Clinical Cancer Research

The Akt Inhibitor ISC-4 Activates Prostate Apoptosis Response Protein-4 and Reduces Colon Tumor Growth in a Nude Mouse Model

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Abstract

Purpose: Prostate apoptosis response protein-4 (Par-4) sensitizes cells to chemotherapy; however, Akt1 inactivates Par-4. Previously we showed that Par-4–overexpressing colon cancer cells responded more readily to 5-fluorouracil (5-FU) than their wild-type counterparts. In this study we investigated (i) the effects of the Akt inhibitor, phenylbutyl isoselenocyanate (ISC-4), on tumor growth in nude mice and (ii) bystander effect of Par-4–overexpressing cells on wild-type tumor growth.

Experimental Design: Mice ($n = 80$) were injected with wild-type HT29 human colon cancer cells in the right flank. Forty of the mice were also injected in the left flank with HT29 cells engineered to overexpress Par-4. The mice were treated with 5-FU, ISC-4, a combination, or vehicle.

Results: ISC-4 reduced tumor growth, with or without 5-FU. When Par-4–overexpressing tumors were present, wild-type tumors grew more slowly compared to when no Par-4–overexpressing tumors were present. The level of Par-4 protein as well as the Par-4 binding protein, GRP78, was increased in wild-type cells growing in the same mouse as Par-4–overexpressing tumors compared with wild-type tumors growing without Par-4–overexpressing tumors.

Conclusions: Par-4–overexpressing tumors exhibited a bystander effect on wild-type tumors growing distally in the same mouse. This suggests that gene therapy need not achieve total penetration to have a positive effect on tumor treatment. Inhibition of Akt with ISC-4 inhibited tumor growth and had a greater effect on cells overexpressing Par-4. The data indicate ISC-4 alone or in combination with Par-4 can greatly reduce tumor growth. *Clin Cancer Res; 17(13); 4474–83. ©2011 AACR.*

Introduction

Colon cancer is the second most common cause of cancer deaths in both men and women in the United States. With current therapeutic strategies, the 5-year survival rate of those with metastatic cancer is between 8% and 12% (1). To address this issue, a number of studies are focused on the search for new and more effective therapy targets. The Prostate apoptosis response protein-4 (Par-4) is a proapoptotic protein that was first identified in prostate cancer cells undergoing apoptosis. Par-4 can increase susceptibility of cancer cells to apoptotic agents such as doxorubicin, TNF-α, and TNF-related apoptosis-inducing ligand (TRAIL; ref. 2–4). The downregulation of Par-4 has been proposed to be a critical event in tumorigenesis (5). Par-4 is downregulated in a number of human cancers, such as endometrial (6), renal cell carcinoma (3), pancreatic (7), lung (8), and colon cancer (9). Furthermore, Par-4 has been shown to be inactivated by Akt1 in human cancers as well as in normal lung embryonic epithelial cells (6, 10). In a number of cell lines, its overexpression is sufficient to induce apoptosis (10–12). In others, increasing Par-4 levels does not cause cell death but increases the apoptotic effect of cell death stimuli (4, 10, 13, 14).

Par-4 activity leads to apoptosis via both intrinsic and extrinsic pathways (15–17). Intrinsic pathways include inhibiting transcriptional regulation by NF-κB (5, 11, 16). The extrinsic pathway involves the activation of TRAIL. In this case, Par-4 exhibits bystander effects, in that cells overexpressing Par-4 can secrete the protein and induce sensitivity to chemotherapy to nearby cancer cells that do not overexpress Par-4 (15). The phosphorylation of Par-4 by Akt1 enables the scaffolding protein 14-3-3 to bind Par-4, causing retention in the cytoplasm (18). Inhibition of Akt1 can result in activated Par-4 and sensitization to apoptotic stimuli.

The PI3K/Akt pathway, together with its associated negative regulator PTEN, is one important signal transduction pathway for chemoprevention and cancer treatment studies. Evidence supporting the importance of the PI3K/Akt signaling pathway in cancer chemoprevention and therapy has been well documented in previous studies (19–22), and has led to development of Akt signaling pathway inhibitors that are able to reduce tumor growth

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Translational Relevance

Par-4 is a tumor suppressor that induces apoptosis in cancer but not in normal cells, and has been shown to reduce tumor growth in mice. A second treatment, inhibition of Akt, reduces tumor growth in mice. The use of ISC-4 to inhibit Akt shows toxicity to cancer cells at low concentrations, but is not toxic to normal cells. ISC-4 has a 2-fold effect: through inhibition of Akt, it results in activation of Par-4 to induce apoptosis and inhibits survival pathways. This 2-pronged approach, systemically applied for cancer treatment, can reduce tumor growth significantly in primary tumors as well as in both occult and frank metastases without damaging normal tissue.

successfully. The entire pathway is deregulated in many human cancers, either by activating mutations, or by deletion of PTEN (23, 24). Specifically, in colon cancer, Akt overexpression has been shown in 57% of sporadic colon tumors, higher than that in many cancers, and upregulation occurs at a premalignant stage (25). Moreover, activation of Akt has been shown in colon cancer cells but not in normal mucosa (25, 26). In this study, we used a new inhibitor of Akt, phenylbutyl isoselenocyanate (Ph–$(CH_2)_4$–N=C=Se; ISC-4; refs. 27, 28), alone and in combination with Par-4, to effect colon tumor regression. ISC-4 was recently developed in our laboratories (28) through extensive structure-activity studies based on naturally occurring phenylalkyl isothiocyanates (Ph–$(CH_2)_n$–N=C=S;ITC), that have been shown to be effective at inhibiting Akt signaling pathways. In both epidemiologic and laboratory investigations, naturally occurring and synthetic ITCs are well-established anticancer agents for cancers at a variety of organ sites (29–37). The lead compounds were optimized and the best Akt inhibitors were obtained by the isosteric replacement of sulfur in ITCs by selenium leading to isoselenocyanate derivatives (Ph–$(CH_2)_n$–N=C=Se).The rationale for this modification was based on the observation that organoselenium compounds have been shown to be effective in retarding tumorigenesis of several cancer types, including colon cancer (38–42), in both animal models and epidemiologic studies. In addition, it has been shown that most cancer patients, including colon cancer patients (43, 44), have lower serum selenium levels than healthy controls. Hence, ISC compounds combined the anticancer properties of both selenium and ITCs. ISC-4 designed by increasing the alkyl chain length and replacing sulfur by selenium in naturally occurring ITCs was identified as the most potent drug-like PI3K/Akt inhibitor (27, 28).

We recently reported that Par-4 overexpression in human colon cancer cells resulted in reduced tumor growth in response to 5-fluorouracil (5-FU) when the cells were implanted into nude mice (45). As cells expressing Par-4 show a bystander effect i*n vitro*, we examined the possibility that this effect may extend to tumor cells that are distally located in a nude mouse model of colon tumor growth. Mice were injected with wild-type (WT) HT29 human colon cancer cells and half of the mice were injected distally with Par-4–overexpressing HT29 cells. Mice were then treated with ISC-4 to establish the efficacy of this drug on tumor growth either with or without the addition of 5-FU.

Materials and Methods

Reagents

ISC-4 was synthesized following a method recently developed by Sharma and colleagues (28). Other reagents: 5-FU (Acros Organics), API-2 (Tocris Biosciences), and phenylbutyl isothiocyanate (PBITC; LKT Laboratories). Cell culture reagents: HT29, SW480, HCT116, and SW620 cells (American Type Culture Collection) and Fugene 6 reagent (Roche Diagnostics). Antibodies were purchased from Santa Cruz Biotechnology, Amersham, and Cell Signaling Technologies.

Cell culture

Human colon cancer cells were cultured in RPMI containing 10% FBS and Pen/Strep at 37°C and 5% CO_2. HT29 cells were transfected with either rat *par-4* cDNA in pCB6+ (a kind gift of Dr. Vivek Rangnekar, University of Kentucky), with the human Par-4 clone in pCMVA6-AC (Origene), or with empty vector using Fugene 6. Human Par-4 was obtained from Origene. Transfectants were selected with G418 (Gibco) and colonies expanded and assayed for Par-4 expression.

Immunoprecipitation and Western blotting

The following antibodies used: Par-4 rabbit polyclonal, caspase 9 rabbit polyclonal, caspase 8 mouse monoclonal (Cell Signaling), and β-actin mouse monoclonal (Sigma). Cells were grown to 80% confluence. Plates were washed with PBS and the cells were lysed into lysis buffer (50 mmol/L HEPES, 100 mmol/L NaCl, 10 mmol/L EDTA, 0.5% NP40, 10% glycerol, 0.0001% Tween 20, supplemented with 0.1 mmol/L phenylmethylsulfonylfluoride, 0.1 mmol/L $NaVO_4$, 0.5 mmol/L NaF, 5 μg/mL leupeptin, 0.1 mmol/L dithiothreitol). In the case of mouse tissues, snap-frozen tissues were homogenized in lysis buffer by using a Fisher Scientific PowerGen homogenizer (Fisher Scientific). The proteins were quantified according to the Bradford Assay and loaded equally onto 10% polyacrylamide gels. For immunoprecipitation, 100 μg protein were incubated with 50 μL Dynabeads (Invitrogen) conjugated to 14-3-3 goat polyclonal antibody (Santa Cruz Biotechnologies). Beads were washed and proteins eluted. Proteins were electrophoresed at 150 V and transferred to nitrocellulose membranes by using a semidry blotter (BioRad). Membranes were blocked with 5% nonfat dry milk for 2 hours and incubated with primary antibody overnight. The blots were washed 3× in TBS-Tween and incubated for 1 hour in appropriate horseradish peroxidase–conjugated secondary antibodies (GE Healthcare). Blots were washed and

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developed by using the ECL chemiluminescent kit (Amersham). The blots were exposed to autoradiography film and scanned.

Cell viability assay

Human colon cancer cells were seeded in a 96-well plate were treated with 3.15 to 50 μmol/L ISC-4, PBITC, or API-2 for 48 hours, or according to text. In addition, HT29 cells, transfected with either Par-4 or empty vector, were treated with ISC-4. *In vitro* cytotoxic efficacy was measured by MTT cell viability assay (Chemicon).

Nude mouse experiments

All mice were treated according to the guidelines set forth by the Association for Assessment and Accreditation of Laboratory Animal Care. Forty 6-week-old female athymic nude mice (Harlan Sprague Dawley) were injected in the right flank with 1×10^7 WT HT29 cells and 20 of the mice were also injected in the left flank with 1×10^7 HT29 cells transfected to overexpress Par-4. Starting at 7 days post-injection, tumors were measured weekly with calipers. Tumor volume was calculated by the formula, $(l \times w^2)/2$. Half of the mice were treated 3× weekly with ISC-4, at 3 ppm in 50 μL dimethyl sulfoxide (DMSO) by intraperitoneal (i.p.) injection. Half of the ISC-4–treated mice were additionally treated by i.p. injection with 5-FU (30 mg/kg in PBS; based on maximum tolerated dose of 30 mg/kg/d; ref. 46) on days 5, 7, 10, 14, 21, and 28 after injection of cells. Once the tumors reached a size of 2 cm in the largest diameter, mice were euthanized, tumors were removed, counted, weighed, and the tissue snap frozen in liquid nitrogen and stored at −80°C. The experiment was repeated with an additional 40 mice. All animal work was done with the full approval of the Penn State Hershey's Institutional Animal Care and Use Committee.

Fluorescent tissue staining

Frozen tumor tissue from mice was sliced and fixed in 4% paraformaldehyde solution for 20 minutes at room temperature. Slides were washed in PBS and tissue sections permeabilized in 0.2% Triton-X-100 and then blocked with 5% FBS, 0.1% Triton-X-100 in PBS for 1 hour. Samples were then incubated overnight at 4°C in primary antibody against Par-4, 1:200 dilution in 1% bovine serum albumin and 0.05% Triton-X-100 in PBS. Sections were washed in PBS and secondary antibody conjugated to Cy2 was applied and incubated in the dark for 1 hour at room temperature. Slides were mounted with mounting medium (60% glycerol) and stored in the dark. The images were collected by a Leica TCS SP2 AOBS confocal microscope with ×63 oil immersion optics. (Leica Microsystems Inc.). To avoid cross-talk between the 2 channels, sequential scanning of the tissue sample mounts was done.

Statistical methods

The statistical software program R version 2.11.1 (http://www.r-project.org/) was used to conduct the statistical analysis. A repeated measures analysis of variance was used to test for an overall significance in treatment effect as well as at individual concentrations of the treatments for the *in vivo* studies. A 2-sample t test with unequal variances was used to test 2 individual treatments at specified concentrations. The robust Mann–Whitney 2-sample nonparametric test was calculated for comparisons. The IC_{50} values were computed with the drc (analysis of dose–response curves) package (version 2.1.1) by using R (version 2.12.2; refs. 47, 48). Samples were normalized to the WT cells treated with DMSO only in each experiment.

Results

ISC-4 induces cell death in human colon cancer cells

Akt inhibitors have been well studied as therapeutic options for cancer treatment. As a downstream target of Akt1, Par-4 may play a role in this process. ISC-4 (Fig. 1A) induces apoptosis at very low concentrations in cancer cells but not in normal cells (27). We investigated the relative potency of ISC-4 and the sulfur analog, PBITC, with a commercially available Akt inhibitor, API-2, in HT29 cells (Fig. 1B). The human colon cancer cell line, HT29, was used for the experiments in this study for its high tumorigenicity in nude mice. The results show ISC-4, with an IC_{50} value of 6.57 μmol/L, to be more potent than either PBITC or API-2, with IC_{50} values of 38.1 and greater than 50 μmol/L, respectively (Fig. 1B).

Relative absorbance in the MTT assay was analyzed with a repeated measures analysis of variance that included the predictor variables treatment, concentration, and a treatment by concentration interaction effect. Both treatment and concentration had a significant effect on cellular response. An analysis of variance at individual concentrations shows no significant difference among the DMSO groups ($P = 0.684$) or at concentrations less than 12.5 μmol/L, but a significant difference is observed between ISC-4 and the other 2 treatments at concentrations of 12.5 μmol/L ($P = 0.0017$), 25 μmol/L ($P < 0.001$), and 50 μmol/L ($P = 0.0035$). The differences among the 3 treatment groups as varied by concentration are depicted in the graph in Figure 1B, along with SE bars. The higher concentrations of ISC-4 treatment yielded the smallest absorbances, and individual comparisons of ISC-4 to the 2 other treatments yielded statistically significant differences.

A number of human colon cancer cell lines, HCT116, HT29, KM12C, SW480, and SW620, were compared for relative sensitivity to ISC-4. In all cases, ISC-4 inhibited cell growth in a dose-dependent manner at the concentrations tested, with IC_{50} values of 9.15, 8.05, 13.07, 11.79, and 9.31, respectively (Fig. 1C), indicating that the effect of ISC-4 is not specific to only 1 or 2 colon cancer cell lines. The levels of Par-4 and phospho-Akt (pAkt) proteins were compared by Western blot analysis between cell lines, and correlated to the sensitivity of the cells to ISC-4. Although there is little variation in the Par-4 levels of these cells, the amount of pAkt varies more widely. The upper band present most notably in HT29 and SW620 represents the Akt1 isoform (Fig. 1C). Inhibition of this protein would

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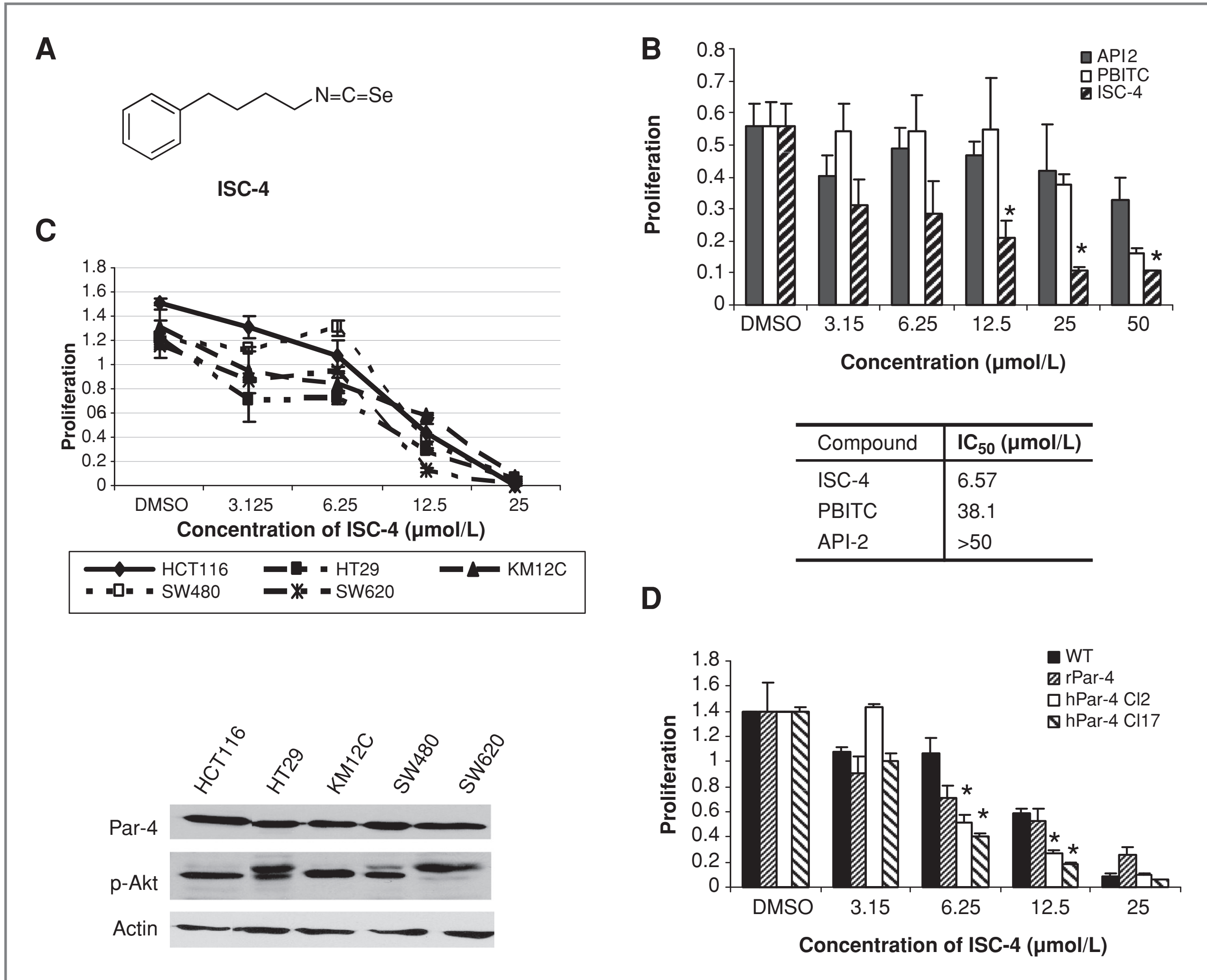

Compound	IC_{50} (μmol/L)
ISC-4	6.57
PBITC	38.1
API-2	>50

Figure 1. ISC-4 reduces cell viability more potently than other Akt inhibitors. A, structure of ISC-4. B, HT29 cells were treated for 48 hours with increasing concentrations of Akt inhibitors, or with DMSO vehicle. ISC-4 was significantly more potent than were API-2 and PBITC at doses of 12.5 and 25 μmol/L. Both PBITC and ISC-4 compounds were more potent than API-2 at 50 μmol/L ($P = 0.000585$). The table depicts IC_{50} values of compounds from MTT assay. C, a panel of colon cancer cell lines was treated with increasing concentrations of ISC-4. Data show a dose-dependent response in each cell line. All cells were dead at 50 μmol/L ISC-4. The Western blot analysis shows Par-4 and p-Akt protein expression in all cell lines. The upper band in the pAkt lane represents Akt1, the isoform responsible for Par-4 inhibition. D, ISC-4 treatment of WT or Par-4–transfected cells with increasing doses of ISC-4 or DMSO vehicle for 48 hours showed that Mock-transfected cells and cells transfected with rat par-4 were less sensitive to treatment than human Par-4–transfected cells (clones 12 and 17; $P = 0.014177$ – overall treatment effect). Compare Par-4–transfected cells to Mock-transfected cells at specific dose levels (hPar-4 cl 12, $P = 0.0304$ and 0.015; hPar-4 cl 17, $P = 0.0014$ and 0.0038 for 6.25 μmol/L and 12.5 μmol/L ISC-4, respectively). Asterisks indicate cells with significantly increased sensitivity to ISC-4 over Mock-transfected cells.

be expected to result in activation of Par-4, sensitizing the cells to apoptosis. However, it is difficult to say from these data that the pAkt levels affect sensitivity to ISC-4.

ISC-4 has been previously shown to increase the binding of Par-4 to NF-κB and decrease the binding to 14-3-3, indicating that ISC-4 causes inhibition of Akt1 and subsequent activation of Par-4 (45). As our earlier data on Par-4 were collected by using the rat *par-4* gene, the *in vivo* experiments in this study were conducted by using the same cells transfected for continuity. We transfected HT29 cells with the human *PAR-4* gene for comparison with the rat *par-4* gene. HT29 cells transfected with the plasmid for expression of either rat or 2 selected clones of human Par-4, or with an empty vector (Mock), were incubated with ISC-4. The overexpression of human Par-4 in the cells resulted in a reduction of the IC_{50} value to half that of the Mock-transfected cells in this experiment, with IC_{50} values of 11.0 for Mock cells and 5.64 and 4.6 for hPar-4 clones 12 and 17, respectively (Fig. 1D).

A repeated measures analysis of variance was used to compare overall effects of the Mock and Par-4 treatments yielding a statistically significant effect due to treatment ($P = 0.0141$) and concentration level ($P = 0.0017$), with no significant interaction effect ($P = 0.7686$). The

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Table 1. Number of mice in each treatment group in the *in vivo* tumor growth experiment

Compound	WT only	WT + Par-4
Vehicle	5	5
5-FU	5	5
ISC-4	5	5
5-Fu + ISC-4	5	5

NOTE: Tumor injections and drug treatments of each of the 8 mouse groups.

individual significant differences between clones were analyzed with a 2 sided *t* -test, and were only observed at the higher concentrations of 12.5 μmol/L and 6.25 μmol/L for the 2 human Par-4 clones.

ISC-4 reduces tumor growth in nude mice

As ISC-4 inhibits tumor cell viability but not normal cell viability *in vitro* (27), both the effects of ISC-4 on colon tumor growth and the toxicity of ISC-4 in mice were tested. Mice were injected with WT HT29 tumor cells only or with WT cells plus Par-4–overexpressing cells in opposite flanks. Mice were treated by i.p. injection 3 times weekly for 5 weeks with 3 ppm ISC-4 in DMSO, or with DMSO only. Table 1 outlines the experimental groups. Tumors were measured weekly, and the tumor volumes calculated. The tumor growth rate was assessed in 2 ways. One assessment was a comparison of tumor volumes at a time point when all of the mice were still alive, that is, week 3 (Fig. 2A). Expectedly, tumors formed by Par-4–overexpressing HT29 cells were smaller than tumors formed by WT HT29 cells. This is consistent with our previous findings that Par-4–overexpressing tumors grew more slowly than did WT tumors (49). Par-4 tumors showed a great response to ISC-4, particularly in conjunction with 5-FU. In 20% of the cases, the Par-4 tumors treated with ISC-4 disappeared altogether. In these cases, the WT tumors in those mice grew as rapidly as WT tumors in other mice that had not been injected with Par-4–overexpressing tumor cells. The rate of tumor growth both with and without ISC-4 treatment was determined through week 4 (Fig. 2B). After week 4, the number of mice remaining in the treatment groups was not large enough for statistically valid comparisons of tumor volumes. Results showed that mice treated with ISC-4 showed significantly retarded tumor growth compared with mice receiving no ISC-4 ($P = 0.042$). Asterisks indicate those tumors that are significantly smaller than the corresponding tumors in other treatment groups. The second assessment was a comparison of the length of time it took for the tumors to exceed a maximum allowable diameter of 2 cm (Fig. 2C). The growth rate, including both tumor volume and time to a

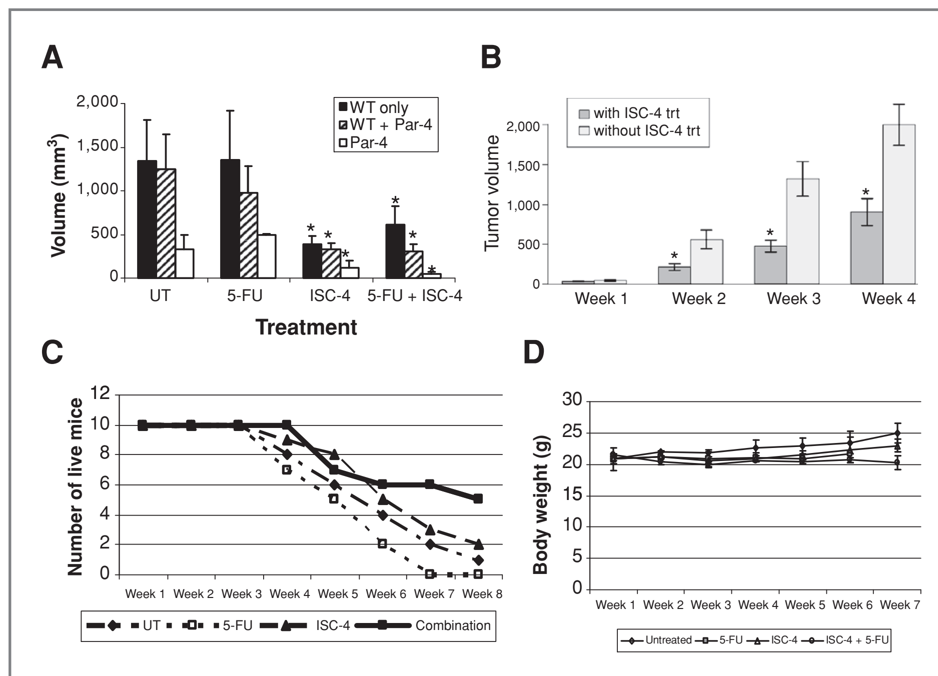

Figure 2. Treatment of mice with ISC-4 to determine effects on tumor volume. A and B, mice were treated with ISC-4, 5-FU, combination, or neither starting 1 week after injection of tumor cells. Three weeks after tumor cell injection, tumors were measured and volumes shown for each tumor type across all treatment levels. Asterisks indicate that corresponding tumors in mice treated with ISC-4 were smaller than those in mice without ISC-4 (WT alone tumors, $P = 0.005$; WT + Par-4 tumors, $P = 0.0038$; Par-4 tumors, $P = 0.0193$). B, tumor volumes were compared for growth rates up through week 4. Asterisks indicate statistical differences between ISC-4–treated and ISC-4–untreated samples from the same week. Nonparametric values at weeks 2, 3, and 4 are 0.008, 0.0003, and 0.0006, respectively. C, measurement of time until tumors reached 2 cm at the largest diameter, resulting in euthanasia. D, mice were weighed once weekly prior to drug injection and weights recorded.

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size of 2-cm diameter indicated that tumors in mice treated with ISC-4 grew more slowly than did tumors in mice that did not receive ISC-4. The drug had no severe systemic effects on the mice, as no mice sickened and died as a result of treatment and no mice showed weight loss during the experiment, although the mice treated with the combination of ISC-4 and 5-FU showed a lack of weight gain (Fig. 2D). Interestingly, the mice treated with 5-FU alone had the fastest WT tumor growth, indicating that 5-FU had no positive effect on WT tumor regression or growth inhibition. This trend was repeatable when the experiment was repeated, as mice with the combination treatment presented the slowest growing tumors and those with 5-FU treatment had the fastest growing tumors. Finally, for the mice with combination treatment, 5-FU was stopped after week 6, and the tumors did not seem to increase in growth significantly. In the future, treatment can be stopped earlier to detect more variation. Potentially, HT29 cells are resistant to 5-FU, although the reason for a growth stimulatory effect is not clear. However, 5-FU alone did retard the growth of Par-4–overexpressing tumors.

Par-4 tumors had a bystander effect on wild-type tumors growing in the same mice

WT tumors in mice were examined prior to administration of therapeutic drugs. At 7 days after injection of cells, the tumors were measured and volumes calculated. All tumors growing from WT cells in mice with no other tumor were larger than every WT tumor growing in a mouse that had also been implanted with Par-4–overexpressing cells (Fig. 3A). Similar results were obtained when the experiment was repeated. The tumor volume ratio of WT only/WT with Par-4 in the same mouse in the first experiment was 1.8, whereas in the second experiment the ratio was 2.0. Furthermore, at the time of euthanasia, the size of the WT tumors growing in the mice was inversely proportional to the size of the Par-4 tumor growing in the same mouse, indicating a dose-dependent bystander effect of Par-4–overexpressing cells on WT cells (Fig. 3B). This also indicates that the bystander effect functions effectively in distally growing tumors.

To examine the role of Par-4 with both treatment elements, ISC-4 and 5-FU, the WT tumors in all mice with both treatments were compared. The WT tumors in mice that also had Par-4 tumors grew significantly more slowly than the WT tumors growing alone in mice (Fig. 3C). 5-FU alone did not show a growth reduction of tumors. This suggests that the apoptotic inducement of 5-FU alone was not sufficient to fully induce Par-4 mediated apoptosis in WT cells as Par-4 may still have been inhibited by Akt1 activity. However, with both agents together, tumor growth was significantly slowed. On the contrary, the growth of Par-4–overexpressing tumors was retarded by treatment with 5-FU as compared with vehicle-treated tumors (data not shown).

ISC-4 downregulates Akt1 in mouse tumors

As ISC-4 downregulates Akt activity and Akt1 activity is important for the inhibition of Par-4 activity, the effects of

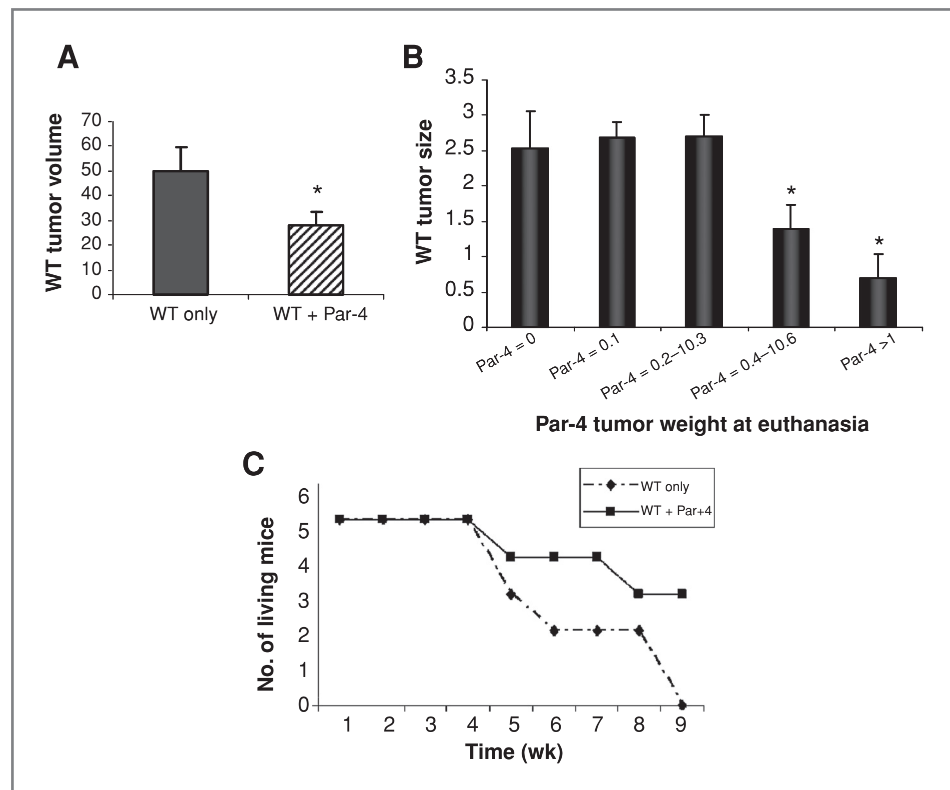

Figure 3. Bystander effects of Par-4. A, volume of WT tumors 1 week after cell injection, prior to treatment. $P = 0$. B, comparison of the WT tumor weights at euthanasia with the Par-4 tumor weight in the same mouse. Tumors were excised and weighed. The larger Par-4 tumors were present in mice with smaller WT tumors in an inverse proportional scale. Asterisks indicate significant reduction in WT tumor size with the presence of Par-4 tumors. C, time to maximum tumor size of WT tumors growing alone compared with WT tumors growing in mice that also had Par-4 tumors. The comparison is made in animals receiving both 5-FU and ISC-4. At the end of 9 weeks, 60% of mice with Par-4 tumors growing in them had WT tumors less than 2 cm in diameter, whereas all the tumors growing in mice without Par-4 tumors had reached maximum diameter.

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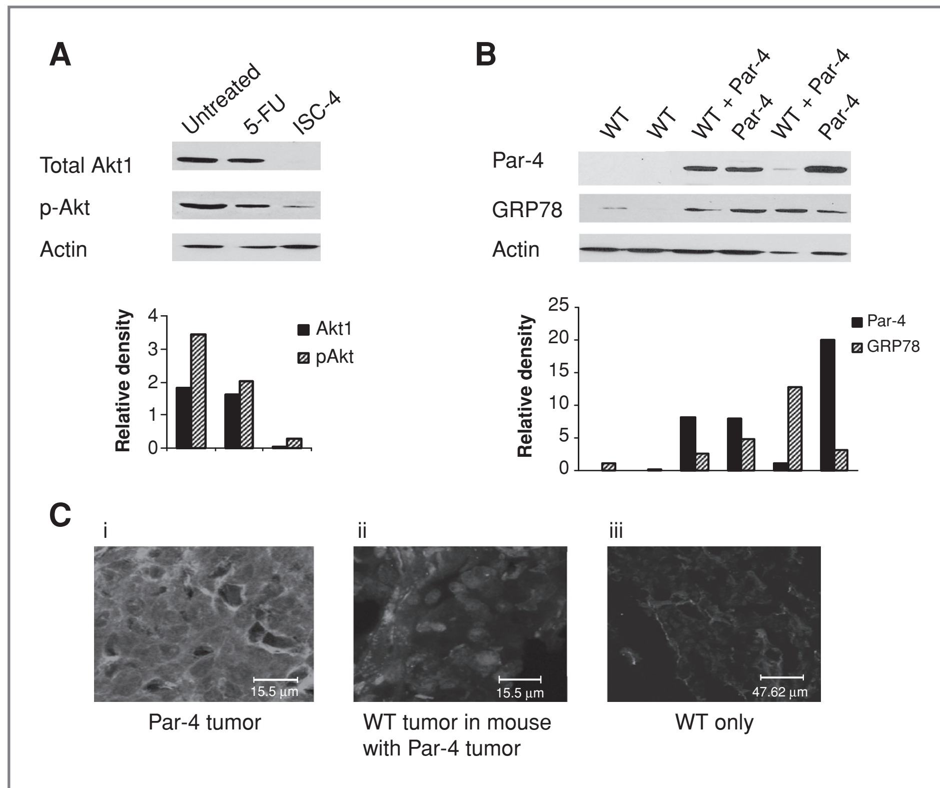

Figure 4. Analysis of proteins expressed in mouse tumors. A, Western blot analysis of total Akt1 and p-Akt (pan-Akt) in tumors treated with 5-FU, ISC-4, or neither. Akt1 expression is reduced to negligible levels in ISC-4–treated tumors (top). B, Par-4 (top) and GRP78 (middle) expression in WT tumors growing alone and paired WT and Par-4 tumors growing in the same mouse; bottom, β-actin for loading control. Densitometric analysis of banding is shown, normalized against β-actin. C, fluorescent staining of tumor thin slices for Par-4 (green): i, Par-4–overexpressing tumors; ii, WT tumor growing in mouse with Par-4 tumor; iii, WT tumors growing alone.

ISC-4 on Akt1 expression and Akt phosphorylation in tumor tissues was examined. Lysates were made from tumor tissue taken from mice at euthanasia. The tumor lysates were assayed by Western blot for expression of Par-4, Akt1, p-Akt, and β-actin for control. Figure 4A shows that administration of ISC-4 to the mice downregulates both the protein levels and the phosphorylation levels of Akt1 in mouse tumors. Potentially the faint band in the p-Akt lane under ISC-4 treatment is the result of Akt2 or Akt3, which are present in small amounts in these cells. Shown beneath the Western blots are densitometric analyses of the band densities.

Par-4 protein levels can increase in wild-type tumors growing in mice with Par-4 tumors

GRP78 is a protein expressed in the endoplasmic reticulum of cells. However, GRP78 is also present on cell surfaces where it acts as a receptor for soluble ligands (50), including exogenous Par-4 (15). Under conditions of ER stress, Par-4 mediates translocation of GRP78 to the cell surface. When GRP78 is present on the cell surface, it can be bound by exogenous Par-4, activating the apoptotic machinery within the cell (15). Therefore, we asked the question of whether GRP78 is present in the tumor cells, and whether the presence of Par-4 alters GRP78 expression. We examined the WT tumors from mice with only WT tumors and WT tumors from mice with paired Par-4 tumors, as well as Par-4 tumors themselves. Fig. 4B (top),shows Par-4 levels in tumors excised from mice at euthanasia. Lanes 1 and 2 are WT tumors from mice with only WT tumors (labeled WT), lanes 3 and 4 are WT and Par-4 tumors from the same mouse (labeled WT + Par-4 and Par-4, respectively), and lanes 5 and 6 paired WT + Par-4 and Par-4 tumors from a different mouse. The Western blot shows that WT tumors growing in mice with only WT tumors have very little Par-4 or GRP78. However, when Par-4 is overexpressed in tumors (lanes 4 and 6), GRP78 is increased. Likewise, in WT tumors growing in mice that also have Par-4 tumors (lanes 3 and 5) GRP78 is also increased.

Fluorescence microscopy was used to determine subcellular localization of Par-4 in tumor cells, as well as to validate the results of Western blotting. Sections were made from frozen tumor samples and stained with a primary antibody against Par-4. The secondary antibody contained a Cy-2 fluorescent tag and the images were collected by a Leica TCS SP2 AOBS confocal microscope. Results showed that Par-4 was highest in tumors overexpressing Par-4 [Fig. 4C (i)] and was also increased in WT tumors growing in the same mouse [Fig. 4C(ii)] as compared with WT tumors growing in mice that had no Par-4 tumors [Fig. 4C(iii)].

Par-4 causes apoptosis in tumors through both intrinsic and extrinsic pathways

Par-4 protein in cells acts through both intrinsic and extrinsic pathways. To examine which pathway plays a role in apoptosis in the mouse tumors, the cleavage of caspase 8 and caspase 9 were examined. In WT tumors, no caspase 9 was cleaved, yet in Par-4–overexpressing tumors caspase 9 was cleaved (Fig. 5A, note band at 37 kDa in the second panel), particularly when no chemotherapy treatment was

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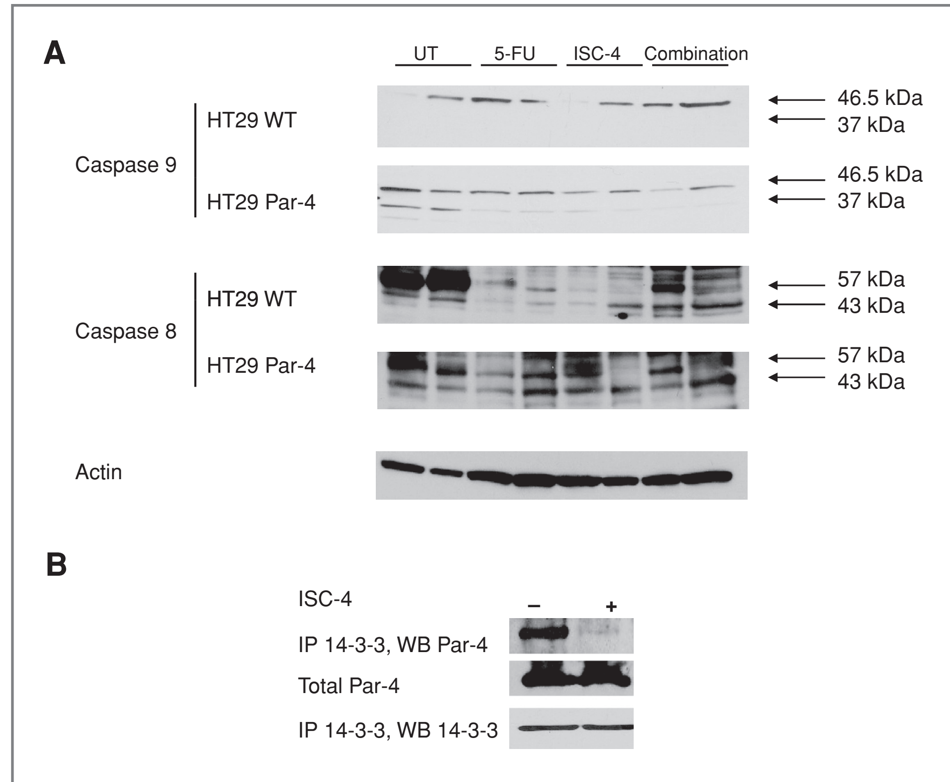

Figure 5. Both extrinsic and intrinsic pathways are active in mouse tumors. A, caspase 9 cleavage in WT and Par-4 tumors indicates activation of the intrinsic pathway by Par-4 overexpressing, but not in WT HT29 tumors. Caspase 8 cleavage increases with chemotherapeutic treatment in all tumors, possibly overcoming the activity of caspase 9 in Par-4–overexpressing cells. B, addition of chemotherapy causes release of Par-4 from 14-3-3. IP, immunoprecipitation; WB, Western blot.

administered. This indicates that Par-4 alone can induce apoptosis through the intrinsic pathway. However, when apoptotic stimuli is added, potentially the extrinsic pathway takes over apoptotic activities, as evidenced by the fact that caspase 8 is cleaved in both WT and Par-4–overexpressing tumors that were treated with either 5-FU or ISC-4, or both (Fig. 5A, note band at 43 kDa). Finally, ISC-4 given to mice results in release of Par-4 from 14-3-3 in the tumors, allowing it to become active for induction of apoptosis (Fig. 5B).

Discussion

5-FU has been used as a component of the therapeutic regimen for colon cancer patients for decades (51). However, there is a need for a more effective regimen, because even when using a combination of 5-FU with other chemotherapeutic agents, the clinical response rate for patients with metastatic disease remains at 20% to 39% (52). Recent studies have shown that the tumor suppressor, Par-4, may play a role in response to colon cancer treatment. Par-4 levels have been shown to be reduced in human colon cancer cells as compared with normal colon tissue. However, although Par-4 with no chemotherapy seems to retard tumor growth, simply increasing Par-4 protein levels may not provide optimal desired therapeutic effects. Maintaining Par-4 in an active state is important to the apoptotic activity of Par-4 in tumor cells. As Akt1 results in inactivation of Par-4, it is necessary to inhibit Akt1. This allows not only for the activation of Par-4, but also for inhibition of additional prosurvival downstream targets of Akt1. ISC-4 is an Akt inhibitor that has been shown to cause apoptosis in cancer cells, but not in normal cells and reduce tumor growth with no toxicity in mice at effective doses (27, 28), and is, therefore, a suitable compound to use for *in vivo* inhibition of Akt1. A comparison of ISC-4 with other Akt inhibitors showed ISC-4 to be more effective in cultured cells.

The only effect of Akt inhibition that we tested in this study was the activity of Par-4. However, ISC-4 is a pan-Akt inhibitor, so it inhibits Akt2 and Akt3 as well as Akt1. Inhibition of all Akt isoforms can have an effect on tumor growth, regardless of Par-4 status. Although Western blot analysis showed very little Akt2 or Akt3 in these cells, there may still be an effect of inhibiting their activity. In addition, Akt1 affects additional pathways that regulate apoptosis and survival. This may explain why the use of ISC-4 had a similar effect on WT tumors growing alone in mice as WT tumors growing in mice that also had Par-4 tumors growing in them.

Tumors from Par-4–overexpressing cells grew more slowly from the beginning than the WT tumors, although equal numbers of viable cells were injected. This suggests that Par-4 affects tumor growth from the point of initiation even without chemotherapy, and may, therefore, be a natural inhibitor of the formation of metastatic lesions. One confounding factor of the rapid tumor regression of Par-4–overexpressing tumors is that once those tumors

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shrank, the WT tumors in those mice began to grow. For this reason, a method of reintroducing Par-4 into tumor cells needs to be developed. The significance of the bystander effect is that there need not be 100% transfection efficiency to elicit a profound effect on the tumor. This laboratory is exploring those possibilities. The finding that the bystander effect functions distally to the cells overexpressing Par-4 has great significance for offering a therapeutic value of gene therapy using Par-4, in that transfected cells need not be proximally located to have an effect on untransfected tumor cells. Not only known tumor burden but also distant metastases can be affected by systemically released Par-4. In this study, as Par-4–overexpressing tumors decreased in size, the WT tumors in the same mice grew more rapidly. Therefore, to be effective in the long-term therapy outcome, the Par-4 must continue to be released, meaning that a method of *in vivo* transfection of cells with Par-4 must be repeated periodically. The use of nanotechnology to deliver Par-4 to cells has been and continues to be explored.

In conclusion, ISC-4 alone is a potent and safe inhibitor of colon tumor growth in a xenograft model when used as a single therapy. The addition of the current standard of care, 5-FU, enhances the growth inhibition of ISC-4. This suggests that tumors that are resistant to 5-FU treatment can be alternately treated with ISC-4 alone or can be sensitized to 5-FU through combination with ISC-4. Finally, when Par-4 is added to the cells, either from overexpression within the tumor or exogenously applied, tumor growth is further slowed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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