Published in final edited form as:
*Transl Stroke Res*. 2011 March ; 2(1): 51–59. doi:10.1007/s12975-010-0034-4.

# CeeTox™ Analysis of CNB-001 a Novel Curcumin-Based Neurotrophic/Neuroprotective Lead Compound to Treat Stroke: Comparison with NXY-059 and Radicut

**Paul A. Lapchak** and
Department of Neurology, Cedars-Sinai Medical Center, Burns and Allen Research Institute, 110 N. George Burns Road, D-2091, Los Angeles, CA 90048, USA

**James M. McKim Jr.**
CeeTox Inc, 4717 Campus Dr., Kalamazoo, MI 49008, USA

Paul A. Lapchak: Paul.Lapchak@cshs.org

## Abstract

In the present study, we used a comprehensive cellular toxicity (CeeTox) analysis panel to determine the toxicity profile for CNB-001 [4-((1E)-2-(5-(4-hydroxy-3-methoxystyryl-)-1-phenyl-1H-pyrazoyl-3-yl)vinyl)-2-methoxy-phenol)], which is a hybrid molecule created by combining cyclohexyl bisphenol A, a molecule with neurotrophic activity and curcumin, a spice with neuro-protective activity. CNB-001 is a lead development compound since we have recently shown that CNB-001 has significant preclinical efficacy both in vitro and in vivo. In this study, we compared the CeeTox profile of CNB-001 with two neuroprotective molecules that have been clinically tested for efficacy: the hydrophilic free radical spin trap agent NXY-059 and the hydrophobic free radical scavenger edaravone (Radicut). CeeTox analyses using a rat hepatoma cell line (H4IIE) resulted in estimated $C_{Tox}$ value (i.e., sustained concentration expected to produce toxicity in a rat 14-day repeat dose study) of 42 μM for CNB-001 compared with >300 μM for both NXY-059 and Radicut. The CeeTox panel suggests that CNB-001 produces some adverse effects on cellular adenosine triphosphate content, membrane toxicity, glutathione content, and cell mass (or number), but only with high concentrations of the drug. After a 24-h exposure, the drug concentration that produced a half-maximal response ($TC_{50}$) on the measures noted above ranges from 55 to 193 μM. Moreover, all CNB-001-induced changes in the markers were coincident with loss of cell number, prior to acute cell death as measured by membrane integrity, suggesting a cytostatic effect of CNB-001. NXY-059 and Radicut did not have acute toxic effects on H4IIE cells. We also found that CNB-001 resulted in an inhibition of ethoxyresorufin-*o*-deethylase activity, indicating that the drug may affect cytochrome P4501A activity and that CNB-001 was metabolically unstable using a rat microsome assay system. For CNB-001, an estimated in vitro efficacy/toxicity ratio is 183–643-fold, suggesting that there is a significant therapeutic safety window for CNB-001 and that it should be further developed as a novel neuroprotective agent to treat stroke.

### Keywords

Stroke; Translational; Development; Neuroprotective; In vivo; In vitro

Correspondence to: Paul A. Lapchak, Paul.Lapchak@cshs.org.

## Introduction

There have been many failed attempts at neuroprotection to treat acute ischemic stroke (AIS), some of which are documented in recent scientific reviews [1–5]. Since it is imperative to continue to develop novel strategies to treat AIS, we have used a translational development standard operating development procedure (SOP), which includes rational molecule design, and multi-tier in vitro and in vivo testing for both efficacy and safety [1,6]. One compound designated CNB-001 [4-((1E)-2-(5-(4-hydroxy-3-methoxystyryl-)-1-phenyl-1H-pyrazoyl-3-yl)vinyl)-2-methoxy-phenol)], which is a curcumin-based analog, is our current lead development compound [7,8].

Based upon in vitro analysis, CNB-001 has been shown to promotes cellular survival in a dose-dependent manner when applied to cell exposed to the mitochondrial neuro-toxin, iodoacetic acid [9], an affect that may be related to a CNB-001 increase in the cellular content of adenosine triphosphate (ATP) [7,10]. CNB-001 is also effective against toxicity mediated by the excitatory amino acid glutamate, which causes HT22 cell death by an oxytosis mechanism [7,8], a non-excitotoxic mechanism [7,11] where cell death is mediated by the depletion of intra-cellular glutathione (endogenous antioxidant) via the inhibition of the cystine/glutamate antiporter. In addition, CNB-001 has neuroprotective or neurotrophic factor activity in a trophic factor withdrawal assay [12]. CNB-001 promotes the survival of freshly plated low-density cultured rat cortical neurons in F12/DMEM plus N2 medium, a serum-free culture medium frequently used for assaying neurotrophic factors [12]. In all three preclinical in vitro development assays, the $EC_{50}$ value for CNB-001-induced cell survival is 0.3–0.7 μM.

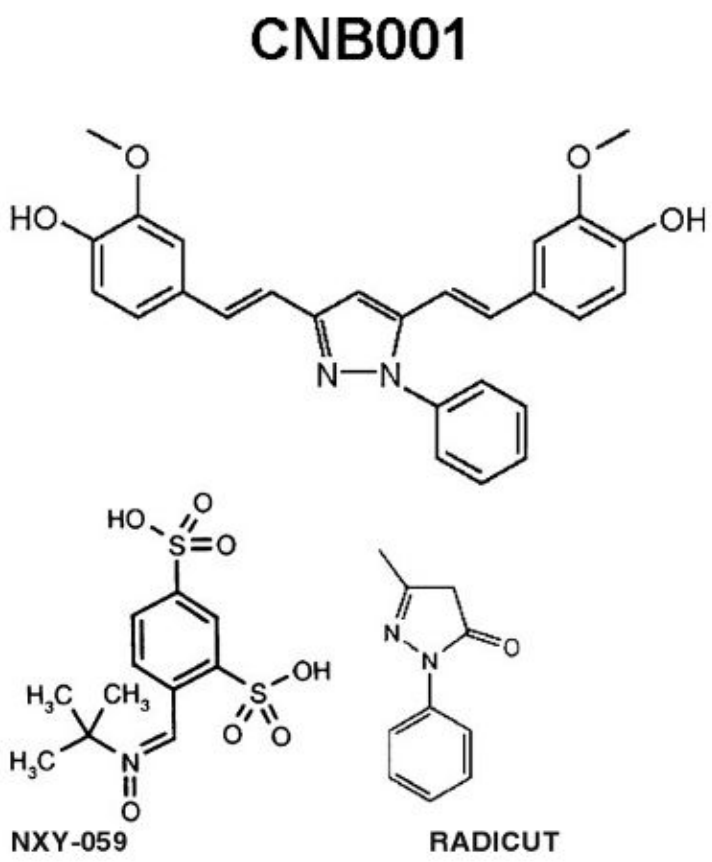
Moreover, we recently showed that CNB-001 is neuro-protective in vivo using the rabbit small clot embolism model, where post-embolization treatment with CNB-001 significantly improved behavior or reduced embolism-induced clinical deficits [10]. Because CNB-001 is a lead compound for further investigation, it was extremely important to determine if there are toxicities related to the compound. For this, we used the current industry standard, the CeeTox assay system (www.CeeTox.com) which has recently been reviewed [13]. For comparison, we chose two small molecule neuroprotective compounds that have been tested in preclinical stroke models as well as in AIS clinical trials. The first compound is NXY-059, the water-soluble spin trap antioxidant that failed in the SAINT II clinical trial [14–16]. In addition, we studied Radicut (Edaravone), a lipid soluble antioxidant that is currently approved in Japan for the treatment of AIS when administered within 24 h of a stroke [17–22], even though there has not been a randomized double blind international trial to support the worldwide use of the drug [23].

CeeTox analysis is an effective in vitro predictive toxicity screening assay to determine the toxicity profile of drugs being developed for human use [13,24] so that drug development can be de-risked during early development stages. Thus, in the present study, using CeeTox analysis [13], we determined the safety profile of the lead CNB-001, which was compared with two other neuro-protective compounds, NXY-059 and Radicut.

## Materials and Methods

### Neuroprotective Compounds

Chemical structure of the three study compounds.




## Experimental Protocol

All assays utilizing the rat hepatoma (H4IIE) cell line as the test system were conducted by CeeTox Inc Kalamazoo, MI, USA, as described in detail by McKim [13] according to SOP 9066-091/092. CeeTox Inc. did not have access to chemical structures or identities during assay.

**Drug Synthesis and Preparation for Assay—**CNB-001 was synthesized and purified according to a previously published procedure [7]. Disodium 4-[[(1,1,-dimethylethyl) imino]methyl]benzene-1,3-disulfonate *N*-oxide (NXY-059; CAS 168021-79-2) was synthesized and purified according to a previously published procedure, and pharmacological testing has previously been reported and reviewed [1,25,26]. 3-Methyl-1-phenyl-2-pyrazolin-5-one (Radicut; CAS 89-25-8) was purchased from Sigma Inc. (St. Louis, MO, USA). Pharmacological testing of Radicut has previously been reported and reviewed [1,27].

Since CNB-001 and RADICUT are lipophilic compounds, dimethylsulfoxide (DMSO) was used to prepare 20-mM stock solutions that were then further diluted in DMSO to prepare 2-mM stock solutions. NXY-059 is water soluble and was made up in purified, sterile water at concentrations of 20 and 2 mM. For all drugs, the 20- and 2-mM stocks were used to prepare dosing solutions of 1–300 μM in culture medium. The final concentration of DMSO in the 0–100-μM solutions was 0.5% and at the 300-μM solution DMSO was 1.5%. The final dosing solutions were prepared in Eagle's minimum essential medium. All experiments that used DMSO as the drug solvent also included a DMSO negative control. Negative controls

of medium plus DMSO (0.5%) were included with and without cells. A positive control for complete cell death received 1 mM digitonin in medium on the day of dosing [13].

For solubility, the test compounds were prepared in DMSO, and the appropriate amounts were then added to complete medium containing 10% bovine serum and 10% calf serum at 37°C. The samples were evaluated using light scattering with a Nepheloskan instrument. A reading that was greater than or equal to three times the background was considered the limit of solubility [13].

**H4IIE Cell Line—**Rat hepatoma-derived H4IIE cells were used as the test system because the cells have a rapid doubling time in culture (i.e., 22 h) [13]. The culture medium used for these cells was Eagle's minimum essential medium with 10% bovine serum and 10% calf serum (Invitrogen). H4IIE cells were seeded into 96-well plates and allowed to equilibrate for approximately 48 h before drug assay to allow cells to move into a stable growth phase prior to treatment. Following the equilibration period, the cells were exposed to CNB-001, NXY-059, and Radicut at concentrations of 1–300 μM. Three to seven replicates were done for each assay to construct concentration–response curves. Solubility was determined by nephelometry techniques immediately after dosing and prior to harvesting the cells at 6 or 24 h. Following the incubation of cells with CNB-001, NXY-059, or Radicut, the cells or their supernatant (culture medium) was analyzed for changes in cell proliferation (cell mass), membrane leakage, mitochondrial function, oxidative stress, and apoptosis. The resultant exposure concentration–response curves were graphed and analyzed to determine the concentration (μM) that produced a half-maximal response or $TC_{50}$ [13].

**General Cellular Measures of Toxicity:** (1) Cell mass: Cell mass in each well was measured with a modified propidium iodide (PI) [28], a specific nucleic acid binding dye that fluoresces when intercalated within the nucleic acids. The 15-nm shift enhances PI fluorescence approximately 20 times while the excitation maxima are shifted 30–40 nm. Triton-X-100 was used to permeabilize the H4IIE cells, thereby allowing the PI access to intracellular RNA and DNA. Fluorescence was measured using a Packard Fusion plate reader at 540-nm excitation and 610-nm emission [13]. Data are collected as relative fluorescent units (RFU) and expressed as percent change relative to control. (2) Membrane toxicity: The presence of α-glutathione *S*-transferase (α-GST), an enzyme leakage marker, was measured in the culture medium using an ELISA assay purchased from Argutus Medical [13,29]. At the end of the exposure period, the medium covering the cells in each well was removed and stored at 80°C until assayed. Absorbance values were measured with a Packard SpectraCount™ reader at 450 nm and reference absorbance at 650 nm. Leakage of α-GST from the cell into the culture medium was determined by collecting the culture medium at the end of the exposure period. Thus, the values measured represent total enzyme leakage lost over the exposure period. 3-*[*4,5-*Dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT)*: After the medium was removed from a plate for α-GST analysis, the cells remaining in each well were evaluated for their ability to reduce soluble-MTT (yellow) to formazan-MTT (purple) [13,30,31]. An MTT stock solution was prepared in complete medium just prior to use and warmed to 37°C. Once the medium was removed from all wells, MTT solution was added to each well and the plate was allowed to incubate at 37°C for 3–4 h. Following incubation, all medium was removed and the purple formazan product was extracted using anhydrous isopropanol. Sample absorbance was read at 570 nm and reference absorbance at 650 nm with a Packard Fusion reader. The control for 100% dead or maximum enzyme release was based on cells treated with 1 mM digitonin at the time of dosing. Percent dead cells relative to digitonin-treated cells was determined and then subtracted from 100% to yield the percent live cells. (3) Cellular ATP content: ATP content was determined using a modification of a standard luciferin/ luciferase luminescence assay [32] based on a reaction between ATP+Dluciferin+oxygen catalyzed by luciferase to yield

oxyluciferin+AMP+PPi+$CO_2$+light. The emitted light is proportional to the amount of ATP present [13]. At the end of the 24-h exposure period, the medium was removed from the cells and the ATP cell lysis buffer was added to each well. Plates were analyzed immediately or stored at −20°C until needed. On the day of analysis, the plates were thawed and calibration curve was prepared with ATP in the same liquid matrix as samples. ATP was quantified by adding ATP substrate solution and then reading luminescence on a Packard Fusion Luminescence reader. ATP levels (picomoles ATP per million cells) in treated cells were extrapolated using the regression coefficients obtained from the linear regression analysis of the calibration curve. Background-corrected luminescence was used to determine percent change relative to controls by dividing treated values by control values and multiplying by 100.

**Oxidative Stress:** (1) Intracellular glutathione (GSH) levels: Intracellular glutathione levels were determined using a modification of the procedure published by Griffith [33]. Briefly, the sulfhydryl group of GSH reacts with DTNB (5,5′-dithio-bis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405 or 412 nm provides an accurate estimation of GSH in the sample. At the end of the exposure period, the medium was removed from the cells and metaphosphoric acid was added to each well. Plates were then shaken for 5 min at room temperature and stored at −20°C until needed. The sample plates were thawed just prior to analysis and centrifuged at >2,000×*g* for 2 min. Sample aliquots were removed and transferred to a clean 96-well plate along with appropriate standard curve controls. Sample pH was neutralized just prior to analysis, and each well received an aliquot of PBS reaction buffer containing Ellman's reagent, NADPH, and glutathione reductase. The plates were shaken for 15–30 min at room temperature, and glutathione content was determined colorimetrically with a Packard Fusion reader at 415 nm. The assay is based on the concept that all GSH is oxidized to GSSG by DTNB reagent. Two molecules of GSH are required to make one molecule of GSSG (oxidized glutathione). Total GSH was determined by reducing GSSG to 2GSH with glutathione reductase. A standard curve was prepared with oxidized glutathione (GSSG) over a range of concentrations. These concentrations are then converted to glutathione equivalents (GSX) essentially by multiplying the GSSG standard concentrations by 2. The amount of GSX expressed as picomoles/well was determined using the standard curve and regression analysis and are expressed as percent of control. (2) Lipid peroxidation measured as 8-isoprostane (8-ISO or 8-epi PGF2α): 8-ISO levels were determined using an ELISA (Cayman Chemical Inc). 8-ISO is a member of a family of eicosanoids produced nonenzymatically by random oxidation of tissue phospholipids by oxygen radicals. Therefore, an increase in 8-ISO is an indirect measure of increased lipid peroxidation [34]. At the end of the exposure period, plates were either analyzed immediately or stored at −80°C until needed for analysis. Color development, which is indirectly proportional to the amount of 8-ISO present in the sample, was read on a Packard Fusion or equivalent plate reader at 415 nm [13]. Background absorbance produced from Ellman's reagent is subtracted from all wells. Non-specific binding is subtracted from the maximum binding wells to give a corrected maximum binding expressed as $B_o$. The percent of bound ($B$) relative to maximum binding capacity ($B_o$) for all unknown samples and for standards was determined an expressed as ($\%B/B_o$). The $\%B/B_o$ for standards was plotted against the log of 8-ISO added to yield the final standard curve. This curve was used to convert $\%B/B_o$ to pg 8-ISO/mL of sample.

**Apoptosis:** Caspase 3 activity was determined using a caspase substrate (DEVD, Asp-Glu-Val-Asp) labeled with a fluorescent molecule, 7-amino-4-methylcoumarin (AMC). Caspase

3 cleaves the tetrapeptide between D and AMC, thus releasing the fluorogenic green AMC [13]. Following the test article exposure to cells in 96-well plates, medium was aspirated from plates and PBS added to each well. Plates were stored at −80°C to lyse cells and store samples until further analysis. On the day of analysis, plates were removed from freezer and thawed. Caspase buffer with fluorescent substrate was added to each well and incubated at room temperature for 1 h. AMC release was measured in a spectrofluorometer at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Values are expressed as RFU. After sample plates were completely thawed, the caspase substrate buffer mix was added to each plate. Plates were incubated at room temperature for 1 h, shielded from light. Plates were read using a spectrofluo-rometer at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Values were expressed as RFU.

**P-glycoprotein Binding Using the MTT Assay:** The H4IIE cells possess high levels of P-glycoprotein (PgP) protein in the outer membrane and be used effectively for evaluation of drug binding to PgP [13,35–37]. For this assay, cells are incubated with and without cyclosporin A (CSA) (a PgP inhibitor) at a single exposure concentration (50 μM), and the difference in toxicity is determined with the MTT assay. Compounds with increased toxicity in the presence of CSA have a high probability of binding to PgP proteins. However, compounds of low toxicity will typically not show a difference relative to the addition of CSA, regardless of whether they bind to PgP. At the end of the 24-h exposure period, the culture medium was removed and the remaining attached cells were assayed for their ability to reduce MTT. Viable cells will have the greatest amount of MTT reduction and highest absorbance values. Percent control values were determined by dividing the mean absorbance/fluorescence of the treatment group by the mean absorbance of the control group and multiplying by 100.

**Metabolic Stability:** Metabolic stability was conducted using pooled microsomes from non-induced male Sprague–Dawley rats [13]. The test compounds were incubated for 30 min at 37°C at concentrations of 1 μM. Subsequent HPLC analysis using a Waters Alliance 2795 in combination with a Waters Quattro Premier mass spectrometer measured disappearance of the parent molecule. The compounds were run on a Waters X-BridgeC18 (186003021) 50×2.1-mm column with 3.5-μm particle packing at a flow rate of 1 ml/min and with the temperature maintained at 50°C. Solvent A was water with 0.1% formic acid. Solvent B was acetonitrile with 0.07% formic acid. The data for three replicates are expressed as percent of parent remaining. Metabolism rankings are based on extent of metabolism with a percent remaining breakdown of 100–65% as low, 65–45% as moderate, and <45% as high.

**Cytochrome P450 1A1/2 Activity:** Ethoxyresorufin-*o*-deethylase (EROD) activity was evaluated using a fluorescent substrate, ethoxyresorufin [38]. Fluorescent-based assays were read using a spectrofluorometer at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Values were expressed as RFU, and fold inductions were calculated relative to controls after background subtraction.

## Results

1. Drug solubility: CNB-001 was soluble up to and including 100 μM in the culture medium system, but was not completely soluble at 300 μM. However, both NXY-059 and Radicut were soluble up to and including 300 μM in the assay system. The solubility profile will later be discussed in reference to the cell toxicity results in Fig. 1.
2. Effects on cellular toxicity: Fig. 1 shows dose–response profiles for the effects of CNB-001 on cellular toxicity using a 24-h analysis endpoint. For CNB-001 (Fig. 1

and Table 1), cell death ($TC_{50}$=193 μM) measured using the release of α-GST into the culture medium was observed at the highest exposure concentration (300 μM). However, it must be noted that this was outside the range of complete solubility of the compound. There was also a decrease in cell number or mass after incubation with CNB-001 that occurred with a $TC_{50}$ of 88 μM. As shown in Fig. 1, other parameters had similar concentration–response curves in culture, with significant decreases in cellular ATP content ($TC_{50}$=55 μM), GSH content ($TC_{50}$=76 μM), and MTT reductase activity ($TC_{50}$=131 μM). For CNB-001, a reduction in cell proliferation was observed between 20 and 100 μM that was tracked by the other biochemical markers, suggesting a cytostatic effect (i.e., inhibition of cell growth and division), rather than a direct effect on mitochondria. In contrast to the results obtained for CNB-001, NXY-059 (Table 1) was not acutely toxic and had no significant effect on the subcellular markers of acute toxicity across the exposure range tested up to 300 μM. In all five assays described above, the $TC_{50}$ values were >300 μM. The same is true for Radicut (Table 1), which had $TC_{50}$ values >300 μM for all assayed markers with the exception of a 30% decrease in cell proliferation observed at the 300-μM exposure.

3. Effects on oxidative stress markers: Fig. 1 shows a comparison of two markers used to determine the effects of CNB-001 on markers of oxidative stress. For this measure, we used intracellular GSH content and lipid peroxidation measured as 8-isoprostane [13]. CNB-001 produced a significant reduction in intracellular GSH content with a $TC_{50}$ of 76 μM, but did not have any effect on 8-isoprostane. There was no effect of either NXY-059 or Radicut on either marker.

4. Effect on apoptosis or caspase-3 activity: As a measure of the effects of the study drugs on apoptotic mechanisms, we used caspase-3 activity [13], which is a key mediator of apoptosis in neuronal cells, but also has non-apoptotic functions [39]. None of the drugs affected caspase-3 activity (not shown).

5. Drug PgP Binding: Table 2 shows that all three compounds showed low interaction with the permeability-glycoprotein when cells are incubated with and without CSA, a PgP inhibitor at a single exposure concentration (50 μM). Compounds with increased toxicity in the presence of CSA have a high probability of binding to PgP proteins. However, compounds of low toxicity will typically not show a difference relative to the addition of CSA, regardless of whether they bind to PgP.

6. Metabolic stability of CNB-001: CNB-001 was metabolically unstable that is it was highly metabolized with only 42% of the parent compound remaining after incubation with rat microsomes (phase 1 metabolism) for 30 min at 37°C measured using HPLC/MS. It is important to note that H4IIE cells have low constitutive metabolic activity, but is sensitive to CYP1A1/2 induction. Compounds that induce CYP1A enzymes may also be substrates and undergo metabolism in the H4IIE system. NXY-059 was found to be metabolically stable with low levels of metabolism; 93% of the parent compound remained after incubation, whereas Radicut was also metabolically unstable with only 28% of parent remaining.

Since CNB-001 is a lead compound, we determined if the compound would affect cytochrome P450 1A1/2 activity. As shown in Fig. 2, there was inhibition of EROD activity with an $IC_{50}$ of approximately 25 μM. The greater reduction in activity noted at 50- and 100-μM exposure concentrations may be due to general cellular toxicity.

1. $C_{Tox}$ ranking: For each compound studies, a $C_{Tox}$ value was generated by CeeTox Inc., using a patented proprietary algorithm [40]. $C_{Tox}$ values were generated from the $TC_{50}$ values in this report. The $C_{Tox}$ ranking for CNB-001, which is an estimate of a sustained concentration expected or necessary to produce toxicity in a rat 14-

day repeat dose study, was 42 μM, whereas the $C_{Tox}$ ranking for NXY-059 and Radicut were both >300 μM. The results are shown in Fig. 3.

## Discussion

The present study determined the in vitro toxicity profile of CNB-001, a lead neurotrophic/neuroprotective compound currently being developed for the treatment of AIS. The treatment of AIS with a neuroprotective compound is a formidable challenge that has gone unmet for 25 years. There are many reasons for the failure of neuroprotectives that have been tested in AIS clinical trials, primarily lack of efficacy, but also there has been dose-limiting toxicity [1–3,41–43]. In order to advance the development of CNB-001, we used the CeeTox panel to de-risk the further development of the drug. The $C_{Tox}$ value or sustained concentration expected to produce toxicity in a rat 14-day repeat dose study was estimated to be 42 μM for CNB-001 compared with >300 μM for both NXY-059 and Radicut. The $C_{Tox}$ value suggests that there is a moderate probability of in vivo toxicity effects related to chronic long-term administration of CNB-001, but there are numerous caveats associated with the interpretation of the CeeTox panel assay, especially the fact that the $C_{Tox}$ value is for 14-day repeat dosing.

The general cellular health panel suggests that CNB-001 produces some adverse effects with high concentrations of the drug, with calculated TC50 values in the range of 55–193 μM. From a drug development perspective, it is interesting to note that CNB-001 is efficacious in vitro using HT22 cells and primary cortical cells with an EC50 values in the range of 0.3–0.7 μM [7,10]. A simple view of the efficacy/toxicity ratio indicates that there is a significant therapeutic safety window of 183–643-fold. CNB-001 has also been studied in vivo in two different animal models: A [1] rabbit embolic stroke model where CNB-001 given at 100 mg/kg as a single bolus injection was shown to produce significant behavioral improvement without any signs of acute toxicity [10] and a [2] rat cognition assay where CNB-001 at 10 mg/kg by gavage significantly increased the object recognition index [8].

It is also interesting to compare the effects of CNB-001 on GSH levels in the rat hepatoma-derived H4IIE cell line and HT22 cells [7]. In the “toxicity” study, CNB-001 was found to produce a decrease of GSH with a TC50 of 76 μM, whereas Liu and colleagues showed that CNB-001 was neuroprotective with an EC50 of 0.7 μM when studied in an HT22 cell “oxytosis” assay [11] induced by the addition of glutamate (5 mM) to induce cell death via a non-excitotoxic mechanism [7,11]. In the assay, elevated levels of extracellular glutamate interfere with cystine uptake through the cystine/glutamate antiporter, which normally carries cystine into cells at the expense of the outflow of glutamate [11]. This results in the depletion of intracellular GSH levels. The results from the HT22 cell assay suggest that CNB-001-induced neuroprotection is mediated via normalization of GSH; however, we have found that CNB-001 had no effect on GSH levels in HT22 cells [10]. Thus, CNB-001-induced neuroprotection does not appear to be associated with normalization of GSH in HT22 cells.

In H4IIE cells, CNB-001 also produced a reduction of cellular ATP content with a TC50 of 55 μM. The decrease in both GSH and ATP measured after 24-h incubation was coincident with a decrease in cell mass, indicating that CNB-001 did not result in oxidative stress or mitochondrial toxicity. Taken together, it appears that the high dose effects of CNB-001 on H4IIE cells may be due to a cytostatic effect of the drug because there was a significant decrease in cell mass or number at the high end of the concentration–response curve.

In this study, we also compared the toxicity profile of CNB-001 with NXY-059 and Radicut, two free radical scavenging drugs that have different chemical and pharmacological

properties [18,27,44–48]. NXY-059 has undergone an extensive preclinical and clinical development plan, where it was shown to be safe, but failed to be efficacious in a double-blind randomized clinical trial whether administered as a monotherapy or in combination with the tPA [14–16]. Using CeeTox analysis, there were no indications of NXY-059 toxicity to H4IIE cells in culture confirming that safety profile of the drug observed in animals and AIS patients. Using the hepatoma cells, there were also no indications of Radicut toxicity, a drug that is formally approved by the Japanese health ministry for the treatment of AIS if administered within 24 h of a stroke [1,27]. The results with the three molecules support the usefulness of the CeeTox assay as a primary de-risking assay. Even though Radicut was not toxic to H4IIE cells in culture, there have been numerous reports of renal toxicity associated with Radicut administration to stroke patients [49]. It has been reported that approximately 45% of patients with Radicut-induced renal toxicity recover renal function after Radicut treatment is stopped [49].

The metabolic stability of 1 μM of CNB-001, NXY-059, and Radicut were compared using a male Sprague–Dawley rat microsomal preparation. The assay measured disappearance of the parent molecule, but did not measure metabolites of any of the compounds. The assay suggested that CNB-001 and Radicut were metabolically unstable and that in vivo bioavailability may be low after single dosing, and this must be taken into consideration when developing the compounds to treat AIS. NXY-059 was very stable metabolically and thus is not as much a concern. In vivo studies using the rabbit embolic stroke model show that a single bolus injection of CNB-001, Radicut, and NXY-059 was sufficient to produce significant behavioral improvement when behavior was measured 24–48 h following embolization [10,26,27]. Thus, for acute administration, all three compounds were effective in producing a significant behavioral improvement when administered up to 1 h following embolization [10,26,27]. For NXY-059, the maximum therapeutic window was 1 h [26]; Radicut was also active when administered 3 h following embolization [27].

The results of the rat microsomal preparation analyses must be addressed in the context of the general cellular toxicity assays done using H4IIE cells. It is important to note that rat H4IIE constitutively expresses several key cytochrome P (CYP) 450 enzymes including CYP1A, CYP2B, CYP2C, and CYP3A activity in the H4IIE cells. In addition, there is both glucuronide and glutathione conjugation capability. The cell line used in this study is sensitive to inducers of CYP1A [13]. However, we found that CNB-001 resulted in an inhibition of EROD activity relative to controls, but much of this may be attributed to cell death at the higher concentrations. In most instances, the results obtained with H4IIE cells reflect toxicity due to the non-metabolized forms of the test compounds. However, it cannot be excluded that situations may occur where the test compound both induces CYP and is also metabolized by one of the constitutively expressed CYP isoforms; thus, toxicity may be the result of metabolism and/or conjugation.

In conclusion, CeeTox analysis proved useful as a de-risking tool and provided useful information concerning the in vitro concentration where cellular toxicity may occur, compared with the neuroprotection profile of the drug. The observation that the $C_{\mathrm{Tox}}$ value for CNB-001 is 42 μM indicates that CNB-001 should continue to be developed as a lead compound. It is generally viewed that acute or sub-chronic administration of neuroprotective compounds will be used for the treatment of AIS during the first 24 h after an AIS [1]. Thus, even though the $C_{\mathrm{Tox}}$ value is representative of a 14-day exposure, the drug may be administered for a shorter duration, thus even further reducing the possibility of in vivo toxicity, which may be exposure dependent. Ultimately, with the continued development of CNB-001, toxicity will be determined using a two-species GLP toxicity study as required by the FDA [50].

## Acknowledgments

This work was supported by a U01 Translational research grant NS060685 to PAL. Paul Wilga, MS of CeeTox Inc., is thanked for his technical contribution to this manuscript.

## References

1. Lapchak PA. Translational stroke research using a rabbit embolic stroke model: a correlative analysis hypothesis for novel therapy development. Translational Stroke Research. 2010; 1(2):96–107. Perspective Review. 10.1007/s12975-010-0018-4 [PubMed: 20539748]
2. O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells DW. 1,026 experimental treatments in acute stroke. Ann Neurol. 2006; 59(3):467–77. [PubMed: 16453316]
3. Feuerstein GZ, Zaleska MM, Krams M, Wang X, Day M, Rutkowski JL, et al. Missing steps in the STAIR case: a Translational Medicine perspective on the development of NXY-059 for treatment of acute ischemic stroke. J Cereb Blood Flow Metab. 2008; 28(1):217–9. [PubMed: 17579658]
4. Green AR, Ashwood T. Free radical trapping as a therapeutic approach to neuroprotection in stroke: experimental and clinical studies with NXY-059 and free radical scavengers. Curr Drug Targets CNS Neurol Disord. 2005; 4(2):109–18. [PubMed: 15857295]
5. Durukan A, Tatlisumak T. Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. Pharmacol Biochem Behav. 2007; 87(1): 179–97. [PubMed: 17521716]
6. Maher P, Salgado KF, Zivin JA, Lapchak PA. A novel approach to screening for new neuroprotective compounds for the treatment of stroke. Brain Res. 2007; 1173:117–25. [PubMed: 17765210]
7. Liu Y, Dargusch R, Maher P, Schubert D. A broadly neuroprotective derivative of curcumin. J Neurochem. 2008; 105(4):1336–45. [PubMed: 18208543]
8. Maher P, Akaishi T, Schubert D, Abe K. A pyrazole derivative of curcumin enhances memory. Neurobiol Aging. 2008; 31:706–9. [PubMed: 18639368]
9. Lapchak PA, Maher P, Schubert D, Zivin JA. Baicalein, an antioxidant 12/15-lipoxygenase inhibitor improves clinical rating scores following multiple infarct embolic strokes. Neuroscience. 2007; 150(3):585–91. [PubMed: 17942241]
10. Lapchak PA, Schubert D, Maher P. The curcumin derivative CNB-001 attenuates ischemia-induced cell death in vitro and reduces embolism-induced clinical deficits in vivo in a rabbit embolic stroke model: neuroprotection is mediated via an erk-orp150 pathway. Stroke. 2010; e276:85.
11. Tan S, Schubert D, Maher P. Oxytosis: A novel form of programmed cell death. Curr Top Med Chem. 2001; 1(6):497–506. [PubMed: 11895126]
12. Abe K, Takayanagi M, Saito H. Effects of recombinant human basic FGF and its modified protein CS23 on survival of primary cultured neurons from various regions of fetal rat brain. Japan Journal of Pharmacology. 1990; 53:221–7.
13. McKim JM Jr. Building a tiered approach to in vitro predictive toxicity screening: a focus on assays with in vivo relevance. Comb Chem High Throughput Screen. 2010; 13(2):188–206. [PubMed: 20053163]
14. Bath PM, Gray LJ, Bath AJ, Buchan A, Miyata T, Green AR. Effects of NXY-059 in experimental stroke: an individual animal meta-analysis. Br J Pharmacol. 2009; 157(7):1157–71. [PubMed: 19422398]
15. Diener HC, Lees KR, Lyden P, Grotta J, Davalos A, Davis SM, et al. NXY-059 for the treatment of acute stroke: pooled analysis of the SAINT I and II Trials. Stroke. 2008; 39(6):1751–8. [PubMed: 18369171]
16. Shuaib A, Lees KR, Lyden P, Grotta J, Davalos A, Davis SM, et al. NXY-059 for the treatment of acute ischemic stroke. N Engl J Med. 2007; 357(6):562–71. [PubMed: 17687131]
17. Yoshida H, Yanai H, Namiki Y, Fukatsu-Sasaki K, Furutani N, Tada N. Neuroprotective effects of edaravone: a novel free radical scavenger in cerebrovascular injury. CNS Drug Rev. 2006; 12(1): 9–20. [PubMed: 16834755]

18. Watanabe T, Tahara M, Todo S. The novel antioxidant edaravone: from bench to bedside. Cardiovasc Ther. 2008; 26(2):101. [PubMed: 18485133]

19. Toyoda K, Fujii K, Kamouchi M, Nakane H, Arihiro S, Okada Y, et al. Free radical scavenger, edaravone, in stroke with internal carotid artery occlusion. J Neurol Sci. 2004; 221(1–2):11–7. [PubMed: 15178207]

20. Mishina M, Komaba Y, Kobayashi S, Tanaka N, Kominami S, Fukuchi T, et al. Efficacy of edaravone, a free radical scavenger, for the treatment of acute lacunar infarction. Neurol Med Chir (Tokyo). 2005; 45(7):344–8. discussion 8. [PubMed: 16041179]

21. Inatomi Y, Takita T, Yonehara T, Fujioka S, Hashimoto Y, Hirano T, et al. Efficacy of edaravone in cardioembolic stroke. Intern Med. 2006; 45(5):253–7. [PubMed: 16595989]

22. Otomo E. Effect of a novel free radical scavenger, edaravone (MCI-186), on acute brain infarction. Randomized, placebo-controlled, double-blind study at multicenters. Cerebrovasc Dis. 2003; 15(3):222–9. [PubMed: 12715790]

23. Lapchak PA. A critical assessment of edaravone acute ischemic stroke efficacy trials: is edaravone an effective neuroprotective therapy? Expert Opinion on. Pharmacotherapy. 2010; 11(10):1753–63.10.1517/14656566.2010.493558

24. Toxicity screening methods. 2009.

25. Lapchak PA, Araujo DM, Song D, Wei J, Purdy R, Zivin JA. Effects of the spin trap agent disodium-[*tert*-butylimino)methyl] benzene-1, 3-disulfonate *N*-oxide (generic NXY-059) on intrace-rebral hemorrhage in a rabbit large clot embolic stroke model: combination studies with tissue plasminogen activator. Stroke. 2002; 33(6):1665–70. [PubMed: 12053009]

26. Lapchak PA, Araujo DM, Song D, Wei J, Zivin JA. Neuro-protective effects of the spin trap agent disodium-[(tert-butylimino) methyl]benzene-1, 3-disulfonate N-oxide (generic NXY-059) in a rabbit small clot embolic stroke model: combination studies with the thrombolytic tissue plasminogen activator. Stroke. 2002; 33(5):1411–5. [PubMed: 11988623]

27. Lapchak PA, Zivin JA. The lipophilic multifunctional antioxidant edaravone (radicut) improves behavior following embolic strokes in rabbits: a combination therapy study with tissue plasminogen activator. Exp Neurol. 2009; 215(1):95–100. [PubMed: 18851964]

28. Hopkinson K, Williams EA, Fairburn B, Forster S, Flower DJ, Saxton JM, et al. A MitoTracker Green-based flow cytometric assay for natural killer cell activity: variability, the influence of platelets and a comparison of analytical approaches. Exp Hematol. 2007; 35(3):350–7. [PubMed: 17309815]

29. Vickers AE. Use of human organ slices to evaluate the biotransfor-mation and drug-induced side-effects of pharmaceuticals. Cell Biol Toxicol. 1994; 10(5–6):407–14. [PubMed: 7697504]

30. Bernas T, Dobrucki J. Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry. 2002; 47(4):236–42. [PubMed: 11933013]

31. Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys. 1993; 303(2):474–82. [PubMed: 8390225]

32. Lapchak PA, De Taboada L. Transcranial near infrared laser treatment (NILT) increases cortical adenosine-5′-triphosphate (ATP) content following embolic strokes in rabbits. Brain Res. 2010; 1306:100–5. [PubMed: 19837048]

33. Griffith OW. Determination of glutathione and glutathionedisulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem. 1980; 106:207–12. [PubMed: 7416462]

34. Vacchiano CA, Tempel GE. Role of nonenzymatically generated prostanoid, 8-iso-PGF2 alpha, in pulmonary oxygen toxicity. J Appl Physiol. 1994; 77(6):2912–7. [PubMed: 7896640]

35. Muller H, Klinkhammer W, Globisch C, Kassack MU, Pajeva IK, Wiese M. New functional assay of P-glycoprotein activity using Hoechst 33342. Bioorg Med Chem. 2007; 15(23):7470–9. [PubMed: 17890094]

36. Gao GL, Wan HY, Zou XS, Chen WX, Chen YQ, Huang XZ. Relationship between the expression of P-glycoprotein, glutathione S-transferase-pi and thymidylate synthase proteins and adenosine

triphosphate tumor chemosensitivity assay in cervical cancer. Zhonghua Fu Chan Ke Za Zhi. 2007; 42(3):201–5. [PubMed: 17537309]

37. Li QY, Wang Y, Yin ZF, Wu MC. Application of the improved MTT assay in predicting the intrinsic drug resistance of liver cancer. Zhonghua Yi Xue Za Zhi. 2007; 87(5):333–5. [PubMed: 17456363]
38. Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T, Mayer RT. Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. Biochem Pharmacol. 1985; 34(18):3337–45. [PubMed: 3929792]
39. D'Amelio M, Cavallucci V, Cecconi F. Neuronal caspase-3 signaling: not only cell death. Cell Death Differ. 2009; 17:1104–14.10.1038/cdd.2009.180 [PubMed: 19960023]
40. Toxicity screening methods. 2007.
41. Green AR. Why do neuroprotective drugs that are so promising in animals fail in the clinic? An industry perspective. Clin Exp Pharmacol Physiol. 2002; 29(11):1030–4. [PubMed: 12366397]
42. Legos JJ, Tuma RF, Barone FC. Pharmacological interventions for stroke: failures and future. Expert Opin Investig Drugs. 2002; 11(5):603–14.
43. Liebeskind DS, Kasner SE. Neuroprotection for ischaemic stroke: an unattainable goal? CNS Drugs. 2001; 15(3):165–74. [PubMed: 11463125]
44. Higashi Y, Jitsuiki D, Chayama K, Yoshizumi M. Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a novel free radical scavenger, for treatment of cardiovascular diseases. Recent Patents Cardiovasc Drug Discov. 2006; 1(1):85–93.
45. Lapchak PA. NXY-059. Centaur Curr Opin Investig Drugs. 2002; 3(12):1758–62.
46. Lapchak PA, Araujo DM. Development of the Nitrone-Based Spin Trap Agent NXY-059 to treat acute ischemic stroke. CNS Drug Rev. 2003; 9(3):253–62. [PubMed: 14530797]
47. Maples KR, Green AR, Floyd RA. Nitrone-related therapeutics: potential of NXY-059 for the treatment of acute ischaemic stroke. CNS Drugs. 2004; 18(15):1071–84. [PubMed: 15581379]
48. Maples KR, Ma F, Zhang YK. Comparison of the radical trapping ability of PBN, S-PPBN and NXY-059. Free Radic Res. 2001; 34(4):417–26. [PubMed: 11328677]
49. Hishida A. Clinical analysis of 207 patients who developed renal disorders during or after treatment with edaravone reported during post-marketing surveillance. Clin Exp Nephrol. 2007; 11(4):292–6. [PubMed: 18085390]
50. FDA. Guidance for Industry M4S: The CTD—Safety Appendices. 2001. http://www.fda.gov/cder/guidance/index.htm

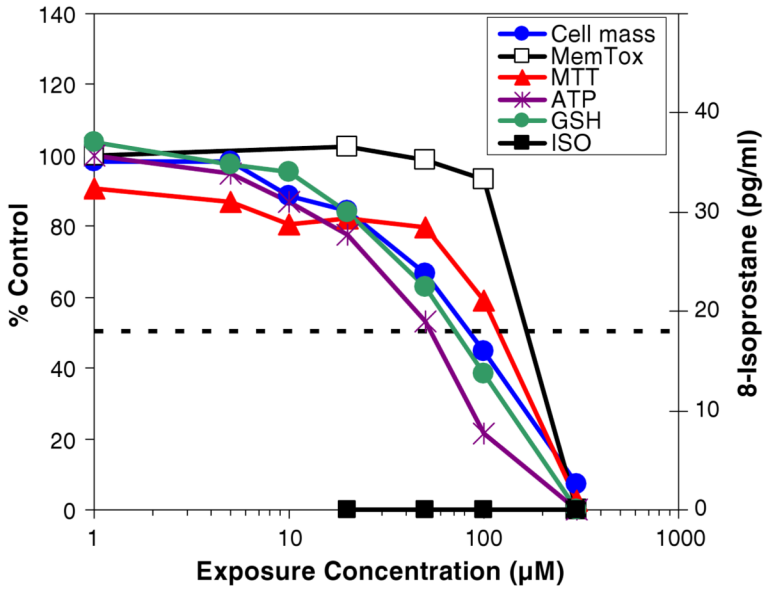



**Fig. 1.**

CeeTox analysis of CNB-001. Effects of CNB-001 on cellular toxicity and oxidative stress following incubation of rat hepatoma-derived H4IIE cells with CNB-001. Data are expressed as % control for cell mass (*blue line*), membrane toxicity (*black line open square*), MTT assay (*red line*), ATP (*purple line*), GSH content (*green line*), or pg/ml for 8-isoprostane (*black line*). CNB-001 produced significant (*$p<0.05$) decreases in all markers except for 8-isoprostane with $TC_{50}$ values in the range of 55–193 μM

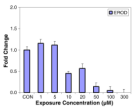



**Fig. 2.**

EROD activity for CNB-001. Effect of CNB-001 on EROD activity. EROD activity is presented as vs. exposure concentration (μM). There was apparent inhibition of EROD activity at CNB-001 doses above 10 μM that was associated with the cytostatic effect of CNB-001

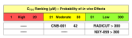


**Fig. 3.**
$C_{Tox}$ ranking profile

## Table 1
### Comparison of CNB-001 with NXY-059 and Radicut on cell toxicity

| Compound | Cell number (cell mass) $TC_{50}$ (μM) | MemTox $TC_{50}$ (μM) | MTT $TC_{50}$ (μM) | ATP $TC_{50}$ (μM) |
|---|---|---|---|---|
| CNB-001 | 88 | 193 | 131 | 55 |
| NXY-059 | >300 | >300 | >300 | >300 |
| RADICUT | >300 | >300 | >300 | >300 |

$TC_{50}$=concentration that produced a half-maximal response. Cell number (or mass) (*n*=7 replicates). MemTox=membrane toxicity measured using GST leakage (*n*=3 replicates). MTT=3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (*n*=7 replicates). ATP=adenosine triphosphate content (*n*=7 replicates)

**Table 2**

**Drug P-glycoprotein (PgP) binding**

| Compound | % Control (compound) | % Control (compound+CSA) | % Difference |
|---|---|---|---|
| CNB-001 | 79.9 | 79.0 | NC |
| NXY-059 | 100.0 | 93.6 | NC |
| RADICUT | 95.9 | 94.8 | NC |

PgP interaction ranking (based on % difference in the absence and presence of CSA, *n*=7 replicates)

*NC* no change