Targeting Survivin Inhibits Renal Cell Carcinoma Progression and Enhances the Activity of Temsirolimus

Jennifer S. Carew, Claudia M. Espitia, Weiguo Zhao, Monica M. Mita, Alain C. Mita, and Steffan T. Nawrocki

Abstract

Elevated expression of the antiapoptotic factor survivin has been implicated in cancer cell survival and disease progression. However, its specific contribution to renal cell carcinoma (RCC) pathogenesis is not well defined. We investigated the roles of survivin in RCC tumor progression, resistance to mTOR inhibitors, and evaluated the therapeutic activity of the survivin suppressant YM155 in RCC models. Here, we report that survivin expression levels were significantly higher in RCC cell lines compared with normal renal cells. Stable targeted knockdown of survivin completely abrogated the ability of 786-O RCC tumors to grow in mice, thus demonstrating its importance as a regulator of RCC tumorigenesis. We next explored multiple strategies to therapeutically inhibit survivin function in RCC. Treatment with the mTOR inhibitor temsirolimus partially diminished survivin levels and this effect was augmented by the addition of YM155. Further analyses revealed that, in accordance with their combined anti-survivin effects, YM155 significantly improved the anticancer activity of temsirolimus in a panel of RCC cell lines in vitro and in xenograft models in vivo. Similar to pharmacologic inhibition of survivin, shRNA-mediated silencing of survivin expression not only inhibited RCC tumor growth, but also significantly sensitized RCC cells to temsirolimus therapy. Subsequent experiments demonstrated that the effectiveness of this dual survivin/mTOR inhibition strategy was mediated by a potent decrease in survivin levels and corresponding induction of apoptosis. Our findings establish survivin inhibition as a novel approach to improve RCC therapy that warrants further investigation.

Introduction

Survivin (BIRC5) is an inhibitor of apoptosis protein (IAP) family member with key roles in cell division and apoptosis suppression (1, 2). Although it is highly expressed during embryonic and fetal development, survivin is not expressed in differentiated normal tissue except for the thymus, placenta, stem cell compartment, and basa epithelium of the colon (3). A number of studies have demonstrated the frequent elevation of survivin expression levels across multiple tumor types and this aberrant expression is correlated with aggressive disease and shorter overall patient survival (4–6). Because of its selected overexpression patterns and critical involvement in the control of cell proliferation and cell death, survivin is a promising target for therapeutic inhibition.

Targeted treatment options for renal cell carcinoma (RCC) include the mTOR inhibitors temsirolimus and everolimus and the multitargeted kinase inhibitors sorafenib and sunitinib. These agents have improved clinical outcomes, but drug resistance continues to be a major problem highlighting the need for novel therapeutic strategies (9, 10). mTOR is a key regulator of protein synthesis that effects many cellular functions, including cell division, angiogenesis, and apoptosis. It is also an important downstream mediator of the PI3K–AKT cell survival pathway that is deregulated in many cancers (11). Activation of mTOR induces the translation of various oncogenic proteins such as c-Myc and hypoxia-inducible factor-1α (HIF1α) that results in an increase in genes that promote angiogenesis (VEGF) and cell survival (survivin; refs. 12–14). Consistent with mTOR's role in these processes, we have previously shown that inhibition of mTOR decreases survivin levels, which significantly contributes to its anticancer activity (15).

We hypothesized that targeting survivin may be an effective strategy for RCC therapy that will increase the benefit of mTOR inhibition. Here we report, that survivin is widely overexpressed in RCC cell lines and that its expression is required for RCC tumor progression as survivin silencing completely abrogated tumor growth. We further demonstrated that survivin expression directly regulated temsirolimus sensitivity using knockdown and overexpression approaches. The survivin suppressant YM155 exhibited promising activity in RCC models and augmented the in vitro and in vivo activity of temsirolimus through a mechanism characterized by cooperative depletion of survivin levels. Collectively, our findings establish survivin as an essential regulator of both RCC progression and therapeutic sensitivity to temsirolimus. Clinical studies focused on targeting survivin expression as a new strategy to improve clinical outcomes for patients with RCC are warranted.
Materials and Methods

Cells and cell culture

786-O, A498, Caki-1, Caki-2, and Achn renal cancer cell lines were obtained from the ATCC. RCC4 cells were obtained from Dr. Sunil Sudarshan (University of Alabama at Birmingham, Birmingham, Alabama). Human renal cancer cell lines were maintained in RPMI supplemented with 10% FBS in a humidified incubator 37°C with 5% CO₂. Human normal renal proximal tubule epithelial cells (RPTC) were purchased from Clonetics and cultured in REGM media (REGM BulletKit, Clonetics). Cell lines were authenticated by the source banks using DNA profiling techniques and were used in this study in accordance with AACR guidelines.

Antibodies and reagents

Antibodies were obtained from the following commercial sources: anti-survivin (Santa Cruz Biotechnology); anti-cleaved caspase-3, anti-clAP-1, anti-clAP-2, anti-XIAP (Cell Signaling Technology); anti-β-tubulin (Sigma-Aldrich); anti-proliferating cell nuclear antigen (PCNA; Dako); goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Laboratories); Rat anti-mouse IgG2a-HRP (Serotec); and sheep anti-mouse-HRP and donkey anti-rabbit-HRP (Amersham). YM155 was kindly provided by Astellas Pharmaceuticals. Temsirolimus was obtained from the CTRC pharmacy.

qRT-PCR

cDNA from renal cells was used for relative quantification by RT-PCR analyses. First-strand cDNA synthesis was performed from 1 μg RNA in a 20-μL reaction mixture using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems). BIRC5 transcripts were amplified using commercially available TaqMan Gene expression assays (Applied Biosystems). GAPDH was used as a housekeeping gene.

Quantification of drug-induced cytotoxicity

Cell viability was assessed by MTT assay. Cells were seeded into 96-well microculture plates at 10,000 cells per well and allowed to attach for 24 hours. Cells were then treated with YM155, temsirolimus, or the combination for 24 hours. Following drug treatment, MTT was added and cell viability was quantified by enhanced chemiluminescence. The proapoptotic effects of YM155 and temsirolimus were quantified by PI staining and FACS analysis of sub-G₀/G₁ DNA and quantification of active caspase-3-positive cells by flow cytometry using a commercial kit (BD Biosciences) as previously described (16).

Preparation and transfection of survivin shRNA and overexpression constructs

Survivin lentiviral shRNA and nontarget control shRNA were obtained from Dharmacon. Cells were infected with lentiviral particles in media with 5 μg/mL polybrene and incubated at 37°C for 24 hours. Cells were cultured in 1 μg/mL puromycin to select for cells with stable knockdown of survivin expression. Knockdown of survivin levels was determined by immunoblotting and quantified using ImageJ software. The following target sequences were used:

<table>
<thead>
<tr>
<th>Survivin shRNA</th>
<th>Gene target sequence</th>
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<tbody>
<tr>
<td>Survivin #1</td>
<td>CACCTGAAAGCTTCCTCGA</td>
</tr>
<tr>
<td>Survivin #2</td>
<td>GAGTGATAGGAAGCGTCTG</td>
</tr>
<tr>
<td>Survivin #3</td>
<td>ATCTGTTCAAAAGCCGTA</td>
</tr>
</tbody>
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Survivin overexpression was carried out using the pORF5-hSurvivin expression vector (Invitrogen). Lyophilized GT100 E. coli bacteria transformed by pORF5-hSurvivin and pORF-mcs were streaked on an E. coli Fast-Media Amp plate (Invitrogen). After incubation at 37°C overnight, colonies from each construct were grown in LB supplemented with ampicillin using the Fast-Media Amp liquid (Invitrogen). Plasmids were isolated using a Qiagen mini-prep plasmid isolation kit (Qiagen Inc.). 786-O cells were transiently transfected with pORF5-hSurvivin and the empty vector pORF-mcs using the TransFast reagent (Promega) according to the manufacturer’s instructions. Briefly, 1 μg of each plasmid was diluted in serum-free medium. The diluted plasmids were vortexed before the addition of 3 μL Transfast reagent to bring the Transfast/DNA ratio to 1:1. DNA/Transfast mixtures were incubated at room temperature for 30 minutes to allow the Transfast/DNA complex formation. Cells were washed once with serum-free medium before adding DNA/Transfast mixtures. Cells in Transfast/DNA mixture were then incubated at 37°C for 1 hour. Following this incubation period, serum-containing medium was added to each well, and cells were incubated at 37°C overnight. Cells were then treated with drugs for 72 hours and apoptosis was measured by PI-FACS analysis. Survivin overexpression was confirmed at 72 hours by immunoblotting.

Immunoblotting

Renal cancer cells for immunoblotting were collected and lysed as previously described (16). Briefly, approximately 50 μg of total cellular protein from each sample were subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose membranes and blocked with 5% nonfat milk in a TBS solution containing 0.1% Tween-20 for 1 hour. The blots were then probed overnight with the relevant primary antibodies, washed, and probed with species-specific secondary antibodies coupled to HRP. Immunoreactive material was detected by enhanced chemiluminescence (West Pico, Pierce, Inc.).

Xenograft studies

786-O control and survivin shRNA cells (1 × 10⁶ per mouse) were collected, washed in PBS, and suspended in HBSS with Matrichel (BD BioSciences). Cells were then subcutaneously implanted into female nude mice (BALB/c background) from Harlan. Mice were monitored daily and tumors were measured twice weekly for 100 days or euthanized when mean tumor burden reached approximately 1,000 mm³. For treatment studies, 786-O and Caki-1 renal cancer cells (1 × 10⁶) were suspended in a mixture of HBSS and Matrichel and subcutaneously implanted into female nude mice (BALB/c background). Tumor-bearing animals from each cell line xenograft were randomized into treatment groups and treated with drugs twice weekly for 100 days or euthanized when mean tumor burden reached approximately 1,000 mm³. 786-O and Caki-1 renal cancer cells (1 × 10⁶) were suspended in a mixture of HBSS and Matrichel and subcutaneously implanted into female nude mice (BALB/c background). Tumor-bearing animals from each cell line xenograft were randomized into treatment groups and treated with drugs twice weekly for 100 days or euthanized when mean tumor burden reached approximately 1,000 mm³.

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groups. Mice were treated with vehicle, temsirolimus (2 mg/kg IV, QDx21), YM155 (5 mg/kg administered as a 7-day continuous infusion using an implanted Alzet micro-osmotic pump), or both agents. Mice were monitored daily and tumor volumes were measured twice weekly. On day 11, tumors from representative animals were excised from each group, formalin fixed and paraffin embedded for IHC analysis.

IHC

Paraffin-embedded tumor sections were mounted on slides, which were used to determine survivin, PCNA, and cleaved caspase-3 expression levels. Sections were deparaffinized in xylene, treated with a graded series of alcohol [100%, 95%, and 80% ethanol/double-distilled H$_2$O (v/v)] and rehydrated in PBS (pH 7.5). Heat-induced epitope retrieval was performed by microwaving slides in a citrate buffer for 5 minutes. The slides were allowed to cool and endogenous peroxides were blocked with a 3% hydrogen peroxide solution for 10 minutes. Slides were then incubated in a protein block solution (5% horse and 1% goat serum in PBS) for 1 hour at ambient temperature. Positive reactions were visualized by immersing the slides with stable 3,3'-diaminobenzidine (Research Genetics) for 10 minutes. The sections were rinsed with distilled water and counterstained with Gill's hematoxylin (Sigma). Images were captured using an Olympus fluorescent microscope with a DP71 camera and a ×20 objective. Image-Pro Plus software Version 6.2.1 (MediaCybernetics) was used for image acquisition and survivin expression was quantified by densitometric analysis of five random high-power fields containing viable tumor cells. Quantification of cleaved caspase-3 and PCNA was conducted by manually counting the number of positive cells in five random fields.

Figure 1. Survivin is overexpressed in RCC cell lines and is essential for RCC tumor growth. A, survivin is overexpressed in RCC cell lines. Cells were collected and survivin expression was determined by qRT-PCR. Mean ± SD, n = 3. B, survivin is overexpressed at the protein level in RCC cells. Survivin expression was measured by immunoblotting in normal RPTEC cells and in a panel of RCC cell lines. C, silencing survivin expression using three different shRNA constructs. 786-O RCC cells were transfected with nontarget control or survivin shRNA and cultured in puromycin-containing media to produce cells with stable survivin knockdown. Immunoblotting confirmed a reduction in survivin levels, which was quantified by densitometry. D, potent knockdown of survivin prevents RCC tumor growth. A total of 1 x 10$^7$ 786-O shRNA control or shRNA survivin cells were implanted into nude mice. Tumors were measured twice weekly until the mean tumor volume was 1,000 mm$^3$ or mice reached day 100. Mean ± SEM, n = 5.
Statistical analyses
Statistical significance of differences observed between samples was determined using the Student t test or the ANOVA Tukey–Kramer comparison test where appropriate. Differences were considered significant in all experiments at P < 0.05.

Results
Potent knockdown of survivin is required to inhibit RCC tumor growth
Survivin plays a key role in promoting cell-cycle progression and inhibiting apoptosis (1). In accordance with this, high survivin expression has been observed in many different cancer types and has been associated with tumor progression, drug resistance, and poor prognosis (17–21). Therefore, targeting survivin expression may have potent anticancer activity. We first evaluated survivin expression in a panel of RCC cell lines and in normal RPTEC. Analysis of survivin expression by qRT-PCR (Fig. 1A) and immunoblotting (Fig. 1B) revealed that survivin was significantly overexpressed in all RCC cell lines tested with the highest expression observed in 786-O, A498, and Caki-1. To investigate the importance of survivin expression as a regulator of RCC tumor progression, we knocked down survivin levels in 786-O cells using three different lentiviral shRNA constructs. Survivin levels were potently silenced in stable cell lines generated from shRNA construct #1 (>99% knockdown) and #2 (>95% knockdown) and moderately reduced in construct #3 (approximately 60% knockdown; Fig. 1C). In agreement with the key role of survivin...
in tumor proliferation, cells with highly silenced survivin expression were unable to form tumors in mice (Fig. 1D). Interestingly, moderate survivin knockdown did not alter tumor formation or tumor growth compared with cells transfected with control shRNA constructs. This suggests that even modest aberrant expression of survivin is sufficient to promote RCC pathogenesis.

Survivin expression regulates sensitivity to temsirolimus

Although temsirolimus has demonstrated clinical benefit in RCC, drug resistance continues to be a major problem (22). We hypothesized that survivin levels may control sensitivity to mTOR inhibitors. Temsirolimus treatment stimulated a dose-dependent reduction in survivin expression in RCC cell lines (Fig. 2A). However, high-dose temsirolimus was insufficient to completely reduce survivin expression. We further investigated the role of survivin as a regulator of temsirolimus sensitivity by assessing the impact of differential survivin expression on temsirolimus activity in paired 786-O cells with shRNA-mediated knockdown and forced overexpression. As expected, survivin knockdown significantly augmented temsirolimus-mediated apoptosis (Fig. 2B) and overexpression promoted drug resistance (Fig. 2C). These results suggest that targeting survivin levels may be an effective approach to enhance the anticancer activity of temsirolimus.

YM155 decreases survivin levels and induces apoptosis in RCC cells

Considering that genetic inhibition of survivin yielded significant anti-RCC effects in our initial series of experiments, we next investigated the activity of the clinical survivin suppressant YM155 (Fig. 3A). Treatment with YM155 selectively reduced survivin levels without significantly altering the expression levels of other IAPs, including cIAP-1, cIAP-2, and XIAP (Fig. 3B). Consistent with the drug-induced reduction in survivin levels, MTT assays demonstrated that YM155 treatment diminished RCC cell viability in a dose-dependent manner (Fig. 3C). YM155 also promoted apoptosis in RCC cells as measured by PI-FACS analysis (Fig. 3D) and caspase-3 activation (Fig. 3E). Collectively, these data demonstrate that targeting survivin expression with YM155 is an effective strategy to stimulate RCC cell death.
YM155 and temsirolimus cooperate to diminish survivin levels and promote enhanced apoptosis

Considering that our data demonstrated that survivin overexpression is sufficient to confer resistance to temsirolimus, we next investigated the ability of YM155 and temsirolimus to cooperate in RCC cancer cell lines. Treatment with either YM155 or temsirolimus alone triggered a partial reduction in survivin expression (Fig. 4A). However, combination treatment resulted in a very substantial decrease in survivin levels (Fig. 4A) that directly correlated with reduced RCC cell viability compared with single-agent therapy (Fig. 4B). Formal synergy analysis demonstrated that combinations of 50 nmol/L temsirolimus and 10 nmol/L YM155 produced combination indices (CI) lower than one in all six RCC cell lines—786-O = 0.75, Caki-1 = 0.83, Caki-2 = 0.62, A498 = 0.68, Achn = 0.73, and RCC4 = 0.64—thus demonstrating the synergistic anticancer activity of this combination. To determine whether the decrease in cell viability was due to the induction of apoptosis, cell death was measured by PI-FACS analysis and the determination of active caspase-3 levels. Consistent with the observed potent decrease in survivin expression induced by the YM155 and temsirolimus combination, apoptosis was significantly increased in all RCC cell lines following combination therapy (Fig. 4C and D).

YM155 augments the anticancer activity of temsirolimus in RCC xenografts

To further investigate the potential benefit of the temsirolimus and YM155 combination, xenograft studies were performed by implanting 786-O and Caki-1 cells into nude mice. Tumor-bearing animals were randomized into groups and given 2 mg/kg temsirolimus i.v. QDx21, 5 mg/kg YM155 by 7-day continuous infusion, or the combination. Treatment with temsirolimus alone resulted in a dramatic decrease in mean tumor volume in 786-O and Caki-1 compared with the vehicle-treated controls (Fig. 5A and B). YM155 monotherapy also stimulated a reduction in tumor growth. Importantly, the combination significantly and more effectively decreased tumor burden in both 786-O and Caki-1 models compared with either single-agent alone (Fig. 5A and B).
In addition, drug treatment with the combination was very well tolerated, as no significant animal weight loss was observed at the completion of the study (Fig. 5C).

YM155 and temsirolimus decrease survivin expression, inhibit tumor cell proliferation, and stimulate apoptosis in vivo

Two RCC xenograft studies demonstrated that YM155 augments the antitumor activity of temsirolimus in vivo. Because survivin is a key regulator of cell division and apoptosis and has been linked to drug resistance, we determined its expression in tumor specimens following drug treatment. IHC revealed that survivin levels were significantly reduced in tumors collected from temsirolimus and YM155 treated mice and the combination of both agents produced a further decrease in survivin expression (Fig. 6A). The substantial reduction in survivin levels was linked to a significant decrease in tumor cell proliferation (Fig. 6B) and corresponding increase in tumor cell apoptosis (Fig. 6C) in tumors obtained from mice treated with the combination. Taken together, our data demonstrate that targeting survivin expression with temsirolimus and YM155 is a promising antitumor strategy for RCC.

Discussion

The incidence and mortality of RCC have been increasing over the last several decades (23). Current treatment options include mTOR inhibitors such as temsirolimus and the multitargeted kinase inhibitors sorafenib and sunitinib (24). However, resistance to these agents is an emerging problem and new therapeutic strategies to circumvent and/or overcome drug resistance are clearly needed. Survivin is a key regulator of cell division, an inhibitor of apoptosis, and has also recently been identified as an important mediator of drug resistance (20, 25, 26). Recent studies have suggested that survivin may play a key role in RCC pathogenesis as its high expression levels were linked to tumor aggressiveness and poor prognosis (6, 27, 28). Loss-of-function of the von Hippel–Lindau (VHL) tumor suppressor gene results in constitutive HIF expression and has been well documented in RCC (29). However, a direct relationship between VHL status and response to
VEGF or mTOR inhibitors has not been observed in clinical studies (30, 31). Furthermore, the absence of RCC tumorigenesis in VHL-deficient mice along with genetic data collected from humans suggests that RCC development likely requires additional cofactors that may synergize with VHL loss to trigger oncogenic transformation (32–34). Interestingly, increased survivin levels were recently reported to be associated with hypoxia (35). Therefore, it is possible that high HIF expression also contributes to survivin overexpression in RCC. Consistent with these findings, we also determined that survivin levels were significantly higher in a panel of RCC cell lines compared with normal renal cells.

To evaluate the potential contribution of survivin to RCC pathogenesis, we generated three stable cell lines with varying degrees of survivin expression. We selected 786-O cells as this cell line exhibited the highest survivin levels in our RCC cell line panel. Efficient knockdown of survivin (>95%) completely eliminated in vivo tumor growth capacity, thus demonstrating proof-of-principle that targeting survivin expression is a promising approach for RCC treatment. Our findings are in agreement with previous reports demonstrating that survivin-deficient cells have low tumor formation in xenografts (36, 37). Interestingly, a partial decrease (60% knockdown) in survivin levels did not alter
tumor growth compared with control shRNA-transfected cells suggesting that a high threshold of survivin depletion must be reached in order to achieve therapeutic benefit.

Although a plethora of studies confirm the importance of survivin as a catalyst of cancer progression, few agents that selectively target its expression currently exist. Several approaches to disrupt its function have been used, including the use of antisense oligonucleotides such as SPC3042 and small-molecule inhibitors such as YM155 and terameprocol (EM1421; refs. 38, 39). YM155 was identified in a high-throughput screen to identify agents that selectively block survivin promoter activity and is the most clinically advanced of this new class of agents (40). It has demonstrated activity against various tumor types in preclinical studies (19, 40–43). We selected the VHL null 786-O and the VHL wild-type Caki-1 cell lines as models for our xenograft studies as they both displayed high basal survivin expression. Because of its short half-life, YM155 was administered by continuous infusion using an Alzet mini-pump over a period of 7 days. Continuous treatment with YM155 resulted in stable disease burden in both RCC tumor models. However, the 786-O tumors eventually progressed in the absence of YM155 (day 17). Interestingly, Caki-1 tumor burden remained relatively stable even after YM155 withdrawal, suggesting that this tumor type may be more sensitive to survivin depletion. We plan to further evaluate targeted survivin suppression in additional models of RCC with differing VHL status.

Several clinical studies of YM155 revealed that it was very well tolerated, but likely would be best utilized in combination chemotherapy due to its limited single-agent efficacy (44–48). Multiple combination studies with YM155 were conducted with paclitaxel or docetaxel regimens based on the rationale that YM155 may be able to overcome taxane-mediated upregulation of survivin expression, which promotes drug resistance (44, 48–50). However, the lack of efficacy that was observed in these clinical studies may have been due to incomplete suppression of survivin levels in the tumors. As demonstrated by our survivin silencing studies, a very high degree of survivin depletion is required to achieve antitumor efficacy. We hypothesized that combining YM155 with another agent that reduces survivin expression may yield a more potent decrease in survivin expression and improve efficacy. Inhibitors of PI3K, AKT, mTOR, histone deacetylase (HDAC), and MEK/ERK signaling have all been reported to modulate survivin expression (15, 17). We focused on the mTOR inhibitor temsirolimus rather than the VEGF/multi-tyrosine kinase inhibitors (sunitinib and sorafenib) due to its ability to significantly decrease survivin expression in our preclinical models and its considerable activity against RCC. Consistent with our hypothesis, the addition of YM155 to temsirolimus synergistically reduced survivin expression in RCC cell lines and significantly decreased tumor burden in both tested RCC xenograft models compared with single-agent treatment. Analysis of tumor specimens taken following treatment revealed highly depleted survivin levels and increased induction of apoptosis indicating that tumor cell death directly contributed to the activity of this combination. In our study, temsirolimus exhibited limited in vitro proapoptotic activity, but displayed potent anticancer activity in vivo. This observation may be, in part, due to the ability of mTOR inhibitors to block angiogenesis, which results in a more robust antitumor response in xenograft studies. Importantly, we demonstrated that the limited cytotoxicity of temsirolimus could be enhanced through depletion of survivin levels.

Our collective findings demonstrate that survivin expression is essential for RCC progression and is a key mediator of resistance to temsirolimus. Our data provide a strong rationale for the design of clinical trials to evaluate the efficacy of YM155 in combination with mTOR inhibitors or other agents that have previously been identified to reduce survivin levels, such as PI3K, AKT, HDAC, or MEK/ERK inhibitors.

Disclosure of Potential Conflicts of Interest

S.T. Nawrocki reports receiving a commercial research grant from Astellas Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: J.S. Carew, M.M. Mita, A.C. Mita, S.T. Nawrocki


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.S. Carew, C.M. Espitia, W. Zhao, S.T. Nawrocki

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.S. Carew, C.M. Espitia, M.M. Mita, A.C. Mita, S.T. Nawrocki

Writing, review, and/or revision of the manuscript: J.S. Carew, C.M. Espitia, M.M. Mita, A.C. Mita, S.T. Nawrocki

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.M. Espitia, W. Zhao, S.T. Nawrocki

Study supervision: A.C. Mita, S.T. Nawrocki

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References


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