Biochemical Pharmacology 82 (2011) 1066–1072

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Mcl-1 downregulation by YM155 contributes to its synergistic anti-tumor activities with ABT-263

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ARTICLE INFO

Article history:
Received 9 June 2011
Received in revised form 1 July 2011
Accepted 6 July 2011
Available online 20 July 2011

Keywords:
YM155
Mcl-1
Survivin
ABT-263
Cell death

ABSTRACT

YM155, a small-molecule survivin suppressant, exhibits anti-tumor activities *in vitro*, *in vivo* and in clinical trials. However, the mechanism of YM155 action remains unclear. In this study, YM155 was administered to a panel of cell lines and the effects of YM155 on Bcl-2 family members were analyzed. Our results show that YM155 strikingly downregulates Mcl-1 in a broad spectrum of cancer cell lines and that the Mcl-1 modulation occurs at the transcriptional level, independently of survivin modulation or caspase activity. Furthermore, analysis of the contribution of Mcl-1 or survivin downregulation to YM155-induced cell death *in vitro* showed that knockdown of Mcl-1 sensitizes cells to YM155-induced cytotoxicity. Finally, our data demonstrate that downregulation of Mcl-1 by YM155 synergistically lowers the threshold of Bcl-2 family member inhibitor ABT-263-induced cell death. Our findings reveal a novel mechanism by which survivin-independent Mcl-1 suppression plays a critical role in YM155-mediated anti-tumor activities. YM155 treatment in combination with ABT-263 thus affords a new strategy for cancer treatment.

1. Introduction

Inhibitors of apoptosis (IAP) family members are negative regulators of apoptosis that suppress the activation of cell death executioners, or caspases. Survivin is the smallest member of the IAP family, consisting of 142 amino acids. It is absent or minimally expressed in most normal, differentiated adult tissues but is upregulated in most cancers. Studies have shown that survivin not only suppresses cellular apoptosis by inhibiting caspase activation but also promotes cellular proliferation by facilitating accurate mitotic progression [1–3]. Survivin is thus an attractive target for treating cancer, and using small-molecule antagonists to inhibit survivin is a strategy to treat malignant tumors.

The first-in-class survivin inhibitor is YM155, a small imidazolium-based compound. YM155 effectively blocks survivin expression via inhibition of its promoter activity [4]. YM155 causes cell death *in vitro*, induces tumor regression of lymphoma, prostate cancer and non-small cell lung cancer (NSCLC) *in vivo* [4,5] and has been evaluated in phase I and II clinical trials [6,7]. Though YM155 downregulates survivin and induces apoptosis in various cancers, our unpublished data and other studies suggest that survivin downregulation is not sufficient to cause apoptosis in some cases [8]. Consistent with these observations, YM155 is thought to primarily function in the regulation of cell division rather than in the control of apoptosis [9,10]. Therefore, the mechanism of YM155-induced apoptosis remains unclear. Further studies are needed to elucidate the mechanism by which YM155 induces apoptotic cell death and tumor regression.

Cellular apoptosis is a well-characterized form of programmed cell death that plays a vital role in development and homeostasis. It can be triggered via intrinsic (mitochondrial) and/or extrinsic (TNF-death receptor) stimuli. In the intrinsic pathway, apoptosis is tightly regulated by the Bcl-2 family members (e.g., Bcl-2, Bcl-XL, Mcl-1 and Bax) at the mitochondrial level and by the inhibitor of apoptosis (IAP) family members (e.g., XIAP, c-IAP1/2 and survivin) at the caspase activation level [11,12]. Multiple internal interactions between proapoptotic and prosurvival molecules control the initiation of cellular apoptosis. To determine whether YM155 causes apoptosis by affecting Bcl-2 family proteins, we analyzed the changes of Bcl-2 family members in various cancer cell lines following administration of YM155, and show that Mcl-1 is

Abbreviations: IAP, inhibitors of apoptosis; NSCLC, non-small cell lung cancer; XIAP, X-linked inhibitor of apoptosis protein; DMSO, dimethyl sulfoxide; PARP, poly (ADP-ribose) polymerase; STAT3, signal transducer and activator of transcription 3.

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doi:10.1016/j.bcp.2011.07.064

downregulated in a wide range of established cancer cell lines. We further demonstrate that the downregulation of Mcl-1 contributes to YM155-induced cell cytotoxicity.

2. Materials and methods

2.1. Cells and reagents

PC3, H28, NCI-H661 [H661], NCI-H157 [H157], DLD-1, D37, U251 and SCC9 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). PC3, H28, H661, H157 and DLD-1 were cultured in RPMI-1640 supplemented with 10% FBS, while D37, U251 and SCC9 cells were maintained in DMEM supplemented with 10% FBS. ABT-263 and PS341 (purity > 99% by HPLC) were purchased from Chemietek (Indianapolis, IN, USA). General caspase inhibitor Z-VAD-FMK was obtained from R&D Systems (Minneapolis, MN, USA). Cycloheximide and actinomycin D were purchased from Sigma (St. Louis, MO, USA). YM155 (Supplementary data 1) was prepared by Chemietek (Indianapolis, IN, USA) according to literature procedures (US Patent Application: US 2006/0223831A1). Chemical purity: >99% (by HPLC). Analytical data: ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.94 (d, J = 1.2 Hz, 1H), 8.63 (d, J = 2.4 Hz, 1H), 8.54 (dd, J = 1.6, 2.4 Hz, 1H), 8.14 (dd, J = 1.6, 6.8 Hz, 1H), 8.09 (m, 1H), 7.97–7.94 (m, 2H), 6.16 (s, 2H), 4.89 (t, J = 5.2 Hz, 2H), 3.81 (t, J = 5.2 Hz, 2H), 3.24 (s, 3H), 2.97 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 175.1, 175.0, 154.6, 149.1, 145.0, 144.7, 144.5, 135.8, 135.6, 132.2, 131.8, 130.7, 130.5, 127.4, 127.2, 70.0, 59.0, 49.3, 48.1, 11.2. MS: m/z: 362.90 [M^+].

2.2. Cell viability detection with trypan blue exclusion staining

Cells were treated with DMSO or YM155 for 24 h. Cells were then harvested and stained with trypan blue and live cells counted by TC10 (Bio-Rad, Richmond, CA, USA). Each experiment was performed in triplicate at least three times.

2.3. Cytochrome c release analysis

Following treatment with YM155, ABT-263 or both drugs for 24 h, 4×10^6 cells per condition were resuspended in 100 μl of permeabilization buffer (75 mM NaCl, 8 mM Na_2PO_4, 1 mM NaH_2PO_4, pH 7.4, 250 mM sucrose, 1 mM EDTA, 700 μg/ml digitonin). Cells were incubated in the above permeabilization buffer at room temperature for 1 min, after which the supernatant containing cytochrome c protein was obtained following centrifugation for 3 min at 13,000 × g. For detection of cytochrome c, 3 μg of the cytosolic fraction was supplemented with SDS-PAGE loading buffer and subjected to western blot analysis. Blots were then probed with anti-cytochrome c antibody.

2.4. Western blotting analysis

Whole cell lysates were prepared, separated by SDS-PAGE and transferred to PVDF membrane. Blots were probed with polyclonal or monoclonal antibodies against Mcl-1, survivin, Bcl-XL, XIAP, cleaved Caspase 3, cleaved PARP (Cell Signaling Technologies, Beverly, MA, USA) and β-actin (Sigma, St. Louis, MO, USA).

2.5. Real-time PCR

Total RNA was extracted from the samples with Trizol reagent (Invitrogen, Carlsbad, CA, USA), and first-strand cDNA was generated using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR was done with SYBR GreenER qPCR Supermix for ABI PRISM (Invitrogen, Carlsbad, CA, USA) using the ABI 7300 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Survivin primer sequences: 5′-CTGCCTGGCAGCCCTTT-3′ (forward) and 5′-CCTCCAAGAAGGGCCAGTTC-3′ (reverse). Mcl-1 primer sequences: 5′-GGGCAGGATTGTGACTCTCATT-3′ (forward) and 5′-GATGCAGCTTTCTTGGTTTATGG-3′ (reverse). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a housekeeping gene for the purpose of normalization.

2.6. Plasmid construction

FUGW-survivin was generated from the FUGW-GFP plasmid and the pCDNA3.1-survivin cDNA (provided by Dr. Fengzhi Li) using standard molecular cloning techniques. The pAD-2HA-Mcl-1 plasmid was a gift of Dr. Shengbing Huang (Mayo Clinic, MN, USA). The pGreenPuro™ shRNA plasmid, containing the H1 RNA polIII promoter, was obtained from System Biosciences (SBI, Mountain View, CA, USA). Construction of the shRNA lentivector was performed according to the manufacturer's instructions. Mcl-1 shRNA (sh-Mcl-1) oligonucleotide: 5′-GGACTTTTATACCTGTTAT-3′. An shRNA template oligonucleotide targeting luciferase was used as a negative control (sh-ctrl): 5′-GTGCGTTGTTAGTACTAA-3′.

2.7. Lentivirus production and infection

VSV-glycoprotein pseudotyped lentiviral vector particles were produced by co-transfection of 293TN cells with lentiviral expression vector, packaging and envelope plasmids (3 μg, 2.5 μg and 2.5 μg/6 cm dish, respectively) using the Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA). After 24 h of co-transfection, the media was changed, and the virus-containing supernatant was harvested at 48 h. Viral supernatants were titrated on 293TN cells, and transductions were conducted in the presence of 4 μg/ml polybrene (Sigma, St. Louis, MO) with a multiplicity of infection (MOI) of 5. siRNA of survivin (si-survivin) and negative control (si-ctrl) were purchased from Ambion Inc. (Austin, TX, USA) and a final concentration of 20 nM was used for transfections.

2.8. Statistical analysis

All data are expressed as the mean and standard deviation from at least three individual experiments. Differences between groups were compared using an unpaired two-tailed t test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Treatment with YM155 results in a time- and dose-dependent downregulation of Mcl-1

Previous studies showed that YM155 inhibited survivin expression and induced apoptosis [4]. To determine the effect of YM155 on Bcl-2 family proteins, H28 (mesothelioma) and PC3 (prostate cancer) cells were treated with a range of YM155 concentrations (from 1 to 1000 nM) for 24 h after which the expression of Bcl-2 family proteins was examined by immunoblotting. Consistent with previous reports [4], survivin was downregulated in a dose-dependent manner. Surprisingly, Mcl-1 was also downregulated in H28 and PC3 cells in a time- and dose-dependent manner (Fig. 1A and B). No changes of other Bcl-2 family members were detected (Fig. 1A and data not shown). To determine whether downregulation of Mcl-1 by YM155 was a cell line-specific effect, D37, U251 (Glioblastoma), H661, H157 (lung cancer), DLD-1 (colorectal carcinoma) and SCC9 (Head and Neck squamous carcinoma) cells were treated with 100 nM YM155 for 8 h. As shown in Fig. 1C, Mcl-1 was decreased in all the cell lines

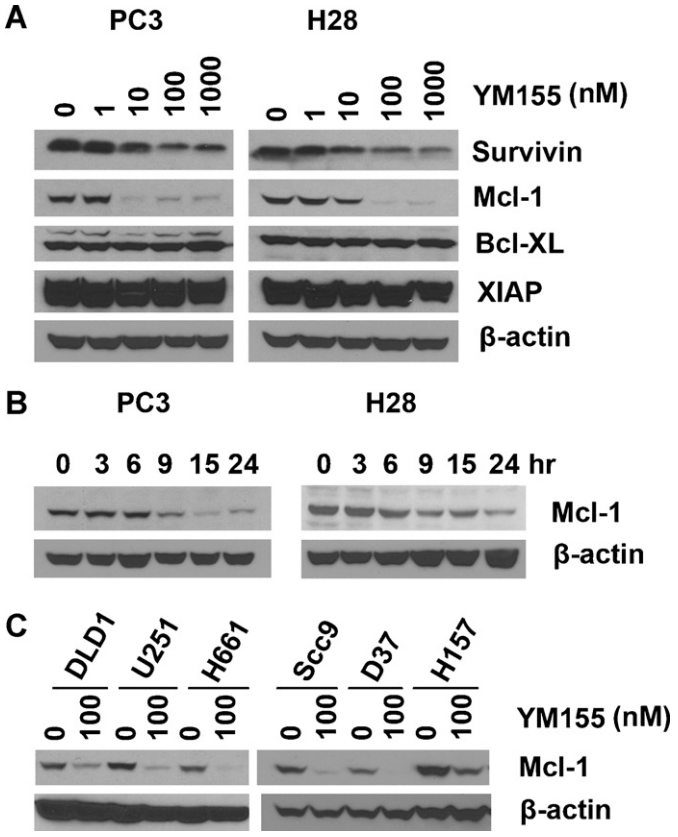

Fig. 1. Mcl-1 is downregulated by treatment of YM155 in a time- and dose-dependent manner. (A) PC3 and H28 cells were treated with YM155 as indicated concentration for 24 h, the changes of proapoptotic and prosurvival proteins were detected. (B) PC3 and H28 cells were administrated with 50 nM YM155 for indicated periods after which cell lysates were detected against Mcl-1. (C) Cells were cultured at the presence or absence of 100 nM YM155 for 8 h for Mcl-1 expression analysis.

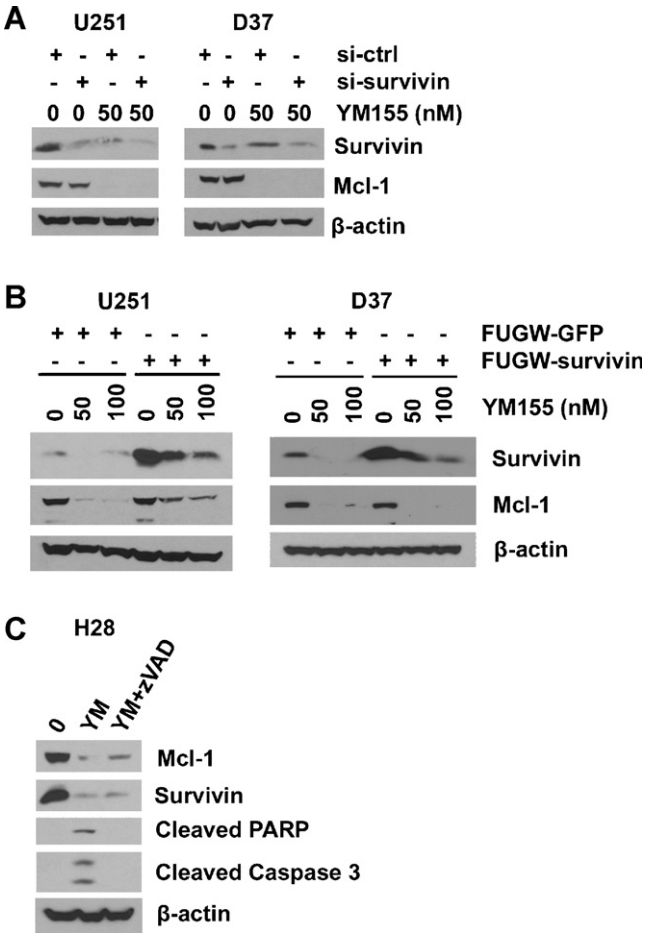

Fig. 2. Decreased Mcl-1 expression by YM155 is independent of either survivin status or caspase activity. (A) U251 and D37 cells were transfected with siRNA of survivin (si-survivin) or control (si-ctrl), following by treatment with or without YM155 for 24 h. Expression of survivin and Mcl-1 were monitored. (B) U251 and D37 cells were infected with FUGW-GPF or FUGW-survivin at MOI of 5, then cells were administrated with indicated dose of YM155 for 24 h. After that, survivin and Mcl-1 level were examined. (C) H28 cells were treated with 100 nM YM155 at the presence or absence of 25 μM Z-VAD-FMK for 8 h. Cells were harvested and cell lysate were analyzed against Mcl-1, survivin, cleaved PARP and cleaved caspase 3 by Western blotting. β-Actin were loaded as endogenous control.

tested. These data indicate that YM155 downregulates Mcl-1 expression in various cancer cell types.

3.2. Mcl-1 downregulation by YM155 is independent of survivin expression and caspase activity

YM155-mediated inhibition of survivin was first characterized by suppression of survivin promoter activity [4]. To test whether the downregulation of Mcl-1 by YM155 is dependent on changes in survivin expression, D37 and U251 cells were transfected with survivin siRNA. As shown in Fig. 2A, there was no change in the protein level of Mcl-1 following survivin knockdown in U251 and D37 cells. Mcl-1 was only decreased in response to YM155 treatment. Ectopic overexpression of survivin also had no effect on Mcl-1 protein level (Fig. 2B). These data indicate that the downregulation of Mcl-1 by YM155 is independent of the level of survivin expression. Interestingly, we found that even the ectopic expression of survivin was downregulated by treatment of YM155.

YM155 induces tumor regression in various cancer cells or xenografts through apoptotic cell death. As previous studies showed that Mcl-1 could be cleaved during apoptosis by active caspase [13], we examined the effect of caspase activity on the downregulation of Mcl-1 by YM155. H28 cells were treated with 50 nM YM155 in the presence or absence of the general caspase inhibitor Z-VAD-FMK for 24 h. Even though caspase 3 and PARP cleavage were inhibited, Mcl-1 and survivin were still downregulated (Fig. 2C), suggesting the downregulation of Mcl-1 and survivin by YM155 is independent of caspase activity.

3.3. Mcl-1 contributes to YM155-induced cell death

We further evaluated the role of Mcl-1 downregulation in YM155-induced cell death. U251 and H28 cells were stably infected with Mcl-1 shRNA (sh-Mcl-1) or shRNA control (sh-ctrl) lentivirus. As shown in Fig. 3A and B, compared to sh-ctrl-infected cells, downregulation of Mcl-1 increased YM155-induced cell death in both U251 (61% cell viability in sh-ctrl cells vs. 36% in sh-Mcl-1 cells at 50 nM YM155, $P < 0.05$) and H28 cells (55% cell viability in sh-ctrl cells vs. 40% in sh-Mcl-1 cells at 50 nM YM155, $P < 0.05$), shown by decreased cellular viability and increased PARP cleavage. Furthermore, the double knockdown of Mcl-1 and survivin in U251 cells further enhanced YM155-triggered cell death, indicating that Mcl-1 plays an important role in YM155-induced cytotoxicity. We then performed the converse experiment to test whether upregulated Mcl-1 could protect cells from YM155-induced cell death. Interestingly, overexpression of Mcl-1 only slightly increased cellular resistance to YM155-induced cell death (data not shown). That may be because ectopic overexpression of Mcl-1 was also downregulated by treatment of YM155 (Fig. 3C). Moreover, the survivin level was not affected by

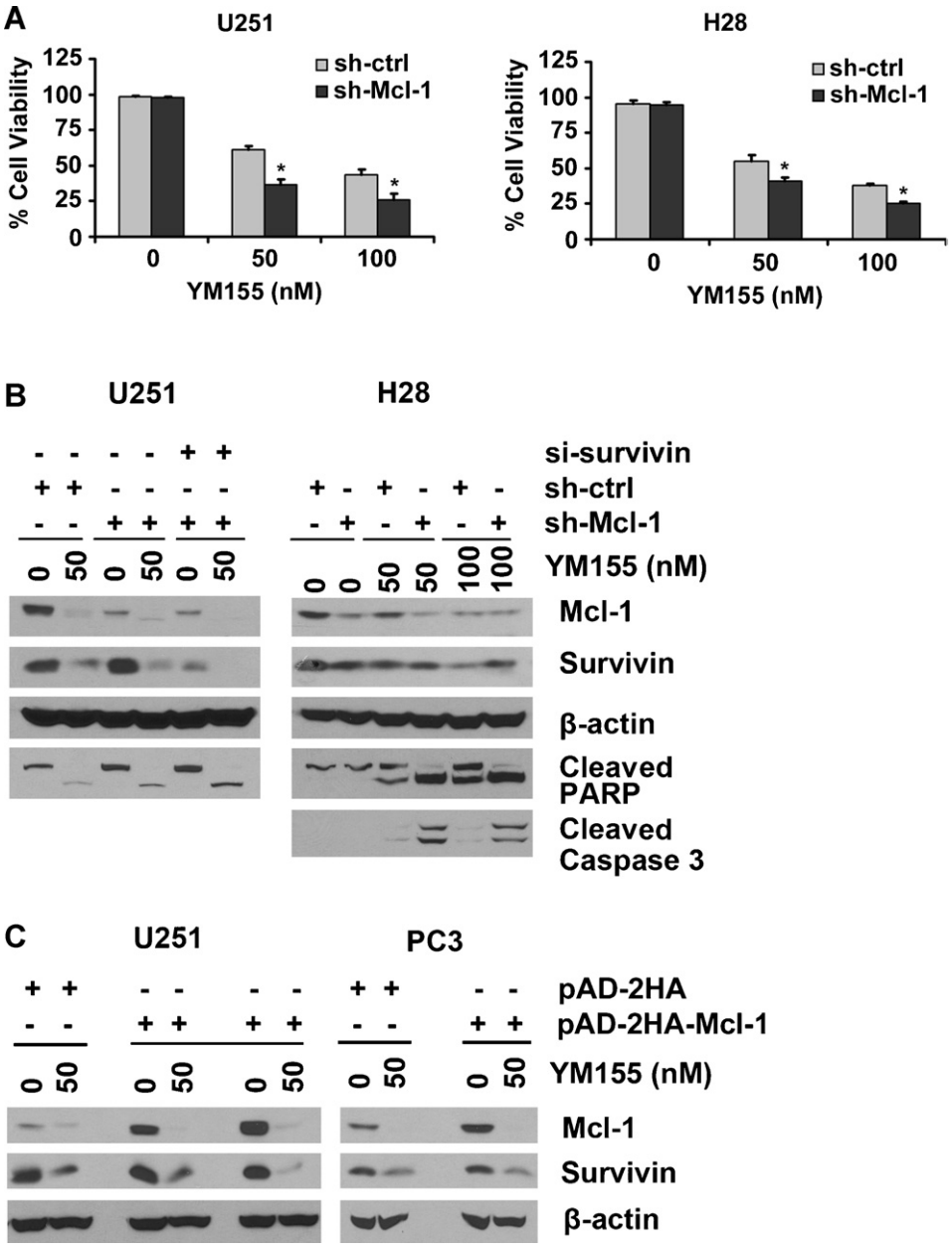

Fig. 3. Mcl-1 downregulation sensitized cells to YM155-induced cell death. U251 and H28 cells were knocked down with Mcl-1 (sh-Mcl-1) or control shRNA (sh-ctrl), following by treatment with YM155 for 24 h. (A) Cell viability rates were analyzed and normalized to vehicle treatment cells. Data represents means ± standard deviation of three independent experiments (*$P < 0.05$, compared with vehicle treatment control); (B) whole cell lyaste were prepared and analyzed with antibodies against Mcl-1, survivin, cleaved PARP and cleaved caspase 3 by Western blotting. (C) U251 and PC3 cells were infected with Mcl-1 or empty overexpression vector followed by treatment with YM155 or vehicle for 24 h. Expression level of Mcl-1 and survivin were tested by Western blotting. β-Actin were used as endogenous control.

the downregulation or overexpression of Mcl-1, indicating that survivin downregulation by YM155 is also independent of Mcl-1.

3.4. YM155 downregulates Mcl-1 through inhibition of transcription

To determine how Mcl-1 was downregulated by YM155, PC3 cells were first treated with proteasome inhibitor PS341 (10 nM) in the presence or absence of 100 nM YM155. The downregulation of Mcl-1 was quicker in the presence of YM155, indicating that YM155 downregulated Mcl-1 at either the level of translation or transcription (Fig. 4A). To further characterize the mechanism of Mcl-1 downregulation by YM155, PC3 cells were treated with either cycloheximide (100 μM) or actinomycin D (5 μg/ml) in the presence or absence of YM155. The presence of YM155 did not contribute to Mcl-1 downregulation by cycloheximide or actinomycin D, suggesting that the inhibition of Mcl-1 expression by YM155 occurs at the transcriptional level (Fig. 4B and C). Next, we examined Mcl-1 mRNA using real-time PCR. After 6 h of treatment with YM155, Mcl-1 mRNA was decreased to 38% of vehicle-treated control ($P < 0.05$), similar to survivin, which was downregulated to 32% of control ($P < 0.05$) (Fig. 4D).

3.5. YM155 enhances ABT-263-triggered cell death via downregulation of Mcl-1

ABT-263 is an orally bioavailable Bcl-2 family member inhibitor that possesses high affinity for Bcl-XL, Bcl-2 and Bcl-w, but not for Mcl-1 [14]. Previous studies showed that downregulation of Mcl-1

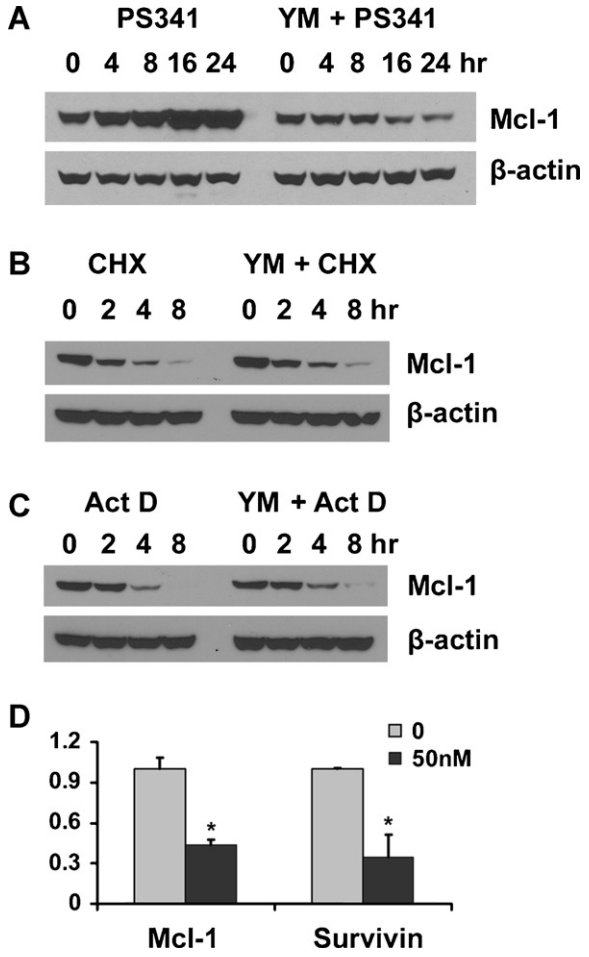

Fig. 4. Mcl-1 is downregulated by YM155 at transcription level. PC3 cells were exposed to 10 nM PS341 (A), 10 μM cycloheximide (B) and 5 μg/ml actinomycin D (C) in the presence or absence of 100 nM YM155 for indicated periods, then whole cell lysates were prepared and detected against Mcl-1 antibodies. β-Actin were used as endogenous control. (D) PC3 cells were treated with 50 nM YM155 for 8 h, after that total RNA was extracted and Mcl-1 and survivin mRNA level were quantified by real time PCR. Data represents means ± standard deviation of three independent experiments (*$P < 0.05$, compared with vehicle control).

could enhance ABT-263-induced apoptotic cell death in a wide range of cancer cells [15–17]. Here, we found that knockdown of Mcl-1 by shRNA significantly enhanced ABT-263-induced cell death in U251 and H28 cells (Fig. 5A), consistent with the previous report. Because Mcl-1 could be downregulated by treatment of YM155, we tested the effects of combination treatment with YM155 and ABT-263 on U251 and H28 cells. As indicated in Fig. 5B, YM155 dramatically potentiated ABT-263-induced cell cytotoxicity in both U251 and H28 cells. Furthermore, the combination index (calculated by the median effect method) was lower than 1 in a wide YM155 dose range suggesting a synergistic interaction between YM155 and ABT-263 (Fig. 5C). Enhancement of the cell killing ability of ABT-263 by YM155 was also observed by western blotting. As shown in Fig. 5D, the combination treatment of ABT-263 and YM155 induced greater cytochrome *c* release and cleavage of caspase 3 and PARP than either drug did alone, further demonstrating a synergistic interaction between YM155 and ABT-263. We also examined the contribution of survivin expression to the enhancement of ABT-263 induced cell death by YM155. As shown in Fig. 5E, downregulation of survivin by siRNA failed to sensitize U251 or H28 cells to ABT-263-triggered cell death, indicating that the enhancement of ABT-263-induced cell death by YM155 is due to downregulation of the novel target Mcl-1.

4. Discussion

YM155, a small-molecule survivin inhibitor, demonstrates potent anti-tumor activity in a wide range of human cancer cell lines and xenograft mouse models [4,5,18]. A better understanding of the mechanism by which YM155 triggers apoptosis will facilitate the usage of YM155 in clinical therapy and its combination with other targeted agents to treat tumors. In the present study, we demonstrate that downregulation of Mcl-1 is a key process in YM155-mediated apoptosis. Furthermore, we demonstrate that downregulation of Mcl-1 by YM155 is independent of survivin downregulation and has no relationship to caspase activity. We also show that the downregulation of Mcl-1 plays a vital role in YM155-induced cytotoxicity, and sensitizes cells to ABT-263-triggered apoptotic cell death.

Mcl-1 is a widely expressed prosurvival Bcl-2 family member that protects cells from stimuli-triggered apoptosis. The turnover of Mcl-1 usually correlates with induction of cellular apoptosis [19]. Here, we demonstrate that Mcl-1 downregulation by YM155 is not a cell line-specific phenomenon but is detectable in a wide range of human tumor cell types. Given the known role of Mcl-1 in apoptosis, our data indicate that Mcl-1 downregulation plays a vital role in the apoptotic effect of YM155.

Interestingly, decreased Mcl-1 and survivin expression are independent events triggered by YM155. Given that overexpression of Mcl-1 and survivin is frequently observed in various human cancers and that Mcl-1 is also involved in cell cycle regulation [20], Mcl-1 and survivin may possibly be regulated in parallel by the same cellular mechanism. For example, both Mcl-1 and survivin are target genes of STAT3, and could be simultaneously regulated by STAT3 in response to multiple stimuli [21,22]. The administration of indirubin, an active component of a traditional Chinese herbal medicine, inhibits STAT3 signaling, resulting in the downregulation of Mcl-1 and survivin followed by induction of apoptotic cell death [23]. Downregulation of Mcl-1 and survivin is associated with decreased cell proliferation and induction of cell death.

Mcl-1 has a very short half-life, and its expression can be regulated at the transcriptional, translational and/or post-translational level in response to various cellular stimuli [19,24]. In the present study, we demonstrate that Mcl-1 is downregulated by YM155 at the transcriptional level, similar to survivin. Given the evidence that Mcl-1 and survivin share the same trans-regulatory elements and are regulated by the same effectors, such as STAT3 [21–23], it is possible that Mcl-1 is negatively regulated by YM155 via inhibition of promoter activity, at least in PC3 cells. Further study of the mechanism by which YM155 downregulates Mcl-1 expression could inform more rational development of this agent by administration either alone or in combination with other targeted agents in the treatment of human cancers.

ABT-263 is a Bcl-2 family member small molecule inhibitor that possesses high affinity for Bcl-2, Bcl-xL, and Bcl-w ($K_i < 1$ nM), but not for Mcl-1 (K_i = 550 nM)and A1 (K_i = 354 nM) [14]. Neutralization of Mcl-1 is a general method to enhance the anti-tumor activity of ABT-263 or ABT-737, the first generation Bcl-2 family inhibitor [25]. Because Mcl-1 expression could be downregulated by treatment of YM155, we hypothesized that treatment with YM155 would sensitize cells to ABT-263-induced cell apoptosis. Though survivin could inhibit apoptosis by blocking caspase activation, downregulation of survivin did not contribute to ABT-263-triggered cell death, indicating that downregulation of Mcl-1 by YM155 plays a key role in the cell death enhancement caused by the co-treatment of YM155 and ABT-263. In summary, YM155, a small-molecule survivin inhibitor, suppresses Mcl-1 expression in a wide spectrum of cancer cell lines. The downregulation of Mcl-1 provides a rationale for new avenues of YM155 combination therapy with other targeted or chemotherapeutic agents for preclinical or clinical studies.

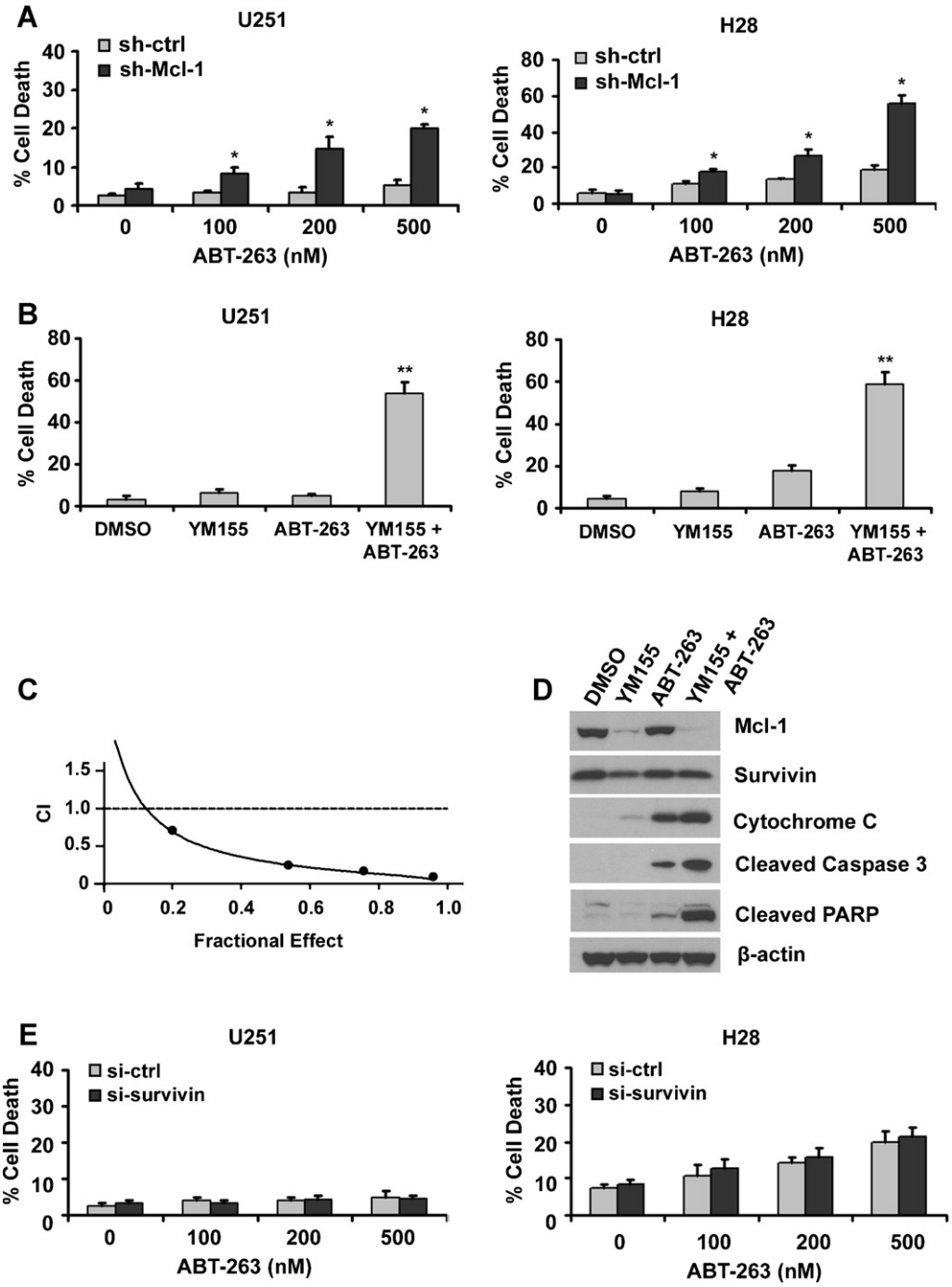

Fig. 5. YM155 enhanced ABT-263-induced cell death via downregulation of Mcl-1. (A) U251 and H28 cells infected with sh-Mcl-1 or sh-ctrl were treated with graded-dose of ABT-263 for 24 h. Cell death rates were analyzed (*$P < 0.05$, compared with sh-ctrl). (B) U251 and H28 cells were administrated with YM155 (20 nM) and/or ABT-263 (500 nM) as indicated for 24 h, YM155 significantly enhanced ABT-263-triggered cell death (**$P < 0.001$, compared with single drug treatment). (C) Value of Combination Index (CI) of YM155 and ABT-263 for cell killing in H28 cells was calculated in relation to the fractional effect. (D) H28 cells were cultured at the presence or absence of YM155 (20 nM) and/or ABT-263 (500 nM) as indicated for 24 h. The change of Mcl-1, survivin, cleaved caspase 3, cleaved PARP and cytochrome *c* release were detected via Western blotting. (E) U251 and H28 cells infected with si-ctrl or si-survivin were treated with graded-dose of ABT-263 for 24 h. *$P < 0.05$, compared with vector control. All the above cell death rates represent means ± standard deviation of three independent experiments.

Conflict of interest

The authors state no conflict of interest.

Acknowledgements

We thank Dr. Fengzhi Li (Roswell Park Cancer Institute, NY) and Dr. Shengbing Huang (Mayo Clinic, MN) for providing plasmids. This work was supported by Guangdong Province Nature Science Foundation (10151008901000025) and Guangdong Province Developing Program (2009B050700024 and 2009B060700096).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.07.064.

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