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ORIGINAL ARTICLE

In vitro and *in vivo* neuroprotective activity of the cardiac glycoside oleandrin from *Nerium oleander* in brain slice-based stroke models

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Abstract

The principal active constituent of the botanical drug candidate PBI-05204, a supercritical CO_2 extract of *Nerium oleander*, is the cardiac glycoside oleandrin. PBI-05204 shows potent anticancer activity and is currently in phase I clinical trial as a treatment for patients with solid tumors. We have previously shown that neriifolin, which is structurally related to oleandrin, provides robust neuroprotection in brain slice and whole animal models of ischemic injury. However, neriifolin itself is not a suitable drug development candidate and the FDA-approved cardiac glycoside digoxin does not cross the blood–brain barrier. We report here that both oleandrin as well as the full PBI-05204 extract can also provide significant neuroprotection to neural tissues damaged by oxygen and glucose deprivation as occurs in ischemic stroke. Critically, we show that the neuroprotective activity of PBI-05204 is maintained for several hours of delay of administration after oxygen and glucose deprivation treatment. We provide evidence that the neuroprotective activity of PBI-05204 is mediated through oleandrin and/or other cardiac glycoside constituents, but that additional, non-cardiac glycoside components of PBI-05204 may also contribute to the observed neuroprotective activity. Finally, we show directly that both oleandrin and the protective activity of PBI-05204 are blood brain barrier penetrant in a novel model for *in vivo* neuroprotection. Together, these findings suggest clinical potential for PBI-05204 in the treatment of ischemic stroke and prevention of associated neuronal death.

Keywords: biolistics, brain slice, cardiac glycoside, Na^+, K^+-ATPase, neuroprotection.

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Stroke continues to represent a major health problem in the United States, with some 800 000 people experiencing a new or recurrent stroke each year (Lloyd-Jones *et al.* 2010). Moreover, stroke incidence has more than doubled in low- and middle-income countries over the past 40 years, and in the past decade has exceeded the incidence of stroke in high-income countries for the first time (Feigin *et al.* 2009). Despite this urgent medical need, the thrombolytic drug tissue plasminogen activator remains the only clinical therapy approved by the FDA for treatment of acute stroke, and generally must be administered within 3–4.5 h after onset of stroke symptoms (Krishnan *et al.* 2010). However, less than 6% of stroke victims are typically eligible for tissue plasminogen activator treatment because they are not able to access emergency medical care within this restricted therapeutic time window (Krishnan *et al.* 2010).

Although recent and exciting progress has been made in neuroregenerative and neuroresuscitative strategies (Cramer and Riley 2008), such as antibody-mediated antagonism of neurite outgrowth inhibitor (Nogo) receptors (Papadopoulos

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Abbreviations used: ACSF, artificial CSF; BBB, blood–brain barrier; DMSO, dimethylsulfoxide; OGD, oxygen–glucose deprivation; PND, postnatal day; YFP, yellow fluorescent protein.

et al. 2002; Tsai *et al.* 2011), direct provision of neuroprotection to neural tissues damaged in ischemic stroke remains a problematic area for therapeutic development. Indeed, the failure of numerous stroke clinical trials over the last two decades emphasizes the need for the identification of new and more effective candidate drugs and drug targets for neuroprotective strategies in stroke (Gladstone *et al.* 2002; Fisher *et al.* 2009).

In this context, we report here the identification and analysis of a neuroprotective activity provided by the botanical drug candidate PBI-05204, derived from *Nerium oleander*, currently in phase I clinical trial for patients with solid tumors (Newman *et al.* 2008). The principal active anticancer component of PBI-05204 is the cardiac glycoside oleandrin, and as such was introduced for cancer treatment as part of a growing appreciation for the multiple anticancer mechanisms that can be mediated by lipid soluble cardiac glycoside drugs (Newman *et al.* 2008; Prassas and Diamandis 2008).

In a recent chemical biology screen using a brain slice-based assay for ischemic stroke, we found that certain cardiac glycoside compounds can also provide direct neuroprotection in the context of ischemic injury, both in brain slices as well as in two independent whole animal models for stroke (Wang *et al.* 2006). In this study, we found that while the only two FDA-approved cardiac glycoside drugs, digoxin and digitoxin, were able to provide neuroprotection in the brain slice stroke assay (although with lower apparent potency than the primary hit from this screen, the related cardiac glycoside neriifolin), these drugs are unlikely to be useful as treatments for clinical stroke because they are substrates for P-glycoprotein and are thereby actively excluded from the brain by the blood–brain barrier (BBB) (Schinkel 1999). Indeed, we subsequently showed that therapeutic levels of digoxin could not be attained in the CNS via systemic delivery in a perinatal rat model for focal ischemia (M. Homi and D. Warner, personal communication). In contrast, the phase I PBI-05204 drug candidate and its constituent oleandrin are BBB-penetrant, motivating the studies described here to investigate the potential neuroprotective benefit of PBI-05204 in brain slice-based assays for focal ischemia.

Materials and methods

Materials

The botanical drug PBI-05204 was prepared as an ethanol modified supercritical CO_2 extract of organically grown *Nerium oleander* (Newman *et al.* 2008) and was provided by Phoenix Biotechnology (San Antonio, TX, USA). The extract was specifically formulated for oral administration. The resulting product, PBI-05204, was introduced into clinical phase I trial at the University of Texas M. D. Anderson Cancer Center (Houston, TX, USA). Oleandrin content of the botanical drug product was measured using a validated HPLC method. Oleandrin was purchased from ChromaDex (Irvine, CA, USA). Both compounds were dissolved into a series of dimethyl sulfoxide (DMSO) stocks and diluted 1000-fold to their final indicated concentrations for the brain slice studies; vehicle only (0.1% DMSO) was added to all control conditions. Digibind was purchased from GlaxoSmithKline (Research Triangle Park, NC, USA); pre-immune whole sheep IgG from Jackson Laboratories (Bar Harbor, ME, USA).

DART-MS analyses of *Nerium oleander* extract

A JEOL AccuTOF-DART mass spectrometer (Jeol USA, Peabody, MA, USA) was used to determine the basic composition of the supercritical CO_2 *N. oleander* extract. Analyses were conducted in a positive ion mode (DART+) giving masses corresponding to the $M+H^+$ ions generated by the DART-MS. A range of settings on the instrument was used to determine optimal conditions for *N. oleander* analyses. The general settings for DART+ included: needle voltage 3500 V; orifice 1, 2–20 V; ring lens 2–5 V; orifice 2, 2–5 V; and peak voltage 1000 V. Calibrations were performed internally with each sample using a 10% solution of PEG 600 which provides mass markers throughout the required mass range of 100–1000 mass units. Other analyses were undertaken in the DART- mode and these consisted of: needle voltage 3500 V; heating element 250°C; electrode 1, −150 V; electrode 2, −250 V; He gas flow rate 3.79 LPM. Mass spectrometer settings: MCP 2600 V; orifice 1, −15 V; ring lens −5 V, orifice 2, −5 V; and peak voltage 1000 V. Calibrations were performed internally with each sample using a perfluorinated carboxylic acid solution that provides markers throughout the required mass range of 100–1000 mass units. The *N. oleander* samples were introduced neat into the DART helium plasma using the closed end of a borosilicate glass melting point tube. The capillary tube was held in the He plasma for approximately 3–5 s per analysis. Molecular formulas were confirmed by elemental composition and isotope matching programs provided with the JEOL AccuTOF DART-MS instrument. A searchable database of *N. oleander* constituents, developed by HerbalScience (Naples, FL, USA) was used.

Brain slice explant cultures and oxygen–glucose deprivation

Coronal brain slices were prepared from postnatal day (PND) 10 Sprague–Dawley rat pups (Charles River, Wilmington, MA, USA). Animals were killed in accordance with NIH guidelines and under Duke IACUC approval and oversight. Briefly, tissue from the central third of the brain was cut into 250 μm thick sections using vibratomes (Vibratome; Bannockburn, IL, USA) and placed in short-term organotypic culture as previously described (Braithwaite *et al.* 2010). Brain tissues were bathed in cold artificial CSF (ACSF, containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 24 mM D-glucose, and 10 mM HEPES) supplemented with 1 μM MK-801 during the cutting procedure. Oxygen–glucose deprivation (OGD) was then administered to a portion of the brain slices for 5.5 min by suspension in glucose-free, N_2-bubbled ACSF containing low O_2 (< 0.5%). Control and OGD-treated brain slices were then plated individually in 12-well multiwell plates atop a semi-solid substrate consisting of culture medium (Neurobasal A medium supplemented with 15% heat-inactivated horse serum, 10 mM KCl, 10 mM HEPES, 100 U/mL penicillin/streptomycin, 1 mM sodium pyruvate, and 1 mM L-glutamine) set in 0.5% reagent-grade agarose, in an interface culture configuration analogous to that

done using transwell inserts (Stoppini *et al.* 1991). Brain slices were biolistically transfected, incubated for 24 h at 37°C under 5% CO_2 in humidified chambers, and then assessed for protection against neurodegeneration (see sections below). Test compounds were added to culture medium at the indicated concentrations at the time of brain slice preparation.

Biolistic transfection

One hour after their preparation ± OGD treatment, brain slice explants were subjected to particle-mediated gene transfer, or biolistics, using 1.6 μm elemental gold particles and a helium gas pressure of 95 psi on a Helios gene gun device (Bio-Rad, Hercules, CA, USA), as detailed previously (Lo 1999). To label a subpopulation of pyramidal neurons in the cortical regions of each coronal brain slice explant, biolistic particles were used to deliver a plasmid expression construct expressing yellow fluorescent protein (YFP) driven by the cytomegalovirus (CMV) promoter-enhancer in the gWiz expression vector (Genlantis, San Diego, CA, USA).

Assessment of neuroprotection

Twenty-four hours after brain slice preparation ± OGD, numbers of healthy pyramidal neurons in the cortical regions of the brain slices were assessed using fluorescence stereomicroscopes (Leica, Buffalo Grove, IL, USA) as previously described (Wang *et al.* 2006; Braithwaite *et al.* 2010). Briefly, pyramidal neurons were identified based on their characteristic morphology and positioning within the cortical plate, and were scored as healthy if they exhibited: (i) a robust and brightly labeled cell body; (ii) a clear apical dendrite extending radially towards the pial surface of the slice; (iii) > 2 clear basal dendrites > 2 cell body diameters long extending directing from the cell soma; and (iv) clear and continuous cytoplasmic labeling with the YFP visual marker in the cell soma as well in all dendrites and the axon. Numbers of healthy cortical pyramidal neurons per brain slice are expressed as means ± SD, or as a percentage of those present in the internal positive control condition in Figs 1f and 4a, b.

Western blotting

Fresh or explanted brain slices, at the indicated times, were washed three times with phosphate-buffered saline on ice and homogenized by trituration through a 25 g needle in homogenization buffer (1% NP-40 in 50 mM Tris–HCl pH 7.6, 150 mM NaCl, 2 mM EDTA) supplemented with Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). Samples were cleared twice by centrifugation at 3000 *g*, separated on 10–20% Tricine gels (Invitrogen, Carlsbad, CA, USA), and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). Immunoblotting was done using the 05-369/C464.6 mouse monoclonal against the α1 subunit of the Na^+, K^+-ATPase (Upstate/Millipore, Billerica, MA, USA), or the MA3-915 mouse monoclonal against α3 subunit (Affinity Bioreagents/Thermo Fisher Scientific, Waltham, MA, USA). Expression of the α-subunits in each sample was normalized to internal neuron-specific tubulin βIII levels detected with the monoclonal antibody 5G8 (Promega, Madison, WI, USA) on the same immunoblots. Horseradish peroxidase-conjugated secondary IgG antibodies were used for ECL visualization (Amersham/GE Healthcare, Piscataway, NJ, USA) on a Bio-Rad VersaDoc MP5000.

In vivo neuroprotection studies

PBI-05204, dissolved in DMSO, was administered to rat pups via i.p. injection at the indicated doses, with a total injection volume of 50 μL. Control pups received an i.p. injection of 50 μL DMSO only. Pups were returned to their dams, and used for brain slice preparation 24 h later as described above except that no neuroprotectant (MK-801) was added to the ACSF during the brain slicing process.

In vivo biodistribution studies

PBI-05204 (38 mg/kg containing 0.8 mg/kg oleandrin, 50 μL) and oleandrin (3 mg/kg, 100 μL) were administered to male CD1 mice i.p. in DMSO : PEG400 vehicle, 50 : 50 v/v (PBI-05204, 28.6 mg/mL) or 25 : 75 v/v (oleandrin, 1 mg/mL). Blood was collected in Na citrate containing-tubes and brain tissue harvested, blotted and weighed from groups of animals at the following time points post-administration: 0.5, 1, 2, 8, and 24 h ($n = 5$ per time point). Samples were also collected from a control group of five animals per drug arm that received DMSO : PEG400 vehicle alone. Plasma and brain tissue samples were frozen at −80°C until analyzed. Oleandrin was isolated using a liquid : liquid extraction procedure and quantified using LC/MS/MS with internal standardization. A 900 μL aliquot of cinobufotalin internal standard solution in 50 : 50 methanol : 0.2% formic acid (25 ng/mL) was added to 100 μL plasma or tissue homogenate (100 mg/mL tissue wt. per vol. in phosphate-buffered saline). Diluted samples were then extracted with 5 mL of methyl tertiary butyl ether. The organic layer was collected, evaporated with a stream of nitrogen and reconstituted in 100 μL of methanol : 0.2% formic acid (1 : 1, v/v). Oleandrin was separated using an aqueous formic acid : methanol gradient mobile phase and Phenomenex Luna C8(2) 3 μm, 2 × 100 mm analytical column. Samples were maintained continuously at 5°C. The detection of oleandrin was achieved using a Waters QuattroUltima triple quadrupole mass spectrometer operated in electrospray positive ionization and selected reaction monitoring mode. The mass transition of m/z 577.3 > 373.2, specific for oleandrin (M+H), was monitored and response quantified using Waters QuanLynx software. The dynamic quantification range of the assay was 0.50–1000.0 ng/mL.

Statistical analysis

For determination of neuroprotection in brain slice assays, statistical significance was tested by ANOVA followed by Dunnett's *post hoc* comparison test at the 0.05 confidence level. For the *ex vivo* brain slice explants studies, 12 brain slices were per tested per experimental condition, with each set of 12 brain slices selected at random from the total pool of brain slices prepared from 12 or 24 rat pups per experimental run. Each experiment was carried out at least three times with similar results. For the *in vivo* PBI-05204 treatment studies, a total of 48 brain slices from four rat pups for each experimental condition was used for statistical analysis.

Results

To assess the potential neuroprotective activity of PBI-05204, we used a brain slice-based assay in which transient OGD is used to induce neuronal degeneration. Such brain-slice OGD assays have been widely used for *in vitro*

studies of ischemic damage and mechanisms (Cho *et al.* 2007), and we previously used a high-throughput version of this brain slice approach in a chemical biology screen that identified the neuroprotective activity of neriifolin and other cardiac glycoside molecules (Wang *et al.* 2006). In this assay, 5–8 min of OGD in nitrogen-bubbled artificial CSF is sufficient to induce > 50% neurodegeneration and cell death over the subsequent 24 h period. Such degeneration is measured via biolistic transfection of the vital fluorescent reporter, YFP, which creates a dispersed 'sentinel' population of cortical pyramidal neurons that can be used to quantify neuronal vitality and numbers, as we have previously described (Wang *et al.* 2006). This method has a significantly greater dynamic range and linearity than bulk cell survival assessments (e.g. propidium iodide staining) (Cho *et al.* 2007), and has the further advantage that morphological robustness of surviving neurons is directly assessed.

As can be seen in Fig. 1a and b, subjecting YFP-transfected coronal brain slices to transient OGD results in the degeneration and clearance of a large proportion of cortical pyramidal neurons by 24 h following brain slice preparation and OGD treatment. Fig. 1c shows that application of a single bolus of 23 μg/mL PBI-05204 to OGD-treated brain slices over this 24 h period provided clear neuroprotection to YFP-transfected cortical neurons. This concentration of PBI-05204 was chosen to approximate ~1 μM of its principal cardiac glycoside component, oleandrin, based on MS analysis (Table 1), and to correspond to the concentration range of neriifolin and other cardiac glycosides that we have previously shown to provide neuroprotective activity in this assay (Wang *et al.* 2006).

Figure 1e shows that 23 μg/mL PBI-05204 provided near complete neuroprotection as assessed 24 h after OGD, and that a 3-fold higher concentration of PBI-05204 did not increase levels of neuroprotection further. However, a 10-fold higher concentration of PBI-05204 was ineffective, consistent with the development of toxic effects at high levels of pharmacological inhibition of the target of cardiac glycosides, the Na^+, K^+-ATPase (Kaplan 2002).

Next, we asked if PBI-05204 was still able to provide neuroprotection to brain slices if its administration was delayed relative to the timing of the OGD treatment. Because the majority of stroke victims are not able to access emergency medical care until 2–3 or more hours after stroke onset (Adams

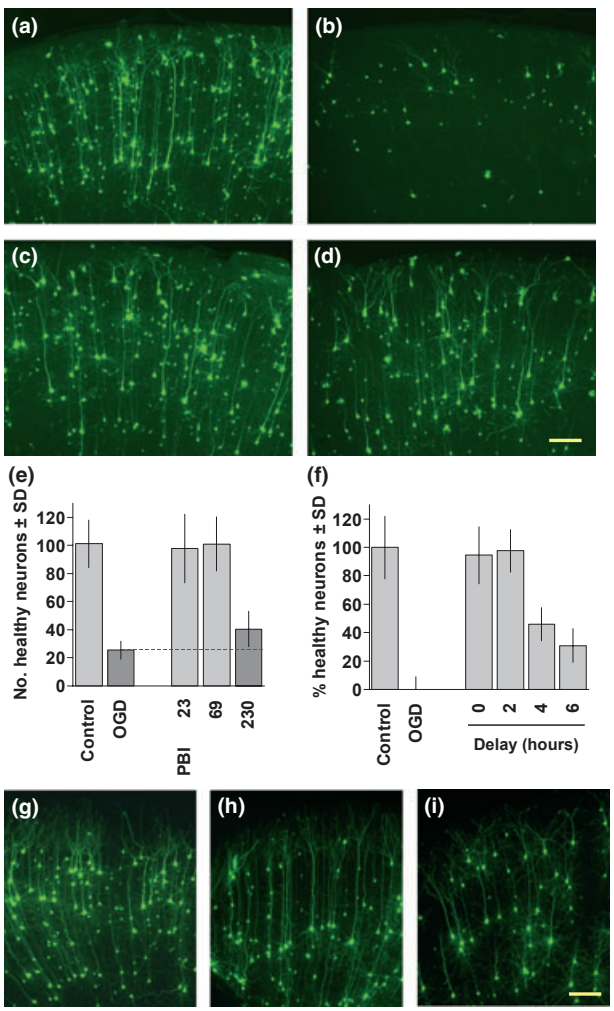

Fig. 1 PBI-05204 provides protection against neurodegeneration induced by oxygen-glucose deprivation (OGD) in a brain slice model. (a–d) Typical levels of neuronal survival in the cortical regions of coronal brain slice explants 24 h following their preparation and biolistic transfection with YFP. Panel (a) shows a control brain slice treated with 0.1% DMSO only; whereas panels (b–d) show brain slices subjected to 5.5 min of transient OGD at the time of brain slice preparation, and treated with 0.1% DMSO (b), 23 μg/mL PBI-05204 (c), or 1 μM oleandrin (d). Note that these are bulk concentrations of these compounds in the culture medium supporting the brain slice explants; actual concentrations in the interior of the brains slices may be significantly lower. Photomicrographs taken using a Zeiss SteREOLumar microscope at 50× magnification; scale bar shows 200 μm. (e) Quantification of neuronal survival in DMSO ('OGD')- versus PBI-05204 ('PBI')-treated brain slice explants; concentrations of PBI-05204 shown in μg/mL. Mean ± SD total numbers of healthy pyramidal neurons in the cortical regions of brain slice explants are shown for $n = 12$ brain slices per treatment condition. Light gray bars denote statistically significant difference with respect to the OGD condition by ANOVA followed by Dunnett's *post hoc* comparison test at the 0.05 confidence level. (f) Treatment with 23 μg/mL PBI-05204 still provided significant neuroprotection after 2–6 h delay of compound administration to brain slices after OGD treatment. Relative survival (%) is shown, with the OGD negative control set at 0% and the non-OGD positive control set at 100%. All time points showed significant differences with respect to the OGD negative control by ANOVA followed by Dunnett's *post hoc* comparison test at the 0.05 confidence level. (g–i) Example photomicrographs showing levels of neuronal survival provided by 23 μg/mL PBI-05204 applied after a 2 h (g), 4 h (h), or 6 h (i) time delay following OGD treatment. Scale bar shows 200 μm.

Table 1 Principal cardiac glycoside and other major components of PBI-05204 as determined by MS-DART TOF analysis. Other triterpenes known to be present in *Nerium oleander* (e.g. Siddiqui *et al.* 1995) were also identified under these specific DART-MS conditions

Cardiac glycoside component	Calculated	Measured	Relative abundance (%)
Oleandrin	577.3377	577.3424	2.99
Oleandrigenin	433.2590	433.2588	3.31
Ursolic acid/betulinic acid	457.3682	457.3800	15.29
Odoroside	535.3271	535.3265	0.80
Oleanolic acid	471.3678	471.3529	0.60
Urs-12-ene-3β, 28-diol/betulin	443.3889	443.3954	5.44
3β,3β-hydroxy-12-olean en-28-oic acid	441.3732	441.3768	14.26
28-norurs-12-en-3β-ol	413.3783	413.3915	4.94
urs-12-en-3β-ol	427.3940	427.3865	4.76

et al. 2007), a priority for candidate stroke therapies is the retention of neuroprotective activity when administered after ischemic damage by at least several hours. Figure 1f shows that a 2 h delay of compound administration was well tolerated, showing similar levels of neuroprotection to those attained with application of PBI-05204 immediately following OGD treatment; example photomicrographs are shown in Fig. 1g–i. Neuroprotective benefit was reduced with 4–6 h of delay of PBI-05204 administration, but at levels of neuroprotection that were still statistically significant (Fig. 1f). As we have previously shown that compounds that inhibit initiating events in ischemic neuronal damage, such as the blockage of excitotoxic calcium entry by MK-801, cannot tolerate such long delays of compound administration in this brain slice assay (Wang *et al.* 2006), these results suggest that the neuroprotective activity of PBI-05204 may intercede downstream of these initial ischemic events.

The active constituent of PBI-05204 mediating its anticancer effects is the cardiac glycoside oleandrin (Newman *et al.* 2008; Yang *et al.* 2009), bearing close structural relation to neriifolin (Fig. 2A) which we have previously found to be neuroprotective in *in vitro* and *in vivo* models of focal ischemia (Wang *et al.* 2006). We thus hypothesized that oleandrin and/or other cardiac glycoside components of PBI-05204 may also underlie the neuroprotective effects we have observed in the brain slice OGD assay. In support of this hypothesis, we showed that both the α1 and α3 isoforms of the Na^+, K^+-ATPase α-subunit are expressed in these brain slice explants (Fig. 2b). Moreover, expression of the α3-subunit increased significantly over a 2-day period following explantation of brain slices into organotypic culture, with significant increases in α3 : α1 expression ratios associated with time after explantation and/or OGD treatment. In fact, we have recently shown that higher ratios of expression of the α3- to α1-subunit are associated with differential, increased sensitivity of tumor cells to cardiac glycoside toxicity (Yang *et al.* 2009).

As oleandrin is the principal cardiac glycoside component of PBI-05204 (Table 1), we further hypothesized that oleandrin itself may account for the neuroprotective activity of PBI-05204 shown in Fig. 1. To test this, we first asked if a pure preparation of oleandrin was sufficient to provide neuroprotection in the brain slice OGD assay. In fact, we found that oleandrin provided robust, concentration-dependent neuroprotection to OGD-treated brain slices, to levels that were as high if not higher than neriifolin itself, our benchmark cardiac glycoside from previous studies (Figs 1d and 3a).

Next, we asked if these neuroprotective effects were mutually occlusive; that is, if oleandrin is the active neuroprotective component of PBI-05204, we would expect that just-maximally effective concentrations of PBI-05204 and oleandrin should not be additive. We found, in fact, that a combination of 23 μg/mL PBI-05204 and 1 μM oleandrin provided no more neuroprotection to OGD-treated brain slices than that provided by either compound alone (Fig. 3b).

To test necessity, we asked if oleandrin and/or other cardiac glycoside components of PBI-05204 are required for the neuroprotective activity of PBI-05204. We approached this by depleting PBI-05204 of cardiac glycoside components using antibody Fab fragments directed at cardiac glycosides. Such Fab preparations, commercialized as Digibind, are used clinically as an antidote for accidental digoxin intoxication (Hardman *et al.* 1996). Digibind has been shown to bind to and neutralize the activity of oleandrin and a number of other related cardiac glycosides in addition to digoxin and digitoxin (Dasgupta and Emerson 1998; Flanagan and Jones 2004; Dasgupta *et al.* 2008).

We found, surprisingly, that even very high concentrations of Digibind Fab fragments were not able to inhibit fully the neuroprotective activity of 23 μg/mL PBI-05204 (Fig. 3c). In contrast, much lower concentrations of Digibind were able to neutralize the equivalent amount of activity of oleandrin (1 μM) based on MS analysis (Fig. 3d). Together, these experiments suggested that there may be other constituents of PBI-05204, in addition to oleandrin, that provide neuroprotection to OGD-treated brain slice explants.

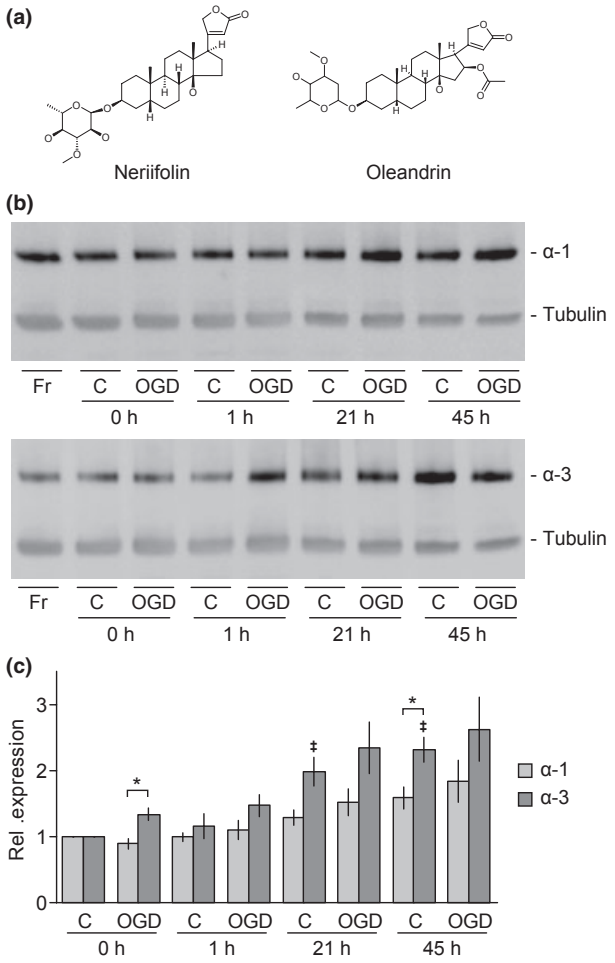

Fig. 2 The α3 subunit of the Na^+, K^+-ATPase, which preferentially binds oleandrin, is expressed in rat brain slices and increases with time in explant culture. (a) Close structural relationship between oleandrin and neriifolin, which we have shown in previous studies (Wang *et al.* 2006) to provide neuroprotection to OGD-treated brain slices and in two independent whole-animal models of focal ischemia. (b) Western blots of rat brain slice lysates harvested at the indicated times in explant culture compared with those that were freshly cut ('Fr'). Expression of the α1 subunit of the Na^+, K^+-ATPase appears to increase slightly over the first 2 days in explant culture, whereas the α3 subunit increases more dramatically, compared with the internal tubulin control. Six brain slices were included in each sample; 'C', control brain slices; 'OGD', brain slices subjected to 5.5 min OGD at the time of brain slice preparation. (c) Densitometric analysis of three independent Western blotting experiments as shown in panel (b), relative to control expression levels at the time of initial brain slice preparation, and normalized to internal tubulin controls. ‡Significant increases in relative expression level of α3 compared with 0 h; and *significant increases in the α3 : α1 ratio of expression, by a Student's *t*-test at the 0.05 significance level.

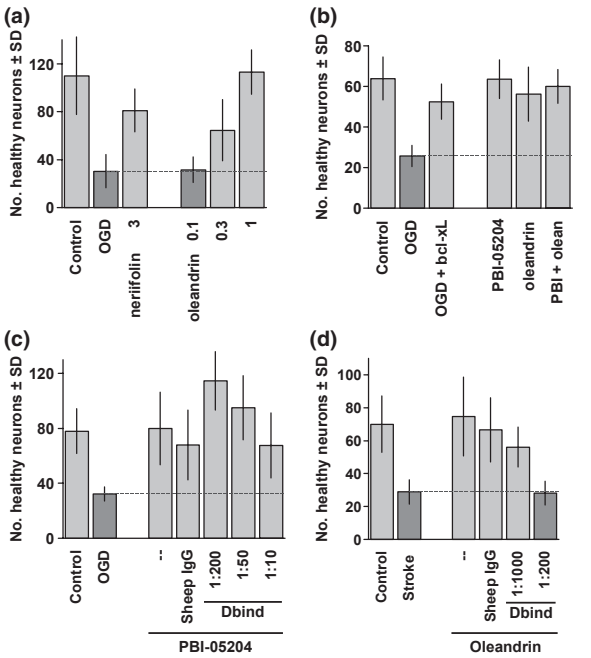

Fig. 3 Oleandrin is a neuroprotective component of PBI-05204, but other neuroprotective constituents of PBI-05204 may also be active in the brain slice assay. (a) Oleandrin provides significant neuroprotection in the OGD brain slice assay, to levels that equal or exceed that provided by neriifolin. Concentrations shown in μM. (b) Co-treatment using just sub-maximal concentrations of PBI-05204 (23 μg/mL) and oleandrin (1 μM) are not additive compared with levels of neuroprotection provided by each compound alone. The PBI-05204, oleandrin, and PBI-05204 + oleandrin conditions were not significantly different from each other. Internal positive control was co-transfection with the anti-apoptotic gene *bcl-xL*. (c, d) Fab fragments targeting digoxin and related cardiac glycosides including oleandrin (Digibind, 'Dbind') were ineffective in inhibiting the neuroprotective effects of 23 μg/mL PBI-05204 through the indicated dilution range (c). In contrast, low dilutions of Digibind in a similar concentration range were able to block completely the neuroprotective effects of 1 μM pure oleandrin (d). No Digibind added ('– –') and pre-immune whole sheep IgG were the internal controls. For (a–d), light gray bars denote statistically significant difference with respect to the OGD condition by ANOVA followed by Dunnett's *post hoc* comparison test at the 0.05 confidence level.

A more extended analysis of the concentration–response relations of oleandrin and PBI-05204 supported this hypothesis. First, we found that the concentration–response relation for oleandrin in the brain slice OGD assay showed a similar profile to that which we previously established for neriifolin (Wang *et al.* 2006), though with some 10-fold greater apparent potency (Fig. 4a). However, we found that PBI-05204 had a considerably broader concentration range over which neuroprotection was provided, spanning some four orders of magnitude (Fig. 4b). Assuming that PBI-05204 is composed of ~3% oleandrin as per MS analysis (Table 1), superimposition of the oleandrin concentration–response relation would suggest that only the upper range of the concentration–response relation for PBI-05204 can be accounted for by the neuroprotective activity of oleandrin (Fig. 4b).

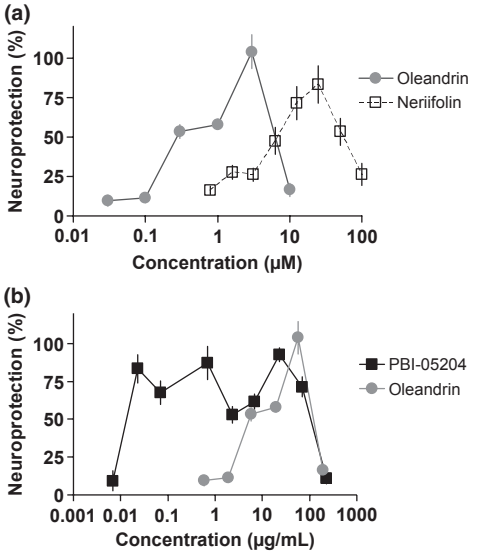

Fig. 4 PBI-05204 shows a broader concentration-response relation for neuroprotection than does oleandrin. (a) Concentration-response relation for oleandrin (gray filled circles). Compendium of six runs normalized to the difference between internal positive (non-OGD) and negative (OGD) controls for each run set to 100%; means ± SEM are shown. The concentration relation for neriifolin (open squares) is reprinted here from Wang *et al.* (2006) for ease of comparison. (b) Concentration-response for PBI-05204 (black filled squares). Compendium of 13 runs normalized to the difference between internal positive (non-OGD) and negative (OGD) controls for each run set to 100%; means ± SEM are shown. Data for oleandrin from panel (a) are replotted here on the same concentration axis assuming 3% composition for oleandrin in the PBI-05204 extract (see Table 1).

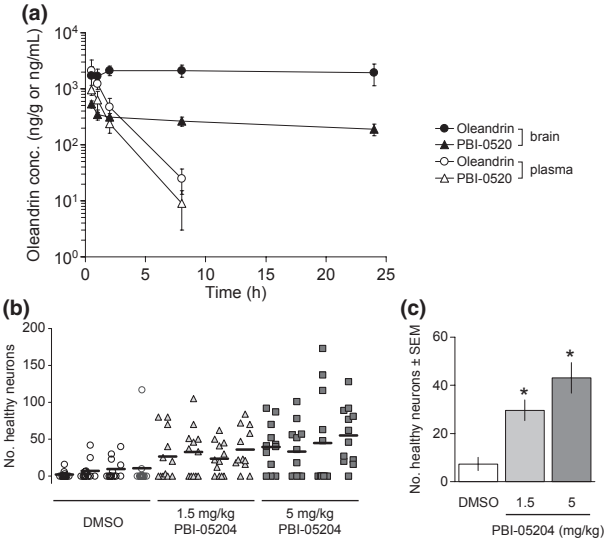

Fig. 5 *In vivo* systemic administration of PBI-05204 provides lasting neuroprotection to CNS tissues. (a) Concentration–time relation in plasma and brain tissue for oleandrin over 24 h post i.p. administration of PBI-05204 (triangles) and oleandrin (circles) in CD1 mice. Mean ± SD oleandrin concentration in brain (ng/g) and plasma (ng/mL) for five mice per time point are shown. PBI-05204 (38 mg/kg containing 0.8 mg/kg oleandrin, 50 μL) and oleandrin (3 mg/kg, 100 μL) were dissolved in DMSO : PEG400 vehicle, 50 : 50 v/v (PBI-05204, 28.6 mg/mL) or 25 : 75 v/v (oleandrin, 1 mg/mL); control groups received DMSO : PEG400 vehicle alone. At 0.5, 1, 2, 8 and 24 h, plasma and brain were harvested for LC/MS/MS analysis. Rapid and sustained CNS penetration was observed following both PBI-05204 and oleandrin administration. (b) PBI-05204, 1.5 or 5 mg/kg, dissolved in 50 μL DMSO, was injected i.p. into PND 9 rat pups; control pups received DMSO only. Twenty-four hours later, brain slices were prepared from these rat pups, in the absence of any neuroprotective agents during the cutting procedure, then biolistically transfected with YFP and placed into explant culture for an additional 24 h. Under these conditions, control brain slices show minimal survival. However, brain slices taken from rat pups treated with PBI-05204 were significantly neuroprotected, assessed using 12 brain slices prepared from each rat pup. Numbers of healthy pyramidal neurons in each of 12 brain slice explants prepared from each rat pup are shown for the 4 individual rat pups in each experimental condition. Representative run of three runs with similar results. (c) Brain slice data from panel (b) were pooled by treatment condition. Mean ± SEM numbers of healthy neurons per brain slice are shown; *significant difference with respect to the DMSO control condition by ANOVA followed by Dunnett's *post hoc* comparison test at the 0.05 confidence level.

Finally, we asked if PBI-05204 can provide neuroprotection to CNS tissues when delivered in an *in vivo* context. Unlike digoxin, we found that at least some of the chemical components of PBI-05204, notably oleandrin, can cross the intact BBB (Fig. 5a). In this experiment, systemic administration of oleandrin and PBI-05204 in mice resulted in rapid CNS penetration within 30 min and retention of oleandrin concentrations in brain tissue > 30-fold that in plasma at 8 h (Fig. 5a). Of note, mice were observed to be more sensitive to PBI-05204 compared with equivalent doses of oleandrin alone. PBI-05204 (143 mg/kg; equivalent to 3 mg/kg oleandrin) resulted in clinically observed CNS toxicity within 30 min, although near equivalent oleandrin concentrations were measured in brain tissue (1629 ± 464 ng/mL) compared with that of 3 mg/kg oleandrin alone (1698 ± 786 ng/mL). These data, coupled with concentration–response data described above, further suggest that other bioactive components may contribute to the pharmacologic effect of PBI-05204, and that optimal neuroprotection may be achievable at lower oleandrin-equivalent doses of extract compared with oleandrin alone.

To test functionally whether sufficient PBI-05204 can be delivered to brain via systemic administration to provide neuroprotection to CNS tissues, PBI-05204 was injected i.p. into rat pups on PND 9. Twenty-four hours later, at PND 10, brain slices were prepared and transfected as above, except that the neuroprotectant usually applied during brain tissue slicing, MK-801, was omitted. This protocol results in pathophysiological damage to brain slices that is similar to that resulting from OGD, but minimizes the numbers of steps in

the experimental procedure. As expected, brain slices taken from control pups injected with DMSO only showed very few viable neurons after YFP transfection and 24 h of *in vitro* incubation (Fig. 5b and c). In contrast, brain slices prepared from pups injected with 1.5 or 5 mg/kg PBI-05204 (1/6 and 1/2 of maximally tolerated dose, respectively) showed significant levels of neuroprotection, with higher mean levels of neuroprotection provided by the higher dose (Fig. 5b and c). These results indicated that systemic delivery of PBI-05204 was capable of conferring neuroprotection to CNS tissues that persisted even after their removal from the animal and withdrawal from any continuing source of PBI-05204 for at least a 24-h period.

Discussion

We have shown that the cardiac glycoside oleandrin can provide robust neuroprotection in a brain tissue-based model for ischemic injury, reminiscent of that which we have previously established for the related cardiac glycoside, neriifolin, in this brain slice model as well as in two independent whole-animal models of ischemic stroke (Wang *et al.* 2006). Interestingly, the effectiveness of oleandrin in inhibiting human cancer cell growth is associated the expression of the α3 subunit of the Na^+, K^+-ATPase (Yang *et al.* 2009), and whereas three of the four known α-subunit Na^+, K^+-ATPase isoforms are expressed in brain (all but α4), the α3 isoform shows primarily neuronal expression in the adult CNS (McGrail *et al.* 1991; Watts *et al.* 1991). Together with similar neuroprotective properties we previously reported for digoxin, digitoxin, and ouabain in the brain slice model (Wang *et al.* 2006), we suggest that this emerging pharmacology of neuroprotection indicates the Na^+, K^+-ATPase (Kaplan 2002) as a likely target of action of oleandrin, as well as of the botanical drug PBI-05204, derived from an extract of the *Nerium oleander* plant, whose principal active component is oleandrin.

Intriguingly, PBI-05204 was originally developed as a clinical candidate for the treatment of cancer, and represents an increasing appreciation of the utility of target engagement with the Na^+, K^+-ATPase enzyme in cancer therapy (Newman *et al.* 2008; Prassas and Diamandis 2008). The benefits of cardiac glycosides in cancer and focal ischemia may be mediated through inhibition of the classically known function of Na^+, K^+-ATPase, namely, the active transport of Na^+ and K^+ ions across the plasma membrane (Kaplan 2002). As the Na^+, K^+-ATPase accounts for 40–70% of ATP consumption in the brain (Astrup 1982; Clausen *et al.* 1991; Friberg and Wieloch 2002), one possible neuroprotective mechanism in stroke is that inhibition of Na^+, K^+-ATPase preserves ATP levels at a time when catastrophic decreases in ATP levels are leading to necrotic and other forms of neuronal cell death (Golstein and Kroemer 2007; Besancon *et al.* 2008). Such a mechanism as part of a metabolic 'arrest' of transmembrane ionic gradients was proposed by Hochachka (1986) as a defense strategy against hypoxia across the animal kingdom.

Additionally, while excess Ca^{2+} entry is a principal component of excitotoxicity (Choi 1995; Pettmann and Henderson 1998), drastic decreases in cytoplasmic Ca^{2+} can also be damaging, especially in the penumbra of stroke (Franklin and Johnson 1992; Johnson *et al.* 1992; Lee *et al.* 2000). In fact, abnormally low levels of intracellular Ca^{2+} have been reported in models of transient ischemia in a 1–3 day period corresponding to the progression of cell death in the penumbra (Connor *et al.* 1999). Inhibition of the Na^+, K^+-ATPase would likely lead to increases in neuronal intracellular Ca^{2+} by the same mechanism as the inotropic effect of digitalis compounds in treating congestive heart failure, namely, the secondary inhibition of the Na^+/Ca^{2+} exchanger via erosion of the Na^+ transmembrane gradient (Hardman *et al.* 1996). In fact, the Na^+/Ca^{2+} exchanger itself has been directly implicated in neuronal survival in stroke models, although the mechanism remains unclear (reviewed in Besancon *et al.* 2008).

However, it is also possible that modulation of the pleiotropic signaling functions of the Na^+, K^+-ATPase may be involved in the neuroprotective effects reported here. There is a growing appreciation, for example, that Na^+, K^+-ATPase does not operate in isolation as a simple transmembrane pump, but rather forms the central core of a complex signalosome which has been described as a vesicular, multimolecular signaling complex that is assembled in caveolae and delivers signals to specific intracellular components such as mitochondrial membranes (Garlid *et al.* 2009). Other activities associated with the Na^+, K^+-ATPase signalosome include elevated intracellular Ca^{2+}, activation of Src and the ERK1/2 pathways, as well as activation of phosphoinositide 3-kinase and protein kinase B (Akt) (Schoner and Scheiner-Bobis 2007). Additional involvement of one or more of these alternative, Na^+, K^+-ATPase signalosome-associated pathways in oleandrin-mediated neuroprotection cannot be excluded at present.

We have also provided indirect evidence for additional non-cardiac glycoside components contained in PBI-05204 that provide neuroprotection in the brain slice stroke assay. In future studies, it will be of interest to determine whether molecular components mediating these neuroprotective effects, distinct from oleandrin and other cardiac glycoside constituents of PBI-05204, can be isolated and identified. Alternatively, it is possible that these additional components in PBI-05204 may act as co-factors in cardiac glycoside action at the Na^+, K^+-ATPase, perhaps binding to different sites in the extended Na^+, K^+-ATPase receptor complex or modulating enzyme-associated signalosome activities and function.

Finally, although a full, *in vivo* study of PBI-05204 in whole-animal models of stroke was beyond the scope of the

present report, we have shown here that lasting neuroprotective benefit can be conferred by PBI-05204 via its systemic delivery in an *in vivo* setting in which the BBB remains intact. In contrast, digoxin, the only cardiac glycoside in current clinical usage in the U.S., does not cross the BBB. Although this approach could not be used to determine directly an *in vivo* time window for therapeutic benefit following focal ischemia, we could show in explanted brain slice assays that the neuroprotective benefit of PBI-05204 is maintained for up to a 4–6 h delay of compound administration after OGD. Collectively, these attributes of PBI-05204-mediated neuroprotection suggest its potential use in the treatment of clinical stroke, and extend previous reports from us and others implicating inhibition of the Na^+, K^+-ATPase in providing neuroprotection against ischemic injury (Wang *et al.* 2006; Tzen *et al.* 2007; Gottron and Lo 2010).

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Conflict of interest disclosure

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