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 Δ4-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 cell lines; 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.

Introduction

Metastatic prostate cancer is initially treated with gonadal testosterone deprivation, which typically initially leads to a tumor response (1). Nearly all tumors eventually progress despite low serum testosterone concentrations, as “castration-resistant” prostate cancer (CRPC), through mechanisms that require the intratumoral synthesis of dihydrotestosterone (DHT) from precursor steroids (2–8). The synthesis of all androgens is dependent on CYP17A1 enzymatic activity (9). Treatment with abiraterone (abi) acetate, which inhibits 17α-hydroxylase/17,20-lyase (CYP17A1), leads to frequent prostate-specific antigen (PSA) declines and prolongs survival of men with docetaxel-treated CRPC, leading to approval by the U.S. Food and Drug Administration (FDA) in April 2011 (10–12). However, not all tumors respond, and most tumors that respond eventually become resistant to abi. Clinical resistance to abi is now frequently encountered in clinical oncology. Urinary androgen metabolites are detectable in patients treated with abi, suggesting that CYP17A1 inhibition with abi is incomplete, permitting the synthesis of residual androgens (13). Moreover, initial preclinical studies suggest that abi resistance is mediated at least in part by upregulation of steroidogenic enzymes and sustained intratumoral DHT synthesis (14, 15). Together, these data suggest that concomitantly blocking a second enzyme required for DHT synthesis might yield...
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 the American Type Culture Collection and maintained in RPMI-1640 containing 10% FBS. The LNCaP 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]-Δ4-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 1,000,000 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.

Gene expression and immunoblotting

After treatment with the indicated steroids, total RNA was collected using the RNeasy 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 (RPLPO; 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 RPLPO 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 with human adrenal physiology. Two days later, 7 × 106 LNCaP 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 abi and vehicle treatment groups was assessed by ANOVA based on a mixed-effect model.

Chemical and radiotracic synthesis

The structures and radioactive scheme for abi and Δ4-abi 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 (108 cpm) in methanol (20 mL) and KOH (200 mg) was refuxed 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 1 (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), 6.0(s, 1H), 7.21–7.25(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-diene-3β-ol (abiraterone, 4): A solution of 3 (180 mg, 0.46 mmol) in methanol (20 mL) and KOH (200 mg) was refuxed 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), 6.0(s, 1H), 7.21–7.25(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, 47.37, 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-diene-3-one (Δ4-abiraterone, 5): A solution of 4 (12 mg, 0.034 mmol) in toluene with N-methylpyrrolidone (1 mL) was refuxed 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 refuxed 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, 122.54, 123.28, 129.49, 133.95, 140.26, 148.19, 151.71, 171.29, 199.76.

Diethyl-3-pyridylborane 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, 1H), 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).

1H-[3H]-A5diol was prepared by reducing [1H]-DHEA (100 cpm) 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). [3H]-pregnenolone (100,000 cpms, 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 BioSafeII 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 Δ⁴-androstenedione
3βHSD enzymatic activity is required for the transformation of DHEA to Δ⁴-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 abiraterone. 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://example.com/figure1.png)

Figure 1. Abi blocks enzymatic conversion of DHEA to AD by 3βHSD in CRPC cell lines. A, 3βHSD is required for the conversion of DHEA to AD. B, DHEA depletion (left) and AD accumulation (right) occur in LNCaP cells and are inhibited by abi in dose- and time-dependent manners. Treatment is with 100 nmol/L [3H]-DHEA (top), which may reflect the physiologic contribution of sulfated DHEA in man and 20 nmol/L [3H]-DHEA (below), reflecting physiologic concentrations of unconjugated DHEA in serum. C, abi similarly blocks conversion from DHEA — AD in LAPC4. AD accumulation reaches a steady state by 7 hours, possibly reflecting more rapid 5α-reduction of AD to 5α-dione in LAPC4. In B and C, experiments were carried out in triplicate by TLC. The y-axis denotes the proportion of total steroid signal. Representative experiments are shown, and error bars represent the SE. T, testosterone.
transcripts encoding both proteins have been detected in prostate cancer (2, 22). To determine the effects of abi on 3βHSD1 and 3βHSD2, recombinant enzymes were expressed in yeast microsomes, and the effects of abi on enzymatic activity were determined by incubation with [3H]-pregnenolone and quantitating Δ5—Δ4 conversion to progesterone (Fig. 2A and B). Abi inhibited recombinant human 3βHSD1 and 3βHSD2 activity with competitive Ki values of 2.1 and 8.8 μmol/L. In contrast to 3βHSD1, inhibition of 3βHSD2 was mixed competitive–noncompetitive at 20 μmol/L. Given its Δ5 structure, we also determined whether abi is a substrate for both human 3βHSDs. The oxidation/isomerization of abi to its Δ4-3-keto-congener (Δ4-abi, Fig. 2E) was catalyzed by 3βHSD1 (Km = 0.45 μmol/L, Vmax = 0.001 μmol/min/mg; Fig. 2C) and 3βHSD2 (Km = 0.76 μmol/L, Vmax = 0.004 μmol/min/mg; Fig. 2D).

Abiraterone inhibition of 3βHSD blocks DHT synthesis and the androgen receptor response

We next sought to determine the effects of abi on the steroioidogenic pathway downstream of 3βHSD, 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 5α-dione and DHT in LNCaP and LAPC4 (Fig. 3A and B). Furthermore, 10 μmol/L abi was sufficient to completely block synthesis of 5α-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 A5diol with and without abi pretreatment. Abi completely blocked androgen receptor nuclear translocation induced by both of these Δ4-precursors (Fig. 3C). DHEA-induced expression of the androgen receptor–responsive genes, PSA, FKBP5, and TMPRSS2, are all inhibited by ab in a dose-dependent manner (Fig. 3D).

Abiraterone inhibits the conversion of Δ4-androstenediol to testosterone

A5diol has been implicated in the development of CRPC, and some studies have detected this Δ4-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-keto reduction to A5diol (24), generating another potential substrate for 3βHSD that can be converted to testosterone (Fig. 4A). To determine whether abi can also block conversion of A5diol to testosterone, LNCaP and LAPC4 cells were treated with [3H]-A5diol and flux to downstream metabolites was assessed. Abi effectively blocks testosterone synthesis in both LNCaP and LAPC4 (Fig. 4B and C).

Figure 2. Abi as substrate and inhibitor for recombinant human 3βHSD1 and 3βHSD2. A and B, Lineweaver–Burk plots of pregnenolone metabolism and abiraterone inhibition for 3βHSD1 and 3βHSD2, respectively, showing competitive inhibition for 3βHSD1 and mixed competitive–noncompetitive inhibition for 3βHSD2 (r² > 0.99 for all lines). C and D, abiraterone as substrate for 3βHSD1 and 3βHSD2, respectively, with curve fits to the Michaelis–Menten equation (r² = 0.99 and 0.95, respectively). E, HPLC chromatograms of abiraterone (top), its Δ4-3-keto homolog (middle), and products of incubations with 3βHSD2 and abiraterone with NAD+ (bottom), showing conversion to its Δ4-3-keto homolog.
**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 (ab) 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 ab cohorts, and tumor volume relative to pretreatment values was monitored over 4 weeks of treatment. The 0.5 mmol/kg/d ab 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 ab 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 ab is probably the clearest demonstration of the continued requirement for androgens in driving CRPC (11). However, CYP17A1 inhibition with ab 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, ab also inhibits the conversion of DHEA to AD by 3β-HSD, leading to decreased synthesis of downstream 5α-dione and DHT. Ab 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 mix-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, Vianerx Pharmaceuticals, Biomarin Pharmaceuticals, Orphanal Pharmaceuticals, and Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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