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ENZYME INHIBITION AND THE TOXIC ACTION OF MONILIFORMIN AND OTHER VINYLOGOUS α-KETOACIDS

LEO T. BURKA,* JOHNIENE DORAN and BENJAMIN J. WILSON
Department of Biochemistry and Center in Environmental Toxicology, Vanderbilt University School of Medicine, Nashville, TN 37232, U.S.A.

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Abstract—The inhibition of two thiamine-requiring enzymes by the potent mycotoxin, moniliformin (1-hydroxycyclobutene-3,4-dione), was investigated. Rat brain transketolase and pyruvate dehydrogenase were inhibited 25 percent by 10^{-9} M moniliformin. Studies carried out to determine if moniliformin causes enzyme inhibition by reaction with thiamine were negative. Varying the hydroxycyclobutenedione structure by substitution or ring expansion resulted in loss of toxicity and inhibition.

Moniliformin is a highly toxic fungal metabolite first isolated from *Fusarium moniliforme* grown on corn [1]. Recently both *F. moniliforme* and *F. fusarioides* have been found to produce moniliformin when cultured on corn [2]. Both fungi are common fungal contaminants of grain.

Thiel [3] has reported that the molecular mechanism for the toxic action of moniliformin involves selective inhibition of mitochondrial pyruvate and α-ketoglutarate oxidations. This author suggested that moniliformin may inhibit oxidation of α-ketoacids by inactivating lipoate in analogy to inactivation of this cofactor by arsenicals. Enzyme systems responsible for oxidation of α-ketoacids, however, required other coenzymes, and moniliformin could interfere at any of a number of sites. Moniliformin is, in fact, a vinylogous α-ketoacid and might be expected to interact much like pyruvate or α-ketoglutarate with enzymes or coenzymes.

A series of experiments with moniliformin and similar compounds (Fig. 1) containing the vinylogous ketoacid structure (COCOC=C—OH) has been carried out to further delineate the interaction of moniliformin with two enzymes that require thiamine pyrophosphate as a cofactor.

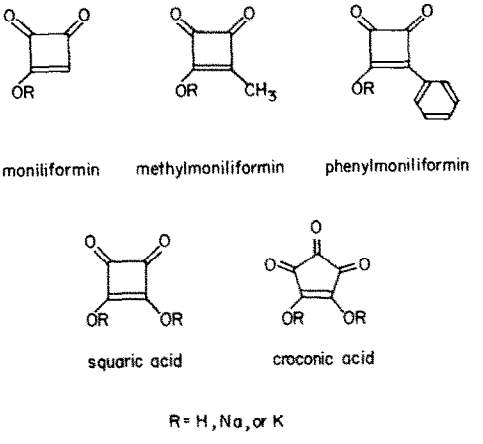

Fig. 1. Structures of moniliformin and analogs.

MATERIALS AND METHODS

Moniliformin was synthesized as described by Springer *et al.* [4]. Methyl moniliformin [5], phenylmoniliformin [5], and croconic acid [6] were prepared according to literature procedures. Squaric acid was obtained from the Aldrich Chemical Co. (Milwaukee, WI). All analogs except croconic acid were converted to the potassium salts and recrystallized from water or methanol–water. Croconic acid was used as the disodium salt.

Sprague–Dawley rats (Harlan Animal Industries, Indianapolis, IN) weighing about 200 g were decapitated. Brains and livers were removed and separately homogenized at 4° in 5–10 vol. of 0.2 M sucrose using a Teflon and glass homogenizer. The homogenate was used directly for pyruvate dehydrogenase assay. For transketolase studies the homogenate was centrifuged at 105,000 *g* for 60 min at 4°. The supernatant fraction was transferred to dialysis tubing (6000–8000 MWCO) and dialyzed overnight at 4° against distilled water. Protein concentrations were determined by the method of Lowry *et al.* [7].

Pyruvate dehydrogenase assays were based on the reduction of ferricyanide to ferrocyanide. The procedure used is an adaptation of that of Itokawa [8]. Incubation mixtures consisted of 150 μmoles of potassium phosphate buffer (pH 6.5), 1 μmole of calcium chloride, 0.2 μmole of thiamine pyrophosphate and 50 mg of tissue homogenate. Moniliformin was added in 0.1 ml of solution to give the desired concentration. Sufficient water was added to give 2.8 ml. After preincubation at 37° for the specified time, potassium ferricyanide (10 μmoles) and potassium pyruvate (50 μmoles) were added. Zero time

* Author to whom all correspondence should be addressed: Dr. Leo T. Burka, Department of Chemistry, Vanderbilt University, Nashville, TN 37235, U.S.A.

controls were quenched immediately with 1 ml of 7.5% trichloroacetic acid. After incubation for the specified time at 37°, the remaining incubation mixtures were quenched in the same way. The quenched mixtures were centrifuged at 1800 rpm for 10 min and ferric chloride (3.3 μmoles) was added to 2 ml of the supernatant fraction. The absorbance at 660 nm was determined after the mixture stood at room temperature for 10 min. In one experiment the tissue homogenate and moniliformin were preincubated in the buffer mixture for 20 min at 37°, and then dialyzed against the buffer solution for 24 hr. After this time protein concentration was determined, thiamine pyrophosphate was added, and the assay was performed as usual using a 15-min preincubation time and a 15-min incubation time.

Transketolase assays were based on the conversion of ribose to sedoheptulose. The procedure used for the incubations is an adaptation of that of Abe and Itokawa [9]. The incubation mixture consisted of 0.1 ml of the dialyzed supernatant fraction (containing 1 mg of protein), 0.1 ml of 1 mM thiamine pyrophosphate and 0.5 ml of a solution containing 25.7 μmoles of potassium phosphate (pH 7.4), 4.8 μmoles of sodium chloride, 140 μmoles of potassium chloride and 1.2 μmoles of magnesium sulfate. Moniliformin was added in 0.1 ml of solution to give the desired final concentration. The volume of the incubation mixture was adjusted to 1 ml with distilled water. After preincubation at 37° for the specified time, 0.2 ml of 17.6 mM ribose-5-phosphate was added. The zero time control was stopped at this time by adding 3 ml of 7.5% trichloroacetic acid. After incubation for the specified time at 37°, the remaining mixtures were quenched. The quenched mixtures were centrifuged at 1800 rpm for 10 min and a 0.1-ml aliquot of the supernatant fraction was diluted and assayed for sedoheptulose by the method of Dische [10].

The ^{13}C-n.m.r. spectrum (JEOL FX90Q) of thiamine pyrophosphate was measured as a 0.05 M solution in 1.0 M phosphate buffer (pH 8.8). ^{13}C Spectra

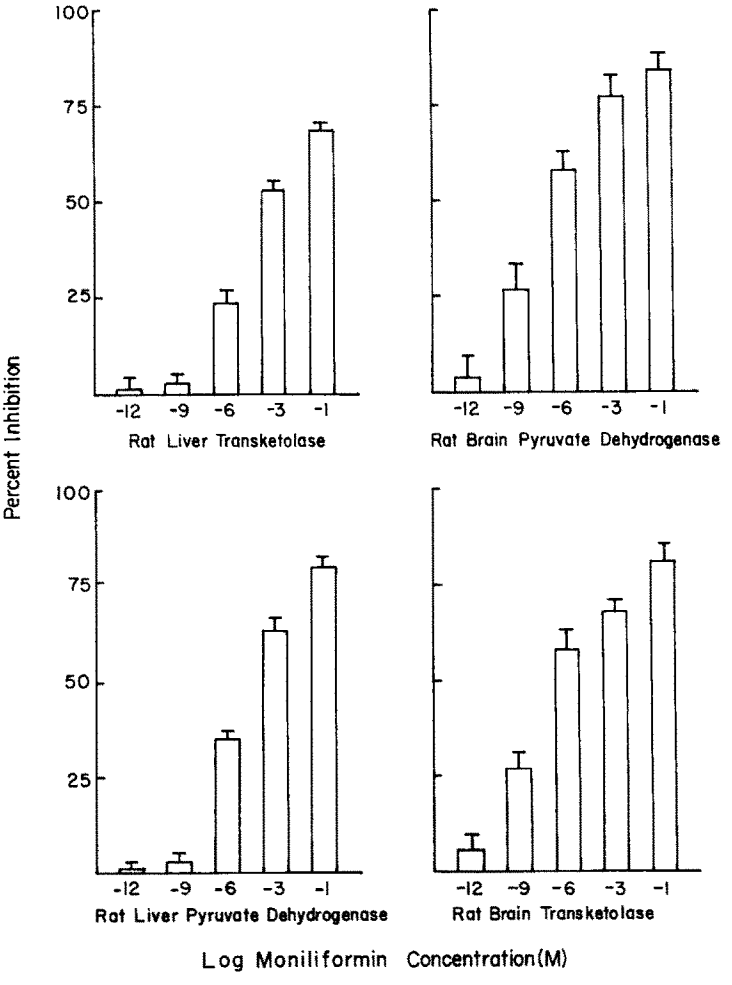

Fig. 2. Effect of moniliformin on transketolase and pyruvate dehydrogenase. Moniliformin and thiamine pyrophosphate were preincubated with the homogenate or supernatant fraction for 15 min before adding ferricyanide and pyruvate or ribose-5-phosphate. Pyruvate dehydrogenase incubation time was 15 min; transketolase incubation time was 20 min. Each bar is the mean ± S.E.M. for six to eighteen triplicate determinations. Uninhibited activities [nmoles·(mg protein)$^{-1}$·hr^{-1}] were rat liver transketolase, 1540 ± 30; rat brain transketolase, 426 ± 7; rat liver pyruvate dehydrogenase, 1200 ± 30; and rat brain pyruvate dehydrogenase, 377 ± 16.

were also obtained on a solution 0.05 M in both thiamine pyrophosphate and moniliformin and also on a solution 0.1 M in thiamine pyrophosphate and 0.05 M in moniliformin. Phosphate buffer (1.0 M, pH 8.8) was used as the solvent. Acquisition of each spectrum took about 1 hr; the sample temperature was 35–40° during this time.

The catalytic decarboxylation of pyruvate by thiamine was performed as described by Yatco-Manzo *et al.* [11]. A mixture of 0.01 M thiamine hydrochloride and 0.5 M sodium pyruvate in 0.0125 M borate buffer (pH 8.7) with or without 0.01 M moniliformin was incubated at 50° for the specified time. Acetoin was measured by the procedure of Westerfield [12].

Lethality studies were performed on 19- to 22-g mice (Harlan Animal Industries). The animals were acclimatized to the holding facility for at least 24 hr before beginning the study. The compounds were dissolved in sterile saline such that 0.1 ml contained the required dose. The following dose rates (mmoles/kg) were given intraperitoneally to each of six mice: moniliformin, 0.13, 0.15, 0.18, 0.22; methylmoniliformin, 0.55, 0.93, 1.5, 2.2; phenylmoniliformin, 0.44, 0.89, 1.8, 3.5; squaric acid, 0.22, 0.44, 0.89, 1.8; and croconic acid, 0.22, 0.44, 0.88, 1.8. The animals were observed for 1 week after injection. The moving average method described by Weil [13] was used to determine the LD_{50}.

RESULTS

The inhibition of pyruvate dehydrogenase and transketolase from rat liver and brain by moniliformin is shown in Fig. 2. The inhibition of the two enzymes is approximately linear with the log of moniliformin concentration over several orders of magnitude. Thiel [3] reports that a 5×10^{-6} M concentration of moniliformin causes a 50 percent inhibition of oxygen uptake by rat liver mitochondria with pyruvate as the substrate. In our assays 50 percent inhibition of rat liver pyruvate dehydrogenase, as measured by reduction of ferricyanide, occurs at about 10^{-4} M moniliformin.

The effect of varying the preincubation time of the enzymes with moniliformin and thiamine is shown in Tables 1 and 2. In all cases there was a diminution of enzyme activity with increased preincubation time. The percent inhibition was essentially constant in the transketolase experiments; however, a gradual increase in inhibition with increased preincubation time was evident in the pyruvate dehydrogenase results. Enzyme activity after a given preincubation time was not dependent on the time that thiamine and moniliformin were incubated together. For instance, rat brain pyruvate dehydrogenase gave 60 ± 10 nmoles·(mg protein)$^{-1}$·hr^{-1} regardless of whether thiamine was present only in the last 15 min or present for the entire hour of the preincubation time.

Preincubation of pyruvate dehydrogenase from brain and liver with 10^{-3} M moniliformin for 20 min followed by dialysis for 24 hr against the buffer solution resulted in a decrease in inhibition. For brain, pyruvate dehydrogenase inhibition decreased from 49 ± 7 to 18 ± 3 percent; likewise, liver enzyme inhibition decreased from 49 ± 3 to 18 ± 3 percent after dialysis. The measured decrease in inhibiton was a composite of a 25–30 percent decrease in

Table 1. Effect of varying preincubation on the inhibition of pyruvate dehydrogenase by moniliformin*

Total preincubation time (min)	Preincubation with moniliformin (min)	Preincubation with thiamine (min)	Substrate converted [nmoles·(mg protein)$^{-1}$·hr^{-1}]
Liver			
15		15	910 ± 40
15	15	15	350 ± 20
30		15	810 ± 30
30	30	15	260 ± 30
45		15	740 ± 50
45	45	15	210 ± 20
30		30	860 ± 40
30	30	30	280 ± 20
60		60	650 ± 40
60	60	60	130 ± 30
Brain			
15		15	280 ± 10
15	15	15	160 ± 10
30		15	240 ± 10
30	30	15	100 ± 10
60		15	180 ± 10
60	60	15	70 ± 10
30		30	280 ± 20
30	30	30	120 ± 10
60		60	200 ± 10
60	60	60	60 ± 10

* Moniliformin and thiamine pyrophosphate were preincubated with the tissue homogenate for the specified time before adding ferricyanide and pyruvate. Incubation time was 5 min. The results are the means ± S.D. of triplicate determinations.

Table 2. Effect of varying preincubation on the inhibition of transketolase by moniliformin*

Total preincubation time (min)	Preincubation with moniliformin (min)	Preincubation with thiamine (min)	Substrate converted [nmoles·(mg protein)$^{-1}$·hr^{-1}]
Liver			
15		15	890 ± 60
15	15	15	660 ± 30
30		15	780 ± 40
30	30	15	600 ± 20
60		15	560 ± 40
60	60	15	410 ± 30
30		30	900 ± 50
30	30	30	650 ± 40
60		60	680 ± 30
60	60	60	490 ± 30
Brain			
15		15	400 ± 20
15	15	15	270 ± 20
30		15	380 ± 20
30	30	15	240 ± 20
60		15	340 ± 20
60	60	15	180 ± 10
30		30	330 ± 10
30	30	30	220 ± 10
60		60	290 ± 10
60	60	60	180 ± 10

* Moniliformin and thiamine pyrophosphate were preincubated with the supernatant fraction for the specified time before adding ribose-5-phosphate. Incubation time was 7.5 min. The results are the means ± S.D. of triplicate determinations.

activity in controls after dialysis and a 15–20 percent increase in activity in the moniliformin-containing preparations after dialysis.

The ^{13}C-n.m.r. spectrum of thiamine pyrophosphate had resonances at δ 12.0, 24.7, 28.7, 51.7, 65.0, 105.6, 136.4, 143.9, 154.8, 157.1 162.8 and 169.4. The spectrum was unchanged upon addition of one-half or one equivalent of moniliformin except for the appearance of resonances at δ 169.3 and 203.7 due to moniliformin.

The effect of moniliformin on the catalytic decarboxylation of pyruvate by thiamine is shown in Table 3. An equimolar concentration of moniliformin had no effect on the formation of acetoin in this system.

The effects of moniliformin analogs on pyruvate dehydrogenase and transketolase are shown in Tables 4 and 5. Phenylmoniliformin showed a concentration-dependent inhibition on pyruvate dehydrogenase. The other analogs had no significant effect.

Table 3. Effect of moniliformin on the catalytic decarboxylation of pyruvate by thiamine*

Time (hr)	Pyruvate concn	Moniliformin concn	Acetoin (μmoles)
3	0.01 M	0	12
3	0.01 M	0.01 M	12
22	0.01 M	0	55
22	0.01 M	0.01 M	56

* Two milliliters of 0.0125 M borate buffer (pH 8.7) containing 0.01 M thiamine hydrochloride and 0.5 M sodium pyruvate and with or without 0.01 M moniliformin was incubated at 50° for the specified time. The average of duplicate experiments is reported.

The i.p. LD_{50} of moniliformin in mice was determined to be 20 ± 2 mg/kg (0.15 mmole/kg). Farb *et al.* [14] report an LD_{50} of 24 mg/kg in mice. All deaths occurred in less than 12 hr. Surviving animals showed no ill effects in the 1-week observation period. None of the analogs was lethal at any dose given. Single i.p. doses of methylmoniliformin, 334 mg/kg (2.2 mmoles/kg); phenylmoniliformin, 752 mg/kg (3.5 mmoles/kg); squaric acid, 336 mg/kg (1.8 mmoles/kg); and croconic acid, 328 mg/kg (1.8 mmoles/kg) gave no obvious toxic effect.

Mice injected intraperitoneally with moniliformin soon developed signs of rapidly progressing muscular weakness with sternal recumbency and failure to move when prodded. Lethal doses caused blanching of the normal retinal color, respiratory distress, and apparent coma preceding death that occurred within 2–3 hr following injection.

DISCUSSION

Moniliformin is a potent inhibitor of the two enzymes studied. Concentrations as low as 10^{-9} M resulted in 25 percent inhibition of rat brain pyruvate dehydrogenase and transketolase. Although both these enzymes act on carbonyl-containing substrates and require thiamine as a cofactor, transketolase does not require lipoic acid as a cofactor. Thus, if moniliformin inhibits pyruvate and α-ketoglutarate oxidation by interaction with lipoate as suggested by

Table 4. Effects of moniliformin analogs on pyruvate dehydrogenase*

	Liver pyruvate dehydrogenase (% of control ± S.E.M.)	Brain pyruvate dehydrogenase (% of control ± S.E.M.)
Moniliformin		
10^{-3} M	43 ± 1	52 ± 1
Phenylmoniliformin		
10^{-3} M	85 ± 1	84 ± 2
10^{-1} M	69 ± 1	71 ± 1
Methylmoniliformin		
10^{-3} M	97 ± 1	103 ± 1
10^{-1} M	98 ± 2	101 ± 1
Croconic acid		
10^{-3} M	102 ± 1	97 ± 1
10^{-1} M	103 ± 1	98 ± 1
Squaric acid		
10^{-3} M	101 ± 1	101 ± 1
10^{-1} M	99 ± 1	100 ± 1

* Moniliformin analog and thiamine pyrophosphate were preincubated with the tissue homogenate for 15 min before adding ferricyanide and pyruvate. Incubation time was 15 min. The results are the means ± S.E.M. for triplicate determinations for five animals.

Thiel, this cannot be the only mechanism of inhibition by moniliformin.

Interaction with thiamine is a possibility since it is a required cofactor for the enzymes inhibited. Moniliformin is known to react readily with primary amines to form vinylogous amides [15]. The reaction of moniliformin with the primary amino group of thiamine might result in the observed inhibitory effect. No observable change occurred in the ^{13}C-n.m.r. spectrum of thiamine pyrophosphate upon addition of moniliformin. One would expect a shift of the resonance at δ 169.4 if the amino nitrogen were converted to an amido nitrogen. Likewise, a shift in the resonance at δ 154.8 should occur if moniliformin reacted irreversibly with the thiazolium carbon responsible for the catalytic activity of thiamine. Additionally, the chemical shifts of moniliformin were unchanged in the presence of thiamine. Varying the time that moniliformin and thiamine were incubated together in the presence of the enzyme had little effect on inhibition as shown in Tables 1 and 2. Moniliformin also had no effect on the catalytic decarboxylation of pyruvate by thiamine hydrochloride.

The inhibition of pyruvate dehydrogenase was reversed by removing the moniliformin by dialysis although the enzyme activity was still not up to control levels after 24 hr of dialysis. Thus, it would appear that inhibition involved some reversible interaction with the enzyme complex itself and not a chemical reaction with one of the cofactors.

The effect of varying the substituents on the hydroxycyclobutenedione structure was striking. All the analogs studied are at least two orders of magnitude less potent toxins (on a molar basis) than moniliformin. The analogs did not inhibit transke-

Table 5. Effects of moniliformin analogs on transketolase*

	Liver transketolase (% of control ± S.E.M.)	Brain transketolase (% of control ± S.E.M.)
Moniliformin		
10^{-3} M	45 ± 1	41 ± 2
Phenylmoniliformin		
10^{-3} M	98 ± 1	98 ± 2
10^{-1} M	99 ± 1	96 ± 1
Methylmoniliformin		
10^{-3} M	97 ± 1	96 ± 1
10^{-1} M	98 ± 2	99 ± 3
Croconic acid		
10^{-3} M	99 ± 1	96 ± 1
10^{-1} M	96 ± 1	96 ± 2
Squaric acid		
10^{-3} M	100 ± 1	99 ± 1
10^{-1} M	100 ± 1	100 ± 2

* Moniliformin analog and thiamine pyrophosphate were preincubated with the dialyzed supernatant fraction for 15 min before adding ribose-5-phosphate. Incubation time was 20 min. The results are the means ± S.E.M. for triplicate determinations for five animals.

tolase at 10^{-1} M concentration. Phenylmoniliformin inhibited pyruvate dehydrogenase but it was much less potent than moniliformin. The other analogs had no effect on pyruvate dehydrogenase at 10^{-1} M concentration. This last result is intriguing since, if moniliformin were occupying an active site or an allosteric site on the enzyme complex, methylmoniliformin, rather than phenylmoniliformin with a bulky, electron-rich aromatic substituent, might be expected to behave more like moniliformin. It should be pointed out that all the hydroxycyclobutenediones have pK_a values near zero and therefore should be completely ionized at physiological pH. The apparent correlation of decreased toxicity of the analogs with decreased pyruvate dehydrogenase inhibition offers support to Thiel's suggestion that inhibition of the tricarboxylic acid cycle is the mechanism of toxicity for moniliformin. However, the transketolase results are also consistent with interference in carbohydrate metabolism being the mechanism of toxicity. Considering the short period of time required for death with moniliformin, it is our opinion that interference with the tricarboxylic acid cycle is a more likely cause of death than inhibition of transketolase.

Fusarium moniliforme is well established as the biological agent on corn that causes leucoencephalomalacia in equines (ELEM) [16, 17]. If moniliformin or a structurally similar compound does interfere with the energy-producing tricarboxylic acid cycle, it could explain the formation of malacic areas in the equine brain. However, no moniliformin has ever been detected in samples of disease-incriminated corn [18]. In one study, repeated intravenous administration of subacute doses of moniliformin to a donkey resulted in acute death with no promonitory signs. No gross neural lesions were noted, but mild microscopic lesions were seen which were somewhat similar to those occurring in both the natural and experimentally produced cases of ELEM [19]. In view of this contradictory evidence, the possible role of moniliformin in ELEM is unclear.

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