Cellular Detoxification of Tripeptidyl Aldehydes by an Aldo-Keto Reductase*

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Calpain inhibitor I, N-acetyl-leucyl-leucyl-norleucinal (ALLN), a cell-permeable synthetic tripeptide with an aldehyde at its C terminus specifically inhibits the activity of cysteine proteases. Since the regulated degradation of 3-hydroxy-3-methylglutaryl-CoA reductase in Chinese hamster ovary (CHO) cells is blocked by ALLN and ALLN has a cytotoxic effect on cells, we attempted to isolate ALLN-resistant cells that overproduce an ALLN-sensitive protease(s). However, we obtained an ALLN-resistant cell line that overproduced P-glycoprotein (Sharma, R. C., Inoue, S., Rotieman, J., Schimke, R. T., and Simoni, R. D. (1992) J. Biol. Chem. 267, 5731-5734). To circumvent the multidrug resistance (MDR) phenotype during selection, we have stepwise selected an ALLN-resistant cell line of CHO cells in the presence of verapamil, a competitive inhibitor of P-glycoprotein. These non-MDR ALLN-resistant cells overexpress a 35-kDa protein and have increased aldo-keto reductase activity. Partial amino acid sequences of the 35-kDa protein are highly homologous to members of the aldo-keto reductase superfamily. The aldo-keto reductases are NADPH-dependent oxidoreductases and catalyze reduction of a wide range of carbonyl compounds such as aldehydes, sugars, and ketones. Our findings support the concept that a physiological function for aldo-keto reductases may be detoxification.

N-Acetyl-leucyl-leucyl-norleucinal (ALLN),1 a cytotoxic synthetic tripeptide with an aldehyde group at its C terminus, is a potent inhibitor of cysteine proteases including calpains and lysosomal cathepsins (1). The aldehyde moiety is crucial for inhibition of protease activity (2). We have recently shown that ALLN and its closely related analog N-acetyl-leucyl-leucyl-methioninal (ALLM) inhibit the endoplasmic reticulum-localized degradation of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol biosynthesis (3, 4).

To characterize the ALLN-sensitive protease(s) involved in the degradation of HMG-CoA reductase and HMGal, we attempted to isolate a cell line resistant to ALLN with the anticipation of obtaining cells expressing elevated levels of the ALLN-sensitive protease(s). The first ALLN-resistant cell line we obtained showed a classical MDR phenotype, resulting from amplification of the mdrl gene (5). These results suggested that the apparent resistance of these cells to ALLN is due to the outward pumping activity of P-glycoprotein (5). To circumvent the selection for the MDR phenotype, we adapted CHO cells to grow in increasing amounts of ALLN in the presence of verapamil, a competitive inhibitor of P-glycoprotein (6, 7). Verapamil at 5-20 μM reverses the MDR phenotype in MDR cell lines but has no effect on cell growth rate (5). The ALLN-resistant (SI100) cells we have obtained in this study show no detectable elevation in P-glycoprotein. Rather, these cells express elevated levels of a 35-kDa protein that belongs to the aldo-keto reductase superfamily. The aldo-keto reductases are NADPH-dependent oxidoreductases that catalyze the reduction of aldehydes and ketones to corresponding alcohols (8). Elevated levels of aldo-keto reductase in SI100 cells can inactivate ALLN by reducing its active aldehyde group to corresponding alcohols (2). This may be the mechanism of ALLN resistance in these cells.

Enzymes of the aldo-keto reductase superfamily such as aldose reductase (EC 1.1.1.21) and aldehyde reductase (EC 1.1.1.12) have been classified on the basis of a preferential, but not exclusive, substrate specificity (8). Recently, several cDNA clones of aldo-keto reductases have been isolated and their primary structures were determined. Human liver aldehyde reductase (9) has strong homology to aldose reductases of mouse vas deferens (10), rat lens (11), and human placenta (9). The calculated molecular mass of these enzymes is about 35-37 kDa, similar to 35-kDa aldo-keto reductase obtained in the present study.

Our data suggest that resistance to a tripeptide with an aldehyde group at its C terminus results from overaccumulation of an aldo-keto reductase under conditions that do not allow overexpression of P-glycoprotein. Thus, elevated levels of an aldo-keto reductase may constitute a general mechanism for acquired resistance to toxic agents whose active form requires an aldehyde or keto group. These results support the concept that members of the aldo-keto reductase family serve as a detoxifying system (12).

EXPERIMENTAL PROCEDURES

Materials—Calpain inhibitor I (ALLN) and II (ALLM) were purchased from Boehringer Mannheim or Calbiochem. All other commercially available reagents were from Sigma. Calpeptin (benzyloxycarbonyl-leucyl-leucyl-norleucinal) was a generous gift of Dr. To Shimasa Tsujinaka (Osaka University, Japan) and Dr. Takaharu Tanaka (Suntory Research Center, Japan). E-64-d (ethyl(2S,3S)-3-[1a]-3-methyl-1-(3-methylbutylcarbamoyl)butylcarbamoyl)oxirane-
2-carboxylate) was kindly provided by Dr. Masaharu Tamai (Taisho Pharmaceutical Co., Japan).

**Cell Culture**—CHO-K1 cells were grown in minimal essential medium supplemented with 5% fetal calf serum at 37 °C in a 5% CO₂ atmosphere. For stepwise selection to ALLN resistance, exponentially growing cells were treated with 10 μM ALLN in the presence of 20 μM verapamil. The medium was changed every 2–3 days for 1–2 weeks, at which time cells were trypsinized and replated in the same medium. After about 1 month of growth, cells became resistant to 10 μM ALLN in the presence of verapamil. The concentration of ALLN was increased in a stepwise fashion (10–20 μM increments) up to 100 μM ALLN, always in the presence of 20 μM verapamil, a process that required about 3–4 months. These cells are designated S1100 cells. In a similar manner, we selected cells which grew in 260 μM ALLN, designated S1260 cells. Cytotoxicity assays by the clonogenic method were performed as described (5). Transfection of wild-type CHO and SI100 cells with the gene for HMGal was carried out as described (13), and G418 at 250 μg/ml was added to the medium to maintain a stable HMGal phenotype. These cells are designated CHO-HMGal and SI1100-HMGal cells, respectively.

**HMGal Assay**—Activity of HMGal was determined as described by Skalnik et al. (13). Specific activity is expressed as Azo₄₆₅/h/mg of protein.

**Measurement of P-glycoprotein**—Amounts of P-glycoprotein were determined as described by Sharma et al. (5).

**Preparation of Cell Extracts**—Unless specified otherwise, the following procedures were carried out at 4 °C. About 100 mg of cells (1–2 × 10⁶ cells) were homogenized by 40 strokes in a Dounce homogenizer in 500 μl of 10 mM sodium phosphate, pH 7.4, containing 0.5 mM EDTA and 2 mM β-mercaptoethanol. The homogenate was centrifuged at 1,000 g for 10 min. The supernatant (total cell extract) was centrifuged at 100,000 g for 30 min. The resultant supernatant was removed and designated "cytosolic fraction." The pellet was resuspended in 200 μl of the same buffer, sonicated 3 times for 10 s, and used as a "membrane fraction." Both cytosolic and membrane fractions were dialyzed against 500 ml of 10 mM sodium phosphate, pH 7.4, containing 0.5 mM EDTA and 2 mM β-mercaptoethanol.

**Assay of Aldo-Keto Reductase**—Reaction mixture, in a total volume containing an aldehyde group at its terminus (1). The reaction mixture contained 50 μl of cytosolic fraction of cell extracts, 0.1 mM NADPH, and various cytotoxicity inhibitors. The reaction was monitored at 210 nm. Isolated peptides were sequenced on an Applied Biosystems 473 protein sequencer in the presence of 3 μg of Polybrene.

**RESULTS AND DISCUSSION**

**Characterization of SI100 Cells**—A cell line resistant to ALLN was selected in the presence of verapamil, as described under "Experimental Procedures." These SI100 cells are 30-fold more resistant to ALLN than the parental CHO cells (Fig. 1 and Table I). Since ALLN is a specific inhibitor of cysteine proteases such as calpains (Ca²⁺-dependent neutral cysteine proteases) and lysosomal cathepsins (1), we determined whether SI100 cells are also resistant to other cysteine protease inhibitors (Table I). ALLN is a synthetic tripeptide containing an aldehyde group at its C terminus (1). The cytotoxicity of ALLN to CHO cells is less than that of ALLM, and SI1100 cells were also at least 3-fold more resistant to ALLM than CHO cells. However, with other cysteine protease inhibitors (calpeptin and E-64-d), SI1100 and CHO cells showed similar toxicities (Table I). Calpeptin is a dipetide containing an aldehyde group at its C terminus (16, 17), and E-64-d is the cell-permeable inhibitor derived from E-64 and does not contain an aldehyde group (2, 18). These results show that the resistance of SI1100 cells to cysteine protease inhibitors is specific for ALLN and ALLM.

The ALLN-resistant cells that we have previously obtained exhibit the classical multidrug resistance phenotype (5). In this study, we isolated the ALLN-resistant cells in the presence of verapamil to prevent selection of the MDR phenotype, since verapamil reverses the MDR phenotype as a competitive inhibitor of drug transport by P-glycoprotein (6). As shown in Table I, SI1100 cells have a similar sensitivity to pleiotropic drugs (doxorubicin, etoposide, colchicine) as do CHO cells. In addition, SI1100 and CHO cells had the same amounts of P-glycoprotein (data not shown) as measured by quantitative immunoblotting using the C219 anti-P170 monoclonal antibody (5). These results confirm that SI1100 cells do not display the classical MDR phenotype.

**A Cystolic 35-kDa Protein Is Highly Overproduced in SI1100 Cells**—When proteins in total cell extracts are separated by SDS-PAGE, we found that the amount of a 35-kDa protein is dramatically increased in SI1100 cells (Fig. 2). This 35-kDa protein is cystolic, and its amount is still higher in S1260 cells, which are selected in the presence of 260 μM ALLN with 20 μM verapamil. Two-dimensional gel electrophoresis of total cell extracts shows that only one protein band is clearly overproduced in SI1100 cells (Fig. 3). After blotting to a polyvinylidene difluoride membrane, we cut out the 35-kDa

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**Fig. 1. Cytotoxicity of ALLN**. Exponentially growing cells were trypsinized and plated (500 cells/6-cm Petri dish) in 5 ml of complete medium containing various concentrations of ALLN. After 7–10 days of incubation, colonies were fixed with ethanol, stained with crystal violet, and counted. •, CHO cells; ▲, SI100 cells.

**Table I**

| Inhibitors | LD₅₀ (μM) |
|-----------|----------|
| ALLN      | 5        |
| ALLM      | 140 (>,500) |
| Calpeptin | 50       |
| E-64-d    | 200      |
| Doxorubicin| 0.05    |
| Etoposide | 0.08     |
| Colchicine| 0.06     |

*ALLM is not soluble in aqueous solution at a concentration higher than 500 μM.*
protein was digested with trypsin, and the amino acid sequences of the two peptides obtained are Ile-Leu-Asn-Lys-

sequences of isolated peptides were determined. The amino acid sequences of the two peptides obtained are Ile-Leu-Asn-Lys-

SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue R-250. Total cell extracts (lanes 2, 5, and 9), cytosolic fractions (lanes 3, 6, and 9), membrane fractions (lanes 4, 7, and 10), and molecular mass markers (lanes 1 and 10) are shown. Arrowheads indicate a 35-kDa protein highly expressed in SI100 and SI260 cells.

**Fig. 2.** Increased level of 35-kDa protein in ALLN-resistant cells. Crude cell extracts (50 µg/lane) of CHO (lanes 2–4), SI100 (lanes 5–7), and SI260 (lanes 7–10) were electrophoresed in 5–10% gradient SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue R-250. Total cell extracts (lanes 2, 5, and 8), cytosolic fractions (lanes 3, 6, and 9), membrane fractions (lanes 4, 7, and 10), and molecular mass markers (lanes 1 and 10) are shown. Arrowheads indicate a 35-kDa protein highly expressed in SI100 and SI260 cells.

**Fig. 3.** Two-dimensional gel electrophoresis analysis. The total cell extract (260 µg of protein) of CHO (A) and SI100 (B) was subjected to two-dimensional gel electrophoresis, as described under "Experimental Procedures." First dimension, nonequilibrium pH gradient electrophoresis (NEPHGE); second dimension, 5–10% gradient SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue R-250. An arrow indicates the 35-kDa protein abundant in SI100 cells (B). No band was detected in the same position in CHO cell extract (A).

protein spot, and amino acid analysis of this protein was performed. Since its N-terminal amino acid was blocked, the protein was digested with trypsin, and the amino acid sequences of isolated peptides were determined. The amino acid sequences of the two peptides obtained are Ile-Leu-Asn-Lys-Pro-Gly-Leu and Pro-Val-Tyr-Asn-Gln-Val-Glu-X-Pro-Tyr-Leu, respectively. A search of current protein data bases revealed that these two sequences have significant homology with the aldo-keto reductase superfamily (9). An alignment of the amino acid sequence of the peptides of the 35-kDa protein with that of several members of the superfamily is shown in Fig. 4. The comparison shows that the peptide sequences of 35-kDa protein are highly homologous to chlordecone reductase (19), aldose reductase of several species (9–11), prostaglandin F synthase (20), and 3α-hydroxysteroid dehydrogenase, and less homologous to aldehyde reductase (9). All these enzymes are members of a large family of proteins capable of reducing carbonyl groups on a wide variety of compounds, including sugars, aldehyde metabolites, steroid hormones, as well as xenobiotic aldehydes and ketones (8). These proteins are cytosolic, NADPH-dependent oxidoreductases and have a similar molecular mass of 35–37 kDa (8). The amino acid composition of the 35-kDa protein is shown in Table II, which indicates a strong resemblance to those of aldose and aldehyde reductases. The PI value of 35-kDa protein on isoelectric focusing gel electrophoresis (PI = 6.5, data not shown) is also similar to the PI value of aldose reductases of human (9), mouse (10), rat (11), and aldehyde reductase of human (9) as estimated from their amino acid compositions. These results suggest that the 35-kDa protein overproduced in SI100 cells is an aldo-keto reductase.

**Fig. 4.** Comparison of partial amino acid sequences of 35-kDa protein to aldo-keto reductase superfamily. The amino acid sequences of two tryptic peptides of the 35-kDa protein were determined as described under "Experimental Procedures." The references of amino acid sequences are chlordecone reductase (19), mouse aldose reductase (10), rat aldose reductase (11), human aldose reductase (9), bovine prostaglandin F synthase (20), rat 3α-hydroxysteroid dehydrogenase (28, 29), and human aldehyde reductase (9), respectively. Identical residues to the sequences of 35-kDa protein are boxed. The dash represents a gap.
this possibility, stable transfectants of HMGal gene were isolated, and the effect of ALLN on the mevalonate-accelerated degradation of HMGal was determined. Fig. 5 shows that the activity of HMGal in both CHO and SI100 cells decreases to about 20% of control when 20 mM mevalonate is added to cells. This decrease in activity is due to accelerated degradation of HMGal (4, 13, 23, 24). Addition of ALLN to CHO cells causes a dose-dependent increase in HMGal activity, indicating that the mevalonate-accelerated degradation is inhibited by ALLN with an IC_{50} of approximately 50 μM (Table IV). In the SI100 cells, however, the inhibitory effect of ALLN on the degradation of HMGal is markedly less than that of CHO cells. The IC_{50} of ALLN in SI100 cells is about 300 μM. This higher IC_{50} for inhibition of the degradation of HMGal reflects the inactivation of ALLN in SI100 cells. Since the aldehyde group of ALLN is crucial for the inhibition of cysteine proteases (2), this result is consistent with the elevated level of aldo-keto reductase in SI100 cells. Essentially the same effect of ALLN on the degradation of HMGal was observed. Calpeptin and E-64-d also inhibit the mevalonate-accelerated degradation of HMGal in CHO cells with IC_{50} of 150 and 400 μM, respectively (Table IV). However, these inhibitory effects do not differ significantly between sensitive and resistant cells even though calpeptin has an aldehyde group and is capable of being a substrate of aldo-keto reductase (22). Similarly, calpeptin is not differentially cytotoxic to sensitive CHO cells compared with SI100 cells (Table I). One possible reason to explain the lack of an effect of calpeptin either on cytotoxicity or HMGal degradation is that it is efficiently reduced to the inactive form by aldo-keto reductase even in CHO cells. It is also possible that the cell-

### Table II
Comparison of amino acid composition

| Amino acid | 35-kDa protein* | Aldose reductase* (mouse) | Aldehyde reductase* (human) | mol % |
|-----------|-----------------|---------------------------|-----------------------------|------|
| Ala       | 6.9             | 8.2                       | 8.9                         |
| Val       | 7.5             | 7.9                       | 7.4                         |
| Leu       | 10.2            | 10.1                      | 11.4                        |
| Ile       | 5.8             | 6.4                       | 4.6                         |
| Pro       | 6.2             | 6.1                       | 7.7                         |
| Met       | 1.0             | 1.6                       | 1.5                         |
| Phe       | 4.0             | 4.4                       | 2.5                         |
| Trp       | ND              | 1.6                       | 2.2                         |
| Gly       | 6.1             | 4.1                       | 5.5                         |
| Ser       | 3.8             | 8.4                       | 4.9                         |
| Thr       | 4.7             | 3.8                       | 3.7                         |
| Cys       | ND              | 1.6                       | 1.9                         |
| Tyr       | 3.9             | 3.5                       | 4.3                         |
| Asx       | 9.0             | 9.8                       | 8.9                         |
| Gix       | 15.0            | 12.7                      | 10.8                        |
| Lys       | 8.7             | 9.5                       | 6.2                         |
| Arg       | 3.5             | 3.2                       | 4.9                         |
| His       | 3.9             | 2.9                       | 2.8                         |

* Amino acid composition of 35-kDa protein was determined as described under "Experimental Procedures" and presented as apparent mol %, since Trp and Cys were not determined (ND).

### Table III
Specific activity of aldo-keto reductase

Specific activity of NADPH-dependent aldo-keto reductase of cytosolic fraction of cell extracts was determined spectrophotometrically. The values are the means of three independent experiments. When NADH was used instead of NADPH, no activity was detected (<0.05 nmol/min/mg of protein).

| Substrate       | CHO SI100 SI260 |
|-----------------|-----------------|
|                  | n mol/min/mg of protein |
| D-Xylose (100 μM) | 1.3 ± 0.3       | 5.0 ± 1.8       | 7.3 ± 2.7       |
| DL-Glyceraldehyde (500 μM) | 1.5 ± 0.2       | 7.0 ± 0.2       | 14.7 ± 1.3      |
| ALLN (200 μM)    | 0.9 ± 0.1       | 3.4 ± 1.5       | 9.0 ± 1.8       |
| ALLM (200 μM)    | 0.1 ± 0.1       | 0.5 ± 0.1       | 1.5 ± 0.1       |
| Calpeptin (270 μM) | 1.4 ± 0.5       | 23.4 ± 6.6     | 46.8 ± 5.3     |
| E-64-d (300 μM)  | <0.05           | <0.1            | <0.1            |
| Without substrate | <0.05           | <0.1            | <0.1            |

### Table IV
Inhibition of mevalonate-accelerated degradation of HMGal

Inhibition of mevalonate-accelerated degradation by cysteine protease inhibitors was determined as described in the legend to Fig. 5. IC_{50} was calculated from two independent experiments.

| Inhibitors | CHO-HMGal IC_{50} μM | SI100-HMGal IC_{50} μM |
|------------|-----------------------|------------------------|
| ALLN       | 50                    | 300                    |
| ALLM       | 330                   | >500                   |
| Calpeptin  | 150                   | 180                    |
| E-64-d     | 400                   | 500                    |

* ALLM is not soluble in aqueous solution at a concentration higher than 500 μM.
penetrating efficiency of calpeptin is lower than that of ALLN.

In order to see if an elevated level of aldo-keto reductase in SI100 cells represents a stable phenotype, SI100 cells were grown in medium without drugs. The phenotype of SI100 cells (ALLN resistance and elevated level of 35-kDa protein) did not change after 1 month of growth in medium without drug; however, after 2 months the amount of 35-kDa protein was two-thirds of original SI100 cells (data not shown). This indicates that the drug resistance phenotype of SI100 cells is relatively stable. Currently we have no information concerning the mechanism of overexpression of this aldo-keto reductase.

Our results show that cells can become resistant to cysteine protease inhibitors requiring an active aldehyde sink as well as overproduction of an aldo-keto reductase. Interestingly, Molowa et al. (22) have reported differences in aldo-keto reductase levels in human liver. These results suggest that the drugs that require aldehyde or keto groups for activity may be subject to acquired resistance due to differences in aldo-keto reductase levels in human liver. These differences may contribute to differential responsiveness within a population based on differing levels of this drug-inactivating system.

The existence of multiple mechanisms of resistance to a single agent appears to be a common theme in cultured animal cells, as indicated by resistance to ALLN. There are multiple mechanisms for resistance to antifolates (25, 26), and individual cells may have more than a single mechanism (27). This indicates the complexity of understanding and identifying mechanisms of potential importance in clinical resistance to cancer chemotherapeutic agents.

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