Abiraterone Inhibits 3β-Hydroxysteroid Dehydrogenase: A Rationale for Increasing Drug Exposure in Castration-Resistant Prostate Cancer

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Abstract

Purpose: Treatment with abiraterone (abi) acetate prolongs survival in castration-resistant prostate cancer (CRPC). Resistance to abi invariably occurs, probably due in part to upregulation of steroidogenic enzymes and/or other mechanisms that sustain dihydrotestosterone (DHT) synthesis, which raises the possibility of reversing resistance by concomitant inhibition of other required steroidogenic enzymes. On the basis of the 3β-hydroxyl, Δ5-structure, we hypothesized that abi also inhibits 3β-hydroxysteroid dehydrogenase/isomerase (3βHSD), which is absolutely required for DHT synthesis in CRPC, regardless of origins or routes of synthesis.

Experimental Design: We tested the effects of abi on 3βHSD activity, androgen receptor localization, expression of androgen receptor–responsive genes, and CRPC growth in vivo.

Results: Abi inhibits recombinant 3βHSD activity in vitro and endogenous 3βHSD activity in LNCaP and LAPC4 cells, including conversion of [3H]-dehydroepiandrosterone (DHEA) to Δ5-androstenedione, androgen receptor nuclear translocation, expression of androgen receptor–responsive genes, and xenograft growth in orchietomized mice supplemented with DHEA. Abi also blocks conversion of Δ5-androstenediol to testosterone by 3βHSD. ABI inhibits 3βHSD1 and 3βHSD2 enzymatic activity in vitro; blocks conversion from DHEA to androstenedione and DHT with an IC50 value of less than 1 μmol/L in CRPC cells; inhibits androgen receptor nuclear translocation; expression of TMPRSS2, prostate-specific antigen, and FKBP5; and decreases CRPC xenograft growth in DHEA-supplemented mice.

Conclusions: We conclude that abi inhibits 3βHSD-mediated conversion of DHEA to active androgens in CRPC. This second mode of action might be exploited to reverse resistance to CYP17A1 inhibition at the standard abi dose by dose-escalation or simply by administration with food to increase drug exposure. Clin Cancer Res; 18(13); 3571–9. ©2012 AACR.
Translational Relevance

The development and progression of “castration-resistant” prostate cancer (CRPC) is dependent on intratumoral steroidogenesis, resulting in androgen synthesis and activation of the androgen receptor. Abiraterone acetate blocks the synthesis of androgens by inhibiting CYP17A1 and extends survival in men with metastatic CRPC. However, resistance occurs and may be attributable in part to continued steroidogenesis. Here, we show that abiraterone also blocks 3β-hydroxysteroid dehydrogenase (3βHSD), an enzyme that is absolutely required for the synthesis of biologically active androgens. This second activity may be clinically exploited to reverse resistance by increasing the dose of abiraterone acetate or administration with a high fat meal to increase drug exposure, permitting cooperative dual enzyme inhibition to more potently block androgen synthesis. Our work provides a rationale for clinical trials that test the effects of increased drug exposure in CRPC.

Materials and Methods

Cells and culture conditions

The LNCaP prostate cancer cell line was purchased from American Type Culture Collection and maintained in RPMI-1640 containing 10% FBS. The LAPC4 prostate cancer cell line was generously provided by Charles Sawyers (Memorial Sloan Kettering Cancer Center, New York, NY) and was grown in Iscove’s Modified Dulbecco’s Medium with 10% FBS.

Steroid metabolism

Cells were seeded in 12-well dishes at 100,000 cells per well and incubated with [3H]-DHEA or [3H]-Δ5-androstenediol (A5diol, see Supplementary Data; 400,000–1,000,000 cpm/well; PerkinElmer) added in less than 0.2% ethanol at 20 or 100 nmol/L, and aliquots of medium (0.25 or 0.5 mL) were collected at the indicated time points. Collected medium was treated with 1,000 units of β-glucuronidase (Helix Pomatia) and was grown in Iscove’s Modified Dulbecco’s Medium under nitrogen. For high-performance liquid chromatography (HPLC) analysis, the dried samples were dissolved in methanol and injected on a Breeze 1525 system equipped with model 717 plus autoinjector (Waters Corp.) or Agilent 1260 and a Kinetex 100 × 2.1 mm, 2.6 μmol/l, C18 reverse-phase column (Phenomenex) and methanol/water gradients at 30°C. The column effluent was analyzed using a dual-wavelength UV-visible detector set at 254 nm or β-RAM model 3 in-line radioactivity detector (IN/US Systems, Inc.) using Liquisint scintillation cocktail (National Diagnostics). Alternatively, dried samples were applied to plastic-based silica gel plates (Whatman) and separated by thin-layer chromatography (TLC) using a mobile phase of 3:1 chloroform:ethyl acetate, followed by exposure of the plates to a phosphorimager screen and quantitation with a Storm model 860 phosphorimager (Applied Biosystems). All HPLC and TLC studies were conducted in triplicate and repeated in independent experiments.

Gene expression and immunoblotting

After treatment with the indicated steroids, total RNA was collected using the RNaseasy system (QIAGEN), and 1 μg RNA was used in a reverse transcription reaction using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR (qPCR) analysis was conducted in triplicate using previously published primers for PSA, TMPRSS2, FKBP5, and the housekeeping gene large ribosomal protein P0 (RPLP0; ref. 18). The thermocycling reaction was carried out in an ABI 7500 Real-Time PCR machine (Applied Biosystems) using iTaq Fast SYBR Green Supermix with ROX (Bio-Rad) in 96-well plates at 20 μL final reaction volume. Accurate quantitation of each mRNA was achieved by normalizing the sample values to RPLP0 and to vehicle-treated cells. Subcellular protein localization studies were done as described previously (18). Briefly, cells were pretreated with abi for 30 minutes, followed by treatment with the indicated steroids for 6 hours and cell fractionation was done with a nuclear and cytoplasmic fractionation kit (ThermoScientific). Proteins were resolved by electrophoresis, transferred to a nitrocellulose membrane and incubated with anti-androgen receptor and anti-lamin B (Santa Cruz) antibodies, LI-COR secondary antibodies (LI-COR Biosciences), and scanned with the LI-COR Odyssey IR Imaging System.

Mouse xenograft studies

Male NOD/SCID mice 6 to 8 weeks of age were obtained from the University of Texas Southwestern Medical Center Animal Resources Center, and studies were conducted under an Institutional Animal Care and Use Committee-approved protocol. Mice were surgically orchietomized and implanted with a 5 mg 90-day sustained release DHEA pellet (Innovative Research of America) to mimic CRPC physiology. Two days later, 7 × 106 LAPC4 cells were injected subcutaneously with Matrigel. Tumor dimensions were measured 2 to 3 times per week, and volume was calculated as length × width × height × 0.52. Once tumors reached 300 mm3, mice were randomly assigned to vehicle or abi treatment groups. Mice in the abi
group were treated with 5 ml/kg intraperitoneal injections of 0.5 mmol/kg/d (0.1 mL 5% benzyl alcohol and 95% safflower oil solution) and control mice with vehicle only, once daily for 5 days per week over a duration of 4 weeks (n = 8 mice per treatment). Statistical significance between ani and vehicle treatment groups was assessed by ANOVA based on a mixed-effect model.

Chemical and radiocochmical synthesis

The structures and synthetic scheme for ani and Δ4-ani are in Supplementary Fig. S1. Androst-5-ene-3β-ol-17-one-3-acetate (1) discussed in the work of (19): To a solution of DHEA (3.24 g, 11.2 mmol) in pyridine (60 mL) was added acetic anhydride (4 mL) and dimethylaminopyridine (13.68 mg, 0.112 mmol). After stirring at room temperature for 18 hours, water was added, and the products were extracted into ether, which was washed with 1N HCl to remove excess pyridine, dried with MgSO4, and concentrated under reduced pressure. Column chromatography on silica gel gave 1 (3.4 g, 92% yield).

1H NMR (400 MHz, CDCl3): δ = 0.87(s, 3H), 1.08(s, 3H), 1.08–1.20(m, 2H), 1.20–1.34(m, 2H), 1.33–2.15(m, 20H), 2.22–2.38(m, 2H), 2.42–2.50(m, 1H), 4.54–4.65 (m, 1H), 5.03–5.50(m, 1H) ppm.

3β-Acetoxyandrosta-5,16-dien-3-one (2): A solution of 1 (0.498 g, 1.51 mmol) in tetrahydrofuran at −78°C was treated with potassium hexamethyldisilazane (KHMS, 0.5 mol/L, 3.0 mL, 1.5 mmol) and stirred for 1 hour at −78°C. N-Phenyl-bis(trifluoromethanesulfonimide) (0.646 g, 1.81 mmol), was added, and the reaction was stirred at −78°C for 2 hours, then warmed slowly to room temperature and quenched with saturated NH4Cl. The reaction mixture was concentrated under reduced pressure, and the residue was extracted with ethyl acetate, which was washed with brine, dried with MgSO4, and concentrated under reduced pressure. Column chromatography on silica gel gave 2 (602 mg, 86% yield).

1H NMR (400 MHz, CDCl3): δ = 0.99(s, 3H), 1.05(s, 3H), 1.09–1.24(m, 2H), 1.40–2.08(m, 18H), 2.10–2.40(m, 5H), 4.52–4.66(m, 1H), 5.32–5.42(m, 1H), 5.58(bs, 1H) ppm.

3β-Acetoxy-17-(3-Pyridyl)androsta-5,16-dien-3-one (abiraterone, 3): A suspension of 2 (846 mg, 1.82 mmol), diethyl-3-pyridylborane (411 mg, 2.79 mmol), bis(triphenylphosphine)palladium(II) chloride (12 mg, 0.018 mmol), and saturated Na2CO3 (0.364 mL) in 20 mL tetrahydrofuran was refluxed under nitrogen for 1 hour. The reaction was concentrated under reduced pressure, and the residue was extracted into diethyl ether, which was washed with brine, dried over MgSO4, and concentrated under reduced pressure. Column chromatography on silica gel gave 3 (602 mg, 86% yield).

1H NMR (400 MHz, CDCl3): δ = 0.87–0.91(m, 6H), 1.09–1.39(m, 10H), 1.44–1.96(m, 15H), 1.99–2.10(m, 8H), 2.22–2.44(m, 4H), 2.50–2.78(m, 2H), 3.10–3.37(m, 4H), 3.42–3.56(m, 1H), 5.36–5.42(m, 1H), 6.08(s, 1H), 7.21–7.27(m, 1H), 7.50–7.62(m, 1H), 8.38–8.62(m, 2H) ppm.

13C NMR (125 MHz, CDCl3): δ = 16.82, 19.50, 21.05, 21.69, 27.97, 30.63, 31.74, 32.03, 35.43, 37.02, 37.15, 38.37, 47.56, 50.48, 57.69, 74.10, 77.01, 77.27, 77.52, 122.54, 123.28, 129.49, 133.95, 140.26, 148.08, 148.14, 151.89, 170.82 ppm.

17-(3-Pyridyl)androsta-5,16-dien-3β-ol (abiraterone, 4): A solution of 3 (180 mg, 0.46 mmol) in methanol (20 mL) and KOH (200 mg) was refluxed for 3 hours, and the reaction mixture was neutralized with 1N HCl. After concentrating under reduced pressure, the residue was extracted into ether, which was washed with brine, dried over MgSO4, and concentrated under reduced pressure. Column chromatography on silica gel gave 4 (142 mg, 88% yield).

1H NMR (400 MHz, CDCl3): δ = 0.86–1.36(m, 16H), 1.38–1.94(m, 20H), 1.96–2.32(m, 6H), 2.36–2.48(m, 6H), 3.42–3.56(m, 1H), 5.36–5.42(m, 1H), 6.08(s, 1H), 7.21–7.27(m, 1H), 7.50–7.62(m, 1H), 8.38–8.62(m, 2H) ppm.

13C NMR (125 MHz, CDCl3): δ = 16.83, 19.59, 21.11, 30.67, 31.76, 31.86, 32.05, 35.48, 36.93, 37.41, 42.54, 47.57, 50.58, 57.78, 71.93, 121.59, 129.48, 133.90, 141.37, 148.06, 148.13, 151.91 ppm.

17-(3-Pyridyl)androsta-4,16-dien-3-one (Δ4-abiraterone, 5): A solution of 4 (12 mg, 0.034 mmol) in toluene with N-methylpiperidone (1 mL) was refluxed until 2 mL of liquid was distilled off using a Dean–Stark apparatus. Aluminium isopropoxide (10.5 mg, 0.051 mmol) was added, and the reaction was refluxed for an additional 6 hours. The products were extracted into ethyl acetate, which was washed with brine, dried over MgSO4, and concentrated under reduced pressure. Column chromatography on silica gel gave 5 (10 mg, 83% yield).

1H NMR (400 MHz, CDCl3): δ = 0.97–1.08 (m, 6H), 1.09–1.28(m, 6H), 1.36–1.75 (m, 8H), 1.75–1.95(m, 2H), 1.955–2.13(m, 3H), 2.21–2.50(m, 5H), 5.70–5.72(bs,1H), 5.94–6.00(m, 1H), 7.16–7.27(m, 2H), 7.58–7.65(m, 1H), 8.43–8.48(m, 1H), 8.58–8.62(m, 1H).

13C NMR (125 MHz, CDCl3): δ = 16.86, 17.48, 21.12, 31.89, 31.99, 33.01, 34.18, 34.34, 35.28, 35.81, 38.94, 47.53, 54.15, 57.00, 123.33, 124.26, 129.30, 129.33, 133.00, 133.95, 148.06, 148.19, 151.71, 171.26, 199.76.

Diethyl-3-pyridyldiborane used in the synthesis of 3 was made following a published procedure (20). Starting from 3-bromopyridine (322 mg, 2.03 mmol), diethyl-3-pyridylborane was obtained (256 mg, 84%).

1H NMR (400 MHz, CDCl3): 0.47(m, 6H), 0.58–0.78(m, 4H), 7.19–7.26(m, 1H), 7.57(s, 1H), 7.72(d, J = 6Hz, 1H), 8.019(d, J = 4.4Hz, 1H).

The [1H]-A5diol was prepared by reducing [1H]-DHEA (100 ppm) in methanol (~0.1 mL) with a few crumbs of NaBH4 at room temperature for 30 minutes. The crude reaction mixture was evaporated under a nitrogen stream and purified on a pipette column of silica gel.

Enzyme assays

Incubations testing abiraterone as an inhibitor contained recombinant human 3βHSD1 or 3βHSD2 (in yeast microsomes, 3.2 or 25 μg protein per incubation,
respectively). $[^{3}H]$-pregnenolone (100,000 cpmp, 1–20 μmol/L), and abiraterone (5–20 μmol/L) or ethanol vehicle in 0.2 to 1 mL of potassium phosphate buffer. After preincubation at 37°C for 1 to 3 minutes, NAD$^+$ (1 mmol/L) was added, and the incubation was conducted at 37°C for 15 minutes. The reaction was stopped by addition of 1 to 2 mL ethyl acetate:isooctane (1:1) and extracting the steroids into the organic phase. The dried extracts were resolved either by TLC on plastic-backed silica gel plates using 3:1 chloroform:ethyl acetate or by HPLC. For TLC, regions of the plates containing steroids were identified with iodine vapor, excised with scissors, and quantitated by liquid scintillation counting as described (21). For HPLC, pregnenolone radioactivity was quantitated with BioSafe scintillation cocktail. Incubations testing abiraterone as a substrate were carried out as above but substituting 0.1 to 5 μmol/L unlabeled abiraterone for pregnenolone and quantitating conversion by HPLC.

Results

Abiraterone inhibits conversion of DHEA to Δ^4-androstenedione

3βHSD enzymatic activity is required for the transformation of DHEA to Δ^4-androstenedione (AD; Fig. 1A). To establish the effect of abi on this initial step in the conversion from adrenal DHEA to DHT, conversion from [3H]-DHEA to AD in LNCaP and LAPC4 cells was determined in the presence of abi. DHEA depletion and AD accumulation were inhibited by abi in LNCaP, with an IC50 < 1 μmol/L (Fig. 1B). In LAPC4, although AD accumulation reaches a steady state by 7 hours, probably attributable in part to more robust downstream conversion to 5α-androstane-dione (5α-dione; ref. 5), abi similarly blocked conversion from DHEA to AD (Fig. 1C).

3βHSD1 is traditionally thought to be the peripherally expressed isoenzyme and 3βHSD2 is required for enzyme activity in the adrenals and gonads (16, 17). However,

![Figure 1](https://cancerres.aacrjournals.org/content/18/13/3574/F1.large.jpg)
transcripts encoding both proteins have been detected in prostate cancer (2, 22). To determine the effects of abi on $3\beta$HSD1 and $3\beta$HSD2, recombinant enzymes were expressed in yeast microsomes, and the effects of abi on enzymatic activity were determined by incubation with [$^{1}H$]pregnenolone and quantitating enzymatic activity were determined by incubation with [3H]-pregnenolone and quantitating $3\beta$HSD expression in yeast microsomes, and the effects of abi on $3\beta$HSD and abiraterone with NAD$^+$, with curve fits to the Michaelis-Menten equation ($r^2 = 0.99$ and 0.95, respectively). E, HPLC chromatograms of abiraterone (top), its $\Delta^{4}$-3-keto homolog (middle), and products of incubations with $3\beta$HSD2 and abiraterone with NAD$^+$ (bottom), showing conversion to its $\Delta^{4}$-3-keto homolog.

**Abiraterone inhibition of $3\beta$HSD blocks DHT synthesis and the androgen receptor response**

We next sought to determine the effects of abi on the steroidogenic pathway downstream of $3\beta$HSD, all the way to DHT and regulation of the androgen receptor response. In addition to blocking AD accumulation, treatment with abi inhibited the downstream synthesis of $5\alpha$-dione and DHT in LNCaP and LAPC4 (Fig. 3A and B). Furthermore, 10 μmol/L abi was sufficient to completely block synthesis of $5\alpha$-dione and DHT in both cell lines. To determine the effect of abi on androgen receptor nuclear translocation, LNCaP cells were treated with DHEA and $5\alpha$-dione with and without abi pretreatment. Abi completely blocked androgen receptor nuclear translocation induced by both of these $\Delta^{4}$-androgen precursors (Fig. 3C). DHEA-induced expression of the androgen receptor–responsive genes, PSA, FKB15, and TMPRSS2, are all inhibited by abi in a dose-dependent manner (Fig. 3D).

**Abiraterone inhibits the conversion of $\Delta^{4}$-androstenediol to testosterone**

$5\alpha$-dione has been implicated in the development of CRPC, and some studies have detected this $\Delta^{4}$-precursor steroid in prostate cancer tissue after gonadal testosterone deprivation (23). Furthermore, blocking conversion of DHEA to androstenedione might instead lead to diversion of DHEA by $17\beta$-reduction to $5\alpha$-dione (24), generating another potential substrate for $3\beta$HSD that can be converted to testosterone (Fig. 4A). To determine whether abi can also block conversion of $5\alpha$-dione to testosterone, LNCaP and LAPC4 cells were treated with [$^{3}H$]-$5\alpha$-dione and flux to downstream metabolites was assessed. Abi effectively blocks testosterone synthesis in both LNCaP and LAPC4 (Fig. 4B and C).
In vivo CRPC growth in mice supplemented with DHEA is inhibited by abiraterone

In contrast to humans, rodents generally lack adrenal CYP17A1 expression and consequently do not make adrenal androgens (25). To mimic DHEA production in the human to test the effect of abiraterone (abi) on 3β-HSD in CRPC, LAPC4 xenografts were developed in orchiectomized mice that were implanted with 90-day sustained release DHEA pellets. Tumors reaching the threshold volume of 300 mm³ were randomly assigned to vehicle or abi cohorts, and tumor volume relative to pretreatment values was monitored over 4 weeks of treatment. The 0.5 mmol/kg/d abi treatment dose was previously shown to yield serum concentrations of about 0.5 to 1 μmol/L (26). Xenograft tumor growth in the control group was widely variable, with some tumors growing slowly and only a subset of tumors exhibiting robust growth. As shown in Fig. 5, treatment with abi significantly inhibited CRPC progression in the robustly growing subset, effectively putting a ceiling on tumor growth over 4 weeks of treatment ($P < 0.00001$).

Discussion

Mechanisms that permit the intratumoral synthesis of DHT play a major role in driving CRPC despite low serum testosterone. The clinical and survival benefit of CYP17A1 inhibition with abiraterone is probably the clearest demonstration of the continued requirement for androgens in driving CRPC (11). However, CYP17A1 inhibition with abiraterone is not complete, possibly leaving the door open for intratumoral mechanisms that permit sustained steroidogenesis, even with lower concentrations of precursor steroids (13–15). Conversion from Δ5- to Δ4-steroids by 3β-HSD is required for the synthesis of testosterone and DHT whether this reaction occurs through adrenal 19-carbon precursors or with 21-carbon steroids through de novo steroidogenesis (Fig. 6; refs. 18, 27).

Here, we show that in addition to CYP17A1 inhibition, abi also inhibits the conversion of DHEA to AD by 3β-HSD, leading to decreased synthesis of downstream Δ5-dione and DHT. Abi also blocks the conversion of A5diol, which has been implicated in some studies of CRPC (23), to...
testosterone by 3βHSD. These studies also further support our previous studies that the major mechanism of A5diol occurs through its conversion to testosterone (18). The mechanism of 3βHSD1 inhibition by abi is mainly competitive in nature, whereas for 3βHSD2 the nature of competition appears to be mixed, at least at a higher concentration of abi. Abi is also a somewhat more potent inhibitor of 3βHSD1 compared with 3βHSD2. The results with intact prostate cancer cells show that there is greater than 50% inhibition with 1 μmol/L abi, which is approached but not met by the concentrations achieved in the fasting state in the phase I trials (10, 12). Standard treatment is to administer abi when fasting (28). On the other hand, plasma concentrations meet or exceed 1 μmol/L when abi is given with a high fat meal (10, 12). Inhibition of 3βHSD by abi blocks androgen receptor nuclear translocation and expression of androgen-responsive genes. Furthermore, abi blocks CRPC growth in mice supplemented with DHEA to mimic human adrenal physiology. The 1,000 mg dose of abi in the phase III trials was chosen based on the plateau of 21-carbons steroids that suggests effective CYP17A1 inhibition (10). Together, these results suggest that increasing drug exposure to block both 3βHSD and CYP17A1 may yield clinical responses in patients with initial or acquired abi resistance.

Intratumoral synthesis of DHT is a central mechanism that drives CRPC (2–5). Although survival is extended in men with CRPC by treatment with abi, initial or acquired

Figure 4. Conversion of A5diol to testosterone (T) by 3βHSD is blocked by abi. A, 3βHSD is required for the conversion of A5diol to testosterone. B, depletion of A5diol (left) and conversion to testosterone (middle) in LNCaP cells are blocked by abi in dose- and time-dependent manners. Treatment is with 20 nmol/L A5diol and the indicated concentrations of abi. C, conversion of A5diol to testosterone in LAPC4 cells after 24 hours of treatment is similarly blocked by abi. B and C, experiments were carried out in triplicate by HPLC. In the bar graphs, the y-axis denotes the proportion of total steroid signal. Representative tracings with and without abi are shown, and error bars represent the SE.

Figure 5. Abi inhibits CRPC growth in orchiectomized mice supplemented with DHEA. Subcutaneous LAPC4 xenografts were grown in orchiectomized mice implanted with subcutaneous 90-day sustained release DHEA pellets. Once tumor volume reached 300 mm3, mice were randomized to vehicle or abi treatment groups. Tumor volume was assessed for the subsequent 4 weeks, and change in tumor size over time is shown in box plots relative to pretreatment tumor volume. Statistical significance for differences between treatment groups was assessed by ANOVA based on a mixed-effect model with a P value of P < 0.00001.
resistance occurs, and analysis of urinary androgen metabolites shows that CYP17A1 inhibition is incomplete, suggesting the possibility of sustained steroidogenesis (11, 13). Our findings suggest that abi may also have a second, weaker action, by blocking 3βHSD enzymatic activity, downstream synthesis of DHT, the androgen receptor response, and CRPC progression in vivo. These results suggest that increasing abi drug exposure by administration with a high fat meal (28), increasing dose, or both, may have therapeutic use and might reverse resistance to abi. Notably, 3βHSD inhibition would be effective no matter the relative contributions of adrenal precursors versus de novo steroidogenesis from cholesterol (18).

Disclosure of Potential Conflicts of Interest
N. Sharifi has been compensated as a consultant/advisory board member for Janssen. R.J. Auchus has been compensated as a consultant/advisory board member for Janssen, Viamet Pharmaceuticals, Biomarin Pharmaceuticals, Orphagen Pharmaceuticals, and Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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