RESEARCH ARTICLE

A Clinically Relevant Androgen Receptor Mutation Confers Resistance to Second-Generation Antiandrogens Enzalutamide and ARN-509

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ABSTRACT

Despite the impressive clinical activity of the second-generation antiandrogens enzalutamide and ARN-509 in patients with prostate cancer, acquired resistance invariably emerges. To identify the molecular mechanisms underlying acquired resistance, we developed and characterized cell lines resistant to ARN-509 and enzalutamide. In a subset of cell lines, ARN-509 and enzalutamide exhibit agonist activity due to a missense mutation (F876L) in the ligand-binding domain of the androgen receptor (AR). AR F876L is sufficient to confer resistance to ARN-509 and enzalutamide in vitro and in vivo models of castration-resistant prostate cancer (CRPC). Importantly, the AR F876L mutant is detected in plasma DNA from ARN-509-treated patients with progressive CRPC. Thus, selective outgrowth of AR F876L is a clinically relevant mechanism of second-generation antiandrogen resistance that can potentially be targeted with next-generation antiandrogens.

SIGNIFICANCE: A missense mutation in the ligand-binding domain of the androgen receptor F876L confers resistance to the second-generation antiandrogens enzalutamide and ARN-509 in preclinical models of AR function and prostate cancer and is detected in plasma DNA from ARN-509–treated patients with progressive disease. These results chart a new path for the discovery and development of next-generation antiandrogens that could be coupled with a blood-based companion diagnostic to guide treatment decisions. Cancer Discov; 3(9): 1–10. © 2013 AACR.

See related commentary by Nelson and Yegnasubramanian, p. xxx.
RESULTS
Identification of AR F876L in Second-Generation Antiandrogen-Resistant Cell Lines

To determine potential mechanisms underlying acquired second-generation antiandrogen resistance, we developed cell lines resistant to ARN-509 and enzalutamide. Through prolonged in vitro selection, we generated six prostate cancer cell lines resistant to ARN-509 [two each of LNCaP, LNCaP/AR(cs), and LNCaP/AR-Luc; see Methods section] and four enzalutamide-resistant cell lines [two each of LNCaP/AR(cs) and LNCaP/AR-Luc]. Consistent with published data, in vitro, both ARN-509 and enzalutamide were full proliferative and transcriptional antagonists in all three LNCaP parental cell lines (8, 9). However, in two LNCaP lines selected in ARN-509 (LNCaP ARN-509r1 and 2) and one LNCaP/AR-Luc line selected in the presence of enzalutamide (LNCaP/AR-Luc ENZ2), both ARN-509 and enzalutamide stimulated cell proliferation and transcription on a subset of the 15 AR target genes analyzed (Fig. 1A and B and Supplementary Fig. S1). The partial agonist activity was independent of the compound used for selection; ARN-509 and enzalutamide displayed partial agonist activity in all three cell lines regardless of the compound used to derive the resistant variants. Consistent with proliferative activity, ARN-509 or enzalutamide only partially antagonized androgen-dependent growth of these resistant lines (Supplementary Fig. S1). The conversion of enzalutamide and ARN-509 to partial agonists in these cell lines is probably not due to AR overexpression, as these three cell lines expressed AR at levels approximating or lower than the parental cell line (Supplementary Fig. S2).

Given the phenotypic similarities between these three ARN-509/enzalutamide-resistant cell lines and prostate cell lines expressing ligand-specific resistance mutations in AR (e.g., L701H, W741C, H874L, T877A), we hypothesized that a novel AR mutation(s) may be the resistance driver (6, 7, 13, 14). Therefore, we sequenced the AR ligand-binding domain (c2013-2757) from the cell lines in which enzalutamide and ARN-509 showed partial agonist activity. In all three cell lines, we identified the identical missense mutation of thymine to cytosine at position 2626 of the AR mRNA resulting in the conversion of phenylalanine 876 to leucine (Fig. 1C–E). In addition, sequencing of individual subcloned PCR products from the LNCaP/AR-Luc ENZ2 cell line indicated that in all cell lines the F876L mutation arose in the endogenous, T877A-containing AR allele. The identification of the same mutation in the three second-generation antiandrogen-resistant cell lines supports the hypothesis that nonsteroidal antiandrogen treatment selects for compound-specific gain-of-function AR mutations not observed at a high frequency in the untreated population (15) and suggests that the F876L mutation is responsible for the partial agonist activity of ARN-509 and enzalutamide observed in the resistant cell lines. Transcriptional reporter-based studies comparing the activities of an AR mutant harboring the single point F876L or the F876L/T877A double mutant found in the in vitro selected lines with wild-type AR or other mutations commonly found in patients with CRPC confirmed that mutation of AR F876 to L is sufficient to confer agonist activity to ARN-509 and enzalutamide (Fig. 2A and B and Supplementary Figs. S3 and S4).

Although the AR F876L mutation resides in a hotspot for CRPC AR mutations, this particular mutation has not been reported in prostate cancer or androgen-insensitive populations. F876 is located in helix 11 of the AR ligand-binding domain and contributes to a small hydrophobic core formed by residues in helix 11 (F891, L880), the loop between helices 11 and 12 (F891), and helix 3 (F697, L701; Supplementary Fig. S5). Unlike the neighboring residue T877, which coordinates hydrogen bonding to the 17β-OH group of dihydrotestosterone, F876 more likely contributes indirectly to binding of the ligand by influencing ligand-induced conformational changes in helix 12. By affecting protein interactions or the N-C terminal interaction mediated by helix 12, the F876L mutation might affect the off-rate of certain ligand-binding interactions as well as the response of AR to these ligands. Consistent with these predictions, in equilibrium AR-binding assays, ARN-509 and enzalutamide bound to the F876A mutant with 30- and 48-fold higher affinity than to wild-type AR, respectively (Supplementary Fig. S6 and Supplementary Table S1).

F876L Confers Ligand-Selective Resistance In Vitro

To further characterize the mechanism of action of the AR F876L mutant, we engineered two LNCaP cell lines to overexpress AR F876L at levels comparable with the LNCaP/AR(cs) model (Supplementary Fig. S7). ARN-509 and enzalutamide had little transcriptional or proliferative activity in LNCaP/AR(cs) and blocked R1881-induced transcription and proliferation to levels consistent with their agonist activity at the highest concentration (Fig. 3A and B and Supplementary Fig. S8). In contrast, both enzalutamide and ARN-509 showed robust transcriptional and proliferative agonist activity in AR F876L-expressing cells (Fig. 3A and B and Supplementary Figs. S8 and S9).

Transcriptional activation of androgen-regulated AR target genes requires agonist-induced DNA binding and subsequent recruitment of transcriptional coregulators (16). To confirm the VP16-AR reporter results indicating ARN-509 and enzalutamide stimulate AR F876L DNA binding, we conducted chromatin immunoprecipitation (ChIP) analysis of six AR target genes requires agonist-induced DNA binding and subsequent recruitment of transcriptional coregulators (16). To confirm the VP16-AR reporter results indicating ARN-509 and enzalutamide stimulate AR F876L DNA binding, we conducted chromatin immunoprecipitation (ChIP) analysis of six AR target genes from cells treated with R1881 and/or each antagonist. In the LNCaP/AR(cs) cells, R1881 promoted AR DNA binding (Fig. 3C). Consistent with the VP16-AR reporter data, both ARN-509 and enzalutamide promoted AR DNA binding in the LNCaP/SRαF876L cells. In the presence of R1881, all antagonists inhibited R1881-stimulated AR DNA binding to levels consistent with their partial agonist or antagonist activity in both cell lines (Supplementary Fig. S10).

Many AR full and partial agonists stimulate an interaction between a hydrophobic motif in the AR amino terminal domain and AF-2, a protein interaction surface formed by helix 12 in the AR C-terminal ligand-binding domain (N-C interaction; ref. 17). Consistent with the proposed regulation of ligand-stimulated changes in H12 conformation by the F876L mutation, enzalutamide and ARN-509 promoted the N-C interaction of AR F876L but not wild-type AR (Fig. 3D). Thus, the agonist activity of ARN-509 and enzalutamide on AR F876L seems to be associated with an agonist-like AF-2 conformation.
Figure 1. ARN-509 and enzalutamide resistance. A, LNCaP and LNCaP ARN-509r1 cell proliferation. LNCaP and LNCaP ARN-509r1 cells were cultured in the presence of hormone-depleted medium for 2 days followed by ligand addition. Proliferation is quantified by CellTiter-Glo luminescence-based viability assay after 7-day compound treatment. B, quantitative PCR analysis of AR-regulated gene expression. LNCaP, LNCaP/AR(cs), LNCaP/AR–Luciferase and three second-generation antiandrogen-resistant cell lines were cultured for 3 days in hormone-depleted medium followed by treatment with vehicle, 1 nmol/L R1881, or 30 μmol/L compound. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase. C, Sanger sequence identification of F876L mutation. DNA sequence chromatograms obtained from direct sequencing of AR–LBD PCR products from three ARN-509 and enzalutamide gain-of-function resistant cell lines. Sequence analysis shows the presence of heterogeneous c.2626 T > C (encoding F876L) mutation in cell lines showing ARN-509 and enzalutamide gain-of-function activity. D, AR amino acid and protein sequence indicating F876L and T877A AR mutations. E, schematic representation of AR domain structure showing amino acids that when mutated display altered ligand activity in CRPC. NTD, N-terminal domain; DBD, DNA binding domain; LBD, ligand binding domain.
**F876L Conveys Resistance to Second-Generation Antiandrogens In Vivo**

To determine whether the F876L alteration conveys resistance to enzalutamide and ARN-509 in vitro, LNCaP cell lines stably expressing AR F876L were injected (subcutaneously) into castrated immune-deficient mice [severe combined immunodeficient (SCID) Hairless Outbred; SHO] and tumors established. Consistent with the in vitro data, neither ARN-509 nor enzalutamide at 30 mg/kg/day affected the growth of LNCaP/SRxF876L tumors (Fig. 4). This lack of activity was not a function of unexpectedly low compound exposure, as day 28 plasma drug levels were quantified and were consistent with previous LNCaP/AR xenograft studies (ref. 9; Supplementary Table S2). In addition, in a parallel experiment, 30 mg/kg/day ARN-509 exhibited robust anti-tumor activity in the LNCaP/AR(cs) model, consistent with previous results (ref. 9; Fig. 4).

**F876L-Encoding Mutations Are Found in ctDNA from ARN-509–Treated Patients with Progressive Disease**

To determine whether the F876L mutation is likely a determinant of acquired resistance to second-generation antiandrogens in patients with CRPC, we analyzed circulating tumor DNA (ctDNA) in plasma from patients who participated in the phase I portion of a clinical trial of ARN-509 using the sensitive, emulsion PCR-based BEAMing (Beads, Emulsions, Amplification, and Magnetics) method (18). BEAMing has been successfully used to detect a variety of tumor-derived mutations in driver oncogenes such as PI3K3A and KRAS in ctDNA derived from human plasma (19). The phase I study was a first-in-humans dose-escalation study that assessed safety, tolerability, pharmacokinetics, pharmacodynamics, and antitumor activity of ARN-509 in men with metastatic CRPC across nine dose levels (12). Figure 5A depicts the PSA response (% change from baseline) for each individual patient. At 12 weeks, 46.7% of patients had 50% or more decline in PSA as compared with baseline. Pretreatment and during-treatment plasma samples were analyzed. Time of BEAMing analysis is indicated by the terminal end of the PSA response line. Eighteen of the 29 patients had PSA above baseline at time of analysis, indicating either intrinsic or acquired resistance to ARN-509.

Three probes were designed to monitor the three nucleotide changes that can encode for the F876L amino acid substitution (Supplementary Table S3). Dilution mixing experiments with the mutant sequence and wild-type DNA indicated a technical sensitivity of 0.02% (potential to detect one mutant among 5,000 wild-type). In an initial screen of plasma samples from the 29 ARN-509–treated patients, evidence of the mutation was detected in three patients (Table 1 and Supplementary Table S4). At the time of BEAMing analysis, patients 7 and 10 had PSA levels above baseline, whereas patient 13 had evidence of rising PSA above the treatment nadir (Supplementary Fig. S11). In all three patients, the nucleotide change c2628a was detected. In one of these three patients (patient 10), the c2626c mutant was also detected, indicative of polyclonal
Figure 3. The F876L AR mutation confers partial agonist activity to ARN-509 and enzalutamide. A, quantitative PCR analysis of AR-regulated gene expression. LNCaP/AR(cs) and LNCaP/SRαF876L were cultured for 3 days in hormone-depleted medium followed by treatment with vehicle, 1 nmol/L R1881, and 1, 3, 10, and 30 μmol/L compound in the presence or absence of 1 nmol/L R1881. B, LNCaP/AR(cs) and LNCaP/SRαF876L cell proliferation. Cells were cultured in the presence of hormone-depleted medium for 2 days followed by ligand treatment for 7 days. Proliferation is quantified by CellTiter-Glo luminescence-based viability assay. D, ChIP assay of AR. ChIP assays were conducted on LNCaP/AR(cs) and LNCaP/SRαF876L cells incubated for 3 days in hormone-depleted medium followed by 4-hour ligand treatment. C, AR N-C interaction assay. Ligand-induced N-C interaction was monitored via mammalian two-hybrid assay in HepG2 cells. Antagonists were assayed at 8 μmol/L, R1881 at 1 nmol/L.

...disease. F876L-encoding mutations were not detected in any of pretreatment samples (0/29), suggesting that if they were present before ARN-509 treatment, they were below the limit of detection, or the mutations arose de novo during ARN-509 treatment. In either scenario, the data support the hypothesis that the selective outgrowth of lesions bearing the mutant allele to levels sufficient to detect in ctDNA is dependent on chronic exposure to ARN-509 and is associated with rising PSA. To further establish the association of F876L with progressive disease, we analyzed plasma samples taken at additional time points from the three patients who scored positive during the initial screen (Table 1). In patient 10, the mutation was not detected at one other time point analyzed (cycle 4; PSA 102% of baseline). In patient 13, the mutation was not detected at cycle 4 (PSA 16.2% of baseline; patient was scored positive at cycle 11). The mutant sequence at cycle 11 was at the limit of detection and would be estimated to arise via amplification of a single mutant molecule. Although the PSA of patient 13 was slowly rising from the treatment nadir at cycles 11 and 12, at both time points the PSA was still more than 60% below study start, and thus frank resistance had not yet emerged. Identification of the mutant sequence at the limit of detection likely reflects the presence of a relatively rare, mutant clone that has the potential to expand...
under continued selective pressure and eventually drive progressive disease. Alternatively, the correlation between F876L detection and PSA levels may be compromised by the use of plasma preparation protocols that have not been optimized to enrich for ctDNA, and thus the analyzed samples may contain variable levels of contaminating lymphocyte DNA that could diminish the tumor-derived signal (Methods).

Given the relatively long duration of treatment of patient 7, plasma collected at selected time points while the patient was responding to treatment (>90% decline from baseline; cycle 4, 8, and 10) or starting to exhibit progression (cycle 15 and 19; Fig. 5B) were analyzed. Interestingly, mutations were not detected in the three samples from treatment cycles 4, 8, and 10, whereas the c2628a mutation was detected in the two samples analyzed from cycles 15 and 19. These clinical data are consistent with the preclinical data indicating that the F876L amino acid change is sufficient to convey resistance to ARN-509.

**DISCUSSION**

The clinical activity of first- and second-generation hormonal therapy in both castration-sensitive and castration-resistant prostate cancer underscores the central role of the AR across the full spectrum of prostate cancer disease states. Understanding the mechanisms of acquired resistance to first-generation antiandrogens (10) was the first step toward the discovery and development of two second-generation agents, enzalutamide and apalutamide.
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and ARN-509 (8, 9). It follows that understanding mechanisms of acquired resistance to the second-generation antiandrogens will inform design, discovery, and development of next-generation antiandrogens with broad activity across multiple resistant states. In both preclinical functional studies and a correlative clinical study, we describe an amino acid change in resistant states. In both preclinical functional studies and a correlative clinical study, we describe an amino acid change in resistant states.

The discovery of a novel AR mutation that confers resistance to both enzalutamide and ARN-509 underscores the ligand-selective nature of such mutations and indicates that late-stage CRPCs bearing such mutations are still dependent on AR signaling for growth and survival. On the basis of the hypothesis that gain-of-function mutations could contribute to acquired second-generation antiandrogen resistance, Balbas and colleagues (20) recently described a forward genetic screen for AR mutations that confer agonist activity to enzalutamide. Consistent with our results, F876 was the only amino acid residue identified in the screen capable of inducing an antagonist to agonist switch. These results suggest that it may be possible to therapeutically target the majority of second-generation antiandrogen resistance arising through AR mutation by developing AR antagonists that function in the context of AR (wild-type) overexpression as well as the F876L mutation.

Balbas and colleagues (20) used a rational drug design approach to identify the novel thiohydantoin-based F876L AR antagonist, DR103, which was engineered to accommodate structural changes introduced by the F876 mutation. DR103 stands as the first published proof-of-concept of the approach to target AR F876L in the setting of acquired resistance to ARN-509 and enzalutamide.

Using the BEAMing technique on ctDNA from patient plasma, mutations encoding F876L were detected in three of 29 patients who had received the second-generation antiandrogen ARN-509 as part of a phase I dose-escalation study and had ctDNA samples analyzed. Across doses ranging from 30 to 480 mg per day, 14 of 30 patients exhibited 50% or more reduction of PSA at 12 weeks (12). Consistent with the F876L mutation as a driver of acquired resistance to ARN-509, the mutation was detected in 0 of 29 pretreatment samples. Two of the mutant-bearing patients had frank PSA elevation, whereas the PSA of the third mutant-bearing patient was rising from treatment nadir at the time of BEAMing analysis. Given the wide range in dose, and the heterogeneity with respect to intrinsic resistance and therefore duration of treatment (selective pressure) of the patients screened in this study, it is difficult to estimate the prevalence of the F876L-encoding mutations in patients who will progress on second-generation antiandrogens. Although this initial study was limited to screening of ARN-509–treated patients, based on our preclinical work and the work of Balbas and colleagues (20), F876L-encoding mutations are predicted to arise in enzalutamide-treated patients with progressive disease with similar frequency and clinical impact. A more accurate delineation of the frequency of F876L in patients who develop progressive disease while treated with second-generation antiandrogens will require a larger study of patients, preferably in an early stage of CRPC such as prechemotherapy or nonmetastatic CRPC, where intrinsic resistance will be minimal and treatment duration relatively long (21).

Although BEAMing is a very sensitive technique to detect mutations in ctDNA, it probes only for a priori defined changes. It is possible that additional resistance-conferring mutations arise in enzalutamide- and ARN-509–treated patients that were not defined in the preclinical studies, and that with the use of emergent ctDNA-based next-generation sequencing approaches, other genetic changes will be uncovered. These data are essential to the development of next-generation antiandrogens that target the broadest spectrum of resistance-conferring mutations.

The identification of a novel amino acid change that bestows resistance to the second-generation antiandrogens enzalutamide and ARN-509 enables the design and/or discovery of next-generation agents. Given the multifocal and thus potentially polyclonal nature of CRPC, next-generation agents must have potent activity on both wild-type and mutant AR along
with good pharmacokinetics to ensure full receptor occupancy, and AR selectivity to minimize undesirable off-target effects (9, 22). The real-time clinical identification of F876L (and potentially other mutations) in patients who have progressed on second-generation androgens using a non-invasive cdDNA-based companion diagnostic would distinguish patients most likely to benefit from next-generation androgen therapy and guide treatment decisions.

METHODS

Cell Culture and Reagents

LNCaP [American Type Culture Collection (ATCC), purchased 2009], LNCaP/AR(cs) [an LNCap cell line engineered to overexpress wild-type AR three- to fivefold higher than the parental LNCap cell line, gift from Charles Sawyer (Memorial Sloan-Kettering Cancer Center), 2009; ref. 23] and LNCaP/AR-Luc (an LNCap/AR(cs) cell line containing a stable integrated androgen-responsive ARRE2-Po-luc reporter, gift from Charles Sawyer, 2009; ref. 24), and CV1 (ATCC, purchased 2010) cells were maintained in RPMI-1640 supplemented with 10% FBS (HyClone). HepG2 cells were obtained from ATCC in 2009 and maintained in minimum essential medium plus 10% FBS. The cell lines used have not been further authenticated. All media also contained 1 mmol/L sodium pyruvate and 1× nonessential amino acids. Unless otherwise noted, all tissue culture media were purchased from Mediatech. DNA and plasmids are detailed in the Supplementary Materials and Methods.

ARN-509 and enzalutamide were synthesized at Aragon Pharmaceuticals. Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich.

Resistant Line Selection

LNCaP, LNCaP/AR(cs), or LNCaP/AR(cs)-Luc cells were cultured in increasing concentrations of either ARN-509 or enzalutamide (800 nmol/L–6 μmol/L) over a course of 6 months. After selection, the cells were maintained in RPMI plus 10% FBS and 6 μmol/L ARN-509 or enzalutamide.

Stable Cell Line Generation

Detailed protocols used to generate the PC3 and LNCaP cell lines stably expressing AR and AR F876L are provided in the Supplementary Materials and Methods.

Whole-Cell Extract Binding Assay

Binding assays were conducted as described in Clegg and colleagues (9). Details of the proliferation assays are provided in the Supplementary Materials and Methods.

Transcriptional Reporter Assays

Transcriptional reporter assays were conducted as described in Clegg and colleagues (9). Specific details of the individual assays are presented in the Supplementary Materials and Methods.

RNA Isolation and Real-Time PCR

RNA isolation and quantitative PCR (qPCR) were conducted as described in the study conducted by Clegg and colleagues (9). Expression data were log2 standardized and graphed in cell plot using JMP8 software (SAS). Real-time PCR primer sequences are listed in Supplementary Table S5.

Chromatin Immunoprecipitation Assay

ChiP assays were conducted as described previously (26). Specific details are presented in Supplementary Materials and Methods. Real-time PCR primer sequences used in the ChiP assays are listed in Supplementary Table S6.

In Vivo Pharmacology

All animal studies were carried out at Aragon Pharmaceuticals, Inc., under protocols approved by the Institutional Animal Care and Use Committee, and institutional guidelines for the proper, humane use of animals in research were followed. In vivo xenograft experiments were carried out in SHO male mice (Charles River Laboratories). Mice were orchiectomized under isoflurane anesthesia and were given 7 to 10 days to recover. LNCaP/AR(cs) or LNCaP/SROF876L cells were suspended in 50% RPMI, 50% Matrigel (BD Biosciences), and 3 × 10^6 cells per xenograft were injected in a volume of 100 μL. Animals were observed weekly until tumor growth was apparent. After 40 to 60 days postinjection, animals were randomized into cohorts with equivalent mean tumor burden and range (150–250 mm^3). All compounds were administered daily by oral gavage. For all LNCaP/AR(cs) xenograft studies, ARN-509 and enzalutamide drug stocks were prepared in 18% PEG-400, 1% Tween-80, and 1% povidone, and were formulated for dosing in 15% Vitamin E-TPGS and 65% of a 0.5% w/v CMC solution in 20 mmol/L citrate buffer (pH 4.0). ARN-509 and enzalutamide pharmacokinetics were assessed at the end of study as described previously (9).

BEAMing Assay

All BEAMing assays were conducted by Inostics GmbH. Plasma was collected using a standard clinical laboratory method. Briefly, blood was collected in K2-EDTA-evacuated tubes and mixed thoroughly by slowly inverting several times. Within 30 minutes of collection, tubes were spun at 2,000 × g for 15 minutes. Plasma was decanted and transferred to cryo storage tubes. Within 90 minutes of decantation, plasma was stored at ~70°C or lower until analysis. DNA was purified from 300 to 500 μL plasma aliquots and extracted as previously described (19). Mutation detection was conducted according to BEAMing technology as previously described (19). Briefly, in the initial PCR step, the target region (~100 bp) was amplified using gene-specific primers with tag sequences and subjected to an emulsion PCR containing primer-coated magnetic beads. After emulsion, PCR discrimination of wild-type and mutant beads was conducted by allele-specific hybridization followed by flow cytometry. Flow cytometry results were analyzed using FCS Express (De Novo Software), resulting in the quantification of the ratio of the mutant allele over the wild-type alleles.

Disclosure of Potential Conflicts of Interest

J.D. Joseph is employed (other than primary affiliation; e.g., consulting) as a principal scientist and has ownership interest (including patents) in Aragon Pharmaceuticals. N. Lu is an employee (other than primary affiliation; e.g., consulting) and has ownership interest (including patents) in Aragon Pharmaceuticals. E.C. Maneval is employed (other than primary affiliation; e.g., consulting) as Vice President, Clinical Development, and has ownership interest (including patents) in Aragon Pharmaceuticals. I. Eden is employed (other than primary affiliation; e.g., consulting) as Chief Medical Officer and has ownership interest (including patents) in Aragon Pharmaceuticals. B. Darimont has ownership interest (including patents) in Aragon Pharmaceuticals.
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Consulting) as Senior Director of Biology and has ownership interest (including patents) in Aragon Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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