NEOPLASIA
www.neoplasia.com

Volume 29 Number xxx Month 2022 pp. 100798

Mcl-1 levels critically impact the sensitivities of human colorectal cancer cells to APG-1252-M1, a novel Bcl-2/Bcl-X_L dual inhibitor that induces Bax-dependent apoptosis

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Abstract

New treatment options, such as targeted therapies, are urgently needed for the treatment of colorectal cancer (CRC), the third leading cause of cancer-related deaths worldwide. The current study focuses on demonstrating the therapeutic efficacies of APG-1252-M1 (an active form of the prodrug, APG-1252 or pelcitoclax), a highly potent Bcl-2/Bcl-X_L dual inhibitor in clinical trials, against CRC and understanding the underlying mechanisms. APG-1252-M1 effectively decreased the survival of CRC cell lines, particularly those expressing relatively low levels of Mcl-1, with the induction of apoptosis. High levels of Mcl-1 were significantly correlated with decreased sensitivity of CRC cell lines to APG-1252-M1. When combined with an Mcl-1 inhibitor, APG-1252-M1 synergistically decreased the survival and induced apoptosis of APG-1252-M1-insensitive cell lines with high levels of Mcl-1. This combination further decreased the survival and enhanced apoptosis even in sensitive cell lines with relatively low levels of Mcl-1, whereas enforced expression of ectopic Mcl-1 in these cells abrogated APG-1252-M1's effects on decreasing cell survival and inducing apoptosis, which could be reversed by Mcl-1 inhibition. APG-1252-M1 rapidly induced cytochrome C and Smac release from mitochondria with caspase-3 and PARP cleavage. Deficiency of Bax in CRC cells abolished APG-1252-M1's ability to induce apoptosis, indicating that APG-1252-M1 induces Bax-dependent apoptosis. The current study thus demonstrates the potential of APG-1252-M1 as a monotherapy in the treatment of CRC, particularly those with low Mcl-1 expression, or in combination with an Mcl-1 inhibitor, warranting further evaluation *in vivo* and in the clinic.

Neoplasia (2022) 29, 100798

Keywords: Bcl-2, Bcl-X_L, APG-1252-M1 (APG-1252), Mcl-1 apoptosis, Colorectal cancer

Introduction

In the United States, colorectal cancer (CRC) is the third most common cancer diagnosed and the third leading cause of cancer-related deaths in men and women, with about 52,980 deaths expected during 2021 [1]. For the past 2 decades, fluoropyrimidine-based chemotherapy has remained the foundation of treatment in the metastatic setting [2]. Thus, new treatment options, such as targeted therapies, are urgently needed. Fortunately, advances in understanding the biology of the disease and in the development of cost-effective technologies that can precisely determine the molecular profiling of the disease will allow us to develop various targeted therapies against CRC, particularly metastatic CRC [3], some of which have been used to treat CRC in the clinic [2,4,5].

Evasion of apoptosis, a form of programmed cell death that is essential for tissue homeostasis, is known to be one of the major hallmarks of cancer. Apoptosis is a particularly important process in the colon where it participates in intestinal turnover. Compromised apoptosis facilitates transformation, tumor progression and therapeutic resistance [4,6]. Bcl-2 family proteins are known for their key roles in regulation of the intrinsic apoptotic pathway. Elevation of anti-apoptotic proteins such as Bcl-2 is commonly associated with various cancers including CRC. Thus, targeting Bcl-2 family proteins has emerged as a novel and promising cancer therapeutic strategy. As a result, small molecule inhibitors named 'BH3-mimetics' (Bcl-2 homology 3 mimetics) have been developed and some of these, such as ABT199 and

Abbreviations: CRC, colorectal cancer; Cyt C, cytochrome C; KO, Knockout; SRB, sulforhodamine B; CI, combination index.

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Received 28 January 2022; received in revised form 8 March 2022; accepted 8 April 2022

https://doi.org/10.1016/j.neo.2022.100798

ABT263, are approved or under clinical investigation [4,7]. The Bcl-2 family of proteins has been implicated in CRC initiation, progression and resistance to therapy and thus should be a valid target for the treatment of CRC as well [4].

APG-1252-M1 (or APG-1244) is the *in vivo* active metabolite of APG-1252 (pelcitoclax) developed by Ascentage Pharma and functions as a dual inhibitor of Bcl-2 and Bcl-X_L. In preclinical studies, it had remarkable single-agent antitumor effects in acute myeloid leukemia, small cell lung cancer and gastric cancer [8–10] and in overcoming intrinsic and acquired resistance to ABT-199 in multiple myeloma cells [11]. It showed enhanced activity against the growth of gastric cancer xenografts when combined with the chemotherapeutic agent 5-FU [10], and against nasopharyngeal carcinoma when combined with gemcitabine [12]. Clinical trials are currently underway to test the activity of APG-1252 as a single agent or in combination with chemotherapeutic drugs (NCT03387332, NCT04354727, NCT04001777, NCT04210037, and NCT03080311). A serious concern of the Bcl-2/Bcl-X_L inhibitors is the platelet toxicity that causes thrombocytopenia in patients [13,14]. APG-1252 was synthesized based on the strategy of pro-drug design. As so, it has limited cell permeability during circulation when given through iv route and will be converted to the more active metabolite, APG-1252-M1, once in tumors/tissues. This feature makes it a unique Bcl-2/Bcl-X_L inhibitor with a favorable and manageable platelet toxicity profile in patients as demonstrated in clinical trials [15,16].

The current study focused on determining its activity against CRC cells, revealing the factors that determine cell sensitivity to APG-1252-M1 and understanding the underlying molecular mechanisms. Consequently, we have demonstrated the potential of APG-1252-M1 as a monotherapy in the treatment of CRC, particularly those with low Mcl-1 expression, or in combination with an Mcl-1 inhibitor via the potent induction of Bax-mediated apoptosis.

Materials and methods

Reagents

APG-1252-M1, APG-1252 (prodrug of APG-1252-M1 for *in vivo* study) and APG-3526 were provided by Ascentage Pharma Group Inc. (Suzhou, China). ABT263, ABT199 and S63845 (MIK665) were purchased from MedChemExpress (Monmouth Junction). AZD5991 was purchased from Selleckchem (Houston, TX). The caspase inhibitors, CBZ-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) and Z-lle-Glu(Ome)-Thr-Asp(OMe)-fluoromethyl ketone (z-IETD-fmk), were purchased from Enzyme System Products (Livermore, CA). CYD-2-11 was provided by Dr. X. Deng (Emory University, GA). BMH was purchased from Thermo Scientific (Rockford, IL). Rabbit antibodies against caspase-8, caspase-3, PARP, Smac, PUMA and DR5 (D4E9) were purchased from Cell Signaling Technology (Beverly, MA). Mouse antibodies against Bcl-2, Bax (N-20), Mcl-1, cytochrome C (Cyt C) and rabbit anti-Bcl-X_L antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas). Bim antibody was purchased from EMD Millipore (Burlington, MA). Mouse monoclonal DR4 antibody (B-N28) was purchased from Cell Science (Newburyport, MA). Rabbit polyclonal microtubule-associated protein light chain 3 (LC3) antibody was purchased from Novus Biologicals (Littleton, CO). GAPDH antibody was purchased from Trevigen (Gaithersburg, MD). Mouse monoclonal tubulin and actin antibodies were purchased from Sigma Chemical (St. Louis, MO).

Cell lines and cell culture

Human colon cancer cell lines, HCT116, HCT15, SW480, SW620, KM12, KM12SM, HT29, HCC2998 and DLD were obtained from ATCC (Manassas, VA). HCT116/V, HCT116/Mcl-1, HCC2998/V and HCC2998/Mcl-1 cell lines were established using lentiviral Mcl-1 expression system as described in our previous study [17]. HCT116/Bax-KO, HCT116/PUMA-KO, HCT116/Smac-KO, and HCT116/FADD-KO cell lines were kindly provided by Drs. J. Yu and L. Zhang (University of Pittsburgh, Pittsburgh, PA). HCT116/Bim-KO cell lines were established as described previously [18]. These cell lines were not authenticated. The cell lines were cultured in RPMI 1640 or DMEM medium containing 5% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Cell survival and apoptosis assays

Cells in 96-well plates were treated with the tested agents for 3 days. Cell numbers were then estimated with sulforhodamine B (SRB) assay as described previously [19] or by CellTiter-Glo Luminescent Cell Viability Assay (Promega; Madison, WI). The combination index (CI) for drug interaction was calculated using CompuSyn software (ComboSyn, Inc; Paramus, NJ). Apoptosis was detected with flow cytometry-based apoptotic assay using an annexin V/7-AAD kit purchased from BD Biosciences (San Jose, CA) according to the manufacturer's protocol. Protein cleavage was detected using Western blotting as an additional indication of apoptosis.

Western blot analysis

Whole-cell protein lysates were prepared and Western blotting was conducted as previously described [20].

Gene knockdown

Lentiviruses carrying scramble and Mcl-1 shRNA, respectively, were purchased from Dharmacon, Inc (Lafayette, CO) and used as instructed by the company. Knockdown efficiency was determined by Western blotting.

Mitochondrial isolation and Bax oligomerization assay

Digitonin-based mitochondrial isolation [21] was used to detect Cyt C release and Bax oligomerization. In brief, cells were washed with PBS and then incubated with permeabilization buffer (20 mM HEPES/KOH, pH 7.5, 100 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl and 0.025% digitonin) with protease inhibitor cocktail for 5 min on ice. Supernatant and membrane pellet fractions were separated by centrifugation at 13,000 g for 5 min. The supernatant (cytosolic fraction) was prepared for Western blot to detect Cyt C and Smac. The membrane fraction was treated with crosslinker BMH (0.5 mM) in crosslinking buffer (20 mM HEPES/KOH, pH 7.0, 100 mM sucrose, 2.5 mM $MgCl_2$ and 50 mM KCl) for 30–60 min at room temperature. The reaction was quenched by the addition of reducing sample buffer, boiled for 5 min and then subjected to SDS-PAGE gels and analyzed by western blotting using Bax antibody [22].

Statistical analysis

Two-sided unpaired Student's t-test that analyzes significant difference between two groups, one-way ANOVA test that determines differences among multiple groups and correlation analysis were conducted with GraphPad Prism 9 (GraphPad Software, San Diego, CA). Results were considered statistically significant when P values were less than 0.05.

Results

APG-1252-M1 effectively decreases survival and induces apoptosis in CRC cell lines with low levels of Mcl-1

We first determined the effects of APG-1252-M1 on the growth of a panel of CRC cell lines and found that the tested cell lines exhibited varied

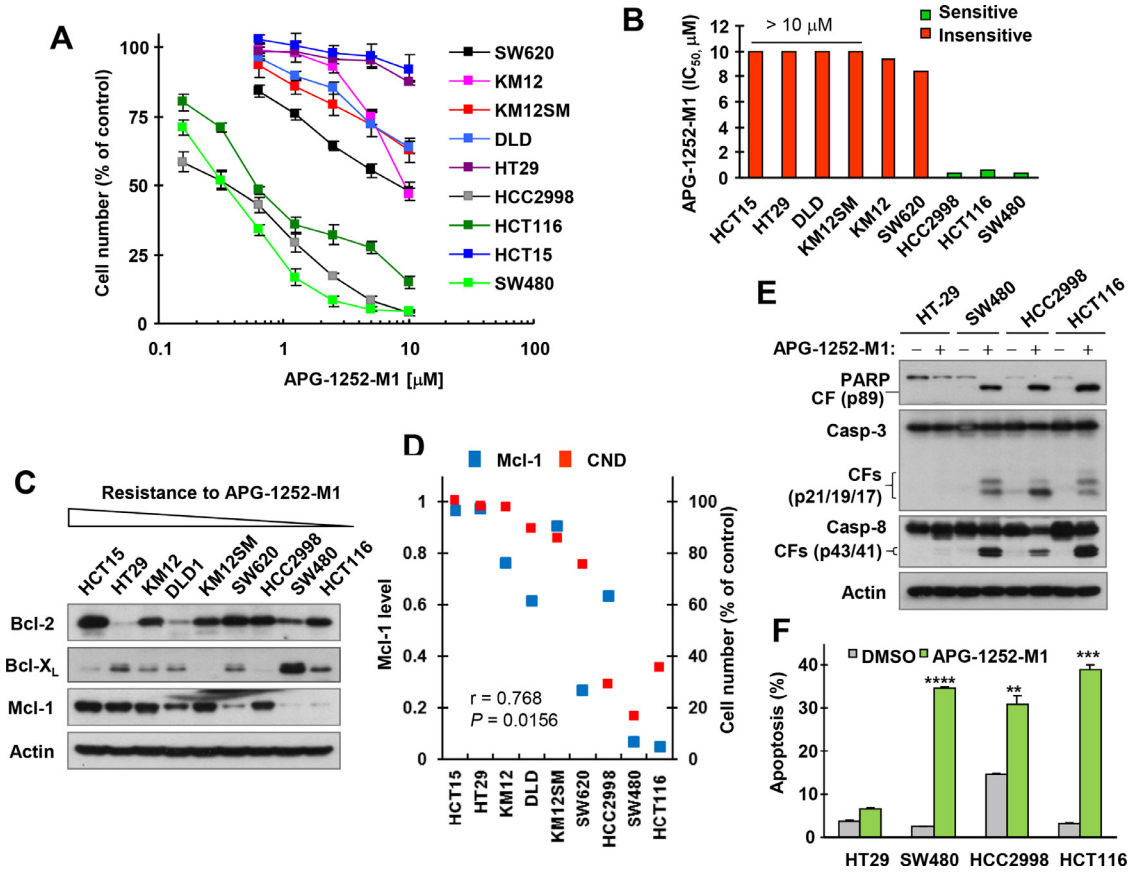

Fig. 1. APG-121252-M1 shows varied activities against the growth of human CRC cells (A and B), which have different expression levels of Bcl-2, Bcl-X_L and Mcl-1 (C) that impact cell responses to APG-1252-M1 (D), with induction of apoptosis (E and F). A, The indicated cell lines were exposed to varied concentrations of APG-1252-M1 for 3 days. Cell numbers were estimated with the SRB assay. The data are means ± SDs of four replicate determinations. B. IC_{50}s were generated from the concentration-dependent curves of the indicated cell lines exposed to the given inhibitors for 3 days presented in A. C, Basal levels of the given proteins in the indicated cell lines with similar cell densities were detected with Western blotting. D, Correlation between the expression levels of Mcl-1 and cell number decrease (CND) caused by 1.25 μM APG-1252-M1. E and F, The indicated cell lines were exposed to DMSO or 1 μM APG-1252-M1 for about 24 h and then harvested for Western blotting to detect different proteins as indicated (E) and annexin V/flowcytometry (F). CF, cleaved form. **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$ compared with DMSO control.

sensitivities to APG-1252-M1: some cell lines (e.g., HCC2998, HCT116 and SW480) were very sensitive to APG-1252-M1 with IC_{50}s < 1 μM, whereas others (e.g., HT29, KM12SM and HCT15) were very resistant with IC_{50}s > 10 μM (Fig. 1A and B). Given that APG-1252-M1 is a Bcl-2/Bcl-X_L dual inhibitor, we then checked basal levels of Bcl-2 and Bcl-X_L in these cell lines in order to determine whether the levels of these proteins impact cell responses to APG-1252-M1. In fact, we did not see a clear and significant correlation between the expression of Bcl-X_L or particularly Bcl-2 and cell sensitivity to APG-1252-M1 (Fig. 1C and D and S1). Mcl-1 is an anti-apoptotic protein that, functionally like Bcl-2 and Bcl-X_L, negatively regulates intrinsic apoptotic pathway [23,24]. We also checked Mcl-1 levels in these cell lines and found that APG-1252-M1-senstive cell lines tended to have lower levels of Mcl-1 than those insensitive to APG-1252-M1, showing a significant negative correlation between Mcl-1 abundance and cell response to APG-1252-M1 (Fig. 1C and D). Finally, we determined whether APG-1252-M1 induces apoptosis of CRC cell lines. While HT29 cells were resistant, three sensitive cell lines, HCC2998, HCT116 and SW480, underwent rapid apoptosis after a 24 h treatment as evidenced by detection of increased cleavage of caspase-3, caspase-8 and PARP (Fig. 1E) and annexin V-positive cell populations (Fig. 1F). Hence, APG-1252-M1 effectively decreases the survival of some sensitive CRC cell lines with the induction of apoptosis.

We further checked the effects of ABT199, a Bcl-2 inhibitor, and ABT263, a Bcl-1/Bcl-X_L/Bcl-w inhibitor [7, 25], on the growth of this panel of CRC cell lines. In general, both inhibitors, particularly ABT263, generated similar cell response patterns to APG-1252-M1 (Fig. S2). However, APG-1252-M1, in general, had even lower IC_{50}s than these agents in decreasing the survival of the sensitive cell lines although ABT263 showed better activity than APG-1252-M1 against the insensitive cell lines (Fig. S2).

APG-1252-M1 combined with an Mcl-1 inhibitor synergistically decreases the survival and induces apoptosis of APG-1252-M1-insensitive cell lines with high levels of Mcl-1

Given the finding that high baseline expression of Mcl-1 is significantly associated with cell resistance to APG-1252-M1, we asked whether Mcl-1 inhibition (e.g., with a Mcl-1 inhibitor) would sensitize the insensitive cell lines with high levels of Mcl-1 to APG-1252-M1. When combined with any of the three Mcl-1 inhibitors, S63845, AZD5991 and APG3526, APG-1252-M1 synergistically decreased the survival of the tested insensitive CRC cell lines with CIs far smaller than 1 (Fig. 2A). The combination of APG-1252-M1 with either S63845 or AZD5991 effectively enhanced cleavage of caspase-3, caspase-8 and PARP and increased annexin V-positive

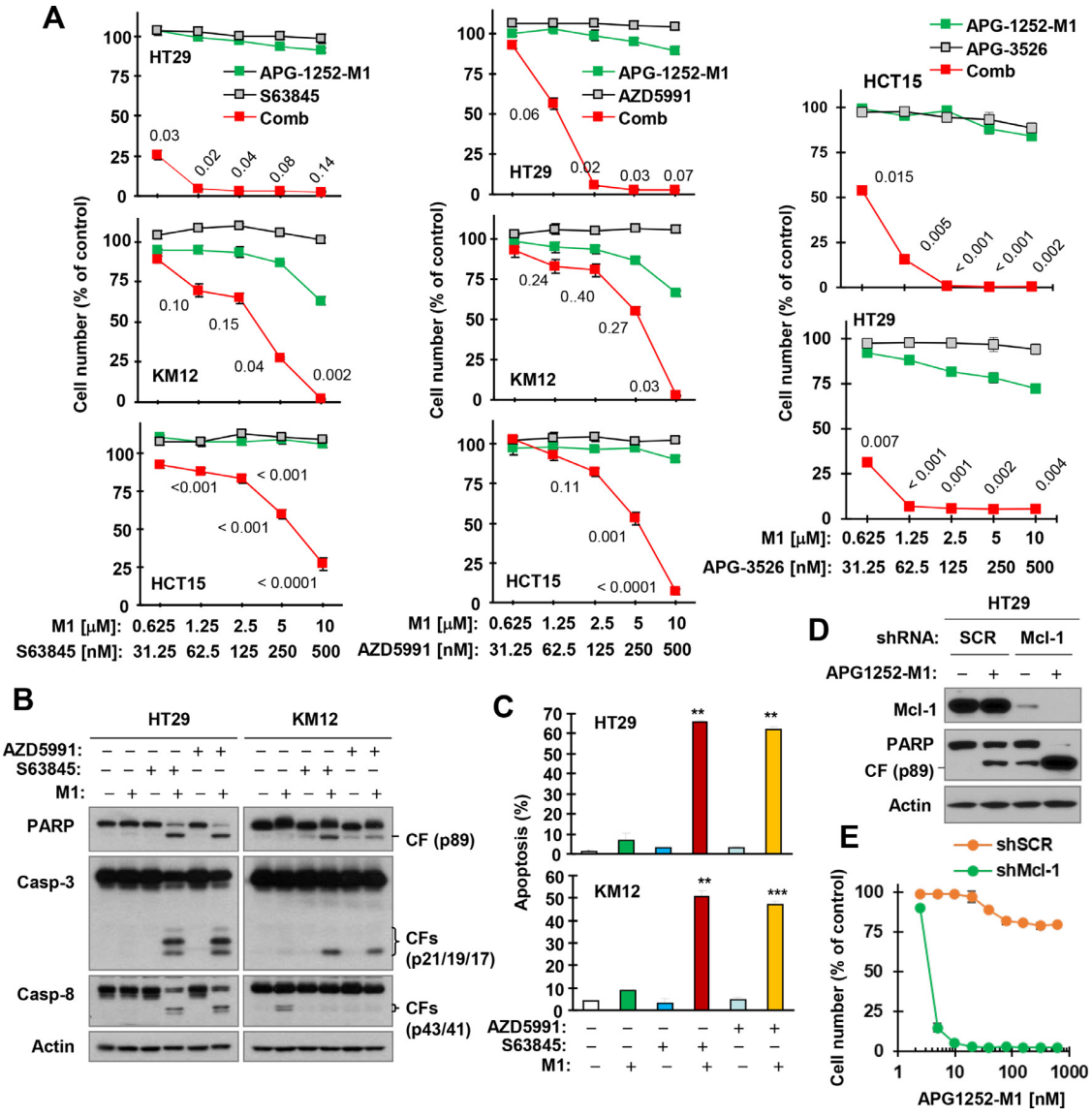

Fig. 2. APG-1252-M1 in combination with an Mcl-1 inhibitor synergistically decreases the survival (A) and enhances apoptosis (B and C) of APG-1252-M1-insensitive CRC cell lines; this is in agreement with the data generated with Mcl-1 knockdown (D and E) A, The indicated cell lines were exposed to varied concentrations of APG-1252-M1 (M1) alone, S63845 alone, AZD5991 alone, APG-3526 alone, APG-1252-M1 plus S63845, APG-1252-M1 plus AZD5991 or APG-1252-M1 plus APG-3526 for 3 days. Cell numbers were estimated with the SRB assay. The data are means ± SDs of four replicate determinations. The numbers inside the graphs are CIs. B and C, The indicated cell lines were treated with DMSO, 5 μM APG-1252-M1, 0.5 μM S63845 or AZD5991, or APG-1252-M1combined with S64845 or AZD5991. After about 25 h (B) or around 30 h (C), the cells were harvested for Western blotting (B) or annexin V/flow cytometry (C). CF. cleaved from. **, $P < 0.01$; and ***, $P < 0.001$ at least compared with either single agent alone. D and E, HT29 cells were infected with lentivisuses carrying shMcl-1 for 40 h followed by treatment with 0.5 μM APG1252-M1 for 16 h (D) or different concentrations of APG1252-M1 for 3 days (E). The data in E are the means ± SDs of triplicate determinations.

cell populations while each agent alone at the tested concentrations did so minimally or not detectably (Fig. 2B and C), indicating that Mcl-1 inhibition combined APG-1252-M1 restores the sensitivity of insensitive CRC cell lines to undergo APG-1252-M1-induced apoptosis. We further knocked down Mcl-1 expression in HT29 cells (Fig. 2D) and then checked their responses to APG-1252-M1. In agreement with the effects of Mcl-1 inhibitors, Mcl-1 knockdown made the cells very sensitive to APG-1252-M1 as determined by cell viability (Fig. 2E) and PARP cleavage (Fig. 2D). Similar results were also generated in SW620 cells (data not shown). Thus genetic inhibition of Mcl-1 further demonstrate the critical role of Mcl-1 in conferring cell resistance to APG-1252-M1.

Interestingly, we found that ABT263, but not ABT199, when combined with S63845 synergistically decreased the survival of APG-1252-M1-insensitive CRC cells (Fig. S3), suggesting that APG-1252-M1 and ABT263 may share similar mechanisms in inducing the death of CRC cells.

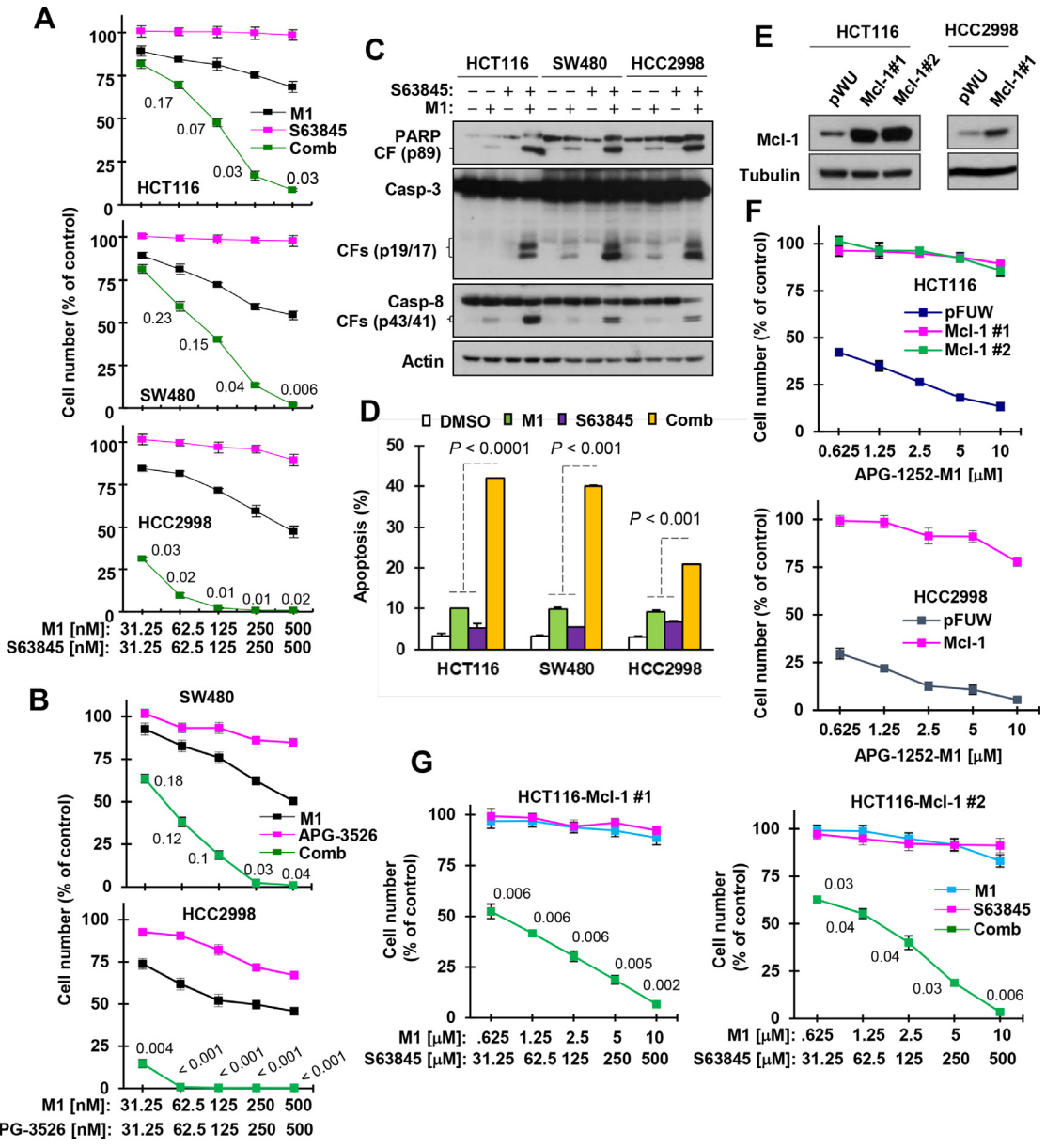

Fig. 3. APG-1252-M1 combined with an Mcl-1 inhibitor synergistically decreases the survival (A and B) and enhances apoptosis (C and D) of APG-1252-M1-senstive CRC cell lines, whereas enforced expression of ectopic Mcl-1 in the sensitive cells abrogates APG-1252-M1's ability to decrease cell survival (E and F), which can be reversed by Mcl-1 inhibition (G). A and B, The indicated cell lines were treated with varied concentrations of APG-1252-M1 (M1) alone, S63845 alone, APG3526 alone, APG-1252-M1 plus S63845, or APG-1252-M1 plus APG3526 for 3 days. Cell numbers were estimated with the SRB assay. The data are means ± SDs of four replicate determinations. C and D, The indicated cell lines were treated with DMSO, 0.25 μM APG-1252-M1, 0.5 μM S63845 or APG-1252-M1 combined with S64845. After around 20 h, the cells were harvested for Western blotting (C) or annexin V/flow cytometry (D). CF. cleaved from. E, Ectopic Mcl-1 expression in the given cell lines was detected with Western blotting. F and G, The different cell lines were exposed to varied concentrations of APG-1252-M1 as indicated for 3 days or treated with indicated concentrations of APG-1252-M1 alone, S63845 alone or their combination for 3 days. Cell numbers were estimated with the SRB assay. The data are means ± SDs of four replicate determinations.

Inhibition of Mcl-1 further enhances APG-1252-M1's effects on decreasing the survival and inducing apoptosis of APG-1252-M1-sensitive cells expressing low levels of Mcl-1, whereas enforced expression of ectopic Mcl-1 in the cells abrogates APG-1252-M1's therapeutic efficacy, which can be reversed by Mcl-1 inhibition

We were also curious to know whether Mcl-1 inhibition further sensitizes the sensitive CRC cells to APG-1252-M1. As we observed in the resistant CRC cells, APG-1252-M1 combined with either S63845 (Fig. 3A) or APG-3526 (Fig. 3B) synergistically decreased the survival of the tested sensitive CRC cell lines. Consistently, the combination of APG-1252-M1 and S63845 was much more potent than either agent alone in inducing the cleavage of caspases and PARP (Fig. 3C) and in increasing annexin V-positive cells (Fig. 3D) in each tested sensitive CRC cell line. To further leverage the critical role of Mcl-1 in impacting cell sensitivity to APG-1252-M1, we enforced the elevation of Mcl-1 levels in two sensitive cell lines (HCT116 and HCC2998) via lentiviral overexpression of ectopic Mcl-1 as confirmed by Western blotting (Fig. 3E). Here, HCT116/Mcl-1 #1 and #2 represent two different cell populations expressing ectopic Mcl-1 generated from HCT116 cells duplicated for Mcl-1 lentiviral infection. These Mcl-1-expressing cell lines were fully resistant to APG-1252-M1 (Fig. 3F). However, the presence of S63835 restored the sensitivities of both HCT116/Mcl-1 #1 and #2 cells to APG-1252-M1 (Fig. 3G). These results together convincingly demonstrate the critical role of Mcl-1 in determining the sensitivity of CRC cells to APG-1252-M1.

APG-1252-M1 activates caspase-8 and caspase-3 and induces release of Cyt C and Smac from mitochondria, accompanied with varied effects on Mcl-1 levels in APG-1252-M1-sensitive CRC cells

APG-1252-M1 obviously induces caspase- 8 activation while activating caspase-3 as shown above in Fig. 1E. To better understand the role of caspase-8 activation, we conducted detailed time-course analyses of caspase-8 cleavage in comparison with caspase-3 activation in the sensitive CRC cells. We detected clear cleavage of caspase-8, caspase-3 and PARP with similar degrees early at 4 h post APG-1252-M1 treatment in both HCT116 and HCC2998 cells (Fig. 4A and B). We also clearly detected the release of Cyt C and Smac from mitochondria in HCT116 cells exposed to APG-1252-M1 at 2 h post APG-1252-M1 treatment (Fig. 4C). To gain insight into the dynamic activation of caspases and release of Cyt C and Smac, we then performed an even shorter time-course analyses of these events within a 0–3 h treatment period in HCT116 as shown in Fig. 4D. We started to observe clear cleavage of caspase-8, caspase-3 and PARP at 1.5 h post APG-1252-M1 treatment while detecting clear Cyt C and Smac in the cytosol early at 0.5 h post treatment, indicating the release of Cyt C and Smac ahead of caspase-8, caspase-3 and PARP cleavage.

We also determined whether APG-1252-M1 exerts any effects on the levels of several apoptosis-regulating proteins and on inducing autophagy. We found that APG-1252-M1 treatment did not apparently alter the levels of Bcl-2, Bcl-X_L, Bim, Bax, DR4, DR5 and LC3 II in HCT116 cells, but we clearly detected increased levels of Mcl-1 and Bid from 2 h to 24 h (Fig. 4A). APG-1252-M1 did not alter LC3 II levels in HCC2998 cells either (Fig. 4B), suggesting that APG-1252-M1 does not induce autophagy while inducing apoptosis. Interestingly, we found that APG-1252-M1 deceased Mcl-1 levels in the sensitive HCC2998 cells starting at 8 h post treatment (Fig. 4B). Given the varied effects of APG-1252-M1 on modulation of Mcl-1 levels, we further checked its effects on Mcl-1 in additional CRC cell lines and found that APG-1252-M1 decreased Mcl-1 levels in sensitive SW480 cells, but elevated Mcl-1 levels in resistant HT29 cells (Fig. 4E). Hence, it appears that APG-1252-M1 exerts cell line-dependent effects on modulation of Mcl-1 levels.

The same category of compound, ABT263, basically exerted very similar effects as APG-1252-M1 did on facilitating release of Cyt C and Smac, inducing cleavage of caspase-8, caspase-3 and PARP, and modulating the levels of Mcl-1 even at 4 h post treatment. We detected clear release of Cyt C and Smac, but weak or no cleavage of caspase-3, caspase 8 and PARP in cells exposed to ABT199 under the same tested conditions (Fig. S4A and B), suggesting that ABT199 may have relatively weak activity in the induction of apoptosis. Similarly, ABT263 and ABT199 did not alter the levels of other apoptosis-regulating proteins including Bcl-2, Bax, Bim, DR4 and DR5 (Fig. S4A).

APG-1252-M1 or its combination with Mcl-1 inhibition induces Bax-dependent apoptosis

To determine whether APG-1252-M1 indeed initiates apoptosis through activation of the intrinsic apoptotic pathway, we first checked the effect of caspase inhibition on induction of apoptosis by APG-1252-M1. The presence of the pan caspase inhibitor, VAD-FMK, or the caspase-8 inhibitor, IETD-FMK, abrogated the ability of APG-1252-M1 to induce cleavage of caspase-3, caspase-8 and PARP (Fig. 5A), indicating caspase-dependent apoptosis. Similar results were also generated with ABT263 (Fig. S5). Following this study, we then checked the effect of deficiency of Bax, Bim, Smac, Puma or FADD, which are critical components in the regulation of both intrinsic and extrinsic apoptotic pathways [26], on protection of cells from APG-1252-M1-induced killing or apoptosis using a panel of isogenic cell lines derived from HCT116. In both cell survival and apoptotic assays, we found that BAX-KO cells were fully resistant to APG-1252-M1, whereas FADD-KO cells behaved like the parental cells to fully respond to APG-1252-M1. Deficiency in Smac, Puma or Bim was partially resistant to APG-1252-M1 with varied degrees (Fig. 5B and C). By comparing cleavage of caspases and PARP between HCC116 and either of the deficient cell lines, we generated similar results (Fig. 5D). Beyond APG-1252-M1 alone, the combination of APG-1252-M1 with S63845 showed enhanced effect on decreasing the survival of HCT116 cells, but this effect was lost in HCT116/Bax-KO cells (Fig. 5E). Hence, it is clear that APG-1252-M1 or its combination with Mcl-1 inhibition induces Bax-dependent apoptosis. To further support this conclusion, we also determined the effect of APG-1252-M1 on Bax activation by assessing the formation of Bax oligomerization including dimers and trimers and found that increased levels of Bax dimers and trimers were detected in cells exposed to APG-1252-M1, particularly at early times starting at 4 h (Fig. 5F). Here we used CYD-2-11, a Bax activator known to activate Bax [27], as a positive control. Thus, APG-1252-M1 apparently activates Bax in CRC cells.

Caspase-8 activation is likely secondary to mitochondrial activation of caspase-3 and further facilitates mitochondria-dependent apoptosis induced by APG-1252-M1

We found that enforced overexpression of ectopic Mcl-1 in HCT116 prevented caspase-8 from being cleaved or activated with treatment with either APG-1252-M1 or ABT263 (Fig. 6A), suggesting an event downstream of mitochondrial apoptosis. Thus, we further determined the effects of caspase inhibition on Cyt C and Smac release from mitochondria induced by APG-1252-M1 or ABT263, which are events that lead to activation of caspase-3. Interestingly, we found that both the pan-caspase inhibitor, Z-VAD-FMK, and the caspase-8 inhibitor, Z-IETD-FMK, partially attenuated the ability of either APG-1252-M1 or ABT263 to induce the release of Cyt C and Smac from mitochondria (Fig. 6B), suggesting an impact of caspase-8 activation on induction of Cyt C and Smac release and subsequent apoptosis.

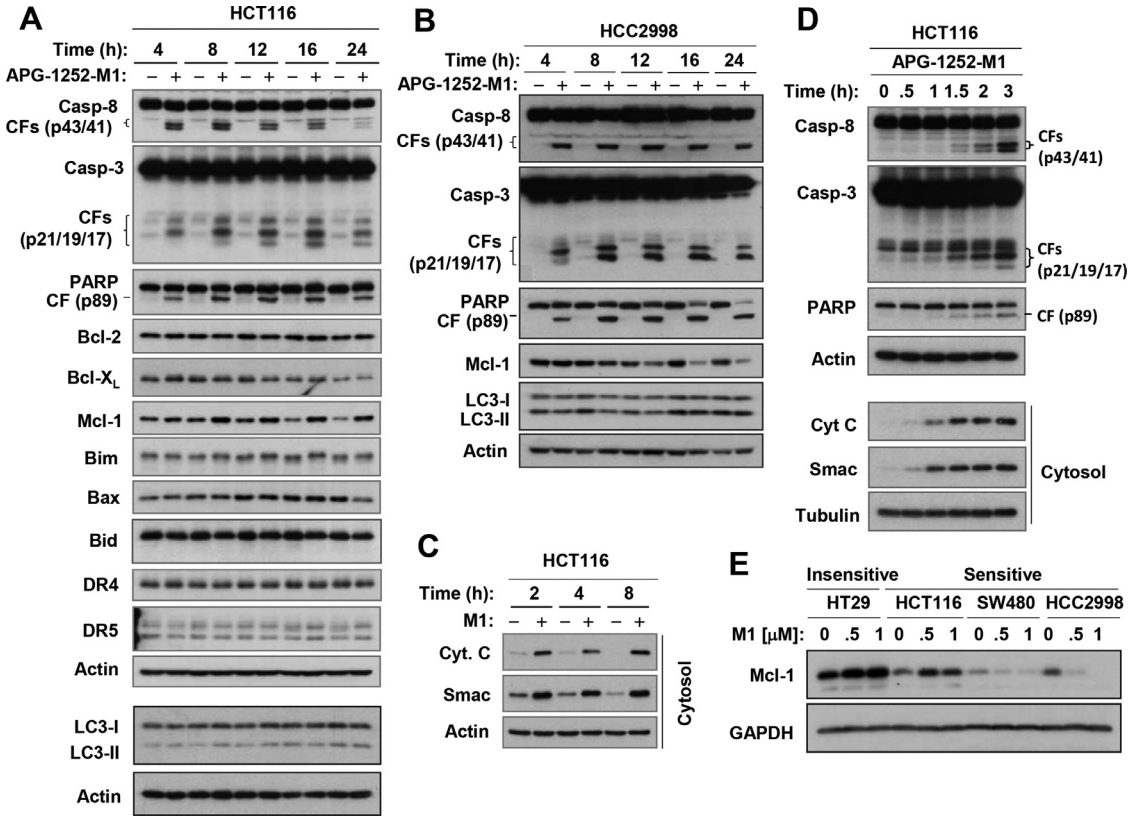

Fig. 4. APG-1252-M1 rapidly induces cleavage of caspase-8, caspase-3 and PARP and release of Cyt. C and Smac from mitochondria (A-D) with different effects on Mcl-1 levels (A, B and E) in APG-1252-M1-senstive cells. A-D, HCT116 or HCC2998 cells were exposed to DMSO or 1 μM APG-1252-M1 (M1) for different times as indicated and then harvested for preparation of whole-cell protein lysates or cytosolic fraction and subsequent Western blotting. CF, cleaved from. E, The tested cell lines were exposed to different concentrations of APG-1252-M1 as indicated for 24 h. Proteins of interest were detected with Western blotting.

Discussion

The success of targeted cancer therapies largely relies on the identification of patient populations who are most likely to respond to a given targeted therapy. In this end, it is essential to identify the biomarker(s) that can be used to select these patients. As a novel Bcl-2/Bcl-X_L dual inhibitor, APG-1252-M1 effectively decreased the survival and induced apoptosis of a few CRC cell lines with IC_{50}s < 1 μM, while weakly decreasing the survival of most other CRC cell lines (IC_{50}s > 10 μM). Both Bcl-2 and Bcl-X_L were universally expressed in the tested CRC cell lines and the baseline expression levels of either Bcl-X_L or particularly Bcl-2 did not correlate with cell sensitivities to APG-1252-M1. The interesting and important finding is that the baseline levels of Mcl-1 varied among these CRC cell lines with a significant negative correlation with cell responses to APG-1252-M1: i.e., low baseline expression was significantly correlated with increased cell sensitivity to APG-1252-M1. Hence, our findings suggest that baseline Mcl-1 expression may be a biomarker to predict CRCs that may respond to APG-1252-M1 monotherapy and likely other similar Bcl-2/Bcl-X_L dual inhibitors such as ABT263 since both APG-1252-M1 and ABT263 shared some similarities in suppressing the growth of CRC cells.

In relation to this finding, APG-1252-M1, when combined with an Mcl-1 specific inhibitor, synergistically decreased the survival of the insensitive CRC cell lines with high levels of Mcl-1 with enhanced induction of apoptosis. The activity of APG-1252-M1 against those sensitive CRC cell lines with low baseline levels of Mcl-1 could be even further augmented when combined with an Mcl-1 inhibitor. The direct evidence for the critical role of Mcl-1 in determining the sensitivity of CRC cells came from the following findings: (1) enforced overexpression of ectopic Mcl-1 in the sensitive CRC cell lines rendered them resistant to APG-1252-M1; and (2) addition of an Mcl-1 inhibitor to these Mcl-1-expressing cell lines fully restored APG-1252-M1 sensitivity. These data strongly support the notion that Mcl-1 is a critical molecular determinant for CRC cells to respond to APG-1252-M1 monotherapy. Fortunately, APG-1252-M1 combined with an Mcl-1 inhibitor exerted synergistic effects on decreasing the survival and inducing apoptosis of CRC cells regardless of Mcl-1 level. Therefore, developing this combination regimen may have potential to maximize the therapeutic efficacy of APG-1252-M1, and likely other similar inhibitors such as ABT263, against CRCs. Our findings with APG-1252-M1 in CRC cells are in agreement with previous reports demonstrating the negative impact of Mcl-1 on cell sensitivity to other Bcl-2 inhibitors such as ABT263 [28–31].

Another interesting finding is that APG-1252-M1 modulated Mcl-1 levels in a cell context-dependent manner. i.e., it elevated Mcl-1 in some cell lines (e.g., HCT116) while decreasing Mcl-1 levels in others (e.g., HCC2998). This is likely also the case for other similar inhibitors such as ABT263. The underlying mechanisms accounting for the cell-dependent modulation of Mcl-1 are unclear and thus deserve further investigation in the future. Indeed, recent studies have also shown the induction of Mcl-1 due to inhibition of Bcl-2 or Bcl-X_L with BH3 mimetics, resulting in a rescue loop triggering cell protective effect [32,33]. It is likely that APG-1252-M1-induced Mcl-1 elevation in some cell lines may evade apoptosis and counteract its therapeutic efficacy. Hence, this finding further supports the rationale of co-targeting Mcl-1 to enhance the therapeutic efficacies of APG-1252-M1 and possibly

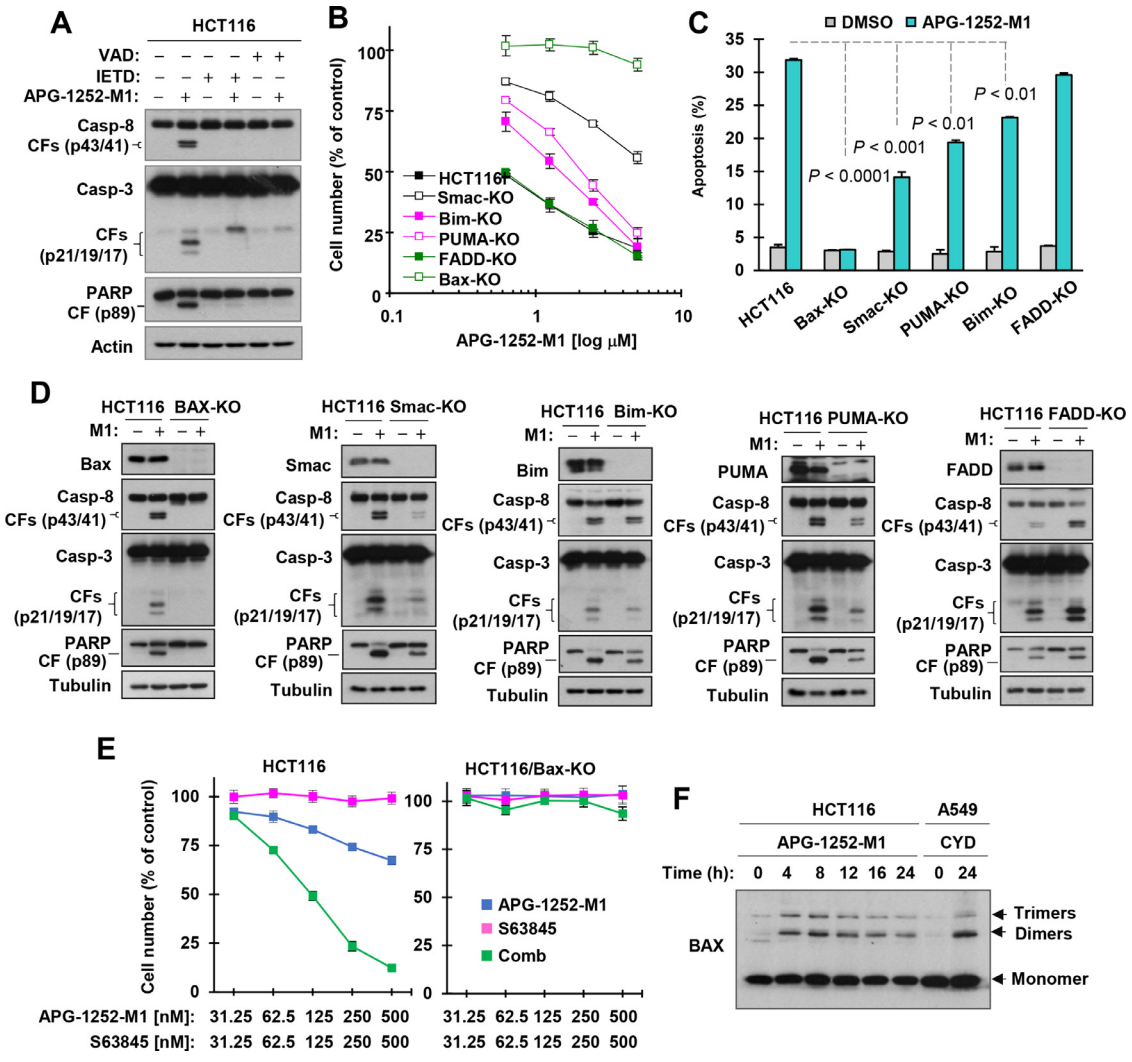

Fig. 5. APG-1252-M1 induces Bax-dependent, but FADD-independent, apoptosis, in which Smac, Puma and Bim are partially involved. A, HCT116 cells were pre-treated with 40 μM Z-VAD or Z-IETD for 30 min and then co-treated with 1 μM APG-1252-M1 for additional 4 h. Proteins of interest were detected with Western blotting. B and C, The indicated isogeneic cell lines derived from HCT116 were exposed to varied concentrations of APG-1252-M1 for 3 days (B) or to DMSO or 1 μM APG-1252-M1 for 24 h (C). Cell numbers were estimated with the SRB assay (B) and apoptosis was detected with annexin V/ flow cytometry (C), respectively. The data are means ± SDs of four replicate (B) or duplicate (C) determinations. D, the given cell lines were exposed to DMSO or 1 μM APG-1252-M1 (M1) for 4 h. Different proteins were detected with Western blotting. E, The tested cell lines were treated with indicated concentrations of APG-1252-M1 alone, S63845 alone or their combination for 3 days. Cell numbers were estimated with the SRB assay. The data are means ± SDs of four replicate determinations. F, HCT116 cells were exposed to 1 μM APG-1252-M1 for different times as indicated. Moreover, A549 cells were exposed to 5 μM CYD-2-11 (CYD) for 24 h. The cells were then harvested for detection of Bax oligomerization. CYD-2-11 treatment here serves as a positive control.

other similar inhibitors in the treatment of CRCs, particularly those with treatment-induced Mcl-l elevation.

We noted that, in contrast to APG-1252-M1 and ABT263, the combination of ABT199 and S63845 did not have or had very weak enhanced effects on decreasing the survival of CRC cell lines. Similar finding was also observed in cervical cancer cells [31]. Beyond, synergy between co-targeting Bcl-X_L and Mcl-1 in decreasing the survival of different types of cancer cells was also reported [34,35]. Thus, it is very likely that Bcl-X_L inhibition plays a dominant role in mediating synergistic effects of APG1252-M1 combined with an Mcl-1 inhibitor in decreasing the survival and inducing apoptosis of CRC cells.

It is well established that Bcl-2-like family proteins such as Bcl-2, Bcl-X_L and Mcl-1 inhibit Bax activation via direct interaction, resulting in the negative regulation of Bax-mediated apoptosis, whereas BH3-only proteins such as Bim and Puma facilitate Bax-dependent apoptosis through interacting and sequestering these Bcl-2-like family proteins [26, 36]. Smac, as a protein released from mitochondria during apoptosis, promotes caspase-9/caspase-3 activation via suppression of the function of the inhibitors of apoptosis (IAPs) such as XIAP [26,36]. Therefore, we can speculate that APG-1252-M1, as a Bcl-2/Bcl-X_L dual inhibitor, should induce apoptosis via eventual activation of Bax-dependent apoptosis. Indeed, our results have demonstrated that APG-1252-M1 or its combination with Mcl-1 inhibition induces Bax-dependent apoptosis since Bax deficiency in CRC cells abolished the ability of APG-1252-M1 or its combination with a Mcl-1 inhibitor to decrease cell survival and induce apoptosis. Consistently, deficiency of Puma, Bim or Smac partially protected the tested CRC cells from APG-1252-M1-induced

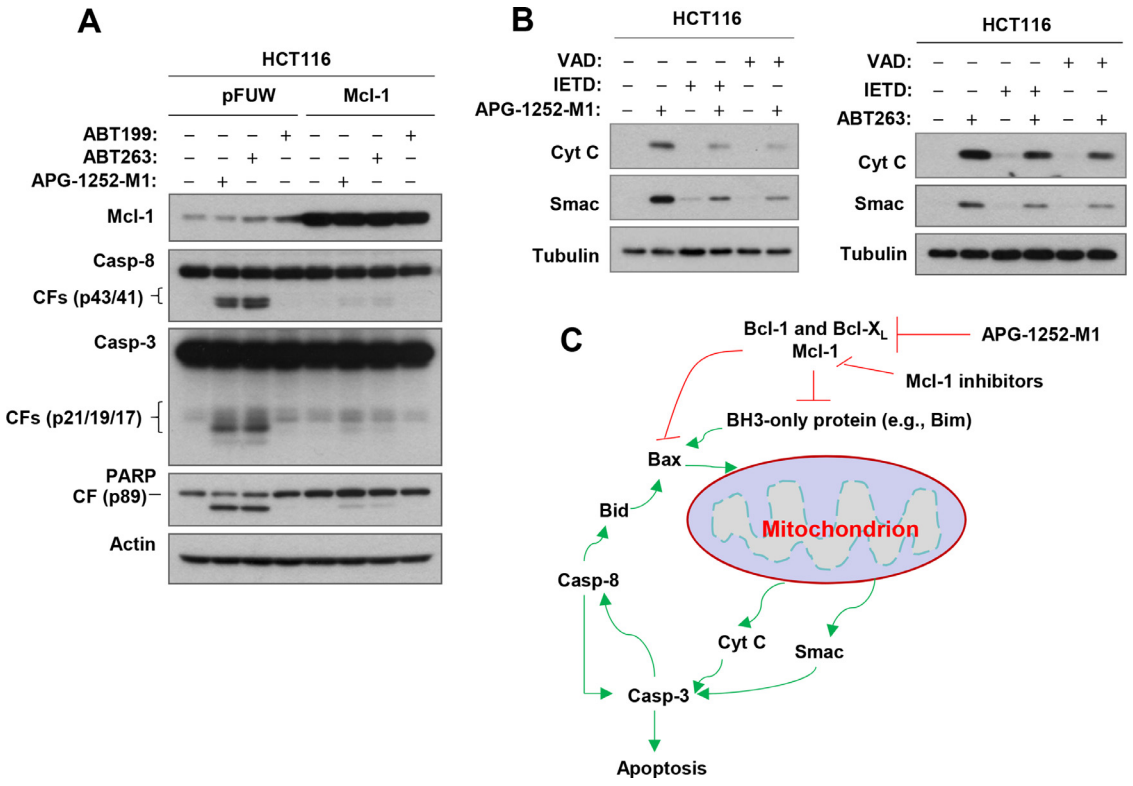

Fig. 6. Caspase-8 activation induced by APG-1252-M1 is likely secondary to Bax-dependent activation of caspase-3 and facilitates activation of the mitochondrial apoptotic pathway. A, The given cell lines were exposed to DMSO, 1 μM APG-1252-M1, 3 μM ABT199 and 3 μM ABT263 for 4 h. The proteins of interest were detected with Western blotting. CF, cleaved from. B, HCT116 cells were pre-treated with 40 μM Z-VAD or Z-IETD for 30 min and then co-treated with 1 μM APG-1252-M1 or 3 μM ABT263 for additional 4 h. The cells were then harvested for preparation of cytosolic fraction and subsequent Western blotting. C, A schema for a working model suggesting feedback activation of caspase-8 by caspase-3 and subsequent feedforward facilitation of the mitochondrial apoptotic pathway during APG-1252-M1-induced apoptosis. The combination of APG-1252-M1 with Mcl-1 inhibition has maximal effect on the induction of Bax-mediated apoptosis.

apoptosis. Thus, we have convincingly demonstrated that APG-1252-M1 activates the intrinsic apoptotic pathway, resulting in Bax activation-mediated apoptosis in CRC cells.

In this study, APG-1252-M1 as well as ABT263 quickly and robustly induced cleavage of caspase-8, indicating activation of caspase-8 in CRC cells; this event may also play a critical role in mediating or enhancing APG-1252-M1-induced apoptosis since the caspase-8 inhibitor, IETD-FMK, effectively abrogated cleavage of caspase-3 and PARP induced by APG-1252-M1. FADD plays an essential role in mediating death receptor-induced activation of extrinsic apoptosis via recruitment and activation of caspase-8 [37,38]. However, FADD knockout failed to provide any protective effect on APG-1252-M1-induced apoptosis, suggesting no involvement of activation of the extrinsic apoptotic pathway. It is known that caspase-3 can activate caspase-8, leading to facilitation or amplification of Bax-dependent apoptosis through Bid cleavage [39–41]. In this study, release of Cyt C and Smac occurred early before cleavage of caspase 3 and caspase-8 in the sensitive CRC cells exposed to APG-1252-M1. Moreover, caspase-8 inhibition such as with a caspase inhibitor compromised the ability of APG-1252-M1 to induce the release of both Cyt C and Smac. Hence, it is plausible to speculate that caspase-8 activation induced by APG-1252-M1 in CRC cells is likely secondary to Bax-mediated activation of caspase-3; this feedback activation of caspase-8 will feed forward further activation of the mitochondrial apoptotic signaling, enhancing apoptotic cell death (Fig. 6C). Bid is known to be a substrate of caspase-8 and facilitate Bax activation via its truncate form [42,43]. In this study, we detected Bid reduction in cells exposed to APG1252-M1 in parallel to caspase 8 cleavage albeit without cleaved band detected. In the same cells exposed to TRAIL that is known to induce caspase-8 activation and Bid cleavage, we did not detect cleaved band of Bid either albeit substantial reduction of Bid levels (Fig. S6). It is likely that Bid antibody we used does not capture cleaved band well. Hence, Bid reduction in cells exposed to APG-1252-M1 is likely due to Bid cleavage,

One interesting observation is that we clearly detected release of Cyt C and Smac from mitochondria, but not increased cleavage of caspases and PARP in CRC cells exposed to ABT199 for a short time (4 h; Fig. S4). It is possible that other unknown factors, which negatively regulate events leading to activation of caspases downstream of Cyt C and Smac, may not be effectively removed by ABT199 during the short period of treatment. It is also possible that it may take a longer time for ABT199 to induce caspase activation and PARP cleavage although it quickly triggers Cyt C and Smac release. Nonetheless, this deserves a further investigation.

In summary, the current study has demonstrated the therapeutic potential of the new Bcl-1/Bcl-X_L dual inhibitor, APG-1252-M1, either alone or in combination with Mcl-1 inhibition in the treatment of CRC via effective induction of the intrinsic apoptotic pathway. It is clear that the level of Mcl-l critically determines the response of CRC cells to APG-1252-M1 when used as a monotherapy. Findings in this study warrant further *in vivo* studies of the therapeutic efficacy against CRC.

Funding

This study was supported by a research fund from Ascentage Pharma Corp Ltd (to SYS).

Declaration of Competing Interest

Y. Zhai is an equity shareholder of Ascentage Pharma Group International and a full-time employee of one or more of its affiliates. S. Wang is a co-founder, chief-scientific advisor and board member of Ascentage Pharma Group, which has licensed APG-1252 from the University of Michigan. He is also a paid consultant of Ascentage, owns shares in Ascentage, is a co-inventor of APG-1252 and receives royalty from the University of Michigan. S-Y. Sun received research fund from Ascentage Pharma Group Corp Ltd, an affiliate of Ascentage Pharma Group International. Others disclose no potential conflicts of interest.

CRediT authorship contribution statement

Weilong Yao: Conceptualization, Visualization, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Longchuan Bai:** Conceptualization, Visualization, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Shaomeng Wang:** Conceptualization, Visualization, Project administration, Writing – original draft, Writing – review & editing. **Yifan Zha:** Conceptualization, Visualization, Project administration, Writing – original draft, Writing – review & editing. **Shi-Yong Sun:** Conceptualization, Visualization, Project administration, Writing – original draft, Writing – review & editing.

Acknowledgements

We are thankful to Drs. J. Yu and L. Zhang (University of Pittsburgh, Pittsburgh, PA) for providing some KO cell lines. We are also grateful to Dr. A. Hammond in our department for editing the manuscript. SYS is a Georgia Research Alliance Distinguished Cancer Scientist and a Halpern Research Scholar.

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