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Aspirin overcomes Navitoclax-resistance in hepatocellular carcinoma cells through suppression of Mcl-1

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ABSTRACT

Small-molecule Bcl-2/Bcl-xL inhibitor Navitoclax represents a promising cancer therapeutic since preclinical and clinical studies with Navitoclax have demonstrated strong anticancer activity in several types of cancers. However, because Navitoclax has a low binding affinity to Mcl-1, anticancer activity by Navitoclax is often attenuated by the elevated expression of Mcl-1 in hepatocellular carcinoma (HCC) and other cancers, posing a serious problem for its potential clinical utilities. Therefore, approaches that suppress the expression of Mcl-1 are urgently needed to overcome Navitoclax-resistance in these cancers. Here, we reported that aspirin markedly suppressed Mcl-1 expression, and significantly enhanced Navitoclax-mediated cell viability inhibition and apoptosis induction in HCC cells. We further showed that aspirin robustly enhanced Navitoclax-triggered cytosolic cytochrome c release, activation of initiator caspase-9 and effector caspase-3, and cleavage of PARP. Importantly, the cell death induction by the combination could be rescued by a cell-permeable caspase-9 inhibitor Z-LEHD-FMK, indicative of an indispensable role of mitochondrial apoptosis pathway during the combination effect. Taken together, our study suggests that aspirin can be used to enhance Navitoclax-mediated anticancer activity via suppression of Mcl-1. Since aspirin is one of the most commonly used medicines, our findings therefore have translational impacts on Navitoclax-based therapy for HCC.

1. Introduction

Human hepatocellular carcinoma (HCC) is one of the most common malignancies. Although surgical resection, local treatment and liver transplantation could provide chances for a cure in a small fraction of patients whose diseases were diagnosed at early stage, majority of patients with advanced stage of HCC were not suitable for surgery and chemotherapy remains the only modality for them [1,2]. Unfortunately, inherent resistance to chemotherapeutics by HCC cells often leads to treatment failure. Therefore, novel therapeutic strategies are urgently needed to improve the survival of HCC patients.

Apoptosis is the process of programmed cell death that occurs in multicellular organisms, and plays a key role in maintenance of cell homeostasis and differentiation [3,4]. Deficiency in apoptotic cell death induction is one of the hallmarks in most types of cancers, including HCC [1,2,5]. Apoptosis is tightly regulated by proapoptotic and antiapoptotic proteins. For instance, apoptosis signaling mediated by mitochondria is controlled by Bcl-2 family proteins, which share the Bcl-2 homology (BH) domains, and are divided into proapoptotic and antiapoptotic groups [6,7]. The antiapoptotic Bcl-2 members, including Bcl-2 itself, Bcl-xL, Mcl-1 and several others, inhibit apoptosis by sequestering two multidomain proapoptotic Bcl-2 members (Bax and Bak) [8]. Bcl-2, Bcl-xL and Mcl-1 were found highly expressed in many types of cancer cells and cancer tissues, and are considered as the major contributing factors for the resistance of these cancers toward conventional therapies [9,10].

On the other hand, the antiapoptotic function of Bcl-2, Bcl-xL and Mcl-1 can be antagonized by the "BH3-only" pro-apoptotic Bcl-2 proteins (e.g., Bad, Bim, Puma, Noxa, etc.) and synthetic peptides or small molecules that structurally and functionally mimic BH3 proteins (BH3 mimetics) [7,8]. Navitoclax (ABT-263) is a small-molecule BH3 mimetics, which was developed by Abbott Laboratories as a novel anticancer drug [11]. Navitoclax specifically binds to Bcl-xL and Bcl-2 with high affinities. Cellular experiments demonstrated that Navitoclax efficiently antagonizes Bcl-2/Bcl-xL-

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mediated sequestration of Bax and Bak, allowing Bax and Bak to permeabilize the outer mitochondrial membrane, which results in cytochrome c released from the mitochondrial intermembrane space into cytosol. Once in cytosol, cytochrome c induces initiator procaspase-9 autoactivation, and subsequently leading to effector caspase-3 activation and eventually cell death [11–14]. Both preclinical and clinical studies showed that Navitoclax potently induces apoptosis in cancer cells and elicited strong anticancer activity in small cell lung cancer and multiple types of hematological malignancies [11,15–18]. Therefore, Navitoclax represents a promising anticancer therapeutics.

Since Bcl-xL is frequently expressed by HCC cells and HCC tumor tissues at high levels, recent studies have investigated the anticancer effect of Navitoclax and or its analog ABT-737 on HCC [17,19,20]. However, because both Navitoclax and ABT-737 have a very low binding affinity to Mcl-1, the apoptosis signaling triggered by these two compounds often was neutralized by the elevated expression of Mcl-1 in HCC cells, which consequently cause resistance of HCC cells to these compounds [11,16,18,21]. To increase Navitoclax sensitivity in HCC cells and other cancer cells, attention has been focused on combining Navitoclax/ABT-737 with approaches that suppress the expression of Mcl-1 by cancer cells.

Aspirin (acetylsalicylic acid) is a common non-steroidal anti-inflammatory drug (NSAID) that has antipyretic, analgesic and anti-inflammatory effect. As with other NSAIDs, Aspirin shows strong anticancer activities in multiple cancer cell types. Notably, recent studies demonstrated the anticancer activity by aspirin is associated with down-regulation of Mcl-1 in colorectal cancer, oral squamous cancer, cervical cancer and leukemia cells [14,22–24]. However, whether aspirin could suppress Mcl-1 in HCC cells, and whether aspirin could be used to overcome the resistance of Navitoclax in HCC cells remain elucidated.

2. Materials and methods

2.1. Cell culture and reagents

HCC cell lines HepG2 and BEL-7402 were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), and maintained in high-glucose DMEM (HyClone/Thermo Fisher Scientific, Beijing, China) supplemented with 10% heat-inactivated fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd, Hangzhou, China) in a humidified atmosphere of 95% air plus 5% CO_2 at 37 °C incubator. Navitoclax was purchased by Biochempartner (Shanghai, China) and compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma–Aldrich) at 20 mmol/L and stored at −20 °C. Aspirin was purchased from Shandong Xinhua Pharmaceutical Co., Ltd (Zibo, China), and was dissolved in DMSO 1.5 mol/L and stored at −20 °C. The Mcl-1, β-actin primary antibodies were purchased from Santa Cruz Biotechnology; PARP, caspase-9, caspase-3 primary antibodies were obtained from Cell Signaling Technology (Danvers, USA). Bcl-2, Bcl-xL, XIAP and Survivin primary antibodies were purchased from Wuhan Boster Bio-engineering Limited Company (Wuhan, China).

2.2. Cytotoxicity assay

The anticancer activity of combination treatment with aspirin and Navitoclax was measured by 3-[4,5-dimethylthiazol-2-thiazolyl]-2,5-diphenyl-tetrazolium bromide (MTT) assay as described previously [20].

2.3. Analysis of cell death and apoptosis

Cell death was evaluated by trypan blue exclusion assay. Apoptosis was measured using the Annexin V-FITC/PI apoptosis detection kit (KeyGEN Biotech, Nanjing, China) according to the manufacturer's instructions. Before flow cytometric analysis, cells were harvested and resuspended in 500 μl binding buffer, the cell suspension was added to Annexin V/FITC (5 μl) and homogeneous mixing, 5 μl of Propidium Iodide (PI) was added to the solution and the samples were incubated at room temperature in the dark for 15 min. Each sample containing $1–3 \times 10^5$ cells were measured using a BD LSR II system (BD Biosciences) analyzed by the DiVA software (version 4.1.2; BD Biosciences).

2.4. Western blotting analysis

Cells treated with aspirin, Navitoclax alone and the combination for 48 h were lysed by RIPA lysis buffer (Beyotime Biotech, Nantong, China) supplement with protease inhibitor (Roche Pharmacy, Shanghai, China) for 30 min. Equal amounts of sample were separated by 12% Tris–glycine gel, transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, USA) and incubated with antibodies. Immunoblots were visualized on film (Kodak, Beijing, China) using ECL (Thermo Scientific, Rockford, USA).

2.5. Cytochrome c release analysis

Cells were treated with Navitoclax, aspirin alone, or the combination for 48 h, cytosolic and mitochondrial fractions were collected using a digitonin-based subcellular fractionation technique [25]. For detection of cytochrome c, 30 μg of the cytosolic fraction was supplemented with 2× SDS–PAGE loading buffer, and then subjected to Western blotting analysis. Blots were probed with anti-cytochrome c and β-actin antibodies.

2.6. Statistical analysis

One-way ANOVA was carried out to analyze the differences, using SPSS for Windows version 13.0 (SPSS Inc, Illinois, USA). $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Aspirin suppresses Mcl-1 expression in HCC cells

Previous studies have shown that aspirin induces down-regulation of a number of antiapoptotic proteins [14,22–24]. We therefore examined the effect of aspirin on the expression of three antiapoptotic Bcl-2 family members, including Bcl-2, Bcl-xL and Mcl-1, and two important members of inhibitors of apoptosis (IAP), including X-linked IAP and Survivin in HepG2 and BEL-7402. We treated HCC cells with a non-toxic concentration range (SI Fig. 1) of aspirin at 2.5, 5 and 10 mM, since previous studies showed that similar concentrations of aspirin efficiently suppressed Mcl-1 in cervical cancer, oral squamous cancer and leukemia cells [14,22–24]. We noted that aspirin had negligible effect on the expression of Bcl-2, Bcl-xL, XIAP and Survivin in two HCC cell lines (Fig. 1A and B). Of note, we observed that treatment with aspirin for 48 h suppressed Mcl-1 expression in both HCC cell lines in a dose dependent manner. Aspirin partially inhibited Mcl-1 expression at 2.5 mM, markedly reduced Mcl-1 expression at 5 mM and completely suppressed Mcl-1 expression at 10 mM in both HCC cell lines. We also found that suppression of Mcl-1 by aspirin occurred very rapidly, as Western blotting analysis showed that treatment with aspirin at 5 mM for 8 h markedly reduced the

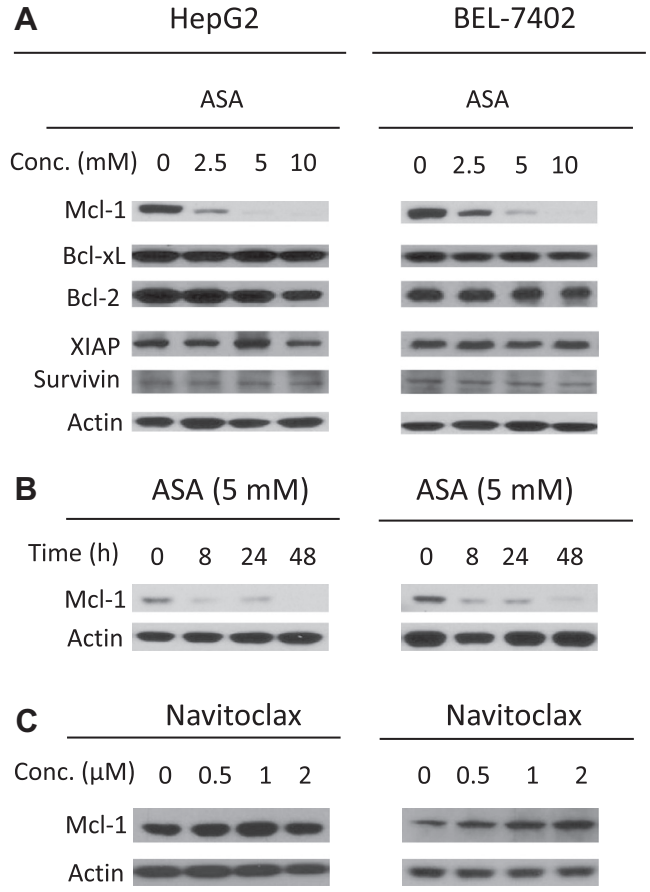

Fig. 1. Aspirin inhibits Mcl-1 expression in HCC cells in a dose-dependent manner. (A) BEL-7402 and HepG2 cell lines were treated with 2.5 mM, 5 mM, 10 mM of aspirin (ASA) for 48 h, cells were harvested and cell lysates were examined by Western blotting assays for expressions of Bcl-2, Bcl-xL, Mcl-1, XIAP and Survivin. Actin was used as a loading control. (B) HCC cells treated with 5 mM of ASA for 8, 16, 48 h, Mcl-1 expression was examined by Western blotting analysis in cell lysates. Actin was used as a loading control. (C) HCC cells treated with 0.5, 1, 2 μM Navitoclax for 48 h, Mcl-1 expression was examined by Western blotting analysis in cell lysates. Actin was used as a loading control.

level of Mcl-1 in HCC cell lines (Fig. 1B). Meanwhile, we examined the effect of Navitoclax on the expression of Mcl-1 in HCC cell lines. We found that Navitoclax at 0.5, 1 and 2 μM, a non-toxic concentration-range had no effect on Mcl-1 in HepG2 cell line, and modestly increased the level of Mcl-1 in BEL-7402 HCC cell line upon treatment for 48 h (Fig. 1C).

3.2. Aspirin enhances Navitoclax-mediated cell viability inhibition in HCC cell lines

Since Mcl-1 is a critical mediator of Navitoclax-resistance in cancer cells, we reasoned that suppression of Mcl-1 expression by aspirin could be exploited to enhance Navitoclax-mediated anticancer activity in HCC cell lines. We thus treated HCC cell lines with aspirin alone, Navitoclax alone or both for 48 h, and examined cell viability inhibition by MTT assays. We found aspirin alone or Navitoclax alone had a negligible cytotoxic effect in two HCC cell lines. In contrast, their combination considerably inhibited cell viability in both of the two HCC cell lines. Notably, the combination effect was closely correlated with the magnitude of Mcl-1 down-regulation by aspirin. For instance, as shown in Fig. 1A, aspirin at 2.5 mM, an concentration partially suppressing Mcl-1 expression, led to a modest enhancement of Navitoclax-mediated the cell viability inhibition, while aspirin at 5 and 10 mM, concentrations achieving almost complete suppression of Mcl-1 expression, in combination with Navitoclax could cause 80–100% cell viability inhibition in HCC cell lines (Fig. 2A and B). Thus, these data demonstrated that aspirin substantially enhanced Navitoclax-mediated cell viability inhibition in a concentration-range that could induce down-regulation of Mcl-1.

3.3. Aspirin enhances Navitoclax-mediated anticancer activity through apoptotic cell death induction

We next investigated whether aspirin enhanced the anticancer activity of Navitoclax in HCC cells through apoptosis induction. We used aspirin at 5 mM since this concentration markedly suppresses Mcl-1 expression and had minimal single-agent activity in HCC cells. We treated HCC cells with aspirin alone, Navitoclax alone or their combination for 48 h. Then we stained HCC cells with Annexin-V/PI, and analyzed apoptosis with flow cytometery assay. We found that as compared to DMSO control, treatment with either aspirin or Navitoclax alone slightly induced apoptosis in both HCC cell lines (Fig. 3A). In striking contrast, their combination induced 89% and 81% of cells apoptosis in BEL-7402 and HepG2 cell lines, respectively (Fig. 3A). Western blotting analysis was performed to further examine the apoptosis biomarkers. As shown in Fig. 3B, treatment with either single agent induced minimal PARP cleavage and caspase-9, -3 activation, while their combination induced robust accumulation of cleaved PARP and activated caspase-9, -3 in both of these two cell lines (Fig. 3B).

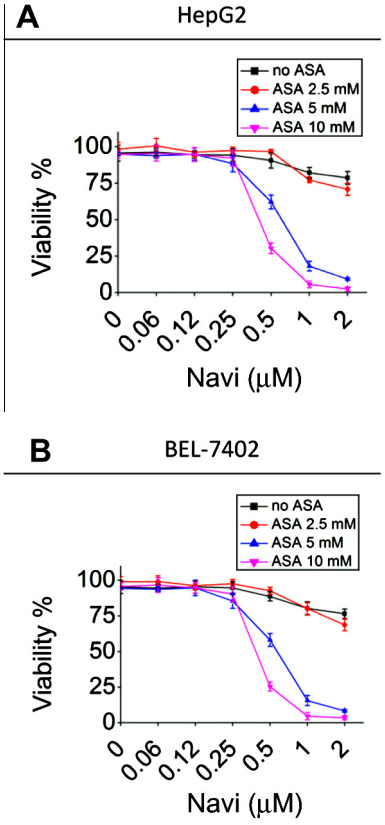

Fig. 2. ASA enhances Navitoclax-mediated cell viability inhibition in HepG2 and BEL-7402 cell lines. (A) HepG2 and (B) BEL-7402 cell lines were treated with different concentrations of Navitoclax (Navi) alone, or allied with ASA at 2.5, 5, 10 mM for 48 h, cell viability inhibition was determined using an MTT assay.

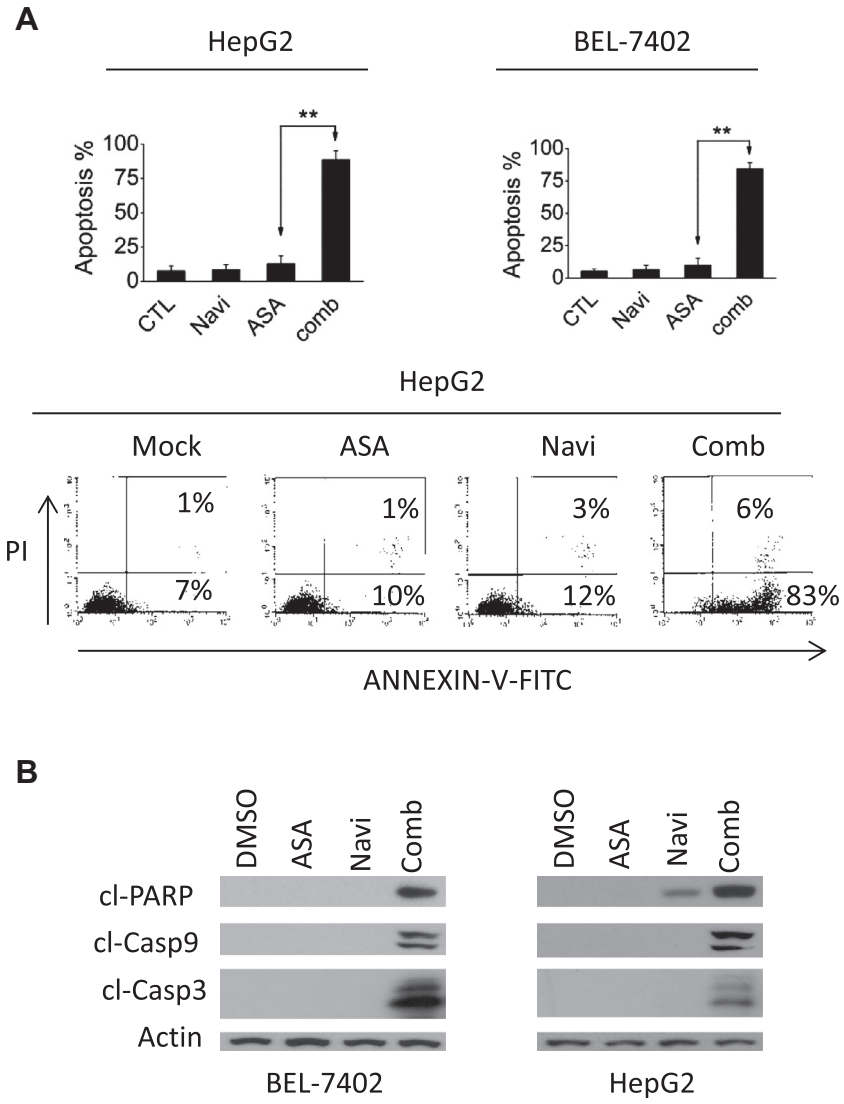

Fig. 3. ASA enhances Navitoclax-induced apoptosis in HepG2 and BEL-7402 cell lines. (A) HepG2 and BEL-7402 cell lines were treated with aspirin (ASA) at 5 mM alone, Navitoclax (Navi) at 1 μM alone or both for 48 h, cells were examined with Annexin V/PI staining and examined by flow cytometry assay. (top panels) Data of Annexin V-positive cells show means ± S.D. of three experiments. $^{**}p < 0.01$. (lower panel) Representative dot plot of apoptosis assay for HepG2 cell line. (B) HepG2 and BEL-7402 cell lines were treated with ASA at 5 mM alone, Navi at 1 μM alone or both for 48 h, PARP cleavage, activation of caspase-9 and caspase-3 were detected with Western blotting analysis and indicated antibodies. Actin was used as loading control.

3.4. Aspirin enhances Navitoclax-mediated anticancer activity dependent on mitochondrial apoptosis signaling pathway

It was revealed that Bcl-2/Bcl-xL inhibitors exhibit the anticancer activity relying on mitochondrial apoptosis pathway in other tumor types. We thus investigated whether combination treatment with aspirin and Navitoclax would induce cytosolic release of cytochrome c, a hallmark of mitochondrial apoptosis signaling activation in BEL-7402 and HepG2 cell lines. As shown in Fig. 4A, cytochrome c was undetectable in the cytosol of HCC cells treated with either signal agent. By striking contrast, cotreatment with the two drugs resulted in robust release of cytochrome c in the two cell lines, indicating the combination strongly activated mitochondrial apoptosis signaling pathway.

Since caspase-9 plays a key role in initiation of mitochondrial apoptosis signaling, we therefore employed Z-LEHD-FMK, a pharmacological caspase-9 inhibitor to investigate whether the combination anticancer activity is dependent on mitochondria-mediated apoptotic cell death induction in HCC cells. We pretreated the HCC cells with the caspase-9 inhibitor for 1 h, then added aspirin (5 mM) and Navitoclax (1 μM) simultaneously (Fig. 4B). We found that inhibition of caspase-9 activity by Z-LEHD-FMK almost completely blocked the cell death induction by the combination, evidently indicating that mitochondrial apoptosis pathway is required in the anti-HCC effect by the combination.

Collectively, these results demonstrated that aspirin substantially enhanced Navitoclax-triggered apoptotic cell death in these two HCC cell lines.

3.5. Knockdown of Mcl-1 by siRNA phenotypes aspirin in enhancing Navitoclax-mediated anticancer activity in HCC cells

To validate the contribution of Mcl-1 suppression by aspirin in enhancement of Navitoclax in HCC cells, we employed a specific Mcl-1 siRNA. As shown in SI Fig. 2, siRNA efficiently suppressed the expression of Mcl-1 in HCC cell lines, and achieved a similar effect as 5 mM of aspirin in enhancing Navitoclax-induced cell death in both of the two cell lines. This result indicated that Mcl-1 suppression by aspirin is essential in enhancing Navitoclax-mediated anticancer activity in HCC cell lines.

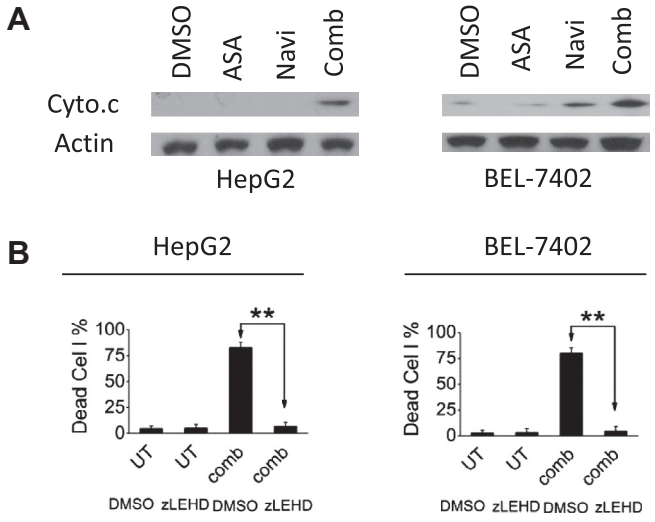

Fig. 4. Aspirin potentiates Navitoclax-mediated anticancer activity dependent on mitochondrial apoptosis signaling pathway. (A) BEL-7402 and HepG2 cell lines were treated with Navitoclax (Navi) at 1 μM a lone, aspirin (ASA) at 5 mM alone, their combination for 48 h, cells were harvested and fractioned. The expression of cytochrome c in cytoplasm was examined by Western blotting and specific antibody. Actin was used as control. (B) BEL-7402 and HepG2 cell lines were pretreated with 50 μM of caspase-9 inhibitor (Z-LEHD.fmk) for 1 h before the combination (comb) treatment with aspirin (5 mM) alone and Navitoclax (1 μM), cell death induction was examined with a trypan blue exclusion assay. $^{**}p < 0.01$.

4. Discussion

Aspirin is well known for its antipyretic, analgesic and anti-inflammatory effect. However, recent evidences indicated that as with other NSAIDs, aspirin also has strong anticancer activity [26–30]. Notably, a number of studies showed that the anticancer activity by aspirin was associated with the suppression of antiapoptotic Bcl-2 proteins, such as Bcl-2, Bcl-xL, Mcl-1 and inhibitors of apoptosis member XIAP and Survivin in cancer cells. We here observed that aspirin markedly represses the expression of Mcl-1 in HCC HepG2 and BEL-7402 cell lines, but has minimal effect on the expression of other antiapoptotic proteins. These discrepancies between our observation and previous studies may be caused by the different concentrations of aspirin employed in the studies, and may also reflect the diverse response of different cancer types to aspirin.

The Bcl-2 family of proteins consists of both pro- and antiapoptotic members, and controls the mitochondrial pathway of apoptosis [8]. Several antiapoptotic Bcl-2 family proteins, such as Bcl-xL and Mcl-1 are expressed at high levels in HCC and many other cancer types [9,10,20]. Therefore, targeting these antiapoptotic proteins in cancer cells is of great interest in the management of these cancers. In this regard, orally bioavailable small molecule Bcl-2 inhibitor Navitoclax (ABT-263) and its analog ABT-737 have attracted special attention. However, since Navitoclax and ABT-737 have low binding affinities to Mcl-1, they exhibit very weak single-agent activity against HCC and many other solid cancer cells in which elevated level of Mcl-1 are often detected. Accordingly, strategies that target Mcl-1 in cancer cells are explored for more broadly using Navitoclax and ABT-737 in cancer therapy [11–13,21]. For instance, we have recently shown that Norcantharidin, a major bioactive constituent of Chinese blister beetle Mylabris, repressed Mcl-1 and potently enhanced ABT-737-mediated apoptosis in HCC cells [20]. In the current study, we found that down-regulation of Mcl-1 by aspirin could be exploited to enhance Navitoclax-mediated anti-HCC activity. Since aspirin is one of the most commonly used medicines, our findings therefore have significant clinical implications and potential translational impacts on HCC treatment.

Our data support that repression of Mcl-1 is responsible for the enhancement of Navitoclax-mediated anticancer activity by aspirin. Firstly, we observed that the enhancement of Navitoclax-mediated cell viability inhibition by aspirin is well correlated with the ability of aspirin to inhibit the expression of Mcl-1 in two HCC cell lines; Secondly, consistent with previous studies [20,31], knock-down of Mcl-1 by siRNA phenotypes aspirin in enhancing Navitoclax-triggered cell death induction in HCC cell lines.

Our data also demonstrated that the enhancement of Navitoclax-mediated anticancer activity by aspirin relies on the mitochondria-dependent apoptosis induction. For instance, flow cytometric analysis showed that the combination treatment led to massive cells undergoing apoptosis in BEL-7402 and HepG2 cell lines. Western blotting analysis further showed that combination treatment induced activation of several critical biomarkers of mitochondria-mediated apoptosis, including cytosolic cytochrome c release, activation of initiator caspase-9 and effector caspase-3 and PARP cleavage. Importantly, we found that cell death induction by the combination could be rescued by the pharmacological caspase-9 inhibitor Z-LEHD-FMK, indicated the combination effect of aspirin and Navitoclax is chiefly dependent on the activity of caspase-9, the initiator caspase of mitochondrial apoptosis pathway. These findings are in agreement with previous reports that combinational effect of ABT-737 with agents targeting Mcl-1 in breast cancer, melanoma and many other cancers are dependent on caspase activation and mitochondrial apoptosis induction [20,21,32,33].

Competing interest

The authors declare that they have no competing interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.018.

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