ANTIOXIDANTS & REDOX SIGNALING
Volume 10, Number 4, 2008

DOI: 10.1089/ars.2007.1816

Original Research Communication

Relation Between Triketone Structure, Generation of Reactive Oxygen Species, and Selective Toxicity of the Diabetogenic Agent Alloxan

MATTHIAS ELSNER, EWA GURGUL-CONVEY, and SIGURD LENZEN

ABSTRACT

The diabetogenic agent alloxan is a triketone that selectively destroys pancreatic beta cells. To investigate the importance of the triketone structure of alloxan for its cytotoxic potency, alloxan was compared with ninhydrin, also a triketone, and the amino derivative of alloxan uramil, which is not a triketone, because the 5-keto group of the alloxan is replaced by an amino group. Both compounds are cytotoxic but not diabetogenic. Ninhydrin was capable of generating cytotoxic reactive oxygen species (ROS) through redox cycling with dithiols, and uramil could also generate cytotoxic ROS. Both ninhydrin and uramil could not redox cycle with glutathione (GSH) and were not selectively toxic to beta cells; their structure does not allow selective cellular uptake *via* the GLUT2 glucose transporter. Thus, the results show that the 5-keto group in the pyrimidine ring structure of the triketone alloxan is crucially important for its ability to be selectively taken up into the beta cells via the specific glucose transporter GLUT2. The 5-keto group of the molecule enables redox cycling of alloxan through reaction with glutathione (GSH), thereby generating the cytotoxic ROS. Thus, the unique combination of these two properties confers on alloxan the beta cell–selective toxicity and diabetogenicity. Replacement of the 5-keto group by an amino group, as in uramil, abolishes selective beta cell toxicity because of the loss of the glucose analogue structure and the capability to generate ROS *via* redox cycling with GSH and cysteine. *Antioxid. Redox Signal.* 10, 691–699.

INTRODUCTION

The diabetogenic agent alloxan (2,4,5,6[1H, 3H]-pyrimidinetetrone), a triketone, can generate reactive oxygen species (ROS) in a cyclic reaction between this substance and its reduction product, dialuric acid. Reduction of alloxan to dialuric acid in the cell requires the presence of a suitable thiol, typically the tripeptide glutathione (GSH) (4, 7, 18, 31).

In the present investigation, we studied the importance of the triketone structure of alloxan (Fig. 1) for its cytotoxic potency. For this purpose, we compared alloxan with ninhydrin, also a cytotoxic triketone (see Fig. 1), which, however, is not diabetogenic (5, 12). Furthermore, we compared alloxan with the closely related nondiabetic compound uramil (5), the amino derivative of alloxan (16), which is not a triketone because the 5-keto group of the alloxan molecule is replaced by an amino group in this compound (see Fig. 1).

We show in this study that the 5-keto group of the triketone structure of alloxan is not only crucially important for its ability to initiate redox cycling through reaction with the monothiol glutathione, thereby generating cytotoxic reactive oxygen species but also that the 5-keto group is an integral element of the chemical structure of this pyrimidine derivative, which confers on it the similarity with the glucose molecule that is required for the selective uptake of alloxan into the pancreatic beta cell and thus for its selective toxicity and diabetogenicity.

Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany.

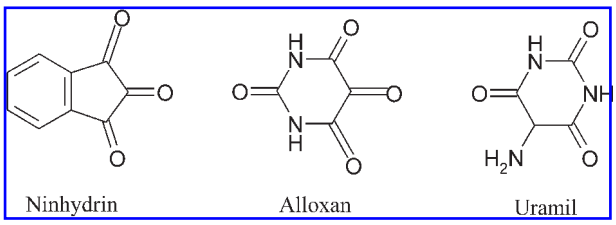

FIG. 1. Chemical structures of the triketone compounds ninhydrin, alloxan, and uramil.

MATERIALS AND METHODS

Materials

Alloxan monohydrate, uramil (5-aminobarbituric acid), reduced L-glutathione (GSH), L-cysteine, L-dithiotreitol (DTT), and catalase (from bovine liver) were obtained from Sigma (Munich, Germany); streptozotocin, from Alexis Biochemicals (Lausen, Switzerland); and superoxide dismutase (SOD), from bovine erythrocytes from Roche Diagnostics (Mannheim, Germany). Geneticin (G 418), zeocin, lipofectamine, and all other tissue-culture equipment were from Invitrogen (Karlsruhe, Germany). All other reagents of analytic grade were from Merck (Darmstadt, Germany).

Tissue culture

The RINm5F insulin-producing tissue culture cells were established by Gazdar *et al*. (10) by a radiation-induced rat pancreatic islet tumor. The cells (passages 55–75) were cultured in RPMI 1640 medium, supplemented with 10 m*M* glucose, 10% (vol/vol) fetal calf serum, penicillin, and streptomycin in a humidified atmosphere at 37°C and 5% CO_2.

Stable overexpression in RINm5F cells

Overexpressing RINm5F cells were generated through stable transfection of the respective cDNA under the control of the cytomegalovirus promoter in the pcDNA3 vector by using lipofectamine (29). Transfection of rat GLUT2 glucose transporter cDNA in RINm5F cells was achieved as described (8). The GLUT2 protein expression in cell clones was compared with the expression level in rat liver (defined as 100%; $n = 4$). In GLUT2-overexpressing RINm5F insulin-producing cells, the expression reached the same level as in liver [104 ± 7% ($n = 4$)], whereas in control RINm5F cells, it was negligible (<1% of control), as shown before (8). Transfection of rat cytoplasmic Cu/Zn SOD cDNA and of human catalase cDNA alone, as well as double transfection of Cu/Zn SOD cDNA plus catalase cDNA in RINm5F cells, was performed as described (30). Transfected clones were selected through resistance against geneticin (29) and, in the case of the double transfection, additionally against zeocin (30).

Exposure to chemical compounds

Control and overexpressing RINm5F cells were seeded at a concentration of 2.5×10^4 cells/well in 100 μl culture medium in 96-well microplates and allowed to attach for a period of 24 h at 37°C before incubation with the different test compounds. Test compounds were dissolved in 10 m*M* HCl immediately before the experiment and added to the incubation medium (20 m*M* Hepes-supplemented Krebs–Ringer bicarbonate medium, pH 7.4, without glucose) (15). The pH of the buffer was not affected by these additions. Cells were incubated with different concentrations of alloxan, ninhydrin, uramil or streptozotocin, and glutathione, cysteine, or DTT for 1 h at 37°C in the incubation medium and, after removal of the test compounds, for another 24 h in RPMI 1640 medium at 10 m*M* glucose. In some sets of experiments, antioxidative enzymes were added to the incubation medium. After the overnight incubation period, the viability of the cells was determined by using a microplate-based MTT assay (21). Viability was expressed as percentage of the untreated samples.

Oxygen uptake

Oxygen uptake by alloxan, ninhydrin, uramil, and streptozotocin, and in the presence of thiols was measured by using a WTW Oxi 340/SET dissolved oxygen meter (WTW, Weilheim, Germany). The buffer used was 50 m*M* potassium phosphate at pH 7.4, and the temperature was maintained at 25°C. Recordings of oxygen uptake were started immediately after addition of the test compounds to the buffered thiol solutions; in experiments in which the effects of superoxide dismutase and catalase were investigated, these were added before the test substances.

Enzymatic glucose phosphorylation capacity of control RINm5F and GLUT2 RINm5F cell homogenates after exposure to alloxan, ninhydrin, and uramil

Control RINm5F and GLUT2 RINm5F cells were seeded at a concentration of 3×10^6 cells and allowed to attach for a period of 24 h at 37°C before incubation with alloxan (10 m*M*), ninhydrin (0.25 m*M*), or uramil (5 m*M*) (23). Cells were incubated with test compounds for 5 min at 37°C (23). After removal of the incubation medium, the cells were washed twice with PBS and trypsinized. Thereafter, the cells were homogenized by sonication (60 W, three bursts of 10 sec each) and immediately used for spectrophotometric measurement of glucose phosphorylation capacity in an enzyme-coupled assay containing 25 m*M* glucose, as described previously (19).

Statistical analyses

Data are expressed as mean ± SEM. Statistical analyses were done by using one-way ANOVA followed by Dunnett's test or Tukey–Kramer test for multiple comparisons. EC_{50} values were calculated from nonlinear regression analyses using the one-phase exponential decay algorithm of the Prism4 analysis program (GraphPad, San Diego, CA).

RESULTS

Toxicity of alloxan, uramil and ninhydrin in the presence of thiols in RINm5F insulin-producing cells

Alloxan, even at high millimolar concentrations (up to 20 m*M*), was not toxic to RINm5F insulin-producing cells lacking the expression of the GLUT2 glucose transporter (Table 1 and

Fig. 2A). In contrast, alloxan was toxic to RINm5F insulin-producing cells expressing the GLUT2 glucose transporter, with a half maximally effective concentration (EC_{50}) of alloxan, at which 50% of the cells died, of 3.9 m*M* (see Table 1). In the presence of GSH (1, 5, or 10 m*M*), alloxan became very toxic to RINm5F cells, irrespective of the expression of the GLUT2 glucose transporter. The EC_{50} values of alloxan under these conditions were between 17–39 μM for both RINm5F control and RINm5F-GLUT2 cells. Interestingly, in the combination of alloxan with GSH, the toxicity decreased with increasing GSH concentrations between 1 and 10 m*M*, resulting in a doubling of the EC_{50} value (see Table 1) confirming that the ratio of alloxan to GSH concentration is important for the toxicity (7). Similar effects were observed with the thiols cysteine and DTT. The EC_{50} values for these compounds with alloxan were between 17 and 43 μM, whereas the toxicity decreased again at higher concentrations of the thiols (5 m*M*) (see Table 1).

Ninhydrin, with EC_{50} values of 19 and 25 μM, respectively, showed a prominent toxicity against RINm5F control and RINm5F-GLUT2 cells (Table 1; Figs. 2A and 3A). Addition of 5 or 10 m*M* GSH to the incubation media did not affect the toxicity significantly. Only at 1 m*M* GSH was a slight increase of the EC_{50} (~40–50 μM) detectable. Cysteine abolished the toxic effect of ninhydrin totally up to concentrations of 250 μM (see Table 1). The reason for this loss of the toxic effect is likely an adduct formation of ninhydrin and cysteine (18). In contrast, DTT (1 and 5 m*M*) had a strong enhancing effect on the toxicity of ninhydrin to RINm5F insulin-producing cells as well as to RINm5F cells expressing the GLUT2 glucose transporter. The EC_{50} was between 2 and 5 μM ninhydrin (see Table 1).

Uramil had a comparable toxic effect both on RINm5F control and RINm5F-GLUT2 cells with EC_{50} in the range of 121–137 μM (see Table 1). In contrast to the other analyzed compounds, the toxicity of uramil was inhibited by all three thiols used. GSH (1, 5, and 10 m*M*) strongly reduced or even completely suppressed the uramil toxicity in a concentration-dependent manner. The 10 m*M* GSH together with 500 μM uramil did not cause significant cell death. In incubations with uramil and cysteine (1 and 5 m*M*), the EC_{50} was ~400 μM. An addition of DTT (1 and 5 m*M*) to the uramil-containing medium (up to 500 μM) inhibited the toxic effect of the compound completely (see Table 1). The reason for this loss of toxicity in the presence of the thiols is again likely adduct formation. The toxicity of streptozotocin was not affected by addition of thiols (data not shown).

TABLE 1. HALF MAXIMALLY EFFECTIVE CONCENTRATIONS (EC_{50}) FOR THE TOXICITY OF ALLOXAN, URAMIL, AND NINHYDRIN IN THE ABSENCE OR PRESENCE OF GSH, CYSTEINE, OR DTT IN RINM5F INSULIN-PRODUCING CELLS AND RINM5F INSULIN-PRODUCING CELLS EXPRESSING THE GLUT2 GLUCOSE TRANSPORTER

	EC50 (μM)	
Test compounds	*Control RIN m5F cells*	*GLUT2 RINm5F cells*
Alloxan	No inhibition up to 20 m*M* (4)	3,923 ± 313 (8)
Alloxan + 1 m*M* GSH	22 ± 2 (8)[b]	17 ± 1 (8)[b]
Alloxan + 5 m*M* GSH	22 ± 1 (4)[b]	28 ± 1 (4)[b]
Alloxan + 10 m*M* GSH	39 ± 2 (4)[b]	38 ± 1 (4)[b]
Alloxan + 1 m*M* cysteine	43 ± 2 (4)[b]	32 ± 2 (4)[b]
Alloxan + 5 m*M* cysteine	57 ± 7 (7)[b]	54 ± 3 (7)[b]
Alloxan + 1 m*M* DTT	17 ± 2 (4)[b]	20 ± 4 (4)[b]
Alloxan + 5 m*M* DTT	46 ± 1 (4)[b]	47 ± 1 (4)[b]
Ninhydrin	19 ± 0 (12)	25 ± 0 (12)
Ninhydrin + 1 m*M* GSH	40 ± 4 (4)	49 ± 4 (4)
Ninhydrin + 5 m*M* GSH	21 ± 0 (8)	23 ± 1 (8)
Ninhydrin + 10 m*M* GSH	20 ± 1 (8)	23 ± 2 (8)
Ninhydrin + 1 m*M* cysteine	>250 (4)[b]	>250 (4)[b]
Ninhydrin + 5 m*M* cysteine	>250 (4)[b]	>250 (4)[b]
Ninhydrin + 1 m*M* DTT	2 ± 0 (8)	2 ± 0 (8)
Ninhydrin + 5 m*M* DTT	5 ± 0 (4)	4 ± 1 (4)
Uramil	121 ± 4 (12)	137 ± 2 (12)
Uramil + 1 m*M* GSH	146 ± 8 (8)[b]	166 ± 6 (8)[a]
Uramil + 5 m*M* GSH	246 ± 10 (12)[b]	222 ± 10 (12)[b]
Uramil + 10 m*M* GSH	>500 (4)[b]	>500 (4)[b]
Uramil + 1 m*M* cysteine	413 ± 60 (4)[b]	416 ± 31 (4)[b]
Uramil + 5 m*M* cysteine	400 ± 43 (8)[b]	360 ± 32 (8)[b]
Uramil + 1 m*M* DTT	>500 (8)[b]	>500 (8)[b]
Uramil + 5 m*M* DTT	>500 (4)[b]	>500 (4)[b]

Data are expressed as mean ± SEM with the numbers of experiments given in parentheses. The EC_{50} values were calculated by nonlinear regression analyses. Viability of the cells was determined by the MTT assay.

[a]$p < 0.05$; [b]$p < 0.01$. Statistical analyses were performed by using one-way ANOVA followed by Dunnett's test for multiple comparisons.

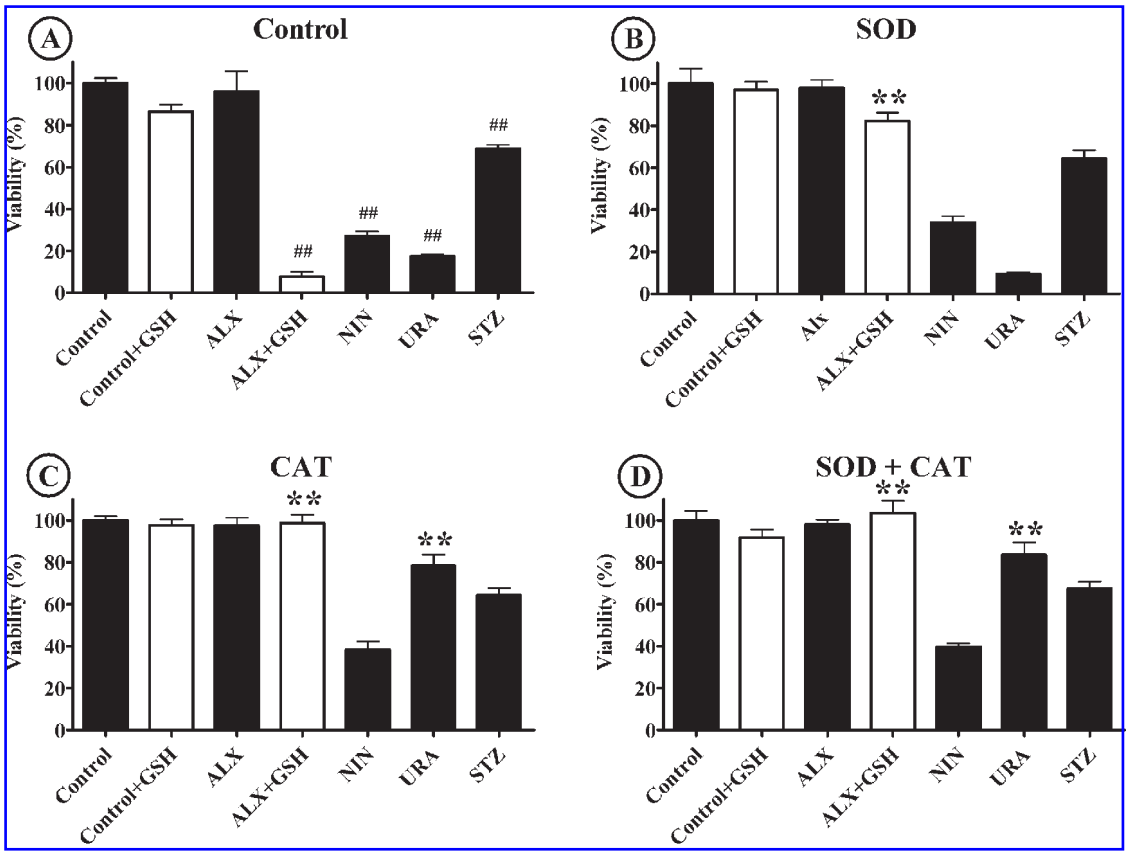

FIG. 2. Toxicity of alloxan (ALX), ninhydrin (NIN), uramil (URA), and streptozotocin (STZ) in RINm5F insulin-producing cells, potentiation by GSH, and protection by SOD catalase (CAT). Cells were incubated for 1 h with alloxan (50 μM), ninhydrin (50 μM) or uramil (250 μM), or streptozotocin (10 m*M*) in the presence (*white columns*) and absence (*black columns*) of GSH (5 m*M*), SOD (125 μg/ml), and/or CAT (500 μg/ml). Cell viability was measured by the MTT assay and expressed as a percentage of the untreated cells. Data are expressed as mean ± SEM from eight individual experiments. Statistical analyses were performed by using one-way ANOVA followed by Tukey–Kramer test for multiple comparisons. $^{\#\#}p < 0.01$, Alx+GSH *vs.* Control+GSH; NIN, URA, STZ *vs.* control. $^{**}p < 0.01$ *vs.* treated control cells.

Effect of the antioxidative enzymes SOD and catalase on the toxicity of alloxan, uramil, and ninhydrin in RINm5F insulin-producing cells

Addition of SOD (125 μg/ml) to the incubation medium did not affect the toxicity of alloxan in the absence of GSH (5 m*M*). Both in control RINm5F insulin-producing cells (Fig. 2B) and in RINm5F insulin-producing cells expressing the GLUT2 glucose transporter (Fig. 3B), alloxan was also not toxic in the presence of SOD. In the presence of GSH (5 m*M*), however, which increases the toxicity of alloxan strongly, addition of SOD (125 μg/ml) to the incubation medium significantly decreased the toxicity of alloxan, both in control (Fig. 2B) and in RINm5F-GLUT2 insulin-producing cells (Fig. 3B).

No protective effect by SOD was detectable in the experiments with ninhydrin, uramil, or streptozotocin. But streptozotocin, in contrast to ninhydrin and uramil, showed, like alloxan, specificity of the toxic action for RINm5F-GLUT2 insulin-producing cells. The viability of RINm5F control cells after exposure to 10 m*M* streptozotocin was around 65%, whereas the viability of RINm5F-GLUT2 after incubation with 5 m*M* streptozotocin was only around 7%. The higher toxicity of streptozotocin to RINm5F-GLUT2 cells in comparison with RINm5F control cells can be explained by the preferential uptake of this compound into the cell by the GLUT2 glucose transporter (6).

Addition of catalase (500 μg/ml) to the incubation medium significantly reduced the toxicity of alloxan, ninhydrin, and uramil in RINm5F control (see Fig. 2C) and RINm5F-GLUT2 cells (see Fig. 3C), whereas the toxicity of streptozotocin remained unaffected by catalase addition (Figs. 2C and 3C).

A combination of SOD and catalase did not provide more protection than catalase alone against the toxicity of alloxan, ninhydrin, and uramil, whereas toxicity against streptozotocin again remained unaffected (Figs. 2D and 3D).

To evaluate the importance of the intracellular antioxidative defense for protection of insulin-producing cells against the toxicity of ROS generated by alloxan, ninhydrin, uramil, and streptozotocin, the protective effect of overexpression of the antioxidative enzymes SOD and catalase, alone and in combination, in the cytoplasm of RINm5F insulin-producing cells was

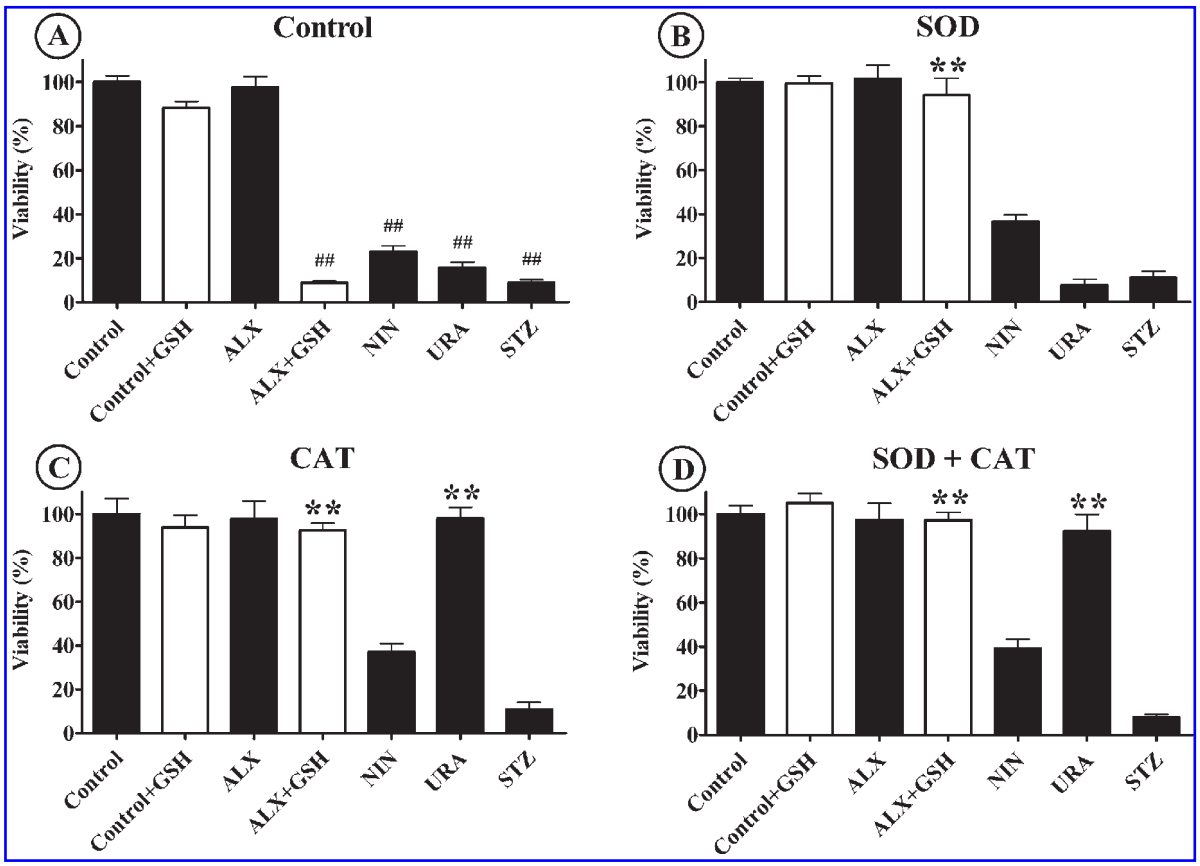

FIG. 3. Toxicity of alloxan (ALX), ninhydrin (NIN), uramil (URA), and streptozotocin (STZ) in RINm5F insulin-producing cells expressing GLUT2 glucose transporter, potentiation by GSH, and protection by SOD and catalase (CAT). Cells were incubated for 1 h with alloxan (50 μM), ninhydrin (50 μM), uramil (250 μM), or streptozotocin (10 mM), in the presence (*white columns*) and absence (*black columns*) of GSH (5 mM), SOD (125 μg/ml), and/or CAT (500 μg/ml). Cell viability was measured by the MTT assay and expressed as a percentage of the untreated cells. Data are expressed as mean ± SEM from eight individual experiments. Statistical analyses were preformed by using one-way ANOVA followed by Tukey–Kramer test for multiple comparisons. $^{\#\#}p < 0.01$, Alx+GSH *vs.* Control+GSH; NIN, URA, STZ *vs.* control. $^{**}p < 0.01$ *vs.* treated control cells.

analyzed. The level of SOD expression was 2.5 times higher, and the level of catalase expression was around 40 times higher than that in control cells (7).

SOD overexpression provided protection against alloxan (50 μM) in the presence of GSH (5 mM). The viability increased from 6% (Fig. 4A) to 66% (Fig. 4B). The protection by SOD against ninhydrin and uramil was only marginal (Fig. 4B).

Catalase overexpression also provided significant protection against the toxicity of alloxan in the presence of GSH (5 mM) (see Fig. 4C), but against the toxicity of ninhydrin and uramil, the protection of catalase was more prominent than the protection of SOD (Fig. 4C). The viability of RINm5F cells overexpressing catalase was approximately 3 times higher than that of SOD-overexpressing cells exposed to these three toxins (Fig. 4C).

A combined overexpression of SOD plus catalase in RINm5F insulin-producing cells provided a slight additional protective effect against alloxan toxicity (50 μM), but no additional protective effect against ninhydrin (50 μM) and uramil (250 μM) toxicity in comparison with catalase-overexpressing cells (see Fig. 4D). Against the toxicity of streptozotocin, the overexpression of antioxidant enzymes had no significant protective effect (see Fig. 4A–D).

Oxygen uptake by alloxan, ninhydrin, and uramil in the presence of thiols

To obtain direct evidence for redox cycling of the toxins with the thiols GSH, cysteine, and DTT, the initial oxygen uptake was determined. Oxygen was consumed in a solution of alloxan (50 μM) with all three thiols (1 mM) (Table 2), confirming earlier publications (18). In the reactions of ninhydrin (50 μM) with the thiols (1 mM), only the combination with DTT resulted in a very high rate of oxygen uptake (see Table 2). Thus, ninhydrin apparently does not redox cycle with GSH and cysteine. Uramil (250 μM) caused virtually no effect on oxygen uptake with the three thiols (see Table 2). This indicates that uramil does not redox cycle with these thiols (see Table 2). Streptozotocin (50 μM) also did not cause oxygen uptake with the three thiols (data not shown).

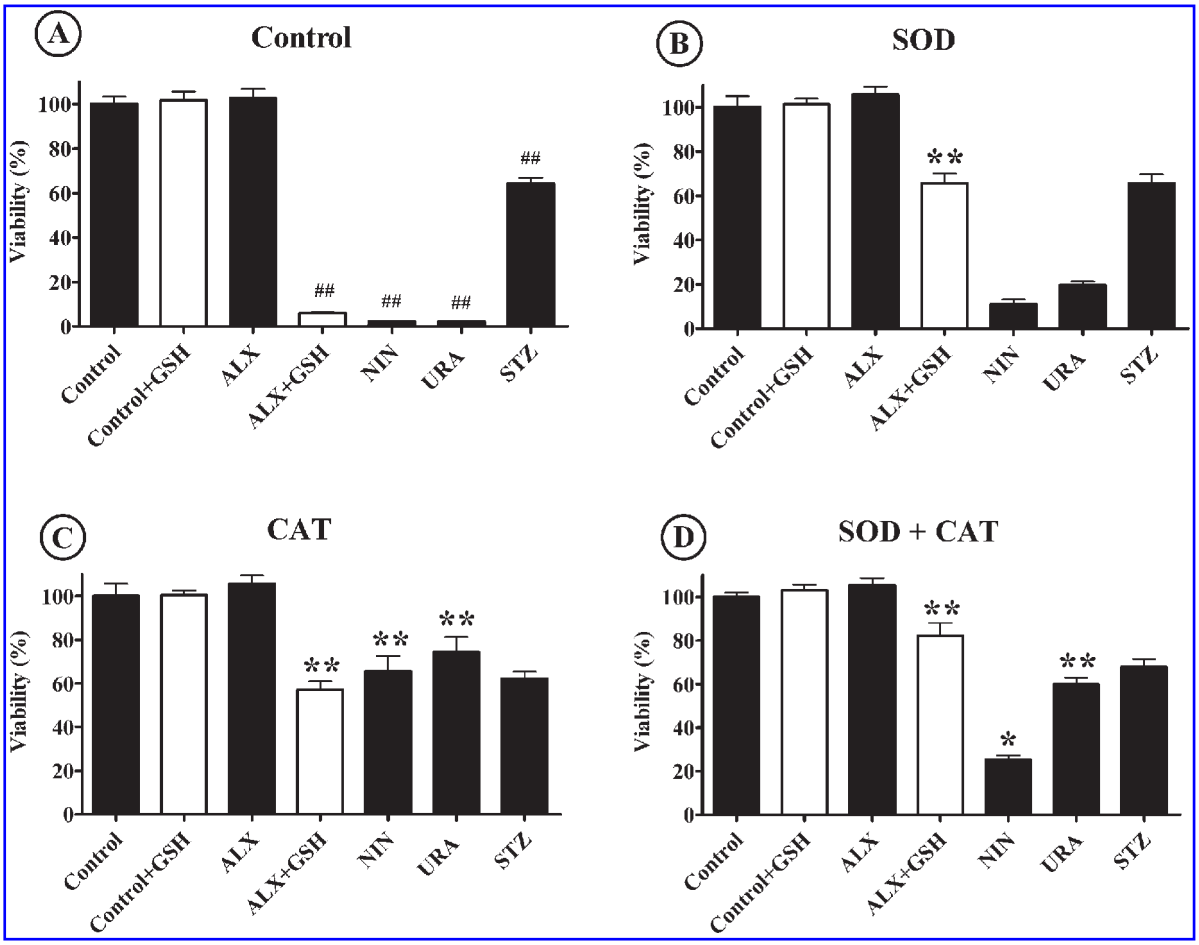

FIG. 4. Toxicity of alloxan (ALX), ninhydrin (NIN), uramil (URA) and streptozotocin (STZ), and uramil (URA), and its potentiation by GSH in RINm5F cells overexpressing CAT, SOD or CAT and SOD in combination. Cells were incubated for 1 h with alloxan (50 μM), dialuric acid (50 μM), alloxantin (50 μM), ninhydrin (50 μM), or uramil (250 μM), in the presence (*white columns*) and absence (*black columns*) of GSH (5 m*M*). Cell viability was measured by the MTT assay and expressed as a percentage of the untreated cells. Data are expressed as mean ± SEM from eight individual experiments. Statistical analyses were performed by using one-way ANOVA followed by Tukey–Kramer test for multiple comparisons. $^{\#\#}p < 0.01$, Alx+GSH *vs.* Control+GSH; NIN, URA, STZ *vs.* control. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* treated control cells.

TABLE 2. OXYGEN UPTAKE BY ALLOXAN, NINHYDRIN, AND URAMIL IN THE PRESENCE OF GSH, CYSTEINE, OR DTT

	Initial rate of oxygen uptake (μmol/L/min)		
	GSH	*Cysteine*	*DTT*
Alloxan	10.6 ± 1.5 (4)[a]	14.4 ± 2.2 (4)[b]	23.7 ± 1.1 (4)[a,b]
Ninhydrin	0.8 ± 0.2 (6)[c]	1.3 ± 0.2 (6)[d]	169.5 ± 18.7 (6)[c,d]
Uramil	0.1 ± 0.2 (6)	−0.2 ± 0.2 (4)	0.0 ± 0.1 (4)

The oxygen uptake by alloxan (50 μM), ninhydrin (50 μM), and uramil (250 μM) in the presence of GSH, cysteine, and DTT (all 1 m*M*) was measured with a Clark electrode. The initial rates of oxygen uptake were calculated by nonlinear regression analyses. Data are expressed as mean ± SEM with the numbers of experiments given in parentheses. Statistical analyses were performed by using one-way ANOVA followed by the Tukey–Kramer test for multiple comparisons.

[a] $p < 0.01$ alloxan + GSH *vs.* alloxan + DTT.
[b] $p < 0.01$ alloxan + cysteine *vs.* alloxan + DTT.
[c] $p < 0.01$ ninhydrin + GSH *vs.* ninhydrin + DTT.
[d] $p < 0.01$ ninhydrin + cysteine *vs.* ninhydrin + DTT.

TABLE 3. ENZYMATIC GLUCOSE PHOSPHORYLATION CAPACITY OF CONTROL RINM5F AND GLUT2 RINM5F CELL HOMOGENATES AFTER EXPOSURE TO ALLOXAN, NINHYDRIN, AND URAMIL

	Glucose phosphorylation capacity (%)	
Test compounds	*Control RINm5F cells*	*GLUT2 RINm5F cells*
Control	100 ± 4 (7)	100 ± 5 (7)
Alloxan (10 m*M*)	87 ± 3 (5)	33 ± 7 (5)[a,b]
Ninhydrin (0.25 m*M*)	51 ± 2 (5)[a]	47 ± 3 (5)[a]
Uramil (5 m*M*)	59 ± 5 (6)[a]	61 ± 9 (6)[a]

Data are expressed as mean ± SEM with the numbers of experiments given in parentheses. The enzymatic glucose phosphorylation capacity of control RINm5F and GLUT2 RINm5F cell homogenates was determined by a spectrophotometric assay. Data are expressed in percentage of the enzyme activities of untreated cell homogenates. Statistical analyses were performed by using Student's *t* test.

[a]$p < 0.01$ *vs*. with untreated control cell homogenates. Statistical analyses were performed using one-way ANOVA followed by Dunnett's test for multiple comparisons.

[b]$p < 0.01$ *vs*. to control RINm5F cell homogentates treated with the same test compound.

Enzymatic glucose-phosphorylation capacity of control RINm5F and GLUT2 RINm5F cell homogenates after exposure to alloxan, ninhydrin, and uramil

To prove that alloxan is taken up into the cell via the GLUT2 glucose transporter, RINm5F control and RINm5F-GLUT2 cells were incubated with alloxan (10 m*M*), ninhydrin (0.25 m*M*), or uramil (5 m*M*). Enzymatic glucose phosphorylation capacity was decreased to the same extent after ninhydrin and uramil incubation both in RINm5F control and RINm5F-GLUT2 cells (Table 3). In contrast, after incubation with alloxan, a significant reduction in the glucose phosphorylation capacity was noted only in RINm5F-GLUT2 cells but not in RINm5F control cells (see Table 3).

DISCUSSION

The results make it possible to define the crucial importance of the triketone structure of alloxan for its selective pancreatic beta cell toxic and diabetogenic action as follows:

1. The 5-keto group of the alloxan molecule is an integral element of the chemical structure of this pyrimidine derivative and thus a crucial feature that confers on it the similarity with the glucose molecule that is required for the selective uptake of alloxan into the pancreatic beta cell *via* the low affinity GLUT2 glucose transporter in the plasma membrane (8, 9) and thus for its selective toxicity to insulin-producing cells and its diabetogenicity. This was confirmed in the present study by showing that only alloxan significantly increased the inhibition of glucose phosphorylation in GLUT2 glucose transporter–expressing cells, whereas the inhibitory effect of ninhydrin and uramil on glucose phosphorylation (16, 17) was not increased.
2. Uramil, the amino derivative of alloxan (16), in which the 5-keto group of the alloxan molecule is replaced by an amino group, is not selectively beta cell toxic, as shown by the observation that, in contrast to alloxan, it is not more toxic in RINm5F insulin-producing cells expressing the GLUT2 glucose transporter than in cells that do not express this transporter. The reason for this is that through this chemical modification, the molecule loses the required similarity with the glucose molecule, so that it is no more selectively taken up into the cell via the GLUT2 glucose transporter.
3. However, the triketone structure of alloxan confers on this molecule the ability to initiate redox cycling through reaction with the monothiol GSH, as well as the amino acid cysteine, which provides the reactive SH group to the tripeptide GSH, confirming earlier observations that showed that the formation of the reduction product in this reaction between alloxan and GSH is accompanied by characteristic ultraviolet spectral changes (3, 18). As shown diagrammatically, this results in the formation of the toxic ROS (Fig. 5). From this originates the toxicity of alloxan. Replacement of the keto group of the alloxan molecule by the amino group, as in the case of uramil, abolishes the ability to redox cycle not only with GSH but also with cysteine and DTT. In the same way, many other pyrimidine derivatives lacking a keto group in position C-5 have been found before to be inactive (16). The interaction of uramil with these thiols causes for-

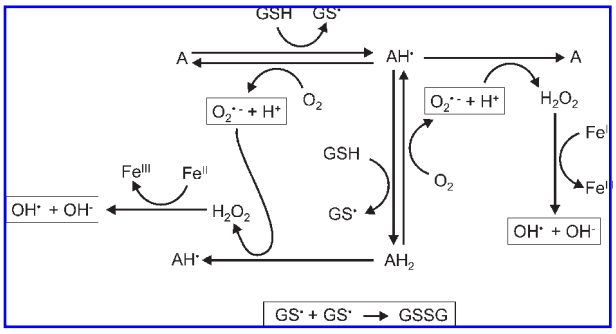

FIG. 5. Model of redox cycling and ROS generation of alloxan with glutathione A, alloxan; AH·, alloxan radical; AH_2, dialuric acid.

mation of adducts with these thiols (unpublished observation), thereby forming inactive and nontoxic compounds, because the reactive 5-keto group is lost through this adduct formation. This observation is in agreement with observations that whenever adducts are formed and result in a loss of the triketone structure, the biologic activity of alloxan and ninhydrin is lost (3, 18).

4. The experiments with extracellular addition of GSH show that alloxan also can be toxic when it is able to redox cycle extracellularly, even if the GLUT2 transporter for uptake is not present in the plasma membrane. During the redox cycling, superoxide radicals and hydrogen peroxide are generated. These can be inactivated extracellularly through addition of SOD and catalase. Otherwise, the hydrogen peroxide molecules formed extracellularly can pass the plasma membrane, and toxic hydroxyl radicals can be generated intracellularly in the Fenton reaction. (7).
5. The fact that catalase provides protection against uramil toxicity but not against streptozotocin toxicity indicates that, although not through redox cycling with the monothiols GSH and cysteine and with the dithiol DTT, uramil may be cytotoxic also through generation of ROS, even though it is not possible to explain at present the exact underlying mechanism.
6. The triketone ninhydrin, in contrast to alloxan, does not redox cycle with these monothiols. With the amino acid cysteine, it reacts in its typical manner as an amino group reagent (1), thereby being inactivated through adduct formation (26). But it redox cycles very vigorously with the dithiol DTT, confirming earlier observations (18). DTT can be considered a model dithiol for dithiols present in many cellular proteins, thus indicating that triketones can cause cell damage in principle through generation of ROS by reaction with many different cellular thiols. Overall, however, the ability of ninhydrin to react with many biologic structures in multiple ways (1, 20, 28) makes this compound a less-specific toxic agent.
7. Conversely, although it is a triketone, ninhydrin is not selectively pancreatic beta cell toxic, because it is apparently not sufficiently similar to the glucose molecule and therefore not taken up selectively into the pancreatic beta cell *via* the GLUT2 glucose transporter.
8. Thus, both uramil and ninhydrin are, like alloxan, also toxic through generation of ROS. This conclusion is supported by the fact that catalase provides protection against toxicity of uramil and ninhydrin. However, because SOD does not provide protection, this indicates that, at variance from the situation with alloxan (7, 22, 24, 31), ROS-generating reactions not depending on superoxide radical ($O_2^{\cdot -}$) formation come into action. The fact that catalase provides protection indicates that removal of hydrogen peroxide (H_2O_2) is responsible for this protection, thereby preventing formation of the hydroxyl radical ($^{\cdot}OH$), which is ultimately responsible for cell death (22, 31). Both compounds, in contrast to alloxan, are not glucose analogues and therefore are not selectively pancreatic beta cell toxic and thus also not suited for induction of experimental diabetes (27).
9. Streptozotocin, although it does apparently not redox cycle with typical thiols in the cells (unpublished observation), is, like alloxan, another prominent selectively beta-cell toxic and diabetogenic agent (27). It is cytotoxic through its alkylating potency (2, 6, 11, 14, 25) and shares with alloxan the ability to interfere with the beta-cell O-GlcNAc pathway (13). These results thus prove that different mechanisms of toxic action can result in selective beta-cell toxicity and thus diabetogenicity, as long as a chemical similarity with the glucose molecule secures a selective uptake into the pancreatic beta cell.

ACKNOWLEDGMENTS

The skillful technical assistance of Maren Böger is gratefully acknowledged.

ABBREVIATIONS

CAT, catalase; CuZnSOD, copper zinc superoxide dismutase; DTT, dithiothreitol; EC_{50}, half maximally effective concentration; G 418, geneticin; GSH, glutathione; H_2O_2, hydrogen peroxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MnSOD, manganese superoxide dismutase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; O_2, oxygen; $O_2^{\cdot -}$, superoxide radical; $^{\cdot}OH$, hydroxyl radical; ROS, reactive oxygen species; SOD, superoxide dismutase.

REFERENCES

1. Abderhalden R. Über die Einwirkung von Ninhydrin, Alloxan und Isatin auf Aminosäuren und Polypeptide. *Zeitschr Physiol Chem* 252: 81–94, 1938.
2. Bennett RA and Pegg AE. Alkylation of DNA in rat tissues following administration of streptozotocin. *Cancer Res* 41: 2786–2790, 1981.
3. Brömme HJ, Mörke W, Weinandy R, Peschke D, and Peschke E. Formation of compound 305 requires the simultaneous generation of both alloxan and GSH radicals. *Horm Metab Res* 34: 62–66, 2002.
4. Brömme HJ, Weinandy R, and Peschke E. Influence of oxygen concentration on redox cycling of alloxan and dialuric acid. *Horm Metab Res* 37: 729–733, 2005.
5. Brückmann G and Wertheimer E. Alloxan studies: the action of alloxan homologues and related compounds. *J Biol Chem* 168: 241–256, 1947.
6. Elsner M, Guldbakke B, Tiedge M, Munday R, and Lenzen S. Relative importance of transport and alkylation for pancreatic beta-cell toxicity of streptozotocin. *Diabetologia* 43: 1528–1533, 2000.
7. Elsner M, Gurgul-Convey E, and Lenzen S. Relative importance of cellular uptake and reactive oxygen species for the toxicity of alloxan and dialuric acid to insulin-producing cells. *Free Radic Biol Med* 41: 825–834, 2006.
8. Elsner M, Tiedge M, Guldbakke B, Munday R, and Lenzen S. Importance of the GLUT2 glucose transporter for pancreatic beta cell toxicity of alloxan. *Diabetologia* 45: 1542–1549, 2002.
9. Elsner M, Tiedge M, and Lenzen S. Mechanism underlying resistance of human pancreatic beta cells against toxicity of streptozotocin and alloxan. *Diabetologia* 46: 1713–1714, 2003.
10. Gazdar AF, Chick WL, Oie HK, Sims HL, King DL, Weir GC, and Lauris V. Continuous, clonal, insulin- and somatostatin-secreting cell lines established from a transplantable rat islet cell tumor. *Proc Natl Acad Sci U S A* 77: 3519–3523, 1980.

11. Green MH, Lowe JE, Petit-Frere C, Karran P, Hall J, and Kataoka H. Properties of N-methyl-N-nitrosourea-resistant, Mex- derivatives of an SV40-immortalized human fibroblast cell line. *Carcinogenesis* 10: 893–898, 1989.
12. Hidy PH. Structural requirements for diabetogenic action in alloxan and related compounds. *J Biol Chem* 163: 307–311, 1946.
13. Konrad RJ and Kudlow JE. The role of O-linked protein glycosylation in beta-cell dysfunction. *Int J Mol Med* 10: 535–539, 2002.
14. Ledoux SP, Woodley SE, Patton NJ, and Wilson GL. Mechanisms of nitrosourea-induced beta-cell damage: alterations in DNA. *Diabetes* 35: 866–872, 1986.
15. Lenzen S. Insulin secretion by isolated perfused rat and mouse pancreas. *Am J Physiol* 236: E391–E400, 1979.
16. Lenzen S, Brand FH, and Panten U. Structural requirements of alloxan and ninhydrin for glucokinase inhibition and of glucose for protection against inhibition. *Br J Pharmacol* 95: 851–859, 1988.
17. Lenzen S, Freytag S, Panten U, Flatt PR, and Bailey CJ. Alloxan and ninhydrin inhibition of hexokinase from pancreatic islets and tumoural insulin-secreting cells. *Pharmacol Toxicol* 66: 157–162, 1990.
18. Lenzen S and Munday R. Thiol-group reactivity, hydrophilicity and stability of alloxan, its reduction products and its N-methyl derivatives and a comparison with ninhydrin. *Biochem Pharmacol* 42: 1385–1391, 1991.
19. Lenzen S, Tiedge M, and Panten U. Glucokinase in pancreatic B-cells and its inhibition by alloxan. *Acta Endocrinol (Copenh)* 115: 21–29, 1987.
20. McCaldin DJ. The chemistry of ninhydrin. *Chem Rev* 60: 39–51, 1960.
21. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55–63, 1983.
22. Munday R. Dialuric acid autoxidation: effects of transition metals on the reaction rate and on the generation of "active oxygen" species. *Biochem Pharmacol* 37: 409–413, 1988.
23. Munday R, Ludwig K, and Lenzen S. The relationship between the physicochemical properties and the biological effects of alloxan and several N-alkyl substituted alloxan derivatives. *J Endocrinol* 139: 153–163, 1993.
24. Munday R and Winterbourn CC. Reduced glutathione in combination with superoxide dismutase as an important biological antioxidant defence mechanism. *Biochem Pharmacol* 38: 4349–4352, 1989.
25. Murata M, Takahashi A, Saito I, and Kawanishi S. Site-specific DNA methylation and apoptosis: induction by diabetogenic streptozotocin. *Biochem Pharmacol* 57: 881–887, 1999.
26. Prota G and Ponsiglione E. On the reaction of ninhydrin with cysteine and analogues: a revision. *Tetrahedron* 29: 4271–4274, 1973.
27. Rerup CC. Drugs producing diabetes through damage of the insulin secreting cells. *Pharmacol Rev* 22: 485–518, 1970.
28. Schönberg A and Singer A. Die Chemie des Ninhydrins und anderer cyclischer 1,2,3-Tricarbonylverbindungen. *Tetrahedron* 34: 1285–1300, 1978.
29. Tiedge M, Lortz S, Drinkgern J, and Lenzen S. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* 46: 1733–1742, 1997.
30. Tiedge M, Lortz S, Munday R, and Lenzen S. Complementary action of antioxidant enzymes in the protection of bioengineered insulin-producing RINm5F cells against the toxicity of reactive oxygen species. *Diabetes* 47: 1578–1585, 1998.
31. Winterbourn CC and Munday R. Glutathione-mediated redox cycling of alloxan: mechanisms of superoxide dismutase inhibition and of metal-catalyzed OH. formation. *Biochem Pharmacol* 38: 271–277, 1989.

Address reprint requests to:
Dr. Matthias Elsner
Institute of Clinical Biochemistry
Hannover Medical School,
30623 Hannover
Germany

E-mail: Elsner.Matthias@mh-hannover.de

Date of first submission to ARS Central, July 2, 2007; date of final revised submission, October 1, 2007; date of acceptance, October 14, 2007.

This article has been cited by:

1. S. M. J. Scullion, E. Gurgul-Convey, M. Elsner, S. Lenzen, P. R. Flatt, N. H. McClenaghan. 2012. Enhancement of homocysteine toxicity to insulin-secreting BRIN-BD11 cells in combination with alloxan. *Journal of Endocrinology* **214**:2, 233-238. [CrossRef]
2. Rita Kakkar, Mamta Bhandari, Ritu Gaba. 2012. Tautomeric transformations and reactivity of alloxan. *Computational and Theoretical Chemistry* **986**, 14-24. [CrossRef]
3. Verônica B. Brito, Vanderlei Folmer, Inês Isabel R. Guerra, João Batista T. Rocha. 2011. Kinetics of Alloxan-Induced Inhibition on #-Aminolevulinate Dehydratase Activity in Mouse Liver Homogenates. *Applied Biochemistry and Biotechnology* . [CrossRef]
4. N P Dhammika Nanayakkara, Kevin K Schrader. 2011. Evaluation of free radical-generating compounds for toxicity towards the cyanobacterium Planktothrix perornata, which causes musty off-flavour in pond-raised channel catfish (Ictalurus punctatus). *Aquaculture Research* no-no. [CrossRef]