Antitumor Activity of YM155, a Selective Small-Molecule Survivin Suppressant, Alone and in Combination with Docetaxel in Human Malignant Melanoma Models

Kentaro Yamanaka, Takahito Nakahara, Tomohiro Yamauchi, Aya Kita, Masahiro Takeuchi, Fumiko Kiyonaga, Naoki Kaneko, and Masao Sasamata

Abstract

Purpose: Aggressive cell growth and chemoresistance are notorious obstacles in melanoma therapy. Accumulating evidence suggests that survivin is preferentially expressed in cancer cells and plays a crucial role in cell division and apoptosis dysfunction. Here, we evaluated the therapeutic potential of YM155, a selective survivin suppressant, alone and in combination with docetaxel using human melanoma models.

Experimental Design: A375 and SK-MEL-5 human malignant melanoma cells were treated with siRNA, YM155, and/or docetaxel, and cell viability, mRNA and protein expression levels, cell-cycle distribution, and immunohistochemical staining were then evaluated. Furthermore, the efficacy of YM155 combined with docetaxel was further examined in established xenograft models.

Results: Survivin suppression was sufficient to induce spontaneous apoptosis of melanoma cells. YM155 showed nanomolar antiproliferative effects and induced tumor regression in established melanoma xenograft models. Docetaxel showed antitumor activity against melanoma cells, although it also induced survivin upregulation and G2/M mitotic arrest; however, cotreatment with YM155 decreased survivin expression below basal levels. Combination treatment of YM155 and docetaxel induced a greater rate of apoptosis than the sum of the single-treatment rates and promoted tumor regression without enhanced body weight loss in the melanoma xenograft models.

Conclusions: Survivin is responsible for the inherent low levels of spontaneous apoptosis in melanoma cells. The concomitant combination of YM155 with docetaxel diminished the accumulation of survivin in G2/M mitotic arrest, and induced more intense apoptosis compared with each single treatment. YM155 in combination with docetaxel is well tolerated and shows greater efficacy than either agent alone in mouse xenograft models.

Introduction

Malignant melanoma is the most lethal form of skin cancer with a median overall survival of only 6 months once distant metastasis occurs (1). Despite considerable research efforts aimed at developing alternative therapeutic approaches, including multiagent regimens, most cytotoxics have failed to improve overall survival of melanoma patients (2). Advanced melanoma patients generally have a poor prognosis with currently approved therapeutic agents, underscoring the need for more effective treatment options.

Defects in apoptotic signaling promote melanoma development and progression, which leads to metastasis and the high mortality rate associated with melanoma (3). Patient-derived melanoma cells often show inherently low levels of spontaneous apoptosis (4). Apoptosis resistance correlates with increased metastatic potential of melanoma, suggesting that acquisition of apoptosis resistance facilitates the vertical growth and deep invasion of melanoma (5). As most cytotoxic agents exert their effects through induction of apoptosis, their limited efficacy against melanoma is likely due to dysfunctional apoptosis signaling. In this context, the development of agents capable of triggering cancer cell apoptosis may represent a novel therapeutic approach for melanoma treatment.

Survivin is a member of the inhibitor of apoptosis (IAP) family proteins (6, 7) and regulates cell division as a component of the spindle checkpoint machinery (8). Unlike other antiapoptotic proteins, survivin expression is upregulated in malignant melanoma and yet is undetectable in normal melanocytic nevi and normal differentiated skin tissues (3, 9). Retrospective analyses of melanoma patients have revealed that survivin upregulation is correlated with decreased survival rate, increased relapse, and chemoresistance (9, 10). Biologically, survivin is upregulated in the G2/M phase of the cell cycle and physically

Authors' Affiliation: Drug Discovery Research, Astellas Pharma, Inc., Tsukuba-shi, Ibaraki, Japan

Corresponding Author: Kentaro Yamanaka, Institute for Drug Discovery Research, Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba-shi, Ibaraki 305-8585, Japan. Phone: 81-29-863-7058; Fax: 81-29-852-5391; E-mail: kentaro.yamanaka@jp.astellas.com
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Translational Relevance

The apoptosis resistance of melanoma is an area of intense investigation. As most chemotherapeutic agents act through the induction of apoptosis, dysregulation of apoptosis represents a large obstacle in melanoma therapy. Therefore, novel anticancer drugs that selectively counteract survivin expression are desirable not only to induce apoptosis but also to chemo-sensitize melanoma. We previously showed that monotherapy with YM155, a first-in-class selective small-molecule survivin suppressant, resulted in modest anticancer activity and an acceptable toxicity profile in patients with melanoma. In this study, we found that the in vitro combination of YM155 and docetaxel induced a higher rate of apoptosis than the sum of the single treatment rates. In established melanoma xenograft models, combination treatment induced complete tumor regression without an enhancement of body weight loss. These results suggest that treatment with YM155 in combination with docetaxel could safely improve the poor prognosis of patients with metastatic melanoma.

bonds to interphase microtubules, and the mitotic spindle and centrosome during mitosis (11). Increased survivin expression confers paclitaxel chemoresistance to cancer cells (12, 13). Given its preferential expression in cancer cells, function in cancer cell division and chemoresistance, and correlation with poor patient prognosis, survivin has been proposed as a biomedical target for cancer therapy (6, 14).

Unlike most conventional chemotherapies, taxanes, such as docetaxel and paclitaxel, have been shown to upregulate survivin expression levels, which is attributed to mitotic arrest at the G2/M stage and the augmented stability of survivin protein through phosphorylation at Thr34 by p34cdc2 (15). It is also postulated that the transcriptional activation of the survivin promoter via PI3K/AKT- and ERK1/2 pathways account for paclitaxel-induced survivin upregulation (16). On the basis of these findings, we speculated that inhibition of survivin could enhance the response of melanoma cells to microtubule-stabilization agents. However, the paucity of agents with survivin-suppressive activity has hampered characterization of the significance of the survivin-mediated apoptotic blockade.

We previously identified a small molecule survivin suppressant YM155 using cell-based high-throughput screening and lead optimization. YM155 selectively suppresses survivin expression, resulting in activation of caspases and apoptosis induction in hormone refractory prostate cancer cells. Continuous infusion of YM155 has also been found to induce tumor regression and intratumoral survivin suppression in established human hormone refractory prostate cancer (HRPC), non-Hodgkin lymphoma (NHL), and non–small cell lung cancer (NSCLC) tumor xenografts (17–19). In clinical settings, YM155 was shown to be tolerable in phase I studies with advanced cancer patients and showed antitumor activity in NHL and HRPC subjects (20, 21). Multicenter phase II trials showed the safety and tolerability of YM155 in patients with unresectable melanoma (22) and advanced refractory NSCLC (23). Given that survivin is intimately involved in spindle microtubule behavior and apoptosis, and that YM155 showed efficacy against human cancers in both preclinical and clinical settings, we postulated that a combination of YM155 with the antimitic agent docetaxel would show promising efficacy against melanoma.

Here, we evaluated the therapeutic potential of YM155 alone and in combination with docetaxel in preclinical melanoma models, both in vitro and in vivo.

Materials and Methods

Cell culture

The human malignant melanoma cell lines A375 (CRL-1619), SK-MEL-5 (HTB-70), RPMI-7951 (HTB-66), SK-MEL-28 (HTB-72), and SK-MEL-2 (HTB-68) were obtained from the American Type Culture Collection. The cells were routinely cultured at 37°C in a 5% CO2 atmosphere in RPMI 1640 medium supplemented with 10% heat-inactivated FBS.

Reagents

YM155 monobromide was synthesized at Astellas Pharma, Inc. For in vitro experiments, paclitaxel, docetaxel, and dacarbazine were purchased from Sigma-Aldrich. Test compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium [DMSO final concentration, 0.1% (v/v)]. For in vivo studies, the dose levels of YM155 are expressed as the cationic moiety of the drug substance. Docetaxel (Taxotere INJ) was purchased from Sanofi Aventis Co., Ltd. Drugs were dissolved and diluted in saline immediately prior to administration.

In vitro cell proliferation assay

The antiproliferative activities of YM155, dacarbazine, and paclitaxel were determined via the method used at the National Cancer Institute. Briefly, after drug treatment for 48 hours, the cell count was determined using a sulforhodamine B assay. The 50% growth inhibition value (GI50; drug concentration resulting in a 50% reduction in net protein increase in control cells during drug incubation) was calculated by logistic analysis. The assay was done in triplicate, and the mean GI50 value was obtained from the results of 4 independent assays.

Transient transfection of siRNAs

Cells were transfected with 10 nmol/L siRNAs using RNAiMax (Invitrogen) according to the manufacturer’s instructions. siRNAs with the following sequences were purchased from Sigma-Aldrich: negative control siRNA (CGGCCGCGC- AAUCGAAUGCAGACG), survivin siRNA#1 (CUUGAGAUGU-
in ice-cold 70% ethanol and incubated at 4°C. Cells were resuspended by using an ABI PRISM 7900 sequence detection system.

Western blot analysis
Protein was extracted using radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. Extracted protein (20 μg) samples were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking at room temperature with TBS-T buffer containing 10% Blocking One (Nakalai Tesque), each membrane was incubated overnight at 4°C with one of the following primary antibodies: affinity-purified rabbit anti-survivin, goat anti-c-IAP2, rabbit anti-XIAP (R&D Systems), rabbit anti-PARP, rabbit anti-Bcl-2, rabbit anti-Bcl-xl, rabbit anti-Bad, mouse SmaC-Diablo (Cell Signaling), mouse anti-β-tubulin (Santa Cruz), or rabbit anti-actin (Sigma-Aldrich). The membranes were then washed with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Proteins of interest were visualized by enhanced chemiluminescence using ECL (Amersham).

In vitro assay for cell viability and caspase activity
Cell viability and caspase activity were determined using a CellFitter-Glo luminescent cell viability assay and Caspase-Glo3/7 luminescent assay (Promega), respectively. The luminescence signals for the drug-treated cells were measured in a plate-reading luminometer (EnVision; Perkin–Elmer) with volume to minimize intragroup and intergroup variations.

cDNA synthesis and quantitative real-time PCR
The first day of drug treatment was designated as day 0, and observation continued until the day 21. YM155 was administered as a 3-day continuous infusion every week for 2 weeks or as a 7-day continuous infusion using a micro-osmotic pump (Alzet Model 1003D and 1007D; Durect). Dacarbazine was intraperitoneally administered daily for 5 consecutive days. Docetaxel was administered i.v. once or 3 times (once every 4 days). Maximum tolerated dose (MTD) was determined in a separate study. Body weight and tumor diameter were measured twice a week, and tumor volume was determined (length × width² × 0.5). Complete regression (CR) was defined as tumor regression to below the limit of palpation.

Statistical analysis
Cell viability, caspase activation level, and relative mRNA expression levels for all survivin siRNA- or YM155-treated groups were compared with the control group using Dunnett’s test. For in vivo studies, statistically significant differences were determined using Dunnett’s or Student’s t test. All data analyses were done using SAS software (SAS Institute, Inc.) and P less than 0.05 was considered statistically significant.

Results

Effects of survivin gene knockdown on cell growth and spontaneous apoptosis
To assess the validity of targeting survivin for melanoma therapy, we first examined the effect of siRNA-mediated
survivin knockdown on melanoma cell growth and apoptosis. Human A375 malignant melanoma cells were transfected with one of 3 siRNAs (10 nmol/L) targeting different survivin mRNA sequences. After 48 hours posttransfection, survivin mRNA was reduced by more than 80% compared with cells transfected with negative control siRNA (Fig. 1A). The downregulation resulted in reduced viable cell counts and a 5.3- to 13.4-fold increase in caspase activities at 96 hours posttransfection (Fig. 1B and C). Western blot analysis showed that survivin gene knockdown was accompanied by an increase of cleaved PARP, indicating that survivin suppression induced spontaneous apoptosis in melanoma cells. No apparent decrease was observed in the levels of XIAP or Smac/Diablo (Fig. 1D). Similar effects were observed in SK-MEL-5 human malignant melanoma cells (data not shown).

**Antiproliferative activity of YM155 against human malignant melanoma cell lines**

We next examined the antiproliferative activities of YM155, paclitaxel, and dacarbazine against human malignant melanoma cell lines. Both YM155 and paclitaxel inhibited the growth of the human melanoma cell lines with GI50 values ranging from 3.2 to 11 nmol/L and 5.8 to 29 nmol/L, respectively, whereas dacarbazine showed no antiproliferative effect up to 10 μmol/L (Fig. 2A). We further confirmed the in vitro effect of YM155 on survivin expression in the A375 cell line. YM155 dose-dependent suppressed endogenous survivin expression at both the mRNA and protein levels (Fig. 2B and C) but had no apparent effects on the protein expression of c-IAP2, XIAP, Bcl-xl, Bad, actin, or tubulin up to 40 nmol/L (Fig. 2C). YM155 treatment did result in a slower migrating Bcl-2 band, although the predominant Bcl2 band was similar to the control. These findings showed that YM155 selectively inhibited survivin expression without significantly affecting the levels of other proteins in melanoma cells.

**YM155-induced CR in human malignant melanoma SK-MEL-5 xenograft tumors**

To compare the in vivo antitumor activities of YM155 and dacarbazine monotherapies, the 2 therapies were evaluated in an SK-MEL-5 mouse xenograft model. Continuous infusion of YM155 at 3 and 10 mg/kg/d significantly inhibited tumor growth and induced tumor regression, and 3 of 5 mice experienced CR at the MTD. No significant decreases in body weight were observed at any dose of YM155 (data not shown). In contrast, dacarbazine showed no statistically significant antitumor activity, even at the MTD (Fig. 3). These results suggest that the in vivo antitumor activity of YM155 against human malignant melanoma may be more potent than that of dacarbazine.

**YM155 abrogates docetaxel-induced survivin upregulation in A375 and SK-MEL-5 human melanoma cells**

To define the relationship between apoptosis induction and docetaxel-induced survivin expression, we evaluated caspase activity and survivin protein levels in A375 and SK-MEL-5 cells treated with docetaxel at various concentrations. Docetaxel dose-dependently increased caspase-3 and -7 activities at concentrations from 1 to 30 nmol/L, whereas a bell-shaped dose-dependence curve was observed at concentrations exceeding 30 nmol/L in
SK-MEL-5 cells (Fig. 4). In A375 cells, caspase activity peaked at docetaxel concentrations exceeding 30 nmol/L and did not decrease at higher concentrations. Western blot analysis confirmed that 30 nmol/L docetaxel-induced upregulation of survivin in both melanoma cell lines (Fig. 4). These results suggested that docetaxel induces apoptosis via microtubule stabilization, but its efficacy is limited by the induction of survivin expression, which triggers a proliferation pathway to counteract induced apoptosis.

To clarify whether YM155 attenuates the increase of survivin expression by docetaxel, melanoma cells were treated for 48 hours with either docetaxel alone or in combination with YM155. Western blot analysis revealed that docetaxel-induced survivin upregulation was reduced below the basal level on combination treatment with YM155 in both cell types (Fig. 4). These data indicated that the concomitant combination of YM155 with docetaxel diminished the accumulation of survivin, which is reported to inhibit the effectors proteases caspase-3 and -7 and suppress apoptosis (7).
YM155 enhances docetaxel-induced apoptosis and decreases survivin upregulation at G2/M mitotic arrest in SK-MEL-5 human melanoma cells

SK-MEL-5 cells treated with either YM155, docetaxel, or both agents were analyzed using flow cytometry and immunohistochemical staining to assess the in vitro effects on cell-cycle distribution, apoptosis induction, survivin expression levels, and phenotypic changes (Fig. 5). Cell-cycle analysis revealed that exposure to 10 and 30 nmol/L YM155 increased the distribution of the subG1 apoptotic fraction.

Figure 4. Dose response for caspase activity and survivin expression induced by docetaxel in human malignant melanoma cell lines. Cells were treated with docetaxel from 1 to 1,000 nmol/L for 48 hours (control: 0.1% DMSO). Each bar represents the mean ± SE (n = 4) caspase-3 and -7 activity. All values were normalized to cell number and expressed as the fold increase over the control. For Western blot analysis, cells were treated with docetaxel with or without YM155 at the indicated concentration for 48 hours (control: 0.1% DMSO), and protein expression was visualized with the indicated antibodies.

Figure 5. In vitro effects of combined YM155 and docetaxel treatment on cell-cycle distribution, phospho-survivin expression, and cell phenotype. A, cell-cycle distribution was determined based on cellular DNA content using flow cytometry after 48 hours drug treatment. Each bar represents the mean ± SE from 3 separate experiments. B, immunohistochemical analysis was conducted in SK-MEL-5 cells treated with the indicated drugs for 48 hours. Cells were fixed and subjected to immunofluorescent staining with anti-p-Survivin (Thr 34) or anti-α-tubulin. DNA was stained with propidium iodide, and cells were visualized using confocal microscopy.
fraction by 22.7% and 68.5%, respectively (Fig. 5A), although no apparent mitotic defects, polyploidy, or specific cell-cycle arrest in the G2/M phase were induced (Fig. 5A and B). In contrast, treatment with 30 and 100 nmol/L docetaxel increased the G2/M fractions by 36.5% and 43.7%, respectively, while the subG1 apoptotic fractions were increased by 39.2% and 31.0%, respectively (Fig. 5A). Immunohistochemical analysis confirmed that 10 nmol/L YM155 reduced p34Thr-survivin protein levels, whereas docetaxel treatment increased p34Thr-survivin protein and multinucleated cells (Fig. 5B). Furthermore, docetaxel-induced p34Thr-survivin upregulation was apparently decreased by the concomitant treatment with 10 nmol/L YM155 (Fig. 5B). The augmented cell numbers at the G2/M phase caused by docetaxel exposure were diminished by the concomitant treatment of YM155, whereas the proportion of apoptotic cells in the combination treatment was 76.6%, which was greater than the sum in either treatment alone (Fig. 5A).

**Antitumor effects of YM155 combined with docetaxel in A375 and SK-MEL-5 human melanoma xenograft models**

The efficacy of YM155 in combination with docetaxel was examined in A375 and SK-MEL-5 human malignant melanoma xenografts. YM155 was administered at 2 mg/kg/d with 7 days infusion to recapitulate clinical dosing and the steady-state serum concentrations in humans. YM155 in concomitant combination with docetaxel significantly inhibited tumor growth compared with each single-compound group (P < 0.01) in both A375 and SK-MEL-5 established tumors. However, each monotherapy treatment induced tumor regression, which was followed by successive tumor regrowth during the observation period. No significant decrease in body weight was observed in the combination group as compared with the docetaxel group. These results indicated that YM155 in combination with docetaxel was tolerated in mice and enhanced the in vivo tumor response to docetaxel.

**Discussion**

Here, we examined the validity of targeting survivin and the therapeutic potential of YM155 alone and in combination with docetaxel using preclinical melanoma models. We found that survivin suppression induced spontaneous apoptosis in human malignant melanoma cell lines, and that YM155 exhibited nanomolar antiproliferative activity and showed tumor regression in established xenograft refractory to dacarbazine. In an *in vitro* combination study, YM155 decreased docetaxel-induced survivin accumulation at G2/M cell-cycle arrest and synergistically enhanced antitumor activity of docetaxel in melanoma cells. In addition, YM155 concomitantly combined with docetaxel resulted in greater tumor reduction than each single treatment in established xenograft models. Taken together, these findings suggest that YM155 may be a promising candidate for melanoma therapy as a novel apoptosis inducer with survivin-suppressive activity.

As cancer cells develop heterogeneity through a series of accumulated genetic changes, it was unclear whether the cytoprotective potential of survivin and therapeutic potential of YM155 would be differentiated by tumor type, specific somatic mutation, or cell-cycle progression. Our present findings suggest that melanoma cells may acquire a cytoprotective phenotype by mitigating apoptosis induction through overexpression of survivin. In addition, melanoma cells became sensitive to apoptosis induction when survivin was downregulated using siRNA and YM155. Among the acquired genetic alterations in melanoma cells, mutations in BRAF, NRAS, and p53 are frequently observed in human melanoma (24). As these somatic mutations are capable of modulating survivin promoter activity via MAPK kinase, the PI3-Akt kinase pathway, and direct promoter binding (25), they may predominantly exploit survivin-mediated apoptosis dysfunction and serve as surrogate classifiers for the efficacy of YM155 in human melanoma. Further extensive research for survivin-mediated apoptosis defects may allow the rational stratification of melanoma patients who would most likely respond to YM155.

Combination chemotherapy is typically employed to achieve a better response rate than that of monotherapy and is generally designed empirically using drugs that act through different cytotoxic mechanisms with less overlapping toxicity. Here, docetaxel induced cell-cycle arrest at the G2/M stage and elevated the expression and stabilization of survivin. When YM155 was concomitantly combined with docetaxel, the accumulation of survivin at G2/M arrest was diminished and more intense apoptosis was observed compared with each single treatment (Figs. 4 and 5). In addition, YM155 potentiated antitumor activity of docetaxel without an enhancement of body weight loss in established xenograft models (Fig. 6). Our previous analysis also revealed that tumor regression induced by YM155 in combination with docetaxel was accompanied by decreased intratumoral survivin and increased apoptosis rate compared with either treatment alone, and both the concomitant and sequential treatment with YM155 and docetaxel enhanced the antitumor activity of docetaxel against established human NSCLC xenograft tumors (19). It was also reported that adenovirus-mediated inhibition of survivin expression sensitizes human prostate cancer cells to taxane, both *in vitro* and *in vivo* (11). These results lead us to a conjecture that elevated survivin expression in G2/M mitotic arrest caused by docetaxel treatment engenders apoptosis resistance. In this context, YM155 may sensitize human melanoma to docetaxel via the inhibition of docetaxel-induced survivin cytoprotection.

In clinical settings, docetaxel treatment is effective against several types of human cancers. Despite its clinical benefits, the long-term treatment of docetaxel is often limited due to development of drug resistance and cumulative side effects, primarily neuropathy and myeloid toxicity (26, 27). Therefore, drugs that circumvent docetaxel...
resistance without overlapping side effects represent ideal candidates for developing novel combinatorial therapeutic regimen for treating melanoma. Although cancer cells can acquire resistance to docetaxel through various mechanisms, our findings suggest that survivin may be involved in the mitotic survival pathway and counteract docetaxel-induced apoptosis in melanoma cells. YM155 monotherapy showed modest anticancer activity with an acceptable toxicity in patients with melanoma (22, 28). As no serious hematologic and neurologic events were reported following YM155 monotherapy (20, 21), the combination regimen of YM155 with docetaxel may be beneficial for cancer treatment. Further clinical investigation of YM155 as an apoptosis inducer, either alone or in combination with docetaxel, for the treatment of malignant melanoma is warranted.

Disclosure of Potential Conflicts of Interest

All authors are employees of Astellas Pharma, Inc. and have no other relevant conflicts of interest to disclose.

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