ABT-869 Inhibits the Proliferation of Ewing Sarcoma Cells and Suppresses Platelet-Derived Growth Factor Receptor β and c-KIT Signaling Pathways

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Abstract

The Ewing Sarcoma (EWS) family of tumors is one of the most common tumors diagnosed in children and adolescents and is characterized by a translocation involving the EWS gene. Despite advances in chemotherapy, the prognosis of metastatic EWS is poor with an overall survival of <30% after 5 years. EWS tumor cells express the receptor tyrosine kinases, platelet-derived growth factor receptor (PDGFR) and c-KIT. ABT-869 is a multitransfected small-molecule inhibitor that targets Fms-like tyrosine kinase-3, c-KIT, vascular endothelial growth receptors, and PDGFRs. To determine the potential therapeutic benefit of ABT-869 in EWS cells, we examined the effects of ABT-869 on EWS cell lines and xenograft mouse models. ABT-869 inhibited the proliferation of two EWS cell lines, A4573 and TC71, at an IC₅₀ of 1.25 and 2 μmol/L after 72 h of treatment, respectively. The phosphorylation of PDGFRβ, c-KIT, and extracellular signal-regulated kinases was also inhibited. To examine the effects of ABT-869 in vivo, the drug was given to mice injected with EWS cells. We observed inhibition of growth of EWS tumor cells in a xenograft mouse model and prolonged survival in a metastatic mouse model of EWS. Therefore, our in vitro and in vivo studies show that ABT-869 inhibits proliferation of EWS cells through inhibition of PDGFRβ and c-KIT pathways. Mol Cancer Ther; 9(3): 653–60. ©2010 AACR.

Introduction

Ewing sarcoma (EWS) is the second most common primary bone tumor in childhood and is characterized by the EWS/FLI-1 translocation (1 2 3 4). Despite multimodal approaches to therapy, only 60% of patients with localized disease are cured. Approximately 30% of patients with metastatic disease have long-term survival beyond 5 years (5). The t(11;22)(q24;q12) translocation is identified in over 95% of EWS tumors and results in the formation of the EWS/ETS fusion gene (6). Of these translocations, EWS/FLI-1 is the most common, consisting of over 85% of these aberrations. The EWS/FLI-1 fusion gene encodes for a transcription factor, which results in abnormal growth. Chemotherapy, surgery, and radiation therapy are standard approaches to treat EWS; however, given the toxicities of treatment and poor prognosis of progressive disease, alternative modes of therapy are needed.

Several approaches have been used to target EWS cells for therapy. Because the EWS/ETS translocation is not expressed in normal cells and is unique to Ewing Sarcoma Family Tumors, it provides an attractive target for therapy. The inhibition of EWS/FLI-1 by either antisense oligonucleotides or small interfering RNAs has shown antitumor effects in vitro. However, due to the poor cellular penetration of small interfering RNAs and susceptibility to degradation, their activity has not been successful in in vivo models (7). Antisense oligonucleotides encapsulated in nanocapsules have inhibited growth of tumors in a mouse xenograft model (8). Rapamycin has been shown to downregulate EWS/FLI-1 and inhibit cell growth in vitro (9), suggesting that the inhibition of mammalian target of rapamycin and phosphatidylinositol 3-kinase are potential targets for therapy.

Platelet-derived growth factor receptor-β (PDGFRβ) is expressed on EWS cells, and its downstream signaling pathways are important for growth of tumor cells (10). The c-KIT tyrosine kinase receptor pathway has also

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been shown to be critical for growth and progression in EWS (11). Previous studies show that both pathways are activated in Ewing Sarcoma family tumors (12) and are potential molecular targets. Autophosphorylation of c-KIT is inhibited by imatinib, a receptor tyrosine kinase inhibitor, at an IC₅₀ of 0.1-0.5 μmol/L, whereas in vitro testing of cell lines showed that 50% growth inhibition required higher doses of imatinib at 10 μmol/L (11, 13, 14). This suggests that the effect of imatinib on the growth of EWS cells was not exclusively mediated by c-KIT, but by other pathways (15).

ABT-869 is a multitargeted small-molecule inhibitor that binds the ATP binding site of several receptor tyrosine kinases, including FLT3, c-KIT, vascular endothelial growth factor receptor (VEGFR)1-3, and PDGFα and β receptor family members (16). Preclinical studies have shown the efficacy of ABT-869 in acute myelogenous leukemia (AML), human fibrosarcoma, breast, colon, and small-cell lung carcinoma xenograft models, as well as in orthotopic breast, prostate, and glioma models (17). In AML cell lines, ABT-869 was shown to inhibit phosphorylation of signal transducers and activators of transcription 5, extracellular signal-regulated kinase (ERK), KIT, and Pim-1 (16). The drug was also able to inhibit tumor growth in mouse xenograft models of two AML cell lines with daily oral administration. Given similar targets in EWS cells, we hypothesized that ABT-869 might be active against this tumor in vitro and in vivo.

In this article, we report the effects of ABT-869 on EWS cell proliferation and signaling. The drug was tested in vitro and in vivo and was shown to inhibit the proliferation of EWS cells. Both c-KIT and PDGFβ receptors, as well as downstream kinases, were inhibited by ABT-869. Furthermore, the treatment of EWS cells in xenograft models resulted in prolonged survival. Our results suggest that ABT-869 is active against EWS tumor cells in vitro and in vivo.

Materials and Methods

Cell Lines and Culture Conditions

The EWS tumor cell lines, TC71 and A4573, were kindly provided by Timothy Triche (Children’s Hospital of Los Angeles, Los Angeles, CA). The cells were cultured on collagen-coated tissue culture plates in DMEM (Life Technologies/Invitrogen) containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine (Sigma, Inc.), and 10% fetal bovine serum (FBS; Life Technologies/Invitrogen). Adherent monolayers were passaged every 3 to 5 d and grown at 37°C in a humidified atmosphere with 5% CO₂.

ABT-869 Drug

ABT-869 is a receptor-tyrosine kinase inhibitor (Abbott Laboratories, Inc.). For in vitro analysis, this compound was dissolved in DMSO at a 10-mmol/L concentration and aliquoted in desired working volumes of 20 μL and stored at -20°C. The drug was further diluted in

DMSO and used at 1:1,000 dilutions in cell culture experiments. For in vivo analysis, the compound was suspended in corn oil and was administered by oral gavage at the dose of 40 mg/kg/d. This dose has shown to be well tolerated and sustain murine serum level of >1 μmol/L, 8 h after the dose was given (16, 17). The oral, once-daily dosing regimen would be easier for patients and is currently being studied in adult clinical trials.

Proliferation Studies

Dose response of the cell lines treated with ABT-869 was analyzed to determine the IC₅₀. To determine the rate of proliferation, cell counts were analyzed by the trypan blue exclusion method on a Beckman-Coulter Vi-CELL XR. Cells were seeded at 1 × 10⁵ cells/mL in triplicate in 1 mL on 24-well culture plates. The next day, the medium was replaced and the cells were incubated with various concentrations of ABT-869 for 72 h. The medium was removed, cells were washed with 1× PBS, and were trypsinized. The cells were washed off the plate with the culture medium and the entire sample was analyzed.

Immunoprecipitation and Western Blot Analysis

The expression of PDGFβ, c-KIT, and their signaling pathways was determined by Western blot analysis. Both A4573 and TC71 cell lines were seeded at 1 × 10⁵ cells/mL on 100-mm plates. The next day, the medium was replaced and the cells were incubated with the IC₅₀ dose of ABT-869 for 72 h. Before cell lysis, the cultures were treated with ligand for 10 min to induce the phosphorylation of the receptor tyrosine kinases and to activate their signaling pathways. EWS cells were treated with recombinant human PDGF-BB (Peprotech) at 100 μmol/L concentration or recombinant human Stem Cell Factor (R&D Systems) at 100 μmol/L concentration.

Cell lysates were obtained by washing the plates twice with 1× PBS then freezing at -20°C. The plates were thawed on ice and 0.5 mL radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris (pH 8.0)] containing 1% phosphatase inhibitor cocktail (Sigma, Inc.) and 1% protease inhibitor cocktail (Sigma, Inc.) was added to the plates. The plates were allowed to incubate on ice for ~10 min. The cells were scraped and an additional 0.2 mL of radioimmunoprecipitation assay buffer was added to wash the plates. The cells were sheared by passing the lysates through a 21-1/2-gauge then a 27-1/2-gauge syringe. The lysates were incubated and rotated at 4°C for 30 min. The cells were centrifuged at 14,000 × g for 10 min at 4°C. Protein concentrations were determined using the BCA protein assay reagent (Pierce Biotechnologies).

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For immunoprecipitations, the Catch and Release v2.0 kit (Upstate Biotechnology, Inc.) was used as directed, loading 500 μg to 1 mg of whole-cell lystate and 4 μg of specific primary antibody. The columns were incubated overnight at 4°C on a rotator. The columns were spun down and the eluate was used for Western blot analysis. The bound proteins were eluted with 40 μL denaturing elution buffer. Boiling Laemmli buffer [1 mol/L Tris-HCl (pH 6.8), 10% SDS, 0.5% bromophenol blue, 50% glycerol, 5% βME] was added to bring the total volume of eluted proteins to 60 μL. The immunoprecipitated samples were resolved on a 5% SDS-PAGE gel and transferred to nitrocellulose membranes, incubated with specific antibodies, and visualized by chemiluminescence. Other proteins (50 μg/lane) were resolved on an 8% or 10% SDS-PAGE gel.

The antibodies used for immunoprecipitation were c-KIT (MS-289, Thermo Scientific) and PDGFRβ (sc-432, Santa Cruz Biotechnology). The antibodies used to characterize the phosphorylation status of PDGFRβ and KIT were c-KIT (A4502, DAKO), phospho-c-KIT (ab5631, Abcam), PDGFRβ (sc-432, SCBT), and phospho-tyrosine (sc7020, SCBT). The antibodies used to characterize the activation of the downstream signaling pathways were pan AKT, phospho-AKT(p-thr), p42/p44-mitogen-activated protein kinase (MAPK), phospho-p42/p44-MAPK, GSK3β, and phospho-GSK3β. Unless otherwise noted, all antibodies were purchased from Cell Signaling Technology, Inc.

**Xenograft Model of EWS in Nonobese Diabetic/Severe Combined Immunodeficient Mice**

TC71-GFP/LUC and A4573-GFP/LUC cells were grown in DMEM with 10% FBS, antibiotics (penicillin/streptomycin), and 1-glutamine to a density of 75% to 90%. To prepare for injection, cells were trypsinized from the tissue culture plates and washed twice with PBS. Cells were counted and viability was tested using the trypan blue exclusion method. Immediately before injection, the cells were resuspended in a serum-free, antibiotic-free medium. Only cells that were growing with a viability of >90% were used.

Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were 6 to 8 wk of age at the time of injection. Each mouse was injected with 5 × 10⁶ TC71-GFP/LUC or A4573-GFP/LUC cells suspended in equal volume of DMEM (without FBS or antibiotics) and Matrigel in 0.2 mL. The mixture was injected using a 28-1/2-guage needle s.c., dorsally off the midline. The mice were treated in three separate experimental groups: ABT-869 treatment provided immediately, a delayed ABT-869 treatment group, and a group treated with corn oil vehicle control. The delayed group was initially given corn oil until the mice had a tumor volume of 300 mm³, then ABT-869 treatment was initiated. All mice were euthanized when the vehicle control mice reached a tumor volume of 2.5 cm³. The mice were treated according to the NIH Guidelines for Animal Care and as approved by...
the University of California at Los Angeles Institutional Animal Care and Use Committee.

Metastatic EWS Model in NOD/SCID Mice and Bioluminescence Imaging

TC71-GFP/LUC and A4573-GFP/LUC cells were grown in DMEM with 10% FBS, antibiotics (penicillin/streptomycin), and l-glutamine. To prepare for injection, cells were trypsinized from the tissue culture plates and washed twice with PBS. Cells were counted and viability was tested using the trypan blue exclusion method. Immediately before injection, the cells were resuspended in a serum-free, antibiotic-free medium. Only cells >90% viable were used.

All NOD/SCID mice were 6 to 8 wk of age at the time of injection. Each mouse was injected with 5 × 10⁶ TC71-GFP/LUC or A4573-GFP/LUC cells suspended in 0.1 mL DMEM (without FBS or antibiotics) through the tail vein using a 28 1/2-gauge needle. All experimental manipulations with the mice were done under sterile conditions in a laminar flow hood. The mice were treated in two separate experimental groups: immediate ABT-869 and corn oil vehicle. Six mice per treatment group were analyzed.

After the injection of cells, the mice were imaged at various time points to ensure the presence of disease using an in vivo IVIS 100 bioluminescence/optical imaging system (Xenogen). D-Luciferin (30 mg/mL; Xenogen) dissolved in PBS was injected i.p. at a dose of 100 μL/mouse 15 min before measuring the light emission. General anesthesia was induced with 2.5% isoflurane and continued during the procedure with 2% isoflurane.

After acquiring photographic images of each mouse, luminescent images were acquired with various (5–60 s) exposure times. The resulting grayscale photographic and pseudocolor luminescent images were automatically superimposed by the IVIS Living Image (Xenogen) software to facilitate matching the observed luciferase signal with its location on the mouse.

Immunohistochemistry

All tumors were harvested from the mice. The tumor sections were fixed in formalin and submitted to the University of California at Los Angeles Department of
Pathology & Laboratory Medicine for sectioning and staining. The slides were stained with H&E and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) antibodies purchased from Cell Signaling Technology, Inc. Digital images of representative slides were taken.

Results

**ABT-869 Inhibits Proliferation of EWS Cells In vitro**

To assess the effects of ABT-869 on EWS cell growth, we analyzed two EWS cell lines, A4573 and TC71, after treatment at various concentrations of the drug from 10 nmol/L to 10 μmol/L by the trypan blue exclusion method. Initial testing showed that the IC_{50} value for cellular proliferation for both A4573 and TC71 EWS cells were between 1 and 10 μmol/L (Fig. 1A). Further testing showed that ABT-869 significantly inhibited the growth of both EWS lines at concentrations between 1 and 2 μmol/L after 72 h of treatment. The IC_{50} value for cellular proliferation of the A4573 cells was 1.25 μmol/L, whereas IC_{50} value for cellular proliferation of the TC71 cells was 2 μmol/L (Fig. 1B). Similarly, MTT assays confirmed that ABT-869 inhibited growth of both A4573 and TC71 cells at the same IC_{50} concentrations (data not shown).

**ABT-869 Inhibits Activation of the PDGFRβ and c-KIT Signaling Pathways**

Previous studies showed that EWS cell lines overexpress the receptor tyrosine kinases, PDGFRβ, and c-KIT (14). To determine whether inhibition of PDGFRβ and c-KIT pathways participate in the proliferation of EWS cells, we analyzed the activation of PDGFRβ and c-KIT after treatment of two human EWS cell lines, TC71 and A4573, with ABT-869. Immunoprecipitations were done with PDGFRβ or c-KIT antibody. Treatment with the PDGFRβ ligand, PDGF-BB, at 100 μmol/L concentration resulted in significant phosphorylation of PDGFRβ (Fig. 2A) in both cell lines, but pretreatment for 72 hours with their respective IC_{50} concentrations of ABT-869 blocked PDGF-BB–mediated PDGFRβ phosphorylation. Similarly, SCF-induced c-KIT phosphorylation was blocked by ABT-869 pretreatment in both cell lines (Fig. 2B). We also examined cells that were not treated or stimulated with PDGF or c-KIT ligand and there was no difference compared with nontreated and stimulated (data not shown). These results show that PDGFRβ and c-KIT activation are inhibited by ABT-869.

Activation of PDGFRβ and c-KIT initiates signaling pathways critical to cell proliferation, survival, angiogenesis, and blood vessel maturation (10). Two critical pathways downstream of PDGFRβ and c-KIT include ERK and phosphatidylinositol 3-kinase/AKT. Both pathways are controlled by several other receptor tyrosine kinases, including IGFR and VEGFR2. To assess whether ABT-869 could inhibit the activation of ERK or AKT pathways downstream of PDGFRβ and c-KIT in EWS cells, we treated TC71 and A4573 cells with the ligands for PDGFRβ and c-KIT in the presence of the drug or vehicle control and did Western blot analyses with phosphospecific antisera. ABT-869 inhibited activation of ERK in the PDGF-BB and SCF stimulated lysates, whereas the phosphorylation of AKT was partially inhibited by drug treatment in A4573 cells (Fig. 3). Our results suggest that ABT-869 treatment inhibits the activation of p42/p44MAPK and in certain EWS cells, AKT.

**ABT-869 Inhibits the Growth and Progression of EWS Cells In vivo**

To determine whether the inhibition of PDGFRβ and c-KIT induced by ABT-869 inhibits tumor growth in vivo, NOD/SCID mice were inoculated s.c. with TC71 or A4573 cells. Mice were treated daily by oral gavage with either ABT-869 at 40 mg/kg or a corn oil vehicle control. The delayed treatment group received ABT-869 at 40 mg/kg/d when the tumors reached a volume of

![Figure 4. ABT-869 treatment in a xenograft model of EWS suppresses tumor growth in vivo. Points, mean tumor volumes in SCID mice (3–5 per group) injected with 5 × 10^6 A4573 cells (A) or TC71 (B) in the flank with Matrigel; bars, SEM. Mice were given 40 mg/kg/d of ABT-869 orally every day. Mice gavaged with corn oil vehicle alone had a 28- to 50-fold greater tumor volume at 25 d compared with mice treated at the time of injection. In mice treated when tumor volume was 300 mm^3 (arrow), log linear tumor growth rates tended to be slower than control after the start of this delayed treatment.](http://www.aacrjournals.org/doi/10.1158/1535-7163.MCT-09-0812)
300 mm$^3$. Previous studies showed that the drug does not affect normal organ function (17). We did not observe any signs of physical distress (lethargy, ruffled fur) or weight loss during the course of treatment with ABT-869 during our experiments (data not shown).

Treatment with ABT-869 directly after inoculation resulted in activity preventing tumor formation from injected cells. In previous experiments, treatment with the drug after significant tumor burden did not result in improved survival (data not shown). Therefore, this experiment was done to assess the effects of drug in a setting of microscopic disease, before the onset of significant metastatic disease. One of the difficulties with eradicating EWS disease is that there are residual cells that are resistant to chemotherapy, which increase the risk of relapse. Tumor growth was significantly inhibited following delayed treatment of drug at 40 mg/kg/d (Fig. 4A and B). Geometric mean tumor volumes at 25 days after injection with TC71 cells were 22% and 2.0% of vehicle control under delayed and immediate treatment, respectively ($P < 0.01$ for delayed versus immediate and for both comparisons to control volume). Similarly, geometric mean volumes using the A4573 cell line were 23% and 3.6% of control, respectively ($P < 0.01$ for all comparisons). By H&E staining, the histology showed that tumors from mice treated with ABT-869 had increased evidence of necrosis and inflammation compared with vehicle controls (Fig. 5A and B). TUNEL staining showed increased apoptosis in the immediate and delayed treatment groups compared with the vehicle controls for both cell lines (Fig. 5C and D). There were no differences in the cell cycle profile of cells treated with ABT-869 compared with vehicle control (data not shown). Therefore, ABT-869 is effective in suppressing growth and inducing cell death of EWS cells in vivo.

ABT-869 Inhibits Progression of Tumor Cells in a Metastatic EWS Model

To analyze the potential effects of ABT-869 on a metastatic model of EWS, green fluorescent protein (GFP)/Luciferase-expressing A4573 and TC71 cells (A4573-GFP/LUC, TC71-GFP/LUC) were generated through lentiviral transduction followed by sorting for GFP. The sorted cells were cultured and injected through the tail vein into female NOD/SCID mice. Six mice were analyzed per treatment group. Engraftment and disease progression were monitored by acquiring in vivo bioluminescent images at least once per week. The mice began treatment the day after injection. Kaplan-Meier analysis showed a survival benefit in the treatment group compared with the vehicle control group with both the A4573 GFP/LUC cell lines ($P = 0.015$; Fig. 6A) and TC71-GFP/LUC ($P = 0.002$; Fig. 6B). Furthermore, the tagged cells showed evidence of more aggressive disease in mice treated with ABT-869 compared with nontreated mice (Fig. 6C). As previously observed, the mice tolerated the ABT-869 well and maintained their normal activity levels and weight (16). These results suggest that survival...
is prolonged and disease progression is suppressed in mice treated with ABT-869.

Discussion

The use of a multimodal approach to the treatment of EWS has resulted in improved outcomes. However, patients with metastatic, relapsed, or resistant EWS continue to have poor prognoses. Therefore, improved therapeutic modalities are warranted. Previous work showed that tyrosine kinases, c-KIT and PDGFRβ, are both expressed in EWS cells and are potentially important targets for therapy (10, 11). Both of these receptor tyrosine kinases and their downstream targets seem to be crucial for the growth of EWS tumors (10, 11). This is the first report that shows that targeting c-KIT and PDGFRβ through a multitargeted receptor tyrosine receptor kinase inhibitor is effective in suppressing the growth of EWS cells in vitro and in vivo.

We previously published that ABT-869 inhibited phosphorylation of constitutively active receptor tyrosine kinase, fms-like tyrosine kinase internal tandem duplication (FLT3-ITD) in AML cells (16). In this article, we show that a multitargeted small-molecule receptor tyrosine kinase inhibitor, ABT-869, also inhibits the phosphorylation of receptor tyrosine kinases in EWS cells and inhibits growth of tumor cells in vitro and in vivo.

Previous reports have shown the inhibition of EWS cell proliferation by targeted therapies. Gefitinib and vandetanib are potent inhibitors of EGFR and VEGFR-2, respectively. When tested against the EWS cell line TC71,
the IC_{50} was relatively high at 10 μmol/L, compared with the nanomolar concentrations that inhibit EGFR and VEGFR-2 kinase activity in vitro (18). This suggests that the EGFR inhibition alone is most likely not sufficient to have an effect on the growth of EWS cells as a single agent. In the two cell lines that were tested, gefitinib and vandetanib did not inhibit the phosphorylation of p42/44 MAPK (Erk 1/2) and AKT-1, nor did they affect levels of cyclin D1 and c-myc (18). In our studies, ABT-869 at low micromolar concentrations showed decreased phosphorylation of ERK 1/2 in both the TC71 and A4573 cell lines and also showed decreased phosphorylation of AKT in the A4573 cell line. Given the higher IC_{50} of ABT-869 in EWS compared with in AML cells, our results suggest that the drug inhibits proliferation at least in part through suppressing the activation of the PDGFβ and c-KIT receptors and their downstream targets. However, these pathways do not seem to be strong drivers of EWS cell proliferation. Additional pathways or kinases, such as VEGFR, involving angiogenesis, may be alternative mechanisms by which ABT-869 inhibits EWS cells in vitro (17). Imatinib, another receptor tyrosine kinase inhibitor, has been shown to decrease autophosphorylation of c-KIT in vitro, but its effects on the growth of EWS cells required a dose that was much higher than ABT-869, with most cell lines requiring >10 μmol/L (11, 13, 14). This suggests that c-KIT inhibition alone is insufficient to provide a therapeutic effect in EWS.

Our results with xenograft models showed that treatment with ABT-869 resulted in decreased tumor growth. The fact that ABT-869 is not a general antiproliferative drug, but rather inhibits both proliferation and induces cell death, is consistent with previous reports (17). Results using luciferase-tagged EWS cells suggest that ABT-869 prolongs survival and maintains stable disease. This may have clinical significant because survival of patients with metastatic EWS is poor despite multimodal chemotherapy. Thus, our data suggest that use of ABT-869 may be useful for patients with metastatic disease. However, we did observe a difference in the xenograft model compared with the metastatic model. This difference is most likely due to the greater tumor burden in the metastatic disease model. Very little toxicity was observed in mice, suggesting that this drug could be potentially used to treat patients with EWS. Previous studies showed that imatinib sensitizes EWS cells to vincristine and doxorubicin (11). Future experiments will examine combined therapy with ABT-869 and chemotherapy or other small molecules that target additional signaling pathways.

Disclosure of Potential Conflicts of Interest

K.M. Sakamoto: commercial research grant, Abbott Laboratories. No other potential conflicts of interest were disclosed.

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