Small-Molecule ONC201/TIC10 Targets Chemotherapy-Resistant Colorectal Cancer Stem–like Cells in an Akt/Foxo3a/TRAIL-Dependent Manner

Varun V. Prabhu1,2, Joshua E. Allen3, David T. Dicker1,2, and Wafik S. El-Deiry1,2

Abstract

Self-renewing colorectal cancer stem/progenitor cells (CSC) 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 antitumor 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 antitumor effect of ONC201/TIC10 in colorectal cancer involves targeting CSCs and bulk tumor cells. ONC201/TIC10 depletes CD133–, CD44+, and Aldefluor+ cells and 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 in vitro and 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. Cancer Res; 75(7); 1423–32. ©2015 AACR.

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-like cells termed cancer stem cells (CSC; ref. 4–8). CSCs are capable of self-renewal and are essential for long-term sustenance of the tumor. CSCs not only self-renew but also are resistant to chemotherapy. The CSC hypothesis suggests that targeting of CSCs along with bulk tumor cells could lead to more effective treatment regimens (3, 9, 10).

The endogenous 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 in 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

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.

1Penn State Hershey Cancer Institute, Department of Medicine (Hematology/Oncology), Penn State College of Medicine, Hershey, Pennsylvania. 2Laboratory of Translational Oncology and Experimental Cancer Therapeutics, Department of Medical Oncology and Molecular Therapeutics Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania. 3Oncoceutics, Inc., Hummelstown, Pennsylvania.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

The work was presented in part at the 104th Annual Meeting of the American Association for Cancer Research (April 2013).

Corresponding Author: Wafik S. El-Deiry, 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; E-mail: wafik.eldeiry@fccc.edu
doi: 10.1158/0008-5472.CAN-13-3451
©2015 American Association for Cancer Research.

www.aacrjournals.org
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, as it also leads to TRAIL receptor DR5 induction (12).

In this study, we hypothesized that the potent antitumor 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 require upstream Akt and ERK inhibition, followed by Foxo3a activation. These findings provide further insight into the potent antitumor 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 ATCC and maintained in DMEM or McCoy 5A (Invitrogen) containing 10% FBS 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 morphologic observation. ONC201/TIC10 (NSC350625) was obtained from the NCI Developmental Therapeutic Program and from Oncocentrics, 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 Biosciences, 1:20), TRAIL (Abcam, 1:50 or 1:100), DR5 (Imgenex, 1:100), or normal IgG control (Invitrogen) overnight or for 2 hours 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 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/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 to 45 minutes at room temperature or 37°C. Finally, cells were washed and resuspended in Aldefluor buffer, and Aldefluor bright cells [Aldefluor⁻ cells, Aldefluor⁺] and Aldefluor low cells [Aldefluor⁻ low 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, whereas 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) were added. For nonadherent growth conditions, colonospheres were cultured in ultra-low attachment plates (Corning). Cells were suspended in Mammocult medium and 1,000 to 20,000 cells were seeded per well (depending on 6-/24-well plate and cell line). Cells were immediately treated with DMSO or ONC201/TIC10. Colonospheres (>60 µm) were counted and imaged after 3 to 7 days. Medium was replaced every 3 to 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-µL 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 [intraperitoneally (i.p.)]. Doses were administered posttumor implantation on days 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 [(length + 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.) for 2 hours with intermittent vortexing. Digested tumor cells from each group were filtered through a 100-µm filter. Cells were reinjected into mice as described above to determine tumor formation. Tumor initiation and growth were monitored after 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 were 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 hours. 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), phosho (p)-Akt (Cell Signaling), ERK (Cell Signaling), pERK (Cell Signaling), FXX3a (Abcam), pS253 FXX3a (Cell Signaling), pS294 FXX3a (Cell Signaling), c-FLIP (Cell Signaling), ALDH (BD Biosciences), cleaved (cldv) caspase-8 (C8) (Cell Signaling), cldv PARP (Cell Signaling), actin (Sigma), and Ran (BD Biosciences).

siRNA transfection
siRNA [control, DR5 (Santa Cruz Biotechnology) or FXX3a (Dharmacon)] transfection of cells was performed with OptiMEM 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 ± SD (or SEM) of data from three or more independent experiments. For pairwise analysis, we analyzed the data using the Student 2-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 3 cell lines. ONC201/TIC10 depleted Aldefluor+ cells in HCT116, DLD1, and SW480 cell lines (Fig. 1B and C and Supplementary Fig. S3C). In HCT116 cells, 5-FU, a first-line chemotherapeutic drug for colorectal cancer (14), did not significantly reduce the Aldefluor+ population, whereas ONC201/TIC10 significantly depleted Aldefluor+ cells (Fig. 1B). ONC201/TIC10 also depleted CD44+ cells in SW480, DLD1, and HCT116 cell lines (Figs. 1A, 4D, and 5C). In our experiments, the percentage of CD44+ cells is relatively high in SW480 and HCT116 cell lines compared with the other markers used for CSCs. Our data are 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. On injection of the Aldefluor+ population, whereas ONC201/TIC10 significantly depleted Aldefluor+ cells (Fig. 1B). ONC201/TIC10 also depleted CD44+ cells in SW480, DLD1, and HCT116 cell lines (Figs. 1A, 4D, and 5C). In our experiments, the percentage of CD44+ cells is relatively high in SW480 and HCT116 cell lines compared with the other markers used for CSCs. Our data are 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 to 10 μmol/L (Fig. 1D). At the 5 μmol/L dose, ONC201/TIC10 depleted CD133 expression in a time-dependent manner, with maximal effects observed 72 hours following treatment (Fig. 1D). Representative flow cytometric images of CD44 (Supplementary Fig. S1B) and CD133 (Supplementary Fig. S1A) staining are shown. Thus, ONC201/TIC10 depletes markers of colorectal CSCs in a dose- and time-dependent manner.

TIC10 prevents colonospheres formation in vitro
Colonospheres 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 colonospheres formation. ONC201/TIC10 significantly reduced colonospheres formation of SW480 and DLD1 cells (Fig. 2A). In DLD1 cells, the colonospheres formed upon ONC201/TIC10 treatment were smaller compared with the control (Fig. 2B).

Next, we tested the effects of ONC201/TIC10 on sorted Aldefluor+ cells. Aldefluor+ cells formed significantly more colonospheres compared with Aldefluor− cells (Fig. 2C). Post-sort validation was performed to confirm the enrichment of Aldefluor+ cells versus Aldefluor− cells (Supplementary 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 reinfected into mice to assess tumor formation. As expected, Aldefluor+ cells formed tumors more often than Aldefluor− cells (Supplementary Fig. S2C). Aldefluor− cells formed one tumor in three injections, whereas Aldefluor+ cells formed tumors in every injection. The Aldefluor+ CSC-initiated tumors retained the Aldefluor+ phenotype 3 weeks postinjection (Supplementary 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. 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 (Supplementary 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 reinfected into mice to assess tumor formation. As expected, Aldefluor+ cells formed tumors more often than Aldefluor− cells (Supplementary Fig. S2C). Aldefluor− cells formed one tumor in three injections, whereas Aldefluor+ cells formed tumors in every injection. The Aldefluor+ CSC-initiated tumors retained the Aldefluor+ phenotype 3 weeks postinjection (Supplementary 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 and B and Supplementary Fig. 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 (Figs. 3D and 5A). At the endpoint, single cells were isolated from initial tumors and equal numbers of viable cells were reinfected into mice to assess tumor formation. As expected, Aldefluor+ cells formed tumors more often than Aldefluor− cells (Supplementary Fig. S2C). Aldefluor− cells formed one tumor in three injections, whereas Aldefluor+ cells formed tumors in every injection. The Aldefluor+ CSC-initiated tumors retained the Aldefluor+ phenotype 3 weeks postinjection (Supplementary 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 and B and Supplementary Fig. 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 (Figs. 3D and 5A). At the endpoint, single cells were isolated from initial tumors and equal numbers of viable cells were reinfected into mice to assess tumor formation. As expected, Aldefluor+ cells formed tumors more often than Aldefluor− cells (Supplementary Fig. S2C). Aldefluor− cells formed one tumor in three injections, whereas Aldefluor+ cells formed tumors in every injection. The Aldefluor+ CSC-initiated tumors retained the Aldefluor+ phenotype 3 weeks postinjection (Supplementary Fig. S2D). These data suggest that Aldefluor+ cells with enhanced self-renewal represent the colorectal CSC population.

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-fluorouracil–treated Aldefluor+ CSCs. ONC201/TIC10 reduced tumor initiation at three different dilutions of injected Aldefluor+ CSCs, whereas 5-FU 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 endpoint tumor volume (Supplementary 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+s and CD44+s cells. Surface TRAIL+s cells within the CD133+s population were significantly elevated upon ONC201/TIC10 treatment in HCT116 cells (Fig. 4A). Mean surface TRAIL fluorescence in the CD133+s population was also elevated significantly (Fig. 4A). ONC201/TIC10 treatment increased TRAIL+s cells within the CD44+s population in a dose-dependent manner in SW480 cells (Fig. 4B). Representative flow cytometric images of CD44 and TRAIL staining (Supplementary Fig. S1B) are shown. We simultaneously observed ONC201/TIC10-mediated TRAIL induction in the total population (Supplementary 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 2 populations. ONC201/TIC10 significantly induced surface TRAIL in both CD133+s and CD133− cells (Fig. 4E). TRAIL was also upregulated in Aldefluor+s 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- (Supplementary Fig. S3D) and recombinant TRAIL-mediated tumor cell death (12). ONC201/TIC10-mediated inhibition of Aldefluor− and Aldefluor+/CD44+ cells was impaired when cells were treated with RIK-2 (Fig. 4C and D and Supplementary Fig. S3C). The fold depletion of Aldefluor− cells by ONC201/TIC10 was significantly reduced in SW480 and DLD1 cells treated with RIK-2 as compared with 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. (Supplementary Fig. S5). DR5 knockdown rescued ONC201/TIC10-mediated colonosphere inhibition and depletion of CD44+ cells (Supplementary Fig. S6). The data provide 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 (Supplementary
These changes were associated with dephosphorylation of Foxo3a at the direct phosphorylation sites of Akt (S253; ref. 18) and ERK (S294; ref. 19; Supplementary 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 cldv C8 and cldv 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; Supplementary 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 (Supplementary Fig. 5D). 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 with 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 with WT (Fig. 5E and Supplementary Fig. 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 (Supplementary Fig. S6). Thus, Akt/ERK inhibition and Foxo3a activation are involved in TRAIL-dependent ONC201/TIC10-mediated anti-CSC effects.
Discussion

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 multidrug resistance transporters responsible for drug efflux (20–21). In our study, we validated our experimental CSC model in vitro and 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-fluorouracil as compared with sorted non-CSCs. In addition, chemotherapy had a limited effect on CSC-mediated tumor initiation 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 antiapoptotic 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 in vitro and 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 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 with 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 suggest 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 and colleagues, which demonstrated that combined inhibition of MEK/ERK and PI3K/Akt/mTOR efficiently targets glioblastoma stem cells as compared with 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.

Disclosure of Potential Conflicts of Interest

J.E. Allen is Director of Development with Oncoceutics and has ownership interest including patents) in Oncoceutics. W.S. El-Deiry has ownership interest (including patents) in and is a consultant/advisory board member of Oncoceutics. No potential conflicts of interest were disclosed by the other authors.

References

15. de la Roche M, Worm J, Bienz M. The function of BCL9 in Wnt/beta-catenin signaling and colorectal cancer cells. BMC Cancer 2008;8:199.

Authors’ Contributions

Conception and design: V.V. Prabhu, J.E. Allen, W.S. El-Deiry Development of methodology: V.V. Prabhu, D.T. Dicker Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V.V. Prabhu, D.T. Dicker Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V.V. Prabhu, D.T. Dicker Writing, review, and/or revision of the manuscript: V.V. Prabhu, J.E. Allen, D.T. Dicker, W.S. El-Deiry Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V.V. Prabhu Study supervision: W.S. El-Deiry

Acknowledgments

The authors thank Yifu Ding and Arunasalam Navaraj for assistance with colonosphere culture, Nathan G. Dolloff for providing myristoylated Akt-over-expressing cells, and the Penn State Hershey Flow Cytometry Core for help with flow sorting experiments. We apologize to those colleagues whose work could not be cited because of space constraints.

Grant Support

This work was supported by grants from the NIH (CA173453-02) and the American Cancer Society (W.S. El-Deiry) and Penn State Hershey Cancer Institute laboratory start-up funds (W.S. El-Deiry). This work was also supported by Oncoceutics and a Pennsylvania Department of Health grant awarded to Oncoceutics. W.S. El-Deiry is an American Cancer Society Research Professor. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 29, 2013, revised January 9, 2015; accepted January 30, 2015; published OnlineFirst February 20, 2015.


Small-Molecule ONC201/TIC10 Targets Chemotherapy-Resistant Colorectal Cancer Stem–like Cells in an Akt/Foxo3a/TRAIL–Dependent Manner


Cancer Res 2015;75:1423-1432. Published OnlineFirst February 20, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-13-3451

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2015/02/20/0008-5472.CAN-13-3451.DC1

Cited articles
This article cites 47 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/75/7/1423.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/75/7/1423.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.