J. Enzyme Inhibition, 1993, Vol. 7, pp. 249-256
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STUDIES ON ALDOSE REDUCTASE INHIBITORS FROM FUNGI. II. MONILIFORMIN AND SMALL RING ANALOGUES

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(Received 19 July 1993)

The fungal metabolite moniliformin and several small ring analogues were evaluated for potential substrate and inhibitory activity in the rat lens aldose reductase (AR) assay. Even though all of these compounds possess carbonyl moieties and structural similarities to AR substrates, none were found to function as substrates over a concentration range of 1.0 mM to 10 μM. All of the compounds did display inhibitory activity with IC_{50}s ranging from 19–110 μM. The most inhibitory compounds were the four-membered ring moniliformin (IC_{50} 19 μM), the five-membered analogue croconic acid (IC_{50} 28 μM) and six-membered derivative tetrahydroxy *p*-benzoquinone (IC_{50} 23 μM). Modification of moniliformin by methylation (methyl moniliformin) or hydroxylation (squaric acid) resulted in a significant decline in inhibitory activity. All of the compounds evaluated except moniliformin displayed uncompetitive, non-competitive or mixed-type kinetics relative to the substrate (glyceraldehyde) and cofactor (NADPH), kinetic profiles commonly observed for inhibitors of AR.

KEY WORDS: Aldose reductase, moniliformin, croconic acid, inhibitors

INTRODUCTION

There is a considerable amount of evidence implicating the enzyme aldose reductase (EC.1.1.1.21) in the pathogenesis of the complications of chronic diabetes[1]. Aldose reductase (AR), along with sorbitol dehydrogenase (SDH), form the polyol pathway which converts glucose to fructose in many mammalian tissues. In this pathway, AR catalyzes the NADPH-dependent reduction of glucose to sorbitol, while SDH uses NAD^+ to oxidize sorbitol to fructose. During the hyperglycemia associated with diabetes there is increased flux of glucose through the polyol pathway and AR-catalyzed reduction to sorbitol. Since sorbitol is not efficiently oxidized by SDH it accumulates intracellularly and this accumulation is linked to a number of biochemical changes including hyperosmosis, myoinositol depletion and decreased activity of Na^+/K^+ ATPase. These biochemical changes ultimately are manifested as chronic diabetic pathologies including cataracts, retinopathy, neuropathies and nephropathies.

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Since AR appears to initiate this sequence of events, this enzyme has become an attractive target for pharmacological intervention to prevent or delay the onset of diabetic complications. Various AR inhibitors (ARIs) have been discovered in *in vitro* models, and a number of these have been found to reverse the biochemical changes associated with chronic diabetes and to mitigate the disease-associated pathologies[2].

Aldose reductase inhibitors (ARIs) discovered to date have been classified by structural type as carboxylic acids, cyclic imides and phenolic compounds. Most of the cyclic imide ARIs are spirohydantoins and include sorbinil, alconil and AL-1576. Carboxylic acid ARIs typically include the N-aroylglycine fragment and alrestatin, tolrestat, ponalrestat and isosteric arylsulfonylglycines are representative examples. Most phenolic ARIs are natural products such as the flavonoid quercitrin[3], the benzopyranone ellagic acid[4], and the bis-naphthyl gossypol[5]. Other synthetic poly-phenols, such as 2, 2′, 4, 4′-tetrahydroxybenzophenone[6], are also known to inhibit AR.

In an earlier study we found that the fungal metabolite citrinin and semisynthetic derivatives of citrinin displayed good inhibitory activity in vitro versus AR isolated from rat lenses[7]. These compounds were selected for study since they possess an acidic carboxyl group, a phenolic-like moiety and benzopyran ring system found in many ARIs. Recently, high ARI activity has been reported for the alpha-hydroxybutenolide WF-3681, a metabolite of the fungus *Chaetomella raphigera*[8].

Our screening of fungal metabolites for ARI activity has revealed good activity in moniliformin (semisquaric acid, **1**), which bears some structural analogy to an alpha-hydroxybutenolide, and which, like citrinin, is known to exert a variety of biological effects in animal and plant systems[9]. As part of our continuing studies to identify basic structural elements associated with AR inhibition, we also have examined a number of synthetic four, five and six-membered ring polycarbonyl-enol analogues of moniliformin. Thus we have studied the effects of moniliformin (3-hydroxy-cyclobut-3-ene-1, 2-dione, **1**), methylmoniliformin (3-hydroxy-4-methyl-cyclobut-3-ene-1, 2-dione, **2**), squaric acid (3, 4-dihydroxy-cyclobut-3-ene-1, 2-dione, **3**), disodium croconate (4, 5-dihydroxy-cyclopent-4-ene-1, 2, 3-trione disodium salt, **4**), rhodizonic acid (5, 6-dihydroxy-cyclohex-5-ene-1, 2, 3, 4-tetraone, **5**) and tetrahydroxy-1, 4-quinone (**6**) on aldose reductase isolated from rat lens (Figure 1).

MATERIALS AND METHODS

Moniliformin and Derivatives

Synthetic moniliformin and methylmoniliformin were gifts from Ciba-Geigy. Tetrahydroxy *p*-benzoquinone, rhodizonic acid and squaric acid were obtained commercially (Aldrich Chemical Co., Milwaukee, WI). Disodium croconate was prepared from glyoxal via the sodium salt of tetrahydroxy *p*-benzoquinone according to the method of Fatiadi *et al.*[10]

Enzyme isolation and assay

Aldose reductase was isolated from the lenses of rat eyes as described earlier[11]. Enzyme activity was assayed spectrophotometrically at 30°C by determining the

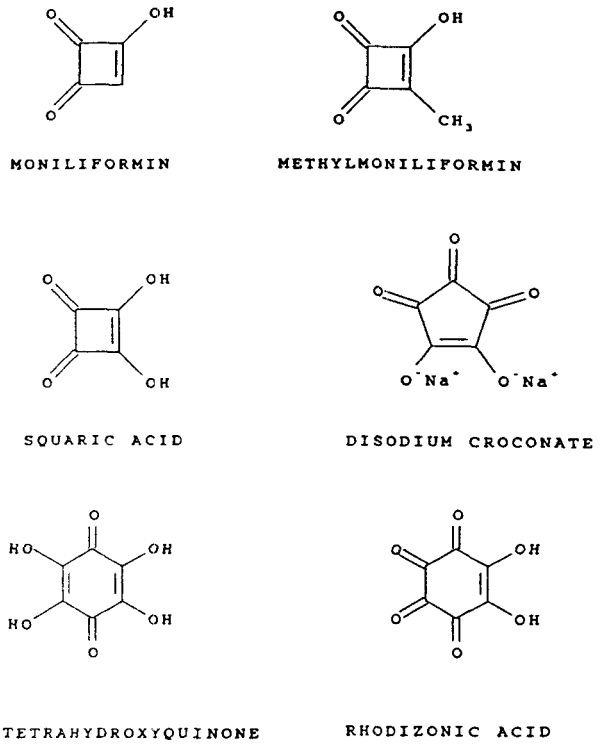

FIGURE 1 Polycarbonyl-Enol Analogues of Moniliformin

decrease in NADPH absorbance at 340 nm in a Shimadzu UV-160 spectrophotometer equipped with a thermo-controlled multi-cell positioner. The control reaction mixture contained 0.104 mM NADPH (Sigma Type I) in 0.1 M phosphate buffer (pH 6.2), 10 mM D,L-glyceraldehyde (Sigma Chemical Co.), 0.2 ml of enzyme solution and distilled water in a total volume of 2.0 ml. A solvent blank was used and contained all of the above reagents except glyceraldehyde to correct for any oxidation of NADPH not associated with reduction of substrate. The reaction was initiated by the addition of substrate and was monitored for 3 min following a 45 s initiation period. Enzyme activity was adjusted by dilution of the enzyme solution with distilled water such that 0.2 ml of supernatant gave an average reaction rate for the control reaction of 0.0120 ± 0.0020 absorbance units/min.

Effects of Inhibitors

Enzyme activity was determined by including 0.2 ml of an aqueous solution of the inhibitor at the desired concentration in the reaction mixture. The inhibitors were screened over a wide concentration range (1–200 μM) to obtain inhibitory data required to construct log concentration-percent inhibition curves. Each compound was

tested at no fewer than five different concentrations with a minimum of four determinations at each concentration. The percent inhibition for each inhibitor was calculated at all concentrations by comparing the rate of reactions containing inhibitor to that of control reactions with no inhibitor. Inhibitor IC_{50} values were then obtained by least squares analyses of the linear portion of the log inhibitor concentration versus percent inhibition curves using the LINEFIT program[12].

Kinetic Studies

Kinetic studies were carried out using two to four concentrations of each inhibitor. For substrate kinetics, the concentrations of the substrate D,L-glyceraldehyde range from 0.16–5.0 mM and for cofactor kinetics, the concentrations of NADPH were varied from 3.25–104 μM. The nature of inhibition produced by each concentration of inhibitor was determined by analysis of double reciprocal plots of enzyme velocity versus D,L-glyceraldehyde or NADPH concentration as generated by least squares fit of the data using the LINEFIT program[12].

RESULTS AND DISCUSSION

AR catalyzes the NADPH reduction of a variety of aryl and alkyl aldehydes and aldoses, presumably in their ring opened, aldehyde forms. The reduction of aldoses by AR is believed to be a sequential, ordered (bi bi) process that involves initial formation of an enzyme-cofactor complex (E-NADPH), followed by binding of the substrate[13]. The resultant enzyme-cofactor-substrate complex rearranges with reduction of the substrate carbonyl moiety to yield an enzyme-product (alcohol)-$NADP^+$ complex. This complex then releases the product alcohol, leaving the enzyme-$NADP^+$ complex which regenerates the free enzyme. All of the compounds included in this study possess two or more ketone carbonyl moieties and thus have the potential to function as AR substrates via the catalytic mechanism described above. Furthermore, all of these compounds, possess hydroxyl moieties similar to the aldose substrates, and the 5- and 6-membered ring compounds also bear some steric resemblance to the sugar substrates. Thus each compound was screened initially as a potential substrate in the presence of AR and the cofactor NADPH. Also, each compound was tested over a wide concentration range (10–1000 μM) to identify potential substrate activity and to avoid the possibility that catalytic activity may be masked by substrate inhibition at higher concentrations.

In the presence of AR and NADPH, none of the cyclic ketones displayed significant substrate activity over the entire concentration range. A low level of NADPH oxidation was observed when rhodizonic acid and the tetrahydroxyquinone were assayed with AR; this activity reached only 20% of the maximal velocity observed with glyceraldehyde as the substrate. However, further studies revealed that this low level of apparent activity was also present with rhodizonic acid or the tetrahydroxyquinone in the presence of NADPH alone. Thus the consumption of NADPH is not the result of AR-catalyzed reduction of these ketones, but rather some non-specific event such as direct reduction by NADPH.

TABLE I
Aldose reductase inhibitory activity of moniliformin and small ring derivatives.

COMPOUND	IC_{50}, μM (95% CL)	KINETICS	
		SUBSTRATE	COFACTOR
Moniliformin (**1**)	19 (11–30)	Mixed	Comp.
Methylmoniliformin (**2**)	110 (80–170)	Mixed	Mixed
Squaric acid (**3**)	81 (50–130)	Mixed	Non-Comp.
Croconate (**4**)	28 (10–70)	Non-Comp.	Non-Comp.
Rhodizonic acid (**5**)	82 (50–150)	Uncomp.	Uncomp.
Tetrahydroxyquinone (**6**)	23 (15–40)	Uncomp.	Uncomp.

The inability of AR to reduce these ketones is consistent with the published substrate profile for this enzyme[14]. To date there are no reports of AR catalyzing the reduction of any other substrate other than those possessing an aldehyde moiety, or latent aldehyde as in the aldoses. These results are also consistent with data reported by Mayfield and DeRuiter with a series of acetophenone derivatives[15]. These ketones were found to be inert to AR reduction and, in fact, to possess weak competitive AR inhibitory activity.

After substrate analysis, the cyclic carbonyl compounds were screened as potential inhibitors. Each compound was tested over a wide concentration range (1–200 μM) in the presence of AR, NADPH and the substrate glyceraldehyde. For each analysis, appropriate blanks consisting of AR and NADPH, as well as AR, NADPH and inhibitor compound were included for substrate non-specific activity. These data were used to construct log-concentration-inhibition curves from which inhibitor IC_{50} values were calculated. The results of these evaluations are presented in Table 1.

These data demonstrate that representative members of each ring class possess modest ARI activity. In the four-membered ring series, moniliformin is most inhibitory with an IC_{50} of 19 μM. It is interesting to note that simple substitution of a methyl group as in methylmoniliformin, or a hydroyl group, as in squaric acid, results in a four to six fold decrease in activity. With these limited number of compounds, it is unclear if the reduction of ARI activity observed upon substitution is a result of substituent electronic or steric factors, or possibly a combination of these factors.

The five-membered ring analogue, croconic acid (as the disodium salt) was found to be nearly as active as moniliformin. Also, of the two six-membered ring compounds screened, only tetrahydroxy *p*-benzoquinone displayed inhibitory activity comparable to moniliformin. This is interesting since tetrahydroxy *p*-benzoquinone and rhodizonic acid differ from each other only in the degree of ring oxidation. The former possesses a para-quinone moiety flanked by four enolic hydroxy gorups; the latter has four adjacent keto functionalities flanked by two enolic moieties. Thus the degree of ring oxidation appears to be important for AR affinity and inhibitory activity. However, further interpretation of structure-inhibition trends is complicated by the fact that all of the compounds tested behave as strong acids in aqueous solution, yielding anionic species, some of which would be aromatic, depending on their degree of deprotonation[16].

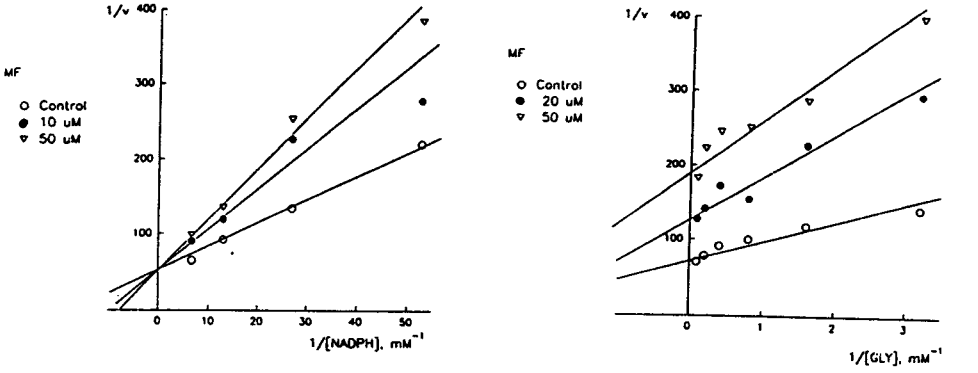

FIGURE 2 Lineweaver-Burk double reciprocal plots of initial enzyme velocity versus concentration of (a) the cofactor NADPH or (b) the substrate D,L-glyceraldehyde in the presence of moniliformin.

It might be mentioned in passing that a study by Takeuchi[17], comparing the "anti-diabetic" properties (in terms of mesoxalic acid-like activity) in rabbits of rhodizonic acid, tetrahydroxy *p*-benzoquinone, and croconic acid showed rhodizonic acid to be considerably more potent that either of the other compounds.

All of the compounds were also evaluated to determine their kientic profile of inhibition relative to both the substrate (glyceraldehyde) and the cofactor NADPH. The compounds displayed varying kinetic profiles: the six-membered ring compounds produced uncompetitive inhibition relative to both substrate and cofactor, while the five-membered croconate displayed non-competitive kinetics versus both glyceraldehyde and NADPH (Figures 3 and 4). Each of the four-membered compounds varied somewhat from each other in its kinetic profiles. Perhaps most interesting is the competitive nature of moniliformin with the cofactor NADPH (Figure 2).

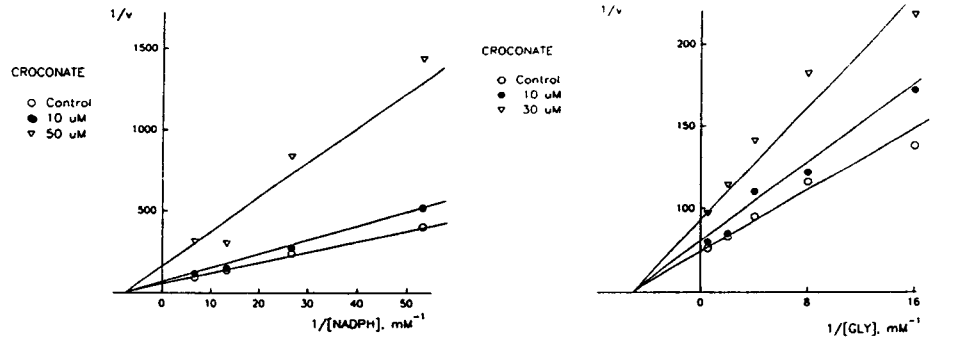

FIGURE 3 Lineweaver-Burk double reciprocal plots of initial enzyme velocity versus concentration of (a) the cofactor NADPH or (b) the substrate D,L-glyceraldehyde in the presence of disodium croconate.

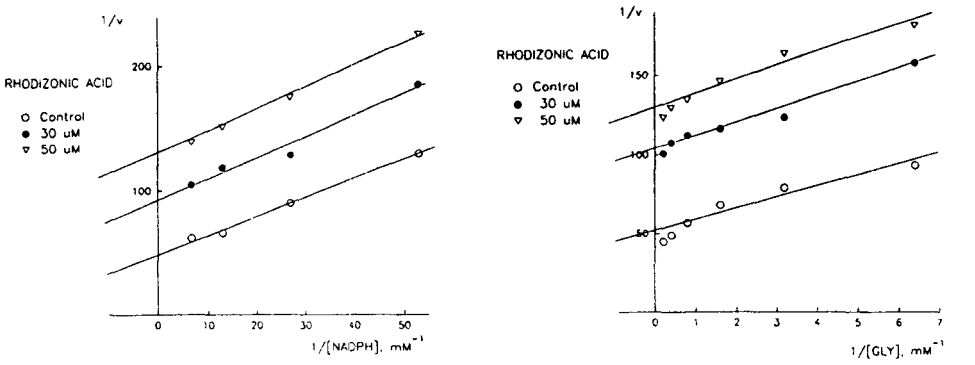

FIGURE 4 Lineweaver-Burk double reciprocal plots of initial enzyme velocity versus concentration of (a) the cofactor NADPH or (b) the substrate D,L-glyceraldehyde in the presence of rhodizonic acid.

Many ARIs are reported to display non-competitive, uncompetitive or mixed kinetic profiles with respect to both substrate and cofactor as was observed for most of the small ring carbonyl compounds of this study. These types of kinetic data have been interpreted to indicate inhibitor binding at a site distinct from the substrate and cofactor binding site, or binding at sites that only partially overlap those sites responsible for binding of endogenous ligands[18]. Thus it appears that the modest AR inhibition produced by moniliformin and small ring analogues is a result of interaction at a site distinct from the substrate binding site.

Acknowledgements

We thank Dr. Hp. Fischer, Ciba-Geigy, Switzerland, for his generous gifts of moniliformin and methylmoniliformin.

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