

Report

The FLT3 inhibitor tandutinib (formerly MLN518) has sequence-independent synergistic effects with cytarabine and daunorubicin

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AML remains a difficult disease to treat. Despite response to induction chemotherapy, most patients ultimately relapse. Further, among elderly patients, aggressive therapy options are often limited due to other medical conditions and decreased tolerance of these patients to conventional chemotherapy.

Internal tandem duplications (ITD) of the FLT3 juxtamembrane domain occur in 20–30% of AML patients and predict poor outcome. First clinical data with the FLT3 inhibitor tandutinib demonstrated antileukemic activity in approximately half of the patients—predominantly with FLT3 ITD positive AML. But the data also show that optimal use of tandutinib will require combination therapy with cytotoxic agents. Notably, single agent tandutinib has not been associated with myelosuppression, mucositis or cardiac toxicity—the dose limiting toxicities of AML chemotherapy.

We determined the feasibility of combining tandutinib with the standard “3 + 7” induction regimen in AML and show that, in contrast to other structurally unrelated FLT3 inhibitors recently evaluated in clinical trials, the use of tandutinib displayed application sequence independent synergistic antileukemic effects in combination with cytarabine and daunorubicin. Strong synergistic antiproliferative and proapoptotic effects were thereby predominantly seen on FLT3 ITD positive blasts. Further we demonstrate, that addition of tandutinib may allow dose reduction of chemotherapy without loss of overall antileukemic activity—resulting in a potential decrease of side effects. This approach might be an interesting novel strategy especially in the treatment of elderly/comorbid patients.

Our data provide a rationale for combining tandutinib with induction chemotherapy in intensified as well as in dose reduction protocols for FLT3 ITD positive AML.

Introduction

Acute myeloid leukemia (AML) is the most common type of acute leukemia in adults. Patients typically respond to initial treatment with anthracycline and cytarabine-based induction chemotherapy, but most patients ultimately relapse and die of their disease.¹ Treatment of AML is further complicated by the epidemiology of the disease: AML occurs predominantly in elderly patients and incidence rises with age. Among the expanding population of elderly AML patients, aggressive therapy options are often limited due to other medical conditions and the decreased tolerance of these patients to conventional therapy.² The inadequacy of conventional medical therapy for AML has led to the desire to develop novel biological/molecular therapies that would improve treatment outcomes and/or have a more favorable side effect profile.

The FMS-like tyrosine kinase 3 (FLT3) protein is a receptor tyrosine kinase that is expressed at high levels in 70–100% of cases of AML and has been identified as potential target for molecular therapy. Notably, gain-of-function mutations (mostly internal tandem duplications (ITD) of the juxtamembrane domain) occur regularly in de novo AML, resulting in constitutive activation of FLT3 tyrosine kinase activity. There is substantial evidence to support the notion that these mutations play an important role in the initiation and/or maintenance of AML in a subset of patients.³⁻⁷ In addition, the presence of FLT3 ITD mutations predict for a worse outcome,⁸⁻¹¹ particularly for those with a high mutant to normal FLT3 allelic ratio.^{12,13}

Several FLT3 tyrosine kinase inhibitors (TKIs) have been or are currently being studied in phase I/II clinical trials of patients with AML, but thus far these agents have demonstrated only modest single agent activity.¹⁴⁻¹⁸ We and others have therefore suggested that treatment strategies combining conventional chemotherapy with molecular targeted therapies are needed to sustainably

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improve outcome.¹⁹⁻²¹ Although, previous studies have suggested that the timing of drug application may be crucial when adding FLT3-inhibitors to polychemotherapy regimens.^{20,22} This circumstance may complicate the actual transition of such protocols into the clinic.

Tandutinib (formerly MLN518) is a 4-piperazinyl quinazoline compound that is a potent inhibitor of type III receptor tyrosine kinases (RTKs) with a cellular IC_{50} of 200 nM for FLT3. In a phase II study, tandutinib demonstrated antileukemic activity in approximately half of the evaluable patients, but optimal use of tandutinib will require combination therapy with cytotoxic agents. Notably, single agent Tandutinib has not been associated with myelosuppression, mucositis or cardiac toxicity—the dose limiting toxicities of AML chemotherapy.

In our study, we wished to investigate the biological effects of tandutinib^{17,23,24} when combined with cytarabine and daunorubicin, the current cornerstones of medical therapy for AML.¹ We will show, that combination therapy strategies including tandutinib display beneficial antileukemia activity in FLT3 ITD positive leukemias in settings simulating standard induction therapy regimens—as well as in dose reduced protocols, which may provide a true therapeutic option for elderly/comorbid patients. Of note, extensive cell cycle experiments will demonstrate that the sequence of tandutinib administration has no impact on the efficacy of combination therapy in these patient subgroups—which is in contrast to the findings with other, structurally unrelated FLT3 inhibitors, in previous studies.^{20,25}

Results

Ex vivo effects of tandutinib in combination with cytarabine or daunorubicin on primary human AML cells. Griswold et al. previously reported that tandutinib preferentially inhibits cellular proliferation and induces apoptosis of primary AML blasts with FLT3 ITD mutations when compared with FLT3 WT leukemic blasts or normal hematopoietic progenitors.²⁴ We tested whether the growth inhibitory effects of tandutinib would result in an additive effect when combined with standard chemotherapy.

The FLT3 ITD status of twelve patients with refractory and/or multiply relapsed AML was determined by D-HPLC and DNA sequencing, nine AML patients were diagnosed with a unique FLT3 ITD and three patients displayed AML without associated mutations of the FLT3 juxtamembrane or activation loop domains. Low-density mononuclear cells were cultured for 72–96 hours in the presence or absence of tandutinib and/or cytarabine or daunorubicin. To determine if inhibition of FLT3 kinase by tandutinib might potentiate the effects of cytarabine or daunorubicin, we performed experiments with fixed doses of tandutinib. The effects of single agent or combination treatment on cellular proliferation are depicted in Figure 1. Cytarabine (doses of 250–8,000 nM) or daunorubicin (doses 10–320 nM) significantly inhibited the proliferation of AML blasts regardless of the FLT3 mutational status. In contrast, single agent tandutinib (100–500 nM) significantly inhibited the proliferation of primary AML blasts with FLT3 ITD mutations but displayed only minor effects on the proliferation of AML cells expressing WT FLT3. Consequently, treatment

of FLT3 ITD leukemic cells with a combination of tandutinib and cytarabine (or daunorubicin) resulted in greater inhibition of proliferation than either agent used alone. In contrast, there was no obvious effect of adding tandutinib to chemotherapy in patient AML samples lacking a FLT3 ITD mutation.

Effects of simultaneous combination of tandutinib with cytarabine and/or daunorubicin on cellular proliferation and induction of apoptosis in three FLT3 ITD cell line models. Despite these intriguing results, a number of technical and biological issues were identified in our experiments. First, considerable inter-patient variation in the sensitivity of AML blasts to the chemoagents was noted. As the limited number of ex vivo blasts precluded repetitive studies and/or the use of multiple different ratios of tandutinib and chemotherapy, statistical interpretation was aggravated. Further, contamination with non-neoplastic mononuclear cells had to be accepted in favor of a blast-isolation method (Ficoll centrifugation) that would keep native cells as stress-free as possible. Therefore, maximal antiproliferative effects measured by an XTT-based assay have been obscured by the presence of non-neoplastic XTT-responsive cells.

To address these problems, we chose a highly reproducible in vitro cell-line based assay to study the biological effects of combining tandutinib with standard chemotherapy, i.e., cytarabine plus anthracyclines.¹ We performed fixed ratio dilution experiments to create isobolograms, which allow mathematical analysis to compute combination indices (CI) for various levels of drug activity. Three cell line models harboring unique juxtamembrane tandem duplications in FLT3 (see “Methods”) were employed to reduce potential bias from idiosyncrasies of any individual cell line.^{19,32}

First, to determine the concentrations of cytarabine, daunorubicin or tandutinib capable of decreasing proliferation by 50% (ED_{50}) dose effect plots were computed. Specifically, ED_{50} of tandutinib was ~100 nM for all three cell lines (X-intercept values in Fig. 2A–F); the ED_{50} of cytarabine was ~2,200 nM (MV4:11), ~240 nM (MOLM14) and ~1,400 nM (Ba/F3 FLT3 ITD). The ED_{50} for daunorubicin was 5 nM (MV4:11), ~30 nM (MOLM14) and ~100 nM (Ba/F3 FLT3 ITD), respectively.

Using these results, we treated cells with combinations of tandutinib and cytarabine or daunorubicin with fixed ratios in relationship to the individual agent ED_{50} . Isobolograms were computed for ED_{50} , ED_{75} and ED_{90} (see “Methods”), revealing the greatest impact at ED_{90} (Fig. 2A–F). The actual concentrations of tandutinib plus cytarabine necessary to inhibit cells by 90% (ED_{90}) fell far to the left of the line of dose equivalence for all tested cell lines. Thus, adding tandutinib at low nanomolar doses markedly reduced the amount of cytarabine necessary to achieve any given amount of anti-proliferative effect. CI values for the observed effects on proliferation at ED_{90} was ~0.5 for all cell lines, suggesting that combination of tandutinib and cytarabine was strongly synergistic. Likewise, combining tandutinib with daunorubicin had a similar potent antiproliferative effect, with values falling significantly to the left of the lines of equivalence for doses capable to inhibit 90% of cellular proliferation in all cell lines. These results indicate that the combination has a synergistic

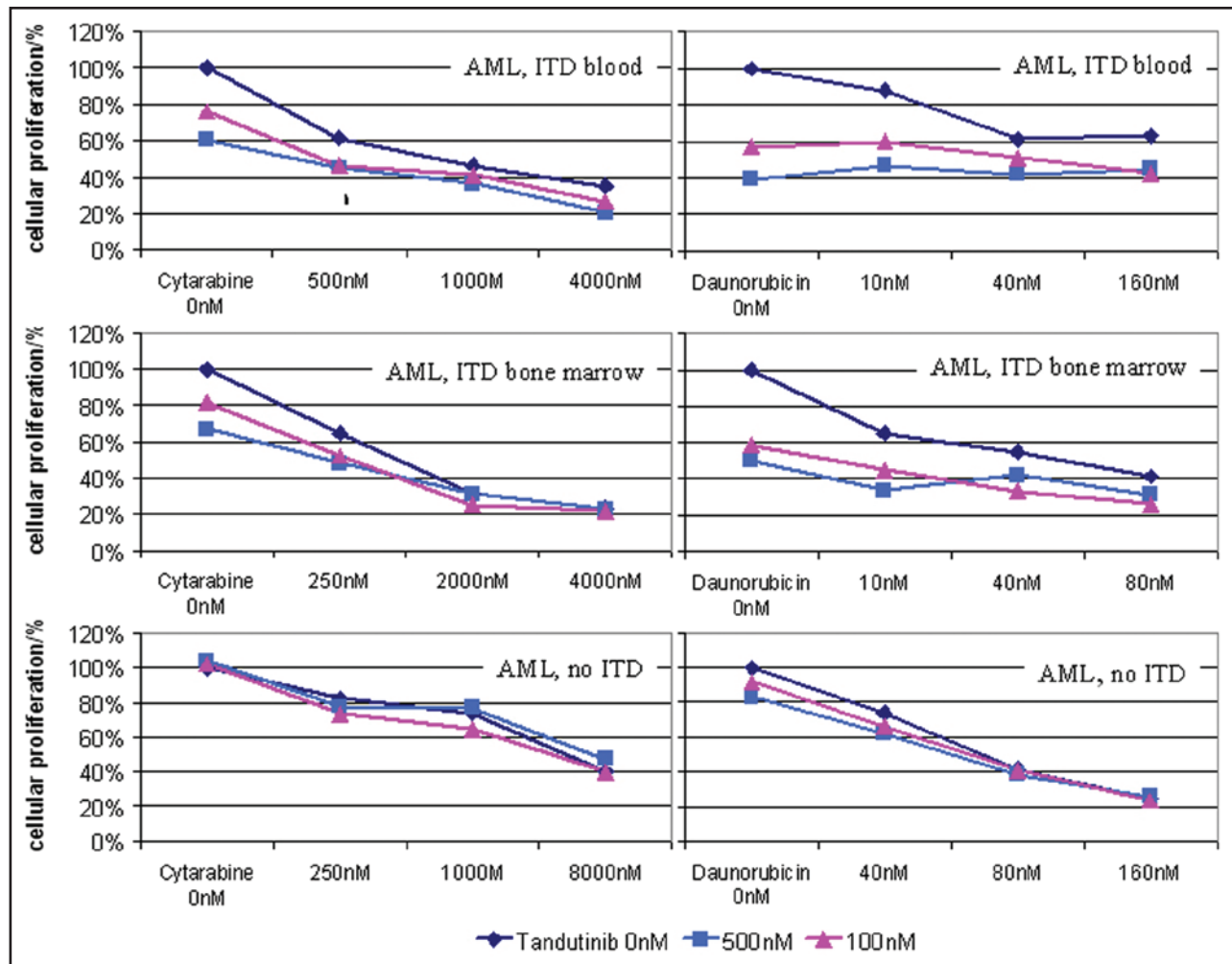


Figure 1. Effects of tandutinib and/or cytarabine or daunorubicin on the proliferation of primary AML cells. Low-density peripheral blood or bone marrow mononuclear cells from patients with refractory and/or relapsed AML were incubated with cytarabine (250–8,000 nM) or daunorubicin (10–160 nM) and/or tandutinib (100–500 nM) for 72–96 hours and cellular proliferation was assessed with an XTT-based assay. Single agent tandutinib significantly inhibited the proliferation of FLT3 ITD AML cells but not FLT3 WT AML cells. Further, the addition of tandutinib to cytarabine or daunorubicin increased the antiproliferative effects of the chemotherapeutic agents on FLT3 ITD AML. Again, no significant effect was seen on FLT3 WT AML cells. Representative data is shown from a total of 12 patients that were studied.

antiproliferative effect, which is also reflected in the calculated CI values that are significantly less than one.

Besides the antiproliferative capacity of FLT3 inhibitors in AML models, previous studies demonstrated a potent and preferential induction of apoptosis in cells expressing FLT3 ITD mutations compared to the FLT3 wildtype isoform.^{23,32,36,37} Further, the combination of various FLT3 inhibitors with standard anti-leukemia agents was previously demonstrated to have a beneficial overall antitumor effect.^{19,38,39} We herein show that tandutinib in combination with cytarabine or daunorubicin not only potentially inhibits cellular growth but synergistically increases cell killing. The ED₅₀ doses of tandutinib for induction of apoptosis were ~300 nM (MV4:11/MOLM14) and ~100 nM (Ba/F3 FLT3 ITD). The ED_{50s} doses of cytarabine were ~1,000 nM (MV4:11), ~700 nM (MOLM14) and ~500 nM (Ba/F3 FLT3 ITD). The ED_{50s} doses of daunorubicin were ~10 nM (MV4:11), ~20 nM (MOLM14) and ~5 nM (Ba/F3 FLT3 ITD),

respectively. The experimental combination results for ED₅₀, ED₇₅ and ED₉₀ again demonstrate a strong synergistic proapoptotic effect of tandutinib combined with cytarabine, and an additive to synergistic interaction of daunorubicin combined with tandutinib in inducing apoptosis in these FLT3 ITD positive cell lines. Again, the greatest impact on CI values was seen at ED₉₀, indicating beneficial potency for the combination of tandutinib with these traditional cytotoxic agents (Fig. 2G–L).

To more closely mimic clinical conditions where two or more chemotherapy agents are administered at the same time (e.g., the “3 + 7” regimen combining cytarabine with daunorubicin), we further tested whether a triple drug combination of tandutinib with cytarabine and daunorubicin was synergistic for induction of apoptosis. As the interaction of a triple drug combination can not be depicted using a 2-dimensional isobologram blot, only the numerical data are presented (Table 1). The experimental combination results for ED₅₀ indicate an additive to synergistic

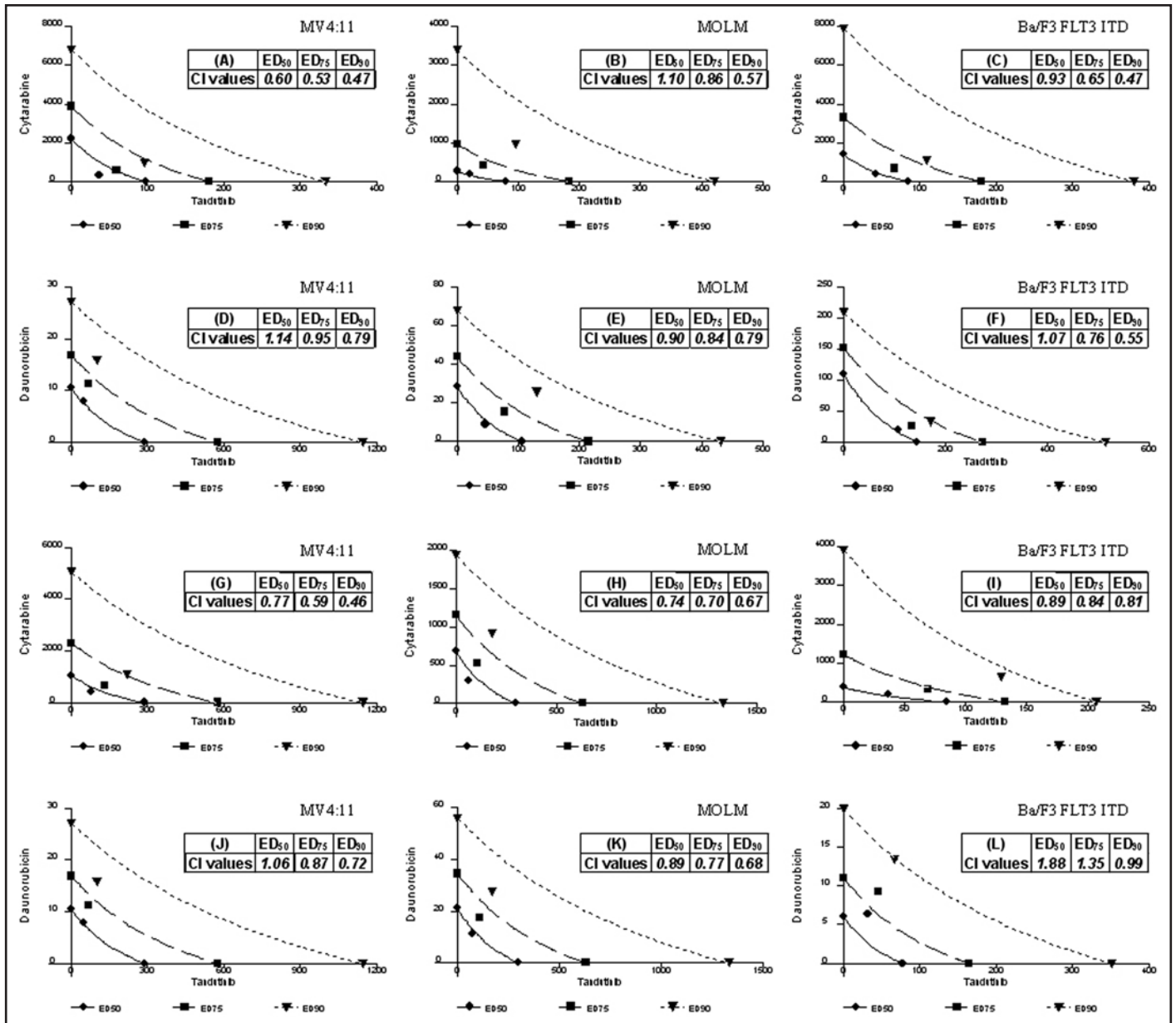


Figure 2. Isobolograms for the effects of tandutinib and cytarabine or daunorubicin on cellular proliferation and induction of apoptosis. Proliferation was assessed using an XTT-based assay and the results were analyzed using Calcsyn software. Representative results are displayed for experiments with the MV4:11, MOLM14 and Ba/F3 FLT3 ITD cell lines (concentrations of agents are expressed in nM). (A–C) Cells were incubated with cytarabine, tandutinib, or both for 72 hours. The combination of tandutinib and cytarabine had an additive to synergistic effect of the drugs using ED₅₀ as the experimental end point. For 90% inhibition (ED₉₀), the experiment points fall to the left of the predicted line of additive effect, indicating a strongly synergistic interaction of cytarabine and tandutinib. (D–F) Analogous, treatment of cells with daunorubicin and tandutinib demonstrates an additive effect when using ED₅₀ as the experimental endpoint and a synergistic effect when using ED₉₀ as the experimental endpoint. The computed combination indices for these experiments are depicted next to each figure. Similar, the apoptotic effects of the combination of tandutinib with cytarabine or daunorubicin, were measured by an Annexin V-based assay and isobolograms were computed as described above. (G–I) The experimental points fall to the left of the predicted line of additive effect, indicating a strongly synergistic interaction of cytarabine and tandutinib for ED₅₀ as well as ED₉₀. This is reflected by CI values well smaller than one. (J–L) Treatment of cells with daunorubicin and tandutinib demonstrates an additive effect when using ED₅₀ as the experimental endpoint and an additive to synergistic effect when using ED₉₀ as the experimental endpoint.

interaction of cytarabine, daunorubicin and tandutinib. Using ED₇₅, or ED₉₀ as the desired experimental endpoint, the combination indices (CI) were all much less than one, indicating strong synergy for the triple drug combination. Of note, synergy was seen with all tested FLT3 ITD positive cell lines as well as with a sample

of primary AML blasts obtained from a patient with a confirmed FLT3 ITD mutation.

In vitro modeling of the use of tandutinib to reduce chemotherapy doses for elderly and/or comorbid patients with AML. Among the expanding population of elderly AML patients,

Table 1 Combination indices (CI) values for the effects of tandutinib (Tn) in combination with cytarabine (Cy) and daunorubicin (Dn) on induction of apoptosis

	CI values	ED ₅₀	ED ₇₅	ED ₉₀
Cy + Dn + Tn	MV4-11	1.02	0.88	0.76
	MOLM14	0.75	0.68	0.61
	Ba/F3 FLT3 ITD	0.85	0.54	0.42
	AML, ITD pos.	0.70	0.52	0.42

Combination indices (CI) values for the effects of tandutinib (Tn) in combination with cytarabine (Cy) and daunorubicin (Dn) on induction of apoptosis.

aggressive therapy options are often limited due to other medical conditions and the decreased tolerance of these patients to conventional chemotherapy. We show that the use of tandutinib may allow dose reduction of chemotherapy with resultant decreased side effects, but without loss of antileukemic activity.

Cells were treated with tandutinib in combination with cytarabine and daunorubicin with fixed ratios in relationship to the individual agent ED₅₀. Induction of apoptosis was analyzed by AnnexinV/PI-staining and flow cytometry. Isobologram analysis of the triple combination with tandutinib, cytarabine and daunorubicin allowed calculation of dose reduction indices (DRI) for each of the single agents (Calculusyn[®] software) (Table 2).

Taking ED₅₀ as primary endpoint, our data show, that addition of tandutinib to standard “3 + 7” induction chemotherapy may allow a dose reduction for cytarabine up to 4-fold, daunorubicin up to 3,5-fold and tandutinib up to 8-fold of the concentrations, which would otherwise be necessary to achieve the single agent ED₅₀.

For ED₉₀ as primary endpoint of the triple combination, the beneficial combination effect was even more pronounced with DRI for cytarabine and tandutinib each up to ~12-fold.

We next isolated native FLT3 ITD positive blasts from an AML patient and treated the cells *ex vivo* with cytarabine, daunorubicin and tandutinib. The treated cells were analyzed in an XTT-based assay to analyze inhibition of cellular proliferation. As it turned out, the *ex vivo* blasts were quite insensitive towards cytarabine with an estimated computed IC₉₀ in unphysiological concentrations. Monotherapy with tandutinib up to 1,000 nM did only reveal a moderate antiproliferative effect and the estimated computed ED₉₀ was again in a non-pharmacologically relevant concentration. In contrast, our dose reduction model revealed (Table 2), that combination of cytarabine, daunorubicin and tandutinib would allow significant dose reductions of all three agents with achievable ED_{90s} in physiological concentrations.

We conclude, that dose reduction strategies by adding a FLT3-inhibitor such as tandutinib may be feasible—particularly in the elderly or patients with co-existent illnesses that would predict for a poor tolerance of standard chemotherapy regimens.

Effect of sequential application of tandutinib, cytarabine and daunorubicin on cell cycle progression, cellular proliferation and induction of apoptosis. Previous *in vitro* studies utilizing the FLT3 inhibitors lestaurtinib (CEP-701) and midostaurin (PKC412),³⁹ demonstrated the impact of cell cycle effects caused by FLT3

inhibitors and importance of treatment sequencing for optimal killing of AML cells: For midostaurin (PKC412) a G₁/G₀ arrest in FLT3 ITD positive cells was reported—whereas a G₂/M arrest was seen in FLT3 WT cells. Furthermore, treatment with lestaurtinib with or following chemotherapy was found to be synergistic, whereas treatment with lestaurtinib followed by chemotherapy was generally antagonistic.²⁰ As combination of “cell cycle incompatible” drugs could result in antagonistic effects on killing of cancer cells,^{20,35,40-42} we examined cell cycle effects of the structurally unrelated FLT3 inhibitor tandutinib in combination with anti-neoplastic agents of the standard “3 + 7” regimen to define an optimal sequence for drug treatment.

For our experiments, we used propidium iodide DNA-staining to differentiate cell cycle phases of FLT ITD positive cells by the DNA content of cells. As shown in Figure 3A, cytarabine, a potent inhibitor of DNA-polymerases, lead to accumulation of cells in S-phase. Treatment of cells with daunorubicin, a topoisomerase II-inhibitor, resulted in accumulation of cells in the G₂/M compartment (Fig. 3A). Higher concentrations lead to the expected induction of apoptosis as assessed by an increasing fraction of cells with sub-G₁ DNA content, indicating dead or apoptotic cells.

Treatment with tandutinib induced a concentration-dependent accumulation of cells in the G₁ compartment or, in higher concentrations, induction of apoptosis. In contrast, tandutinib treatment of the FLT3 WT HL60 human AML cell line did not produce any significant cell cycle effects or induction of apoptosis—even when using tandutinib concentrations up to 10 μM (data shown as Suppl. Fig. 4). This is in line with findings for FLT3 WT in a Ba/F3 background. Next, we tested different temporal sequences of combining cytarabine or daunorubicin in combination with tandutinib with regard to antiproliferative effects. All of the tested therapy regimens displayed an additive or synergistic effect as demonstrated by combination indices (CI) in the range or less than one for ED₅₀ and ED₉₀ (Table 3), indicating that combination regimens involving tandutinib are favorable regardless of the sequence of drug application chosen.

Looking at cell cycle distribution, cells treated with tandutinib followed with delayed (24 hours) addition of cytarabine had an increased population of cells in the G₁ compartment that was not present in cells treated with cytarabine with delayed addition of tandutinib. However, there was no significant difference in the effectiveness of either regimen to induce apoptosis/cellular death. The combination of tandutinib followed by delayed addition of daunorubicin or the reverse sequence combination were both very potent for the induction of apoptosis: treatment with tandutinib for 24 hours before adding daunorubicin had equal potency for inducing apoptosis when compared with the reverse sequencing (daunorubicin 24 hours before adding tandutinib) (Fig. 3B). As was observed for tandutinib and cytarabine, both sequential combinations of tandutinib and daunorubicin were more effective in inducing apoptosis than monotherapy with either agent. Noteworthy, in this assay, the sub-G₁ fraction seen after propidium iodide staining is not specific for apoptotic cells alone, but also includes dead/necrotic cells. However, these results are consistent

Table 2 **In vitro modeling of the use of tandutinib (Tn) to reduce chemotherapy doses**

Induction of apoptosis at ED ₅₀	Single drug (nM)			DRI			Combination (nM)		
	Cy	Dn	Tn	Cy	Dn	Tn	Cy	Dn	Tn
MV4:11	1121.3	10.6	289.6	4.9	1.5	6.4	227.6	7.3	45.5
MOLM14	588.1	21.3	298.1	3.6	3.5	7.9	188.6	6.0	37.7
Ba/F3 FLT3 ITD	386.2	8.4	84.5	2.6	1.8	2.9	146.7	4.7	29.3
Induction of apoptosis at ED ₉₀	Single drug (nM)			DRI			Combination (nM)		
	Cy	Dn	Tn	Cy	Dn	Tn	Cy	Dn	Tn
MV4:11	4273.7	27.2	1145.6	8.7	1.7	11.7	491.2	15.7	98.2
MOLM14	1950.0	56.1	1334.1	3.7	3.4	12.7	523.0	16.7	104.6
Ba/F3 FLT3 ITD	3917.7	23.9	207.0	12.5	2.4	3.3	312.7	10.0	62.5
Inhibition of proliferation at ED ₉₀	Single drug (nM)			DRI			Combination (nM)		
	Cy	Dn	Tn	Cy	Dn	Tn	Cy	Dn	Tn
AML FLT3 ITD+	2.9 x 10 ⁵	521.0	1.6 x 10 ⁸	9.0	3.2	2.3 x 10 ⁶	32222.0	162.8	69.6

Cells were treated with Tn in combination with cytarabine (Cy) and daunorubicin (Dn) with fixed ratios in relationship to the individual agent ED₅₀. Induction of apoptosis was analyzed by AnnexinV/PI-staining and flow cytometry. Isobologram analysis of the triple combination with Tn, Cy and Dn allowed to calculate dose reduction indices (DRI) for each of the single agents. Our data demonstrates that addition of Tn to Cy and Dn may allow dose reduction of the agents without loss of the overall cytotoxic effect.

Table 3 **CI values for the antiproliferative effects of a sequential application regimen with cytarabine, daunorubicin and tandutinib on FLT3 ITD positive cells**

	Cy (Inc. 72 h) IC ₅₀ (nM)	Tn (Inc. 72 h) IC ₅₀ (nM)	Cy + Tn after 24 h CI (ED ₅₀)	Tn + Cy after 24 h CI (ED ₅₀)	Cy + Tn after 24 h CI (ED ₉₀)	Tn + Cy after 24 h CI (ED ₉₀)
MV4-11	2130	88	0.50	1.14	0.40	0.65
MOLM14	273	139	1.26	0.88	0.43	0.54
Ba/F3 FLT3 ITD	700	49	0.74	1.21	0.28	0.45
	Dn (Inc. 72 h) IC ₅₀ (nM)	Tn (Inc. 72 h) IC ₅₀ (nM)	Dn + Tn after 24 h CI (ED ₅₀)	Tn + Dn after 24 h CI (ED ₅₀)	Dn + Tn after 24 h CI (ED ₉₀)	Tn + Dn after 24 h CI (ED ₉₀)
MV4-11	6	65	1.21	1.31	0.66	0.44
MOLM14	18	243	0.87	1.50	0.55	0.52
Ba/F3 FLT3 ITD	6	40	0.93	0.68	0.56	0.41

Cells were incubated with cytarabine (Cy) or daunorubicin (Dn) followed by tandutinib (Tn) after 24h, or vice versa. Effects on cellular proliferation were assessed using an XTT-based assay. Representative results are displayed above. The doses necessary to produce a given effect suggest a potent additive to synergistic activity for the addition of cytarabine or daunorubicin to tandutinib for all tested drug sequencing regimens (light gray shading) using ED₉₀ as the experimental endpoint. Results for ED₅₀ are shown in the light gray shading and for ED₉₀ in the darker shading. IC₅₀s for the single agents are also listed.

with our previous experiments using an annexin V-based flow cytometric method to assess induction of apoptosis.

In conclusion, using appropriate concentrations, all three agents could induce apoptosis in FLT3 ITD positive cells—regardless of the cell cycle effects seen with sub-cytotoxic concentrations. Consequently, regardless of the application scheme chosen, combination regimens including tandutinib with cytarabine and/or daunorubicin are potent inducers of programmed cell death with synergizing cytotoxic effects. Importantly, in contrast to previous reports with structurally unrelated FLT3 inhibitors, there was no significant benefit of either sequential regimen to induce cellular apoptosis.

Discussion

The success of the small molecule compound imatinib mesylate (Gleevec[®], Gleevec[®]) in the treatment of human malignancies

driven by mutant tyrosine kinases (e.g., BCR-ABL in CML⁴³ or KIT in GIST⁴⁴), has led to an extensive search for compounds that might target mutations of the FLT3 receptor tyrosine kinase—especially FLT3 ITD mutations in the JM-domain. FLT3 mutations are the most frequently found oncogenic class I mutations identified in AML. The potential relevance of this target is underscored by the observation that patients harboring FLT3 ITD mutations typically have inferior post-remission outcomes after standard chemotherapy compared to patients whose blasts express a wild-type FLT3 kinase isoform.⁴⁵

Phase I/II studies of structurally divergent FLT3 inhibitors including tandutinib (MLN518),¹⁷ sunitinib (SU11248)¹⁴⁻¹⁶ and lestaurtinib (CEP-701)^{18,46} demonstrated that FLT3 inhibitors potently inhibit autoactivating FLT3 ITD mutations in vivo and are able to induce high rates of peripheral blood clearing of myeloblasts. For example, an anti-leukemic activity

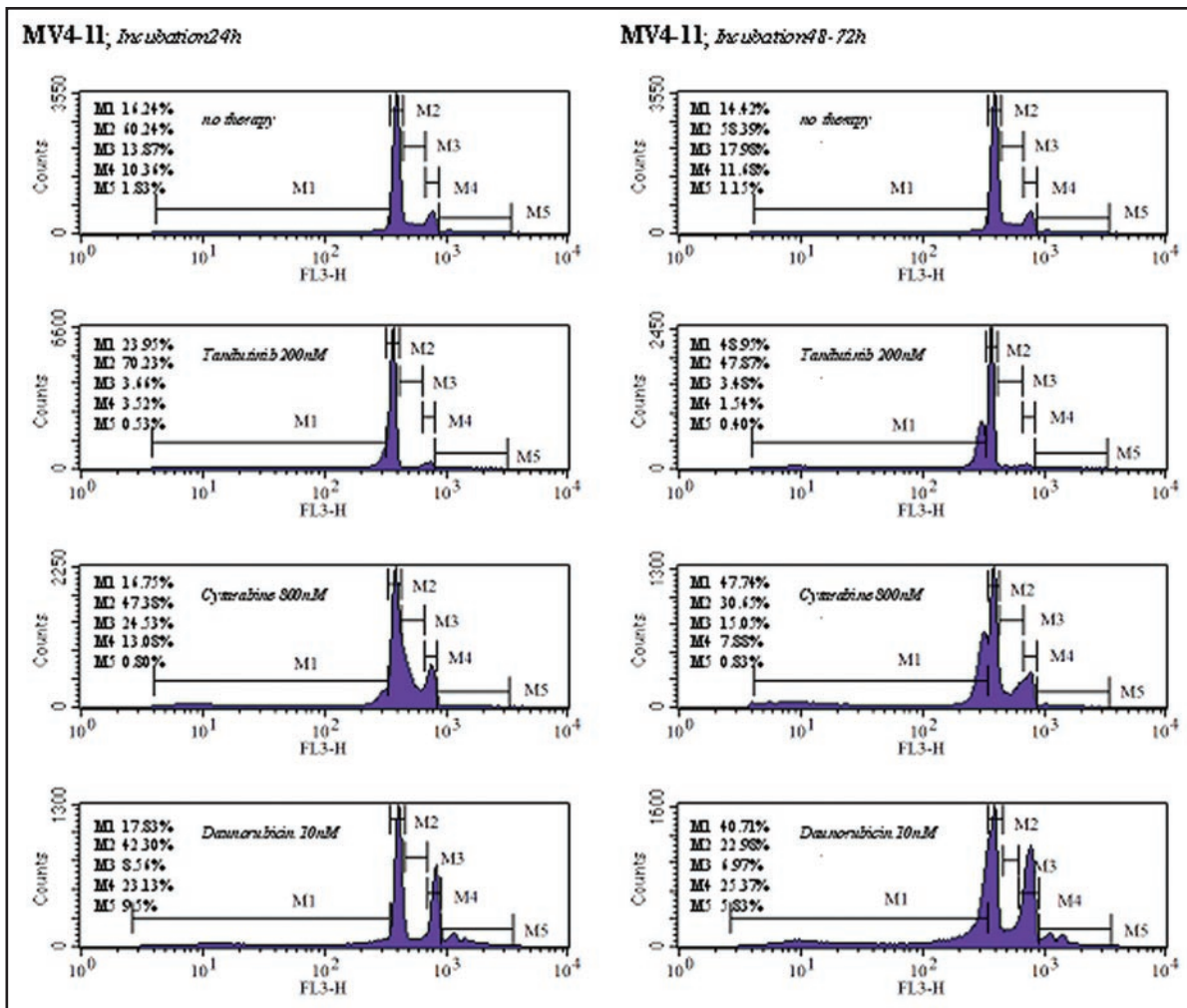


Figure 3. Effects of monotherapy or sequential combination therapy on cell cycle distribution and induction of apoptosis. (A) MV4:11 cells were incubated with different doses of control media, tandutinib, cytarabine or daunorubicin. Cells were analyzed for cell cycle distribution at 0, 24 and 48 hours. As shown here, each of the three agents alters the cell cycle distribution: tandutinib treatment caused an accumulation in G₁-phase, cytarabine treatment induced accumulation in S-phase, and daunorubicin induced accumulation in G₂/M-phase. Each of the three agents induced cellular death as evidence by an increase in the sub-G₀ MV4:11. Similar results were obtained using the MOLM14 and transfected Ba/F3 FLT3 ITD cell lines. Key: M1: dead and apoptotic cells; M2: G₀/G₁-phase; M3: S-phase; M4: G₂/M-phase; M5: polyploid cells.

of tandutinib was seen in ~40% of the FLT3 ITD positive subgroup of patients in a phase I study, although there were no complete hematologic remissions.¹⁷ This is in line with our ex vivo data for native blasts showing significant antitumor activity preferably for FLT3 ITD positive samples—but not the wild type isoform subgroup. We therefore concentrated our in vitro synergy experiments on the clinically most relevant FLT3 ITD positive subgroup.

Recently, we and others have reported that the FLT3 inhibitors sunitinib (SU11248),¹⁹ midostaurin (PKC412)³⁹ or lestaurtinib (CEP-701)²⁰ can be combined with standard antileukemic drugs such as cytarabine or daunorubicin to produce synergistic anti-proliferative and pro-apoptotic effects on FLT3 ITD cells. We obtained similar results for the administration of tandutinib in combination with cytarabine and/or daunorubicin.

It was previously reported that the application sequence of the structurally unrelated FLT3 inhibitor lestaurtinib (CEP-701)

in combination with chemotherapy is important for the overall cytotoxic effect and lestaurtinib-induced G₁-phase arrest might diminish chemotherapy induced cytotoxicity.²⁰ We therefore tested whether the structurally unrelated FLT3 inhibitor tandutinib shows similar effects in sequencing schemes combining the standard “3 + 7” chemotherapy regimen with tandutinib. Surprisingly, all tested combination regimens had synergistic cytotoxic effects that were irrespective of the sequencing of the various agents. Therefore, unlike lestaurtinib, the synergistic effect of tandutinib in combination with cytarabine and/or daunorubicin appears to be independent of the sequence of drug administration. The reasons for the difference of our results compared to those previously reported for lestaurtinib are not clear and might be due to technical differences in these complex experimental procedures. Alternatively, the disparate results may be due to off-targets and different biological effects of these two structurally unrelated FLT3 TKIs.

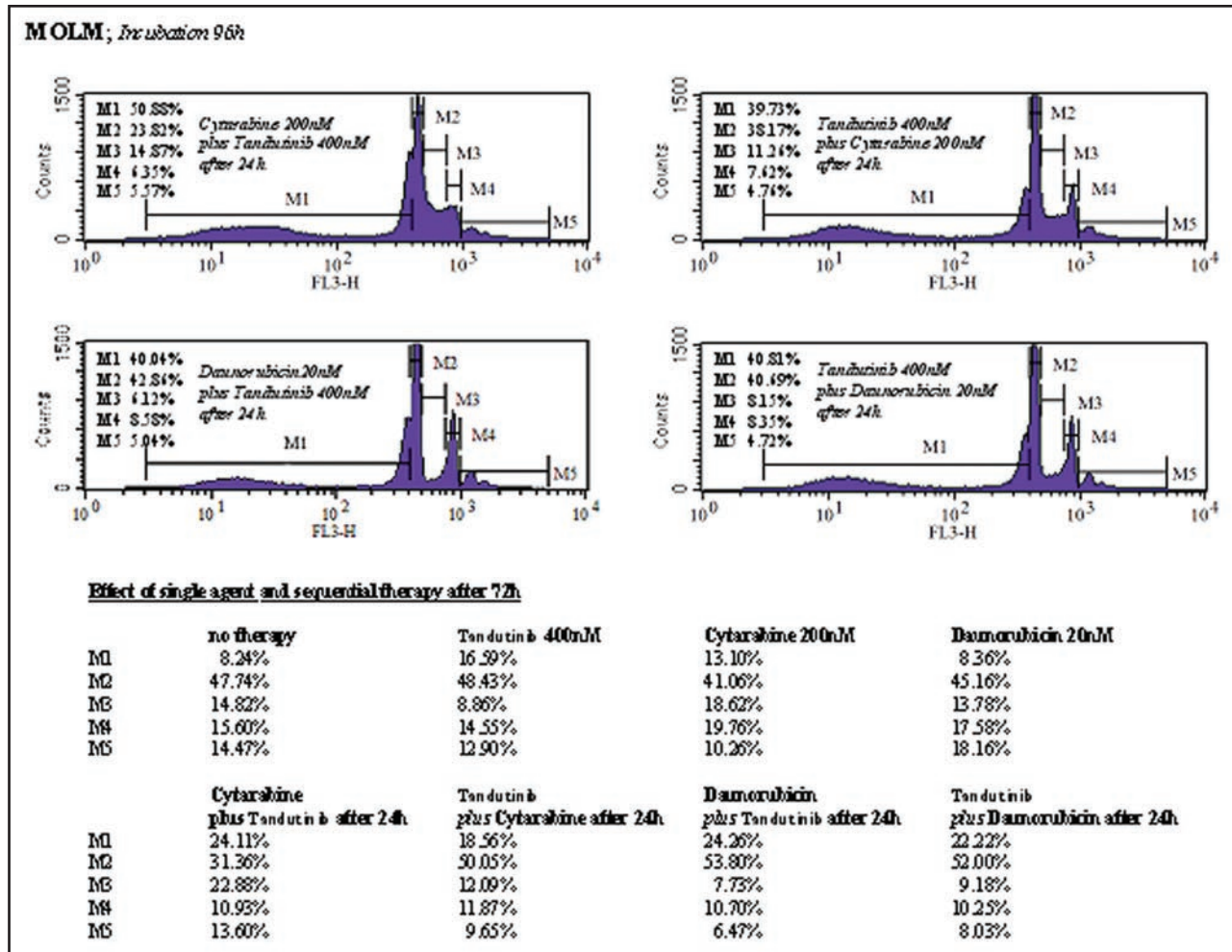


Figure 3. Effects of monotherapy or sequential combination therapy on cell cycle distribution and induction of apoptosis. (B) Cells were incubated with equally potent doses of cytarabine or daunorubicin and tandutinib was added after 24 h. Experiments using the reverse sequencing (tandutinib first, with delayed addition of chemotherapy agent) were performed simultaneously. Cell cycle analysis was performed after 0 h, 24 h, 48 h, 72 h and 96 h. The figure shows data using the 96 hour endpoint for combination regimens. The results shown were obtained using the MOLM14 cell line. Similar results were obtained using the MV4:11 and Ba/F3 FLT3 ITD cell lines. Key: M1: dead and apoptotic cells; M2: G₀/G₁-phase; M3: S-phase; M4: G₂/M-phase; M5: polyploid cells.

Our findings suggest, that addition of tandutinib to standard chemotherapy used in AML induction chemotherapy could result in an enhancement of the overall anti-leukemic effect in patients with FLT3 ITD positive AML. The primary dose-limiting toxicity of tandutinib is reversible generalized muscular weakness and/or fatigue,¹⁷ suggesting that combining tandutinib with traditional anti-leukemic agents might improve therapy outcome without increasing toxicity.

Furthermore elderly patients who often display poor tolerance of standard AML chemotherapy regimens may benefit from integrating a relatively non-toxic TKI such as tandutinib into treatment regimens: As indicated by our dose reduction experiments, addition of tandutinib may allow considerable dose reductions of cytarabine and daunorubicin—without loss of the overall cytotoxic effect on AML cells. These data may be useful in the design of future clinical trials to test this concept.

In conclusion, regardless of the application scheme chosen, combining tandutinib with cytarabine and/or daunorubicin is

more efficacious than chemotherapy alone for FLT3 ITD positive AML. Currently, the combination of tandutinib plus standard “3 + 7” induction chemotherapy is being tested in a phase I/II study for treatment of newly diagnosed AML.

Materials and Methods

Cell culture. HL60, MV4:11 and Ba/F3 cell lines were obtained through the American Type Culture Collection (ATCC, Manassas; VA) and the MOLM14 cell line was acquired through the Fujisaki Cell Center (Okayama, Japan). Wild type (WT) Ba/F3 cells were grown in RPMI 1640 supplemented with 10% FBS (fetal bovine serum) and 10% WEHI supernatant. MOLM14 and MV4:11 cells were maintained in RPMI 1640 supplemented with 10% FBS. HL60 cells were maintained in RPMI 1640 supplemented with 20% FBS. MV4:11 cells are hemizygous for an ITD of amino acids VDFREYEYDH at position 592–601,²⁶ while MOLM14 cells (sister cell line to MOLM 13) are heterozygous for an ITD of DFREYE at amino acid position 593–598.²⁷ The

cDNA for human FLT3 was cloned into the pLXSN retroviral vector.²⁸ Mutations encoding for a FLT3 ITD (reduplication of YEYDLK at amino acid positions 597–602)²⁹ were produced by site directed mutagenesis and confirmed by bi-directional sequencing. Retroviral packaging cells were transfected with plasmids encoding FLT3 WT or ITD and retroviral supernatants were used to transduce Ba/F3 cells.³⁰ Cells were selected for factor-independent growth (ITD FLT3) or FL-dependent growth (FLT3 WT) as previously described.³¹ Ba/F3 FLT3 WT cells were maintained in RPMI 1640 + 10% FBS supplemented with 10 ng/ml of recombinant human FL (R&D Systems, Minneapolis, MN). Factor-independent Ba/F3 FLT3 ITD cells were maintained in RPMI 1640 supplemented with 10% FBS.

Patient specimens. Patients were consented for the use of residual blood specimens remaining after completion of clinical testing to determine FLT3 ITD status. The Institutional Review Boards of both the Oregon Health & Science University and the Portland VA Medical Center approved the studies involving patient specimens. Samples were de-identified prior to use in the *in vitro* experiments and the FLT3 ITD status of the de-identified specimens was independently determined by D-HPLC and direct sequencing as previously described.³²

Mononuclear cells were isolated by Ficoll-Hypaque density centrifugation and were plated in the presence or absence of tandutinib and/or cytarabine and/or daunorubicin. The effect of tandutinib and/or cytarabine and/or daunorubicin on the proliferation of primary AML myeloblasts was determined after 72–96 hours of treatment using an XTT-based assay. In the experiments involving a combination of tandutinib and cytarabine and/or daunorubicin, the two agents were added simultaneously to the cells. *In vitro* data were compared with the clinical outcome of each patient.

Reagents. The piperazinyl quinazoline class compound tandutinib (formerly CT53518/COR53518/MLN518)^{23,24} was obtained from Millenium Pharmaceutical and dissolved in DMSO to create 10 mM stock solutions, and kept frozen at -20°C. Cytarabine (Cytosine β -D-Arabinofuranoside) and Daunorubicin Hydrochloride were obtained from Sigma (St. Louis, MO).

Proliferation assays. Cells were added to 96 well plates at densities of 30,000 cells/well for MV4:11, MOLM14, and Ba/F3-derived cells. Test compounds were added and proliferation was measured at 72 hours using an XTT-based assay (Roche Molecular Biochemicals; Indianapolis, IN).³³

Apoptosis assays. Cells were rinsed with PBS and incubated in RPMI supplemented with 10% FBS. 1×10^6 cells were incubated with test compounds for 48 hours and translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane as an early indicator of apoptosis was analyzed using an AnnexinV-FITC kit (Immunotech, Marseille, France)³³ and a FACScalibur flow cytometer loaded with CellQuest analysis software (BD, Heidelberg, Germany).

Data analysis. Results from experiments were analyzed using the median effect method of Chou and Talalay³⁴ (Calculusyn software available from Biosoft, Cambridge, UK) as described previously.¹⁹ Cells were treated with fixed ratios in relationship to the individual

agent ED₅₀ and the resultant data was analyzed using the method of Chou and Talalay to produce isobolograms. This allowed calculation of combination indices (CI). The CI provides a numerical description of the effects of a combination treatment. Specifically, a CI < 1 indicates synergy, a CI = 1 indicates an additive effect, and a CI > 1 indicates antagonism between the two agents.

Isobologram analysis further allowed to compute dose reduction indices (DRI), which describes the potency of how much a certain dose of drug A can be reduced when drug B is added to the system at a certain concentration. The overall cytotoxicity thereby remains unchanged.

Sequential therapy analysis. We addressed the question of whether or not the specific schedule of drug application influences cytotoxic efficacy. Sequential application schemes were evaluated with either tandutinib as initial therapy followed by cytarabine or daunorubicin after 24 or 48 hours or vice versa. Proliferation assays were performed to create isobolograms and combination indices were computed as described above. Cell cycle experiments were assessed, as we have previously described, by flow cytometry after ethanol fixation and staining cells with propidium iodide (PI) (Fluka, Buchs, Switzerland).³⁵ Samples were pre-treated with RNase to reduce RNA interference. We measured untreated, mono-treated and sequentially treated samples at 0 h, 24 h, 48 h, 72 h and 96 h-timepoints for dynamic analysis of cell cycle. The data were analyzed using a FACScalibur flow cytometer loaded with CellQuest analysis software (BD, Heidelberg, Germany).

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/SchittenhelmCC8-16-Sup.pdf

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