

Impaired S-Phase Arrest in Acute Myeloid Leukemia Cells with a FLT3 Internal Tandem Duplication Treated with Clofarabine

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Abstract Purpose: Acute myeloid leukemia cells with an internal tandem duplication mutation of FLT3 (FLT3-ITD) have effective DNA repair mechanisms on exposure to drugs. Despite this, the phenotype is not associated with primary resistant disease. We show defects in the response of mutant FLT3 AML cells to the S-phase drug clofarabine that could account for the apparent contradiction.

Experimental Design: We studied responses of AML cells to clofarabine *in vitro*.

Results: When treated with a short pulse of clofarabine, FLT3-ITD-harboring MOLM-13 and MV4.11 cells undergo similar damage levels (γ H2AX foci) to wild-type cells but have a better repair capability than wild-type cells. However, whereas the wild-type cells undergo rapid S-phase arrest, the S-phase checkpoint fails in mutant cells. Cell cycle arrest in response to DNA damage in S phase is effected via loss of the transcriptional regulator cdc25A. This loss is reduced or absent in clofarabine-treated FLT3 mutant cells. Furthermore, cdc25A message levels are maintained by the FLT3-ITD, such that message is reduced by 87.5% on exposure to FLT3 small interfering RNA. Primary FLT3-ITD samples from untreated patients also display impaired cell cycle arrest and show enhanced sensitivity on prolonged treatment with clofarabine compared with wild-type samples.

Conclusion: There is a reversal of phenotype in mutant FLT3 cells dependent on the length of exposure to clofarabine. Efficient DNA repair may render the cells resistant to a short pulse of the drug, but a failure of cell cycle checkpoint(s) in S phase renders the cells sensitive to prolonged exposure. (Clin Cancer Res 2009;15(23):7291–8)

FLT3 is one of the most commonly mutated genes in acute myeloid leukemia (AML). It is a member of the type III tyrosine kinase receptor family, which promotes cell growth. Mutated *FLT3* can lead to constitutive activation of the receptor and of multiple downstream genes and pathways. Approximately 30% of AML patients have mutations of the *FLT3* gene in their blast cells, composed of either *FLT3*-internal tandem duplication (FLT3-ITD) mutations (24%; ref. 1) or *FLT3* activation loop mutations (7%; ref. 1). *FLT3* mutations are associated with a relatively poor prognosis in AML due to an increased relapse rate (2–4).

Clofarabine is a second-generation purine nucleoside analogue with favorable pharmacologic properties (resistance to

deaminase degradation and stability in gastric acid) and is in increasing clinical use for the treatment of AML (5–9). Because of the potential oral availability and its acceptable toxicity with respect to mucositis and alopecia, its use has recently been explored in older AML patients with promising results.³

Mechanisms supporting heterogeneity in sensitivity and resistance to clofarabine have been reported in the literature; particularly, a variability in the cellular accumulation of clofarabine triphosphate in circulating blasts has been recorded (6, 7). We have previously shown efficient DNA repair following anthracycline exposure in AML cells with a *FLT3*-ITD (10) and were thus initially interested to discover whether DNA damage and repair mechanisms are important in the response of *FLT3*-ITD cells to clofarabine. There is a well-characterized assay (the measurement of γ H2AX) that can be used as a biomarker of the effectiveness of clofarabine at reaching its DNA target and inducing a damage response (7, 11). The use of this assay has enabled us to focus on events subsequent to clofarabine-induced DNA damage and to dissect mechanisms by which cells proceed to apoptosis or recovery subsequent to the initial damage response signals.

The damage response pathway is closely linked to cell cycle arrest mechanisms. In a well-documented but fast-evolving model of cell cycle arrest in S phase (the phase in which

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Translational Relevance

We have shown that the resistance of AML cells with an internal tandem duplication mutation of FLT3 to clofarabine in short-term culture is reversed on prolonged culture due to a failure of S-phase arrest, which allows prolonged drug accumulation. This paves the way for studies of *in vivo* S-phase responses, as knowledge of these kinetics might be used to therapeutic advantage in terms of length of infusion or timing combinations of drugs that are thought to be synergistic. Our study may be just the first of many examples of activating mutations that affect the S-phase checkpoint response to a range of S-phase drugs.

clofarabine-treated cells would be expected to arrest), stalled replication forks lead to ATR-dependent induction of Chk1 (12). Chk1 phosphorylates and thus inactivates cdc25A (13, 14). Cdc25A is an essential regulatory protein and its degradation in response to DNA damage is crucial for cell cycle arrest. The protein has multiple phosphorylation sites: most of these are in the regulatory domain and allow the protein to be targeted for ubiquitinylation and proteolysis, such that Cdc25A only has a half-life of 20 to 30 min (15). This keeps cdc25A levels at a physiologic threshold that allows normal S-phase progression. Cdc25A dephosphorylates and activates the cyclin E/A-CDK2 complex. Cells unable to degrade cdc25A fail to undergo S-phase arrest in response to DNA damage (13, 16, 17). We therefore examined expression of this crucial protein in cells treated with clofarabine.

Materials and Methods

Materials

Materials were from Sigma unless otherwise stated below.

Cells

Leukemic cell lines and samples from untreated patients with AML were used in these experiments. The KG1 and MV4.11 cell lines were obtained from the American Type Culture Collection. HL-60 cells were from the European Collection of Animal Cell Cultures. MOLM-13 cells were from the German Collection of Microorganisms and Cell Cultures. HL-60, MOLM-13, and MV4.11 were maintained in RPMI 1640 with 10% FCS (First Link), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. KG1 cells were maintained as above but with 20% FCS. All cultures were at 37°C in 5% CO₂. All experiments were done with cell lines in log phase. Continued testing to authenticate these cell lines was done using a panel of monoclonal antibodies and FLT3 mutational analysis toward the final passage of each batch thawed. Presentation blood or bone marrow samples from AML patients were obtained with informed consent and used according to a protocol ratified by Nottingham 1 Research Ethics Committee. White cells were isolated using a standard density gradient/centrifugation method with Histopaque and cryopreserved in liquid nitrogen. For analysis, cryopreserved samples were thawed and rested in culture medium enriched to 20% FCS for 90 min before experimental procedures. Only samples with >85% post-rest viability were used. Primary AML samples were cultured at 10⁶/mL in RPMI 1640 with 10% FCS, 2 mmol/L L-glutamine supplemented with 20 ng/mL interleukin-3, 20 ng/mL SCF, 20 ng/mL interleukin-6 + 25 ng/mL granulocyte colony-stimulating factor (R&D Systems) + 0.07 µL/mL β-mercaptoethanol. At 48 h, they were counted and replated for functional assays. Only samples with a 48 h count of at least 4 × 10⁵/mL were used.

Clofarabine response assays

Leukemic cells were suspended in sterile Falcon tubes at 5 × 10⁵/mL for cell lines and 8 × 10⁵/mL for patient cells in the medium described above. After preliminary dose-finding studies, they were cultured with clofarabine at doses specified elsewhere for the assays described below. Clofarabine was a gift from Bioenvision (now part of Genzyme) and was maintained at room temperature as a 3 mmol/L stock solution in saline. In pulsing experiments, cells were cultured with clofarabine, rinsed twice at 4°C in RPMI 1640, and resuspended in fresh medium for the remainder of the culture.

Assays for apoptosis

Apoptosis in cultured cells was assessed using flow cytometric 7-amino actinomycin D (7-AAD) and forward scatter measurements (18, 19). 7-AAD (5 µg/mL) was added to each tube 15 min before the end of culture. 7-AAD fluorescence was measured using the FL3 channel of a FACSCalibur (Becton Dickinson).

γH2AX assays

Flow cytometry. Cells were incubated at 5 × 10⁵/mL for 1 h with and without the specified doses of clofarabine followed by two rinses in ice-cold RPMI 1640. A portion of the cells was then fixed immediately to assess baseline damage, and a portion was resuspended in fresh culture medium and returned to the incubator to allow time for repair to take place. Cells were harvested and fixed after 2 h further incubation. H2AX phosphorylation was measured with a kit from Upstate according to the manufacturer's instructions. Counterstaining to estimate intracellular DNA content was with 25 µg/mL 7-AAD in PBS. For quantitative analysis, fluorescence values obtained from untreated control cells were subtracted to ensure specificity.

Immunocytochemistry. Clofarabine-treated and control cells were fixed to glass slides using the Liqui-Prep Cytology system (LGM International), fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Unconjugated monoclonal γH2AX antibody (Upstate) was applied for 1 h. Foci were visualized using the Mach4 horseradish peroxidase system (BioCare Medical). As the number of foci varied per cell, these were quantified using a modification of the H score system originally developed to determine estrogen receptor status in breast carcinomas (20). In each field, the number of cells staining positively was assessed and divided into groups according to the number of foci present: N (no foci), L (1-6 foci per cell), M (7-12 foci), H (≥13 foci), and C (completely damaged, individual foci not countable). A total of 100 cells per slide were assessed and the H score was calculated as follows: N + 2L + 3M + 4H + 5C. The H score of untreated cells was subtracted from that of treated cells.

Bromodeoxyuridine incorporation assay

Cells at 5 × 10⁵/mL (cell lines) or 8 × 10⁵/mL (patient samples) were cultured with clofarabine for the times specified in Results. Following two washes, 100 µmol/L bromodeoxyuridine (BrdUrd; DAKO) was incubated with the cells for 45 min at 37°C. The cells were then washed in PBS + 1% glucose, fixed and permeabilized in cold 70% ethanol, and stored at -20°C overnight or for up to 6 days. The cells were then pelleted, incubated for 30 min in 2 mL of 2 mol/L HCl at room temperature, pelleted, incubated in 2 mL of 0.1 mol/L borax (pH 8.5) for 5 min at room temperature, and washed in PBS + 1% glucose. Immunostaining was with anti-BrdUrd (DAKO). Isotype-matched control and secondary antibodies were also from DAKO. Counterstaining to estimate intracellular DNA content was with 25 µg/mL 7-AAD in PBS.

Cdc25A measurements

Cdc25A was measured flow cytometrically using a monoclonal primary antibody from Novus (DCS120/121) or isotype control. Cells were fixed and permeabilized with leucoperm (Ab Serotec) and incubated for 60 min with 5 µL primary antibody. Rabbit anti-mouse FITC (DAKO) was used as second layer.

Small interfering RNA nucleofection

Small interfering RNA (Hs_FLT3_2_HP, 1423109; control siRNA, 1022076; Qiagen) was introduced into 4 × 10⁶ cells in logarithmic

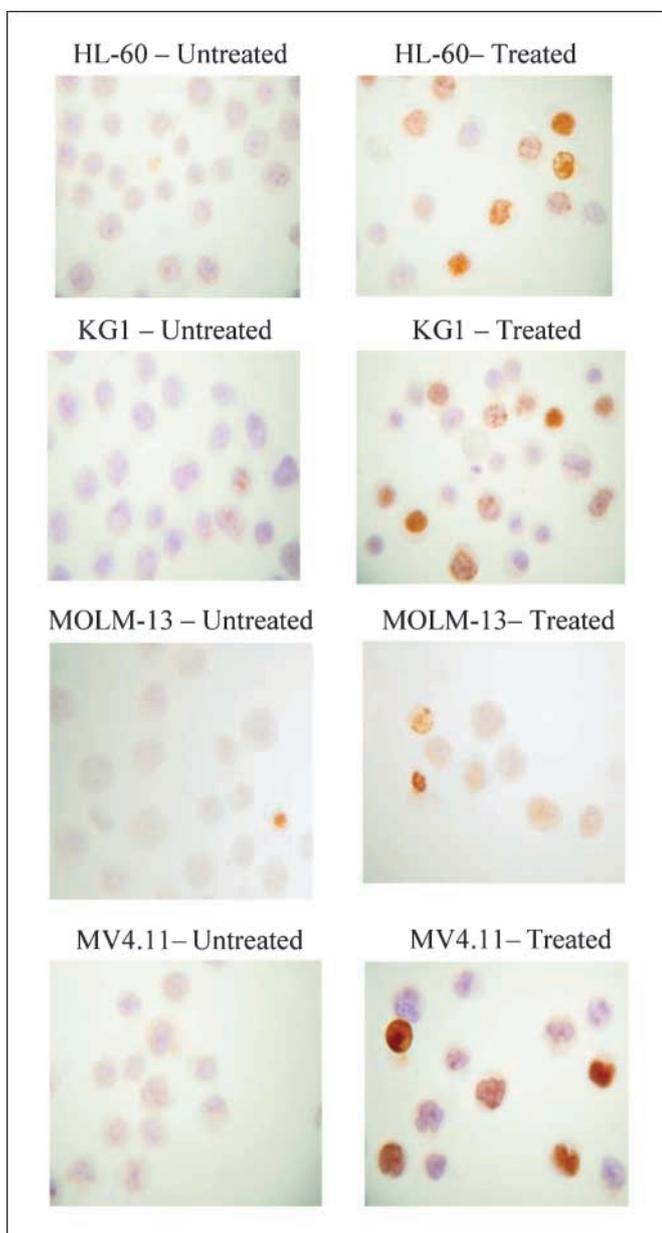


Fig. 1. γ H2AX phosphorylation in leukemic cell lines. Cells were cultured for 1 h with equitoxic clofarabine (0.3 μ mol/L for HL-60 and MOLM-13 and 1 μ mol/L for KG1 and MV4.11). γ H2AX was measured directly after treatment.

growth phase using nucleofection (Amaxa). Cells were nucleofected according to the manufacturer's guidelines; solution R (Amaxa) and program X-001 were used for the MV4-11 cell line, and solution V (Amaxa) and program T-019 were used for HL-60 nucleofection.

FLT3 mutation analysis

FLT3-ITDs were analyzed by previously described methods (10). ITD mutations were confirmed by analysis on a 3130 Genetic Analyzer (Applied Biosystems). Samples were also analyzed for the presence of a point mutation in the tyrosine kinase domain at codon position 835 (Asp; ref. 21).

Quantitative PCR

RNA was prepared from cell lines using QIAamp RNA kits with DNase treatment according to the manufacturer's instructions (Qiagen). Up to 2 μ g RNA was used in a reverse transcription reaction with MMLV reverse transcriptase (Invitrogen) and random hexamers (GE Healthcare). Quantitative PCR was done on an ABI Prism 7700 (Applied

Biosystems) using Excite Real-time Mastermix with SYBR Green (Biogene). Thermal cycler conditions included incubation at 95°C 10 min followed by 40 cycles of 95°C 15 s and 60°C 1 min. Following the 40 cycles, the products were heated from 60°C to 95°C over 20 min to allow melting curve analysis to be done. This allowed the specificity of the products to be determined (single melting peak) and confirmed the absence of primer-dimers.

To enable the levels of transcripts to be quantified, standard curves were generated using serial dilutions of KG1a cDNA for *cdc25a* and MV4.11 for *FLT3*. The housekeeping gene β ₂-microglobulin (β 2M) was used to standardize the samples and the relative expression level of *cdc25a* was therefore calculated as the ratio between the level of *cdc25a* and the level of β 2M. The sequences of the primers used to quantify β 2M have been published previously (22); primers for *cdc25a* were purchased from Qiagen (QT00001078). Negative controls (no template) were included in each experiment and all reactions were run in triplicate.

Results

DNA damage response in clofarabine-treated cells. For this study, we used MV4.11 cells that are homozygous for the *FLT3*-ITD, MOLM-13 that has one mutant and one wild-type allele, and two *FLT3* wild-type cell lines: KG1 and HL-60. All four cell lines express *FLT3* message and protein, but only the

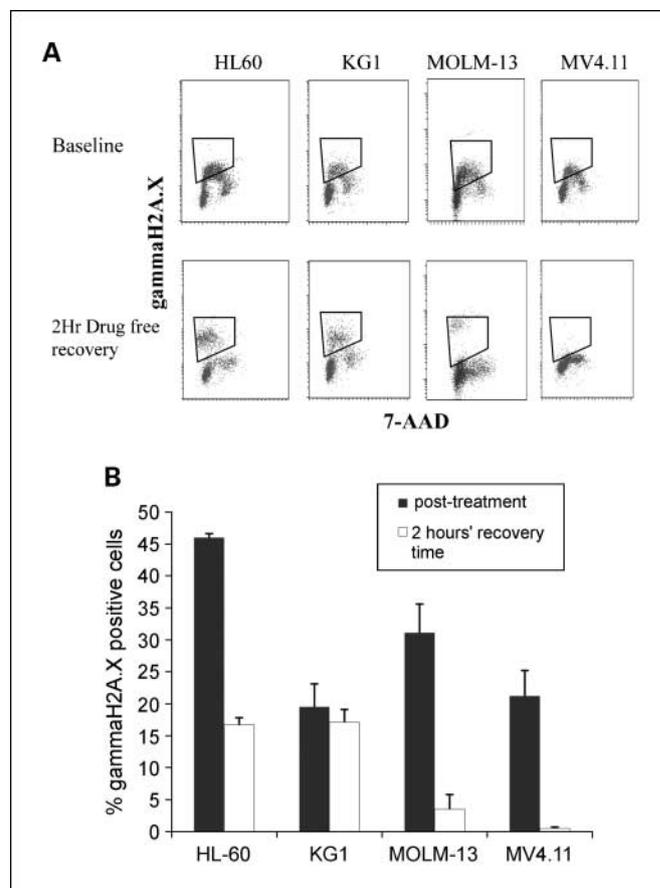


Fig. 2. Flow cytometric analysis γ H2AX phosphorylation. γ H2AX was measured directly after treatment and following 2 h additional culture in drug-free medium to allow recovery. A, representative flow cytometry dot-plots. γ H2AX fluorescence on the Y axis, plotted against DNA content (7-AAD) on the X axis, directly after drug treatment and following 2 h recovery time. B, as in A. Mean of two to three independent flow cytometry experiments.

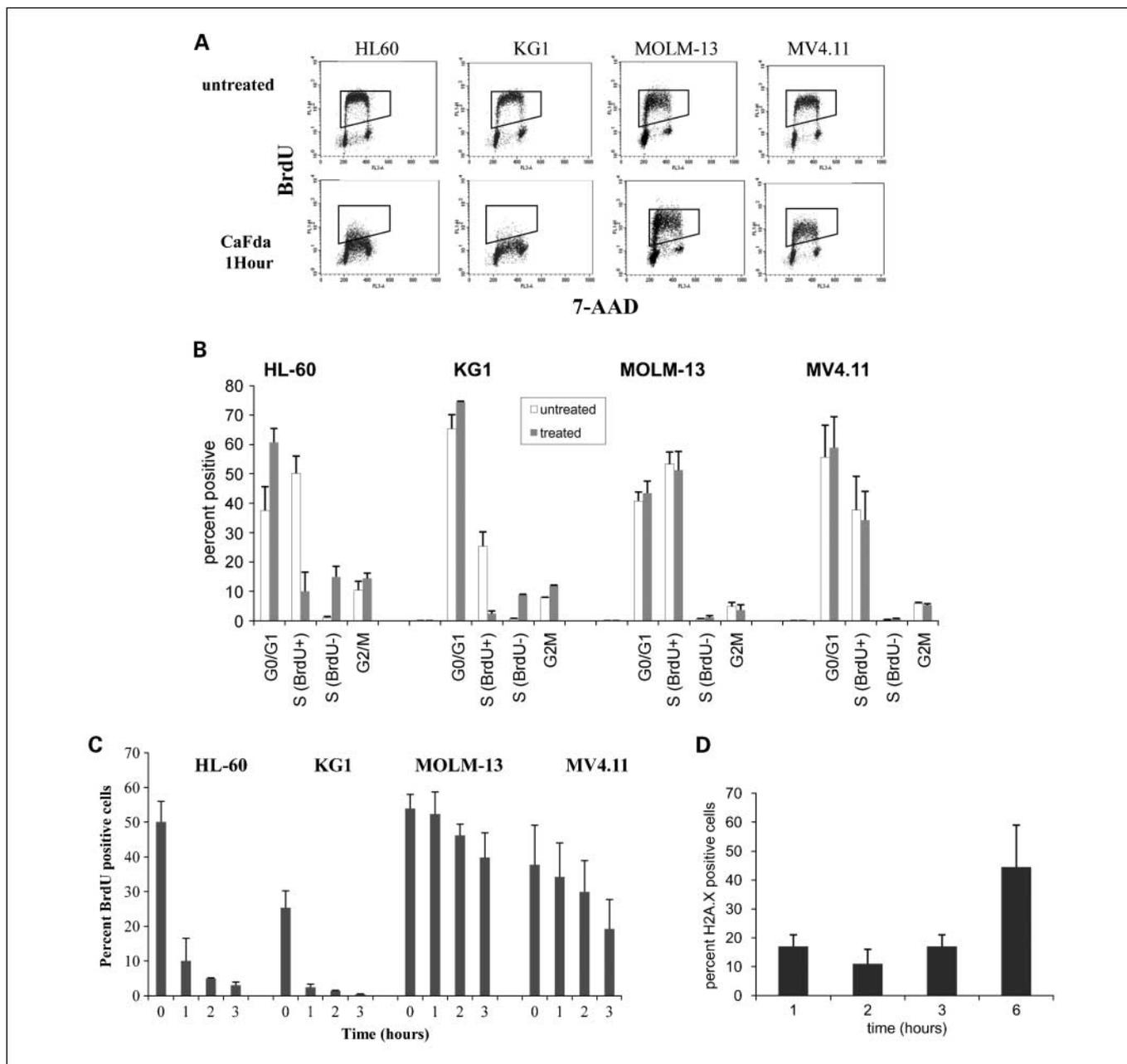


Fig. 3. Inhibition of BrdUrd incorporation by clofarabine. Cells were cultured for the times stated with equitoxic clofarabine (0.3 $\mu\text{mol/L}$ for HL-60 and MOLM-13 and 1 $\mu\text{mol/L}$ for KG1 and MV4.11). **A**, representative dot-plots of anti- BrdUrd fluorescence (Y axis) plotted against 7-AAD. **B**, summary histogram illustrating the proportion of cells in each phase of the cell cycle following 1 h treatment (mean \pm SD). **C**, summary histogram illustrating the percentage of BrdUrd-positive cells over time (mean \pm SD). **D**, γH2AX expression over time in MV4.11 in continuous culture with clofarabine.

MOLM-13 and MV4.11 express phosphorylated (constitutively activated) FLT3 (data not shown). We first established a time point and doses for early cell death induced by clofarabine. We initially cultured cells for 2, 4, and 6 h with up to 1 $\mu\text{mol/L}$ clofarabine, a dose that corresponds to clinically achievable plasma levels in AML patients (6, 23). We noted cell death at 6 h and established a dose for each cell line that will kill 10% to 30% of cells (data not shown). All the cells used undergo extensive damage with longer incubations (data not shown), but we deliberately chose doses that induce early cell death because a high amount of cell death can obscure the pathways used by the cell to deal with toxic insults.

As resistance to clofarabine has been associated with failure of cells to incorporate the drug, it was important for us to establish whether cells were damaged by exposure to clofarabine. H2AX is a histone variant that becomes phosphorylated on serine 139 early in the response to DNA damage (24, 25). (H2AX with phosphorylated serine 139 is also known as γH2AX .) γH2AX is important in the formation of a stable repair complex at the site of DNA damage (26) and dephosphorylation of γH2AX indicates repair (25). We pulsed cells with clofarabine for 1 h before measuring damage foci by immunocytochemistry using the H score (see Materials and Methods). The H score was 131 in MV4.11 cells and 69 in MOLM-13,

compared with 117 in KG1 and 107 in HL-60, indicating damage in all four cell lines (Fig. 1). We also pulsed cells with clofarabine for 1 h and measured γ H2AX after 2 h recovery in drug-free medium using flow cytometry. In FLT3 mutant MOLM-13 and MV4.11 cells, the γ H2AX positivity found in cells immediately after treatment had almost completely disappeared 2 h later (Fig. 2). In contrast KG1 cells, subjected to the same procedure, only lost 12% of γ H2AX fluorescence after 2 h recovery time. The HL-60 response was intermediate. These results indicate that the FLT3-ITD cell lines efficiently repair clofarabine-induced damage as assessed by the decrease of γ H2AX with time.

Clofarabine-induced reduction in DNA replication. Using BrdUrd and 7-AAD, we also measured the extent to which clofarabine inhibits DNA synthesis. We found that clofarabine reduced the proportion of cells synthesizing DNA by 91% after 1 h in KG1 cells and 80% in HL-60 but by only 1% in MOLM-13 and 9% in MV4.11 cells (Fig. 3A and B). After 1 h treatment, numerous BrdUrd-negative S-phase cells can be observed on the FACS plots of HL-60 and KG1, but not MOLM-13 or MV4.11 cells, indicating cell cycle arrest in S phase in the former but not the latter. Figure 3C shows that the continued presence of clofarabine over 3 h gradually reduces the proportion of BrdUrd-positive cells in the FLT3 mutant cell lines. This suggests that, over a prolonged period, these cells continue to synthesize DNA and therefore remain vulnerable to clofarabine incorporation. It seemed likely that, despite efficient repair processes, clofarabine-induced damage would eventually become overwhelming. We therefore monitored the proportion of γ H2AX-positive MV4.11 cells over time in the continued presence of clofarabine. After a nadir at 2 h continuous culture, the proportion of γ H2AX-positive cells starts to increase from 11% of total cells at 2 h to 44.5% at 6 h (Fig. 3D).

FLT3-cdc25A pathway in DNA synthesis. Aberrant activity of the cell cycle phosphatase cdc25a driven by mutant FLT3 could account for the defect in S-phase arrest in the ITD cells. Phosphorylation of cdc25A regulatory sites following DNA damage accelerates the proteolysis of cdc25A; thus, we would expect drugs that induce cell cycle arrest in S phase to induce a decrease in protein. We documented a clofarabine-induced decrease in cdc25A in the FLT3 wild-type cell lines following a 1 h exposure to clofarabine (Fig. 4A). This decrease was >50% weaker in MOLM-13 cells and was not observed in MV.11 cells. Moreover, we found that siRNA to FLT3 decreases cdc25A transcript expression in MV4.11 cells by 87.5% (Fig. 4B), suggesting that the protein levels might be sustained, at least in part, by aberrant transcription. These data combine to suggest aberrant expression of cdc25A in FLT3-ITD cells sufficient to support the continued cycling of damaged cells.

Primary cells with a FLT3-ITD are sensitive to clofarabine in continuous culture compared with wild-type samples. Finally, we asked whether the defect in cell cycle arrest in damaged MOLM-13 and MV4.11 cells would also be noted in samples from patients presenting with AML. We cultured 12 primary AML samples with clofarabine for 24 h (preliminary studies having shown no response at 6 h). Two samples were excluded from analysis because of high background apoptosis in untreated cells. Both of these samples had FLT3-ITDs, which was unsurprising because we have shown previously that this phenotype has heightened susceptibility to *in vitro* apoptosis (27). Three of 10 remaining samples had FLT3-ITDs and one

had a point mutation. The rate of proliferation in untreated cells was a major determinant of sensitivity to clofarabine ($P = 0.02$). Furthermore, in a subset of samples, we established, using γ H2AX, that clofarabine induces DNA damage (Fig. 5A).

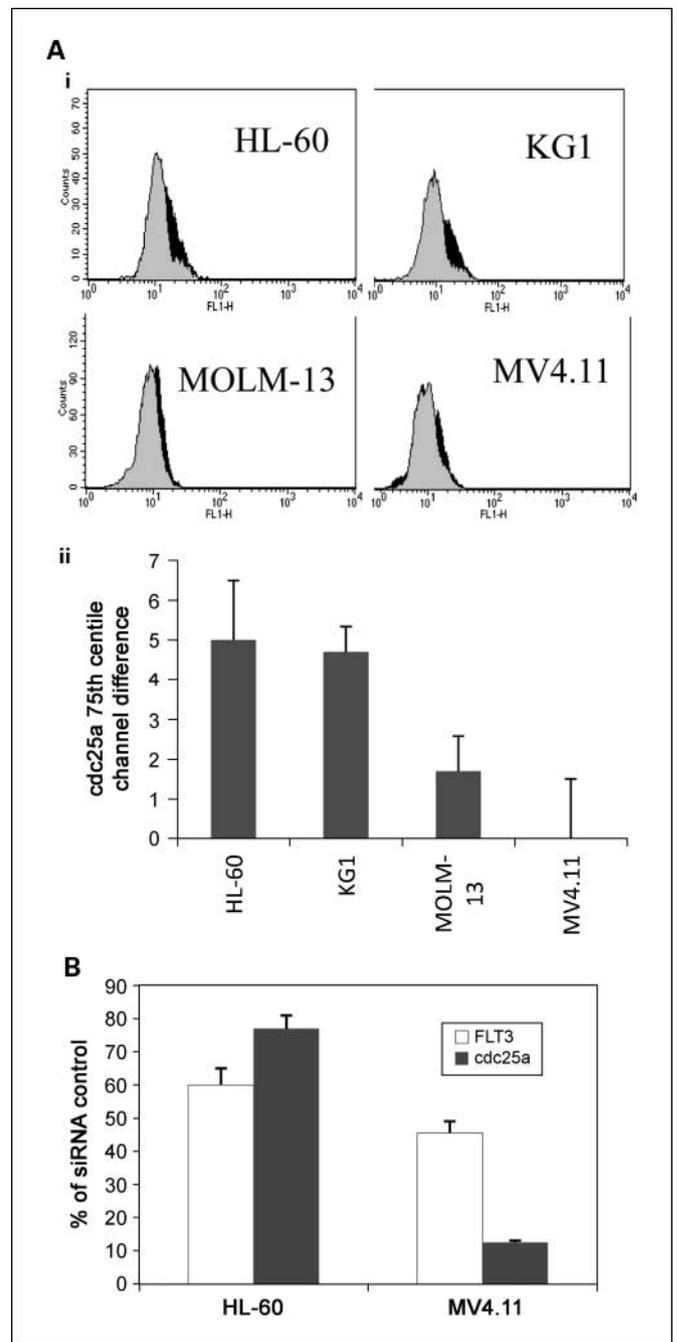


Fig. 4. cdc25 expression. *A*, Cdc25a expression by flow cytometry before (black histograms) and following (gray histograms) 1 h incubation with clofarabine. *i*, representative histograms; *ii*, as cdc25a is only detected in S and G₂-M phases by this method, the median channel difference between treated and untreated cells does not reflect any drug-induced change: this summary chart therefore documents the channel (fluorescence) difference at the 75th centile between treated and untreated cells (mean \pm SE of three experiments). *B*, effect of FLT3 siRNA inhibition on FLT3 and cdc25a message levels. Cdc25a and FLT3 message levels were measured 24 h after treating MV4.11 and HL-60 cells with siRNA to FLT3. Control cells were treated with nonspecific siRNA. Means and mean deviations of two experiments are shown.

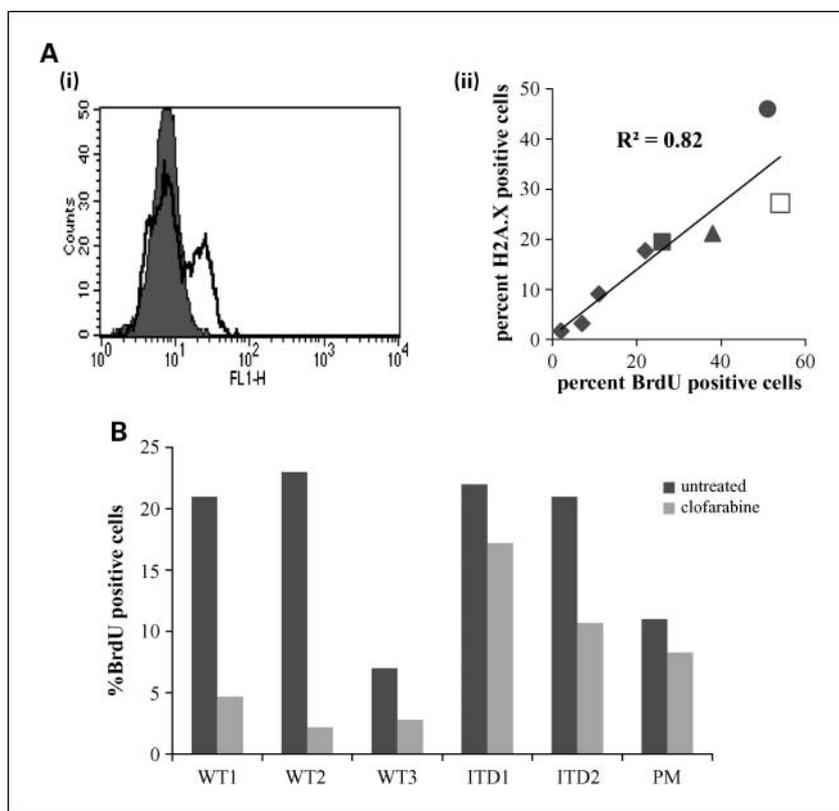


Fig. 5. Sensitivity of primary AML cells with and without mutant FLT3 to 0.3 μmol/L clofarabine. *A, i*, flow cytometric plot illustrating γH2AX expression in a clofarabine-treated AML patient sample (unfilled histogram) and in untreated cells (filled histogram); *ii*, scatter-plot indicating the percentage of BrdUrd incorporated and the percentage of γH2AX-positive cells (after 1 h for cell lines and 2 h for patient cells). Patient samples are indicated by filled diamonds, HL-60 cells by a circle, KG1 by a filled square, MOLM-13 by an unfilled square, and MV4.11 by a triangle. *B*, BrdUrd incorporation with and without 1 h exposure to 0.3 μmol/L clofarabine in individual patient samples: three wild-type samples (WT1-WT3), two ITD samples (ITD1 and ITD2), and one sample with a FLT3 D835Y point mutation (PM).

After 2 h clofarabine treatment, the percentage of γH2AX-positive cells was, in each case, only slightly less than the percentage of cycling cells at 1 h; that is, most, if not all, cycling cells accumulate clofarabine-induced damage. The concentration of clofarabine was not a limiting response determinant: there was no increased sensitivity to clofarabine when the dose was increased from 0.3 to 1 μmol/L (mean % cell death, 11.7% at 0.3 μmol/L and 9.8% at 1 μmol/L). These data combine to indicate that, at 0.3 μmol/L, clofarabine metabolites are able to saturate their targets in S-phase cells and induce DNA damage, but cells not in S phase are unaffected, even when the clofarabine concentration is increased. However, we also noted that the three FLT3 mutant samples with at least 5% BrdUrd positivity in untreated cells appeared particularly sensitive to clofarabine in this culture system (Table 1). To determine whether the

impaired cell cycle arrest in S phase observed in MOLM-13 and MV4.11 cells would also apply to patient cells harboring FLT3 mutations, we selected the six samples in which there was at least 5% BrdUrd positivity in untreated cells and measured BrdUrd inhibition by clofarabine after 1 h treatment. In the two FLT3-ITD samples, 21.9% and 49% BrdUrd inhibition was recorded; there was 24.3% inhibition in the sample with a point mutation (Fig. 5B), whereas, in the wild-type samples, BrdUrd was inhibited by 60.3%, 77.5%, and 90.5%. The difference in BrdUrd inhibition between the three wild-type and the three mutant samples was statistically significant ($P = 0.05$). The rapid S-phase inhibition found in primary FLT3 wild-type cells could account for their protection from clofarabine in prolonged culture in contrast to the impaired S-phase inhibition in the sensitive FLT3 mutant cells.

Table 1. Response of primary AML cells to clofarabine

Trial no.	Sample	% Blasts*	% Loss of viable cells (0.3 μmol/L clofarabine)	% BrdUrd-positive cells before treatment	FLT3 status
9452	Bone marrow	91	46	22	ITD
9437	Peripheral blood	92	7	2	Wild-type
9436	Peripheral blood	84	0	7	Wild-type
9444	Peripheral blood	82	18	11	D835Y
9425	Peripheral blood	86	9	21	Wild-type
9416	Bone marrow	64	8	23	Wild-type
9397	Peripheral blood	82	25	21	ITD
9373	Peripheral blood	82	0	1	Wild-type
9356	Peripheral blood	87	4	2	Wild-type
9315	Peripheral blood	85	0	4	ITD

*The percentage of blasts was ascertained flow cytometrically as CD45 intermediate/side-scatter low cells.

Discussion

We have shown previously that AML cells with FLT3-ITDs mount an effective repair response on treatment with daunorubicin (10). Despite this, the poor risk FLT3-ITD phenotype is associated with an enhanced relapse risk rather than impaired remission rate (3, 4) and there is no evidence that the phenotype contributes to primary chemoresistance in randomized trial patients. The initial focus of this study was to analyze heterogeneity in the DNA damage response to clofarabine in AML, and we used the γ H2AX reporter assay to show that clofarabine induces DNA damage and that FLT3 mutant cells are particularly efficient at repairing the damage following a short exposure. However, sensitivity to S-phase drugs is likely to depend, at least in part, on the cycle status of the cell, and we therefore started measuring BrdUrd and uncovered the marked difference between ITD and wild-type cells in their ability to arrest in S phase following clofarabine treatment.

We found that downregulation of FLT3-ITD by siRNA reduced cdc25A message ~10-fold in the mutated MV4.11 cell line. Cdc25A is a key regulator of cell cycle progression. Cdc25A inactivation through Chk1 mediates rapid cell cycle arrest that is independent of p53 and does not require protein synthesis (28). MV4.11 cells express wild-type p53, and a p53-dependent pathway of cell cycle arrest in S phase has recently been described, mediated by p21 and subsequent downregulation of cdc25A at the transcriptional level (29). However, we did not find that clofarabine downregulated cdc25A message in MV4.11 cells (data not shown). It is interesting that cdc25A is the key effector molecule on which the different S-phase pathways converge. In this context, the cdc25A-null phenotype is embryonically lethal and the (+/-) embryonic fibroblast shows reduced ability to undergo oncogenic transformation (30, 31).

We were motivated to investigate cdc25A because its expression is driven by c-myc (32), which is known to be aberrantly upregulated in FLT3-ITD cells (33). However, this might not be the relevant pathway: it is also of note that Pim-1, another major transcriptional target of FLT3-ITDs (34), is reported to activate cdc25A (35), although the mechanism is unclear

(36). Recently, a phosphoinositide 3-kinase inhibitor has been used to downregulate cdc25A expression in Ba/F3 cells expressing a FLT3-ITD (37). Thus, there may be more than one pathway by which FLT3-ITDs upregulate cdc25A expression.

We have shown that the sensitivity of FLT3 mutant cells to clofarabine in continuous application of drug follows on from the failure to inhibit DNA synthesis, which thus prolongs the time over which drug can be incorporated into DNA. Data from primary cells studied *in vitro* backed up this finding, but now the exact kinetics of abnormal proliferation in the face of DNA-damaging agents needs to be worked out in cells taken from patients undergoing treatment, as knowledge of these kinetics might be used for therapeutic advantage in terms of length of infusion or timing combinations of drugs that are thought to be synergistic: there is a lot of interest in clinically effective combinations involving clofarabine, and a trial of clofarabine with cytarabine has recently been reported (9).

Finally, there is no reason to assume that mutant FLT3 is unique in activating cdc25A in leukemia cells or that clofarabine has unique effects. Findings analogous to our own have been found in other systems; for example, cdc25A depletion after cytarabine treatment was found to be dependent on another component of the pathway (rad9), such that rad9^{-/-} mouse embryonic stem cells show increased sensitivity to cytarabine (38). While this article was being prepared, an article was published indicating that NPM/ALK and BCR/ABL as well as FLT3-ITD are associated with constitutive expression of cdc25A (37). Our study thus may provide a paradigm for exploring the effect of other activating mutations on the S-phase checkpoint response to a range of S-phase drugs.

Disclosure of Potential Conflicts of Interest

M. Pallis received a grant from Bioenvision for research on clofarabine. The other authors disclosed no potential conflicts of interest.

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