In Vitro and in Vivo Activity of Protein Kinase C Inhibitor Chelerythrine Chloride Induces Tumor Cell Toxicity and Growth Delay in Vivo

Steven J. Chmura, M. Eileen Dolan, Amy Cha, Helena J. Mauceri, Donald W. Kufe, and Ralph R. Weichselbaum

Departments of Pathology [S. J. C., A. C.], Radiation and Cellular Oncology [H. J. M., R. R. W.], and Medicine [M. E. D.], Division of Biological Sciences, University of Chicago and the Pritzker School of Medicine, Chicago, Illinois 60637, and Cancer Pharmacology, Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115 [D. W. K.]

ABSTRACT

Although clonogenic or divisional death is the main mechanism by which DNA-damaging agents demonstrate antitumor activity, recent data indicate that strategies specifically designed to trigger apoptosis may also prove to be useful antitumor agents. Protein kinase C (PKC) isoenzymes are involved in the regulation of cell proliferation, differentiation, and survival. Whereas pharmacological inhibition of PKC activity triggers apoptosis in most mammalian cells, cell line and tissue differences in sensitivities to these inhibitors remain. Whereas PKC inhibitors have potential as antitumor agents, issues of kinase specificity and solubility have remained obstacles to their clinical use. In this report, we investigated the antitumor activity of the PKC inhibitor chelerythrine chloride (chelerythrine), a selective inhibitor of group A and B PKC isozymes. Chelerythrine exhibited cytotoxic activity against nine human tumor cell lines tested in vitro. On the basis of the finding that radioresistant and chemoresistant squamous cell carcinoma lines (HNSCC) undergo apoptosis rapidly after treatment with chelerythrine in vitro, we assessed the effects of this agent on p53-deficient SQ-20B HNSCC cells in vivo. The results demonstrate that chelerythrine treatment of nude mice bearing SQ-20B is associated with significant tumor growth delay. Significantly, treatment with chelerythrine resulted in minimal toxicity. These findings demonstrate a potential for chelerythrine as an antitumor drug against squamous cell carcinoma.

INTRODUCTION

PKC isozymes comprise a family of at least 11 serine/threonine protein kinases. All PKC family members contain an amino-terminal regulatory domain that includes PS and phorbol ester binding sites (1). The COOH-terminal catalytic domain binds both ATP and substrate and contains autophosphorylation sites. The COOH-terminal domain represents a main target for pharmacological inhibition of PKC isoforms.

Nonspecific inhibition of serine-threonine kinases, such as the fungal alkaloid staurosporine, has been used to study the role of PKC inhibition in the induction of apoptosis as first described by Bertrand et al. (2). The results demonstrate that kinase inhibition triggers apoptosis of nucleated mammalian cells throughout different phases of the cell cycle (3–6). Apoptosis is also induced after cellular exposure to other PKC inhibitors, such as naphthalene sulfonamides (7 and 8) and the ceramide metabolite sphingosine (9–12). Significantly, numerous reports have demonstrated little if any selectivity of these inhibitors for PKC compared with other intracellular kinases (13, 14). Therefore, the potential clinical role for selective PKC inhibitors remains unclear.

In the induction of apoptosis, several intracellular events are altered prior to the activation of caspases. These events include release of free Ca$^{2+}$ from intracellular stores, down-regulation of Bcl-2, (15–17), and p34$^{\text{cdc2}}$ activation outside of the G2-M cell cycle phase (18–21). PKC activity is important in suppressing sphingomyelin hydrolysis and the subsequent induction of apoptosis by ceramide (22, 23). In this context, inhibition of PKC with chelerythrine chloride induces apoptosis by activation of a neutral sphingomyelinase, accumulation of ceramide, and depletion of sphingomyelin (12, 24–26). These findings suggest that PKC inhibition may also trigger apoptosis through the activation of the ceramide-signaling cascade.

Alternatives to staurosporine have been isolated in an attempt to obtain more specific inhibition of PKC. One of the most specific PKC inhibitors developed is chelerythrine chloride (27), a benzophenanthridine alkaloid. In contrast to staurosporine, chelerythrine is at least 100-fold more selective for PKCs than for other kinases (i.e., PKA, PKG). Chelerythrine competes for the conserved catalytic sites of PKC and seems to be a potent and specific inhibitor of the group A and group B

Received 8/4/99; revised 9/16/99; accepted 11/4/99.

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1 Supported by NIH Grants C50 DE/CA11921, GM07183, 5-R01-CA41068, 5-R01-CA42596, PO1-CA-19266, and HD-07009. S. J. C., D. W. K., and R. R. W. have a direct or indirect financial interest in Ilex Research.

2 To whom requests for reprints should be addressed, at University of Chicago Hospitals, Department of Radiation Oncology, 5841 S. Maryland Avenue, MC 1089, Chicago, IL 60637. Phone: (312) 702-0817; Fax: (312) 702-1968; Email: rrw@rover.uchicago.edu.

3 The abbreviations used are: PKC, protein kinase C; DAG, diacylglycerol; PS, phosphatidylserine; DAPI, 4’,6-diamino-2-phenylindole; PI, propidium iodide; PKA, cyclic-AMP dependent protein kinase; PMA, phorbol 12-myristate 13 acetate.
kinases (27–30). Unlike H7 and staurosporine, chelerythrine does not inhibit other kinases or activate phospholipase D at concentrations that induce apoptosis. The specificity of chelerythrine for PKC has prompted the use of this agent to study PKC function in cells (31).

We report herein that chelerythrine chloride (chelerythrine) is cytotoxic to nine human tumor cell lines. Chelerythrine was chosen for its kinase specificity, ease of delivery in vivo, ability to trigger ceramide accumulation, and inhibition of PKC in an ATP-independent manner (27, 30, 32). We selected a p53-deficient chemo/radiation-resistant tumor cell line, SQ-20B, to test whether chelerythrine induces growth delay or tumor regression in vivo. We identified apoptosis as the predominant mechanism of chelerythrine-induced cell killing in vitro. Although previous reports have failed to demonstrate in vivo antitumor activity for chelerythrine in leukemia models (33, 34), treatment of nude mice bearing SQ-20B xenograft tumors with i.p. chelerythrine produced growth delay. Importantly, chelerythrine exhibited minimal systemic toxicity. Our preclinical findings suggest that chelerythrine or other similar compounds may be effective against certain human tumors that are otherwise resistant to standard regimens.

MATERIALS AND METHODS

Drugs and Reagents. Chelerythrine chloride, ATP, PBS, and PI were purchased from Sigma Chemical Corp. (St. Louis, MO). Chelerythrine was dissolved in sterile water for in vitro experiments or in PBS for animal studies immediately prior to use.

Cell Culture. Human head and neck squamous cell carcinoma line (SQ-20B) was grown in DMEM:F-12 (3:1), 20% fetal bovine serum (Life Technologies, Inc, Grand Island, NY), 1% penicillin-streptomycin, and 1% hydrocortisone at 37°C in a humidified atmosphere containing 5% CO2. The human colon carcinoma cell line HT29 was a gift from Dr. L. C. Erickson, Loyola Medical Center, Maywood, IL, and was grown in DMEM supplemented with 10% fetal bovine serum and 2 mM l-glutamine. MCF7 cells resistant to adriamycin (MCF7 ADR) originated in Dr. K. Cowan’s laboratory (National Cancer Institute, Bethesda, MD). All MCF7 cells were grown in Richter’s improved MEM supplemented with 10% fetal bovine serum and 2.2 g/l sodium bicarbonate. Daoy cells were grown in Richter’s improved MEM zinc option medium supplemented with 10% fetal bovine serum, 2.2 g/l sodium bicarbonate, 40 µg/ml gentamicin, 20 mM HEPES, and 10 mM l-glutamine. SQ20B, SCC61, JSQ3, and SCC35 were grown in 75% DMEM, 25% Ham’s F-12K medium supplemented with 20% fetal bovine serum, 0.4 µg/ml hydrocortisone, and 100 units/ml penicillin-
Chelerythrine induces growth delay. Athymic (nude) mice were injected s.c. in the right hind limb with 5 × 10⁶ SQ20B cells. Tumors were allowed to grow for 8 days, at which time treatment was started. Control mice were injected i.p. with PBS on days 8, 10, and 12. Treatment group 2 mice (n = 10) were injected once i.p. on days 8, 10, and 12 with 2.5 mg/kg chelerythrine chloride, treatment group 3 mice (n = 8) were injected once i.p. on days 8, 10, and 12 with 5 mg/kg chelerythrine chloride, and treatment group 4 mice (n = 10) were injected once i.p. on days 8, 11, 14, with 5 mg/kg chelerythrine chloride. Tumor volume was determined as described above, with each data point representing the mean of one to three individual experiments (±SEM). *p < 0.05; Mann-Whitney Rank Sum Test.

**PKC Assay via Phosphotransferase.** Two assays were used to quantitate PKC phosphotransferase activity after treatment of cells with chelerythrine. Both assays rely on the ability of activated PKC from crude cell extracts to phosphorylate a substrate in vitro. Specificity is derived from competition with a peptide inhibitor that binds the pseudosubstrate site of PKC pseudosubstrate inhibitor, and normalized to 10⁵ cells per assay.

**PI Exclusion Assay for Apoptosis.** Cells (2.5–3.5 × 10⁶) were cultured in 24-well tissue culture plates (2 ml) for all experiments. Cells were treated with varying concentrations of chelerythrine and incubated for 4 h at 37°C, and then the media was washed off and replaced. At the indicated time points, cells were harvested, washed once, and resuspended in PBS containing 50 μl of 100 μg/ml PI. Viability was analyzed by flow cytometry (FACS) on a FACScan (Becton-Dickinson) using Lysis II software.

**Growth of Human Tumor Xenografts.** SQ-20B (1–5 × 10⁶) tumor cells were injected into the right hind limbs of Sprague-Dawley nude mice (Frederick Cancer Research Institute, Frederick, MD). Xenografts were grown for 2–3 weeks, at which time animals were sorted into treatment groups, such that the mean tumor volume was 45 ± 4.5 mm³ (±SD). At day 0, initial tumor volume was determined by direct measurement with calipers and calculated using the equation \( l \times W^2 \times 0.5 \) (36). During treatment, tumor volumes were measured twice weekly and are presented as percent of original tumor volume. Data are presented as fractional tumor volume ± SEM. Significance was analyzed using the Mann-Whitney Rank Sum Test (*p < 0.05) and denoted in the graphs by an asterisk (*).

Control mice (n = 12) were weighed and injected i.p. with PBS on days 8, 10, and 12. To determine the proper injection volume, the weight of a mouse (in grams) was divided by 100, and the resulting number (in microliters) was used. Chelerythrine was dissolved in the corresponding volume of PBS. Mice in treatment group 2 (n = 10) were injected i.p. on days 8, 10, and 12 with 2.5 mg/kg chelerythrine. Treatment group 3 mice (n = 8) were injected i.p. on days 8, 10, and 12 with 5 mg/kg chelerythrine. Treatment group 4 mice (n = 10) were injected i.p. on days 8, 11, 14, and 15 with 5 mg/kg chelerythrine. Volume and body weight were determined on days 8, 10, 13, 15, 17, 21, 24, 27, 30, and 34. Each data point represents the mean of one to three individual experiments.

**RESULTS**

**Chelerythrine Inhibits PKC Activity in Vivo.** Although previous reports demonstrate that chelerythrine is a potent inhibitor of PKC in vitro and in WEHI-231 cells in vivo (24), the following studies were conducted to ensure that PKC activity was inhibited in whole cell lysates from treated SQ-20B peptide was determined in a liquid scintillation counter (35). Enzyme activity is reported as picomoles of 32P incorporated per minute, subtracted from the activity with the addition of the pseudosubstrate inhibitor, and normalized to 10⁵ cells per assay.

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor Growth</th>
<th>Day to Reach Five-fold Initial Tumor Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/kg 48 h 3× PBS (Control)</td>
<td>n</td>
<td>12</td>
</tr>
<tr>
<td>2.5 mg/kg 48 h 3× (Group 2)</td>
<td>n</td>
<td>10</td>
</tr>
<tr>
<td>5 mg/kg 48 h 3× (Group 3)</td>
<td>n</td>
<td>8</td>
</tr>
<tr>
<td>5 mg/kg 72 h 3× (Group 4)</td>
<td>n</td>
<td>10</td>
</tr>
</tbody>
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**Figure 3**

Fractional tumor growth over time after treatment with chelerythrine. Squares depict the mean percent of original tumor volume ± SEM.
cells. Cells were treated with increasing concentrations of chelerythrine, lysed, and assayed for the phosphotransferase activity of PKC using a peptide derived from glial fibrillary acidic protein (37). The reaction was carried out in the presence of phosphatidylserine and Ca$^{2+}$ for the experimental group to ensure PKC group A and group B activity. Within 30 min after the addition of chelerythrine, the shortest exposure time that produced detectable growth inhibition in the in vitro assays, PKC activity decreased in a dose-dependent manner, with the phosphotransferase activity of PKC abolished after treatment of cells with 10 μM chelerythrine. The Nonradioactive Protein Kinase Assay kit (Panvera) also was used to confirm the results, and a representative experiment is shown in Fig. 1. Thus, chelerythrine inhibits the PKC group A and group B isoforms in the dose range used for the following in vitro experiments.

Chelerythrine Exhibits a Broad Range of Cytotoxic Activity in Vitro. The cytotoxic effects of chelerythrine were evaluated in nine human tumor cell lines to assess its spectrum of activity. Chelerythrine decreased cell viability as determined by the MTT assay in a dose-dependent manner in MCF7 breast (wt p53; Ref. 38), MCF7ADR breast (resistant to adriamycin), HT29 colon (mutant p53; Ref. (39), DaOY brain (mutant p53; Ref. 40), and LnCaP (mutant p53; Ref. 41) prostate cells. The ED$_{50}$ ranged between 2 and 5 μM upon exposure to drug for 4 h (not shown). Exposure beyond 4 h up to a 56-h exposure did not alter the ED$_{50}$. Equivalent dose-response curves (ED$_{50}$, 4.0–5.2 μM) were found for several radio and/or chemo-resistant oral tumor cell lines, including SQ-20B, JSQ-3, and SCC-35 (all mutant p53; Ref. 42) and for the radiosensitive SCC61 line (wild type p53; Refs. 43, 44).

On the basis of this initial screening, we then examined the effects of 4 h of chelerythrine exposure on clonogenic survival and apoptosis in the oral tumor cell lines as shown in Fig. 2, A and B. The results demonstrate that at concentrations of chelerythrine <3 μM, the percentage of apoptotic cells 24 h after exposure of the cell lines to chelerythrine, as determined by flow cytometry and PI exclusion, correlates well with clonogenic survival. However, the SCC35 cells proved to be more resistant to cell killing, as determined by the clonogenic assay at concentrations of chelerythrine greater than 3 μM. These data suggest that apoptosis is the predominant mechanism of cell death in all of the oral cell lines. We selected the SQ-20B cell line for the subsequent apoptosis studies and animal experiments as an example of a radio/chemo-resistant human epidermoid carcinoma line (ED$_{50}$, 3.7 μM with chelerythrine; Refs. 45, 46). In comparison to other commonly used cytotoxic drugs, such as paclitaxel and cisplatin, in SQ20B cells, our laboratory and others have demonstrated substantial clonogenic killing between 1 and 5 μM. For example, paclitaxel inhibits growth of SQ20B cells in the 600–900-nM range (47). These results suggest that chelerythrine is similar in potency to commonly used chemotherapeutic agents (48).

Chelerythrine Exhibits Antitumor Activity in SQ-20B Xenografts. We selected a dosing schedule for mice based on our initial toxicity studies. Because rapid bruising appeared on most mice given single-dose toxic concentrations (>25 mg/kg), we split the dosing schedule to minimize toxicity. In a previous report using the p388 mouse leukemia model, chelerythrine after a single dose did not result in an antileukemic effect (33). Mice bearing SQ-20B xenografts (n > 8 for all groups) were injected i.p. with 2.5 mg/kg on days 8, 10, and 12 (group 2, n = 10), 5 mg/kg on days 8, 10, and 12 (group 3, n = 8), and 5 mg/kg on days 8, 11, and 14 (group 4, n = 10). As demonstrated in Fig. 3, chelerythrine resulted in tumor growth delay in all treatment groups. The mean tumor volume on day 13 for group 2, on day 10 for group 3, and on day 13 for group 4 was statistically
significantly reduced ($P < .05$). By day 21, the mean tumor volumes measured 370 mm$^3$ in control group, 197 mm$^3$ in group 2, 215 mm$^3$ in group 3, and 115 mm$^3$ in group 4. Animals were removed from the control group if measurements exceeded 1.5 cm$^3$ and were terminated on day 34 because of tumor burden in the untreated group. No animals were removed from the treatment groups. These data demonstrate that chelerythrine inhibits tumor growth.

To assess growth delay, we compared the time taken for tumors in each group to reach five times their original volume. As shown in Table 1, chelerythrine inhibited tumor growth in all treatment groups compared with control studies. At no time did the body weight of any experimental animal fall below 90% of the initial weight at the beginning of treatment (Fig. 4). There were no treatment-related deaths at these concentrations of chelerythrine. Similar results were obtained in two independent experiments. These results suggest that chelerythrine has the potential to delay tumor growth at concentrations that produce acceptable weight loss.

**DISCUSSION**

Our data demonstrate that chelerythrine is cytotoxic to tumor cell lines regardless of their p53 status. It is interesting to note that doses of chelerythrine are comparable to that of other agents such as cisplatin and paclitaxel in SQ-20B cells (47, 48). We also show that chelerythrine produces tumor growth inhibition in an HNSCC tumor model of SQ-20B xenografts in mice. These data coupled to the <10% weight loss in the treatment groups suggests that chelerythrine may be a useful antitumor agent. We recognize the potential limitations of xenograft and in vitro models as screening tools for antineoplastic agents. However, the present findings suggest that chelerythrine chloride produces antineoplastic effects against diverse histological subtypes of human tumors. The development of inhibitors of PKC has interest in clinical oncology. For example, NK109, a chelerythrine analogue, is in Phase 2 trial in Japan and UCN-01 (7-hydroxystaurosporine) a PKC inhibitor, in Phase I trials in the United States.

Chelerythrine chloride produces its antineoplastic effect in part through the induction of apoptosis secondary to inhibition of PKC, activation of sphingomyelinase, and induction of ceramide production (25, 49). The demonstration that UNC-01 induces antitumor effects by mechanisms distinct from those identified for chelerythrine (50) suggests that chelerythrine may exhibit a different spectrum of activity. Other selective PKC inhibitors, such as calphostin C, which appears to act in part as a competitive inhibitor of DAG, are also under investigation for use with photodynamic therapy in bladder cancer (51, 52). Thus, chelerythrine is a potentially useful antineoplastic agent against HNSCC tumors alone or as previously demonstrated in combination with ionizing radiation (49).

**REFERENCES**

Chelerythrine Induces Apoptosis and Tumor Regression


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