

## Research paper

# CNB-001, a pleiotropic drug is efficacious in embolized agyrencephalic New Zealand white rabbits and ischemic gyrencephalic cynomolgus monkeys

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## ABSTRACT

Ischemic stroke is an acute neurodegenerative disease that is extremely devastating to patients, their families and society. Stroke is inadequately treated even with endovascular procedures and reperfusion therapy. Using an extensive translational screening process, we have developed a pleiotropic cytoprotective agent with the potential to positively impact a large population of brain ischemia patients and revolutionize the process used for the development of new drugs to treat complex brain disorders. In this unique translational study article, we document that the novel curcumin-based compound, CNB-001, when administered as a single intravenous dose, has significant efficacy to attenuate clinically relevant behavioral deficits following ischemic events in agyrencephalic rabbits when administered 1 h post-embolization and reduces infarct growth in gyrencephalic non-human primates, when administered 5 min after initiation of middle cerebral artery occlusion. CNB-001 is safe and does not increase morbidity or mortality in either research species. Mechanistically, CNB-001 inhibits human 5- and 15-lipoxygenase *in vitro*, and can attenuate ischemia-induced inflammatory markers, and oxidative stress markers, while potentially promoting synaptic plasticity mediated by enhanced brain-derived neurotrophic factor (BDNF).

## 1. Introduction

Ischemic brain diseases are not effectively treated due to the lack of therapeutic tools at the physician's disposal; this in part is due to high cost of drug development and the lack of dedicated focused funding for translational research (Lapchak and Zhang, 2017). Acute ischemic stroke (AIS) is the fifth leading cause of death and the leading cause of adult disability in the USA with an estimated cost of \$68.9 billion annually (Lapchak and Zhang, 2017; Writing Group M et al., 2016; Benjamin et al., 2017). Each year there are approximately 800,000 new stroke victims in the USA; the majority of stroke victims suffer from cardiovascular disease and other chronic conditions at the time of the stroke, primarily diabetes and/or hypertension (AHA Writing Group M et al., 2016; Go et al., 2013a,b). In the USA, stroke kills 130,000 victims annually, and incidence rate has stabilized at 795,000 victims annually (Benjamin et al., 2017; Lapchak and Zhang, 2018). In the United Kingdom, stroke is the 4th largest cause of death with an annual incidence of 152,000 and 12.5% of stroke victims die within 30 days.

For stroke patients, a single thrombolytic reperfusion treatment strategy has been utilized to provide an option to patients (Fang et al., 2010; Reeves et al., 2005; Schwamm et al., 2013; Lyden, 2015), but only 7–10% of all patients receive or are eligible for treatment. The current standard-of-care and physicians' treatment of choice for acute ischemic is tissue plasminogen activator (tPA), a thrombolytic that promotes recanalization, and is so far the only Food and Drug Administration (FDA)-approved treatment for stroke (Lapchak, 2002; Schellinger et al., 2001a; Schellinger et al., 2001b; Verstraete, 1999) that improves clinical function measured using either the National Institutes of Health Stroke Scale (NIHSS) or modified Rankin scores (mRS) (NINDS, 1995; Lees et al., 2010; Hacke et al., 2008; Savitz et al., 2007). More recently, endovascular approaches to remove the embolus in large vessel-occluded patients has also proven beneficial in a select patient population as demonstrated in a series of recent trials (Berkhemer et al., 2015; Goyal et al., 2015; Campbell et al., 2015; Jovin et al., 2015; Saver et al., 2015a; Rebello et al., 2016). In the select LVO patient population, endovascular procedures can be used as late as 24 h after a stroke, but

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the procedures are not typically applied to non-LVO strokes, leaving the vast majority of strokes inadequately treated (Desai et al., 2018; Jovin et al., 2018; Nogueira et al., 2018).

The lack of clinically effective cytoprotective therapeutics may be due to the historical use of a single drug target development strategy (i.e. target-centric), which cannot effectively address the complex pathophysiology of stroke in the clinical setting, and an abundance of research conducted in small animals, rats and mice. There is a gap in translational knowledge that limits rodent modeling, thereby warranting stroke studies be conducted in rabbits and non-human primates. Historically, there have been many failed attempts at neuroprotection (O'Collins et al., 2006; Lapchak and Boitano, 2017), most of which have been ill-designed clinical trials with inappropriate drug candidates and or drug targets (O'Collins et al., 2006; Lapchak and Boitano, 2017). However, scientific advances and a greater understanding of the complex processes underlying ischemic events is only now allowing researchers to design efficacious molecules, that remain to be tested in clinical trials (See (Lapchak and Zhang, 2017; Lapchak and Uteshev, 2017; Lapchak and Zhang, 2012; Lapchak and Boitano, 2017; Lapchak, 2017a,b)).

The initial ischemic event activates and propagates, in a time-dependent fashion, a series of detrimental and devastating insults, including excitotoxicity, mitochondrial dysfunction and reduced energy metabolism, oxidative stress, inflammation, and loss of trophic support (O'Collins et al., 2006; Lapchak and Boitano, 2017; Prior et al., 2014; Pangalos et al., 2007; Swinney and Anthony, 2011), amongst others. Our data suggests that a novel strategy to effectively attack multiple cascade targets has numerous potential advantages to treat stroke victims (Lapchak and Zhang, 2017; Dirnagl, 2012; Dirnagl et al., 1999; Lo and Ning, 2016; Terasaki et al., 2014; Fisher, 2011; Lok et al., 2015; Xing et al., 2012; O'Collins et al., 2011, 2012; Wahlgren and Ahmed, 2004). Thus, a single pleiotropic molecule or a cocktail of molecules that blocks multiple pathways of the ischemic cascade, provides protection to the neurovascular unit, and possibly even promote plasticity, tissue repair or stimulates endogenous stem cells would be beneficial to patients (Han-Sen et al., 2017; Amar et al., 2015; Lapchak, 2011; Cramer et al., 2011; Moskowitz et al., 2010).

We have synthesized and extensively characterized the curcumin-based pleiotropic compound, CNB-001 [4-((1E)-2-(5-(4-hydroxy-3-methoxystyryl)-1-phenyl-1H-pyrazoyl-3-yl)vinyl) -2-methoxy-phenol], a cytoprotective compound with potent neurotrophic and neuroprotective cell survival activities in vitro (Liu et al., 2008a). CNB-001 is an attractive drug candidate that is the first in class candidate from a new series of compounds that may be utilized as a treatment for ischemic disease; this may be a seminal advancement in stroke research.

In this article, we describe a drug development platform and screening methodology demonstrating that CNB-001 has significant efficacy to attenuate behavioral deficits following ischemic events in two agyrencephalic and gyrencephalic stroke models. In the present study, executed in a manner consistent with current STAIR (Fisher, 2003; STAIR, 1999) and RIGOR guidelines (Lapchak, 2013; Landis et al., 2012), we systematically studied the potential benefit of intravenously-administered CNB-001 as a drug candidate in stroke models, with all treatment given intravenous (IV) post-ischemia to simulate most likely clinical use. The models include the standardized positive-controlled rabbit small clot embolic stroke model (RSCM) that was critical to the FDA approval of rt-PA (Zivin et al., 1985; Lapchak, 2015; Lapchak, 2010), and a non-human primate (NHP) middle cerebral artery occlusion (MCAO) model of penumbral infarct expansion using diffusion-weighted magnetic resonance imaging (DWI MRI) analysis (Cook and Tymianski, 2012; Cook et al., 2012a,b). The models that we have chosen for the development of CNB-001 have the advantage of effectively simulating ischemic stroke in two large animal species, and may be representative of the diverse patient population that would eventually be enrolled in clinical trials (NINDS, 1995; Hacke et al., 2008; Jickling and Sharp, 2014; Lapchak, 2013, Lapchak, 2015,

Lyden and Lapchak, 2012) to further study CNB-001.

The study demonstrates that the pleiotropic compound, CNB-001, has significant efficacy in male rabbits and NHP's following cerebral ischemia. Moreover, a survey of mechanism studies from in vitro and in vivo assays show that CNB-001 is a potent 5- and 15-LOX inhibitor, anti-inflammatory, regulator of neurotrophic factor and synaptic plasticity pathways, and can attenuate the spread of the ischemic penumbra in a permanent occlusion in NHP's. These mechanistic insights form the basis for additional in-depth mechanism studies.

## 2. Materials and methods

### 2.1. Animals and animal welfare

All procedures were approved by the Cedarsinai Institutional Animal Care Committee (IACUC) and overseen by a veterinarian. This study utilized male New Zealand white rabbits (*Oryctolagus cuniculus*) obtained from a commercial supplier in California. Rabbits were supplied food (alfalfa cubes) and water ad libitum while under quarantine in an enriched environment for at least 3 days prior to experimental use. Rabbits were humanely euthanized if they were in pain, showed extreme discomfort, or if they were unable to reach food or water after embolization or ischemia. Studies were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

This study also utilized male non-human primates, cynomolgus monkeys (5–6 kg) obtained from a commercial vendor. All animals were purchased under a Queen's University IACUC-approved protocol for both surgical and treatment procedures used in this study and overseen by a primate veterinarian.

### 2.2. Drug preparation & drug administration

CNB-001 was synthesized by AQ BioPharma Co., Ltd. (Shanghai, China) according to Liu et al., 2008 (Liu et al., 2008b). Purity is > 99% using HPLC analysis (not shown), and the molecule was confirmed by NMR analysis. CNB-001 was dissolved in 30% Solutol HS15/70% normal saline (Vehicle, a vehicle that has been used clinically to solubilize a variety of drugs (Strickley, 2004)) and maintained at 37–39 °C prior to intravenous (IV) injection (slow bolus). The vehicle was also maintained at 37–39 °C prior to IV injection.

#### 2.2.1. In vitro assays

2.2.1.1. *Enzyme inhibition assay.* 1) 5-LOX: Human peripheral blood mononuclear lymphocytes (PBML) cells were pre-incubated for 15 min at 37 °C in Hank's balanced salt solution and then 30 μM arachidonic acid (AA) was added for another 15 min in the presence of increasing concentrations of CNB-001 or nordihydroguaiaretic acid (NDGA), as a positive control. Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) was quantified by LC/MS and data is presented as percentage of inhibition of LTB<sub>4</sub> generation; 2) COX-1 and 12-LOX: Human platelets were used with substrate 100 μM arachidonic acid or 30 μM AA, respectively. PGE<sub>2</sub> are 12-HETE were measured as enzyme products, respectively; 3) COX-2: Human recombinant insect Sf21 cells were used with substrate 0.3 μM arachidonic acid and PGE<sub>2</sub> was measured; 4) 15-LOX: Rabbit reticulocytes were used with substrate 260 μM linoleic acid and 13-HPODE was quantified.

#### 2.2.2. In vivo assays

Pharmacokinetic (PK) Profile in New Zealand White Rabbits. All animals were purchased under the Pharmaron Inc. IACUC-approved protocol for treatment procedures used in the PK study. The PK profile of IV-administered CNB-001 was determined by Pharmaron Inc. (Irvine, CA). Since IV dosing is a preferred route of administration for a stroke victim, we have completed the PK analysis of IV-administered CNB-001 in male New Zealand white rabbits (2–3 months old) that were given a

bolus IV injection of CNB-001 (10 mg/kg) and blood was drawn at 9 specific time points; 5 min, 15 min, 30 min, 1, 2, 4, 6, 8, and 24 h post dose to determine the half-life ( $t_{1/2}$ ) of the compound. Brain content was measured at 1, 3 and 6 h in each species following a single IV dose administration.

**2.2.2.1. Rabbit small clot embolic stroke model (RSCEM) studies.** The procedure for small clot embolic stroke was performed as described previously (Lapchak et al., 2007; Lapchak et al., 2004a; Lapchak, 2015; Lapchak et al., 2015). Briefly, rabbits were anesthetized with isoflurane and the right internal carotid artery (ICA) was exposed, and the external carotid artery (ECA) and the common carotid artery (CCA) were ligated. A Becton, Dickinson and Company (B-D) plastic catheter oriented toward the brain was inserted into the CCA positioned toward the brain and secured with ligatures. The incision was closed around the catheter so that the distal end was accessible outside. The catheter was filled with heparinized saline and plugged with injection caps. The animals were then allowed to recover from anesthesia for at least 2 h before embolization.

Blood was drawn from one or more donor rabbits and allowed to clot for 2–3 h at 37 °C. The large blood clot was then suspended in phosphate-buffered saline pH 7.4 (PBS) with 0.1% bovine serum albumin (BSA) and Polytron-generated fragments were sequentially passed through sizing screens to result in a suspension of small-sized blood clots, which were suspended in PBS containing 0.1% bovine serum albumin. The blood clot suspension was then spiked with  $^{57}\text{Co}$ -labeled microspheres (Perkin-Elmer Inc.) to allow for tracking into the brain. For embolization, rabbits were placed in a restrainer and 1 ml of a clot particle suspension was injected through the carotid catheter, which was then flushed with sterile saline. Rabbits are in the absence of anesthetics, and analgesics, and fully conscious during the embolization procedure and they are self-maintaining (i.e. they do not require artificial respiration or other external support) throughout the study. This has the benefit of allowing for immediate observation of the effects of clot embolization on behavior at the time of embolus injection and thereafter. After the embolization process was completed, the catheter was heat-sealed ligated close to the neck.

For test substance administration, rabbits were placed in a Plexiglas restrainer (Plaslabs Inc.) for the duration of the treatment. For the post-embolization limited therapeutic window study, rabbits were administered 10 mg/kg CNB-001 either 5 or 60 min post-embolization. For the dose-response curve study, embolized rabbits were administered CNB-001 1–50 mg/kg, 1 h post-embolization, with concurrent vehicle-treated embolized rabbits run in parallel. For Western blot and ELISA studies, drug was administered IV 1 h post-embolization, and cortical tissues were removed 6 h post-dosing.

**2.2.2.2. RSCEM: Embolic stroke quantal analysis.** The use of an embolic stroke model with clinical rating scores is a highly desirable primary endpoint to use when a novel therapeutic is being tested for further development and to support a clinical trial (Zivin et al., 1985; Jickling and Sharp, 2014; Lapchak, 2010; Turner et al., 2011; Zivin and Waud, 1992). Clinical scores in combination with quantal analysis is a sophisticated statistical analysis method to determine how a large population of stroke “patients”, in this case, rabbits, will respond to a treatment.

To evaluate the quantitative relationship between clot dose in brain and behavioral deficits or clinical scores, logistic sigmoidal (S-shaped) quantal analysis curves are fit to the dose-response data as originally described by Waud (Waud, 1972) and thereafter (Lapchak, 2002; Lapchak et al., 2007; Lapchak et al., 2004a; Lapchak et al., 2004b; Lapchak et al., 2018). Embolized rabbits are scored as abnormal if they have one or more of the following symptoms: ataxia, leaning, circling, lethargy, nystagmus, loss of balance, loss of limb/ facial sensation and occasionally, hind-limb paraplegia. Using this rating system with low inter-rater variability (< 5%), each surviving animal is rated as either

behaviorally normal or abnormal by an observer naïve to the study design and/or treatment groups. Death is also on the continuum of ratings. Because this study was conducted randomized and blinded, there was no investigator bias in behavioral scoring of embolized rabbits.

A separate quantal curve is generated for each treatment condition and a statistically significant increase in the  $P_{50}$  value or the amount of microclots in brain that produce neurologic dysfunction in 50% of a group of animals compared to control is indicative of a behavioral improvement and cytoprotection.

**2.2.2.3. Non-human primate (NHP) ischemia model.** The NHP middle cerebral artery occlusion (MCAO) model previously described by Cook et al. was also used to evaluate the efficacy of CNB-001 (Cook et al., 2012a). All procedures were approved by the Queen's University Animal Care Committee and overseen by a primate veterinarian. Baseline structural and functional neuroimaging were acquired prior to MCAO to provide baseline data. Animals underwent right MCAO using a pterional craniotomy approach under general anesthesia. Body temperature, blood pressure, blood glucose were maintained within normal physiologic ranges during the procedures (Cook et al., 2012a). CNB-001 was administered IV 5 min following the start of MCAO. MRI was performed during the MCAO to determine the perfusion deficit (tissue at risk) and to evaluate early stroke volume on diffusion weighted imaging, providing a measure of ischemic penumbra volume (Perfusion Volume minus Diffusion Weighted Infarct volume).

**2.2.2.4. NHP MRI imaging.** MRI imaging was used to obtain baseline structural data on T1 and T2 weighted images. Acute MRI imaging following stroke included perfusion and diffusion weighted imaging (PWI and DWI respectively) (Cook et al., 2012b; Toth and Albers, 2009). After the ischemia procedure, anesthetized animals were positioned prone in a cylindrical bed and imaged in a 3 T Siemens Trio MRI. Stacks of axial T2- and diffusion-weighted (three orthogonal diffusion directions at  $b = 1000 \text{ s/mm}^2$ ) images were obtained. Diffusion-weighted images were post-processed with MATLAB to produce average diffusion-weighted images from the three directions obtained. Hyperintense regions were identified, and regions of interest (ROIs) were traced around each lesion. The ROIs were determined and their volumes were calculated by multiplying by MRI slice thickness (2 mm). DWI volume was obtained serially over 6 h to provide a response curve for DWI hyperintensity accumulation. An arterial spin labeled PWI of the whole brain was acquired during the period of ischemia to confirm complete MCA occlusion and define the region of tissue at risk of infarction.

### 2.2.3. Target engagement and mechanism of action survey studies

We utilized an in vitro panel of enzyme assays, and a rabbit embolic stroke model to provide some insight into possible important mechanisms of action of CNB-001. For Western blot (WB) and ELISA, we utilized a common 6 h tissue collection time to harvest tissues for assay. The primary antibodies used for WB, and ELISA kits used in this study are provided in Table 1.

**2.2.3.1. Western blot.** For WB analysis, we harvested rabbit cortical tissues from the ipsilateral ischemic hemispheres were removed 6 hours post-drug treatment (i.e.: 7 hours post-embolization). Tissue was maintained at  $-80 \text{ }^\circ\text{C}$  until they were homogenized in 10 volumes of Tris-buffered saline (TBS) containing a cocktail of protease inhibitors [20 mg/ml each of pepstatin A, aprotinin, phosphoramidon, and leupeptin; 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF); 1 mM EGTA; 5 mM fenvalerate; and 5 mM cantharidin as described previously (Lapchak et al., 2011a). Samples were sonicated  $2 \times 10 \text{ s}$ . Protein concentrations were determined using the BCA protein assay (Pierce). Equal amounts of protein were solubilized in  $2.5 \times$  SDS-sample buffer, separated on 12% SDS-

**Table 1**  
Antibodies and ELISA kits.

Antibody	Species (cross-reactivity)	Dilution	Source
COX-2	Rabbit	1:1000	Santa Cruz
VCAM	Rabbit	1:1000	Santa Cruz
P-ERK	Rabbit	1:1000	Santa Cruz
5-LOX	Rabbit	1:1000	BD
Pro-BDNF	Rabbit	1:1000	Santa Cruz
BDNF	Rabbit	1:1000	MyBiosource
Actin	Rabbit	1:1000	Cell signaling
ELISA	Species (cross-reactivity)	ID number	Source
BDNF	Rabbit	ABIN572355	Cloud Clone
NT-3	Rabbit	ABIN775293	Blue Gene

polyacrylamide gels, transferred to nitrocellulose, developed, and scanned for quantitation. Protein levels were normalized to actin levels. ANOVA assays with the Tukey post-hoc test were used to determine differences between means. Two-way ANOVA with repeated measures were used and Tukey or Dunnett post hoc tests were employed for multiple comparisons. Significance was rejected at the  $p > .05$  level.

For rabbit cortical tissues, we also conducted BDNF (Brain-derived neurotrophic factor: SEA011Rb, Cloud Gene) and NT-3 (Neurotrophin 3: ABIN775293, Supplier: Blue Gene) ELISA's according to the manufacturer's instructions. We were unable to detect NGF in rabbit cortex using the antibody competition assay (ABIN775289, Supplier: Blue Gene; not shown), but both BDNF and NT-3 were measurable under all conditions using the sandwich ELISA.

#### 2.2.4. RIGOR, power and statistical analysis

The studies were conducted randomized and blinded per STAIR (Fisher, 2003; Lapchak and Zhang, 2018, STAIR, 1999) and RIGOR guidelines and criteria (Spengos et al., 2000; Lapchak and Zhang, 2018, Schulz et al., 2010). For all experiments, rabbits and NHP's were randomly allocated into treatment groups before the surgical/stroke procedure, with concealment of the randomization guaranteed by using an independent party. The randomization sequence was not revealed until all analyses were complete.

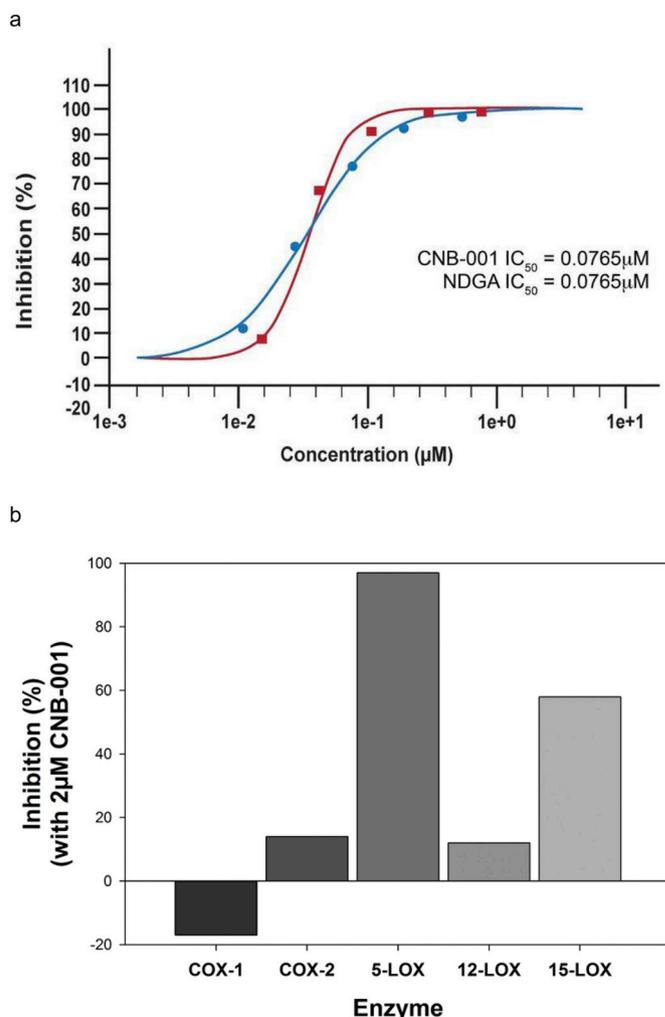
**2.2.4.1. Rabbits.** Power analysis of the quantal dose-response curves for rabbits indicates that, assuming  $\alpha = 0.05$ , a standard deviation of 25%, and a sample size of 14 animals per treatment group, we will have 90% power to detect a 50% change in mean  $P_{50}$  values.  $P_{50}$  (Lapchak et al., 2004a; Zivin and Waud, 1992). Behavioral data are analyzed for significance using the unpaired  $t$ -test (MedCalc Version 9.4.1.0) (Lapchak et al., 2011a; Lapchak et al., 2008; Lapchak et al., 2007).

**2.2.4.2. NHP.** Power analysis was based upon assuming  $\alpha = 0.05$  and a beta of 0.8. Assuming a difference in final volume of stroke of 30% in treated animals with a standard deviation of 15% we determined that 4 animals per group were required to power the experiment (Cook et al., 2012a). Repeated measures two-way ANOVA was used to assess statistical significance.

### 3. Results

#### 3.1. CNB-001 pharmacological characteristics

CNB-001 has medicinal chemistry characteristics associated with a drug that is lipophilic ( $\text{clog}P = 4.67$ ) and can penetrate the blood brain barrier (BBB) (Pajouhesh and Lenz, 2005). An actual  $\log P$  value of 3.75 was measured by Pharmaron Inc. (Irvine, CA). During initial screens, we established that CNB-001 is also a high potency human 5-LOX inhibitor in vitro (Fig. 1a). The  $IC_{50}$  for CNB-001 is 0.076  $\mu\text{M}$  and a high affinity positive control, NDGA 0.076  $\mu\text{M}$ . However, CNB-001 did not



**Fig. 1. CNB-001 In vitro Activity:** a) In vitro 5-LOX inhibition by CNB-001 and the positive control inhibitor NDGA. The  $IC_{50}$  for CNB-001 is 0.076  $\mu\text{M}$  and a high affinity positive control, NDGA 0.076  $\mu\text{M}$ . b) CNB-001 also inhibits 15-LOX, but not 12-LOX, COX-1 or COX-2. Controls were indomethacin for COX-2 ( $IC_{50}$  0.050  $\mu\text{M}$ ; COX-2 Rofecoxib  $IC_{50}$  0.101  $\mu\text{M}$ ; 12-LOX Baicalein  $IC_{50}$  0.68  $\mu\text{M}$ ; 15-LOX PD-146176  $IC_{50}$  0.76  $\mu\text{M}$ ). The assays were conducted by MDS Pharma Services during initial compound selection screening.

inhibit human 12-LOX when tested with concentrations as high as 2  $\mu\text{M}$  (Fig. 1b), but does inhibit 15-LOX, but CNB-001 is a less effective inhibitor for this enzyme. CNB-001 did not affect COX-1 or COX-2 activity.

PK analysis of CNB-001 indicated that the  $t_{1/2}$  was  $3.37 \pm 0.52$  h ( $n = 3$ ) in rabbits and the clearance rate was 44.2 ml/min/kg in rabbits, respectively. In separate groups of rabbits ( $n = 3$ ), we measured brain CNB-001 content, after an IV injection (10 mg/kg) and found that CNB-001 accumulated in brain within 1 h. Quantitative data showed that CNB-001 measured 1480 ng/g brain tissue at 1 h and decreased to 240 ng/g brain tissue by 6 h. We did not conduct PK analysis in NHP's because of the acute treatment and study assay design used to assess the effects of CNB-001 on MRI measures.

#### 3.2. Safety and toxicity profile

The safety profile of CNB-001 was also determined rabbits by Pharmaron Inc. using single IV dose of CNB-001 (10 mg/ml). As shown in Table 2, there were no gross behavioral abnormalities associated with CNB-001 administration. In addition, we used the Ames test (Ames et al., 1973a; Ames, 1973; Ames et al., 1973b; Lapchak, 2012)

**Table 2**  
CNB-001 clinical toxicology observations (conducted by Pharmaron Inc. during the PK study).

Clinical observation	IV dose (10 mg/kg)
	Rabbits
1. Convulsions- uncontrollable muscle contractions	No
2. Uncoordinated movement	No
3. Discoloration	No
4. Torpidity	No
5. Temperature change	No
7. Disuse of hind limbs, forelimbs	No
8. Dyspnea (labored breathing)	No
9. Frothing from the mouth	No
10. Ptosis, eyelid	No
11. Soft stool	No
12. Skin lesions	No
13. Unnatural Secretions	No
14. Obvious Weight drop	No
15. Mortality	No

studies were done as described previously (Lapchak et al., 2011b) using 2 strains of bacteria, specifically *S. typhimurium* TA989: hisD3052, rfa, uvrB/pKM101, which detects frameshift mutations and *S. typhimurium* TA100: hisG45, rfa, uvrB/pKM101, which detects base-pair substitutions either in the presence or absence of an S9 fraction from the livers of Aroclor 1254-treated rats (Aly and Domenech, 2009; Aly et al., 2009).

### 3.3. CNB-001 efficacy in embolized rabbits

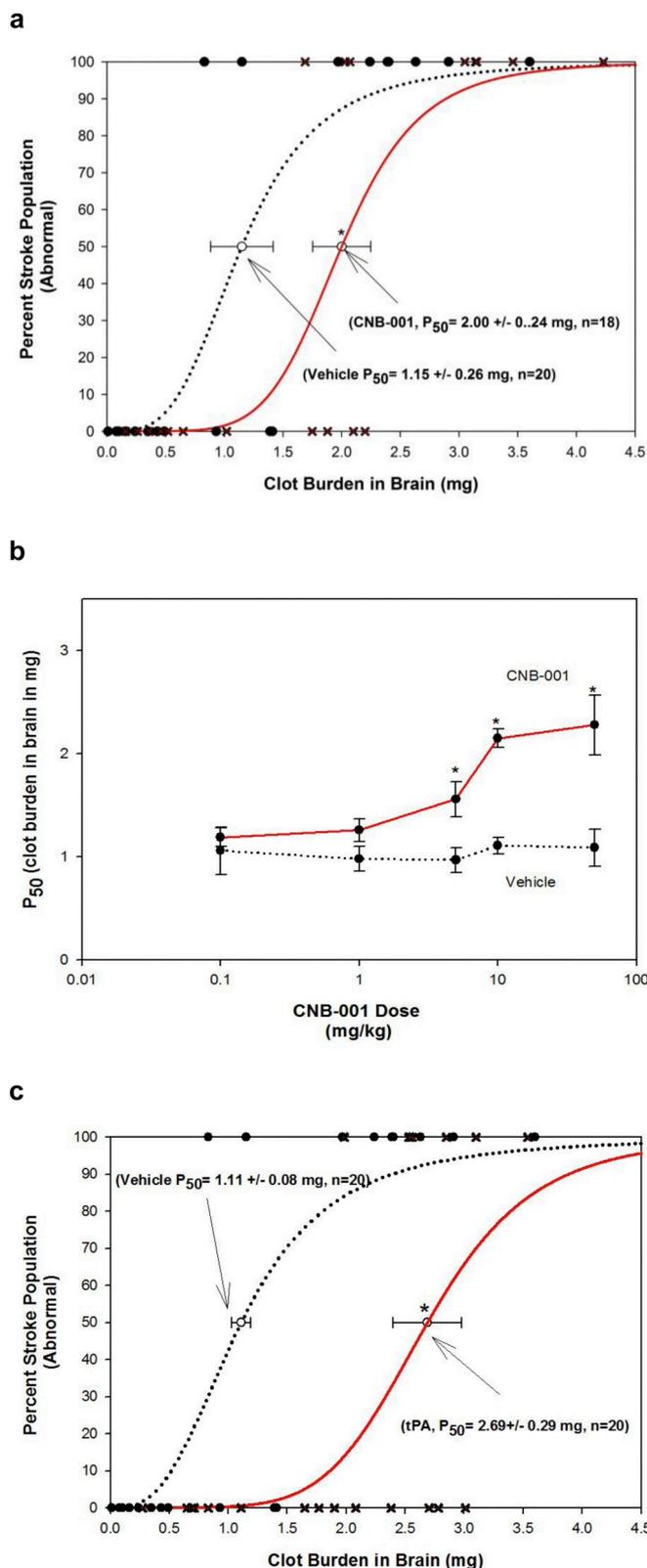
In male New Zealand white rabbits, CNB-001 improved clinical function in embolized rabbits. Rabbits underwent catheterization and small clot embolization followed by CNB-001 (10 mg/ml, IV) administration. In Fig. 2a, we show that CNB-001 administered 60 min after embolization significantly improved behavior measured 48 h following embolization (\**p* < .05) and increased P<sub>50</sub> by 74%. The increase in P<sub>50</sub> indicated that a CNB-001-treated rabbit was behaviorally normal when clot burden in brain increased. (See Table 3 for raw data). Note, CNB-001 was also effective and increased P<sub>50</sub> to 2.49 ± 0.19 (*n* = 21) when administered 5 min post-embolization (not shown). Fig. 2b shows that CNB-001 doses between 5 and 10 mg/ml are effective drug doses to significantly improve behavior measured 48 h after embolization (\**p* < .05), when the drug was administered 1 h after embolization, a time when standard-of-care therapy, tPA also significantly improves behavioral function (Zivin et al., 1985; Lapchak et al., 2004b). In this study, a 3.3 mg/kg tPA positive control group, run in parallel, had a P<sub>50</sub> value of 2.69 ± 0.29 mg (*n* = 20), which was statistically significant (*p* < .05) from the vehicle control curve for the 1 h treatment group (Fig. 2c).

### 3.4. CNB-001 reduces stroke expansion in NHP ischemia model

For this assay to determine the efficacy of CNB-001 in a NHP model, permanent occlusion of the MCAO was used in monkeys. Five minutes after the occlusion, CNB-001 (10 mg/kg) or vehicle was administered and serial diffusion weighted images were captured. As shown in Fig. 3a, with vehicle treatment there was a greater increase in the fraction of diffusion volume measured over time compared to the CNB-001-treated group. While there was some heterogeneity in response, with *n* = 4 per group, by approximately 6 h, the Mean ± SD for the ratio was 0.185 ± 0.0485 for CNB-001-treated vs 0.314 ± 0.016 for vehicle-treated (*p* = .0017) demonstrating reduced infarct expansion in CNB-001-treated macaques. Fig. 3b shows representative MRI sections from the vehicle and CNB-001-treated groups for 1, 2.5 and 6 h. Table 4 provides raw data for the MRI study.

### 3.5. Target engagement mechanism studies

To provide some insight into target engagement and the mechanisms involved in CNB-001-induced behavioral improvement when administered after an ischemic event, we used the rabbit embolic stroke



(caption on next page)

**Fig. 2. CNB-001 improves clinical function in embolized rabbits:** Rabbits underwent catheterization and small clot embolization followed by CNB-001 (10 mg/kg, IV) (a) 60 min after embolization. CNB-001 (red X) significantly improved behavior and increased the P<sub>50</sub> value (i.e. clot burden) measured 48 h following embolization (\*p < .05) compared to vehicle (black circle). Panel b shows that CNB-001 (5–10 mg/kg) is the minimally effective dose of drug to significantly improve behavior measured 48 h after embolization (\*p < .05) (Red line is CNB-001 compared to the dotted line for the vehicle); n = 18–21 per group for the dose-response curve. Panel c shows that standard-of-care therapy, tPA as a positive control, which improves clinical function in embolized rabbits when tPA (red line and X symbols) was administered at 3.3 mg/kg, IV (20% bolus followed by 80% infused over 30 min) compared to the dotted line and black circles. For the superimposed graphs, behaviorally normal animals are plotted on the y-axis at 0 and abnormal animals are plotted at 100. There is positive correlation between the raw data and the statistically fit sigmoidal quantal curves. tPA significantly improved behavior and increased the P<sub>50</sub> value (i.e. clot burden) measured 48 h following embolization (\*p < .05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

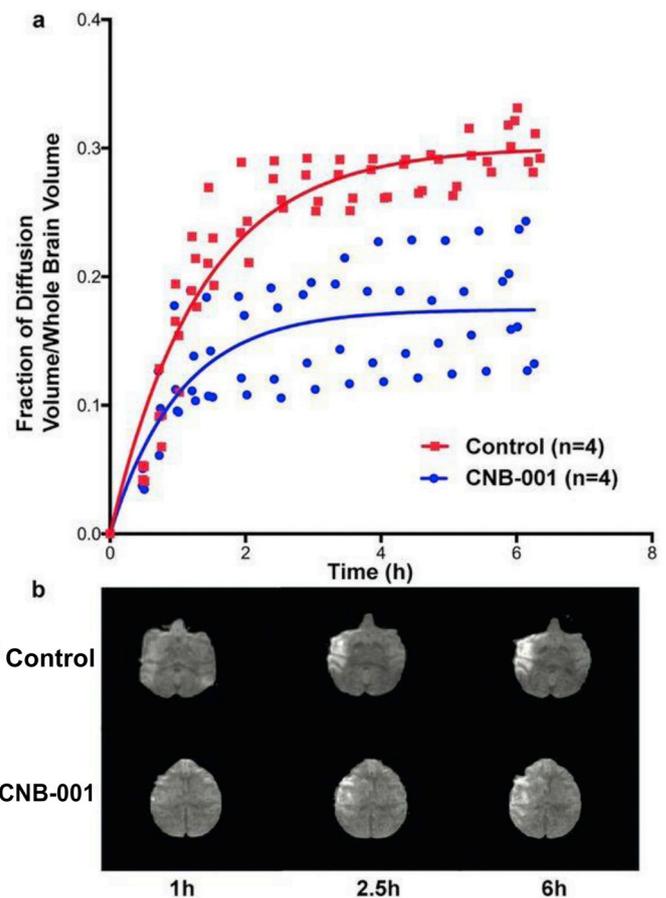
**Table 3**  
Raw quantal curve data: rabbit embolic stroke- data for 1-h treatment quantal analysis.

Control		CNB-001 (10 mg/kg)		tPA (3.3 mg/kg)	
Clot weight (mg)	Normal (0)/Abnormal (100)	Clot weight (mg)	Normal (0)/Abnormal (100)	Clot weight (mg)	Normal (0)/Abnormal (100)
0.01	0.00	0.16	0.00	0.27	0.00
0.08	0.00	0.26	0.00	0.69	0.00
0.10	0.00	0.36	0.00	0.71	0.00
0.16	0.00	0.46	0.00	0.83	0.00
0.24	0.00	0.52	0.00	1.11	0.00
0.35	0.00	0.65	0.00	1.65	0.00
0.43	0.00	1.02	0.00	1.77	0.00
0.49	0.00	1.69	100.00	1.90	0.00
0.83	100.00	1.75	0.00	1.98	100.00
0.93	0.00	1.88	0.00	2.08	0.00
1.15	100.00	2.03	100.00	2.38	0.00
1.39	0.00	2.07	100.00	2.53	100.00
1.41	0.00	2.10	0.00	2.54	100.00
1.97	100.00	2.20	0.00	2.56	100.00
2.24	100.00	3.05	100.00	2.70	0.00
2.39	100.00	3.14	100.00	2.78	0.00
2.40	100.00	3.15	100.00	2.85	100.00
2.63	100.00	3.46	100.00	3.01	0.00
2.91	100.00	4.23	100.00	3.10	100.00
3.60	100.00				

**Definition:** Embolized rabbits are scored as abnormal if they have one or more of the following symptoms: ataxia, leaning, circling, lethargy, nystagmus, loss of balance, loss of limb/facial sensation and occasionally, hind-limb paraplegia.

model. Ischemic stroke is associated with excitotoxicity, increased inflammation, oxidative stress and free radical production, and the loss of trophic factor support (Dirnagl et al., 1999; Moskowitz et al., 2010; Lo and Rosenberg, 2009).

To determine the effect of CNB-001 on some of these parameters, we used WB blotting to examine the expression of relevant proteins in the rabbit cortex. In Fig. 4a and b, we demonstrate that CNB-001 decreases COX-2 in rabbit cortical tissues removed from embolized rabbits, attenuated a stroke-induced increase in 5-LOX expression, and increased BDNF compared to levels measured in vehicle-treated rabbits. Fig. 5 provides data for using ELISA analysis of cortical BDNF and NT-3 contents. We found that CNB-001 significantly increased BDNF content compared to vehicle-treated embolized cortex, but there was no significant effect of either embolization or CNB-001 on cortical NT-3 content at the time point used for study.



**Fig. 3. CNB-001 reduces penumbral expansion in a gyrencephalic NHP ischemia model:** a) CNB-001 slows stroke progression and preserves brain following middle cerebral artery occlusion in cynomolgus macaques. CNB-001 (10 mg/kg) or vehicle (control) was administered IV 5 min after onset of permanent middle cerebral artery occlusion (MCAO). Animals were immediately transferred to the MRI where diffusion weighted imaging was acquired serially over 6 h. b) Representative MRI sections for the vehicle (control) and CNB-001-treated animals. Results are reported as volume of DWI hyperintensity as a fraction of the whole brain volume. There was a significant reduction in DWI volume in the CNB-001 treated group at 6 h (p = .0017, Repeated measures two-way ANOVA). c) presents all raw data with Mean ± SD, n = 4).

3.6. Exclusions and mortality rate

For rabbit embolic stroke behavioral studies, 4 rabbits were excluded from quantal analysis due to severe strokes requiring immediate euthanasia per IACUC protocol, and 3 rabbits were excluded due to technical issues with catheter patency prior to embolization. For rabbit embolic stroke mechanism studies (Western blot and ELISA), 1 rabbit was also excluded due to a severe stroke requiring immediate euthanasia per IACUC protocol. For NHP studies, all assigned macaques were included in the studies (See Table 5).

4. Discussion

In this study, we used a two-large animal research design to further enable the development of CNB-001 to treat stroke. The overall strength of this work is the translational value of utilizing two large species with varied study design to demonstrate significant efficacy of the designed curcumin compound CNB-001. While curcumin itself is not an optimal therapeutic for ischemic disease treatment due to poor pharmacokinetic characteristics (Anand et al., 2007), CNB-001 is BBB-penetrable, has good PK profiles in multiple species, is pleiotropic and efficacious in agyrencephalic and gyrencephalic animals. With this data set the

**Table 4**  
Raw Quantal Curve Data: Non-human primate ischemia- Data for 5 min post-ischemia treatment regimen.

CNB-001-treated group							
Time Post MCAO (h)	CNB1 (DWI/Whole Brain Volume)	Time Post MCAO (h)	CNB2 (DWI/Whole Brain Volume)	Time Post MCAO (h)	CNB3 (DWI/Whole Brain Volume)	Time Post MCAO (h)	CNB4 (DWI/Whole Brain Volume)
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.485	0.051	0.505	0.035	0.475	0.037	0.495	0.053
0.728	0.061	0.758	0.068	0.713	0.127	0.743	0.098
0.970	0.112	1.010	0.095	0.950	0.177	0.990	0.096
1.213	0.111	1.263	0.104	1.188	0.189	1.238	0.138
1.455	0.107	1.515	0.106	1.425	0.184	1.485	0.142
1.940	0.121	2.020	0.108	1.900	0.185	1.980	0.170
2.425	0.120	2.525	0.106	2.375	0.191	2.475	0.176
2.910	0.133	3.030	0.112	2.850	0.186	2.970	0.195
3.395	0.144	3.535	0.117	3.325	0.194	3.465	0.215
3.880	0.133	4.040	0.118	3.800	0.189	3.960	0.227
4.365	0.140	4.545	0.121	4.275	0.189	4.455	0.229
4.850	0.148	5.050	0.124	4.750	0.181	4.950	0.228
5.335	0.155	5.555	0.127	5.225	0.188	5.445	0.236
5.917	0.159	6.161	0.127	5.795	0.196	6.039	0.237
6.014	0.161	6.262	0.132	5.890	0.202	6.138	0.243

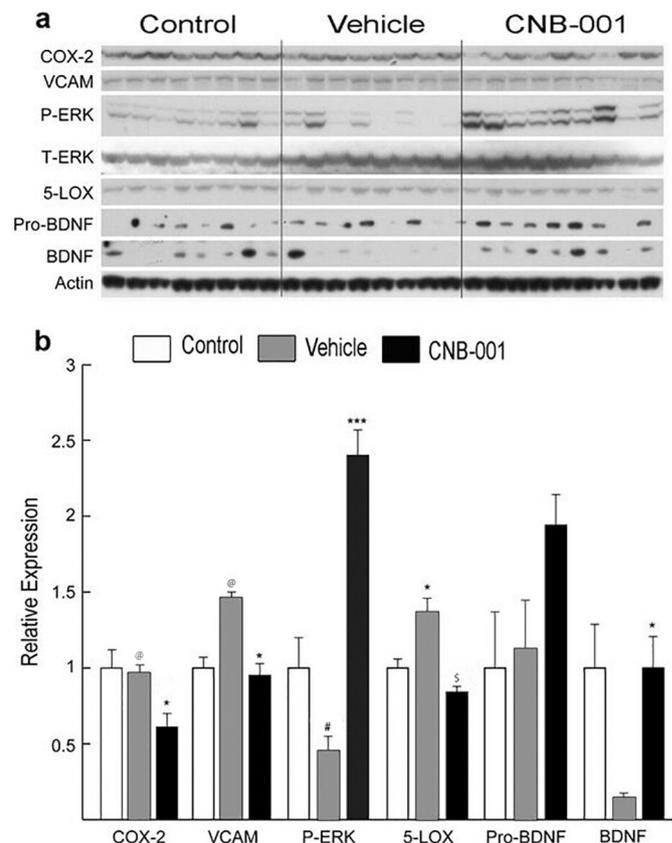
Vehicle-treated group							
Time Post MCAO (h)	CON1 (DWI/Whole Brain Volume)	Time Post MCAO (h)	CON2 (DWI/Whole Brain Volume)	Time Post MCAO (h)	CON3 (DWI/Whole Brain Volume)	Time Post MCAO (h)	CON4 (DWI/Whole Brain Volume)
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.512	0.041	0.482	0.053	0.506	0.052	0.491	0.042
0.768	0.092	0.723	0.091	0.759	0.068	0.737	0.129
1.024	0.110	0.964	0.165	1.012	0.154	0.983	0.194
1.280	0.177	1.204	0.189	1.265	0.214	1.228	0.231
1.536	0.193	1.445	0.210	1.519	0.230	1.432	0.269
2.047	0.211	1.927	0.234	2.025	0.243	1.909	0.289
2.559	0.253	2.409	0.276	2.531	0.260	2.386	0.290
3.071	0.259	2.891	0.279	3.037	0.251	2.863	0.292
3.583	0.261	3.373	0.279	3.543	0.251	3.341	0.291
4.095	0.262	3.854	0.283	4.049	0.261	3.818	0.292
4.607	0.267	4.336	0.288	4.556	0.265	4.444	0.291
5.118	0.270	4.735	0.295	5.062	0.263	4.937	0.291
5.630	0.281	5.300	0.315	5.568	0.290	5.431	0.294
6.245	0.281	5.878	0.318	6.175	0.289	6.024	0.301
6.347	0.292	5.974	0.321	6.277	0.311	6.122	0.331

Raw data for individual macaques enrolled in the MRI study. Note that the time post-MCAO is different for each animal due to the time required for placement of each macaque in the scanner following clip placement.

development of CNB-001 can be further pursued in a clinical trial.

We found that CNB-001 produces statistically significant behavioral improvement in embolized rabbits when administered with a 1-h delay after stroke onset. Using multiple endpoint analysis, we found that CNB-001 improved overall clinical function in embolized rabbits and in non-human primates, CNB-001 administration significantly attenuated penumbral expansion following permanent occlusion during the critical early phase following ischemia induction. In embolized rabbits, the behavioral effect of CNB-001 was associated with modulation of pathways involved in ischemia-induced inflammatory responses, free radical production and synaptic plasticity. While this study utilized a 1-h administration time for rabbit embolic studies, additional temporal studies to define the therapeutic window are required. In a previous article (Lapchak, 2010), a correlation was hypothesized between rabbit and man indicating that efficacy at 1 h in the rabbit model would correlate to approximately 3 h in man.

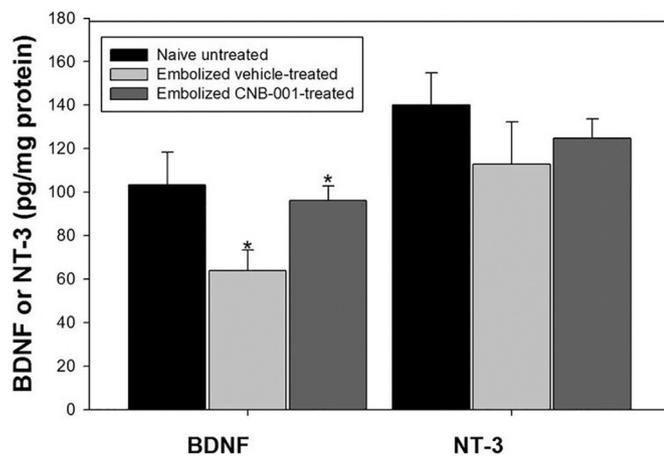
Recent studies done in a variety of disease models have shown



**Fig. 4. CNB-001 regulates oxidative stress and neurotrophic markers in rabbit cerebral cortex. Western Blot analysis:** Rabbits were embolized with a small clot suspension, administered vehicle or CNB-001 (10 mg/kg) 1 h post embolization and 6 h post-drug treatment, ipsilateral cortical tissues were removed and assayed by Western blot analysis ( $n = 8$  per group). Panels A and B show that CNB-001 attenuates embolization-induced increases in COX-2, VCAM, 5-LOX and reverses decreases on P-ERK and BDNF ( $*p < .05$ ). For COX-2, comparisons between vehicle-treated and CNB-001-treated ( $@p < .05$ ). For VCAM,  $@p < .05$  for comparison between control (naïve) and vehicle-treated, and  $*p < .05$  comparison between vehicle-treated and CNB-001-treated. For P-ERK,  $#p < .001$  comparison between control (naïve) and vehicle-treated, and  $***p < .0001$  comparison between vehicle-treated and CNB-001-treated. For 5-LOX,  $*p < .05$  for comparison between control (naïve) and vehicle-treated, and  $^{\$}p < .01$  for comparison between vehicle-treated and CNB-001-treated. Pro-BDNF  $p > .05$  for all comparisons between the 3 groups. For BDNF,  $p > .05$  for comparison between control (naïve) and vehicle-treated, and  $*p < .05$  for comparison between vehicle-treated and CNB-001-treated.

that CNB-001 decreases reactive oxygen species formation, reduces lipid peroxidation, maintains normal physiological mitochondrial membrane potential, reduces apoptosis and has potent anti-inflammatory effects (decreases IL-6, TNF-alpha, COX-2), all characteristics that would indicate that CNB-001 would have utility in the treatment of stroke (Panzhinskiy et al., 2014; Wu et al., 2011; Sharma et al., 2010; Maher et al., 2010; Valera et al., 2013a; Jayaraj et al., 2013). CNB-001 has the unique property of being a potent neuroprotectant against a wide variety of insults in vitro and stimulates the production of pro-survival neurotrophic factors or activates pathway involved in cell survival (Liu et al., 2008a; Lapchak et al., 2011a; Wu et al., 2011; Sharma et al., 2010; Valera et al., 2013a).

The most important observations of this study are that single IV dose administration of CNB-001 at the effective dose (i.e.10 mg/kg) determined in embolized rabbits, promotes significant and durable behavioral improvement in rabbits for 48 h after a single dose despite a plasma half-life of 3.37 h. The most dramatic effects of CNB-001 were



**Fig. 5. Rabbit ELISA assay- CNB-001 restores BDNF content in rabbit cortex, but not NT-3 levels:** Rabbits were embolized with a small clot suspension, administered vehicle or CNB-001 (10 mg/kg) 1 h post embolization and 6 h post-drug treatment, ipsilateral cortical tissues were removed and assayed by ELISA ( $n = 6$  per group). The panel shows that CNB-001 reverses embolization-induced BDNF deficits, but does not affect NT-3 levels at the time point studies (\* $p < .05$ ).

**Table 5**  
Mortality and exclusion rate.

Species	Assay	n	Mortality	Exclusions- note
Rabbit	PK studies	9	0	
Rabbit	RSCEM- Quantal Analysis	295	7	7 excluded
Rabbit	RSCEM- WB	24	01	1 rabbit excluded
Rabbit	RSCEM- ELISA	18	0	
NHP	MRI Analysis	8	0	

observed using the embolic stroke model where beneficial effects are measured at 48 h (Fig. 2), in the second model where early treatment following a permanent occlusion in NHP's significantly reduced stroke expansion during the initial critical 6 h period following ischemia (Fig. 3).

The results suggest that engagement of multiple pathways by a single CNB-001 dose is sufficient to produce a durable response. While initial screening data showed that CNB-001 was a potent 5-LOX inhibitor in vitro, and also inhibits 15-LOX; both enzymes have been recognized as potential therapeutic targets for stroke to confer vascular and neuronal protection (van Leyen et al., 2014; van Leyen, 2013; Singh and Chopra, 2014; Jatana et al., 2006), we further investigated cellular responses in vivo to single dose administration of CNB-001 using the rabbit embolic stroke model to parallel a common type of stroke in patients. Changes in behavior and significant reduction in ischemia could be associated with a variety of ischemic cascade pathways.

Additional markers for inflammation are COX-2 and glial fibrillary acidic protein (GFAP). COX-2 produces prostaglandins that are pro-inflammatory, and COX-2 inhibitors have been shown to be neuroprotective in several animal models of ischemic stroke [see Hurley (Hurley et al., 2002)]. While CNB-001 is not a direct COX-2 inhibitor (Fig. 1b), CNB-001 significantly lowers the amount of COX-2 in embolized rabbits (Fig. 4a). GFAP is an indirect marker for inflammation because when activated, astrocytes make pro-inflammatory cytokines and chemokines. Increased numbers of astrocytes are associated with stroke, and GFAP is increased in stroked rats in the absence CNB-001 treatment. However, CNB-001 significantly reduced GFAP expression in these animals (Fig. 4b).

In embolized rabbits where 5-LOX expression is increased, CNB-001 does attenuate the stroke-induced increase (Fig. 4). Another pro-

oxidant enzyme is 5-LOX. 5-LOX is abundant in the brain and its product, 5-HPETE, is converted to leukotrienes that mediate inflammation and as well as oxidative stress. 5-LOX inhibitors are neuroprotective in models of stroke (Jatana et al., 2006). 5-LOX itself is a major producer of ROS and both COX and 5-LOX inhibitors protect from microglial activation (Klegeris and McGeer, 2002), which is a major pathological component of ischemia. The major substrate for 5-LOX, arachidonic acid (AA), is derived from phospholipids by phospholipase  $A_2$ , which has been implicated in ischemia (Muralikrishna Adibhatla and Hatcher, 2006). Therefore, products of 5-LOX and the molecular pathways they alter are critical compounds of ischemic stroke. We have previously shown that CNB-001 decreases 5-LOX expression in both normal and Alzheimer's disease transgenic mice (Valera et al., 2013b). Further studies would be essential to confirm a therapeutic contribution from each of these putative pathways described above.

In conclusion, we have demonstrated that the drug candidate CNB-001 is cytoprotective in 2 large animal species that are critical for the testing and development of novel cytoprotective approaches to treat stroke victims. CNB-001 produces acute and durable improvement of ischemia-induced motor function deficits in embolized rabbits. We have linked behavioral improvement to a rapid decrease of inflammatory marker production in the ischemic brain, and normalization of BDNF levels in embolized rabbit cortex (Figs. 4 and 5), suggesting a role for neuroplasticity events in the beneficial effects of CNB-001.

The observation that a novel curcumin-based drug like CNB-001 can effectively attenuate ischemia-induced clinical function deficits and reduce infarct expansion in large animal species supports the hypothesis that pleiotropic compounds may be the "class" of compound that should be developed for the treatment of ischemic disease. There are a number of important circumstances where a safe, brain-penetrable drug such as CNB-001 can be useful to stroke and potential ischemia victims (Lapchak, 2011). First, since CNB-001 reduces clinical deficits in ischemia models when administered pre- and post- ischemic insult, it is feasible to use CNB-001 to halt the initiation and/or progression of ischemic damage administered to stroke patients. Second, as previously hypothesized by Bratane and colleagues, it may be possible to "freeze" penumbra in the field prior to thrombolysis and/or embolectomy (Bratane et al., 2011). The clinical impact of an efficacious cytoprotectant like CNB-001 cannot be overestimated as it may serve as an effective bridge to reperfusion interventions (i.e., tPA and embolectomy), and even be used in combination with tPA (Lapchak and Boitano, 2014) or embolectomy with or without tPA. An effective cytoprotectant given promptly in the field in the first 3-4 h could significantly promote the population of patients successfully treated with tPA or embolectomy. Rapid-response ambulance personnel can now rapidly deliver IV medications to stroke victims early after stroke onset when they may be most beneficial (Saver, 2006; Saver et al., 2010). In the FAST-MAG study (Saver et al., 2015b), IV drug was successfully delivered to stroke patients in the field within 1 h – 2 h (73.4 and 99%, respectively). This was also the strategy utilized for postsynaptic density (PSD-95) inhibitors (FRONTIER, n.d.), scaffolding protein members of membrane-associated guanylate kinase (MAGUK) superfamily, a key mechanism involved in glutamate neurotoxicity. Lastly, the need for cytoprotectants that reduce infarct growth and volume and potentially increase the window for reperfusion is important (Lapchak and Boitano, 2014; Lapchak, 2015; Lyden et al., 2016), and concepts for their introduction into clinical trials have been established (Goyal et al., 2016).

**Limitations:** Even though we utilized 2 large animal species in this extensive study of CNB-001, there are limitations of the study. First, this proof-of-concept or pilot study utilized young male rabbits for most of the pharmacological characterization, and young NHP's for MRI analysis. Because stroke is predominantly a disease of the aged brain (Lapchak and Zhang, 2017; Lapchak and Zhang, 2018) with an equal balance between males and females (Benjamin et al., 2017), additional confirmatory gender analysis studies should be conducted in aged animals (Lapchak and Zhang, 2018) when funding has been secured to do

so. Second, in the study we tested CNB-001 as a monotherapy, but there would be substantial value to combine a CNB-001 with current reperfusion therapy techniques (Lapchak and Zhang, 2018; Lapchak and Boitano, 2014). Thus, additional combination studies should be conducted to determine efficacy and safety.

Moreover, with the mechanism of action, and target engagement studies described herein, we have provided a strong basis for further elucidation of mechanisms of cytoprotection and repair pathways activated by CNB-001. Additional temporal studies are warranted in aged rodents.

Taken together, CNB-001 is a prime candidate for the treatment of ischemic stroke and should be further pursued in well-designed clinical trials.

#### Author contribution

PAL designed and wrote the main manuscript text. DJC and DRS edited the manuscript. PAL, RB, DJC, DRS designed the studies. PAL, PDB, RB, SD, JML, and DRS conducted technical aspects of the studies.

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#### Conflict of interest statement

No conflicts to report.

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All listed authors participated in one or more of the following: intellectual contribution, experiments, study design, data interpretation, data analysis, and/or manuscript writing resulting in the scientific content of this manuscript. All authors have approved the manuscript and the senior authors (PAL, DRS) reviewed all raw data incorporated into this article.

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