Mechanistic Studies on the Inactivation of the Proteasome by Lactacystin in Cultured Cells*

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The natural product lactacystin exerts its cellular antiproliferative effects through a mechanism involving acylation and inhibition of the proteasome, a cytosolic proteinase complex that is an essential component of the ubiquitin-proteasome pathway for intracellular protein degradation. In *vitro*, lactacystin does not react with the proteasome; rather, it undergoes a spontaneous conversion (lactonization) to the active proteasome inhibitor, *clasto*-lactacystin β-lactone. We show here that when the β-lactone is added to mammalian cells in culture, it rapidly enters the cells, where it can react with the sulfhydryl of glutathione to form a thioester adduct that is both structurally and functionally analogous to lactacystin. We call this adduct lactathione, and like lactacystin, it does not react with the proteasome, but can undergo lactonization to yield back the active β-lactone.

Lactacystin is a natural product that was originally discovered for its ability to induce neurite outgrowth and differentiation in a mouse neuroblastoma cell line, Neuro 2A (1). Subsequent work (2, 3) demonstrated that the biological effects of lactacystin result from its ability to acylate and inhibit the proteasome, a ubiquitous intracellular protein-degrading machine (for reviews, see Refs. 4–10). Because the proteasome is involved both in the normal turnover of cellular proteins (11) and in the processing and degradation of regulatory proteins that control cell growth and metabolism (12), proteasome inhibitors can have profound biological consequences.

As part of a program to develop proteasome inhibitors into novel therapeutic agents, we have undertaken detailed studies of the mechanism of lactacystin. In a recent report (13), we demonstrated that lactacystin *per se* is not a proteasome inhibitor. Rather, lactacystin in aqueous solution can spontaneously undergo an intramolecular reaction (lactonization) to form the active proteasome inhibitory species, *clasto*-lactacystin β-lactone. Herein we have extended these studies to examine the mechanism of proteasome inhibition by lactacystin in cultured cells.

The values for the rate constants given in Scheme 1 were estimated based on measurements of the GSH dependence of the steady-state rate constant for lactathione hydrolysis under the same conditions. The spectrophotometric assays and data analysis were the same as those used in a previous work to study lactacystin hydrolysis (13).

EXPERIMENTAL PROCEDURES

Synthesis—Lactacystin and *clasto*-lactacystin β-lactone were prepared by the method of Corey et al. (14, 15). Lactathione was prepared by mixing β-lactone (49 mg, 0.229 mmol) in 10 ml of acetonitrile with glutathione (360 mg, 1.17 mmol) and 1.35 ml of 1.00 N NaOH (1.35 mmol). The mixture was stirred for 4 h at room temperature, and 1 N HCl was added to obtain pH 4. The solution was concentrated on a rotovap and then lyophilized. The dry material was resuspended in a small volume of water and subjected to reverse-phase HPLC on a 19 × 300-mm Delta-Pak C18 column (Waters). Lactathione and the lactathione isomer (see Fig. 1) were collected in separate fractions eluting from the column. Lyophilization of the fractions afforded 48 mg of lactathione and 7.8 mg of the lactathione isomer. The structure of the synthetic materials was confirmed by proton magnetic resonance spectrometry and electrospray ionization mass spectrometry. β-[3H]Lactone at a specific activity of 3.4 Ci/mmol was prepared from β-lactone by the method described (3).

HPLC Analyses of Lactathione and Lactacystin—The HPLC methodology has been described in detail (13). Briefly, the stationary phase was a C18 column (Vydac, 218TP54), and the mobile phase consisted of a gradient of methanol (0.05% trifluoroacetic acid) in water (0.06% trifluoroacetic acid).

Analysis of Lactathione Hydrolysis—The values for the rate constants given in Scheme 1 were estimated based on measurements of the GSH dependence of the steady-state rate constant for lactathione hydrolysis in phosphate-buffered saline at pH 7.4 and 37 °C and on separate measurements of β-lactone hydrolysis under the same conditions. The spectrophotometric assays and data analysis were the same as those used in a previous work to study lactacystin hydrolysis (13).

Lactathione Inactivation of Proteasome Activity—The 20 S proteasome and PA28 activator were purified from rabbit reticulocytes as described (13). Hydrolysis of the substrate succinyl-Leu-Leu-Val-Tyr

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7-amino-4-methylcoumarin by the 20 S proteasome-PQA28 complex was assayed by continuously monitoring the fluorescence of the liberated 7-amino-4-methylcoumarin. Fluorescence values for lactacystin were estimated by analysis of progress curves obtained upon addition of lactacystin (13) at a final concentration of 2 μM.

General Cell Culture—Cell lines were obtained from American Type Culture Collection. Jurkat cells (human T cell leukemia, clone E6-1) were grown in RPMI 1640 medium (JRH Biosciences) supplemented with 10% heat-inactivated FBS (Sigma) and antibiotics (1000 units/ml penicillin and 100 μg/ml streptomycin) (Sigma). HeLa cells (human erythroid carcinoma) were grown in Dulbecco’s modified Eagle’s medium (DME/10% high glucose) (JRH Biosciences) supplemented with 10% FBS and antibiotics as described above. C2C12 cells (mouse myoblast) were grown in Dulbecco’s modified Eagle’s medium (DMEM/HIGH modified) supplemented with 10% heat-inactivated FBS and antibiotics described as above. All cells were grown at 37 °C in an atmosphere of 5% CO2. Cells were passaged biweekly and used for experimental purposes in exponential growth phase.

GSH Depletion Studies—For GSH depletion, Jurkat cells were kept at low density (≤5 × 10^6 cells/ml) for 24 h in the presence of 1 μM buthionine sulfoxime (16). Following this treatment, the cells were harvested, resuspended in fresh medium, and aliqutoned into 24-well plates at a density of 5 × 10^5 cells/ml for measurement of lactacystin accumulation or 7 × 10^4 cells/ml for measurement of IL-2 production. Aliquots of 2 × 10^4 cells were harvested and washed, and total intracellular glutathione was measured enzymatically using the GSH-GSSG recycling assay (17).

Extraction of Lactathione from Cells—Following exposure to β-lactone or lactacystin at the concentrations and times indicated in the figure legends, cells were harvested and washed twice with cold PBS. To each cell pellet (5 × 10^5 cells) in a microcentrifuge tube was added 200 μl of cold methanol with 0.1% trifluoroacetic acid, and the pellet was homogenized with a motorized pestle. The tubes were centrifuged at 16,000 × g for 10 min at 4 °C. The supernatant was collected and dried in a vacuum centrifuge. The dried samples were resuspended in water with 0.06% trifluoroacetic acid and subjected to reverse-phase HPLC (see above).

Assay of 20 S Proteasome Activity in Crude Cell Extracts—Jurkat cells at 7 × 10^6 cells/ml in medium were treated for 30 min with different concentrations of β-lactone. The cells were harvested and washed twice with cold PBS. The resulting pellets were ground with a motorized pestle in 50 μl of cold 5 mM HEPES, pH 7.5, with 1 mM Dithiothreitol. The samples were centrifuged for 10 min at 16,000 × g in the cold, and the supernatants were collected. The total soluble protein was measured using the Coomassie Plus Protein Assay (Pierce). For measurement of 20 S proteasome peptidase activity, 10 μl of each sample (40–80 μg of total protein) was diluted into a cuvette containing 2 ml of 0.06% trifluoroacetic acid in water. A 100 μl aliquot of the sample was subjected to reverse-phase HPLC as described under “Experimental Procedures.”

RESULTS AND DISCUSSION

Lactathione Formation In Vitro—In aqueous solution, clasto-lactacystin β-lactone readily reacts with N-acetyl-L-cysteine (NAC) to form the thiostere adduct that is lactacystin (13). This prompted us to speculate that an analogous thiostere adduct would form upon mixing β-lactone with GSH. Fig. 1 shows the UV chromatograms of a reaction mixture containing β-lactone and GSH along with controls for the two components incubated separately. Two new peaks were observed in the chromatogram of the reaction mixture. In addition, the peak corresponding to the β-lactone had disappeared, but no peak corresponding to clasto-lactacystin dihydroxy acid was detected, indicating that the lactone had not hydrolyzed. The two new products were subsequently prepared in quantities sufficient for protein, NMR and mass spectrometric analysis (data not shown). The larger of the two peaks (M + H = 521.8) corresponds to the predicted thiostere adduct (Scheme 1), for which we suggest the trivial name lactathione. The smaller peak (M + H = 521.8) represents an isomer of lactathione, whose exact structure and origin are currently under investigation.

Aqueous Hydrolysis of Lactathione—In aqueous solution, lact-
Lactathione undergoes spontaneous hydrolysis to yield NAC and clasto-lactacystin dihydroxy acid. As shown in our previous work (13), this process proceeds exclusively through the intermediacy of the $\beta$-lactone, and this latter species is solely responsible for proteasome inactivation. The hydrolysis of lactathione was studied employing the same methodology, and we obtained similar results as summarized in Scheme 1. Also, the inactivation of 20 S proteasome chymotrypsin-like activity by lactathione was examined as a function of GSH concentration as shown in Fig. 2. Increasing the GSH concentration, which will lower the steady-state level of $\beta$-lactone (Scheme 1), also slows proteasome inactivation (Fig. 2). Likewise, at pH 6.5, where lactathione is stable because lactonization is suppressed (data not shown), the rate of proteasome inactivation by lactathione is greatly reduced ($k_{\text{inact}}/[I] \approx 0.04 \text{ mM}^{-1} \text{s}^{-1}$). Thus, the properties of lactathione in vitro are entirely analogous to lactacystin. Like lactacystin, lactathione does not function as a proteasome inhibitor, but it can serve as a source of the active species, clasto-lactacystin $\beta$-lactone.

**Lactathione Formation in Cells**—Of particular interest in Scheme 1 is the second-order rate constant for combination of $\beta$-lactone with glutathione to form lactathione, 3.6 $\text{M}^{-1} \text{s}^{-1}$, measured in PBS at 37°C. This result suggested that in the physiological range of intracellular GSH ([GSH] $\geq 1 \text{ mM}$), intracellular $\beta$-lactone would react rapidly ($t_{1/2} \approx 3 \text{ min}$) to form lactathione. Fig. 3 shows chromatographic analyses of extracts from Jurkat cells that had been exposed to $\beta$-lactone, lactacystin, or vehicle (dimethyl sulfoxide). In the extracts from both $\beta$-lactone- and lactacystin-treated cells, a peak with the same retention time as lactathione (21.4 min) was observed. This component is absent in the extract from vehicle-treated cells. The identity of this component as lactathione was confirmed in separate experiments by collecting it and subjecting it to mass spectral analysis. The major component that was detected had a mass identical to synthetic lactathione (see above). Interestingly, only lactathione, and not lactacystin, could be detected in the extracts from lactacystin-treated cells. Although the absence of lactacystin in extracts from lactacystin-treated cells is a negative result, control experiments suggest that it is significant. The retention time of lactathione in this chromatographic system is 24 min, which is well resolved from any of the background peaks. Also, as a control for recovery, we supplemented extracts from naive cells with lactathione and lactacystin and then prepared the extracts for chromatography. The peaks corresponding to both lactathione and lactacystin were observed (data not shown), and both were obtained with 79% overall recovery through the procedure.

In light of these control experiments, we feel that our inability to detect lactacystin in extracts from lactacystin-treated cells suggests that either lactacystin does not enter these cells or that it enters the cells and is very rapidly converted to lactathione (e.g., the conversion might be catalyzed by a GSH transferase). To test these possibilities, we examined the effect of glutathione depletion on accumulation of lactathione inside the cells. A treatment of Jurkat cells with the $\gamma$-glutamylcysteine synthase inhibitor, buthionine sulfoxime, that was sufficient to lower intracellular GSH concentrations by $\approx 95\%$ had the expected effect of lowering lactathione accumulation in both $\beta$-lactone- and lactacystin-treated cells (Table I). Nevertheless, no lactacystin could be detected by chromatographic analysis of the GSH-depleted, lactacystin-treated cells. Additionally, we tested the ability of a commercially available rat liver GSH transferase preparation to catalyze lactathione formation. The integrity of this enzyme preparation was confirmed by monitoring the conjugation of 1-chloro-2,4-dinitrobenzene in a standard assay (18). Nevertheless, no lactathione formation could be detected when lactacystin was tested as a substrate (data not shown). Also, lactacystin showed no ability to inhibit GSH transferase activity in the standard assay (data not shown).

We next examined the kinetics of lactathione accumulation in...
in Jurkat cells treated with β-lactone or lactacystin using the HPLC assay. Representative data are shown in Fig. 4A. Lactathione accumulation in β-lactone-treated cells could be detected at the earliest time point that we were capable of obtaining. This time point is ~2 min, which is the time it takes to harvest and wash the cells after adding the drug. The intracellular lactathione concentration reaches a maximum at 15–30 min and then decays with a $t_{1/2}$ of ~30 min. As a rough approximation, we can estimate the intracellular volume from the mass of the cell pellet assuming 1 mg of cell mass has a volume of 800 μm$^3$. Thus, the intracellular concentration of lactathione at the peak is ~140 μM, which is higher than the starting concentration (50 μM) of β-lactone in the medium. We have also performed this experiment with HeLa cells, and the shape of the time course is nearly identical (data not shown). Nevertheless, the peak concentrations of lactathione observed in HeLa cells is lower by about two-thirds compared with Jurkat cells for the same dose of β-lactone. This could reflect differences in the permeability of the drug in the different cell types; however, we suspect that this observation most likely reflects the condition that the HeLa cells are adherent cells and the Jurkat cells grow in suspension. On one occasion, we first harvested HeLa cells and then treated them with β-lactone in suspension, and the lactathione accumulation was three times higher than when the cells were treated on the plates.

The time course for lactathione accumulation in cells treated with lactacystin differed substantially from that with β-lactone (Fig. 4A). The peak level was lower, and it was obtained at a longer time (45–60 min). Nevertheless, the decay was slower, so that at 60 min, the concentrations of lactathione in the lactacystin-and β-lactone-treated cells are equal, and at longer times, the lactathione concentration is higher in the lactacystin-treated cells. The same observations were made in experiments with HeLa cells (data not shown). These results, together with consideration of the mechanism of lactacystin hydrolysis as elucidated in our previous work (13), suggested a model whereby only lactacystin, but not lactathione, can enter cells. To illustrate this point, we have performed a calculation of the time dependence of β-lactone concentration in aqueous medium starting at zero time with equal concentrations of either β-lactone or lactacystin (Fig. 4B). Starting with β-lactone, the β-lactone concentration decays in a first-order process due to hydrolysis. Stating with lactacystin, the β-lactone concentration is initially zero and with time shows a transient accumulation. This represents the balance between production of β-lactone via the lactonization of lactacystin and β-lactone destruction via hydrolysis. The concentrations of lactathione relevant to the cell culture experiments are relatively low (<50 μM), so that the NAC produced in the lactonization step never accumulates to a level that is high enough to drive the back-reaction (i.e. re-formation of lactacystin from β-lactone and NAC) to an appreciable extent. Thus, one need only consider a simple model of two sequential first-order reactions for the calculation of β-lactone concentration when starting with lactacystin. There are two crucial features of this system (Fig. 4B) that are relevant for consideration of the data (Fig. 4A). First, the concentration of β-lactone will eventually be higher when starting with lactacystin. The timing of this (i.e. the time point at the intersection of the two curves) will depend on the relative magnitudes of the rate constants for lactonization and hydrolysis. Second, because the hydrolysis of lactacystin proceeds exclusively through the intermediary of β-lactone, the areas under the curves are equal. Both of these features parallel the lactathione accumulation data. First, we observed that at longer times (~60 min), lactathione concentration was higher in the lactacystin-treated cells. Second, the areas under the curves for the data in Fig. 4A (calculated by a model-independent approach (linear trapezoidal rule)) are 470 nmol/10$^5$ cells × min for the β-lactone-treated cells and 410 nmol/10$^5$ cells × min for the lactacystin-treated cells. Thus, these kinetics suggest that a relationship of proportionality exists between intracellular lactathione accumulation and the extracellular concentration of β-lactone. These results led us to propose the model
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PROTEASOME

LACTATONE

LACTACYSTIN

SCHEME 2. Mechanism of proteasome inhibition by lactacystin in cells.

The activation of the transcription factor NF-κB and, subsequently, the expression of the genes that NF-κB controls are dependent on the proteasome (12). The proteasome functions in this pathway by degrading IkBα, an inhibitor of NF-κB that, under basal conditions, binds to NF-κB and sequesters it in the cytoplasm. The mechanism involves a signal-induced phosphorylation and ubiquitination of IkBα, which targets this protein for degradation via the ubiquitin-proteasome pathway (19). Expression of the human IL-2 gene depends on NF-κB activation (20) and thus provides a useful system for studying a specific biological role of the proteasome and a specific consequence of proteasome inhibition (i.e. inhibition of IL-2 production). Fig. 6A shows the ability of lactacystin to suppress IL-2 production by stimulated Jurkat cells as a function of NAC concentration. The rationale for this experiment can be understood by reference to Scheme 2. Extracellular NAC will lower the extracellular β-lactone concentration by driving the back-reaction to form lactacystin. Thus, unless lactacystin can enter the cells, the extracellular NAC should prevent proteasome inactivation. In the absence of NAC, 10 μM lactacystin applied 30 min prior to the stimulus decreased IL-2 production to ~10% of the control value (Fig. 6A). With increasing concentrations of NAC, IL-2 production was restored in the lactacystin-treated cells, reaching ~80% of the control value at 5 mM NAC. We also measured lactathione accumulation under these same conditions, and the results are presented in Fig. 6B. Addition of NAC suppressed intracellular lactathione accumulation in lactacystin-treated cells, and the dose response mirrors the restoration of IL-2 production (i.e. prevention of proteasome inhibition) in the cells. Independently of the effect of lactacystin, NAC suppressed IL-2 production by about one-third at 5 mM. This is most likely due to the well documented ability of NAC to prevent NF-κB activation (21), presumably reflecting the involvement of reactive oxygen intermediates in the signaling pathway and the ability of antioxidants such as NAC to buffer them (22).
β-lactone is a common mechanism for detoxification of these compounds. In this aspect, the formation of lactathione upon entry of β-lactone into cells may serve as a buffer against β-lactone-mediated proteasome inactivation. Alternatively, the ability of lactathione to spontaneously regenerate the β-lactone (via lactonization) suggests that lactathione could serve as a reservoir for the prolonged release of the active species. The rapid accumulation of lactathione in β-lactone-treated cells (Fig. 4A) suggests that the cells are freely permeable to this molecule, whereas the observation that lactathione accumulates to a level that exceeds that of extracellular β-lactone suggests that the cells are relatively impermeable to lactathione. Thus, lactathione formation concentrates β-lactone inside the cell, albeit in an inactive form. However, this concentrating effect may be counterbalanced by subsequent metabolism of the lactathione. In vitro at physiological GSH concentrations (≈1 mM), lactathione is very stable ($t_{1/2}$ ≥ 15 h).

In Jurkat or HeLa cells, lactathione disappears, with a half-time of ≈30 min (e.g., Fig. 4A). This loss of lactathione from the cells may be mediated by a glutathione sulfur conjugate export pump in the cell membrane (23–25). Irrespective of the mechanism, it is clearly too fast to be accounted for by spontaneous lactonization of lactathione and subsequent hydrolysis of the β-lactone. To examine the fate of intracellular lactathione, we prepared radiolabeled β-lactone and performed pulse-chase experiments in HeLa cells. Fig. 7A shows the time course for the appearance of tritium in the medium from cells following a 30-min pulse of [3H]lactone. These data are superimposed upon the time course for lactathione accumulation in HeLa cells treated with β-lactone measured in a separate experiment. The appearance of tritium in the medium mirrored the loss of lactathione from inside the cells, suggesting that intracellular lactathione was transported out of the cells. To ascertain the identities of the tritiated species, aliquots of chase medium were subjected to reverse-phase HPLC, fractions were collected, and the column fractions were subjected to liquid scintillation counting (Fig. 7B). The major peak of radioactive material had a retention time coincident with lactathione (21–22 min). A second broad peak of radioactive material at an earlier retention time (8–11 min) may correspond to clasto-lactacycst dihydroxy acid (retention time = 8 min; see Fig. 1), which would be produced from lactonization and hydrolysis of lactathione during the chase interval. However, the peak fraction is at 10–11 min, which more closely corresponds to the...
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cysteine sulfur adduct of the \( \beta \)-lactone (i.e. desacetyllactacystin), which has a retention time of 10 min in this chromatographic system (data not shown). This species could potentially be generated via the sequential actions of \( \gamma \)-glutamyl transpeptidase and dipeptidases upon export of lactathione from the cells (for review, see Ref. 26).

Near the beginning of the chase interval, the major portion of the tritium was found associated with the lactathione peak (retention time = 24 min). At longer times, the major portion of the tritium was found associated with the earlier eluting peaks (retention time = 8–11 min). These kinetics suggest that lactathione is the predominant form being exported from the cells, with subsequent generation of the other species by either spontaneous chemical conversion (i.e. lactonization and hydrolysis) or enzyme-catalyzed reactions taking place outside the cells. A final point worth noting is that in vivo, the pathway for detoxification that is initiated by GSH conjugation is completed by uptake of the resulting cysteine sulfur conjugates into kidney tubules, where they are acted upon by \( N \)-acetyltransferases to produce mercapturic acids that are excreted in urine. Thus, the ultimate fate of lactathione in vivo is predicted to be excretion of the mercapturate derived from lactathione, i.e. lactacystin.\(^2\)

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\(^2\) We can call this hypothesis “Metabolic Irony.”
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