Insulin-like growth factor-I receptor inhibition by specific tyrosine kinase inhibitor NVP-AEW541 in endometrioid and serous papillary endometrial cancer cell lines

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ABSTRACT

Purpose. The role of the insulin-like growth factor (IGF) system in endometrial cancer has been well established. The IGF-I receptor (IGF-IR) emerged as a promising therapeutic target in a number of cancers. NVP-AEW541 (Novartis Pharma) is a pyrrolo(2,3-d)pyrimidine derivative with specific IGF-IR tyrosine kinase inhibitory activity. NVP-AEW541 has been shown to abrogate IGF-I-mediated IGF-IR autophosphorylation and to reduce activation of the IGF-IR signaling pathways. The aim of the present study was to investigate the anti-proliferative activity of NVP-AEW541 in Type I (endometrioid) and Type II (uterine serous papillary endometrial carcinoma, USPC) endometrial cancer cell lines.

Methods. Type I (ECC-1, Ishikawa) and Type II (USPC-1, USPC-2) endometrial cancer cell lines were treated with NVP-AEW541 in the presence of IGF-I, and the following parameters were measured: IGF-IR, AKT and ERK phosphorylation, apoptosis, proliferation, cell cycle progression and IGF-IR internalization.

Results. Results obtained showed that NVP-AEW541 abolished the IGF-I stimulated IGF-IR phosphorylation in all of the cell lines investigated, whereas it abolished AKT and ERK phosphorylation preferentially in ECC-1 and USPC-1 cells. Furthermore, the inhibitor prevented from IGF-I from exerting its antiapoptotic effect in ECC-1, USPC-1 and USPC-2 cells. In addition, proliferation assays showed that NVP-AEW541 caused a decrease in proliferation rate in all of the cell lines. NVP-AEW541 had no major effect on the insulin receptor.

Conclusion. Our results suggest that specific IGF-IR inhibition by NVP-AEW541 might be a promising therapeutic tool in endometrial cancer.

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Introduction

Endometrial cancer is the most widespread gynecologic cancer in Western countries, accounting for 6% of all cancers in women [1]. Women have an approximate 2.5% lifetime risk of developing endometrial cancer and the incidence of the disease has been increasing in recent years as a result of the growing obesity epidemics. Hence, endometrial cancer constitutes a major public health issue and there is an urgent need to identify new therapeutic regimens. However, in spite of a huge advance in our understanding of endometrial cancer biology, therapeutic modalities haven’t significantly changed over the past 40 years. Endometrial cancers are classified into two major groups, Type I and Type II, with Type I being the most frequent (more than 80% of cases) [2,3]. Type I tumors are usually estrogen-dependent, low-grade neoplasms, with an endometrioid, well-differentiated morphology, and are generally associated with a relatively good prognosis. Type II tumors appear at an advanced age, are not associated with exposure to estrogens, display a less differentiated phenotype, and have a worst prognosis. Uterine serous papillary endometrial carcinoma (USPC) constitutes the predominant histological class among Type II tumors [4,5]. USPCs represent 10% of all endometrial carcinomas, are diagnosed at an advanced stage, and account for 50% of all relapses of the endometrial cancers, with a 5-year survival rate of 55% [6,7].

The insulin-like growth factors (IGF-I, IGF-II) are a family of growth factors, binding proteins, and receptors that play a key role in regulating growth, resistance to apoptosis, and differentiation [8]. The IGF system has been implicated in the etiology of a number of malignancies, including endometrial cancer [9–11]. In the uterus, cyclic changes in IGF-I expression play an important role in regulating the transition of premenopausal endometrium through proliferative, secretory, and menstrual cycles [12,13]. In addition, there is evidence for a correlation between expression and activation of IGF axis components and endometrial cancer risk [14,15]. Furthermore, epidemiological studies have demonstrated a link between serum IGF-I and insulin values and endometrial cancer risk [12,16]. This correlation was further increased in obese patients [17]. A recent study suggested that the estradiol-induced proliferation of Ishikawa endometrial cancer cells was mediated by the MAPK pathway via autocrine stimulation of IGF-I. Importantly, the authors...
observed that IGF-I increased cell proliferation and an anti-IGF-I receptor (IGF-IR) antibody inhibited the estradiol-induced proliferation [18].

Consistent with the central role for the IGF axis in cancer development, the IGF-IR emerged in recent years as a promising therapeutic target [9,19,20]. Multiple approaches are being utilized to abrogate IGF-IR signaling in vitro and in vivo, including blocking antibodies, IGF-IR antisense oligonucleotides, small interference RNA, and tyrosine kinases inhibitors [8]. Several ongoing multicenter studies are being conducted to evaluate the efficacy and safety of IGF-IR inhibitors in ovarian cancer. To the best of our knowledge, no study has so far evaluated the impact of IGF-IR targeting in USPC. NVP-AEW541 (Novartis Pharma) is a pyrrolo(2,3-d) pyrimidine derivative with specific IGF-IR tyrosine kinase inhibitory activity. NVP-AEW541 has been shown to abrogate IGF-I-induced IGF-IR autophosphorylation and to reduce activation of the IGF-IR signaling pathways. The inhibitor has shown efficacy in various experimental tumor models, including fibrosarcoma, neuroblastoma, Ewing’s sarcoma and ovarian cancer [21-24]. Given that IGF-IR activation correlates with the development of endometrial cancer, the aim of the present study was to investigate the anti-proliferative potential of NVP-AEW541 as a targeted therapy approach against the IGF-IR in Type I and Type II endometrial cancers.

Materials and methods

Cell lines and treatments

Human uterine serous papillary endometrial carcinoma (USPC) and endometrioid endometrial cancer cell lines were used in this study. For USPC, the USPC-1 and USPC-2 cell lines were employed, and for endometrioid endometrial cancer, the ECC-1 and Ishikawa lines were used. The USPC cell lines were provided by Dr. A. D. Santin, Yale University School of Medicine, New Haven, CT, USA. The ECC-1 and Ishikawa cell lines were obtained from Dr. Y. Sharoni, Ben Gurion University, Beer-Sheva, Israel. ECC-1 and Ishikawa cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/l d-glucose (Gibco BRL®, Paisley, Scotland), and USPC cells were maintained in RPMI-1640 medium (Biological Industries, Kibbutz Beit Haemek, Israel). Media were supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 50 μg/ml gentamicin sulfate. All reagents were purchased from Biological Industries. In addition, 5.6 g/l fungizone was added (Sigma-Aldrich, St. Louis, MO, USA). The NVP-AEW541 selective IGF-IR inhibitor was obtained from Novartis Pharma (Basel, Switzerland) and kept as a stock solution (10 mM) in DMSO and stored at −20 °C. In all of the experiments, cells were treated with IGF-I (50 ng/ml) (Cytolab Ltd., Rehovot, Israel), in the absence or presence of the IGF-IR inhibitor (1 μM and 10 μM).

Western immunoblots

Cells were serum starved overnight and then incubated with 1 μM or 10 μM of NVP-AEW541, in the presence or absence of IGF-I. Cells were then harvested, and whole cell lysates were prepared. Samples were subjected to 10% SDS-PAGE, followed by electrophoretic transfer of the proteins to nitrocellulose membranes. After blocking with 3% BSA or milk in 20 mM Tris·HCl (pH 7.5), 135 mM NaCl, and 0.1% Tween 20, blots were incubated with polyclonal human IGF-IR (β-subunit or insulin receptor (IR) antibodies (Cell Signaling, Danvers, MA, USA), washed extensively with 20 mM Tris·HCl (pH 7.5), 135 mM NaCl, and 0.1% Tween 20, and incubated with a horseradish peroxidase-conjugated secondary antibody. Proteins were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). In addition, blots were probed with antibodies against Erk1, phospho-Erk1/2 (Thr202/Tyr204), Akt, phospho-Akt (Ser473), poly(ADP-ribose) polymerase (PARP) (Cell Signaling), and tubulin (Sigma-Aldrich). The antibodies were diluted according to manufacturer’s protocols.

Immunoprecipitation (IP) assays

Cells were treated, harvested, and lysed as described above. Lysates were precipitated overnight with 2 μg/ml of anti-human IGF-IIR β-subunit antibody or anti-human IR at 4°C. The precipitates were then incubated with protein A/G beads (sc-2003, Santa Cruz Biotechnology), for 3 hr. Immunoprecipitates were pelleted by centrifugation at 2500 rpm and then washed three times with sample washing buffer. Finally, pellets were dissolved in 30 μl of sample buffer and boiled for 10 min. Immunoprecipitates were resolved on 8% SDS-PAGE and immunoblotted with an anti-phospho-tyrosine antibody.

RT-PCR for IGF-IR mRNA expression

Total RNA was prepared from endometrial cancer cells using Trizol (Sigma-Aldrich). 2.5 μg of total RNA was reverse transcribed and amplified by PCR. The primers used for IGF-IR mRNA were: sense, GAA-CTG-GAA-CCC-CTC-CTC-TC; antisense, CTT-CTC-GGC-TAG-ATT-TTT-GG. The size of the band was 275 bp. For control purposes, levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were measured using the following primers: sense, 5’-ACCAAGTCCTCATGC-CATCAC-3’; antisense, 5’-TCCACACCCCTGTTGTCGTA-3’. The size of the amplified GAPDH mRNA fragment was 452 bp.

Proliferation assays

Cells were seeded in 24 well plates (2 × 10⁴ cells/well), and exposed to increasing doses of NVP-AEW541 and/or IGF-I (50 ng/ml) for 72 h, in triplicate dishes. The proliferation rate was determined by

Fig. 1. Expression of endogenous IGF-IR in endometrial cancer cells. (A) Western blot analysis of IGF-IR levels in endometrial cancer cell lines. Cells were lysed and extracts were electrophoresed through SDS-PAGE, followed by transfer and incubation with an anti-IGF-IR β-subunit. (B) Total RNA was prepared from endometrial cancer cells and IGF-IR and GAPDH mRNA levels were evaluated by a semiquantitative RT-PCR assay.

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MTT assays. Every 24 h, 100 μl of Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma-Aldrich) was added to each well and incubated at 37 °C for 1 h, after which the medium was removed and 300 μl of DMSO was added. The color developed was quantitated by measuring absorbance at a wavelength of 530 nm and reference wavelength of 630 nm on an UVmax Kinetic Microplate Reader (Molecular Devices, Spectra Max 190).

Cell cycle analysis

For cell cycle analysis, cells were seeded in 6-well plates (1x10⁶), serum-starved for 24 h and then incubated in the presence of NVP-AEW541 and IGF-I for 24 h. At the end of this period, cells washed three times with cold PBS, trypsinized, permeabilized with Triton X 100 (4%), and stained with propidium iodide (50 μg/ml). Stained cells were analyzed using a FACSort Flow Cytometer (Beckton Dickinson, CA, USA).

Confocal microscopy

ECC-1 and USPC-2 cells were plated on cover slips in 6-well plates (5 x 10⁵ cells/well) for 24 h. Cells were transfected using the JetPEI™ reagent (Polyplus Transfection, Illkirch, France), according to manufacturer’s recommendations, with a plasmid containing an IGF-IR cDNA fused to a green fluorescence protein (GFP) (1 μg) marker. The IGF-IR-GFP cDNA plasmid was kindly provided by Prof. Rosemary O’Connor (University of Cork, Ireland). After 48 h cells were treated with NVP-AEW541 for 60 min and washed with PBS. Fixation was done with 3% paraformaldehyde.

Fig. 2. Effect of NVP-AEW541 on the IGF-I-stimulated IGF-IR, Akt, and ERK phosphorylation. ECC-1 and Ishikawa endometrioid cells (A) and USPC-1 and USPC-2 cells (B), were treated with increasing amounts of NVP-AEW541 for 2 h, followed by IGF-I treatment for 10 min. At the end of the incubation period, cells were lysed and the levels of phosphorylated and total proteins were measured by Western blot analysis. (C–E) Optical density was expressed as pIGF-IR, pAKT and pERK values normalized to the corresponding total proteins. A value of 100% was given to the optical density of IGF-I treated cells. The bars represent the mean ± S.E.M. of four independent experiments. *p < 0.05 versus IGF-I treated cells.

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performed with 100% methanol for 20 min at 20 °C and washed with PBS. Cells were dyed with DAPI (5 μg/ml) (Sigma-Aldrich), for 5 min and cover slips were mounted on microscope slides. Imaging was done using a Leica SP5 confocal microscope.

**Statistical analysis**

The statistical significance of the differences observed between groups was assessed using the T-test (two samples, equal variance). P<0.05 was considered statistically significant.

**Results**

**IGF-IR expression in endometrial cancer cell lines**

To begin to investigate the potential inhibition of IGF-IR signaling by NVP-AEW541, we measured the basal IGF-IR levels in two Type I and two Type II endometrial cancer cell lines. Western blot analysis revealed that ECC-1 and USPC-1 cells express high levels of IGF-IR whereas Ishikawa and USPC-2 cells express reduced IGF-IR levels (Fig. 1A). To assess whether the IGF-IR levels were associated with corresponding trends in mRNA expression, IGF-IR mRNA levels were determined using quantitative RT-PCR.
Effect of IGF-IR inhibition on proliferation

To assess the impact of the NVP-AEW541 inhibitor on cell proliferation, cells were seeded in 24 well plates (2×10⁴ cells/well), and exposed to increasing doses of the inhibitor and IGF-I (50 ng/ml) for 72 h. The proliferation rate was determined by MTT assays. Results obtained showed that addition of NVP-AEW541 on top of IGF-I in all of the cell lines caused a significant decrease in proliferation rate compared with the IGF-I-induced rate seen in control cells (Fig. 3B). Thus, in ECC-1 cells, the inhibitor caused a 23%, 70%, and 108% decreases in proliferation rate at 24 h, 48 h, and 72 h, respectively, at the high dose, compared with IGF-I treated cells, respectively (Fig. 2C). In addition, the inhibitor abolished Akt and ERK phosphorylation only in the ECC-1 and USPC-1 cell lines (60% and 43% decreases in pAKT and 55% and 41% decreases in pERK at the high dose, compared with IGF-I treated cells, respectively (Fig. 2D and E).

Table 1
Inhibition of IGF-I-stimulated cell cycle progression by NVP-AEW541.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control</th>
<th>IGF-I (50 ng/ml)</th>
<th>IGF-I + AEW541 (1 μM)</th>
<th>IGF-I + AEW541 (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. ECC-1</td>
<td>G0/G1</td>
<td>78.8</td>
<td>62.2</td>
<td>81.7</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>8.2</td>
<td>18.7</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>13</td>
<td>19.1</td>
<td>12.3</td>
</tr>
<tr>
<td>B. Ishikawa</td>
<td>G0/G1</td>
<td>60.4</td>
<td>57.3</td>
<td>61.8</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>15.6</td>
<td>14.8</td>
<td>14.4</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>24</td>
<td>27.9</td>
<td>23.8</td>
</tr>
<tr>
<td>C. US-1</td>
<td>G0/G1</td>
<td>60.6</td>
<td>54.8</td>
<td>73.1</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>9.9</td>
<td>15.9</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>29.5</td>
<td>29.3</td>
<td>15.9</td>
</tr>
<tr>
<td>D. US-2</td>
<td>G0/G1</td>
<td>64.4</td>
<td>62</td>
<td>73.5</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>11.4</td>
<td>12</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>24.2</td>
<td>26</td>
<td>17</td>
</tr>
</tbody>
</table>

Endometrial cancer cell lines were seeded in 6-well plates, serum-starved for 24 h and then incubated in the presence of NVP-AEW541 and IGF-I for 24 h. At the end of this period cells were examined for their cell cycle profile using propidium iodide. The table quantitatively shows the percentage of cells at the different cell cycle phases. The results represent the values of a typical experiment, repeated three times.

* Significantly different versus IGF-I-treated cells (p<0.05).

Effect of IGF-IR inhibition on apoptosis

To evaluate the effect of IGF-IR inhibition on apoptosis, endometrial cancer cell lines were serum starved for 24 h, after which they were treated with IGF-I (50 ng/ml) for 2 h or 24 h in the absence or presence of NVP-AEW541. At the end of the incubation period cells were lysed and levels of poly (ADP-ribose) polymerase (PARP) were measured by Western blots. Appearance of an ~85 kDa band, a proteolytic cleavage product of the full-length ~116 kDa PARP protein, is considered an early marker of apoptosis. Results obtained showed that, after 2 h, IGF-I treatment diminished the intensity of the ~85 kDa band in all of the cell lines (Fig. 3A). However, addition of NVP-AEW541 on top of IGF-I prevented from IGF-I from exerting its antiapoptotic effect. This effect was evident after 24 h only in ECC-1 and USPC-1 cells.

Effect of IGF-IR inhibition on apoptosis

To assess the impact of IGF-IR inhibition on apoptosis, endometrial cancer cell lines were serum starved for 24 h, after which they were treated with IGF-I (50 ng/ml) for 2 h or 24 h in the absence or presence of NVP-AEW541. At the end of the incubation period cells were lysed and levels of poly (ADP-ribose) polymerase (PARP) were measured by Western blots. Appearance of an ~85 kDa band, a proteolytic cleavage product of the full-length ~116 kDa PARP protein, is considered an early marker of apoptosis. Results obtained showed that, after 2 h, IGF-I treatment diminished the intensity of the ~85 kDa band in all of the cell lines (Fig. 3A). However, addition of NVP-AEW541 on top of IGF-I prevented from IGF-I from exerting its antiapoptotic effect. This effect was evident after 24 h only in ECC-1 and USPC-1 cells.

Effect of IGF-IR inhibition on proliferation

To assess the impact of the NVP-AEW541 inhibitor on cell proliferation, cells were seeded in 24 well plates (2×10⁴ cells/well), and exposed to increasing doses of the inhibitor and IGF-I (50 ng/ml) for 72 h. The proliferation rate was determined by MTT assays. Results obtained showed that addition of NVP-AEW541 on top of IGF-I in all of the cell lines caused a significant decrease in proliferation rate compared with the IGF-I-induced rate seen in control cells (Fig. 3B). Thus, in ECC-1 cells, the inhibitor caused a 23%, 70%, and 108% decreases in proliferation rate at 24 h, 48 h, and 72 h, respectively, at the low dose (1 μM), and a 50%, 95%, and 140% decreases at the high dose (10 μM). On the other hand, in Ishikawa cells the low dose led to minimal decreases in cell numbers compared with IGF-I treated cells,
whereas the high dose resulted in significant decreases in cell numbers (41%, 50%, and 111% at 24 h, 48 h and 72 h respectively). Furthermore, decreases in cell proliferation were observed in USPC-1 and USPC-2 cells with the high dose of NVP-AEW541 (66%, 114%, 184% and 50%, 83%, 122% at 24 h, 48 h and 72 h, respectively).

**Effect of IGF-IR inhibition on cell cycle progression**

To examine whether the IGF-IR inhibitor caused changes in IGF-I-induced cell cycle events, endometrial cancer cells were serum-starved and then incubated with IGF-I (50 ng/ml) for 24 h in the presence or absence of NVP-AEW541. At the end of the incubation period, cells were harvested, propidium iodide was added, and samples were analyzed by flow cytometry. Cell cycle analysis revealed that in all of the cell lines the inhibitor reduced the IGF-I-induced proportion of cells at the G2/M phase and caused an increase in the proportion of cells at G0/G1 phase by ~20% in ECC-1 and USPC-1 cells and by ~16% in USPC-2 cells (Table 1).

**Effect of IGF-IR inhibition on IGF-IR internalization**

To assess the effect of the inhibitor on IGF-IR internalization, ECC-1 and USPC-2 cells were transiently transfected with an IGF-I-expression plasmid fused to a GFP tag. After 48 h, cells were treated with the inhibitor for 1 h. Microscopic analyses were done using confocal microscopy. Results obtained showed that NVP-AEW541 triggered significant IGF-IR internalization in ECC-1 and USPC-2 cells (Fig. 4). Thus, whereas the receptor displayed a mainly membrane localization of NVP-AEW541 by assessing its effects on IR activation. Bars are mean±SEM of 3 independent experiments.

**Discussion**

Due to the aggressive course of advanced/recurrent endometrial adenocarcinoma and USPC, and given the lack of effective therapeutic strategies, there is a need for development of novel approaches for improving patient outcome. Because the clinical course of USPC is similar to that of serous papillary ovarian cancer, and in view of the rarity of this type of endometrial tumor, most of the management of this disease is based on experience gained with ovarian cancer. Of interest, several studies have shown a correlation between some of the major components of the IGF system and endometrial cancer risk. Thus, Ayabe et al. [25] reported higher IGF-I and lower IGFBP-1 circulating levels in postmenopausal patients with endometrial cancer compared with controls. Petridou et al. [26] reported that endometrial cancer was positively associated with IGF-II blood levels and inversely correlated with IGF-I. In addition, McCampbell et al. [15] reported a large increase in IGF-IR levels in biopsies from hyperplastic endometrium and endometrial carcinoma compared with proliferative endometrium. Likewise, IGF-IR and IGF-II expression and endometrial cancer stage was investigated in a study that included 59 endometrial adenocarcinomas, 10 endometrial hyperplasias, and 7 normal tissues [13]. A higher expression of IGF-IR and IGF-II in malignant tissue at advanced stages (stages III-IV) compared with early stages or endometrial hyperplasia was reported. In the present study we demonstrate that the selective IGF-IR inhibitor NVP-AEW541 effectively blocks IGF-IR activity in all of the endometrial cancer cell lines investigated. Furthermore, the inhibitor also abolished the IGF-I-mediated signaling cascades, mainly in ECC-1 and USPC-1 cancer cells. Cell cycle analyses revealed that NVP-AEW541 caused a progressive accumulation of ECC-1, USPC-1 and USPC-2 cells in G0/G1 phases compared to IGF-I-treated cells, with a marked decrease in the percentage of cells in S and G2/M phases.

In addition to its effect on cell cycle progression, Western blot and MTT analyses revealed that NVP-AEW541 led to apoptotic cell death and exhibited antiproliferative effects. Thus, addition of NVP-AEW541 on top of IGF-I induced PARP cleavage and prevented from IGF-I from exerting its antiapoptotic effect after 24 h in ECC-1 and USPC-1 cells. The inhibitor also caused a significant decrease in the IGF-I-induced proliferation rate in all of the cell lines. Finally, our results revealed that NVP-AEW541 may trigger IGF-IR internalization in ECC-1 and USPC-2 cells. Particularly potent effects of the inhibitor were seen in the ECC-1 and USPC-1 endometrial cancer cells, probably because of the high IGF-IR expression in these cancer cells. We may speculate that Ishikawa cells, that have a constitutively active PI3K-AKT pathway due to PTEN mutations, are likely to be relatively resistant to IGF-IR targeted therapy.
A serious obstacle to specific IGF-IR targeting is the close homology between the IGF-IR and the IR kinase domains. Therefore, it is very important that any strategy designed to block IGF-IR signaling exhibits specificity for IGF-IR and does not affect IR signaling. Immunoprecipitation analysis revealed that the small molecule NVP-AEW541 blocks IGF-I-signaling without detectable effects on IR activation. Of interest, a slight reduction in IR activity was seen in the USPC-1 cell line, however this reduction is probably not significant because of the low basal IR expression in this cell line. Together, these data suggest that NVP-AEW541 might be a promising anticancer therapeutic strategy in endometrial cancer.

Conflict of interest statement
The authors declare that there are no conflict of interests.

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