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Prolonged cellular midostaurin retention suggests potential alternative dosing strategies for FLT3-ITD-positive leukemias

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Small molecule tyrosine kinase inhibitors (TKI) have become a valuable therapeutic tool for the treatment of a variety of malignancies. Inhibition of constitutively activated tyrosine kinases induces apoptotic cell death in transformed cells, although leaving their normal counterparts largely unaffected. It is widely accepted that continuous target inhibition is a prerequisite for efficient cytotoxic activity of TKIs. Recently, several groups have demonstrated that *BCR–ABL* transformed cells can undergo apoptosis through prolonged intracellular drug accumulation and retention upon high-dose TKI pulse exposure.[1–5] This suggests that a given TKI might still be present at active concentrations in the target cells, although the drug has already been cleared from the patient's plasma. Our group has pioneered the measurement of intracellular TKI accumulation using both direct drug detection and functional read outs.[1,2] We propose that a TKI's intracellular accumulation potential is an important pharmacological property that can be tested systematically. We further suggest that intracellular TKI accumulation potential should be considered when testing different dosing strategies in future clinical trials.

In acute myeloid leukemia (AML), activating *FLT3* mutations are found in ~30–35% of patients.[6,7] The most common mutation type is represented by internal tandem duplications (*FLT3*-ITD) usually located within the juxtamembrane domain or the tyrosine kinase domain 1.[8] *FLT3*-ITDs lead to constitutively activated intracellular signaling, resulting in malignant transformation.[9] Several FLT3-TKIs are currently in clinical development, demonstrating short-term biological and clinical activity as monotherapy.[10–12] Sorafenib has been reported to improve event-free survival in unselected AML patients aged <60 years in combination with standard chemotherapy, and recent reports highlighted the efficacy of midostaurin in combination with standard chemotherapy in *FLT3*-mutated AML.[13–15] Midostaurin is a multikinase inhibitor derivative of staurosporine that shows activity against several kinases, including FLT3, KIT, several members of the PKC enzyme family, KDR and PDGFR-β.[16] Recently reported results of an international, randomized, placebo-controlled phase III clinical trial (RATIFY) strikingly demonstrated a highly significant and clinically relevant increase in overall and event-free survival in adults aged 18–60 years with newly diagnosed *FLT3*-mutated AML treated with midostaurin in addition to standard chemotherapy.[15] This survival benefit will likely lead to the approval of midostaurin for the treatment of *FLT3*-mutated AML in the near future.

To study the effects of high-dose midostaurin pulse treatment, we examined the induction of apoptotic cell death in hematopoietic mouse Ba/F3 cells (DSMZ, Braunschweig, Germany) stably transfected with mutant *FLT3*-ITD (Ba/F3-FLT3-ITD). To rule out a cell context-specific phenomenon, similar experiments were performed in mutant FLT3-ITD expressing human myelomonocytic MV4–11 leukemic cells (DSMZ, Braunschweig, Germany). Cells were treated with different biologically relevant concentrations of midostaurin for 2 h (that is, 35, 100 and 3500 nM),[17] extensively washed as described[1,2] and then maintained in TKI-free cell culture medium for a total of 24 h. Untreated cells and cells treated continuously with midostaurin at the above mentioned concentrations served as controls. High-dose pulse exposure (2 h) of Ba/F3-FLT3-ITD cells using 3500 nM midostaurin resulted in $>80\%$ apoptotic cells at 24 h, whereas, in contrast, 2 h treatment using 35 and 100 nM midostaurin, respectively, did not increase apoptosis levels (Figure 1a). Effects mediated by off-target activity of midostaurin were ruled out by using Ba/F3-FLT3-wild-type cells as a control. In this control setting, no increase of apoptosis was detected upon 2 h pulse exposure at any midostaurin concentration tested (data not shown). Interestingly, caspase 3 cleavage kinetics in Ba/F3-FLT3-ITD cells were similar across the different midostaurin concentrations used, which suggested a common specific mechanism of apoptosis induction operating in cells exposed to either low or high midostaurin concentrations, and thus confirmed previous findings obtained with BCR–ABL TKIs (Figure 1b).

We then employed our previously developed experimental design consisting of repeated washing and replating procedures to demonstrate intracellular drug accumulation and retention upon high-dose TKI pulse exposure (Figures 1c).[1,2] Three consecutive rounds of thorough medium exchange (each consisted of 2× drug wash out) at 2 h intervals were performed followed by measurement of apoptotic cell death at 24 h. Untreated cells were incubated with the supernatants derived from the three corresponding washing steps.[1,2] In line with data presented above (Figure 1a), high-dose midostaurin pulse exposure induced apoptotic cell death at the 24 h endpoint in both *FLT3*-mutant cell lines (Figures 1d and e; upper panel: red bars (1×)). However, when cells were extensively washed a second (2 h post the initial media exchange) and a third time (4 h post initial media exchange), this effect was largely abolished and we observed only a slight increase in apoptotic cell death as compared with the baseline apoptosis levels (Figures 1d and e; upper panel: orange (2×) and yellow (3×) bars). Incubation of previously untreated cells with cell culture supernatants derived from the second media exchange (S2) resulted in a substantial increase in the fraction of apoptotic cells (Figures 1d and e; lower panel).

In BCR–ABL-positive cells, we and others have demonstrated that the extent of suppression of STAT5 and ERK phosphorylation indicates the presence of functionally relevant levels of TKI.[1–5] Similarly, STAT5 signaling is aberrantly activated in FLT3-ITD-positive cells and confers important survival signals in these cells.[18,19] Therefore, we investigated the phosphorylation kinetics of both STAT5 and ERK in response to high-dose midostaurin pulse exposure. Complete absence of both, phosphorylated ERK and phosphorylated STAT5, was observed following the first round of drug wash out after high-dose midostaurin pulse exposure (1×). However, in concordance with our results on induction of apoptosis (Figures 1d and e), we observed gradual reappearance

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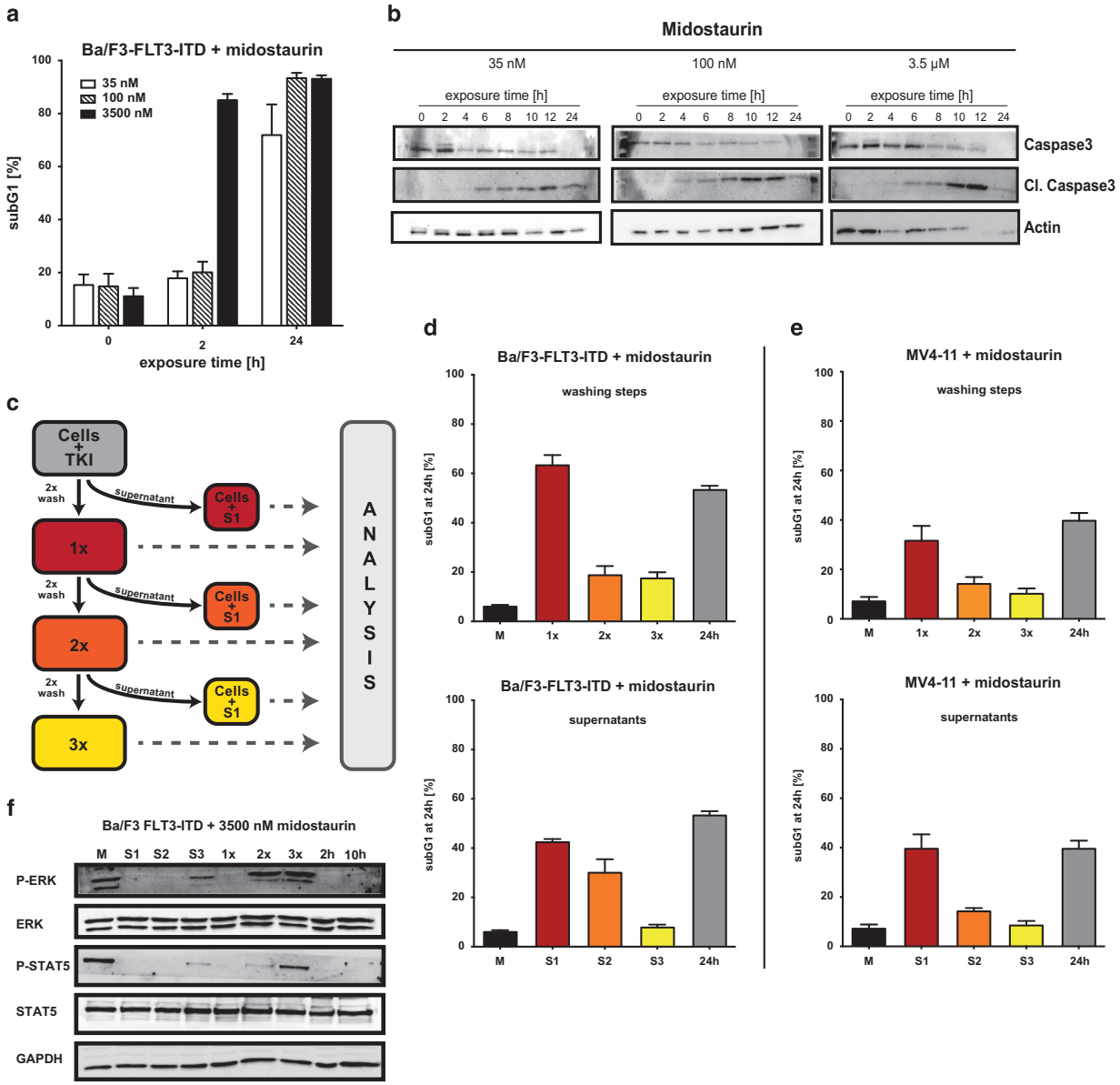

Figure 1. High-dose midostaurin pulse exposure leads to prolonged inhibition of oncogenic signaling and induction of apoptosis. (**a**) Ba/F3 cells expressing a *FLT3* internal tandem duplication (Ba/F3-FLT3-ITD)[22] were seeded into six-well plates (5×10^4 cells per ml, total volume 2 ml) in RPMI 1640+10% FCS. Cells were treated for 2 h or 24 h with midostaurin as indicated. Cells exposed to 0.35% DMSO (0 h) served as controls. Cells treated for 2 h and control cells were washed 2× with 2 ml phosphate-buffered saline (PBS) at room temperature and replated in fresh cell culture media (2 ml final volume). Twenty four hours after start of the experiment percentage of cells in subG1 phase was measured by flow cytometry after propidium iodide staining (PI). Experiments were performed in biological triplicates. Depicted is the mean percentage of cells in subG1 phase +s.e.m. of biological triplicates. (**b**) Ba/F3-FLT3-ITD cells were treated with 35, 100 and 3500 nM midostaurin for different periods of time as indicated. Upon treatment, cells were lysed immediately in lysis buffer. Cellular lysates were subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE) and subsequently immunoblotted with antibodies against cleaved caspase 3 (#9664; Cell Signaling Technology, Danvers, MA, USA), caspase 3 (#7148; Santa Cruz, Dallas, TX, USA) and beta-actin (#A5441-2, Sigma-Aldrich, Munich, Germany). (**c**) Schematic overview of experimental setup for high-dose pulse exposure experiments. (**d**, **e**) Cells (**d:** Ba/F3-FLT3-ITD; **e:** MV4–11) were treated with midostaurin for 2 h. Cells initially pulse-exposed to midostaurin were washed twice with 2 ml PBS at room temperature and were then replated in 2 ml fresh media (density: 5×10^4 cells per ml) as described in Figure 1A (1×). To test for residual TKI activity, the cell culture supernatant was transferred to previously untreated cells (S1), which were subsequently incubated for 24 h. Two hours after replating, a second drug wash out was performed (2× 2 ml PBS). Cells were again replated in 2 ml fresh media (2×). Again, supernatants were transferred to previously untreated cells (S2). This procedure was repeated for a third time (3×, S3). Cells exposed to 0.35% DMSO (M) or to midostaurin for 24 h served as controls. Twenty four hours after start of TKI exposure percentage of cells in subG1 phase was measured by flow cytometry after PI. Depicted is the mean percentage of cells in subG1 phase +s.e.m. of biological triplicates. (**f**) Ba/F3-FLT3-ITD cells were treated with 3500 nM midostaurin as described in **c**. Untreated cells served as positive controls for phosphorylation signals (M). Cells treated continuously with TKI for 2 h or 10 h (2 and 10 h) served as positive controls for midostaurin activity. Lysates were prepared 2 h post drug wash out, allowing recovery of phosphorylation signals in case of absent midostaurin activity. S1, S2 and S3: lysates were prepared upon incubation of previously untreated cells for 2 h with the respective cell culture supernatants obtained upon each washing procedure. Lysates were subjected to 10% SDS–PAGE and subsequently immunoblotted with antibodies against phospho-ERK (#9106; pThr202/pTyr204; Cell Signaling) and phospho-STAT5 (#05-495; pY694/pY699; Merck Millipore, Darmstadt, Germany). To control for equal loading, blots were stripped and reprobed with anti-ERK (#9102; Cell Signaling), anti-STAT5 (#1081; Santa Cruz) and anti-GAPDH (#H86504M; Meridian Lifescience, Memphis, TN, USA) antibodies.

of phosphorylated ERK and phosphorylated STAT5 signals after the second round of drug wash out (2×) reaching almost complete recovery of phosphorylation signals upon the third round (3×) of media exchange (Figure 1f; 1–3×). In line with this, incubation of previously untreated cells with supernatants derived from the corresponding washing steps 1 (S1), 2 (S2) and 3 (S3), respectively, almost completely inhibited phosphorylation of ERK and STAT5, indicating abundance of active TKIs in the supernatants (Figure 1f; S1–S3). This suggests that STAT5 phosphorylation may prove useful as a surrogate parameter to monitor target inhibition in *FLT3*-mutant AML, but also as a potential drug target in combination with TKIs as suggested by Schafranek *et al.*[1,5]

To test whether redistribution of midostaurin occurs from treated cells into the extracellular medium, midostaurin concentrations were determined in cellular supernatants by high-performance liquid chromatography with diode array and fluorescence detector. Ba/F3-FLT3-ITD cells, as well as leukemic blasts from two patients with AML and normal CD34+ cells were used for this analysis. High-dose midostaurin (3500 nM) pulse exposure (2 h) was followed by thorough drug wash out and midostaurin concentrations were measured in the cell culture media at several time points after drug wash out (0, 15, 30, 60 and 120 min). Midostaurin concentrations increased over time in the cell culture supernatants that proved midostaurin release from treated cells into the medium. Already 15 min after initial drug wash out, the midostaurin concentration in the cell culture medium reached levels above the half maximal inhibitory concentration (IC50) for Ba/F3-FLT3-ITD cells and reached >100 nM, corresponding to about $3\times$IC50, after 120 min. The redistribution kinetics were highly similar across the different cell types tested (Figures 2a–c).

Together, these data demonstrate intracellular midostaurin accumulation upon high-dose pulse exposure and subsequent slow release into the extracellular media over time in a manner similar to what has been described for BCR–ABL TKIs.

From the clinical perspective, our findings may prove useful for the future development of therapeutic TKI dosing schedules: selection of TKI lead compounds for clinical development is typically based on pharmacodynamic and pharmacokinetic characteristics. Recently, published data demonstrated that a short plasma half-life of a given compound may not necessarily predict a deficit in terms of clinical usefulness.[20] Our *in vitro* model of high-dose midostaurin pulse treatment revealed a tight interplay between extracellular and intracellular midostaurin concentrations. The data presented here is consistent with a model whereby intracellular accumulation and retention of a TKI *in vivo* translates into significantly higher intracellular TKI concentrations as compared with the extracellular medium. It is conceivable that in the setting of high-dose pulse exposure this may then result in prolonged intracellular TKI exposure, significantly exceeding plasma half-life of the TKI as, for example, observed in a clinical trial with dasatinib.[20,21] In the most recent clinical trial testing midostaurin in combination with standard chemotherapy for AML (RATIFY), midostaurin was given at a dose of 50 mg twice per day.[15] Taking into account the data presented here, it might be worthwhile to consider investigating alternative dosing schedules with only once daily drug administration mimicking the high-dose pulse exposure setting described here. Such a clinical trial needs to be paralleled by close clinical, pharmacokinetic and pharmacodynamic monitoring to thoroughly assess safety and efficacy. As seen with dasatinib, high-dose pulse treatment might be of advantage with respect to side effects and most prominently with respect to the patient's compliance to the prescribed medication. Anyhow, as it cannot be ruled out that unwanted and potentially severe side effects such as QT interval prolongation might occur even more frequently upon high-dose pulse treatment, acute and chronic

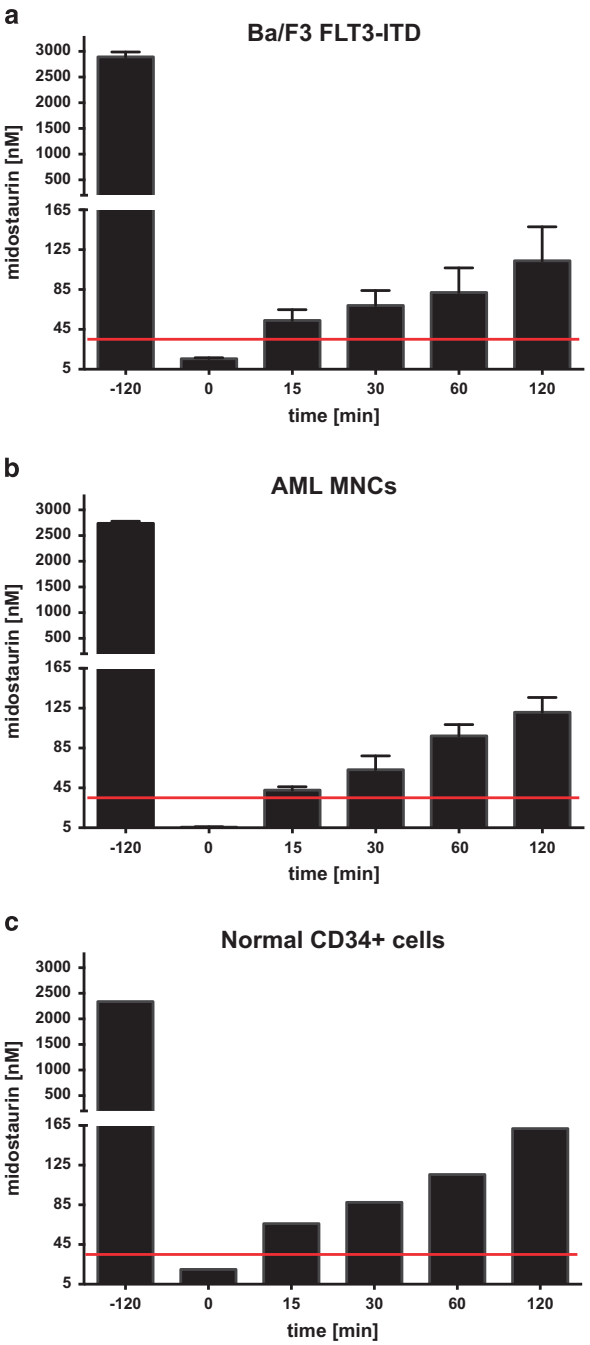

Figure 2. Measurement of midostaurin concentrations released into cell culture medium upon high-dose exposure. Midostaurin concentrations were measured in cell culture supernatants by high-performance liquid chromatography after exposure of cells to 3500 nM midostaurin for 2 h. The red lines represent the IC50 for midostaurin (35 nM) in Ba/F3-FLT3-ITD cells. Results obtained from Ba/F3-FLT3-ITD cells (**a**, $N=3$), leukemic blasts from acute myeloid leukemia (AML) patients carrying FLT3-ITD mutations (AML MNCs) (**b**, $N=2$), and normal CD34+ hematopoietic progenitor cell enriched leukapheresis product (**c**, $N=1$) are depicted. Supernatant 1 (S1) was measured and served as a positive control (−120 min). Measurements of cell culture media samples taken immediately upon replating served as a control for efficacy of drug wash out (0 min). Depicted are mean values +s.e.m. were applicable. The acquisition and experimental use of human samples was approved by the institutional review board of the University Medical Center and all individuals gave written informed consent.

toxicity of such a treatment regimen has to be assessed thoroughly in clinical trials. In summary, our data sets a new cornerstone for future developments of TKI therapy in AML: we propose to analyze both, plasma and intracellular pharmacokinetics to optimize dosing schedules in upcoming clinical trials testing TKIs.

CONFLICT OF INTEREST

DBL received honoraria from Novartis and BMS; TF received honoraria from Novartis; and MCW and MD have no relevant conflicts of interest to disclose.

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AUTHOR CONTRIBUTIONS

DL and TF: designed the research, analyzed and interpreted data, and wrote the paper. MCW: performed the research, analyzed data and contributed to writing the paper. MD: performed and analyzed the TKI measurements, and contributed to writing the paper.

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