Preclinical Development

Targeting the Insulin Growth Factor and the Vascular Endothelial Growth Factor Pathways in Ovarian Cancer

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Abstract

Antiangiogenic therapy is emerging as a highly promising strategy for the treatment of ovarian cancer, but the clinical benefits are usually transitory. The purpose of this study was to identify and target alternative angiogenic pathways that are upregulated in ovarian xenografts during treatment with bevacizumab. For this, angiogenesis-focused gene expression arrays were used to measure gene expression levels in SKOV3 and A2780 serous ovarian xenografts treated with bevacizumab or control. Reverse transcription-PCR was used for results validation. The insulin growth factor 1 (IGF-1) was found upregulated in tumor and stromal cells in the two ovarian xenograft models treated with bevacizumab. Cixutumumab was used to block IGF-1 signaling in vivo. Dual anti-VEGF and IGF blockade with bevacizumab and cixutumumab resulted in increased inhibition of tumor growth. Immunohistochemistry measured multivessel density, Akt activation, and cell proliferation, whereas terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay measured apoptosis in ovarian cancer xenografts. Bevacizumab and cixutumumab combination increased tumor cell apoptosis in vivo compared with therapy targeting either individual pathway. The combination blocked angiogenesis and cell proliferation but not more significantly than each antibody alone. In summary, IGF-1 activation represents an important mechanism of adaptive escape during anti-VEGF therapy in ovarian cancer. This study provides the rationale for designing bevacizumab-based combination regimens to enhance antitumor activity. Mol Cancer Ther; 11(7); 1576–86. © 2012 AACR.

Introduction

Ovarian cancer arises from the epithelial layer covering the surface of ovaries and metastasizes intraperitoneally (i.p.). Tumor growth and metastasis rely heavily on efficient development of new blood vessels (1). The peritoneal environment is rich in secreted factors, including VEGF (2), lysophosphatic acid (3), platelet-derived growth factor (PDGF; ref. 4), TGF-β, and others, which stimulate angiogenesis and growth of ovarian tumors. Angiogenic signatures correlate tightly with survival of patients with ovarian cancer (5), underscoring the significance of angiogenesis to ovarian tumor progression. Of these factors, VEGF plays a significant role. Immunostaining detects VEGF expression in most ovarian tumors and omental metastases, and VEGF is measurable in ovarian cancer patients’ ascites and serum (6). VEGF and VEGFR-2 expression levels in ovarian tumors are independent poor prognostic factors (7–9) and correlate with surgical stage (10). VEGF is secreted in malignant ascites at concentrations 50 to 200 times higher than in nonmalignant fluid (11) and serum VEGF concentrations correlate with prognosis (12).

Antiangiogenic therapy (AAT) and particularly anti-VEGF strategies have direct inhibitory effects on blood vessels and may also block the proliferation of ovarian cancer cells (13). Anti-VEGF agents inhibit angiogenesis, destroy existent vessels, and facilitate normalization of abnormally tortuous and fenestrated vessels (14, 15). The effects on blood vessels lead to increasing hypoxia, nutrient deprivation, and other dynamic changes in the tumor microenvironment causing arrest of proliferation and/or apoptosis of cancer cells (16).

Recent phase II studies have indicated that AAT is highly effective in ovarian cancer (17–20). On the basis of those results, bevacizumab, a humanized monoclonal antibody to VEGF was investigated in the upfront treatment of advanced stage ovarian cancer along with chemotherapy (GOG protocol 218 and ICON 7; refs. 21, 22). The recent results of these studies showed improvement in progression-free survival for women treated with bevacizumab in combination with standard chemotherapy, bringing the agent to the forefront of ovarian cancer adjuvant therapy. However, although patients with ovarian cancer derive initial clinical benefit from bevacizumab,
this is usually transitory and has not translated into improved long-term survival (21, 22).

It has been speculated that under the pressure of AAT, adaptive escape and survival pathways emerge, facilitating alternative mechanisms of growth or metastasis. For instance, in one study using glioblastoma and pancreatic islet tumors, anti-VEGF therapy elicited activation of the Met pathway leading to increased tumor invasiveness (23–25). In another study using melanoma and sarcoma models, treatment with anti-VEGF antibody increased recruitment of myeloid cells (Gr1^+^) to the necrotic areas of tumors, altering the proinflammatory milieu (26, 27) that led to increased propensity to metastasis. We hypothesized that such adaptive mechanisms are activated in ovarian tumors treated with bevacizumab and set out to explore alterations of angiogenesis-related gene expression levels in ovarian cancer xenografts treated with bevacizumab by using pathway-focused gene expression arrays.

Here, we report that the insulin growth factor-1 (IGF-1) is upregulated during treatment with bevacizumab of ovarian xenografts and show that dual anti-VEGF and IGF blockade results in enhanced tumor growth inhibition compared with therapy targeting a single pathway. These results point to IGF-1 upregulation as representing an important mechanism of adaptive escape during anti-VEGF therapy in ovarian cancer and provide the rationale for designing bevacizumab-based combination regimens to increase antitumor activity.

**Materials and Methods**

**Ovarian cancer cell lines and materials**

SKOV3 ovarian cancer cells were from the American Type Culture Collection. A2780 cells were a gift from...
Dr. J. Turchi of Indiana University (Indianapolis, IN) and human umbilical vein endothelial cell (HUVEC) cells were from Cell Applications. The cells were not authenticated. SKOV3 cells were cultured in growth media containing 1:1 MCDB 105 (Sigma) and M199 (CellGro), A2780 cells were grown in Dulbecco’s Modified Eagle’s Medium (Sigma), and HUVEC cells were cultured in endothelial growth media supplemented with growth factors, as per provider’s instructions (Cell Applications). Cancer cell media was supplemented with 10% heat-inactivated FBS (CellGro) and 1% antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) and cells were grown at 37°C in a humidified 5% CO2 atmosphere. Cixutumumab was provided by Dr. Ruslan Novosyadlyy of Imclone and bevacizumab was purchased from Indiana University Pharmacy.

**In vitro treatment with cixutumumab and bevacizumab**

Used 20 and 75 μg/mL, respectively, for 3 days.

**In vivo anti-VEGF and IGF therapy**

Seven to 8-week-old female nude mice (nu/nu BALB/c) were purchased from Harlan. Animal procedures were approved by the Indiana University School of Medicine Animal Care and Use Committee and were in accordance with federal regulations. After acclimatization, A2780 cells or SKOV3 cells (5 × 10⁶ cells/mouse) were injected subcutaneously in the right flank. Tumors were measured weekly with calipers, and tumor volume in mm³ was calculated according to the formula: volume = width² × length/2. Treatments were started when tumors became measurable, usually within 1 week of s.c. tumor implantation. To study tumor response to bevacizumab, the agent was administered i.p. at a dose of 5 mg/kg twice a week. Control mice were treated with 0.9% sodium chloride. Treatment was continued until tumors exceeded 2 cm³ or animals showed signs of distress, at which point mice were sacrificed and tumors were harvested, measured, and snap frozen. When possible, tumors from each group were minced in the presence of hyaluronidase at a concentration of 1 mg/mL and cultured. The xenograft-derived cultures were characterized by proliferation (MTT) and clonogenic assays.

To measure response to bevacizumab and cixutumumab, mice were treated with bevacizumab (5 mg/kg, i.p., twice a week); cixutumumab (60 mg/kg, i.p., twice a week); bevacizumab and cixutumumab (5 mg/kg, i.p., and 60 mg/kg, i.p., respectively, twice a week), or v/v saline control i.p. Each experimental group consisted of 5 to 6 animals. Both cell lines (SKOV3 and A2780) were tested. Treatment continued for up to 3 weeks (A2780 cells) and 6 weeks (SKOV3 cells), at which point mice were euthanized, tumors harvested and paraffin embedded for future studies.

**Angiogenesis PCR array**

RNA was extracted from tumors harvested and snap frozen from control and bevacizumab-treated animals (n = 3 per group and per cell line), by using the RNA STAT-60 reagent (Tel-Test, Inc.). RNA was reverse-transcribed using the RT2 First Strand Kit (C-03; SA Biosciences), according to the manufacturer’s instructions. Genomic DNA contamination was eliminated by DNAse treatment by using RNEasy Micro Kit (Qiagen GmbH). Human Angiogenesis RT2 Profiler PCR Array and RT2 Real-Time SyBR Green/ROX PCR Mix were purchased from SA Bioscience Corporation. PCR was carried out on ABI Prism 7900 Sequence Detector (Applied Biosystems). For data analysis, the ΔΔCt method was used; for each gene, fold-changes were calculated as difference in gene expression between control and bevacizumab-treated groups. A positive value indicated gene upregulation and a negative value indicated gene downregulation.

**Reverse transcriptase PCR**

RNA extracted from xenografts by using the RNA STAT-60 reagent (Tel-Test, Inc.) was reverse-transcribed...
using the iScript cDNA synthesis kit (Bio-Rad). The PCR reactions used Taq polymerase (Promega) and the following primers: IGF-1 (forward): 5'-CCTGCGCAATGGAA-TAAAGT-3', (reverse): 5'-TCAAATGTACTTCCTTCTGGTC-3'; GAPDH (forward): 5'-GATTCCACCTGTGGCAAATTCC-3'; (reverse): 5'-CACGTTGGCAGTG-GGGAC-3'. For real-time PCR, we used the FastStart TaqMan Probe Master (Rox, Roche) on an ABI Prism 7900 platform (Applied Biosystems) according to the manufacturer’s procedures. The primers and probes used are enclosed in Supplementary Table S1. At the end of the PCR reaction, a melting curve was generated and the cycle threshold (Ct) was recorded for the reference gene and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative expression of gene of interest was calculated as ΔΔCt, measured by subtracting the Ct of the reference gene from that of the control. Results are presented as mean ± SD of replicates. Each measurement was carried out in duplicate and repeated in independent conditions.

Western blotting
Tumors were minced on ice and resuspended in radiolysis precipitation assay buffer containing protease inhibitors leupeptin (1 μg/mL), aprotinin (1 μg/mL), phenylmethylsulfonyl fluoride (PMSF; 400 μmol/L), and sodium orthovanadate (Na3VO4; 1 mmol/L). Tumor or cell lysates were sonicated briefly and subjected to centrifugation at 14,000 rpm for 15 minutes at 4°C to sediment particulate material. Equal amounts of protein (50 μg) were separated by SDS–PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore). After blocking, membranes were probed with primary antibody overnight at 4°C with gentle rocking. Antibodies used are GAPDH (1:5,000 dilution; Biodesign International), total IGF-1 receptor β, phospho-IGF-1 receptor β (Tyr1150/1151), phospho-IRS-1 (Ser307), VEGFR-2 (Cell Signaling), and Stat-5B (1:1,000; Santa Cruz; SC-1656). After incubation with specific horseradish peroxidase–conjugated secondary antibody, antigen–antibody complexes were visualized using the enhanced chemiluminescence detection system (Amersham Biosciences). Images were captured by a luminescent image analyzer with a CCD camera (LAS 3000, Fujifilm).

Immunohistochemistry
Immunohistochemistry evaluated multivessel density (MVD) by staining for CD31, cell proliferation by staining...
for Ki67, and measured phospho-Ser^{273}\)-Akt on paraffin-embedded sections. Antibodies were from Cell Signaling (phospho-Akt, 1:100 dilution), Dako (Ki67, clone M7248, 1:100 dilution), and from Abcam (CD31, 1:250 dilution). After incubation with primary antibodies overnight at 4°C, detection was obtained with the avidin-biotin peroxidase technique using the DAKO Detection Kit (DAKO-Cytomation). For phospho-Akt, scoring used a 0–3+ scale for intensity and recording of the percentage of cells staining. An H score was calculated as the product between intensity and percentage of cells staining and compared between groups. For Ki67, the numbers of positive cells were counted per 5 high-power fields (HPF) per specimen and average values were compared between groups. MVD was assessed by counting the number of vessels lined by CD31-positive endothelial cells in 5 areas that appeared to have the highest vessel density per specimen. Numbers of vessels were averaged and compared between groups. Cell apoptosis was analyzed on frozen 5-μm thick tumor sections with In Situ Cell Death (Apoptosis) Detection allowing terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) labeling (Roche Applied Science), according to the manufacturer’s instructions. Numbers of cells staining under UV excitation in 5 HPF per specimen were averaged for comparison between groups.

**Statistical analysis**

Statistical analyses were conducted using SAS version 9.3. Shapiro–Wilk and Levene tests were used to test normality and equal variance of the data, respectively. When normality and equal variance assumptions held, groups were compared using t tests or one-way ANOVA with significant overall tests followed by pairwise tests adjusted for multiple comparisons using the Student Newman–Keuls approach; otherwise Kruskal–Wallis tests were done with significant overall tests followed by pairwise exact Wilcoxon rank sum tests adjusted for multiple comparisons using the Hochberg–Bonferroni Step-Up approach. A P value of less than 0.05 was deemed significant. When an overall test was not significant, post hoc tests (t tests or exact Wilcoxon rank sum tests) were done to compare selected group combinations.
Results

IGF-1 is induced by treatment with bevacizumab in ovarian xenografts

Quantitative pathway-focused gene expression analysis was carried out in xenografts derived from 2 serous ovarian cancer cell lines (SKOV3 and A2780) after treatment with bevacizumab or control by using angiogenesis gene expression superarrays (Fig. 1). These arrays permit quantification of 92 angiogenesis-related genes and 6 housekeeping genes through quantitative RT-PCR methodology. Xenografts derived from A2780 cells had a doubling time of 4 days (control) versus 8 days (bevacizumab); whereas xenografts derived from SKOV3 cells had a doubling time of 7 days (control) versus 28 days (bevacizumab). Six genes were significantly upregulated in A2780 xenografts treated with bevacizumab compared with control, at a P < 0.05 level of significance: ID-1, TNFa-induced protein 2, IGF-1, heparinase, thrombospondin-1 and 2, and tissue inhibitor of metalloproteinase (TIMP)-1 (Table 1, Fig. 2A). Five genes were upregulated in SKOV3 xenografts treated with bevacizumab compared with control, at a P < 0.05 level of significance: angiopoietin-like 3, collagen type 4α3, IGF-1, interleukin-1β (IL-1β), and sphingosine 1 phosphate receptor (Table 1, Fig. 2A).

Next, the activity of cixutumumab, of bevacizumab, and of the combination of 2 antibodies was tested in vitro in HUVEC and SKOV3 cells, consistent with this antibody’s known mechanism of action (Fig. 3A and B). As anticipated, treatment with bevacizumab did not affect IGF-1 receptor expression level. Interestingly, in HUVEC cells, bevacizumab and cixutumumab single agents also induced downregulation of VEGF receptor 2, the main receptor involved in VEGF signaling. The combination of cixutumumab and bevacizumab significantly downregulated VEGFR2 expression in HUVEC cells, as compared with each agent alone (Fig. 3A). These data suggest that cixutumumab has a direct effect on VEGFR2 expression levels and that this effect is synergistic with bevacizumab’s effects.

Effects on tumor angiogenesis were measured by assessing MVD in SKOV3- and A2780-derived xenografts. Numerically, both single agents cixutumumab and bevacizumab decreased MVD in ovarian xenografts and the combination regimen further reduced MVD compared with each agent alone; however, the overall test was not statistically significant (Fig. 4A and B, P = 0.05 and Supplementary Fig. S2A and S2B). Apoptosis was measured by TUNEL staining in xenografts treated with control, cixutumumab, bevacizumab, and the 2 antibody combination (Fig. 4C and D, P < 0.05 for overall test). Increased apoptosis was induced by the combination bevacizumab and cixutumumab, but not by single agent anti-VEGF or anti-IGF-1R antibody.
**Discussion**

Blockade of tumor angiogenesis represents a promising targeted therapy in ovarian cancer, however its effects are transitory and impact on overall survival is not proven (22). Identification of rational combinations to improve the efficacy of antiangiogenic drugs is therefore imperative and represents an unmet need. The goal of this study was to gain a deeper understanding of the adaptive response to anti-VEGF therapy in ovarian cancer models and to identify pathways that are potentially responsible for tumor escape under continued treatment with bevacizumab.

Because one of the proposed resistance mechanisms to anti-VEGF therapy is switch to and reliance on alternative angiogenic pathways, we focused on discovering angiogenesis-related genes altered in tumors exposed to bevacizumab. Interestingly, gene expression array analysis did not identify altered expression of the VEGF family factors during bevacizumab treatment and this was confirmed by qRT-PCR for VEGFA in ovarian xenografts. However, IGF-1 upregulation was detected and was the common denominator in 2 independent xenograft models tested versus the corresponding vehicle-treated controls. Interestingly, IGF-1, as measured by gene expression arrays, was not significantly upregulated in SKOV3 xenografts after 4 weeks of bevacizumab treatment (e.g., at the point of maximal tumor response, Fig. 1); but emerged as an upregulated transcript in tumors resistant (A2780) or persistent (SKOV3) during treatment with bevacizumab.

The interest in this candidate gene was further increased by the availability of several agents targeting this pathway, currently being evaluated in clinical trials (32) and the significance of the pathway in ovarian cancer. IGF-1 stimulates growth of ovarian xenografts (33) and inhibition of the pathway with a neutralizing anti-IGF-1R antibody blocked tumor growth and reduced receptor signaling through IRS-1, PKC, mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K)-AKT pathways (33, 34). IGF-1 expression in ovarian tumors is associated with aggressive disease progression and increased angiogenesis through both autocrine and paracrine effects (35). Furthermore, IGF-1 receptor expression levels correlate with cisplatin resistance in ovarian cancer cell lines (34). Collectively, these data support a role of the IGF pathway in progression of ovarian cancer. To our knowledge, our study links for the first time IGF-1 to tumor escape under continued treatment with bevacizumab.

For the experiments included in this report, we used cixutumumab, an antibody against IGF-1 receptor. Cixutumumab downregulated the expression levels of IGF-1 receptor in HUVEC and in SKOV3 cells and downregulated VEGFR-2 in HUVEC cells, acting in a synergistic manner with bevacizumab. The observed synergistic effect of bevacizumab and cixutumumab growth in vivo, by suppressing angiogenesis, cell proliferation, and enhancing apoptosis.

**Figure 3.** Effects of bevacizumab, cixutumumab, and combination bevacizumab and cixutumumab in vitro and in vivo. A, Western blotting for IGF-1 receptor (β), VEGFR-2, and phospho-IGF-1 receptor (β) in HUVEC cells treated with cixutumumab (20 μg/mL), bevacizumab (75 μg/mL), and the combination of cixutumumab and bevacizumab. GAPDH was used as control. B, Western blotting for IGF-1 receptor (β), VEGFR-2, and phospho-IGF-1 receptor (β) in SKOV3 cells treated with cixutumumab (20 μg/mL), bevacizumab (75 μg/mL), and the combination. GAPDH was used as control. C, growth curve for SKOV3 xenografts. Results are means ± SE of 5 mice per group, for overall ANOVA test at week 6; cixutumumab was different from control (P < 0.05 for overall Kruskal-Wallis test at week 6). D, growth curve for A2780 xenografts. Results are means ± SE of 5 mice per group, P < 0.05 for overall ANOVA test at day 17. Control was different from all other groups and cixutumumab alone was different from bevacizumab and the combination therapy.

Treatment with bevacizumab and with cixutumumab also inhibited cell proliferation, as assayed by Ki67 staining in SKOV3 and A2780 xenografts. The combination cixutumumab and bevacizumab suppressed Ki67, but not to a greater extent than each agent alone (Fig. 5A and B, and Supplementary Fig. S3C, P < 0.05 for overall test). Decreased in Akt phosphorylation as measured by immunohistochemistry was induced numerically by both cixutumumab and bevacizumab, but the results were not statistically significant (Fig. 5C and D). These data suggest that cixutumumab and bevacizumab inhibit tumor growth in vivo, by suppressing angiogenesis, cell proliferation, and enhancing apoptosis.
in vivo supports the hypothesis that IGF-1 induction is part of a key adaptive mechanism for tumor cells, potentially under the selection pressure in the microenvironment of bevacizumab-treated tumors. While our original screen was aimed at the human cellular component of the tumors, and revealed upregulation of human IGF-1, our model affords the investigation of the stromal contribution using species-specific primers. Thus, we confirmed that stroma represents a major source of IGF-1 in the resistant tumors. As cixutumumab cross-reacts with the mouse IGF-1 binding to receptors on the tumor cells, this component is also neutralized by the antibody used, and may contribute to the antitumor effect observed. However, at this point we cannot reliably separate the specific contributions of these 2 compartments to the tumor supporting IGF-1 pool.

The molecular mechanism of IGF-1 induction in our models remains an open question. The complexity of microenvironmental stress potentially relevant for tumor adaptation to bevacizumab (increased hypoxia, nutrient deprivation, more acidic pH) poses a considerable challenge for reconstructing this model in vitro. For example, exposure of ovarian cancer cells in vitro to hypoxia or to glucose deprivation failed to induce IGF-1 (not shown). Exposure of ovarian cancer cells to both hypoxia and glucose deprivation caused cell death in less than 6 hours. However, the relevance of hypoxia to the mechanism identified here cannot be ruled out simply based on this simplified approach. We have documented hypoxia in ovarian cancer xenografts exposed to bevacizumab by measuring expression levels for 2 known hypoxia-inducible target genes, carbonic anhydrase 9 (CA9), and microRNA-210 (Supplementary Fig. S3). Furthermore, it is documented that conditions in the tumor microenvironment, such as hypoxia, can lead to enhanced responsiveness to IGF-1 (36), which might explain the reliance on this specific pathway as an adaptive mechanism to antiangiogenic treatment.

In addition, a mechanistic clue for IGF-1 induction may be provided by STAT-5b, which we found to be robustly upregulated in bevacizumab-treated tumors. STAT-5b is both a hypoxia-inducible factor (37, 38) and a well-recognized trigger for IGF-1 secretion (29, 39). STAT-5b is an important regulator of cancer cell survival through modulation of antiapoptotic signals via Bcl-xL in prostate and breast cancer and leukemia (40, 41, 42). STAT-5b along with NF-xB complex’s Rel-A subunit have also been...
implicated in carboplatin resistance in ovarian cancer (43), suggesting that this is an important survival pathway in ovarian tumors. In that study, STAT-5b and Rel-A were upregulated in recurrent postchemotherapy ovarian specimens compared with primary tumors, and the simultaneous knockdown of the 2 transcription factors sensitized tumor cells to carboplatin (43). STAT-5b regulates other angiogenic factors and members of the extracellular matrix, such as VEGF-C and collagen type VI and XII (42), and interestingly the JAK-STAT5 signaling axis also regulates ID-1 expression (42, 44). We found ID-1 to be upregulated in bevacizumab-treated xenografts and it is possible that its regulation occurs downstream of STAT signaling under these conditions. Interestingly, at least based on our qRT-PCR data, STAT-5b induction appears more robust in the stromal component of tumors compared with tumor cells, consistent with higher induction of murine IGF-1 in bevacizumab-treated tumors. These observations argue for the importance of the tumor microenvironment in the adaptive response to AAT.

A limitation of our study is the use of subcutaneous, rather than intraperitoneal xenografts. Ovarian tumors usually develop in the peritoneal space that has unique characteristics favoring tumor development. The peritoneum consists of mesothelial cells that cover and protect the viscera. The subperitoneal stroma contains a collagen-based matrix, immune cells, activated fibroblasts, blood vessels, and lymphatics. Several studies have shown an "activated" phenotype associated with ovarian cancer, as opposed to its quiescent state in benign conditions (45). The proinflammatory signature associated with cancer consists of chemokines, cytokines, and growth factors commonly upregulated in the peritoneal milieu (45–47). While tumor cells play a role in the secretion of factors that modulate angiogenesis, more recently it has become accepted that tumor infiltrating cells such as fibroblasts, myeloid cells, immune cells, and endothelial precursors play a crucial role modulating neovascularization (48). Future studies will focus on intraperitoneal xenografts in an effort to better mimic the development of ovarian tumors and metastases in the peritoneal space.

We show that cixutumumab and bevacizumab synergize in vivo blocking ovarian tumor growth and angiogenesis. To our knowledge, these data provide the first proof of principle that a combination between anti-VEGF therapy and inactivation of the IGF pathway may provide significant benefit over antiangiogenic drugs as single
agents and support further testing of this or similar combinations in patients with ovarian cancer.

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

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