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BIOKINETICS

Inhibition of ligand–protein complex isomerization by some dopamine transporter inhibitors

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Abstract. Kinetic analysis of the interaction of four *N*-substituted nortropane derivatives with the dopamine transporter was made to characterize the mechanism of the binding process. The kinetics were studied by radioligand binding experiments. It was found that the studied compounds initiate a slow isomerization process of the initially formed ligand–transporter complex, but at higher concentrations, the same compounds inhibit the isomerization process. The results suggest that the studied ligands interact with two distinct binding sites of the transporter protein that have different ligand binding specificities. The interaction of ligands with different binding sites must be taken into consideration when analyzing the ligand recognition patterns of the transporter protein.

Keywords: biokinetics, dopamine transporter, DAT, tropane derivatives, ligand binding kinetics, isomerization of ligand–protein complex.

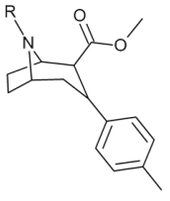
INTRODUCTION

The interaction of ligands with their target sites on proteins is commonly described as a rapid binding equilibrium, but may also occur as a two-step process, where the rapid initial binding step is followed by a slow formation of the formed complex. The latter two-step interaction mechanism was initially discussed by Strickland et al. in 1975 [1], and the formation of the second complex was referred to as the "isomerization" step [2]. Importantly, the presence of the slow isomerization step can be proven by kinetic analysis of the ligand binding process. This kinetically distinguishable isomerization step was identified in the case of different membrane proteins, including the glutamate receptor [3], low density lipoprotein receptor [4], β-adrenergic receptor [5], muscarinic acetylcholine receptor [2] and dopamine D_2 receptor [6].

Kinetic analysis of the ligand binding mechanism was initially used in direct radioligand binding experiments, which allow straightforward assay of the isomerized complex due to the slowness of ligand off-rate [7]. However, the applicability of the kinetic analysis was further expanded to study non-radioactive substances by using selective radioligands [7].

In the case of the dopamine transporter (DAT), both options – the rapid equilibrium binding and the mechanism including the slow isomerization step – have been documented [8,9]. It has been reported that relatively small changes in the ligand structure govern the shift between these two mechanisms [9].

In this paper, the results of a kinetic analysis of the DAT interaction with four *N*-substituted nortropane derivatives (**1**–**4**) are reported:



1 – R = methyl; **2** – R = ethyl; **3** – R = *n*-propyl; **4** – R = *n*-butyl.

It was found that these compounds initiate the isomerization of the ligand–DAT complex, but, at a higher concentration, the same ligands also inhibit the isomerization process. This manifests in slowing down the observed binding rate constants. These results demonstrate that there exists a third mechanism of the ligand interaction with DAT, which has not been documented before. This mechanism seems to be analogous with the substrate inhibition phenomenon described in enzyme catalysis [10].

MATERIALS AND METHODS

Compound **1** was prepared, as described previously [11, 12], by the treatment of anhydroecgonine with *p*-tolylmagnesiumbromide. Compounds **2**, **3** and **4** were synthesized from 2*β*-carbomethoxy-3*β*-(4′-methylphenyl)nortropane and the corresponding alkylhalide by following the general alkylation method described elsewhere [9,13].

Ligand binding kinetic experiments with DAT sites on mouse striatal membranes were performed at 25 °C using a fixed 3 nM concentration of [^{3}H]PE2I (*N*-(3-iodoprop-2*E*-enyl)-2*β*-carbo-[^{3}H]methoxy-3*β*-(4′-methylphenyl)nortropane) as the radioligand [14,15] with various concentrations of unlabeled ligands, as described previously [9,16]. Aliquots from the binding experiments were taken at various time points, the reaction was stopped with a cold buffer and the suspension was filtered through a glass fiber filter. The radioactivities of the glass filters were measured to yield time curves of the binding reaction, which were then analyzed using an exponential rate equation, and the observed rate constants (k_{obs}) were calculated. The NMR spectra were measured using either 700 MHz Bruker Avance-III or 200 MHz Bruker Avance-II NMR spectrometers, while HRMS analyses were performed with Varian 910-FT-ICR-MS spectrometer. Data processing was made with GraphPad Prism, version 4.00.

The synthesized compounds were characterized as follows:

2*β*-Carbomethoxy-3*β*-(4′-methylphenyl)tropane (**1**)
^{1}H NMR (200 MHz, $CDCl_3$ + TMS, δ): 1.56–1.79 (m, 3H), 2.00–2.19 (br, 2H), 2.23 (s, 3H), 2.29 (s, 3H), 2.58 (td, 1H, *J* = 2.7, 12.4 Hz), 2.87–3.05 (m, 2H), 3.36 (br, 1H), 3.50 (s, 3H), 3.55 (br, 1H), 7.11 (q, 4H, *J* = 8.2 Hz). ^{13}C NMR (50 MHz, $CDCl_3$ + TMS, δ): 21.0, 25.2, 26.0, 33.4, 34.2, 42.0, 51.0, 52.9, 62.4, 65.4, 127.2, 128.7, 135.2, 140.0, 172.2. HRMS (ESI): calculated ([M+H]$^+$) 274.18016, found 274.17991 (0.89 ppm error).

N-Ethyl-2*β*-carbomethoxy-3*β*-(4′-methylphenyl)nortropane (**2**)
^{1}H NMR (700 MHz, $CDCl_3$ + TMS, δ): 0.99 (t, 1H, *J* = 7.1 Hz), 1.61 (ddd, 1H, *J* = 4.2, 9.2, 12.8 Hz), 1.65 (dt, 1H, *J* = 3.7, 12.3 Hz), 1.71 (ddd, 1H, *J* = 4.4, 9.3, 13.2 Hz), 1.96–2.01 (m, 2H), 2.05–2.10 (m, 2H), 2.24–2.27 (m, 1H), 2.28 (s, 3H), 2.32–2.37 (m, 1H), 2.58 (td, 1H, *J* = 2.7, 12.5 Hz), 2.90 (t, 1H, *J* = 4.1 Hz), 3.00 (dt, 1H, *J* = 5.0, 12.9 Hz), 3.41 (br, 1H), 3.49 (s, 3H), 3.73 (br, 1H), 7.06 (d, 2H, *J* = 8.0 Hz), 7.15 (d, 2H, *J* = 8.1 Hz). ^{13}C NMR (176 MHz, $CDCl_3$ + TMS, δ): 14.0, 21.0, 25.7, 26.2, 34.0, 34.3, 47.2, 50.9, 52.9, 61.6, 61.9, 127.2, 128.6, 135.1, 140.2, 172.1. HRMS (ESI): calculated ([M+H]$^+$) 288.19581, found 288.19563 (0.60 ppm error).

N-Propyl-2*β*-carbomethoxy-3*β*-(4′-methylphenyl)nortropane (**3**)
^{1}H NMR (700 MHz, $CDCl_3$ + TMS, δ): 0.87 (t, 3H, *J* = 7.2 Hz), 1.39 (m, 2H), 1.59 (br, 1H), 1.65 (d, 1H, *J* = 12.0 Hz), 1.71 (m, 1H), 1.99 (m, 1H), 2.07 (m, 1H), 2.20 (m, 2H), 2.29 (s, 3H), 2.58 (t, 1H, *J* = 12.2 Hz), 2.90 (s, 1H), 2.98 (m, 1H), 3.38 (br, 1H), 3.49 (s, 3H), 3.68 (br, 1H), 7.07 (d, 2H, *J* = 7.6 Hz), 7.15 (d, 2H, *J* = 7.6 Hz). ^{13}C NMR (176 MHz, $CDCl_3$ + TMS, δ): 11.8, 21.0, 22.2, 25.9, 26.2, 33.9, 34.2, 50.9, 52.9, 55.6, 61.9, 62.6, 127.2, 128.6, 135.1, 140.3, 172.2. HRMS (ESI): calculated ([M+H]$^+$) 302.21146, found 302.21131 (0.47 ppm error).

N-Butyl-2*β*-carbomethoxy-3*β*-(4′-methylphenyl)nortropane (**4**)
^{1}H NMR (700 MHz, $CDCl_3$ + TMS, δ): 0.88 (t, 3H, *J* = 7.1 Hz), 1.28–1.39 (m, 4H), 1.60 (ddd, 2H, *J* = 4.0, 9.2, 12.5 Hz), 1.64 (dt, 1H, *J* = 3.6, 12.1 Hz), 1.71 (ddd, 1H, *J* = 4.4, 9.3, 13.0 Hz), 1.98 (m, 1H), 2.07 (m, 1H), 2.21 (m, 1H), 2.25 (m, 1H), 2.29 (s, 3H), 2.57 (td, 1H, *J* = 2.1, 12.6 Hz), 2.89 (t, 1H, *J* = 3.9 Hz), 2.98 (dt, 1H, *J* = 4.7, 12.9 Hz), 3.38 (br, 1H), 3.49 (s, 3H), 3.68 (br, 1H), 7.07 (d, 2H, *J* = 7.9 Hz), 7.15 (d, 2H, *J* = 8.0 Hz). ^{13}C NMR (176 MHz, $CDCl_3$ + TMS, δ): 14.0, 20.3, 21.0, 25.9, 26.1, 31.3, 33.9, 34.2, 50.9, 53.0, 53.3, 61.8, 62.7, 127.3, 128.6, 135.1, 140.3, 172.1. HRMS (ESI): calculated ([M+H]$^+$) 316.22711, found 316.22706 (0.13 ppm error).

RESULTS AND DISCUSSION

A specific DAT inhibitor [^{3}H]PE2I was used as the radioligand, and the observed rate constants (k_{obs}), characterizing the interaction of this ligand with the transporter, were determined in the presence of various concentrations of compounds **1**–**4** (Fig. 1). While typically the effect of ligand concentration to the apparent rate constant has been either ascent or descent of the k_{obs} vs the concentration plot to a plateau [9,16], the results of this series demonstrate a two-phase dependence. Such biphasic plots have never been observed for membrane proteins or receptors before, although the dose-dependent increase or decrease of the

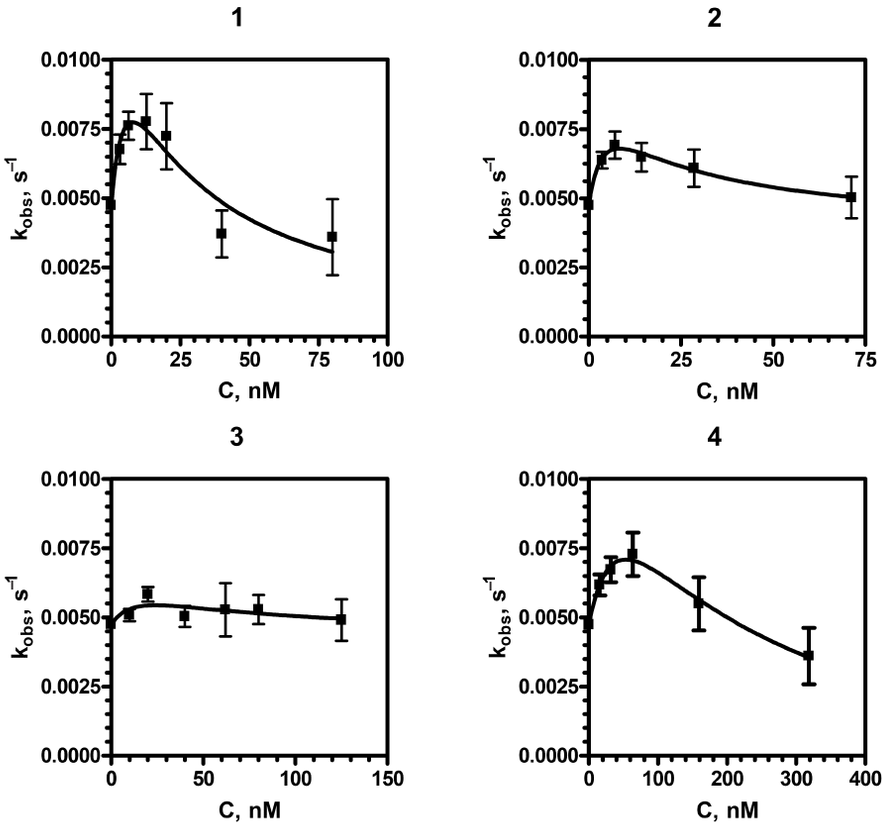


Fig. 1. Influence of increasing concentration of compounds **1**–**4** on observed rate constant (k_{obs}) of [^{3}H]PE2I binding with DAT.

k_{obs} values have been used to differentiate between the two ligand binding mechanisms. Firstly, the dose-dependent decrease of the k_{obs} value proves the fast and competitive binding of the non-radioactive ligand [7,16]. Secondly, the dose-dependent increase in the k_{obs} value proves the isomerization mechanism for the non-radioactive ligand because the DAT sites are consumed faster in two simultaneous binding reactions [7,16].

The biphasic shape of the k_{obs} vs the concentration plot can formally be explained by the interaction of the ligand with two different DAT sites of different functionality. Ligand binding in the first site is accompanied by isomerization, while the second complex inhibits this process. These possibilities were mathematically simulated by solving partial differential equations for a general reaction scheme, describing the interaction of different ligands with multiple binding sites on the target protein and considering the possibility of isomerization of these complexes [17]. Following this analysis and taking into consideration that the concentration of the reporter ligand was constant in all experiments, the biphasic k_{obs} vs the ligand concentration plot can be analyzed by superposition of the two functions, which describe the ascent and descent of the dependence, respectively:

$$k_{obs} = \frac{a[B]}{K_1 + [B]} + \frac{b}{1 + K_2/[B]} + c. \quad (1)$$

In this equation, $[B]$ stands for ligand concentration, K_1 and K_2 characterize $[B]$ interaction with the isomerizing site and the inhibitory site, respectively, and the parameters a and b take into consideration the isomerization steps of ligand–DAT complexes. This equation was used for data processing by means of non-linear regression analysis and for drawing the graphs shown in Fig. 1.

It was found that the parameters a and b had similar values for all ligands (**1**–**4**), ranging from 0.01 to 0.07. On the other hand, the parameters K_1 and K_2 depended on the ligand structure and were 11 nM, 6 nM, 26 nM, 88 nM and 14 nM, 16 nM, 35 nM, 123 nM for the same ligand series (**1**–**4**), respectively.

Parameters K_1 and K_2 represent the binding affinity of the studied *N*-substituted nortropane derivatives of the two distinct sites: the former causes isomerization of the ligand–protein complex and the latter inhibits it by binding another ligand. This explains the biphasic shape of the k_{obs} vs $[B]$ plots as the ratio $K_1 < K_2$ holds for all studied ligands; however, the differences between these affinities are small. For other previously studied *N*-substituted

nortropane derivatives of this series [9], the difference between K_1 and K_2 may be smaller or greater, and therefore these binding sites cannot be revealed in the same kinetic experiments.

The kinetic evidence for the presence of two binding sites of different functionality, interacting with *N*-substituted nortropanes, is the main finding in this study. Understandably, information about the structure and positioning of these sites on the transporter protein cannot be obtained by this analysis. Therefore, it is interesting to compare these results with other ideas about the presence of distinct binding sites on DAT, based on the facts that different types of compounds interact with this transporter, but also on the results of structural studies and molecular modeling, as summarized in a recent review [18]. Apart from the two main isosteric binding sites of DAT ligands, which have been noted as high- and low-affinity sites, allosteric sites have also been reported [19,20]. The high- and low-affinity sites have been evident with early DAT radioligands, e.g., [^{3}H]WIN 35,428 ([^{3}H]2β-carbomethoxy-3β-(4-fluorophenyl)tropane) and [^{3}H]cocaine, while the [^{3}H]PE2I has selectivity for a homogenous population of DAT sites [14,15]. The results of this study suggest that an additional site is present, but whether it is allosteric or isosteric cannot be differentiated with the approach described here.

The allosteric sites are believed to have significant pharmacological potential [18]. However, despite the great practical interest, there seems still to be a discrepancy in understanding the role of different documented binding sites of DAT and the distinction between the actual and hypothetical sites [18]. Therefore, it is important to recapitulate the importance and uniqueness of kinetic studies, which describe the mechanism of ligand binding with their target sites and prove the formation of different complexes.

CONCLUSIONS

In addition to the two previously known modes of DAT interaction with *N*-substituted nortropane derivatives, which include or do not include the slow isomerization step, a third kinetic mechanism of ligand binding was discovered in this study. This mechanism involves slow isomerization of the ligand–DAT complex, which is inhibited by another site at high ligand concentration. The transition between these kinetic mechanisms is governed by the ligand structure. This aspect of DAT specificity, as well as the positioning of these binding sites in the DAT structure, needs further analysis as this information may have great potential for the design of dopaminergic drugs.

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Photo: private collection/ Margit Partei

Siim Kukk defended his PhD at the University of Tartu in 2017 under the supervision of Prof. Jaak Järv. In parallel to his studies in academia, he started his industry career in 2010 at PharmaSynth as a synthetic organic chemist. In 2020–2022, he was employed abroad as a scientist at the contract research organization Symeres, in a chemical library synthesis group. In 2022, he returned to PharmaSynth as a head of R&D. From 2023, he is also a visiting lecturer at the University of Tartu. His research interests are synthesis of biologically active compounds and ligand binding kinetics.

REFERENCES

1. Strickland, S., Palmer, G. and Massey, V. Determination of dissociation constants and specific rate constants of enzyme–substrate (or protein–ligand) interactions from rapid reaction kinetic data. *J. Biol. Chem.*, 1975, **250**(11), 4048–4052. https://doi.org/10.1016/S0021-9258(19)41384-7
2. Järv, J., Hedlund, B. and Bartfai, T. Isomerization of the muscarinic receptor–antagonist complex. *J. Biol. Chem.*, 1979, **254**(13), 5595–5598. https://doi.org/10.1016/S0021-9258(18)50452-X
3. Abele, R., Keinänen, K. and Madden, D. R. Agonist-induced isomerization in a glutamate receptor ligand-binding domain: a kinetic and mutagenetic analysis. *J. Biol. Chem.*, 2000, **275**(28), 21355–21363. https://doi.org/10.1074/jbc.M909883199
4. Chappell, D. A., Fry, G. L., Waknitz, M. A. and Berns, J. J. Evidence for isomerization during binding of apolipoprotein-B100 to low density lipoprotein receptors. *J. Biol. Chem.*, 1992, **267**(1), 270–279. https://doi.org/10.1016/S0021-9258(18)48489-X
5. Schliebs, R. and Bigl, V. Kinetics of the interaction of dihydroalprenolol with beta-adrenergic receptors in rat cerebral cortex. *Gen. Physiol. Biophys.*, 1984, **3**(1), 31–46.
6. Lepiku, M., Rinken, A., Järv, J. and Fuxe, K. Kinetic evidence for isomerization of the dopamine receptor–raclopride complex. *Neurochem. Int.*, 1996, **28**(5–6), 591–595. https://doi.org/10.1016/0197-0186(95)00123-9
7. Järv, J. Neurotoxic agents interacting with the muscarinic acetylcholine receptor. In *Selective Neurotoxicity* (Herken, H. and Hucho, F., eds). Springer, Berlin, Heidelberg, 1994, 659–680. https://doi.org/10.1007/978-3-642-85117-9_18

8. Stepanov, V. and Järv, J. Slow isomerization step in the interaction between mouse dopamine transporter and dopamine re-uptake inhibitor N-(3-iodoprop-2E-enyl)-2β-carbo-[3H]methoxy-3β-(4′-methylphenyl)nortropane. *Neurosci. Lett.*, 2006, **410**(3), 218–221. https://doi.org/10.1016/j.neulet.2006.10.007
9. Kukk, S. and Järv, J. Small structural changes at the *N*-position of the tropane core control the mechanism of nortropane derivatives binding to dopamine transporter. *ChemistrySelect*, 2018, **3**(23), 6581–6584. https://doi.org/10.1002/slct.201801532
10. Reed, M. C., Lieb, A. and Nijhout, H. F. The biological significance of substrate inhibition: a mechanism with diverse functions. *BioEssays*, 2010, **32**(5), 422–429. https://doi.org/10.1002/bies.200900167
11. Emond, P., Garreau, L., Chalon, S., Boazi, M., Caillet, M., Bricard, J. et al. Synthesis and ligand binding of nortropane derivatives: N-substituted 2β-carbomethoxy-3β-(4‘-iodophenyl)nortropane and *N*-(3-Iodoprop-(2*E*)-enyl)-2β-carbomethoxy-3β-(3‘,4‘-disubstituted phenyl)nortropane. New high-affinity and selective compounds for the dopamine transporter. *J. Med. Chem.*, 1997, **40**(9), 1366–1372. https://doi.org/10.1021/jm960795d
12. Xu, L. and Trudell, M. L. Stereoselective synthesis of 2β-carbomethoxy-3β-phenyltropane derivatives. Enhanced stereoselectivity observed for the conjugate addition reaction of phenylmagnesium bromide derivatives with anhydro dichloromethane. *J. Heterocycl. Chem.*, 1996, **33**(6), 2037–2039. https://doi.org/10.1002/jhet.5570330676
13. Moore, J. L., Taylor, S. M. and Soloshonok, V. A. An efficient and operationally convenient general synthesis of tertiary amines by direct alkylation of secondary amines with alkyl halides in the presence of Huenig’s base. *Arkivoc*, 2005, **2005**(6), 287–292. https://doi.org/10.3998/ark.5550190.0006.624
14. Page, G., Chalon, S., Emond, P., Maloteaux, J.-M. and Hermans, E. Pharmacological characterisation of (*E*)-*N*-(3-iodoprop-2-enyl)-2β-carbomethoxy-3β-(4′-methylphenyl)nortropane (PE2I) binding to the rat neuronal dopamine transporter expressed in COS cells. *Neurochem. Int.*, 2002, **40**(2), 105–113. https://doi.org/10.1016/S0197-0186(01)00086-9
15. Stepanov, V., Schou, M., Järv, J. and Halldin, C. Synthesis of 3H-labeled *N*-(3-iodoprop-2*E*-enyl)-2*β*-carbomethoxy-3*β*-(4-methylphenyl)nortropane (PE2I) and its interaction with mice striatal membrane fragments. *Appl. Radiat. Isot.*, 2007, **65**(3), 293–300. https://doi.org/10.1016/j.apradiso.2006.09.003
16. Kukk, S. and Järv, J. Differentiating between drugs with short and long residence times. *MedChemComm*, 2016, **7**(8), 1654–1656. https://doi.org/10.1039/C6MD00269B
17. Kukk, S., Järv, J. and Miidla, P. Kinetic tools for the identification of ligand–receptor interaction mechanisms. *Proc. Est. Acad. Sci.*, 2017, **66**(2), 202–213. https://doi.org/10.3176/proc.2017.2.08
18. Nepal, B., Das, S., Reith, M. E. and Kortagere, S. Overview of the structure and function of the dopamine transporter and its protein interactions. *Front. Physiol.*, 2023, **14**, 1150355. https://doi.org/10.3389/fphys.2023.1150355
19. Rothman, R. B., Dersch, C. M., Ananthan, S. and Partilla, J. S. Studies of the biogenic amine transporters. 13. Identification of “Agonist” and “Antagonist” allosteric modulators of amphetamine-induced dopamine release. *J. Pharmacol. Exp. Ther.*, 2009, **329**(2), 718–728. https://doi.org/10.1124/jpet.108.149088
20. Rothman, R. B., Ananthan, S., Partilla, J. S., Saini, S. K., Moukha-Chafiq, O., Pathak, V. et al. Studies of the biogenic amine transporters 15. Identification of novel allosteric dopamine transporter ligands with nanomolar potency. *J. Pharmacol. Exp. Ther.*, 2015, **353**(3), 529–538. https://doi.org/10.1124/jpet.114.222299

Ligand-valk kompleksi isomerisatsiooni inhibeerimine mõnede dopamiini transpordi inhibiitorite poolt

Siim Kukk

Ligand-valk kompleksi seondumise kineetika uurimine on võimaldanud, lisaks paljule muule, määrata ravimainete seondumise mehhanismi. Tropaani derivaatide seas on täheldatud nii tavapärast ühe staadiumiga seondumist kui ka kahe staadiumiga seondumist, kus teises etapis ligand-valk kompleks isomeriseerub, st tekib kompleksi konformatsiooni muutus, mis dissotsieerub oluliselt aeglasemalt võrreldes kiire staadiumiga ning seeläbi suurendab seondumise efektiivsust.

Uuringus tuvastati radioligandi seondumise kineetika eksperimentidega neli ligandi, millel on ebatüüpiline seondumise kineetika: nimelt eksisteerib vähemalt üks kiire lisastaadium, kus juba seondunud ligand-valk kompleksile seondub veel üks ligandi molekul, mis pärsib aeglase staadiumi teket. Ebatüüpilist seondumise kineetikat iseloomustab kahe-faasiline kineetika kõver, kus ligandi madalamatel kontsentratsioonidel radioligandi seondumine kiireneb märgistamata ligandi kontsentratsiooni suurenedes, misjärel toimub üleminek teise faasi, kus ligandi kontsentratsiooni suurenedes seondumise kiirus aeglustub. Selline kahe-faasiline ligandi kontsentratsioonist tingitud radioligandi seondumise kiiruse sõltuvus on võimalik vaid juhul, kui eksisteerib veel vähemalt üks kiire staadium, kus ligand-valk kompleksiga seondub teine ligand. Ühendi struktuur määrab ülemineku ühelt mehhanismilt teisele. Kuna uuring ei anna infot, kuhu seondub valgus teine ligand teadaolevate seondumistaskute suhtes, peavad selle küsimuse lahendama tulevased analüüsid.