Proc. Estonian Acad. Sci. Chem., 2006, **55**, 3, 166–172
https://doi.org/10.3176/chem.2006.3.05

Interaction of tritium-labelled dopamine transporter inhibitor PE2I with mice striatal membrane fragments

Vladimir Stepanov* and Jaak Järv

Institute of Organic and Bioorganic Chemistry, University of Tartu, Jakobi 2, 51014 Tartu, Estonia

Received 7 April 2006

Abstract. Interaction of ^{3}H-labelled *N*-(3-iodoprop-2*E*-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)nortropane ([^{3}H]PE2I), a novel tritium-labelled ligand for tracing dopamine transporter protein, with mice striatal membrane fragments was studied under equilibrium conditions. Radioligand binding with a homogeneous population of binding sites was observed in these brain membrane fragments and characterized by the K_d value 22 ± 5 nM and $B_{max} = 0.12 \pm 0.02$ pmol/mg tissue. The specific binding of [^{3}H]PE2I was effectively displaced by unlabelled PE2I as well as GBR 12935, also known as an inhibitor of the transporter protein. Rather similar pIC_{50} values, 7.1 ± 0.3 and 6.9 ± 0.3, respectively, were obtained for these ligands in displacement experiments. This is in agreement with similar pharmacological effects of these ligands on dopaminergic neurons. After correction by the Cheng–Prusoff equation the displacement study yielded the K_d value of 40 nM for PE2I. The difference between the K_d values for PE2I obtained from the direct binding study and displacement experiments seems to point to some specific feature of the mechanism of PE2I interaction with the transporter sites and will be clarified through systematic kinetic study of the radioligand binding.

Key words: dopamine transporter, radioligand analysis, [^{3}H]PE2I, DA_T inhibitor, GBR 12935.

INTRODUCTION

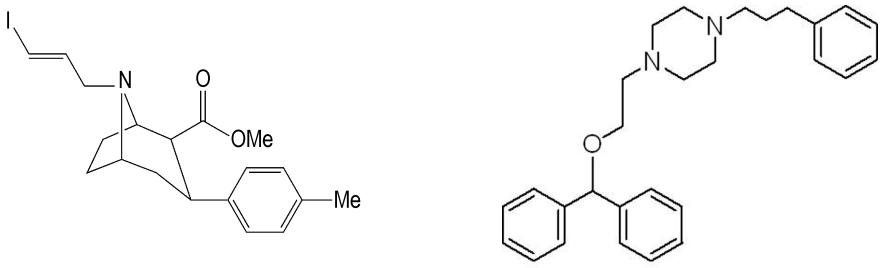
Dopamine is a neurotransmitter, which activates dopamine receptors in brain, and disorders of this dopaminergic system affect movement control and cause a decline in neurocognitive functions [1, 2]. The action of dopamine is terminated primarily through its binding to a dopamine transporter (DA_T) and the following translocation of the ligand back into dopaminergic neurons [3, 4]. Thereby the

* Corresponding author, vladimir.stepanov@ut.ee

transporter serves as an important target of drugs [5], and its selective labelling by radioligands is used for tracing the dopaminergic nerve terminals in brain by positron emission tomography (PET) or single photon emission computed tomography (SPECT) [6, 7].

Interaction of drugs with DA_T can be investigated by radioligand binding techniques in vitro and the key point for these studies is the availability of selective radioactive ligands. Although the list of drugs interacting with DA_T is rather long and covers many tropane, benztropine, piperazine, methylphenidate, mazindol, and phencyclidine derivatives, the majority of these compounds interact also with other monoamine transporters, primarily with the serotonine transporter. Therefore the radioligand analysis of DA_T binding properties had only a limited applicability until (*E*)-*N*-(3-iodoprop-2-enyl)-2β-(4'-tolyl)nortropane with the code name PE2I (Fig. 1), belonging to a new generation of potent and selective dopamine transporter inhibitors, was found to meet the basic criteria for being a selective DA_T radioligand [8–10]. The labelling of this ligand by iodine-123 (half-life 13.1 h) and carbon-11 (half-life 20 min) has yielded radioactive ligands, already tested in vivo in PET studies [11, 12]. In parallel, binding properties of the iodine-labelled PE2I have been investigated [8, 10, 13].

In our previous paper we described eight-step synthesis of the tritium-labelled PE2I [14]. This novel radioligand was synthesized for a more convenient assay of DA_T binding properties in vitro. In this report we present data about testing the binding properties of this new radioligand with membrane fragments of mice striatum, the brain area rich in dopaminergic nerve terminals [1]. For comparison, displacement of the radioligand from these binding sites by unlabelled PE2I, as well as by another well-known DA_T antagonist GBR 12953 (Fig. 1), was studied.



PE2I GBR 12935

Fig. 1. Comparison of the structures of the dopamine transporter inhibitors used in this study. *Left*: PE2I – *N*-(3-iodoprop-2*E*-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)nortropane. *Right*: GBR 12935 – 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine.

EXPERIMENTAL

N-(3-iodoprop-2*E*-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)nortropane (PE2I) and its ^{3}H-labelled derivative ([^{3}H]PE2I) were synthesized and characterized by NMR spectra, HRMS, and HPLC as described previously [14]. 1-[2-(Diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine (GBR 12935) was purchased as HCl salt from Sigma-Aldrich. Other chemicals of at least analytical grade were purchased from Fluka, Riedel-Hädel, and Sigma-Aldrich and were used without purification.

Striatal membrane fragments of 3-month-old female white mice were prepared as follows: mice were decapitated, their brains removed and striatum tissue rapidly dissected, snap-frozen in liquid nitrogen, and stored at −83 °C. For radioligand binding studies the frozen tissue was homogenized in ice-cold 70 mM Tris/HCl buffer (pH 7.4) containing 50 mM NaCl and 5 mM KCl. The same buffer was used through all experiments with these membrane fragments. The samples were centrifuged at 40000 *g* for 15 min, and resuspended in the same buffer. This procedure was repeated three times. Finally a suspension of the membrane fragments was obtained at a concentration of 10 mg of wet tissue per mL. The membrane suspension was additionally diluted with buffer before assays.

The [^{3}H]PE2I binding assay was performed as follows: 150 µL of the suspension of striatal membranes (33 µg wet tissue in standard assay), 100 µL of [^{3}H]PE2I solution of different concentration, ranging from 0.9 to 31 nM, and 50 µL of buffer or 60 µM of PE2I solution to determine non-specific binding of [^{3}H]PE2I were mixed and incubated at 25 °C during 60 min. Then the samples were rapidly filtered on Whatman GF/B filters, washed twice with 5 mL of ice-cold assay buffer, and filter-bound radioactivity was measured on a LKB Wallac 1219 Rackbeta liquid scintillation counter (57% ^{3}H counting efficiency with OptiPhase 'HiSafe' 3 liquid scintillation cocktail from Fisher Chemicals, UK). The filters were equilibrated in scintillation cocktail before counting for 12 h. The specific binding was determined as difference between the total and non-specific binding of [^{3}H]PE2I.

Displacement of [^{3}H]PE2I from striatal membranes by unlabelled PE2I and GBR 12935 was conducted as follows: 150 µL of the suspension of striatal membranes (33 µg wet tissue), 50 µL of [^{3}H]PE2I (final concentration 10 nM), and 50 µL of solutions of unlabelled PE2I and GBR 12935, correspondingly at appropriate final concentrations, were mixed and incubated at 25 °C during 60 min. The samples were rapidly filtered on Whatman GF/B filters, washed twice with 5 mL of ice-cold assay buffer, and filter-bound radioactivity was measured.

Data processing was performed using the software package GraphPad Prism 4 (San Diego, USA).

RESULTS AND DISCUSSION

Specific binding of the tritium-labelled PE2I with mice striatal membranes can be observed already at nanomolar concentrations of the radioligand (Fig. 2). The binding data obtained were described by a conventional binding isotherm:

$$B_{eq} = \frac{B_{max}\left[\,[^3\mathrm{H}]\mathrm{PE2I}\,\right]}{K_d + \left[\,[^3\mathrm{H}]\mathrm{PE2I}\,\right]},$$

where B_{eq} stands for specifically bound [^{3}H]PE2I, B_{max} is the maximal observed radioligand binding with the membrane fragments, and K_d is the appropriate dissociation constant. Within the concentration interval of [^{3}H]PE2I used, a homogeneous population of the binding sites was observed. This conclusion is in agreement with the pharmacological data about the selectivity of this ligand against DA_T, if compared with other binding sites for biogenic amines in brain membranes [15].

The apparent K_d value 22 ± 5 nM, calculated for [^{3}H]PE2I from these binding data, remains within the common range of dissociation constants, characteristic of neurotransmitter antagonists in general. Application of [^{125}I]PE2I for in vitro study with rat striatal membranes revealed the K_d value 4 nM [8], remaining in the same range of affinity. A similar value, $K_d = 3.8$ nM, was obtained for [^{125}I]PE2I interaction with the rat neuronal DA_T expressed in COS cells [10].

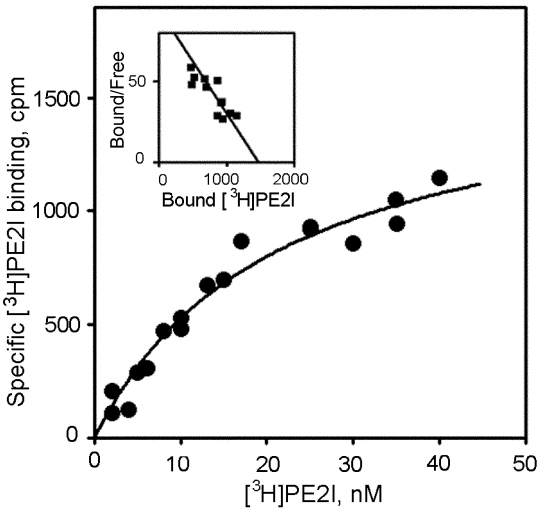


Fig. 2. Specific binding of [^{3}H]PE2I with mice striatal membrane fragments (33 µg of wet tissue per sample, assay medium 70 mM Tris/HCl buffe r, pH 7.4, 50 mM NaCl and 5 mM KCl, 25 °C). The insert shows the Scatchard plot of the binding data.

The radioligand [^{3}H]PE2I was also used in a displacement study with unlabelled PE2I and another DA_T inhibitor GBR 12935. The results of this study are shown in Fig. 3. The pIC50 values calculated from these displacement curves for PE2I and GBR 12935 were 7.1 ± 0.3 and 6.9 ± 0.3, respectively. Thus, although the chemical structure of these compounds is rather different (see Fig. 1), their affinity for the DA_T binding sites in striatal membranes was found to be quite similar on mice striatal membrane fragments.

Correction of the IC_{50} values by the Cheng–Prusoff equation [16] yielded the K_i values 26 nM for PE2I and 42 nM for GBR 12935, respectively. For PE2I this constant is in rather good agreement with the dissociation constant $K_d = 22$ nM, calculated from the direct binding study with the tritium-labelled ligand. Moreover, as reported before [10], PE2I displaced the specific binding of [^{3}H]GBR 12935 on membrane fragments of transfected COS cells, expressing the rat neuronal dopamine transporter, with the pK_i value of 7.7 ± 0.3. The K_i value (20 nM) calculated from this experiment, using different radioactive ligands, is also in good agreement with the present results for PE2I.

On the other hand, the K_i value for GBR 12935 in the displacement experiment (42 nM) is close to the EC_{50} value for this ligand in the functional assay of dopamine uptake by the transfected COS cells (50 nM) made in [10]. However, both these results remain significantly different from the $K_d = 0.4$ nM obtained for [^{3}H]GBR 12935 in the binding study made in the same report [10]. Interpretation of this discrepancy is hampered by the fact that a significant binding of

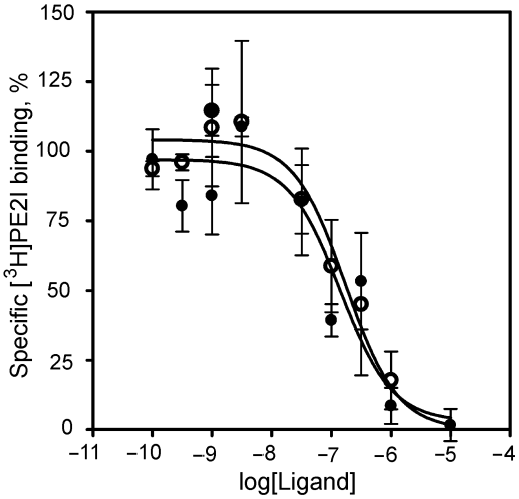


Fig. 3. Displacement of [^{3}H]PE2I from its binding sites on mice striatal membrane fragments (33 µg of wet tissue per sample, assay medium 70 mM Tris/HCl buffer, pH 7.4, 50 mM NaCl and 5 mM KCl, 25 °C) by unlabelled PE2I (filled circles) and GBR 12935 (empty circles).

GBR 12935 with other cell components, primarily with cytochrome P450, takes place in cell cultures [10]. Differently from GBR 12935, no such interaction was observed in the case of PE2I [10]. Therefore [^{3}H]PE2I may be considered as a useful tool for further investigations with DA_T, including kinetic study of the mechanism of the interaction of this transporter antagonist with its target protein.

ACKNOWLEDGEMENTS

This work was supported by the Estonian Science Foundation (grant 6698), the Ministry of Education and Research (grant 0182592s03), and by a grant to VS from Pharmasynth AS.

REFERENCES

1. Dailly, E., Chenu, F., Renard, C. E. & Bourin, M. Dopamine, depression and antidepressants. *Fundam. Clin. Pharmacol.*, 2004, **18**, 601–607.
2. Harvey, B. H. & Bouwer, C. D. Neuropharmacology of paradoxic weight gain with selective serotonin reuptake inhibitors. *Clin. Neuropharmacol.*, 2000, **23**, 90–97.
3. Bannon, M. J. The dopamine transporter: role in neurotoxicity and human disease. *Toxicol. Appl. Pharmacol.*, 2005, **204**, 355–360.
4. Kanner, B. I. Sodium-coupled neurotransmitter transport: structure, function and regulation. *J. Exp. Biol.*, 1994, **196**, 237–349.
5. Newman, A. H. & Kulkarni, S. Probes for the dopamine transporter: new leads toward a cocaine-abuse therapeutic – a focus on analogues of benztropine and rimcazole. *Med. Res. Rev.*, 2002, **22**, 429–464.
6. Brooks, D. J., Frey, K. A., Marek, K. L., Oakes, D., Paty, D., Prentice, R., Shults, C. W. & Stoessl, A. J. Assessment of neuroimaging techniques as biomarkers of the progression of Parkinson's disease. *Exp. Neurol.*, 2003, **184**, S68–79.
7. Piccini, P. P. Dopamine transporter: basic aspects and neuroimaging. *Mov. Disord.*, 2003, **18**, S3–8.
8. Chalon, S., Garreau, L., Emond, P., Zimmer, L., Vilar, M. P., Besnard, J. C. & Guilloteau, D. Pharmacological characterization of (*E*)-*N*-(3-iodoprop-2-enyl)-2beta-carbomethoxy-3beta-(4'-methylphenyl)nortropane as a selective and potent inhibitor of the neuronal dopamine transporter. *J. Pharmacol. Exp. Ther.*, 1999, **291**, 648–654.
9. Emond, P., Garreau, L., Chalon, S., Boazi, M., Caillet, M., Bricard, J., Frangin, Y., Mauclaire, L., Besnard, J. C. & Guilloteau, D. Synthesis and ligand binding of nortropane derivatives: *N*-substituted 2beta-carbomethoxy-3beta-(4'-iodophenyl) nortropane and *N*-(3-iodoprop-(2*E*)-enyl)-2beta-carbomethoxy-3beta-(3',4'-disubstituted phenyl)nortropane. New high-affinity and selective compounds for the dopamine transporter. *J. Med. Chem.*, 1997, **40**, 1366–1372.
10. Page, G., Chalon, S., Emond, P., Maloteaux, J. M. & Hermans, E. Pharmacological characterization of (*E*)-*N*-(3-iodoprop-2-enyl)-2beta-carbomethoxy-3beta-(4'-methylphenyl)nortropane (PE2I) binding to the rat neuronal dopamine transporter expressed in COS cells. *Neurochem. Int.*, 2002, **40**, 105–113.
11. Bergstrom, K. A., Tupala, E. & Tiihonen, J. Dopamine transporter in vitro binding and in vivo imaging in the brain. *Pharmacol. Toxicol.*, 2001, **88**, 287–293.
12. Halldin, C., Erixon-Lindroth, N., Pauli, S., Chou, Y. H., Okubo, Y., Karlsson, P., Lundkvist, C., Olsson, H., Guilloteau, D., Emond, P. & Farde, L. [^{11}C]PE2I: a highly selective radioligand

for PET examination of the dopamine transporter in monkey and human brain. *Eur. J. Nucl. Med. Mol. Imaging*, 2003, **30**, 1220–1230.
13. Hall, H., Halldin, C., Guilloteau, D., Chalon, S., Emond, P., Besnard, J., Farde, L. & Sedvall, G. Visualization of the dopamine transporter in the human brain postmortem with the new selective ligand [^{125}I]PE2I. *Neuroimage*, 1999, **9**, 108–116.
14. Stepanov, V. Synthesis of tritium-labeled *N*-(3-iodoprop-2*E*-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)nortropane. In *29th Estonian Chemistry Days: Abstracts of Scientific Conference*. Tallinn University Press, 2005, 107.
15. Rothman, R. B. & Baumann, M. H. Monoamine transporters and psychostimulant drugs. *Eur. J. Pharmacol.*, 2003, **479**, 23–40.
16. Cheng, Y. & Prusoff, W. H. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, 1973, **22**, 3099–3108.

Dopamiini transportvalgu triitiumiga märgistatud inhibiitori PE2I seostumine hiire aju juttkeha membraanifragmentidega

Vladimir Stepanov ja Jaak Järv

On uuritud dopamiini transportvalgu triitiumiga märgistatud inhibiitori *N*-(3-iodoprop-2*E*-enüül)-2β-karbometoksü-3β-(4-metüülfenüül)nortropaani (koodnimetusega PE2I) seostumist hiire aju juttkeha membraanifragmentidega. Mõõtmised on teostatud ligandi seostumisel tasakaalutingimustes. On leitud, et uuritav radioligand toimib juttkeha membraanide sidumiskohtade homogeense populatsiooniga ja sidumise protsessi kirjeldab K_d väärtus 22 ± 5 nM. Sidumiskohtade arv membraanifragmentidel (B_{max}) on $0{,}12 \pm 0{,}02$ pmol/mg. Samuti on leitud, et membraanidega spetsiifiliselt seostunud radioligandit on transportvalgu inhibiitorite PE2I ning GBR 12935 abil võimalik tekkinud kompleksist välja tõrjuda ja nende ligandite toimet kirjeldavad pIC_{50} väärtused $7{,}1 \pm 0{,}3$ ning $6{,}9 \pm 0{,}3$. Peale väljatõrjumiskatsete tulemuste korrigeerimist Chengi–Prusoffi võrrandi abil on [^{3}H]PE2I väljatõrjumise andmetest lähtudes saadud PE2I ja GBR 12935 jaoks K_i väärtused 26 nM ja 42 nM. Saadud tulemused näitavad, et triitiumiga märgistatud PE2I on uus perspektiivne radioligand, mille abil on võimalik uurida dopamiini transportvalgu omadusi, sealhulgas ka antagonistiga seostumise protsessi kineetikat ja mehhanismi.