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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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Statement of 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 two fold effect: through inhibition of Akt, it results in activation of Par-4 to induce apoptosis and inhibits survival pathways. This two-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.
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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-FU than did wild type counterparts. In this study we investigated: 1) the effects of the Akt inhibitor, phenylbutyl isoselenocyanate (ISC-4), on tumor growth in nude mice and 2) bystander effect of Par-4 overexpressing cells on wild type tumor growth.

Experimental design: Mice (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. 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 than 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 to 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.
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**Introduction**

Colon cancer is the second most common cause of cancer deaths in both men and women in the US. 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 pro-apoptotic 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, tumor necrosis factor alpha (TNF-α), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (2-4). The down-regulation of Par-4 has been proposed to be a critical event in tumorigenesis (5). Par-4 is down-regulated in a number of human cancers, namely, 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 NFκB (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
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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 literature (19-22), and has led to development of Akt signaling pathway inhibitors that are able to reduce tumor growth 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 over-expression has been shown in 57% of sporadic colon tumors, higher than in many cancers, and upregulation occurs at a pre-malignant 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) (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; ITCs), that have been shown to be effective at inhibiting Akt signaling pathways. In both epidemiological and laboratory investigations, naturally occurring and synthetic ITCs are well established anti-cancer 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,
ISC-4 and Par-4 inhibit colon tumor growth including colon cancer (38-42), in both animal models and epidemiological studies. In addition, it has been demonstrated 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 reported recently 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 in 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 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 *et al.* (28). Other reagents obtained from: 5-FU Acros Organics (Geel, Belgium), API-2 (Tocris Biosciences, Ellisville, MO), and PBiTc (LKT Laboratories, St. Paul, MN). Cell culture reagents: HT29, SW480, HCT116, and SW620 cells (American Type Culture Collection, Manassas, VA) and
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Fugene 6 reagent (Roche Diagnostics, Indianapolis, IN, USA). Antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, Amersham, Piscataway, NJ, and Cell Signaling Technologies, Boston, MA.

**Cell culture**

Human colon cancer cells were cultured in RPMI containing 10% FBS and Pen/Strep at 37°C and 5% CO₂. 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, Rockville, MD), or with empty vector using Fugene 6. Human Par-4 was obtained from Origene. Transfectants were selected with G418 (Gibco, Carlsbad, CA) and colonies expanded and assayed for Par-4 expression.

**Immunoprecipitation and Western blotting**

Antibodies used were: Par-4 rabbit polyclonal, Caspase 9 rabbit polyclonal, Caspase 8 mouse monoclonal (Cell Signaling, Danvers, MA), and β-actin mouse monoclonal (Sigma, Saint Louis, MO). Cells were grown to 80% confluence. Plates were washed with PBS and the cells were lysed into lysis buffer (50 mm HEPES, 100 mm NaCl, 10 mm EDTA, 0.5% NP40, 10% glycerol, 0.0001% Tween 20, supplemented with 0.1 mm PMSF, 0.1 mm NaVO₄, 0.5 mm NaF, 5 μg/ml leupeptin, 0.1 mm DTT). In the case of mouse tissues, snap-frozen tissues were homogenized in lysis buffer using a Fisher Scientific PowerGen homogenizer (Fisher Scientific, Pittsburg, PA). 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, Carlsbad, CA) conjugated to 14-3-3 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Beads were washed and proteins eluted. Proteins were electrophoresed at 150 v and transferred to nitrocellulose membranes using a semi-dry blotter.
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**Cell viability assay**

Human colon cancer cells were seeded in a 96 well plate were treated with 3.15 to 50 μM 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 using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) cell viability assay (Chemicon, Temecula, CA).

**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, Indianapolis, IN) were injected in the right flank with 1 X10^7 wild type HT29 cells and 20 of the mice were also injected in the left flank with 1 X10^7 HT29 cells transfected to overexpress Par-4. Starting at 7 days post injection, tumors were measured weekly with calipers. Tumor volume was calculated using the equation; (l x w^2)/2. Half of the mice were treated 3X weekly with ISC-4, at 3 PPM in 50 μl DMSO by intraperitoneal (IP) injection. Half of the ISC-4 treated mice were additionally treated by IP injection with 5-FU (30 mg/kg in PBS) (based on maximum tolerated dose of 30 mg/kg/day (46)) on day 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...
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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 performed 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 min
at room temperature. Slides were washed in PBS and tissue sections permeabilized in 0.2%
Triton-X-100 then blocked with 5% FBS, 0.1% Triton-X-100 in PBS for 1h. Samples were then
incubated overnight at 4°C in primary antibody against Par-4, 1:200 dilution in 1% BSA 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 1h at room temperature. Slides were mounted
with mounting medium (60% glycerol) and stored in the dark. The images were collected using a
Leica TCS SP2 AOBS confocal microscope with x63 oil immersion optics. (Leica Microsystems
Inc., Bannockburn, IL). To avoid crosstalk between the two 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/](http://www.r-project.org/)) was used to
perform 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 two-sample t-test with unequal variances was used to test two
individual treatments at specified concentrations. The robust Mann-Whitney two-sample
nonparametric test was calculated for comparisons. The IC50 values were computed with the drc
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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, phenylbutyl isothiocyanate (PBITC), with a commercially available Akt inhibitor, API2, 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$_{50}$ = 6.57 µM, to be more potent than either PBITC or API-2 with IC$_{50}$ of 38.1 µM and >50 µM, 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=.684) or at concentrations less than 12.5 µM, but a significant difference is observed between ISC-4 and the other two treatments at concentrations of 12.5 µM (p=.0017), 25 µM (p<.001), and 50 µM (p=.0035). The differences among the three treatment groups as varied by concentration are graphed in Figure 1B along with standard error bars. The higher concentrations of ISC-4 treatment yielded the smallest absorbances, and
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individual comparisons of ISC-4 to the two 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 IC50s 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 one or two 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. While 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 be expected to result in activation of Par-4, sensitizing the cells to apoptosis. However, it is difficult to say from this data that the pAkt levels affect sensitivity to ISC-4.

ISC-4 was shown previously to increase the binding of Par-4 to NFκB 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 was collected using the rat par-4 gene, the in vivo experiments in this study were performed 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 two 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 IC50 to half that of the mock transfected cells in this
ISC-4 and Par-4 inhibit colon tumor growth 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=.0141) and concentration level (p=.0017), with no significant interaction effect (p=.7686). The individual significant differences between clones were analyzed with a two sided T-Test, and were only observed at the higher concentrations of 12.5 μM and 6.25 μM for the two 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 wild type (WT) HT29 tumor cells only or with WT cells plus Par-4 overexpressing cells in opposite flanks. Mice were treated by IP 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 tumor volumes calculated. The tumor growth rate was assessed in two ways. One assessment was a comparison of tumor volumes at a time point when all of the mice were still alive, i.e. week 3 (Fig. 2A). Expectedly, tumors formed by Par-4 overexpressing HT29 cells were smaller than tumors formed by wild type 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
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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). 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 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 demonstrated weight loss during the experiment, although those 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 WT tumors growing in the same mice
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Wild type 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, while 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 wild type tumors in all mice with both treatments were compared. The wild type tumors in mice that also had Par-4 tumors grew significantly more slowly than did the wild type 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 other hand, the growth of Par-4 overexpressing tumors was retarded by treatment with 5-FU as compared to vehicle treated tumors (data not shown).

**ISC-4 downregulates Akt1 in mouse tumors**
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As ISC-4 downregulates Akt activity and Akt1 activity is important for the inhibition of Par-4 activity, the effects of 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, phospho 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 phospho-Akt lane under ISC-4 treatment is the result of Akt 2 or 3, 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 WT 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, upper panel, 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...
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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 using a Leica TCS SP2 AOBS confocal microscope. Results showed that Par-4 was highest in tumors overexpressing Par-4 (Fig. 4C, panel i) and was also increased in WT tumors growing in the same mouse (panel ii) as compared to WT tumors growing in mice that had no Par-4 tumors (panel 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 wild type tumors, no caspase 9 was cleaved, yet in Par-4 overexpressing tumors caspase 9 was cleaved (Figure 5A, note band at 37 kDa in second panel), particularly when no chemotherapy treatment was 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, ISC-4, or both (Figure 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).
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**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, as even when using a combination of 5-FU with other chemotherapeutic agents, the clinical response rate for patients with metastatic disease remains at 20-39% (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 to normal colon tissue. However, although Par-4 with no chemotherapy appears 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 pro-survival 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 Akt 2 and Akt 3 as well as Akt 1. Inhibition of all Akt isoforms can have an effect on tumor growth, regardless of Par-4 status. While Western blot analysis showed very little Akt 2 or 3 in these cells, there may still be an effect of inhibiting their activity. In addition, Akt 1 affects additional pathways that regulate apoptosis and survival. This
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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 did wild type 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 shrank, the wild type 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 long-term therapy outcome, the Par-4 must continue to be released, meaning that a method of \textit{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,
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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.
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References

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Figure legends

**Fig. 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 API2 and PBITC at doses of 12.5 and 25 μM. Both PBITC and ISC-4 compounds were more potent than API2 at 50 μM (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 μM ISC-4. The Western blot analysis shows Par-4 and phosphoAkt (pAkt) 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 wild type 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 were 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 μM and 12.5 μM ISC-4, respectively). Asterisks indicate cells with significantly increased sensitivity to ISC-4 over Mock transfected cells.

**Fig. 2.** Treatment of mice with ISC-4 to determine effects on tumor volume. A, mice were treated with ISC-4, 5-FU, combination, or neither starting one 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. Tumors in mice treated with ISC-4 were smaller than
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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). Tumor volumes were compared for growth rates up through week 4. 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.

**Fig. 3.** Bystander effects of Par-4. A, volume of WT tumors one week after cell injection, prior to treatment. p=0. B, comparison of the wild type 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 wild type tumors in an inverse proportional scale. C, time to maximum tumor size of wild type tumors growing alone compared with wild type 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 wild type tumors less than 2 cm in diameter, while all the tumors growing in mice without Par-4 tumors had reached maximum diameter.

**Fig. 4.** Analysis of proteins expressed in mouse tumors. A, western blot analysis of total Akt1 and phospho 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 panel). B, Par-4 (upper panel) and GRP78 (middle panel) expression in wild type tumors growing alone and paired wild type and Par-4 tumors growing in the same mouse. Lower panels show β-actin for loading control. Densitometric analysis of banding is shown, normalized against β-actin. C, fluorescent staining
ISC-4 and Par-4 inhibit colon tumor growth

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.

Fig. 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.

Table 1. Treatment groups in the in vivo tumor growth experiment. Table 1 outlines the tumor
injections and drug treatments of each of the 8 mouse groups.

Table 1. Number of mice in each treatment group.

<table>
<thead>
<tr>
<th>Compound</th>
<th>WT only</th>
<th>WT + Par-4</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>5-FU</td>
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<td>5</td>
</tr>
<tr>
<td>ISC-4</td>
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<td>5</td>
</tr>
<tr>
<td>5-Fu + ISC-4</td>
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</table>
Figure 1

A

\[ \text{ISC-4} \]

B

<table>
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<tr>
<th>Concentration in ( \mu \text{M} )</th>
<th>DMSO</th>
<th>3.15</th>
<th>6.25</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
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<td><strong>Concentration of ISC-4 in ( \mu \text{M} )</strong></td>
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<td>3.15</td>
<td>6.25</td>
<td>12.5</td>
<td>25</td>
<td>50</td>
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<tr>
<td><strong>Compound</strong></td>
<td><strong>IC(_{50}) (( \mu \text{M} ))</strong></td>
<td>6.57</td>
<td>38.1</td>
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C

D

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<th>Concentration of ISC-4 in ( \mu \text{M} )</th>
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<tr>
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<td><strong>IC(_{50}) (( \mu \text{M} ))</strong></td>
<td>6.57</td>
<td>38.1</td>
<td>&gt;50</td>
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</table>
Figure 3

A

WT tumor volume

WT only

WT + Par-4

B

WT tumor size

Par-4 tumor weight at euthanasia

Par-4 = 0

Par-4 = 0.1

Par-4 = 0.2-0.3

Par-4 = 0.4-0.6

Par-4 > 1

C

# of living mice

WT only

WT + Par-4

Time in weeks
Figure 4

A

<table>
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<tr>
<th></th>
<th>Untreated</th>
<th>5-FU</th>
<th>ISC-4</th>
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<td>Actin</td>
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B

<table>
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<tr>
<th></th>
<th>WT</th>
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<tr>
<td>Par-4</td>
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<tr>
<td>GRP78</td>
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<tr>
<td>Actin</td>
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C

i) Par-4 tumor
ii) WT tumor in mouse With Par-4 tumor
iii) WT only
Figure 5

A

<table>
<thead>
<tr>
<th></th>
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<th>5-FU</th>
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B

<table>
<thead>
<tr>
<th></th>
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<td>IP 14-3-3 WB Par-4</td>
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<tr>
<td>Total Par-4</td>
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<tr>
<td>IP 14-3-3, WB 14-3-3</td>
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The Akt inhibitor ISC-4 activates Prostate apoptosis response protein-4 and reduces colon tumor growth in a nude mouse model

Arun K. Sharma, Christina L. Kline, Arthur Berg, et al.

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