Trapoxin, an Antitumor Cyclic Tetrapeptide, Is an Irreversible Inhibitor of Mammalian Histone Deacetylase*

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Trapoxin (cyclo-(L-phenylalaninyl-L-phenylalaninyl-D-pipecolinyl-L-2-amino-o-oxo-9,10-epoxy-decanoyl)) is a fungal product that induces morphological reversion from transformed to normal in sis-transformed NIH3T3 fibroblasts. Trapoxin was found to cause accumulation of highly acetylated core histones in a variety of mammalian cell lines. In vitro experiments using partially purified mouse histone deacetylase showed that a low concentration of trapoxin irreversibly inhibited deacetylation of acetylated histone molecules. Chemical reduction of an epoxide group in trapoxin completely abolished the inhibitory activity, suggesting that trapoxin binds covalently to the histone deacetylase via the epoxide. In contrast, inhibition by trichostatin A, a known potent inhibitor of histone deacetylase, was reversible. Despite the different mode of inhibition, trapoxin and trichostatin A induced almost the same biological effects on the cell cycle and differentiation. These results strongly suggest that the in vivo effects commonly induced by these agents can be attributed to histone hyperacetylation resulting from the inhibition of histone deacetylase.

Histones in the nucleosome core particle are subject to reversible acetylation at the ε-amino group of the specific lysine residues (1, 2). The acetylation and deacetylation are catalyzed by specific enzymes, histone acetyltransferase and deacetylase, respectively, and the net level of the acetylation is controlled by equilibrium between these enzymes (2-4). Since the discovery of histone acetylation, the reaction has been suggested to play a crucial role in modulating interactions between histones and DNA to facilitate gene expression. Recent genetic, biochemical, and immunological approaches have provided substantial evidence indicating that histones associated with actively transcribed genes are more highly acetylated than those from nontranscribed regions (5-7). Genetic analyses using mutant strains of yeast in which individual lysines in the N-terminal region of histone H4 are substituted by other amino acids demonstrated that substitution of 3 or 4 of the lysine residues inhibited the induced expression of GAL1, PHO5, and CUP1 genes (8) and lysine 16 was essential for the correct expression of the mating-type genes (9, 10).

Another approach to elucidating the precise function of reversible histone acetylation is the use of the specific inhibitors of the enzymes controlling the level of acetylation. n-Butyrate at millimolar cell concentrations was shown to inhibit the histone deacetylase in a noncompetitive manner and induced the intracellular accumulation of hyperacetylated histones (11, 12). A variety of biological phenomena such as differentiation and cell cycle arrest induced by n-butyrates (13, 14) may be ascribed to the induced hyperacetylation of histones. However, the involvement of histone acetylation in the biological effects of n-butyrate is still unclear since a high concentration of the agent causes nonspecific effects on other enzymes, cytoskeleton, and cell membranes (15, 16). Trichostatin A, originally reported as a fungistatic antibiotic by Tsuji et al. (17), was found to be a very potent inducer of erythroid differentiation in murine erythroblasts. Further studies revealed that the agent blocked the cell cycle progression in the normal fibroblast cells specifically at both G1 and G2 phases (19) and induced phenotypic reversion in sis-transformed fibroblast cells (20). Recently, we demonstrated that trichostatin A caused potent and irreversible inhibition of the mammalian histone deacetylase at a nanomolar concentration both in vivo and in vitro. We also found that a trichostatin A-resistant mutant cell line possessed a trichostatin A-resistant histone deacetylase, indicating that the primary target molecule of the agent in vivo is the histone deacetylase itself (21). Thus, trichostatin A is now available as a more specific inhibitor of the enzyme than n-butyrate to elucidate the role of histone acetylation.

Here we describe another type of the specific inhibitor of the histone deacetylase, trapoxin, a cyclic tetrapeptide that was reported to induce morphological reversion from transformed to normal in v-sis-transformed NIH3T3 cells (22, 23). Unlike trichostatin A, the inhibition by trapoxin was found to be irreversible. However, experiments using the trichostatin A-resistant mutant cell line suggested that inhibition of the enzyme was the primary reason for the inhibitory effect of trapoxin on cell proliferation. Almost all biological activities of trapoxin are very similar to those of trichostatin A, suggesting that histone acetylation control is involved in cell cycle progression, differentiation, and sis oncogene-mediated transformation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trapoxin was isolated from the culture broth of Heli- comona ambiens RF-1023 (22) and was dissolved in dimethyl sulfoxide as stock solutions (0.1-10 mg/ml). Trichostatin A isolated from the culture broth of Streptomyces hygroscopicus Y-50 (17) was used as a

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control. Hydroxyurea, adriamycin, vinblastine, Colcemid, and propidium iodide were purchased from Sigma, and sodium n-butyrate was from Weko Pure Chemical Industries Ltd. Human plasmogen was from Boehringer Mannheim; skim milk and agae noble were from Difco, and fetal bovine serum was from Cell Culture Laboratories. [3H]Acetyl-labeled histones (12 Ci/mmol) was purchased from Amerham Corp.

Chemical Reduction of an Epoxide Group in Trapoxin—Trapoxin was reduced to cyclo-(t-phenylalanyl-t-phenylalanyl-p-pipe Colinyl-L-2-amino-8-oxo-10-hydroxy-decanoil) by the method described previously (22). Briefly, trapoxin (10 mg) dissolved in 100 μl of ethanol/tetrahydrofuran (1:1) was mixed with a reducing agent solution consisting of 8 mg of diphenyl diselenide, 1.9 mg of NaBH4, 0.2 ml of EtOH, and 0.5 μl of acetic acid. The reaction mixture was stirred at room temperature for 30 min and then extracted with 2.5 ml of ethyl acetate. The extract was evaporated in vacuo to dryness, and the residue was purified by preparative TLC (Merck KGF, CH2Cl2/methanol 1:1, Rf = 0.5) yielding 8 mg of reduced trapoxin.

Cells and Culture Conditions—A mouse mammary gland tumor cell line FM3A and its trichostatin A-resistant mutant cell line TR303 (21) were cultured in a humidified atmosphere of 5% CO2, 95% air at 37 °C in ES medium (Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with 2% calf serum. A rat embryonic fibroblast cell line 3Y1-B, a mouse teratocarcinoma cell line F9, a mouse Friend leukemia cell line MEL, and a human cervix carcinoma cell line HeLa were cultured in minimal essential medium (MEM) supplemented with 12% fetal bovine serum. In the case of F9 cells, gelatin-coated plastic dishes (Corning) were used for both subculturing and the assay for plasmogen activator.

Cell Synchronization—To synchronize 3Y1 cells at G0 phase, resting cells grown to confluence were trypsinized and seeded at 5 × 106 cells/ml 35-mm dish in MEM containing 0.5% serum but lacking glutamine. Most of the cells were introduced into Go phase after 36 h of incubation in this medium. The Go-starting synchronous culture was obtained by transferring these cells into complete MEM containing 12% serum. To obtain synchronization at early S phase, the Go cells were cultivated in MEM supplemented with 12% serum for 10 h without drugs and for a further 14 h with 1 mM hydroxyurea.

Flow Cytometry—Flow cytometry was used to analyze the distribution of DNA contents in the cell populations. Measurements were performed with isolated nuclei stained with propidium iodide according to the method of Noguchi and Browne (24). Total fluorescence intensities were determined by quantitative flow cytometry using an Epics C system (Coulter Electronics Inc.) equipped with a 5-watt argon ion laser operated at 200 miliwatts at a wavelength of 488 nm.

Assay for Plasmogen Activator—Plasmogen activator activator sized and secreted by F9 cells was assayed as described by Nishimune et al. (25). Briefly, F9 cells on gelatin-coated plastic dishes were exposed to various concentrations of trapoxin for 24 h and were then harvested using a rubber policeman, collected by centrifugation at 700 × g for 10 min, and washed once with phosphate-buffered saline. Cells were then suspended in 1 ml of ice-cold lysis buffer (10 mm Tris-HCl, 50 mm sodium bisulfite, 1% Triton X-100, 10 mm MgCl2, 8.6% sucrose, pH 6.5) and homogenized. The nuclei were collected by centrifugation at 500 × g for 10 min and washed three times with the lyase buffer and once with Tris-HCl, 15 mm EDTA (pH 7.4) successively. The pellet was suspended in 0.1 ml of ice-cold H2O, and concentrated H2SO4 was added to give a concentration of 0.4 N. After incubation at 4 °C for at least 1 h, the suspension was microcentrifuged for 5 min at 15,000 rpm, and the supernatant was mixed with 1 ml of acetone. After overnight incubation at −20 °C, the precipitated materials were collected by microcentrifugation. Protein was measured using a protein assay kit (Bio-Rad).

Acid Urea Triton Gel Electrophoresis—The level of acetylation of histones was analyzed by slab gel electrophoresis using acid urea Triton (AUT) gels (12). The procedure was modified by incorporating an upper gel (1 M acetic acid, 0.3 M urea, 4.4% acrylamide) onto the separating gel (1 M acetic acid, 8% urea, 0.5% Triton X-100, 45 mm NH4, 16% acrylamide). The histone solution was incubated with the same volume of loading buffer (7.4 M urea, 1.4 M NH4, 10 mm dithiothreitol) for 5 min and was then mixed with a 1:8 volume of 0.1% pyronin G in glacial acetic acid. They were electrophoresed in 0.2 M glycine and 1 M acetic acid. The gel was stained with Coomassie Brilliant Blue.

Assay of Histone Deacetylation—The mouse histone deacetylase was partially purified from FM3A cells as described previously (19). A buffer consisting of 50 mm potassium phosphate (pH 7.5), 10% glycerol, 1 mm EDTA, and 0.1 mm phenylmethylsulfonyl fluoride was used for dialysis and pretreatment of the enzyme preparation with inhibitors. Phenylmethylsulfonyl fluoride itself does not cause any effect on enzyme activity. To obtain [3H]acetyl-labeled histones as the substrate for the histone deacetylase assay, 2 × 106 cells of FM3A in 100 ml of ES medium were incubated with 0.5 μCi/ml [3H] acetate in the presence of 5 mM sodium butyrate for 30 min, and the labeled histone fraction was immediately extracted as described above. Radioactivity was determined using a liquid scintillation spectrometer (Aloka Co.). The specific activity was determined to be 1.2 μCi/mg histones. The assay for in vitro deacetylation of histones was basically according to that as proposed by Inoue and Fujimoto (3). For the standard assay, 10 μl of [3H]acetyl-labeled histones (15,000 cpm/11 μg) was added to 90 μl of the enzyme fraction, and the mixture was incubated at 37 °C for 10 min. The reaction was stopped by the addition of 10 μl of concentrated HCl. The released [3H]acetic acid was extracted with 1 ml of ethyl acetate, and 0.9 ml of the solvent layer was taken into 5 ml of toluene scintillation solution for determination of radioactivity.

RESULTS

Effect of Trapoxin on Histone Acetylation in Vivo—Trapoxin, a cyclic tetrapeptide (Fig. 1), was found as an agent that induces morphological normalization in v-sis-transformed NIH3T3 cells, whose activity was very similar to that of trichostatin A on the sis-transformed cells (20, 23). We previously demonstrated that trichostatin A was a potent and specific inhibitor of mammalian histone deacetylase. To examine the similarity between the molecular actions of these agents, we analyzed the effect of trapoxin on the intracellular level of histone acetylation. Fig. 2 shows the profiles of histones on AUT gel electrophoresis that separates each histone with different extents of acetylation due to slower migration rates of the acetylated species. Slower moving ladders of core histones, especially H3 and H4, were observed upon trapoxin treatment of all of the cell lines tested. In particular, the H4 band was followed by four additional bands corresponding to mono- di-, tri-, and tetra-acetylated forms, among which the tri- or tetra-acetylated form was predominant. On the other hand, no acetylation itself was detected in histone H1, which is known to be phosphorylated but not acetylated. These patterns in AUT gel electrophoresis were almost identical to those observed with trichostatin A treatment.

![Structure of trapoxin and trichostatin A](image-url)
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Histone Deacetylase Activity Is Inhibited by Trapoxin in Vitro—The level of histone acetylation is mainly controlled by the equilibrium between acetyltransferase and deacetylase. The most likely reason for the induction of histone hyperacetylation by trapoxin is the direct inhibition of histone deacetylase. This possibility was tested by analyzing the in vitro effect of trapoxin on the partially purified histone deacetylase from mouse FM3A cells. The enzymatic activity was monitored by measuring the amounts of [3H]acetic acid released from the 3H-acetylated histones as the substrate. As shown in Fig. 3A, the radioactivity was linearly released by the control enzyme during incubation at 37 °C within 10 min. The release of [3H]acetate was inhibited by the presence of trapoxin in the reaction mixture in a dose-dependent manner. However, the reaction rate in the presence of trapoxin was not linear, and the steady-state velocity was slower than that of the initial velocity (Fig. 3A), suggesting that trapoxin is classified into “slow binding” inhibitors (26). We next analyzed the effect of preincubation of the enzyme preparation with trapoxin at 4 °C on the inhibitory activity. The residual enzymatic activity after various lengths of preincubation time was determined by measuring the release of [3H]acetate from the labeled histones during 10 min. The inhibitory effect was enhanced by the prolonged preincubation of the enzyme preparation with trapoxin at 4 °C and reached a plateau by 1 h (Fig. 3B). Such enhancement of the inhibitory activity depending on the time of preincubation was not observed with trichostatin A.

Inhibition of Histone Deacetylase by Trapoxin Is Irreversible—We next tested the reversibility of the enzyme inhibition by trapoxin. The mouse histone deacetylase was incubated with 2 nM trapoxin or 10 nM trichostatin A for 6 h at 4 °C. The treated enzyme preparations were then dialyzed against a buffer consisting of 50 mM potassium phosphate (pH 7.5), 10% glycerol, 1 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride for 6 or 18 h, and their residual activities were determined as shown in Fig. 4. The activity of the control enzyme without pretreatment with drugs did not significantly decrease during dialysis. The residual enzymatic activity after the treatment with trichostatin A increased gradually during dialysis and reached to about 60% of the initial enzyme activity within 18 h. On the other hand, the enzyme treated with trapoxin no longer recovered its activity even after dialysis for 18 h. These results suggest that the effect of trapoxin is irreversible and that the mechanism of enzyme inhibition by trapoxin is different from that by trichostatin A. This was confirmed by the kinetic analyses as shown in Fig. 5. The EV plots of initial velocity versus relative enzyme concentration showed that the activity of control enzyme increased almost proportionally to the increased amounts of the enzyme added. The addition of 10 nM trichostatin A into the enzyme preparation dramatically reduced the initial ve-

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**Fig. 2.** Effect of trapoxin on histone acetylation in various cell lines. Cells were incubated without (−) or with (+) 100 nM trapoxin for 6 h. Each histone fraction was extracted and analyzed on an AUT gel as described under “Experimental Procedures.” The gel was stained with Coomassie Brilliant Blue. 3Y1, a normal fibroblast cell line cloned from Fisher rat embryo; FM3A, a mouse mammary tumor cell line; F9, a mouse teratocarcinoma cell line; MEL, a mouse erythroleukemia cell line; and HeLa, an epithelia-like carcinoma cell line from human cervix.

**Fig. 3.** Effect of trapoxin on activity of partially purified mouse histone deacetylase. Nuclear histone deacetylase was partially purified from cell extracts of mouse FM3A cells, and its enzymatic activity was determined as described under “Experimental Procedures.” A shows progress curves for the inhibition of histone deacetylase. Various concentrations of trapoxin were added to the enzyme preparation simultaneously with 3H-acetylated histones, and the reaction mixtures were incubated at 37 °C. The amount of [3H]acetic acid released from the labeled acetylated histones was determined at the indicated times. Trapoxin added was 0 (○), 1 (▲), and 10 (■) nM. B shows the effect of preincubation with trapoxin on its inhibitory activity. The enzyme preparation was pretreated with various concentrations of trapoxin or trichostatin A at 4 °C, and the residual activity was determined at the indicated times by measuring the amount of [3H]acetic acid released during the incubation for 10 min at 37 °C. Trapoxin added was 0 (○), 2 (▲), and 10 (■) nM. Trichostatin A added was 10 nM (●).
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**FIG. 4.** Reversibility of enzyme inhibition by trapoxin. The partially purified histone deacetylase preparation was pretreated with 0.01% dimethyl sulfoxide (Control), 2 nM trapoxin, and 10 nM trichostatin A for 6 h. The treated enzyme preparations were dialyzed against drug-free buffer, and the residual enzyme activity was determined at the indicated times. The open, hatched, and closed bars represent 0, 6, and 18 h of dialysis, respectively.

**FIG. 5.** Kinetic analysis of inhibition by trapoxin of mouse histone deacetylase. Radioactivities released from the labeled histone substrate (15,000 cpm) by the enzyme activities of various concentrations of partially purified histone deacetylase preparation in the presence or absence of 2 nM trapoxin were determined. The data, together with those obtained by a similar experiment using trichostatin A (10 nM), are presented as an EV plot of initial velocity versus relative enzyme concentration.

Locality, but the residual activity still increased proportionally as the enzyme concentration increased, indicating that trichostatin A inhibits the enzyme activity at a constant ratio at each enzyme concentration. This was consistent with the previously described result that trichostatin A was a noncompetitive inhibitor with the $K_I$ value of about 3 nM (21). In contrast, 2 nM trapoxin caused reduction of the enzyme activity by a fixed extent irrespective of the enzyme concentrations, leading to the parallel shift of the EV plot line from the control line toward the right. This result suggests that a fixed amount of the histone deacetylase corresponding to the amount of trapoxin was irreversibly inactivated.

**An Epoxide Group in Trapoxin Is Essential for Enzyme Inhibition**—The above results suggest that trapoxin inhibits the enzyme activity by binding tightly to histone deacetylase. Since only the epoxide group in the chemical structure of trapoxin is a chemically reactive moiety (Fig. 1), this moiety seems likely to play a role in forming a covalent bond between trapoxin and the enzyme molecule. We therefore conducted reduction of the epoxide group in trapoxin and analyzed the inhibitory activity of the reduced product. The reduced form of trapoxin, cyclo-(L-phenylalanyl-L-phenylalanyl-D-pipelicolinyl-2-aminol-8-oxo-10-hydroxydecanoyl), was obtained by reaction with NaBH$_4$, and its effects on histone acetylation in vivo and on the enzymatic activity of the histone deacetylase in vitro were examined (Fig. 6). Highly acetylated histone H4 was not detected in the mouse FM3A cells treated with the reduced trapoxin for 6 h in contrast to the hyperacetylation in the trapoxin-treated cells. The reduced trapoxin did not cause any inhibitory effect on the partially purified histone deacetylase even at a high concentration (100 nM). These results strongly suggest that trapoxin inactivates the histone deacetylase by binding the enzyme via its epoxide group.

**Cross-resistance to Trapoxin of Trichostatin A-resistant Mutant Cell Line**—We previously isolated a mutant cell line of

**FIG. 6.** The role of an epoxide group in inhibitory activity of trapoxin. The reduced form of trapoxin was obtained according to the procedure described under “Experimental Procedures,” and its effects on histone acetylation in vivo and on the deacetylase activity in vitro were examined. Panel A shows the chemical structures of native and reduced forms of trapoxin. Panel B, FM3A cells were treated with various concentrations of reduced trapoxin for 6 h, and the level of histone H4 acetylation was compared with that of trapoxin-treated cells on an AUT gel. Panel C, the partially purified histone deacetylase preparation was treated with various concentrations of native and reduced trapoxin for 6 h, and the residual enzyme activities were determined.
FM3A showing resistance to trichostatin A in which the histone deacetylase was resistant to trichostatin A (21). This mutant cell line (TR303) did not show the phenotype of multidrug resistance, but it showed the phenotype of trichostatin A-specific resistance, which was attributed to a mutation in the histone deacetylase itself. As shown in Table I, the minimal inhibitory concentration of trapoxin for cell growth of the mutant cells was 25-fold higher than that for wild-type cells. On the other hand, the cytotoxicity of other antitumor drugs to the mutant was essentially the same as that to wild type. Cross-resistance of the mutant cell line to trapoxin suggests that an alteration of the enzyme structure by the mutation causes the altered sensitivity to both trichostatin A and trapoxin.

**Trapoxin Causes Arrest of the Cell Cycle in 3Y1 Fibroblasts and Induction of Plasminogen Activator Synthesis in F9 Teratocarcinoma Cells**—We reported that trichostatin A induced specific G1 and G2 arrest in the cell cycle of rat 3Y1 fibroblasts (19) and induction of plasminogen activator synthesis in F9 teratocarcinoma cells (27). Similar effects were also reported with n-butyrate, a nonspecific and weak inhibitor of the histone deacetylase (14, 25). If histone acetylation does play a crucial role in the cell cycle control and differential gene expression, trapoxin should also show similar activities. The effects of trapoxin (10 nM) on the cell cycle of rat 3Y1 fibroblasts were analyzed by measuring the DNA content distribution in the cells treated with trapoxin for 24 h (Fig. 7). When the cells arrested at G0 phase by serum starvation were transferred to the fresh medium containing 12% serum, proliferation of the cells occurred synchronously through G1 into S phase. However, the culture treated with trapoxin at 0 h showed no change in the distribution pattern of DNA at 24 h, indicating that trapoxin inhibits the progression of the cell cycle from G0 to S (Fig. 7A). To analyze the effect of trapoxin on the progression through S, G2, and M, we next used a synchronous culture from early S phase. Cells treated with hydroxyurea are arrested at early S phase; removal of the agent causes resumption of the cell cycle synchronously. When cells in the synchronous culture were treated with trapoxin at 0 h, most of the cells were accumulated as the 4C DNA cells within 24 h (Fig. 7B). The absence of M phase cell under phase-contrast microscopy indicated that these cells were arrested at the G2 phase (data not shown). The effect of trapoxin on the cell cycle progression in a randomly growing culture was also analyzed using flow cytometry. The random culture before addition of trapoxin showed a typical profile of DNA content distribution in which the broad peak of the S phase cells intervened between 2C and 4C peaks representing the G1 and G2/M phases. Exposure of the culture to trapoxin caused considerable accumulation of cells at both G1 and G2 phases and a decrease in the number of S phase cells (Fig. 7C). These results clearly indicate that the cell cycle of fibroblast cells was arrested by trapoxin in both G1 and G2 phases.

We next analyzed the effects of trapoxin on mouse teratocarcinoma F9 cells. When F9 cells were treated with 10 nM trapoxin for 24 h, dramatic morphological changes were observed as shown in Fig. 8A. To confirm induced production of plasminogen activator, a generally accepted marker of endoderm cells, in the trapoxin-treated F9 cells, we overlaid the treated cells with a medium containing skim milk and plasminogen. As shown in Fig. 8B, halo-forming colonies were detected in the trapoxin-treated cultures, and more than 80% of the colonies produced plasminogen activator at 100 nM of trapoxin. On the other hand, no halo was formed in the control cultures without the agent or with the agent but without plasminogen.

### Table I

| Drug           | Minimal inhibitory concentration (ng/ml) |
|----------------|------------------------------------------|
|                | FM3A | TR303 |
| Trichostatin A | 20   | 800   |
| Trapoxin       | 2    | 50    |
| Adriamycin     | 1000 | 1000  |
| Vinblastine    | 10   | 5     |
| Colcemid       | 10   | 10    |

**Fig. 7. Effect of trapoxin on the cell cycle progression in 3Y1 fibroblast cells.** Rat normal embryonic fibroblast 3Y1 cells were synchronized at G0 (A) and early S (B) by serum starvation and treatment with hydroxyurea, respectively. The effect of trapoxin on the cell cycle progression in each synchronous culture was determined by measuring the DNA content distribution using flow cytometry after treatment for 24 h. The effect on the DNA distribution pattern in the exponentially growing culture was also determined (C).

**Discussion**

This paper describes a new additional member of inhibitors of mammalian histone deacetylase, trapoxin, whose chemical structure is totally different from those of the other known inhibitors, trichostatin A and n-butyrate. The most characteristic property of trapoxin is the irreversible inhibition. Present results strongly suggest that trapoxin binds covalently to the histone deacetylase via its epoxide moiety. However, it also seems possible that the binding is not covalent and the slow dissociation rate from enzyme-inhibitor complex.
results in apparent enzyme inactivation, since no kinetic distinction can be made between inactivation and tight binding inhibition. In fact, the progress curves of the enzyme reaction with trapoxin suggest that trapoxin is classified into slow binding inhibitors (Fig. 3), some of which are the tight binding inhibitors (26). A binding assay using labeled trapoxin would be necessary for determining whether the irreversible inhibition by trapoxin is really due to covalent binding. In contrast, trichostatin A shows reversible inhibition with the $K_i$ value of 3.4 nM (21), and pretreatment is not necessary for inhibition. Thus, we now possess a set of potent inhibitors of the histone deacetylase with a different mode of action, which will be very useful tools for the analysis of the mechanism of the enzyme reaction.

The trichostatin A-resistant mutant cells that possess the mutated histone deacetylase showed cross-resistance to trapoxin (Table I). This finding indicates that the inhibition of histone deacetylase is the primary reason for the inhibition of cell proliferation by not only trichostatin A but also trapoxin. It seems probable that the gross conformational change of the mutated enzyme molecule affects both of the affinities at the different binding sites.

The biological role of histone acetylation is still unclear. In the present study, we demonstrated that trapoxin induced specific arrest of the cell cycle of rat 3Y1 fibroblasts in both G$_1$ and G$_2$ phases and morphological and biochemical changes in the phenotypes of F9 teratocarcinoma cells, which are very similar to differentiation to endoderm cells. These effects have been commonly observed with trichostatin A (19, 27) and n-butyrate (14, 25). It was also reported that the reversion of the transformed phenotype to the normal one was induced in sis oncogene-transformed fibroblasts by the treatment with trapoxin (23), trichostatin A (20), and n-butyrate. These results strongly suggest that all biological effects at least commonly induced by three different inhibitors, trapoxin, trichostatin A, and n-butyrate, are the direct results of the accumulation of hyperacetylated histones due to inhibition of histone deacetylase.

Various lines of experimental evidence have shown that chromatin preparations that are enriched in transcribed genes contain highly acetylated histones and that acetylated chromatin is enriched in transcriptionally active genes (5, 6, 28). For example, experiments using Hg-agarose affinity chromatography revealed that DNA sequences of active genes were copurified with highly acetylated histones (5, 29). Tazi and Bird (7) showed that short chromatin fragments enriched in CpG-rich DNA that were frequently found upstream from many genes contained highly acetylated histones. Thus, the intimate correlation between transcriptional activity and increased histone acetylation is established. However, whether n-butyrate causes transcriptional activation through the induced acetylation of histones is still controversial. An enhancer-dependent increase in gene expression was observed in butyrate-treated cells that had been transfected with recombiant plasmids (30), and the effect was further enhanced by the presence of scaffold-attached regions adjacent to the reporter gene (31). On the other hand, steroid hormone-dependent activation of genes, such as induction of egg white mRNA in chick oviduct, was inhibited by n-butyrate (32, 33). Experiments using n-butyrate have to be interpreted with caution because of the pleiotropic effects of this drug. Therefore, the use of trapoxin or trichostatin A or both seems to be important for obtaining the conclusive results in these experiments.

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