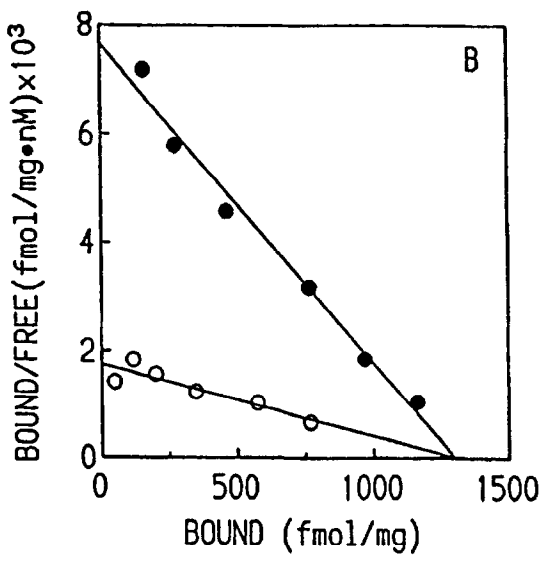


Fig. 3. (A) Inhibition of [^{3}H]paroxetine binding by ASP. (B) Kinetics of [^{3}H]paroxetine binding. Key: (●) control: B_{max}, 1304 fmol/mg; K_d, 0.170 nM; and (○) ASP, 55 nM: B_{max}, 1306 fmol/mg; K_d, 0.748 nM.

pramine, which was used subsequently for labelling its binding site. Two-dimensional electrophoresis revealed the presence of several photo-labelled proteins, two of them (40 and 60 kDa) being most prominent. Unfortunately, neither the chemical properties nor the pharmacological profile and specificity of the synthesized compound were reported. Ransom *et al.* [12] described the synthesis of a non-radioactive aryl-azido derivative of 5-HT that was a photo-dependent inhibitor of 5-HT uptake. It remains to be established what particular proteins can be identified with this label. A potential disadvantage of this ligand is that it may label other 5-HT binding proteins as well; some 5-HT receptors have nanomolar affinity for 5-HT and its derivatives. Wennogle *et al.* [13] described 2-nitroimipramine as an effective photo-affinity probe that nonspecifically labelled numerous proteins of human platelet, rat brain and liver membranes. Only one minor protein component (30 kDa) was selectively labelled by this probe in that the labelling was effectively abolished by various 5-HT uptake inhibitors. Most recently, Launay and colleagues [14] reported partial purification of platelet 5-HT transporter, which was identified by photo-affinity labelling with [^{3}H]paroxetine. However, paroxetine is not a photo-label *per se;* hence, the cross-linking of this ligand required irradiation with a high energy UV laser. It is clear that such a procedure may affect the structure and, subsequently, the properties of the 5-HT transporter protein by disruption of its multiple double bonds, leading to the formation of transient intermediate radicals and photo-oxidation.

Such problems can be avoided using ASP. The synthesis of this compound is relatively simple and can be readily performed in most laboratories. The azidosalicy-

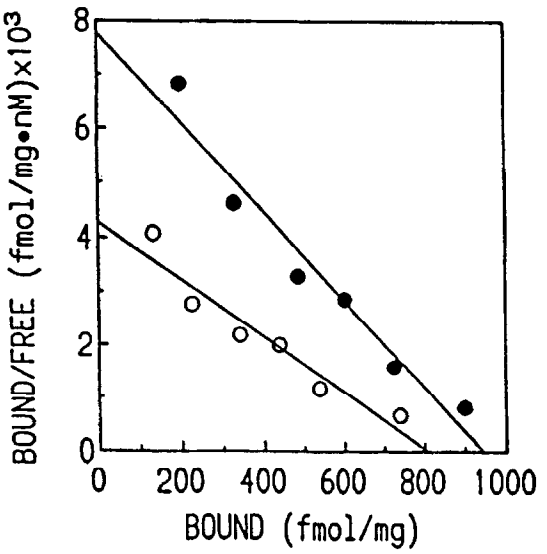


Fig. 4. Scatchard analysis of [^{3}H]paroxetine binding after photo-inactivation in the presence of ASP. Key: (●) control: B_{max}, 945 fmol/mg; K_d, 0.122 nM; and (○) ASP-photo-labelled membranes: B_{max}, 808 fmol/mg; K_d, 0.188 nM.

lyl radical is linked to the nitrogen atom of the piperidinyl residue of paroxetine. The substrate azidosalicylylsuccinate is commercially available and has been used frequently as a cross-linking agent [15]. The straight-forward condensation reaction yields only one product, which can be identified by its distinct chromatographic properties and UV spectrum.

Photodecomposition of substituted aryl azides occurs readily at wavelengths above 300 nm. The major intermediate products of photolysis in organic solvents are aryl nitrens (88% singlet, 12% triplet [16]), which rapidly react with the O—H bonds of water (or ethanol). The resulting products have different UV spectra than their respective parent compounds. ASP is also a photosensitive compound. It readily decomposes upon irradiation (less than 1 min) at the UV range manifested by UV spectroscopy.

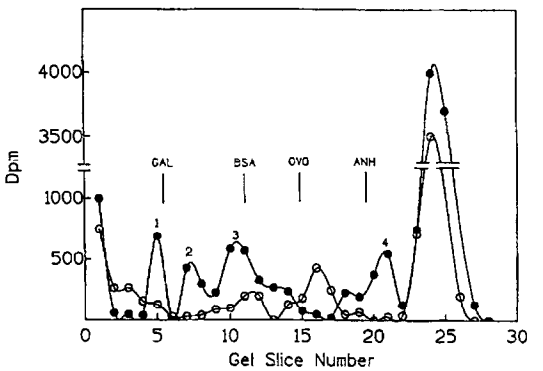


Fig. 5. Electrophoretic separation of platelet membrane proteins prelabelled with [^{3}H]ASP in the presence (●) or absence (○) of 1 mM fluoxetine. GAL: β-galactosidase, 116.2 kDa; BSA: bovine serum albumin, 66.2 kDa; OVO: ovalbumin, 45 kDa; and ANH: carbonic anhydrase, 31 kDa.

In biochemical photolysis experiments that are carried out in aqueous solutions, usually at much lower photolabel concentrations, the nature of the reactions in labelling experiments cannot be readily predicted. The aryl nitrens produced by photolysis could be covalently attached to proteins by insertion into C—H or N—H bonds, formation of an *azo* compound, aromatic substitution, and nucleophilic attack by sulfhydryl or amino compounds.

ASP irreversibly inactivates the paroxetine binding site upon UV irradiation in a time- and concentration-dependent fashion (data not shown). The mechanism of such a reaction is unknown. The photo-label also potently inhibits [^{3}H]paroxetine binding and 5-HT uptake in platelets at equilibrium. Hence, ASP is apparently a specific probe for the [^{3}H]paroxetine and 5-HT recognition site on 5-HT transporter.

Covalent linking of [^{3}H]ASP to human platelet membranes in the presence or absence of 1 mM fluoxetine followed by PAGE revealed that four fractions (120, 100, 71 and 25 kDa) were associated with the synthesized probe. Cloning of the cDNA encoding human platelet and brain 5-HT transporter predicts its molecular weight to be 68 kDa [3, 4]. This is in good agreement with the estimated molecular weight of Fraction No. 3 (71 kDa) detected in PAGE of the platelet membrane protein extract labelled with [^{3}H]ASP. It has been established that the 5-HT transporter is a glycoprotein [17]. Thus, its molecular weight will be higher than 68 kDa (deduced from its amino acid composition).

The results presented in this paper do not warrant any speculation regarding the nature of the three other protein fractions (120, 100 and 25 kDa), which were also found to be labelled with [^{3}H]ASP. Fractions No. 1 and 2 may likely constitute experimental artifacts. Fraction No. 4 (25 kDa) may represent a degradation product of the transporter protein. Although the solubilization mixture contained proteolytic enzyme inhibitors (refer to Materials and Methods), the transporter protein may not have been protected against proteolytic digestion by acid proteases (e.g. cathepsin D or B) of the platelet lysosomal system. Interestingly, a protein fraction of a similar molecular weight (*ca.* 30 kDa) was also reported to be specifically labelled with [^{3}H]azido-imipramine [11] or [^{3}H]nitroimipramine [14]. The PAGE separations of the ASP-labelled platelet membrane proteins were reproducible in terms of the distribution of specifically labelled fractions, although the size of these fractions (recovered radioactivity) and their ratios frequently varied.

ASP can be radiolabelled easily with ^{125}I [18]. However, we found that such a probe is less specific and shows very high nonspecific interaction with platelet proteins, probably caused by the iodination of the paroxetine molecule. Therefore, it may be necessary to iodinate the azidosalicylyl residue before linking it to paroxetine. The use of solubilized and partially purified preparations of 5-HT transporter will eliminate much of the spurious interactions. ASP will very likely facilitate purification of the native 5-HT transporter protein from platelets or brain tissue, which is a prerequisite for any future studies on the mechanism of antidepressant interaction with 5-HT transporter.

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