YM155, a novel survivin suppressant, enhances taxane-induced apoptosis and tumor regression in a human Calu 6 lung cancer xenograft model

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Survivin, an apoptotic inhibitor, is overexpressed in the majority of human tumor types and represents a novel target for anticancer therapy. Taxanes induce a mitotic cell-cycle block through the inhibition of microtubule depolymerization, with subsequent elevated expression/stabilization of survivin. We investigated the administration of survivin suppressant YM155 monobromide (YM155), in combination with docetaxel, in a human non-small-cell lung cancer (NSCLC) xenograft model. Animals received a 7-day continuous infusion of YM155, 2 mg/kg, and/or three bolus doses of docetaxel, 20 mg/kg, according to three dosing schedules: YM155 administered concomitantly with docetaxel, before docetaxel, and after docetaxel. YM155 administered either concomitantly with or before docetaxel showed significant antitumor activity (tumor regression $\geq$ 99%), with complete regression of the established human NSCLC-derived tumors in mice (eight of eight and seven of eight animals, respectively). Significantly fewer complete responses (three of eight animals) were achieved when YM155 was administered after docetaxel. No statistically significant decreases in body weight were observed in the combination versus docetaxel groups. YM155 administered concomitantly with docetaxel resulted in significant decreases in mitotic and proliferative indices, and in a significant increase in the apoptosis index. Elevated survivin expression was seen in tumors from mice treated with docetaxel alone; a significant reduction in survivin expression was seen in tumors from mice treated with YM155 alone or in combination with docetaxel, but not in the control group. These results indicate that in a human NSCLC xenograft model YM155 in combination with docetaxel diminished the accumulation of survivin by docetaxel and induced more intense apoptosis and enhanced antitumor activity, compared with single-agent YM155 or docetaxel. *Anti-Cancer Drugs* **22:454–462**
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Introduction

Survivin is a member of the inhibitor of apoptosis family of caspase interactive proteins, of which there are eight known members [1–4]. Survivin is overexpressed in a wide range of human cancers [5–7]. In cancer cells, survivin seems to regulate proliferation not only through inhibition of apoptosis but also by influencing the cell cycle. Overexpression of survivin correlates with a poor prognosis in cancer patients [8–12], with resistance to chemotherapy [13–15] and an increased rate of tumor recurrence [16–18]. By contrast, survivin is generally found in low levels in normal adult tissues, except in those characterized by high rates of self-renewal, including the thymus, testes, placenta, CD34+ cells, and epithelial cells in crypts of the gastrointestinal tract [5–7]. Suppression of survivin has been shown to induce tumor cell death and to render cells sensitive to normal cell-cycle regulation [19–21]. Furthermore, as survivin has been shown to increase tumor resistance to various apoptotic stimuli, primarily through caspase-dependent mechanisms [22], restoring normal apoptosis by inhibition of survivin should enhance the efficacy of concomitant anticancer therapies. Reduction of survivin levels after chemotherapy has recently been suggested as a potential biomarker for the response to chemotherapy in non-small-cell lung cancer (NSCLC) [23] and may also represent a prognostic indicator [24].

Survivin is a microtubule-associated protein that acts post-mitochondrially to inhibit apoptosis and exhibits cell-cycle-regulated expression that peaks at mitosis [25,26]. During mitosis, survivin binds to the microtubules of the mitotic spindle through its carboxy-terminal α helices. Interference in survivin–microtubule interactions, by means of an antisense-mediated reduction in the expression of survivin, results in a failure of the antiapoptotic function of survivin and an increase in caspase-3 activity with subsequent apoptosis [5].

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Docetaxel, a well-established taxane chemotherapy for the treatment of various cancers, stabilizes microtubule assembly, arrests cell cycle in the G2/M phase, and ultimately induces cancer cell apoptosis. The microtubule stabilization induced by taxanes results in the elevated expression of survivin [27], and the efficacy of taxanes has been shown to be enhanced when combined with survivin inhibition [28–30].

YM155 monobromide (YM155) is a novel small molecule that downregulates survivin and has been shown to inhibit the growth of human tumor cell lines, including NSCLC [31]. YM155 shows potent antitumor activity in human lung carcinoma xenograft models and induces in-vivo antitumor activity without showing severe systemic toxicity in mice. Tumor regression induced by YM155 is accompanied by downregulation of survivin messenger RNA (mRNA) and induction of apoptosis [31]. Continuous infusion of YM155 has been shown to induce tumor regression in the human cancer xenograft model, causing few adverse effects to normal tissues [31]. YM155 has also been shown to sensitize human NSCLC cells, both *in vitro* and *in vivo*, to platinum compounds, considered to be the result of DNA repair inhibition and the subsequent enhancement of apoptosis [32]. YM155 has also shown modest single-agent clinical activity in patients with refractory, advanced NSCLC [33]. Given that current management of NSCLC commonly includes the use of taxanes and that YM155 has shown activity against human lung cancer in both preclinical and clinical settings, we postulated that a combination of YM155 with docetaxel would show benefits in treating human NSCLC and various cancers.

Here, we report the results of a series of studies that aimed to evaluate the antitumor activity of YM155 when combined with docetaxel in an experimental human Calu 6 NSCLC xenograft model.

Materials and methods

Reagents and cell cultures

YM155 was synthesized by Astellas Pharma Inc. (Tokyo, Japan). Docetaxel was purchased from Sanofi-Aventis (Paris, France). For the in-vitro studies, reagents were dissolved in dimethyl sulfoxide and diluted in saline to a final concentration of less than 0.1%. For the in-vivo studies, YM155 was dissolved in physiological saline (0.9% sodium chloride) to create dosing solutions. The solutions were loaded into osmotic pumps (Alzet model 1007D Micro-Osmotic Pump, DURECT Co., California, USA), as reported earlier [31]. Docetaxel was diluted with physiological saline immediately before administration. The human lung carcinoma cell line Calu 6 (HTB-56, Lot No. 208280) was obtained from American Type Culture Collection (Manassas, Virginia, USA). Cells were cultured at 37°C in a 5% CO_2 atmosphere in RPMI1640 medium (Life Technologies, Carlsbad, California, USA) supplemented with 10% heat-inactivated fetal bovine serum within 6 months of receipt of the cell lines.

Total RNA preparation and real-time polymerase chain reaction

Total RNA was isolated from the cells and complementary DNA was synthesized as described earlier [31]. All sets of reactions were conducted in triplicate with specific primer pairs of target human genes from survivin and 18S ribosomal RNA (rRNA), a ubiquitously expressed housekeeping gene. Primer sequences were designed using Primer Express version 1.0 (Applied Biosystems, Foster City, California, USA). The following sequences were selected: human survivin (forward) 5′-CTGCCTGGCAGCCCTTT-3′ and (reverse) 5′-CCTCC AAGAAGGGCCAGTTC-3′; human rRNA (forward) 5′-GCC GCTAGAGGTGAAATTCTTG-3′ and (reverse) 5′-CATT CTTGGCAAATG CTTTCG-3′. Real-time polymerase chain reaction (PCR) was performed using the ABI Prism 7900 sequence detection system and the SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's instructions. The PCR parameters were 95°C for 10 min, followed by 95°C for 15 s and 60°C for 30 s, for 45 cycles. Dissociation curve analyses were performed to verify that there was neither unspecific amplification nor formation of primer dimers. Values were calculated on the basis of standard curves generated for each gene. Normalization of samples was determined by dividing copies of survivin transcripts by copies of rRNA. All sets of reactions were conducted in triplicate. The relative expression levels are expressed as a percentage of the indicated control.

In-vivo study animals and measurement

Five-week-old male nude mice [CAnN.Cg-Foxn1nu/CrlCrlj(nu/nu)] were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan) and maintained as described earlier [31]. All animal experiments were approved by an institutional animal care and use committee in Astellas Pharma Inc. On the basis of the approved protocol, animals were killed when the tumor burden exceeded 10% of the body weight of the host animal. Body weight and tumor diameter were measured twice a week using calipers, and tumor volumes were determined according to the following formula: length $\times$ (width^2)/2. Antitumor activity was expressed as the percentage of inhibition of tumor growth and the percentage of regression of the tumor. The percentage of inhibition of tumor growth on day 35 was calculated for each group using the following formula: $100 \times \{1-[(\text{MTV of each group on day 35})-(\text{MTV of each group on day 0})]/[(\text{MTV of the vehicle control group on day 35})-(\text{MTV of the vehicle control group on day 0})]\}$, where MTV is the mean tumor volume. The percentage of regression of the tumor was calculated for all groups with observed tumor regression, using the following formula: $100 \times [1-(\text{MTV of each group on day 35})/(\text{MTV of each group on day 0})]$.

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The number of complete regressions, defined as instances when the tumor burden fell below the limit of palpation in all groups, was noted.

Calu 6 human lung cancer xenograft model *in vivo*

Calu 6 cells were collected with trypsin, suspended in PBS at 6×10^7 cells/ml, and then mixed with an equivalent volume of Matrigel Basement Membrane Matrix (Becton Dickinson Co., Bedford, Massachusetts, USA). Cells were then grafted subcutaneously (s.c.) into the flanks of mice (3×10^6 cells/0.1 ml/mouse). When the tumor volume reached a value greater than 100 mm^3, the mice were randomized into groups using SAS software (SAS Institute, Cary, North Carolina, USA) so as to minimize intragroup and intergroup tumor volume variation. For the evaluation of in-vivo antitumor activity of YM155 as monotherapy, the first day of administration was designated as day 0, and observations continued until day 14 ($n = 6$). YM155 (and vehicle control) was administered s.c. as a 7-day continuous infusion at 0.3–10 mg/kg.

For the evaluation of in-vivo antitumor activity of YM155 in combination with docetaxel, the first day of administration was designated as day 0, and observations continued until day 35 ($n = 8$). YM155 (and vehicle control) was administered s.c. as a 7-day continuous infusion at 2 mg/kg. Docetaxel (and vehicle control) was administered as an intravenous (i.v.) bolus injection at 20 mg/kg once every 4 days for three doses. The following combination dosing schemes were investigated as studies A, B, and C. In study A (concomitant treatment), the following treatment groups were evaluated: control, which received a saline infusion starting from day 0 and a saline i.v. bolus on days 0, 4, and 8; YM155 alone, which received YM155 starting on day 0 and a saline i.v. bolus on days 0, 4, and 8; docetaxel alone, which received a saline infusion starting on day 0 and docetaxel on days 0, 4, and 8; and concomitant, which received YM155 starting on day 0 and docetaxel on days 0, 4, and 8. In study B (sequential treatment of docetaxel followed by YM155), the following treatment groups were evaluated: control, which received a saline infusion starting on day 8 and a saline i.v. bolus on days 0, 4, and 8; YM155 alone, which received YM155 starting from day 8 and a saline i.v. bolus on days 0, 4, and 8; docetaxel alone, which received a saline infusion starting from day 8 and docetaxel on days 0, 4, and 8; and combination, which received YM155 starting from day 8 and docetaxel on days 0, 4, and 8. In study C (sequential treatment of YM155 followed by docetaxel), the following treatment groups were evaluated: control, which received a saline infusion starting from day 0 and a saline i.v. bolus on days 7, 11, and 15; YM155 alone, which received YM155 starting from day 0 and a saline i.v. bolus on days 7, 11, and 15; docetaxel alone, which received a saline infusion starting from day 0 and docetaxel on days 7, 11, and 15; and combination, which received YM155 starting from day 0 and docetaxel on days 7, 11, and 15.

Immunohistochemistry studies

For intratumoral analysis of survivin levels, cell proliferation, and apoptosis, the xenografted Calu 6 tumors were excised from the animals under chloroform anesthesia on each specified day and cleaned of connective tissues. The excised tumor was then fixed in 10% formaldehyde for 24 h at room temperature, rinsed with 70% ethanol, and kept in 70% ethanol at 4°C until use. The 10% formaldehyde-fixed tissues were embedded in paraffin and sectioned at 5-μm thickness.

For survivin staining, the sections were placed in 0.5% Immunosaver (Nisshin EM Corp., Tokyo, Japan) and heated at 100°C for 20 min in a microwave to retrieve antigens. Endogenous peroxidase activity was blocked by 0.3% H_2O_2 in methanol (v/v) for 10 min at room temperature, followed by three changes in 10 mmol/l PBS (pH 7.4). The sections were then incubated with rabbit antisurvivin antibody for 1 h at room temperature. Antibody binding was visualized using EnVision + (Dako Denmark A/S, Glostrup, Denmark) and diaminobenzidine (DAB) (Liquid DAB Substrate Pack, BioGenex, Fremont, California, USA).

The incidence of apoptosis was assessed with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) by using an Apop Tag Plus Peroxidase in-situ Apoptosis kit (Millipore, Billerica, Massachusetts, USA) according to the manufacturer's instructions. TUNEL reaction was visualized by incubation with DAB solution (Liquid DAB Substrate Pack), and the slides were counterstained with Mayer's hematoxylin. The apoptosis index was determined by calculating the number of TUNEL-positive nuclei per 1000 tumor cells containing non-necrotic tumor components.

For the calculation of the mitotic index, the sections stained with Mayer's hematoxylin and eosin were used and the number of mitotic cells per 1000 tumor cells from four microscopic visual fields containing non-necrotic tumor components was assessed. Only nuclei with characteristic morphologic features of prophase, metaphase, anaphase, or telophase were counted as mitotic cells. Apoptotic and karyolytic nuclei, which usually accompany degenerated cytoplasms, were excluded.

For the calculation of the Ki-67 proliferation index, the sections were heat immobilized, dewaxed in xylene, and rehydrated in a graded series of ethanol with a final wash in distilled water. Antigen retrieval was carried out in 0.5% Immunosaver (Nisshin EM) and heated at 100°C for 20 min in a microwave. Endogenous peroxidase activity was blocked by 0.3% H_2O_2 in methanol (v/v) for 10 min at room temperature, followed by three changes in 10 mmol/l PBS (pH 7.4). The sections were

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then incubated with mouse monoclonal anti-Ki-67 antibody, clone MIB-1 (1 : 50; M7240, Dako), for 1 h at room temperature in a humidity chamber. Antibody binding was visualized using EnVision + and incubation with DAB. The sections were counterstained with Mayer's hematoxylin. The Ki-67 proliferation index was determined by calculating the number of Ki-67-positive cells per 1000 tumor cells containing non-necrotic tumor components.

Statistical analysis

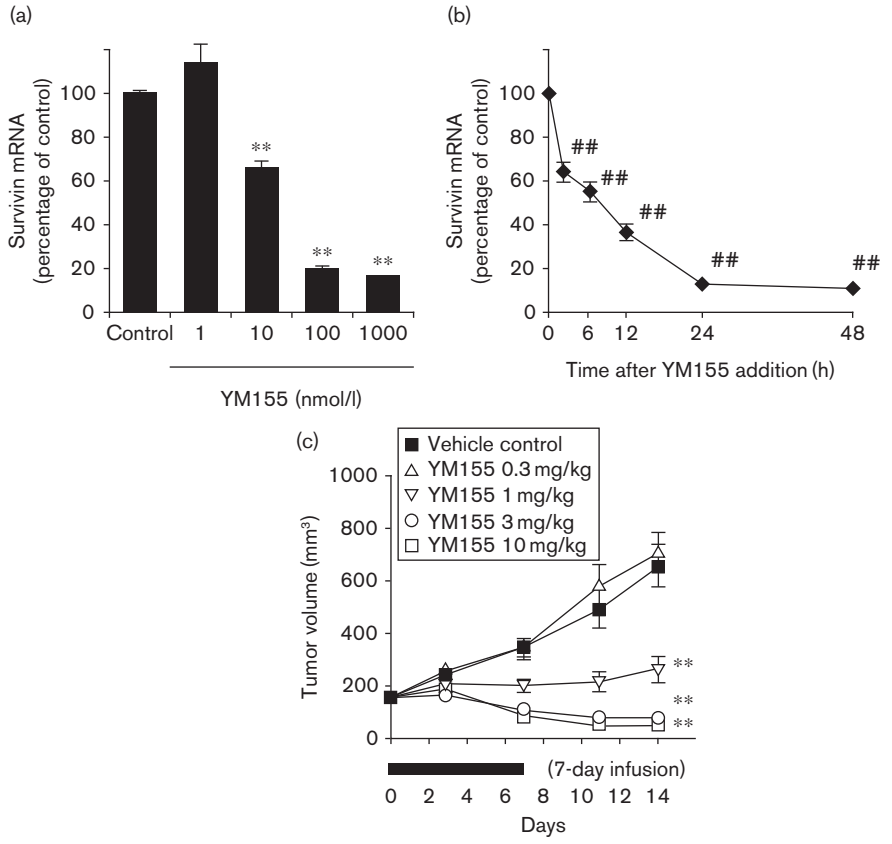
Values were expressed as means ± standard error of the mean. For the mRNA expression levels *in vitro* and monotherapy efficacy evaluation of YM155 *in vivo*, differences between groups were analyzed by Dunnett's test. For combination studies *in vivo*, tumor volume and body weight on day 35 were compared between each single-compound group and combination group using Student's *t*-test. For immunohistochemistry studies, data were analyzed using Student's *t*-test. All data analyses were performed using SAS statistical software (SAS Institute) with *P* values of less than 0.05 considered as significant.

Results

YM155 inhibits survivin expression in Calu 6 human lung cancer cells *in vitro*

YM155 at concentrations from 10 to 1000 nmol/l significantly decreased the expression of survivin mRNA in Calu 6 human lung cancer cells in a dose-dependent manner (Fig. 1a). Survivin mRNA significantly decreased 2 h after the administration of 100 nmol/l YM155 and reached more than 80% inhibition after 24 h (Fig. 1b). The mean log growth inhibition of 50% value of YM155 in

Fig. 1



YM155 suppresses survivin expression in human non-small-cell lung cancer Calu 6 cells and induced significant regression of subcutaneous (s.c.) xenografted Calu 6 tumors in mice. (a) Calu 6 cells ($n=4$) were treated with YM155 (1, 10, 100, and 1000 nmol/l) for 24 h (control: 0.1% dimethyl sulfoxide). (b) Calu 6 cells ($n=4$) were treated with YM155 (100 nmol/l) for the indicated period of time. Cells were analyzed to detect survivin messenger RNA (mRNA) by real-time PCR. Expression levels of survivin mRNA were normalized to that of 18S ribosomal RNA and expressed as relative expression levels percentage of control, or percentage of value at 0 h). Points in both (a) and (b), mean from four separate experiments; bars, standard error (SE); $^{**}P$ value of less than 0.01 versus control (Dunnett's test using within-subject error); $^{\#\#}P$ value of less than 0.01 versus 0 h (Dunnett's test using within-subject error). (c) YM155 was administered as a 7-day s.c. continuous infusion at 0.3, 1, 3, and 10 mg/kg. The vertical bars represent SE ($n=6$). Efficacy in each group was statistically evaluated by Dunnett's test at day 14. $^{**}P$ value of less than 0.01 versus vehicle control.

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Calu 6 cells has been shown to be 6.8 nmol/l [34], a dose at which the current data show YM155-induced suppression of survivin mRNA.

YM155 monotherapy induces significant antitumor activity in the human Calu 6 lung cancer xenograft model *in vivo*

The antitumor effects of YM155 administered as monotherapy were evaluated in the Calu 6 s.c. xenograft model. On day 14, YM155 at 3 and 10 mg/kg completely inhibited tumor growth and induced tumor regression by 50 and 67%, respectively, compared with the initial tumor volumes at day 0 (Fig. 1c). Throughout these studies, no significant decreases in body weight were observed at any dose of YM155 (data not shown).

YM155 enhances docetaxel-induced antitumor activity in the human Calu 6 lung cancer xenograft model *in vivo*

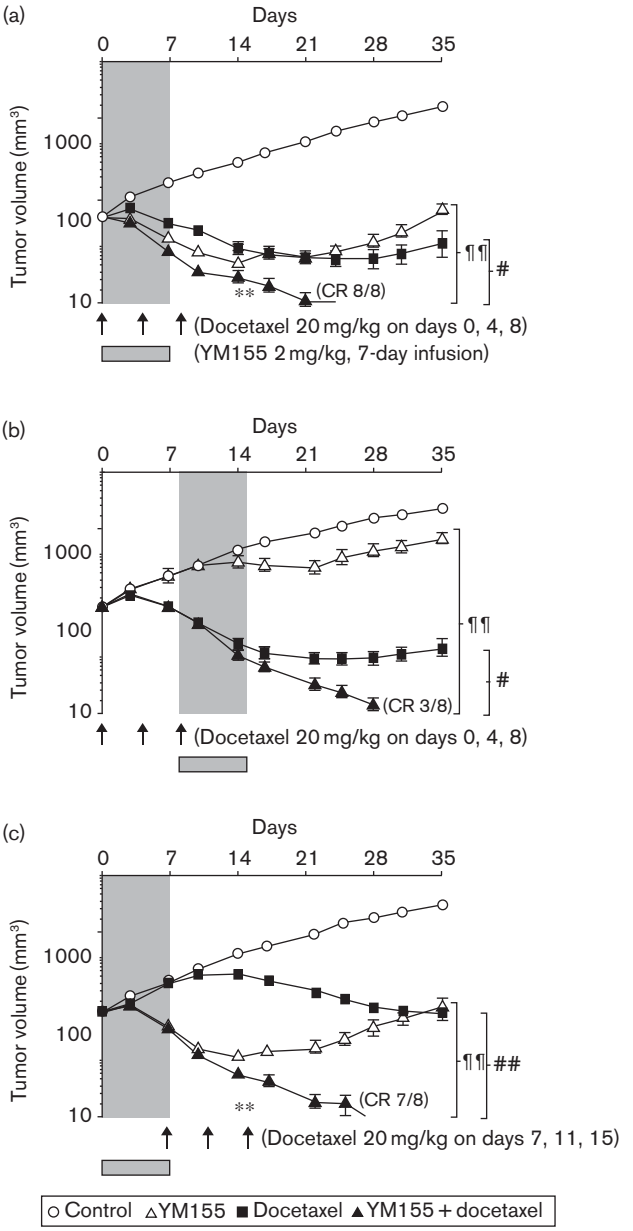
The antitumor effects of YM155 (7-day continuous s.c. infusion at 2 mg/kg) in combination with docetaxel (i.v. bolus at 20 mg/kg) were evaluated in a series of in-vivo studies using the Calu 6 s.c. xenograft model (Table 1; Figs 2 and 3). Although both YM155 and docetaxel monotherapies induced regression of tumors, this was apparent only during the first few weeks and was followed by tumor regrowth in the last few weeks (Fig. 2). Moreover, no complete regressions were achieved with either YM155 or docetaxel monotherapy. In contrast,

Table 1 YM155 potentiated the antitumor activity of docetaxel and induced complete regression of tumors in Calu 6 subcutaneous xenograft model

Treatment groups	Antitumor activity: Inhibition (%)	Antitumor activity: Regression (%)	Number of complete responses
A[a]			
Control	–	–	0
YM155 alone	99	–	0
Docetaxel alone	>100	52	0
YM155 + docetaxel	>100	>100	8/8
B[b]			
Control	–	–	0
YM155 alone	61	–	0
Docetaxel alone	>100	68	0
YM155 + docetaxel	>100	97	3/8
C[c]			
Control	–	–	0
YM155 alone	99	–	0
Docetaxel alone	>100	10	0
YM155 + docetaxel	>100	99	7/8

The number of complete regressions, defined as instances when the tumor burden falls below the limit of palpation in all groups, was noted.
[a]Concomitant treatment: control group [a saline infusion starting from day 0 and a saline intravenous (i.v.) bolus on days 0, 4, and 8]; YM155-alone group (YM155 starting from day 0 and a saline i.v. bolus on days 0, 4, and 8); docetaxel-alone group (a saline infusion starting from day 0 and docetaxel on days 0, 4, and 8); and concomitant group (YM155 starting from day 0 and docetaxel on days 0, 4, and 8).
[b]Sequential treatment of docetaxel followed by YM155: control group (a saline infusion starting from day 8 and a saline i.v. bolus on days 0, 4, and 8); YM155-alone group (YM155 starting from day 8 and a saline i.v. bolus on days 0, 4, and 8); docetaxel-alone group (a saline infusion starting from day 8 and docetaxel, 20 mg/kg, on days 0, 4, and 8); and combination group (YM155 starting from day 8 and docetaxel on days 0, 4, and 8).
[c]Sequential treatment of YM155 followed by docetaxel: control group (a saline infusion of saline starting from day 0 and a saline i.v. bolus on days 7, 11, and 15); YM155-alone group (YM155 starting from day 0 and a saline i.v. bolus on days 7, 11, and 15); docetaxel-alone group (a saline infusion starting from day 0 and docetaxel on days 7, 11, and 15); and combination group (YM155 starting from day 0 and docetaxel on days 7, 11, and 15).

Fig. 2



YM155 potentiated the antitumor activity of docetaxel in the Calu 6 subcutaneous xenograft model. (a) Concomitant treatment with docetaxel and YM155. (b) Docetaxel followed by YM155. (c) YM155 followed by docetaxel. Shaded horizontal bars represent dosing of YM155; arrows represent dosing of docetaxel. The vertical bars represent standard error ($n=8$). Statistical analysis was performed for values on day 35. $^{**}P$ value of less than 0.01; $^{¶¶}P$ value of less than 0.01 versus the YM155 group in the combination group. $^{\#}P$ value of less than 0.05; $^{\#\#}P$ value of less than 0.01 versus the docetaxel group in the combination group. CR, complete regression; NS, no significant difference from the combination group for the docetaxel-alone or YM155-alone groups (Student's *t*-test).

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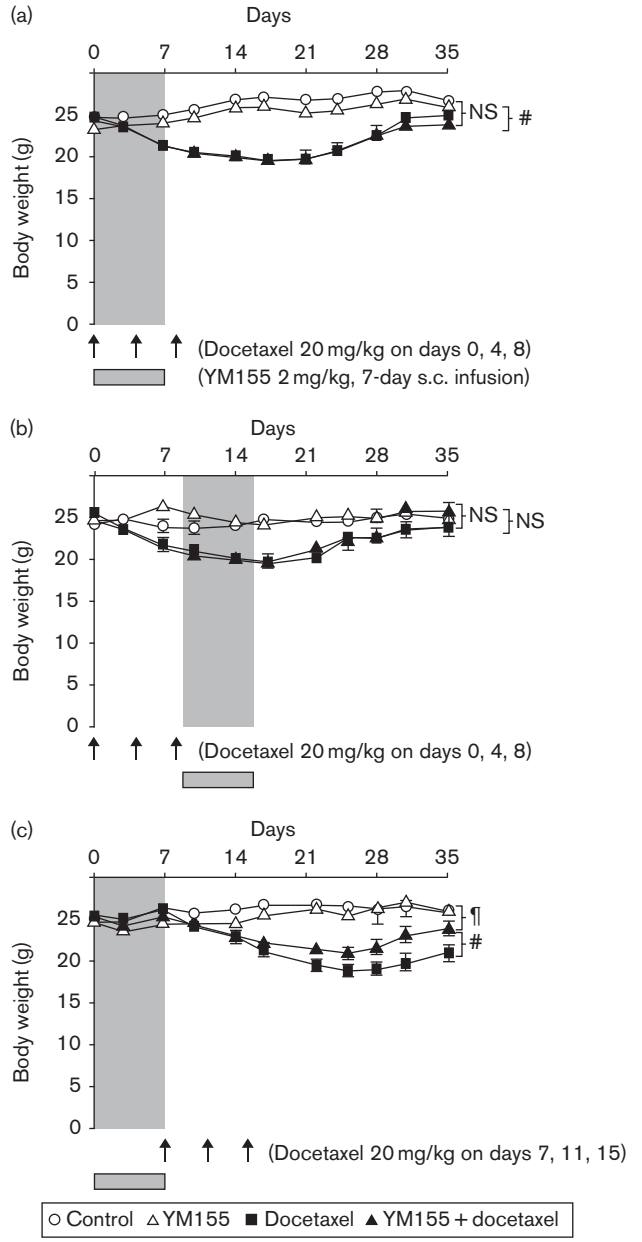
Fig. 3



YM155 induced no significant alteration of docetaxel-induced body weight loss in the Calu 6 lung cancer subcutaneous (s.c.) xenograft model. (a) Concomitant treatment with docetaxel and YM155. (b) Docetaxel followed by YM155. (c) YM155 followed by docetaxel. Shaded horizontal bars represent dosing of YM155; arrows represent dosing of docetaxel. The vertical bars represent standard error ($n=8$). Statistical analysis was performed for values on day 35. $^{¶}P$ value of less than 0.05 versus the YM155 group in the combination group. $^{\#}P$ value of less than 0.05 versus the docetaxel group in the combination group. NS, no significant difference from the combination group for the docetaxel-alone or YM155-alone group (Student's *t*-test).

YM155 given concomitantly with docetaxel significantly inhibited tumor growth compared with either compound given alone, and resulted in a complete regression of established tumors in all treated mice (Fig. 2a). Body weight loss was observed in mice treated with docetaxel but not in those treated with YM155. Docetaxel-induced weight loss was not significantly altered by concomitant YM155 (Fig. 3a). Within the sequential combination regimens, YM155 also significantly potentiated the antitumor activity of docetaxel regardless of the dosing sequence. When bolus dosing of docetaxel was followed by a 7-day continuous infusion of YM155, a complete response was observed in three of eight animals (Fig. 2b). When a 7-day continuous infusion of YM155 was followed by bolus dosing of docetaxel, a complete response was achieved in seven of eight animals (Fig. 2c). No significant alterations in docetaxel-induced body weight loss were observed during the sequential treatment of YM155 and docetaxel (Fig. 3b and c).

YM155 suppresses intratumoral survivin expression *in vivo* and enhances docetaxel-induced apoptosis in the human Calu 6 lung cancer xenograft model *in vivo*

Intratumoral survivin expression levels and apoptosis induction were evaluated in the xenografted Calu 6 tumors in mice that received concomitant treatment with YM155 starting from day 0 and docetaxel on days 0 and 4 (Figs 4 and 5). On day 7, the proliferative status of the xenografted tumors was already significantly altered in both the YM155-alone group and the combination group. The mitotic index for the YM155-alone group was significantly decreased by 86% compared with that in the control group ($P < 0.01$); no decrease was observed in the docetaxel-alone group (Fig. 5a). The mitotic index for the YM155 and docetaxel concomitant group was significantly decreased by 84% ($P < 0.01$) compared with that in the docetaxel-alone group (Fig. 5a). In addition, the proliferation index for the YM155-alone group was significantly decreased by 52% compared with that in the control group ($P < 0.01$); no comparative significant decrease was observed in the docetaxel-alone group (Figs 4 and 5b). The proliferation index for the concomitant group was significantly decreased (63%) compared with that in the docetaxel-alone group ($P < 0.01$), and by 22% compared with that in the YM155-alone group ($P < 0.01$). Although the combination of YM155 and docetaxel did not seem to have a synergistic effect on the tumor proliferation index of the xenografted tumors, there was a significant increase in the apoptotic index compared with the control group ($P < 0.01$), with smaller increases being observed in tumors in the YM155-alone or docetaxel-alone group (Figs 4 and 5c). The apoptosis index in the concomitant group (5.3%) was significantly elevated compared with that in the YM155-alone (2.7%) or docetaxel-alone (4.1%) group ($P < 0.01$).

Intratumoral survivin expression levels in the YM155-alone group were decreased on day 7 compared with those in the control group (Fig. 4). In contrast, slight increases in survivin expression levels were observed when docetaxel was administered alone (Fig. 4). YM155 also

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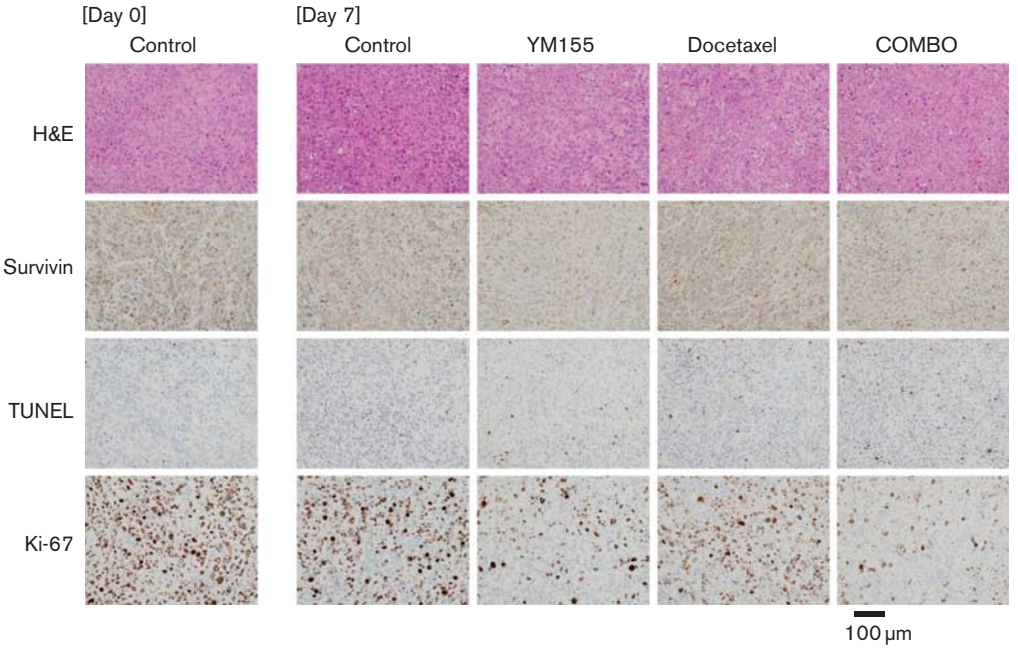
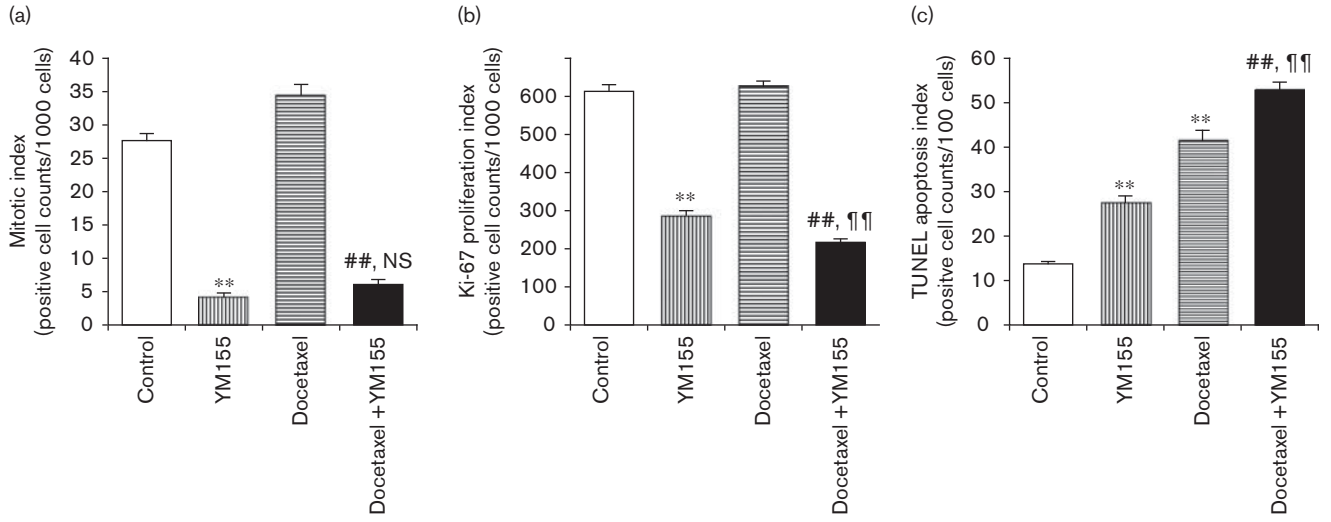
Fig. 4



Representative images of tumor sections from the Calu 6 xenografted mouse model stained with hematoxylin and eosin (H&E), immunohistochemical survivin, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), and Ki-67.

Fig. 5



YM155 suppresses intratumoral survivin expression *in vivo* and enhances docetaxel-induced apoptosis in the human Calu 6 lung cancer xenograft model *in vivo*. (a) Mitotic index, (b) Ki-67 proliferation index, and (c) apoptotic index (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, TUNEL). Data are expressed as mean ± standard error ($n = 8$). $^{**}P$ value of less than 0.01 versus control group, $^{\#\#}P$ value of less than 0.01 versus docetaxel-alone group, $^{\P\P}P$ value of less than 0.01 versus YM155-alone group (all student's *t*-test).

suppressed intratumoral survivin levels in the concomitant combination group (Fig. 4). These results suggest that YM155 inhibits survivin expression in human tumors regardless of the cell-cycle alteration induced by docetaxel.

Discussion

This study has shown the significant efficacy of YM155 in combination with docetaxel in the human Calu 6 xenograft model. Both the concomitant treatment with YM155 and docetaxel and the sequential treatment with

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YM155 followed by docetaxel resulted in complete regression of the vast majority of the tumor xenografts. In those animals in which tumors became impalpable, treatment was considered curative. These animals lived for more than 6 months after treatment (at which point the study was terminated), and no recurrence of disease or deterioration of health was observed during this extended observation period. We have also shown that tumor regression induced by the concomitant administration of YM155 and docetaxel was accompanied by decreased intratumoral survivin and greater apoptosis induction compared with either treatment alone. Although this study was not designed to investigate the effects of YM155 on tumor cell cycle, the mitotic and Ki-67 indices do suggest that YM155 may not significantly alter cell-cycle distribution into G2/M or G0. Interestingly, we have also observed improved efficacy of YM155 (including complete tumor regressions) in combination with docetaxel, paclitaxel, or vinorelbine in Calu 6 NSCLC, A375 human melanoma, and PC-3 human castration-refractory prostate cancer xenograft models (data not shown). The significant antitumor activity of YM155 in combination with G2/M interacting drugs could be partially explained by the fact that YM155 is not competing with them in inducing specific cell-cycle alteration in G2/M and significant cell death by suppressing survivin expression in tumors.

Investigators have shown that inhibition of survivin by a variety of methods can enhance the efficacy of taxanes in suppressing tumor growth. Methylseleninic acid was found to sensitize hormone-refractory prostate cancer cells to apoptosis induced by paclitaxel by downregulating the basal and paclitaxel-induced expression of survivin and Bcl-XL, thus increasing caspase-mediated apoptosis [30]. Survivin antisense RNA was used to block survivin in HL-60 leukemia cells, improving sensitivity to paclitaxel [29]. Downregulation of survivin with antisense oligonucleotides or small interfering RNA has been shown to sensitize NSCLC cells to sulindac/suberoylanilide hydroxamic acid-induced apoptosis [35]. Moreover, upregulation of survivin in highly metastatic ovarian and breast cancer cells through the fibronectin/phosphoinositol-3-kinase/Akt2 pathway has been shown to counteract apoptosis induced by docetaxel [22]. To this end, our findings that tumor regression induced by concomitant administration of docetaxel and YM155, a novel small molecular entity that suppresses survivin, is accompanied by mitotic arrest and greater apoptosis induction, compared with either treatment alone, may indicate that YM155 treatment causes dividing tumor cells to enter a quiescent stage in which they are more sensitive to the proapoptotic effects of docetaxel. Interestingly, in prostate tumors in a transgenic mouse model, the most effective regimens of docetaxel and 2-methoxyestradiol in terms of increased apoptosis and decreased prostate tumor weights were those that increased mitotic cell arrest [36]. This concept is supported by the observation in this study that sequential treatment with YM155 administered before docetaxel was more effective (seven of eight complete responses) than sequential treatment with docetaxel administered before YM155 (three of eight complete responses).

In this study, both concomitant and sequential treatment of YM155 and docetaxel induced no additional increase in the body weights of treated animals over that induced by multiple administrations of docetaxel. This is suggestive of the general tolerability of YM155 and the suitability of survivin as an antitumor therapy target. YM155 has shown little effect on the expression of other inhibitor of apoptosis family proteins or Bcl-2-related proteins *in vitro* [30] and does not show DNA intercalator properties or DNA topoisomerase I inhibitory activity up to 1 μmol/l range *in vitro* (data not shown). Furthermore, in-vitro cell viability assays suggest that YM155 does not induce cell death induction in human normal cultured cell lines (human umbilical vein endothelial cells and human dermal microvascular endothelial cells) up to 10 μmol/l in 10% charcoal-free fetal bovine serum conditions (data not shown). Although we cannot exclude the possibility that YM155 may exert its antitumor effects through targets other than survivin, it does seem to be that the distinct mechanism of action of YM155 is not associated with the systemic adverse effects and body weight typical of conventional chemotherapeutic agents such as docetaxel.

The findings presented here support further investigation of YM155 as a novel antitumor compound and clearly indicate the potential of a YM155–docetaxel combination regimen for the treatment of NSCLC in the clinical setting. Phase 2 clinical studies of YM155 are being conducted in combination with docetaxel in multiple human solid tumors.

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