Induction of Apoptosis by Staurosporine Involves the Inhibition of Expression of the Major Cell Cycle Proteins at the G_2/M Checkpoint Accompanied by Alterations in Erk and Akt Kinase Activities

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Abstract. *Background: Staurosporine is a therapeutic agent that inhibits tumor cell growth by inducing cell death via intrinsic apoptotic pathways. Our previous studies in clinical settings have suggested that certain subpopulations of patients with acute myeloid leukemia (AML) had poor response to chemotherapy. Materials and Methods: The effect of staurosporine on apoptosis and cell cycle distribution in human leukemic cell line U-937 cells was determined. U-937 cells were treated with staurosporine at 0.5 μM for 18 hours or 1 μM for 24 hours. Analyses of cell cycle distribution and apoptosis were performed using flow cytometric analysis. The effects of staurosporine on the targeted proteins were assessed by immunoblot analysis. Results: A blockade of the cell cycle at the G_2/M phase was observed in U-937 cells treated with staurosporine. A concomitant induction of apoptosis and activation of caspase-3 in U-937 cells was also achieved. Treatment of U-937 cells with staurosporine at 1 μM for 24 hours, compared with 0.5 μM for 18 hours, appeared to kill the leukemic more efficiently cells and this dose and duration may specifically target p27, Erk and Akt pathways that are important for cancer cell survival and resistance to treatment. We also show that the effects of stauroporine on cell cycle progression and apoptosis in U-937 cells are closely linked. Conclusion: Our results suggest that induction of apoptosis and inhibitory proliferation and survival pathways are important events induced by staurosporine. Understanding the conditions under which staurosporine shows high specificity and low toxicity in treatment of leukemic cells is of great importance for improving the efficacy of targeted therapeutics and overcoming resistance to chemotherapeutic agents.*

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Acute myeloid leukemia (AML) is a heterogeneous disease that exhibits loss of normal proliferation controls and features of immature cells that are accumulated at immature stages of differentiation (1). To design therapeutic strategies, it is important to identify the drugs that may induce the death of leukemic cells and meanwhile to re-establish normal cell proliferation control.

Staurosporine is a microbial alkaloid, isolated from *Streptomyces* sp. cultures. Staurosporine has demonstrated antiproliferative activity in several human cancer cell lines (2). The predominant effect of staurosporine on cancer cells is to induce G_2/M cell cycle arrest (3, 4) and to modulate G_1 arrest of the cell cycle (5). Stauroporine is a non-selective inhibitor of protein kinase C (PKC) and cyclin-dependent kinase (CDKs) (6). Although the PKC enzyme family has been considered to be a major target of staurosporine, it is unclear how PKC inhibition can cause anticancer activity of the drug, and the most important targets for staurosporine are still obscure. Progression through each cell cycle phase is tightly controlled by the key cell cycle regulators including cyclins, CDKs and CDK inhibitors. Tumors with invasive and metastatic potential often have high levels of cyclins and CDKs (7). Staurosporine inhibits CDKs and is a promising anticancer agent (8).

Understanding the mechanisms by which staurosporine targets CDKs and cell cycle pathways, as well as the signalling network controling proliferation and apoptosis is of great importance for improving the treatment of various types of cancer. Cancer cells can acquire resistance to chemotherapy by a range of mechanisms including the generation of more mutations or overexpressing the targets of the drugs. By utilizing these mechanisms, cancer cells can

inactivate or eliminate the drugs from the cells (9). The dose and conditions under which an anticancer drug is efficient in killing the cancer cells and the specific pathways that control cancer cell survival need to be determined. In addition, it is important to test prospective drug to understand the conditions under which drugs show high specificity and low toxicity. The interaction of effects on both cell cycle progression and the induction of apoptosis as elicited by stauroporine is complex. The G_1 arrest induced by staurosporine is accompanied by hypophosphrylation of pRb (10-12). It has been shown that staurosporine has effects on the activity of phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathways (13). PI3 kinases activate and induce conformational change of AKT, which consequently allow phosphorylation of two regulatory residues: a threonine residue on the kinase domain and a serine residue on the C-terminal hydrophobic domain (Thr308 and Ser472 for AKT1). Phosphorylation of the threonine residue, mediated by phosphositide-dependent kinase 1, is essential for AKT activation, whereas phosphorylation of the serine residue enhances the AKT activity approximately 10-fold (13). In the present study, we studied the effects of the specific conditions of staurosporine on the cell cycle and on specific cyclins and CDKs. We also investigated the effects of staurosporine on apoptosis and the cellular pathways in U-937 leukemic cells.

Materials and Methods

Cell culture and drug treatment. Human leukemic cell line U-937 was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. For the treatment with staurosporine, cells were cultured in the medium containing staurosporine (Sigma, St Louis, MO, USA) at a concentration of 0.5 μM or 1 μM.

Immunoblotting and source of antibodies. Cells were lysed in ice-cold RIPA lysis buffer (120 mM NaCl, 50 mM Tris-HCL pH 7.5, 1% NP-40, 50 mM NaF, 10 mM phenylmethylsulfonyl fluoride (PMSF) and the protease inhibitor cocktail Complete Mini (Roche, Basel, Switzerland), mildly sonicated, and centrifuged twice at 12,000 xg for 30 min at 4˚C. The proteins were then subjected to electrophoresis on a 10% SDS-PAGE gel followed by transfer to nitrocellulose membrane Hybond™ ECL™ (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were probed with primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Life Science, Alesbury, UK) and visualized using the Enhanced Chemi-Luminescence detection system (ECL) and ECL films (Amersham Pharmacia Biotech). The primary antibodies included cyclin B1, caspase-3, Akt, Erk, phosphor-Erk (Cell Signaling, Danvers, MA, USA), cyclin A2, CDK1, CDK2 (Transduction Laboratory, San Jose, CA, USA), P27 (Dako, Glostrup, Denmark), phospho-Akt (ser 473), phospho-Akt (thr 308) and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by HRP-conjugated secondary antibodies (Dako).

Apoptosis analysis. Cells were washed in phosphate-buffered saline and resuspended in binding buffer (0.01 M HEPES pH 7.4, 0.14 M NaCl, 2.5 mM $CaCl_2$) (BD Biosciences, San Jose, CA, USA), stained with 5 μl APC-conjugated annexin V, and 5 μl 7-aminoactinomycin D (7-AAD) (BD Biosciences) in 100 μl binding buffer. The cells (~5×10^5 cells) were incubated for 15 min at room temperture in the dark, and were then subjected to flow cytometric analysis using FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell cycle analysis and flow cytometry. Cells (1×10^6) were harvested after the treatment then fixed and permeabilized. Cells were then washed twice with 1 × PBS containing 10% FCS, resuspended and incubated in 1 × PBS buffer containing 5 μg/ml propidium iodide (Sigma), 100 mM sodium citrate, pH 7.3 and 0.05 mg RNase A (Sigma) for 20 min at 4˚C. The cell fluorescence was measured in a FACS Calibur cytofluorometer (Becton Dickinson) and analyzed using CELL Quest software (Becton Dickinson).

Results

G_2/M cell cycle checkpoint arrest and apoptosis in U-937 cells in response to staurosporine treatment under different conditions. A major obstacle in leukemia therapy is the emergence of treatment-resistant cells. To understand the mechanism underlying the resistance of the leukemic cells in response to staurosporine treatment, we compared the responses of U-937 cells to staurosporine treatment under two different conditions were compared. We firstly exposed U-937 cells to staurosporine at 0.5 μM for 18 hours. Flow cytometric analysis was employed to monitor the changes in cell cycle distribution in control U-937 cells and in cells treated with staurosporine. The proportion of cells in the G_0/G_1 phase significantly decreased, and there was a concomitant increase in the G_2/M phase population in treated cells (data not shown). This suggests that staurosporine induced cell cycle arrest at the G_2/M checkpoint. Since it remained unclear whether staurosporine can sufficiently kill leukemic cells at low toxicity level, we therefore examined the proportion of U-937 cells that were undergoing early apoptosis or U-937 cells at late stage apoptosis. Control U-937 cells or cells treated with staurosporine were stained with APC-conjugated Annexin-V and 7-AAD followed by flow cytometric analysis. The proportion of early apoptotic cells that were the annexin-V^+ and 7-AAD^- was 11.23% after treatment with staurosporine, while the rate of early apoptosis was 6.79% in control cells (Figure 1A), suggesting that staurosporine induced an increase in early apoptosis. The percentage of annexin-V^+ and 7-AAD^+ cells was 6.96% after the treatment with staurosporine compared with 2.93% in control cells, suggesting that staurosporine also induced an equivalent increase in late apoptosis (Figure 1A). However, the rate of total apoptosis induced by staurosporine at 0.5 μM for 18 hours was only 18%, compared with 9% in control cells. These results suggest

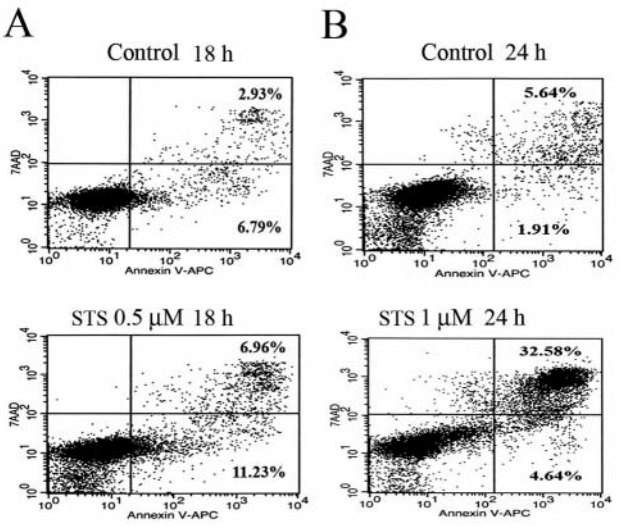

Figure 1. *Evaluation of the rate of apoptosis induced by staurosporine at 0.5 μM for 18 hours or at 1 μM for 24 hours. Control U-937 cells or U-937 cells treated with staurosporine were stained with Annexin-V and 7-AAD followed by flow cytometry analysis. A, Dot blot graph from flow cytometric analysis shows the rate of apoptosis in control U-937 cells (Control) and U-937 cells treated with staurosporine (STS) at 0.5 μM for 18 h. B, Dot blot graph shows the rate of apoptosis in control U-937 cells (Control) and in U-937 cells treated with staurosporine (STS) at 1 μM for 24 h. The percentage of early or late apoptotic cells is indicated.*

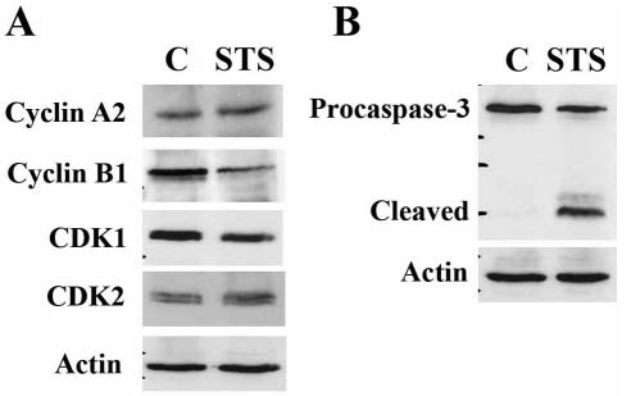

Figure 2. *Evaluation of the effect of staurosporine at 0.5 μM for 18 hours on the expression of cell cycle regulatory factors. A, Immunoblot analysis of cyclin A2, cyclin B1, CDK1 and CDK2 in control U-937 cells (C) and U-937 cells treated with staurosporine (STS). Actin was used as control for equal loading of the samples. A representative blot from the three independent experiments is shown. B, Expression of activated caspase-3 in control U-937 cells (C) and in U-937 cells treated with staurosporine (STS). Antibody to cleaved caspase-3 was used. A representative blot from three independent experiments is shown.*

that staurosporine at this dose and duration did not sufficiently induce a high rate of cell death, although staurosporine sufficiently induced cell cycle arrest at the G_2/M phase.

It is known that U-937 cells lack functional p53 and there is a broad consensus that p53-deficient cells are able to aberrantly re-enter the cell cycle and undergo unchecked DNA replication, leading to increased resistant to chemotherapeutic agent-induced cell death. To further understand the mode of cell death induced by staurosporine, the dose of staurosporine was increased to 1 μM for 24 hours, a low-risk condition at a physiological level. The degree of cell cycle arrest induced by staurosporine under these conditions was similar to that observed in cells treated with 0.5 μM for 18 hours. Staurosporine induced a significant increase in early apoptotic cells. The rate of early apoptosis in cells treated with staurosporine was 2-fold higher compared with that in control cells (Figure 1B). Interestingly, staurosporine induced a much pronounced increase in the rate of late apoptotic cells. The rate of late apoptosis was 32.58%, 5-fold higher level than that in control cells, which was 5.67%. In addition, the total apoptosis induced by staurosporine at 1 μM for 24 hours was 38%, compared with 18% induced by staurosporine at 0.5 μM for 18 hours. This suggests that treatment of U-937 cells with staurosporine at 1 μM for 24 hours may sufficiently induce apoptosis coupled with the induction of cell cycle arrest.

Expression of cyclin A2, cyclin B1, CDK1 and CDK2 in response to the treatment with staurosporine at different conditions. Since we observed that staurosporine under these two different conditions induced similar effects on the cell cycle, but resulted in different degrees of effects on cell death, we investigated the cellular mechanisms underlying the action of staurosporine in U-937 cells by comparing expression of cell cycle regulatory proteins in U-937 cells treated with 0.5 μM staurosporine for 18 hours with those treated with 1 μM staurosporine for 24 hours. Expression of cyclin A2, cyclin B1, CDK and CDK2 following the treatment was examined by immunoblot analysis. We observed a significant decrease in the expression of cyclin B1 and CDK1 in U-937 cells treated with 0.5 μM staurosporine (Figure 2A). Given that cyclin B1 in complex with CDK1 forms the mitotic promoting factor (MPF), which is required for G_2/M-phase progression, the down-regulation of cyclin B1 and CDK1 was an indicator of the successful blockage of G_2/M-phase progression. However, levels of cyclin A2 and CDK2 remained unchanged (Figure 2A), which suggests that staurosporine had no effect on the expression of these two proteins that are involved in DNA synthesis. Apoptosis-triggered by chemotherapeutic agents depends on activation of caspases, which play important roles in the proteolysis of

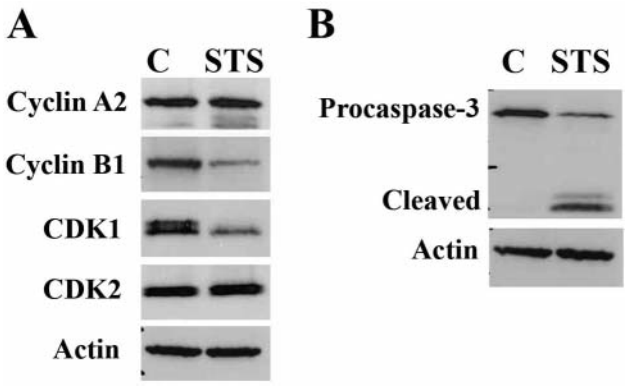

Figure 3. *Evaluation of the effect of staurosporine at 1 μM for 24 hours on the expression of cell cycle regulatory factors. A, Immunoblot analysis of cyclin A2, cyclin B1, CDK1 and CDK2 in control U-937 cells (C) and U-937 cells treated with staurosporine (STS). Actin was used as control for equal loading of the samples. B, Expression of activated caspase-3 in control U-937 cells (C) and in U-937 cells treated with staurosporine (STS). Antibody to cleaved caspase-3 was used. The blots shown in A and B are the representative blots from three independent experiments.*

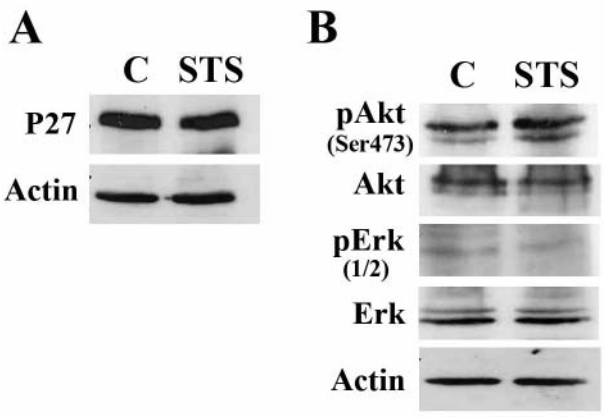

Figure 4. *Effect of staurosporine at 0.5 μM for 18 hours on the expression of P27 and the activity of Akt and Erk1/2. A, Expression of p27 in control U-937 cells (C) and in U-937 cells treated with staurosporine (STS). B, Expression of phosphorylated Akt and phosphorylated Erk1/2 in control U-937 cells and in U-937 cells treated with staurosporine (STS). Antibodies against total Akt and Erk were used as probe to show the equal loading of the samples. The blots shown in A and B are the representative blots from two independent experiments.*

specific targets. We examined the activation of caspase-3 by staurosporine using immunoblot analysis. Treatment of U-937 cells with staurosporine at 0.5 μM readily resulted in cleavage of caspase-3, with the appearance of small fragments (17 and 19 kDa), indicating that increased cleavage of the procaspase was coupled with the observed increase in cell death (Figure 2B). These data suggested that activation of caspase-3 correlated well with the proportion of apoptosis. To examine whether the cell cycle regulatory proteins exhibit a different expression pattern in U-937 cells treated with staurosporine at 1 μM for 24 hours, we performed immunoblot analysis in control U-937 cells and in U-937 cells treated with staurosporine at 1 μM for 24 hours. Similar to what was observed in cells treated with 0.1 μM for 18 hours, there was also a significant decrease in the expression of cyclin B1 and CDK1. The band that represents the phosphorylated form of CDK1 was completely diminished in the cells treated with staurosporine, while expression of cyclin A2 and CDK2 remained unchanged in cells treated with 1 μM staurosporine (Figure 3A). The increased cleavage of the procaspase-3 confirmed the higher degree of apoptosis induced by 1 μM staurosporine (Figure 3B).

AKT and ERK 1/2 signalling pathways in staurosporine-mediated apoptosis and cell cycle arrest in U-937 cells. U-937 cells are p53-deficient cells that are able to undergo unchecked DNA replication. P21 and p27 are the downstream targets of p53. We wanted to compare the expression of p21 and p27 as well as the activation of PI3/Akt and mitogen-activated phosphor-kinase/Erk (MAPK/Erk) pathways in response to the treatment of staurosporine at two different doses and incubation time. Expression of p21 was undetectable in U-937 cells (data not shown). Staurosporine at 0.5 μM for 18 hours did not result in any change in the level of p27 expression (Figure 4A). It is known that PI3/Akt and MAPK/Erk pathways mediate cell survival. The activation of Akt is required phosphorylation of both a threonine residue on the kinase domain and a serine residure on the hydrophobic regulatory domain. We therefore examined the phosphorylation of the threonine residue on the kinase domain of Akt and the serine residue on the hydrophobic domain of Akt by using antibodies specific for Thr 380 and Ser 473 phosphorylated form of Akt in control U-937 cells and in cells treated with 0.5 μM staurosporine for 18 h. The levels of phospho-Akt remained similar to that in the control U-937 cells, indicating that staurosporine treatment at 0.5 μM had no effect on Akt phosphorylation (Figure 4B). Erk was weakly activated in control U-937 cells, but remained unchanged in U-937 cells treated with staurosporine at 0.5 μM for 16 h (Figure 4B). This result suggests that staurosporine treatment under these conditions did not interfere with phosphorylation of the threonine residue in the Akt kinase domain or the serine residue in the hydrophobic domain in the Akt.

We then examined the expression of p27, phosphorylation of Akt and Erk 1/2 in U-937 cells treated with 1 μM for 24 hours. In contrast to what was observed in U-937 cells treated with 0.5 μM, expression of p27 was significantly

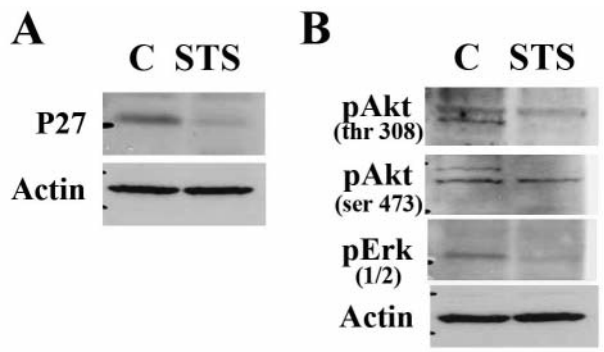

Figure 5. *Effect of staurosporine at 1 μM for 24 hours on the expression of p27 and the activity of Akt and Erk1/2. A, Expression of p27 in control U-937 cells (C) and in U-937 cells treated with staurosporine (STS). B, Expression of phosphorylated Akt and phosphorylated Erk1/2 in control U-937 cells and in U-937 cells treated with staurosporine (STS). Antibody against actin was used as probe to show the equal loading of the samples. The blots shown in A and B are the representative blots from three independent experiments.*

down-regulated in U-937 cells treated with 1 μM staurosporine (Figure 5A). Phosphorylation of Erk 1/2 was significantly reduced in U-937 cells treated with 1 μM staurosporine. Staurosporine at 1 μM also induced changes in the expression patterns of phospho-Akt at Thr 380 or Ser 473 residues in U-937 cells (Figure 5B). These results suggest that 1 μM staurosporine induced sufficient cell death and this action may be mediated through modulating p27 expression and modulating the activities of Akt and Erk 1/2. Furthermore, expression of p27 and the activity of Akt and Erk 1/2 may be functionally linked to the process of cell death induced by staurosporine.

Discussion

Despite improvements in treatment regimens for leukemia during the past decades, there is still a fraction of patient populations who die from the disease or complications of treatment (14). Variability in response to therapy, both with regards to efficacy and to adverse events, is leading the pharmaceutical industry down the path of personalized medicine. However, biological and clinical processes which enable leukemia to become resistance to treatment are poorly understood. Identification and validation of mutliple cellular and molecular pathways associated with the poor response to treatment are of the utmost importance for improving treatment. The aim of our present study was therefore to: i) evaluate the possible association between staurosporine treatment and cell cycle pathways; ii) investigate the specific effect of staurosporine on induction of cell death in leukemic cells; iii) investigate the cellular pathways that may be specifically targeted by staurosporine and the possible functional link between these pathways and cellular resistance to staurosporine treatment. In the present study, we have shown that staurosporine at 0.5 μM for 18 hours or at 1 μM for 24 hours effectively induced G_2/M phase cell cycle arrest. This is consistent with the reported studies in which staurosporine is suggested to target the basic mechanisms of proliferation in cancer cells (15). Our findings further revealed that the cell cycle arrest induced by staurosporine was coupled with the concomitant down-regulation of cyclin B1 and CDK1. Since cyclin B1 and CDK1 are the key proteins that regulate mitosis and cell division, the effect of staurosporine on the exression of cyclin B1 and CDK1 under the conditions studied here suggests that staurosporine is a potent antiproliferative agent in leukemic cells. One of our striking observations is that staurosporine had different effects on apoptosis and several cellular pathways depending on its dose and duration of treatment. Treatment of U-937 cells with staurosporine at 1 μM for 24 hours effectively and specifically induced apoptosis. The total rate of apoptosis induced by staurosporine at this condition was 38% . However, treatment of U-937 cells with staurosporine at 0.5 μM for 18 hours induced only 18% cell death. In the present study, we have demonstrated that although staurosporine at 0.5 μM for 18 hours effectively blocked cell cycle progression, it showed no effect on the expression of p27, phosphorylated Erk or phosphorylated Akt. This suggests that these proteins may not be functionally associated with G_2/M cell cycle pathways in leukemic cells. Interestingly, treatment of U-937 cells with staurosporine at 1 μM for 24 hours led to down-regulation of p27, decreased expression of phosphorylated Erk and lower activity of Akt. Staurosporine at 1 μM for 24 hours induced apoptosis, and altered the expression of p27 and activities of Erk and Akt; this result indicates that induction of apoptosis and the regulation of p27-, Erk- and Akt-associated pathways are intimately related. These multiple cellular events may cooperatively determine the sensibility of U-937 leukemic cells in response to staurosporine treatment. We demonstrated that the induction of a high level of cell death by staurosporine was coupled with an increase in Erk activity and alteration in Akt phosphrylation patterns, suggesting that Erk and Akt kinase activities are critical in mediating the effect of staurosporine treatment of leukemic cells.

A major obstacle in leukemia therapy has been the emergence of a subpopulation of leukemic cells that do not respond to cell death induced by chemotherapeutic agents. Hence, there is an urgent need to identify the cellular pathways responsible for such treatment resistance and to be able to convert the resistant leukemia cells into sensitive ones. There is evidence that activation of the Akt pathways exerts its effects in apoptosis, although the mechanisms remain unknown and it may vary with cell type (16, 17). We have shown that the Erk and Akt phosphorylation patterns

remained unaffected in staturosporine-treated U-937 cells where the extent of apoptosis was only minor. However, the diminished expression of the phosporylated forms of Erk and changed phosphorylated forms in Akt in U-937 cells were observed in U-937 cells where staurosporine induced a high level of apoptosis. It is possible that Erk and Akt survival signalling pathways contribute in part to staurosporine-induced apoptosis. Taken together, our results provide information to help gain insights into the functional role of down-regulation of the Erk or Akt pathways in staurosporine-mediated cell death.

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