

Bryostatin Effects on Cognitive Function and PKC ϵ in Alzheimer's Disease Phase IIa and Expanded Access Trials

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Abstract. Bryostatin 1, a potent activator of protein kinase C epsilon (PKC ϵ), has been shown to reverse synaptic loss and facilitate synaptic maturation in animal models of Alzheimer's disease (AD), Fragile X, stroke, and other neurological disorders. In a single-dose (25 $\mu\text{g}/\text{m}^2$) randomized double-blind Phase IIa clinical trial, bryostatin levels reached a maximum at 1–2 h after the start of infusion. In close parallel with peak blood levels of bryostatin, an increase of PBMC PKC ϵ was measured ($p = 0.0185$) within 1 h from the onset of infusion. Of 9 patients with a clinical diagnosis of AD, of which 6 received drug and 3 received vehicle within a double-blind protocol, bryostatin increased the Mini-Mental State Examination (MMSE) score by $+1.83 \pm 0.70$ unit at 3 h versus -1.00 ± 1.53 unit for placebo. Bryostatin was well tolerated in these AD patients and no drug-related adverse events were reported. The 25 $\mu\text{g}/\text{m}^2$ administered dose was based on prior clinical experience with three Expanded Access advanced AD patients treated with bryostatin, in which return of major functions such as swallowing, vocalization, and word recognition were noted. In one Expanded Access patient trial, elevated PKC ϵ levels closely tracked cognitive benefits in the first 24 weeks as measured by MMSE and ADCS-ADL psychometrics. Pre-clinical mouse studies showed effective activation of PKC ϵ and increased levels of BDNF and PSD-95. Together, these Phase IIa, Expanded Access, and pre-clinical results provide initial encouragement for bryostatin 1 as a potential treatment for AD.

Keywords: Alzheimer's disease, bryostatin 1, controlled clinical trial, expanded access trials, pharmacokinetics, protein kinase C

INTRODUCTION

In addition to amyloid plaques and neurofibrillary tangles, an important pathologic hallmark of Alzheimer's disease (AD) is synaptic loss, which results in cognitive dysfunction and memory loss. In a recent study, protein kinase C isozyme epsilon (PKC ϵ) was found to be deficient in fresh frozen hippocampal brain samples from AD versus control patients [1]. PKC ϵ is a signaling enzyme known to be involved in learning and memory [2] and

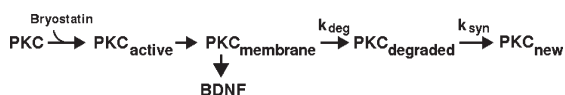
participates in synaptic growth and synaptogenesis [3, 4]. PKC ϵ activators such as bryostatin 1 and DCPLA methyl ester are capable of preventing and/or reversing synaptic loss in various disease models including aged rats [5], AD transgenic mice [6], and rats subjected to neuronal and synaptic loss from ischemia/hypoxia [7, 8]. They elevate BDNF and facilitate synaptic maturation [9] even in normal animals during learning and memory [3]. If these compounds have similar effects in humans, they could be of value for treating the synaptic loss in AD and other neurodegenerative disorders such as Fragile X mental retardation, stroke, and traumatic brain injury, thereby ameliorating loss of memory, restoring other cognitive functions, and preserving the quality of life.

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However, little is known about the safety, pharmacokinetics, or efficacy of bryostatin 1 in AD patients.

Bryostatin 1 activates PKC ϵ by binding to the C1A and C1B domains of conventional and novel isoforms of PKC [10–12], which bind the natural ligand 1,2-diacylglycerol (DAG) [13]. Bryostatin 1 has highest affinity for PKC α and ϵ [14–16]. Like DAG, bryostatin 1 produces a time-dependent biphasic effect on PKC ϵ beginning with an initial activation accompanied by membrane translocation [17, 18]. Once activated, PKC ϵ is proteolytically degraded in a process called downregulation [19], which lasts up to 24 h, followed by *de novo* synthesis that restores normal levels [20].

PKC ϵ activation is an obligatory step prior to downregulation [21, 22]. Thus, downregulation can serve as an indicator of earlier PKC ϵ activation, as the scheme here shows:



Bryostatin 1 has been extensively tested clinically as a treatment for cancer [23–35], for which it was considered as a PKC inhibitor due to its ability to downregulate PKC. Downregulation is particularly significant at higher doses of bryostatin, e.g., >30 $\mu\text{g}/\text{m}^2/\text{week}$. Downregulation may potentially complicate the therapeutic use of bryostatin 1 because it could produce effects that are different or even opposite from activation. Therefore, it is important to optimize clinical trial protocols to maximize the lower dose biochemical effect of PKC ϵ activation. Here, we report on initial human clinical trials of bryostatin 1 in AD including an initial Phase IIa safety and tolerability study and Expanded Access trials. In addition to activation, we demonstrate here in AD patients that bryostatin also increases the levels of PKC ϵ (see below) that are associated with cognitive improvement. Results are also included from pre-clinical testing of bryostatin 1 in mice from which blood-brain distribution parameters, pharmacokinetics, and dose-dependent mechanism(s) of action guided the clinical trial protocols described here.

MATERIALS AND METHODS

Drug manufacturing and administration

Bryostatin 1 for animal experiments and biochemical testing was obtained from Tocris. Bryostatin 1

for clinical trials was generously provided by the National Cancer Institute. Sterile injectable bryostatin 1 and diluent were manufactured by Lyophilization Technology, Inc., Ivyland, PA. Bryostatin 1 was produced as a sterile lyophilized cake in 10 ml lyophilized sterile vials containing 50 μg bryostatin 1 and 2.5 mg Povidone C17, USP. Placebo was prepared identically except that bryostatin 1 was omitted. Sterile PET Diluent was made in 10 ml sterile vials and contained 1 ml solution of polyethylene glycol 400 (60%), dehydrated ethanol, USP (30%), and Polysorbate 80, USP (10%), v/v. Povidone C17 was a generous gift of Ashland Chemical Co., Wayne, NJ. Pure bryostatin 1 was stored at -20°C . The bryostatin 1, placebo, and PET diluent drug product were stored in controlled access chambers at +2 to $+8^\circ\text{C}$. The identity, purity, and stability of the drug substance and drug product were confirmed by mass spectrometry [36], HPLC, UV spectroscopy, and IR spectroscopy at 6-month intervals. The identity, ethanol content, and viscosity of the PET diluent were also confirmed at 6-month intervals. Moisture content was measured at 6-month intervals by pulsed coulometric KF titration using Hydranal Coulomat AK (Sigma-Aldrich) in a Mettler Toledo C20 titrator. Sterility and endotoxin content were confirmed every 6 months by Alcami, Wilmington, NC.

Bryostatin 1 or placebo was administered by dissolving the contents of one vial in 1 ml PET diluent, followed by dilution with 9 ml of 0.9% sodium chloride injection, USP. This solution was then injected into a 250-ml Baxter non-DEHP-non-PVC Intravia polyolefin-lined saline IV bag. Hospira polyethylene lined tubing was used. The bryostatin 1 was administered to the EA patient by drip infusion over a period of 1 h. PVC tubing and bags were avoided because previous research showed that bryostatin 1 could bind to PVC.

Stability tests showed that the bryostatin 1 concentration thus prepared was stable at room temperature in PVC-free infusion bags for at least 21 days. Bryostatin was stable in glass vials containing 9 ml saline for at least 10 days at room temperature. Aqueous solutions of bryostatin were found to bind to PVC containers but not to polypropylene or glass. Bryostatin dissolved as 10% solutions in ethanol or DMSO was stable for at least 30 days in glass containers.

Phase IIa trial: Inclusion and exclusion criteria

Patients over 50 years of age with Mini-Mental State Examination (MMSE) score of 12–26 and a

diagnosis of AD supported by CT or MRI scan, living outside an institution, but with a caregiver were considered eligible. Exclusion criteria included dementia due to any condition other than AD, including vascular dementia (modified Hachinski Ischemic Scale ≥ 5), cerebral tumor, Huntington's disease, Parkinson's disease, major depression, psychotic episodes, agitation, epilepsy, uncompensated congestive heart failure, pregnancy, lack of caregiver, or alcohol or drug dependence; evidence of clinically significant unstable cardiovascular, renal, hepatic, gastrointestinal, neurological, or metabolic disease within the past 6 months; change in use of any drug within 14 days prior to randomization; any medical or psychiatric condition that may have required medication or surgical treatment during the study; life expectancy less than 6 months; any clinically significant screening laboratory values outside the normal; use of an investigational drug within 30 days prior to the screening visit or during the entire study; or blood pressure $>180/100$.

Efficacy and safety endpoints

The primary objective was an evaluation of the safety and tolerability of bryostatin. Secondary objectives were a preliminary assessment of pharmacokinetics and effects on cognitive function. Behavioral outcome measures were MMSE, Alzheimer's Disease Assessment Scale-Cognitive (ADAS-cog), Clinician's Interview-Based Impression of Change-plus (CIBIC-plus), Clinical Dementia Rating (CDR), Severe Impairment Battery (SIB), Hopkins Verbal Learning Test Revised (HVLT-R), and Alzheimer's disease Cooperative Study Activities of Daily Living (ADCS-ADL) at 24, 48, and 72 h post dose. Subjects were given baseline EKG, CBC, hematology, HIV/Hepatitis B screening and urinalysis for blood alcohol, drug and pregnancy screening. Vital signs were monitored at baseline and at intervals up to 24 h post infusion. A follow up examination was conducted four weeks after treatment. Blood was drawn for PKC within 30 min prior to infusion and at 15, 30, and 60 min after start of infusion, and 20 min, 1, 2, 6, 24, 48, and 72 h post infusion. Peripheral blood mononuclear cells (PBMCs) were isolated from blood on site and shipped on dry ice to our laboratory for analysis within 48 h. Blood was also drawn at the same time points and shipped to a commercial GLP lab for bryostatin 1 pharmacokinetic measurement.

Human clinical trials

The Single Patient Expanded Access study (BRY-SPEA-JM) and the Phase IIa clinical trial (NTRP101-201) were conducted under a U.S. IND (IND 71,276) and received approval from the relevant Institutional Review Boards. Expanded access patient #2 was treated at Marshall University, Charleston, WV. Expanded access patient #3 was treated at Via Christi Hospitals, Wichita, KS. The Phase IIa trial was conducted by the CRO Parexel at Glendale Adventist Hospital, Glendale, CA under the supervision of our corporate partner, Neurotrope BioScience, Inc. Human studies were performed in compliance with the protocol and in accordance with Good Clinical Practice (GCP) (International Conference on Harmonization [ICH], Guidance E6, 1996, principles of human subject protection), and applicable country-specific regulatory requirements.

Subjects

The Phase II safety study (*Identifier NCT02221947*) included nine subjects (4 male and 5 female) ranging in age from 62 to 82 with a clinical diagnosis of probable AD. The mean age was 71.8 ± 7.4 . MMSE was measured 3 h, 4 days, and 15 days after infusion; however, based on earlier animal studies [37], only short-term improvements in cognition from a single dosing were expected.

Bryostatin 1 administration in mice

Bryostatin 1 (Tocris, 10 $\mu\text{g}/\text{vial}$) was dissolved in 10 μl dimethyl sulfoxide (DMSO) and diluted to 100 μl in sterile normal saline. We administered bryostatin 1 to groups of 3 to 6 male C57BL/6N mice through the lateral tail vein and samples were collected after a delay ranging from 1 h to 28 d. The injection schedules and dosages are shown in Table 1. Twelve control groups of 3 or 4 mice each, injected with vehicle on the same days as the treated groups, were also used. All animal studies were conducted in accordance with AAALAC guidelines.

Mouse assays

PSD-95, pPSD-95, and synaptophysin were measured by western blotting. We also measured brain BDNF, pro BDNF, and neuron-specific enolase (NSE) in unfractionated brain extracts using

Table 1
Preclinical mouse dosing schedule

Group Name	Dose $\mu\text{g}/\text{m}^2$	Group ID		Week					
		1 h Delay	24 h Delay	1	2	3	4	5	6
Weekly 10 $\mu\text{g}/\text{m}^2$	10	7	6	✓	✓	✓X			
	10		8	✓	✓	✓	✓	✓	✓X
Weekly 15 $\mu\text{g}/\text{m}^2$	15	17	18	✓	✓	✓X			
	Weekly 25 $\mu\text{g}/\text{m}^2$	25	21	22	✓X				
25		23	24	✓	✓X				
25		25	4	✓	✓	✓X			
Semiweekly	15		39	✓✓	✓✓X				
	20		40	✓✓	✓✓X				

Injection schedule of bryostatin in preclinical mouse experiment. Bryostatin 1 was injected into the tail vein of 3 to 6 male C57BL/6N mice once or twice per week at the indicated dosage. At the specified delay after the last injection, blood PBMCs and brain were collected and PKC ϵ , BDNF, and PSD-95 were measured as described in Methods. Equal numbers of control animals were injected with vehicle on the same schedule. ✓ = Injection of bryostatin at the indicated dose or a vehicle control. X = Collection date at specified delay (1 or 24 h) after last injection. N = 3 to 6 animals/group.

commercial ELISA kits (Abnova, Abnova, and MyBioSource, respectively).

PKC ϵ measurement

PBMCs were isolated from blood in animal experiments by centrifugation in Ficoll-Paque (GE Healthcare) in 12 ml Accuspin tubes (Sigma-Aldrich). PBMCs were isolated in the clinical experiments from blood drawn into 8 ml Vacutainer Cell Preparation Tubes (CPT Tubes) containing sodium heparin and Ficoll (BD, red/green) and crude PBMCs were isolated according to the manufacturer's instructions. Due to the lability of PKC, only a single step of purification was performed. PBMCs were stored at -70 to -80°C for up to 72 h and homogenized by sonication in 330 μl PBS and 10 μl were taken for protein measurement. Brain tissue was homogenized by sonication in 3 volumes of ice-cold $1\times$ phosphate-buffered saline (pH 7.4) using a 1/16" microprobe and Misonix S-4000 ultrasonic sonicator set at amplitude = 10% (32 μm) for 30 s (approx. 113 joules).

A portion of the homogenate was fractionated by ultracentrifugation (10 min, 100000 \times g, Beckman Max-XP) for PKC ϵ measurement. The cytosol fraction (320 μl in PBS) was put on ice and the particulate fraction was re-suspended to its original volume (320 μl) by adding cold PBS + 1% Tween-20 and sonicating on ice for 5 s. Because PKC is very labile, samples were not processed further but applied immediately to isozyme-specific ELISA plates (USCNK Life Science, Inc.) which had been coated with flat black paint on the outside to minimize cross-talk. Each measurement was done with 4 or 8 replicates. Samples were diluted with appropriate buffer to make the final Tween-20 concentrations the same.

Plates were processed according to the directions of the manufacturer except that the plates were washed five times and they were centrifuged in an Eppendorf 5804 refrigerated centrifuge with an A-2-DWP swinging bucket rotor for 10 min at 2250 \times g after each wash. Plates were developed with Thermo Supersignal Femto reagent and chemiluminescence was recorded in a BioTek Synergy HT microplate reader. The microplate reader was calibrated weekly using a luminescence test plate (BioTek). Subcellular localization of PKC ϵ in brain was calculated as the ratio of PKC ϵ in membrane / (membrane + cytosol) fractions. All measurements were compared with control mice injected over the same period of time with vehicle only. The PKC ϵ method was validated according to FDA Guidance document on Bioanalytical Method Validation (2001).

Bryostatin measurement

Bryostatin 1 in the EA subject was measured by LCMS using a Thermo/Dionex NCP-3200RS gradient nanopump attached to an Acclaim Pepmap 100 180 $\mu\text{m} \times 5$ cm C18 3 μm capillary column (100 Å particle size) at a flow rate of 1.5 $\mu\text{l}/\text{min}$. Samples were passed into a Nanospray Flex Ion ESI source and analyzed with a Thermo TSQ triple-quadrupole mass spectrometer in SRM mode as previously described [36]. Bryostatin in the Phase IIa samples was measured by a commercial cGLP laboratory using the method of Zhao et al. [38].

Statistical analysis

P-values were calculated using Student's *t* test. PKC ϵ ratio values were calculated using standard

error propagation techniques. Pharmacokinetic half-life parameters were calculated by nonlinear least-squares analysis using custom software (Xdata). Other statistics, including power analysis, were calculated in R. ANOVAs were calculated using R and followed up with Tukey HSD multiple comparisons of means (95% family-wise confidence level). One-way robust repeated measures ANOVAs were calculated by the method of Wilcox [39].

RESULTS

Human Phase IIa clinical trial

We conducted a parallel randomized double-blind Phase IIa trial with 6 subjects receiving a single i.v. dose of bryostatin 1 at $25 \mu\text{g}/\text{m}^2$ and 3 subjects receiving a placebo. The primary objective was an evaluation of the safety and tolerability of bryostatin 1. The secondary objective was a preliminary assessment of pharmacokinetics, pharmacodynamics, and effects on cognitive function in patients with AD.

No deaths or SAEs occurred and no subject had an AE leading to withdrawal from the study. No myalgia was reported. Three subjects experienced minor adverse events (headache, dizziness, or rash). The headache occurred in a bryostatin patient and was not considered related to the drug. All hematology, blood chemistry, coagulation, renal function, liver function, and cardiac assessments were unremarkable after treatment. There were no clinically significant changes in any vital signs.

Bryostatin reached peak plateau level within 1 h for all 6 patients infused with the drug (Fig. 1). Bryostatin 1 treatment increased MMSE score at 3 h after the end of infusion ($p=0.041$, paired t -test; Table 2, Fig. 2) but not at later times (not shown). MMSE scores of the placebos at 3 h were not statistically significant from baseline values. Although the change in the treated group at 3 h was statistically significant, considerably larger numbers of subjects would be needed to draw definitive conclusions about efficacy. Based on earlier animal studies [37], only short-term improvements in cognition from a single dosing were expected. Other tests (ADAS-cog, CIBIC-plus, CDR, SIB, HVLT-R, and ADCS-ADL) were either hampered by incomplete data or showed no statistically significant change in cognitive level.

Based on the 1 h bryostatin results, we analyzed PBMC PKC ϵ levels at 1 h. Bryostatin 1 treatment increased PBMC PKC ϵ levels at 1 h ($p=0.0185$, repeated measures ANOVA, Fig. 3a) followed by

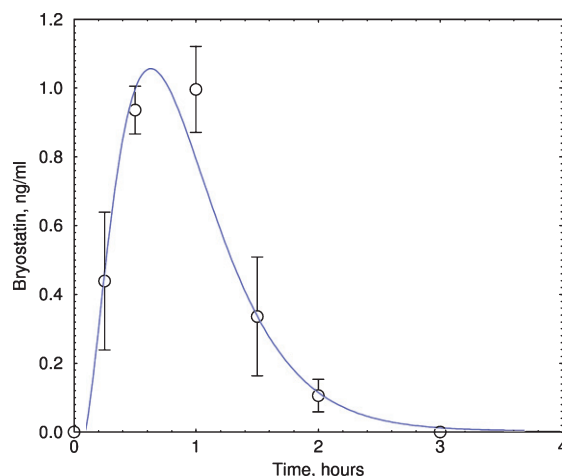


Fig. 1. Bryostatin elevation in Phase IIa subjects. Plasma bryostatin was measured by LCMS. All 6 bryostatin-infused (1-h infusion of $25 \mu\text{g}/\text{m}^2$, single dose) subjects showed peak elevation of bryostatin at 1-2 h after the start of infusion. Y-axis = ng bryostatin / ml. Each data point represents mean \pm SEM of six patients. Only samples from bryostatin-treated patients were measured.

Table 2
MMSE scores

	Baseline	3 h Post	Δ MMSE at 3 h
Placebo ($n=3$)	22.00 ± 1.53	21.00 ± 2.00	-1.00 ± 1.53
Bryostatin ($n=6$)	21.33 ± 1.50	23.16 ± 1.89	$+1.83 \pm 0.70$

Bryostatin Efficacy on Cognitive Function in blinded Phase IIa trial. A single i.v. infusion was followed 3 h, 4 d, and 15 d after infusion by a cognitive evaluation with the Mini-Mental State Examination-2 (MMSE-2). MMSE scores in bryostatin-treated subjects were increased at 3 h ($p=0.041$, paired t -test). MMSE scores of the treated groups at other times or of the placebos at 3 h were not significantly different from the corresponding baseline values.

a long-term downregulation between 12 and 72 h ($p=0.0296$, two-tailed paired t -test, Fig. 3b). PKC ϵ values in PBMC samples were fairly reproducible from a given patient over time, but PBMCs from different patients had widely different PKC values. Thus the differences in $t=0$ values are not significant and the relatively large error bars shown in Fig. 3 are the result of large patient-to-patient variation. The highest PKC ϵ level occurred 1 h post infusion, in close correspondence to the peak levels of bryostatin measured in the patient's blood samples. Based on the preclinical data described below, the dose chosen, $25 \mu\text{g}/\text{m}^2$ i.v., was predicted to be sufficient to activate PKC ϵ and induce increased synthesis of synaptogenic factors such as BDNF and PSD-95 (See *Preclinical Experiments* below).

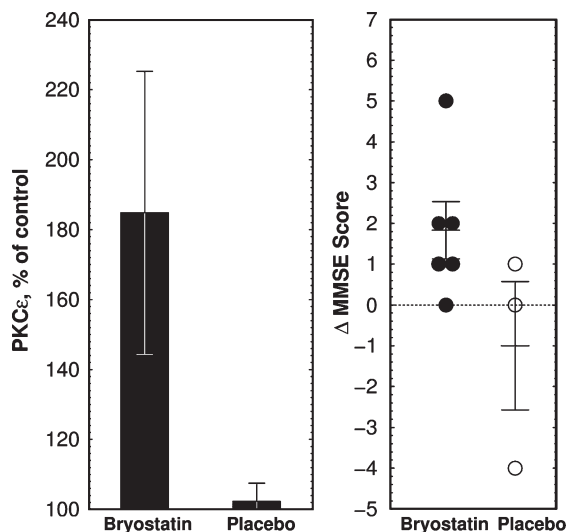


Fig. 2. Clinical trial outcome of bryostatin 1 treatment in Phase IIa AD trial. A) Mean PKC ϵ changes above preinfusion level at 1 h measured in PBMCs collected from 6 bryostatin 1 and 3 placebo-treated patients. Note expanded y-axis. PKC ϵ values are percent of blank-subtracted control in chemiluminescence units (mean \pm SEM). B) Changes in MMSE scores. MMSE is scored in discrete integers on a scale from 0 (severe dementia) to 30 (normal). MMSE scores in bryostatin-treated subjects were increased at 3 h ($p=0.041$, paired t -test) but not at 4d or 15d (not shown).

Human Expanded Access (EA) subjects

In contrast to the Phase IIa trial, in which subjects received a single infusion, EA subjects received up to 26 infusions over a period of up to 46 weeks (Table 3). Based on earlier pre-clinical studies of cognitive enhancement and neuroprotection in three different AD transgenic mouse strains, and also based on earlier clinical studies for oncologic indications, we hypothesized that an optimal bryostatin treatment protocol would be a dose of 25 $\mu\text{g}/\text{m}^2$ for 3 successive weeks followed by several lower doses of gradually increased spacing. These doses are 4.8–12 \times lower than many doses given previously in some cancer trials [23, 24] but comparable to others [40–42]. Bryostatin infusion produced clinically relevant improvements in assessed behavior in all three patients (Table 3). Psychometric assessment was not possible in subjects #1 and #2 because both were non-verbal.

Subject #1

EA subject #1 was given multiple infusions over a period of 5 months. He became more alert and

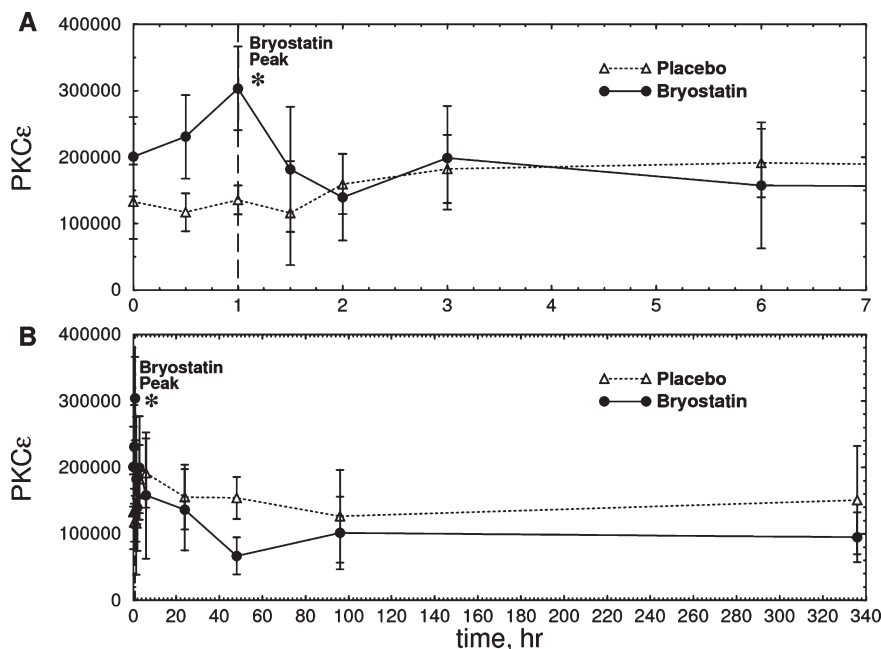


Fig. 3. Time course of total PKC ϵ measured in PBMC samples from six Phase IIa subjects treated with bryostatin 1. A) Expanded scale (0–7 h). B) Full scale (0–340 h). There was an increase in total PKC ϵ in the bryostatin group but not in the placebo at 1 h after the start of infusion (Bryostatin $F(7,013, 21,039) = 3.026$, $p=0.023$; placebo $F(10,20) = 0.75$, $p=0.67$, repeated measures ANOVA; $p=0.0185$ at 1 h, two-tailed matched pair t -test) Bryostatin also decreased PKC ϵ between 12 h and 72 h ($p=0.0296$, two-tailed matched pair t -test, average of 12, 48, and 72 h points). Data points are mean PKC ϵ in arbitrary units \pm SEM (Placebo, $n=3$; Bryostatin 1, $n=6$). The treated and placebo groups are not significantly different at $t=0$.

Table 3
Compassionate use patient details

Subj.	Age	Sex	No. of doses	Clinical Profile	Course / Duration	Adverse events	Outcome
1	95	M	4	Disoriented, intermittent coma, non-verbal	Became alert, attentive; remembered date, place, time; mind active, engaged, watched TV, requested return to work	None reported	Treatment discontinued due to infections unrelated to study drug.
2	38	F	11	Familial early-onset AD due to PSEN1 mutation. Non-verbal, drooling, unable to swallow (fed with gastronomy), attention grossly impaired, spasticity, inability to move	Return of some language and vocalization, swallowing, increased attentiveness to environment and persons, increased range of motion. Treatment stopped due to illness after 5 months; vocalizations also stopped, decreasing alertness, unresponsive, C-S respiration noted.	Aspiration pneumonia, not treatment related; improvements maintained throughout hospitalization while treatment paused.	After 5 months, a series of infections including sepsis, urinary tract and pneumonia prevented continuation of the study. Treatment discontinued due to infections unrelated to study drug.
3	76	M	26	MMSE 2-3; ADCS-ADL-Sev 18; hallucinations	MMSE improved to 10-12; ADCS-ADL-Sev 33; hallucinations reduced, return of complex motor skills incl. swimming, billiards. After 7 months, CIBIC-Plus score returned to 'minimally improved.'	Grade 1 and 2 myalgia, relieved with ibuprofen	Dosage frequency reduced over 7 months but MMSE score remained above baseline. Treatment discontinued due to change of residential facility.

engaged, watched TV, began speaking, and requested to return to work. The improvements lasted for several weeks, after which the treatment was discontinued due to non-drug-related infections.

Subject #2

EA subject #2 was given 11 infusions over 6 months as described previously [43] (identified as IV-18). The patient showed increased vocalization of words, responses to verbal commands, increased swallowing (after many months of requiring tube-feeding), primitive conversational interaction, and some increased range of limb motion. Treatment was interrupted twice for hospitalizations due to severe pneumonia which was not considered treatment-related. The improvements lasted for roughly 8 weeks from trial initiation and gradually diminished as dosing became intermittent and eventually was discontinued due to sepsis, urinary tract infections, and severe pneumonia.

Subject #3

Subject #3 was a 76-year-old Hispanic male diagnosed with AD and rated as severely demented (baseline MMSE = 3 on the day of and before the 1st dose, and MMSE = 2 during initial evaluation) and with no family history of AD. Concomitant medications included Simvastatin, Felcainide, Lexapro, Namenda, and Clonazepam. The subject was given 25 infusions of bryostatin 1 in a dose-ranging study over a period of 46 weeks. PBMCs were isolated from Subject #3 on site 60 min after the start of drug infusion and total PKC ϵ was measured in cell extracts within 48 h. Additional blood plasma samples for measurement of drug levels were collected before and after infusion.

Within 3 h of the first infusion, Subject #3's MMSE rose from a baseline of 3 to 12. The MMSE score remained within the 10-12 range for 1-2 weeks, and remained between 5 and 12 thereafter (Fig. 4, green). These improvements of the MMSE roughly paralleled the changes of the ADCS-ADL. Improvements in cognitive function early in the course of treatment were also noted by the health care workers and caregivers. The patient, who had previously been largely immobile and absorbed in constant hallucinations that had occurred for many months prior to the bryostatin trial, became free of hallucinations, became mobile, able to feed himself, care for bodily functions, speak and recognize words, interact

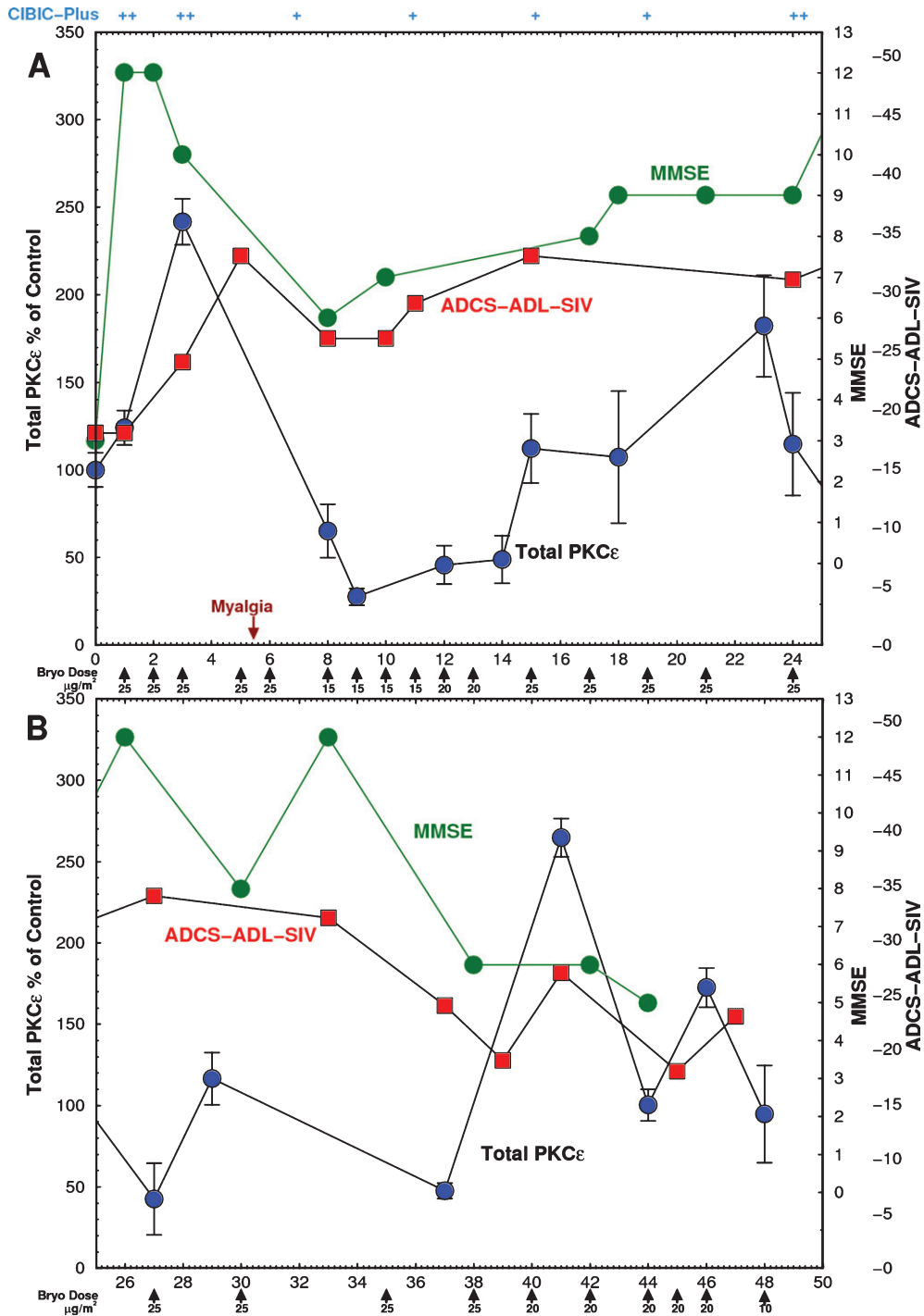


Fig. 4. Time course of Expanded Access Subject PKC ϵ and psychometric scores. Subject was infused with the indicated doses on 26 occasions over 46 weeks with bryostatin 1 as indicated by the arrows. The dose rate was gradually lowered from 25 to 10 $\mu\text{g}/\text{m}^2/\text{week}$. Blood was drawn immediately before and after infusion and PBMCs were isolated on site and frozen. PBMCs were obtained from blood samples before each bryostatin dose and PKC ϵ was measured by ELISA within 48 h. (Green) MMSE scores, measured 3 h after infusion, increased after the first dose and remained above baseline for 44 weeks. (Blue) Postinfusion PBMC PKC ϵ . Bryostatin 1 infusion increased PKC ϵ on the day of infusion followed by rapid downregulation. (Red) ADCS-ADL-SIV scores. PKC values are mean \pm SD of 4 to 8 replicate measurements. A) 0–25 weeks. B) 25–50 weeks.

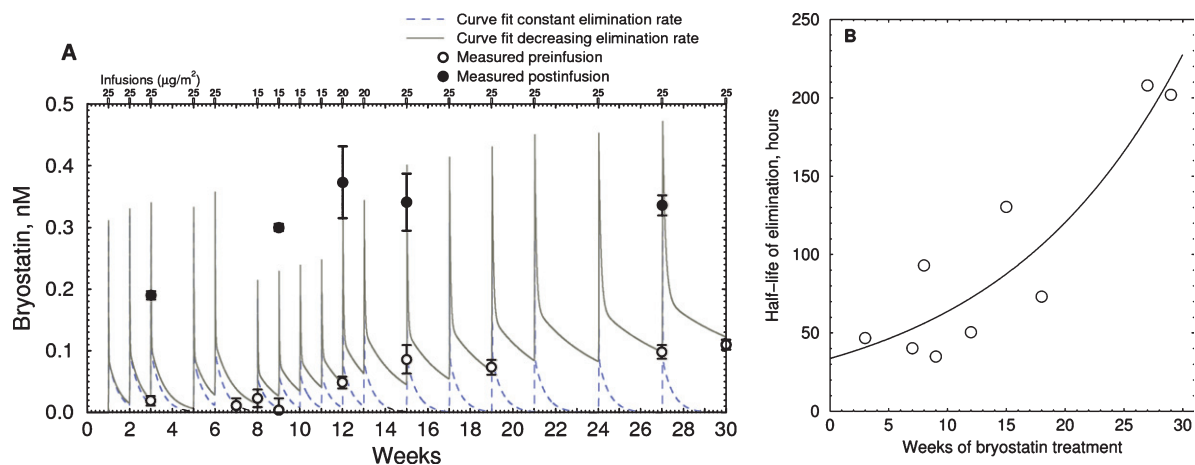


Fig. 5. Time course of bryostatatin elevation with successive infusions in EA subject. A) Blood plasma bryostatatin was measured by LCMS in Expanded Access patient #3 after 1 h i.v. of 15 to 25 $\mu\text{g}/\text{m}^2$ infusions during successive weeks. Doses are indicated in small rectangles along the top. Values are mean \pm SD of three replicate measurements. Blood plasma sample collection was discontinued after 30 wk. Open = preinfusion concentrations, closed = post-infusion concentrations. Dashed line = polyexponential least squares fit assuming constant elimination rate. Solid line = least squares fit with increasing elimination half-life. Parameters: k_{inj} = rate constant injection site to blood = 0.09 min^{-1} ; $k_{\text{compartment}1}$ = rate constant fast elimination = 0.010 min^{-1} ; $k_{\text{compartment}2}$ = rate constant of slow elimination = 0.00012 min^{-1} ; v_r = volume ratio of compartment 1 to 2 = 3.44. B) Plot of calculated elimination half-life of bryostatatin in compartment 2 estimated in (A) versus weeks of infusion.

conversationally with others, and engage successfully in complex physical activities such as swimming and billiards. CIBIC-Plus score returned to 'minimally improved' after 7 months. By 9 months, the MMSE score declined to 5. As dosing became much less frequent his deficits and symptoms, including hallucinations, returned. Treatment was terminated when caregivers requested a change to a different residential facility.

After the first 3 doses administered to Subject #3, the pre-infusion levels of PKC ϵ increased by 140%, and then became profoundly downregulated to about 25% of the baseline value, gradually returning to and finally exceeding baseline levels (Fig. 4, blue). These levels, consistent with downregulation, recovered gradually as the intervals between bryostatatin doses were progressively increased. The blue curve in Fig. 4 shows only the values of the preinfusion total PKC ϵ before each dose. There was an apparent correspondence in the first 24 weeks between the changes observed in the MMSE and the ADCL-ADL and the changes of PBMC PKC ϵ (Fig. 4a); however, the correlation was not statistically significant. In the second part of this trial, exploration of lower and less frequent doses was conducted. These doses resulted in lower efficacy (Fig. 4b). As an exploratory trial, these interval changes were made in an effort to optimize PKC ϵ induction and activation and to minimize downregulation. No changes in serum chemistry, CBC, or

vital signs except for moderate weight loss (82.7 to 75.9 kg) were noted throughout the treatment. The only AE was Grade 1 mild myalgia at 6 weeks, which roughly coincided with the period of greatest PKC ϵ downregulation. It resolved completely after two 500 mg tablets of Tylenol. Myalgia has been frequently observed in cancer trials after bryostatatin 1 treatment and is the dose-limiting toxicity at higher doses [41, 44, 45].

Bryostatatin 1 blood plasma levels in Subject #3, measured by LCMS [35], reached a maximum of 0.4–0.5 nM within 1 h after infusion (Fig. 5). Over the course of the treatment we observed a gradual increase in the baseline level of bryostatatin measured between infusions. To determine whether this apparent increase in half-life could be accounted for by accumulation of bryostatatin 1, we created a two-compartment pharmacokinetic model based on our earlier mouse results [36]. The pharmacokinetic parameters were calculated using our previous data on the time course of bryostatatin 1 in mouse blood plasma, measured by LCMS (Fig. 6). The fitted parameters were comparable to those measured in mice by Zhang et al. [46]. The lower curve represents the bryostatatin in brain (solid triangles = measured values; solid line = fitted curve).

The simulation showed that the accumulation of bryostatatin was due a decrease in the rate constant of elimination from the slower compartment. The

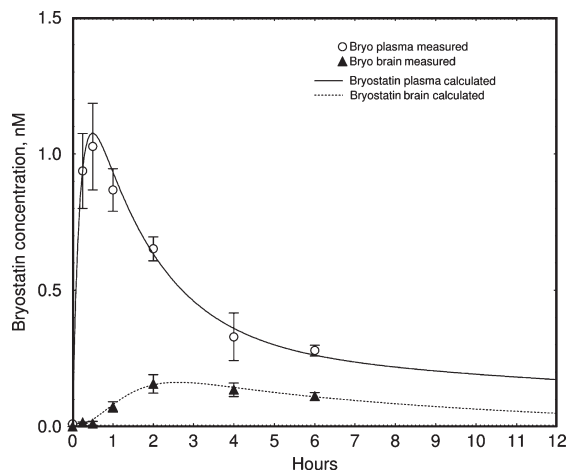


Fig. 6. Pharmacokinetic simulation of bryostatins 1 blood plasma concentration in mouse. Upper curve = blood plasma. Lower curve = brain. Values are mean \pm SEM, $n = 3-6$ mice per group. The lower curve was fitted to measured values using a simple saturable brain uptake model using parameters of $V_{m_{\text{uptake}}} = 0.017$ nM min^{-1} , $K_{m_{\text{uptake}}} = 1.5$ nM, and rate constant of elimination = 0.027 min^{-1} .

apparent half-life of elimination, calculated by comparing pre- and post-dose blood plasma levels for each administration, increased from 32 to 200 h during the course of treatment (Fig. 5B). Post-infusion values (filled circles) were not at the peak of the simulated values in all cases, indicating that these blood samples were drawn near but not precisely at the point of peak plasma concentration.

Preclinical experiments

To test optimization of the doses used both in the Phase IIa and the Expanded Access trials, we conducted exploratory tests of different protocols by administering bryostatins 1 to male C57BL/6N mice with i.v. bolus injections (each over 20 s) through the tail vein. We used the mouse results to develop our model, which was needed to interpret the long-term human results. We also used the mouse PKC ϵ results (below) to inform the dosing schedule. Groups of mice were injected biweekly, weekly, semiweekly (1 on/1 off), or intermittently (3 on/3 off) using up to six doses of 10, 15, 20, or 25 $\mu\text{g}/\text{m}^2$ (see Table 1 for injection schedules). Blood PBMCs and brain were collected at 1 h or 24 h after the last dose. We measured total PKC ϵ by isozyme-specific ELISA in brain extracts and PBMCs isolated from blood. Subcellular localization of PKC ϵ in brain was calculated as the ratio of PKC ϵ in membrane / (membrane + cytosol) fractions. We also measured brain BDNF, proBDNF,

PSD-95, pPSD-95, and synaptophysin in unfractionated brain extracts. All measurements were compared with control mice injected on the same days with vehicle only.

Mouse brain BDNF

As reported earlier [5, 47, 48], bryostatins 1 increased the brain levels of total BDNF (mature + pro-BDNF forms) by up to 100% (Fig. 7, top). The elevation in BDNF increased linearly with the number of successive injections given at one week intervals, with the largest effects observed 24 h after 3 weeks of 25 $\mu\text{g}/\text{m}^2$ bryostatins 1 weekly. Increasing the dose frequency to twice per week elevated BDNF at lower doses (15 and 20 $\mu\text{g}/\text{m}^2$), indicating that repeated dosing has a cumulative effect. Other dosing schedules, including 1 on/1 off (alternating weeks), and 2 doses of 25 $\mu\text{g}/\text{m}^2$ followed by alternating weeks of 20 $\mu\text{g}/\text{m}^2$ (2 \times 25 + 20 1 on/1 off), produced little or no elevation of BDNF (not shown). Little change in BDNF was observed in samples collected 1 h after injection (not shown). No significant changes were observed for BDNF in blood plasma or for proBDNF in brain (not shown).

The nonlinearity of the dosage response relationship suggested the possibility that the elevation of BDNF could be a toxic effect. However, no changes in blood plasma neuron-specific enolase, a marker for neuronal death [49, 50], were observed in any sample. Thus, we conclude that bryostatins 1 needs to be above a threshold level to produce an increase in BDNF, and that BDNF is not a response to toxic cell injury by bryostatins 1.

Mouse brain PSD-95

PSD-95 also increased after successive doses of 25 $\mu\text{g}/\text{m}^2$ bryostatins 1 treatment (Fig. 7, middle). No other statistically significant correlation between PKC ϵ and BDNF, PSD-95, or synaptophysin was observed at either 1 h or 24 h. No changes were observed for pPSD-95 (not shown).

Mouse brain PKC ϵ

While single infusions in humans produced a transient elevation of PKC ϵ , the principal observable effect of bryostatins 1 injected at 25 $\mu\text{g}/\text{m}^2$ in mouse, measured both 1 h and 24 h after the last dose, was downregulation of total brain PKC ϵ ($F(40,74) = 12.48$, $p < 0.0001$, ANOVA) (Fig. 7,

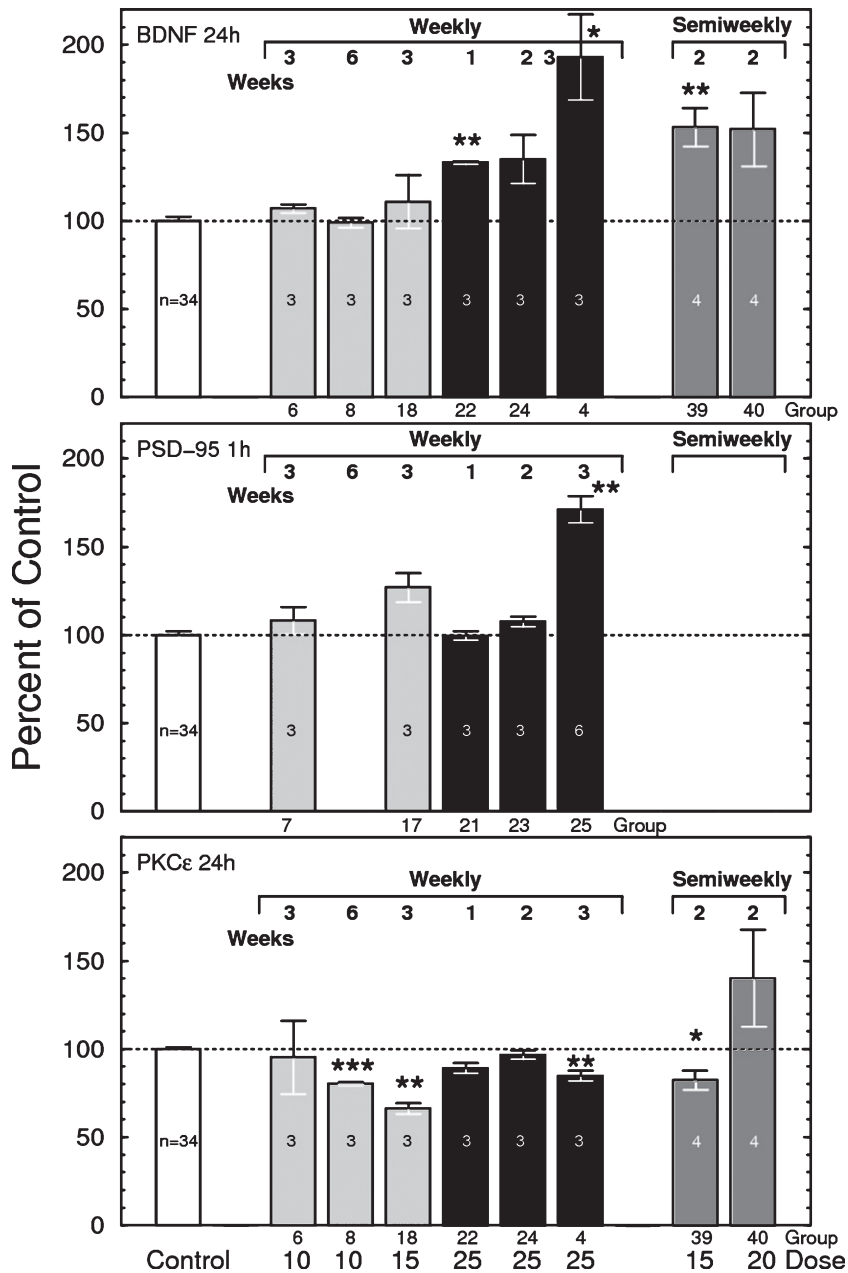


Fig. 7. Top: Total brain BDNF measured 24 h after i.v. injections of bryostatin 1 in mice. Only weekly treated groups are shown. Each bar represents 3 to 6 mice except control = 31 mice ($F(40,74) = 12.48, p < 0.0001$, ANOVA, includes all thirteen 24-h treated groups). Bars are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$. See Table 1 for dosing schedule. Middle: Effects of bryostatin 1 on mouse brain PSD-95 measured 1 h after i.v. injection of bryostatin. Each bar represents 3 to 6 mice except control = 31 mice ($F(20,53) = 4.028, p < 0.0001$). Bars are mean \pm SEM. ** $p < 0.01$. Bottom: Total brain PKCε measured by PKCε-specific ELISA 24 h after i.v. injection of bryostatin 1 in mice. Only weekly treated groups are shown. Each bar represents 3 to 6 mice except control = 31 mice ($F(40,74) = 12.48, p < 0.0001$, ANOVA). Bars are mean \pm SEM. ** $p < 0.01$, *** $p < 0.001$.

bottom). This is consistent with the known effects of C1-domain PKC activators, whereby enzyme activation and translocation are followed by rapid downregulation due to proteasomal activity. Enzymatic PKC activity was generally consistent with

the ELISA results (not shown). PKCε downregulation was more severe in PBMC cells, but also more variable (not shown).

We also measured membrane translocation, which is accepted as an index of PKC activation.

Translocation is defined as PKC in membrane / Total PKC; thus a value of 1.00 signifies complete membrane translocation. Bryostatin 1 produced maximal membrane translocation at $15 \mu\text{g}/\text{m}^2$ for 3 weeks and $25 \mu\text{g}/\text{m}^2$ for 2 weeks when measured 24 h after administration (not shown). Mice with higher translocation generally also had higher downregulation, consistent with the known life cycle for PKC. In mouse brain the baseline level of translocation was 0.775 ± 0.032 (mean \pm SE, $N = 33$); therefore, a 29% increase in the ratio would represent essentially 100% membrane localization.

DISCUSSION

This was the first clinical study of bryostatin 1 in AD patients. Bryostatin 1 produced large changes in PKC ϵ during the early course of treatment both in mice and in the EA patient, indicating effective target engagement. Peak levels of PKC ϵ occurred within 1 h of infusion onset. Long-term treatment induced downregulation of PKC ϵ that was dependent on dosing levels and duration. Administration of the highest doses used here ($25 \mu\text{g}/\text{m}^2$) for 5 of 6 successive weeks produced measurable downregulation. Downregulation is a well-documented phenomenon that results from the transient nature of PKC ϵ C1 domain activation in neurons [11, 12], after which PKC becomes dephosphorylated, ubiquitinated, and degraded primarily by the proteasome. Our previous results showed that a brief pulse of PKC ϵ activation produces prolonged protein synthesis that lasts for at least one week [51]. Thus, PKC ϵ activators can produce long-term changes that continue well after the drug has been eliminated. Bryostatin produces a marked improvement in performance of 5XFAD mice in the water maze memory task, and also improves performance in unimpaired wild-type animals [3]. Further research on the mechanisms of PKC ϵ biosynthesis and degradation will be necessary in order to derive the numerical relationship between PBMC PKC ϵ , brain PKC ϵ , brain BDNF, and synaptogenesis that is needed for PKC ϵ to be useful as a biomarker.

The Phase IIa trial results are consistent with our animal testing [3, 52] which indicated an early cognitive enhancement within hours of the first dosing. In EA Subject #3, the PKC ϵ appeared to correlate with the psychometric scores in the early part of treatment, even though the correlation did not reach statistical significance. The threshold effect on BDNF noted

in our preclinical results suggests that the frequency of dosing might be a critical factor in determining effectiveness of bryostatin.

Repeated infusions of bryostatin over several months in EA Subject #3 led to an accumulation of blood plasma levels reaching 0.1 nM up to 2 weeks after infusion. Simulations showed that this accumulation could not be described by a fixed elimination rate, and that a 6-fold decrease in elimination rate was necessary to account for the increased pre-infusion levels. This could be attributed to disease progression or to gradual drug accumulation in a slowly equilibrating compartment. By contrast, in the Phase IIa trial, in which a single infusion was given, the time course was characterized by rapid elimination. Although samples in the Phase IIa study were collected out to 14 days, the sensitivity of the assay used in the Phase IIa trial (lower limit of quantitation = 0.2 ng/ml) was insufficient to perform detailed pharmacokinetic modeling. The increased half-life of bryostatin 1 when administered over long periods suggests that future trials should consider possible bryostatin accumulation over long intervals of dosing.

The neuroprotective effects of bryostatin 1 may be due to its ability to induce synaptogenesis by elevating levels of synaptic growth factors in brain such as BDNF [47, 53] and other synaptic growth factors. BDNF is a potent neurotrophic factor, but is unable to cross the blood-brain barrier [54]. Our previous animal experiments demonstrated that bryostatin 1 is capable of crossing the blood-brain barrier and restoring water maze learning in aged rats and transgenic mice used as models for many types of neurodegenerative diseases [7, 48, 55, 56]. However, other mechanisms of neuroprotection, such as a direct effect of bryostatin-binding proteins on dendritic spine architecture [57], anti-apoptosis, or interference with amyloid- β toxicity through apolipoprotein E, [58], endothelin convertase [59], or neprilysin [60] may also be important.

These preliminary studies provide valuable guidance for the design of larger clinical trials. Bryostatin was well tolerated in AD patients. Although this study was insufficiently powered to draw any definitive conclusions about efficacy, bryostatin appeared to produce a short-duration improvement of the MMSE psychometric scores. The long half-life of bryostatin 1 and high between-patient variability in the PKC biomarker suggest that protocols may need to accommodate bryostatin elevation over long intervals of dose administration.

The findings here of safety, favorable pharmacokinetics, target engagement, initial cognitive improvement with even a single dose in the Phase IIa trial, and improvements of cognitive functions in the Compassionate Use trials, collectively suggest that bryostatin may be a promising candidate drug for the treatment of AD.

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