Small molecule ONC201/TIC10 targets chemotherapy-resistant colorectal cancer stem-like cells in an Akt/Foxo3a/TRAIL-dependent manner

Varun V. Prabhu\textsuperscript{1,2}, Joshua E. Allen\textsuperscript{3}, David T. Dicker\textsuperscript{1,2} and Wafik S. El-Deiry\textsuperscript{1,2,*}

\textsuperscript{1} Penn State Hershey Cancer Institute, Department of Medicine (Hematology/Oncology), Penn State College of Medicine, Hershey, PA, USA

\textsuperscript{2} Laboratory of Translational Oncology and Experimental Cancer Therapeutics, Department of Medical Oncology and Molecular Therapeutics Program, Fox Chase Cancer Center, Philadelphia, PA, USA

\textsuperscript{3} Oncoceutics, Inc., 1214 Research Boulevard, Hummelstown, PA 17036

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**Corresponding Author**: Laboratory of Translational Oncology and Experimental Cancer Therapeutics, Department of Medical Oncology and Molecular Therapeutics Program, Fox Chase Cancer Center, 333 Cottman Avenue, Room P2035, Philadelphia, PA 19111. Phone: 215-214-4233; Fax: 215-214-1590; Email: wafik.eldeiry@fccc.edu

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Abstract

Self-renewing colorectal cancer stem/progenitor cells (CSCs) contribute to tumor maintenance
and resistance to therapy. Therapeutic targeting of CSCs could improve treatment response and
prolong patient survival. ONC201/TIC10 is a first-in-class anti-tumor agent that induces TRAIL
pathway mediated cell death in cancer cells without observed toxicity. We have previously
described that ONC201/TIC10 exposure leads to transcriptional induction of the TRAIL gene via
transcription factor Foxo3a, which is activated by dual inactivation of Akt and ERK. The Akt
and ERK pathways serve as important targets in CSCs. Foxo3a is a key mediator of Akt and
ERK-mediated CSC regulation. We hypothesized that the potent anti-tumor effect of
ONC201/TIC10 in colorectal cancer involves targeting CSCs and bulk tumor cells.
ONC201/TIC10 depletes CD133(+), CD44(+) and Aldefluor(+) cells \textit{in vitro} and \textit{in vivo}. TIC10
significantly inhibits colonosphere formation of unsorted and sorted 5-Fluorouracil-resistant
CSCs. ONC201/TIC10 significantly reduces CSC-initiated xenograft tumor growth in mice and
prevents the passage of these tumors. ONC201/TIC10 treatment also decreased xenograft tumor
initiation and was superior to 5-Fluorouracil treatment. Thus, ONC201/TIC10 inhibits CSC self-
renewal \textit{in vitro} and \textit{in vivo}. ONC201/TIC10 inhibits Akt and ERK, consequently activating
Foxo3a and significantly induces cell surface TRAIL and DR5 expression in both CSCs and non-
CSCs. ONC201/TIC10-mediated anti-CSC effect is significantly blocked by the TRAIL
sequestering antibody RIK-2. Overexpression of Akt, DR5 knockdown and Foxo3a knockdown
rescues ONC201/TIC10-mediated depletion of CD44(+) cells and colonosphere inhibition. In conclusion, ONC201/TIC10 is a promising agent for colorectal cancer therapy that targets both non-CSCs and CSCs in an Akt-Foxo3a-TRAIL-dependent manner.

**Introduction**

Colorectal cancer is the third leading cause of cancer-related deaths in the United States [1]. Despite the approval of new agents, overall survival has not dramatically improved for patients with advanced unresectable metastatic colorectal cancer [2]. There is a major unmet need for new tumor-specific therapeutic regimens to overcome the resistance and toxicity associated with current therapies.

Similar to normal proliferative tissues, tumors are composed of cells at various stages of differentiation most of which are incapable of self-renewal [3]. Emerging evidence suggests that colorectal tumors contain a small population of stem cell-like cells termed cancer stem cells (CSCs) [4-8]. CSCs are capable of self-renewal and are essential for long-term sustenance of the tumor. CSCs not only self-renew, but are also resistant to chemotherapy. The cancer stem cell hypothesis suggests that targeting of CSCs along with bulk tumor cells could lead to more effective treatment regimens [3, 9, 10].

The endogenous TNF-related apoptosis-inducing ligand (TRAIL; Apo2L) protein selectively induces cell death in cancer cells while sparing normal cells. Recombinant TRAIL and agonistic anti-TRAIL receptor antibodies have been or are in clinical trials to combat human cancer [11]. This approach is particularly attractive for targeting CSCs as it potentially avoids toxic effects in normal stem/progenitor cells in the colonic crypt.
ONC201/TIC10 is a novel TRAIL pathway inducing small molecule with potent antitumor efficacy in vitro and in vivo. ONC201/TIC10 is entering phase I/II clinical trials for patients with advanced cancer. We have previously shown that ONC201/TIC10 induces TRAIL-mediated apoptosis in several tumor types, including breast, colon and glioblastoma multiforme tumors, without toxicity. ONC201/TIC10 inactivates both Akt and ERK signaling to induce Foxo3a nuclear translocation and TRAIL-gene transcription, independent of p53 status. ONC201/TIC10 improves the half-life, tissue distribution, route of administration and spectrum of activity and overcomes the therapeutic limitations of recombinant TRAIL and TRAIL-agonist antibodies. ONC201/TIC10 activates the TRAIL pathway in a manner that goes beyond upregulation of the TRAIL gene since it also leads to TRAIL receptor DR5 induction [12].

In this study, we hypothesized that the potent anti-tumor effect of ONC201/TIC10 in colon cancer involves targeting the CSC population along with bulk tumor cells. We demonstrate that ONC201/TIC10 depletes colorectal CSC markers and colonosphere formation in vitro. ONC201/TIC10 inhibits self-renewal of 5-Fluorouracil (5-FU)-resistant CSCs in vitro and in vivo. ONC201/TIC10 targets CSCs in a TRAIL-dependent manner. ONC201/TIC10 mediated anti-CSC effect and TRAIL induction in CSCs and bulk tumor cells requires upstream Akt and ERK inhibition, followed by Foxo3a activation. These findings provide further insight into the potent anti-tumor efficacy of ONC201/TIC10 and further strengthen the preclinical evidence for the translation of this novel small molecule into the clinic. The identification of CSCs as a target for ONC201/TIC10 provides an innovative biomarker that could be monitored in the clinic through analysis of tumor biopsies or circulating tumor cells.

Materials and Methods
Cell culture and Reagents

SW480, DLD1 and HCT116 cells were obtained from American Type Culture Collection and maintained in Dulbecco’s modified Eagle medium or McCoy’s 5A (Invitrogen) containing 10% fetal bovine serum and penicillin/streptomycin at 37°C in 5% CO₂. HCT116 cells overexpressing myristoylated Akt were obtained from Dr. Nathan G. Dolloff. Cells were regularly tested and authenticated every month by bioluminescence, growth and morphological observation. ONC201/TIC10 (NSC350625) was obtained from the NCI Developmental Therapeutic Program and from Oncoceutics, Inc. The anti-TRAIL RIK-2 antibody (Santa Cruz Biotechnology) was used at 1 mg/ml. Chemotherapeutic drug 5-FU was obtained from Sigma.

Flow cytometry for cell surface markers and sub-G1 analysis

Cells were trypsinized or harvested with cell dissociation buffer (Invitrogen), washed with PBS and fixed with 4% paraformaldehyde. Next cells were washed and incubated with CD133 (Miltenyi Biotec, 1:25), CD44 (BD Bioscience, 1:20), TRAIL (Abcam, 1:50 or 1:100), DR5 (Imgenex, 1:100) or normal IgG control (Invitrogen) overnight or for 2 hours (h) at 4°C. Next, cells were washed and incubated with secondary antibody (Invitrogen, 1:200). Finally, cells were washed, resuspended in PBS and analyzed by flow cytometry. Flow cytometry was performed using an Elite ESP flow cytometer (Beckman-Coulter). For Sub-G1 analysis, cells were treated, trypsinized, ethanol-fixed, stained with propidium iodide (Sigma) and analyzed by flow cytometry as previously described [12].

Aldefluor assay and Fluorescence activated cell sorting (FACS)
The Aldefluor assay was performed as described in the manufacturer’s protocol (ALDEFLUOR Kit, STEMCELL Technologies). Cells were trypsinized or harvested with cell dissociation buffer (Invitrogen), washed with PBS and counted. Cells were resuspended (1 million cells per ml) in Aldefluor assay buffer with activated Aldefluor reagent (2.5-5 µl per ml). Immediately, half of the resuspended cells were added to a tube with Aldefluor DEAB reagent, an inhibitor of ALDH activity (5 µl for 0.5 ml), to serve as a negative control. All samples were incubated for 30-45 minutes at room temperature or 37ºC. Finally, cells were washed and resuspended in Aldefluor buffer and Aldefluor bright cells (Aldefluor positive (+) cells, Aldefluor(+) ) and Aldefluor low cells (Aldefluor negative (-) cells, Aldefluor(-)) were detected by flow cytometry.

Preparative FACS sorting was performed at the Penn State Hershey flow cytometry core with the Becton-Dickinson FACSAria SORP Cell Sorter. Aldefluor(+) and Aldefluor(-) cells were gated according to the DEAB control. Sorted cells (99% purity) were collected in media on ice. Aldefluor(+) cells were maintained as colonosphere cultures, while Aldefluor(-) cells were maintained as adherent cultures.

**Colonosphere culture**

Colonospheres were cultured using the MammoCult™ Human Medium Kit (STEMCELL Technologies). Mammocult medium was prepared for each experiment by adding the supplement provided in the kit, Heparin sodium (Sigma, 4 µg/ml) and Hydrocortisone (BD, 0.5 µg/ml). For non-adherent growth conditions, colonospheres were cultured in Ultra Low attachment plates (Corning). Cells were suspended in Mammocult medium and 1000-20,000 cells were seeded per well (depending on 6-well/24-well plate and cell line). Cells were immediately treated with
DMSO or ONC201/TIC10. Colonospheres (> 60 µm) were counted and imaged after 3-7 days. Medium was replaced every 3-7 days for sorted cells in culture.

**In vivo studies**

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Penn State Hershey Medical Center. For subcutaneous xenografts, 4- to 6-week-old female athymic nu/nu or hairless SCID mice (Charles River Laboratories) were used. Sorted DLD1 Aldefluor(+) and Aldefluor(-) cells were subcutaneously injected into the right and left flank of the mice as a 200 ml suspension of 1:1 Matrigel (BD) and PBS. All subcutaneous tumors were allowed to reach a detectable volume (~125 mm³) before initiating ONC201/TIC10 treatment. Upon tumor formation, mice were administered either vehicle or ONC201/TIC10 50 mg/kg (i.p.). Doses were administered post-tumor implantation on day 7, 14 and 22. Tumor growth was monitored until the endpoint. Tumor growth was determined by measuring the length and width of the tumor with a caliper and tumor volume was calculated as \(((\text{length}+\text{width})/2)^3\).

For in vivo passage, tumors were harvested immediately following sacrifice at the endpoint and subjected to digestion using Collagenase type 3 (Worthington, 155 units/ml) in sterile serum- and antibiotic-free RPMI (Mediatech, Inc. Herndon, VA) for 2 h with intermittent vortexing. Digested tumor cells from each group were filtered through a 100 µm filter. Cells were re-injected into mice as described above to determine tumor formation. Tumor initiation and growth was monitored post tumor implantation.

Tumors were harvested from euthanized mice and homogenized in lysis buffer for western blot analysis or fixed in 4% paraformaldehyde in PBS for immunohistochemistry. Paraffin-embedding, sectioning and hematoxylin and eosin staining was performed by the Histology Core
Facility at Penn State Hershey Medical Center. Immunohistochemistry was performed as previously described [12]. The following antibodies were used: TRAIL (Abcam), CD44 (Cell Signaling) and CD133 (Santa Cruz Biotechnology).

**Western blot**

Western blotting was performed as described previously [12]. Sorted Aldefluor(+) cells were treated with DMSO or ONC201/TIC10 for 72 h. After treatment, protein lysates were collected and a protein assay (Biorad) was performed. Protein lysates were normalized for equal total protein, LDS sample buffer and reducing agent (Invitrogen) were added and the samples were used for SDS-PAGE. After transfer, primary and secondary antibody incubations were performed, and the signal was detected by using a chemiluminescent detection kit, followed by autoradiography. The following antibodies were used: Akt (Cell Signaling), phospho (p)-Akt (Cell Signaling), ERK (Cell Signaling), pERK (Cell Signaling), Foxo3a (Abcam), pS253 Foxo3a (Cell Signaling), pS294 Foxo3a (Cell Signaling), c-FLIP (Cell Signaling), ALDH (BD Biosciences), cleaved (clvd) caspase-8 (C8) (Cell Signaling), clvd PARP (Cell Signaling), Actin (Sigma) and Ran (BD Biosciences).

**siRNA transfection**

siRNA (control, DR5 (Santa Cruz Biotechnology) or Foxo3a (Dharmacon)) transfection of cells was performed with Opti-MEM and Lipofectamine RNAiMAX (Invitrogen) using media without antibiotics. After overnight siRNA incubation the cells were treated with complete medium containing ONC201/TIC10.

**Statistical analysis**
Results are presented as the mean ± standard deviation (or standard error of mean) of data from three or more independent experiments. For pairwise analysis, we analyzed the data using the Student’s two-tailed t-test in Excel (Microsoft). Statistically significant changes with p-values are presented in the figures.

Results

**ONC201/TIC10 depletes colorectal CSC markers in vitro**

To determine the effects of ONC201/TIC10 on the CSC population, we evaluated CSC marker expression in response to ONC201/TIC10 treatment. Three different markers of colorectal CSCs, Aldefluor [6], CD44 [13] and CD133 [5] were tested in three cell lines. ONC201/TIC10 depleted Aldefluor(+) cells in HCT116, DLD1 and SW480 cell lines (Fig 1B, 1C and S3C). In HCT116 cells, 5-FU, a first-line chemotherapeutic drug for colorectal cancer [14], did not significantly reduce the Aldefluor(+) population, while ONC201/TIC10 significantly depleted Aldefluor(+) cells (Fig 1B). ONC201/TIC10 also depleted CD44(+) cells in SW480, DLD1 and HCT116 cell lines (Fig 1A, 4D and 5C). In our experiments, the percentage of CD44(+) cells is relatively high in SW480 and HCT116 cell lines compared to the other markers used for CSCs. Our data is consistent with other studies that have also observed high levels of CD44 mRNA [15] and protein [16] in SW480 and HCT116 cells. Next, we tested the effects of ONC201/TIC10 on CSC marker expression in a dose- and time-dependent manner. In DLD1 cells, ONC201/TIC10 depleted CD133 expression in a dose-dependent manner at doses ranging from 2.5-10 µM (Fig 1D). At the 5 µM dose, ONC201/TIC10 depleted CD133 expression in a time-dependent manner, with maximal effects observed 72 h following treatment (Fig 1D). Representative flow
cytometry images of CD44 (Fig S1B) and CD133 (Fig S1A) staining are shown. Thus, ONC201/TIC10 depletes markers of colorectal CSCs in a dose- and time-dependent manner.

**TIC10 prevents colonosphere formation in vitro**

Colonosphere cultures have been shown to enrich for CSCs [17]. To test the anti-CSC effects of ONC201/TIC10 in a functional *in vitro* model of CSC self-renewal, we tested the ability of ONC201/TIC10 to prevent colonosphere formation. ONC201/TIC10 significantly reduced colonosphere formation of SW480 and DLD1 cells (Fig 2A). In DLD1 cells, the colonospheres formed upon ONC201/TIC10 treatment were smaller compared to the control (Fig 2B).

Next, we tested the effects of ONC201/TIC10 on sorted Aldefluor(+) cells. Aldefluor(+) cells formed significantly more colonospheres compared to Aldefluor(-) cells (Fig 2C). Post-sort validation was performed to confirm the enrichment of Aldefluor(+) cells versus Aldefluor(-) cells (Fig S2A). 5-FU did not prevent colonosphere formation of Aldefluor(+) cells (Fig 2D). ONC201/TIC10 prevented colonosphere formation of Aldefluor(+) cells in a dose-dependent manner (Fig 2E and 2F). Thus, ONC201/TIC10 prevents self-renewal of chemotherapy-resistant CSCs.

**ONC201/TIC10 prevents CSC-mediated xenograft tumor growth and self-renewal in vivo**

To confirm that Aldefluor(+) cells represent the CSC population *in vivo*, we passaged sorted tumor cells in athymic nude mice. Post-sort validation was performed to confirm the enrichment of DLD1 Aldefluor(+) and Aldefluor(-) cells (Fig S2A). Sorted Aldefluor(+) and Aldefluor(-) cells were injected into the opposite flanks of the same mouse to compare tumor formation. Upon initial injection post-sort, both Aldefluor(+) and Aldefluor(-) cells initiated tumors. At the
end point, single cells were isolated from the initial tumors and equal numbers of viable cells were re-injected into mice to assess tumor formation. As expected, Aldefluor(+) cells formed tumors more often than Aldefluor(-) cells (Fig S2C). Aldefluor(-) cells formed one tumor in 3 injections while Aldefluor(+) cells formed tumors in every injection. The Aldefluor(+) CSC-initiated tumors retained the Aldefluor(+) phenotype three weeks post-injection (Fig S2D). These data suggest that Aldefluor(+) cells with enhanced self-renewal represent the colorectal CSC population.

Next, we determined whether ONC201/TIC10 could prevent Aldefluor(+) CSC-mediated tumor growth and self-renewal in vivo. Sorted Aldefluor(+) cells were injected subcutaneously into athymic nude mice and the mice were administered 3 doses of vehicle or ONC201/TIC10 once a week. ONC201/TIC10 significantly reduced the growth of the CSC-initiated tumors (Fig 3A, 3B and S2B). We did not observe any significant changes in the body weight of mice in response to ONC201/TIC10 treatment (Fig 3C). CSC markers ALDH, CD44 and CD133 were also downregulated upon ONC201/TIC10 treatment (Fig 3D and 5A). At the end point, single cells were isolated from initial tumors and equal numbers of viable cells were re-injected into mice. The ONC201/TIC10 treated cells formed 1 tumor in 5 injections while vehicle treated cells formed 4 tumors in 5 injections (Fig S2C). Thus, ONC201/TIC10 treatment prevented the self-renewal of Aldefluor(+) CSCs in vivo without apparent toxicity.

To conclusively determine the effect of ONC201/TIC10 on CSC self-renewal in vivo we performed a limiting dilution tumor initiation assay with vehicle, ONC201/TIC10 and 5-FU treated Aldefluor(+) CSCs. ONC201/TIC10 reduced tumor initiation at three different dilutions of injected Aldefluor(+) CSCs while 5-Fluorouracil had a modest effect on tumor initiation (Fig 3E). ONC201/TIC10 treatment significantly increased the number of days required for tumor
initiation and the number of days to end point tumor volume (Fig S2E). Thus, ONC201/TIC10 significantly reduced CSC-mediated tumor initiation and tumor growth.

ONC201/TIC10-mediated anti-CSC effect involves induction of cell surface TRAIL and DR5

We examined ONC201/TIC10-mediated TRAIL induction specifically in CSCs. Surface TRAIL induction was determined in CD133(+) and CD44(+) cells. Surface TRAIL(+) cells within the CD133(+) population were significantly elevated upon ONC201/TIC10 treatment in HCT116 cells (Fig 4A). Mean surface TRAIL fluorescence in the CD133(+) population was also elevated significantly (Fig 4A). ONC201/TIC10 treatment increased TRAIL(+) cells within the CD44(+) population in a dose-dependent manner in SW480 cells (Fig 4B). Representative flow cytometry images of CD44 and TRAIL staining (Fig S1B) are shown. We simultaneously observed ONC201/TIC10-mediated TRAIL induction in the total population (Fig S3A and S3B). To further confirm our hypothesis that ONC201/TIC10 induces TRAIL in both CSCs and non-CSCs we examined TRAIL induction in the two populations. ONC201/TIC10 significantly induced surface TRAIL in both CD133(+) and CD133(-) cells (Fig 4E). TRAIL was also upregulated in Aldefluor(+) CSC-initiated xenograft tumors upon ONC201/TIC10 treatment (Fig 3D).

To confirm that ONC201/TIC10-mediated anti-CSC effects are TRAIL-dependent we used the TRAIL-blocking antibody RIK-2. We previously demonstrated that RIK-2 prevents ONC201/TIC10- (Fig S3D) and recombinant TRAIL-mediated tumor cell death [12]. ONC201/TIC10 mediated inhibition of Aldefluor(+) and CD44(+) cells was impaired when cells were treated with RIK-2 (Fig 4C, 4D and S3C). The fold depletion of Aldefluor(+) cells by ONC201/TIC10 was significantly reduced in SW480 and DLD1 cells treated with RIK-2 as
compared to control. Thus, TRAIL-dependent anti-CSC effects of ONC201/TIC10 were blocked by TRAIL-sequestering antibody RIK-2.

We have previously reported that ONC201/TIC10 upregulates DR5 in tumor cells and disruption of the DR5 death domain decreases ONC201/TIC10-mediated cell death [12]. ONC201/TIC10 significantly increased cell surface DR5 in CD44(+) and CD44(-) cells. (Fig S5). DR5 knockdown rescued ONC201/TIC10-mediated colonosphere inhibition and depletion of CD44(+) cells (Fig S6). The data provides evidence for a DR5-TRAIL-dependent mechanism of ONC201/TIC10-mediated anti-CSC effects.

**ONC201/TIC10-mediated induction of surface TRAIL in CSCs involves inhibition of Akt and ERK, followed by Foxo3a activation**

To determine the signaling pathway involved in ONC201/TIC10-mediated TRAIL induction in CSCs, we determined the effects of ONC201/TIC10 treatment on previously described regulators of the TRAIL pathway [12]. We determined levels of Akt, ERK and Foxo3a in response to ONC201/TIC10 treatment in sorted Aldefluor(+) cells. ONC201/TIC10 treatment downregulated Akt and pERK in a dose-dependent manner (Fig S4A). These changes were associated with dephosphorylation of Foxo3a at the direct phosphorylation sites of Akt (S253) [18] and ERK (S294) [19] (Fig S4A). We have previously shown that Akt and ERK inhibition results in Foxo3a activation, translocation to the nucleus and TRAIL-induction [12]. Downregulation of pAkt, pERK and a corresponding dephosphorylation of Foxo3a was also observed in xenograft tumors upon ONC201/TIC10 treatment (Fig 5A). ONC201/TIC10 treatment also upregulated the apoptosis markers clvd C8 and clvd PARP in the xenograft tumors (Fig 5A). Next, we compared ONC201/TIC10-mediated signaling in CSCs and non-CSCs. ONC201/TIC10 reduced levels of
Akt, pAkt, pERK, pS294 Foxo3a and pS253 Foxo3a in both CSCs and non-CSCs (Fig 5B). Thus, ONC201/TIC10 targets CSCs and non-CSCs via Akt/ERK inhibition and Foxo3a activation. Upregulation of c-FLIP is one of the mechanisms of resistance to TRAIL-based agents [20, 21]. We observed that ONC201/TIC10 decreased levels of c-FLIP in both CSCs and non-CSCs (Fig 5B).

To confirm the importance of Akt inhibition in ONC201/TIC10-mediated anti-CSC effects, we used HCT116 cells that overexpress myristoylated-Akt (myr-Akt) (Fig S4B). In accordance with our previous findings, the fold induction of TRAIL fluorescence by ONC201/TIC10 in the total population was significantly reduced in myr-Akt cells as compared to wild-type (WT) cells (Fig S4D). ONC201/TIC10-mediated inhibition of CD44(+) cells was overcome by overexpression of myr Akt (Fig 5C). Fold depletion of CD44(+) and Aldefluor(+) cells by ONC201/TIC10 was significantly reduced in myr-Akt cells as compared to WT cells (Fig 5C and 5D). We also determined effects of myr-Akt overexpression on ONC201/TIC10-mediated TRAIL-induction in CSCs. The fold induction of CD44 and surface TRAIL dual positive cells by ONC201/TIC10 was significantly reduced in myr-Akt cells as compared WT (Fig 5E and S4C). To determine the precise role of Foxo3a in ONC201/TIC10-mediated anti-CSC effects we performed siRNA-mediated knockdown of Foxo3a. Foxo3a knockdown rescued ONC201/TIC10-mediated colonosphere inhibition and depletion of CD44(+) cells (Fig S6). Thus, Akt/ERK inhibition and Foxo3a activation are involved in TRAIL-dependent ONC201/TIC10-mediated anti-CSC effects.

Discussion

Cancer stem cells (CSCs) have an enhanced capability for self-renewal and are inherently resistant to cell death induction by radiation and chemotherapeutic agents. Several mechanisms
are involved in CSC resistance such as impaired apoptosis, enhanced DNA damage repair, quiescence and multi-drug resistance transporters responsible for drug efflux [20, 21]. In our study we validated our experimental CSC model \textit{in vitro} and \textit{in vivo} using FACS sorted CSCs and non-CSCs. Sorted CSCs had enhanced capacity for self-renewal in colonosphere assays and were resistant to 5-FU as compared to sorted non-CSCs. Sorted CSCs showed enhanced self-renewal capacity \textit{in vivo}, as they formed more tumors after passaging as compared to non-CSCs. In addition, chemotherapy had a limited effect on CSC-mediated tumor initiation \textit{in vivo}.

Resistance to TRAIL-based agents in CSCs has been described in recent studies [22-24]. The mechanisms involved in CSC resistance to TRAIL-based agents include suppression of Caspase-8, upregulation of c-FLIP and other anti-apoptotic proteins such as XIAP, Mcl-1, Bcl-2 [20, 21]. Several studies have also described alternative approaches with TRAIL-based therapies to target leukemia-initiating cells [25] and CSCs in pancreatic [26], colorectal [27], breast [28-31], glioma [32, 33] and glioblastoma [34, 35] tumors. These approaches include the use of mesenchymal stem cells expressing TRAIL [36], chemotherapy or inhibition of c-FLIP in combination with TRAIL-based therapies and nanoparticle based delivery of TRAIL.

Our study describes a novel TRAIL-inducing small molecule ONC201/TIC10, which targets both non-CSCs [12] and CSCs in colorectal cancer. We observed that ONC201/TIC10 depletes multiple markers of CSCs in colorectal cancer cell lines and xenograft tumors in mice. Thus, the effects of ONC201/TIC10 on CSCs are not limited to specific clones of CSCs. We tested the effects of ONC201/TIC10 on CSC self-renewal \textit{in vitro} and \textit{in vivo}. Functional effects of ONC201/TIC10 on CSC self-renewal were determined using colonosphere assays. ONC201/TIC10 prevented sphere formation and growth of colorectal cancer cells. Weekly dosing with ONC201/TIC10 prevented the growth and passage of xenograft tumors initiated with
sorted CSCs without affecting the body weight of mice. ONC201/TIC10-treatment also decreased xenograft tumor initiation and was superior to 5-Fluorouracil. ONC201/TIC10 induced surface TRAIL in colorectal CSCs and non-CSCs. ONC201/TIC10-mediated TRAIL induction was further confirmed in CSC-initiated xenograft tumors. The anti-CSC effect was dependent on TRAIL induction, as a TRAIL-sequestering antibody blocked the anti-CSC effect. ONC201/TIC10 also downregulates c-FLIP in both CSCs and non-CSCs. We have previously reported that ONC201/TIC10 upregulates DR5 in tumor cells and disruption of the DR5 death domain decreases ONC201/TIC10-mediated cell death [12]. ONC201/TIC10 significantly increased cell surface DR5 in CSCs and non-CSCs. DR5 knockdown rescued ONC201/TIC10-mediated anti-CSC effects indicating a DR5-TRAIL-dependent mechanism. Thus, we provide conclusive evidence of the TRAIL-mediated anti-CSC effect of small molecule ONC201/TIC10 using in vitro and in vivo models.

We have previously shown that combined inhibition of Akt and ERK is important for TIC10-mediated TRAIL induction. Inhibition of both the Akt and ERK pathways results in improved TRAIL induction compared to the inhibition of a single pathway [12]. Akt and ERK activation results in the propagation of CSCs in colon [37, 38], prostate [39], brain [40-42], breast [43], lung tumors [44] and leukemia [45]. Clearly, the Akt and ERK pathways serve as important targets in CSCs. In the current study, we demonstrate that ONC201/TIC10 inhibits the Akt and ERK pathways in sorted colorectal CSCs and non-CSCs. Overexpression of myristoylated Akt prevented ONC201/TIC10-mediated depletion of CSCs and inhibited TRAIL-induction by ONC201/TIC10 in CSCs. Our data suggests that Akt and ERK inhibition is important for TRAIL-dependent anti-CSC effects of ONC201/TIC10. Our findings are in agreement with a report by Sunayama et al, which demonstrated that combined inhibition of MEK/ERK and
PI3K/Akt/mTOR efficiently targets glioblastoma stem cells as compared to either strategy alone [46]. The same group also identified Foxo3a as a key mediator of the ERK- and Akt-mediated regulation of glioblastoma stem cells [47]. Our results show that Foxo3a is activated in colorectal CSCs and non-CSCs upon inhibition of the Akt and ERK pathways by ONC201/TIC10. ONC201/TIC10 activated Foxo3a by preventing Foxo3a phosphorylation at Akt and ERK specific sites. In addition, Foxo3a knockdown rescued ONC201/TIC10-mediated anti-CSC effects. Thus, we provide conclusive evidence for the importance of Foxo3a in ONC201/TIC10-mediated anti-CSC effects. The direct binding target of ONC201/TIC10 and the mechanism upstream of Akt and ERK inhibition remains an area of active study.

Our study specifically investigated the TRAIL-mediated effects of ONC201/TIC10 on CSCs. However, other effects of ONC201/TIC10-mediated Akt and ERK inhibition on CSCs independent of TRAIL-induction remain to be elucidated. We did not observe toxicity in response to the dose of ONC201/TIC10 that produced an anti-CSC effect in vivo. However, in future studies, determining effects of ONC201/TIC10 on normal stem cells in the colonic crypt is worth investigating. In addition, the identification of synergistic combinations of ONC201/TIC10 with current chemotherapeutic and targeted agents to target colorectal CSCs is needed for clinical translation of these studies.

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We apologize to those colleagues whose work could not be cited due to space constraints.

Author contributions

V.V.P., J.E.A. and W.S.E-D. conceived the study and participated in the design, analysis and interpretation of experiments. V.V.P conducted the experiments and wrote the manuscript. D.T.D. conducted flow cytometry analysis. W.S.E-D. supervised the experiments and contributed as senior author including editing of the manuscript and responsibility for oversight of conduct of the research.

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Figure Legends

Figure 1. **ONC201/TIC10 depletes colorectal CSC markers in vitro.** (A) ONC201/TIC10 depletes CD44(+) cells. SW480 cells were treated for 72 h with DMSO or 5 µM ONC201/TIC10. CD44(+) cells were detected by flow cytometry. * indicates p<0.005. (B) ONC201/TIC10 depletes 5-Fluorouracil (5-FU) resistant Aldefluor(+) cells. HCT116 cells were treated for 72 h with DMSO or 5 µM ONC201/TIC10 or 40/80 µM 5-FU. Aldefluor(+) cells were detected by flow cytometry. * indicates p<0.001. (C) Flow cytometry histograms for Aldefluor assay of DLD1 cells treated with DMSO or 2.5/5 µM ONC201/TIC10 for 72 h. Aldefluor(+) cells were determined according to the DEAB negative control. Data is represented as side scatter (SS) or count versus Aldefluor (ALDF1). (D) ONC201/TIC10 depletes CD133(+) cells in a dose- and time-dependent manner. DLD1 cells were treated with DMSO (72 h) or 2.5/5/10 µM ONC201/TIC10 (72 h) or 5 µM ONC201/TIC10 (24/48/72 h). CD133(+) cells were detected by flow cytometry. * indicates p < 0.05.
Figure 2. ONC201/TIC10 prevents colonosphere formation in vitro. (A) ONC201/TIC10 prevents colonosphere formation. DLD1 and SW480 cells (10,000 cells/well) were plated for colonosphere formation in the presence of DMSO or 10 µM ONC201/TIC10. Colonospheres (> 60 µm) were counted at 96 h. (B) Representative image (10X magnification) for DLD1 cells in (A). Each division on the scale is 10 µm. (C) Sorted Aldefluor(+) and Aldefluor(-) HCT116 cells (1000 cells/well) were plated for colonosphere formation. Colonospheres (> 60 µm) were counted after 6 days. (D) Sorted Aldefluor(+) HCT116 cells (1000 cells/well) were plated for colonosphere formation in the presence of DMSO or 40 µM 5-FU. Colonospheres (> 60 µm) were counted after 6 days (p value = 0.446). (E) Sorted Aldefluor(+) SW480 cells (10,000 cells/well) were plated for colonosphere formation in the presence of DMSO or 2.5/5/10 µM ONC201/TIC10. Colonospheres (> 60 µm) were counted after 72 h. * indicates p < 0.006. (F) Representative images (20X magnification) for (E). Each division on the scale is 10 µm.

Figure 3. ONC201/TIC10 prevents CSC-mediated xenograft tumor growth and self-renewal in vivo. (A) ONC201/TIC10 prevents CSC-mediated xenograft tumor growth. Sorted DLD1 Aldefluor(+) cells were subcutaneously injected into the right and left flank of athymic nude mice. Upon tumor formation, mice were administered either vehicle or ONC201/TIC10 50 mg/kg (i.p.). (N = 7 per group) Doses (indicated by triangle) were administered post-tumor implantation on day 7, 14 and 22. Tumor growth was monitored until the endpoint. Data is presented as mean ± standard error of mean. * indicates p < 0.05. (B) Representative image for (A). (C) Body weight of mice in (A) was monitored along with tumor growth until the end point. (D) Immunohistochemistry analysis of harvested tumors from (A) for CSC markers and TRAIL. (E) Sorted Aldefluor(+) DLD1 cells were plated for colonosphere culture in the presence of DMSO or 50 µM 5-FU or 5 µM ONC201/TIC10 for 72 h. Viable cells were counted and the
indicated number of cells were injected into the right and left flank of hairless SCID mice. Tumor initiation was monitored till Day 80 post tumor implantation.

**Figure 4. ONC201/TIC10-mediated anti-CSC effect involves induction of surface TRAIL.** (A) ONC201/TIC10 induces surface TRAIL in CSCs. HCT116 cells were treated with DMSO or 5/10 µM ONC201/TIC10 for 72 h and CD133 and TRAIL staining was detected by flow cytometry. * indicates p < 0.04. (B) SW480 cells were treated with DMSO or indicated doses of ONC201/TIC10 for 72 h. CD44 and TRAIL staining was detected by flow cytometry. * indicates p < 0.03. (C) ONC201/TIC10-mediated anti-CSC effect is TRAIL-dependent. DLD1 cells were treated with DMSO or 5 µM ONC201/TIC10 for 72 h in the presence of control IgG or RIK-2 antibody. Aldefluor(+) cells were detected by flow cytometry. Fold depletion is the ratio of % Aldefluor(+) cells in DMSO and ONC201/TIC10 treatment groups. * indicates p < 0.05. (D) DLD1 cells were treated with DMSO or 5 µM ONC201/TIC10 for 72 h in the presence of control IgG or RIK-2 antibody. CD44(+) cells were detected by flow cytometry. * indicates p < 0.04. (E) HCT116 cells were treated with DMSO or 5/10 µM ONC201/TIC10 for 72 h and CD133 and TRAIL staining was detected by flow cytometry. * indicates p < 0.04.

**Figure 5. ONC201/TIC10-mediated induction of surface TRAIL in CSCs involves inhibition of Akt and ERK followed by Foxo3a activation.** (A) ONC201/TIC10 inhibits Akt, ERK and activates Foxo3a in CSCs. Sorted DLD1 Aldefluor(+) cells were subcutaneously injected into the right and left flank of athymic nude mice. Upon tumor formation, mice were administered either vehicle or ONC201/TIC10 50 mg/kg (i.p.). Tumors were harvested after 72 h of treatment and western blot analysis was performed (B) Western blot analysis of sorted Aldefluor(+) (CSC) and unsorted (non CSC) SW480 cells treated with DMSO or 5 µM ONC201/TIC10 for 72 h (C) Parental (WT) or myristoylated- (myr-) Akt overexpressing...
HCT116 cells were treated with DMSO or 5 µM ONC201/TIC10 for 72 h. CD44 staining was detected by flow cytometry. Fold depletion is the ratio of % CD44(+) cells in DMSO and ONC201/TIC10 treatment groups. * indicates p < 0.02. (D) WT or myr-Akt overexpressing HCT116 cells were treated with DMSO or 5 µM ONC201/TIC10 for 60 h. Aldefluor(+) cells were detected by flow cytometry. Fold depletion is the ratio of % Aldefluor(+) cells in DMSO and ONC201/TIC10 treatment groups. * indicates p < 0.0005. (E) WT or myr-Akt overexpressing HCT116 cells were treated with DMSO or 5 µM ONC201/TIC10 for 72 h. CD44 and TRAIL staining was detected by flow cytometry. Fold induction is the ratio of % CD44(+)TRAIL(+) cells in ONC201/TIC10 and DMSO treatment groups. * indicates p < 0.05.
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(A) Tumor volume (mm$^3$) over days post tumor implantation for Control and ONC201/TIC10 groups.

(B) Images showing Control and ONC201/TIC10 after tumor implantation.

(C) Body weight (g) over days post tumor implantation for Control and ONC201/TIC10 groups.

(D) Immunohistochemistry images for TRAIL, CD44, and CD133 in Control and ONC201/TIC10 groups.

(E) Table showing tumor formation rates for different groups:

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>300,000</td>
</tr>
<tr>
<td>DMSO</td>
<td>8/8</td>
</tr>
<tr>
<td>ONC201/TIC10</td>
<td>3/8</td>
</tr>
<tr>
<td>5-FU</td>
<td>5/6</td>
</tr>
</tbody>
</table>
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(A)

ONC201/TIC10 - +

pAkt
Akt
pERK
ERK
pS294 Foxo3a
pS253 Foxo3a
clvd PARP
clvd C8
ALDH
Actin

(B)

non CSC
CSC

ONC201/TIC10 - + - +

Akt
pAkt
pERK
pS294 Foxo3a
pS253 Foxo3a
c-FLIP
Actin

(C)

Average % CD44(+) by DMSO and ONC201/TIC10

WT
myr Akt

(D)

Fold depletion of Aldefluor(+) cells by ONC201/TIC10

WT
myr Akt

(E)

Fold induction of CD44(+)/TRAIL(+) by ONC201/TIC10

WT
myr Akt
Small molecule ONC201/TIC10 targets chemotherapy-resistant colorectal cancer stem-like cells in an Akt/Foxo3a/TRAIL-dependent manner

Varun V. Prabhu, Joshua E. Allen, David T Dicker, et al.

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