Preclinical Development

Honokiol in Combination with Radiation Targets Notch Signaling to Inhibit Colon Cancer Stem Cells

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Abstract
Cancer stem cells are implicated in resistance to ionizing radiation (IR) and chemotherapy. Honokiol, a biphenolic compound has been used in traditional Chinese medicine for treating various ailments. In this study, we determined the ability of honokiol to enhance the sensitivity of colon cancer stem cells to IR. The combination of honokiol and IR suppressed proliferation and colony formation while inducing apoptosis of colon cancer cells in culture. There were also reduced numbers and size of spheroids, which was coupled with reduced expression of cancer stem cell marker protein DCLK1. Flow cytometry studies confirmed that the honokiol–IR combination reduced the number of DCLK1⁺ cells. In addition, there were reduced levels of activated Notch-1, its ligand Jagged-1, and the downstream target gene Hes-1. Furthermore, expression of components of the Notch-1 activating γ-secretase complex, presenilin 1, nicastrin, Pen2, and APH-1 was also suppressed. On the other hand, the honokiol effects were mitigated when the Notch intracellular domain was expressed. To determine the effect of honokiol–IR combination on tumor growth in vivo, nude mice tumor xenografts were administered honokiol intraperitoneally and exposed to IR. The honokiol–IR combination significantly inhibited tumor xenograft growth. In addition, there were reduced levels of DCLK1 and the Notch signaling–related proteins in the xenograft tissues. Together, these data suggest that honokiol is a potent inhibitor of colon cancer growth that targets the stem cells by inhibiting the γ-secretase complex and the Notch signaling pathway. These studies warrant further clinical evaluation for the combination of honokiol and IR for treating colon cancers. Mol Cancer Ther; 11(4); 963–72. ©2012 AACR.

Introduction
Colon cancer remains incurable warranting discovery of new strategies for therapy. Eradicating drug-resistant colon cancer stem cells is one provocative area of investigation. This article focuses on defining nontoxic approaches toward eradicating colon tumor stem cells. Honokiol (Fig. 1A) is a biphenolic compound from Magnolia officinalis that is used in traditional Chinese and Japanese medicine for the treatment of various ailments including ulcer, allergy, and bacterial infections. It is also used as a muscle relaxant and possesses antithrombotic activity (1, 2). Recent studies have shown that it has antitumor activity with low toxicity (3, 4).

Notch signaling is an evolutionarily conserved mechanism that affects proliferation, differentiation, and apoptotic programs, thereby maintaining tissue homeostasis. Notch signaling also plays a fundamental role in the differentiation and maintenance of stem cells. Aberrant activation of the Notch signaling has been associated with the development of many cancers, including colon cancers (5, 6). More importantly, altered Notch activity has been shown to partially explain the apparent radioresistance present in the stem cell fraction in cancers (7). This suggests that targeting the Notch signaling pathway might affect growth of cancer stem cells. In colon cancers, the levels of Notch-1 expression is associated with the pathologic grade, progression, and metastasis (5). Notch signaling is initiated when a ligand such as Jagged interacts with the notch transmembrane receptor (8), leading to 2 sequential proteolytic events, including one that occurs just inside the membrane by the γ-secretase complex (9). The enzyme complex is made up of 4 proteins, presenilin, nicastrin, APH-1 (anterior pharynx-defective 1), and PEN-2 (presenilin enhancer 2), all of which are essential for activity. Cleavage by the γ-secretase complex releases the Notch intracellular domain (NICD), which in turn translocates into the nucleus of the cells, interacts with the C promoter-binding factor-1 (CBF1) transcriptional

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cofactor, and transactivates target genes, such as those in the hairy and enhancer of split (Hes) and Hes related with YRPW motif (Hey) family proteins (10).

Tumorigenesis in the gut is thought to arise specifically in the stem cell population located at or near the base of the colonic crypts (11–13). Various proteins have been identified as potential markers for stem cells (14–18). We have shown that doublecortin and CaM kinase-like-1 (DCLK-1), a microtubule-associated kinase expressed in postmitotic neurons (19) is an intestinal stem cell marker that is expressed in colon adenocarcinoma (20). In this article, we have determined the effect of combining honokiol and ionizing radiation (IR) on the Notch signaling pathway and on colon cancer stem cells.

Materials and Methods

Cells and reagents

HCT116 and SW480 cells (American Type Culture Collection) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS (Sigma-Aldrich) and 1% antibiotic solution (Mediatech Inc.). Normal colon epithelial cells (FHC, CRL-1831) were grown in Ham’s F12 medium 45%, DMEM 45%, 25 mmol/L HEPES, 10 ng/mL cholera toxin, 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 100 ng/mL hydrocortisone, 10% FBS, and 1% antibiotic solution at 37°C in a humidified atmosphere of 5% CO2. All cells used in this study were within 20 passages after receipt or resuscitation (approximately 3 months of noncontinuous culturing). The cell lines were not authenticated as they came from national repositories. Honokiol was purchased from LKT Laboratories. N-[(N-(3, 5-Difluorophenacetyl)-l-alanyl)-S-phenylglycine t-butyl ester (DAPT), a γ-secretase inhibitor (GSI) was purchased (Sigma-Aldrich; Supplementary Fig. S1).

Proliferation and apoptosis assays

Cells in 96-well plates were treated with honokiol (0–50 μmol/L) followed by IR (0–5 Gy). Proliferation was
analyzed by hexoseaminidase assay (21, 22). For apoptosis, caspase-3/7 activity was measured using the Apo-one Homogeneous Caspase 3/7 Assay Kit (Promega).

**Clonogenicity assay**

Cells were treated with honokiol (25 μmol/L) for 4 hours and then exposed to IR (0–5 Gy). Forty-eight hours following IR, the honokiol-containing medium was removed, and the cells were incubated for an additional 10 days. Treatments were carried out in triplicate. The colonies obtained were formalin fixed and stained with hematoxylin. The colonies were counted and compared with untreated cells.

**Western blot analysis**

Cell lysates were subjected to PAGE and blotted onto Immobilon polyvinylidene difluoride membranes (Millipore). Antibodies were purchased from Cell Signaling Technology, Abcam Inc., GenScript USA Inc., and Santa Cruz Biotechnology Inc., and specific proteins were detected by the enhanced chemiluminescence system (GE Healthcare).

**Flow cytometric analyses**

Twenty-four hours following honokiol (25 μmol/L) and IR (5 Gy) exposure, cells were subjected to direct immunofluorescence staining (phycoerythrin-conjugated DCLK1 or phycoerythrin-conjugated CD133 antibody), followed by flow cytometric analyses. The samples were analyzed using a FACS Calibur analyzer (Becton Dickinson), capturing 10,000 events for each sample. Results were analyzed with ModFit LT software (Verity Software House).

**Colonosphere assay**

Cells were treated with honokiol (25 μmol/L) for 4 hours and then irradiated (5 Gy). After 7 days, the number and size of colonospheres were determined using Celigo (Cyntellect Inc.). For second and third passages, cells were grown in the absence of honokiol and IR.

**Plasmids and transfections**

Cells were transfected with plasmid EF.hICN1.CMV. GFP encoding the NICD or the empty vector EF.v-CMV. GFP (Addgene Inc.) and subsequently treated with honokiol (25 μmol/L) and IR (5 Gy). Cell proliferation and apoptosis were detected using hexoaminidase assay and Apo-one Homogeneous Caspase 3/7 Assay Kit, respectively.

**HCT116 xenograft tumors in mice**

Five-week-old male athymic nude mice (Charles River) were used for in vivo experiments. They were maintained with water and standard mouse chow diet ad libitum and used in studies as per protocols approved by the University’s Animal Studies Committee. Animals were injected with 1 × 10⁶ HCT116 cells into the flank and allowed to develop. Upon identification of a palpable tumor, honokiol (200 μg/kg body weight) was administered intraperitoneally daily for 21 days. Where applicable, animals were also irradiated at 5 Gy once a week for 3 weeks. At the end of treatment, the animals were euthanized, and the tumors were used for histology (hematoxylin & eosin), immunohistochemistry, and gene expression studies.

**Immunohistochemistry**

Four micrometer sections from paraffin-embedded tissues were incubated overnight with primary antibodies, followed by one hour with a broad-spectrum secondary antibody and horseradish peroxidase conjugate. The slides were developed with DAB and counterstained by hematoxylin (Invitrogen). Slides were examined in Nikon Eclipse Ti microscope under a ×40 objective.

**Statistical analysis**

All values are expressed as the mean ± SEM. Data was analyzed using an unpaired 2-tailed t test. A P value of less than 0.05 was considered statistically significant. For multiple comparisons, ANOVA was done using Bonferroni corrections for multiple comparisons. To be considered significant, the P value must be less than 0.017. The SPSS V17 statistical software was used for these analyses.

**Results**

**Honokiol and IR inhibit cell proliferation**

Radiation therapy is commonly used in colorectal cancers either before or after surgery. However, it can have significant side effects suggesting that reducing the dose would be beneficial. Given that honokiol can reduce growth of colorectal cancer cells, we determined its effect when combined with IR. The honokiol–IR combination significantly suppressed proliferation of colorectal cancer cells within 24 hours at a dose of 25 μmol/L, which continued significant suppression over the next 72 hours (Fig. 1B). In contrast, the combination did not affect proliferation of normal colon cells (Fig. 1C). To determine the long-term effect of honokiol–IR treatment, cells were treated with honokiol for 4 hours before exposure to 5 Gy IR. The cells were exposed to honokiol for an additional 24 hours before allowing growth in normal medium. There were fewer colonies in both the two colorectal cancer cells with the combination treatment when compared with cells treated with either treatment alone (Fig. 1D), suggesting that the combination honokiol–IR treatment is effective.

**Honokiol–IR combination induces apoptosis**

Caspase-3/7 are key effector molecules in the apoptosis pathway that initiate events that lead to the hallmarks of apoptosis, including DNA laddering and cellular morphologic changes (23, 24). To determine whether honokiol–IR induced apoptosis, we determined caspase-3/7 activity. There was an increase in the caspase-3/7 activity following treatment with the combination of honokiol and IR (Fig. 2A). This was further confirmed by Western blot
analyses, showing higher levels of activated caspase-3 in both colorectal cancer cells when compared either treatment alone (Fig. 2B). Further confirmation was obtained by Western blot analyses for the antiapoptotic Bcl2 and BclxL, and proapoptotic Bax proteins. Although either honokiol or IR alone inhibited Bcl2 and BclxL, there was a significant inhibition of the two proteins with the combination treatment (Fig. 2C). On the other hand, there was a significant increase in Bax protein with the individual treatment, with a further increase in response to the combination. These data suggested that honokiol is a potent inducer and an enhancer of radiation-induced apoptosis.

Cyclin D1 is a key cell-cycle regulatory protein that functions as a cofactor for several transcription factors (25). Cyclin D1 overexpression has been linked to the development and progression of cancer (26). Similarly, c-myc is upregulated in cancers. In both colorectal cancer cells, honokiol and IR treatment resulted in reduced cyclin D1 and c-myc expression compared with untreated cells or cells treated with honokiol or IR alone. D, lysates from cells incubated with 25 μmol/L of honokiol and exposed to 5 Gy IR were analyzed by Western blotting for cyclin D1, c-myc, and p21 expression. The honokiol–IR combination inhibits cyclin D1 and c-myc expression compared with either honokiol or IR alone while increasing p21 expression.

**Figure 2.** Honokiol and IR induce cancer cell apoptosis. A, HCT116 and SW480 cells were treated with either 25 μmol/L honokiol or 5 Gy IR or both for 48 hours and tested for caspase-3/7 activity. The honokiol and IR combination induces apoptosis in both the cells when compared with untreated controls (*, *P* < 0.05). B, honokiol and IR combination induces caspase-3, an apoptosis mediator. Cells were treated with 25 μmol/L honokiol and 5 Gy IR. After 48 hours, the lysates were analyzed by Western blotting for caspase-3 protein. The combination of honokiol with IR resulted in increased levels of cleaved caspase-3 when compared with either treatment alone. C, lysates from cells treated with the combination of honokiol and IR were analyzed by Western blotting for Bax and Bcl-2 family proteins. The honokiol–IR combination induces proapoptotic protein Bax and reduces expression of antiapoptotic protein Bcl-2 family proteins when compared with untreated cells or cells treated with honokiol or IR alone. D, lysates from cells incubated with 25 μmol/L of honokiol and exposed to 5 Gy IR were analyzed by Western blotting for cyclin D1, c-myc, and p21 expression. The honokiol–IR combination inhibits cyclin D1 and c-myc expression compared with either honokiol or IR alone while increasing p21 expression.

Honokiol–IR targets cancer stem cells

Colorectal tumors are thought to arise specifically in the stem cell population located at or near the base of the intestinal and colonic crypts. Markers used for identification of colon cancer stem cells include CD44, CD133, CD24, CD29, Lgr5, and DCLK-1 (20, 27). This cell population is capable of self-renewal and generating tumors resembling the primary tumor (16). We first determined the effects of honokiol–IR combination on DCLK1 and CD133 expression. Flow cytometric analyses showed a significant decrease in DCLK1+ and CD133+ expression. Flow cytometric analyses showed a significant decrease in DCLK1+ and CD133+ cells in SW480 with honokiol–IR combination (Fig. 3A). Western blot analyses confirmed these findings, as well reduction in other cancer stem cell markers (Fig. 3B). We confirmed these results by carrying out colonosphere formation assays, a hallmark for growth from cancer stem cells. The honokiol–IR combination significantly inhibited primary and secondary SW480 colonosphere formation (Fig. 3C and D). Similar effects were obtained with tertiary colonosphere formation and also in HCT116 cells (data not shown).

**Honokiol–IR inhibits Notch signaling by downregulating the γ-secretase complex**

We next determined the effect of honokiol–IR combination on Notch signaling–related proteins in the 2 colorectal cancer cells. Both Notch-1 and its ligand, Jagged-1, were downregulated by the honokiol–IR combination (Fig. 4A). Further confirmation was obtained when reduced expression of Hey-1 and Hes-1 expression was
observed (Fig. 4A). We next determined whether the γ-secretase complex comprising presenilin, nicastrin, APH1, and PEN2 is affected. Treatment with the honokiol–IR combination resulted in downregulation in the expression of all 4 proteins (Fig. 4B). In addition, the combination inhibited expression of Skip1, a nuclear cofactor in NICD-mediated transcriptional activation (Fig. 4C). These data suggested that honokiol–IR-mediated downregulation of the Notch signaling occurs in part through the inhibition of the γ-secretase complex. In addition, cotreatment of the honokiol–IR combination with DAPT further reduced Hes-1 expression (Fig. 5A) and proliferation (right panel; Fig. 5B).

We next determined whether lack of Notch-1 activation is the reason for reduced growth of colon cancer cells. For this, we expressed the intracellular domain NICD in HCT116 and SW480 cells. Western blot analyses showed increased expression of Hes-1 following ectopic expression of NICD in both cell lines (Fig. 5C). Furthermore, although honokiol or IR alone inhibited the basal levels of Hes-1 expression, NICD rescued this inhibition resulting in increased Hes-1 expression. Moreover, ectopic expression of NICD reversed honokiol- and IR-mediated inhibition of cell proliferation (Fig. 5D, left panel) and induction of apoptosis (Fig. 5D, right panel). Together, these data suggested that honokiol and IR inhibits the γ-secretase complex, thereby affecting Notch signaling.
Honokiol radiosensitizes colon tumor xenografts

To evaluate the role of honokiol–IR combination on tumor growth in vivo, we next examined its effects on growth of HCT116 xenografts. Honokiol was administered intraperitoneally to mice bearing xenografts and irradiated as shown in Fig. 6A. Although treatment with either honokiol or IR inhibited the growth of the tumor xenografts, there was reduction with the honokiol–IR combination (Fig. 6B). The excised tumors from control animals weighed approximately 3,300 mg, those treated with honokiol and IR alone weighed approximately 1,500 and 2,300 mg, respectively. Moreover, the tumors from animals treated with the honokiol–IR combination weighed less than 800 mg (Supplementary Fig. S2A). There was no apparent change in liver, spleen, or body weight in the animals (data not shown). These data implied that the honokiol–IR is a potential therapeutic combination for treating colon cancers but is relatively nontoxic to the animals. We also determined the effect of the combination on tumor vascularization by staining for endothelial-specific antigen CD31. As shown in Supplementary Fig. S2B, treatment with the honokiol–IR combination lead to a significant reduction in CD31 staining and to the obliteration of the normal vasculature compared with either honokiol or IR alone (Supplementary Fig. S2B).

To further investigate whether the honokiol–IR combination affects cancer stem cells, we determined specific marker expression in the tumor tissues. Western blot analyses showed that the honokiol–IR combination significantly reduced the expression of cancer stem cell proteins DCLK1, SOX-9, CD133, and CD44 (Fig. 6C, left panel), which was confirmed by immunohistochemistry (Fig. 6C, right panel). These data suggested that the combination of honokiol and IR targets colon cancer stem cells with high potency. Furthermore, treatment with the honokiol–IR combination resulted in significantly lower levels of activated Notch-1, its ligand Jagged 1, and the downstream target gene Hes-1 (Fig. 6D, left panel). There was also a significant reduction in the expression of γ-secretase complex proteins, presenilin 1 and 2, nicastrin, APH1 and PEN2 in both HCT116 and SW480 cells. Again, further confirmation of the downregulation was obtained by immunohistochemistry for the proteins in the xenograft tissue (Fig. 6D, right panel). These data suggested that the honokiol–IR combination significantly affects the expression of Notch signaling–related proteins, which might contribute to the inhibitory effects of this treatment.

Discussion

Our results indicate that the honokiol–IR combination has a significant potential as an anti-colorectal cancer therapeutic strategy. Unlike other polyphenolic agents, which have been hindered by poor absorption and rapid excretion, honokiol exhibits a desirable spectrum of bioavailability (4). Significant systemic levels of honokiol can be obtained in preclinical models, and it can also cross the blood brain barrier (4). Currently, it is not known whether honokiol has a single major target or several targets. However, it has several activities that make it desirable both as a therapeutic and as a chemopreventive agent. First, it is orally bioavailable and crosses the blood brain barrier. Second, it inhibits NF-κB activity in a manner that is different from other known inhibitors. Honokiol can also cause mitochondrial dysfunction in cancer cells (4).

Honokiol radiosensitizes colon tumor xenografts

To evaluate the role of honokiol–IR combination on tumor growth in vivo, we next examined its effects on growth of HCT116 xenografts. Honokiol was administered intraperitoneally to mice bearing xenografts and irradiated as shown in Fig. 6A. Although treatment with either honokiol or IR inhibited the growth of the tumor xenografts, there was reduction with the honokiol–IR combination (Fig. 6B). The excised tumors from control
These data together suggested that honokiol could be used as an effective agent either alone or in combination with IR and/or chemotherapeutic drugs. Although the compound alone can be administered as a chemopreventive agent, our data shows that it can be used in combination with other modalities such as chemotherapeutic drugs or radiation for therapeutic activity. Of course, there is a need to consider how the honokiol could be administered. For colorectal cancers, one mode could be oral administration, especially because it is water soluble and has been shown to be bioavailable. This would also be an attractive chemopreventive strategy. Another delivery method could be an intravenous route of administration, which could have efficacy in therapeutic paradigms. In this regard, a recent study showed that a biodegradable self-assembled PEG-PCL-PEG micelle encapsulating honokiol can be administered intravenously for effectively targeting colorectal cancers (28).
More importantly, this method was found to be effective, stable, and safe.

Honokiol disrupts many of the characteristic cancer-promoting events. Previous studies have shown that honokiol radiosensitizes lung cancer cells (29). Our current studies show a similar effect on colorectal cancer cells. The honokiol–IR combination inhibited proliferation of colorectal cancer cells and promoted apoptosis at a much higher rate than either honokiol or IR alone (29). Previous studies with LL/2 Lewis lung carcinoma cells showed that honokiol combined with radiotherapy can induce the cells to arrest in the G0/G1 phase and a corresponding decrease in the S-phase (29). Recently, we showed that whereas honokiol induced a G0/G1 phase arrest and that IR alone induces G2/M arrest, the combination induced higher rates of G2/M arrest (30). Given that radiation is known to induce mitotic catastrophe (apoptosis during mitosis), we hypothesize that the compound probably drives the cells through the cell cycle but never makes it out of mitosis because of IR-induced catastrophe. Thorough analyses of the transition of the cell through the various phases of the cell cycle are needed along with the checkpoint-related markers to confirm this phenomenon.

Recent studies have suggested that cancer stem cells have the capacity to drive tumor resistance and...
Honokiol Radiosensitizes Colon Cancer Stem Cells

DCLK1 stem cells are quiescent and also label retaining cells. Honokiol–IR–mediated inhibition of colorectal cancer and releases the NICD from the membrane. Therefore, ligand Jagged1 as well as all 4 essential members of the combination resulted in downregulation of the Notch signaling is also 10- to 30-fold higher in the stem cell renewal and vascular development (39). Studies, we have determined that the honokiol–IR combination is a potent inhibitor cancer stem cell based on 2 approaches. First, we determined that it inhibits the expression of DCLK1, a bonafide stem cell marker of the intestinal epithelial cells. The extracellular domain in DCLK makes it convenient to isolate and grow cancer stem cells in vitro. DCLK1+ intestinal stem cells are quiescent and also label retaining cells. DCLK1+ cells are also numerically rare in human colorectal cancers and these cells are also quiescent. Others have suggested that the stem cell expresses proteins such as LGR5 and SOX9 (36, 37). Unfortunately these are not cell surface expressing proteins and hence the need to resort to Western blotting techniques. Moreover, these cells are not quiescent and are actively dividing. The possibility exist that these proteins are expressed by immediate progenitors of the quiescent stem cells and hence are rapidly dividing daughter cells. Nevertheless, our Western blot analyses have shown that the honokiol–IR combination also inhibits the expression of these proteins along with other such proteins including CD133 and CD44. This was also confirmed in vivo where the combination significantly reduced the expression of these markers and also the growth of the xenografts.

Another method that is commonly used to show stemness is the growth of spheroids or colonies. The honokiol–IR combination inhibited colonosphere formation further suggesting that they target the cancer stem cells.

The Notch pathway plays a critical role in colon cancer (38). Notch also has been shown to be important in stem cell renewal and vascular development (39). Notch signaling is also 10- to 30-fold higher in the stem cells when compared with other cell types (40). In our studies, we have determined that the honokiol–IR combination resulted in downregulation of the Notch ligand Jagged1 as well as all 4 essential members of the γ-secretase complex, the critical enzyme that cleaves and releases the NICD from the membrane. Therefore, honokiol–IR–mediated inhibition of colorectal cancer cell growth is partly mediated via inactivation of Notch-1 activity. This was further confirmed by the combination of a GSI with honokiol–IR, which further inhibited proliferation and induced apoptosis. However, ectopic expression of NICD reversed the effects of honokiol and IR, and partially restored cell growth. Similarly, whereas the combination of honokiol and IR with a GSI further inhibited Hes-1 expression, the ectopic NICD partially rescued Hes-1 expression. Indeed studies have shown that targeting the γ-secretase complex using small molecule GSI inhibitors affect tumor-initiating cells in mouse model of ERBB2 breast cancer (41). It would also be interesting to determine whether there are other clients for the γ-secretase complex and the role of these client proteins in cancer stem cell biogenesis. Our data suggest that ectopic expression of the Notch intracellular domain only partially rescues the honokiol effect. This suggests that there might be other pathways that could also mediate the honokiol–IR effect. In this regard, honokiol has been shown to block TNF-α–induced NF-κB and Akt activation resulting in enhanced TNF-α–mediated cell death (42). A more recent study also showed that honokiol arrests cell cycle, induces apoptosis, and potentiates the cytotoxic effect of gemcitabine by affecting NF-κB (43, 44). These data suggest that NF-κB could also play a role in radiosensitizing colon cancer stem cells.

Recent reports from experimental and clinical studies have also proposed that combinations of radio- or chemotherapy agents with natural preventive agents have greater than additive effects resulting in increased efficacy while reducing potential side effects (45). This study provides evidence that treatment with the honokiol–IR combination results in a growth inhibition in vitro and in vivo. Furthermore, the combination treatment was more potent against colon cancer stem cells. In addition, the honokiol–IR combination significantly suppressed Notch-1 activation. Taken together, these data suggest that the combination of honokiol and radiation to target colon cancer stem cells is an attractive novel potential agent for the treatment and prevention colon cancer. Further studies are warranted to show the efficacy of the honokiol–IR combination in the clinical setting.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: S. Ponnurangam, Y. Zhang, S. Anant, D. Subramaniam, Y. Zhang
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