4-Acetylantroquinonol B inhibits colorectal cancer tumorigenesis and suppresses cancer stem-like phenotype

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Introduction

Colorectal cancer (CRC) is the third most common cancer, the fourth principal cause of cancer-related mortality globally (Armelo and de Pretis, 2014), and the most prevalent gastrointestinal neoplasm in Taiwan (Hsu et al., 2012; Siegel et al., 2012). Currently, in the management of CRC, 5-fluorouracil (5-FU) alone or in combination with other chemotherapeutic agents, such as folinic acid/leucovorin plus oxaliplatin (FOLFOX), irinotecan and capcetabine, or with vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR) antagonists such as bevacizumab and cetuximab (Lam et al., 2014). Despite substantial progress made in the clinical management of colorectal cancer, the preferred therapeutic strategy is still, in most cases, surgical resection. This treatment modality may not be unconnected with the increasing prevalence of cancer stem cells (CSCs). CSCs have been defined as cells within the tumor that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor.
metastasis-free survival (DMFS) and overall survival (OS) rates (Marin et al., 2012). Accumulating evidence seems to support the probable consideration of CRC as stem cell pathology. The CSC model is suggestive of the cancer subset origin and maintenance, as well as, stem cell-driven disease progression. In fact, CRC CSCs have been implicated in disease recurrence, and the identification, as well as therapeutic targeting of this subpopulation of CRC cells has been propounded to have tumor cell differentiation-enhancing, self-renewal pathway blocking and increased chemosensitizing capability (Marin et al., 2012).

Despite progress made with conventional CRC therapeutic strategies, such as surgery, hormonal therapy, anti-angiogenesis therapy, and immunotherapy, there is still a lack of efficacy in terms of long-term outcome because of their failure to target cancer stem cells and non-specific effects on normal cells (Marin et al., 2012). Clearly, novel therapeutic strategies must be developed to eliminate CSCs and dramatically improve positive patient outcomes and disease-free survival in CRC cancer patients. Seminal and molecular evidences strongly support the ability of naturally-occurring compounds to modulate cell proliferation, cell differentiation or cell death signaling pathways and to regulate fundamental cellular bioactivities (Orlikova et al., 2014). Recently, there is increased focus on several natural compounds with promising anticancer and CSC-targeted activities, including curcumin, resveratrol, cyclopamine, and piperine (Botchkina, 2013). 4-Acetyl-antroquinonol B (4-AAQB), closely related to the better known antroquinoninol (AQ), is a bioactive isolate of the mycelia of Antrodia camphorata, a Taiwanese camphor tree mushroom with documented anti-inflammatory, hypoglycemic, vasorelaxation, and recently demonstrated, anti-proliferative activity (Geethangili and Tzeng, 2011; Yang et al., 2009).

In this study, we examined the biological activities of 4-AAQB and validated its CSC-eliminating and -chemosensitizing or -synergizing activities. We demonstrated that 4-AAQB effectively disrupts tumor-essential signaling pathways, inhibits acquisition of the CSC phenotype, down-regulates stemness-associated genes, attenuates tumor aggressiveness and reverses chemoresistance in CRC cells.

Materials and methods

Reagents and chemicals

The compounds antroquinonol (AQ, ≥99% purity) and 4-acetylanthroquinol B (4-AAQB, ≥99% purity) were obtained from New Bellus Enterprises Co., Ltd. (Tainan, Taiwan), AQ or 4-AAQB was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich Corporation, St. Louis, MO, USA). The prepared solutions were then filter-sterilized, aliquoted, and frozen at −20 °C until use. Propidium iodide (PI) from Sigma (St. Louis, MO, USA), TRIzol reagent, Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), and trypsin-EDTA were purchased from Gibco (New York, NY, USA). The QIAGEN one-tube RT-PCR system was obtained from Qiagen (GmbH, Germany). Annexin-V/ FITC apoptosis kit was from R&D systems (Minneapolis, MN, USA).

Cell lines, culture and compound treatments

Human colon carcinoma cell lines derived from different tumors (DLD-1, HCT-116, SW-480, HT-29 and RKO cells). The cancer cell lines including, DLD-1, HCT-116, SW-480, HT-29 and RKO were maintained in RPMI medium and supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Primary human fetal colon epithelial cells (CRL-1831) (ATCC, Manassas, VA, USA) were cultured in a 1:1 mixture of DMEM and Ham’s nutrient mixture, supplemented with 10% fetal calf serum (FCS), 25 mM HEPES, 10 ng/ml cholera toxin, 5 μg/ml insulin, 5 μg/ml transferrin and 100 ng/ml hydrocortisone. Cells were maintained in a humidified incubator with 5% CO2 at 37 °C. Tested cells were seeded in 6 well plates with 104 cells per well for 14 days. Each well contained 10 ml RPMI medium as cultured condition for CRC cells. Cells were grown to approximately 80% confluence before the addition of AQ or 4-AAQB. Subsequently, cells were incubated with increasing concentrations of AQ or 4-AAQB and cell growth was measured using MTT assay (Yeh et al., 2009).

MTT assay

After cells had been seeded in 96-well plates and incubated overnight to allow cell adherence, we replaced culture medium with AQ or 4-AAQB containing-medium as indicated and incubated again for 48 h. We then determined cell viability and proliferation using 1 μg/ml tetrazolium dye MTT 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for 3 h. Medium was suctioned and formed MTT crystals were resuspended in DMSO. TECAN microplate fluorometer determined color intensity at 595 nm. DMSO was used in treating control cells and assigned a value of 100%. The combination (CI) and isobologram for combination treatment were determined using CalcuSyn software ( Biosoft, Cambridge, United Kingdom).

Western blot analysis

DLD-1, HT-29 and HCT116 colonospheres were seeded and treated with 4-AAQB alone for 48 h. Protein expression was determined using the SDS-PAGE and immunoblotting as previously described (Yeh et al., 2013). The primary antibodies recognizing against β-JAK2, JAK2, pSTAT3, STAT3, Nanog, TYK2, pSrc, Src, Lgr5, Suz12, EzH2 and Tri-methyl H3 were from Cell Signaling Technology (Beverly, MA); β-actin was from Sigma-Aldrich. Secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and then visualized by enhanced chemiluminescence (Amersham).

RNA preparation and real-time PCR

Total RNAs from DLD-1 colonospheres were extracted with TRIzol kit (Life Technologies Inc.) according to the manufacturer’s instructions. One microgram of total RNA was used for cDNA synthesis with SuperScript II reverse transcriptase (Life Technologies Inc.) and random hexamers. To quantify the expression of DLD-1 colonosphere-specific mRNA, RT-PCR was performed on total RNA using the Reverse-IT One-Step RT-PCR Kit according to the manufacturer’s instructions.

Soft agar colony formation assay

Freshly sorted DLD-1, HT-29, HCT-116 colonosphere cells were counted and seeded in triplicate at 500 cells/well in six-well plates pre-coated with 1% agarose. After 2 weeks of incubation in culture media with 4-AAQB (5 μM and 10 μM), we assessed the anchorage-dependent growth, by staining with 0.005% crystal violet, observing and manually counting the colonies formed under a microscope.

Colonosphere formation assay

The CRC cells used were DLD-1, HT-29 and HCT-116 and tumor spheroid assay was done as previously described (Yeh et al., 2012). For the formation of tumor spheroids, cells were cultured in HEScGRO serum-free medium (Chemicon) supplemented with 20 ng/ml Hgf, 10 ng/ml hFGF-β and NeuroCult NS-A proliferation supplements. Sorted cells were seeded at low densities (1000 cells/ml) in 12-well low adhesion plates at 1 ml per well. Spheroids (tight, spherical, non-adherent masses ≥ 90 μm in diameter) were counted, and at least 50 spheroids per group were measured with an ocular micrometer. For secondary spheroid-forming assays, primary spheroids were dissociated mechanically and processed as in the primary assay. This process was repeated with the secondary spheroids, for generation of tertiary spheroids. For
the quantification of the percentage of spheroids, cells were seeded at one cell per well in 96-well plates.

**Flow cytometry analysis and isolation of cell CD44+/CD133+ cell subpopulation**

CD133 and CD44 expression profiles of cultured colorectal cancer cells were analyzed by flow cytometry. Briefly, 1 × 10^6 cells suspended in 100 μl of 1% BSA/PBS containing 1 μg of CD133 and CD44 (eBioscience, Inc., San Diego, CA, USA) were incubated on ice for 30 min to block non-specific Fc interaction, and then labeled with FITC-conjugated anti-CD44 and PE-conjugated anti-CD133 antibodies for 1 h. Thereafter, the labeled cells were resuspended in PBS containing 1% FBS, and analyzed by flow cytometry (BD Biosciences). Unstained cells and isotypic IgG were used as negative control. Using the FACS technique, CD44 and CD133 co-labeled DLD1, HCT116, RKO and SW480 cells were sorted for CD133+/CD44+, CD133+CD44+, CD133-CD44+ and CD133-CD44- subpopulations. To exclude dead cells during sorting, propidium iodide (1 μg/ml) (PI, Invitrogen) was added to the suspended cells. Sorted CD133+/CD44+ cells were then used for colonosphere formation assay as described above.

**Flow cytometric analysis of aldehyde dehydrogenase (ALDH) positive cells**

ALDH enzyme expression was used to isolate colon CSC populations in this study. The Aldefluor assay was performed according to the manufacturer’s guidelines (StemCell Technologies). Briefly, single cells obtained from cell cultures were incubated in an Aldefluor assay buffer containing an ALDH substrate (bodipy-aminocetaldehyde, BAAA) for 50 min at 37 °C. As a negative control, a fraction of cells from each sample was incubated under identical conditions in the presence of an ALDH inhibitor (diethylaminobenzaldehyde, DEAB). Flow cytometry was used to measure the ALDH-positive cell population (Yeh et al., 2012).

**Immunofluorescence assay**

DLD1 colonospheres were seeded in 96-well dishes (Corning, NY) and incubated at 37 °C for 3 h. Cells were washed with cold PBS and then fixed with pre-chilled 4% PFA for 20 min at room temperature. After blocking with 1% bovine serum albumin in PBS–Triton 0.1%, cells were incubated with primary antibodies: pSRC (anti-Src (phosphor Y418) antibody (ab4816); Abcam, Inc.), and pSTAT3 (Anti-STAT3 (phosphor Y705) antibody [EP2147Y] (ab76315); Abcam, Inc.) over-night at 4 °C in the dark. Then cells were washed three times with PBS–Triton 0.1% and incubated with Alexa-Fluor-conjugated secondary antibodies against mouse or rabbit (Invitrogen) at room temperature for 1 h in the dark. Cells mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA) and analyzed using a fluorescence microscope.

**Evaluation of tumor inhibitory effects of 4-AAQB in vivo**

NOD/SCID mice were purchased from the National Taiwan University and maintained in compliance with the institutional policy. All animal procedures were approved by the Institutional Animal Care and Use Committee at Taipei Medical University. Colorectal cancer cell line, DLD1 cells sorted for co-expression of CD44 and CD133 positivity were injected into the right flank of the animals at a concentration of 1 × 10^6 cells/50 μl. One week after tumor injection, different treatment regimens were started. Four regimens were performed: control; 4-AAQB alone (2.5 mg/kg/day, intraperitoneal injection); FOLFOX alone (5-FU 10 mg/kg, 5 times/week, and oxaliplatin 5 mg/kg 3 times/week, intravenous injection), and a combination of 4-AAQB (2.5 mg/kg/day, 5 times/week, intraperitoneal injection) + FOLFOX (as described above) for a period of 6 weeks. The tumor size of CD44+/CD133+ DLD1-bearing mice was measured weekly using a standard caliper test. Data are expressed as fold change in mm^2 as compared to its starting volume. Mice humanely killed at the end of experiments.

**Statistical analysis**

Data were calculated in triplicate and expressed as the mean ± standard error of the mean. Comparative analysis was done using either the Student’s t-test, or one-way analysis of variance (ANOVA) post hoc tests. P < 0.05 was considered as a statistically significant result.

**Results**

**Malignant human colorectal cancer cells are enriched with CD133 and CD44**

There is a broad consensus that CD44, CD133 and ALDH1 are CSC markers and help delineate this subset of cells in any heterogeneous tumor cell population. We thus evaluated the relative proportion of cells expressing the surface markers, CD133 and CD44 using the flow cytometric assay. DLD-1, HCT-116, RKO and SW-480 cells were used in the analysis. As shown in Fig. 1 for a single marker, 2.85% of DLD1 cells were CD44 positive while 25.9% were CD133 positive. By comparison, 0.09% of HCT116 cells were CD133 positive, and 96.7% were CD44 positive. In contrast to high percentage of cells expressing for a single marker of CD44 or CD133, the percentage of cells with co-expression of CD44 and CD133 markers on the same cell is lower for most of cell lines. We observed that only 1.65% of SW480 cells and 1.70% of RKO cells expressed CD133 and CD44 concomitantly. In contrast, higher co-expression was noted in DLD1 (14.9%) and HCT116 (3.32%) cell lines. This data corroborates the hypothesis that CSCs are a small subset of the tumor bulk, and demonstrate that a combination of CD133 and CD44 rather than any of the surface markers delineates this CSC subpopulation.

To further investigated the CSC-associated self-renewal potential of the colorectal cells, we performed the tumorsphere formation assay of the CD44+/CD133 sorted cells in ultra-low non-treated culture dish with conditioned medium. After seven (7) to ten (10) days, unlike the CD44+/CD133 double-negative cells, CD44+/CD133 double-positive cells generated many large colonospheres, while fewer and smaller spheres were generated by the CD44-/CD133+ or CD44+/CD133- stained cells (Fig. 1C). This data further demonstrate that CD44+/CD133+ enriched colorectal cells delineate CSCs or tumor-initiating with self-renewal potential.

**4-AAQB inhibits the proliferation of aggressive human colorectal cancer cells**

In order to determine the effect of 4-acetylantroquinoninol B (4-AAQB) and antroquinoninol (AQ) on colorectal cancer cell growth, we treated DLD-1, HCT-116, SW-480, RKO and HT-29 cells with 4-AAQB and AQ. After 48 h of treatment with either of these compounds, the cell viability assay showed reduction in cell number in the treated group, relative to the untreated control (Fig. 2). The effect of 4-AAQB on the cell viability was, however, much stronger than that of AQ. Specifically, 4-AAQB showed greater cell growth inhibitory potential against DLD-1 cells with an IC_{50} of 11.3 μM. The determined IC_{50} of 4-AAQB against HCT-116, SW-480, RKO and HT-29 cells were 34.6, 38.1, 22.3 and 39.2 μM, respectively (Table 1). Compared to AQ, 4-AAQB has a greater anti-proliferative potential in all five colorectal cancer cell lines, evidenced by the 2.4- and 1.3-fold IC_{50} reduction in DLD-1 and HCT-116 cells, respectively. In parallel experiment, after CD44+/CD133+ surface marker sorting, we performed a comparative analysis of the effect of 4-AAQB and AQ on CD133+ DLD-1 cells using the SRB cell viability assay. Results obtained showed that 4-AAQB significantly inhibited viability of the CD133+ DLD-1 cells compared to the
A 4-AAQB-treated group (Fig. 2F). Together, these data portrayed 4-AAQB as the more effective inhibitor of colorectal cancer cell proliferation. In addition, we analyzed the cytotoxic effect of 15 μM of 4-AAQB on non-tumorigenic fetal human colon epithelial cells (CRL-1831) and tumorigenic carcinoma DLD-1, HCT-116 colorectal cell lines in comparison to untreated cells served as control. While 4-AAQB had a very significant cytotoxic effect on the tumorigenic DLD-1 and HCT-116 colorectal carcinoma cells, with approximately 60% (P < 0.001) and 32% (P < 0.01) reduction in cell population, respectively, the non-tumorigenic fetal human colon epithelial cells (CRL-1831) were almost unaffected (Fig. 2G).

4-AAQB enhances chemosensitivity in 5-FU-resistant colorectal cancer stem cells

CSCs play a vital role in disease recurrence and the acquisition of chemoresistance in various carcinoma types (Yeh et al., 2012). Since 4-AAQB potently inhibited the colorectal cancer cell growth (Fig. 2), we then examined its cytotoxic effect on CRC-CSCs represented by colonospheres generated from CD44+/CD133+ double-positive DLD-1, HCT-116 and HT-29 colorectal cells in comparison with chemotherapeutic agents commonly used to treat CRC. The exposure of the cells to 5-fluorouracil (5-FU) and oxaliplatin increased the numbers of ALDH+ CRC CSCs, by 18.7% and 10.7% respectively; suggesting that exposure to these chemotherapeutic agents induces increased enrichment with active ALDH+ CSCs (Fig. 3A). Interestingly, our data indicate that 4-AAQB reversed this trend and increased the sensitivity of CRC cells to chemotherapy; indeed, 5-FU resistant CRC-CSCs were very responsive to 4-AAQB treatment in a dose-dependent manner (Fig. 3B). We noted a 25% and 40% decrease in DLD-1 colonosphere formation, compared to 30% and 60% in parental cells when treated with 10 and 20 μM of 4-AAQB respectively. On the other hand, 10 and 20 μM of 4-AAQB induced a 30% and about 40% reduction in HCT-116 colonospheres, in comparison to the 50% and 62% decrease noted in parental cells. We next determined the IC50 of the inhibitory activity of 4-AAQB in colonosphere CSCs isolated from a variety of human colon cancer cell lines. As shown in Table 2, 4-AAQB showed specific anti-colon CSC activity with an IC50 value ranging from 4.5 μM to 12.9 μM in DLD-1, HCT-116 and HT-29 colonospheres. This result suggests that 4-AAQB enhances the chemosensitivity of 5-FU-resistant CRC-CSCs and is a putative effective CSC-targeting therapeutic agent.

4-AAQB downregulates cancer stem cell activity and expression of stem cell-associated genes in colorectal cancer via disruption of the hedgehog (Hh) and Lgr5–Wnt/β-catenin pathways

Aberrant WNT pathway signaling is an early progression event in 90% of colorectal cancers. To begin addressing the role of constitutive WNT signaling in colorectal cancer, we first looked for expression of WNT genes in colorectal cancer. In all five colorectal cancer cell lines examined, we found expression of 4 members of the WNT family genes in colorectal cancer cell lines. A subsequent RT-qPCR analysis showed that WNT messenger RNA (Wnt2, Wnt4, Wnt5b, and Wnt10b) is abundant mainly in APC-deficient cells (DLD1, HT-29, SW480), although its expression in cells with intact APC (but harboring β-catenin activating mutation: HCT116 and RKO) was still detected (Supplementary Fig. 1). To further understand the role of 4-AAQB in the regulation of CRC-CSCs and the underlying mechanism, we examined the activity of
the canonical Wnt pathway, as well as the Hh pathway in 4-AAQB-treated CRC cells. As shown in Fig. 4, 4-AAQB reduced the expression of smoothened (SMO) mRNA expression in a dose-dependent manner, and consequently disrupts the SMO-dependent Hh pathway. Similarly, treatment with 4-AAQB potentially disrupts the Lgr5–Wnt/β-catenin signaling axis as demonstrated by the dose-dependent downregulation of both Lgr5 and Wnt/β-catenin signaling pathway (Fig. 4A). This was in parallel with the downregulation of VEGF and Sox-2 expression after 4-AAQB treatment. We also observed a 61% or 90% reduction of ALDH-enriched cells when treated with 5 μM or 10 μM of 4-AAQB, respectively (Fig. 4B), reduced clonogenicity (Fig. 4C, D), and attenuated colonosphere formation in DLD-1, HT-29 and HCT-116 CRC cells (Fig. 4E). Our results showed that 4-AAQB dose-dependently reduced the CRC-CSC spheroid viability (Fig. 4F). Taken together, these data are indicative of the ability of 4-AAQB to inhibit ALDH activity, disrupt transcriptional activities of the Hh, Lgr5 or canonical Wnt/β-catenin pathway, and consequently suppress CSC-related activities. Furthermore, we checked its effects on the migration and invasion of colonosphere. 4-AAQB significantly inhibited the migration of DLD1 colonosphere. It also inhibited the cell invasion in a dose-dependent manner.
Collectively, these results indicated that 4-AAQB effectively inhibits the invasiveness hallmarks associated with aggressive colon cancer. 4-AAQB inhibits JAK–STAT signaling pathway and suppresses its downstream target genes

Since CSCs have been associated with increased tumorigenicity, chemoresistance and cancer aggression, we sought to elucidate the role of 4-AAQB in the dysregulation of JAK–STAT signaling axis which has documented critical role in the transduction of extracellular signals from growth factors and cytokines as well as survival of cancer stem-like cells (Kroon et al., 2013). To determine if the 4-AAQB suppressed STAT3-mediated transcription in CRC-CSCs, we generated colonospheres from DLD-1, HT-29 and HCT-116 cells. The colonospheres were passaged until the tertiary generation was generated and subjected to treatment with 10 μM or 20 μM of 4-AAQB. Fig. 5 shows decreased pJak2 and pJak2-modulated pSTAT3 in the colonospheres, with a subsequent suppression of the expression of JAK–STAT pathway downstream genes, including Nanog (Fig. 5A), TYK2, p-Src, and Lgr5 (Fig. 5B). Considering the role of epigenetics in cancer initiation, maintenance and progression, we then probed the effect of 4-AAQB treatment on cancer-associated epigenetic factors. Our findings showed that when treated with 4-AAQB, the DLD-1 spheroid cells lost expression of the tumor-promoting epigenetic factors Ezh2 and Suz12 and increased expression of trimethyl H3 that is typically lost in CRC (Fig. 5B). The inhibitory effect of 4-AAQB on the activation of STAT3 and Src was further confirmed by immunocytochemistry.

Table 1
<table>
<thead>
<tr>
<th>Colon cancer cell line</th>
<th>Histopathological classification</th>
<th>IC_{50}[^a][μM]</th>
<th>4-Acetylantroquinonol B IC_{50}[^a][μM]</th>
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<tr>
<td>DLD-1</td>
<td>Colorectal adenocarcinoma, Duke type C</td>
<td>27.4</td>
<td>11.3</td>
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<td>HCT-116</td>
<td>Colorectal carcinoma</td>
<td>45.2</td>
<td>34.6</td>
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<tr>
<td>SW-480</td>
<td>Colorectal adenocarcinoma, Duke type B</td>
<td>ND[^d]</td>
<td>38.1</td>
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<tr>
<td>RKO</td>
<td>Colorectal carcinoma</td>
<td>ND</td>
<td>22.3</td>
</tr>
<tr>
<td>HT-29</td>
<td>Colorectal adenocarcinoma</td>
<td>ND</td>
<td>39.2</td>
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[^a]: Sigmoidal dose response curves (variable slopes) were generated GraphPad Prism V. 4.02 (GraphPad Software Inc.).
[^b]: Test compound concentration required to inhibit cell proliferation by 50% after 48 h of treatment compared with vehicle control DMSO.
[^c]: Values are the mean of triplicate of at least two independent experiments.
[^d]: ND (not determined) = IC_{50} value > 100 μM.

Fig. 3. Chemotherapy induces increased ALDH enrichment but 4-AAQB reverses this CSC-associated chemoresistance trend. (A) Comparative FACS analysis of ALDH1 activity in adherent CD44+/CD133+ double positive DLD-1 cells. The number of ALDH+ drug resistant CSCs increased after 5-fluorouracil or oxaliplatin chemotherapy. It indicates the importance of CSCs as the main target to overcome 5-fluorouracil resistance. (B) Treatment of DLD-1 and HCT-116 parental and spheroid cells with 4-AAQB induced significant reduction in cell number and moderate inhibition in HT-29, compared to their untreated control counterparts. All experiments were performed in triplicate and data are the mean ± S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001.
Fig. 5C demonstrates the co-localization of p-Src and p-STAT3 in the DLD-1 derived colonospheres, attenuation of p-Src and p-STAT3 expression as well as significant reduction in spheroid cell size after treatment with 4-AAQB using the immunoﬂuorescent (IFC) staining technique. Since EZH2 is highly expressed in colorectal cancer and associations with treatment response and prognosis, we sought to examine whether inhibition of EZH2 enhanced the biological effect of 4-AAQB on cancer stem cell viability in colonospheres. Transduction of EZH2 siRNA in DLD1 colonosphere inhibited EZH2 expression, as shown by the western blot analysis (Fig. 5D). 4-AAQB inhibited cell viability in DLD1 colonosphere in a dose-dependent manner (Fig. 5E). Transduction of EZH2 siRNA further enhanced the inhibitory effect of 4-AAQB on cell viability in colonosphere. These data suggest that inhibition of EZH2 can enhance the chemopreventive effect of 4-AAQB.

4-AAQB augments the anticancer therapeutic effect of 5-FU in colorectal cancer cells

To better understand the effectiveness of 4-AAQB in CRC treatment, we evaluated its anticancer therapeutic effect in combination with a prototypic CRC chemotherapy. When combined with 5-FU, 4-AAQB enhanced the cancer killing potential of 5-FU in CRC cell lines, DLD-1, HT-29 and HCT-116, most signiﬁcantly in the DLD-1 cells (Fig. 6A). To determine whether the combination of 4-AAQB and 5-FU has synergistic or additive activity, we performed isobologram drug combination analysis to assess their combined inhibitory effects and combination indices. As indicated in the isobologram, all dose pairs fell within the isobologram triangle, indicating 5-FU–4-AAQB synergism in the DLD-1, HT-29 and HCT-116 cells (Fig. 6A, B, C). Taken together, these findings indicate that 4-AAQB interacts synergistically with 5-FU to significantly inhibit colon cancer cell growth and colonosphere formation.

4-AAQB inhibits tumor growth and cancer-associated body weight loss in colorectal cancer xenograft nude mice

The enhanced tumor-inhibitory role of 4-AAQB in CRC was further validated by signiﬁcant tumor size reduction in nude mice subjected to 4-AAQB and FOLFOX combination therapy, in comparison to FOLFOX or 4-AAQB monotherapy (Fig. 7A, B). As we alluded above, we inoculated NOD-SCID mice subcutaneously with CD44+/CD133+ double positive DLD-1 cells representing CRC-CSCs and 4-AAQB and/or FOLFOX treatment was commenced a week after injection. Compared to vehicle-treated mice, those treated with FOLFOX or 4-AAQB only, showed limited reduction in tumor size over the experimental time course (P < 0.05 for FOLFOX; P < 0.01 for 4-AAQB), however, 4-AAQB–FOLFOX combination treatment signiﬁcantly affected the tumor burden, evidenced by markedly reduced tumor size (P < 0.001) and suppressed tumor progression (Fig. 7B). We also noted that in contrast to FOLFOX chemotherapy that causes a dramatic weight loss in CRC patients, the 4-AAQB-treated mice showed no loss of body weight, and when combined with FOLFOX, 4-AAQB normalized the body weight over time (Fig. 7C). Consistent with all other data in this study, 4-AAQB

Table 2

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<tr>
<th>Colon sphere</th>
<th>Histopathological classification</th>
<th>IC50 (μM) ± SD</th>
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<tr>
<td>DLD-1sphere</td>
<td>Colorectal adenocarcinoma, Dukes type C</td>
<td>4.5 ± 1.2</td>
</tr>
<tr>
<td>HCT-116sphere</td>
<td>Colorectal carcinoma</td>
<td>9.3 ± 1.3</td>
</tr>
<tr>
<td>HT-29sphere</td>
<td>Colorectal adenocarcinoma</td>
<td>12.9 ± 1.1</td>
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Fig. 4. 4-AAQB downregulates key CSC regulator genes, resulting in lower tumorigenicity and survival. (A) Treatment of DLD-1 cells with 4-AAQB downregulated several genes critical for cancer progression and CSC maintenance, including SMO, VEGF, Sox-2, Lgr5, and β-catenin; GAPDH served as internal control. (B) Reduced number of ALDH+ DLD-1 cells after 48 h 4-AAQB treatment. (C and D) 4-AAQB negatively affects the clonogenicity of DLD-1, HT-29 and HCT-116 cells. (E and F) Colonospheres were incubated with varying concentration of 4-AAQB (0, 10, and 20 μM) for 48 h. 4-AAQB inhibited the self-renewal capacity of the colonospheres derived from DLD-1, HT-29 and HCT-116 cells. Data are expressed as the mean ± S.E.M. and are representative of experiment performed in triplicate.
Fig. 5. 4-AAQB alters pathways critical for cancer survival, invasiveness, and epigenetic control mechanisms. (A) 4-AAQB inhibits activation of Jak2, Stat3 and Nanog in DLD-1, HT-29 and HCT-116 cells. (B) Downregulation of TYK2, Src, Lgr5, Suz12 and Ezh2, with upregulation of Tri-methyl H3 after exposure of DLD-1 cells to 4-AAQB. (C) Co-localization of p-Src and p-STAT3 in the DLD-1 derived colonospheres. Treatment with 4-AAQB attenuates expression of p-Src and p-STAT3 and significantly reduced the sizes of spheroids formed using the immunofluorescent (IFC) staining technique. (D) EZH2 siRNA enhances the inhibitory effects of 4-AAQB on self-renewal capacity of DLD1 colonospheres. DLD1 colonospheres were transduced with scrambled or EZH2 siRNA, and Western blot analyses were performed to examine the expression of EZH2 and β-actin. (E) Scrambled or EZH2 siRNA DLD1 cells were grown for one week. DLD1 colonospheres were harvested and resuspended and cell viability was determined by trypan blue assay. All experiments were performed in triplicate and data are the mean ± S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 6. Drug combination analysis of 4-AAQB and 5-FU. (A–C) Upper panel: Dose–response chart of the colorectal cell lines DLD1, HCT116, and SW480 to 4-AAQB and 5-FU combination therapy show cell viability after 48 h treatment with 2.5 μM to 20 μM 4-AAQB combined with 2.5 μM, 5 μM and 10 μM of 5-FU. Lower panel: Isobologram analysis of combination index, CI of 4-AAQB and 5-FU in the 3 human colorectal cell lines. Results shown are expressed as means ± S.E.M. of experiments performed in triplicate.
significantly reduced the percentage of CD44+/CD133+ DLD-1 cells in the xenografts and enhanced the ability of FOLFOX to do same (Fig. 7D).

Discussion

Colorectal carcinoma (CRC) ranks among the most common malignancies globally, and despite substantial progress made in the clinical management of this disease, in most cases, surgical resection still remains the preferred therapeutic strategy (Armelao and de Pretis, 2014). This treatment modality may not be unconnected with the increasing prevalence of CSC-induced chemoresistance and tumor cell maintenance in CRC (Botchkina, 2013). Colorectal CSCs, characteristically similar to normal colorectal stem cells, initiate and maintain tumor cell growth and proliferation as a result of their ability to self-renew and confer the chemoresistance to cancer cells. Many studies have demonstrated that the presence and activities of cancer stem cells positively correlate with tumor insensitivity to chemotherapy, and consequently facilitate unrestricted proliferation and tumor progression (Botchkina, 2013). However, most contemporary conventional chemotherapeutic agents target and eliminate the non-CSC tumor mass, leaving the so-called tumor initiating cells (TICs) or CSCs, thus, the unabated incidence of disease recurrence and unfavorable clinical outcomes. In most cases, CSCs have been identified based on their expression of specific cell surface markers, including aldehyde dehydrogenase (ALDH1), CD133, and CD44. In a series of recent studies, ALDH1 has been described to play a crucial role in identification of putative CSCs and/or potential cancer-initiating cells in colon cancers. CD133 (also known as prominin-1) is a type I transmembrane glycoprotein that has been very important and extensively used in characterization of CSCs. CD133+ cells have also been found to maintain long-term expression of CD133 when grown in sphere media. O'Brien and colleague were the first to demonstrate that only a small subset of CSCs isolated from a CD133+ population were capable of growing as colonospheres in serum-free sphere media, and these cells could initiate tumor growth in a serial xenograft mouse model (O'Brien et al., 2007). CD44 is a hyaluronan receptor that plays a critical role in the homing and colonization of adult stem cells, CSCs, and metastasizing cancer cells. Similarly, single CD44+ colon cancer cells have been shown to form spheres in serum-free sphere media and have been used to establish xenograft tumor models in vivo (Dalerba et al., 2007). Consequently, CD44 has been reported to be a marker for colon CSCs. While colon cancer cells express both CD133 and CD44, the presence of these markers alone is probably insufficient to identify CSCs (Wang et al., 2012). Some literatures showed that ALDH1 could be added to CD44 to further restrict the phenotypic definition of CSCs and isolate an even more tumorigenic subset. Furthermore, cells expressing CD133+ and CD44+ have exhibited greater tumorigenicity than cells expressing either marker alone (Haraguchi et al., 2008). Taken together, these results suggest that a combination of markers were needed to identify the CSC population in human colon cancer cells. This necessitates urgent discovery and development of novel therapeutic agents with inherent CSC-targeting potential (Findlay et al., 2014). In the recent years, several natural compounds are found to have the ability to kill cancer stem cells, such as salinomycin, curcumin, sulforaphane, and a novel gemini vitamin D analog (BXL0124) (Hu and Fu, 2012).

A. camphorata is a medicinal mushroom whose pharmacological effects are different from those of conventional chemotherapeutical drugs, due to its cancer cell type-based selective killing. Recent studies...
on *A. camphorata* have several demonstrated biological activities including anticancer, anti-inflammatory, hypoglycemic, and vasorelaxation (Geethangili and Tzeng, 2011). AQ and its derivatives, including 4-AAQB, have been shown to induce diverse biological functions in a concentration or cell-type dependent manner (Lin et al., 2010). At low concentrations (1–20 μM), AQ from ethanolic extract of *A. cinnamomea* mycelia showed hepatoprotective effects from ethanol-induced oxidative stress through heme oxygenase-1 and Nrf-2 signaling activation (Kumar et al., 2011). On the other hand, high concentrations of AQ (30 μM) induce a cross-talk between apoptosis, autophagy and senescence in human pancreatic carcinoma cells (Yu et al., 2012). A recent study also showed that 4-AAQB arrests proliferation of human hepatocellular carcinoma HepG2 cell by affecting p53, p21 and p27 levels (Lin and Chang, 2011). Results from a large number of studies have unequivocally evidenced demonstrated that mutant p53 not only plays a pivotal role in the transformation of CRC, but also contributes to the aggressive-invasive nature of CRC. To examine if the ability of 4-AAQB to inhibit cellular growth inhibition in CRC cells dependent on p53 status, we treated colon cancer cell lines DLD1 (mutant p53), HCT116 (WT p53), RKO (WT p53), and SW480 (mutant p53) with different concentrations of 4-AAQB. We found that treatment of cells with different doses of 4-AAQB for 48 h led to potent cellular growth inhibition indicating that these cells underwent growth inhibition independently of their p53-status (Fig. 2). These results support other previous results that AQ induces apoptosis in human cancer cells by a p53-independent mechanism (Chiang et al., 2010). Therefore, additional experiments are required to clarify the role of p53 in the 4-AAQB-induced apoptosis in CRC cells. Additionally, our research group identified various anticancer pure compounds from the extracts of *A. camphorata* (Hsieh et al., 2010, 2011; Lee et al., 2012; Rao et al., 2011; Tsai et al., 2010; Yeh et al., 2009, 2013). 4-AAQB was isolated and purified from the mycelia of *A. camphorata*. In this study, we identified 4-AAQB as an inhibitor of cellular transformation, tumor aggression and progression, as well as chemoresistance in CRC.

This study also compared the anticancer effect of 4-AAQB, AQ and FOLFOX, in colorectal cancer cell lines, DLD-1, HCT-116, HT-29, SW-480, and RKO, as well as in colonospheres generated from the CRC cells. We demonstrated the anti-proliferative effects of 4-AAQB, as monotherapy and in combination with FOLFOX or 5-FU. We showed that treatment of the CRC cells with 4-AAQB resulted in reduced cancer cell viability and CD133⁺ cell population (Fig. 2), and ALDH1 activity (Fig. 3). ALDH1 is a marker for normal and cancer colorectal stem cells, is required for the maintenance of chemoresistant CSCs (Rah et al., 2014), and is reportedly activated or overexpressed after chemotherapy (Januchowski et al., 2013). This corroborates the probable involvement of CSCs, evidenced by increased pool of ALDH enriched cells and upregulation of stemness markers such as SMO, VEGF, Sox-2 and Nanog (Figs. 4 and 5), in the modulation of CRC cells' sensitivity to conventional CRC anticancer therapy, such as the fluoropyrimidine-based chemotherapy with or without the anti-VEGF or EGFR agents. Furthermore, we demonstrated the inhibitory role of 4-AAQB on several tumor-related signaling pathways, including the Lgr5/Wnt/β-catenin, Hh and JAK–STAT pathways (Figs. 4 and 5). This inhibitory potential on the canonical Wnt/β-catenin pathway is important because the regulation of proliferation, self-renewal and terminal fate of pluripotent CRC stem cells is dependent on Wnt/β-catenin signaling (Ling et al., 2009; Nusse, 2008). Also, since SMO plays a critical role in cancer cell invasion and migration (Yoo et al., 2008), inhibiting the expression of SMO protein, and thus, disrupting the sonic Hh pathway, in CRC parental and CSC cells treated with 4-AAQB obviously correlated with attenuated invasive and migratory ability of the colorectal cancer cells and colonospheres (Figs. 4 and 5).

In addition, the inhibition of JAK–STAT signaling by 4-AAQB (Fig. 5) was of no lesser relevance since several studies have demonstrated that STAT3, specifically STAT3, is essential for various aspects of tumor formation, including proliferation, angiogenesis, metastasis, apoptosis, determination of its terminal fate, immune cell mobilization, and invariably, chemosensitivity (Jove, 2000; Levy and Inghirami, 2006; Yu et al., 2009). Associated with dysregulation of these signaling pathways was the inactivation or down-regulation of their downstream target genes or transcriptional factors, such as vimentin, β-catenin, Nanog, and Sox-2, in a dose-dependent manner. Conversely, 4-AAQB induced re-expression of the epithelial marker E-cadherin (data not provided). This is consistent with the CSC hypothesis which suggests that if CSCs are eliminated, chemoresistance is abolished, and responsiveness of cancer cells to therapeutic agents is enhanced. Considering the role of epigenetics in cancer initiation, maintenance and progression, we then probed the effect of 4-AAQB treatment on selected cancer-associated epigenetic factors. Our findings showed that when treated with 4-AAQB, the DLD1 spheroid cells lost expression of tumor-promoting epigenetic factors EzH2 and Suz12 and increased expression of tri-methyl H3 which is typically lost in CRC (Fig. 5).

Conventional first-line treatments for patients with metastatic CRC involve a combination of 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX). FOLFOX has demonstrated good efficacy in phase III trials and is actually employed in younger patients with metastatic CRC (Lenz, 2008). Moreover, neoadjuvant chemotherapy has been combined with antiangiogenic drugs, particularly with bevazcumab (Avastin), which target vascular endothelial growth factor (VEGF), and cetuximab (Erbitux) that inhibits the epidermal growth factor receptor (EGFR) (Cunningham et al., 2004; Hurwitz et al., 2004). Although these types of combination therapies have increased disease-free survival and improved overall survival in patients with CRC, most patients with metastatic disease are not cured. Thus, other approaches for combination therapies are desired. Our current data further demonstrate that 4-AAQB either alone or together with 5-FU could be effective in eliminating CRC spheroids (Fig. 6). 4-AAQB was identified as the most promising supplement for increasing the bioactivity of *A. cinnamomea* during cultivation (Chiang et al., 2013; Hsieh et al., 2011). However, the effects of 4-AAQB on cancer-initiating cells have not been reported. In this study, we found that equal concentrations of 4-AAQB and AQ did not exhibit the same degree of inhibition of cell viability and tumor sphere-forming capacity in these cells; the inhibitory effects of 4-AAQB were more potent. Finally, flow cytometric isolation of ALDH1-expressing CRC cells implanted into NOD-SCID mice and treated with 4-AAQB, generated tumor xenografts of less quantity and smaller sizes, in contrast to those formed in untreated mice (Fig. 7). Our data showed that 4-AAQB was able to reduce tumor volume and growth in treated mice while tumor growth and volume increased in control mice.

Taken together, we identified and provided evidence that 4-acetylantroquoninol B (4-AAQB) is a potent anti-proliferative and anti-CSC therapeutic agent in CRC. We demonstrated that 4-AAQB effectively reverses or attenuates resistance of cancer cells to anticancer therapy, thus, enhancing chemosensitivity, both *in vitro* and *in vivo*. We also show that the STAT3 pathway may play an important role in the molecular mechanism of 4-AAQB-mediated suppression of colon cancer-initiating cells and thus is a potential therapeutic target in the treatment of colorectal cancer. Concisely, 4-AAQB, a novel compound, projects as a potent therapeutic agent for monotherapy or as a component of standard combination chemotherapy.

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**Conflict of interest statement**

The authors declare that there are no potential conflicts of interest.

**Transparency document**

The Transparency document associated with this article can be found online.
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References