Synergism between Etoposide and 17-AAG in Leukemia Cells: Critical Roles for Hsp90, FLT3, Topoisomerase II, Chk1, and Rad51

Qing Yao,1 Brenda Weigel,2 and John Kersey1,2

Abstract  Purpose: DNA-damaging agents, such as etoposide, while clinically useful in leukemia therapy, are limited by DNA repair pathways that are not well understood. 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG), an inhibitor of the molecular chaperone heat shock protein 90 (Hsp90), inhibits growth and induces apoptosis in FLT3+ leukemia cells. In this study, we evaluated the effects of etoposide and 17-AAG in leukemia cells and the roles of Hsp90, FMS-like tyrosine kinase 3 (FLT3), checkpoint kinase 1 (Chk1), Rad51, and topoisomerase II in this inhibition.

Experimental Design: The single and combined effects of 17-AAG and etoposide and the mechanism of these effects were evaluated. FLT3 and the DNA repair-related proteins, Chk1 and Rad51, were studied in small interfering RNA (siRNA) – induced cell growth inhibition experiments in human leukemia cells with wild-type or mutated FLT3.

Results: We found that etoposide and the Hsp90/FLT3 inhibitor 17-AAG, had synergistic inhibitory effects on FLT3+ MLL-fusion gene leukemia cells. Cells with an internal tandem duplication (ITD) FLT3 (Molm13 and MV4;11) were more sensitive to etoposide/17-AAG than leukemias with wild-type FLT3 (HPB-Null and RS4;11). A critical role for FLT3 was shown in experiments with FLT3 ligand and siRNA targeted to FLT3. An important role for topoisomerase II and the DNA repair-related proteins, Chk1 and Rad51, in the synergistic effects was suggested from the results.

Conclusions: The repair of potentially lethal DNA damage by etoposide in leukemia cells is dependent on intact and functioning FLT3 especially leukemias with ITD-FLT3. These data suggest a rational therapeutic strategy for FLT3+ leukemias that combines etoposide or other DNA-damaging agents with Hsp90/FLT3 inhibitors such as 17-AAG.

Combination chemotherapy remains the primary treatment modality for cancer patients. DNA-damaging agents, such as etoposide, are currently in clinical use for therapy of leukemia. Etoposide, a topoisomerase II inhibitor, induces double strand breaks (DSB) during DNA replication (1). DSBs can initiate a strong proapoptotic signal when damaged DNA is left unrepaired as cell survival relies on the efficiency of DNA repair (2). To prevent DNA damage – induced apoptosis, the breaks must be repaired by different components of the DNA repair machinery (3, 4). In proliferating cells, homologous recombination DNA repair (HRR) predominates, whereas quiescent cells use nonhomologous end joining (5). The major enzymatic component of HRR in eukaryotic cells is Rad51, as cells deficient in Rad51 accumulate DSBs after replication or at stalled replication forks (6). Etoposide induces increased expression of Rad51 (6). The level of the Rad51 protein positively correlates with cellular etoposide resistance, and overexpression of Rad51 confers etoposide resistance (7). Checkpoint kinase 1 (Chk1) is an important regulator of genome maintenance by the HRR system. Chk1 interacts with Rad51; Chk1-depleted cells fail to form Rad51 nuclear repair foci after hydroxyurea-induced DSBs and inhibition of DNA replication (8). Chk1 can also be activated by a number of replication inhibitors, including etoposide (9–13). Chk1 gene deletion sensitizes cells to DNA replication inhibitors (13, 14–16). Collectively, it is possible that disrupting Chk1-Rad51 signaling might enhance etoposide cytotoxicity in tumor cells.

FMS-like tyrosine kinase 3 (FLT3), a receptor tyrosine kinase with an important role in early hematopoiesis, is expressed and often mutated in human leukemias. Patients with acute myelogenous leukemia (AML) harboring internal tandem duplications (ITD) of the FLT3 receptor have a poor prognosis compared with patients lacking such mutations. The ITD mutations of FLT3 occur in ~30% of de novo AML (17, 18), including many of the subgroup of AML that also have MLL fusion genes (19, 20). We and others have previously shown that FLT3 is a client of heat shock protein 90 (Hsp90) (20, 21). The Hsp90 complex is inhibited by the benzoquinone ansamycin antibiotic, geldanamycin and its derivatives, including 17-(allylamino)-17-demethoxygeldanamycin (17-AAG; refs. 22, 23). Cells with ITD-FLT3 are particularly sensitive to the geldanamycin family of Hsp90 inhibitors. 17-AAG has entered clinical trials in patients with solid tumors (23, 24) and leukemia, including those with ITD-FLT3.3

Authors’ Affiliations: 1The Cancer Center, University of Minnesota MMC 806, 420 Delaware St. SE, and 2 Division of Pediatric Hematology-Oncology and Bone Marrow Transplantation, University of Minnesota, Minneapolis, Minnesota

Received 7/18/06; revised 10/18/06; accepted 10/19/06.

Grant support: National Cancer Institute (CA087053) (J. Kersey) and the Children’s Cancer Research Fund (J. Kersey).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: John H. Kersey, MMC 806, 420 Delaware St. SE, Minneapolis, MN 55455. Phone: 612-625-4659; Fax: 612-626-3069; E-mail: kerse001@umn.edu.

©2007 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-06-1750

3 Unpublished data.
Previous research has shown an important role for other receptor tyrosine kinases, the epidermal growth factor receptor (EGFR; refs. 25–27) and the insulin-like growth factor receptor (IGF-IR; refs. 28, 29) in DNA repair. In the present studies, we found that MLL-fusion gene leukemia cell lines with ITD-FLT3 had reduced cellular levels of the DNA repair proteins Chk1 and Rad51 and were more sensitive to etoposide in cell growth experiments than leukemias with wild-type FLT3 (HPB-Null and RS4;11). Furthermore, etoposide, in combination with the Hsp90/FLT3 inhibitor 17-AAG, had synergistic inhibitory effects in FLT3+ cells. These studies raise the possibility that DNA repair may be dependent on FLT3 in FLT3+ leukemia cells. In this study, we used small interfering RNA (siRNA) targeted to FLT3, Chk1, and Rad51 and showed a critical role for each of these molecules in the synergistic effects of etoposide and the Hsp90/FLT3 inhibitor, 17-AAG.

### Materials and Methods

**Reagents.** 17-AAG was provided by the Developmental Therapeutics Program of the National Cancer Institute. Both 17-AAG and etoposide were dissolved in DMSO to a 10 mmol/L stock solution. “SMARTpool” for human FLT3, a combination of four synthetic siRNA duplexes each of which was designed to reduce mRNA for human FLT3, human Chk1, human Rad51, and nonsilencing negative control siRNA, were obtained from Dharmacon (Lafayette, CO).

**Cell culture and cell proliferation assay.** Human leukemia cell lines, MV4;11, Molm13, HPB-Null, and RS4;11 were described previously (20). MV4;11 and Molm13 have previously been found to have ITD-FLT3 mutations, whereas RS4;11 and HPB-Null have wild-type FLT3 as shown in Table 1. The cell lines were maintained in RPMI 1640 containing 10% fetal bovine serum. For cell proliferation assays using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, cells were seeded in replicates of six at a density of $1 \times 10^5$.

### Table 1. Inhibition of proliferation of leukemia cell lines by 17-AAG and etoposide correlates with mutation/expression of FLT3

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Lineage</th>
<th>FLT3 status</th>
<th>MLL fusion gene</th>
<th>IC$_{50}$ of 17-AAG (nmol/L)</th>
<th>IC$_{50}$ of etoposide (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molm13</td>
<td>Myeloid</td>
<td>ITD</td>
<td>Yes</td>
<td>31 ± 3</td>
<td>38 ± 9</td>
</tr>
<tr>
<td>MV4;11</td>
<td>Myeloid</td>
<td>ITD</td>
<td>Yes</td>
<td>40 ± 2</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>RS4;11</td>
<td>Pro-B</td>
<td>Wild-type</td>
<td>Yes</td>
<td>700 ± 52</td>
<td>102 ± 19</td>
</tr>
<tr>
<td>HPB-NULL</td>
<td>Pro-B</td>
<td>Wild-type</td>
<td>No</td>
<td>470 ± 36</td>
<td>73 ± 8</td>
</tr>
<tr>
<td>THP-1</td>
<td>Myeloid</td>
<td>Wild-type</td>
<td>Yes</td>
<td>800 ± 48</td>
<td>855 ± 58</td>
</tr>
<tr>
<td>KM3</td>
<td>Myeloid</td>
<td>Wild-type</td>
<td>No</td>
<td>2200 ± 164</td>
<td>124 ± 17</td>
</tr>
<tr>
<td>IE8</td>
<td>Pre-B</td>
<td>Negative</td>
<td>No</td>
<td>2100 ± 196</td>
<td>111 ± 24</td>
</tr>
<tr>
<td>U937</td>
<td>Myeloid</td>
<td>Negative</td>
<td>No</td>
<td>4500 ± 321</td>
<td>11538 ± 458</td>
</tr>
</tbody>
</table>

Abbreviation: ITD; FLT3 internal tandem duplication mutation.

**Fig. 1.** Combination effects of 17-AAG and etoposide in human leukemia cell lines. The median effects of drug combination for MV4;11, Molm13, RS4;11 and HPB-Null are shown. Cells were incubated with 17-AAG, etoposide, or both for 72 h, and proliferation was assessed using MTT assays. The experiments were repeated at least thrice.
cells/mL in 96-well plates in the presence or absence of various concentrations of 17-AAG and etoposide. Plates were incubated for the indicated hours at 37°C, 5% CO₂, and proliferation was measured using CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay (Promega, Madison, WI). Results are reported as means ± SE for each point on the curve.

Transfection with siRNA. The cells were washed twice and resuspended in OptiMEM medium (Invitrogen, San Diego, CA). Five million cells were then mixed with 10 μg of siRNA duplexes. Electroporation was done using a BTX Electro Square Porator ECM830 (BTX, Holliston, MA) with one pulse at 240 V. After electroporation, cells were immediately mixed with RPMI 1640 containing 10% fetal bovine serum. Cell growth was calculated with the viable cell numbers detected by trypan blue exclusion test.

Western blotting. Cells were lysed by sample buffer [62.5 mmol/L Tris (pH 7.4), 2% SDS, 10% glycerol, and the protease inhibitor “Complete Mini” (Roche, Indianapolis, IN)], and the cell lysate was clarified by centrifugation. About 20 μg of cell lysate of each sample were electrophoresed by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with different antibodies. Antibodies were purchased as follows: Hsp90 and Hsp70 from BD PharMingen (San Diego, CA); FLT3, Chk1, Rad51, and poly(ADP-ribose) polymerase (PARP) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); p-Chk1 (S317) from Upstate Biotechnology, Inc. (Lake Placid, NY); and actin from Sigma (St. Louis, MO). For detection, the blots were incubated with horseradish peroxidase–conjugated anti-immunoglobulin G antibody (Promega) and developed using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Arlington Heights, IL). The densitometric analysis was done using Molecular Analyst (Bio-Rad, Hercules, CA).

Analysis of combined effects of drugs. Each of the cell lines were simultaneously exposed to 17-AAG and etoposide for 72 h, and inhibition of cell proliferation was determined by CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay. To calculate combined drug effects, the combination index (CI) method was used. The CI values were determined at different effect levels of growth inhibition as a quantitative measure of the degree of drug interaction using the computer software CalcuSyn (BioSoft, Ferguson, MO). Combined drugs were used at fixed molar ratios. The drug doses that produced about 50% of growth inhibition in single drug experiments were chosen to determine an appropriate fixed molar ratio of two combined drugs.

Detection of apoptosis. Cells cultured under the various conditions were harvested and labeled using the CaspaTag caspase activity kit (Serologicals Corporation, Norcross, GA). Briefly, harvested cells were incubated with FAM-VAD-FMK that irreversibly binds to caspases-1 to caspase-9. After two washes, propidium iodide (PI) was added, and cells were analyzed on a FACS Calibur flow cytometer.
Cell cycle analysis. One million cells were suspended in 1 mL solution containing 50 mg/mL PI, 0.1% sodium citrate, and 0.1% Triton X-100. The PI-stained samples were analyzed within 24 h. The cell cycle distribution and apoptosis were determined by the analysis of nuclear DNA content using CellQuest-Pro software (Becton Dickinson, Mountain View, CA).

Results

17-AAG and etoposide are synergistic in the inhibition of cell proliferation of cell lines expressing FLT3, especially in cells with mutated FLT3. Our initial studies addressed the relationship between FLT3 mutation and sensitivity to 17-AAG and etoposide. We examined human leukemia cell lines for expression of FLT3 and inhibition of cell growth by 17-AAG and etoposide using MTT cell proliferation assay. Results are shown in Table 1. Inhibition by 17-AAG as reported in our previous study shows greatest sensitivity of cells with mutated FLT3 and least sensitivity of cells that lack FLT3 (20). Cell lines expressing ITD-FLT3 also had the greatest sensitivity to etoposide. The IC50s of etoposide for Molm13 and MV4;11 cells were 38 and 46 nmol/L, respectively. The cell lines expressing wild-type FLT3 showed intermediate sensitivity to 17-AAG and etoposide. Cell lines that were FLT3 negative were very resistant to 17-AAG.

To determine the combination effect of 17-AAG and etoposide, we compared the cells that express ITD-FLT3 with cells that express wild-type FLT3. Each of the cell lines were exposed to varying concentration of 17-AAG and etoposide at a fixed molar ratio for 72 h, and inhibition of cell proliferation was determined. CIs were plotted against fractional effects as described in Materials and Methods. CI values significantly <1 indicate synergy; values close to 1 indicate an additive effect; and values significantly >1 indicate an antagonistic effect of the two agents.

As seen in Fig. 1, 17-AAG and etoposide synergistically inhibited cell proliferation of ITD-FLT3 cells (MV4;11 and

Fig. 3. FLT3 siRNA and/or etoposide mediated inhibition on cell growth of MV4;11 cells. A, Western blot analysis showed that protein expression levels in MV4;11 cells transfected with control siRNA, 50, 100, 200, and 400 pmol FLT3 siRNA after 24 h. B, cell growth of MV4;11 cells after transfected with control siRNA, 50, 100, 200, and 400 pmol FLT3 siRNA at days 0, 1, 2, and 3. C, effect of etoposide on cell growth of MV4;11 cells transfected with control siRNA and 400 pmol FLT3 siRNA after 72 h. The experiments were repeated at least thrice.

Cancer Therapy: Preclinical
Synergistic Effects of Hsp90 Inhibitor and Etoposide

The role of intact Hsp90 in control of Chk1 and Rad51 and growth of cells with wild-type FLT3. We initiated a series of experiments to evaluate the role of Hsp90/FLT3, Chk1, and Rad51 in cells that were stimulated to proliferate by FLT3 ligand (FL). Cells with wild-type FLT3 (RS4;11) proliferate more in the presence of FL than the cells without FL. FLT3 siRNA inhibits cell proliferation induced by FL (Fig. 2A). At 6 h after FL stimulation, phosphorylation of both FLT3 and Chk1 were inhibited by FLT3 siRNA (Fig. 2B). The early effects induced by FL on Chk1 suggest that the phosphorylation of Chk1 may possibly be directly downstream of phosphorylated FLT3. At 48 h after FL stimulation, the phosphorylation of Chk1 is enhanced more than that at 6 h (Fig. 2D). Etoposide inhibited cell growth as shown in Fig. 2C, but did not significantly affect phosphorylation of Chk1, expression of Rad51 or total FLT3 as shown in Fig. 2D. In contrast, the Hsp90/FLT3 inhibitor, 17-AAG, which inhibited cell growth to the same extent as etoposide significantly reduced the total amount of FLT3, Chk1, and Rad51. Cell growth was most inhibited by the combination of 17-AAG and etoposide (Fig. 2C). This was reflected in more apoptosis as an increase of the cleaved PARP (Fig. 2D).

In conclusion, in cells with wild-type FLT3, FL induced phosphorylation of FLT3, Chk1, and expression of Rad51. In these cells with wild-type FLT3, 17-AAG but not etoposide resulted in down-regulation of Chk1 and Rad51. The combination of 17-AAG and etoposide resulted in the greatest inhibition of cell growth and apoptosis.

The role of FLT3 and Hsp90 in survival of ITD-FLT3 cells. As shown in Table 1 and Fig. 1, cells with mutated FLT3 were found to be more sensitive to 17-AAG and etoposide than cells with wild-type FLT3. Thus, we evaluated the mechanism of synergism between 17-AAG and etoposide in cells that have mutated FLT3. Cells with ITD-FLT3 have constitutive activation of FLT3. We did FLT3 knockdown to evaluate its role in cell survival. ITD-FLT3 (MV4;11) cells were transfected with FLT3 siRNA as described in Materials and Methods. Small RNAs reduced FLT3 expression by 84% by densitometry at 24 h (Fig. 3A). In addition, as shown in Fig. 3A, FLT3 siRNA resulted in a reduction of p-AKT, total and phosphorylated Chk1, and total Rad51, but had no effects on total AKT, Hsp90, or Hsp70. Cell growth experiments showed that FLT3 siRNA inhibited cell growth in a dose-dependent manner (Fig. 3B), and apoptosis was shown by increased PARP in Fig. 3A. FLT3 siRNA, combined with etoposide, induced additional inhibition of cell growth in MV4;11 cells (Fig. 3C). In summary, these experiments show that tumor cell growth is critically dependent on FLT3 signaling in leukemia cells with ITD-FLT3.

We next conducted experiments to further evaluate the mechanism of the synergism between the Hsp90 inhibitor 17-AAG and etoposide in ITD-FLT3 cells, with emphasis on DNA repair and cell signaling pathways. As shown in Fig. 4, a marker of DSBs, γ-H2A.X (30, 31), was induced by 17-AAG or etoposide and more by the combination. Of interest, cells incubated with the Hsp90 inhibitor, 17-AAG, had significantly less phosphorylation of Chk1, expression of Rad51 or total AKT, Hsp90 and Cdc37 were also analyzed using Western blotting. Hsp90 and Cdc37 were not significantly altered by 17-AAG alone or in combination with etoposide. As reported in earlier studies (20, 32) Hsp70 was not inhibited by 17-AAG. It is known that topoisomerase IIα is increased at G2-M arrest induced by etoposide treatment (33), and this was the case in our experiments as shown in Fig. 4. In contrast, topoisomerase IIα was inhibited by 17-AAG alone and in combination with

![Fig. 4. Combinations of 17-AAG and etoposide down-regulate DNA topoisomerase (Topo IIα). DNA repair proteins (Chk1 and Rad51), signal transduction kinases (FLT3 and AKT) and induced more apoptosis (cleavage of PARP) than either agent alone. Western blot analysis of proteins in cell lysate from MV4;11 cells after treatment of 200 nmol/L 17-AAG and 200 nmol/L etoposide alone and in combination for 0, 24, and 48 h is shown.](www.aacrjournals.org)
etoposide. Thus, clearly, 17-AAG abolished the increase of topoisomerase IIα induced by etoposide.

In summary, these results from Western blot analysis show that 17-AAG results in reduced total levels of Chk1 and Rad51, both important in DNA repair following etoposide-induced DNA damage. As an end result, the combination of 17-AAG and etoposide resulted in more unrepaired DNA damage, and this combined with reduced topoisomerase IIα most likely explains...

Fig. 5. Effects of 17-AAG or etoposide alone and the combination of two agents on cell cycle distribution and apoptosis for ITD-FLT3 leukemia cell lines. A, MV4;11 cells were treated with 200 nmol/L 17-AAG or 200 nmol/L etoposide alone and the combination of two agents. Cell cycle arrest was determined for 0, 1, and 2 days with PI staining and fluorescence-activated cell sorting analysis. B, MV4;11 cells were treated with 200 nmol/L 17-AAG or 200 nmol/L etoposide alone and the combination of two agents for 48 h. Apoptosis was determined with PI/Caspatag staining and fluorescence-activated cell sorting analysis. The percentage of cells: bottom left, viable cells; top right, caspase/PI-positive dead cells; bottom right, caspase-positive cells. The experiments were repeated at least thrice.
the inhibition of cell growth and increased apoptosis resulting from the combination of the two agents.

We determined in more detail the effect of 17-AAG and etoposide on cell cycle and apoptosis of ITD-FLT3 cells using MV4;11 cells. As shown in Fig. 5A, 17-AAG resulted in the accumulation of cells in G0-G1. After treatment with 200 nmol/L 17-AAG for 48 h, MV4;11 cells were arrested in G0-G1 phase from a baseline of 71% to 88%. S-phase cells decreased from a baseline of 22% to 10%, and <2N portion (DNA fragmentation) cells were increased from 24% to 33%. In contrast to 17-AAG, etoposide (200 nmol/L) induced more cell arrest in G2-M phase (8% to 34%), decreased DNA synthesis (S phase) from 22% to 12%, and induced more <2N portion cells. Interestingly, the combined treatment had the greatest effect in decreasing S phase from cells and induced more <2N cells than those treated with 17-AAG or etoposide alone. We also used CaspaseTag/PI assay to compare the induction of apoptosis by the individual agents and the 17-AAG–etoposide combination. Consistent with the PARP and <2N studies described earlier, more caspase activity was detected in cells treated with the drug combination than in cells treated with either drug alone. As shown in Fig. 5B, after treatment with 17-AAG for 48 h, early apoptotic (CaspaseTag positive/PI negative) cells increased, late apoptotic (CaspaseTag/PI double positive) cells increased and necrotic (CaspaseTag negative/PI positive) cells increased compared with control cells. With etoposide treatment, early apoptotic cells increased, late apoptotic cells increased, and necrotic cells were increased. Combination treatment resulted in increased early apoptotic cells, increased late apoptotic cells, and increased necrotic cells compared with control cells. Thus, more apoptotic cells were observed with the combination of 17-AAG and etoposide than either agent alone. A second ITD-FLT3 cell line, Molm 13, showed very similar effects of 17-AAG and etoposide in combination (data not shown).

**Etoposide increases the inhibition of cell growth induced by Chk1 or Rad51 depletion.** Next, we tested the effect of Chk1 inhibition on cell growth and Rad51 expression. Transfection of Chk1 siRNA into ITD-FLT3 MV4;11 cells resulted in a 79% reduction of Chk1 based on densitometry (Fig. 6A). Chk1 siRNA reduced the level of Rad51 (Fig. 6A), inhibited cell growth (Fig. 6B), and induced cleavage of PARP (Fig. 6A) in a dosage-dependent manner. More inhibition of cell growth was induced by the combination of Chk1 siRNA and etoposide than etoposide alone in MV4;11 cells as shown in Fig. 6C.
Rad51 siRNA resulted in inhibition of cellular Rad51 (Fig. 7A shows an 87% reduction based on densitometry). Cell growth was inhibited in a dosage-dependent manner by Rad51 siRNA (Fig. 7B). As shown in Fig. 7A, Rad51 siRNA had no effects on the expression of FLT3, Chk1, Hsp90, or Hsp70. There was an increase in the cleavage of PARP by Rad51 siRNA. In addition, the growth of the cells transfected with Rad51 siRNA were inhibited more by etoposide than the control cells (Fig. 7C).

Expression levels of Chk1 and Rad51 differ in ITD-FLT3 and wild-type FLT3 leukemia cells. Because the experiments above showed differences between ITD-FLT3 and wild-type FLT3 in effects of the Hsp90 inhibitor, 17-AAG, and etoposide, we looked for baseline differences between wild-type FLT3 and ITD-FLT3.

Fig. 7. Rad51 siRNA and/or etoposide mediated inhibition on cell growth of MV4;11 cells. A, Western blot analysis showed that protein expression levels in MV4;11 cells transfected with control siRNA, 100, 200, 400, and 800 pmol Rad51 siRNA after 24 h. B, cell growth of MV4;11 cells after transfected with control siRNA, 100, 200, 400, and 800 pmol Rad51 siRNA at days 0, 1, 2, and 3. C, effect of etoposide on cell growth of MV4;11 cells transfected with control siRNA and 800 pmol Rad51 siRNA after 72 h. D, expression levels of Chk1 and Rad51 in Molm13, MV4;11, HPB-Null, and RS4;11 leukemia cell lines. The experiments were conducted in triplicates.
ITD-FLT3 cells. In these experiments, Western blotting was used to determine the quantity of the DNA repair-associated proteins Chk1 and Rad51 in cells. As shown in Fig. 7D, Chk1 and Rad51 were found at higher levels in etoposide/17-AAG–insensitive wild-type FLT3 (HPB-Null and RS4;11) cells than in etoposide/17-AAG–sensitive ITD-FLT3 cells (Molm13 and MV4;11). In addition, both wild-type FLT3 HPB-Null and RS4;11 showed fewer <2N apoptotic or necrotic cells (3.6% and 5.2%, respectively) than ITD-FLT3 Molm13 and MV4;11 cells (17.3% and 22.1%, respectively) in a representative experiment.

References

24. Workman P. Auditing the pharmacological accounts

Discussion

Results from this study provide evidence that leukemia cell death produced by the topoisomerase II inhibitor, etoposide, and the specific inhibitor of Hsp90, 17-AAG, are synergistic. Importantly, the sensitivity and synergism between these two agents is greatest in cells with mutated FLT3. Previously, we and others reported that both ITD-FLT3 and wild-type FLT3 are client proteins of Hsp90, and treatment with Hsp90 inhibitors, 17-AAG or herbimycin, resulted in depletion of both proteins (20, 21, 32). The FLT3 inhibitor, GTP14564, was previously found to have a synergistic effect in both ITD-FLT3 and wild-type FLT3 cells (34). These and other data suggest that FLT3 is potentially an important target for leukemia therapy.

In the current research, we first studied the mechanisms of the combined effects of etoposide and inhibition of FLT3 in cells with wild-type FLT3. We show for the first time that wild-type FLT3 RS4;11 leukemia cells are dependent on FLT3 ligand for optimal growth and survival. We also used siRNA to specifically examine the role of wild-type FLT3 in RS4;11 cells (Fig. 2A and B). Small RNAi uses double-stranded RNA complexes to target specific genes for silencing. A short synthetic duplex RNA long enough to induce gene-specific silencing but short enough to evade host responses was used (35). We used transient transfection with siRNA to knock down FLT3 gene expression. FLT3 siRNA significantly inhibited cell proliferation in cells with wild-type FLT3 (Fig. 2A).

Etoposide is a potent inducer of DSBS and is clinically highly effective in AML therapy. ITD mutations of FLT3 are found in about 30% of cases of de novo AML. The mechanisms of ITD-FLT3 and FLT3 have been explored, and agents capable of inhibiting FLT3 kinase have been developed and display promising preclinical activity (36–44). The combinations of FLT3 inhibitor with other conventional chemotherapies are likely to improve the outcomes of patients with AML (45, 46).

Chk1 is an essential checkpoint kinase that is required for cell cycle delays after DNA damage or blocked DNA replication (47, 48). Chk1 can directly interact with Rad51, and Rad51 is phosphorylated in a Chk1-dependent manner. Depletion of Chk1 results in failed Rad51 nuclear foci formation (8). We observed that depletion of Chk1 also reduced Rad51 expression. In this study, we also found that leukemia cells with wild-type FLT3 express higher levels of Chk1 and Rad51 than ITD-FLT3 cells. This might partially explain why wild-type FLT3 cells have less sensitivity to etoposide because Chk1 and Rad51 are proteins involved in DNA repair during DNA damage induced by etoposide. Our studies showed that Chk1 and Rad51 are down-regulated following FLT3 siRNA. These results are consistent with the previous reports that FLT3 inhibitor, CEP-701, induced a synergistic cytotoxicity with etoposide in ITD-FLT3 leukemia cells (45).

In conclusion, results in the present study show that the combination of 17-AAG and etoposide have synergistic inhibitory effects in ITD-FLT3 cells in vitro. A critical role for FLT3, topoisomerase II, Chk1, and Rad51 were shown in this study. The synergistic activity between the Hsp90 inhibitor, 17-AAG, and the DNA-damaging agent, etoposide, provides useful leads for the design of trials that combine these two distinct classes of agents in vivo.


Levis M, Pham R, Smith BD, Small D. In vitro studies of a FLT3 inhibitor combined with chemotherapy: sequence of administration is important to achieve synergistic cytotoxic effects. Blood 2004;104:1145–50.


Synergism between Etoposide and 17-AAG in Leukemia Cells: Critical Roles for Hsp90, FLT3, Topoisomerase II, Chk1, and Rad51

Qing Yao, Brenda Weigel and John Kersey


Updated version: Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/13/5/1591

Cited articles: This article cites 48 articles, 28 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/13/5/1591.full.html#ref-list-1

Citing articles: This article has been cited by 5 HighWire-hosted articles. Access the articles at:
/content/13/5/1591.full.html#related-urls

E-mail alerts: Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions: To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions: To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.