EXPERIMENTAL RESEARCH

A SIMPLE APPROACH FOR PILOT ANALYSIS OF TIME-DEPENDENT ENZYME INHIBITION: DISCRIMINATION BETWEEN MECHANISM-BASED INACTIVATION AND TIGHT BINDING INHIBITOR BEHAVIOR

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The increase in enzyme inhibition developed during prolonged incubation of an enzyme preparation with a chemical substance may be associated with both the non-covalent and also with covalent enzyme-inhibitor complex formation. The latter case involves catalytic conversion of a mechanism-based irreversible inhibitor (a poor substrate) into a reactive species forming covalent adduct(s) with the enzyme and thus irreversibly inactivating the enzyme molecule. Using a simple approach, based on comparison of enzyme inhibition after preincubation with a potential inhibitor at 4ºC or 37ºC we have analyzed inhibition of monoamine oxidase A (MAO A) by known MAO inhibitors pargyline and pirlindole (pyrazidol). MAO A inhibitory activity of pirlindole (reversible tight binding inhibitor of MAO A) assayed after mitochondrial wash was basically the same for the incubation at both 4ºC and 37ºC. In contrast to pirlindole, the effect of pargyline (mechanism based irreversible MAO inhibitor) strongly depended on the temperature of the incubation medium. At 37ºC the residual activity MAO A in the mitochondrial fraction after washing was significantly lower than in the mitochondrial samples incubated with pargyline at 4ºC. Results of this study suggest that using analysis of both time- and temperature-dependence of inhibition it is possible to discriminate mechanism-based irreversible inhibition and reversible tight binding inhibition of target enzym

Key words: enzyme inhibition; time-dependent and temperature-dependent inhibition; mechanism-based inhibitors; reversible tight binding inhibitors

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INTRODUCTION

The increase in enzyme inhibition developed during prolonged incubation of an enzyme preparation with a chemical substance may be associated with both the non-covalent enzyme-inhibitor complex formation via an induced-fit mechanism and also with covalent enzyme-inhibitor complex formation proceeding via the mechanism based inhibition. The latter case involves catalytic conversion of a mechanism-based irreversible inhibitor (a poor substrate) into a reactive species forming covalent adduct(s) with the enzyme and thus irreversibly inactivating the enzyme molecule. Since catalytic activity usually demonstrates higher temperature-dependence than non-covalent enzyme-inhibitor complex formation, it appears that preincubation of enzyme preparations with studied inhibitors at different temperatures will help to discriminate tight binding from mechanism-based inhibition.

In this study we have compared inhibition of monoamine oxidase (MAO; EC1.4.3.4) type A (MAO A) by two known inhibitors: pargyline and pirlindole (pyrazidol). Pargyline (N-methyl-N-propargylbenzylamine hydrochloride) is an irreversible mechanism-based inhibitor of both MAO A and MAO B; the radiolabeled [^{3}H]-pargyline has been used for estimation of MAO A and MAO B concentrations in tissue preparations (e.g. [1, 2]). Pirlindole hydrochloride (2,3,3a,4,5,6-hexahydro-8-methyl-1*H*-pyrazino [3,2,1-*j,k*] carbazole hydrochloride), an original Russian antidepressant [3, 4], is a selective MAO A inhibitor [5], which exhibits properties of the reversible tight binding inhibitor [6].

MATERIALS AND METHODS

The radiolabeled substance [^{14}C]5-hydroxytryptamine creatinine sulfate (a MAO A substrate) was from Amersham (England). Other reagents were purchased from Sigma-Aldrich (USA). Monoamine oxidase inhibitors were from the Collection of chemical substances (Laboratory of Pharmacoproteomics, Institute of Biomedical Chemistry).

Rat liver mitochondria were isolated by the conventional method of differential centrifugation [7]. The final mitochondrial pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.4 and stored at -20ºC until analysis.

MAO inhibition by pargyline and pirlindole was investigated by incubating the thawed mitochondrial suspension (protein concentration 3 mg/ml) with pirlindole or pargyline (final concentrations of 5 μM and 2 μM, respectively) at 4ºC or 37ºC. The incubations were performed in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (0.1 mM). The mitochondrial samples were incubated with MAO inhibitors for 0-30 min. After centrifugation (13000 rpm, Eppendorf Centrifuge 5415R, 30 min) the mitochondrial pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.4 (protein concentration of 1 mg/ml) and used for the MAO A assay. Control samples were treated under the same conditions, but in the absence of pargyline or pirlindole.

The activity of MAO A was determined radiometrically using 0.1 mM [^{14}C]5-hydroxytryptamine creatinine sulfate (specific

Table 1. Inhibition of rat liver mitochondrial MAO A activity (%) assayed after incubation with 5 μM pirlindole at 4ºC or 37ºC and subsequent mitochondrial wash

Temperature	Incubation time, min	
	30	60
4ºC	52±7	59±7
37ºC	55±6	55±6

Note. Data represent percent of MAO A inhibition (mean + SEM; n = 7) after sedimentation of the mitochondrial pellet from the incubation mixture. Details are given in the Materials and Methods section.

Table 2. Inhibition of rat liver mitochondrial MAO A activity assayed after incubation with 2 μM pargyline at 4ºC or 37ºC and subsequent mitochondrial wash

Temperature	Incubation time, min				
	0	15	30	45	60
4ºC	0	13±5	9±5	21±4	20±4
37ºC	0	39±5*	42±9*	47±6*	44±4*

Note. Data represent percent of MAO A inhibition (mean + SEM; n = 7) after sedimentation of the mitochondrial pellet from the incubation mixture. Details are given in the Materials and Methods section. The asterisk shows statistically significant difference between MAO A inhibition assayed at 4ºC or 37ºC ($p < 0.01$).

radioactivity of 10 Ci/mole) as a substrate. Previous studies have shown that this concentration of [^{14}C]5-hydroxytryptamine is specifically deaminated by MAO A [7]. Mitochondrial protein content was determined by the method of Bradford [8].

Results were statistically treated using two-tailed Student's *t* test. Differences were considered as statistically significant at $p < 0.05$.

RESULTS

In accordance with results of previous studies (e.g. [6, 9]) pirlindole was a potent MAO A inhibitor (Table 1). Its MAO A inhibitory activity assayed after mitochondrial wash was basically the same for the incubation at both 4ºC and 37ºC (Table 1).

In contrast to pirlindole, the effect of pargyline strongly depended on the temperature of the incubation medium (Table 2). At 37ºC the residual activity MAO A in the mitochondrial fraction after washing was significantly lower than in the mitochondrial samples incubated with pargyline at 4ºC ($p < 0.01$).

DISCUSSION

A study of a mode of drug-target enzyme interaction is an important step during characterization of mechanisms of the drug action in the context of both its effectiveness and also safety. (In the case of MAO inhibitors this was proved in numerous studies [10, 11].) Frequently, initial suggestion on the mode of enzyme inhibition (reversible or irreversible) comes from a time dependent increase in the enzyme inhibition resulted in a IC_{50} decrease, which is used for calculation of k_{inact} [12], However, some reversible inhibitors (e.g. befloxatone, a reversible MAO A inhibitor [13]) also demonstrate time-dependent inhibition.

Results of this study indicate that using a simple approach, analysis of both time- and temperature-dependence of inhibition it is possible to discriminate mechanism-based irreversible inhibition and reversible tight binding inhibition. The latter demonstrates less pronounced (if any) temperature dependence of enzyme inhibition.

COMPLIANCE WITH ETHICAL STANDARDS

The experiments were carried out in compliance with generally accepted norms of the humane care of laboratory animals.

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CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

REFERENCES

1. *Anderson M., Tipton K.,* Estimation of monoamine oxidase concentrations in soluble and membrane-bound preparations by inhibitor binding. J. Neural Transm. 1994, **41** (Suppl), 47-53.
2. *Buneeva O.A., Medvedeva M.V., Medvedev A.E.* (2008) Analysis of ubiquitin-dependent regulation of the monoamine oxidase sensitivity to proteolysis and specific inhibition by pargyline. Biomed. Khim., **54**(6), 720-726.
3. *Mashkovskii M.D., Gorkin V.Z., Andreeva N.I., Verevkina I.V., Siniukhin V.N.* (1975) Study of the mechanism of action of the new antidepressant pyrazidol. Farmakol Toksikol. **38**(5), 531-536
4. *Macedo, A., Leiria, E. and Filipe, A.* (2011). Pirlindole in the Treatment of Depression. Clin. Drug Investig. **31,** 61–71
5. *Gorkin V.Z.* (1985) Studies on the nature and specific inhibition of monoamine oxidases. In: Neuropharmacology-85, Kelemen K., Magyar K., Vizi E.S, Budapest: Akamediai Kiado, pp. 9-14.
6. *Medvedev A.E., Shvedov V.I., Chulkova T.M., Fedotova O.A., Saederup E., Squires R.F.* The influence of antidepressant pirlindole and its dehydro-derivative on the activity of monoamine oxidase A and $GABA_A$ receptor binding. J. Neural Transm. 1998, **52** (Suppl), 337-342.
7. *Medvedev A.E., Kirkel A.Z., Kamyshanskaya N.S., Axenova L.N., Moskvitina T.A., Gorkin V.Z., Andreeva N.I., Golovina S.M., Mashkovsky M.D.* (1994) Inhibition of monoamine oxidase by novel antidepressant tetrindole. Biochem. Pharmacol., **47**, (2), 303-308.
8. *Bradford, M.* (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., **72** (1–2), 248–254.
9. *Medvedev A.E., Veselovsky A.V., Shvedov V.I., Tikhonova O.V., Moskvitina T.A., Fedotova O.A., Axenova L.N., Kamyshanskaya N.S., Kirkel A.Z., Ivanov A.S.* (1998) Inhibition of monoamine oxidase by pirlindole analogues: 3D-QSAR and CoMFA analysis. J. Chem. Inf. Comput. Sci., **38**, 1137-1144.
10. *Youdim M.B., Edmondson D., Tipton K.F.* (2006) The therapeutic potential of monoamine oxidase inhibitors. Nat. Rev. Neurosci., **7**(4), 295-309.
11. *Flockhart D.A.* (2012) Dietary restrictions and drug interactions with monoamine oxidase inhibitors: an update. J. Clin. Psychiatry;73 (Suppl 1), 17-24.
12. *Krippendorff B-F., Neuhaus R., Lienau P., Reichel A. and Huisinga W.* (2009) Mechanism-based inhibition: deriving K_i and k_{inact} directly from time-dependent IC_{50} values. J. Biomol. Screen., 14(8), 913-923.
13. *Curet O., Damoiseau G., Aubin N., Sontag N., Rovei V., Jarreau F.X.* (1996) Befloxatone, a new reversible and selective monoamine oxidase-A inhibitor. I. Biochemical profile. J. Pharmacol. Exp. Ther. 277(1), 253-264.

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ПРОСТОЙ ПОДХОД К ПЕРВИЧНОМУ АНАЛИЗУ ЗАВИСИМОГО ОТ ВРЕМЕНИ ИНГИБИРОВАНИЯ ФЕРМЕНТА: РАЗЛИЧЕНИЕ МЕЖДУ ДЕЙСТВИЕМ МЕХАНИЗМ-АКТИВИРУЕМОГО И ПРОЧНО-СВЯЗАННОГО ИНГИБИТОРА

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Увеличение ингибирования фермента при длительной инкубации ферментного препарата с химическим веществом, может быть связано как с формированием нековалентного комплекса фермент-ингибитор, так и с образованием ковалентного фермент-ингибиторного комплекса. Последний включает каталитическое превращение необратимого ингибитора («плохого» субстрата) в реакционноспособный продукт, который образует ковалентный аддукт с ферментом и, таким образом, необратимо инактивирует молекулу фермента. Используя простой подход, основанный на сравнении ингибирования фермента после преинкубации с потенциальным ингибитором при 4ºС или 37ºС, мы проанализировали ингибирование моноаминоксидазы А (МАО А) известными ингибиторами МАО паргилином и пирилиндолом (пиразидолом). МАО А ингибирующая активность пирлиндола (обратимого прочно связанного ингибитора МАО А) после промывки митохондрий, была практически одинаковой для инкубации как при 4°С, так и при 37°С. В отличие от пирлиндола, эффект паргилина (необратимого механизм-активируемого ингибитора МАО) сильно зависел от температуры инкубационной среды. При 37ºС остаточная активность МАО А после промывки митохондриальной фракции была значительно ниже, чем в образцах митохондрий, инкубированных с паргилином при 4ºС. Результаты данного исследования показывают, что с помощью анализа зависимости ингибирования как от времени, так и от температуры можно различать действие необратимых механизм-активируемых ингибиторов и обратимых прочносвязанных ингибиторов целевого фермента.

Ключевые слова: ингибирование фермента; зависимое от времени и температуры ингибирование; механизм-активируемые ингибиторы; обратимые прочносвязанные ингибиторы

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