Depletion of the erbB-2 Gene Product p185 by Benzoquinoid Ansamycins

Penny Miller, Catherine DiOrio, Mikel Moyer, Rodney C. Schnur, Arthur Bruskin, Walter Cullen, and James D. Moyer


ABSTRACT

Herbimycin A, a benzoquinoid ansamycin antibiotic, was originally isolated on the basis of its herbicidal activity (1) and subsequently reinsolated on the basis of its ability to revert to normal the morphology of Rous sarcoma virus-transformed fibroblasts (2). Herbimycin A is closely related in structure to two other benzoquinoid ansamycins, geldanamycin (3) and mabecin (4). Analogues of herbimycin A and geldanamycin have been prepared and shown to be active in cellular growth inhibition assays and murine tumor models (5–7).

Herbimycin A inhibits the growth of fibroblasts transformed by a variety of oncogenes encoding protein tyrosine kinases such as src, yes, fps, or erbB1 (2, 8, 9) and also reverts the morphology of the cells from “transformed” to “normal” (2, 8, 9). The mechanism of this effect is unclear but may result from a reduction in the level of the tyrosine kinases as well as direct inhibition of the tyrosine kinase reaction. The activity of src kinase in lysates of cells treated with herbimycin was reduced by 50% (2), probably due at least in part to an increase in turnover of pp60src (10), and was reduced to an even greater extent in human colon tumor cells (11). Similarly, the level of lck tyrosine kinase in T-cells was decreased 68% after overnight incubation of the cells with 1 μM herbimycin A (12). Although several reports conclude that herbimycin A does not directly inhibit the tyrosine kinase reaction (2, 12), this conclusion is called into question by a report that reducing agents present in the commonly used kinase reaction mixtures may directly inactivate herbimycin A (13, 14). Thus, it remains possible that some of the effects of herbimycin may result from its direct inhibition of the tyrosine kinase reaction, and inactivation of tyrosine kinases by direct alkylation of enzyme sulfhydryl groups by herbimycin A has been proposed as a mechanism of this inhibition (15). Although the mechanism is unclear, herbimycin (0.88 μM) reduces protein phosphotyrosine levels in treated cells transformed by src, ros, or erbB oncogenes (8, 10), and pretreatment with herbimycin prevents the increase in phosphotyrosine of T-cells exposed to anti-CD3 monoclonal antibody (12). Thus, herbimycin A appears to have an effect on a number of protein tyrosine kinases and has been widely used as a tyrosine kinase inhibitor, but its mechanism of action remains unclear.

Herbimycin A has been reported to have antiangiogenic effects (16), to inhibit c-myc expression (17), to increase cellular fibronectin expression (18), and to inhibit the association of p60 src with the cytoskeleton (19). The relationship of these effects, if any, to the inhibition of tyrosine kinases remains unclear. Herbimycin also induces the differentiation of K562 human myelogenous leukemia cells, possibly by inhibiting the abl tyrosine kinase that is strongly implicated in this disease (20, 21), and induces differentiation and cell death in several cell lines of neural origin by a mechanism that may be unrelated to inhibition of tyrosine kinases (22, 23). Herbimycin A, geldanamycin, and several analogues have been shown to have antitumor effects in vivo, although the relation of these effects to the inhibition of tyrosine kinases is unclear (5, 23–25). A better understanding of the mechanism of benzoquinoid ansamycin action is necessary to assess the potential of these compounds as antitumor agents.

erbB2 encodes a Mr 185,000 receptor-like protein (p185) with tyrosine kinase activity and a high degree of homology to the epidermal growth factor receptor (26). erbB2 is overexpressed in many breast tumors and is associated with a poor prognosis (27). Transfection of breast epithelial cells with erbB2 transforms these cells (28), and growth of some breast cancer cells can be inhibited by antibodies to p185 (29), suggesting that inhibitors of p185 may be of value in the treatment of breast cancer. As part of a program seeking inhibitors of erbB2, we have examined the effects of herbimycin A and related benzoquinoid ansamycins on p185 in breast cancer cells and report here that these compounds rapidly deplete p185 levels.

MATERIALS AND METHODS

Chemicals. Herbimycin A (Fig. 1) and geldanamycin were prepared by fermentation from Streptomyces as described previously (1, 3). Geldanamycin analogues CP127374, CP144365, and CP168517 were prepared by methods to be reported elsewhere. [3H]Uridine and L-[3H]Valine were from DuPont New England Nuclear (Boston, MA). L-[35S]Methionine was from Amersham (Arlington Heights, IL).

Cell Culture. SKBr3 and BT474 human breast carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in McCoy’s modified 5A medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Four Rat-1 fibroblast lines were obtained from Oncogene Science, Inc. (Uniondale, NY). Rat-1 1173 and 714 are sibling clones of the 1174 and 711 lines, respectively, described by Maier et al. (30). Rat-1 9–24c and 10–24k are described in the same reference. Rat cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Immunoblotting. Cells were seeded in 6-well plates at 5 × 10^4 cells/well. On the following day, 0.25% dimethyl sulfoxide (vehicle) or a compound was

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added and cells were incubated at 37°C. After 6 h, the cells were washed twice with 10 mM Tris, pH 7.4-150 mM NaCl-4.5 mM KCl-0.5 mM Na3VO4 and lysed in 5 mM hot sample buffer (60 mM Tris, pH 6.8-2% sodium dodecyl sulfate-10% glycerol-0.02% bromophenol blue), and the lysate was placed in a boiling bath for 5 min. Proteins were resolved by electrophoresis on 4—20% polyacrylamide gels (Integrated Separation Systems) and transferred to Immobilon polyvinylidene difluoride membranes with a Minifold Manifold ( Hoefer). Membranes were blocked for 1 h in blocking buffer (10 mM Tris, pH 7.4-150 mM NaCl-5% bovine serum albumin-1% ovalbumin) and then incubated for 2 h with the primary antibody diluted in blocking buffer. Primary antibodies included anti-phosphotyrosine monoclonal antibody PY69 (ICN Radiochemicals, Irvine, CA), 1 μg/ml; a polyclonal antibody to the carboxy terminus of p185, NT1 (Oncogene Science), 1:1000; a polyclonal antibody to the kinase domain of p185, AB-1 (Oncogene Science), 5 μg/ml; and a monoclonal antibody to the external domain of p185, OD3 (DuPont New England Nuclear), 5 μg/ml. Membranes were washed twice in rinse buffer (10 mM Tris, pH 7.4-150 mM NaCl) once in rinse buffer plus 0.05% Triton X-100, and twice more in rinse buffer. All washes were for 10 min in a volume of 200 ml. Membranes were next incubated with 1 μCi/ml 125I-goat anti-mouse IgG (ICN), 125I-protein A (ICN), or 125I-goat anti-mouse IgM (DuPont New England Nuclear) in blocking buffer for 30 min, washed as described above, dried, and exposed to film. Quantitation was by scintillation counting of excised gel bands or densitometric scanning of autoradiograms. Alternatively, p185 immunoblotting was performed with a monoclonal antibody to a Mr 67,000 polypeptide from the carboxy terminus of p185 (Molecular Oncology, Gaithersburg, MD) and a horseradish peroxidase-conjugated goat anti-mouse IgG (Boehringer Mannheim). Bound secondary antibody was detected by enhanced chemiluminescence (Amersham). IC50 values for the reduction of p185 were determined by immunoblotting with NT1 antibody as described above, except that cellular proteins were immobilized by filtration onto Immobilon polyvinylidene difluoride membranes with a Minifold Manifold (Schleicher and Schuell, Keene, NH) without prior separation of proteins by electrophoresis.

**ELISA.** Rat-1 cells were seeded and incubated with compound as described for immunoblotting. Following this treatment, cells were washed twice with Hanks' balanced salt solution and lysed with 0.5 ml 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5-150 mM NaCl-1.5 mM CaCl2-1 mM [ethylenebis(oxyethylenenitrilo)tetraacetic acid-1% glycero1-1% Triton X-100-1 mM Na3VO4-10 mM NaF-10 μg/ml leupeptin-25 μg/ml soybean trypsin inhibitor-50 mg/ml phenylmethylsulfonyl fluoride. Lysates were analyzed for p185 protein content using the protocol and reagents contained in a new ELISA kit (Oncogene Science).

**Separation and Visualization of SKBr3 Glycoproteins.** SKBr3 cells were seeded in 10-cm plates (3 x 105 cells/plate) supplemented with 50 μCi/ml of [35S]methionine and incubated for 17 h. The medium was removed by aspiration, and the cells were washed twice with phosphate-buffered saline. The cells were harvested by the addition of 1 ml of a solution of 125 mM Tris-HCl, pH 7.4-375 mM NaCl-25% glycerol-2.5 mM [ethylenebis(oxyethylenenitrilo)tetraacetic acid-2.5% Triton X-100-125 mM NaF, followed by gentle scraping and storage at —70°C. Wheat germ lectin Sepharose 6MB beads (Pharmacia LKB) were prepared by dispensing 200 μl of a 50% suspension into 15-ml tubes and washing twice with rinse buffer (50 mM Tris-HCl, pH 7.4-150 mM NaCl-0.2% Triton X-100-10 mM NaF-0.1 mM sodium o-vanadate). The washed beads were diluted with 1.9 ml rinse buffer, 600 μl of cell lysate were added, and this suspension was incubated in a rotating mixer at 4°C for 1 h. The beads were then collected by centrifugation and washed 3 times with rinse buffer. Glycoproteins were eluted with 400 μl rinse buffer containing 0.5 M N-acetyl-d-glucosamine. Equal portions of protein from each sample were separated by gel electrophoresis, and duplicate gels were analyzed by autoradiography and Western blotting as described above.

**Measurement of Protein, RNA, and DNA Synthesis, ATP Pools, and erbB2 mRNA Level.** Total protein synthesis was measured by [3H]valine incorporation. [3H]Valine was added to the medium for the last 3 h of incubation with herbimycin A. In some samples, 10 μg/ml cycloheximide was used in place of herbimycin A as a positive control for the inhibition of protein synthesis. erbB2 mRNA levels were determined by dot blots of total RNA extracts, probed with a [32P]-labeled 40-mer from the fourth erbB2 exon (Oncogene Science). Total RNA synthesis was measured by 5-3H]uridine incorporation. [3H]Uridine was added to the medium for the last hour of incubation with herbimycin A. Actinomycin D (5 μg/ml) as a positive control inhibited RNA synthesis by 73% under the conditions used. ATP and ADP levels were determined by high pressure liquid chromatography of trichloroacetic acid extracts of cells as described previously (31).

**RESULTS**

SKBr3 human breast cancer cells express high levels of the erbB2 gene product p185 which has a basal level of tyrosine phosphorylation attributable to autophosphorylation. Exposure of SKBr3 cells to 0.35 μM herbimycin A produced a rapid and nearly complete depletion of the tyrosine phosphorylation of p185 and a somewhat delayed depletion of the p185 protein itself (Fig. 2). Greater than 50% depletion of p185 occurs within 3 h of exposure to herbimycin A. The depletion of p185 protein was documented by Western blotting with antibodies to the COOH-terminal (Fig. 2B), extracellular (Fig. 2C), and kinase (Ab-1; data not shown) domains. The loss of the p185 protein was also confirmed with a commercial sandwich ELISA (Oncogene Science) that captures and quantitates p185 with monoclonal antibodies to the p185 external domain which react with distinct epitopes (data not shown).

A similar reduction of p185 protein on exposure to herbimycin A was observed in BT474, another breast cancer line that overexpresses p185. The depletion of p185 was time and concentration dependent with an IC50 of ~0.1 μM evaluated at 6 h of exposure (Fig. 3). The related benzoquinoid ansamycin, gebdanamycin, produced a similar depletion of the p185 protein itself (Fig. 2). Greater than 50% depletion of p185 in these cells (data not shown). Reduction of the p185 protein and phosphotyrosine by herbimycin A and 17-allylaminodendamethoxygeldanamycin (CP127374) was also observed in B104—1, a mouse fibroblast line expressing an activated form of the erbB2 gene product p185 which has a basal level of tyrosine phosphorylation attributable to autophosphorylation. Exposure of SKBr3 cells to 0.35 μM herbimycin A produced a rapid and nearly complete depletion of the tyrosine phosphorylation of p185 and a somewhat delayed depletion of the p185 protein itself (Fig. 2). Greater than 50% depletion of p185 occurs within 3 h of exposure to herbimycin A. The depletion of p185 protein was documented by Western blotting with antibodies to the COOH-terminal (Fig. 2B), extracellular (Fig. 2C), and kinase (Ab-1; data not shown) domains. The loss of the p185 protein was also confirmed with a commercial sandwich ELISA (Oncogene Science) that captures and quantitates p185 with monoclonal antibodies to the p185 external domain which react with distinct epitopes (data not shown).

The effects of benzoquinoid ansamycins on p185 were also studied using MDA MB 453, another breast cancer line that overexpresses p185, and Rat-1 714, a rat fibroblast line transfected with human erbB2 activated by a point mutation in the transmembrane domain (30). In these cell lines, p185 phosphotyrosine and protein levels declined simultaneously on exposure to 0.17 μM CP127374 (Fig. 4). These effects are not specific to CP127374 because the related benzoquinoid ansamycins, gebdanamycin, produced a similar depletion of the p185 protein in these cells (data not shown). This effect of the benzoquinoid ansamycins is therefore not restricted to breast cancer cells.

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Fig. 2. Depletion of p185 as measured by Western blotting. SKBr3 cells in culture were exposed to 0.35 μM herbimycin A for the indicated time and then extracted as described in "Materials and Methods." A, p185 phosphotyrosine levels measured by immunoblotting with monoclonal antibody PY69; B, p185 protein levels were measured using NT1, a polyclonal antibody to the carboxy terminus of p185; C, p185 protein levels were also measured using OD3, a monoclonal antibody to the external domain of p185. Ordinate, molecular weights of protein standards in thousands.

Herbimycin A contains a p-benzoquinone structure as part of its macrolide ring, and it has been suggested that this moiety alkylates key sulfhydrols of the tyrosine kinases (14, 15). Therefore, we examined the effects of p-benzoquinone and found that at high concentrations it depleted p185 tyrosine phosphorylation and the p185 protein in SKBr3 cells (Fig. 5). It is therefore likely that the benzoquinone nucleus is important for the reduction of p185 by herbimycin A, but the rest of the molecule provides a >100-fold increase in potency over benzoquinone alone. Some modifications of the benzoquinone moiety are well tolerated. For example, amino substitution at position 17 of geldanamycin as in CP127374 afforded a compound with ~3-fold greater potency (Fig. 6). However, reduction of the quinone to the hydroquinone resulted in decreased potency (data not shown). Since these two forms may be interconvertible in culture, we sought to determine which form is active by locking the hydroquinone moiety in its reduced state through acylation. This modification found in CP168157 led to loss of activity (Fig. 6). The quinone moiety is not the only essential moiety for activity, however, because removal of the carbamyl group at position 7 of geldanamycin resulted in loss of activity (Fig. 6), as does reduction to 2,3,4,5-tetrahydrogeldanamycin (IC50 >3500 nM). These data indicate that the reduction of p185 levels by herbimycin A and geldanamycin at submicromolar concentrations decreased tyrosine phosphorylation of p185 is apparent prior to reduction in p185 protein.

Fig. 3. Potency of herbimycin A in the depletion of p185. BT474 and SKBr3 cells were treated for 6 h with the indicated concentrations of herbimycin A. Extracts of the cells were separated by electrophoresis, and p185 was measured by immunoblotting with polyclonal antibody NT1. There was no significant change in p185 protein in the control cultures without herbimycin during this time.

Fig. 4. Effect of CP127374 on p185. MDA MB 453 cells (A) or Rat-1 714 cells (B) were incubated with 0.27 μM CP127374 for the indicated times at 37°C, lysed in hot sample buffer, and analyzed for p185 protein and phosphotyrosine (PY) by immunoblotting with polyclonal antibody NT1 and monoclonal antibody PY69, respectively.
BENZOQUINOID ANSAMYCIN DEPLETION OF p185

results from a specific interaction involving several regions of this inhibitor, rather than simply the chemical reactivity of the quinone moiety.

The effect on p185 was not simply a consequence of cytotoxicity because SKBr3 cell morphology, as well as viability assessed by trypan blue permeability, was not affected after a 6-h exposure to herbimycin A. In order to understand the basis for the loss of p185 in cells treated with benzoquinoid ansamycins we examined several aspects of cellular metabolism. Cellular RNA and protein synthesis, ATP pools, and gross metabolism as measured by ATP/ADP ratios were all near normal after a 6-h exposure to 0.35 μM herbimycin A, at which time p185 is substantially depleted (Table 1). Furthermore, erbB2 mRNA levels were not decreased at this time. A near complete (93%) inhibition of protein synthesis with cycloheximide (10 μg/ml) lowered p185 levels in SKBr3 cells only 14% in 6 h (data not shown), in agreement with reports that p185 has a half-life of 7 h (33). The loss of p185 therefore cannot be attributed to a general effect on ATP availability, protein synthesis, or erbB2 transcription but appears to result from an increased rate of degradation of p185.

The specificity of the benzoquinoid ansamycins for depletion of p185 was examined by measuring their effect on 35S-labeled cell glycoproteins. Treatment of SKBR3 cells with 0.4 μM CP127374 for 6 h depleted p185 by 95%, whereas the other major cell glycoproteins

Fig. 5. Effect of benzoquinone on p185. SKBr3 cells were treated for 6 h with the indicated concentrations of p-benzoquinone. Extracts of the cells were separated by electrophoresis and p185 (erbB2) protein and phosphotyrosine (pTyr) levels were measured by immunoblotting with the appropriate antibodies.

Fig. 6. Structure activity relationships of geldanamycins. The IC50 values were determined by measurement of p185 concentrations of SKBr3 cells after a 6-h exposure to compound (CP) as described in "Materials and Methods."
Table 1  Effects of herbimycin A on SKBr3 cells
Treatments were for 6 h with 0.35 μM herbimycin A unless noted otherwise. All measurements were made as described in "Materials and Methods.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% of control</th>
</tr>
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<tbody>
<tr>
<td>p185 protein</td>
<td>35</td>
</tr>
<tr>
<td>p185 tyrosine phosphorylation</td>
<td>5</td>
</tr>
<tr>
<td>erbB2 mRNA</td>
<td>130</td>
</tr>
<tr>
<td>Protein synthesis</td>
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</tr>
<tr>
<td>RNA synthesis (1.75 μM)</td>
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<tr>
<td>ATP/ADP</td>
<td>108</td>
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<tr>
<td>ATP</td>
<td>99</td>
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were largely unaffected (Fig. 7A). The only conspicuous change in the pattern of proteins seen after wheat germ lectin affinity purification and electrophoresis was a decreased band at Mr ~180,000 that may be p185 itself. This indicates that under conditions of near complete depletion of p185, most other major cellular glycoproteins are unaffected.

Further evidence for selectivity in the depletion of p185 by the benzoquinoid ansamycins was obtained by the use of cells transfected with genetically engineered mutant forms of erbB2. Four clones of Rat-1 cells transfected with distinct forms of erbB2, engineered as described by Maier et al. (30), were examined in these experiments. The structures of the proteins encoded by the transfected genes are described in Fig. 8A. The lines express either normal human c-erbB2 (line 1173), mutant erbB2 with an activating point mutation in the transmembrane domain (line 714), erbB2 lacking amino acids 751–979 in the kinase domain (line 9-24C), or erbB2 truncated at amino acid 684 and therefore lacking nearly all the intracellular domain (line 10-24K). Although the latter proteins lack tyrosine kinase activity, they were shown to localize to the membrane and to be endocytosed in response to anti-p185 antibody in a manner similar to that of the wild-type c-erbB2 product. Together these lines allowed us to examine the requirement for the kinase domain in the action of the benzoquinoid ansamycins.

On treatment with CP127374, the cell lines expressing p185 with an intact kinase domain lose p185 within 6 h, whereas both lines lacking the kinase domain of p185 continue to express the respective mutant erbB2 gene products (Fig. 8). These measurements were made with the p185 ELISA kit (Oncogene Sciences) that uses antibodies to the external domain of p185. These epitopes are conserved in all the mutant forms of p185 expressed in these Rat-1 cells. Similar experiments with the 1173 and 9–24C lines, in which Western blotting with an antibody to the COOH-terminal polypeptide was used for the measurements, confirmed the findings with the ELISA assay (data not shown).
DISCUSSION

The identification of erbB2 as a transforming gene for breast epithelial cells has stimulated efforts to identify inhibitors of p185 for evaluation as antitumor agents. We report here that herbimycin A, a compound used widely as a "tyrosine kinase inhibitor," not only reduces the activity of p185 tyrosine kinase in cells but also depletes the cell of the p185 protein.

We have attempted to demonstrate direct inhibition of p185 kinase activity in vitro by incubation of ansamycins with p185 immune complexes prepared from SKBr3 extracts and by incubation of ansamycins with SKBr3 membranes, followed by incubation with ATP and measurement of p185 autophosphorylation. Although others have reported that isolated v-src kinase is inhibited by herbimycin A in vitro if reducing agents are avoided in the reaction buffer, we observed partial inhibition of p185 autophosphorylation only at concentrations far exceeding (~100-fold) those shown to reduce p185-associated phosphotyrosine and protein in cells. This evidence supports our conclusion that the primary action of ansamycins in cells is not direct inhibition of tyrosine kinases.

Specific structure activity relationships were observed in the ansamycins for their potency in the reduction of p185 levels of cells. Potency was increased by replacement of the 17-methoxy group by the allyl amino group (Fig. 6). The amino group is known to destabilize the hydroquinone redox state versus the quinone form (34). Thus, the greater potency seen with CP127374 may be the result of preservation of the quinone moiety in culture. The results with p-benzoquinone, CP168157, and geldanamycin hydroquinone suggest that the quinone substructure is required for p185 inhibition. Activity is not altered by methoxy substitution at positions 11 and 15, as seen by the comparison of activity of geldanamycin with herbimycin A. Finally, the carbamate substructure, missing in CP144365, is essential for the activity of ansamycins. Thus, while it has been observed that the ansamycins produce multiple responses in cells, their potency for the reduction of p185 is markedly changed by subtle structural changes, suggesting that a specific cellular receptor mediates this effect.

The loss of p185 occurs within 3 h (Fig. 2) and reflects an increase in p185 protein degradation rather than an effect on synthesis, because protein and RNA synthesis and steady state erbB2 mRNA levels are not substantially reduced by herbimycin during this time (Table 1). Furthermore, the loss of p185 occurs much faster than the normal turnover of the protein under conditions of complete inhibition of protein synthesis. The increased rate of turnover is selective for a small subset of proteins because most cellular glycoproteins are not depleted (Table 1). Previous reports have shown that other protein tyrosine kinases, such as pp59α, pp56κ, and pp60+*, are also depleted on treatment with herbimycin A (10–12). Taken together, these results indicate that benzoquinoid ansamycins initiate a selective degradation of some tyrosine kinases or alternatively of proteins containing tyrosine phosphate residues.

To further identify the basis for the selective loss of p185, we examined cells expressing an activated mutant form and two kinase-negative mutants with deletions of internal domains. Our results (Fig. 8) show that the kinase domain is necessary for depletion by the benzoquinoid ansamycins. At present we have not established whether a portion of the deleted sequence (amino acids 751–979) is required or if the requirement is simply for tyrosine phosphorylation. A more precise definition of the sequence required for ansamycin action would be of interest. Mutants with smaller deletions spanning the kinase domain as well as point mutants would enable us to address this question.

It will be of interest to determine whether the process by which the benzoquinoid ansamycins deplete tyrosine kinase levels is operative under physiological conditions as a mechanism of regulation.

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