Cancer Chemother Pharmacol (2008) 62:439–448
DOI 10.1007/s00280-007-0623-4

ORIGINAL ARTICLE

The FLT3 inhibitor PKC412 in combination with cytostatic drugs in vitro in acute myeloid leukemia

Lars Möllgård · Stefan Deneberg · Hareth Nahi · Sofia Bengtzen · Kerstin Jonsson-Videsäter · Thoas Fioretos · Anna Andersson · Christer Paul · Sören Lehmann

Received: 3 July 2007 / Accepted: 1 October 2007 / Published online: 25 October 2007

Abstract An internal tandem duplication of FLT3 (FLT3/ITD) occurs in approximately 25% of newly diagnosed AML. PKC412 inhibits the growth of leukemic cell lines with FLT3 mutations such as the MV4-11. This study evaluated the in vitro effects of the combination of PKC412 and ara-C or daunorubicin, studying the effect of co-incubation, pre-incubation and sequential incubation of the drugs in patient samples and cell lines. Thirty-three patients with AML were included. Two cell lines were studied; MV4-11 that expresses the FLT3/ITD and HL-60 that does not. In the patient cells PKC412 exerted its effect at concentrations between 0.1 and 2.0 μM. For MV4-11 cells concentrations down to 1 nM were effective. In patient samples, the results of co-incubation of PKC412 with ara-C were synergistic in 5%, additive in 67%, sub additive in 17% and antagonistic in 11% of the cases. In patient cells, incubations with ara-C and PKC412 resulted in synergistic effects in 17% of the FLT3/ITD positive samples compared to 0% synergistic in the FLT3/ITD negative samples ($p < 0.01$). Antagonistic effects were more common in the FLT3/ITD negative samples. The timing of the drugs had little impact on the effect. In cell lines, antagonistic effects were seen frequently in HL-60 (90%) and less so in MV4-11 (60%) regardless of sequence or timing of the drugs. The combination of daunorubicin and PKC412 resulted in more synergistic and less antagonistic effects compared to combinations with ara-C, in both patient material and cell lines. The combination of Lonafarnib, a farnesyl-transferase inhibitor (FTI) and PKC412 had additive and synergistic effects in both FLT3/ITD positive and negative cell lines. In conclusion, the combination of PKC412 together with chemotherapeutic drugs is more effective in FLT3/ITD positive AML cells. Antagonistic effects can be seen, especially in patient samples without FLT3/ITD. Also, the combination of PKC412 and the farnesylinhibitor lonafarnib should be further explored.

Keywords Acute myeloid leukemia · FLT3 · PKC412 · Cytarabine · Daunorubicin · Lonafarnib

Introduction

Fms-like tyrosine kinase 3 (FLT3) is a member of the class III tyrosine kinase receptor family. FLT3 is predominantly expressed on hematopoietic progenitor cells but is also found in other tissues such as placenta, gonads and brain [22, 30]. Interaction with its ligand (FL) results in receptor dimerization, autophosphorylation and subsequent phosphorylation of cytoplasmic substrates that are involved in signalling pathways regulating the proliferation of pluripotent stem cells, early progenitor cells and immature lymphocytes [21].

A majority of acute myeloid leukemias (AML) and lymphoblastic leukemias (ALL) express FLT3 [4]. FLT3 is mutated and activated in approx. 30% of all patients with AML [9]. The mutations involve either an internal tandem

Lars Möllgård and Stefan Deneberg contributed equally to the manuscript.

L. Möllgård · S. Deneberg (✉) · H. Nahi · S. Bengtzen · K. Jonsson-Videsäter · C. Paul · S. Lehmann
Department of Hematology,
Karolinska University Hospital Huddinge,
141 86 Stockholm, Sweden
e-mail: stefan.deneberg@karolinska.se

T. Fioretos · A. Andersson
Department of Clinical Genetics,
University Hospital, Lund, Sweden

duplication (FLT3/ITD) in approx. 25% of AML patients or a point mutation in the activating loop in approx. 7% of patients. Both types of mutations result in a ligand-independent receptor dimerization, phosphorylation and constitutive activation of downstream signalling pathways [16]. FLT3/ITD is not expressed in chronic myeloid leukemia, chronic lymphocytic leukemia, non-Hodgkin's lymphoma or multiple myeloma [38]. The presence of FLT3/ITD in adult AML patients seems to have little or no impact on the ability to achieve complete remission. The most significant impact of FLT3/ITD found in several studies is its association with increased relapse risk, decreased disease-free survival (DFS) and overall survival (OS) [8, 13, 15]. However, two large studies failed to show a decreased OS in AML patients with FLT3/ITD [31, 33].

PKC412 (*N*-benzoyl-sturosporine) was developed originally as a protein kinase C and vascular growth factor receptor inhibitor and has been used in phase I trials in solid tumors [25, 27]. Subsequently, this compound was found to specifically and potently inhibit the growth of leukemic cell lines expressing FLT3/ITD or an activating loop mutation of FLT3. Moreover, PKC412 prolonged survivial in transgenic mice with a FLT3/ITD-induced-myeloproliferative-like syndrome [36]. PKC412 has also been used in a phase II trial in advanced AML patients with the FLT3/ITD [32]. In the latter study, 14 of the 20 patients experienced at least a transient 50% reduction in the number of peripheral blasts. This response is comparable to that observed with imatinib in blast crisis of chronic myeloid leukemia [5]. To clarify the role of PKC412 in AML new treatment strategies have to be developed, including combinations with conventional cytostatic drugs used in AML. Furukawa et al. recently published results of co-incubation of PKC412 with eight different cytotoxic agents in human leukemia cell lines, showing synergistic results with all agents in the cell lines with an activating FLT3/ITD mutation. In the cell lines expressing wild type FLT3, antagonistic results of co-incubation were frequent [7].

Lonafarnib, SCH-66336, is a farnesyl-transferase inhibitor (FTI) that has been shown to inhibit farnesylation and activation of Ras. It has been suggested that Lonafarnib also acts through inhibition of the farnesylation of RhoB, centromere-binding proteins (CENP)-E and -F as well as other not yet identified proteins [26]. Lonafarnib has shown effect as a single drug in a Phase II study in patients with MDS and CMML. Of 42 evaluable patients, 12 responded, 2 of whom had complete response and 10 had a hematological improvement. In addition, 16 of 37 patients who had bone marrow (BM) blasts of 45% at baseline showed a reduction of ≥50% in BM blasts [6].

We undertook an in vitro study where the combination of PKC412 and cytostatic drugs in samples from AML patients was evaluated. The AML cell lines MV4-11 and HL-60 were examined in a similar way. The study was designed to evaluate the role of different timing of the combined drugs. Also, cell lines were also exposed to the combination of FTI and PKC412.

Material and methods

Patient samples and cell lines

Peripheral blood or bone marrow was collected from 33 consecutive patients with AML. The characteristics of the patients are shown in Table 1. Peripheral blood or bone marrow were collected in heparinized tubes before the start of the treatment. The leukemic blast cells were separated by centrifugation (400*g*, 30 min) on Lymphoprep. The cells were then washed in RPMI 1640. MV4-11 is a FLT3/ITD positive cell line expressing only the mutated version of the gene, making it a model cell line for FLT3/ITD-related research. HL-60 is a cell line derived from a patient with acute promyelocytic leukemia. It expresses wild type FLT3 [28]. The study was approved by the Medical Research Ethics Committee and the institutional review board of the Karolinska University Hospital.

Incubations and culturing

Cells from fresh samples ($n = 24$) and freshly frozen cells ($n = 9$) were used for analysis. The cells (2.0×10^5 cells/ml) were incubated in a medium consisting of RPMI 1640 supplemented with Glutamax, 10% fetal bovine serum (FBS) and the cystostatic drug. In the PKC412 sensitivity study, we used drug concentrations between 0.001 and

Table 1 Patient characteristics

Variable	FLT3/ITD positive	FLT3/ITD negative
N	10	23
Age, median (range)	62.4 (30–79)	68.2 (23–88)
Gender (M/F)	5/5	7/16
Diagnosis (FAB)		
M0/M1/M2	0/3/3	0/7/6
M3/M4/M5	1/2/1	0/5/2
M6/M7/MDS-AML	0/0/0	0/0/2
Cytogenetic risk group		
Low	1	0
Intermediate	9	16
Poor	0	3
No data	0	4
Blast percentage in bone marrow at diagnosis (p = NS) % (range)	74.2 (25–94)	60.5 (28–90)

2.0 μM. In the synergism and timing study, we used the following concentrations: PKC412 0.2 μM continuously, ara-C 0.5 μM continuously and daunorubicin 0.15 μM for 1 h. The 1 h incubation of daunorubicin was chosen to mimic the in vivo situation [34]. In the first part of the synergism study, a 4 days co-incubation of PKC412 and ara-C or daunorubicin was performed. In the second part, two different in vitro timing models were used. In the pre-incubation model, the incubation time for the first drug was 4 days and the second drug was added after 1 day. In the sequential exposure model, the cells were incubated for 2 days with the first drug, then centrifuged and resuspended and incubated with the second drug for another 2 days. The different incubation models are illustrated in Fig. 1. Cell lines were incubated as described above at a cell concentration of 0.5 × 10(5)/ml. The following drug concentrations were used for HL-60: Ara-C at 0.05 or 0.1 μM and PKC 412 at 0.2 or 0.5 μM continuously and in the sequential exposure analysis. For daunorubicin, cells were exposed to 0.1 μM of the drug for 1 h. FTI at 0.25–0.5 μM was used continuously in co-incubation with PKC at 0.2–0.5 μM for four days. For MV4-11, Ara-C at 0.01 μM and PKC412 at 0.005 μM were used in the co- and pre-incubation studies and Ara-C at 0.05 μM and PKC412 at 0.01 μM in the sequential incubations. Daunorubicin at 0.03 μM was incubated for 1 h. Two different concentrations were used for the FTI-PKC412 4 day co-incubations: 1 nM PKC412 + 250 nM FTI and 5 nm PKC412 + 0.5 μM FTI. The concentrations were chosen on the basis of the IC50 curves.

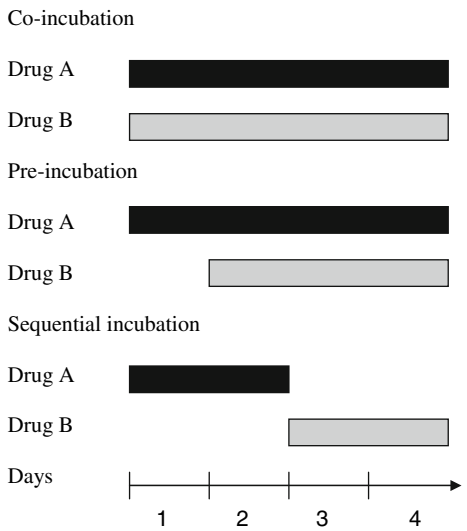

Fig. 1 Schematic illustration of the three different types of incubation used in this study. The combination of PKC412 0.2 mM continuously and ara-C 0.05 mM continuously or daunorubicin 0.15 mM for 1 h. In cell lines the concentrations of the drugs were chosen according to IC-50 curves (data not provided). The 1 h incubation of daunorubicin was chosen to mimic the in vivo situation with 1 h infusion

ATP bioluminescence assay

Extraction of ATP in leukemic cells was performed by mixing equal volumes (2 ml) of cell-suspension and 2.5% trichloracetic acid (TCA). The extracts were assayed immediately or stored in a freezer (−20°C) until analysis. For the assessment of cell viability we used a bioluminiscence assay measuring ATP content [3, 12, 24, 29]. The measurements were performed automatically in an Athos Lucy 1 luminometer (Hettich Labinstrument AB, Sweden). An ATP Kit SL 144-041 (Bio Thema, Dalarö, Sweden) was used for the reaction. The amount of ATP (given as nmol ATP/sample) and the percentage ATP in a sample when compared to the drug-free control was calculated. The result at each concentration represents a mean of two parallel experiments.

Analysis of FLT3 mutations

Polymerase chain reaction (PCR) was performed to identify patient samples with FLT3/ITD and point mutation D835 in the second tyrosine kinase domain (ATKD) as previously described [1]. FLT3/ITD status in the cell lines have been explored previously [28] and were verified with PCR.

The additive model

In order to study the cytotoxic effects on cells incubated with the drug-combination, the additive model was used [11, 35]. This model predicts that the effect of a combination will be equal to the product of the effects of its constituents. For example, if a drug combination was composed of drugs producing a cell viability of 40 and 60%, respectively, the combination would be expected to result in 24% viable cells (0.4 × 0.6). An observed combination effect that is larger than predicted by the additive model indicates synergism, whereas a smaller effect represents a subadditive effect. A ratio between the observed viability and the viability predicted by the additive model was calculated for all combinations. If the ratio exceeded 1.2, the interaction was classified as subadditive and if it was below 0.8, the interaction was classified as synergistic. Ratios between 0.8 and 1.2 were considered to indicate additive interactions, and this interval was set to take into account the intra-assay variability [17]. A cell viability value for the combination exceeding the cell viability of the most effective drug alone was classified as antagonism.

Statistics

The *t* test for independent samples was used to evaluate the differences in effect between FLT3/ITD positive and negative samples in each tested concentration of PKC412 and when comparing the individual drugs and the combination of ara-C/daunorubicin and PKC412 in FLT3/ITD positive

and negative samples. We used the Chi-square algorithm to find differences in the distribution of synergistic, additive, subadditive, and antagonistic effects in the FLT3/ITD positive and negative group, respectively. The Pearson correlation test was used to evaluate correlation of sensitivity of individual patient samples to different drug combinations.

Results

FLT3/ITD analysis

The PCR analysis showed that 10 of the 33 patients (30%) were FLT3/ITD positive. Four of these patients were included in the initial experiments to find the optimal concentration PKC412 and 10 were included in the timing study. No sample harboured a point mutation D835.

PKC412 sensitivity in AML blast cells and cell-lines

In ten patients, different concentrations of PKC412 were tested in order to find concentrations appropriate for the synergism experiments (Fig. 2). Nine different concentrations between 0.001 and 2.0 μM were used. Four patients were tested in the lower interval (0.001–1.0 μM) and six in the higher interval (0.05–2.0 μM). The therapeutic range of PKC412 was found to be within the interval 0.1–2.0 μM. When all combination experiments using PKC412 and Ara-C were summarized, the FLT3/ITD positive samples were significantly more sensitive to PKC412 compared to the FLT3/ITD negative ($p < 0.05$) (Fig. 3).

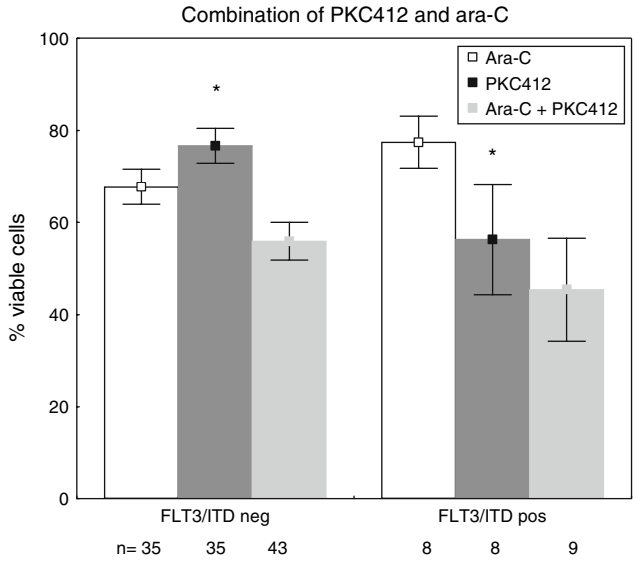

Fig. 3 This figure summarise the results of 138 different incubations from 18 patient samples used in the PKC/ara-C synergy study, comparing FLT3/ITD negative and positive AML samples (mean ± SEM). The concentrations ara-C 0.5 mM and PKC412 0.2 mM were used. * $p < 0.05$

IC50 curves for the cell lines HL-60 and MV4-11 shows that the FLT3/ITD negative cell line HL60 the therapeutic range is 0.2–0.5 μM and in the FLT3/ITD positive cell line MV4-11 it is 5–10 nM. (Fig. 4)

Co-incubation with ara-C or daunorubicin and PKC412

In 24 samples, co-incubation with PKC412 and ara-C was performed. Seventeen FLT3/ITD negative patient samples

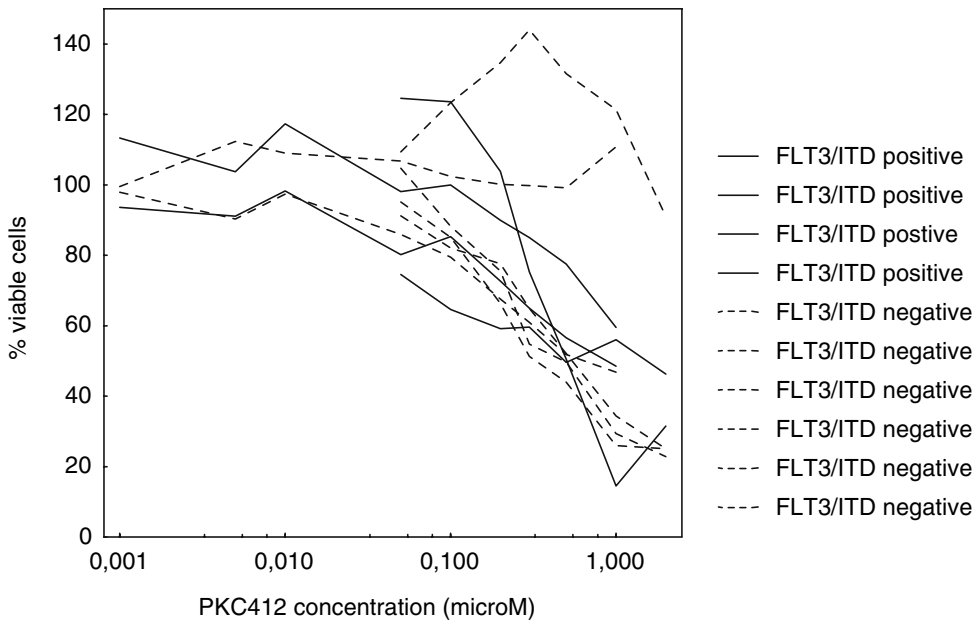

Fig. 2 The in vitro effect of PKC412 in samples from ten patients with FLT3/ITD negative (*dashed lines*) or positive (*solid lines*) AML. Four patients were tested in the lower interval (0.001–1.0 mM) and six in the higher interval (0.05–2.0 mM). For each concentration there was no significant difference between FLT3/ITD positive and negative samples

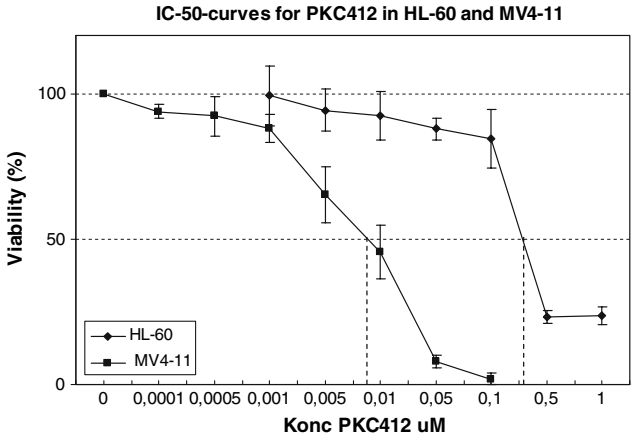

Fig. 4 IC-50 curves showing the difference in sensitivity for PKC412 between HL-60 and MV4-11 [mean values(±SEM)]

were investigated in the synergy study with co-incubation of PKC412 and ara-C. In 11 samples an additive interaction was seen, in four a sub additive and in two an antagonistic effect. In the seven FLT3/ITD positive samples, five showed an additive, one a synergistic effect and one a sub additive effect. In this patient subgroup, no sample with an antagonistic effect was found. The proportions of each type of response are shown in Fig. 5. PKC412 and daunorubicin were co-incubated in 26 patients, 18 FLT3/ITD negative and 8 FLT3/ITD positive. The combination with daunorubicin and PKC412 tended to have more synergistic effects (25 vs. 17%) and less antagonistic (0 vs. 17%) in the FLT3/ITD positive samples compared to the FLT3/ITD negative samples. When co-incubation with ara-C was compared with co-incubation with daunorubicin in the FLT3/ITD negative group, only co-incubation with daunorubicin resulted in synergistic effects (Fig. 5). Figure 6 shows the individual results for the combination incubations with PKC412 with ara-C versus daunorubicin. As shown by the figure, there was a significant correlation in the sensitivity between the two incubations ($p < 0.05$) There were no differences in cytotoxic effects between fresh cells and cells that had been cryoperserved. This was evaluated for all tested drugs as well as for cells with or without FLT3-ITD (data not shown).

Co-incubation with ara-C or daunorubicin and PKC412 in cell lines

In the wild type FLT3 HL-60 cell line, co-incubation of Ara-C and PKC412 resulted in antagonistic effects in three out of three independently performed experiments. In the FLT3/ITD positive MV4-11 cell line, the effect was additive in two out of three and antagonistic in one out of three experiments (Fig. 7). Co-incubation of daunorubicin and PKC412 rendered additive effects in two and a synergistic effect in one out of three tests with MV4-11. In HL-60, two

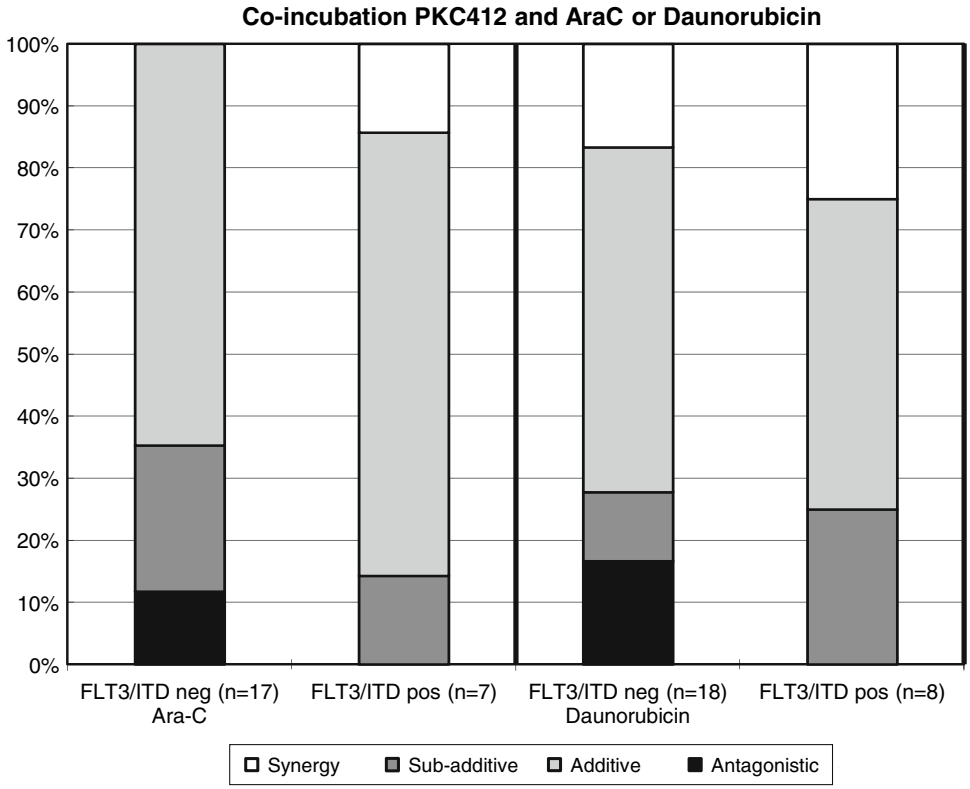

Fig. 5 Combination results of co-incubations with PKC412 (0.2 mM) and Ara-C (0.5 mM) or daunorubicin (0.15 mM) for 4 days on primary blast cells from patients with AML

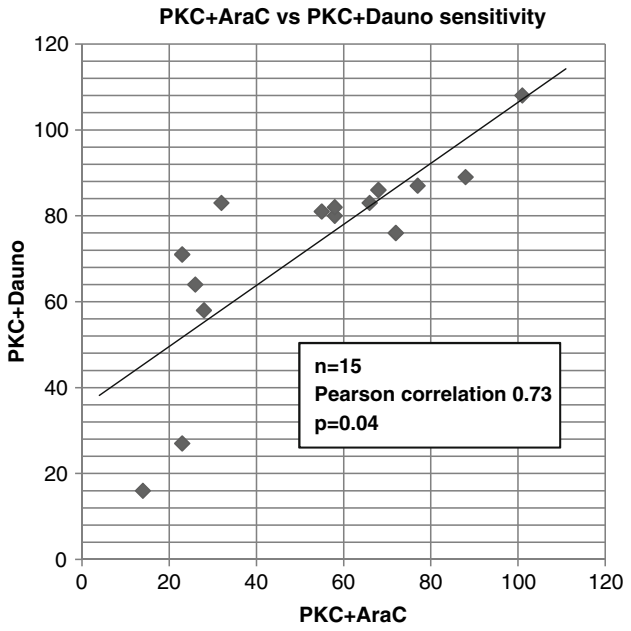

Fig. 6 Correlation of sensitivity between combinations of PKC+Ara-C and PKC + daunorubicin in the 15 patients (4 FLT3–ITD, 11 FLT3–WT) . Cells were co-incubated with both combinations for 4 days and the figure shows cell viability compared to unexposed controls

tests were sub additive and one out of three was antagonistic. The results of co-incubation in MV4-11 and HL60 cells are shown in Fig. 7.

Pre-incubation with ara-C and daunorubicin in combination with PKC412

When incubation was started with ara-C, a synergistic or additive effect was seen in 13 of 21 samples, 8/15 of the FLT3/ITD negative and 5/6 of the FLT3/ITD positive ones. When the incubation was started with PKC412 a synergistic or additive effect was seen in 13 of 21 samples, 7/15 of the FLT3/ITD negative and 6/6 of the FLT3/ITD positive ones. There results suggest that pre-incubation with PKC412 does not adversely effect the antileukemic effect in patient samples but it confirms that cells with FLT3/ITD are more sensitive the exposure of PKC412. The combination effects in the different groups and incubation modes are presented in Fig. 8. Eight patient samples were studied in the sequential incubation model. No differences in cytotoxic effect was found if samples were sequentially exposed to either PKC or Ara-C for day 1–2 and then the other drug day 3–4 (data not shown).

In all, 82 different incubations with ara-C and PKC412 were performed. If the results are summarized, not taking into consideration the type of incubation we found that in the FLT3/ITD negative group 56% resulted in a synergistic or additive effect and 44% in a sub additive or antagonistic effect as compared to 91 and 9%, respectively in the FLT3/ITD positive group ($p < 0.05$). In the FLT3/ITD positive group, 17% of the incubations resulted in a synergistic effect as compared to 0% in the FLT3/ITD negative group ($p < 0.01$).

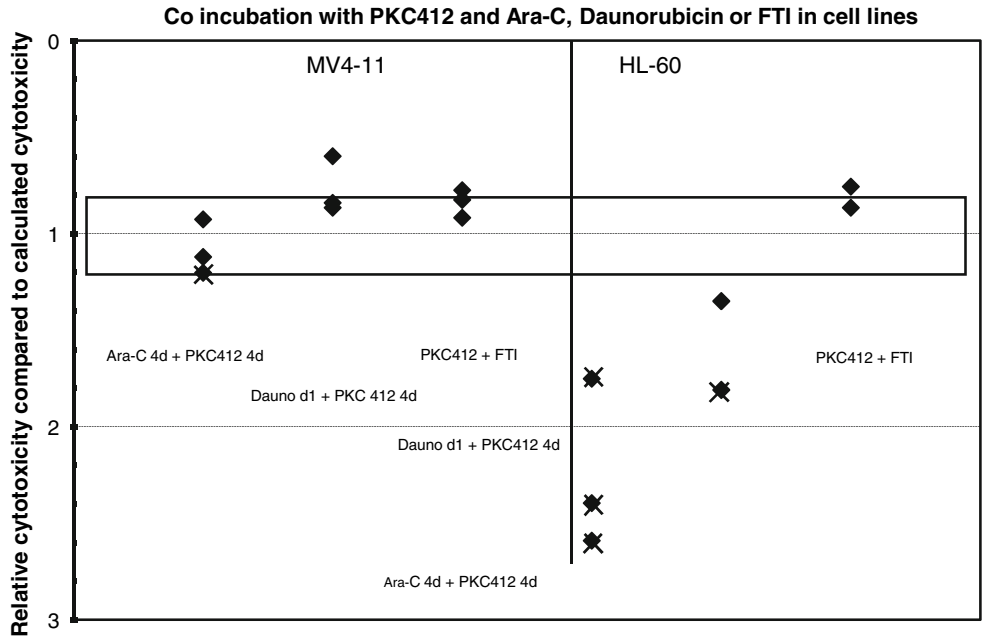

Fig. 7 Results of individual experiments on cell lines co-incubated with PKC412 (1–5 nM in MV4-11 and 0.2–0.5 mM in HL-60) and either Ara-C (0.01 mM in MV4-11 and 0.05–0.1 mM in HL-60), daunorubicin (0.03 mM in MV4-11 and 0.1 mM in HL-60) or FTI (0.25–0.5 mM in MV4-11 and HL-60). The ratio between the calculated effect (from the single drug incubations) and the found effect of co-incubation is plotted on the y-axis. According to the additive model values between 0.8 and 1.2 are additive (within the box in the figure). Values below 0.8 are synergistic (up in the figure) and values above 1.2 are sub-additive (down in the figure). Antagonism is defined as a result where the combination of the two drugs is less effective than with either drug alone. ✱ shows antagonistic results

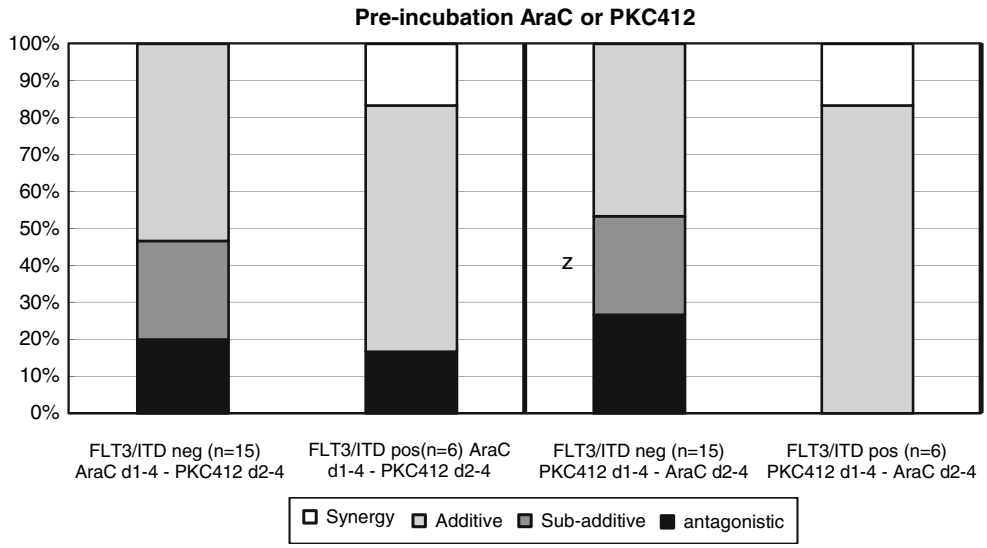

Fig. 8 Combination effect of PKC412 0.2 mM and Ara-C 0.5 mM using different time schedules where cells where pre-incubated in one drug for 24 h before adding the next drug. The experiments were performed in patient AML blast cells

Co-incubation with FTI in combination with PKC 412 in cell lines

In contrast to the two-log difference in sensitivity to PKC 412 there was no difference in sensitivity to FTI between MV4-11 and HL60 cells. The drugs had only additive and synergistic effects, no sub-additive or antagonistic effects were seen. This contrasts to the results of the other incubations with PKC412 and conventional cytostatic drugs where antagonism was seen in both MV4-11 and HL60 (Fig. 7).

Discussion

Although the outcome of patients with acute myeloid leukemia has improved as a result of intensive cytarabine- and anthracycline-based chemotherapy in combination with advanced supportive care and introduction of allogeneic stem-cell transplantation, relapse continues to represent the leading cause of death in the majority of the patients [2, 20]. The prognosis in the older AML population is worse due to substantially greater treatment-related toxicity, lower CR rate and fewer durable remissions. The challenge has been to find other strategies focusing on new drugs with new mechanisms of action compared to conventional chemotherapy. An important step forward in hematology has been the development of the tyrosin kinase inhibitor imatinib in chronic myeloid leukaemia, which has further increased the efforts to find new therapies that target fundamental molecular abnormalities in AML [5].

FLT3 mutations are among the most common molecular abnormalities in AML making them attractive molecular targets for antileukemic treatment. PKC412, SU11248 and CEP-701 are examples of tyrosine kinase inhibitors that inhibit the growth of leukemic cells carrying the FLT3/ITD [18, 36, 37]. The experience so far suggests that these drugs need to be combined with other anti-leukemic treatments, e.g. conventional chemotherapy, in order to obtain clinically relevant anti-leukemic effects. Theoretically, the new FLT3-inhibitors and conventional cytostatic drugs target different mechanisms of action, potentially leading to synergistic effects when they are combined. From a clinical point of view, the toxicity profiles of the new drugs are also important, so that relevant combinations can be tested in clinical trials. The timing of the drugs in combination therapy may also be of importance. With different mechanisms of action one can hypothesise that pre-treatment with one drug activates or inhibits different intracellular signalling pathways, making the malignant clone more sensitive to the next drug.

In the first part of this study, the PKC412 sensitivity in AML blast cells was explored in order to find appropriate concentrations for the synergism study (Fig. 2). When accumulating all experiments performed on primary AML blastcells, FLT3/ITD-positive cells were significantly more sensitive to PKC412 compared to FLT3/ITD negative samples (Fig. 3). In cell lines, the difference in sensitivity between the FLT3/ITD positive and negative cell lines was in the two-log range. This is in accordance with finding by other groups such as Hunter et al. [10] who previously have shown that different FLT3 inhibitors, including PKC412, were more potent in killing AML cells in vitro harbouring the FLT3/ITD as compared to cells with wild-type receptor.

In our subsequent co-incubation study where primary AML blasts were continuously exposed to PKC and Ara-C

for 4 days, synergistic and additive effects were more common in the FLT3/ITD positive samples (91%) compared to the FLT3/ITD negative group (56%). No antagonistic effects were seen in the FLT3/ITD positive samples whereas 2 out of 17 of the FLT3/ITD negative samples showed antagonistic effects. Interestingly, the combination effects were less encouraging in cell lines, where the FLT3/ITD negative HL-60 cells showed a high degree of antagonism from the combination of PKC412 and Ara-C. This, together with our results from the primary AML cells suggests that combinations with PKC412 and Ara-C should be avoided in FLT3/ITD negative patients.

Surprisingly, many experiments showed antagonistic effects also in the FLT3/ITD positive MV4-11 cells. Yee et al found synergistic effects of the FLT3 inhibitor SU11248 and cytarabine in primary AML myeloblasts from three patients expressing FLT3/ITD but not in two patients expressing WT FLT3 protein. In this study we found similar results with another FLT3 inhibitor, PKC412, in a larger patient group. In a co-incubation study by Knapper et al. [14] synergistic and antagonistic effects were found combining PKC412 and Ara-C in both FLT3/ITD positive and FLT3/ITD negative samples. No statistically significant difference was seen between the groups though. This seems, in part, to contradict our results. However, the method of defining synergy by Knapper et al differs from ours, which may account for the difference. Also, the incubation time differs compared to our study. Furukawa et al found that PKC412 showed synergistic effects in combination with seven chemotherapeutic agents studied in FLT3-mutated cell lines. In cell lines without FLT3 mutation, PKC412 showed antagonistic effects when combined with most drugs, except for cyclophosphamide and vincristine [7]. These results are compatible with ours.

A major question for our study was the impact of the timing of the exposure of PKC412 and the combination drug on the cytotoxic effect in the primary AML cells. Indolocarbazoles such as PKC412 and CEP-701, are known to induce G1 cell cycle arrest, an action that potentially could counteract the activities of cell cycle specific chemotherapeutic agents when used in combinations. Importantly, previous studies has shown synergistic or antagonistic effects when cells are exposed to indolocarbazoles either after or before a chemotherapeutical agent, respectively [18]. We first used a time schedule where the cells were pre-incubated for 24 h in either PKC412 or the combination drug before exposure of the other drug. The results confirmed that the FLT3/ITD positive cells are more sensitive to PKC412 combinations. However pre-incubation of any of the drugs did not effect the antileukemic effect in either an adverse or advantageous way. Secondly, we performed a sequential scheme by exposing the cells to only PKC412 for 48 h and then only Ara-C for another 48 h. Also by using this timing schedule, no differences could be seen if the cells were either first exposed to PKC412 an then Ara-C or vice versa. From these experiments we conclude that the timing of the drugs in vitro, as performed in this work, does not affect the antileukemic effects of combination between PKC412 and Ara-C or daunorubicin on primary AML samples. However, our model does not take account the possible effect of the active metabolite of PKC412, CGP52421. As recently shown by Levis et al., this metabolite is more cytotoxic and acts with another, less specific, kinase activity in AML blast cells compared to its parent compound [19]. This, as well as the extensive protein binding of PKC412, will potentially alter the effect in vivo. This has to be taken into consideration while extrapolating our results to the clinical situation. The role of the active metabolite CGP52421 and its for the clinical effect of PKC412 should be further studied.

The farnesyl transferase inhibitor lonafarnib inhibits the activity of RAS. RAS is a downstream target of FLT3 activation [23] and based on this rational, we tested the combination effects of lonafarnib and PKC412. This combination has the potential to be effective both in FLT3-ITD and FLT3-WT cells. Two out of three experiments showed clear synergism suggesting that this combination of two orally available drugs is highly interesting for further studies.

These in vitro results are more heterogeneous compared to other studies where other FLT3 inhibitors have been used [18, 37]. This heterogeneity is probably a reflection of the genetic complexity of primary AML samples compared to well-defined cell lines rather than due to differences between the different drugs. One reason could be that the effects of FLT3 inhibitors are more complex in patient AML samples and that they interfere with various intracellular signalling pathways and the cell cycle status, resulting in modulation of the effects of the cytostatic drug. Promising results were found with the combination of PKC412 and the farnesyl transferase inhibitor Lonafarnib. These effects should be further explored.

In conclusion, to our knowledge, this is the first in vitro study on human AML blasts trying to explore the combination and timing of PKC412 and conventional cytostatic drugs. The study show that the combination of PKC412 and ara-C or daunorubicin can result in synergistic, additive, sub additive as well as antagonistic effects. The results confirm that FLT3/ITD positive cells are more sensitive and suggests that combinations with PKC412 and chemotherapeutic drugs should be used with caution in the FLT3/ITD negative patients. Despite previous results in cell lines by other groups, we could not confirm that the timing of the exposure of the drugs is important for the anti-leukemic effect in primary AML-cells.

The development of FLT3 inhibitors is an important step in finding new ways to improve the treatment results in

AML. Hopefully, ongoing and future clinical trials will clarify how the FLT3 inhibitors should be used in combination with conventional chemotherapy and/or other new non-chemotherapeutic drugs.

References

1. Andersson A, Johansson B, Lassen C, Mitelman F, Billstrom R, Fioretos T (2004) Clinical impact of internal tandem duplications and activating point mutations in FLT3 in acute myeloid leukemia in elderly patients. Eur J Haematol 72:307–313
2. Burnett AK (2002) Acute myeloid leukemia: treatment of adults under 60 years. Rev Clin Exp Hematol 6:26–45, discussion 86–7
3. Cree IA, Neale MH, Myatt NE, de Takats PG, Hall P, Grant J, Kurbacher CM, Reinhold U, Neuber K, MacKie RM, Chana J, Weaver PC, Khoury GG, Sartori C, Andreotti PE (1999) Heterogeneity of chemosensitivity of metastatic cutaneous melanoma. Anticancer Drugs 10:437–44
4. Drexler HG (1996) Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells. Leukemia 10:588–99
5. Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, Capdeville R, Talpaz M (2001) Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. N Engl J Med 344:1038–1042
6. Feldman E, Cortes J, Holyoak T (2003) Continuous oral lonafarnib (Sarasar) for the treatment of patients with myelodysplastic syndrome. Blood 102:421a
7. Furukawa Y, Vu HA, Akutsu M, Odgerel T, Izumi T, Tsunoda S, Matsuo Y, Kirito K, Sato Y, Mano H, Kano Y (2007) Divergent cytotoxic effects of PKC412 in combination with conventional antileukemic agents in FLT3 mutation-positive versus-negative leukemia cell lines. Leukemia 21:1005–1014
8. Frohling S, Schlenk RF, Breitruck J, Benner A, Kreitmeier S, Tobis K, Dohner H, Dohner K (2002) Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: a study of the AML Study Group Ulm. Blood 100:4372–80. Epub 2002 Aug 08
9. Gilliland DG, Griffin JD (2002) The roles of FLT3 in hematopoiesis and leukemia. Blood 100:1532–1542
10. Hunter HM, Pallis M, Seedhouse CH, Grundy M, Gray C, Russell NH (2004) The expression of P-glycoprotein in AML cells with FLT3 internal tandem duplications is associated with reduced apoptosis in response to FLT3 inhibitors. Br J Haematol 127:26–33
11. Jonsson E, Fridborg H, Nygren P, Larsson R (1998) Synergistic interactions of combinations of topotecan with standard drugs in primary cultures of human tumor cells from patients. Eur J Clin Pharmacol 54:509–514
12. Kangas L, Gronroos M, Nieminen AL (1984) Bioluminescence of cellular ATP: a new method for evaluating cytotoxic agents in vitro. Med Biol 62:338–343
13. Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, Asou N, Kuriyama K, Jinnai I, Shimazaki C, Akiyama H, Saito K, Oh H, Motoji T, Omoto E, Saito H, Ohno R, Ueda R (1999) Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. Blood 93:3074–3080
14. Knapper S, Mills KI, Gilkes AF, Austin SJ, Walsh V, Burnett AK (2006) The effects of lestaurtinib (CEP701) and PKC412 on primary AML blasts: the induction of cytotoxicity varies with dependence on FLT3 signaling in both FLT3-mutated and wild-type cases. Blood 108:3494–3503
15. Kottaridis PD, Gale RE, Frew ME, Harrison G, Langabeer SE, Belton AA, Walker H, Wheatley K, Bowen DT, Burnett AK, Goldstone AH, Linch DC (2001) The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood 98:1752–1759
16. Kottaridis PD, Gale RE, Linch DC (2003) Flt3 mutations and leukaemia. Br J Haematol 122:523–538
17. Lepri E, Barzi A, Menconi E, Portuesi MG, Liberati M (1991) In vitro synergistic activity of PDN-IFN alpha and NM + IFN alpha combinations on fresh bone-marrow samples from multiple myeloma patients. Hematol Oncol 9:79–86
18. Levis M, Pham R., Smith BD, Small D (2004) In vitro studies of a FLT3 inhibitor combined with chemotherapy: sequence of administration is important to achieve synergistic cytotoxic effects. Blood 104:1145–1150. Epub 2004 May 4
19. Levis M, Brown P Smith BD, Stine A, Pham R, Stone R, Deangelo D, Galinsky I, Giles F, Estey E, Kantarjian H, Cohen P, Wang Y, Roesel J, Karp JE, Small D (2006) Plasma inhibitory activity (PIA): a pharmacodynamic assay reveals insights into the basis for cytotoxic response to FLT3 inhibitors. Blood 108(10):3477–3483
20. Lowenberg B, Downing JR, Burnett A (1999) Acute myeloid leukemia. N Engl J Med 341:1051–1062
21. Lyman SD (1995) Biology of flt3 ligand and receptor. Int J Hematol 62:63–73
22. Maroc N, Rottapel R, Rosnet O, Marchetto S, Lavezzi C, Mannoni P, Birnbaum D, Dubreuil P (1993) Biochemical characterization and analysis of the transforming potential of the FLT3/FLK2 receptor tyrosine kinase. Oncogene 8:909–918
23. Mizuki M, Fenski R, Halfter H, Matsumura I, Schmidt R, Muller C, Gruning W, Kratz-Albers K, Serve S, Steur C, Buchner T, Kienast J, Kanakura Y, Berdel WE, Serve H (2000) Flt3 mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways. Blood 96(12):3907–3914
24. Mollgard L, Tidefelt U, Sundman-Engberg B, Lofgren C, Paul C (2000) In vitro chemosensitivity testing in acute non lymphocytic leukemia using the bioluminescence ATP assay. Leuk Res 24:445–452
25. Monnerat C, Henriksson R, Le Chevalier T, Novello S, Berthaud P, Faivre S, Raymond E (2004) Phase I study of PKC412 (*N*-benzoyl-staurosporine), a novel oral protein kinase C inhibitor, combined with gemcitabine and cisplatin in patients with non-small-cell lung cancer. Ann Oncol 15:316–323
26. Morgillo F, Lee HY (2006) Lonafarnib in cancer therapy. Expert Opin Investig Drugs 15:709–719
27. Propper DJ, McDonald AC, Man A, Thavasu P, Balkwill F, Braybrooke JP, Caponigro F, Graf P, Dutreix C, Blackie R, Kaye SB, Ganesan TS, Talbot DC, Harris AL, Twelves C (2001) Phase I and pharmacokinetic study of PKC412, an inhibitor of protein kinase C. J Clin Oncol 19:1485–1492
28. Quentmeier H, Reinhardt J, Zaborski M, Drexler HG (2003) FLT3 mutations in acute myeloid leukemia cell lines. Leukemia 17:120–124
29. Rhedin AS, Tidefelt U, Jonsson K, Lundin A, Paul C (1993) Comparison of a bioluminescence assay with differential staining cytotoxicity for cytostatic drug testing in vitro in human leukemic cells. Leuk Res 17:271–276
30. Rosnet O, Mattei MG, Marchetto S, Birnbaum D (1991) Isolation and chromosomal localization of a novel FMS-like tyrosine kinase gene. Genomics 9:380–385
31. Schnittger S, Schoch C, Dugas M, Kern W, Staib P, Wuchter C, Loffler H, Sauerland CM, Serve H, Buchner T, Haferlach T, Hiddemann W (2002) Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics FAB subtype, and prognosis in the AMLCG study and usefulness

as a marker for the detection of minimal residual disease. Blood 100:59–66
32. Stone RM, DeAngelo DJ, Klimek V, Galinsky I, Estey E, Nimer SD, Grandin W, Lebwohl D, Wang Y, Cohen P, Fox EA, Neuberg D, Clark J, Gilliland DG, Griffin JD (2005) Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor PKC412. Blood 105:54–60. Epub 2004 Sep 2
33. Thiede C, Steudel C, Mohr B, Schaich M, Schakel U, Platzbecker U, Wermke M, Bornhauser M, Ritter M, Neubauer A, Ehninger G, Illmer T (2002) Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood 99:4326–4335
34. Tidefelt U, Sundman-Engberg B, Rhedin AS, Paul C (1989) In vitro drug testing in patients with acute leukemia with incubations mimicking in vivo intracellular drug concentrations. Eur J Haematol 43:374–384
35. Valeriote F, Lin H (1975) Synergistic interaction of anticancer agents: a cellular perspective. Cancer Chemother Rep 59:895–900
36. Weisberg E, Boulton C, Kelly LM, Manley P, Fabbro D, Meyer T, Gilliland DG, Griffin JD (2002) Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412. Cancer Cell 1:433–443
37. Yee KW, Schittenhelm M, O'Farrell AM, Town AR, McGreevey L, Bainbridge T, Cherrington JM, Heinrich MC (2004) Synergistic Effect of SU11248 with Cytarabine or Daunorubicin on FLT3-ITD Positive Leukemic Cells. Blood 10:10
38. Yokota S, Kiyoi H, Nakao M, Iwai T, Misawa S, Okuda T, Sonoda Y, Abe T, Kahsima K, Matsuo Y, Naoe T (1997) Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. Leukemia 11:1605–1609