Potentiation of DNA Damage and Cytotoxicity by Calmodulin Antagonists

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Many physical and chemical agents used in anti-neoplastic therapy are known to act by effecting DNA damage. The possibility that calmodulin may be an important modulator of the response to DNA damage in eukaryotic cells has been explored in a number of studies which use calmodulin antagonists in combination with agents known to damage DNA. We review these studies and discuss the therapeutic potential of calmodulin antagonists in combination therapy.

Many physical and chemical anti-neoplastic agents are known to produce their therapeutic effects by damaging DNA. The potential to enhance therapeutic efficacy by the potentiation of DNA damage, either directly or via the inhibition of DNA repair processes, suggests novel opportunities for the development of anti-neoplastic regimens in more effective treatment of patients with cancer. Recent research using calmodulin antagonists has suggested that calmodulin may be important in the damage and repair of DNA. We reviewed the data examining the interactions of calmodulin antagonists with agents known to induce DNA damage.

In recent years the existence of a calcium messenger system as a fundamental means of regulating cellular functions has been well established. The large gradient between extracellular Ca++ concentrations (1 mM) and intracellular free calcium concentration (100–200 nanomolar) is maintained by a complex series of homeostatic mechanisms. Small changes in intracellular calcium concentrations affect a wide variety of cellular functions as part of a “second messenger” system analogous to the cyclic adenosine 3',5-monophosphate (cAMP) messenger system. A small calcium-binding protein, calmodulin, plays an important role in the calcium messenger system [1,2].

Calmodulin was discovered in the late 1960s and has been reviewed extensively in recent years [3,4,5,6,7,8,9]. It is a small (148 amino acid, molecular weight 16,700), acidic (pI 3.9), heat-stable protein present in all eukaryotic cells examined to date. It has been highly conserved across evolutionary lines, and only subtle differences have been identified in the sequences of calmodulins purified from species which are widely separated phylogenetically, a factor which implies that calmodulin is part of a regulatory system common to all eukaryotic cells.

Each molecule of calmodulin has four structurally similar domains, each of which can bind a single Ca++ ion in response to changes in the intracellular calcium concentration (kd $3.5 \times 10^{-6}$M). The binding of Ca++ to calmodulin produces

Abbreviations: AP: apyrimidinic/apurinic cAMP: cyclic adenosine 3',5-monophosphate CHO: Chinese hamster ovary C-kinase: Ca++ stimulated protein kinase J/m²: Joules/meter² ssb: single-strand breaks UV: ultraviolet

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conformational changes, which allow the specific interaction of calmodulin with a number of target proteins. In this manner, activity of the target proteins is increased, causing changes in a variety of cellular functions.

Strict criteria for the designation of calmodulin-dependent processes have been set forth by Cheung, one of the first to describe the protein [10]. Enzymes which have met the stringent criteria include those involved in cyclic nucleotide metabolism [11], protein phosphorylation [12], energy metabolism [13], and other key cellular functions. The list of processes in which calmodulin has been implicated as a regulator is much longer and includes such processes as DNA synthesis [14], cell growth and differentiation [15], and microtubular function [16], which are important in consideration of the role calmodulin may play in neoplastic cells.

A large number of compounds have been demonstrated to be calmodulin antagonists (for reviews, see [17,18,19]. The phenothiazine family of compounds, used clinically as anti-psychotics, were the first compounds to be used experimentally as calmodulin inhibitors. A wide variety of compounds from different structural classes has subsequently been shown to have calmodulin antagonist activity, including the napthalene-sulfonamide, or "W" compounds.

The large body of research that examines the role of calmodulin in neoplasia has been discussed in several reviews [15,20,21,22]. Hait and Lazo have summarized a large number of investigations in which calmodulin antagonists have been shown to inhibit cell growth and produce cytotoxicity [22]. Most experiments have involved work with malignant cell lines in culture, although some in vivo animal experiments have been reported and clinical trials are currently in progress. Many of the studies summarized demonstrated a good correlation between the concentration of calmodulin inhibitors required to inhibit clonogenicity and the concentration required to inhibit calmodulin in cell-free assay systems.

Several lines of research address the role that calmodulin may play in neoplastic cells. Increased concentrations of calmodulin have been reported in some but not all studies in which the calmodulin content of hyperproliferative and malignantly transformed cells has been compared to that of controls [15]. Cell-cycle specific elevations in calmodulin content have been reported, and progression through the cell cycle has been shown to be inhibited by calmodulin antagonists [23,24]. The capacity of calmodulin to alter protein activity by phosphorylation leaves open, however, the possibility that calmodulin may specifically affect neoplastic cells via the regulation of as yet unidentified processes. As mentioned previously, data implicate calmodulin as a regulator of many more processes than are at present definitively categorized as calmodulin-dependent.

The following review attempts to summarize the data linking calmodulin to the fundamental cellular process of DNA repair. Experiments have been conducted using physical factors known to induce DNA damage, such as ultraviolet (UV) light, X-irradiation, and hyperthermia, in conjunction with calmodulin antagonists. In addition, many chemotherapeutic agents currently in use are thought to exert their therapeutic effects by inducing DNA damage, and experiments have been performed which examine their interactions with calmodulin antagonists. The potentiation of therapeutic effects produced by DNA damage in malignant cells may be mediated by the inhibition of DNA repair processes. Thus, the findings that calmodulin antagonists may be regulators of DNA repair indicate that there may be a role for calmodulin antagonists as components of new regimens in anti-neoplastic therapy.
CALMODULIN ANTAGONISTS AND BLEOMYCIN

Using Chinese hamster ovary (CHO) cells growing exponentially in culture, Chafouleas, Bolton, and Means examined the effects of calmodulin inhibitors in combination with bleomycin, an anti-tumor antibiotic thought to act by fragmenting DNA [25]. The authors noted that when CHO cells were treated with non-toxic concentrations of bleomycin in combination with non-toxic concentrations of W-13, a naphthalenesulfonamide calmodulin inhibitor, a dramatic decrease in the fraction of surviving cells occurred. There was a dose-dependent decrease in the surviving fraction of cells as the concentration of W-13 was increased. This effect was not noted when compound W-12, an analog of W-13, which is not active as a calmodulin antagonist, was used. The effect of this combination treatment was most prominent when cells were first exposed to bleomycin, then allowed to recover from bleomycin-induced lethal damage in the presence of the phenothiazine calmodulin inhibitor, trifluoperazine.

The authors postulated that their results were due to the inhibition of a calmodulin-dependent process of DNA repair. In support of this hypothesis, they found a decrease in the nucleoid sedimentation migration rate, a measure shown to correlate with DNA damage, in nucleoids which had been prepared from cells treated with bleomycin. In cells treated with bleomycin and then allowed to recover in either fresh medium or medium containing the inactive congener W-12, recovery of the nucleoid sedimentation migration rate was no different from that of controls. In cells treated with bleomycin which were allowed to repair in medium containing the calmodulin inhibitor W-13, however, the nucleoid sedimentation migration rate was approximately that of the control cells treated with bleomycin alone, which were not given the opportunity for repair. The authors concluded that DNA repair had not taken place in presence of the calmodulin antagonist W-13 but had occurred in the presence of the inactive compound, W-12.

In addition, Chafouleas and his colleagues found that W-13 did not potentiate DNA damage induced by bleomycin in E. coli [25]. Unlike eukaryotes, E. coli and other prokaryotes do not contain calmodulin. This system provided additional support for their contention that the potentiation of bleomycin cytotoxicity by calmodulin antagonists was due to the inhibition of calmodulin.

Lazo, Hait, and their colleagues also reported the effects of calmodulin antagonists in combination with bleomycin on the viability of malignant cells [26,27,28]. For example, in L1210 murine leukemia cells, bleomycin cytotoxicity was enhanced by a variety of different structural classes of calmodulin antagonists including the naphthalenesulfonamide, W-7, the phenothiazines, trifluoperazine and chlorpromazine, the diphenylbutylpiperidine, pimozide, and the bee venom peptides, melittin and mastoparan. The potentiation of bleomycin-induced cytotoxicity was not noted when the inactive congeners of naphthalenesulfonamide, W-5, or phenothiazine, chlorpromazine sulfoxide, were used.

The potentiation of bleomycin cytotoxicity was more prominent in cell lines naturally resistant to bleomycin. For example, Lazo et al. demonstrated a dose-dependent enhancement of bleomycin-induced cytotoxicity in human SK-OV ovarian carcinoma cells using the calmodulin inhibitors chlorpromazine, melittin, and pimozide but found no enhancement in A-253 cells, a human squamous cell carcinoma from the head and neck region, which is more sensitive to bleomycin [28].
In addition to noting the enhancement of cytotoxicity, these experimenters directly examined the effects of bleomycin and calmodulin antagonists on DNA [27]. Using alkaline elution techniques to examine DNA from the L1210 cells, the authors noted an increased number of DNA breaks in cells treated with bleomycin in conjunction with pimozide as compared to cells treated with bleomycin alone. DNA from cells treated with pimozide alone did not have an increased number of breaks when compared to DNA from untreated cells. In a cell-free system using plasmid DNA, the authors noted no potentiation of bleomycin-induced DNA breakage with the addition of pimozide.

These studies suggested that the augmentation of bleomycin-induced cytotoxicity by calmodulin antagonists was secondary to increased damage to cellular DNA. This increased damage could have been a direct effect of the combination or an indirect effect mediated by inhibition of a calmodulin-mediated process of DNA repair, as suggested by Chafouleas, Bolton, and Means [25].

Although the concentrations of calmodulin antagonists required to enhance cytotoxicity are similar to the concentrations required to inhibit calmodulin, the lack of specificity of currently utilized calmodulin antagonists makes it critical that these data be interpreted cautiously. The strict criteria required in order to implicate calmodulin when these drugs are used have been set forth by Hait and Lazo [22]. When followed, these criteria will allow the interpretation that the observed potentiation of bleomycin-induced DNA damage is consistent with inhibition of calmodulin-sensitive DNA repair mechanism(s) by calmodulin antagonists.

CALMODULIN ANTAGONISTS AND X-IRRADIATION

In an attempt to examine further the hypothesis that calmodulin antagonists inhibited DNA repair processes, Lazo, Hait, et al. also treated cells with calmodulin antagonists in combination with other agents known to cause repairable DNA damage. Using calmodulin antagonists in combination with X-irradiation and the chemotherapeutic agent etoposide, the authors were unable to demonstrate cytotoxic augmentation in L1210 cells [27].

The absence of a synergistic effect between calmodulin antagonists and X-irradiation to increase cytotoxicity and DNA damage or to decrease DNA repair was also shown by Ridinger and his colleagues in experiments utilizing a murine mammary carcinoma cell line. The investigators found that addition of the calmodulin inhibitor W-13 to cell cultures prior to X-irradiation had no effect either on cell survival, the amount of DNA damage induced, or the ability of the cells to repair the X-ray-induced DNA damage [29].

In in vivo experiments, however, George and Singh noted different effects using X-irradiation in combination with calmodulin antagonists. When these researchers exposed mice bearing a transplantable murine fibrosarcoma to the phenothiazine-type drugs, promethazine, prochlorperazine, and trimeprazine, they noted no change in tumor growth rate. When the same compounds were administered to mice prior to X-ray treatments, however, these drugs potentiated the previously documented growth inhibitory effects of X-irradiation [30]. Though not yet confirmed by in vitro data, these experiments clearly suggest a possible role for phenothiazine calmodulin antagonists in the potentiation of X-irradiation-induced cytotoxicity, perhaps through inhibition of certain DNA repair processes.
CALMODULIN ANTAGONISTS AND HYPERTHERMIA

Increased attention has recently been focused on the potential for the use of hyperthermia in cancer therapy since the recognition that tumor cells were more sensitive to heat than normal cells [31]. The mechanism by which cell death is induced by hyperthermia is not certain, but effects on DNA repair, synthesis, and replication have been reported [32,33]. Interest has also developed in the use of hyperthermia in conjunction with calmodulin antagonists.

Using Reuber H35 rat hepatoma cells and N3A neuroblastoma cells, Wiegant and colleagues demonstrated significant potentiation of hyperthermic cytotoxicity when cells were simultaneously treated with trifluoperazine [34]. Concentrations of trifluoperazine (5 μm and 10 μm), which had been shown to be non-cytotoxic alone, were found to enhance the cytotoxic effect of hyperthermia (43°C for one hour). Similar results were obtained for both the neuroblastoma and hepatoma cell lines. George and Singh found that in vivo treatment of a transplantable murine fibrosarcoma with non-toxic doses of chlorpromazine prior to treatment with hyperthermia delayed tumor growth to a greater degree than did hyperthermia alone [35]. Again, the calmodulin antagonists appear to play a role in inhibiting DNA repair or potentiating DNA damage, though whether this apparent effect is due to the inhibition of a calmodulin-dependent process or is secondary to one of the many other effects of the phenothiazine-type calmodulin antagonists is not yet clear.

CALMODULIN ANTAGONISTS WITH BLEOMYCIN AND HYPERTHERMIA

Given that interactions of bleomycin and hyperthermia [36], hyperthermia and calmodulin antagonists, and bleomycin and calmodulin antagonists have all been shown to enhance cytotoxicity, it is not surprising that combinations of all three agents have been studied. Smith, Mircheva, and Bleezen, using murine EMT6 mammary tumor cells, found that the addition of a non-toxic dose of trifluoperazine to bleomycin enhanced the degree of cytotoxicity by a factor of 1.3, while the addition of a non-toxic exposure to hyperthermia to bleomycin enhanced cytotoxicity by a factor of 19 [37,38]. The combination of bleomycin with trifluoperazine and hyperthermia produced enhancement of cytotoxicity by a factor of 112, a greater than additive effect. Unfortunately, the authors did not present data showing the effect of hyperthermia and trifluoperazine in combination without concurrent bleomycin administration.

Smith et al. examined the DNA damage caused by bleomycin, hyperthermia, and trifluoperazine by the nucleoid sedimentation and alkaline denaturation assays [39]. The nucleoid sedimentation assay preferentially detects single-strand breaks in DNA, whereas the alkaline denaturation assay detects both phosphodiester bond breaks in the DNA as well as alkali-labile lesions. The alkali-labile lesions are due to the presence of apyrimidinic/apurinic sites (AP sites) rendering the intact DNA molecule susceptible to alkaline denaturation. Analysis of results from the two different assays of DNA damage showed that hyperthermia diminished the repair of bleomycin-induced DNA damage by depressing the repair of both single-strand breaks (ssbs) and AP sites, whereas trifluoperazine selectively interfered with the repair of AP sites. Since the calmodulin antagonist, trifluoperazine, and hyperthermia appear to inhibit the repair of DNA damage by separate mechanisms, there is a rational basis for the synergistic enhancement of the cytotoxic effect of bleomycin cited above.
CALMODULIN ANTAGONISTS AND 254 nm UV LIGHT

The cyclobutane dimer formed by adjacent pyrimidine bases in DNA upon absorption of ultraviolet (UV) light in the 254 nm range is a well-understood model of DNA damage, and has been described as the "classic test lesion" for the study of DNA repair [40]. Research done with this experimental system assumes special importance because the mechanism of UV-induced DNA damage and repair is so well understood.

Charp and Regan examined the effects of trifluoperazine in combination with 254 nm UV light on normal-human-skin fibroblasts in culture [41]. Having prelabeled the cells with $^3$H thymidine, they administered 20 Joules/meter$^2$ (J/m$^2$) of 254 UV light to initiate the formation of cyclobutane pyrimidine dimers. Immediately after UV exposure, either 25 $\mu$M of trifluoperazine or a control solution was added, and the percentage of pyrimidine dimers remaining was followed by two-dimensional paper chromatographic analysis of hydrolyzed DNA from the exposed fibroblasts. After 24 hours of post-exposure incubation, the authors noted a significant difference in the pyrimidine dimers remaining in the DNA of cells treated with trifluoperazine compared to the control cells.

The authors tried to determine which step of the DNA repair process was affected by trifluoperazine. Further studies with bromodeoxyuridine photolysis demonstrated normal function of exonuclease and polymerase activity in the presence of trifluoperazine. These results led the authors to deduce that calmodulin antagonists acted upon the incision step of the DNA repair process. Additional studies using cytosine-arabinoside to inhibit DNA polymerase supported this conclusion.

To determine whether or not the combination of UV light with calmodulin antagonists might be applied to the treatment of malignancies involving the skin, such as cutaneous T-cell lymphoma, we studied the effect of trifluoperazine and melittin with UV light on the L1210 murine lymphocyte leukemia. These studies failed to demonstrate an effect of trifluoperazine and melittin on the inhibition of cellular proliferation produced by 254 nm UV light [42]. A possible reason for this discrepancy might be that many rodent cell lines are deficient in the repair of UV-induced pyrimidine dimers. Mouse L cells have, however, been shown to have excision repair mechanisms in place, although perhaps at reduced levels [43,44]. In addition, we obtained similar results using the combination of UV exposure and trifluoperazine with human CEM T-cell lymphoblastic leukemia cell line.

CALMODULIN ANTAGONISTS AND OTHER CHEMOTHERAPEUTIC AGENTS

Kwok and Twentyman examined the effects of a number of cytotoxic chemotherapeutic agents in combination with trifluoperazine [45]. They used small spheroids of murine EMT6/Ca/VJAC mammary tumor cells treated with bleomycin, CCNU, nitrogen mustard, and X-irradiation followed by exposure to non-toxic concentrations of trifluoperazine. No difference was noted in the surviving fraction of cells exposed to trifluoperazine when that was compared to the surviving fraction of cells exposed to the cytotoxic agent alone.

Darkin and his colleagues found that DNA isolated from PY815 cell nuclei was damaged after treatment with the DNA intercalating agent m-AMSA [46]. The DNA
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was resealed when the nuclei underwent subsequent incubation in drug-free medium. The degree of repair was, however, significantly inhibited when incubation took place in the presence of chlorpromazine.

Interactions between doxorubicin and related anthracyclines with calmodulin antagonists have been explored by Ganapathi and his associates [47,48]. Much of their work has involved the use of a doxorubicin-resistant P388/DOX murine leukemia line, where they have demonstrated up to a 100-fold increase in anthracycline-induced cytotoxicity with the use of trifluoperazine. The mechanisms underlying the resistance of these cells to doxorubicin and other compounds are unclear (for a recent review, see [49]). Cytotoxic enhancement was also noted in experiments with the non-resistant P388/S cell line. These results were dose-dependent and were noted with both trifluoperazine and chlorpromazine at concentrations appropriate to the potency of the two compounds as calmodulin antagonists [44].

In further experiments with the non-resistant P388/S cell line, Ganapathi and his associates noted that calmodulin antagonists preferentially enhanced the cytotoxicity of strong DNA binding agents, including the chemotherapeutic agents dactinomycin and AMSA, in addition to the anthracyclines doxorubicin and daunorubicin [50]. In contrast, they did not note enhancement of cytotoxicity when weakly DNA-binding anthracycline analogs were used. The mechanism of cytotoxic enhancement in the sensitive P388/S cell line is less well understood than in the resistant phenotype, where attention has been focused on differences in cellular uptake and retention of the anthracyclines [44,45]. The existence of cytotoxic enhancement of strong DNA binding agents from a variety of different structural classes by calmodulin antagonists again suggests, however, that calmodulin may be involved in DNA damage and repair.

The studies of agents that induce DNA damage with calmodulin antagonists are summarized in Table 1. Several explanations are possible for the inconsistent results obtained. In many of these experiments assessing cytotoxicity, several different assay systems are used to determine dose-response effects, such as cell count by coulter counter to quantitate cell growth inhibition, the clonogenic assay which looks at the number of colonies formed to quantitate cell survival, or growth delay as used in in vivo tumor systems, each having different sensitivities and specificities [51]. Also, individual cell lines display a variety of sensitivities to agents used, as demonstrated by Lazo and associates in their studies using bleomycin in conjunction with calmodulin antagonists [28].

Other difficulties arise in definitively ascribing effects obtained with calmodulin antagonists to the inhibition of calmodulin. Known calmodulin antagonists suffer from a lack of specificity and are known to exert effects on cellular components such as membranes and hormone receptors, in particular the phorbol ester receptor believed to be the phospholipid, Ca++ stimulated protein kinase (C-kinase), which are not directly linked to their effects as calmodulin antagonists. This action complicates their use as tools for the elucidation of calmodulin-dependent processes [18]. Interpretation of experimental data is also complicated by the lack of precise techniques for the assay of DNA repair inhibition, necessitating the use of biological end-points in the place of more specific assays [52]. Future studies using the newly synthesized calmodulin antagonist, CGS-9343B, a drug shown to inhibit calmodulin-sensitive enzymes out of proportion to its effect on protein kinase C and to its effect on dopamine receptors,
TABLE 1
Summary of Studies Using Calmodulin Antagonists in Combination with Physical and Chemical Agents Which Damage DNA

| DNA-Damaging Agent | Experimental System | Calmodulin Antagonist | Result          | Reference |
|--------------------|---------------------|-----------------------|-----------------|-----------|
| Bleomycin          | CHO cells           | W-13, TFP             | Enhancement noted | [25]      |
|                    | L1210 cells         | W-7, TFP, CPZ, Mel, Mas | Enhancement noted | [26,27]  |
|                    | Plasmid DNA         | PIM                   | No enhancement noted | [26]      |
|                    | Sk-OV cells         | CPZ, PIM, Mel         | Enhancement noted | [28]      |
|                    | A-253 cells         | CPZ, PIM, Mel         | No enhancement noted | [28]      |
|                    | Bone marrow progenitor cells | CPZ, PIM, Mel | Enhancement noted | [28]      |
|                    | EMT6 cell spheroids | TFP                   | No enhancement noted | [45]      |
| X-Irradiation      | EMT6 cells 67 (P)   | W-13                  | No enhancement noted | [29]      |
|                    | L1210               | PIM, Mel              | No enhancement noted | [26]      |
|                    | EMT6 cell spheroids | TFP                   | No enhancement noted | [45]      |
|                    | In vivo murine fibrosarcoma | PMZ, PPZ, TPZ | Enhancement noted | [30]      |
| Hyperthermia        | Neuroblastoma       | TFP                   | Enhancement noted | [34]      |
|                    | N3A cells, H35 hepatoma cells | Calmidazolium | Enhancement noted | [35]      |
|                    | In vivo murine fibrosarcoma | CPZ                   | Enhancement noted | [35]      |
| Bleomycin and Hyperthermia | EMT6 cells | TFP                   | Enhancement noted | [37,38]  |
| Ultraviolet Light  | Normal human fibroblasts | TFP                  | Enhancement noted | [41]      |
| (254 nm)            | L1210 cells         | TFP, Mel              | No enhancement noted | [42]      |
|                    | CEM cells           | TFP                   | No enhancement noted | [42]      |
|                    | AMSA                | CPZ                   | Enhancement noted | [46]      |
|                    | P388/S cells        | TFP                   | Enhancement noted | [50]      |
| Doxorubicin         | P388/S cells        | TFP                   | Enhancement noted | [50]      |
|                    | EMT6 cell spheroids | TFP                   | No enhancement noted | [45]      |
| Dactinomycin        | P388/S cells        | TFP                   | Enhancement noted | [50]      |
|                    | CCNU                | TFP                   | No enhancement noted | [45]      |
| Nitrogen Mustard    | EMT6 cell spheroids | TFP                   | No enhancement noted | [45]      |
| Etoposide           | L1210 cells         | PIM, Mel              | No enhancement noted | [26]      |

Abbreviations: TFP, trifluoperazine; CPZ, chlorpromazine; PIM, pimozone; Mel, melittin; Mas, mastoparan; PMZ, promethazine; PPZ, prochlorperazine; TPZ, trimeprazine

should help to elucidate the role of calmodulin [54]. Furthermore, studies using isolated nuclei and purified nuclear enzymes should help to define precisely the proposed calmodulin-dependent processes.

In spite of these difficulties in interpreting experimental data, there is reason to be optimistic about the future role of calmodulin antagonists in anti-neoplastic therapy. Calmodulin antagonists such as trifluoperazine have already been demonstrated to be
relatively well tolerated and non-toxic in their clinical use for psychiatric disorders. Some have already begun to be used in combination with other agents in clinical trials. Based on the observed in vitro potentiation of bleomycin-induced cytotoxicity by calmodulin antagonists, a phase I–II study using trifluoperazine in combination with bleomycin was conducted. Patients with a variety of malignancies, including mycosis fungoides and gynecologic malignancies, were enrolled in the study. Although neuro-psychiatric side effects and other toxicities were noted, 4 of 19 patients had measurable responses to the experimental regimen, warranting further clinical evaluation [53].

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