Histone deacetylase–mediated transcriptional activation reduces proviral loads in HTLV-1–associated myelopathy/tropical spastic paraparesis patients

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Epigenetic modifications of chromatin may play a role in maintaining viral latency and thus persistence of the human T-lymphotropic virus type 1 (HTLV-1), which is responsible for HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP). A major determinant of disease progression is increased peripheral blood proviral load (PVL), possibly via the accumulation of infected cells in the central nervous system (CNS) creating a damaging inflammatory response. Current therapeutic approaches that focus on reducing either cell proliferation, viral replication, or tissue invasion are still unsatisfactory. Contrasting with these inhibitory strategies, we evaluated the efficacy of a novel approach aimed, paradoxically, at activating viral gene expression to expose virus-positive cells to the host immune response. We used valproate (VPA), a histone deacetylase inhibitor that has been used for decades as a chronic, safe treatment for epileptic disorders. Based on in vitro and in vivo data, we provide evidence that transient activation of the latent viral reservoir causes its collapse, a process that may alleviate the condition of HAM/TSP. This represents the first such approach to treating HAM/TSP, using gene activation therapy to tilt the host-pathogen balance in favor of an existing antiviral response. This trial is registered at http://clinicaltrials.gov/as no. NCT00519181.

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Introduction

Of the 10 to 20 million human T-lymphotropic virus 1 (HTLV-1)–infected people worldwide, a significant fraction (approximately 2%-3%) will develop adult T-cell leukemia (ATL), while a similar proportion will develop HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP). A major determinant of disease progression is increased peripheral blood proviral load (PVL), possibly via the accumulation of infected cells in the central nervous system (CNS) creating a damaging inflammatory response. Current therapeutic approaches that focus on reducing either cell proliferation, viral replication, or tissue invasion are still unsatisfactory. Contrasting with these inhibitory strategies, we evaluated the efficacy of a novel approach aimed, paradoxically, at activating viral gene expression to expose virus-positive cells to the host immune response. We used valproate (VPA), a histone deacetylase inhibitor that has been used for decades as a chronic, safe treatment for epileptic disorders. Based on in vitro and in vivo data, we provide evidence that transient activation of the latent viral reservoir causes its collapse, a process that may alleviate the condition of HAM/TSP. This represents the first such approach to treating HAM/TSP, using gene activation therapy to tilt the host-pathogen balance in favor of an existing antiviral response. This trial is registered at http://clinicaltrials.gov/as no. NCT00519181.

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process is driven by the viral Tax oncoprotein, and (3) that infected cells escape from immune surveillance after silencing virus transcription.

There are thus 2 main categories of infected lymphocytes: those expressing viral proteins that are rapidly eliminated by host immunity and those carrying silent provirus that have the capacity to accumulate by cell mitosis. Although cells of the former type potentially generate inflammation, major problems result from the long-term accumulation of provirus-carrying cells. When a threshold level of these cells is reached in peripheral blood, a proportion migrates into the CNS.23 Since a close correlation exists between the number of proviral copies and the amount of expressed viral RNAs,24 an increase in the PVL is expected to worsen indirectly inflammation, although there is an overlap in the absolute levels of proviral load between patients with HAM/TSP and asymptomatic carriers. Inversely, a long-term reduction in the number of provirus-carrying cells would be protective against collateral damage responsible for HAM/TSP. In this context, we postulated that transient transcriptional activation of this viral reservoir out of hiding would result in rapid depletion of the infected cells by the host immune system.

Patients, materials, and methods

HAM/TSP patients included in the study were followed at the University Hospital of Fort-de-France, French West Indies. The study was approved by the local and Regional Research Ethics Committee, and all procedures were carried out with informed consent obtained in accordance with the Declaration of Helsinki.

Gene reporter assays

HeLa cervix epithelial cells were grown in minimal essential medium (Sigma, St Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U penicillin, and 100 μg streptomycin per milliliter. Twenty-four hours prior to transfection, cells were cultured at a density of 3 × 10^5 cells per well (6-well dish; Nunc, Rochester, NY) and transfected with different plasmid combinations. The reporter plasmid (pCRLuc, a gift of Ralph Grassmann, Erlangen, Germany) contains the HTLV-1 long terminal repeat promoter cloned upstream the luciferase gene into pGem-luc (Promega, Madison, WI). The pCMVTax1 effector plasmid (provided by François Bex, Free University of Brussels, Brussels, Belgium) encodes the HTLV-1 Tax transcriptional activator protein from a cytomegalovirus (CMV) promoter. Cells were transfected with pCRLuc and pCMVTax1 or pCMV empty vector using GeneJammer reagent (Stratagene, La Jolla, CA) following the manufacturer’s protocol. Different amounts and ratios of effector and reporter plasmids were tested in preliminary assays: 1 μg pCRLuc and 25 ng pCMVTax1 were found to be optimal. After transfection, cells were grown at 37°C for 18 hours in the presence of different concentrations of valproate (VPA; 0, 1, 5 mM) and luciferase enzyme activity was measured using the dual-luciferase assay system (Promega). Data (in mean light arbitrary units ± standard deviation) are derived from 3 independent experiments.

Jurkat T lymphocytes were cultivated in OptiMEM (Invitrogen, Frederick, MD) supplemented with 20% heat-inactivated FCS, 2 mM L-glutamine, 100 U penicillin, and 100 μg streptomycin per milliliter. Cells (4 × 10^5) were transfected with 2 μg pCRLuc reporter in the presence of 10 ng plasmid pCMVTax1 or control pCMV. Transfected cells were cultivated for 24 hours with VPA (0, 1, 5 mM) and luciferase activity was measured.

Analysis of apoptosis by flow cytometry

Peripheral blood mononuclear cells from HAM/TSP or ATL patients were isolated by Ficoll density centrifugation (GE Healthcare, Little Chalfont, United Kingdom) and either frozen in liquid nitrogen or directly cultivated in RPMI medium (data of Figure 2) containing 15% (vol/vol) fetal bovine serum, 2 mM L-glutamine, 100 U penicillin, and 100 μg streptomycin per milliliter (Eurobio, Paris, France). Preliminary experiments with frozen or fresh cells fixed the optimal times of culture (up to 96 hours) and concentrations of VPA (in a range of 0 to 10 mM). After culture, cells were labeled with either a CD4- or a CD8-specific antibody conjugated to allophtocyanin (APC; BD Biosciences, San Jose, CA). Apoptotic cells were identified after staining with fluorescein-isothiocyanate (FITC)-labeled annexin V (annexin) and propidium iodide (PI; BD Biosciences). Ten-thousand events of dual- or triple-labeled sample were collected by a flow cytometer (FACSCalibur; BD Biosciences) using the FSC/SSC, FL1 (annexin), FL2 (PI), and FL4 (APC) detectors. Proportions of live and apoptotic cells were determined using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA) after appropriate compensation. Apoptotic rates were expressed as mean plus or minus standard deviation and comparisons made by Wilcoxon signed rank test at 95% confidence. Similar cultures and assays were also performed with the HTLV-I-infected MT2 cells (ECACC 93121518).

Western blot analysis for histone H3 acetylation and p19 viral core protein ELISA

Primary PBMCs freshly isolated from 3 HAM/TSP patients and 3 controls were cultured with an optimal concentration of 2 mM VPA for 48 hours. Corresponding cell culture supernatants were collected, and the viral core 19 protein was analyzed by enzyme-linked immunosorbent assay (ELISA, Retrotek; Zephticientrix, Buffalo, NY) according to the manufacturer’s protocol. Absolute concentrations of p19 were determined with a standard purified antigen dilution curve. Cells were then fractionated and analyzed by flow cytometry to determine apoptotic rates (as described in the previous paragraph) or by Western blot to evaluate the levels of histone H3 acetylation. After 3 washes in phosphate-buffered saline, cells were lysed in Tris 20 mM (pH 7.5), 0.1% Triton X-100, and a cocktail of proteases (Complete; Roche, Indianapolis, IN). Protein (10 μg) was migrated on a denaturing sodium dodecylsulfate polyacrylamide gel and transferred onto PVDF membranes. After saturation of the filters in Tris-buffered saline containing 1% blocking solution (Roche), proteins were revealed with anti-acetylated histone H3 (Upstate, Lake Placid, NY) or antiactin (Sigma) antibodies in association with a goat antirabbit alkaline phosphatase conjugate (Dako Cytomation, Carpinteria, CA). After incubation with chuminolinescent substrate (Perkin Elmer, Walkham, MA), immunoblots were exposed to autoradiographic films (GE Healthcare) or scanned by the Kodak Digital Science image station type 440 (Roche, NY). After background subtraction, a ratio was calculated between the light intensities (in arbitrary units) generated by acetylated histone H3 and the signal corresponding to actin used to normalize protein levels.

VPA trial in HAM/TSP patients

The study was approved by the Local and Regional Research Ethics Committee and all procedures were carried out in accordance with the Declaration of Helsinki. After having obtained written consent, 20 patients suffering from HAM/TSP were recruited according to World Health Organization criteria25 from a cohort at the “Centre Hospitalier Universitaire” of the Neurology department in Fort-de-France (Martinique). All patients presented with long-term mobility impairment with a DSS score (expanded disability status scale) of between 5 and 7.5. VPA was administrated orally at a maximal dose of 20 mg/kg/day. Serum concentrations of VPA were measured at day 15 by the EMIT method (SYVA reagents; Dade- Behring, Dundingen, Switzerland) with a Cobas Mira analyzer (Roche). Regular clinical evaluation included determination of the DSS score as well as a more sensitive estimation of mobility based on the duration of an 8-meter walk, the shortest time of 2 tests being considered.

Measurement of HTLV-1 proviral load in PBMCs

HTLV-1 proviral load in PBMCs was quantified in the HAM/TSP patients and asymptomatic carriers. PBMCs were isolated from EDTA-containing blood by density gradient centrifugation and cryopreserved until use.
Inhibitors of histone deacetylase (HDAC) efficiently activate gene transcription by modulating the levels of acetylation of histone and nonhistone proteins. Apart from efficacy, a prerequisite for clinical use of this type of molecule would be a good tolerability and bioavailability. In contrast to other HDAC inhibitors, VPA has demonstrated good safety profile for 35 years of use as long-term therapy for epileptic disorders. VPA is very well tolerated at millimolar concentrations and, with a serum half-life of 9 to 18 hours, has acceptable pharmacokinetic properties. For these reasons, VPA was selected as a tool to activate gene transcription.

First, the potency of VPA to initiate viral expression was evaluated using an HTLV-1 promoter-luciferase reporter assay. Viral promoter activity was found to be increased in HeLa and Jurkat cell lines following stimulation with different concentrations of VPA both in the absence or presence of ectopically expressed Tax transcriptional activator (Figure 1: basal and + Tax, respectively). Real time reverse-transcription (RT)–PCR amplifications demonstrated that VPA activates transcription of the luciferase gene (data not shown). These results thus demonstrate that VPA efficiently activates HTLV-1 promoter-driven transcription.

Since HDAC inhibitors are known to induce apoptosis, initial time course and dose response experiments were performed in an HTLV-1–infected cell line (MT2) as well as in primary PBMCs isolated from HAM/TSP or ATL patients (data not shown). A VPA concentration of 2 mM for 48 hours, found to induce moderate levels of apoptosis in HTLV-TSP PBMCs, was selected and added to primary cell cultures derived from 3 HAM/TSP patients and 3 noninfected controls. VPA induced hyperacetylation of histone H3 tails, used as an indirect marker of increased general acetylation within these cells (Figure 2A). In parallel, expression of the HTLV-1 core protein p19 was increased in the corresponding supernatant (Figure 2B). Moreover, CD4+ T lymphocytes underwent moderate rates of apoptosis (Figure 2C,D). In other words, under hyperacetylated conditions, VPA concomitantly stimulated both viral expression and apoptosis.

To assess the proapoptotic potency of VPA in CD4+ cells, the main provirus reservoir, as well as in CD8+ T lymphocytes, which are thought to be major contributors of collateral damage leading to inflammation, levels of apoptosis were evaluated concurrently in PBMCs from 11 HAM/TSP patients (Figure 3A,B) harboring different PVL levels (Figure 3C). Mean apoptotic rates measured in the CD4+ T-cell population increased in the presence of VPA (16.3 compared with 5.0 in controls; Figure 3A, Table 1); this difference was statistically highly significant (*** Wilcoxon 2-tailed signed rank test, P = .002; Table 1). In the same samples, CD8+ T lymphocytes also underwent significant apoptosis in the presence of VPA (40.7 versus 11.2 for controls; ** Wilcoxon 2-tailed signed rank test, P = .007; Table 1). Of note, VPA was also proapoptotic in the non–T-cell population as well as to lymphocytes isolated from healthy individuals (B and NI, respectively; Table 1).

Thus the ex vivo data provide compelling evidence that VPA activates viral expression and triggers apoptosis of T lymphocytes isolated from HAM/TSP patients. If our working model is valid, these mechanisms would allow the elimination of many infected cells in vivo. To assess this concept, we conducted an exploratory trial with standard doses of VPA orally administered to 4 HAM/TSP patients at late clinical stages (DSS score of 5–7.5). Serum concentrations of VPA reached steady-state levels of 45 to 93 μg/mL a few days after initiation of treatment (Table 2). Except for HAM-2 who showed a brief increase in the DSS score at day 30 (which prompted therapy interruption), no major alteration of clinical symptoms was observed during the 2-month trial period. A more sensitive parameter of ambulation, the time required to walk 8 meters, worsened transiently at day 15 most likely due to a reduction in spasticity.

PVLs reported to be very stable in HAM/TSP patients; in contrast we observed a transient increase in the PVL in all patients independent of the duration of VPA treatment (3–4 or 8 weeks; Figure 4A). However, around day 45, PVL collapsed, typically when the VPA treatment had been maintained for 2 months (HAM 3–4, Figure 4A). The increase in PVL with VPA from day 0 to 30, the decrease from day 30 to 60, and the decrease from day 0 to 60 were all significantly greater than normal fluctuations seen in PVL in 36 untreated HTLV-1–infected subjects (P = .042, P = .009, P = .027, respectively, Wilcoxon Mann-Whitney 2-tailed test; Figure 4B). This did not appear related to unusually unstable PVL in the subjects treated since the difference in PVL observed in 3 of the subjects prior to treatment with VPA (Figure 4A) was well
A

B

C

D

Figure 2. VPA induces hyperacetylation, triggers viral expression, and is proapoptotic for primary cells freshly isolated from HTLV-I–infected patients with tropical spastic paraparesis. (A) Histone H3 is hyperacetylated in the presence of VPA. Peripheral blood mononuclear cells were isolated from 3 HTLV-infected, untreated HAM/TSP patients (1-3) and 3 noninfected (NI) healthy controls (4-6) and directly cultivated (ie, without cryopreservation) for 48 hours in the absence (–) or the presence (+) of 2 mM VPA. Cell lystate protein (10 μg) was analyzed by Western blot using an antibody specific for the acetylated forms of histone H3 (Ac H3) or, as a control for normalization of the protein levels, an antiactin antisera (actin). After incubation with the appropriate alkaline phosphatase–linked conjugates, the blots were revealed by chemiluminescence and autoradiography (top 2 panels). After quantification of the luminescence signals with a luminometer and subtraction of the background, a ratio between the mean intensities generated by each antibody was calculated (Ac H3/actin ratio). (B) VPA activates expression of the viral core protein p19 in the supernatant of these primary T-lymphocyte cultures. The culture supernatals were collected, and expression of p19 was determined by real-time PCR (in pg per mL) or the presence (+) of 2 mM VPA. Apoptotic cells were identified by flow cytometry based on CD4/annexin/PI (A) or CD8/annexin/PI (B) triple labeling, and a ratio between apoptotic and live cells was calculated. (C) PVL of the 11 HAM/TSP subjects determined by real-time PCR (in number of HTLV copies per 10^6 PBMCs).

Discussion

Our findings show that treatment of HAM/TSP patients with a standard clinical dose of VPA produces a substantial decline in the PVL. Contrasting with current unsatisfactory therapies based on inhibition of inflammation, cell proliferation, or viral replication, we demonstrate the efficacy of a novel approach aimed, paradoxically, at activating viral gene expression to expose virus-positive cells to the host immune response. The basic principle of this approach is that treatment of HAM/TSP patients with a standard clinical dose of VPA produces a substantial decline in the proviral load.

Table 1. Apoptotic rates from B cells and PBMCs

<table>
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<tr>
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<th>CD4</th>
<th>CD8</th>
<th>B</th>
<th>NI</th>
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<tr>
<td>Control</td>
<td>5.0 (± 2.4)**</td>
<td>11.2 (± 10.1)**</td>
<td>4.2 (± 3.4)*</td>
<td>1.1 (± 0.4)**</td>
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<tr>
<td>VPA</td>
<td>16.3 (± 10.9)**</td>
<td>40.7 (± 37.2)**</td>
<td>10.9 (± 13.0)*</td>
<td>1.6 (± 0.5)**</td>
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The mean apoptotic rates (± standard deviation) were calculated from the data of Figure 3A (CD4+) and 3B (CD8+), from the B-cell population (B) and from noninfected (NI) PBMCs. The data were statistically compared with the Wilcoxon 2-tailed signed rank test. Note that, in contrast to the results of Figure 2, the data presented here were obtained with cryopreserved cells.

** and *** indicate levels of statistical significance.
approach is to temporarily force the latent virus out of hiding to displace the dynamic equilibrium modulating its long-term persistence and thereby decrease the PVL. For the first time, we provide evidence that VPA leads to depletion of HTLV-1–infected cells in vivo, most probably via activation of viral expression and subsequent destruction by the host immune system.

Triggering expression of viral proteins and more particularly Tax could, in principle, induce proliferation of the infected cell and indirectly increase the proviral loads. Besides, VPA at a concentration of 5 mM has recently been shown to interfere with CD8⁺ cell-mediated lysis of Tax-expressing cells ex vivo, suggesting that the efficiency of CTL surveillance of HTLV-1 might be reduced in vivo. The net outcome of these 2 processes (immune control and Tax-induced cell proliferation) could not be accurately predicted from cell culture assays. Our present data demonstrate that VPA at a pharmacologically relevant dose of approximately 1 to 3 mM effectively decreases the proviral loads in all HTLV-1–infected subjects. It is noteworthy that the PVL transiently increased in some patients (Figure 4), possibly indicating an imbalance between clonal expansion and immune surveillance in favor of the former mechanism.

Since a close relationship exists between increased PVL and clinical deterioration, a transient burst in viral replication and/or expression might temporarily exacerbate inflammation. Although long-term VPA treatment is possible, adverse effects potentially occurring during treatment (HAM-2 at day 30 [Table 2]; S.O., ongoing clinical trial, begun May 2006) could benefit from combination therapy with molecules reducing inflammation (prednisolone), viral replication (AZT/3TC), or cell proliferation (IFN-β). However, we think that VPA treatment alone would be particularly useful before irreversible CNS damage occurs, although HDAC inhibitors may harbor interesting neurologic properties. The idea is to avoid high levels of PVL in the 3% to 5% of infected patients who are evolving toward HAM/TSP. Importantly, long-term treatment (over years) with pharmacologically relevant doses of VPA as low as 20 mg/kg/day is possible, and this approach

<table>
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<th>Patient code</th>
<th>Age, y</th>
<th>Duration of TSP, y</th>
<th>Duration of VPA treatment, d</th>
<th>Seric concentration of VPA at day 15, µg/mL</th>
<th>DSS score (at day)</th>
<th>8 meters walking time (at day)</th>
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<tr>
<td>HAM-1</td>
<td>58</td>
<td>7</td>
<td>21</td>
<td>58.2</td>
<td>5.0 5.0 5.0</td>
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<td>70</td>
<td>11</td>
<td>30</td>
<td>45.9</td>
<td>6.5 7.0 6.5</td>
<td>60 106 69</td>
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<tr>
<td>HAM-3</td>
<td>55</td>
<td>19</td>
<td>60</td>
<td>93.1</td>
<td>6.5 6.5 6.5</td>
<td>19 24 21</td>
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<tr>
<td>HAM-4</td>
<td>75</td>
<td>11</td>
<td>60</td>
<td>71.4</td>
<td>7.5 7.5 7.5</td>
<td>ND ND ND</td>
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</table>

VPA was taken orally by 4 volunteers with tropical spastic paraparesis at a dose of 20 mg/kg of weight per day for 3 (HAM-1), 4 (HAM-2), or 8 (HAM-3 and HAM-4) weeks. The steady-state seric concentrations of VPA in micrograms per milliliter were measured at day 15. All HAM/TSP patients had experienced mobility impairments for 7 to 19 years, and, as indicated, their DSS scores (expanded disability status scale) were between 5 and 7.5. A more sensitive estimation of mobility was calculated based on the time (in seconds) required to walk more than 8 meters. ND indicates not determined.

Figure 4. Pilot study of valproate-based therapy in HAM/TSP patients. (A) The PVLs were determined by real-time PCR at the indicated days of the valproate treatment. The PVLs are represented in number of copies per 1000 cells. ND indicates not determined. (B) The PVLs at 4-year intervals were determined by real-time PCR in 36 HAM/TSP patients. The PVLs are represented in number of copies per 1000 cells. (C) Three HAM/TSP patients (HAM-5, HAM-6, and HAM-7) were treated with AZT + 3TC for 2 months. The PVLs were determined by real-time PCR at the indicated days and are represented in number of copies per 1000 cells.
might thus also have biologic effects directly on HAM/TSP. In this context, we should mention that VPA reduced spasticity in all patients shortly after initiation of their treatment. Our present report does not stress the long-term clinical outcome of VPA treatment in HAM/TSP. Whether the elimination of the silent population of virus will affect the course of disease is currently unknown. However, since there is a clear relationship between the evolution of the proviral loads and occurrence of HAM/TSP,34 the idea is to restrict the number of infected cells to reduce the probability of disease onset.

Although we favor an immunologic mechanism for the action of VPA, an alternative but not exclusive paradigm would be that induction of histone acetylation results in transcriptional activation of critical nonviral genes. In this context, a selective proapoptotic effect of VPA against tumor cells has recently been associated with death receptor signaling.32,33 If virus independent, VPA therapy might also be effective in other Th1 cytokine–driven inflammatory, demyelinating, neurodegenerative CNS diseases such as multiple sclerosis (MS). Indeed, recent evidence in an animal model of MS (experimental autoimmune encephalomyelitis) demonstrated that another HDAC inhibitor, trichostatin A, reduced chemotactic, pro-Th1, and proproliferative mRNAs concomitantly with clinical improvement in mice.34

In summary, we propose a novel therapeutic approach to HAM/TSP that significantly decreased proviral load and reduced muscle spasticity in all subjects. This is a proof-of-concept study for gene activation therapy for HAM/TSP using the HDAC inhibitor VPA that should spur phase 1 double-blind placebo-controlled crossover trials. After several decades of clinical use for epilepsy, this novel application for this old drug in a neurodegenerative disease further broadens its potential therapeutic use as already proposed in cancer.11,28,35

Acknowledgments

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Authorship

Contribution: A.L. and L.W. designed research and wrote the paper; N. Gillet, N. Grandvaux, O.V., G.B., and M.C.B. performed experiments; S.O., A.S., and D.S. performed the clinical trials; B.A. performed statistical analyses; J.H., A.B., and R.C. participated in analyses. R.C. and L.W. contributed equally to this study. Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

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