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Clavulanic acid does not affect convulsions in acute seizure tests in mice

Maciej Gasior · Katarzyna Socała · Dorota Nieoczym · Piotr Wlaź

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Abstract Clavulanic acid (CLAV) inhibits bacterial β-lactamases and is commonly used to aid antibiotic therapy. Prompted by the initial evidence suggestive of the potential anticonvulsant and neuroprotective properties of CLAV, the present study was undertaken to systematically evaluate its acute effects on seizure thresholds in seizure tests typically used in primary screening of potential antiepileptic drugs (AEDs). In the present study, 6-Hz seizure threshold, maximal electroshock seizure threshold (MEST) test, and intravenous pentylenetetrazole (i.v. PTZ) seizure tests were used to determine anticonvulsant effects of intraperitoneally (i.p.) administered CLAV in mice. Acute effects on motor coordination and muscle strength were assessed in the chimney and grip-strength tests, respectively. Doses of CLAV studied in the present study were either comparable or extended the doses reported in the literature to be effective against kainic acid-induced convulsions in mice or behaviorally active in rodents and monkeys. CLAV had no effect on seizure thresholds in the 6-Hz (64 ng/kg to 1 mg/kg) and MEST (64 ng/kg to 5 mg/kg) seizure tests. Similarly, CLAV had no effect on seizure thresholds for i.v. PTZ-induced myoclonic twitch, clonic convulsions, and tonic convulsions (64 ng/kg to 5 mg/kg). Finally, CLAV (64 ng/kg to 5 mg/kg) had no effect on the motor performance and muscle strength in the chimney and grip-strength tests, respectively. In summary, CLAV failed to affect seizure thresholds in three seizure tests in mice. Although the results of the present study do not support further development of CLAV as an AED, its beneficial effects in chronic epilepsy models warrant further evaluation owing to its, for example, potential neuroprotective properties.

Keywords Pentylenetetrazole · 6-Hz seizure · Maximal electroshock · Seizure threshold · Antiepileptic drugs · β-Lactamase inhibitor

M. Gasior (✉)
Clinical Research, Cephalon, Inc.,
41 Moores Road, Frazer, PA 19355, USA
e-mail: NextPharma@gmail.com

K. Socała · D. Nieoczym · P. Wlaź (✉)
Department of Animal Physiology, Institute of Biology,
Maria Curie-Skłodowska University, Akademicka 19,
20-033 Lublin, Poland
e-mail: piotr.wlaz@umcs.lublin.pl

Introduction

With the exception of vigabatrin developed specifically to increase the levels of γ-aminobutyric acid in the brain (Lippert et al. 1977) and levetiracetam's analog brivaracetam optimized for its activity at a high-affinity ligand for the synaptic vesicle protein, SV2A (Matagne et al. 2008), this rational, target-based drug discovery and optimization approach has not yet been as successful as the target-agnostic approach in bringing antiepileptic drugs (AEDs) to the patients (Gasior and Wiegand 2011). Very often, compounds effective in pre-clinical seizure tests and epilepsy models become approved drugs long before their molecular actions are discovered (e.g., gabapentin and its activity at $\alpha_2\delta$ proteins) (Gee et al. 1996).

Clavulanic acid (CLAV), a competitive inhibitor of bacterial β-lactamases which downgrade β-lactam antibiotics (Fig. 1), has been used clinically to support antibiotic therapy for over 30 years (Reading and Cole 1977; Reading et al. 1983; Payne et al. 1994). CLAV readily penetrates into the brain (Nakagawa et al. 1994) and has recently been reported to produce potent anxiolytic effects with minimal side effects in primates and rodents (Kim

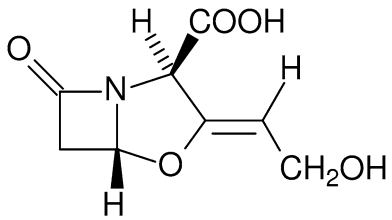

Fig. 1 Chemical structure of CLAV

et al. 2009). Of note, no significant binding of CLAV to any of the sixty-three primary molecular targets was detected to support its pharmacological effects in vivo, including targets typically implicated in mediating anti-anxiety effects (Kim et al. 2009). Further supporting the actions of CLAV within the central nervous system, which translate into measurable in vivo effects were the recent reports of its stimulatory effect on sexual behavior in rats (Chan et al. 2009) and beneficial effect on the behavioral deficit in rodent models of neurodegenerative diseases (Huh et al. 2010). The latter study also demonstrated efficacy of CLAV against kainic acid-induced convulsions and lethality in mice (Huh et al. 2010). That study, together with the reported efficacy of CLAV in a planarian assay of glutamate- and cocaine-induced convulsions, prompted the evaluation of CLAV in acute seizure assays with predictive validity of clinical efficacy and, therefore, recommended for the primary screen of AED candidates (Smith et al. 2007; Willis et al. 2009; Gasior and Wiegand 2011).

Materials and methods

Animals

Experimentally naive male Albino Swiss mice weighing 25–30 g were obtained from a commercial breeder (Laboratory Animals Breeding, Słaboszów, Poland). The animals were housed in colony cages under controlled laboratory conditions (ambient temperature, 22–23°C, relative humidity, 45–55%, 12 h light/dark cycle, lights on at 6:00 a.m.). Chow pellets (Agropol S.J., Motycz, Poland) and tap water were continuously available. The experimental protocol was approved by the Local Ethics Committee at the Medical University of Lublin (license number 46/2010), and all the procedures were in strict compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drugs

Clavulanic acid (CLAV, as potassium salt) and pentylenetetrazole (PTZ) were used in the present study. All the compounds were obtained from Sigma-Aldrich (Poznań, Poland). CLAV was dissolved in saline and administered intraperitoneally (i.p.) in a volume of 10 ml/kg. PTZ was dissolved in saline and administered intravenously (i.v.). Pretreatment time for CLAV (60 min) was selected to correspond to the time to achieve maximal in vivo effect as confirmed by literature search and in pilot studies.

Psychomotor (6-Hz) seizure threshold test

Psychomotor seizures (6-Hz seizures) were induced by applying square-wave alternating current (frequency, 6 Hz; duration, 3 s) via corneal electrodes delivered from a Grass S48 stimulator (Grass Technologies, West Warwick, RI, USA). Ocular anesthetic, 1% solution of lidocaine hydrochloride (Abbott, Abbott Park, IL, USA), was applied onto animals' corneas 1 min before stimulation and 0.9% saline was used to wet electrodes immediately before testing to ensure good electrical contact. Each mouse was manually restrained during stimulation and placed in a Plexiglas arena (35 cm × 20 cm × 14 cm) for behavioral observation immediately following the stimulation. Convulsions were characterized by a stunned (fixed) posture, which was often followed by rearing, forelimb clonus, and twitching (Barton et al. 2001; Giardina and Gasior 2009). At the end of the convulsions, the animals resumed normal exploratory behavior. Control animals always exhibited more than 10 s of abnormal behavior whereas treated animals were considered to be protected if the abnormal behavior was less than 10 s in duration.

To determine the threshold for 6-Hz seizures, groups of 19–20 mice were stimulated with different current intensities according to an 'up-and-down' method (Kimball et al. 1957; Giardina and Gasior 2009). Each mouse was stimulated only once at any given current intensity and the presence or absence of seizure activity was judged as described above. If the mouse responded with convulsions (as described above), the next mouse was stimulated with a current of an intensity 0.06-log step lower than the previous one. If, however, the mouse did not respond with convulsions, the next mouse was stimulated with a current of an intensity 0.06-log step higher than the previous one. After all mice from a given treatment group were tested, the median current strength (CS_{50} in mA) with corresponding standard error of the mean (SEM) was calculated as described elsewhere (Kimball et al. 1957; Giardina and Gasior 2009). Each CS_{50} represented a current strength of the 6-Hz stimulation that was predicted to induce seizures in 50% of the animals tested.

Maximal electroshock seizure threshold (MEST) test

Generalized tonic-clonic convulsions were induced by applying a sine-wave alternating current (maximal output

voltage 500 V, 50 Hz for 0.2 s) via corneal electrodes from a rodent shocker (type 221; Hugo Sachs Elektronik, Freiburg, Germany). Sterile saline was used to wet electrodes immediately before testing to ensure good electrical contact. Mice were manually restrained during stimulation and immediately after the stimulation. Tonic hind limb extension (tonus; i.e., the hind limbs of animals out-stretched 180° to the plane of the body axis), which typically occurred within less than 10 s post stimulation, was taken as the behavioral endpoint reflecting seizure activity (Löscher et al. 1991; Giardina and Gasior 2009). The electrical system of the stimulator was self-adjustable so that the changes in impedance did not result in the alterations of current intensity (i.e., the system provided constant current stimulation).

The threshold for maximal electroconvulsions was determined using the same methodological principles as described earlier for the "up-and-down" method in the case of the 6-Hz seizure threshold test (Kimball et al. 1957; Giardina and Gasior 2009) except for employing different behavioral endpoint of seizure activity (tonus vs. psychomotor seizures) and specific parameters of electrical stimulation such as the shape (sine vs. square), frequency (50 vs. 6 Hz), and duration (0.2 vs. 3 s) of the stimulating current between the maximal electroconvulsions and 6-Hz stimulation, respectively. Seizure threshold in the MEST test, as described earlier, was expressed as the median current strength (CS_{50} in mA) that was predicted to induce seizures in 50% of the animals tested; corresponding SEM was calculated for statistical analysis (Kimball et al. 1957; Giardina and Gasior 2009).

Intravenous PTZ seizure threshold test

Mice were placed in a cylindrical plastic restrainer (12-cm long, 3-cm inner diameter) with a plunger for restraint. The lateral tail vein was catheterized with a 2-cm long 27-gauge needle attached to a 25-cm length of polyethylene tubing PE20RW (Plastics One Inc., Roanoke, VA, USA). After correct needle placement into the tail vein, which was verified by the appearance of blood in the tubing, the needle was secured gently to the tail using adhesive tape. The tubing was attached to a 5-ml plastic syringe containing PTZ solution. The syringe was mounted on an infusion pump (model Physio 22, Hugo Sachs Elektronik–Harvard Apparatus GmbH, March-Hugstetten, Germany). Following catheterization, the mice were released from the restrainer and placed in a Plexiglas arena for behavioral observation during the infusion. PTZ (10 mg/ml) was infused at a rate of 0.2 ml/min. These infusion parameters were determined to be optimal for the reliable assessment of behavioral manifestation of i.v. PTZ-induced seizures. Specifically, three sequentially-occurring behavioral endpoints of seizure activity were used to determine the threshold for seizure induction: (1) first myoclonic twitch (rapid upward flick of rigid tail), (2) clonus (repeated jerking movements of all four limbs lasting at least 5 s) with loss of the righting reflex (clonic convulsions, or clonus), and (3) tonic forelimb extension (tonic convulsions, or tonus). The times between the start of the infusion and the onset of these endpoints were recorded and used to calculate seizure thresholds for each endpoint separately using the following formula: threshold dose of PTZ (mg/kg) = (PTZ concentration (mg/ml) × infusion rate (ml/s) × infusion duration (s) × 1000)/body weight (g). Seizure threshold was expressed as a mean dose (±SEM) of PTZ (in mg/kg) needed to produce a given endpoint as noted above. The infusion was stopped at the beginning of the tonic convulsions, which was usually lethal. All surviving animals were euthanized immediately after the end of the infusion.

Grip test

The acute effect of a range of doses of CLAV on muscular strength was quantified by the grip-strength test as described elsewhere (Nieoczym et al. 2010). The pretreatment time for CLAV in the grip-strength test matched that for seizure tests. The grip-strength apparatus (BioSeb, Chaville, France) comprised a steel wire grid (8 × 8 cm) connected to an isometric force transducer. The mice were lifted by the tails so that they could grasp the grid with their forepaws. The mice were then gently pulled backward by the tail until they released the grid. The maximal force (in newtons, N) exerted by the mouse before losing the grip was recorded. Three consecutive measurements were collected and their means were calculated for each animal. Then, the mean maximal force (±SEM) for the treatment group was calculated.

Chimney test

The chimney test was used to assess the acute adverse effects of a range of doses of CLAV on motor performance (Boissier et al. 1960). The pretreatment time for CLAV matched that for seizure tests. In this test, the inability of an animal to climb backward up through a Plexiglas tube (3 cm, inner diameter × 30 cm, length) within 60 s was an indication of motor impairment. Eight mice per treatment were subjected to this testing procedure.

Statistical analysis

Threshold values obtained in the i.v. PTZ seizure test are expressed as group means ±SEM and represent doses of PTZ (in mg/kg i.v.) necessary to induce myoclonic twitch,

clonus, or tonus. An "up-and-down" method was used to calculate CS_{50} values, ±SEM, and 95% confidence intervals (CI) in the 6-Hz threshold and MEST tests (Kimball et al. 1957; Giardina and Gasior 2009). Except for the categorical data from the chimney test (the Fisher's exact test), all the other experimental data from pharmacodynamic assays were statistically analyzed with either Student's *t* test or one-way analysis of variance (ANOVA) followed by Dunnett's test for specific post-hoc comparisons, where appropriate. Differences were considered statistically significant at $p < 0.05$. All statistical tests were conducted using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Effects of CLAV on seizure threshold in the 6-Hz test

The control CC_{50} value for psychomotor seizures was 17.4 ± 1.0 (CI 16.7–18.1) mA in the 6-Hz seizure test. CLAV (64 ng/kg to 1 mg/kg) had no effect on the seizure threshold in the 6-Hz test ($F(6, 61) = 1.343$, $p = 0.252$, Fig. 2).

Effects of CLAV on seizure threshold in the MEST test

The effect of CLAV on seizure threshold for generalized tonus in the MEST test was tested in two separate experiments at doses ranging from 64 ng/kg to 40 µg/kg (experiment 1) and from 200 µg to 5 mg/kg (experiment 2). Each experiment included its own well-powered control group treated with vehicle. Effects of CLAV versus the matching control group were analyzed separately in each experiment in order to avoid creating an unbalanced number of mice in the control group if both control groups would have been combined into one control group.

The control CC_{50} value for generalized tonus was 7.9 ± 1.0 (CI 7.2–8.7) mA and 9.3 ± 1.0 (CI 8.5–10.2) mA in experiment 1 and 2, respectively ($p = 0.0196$; *t* test, Fig. 2). CLAV (64 ng/kg to 5 mg/kg) had no effect on the seizure threshold in the MEST test (experiment 1: $F(3, 32) = 1, 492$, $p = 0.236$; experiment 2: $F(3, 33) = 0.458$, $p = 0.714$).

Effects of CLAV on seizure threshold in the i.v. PTZ test

The effect of CLAV on seizure threshold for the onset of myoclonic twitches, generalized clonus, and generalized tonus in the i.v. PTZ test (Fig. 3) was tested at doses ranging from 64 ng/kg to 40 mg/kg (experiment 1) and from 0.2 to 5 mg/kg (experiment 2). As in the MEST test (see above), each experiment included its own control group treated with vehicle and statistical analyses were performed separately for each experiment.

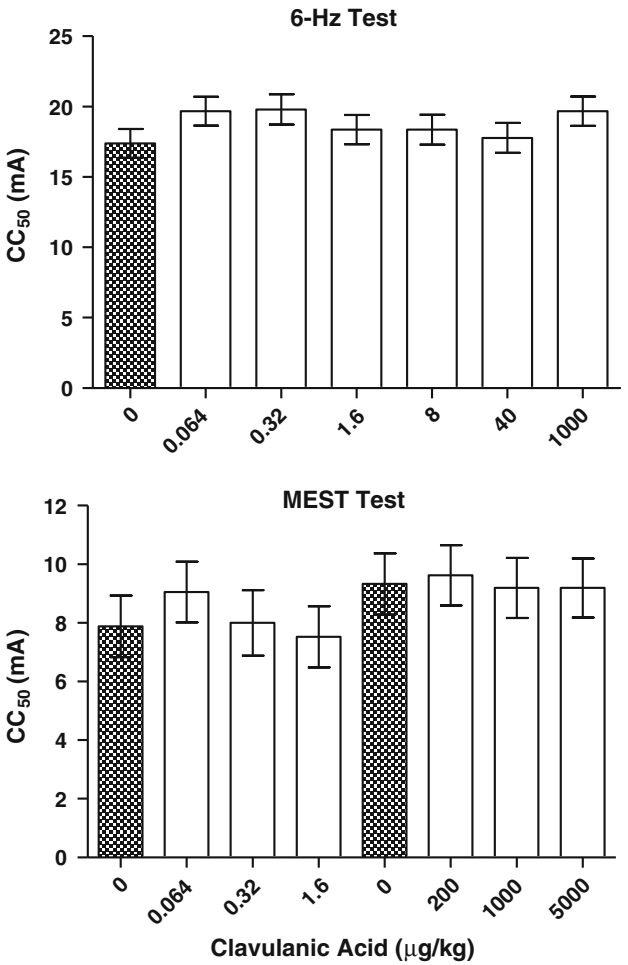

Fig. 2 Effects of CLAV on seizure thresholds in the 6-Hz and MEST seizure tests. CLAV was administered at doses ranging from 64 ng/kg to 5 mg/kg (i.p.) 60 min before seizure testing (*open bars*); control groups received saline instead of CLAV (*shaded bars*). Each group consisted of 19–20 animals. Data are presented as CC_{50} values (±SEM) in mA. Each CC_{50} value represents current intensity predicted to produce convulsions in 50% of mice tested at a given dose of CLAV (Kimball et al. 1957)

In vehicle-treated control group, the PTZ thresholds for the onset of myoclonic twitches were 40.7 ± 1.9 and 40.4 ± 1.3 mg/kg ($p > 0.05$; *t* test), for generalized clonus were 52.7 ± 2.6 and 47.7 ± 1.7 mg/kg ($p > 0.05$; *t* test), and for generalized tonus were 97.5 ± 6.4 and 100.1 ± 7.7 mg/kg ($p > 0.05$; *t* test) in experiments 1 and 2, respectively. CLAV had no effect on the threshold for the onset of myoclonic twitches (experiment 1: $F(5, 65) = 0.725$, $p = 0.202$; experiment 2: $F(3, 51) = 0.506$, $p = 0.680$), generalized clonus (experiment 1: $F(5, 65) = 2.109$, $p = 0.075$; experiment 2: $F(3, 51) = 0.506$,

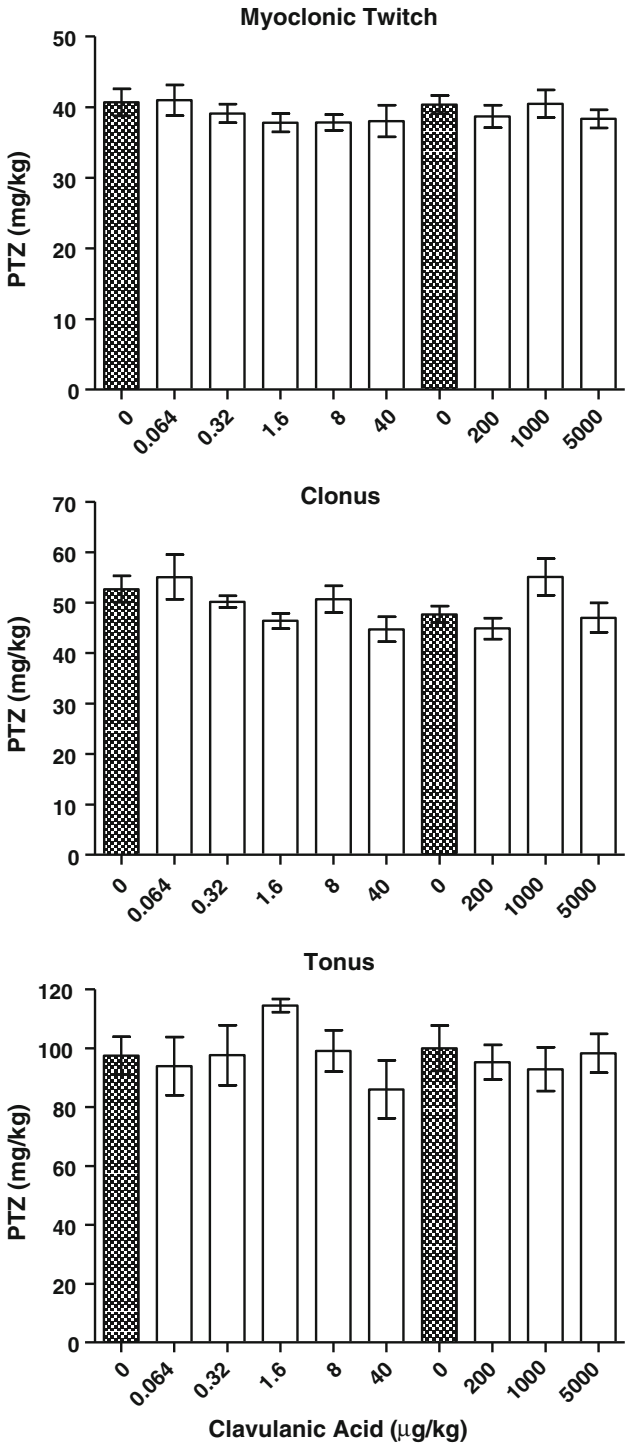

Fig. 3 Effect of CLAV on the threshold for the onset of first myoclonic twitch, clonus, and tonus in the i.v. PTZ seizure threshold test in mice. Each group consisted of 10–14 animals. Data are presented as means (±SEM) in mg/kg of i.v. PTZ necessary to induce each of the three behavioral endpoints of the i.v. PTZ seizure test (Giardina and Gasior 2009)

$p = 0.052$), and generalized tonus (experiment 1: $F(5, 64) = 1.368$, $p = 0.248$; experiment 2: $F(3, 51) = 0.209$, $p = 0.889$) (Fig. 3).

Effects of CLAV on motor performance and muscle strength in the chimney test and grip strength tests, respectively

CLAV (64 ng/kg to 5 mg/kg) had no behavioral effect in the chimney test and grip strength tests ($p > 0.005$; data not shown).

Discussion

Two studies, one in rats (Huh et al. 2010) and one in planarians (Rawls et al. 2010), provided the initial evidence suggestive of the anticonvulsant properties of CLAV and its potential of becoming a novel anticonvulsant/neuroprotective agent. CLAV was tested in the present study at comparable and extended ranges of doses (64 ng/kg to 5 mg/kg, i.p.) demonstrated to be anticonvulsant and neuroprotective against seizures and the death of hippocampal neurons induced by kainic acid in rats (0.01 mg/kg, i.p.), neuroprotective in a model of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced loss of dopaminergic neurons in mice (0.1 mg/kg, i.p.), or efficacious in behavioral assays in rats (0.001 mg/kg, i.p.; 0.01–1.0 mg/kg, p.o.), Syrian golden hamsters (10 ng/kg to 1 µg/kg, i.p.), or cotton-top tamarind monkeys (1 mg/kg, i.p.) (Kim et al. 2009; Chan et al. 2009; Huh et al. 2010). Nevertheless, CLAV showed no acute anticonvulsant properties in three seizure tests employed in the present study. Although specific pharmacokinetic parameters of CLAV in Albino Swiss mice have not been studied, the fact that its CNS-mediated pharmacological effects in vivo were evident across a variety of species (including a number of rodent species) would suggest that the failure of CLAV to affect seizure sensitivity in the present study was not due to its limited penetration into the CNS in Swiss Albino mice.

Of note, there is not a single approved or under-development AED that would not show efficacy in at least one of the tests employed in the present study (Smith et al. 2007; Giardina and Gasior 2009; Bialer et al. 2010). Thus, the results of the present study do not generally support further consideration of CLAV as an AED. However, the possibility still exists that testing of CLAV after chronic exposure might uncover its anticonvulsant effects as was the case for some of its behavioral effects (Chan et al. 2009). Even if not by directly affecting seizure susceptibility, there still is a potential that the proposed neuroprotective properties of CLAV (Huh et al. 2010) might benefit, for example, the treatment of temporal lobe epilepsy. Thus, further comprehensive testing of CLAV in seizure tests relevant to specific molecular mechanisms (e.g., acute seizure tests employing convulsant agents with specific pharmacological actions for further differentiation of

anticonvulsant properties) and/or epilepsy models involving mechanism resulting, among others, from neurodegenerative processes is warranted (Stafstrom and Sutula 2005; Acharya et al. 2008; Gasior and Wiegand 2011; Löscher 2011).

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Conflict of interest We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. M. Gasior is a full-time employee of Cephalon, Inc. at the time of this submission, but this work was not supported by Cephalon, Inc. The authors declare no conflict of interest.

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