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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Screening and evaluation of rat kidney aldose reductase inhibitory activity of some pyridazine derivatives

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Abstract Aldose reductase (AR) is an enzyme that catalyzes the conversion of glucose to sorbitol, which is in turn converted to fructose by sorbitol dehydrogenase. The increased glucose flux through this metabolic pathway has been linked to the development of diabetic complications such as neuropathy, nephropathy, retinopathy, and cataract. Inhibitors of AR thus seem to have the potential to prevent or treat diabetic complications. AR inhibitors belong to different chemical classes, one of which comprises pyridazinone analogues. At present, however, side effects and/or insufficient pharmacokinetic profiles have made most of the drug candidates undesirable. We evaluated a series of 2*H*-pyridazine-3-one and 6-chloropyridazine analogues via an *in vitro* spectrophotometric assay for their ability to inhibit rat kidney AR. The study showed that the introduction of a pyrazole ring on pyridazinone led to a marked decrease in AR inhibitory potency. Moreover, introduction of an acetic acid side chain on 2*H*-pyridazine-3-one and 6-chloropyridazine did not improve the AR inhibitory activity, which was an unexpected result. On the basis of preliminary AR inhibitory screening results on 2*H*-pyridazine-3-one and 6-chloropyridazine derivatives, we embarked on the synthesis of more derivatives to discover more active molecules.

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Introduction

The enzyme aldose reductase (AR) belongs to the superfamily of aldo-keto reductases. As a key enzyme in the polyol pathway, its physiological role involves the reduction of glucose to sorbitol followed by the subsequent NAD^+-dependent oxidation of sorbitol to fructose by sorbitol dehydrogenase (Fig. 1). In situations of increased glucose flux, sorbitol can accumulate in cells of insulin-dependent glucose uptake, leading to increased osmotic pressure in the cells (Klebe *et al.*, 2004). The pathophysiological activity of AR plays a key role in the development of degenerative long-term diabetic complications such as neuropathy, nephropathy, retinopathy, cardiomyopathy, and cataract formation (Suzen and Buyukbingol, 2003).

In vivo and *in vitro* studies suggest a clear benefit of the administration of AR inhibitors (ARIs) in various model systems exposed to high levels of glucose as well as in the treatment of diabetic patients (Sarges and Oates, 1993; Mylari *et al.*, 2003; Steuber *et al.*, 2006). Significant effort has been spent in the discovery of potent ARIs resulting in a large variety of chemical classes. At present, however, side effects related to toxicity or inadequate pharmacokinetic profiles have rendered most of the drug candidates undesirable (Suzen and Buyukbingol, 2003). Up to now, most of the carboxylate type and hydantoin type ARIs developed during the last two decades (Fig. 2) have failed in clinic trails, probably owing to insufficient physiochemical or selectivity properties (Steuber *et al.*, 2006). Enzyme inhibitors are molecular agents that interfere with catalysis, slowing or halting enzymatic rections. The study of enzyme inhibitors also has provided valuable information about enzyme mechanisms and has helped define some metabolic pathways.

It has been shown that one of the important cyclooxygenase-2 (COX-2) inhibitors, pyridazinone compounds, have the ability to inhibit AR, lower sorbitol levels, and thus lower fructose levels, and/or treat or prevent diabetic complications (Banavara, 2005). The changes in vascular prostaglandin (PG) production are implicated in the derangement of vascular reactivity in diabetes. Although the mechanism of altered PG production in diabetes is largely unknown, the results suggest that the augmenting effect of high glucose on PG production and COX-2 expression is, at least in part, due to increased glucose metabolism via sorbitol pathway (Fig. 1) (Lee *et al.*, 2000). It has also been

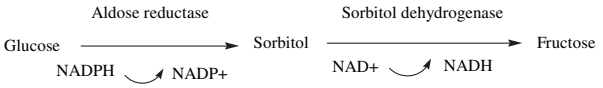


Fig. 1 Conversion of glucose to sorbitol via the sorbitol pathway

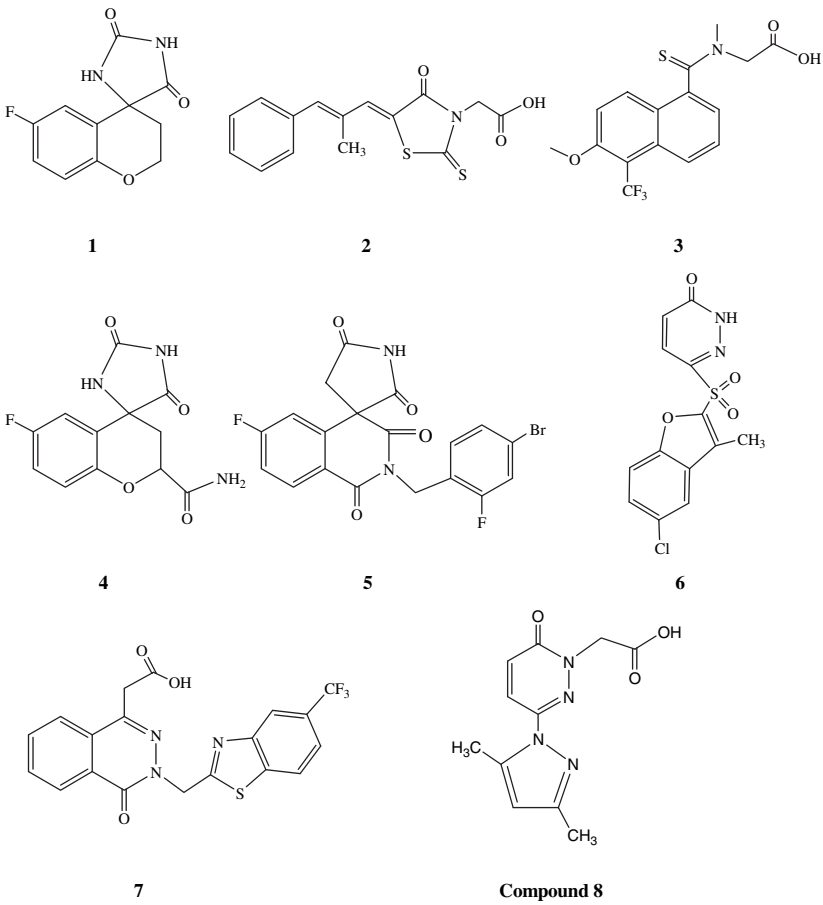


Fig. 2 Chemical formulas of cyclic amide type (sorbinil 1, fidarestat 4, minalrestat 5), carboxylic acid type (epalrestat 2, tolrestat 3) and pyridazinone type (a novel pyridazinone derivative 6 (Steuber *et al.*, 2006), zopolrestat 7, compound 8) aldose reductase inhibitors

found that transcriptional up-regulation of COX-2 in vascular smooth muscle is partially responsible for vascular hyperreactivity in mice. As abnormal vascular smooth muscle responses may contribute to the etiology of diabetic vascular complications, there is a possibility that the selective inhibition of vascular smooth muscle COX-2 activity may help prevent vascular complications in type 2 diabetes (Guo *et al.*, 2005).

Because of a shortage of drugs currently available for the treatment diabetic complications, the search for new ARIs with more favorable biological properties is still a major pharmaceutical challenge (Pau *et al.*, 2004). Since some ARIs showing a pyridazinone anchor group were reported to possess excellent affinity and tissue penetration properties, significant research has been conducted (Courdet *et al.*, 1991, 1992; Buyukbingol *et al.*, 1994; Constantino *et al.*, 1999; Mavvari *et al.*, 2005; Steuber *et al.*, 2006). In light of

these findings, a series of 6-pyrazole-2*H*-pyridazin-3-one and 6-chloropyridazine derivatives (Fig. 3) that have COX-2 inhibitor activity (Banoğlu *et al.*, 2004, 2005; Şüküroğlu *et al.*, 2005) were evaluated via an *in vitro* spectrophotometric assay for their ability to inhibit rat kidney AR.

Materials and Methods

All chemicals were obtained from Aldrich, Deisenhofen (Germany). Uncorrected melting points were determined via a Buchi 510 capillary melting point apparatus. The ^{1}H nuclear magnetic resonance (NMR) spectra were measured via a Varian Mercury 400 FT-NMR spectrometer. All chemical schifts were reported as δ (ppm) relative to TMS as internal standart. Dimethyl sulfoxide (DMSO)-d_6 was used as solvent. The elemental analyses (C, H, N) were performed at the Scientific and Technical Council of Turkey, Instrumental Analysis Center (Ankara, Turkey) and were within ± 0.4% of the therotical values.

Male albino rats weighing 200 to 250 g and receiving a standart diet were used. Thirty rats were killed and kidney tissues were obtained. AR (ALR2) activity was determined after isolation from kidney tissue. All enzyme experiments were performed in triplicate. Procedures involving the animals and their care conformed to institutional guidelines, in compliance with national and international laws and guidelines for the use of animals in biomedical research.

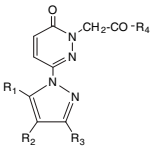
Chemistry

Synthesis of compounds. Synthesis and anti-inflammatory activity of compounds **1** to **7** (Banoğlu *et al.*, 2004), **8** to **14** (Banoğlu *et al.*, 2005), and **15** to **20** (Şüküroğlu *et al.*, 2005) were published earlier by our research group. Preparation and characterization of compounds **22**, **23**, and **24** are as follows.

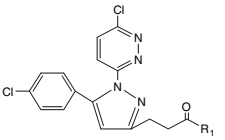
General synthesis of compounds ***22, 23, 24.***

A reaction mixture containing compound **21** (US Patent 5242940) (0.01 mol) and triethylamine (0.011 mol) in 20 ml of anhydrous dichloromethane at 0°C (ice bath) was treated with ethyl chloroformate (0.01 mol) with stirring at 0°C for 15 min. The mixture was then treated with an appropriate amine derivative (0.011 mol) with continued stirring at room tempetature overnight. After evaporation to dryness, acetone was added and all undissolved salts were filtered off. The filtrate was then evaporated to dryness and the residue was recrystallized from the appropriate solvent.

N-(4-chlorophenyl)-3-[5-(4-chlorophenyl)-1-(6-chloropyridazin-3-yl)-1*H*-pyrazol-3-yl]propanamide (**22**); ^{1}H NMR in DMSO-d_6 δ: 2.79 (2H, t, $CH_2C\underline{H}_2CONH$), 3.00 (2H, t, $C\underline{H}_2CH_2CONH$), 6.67 (1H, s, pyrazole-H^4), 7.34 (4H, m, phenyl-$H^{2,6}$+ phenyl-$H^{2',6'}$), 7.44 (2H, d, phenyl-$H^{3,5}$), 7.63 (2H, d, phenyl-$H^{3',5'}$), 8.11(1H, d, pyridazine-H^4), 8.15(1H, d, pyridazine-H^5),



Compounds **1**-**20**



Compounds **21**-**24**

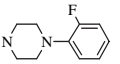
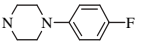
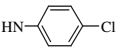
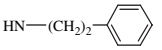
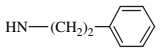
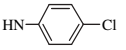
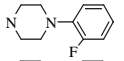
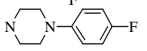
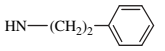
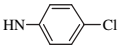
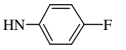
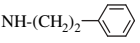
No	R_1	R_2	R_3	R_4
1	CH_3	H	Ph	OH
2	CH_3	H	Ph	OC_2H_5
3	CH_3	H	Ph	
4	CH_3	H	Ph	
5	CH_3	H	Ph	
6	CH_3	H	Ph	
7	CH_3	H	Ph	
8	CH_3	H	CH_3	OH
9	CH_3	H	CH_3	OC_2H_5
10	CH_3	H	CH_3	
11	CH_3	H	CH_3	
12	CH_3	H	CH_3	
13	CH_3	H	CH_3	
14	CH_3	H	CH_3	
15	CH_3	Cl	CH_3	OH
16	CH_3	Cl	CH_3	OC_2H_5
17	CH_3	Cl	CH_3	
18	CH_3	Cl	CH_3	
19	CH_3	Cl	CH_3	
20	CH_3	Cl	CH_3	
21	OH	–	–	–
22		–	–	–
23		–	–	–
24		–	–	–

Fig. 3 Formulas of 6-pyrazole-2*H*-pyridazin-3-one and 6-chloropyridazine derivatives tested for ARI activity

10.17(1H, s, NH). Anal. calcd for $C_{22}H_{16}Cl_3N_5O$ C, 55.89; H, 3.41; N, 14.81. Found: C,55.14; H, 3.43; N, 14.78.

N-Phenethyl-3-[5-(4-chlorophenyl)-1-(6-chloropyridazin-3-yl)-1*H*-pyrazol-3-yl]propanamide (**23**) ; ^{1}H NMR in DMSO-d_6 δ: 2.46 (2H, m, $CH_2\underline{CH_2}CONH$, under H_2O), 2.67 (2H, t, $CH_2\underline{CH_2}Ph$), 2.88 (2H, t, $\underline{CH_2}\underline{CH_2}CONH$), 3.27 (2H, q, $NHC\underline{H_2}CH_2$), 6.60 (1H, s, pyrazole-H^4), 7.16-7.23 (5H, phenyl), 7.32 (2H, d, phenyl-H^{2,6}), 7.42 (2H, d, phenyl-H^{3,5}), 8.01 (1H, t, NH), 8.08 (1H, d, pyridazine-H^4), 8.14 (1H, d, pyridazine-H^5). Anal. calcd for $C_{24}H_{21}Cl_2N_5O$ C, 61.81; H, 4.54; N, 15.02. Found: C,61.79; H, 4.39; N, 15.04.

4-{3-[5-(4-chlorophenyl)-1-(6-chloropyridazin-3-yl)-1*H*-pyrazol-3-yl]propanoyl}piperidine (**24**); ^{1}H NMR in DMSO-d_6 δ: 2.73 (2H, t, $CH_2\underline{CH_2}CONH$), 2.88 (2H, t, $\underline{CH_2}CH_2CONH$), 3.41 (4H, m, piperidine-H^{2,6}), 1.38-1.56 (6H, m, piperidine-H^{3,4,5}), 6.68 (1H, s, pyrazole-H^4), 7.32 (2H, d, phenyl-H^{2,6}), 7.42 (2H, d, phenyl-H^{3,5}), 8.08 (1H, d, pyridazine-H^4), 8.15 (1H, d, pyridazine-H^5). Anal. calcd for $C_{21}H_{21}Cl_2N_5O$ C, 58.61; H, 4.92; N, 16.27. Found: C,58.69; H, 4.79; N, 16.12.

Enzyme section

Isolation of aldose reductase enzyme.

The AR (ALR2) enzyme was isolated by the method (Cerelli *et al.*, 1986) described below. Pooled kidney were thawed on ice and homogenized with 3 volumes of distilled water, followed by centrifugation at $10{,}000 \times g$ for 20 min. Saturated ammonium sulfate was added to the supernatant to 40% saturation. The thick suspension had been stirred for 15 min, followed by centrifugation at $10{,}000 \times g$ for 20 min. The inert protein left in the supernatant was removed by increasing the ammonium sulfate concentration to 50% saturation followed by centrifuging the mixture at $10{,}000 \times g$ for 20 min. The AR enzyme was precipitated from the 50% saturated solution by adding powdered ammonium sulfate to 75% saturation and was recovered by centrifugation at $10{,}000 \times g$ for 20 min. Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as the standard.

Determination of aldose reductase activity.

AR activity of the freshly prepared supernatant was assayed spectrophotometrically by determining the decrease in NADPH concentration at 340 nm via a UV-1700 Visible spectrophotometer. DL-Glyceraldehyde was used as the substrate. The enzyme was dissolved in 10 ml of 0.05 M NaCI solution and 0.1 ml was added to a quartz cuvette containing 0.1 ml of phosphate buffer (0.067 M, pH 6.2), 0.1 ml of NADPH (2×10^{-5} M final concentration), 0.1 ml of the test drug (MEB), and 2.4 ml of distilled water to obtain a 2.9-ml solution. The reaction was started by the addition of 0.1 ml of DL-glyceraldehyde (5×10^{-5} M final concentration) to the cuvette, and the decrease in

Table 1 Results of aldose reductase inhibition by 2*H*-pyridazin-3-one and 6-chloropyridazine derivatives

Compounds	İnhibition (%)
1	0.76 ± 0.84
2	0.00 ± 0.00
3	1.85 ± 0.59
4	0.00 ± 0.00
5	3.37 ± 1.99
6	0.00 ± 0.00
7	0.00 ± 0.00
8	7.73 ± 2.19
9	0.00 ± 0.00
10	0.00 ± 0.00
11	0.00 ± 0.00
12	0.00 ± 0.00
13	0.00 ± 0.00
14	1.85 ± 9.25
15	0.98 ± 1.92
16	0.00 ± 0.00
17	0.00 ± 0.00
18	0.00 ± 0.00
19	0.00 ± 0.00
20	0.00 ± 0.00
21	0.76 ± 0.84
22	0.00 ± 0.00
23	0.00 ± 0.00
24	0.00 ± 0.00

Values represent the means ± SD of three individual experiments

NADPH concentration was recorded at 340 nm for 5 min at 37°C. Readings were taken at intervals in the periods when the changes in absorbance were linear. The results are shown in Table 1.

Results and Discussion

The AR inhibition data reported in Table 1. Results clearly showed that introduction of a substituted pyrazole ring on 2*H*-pyridazin-3-one and 6-chloropyridazine led to a marked decrease in AR inhibitory potency. This finding is in contrast to many effective AR inhibitory compounds possessing ring systems similar to that of pyrazole such as thiazole (Klebe *et al.*, 2004; Maccari *et al.*, 2005), oxadiazole (Klebe *et al.*, 2004), pyrolidine (Pau *et al.*, 2004), hydantoin, and related cyclic imides (Buyukbingol *et al.*, 1994) (Fig. 2).

Moreover, introduction of an acetic acid side chain to 2*H*-pyridazin-3-one and 6-chloropyridazine did not improve the AR inhibitory activity, showing very slight inhibitory activities indicated as percent inhibitor potency at a concentration of 10^{-4} M. Since carboxylic acid derivatives are typified by Epalrestat (the only AR available on the market), Alrestatin, Tolrestat, and Zopolrestat (bearing a pyridazinone ring) (Fig. 2), the unsatisfactory inhibition rates obtained from compounds **1**, **8**, **15** were quite surprising. Costantino *et al.* (1996, 1999) described the importance of the acidic side chain by insertion of an acetic or longer acidic chain on pyridazinone ring.

Thus AR has long been recognized as an important target for preventing of diabetic complications (Sarges and Oates, 1993). At present, Epalrestat is the only ARI available on the market (Maccari *et al*., 2005), and the research area requires further work. On the basis of our preliminary AR inhibitory screening results on 2*H*-pyridazine-3-one and 6-chloropyridazine derivatives, we embarked on the synthesis of more derivatives to discover more active molecules.

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