Src as a Therapeutic Target in Biliary Tract Cancer

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Abstract

Src, a nonreceptor tyrosine kinase, is involved in a number of cancer-related signaling pathways and aberrantly activated in biliary tract cancer (BTC). This study aimed to elucidate the potential role of Src as a therapeutic target in BTC. We tested bosutinib, an orally active c-Src/Ab1 kinase inhibitor, alone or in combination with cytotoxic agents using 9 human BTC cell lines: SNU-245, SNU-308, SNU-478, SNU-869, SNU-1079, SNU-1196, HuCCT1, TFK-1, and EGI-1. Of these, SNU-308 and SNU-478 were relatively sensitive to bosutinib. Bosutinib abrogated phosphorylation of Src and its downstream molecules, and significantly increased G1 cell-cycle arrest and apoptosis. Bosutinib significantly inhibited cell migration and invasion and decreased epithelial–mesenchymal transition markers. Bosutinib combined with gemcitabine or cisplatin showed synergistic antiproliferative and antimigratory effects. In addition, this combination further inhibited phosphorylation of Src and its downstream molecules and decreased epithelial–mesenchymal transition marker expression compared with bosutinib alone. We established a SNU-478 xenograft model for in vivo experiments, because SNU-478 was more tumorigenic than SNU-308. Bosutinib combined with gemcitabine or cisplatin showed significantly more potent antitumor effects than bosutinib alone. Bosutinib combined with gemcitabine further decreased Ki-67 expression and Src phosphorylation, and further increased TUNEL expression. Our data suggest that Src might be a potential therapeutic target in BTC. Bosutinib demonstrated promising antitumor activity alone or in combination with gemcitabine or cisplatin in BTC cells, which supports further clinical development in patients with advanced BTC. Mol Cancer Ther; 15(7); 1515–24. ©2016 AACR.

Introduction

Src, a nonreceptor tyrosine kinase, is involved in a number of cancer-related signaling pathways including FAK, PI3K, ERK, and STAT3, and promotes the proliferation, adhesion, migration, invasion, and metastasis of cancer cells (1–4). Its activity is increased in a variety of malignancies such as lung, skin, colorectal, breast, ovarian, and head and neck cancer (3–6). In addition, Src activation conferred therapeutic resistance to imatinib treatment for chronic myelogenous leukemia (7, 8), hormone treatment for breast and prostate cancer (9, 10), and trastuzumab treatment for breast cancer (11, 12). On the basis of these findings, Src-targeted agents have recently been investigated in preclinical and clinical studies in a variety of malignant diseases.

Biliary tract cancer (BTC) is a relatively uncommon malignancy with a poor prognosis (13). Most patients are diagnosed at an advanced stage and experience relapse despite radical surgery (14). Although the recent development of targeted therapeutics has significantly improved the clinical outcome of patients with advanced solid tumors, still there is no validated therapeutic target for advanced BTC and the prognosis of patients with advanced BTC remains still disappointing (15, 16). In recent years, only gemcitabine plus cisplatin has been established as a standard chemotherapy for BTC patients (17). Therefore, there is still an urgent unmet need for the development of novel therapeutic strategies for the treatment of advanced BTC, based on specific targets on cancer cells.

A recent study demonstrated that Src is also frequently overexpressed in BTC as in other malignant diseases, although its relationship with clinicopathologic parameters or histologic origin was not significant (18). In addition, blocking Src activity by novel Src inhibitors such as saracatinib (AZD-0530) and AZM555130 reduced the proliferative and invasive potential of human BTC cell lines (18, 19). However, these studies used only a limited number of BTC cell lines and only tested Src inhibitor monotherapy, without investigating combination strategies with other chemotherapeutic agents that are already approved for BTC treatment. Therefore, further preclinical studies are necessary before designing clinical studies using Src inhibitors.

The aim of our study was to investigate Src as a potential therapeutic target in BTC. We evaluated the therapeutic potential of bosutinib (4-anilino-3-quinolinecarbonitrile, also known as...
Materials and Methods

Human BTC cell lines
A total of 9 human BTC cell lines were used in this study. SNU-245, SNU-308, SNU-478, SNU-869, SNU-1079, and SNU-1196 cell lines were purchased from the Korean Cell Line Bank in March 2007 (21). HuCCT1 and TKF-1 cell lines were obtained from the RIKEN BioResource Center in March 2007. EGI-1 cell line was obtained from the Leibniz-Institut DSMZ (German Collection of Microorganisms and Cell Cultures) on March 2007. The origin of each cell line was as follows: SNU-245, extrahepatic cholangiocarcinoma (common bile duct); SNU-308, gallbladder adenocarcinoma; SNU-478, ampulla of Vater adenocarcinoma; SNU-869, ampulla of Vater adenocarcinoma; SNU-1079, intrahepatic cholangiocarcinoma; SNU-1196, extrahepatic cholangiocarcinoma (hepatic duct bifurcation); HuCCT1, intrahepatic cholangiocarcinoma; TKF-1, extrahepatic cholangiocarcinoma; and EGI-1, extrahepatic cholangiocarcinoma (21–24). Most recent authentication of each cell line was performed using “AmpFLSTR Identifiler PCR Amplification Kit (catalog no. 4322288; Applied Biosystems)” by the Korean Cell Line Bank on March 8, 2016. The 3530xl DNA Analyzer (Applied Biosystems) and the Gene-Parser cycler were used for DNA fingerprinting analysis. SNU-245, SNU-308, SNU-478, SNU-869, SNU-1079, SNU-1196, HuCCT1, and TKF-1 cell lines were maintained in RPMI1640 media containing 10% FBS (Welgene Co., Ltd.).

Tested agents
Bosutinib was purchased from Selleck Chemicals LLC for in vitro experiments and provided by Pfizer Inc. for in vivo experiments. The compound was dissolved in DMSO. Gemcitabine was purchased from Lilly Korea Co. Cisplatin was purchased from JW Pharmaceutical Co.

Cell growth inhibition assay
Cells were seeded in 96-well plates and exposed to increasing concentrations of targeted or cytotoxic agents for 72 hours. After drug treatment, tetrazolium dye (MTT; Sigma-Aldrich) was added to each well and incubated for 4 hours at 37°C. Then, the solution was removed carefully and DMSO was added. Cell viability was determined by measuring the absorbance at 570 nm with a VersaMax Microplate Reader (Molecular Devices). The half-maximal inhibitory concentration (IC50) of chemotherapeutic agents was calculated.

Cell-cycle analysis
Cells treated with bosutinib at various concentrations for 24 hours were harvested, fixed with cold 70% ethanol, and stored at −20°C. The fixed cells were harvested by centrifugation and washed in 20 mg/mL RNase A (Invitrogen) and incubated at 37°C for 10 minutes. Next, the cells were stained with 20 μg/mL propidium iodide (PI; Sigma-Aldrich). The DNA content of 10,000 cells per each experimental group was analyzed using a FACS Calibur Flow Cytometer (BD Biosciences). Three independent experiments were performed for each condition.

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The top surface of the insert membrane was coated with a uniform layer of dried murine laminin I matrix. Cells were serum starved for 24 hours then a cell suspension containing $1 \times 10^6$ cells/mL in serum-free media alone or serum-free media with 1.0 μmol/L of bosutinib was added to the inside of each insert. Each insert was then transferred to a bottom well of the plate filled with media containing 20% FBS. After incubation for 24 hours, the invading cells were stained and extracted, and then quantified by measuring the absorbance at 560 nm with a VersaMax microplate reader (Molecular Devices). The data presented are representative of two independent experiments.

**In vivo study**

Animal experiments were performed at the Biomedical Center for Animal Resource Development of Seoul National University (Seoul, Korea) according to the institutional guidelines with prior approval from the Institutional Animal Care and Use Committee. Of SNU-308 and SNU-478 cell lines that were sensitive to bosutinib in this study, we used the SNU-478 xenograft model for *in vivo* experiments, as the SNU-478 cell line was more tumorigenic in female Balb/c athymic nude mice than the SNU-308 cell line. A total of 30 female Balb/c athymic nude mice ages 4 to 6 weeks were supplied from Central Lab Animal, Inc. The mice were adapted to local conditions for 1 week, and then injected subcutaneously in the right flank with $1 \times 10^7$ SNU-478 cells in 100 μL of PBS. After implantation of the tumor cells, the tumor volume was measured every week using calipers and calculated using the following formula: $(\text{width}^2 \times \text{height})/2$. When the tumor volume reached 200 mm³, the mice were randomly divided into 6 treatment groups: control, bosutinib, gemcitabine, cisplatin, bosutinib plus gemcitabine, and bosutinib plus cisplatin. The control group was treated with 0.5% methanol and 0.4% Tween 80 in deionized water via oral gavage. Bosutinib at a dose of 150 mg/kg was administered via oral gavage once daily for 28 days. Gemcitabine (100 mg/kg) and cisplatin (4.5 mg/kg) were injected intraperitoneally twice a week for 28 days. When the tumor volume reached 1,500 mm³, the mice were euthanized with CO₂. The tumors were analyzed as follows: Src, 60 kDa; FAK, 125 kDa; AKT, 60 kDa; ERK, 42/44 kDa; STAT3, 79/86 kDa; and β-actin, 42 kDa.

**Figure 1.**

Bosutinib inhibits the proliferation of BTC cells via the inhibition of Src phosphorylation. A, the effects of bosutinib on BTC cell proliferation were evaluated by MTT assays. Cells were treated with increasing doses of bosutinib for 72 hours. B, a Matrigel-embedded three-dimensional culture system was used to investigate the antiproliferative activity of bosutinib in SNU-308 and SNU-478 cells. Cells were treated with 0 (DMSO), 0.1, and 0.5 μmol/L of bosutinib every 3 days for 12 days, and then examined under light microscopy. C, Western blot analysis was performed to evaluate the effect of bosutinib on Src signaling pathways in BTC cells. SNU-308, SNU-478, and TFK-1 cells were treated with increasing concentrations of bosutinib [0 (DMSO), 0.1, 0.5, and 1.0 μmol/L] for 48 hours, after which protein extracts were immunoblotted with the indicated antibodies. The molecular weight of each protein is as follows: Src, 60 kDa; FAK, 125 kDa; AKT, 60 kDa; ERK, 42/44 kDa; STAT3, 79/86 kDa; and β-actin, 42 kDa.
excised and stored in liquid nitrogen for further Western blot analysis or immunohistochemical staining.

IHC

Four-micron thick sections from paraffin-embedded xenograft tumor tissues were deparaffinized and dehydrated. IHC detection of proliferating cells was conducted using an anti-Ki-67 antibody (GeneTex, Inc.) at a dilution of 1:100. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays were conducted for the IHC detection of apoptosis using an ApopTag In situ Apoptosis Detection Kit (EMD Millipore), in accordance with the manufacturer’s protocol. A phosphorylated Src antibody was used at a dilution of 1:200 and was purchased from Cell Signaling Technology.

Statistical analysis

Experimental data were expressed as the mean ± SE and compared using the Student t test. Data were analyzed and displayed using SigmaPlot software (Systat Software, Inc.). All statistical tests were two-sided, with significance defined as P < 0.05.

Results

Bosutinib inhibits BTC cell proliferation by inhibition of Src phosphorylation and abrogation of its downstream signaling pathways

A total of nine BTC cell lines were treated with bosutinib. Among them, SNU-308 and SNU-478 cells were sensitive to bosutinib with IC_{50} values of 0.65 ± 0.06 and 0.63 ± 0.03 μmol/L, respectively, compared with other cells (Fig. 1A; Supplementary Table S1). In contrast, TFK-1 cells were relatively resistant to bosutinib with an IC_{50} value of 4.45 μmol/L. In a three-dimensional culture system, bosutinib also showed an antiproliferative effect that was dose dependent in both SNU-308 and SNU-478 cells (Fig. 1B).

Next, Western blot analysis was performed to evaluate the effect of bosutinib on the downstream signaling pathways of Src (Fig. 1C). In SNU-308 and SNU-478 cells, bosutinib abrogated Src Tyr416 and FAK Tyr397 phosphorylation in a dose-dependent manner. In contrast, FAK Tyr925 phosphorylation was slightly inhibited by high-dose bosutinib in the SNU-308 cell line only, whereas it was upregulated in SNU-478 and TFK-1 cell lines. In addition, the phosphorylation of AKT and ERK in SNU-308 and SNU-478 cells was decreased with increasing concentrations of bosutinib. Bosutinib treatment decreased STAT3 phosphorylation in SNU-478 cells but it was increased in SNU-308 and TFK-1 cells. In TFK-1 cells, bosutinib did not significantly influence the phosphorylation of Src, AKT, and ERK when compared with SNU-308 and SNU-478 cells.

Inhibition of Src by bosutinib induces G_1 cell-cycle arrest in sensitive BTC cell lines

The effects of bosutinib on the cell cycle of SNU-308, SNU-478, and TFK-1 cells were evaluated using flow cytometry. Bosutinib significantly increased G_1 cell-cycle arrest in a dose-dependent manner in both SNU-308 and SNU-478 cells (Fig. 2A). The sub-G_1 fraction was significantly increased by bosutinib treatment in SNU-478 cells (P = 0.008), and showed a tendency to be increased in SNU-308 cells with borderline significance (P = 0.062). In TFK-1 cells, G_1 cell-cycle arrest was significantly increased after 1 μmol/L bosutinib treatment but this was not observed at lower concentrations. The sub-G_1 fraction in TFK-1 cells was not significantly increased by bosutinib treatment (P = 0.572). After 1 μmol/L bosutinib treatment for 48 hours, the Annexin V-FITC/PI stain revealed that bosutinib significantly induced both early and late apoptosis in SNU-308 and SNU-478 cells (Fig. 2B). However, the tendency was not apparent in TFK-1 cells. Bosutinib decreased cyclin D, cyclin E, cyclin A, and cyclin B expression, and increased p27 expression in both SNU-308 and SNU-478 cells, but not in TFK-1 cells (Fig. 2C). Of note, the nuclear expression of p27 was significantly increased in both SNU-308 and SNU-478 cells, but not in TFK-1 cells (Fig. 2D).

Bosutinib inhibits the migration and invasion of BTC cells

Migration and invasion assays were performed to determine the effect of bosutinib on the migration and invasion activity of BTC cells. The migration assays indicated that bosutinib significantly inhibited the migratory activity of both SNU-308 and SNU-478 cells (Fig. 3A). Furthermore, bosutinib significantly inhibited the invasive activity of SNU-478 cells (Fig. 3B).

Next, we performed Western blot analysis to investigate the effect of bosutinib on the expression levels of proteins related to epithelial–mesenchymal transition (EMT) in SNU-308 and SNU-478 cells. Bosutinib did not influence the expression levels of E-cadherin and β-catenin in both cell lines. However, Vimentin levels were slightly decreased and Snail expression was potently abrogated by bosutinib in a dose-dependent manner (Fig. 3C).

Src inhibition enhances the antiproliferative and antimigratory effects of cytotoxic agents in BTC

Because gemcitabine and cisplatin are standard chemotherapeutic agents in patients with advanced BTC, we investigated the combined effects of bosutinib with either gemcitabine or cisplatin in BTC cells. Bosutinib demonstrated synergistic antiproliferative effects in combination with gemcitabine or cisplatin in SNU-308 and SNU-478 cells (Fig. 4A). The migratory
The effects of cytotoxic agents were potentiated by the addition of bosutinib (Fig. 4B). In addition, combination treatment of bosutinib with gemcitabine or cisplatin for 72 hours significantly inhibited the migration of SNU-478 cells when compared with bosutinib monotherapy. In SNU-478 cells, combination treatment of bosutinib with gemcitabine or cisplatin further abrogated Src phosphorylation compared with bosutinib alone, and further downregulated Vimentin and Snail expression compared with bosutinib, gemcitabine, or cisplatin alone (Fig. 4C).

Figure 3.
Bosutinib inhibits the migration and invasion of BTC cells. A, migration assays were performed to visualize the effects of bosutinib on migration activity in BTC cells. SNU-308 and SNU-478 cells were seeded in a 6-well plate. After 24 hours, cell monolayers were scratched with a sterile 200-µL pipette tip and incubated in culture medium alone or with bosutinib. After 72 hours for SNU-308 cells and 24 hours for SNU-478 cells, the cell migration activity was observed under light microscopy. **, *P* < 0.005. B, invasion activity was examined using a chamber system coated with laminin I matrix. The invaded cells were stained and quantified at 560 nm. **, *P* < 0.005. C, Western blot analysis of proteins associated with EMT was performed after treatment with increasing concentrations of bosutinib [0 (DMSO), 0.1, 0.5, and 1.0 µmol/L].
Src inhibition has potent antitumor effects in a xenograft model

A SNU-478 xenograft model using female Balb/c athymic nude mice demonstrated that bosutinib alone significantly delayed tumor growth compared with controls (Fig. 5A). Bosutinib combined with gemcitabine or cisplatin showed significantly more potent antitumor effects when compared with bosutinib, gemcitabine, or cisplatin monotherapy. Moreover, the combination treatment did not significantly influence the body weight of mice (data not shown).

Tumors treated with bosutinib alone exhibited an apparent decrease in cell proliferation by Ki-67 assays and an apparent increase in apoptosis by TUNEL assays (Fig. 5B). In addition, bosutinib treatment apparently decreased Src phosphorylation. Combination treatment of bosutinib and gemcitabine further decreased Ki-67 expression and Src phosphorylation, and further

Figure 4.
Bosutinib enhances the antiproliferative and antimigratory effects of cytotoxic agents. A, antiproliferative activity of 0.5 μmol/L bosutinib (Bos) in combination with 0.01 μmol/L gemcitabine (Gem) or 1.0 μmol/L cisplatin (Cis) was evaluated by MTT assays. *, P < 0.01; **, P < 0.005; N.S., not significant. B, for migration assays, SNU-478 cell monolayers were scratched with a sterile 200-μL pipette tip, and then treated with 0.1 μmol/L bosutinib, 0.1 μmol/L gemcitabine, or 2.0 μmol/L cisplatin alone or in combination. The distance between the cells was measured. **, P < 0.005. C, in SNU-478 cells, the Western blot analysis of molecules associated with Src signaling pathways and EMT was performed after treatment with 1.0 μmol/L bosutinib, 0.1 μmol/L gemcitabine, or 2.0 μmol/L cisplatin alone or in combination for 48 hours. Bos+C, bosutinib plus cisplatin; Bos+Gem, bosutinib plus gemcitabine; C and Con, control.
increased TUNEL expression. In tumor lysates, the phosphorylation of Src and STAT3 was downregulated by bosutinib monotherapy. Moreover, when bosutinib was combined with gemcitabine, a greater decrease in Src and STAT3 phosphorylation was observed (Fig. 5C).

Discussion

Our data suggested that Src might be a potential therapeutic target in BTC. A previous study indicated that about 80% of BTC specimens expressed an activated Src protein similar to other malignancies (3–6, 18). In our study, a total of nine BTC cell lines were tested, and the IC50 values ranged from 0.63 to 4.45 μmol/L in 3-day MTT assays. Our data support that a subset of BTC may be sensitive to the Src inhibitor, bosutinib.

Bosutinib monotherapy inhibited the phosphorylation of Src Tyr416 and FAK Tyr397, which is an autophosphorylation site for integrins (25–27). In contrast, bosutinib did not inhibit the phosphorylation of FAK Tyr925 residue, which is a Src-specific phosphorylation site. Previous preclinical studies of bosutinib showed conflicting results in terms of FAK phosphorylation. In colon cancer and breast cancer, bosutinib decreased FAK Tyr925 phosphorylation, whereas phosphorylation of the Tyr925 residue was unchanged (28, 29). In contrast, FAK Tyr925 phosphorylation was significantly decreased by bosutinib in thyroid cancer (30). Another Src/Abl inhibitor dasatinib also inhibited FAK Tyr397 phosphorylation in colon cancer and pancreatic cancer (31, 32). The precise molecular mechanisms for these conflicting results need to be investigated further to determine how to abrogate the downstream signaling pathways of Src using Src inhibitors more effectively. In addition, bosutinib decreased AKT and ERK phosphorylation in a dose-dependent manner in SNU-308 and SNU-478 cells, which were relatively sensitive to bosutinib. In contrast, conflicting results were shown for STAT3 phosphorylation. In SNU-478 cells, STAT3 phosphorylation was downregulated by bosutinib. However, in SNU-308 and TFK-1 cells, it was upregulated. This finding is in line with a previous study that demonstrated sustained Src inhibition induced altered JAK–STAT3 binding, leading to aberrant STAT3 activation (33). These results suggest that a STAT3 targeting strategy might be an option to increase the therapeutic efficacy or to overcome the resistance of Src inhibitors.

Cell-cycle analysis demonstrated that bosutinib induced G1 arrest and increased the sub-G1 fraction in a dose-dependent manner in SNU-308 and SNU-478 cells. Bosutinib decreased cyclin D, cyclin E, cyclin A, and cyclin B expression, and
increased p27 expression in both sensitive BTC cell lines. In particular, the nuclear stabilization of p27 was also observed after bosutinib treatment, which has been suggested to be a mechanism of apoptosis induced by the Src inhibitor (34). Moreover, bosutinib monotherapy demonstrated potent anti-migratory and anti-invasive activity against BTC cell lines, and downregulated the expression of proteins related to EMT, which is associated with cancer progression and metastasis and often mediated by the MAPK and PI3K/AKT pathways (35, 36). These findings are also consistent with previous findings of bosutinib in other type of cancers (29, 30, 37, 38). The results of our preclinical studies support bosutinib as a single agent with promising antitumor activity in BTC, which warrants further clinical studies.

Our in vitro and in vivo models also demonstrated that bosutinib has synergistic antiproliferative activity in combination with cytotoxic agents including gemcitabine and cisplatin, which are currently the standard treatment in patients with advanced BTC. Importantly, there was no significant effect on body weight in the in vivo models (15). The combination treatment further inhibited the migratory activity of BTC cells, decreased the phosphorylation of Src and its downstream molecules, decreased EMT marker expression levels such as Vimentin and Snail, and increased apoptosis in BTC cells. These results indicate that this novel combination strategy is worthy of further clinical studies in patients with advanced BTC, because there is currently no validated therapeutic target for advanced BTC despite the poor prognosis of these patients.

In spite of these promising results, alternative cancer survival and growth machineries are serious issues in anticancer drug discovery as it may eventually induce acquired resistance to Src inhibitors. Similarly, a previous study using a murine melanoma model indicated that adoptive cell transfer therapy with cytotoxic T cells unexpectedly induced treatment resistance through an inflammation-induced reversible loss of melanocytic antigens (39). More recently, Obenauf, and colleagues demonstrated that targeted therapy induces secretome changes in drug-sensitive cancer cells, paradoxically generating a tumor microenvironment that promotes the growth of drug-resistant clones (40). In case of Src inhibition, only few have been known about acquired resistance mechanisms. Aforementioned aberrant STAT3 activation by bosutinib could be a resistance mechanism of Src inhibitors (33). Recently, Lu and colleagues demonstrated that IGFBP2/FAK pathway may be associated with dasatinib resistance in non-small cell lung cancer cells (41). Aberrant deregulation of FAK or its downstream molecules by alternative pathway activation may contribute to Src inhibitor resistance. Therefore, further preclinical studies should focus on the identification and overcome of the alternative cancer survival and growth machineries of Src inhibitors in BTC.

A recent study identified that a bosutinib isomer (Bos-I) was more potent to inhibit Chk1 and Wee1 than "authentic" bosutinib and synergized with gemcitabine in a pancreatic cancer cell line (42). However, the ability of Bos-I to inhibit Src and Abl was relatively less potent compared with "authentic" bosutinib. Nevertheless, as Bos-I showed greater antitumor activity than "authentic" bosutinib toward pancreatic cancer cells when combined with gemcitabine, further preclinical or clinical studies are needed to focus on the antiproliferative activity of Bos-I combined with gemcitabine or cisplatin in BTC cells.

In summary, our data suggest that Src might be a potential therapeutic target in BTC. Bosutinib, an orally active small molecule c-Src/Abl kinase inhibitor, demonstrated promising antitumor activity alone or in combination with gemcitabine or cisplatin by significantly inhibiting the phosphorylation of Src and its downstream molecules, inducing G1 cell-cycle arrest via the nuclear stabilization of p27 in part, decreasing migration, invasion, and EMT, and increasing apoptosis in BTC in vitro and in vivo models. The results of our preclinical study support the further clinical development of bosutinib monotherapy or combination therapy with other cytotoxic agents in patients with advanced BTC.

Disclosure of Potential Conflicts of Interest
S.-A. Im is a consultant/advisory board member for AstraZeneca, Hammi, Novartis, and Roche. No potential conflicts of interest were disclosed by the other authors.

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