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A point mutation produced a class 3 aldehyde dehydrogenase with increased protective ability against the killing effect of cyclophosphamide☆

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ABSTRACT

Cyclophosphamides are pro-drugs whose killing agent is produced from an aldehyde that is formed by the action of a P450 oxidation step. The mustard from the aldehyde can destroy bone marrow cells as well as the tumor. Aldehyde dehydrogenase (EC 1.2.1.3) can oxidize the aldehyde and hence inactivate the cytotoxic intermediate but bone marrow has little, if any, of the enzyme. Others have shown that over-expression of the enzyme can afford protection of the marrow. A T186S mutant of the human stomach enzyme (ALDH3) that we developed has increased activity against the aldehyde compared to the native enzyme and HeLa cells transformed with the point mutant are better protected against the killing effect of the drug. It took threefold more drug to kill 90% of the cells transformed with the mutant compared to the native enzyme (15.8 compared to 5.1 mM of a precursor of the toxic aldehyde). Analysis of molecular models makes it appear that removing the methyl group of threonine in the T186S mutant allows the bulky aldehyde to bind better. The mutant was found to be a poorer enzyme when small substrates such as benzaldehyde derivatives were investigated. Thus, the enzyme appears to be better only with large substrates such as the one produced by cyclophosphamide.

1. Introduction

Cyclophosphamide is a commonly used alkylating agent in chemotherapeutic regimens because it has a broad antitumor spectrum. Like many other chemotherapeutic drugs, in addition to killing cancer cells it also is damaging bone marrow cells [1–4]. The cytotoxic agent phosphoramide mustard formed after the bio-activation of the parent compound can covalently bind to DNA resulting eventually in cell death [5–8]. Cyclophosphamide-resistant tumor cells often display a higher content of aldehyde dehydrogenase (ALDH)[1] and over-expression of this enzyme in cell lines can result in variable levels of resistance to cyclophosphamide [9–17]. ALDH is thought to provide cellular protection by converting aldophosphamide, an aldehyde precursor of the phosphoramide mustard, to an acid which does not attack DNA (Fig. 1). Of the 19 forms of human ALDHs, ALDH1 and ALDH3 have been identified with cellular resistance to cyclophosphamide [18–23] with ALDH1 and ALDH3 being equally effective [22]. Liver cytosolic ALDH1 cDNA has been used successfully to induce cyclophosphamide-resistance in bone marrow cells [24–27]. Success, however, has not been reported using stomach cytosolic ALDH3 cDNA. Compared with stomach ALDH3, liver ALDH1 is a better catalyst for

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[1] Abbreviations: ALDH, aldehyde dehydrogenase; ALDH1 and ALDH3 refer to the liver and stomach cytosolic isozymes of the enzyme, respectively.

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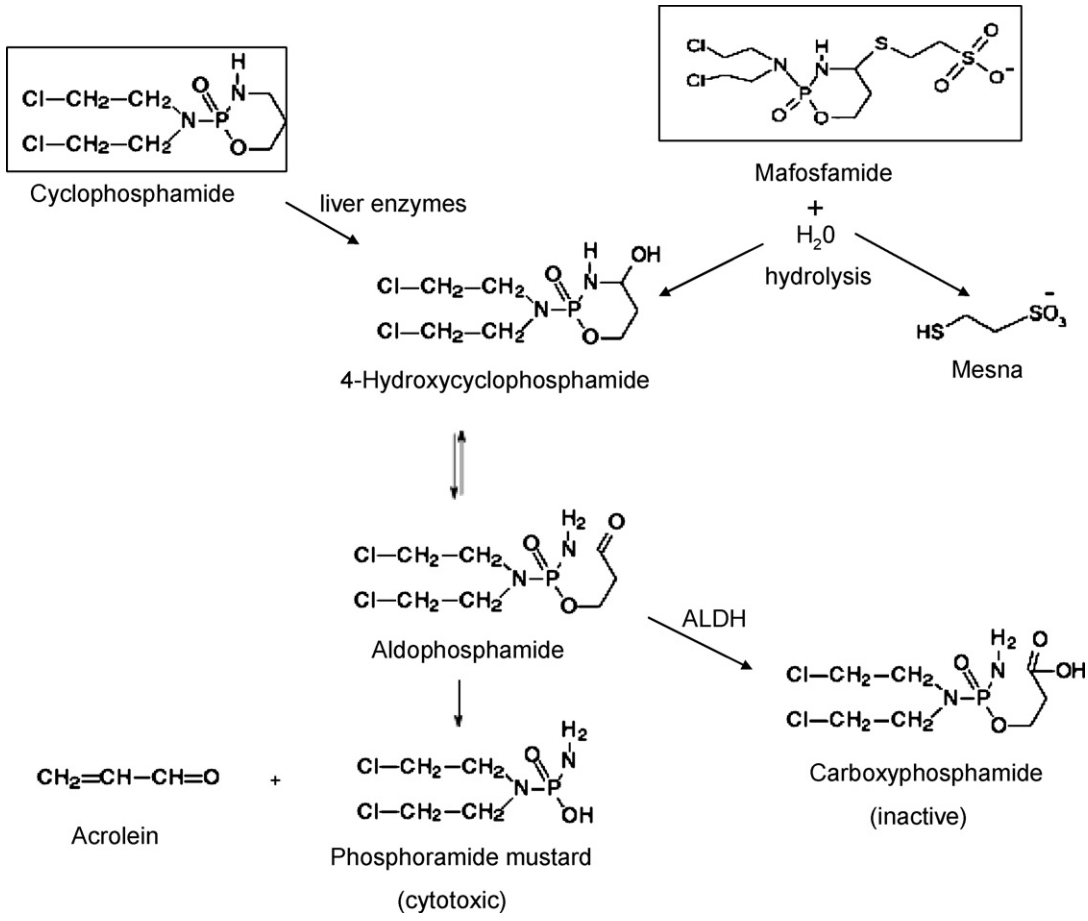

Fig. 1 – Pathway for the conversion of cyclophosphamide to aldophosphamide, the toxic mustard. Mafosfamide (upper right) will spontaneously form aldophosphamide which is the actual substrate for aldehyde dehydrogenase. Cyclophosphamide is enzymatically converted to aldophosphamide.

aldophosphamide oxidation as it has a lower K_m value for the substrate [28–30]. Also, ALDH3 isolated from some tumors has been found to have little or no activity for aldophosphamide [11,12,17,20]. Thus, the failure to protect bone marrow cells by stomach ALDH3 could be partly due to its low catalytic efficiency toward aldophosphamide or a different ALDH3 isozyme is required for the protection.

Here, we report on a different approach to protect the cells using ALDH3. Rather than using a native ALDH3, we made a mutant of the enzyme with the aim of being able to protect transformed cells with sufficient catalytic activity to destroy the cytotoxic aldophosphamide derived from mafosfamide when isolated cells are used. The latter is a compound that spontaneously decomposes to form aldophosphamide.

2. Materials and methods

2.1. *Cloning of ALDH3*

A cDNA clone of human stomach ALDH3 in pT7-7 [31] was used as a template for subcloning into pET-24a (+) (Novagen, USA). The latter was designed such that a 6 His-tag could be placed in the C-terminus of the protein to facilitate protein purification on a nickel affinity column. A point mutation, T186S, was introduced into the ALDH3 sequence, using a Quick Change II Site-Directed Mutagenesis kit (Stratagene, USA). For transfection into HeLa cells, the cDNA sequences from ALDH3 and its mutant were cloned separately into a mammalian expression vector, pEGFPN1 (Clontech, USA) as described [32] with the cDNA for ALDH3 replacing the cDNA for GFP in the original clone.

2.2. *Expression and purification of the native ALDH3 and the T186S mutant*

E. coli BL21 cells (Novagen, USA) were transformed with the native ALDH3 and the T186S mutant cloned in pET-24a (+) expression vectors [33]. Transformed cells were grown at 37 °C for 2–3 h and isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma, USA) was added. After further overnight grown at18 °C, cells were harvested and lysed [34]. The lysate was centrifuged at 15,000 rpm and the resulting supernatant was made to 1% (w/v) protamine sulfate (Sigma, USA) for DNA precipitation. The solution was centrifuged again and the resulting supernatant was applied to a nickel affinity column (Qiagen) equilibrated with a 50 mM sodium phosphate buffer,

pH 7.4 containing 500 mM NaCl and 1 mM β-mercaptoethanol (Sigma, USA). The column was washed with the phosphate buffer containing also 50 mM imidazole (Sigma, USA) to elute the unbound materials. The bound proteins were eluted using a 50–500 mM imidazole gradient in the phosphate buffer. The fractions containing the His-tagged enzymes were homogeneous as judged by SDS-PAGE [35]. The purified enzymes were pooled and further concentrated using a 100,000 molecular weight cut-off centrifugal filter (Amicon, USA). Prior to subsequent characterization, the concentrated enzymes were stored in 50% glycerol at −20 °C and appeared to be stable for at least 6 months.

2.3. Fluorescence assay for dehydrogenase activity

The dehydrogenase activity assays were performed by measuring the rate of increase in the fluorescence of $NADP^+$ (Sigma, USA) reduction [34]. The K_m and k_{cat} values for benzaldehyde (Sigma, USA) and mafosfamide were determined from at least triplicate measurements and the values averaged.

2.4. Transfection of HeLa cells

HeLa cells obtained from Prof. Philip Low at Purdue University were grown in monolayer cultures [32]. The cells were grown to about 80% confluence in a complete medium and then transfected with plasmids expressing ALDH3 or its mutant. Transfection was achieved by using Lipofectin (Roche, USA). After 24 h of transfection, G418 (Invivogen, USA) (0.8 mg/ml) was added to the growth medium and the transfected cells were allowed to grow for a week. The cells were then used for cytotoxicity assays.

2.5. Mafosfamide cytotoxicity assay for transfected cells

Mafosfamide (ASTA Z 7557) was obtained from Asta Werke, Bielefeld, Germany. The cytotoxicity assay was a modification of the protocol described by Giorgianni et al. [28]. Transfected HeLa cells at a density of 4×10^5 ml^{-1} were centrifuged and resuspended in a serum-free medium at a density of 1.0×10^5 ml^{-1}. Mafosfamide was added at increasing concentrations (0–20 mM) and the cells were incubated at 37 °C in a tissue culture incubator with frequent agitation. After 30 min incubation, the cells were spun down and washed twice in the serum-free medium. Then, the cells were resuspended in a complete growth medium and returned to the incubator. After 10–14 days, the cell colonies were counted and the survivors determined.

2.6. Western blotting

Cell extracts were prepared from transfected HeLa cells and analyzed by Western immunoblotting [36,37]. Protein bands on the nitrocellulose filters were detected using rabbit polyclonal antibodies raised against human stomach ALDH3 and goat anti-rabbit second antibody conjugated with alkaline phosphatase. Color development resulting from the alkaline phosphatase activity was mediated by 5-bromo-4-chloro-3-indolyl phosphate (Sigma, USA) and nitro blue tetrazolium (Sigma, USA) [38].

2.7. Determination of protein concentration

Protein assays were conducted using a BCA™ protein assay kit (Pierce, USA) according to the manufacturer's instructions. Bovine serum albumin (Sigma, USA) was used as a protein standard.

2.8. Modeling and substrate docking for the human ALDH3

The model structure for the human ALDH3 (SwissProtein database accession number P30838) was generated using Geno3D [39], an automated Web server for protein molecular modeling (http://geno3d-pbil.ibcp.fr). The model structure was built on the basis of the solved crystal structure of the rat liver ALDH3 (Protein Data Bank ID 1AD3). The putative NAD^+ binding site in the model structure was identified by superposing the rat enzyme structure onto the human ALDH3 model. The 3D atomic coordinates of the aldophosphamide and the benzaldehyde in pdb format were obtained using the Dundee Prodrg2 Server (http://davapc1.bioch.dundee.ac.uk/programs/prodrg/prodrg.html) [40]. For docking of aldophosphamide or benzaldehyde into the human ALDH3 and sheep ALDH1 (Protein Data Bank 1BXS) models, we employed the ArgusLab software, which makes use of the AScore scoring function to determine the low-energy binding mode with a grid encompassing cysteine 243 as a binding group. Molecular graphics images were reproduced using the UCSF Chimera software (http://www.cgl.ucsf.edu/chimera/) [41].

3. Results and discussion

3.1. Attempts to make a better enzyme (ALDH)

To make an enzyme more active, one needs to increase the specific activity of the enzyme. This can, in theory, be accomplished by increasing the velocity of the rate-limiting step. It has proven to be easier to determine the rate-limiting step than to increase it. One technique that has been used in the past by various investigators to accomplish this was a random mutagenesis approach, dubbed directed evolution, to try to produce an enzyme with altered properties [42]. We tried this approach but were not successful partly due to our inability to design a functional screening test to select for a mutant enzyme with more activity than the native enzyme. In addition, we inserted the library of mutant ALDHs prepared for the random mutagenesis study into both *E. coli* and yeast cells and tried to search for a better enzyme by screening for survivors that grew in the presence of mafosfamide. Unfortunately, this approach did not produce any surviving clones. In a study to change the rate-limiting step of ALDH1, we found, using the random mutagenesis approach, that changing a threonine at position 244 to a serine accomplished this [43]. Since such a dramatic change in enzyme property was found, we modeled the point mutation into the structure of both ALDH1 and ALDH3 to determine if the binding pocket for a large substrate would change. The T244 residue in ALDH1 corresponds to T186 in ALDH3. It was found that this mutation might open the binding site so a large mafosfamide-like

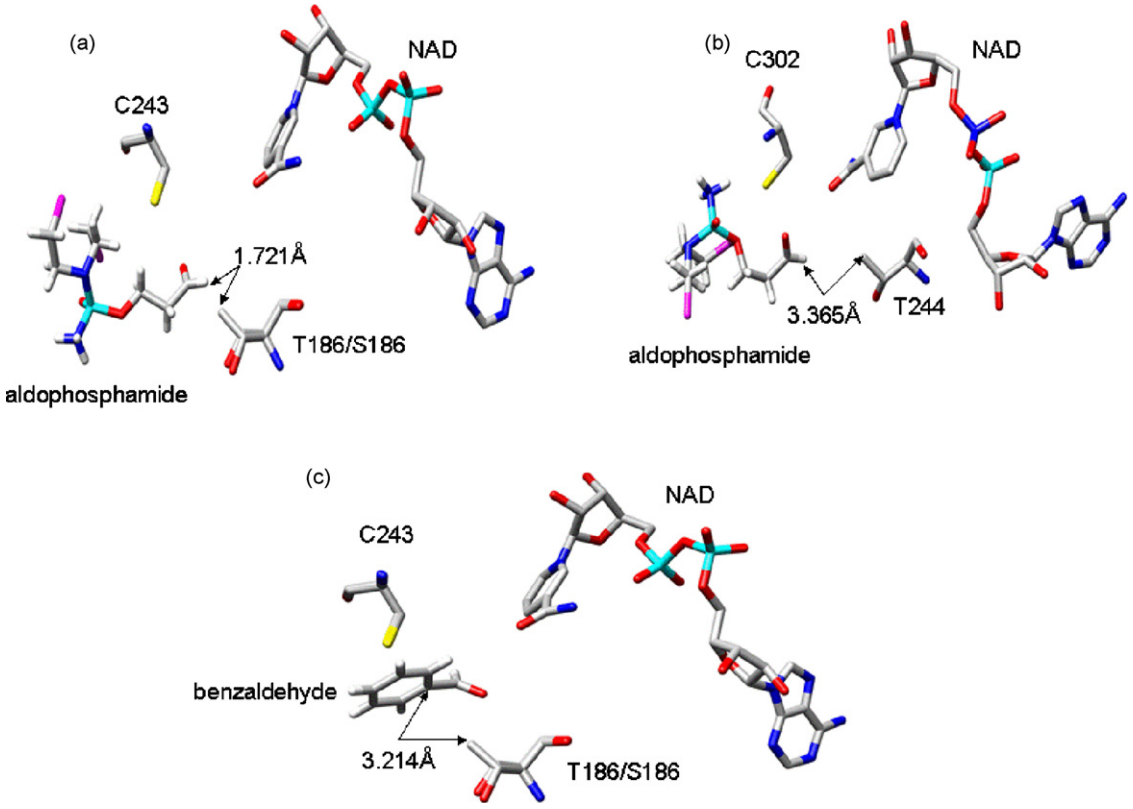

Fig. 2 – Molecular models of the native and the threonine to serine mutant form of aldehyde dehydrogenases. The methyl group of the threonine appears to interact favorably with benzaldehyde (c) but could interfere with the binding of aldophosphamide with ALDH3 (a). Mutating the residue to a serine removed both the favorable and the unfavorable interactions, respectively, consistent with the observation that the mutant was a better enzyme when mafosfamide was the substrate and a poorer one when benzaldehyde was the substrate. In contrast, the binding cavity for ALDH1 was already large so removing the methyl group of the threonine did not make the mutant appear to be a better enzyme for aldophosphamide (b).

substrate could bind well to ALDH3. ALDH1 already possessed a large substrate-binding cavity that can accommodate the bulky substrate and no improvement in binding properties was apparent by making the point mutation (Fig. 2). Based upon this observation we made the T186S mutation to ALDH3 and found that the mutant had higher activity towards mafosfamide and will show below that the ''improved enzyme'' can protect HeLa cells against the drug.

3.2. *Kinetic properties of the T186S mutant*

The native ALDH3 and the T186S mutant were expressed in *E. coli* and purified on a nickel affinity column as described in Section 2. The T186S mutant had ca. twice the activity of the native enzyme when assayed with mafosfamide as a substrate. Since the T186S mutant appeared to be more active, additional detailed measurements of activity were made. The V_m increased from 1100 ± 20 to 1500 ± 35 (nmol/(min mg)) while the K_m for substrate decreased from 189 ± 7 to 148 ± 4 (μM) for the mutant. The V_{max}/K_m ratio went from 5.8 to 10.1 showing that the mutant was approximately 1.7-fold better enzyme than native ALDH3 when mafosfamide was the substrate. Aromatic aldehydes are typical substrates for ALDH3 [44]. Three derivatives of benzaldehyde were employed and the kinetic constants are presented in Table 1. Much to our surprise, the T186S mutant that was employed because it

Table 1 – Kinetic properties of the native and T186S mutant ALDH3

ALDH3 activity	Benzaldehyde	*p*-Nitrobenzaldehyde	*p*-Hydroxybenzaldehyde
Native	2400	1900	130
T186S mutant	210 (11%)	54 (3%)	8 (6%)

Activity assays were performed by measuring the rate of increased fluorescence due to the formation of NADPH in 100 mM sodium phosphate (pH 7.4) at 25 °C. The percents are for the activity of the mutant compared to the native enzyme. Units for activity are nmol/(mg min). For the three substrates, the T186S mutant averaged just 7% the activity of the native enzyme.

oxidized mafosfamide faster than did the native enzyme turned out to be less active when conventional substrates were used. This implies that the T186S mutant was not really a better enzyme, but happened to have more activity against a selected substrate. Our results with the native enzyme are similar to those previously reported [28].

3.3. Protection of HeLa cells

To test the ability of the T186S mutant to protect cells from the killing action of mafosfamide, HeLa cells were transformed with either the native or the mutant ALDH3. Cells were grown, and then exposed to the drug and the survivors calculated. The data showed that indeed the T186S mutant was more than three times as effective at protecting the transformed cells as compared to the native enzyme. It took 15.8 mM mafosfamide to kill 90% of the cells transformed with T186S mutant compared to just 5.1 mM to kill 90% of the cell transformed with native ALDH3. (It took ca. 50 μM mafosfamide to kill 90% of the untransformed HeLa cells). The results are shown in Fig. 3. Other investigators have reported similar protective effects of transfected cells against cyclophosphamide only by over-expression of the native ALDH3 [13,14,17]. We found that using a more active enzyme accomplished this same event without over-expression. It might prove to be possible to use the better enzyme to protect bone marrow cells when a patient is undergoing chemotherapy using a cyclophosphamide drug.

To ensure that the protection we found against the killing effect of the drug was not due to higher levels of enzyme expression, Western blots were run to determine if the level of expression was similar in cells expressing the enzymes (Fig. 4). Both the native and mutant forms of ALDH3 were found to be expressed at essentially the same level showing that the protection afforded by the T186S mutant was related to its increased catalytic activity. Extracts from cells transformed with an empty vector did not show the presence of ALDH or any ALDH activity.

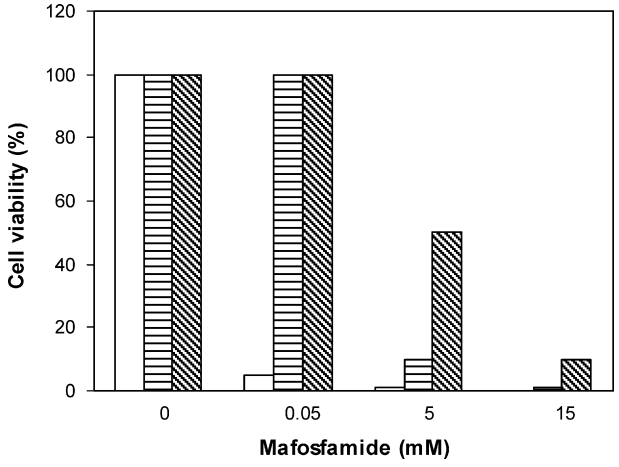

Fig. 3 – Protection of HeLa cells from the killing effects of mafosfamide. Cells were incubated with the compound and the survivors calculated as described in Section 2. Open box represents cells transformed with an empty vector. Box with parallel lines represents cell transformed with native ALDH3. Lastly, the box with diagonal lines represents cells transfected with the T186S mutant of ALDH3.

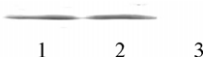

Fig. 4 – Extracts from HeLa cells that were either transfected with the native (lane 1) or T186S mutant form (lane 2) of ALDH3 were analyzed by Western Blotting with anti-ALDH3 antibodies, to confirm the expression levels of the two were comparable. Untransfected cells showed neither ALDH activity nor a protein band after Western blotting (lane 3). The same amount of total protein was added to each lane.

3.4. Mechanism of protection

The rate-limiting step for ALDH3 is the transfer of a hydride from aldehyde to NAD^+ [31]. The threonine residue at position 186 is located near NAD^+ in the active site and is relatively near the substrate-binding site. The methyl group of the theonine appears to be close enough to the substrate so that it could interact, perhaps by a hydrophobic interactions, with aromatic ring of benzaldehyde in the native enzyme. Replacing the methyl containing threonine with a serine in the T186S mutant would disrupt this interaction and could result in a destabilization of the transition state that would lead to the observed lower activity. From the model presented in Fig. 2, it can be seen that a large substrate might fit better into the active site of the enzyme when the methyl group of threonine was removed as the methyl group seems to interfere with the binding of mafosfamide. Thus, with the serine mutant a better interaction with the active site of the enzyme could occur. Finding that benzaldehyde derivatives were not better substrates for the mutant is consistent with the fact that the mutant is not a better enzyme but that it just has a greater specific activity with the large cytotoxic aldehyde derived from cyclophosphamide.

3.5. Conclusion

It is felt that the approach used in this study can be used with other drugs whose side effect is to destroy bone marrow cells. Virtually every drug is inactivated by some enzyme-catalyzed step. In essence, a key enzyme involved in the bio-inactivation of a drug could be placed into the marrow to destroy the cytotoxic chemical produced from the drug. The advantage of this approach is that a patient's own bone marrow could be transformed in tissue culture and the modified cells could be put back in the patient. Though this approach can still be considered to be gene therapy, it has the advantage of introducing the new protein in tissue culture and the cells can be determined to be viable prior to re-introducing them back into the patient. It is difficult to predict what could be the affect of increasing the concentration of an enzyme in the cell. An advantage of using a more active mutant of the enzyme is that over-expression to obtain more activity would not be necessary.

In this study, we show that it is not necessary to use the native form of an enzyme to protect the cell against a cytotoxic metabolite. In the case of ALDH3 it was necessary to only make a single amino substitution to enhance the activity of the enzyme twofold. It is impossible to know if other enzymes can be improved upon with just one or two amino acid substitutions. It is, though, tempting to speculate that it would be possible to enhance the activity of other human enzymes just two- to fivefold so that they too could be used in future gene or protein therapy.

Acknowledgement

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