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Adduct formation in liquid chromatography-triple quadrupole mass spectrometric measurement of bryostatin 1

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ABSTRACT

Bryostatin 1, a potential anti-Alzheimer drug, is effective at subnanomolar concentrations. Measurement is complicated by the formation of low *m/z* degradation products and the formation of adducts with various cations, which make accurate quantitation difficult. Adduct formation caused the sample matrix or mobile phase to partition bryostatin 1 into products of different mass. Degradation of the 927 $[M+Na]^+$ ion to a 869 *m/z* product was strongly influenced by ionization conditions. We validated a bryostatin 1 assay in biological tissues using capillary column HPLC with nanospray ionization (NSI) in a triple-quadrupole mass spectrometer in selected reaction monitoring (SRM) mode. Adduct formation was controlled by adding 1 mM acetic acid and 0.1 mM sodium acetate to the HPLC buffer, maximizing the formation of the $[M+Na]^+$ ion. Efficient removal of contaminating cholesterol from the sample during solvent extraction was also critical. The increased sensitivity provided by NSI and capillary-bore columns and the elimination of signal partitioning due to adduct formation and degradation in the ionization source enabled a detection limit of 1×10^{-18} mol of bryostatin 1 and a LLOQ of 3×10^{-18} mol from 1 μl of sample. Bryostatin 1 at low pmol/l concentrations enabled measurement in brain and other tissues without the use of radioactive labels. Despite bryostatin 1's high molecular weight, considerable brain access was observed, with peak brain concentrations exceeding 8% of the peak blood plasma concentrations. Bryostatin 1 readily crosses the blood–brain barrier, reaching peak concentrations of 0.2 nM, and specifically activates and translocates brain PKCε.

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1. Introduction

Bryostatin 1 (Fig. 1**a**) is a macrocyclic lactone extracted from *Bugula neritina*, a marine bryophyte [1,2]. Bryostatin 1 is the most abundant of numerous bryostatins, which are synthesized by bacteria that exist in a symbiotic relationship with the bryophyte [3]. Bryostatin 1 is a potent activator of protein kinase C (PKC), particularly the ε and α isozymes, with a K_d of 1.4 nM [4], and binds to the C1a and C1b domains, competing with the natural ligand, diacylglycerol [5–7].

Early studies on bryostatin 1 suggested possible benefits of moderate to high doses, which produce mainly downregulation of PKC, as anti-tumor agents [8–10]. However, clinical trials of bryostatin 1, both alone and in combination with conventional anti-cancer drugs including 1-β-D-arabinofuranosylcytosine [11], cisplatin [12], and paclitaxel [13], have so far shown little or no clinical benefit. This may be attributable to our incomplete understanding of the roles of various PKC isozymes in cancer, or to an incomplete understanding of the dynamics of differential activation and downregulation of PKC isozymes by bryostatin 1 and other C1a/C1b activators at different concentrations.

Bryostatin 1 also shows some promise as a possible treatment for neurodegenerative diseases such as Alzheimer's disease (AD). In Tg2576 transgenic mice, used as a model for AD, bryostatin 1 improves learning in Morris water maze tasks. Activators of PKCε such as bryostatin 1 have demonstrated neuroprotective activity in animal models of AD [14], depression [15], and stroke [16], and can protect or enhance memory in rodents, rabbits, and invertebrates [15,17–19]. This memory enhancement is accompanied by increase in levels of synaptic proteins and structural changes in synaptic morphology [20]. PKCε also reduces Aβ levels by activating Aβ-degrading enzymes including endothelin-converting enzyme [21,22]. Thus, PKCε activators may be an effective adjunct to Aβ-reduction therapy. PKCε activators also induce synaptogenesis in the CA1 stratum radiatum of the dorsal hippocampus of young adult rats [20]. The mechanism of synaptogenesis involves arachidonic acid signaling through PKC, triggered by integrin receptors and astrocytic factors [23]. PKCε also mediates the neuroprotective effects of ApoE/cholesterol [24], which is a synaptogenic signal

Abbreviations: LLOQ, lower limit of quantitation; PKC, protein kinase C; AD, Alzheimer's disease; Aβ, beta-amyloid peptide; MTBE, methyl t-butyl ether; NSI, nanospray ionization.

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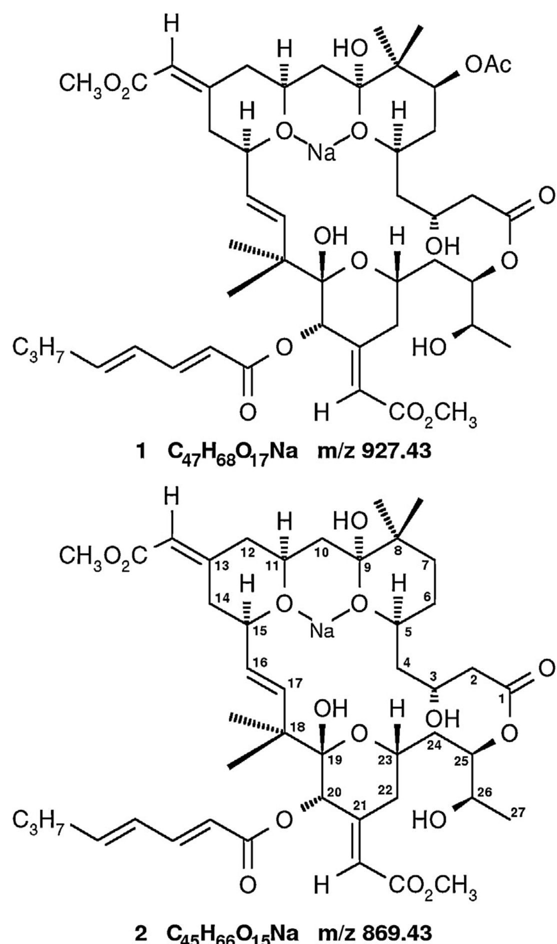

Fig. 1. Structures of bryostatin 1 derivatives. **1** Bryostatin 1 sodium adduct (monoisotopic mass, 927.44; average molecular weight 928.02), **2** the 869.43 (monoisotopic *m/z*) product proposed by Manning et al. [2].

secreted by astrocytes [25]. These results indicate that activation of endogenous PKCε protects against synaptic loss and facilitates synaptogenesis.

Before proceeding to clinical trials, it is necessary to obtain a clear understanding of the pharmacokinetics of bryostatin 1 and its time- and concentration-dependent effects on various PKC isozymes. Because bryostatin 1 is effective at subnanomolar concentrations, these experiments require highly sensitive assays. Zhao et al. [26] used a triple quadrupole mass spectrometer to detect bryostatin 1 with a LLOQ of 55 pM. Fluorescence derivatization methods have also been developed [27]. However, to achieve adequate sensitivity for animal experiments and clinical trials of bryostatin 1, where sample sizes are limited, even higher levels of sensitivity are required. In this article, we report that control of adduct formation and artifactual degradation in the ionization source are essential to obtaining accurate quantitative measurements of bryostatin 1 in biological samples.

2. Materials and methods

Materials—Bryostatin 1 was obtained from Tocris Bioscience (R&D Systems, Minneapolis, MN, USA). Acetonitrile was obtained from ThermoFisher. Paclitaxel, MTBE, and other chemicals were obtained from Sigma–Aldrich.

2.1. Enzyme assays

Protein kinase C assay—Cells were scraped in 0.2 ml homogenization buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaF, 1 μg/ml leupeptin, and 0.1 mM PMSF) and homogenized by sonication in a Marsonix microprobe sonicator (5 s, 10 W). To measure PKC, 10 μl of cell homogenate or purified PKC isozyme was incubated for 15 min at 37 °C in the presence of 10 μM histones, 4.89 mM $CaCl_2$, 1.2 μg/μl phosphatidyl-L-serine, 0.18 μg/μl 1,2-dioctanoyl-sn-glycerol, 10 mM $MgCl_2$, 20 mM HEPES (pH 7.4), 0.8 mM EDTA, 4 mM EGTA, 4% glycerol, 8 μg/ml aprotinin, 8 μg/ml leupeptin, and 2 mM benzamidine. 0.5 μCi·[γ^{32}P]ATP was added and ^{32}P-phosphoprotein formation was measured by adsorption onto phosphocellulose as described previously [28].

For measurements of activation by bryostatin 1 and similar compounds, PKC activity was measured in the absence of diacylglycerol and phosphatidylserine, as described by Kanno et al. [29], and PKCδ, ε, and α were measured in the absence of added EGTA and $CaCl_2$, as described by Kanno et al. [29]. Low concentrations of Ca^{2+} are used because high Ca^{2+} interacts with the PKC phosphatidylserine binding site and prevents activation. For measurements of bryostatin 1 activation, 1,2-diacylglycerol was omitted unless otherwise stated.

PKC isozyme translocation—Activation and translocation of PKCε were measured by western blotting after subcellular fractionation into cytosol and particulate fractions. Homogenates were centrifuged at 100,000 × *g* for 20 min and cytosolic and particulate fractions were separated on 4–20% Tris–glycine SDS polyacrylamide gels, blotted onto nitrocellulose, and probed with isozyme specific antibodies. The blots were photographed in a GE ImageQuant at 16 bits/pixel and analyzed by vertical strip densitometry using Imal Unix software.

2.2. Sample preparation and bryostatin 1 measurement

Bryostatin 1 extraction from tissue—Tissue samples were sonicated in 2 vol. of homogenization buffer (30 s, 10 W), and 0.1–0.5 ml homogenate was extracted 2× with 0.3 ml methyl t-butyl ether (MTBE) and methanol as described below for extraction from blood plasma.

Bryostatin 1 Extraction from blood plasma—Blood plasma was extracted using a modification of the MTBE-methanol method of Matyash et al. [30]. Methanol (100 μl) and MTBE (300 μl) were added to 100 μl EDTA-treated blood plasma in a 1.5-ml polypropylene centrifuge tube. The sample was vortexed and centrifuged for 10 min at 15,000 × *g*. The upper phase was transferred to a new 1.5-ml polypropylene centrifuge tube, 300 μl MTBE were added, the sample was vortexed and centrifuged again, and the upper phase was combined with the first extraction. The sample was evaporated to dryness by evaporation under nitrogen in a water bath at 50 °C. The sample was then redissolved in 0.1 ml ethanol and vortexed. The ethanol was evaporated to dryness and the sample was redissolved in 0.1 ml 50% acetonitrile in water, centrifuged for 1 min at 11,000 × *g*, and transferred to a glass-insert mass spectrometry vial. Samples were stored at −20 °C until use, warmed in a 37 °C water bath, and vortexed before loading onto the autosampler, which was maintained at 25 °C.

Sample quantities— For serum samples, 100 μl serum was used. For brain samples, 0.5 ml of 1→3 homogenate, containing approximately 166 μg protein, was used. The MTBE/methanol extracts of samples and standards were dissolved in 100 μl of 50% acetonitrile in water and the entire sample was transferred to an autosampler vial containing a 0.2 ml glass insert and Teflon screw-cap. A 1.0 μl aliquot was injected for all samples. Quantitation results are reported either as mol measured in the 1 μl that was injected onto the LCMS instrument, or as mol/liter or mol/kg of original tissue, as appropriate, except where noted.

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