

Inhibition of Cysteine Cathepsin Protease Activity Enhances Chemotherapy Regimens by Decreasing Tumor Growth and Invasiveness in a Mouse Model of Multistage Cancer

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Abstract

Increases in protease expression and activity are associated with malignant progression and poor patient prognosis in a number of human cancers. Members of the papain family of cysteine cathepsins are among the protease classes that have been functionally implicated in cancer. Inhibition of the cysteine cathepsin family using a pan-cathepsin inhibitor, JPM-OEt, led to tumor regression in the RIP1-Tag2 (RT2) mouse model of pancreatic islet cell tumorigenesis. The present study was designed to determine whether this cathepsin inhibitor, when used in combination with chemotherapy, would increase antitumor efficacy. RT2 mice were treated in a late-stage regression trial with three different chemotherapy regimens, alone or in combination with the cathepsin inhibitor, JPM-OEt. Cyclophosphamide was administered in either a maximum tolerated dose (MTD) regimen, a “metronomic” continuous low-dose regimen, or a “chemo-switch” regimen consisting of MTD followed by metronomic dosing. Mice were sacrificed at a defined end point and tumor burden was assessed followed by a detailed analysis of cell proliferation, apoptosis, vascularization, and invasiveness in the treated and control lesions. An additional cohort of mice was followed for survival analysis. The cathepsin inhibitor plus the chemo-switch regimen of cyclophosphamide led to the most pronounced reduction in tumor burden and greatest increase in overall survival. Cysteine cathepsin inhibition resulted in a significant decrease in tumor invasiveness, which was further augmented in combination with each of the chemotherapy dosing regimens. These results encourage the development and continuing evaluation of cysteine cathepsin inhibitors as cancer therapeutics. [Cancer Res 2007;67(15):7378–85]

Introduction

Among the critical “hallmark capabilities” that cancers must acquire in their progression to malignancy are the ability to switch on angiogenesis, activate invasion, and promote metastasis (1). Proteolysis is central to all of these processes. Controlled degradation of the extracellular matrix (ECM) and vascular or epithelial basement membranes (BM) is necessary for angiogenesis

and invasion of tumor cells, both into the surrounding normal tissue and the blood and lymphatic systems. BM/ECM remodeling is mediated in an orchestrated manner by several families of matrix-degrading enzymes, including proteases of the cysteine, serine, and matrix metalloproteinase (MMP) classes, as well as endoglycosidases, such as heparanase. However, tumor cell growth, invasion, and metastasis rely not only on capabilities intrinsic to the cancerous cells but also on the ability of the tumor to effectively interact with and modify the tissue microenvironment, both locally and at distant metastatic sites. The tumor microenvironment consists of a multitude of cells including innate and adaptive immune cells, stromal fibroblasts, and blood and lymphatic vascular networks. Increasing awareness of the complex and reciprocal interactions between cancerous and stromal cells has broadened our understanding of how tumors develop and progress (2, 3) and, in the case of the tumor-associated vasculature, has already led to effective treatments (reviewed in ref. 4).

In many cases, it is the host stromal cells, rather than the cancer cells, that produce proteolytic enzymes in the tumor microenvironment, and several studies in preclinical cancer models have shown the critical importance of host-supplied proteases to tumor progression (5–7). Furthermore, numerous clinical reports have documented a significant association between the increased expression and/or activity of several members of these proteolytic enzyme families and poor prognosis (reviewed in refs. 8–10). Thus, proteolytic enzymes produced by tumor cells and/or surrounding tumor-associated stromal cells represent attractive candidates for anticancer therapies. However, clinical trials of MMP inhibitors were unsuccessful due to poor efficacy and toxicity (11), and preclinical trials involving xenograft tumor models were equivocal. More recent studies involving genetically engineered mouse models of cancer suggest that MMP inhibitors could have utility, if administered in the correct regimens and likely at early stages of tumor development (12, 13), providing that toxicities associated with MMP inhibition in normal tissues can be limited. Alternatively, the papain family of cysteine cathepsin proteases has been reported recently as a new class with potentially broader mechanistic efficacy and limited toxicity (14).

The human family of cysteine cathepsins includes 11 members (cathepsins B, C, F, H, K, L, O, S, V, W, and X), which share a conserved active site (15, 16). They primarily function as intracellular proteases mediating terminal nonspecific bulk proteolysis in the acidic environment of lysosomes (15). However, in a variety of malignant tumors, cysteine cathepsins are overexpressed and have altered localization to the invasive tumor margin (reviewed in ref. 16). Furthermore, their increased expression correlates with more aggressive tumors and poorer prognoses for

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patients (reviewed in ref. 8). *In vitro* studies have shown that inhibition of cysteine cathepsin activity can reduce tumor cell invasiveness and growth (17, 18). In addition, recent experiments in which *cathepsins B, L, or S* were deleted in transgenic mouse models of pancreatic islet or mammary cancer resulted in significant reductions in tumor burden (7, 19–21) and reduced invasiveness in the RIP1-Tag2 (RT2) model (19), thus validating these proteases as critical targets of the broad-spectrum cysteine cathepsin inhibitor, JPM-OEt. We showed previously that treatment with JPM-OEt impaired tumor growth, vascularity, and invasiveness; there was, however, no significant effect on tumor cell apoptosis (14). We hypothesized, therefore, that the addition of cytotoxic chemotherapy to cysteine cathepsin inhibition might improve antitumor efficacy by targeting these distinctive hallmark capabilities.

Cytotoxic chemotherapy has classically been given in a maximum tolerated dose (MTD) regimen. Large doses of chemotherapy are given in episodic dosing schedules to allow for recovery from cytotoxic effects on normal tissues, most notably in the rapidly dividing hematopoietic and mucosal lining cells (22). Recently, an alternative chemotherapeutic regimen, involving “metronomic” dosing, has been receiving increasing attention (23–25). Metronomic dosing provides a frequent, low dose of chemotherapy, typically administered daily or continuously. This dosing regimen was initially developed for its apoptotic effects on the tumor vasculature (25), but it has also been shown to have tumoricidal effects (26, 27). Prior studies showed that metronomic dosing of cyclophosphamide is a safe and efficacious treatment option in the mouse model of multistage cancer used in the present studies (28). Additional experiments have shown that a combination “chemo-switch” regimen using MTD cyclophosphamide followed by metronomic dose cyclophosphamide is more effective than either individual dosing schedule at reducing tumor growth and increasing survival in RT2 mice (29).

The RT2 transgenic mouse model of pancreatic islet cell carcinogenesis provides a highly reproducible model of tumorigenesis that progresses through defined sequential stages, including hyperplastic/dysplastic islets, angiogenic islets, and progressive grades of carcinoma (30). This model has been used extensively in preclinical trials to test the efficacy of various chemotherapeutic and antiangiogenic regimens when given at defined windows during the course of tumor progression (29, 31, 32). In the present study, we have chosen to use the regression trial design with treatment between 12 and 16 weeks of age to focus our investigation on well-established carcinomas. This situation is most comparable with the clinical setting in which a patient presents for treatment with a significant tumor burden. The chemotherapy regimen of cyclophosphamide, although not typically used for pancreatic neuroendocrine tumors, was chosen based on prior studies showing efficacy of this agent in this model system (28, 29). The cysteine cathepsin inhibitor JPM-OEt, which binds covalently in the active site and irreversibly inhibits the cysteine cathepsin family (14, 33), was used alone or in combination with the cyclophosphamide treatment regimens. In the present study, cathepsin inhibition was investigated in combination with cyclophosphamide when given in MTD, metronomic, or chemo-switch dosing regimens.

Materials and Methods

Experimental animals. The generation of RT2 mice as a model of pancreatic islet cell carcinogenesis has been reported previously (30). Mice

were maintained in accordance with institutional guidelines at Memorial Sloan-Kettering Cancer Center (MSKCC) and University of California at San Francisco governing the care of laboratory animals. From 12 weeks of age, all RT2 mice received 50% sugar food (Harlan Teklad) and 5% sugar water to relieve hypoglycemia induced by the insulin-secreting tumors.

Drugs and treatments. The cysteine cathepsin inhibitor JPM-OEt was synthesized (34) and administered as described previously (14). JPM-OEt was administered in 20% DMSO/80% H₂O twice daily by i.p. injection for a total dose of 50 mg/kg/d. The MTD regimen of cyclophosphamide (Sigma) was delivered as a previously reported 21-day cycle of 105 mg/kg administered i.p. every other day for a total of three doses, followed by 2 weeks of rest (24). An additional three doses were given to complete the 4-week treatment period in the regression study. For the survival studies, the 3-week cycle (1 week MTD, 2 weeks rest) was repeated as necessary. The metronomic dosing of cyclophosphamide was administered via the drinking water as reported previously (29), resulting in a dose of 10 mg/kg/d for each mouse. For the survival studies, metronomic dosing was continued without interruption. The chemo-switch regimen incorporated an initial three doses of MTD treatment followed by metronomic dosing of cyclophosphamide for the remainder of the 4-week treatment interval in the regression trial. For the survival studies in the chemo-switch-treated mice, metronomic dosing was continued without interruption after the initial MTD dosing in week 12.

Experimental trials and tumor burden analysis. Drug treatments were started when mice reached the age of 12 weeks and continued until mice were 16 weeks for the regression trials. For the survival trials, mice were continuously monitored for signs of hypoglycemic shock or drug side effects and were sacrificed if found moribund and cachexic or if body weight loss exceeded 15%. Mice were anesthetized and underwent heart perfusion using 10 mL PBS followed by 10 mL of 10% zinc-buffered formalin (Medical Chemical Corp.). The pancreas and spleen of the mice were dissected and macroscopic tumors ($\geq 1 \times 1$ mm) were excised and measured. Tumor volume was calculated using the formula $V = a \times b^2 \times \pi / 6$ where a and b equal the longer and shorter diameter of the tumor, respectively. The volumes of all tumors from each mouse were added to give the total tumor burden per animal.

Tissue preparation and immunohistochemistry. Tissues were prepared as described previously for frozen and paraffin embedding (35). FITC-lectin (36), bromodeoxyuridine (BrdUrd) staining, and H&E grading (35) were done as described previously. Microvasculature in mice not perfused with FITC-lectin was visualized using a purified rat anti-mouse pan-endothelial cell antigen monoclonal antibody (mAb; MECA-32, BD PharMingen) and a donkey anti-rat Alexa Fluor 488 secondary antibody (Molecular Probes). Apoptotic cells were visualized using a rabbit anti-cleaved caspase-3 antibody (Cell Signaling Technology) and a goat anti-rabbit Alexa Fluor 568 secondary antibody (Molecular Probes). Tissues were counterstained with 4',6-diamidino-2-phenylindole-containing mounting medium (Vector Laboratories).

Analysis of cell proliferation, apoptosis, tumor vasculature, and tumor invasion. In all histologic analyses, 16-week-old treated tumors were compared with untreated controls at the 12-week-old starting point of the regression trial because control RT2 mice do not survive to 16 weeks in the absence of treatment. In mice bearing more than five tumors, five tumors were selected at random for analysis. For mice with five tumors or less, all tumors were analyzed. Cell proliferation was quantified as the percentage of BrdUrd-positive cells out of the total number of tumor cells per high-power ($\times 400$) field. Tumors were analyzed from three to six mice per treatment group with the exception of the metronomic treatment group for which there was only one mouse available for BrdUrd analysis (total, 434 fields). Tumor cell apoptosis was quantified by counting the number of cleaved caspase-3-positive cells per high-power ($\times 400$) field. Tumors were analyzed from three to five mice per treatment group (total, 472 fields). Microvessel density (MVD) was quantified by counting the total number of FITC-lectin-positive or MECA-32-positive structures observed in each low-power ($\times 200$) field. Vessel branching was quantified by dividing the number of vessel segments (defined as segments between successive branchpoints) over the total vessel length, essentially as described previously (37). For MVD and branching analyses, two comparisons were done: first, lesions of

all sizes were analyzed and second, a subgroup of 'larger' tumors (defined as area $\geq 3 \times 200$ microscope fields, corresponding to $\sim 45\%$ of all lesions analyzed; tumor volume, $\geq 3 \text{ mm}^3$) were separately analyzed. Tumors were analyzed from two to six mice per treatment group, for a total of 344 microscope fields ($\times 200$), using Volocity 4.0 imaging software (Improvision). For analysis of tumor invasion, tissue sections were stained by H&E and graded as described previously (35). All tumors in each of the treatment and control groups were analyzed (total, 360 tumors). For all analyses, tumors were scored and graded blindly by K.M.B.M, A.L.G., or J.A.J. and subsequently decoded.

Statistical analysis. For analysis of tumor burden, cell proliferation, apoptosis, MVD, and vessel branching, means and SEs were calculated for all tumors from each treatment group using the Kruskal-Wallis test followed by the Dunn's multiple comparisons test (InStat, GraphPad Software, Inc.). Statistical comparison of tumor grades was done using a cumulative logit model (38) with generalized estimating equations to correct for correlations within individual mice.

Results

Cathepsin inhibition in combination with a chemo-switch regimen results in significant tumor regression and increased overall survival. Treatment of RT2 mice was started at 12 weeks of age, at a point when the mice had a substantial tumor burden, and

at best only a few weeks to live. Cohorts of control RT2 mice were sacrificed at 12 weeks of age to determine the initial baseline tumor burden at the starting point of the trial, and also at 13.5 weeks, which is end stage in the absence of treatment (Fig. 1A). As shown in Fig. 1A, tumors progress rapidly in untreated RT2 mice with the mean tumor burden increasing from 30.23 to 82.46 mm^3 in 1.5 weeks. We could not observe control RT2 mice beyond the 13.5-week time point because they become ill from their tumor burden and die shortly thereafter in the absence of treatment. Mice treated with MTD chemotherapy showed a widely variable tumor burden (Fig. 1A), as some tumors seemed to become resistant to this treatment with time, whereas others continued to respond, as has been reported previously in this model (29). This phenomenon is also seen in the clinic as patients' tumors become resistant to MTD cytotoxic regimens and often recur aggressively over time. Consistent with previously reported results, daily treatment with the cysteine cathepsin inhibitor JPM-OEt reduced tumor burden when compared with the 13.5-week controls (Fig. 1A). The combination of JPM-OEt with the MTD regimen resulted in a more consistent response from all tumors, compared with MTD treatment alone, and a significant decline in tumor burden compared with the 13.5-week controls ($P < 0.05$; Fig. 1A).

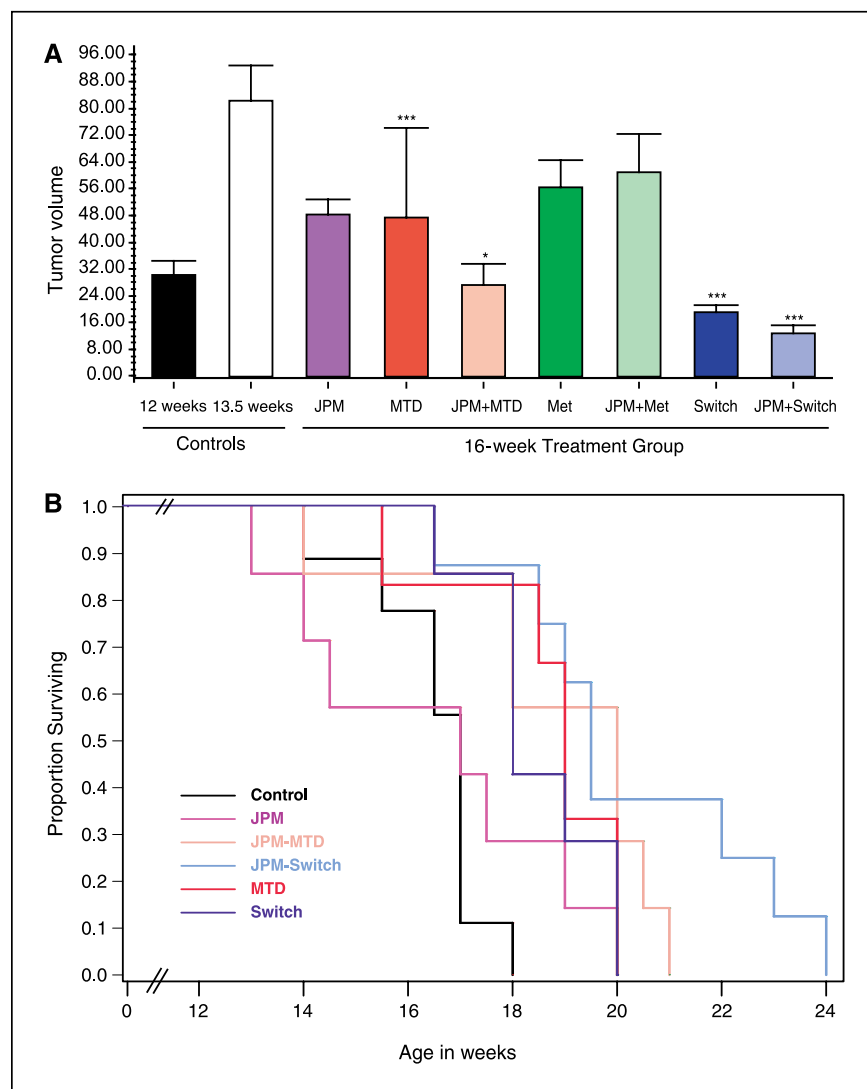


Figure 1. Effects of cathepsin inhibition combined with distinct chemotherapy dosing regimens on tumor burden and overall survival. **A**, tumor volume of untreated RT2 mice at 12 wks (control; $n = 15$) and 13.5 wks (control; $n = 8$) and mice at 16 wks after 4 wks of treatment with the pan-cysteine cathepsin inhibitor JPM-OEt (*JPM*; $n = 12$), MTD cyclophosphamide (*MTD*; $n = 17$), JPM-OEt plus MTD cyclophosphamide (*JPM+MTD*; $n = 12$), metronomic dose cyclophosphamide (*Met*; $n = 11$), JPM-OEt plus metronomic dose cyclophosphamide (*JPM+Met*; $n = 10$), the chemo-switch regimen of MTD cyclophosphamide followed by metronomic dose cyclophosphamide (*Switch*; $n = 12$), or JPM-OEt plus the chemo-switch regimen (*JPM+Switch*; $n = 13$). *, $P < 0.05$; ***, $P < 0.001$. **B**, survival of RT2 mice after no treatment (control; $n = 9$) or after treatment with the pan-cysteine cathepsin inhibitor JPM-OEt ($n = 7$), MTD cyclophosphamide ($n = 6$), JPM-OEt plus MTD cyclophosphamide ($n = 7$), the chemo-switch regimen of MTD cyclophosphamide followed by metronomic dose cyclophosphamide ($n = 7$), or JPM-OEt plus the chemo-switch regimen ($n = 8$).

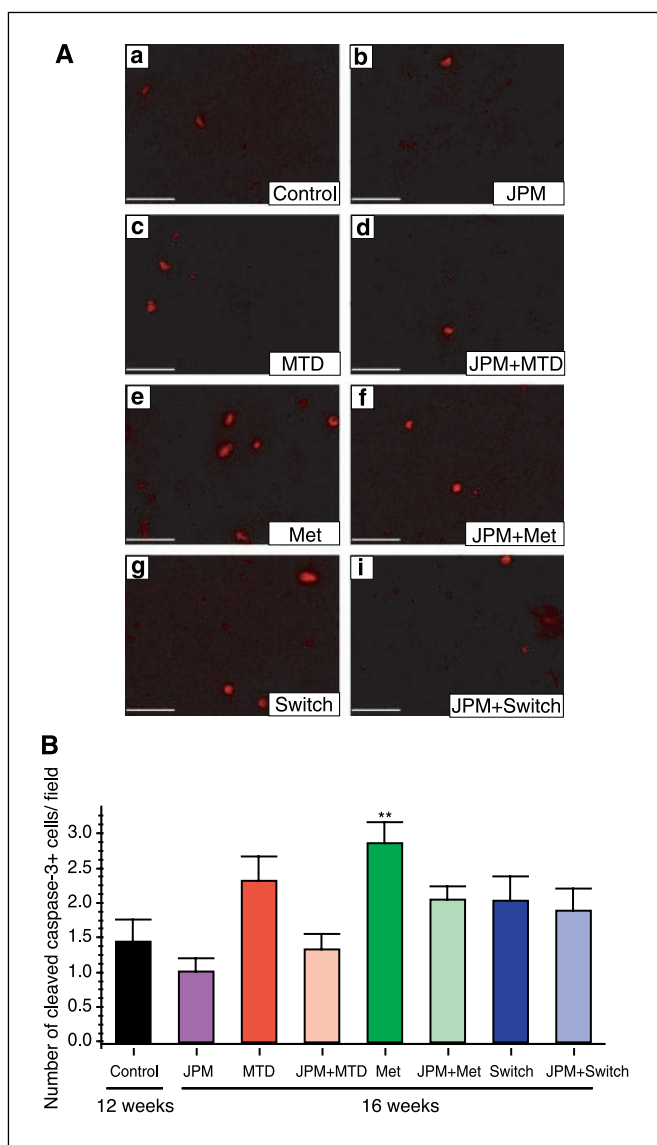


Figure 2. Effects of cathepsin inhibition combined with distinct chemotherapy dosing regimens on apoptosis. **A**, apoptotic cells were detected using an antibody against cleaved caspase-3 and representative images are shown for control 12-week-old RT2 mice (**a**) and 16-week-old RT2 mice treated with JPM-OEt (**b**), MTD cyclophosphamide (**c**), JPM-OEt plus MTD cyclophosphamide (**d**), metronomic cyclophosphamide (**e**), JPM-OEt plus metronomic cyclophosphamide (**f**), chemo-switch cyclophosphamide (**g**), and JPM-OEt plus chemo-switch cyclophosphamide (**h**). Bar, 50 μ m. **B**, the relative numbers of apoptotic cells (cleaved caspase-3 positive) per high-power field ($\times 400$) in 16-week-old treated mice versus 12-week-old control mice. Columns, mean; bars, SE. **, $P < 0.01$.

Metronomically dosed cyclophosphamide administered either alone or in combination with JPM-OEt did not have a significant effect on total tumor volume (Fig. 1A). The most pronounced decline in tumor burden was observed in the chemo-switch groups ($P < 0.001$; Fig. 1A). These results indicate the initial MTD chemotherapy is able to debulk the tumor, whereas the metronomic therapy evidently reduces regrowth/relapse and tumor progression. Notably, the addition of JPM-OEt to the chemo-switch regimen led to an even further regression in tumor burden ($P < 0.001$; Fig. 1A).

Given the intriguing effects on reducing tumor volume following the MTD and chemo-switch regimens in combination with cysteine

cathepsin inhibition, further studies were done to analyze the potential outcome of these treatments on overall survival. Whereas the median overall survival was not significantly increased in mice treated with JPM-OEt compared with controls, we observed a trend toward increased survival when JPM-OEt was given in combination with MTD cyclophosphamide, and the greatest improvement in overall survival was seen in the chemo-switch plus JPM-OEt-treated animals (Fig. 1B).

In addition to significant reductions in tumor burden and increased overall survival in some of these treatment groups, it is important to note that toxicity was minimal (as assessed by weight loss, lethargy, cachexia, etc.) in the various treatment arms involving metronomic or chemo-switch cyclophosphamide alone or in combination with JPM-OEt. As expected, there was some toxicity associated with the repeated MTD regimen when administered for more than one cycle, either alone or in combination with JPM-OEt.

The combination of cyclophosphamide and the cathepsin inhibitor, JPM-OEt, increased apoptosis compared with JPM-OEt treatment alone. Previous studies using JPM-OEt in regression trials in the RT2 model showed that, while reducing cell proliferation, angiogenesis, and invasion, it did not increase the frequency of tumor apoptosis (14). We hypothesized that the addition of chemotherapy would lead to an increase in cell death and thus further reduce tumor growth. In this histopathologic analysis, as well as in those described below, we analyzed tumors from all treatment arms at the defined end point of +4 weeks after initiating treatment at 12 weeks of age. Consistent with prior results (14), JPM-OEt treatment alone did not significantly affect apoptosis in 16-week RT2 tumors when compared with 12-week-old control tumors (Fig. 2). A trend toward increased levels of apoptosis was seen in the cyclophosphamide-treated groups (MTD, metronomic, and chemo-switch regimens) when analyzed at the defined 16-week end point, although the only statistically significant difference was in the metronomic cyclophosphamide-treated animals when compared with the 12-week controls (2-fold increase in apoptosis; $P < 0.01$; Fig. 2B). We anticipate that an overall time course of the pathologic response and future analyses of early time points in particular will reveal increased apoptosis for those regimens involving high-dose chemotherapy. We found that the addition of the cathepsin inhibitor to the MTD regimen or the metronomic dose regimen actually resulted in a trend toward fewer apoptotic cells when compared with the chemotherapy treatment alone. Based on recent data showing that RT2 mice lacking *cathepsin B*, *S*, or *L* had increased levels of tumor cell death (19), this result may seem surprising. However, there are several reports of cathepsins acting as effectors of apoptotic pathways (39, 40); thus, it is possible that inhibiting certain members of the cysteine cathepsin family may block apoptosis in some settings.

Cathepsin inhibition in combination with either MTD or metronomic dosing of cyclophosphamide further reduces tumor cell proliferation. The cysteine cathepsin inhibitor JPM-OEt used as monotherapy has been shown previously to decrease the proliferative capacity of tumor cells as measured by BrdUrd incorporation (14). Therefore, we analyzed tumors from all groups at the +4-week treatment end point for proliferation rates. The current results with JPM-OEt alone (Fig. 3A, b) are consistent with our previous study. Cyclophosphamide, when given in MTD fashion, also reduced proliferation ($P < 0.001$; Fig. 3A, c), more so than JPM-OEt alone. The combination was even more pronounced ($P < 0.001$; Fig. 3A, d). Whereas metronomic cyclophosphamide alone did not seem to affect proliferation (Fig. 3A, e), the

combination of metronomic cyclophosphamide with JPM-OEt reduced proliferation further than JPM-OEt alone ($P < 0.001$; Fig. 3A, f). The chemo-switch (Fig. 3A, g) and JPM-OEt plus chemo-switch cyclophosphamide (Fig. 3A, h) regimens did not significantly alter the proliferative capacity of the tumors evident at the +4-week defined treatment end point when compared with control tumors at the 12-week starting point (Fig. 3B). Several possible explanations may account for these apparently counterintuitive observations, which will be addressed further in the discussion.

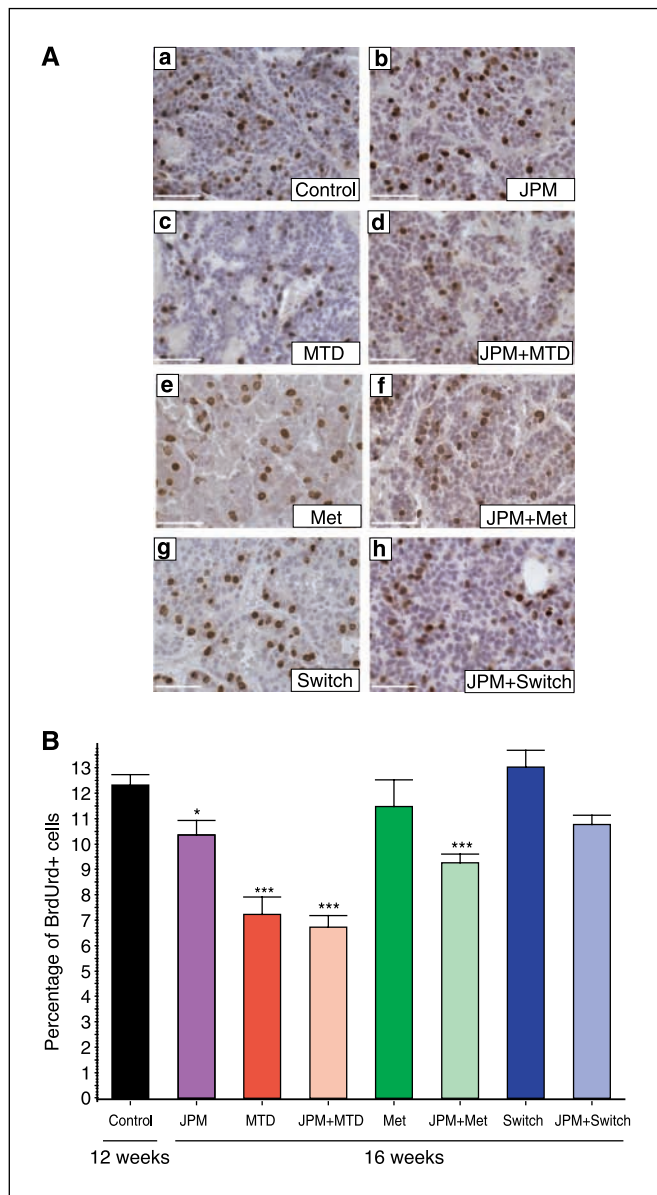


Figure 3. Cell proliferation is reduced following JPM or MTD cyclophosphamide administered alone or in combination. A, proliferating cells were detected by BrdUrd incorporation and representative images are shown for control 12-week-old RT2 mice (a) and 16-week-old RT2 mice treated with JPM-OEt (b), MTD cyclophosphamide (c), JPM-OEt plus MTD cyclophosphamide (d), metronomic cyclophosphamide (e), JPM-OEt plus metronomic cyclophosphamide (f), chemo-switch cyclophosphamide (g), and JPM-OEt plus chemo-switch cyclophosphamide (h). Bar, 50 μ m. B, the percentages of proliferating (BrdUrd positive) cells per high-power field ($\times 400$) in 16-week-old treated mice versus 12-week-old control mice. Columns, mean; bars, SE. *, $P < 0.05$; ***, $P < 0.001$.

Reduced tumor vascularity following cathepsin inhibition.

Next, the tumor vasculature was assessed among the various treatment groups at the defined end point of the trial by quantifying the MVD, as a measure of angiogenesis inhibition (Fig. 4). Whereas the metronomic and chemo-switch dosing of cyclophosphamide both alone and in combination with JPM-OEt showed a trend toward declining numbers of vessels, there was no change in the RT2 mice treated with combined MTD cyclophosphamide plus JPM-OEt. In fact, only the JPM-OEt treatment alone resulted in a statistically significant decline in tumor vascularity (56% reduction; $P < 0.01$; Fig. 4B) when compared with the 12-week control tumors, in an analysis that included lesions of all sizes. However, as there is not necessarily a linear correlation between tumor size and tumor vessel density (41), and as the dependence on angiogenesis may occur only when a tumor reaches a critical size, it is perhaps not surprising that MVD is not substantially affected in several of these treatment groups at the 16-week end point, as their tumor burden is significantly reduced compared with controls. Indeed, when we did the comparative analyses on 'larger' tumors only (tumor volume, $\geq 3 \text{ mm}^3$) in which there may be a greater dependency on angiogenesis, the reduction in MVD was more pronounced in the JPM-OEt plus metronomic and JPM-OEt plus switch chemotherapy treatment groups when compared with those chemotherapy regimens in the absence of JPM-OEt (Fig. 4B).

In certain combinations, the addition of cyclophosphamide seemed to abrogate the effect of cathepsin inhibition alone on decreasing tumor microvasculature. In fact, JPM-OEt plus MTD cyclophosphamide or MTD cyclophosphamide alone had the greatest number of microvessels seen among the treatment groups (Fig. 4B). One possible explanation for this result is that MTD cyclophosphamide treatment has been shown previously to enhance endothelial progenitor cell mobilization (42), which could effectively interfere with the ability of JPM-OEt to inhibit tumor vascularization. Alternatively, in other situations, the blood vessels dictate tissue size by maintaining a fixed vascular density, whereby reduced capability for angiogenesis restrains growth and size without evidencing reduced vascularity (43).

We next asked whether, in addition to decreased MVD, the tumor vessels in the different treatment groups showed any evidence of 'vessel normalization'. Tumor vessel normalization has been observed following several antiangiogenic therapies and proposed as a mechanism by which the efficacy of standard chemotherapy or radiotherapy is increased when combined with antiangiogenic agents, in part due to improved drug delivery (44). Tumor vessels are typically leaky, chaotic, tortuous, and disorganized, and increased vessel branching is used as a hallmark of abnormal vasculature (45). Thus, we analyzed vessel branching in the different treatment groups compared with the control group and found a decrease ranging from 22% to 31% (Fig. 4C). Again, when vessel branching was compared in 'larger' tumors only, this reduction was most pronounced in the JPM plus chemo-switch treatment group (48% reduction; $P < 0.001$; Fig. 4C), the treatment group in which tumor burden was also most significantly reduced (Fig. 1A). It should be noted that whereas the treatment groups that would be expected to be antiangiogenic (JPM-OEt, metronomic, and chemo-switch) all had decreased vessel branching, the MTD-alone group did too, albeit with a smaller reduction than in each of the other groups (Fig. 4C). This may reflect, in part, changes in the vasculature that are secondary to effects on tumor cell metabolism, as discussed by Hlatky et al. (41).

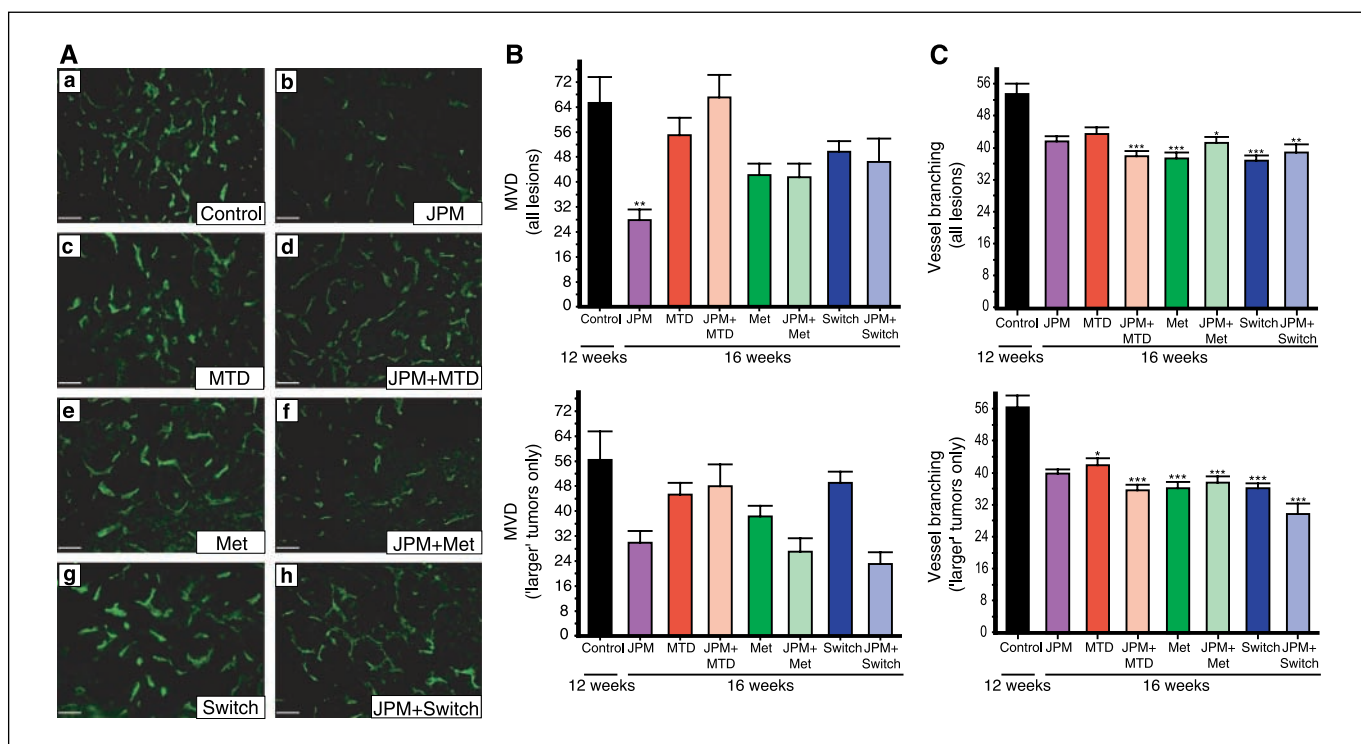


Figure 4. Tumor vascularity and vessel branching are reduced following cathepsin inhibition. **A**, the tumor vasculature was visualized by perfusion of mice with FITC-lectin or by staining with a pan-endothelial cell antigen mAb (MECA-32). Representative images are shown for 12-week-old control mice (**a**) and 16-week-old mice treated with JPM-OEt (**b**), MTD cyclophosphamide (**c**), JPM-OEt plus MTD cyclophosphamide (**d**), metronomic cyclophosphamide (**e**), JPM-OEt plus metronomic cyclophosphamide (**f**), chemo-switch cyclophosphamide (**g**), and JPM-OEt plus chemo-switch cyclophosphamide (**h**). Bar, 50 μ m. **B**, MVD quantified per low-power field ($\times 200$) in 16-week-old treated mice versus 12-week-old control mice. *Top*, MVD in all lesions analyzed; *bottom*, the comparison for the subgroup of 'larger' tumors alone (defined as area $\geq 3 \times 200$ microscope fields, corresponding to $\sim 45\%$ of all lesions analyzed; tumor volume, ≥ 3 mm³). **C**, vessel branching quantified per low-power field ($\times 200$) by dividing the number of vessel segments (as defined between successive branchpoints) over the total vessel length in millimeters. *Top*, vessel branching in all lesions analyzed; *bottom*, the comparison for the subgroup of 'larger' tumors alone. Columns, mean; bars, SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Cathepsin inhibition is necessary and sufficient to reduce tumor invasion. Tumor invasion was assessed by grading lesions as encapsulated (Tum), microinvasive (IC1), or invasive (IC2), and representative examples of each type of tumor are shown in Fig. 5A (*a-c*). As graphed in Fig. 5B, the majority of tumors in the untreated control animals were either microinvasive or invasive. Cyclophosphamide had no effect on the prevalence of invasive carcinomas, irrespective of the dosing regimen (MTD, metronomic, chemo-switch). In contrast, and consistent with previous studies involving JPM-OEt (14) or *cathepsin* gene knockouts (19), cathepsin inhibition significantly shifted the distribution of tumors to less invasive types ($P < 0.05$; Fig. 5B). This effect was seen in all treatment regimens containing JPM-OEt regardless of the dosing schedule of cyclophosphamide that was given in combination with the cathepsin inhibitor. Notably, the shift toward encapsulated tumors was most pronounced in the JPM-OEt plus MTD cyclophosphamide and the JPM-OEt plus chemo-switch regimen groups ($P < 0.001$), the two treatment groups that also showed the trend toward greatest overall survival (Fig. 1B).

Discussion

In this report, we show for the first time that a novel biological targeting agent, an inhibitor of cysteine cathepsin protease activity, has combinatorial efficacy with both traditional and experimental chemotherapy regimens. We compared three dosing schedules for the chemotherapeutic drug, cyclophosphamide, and found that the

chemo-switch regimen (29) produced the best responses in terms of reducing tumor size and malignancy, and increasing survival, which may provide important insights into the design of future clinical trials with these agents.

Whereas the combination regimen of chemo-switch cyclophosphamide and cathepsin inhibition was most effective in reducing tumor burden and extending survival in this mouse model of tumorigenesis, some of the effects of the regimen on apoptosis, proliferation, and tumor vasculature at the 16-week end point were not as pronounced compared with the other treatment arms of the study that were less efficacious in reducing overall tumor burden. This counterintuitive observation may reflect the defined time point of the analyses after 4 weeks of treatment, which may not reveal important but transitory effects occurring earlier in the therapeutic regimen. Future studies are planned to assess these parameters at earlier time points following the initiation of treatment at 12 weeks to determine if the biological effects of cathepsin inhibition in combination with chemo-switch chemotherapy are more pronounced initially. For example, it is possible that there is an initial spike of increased apoptosis and decreased cell proliferation, which debulks the tumor mass, perhaps inducing those tumors to then enter a dormant state by the time they are assessed histologically at the 16-week end point.

The ability of the cysteine cathepsin inhibitor JPM-OEt to significantly reduce tumor invasiveness may ultimately be the most important benefit of this targeted agent. Prior *in vitro* studies showed inhibition of tumor cell invasiveness in the

presence of antibody and small-molecule inhibitors targeting cathepsins (17, 18) but did not assess potential targets. Conceivable mechanisms of action of the inhibitor may include impairment of cathepsin-dependent remodeling and hence structural maintenance of the ECM and BM components, preservation of E-cadherin on cancer cells at the tumor border, perturbed cleavage of additional proteins involved in the metastatic process, or inhibition of tumor-associated macrophage function (20). Cathepsins have been shown to degrade components of the ECM and BM (46, 47), and it is likely that cathepsin inhibitors allow the local tissue environment to retain structural integrity, thus impairing tumor cell invasion.

Indeed, we have shown recently that certain cathepsins cleave the cell adhesion protein, E-cadherin, *in vitro* and *in vivo* (19, 20). Levels of E-cadherin were maintained in RT2 mice lacking *cathepsins B, L, or S*, all of which had significant reductions in tumor invasion (19). Further investigation is currently under way to delineate the exact mechanism whereby cysteine cathepsin inhibitors decrease the invasive potential of tumors in combination with chemotherapy, a unique and important mode of action of these agents. One explanation may be that combining chemotherapy with JPM-OEt simply further reduces the overall pool of tumor cells that could ultimately develop invasive behavior when compared with JPM-OEt alone. However, cathepsin inhibition is clearly required for the reduction in invasiveness in the combined treatment groups, as none of the three chemotherapy alone regimens had any effect on tumor invasion.

Translational considerations. Recent pharmacokinetic studies of the JPM-OEt inhibitor have revealed that this ostensibly cell-permeable ethyl ester is converted to the carboxylic acid form *in vivo* by esterases in the serum (48) and therefore is not expected to be cell permeable. Indeed, we infer that the relatively low toxicity of JPM-OEt in treated mice can be attributed to its decreased

ability to enter cells and inhibit normal lysosomal cathepsin activity. Moreover, we suspect that JPM-OEt is preferentially targeting cell surface or secreted cathepsins in the tumor microenvironment, effectively producing a 'therapeutic window' that maximizes inhibition of aberrant (tumor promoting) extracellular cathepsin activity while minimizing toxic side effects by limiting inhibition of the normal lysosomal pool of cathepsin activity.

In addition to the development of novel targeted inhibitors, such as JPM-OEt, changes in the dosing schedule of 'traditional' anticancer therapies, such as chemotherapy or radiation, have been investigated as a means to improve their therapeutic efficacy. For example, in recent years, metronomic dosing of chemotherapeutic agents has gained increasing attention (25, 49). Although the idea of a chronic low dose of chemotherapy that would be relatively nontoxic to patients yet still effective against tumor cells and tumor endothelium sounds promising, comparatively few patient responses have been seen with this type of dosing schedule when given alone. Clinical trials using metronomic dose schedules have also rarely directly compared the metronomic regimen with an alternative MTD regimen or with a regimen combining the two, comparable with the chemo-switch dosing in this study. In one ongoing trial, the Southwest Oncology Group (SWOG) recently reported promising results using a chemo-switch regimen design in the neoadjuvant treatment of breast cancer patients (50). In this trial, patients were randomized to receive the standard MTD Adriamycin and cyclophosphamide followed by paclitaxel versus a chemo-switch regimen of weekly Adriamycin and daily low-dose cyclophosphamide followed by paclitaxel. Patients receiving the chemo-switch regimen had significantly more pronounced pathologic responses in their primary tumors and were more likely to have no lymph node involvement at the time of surgery (50). A similar study in the adjuvant setting is ongoing in the SWOG 0221 trial. Based on our

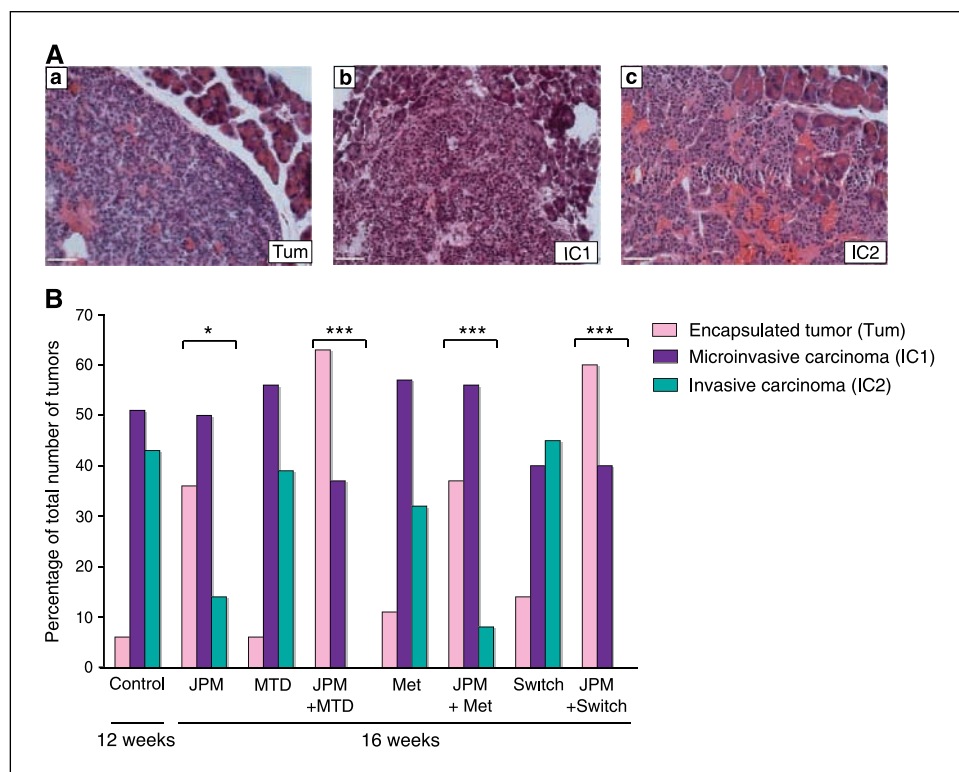


Figure 5. Cathepsin inhibition significantly reduces tumor invasion. **A**, representative images showing an encapsulated tumor (**a**), a microinvasive carcinoma (**b**), and an invasive carcinoma (**c**). Bar, 50 μ m. **B**, relative proportions of encapsulated (*Tum*), microinvasive (*IC1*), and highly invasive (*IC2*) carcinomas in 12-week RT2 controls compared with 16-week-old RT2 mice after treatment with JPM-OEt (*JPM*), MTD cyclophosphamide (*MTD*), JPM-OEt plus MTD cyclophosphamide (*JPM+MTD*), metronomic cyclophosphamide (*Met*), JPM-OEt plus metronomic cyclophosphamide (*JPM+Met*), chemo-switch cyclophosphamide (*Switch*), and JPM-OEt plus chemo-switch cyclophosphamide (*JPM+Switch*). Fewer invasive tumors were seen in all treatment groups receiving JPM-OEt. No decrease in invasiveness was seen in any of the cyclophosphamide treatment groups alone, regardless of the dosing schedule. *, $P < 0.05$; ***, $P < 0.001$.

results with a preclinical model in the present study, we would encourage wider use of such chemo-switch regimen designs in future clinical trials of anticancer agents to maximize therapeutic efficacy.

In conclusion, we have shown that cysteine cathepsin inhibition in combination with two distinct regimens of chemotherapy administration (MTD or chemo-switch) results in tumor regression, decreased tumor invasiveness, and increased survival. Ongoing experiments in preclinical models of other tumor types should reveal the general therapeutic feasibility of cathepsin inhibition, alone and in combination with chemotherapy. We propose that the addition of JPM-OEt to currently used cytotoxic chemotherapy regimens in the clinic has the potential to add significant benefits both in terms of a reduction in tumor burden and an increase in

overall survival and that this may largely be mediated by the ability of JPM-OEt to reduce the invasive capability of tumors.

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