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Aldose Reductase-Catalyzed Reduction of Acrolein: Implications in Cyclophosphamide Toxicity

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SUMMARY

Acrolein, a highly cytotoxic aldehyde, is a metabolic by-product of the antineoplastic agent cyclophosphamide and is responsible for the development of hemorrhagic cystitis, a serious side effect of cyclophosphamide therapy. Aldose reductase (EC 1.1.1.21), a member of the aldo-keto reductase superfamily, catalyzes the NADPH-dependent reduction of acrolein to allyl alcohol (K_m = 80 μM, k_{cat} = 87 min^{-1}). Aldose reductase is expressed at different levels in individuals. This suggests that individual differences in the reductive metabolism of acrolein may be a determinant of acrolein toxicity. In addition to being a substrate, acrolein also produces a time-dependent 7–20-fold increase in the activity of aldose reductase toward a variety of substrates. This involves initial binding of acrolein to a second site (K_s = 58 μM). Acrolein activation of aldose reductase results not only in higher k_{cat} values for all substrates but also in higher K_m values and decreased catalytic efficiencies. Acrolein activation of aldose reductase reduces its affinity for aldose reductase inhibitors.

Acrolein (prop-2-enal), an environmental pollutant found in tobacco smoke, automobile exhaust, and burnt animal fats (1–4), is also generated within cells during lipid peroxidation (4, 5). Acrolein is a highly reactive molecule known to cause single-strand DNA breaks (6). It is mutagenic to *Salmonella* and to human fibroblasts from patients with xeroderma pigmentosum (7, 8). Acrolein has been reported to produce bladder tumors (3) and has also been shown to have hepatotoxic effects (9, 10).

An important source of human exposure to acrolein is cyclophosphamide. Cyclophosphamide is a widely used antineoplastic agent that is also used as an immunosuppressant. Specifically, it is used in the treatment of B cell malignant diseases, solid malignancies, and disorders with an autoimmune component such as multiple sclerosis and in preconditioning for bone marrow transplantation (11). A dose-limiting side effect of cyclophosphamide administration is hemorrhagic cystitis, which is diffuse or insidious bleeding within the bladder wall. Acrolein has been identified as the metabolite of cyclophosphamide responsible for hemorrhagic cystitis (12). The risk of death from resultant massive hemorrhage is high without intervention (13–15). No common risk factors for patients who will develop hemorrhagic cystitis have been identified.

The aldo-keto reductases are a widely distributed enzyme family with a broad specificity for endogenous and exogenous aldehydes and ketones (4, 16). They are expressed at variable levels among individuals (16). However, whether acrolein is a substrate for any of the aldo-keto reductases has not been investigated. Aldose reductase is an NADPH-dependent oxidoreductase and a member of the aldo-keto reductase enzyme family. It is the first enzyme in the polyol pathway, where it catalyzes the reduction of glucose to its corresponding alcohol, sorbitol, an intermediate in D-fructose formation. However, aldose reductase is not strictly specific for aldoses. It also has broad specificity for many aldehydes (16–18). Therefore, it has been postulated to have a role as a detoxification enzyme.

Reductive metabolism of acrolein catalyzed by aldo-keto reductases may be a determinant of acrolein toxicity. As a first step in testing this hypothesis, we analyzed the substrate properties of aldose reductase for acrolein. We report that acrolein is an excellent substrate for aldose reductase. We have also found that acrolein is an activator of aldose reductase, and we have investigated the molecular mechanism of this activation.

Experimental Procedures

Materials. Human psoas muscle was obtained (within 24 hr after death) through the Office of the Medical Investigator, State of New Mexico. Samples were stored at −70° until used. NADPH and DL-glyceraldehyde were obtained from Sigma. Acrolein, cinnamaldehyde, crotonaldehyde, allyl alcohol, methylglyoxal, and phenylglyoxal were from Aldrich. Sorbinil was obtained from Pfizer, tolrestat from Ayerst, and statil from ICI. Red Sepharose CL-6B, PD-10 desalting columns, and PBE-94 chromatofocusing resin were from Pharmacia LKB Biotechnology, Inc. YM-10 pressure filtration membranes were from Amicon. Acrolein was purified by fractional distillation.

Enzyme purification. Aldose reductase was purified to homoge-

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ABBREVIATION: PLP, pyridoxal-5′-phosphate.

neity from human psoas muscle as described previously (18). Aldose reductase activity was extracted from the 100,000 × *g* supernatant fraction onto a Red Sepharose column, followed by chromatofocusing on a Pharmacia PBE-94 column and chromatography on a Bio-Gel hydroxylapatite high performance liquid chromatography column. Purified enzyme was stored at −20° until use, without the addition of sulfhydryl reagents. This procedure affords aldose reductase that retains its native kinetic properties.

Assay of enzyme activity. Routine analysis of aldose reductase was carried out by the addition of enzyme samples to 1-ml volumes of 0.1 M sodium phosphate buffer, pH 7, containing 10 mM DL-glyceraldehyde and 0.1 mM NADPH. Initial velocity studies were conducted at 25° in 1-cm pathlength quartz cuvettes, by monitoring the enzyme-dependent absorbance decrease at 340 nm as NADPH was oxidized to $NADP^+$. Measurements were taken using a Perkin-Elmer Lamda 6 UV/visible spectrophotometer. Kinetic studies of substrates and inhibitors were carried out in the same buffer. K_m and k_{cat} values were determined by nonlinear regression analysis of initial rate data with the ENZFITTER program (Elsevier-Biosoft). K_i values were determined with Dixon plots, using linear regression analysis.

Identification of product. The product of the aldose reductase-catalyzed reduction of acrolein was extracted into ether and derivatized with benzoyl chloride. The product was analyzed by gas chromatography and compared with standards of allyl benzoate and propyl benzoate. Only allyl benzoate was found in the reaction, confirming that the product of the aldose reductase-catalyzed reduction of acrolein is allyl alcohol.

Activation of aldose reductase activity with acrolein. Aldose reductase was incubated with varying concentrations of acrolein in 0.1 M sodium phosphate buffer, pH 7, at room temperature. Aliquots were taken at intervals to determine the activity of aldose reductase. Activation of aldose reductase by PLP, crotonaldehyde, and cinnamaldehyde was similarly determined.

Results

Evidence that acrolein is a substrate for aldose reductase. The ability of aldose reductase to catalyze the NADPH-dependent reduction of acrolein to allyl alcohol was tested as described in Experimental Procedures. Acrolein followed Michaelis-Menten kinetics, with a k_{cat} of 87 min^{-1} and a K_m of 80 μM (Fig. 1). The catalytic efficiency (k_{cat}/K_m) for acrolein was 1.1×10^6 M^{-1} min^{-1}, a value quite comparable to those for other aldose reductase substrates (Table 1). Aldose reductase is best known for its putative role in the development of diabetic complications through its catalysis of the reduction of glucose to sorbitol. The catalytic efficiency of aldose reductase for

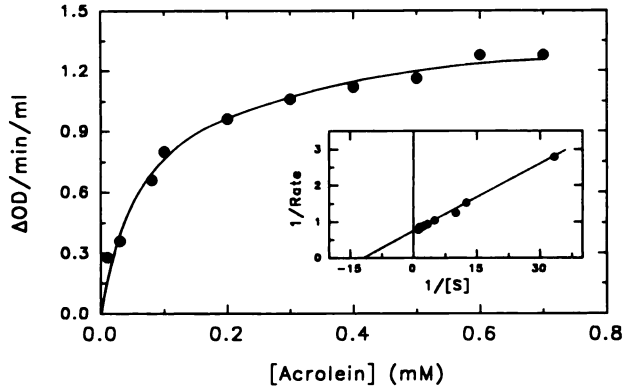

Fig. 1. Kinetic profile of the aldose reductase-catalyzed reduction of acrolein. Acrolein follows normal hyperbolic kinetics with human muscle aldose reductase. The K_m and k_{cat} values are shown in Table 1. *Inset*, double-recrocal plot.

TABLE 1

Substrate specificity of aldose reductase

All data were collected at 25° and pH 7.

Substrate	K_m	k_{cat}	k_{cat}/K_m
	mM	min^{-1}	M^{-1} min^{-1}
DL-Glyceraldehyde[a]	0.013	133	1.0×10^7
Methylglyoxal[b]	0.008	142	1.8×10^7
Phenylglyoxal	0.0036	60	1.7×10^7
Glucose[a]	68	78	1.1×10^3
Acrolein	0.08	87	1.1×10^6
Crotonaldehyde	1.23	118	9.6×10^3
Cinnamaldehyde	0.008	72	9.0×10^6

[a] From Ref. 18.
[b] From Ref. 31.

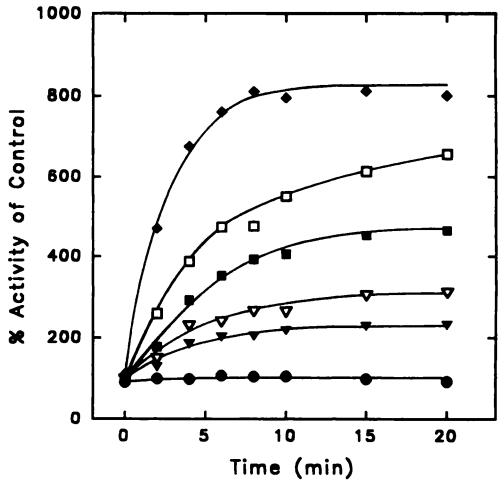

Fig. 2. Reaction of aldose reductase with acrolein. Aldose reductase was incubated at 25° and pH 7 with acrolein at 1 μM (▼), 5 μM (▽), 10 μM (■), 20 μM (□), or 50 μM (♦). Aldose reductase was incubated with buffer only to serve as the control (●). Activities were measured using DL-glyceraldehyde as substrate.

acrolein is 1000 times greater than that for glucose. Therefore, we conclude that the reduction of acrolein catalyzed by aldose reductase may be physiologically important.

Substrate specificity of aldose reductase for other α,β-unsaturated aldehydes. Crotonaldehyde and cinnamaldehyde were also tested as substrates for aldose reductase (Table 1). The Michaelis constants (K_m) ranged from 8.0 μM for cinnamaldehyde to 1.23 mM for crotonaldehyde. The catalytic constants (k_{cat}), however, were very similar to that for acrolein. The catalytic constants for this series of substrates also compared very well with those for well known aldose reductase substrates (18, 19). All catalytic constants were within a factor of approximately 2. Aldose reductase follows a sequential ordered mechanism, with release of $NADP^+$ as the last step. The enzyme has been postulated to undergo a conformational change before release of cofactor (20). The relative invariability of k_{cat} across a wide variety of substrates provides evidence for the idea that this conformational change is at least partially rate limiting.

Acrolein activation of aldose reductase. Acrolein is known to covalently modify and inactivate a number of enzymes (21, 22). Therefore, we incubated aldose reductase in 0.1 M sodium phosphate buffer, pH 7, with varying concentrations of acrolein. Aliqouts were taken at timed intervals and activities were measured. Results are shown in Fig. 2.. In contrast to acrolein modification of other enzymes, which leads to inacti-

vation, up to 8-fold increases in activity, compared with that of controls, were seen with DL-glyceraldehyde as the substrate. Activation was time and concentration dependent. A plot of initial rates of activation versus acrolein concentration gave a hyperbolic curve (Fig. 3), with half-maximal activation occurring with 58 μM acrolein. These results suggest that acrolein first binds to aldose reductase and then activates the enzyme. At 50 μM acrolein, maximum activation occurred within 10 min. Acrolein-modified aldose reductase was stable for approximately 15 min; however, activity then decreased, presumably due to further modification by acrolein.

Other α,β-unsaturated aldehydes were also incubated with aldose reductase, as before, to test for their abilities to activate the enzyme. Crotonaldehyde was a mild activator. Only a 2-fold increase in activity was seen with crotonaldehyde concentrations as high as 500 μM (data not shown). Cinnamaldehyde showed no ability to activate aldose reductase. Other α,β-unsaturated compounds without aldehyde functions, specifically allyl alcohol and acrylamide, did not activate the enzyme. Methylglyoxal, a three-carbon aldehyde, was also tested, and again no increase in activity was seen. Thus, it appears that activation is selective for certain aldehydes.

PLP activation of aldose reductase. PLP was previously reported to activate aldose reductase by forming a reversible Schiff base with a lysine residue, which has been identified as residue 262 in the human enzyme (23). Incubation of aldose reductase with PLP was carried out as with acrolein, and we obtained similar results (Fig. 4). PLP activation could increase activity approximately 3-fold at 250 μM. As with acrolein, activation was time dependent, and initial rates of activation increased with increasing PLP concentrations.

Incubation of acrolein-activated aldose reductase with PLP. The effect of incubation of acrolein-activated aldose reductase with PLP was studied by incubating the enzyme with 50 μM acrolein for 10 min to achieve maximal activation. After 10 min PLP was added (20, 50, or 250 μM). Results are shown in Fig. 5. The addition of PLP caused a time-dependent decrease in the activity of the activated enzyme. Increasing the concentration of PLP increased the amount of deactivation. PLP at 20 μM was able to decrease the activity by 15% in 20 min, and PLP at 250 μM was able to reduce the activity of the activated enzyme by 80% in 20 min. We conclude that acrolein forms a Schiff base with lysine-262 and that the decrease in activity seen upon addition of PLP to acrolein-activated aldose reductase is due to displacement of acrolein by PLP. Because PLP is only a modest activator of aldose reductase, replacement of acrolein by PLP would result in decreased activity.

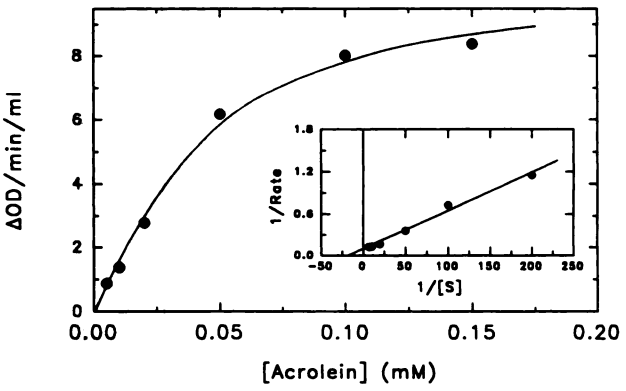

Fig. 3. Plot of the rate of activation of aldose reductase versus acrolein concentration. *Inset*, double-reciprocal plot. Initial rates of activation were calculated from the data in Fig. 2.

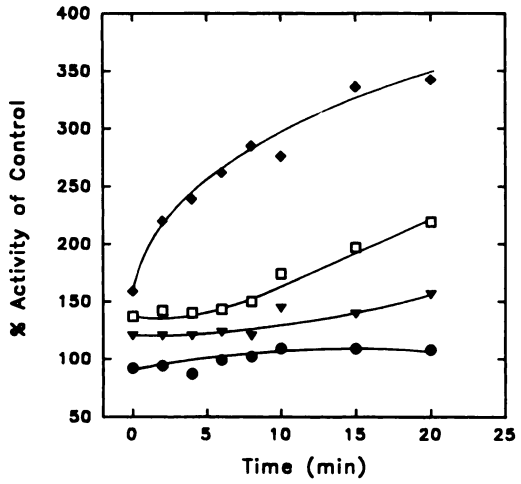

Fig. 4. Reaction of aldose reductase with PLP. Aldose reductase was incubated at 25° and pH 7 with PLP at 20 μM (▼), 50 μM (□), or 250 μM (♦). Aldose reductase was incubated with buffer only as the control (●). Activities were measured using DL-glyceraldehyde as substrate.

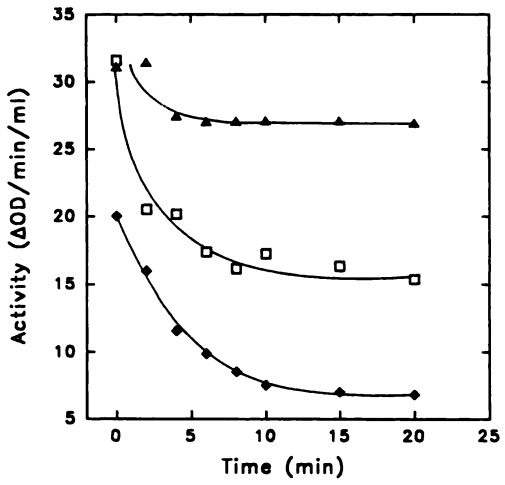

Fig. 5. Effect of incubation of acrolein-activated aldose reductase with PLP. Aldose reductase was incubated at 25° and pH 7 with 50 μM acrolein for 10 min, at which time PLP was added (time 0) at 20 μM (▲), 50 μM (□), or 250 μM (♦).

NADP protection of aldose reductase from activation with acrolein. Addition of $NADP^+$ to a final concentration of 0.01 mM before incubation with 50 μM acrolein provided complete protection of aldose reductase from acrolein-meditated activation.

Substrate specificity of acrolein-activated aldose reductase. The substrate specificity of acrolein-activated aldose reductase was studied. Aldose reductase was incubated with 50 μM acrolein, to achieve maximal activation, and then placed on ice, where the activated enzyme was stable for up to 30 min. The activated enzyme continued to follow Michaelis-Menten kinetics with acrolein as substrate. However, whereas k_{cat} increased from 87 to 526 min^{-1}, K_m also increased from 80 μM to 24 mM, resulting in decreased catalytic efficiency (k_{cat}/K_m = 2.2×10^4 M^{-1} min^{-1}). Therefore, in spite of dramatic increases in k_{cat}, the increase in K_m was great enough to decrease the overall efficiency of the enzyme.

Other aldose reductase substrates were also tested with acrolein-activated aldose reductase (Table 2). Results for all substrates were similar to those seen with acrolein. Dissociation constants increased 13–300-fold, and catalytic constants increased 7–20-fold. For each substrate the catalytic efficiency decreased by a factor of at least 10. Despite the dramatic increases, k_{cat} remained relatively constant. We conclude that, even at this increased rate of catalysis, the conformational change of the enzyme before release of cofactor is still partially rate determining. Additionally, the K_m for NADPH increased from 2.2 μM for the unactivated enzyme (24) to 8.2 μM for acrolein-activated aldose reductase.

Inhibition of acrolein-activated aldose reductase. The dissociation constants for various aldose reductase inhibitors were determined for acrolein-activated aldose reductase. The results are shown in Table 3. For the inhibitors sorbinil and statil, the dissociation constants with the activated enzyme were higher than those with the unactivated enzyme, similar to the pattern seen with the substrates. However, with tolrestat the dissociation constant was essentially the same for the activated and unactivated enzymes.

Discussion

Incubation of aldose reductase with low concentrations of acrolein causes large increases in k_{cat} as well as large increases in K_m for all substrates tested, which results in decreased catalytic efficiency for all substrates, including acrolein. Our results parallel those reported for PLP activation of aldose reductase through formation of a Schiff base with the ε-amino group of lysine-262 (23) and those reported for a mutant aldose reductase in which lysine-262 was replaced with methionine (25). Both of these studies described enzymes with higher catalytic constants and higher Michaelis constants for substrates and cofactor, with decreased catalytic efficiency. Lysine-262 of aldose reductase is part of the tetrapeptide isoleucine-proline-lysine-serine (23). This motif, which is highly conserved among aldo-keto reductases (26), is thought to be important in cofactor binding. Crystallography data show that the ε-amino group of lysine-262 forms a salt link and a hydrogen bond to the monophosphate of NADPH (27). This would explain why modification or mutation of lysine-262 results in decreased binding of NADPH to the enzyme (23, 25). Upon cofactor binding, aldose reductase is known to undergo a conformational change in which a belt or loop of 11 residues (residues 262–272) folds over and locks the cofactor in place (27, 28). Lysine-262 is one residue involved in forming salt links to the belt to stabilize its formation.

We suggest that acrolein acts in a manner similar to that of PLP, namely, formation of a Schiff base with lysine-262 (23). The lack of activation by allyl alcohol or acrylamide, which both lack the aldehyde but retain the double bond, provides evidence against the modification of aldose reductase by acrolein through Michael addition by the nucleophile aldose reductase to the double bond of acrolein. The slow conformational change before cofactor release has already been established (20). The belt must move out of the way for $NADP^+$ release. We suggest that modification of lysine-262 of aldose reductase by acrolein increases the rate of the conformational change through destabilization of the salt link between lysine-262 and $NADP^+$. Additional evidence for acrolein modification at lysine-262 is provided by competition between acrolein and PLP, in which PLP appears to displace acrolein. This observation also provides evidence against modification of aldose reductase by Michael addition, which would lead to a stable covalent derivative.

Why acrolein is a much better activator of aldose reductase than is crotonaldehyde or PLP is not clear. Plotting of initial rates of acrolein activation versus acrolein concentration yields a hyperbolic curve, suggesting that there is a binding site for acrolein. This binding site appears to have affinity for unsaturated aldehydes. The value of the dissociation constant of aldose reductase for PLP is reported to be 0.17 mM (23). Of the compounds we tested, acrolein has the highest affinity for the activation site, with a dissociation constant of 58 μM. Further studies should investigate the chemical properties that contribute to binding at the activation site.

This activation of aldose reductase by acrolein is unlikely to occur *in vivo*. Addition of $NADP^+$ before the addition of acrolein protected the enzyme against activation. The low Michaelis constants of $NADP^+$ and NADPH for aldose reductase (0.5 and 2.2 μM, respectively) ensure that the enzyme is saturated under physiological conditions (24), which should protect against activation.

Acrolein is known to be metabolized by conjugation to glutathione. This involves addition of the sulfhydryl group to the α,β-double bond of acrolein, either nonenzymatically or, more likely, catalyzed by glutathione transferase (21). The resulting adduct may still have toxic potential, because it still has an intact aldehyde functional group. The acrolein-glutathione adduct has been shown to cause nephrotoxicity in rats (29). The acrolein-glutathione adduct can undergo further metabolism to yield 3-hydroxypropylmeracpturic acid (30), which has been isolated in the urine of rats treated with acrolein (31). However, because 3-hydroxypropylmercapturic acid is also detected in the urine of rats treated with allyl alcohol, it is possible that acrolein may be reduced to allyl alcohol before conjugation with glutathione. The acrolein-glutathione adduct has also been suggested to be a substrate for aldehyde dehydrogenase (4). However, the fate of *S*-(2-carboxyethyl)glutathione has not yet been determined.

TABLE 2

Substrate specificity of acrolein-activated aldose reductase

All data were collected at 25° and pH 7.

Substrate	K_m	k_{cat}	k_{cat}/K_m
	mM	min^{-1}	$M^{-1}\ min^{-1}$
DL-Glyceraldehyde	2.2	936	4.3×10^5
Methylglyoxal	1.0	1319	1.3×10^3
Phenylglyoxal	0.26	1156	4.4×10^6
Acrolein	24	526	2.2×10^4

TABLE 3

Inhibitor binding to unactivated aldose reductase and to acrolein-activated aldose reductase

All data were collected at 25° and pH 7.

Inhibitor	K_i	
	Unactivated enzyme[a]	Activated enzyme
	μM	
Sorbinil	0.22	1.1
Statil	0.035	7.1
Tolrestat	0.017	0.010

[a] From Ref. 18.

A significant amount of acrolein is formed in the urine by base-catalyzed β-elimination from 4-hydroxycyclophosphamide, the primary metabolite of cyclophosphamide (32). Phosphoramide mustard, which is also formed in this β-elimination, does not have any toxic activity in the bladder. Treatment of experimental animals with diethylcyclophosphamide, which can be metabolized to acrolein but not phosphoramide mustard, provides additional evidence that acrolein is the metabolite responsible for hemorrhagic cystitis (12). We have shown that acrolein is an excellent substrate for aldose reductase. The catalytic efficiency of aldose reductase for acrolein is 1000 times greater than that for glucose. Therefore, we suggest that the physiological reduction of acrolein to allyl alcohol is a likely reaction. However, the toxicity of allyl alcohol, especially in the bladder, needs to be investigated. Published data show that the toxicity of allyl alcohol is due to alcohol dehydrogenase-catalyzed oxidation of allyl alcohol to acrolein (10, 33). Whether allyl alcohol exists in the urine of cyclophosphamide-treated animals or patients is not known. If allyl alcohol is significantly less toxic than acrolein, then acrolein reduction catalyzed by aldo-keto reductases may be beneficial. Aldose reductase is widely distributed in human tissues, including kidney medulla and cortex (34). However, levels of expression vary widely. Alcohol dehydrogenase is also widely distributed and has also been characterized in rat kidney (35). Whether these enzymes are expressed in the lining of the bladder remains to be determined. One possibility is that the levels of expression of aldose reductase and alcohol dehydrogenase in the epithelial lining of the bladder determine whether acrolein derived from cyclophosphamide is toxic to individual patients. Patients who express low levels of aldose reductase, or high levels of alcohol dehydrogenase, may be prone to acrolein accumulation. With these individuals, measures to protect against acrolein toxicity may need to be taken.

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