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Deguelin, A PI3K/AKT inhibitor, enhances chemosensitivity of leukaemia cells with an active PI3K/AKT pathway

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Summary

Activation of the phosphoinositide 3 kinase (PI3K)/Akt signalling pathway has been linked with resistance to chemotherapeutic drugs, and its downregulation, by means of PI3K inhibitors, lowers resistance to various types of therapy in tumour cell lines. Recently, it has been reported that deguelin, a naturally occurring rotenoid, is a powerful inhibitor of PI3K. We investigated whether or not deguelin could enhance the sensitivity to chemotherapeutic drugs of human U937 leukaemia cells and acute myeloid leukaemia (AML) blasts with an activated PI3K/Akt network. Deguelin (10 nmol/l) induced S phase arrest with interference of progression to G2/M, and at 100 nmol/l significantly increased apoptotic cell death of U937. At 10–100 nmol/l concentrations, deguelin downregulated Akt phosphorylation of leukaemia cells and markedly increased sensitivity of U937 cells to etoposide or cytarabine. A 10 nmol/l concentration of deguelin did not negatively affect the survival rate of human cord blood $CD34^+$ cells, whereas it increased sensitivity of AML blasts to cytarabine. Deguelin was less toxic than wortmannin on erythropoietin- and stem cell factor-induced erythropoiesis from $CD34^+$ progenitor cells. Overall, our results indicate that deguelin might be used in the future for increasing sensitivity to therapeutic treatments of leukaemia cells with an active PI3K/Akt signalling network.

Keywords: apoptosis, cell signalling, drug resistance, leukaemia, $CD34^+$ cells.

Phosphoinositide 3 kinase (PI3K), a lipid kinase, generates 3′-phosphoinositides that recruit proteins containing lipid-recognition domains (pleckstrin homology domains, FYVE domains) to the plasma membrane. PI3K signalling regulates cell growth, motility and survival (Lu *et al*, 2003; Fresno Vara *et al*, 2004). The 3′-phosphoinositides attract phosphoinositide-dependent kinase 1 (PDK1) to the plasma membrane and Akt. PDK1 activates Akt by phosphorylation of Thr 308 residue in the activation loop, although full activation requires phosphorylation of Ser 473 in the C-terminal hydrophobic motif of Akt. The latter phosphorylation step is effected by a kinase that has not been conclusively identified to date (Brazil *et al*, 2004; Hanada *et al*, 2004). The serine/threonine kinase Akt is the major mediator of survival signals that protect cells from apoptosis because of its ability to phosphorylate and inactivate several downstream targets such as pro-apoptotic protein BAD, caspase-9, ASK1, and transcription factors of the FoxO family (Brazil & Hemmings, 2001; Mitsiades *et al*, 2004). Because Akt and its upstream regulators are deregulated in a wide range of solid tumours and haematological malignancies (Kim *et al*, 2005), the PI3K/Akt signalling network is considered as a key determinant of the biological aggressiveness of these tumours, as well as a major potential target for novel anti-cancer therapies (Mitsiades *et al*, 2004). Pharmacological inhibitors that target PI3K (LY294002, wortmannin) have been widely used for *in vitro* studies on cancer cell lines, in which

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they induce apoptosis or increase sensitivity to chemotherapeutic drugs (or both) (West *et al*, 2002). However, neither wortmannin nor LY294002 is entirely specific for the PI3K/Akt pathway, as wortmannin also inhibits phospholipases C, D and A_2, while LY294002 downregulates casein kinase 2 activity (West *et al*, 2002). Furthermore, the effect of LY294002 on PI3K is rapidly reversible, and problems with the toxicity and pharmacokinetics of these drugs have been encountered (West *et al*, 2002). For these reasons, it seems unlikely that LY294002 or wortmannin may be fully developed as cancer therapeutics (Gills & Dennis, 2004).

Very recently, it has been shown that the naturally occurring rotenoid, deguelin, inhibited PI3K activity and reduced Akt phosphorylation in premalignant and malignant bronchial epithelial cells (Chun *et al*, 2003). *In vitro* deguelin treatment, at doses attainable *in vivo* (Udeani *et al*, 2001), inhibited the growth and induced apoptosis of premalignant and malignant bronchial cells but had minimal effect on normal bronchial cells.

Considering that activation of the PI3K/Akt pathway is being increasingly recognized as a factor undermining the successful therapeutic treatment of human leukaemias (Neri *et al*, 2003; Xu *et al*, 2003; Tazzari *et al*, 2004; Zhao *et al*, 2004), we decided to investigate whether or not deguelin could be employed to enhance the sensitivity of human leukaemia cells with an active PI3K/Akt pathway to chemotherapeutic drugs employed for the treatment of acute myeloid leukaemia (AML). Indeed, it is well established that activation of the PI3K/Akt pathway contributes to the resistance of human leukaemia cells to chemotherapeutic agents (Martelli *et al*, 2003; Xu *et al*, 2003; Tabellini *et al*, 2004).

Here, we showed that deguelin, when employed for 24 h at 10 nmol/l concentration, arrested proliferation of U937 cells without inducing apoptosis. At this concentration, deguelin dephosphorylated Akt and increased U937 cell sensitivity to etoposide and cytarabine. Remarkably, at 10 nmol/l deguelin enhanced the sensitivity to cytarabine of AML blasts with an active PI3K/Akt pathway.

These data suggest that deguelin might be employed in the future for enhancing the sensitivity of leukaemia cells to conventional forms of therapy.

Experimental procedures

Chemicals and antibodies

Etoposide, cytarabine, bovine serum albumin (BSA, Fraction V), fetal calf serum (FCS), Roswell Park Memorial Institute (RPMI) 1640 medium, monoclonal antibody to β-tubulin and peroxidase-conjugated secondary antibodies were from Sigma, St Louis, MO, USA. The Complete Protease Inhibitor Cocktail, and the Lumi-Light^{Plus} enhanced chemiluminescence (ECL) detection kit were from Roche Applied Science, Milan, Italy. Wortmannin was from Calbiochem, La Jolla, CA, USA. Deguelin was purchased from Alexis Biochemical, Laufelfingen, Switzerland. The Protein Assay kit (detergent compatible) was from Bio-Rad, Hercules, CA, USA. Rabbit polyclonal antibodies to total Akt, Thr 308 phosphorylated Akt (p-Akt), Ser 473 p-Akt, total p44/42 mitogen-activated protein (MAP) kinase, Thr 202/Tyr 204 p-p44/42 MAP kinase, total p38 MAP kinase, and Thr 180/Tyr 182 p-p38 MAP kinase were from Cell Signalling Technology, Beverly, MA, USA. Phycoerythrin (PE)–conjugated anti-glycophorin A (GPA) monoclonal antibody and fluorescein isothiocyanate (FITC)–conjugated anti-CD71 monoclonal antibody were from DAKO AS, Glostrup, Denmark.

Cell culture

U937 and HL60PT (for parental, obtained from the American Type Culture Collection-CCL 240) cells were routinely maintained in RPMI 1640 medium supplemented with 10% FCS at an optimal cell density of 3–8 × 10^5 cells/ml.

Isolation of human umbilical cord blood CD34$^+$ cells

Cord blood (CB) samples were collected according to the institutional guidelines after informed consent. The CB mononuclear cells, isolated by density gradient centrifugation (Ficoll/Histopaque, 1077 g/ml), were left to adhere to plastic for at least 2 h at 37°C. After removal of adherent cells, CD34$^+$ cells were isolated using a magnetic cell-sorting program (Mini-MACS) and the CD34 isolation kit (Miltenyi Biotec, Bergish Gladbach, Germany) following the manufacturer's instructions. The purity of CD34$^+$ selected cells ranged between 93% and 98%, and was determined by flow cytometry using a PE-conjugated monoclonal antibody that recognizes a separate epitope of the CD34 molecule (581, Beckman Coulter, Miami, FL, USA). Cord blood CD34$^+$ cells were cultured in Ex-vivo-20 (BioWhittaker, Walkersville, MD, USA) serum-free medium supplemented with nucleosides (10 μg/ml each), 0·5% BSA, 10 μg/ml insulin, 200 μg/ml iron-saturated transferrin, 50 μmol/l 2-mercaptoethanol, 5 ng/ml interleukin (IL)−3, 50 ng/ml stem cell factor (SCF), and 10 ng/ml IL-6 (complete medium). For erythroid differentiation, cells were incubated for 6 d in complete medium containing 5 U/ml erythropoietin (EPO) and 50 ng/ml SCF without IL-3 and IL-6. Samples were then double stained with anti-CD71-FITC and anti-glycophorin A and analysed by flow cytometry.

Isolation and culture of AML blasts

Samples from AML patients were obtained at diagnosis from peripheral blood, following informed consent according to the ethical standards of the institutional guidelines. The AML cases were defined according to the classification of the French-American-British (FAB) committee. All samples had >80% blasts after Ficoll-Hypaque density-gradient centrifugation, as assessed by flow cytometric analysis with anti-CD13, CD33, CD34 antibodies (alone or in combination). AML blasts

(1×10^6/ml) were cultured in methylcellulose medium (Methocult, Stem Cell Technologies, Vancouver, Canada) supplemented with human recombinant growth factors: IL-3 (20 ng/ml), IL-6 (20 ng/ml), SCF (50 ng/ml).

Induction and detection of apoptosis

Cell lines were treated with etoposide (5 μg/ml) or cytarabine (0·3 μmol/g) for 6 h at 37°C. In some experiments, prior to apoptosis induction, cells had been pre-incubated for 24 h with wortmannin (100 nmol/l) or deguelin at the indicated concentrations. For experiments on $CD34^+$ cells and AML blasts, cytarabine was employed at 0·1 μmol/l (Frelin *et al*, 2005). For detection of apoptosis, samples containing 2×10^5 cells were harvested by centrifugation at 200 g for 10 min, fixed with 70% cold ethanol for 1 h, and subsequently stained with propidium iodide (PI, DNA-Prep kit, Beckman Coulter). The subdiploid DNA content was calculated using an Epics XL flow cytometer with the appropriate software (Beckman Coulter). At least 10 000 events/sample were acquired. In some cases, cells were also stained with Annexin V-FITC (Roche Applied Science) as reported elsewhere (Zhao *et al*, 2004). Briefly, cells were washed in phosphate-buffered saline (PBS, pH 7·4) and resuspended in 100 μl of binding buffer containing Annexin V-FITC. Cells were analysed by flow cytometry after addition of PI. Annexin V-FITC binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of those cells with a compromised cell membrane. This enabled live cells (unstained with either fluorochrome) to be discriminated from apoptotic cells (stained only with Annexin V-FITC) and necrotic cells (stained with both Annexin V and PI) (Vermes *et al*, 1995). Results obtained with Annexin V-FITC technique were always nearly superimposable to those given by the subdiploid DNA content analysis, as far as the percentage of apoptotic cells was concerned.

Protein concentration assay

This was performed according to the manufacturers' instructions using the detergent compatible Bio-Rad Protein Assay.

Preparation of cell homogenates and Western blot analysis

Cells were washed twice in PBS, then lysed (10^7/ml) in 10 mmol/l Tris–HCl, pH 7·4, 1 mmol/l $MgCl_2$, 1 mmol/l EGTA, 1% Triton X-100, 0·25 mol/l sucrose, containing the Complete Protease Inhibitor Cocktail supplemented with 50 mmol/l NaF, 1 mmol/l 2-glycerophosphate, 25 mmol/l NaPPi. After 15 min on ice, the cells were homogenized by 10 passages through a 25 gauge needle, and centrifuged in a microfuge (9300 ***g***) for 10 min at 4°C. An aliquot of the supernatant was saved for protein assay. Supernatants were mixed with 4 × electrophoresis sample buffer, then protein (80 μg/lane), was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose sheets using a semi-dry blotting apparatus. Sheets were saturated in PBS containing 5% normal goat serum, 4% BSA, 0·25 non-fat dried milk (blocking buffer) for 60 min at 37°C, then incubated overnight at 4°C in blocking buffer containing the primary antibody. After four washes in PBS containing 0·1% Tween-20, the sheets were incubated for 30 min at room temperature with the appropriate peroxidase-conjugated secondary antibody, diluted 1:5000 in blocking buffer, and washed as above. Bands were visualized by the ECL method.

Statistical evaluation

The data are given as mean values ± standard error of the mean (SEM). For statistical analyses, the Student's *t*-test for unpaired samples at the level of significance of 0·01 was used.

Results

Effects of deguelin on the cell cycle of human leukaemia cell lines

As experimental systems, we chose U937 cells, which were reported to have elevated levels of p-Akt (Matsuoka *et al*, 2003), and HL60PT cells, which do not have an active PI3K/Akt pathway (Martelli *et al*, 2003; Neri *et al*, 2003). We assessed whether deguelin affected cell growth by determining the effects of drug on the cell cycle by flow cytometry of PI-stained samples. When employed for 24 h at 1 nmol/l, deguelin did not alter the cell cycle of U937 cells. However, at 10 nmol/l, deguelin significantly increased the percentage of G2/M cells and decreased that of S phase cells (Table I). A 100 nmol/l concentration of deguelin induced a significant increase of apoptotic cells in U937 cells, as demonstrated by Annexin V-FITC staining. In contrast, deguelin (either 10 or 100 nmol/l) did not affect the cell cycle and apoptosis of HL60 PT cells (Table I).

Deguelin downregulates Akt phosphorylation in leukaemia cell lines with an active PI3K/Akt axis

Deguelin is a recently recognized PI3K inhibitor. Therefore, we decided to investigate the effects of deguelin on the phosphorylation levels of Akt, a downstream target of PI3K. As given in Fig 1, Western blot analysis with phospho-specific antibodies confirmed the presence, in these two cell lines, of high levels of both Thr 308 p-Akt and Ser 473 p-Akt. Akt phosphorylation was downregulated by wortmannin. A 24-h incubation with 1 nmol/l deguelin did not decrease the Akt phosphorylation, and consistently showed lack of effect on the cell cycle. However, if the concentration was raised to 10 or 100 nmol/l, the effects of deguelin on the p-Akt levels were very evident. At 10 or 100 nmol/l, deguelin was more effective than wortmannin (100 nmol/l) in inhibiting Akt phosphory-

Table I. Flow cytometric analysis of cell cycle in response to deguelin. Cells were incubated with the indicated concentrations of deguelin for 24 h, then analysed for DNA content and for the percentage of cells in specific phases of the cell cycle by means of PI staining, whereas for detection of apoptotic cells the Annexin-FITC staining was employed. For this reason, the sum of apoptotic cells plus cells in various phases of the cell cycle exceeds 100%. Results are the mean value of three different experiments ± standard deviation.

	Control	1 nmol/l	10 nmol/l	100 nmol/l
U937				
Apoptotic	5·3 ± 2·1	5·1 ± 2·3	8·4 ± 3·1	25·6 ± 4·8*
G1	54·3 ± 6·2	53·7 ± 6·9	48·7 ± 6·9	59·5 ± 7·3
S	21·4 ± 3·5	19·9 ± 2·7	12·6 ± 2·0*	9·3 ± 1·2*
G2/M	20·8 ± 3·3	22·2 ± 3·6	32·9 ± 4·3*	11·1 ± 1·3*
HL60PT				
Apoptotic	6·1 ± 2·5	6·6 ± 2·9	7·9 ± 3·2	6·7 ± 2·3
G1	53·9 ± 5·8	52·9 ± 5·6	52·7 ± 6·8	51·6 ± 5·7
S	24·7 ± 3·9	22·6 ± 3·0	21·0 ± 3·6	22·9 ± 4·3
G2/M	17·2 ± 3·0	19·6 ± 2·5	21·3 ± 2·9	20·9 ± 2·4

*Significant differences ($P < 0·01$) in comparison with untreated (control) cells.

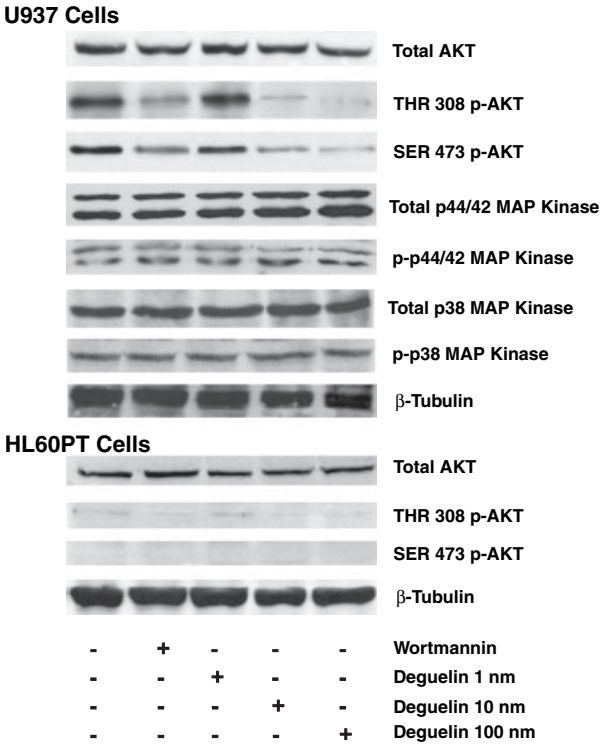

Fig 1. Deguelin inhibits Akt phosphorylation in U937 cells. Western blot analysis for expression of total Akt, Thr 308 p-Akt, Ser 473 p-Akt, total p44/42 MAP kinase, Thr 202/Tyr 204 p-MAP kinase, total p38 MAP kinase and Thr 180/Tyr 182 p-p38 MAP kinase. Cells were incubated with the pharmacological inhibitors for 24 h prior to lysate preparations. Wortmannin was present at 100 nmol/l. Protein (80 μg/lane) was separated by SDS-PAGE and blotted to nitrocellulose sheets. Immunoreactive bands were visualized by the ECL technique. Immunostaining with a monoclonal antibody to β-tubulin confirmed equal loading. Blots are representative of three separate experiments.

lation. Total Akt expression was unchanged by deguelin. Moreover, deguelin did not affect the expression or the phosphorylation levels of either p44/42 or p38 MAP kinases in U937 cells. Total Akt expression was not affected by deguelin also in HL60PT cells. These cells displayed almost undetectable levels of p-Akt, in agreement with our own previous results (Martelli *et al*, 2003; Neri *et al*, 2003).

Deguelin increases sensitivity of human leukaemia cells to chemotherapeutic drugs

As the PI3K/Akt signalling pathway plays a fundamental role in cell survival, we wanted to verify whether deguelin, at concentrations which promote Akt dephosphorylation, could enhance sensitivity of U937 cells to chemotherapeutic drugs, i.e. a treatment known to induce cell death through apoptosis.

As presented in Fig 2A, 1 nmol/l deguelin did not increase the rate of apoptotic U937 cells. When employed at 10 nmol/l it only slightly enhanced the number of apoptotic U937 cells. In contrast, if the concentration was raised to 100 nmol/l, deguelin alone significantly increased the percentage of apoptotic U937 cells. However, deguelin (10 or 100 nmol/l), when used in combination with either etoposide or cytarabine, dramatically increased the number of U937 cells that underwent apoptosis. In this respect, deguelin, at either concentration employed, was more effective than wortmannin. At 1 nmol/l, deguelin was not effective in sensitizing the cells to drugs (Fig 2A).

The concept of targeting the PI3K/Akt pathway would have more validity if the observed increases in apoptosis were greater in cells with an over-activated pathway than in cells with low levels of Akt phosphorylation. To address this issue, we employed HL60PT cells, which have low levels of p-Akt and are very sensitive to etoposide and cytarabine (Neri *et al*, 2003). As given in Fig 2B, deguelin (10–100 nmol/l) did not increase the percentage of apoptotic HL60PT cells in response to chemotherapeutic drugs.

Effect of deguelin on survival, Akt phosphorylation, and on EPO- and SCF-induced erythropoiesis from CD34$^+$ progenitor cells

We next sought to assess the influence of deguelin on the survival of human umbilical CB CD34$^+$ cells cultured in the presence of a cocktail of cytokines. As demonstrated in Fig 3A, wortmannin (100 nmol/l) did not increase in a significant manner the percentage of apoptotic cells after 1 or 2 d of culture. Deguelin gave a similar result when employed at 10 nmol/l. However, if it was used at 100 nmol/l, the percentage of apoptotic cells significantly rose, even after 1 d of culture.

We then investigated the effect of deguelin on the phosphorylation state of Akt expressed by CD34$^+$ cells. As shown in Fig 3B, Akt was not phosphorylated on Thr 308 or Ser 473 in freshly isolated CD34$^+$ cells. After 1 d of culture,

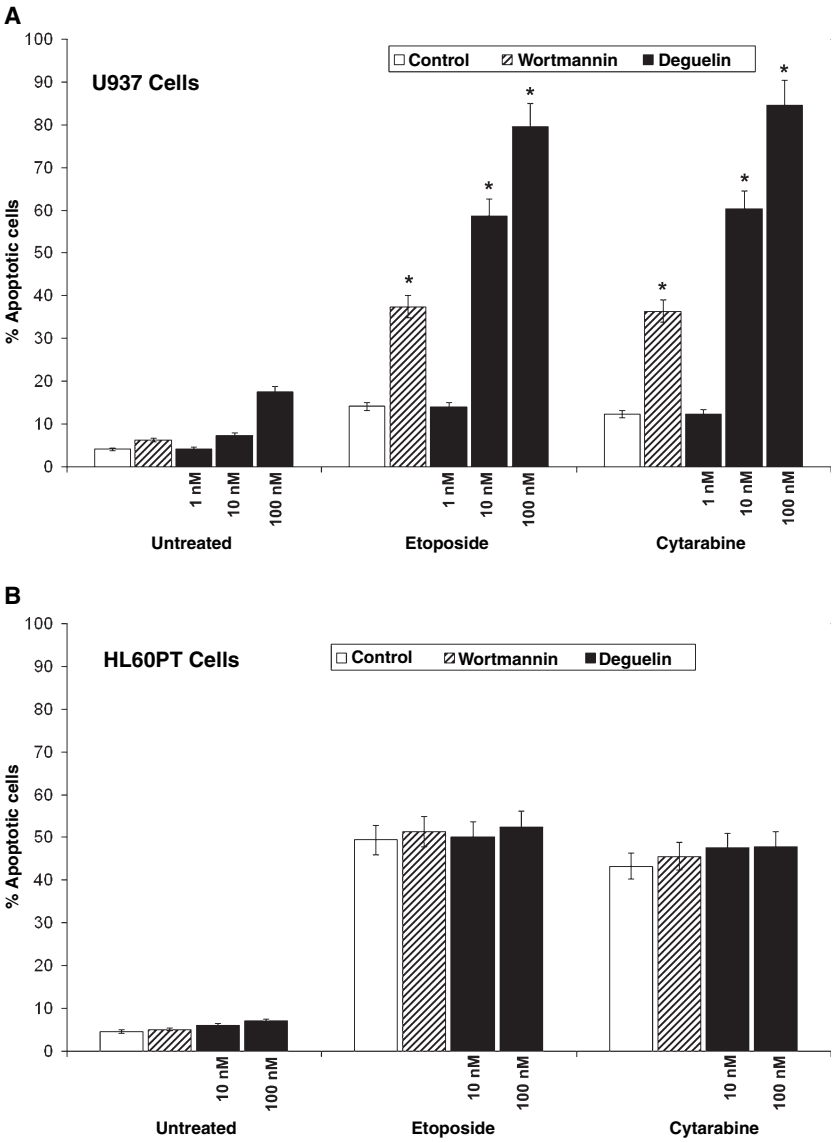

Fig 2. Deguelin increases the sensitivity of leukaemia cells to chemotherapeutic drugs. (A) U937 cells. Cells were treated with etoposide (5 μg/ml) or cytarabine (0·3 μmol/l) for 6 h. (B) HL60PT cells. In (A) and (B) the pharmacological inhibitors had been present for 24 h prior to treatment with the apoptosis inducers. Wortmannin concentration was 100 nmol/l. Apoptotic cells were evaluated by flow cytometric analysis as a subdiploid peak in PI-stained samples. Results are the mean of three different experiments ± standard deviation. *Significant differences ($P < 0{\cdot}01$) in comparison with untreated (control) cells.

there was a dramatic increase in Akt phosphorylation, most probably because of the stimulation exerted by SCF on PI3K (Sui *et al*, 2000). Deguelin markedly inhibited Akt phosphorylation when employed at 10 nmol/l. At 100 nmol/l deguelin, Akt was completely dephosphorylated.

As previous findings indicated that the PI3K inhibitor LY294002 counteracted the EPO-dependent erythroid differentiation of $CD34^+$ progenitor cells (Myklebust *et al*, 2002), we investigated whether or not deguelin negatively affected the formation of GPA^+ erythroid cells from CB $CD34^+$ haematopoietic progenitor cells. The $CD34^+$ cells were cultured for 6 d in serum-free medium supplemented with EPO and SCF. Over the time course of the incubation, the percentage of $CD34^+$ cells decreased from 95·5% to 18·6%, in agreement with previous reports (e.g. Edvardsson *et al*, 2004). In control samples, approximately 41% of GPA^+ cells were detected (Fig 3C). EPO- and SCF-induced formation of GPA^+ erythroid cells was severely impaired by wortmannin (8%). Deguelin (10 nmol/l) was less effective than wortmannin in maintaining an immature phenotype, as there were 21% GPA^+ cells after 6 d of culture.

Deguelin dephosphorylates Akt and increases cytarabine sensitivity of AML blasts but not of CB $CD34^+$

To test the effect of deguelin in primary AML blasts expressing high levels of p-Akt, we incubated cells for 24 h in serum-free medium supplemented with a cocktail of cytokines, because previous results have indicated that incubation of AML blasts in serum-free medium with no cytokines led to a decrease of Akt phosphorylation (Zhao *et al*, 2004). The presence of deguelin (10 nmol/l) during the 24-h incubation produced a dramatic decrease in the levels of p-Akt in all of the four investigated patients (Fig 4), whereas total Akt levels were unaffected. We then investigated whether deguelin might increase AML blast sensitivity to cytarabine. As given in Fig 5A, after a 24-h

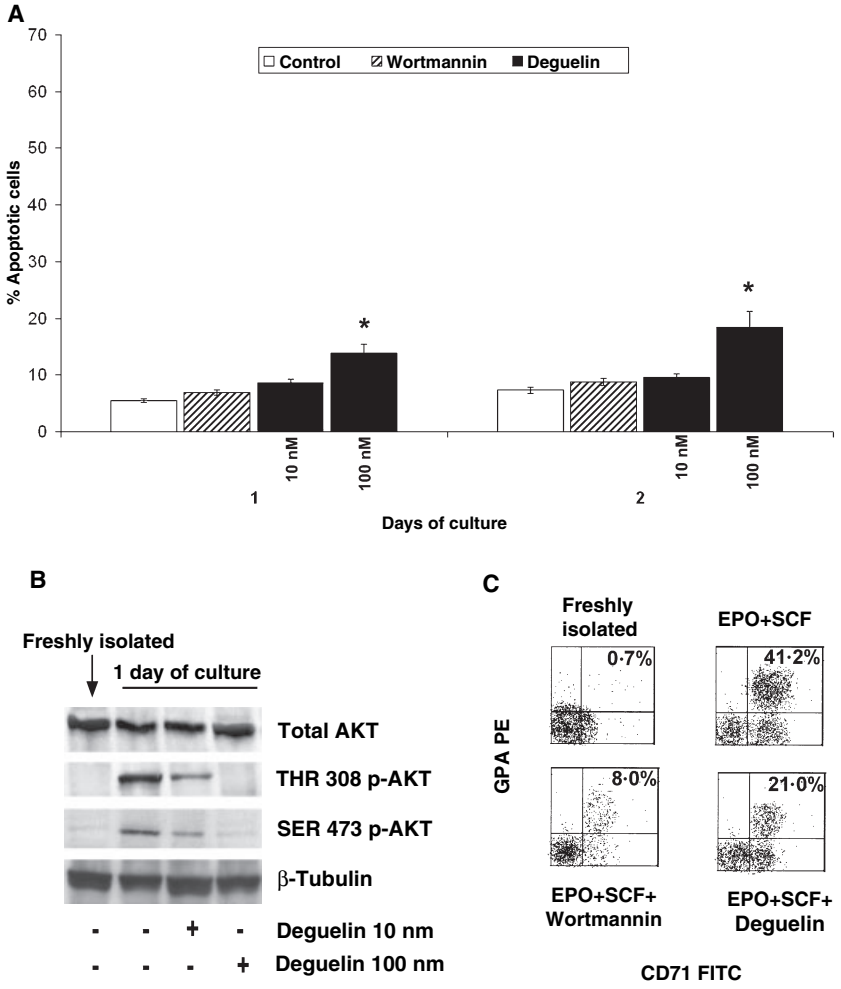

Fig 3. Effects of deguelin on survival, Akt phosphorylation, and on EPO- and SCF-dependent erythroid differentiation of cultured human CB $CD34^+$ cells. (A) effect on apoptosis. Cells were plated in 24-well dishes at 2×10^6 cells/ml in complete medium lacking or containing the indicated inhibitors, and incubated for up to 2 d. Wortmannin concentration was 100 nmol/l. Apoptotic cells were evaluated by flow cytometry analysis. Results are the mean of three different experiments ± standard deviation. The asterisks indicate significant differences ($P < 0{\cdot}01$) in comparison with untreated (control) cells. (B) Western blot analysis for Akt phosphorylation of $CD34^+$ cells. Protein samples blotted to nitrocellulose sheets were probed either with antibodies to total Akt or to p-Akt forms. The blots in B are representative of three separate experiments. In A and B the Akt inhibitors had been present since cell plating. (C) $CD34^+$ cells were immunophenotyped immediately after cell isolation or cultured for 6 d in the presence of 5 U/ml erythropoietin (EPO) and 50 ng/ml SCF. Wortmannin (100 nmol/l) or deguelin (10 nmol/l) were added at time 0 and after 3 d. Samples were analysed by flow cytometry for GPA and CD71 expression. The percentage of GPA^+ cells is indicated in each histogram.

incubation in serum-free medium plus cytokines, most of AML blasts were in G0/G1 phase of the cell cycle as expected (Xu *et al*, 2003). Cytarabine (0·1 μmol/l for 24 h) slightly increased the percentage of sub-G1 (apoptotic cells). Deguelin (10 nmol/l for 24 h) did not significantly alter the cell cycle of AML blasts. However, when cells treated with deguelin were exposed to cytarabine, the percentage of apoptotic cells rose substantially. We next investigated whether deguelin could increase sensitivity to cytarabine of normal $CD34^+$ cells (Fig 5B). After a 24-h treatment, the combination of cytarabine and deguelin did not increase the percentage of apoptotic cells over cytarabine alone, as shown by Annexin V staining. These results demonstrated a fundamental difference in the sensitivity to deguelin of leukaemic cells *versus* normal $CD34^+$ progenitors.

Discussion

The resistance of many types of neoplasia to conventional therapies is a major factor undermining successful cancer treatment. The PI3K/Akt pathway is rapidly emerging as a key mediator of drug resistance in tumour cells. As Akt is usually activated through PI3K, inhibitors that target PI3K (LY294002, wortmannin) have been extensively investigated as anti-cancer molecules. Numerous studies have established the potential utility of these PI3K inhibitors as cancer therapies. However, the toxicity of these PI3K inhibitors and their scarce water solubility has precluded their therapeutic use to date (West *et al*, 2002).

Deguelin is a naturally occurring rotenoid isolated from several plant species that has been recently demonstrated to be capable of inhibiting PI3K in premalignant and malignant human bronchial epithelial cells (Chun *et al*, 2003).

Here, we investigated whether or not deguelin could increase sensitivity to chemotherapeutic drugs of human acute leukaemia cell lines with an active PI3K/Akt pathway. We found that deguelin, when employed for 24 h at 10 nmol/l, caused an S phase arrest of U937 cells, with interference of progression to G2/M phase. This is in agreement with Chun *et al* (2003). Deguelin, when employed alone up to a concentration of 10 nmol/l for 24 h, did not significantly increase the apoptotic rate of U937 cells. However, when the concentration was increased to 100 nmol/l, deguelin induced apoptotic cell death in these cells. This is consistent with the observation that deguelin increased apoptosis of human bronchial cells (Chun *et al*, 2003). It is very interesting that these concentrations fall within a range that is attainable *in vivo* (Udeani *et al*, 2001).

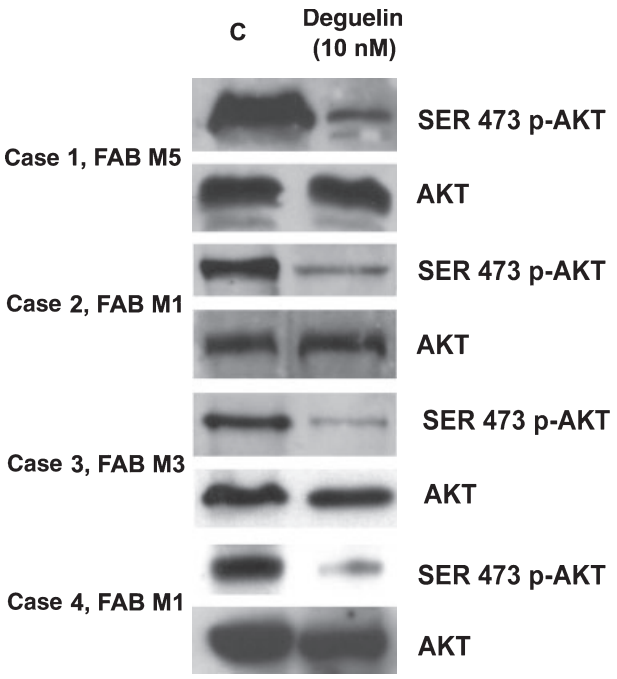

Fig 4. Deguelin induces Akt dephosphorylation in AML blasts. Western blot analysis for expression of total Akt, and Ser 473 p-Akt. Cells were incubated with deguelin (10 nmol/l) for 24 h prior to lysate preparations. Protein (80 μg/lane) was separated by SDS-PAGE and blotted to nitrocellulose sheets. Blots are representative of three separate experiments.

Deguelin (10–100 nmol/l) induced Akt dephosphorylation, however, it did not downregulate either p44/42 or p38 MAP kinase phosphorylation levels. Deguelin was able to sensitize U937 cells to two chemotherapeutic drugs used for the treatment of AML. Moreover, deguelin sensitized AML blasts to cytarabine, even if it did not markedly affect the cell cycle of these cells.

Previous results have suggested that pharmacological inhibitors of PI3K such as wortmannin and LY294002, negatively affect the survival of leukaemic cells, while sparing normal bone marrow or umbilical CB $CD34^+$cells (Xu *et al*, 2003). Our findings, obtained with CB $CD34^+$ cells, have confirmed that wortmannin (100 nmol/l) is not toxic for these cells. They have also shown that deguelin, if used at 10 nmol/l, did not negatively affect the survival rate of haematopoietic progenitors. In contrast, if deguelin was used at 100 nmol/l, it caused a significant increase in the percentage of apoptotic CB $CD34^+$ cells. It is important to emphasize here, that a 10 nmol/l concentration of deguelin lowered the levels of p-Akt in CB $CD34^+$ cells, whereas a 100 nmol/l caused a complete dephosphorylation of Akt. Conceivably, a total loss of p-Akt decreases the survival rate of CB $CD34^+$ cells. In this context, it should not been overlooked that a functioning PI3K/Akt signalling is required for normal haematopoiesis, as erythropoietin activates Akt in $CD34^+$ haematopoietic progenitor cells (Myklebust *et al*, 2002), while inhibition of this pathway suppresses myeloid progenitor cell differentiation (Lewis *et al*, 2004). It has been shown that LY294002, a PI3K inhibitor, severely impairs the SCF- and EPO-driven erythroid differentiation of $CD34^+$ haematopoietic progenitor cells (Myklebust *et al*, 2002). For this reason, we analysed the effect of deguelin on EPO- and SCF-induced erythroid differentiation of CB $CD34^+$ cells. Deguelin (10 nmol/l) was found to be far less effective than wortmannin in blocking the differentiation process of $CD34^+$ haematopoietic progenitors. It might be that the different effects of deguelin and wortmannin on *in vitro* erythropoiesis depend on the fact that wortmannin, apart from PI3K, also inhibits phospholipase C, D and A_2, whose activities are stimulated by EPO and SCF (Mason-Garcia & Beckman, 1992; Clejan *et al*, 1996; Kozawa *et al*, 1997; Roberts *et al*, 1999; Boudot *et al*, 2003).

However, a concentration of 10 nmol/l of deguelin was sufficient to markedly increase the sensitivity of human leukaemia cells to chemotherapeutic drugs. Moreover, a 10 nmol/l deguelin concentration dramatically decreased p-Akt levels of AML blasts and increased the sensitivity of these cells to cytarabine. Although these experiments were performed *in vitro* and the number of investigated patients was limited, taken together they suggest that deguelin, when used at certain concentrations, might not exert a strong toxic effect on normal haematopoietic cells *in vivo*, as suggested also by the experiments in which deguelin was combined with cytarabine treatment in $CD34^+$ cells. One of the patients employed in this study had promyelocytic leukaemia. Usually, this form of leukaemia is sensitive to treatment with retinoids, although resistance to retinoids has been reported (Gallagher, 2002). Thus, it might be envisaged that, in the future, a combination therapy consisting of traditional chemotherapy plus inhibitors of the PI3K/Akt would be of clinical benefit for these patients. It might also be that deguelin would be useful to overcome retinoid resistance, as we have shown for other inhibitors of the PIK3/Akt axis (Martelli *et al*, 2003; Neri *et al*, 2003).

Overall, the present results are in agreement with our own findings obtained with selective Akt inhibitors, which enhanced sensitivity of cells with an active PI3K/Akt pathway to chemotherapeutic drugs (Martelli *et al*, 2003; Tabellini *et al*, 2004). However, an aspect, which needs to be emphasized is that, at present, there is no information about the pharmacokinetics of these Akt inhibitors. Moreover, it is not known whether it is possible to administer them intravenously or if they will cause serious side effects. These Akt inhibitors belong to the same family of chemicals as DPIEL (D-3-Deoxyphosphatidyl-myoinositol ether lipid), which, when given intravenously to rats, caused severe haemolysis (Gills & Dennis, 2004). In contrast, deguelin could be safely administered intravenously and studies on its pharmacokinetics have already been performed in rats (Udeani *et al*, 2001). The results of this investigation showed that a 1 μmol/l concentration of deguelin was achievable in a variety of tissues *in vivo* after intravenous delivery. Moreover, this dosage was not toxic to rats.

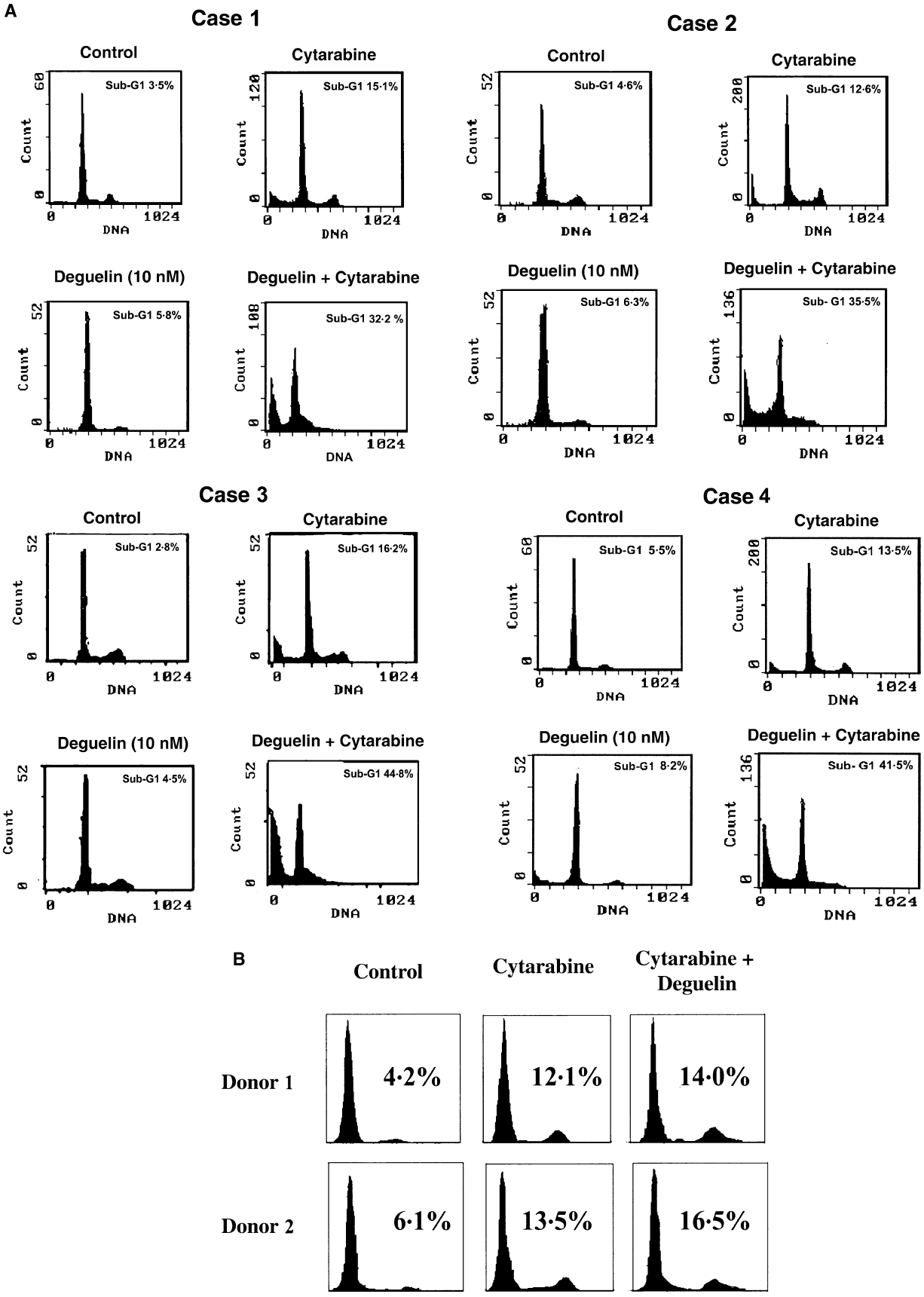

Fig 5. Deguelin increases the sensitivity of AML blasts, but not of normal $CD34^+$ haematopoietic progenitors cells, to cytarabine. (A) AML blasts were treated with deguelin for 24 h, then with cytarabine (0·1 μmol/l) for 24 h. Apoptotic cells were evaluated by flow cytometric analysis as a subdiploid peak in PI-stained samples. A representative of three separate experiments is shown for each case. (B) $CD34^+$ cells were treated with deguelin (10 nmol/l) for 24 h, then for an additional 24 h with cytarabine (0·1 μmol/l). Apoptotic cells were evaluated by Annexin V staining. A representative of three separate experiments is shown.

To date, the effects of PI3K/Akt inhibition are unknown in humans. Tumour cells may be selectively sensitive to inhibition because they often grow in hostile environments and would therefore be likely to have a higher reliance on signalling pathways for survival than normal cells. However, should PI3K/Akt inhibitors cause severe toxicity as single agents, these drugs may also find use as sensitizers to other forms of therapy (as demonstrated here) and for this purpose lower, less toxic doses might be efficacious.

Future work will need to address whether the potential of deguelin as a PI3K/Akt inhibitor may be fully realized in cancer treatment. If so, the combination of deguelin with conventional or novel therapeutic agents may provide better treatment for defeating those leukaemias that have become resistant to therapy because of an upregulation of PI3K/Akt signalling.

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