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ORIGINAL ARTICLE

Targeting Mcl-1 inhibits survival and self-renewal of hepatocellular cancer stem-like cells

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Summary Myeloid cell leukemia-1 (Mcl-1) is highly expressed in tumor tissues and cells of hepatocellular carcinoma (HCC), yet the role of Mcl-1 in cancer stem-like cells (CSLCs) remains largely unclear. Herein, we showed that knockdown of Mcl-1 significantly inhibited HCC cells to form spheres under ultra-low attachment condition in serum-free medium, and also attenuated clone formation. Inhibition of Mcl-1 by specific inhibitors S63845 or A-1210477 hindered secondary sphere formation, triggered apoptosis signaling and reduced the level of stem cell transcription factor Nanog, Sox2 and KLF4 in HCC spheroids cells. This study suggests that Mcl-1 is an essential factor for the survival and self-renewal of HCC CSLCs.

Background

Hepatocellular carcinoma (HCC) is a common cancer worldwide with a very dismal prognosis. Recent studies have revealed that HCC contains a small subset of cells with the capabilities of self-renewal and differentiation to hetero-

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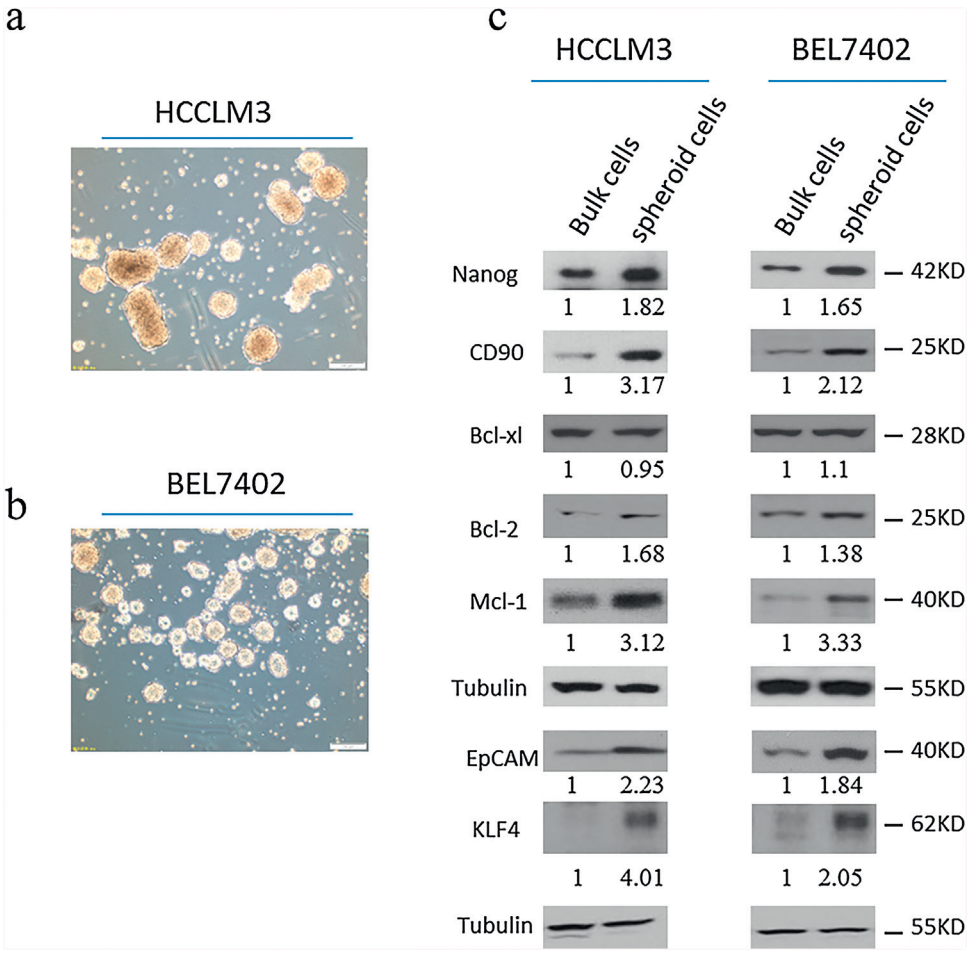

Figure 1 Spheroid HCC cells express higher levels of CD90, Nanog, EpCAM, KLF4 and Mcl-1 than bulk cells. Single cells from HCCLM3 and BEL7402 cell lines were seeded in ultra-low attachment 10 cm petri dishes (100,000 cells per dish), and cultured in serum-free medium for 7 days. The spheres were examined under microscopy and photographed; a, b: Representative spheres were shown (Scale bar 200 μm); c: The expression of Nanog, CD90, EpCAM, Bcl-xl, Bcl-2 Mcl-1 and KLF4 were examined in the lysates of 7-day spheroid cells and bulk cells. Tubulin was used as a loading control. Sphere formation assay was performed for at least three independent times and western blot assay was performed for two independent times.

geneous tumor cells. These so-called cancer stem-like cells (CSLCs) are responsible for tumorigenesis, early metastasis, relapse and drug resistance [1,2]. In order to improve the treatment outcome for patients with HCC, it is imperative to explore the signaling pathways underlying CSLCs' self-renewal and survival that can be targeted in HCC treatment [1,2].

Myeloid cell leukemia-1 (Mcl-1) is a member of the Bcl-2 family proteins. Mcl-1 is well-known for its anti-apoptotic function by sequestering the pro-apoptotic proteins Bax and Bak, stabilizing mitochondrial outer membrane potential, and ultimately blocking apoptosis signaling [3]. In addition, it has been proposed that Mcl-1 is an important factor for the survival and self-renewal of CSLCs in leukemia and several solid types of cancers [4–6]. Mcl-1 is found to be highly expressed in HCC tumor tissues and HCC cells. The high expression of Mcl-1 is closely associated with early metastasis and rapid tumor progression in HCC [7,8]. Besides, Gao and Ren also showed that inhibition of Mcl-1 triggered apoptosis in HCC cells [9,10]. These findings suggest that Mcl-1 represents a therapeutic target for HCC treatment. Nonetheless, whether Mcl-1 has a role in sustaining the growth and survival of HCC cancer stem-like cells has not been addressed unambiguously. We herein addressed this issue through RNA interference and a pharmaceutical Mcl-1 inhibitor.

Materials and methods

Cell culture and reagents

HCCLM3 and BEL7402 cell lines obtained from China Center for Type Culture Collection (Wuhan, China) were maintained in Dulbecco's Modified Eagle's medium (DMEM) (HyClone/Thermo Fisher Scientific, Beijing, China) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd, Hangzhou, China) at 37 °C in a humidified incubator containing 5% CO2. S63845 and A-1210477 were purchased from MedChemExpress (Shanghai, China).

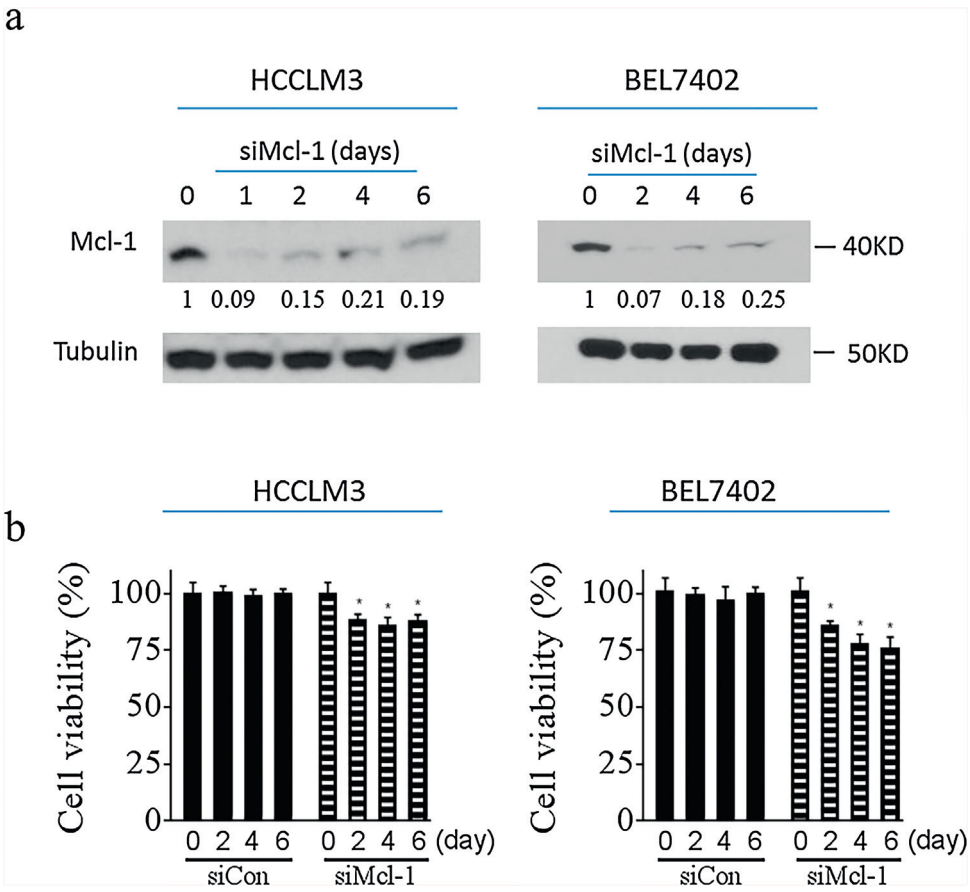

Figure 2 Knockdown of Mcl-1 modestly suppresses cell viability in HCC cells under regular culture condition. HCCLM3 and BEL7402 cell lines were transfected with siRNA against Mcl-1 (siMcl-1) or non-targeting control siRNA (siCon); a: Cells were harvested at different post-transfection time point, and the transfection efficacy was examined by western blotting analysis. Tubulin was used as a loading control; b: Cell viability was determined by CCK-8 assay at indicated time points. CCK-8 assay was repeated for three independent times with triplicates in each experiment. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Cell viability assays

Cells were seeded in triplicate, 5,000/well, in a 96-well plate and treated as indicated in text for 48 h. Cell viability was examined by Cell Counting Kit-8 (CCK-8) assay (Dojindo, Shanghai, China). Results were analyzed and curves were plotted with Prism5 software.

Sphere culture of HCC cells

Single cells (1×1000 per well in 6-well plate or $2-3 \times 10{,}000$ per 10 cm petri dish) dissociated from HCC cell lines were seeded and cultured in ultra-low attachment 6-well plate (Corning) with DMEM-F12 medium supplemented with 20 ng/μL/hEGF (Gibco), 20 ng/μl/bFGF (Gibco), $1 \times$ B27 (Invitrogen), $1 \times$ Insulin-Transferrin-Selenium A (Invitrogen). Half of the medium was replaced with fresh medium every 3 days. Quantification of spheres was performed by transferring a fraction (100 μL) of the culture medium with spheres into 96-well plates and counting the spheres of at least 50 μm under microscope. The total spheres were enumerated by multiplying the total volume of culture medium.

Western blot analysis

Western blot analysis was examined as described previously [9]. Primary antibodies were obtained from: Mcl-1 Clone 22 mouse antibody (#559027) from BD Biosciences-CN (Shanghai, China); Bcl-2 (#15071), Bcl-xl (#2764), PARP (#9542), Caspase-3 (#9662), Nanog (#3580), cMyc (#13987), Sox2 (#14962), KLF4 (#12173), EpCAM (#3599) and Tubulin (#2146) were obtained from Cell Signaling Technologies(Shanghai, China). ImageJ software (NIH) was used to quantify western blot bands.

Small interfering RNA (siRNA) transfection

HCC cells were transfected with siRNA oligos targeting Mcl-1 (SMARTpool: ON-TARGETplus, GE Dharmacon, Shanghai, China) or non-targeting control siRNA with RNAiMax transfection Reagent (Thermo Fisher Scientific, Beijing, China).

Statistical analysis

The results were presented as means ± SEM of three independent experiments. For statistical tests, Prism 5.0

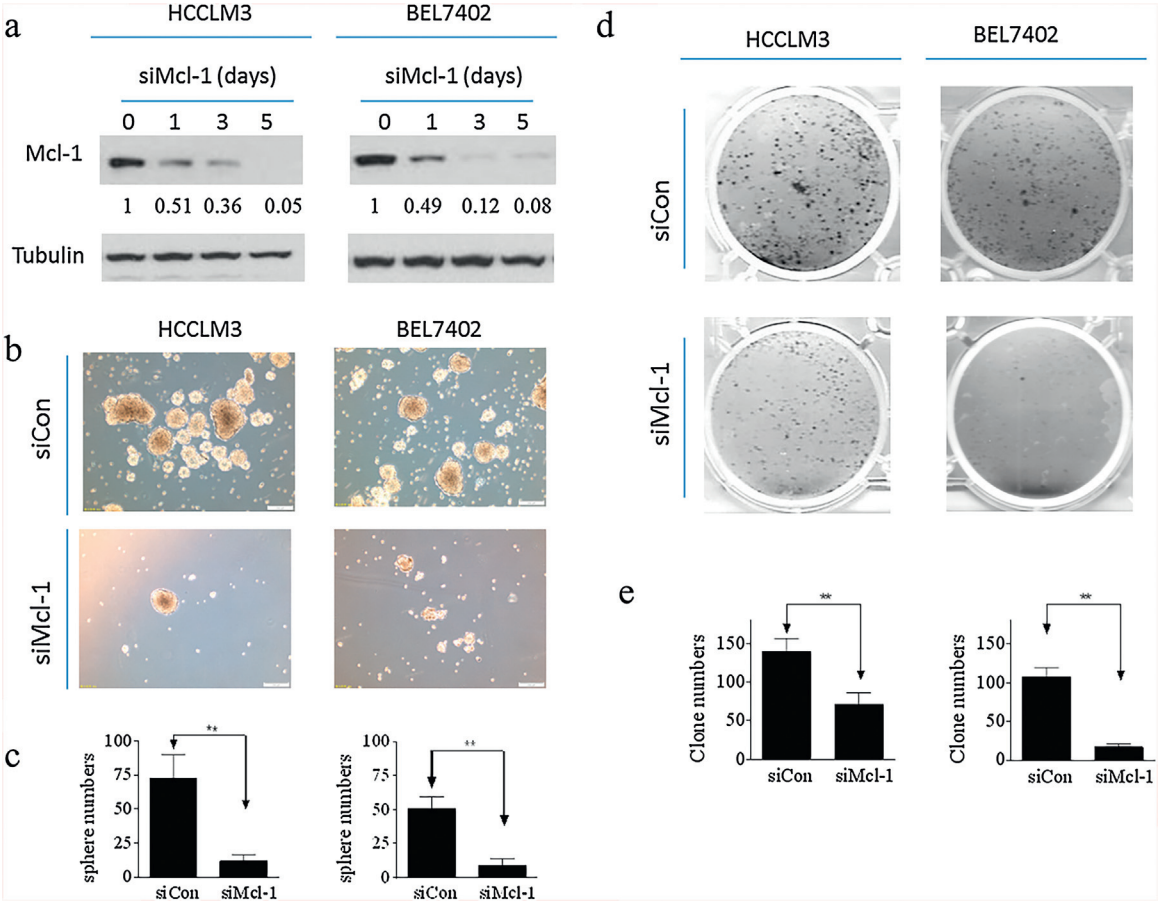

Figure 3 Knockdown of Mcl-1 significantly inhibits the ability of HCC cells to form spheres and clones. HCCLM3 and BEL7402 cell lines were transfected with siRNA against Mcl-1 (siMcl-1) or non-targeting control siRNA (siCon); a: single cells dissociated from transfected cells on day 2 were grown in ultra-low attachment, serum-free medium condition. Spheres were harvested at different post-transfection time point, and the transfection efficacy was examined by western blotting analysis. Tubulin was used as a loading control. Spheres were examined under microscopy after culture for 7 days; b,c: representative spheres were photographed (scale bar 200 μm), and the relative spheres numbers from 3 plates were calculated manually and plotted; d,e: cells were seeded in 6-well plate at 1000 cells per well, grew for 14 days. Clones were stained 0.25%(w/v) Coomassie blue R in methanol/acetic acid/water, and counted manually, and clone numbers from three different experiments were plotted. Each experiment was repeated for three independent times. $^{*}P < 0.05$ and $^{**}P < 0.01$.

(GraphPad Software, SanDiego, CA, USA) was used. *P*-values less than 0.05 were considered statistically significant and less than 0.01 statistically strong significant.

Results

HCC spheroid cells display characteristics of CSLCs and express a higher level of Mcl-1 than bulk cells

CSLCs are able to grow in serum-free suspension culture for clonal proliferation (forming spheres). Therefore, sphere formation assay is widely used in selection and enrichment of CSLCs, also known as tumor initiating cells (TICs) [11]. We thus initiated our study by growing spheres of two HCC cell lines under ultra-low attachment condition in serum-free culture medium. It was found that single cells from HCCLM3 and BEL7402 cell lines formed spheres within 3 days in suspension and continued to grow in mass and number within 7-9 days (Fig.1a,b).

Spheroid cells were harvested on day 7 and western blotting analysis were conducted to examine CSLCs biomarkers in the spheroid cells and cells cultured in regular condition (bulk cells). The results showed that spheroid cells expressed much higher levels of Nanog, CD90, EpCAM and KLF4 than bulk cells (Fig.1c). Since these four proteins have been identified as putative biomarkers of CSLCs in HCC cells [11], these results indicated that CSLCs were substantially enriched in spheres.

It has been reported that CSLCs have survival advantage compared to more differentiated cells, and this is thought to be mediated by high expression of pro-survival proteins such as anti-apoptotic Bcl-2 family proteins [12,13]. Accordingly, we examined the expression of three key anti-apoptotic Bcl-2 family members in spheroid cells and bulk cells with western blotting. The densitometric analysis of the western blot bands showed that Bcl-xl was expressed at a comparable level in both spheroid cells and bulk cells, and Bcl-2 was expressed at a modestly higher level in spheroid cells than in

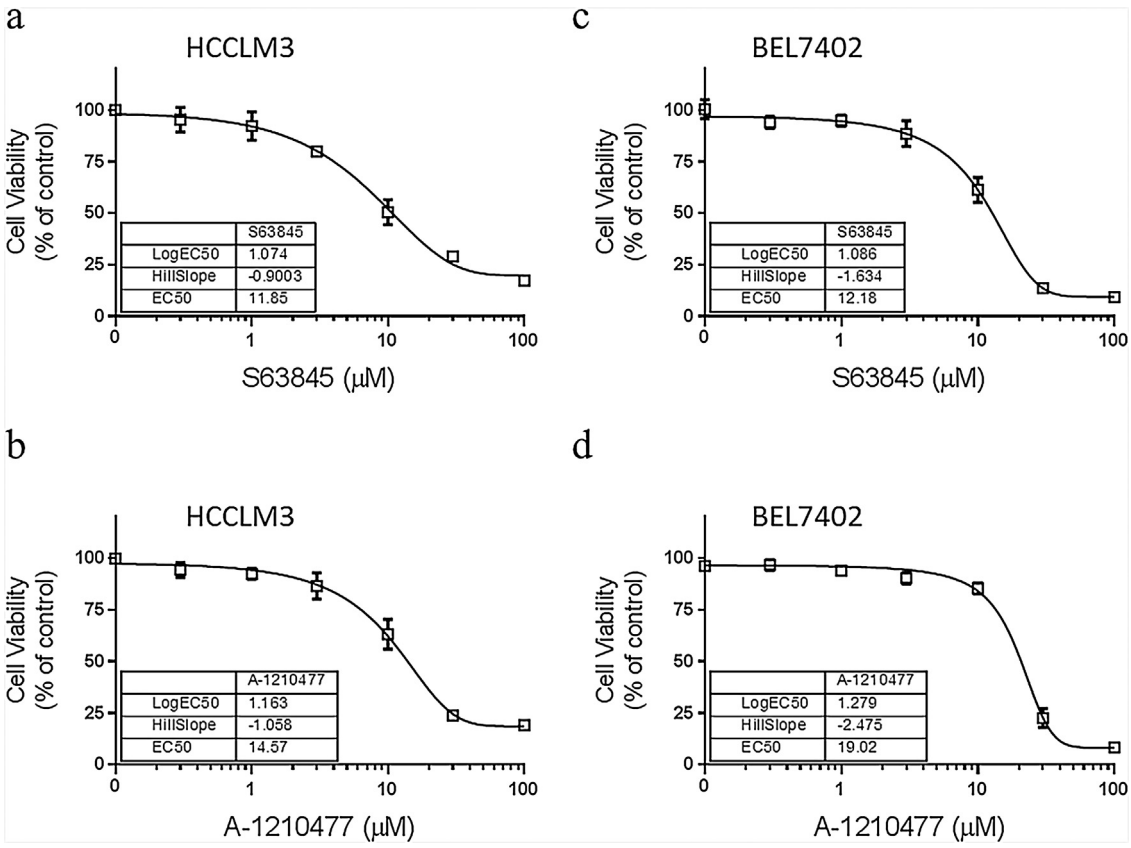

Figure 4 S63845 and A-1210477 modestly inhibit cell viability in HCC cell lines. HCC cells were seeded in 96-well plate (3000 cell per well) and treated with serial dilutions of S63845 or A-1210477 for 72 h. Cell viability was determined by CCK-8 assay. This assay was performed for three independent times. The data were analyzed with Prism5 software and representative curves included EC50 values were shown for each treatment.

bulk cells. It was noteworthy that spheroid cells expressed 3-fold higher level of Mcl-1 compared with the bulk cells (Fig.1c). These suggest that Mcl-1 may play an important role in the survival of CSLCs in HCC among these Bcl-2 family members.

Knockdown of Mcl-1 inhibits sphere formation in HCC cells

We next transfected siRNAs against Mcl-1 genes (siMcl-1) into HCCLM3 and BEL7402 cell lines; and a non-targeting siRNA was used as a control (siCon). Western blotting results showed that siMcl-1 transfection achieved excellent effectiveness in suppressing Mcl-1 expression in both cell lines. As shown in Fig.2a, the protein level of Mcl-1 was substantially reduced within 24—48 h; and the efficiency of siRNA lasted 6 days post-transfection. CCK-8 assays showed that Mcl-1 knockdown indeed significantly inhibited cell viability under regular culture condition with FBS containing medium in the HCC cells (Fig.2b). This is consistent with the notion that Mcl-1 plays a role in the survival of HCC cells [9,10]. Almost completely suppressing the expression of the gene for 6 days only reduced the viability by 14 and 22% in HCCLM3 and BEL7402 cell lines, respectively (Fig.2b). This suggests that Mcl-1 plays a modest role in sustaining the survival of the general population of HCC cells.

We further investigated the role of Mcl-1 in the survival of CSLCs in HCC. To that end, HCC cells dissociated from HCC cell lines transfected with siMcl-1 or siCon were grown in serum-free culture medium to allow sphere formation. Western blotting assay showed that the efficiency of siMcl-1 lasted at least for 5 days post-transfection (Fig.3a). Quantification of spheres (> 50 μm) under microscope showed that cells transfected with siMcl-1 formed significantly fewer spheres than cells with siCon in both HCC cell lines (Fig.3b,c). For instance, in HCCLM3 cell line, siCon transfected cells formed 75.6 ± 11.5 spheres per well in 6-well plate, while siMcl-1 transfected cells formed only 13.5 ± 5.9 spheres, showing an > 80% reduction. These observations indicated that Mcl-1 played a critical pro-survival role in the growth and self-renewal in HCC cells.

As compared to the bulk cells, CSLCs more readily form clones in cell culture [4,5]. We next investigated whether knockdown of Mcl-1 inhibited clonogenic potential of HCC cells. We plated and grew transfected HCC cells at low density (1000 cell/per well in 6-well plate) in FBS containing medium, and allowed the cells growing for another 14 days to form clones. Decent numbers of clones were formed in wells treated by siCon. However, cells transfected with siMcl-1 formed significantly less clones than in cells transfected with siCon in both cell lines (Fig.3d,e). These results altogether suggest that the Mcl-1 is a critical factor in sustaining the survival and growth of HCC CSLCs.

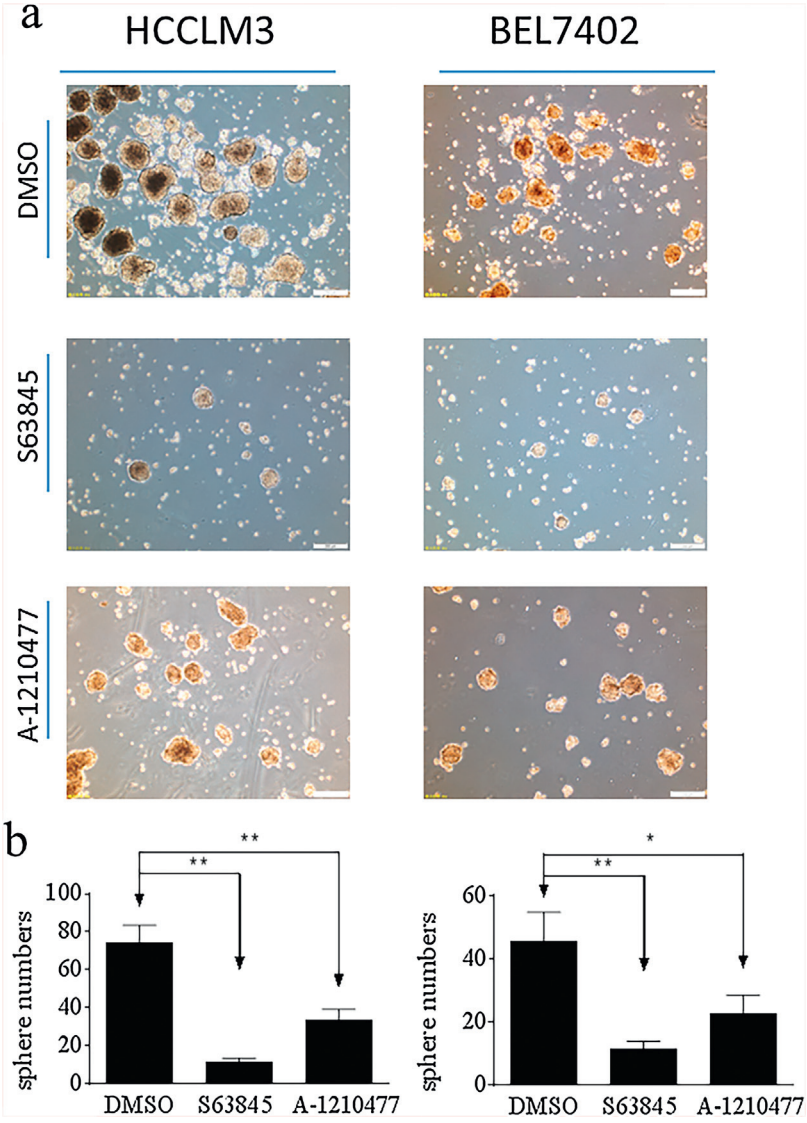

Figure 5 S63845 and A-1210477 inhibit the self-renewal of CSLCs in HCC cell lines. HCC cells were grown in ultra-low attachment plate in serum-free medium for 6 days to allow the formation of primary spheres; then treated the spheres by DMSO or 3 μM S63845 and A-1210477 for 24 h. Single cells dissociated from the treated primary spheres were seeded in ultra-low attachment plate in serum-free medium to allow the formation of secondary spheres. Secondary spheres were examined under microscopy on day 7; a: representative spheres were photographed (scale bar 200 μm); b: the relative spheres numbers from 3 plates from three independent experiments were calculated manually and plotted. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Mcl-1 inhibitors S63845 and A-1210477 attenuate the self-renewal ability of CSLCs in HCC cells

Recently, Mcl-1 has gained intense interest as an attractive target for anticancer therapy [6]. As such, a number of small molecule Mcl-1 inhibitors have been developed up to now. In particularly, S63845 and A-1210477 have drawn increasingly attention among those inhibitors in molecular cancer targeted therapeutic field [14,15]. Both compounds demonstrated much improved selective inhibition of Mcl-1 over Bcl-2 and Bcl-xl as compared to other inhibitors and effective in diverse cancer models [14,15]. Moreover, clinical trials with S63845 was carried out in patients with acute myeloid leukaemia or myelodysplastic syndrome (https://clinicaltrials.gov/ct2/show/NCT02979366). These facts demonstrate that specific Mcl-1 inhibitors represent a promising cancer therapeutic. To further elucidate the role of Mcl-1 in the self-renewal of CSLCs and explore the anti-HCC potential by targeting Mcl-1, we thus used these small molecules to inhibit the function of Mcl-1 in HCC cells and examined the effect on the self-renewal capacity CSLCs with secondary sphere formation assay [16]. We first performed CCK-8 cell viability assay under regular cell culture and found that both compounds modestly inhibit cell viability. S63845 had EC50 values 11.85 μM and 12.18 μM in HCCLM3 and BEL7402 cell lines, respectively. A-1210477 had

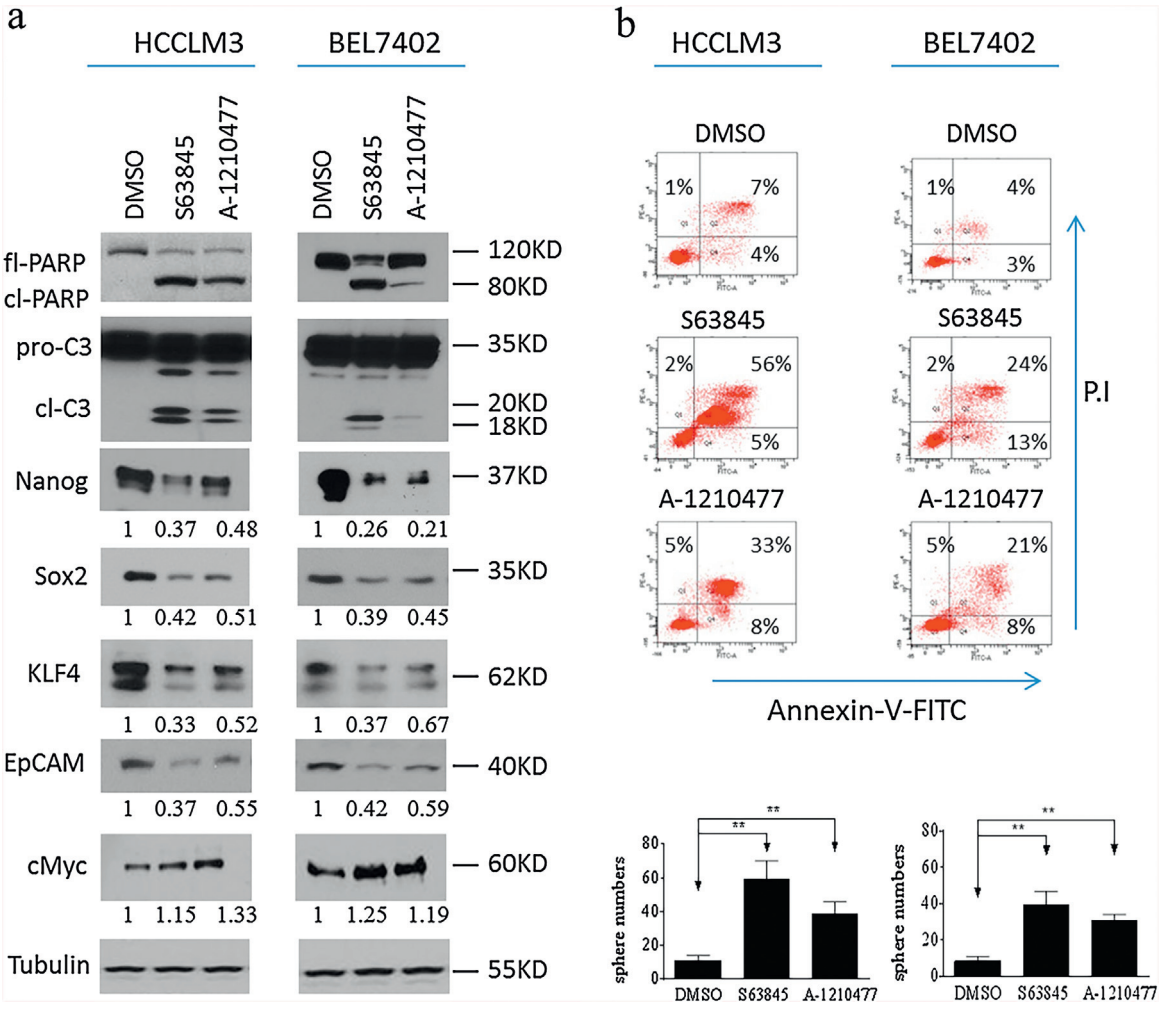

Figure 6 S63845 and A-1210477 trigger apoptosis signaling and reduce the levels of putative stem biomarkers in HCC sphere cells. HCC cells were grown in ultra-low attachment plate in serum-free medium for 6 days to allow the formation of primary spheres; then treated the spheres by DMSO or 3 μM S63845 and A-1210477 for 24 h. a: the treated spheroid cells were harvested and lysed. The expression levels of full-length PARP (fl-PARP), cleaved PARP (cl-PARP), pro-caspase-3 (pro-C3), cleaved caspase-3 (cl-C3), Nanog, Sox2, EpCAM, KLF4 and cMyc were examined by western blotting analysis. Tubulin was used as a control; b: single cells dissociated from the treated cells were stained with Annexin-V-FITC/P.I. Apoptosis were examined by flow cytometry, and representative plots of three experiments were shown for two HCC cell lines. Each experiment was repeated for three independent times. $^{*}P < 0.05$ and $^{**}P < 0.01$.

EC50 values 14.57 μM and 19.02 μM in HCCLM3 and BEL7402 cell lines, respectively (Fig.4a—d).

We grew primary spheres for 6 days, then treated by DMSO, or by S63845 and A-1210477 at 3 μM (a concentration having modest activity in inhibition of cell viability) for 24 h. Single cells dissociated from the treated primary spheres were cultured under ultra-low attachment condition again to form secondary spheres. The results showed that cells from DMSO-treated primary spheres still formed compact and round-shaped spheres with well delimited borders (Fig.5a,b). However, much fewer spheres, or just loosely attached spheres formed from cells isolated from primary spheres treated by the inhibitors. These observations suggest that Mcl-1 is necessary in maintaining the self-renewal ability (stemness) of CSLCs in HCC cells.

S63845 and A-1210477 trigger apoptosis signaling in HCC CSLCs

Mcl-1 is well-known for its anti-apoptotic function. We next investigated whether Mcl-1 was associated with apoptosis signaling of CSLCs in HCC cells by treating 5-day primary spheres with S63845 (3 μM) or A-1210477 (3 μM) and analyzing the alteration of apoptosis signaling by western blotting. It was found that treatment with S63845 or A-1210477 for 24 h triggered PARP cleavage and caspase-3 activation in spheroid cells (Fig.6a). We further dissociated the treated spheres to single cells and analyzed apoptosis by flow cytometry assay in HCCLM3 and BEL7402 cell lines. The results showed that treatment with S63845 or A-1210477 significantly increased cells positively stained with Annexin-V-FITC as compared to DMSO treatment in the cell line (Fig.6b). These

data suggest that apoptosis is involved in the anti-CSLCs activity by Mcl-1 inhibitors in HCC cell lines.

S63845 and A-1210477 reduce the protein levels of several transcriptional factors in HCC CSLCs

Several transcriptional factors, including Nanog, Sox2, KLF4 and c-Myc were reported to have an important role in the maintenance of CSLC's self-renewal [11]. We next investigated whether targeting Mcl-1 would lead to the inhibition of these transcriptional factors in HCC spheroid cells. Western blotting results showed that treatment by 3 μM S63845 or 3 μM A-1210477 markedly reduced the protein levels of Nanog, Sox2 and KLF4 in the primary spheres (Fig.6a). These data thus suggest that reduction of these transcriptional factors may contribute to self-renewal inhibition of HCC CSLC mediated by these Mcl-1 inhibitors. However, c-Myc was slightly increased in the spheres treated by the inhibitors.

EpCAM, a molecule mediating cell-cell adhesion, was also regarded as a biomarker of HCC CSLCs. Western blotting showed that S63845 or A-1210477 markedly reduced the protein level of EpCAM in the primary spheres (Fig.6a). This provides further evidence for the targeting by the Mcl-1 inhibitors.

Discussion

Early metastasis, chemotherapeutic resistance and recurrence still remain the biggest clinical challenges for patients with HCC. Evidences from recent studies suggest that HCC CSLCs are responsible, at least partially, for these aggressive malignancy characteristics of HCC and elimination of this subpopulation of HCC cells may greatly improve the outcomes of HCC [11—13]. Nevertheless, undifferentiated HCC CSLCs often have survival advantages compared to differentiated cell subpopulation. Therefore, there is a pressing need for elucidation of the mechanisms sustaining the survival and self-renewal of the CSLCs.

Mcl-1 is an important pro-survival member of the Bcl-2 family. Our data of this study suggest that Mcl-1 may play a critical role in the survival and self-renewal of HCC CSLCs. This is supported by three lines of evidences. Firstly, Mcl-1 is expressed at a much higher level in CSLCs-enriched HCC spheres than in bulk HCC cells. Secondly, inhibition of Mcl-1 through genetic approach (RNA interfering) significantly suppresses the ability of HCC cells to form spheres under the serum-free condition as well as clone generation. Thirdly, inhibition of Mcl-1 with specific pharmacological molecules (S63845 and A-1210477) greatly retards the ability of HCC cells isolated from primary spheres to form secondary spheres, a common biomarker used to measure the self-renewal ability of CSLCs [16]. Therefore, our data suggest that Mcl-1 may be an important therapeutic target in prevention of metastasis and in overcoming chemotherapeutic resistance in the treatment of HCC.

The results that targeting Mcl-1 substantially increases caspase-3 activation and PARP cleavage further suggest that inhibition of apoptosis signaling may chiefly account for the pro-survival role of Mcl-1 in HCC CSLCs. This is in agreement with the well-known concept that Mcl-1 inhibits apoptosis signaling by directly interaction with the pro-apoptosis Bcl-2 family members such as Bax, Bim, Noxa and Puma in cancer cells. Interestingly, our study also shows that targeting Mcl-1 also leads to the inhibition of Nanog, Sox2 and KLF4, three specific stem cells transcriptional factors in HCC CSLCs. Although interaction details between these factors and Mcl-1 remain to be further investigated, these finding indicate that other mechanisms are involved in the role of Mcl-1 in sustaining survival and self-renewal of HCC CSLCs.

In summary, our data demonstrate that HCC CSLCs express a higher level of Mcl-1 than more differentiated HCC cells. This characteristic facilitates HCC CSLCs to gain survival advantage, and to maintain stemness. Our findings suggest that Mcl-1 might be a valuable therapeutic in prevention of HCC metastasis and relapse. Further in vivo study will assist to elucidate the role of Mcl-1 in tumorigenesis of HCC.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgments

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