The Protein Kinase C Inhibitor CGP41251 Suppresses Cytokine Release and Extracellular Signal-regulated Kinase 2 Expression in Cancer Patients

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ABSTRACT

Components of cell signaling pathways provide important targets for anticancer drugs. Protein kinase C (PKC) is a serine/threonine-specific kinase that regulates cell growth and differentiation. It is also implicated in tumor promotion. The staurosporine analogue CGP41251 is a PKC inhibitor, and it is currently in a Phase I clinical trial for treatment of advanced cancer. However, it is difficult to define its biological activity. We have used two approaches to measure the in vivo biological response to CGP41251: (a) sequential whole blood samples were taken from seven patients before and during treatment and incubated with mitogen (PHA), and cytokine release was measured ex vivo; and (b) peripheral blood lymphocytes were isolated from seven of these patients, and the levels of extracellular signal-regulated kinase 2 were measured by Western blotting. Response to PHA was significantly lowered during treatment (P < 0.001 for TNF-α production; P = 0.03 for IL-6). This was most evident at 7 and 28 days after the start of treatment in patients receiving higher doses (150–300 mg/day). TNF-α and P = 0.002, respectively, for TNF-α and P > 0.001 and P = 0.003, respectively, for IL-6 release). Whole blood cytokine production returned to pretreatment levels after drug administration ceased. The levels of extracellular signal-regulated kinase 2 were reduced by 50–97% during treatment in all seven patients tested. These results show for the first time that a PKC inhibitor can block in vivo signaling pathways in cancer patients. The assays we describe complement toxicity studies in selecting relevant doses for Phase II trial of novel agents, particularly when biological activity occurs at doses below those that cause obvious side effects.

INTRODUCTION

Components of cell signaling pathways provide important targets for new anticancer drugs. Protein kinases are integral parts of the intracellular signaling process regulating actions such as growth, differentiation, motility, and survival. PKC2 has a pivotal role in signal transduction and is a logical target for drug intervention (1, 2). It is a specific serine/threonine kinase regulating a variety of homeostatic processes, and it is important in tumor promotion (3). The staurosporine analogue CGP41251 has the PKC-inhibitory properties of the parent molecule, but it is not a broad spectrum kinase inhibitor and has limited toxicity in preclinical models (4). CGP41251 preferentially inhibits the conventional calcium-dependent and diacylglycerol-dependent PKC subtypes and has shown activity against a variety of cancer cell lines in vitro and in vivo (5–7). PKC is also involved in the acquisition/expression of the multidrug resistance phenotype of cancer cells (8, 9). CGP41251 modulates the expression/activation of P-glycoprotein via phosphorylation of the serine/threonine residues and reverses the multidrug resistance phenotype of cancer cells (10, 11).

Conventional Phase I study design requires dose escalation until toxicity is seen. Implicit in this design is the assumption that biological activity will be seen at doses close to those that are toxic. Markers of biological response may be important in determining effective but possibly nontoxic doses of drugs that inhibit signal transduction. In animal xenograft models, PKC inhibition in tumors is associated with tumor growth inhibition during treatment with CGP41251 (4). However, it is difficult to obtain adequate clinical tumor material to perform tissue-based studies in patients. Alternative methods of assessing the action of a drug on its molecular target are needed.

Because PKC is involved in upstream signal pathways causing the release of cytokines such as TNF-α (12) and the induction of IL-6 by TNF-α (13), we reasoned that such responses may be inhibited during a clinical trial of CGP41251. In a Phase I clinical trial, we therefore measured the effect of the drug on the ability of cells from whole blood to release TNF and IL-6 in response to the mitogen PHA, a signaling pathway known to involve PKC. We also measured the levels of ERK2, which is downstream of stress response signaling pathways involving PKC (14).

MATERIALS AND METHODS

Patients and Blood Samples. All patients in the clinical trial attended the Churchill Hospital (Oxford, United Kingdom) and Beatson Oncology Center (Glasgow, United Kingdom). Thirty-two patients, ages 36–76 years (median age, 63 years), with advanced solid tumors were included in the trial. All were white Caucasians with a performance status of 0–1, and all but two had had prior chemotherapy. The tumor types were mainly colorectal cancer, adenocarcinomas of unknown primary, breast cancer, and lung cancer. Patients received CGP41251 p.o., with drug doses ranging from 25 mg daily up to 300 mg daily in divided doses. This report summarizes data from 27 of the patients from whom appropriate samples were obtained. In all figures and tables, the patients are numbered, and the doses they received were as follows: (a) 25 mg daily (patients 2005 and 2006); (b) 50 mg daily (patients 2007–2012); (c) 100 mg daily (patients 2010–2012); (d) 150 mg daily (patients 2013–2016); (e) 225 mg daily (patients 2017–2025); and (f) 300 mg daily (patients 2029, 2031, and 2032).

Whole blood samples were collected from these patients five times during a 28-day treatment cycle (pretreatment, 4 h, 24 h, 7 days, and 28 days). Additional samples were collected from some patients after treatment had finished. Whole blood samples were collected into tubes containing preservative-free heparin (30 units/ml blood; Leo Laboratories Ltd., Princes Risborough, United Kingdom) and PHA [PHA; 2 μg/ml blood; HA 16/17 (Murex Biotech Ltd., Dartford, United Kingdom)] or heparin alone as controls. All samples were collected and processed under sterile and pyrogen-free conditions (15).

Whole Blood Stimulation. The blood samples were incubated at 37°C in a 5% CO2 saturated, humidified incubator. At 2 and 24 h after in vitro stimulation with PHA, the blood samples were cool spun, and the supernatant was separated, flash-frozen, and stored at –20°C.
Cytokine Immunoassays. TNF-α levels were measured using IRMA kits (Medgenix, Brussels, Belgium). The TNF-α IRMA range was 15–500 pg/ml. The assay was calibrated with the international reference preparation (87/650; NIBSC, Poitiers Bar, Hertfordshire, United Kingdom) and used at detection limits of 20 pg/ml plasma. The assay is specific for TNF-α and did not cross-react with IL-1α, IL-1β, TNF-β, granulocyte macrophage colony-stimulating factor, IFN-α, IFN-β, and IL-6. The calibration, standardization, and assay format were developed specifically for measuring TNF-α in plasma/serum samples.

IL-6 was measured in a RIA developed in our laboratory with a polyclonal antiserum G150 BM (NIBSC). The antiserum was used at a final dilution of 1:1,750,000 (equivalent to an initial dilution of 1:350,000). The standard rhIL-6 used in this study was obtained from Sandoz Pharma Ltd. (Basel, Switzerland). rhIL-6 was also used to generate 131I-rhIL-6 using the chloramine T method. The mass of IL-6 contained in the standard was calibrated with an interim standard preparation (88/514; NIBSC). The specificity of the anti-IL-6 antibody for the relevant ligand in the presence of IL-1α, IL-1β, TNF-β, granulocyte macrophage colony-stimulating factor, IFN-α, and IFN-β was confirmed. The IL-6 RIA range was from 20–10,000 pg/ml with a detection limit of 50 pg/ml.

Western Blotting. Peripheral blood lymphocytes were obtained using Lymphoprep (Nycomed Pharma AS, Oslo, Norway) from 10 ml of whole blood taken before treatment and after 24 h, 7 days, and 28 days of treatment. Cells were snap-frozen and stored at −70°C. Cells were lysed in a 1% Triton X-100 lysis buffer, and protein content was estimated by the Bio-Rad Micro Assay procedure. The samples were boiled for 2 min with equivolume sample buffer [100 mM Tris (pH 6.0), 5% SDS, 4% β-mercaptoethanol, 4% SDS, 0.02% bromphenol blue, and 10% glycerol], and 20 μg of estimated protein were loaded onto a 15% SDS-PAGE gel. Each sample was assayed at least twice on different gels. Electrophoresis was performed using standard conditions. The protein lysates ran at 200 V for 4 h on a 15 × 15-mm SDS-PAGE gel using 1× running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS). Human fibroblasts and Cos 7 cells (unstimulated and stimulated with 15 ng/ml epidermal growth factor for 5 min) were used as positive controls. Rainbow markers (Amersham) were run to determine product size. Protein was transferred to nitrocellulose (Hybond-ECL; Amersham) that had been pre-equilibrated in transfer buffer [25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.3)] using a Trans-Blot electrophoretic transfer cell (Bio-Rad) for 2–3 h at 300 mA. Blots were incubated in blocking reagent (5% milk powder) overnight. Proteins were detected using the Amersham enhanced chemiluminescence protocol. The anti-pan ERK monoclonal antibody was obtained from Transduction Laboratories (Lexington, KY) and used at 1 μg/ml. This antibody reacts with both phosphorylated and nonphosphorylated forms of ERK1 and ERK2. Western blots were quantified using NIH Image 1.58 software.

Statistical Analysis. The Generalized Estimating Equations technique (16) was used to assess overall changes in cytokine production. A paired t test was used to assess differences in cytokine level at individual time points.

RESULTS

Effects of CGP41251 Treatment on Whole Blood Production of TNF-α. Whole blood was collected before treatment and at 4, 24 h, 7 days, and 28 days during treatment in 27 of a total of 32 patients receiving CGP41251 in a Phase I clinical trial. Blood was incubated for 2 and 24 h ex vivo, and PHA-stimulated TNF-α release was assayed. As shown in Figs. 1a and 2a, there was considerable patient-to-patient variation in pretreatment PHA-stimulated TNF-α release (range, 75–7636 pg/ml after 24 h of culture), but spontaneous TNF-α release in unstimulated cultures was rarely above the limits of detection (<20 pg/ml).

Fig. 1a compares the absolute values of TNF-α release in 27 patients before treatment and after 4 h of treatment, and Fig. 1b shows the percentage change. Fig. 2a compares the absolute values of TNF-α release in 27 patients before treatment and after 7 days of treatment, and Fig. 2b shows the percentage change. A comparison of Figs. 1 and 2 shows that the ability of whole blood cells to release TNF-α was impaired after 7 days of treatment. The doses given to individual patients are described in “Materials and Methods.”

Taking data from all patients and all time points and using the statistical technique of Generalized Estimating Equations (16), there was a significant decline in the ability of the whole blood cultures to release TNF-α ex vivo in response to PHA during treatment (P < 0.001 in both 2- and 24-h cultures). The effects appeared to be dose related. For instance, a clear decline in TNF-α release was seen after 7 days of treatment in 15 of 17 patients treated with ≥150 mg/day (P < 0.002, paired t test), but only 3 of 8 patients treated at lower doses showed a decrease in TNF-α release (P < 0.76). Similar results were seen with 2-h cultures of whole blood and after 28 days of treatment in both 2- and 24-h cultures (data not shown). The effects were more variable after 4 and 24 h of treatment, but if only the patients treated with ≥150 mg/day were considered, there was still a significant decline in TNF-α release (P = 0.05 and P = 0.01). These differences in response with time are shown in Fig. 3, which compares individual cytokine release levels over the 28-day treatment course in patients treated with <150 mg/day (Fig. 3a) with those receiving ≥150 mg/day (Fig. 3b).

TNF-α Production after the End of Treatment. In five patients (patients 2015, 2019, 2023, 2024, and 2027) receiving between 150 and 300 mg/day, a whole blood assay was also carried out at 2–6 months after the final dose of CGP41251, and TNF-α production was measured. This was to determine whether the decline in TNF-α release while receiving CGP41251 was due to drug treatment or to disease progression. There was a decrease in TNF-α release after 7 days of treatment, but if only the patients treated with ≥150 mg/day (P < 0.002, paired t test), but only 3 of 8 patients treated at lower doses showed a decrease in TNF-α release (P < 0.76). Similar results were seen with 2-h cultures of whole blood and after 28 days of treatment in both 2- and 24-h cultures (data not shown). The effects were more variable after 4 and 24 h of treatment, but if only the patients treated with ≥150 mg/day were considered, there was still a significant decline in TNF-α release (P = 0.05 and P = 0.01). These differences in response with time are shown in Fig. 3, which compares individual cytokine release levels over the 28-day treatment course in patients treated with <150 mg/day (Fig. 3a) with those receiving ≥150 mg/day (Fig. 3b).
production during treatment in all five of these patients that was most marked after 7 days and 28 days of treatment. TNF-α release essentially returned to pretreatment levels in each patient 2–6 months after finishing CGP41251 treatment (see Fig. 4, a and b for two examples).

The Influence of CGP41251 Treatment on Whole Blood Production of IL-6. IL-6 was reliably detected only after a 24-h ex vivo culture with PHA. The effect of treatment was to significantly lower mitogen-stimulated IL-6 production if all patients were considered (P = 0.03, Generalized Estimating Equation). Fig. 5a compares the absolute values of 24 h IL-6 release in the 27 patients before treatment and after 7 days of treatment, and Fig. 5b shows the percentage change. Table 1 summarizes the results from the patients, dividing them into those treated with $150 \text{ mg/day}$ and those receiving lower doses. Again, significant inhibition of IL-6 production occurred at 7 or 28 days of treatment, but only in the patients receiving the higher doses (P = 0.001 and P = 0.003, respectively, paired t test).

Western Blotting of Cell Lysates. Western blotting was used to assess the levels of ERK in lymphocyte lysates from seven of the patients (patients 2008, 2009, 2011, 2013, 2019, 2023, and 2029). Only one band was detected with the anti-pan ERK antibody, and its molecular weight corresponded to the $M_r$ 42,000 nonphosphorylated ERK2. There was a decline in ERK2 levels during treatment in all of

Fig. 2. A, TNF-α release in whole blood cultures before treatment and after 7 days of CGP41251 treatment. B, TNF-α levels before treatment. C, TNF-α levels after 7 days of treatment. B, percentage change from baseline TNF-α release in patients 7 days after the start of CGP41251 treatment. Whole blood was collected from patients before treatment and after 7 days of treatment and placed into tubes containing PHA. Whole blood was incubated at 37°C for 24 h. Tubes were spun at 1000 rpm, and the plasma was stored frozen. TNF-α release in plasma was measured by IRMA. Patient numbers (2005–2032) are shown on the abscissa. The doses received by individual patients are listed in “Materials and Methods.” Patients 2021 and 2026 had insufficient samples for analysis.

Fig. 3. Summary of TNF-α release in whole blood cultures during 28 days of therapy with CGP41251. Whole blood was collected into tubes containing PHA and incubated at 37°C for 24 h. Tubes were spun at 1000 rpm, and the plasma was stored frozen. TNF-α release in plasma was measured by IRMA. Bar, the median of the values. A, patients receiving $<150 \text{ mg/day}$. B, patients receiving 150–300 mg/day. Differences were examined using the paired t test. A, pretreatment versus 4 h of treatment (paired t test), P = 0.24; pretreatment versus 24 h of treatment, P = 0.31; pretreatment versus 7 days of treatment, P = 0.75; pretreatment versus 28 days of treatment, P = 0.16. B, pretreatment versus 4 h of treatment (paired t test), P = 0.05; pretreatment versus 24 h of treatment, P = 0.01; pretreatment versus 7 days of treatment, P = 0.002; pretreatment versus 28 days of treatment, P = 0.02.

Fig. 4. TNF-α release in whole blood cultures from patient 2023 (A) and patient 2027(B) before, during, and after treatment with CGP41251. Treatment ended after 28 days.
after 7 days of therapy. Factors influencing the variability in early time point results may include different absorption rates immediately after dosing, the time needed to achieve steady-state plasma concentrations, and interpatient variability in protein binding.

Pretreatment production of TNF-α and IL-6 varied considerably from patient to patient. Although the disease state might have contributed to this, genetic variability in the production of cytokines is well documented (17). However, the inherent capacity of a patient’s cells to produce TNF-α and IL-6 did not relate to their subsequent response to the drug. High and low cytokine producers were equally inhibited by treatment with CGP41251.

Of particular interest was the fact that significant effects on cytokine release were seen at doses below the maximally tolerated dose of 225 mg CGP41251/day, suggesting that this assay may be useful in determining

Table 1  Relationship between dose administered and IL-6 release from whole blood culture during CGP41251 treatment

<table>
<thead>
<tr>
<th>Dose (mg/day)</th>
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<th>No. of patients</th>
<th>Mean % change from pretreatment</th>
<th>P</th>
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<tr>
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<td>24 h</td>
<td>7</td>
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<td>0.13</td>
</tr>
<tr>
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<td>7 days</td>
<td>8</td>
<td>9.8</td>
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<td>28 days</td>
<td>8</td>
<td>−10.2</td>
<td>0.30</td>
</tr>
<tr>
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<td>4 h</td>
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<td>24 h</td>
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DISCUSSION

These results show for the first time that a PKC inhibitor can block in vivo cell signaling pathways in cancer patients. Our results also suggest that the assays of biological response reported here might be useful in determining the appropriate doses of drugs that inhibit signal transduction and other novel agents. The first assay measured the ability of the patients’ blood cells to respond to a mitogenic signal. This assay is readily applicable to a clinical setting. The fact that the baseline release of cytokine was below the limits of assay detection showed that this technique was reliable and that our methods of sample processing eliminated any ex vivo stimulation of cells with contaminants such as endotoxin.

There was a significant response to the drug that was related to both the dose and the length of exposure to the drug, with maximum inhibition seen when stable circulating levels of drug were achieved. This was variable in the first 24 h but was more consistent after steady-state plasma levels were reached at 7 days or more. This decline ranged from 54–61% and 57–74% in three patients treated with 50 or 100 mg/day at 7 and 28 days, respectively. In the four patients treated with 150–300 mg/day, the decline ranged from 50–94% and 53–97% of pretreatment levels at 7 and 28 days. Four examples of the Western blots are shown in Fig. 6.

Fig. 5. A, IL-6 release in whole blood cultures before treatment and 7 days after the start of CGP41251 administration. B, IL-6 release before treatment. C, IL-6 release 7 days after the start of treatment. D, IL-6 release before treatment. E, IL-6 release 7 days after the start of treatment. Whole blood was collected from patients before treatment and after 7 days of treatment and placed into tubes containing PHA. Blood was incubated at 37°C for 24 h. Tubes were spun at 1000 rpm, and the plasma was stored frozen. IL-6 release in plasma was measured by RIA. Patient numbers (2005–2032) are shown on the abscissa. The doses received by individual patients are listed in “Materials and Methods.” Some patients (2009, 2010, 2011, 2014, 2021, 2023, and 2026) are excluded from this figure because a complete set of samples was not available.

Fig. 6. Western blotting for ERK2 in peripheral blood mononuclear cell lysates from patients before and during CGP41251 administration. The results shown here are for patients 2008, 2009, 2013, and 2023. Samples shown (from left to right) were obtained before treatment, after 24 h of treatment, after 7 days of treatment, and after 28 days of treatment.
the levels of drug to be used in Phase II trials. The most common side effects of CGP41251 were fatigue, nausea, and vomiting. These were observed to some degree at both low and high doses. It is no known whether PKC inhibition is involved in these side effects.

Whereas the recovery of cytokine release to pretreatment levels 2–6 months after cessation of treatment argued against our results being due to underlying disease progression, the decline in cytokine production during treatment could be secondary to hematological toxicity and a reduction in cytokine-producing cells in the whole blood samples. However, there was no significant bone marrow toxicity as measured by the total WBC count or neutrophil count before treatment versus 7 days or 1 month of therapy (18).

We were able to analyze changes in lymphocytes and monocytes in a subset of 10 patients treated across the dose range. There was a significant drop in lymphocyte counts from pretreatment to day 7, falling from a mean of $1.46 \times 10^9$ cells/liter to $1.11 \times 10^9$ cells/liter on day 7, but no further change to 28 days. For monocytes, there was again a significant decline from pretreatment to day 7, but no further significant difference was seen between day 7 and month 1. The mean pretreatment level was $0.45 \times 10^9$ cells/liter, falling to $0.23 \times 10^9$ cells/liter on day 7 and $0.26 \times 10^9$ cells/liter on month 1.

Although these 10 patients were treated at all dose levels, there was no evidence of a dose-response relationship in the change in monocyte or lymphocyte count, in contrast to the clear dose-response effect on cytokine release. Moreover, the extent of suppression of cytokine release was greater than the reduction in lymphocyte or monocyte counts. This is clearly both for the ERK2 expression and for the cytokine responses where the same of the levels fell to 10–20% of pretreatment values at the highest doses. Finally, the ERK2 expression data are based on an analysis of equal amounts of protein from isolated lymphocytes, thus: any decline in lymphocyte counts would be corrected for. In conclusion, some of the reduction in cytokine release could be due to the drop in lymphocyte count and monocyte count, but the majority of the fall and a change in time and dose could not be explained by this.

The whole blood cytokine release assay may also be useful in the study of novel therapeutics, for instance matrix metalloproteinase, or tumor necrosis factor α converting enzyme, inhibitors that inhibit release of cytokines such as TNF-α (19).

To complement the cytokine release assay, we assessed levels of a key enzyme in the stress response signaling pathway that is known to involve PKC (14). A single band corresponding in molecular weight to nonphosphorylated ERK2 was detected by Western blotting. Because protein was extracted from lymphoprep from unstimulated cells, our results showed a decline in the absolute levels of this key enzyme in the signaling pathway. Whereas these preliminary results require further investigation both in vitro and in vivo, they suggest transcriptional or translational regulation of ERK2. Our observations are in keeping with a report that CGP41251 abolishes the increased ERK activity induced by PMA in vitro (20). The reduction of ERK2 levels was detected even at the lower doses (50 mg/day) but was most pronounced and sustained in patients receiving 225 and 300 mg/day. Indeed, these responses would suggest that measuring ERK2 or other components signaling pathways may be the most sensitive assay of response to signaling inhibitors in vivo. Again, this assay can easily be used in a clinical setting. One of the problems with assessing novel drugs, such as signaling inhibitors, in Phase I trials is that dose-limiting toxicity may occur at concentrations higher than those required to modulate the target enzyme. This could lead to inappropriate chronic dosing schedules in Phase II trials. Conversely, transfer from Phase I to Phase II trials is also difficult if the target has not been inhibited in Phase I trials due to inadequate dosing. Ideally, measurement of inhibition of a target enzyme should be carried out on a tumor and its metastases in vivo. The approaches used here are a step toward this, although we do not yet know whether the changes we have observed in a surrogate tissue are equivalent to changes detected in the target tumor tissue. Animal experiments may be useful in this respect. However, the assays we have used are readily available to incorporate into Phase I trials and are less invasive than tumor biopsy. They may be helpful in selecting a dose range for Phase II studies.

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