Immediate Utility of Two Approved Agents to Target both the Metabolic Mevalonate Pathway and its Restorative Feedback loop

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Abstract

New therapies are urgently needed for hematological malignancies especially in relapsed acute myelogenous leukemia (AML) and multiple myeloma (MM) patients. 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 anti-cancer activity. The statin-dipyridamole combination was synergistic and induced apoptosis in MM and AML cell lines and primary patient samples, whereas normal peripheral blood mononuclear cells (PBSCs) 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 (SREBP2). Simultaneously targeting the MVA pathway and its restorative feedback loop is preclinically effective against hematological malignancies. This work provides strong evidence for the immediate evaluation of this novel combination of FDA approved drugs in clinical trials.
Introduction

There is an urgent need for novel therapeutic strategies in treating both acute myelogenous leukemia (AML) and multiple myeloma (MM) especially in heavily pretreated and relapsed patients. Despite recent advances in MM treatment, it is difficult to achieve progression free survival beyond 36 months (1). In AML, survival is poor following relapse and 40-50% of older AML patients and 20-30% of younger AML patients 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 (Figure 1A). Their frequent use in the prevention of adverse cardiovascular events has led to epidemiological evidence suggesting statin use may reduce cancer incidence (4-6). In hematological 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 cancer patients (15, 16). Dysregulation of the MVA pathway’s restorative sterol-feedback response occurs in both MM (8) and AML (17, 18). Taken together, dysregulation of the MVA pathway in hematological 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 form 20 to 80 mg/day (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.

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

Building on promising results of statins as anti-cancer agents in AML and MM, we conducted a pharmacological screen of FDA approved drugs in combination with statins to identify novel combinations with anti-cancer efficacy in hematological malignancies. The screen identified dipyridamole, a commonly used anti-platelet agent, as potentiating the anti-proliferative effects of statins in MM cells. The combination, synergistic and capable of inducing apoptosis at low micromolar doses in AML and MM 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 anti-cancer efficacy of statins, but provided a strong rationale for the immediate utility of statin-dipyridamole therapy for AML and MM patients.

**Materials and Methods**

**Cell Culture and Compounds.** MM 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 was supplemented with 10% fetal bovine serum (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 DNMZ) and are verified by Dr. Mark Minden every 6 months using STR-I profiling. KMS11 and LP1 cells, obtained from Dr. Suzanne Trudel (Toronto, Princess Margaret Cancer Centre) 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% CO₂ 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 2-10 hours, treated for 48 hours. PBSC 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).

**Chemical Screen for Cytotoxic Drugs.** 96-well plates of KMS11 cells (20000 cells/well) were treated with aliquots of a chemical library (25) of 100 drugs dissolved in DMSO (3-50 µM) using a Biomek FX Laboratory Automated Workstation (Beckman Coulter). One plate had been pre-treated with 3.5 µM of atorvastatin. Following 72 hours
of incubation, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) activity was assessed as previously described (25).

**MTT, TUNEL and Annexin V apoptosis assays.** (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (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, 2-3 x 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<sub>50</sub>) values were computed from dose-response curves using Prism (v5.0, GraphPad Software). For TUNEL assays, 2.5 x 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 dUTP nick end labeling (TUNEL) according to the manufacturer’s instructions (APO-BRDU Apoptosis Kit, Phoenix Flow Systems). Annexin V (AV) 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) (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.** 2.5 x 10^5 cells/ml were seeded in 6 well tissue-culture plates and treated as indicated. For PARP, SREBP2, Rap1 and Rap1A detection, cells were
washed with PBS and lysed using boiling hot SDS lysis buffer (1.1% SDS, 11% glycerol, 0.1M 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, Rap1A (Santa Cruz), anti-SREBP2 (BD Pharmingen) and anti-HMGCR (monoclonal A9, in-house).

**Leukemia xenograft models** Severe Combined Immunodeficiency (SCID) male mice (7-9 week old) were subcutaneously injected with 10⁶ OCI-AML2 cells. When tumors became palpable (15 mm³), mice were randomized and treated daily with 120 mg/kg dipyridamole administered intraperitoneally (i.p.) (5 mg/ml dipyridamole in 50mg/ml polyethylene glycol 600, and 2 mg/ml tartaric acid), 50 mg/kg atorvastatin administered orally (p.o.), a combination of dipyridamole and atorvastatin, or vehicle. Tumors were measured every two days using digital calipers and tumor volume was calculated using the following formula: (tumor length x width²)/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 nM, excitation at 420 nM) 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 1), 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 MM 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 Figure 1A). Validation in AML and MM cell lines showed that dipyridamole was capable of significantly potentiating the anti-cancer effects of atorvastatin (Figure 1B). Dipyridamole alone, used at a physiologically achievable concentration of 5 µM, did not have significant effects on MTT activity (Supplementary Figure 1B). Statins are often used interchangeably but structural differences of each statin governing key facets such as metabolism and lipophilicity not only impact their cholesterol-lowering efficacies but also anti-cancer effects. We therefore also evaluated fluvastatin, another lipophilic statin and found that dipyridamole was also able to potentiate its anti-cancer effects (Supplementary Figure 1C).

The combination of statins and dipyridamole is synergistically antiproliferative and induces apoptosis in AML and MM 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 combination index (CI) (27). The combination of atorvastatin or fluvastatin with dipyridamole synergistically decreased MTT activity at multiple effective concentrations in all AML and MM cell lines (Figure 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 MM panel, and measured apoptosis using TUNEL and PARP cleavage. We treated KMS11 (Figure 2A, left panel) and OCI-AML3 (Figure 2B, left panel) 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 (Figure 2A and B, right panels 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 to TUNEL and PARP cleavage, the AV/PI stain requires fewer cells. Primary cells from AML patients were treated with multiple doses of statins and dipyridamole (Table 1). The statin-dipyridamole combination significantly induced apoptosis in primary AML cells (Figure 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 (Figure 2E). Taken together, these data underscore the therapeutic utility of the statin-dipyridamole combination in AML and MM 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 compared to 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 (Figure 3A) and was comparable to the doses used in our cell culture studies. The combination of atorvastatin and dipyridamole significantly decreased final tumor weight (Figure 3B) and tumor volume (Figure 3C).

Dipyridamole enhances the effects of statin-induced MVA pathway inhibition. The mechanism of dipyridamole’s pro-apoptotic activity in combination with statins remained unclear. Dipyridamole at the low micromolar concentrations used to potentiate statin-induced apoptosis, has no anti-cancer 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 (Figure 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 Rap1, a small GTPase that is geranylgeranylated (34). Addition of dipyridamole increased statin-induced accumulation of unprocessed Rap1 in KMS11 and OCI-AML3 cells (Figure 4A and Supplementary Figure 2), shown sixteen hours post treatment but also evident at later time points.
(Supplementary Figure 2A. Addition of dipyridamole also increased statin-induced accumulation of unprocessed Rap1 in LP1 and OCI-AML2 cells (Figure 5F and Supplementary Figure 2C 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 Rap1 results, we further saw that dipyridamole potentiated statin-induced RhoB mRNA increases in KMS11 and OCI-AML3 cells (Figure 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 (Figure 4B).

We next investigated whether the role of dipyridamole as a reported P-glycoprotein (P-gp) inhibitor (36) could be potentiating statin-induced apoptosis. P-gp is an ATP-binding cassette transporter; its overexpression in cancer cells can 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 over-expressing P-gp (8226DOX) (Supplementary Figure 3A). Dose-response curves of doxorubicin, a P-gp substrate, were generated with and without dipyridamole. The doxorubicin IC50 values in the 8226DOX cells were in the high micromolar range upon addition of dipyridamole compared to the nanomolar range in the parental 8226 cells (Supplementary Figure 3B). If dipyridamole were blocking P-gp, then the IC50 value of doxorubicin would decrease in the 8226DOX 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 (Figure 1B an C). The LP1 cells have been previously characterized as being insensitive to the pro-apoptotic 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 (Figure 5A). Remarkably the HMGCR upregulation observed with statin treatment was decreased upon treatment with the statin-dipyridamole combination (Figure 5B and Supplementary Figure 4A), a phenomenon occurring at early time points during treatment and prior to any significant apoptosis induction. As expected, exposure to statins also caused an induction of HMGCS1 and the low-density lipoprotein receptor (LDLR) and this increase was also suppressed with dipyridamole co-treatment (Figure 5C and Supplementary Figure 4B). HMGCS1 protein levels were similarly affected (Figure 5D). As SREBP2 mRNA levels remained unaffected by concomitant statin-dipyridamole treatment when compared to the statin only treatment (Figure 5C) we examined whether SREBP2 cleavage, which occurs prior to translocation into the nucleus, was affected by the statin-dipyridamole combination. Indeed, the statin-dipyridamole combination inhibited statin-induced SREBP2 cleavage (Figure 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.
Importantly, the statin-dipyridamole combination prevented the upregulation of HMGCR mRNA and protein in primary AML cells responsive to the combination treatment (Figure 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 anti-tumor therapeutic strategy.

**Discussion**

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

Like all anti-cancer agents, it is optimal to administer drugs in multimodal and combinatorial treatment strategies to increase tumor-specific anti-cancer effects. Here, we provide a complimentary approach of combining statins with an already FDA-approved agent in the treatment of hematological malignancies. Dipyridamole has been used as part of antithrombotic therapy for decades and its pharmacology has been thoroughly investigated. Dipyridamole is constantly being re-formulated 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 µM) (39). However, much higher dipyridamole
doses have been tolerated in humans as reported from overdose case reports (40), suggesting 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 AML patients and healthy donor samples would have further evaluated the therapeutic efficacy and potential hemotoxic effects of the statin-dipyridamole combination, as these are longer-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) have been done. 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 thorough 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 breast cancer patients demonstrated that anti-tumor 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 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 anti-cancer efficacy.

Our data suggests 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 Figure 6). 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) (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 anti-cancer activity as a single agent. This is in line with the observation that dipyridamole’s anti-proliferative effects were only observed when tumor cells were simultaneously challenged with statin thereby triggering the feedback loop which 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 anti-tumor 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 anti-cancer 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 MM. 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 MM.

**Disclosure of Potential Conflict of Interest**

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Dipyridamole potentiates the anti-cancer effects of atorvastatin, an inhibitor of the MVA pathway. (A) A simplified schematic of the MVA pathway. (B) Dipyridamole potentiated the anti-cancer effects of atorvastatin in AML (OCI-AML3 and OCI-AML2) and MM (KMS11 and LP1) cell lines as assessed by MTT assay following 48 hrs of compound exposure. *p < 0.05 (t-test, unpaired, two-tailed). (C) AML and MM 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 combination index (CI). CI < 1 indicates synergy, CI = 1 indicates additivity and CI > 1 indicates antagonism. The EC50 (50% effective concentration) EC25 and EC75 are shown for atorvastatin (C, left panel) and fluvastatin (C, right panel). *p < 0.05 (one sample t-test, comparing EC values to 1.0) Data represent the mean ± SD of three independent experiments.

Figure 2. The statin-dipyridamole combination induces apoptosis in AML and MM 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 hrs of compound exposure as assessed by TUNEL (left panels). The atorvastatin-dipyridamole combination also caused PARP cleavage (right panels). Data represent the mean ± SD of at least three independent experiments. Primary AML patient cells were treated for 48 hrs with vehicle, 5 µM fluvastatin, 10 µM dipyridamole and the fluvastatin-dipyridamole combination (D, left panel, n = 5). Primary normal hematopoietic cells (PBSCs) (D, right panel, n = 4) were treated for 48 hrs with vehicle, 10 µM atorvastatin, 10 µM 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 post test, the statin-dipyridamole group being significantly different than all other groups).

**Figure 3. The statin-dipyridamole combination delays tumor growth in leukemia xenografts.** (A) Plasma concentrations of dipyridamole 3 hrs post-administration of 120 mg/kg dipyridamole and vehicle intraperitoneally (i.p.) (B) SCID mice were injected subcutaneously with 10⁶ OCI-AML2 cells. After tumors were palpable, mice were randomized into groups and treated daily with 50 mg/kg atorvastatin orally (p.o.), 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 post test, 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.
Figure 4. Dipyridamole enhances the effects of statin-induced MVA pathway inhibition. (A) The addition of 5 µM dipyridamole to 2 µM fluvastatin or atorvastatin (4 µM in KMS11 and 2 µM in OCI-AML3 cells) increased the accumulation of unprocessed (U) relative to processed (P) Rap1 16 hrs post treatment in KMS11 (left panel) and OCI-AML3 (right panel) cells. Immunoblots are representative of three independent experiments. (B) KMS11 and OCI-AML3 cells were treated as indicated for 16 hrs and assayed for RhoB mRNA expression relative to GAPDH. *p < 0.05 (one-way ANOVA with a Tukey post test, 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.

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 µM of dipyridamole (DP) for 48 hrs and apoptosis was evaluated using AV staining. (B) LP1 cells were treated with 4 µM of fluvastatin (Fluv.) and 5 µM dipyridamole (DP) for 8 and 16 hrs and RNA was harvested for HMGCR expression measured relative to GAPDH by real-time PCR. (C) LP1 cells were treated with 10 µM fluvastatin (Fluv.) and 5 µM dipyridamole (DP) for 12 hrs 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 post test). Data represent the mean ± SD of three to six independent experiments. LP1 cells were treated with 10 µM of fluvastatin (Fluv.) and 5 µM dipyridamole (DP.) for 12 hrs and protein was harvested for HMGCS1 (D) and SREBP2 (E) expression. (F) LP1 cells were treated with 4 µM of fluvastatin (Fluv.), 4 µM of atorvastatin (Ator.) and 5 µM dipyridamole (DP.) for 16 hrs and protein was harvested for
Rap1A expression. Immunoblots are representative of at least three independent experiments. (G, left panel) Primary AML cells were treated for 24 hrs with vehicle, 5 µM fluvastatin, 10 µM dipyridamole or the fluvastatin-dipyridamole combination and RNA was harvested and analyzed for HMGCR expression measured relative to GAPDH by real-time PCR (left panel). (G, right panel) Primary patient cells from patient 5 were treated with 20 µM atorvastatin, 20 µM dipyridamole and the atorvastatin-dipyridamole combination for 24 hrs and assayed for HMGCR protein.
**Figure 1**

**A**

![Metabolic pathway diagram involving Acetyl-CoA, HMG-CoA, Mevalonate, Geranyl pyrophosphate, Farnesyl pyrophosphate, Dolichol, Geranylgeranylpyprophosphate, Cholesterol, Ubiquinone, Farnesylated proteins (Ras, RhoB), and Geranylgeranylated proteins (Rap1, Rac).]

**B**

- **OCI-AML3**
  - Atorvastatin
  - Atorvastatin + Dipyridamole
  - % MTT Activity vs. Atorvastatin (uM)

- **KMS11**
  - % MTT Activity vs. Atorvastatin (uM)

- **OCI-AML2**
  - % MTT Activity vs. Atorvastatin (uM)

- **LP1**
  - % MTT Activity vs. Atorvastatin (uM)

- **OCI-AML3**
  - % MTT Activity vs. Atorvastatin (uM)

**C**

- **Atorvastatin + Dipyridamole**
  - Combination Index vs. EC25, EC50, EC75

- **Fluvastatin + Dipyridamole**
  - Combination Index vs. EC25, EC50, EC75
Figure 3

A

[Dipyriramole (µM)]

Vehicle Dipyridamole

Treatment

B

Tumour Weight (g)

Atorvastatin Dipyridamole

- + - + +

C

Tumour volume (mm³)

Vehicle Atorvastatin Dipyridamole Atorvastatin + dipyridamole

0 2 4 6 8 10 12

Days after treatment

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Figure 4

A

KMS11

\[\text{\textbf{Repl}}\]

\[\text{Tubulin}\]

\[\text{Atorvastatin}\]

\[\text{Fluvastatin}\]

\[\text{Olapatibazole}\]

OC1-AML3

\[\text{\textbf{Repl}}\]

\[\text{Tubulin}\]

\[\text{Atorvastatin}\]

\[\text{Fluvastatin}\]

\[\text{Olapatibazole}\]

B

KMS11

\[\text{\textbf{Repl}}\]

\[\text{Tubulin}\]

\[\text{Atorvastatin 1\,\mu M}\]

\[\text{Atorvastatin 2\,\mu M}\]

\[\text{Atorvastatin 5\,\mu M}\]

\[\text{Atorvastatin 10\,\mu M}\]

OC1-AML3

\[\text{\textbf{Repl}}\]

\[\text{Tubulin}\]

\[\text{Atorvastatin 1\,\mu M}\]

\[\text{Atorvastatin 2\,\mu M}\]

\[\text{Atorvastatin 5\,\mu M}\]

\[\text{Atorvastatin 10\,\mu M}\]
Table 1. The statin-dipyridamole combination induces apoptosis in primary AML cells

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<th>Patient 2</th>
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<tr>
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<td>16</td>
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<tr>
<td>Dipyridamole 5 μM</td>
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</table>

* Following treatment for 48 hours, primary cells were assessed for Annexin V (AV)/propidium iodide (PI) staining by flow cytometry, and percent apoptosis was evaluated by summing the AV+PI− and AV+PI+ quadrants.
Patients 1 and 3 were classified into the intermediate prognosis groups based on cytogenetics and patients 2, 4 and 5 into adverse prognosis groups.
ND indicates not determined.
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.

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