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Actions of the selective protein kinase C inhibitor PKC412 on B-chronic lymphocytic leukemia cells *in vitro*

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Background and Objectives. The staurosporine derivative PKC412 (CGP41251) is a more selective inhibitor of the conventional isoforms of protein kinase C (PKC) than is the parent compound. In addition to its growth inhibitory properties, PKC412 reverses the efflux function of the multidrug resistance (MDR)-1 gene product, P-glycoprotein (P-gp).

Design and Methods. The *in vitro* actions of PKC412 were investigated in peripheral blood lymphocytes (PBL) from 4 normal volunteers, B-cell isolates from 3 normal tonsils and 31 patients with B-cell chronic lymphocytic leukemia (B-CLL). Following incubation with PKC412 for 2 days, the viability of B-CLL cells was decreased relative to that of controls (63±23% at 1 μmole/L; 52±30% at 10 μmole/L; n=20). Normal PBL were significantly more resistant to the drug (91±5% viable cells at 1 μmole/L; 73±18% at 10 μmole/L; n=4). Thirteen of the B-CLL patients were treated with oral PKC412 in a phase II trial.

Results. PKC activity in malignant cells from these patients showed a reduction post-treatment of 25-96% of their respective pre-treatment levels. Morphologic analysis, as well as *in situ* assay for DNA strand breaks (TUNEL assay) showed that B-CLL cells were killed by an apoptotic mechanism. In B-CLL cells the mean IC_{50}, for PKC412, as measured by the reduction of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), was 2.1 μmol/L in 16 samples in which the IC_{50} were below the maximum concentration of PKC412 used for the assay. In tonsillar B-cells, the mean IC_{50} was 11 μmol/L whereas PBL cells were resistant. Four of eight and 1/3 B-CLL samples that were resistant to chlorambucil and fludarabine, respectively, were sensitive to PKC412. In 15/31 B-CLL samples a dose-dependent reversal of P-gp-mediated drug efflux by PKC412 was observed. A statistically significant correlation ($p<0.001$) was observed between P-gp protein expression as measured by FACScan analysis and the reversal of efflux activity by either PKC412 or verapamil. PKC412 increased the sensitivity of B-CLL cells to 2'-chlorodeoxyadenosine and chlorambucil.

Interpretation and Conclusions. This study establishes the *in vitro* cytotoxic and multidrug resistance (MDR) modulatory properties of PKC412 towards malignant cells from B-CLL patients. The direct anti-tumor activity combined with the potential for P-gp modulation make PKC412 an attractive drug for the treatment of malignancies expressing the MDR phenotype, or in combination with conventional drugs.

Key words: B-chronic lymphocytic leukemia, protein kinase C, PKC412, multidrug resistance, apoptosis.

The protein kinase C (PKC) family of enzymes is involved in the transduction of signals that regulate cell proliferation and differentiation. It is implicated in a variety of cellular disorders in which signal transduction pathways are dysregulated.[1] The PKC family comprises at least twelve isoenzymes which phosphorylate serine and threonine residues, possess different requirements for optimal activity, are differentially expressed in various tissues and which are independently regulated.[2] Some studies show a relationship between increased PKC expression and malignant transformation.[3]

PKC functions to oppose apoptosis, particularly in hematopoietic cells and various PKC inhibitors are potent inducers of apoptosis in neoplastic cells. The role of PKC in the regulation of apoptosis may involve induction of bcl-2 mRNA and protein,[4] phosphorylation of bcl-2,[5] or modulation of intracellular pH.[6] Upregulation of unidentified NFκB inducible genes[7-9] may also involve PKC. Moreover, there is substantial evidence that inhibition of PKC may increase the susceptibility of neoplastic cells to cytotoxic drug-induced apoptosis.[10] The important roles played by PKC in the regulation of cell proliferation and apoptosis and thereby malignancy suggest that it is a potential target for the development of anti-cancer agents.[11]

Considerable indirect evidence suggests that P-glycoprotein (P-gp), the product of the multidrug resistance (MDR) –1 gene, is phosphorylated by PKC and that phosphorylation increases its ability to extrude cytotoxic drugs from malignant cells.[12] The MDR phenotype of most cancer cells correlates with the level and activity of PKC. PKC activity is elevated in a number of selected MDR cell lines. Treatment of cells expressing P-gp with PKC activators has been shown to increase drug resistance. By contrast, PKC inhibitors decrease drug resistance. PKC is a positive regulator of P-gp ATPase activity and this may account for the increased drug resistance observed in P-gp overexpressing cells following activation of PKC α.[13] Co-immunoprecipitation studies have shown that P-gp interacts selectively with the α, β, γ, ε and θ PKC isoforms.[14]

The naturally occurring compound staurosporine is a potent inhibitor of PKC and displays high antiproliferative and apoptosis-inducing activity.[15,16] However, it does not selectively inhibit specific PKC isoforms. A derivative of staurosporine, PKC412 (N-benzoyl staurosporine) is a less potent PKC inhibitor but shows a higher degree of selectivity towards *conventional* PKC isoforms α, β_1, β_2 and γ.[17] The proliferation of mitogenically stimulated normal human peripheral blood T-lymphocytes is inhibited by staurosporine at an IC_{50} of <0.01 μmole/L and by PKC412 at an IC_{50} of 0.092 μmole/L.[18]

PKC412 has high affinity for P-gp and sensitizes P-gp-expressing cells to cytotoxic agents.[19] It was more efficient than staurosporine in the reversal of anthracycline efflux in the anthracycline-resistant subline of ovarian carcinoma.[20] In the same study with multidrug resistant human tumor cell lines, 150nmole/L PKC412 enhanced the cytotoxic action of adriamycin. The synergistic action was less pronounced or not observed when the P-gp-negative parental cell line was studied.

Preliminary studies show that PKC412 was well absorbed after oral administration and well tolerated by healthy adult male volunteers (*unpublished observations*) and by patients with advanced cancer.[21] Therefore, PKC412 may have therapeutic value in combination with conventional drugs in the treatment of tumors expressing an MDR phenotype.[22,23]

Previous studies on the actions of PKC412 have been on either normal or malignant human cell lines. Studies have shown that P-gp can be detected in chronic lymphocytic leukemia (CLL) cells and that its expression increases with advancing stage post-therapy[24] and in patients treated with P-gp-transportable drugs.[25] We have previously investigated apoptosis in cells from B-CLL[26,27] and MDR in relation to clinical outcome in blasts from acute leukemia[28] patients. The effect of PKC412 as a cytotoxic agent and as a modulator of MDR was, therefore, investigated in B-CLL cells *in vitro*.

Design and Methods

Patient material

Peripheral blood was collected in preservative-free heparin from 31 patients with B-CLL. Their age ranged from 46 - 87 (mean 66) years, 17 were male and 14 were female. Their Binet stages were A=13, B=10 and C=8. Thirteen had received no previous therapy (Binet stage A=8, B=5) while 18 (Binet stage A=5, B=5, C=8) had received previous therapy. The white cell count ranged from 18.7 to 182.5 × 10^9/L. No patient had received chemotherapy or radiotherapy in the three months prior to this study.

Mononuclear cells were separated by density gradient centrifugation on Lymphoprep (Nygaard, Norway). They were depleted of monocytes by adherence to plastic and T-cells were removed by rosetting with sheep erythrocytes. The final B-CLL cell purity was assessed by FACScan analysis (Becton Dickinson, UK) using CD5 and CD2 and found to be greater than 90%.

Peripheral blood lymphocytes (PBL) were also obtained from five normal individuals and three tonsils removed at surgery. The tonsillar cells were teased and T-cells depleted to give a final B-cell population of >90% purity.

Assessment of cell viability and apoptosis

PBL and B-CLL cells were incubated at 2 × 10^6 cells/mL in RPMI 1640 medium supplemented with antibiotics and 15% fetal calf serum (Life Technologies, UK). Following 48h incubation, cell via-

bility was estimated by determining the percentage of cells excluding 0.1% trypan blue. The percentage of apoptotic cells was determined by morphologic analysis of May-Grünwald Giemsa-stained cytospin preparations.[29] Apoptotic cells were also quantified by labeling of DNA strand breaks with terminal deoxynucleotidyl transferase and fluoresceinated dUTP (TUNEL technique),[29] using the Apoptosis Detection Kit (Promega, UK).

In vitro cytotoxicity

In vitro cytotoxicity was studied using the MTT assay at six concentrations of PKC412 or of cytotoxic drugs. Three cytotoxic drugs conventionally used in the treatment of CLL were studied, ie., 2'-chlorodeoxyadenosine, chlorambucil and fludarabine (all from Aldrich-Sigma, UK). The cytotoxic drugs were studied either alone or in combination with 100 nM of PKC412. Results are expressed as the concentration of drug that decreased cell viability by 50% in the MTT assay compared with controls incubated in the absence of drug (IC_{50}, calculated by IC_{50} software). PKC412 was dissolved in DMSO and diluted stepwise in RPMI medium. Further serial dilutions were performed in RPMI and the highest concentration achieved in culture without reprecipitation was 10mmole/L with 0.5% DMSO. This concentration of DMSO did not affect cell viability in the MTT assay when added alone.

MDR profile

P-gp, multidrug resistance associated protein (MRP) and lung resistance protein (LRP) expression was studied on a FACScan (Becton Dickinson) using monoclonal antibodies MRK16 (P-gp, TCS Biologicals, UK), MRPm6 (MRP, Monosan, UK) and LRP56 (LRP, Monosan, UK). The results are expressed as a ratio of mean cell fluorescence (MCF) of the specific antibody relative to the MCF of isotype-matched control serum.

The modulation of drug efflux by 10 μmole/L verapamil (VPM, Aldrich-Sigma, UK) or PKC412 at 10, 50, and 100 nmole/L was studied on a FACScan using the fluorescent dye DiOC, an established substrate for P-gp.[30] A limited number of samples were also studied with 1 μmole/L PKC412. The results are expressed as a ratio of mean cell fluorescence (MCF) values obtained in the presence and absence of the modulator.

PKC activity

PKC activity in malignant cells was assayed as described earlier.[31] Essentially, activity was measured in cell extracts with protamine sulphate as activator and substrate in the presence of ^{32}P-γ-ATP. Activity was expressed as cpm/μg of protein from cell extracts before and after treatment with PKC412.

Statistical methods

Data were analyzed by Student's t test for paired samples.

Results

Malignant cells from B-CLL patients are more sensitive to apoptosis induction by PKC412 than are normal peripheral blood lymphocytes

The actions of PKC412 on normal PBL from five donors are shown in Figure 1. A significant decrease in viability following 2 days of incubation was observed only at a drug concentration of 10 μmole/L (Figure 1A). Although PKC412 induced an increase in the percentage of apoptotic cells, this was not statistically significant at any of the concentrations tested (Figure 1B). The TUNEL assay also failed to reveal a significant induction of apoptosis by 10 μmole/L PKC412 (data not shown).

Malignant cells isolated from different patients showed highly variable sensitivity to the cytotoxic actions of PKC412. When mean values were compared, a significant decrease in viability was observed even at 0.1 μmole/L PKC412 (Figure 1A). However, some isolates were almost entirely resistant to killing by even 10 μmole/L drug while others showed >90% of dead cells at this concentration (Figure 1A). The induction of apoptosis, as detected by morphologic criteria (Figure 1B) or by the TUNEL assay (Figure 1C), was also highly variable. When mean values were compared, significant apoptosis induction was detected at 1 and 10 μmole/L PKC412. However, some isolates were almost entirely resistant to apoptosis induction even at 10 μmole/L drug, whereas others showed a >15% increase in apoptotic cells at 1 μmole/L PKC412 (Figures 1A and 1B). An inverse correlation was observed between percent surviving and percent apoptotic cells (data not shown). No significant correlation was observed between sensitivity to PKC412 *in vitro* and Binet stage, white cell count or previous treatment history. Incubation of CLL cells *in vitro* with human plasma in place of FCS resulted in a substantial decrease in spontaneous and PKC412 induced apoptosis at concentrations up to 10 μmole/L, the maximum concentration that could be achieved *in vitro* (data not shown).

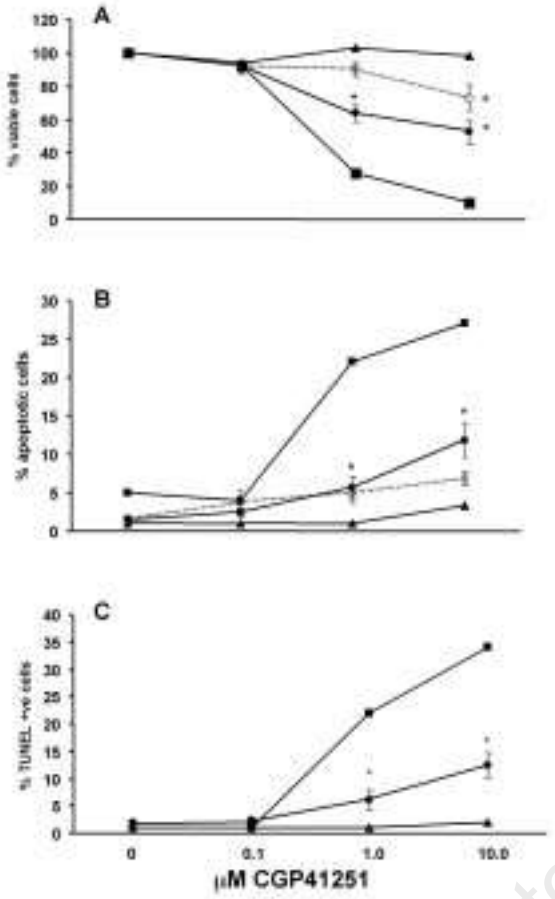

Figure 1. Action of PKC412 on A, viability and B, induction of apoptosis and C, induction of TUNEL-positive cells in normal human mononuclear cells (O) (n=5) and malignant cells from B-CLL patients (n). Bars represent SEM in this and all subsequent figures. An asterisk denotes a significant change relative to control values ($p < 0.01$). Incubations were for 48h. The responses of the most sensitive (■) and most resistant (▲) isolates are also shown to indicate the range of responses obtained in malignant cells. In malignant cells, for control and 10 μmole/L incubations, n=20 in A and n=18 in B and C. For incubations with 0.1 and 1 μmole/L drug, n=20 in A and n=14 in B and C.

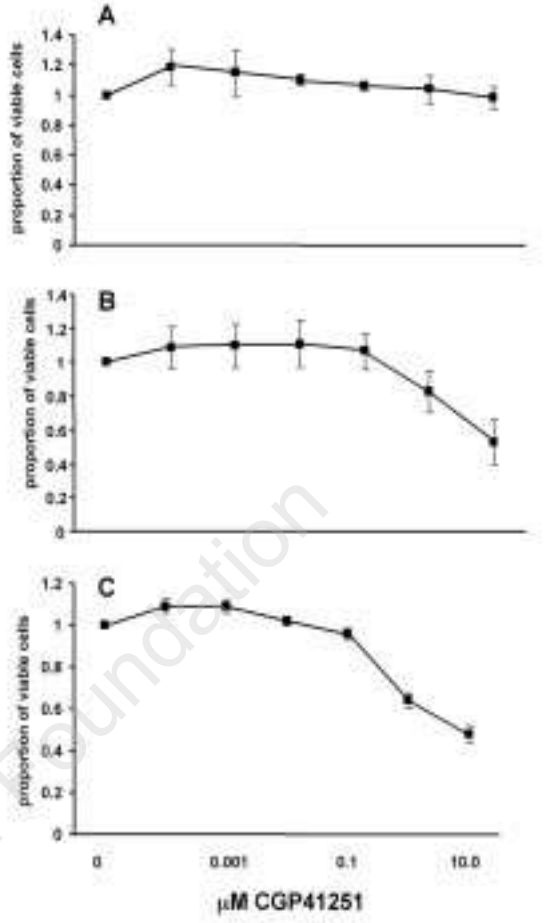

Figure 2. The cytotoxic action of PKC412 in normal PBL (A), tonsil B-cells (B) and B-CLL (C) cells using the MTT assay. Results are expressed as the proportion of viable cells to control cells with no PKC412. Mean values with SEM bars.

PKC412 is cytotoxic to tonsillar B-cells and B-CLL cells but not to normal PBL

The cytotoxic effect of PKC412 was additionally studied in PBL, tonsillar B-cells and B-CLL cells using the MTT assay (Figure 2). Tonsillar B-cells were sensitive to PKC412 with a mean IC_{50} of 11 μmole/L (Figure 2B, calculated on GraphPad Prism software), whereas PBL were resistant up to 10 μmole/L PKC412 (Figure 2A). A majority of B-CLL samples (18/26) were sensitive to PKC412 with a mean IC_{50} value of 2.1 μmole/L (range 0.9–8.1 μmole /L). The remaining samples had IC_{50} values >10 μmole/L, the maximum concentration achieved *in vitro* (Figure 2C). PKC412 at a concentration of 100 nmole/L had no cytotoxic effect on B-CLL cells. However, no correlation was observed between drug sensitivity and

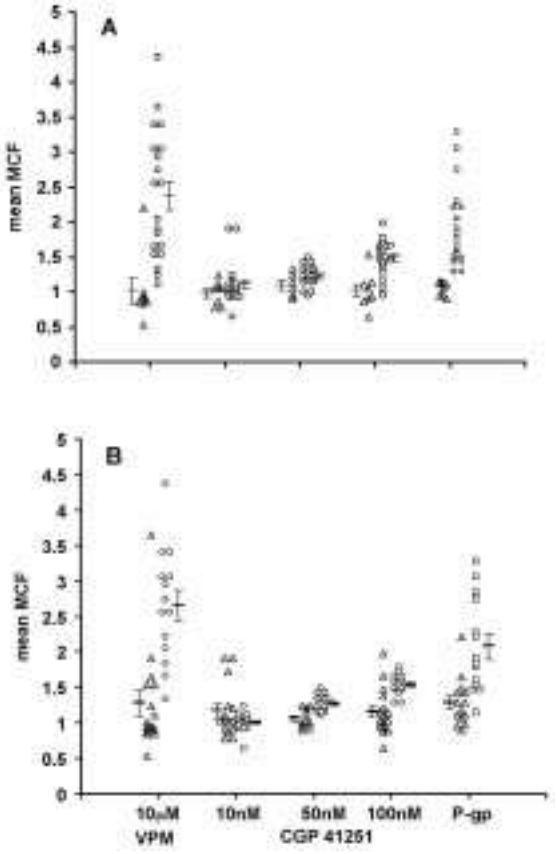

Figure 3. Modulation of efflux of the dye DiOC by 10 μmole/L VPM or PKC412 at 10, 50 and 100 nmole/L in B-CLL cells based on groups of patients divided by either P-gp expression - P-gp⁻ (△) MCF ratio <1.2, P-gp⁺ (○) PKC412 concentration-dependent reversal of efflux (△) -ve concentration dependent, (○) +ve concentration dependent. Means and SEM bars are indicated for each series of data except for P-gp in (A).

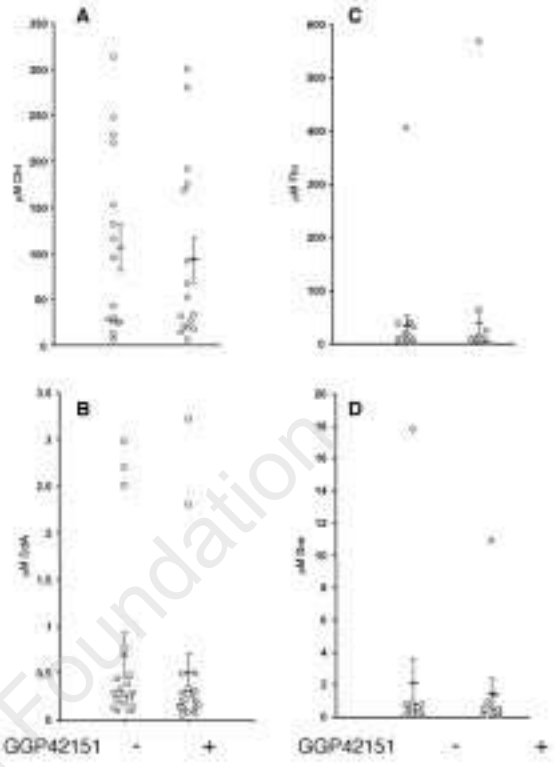

Figure 4. IC_{50} for chlorambucil (A), 2'chlorodeoxyadenosine (B), fludarabine (C), and daunorubicin (D) with and without 100nmole/L PKC412 in B-CLL cells *in vitro*. Concentrations expressed in mmole/L. Mean values with SEM bar are indicated for each series of data.

stage of disease, age or previous treatment. No significant difference was observed in IC_{50} values when the samples were divided into P-gp-positive (MFI >1.2) and P-gp-negative (MFI<1.2) subgroups, or into subgroups showing PKC412 dose-dependent reversal of DiOC efflux and those that did not (data not shown).

PKC412 inhibits PKC activity in B-CLL cells

In 8/13 patients treated with oral PKC412 for 14 days, the PKC activity in the malignant B-cells post-treatment was reduced by 25-96% compared to their respective pre-treatment values. The pre-treatment PKC activity ranged from 496-5684 cpm/μg protein and the post-treatment activity ranged from 331-600 cpm/μg protein.

PKC412 reverses P-gp-mediated efflux of DiOC in B-CLL cells

The role of PKC412 as a modulator of P-gp was investigated in PBL, tonsillar B-cells and B-CLL cells by FACScan analysis of the efflux of DiOC. There was no significant modulation of efflux by either PKC412 or VPM, the latter a known modulator of

P-gp in either PBL or tonsillar B-cells. In B-CLL cells the mean MCF ratio for reversal of efflux by VPM was 1.92. With PKC412 a dose-dependent reversal of efflux was observed with mean MCF ratios of 1.01, 1.18 and 1.34 at 10, 50 and 100 nmole/L respectively. There was a significant correlation between reversal of efflux by PKC412 and that by VPM (p<0.001, r=0.72). A significant correlation was also observed between P-gp expression and reversal of efflux by VPM (p<0.001, r=0.85) and by 100nmole/L PKC412 (p<0.001, r=0.60). When the samples were subdivided on the basis of either P-gp expression (Figure 3A) or dose-dependent reversal of efflux (Figure 3B), the correlation was greatly enhanced.

There was no correlation between efflux modulation by PKC412 at any concentration and expression of either MRP or LRP. Furthermore there was no correlation between P-gp, MRP and LRP expression (data not shown).

PKC412 sensitizes B-CLL cells to some conventional cytotoxic drugs

The mean IC_{50} values for 2'-chlorodeoxyadenosine and chlorambucil showed a small decrease in the presence of 100nmole/L PKC412 *in vitro* (Figures 4A and 4B). This decrease was not statistically significant (2'-chlorodeoxyadenosine alone vs with PKC412 p = 0.052). This effect was not observed for fludarabine (Figure 4C). The mean IC_{50} value for daunorubicin, a substrate for P-gp, also showed a decrease in the presence of PKC412 (Figure 4D) but the decrease was not statistically significant. Although the mean decrease in IC_{50} values with PKC412 was small, subsets of patients were identified (9/18 for 2'-chlorodeoxyadenosine, 5/16 for chlorambucil, 5/19 for fludarabine, 4/11 for daunorubicin) in whom the decrease in IC_{50} values ranged from 32-87%. Additionally, 3/8 and 1/3 samples that were resistant to chlorambucil and fludarabine, respectively, were sensitive to 1.0-1.6 μmole/L PKC412 alone. On the other hand, two samples resistant to chlorambucil and fludarabine were also resistant to PKC412 and one of these samples was also resistant to 2'-chlorodeoxyadenosine and daunorubicin.

Discussion

Previous studies on the actions of PKC412 have been carried out mainly in human and animal tumor cell lines. Studies of its *in vitro* actions on a pure malignant cell population from patients have not been described. This study reports the cytotoxic action and MDR reversal properties *in vitro* on a purified leukemia cell population from patients with B-CLL.

In agreement with previous studies in cell lines, PKC412 had cytotoxic activity in B-CLL cells *in vitro* at concentrations of 1μmole/L and above. Although the mean IC_{50} value for PKC412 in B-CLL cells was 19.1 μmole/L, nearly half the samples had IC_{50} values of less than 2 μmole/L, including some with high lymphocyte counts. In a wide range of cell lines the IC_{50} varied from 15 nmole/L to > 1 μmole/L[31] (*and unpublished data*). In a phase I dose escalation study of 12.5-300 mg/d PKC412 in patients with advanced cancer, the maximal observed plasma concentration of PKC412 was in the range of 0.3-7.0 μmole/L.[21] In addition to the unchanged drug, a metabolite of the drug showing biological activity *in vitro* was also found in patients' plasma at concentrations greater than 10 μmole/L.[21] The phase I study also confirms that although the drug binds to plasma proteins it still had biological activity on cells and tissues *in vivo*. Although PKC activity was not evaluated in the *in vitro* study samples, some of these patients went on to be treated with oral PKC412. The malignant cells from these patients treated with 25-225 mg/d of PKC412 over 2-8 weeks showed a significant reduction in PKC activity (*Virchis et al., submitted*) confirming that PKC412 can inhibit PKC activity in B-CLL cells. B-CLL cells are non-proliferative and are blocked in the G_0/G_1 phase of the cell cycle.[32] The effect of PKC412 on these cells is, therefore, not attributable to actions on the cell cycle but are consistent with a direct action on the apoptotic machinery.

The decrease in spontaneous and PKC412-induced apoptosis *in vitro* in the presence of human plasma may be attributed to at least two factors. First, plasma has been shown to contain unidentified factors that block apoptosis by signaling via the phosphatidylinositol 3'-kinase/AKT pathway.[33] Second, human plasma a1 acid glycoprotein (AAG) binds PKC412 avidly thus blunting its cytotoxic actions. While these *in vitro* observations might suggest that PKC412 may not be effective against B-CLL cells *in vivo*, it is has been shown that sustained exposure resulting from sequential doses in patients results in cytotoxicity of B-CLL cells (*Virchis et al., submitted*), malignant tissues and circulating white cells.[21]

PKC412 at 100 nmole/L displayed a negligible cytotoxic effect on B-CLL cells. Consequently, this concentration was used for studies on *in vitro* reversal of efflux of DiOC in these cells. At this con-

centration PKC412 showed significant reversal of P-gp function in B-CLL cells. Even lower concentrations showed reversal of efflux and a clear dose-dependent reversal was observed in 50% of the samples. The dose-related reversal of efflux also suggests that PKC412 may affect P-gp directly, resulting in retention of DiOC. When samples were grouped on the basis of PKC412 dose-dependent efflux, there was significant correlation with both reversal of efflux induced by VPM and with elevated expression of P-gp. In two samples, a higher concentration of PKC412 (1 μmole/L) was also used in the reversal of efflux studies. The MCF ratio at this higher concentration was comparable to that obtained with 10 μmole/L VPM (data not shown). However, 1 μmole/L PKC412 is not toxic and is well tolerated (*unpublished data*) compared to VPM at 10 μmole/L, a level at which severe cardiac toxicity has been observed in clinical trials.

Incubation of P-gp overexpressing KB 8511 epidermoid carcinoma cells with non-cytotoxic concentrations of PKC412 sensitizes the cells to vinblastine with complete reversal of the MDR phenotype, resulting in a dose-dependent accumulation of the fluorescent dye rhodamine-123.[22] Our preliminary studies with the CEM cell line and its resistant variant VLB using 100nmole/L PKC412 showed similar results, although a complete reversal was not achieved probably due to higher P-gp expression (data not shown). A similar concentration of PKC412 has been used by others for MDR reversal studies in cell lines.[22] Non-toxic concentrations of PKC412 significantly enhanced the cytotoxic properties of doxorubicin, actinomycin D, vinblastine and vincristine but not 5-flourouracil.[34] However, the enhanced intracellular concentrations of doxorubicin did not change P-gp protein expression in human breast carcinoma (MCF-7), murine colon adenocarcinoma (CT-26) or their MDR variant cell lines. PKC412 efficiently reversed anthracycline efflux in an anthracycline-resistant subline (A2780/ADR) of ovarian carcinoma.[20] Furthermore, the antitumor activity of doxorubicin against drug resistant murine carcinoma cells was enhanced by oral administration of PKC412.[23]

The non-significant sensitization of B-CLL cells *in vitro* with a combination of PKC412 and conventional drugs was not surprising. None of these drugs (2'-chlorodeoxyadenosine, chlorambucil and fludarabine) is a substrate for P-gp. However, in a number of samples there was considerable decrease in the IC_{50} value for all the drugs tested. It is, therefore, possible that PKC412 can sensitize B-CLL cells from a subgroup of patients to conventional drugs by mechanisms unrelated to P-gp modulation. It is also possible that consistent sensitization results may be achieved by longer exposure of these cells to PKC412 (see last paragraph). However, a decrease in the IC_{50} value for daunorubicin, a known substrate for P-gp with PKC412, was also not significant. This was surprising considering that a significant correlation was observed between increased P-gp expression and reversal of DiOC efflux in the cohort of samples investigated. This difference may be due to the greater sensitivity of the DiOC efflux assay compared to the MTT assay. However, there was no correlation between PKC412 IC_{50} values and P-gp expression in B-CLL cells. This may be attributed to the narrow concentration window within which PKC412 acts as a modulator of P-gp without causing direct cytotoxic effects.

Although chlorambucil and fludarabine are not substrates for P-gp, treatment with either of these agents in combination with PKC412 can nevertheless be beneficial since PKC may regulate the expresssion of anti-apoptotic proteins.[35] These proteins may oppose the induction of apoptosis by chlorambucil and fludarabine. Signal transduction inhibitors which decrease expression of these proteins via blockade of PKC activity may, therefore, augment the cytotoxic activity of conventional drugs in an additive or synergistic manner. Recent reports also suggest that P-gp in addition to acting as an efflux pump, may also have effects on apoptosis by mechanisms which are yet unclear.[36] In these studies, NIH 3T3 cells transfected with the mdr-1 gene conferred resistance to UV radiation-induced cell death by apoptosis which was reversed by P-gp modulators, VPM or a monoclonal antibody to P-gp.

Since B-CLL cells are non-proliferative, it is not clear whether the cytotoxic effect of PKC412 is via inhibition of PKC. Inhibition of PKC may, however, play a role in the regulation of apoptosis observed in these B-CLL cells. Alternatively, a recent study shows that PKC412 can block *in vivo* signaling pathways in cancer patients by suppressing cytokine release.[37] How this contributes towards apoptosis in B-CLL cells needs investigation. The mechanism of the modulatory effect of PKC412 on MDR is also not clear; it may act directly as a competitive or non-competitive inhibitor of P-gp or may modulate MDR via inhi-

bition of PKC. In cells from AML patients, a highly significant positive correlation was found between MDR1 and PKCβ possibly via modulation of the phosphorylation of P-gp.[34,38]

At present, other PKC inhibitors are being studied *in vitro* and *in vivo* in B-CLL and other malignancies. Bryostatin 1 has completed phase I studies in non-Hodgkin's lymphoma (NHL) and B-CLL; in this context 11/29 patients achieved stable disease for 2-19 months.[39] UCN-01 (7-hydroxy staurosporine) is being tested in phase I clinical trials in patients with refractory neoplasms[40] and has been suggested as a combination agent with fludarabine to improve overall survival in B-CLL.[41] UCN-01 enhances cellular sensitivity to 5-fluorouracil by suppressing thymidylate synthase via downregulation of E2F-1.[42] *In vitro* studies have shown that Safingol enhances the cytotoxic effect of mitomycin C in gastric cancer cells by promoting drug-induced apoptosis.[43] Out of 17 patients with various tumors, minor responses were observed in 3/6 patients with pancreatic cancer and 1/3 with sarcoma in a pilot clinical trial with Safingol (L-threo-dihydrospingosine).[44] Potentiation of 1-β-D-arabinofuranosylcytosine (ara-C) by Safingol or bryostatin 1 by inteference with the mitogen-activated protein kinase (MAPK) cascade has also been demonstrated.[45] However, none of these PKC inhibitors has an effect on the MDR phenotype.

PKC412, an agent with antitumor activity *in vitro* and *in vivo*, offers an attractive combination agent in the treatment of malignancies expressing the MDR phenotype. In addition, its ability to sensitize tumor cells may allow the use of lower concentrations of conventional drugs thus reducing their toxic side effects. In a recent study PKC412 has been shown to sensitize murine cells to ionizing radiation.[46] Sensitization is further confirmed in our studies in cells from B-CLL patients treated with oral PKC412 over a period of 2-8 weeks, such that a significant lowering of the *in vitro* IC_{50} was observed for chlorambucil and fludarabine compared to in pre-treatment samples.[47] In a review on PKC targeting, Jarvis and Grant confirmed the accumulating evidence that selective targeting of PKC improves the efficacy of conventional cytotoxic agents.[48] The statistical discordance between *in vitro* and *in vivo* studies may be attributed to the continuous exposure of B-CLL cells to PKC412 *in vivo* where a significant reduction in circulating malignant cells was observed (*Virchis et al., A novel treatment approach for low grade lymphoproliferative disorders using PKC412, a protein kinase C inhibitor, submitted*).

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KG and RGW: responsibility for the intergrity of work from inception to laboratory analysis, data analysis and data interpretation to submission of manuscript. DTJ, MG, SMH: contributed to a significant proportion of the laboratory work. AEV, HGP, AVH: contributed towards collection and analysis of the clinical data. AM, KC, KCz: contributed towards providing PKC412 data from source and analyzing current data. ABM: contributed towards initial planning of the study and collection of clinical data.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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Peer Review Outcomes

What is already known on this topic

Inhibitors of protein kinase enzymes involved in cell signaling represent an innovative therapeutic strategy in leukemia. Synthetic PKC inhibitors have been developed for clinical use and shown to be active.

What this study adds

Selective inhibition of B-CLL acts through an apoptotic mechanism which apparently spares normal PB lymphocytes.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Francesco Lo Coco, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Professor Lo Coco and the Editors. Manuscript received August 14, 2001; accepted November 11, 2001.

Potential implications for clinical practice

Ongoing phase-II clinical trials should better define the advantages of this naturally occurring agent, particularly in B-CLL cases resistant to chlorambucil and fludarabine. Its role as a chemosensitizer is also clinical interest.

Francesco Lo Coco, Deputy Editor