mTOR inhibitors are synergistic with methotrexate: an effective combination to treat acute lymphoblastic leukemia

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We have previously demonstrated that mTOR inhibitors (MTIs) are active in preclinical models of acute lymphoblastic leukemia (ALL). MTIs may increase degradation of cyclin D1, a protein involved in dihydrofolate reductase (DHFR) synthesis. Because resistance to mtorohetrexate may correlate with high DHFR expression, we hypothesized MTIs may increase sensitivity of ALL to methotrexate through decreasing DHFR by increasing turn-over of cyclin D1. We tested this hypothesis using multiple ALL cell lines and nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice xenografted with human ALL. We found MTIs and methotrexate were synergistic in combination in vitro and in vivo. Mice treated with both drugs went into a complete and durable remission whereas single agent treatment caused an initial partial response that ultimately progressed. ALL cells treated with MTIs had markedly decreased expression of DHFR and cyclin D1, providing a novel mechanistic explanation for a combined effect. We found methotrexate and MTIs are an effective and potentially synergistic combination in ALL. (Blood. 2008;112:2020-2023)

Introduction

Novel and less toxic treatment strategies are needed for patients with acute lymphoblastic leukemia (ALL).1 Previously, we have demonstrated that mTOR inhibitors (MTIs), a class of signal transduction inhibitors, are effective as single agents in preclinical models of ALL.2,3 Combination treatment is the next logical step in the therapeutic use of MTIs. It is important to choose rationally-designed combinations, building on an understanding of the mechanism of action of MTIs and interactions with other agents.

MTIs have been shown to prevent activation and increase degradation of cyclin-dependent kinases, including cyclin D1.4 Cyclin D1 is involved in Rb phosphorylation and release E2Fs which are involved in dihydrofolate reductase (DHFR) synthesis.5-7 Resistance to methotrexate has been shown in tumors that have high DHFR expression.8,9 We hypothesized that MTIs may increase the sensitivity of ALL to methotrexate by decreasing cyclin D1, which would in turn would decrease DHFR.3 We tested this hypothesis using relevant preclinical models.

Methods

In vitro drug testing using ALL cell lines

We used 9 previously characterized ALL cell lines for these experiments, including 4 marine ALL lines (289, 83, 420, and T309) and 5 human ALL lines (Nalm 6, Nalm16, CEM, Molt-4, and Jurkat; the phenotypes are listed in Table S1, available on the Blood website; see the Supplemental Materials link at the top of the online article).3 Cell lines were maintained in culture using published techniques.3 Cells were treated with chemotherapeutic agents, including sirolimus (Wyeth Pharmaceuticals, Philadelphia, PA), temsirolimus (Wyeth), methotrexate (Mayne Pharmaceuticals, Paramus, NJ), L-asparaginase (Merck, Whithouse Station, NJ), doxorubicin (Bedford Labs, Bedford, OH), vincristine (Mayne), dexamethasone (American Regent, Shirley, NY), cytarabine (American Pharmaceutical Partners, Schaumburg, IL), and etoposide (Sicor Pharmaceuticals, Irvine, CA). For each cell line, 1 to 3 × 10⁵ cells/mL were cultured and exposed to drug(s) in triplicate in at least 2 independent experiments. Cell inhibition was assessed using methylthiazoletetrazolium (MTT) and cell death and apoptosis were assessed using annexin-V and 7-amino-actinomycin D (7-AAD) staining as described.2,10 To determine whether combinations were additive, synergistic, or antagonistic we used Chou and Talalay median effects analysis to calculate a combination index (CI) using CalcuSyn v1 software (Biosoft, Cambridge, United Kingdom) as described.11

In vivo drug testing using NOD/SCID xenografts

Under Institutional Animal Care and Use Committee– and Institutional Review Board–approved protocols of the University of Pennsylvania and the Children’s Hospital of Philadelphia and informed consent obtained in accordance with the Declaration of Helsinki, we generated nonobese diabetic/severe combined immunodeficient (NOD/SCID) xenografts from 2 primary human ALL samples as described previously.2 Briefly, mice were randomized to treatment arms as described in “Methods.” The xenograft model was used to measure the activity of the combination treatment in vivo. Mice were killed, and tissues were harvested. Immunobots of harvested cells were performed, and relative protein densities were quantitated as described.2


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Results and discussion

MTIs can be effectively combined with cytotoxic chemotherapeutics

As an initial experiment and to demonstrate potential synergy between MTIs and chemotherapy agents, we tested one mTOR inhibitor, sirolimus, in combination with 7 cytotoxics with known activity in ALL in 3 ALL cell lines (289, 420, and Nalm6). All 3 lines were sensitive to every cytotoxic agent. MTIs had at least an additive effect when combined with methotrexate (MTX), dexamethasone (DEX), L-asparaginase (L-ASP), etoposide (VP-16), and doxorubicin (DOX). The combination of MTIs with vincristine (VCR) and Ara-C (cytarabine) did not add a benefit over either single agent alone. Doses depicted in panel A: sirolimus (0.3 ng/mL), MTX (5 nM), DEX (5 μM), L-ASP (1 μg/μL), VP-16 (1 nM), DOX (1 nM), VCR (1 nM), ARA-C (0.1 μg/mL).

Next, aliquots of cells from 9 ALL lines were treated with methotrexate and 2 MTIs (tepsiroimus (CCI) and sirolimus). (B) MTT data for tepsiroimus and methotrexate in 1 cell line (289), demonstrating a synergistic effect at multiple drug doses. The other cell lines tested showed similar results. (C) Chou and Talalay median effects analysis results for one representative cell line (289), showing a combination index (CI) less than 1 at ED50 (median effective dose to inhibit 50% of cells), ED75, and ED90. (D) A representative example of annexin-V and 7-AAD staining in one cell line (289), demonstrating a synergistic increase in cell death and apoptosis with combined treatment. Doses depicted in panel D: CCI (7.5 ng/mL) and MTX (12.5 nM). Error bars represent SD.

MTIs are synergistic in combination with methotrexate in vitro

Based on our hypothesis that MTIs and methotrexate are synergistic in combination, we tested the 2 clinically available mTOR inhibitors (sirolimus and temsirolimus) in combination with methotrexate using 9 different ALL cell lines. Figure 1B depicts results for 1 cell line (289), demonstrating a synergistic effect when combining methotrexate and temsirolimus as assessed by MTT. We found similar results with combinations of all drug doses in 7 of 9 cell lines using both MTIs. With 2 lines (Molt-4 and Jurkat) we found synergy in combination with all doses except using high doses of both drugs (> IC75). For 5 of the cell lines (289, 83, T309, Nalm 6, and CEM), we performed more extensive experiments and used Chou and Talalay median effects analysis to determine whether the combined effect(s) were mathematically synergistic. We found they were synergistic (CI < 1.0: range 0.33-0.98) in all 5 of the cell lines. Figure 1C depicts a representative example in 1 cell line (289). We assessed apoptosis using 4 cell lines (289, 420, Nalm 6, and CEM) with annexin V staining, comparing cells treated with combinations to individual agents. We found a marked and statistically significant (P < .05) increase in apoptotic (annexin-V-/7-AAD+) and dead (annexin-V+/7-AAD+) cells with combined treatment compared with either single agent. Figure 1D depicts a representative example in 1 cell line (289). These
results confirm that MTIs and methotrexate are synergistic in vitro models of ALL.

**MTIs and methotrexate produce a complete remission in xenografted ALL**

To determine whether MTIs and methotrexate are effective in combination in vivo, we generated xenografts from 2 primary human ALL patient samples (240 and 359). We randomized mice to treatment with temsirolimus 5 mg/kg 5 days a week, temsirolimus 20 mg/kg weekly, methotrexate 5 mg/kg weekly, or temsirolimus and methotrexate combined (with both temsirolimus schedules), after establishment of measurable disease (> 5% blasts in peripheral blood). All drugs were given intraperitoneally. Four to 9 mice were treated in each arm (67 total mice). We focused on the MTI temsirolimus because it is a parenteral agent, simplifying delivery in mice. Mice in all agent groups. The remaining mice were killed 2 months later, after relapse.

We hypothesized MTIs may increase sensitivity of ALL to methotrexate through decreasing DHFR via a decrease in cyclin D1. We treated ALL xenografts from 2 different patient samples (3 treated:3 control from sample 240 and 4 treated:4 control from sample 359) with temsirolimus at 5 mg/kg per day 5 days a week or vehicle control. Mice were exposed for various lengths of time (2 to 21 days) and killed in treatment:control pairs prior to disease relapse. Immunoblots for DHFR and cyclin D1 demonstrated decreased expression of DHFR (> 75%) in all treated animals relative to control animals and decreased expression of cyclin D1 (> 25%) in all treated animals but one over all time periods assessed (Figure 2C). Immunoblots for phospho-S6 were used to confirm down-regulation of a downstream target of mTOR as well (Figure 2C).

In conclusion, we have demonstrated a number of chemotherapeutic agents can be effectively combined with MTIs in ALL cells. We have demonstrated methotrexate and MTIs are synergistic in combination in vitro and in vivo through a novel mechanistic interaction. This work has led to a pilot clinical trial investigating the combination in adults with
relapsed ALL and suggests that this combination should be investigated in future multiinstitutional clinical trials for patients with relapsed/ refractory ALL.

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Authorship

Contribution: D.T.T. and S.A.G. designed the research and drafted the manuscript; V.I.B., M.C., J.F., and A.S. contributed to experimental design; D.T.T., J.H., T.R., Y.C., R.N., and C.S. performed research; D.T.T., S.A.G., M.C., and C.S. analyzed and interpreted data; D.T.T., and C.S. performed statistical analysis; and all authors were involved in critical revision of the manuscript.

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