Stage 2 Combination Testing of Rapamycin with Cytotoxic Agents by the Pediatric Preclinical Testing Program


Abstract

Rapamycin demonstrated broad-spectrum tumor growth inhibition activity against the in vitro panels of childhood tumors used in the Pediatric Preclinical Testing Program (PPTP). Here we have evaluated rapamycin combined with agents used frequently in the treatment of childhood malignancies. Rapamycin was tested in vitro against 23 cell lines alone or in combination with melphalan, cisplatin, vincristine, or dexamethasone (leukemic models only). In vivo, the impact of combining rapamycin with a cytotoxic agent was evaluated using two measures: 1) the therapeutic enhancement measure, and 2) a linear regression model for time-to-event to formally evaluate for sub- and supraadditivity for the combination compared to the agents used alone. Combining rapamycin with cytotoxic agents in vitro gave predominantly subadditive or additive effects, except for dexamethasone in leukemia models for which supra-additive activity was observed. In vivo testing demonstrated that therapeutic enhancement was common for rapamycin in combination with cyclophosphamide and occurred for 4 of 11 evaluable xenografts for the rapamycin and vincristine combination. The combinations of rapamycin with either cyclophosphamide or vincristine were significantly more effective than the respective standard agents used alone at their maximum tolerated doses (MTD) for most evaluable xenografts. The combination of rapamycin and cisplatin produced excessive toxicity requiring cisplatin dose reductions, and therapeutic enhancement was not observed for this combination. Addition of rapamycin to either cyclophosphamide or vincristine at their respective MTDs appears promising, as these combinations are relatively well tolerated and as many of the pediatric preclinical models evaluated demonstrated therapeutic enhancement for these combinations. Mol Cancer Ther; 9(1); 101–12. ©2010 AACR.

Introduction

Rapamycin inhibits the proliferation of many tumor cell lines in vitro including cell lines derived from childhood cancers (1, 2), and showed significant antitumor activity against syngeneic tumor models in the NCI in vitro screening program (3), and against childhood cancer xenografts (2). In our previous study rapamycin induced significant differences in event free survival (EFS) distribution in 27 of 36 solid tumor xenografts and in 5 of 8 ALL xenografts and objective responses were observed in several panels (2). Rapamycin and related mTOR inhibitors have also been shown to have antiangiogenic activity (4).

The rapamycin analogs temsirolimus (CCI-779) and everolimus (RAD001) have been approved for treatment of refractory renal cell carcinoma (5, 6), and temsirolimus demonstrates a high response rate against mantle cell lymphoma at relapse (7). Both temsirolimus and everolimus have completed phase I trials in pediatric patients (8). While the efficacy of rapamycin or its analogs is being assessed in phase II trials, their integration into current chemotherapy regimens used for treatment of childhood...
cancers would appear to be a logical progression in their clinical development.

mTORC1 is negatively regulated by DNA damage and this response is p53-dependent (9). The consequences of continued mTORC1 signaling following DNA damage are somewhat controversial. Murine embryo fibroblasts (MEFs) deficient in TSC2 cannot suppress mTORC1 signaling in the presence of DNA damage, and are more sensitive to methyl methane sulfonate (MMS) than cells wild type for TSC2 (10). In this model, continuing mTORC1 signaling sensitizes cells to DNA damage, putatively by increasing translation of p53, with concomitant induction of apoptosis. Thus, it was postulated that inhibiting mTORC1, by rapamycin for example, would protect cells from DNA damage. The converse was found in the yeast \textit{Saccharomyces cerevisiae}, where TORC1 signaling protected cells from DNA damage as they traversed S-phase and where inhibition of TORC1 sensitized cells to MMS treatment and prevented MMS-induced mutations (11). The proposed mechanism for rapamycin action is that TORC1 signaling is required for synthesis of DNA damage-inducible subunits of ribonucleotide reductase required to increase deoxyribonucleotide pools necessary for error-prone translesion repair by DNA polymerases.

These disparate results raise the concern for the potential of rapamycin-induced G1 ‘arrest’ abrogating the effect of proliferation-dependent cytotoxic agents such as cyclophosphamide, cisplatin or vincristine (12). Limited studies \textit{in vitro} and \textit{in vivo} suggest that rapamycin can potentiate the activity of cisplatin (13–16), and at high concentrations rapamycin appears to modulate MDR1-mediated multidrug resistance \textit{in vitro}, sensitizing cells to agents such as vincristine (17). However, we are unaware of a systematic evaluation of rapamycin combined with agents that are frequently used in the curative therapy of childhood cancers. Here we report the activity of rapamycin combined with DNA damaging cytotoxic agents, cyclophosphamide and cisplatin, the antimitotic agent vincristine, and the anti-leukemia drug dexamethasone.

Materials and Methods

\textit{In vitro Testing}

\textit{In vitro} testing was performed using DIMSCAN, a semi-automatic fluorescence-based digital image microscopy system as previously described (18). Cells were incubated in the presence or absence of rapamycin at a concentration (10 nM) that is clinically achievable and that exceeds the EC\textsubscript{50} for rapamycin by approximately 10-fold, therefore being at or near saturating concentrations for its biological effect on mTOR (2). Rapamycin was tested in combination with cisplatin \((3 \times 10^{-8} - 1 \times 10^{-4} \text{ M})\), melphalan \((3 \times 10^{-8} - 1 \times 10^{-4} \text{ M})\), vincristine \((3 \times 10^{-12} - 1 \times 10^{-8} \text{ M})\), and dexamethasone \((1 \times 10^{-10} - 3 \times 10^{-7} \text{ M})\,\text{leukemic models only}\) with 6 replicates for each concentration evaluated. Mean fluorescence values were determined for each concentration tested and then normalized to the mean of the controls. Data obtained for the agent alone were normalized to controls with no rapamycin present and data obtained for the combination were normalized to controls with 10 nM rapamycin present. Using these normalized data, EC\textsubscript{50} values were determined for the agent in the presence and absence of rapamycin. This testing approach...
allowed the following three possibilities to be distinguished: a) if the effect of rapamycin was additive with the agent being tested, then the EC50 value would be the same whether rapamycin is present or not; b) if the effect of rapamycin was supra-additive with the agent being tested, then the EC50 value would be shifted to lower concentrations when rapamycin is present (EC50 ratio ± rapamycin < 1.0); c) if the effect of rapamycin was supra-additive with the agent being tested, then the EC50 value would be shifted to lower concentrations when rapamycin is present (EC50 ratio ± rapamycin < 1.0).
was subadditive with the agent being tested, then the EC50 value would be shifted to higher concentrations when rapamycin is present (EC50 ratio ± rapamycin > 1.0). The EC50 values were determined using GraphPad Prism Version 4.0 for dose-response curve fitting to the Hill equation. \( P \)-values were two-sided and statistically significant differences, at the 2-sided 0.01 significance level, between the EC50 values, determined with and without rapamycin, were considered evidence for supraadditivity or subadditivity, depending on the direction of shift in EC50 value with the addition of rapamycin. Statistical significance was assessed by calculating the standard normal variate formed from the difference of the two values divided by the SD of that difference (the square root of the sum of the squared SD's of the two individual EC50 values). Differences that fail to achieve statistical significance are consistent with additivity, although there may simply be insufficient data to demonstrate lack of additivity.

**In vivo Testing**

**In vivo Tumor Growth Inhibition Studies.** CB17SC-M \( scid^{+/-} \) female mice (Taconic Farms, Germantown, NY) were used to propagate subcutaneously implanted kidney/rhabdoid tumors, sarcomas (Ewing, osteosarcoma, rhabdomyosarcoma), neuroblastoma, and non-glioblastoma brain tumors, while BALB/c nu/nu mice were used for glioma models, as previously described (19–21). Human leukemia cells were propagated by intravenous inoculation in female non-obese diabetic (NOD)/\( scid^{+/-} \) mice as described previously (22). All mice were maintained under barrier conditions and experiments were conducted using protocols and conditions approved by the institutional animal care and use committee of the appropriate consortium member. Ten mice per group were used for solid tumor models and 8 mice/group for the leukemia models. Tumor volumes (cm³) [solid tumor xenografts] or percentages of human CD45-positive [hCD45] cells [ALL xenografts] were determined as previously described (23). Responses were determined using three activity measures as previously described (23).

**Therapeutic Enhancement.** Therapeutic enhancement represents a therapeutic effect achieved with a tolerated regimen of a combination treatment that exceeds the optimal effect achieved at any tolerated dose of monotherapy associated with the same drugs used in the combination (Rose and Wild, 2004). This definition was operationalized as follows: Therapeutic enhancement was considered present when the tumor growth delay (T-C) for a combination was greater than the tumor growth delay for both of the single agents tested at their maximum tolerated dose (MTD) and when the EFS distribution for the combination treatment was significantly better (\( P < 0.01 \)) than the EFS distributions for both of the single agents tested at their MTD. Testing was considered not evaluable for therapeutic enhancement when the cytotoxic agent used alone and in combination produced excessive toxicity observed in treatment group; NE, not evaluable for therapeutic enhancement; TE, therapeutic enhancement.

**Table 2. Therapeutic enhancement table for solid tumor xenografts**

<table>
<thead>
<tr>
<th>Xenograft</th>
<th>CTX MTD</th>
<th>CTX 0.5MTD</th>
<th>VCR MTD</th>
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<tbody>
<tr>
<td></td>
<td>P-value (combination vs RAP)</td>
<td>P-value (combination vs CTX)</td>
<td>TE</td>
</tr>
<tr>
<td>BT-29</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<td>KT-14</td>
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<td>&lt;0.01</td>
<td>YES</td>
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<tr>
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<td>1.00</td>
<td>NE</td>
</tr>
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<td>&lt;0.01</td>
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<td>&lt;0.01</td>
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<tr>
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<td>1.00</td>
<td>NE</td>
</tr>
<tr>
<td>D645</td>
<td>&lt;0.01</td>
<td>1.00</td>
<td>NE</td>
</tr>
<tr>
<td>D456</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>YES</td>
</tr>
<tr>
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<td>TOX</td>
<td>TOX</td>
</tr>
<tr>
<td>OS-2</td>
<td>TOX</td>
<td>TOX</td>
<td>TOX</td>
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<tr>
<td>OS-31</td>
<td>TOX</td>
<td>TOX</td>
<td>TOX</td>
</tr>
</tbody>
</table>

**NOTE:** \( P \)-values are based on exact log-rank tests. Abbreviations: CTX, cyclophosphamide; VCR, vincristine; CDDP, cisplatin; RAP, rapamycin; TOX, excessive toxicity observed in treatment group; NE, not evaluable for therapeutic enhancement; TE, therapeutic enhancement.
median EFS beyond the observation period. If a treatment group exhibited excessive toxicity (>25% toxic deaths), therapeutic enhancement was not evaluated.

**Model-Based Analysis.** A linear regression model for time-to-event (T) was employed, with testing to determine whether the treatment interaction of the two-drug combination is significantly different from 0. Time-to-event is defined as the interval between study initiation and event (4 times initial tumor volume in solid tumor lines or CD45% ≥25% for leukemia lines). Mice without events at the end of the study period were censored and the corresponding times to event were treated as censored observations in the EFS analysis. The interaction model was as follows: \( T = a_0 + a_1I_1 + a_2I_2 + a_3I_1I_2 \) in which \( I_1 \) and \( I_2 \) are the indicator functions for drug 1 and drug 2, respectively. The coefficients are for the no-treatment control \( (a_0) \), the two drug effects relative to control \( (a_1 \) and \( a_2) \), individually, and the treatment interaction effect of the two drugs relative to control \( (a_3) \). Whether the value of \( a_3 \) is significantly greater or less than 0 \( (P < 0.01) \) indicates supraadditivity or subadditivity of the drug combination, respectively. Otherwise, the drug combination was considered additive. In addition, the estimated values for these coefficients are the estimated times (or additional times) to event associated with the corresponding treatment effects. To allow comparison of the \( a_3 \) values across xenografts and across doses of the standard agents, the values were normalized by using the ratio of \( a_3 \) to the expected additive effect for the combination under the assumption of additivity (i.e., \( a_1 + a_2 \)). This normalized interaction term can be conceptualized as representing the percentage gain or loss of the expected treatment effect observed for the combination under additivity. If any animals were censored in any treatment group, then the combination was not considered evaluable unless the censoring occurred exclusively in the combination group and the combination effect was supra-additive or unless the censoring occurred exclusively in one or both single agent groups and the combination effect was subadditive.

**Drugs and Formulation**

Rapamycin was purchased from LC Laboratories (Woburn, MA). Cyclophosphamide, vincristine and cisplatin were obtained from the NCI Drug Repository. Rapamycin was dissolved in DMSO (5% final concentration) and diluted in 5% Tween 80 in water and administered IP daily × 5 for 6 consecutive weeks at a dose of 5 mg/kg in the solid tumor models and for 4 weeks in ALL models. Cyclophosphamide was administered weekly for 6 weeks by I.P. injection (150 mg/kg q7d × 6; MTD), as was vincristine (1 mg/kg q7d × 6; MTD). Cisplatin was administered at 5.5 mg/kg on day 1 and 21 (MTD). For leukemia models in NOD/scid mice, vincristine was given at 1.0 mg/kg by I.P. injection whether as a single agent or in combination with rapamycin, and the dose of cyclophosphamide was 112.5 mg/kg alone and 84.4 mg/kg in combination with rapamycin. Dexamethasone, given alone, was administered at 30 mg/kg daily ×5 for four consecutive weeks, but due to toxicity the dose was reduced to 7.5 mg/kg when combined with rapamycin. Apart from rapamycin, all drugs were dissolved in physiological saline.

**Results**

**In vitro Testing**

The intent of the *in vitro* studies was to establish whether mTOR inhibition altered the *in vitro* response of the PPTP cell lines to several standard cytotoxic agents. The dose of rapamycin selected by the PPTP for *in vitro* evaluation is one that induces a near plateau effect across PPTP cell lines, as previously demonstrated (2), indicating that the effects observed are relevant to near maximal mTORC1 inhibition. Examples of the shift
in EC<sub>50</sub> values in the presence of rapamycin are shown in Fig. 1, illustrating cell lines and agents for which there was either supraadditivity (EC<sub>50</sub> ratio ± rapamycin significantly < 1) or subadditivity (EC<sub>50</sub> ratio ± rapamycin significantly > 1) or additivity (EC<sub>50</sub> ratio ± rapamycin not significantly different from 1).

Combination testing results for rapamycin with melphalan showed primarily subadditivity, with the median EC<sub>50</sub> ratio ± rapamycin being 1.54 (Table 1A). Fourteen lines showed EC<sub>50</sub> ratios ± rapamycin significantly > 1 indicating subadditivity. Only one line showed an EC<sub>50</sub> ratio ± rapamycin significantly < 1 indicating supraadditivity. A lesser degree of subadditivity was observed for rapamycin in combination with cisplatin, with 9 lines showing EC<sub>50</sub> ratios ± rapamycin significantly > 1 and with 4 lines showing EC<sub>50</sub> ratios ± rapamycin significantly < 1. However, the effects were generally not large and the median EC<sub>50</sub> ratio ± rapamycin was 1.16 (Table 1A). For vincristine, the addition of rapamycin tended to result in subadditive effects, with 9 of 23 cell lines showing EC<sub>50</sub> ratios ± rapamycin significantly < 1 and with 3 of 23 cell lines showing EC<sub>50</sub> ratios ± rapamycin significantly > 1. The median of EC<sub>50</sub> ratios without and with rapamycin present was 1.13. For the combination of dexamethasone and rapamycin, which was only tested in the leukemia cell lines, evidence for supraadditivity was obtained for almost all of the cell lines tested. Six of seven cell lines showed EC<sub>50</sub> ratios ± rapamycin significantly < 1, with the median EC<sub>50</sub> ratio ± rapamycin being 0.42 (Table 1B).

**In vivo Testing**

**Solid Tumors.** Rapamycin was evaluated in combination with cyclophosphamide, cisplatin or vincristine in vivo against twelve solid tumor models chosen for previously demonstrated intermediate sensitivity to these agents (2, 23, 24). Cyclophosphamide and vincristine were administered at their MTDs and also at one-half of their MTDs (0.5 × MTD) with or without concurrent rapamycin treatment. Cisplatin was administered at its MTD and 0.63 × MTD with or without rapamycin. A complete summary of results is provided in Supplementary Table S1, including total numbers of mice, number of mice that died (or were otherwise excluded), numbers of mice with events and median times to event, tumor growth delay, as well as objective responses and EFS T/C. Excessive toxicity was observed for NB-EBe1 in all groups receiving rapamycin (Supplementary Table S1), and so all results for this xenograft are inevaluable and are not described further.

The primary PPTP activity measure for in vivo combination testing is based on the concept of therapeutic enhancement (also known as therapeutic synergy; refs. 25, 26), which is present when the tumor growth delay (T–C) for a combination is greater than the tumor growth delay for both of the single agents tested at their MTD and there is a significant difference (P < 0.01) between the EFS distribution of the combination and the EFS distributions of each of the single agents tested at their MTD. The therapeutic enhancement concept has previously been utilized by Rose and Wild for in vivo combination studies combining a cytotoxic agent with a biologic agent (26).

Therapeutic enhancement was most commonly observed for the combination of rapamycin administered with cyclophosphamide (Table 2 and Supplementary Table S2). Each of the 5 evaluable solid tumor xenografts showed therapeutic enhancement for this combination at the cyclophosphamide MTD. The high level of activity of single agent cyclophosphamide at its MTD prevented evaluation of therapeutic enhancement for 3 xenografts. Figure 2 shows examples of xenografts for which therapeutic enhancement was observed. The combination using cyclophosphamide at 0.5 MTD showed therapeutic enhancement for only 1 of 6 evaluable xenografts. Rapamycin combined with cyclophosphamide at its MTD was effective in significantly prolonging EFS compared to single agent cyclophosphamide at its MTD in each of 5 informative experiments, while the combination using cyclophosphamide at 0.5 MTD prolonged EFS compared to single agent cyclophosphamide at its MTD in 2 of 6 informative experiments.

For vincristine, therapeutic enhancement was observed for 3 of 9 evaluable xenografts for the combination at the vincristine MTD (Table 2 and Supplementary Table S3). The combination using vincristine at 0.5 MTD showed therapeutic enhancement for 2 of 10 evaluable xenografts. Of the 11 xenografts evaluable at one or both of the vincristine doses, 4 showed therapeutic enhancement at at least one of the dose levels. Figure 3 shows examples of xenografts for which therapeutic enhancement was observed: Rh18 (rhabdomyosarcoma, 0.5 MTD), D456 (glioblastoma), and SK-NEP-1 (Ewing sarcoma). Vincristine combined with rapamycin was effective in significantly prolonging EFS compared to single agent vincristine administered at its MTD in 6 of 9 solid tumor models, while vincristine (0.5 MTD) plus rapamycin prolonged EFS compared to single agent vincristine at its MTD in 5 of 10 solid tumor models.

The combination of rapamycin with cisplatin was associated with markedly increased toxicity (Supplementary Table S1). The dose of cisplatin chosen (5.5 mg/kg) was originally considered to be 0.75 × MTD based on our previous study (24). However, this dose of cisplatin was lethal in 13 of 109 mice (11.9%). Hence, in vitro, cisplatin was tested at its MTD (5.5 mg/kg) and 3.5 mg/kg (0.63 × MTD). Toxicity to single agent cisplatin at 5.5 mg/kg was primarily observed in athymic nude mice (12/19 deaths), with scid mice tolerating this dose well as a single agent (1/90 deaths). Both strains tolerated the 3.5 mg/kg dose of cisplatin without toxicity. The addition of rapamycin to cisplatin exacerbated toxicity for athymic nude mice at both the 5.5 mg/kg dose (19/19 deaths) and the 3.5 mg/kg dose (10/19 deaths). Toxicity for scid mice was also exacerbated at both the 5.5 mg/kg dose (27/89 deaths) and at the 3.5 mg/kg dose (18/98 deaths). Therapeutic enhancement at the relatively tolerable dose for the combination (cisplatin at 0.63 × MTD)
was not observed for any of the 7 xenografts evaluable for this measure (Table 2 and Supplementary Table S4). The combination of rapamycin with cisplatin (at 0.63 × MTD) was effective in significantly prolonging EFS compared to single agent cisplatin administered at its MTD for 3 of 7 xenografts. Supplementary Fig. S1 shows results for SK-NEP-1 at the cisplatin single agent MTD where therapeutic enhancement is observed and also shows results for Rh30 at cisplatin 0.63 MTD where enhancement is approached (Supplementary Fig. S1).

A question of biological interest is whether there is evidence for a subadditive, additive, or supra-additive effect for the addition of rapamycin using model based analysis methods. This question was addressed by employing a linear regression model for time-to-event, with testing to determine whether the treatment interaction term ($a_3$) is significantly different from 0. To allow comparison of the $a_3$ values across xenografts and across doses of the standard agents, the values were normalized by using the ratio of $a_3$ to the expected additive effect for the combination under the assumption of additivity. This normalized interaction term can be conceptualized as representing the percentage gain or loss of the expected treatment effect observed for the combination under additivity. A large number of models were inevaluable for these analyses because of either excessive toxicity or because of censoring and hence these analyses should be considered as preliminary estimates.

**Figure 2.** Rapamycin enhances the therapeutic activity of cyclophosphamide. Tumor bearing mice were treated with cyclophosphamide at the MTD (150 mg/kg daily dosing 7 days per week for 6 consecutive weeks), rapamycin (5 mg/kg d × 5 for 6 consecutive weeks) or the combination of cyclophosphamide and rapamycin. Tumor diameters were measured weekly. Panel 1 shows the Kaplan-Meier curves for EFS, control (black), rapamycin (green), cyclophosphamide (blue), or rapamycin + cyclophosphamide (red). Panel 2 shows median relative tumor volumes, control (black), rapamycin (green), cyclophosphamide (blue), or rapamycin + cyclophosphamide (red). Individual tumor growth curves are shown in panel 3, control (light gray), rapamycin (dark lines), and panel 4 cyclophosphamide (light gray), cyclophosphamide + rapamycin (dark lines). A, D456 glioblastoma; B, KT-14 rhabdoid tumor of kidney; C, Rh30 rhabdomyosarcoma.

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of the extent of supra- or subadditivity observed with rapamycin combinations.

Vincristine showed evidence of supraadditivity with rapamycin for three xenografts (SK-NEP-1, Rh18, and D456), while one solid tumor xenograft (BT-29) and two ALL xenografts showed evidence of a subadditive effect for the combination (Table 3). For cisplatin, a supra-additive effect for the combination with rapamycin was observed for SK-NEP-1, while a subadditive effect was observed for KT-14. For cyclophosphamide, analysis for supra- and subadditive effects was compromised by the high level of activity for single agent cyclophosphamide at the dose and schedule studied. For the limited numbers of evaluable xenografts, supraadditivity for the combination was observed for D456, while subadditivity was observed for D645. Two xenografts showed supra-additive combination effects for more than one cytotoxic agent. D456 showed supra-additive effects for vincristine and cyclophosphamide and a trend for supraadditivity for cisplatin. SK-NEP-1 showed a supra-additive effect for vincristine and cisplatin and was inevaluable for cyclophosphamide because of the high level of activity for single agent cyclophosphamide.

**Leukemia Models.** *In vivo* combination testing for the leukemia models used markedly reduced doses of dexamethasone for combination testing due to enhanced toxicity, while similar doses of cyclophosphamide and vincristine were used for both combination and single agent testing. Interpretation of the combination testing

**Figure 3.** Rapamycin enhances the therapeutic activity of vincristine. Tumor bearing mice were treated with vincristine at the MTD (1 mg/kg daily dosing 7 days per week for 6 consecutive weeks) or 0.5MTD, rapamycin (5 mg/kg d × 5 for 6 consecutive weeks) or the combination of vincristine and rapamycin. Tumor diameters were measured weekly. Panel 1 shows the Kaplan-Meier curves for EFS, control (black), rapamycin (green), vincristine (blue), or rapamycin + vincristine (red). Panel 2 shows median relative tumor volumes, control (black), rapamycin (green), vincristine (blue), or rapamycin + vincristine (red). Individual tumor growth curves are shown in panel 3, control (light gray), rapamycin (dark lines), and panel 4 vincristine (light gray), vincristine + rapamycin (dark lines). **A,** Rh18 rhabdomyosarcoma (0.5MTD); **B,** D456 glioblastoma (MTD); **C,** SK-NEP-1 Ewing sarcoma (MTD).
was complicated in several cases by very high single agent activity for the standard agent (e.g., cyclophosphamide for ALL-4 and ALL-8 and vincristine for ALL-19). The combinations of rapamycin with either vincristine or cyclophosphamide did not show therapeutic enhancement (Supplementary Table S5). Despite the much lower dose of dexamethasone used with rapamycin compared to that used as a single agent, therapeutic enhancement was observed for ALL-8 (T-cell) and the T-C value was greater for the combination compared to single agent dexamethasone for ALL-19.

Discussion

Based on our previous testing for single agent activity, twelve solid tumor models and three leukemia models having intermediate sensitivity to each agent were selected for combination testing. For the leukemia models dexamethasone was substituted for cisplatin, as the latter agent has little activity against ALL (24). The intent of the in vitro studies was to use rapamycin to establish whether mTORC1 inhibition altered the in vitro response of the PPTP cell lines to standard cytotoxic agents. The method of analyzing for a shift in EC_{50} for a standard agent in the presence of a test agent is comparable to methods employed in evaluating the effect of RNA interference knockdown of specific gene products on response of cell lines to cytotoxic agents (27–29). The methods have also been applied in combination testing studies analyzing chemical knockdown of activity of therapeutic targets by small molecule inhibitors (29–32). Comparable results have been reported for the shift in EC_{50} method and for the combination indices method (29).

In vitro there was a trend toward subadditive effects when rapamycin was combined with melphalan, cisplatin or vincristine. By contrast, there was strong evidence for supraadditivity for dexamethasone against leukemia cell lines, consistent with previously published results (31). The results obtained for the standard cytotoxic agents suggest that mTORC1 signaling results in enhanced cell death in the presence of these agents. This would be consistent with recent data showing that cells deficient in TSC function (hence with constitutively activated mTORC1 signaling) were more sensitive to the DNA damaging agent methylmethane sulfonate (MMS; ref. 10). However, in S. cerevisiae, inhibition of TORC1

### Table 3. Model-based interaction assessment of combination therapy of rapamycin with standard agents in evaluable xenograft models

<table>
<thead>
<tr>
<th>Agent</th>
<th>Xenograft</th>
<th>Dose</th>
<th>Normalized Interaction Term</th>
<th>Total Effect Expected</th>
<th>Interaction Parameter Estimate</th>
<th>P</th>
<th>R^2</th>
<th>Interpretation</th>
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<tr>
<td>Vincristine</td>
<td>BT-29</td>
<td>MTD</td>
<td>−0.16</td>
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<tr>
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<td>MTD</td>
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<td>−1.960</td>
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<td>SK-NEP-1</td>
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<td>41.760</td>
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signaling reduces survival in cells challenged with MMS or camptothecin (11), and rapamycin significantly decreases clonogenic survival of mammalian cells treated with topotecan in vitro.

For in vivo testing, the addition of rapamycin to cyclophosphamide and to vincristine at their respective MTDs was relatively well tolerated, with little increase in toxicity observed. However, rapamycin significantly potentiated the toxicity of cisplatin administered to scid mice. Athymic nude mice, used for the propagation of glioblastoma models, tolerated cisplatin poorly at the higher dose, with exacerbation of toxicity by the addition of rapamycin.

Rapamycin or its analogs have previously been tested in combination with cytotoxics. Vincristine was reported to be synergistic against several mantle cell lymphoma cell lines in vitro when combined with the rapalog RAD001 (everolimus; ref. 33), although there are no reports evaluating this combination in vivo. However, at high concentrations (>1 μM) rapamycin is a substrate for P-glycoprotein-mediated efflux and hence can reverse vincristine resistance (17). In our study, combination of vincristine with rapamycin produced therapeutic enhancement in 4 of the 11 xenografts evaluable at one or both of the vincristine doses studied. For example, addition of rapamycin converted the response to vincristine from Progressive Disease 2 (PD2) to CR in D456 and Rh18 (0.5MTD), or MCR for SK-NEP-1 tumors. It seems unlikely that at the dose level used, rapamycin is enhancing the antitumor activity of vincristine through reversing P-glycoprotein-mediated drug efflux, as only slight enhanced toxicity was observed (as occurs with most multidrug resistance modulators). In the analysis of clinical trials for drug combinations in which a novel agent is added to a standard agent(s), the combination effect is compared to the effect of the standard agent(s) used alone in the same patient population. Our analysis of vincristine combined with rapamycin shows the combination to be effective in significantly extending EFS compared to single agent vincristine administered at its MTD in 6 of 9 solid tumor models. Thus, the vincristine/rapamycin combination demonstrates promising activity using this clinically relevant comparison.

Cyclophosphamide combined with rapamycin has been evaluated primarily for organ transplantation (34), but not in the context of cancer chemotherapy. In the current study cyclophosphamide showed therapeutic enhancement for all 5 evaluable solid tumor xenografts, although the evaluation of therapeutic enhancement was not possible in some models because of the high level of activity of single agent cyclophosphamide used at its MTD. One important observation is that therapeutic enhancement was rarely observed when cyclophosphamide at 0.5MTD was combined with rapamycin. This highlights the importance of using agents with steep dose response curves like cyclophosphamide at or near their MTD in combination studies. These results suggest that the clinical success of combinations of alkylating agents with mTOR inhibitors will be dependent on whether full doses of the alkylating agents can be administered.

Rapamycin, or the analog CCI-779 (temsirolimus) has been shown previously to potentiate apoptosis induced by cisplatin in several tumor cell models (13, 14), and to potentiate cisplatin antitumor activity in vivo (16). Further, Beuvink et al. (15) have reported increased apoptosis activity when cisplatin was combined with the rapalog everolimus (RAD001) only in tumor cells with wild type p53. The purported mechanism of enhanced cell killing was through rapamycin blocking p53-mediated induction of p21<sup>CIP1</sup>, leading to decreased G1 arrest, and enhanced apoptosis. In our analyzable studies, the combination of rapamycin with cisplatin did not show therapeutic enhancement using the lower, tolerable dose of cisplatin. The mechanism for increased systemic toxicity, when rapamycin is combined with cisplatin is unknown. Potentially, the combination could have similar effects on normal proliferating tissues in the intestine and bone marrow as reported by Beuvink et al. (15). The combination of rapamycin with cisplatin (0.63MTD) was significantly more effective than cisplatin at its MTD in 3 of 7 evaluable models in terms of prolonging EFS.

Rapamycin combined with vincristine, cyclophosphamide or dexamethasone was evaluated against three ALL models. The vincristine and rapamycin combination showed little evidence for superiority in comparison to the single agents, while interpretation of results for the cyclophosphamide and rapamycin combination were complicated by the high level of activity for single agent cyclophosphamide. Although as a single agent dexamethasone was tolerated at 30 mg/kg, in combination with rapamycin the maximum tolerated dose was 7.5 mg/kg. Even at this low dose, therapeutic enhancement was demonstrated for 1 of 3 xenografts. Rapamycin has been shown to reverse dexamethasone resistance in ALL cell lines through modulation of MCL1, but in this previous report, the therapeutic utility was not tested in vivo (31). The value of combining dexamethasone with rapamycin will require examining a larger cohort of leukemia models as well as further evaluation of the increased toxicity.

The most appropriate methodology to apply for in vivo combination testing remains an open question. While formal synergy testing can be performed using isobologram or response surface modeling methods, such methods require testing of several dose levels for each agent and require a very large number of animals for each combination tested against a single xenograft. As our goal is to develop a dataset to inform clinical prioritization for a wide range of histotypes and drug combinations, these formal synergy assessment methods are not suitable. For in vivo combination testing, we favor a fixed-dose drug combination approach, as this minimizes the number of animals required per drug combination. Among fixed-dose approaches, testing for
therapeutic enhancement appears to be the most clinically relevant strategy for evaluating combinations in vivo, as its presence implies an enhanced therapeutic index (i.e., the combination has greater activity than either agent alone administered at the MTD; refs. 25, 26).

Many published combination preclinical studies, particularly with agents lacking intrinsic antitumor activity and used to modulate the activity of cytotoxic agents (e.g., buthionine sulfoxamine given with melphalan, multidrug resistance modulators given with standard chemotherapy agents, etc.), failed to compare the combination effect to the effect of the cytotoxic agent administered at its MTD (35). Clinically, it has been observed that many combinations required substantial reductions in the dose of the standard agent when the novel agent was used at biologically effective doses in humans. The failure of O6-benzylguanine to enhance the clinical activity of nitrosoureas when tested in clinical trials, was primarily the result of the reductions in nitrosourea dosing required to safely administer nitrosoureas with O6-benzylguanine in humans (36, 37). The therapeutic enhancement concept directly addresses this concern by comparing the activity of the combination to the activity of the single agents at their MTD.

The second method of evaluating combination treatments used allows for model based testing for supra- or subadditivity. This approach is conceptually the same as the commonly applied method of summing log-cell kill values for single agents to estimate the log-cell kill expected for additivity. Application of linear regression modeling has the advantage of allowing the statistical evidence for claims of supra- or subadditivity to be quantified. The interesting observation from these analyses is that some xenografts show consistent evidence for supraadditivity, suggesting that there is a subset of tumors for which combinations of cytotoxic agents and rapalogs may be particularly effective. Identifying the biological basis for supraadditivity for these xenografts could have direct clinical implications. One lesson learned from the PPTP evaluations of rapamycin combinations is that optimal application of the model based approach requires selecting durations of treatment for the single agents that are not so effective that treated animals without events are present at the end of the observation period.

Exactly how rapamycin enhances the in vivo antitumor activity of cyclophosphamide and vincristine used at their respective MTDs remains to be explored. Rapamycin enhances some forms of DNA damage, and emerging evidence indicates that the TOR pathway regulates transit through mitosis in yeast. Rapamycin and related mTOR inhibitors also have antiangiogenic activity through inhibition of proliferation of endothelial cells and through impaired VEGF production (4, 38, 39), and hence the interaction may be tumor cell-dependent or independent. There is an apparent discrepancy between the in vitro testing results, which primarily demonstrated subadditivity, and the in vivo testing results, for which therapeutic enhancement was commonly observed. It is important to note, however, that therapeutic enhancement can be present when the true interaction at the cellular level between the agents used in combination ranges from subadditivity to supraadditivity. Particularly when the agents can be administered together at their single agent MTDs, there is the opportunity for a significant increase in efficacy for the combination (i.e., therapeutic enhancement) even when the model based assessment of the interaction shows subadditivity.

In summary, we have evaluated both in vitro and in vivo efficacy for rapamycin combined with standard cytotoxic agents frequently used in treatment of childhood cancer. The predominant interaction in vitro was subadditive activity, with the notable exception of supraadditivity for dexamethasone plus rapamycin for leukemia cell lines. In vivo, there were numerous models that showed therapeutic enhancement for combinations in which rapamycin was administered with either cyclophosphamide or vincristine at their respective MTDs. These results will be useful in planning combination clinical trials with rapalogs in pediatric cancer patients.

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No potential conflicts of interest were disclosed.

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