In-vitro synergism of m-TOR inhibitors, statins, and classical chemotherapy: potential implications in acute leukemia

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Classical chemotherapy has an active, but limited, role in acute leukemia with relapse common in adult patients. Recent evidence has implicated signal transduction pathways in leukemic progression and also in resistance to cytotoxic therapy. We have used a short-term, in-vitro incubation assay with cytotoxic analysis by MTT, confirmed by histone-associated DNA fragmentation, to evaluate both classical and nonclassical combinations of drugs. Isobologram median effect analysis, confirmed by curve shift analysis, was used to identify synergy and antagonism. Fluvastatin, a prenylation inhibitor, demonstrates global enhancement of the effects of classical agents in both AML-193 and KG-1 cell lines. Similarly, the m-TOR inhibitors, RAD-001 (everolimus) and rapamycin, also cause time-dependent global enhancement of cytotoxic agents. At clinically achievable combinations, RAD-001 perturbs the AKT pathway *in vitro*. The unique combination of fluvastatin and an m-TOR inhibitor was synergistic in both cell lines. These effects were independent of whether or not human plasma was used in the assay system. These studies suggest several novel combinations of agents that need to be evaluated in the management of leukemia. *Anti-Cancer Drugs* 19:705–712 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Acute myeloid leukemia (AML) is now recognized as a heterogeneous disease, which responds to classical chemotherapy, but may vary widely in outcome depending upon its subtype and age of the patient on presentation [1–3]. Owing to the limitations of classical cytotoxic drugs in this disease [4] and the emerging molecular biology of signal transduction pathways, pharmaceutical research has looked for targets that block proliferation and block drug resistance [5]. The PI3 kinase (PI3K) pathway is regulated by a variety of cytokines and chemokines that affect white cells [6]. Emerging data has suggested that activation of the PI3K pathway and downstream AKT expression serves as a vital link in the survival of malignant cells and drug resistance [7–9]. Downstream of AKT, m-TOR (mammalian target of rapamycin) binds to one of two scaffold proteins, raptor or rictor, forming complexes critical for cell proliferation and survival [10]. The rictor/m-TOR complex can also phosphorylate AKT at Ser473 [10]. As a consequence, drug inhibition of this pathway is an attractive approach to enhance therapeutic effects [11].

In human AML, activation of m-TOR and consequent S6 ribosomal phosphorylation has been seen in approximately one-half of samples studied and can be suppressed by m-TOR inhibitors [12,13]. In early clinical studies of m-TOR inhibitors, phosphorylation of AKT and Fox01 can be suppressed in 75% of human AML blasts [14]. In addition, the clonogenic properties of AML cells *in vitro* are reduced in the presence of inhibitors of this system [15]. For these reasons, inhibition of the m-TOR pathway has been suggested to be a potential therapeutic approach in treatment of hematologic malignancies [16,17]. However, preclinical work also suggests that m-TOR inhibitors can inhibit cell cycle progression that could be dysfunctional when combined with schedule-dependent cytotoxic agents [18,19]. In addition, short-term culture of human AML cells with a rapamycin analog suggested, by activation of the insulin-like growth factor 1 autocrine loop, a paradoxical upregulation of phosphorylated AKT leading to increased function, thus potentially abrogating any therapeutic benefit from this class of agents [20].

A variety of agents related to rapamycin inhibit downstream signaling from AKT and are currently being evaluated in the clinic [7,21], as are AKT inhibitors [7]. Rapamycin and its analog target the peptidyl-prolyl 'cis trans' isomerase FK506-binding protein (m-TOR) resulting in inhibition of cellular proliferation and induction of apoptosis [22]. An additional pathway seen in JN-DSRCT-1 cells (desmoplastic small round cell tumor) is downregulation by rapamycin of Bcl-xL and upregulation of Bax by a pathway independent of m-TOR inhibition, believed to be related to proteosome inhibition leading to enhanced apoptosis [23].

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The rapamycin analogs are similar in action, but vary in their bioavailability and pharmacokinetics [22]. By itself, RAD-001 has only a minor effect on apoptosis [9]. As a single agent, RAD-001 did not display objective responses in early human clinical trials [24], whereas an additional study with the parent compound rapamycin noted four of nine AML patients responding to treatment [15]. However, it is unclear how best to combine these agents with more classical drugs and the rationale for this report. In addition, the potential role of concurrent inhibition of several signal transduction pathways is not defined.

Another area of research interest is the role of cholesterol in AML proliferation, which acts through a variety of mechanisms including signal transduction of the Mek/Erk pathway [25]. Recent information on the role of increased levels of cholesterol in the plasma membrane of leukemia cells, owing to enhanced synthesis of cholesterol and importation of low-density lipoprotein into these cells, has suggested a resistance mechanism preventing classical cytotoxic agents from causing cell death [26–28]. Cholesterol, but not its precursors, is required for cell proliferation in the leukemia cell lines HL-60 and MOLT-4 [29]. Cytotoxic agents also increase cholesterol fraction in leukemia cells. Cholesterol starvation in the leukemia cell line HL-60 *in vitro* results in formation of polyploidy, multinucleated cells, and slowed the G2 to M cell cycle transition [30]. In KG1a leukemia cells having phenotype $CD34^+ CD38^{+/-}$MDR-1 + , statin exposure downregulates the multiple drug resistance transporter p-glycoprotein and enhances the cytotoxic effects of daunorubicin [31]. Additional studies demonstrate that blockage of cholesterol biosynthesis enhances induction of apoptosis by traditional drugs in both cell lines and in primary AML cell cultures [29]. As single agents, the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have antileukemia activity in preclinical model systems [32–35]. The statins, known prenylation inhibitors, may also work through downregulation of BCL2 [34] in addition to inhibition of geranylgeranylation [36]. Additional antileukemia effects have been seen with specific prenylation inhibitors such as tipifarnib [37]. Fluvastatin was noted to be a strong inducer of differentiation and apoptosis in the NB4 acute promyelocytic cell line [25]. A small phase 1 study of pravastatin, idarubicin, and high-dose cytarabine in AML patients, many with unfavorable genetics, resulted in a 73% complete response rate, thus suggesting the value of this approach [38].

In our previous studies with epithelial malignancies *in vitro*, we identified synergy between the statins and either trastuzumab or anthracyclines using an in-vitro assay and median effect isobologram analysis [39]. The advantage of this assay is that up to three agents can be evaluated together and the dose–response curve analyzed. With the above information, we were prompted to evaluate signal transduction inhibitors (rapamycin and RAD-001) and also the lipid soluble statin, fluvastatin, in combination with classical agents *in vitro*.

Materials and methods

Reagents

Drugs, tissue culture techniques, and median effect analysis were evaluated as previously described [40–43]. Reagent grade fluvastatin and RAD-001 (everolimus) were gifts of Novartis Pharma, Basel, Switzerland. The cytotoxic agents chosen for these studies were drugs already used in the clinical setting. Rapamycin, daunorubicin, and cytosine arabinoside were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Idarubicin was obtained from Calbiochem (San Diego, California, USA). The AML-193 and KG-1 cell lines were obtained from the American Tissue Culture Collection (ATCC, Rockville, Maryland, USA). These cells differ in their phenotype, ability to differentiate in the presence of stimulating factors, and response to chemotherapeutic agents. KG-1 cells are of M1 (undifferentiated myeloid) morphology, phenotype $CD34^+$, $CD33^-$, $CD38^-$ [44], have a point mutation in intron 6 of P53 [45,46], have a loss of chromosome 5 [47], and respond to colony stimulating factors [48,49]. AML-193 cells are of M5 (acute monocytic leukemia) morphology [50], GM-CSF dependent [51], express high affinity FLT3 receptors [52], are FAS sensitive when exposed to interferon [53], produce autocrine granulocyte-macrophage colony-stimulating factor [54], and can be induced to differentiate into granulocytic cells when exposed to retinoic acid [55]. Human serum and fetal calf serum were obtained from Sigma-Aldrich, and ELISA kits for AKT, phospho-AKT, and phospho-S6 from Cell Signaling (Danvers, Massachusetts, USA).

Assessment of cytotoxicity produced by therapeutic agents

Determination of cytotoxicity of the drugs used in this study and median effect analysis were done by previous methods [39–42]. In brief, both the AML-193 and KG-1 cell lines were grown as a suspension in T 150 tissue culture flasks (Corning Glass Works, Corning, New York, USA) using Icoves medium (Invitrogen, Carlsbad, California, USA) with 5% CO_2 and 15% heat-inactivated fetal calf serum for AML-193 cells or 20% for KG-1 cells. In addition, for AML-193 cells only, the medium was supplemented with 0.005 mg/ml insulin and 0.005 mg/ml transferrin. All cultures contained penicillin (100 μg/ml), streptomycin (0.25 mg/ml), and glutamine to a final concentration of 2 mmol/l. All cell lines were periodically tested for mycoplasma contamination. Cell viability tested by trypan blue exclusion was greater than 95%. Harvested cells were aliquoted into 96-well dishes (Falcon 3072) at concentrations of 7000–9000 cells per well. The cells were then cultured for 24 h and cytotoxic

agents or solvent controls were introduced for a 72-h incubation, centrifuged at $3000 \times g$ for 5 min to pellet cells, and cell growth evaluated by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [56] using a BioRad 3550 Microplate Reader (BioRad, Hercules, California, USA). The dose of drug needed to cause cytotoxicity in 50% of the cells (IC_{50} value) was determined using the EZ-ED50 Program (Perrella Scientific, Conyers, California, USA). The concentration of rapamycin or RAD-001 used in the cytotoxicity experiments was fixed at 8 ng/ml [24,57]. All reported values are the means of two to four experiments with each study having three wells per dose level. All drugs were applied to the cells simultaneously. To demonstrate that this test system does not give anomalous results when fetal calf serum is used instead of human serum, the IC_{50} and IC_{70} values of various agents and combinations were compared using either human or fetal calf serum for 48 and 72-h incubations. Differences were analyzed by the *t*-test.

Evaluation of apoptosis

The commercially available Cell Death Detection ELISA Plus kit (Roche Applied Science, Penzberg, Germany) was used to detect DNA fragmentation as previously described [58]. This assay exploits the amount of cytoplasmic histone-associated DNA fragments produced upon cell death. The kit uses mouse monoclonal antibodies against the histone proteins. The drug concentrations used in the combination apoptosis assays were the IC_{50} of the single agent with RAD-001 used at the clinically achievable level of 8 ng/ml [59]. Briefly, cells growing in exponential phase were exposed to the appropriate drug(s) and apoptosis was evaluated at 24, 48, and 72-h intervals. All agents were applied to the culture simultaneously. Cells were then subjected to centrifugation and lysed in 96-well plates. Twenty microliters of the supernatant was transferred to a kit supplied streptavidin-coated plate. This supernatant was incubated for 2 h in the presence of the immune reagent containing the antibodies against the histone proteins and DNA fragments. The complex was then conjugated to form an immuno-complex on the plate and read for optical density at 405 nm with a reference wavelength at 490 nm. Samples were measured in duplicate and a positive control was provided with the kit. The result represents an enrichment factor, which is a relative indicator of the number of cells undergoing apoptosis as calculated by the following formula:

$$\text{Enrichment factor (reflection of apoptosis)} = \text{mU of dying/dead cells/mU of untreated cells}$$

where mU = absorbance (405 nm)–absorbance (490 nm) and is a function of the amount of histones and DNA fragments released into the cytoplasm from the apoptotic cells. Statistical differences were determined by paired *t*-test analysis.

Assessment of drug interaction

Median effect isobologram analysis, a measure of synergism or antagonism based upon the Hill equation, was determined by the method of Chou, using their computer program to determine the combination index (CI), which reflects synergy when less than 1 [60]. The model is most accurate at the Fa_{50}, which is defined as the concentration of drug that results in 50% effect (cytotoxicity as measured by MTT assay) and is not reliable at extremes of Fa [61]. As the model is a linear approximation of a higher order equation, we have previously defined additive effects to be within 1 standard deviation of unity, synergistic effects less than unity, and antagonistic effects greater than unity [62]. Eight fixed drug ratios above and below the IC_{50} with a range of 0.03125–8 N, where N is a value near the IC_{50} of an individual drug, were explored by incubating the drug combinations with cells for 72 h and then determining the degree of cytotoxicity by the MTT assay. Fa is defined as the fraction of cells affected. A plot of log dose versus log (Fa/1 – Fa) gives parallel slopes if no biologic interaction is present (mutually exclusive) or converge if there is an interaction between the drugs (mutually nonexclusive), thus suggesting the appropriate model to determine the CI [60]. Statistical differences were confirmed using the ACT Analysis program (Optimum Therapeutics LLC., Columbus, Ohio, USA) using nonlinear regression of the concentration-effect data. The results were then normalized to IC_{50} equivalents [63]. This method yielded a survival versus IC_{50} equivalent curve for each agent by itself and in combination. A curve shift to the left indicates synergistic drug interactions [63].

Assessment of the effects of m-TOR exposure on AKT and downstream function

The effects of m-TOR inhibition on the concentration of AKT, phospho-AKT, S6, and phospho-S6 (Kit 7170 for total AKT; Kit 7160 for phospho-AKT, which recognizes the phosphate on SER 473 residue; Kit 7205, phospho-S6 ribosomal protein, which recognizes SER 235/236 residues) were determined by ELISA assays. All results were normalized to concentrations at time zero with no drug present. Concentrations of RAD-001 from 4 to 64 ng/ml were applied to KG-1 cells in the presence of serum and incubated over 72 h. After 72 h, cells were centrifuged, washed twice in ice cold phosphate-buffered saline, and then resuspended in 0.5 ml cold lysis buffer plus phenylmethylsulfonyl fluoride to a final concentration of 1 mmol/l for 5 min. Cells were then briefly sonicated and centrifuged at 4°C, and cell supernatant used for ELISA assay. One hundred microliters of cell lysate prepared above was diluted 1 : 1 with the diluent supplied in kit and incubated in appropriate antibody-coated wells for 2 h at 37°C. Diluted lysate was removed,

subsequently washed 4 × in wash buffer, and 100 µl of detection antibody added with further incubation for 1 h at 37°C. The detection antibody was removed, washed 4 × as above, and horseradish peroxidase-linked secondary antibody then incubated in each well for 30 min at 37°C. Cells were then washed 4 × as above and incubated in 100 µl of 3′,3′,5′,5′-tetramethylbenzidine substrate for 10 min at 37°C. A stop solution (100 µl) was added to each well. Results were read within 30 min at 450 nm and compared with an untreated control.

Results

The cytotoxic effect on the cells growing in log phase as measured by a 72-h MTT assay for each individual agent studied is shown in Table 1. Fluvastatin showed minimal activity as a single agent in both cells lines (four logs less active) as compared with the other drugs tested. Daunorubicin and its analog idarubicin showed the greatest sensitivity in both cell lines. IC_{50} values for any given agent were very similar between each cell line with the exception of RAD-001. The KG-1 cell line was an order of magnitude more sensitive to RAD-001 than was the AML-193 cell line.

A previous Developmental Therapeutic Symposium at NCI Frederick had suggested that in-vitro synergism may be modified by the origin of plasma or serum used for culture [64]. To determine that the culture conditions with fetal calf serum did not influence the results obtained in this study, human serum was obtained from a commercial source and used as a replacement for fetal calf in these cytotoxic studies (Table 2). As measured by the *t*-test, there is no statistical difference in the effect of these drugs at two levels of cytotoxic effect or over time whether or not human serum is used.

Table 1 The concentration of drug needed to cause 50% cell death (IC_{50}) after 72-h incubation

	Cell line	
Drug	AML-193	KG-1
Ara-C	4.44 ± 0.28	7.19 ± 1.35
Daunorubicin	0.03 ± 0.01	0.02 ± 0.01
Fluvastatin	92.19 ± 2.09	23.78 ± 3.99
Idarubicin	0.04 ± 0.02	0.06 ± 0.02
RAD-001	2.17 ± 0.10	0.22 ± 0.06
Rapamycin	1.31 ± 0.10	1.64 ± 0.65

Values indicated are given in µmol/l and are means ± SD of three to six experiments.
AML, acute myeloid leukemia; Ara-C, cytosine arabinoside; IC, inhibitory concentration.

The combination studies reflected drug combinations of conventional agents with either an m-TOR inhibitor or fluvastatin. In addition, the potentially unique oral combination of an m-TOR inhibitor and fluvastatin was evaluated. In all cases, plots of the dose versus log (Fa/1 – Fa) indicated mutually nonexclusive interactions suggesting that there were cellular interactions between agents. The nonexclusive criterion is also a more conservative approach in determining antagonistic or synergistic action of two or more drugs [28]. For almost all combinations tested, the addition of either an m-TOR inhibitor or fluvastatin resulted in CI values less than unity indicating synergy. Of particular interest, fluvastatin and either rapamycin or RAD-001 demonstrated additive to synergistic effects in both cell lines (Table 3). Synergism in the cell lines was also noted for both RAD-001 and rapamycin with either an anthracycline or cytosine arabinoside. To confirm the presence of synergy, several combinations were also evaluated using the ACT curve shift analysis program. A typical result using this program is given in Fig. 1.

Triplet combinations were also evaluated in both cell lines (Table 4). Triplet combinations show universal synergism in the two cell lines. The addition of either daunorubicin or idarubicin to the doublet combinations of fluvastatin and rapamycin or RAD-001 and cytosine arabinoside decreased the value of CI, indicating greater synergistic effects of these combinations.

To demonstrate that the effects of the above drug combinations represented true cell death, doublet combinations of RAD-001 with fluvastatin in the KG-1 cell line and RAD-001 with cytosine arabinoside in the AML-193 cell line were evaluated by ELISA cytoplasmic histone-associated DNA fragmentation assay using the clinically achievable concentration of RAD-001 and the IC_{50} of the cytotoxic agents (Fig. 2). The effect of a triplet combination of RAD-001 with idarubicin and

Table 2 Absence of difference in cytotoxic effect using human serum or fetal calf serum at two different cytotoxic concentrations (IC_{50} and IC_{70}) and two different exposure times (48 and 72 h)

KG-1 cell line	IC_{50}FBS	IC_{50}human	*P* value	IC_{70}FBS	IC_{70}human	*P* value
48 h, Fluva + Rapa	42.2 ± 3.6	47.6 ± 4.00	0.288	99.7 ± 9.2	107.4 ± 14.1	0.586
72 h, Fluva + Rapa	12.1 ± 1.0	15.7 ± 1.7	0.125	35.7 ± 3.5	37.6 ± 3.1	0.62
48 h, Fluva + Dauno	43.8 ± 2.5	45.2 ± 3.4	0.67	98.6 ± 7.2	110.4 ± 10.7	0.325
72 h, Fluva + Dauno	19.8 ± 3.4	19.8 ± 1.2	0.981	46.3 ± 8.5	49.6 ± 8.5	0.732
48 h, Ara-C + Rapa	6.8 ± 1.8	7.8 ± 1.9	0.647	24.9 ± 4.1	28.1 ± 5.6	0.578
72 h, Ara-C + Rapa	3.1 ± 0.9	3.8 ± 1.3	0.578	10.9 ± 2.7	13.3 ± 2.6	0.459

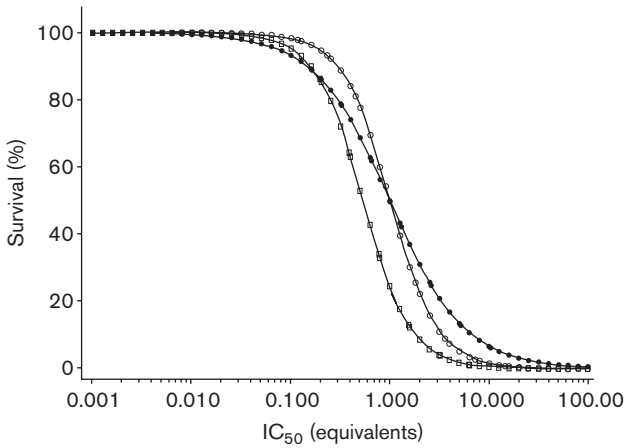
Rapamycin was held constant at 8 ng/ml and the other agent varied. Statistical differences were analyzed by the *t*-test.
Ara-C, cytosine arabinoside; Dauno, daunorubicin; FBS, fetal bovine serum; Fluva, fluvastatin; IC, inhibitory concentration; Rapa, rapamycin.

Table 3 Doublet median effect results for synergy or antagonism following the 72-h incubation of two drugs concurrently, expressed as combination index (CI) value at 50% cytotoxicity (Fa_{50}) ± standard deviation for AML-193 and KG-1 cell lines

	Cell line	
Drug combination	AML-193	KG-1
Fluvastatin + Rapamycin	0.3 ± 0.1	0.8 ± 0.1
Fluvastatin + RAD-001	0.9 ± 0.1	0.5 ± 0.1
Fluvastatin + Ara-C	0.8 ± 0.0	0.7 ± 0.2
Fluvastatin + Daunorubicin	0.4 ± 0.0	0.8 ± 0.1
Fluvastatin + Idarubicin	0.7 ± 0.1	0.9 ± 0.1
RAD-001 + Ara-C	0.9 ± 0.2	0.3 ± 0.1
RAD-001 + Daunorubicin	0.6 ± 0.1	0.7 ± 0.1
RAD-001 + Idarubicin	0.6 ± 0.1	0.5 ± 0.1
Rapamycin + Ara-C	0.3 ± 0.1	0.9 ± 0.1
Rapamycin + Daunorubicin	0.5 ± 0.1	0.3 ± 0.0
Rapamycin + Idarubicin	0.8 ± 0.0	0.3 ± 0.0
Ara-C + Daunorubicin	0.3 ± 0.1	1.3 ± 0.6

Synergistic values are less than 1.0. Rapamycin and RAD-001 concentrations were fixed at 8 ng/ml.
AML, acute myeloid leukemia; Ara-C, cytosine arabinoside; Fa, fraction of cells affected.

Fig. 1



Synergy as measured by nonlinear regression and curve shift analysis for the combination of rapamycin and cytosine arabinoside in the AML-193 cell line. Effects are normalized with a shift of the combination treatment curve to the left compared with the singlet treatment indicating synergy. Open squares □, rapamycin + cytosine arabinoside; closed circle ●, rapamycin; open circle ○, cytosine arabinoside. $P < 0.01$ for the combination versus. single agent.
AML, acute myeloid leukemia; IC, inhibitory concentration.

cytosine arabinoside is shown in Fig. 3. These effects were time dependent with increased exposure time leading to enhanced apoptosis. In addition, manual cell counts using trypan blue exclusion also indicated a dramatic decrease in viable cell numbers over a 72-h incubation period (data not shown).

To demonstrate that the m-TOR inhibitor had an effect on the AKT pathway in this assay system, AKT, phospho-AKT, and phospho-S6 were measured by ELISA assay

Table 4 Triplet median effect results for 72-h incubation of cells with the indicated agents as expressed as combination index (CI) value at 50% cytotoxicity (Fa_{50}) ± standard deviation for AML-193 and KG-1 cell lines

	Cell line	
Drug combination	AML-193	KG-1
Fluvastatin + Ara-C + Daunorubicin	0.7 ± 0.1	0.1 ± 0.1
Fluvastatin + Rapamycin + Ara-C	0.4 ± 0.0	0.6 ± 0.1
Fluvastatin + Rapamycin + Daunorubicin	0.2 ± 0.0	0.5 ± 0.1
Fluvastatin + Rapamycin + Idarubicin	0.2 ± 0.0	0.9 ± 0.1
Fluvastatin + Ara-C + Idarubicin	0.4 ± 0.0	0.5 ± 0.1
RAD-001 + Ara-C + Daunorubicin	0.7 ± 0.0	0.5 ± 0.1
RAD-001 + Ara-C + Idarubicin	0.2 ± 0.1	0.3 ± 0.0
RAD-001 + Daunorubicin + Rapamycin	0.3 ± 0.0	0.7 ± 0.1
Rapamycin + Ara-C + Daunorubicin	0.9 ± 0.1	0.2 ± 0.0
Rapamycin + Ara-C + Idarubicin	0.7 ± 0.1	0.3 ± 0.0

Synergistic values are less than 1.0.
AML, acute myeloid leukemia; Ara-C, cytosine arabinoside; Fa, fraction of cells affected.

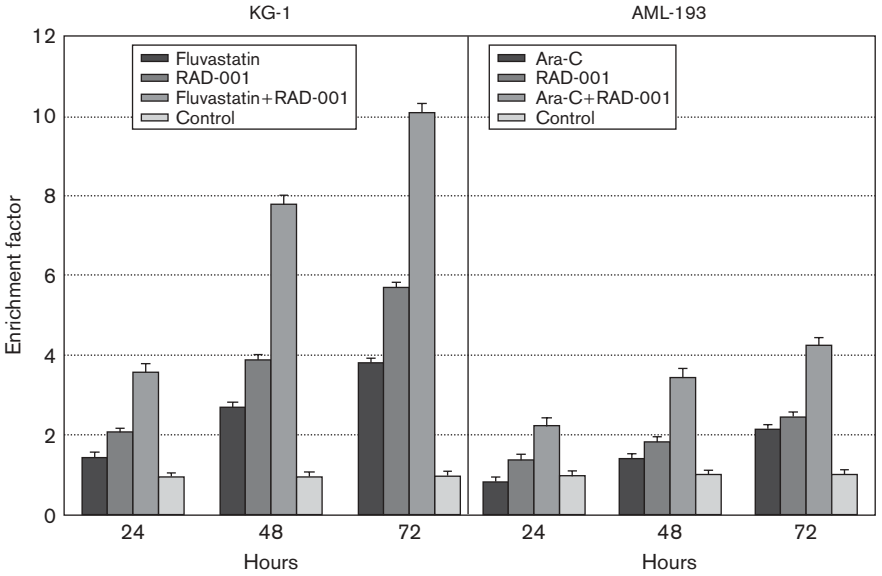
after KG-1 cells were exposed to RAD-001 for 72 h. Both phospho-AKT and phospho-S6 are measures of m-TOR activation. The results, normalized to the level of no drug exposure, are shown in Fig. 4 demonstrating a concentration-dependent effect on the AKT pathway.

Discussion

Redundancy of survival mechanisms and cross-talk between molecular pathways are believed to be two reasons why classical cytotoxic chemotherapy has only a limited role in the treatment of malignant disease [7]. In the case of acute leukemia, resistance to initial therapy portends a poor outcome [65]. Better understanding of proliferation pathways has suggested that AKT activation occurs in at least 60% of cases of AML through activation of the PI3 kinase pathway [11] and is associated with drug resistance. Therefore, inhibition of this pathway has been explored as a potential therapeutic maneuver [15,66,67].

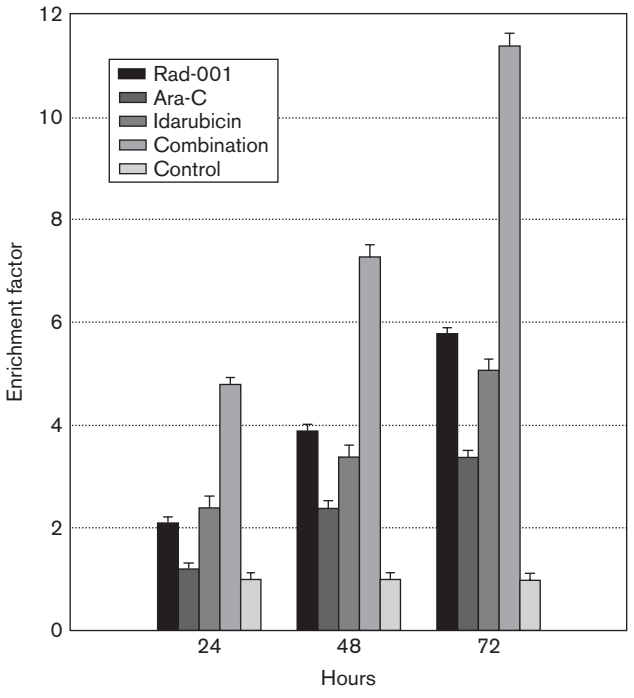
Rapamycin and its analogue RAD-001, a variant with better pharmacological properties [66], inhibit m-TOR, which is downstream from AKT and is involved in the function of this pathway [68]. Initial studies in both preclinical models and in humans have suggested that these compounds exhibit antileukemia activity [67]. Although initially believed to only inhibit raptor/m-TOR complex [10], prolonged culture (24 h) of the rapamycin analogs temsirolimus or RAD-001 in media with serum has resulted in rictor/m-TOR complex inhibition leading to more profound inhibition of the AKT pathway [14]. Activity of the rictor/m-TOR complex can be evaluated by the level of AKT ser 473 phosphorylation in cells [14]. We noted similar findings of lower expression of phosphorylated AKT in our study, which used a 72-h incubation period and fetal calf serum. Therefore, for an m-TOR inhibitor to be effective in blocking the AKT pathway, prolonged exposure may be

Fig. 2



Representative doublet combinations of the nonclassical combination of fluvastatin and RAD-001 in KG-1 leukemia cells and RAD-001 combined with cytosine arabinoside in AML-193 cells. The combinations demonstrate enhanced time-dependent apoptosis ($P<0.01$ for all paired analyses). AML, acute myeloid leukemia; Ara-C, cytosine arabinoside.

Fig. 3



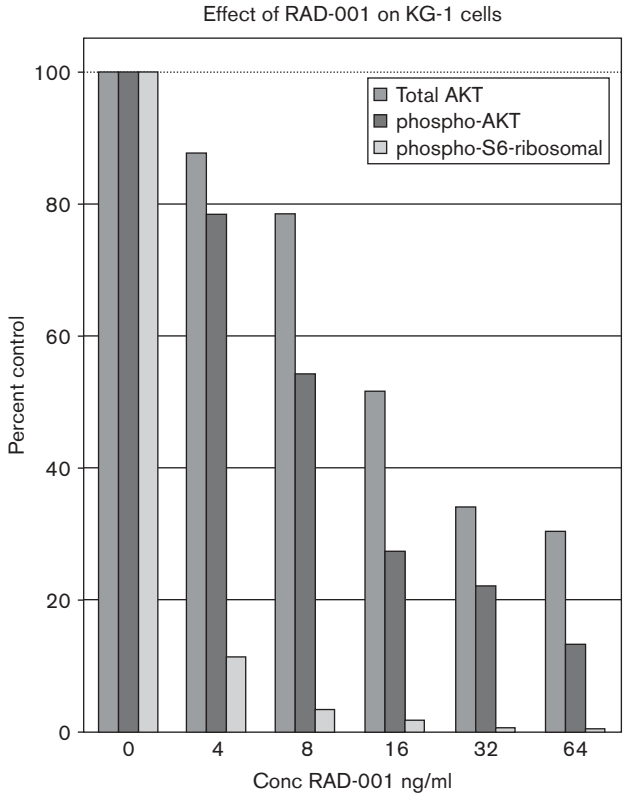
Evaluation of time-dependent apoptotic effects of single agents and the triplet cytosine arabinoside, idarubicin, and RAD-001 in the KG-1 cell line. DNA fragmentation values as measured by ELISA are shown ±standard deviation. $P<0.01$ for all paired analyses. Ara-C, cytosine arabinoside.

necessary. In our in-vitro studies, we also found differences in IC_{50} between rapamycin and RAD-001 in KG-1 cells which was not seen in the more differentiated AML-193 cell line. The reason for this effect remains under investigation.

We have also identified that an m-TOR inhibitor markedly augments the effects of classical cytotoxic agents used alone and in combination with a second cytotoxic agent in leukemia cell lines. No singlet or doublet of cytotoxic drugs used in the clinical treatment of acute leukemia, when combined with an m-TOR inhibitor, demonstrated antagonism in this model system. The triplet combination of RAD-001, cytosine arabinoside, and idarubicin was particularly potent (CI_{50} 0.2–0.3). We have also confirmed in a previous report [9] that in both our cell lines RAD-001 enhances the action of the schedule-dependent drug, cytosine arabinoside. These findings in cell lines suggest that cell cycle delay induced by m-TOR inhibition does not antagonize the cytotoxic action of the commonly used agents for treatment of AML. This effect was further demonstrated in this study to be owing to a time-dependent exposure causing enhanced apoptosis and not just owing to a cytostatic effect.

In a similar manner, the statins have been noted to have preclinical antileukemia activity and to possibly work through several unique mechanisms [26,32–36,69]. Fluvastatin, at clinically achievable plasma levels, enhances

Fig. 4



The effect of RAD-001 on expression of AKT, phospho-AKT, and S6 normalized to control in the AML cell line KG-1. Incubation is for 72 h. AML, acute myeloid leukemia; Conc, concentration.

the effects of trastuzumab in solid tumors *in vitro* and thus may perturb survival pathways [43]. Therefore, these studies are an extension of our prior studies, but utilize an acute leukemia model. We have identified the nonclassical combination of fluvastatin with either rapamycin or RAD-001 as displaying antileukemia synergy *in vitro* and that fluvastatin also enhances the cytotoxic effects of anthracyclines and cytosine arabinoside. This latter finding confirms and extends previous findings of the effect of perturbation of cholesterol biosynthesis in acute leukemia [28].

Therefore, these studies suggest several potential novel combinations of agents that may deserve further testing in acute leukemia. Of particular interest is that the combination of an m-TOR inhibitor with fluvastatin also enhanced apoptotic effects and suggests a potentially less toxic oral combination to be evaluated in elderly patients with acute leukemia who tolerate classical aggressive cytotoxic therapy poorly [70]. The drawbacks of this model system are that it does not reflect either molecular or kinetic heterogeneity of human disease and does not evaluate therapeutic index.

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