Published OnlineFirst July 3, 2014; DOI: 10.1158/0008-5472.CAN-14-0130

Immediate Utility of Two Approved Agents to Target Both the Metabolic Mevalonate Pathway and Its Restorative Feedback Loop

Aleksandra Pandyra[1,2], Peter J. Mullen[1], Manpreet Kalkat[1,2], Rosemary Yu[1,2], Janice T. Pong[1,2], Zhihua Li[1], Suzanne Trudel[1,2], Karl S. Lang[3], Mark D. Minden[1,2], Aaron D. Schimmer[1,2], and Linda Z. Penn[1,2]

Abstract

New therapies are urgently needed for hematologic malignancies, especially in patients with relapsed acute myelogenous leukemia (AML) and multiple myeloma. We and others have previously shown that FDA-approved statins, which are used to control hypercholesterolemia and target the mevalonate pathway (MVA), can trigger tumor-selective apoptosis. Our goal was to identify other FDA-approved drugs that synergize with statins to further enhance the anticancer activity of statins *in vivo*. Using a screen composed of other FDA approved drugs, we identified dipyridamole, used for the prevention of cerebral ischemia, as a potentiator of statin anticancer activity. The statin–dipyridamole combination was synergistic and induced apoptosis in multiple myeloma and AML cell lines and primary patient samples, whereas normal peripheral blood mononuclear cells were not affected. This novel combination also decreased tumor growth *in vivo*. Statins block HMG-CoA reductase (HMGCR), the rate-limiting enzyme of the MVA pathway. Dipyridamole blunted the feedback response, which upregulates HMGCR and HMG-CoA synthase 1 (HMGCS1) following statin treatment. We further show that dipyridamole inhibited the cleavage of the transcription factor required for this feedback regulation, sterol regulatory element–binding transcription factor 2 (SREBF2, SREBP2). Simultaneously targeting the MVA pathway and its restorative feedback loop is preclinically effective against hematologic malignancies. This work provides strong evidence for the immediate evaluation of this novel combination of FDA-approved drugs in clinical trials. *Cancer Res; 74(17); 4772–82. ©2014 AACR.*

Introduction

There is an urgent need for novel therapeutic strategies in treating both acute myelogenous leukemia (AML) and multiple myeloma, especially in heavily pretreated and relapsed patients. Despite recent advances in multiple myeloma treatment, it is difficult to achieve progression-free survival beyond 36 months (1). In AML, survival is poor following relapse and 40% to 50% of older patients with AML and 20% to 30% of younger patients with AML will experience primary inductive failure (2).

Statins, potent inhibitors of the rate-limiting enzyme in the mevalonate (MVA) pathway, HMGCR (3), are used in the treatment of patients with hypercholesterolemia (Fig. 1A). Their frequent use in the prevention of adverse cardiovascular events has led to epidemiologic evidence suggesting that statin use may reduce cancer incidence (4–6). In hematologic malignancies, it has been shown that statins can trigger tumor-specific apoptosis (7–11). These apoptotic effects have been attributed to direct inhibition of HMGCR in tumor cells followed by depletion of fundamental MVA-derived end products such as isoprenoids and cholesterol (9, 12–14). In tumor cells, dysregulation of the MVA pathway has been postulated to be responsible for the observed therapeutic index. Higher tumor expression levels of HMGCR and other MVA pathway enzymes are associated with poor prognosis and reduced survival in patients with cancer (15, 16). Dysregulation of the MVA pathway's restorative sterol feedback response occurs in both multiple myeloma (8) and AML (17, 18). Taken together, dysregulation of the MVA pathway in hematologic malignancies provides a strong rationale for statin therapy.

Early dose-finding prospective clinical trials established that statins can be tolerated at concentrations exceeding cholesterol-lowering doses, which range from 20 to 80 mg/d (19, 20). High doses of statins can be tolerated in the clinical cancer setting, but the ideal dosing regimen remains unclear as efficacy has been observed with high (20) and cholesterol-lowering (21, 22) doses.

[1]Princess Margaret Cancer Centre, Toronto, Ontario, Canada. [2]Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada. [3]Institute of Immunology, University Hospital, University of Duisburg-Essen, Essen, Germany.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Linda Z. Penn, Toronto Medical Discovery Tower, 13th Floor 13-706, 101 College St., Toronto, Ontario, Canada M5G 1L7. Phone: 416-634-8770; Fax: 416-634-8771; E-mail: lpenn@uhnres.utoronto.ca

doi: 10.1158/0008-5472.CAN-14-0130

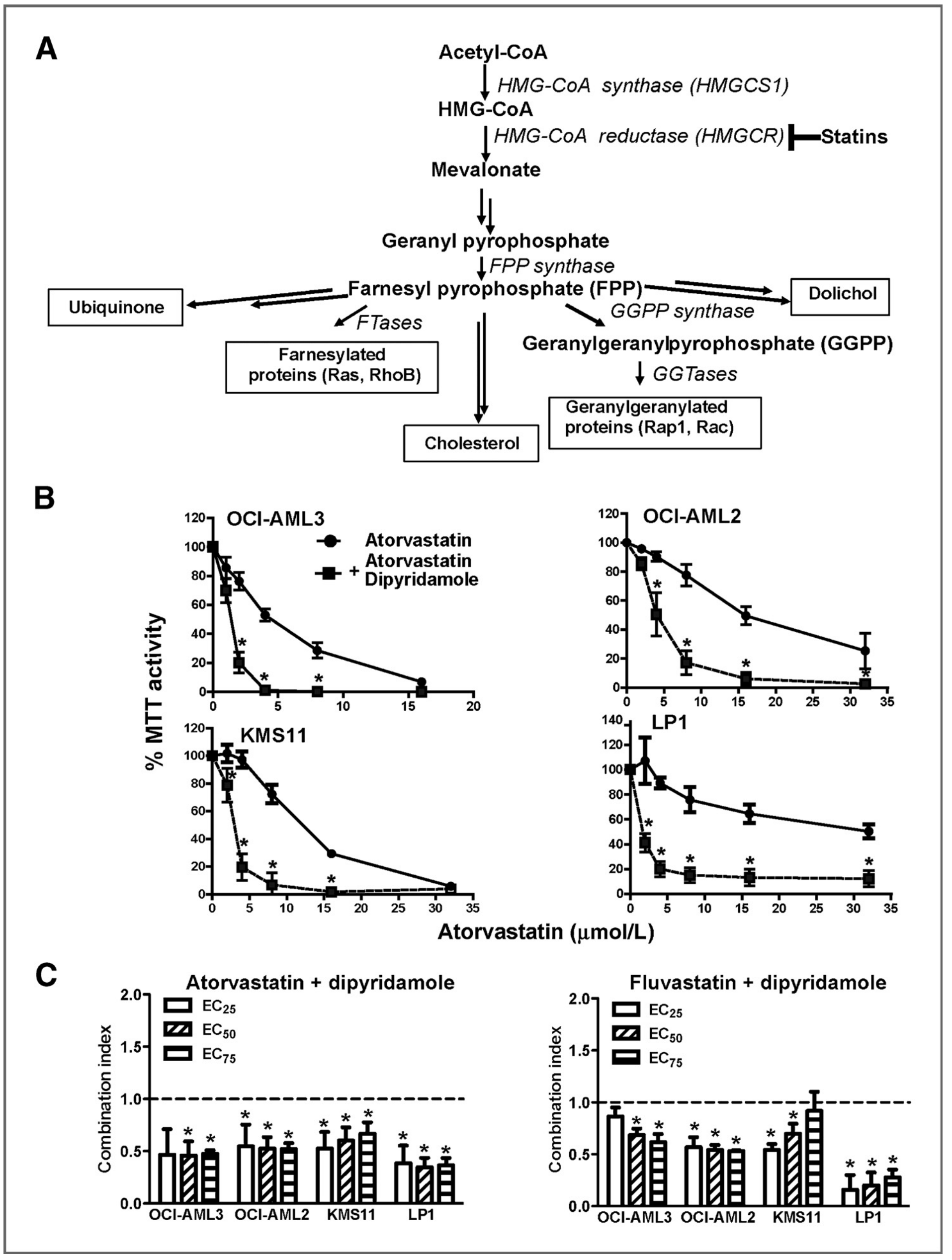

Figure 1. Dipyridamole potentiates the anticancer effects of atorvastatin, an inhibitor of the MVA pathway. A, a simplified schematic of the MVA pathway. B, dipyridamole potentiated the anticancer effects of atorvastatin in AML (OCI-AML3 and OCI-AML2) and multiple myeloma (KMS11 and LP1) cell lines as assessed by MTT assay following 48 hours of compound exposure. *, $P < 0.05$ (t test, unpaired, 2-tailed). C, AML and multuiple myeloma cell lines were exposed to a dose range of atorvastatin, fluvastatin, dipyridamole, and combinations in a fixed ratio. Dose–response curves were generated. Synergy was evaluated using the CI. CI < 1 indicates synergy, CI = 1 indicates additivity, and CI > 1 indicates antagonism. The EC_{50} (50% effective concentration), EC_{25}, and EC_{75} are shown for atorvastatin (C, left) and fluvastatin (C, right). *, $P < 0.05$ (one-sample t test, comparing EC values to 1.0) Data represent the mean ± SD of three independent experiments.

Statins have also been safely combined with the standard-of-care therapy regimens in patients with AML and multiple myeloma without serious side effects in inductive, consolidation, and maintenance therapy (20, 23). While this approach has shown some promise, there remain many nonresponsive patients (24), highlighting an urgent need to develop novel synergistic combinatorial approaches utilizing statin chemotherapy.

Building on promising results of statins as anticancer agents in AML and multiple myeloma, we conducted a pharmacologic screen of FDA-approved drugs in combination with statins to identify novel combinations with anticancer efficacy in hematologic malignancies. The screen identified dipyridamole, a commonly used anti-platelet agent, as potentiating the antiproliferative effects of statins in multiple myeloma cells. The combination, synergistic and capable of inducing apoptosis at low micromolar doses in AML and multiple myeloma cells, slowed tumor growth in a leukemia xenograft model and induced apoptosis in primary AML patient samples. Mechanistically, dipyridamole increased statin efficacy by blunting the MVA restorative feedback response through blocking the regulatory cleavage of the transcription factor, SREBP2. Taken together, these findings have not only uncovered a role for inhibiting MVA pathway feedback regulation as a mechanism to potentiate the anticancer efficacy of statins but also provided a strong rationale for the immediate utility of statin–

Published OnlineFirst July 3, 2014; DOI: 10.1158/0008-5472.CAN-14-0130

dipyridamole therapy for patients with AML and multiple myeloma.

Materials and Methods

Cell culture and compounds

Multiple myeloma cell lines were maintained in RPMI-1640 medium and AML cell lines in Alpha Modified Eagle's Medium (αMEM) and Iscove Modified Dulbecco Medium (IMDM). Media were supplemented with 10% FBS (GIBCO) and penicillin–streptomycin. OCI-AML2 and OCI-AML3 cells were established by and obtained from Drs. McCulloch and Mark Minden (the AML cell lines are proprietary to UHN and available through the German Tissue bank DSMZ) and are verified by Dr. Mark Minden every 6 months using STR-I profiling. KMS11 and LP1 cells, obtained from Dr. Suzanne Trudel (Princess Margaret Cancer Centre, Toronto, ON, Canada) are originally from JCBR and DSMZ cell banks, respectively. The cell lines are authenticated by DNA fingerprinting and multiplex PCR and tested for authenticity every 6 months by Dr. Suzanne Trudel. Cells were incubated at 37°C in 5% CO_2, and cell lines were routinely confirmed to be mycoplasma-free (MycoAlert Mycoplasma Detection Kit, Lonza). Atorvastatin calcium (21 CEC Pharmaceuticals LTD) and fluvastatin (US Biologicals) were dissolved in ethanol. Dipyridamole was dissolved in DMSO (Sigma).

Primary cells

Primary AML patient samples were obtained from consenting patients. PBSCs were obtained from healthy volunteers donating cells for allotransplantation and were granulocyte colony-stimulating factor (GCSF)-mobilized. Mononuclear cells were fractioned by Ficoll-Hypaque gradient sedimentation. Primary cells were cultured in IMDM medium supplemented with 20% FBS and 5% 5367-conditioned medium. Frozen primary cells were thawed and within 2 to 10 hours treated for 48 hours. PBSCs were obtained fresh and treated as indicated and as previously reported (8). Use and collection of human tissue for this study was approved by the University Health Network Institutional Review Board (Toronto, ON, Canada).

Chemical screen for cytotoxic drugs

Plates (96-well) of KMS11 cells (20,000 cells per well) were treated with aliquots of a chemical library (25) of 100 drugs dissolved in DMSO (3–50 μmol/L) using a Biomek FX Laboratory Automated Workstation (Beckman Coulter). One plate had been pretreated with 3.5 μmol/L of atorvastatin. Following 72 hours of incubation, MTS activity was assessed as previously described (25).

MTT, TUNEL, and Annexin V apoptosis assays

MTT assay (26) is a colorimetric assay measuring the reduction of the MTT substrate by oxidoreductase enzymes into formazan and is commonly used as an indirect readout of cellular viability. Briefly, 2×10^5 to 3×10^5 cells/mL were plated in 96-well plates and after 24 hours treated as indicated for 48 hours. Half-maximal inhibitory concentrations (IC_{50}) values were computed from dose–response curves using Prism (v5.0, GraphPad Software). For TUNEL assays, 2.5×10^5 cells/mL were seeded in 6-well plates and treated for 48 hours as indicated. Cells were fixed in ethanol, and staining was performed using terminal deoxynucleotide transferase–mediated dUTP nick end labeling (TUNEL) according to the manufacturer's instructions (APO-BRDU Apoptosis Kit, Phoenix Flow Systems). Annexin V apoptosis assays (Biovision) were carried out as per the manufacturer's protocol. Cells were analyzed for apoptosis by FACS (FACSCalibur cytometer, BD Biosciences).

Drug combination studies

Synergy between statins and dipyridamole was evaluated using the combination index (CI; ref. 27). Dose–response curves were generated for statins and dipyridamole alone and in combination at a constant ratio following compound exposure for 48 hours and assessed by MTT assay. CalcuSyn software (biosoft) was used to evaluate synergy using the median-effect model.

Immunoblotting

A total of 2.5×10^5 cells/mL were seeded in 6-well tissue culture plates and treated as indicated. For PARP (PARP1), SREBP2, total and unprocessed Rap1A detection, cells were washed with PBS and lysed using boiling hot SDS lysis buffer (1.1% SDS, 11% glycerol, 0.1 mol/L Tris, pH 6.8) with 10% β-mercaptoethanol. For HMGCR detection, cells were washed with PBS and lysed as previously described (15). Blots were probed with anti-tubulin (Santa Cruz Biotechnology), anti-PARP (Cell Signaling), anti-Rap1, anti-total and unprocessed Rap1A (Santa Cruz), anti-SREBP2 (BD Pharmingen), and anti-HMGCR (monoclonal A9, in-house).

Leukemia xenograft models

Severe-combined immunodeficiency (SCID) male mice (7- to 9-week-old), obtained from and housed in the Ontario Cancer Institute animal colony, were subcutaneously injected with 10^6 OCI-AML2 cells. When tumors became palpable (15 mm^3), mice were randomized and treated daily with 120 mg/kg dipyridamole administered intraperitoneally (i.p.; 5 mg/mL dipyridamole in 50 mg/mL polyethylene glycol 600, and 2 mg/mL tartaric acid), 50 mg/kg atorvastatin administered orally, a combination of dipyridamole and atorvastatin, or vehicle. Tumors were measured every 2 days using digital calipers and tumor volume was calculated using the following formula: (tumor length $\times$ width^2)/2. Animal work was carried out with the approval of the Princess Margaret Hospital ethics review board in accordance to the regulations of the Canadian Council on Animal Care.

Assessment of dipyridamole levels in serum

Levels of dipyridamole in serum were determined as previously described by spectrofluorometry using differences in the fluorescence of dipyridamole between acidic and basic conditions (28). For standard curves, dipyridamole control solutions were prepared using serum from untreated mice. Fluorescence (490 nmol/L, excitation at 420 nmol/L) was measured using a SpectraMax M5 plate reader (Molecular Devices).

Results

A screen of pharmacologically active drugs identifies dipyridamole as a potentiator of the anticancer effects of atorvastatin

To identify a combination of drugs with novel anticancer activities using an unbiased approach, we screened atorvastatin in combination with a library of 100 on- and off-patent drugs available in Canada (Supplementary Table S1), composed of antimicrobials and metabolic regulators (25). Well-known pharmacokinetic profiles, high achievable plasma concentrations, and a wide therapeutic index characterized the drugs in the library. The KMS11 multiple myeloma cell line was treated for 72 hours with either a sublethal dose of atorvastatin (0%–20% effect on MTS activity), each of the 100 drugs alone, or each in combination with atorvastatin. The combination of dipyridamole, a well-known anti-platelet agent and atorvastatin was found to decrease MTS activity (Supplementary Fig. S1A). Validation in AML and multiple myeloma cell lines

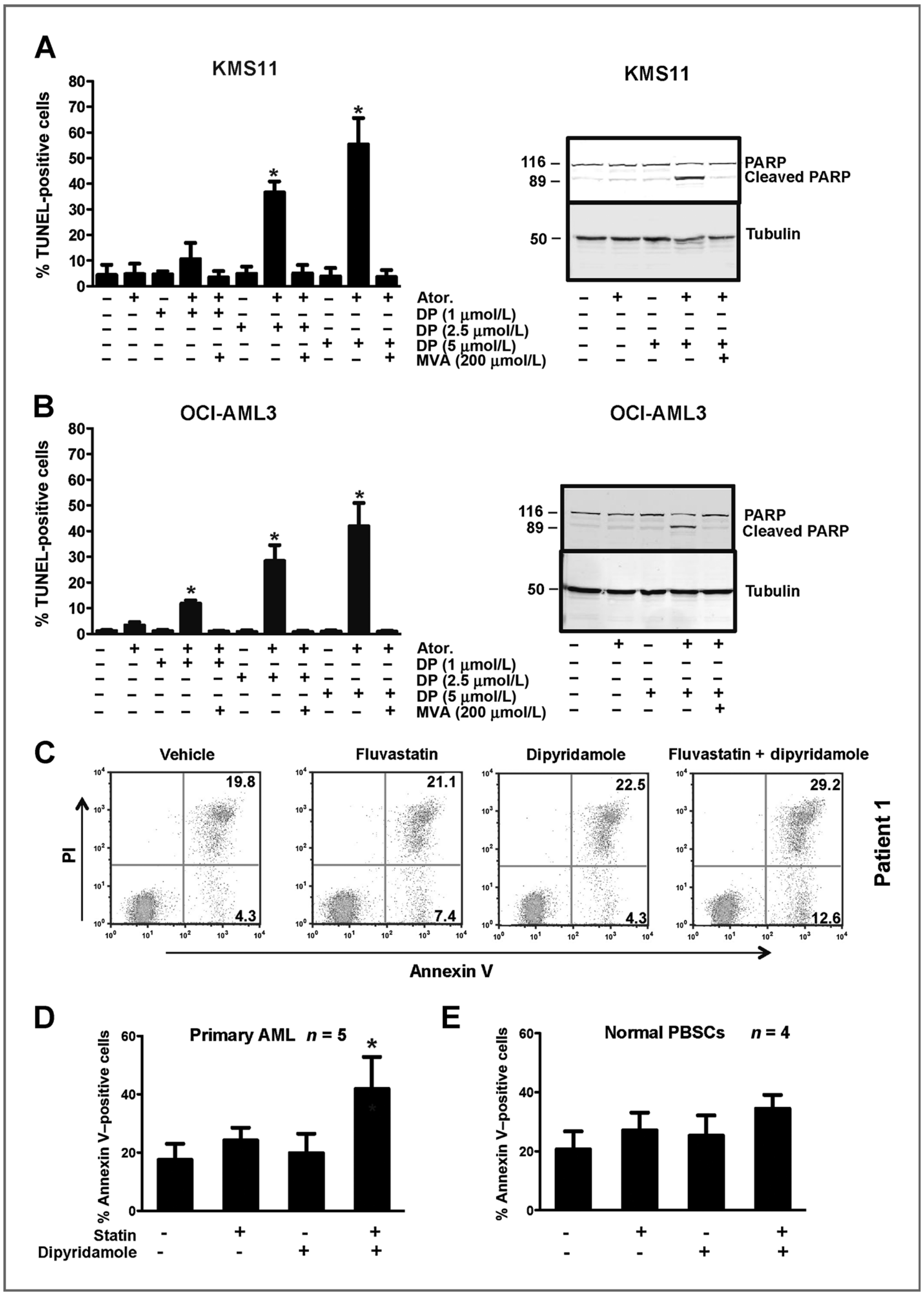

Figure 2. The statin–dipyridamole combination induces apoptosis in AML and multuiple myeloma cell lines and primary AML patient cells. Treatment of the KMS11 (A) or the OCI-AML3 (B) cell lines with low micromolar doses of atorvastatin (Ator.) and dipyridamole (DP) induced apoptosis following 48 hours of compound exposure as assessed by TUNEL (left). The atorvastatin–dipyridamole combination also caused PARP cleavage (right). Data represent the mean ± SD of at least three independent experiments. Primary AML patient cells were treated for 48 hours with vehicle, 5 μmol/L fluvastatin, 10 μmol/L dipyridamole, and the fluvastatin–dipyridamole combination (D, left, $n = 5$). Primary normal hematopoietic cells (PBSCs; D, right, $n = 4$) were treated for 48 hours with vehicle, 10 μmol/L atorvastatin, 10 μmol/L dipyridamole, or the atorvastatin–dipyridamole combination. Primary AML and PBSCs were assessed for AV/PI staining by flow cytometry. Percent apoptosis was evaluated by summing the AV^+/PI^- and AV^+/PI^+ quadrants. A representative primary AML patient sample is shown in C. *, $P < 0.05$ (one-way ANOVA with a Tukey posttest, the statin–dipyridamole group being significantly different than all other groups).

Published OnlineFirst July 3, 2014; DOI: 10.1158/0008-5472.CAN-14-0130

showed that dipyridamole was capable of significantly potentiating the anticancer effects of atorvastatin (Fig. 1B). Dipyridamole alone, used at a physiologically achievable concentration of 5 μmol/L, did not have significant effects on MTT activity (Supplementary Fig. S1B). Statins are often used interchangeably but structural differences of each statin governing key facets such as metabolism and lipophilicity impact not only their cholesterol-lowering efficacies but also anticancer effects. We therefore also evaluated fluvastatin, another lipophilic statin, and found that dipyridamole was also able to potentiate its anticancer effects (Supplementary Fig. S1C).

The combination of statins and dipyridamole is synergistically antiproliferative and induces apoptosis in AML and multiple myeloma cell lines and primary patient cells

We next evaluated whether the statin–dipyridamole combination was synergistic. We treated cells with increasing concentrations of statin and dipyridamole alone and in combination. Synergy at multiple effect levels was evaluated using the CI (27). The combination of atorvastatin or fluvastatin with dipyridamole synergistically decreased MTT activity at multiple effective concentrations in all AML and multiple myeloma cell lines (Fig. 1C).

The limitation of colorimetric assays such as the MTT assay is reliance on mitochondrial enzymes whose rates of conversion of the MTT substrates are used as an indirect measure of cell viability. As these assays do not directly assess apoptosis (29), we chose representative cell lines from the AML and multiple myeloma panel and measured apoptosis using TUNEL and PARP cleavage. We treated KMS11 (Fig. 2A, left) and OCI-AML3 (Fig. 2B, left) cells with atorvastatin and/or dipyridamole and found that there was a dramatic induction of apoptosis when atorvastatin was combined with dipyridamole with no effect of either drug alone. The apoptotic effect was abrogated with the co-administration of MVA and therefore was deemed to result specifically from the inhibition of HMGCR, the target of statins. Cleavage of PARP also occurred in KMS11 and OCI-AML3 cells (Fig. 2A and B, right, respectively) following exposure to the atorvastatin–dipyridamole combination.

To determine whether primary AML cells are sensitive to the combination, we exposed patient samples to statins and/or dipyridamole for 48 hours. Cell death was measured using Annexin V/propidium iodide (AV/PI) staining. As compared with TUNEL and PARP cleavage, the AV/PI stain requires fewer cells. Primary cells from patients with AML were treated with multiple doses of statins and dipyridamole (Table 1). The statin–dipyridamole combination significantly induced apoptosis in primary AML cells (Fig. 2C and D). Death was dose-dependent and observed at similar doses used in cell lines. The effects of the combination were minimal in PBSCs (Fig. 2E). Taken together, these data underscore the therapeutic utility of

Table 1. The statin–dipyridamole combination induces apoptosis in primary AML cells

	% Apoptosis[a]				
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Control	**22**	**21**	**9**	**16**	**20**
Fluvastatin 1.25 μmol/L	ND	ND	11	ND	ND
Fluvastatin 2.5 μmol/L	24	24	17	18	20
Fluvastatin 5 μmol/L	29	24	29	20	20
Atorvastatin 5 μmol/L	28	ND	ND	ND	21
Dipyridamole 2.5 μmol/L	ND	ND	16	16	ND
Dipyridamole 5 μmol/L	23	22	20	16	10
Dipyridamole 10 μmol/L	27	20	25	18	10
Fluvastatin 1.25 μmol/L + dipyridamole 2.5 μmol/L	ND	ND	23	ND	ND
Fluvastatin 2.5 μmol/L + dipyridamole 2.5 μmol/L	ND	ND	39	ND	ND
Fluvastatin 2.5 μmol/L + dipyridamole 5 μmol/L	26	31	38	21	18
Fluvastatin 2.5 μmol/L + dipyridamole 10 μmol/L	32	43	43	21	24
Fluvastatin 5 μmol/L + dipyridamole 5 μmol/L	33	34	ND	21	25
Fluvastatin 5 μmol/L + dipyridamole 10 μmol/L	42	46	55	25	40
Atorvastatin 5 μmol/L + dipyridamole 5 μmol/L	ND	ND	ND	ND	21
Atorvastatin 5 μmol/L + dipyridamole 10 μmol/L	38	ND	ND	ND	29

NOTE: Patients 1 and 3 were classified into the intermediate prognosis groups based on cytogenetics and patients 2, 4, and 5 were classified into adverse prognosis groups.

Abbreviation: ND, not determined.

[a]Following treatment for 48 hours, primary cells were assessed for AV/PI staining by flow cytometry, and percent apoptosis was evaluated by summing the AV^+/PI^- and AV^+/PI^+ quadrants.

Published OnlineFirst July 3, 2014; DOI: 10.1158/0008-5472.CAN-14-0130

the statin–dipyridamole combination in AML and patient samples.

The combination of statins and dipyridamole delays tumor growth in leukemia xenografts

To evaluate the statin–dipyridamole combination *in vivo*, we treated SCID mice harboring established xenografts of OCI-AML2 cells. We chose to orally administer atorvastatin because this is the route of delivery for humans. In addition, atorvastatin has a longer serum half-life than other statins (30) and has previously demonstrated *in vivo* efficacy (8). Gastric pH levels differ between mice and humans, and the highly acidic pH of mice has been reported to impair the oral bioavailability of dipyridamole (31) and so we intraperitoneally administered dipyridamole. The dipyridamole concentration in serum of mice treated with dipyridamole reached micromolar concentrations (Fig. 3A) and was comparable with the doses used in our cell culture studies. The combination of atorvastatin and dipyridamole significantly decreased final tumor weight (Fig. 3B) and tumor volume (Fig. 3C).

Dipyridamole enhances the effects of statin-induced MVA pathway inhibition

The mechanism of dipyridamole's proapoptotic activity in combination with statins remained unclear. Dipyridamole at the low micromolar concentrations used to potentiate statin-induced apoptosis has no anticancer efficacy as a single agent. Furthermore, as the statin–dipyridamole apoptosis was reversible by the concomitant addition of MVA, we wondered whether dipyridamole was influencing the mechanism of statin-induced death at the molecular level. The isoprenylation arm of the MVA pathway (Fig. 1A) is functionally critical for statins to trigger apoptosis of tumor cells (13, 32, 33). We first tested whether dipyridamole contributed to the inhibition of isoprenylation by assessing protein levels of unprocessed Rap1A, a small GTPase that is geranylgeranyled (34). Addition of dipyridamole increased statin-induced accumulation of unprocessed Rap1A in KMS11 and OCI-AML3 cells (Fig. 4A and Supplementary Fig. S2) shown 16 hours posttreatment but also evident at later time points (Supplementary Fig. S2A). Addition of dipyridamole also increased statin-induced accumulation of unprocessed Rap1A in LP1 and OCI-AML2 cells (Fig. 5F and Supplementary Fig. S2C, respectively). Another reported consequence of MVA depletion and the downstream isoprenylation block is the transcriptional upregulation of RHOB (35), a member of the family of small GTPases involved in cytoskeletal motility, vesicle trafficking, and cell adhesion signaling. Consistent with our Rap1A results, we further saw that dipyridamole potentiated statin-induced RhoB mRNA increases in KMS11 and OCI-AML3 cells (Fig. 4B). The dependence of RhoB upregulation on MVA depletion was confirmed by using apoptosis-inducing doses of statins, which also caused similar RhoB mRNA increases as observed with the statin–dipyridamole combination (Fig. 4B).

We next investigated whether the role of dipyridamole as a reported P-glycoprotein (ABCB1, P-gp) inhibitor (36) could be potentiating statin-induced apoptosis. P-gp is an ATP-binding cassette transporter; its overexpression in cancer cells can

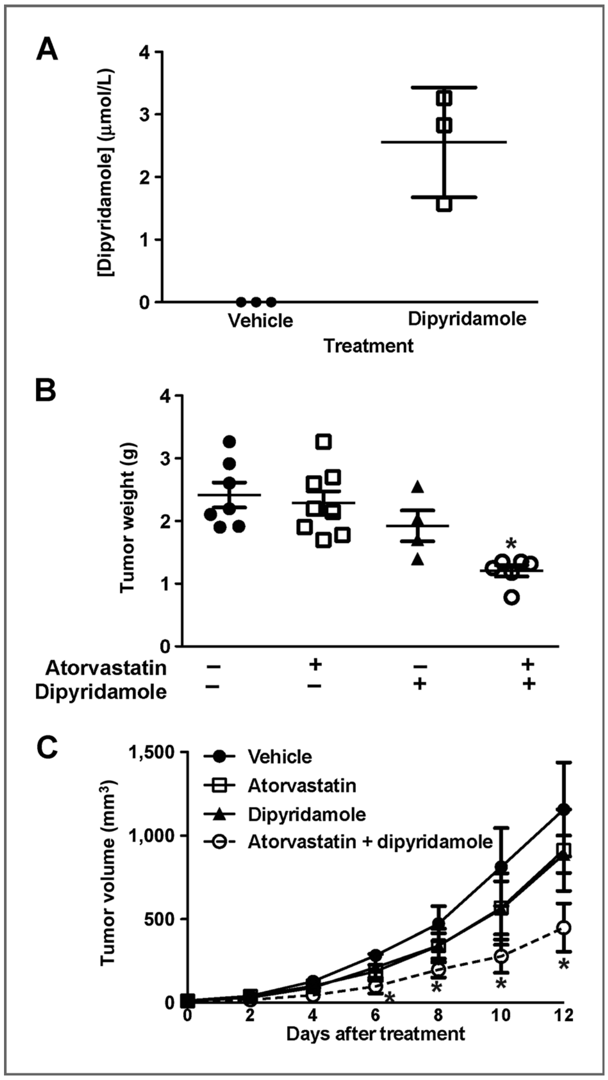

Figure 3. The statin–dipyridamole combination delays tumor growth in leukemia xenografts. A, plasma concentrations of dipyridamole 3 hours postadministration of 120 mg/kg dipyridamole and vehicle i.p. B, SCID mice were injected subcutaneously with 10^6 OCI-AML2 cells. After tumors were palpable, mice were randomized into groups and treated daily with 50 mg/kg atorvastatin orally, 120 mg/kg dipyridamole (i.p.), a combination of dipyridamole and atorvastatin or vehicle. Tumor volume was measured every two days. After 14 days of treatment, mice were sacrificed and tumors were resected and weighed (C). *, $P < 0.05$ (one-way ANOVA with a Tukey posttest, the statin–dipyridamole group being significantly different than all other groups. For tumor weights, the statin-dipyridamole group was significantly different than the PBS and the atorvastatin groups. Data represent the mean ± SD and are representative of two independent *in vivo* experiments, both showing similar results.

contribute to efflux of drugs leading to treatment resistance. We determined whether dipyridamole could modulate P-gp by analyzing a pair of MM 8226 cells lines (37), one parental (8226) and one overexpressing P-gp (8226_{DOX}; Supplementary Fig. S3A). Dose–response curves of doxorubicin, a P-gp substrate, were generated with and without dipyridamole. The doxorubicin IC_{50} values in the 8226_{DOX} cells were in the high

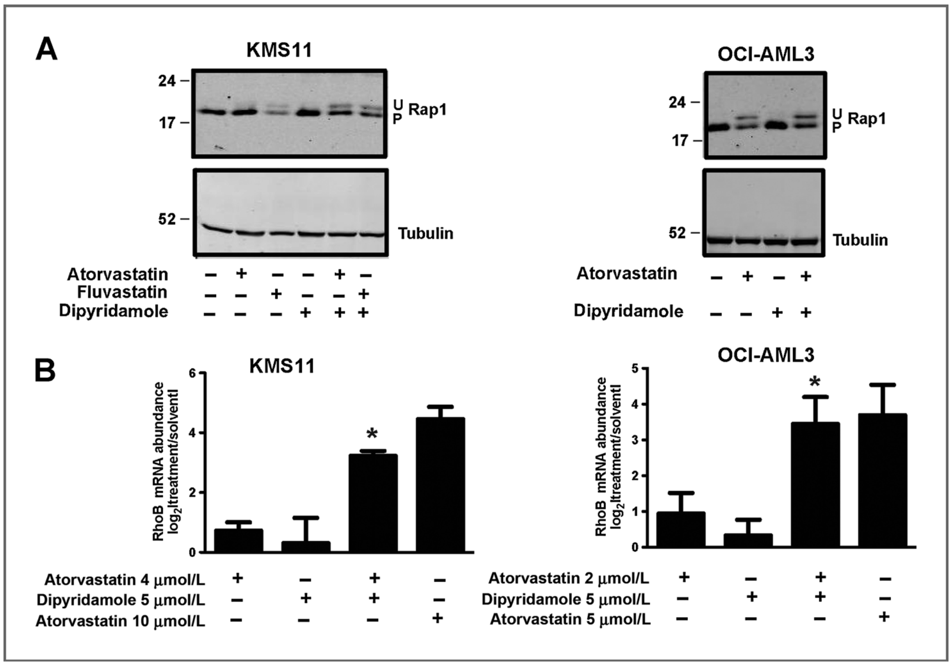

Figure 4. Dipyridamole enhances the effects of statin-induced MVA pathway inhibition. A, the addition of 5 μmol/L dipyridamole to 2 μmol/L fluvastatin or atorvastatin (4 μmol/L in KMS11 and 2 μmol/L in OCI-AML3 cells) increased the accumulation of unprocessed (U) relative to processed (P) Rap1 (RAP1A) 16 hours posttreatment in KMS11 (left) and OCI-AML3 (right) cells. Immunoblots are representative of three independent experiments. B, KMS11 and OCI-AML3 cells were treated as indicated for 16 hours and assayed for RhoB mRNA expression relative to GAPDH. *, $P < 0.05$ (one-way ANOVA with a Tukey posttest, the atorvastatin–dipyridamole group being significantly different than the atorvastatin lower dose and dipyridamole group). Data represent the mean ± SD of at least three independent experiments.

micromolar range upon addition of dipyridamole compared with the nanomolar range in the parental 8226 cells (Supplementary Fig. S3B). If dipyridamole were blocking P-gp, then the IC_{50} value of doxorubicin would decrease in the 8226_{DOX} cells, but this was not evident at the concentrations used in this study. Thus, evidence shows that dipyridamole does not inhibit P-gp, supporting the concept that dipyridamole does not contribute to the observed synergy by blocking statin efflux. Taken together, we have demonstrated that dipyridamole potentiates statin-induced apoptosis by blocking protein isoprenylation in a P-gp–independent manner.

Dipyridamole suppresses the sterol feedback loop through inhibition of SREBP2 cleavage

We were intrigued that the LP1 cells also showed a strong growth reduction in response to the statin–dipyridamole combination (Fig. 1B and C). The LP1 cells have been previously characterized as being insensitive to the proapoptotic effects of statins (32) and this was molecularly linked to a robust upregulation of HMGCR and other sterol-responsive genes following statin exposure (8). In response to sterol depletion, as occurs following statin treatment, feedback mediated by the transcription factor SREBP2 results in the transcriptional induction of sterol-responsive genes such as HMGCR and HMGCS1 (38). Treatment of the LP1 cells with the statin–dipyridamole combination resulted in significant apoptosis induction not achieved with higher statin doses (Fig. 5A). Remarkably, the HMGCR upregulation observed with statin treatment was decreased upon treatment with the statin–dipyridamole combination (Fig. 5B and Supplementary Fig. S4A), a phenomenon occurring at early time points during treatment and before any significant apoptosis induction. As expected, exposure to statins also caused an induction of HMGCS1 and the low-density lipoprotein receptor (LDLR; LDLr) and this increase was also suppressed with dipyridamole co-treatment (Fig. 5C and Supplementary Fig. S4B). HMGCS1 protein levels were similarly affected (Fig. 5D). As SREBP2 mRNA levels remained unaffected by concomitant statin–dipyridamole treatment when compared with the statin-only treatment (Fig. 5C), we examined whether SREBP2 cleavage, which occurs before translocation into the nucleus, was affected by the statin–dipyridamole combination. Indeed, the statin–dipyridamole combination inhibited statin-induced SREBP2 cleavage (Fig. 5E). HMGCR and HMGCS1 statin-induced upregulation was also observed in AML cells and was similarly decreased upon treatment with the statin–dipyridamole combination as was SREBP2 cleavage (Supplementary Fig. S5). Importantly, the statin–dipyridamole combination prevented the upregulation of HMGCR mRNA and protein in primary AML cells responsive to the combination treatment (Fig. 5G). Taken together, we have demonstrated that targeting the MVA pathway using statins, while simultaneously suppressing the feedback whose purpose is to restore the depleted MVA-derived end products, is an effective antitumor therapeutic strategy.

Discussion

Statins demonstrate efficacy in the clinical cancer setting including hematologic malignancies (20, 24). Although the administration of higher than cholesterol-lowering fluvastatin or atorvastatin doses has not yet been evaluated in patients with cancer, the tolerability observed with other statins suggests that elevated doses will be similarly tolerated and that low micromolar range (2–5 μmol/L) doses used in our cell culture studies could be achievable in humans. Evidence shows that even cholesterol-lowering doses can decrease tumor burden in patients with cancer (21, 22). Thus, the optimal dose of statins to use for cancer patient treatment remains

Published OnlineFirst July 3, 2014; DOI: 10.1158/0008-5472.CAN-14-0130

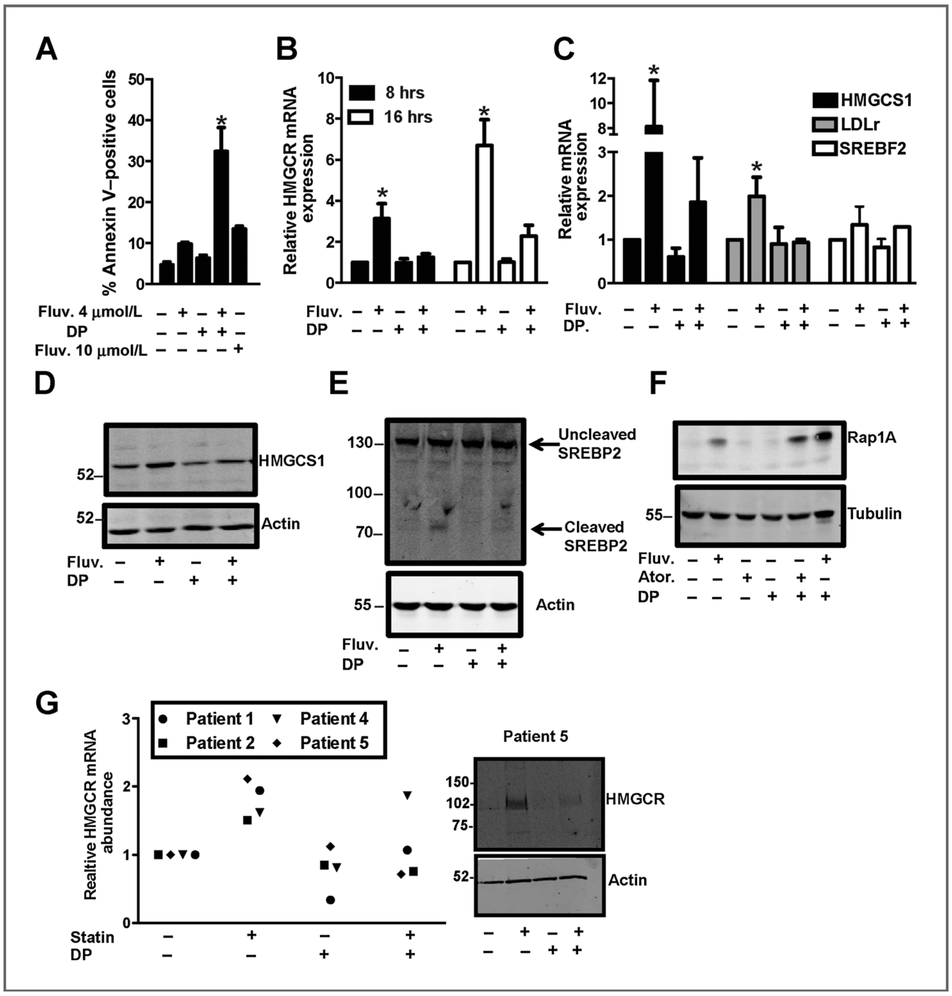

Figure 5. Dipyridamole prevents the statin-induced upregulation of HMGCR through inhibition of SREBP2 cleavage. A, LP1 cells were treated as indicated with fluvastatin (Fluv.) and 5 μmol/L of dipyridamole (DP) for 48 hours, and apoptosis was evaluated using AV staining. B, LP1 cells were treated with 4 μmol/L of fluvastatin (Fluv.) and 5 μmol/L dipyridamole (DP) for 8 and 16 hours, and RNA was harvested for HMGCR expression measured relative to GAPDH by real-time PCR. C, LP1 cells were treated with 10 μmol/L fluvastatin (Fluv.) and 5 μmol/L dipyridamole (DP) for 12 hours and RNA was harvested for HMGCS1, LDLr, and SREBP2 expression measured relative to GAPDH. Changes in mRNA expression are shown relative to vehicle control. *, $P < 0.05$ (1-way ANOVA with a Tukey posttest). Data represent the mean ± SD of three to six independent experiments. LP1 cells were treated with 10 μmol/L of fluvastatin (Fluv.) and 5 μmol/L dipyridamole (DP) for 12 hours, and protein was harvested for HMGCS1 (D) and SREBP2 (E) expression. F, LP1 cells were treated with 4 μmol/L of fluvastatin (Fluv.), 4 μmol/L of atorvastatin (Ator.), and 5 μmol/L dipyridamole (DP) for 16 hours, and protein was harvested for unprocessed RAP1A (Rap1A) expression. Immunoblots are representative of at least three independent experiments. G, left, primary AML cells were treated for 24 hours with vehicle, 5 μmol/L fluvastatin, 10 μmol/L dipyridamole, or the fluvastatin–dipyridamole combination and RNA was harvested and analyzed for HMGCR expression measured relative to GAPDH by real-time PCR (left). G, right, primary patient cells from patient 5 were treated with 20 μmol/L atorvastatin, 20 μmol/L dipyridamole, and the atorvastatin–dipyridamole combination for 24 hours and assayed for HMGCR protein.

unclear, yet evidence strongly suggests that effective dosing can be achieved *in vivo*.

Like all anticancer agents, it is optimal to administer drugs in multimodal and combinatorial treatment strategies to increase tumor-specific anticancer effects. Here, we provide a complimentary approach of combining statins with an already FDA-approved agent in the treatment of hematologic malignancies. Dipyridamole has been used as part of antithrombotic therapy for decades and its pharmacology has been thoroughly investigated. Dipyridamole is constantly being reformulated to maximize systemic exposure and extended release formulations have a reported half-life of 13.6 hours following typical 200 mg twice daily (b.i.d.) dosing with steady-state peak plasma concentrations of 1.0–4.0 μg/mL (2.0–7.9 μmol/L; ref. 39). However, much higher dipyridamole doses have been tolerated in humans as reported from overdose case reports (40), suggesting that dosing could potentially be elevated.

Our apoptosis assays in primary cells demonstrated that the combination of statin and dipyridamole was capable of inducing apoptosis in primary AML patient samples but not in primary normal PBSCs. Ultimately, leukemic progenitor colony formation assays using patients with AML and healthy donor samples would have further evaluated the

Published OnlineFirst July 3, 2014; DOI: 10.1158/0008-5472.CAN-14-0130

therapeutic efficacy and potential hemotoxic effects of the statin–dipyridamole combination, as these are long-term assays that more accurately recapitulate the microenvironment of the disease. However, clinical studies have been conducted that show that the statin–dipyridamole combination is safe and well tolerated in humans when assessed for effects on cardiovascular protection (41) and renal function (42). Therefore, we predict that a safe therapeutic window exists as this combination has been previously safely administered to humans.

Vulnerability of tumor cells to MVA pathway inhibition through statin administration has been attributed to dependence on MVA-derived end products, particularly those utilized for protein isoprenylation. Increased demands for such end products from tumor cells are met through dysregulation of the MVA pathway at multiple levels. The natural homeostatic feedback mechanism triggered in response to MVA pathway inhibition can inhibit statin efficacy (8) by inducing genes such as HMGCR and HMGCS1. Blocking this restorative feedback response through the addition of dipyridamole broadens statins' therapeutic window in tumor cells such as the LP1 cells where the feedback response was previously shown to be an impediment to statin-induced cell death (8). Recently, a window of opportunity clinical trial in patients with breast cancer demonstrated that antitumor responses in patients treated with atorvastatin were correlated with basal HMGCR expression levels (43). Interestingly, HMGCR expression was also elevated post-atorvastatin treatment, leading us to postulate that statin efficacy might also be increased with concomitant block of this feedback response *in vivo* as we observed in our *ex vivo* analyses of AML primary samples. Our results strongly suggest that dipyridamole may be immediately used in combination with statins in cancer patient clinical trials to directly evaluate the hypothesis that blocking the feedback response to statins potentiates anticancer efficacy.

Our data suggest that following statin challenge, dipyridamole inhibits the feedback response by blocking SREBP2 cleavage and nuclear accumulation, thereby resulting in decreased HMGCR and HMGCS1 mRNA expression, a hallmark of the statin and dipyridamole apoptotic response (Supplementary Fig. S6). How dipyridamole contributes to the inhibition of SREBP2 cleavage remains to be elucidated. At the molecular level, dipyridamole is known to inhibit the equilibrative nucleoside transporter (ENT1; ref. 44) and glucose uptake (45). Also, dipyridamole is a multi-isoform phosphodiesterase (PDE) inhibitor with varying degrees of inhibition reported for PDE 5, 6, 7, 8, 10, 11 (46) and has an ability to increase both cAMP and cGMP levels in cell culture and *in vivo*. It is unknown which, if any, of these antithrombotic activities play a role in the potentiation of statin-induced death in tumor cells. Dipyridamole been shown to potentiate classical chemotherapeutic drugs mainly through P-gp modulation and by blocking nucleoside transport (47). Recently, it was shown that dipyridamole alone delays tumor growth in breast cancer xenografts (48), but in our hands, using low micromolar concentrations, dipyridamole did not have appreciable anticancer activity as a single agent. This is in line with the observation that dipyridamole's antiproliferative effects were only observed when tumor cells were simultaneously challenged with statin, thereby triggering the feedback loop that was suppressed by the addition of dipyridamole.

The MVA pathway is targetable in many other tumor types and the statin–dipyridamole combination is likely applicable in other settings. Our work suggests that by combining statins with other agents that block SREBP2 activity antitumor efficacy will be increased. Importantly, we have demonstrated that by effectively dampening a pathway's restorative feedback loop, tumor apoptosis can be maximized. This reinforces the emerging broader concept in cancer treatment strategies that suggests blocking the feedback response to the anticancer agent under investigation can potentiate therapeutic activity and efficacy (49). In summary, we have identified a synergistic combination of two FDA-approved drugs that is preclinically effective in treating AML and multiple myeloma. These studies may serve as a foundation for developing a phase I clinical trial involving the combination of statins and dipyridamole for the treatment of AML and multiple myeloma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The views expressed do not necessarily reflect those of the Ontario Ministry of Health and Long Term Care.

Authors' Contributions

Conception and design: A. Pandyra, A.D. Schimmer, L.Z. Penn
Development of methodology: A. Pandyra, S. Trudel, A.D. Schimmer
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.J. Mullen, M. Kalkat, R. Yu, J.T. Pong, Z. Li, M.D. Minden
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Pandyra, P.J. Mullen, M. Kalkat, A.D. Schimmer, L.Z. Penn
Writing, review, and/or revision of the manuscript: A. Pandyra, P.J. Mullen, M. Kalkat, S. Trudel, K.S. Lang, M.D. Minden, A.D. Schimmer, L.Z. Penn
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Li
Study supervision: L.Z. Penn

Acknowledgments

The authors thank Alessandro Datti and Sumaiya Sharmeen for assistance with the chemical library, Craig Simpson and Marcela Gronda for assistance with animal work and PBSCs, and members of the Penn laboratory for review of the article.

Grant Support

This work was undertaken, in part, thanks to funding from the CRC Program (L.Z. Penn), the OICR through funding provided by the Province of Ontario (L.Z. Penn), the CBCF (A. Pandyra), the Knudson Fellowship (P.J. Mullen), Canadian Stem Cell Network (A.D. Schimmer), and the Ontario Ministry of Health and Long Term Care.

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.

Received January 15, 2014; revised May 15, 2014; accepted June 4, 2014; published OnlineFirst July 3, 2014.

References

1. Ludwig H, Durie BG, McCarthy P, Palumbo A, San Miguel J, Barlogie B, et al. IMWG consensus on maintenance therapy in multiple myeloma. Blood 2012;119:3003–15.
2. Ofran Y, Rowe JM. Treatment for relapsed acute myeloid leukemia: what is new? Curr Opin Hematol 2012;19:89–94.
3. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature 1990;343:425–30.
4. Ahern TP, Pedersen L, Tarp M, Cronin-Fenton DP, Garne JP, Silliman RA, et al. Statin prescriptions and breast cancer recurrence risk: a Danish nationwide prospective cohort study. J Natl Cancer Inst 2011;103:1461–8.
5. Fortuny J, de Sanjose S, Becker N, Maynadie M, Cocco PL, Staines A, et al. Statin use and risk of lymphoid neoplasms: results from the European Case-Control Study EPILYMPH. Cancer Epidemiol Biomarkers Prev 2006;15:921–5.
6. Nielsen SF, Nordestgaard BG, Bojesen SE. Statin use and reduced cancer-related mortality. N Engl J Med 2012;367:1792–802.
7. Wu J, Wong WW, Khosravi F, Minden MD, Penn LZ. Blocking the Raf/MEK/ERK pathway sensitizes acute myelogenous leukemia cells to lovastatin-induced apoptosis. Cancer Res 2004;64:6461–8.
8. Clendening JW, Pandyra A, Li Z, Boutros PC, Martirosyan A, Lehner R, et al. Exploiting the mevalonate pathway to distinguish statin-sensitive multiple myeloma. Blood 2010;115:4787–97.
9. Schmidmaier R, Baumann P, Simsek M, Dayyani F, Emmerich B, Meinhardt G. The HMG-CoA reductase inhibitor simvastatin overcomes cell adhesion-mediated drug resistance in multiple myeloma by geranylgeranylation of Rho protein and activation of Rho kinase. Blood 2004;104:1825–32.
10. Williams AB, Li L, Nguyen B, Brown P, Levis M, Small D. Fluvastatin inhibits FLT3 glycosylation in human and murine cells and prolongs survival of mice with FLT3/ITD leukemia. Blood 2012;120:3069–79.
11. Sassano A, Katsoulidis E, Antico G, Altman JK, Redig AJ, Minucci S, et al. Suppressive effects of statins on acute promyelocytic leukemia cells. Cancer Res 2007;67:4524–32.
12. Li HY, Appelbaum FR, Willman CL, Zager RA, Banker DE. Cholesterol-modulating agents kill acute myeloid leukemia cells and sensitize them to therapeutics by blocking adaptive cholesterol responses. Blood 2003;101:3628–34.
13. Xia Z, Tan MM, Wong WW, Dimitroulakos J, Minden MD, Penn LZ. Blocking protein geranylgeranylation is essential for lovastatin-induced apoptosis of human acute myeloid leukemia cells. Leukemia 2001;15:1398–407.
14. van der Weide K, de Jonge-Peeters S, Huls G, Fehrmann RS, Schuringa JJ, Kuipers F, et al. Treatment with high-dose simvastatin inhibits geranylgeranylation in AML blast cells in a subset of AML patients. Exp Hematol 2012;40:177–86.e6.
15. Clendening JW, Pandyra A, Boutros PC, El Ghamrasni S, Khosravi F, Trentin GA, et al. Dysregulation of the mevalonate pathway promotes transformation. Proc Natl Acad Sci U S A 2010;107:15051–6.
16. Freed-Pastor WA, Mizuno H, Zhao X, Langerod A, Moon SH, Rodriguez-Barrueco R, et al. Mutant p53 disrupts mammary tissue architecture via the mevalonate pathway. Cell 2012;148:244–58.
17. Tatidis L, Gruber A, Vitols S. Decreased feedback regulation of low density lipoprotein receptor activity by sterols in leukemic cells from patients with acute myelogenous leukemia. J Lipid Res 1997;38:2436–45.
18. Banker DE, Mayer SJ, Li HY, Willman CL, Appelbaum FR, Zager RA. Cholesterol synthesis and import contribute to protective cholesterol increments in acute myeloid leukemia cells. Blood 2004;104:1816–24.
19. van der Spek E, Bloem AC, van de Donk NW, Bogers LH, van der Griend R, Kramer MH, et al. Dose-finding study of high-dose simvastatin combined with standard chemotherapy in patients with relapsed or refractory myeloma or lymphoma. Haematologica 2006;91:542–5.
20. Kornblau SM, Banker DE, Stirewalt D, Shen D, Lemker E, Verstovsek S, et al. Blockade of adaptive defensive changes in cholesterol uptake and synthesis in AML by the addition of pravastatin to idarubicin + high-dose Ara-C: a phase 1 study. Blood 2007;109:2999–3006.
21. Minden MD, Dimitroulakos J, Nohynek D, Penn LZ. Lovastatin induced control of blast cell growth in an elderly patient with acute myeloblastic leukemia. Leuk Lymphoma 2001;40:659–62.
22. Garwood ER, Kumar AS, Baehner FL, Moore DH, Au A, Hylton N, et al. Fluvastatin reduces proliferation and increases apoptosis in women with high grade breast cancer. Breast Cancer Res Treat 2010;119:137–44.
23. Hus M, Grzasko N, Szostek M, Pluta A, Helbig G, Woszczyk D, et al. Thalidomide, dexamethasone and lovastatin with autologous stem cell transplantation as a salvage immunomodulatory therapy in patients with relapsed and refractory multiple myeloma. Ann Hematol 2011;90:1161–6.
24. van der Spek E, Bloem AC, Sinnige HA, Lokhorst HM. High dose simvastatin does not reverse resistance to vincristine, adriamycin, and dexamethasone (VAD) in myeloma. Haematologica 2007;92:e130–1.
25. Sharmeen S, Skrtic M, Sukhai MA, Hurren R, Gronda M, Wang X, et al. The antiparasitic agent ivermectin induces chloride-dependent membrane hyperpolarization and cell death in leukemia cells. Blood 2010;116:3593–603.
26. Dimitroulakos J, Ye LY, Benzaquen M, Moore MJ, Kamel-Reid S, Freedman MH, et al. Differential sensitivity of various pediatric cancers and squamous cell carcinomas to lovastatin-induced apoptosis: therapeutic implications. Clin Cancer Res 2001;7:158–67.
27. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984;22:27–55.
28. Oshrine B, Malinin A, Pokov A, Dragan A, Hanley D, Serebruany V. Criticality of pH for accurate fluorometric measurements of dipyridamole levels in biological fluids. Methods Find Exp Clin Pharmacol 2005;27:95–100.
29. Galluzzi L, Aaronson SA, Abrams J, Alnemri ES, Andrews DW, Baehrecke EH, et al. Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differ 2009;16:1093–107.
30. Shitara Y, Sugiyama Y. Pharmacokinetic and pharmacodynamic alterations of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors: drug-drug interactions and interindividual differences in transporter and metabolic enzyme functions. Pharmacol Ther 2006;112:71–105.
31. Kim HH, Sawada N, Soydan G, Lee HS, Zhou Z, Hwang SK, et al. Additive effects of statin and dipyridamole on cerebral blood flow and stroke protection. J Cereb Blood Flow Metab 2008;28:1285–93.
32. Wong WW, Clendening JW, Martirosyan A, Boutros PC, Bros C, Khosravi F, et al. Determinants of sensitivity to lovastatin-induced apoptosis in multiple myeloma. Mol Cancer Ther 2007;6:1886–97.
33. van der Weide K, Korthuis PM, Kuipers F, de Vries EG, Vellenga E. Heterogeneity in simvastatin-induced cytotoxicity in AML is caused by differences in Ras-isoprenylation. Leukemia 2012;26:845–8.
34. Vogt A, Qian Y, McGuire TF, Hamilton AD, Sebti SM. Protein geranylgeranylation, not farnesylation, is required for the G1 to S phase transition in mouse fibroblasts. Oncogene 1996;13:1991–9.
35. Holstein SA, Wohlford-Lenane CL, Hohl RJ. Consequences of mevalonate depletion. Differential transcriptional, translational, and post-translational up-regulation of Ras, Rap1a, RhoA, AND RhoB. J Biol Chem 2002;277:10678–82.
36. Verstuyft C, Strabach S, El-Morabet H, Kerb R, Brinkmann U, Dubert L, et al. Dipyridamole enhances digoxin bioavailability via P-glycoprotein inhibition. Clin Pharmacol Ther 2003;73:51–60.
37. Goard CA, Mather RG, Vinepal B, Clendening JW, Martirosyan A, Boutros PC, et al. Differential interactions between statins and P-glycoprotein: implications for exploiting statins as anticancer agents. Int J Cancer 2010;127:2936–48.
38. Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 1997;89:331–40.
39. Derendorf H, VanderMaelen CP, Brickl RS, MacGregor TR, Eisert W. Dipyridamole bioavailability in subjects with reduced gastric acidity. J Clin Pharmacol 2005;45:845–50.

40. Lagas JS, Wilhelm AJ, Vos RM, van den Dool EJ, van der Heide Y, Huissoon S, et al. Toxicokinetics of a dipyridamole (Persantin) intoxication: case report. Hum Exp Toxicol 2011;30:74–8.
41. Meijer P, Wouters CW, van den Broek PH, Scheffer GJ, Riksen NP, Smits P, et al. Dipyridamole enhances ischaemia-induced reactive hyperaemia by increased adenosine receptor stimulation. Br J Pharmacol 2008;153:1169–76.
42. Kano K, Nishikura K, Yamada Y, Arisaka O. Effect of fluvastatin and dipyridamole on proteinuria and renal function in childhood IgA nephropathy with mild histological findings and moderate proteinuria. Clin Nephrol 2003;60:85–9.
43. Bjarnadottir O, Romero Q, Bendahl PO, Jirstrom K, Ryden L, Loman N, et al. Targeting HMG-CoA reductase with statins in a window-of-opportunity breast cancer trial. Breast Cancer Res Treat 2013;138: 499–508.
44. Hammond JR. Interaction of a series of draflazine analogues with equilibrative nucleoside transporters: species differences and transporter subtype selectivity. Naunyn Schmiedebergs Arch Pharmacol 2000;361:373–82.
45. Hellwig B, Joost HG. Differentiation of erythrocyte-(GLUT1), liver-(GLUT2), and adipocyte-type (GLUT4) glucose transporters by binding of the inhibitory ligands cytochalasin B, forskolin, dipyridamole, and isobutylmethylxanthine. Mol Pharmacol 1991;40:383–9.
46. Schoeffter P, Lugnier C, Demesy-Waeldele F, Stoclet JC. Role of cyclic AMP- and cyclic GMP-phosphodiesterases in the control of cyclic nucleotide levels and smooth muscle tone in rat isolated aorta. A study with selective inhibitors. Biochem Pharmacol 1987;36:3965–72.
47. Burch PA, Ghosh C, Schroeder G, Allmer C, Woodhouse CL, Goldberg RM, et al. Phase II evaluation of continuous-infusion 5-fluorouracil, leucovorin, mitomycin-C, and oral dipyridamole in advanced measurable pancreatic cancer: a North Central Cancer Treatment Group Trial. Am J Clin Oncol 2000;23:534–7.
48. Spano D, Marshall JC, Marino N, De Martino D, Romano A, Scoppettuolo MN, et al. Dipyridamole prevents triple-negative breast-cancer progression. Clin Exp Metastasis 2013;30:47–68.
49. Prahallad A, Sun C, Huang S, Di Nicolantonio F, Salazar R, Zecchin D, et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. Nature 2012;483:100–3.

Published OnlineFirst July 3, 2014; DOI: 10.1158/0008-5472.CAN-14-0130

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

AACR American Association for Cancer Research

Immediate Utility of Two Approved Agents to Target Both the Metabolic Mevalonate Pathway and Its Restorative Feedback Loop

Aleksandra Pandyra, Peter J. Mullen, Manpreet Kalkat, et al.

Cancer Res 2014;74:4772-4782. Published OnlineFirst July 3, 2014.