Photochemical Internalization-Mediated Delivery of Chemotherapeutic Agents in Human Breast Tumor Cell Lines

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ABSTRACT: Breast-conservation surgery (BCS) is now utilized in patients with stage I and II invasive breast cancer. However, positive surgical margins are associated with recurrence, and therefore some form of localized postoperative therapy (radiation/chemotherapy) is necessary to eliminate remaining cancer cells. Existing modalities have significant treatment-limiting side effects; therefore, alternative forms of localized therapy need to be explored. We studied the ex vivo effects of photochemical internalization (PCI) using 4 chemotherapeutic agents: cisplatin, cisplatin analog [D prostanoid, DP], doxorubicin, and bleomycin) on 3 breast cancer cell lines: MCF-7, MDA-MB-435, and MDA-MB-231. Illumination was carried out using a 670-nm diode laser at 5 mW/cm² following incubation in the photosensitizer with aluminum phthalocyanine disulfonate. Toxicity was investigated using colony-forming assays and the mechanism of cell death was determined using Annexin flow-cytometry. We found that toxicity of DP and bleomycin was significantly enhanced by PCI compared with drug alone but was unchanged for cisplatin and doxorubicin. PCI treatment caused a decrease in the percentage of viable cells, predominantly by enhancing apoptosis. The action was synergistic across all 3 cell lines tested for DP and bleomycin. Thus, with appropriate delivery devices and choice of chemotherapeutic agents, PCI holds the promise of enhancing tumor cell toxicity surrounding the cavity of BCS resection sites and thereby decreasing local recurrence.

KEY WORDS: breast cancer chemotherapy, photochemical internalization, photodynamic therapy, chemotherapeutic resistance

ABBREVIATIONS: AlPcS2a, phthalocyanine disulfonate; BCS, breast conservation surgery; BLM, bleomycin; CP, cisplatin; DMSO, dimethyl sulfoxide; Dox, doxorubicin; DP, dichloro(4,4′-dipropyl-2,2′-bipyridine) platinum; FL, linear flow channel; MDR, multiple drug resistance; PBS, phosphate-buffered saline; PCI, photochemical internalization; PDT, photodynamic therapy; PI, propidium iodide; DDM, doublet discriminatory module; RT, radiation

I. INTRODUCTION

Breast conservation surgery (BCS) is now recognized as the preferred therapy for patients with stage I and II invasive breast cancer.1 Numerous studies have shown that positive surgical margins are associated with tumor recurrence and, therefore, approximately 40% of patients undergoing BCS return for a re-excision surgery.2–4 Following BCS, some form of localized postoperative therapy such as radiation (RT), chemotherapy, or both is used in the hope of eliminating any remaining malignant cells.5–9 The use of ionizing RT, though effective, has several limitations, as does the dose-limiting toxicity of many of the chemotherapeutic agents in use as postoperative therapy.10–14 In this study we investigated the ability of a light-based approach known as photochemical internalization (PCI) to increase the local efficacy of cisplatin (CP) and one of its analogs, dichloro(4,4′-dipropyl-2,2′-bipyridine) platinum (DP) on several breast tumor cell lines in vitro. The mechanism of cellular toxicity was in-
vestigated, and these results were compared with the PCI delivery of 2 other chemotherapy agents (doxorubicin [Dox] and bleomycin [BLM]).

Although CP is one of the most effective and most prescribed antitumor agents in use today, its application is limited because of toxic side effects (renal toxicity, emesis, neurotoxicity, bone marrow suppression, and hearing loss). In addition, cellular resistance to platinum has resulted in its limited spectrum of activity against several types of cancer. As a consequence of the limitations of CP, numerous analogs have been developed and characterized with the goal of finding compounds that could be more effective and less toxic. To address these issues and to improve clinical outcome, alternate methods to reduce the limitations of CP and its analogs would be desirable.

PCI is a novel technology that is under development for utilizing the properties of photodynamic therapy (PDT) to enhance the drug delivery of macromolecules in a site-specific manner. Many anticancer agents are limited in their ability to penetrate cell membrane structures and are transported into cells by endocytosis, resulting in their accumulation in intracellular endocytic vesicles (endosomes). Specially designed photosensitizers, such as aluminum phthalocyanine disulfonate (AlPcS2a), which localize preferentially in the cell membrane, therefore will be incorporated in the endosomal membranes inside the cell. Exposure to light leads to endosomal rupture by phototoxic damage and the release of its contents into the cytosol. The released macromolecules can exert their full biological activity instead of being degraded by lysosomal hydrolases. PCI-based relocation and activation of macromolecules has the advantage of reducing drug-induced side effects because the effect is localized to the area exposed to light and because of the possibility of using lower drug concentrations due to increased efficacy. PCI of BLM has proved superior to PDT for sterilizing the tumor bed after cytoreductive surgery.

II. MATERIALS AND METHODS

A. Cell Culture

Human breast cancer cell lines MCF-7 (estrogen receptor–positive breast epithelial adenocarcinoma), MDA-MB-435 (estrogen receptor–negative breast ductal adenocarcinoma), and MDA-MB-231 (estrogen receptor–negative breast epithelial adenocarcinoma) were obtained from the American Culture Type Collection. All cell lines were grown in minimum essential medium (Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum (Invitrogen), penicillin-streptomycin (Invitrogen), and 25 mM HEPES buffer (pH 7.4; Sigma-Aldrich, St. Louis, Missouri). Cells were washed with phosphate buffered saline (PBS; Invitrogen) and treated with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) to allow for detachment from plastic.

B. Drug Preparation

Cis-diaminedichloroplatinum (II) (Sigma) was dissolved in DMSO (≥ 99.9% A.C.S Spectrophotometric grade, Sigma-Aldrich) to obtain a stock solution of 50 mM and diluted in sterile-filtered dimethyl sulfoxide (DMSO) for stock concentrations of 5 mM, 0.5 mM, 0.05 mM, and 0.005 mM. Dox and BLM were purchased from Sigma.

C. PDT/PCI Treatment

Cells from the various cell lines were plated in 35-mm Petri dishes and incubated overnight to allow them to adhere. The cells were then incubated in
1 μg/mL AlPcS\textsubscript{2a} (Frontier Scientific, Inc., Logan, UT) and Dulbecco’s modified Eagle medium for 18 hours. Following incubation, cells were washed 3 times with PBS, and 5 mL of fresh medium was added, followed by 1 to 4 hours of incubation with the drug to be tested at various concentrations. PDT controls received no drug. Irradiation was performed with 670-nm light from a diode laser (Intense, North Brunswick, NJ). The cells were exposed to a range of radiant exposures (0.75–3 J/cm\textsuperscript{2}) delivered at a light irradiance of 5 mW/cm\textsuperscript{2}. Following irradiation, cells were washed with PBS, harvested by trypsinization, resuspended in Dulbecco’s modified Eagle medium supplemented with serum, counted, and plated into 60-mm dishes. Cells were allowed to grow for 11 to 14 days, at which time they were stained with 0.5% crystal violet in 95% ethanol. Colonies containing more than 50 cells were scored as survivors. The number of colonies was normalized to a control group consisting of cells incubated in a photosensitizer (dark control) but that received no light treatment. All experiments were performed in quintuplicate and results are a combination of at least 4 separate experiments.

D. Mechanism of Cell Death

Apoptotic or necrotic cell death was evaluated for MDA-MB-435 and MDA-MB-231 48 hours after PCI treatment using Annexin/propidium iodide (PI)–staining flow cytometry. For PI staining, 40% to 80% confluent cells were incubated at 37°C with 5% CO\textsubscript{2} for 1 hour with CP or DP. After 1 hour, cells were washed with 5 mL PBS and fresh medium added. Cells were incubated at 37°C with 5% CO\textsubscript{2} for 24 hours and 48 hours, at which point cells were harvested, counted, and centrifuged at 500 g for 5 minutes. Cells were then washed in 2.0 mL Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-free PBS. Pellets were then washed in 2.0 mL 1X Annexin-V binding buffer (BD Bioscience, San Jose, CA) and centrifuged at 500 g for 5 minutes. The pellets were treated with Annexin V-FITC conjugate (BD Bioscience, San Jose, CA) and incubated in the dark for 15 minutes. Just before acquisition, the volume of the cell–conjugate mixture was adjusted by the addition of 1X Annexin-V binding buffer. Acquisition to discriminate between apoptotic and necrotic cells was done by staining the cell–conjugate mixture with 10 mL PI solution (BD Bioscience). Acquisitions were done with a FACS Calibur cytometer on the FL1 (Annexin) and FL3 (PI) channels with threshold and Doublet discriminating module set at FL1. The level of shift in events distribution in the Annexin-V only and Annexin-V-PI populations compared with control is indicative of the degree of effectiveness of the treatment agents. A quantitative measure of these event shifts was accomplished by gating.

E. Statistical Analysis

Graph Pad Prism 4.0 (Graph Pad Software, San Diego, CA) was used to graph and run nonlinear regressions on the data. Graph Pad Quick Cales (www.graphpad.com) was used for Student’s t test. P values less than 0.05 were considered significant. All graphical error bars are representative of standard error.

Synergism was calculated when analyzing PCI treatments compared with drug or PDT alone. The equation shown below was used to determine if the PCI effect was synergistic, antagonistic, or additive, where \( \alpha \) is the ratio of the cumulative effect of 2 therapies administered independently to the net
The estrogen-positive cell line MCF-7 proved to be significantly more resistant to Dox than the 2 estrogen-negative cell lines. CP had little effect on cell survival at the concentration range of 0.1 μM to 100 μM. At the highest concentration used (1 mM), cell survival was greatly reduced to less than 12% of control values. In contrast, DP proved to be about 100 times more potent (Fig. 2) than CP on all 3 cell lines. The EC<sub>50</sub> values for CP and DP were compared from the results of clonogenic survival assays for the 2 drugs. Student’s t tests were used to verify significant differences between the EC<sub>50</sub> values of CP and DP (Table 1). The results for DP efficacy compared with CP were significant (P < 0.05) for all 3 lines; MCF-7, the estrogen-positive cell type, was the most resistant of the 3 to DP.

III. RESULTS

A. Effects of Drug Concentration on Clonogenic Survival

The effects of the 4 chemotherapeutic agents on the 3 cell lines’ ability to form daughter colonies following 1 hour incubation is shown in Fig. 2. The estrogen-positive cell line MCF-7 proved to be significantly more resistant to Dox than the 2 estrogen-negative cell lines. CP had little effect on cell survival at the concentration range of 0.1 μM to 100 μM. At the highest concentration used (1 mM), cell survival was greatly reduced to less than 12% of control values. In contrast, DP proved to be about 100 times more potent (Fig. 2) than CP on all 3 cell lines. The EC<sub>50</sub> values for CP and DP were compared from the results of clonogenic survival assays for the 2 drugs. Student’s t tests were used to verify significant differences between the EC<sub>50</sub> values of CP and DP (Table 1). The results for DP efficacy compared with CP were significant (P < 0.05) for all 3 lines; MCF-7, the estrogen-positive cell type, was the most resistant of the 3 to DP.

B. PDT Effect on Clonogenic Survival Curves

Previous experiments have shown that PCI is most efficient when 70% to 80% cells survive AlPcS<sub>2a</sub>.

\[
\alpha = \frac{SF^a \times SF^b}{SF^{ab}}
\]

In this scheme, SF represents the survival fraction for a specific treatment. If 2 treatments are to be compared, the survival fractions of each separate treatment are multiplied together and then divided by the survival fraction when both treatments were applied together. The interaction is calculated based on the dose of each treatment. The resulting number (\(\alpha\)) describes the summative effect as previously described.\(^{28}\) If \(\alpha > 1\), the result is synergistic (supra-additive). If \(\alpha < 1\), the result is antagonistic, and if \(\alpha = 1\) the result is simply additive.

FIGURE 2. Effects of drug concentration on clonogenic survival of MDA-MB-435, MDA-MB-231, and MCF-7 cells. Cells were treated for 1 hour with varying concentrations of (a) cisplatin, (b) dichloro(4,4′-dipropyl-2,2′-bipyridine) platinum, (c) doxorubicin, or (d) bleomycin. Cell survival was assessed by normalizing the number of colonies (≥50 cells) formed for treated cells with controls. Data points are the average of 3 individual clonogenic survival experiments performed in triplicate plates. Error bars denote standard error of the mean.
mediated PDT treatment. The effects of increasing fluence levels on clonogenic survival for all 3 lines are shown in Fig. 3. As can be seen in Fig. 3, increasing fluence levels produced an increase in the cytotoxic PDT effect. At a fluence level of between 0.75 and 1 J/cm², between 75% and 85% of the cells survived for all 3 lines. Therefore, these fluence levels were employed in all subsequent PCI experiments.

Quantitative evaluation of the PCI effect as determined from the degree of interaction (α) between PDT and drug was calculated from the data shown in Fig. 4 and are shown in Table 2. The α values for the effects of PCI-mediated cytotoxicity for CP and Dox were not significantly larger than 1.0, indicating no synergistic effect (P > 0.1). On the other hand, the α values for the effects of PCI-mediated cytotoxicity for DP and BLM ranged from 1.7 to 9.1 for the different cell line and drug combinations tested. This clearly indicated a synergistic effect of PCI. The PCI-mediated increased efficacy for both drugs proved least for the MCF-7 cell line. Drug titration with and without PCI, therefore, were performed with this cell line for both DP and BLM to compare directly the results obtained with PCI of drug to drug alone over the titration range. As can be seen from Fig. 5, the EC₅₀ value (drug concentration corresponding to 50% survival) with and without PCI was 0.08 μM versus 6 μM for DP and 0.07 μg/mL versus 1.2 μg/mL for BLM, respectively.

The results of flow cytometry used to determine the fraction of viable, apoptotic, and necrotic cells for CP and DP on the 2 estrogen-negative cell lines are shown in Fig. 6. The 2 different fluorescent labels used were Annexin V-FITC to distinguish apoptotic cells and PI to label necrosis. Unlabeled cells were assumed to be viable. As expected, PCI treatment caused a decrease in the percentage of viable cells, especially for DP-treated cultures. It also significantly increased the percentage of cells in the apoptotic fraction.

### TABLE 1. EC₅₀ Values determined from clonogenic survival assays

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cell Type</th>
<th>MCF-7</th>
<th>MDA-MB-435</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>305.00 ± 21.21</td>
<td>311.96 ± 57.31</td>
<td>329.36 ± 38.74</td>
<td></td>
</tr>
<tr>
<td>DP</td>
<td>6 ± 0.73*</td>
<td>1.77 ± 0.33*</td>
<td>1.26 ± 0.31*</td>
<td></td>
</tr>
</tbody>
</table>

Values presented are drug concentrations in micromoles.
*Statistically significant compared with cisplatin within cell line, with P < 0.05. DP, dichloro(4,4'-dipropyl-2,2'-bipyridine) platinum.

C. PDT/PCI Effect on Clonogenic Survival

The effects of PCI for the 4 drugs CP, DP, Dox, and BLM on all 3 cell lines were compared with the effects of drug alone for equivalent drug doses. As shown in Fig. 4, PCI of CP or Dox gave no significant increase in efficacy compared with the drug alone. In contrast, PCI of DP or BLM showed significant (P < 0.05) cytotoxic effect for all 3 cell types.

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FIGURE 4. Photodynamic therapy (PDT)/photochemical internalization (PCI) effect on clonogenic survival of MCF-7, MDA-MB-231, MDA-MB-235 cells. PDT, 0.75 J/cm$^2$ for MCF-7 and MDA-MB-435 cells and 1 J/cm$^2$ for MDA-MB-231 cells (at 5 mW/cm$^2$). Drug concentrations: (a) cisplatin (CP), 156 μM and dichloro(4,4′-dipropyl-2,2′-bipyridine) platinum (DP), 1 μM; (b) doxorubicin (Dox), 0.1 μg/ml; bleomycin (BLM), 0.25 μg/ml.

TABLE 2. Quantitative evaluation of the photochemical Internalization effect as determined from the degree of interaction (α) between photodynamic therapy and the drug

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cell Type</th>
<th>MDA-MB-231</th>
<th>MCF-7</th>
<th>MDA-MB-435</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP</td>
<td>2.60 ± 0.26</td>
<td>1.7 ± 0.21</td>
<td>8.2 ± 0.78</td>
<td></td>
</tr>
<tr>
<td>Bleomycin</td>
<td>9.12 ± 0.85</td>
<td>2.8 ± 0.26</td>
<td>4.42 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1.16 ± 0.16</td>
<td>0.97 ± 0.09</td>
<td>0.95 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1.06 ± 0.08</td>
<td>0.83 ± 0.09</td>
<td>1.1 ± 0.08</td>
<td></td>
</tr>
</tbody>
</table>

DP, dichloro(4,4′-dipropyl-2,2′-bipyridine) platinum.

FIGURE 5. Enhancement of chemotherapeutic effect after photochemical internalization (PCI) with (a) dichloro(4,4′-dipropyl-2,2′-bipyridine) platinum (DP) and (b) bleomycin (BLM) MCF-7 cells. 0 J denotes drug with photosensitizer with no illumination; 0.75 J denotes PCI effect for the same drug. PCI resulted in significantly decreased cell survival at given drug concentrations.
IV. DISCUSSION

Breast cancer is the most common cancer and the second leading cause of cancer death among women in developed countries. Treatment modalities include surgical strategies for tumor removal along with adjuvant or neoadjuvant chemotherapy, local RT, hormone receptor modulators, and specific molecular targeting agents such as human epidermal growth factor receptor-2 inhibitors and vascular endothelial growth factor inhibitors. Although surgical strategies have varied from simple lumpectomies to radical mastectomies, BCS is now recognized as the preferred therapy for patients with stage I and II invasive breast cancer. Despite advances in early detection and understanding of the molecular basis of breast cancer biology, 30% of patients with early stage breast cancer have recurrences.

The results of this study clearly demonstrate the sensitivity of 3 breast cancer cell lines to PCI-mediated drug delivery. The cell lines were selected because of their established roles in preclinical breast cancer research. The 4 chemotherapeutic agents were chosen because of their varied mechanisms of cellular uptake and toxicity. The effects of PCI for the 4 drugs—CP, DP, Dox, and BLM—on all 3 cell lines were compared with the effects of drug alone for equivalent drug doses.

PCI of CP or Dox gave no significant increase in efficacy compared with drug alone. CP is an inorganic alkylating agent used for the treatment of a wide variety of malignancies including those of the brain, ovary, breast, head and neck, cervix, and testicles. It is thought to enter the cell by passive diffusion, but there is also evidence to suggest the involvement of transport proteins. Cytotoxicity is due to the formation of DNA adducts. Formation of CP-DNA cross-links distorts the DNA double helix, leading to unwinding and kinking of the DNA. This prevents effective repair, leading to inhibition of DNA replication and transcription and ultimately death.

Dox is an anthracycline antibiotic commonly used for the treatment of a number of cancers including breast, ovarian, small cell lung carcinoma, and acute leukemias. It interacts with membranes containing acidic phospholipids and enters the cell by passive diffusion or carrier-mediated transport. Its mechanism of action is complex and is believed to be due to intercalation with DNA, resulting in inhibition of DNA and RNA synthesis. Because these 2 drugs do not enter cells by endocytosis, PCI would not be expected to have an enhancing effect, which is what was observed. This is in agreement with the negative results obtained with PCI of Dox on the uterine fibrosarcoma cell line MES-SA, as well as that on the MCF-7 cell line reported by Lou et al. On the other hand, Lou et al did find that PCI of Dox could reverse the resistance to the drug of the resistant cell line MCF-7/ADR. Multiple drug resistance (MDR) is a major clinical problem that seriously reduces the efficacy of many chemotherapeutic agents for...
breast cancer. The establishment of an MDR phenotype by cancer cells is a result of complex molecular events and is enhanced by genetic amplification through natural selection of resistant cancer cells following repeated chemotherapy cycles.30 The dominant mechanism includes increased drug sequestration in acidic vesicles, followed by transport to the outer cell wall and extrusion into the external medium coupled with over-expression of cell surface efflux pumps that purge a wide spectrum of chemotherapeutic agents from cells, thereby decreasing their intracellular accumulation.44–46 Pharmacologic inhibition of cell surface efflux pumps as a method to reverse MDR in cancer patients has been studied extensively, but the results generally have been disappointing.30 PCI of a variety of anticancer agents, however, has been reported as a therapeutic strategy to kill MDR cancer cells.42,43,47

In contrast to the results obtained with CP, PCI of CP’s analog DP showed a highly significant (P < 0.05) increase in its cytotoxic effect on all 3 cell types (Figs. 4 and 5). DP is a novel CP analog that was synthesized at the University of Nevada, Las Vegas, in 2007. The addition of the bipyridine ring system to CP doubles its molecular weight and results in a 10-fold increase in lethality in prostate and breast cancer cell lines.37,48 The results shown in Fig. 2 clearly demonstrate a significantly improved efficacy compared with CP. The intrastrand lesions caused by CP typically are repaired by the nucleotide excision repair pathway. The introduction of the 2,2’-bipyridine ligand in DP might inhibit the binding of the nucleotide excision repair pathway proteins, thus preventing or inhibiting the repair of the platinum adducts. Because DP is a larger molecule than CP, it likely depends on active uptake by endocytosis for intracellular transport.48 This would explain the increase in its cytotoxic effect by PCI, which greatly enhances endosomal escape. Even though apoptosis was the dominant method of cell death, its occurrence (versus necrotic cell death) was related primarily to the chemotherapeutic agent used in PCI. A significant increase in apoptosis was observed in response to PCI-mediated delivery of DP in the 2 cell lines examined. One of the cell lines used in this study, MDA-MB-435, is known to have a mutant p53 gene. P53-dependent apoptosis following DNA damage by CP and its analogs has been suggested as an explanation for the sensitivity of this cell line to this drug. Flow cytometry data demonstrated a higher level of apoptosis in MDA-MB-435 cells compared with MDA-MB-231 cells following PCI of DP, suggesting that p53 plays a protective role against the action of the drugs or that the p53 pathway antagonizes the drug mechanism.

The effects of BLM have been shown to be enhanced significantly by PCI on a number of cell types, which is in agreement with the results presented here for breast tumor cell lines.26,27,49 BLM is a large, water-soluble glycopeptide that exerts its cytotoxic effects by inducing single- and double-stranded DNA breaks.50 BLM commonly is used in a number of standard cancer therapies including treatment of squamous cell carcinoma of the head and neck, esophagus, bronchus, skin, and testis and malignant lymphomas. Because of its hydrophilic nature, BLM does not easily penetrate plasma membranes and is therefore transported into cells by endocytosis. Its limited ability to escape from the resulting intracellular endosomes leads to its inactivation, resulting in a relatively low sensitivity to bleomycin.51 However, once in the cytosol, BLM has a significant toxic effect. The ability of PCI to allow the endosomal escape of BLM into the cytosol is a probable explanation for the high α values obtained (Table 2). It has been estimated that as few as 500 BLM molecules can induce cell death.52

Effective PCI-mediated drug therapy in the BCS resection cavity will depend on the development of specialized light delivery devices using balloon applicators and optical fibers. The feasibility of such an approach has been demonstrated previously for brain tumor resection and can be modified easily for use following breast surgery.53–56 Such an indwelling delivery device would be similar to interstitial brachytherapy balloons already in use following BCS with established safety and efficacy profiles.57,58 This type of device would offer the following advantages: it would define the cavity to be irradiated and provide uniform light distribution to the margins; it could be completely implanted under the skin for chronic light delivery, minimizing infection risk and allowing repeated access to the cavity via simple skin puncture; and it could be modified to allow direct delivery of photosensitizer to the target tissue.
V. CONCLUSION

The efficacy of chemotherapeutic agents such as DP and BLM can be enhanced significantly in a site-specific manner using PCI. PCI treatment causes a decrease in the percentage of viable cells predominantly by apoptosis. With appropriately designed delivery devices, PCI holds the promise of enhancing tumor cell toxicity surrounding the cavity of BCS resection sites and decreasing local recurrence.

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