Emodin suppresses proliferation, migration and invasion in ovarian cancer cells by down-regulating ILK in vitro and in vivo

Objective: Although our previous studies have confirmed that 1, 3, 8-trihydroxy-6-methylhaquinone (emodin) inhibits migration and invasion in epithelial ovarian cancer (EOC) cells, the underlying molecular mechanism remains unknown. Here, the aim was to investigate the effects of emodin on EOC cells and to study further the mechanism underlying this process, both in vitro and in vivo.

Materials and methods: Cell proliferation was evaluated by the methylthiazolyl tetrazolium assay. Cell migration and invasion abilities were tested using the transwell assay. The expression of integrin-linked kinase (ILK) and epithelial–mesenchymal transition (EMT)-associated factors were measured with western blotting.

Results: Exogenous ILK enhanced the proliferation, migration and invasion properties of A2780 and SK-OV-3 cells. After treatment with emodin, the survival rate of cells was gradually reduced, including those of SK-OV-3/pLVX-ILK and A2780/pLVX-ILK cells, with increasing emodin concentrations. The migration and invasion abilities of A2780 and SK-OV-3 cells were effectively increased by the transfection of pLVX-ILK, which could be abrogated by following this with 48 hours of emodin treatment. Treatment with emodin significantly downregulated the expression of ILK and EMT-related proteins. So, emodin suppressed proliferation, migration and invasion in ovarian cancer cells by downregulating ILK in vitro. SK-OV-3/pLVX-Con and SK-OV-3/pLVX-ILK cells were used to generate xenografts in nude mice. Tumors grew more rapidly in the SK-OV-3/pLVX-ILK group compared with the control group, and this could be significantly inhibited by emodin. Also, the expression of E-cadherin was downregulated, while the expression of Slug, MMP-9 and Vimentin were upregulated in the SK-OV-3/pLVX-ILK group, and this could be reversed by following treatment with emodin. Emodin did not demonstrate target toxicity on hepatocytes, nephrocytes and cardiomyocytes.

Conclusion: Emodin suppresses proliferation, migration and invasion in ovarian cancer by targeting ILK.

Keywords: emodin, ILK, epithelial ovarian cancer, epithelial–mesenchymal transition, Slug, xenografts in nude mice

Introduction

Epithelial ovarian cancer (EOC) is the most lethal disease among female genital malignant tumors. The lack of effective early detective markers and pathognomonic clinical symptoms means that there is currently no effective technique for the routine screening of the population. As a consequence, 80% of ovarian cancers are diagnosed at the advanced stages with widespread metastases. The prevalence...
of metastasis arises from the poor prognosis of EOC and leads to high mortality rates. Hence, clarifying the molecular mechanisms underlying metastases is the approach with the greatest potential for improving the outcome for EOC patients.

Integrin-linked kinase (ILK) is a 59 kDa serine/threonine protein kinase, which interacts with the cytoplasmic domain of integrin β1 and β3.4,5 ILK is viewed as a core regulator of the phosphoinositide 3-kinase signaling pathway. Once activated, ILK directly activates kinase B/Akt (PKB/Akt) and restrains glycogen synthase kinase 3β (GSK3β) to regulate the extracellular matrix. ILK has been implicated in multiple cellular processes including cell survival, cell cycle progression, inflammation, tumorigenesis and epithelial–mesenchymal transition (EMT).6,7

EMT is a vital process during embryogenesis, tissue remodeling, wound healing and tumor metastasis, where cells lose their epithelial phenotypes, such as E-cadherin, and acquire mesenchymal traits, such as Vimentin.8–10 Overexpression of ILK has been found to occur in prostate cancer,11 bladder cancer,12 colorectal cancer13 and other cancers,14,15 and this promotes cancer cell invasion and migration by regulating EMT-related genes. The overexpression of ILK in rat epithelial cells has been reported to induce cell growth in culture and tumorigenesis.16 Downregulation of ILK inhibits EMT and metastasis in bladder cancer cells.17 The same phenomenon has been found in EOC.18,19 ILK expression is positively correlated with ovarian tumor grade, and ILK modulates the metastatic behavior of ovarian cancer cells.20,21

1,3,8-Trihydroxy-6-methylantraquinone (emodin) has been isolated from the rhizomes of rhubarb, aloes and other plants known to possess anti-bacterial, anti-inflammatory, immunosuppressive and anti-cancer effects.22 Emodin induces apoptosis in human lung adenocarcinoma cells.23 Recent studies have demonstrated that emodin inhibited migration and invasion through the downregulation of MMP-2 and MMP-9 in human breast cancer cells.24 In addition, emodin was shown to repress cell migration and invasion by regulating EMT-related genes in colorectal cancer, head and neck squamous cell carcinoma and ovarian cancer.25–27 Furthermore, our previous studies have demonstrated that emodin inhibits the EMT of EOC cells via the ILK/GSK3β/Slug signaling pathway in vitro.28

The present study was aimed at furthering understanding the role of ILK in EOC cells, and determining whether emodin could inhibit invasion and migration in EOC cells by inhibiting EMT by targeting ILK in vitro and in vivo.

Materials and methods
Cell lines and culture conditions
The human EOC cell lines A2780 and SK-OV-3 cells were purchased from Cell Bank (Chinese Academy of Sciences, Shanghai, People’s Republic of China). A2780 cells were maintained in RPMI-1640 medium (GE Healthcare Life Sciences, HyClone Laboratories, Logan, UT, USA), supplemented with 10% (v/v) fetal bovine serum (FBS; Gibo Life Technologies, Mount Waverley, VIC, Australia). SK-OV-3 cells were cultured in McCoy’s 5A medium (M&C Gene Technology, Beijing, People’s Republic of China) with 10% FBS (v/v). Cells were kept in an incubator at 37°C containing air/CO₂ (95:5, v/v).

Antibodies and chemicals
Emodin and poloxamer 188 were purchased from Sigma Aldrich (St Louis, MO, USA). Dimethyl sulfoxide (DMSO) and propanediol were obtained from Solarbio (Beijing, People’s Republic of China). Emodin was diluted in DMSO in vitro, and the final concentration of DMSO was below 0.1% (v/v). For peritoneal injection, emodin was dissolved in a solution, which was mixed with propanediol/normal saline/poloxamer 188 (1:6:6, v/v). The primary antibodies against E-cadherin (3195S), Vimentin (5741S), Slug (9585S), ILK (3826S) and GAPDH (2118S) were purchased from Cell Signaling Technology (CST, MA, USA). The primary antibody MMP-9(ab76003) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Construction of cells with ILK overexpression
ILK was exogenously expressed in A2780 and SK-OV-3 cells by transfecting them with ILK overexpression lentiviral vector (pLVX-C1-ILK). The lentiviral-containing ILK (pLVX-ILK) or control vector (pLVX-Con) was purchased from the GenePharma Company (Shanghai, People’s Republic of China). The A2780 and SK-OV-3 cells were seeded into 24-well plates with appropriate medium with 10% (v/v) fetal bovine serum (FBS; Gibco Life Technologies, HyClone Laboratories, Logan, UT, USA), supplemented with 10% FBS (v/v), treated for 6 hours and when the cell density was approximately 30%, the cells were transfected with lentiviral, according to the manufacturer’s protocol. After 48 hours of transfection, cells were selected with puromycin for 7–10 days. Then, stable, transfected cells were obtained: A2780/pLVX-ILK, A2780/pLVX-Con, SK-OV-3/pLVX-Con and SK-OV-3/pLVX-ILK.

Methylthiazolyl tetrazolium
Cell viability was assessed using the methylthiazolyl tetrazolium (MTT) assay. To detect the ability of cells to proliferate,
cells were seeded into seven 96-well, flat-bottomed plates at a density of 1,000 cells/well and incubated overnight. Then, every day at the same time point, 10 μL MTT (5 g/L) solution (Sigma Aldrich) was added to each well and then incubated for an additional 4 hours before being treated with DMSO (100 μL/well). Absorbance at 490 nm using a plate reader was used as a measure of the ability of cells to proliferate (Bio-Rad, Hercules, CA, USA). To determine the effects of emodin on cell viability, cells (3,000 cells/well) were seeded into a 96-well plate and incubated overnight. Thereafter, cells were treated with increasing concentrations of emodin (0–80 μM) for 48 hours.

Transwell migration and invasion assays

Transwell migration and invasion assays were performed with 24-well Boyden chambers (Corning Costar, Cambridge, MA, USA). Cells were re-suspended in 100 μL serum-free medium (3x10^4 SK-OV-3 cells or 6x10^4 A2780 cells) and then seeded onto the top of the chamber in 100 μL serum-free medium. The lower chamber was filled with 750 μL medium containing 10% (v/v) FBS. After incubation for 24 hours, the cells that had migrated, on the lower surface of the filter, were fixed with methanol solution for 5 minutes and 3.7% (v/v) paraformaldehyde for 5 minutes and then stained with Giemsa stain. Images (200×) of stained cells were captured with an Olympus IX51 inverted microscope (Olympus Corporation, Tokyo, Japan). Cells were counted in five visual fields per chamber. For the invasion assay, Boyden chambers were pre-coated with 100 μL Matrigel (1:9 dilution in serum free medium; BD Biosciences, San Jose, CA, USA) prior to experimentation. Following this, similar steps were performed as in the migration assay.

Western blot analysis

Total cellular protein and tumor tissue protein were extracted by adding RIPA lysis buffer (Beyotime, Jiangsu, People’s Republic of China) containing 1% (v/v) phenylmethanesulfonyl fluoride (Beyotime) on ice. The proteins were separated in 10% (w/v) tris-glycine gel and transferred electrophoretically to a pure nitrocellulose blotting membrane. Primary antibodies (anti-ILK, anti-E-cadherin, anti-Vimentin, anti-Slug, anti-MMP-9 and anti-GAPDH, 1:1,000) were incubated with membranes overnight at 4°C. The following day, membranes were washed and incubated with the respective horseradish peroxidase (HRP)-conjugated second antibodies (1:3,000; Millipore, Billerica, MA, USA) for 1 hour at 37°C. Antibody-positive bands were detected with Image Quant LAS 4000. ImageJ software was used for densitometric analysis.

Xenografts in nude mice

Four-week-old female BALB/C nude mice were purchased from the HFK Bioscience Company. The treatment protocol followed the guidelines of “Laboratory Animal – Requirements of Environment and Housing Facilities”. Our research was approved by Experimental Animal Ethics Committee of Qilu Hospital of Shandong University (approval number: DWLL-2015-004). Mice were randomized into two groups, and 200 μL re-suspended serum-free PBS with either 1.0x10^7 viable SK-OV-3/pLVX-Con or SK-OV-3/pLVX-ILK cells was injected into the right armpit of mice, according to their group allocation. The volume (V = [length × width^2]/2) of subcutaneous tumors was measured every other day. When tumor volume reached 100 mm^3, each group of mice was randomized in two groups (n=6) to receive different treatments. One group was treated with an intra-peritoneal injection, every day for 21 days, of 50 mg/kg/d of emodin. The other mice were injected with the drug vehicle. On the 21st day after drug injection, blood was sampled from the eyes of the experimental mice to measure liver, renal and cardiac functions. Finally, tumors were removed, weighted and divided into two parts, one for immunohistochemical (IHC) staining and the other for immunoblotting assays.

Immunohistochemistry

Tumor xenografts were stored in 3.7% (w/v) paraformaldehyde. Paraformaldehyde-fixed, paraffin-embedded tissues were cut to produce sections of 4 mm thickness for IHC staining. Tissue sections were probed with anti-ILK (1:200), anti-E-cadherin (1:100), anti-Vimentin (1:200), anti-MMP-9 (1:200) and anti-Slug (1:200) for determining the expression of these proteins in every tissue. Antibody binding was amplified using HRP-conjugated second antibodies for 30 minutes, and the IHC staining was visualized using diaminobenzidine (DAB). Cell nuclei were lightly stained with hematoxylin (Solarbio). Micrographs (400×) were produced using an Olympus IX51 inverted microscope (Olympus Corporation). Positive DAB staining was used to assess tissue sections. The intensity of the protein expression was scored blindly as 0, 1, 2 and 3, which corresponded to negative, weak, moderate, and strong immunoreactivity, respectively. Sections were reviewed by two observers independently in order to verify the IHC scores.

Statistical analysis

GraphPad Prism Version 5.01 was used for all statistical analyses. Data are shown as means ± SD of three independent experiments. The student’s t-test was used to analyze the
differences between any two groups. One-way analysis of variance was used to assess the means among groups. \( P < 0.05 \) was considered to be statistically significant in all analyses.

**Results**

**Exogenous ILK enhanced the proliferation, migration and invasion properties of EOC cells**

Western blotting was conducted in order to test ILK in A2780, A2780/pLVX-ILK, A2780/pLVX-Con, SK-OV-3, SK-OV-3/pLVX-Con and SK-OV-3/pLVX-ILK expression. The expression of ILK was significantly upregulated by the transfection of pLVX-ILK in A2780 and SK-OV-3 cells (Figure 1A and B). Then, the effect of exogenous expression of ILK on the proliferation abilities of human EOC cells was determined and it was found that A2780/pLVX-ILK and SK-OV-3/pLVX-ILK showed much stronger proliferation abilities (Figure 1C and D). Simultaneously, the migration and invasion abilities of human EOC cells were enhanced by the transfection of pLVX-ILK (Figure 1E and F).

**Emodin suppressed the proliferation, migration and invasion of EOC cells**

To examine the effect of emodin on the proliferation of A2780, A2780/pLVX-ILK, A2780/pLVX-Con, SK-OV-3, SK-OV-3/pLVX-Con and SK-OV-3/pLVX-ILK, we treated the cells with a range of doses of emodin (0–80 \( \mu \)M) for 48 hours. We found that emodin suppressed the proliferation of A2780, A2780/pLVX-ILK and A2780/pLVX-Con in a dose-dependent manner (Figure 2A; IC50s were 34.02 \( \mu \)M, 37.05 \( \mu \)M and 42.59 \( \mu \)M for A2780, A2780/pLVX-Con and A2780/pLVX-ILK, respectively). Similar results were
found for SK-OV-3 cells (IC50s were 37.31 μM, 36.84 μM and 43.31 μM for SK-OV-3, SK-OV-3/pLVX-Con and SK-OV-3/pLVX-ILK cells, respectively; Figure 2B). The effect of emodin on the migration and invasion capacity of cells was measured with Transwell assays. The migration and invasion abilities of A2780 and SK-OV-3 cell lines were increased by the transfection of pLVX-ILK, and this effect could be abrogated by following the treatment with emodin (20 μM) for 48 hours (Figure 2C and D). The expression of EMT-related molecules was detected by western blotting analysis to examine whether emodin decreased migration and invasion abilities by inhibiting the EMT of SK-OV-3/pLVX-ILK and A2780/pLVX-ILK. We tested the expression of E-cadherin, Vimentin, MMP-9 and Slug. After transfection of pLVX-ILK, the expression of E-cadherin had decreased, while the levels of Vimentin, MMP-9 and Slug had increased in A2780 and SK-OV-3 cells, which could be counteracted by emodin (Figure 2E and F). Therefore, emodin arrested the migration and invasion abilities of EOC cells by inhibiting EMT through targeting ILK.
Figure 2  Emodin suppressed the proliferation, migration, and invasion of human EOC cells.

Notes: (A) The cells were performed with concentrations of emodin for 48 hours to examine the cell viability. A2780: *p<0.05, **p<0.005, ***p<0.001; A2780/pLVX-Con: nP<0.005, n**p<0.001; A2780/pLVX-ILK: *p<0.05, n**p<0.005. (B) Emodin inhibited SK-OV-3, SK-OV-3/pLVX-Con and SK-OV-3/pLVX-ILK cells growth in a dose-dependent fashion. SK-OV-3: *p<0.005, **p<0.001; SK-OV-3/pLVX-Con: *p<0.005, **p<0.001; SK-OV-3/pLVX-ILK: *p<0.05, **p<0.005, ***p<0.001. (C) Representative transwell migration and invasion assay of A2780, A2780/pLVX-Con, A2780/pLVX-ILK, SK-OV-3, SK-OV-3/pLVX-Con and SK-OV-3/pLVX-ILK cells after being treated with or without emodin (×200 magnification). (D) Quantification of migration and invasion abilities of the EOC cells with and without the treatment with emodin. *p<0.005, **p<0.001; **p<0.005, ***p<0.001. (E) The expression of ILK, E-cadherin, Vimentin, MMP-9 and Slug detected by western blot analysis after being treated with or without emodin are shown. (F) Quantitative analyses of ILK and EMT-related protein expression of SK-OV-3, SK-OV-3/pLVX-Con and SK-OV-3/pLVX-ILK cells. *p<0.05, **p<0.005, ***p<0.001; **p<0.05, ***p<0.005, ***p<0.001.

Abbreviations: Emodin, 1, 3, 8-trihydroxy-6-methylanthraquinone; EOC, epithelial ovarian cancer; ILK, integrin-linked kinase; EMT, epithelial–mesenchymal transition; Con, control.
Emodin repressed the proliferation of human tumor cells in vivo

On the basis of the in vitro findings, we moved on to determine the effects of ILK and the role of emodin in vivo. After transfection of the empty vector or ILK into SK-OV-3 cells, we injected SK-OV-3/pLVX-Con and SK-OV-3/pLVX-ILK cells hypodermically into the armpits of female nude mice. We observed that tumors grew more rapidly in the SK-OV-3/pLVX-ILK group compared with the control group (SK-OV-3/pLVX-Con), and growth could be inhibited significantly by emodin (Figure 3A and B). As expected, the nodules were heavier in the SK-OV-3/pLVX-ILK group, when compared to the control group (Figure 3C and D). At the same time, the effects of overexpression of ILK on EOC cells were counteracted by emodin (Figure 3C and D). The effects of ILK on EOC cells were confirmed by the results in vivo. Simultaneously, the ability of EOC cancer cells to proliferate was decreased by emodin. So, emodin inhibited the proliferation of EOC cancer cells by targeting ILK in vivo.

Emodin inhibited the EMT of tumor cells in vivo

The tumors removed from nude mice were stained immunohistochemically and subjected to western blot assays. It was found that the expression of E-cadherin was downregulated, while the expression of Slug, MMP-9 and Vimentin were upregulated in the SK-OV-3/pLVX-ILK group. Contrary results were obtained from emodin-treated groups (Figure 4A and B). In addition, IHC staining showed that the levels of Slug, MMP-9 and Vimentin were increased, while the level of E-cadherin was decreased after exogenous expression of ILK.

Figure 3 Emodin repressed the proliferation of tumor cells in vivo.

Notes: (A) Tumors grew more rapidly after transfection of exogenous ILK gene, which can be balanced out by emodin in vivo. (B) Quantitative analyses of tumors growth shown in A. *P<0.05, **P<0.005, ***P<0.001; **P<0.005, ***P<0.0001. (C) The nodules were heavier in SK-OV-3/pLVX-ILK group compared with the control group. Exogenous expression of ILK was counteracted by emodin. (D) Quantification of tumors weight shown in C. *P<0.05, **P<0.005, ***P<0.001. 

Abbreviations: Emodin, 1, 3, 8-trihydroxy-6-methylanthraquinone; ILK, integrin-linked kinase; Con, control.
This could be reversed by emodin (Figure 4C–F). Our results demonstrate that ILK was associated significantly with EMT of EOC cells in vivo and that the impact of exogenous ILK could be abrogated by emodin.

The cardiac, liver and renal toxicities of emodin were mild in vivo

The concentrations of serum alanine aminotransferase, aspartate aminotransferase, urea nitrogen, creatinine and
Emodin suppresses proliferation, migration and invasion in EOC cells via ILK pathway

Figure 5. Cardiac, liver, renal toxicity of emodin was mild in vivo. (A) Hematologic toxicity test results showed that emodin did not change liver function indices. (B) The concentrations of UREA and CREA were measured. (C) Emodin was not toxic to heart under applied concentration.

Abbreviations: Emodin, 1, 3, 8-trihydroxy-6-methylanthraquinone; ILK, integrin-linked kinase; UREA, urea nitrogen; CREA, creatinine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Con, control.

Discussion

ILK is a factor that is known to be crucial in regulating the extracellular matrix and cell–cell interactions by phospho-
rylating its downstream target kinases, B/Akt (PKB/Akt) and GSK3β.7 ILK has been found to promote the metastatic behavior of ovarian cancer cells.20,29 ILK has been reported to facilitate cancer cell migration and invasion by regulat-
ing EMT processes.15,30,31 Consistently, the knockdown of ILK inhibited tumor invasion and metastasis by inhibiting the EMT process in bladder cancer and in oral squamous cell carcinoma.17,32 In the present study, we found that the overexpression of ILK of A2780 and SK-OV-3 cells had a greater potential for proliferation, migration and invasion. An in vivo mouse model of exogenous expression of ILK was successfully established, and using this, it was demonstrated that the overexpression of ILK enhanced tumor growth and metastasis in vivo. This means that ILK may play an important role in metastasis in ovarian cancer and it could be a novel marker for the targeting of molecular therapies for advanced ovarian cancer.

Emodin exerts diverse biological activities, including inhibition of proliferation, migration and invasion in many different kinds of cells.21,24,33 EMT plays an essential role in tumor metastasis, where cells downregulate their epithelial markers, such as E-cadherin, and upregulate mesenchymal traits, such as Vimentin.34 Emodin inhibited EMT in head and neck squamous cell carcinoma cells.25 In the present study, it was found that emodin could inhibit the proliferation, migration and invasion abilities of EOC cells by inhibiting EMT, which is in accord with previous studies. The inhibitive effect of emodin on EMT is, therefore, known, but the underly-
ing molecular mechanism still requires elucidation. Recent studies have implied that the effect of emodin on ILK is mediated via the MEK/ERK signaling pathway.35 Emodin was first reported to regulate the expression of ILK in tubular epithelial to myofibroblast transdifferentiation.36 It was subsequently shown to ameliorate high-glucose-induced podocyte EMT through ILK both in vitro and in vivo.37 In addition, emodin was reported to inhibit migration and invasion by promoting mesenchymal–epithelial transition by targeting ILK in human endometrial stromal cells.38 Further, in a previous study, carried out in our own laboratory, it was speculated that emodin inhibits EMT via the ILK/GSK3β/Slug signaling pathway in vitro.28 In our current study, it was also found that emodin decreased the expression of ILK, resulting in a repression

Creatine kinase were measured by extracting eyeball blood. Hematologic toxicity test results showed little or no effects on the functional indices of liver, kidney and heart (Figure 5A–C). Thus, emodin was not toxic at the applied concentrations.
of the abilities of EOC cells to migrate and invade through restraining EMT. Further, the effect of the overexpression of ILK could be reduced by emodin treatment in vitro. In terms of xenografts in nude mice, the tumors grew more quickly in the SK-OV-3/pLVX-ILK group compared to the control group (SK-OV-3/pLVX-Con), and this could be decelerated significantly by emodin. The expression of E-cadherin was downregulated, while the expression of Slug, MMP-9 and Vimentin were upregulated in the tumors of the mice in the SK-OV-3/pLVX-ILK group, which was crosscurrent in the tumors from the emodin-treated group of mice. Hematologic toxicity test results indicated that emodin (50 mg/kg/d) was not toxic and did not alter liver, kidney and cardiac function indices, but a change in stoil characteristics induced by emodin was observed. Taking these new data into account, emodin could be considered to have potential clinical value due to its actions against ovarian cancer in the future.

In summary, exogenous expression of ILK enhanced the proliferation, migration and invasion properties of A2780 and SK-OV-3 cells, and this could be abrogated by emodin in vitro. A xenograft tumor model was used to show that emodin targeted the ILK signaling pathway, thereby inhibiting EOC cell growth and EMT-associated protein expression in vivo. In our experiment, fact that no metastatic lesions were observed attributed to the short-term nature of the observations and the low level of the technology used. The underlying mechanisms by which emodin targets ILK and by which ILK accelerates EMT are still unclear. Additionally, in vivo studies are preferable for the construction of a xenograft tumor model with knockdown ILK and for investigating the therapeutic effects of emodin on ovarian cancer.

Conclusion
In this study it has been demonstrated that ILK has a role in EOC cells, and a molecular basis for further studies has been provided. It has been shown that ILK could enhance the proliferation, migration and invasion abilities of EOC cells, and these effects could be neutralized by emodin in vitro and in vivo. Furthermore, emodin inhibited the migration and invasion abilities of EOC cells by inhibiting the EMT of EOC cells through the targeting of ILK. The findings presented here suggest a novel therapeutic approach to the treatment of ovarian cancer and highlights the possible value of using ILK as a prognostic marker in advanced ovarian cancer.

Acknowledgment
This study was funded by grants from Science and Technology Development Project of Shandong Province (2008GG2NS02017).

Disclosure
The authors report no conflicts of interest in this work.

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