Vasoactivity of AG014699, a Clinically Active Small Molecule Inhibitor of Poly(ADP-ribose) Polymerase: a Contributory Factor to Chemopotentiation In vivo?

Majid Ali,1 Brian A. Telfer,1 Cian McCrudden,2 Martin O’Rourke,2 Huw D. Thomas,3 Marzieh Kamjoo,1 Suzanne Kyle,3 Tracy Robson,2 Chris Shaw,2 David G. Hirst,2 Nicola J. Curtin,3 and Kaye J. Williams1

Abstract Purpose: Poly(ADP-ribose)polymerase (PARP) plays an important role in DNA repair, and PARP inhibitors can enhance the activity of DNA-damaging agents in vitro and in vivo. AG014699 is a potent PARP inhibitor in phase II clinical development. However, the range of the therapeutics with which AG014699 could interact via a DNA-repair based mechanism is limited. We aimed to investigate a novel, vascular-based activity of AG014699, underlying in vivo chemosensitization, which could widen its clinical application.

Experimental Design: Temozolomide response was analyzed in vitro and in vivo. Vessel dynamics were monitored using “mismatch” following the administration of perfusion markers and real-time analysis of fluorescently labeled albumin uptake in to tumors established in dorsal window chambers. Further mechanistic investigations used ex vivo assays of vascular smooth muscle relaxation, gut motility, and myosin light chain kinase (MLCK) inhibition.

Results: AG014699 failed to sensitize SW620 cells to temozolomide in vitro but induced pronounced enhancement in vivo. AG014699 (1 mg/kg) improved tumor perfusion comparably with the control agents nicotinamide (1 g/kg) and AG14361 (forerunner to AG014699; 10 mg/kg). AG014699 and AG14361 relaxed preconstricted vascular smooth muscle more potently than the standard agent, hydralazine, with no impact on gut motility. AG014699 inhibited MLCK at concentrations that relaxed isolated arteries, whereas AG14361 had no effect.

Conclusion: Increased vessel perfusion elicited by AG014699 could increase tumor drug accumulation and therapeutic response. Vasoactive concentrations of AG014699 do not cause detrimental side effects to gut motility and may increase the range of therapeutics with which AG014699 could be combined with for clinical benefit.

and AG014698 caused profound chemopotentiation in xenograft models established in nude mice (2). These promising data led to AG014699 being the first PARP inhibitor to be evaluated in cancer patients. Phase I and II trials have been completed in which AG014699 was combined with the DNA-damaging agent temozolomide (TMZ). These studies have shown excellent PARP inhibition in peripheral blood leukocytes and tumor material at a dose of 12 mg/m^2^ that can be well tolerated in combination with TMZ (100 mg/m^2^) and yields encouraging activity in malignant melanoma (3). Importantly, no AG014699-associated toxicity was observed at doses that caused profound and durable suppression of PARP activity. Given the promising clinical profile of AG014699, broadening its potential clinical application to an increased range of anticancer therapeutics would be an attractive approach.

One major obstacle to effective chemotherapy is poor drug delivery. It remains challenging to adequately deliver chemotherapeutic agents/drugs directly into the tumor microenvironment due to its hostile nature and poor vascular perfusion (4). Fluctuations in tumor blood flow have been observed in patients (5) that could affect microregional oxygenation and drug delivery (6). Tumor blood flow has been correlated with poor outcome following chemotherapy, and also radiotherapy and surgery (7). The latter two observations are likely to be associated with fluctuations in oxygen availability causing tumor hypoxia that results in radiosistance and an aggressive disease phenotype.

The early PARP inhibitors, nicotinamide and the benzamidines, are potent in vivo radio sensitizers, an effect that was achieved at least in part by virtue of their ability to increase tumor blood flow. Indeed, nicotinamide is used clinically in combination with carbogen breathing to improve tumor oxygenation for radiotherapy (8–10). Our initial studies revealed that the PARP inhibitor AG14361, a forerunner of the clinical candidate AG014699, increased the transient perfusion of tumor xenografts by inhibiting spontaneous rhythmic tumor vasoconstriction (11). We postulated that the apparent vasoactivity of AG14361 contributes to its in vivo radiosensitization and chemosensitization. In keeping with this suggestion, AG14361, coupled with TMZ, caused complete regression of SW620 xenografts in vivo, whereas having no effect on TMZ sensitivity in SW620 cells cultured in vitro (11). In this present study, we investigate the vasoactivity of the clinical lead agent AG014699 in vivo and ex vivo compared with AG14361 and the known vasoactive agents nicotinamide and hydralazine and assess its antitumor effects in combination with TMZ.

**Materials and Methods**

**Reagents.** For in vitro studies, we dissolved TMZ (gift from Cancer Research UK), and PARP inhibitors AG14361 and AG014699 (Pfizer Oncology) in DMSO to allow addition to cell cultures at a final DMSO concentration of 1% (v/v). All other chemicals and reagents were from Sigma unless otherwise stated. For in vivo evaluation, we prepared all agents immediately before administration. TMZ was suspended and administered AG143699 and nicotinamide were dissolved in normal saline. AG14361 and AG14447 were prepared as the HCl salt by dissolving in equimolar HCl in saline.

**Cell lines and culture.** We maintained exponentially growing cultures of SW620 (American Type Culture Collection) and HT29 (a kind gift from Professor Caroline Dive, University of Manchester, Manchester, UK) colorectal cancer cells in RPMI 1640 containing 10% (v/v) fetal calf serum. Cells were verified as Mycoplasma free (MycoAlert, Cambrex Bioscience).

**In vitro chemosensitization.** We estimated cell growth inhibition in exponentially growing SW620 cells in 96-well plates exposed to increasing concentrations of TMZ, alone or in combination with AG014699 (0.4 μmol/L) for 5 d before staining with sulphorhodamine B as previously described (12). Cell growth, determined after subtraction of time 0 values, was expressed as a % of the relevant DMSO or AG014699 alone control, as appropriate. GI50 (concentration of drug that inhibited growth by 50%) values were calculated from the computer-generated curves (GraphPad Software, Inc.).

**In vivo TMZ studies.** All of the in vivo experiments were reviewed and approved by the relevant institutional animal welfare committees, and performed according to national law (Scientific Procedures Act 1986) and UK Coordinating Committee on Cancer Research Guidelines. Adult female athymic nude mice (nu/nu) used for antitumor studies were maintained and handled in isolators under specific pathogen-free conditions. For TMZ studies, we implanted 1 × 10^6^ SW620 cells s.c. into one flank of each mouse. When tumors were palpable (10–12 d after implantation), we randomized the mice (five animals per group) to receive five daily doses of TMZ administered p.o. as a suspension in saline at 68 mg/kg either alone or in combination with five daily i.p. administrations of AG014699 at 1.0 mg/kg, normal saline p.o. and i.p. (controls) or normal saline p.o. and AG014699 (10 mg/kg) i.p. Data are presented as median relative tumor volumes (RTV), defined as the calculated tumor volume divided by the calculated tumor volume on the initial day of treatment (day 0). Thus, on day 0, the RTV value is 1 and RTV4 is when the tumor is four times as large as its initial value. Tumor growth delays were calculated as follows:

Tumor growth delay (TGD) = Median time to RTV4 in treated group - median time to RTV4 control

**Vessel mismatch studies.** Once SW620 xenografts (established as described above) had reached an approximate diameter of 10 mm, we administered AG14361 (10 mg/kg), AG014699 (1 mg/kg), nicotinamide (1 g/kg), or saline (control) i.p., followed 30 min later by Hoechst 33342 (i.v., 15 mg/kg, dissolved in PBS) and finally carbocyanine

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**Translational Relevance**

AG014699, a phosphate salt of AG14447, is a potent inhibitor of the DNA-repair enzyme, poly(ADP-ribose) polymerase. Promising preclinical data demonstrating sensitization of tumor cells to DNA-damaging chemotherapy in vitro and in vivo led to the clinical development of AG014699. Phase I and II trials have reported profound and durable suppression of poly(ADP-ribose) polymerase activity with no appreciable toxicity. AG014699 is well tolerated in combination with temozolomide and yields encouraging activity in malignant melanoma. Although these data are promising, the range of agents that AG014699 could enhance through a DNA repair–based mechanism is limited. We report a novel vascular effect of AG014699 that could contribute to the chemosensitization achieved with this compound. By increasing tumor perfusion, AG014699 could improve drug delivery and be beneficial in combination with agents whose clinical efficacy is limited by poor distribution.

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(i.v., 1 mg/kg, dissolved in 75% dimethyl sulphoxide) after a further 20 min. We excised and rapidly froze the tumors 5 min later as described previously (9). Tumor sections (10 μm) were prepared and scanned under a Nikon Eclipse E800 microscope to analyze Hoechst 33342 and carbocyanine positive vessels (excitation wavelengths, 340-380 nm and 450-490 nm; emission wavelengths, 480 nm and 510 nm, for Hoechst and carbocyanine, respectively).

Real-time analysis of tumors established in dorsal window chambers. We implanted dorsal window chambers (DWC), attached to the back of female nude mice as described previously (13), with SW620 or HT29 cells (~50 μL of a 1 × 10⁶/ml stock prepared in serum-free RPMI). At a tumor volume of ~100 mm³, mice were anesthetized and prepared for intravital microscopy. Tumor vasculature images were taken using bright-field microscopy (Nikon Eclipse E800) as well as background fluorescence readings before the i.v. administration of bovine serum albumin (BSA) labeled with Alexa fluorochrome (BSA-647 excitation wavelength of 647 nm, prepared at 1 mg/ml in sterile saline; 0.1 mL/mouse; Molecular Probes, Invitrogen). Changes in fluorescence (emission, 668 nm) were monitored in real-time (Metamorph analysis system) continuously preinjection and postinjection of AG014699 (1-10 mg/kg i.p.).

Fig. 1. The different effects of AG014699 on TMZ-induced growth inhibition of SW620 cells in vitro and xenografts in vivo. A, TMZ alone (•, solid line) caused a concentration-dependent inhibition of cell growth that was not significantly increased by the combination with 0.4 μmol/L AG014699 (○, broken line). Points, mean of five independent experiments; bars, SD. B. A series of SW620 xenografts treated with control vehicle (•, solid line), AG014699 (10 mg/kg daily × 5) alone (○, broken line), TMZ (68 mg/kg daily × 5) alone (△, solid line), or the combination of TMZ with AG014699 at 1 mg/kg (▲, broken line). Data are median RTV from five animals per group.

Ex vivo assay: rat tail artery. Male albino Wistar rats (8- to 12-wk-old; Harlan UK Ltd.) were killed by CO₂ asphyxiation followed by cervical dislocation. We removed the tail from the animal at its most proximal point and peeled back the skin on the ventral surface to reveal the vascular bed containing the tail artery. We removed the membrane overlying the artery in the same manner and bathed the exposed artery in ice-cold Krebs’ solution (118 mmol/L NaCl, 4.7 mmol/L KCl, 25 mmol/L NaHCO₃, 1.15 mmol/L NaH₂PO₄, 2.5 mmol/L CaCl₂, 1.1 mmol/L MgCl₂, 5.6 mmol/L C₆H₁₂O₆) to prevent dehydration. We then introduced a cannula of 1-mm polyethylene gas chromatography tubing into the artery and slid the artery over it until an insertion overlap of ~5 mm was achieved before securing it to the cannula using double-knotted thread. A length of artery ~10 mm beyond the end of the cannula was freed from its vascular bed and cut.

We connected the cannulated artery to an internal/external perfusion apparatus and perfused it with oxygenated Krebs’ (95% O₂/5% CO₂; BOC) at 37°C, increasing the rate of perfusion from 0.25 to 2 ml/min in 0.25-ml/min increments over a period of 1 h. After equilibration of the artery segments for 1 h, we elicited submaximal constriction by perfusing the arterial segments with perfusate that contained 10 μmol/L phenylephrine (PE). We determined the dilatory properties of AG14361/AG014699 by replacing the constricating perfusate with one that contained 10 μmol/L PE plus the relevant concentration of either AG14361 or AG014699. Arterial constriction or dilation was detected by an increase or decrease in pressure detected by transducers (by changes in pressure, monitored by water displacement) connected to a MacLab system (AD Instruments Pty Ltd.), and was visualized on a personal computer. The degree of relaxation elicited by AG14361/AG014699 treatment was expressed as a percentage of the magnitude of constriction that was observed following 10 μmol/L PE treatment.

Rat ileum. We removed a segment of ileum from rats killed as described above. After flushing the fecal matter from the lumen using ice-cold Krebs’ solution, we placed the tissue in fresh, ice-cold oxygenated Krebs’. We mounted 5-mm thick rings of ileum on the pins of isometric force transducers and lowered them into a tissue bath containing Krebs’ at 37°C, with oxygenated Krebs’ flowing through at a rate of 2 ml/min per minute. In this system, one pin is stationary, whereas the second pin is free to move vertically and is used to apply tension to the piece of tissue. With increasing application of tension, the rings begin contracting spontaneously. When the average tension on each ring reached 0.5 grams, we allowed the tissue to equilibrate (average 15 min) before applying the test agent by perfusing the tissue bath with the relevant agent. Intestinal constriction was detected by the transducers connected to a MacLab system (AD Instruments Pty Ltd.) and was visualized on a Macintosh computer. Differences in the magnitude of contractions 15 min following application of the agent were compared with the magnitude of contraction in the lead up to treatment and were expressed as a percentage of the magnitude of contraction before treatment.

In vitro myosin light chain kinase activity analysis. Kinase activity analysis was done using Upstate’s IC₅₀ Profiler Express service. Samples (5 mmol/L) of AG14361 and AG014699 were sent to Upstate’s Drug Discovery Service department. From this sample, a 10-point, 1/2 logarithmic dilution series was generated, resulting in an agent test concentration range of 100 nmol/L to 100 μmol/L as control [1% (v/v) DMSO]. The kinase activity analysis protocol used by Upstate’s Drug Discovery Service department. From this sample, a 10-point, 1/2 logarithmic dilution series was generated, resulting in an agent test concentration range of 100 nmol/L to 100 μmol/L as control [1% (v/v) DMSO]. The kinase activity analysis protocol used by Upstate’s Drug Discovery Service department. From this sample, a 10-point, 1/2 logarithmic dilution series was generated, resulting in an agent test concentration range of 100 nmol/L to 100 μmol/L as control [1% (v/v) DMSO]. The kinase activity analysis protocol used by Upstate’s Drug Discovery Service department. From this sample, a 10-point, 1/2 logarithmic dilution series was generated, resulting in an agent test concentration range of 100 nmol/L to 100 μmol/L as control [1% (v/v) DMSO].

Briefly, in a final reaction volume of 25 μL, myosin light chain kinase (MLCK; h; 5-10 mU) was incubated with 8 mmol/L MOPS (pH 7.0), 0.2 mmol/L EDTA, 0.5 mmol/L CaCl₂, 16 μg/ml calmodulin, 250 μmol/L KCl, NNKRTLSFAEPG, and 10 mmol/L Mgacetate and [γ⁻³²P]-ATP. The reaction was initiated by the addition of the MgATP mix. After incubation for 40 min at room temperature, the reaction was stopped by the addition of 5 μL 3% phosphoric acid solution. The reaction (10 μL) was then spotted onto a P30 filtermat and washed thrice for 5 min in 75 mmol/L phosphoric acid and once

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AG014699 does not enhance TMZ-induced growth inhibition in vitro but profoundly increases the activity of TMZ against SW620 xenografts. SW620 cells express low levels of methylguanine methyl transferase and have functional mismatch repair, and are therefore profoundly sensitive to TMZ. We had previously found that AG14361 did not enhance TMZ-induced growth inhibition in these cells (11) and we confirmed in the current study that there was no statistically significant difference in growth inhibition by TMZ alone or in combination with AG014699 (P = 0.596 from five independent experiments; Fig. 1A). Furthermore, coadministration of a PARP inhibitor did not enhance the cytotoxicity of TMZ in SW620 cells by clonogenic survival assay (Supplementary Fig. S1). However, when AG014699 was combined with TMZ in vivo, a completely different picture emerged. TMZ alone caused transient regression followed by regrowth resulting in a 25-day tumor growth delay (time to RTV4). AG014699 alone had no impact on tumor growth but the combination of TMZ and AG014699 resulted in prolonged regression lasting at least 53 days (time to RTV 4, 60, 63, and 100 days in three mice that relapsed) with two of five mice remaining tumor free at day 100 (Fig. 1B). The difference in in vivo and in vitro chemosensitization by AG014699 was not thought to be due to divergent concentrations of the drug in the two systems, as we have shown previously that concentrations of the parent drug, AG14447, are between 1.5 and 0.6 μmol/L for 24 hours after a single i.p. injection of AG014699 at a concentration of 1 mg/kg (15).

Fig. 2. AG014699 and AG14361 are more potent inhibitors of vessel mismatch than the positive control agent nicotinamide. The perfusion markers Hoechst and carbocyanine were administered i.v. 20 min apart to SW620 tumor-bearing mice that had been pretreated with AG014699 (699; 1 mg/kg), AG14361 (361; 10 mg/kg), nicotinamide (nic; 1 g/kg), or saline (control). Data presented in A are average vessel mismatch values (n = 4 per group, ± SEM). *, P = 0.03 versus saline-treated control. B, the impact of the agents on percentage of vessels closing (Hoechst positive only) or opening (carbocyanine positive only; average values ± SEM).

Fig. 3. Real-time analysis of the effects of AG014699 on the accumulation of BSA labeled with Alexa-647 in SW620 and HT29 tumors established in DWC. A, fluorescence (arbitrary units, FU) was monitored every minute in control (saline; open symbols) and AG014699-treated (1 mg/kg; solid symbols) tumors. B, the distribution of BSA-647 immediately after injection (i), immediately before AG014699 or saline administration (ii), and at the end of the experiment (iii) as illustrated on the uptake curves in A. Bolus injections of saline via i.p. (iv) or i.v. (v) routes do not alter BSA-647 fluorescence.

C.E.A. Arris and N.J. Curtin, unpublished observations.
**PARP inhibitors reduce vessel mismatch in SW620 tumor xenografts in vivo.** To determine why there was such a profound in vivo chemosensitization in the absence of in vitro activity, we investigated whether AG014699 (in comparison AG14361 and nicotinamide) might be increasing the area of tissue that received TMZ through an effect on tumor perfusion. Hoechst 33342 and carbocyanine are routinely used as markers of tumor perfusion. As they have distinct emission wavelengths, they can be analyzed simultaneously in the same tumor. We administered Hoechst 33342 20 minutes before carbocyanine and observed the staining pattern of the tumor blood vessels. Vessels staining with one dye only are denoted as mismatched and have either closed (Hoechst 33342 only) or opened (carbocyanine only) in the period between administration of the dyes. Vessels open throughout the 20-minute interval stain with both dyes. In keeping with our previous findings (11), vessel mismatch was markedly reduced in the AG14361 (10 mg/kg) pretreated SW620 xenografts compared with control (18% versus 51%; Fig. 2A). Vessel mismatch was also reduced following AG014699 (1 mg/kg; 28%) and nicotinamide (1 g/kg; 19%) pretreatment (Fig. 2A). Thus, AG014699 has a similar effect to a 10× higher concentration of AG14361 and a 1,000× higher concentration of nicotinamide. Analysis of the proportion of vessels stained with only one dye revealed subtle differences between the agents used (Fig. 2B). Whereas in saline control–treated animals, 30% of vessels closed between Hoechst and carbocyanine administration; this was reduced to 12% in AG14361 and nicotinamide–treated tumors. Similarly, the number of vessels stained with carbocyanine only was also reduced in tumors treated with AG14361 and nicotinamide, suggesting that for these agents, vessels that were open at the point of Hoechst 33342 injection remained so, such that the proportion of Hoechst 33342 only (closed) and carbocyanine only (opened) vessels was low. For AG014699, the amount of vessel closure was further reduced compared with AG14361 and nicotinamide to only 5% in mice treated with AG014699 (Fig. 2B). Furthermore, the proportion of vessels open at the time of carbocyanine injection was greater in AG014699–treated mice (21%) compared with AG14361 and nicotinamide (7% for both; Fig. 2B), indicating that a substantial amount of the vessel mismatch was a consequence of vessel opening.

The effects of AG014699, AG14361, and nicotinamide on vascular perfusion in tumor xenografts established in DWCs. To further investigate the effects of the PARP inhibitors on tumor
Inhibitors of the DNA-repair enzyme PARP have received much attention recently as a consequence of their potential ability to enhance the effects of a number of DNA-damaging anticancer therapeutics (1). However, although this application holds much promise, our data suggest that the utility of PARP inhibitors could exceed that initially anticipated through an in vivo mechanism influencing tumor perfusion.

AG014699 was the first PARP inhibitor to be used in cancer patients and is currently in phase II trials. Analyzing the chemopotentiating effect of AG014699 in preclinical studies, we observed profound differences between in vitro and in vivo models. SW620 cells are exquisitely sensitive to the chemotherapy agent TMZ. In vitro, AG014699 is unable to enhance this sensitivity, yet in vivo, combination of AG014699 and TMZ yielded pronounced growth delay or tumor cures at doses of TMZ that caused only transient growth delay when administered alone. This suggested that mechanisms other than inhibition of DNA repair were contributing to the observed effects of AG014699 in vivo.

Given the reported vasoactive effects of early PARP inhibitors, we investigated the influence of AG014699 on vascular dynamics using both in vitro and ex vivo models. Comparisons were made with AG14361, the effective forerunner to AG014699 in the development of a clinical lead candidate, and also nicotinamide and hydralazine that acted as positive controls in the model systems used. Tumor vascular effects were confirmed by both vessel mismatch studies and real-time observations of perfusion using tumors grown in DWCs. Compellingly, the relative efficacies of agents showed excellent concordance between the mismatch and DWC studies using SW620 tumors, and have been replicated using other tumor systems and mouse backgrounds (HT29 data here within and data not shown). Furthermore, vasoactivity was proven in excised rat arteries, and both AG014699 and AG14361 were more potent than the control agent hydralazine at causing relaxation of preconstricted arteries. The effect was specific to vascular smooth muscle as neither agent had any significant activity on contraction of rat ileum. Preliminary investigations into the mechanisms of the effect of the two agents revealed that AG014699 inhibited the activity of MLCK at concentrations commensurate with its effects on preconstricted arteries. AG14361 had no effect on MLCK and so its mechanism at this point remains elusive. However, the differential impact of the two agents could contribute to the subtle differences observed in the mismatch studies whereby AG14361 appeared to maintain vessels that were already open in an open state, whereas significant vessel opening continued during AG014699 treatment.

Relating these data to the observed chemosensitization effects, they would strongly support a model whereby AG014699 and AG14361 treatment can increase the extent of tumor exposure to drug by enhancing vascular perfusion. As the efficacy of a number of clinically used chemotherapy agents is limited by their very poor distribution in vivo (4, 6), the overall consequence would be to expose a greater number of tumor cells to drug, thereby increasing the overall antitumor effect. Interestingly, previous studies have reported that PARP inhibitors can sensitize tumor xenografts to doxorubicin (16) and cisplatin (17). As neither agent would be anticipated to be enhanced by PARP inhibition through a classic

**Discussion**

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DNA repair–based mechanism, vasoactivity could have been the basis for the enhancement, exemplifying the application of PARP inhibitors with different classes of agent.

In addition, the ex vivo assays performed suggest that the positive effects of AG014699 treatment on drug delivery would not be confounded by negative effects on gut motility. Gastric toxicity limits the therapeutic benefit of nicotinamide (18), so the absence of an inhibitory effect in intestinal tissues in the cases of AG14361 and AG014699 is an attractive finding. It is also important to note is that AG014699 and AG14361 begin exhibiting vasoactivity at a concentration that is two orders of magnitude lower than the concentration at which nicotinamide elicits these effects.

AG014699 is an attractive drug for anticancer therapy but the range of drug classes with which it synergizes through a DNA repair–based mechanism is quite restricted. However, our data suggest that it may have broader application on the basis of improved tumor perfusion and hence drug delivery. In addition, enhanced perfusion could also contribute to the in vivo radiosensitization previously reported for AG14361 (11) and a focus of our ongoing studies with AG014699. The fact that AG014699 seems to have very little clinical toxicity supports its wider application in the clinical setting. We believe our demonstration of the vasoactive effect of AG014699 justifies further in vivo studies with a range of cytotoxic drugs as a prelude to using potential improvements in drug delivery as a therapeutic maneuver in the clinical setting.

Disclosure of Potential Conflicts of Interest

N. Curtin received a commercial research grant from Agouron/Pfizer and N. Curtin and K. Williams are inventors on a patent from Pfizer. The other authors disclosed no potential conflicts of interest.

Acknowledgments

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References

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