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Inhibitory effect of pyridyloxy- or phenoxylphenoxyalkanate derivatives on rat lens aldose reductase and rat platelet aggregation

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Abstract

The therapeutic potential of aldose reductase inhibitors for the prevention of the secondary complications of diabetes has been extensively reported. On the other hand, the hyperaggregability of platelets in diabetic patients has also been reported as a cause of chronic diabetic complications. The purpose of this study was to develop new compounds with these dual effects from pyridyloxy- or phenoxylphenoxyalkanate synthesized derivatives and examine the effect of their structure–activity relationships on the inhibition of rat lens aldose reductase (RLAR) as well as on platelet aggregation. 2-[4-(2,6-dichloro-3-methyl-phenoxy)-3-nitro-phenoxy]-propionic acid (**3**) exhibited the most potent inhibitory effect ($IC_{50} = 3.0 \pm 0.21\ \mu M$), comparable to tetramethylene glutaric acid ($IC_{50} = 6.1 \pm 0.2\ \mu M$), which is used as a positive control on RLAR, and showed potent inhibitory activities on rat platelet aggregation induced by ADP and collagen ($IC_{50} = 0.093 \pm 0.01$ and $0.032 \pm 0.01\ \mu M$, respectively) comparable with aspirin ($IC_{50} = 0.15 \pm 0.05$ and $0.047 \pm 0.01\ \mu M$, respectively), used as a positive control.

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Introduction

Increased aldose reductase (AR) activity and platelet aggregation are considered to be two causes of diabetic complications. Sorbitol is produced from glucose by AR through the polyol pathway and in diabetes the substantial accumulation of sorbitol in cells causes damage (Galvez et al 2003). This is associated with the development of some chronic diabetic complications, such as cataracts, neuropathy and retinopathy (Tomlinson et al 1994). On the other hand, hyperaggregability of platelets in diabetic patients has been reported as a cause of chronic diabetic complications (Colwell et al 1983; Zatz & Brenner 1986) so it seems likely that normalization of aggregability of platelets in diabetic patients may be beneficial for the prevention or treatment of chronic diabetic complications. We have reported that synthetic flavonoids, including 2′-hydroxychalcones, inhibit rat lens aldose reductase (RLAR) and rat platelet aggregation (Lim et al 2000, 2001).

In a previous paper, we synthesized various derivatives of phenoxylphenoxyalkanoic acid, and their anti-edematous and analgesic activities were tested (Shin et al 1997, 2000).

Kador et al (1985) have shown that the structural requirements for AR inhibitory activity are a generally planar structure with two aromatic hydrophobic regions and a common region susceptible to charge-transfer interactions (Lee et al 1994).

Pyridyloxy- or phenoxylphenoxyalkanate derivatives have almost all of the structural requirements for AR inhibitory activity. This paper describes the inhibitory effect of these compounds on RLAR and on platelet aggregation. Among those compounds tested, only a few were of importance for further investigation.

Materials and Methods

Chemicals

The compounds tested were synthesized by the method described previously (Figure 1; Shin et al 1997, 2000). The sources of other materials were: DL-glyceraldehyde, Sigma Chemical Co.; β-nicotinamide adenine dinucleotide phosphate tetrasodium (β-NADPH), Sigma Chemical Co.; ADP, collagen, aspirin and 3,3′-tetramethyleneglutaric acid (TMG), Aldrich Inc., USA.

Animals

Animal studies were carried out in a pathogen-free barrier zone at Seoul National University Hospital in accordance with the procedure outlined in the Guide for the Care and Use of Laboratory Animals. All animals were acclimated at least for 1 week, caged in groups of three or less, and fed with a diet of animal chow and water ad libitum. They were housed at 23 ± 0.5°C and 10% humidity in a 12-h light/dark cycle.

Preparation of RLAR

Crude RLAR was prepared as follows: rat lenses were removed from Sprague–Dawley rats weighing 250–280 g and frozen until use. The supernatant fraction of the rat lens homogenate was prepared according to Hayman and Kinoshita (1965) and then partially purified according to Inagaki et al (1982). Partially purified enzyme with a specific activity of 6.5 mU mg^{-1} was routinely used to test enzyme inhibition. The partially purified material was separated into 1.0 mL aliquots and stored at −40°C.

Measurements of RLAR activity

RLAR activities were assayed spectrophotometrically by measuring the decrease in absorption of NADPH at 340 nm over a 4-min period with DL-glyceraldehyde as a substrate (Sato & Kador 1990). Each 1.0 mL cuvette contained equal units of enzyme, 0.10 M sodium phosphate buffer (pH 6.2), 0.3 mM NADPH with or without 10 mM substrate and inhibitor. The concentration of inhibitors giving 50% inhibition of enzyme activity (IC_{50}) was calculated from the least-squares regression line of the logarithmic concentrations plotted against the remaining activity.

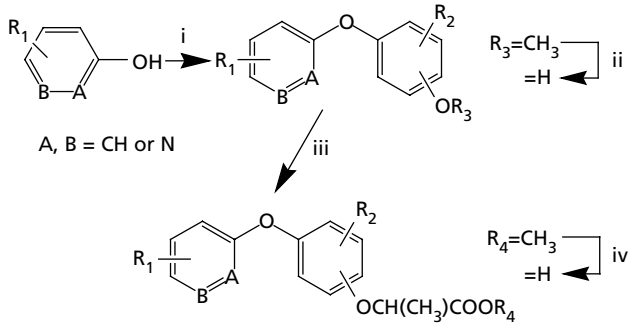


Figure 1 Preparation of pyridyloxy- or phenoxylphenoxy propionic acid derivatives: (i) substituted *p*-bromoanisole, KOH, Cu_2O, 70°C; (ii) 48% HBr, acetic acid, reflux, 5 h; (iii) ethyl-2-bromopropionate, K_2CO_3, methyl ethyl ketone, reflux, 10 h; (iv) EtOH, KOH, room temperature.

Platelet aggregation

Fresh blood, obtained from the abdominal aorta of each rat from Sprague–Dawley male rats weighing 250–290 g, containing 0.38% sodium citrate was centrifuged at 1000 rpm for 10 min at room temperature to obtain a supernatant of platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was prepared by re-centrifugation of the remaining blood at 3000 rpm for 10 min. Each compound was dissolved in DMSO or buffer and 2.5 μL of each was added to 222.5 μL PRP (5×10^5 platelets μL^{-1}). Adensosine 5′-diphosphate (ADP) and collagen were used as inducers at final concentrations of 2.5 μM and 10 μg mL^{-1}, respectively. Platelet aggregation was determined by a turbidimetric method using a platelet aggregometer (Gabbasov et al 1989). A water-soluble sample was dissolved in saline and 2.5 μL of this was added to 222.5 μL PRP, while an insoluble sample in saline was dissolved in DMSO, and 2.5 μL of this was added to 222.5 μL PRP. After incubation at 37°C for 1 min with stirring, platelet aggregation was initiated by addition of 25 μL of inducer. Saline or DMSO was used as a control.

The degree of anti-platelet aggregation (%) was calculated from the following equation: ratio of anti-platelet aggregation (%) = [1 − (platelet aggregation potency of sample/platelet aggregation potency of control)] × 100.

Statistical analysis

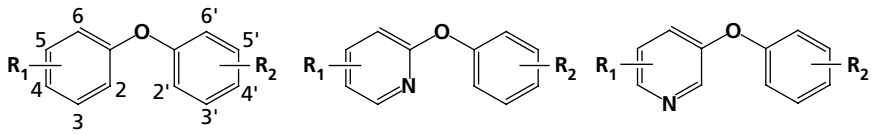
The experiments were performed in triplicate. Data were summarized as mean ± s.d. To analyse the data statistically, we performed a one-way analysis of variance (ANOVA) for repeated measurements of the same variable. We then used Scheffe's multiple range *t*-test to determine which means were significantly different from the mean of the control.

Result and Discussion

Table 1 shows the data of activity of phenoxyl- (series A), 2-pyridyloxy- (series B) or 3-pyridyloxy- (series C) phenoxyalkanate derivatives tested as inhibitors of RLAR with DL-glyceraldehyde as substrate, and of rat platelet aggregation induced by 2.5 μM ADP and 10.0 μg mL^{-1} collagen. Aspirin and TMG are also included for comparison.

By combining the spatial position of the aldose reductase inhibitors (ARIs) with their binding modes and inhibitory activities, the pharmacophore requirements of the ARIs were reported as follows (Figure 2; Lee et al 1998). An ionized portion (R1 = alkanoic acid) of ARI forms hydrogen bonding interactions with the side-chains of

Table 1 Inhibitory effects of pyridyloxy- and phenoxylphenoxyalkanoic acid derivatives on RLAR and rat platelet aggregation



No.		R_1	R_2	RLAR[a] (IC_{50}, μM)	Rat platelet aggregation[b]	
					ADP (IC_{50}, μM)	Collagen (IC_{50}, μM)
A	1	2,6-diCl, 3-CH_3	4′-OCH(CH_3)COOH	35.2 ± 2.2	5.4 ± 0.2	15.4 ± 1.0
	2	2,6-diCl, 3-CH_3	4′-OCH(CH_3)$COOCH_3$	13.5 ± 1.2	66.7 ± 3.0 (0.25 ± 0.1)	67.2 ± 3.8 (0.22 ± 0.01)
	3	2,6-diCl, 3-CH_3	4′-OCH(CH_3)COOH, 2′-NO_2	98.4 ± 4.5 (3.0 ± 0.21)	99 ± 6.9 (0.093 ± 0.01)	100 ± 12.6 (0.032 ± 0.01)
	4	2,6-diCl, 5-CH_3	4′-OCH(CH_3)COOH	55.7 ± 2.9 (9.2 ± 1.23)	21.0 ± 1.9	38.6 ± 5.2
	5	2,6-diCl, 5-CH_3	4′-OCH(CH_3)COOEt	1.2 ± 0.3	5.5 ± 0.2	3.2 ± 0.54
	6	2,6-diCl, 5-CH_3	4′-OCH(CH_3)COOH, 2′-NO_2	78.0 ± 5.2 (5.9 ± 0.36)	19.1 ± 0.3	28.5 ± 1.0
	7	2,6-diCl, 5-CH_3	3′-OCH(CH_3)COOH	50.2 ± 5.8 (9.8 ± 1.1)	19.1 ± 0.9	19.9 ± 1.2
	8	2,6-diCl, 5-CH_3	3′-OCH(CH_3)COOH, 4′-NO_2	39.0 ± 3.6	18.1 ± 1.6	20.5 ± 1.6
	9	2,6-diCl, 5-CH_3	3′-OCH(CH_3)COOEt, 4′-NO_2	20.0 ± 2.2	9.5 ± 1.2	9.1 ± 1.8
	10	2-NO_2	4′-OCH(CH_3)COOH	20.9 ± 4.3	39.1 ± 3.9	58.3 ± 3.2
	11	2-NO_2	4′-OCH(CH_3)COOEt	24.0 ± 3.7	−10.2 ± 1.2	−5.3 ± 0.6
	12	4-CH_3	4′-OH	44.2 ± 8.2 (11.5 ± 1.6)	0.1 ± 0.6	5.0 ± 0.8
	13	4-CH_3	4′-OCH_3	17.6 ± 1.3	0.0 ± 0.2	6.0 ± 1.1
	14	4-CH_3	4-OCH_2COOH	42.4 ± 3.9	21.9 ± 2.1	38.9 ± 3.1
B	15	H	3′-OCH(CH_3)COOH	4.8 ± 0.6	9.5 ± 0.3	10.6 ± 2.1
	16	3-Cl	3′-OCH(CH_3)COOH	50.6 ± 3.8 (9.8 ± 0.5)	29.5 ± 3.9	31.0 ± 4.0
	17	3-Cl	4′-OCH(CH_3)COOH	10.5 ± 0.3	68.4 ± 8.2 (0.23 ± 0.06)	52.5 ± 2.6 (0.27 ± 0.01)
	18	4-Cl	4′-OCH(CH_3)COOH	17.2 ± 0.6	19.6 ± 2.0	25.5 ± 1.9
	19	4-Cl	3′-OCH(CH_3)COOH	53.2 ± 3.9 (9.1 ± 0.5)	−10.0 ± 1.3	−11.5 ± 2.3
	20	4-Cl	2′-OCH(CH_3)COOH	5.8 ± 0.2	1.0 ± 0.2	7.0 ± 0.5
	21	5-Cl	4′-OCH(CH_3)COOH	31.8 ± 0.9	2.8 ± 0.3	18.92 ± 1.3
	22	6-Cl	4′-OCH(CH_3)COOH	11.5 ± 1.9	21.0 ± 2.3	38.2 ± 2.5
	23	3-NO_2	4′-OCH(CH_3)COOEt	31.8 ± 2.8	12.4 ± 1.3	37.2 ± 2.9
	24	5-NO_2	4′-OCH(CH_3)COOH	60.9 ± 3.7 (8.2 ± 0.2)	10.5 ± 0.6	48.4 ± 3.2
	25	5-NO_2	4′-OCH(CH_3)COOEt	0.2 ± 1.2	11.43 ± 0.7	46.3 ± 5.6
	26	3-Cl	4′-OCH_3	6.5 ± 1.6	0.0 ± 0.3	−10.3 ± 2.1
	27	3-Cl	2′,6′-diNO_2, 4′-CO_2H	63.6 ± 2.5 (8.7 ± 1.8)	58.7 ± 5.0 (0.27 ± 0.02)	59.7 ± 6.6 (0.25 ± 0.05)
	28	4-Cl	4′-NO_2, 3′-CO_2H	5.0 ± 0.2	2.9 ± 0.1	19.0 ± 1.1
	29	4-NO_2	4′-OH	24.9 ± 1.9	0.0 ± 0.3	20.6 ± 1.6
	30	4-NO_2	4′-OCH_3	4.0 ± 0.3	1.8 ± 0.9	12.8 ± 1.0
C	31	6-NO_2	4′-OCH_3	11.4 ± 0.8	0.0 ± 1.2	10.6 ± 1.8
	32	4-CH_3	2′-NO_2, 4′-CO_2H	23.5 ± 2.8	62.5 ± 3.3 (0.215 ± 0.02)	56.0 ± 3.9 (0.28 ± 0.02)
	33	2-Cl	2′-NO_2, 4′-CO_2H	19.3 ± 2.6	8.0 ± 0.5	19.0 ± 0.8
	34	2-Cl	4′-NO_2, 3′-CO_2H	16.9 ± 1.3	0.9 ± 0.1	12.7 ± 1.2
	35	4-Cl	2′-NO_2, 4′-CO_2H	20.5 ± 2.9	86.6 ± 5.1 (0.123 ± 0.01)	100.1 ± 9.5 (0.044 ± 0.01)
	36	2-NO_2	2′-NO_2, 4′-CO_2H	33.3 ± 2.5	38.4 ± 3.9	45.3 ± 2.9
	Tetramethylene glutaric acid			78.8 ± 6.4 (6.1 ± 0.2)	62.7 ± 8.8 (0.21 ± 0.03)	84.3 ± 9.5 (0.22 ± 0.02)
	Aspirin				77.2 ± 7.3 (0.15 ± 0.05)	101.2 ± 11.3 (0.047 ± 0.01)

[a]% inhibition at 10^{-5} M. [b]% inhibition at 0.3 mg mL^{-1}.

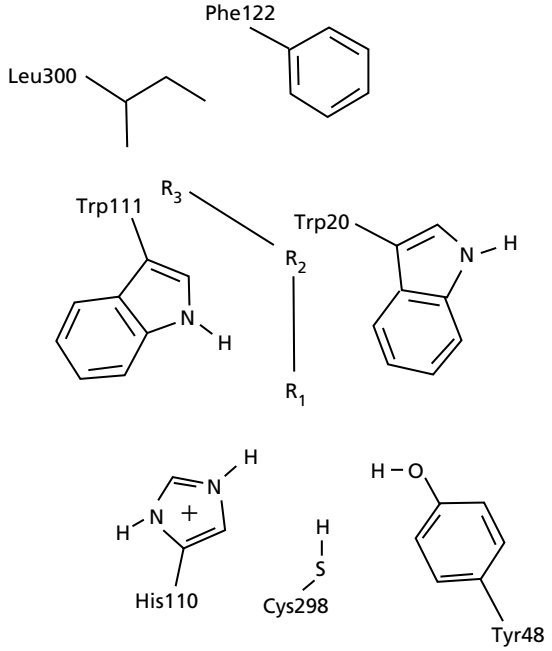


Figure 2 Schematic diagram depicting the pharmacophores of ARIs (Lee et al 1998).

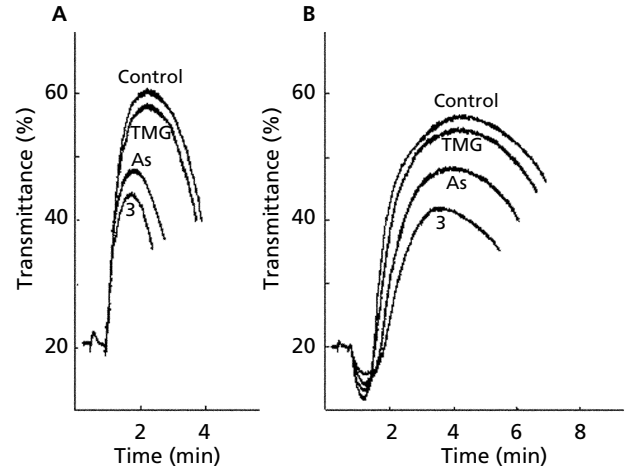


Figure 3 Effect of compound **3**, TMG and aspirin on rat platelet aggregation induced by 2.5 μM of ADP (A) and 10.0 μg mL^{-1} of collagen (B). Platelets were stimulated at the time indicated by the arrows. The concentration of the drugs was 0.1 μM on A and 30 nM on B. Control, TMG, As and **3** indicate vehicle only, tetramethyleneglutaric acid, aspirin and 2-[4-(2,6-dichloro-3-methyl-phenoxy)-3-nitro-phenoxy]-propionic acid, respectively.

Tyr48, His110 and Trp111, while an aromatic portion (R2 = phenoxy) forms aromatic–aromatic interactions with the side-chains of Trp20, Trp111 and Phe122. The aromatic portion also forms hydrogen-bonding interactions with Cys298 and the main chain of the N–H of Leu300. A side-chain (R3) such as a pyridyloxyl or phenoxyl group intercalates between Trp111 and Leu300 to improve the aromatic–aromatic interactions further.

As far as RLAR inhibitory activities are concerned, compounds **3** and **6** of series **A** exhibit the most potent inhibitory effects ($IC_{50} = 3.0 \pm 0.21$ and 5.9 ± 0.36 μM, respectively), comparable to TMG ($IC_{50} = 6.1 \pm 0.2$ μM), which is used as the positive control. Compounds **3** and **6** have a hydrogen-bonding R1 group and a proper R2 group containing nitro, an electron-withdrawing group.

Compounds **24** and **27** are the most potent ($IC_{50} = 8.2 \pm 0.2$ and 8.7 ± 1.8 μM, respectively) in series **B**, however they are less potent than TMG. The other compounds do not show any significant inhibition. Among these, compounds with free acid, such as **4** or **24**, are more potent RLAR inhibitors than the ethylated molecules such as **5** or **25**.

The anti-aggregatory effect of the compounds was more marked when platelet aggregation was induced by collagen rather than by ADP. As far as inhibition of platelet aggregation is concerned, compounds **3** and **35** exhibited a strong inhibitory effect on the rat platelet aggregation induced by ADP ($IC_{50} = 0.093 \pm 0.01$ and 0.123 ± 0.01 μM, respectively) and collagen ($IC_{50} = 32 \pm 10$ and 44 ± 10 nM, respectively), comparable with aspirin ($IC_{50} = 0.15 \pm 0.05$ μM induced by ADP and $IC_{50} = 47 \pm 10$ nM induced by collagen), which is used as positive control, but compound **35** showed relatively weak inhibitory activities on RLAR.

Figure 3 shows the effects of compound **3**, TMG and aspirin on the platelet aggregation rate induced by 2.5 μM of ADP (Figure 3A) and 10 μg mL^{-1} of collagen (Figure 3B). In Figure 3A, a representative aggregometer tracing representing the effect of compounds on ADP-induced platelet aggregation in rat PRP is shown. Compound **3** and aspirin inhibited the rate of platelet aggregation by 38.1 and 29.4%, respectively, at 0.1 μM. TMG, an ARI (May et al 1990), did not show a significant inhibitory effect at 0.1 μM. A representative aggregometer tracing representing the effect of compounds on collagen-induced platelet aggregation in rat PRP is shown in Figure 3B. Compound **3** and aspirin inhibited the rate of platelet aggregation by 41.4 and 25.1%, respectively, at 30 nM. TMG did not show a significant inhibitory effect at 30 nM.

Conclusions

From the in-vitro data, compound **3**, 2-[4-(2,6-dichloro-3-methyl-phenoxy)-3-nitro-phenoxy]-propionic acid, was found to show potent inhibitory activities, therefore this compound could be offered as a leading compound for further study as a new drug for diabetic complications. Further study remains to be done to confirm the action mechanism.

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