



## TCP-FA4: A derivative of tranylcypromine showing improved blood–brain permeability

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### ARTICLE INFO

#### Article history:

Received 7 May 2009

Accepted 31 July 2009

#### Keywords:

Fatty acid chains

Lipoamino acids

Blood–brain barrier (BBB)

Neuroprotection

β-Amyloid

Monoamine oxidase inhibition

### ABSTRACT

A variety of approaches have been taken to improve the brain penetration of pharmaceutical agents. The amphipathic character of a compound can improve its interaction with the lipid bilayer within cell membranes, and as a result improve permeability. Fatty acid chains or lipoamino acids of various lengths were attached to tranylcypromine (TCP), in an attempt to improve the blood–brain barrier (BBB) permeability by increasing the lipophilicity as well as the amphiphatic character of the drug. TCP-FA4, one of the derivatives containing a four carbon alkyl acid chain, showed the greatest improvement in permeability. This molecule was slightly neuroprotective in a β-amyloid-induced neurodegeneration assay and may also be capable of upregulating brain derived neurotrophic factor (BDNF), as indicated by cell culture assays using human umbilical vein endothelial cells. Since decreased levels of BDNF are observed in many CNS disorders, and direct injection of BDNF is not a viable option due to its poor permeability across the BBB, small molecules capable of regulating BDNF that also cross the BBB may be an interesting treatment option.

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## 1. Introduction

Delivery of pharmaceutical agents to the brain is one of the most challenging areas of drug delivery. Tight junctions, minimized surface area, electrostatic interactions and increased metabolism as well as active efflux systems all work together to create the blood–brain barrier (BBB) [1–4]. Different approaches have been taken to improve the brain penetration of pharmaceutical agents. The objective of this specific study was to investigate the effect of the attachment of lipoamino acids (LAAs) or fatty acid (FA) moieties on the BBB permeability of a model compound, tranylcypromine (*trans*-(+)-2-phenylcyclopropanamine, TCP). Although the conjugation of FAs increase the lipophilicity, the addition of LAAs has the added advantage of giving an amphipathic character to the drug molecules, which in turn can facilitate their interaction with cell membranes [5]. This approach to improve the

permeability across biological barriers, including the BBB, has been taken before with some success [6–8]. Various approaches, both computational and experimental, were taken to predict the interaction of TCP derivatives with biomembranes (data not included) [9,10]. The rationale behind this study was that by increasing the amphiphilic character of a compound, its interaction with the lipid bilayer within cell membranes can also be enhanced and as a result improve permeability. The proof of concept was attempted using the irreversible monoamine oxidase inhibitor (MAOI) TCP. Various derivatives of TCP were previously synthesized in which FAs or LAAs of various alkyl chain lengths were conjugated to the TCP amine group [10] (Fig. 1). These TCP derivatives were then tested in an *in vitro* BBB model using bovine brain microvessel endothelial cells (BBMECs). The studies showed that although the TCP derivatives containing LAA were predicted to have better permeability than their FA counterparts, on the basis of computational and experimental models [9], TCP-FA4, one of the derivatives containing a four carbon alkanolic acid residue showed the greatest improvement in permeability. The transport properties of this derivative were thoroughly characterized and are described herein.

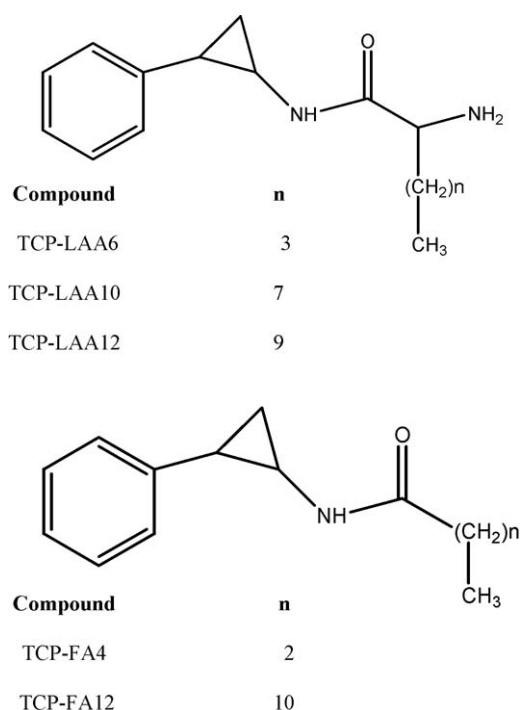
Furthermore, we briefly investigated the neuroprotective properties of TCP-FA4. Although this compound was originally synthesized as a model compound to demonstrate a proof of

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<sup>1</sup> This work is part of K.E.D. Ph.D. Thesis.



**Fig. 1.** TCP conjugates with lipoamino acids (LAAs) or fatty acids (FAs) bearing a side alkyl chain of varying length.

concept, the literature suggested that TCP could have neuroprotective properties [11]. Since TCP-FA4 is predicted to have good BBB permeability, it could prove to be an important discovery if it is neuroprotective. TCP (and potentially its derivatives) could be neuroprotective as a result of their ability to induce synthesis of brain derived neurotrophic factor (BDNF) [11]. BDNF is a 27 kDa protein found within the brain and periphery that supports the survival of existing neurons as well as aids in the growth and differentiation of new neurons [12]. In recent years, BDNF has become a drug target for many CNS disorders including Alzheimer's, Parkinson's, epilepsy [12], as well as cerebral ischemia [11]. Since neurotrophins possess less than optimal pharmacological properties including poor stability and minimal BBB permeation, the discovery of small molecules that can regulate BDNF expression could become beneficial [13].

## 2. Materials and methods

All chemicals and cell culture reagents were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise specified.

### 2.1. Isolation and maintenance of BBMECs

Bovine brain microvessel endothelial cells (BBMECs) were isolated from the gray matter of bovine cerebral cortices by enzymatic digestion followed by centrifugation and then seeded as primary cultures following methods described by Audus and Borchardt [14]. Isolated BBMECs were seeded at a density of approximately 50,000 cells/cm<sup>2</sup> onto 24-well culture plates or 100 mm culture dishes (Corning Costar, Acton, MA) containing Nucleopore<sup>®</sup> polycarbonate membranes (pore size 0.4 μm) (Whatman, Florham Park, NJ) pretreated with rat-tail collagen prepared in house and fibronectin. BBMECs were grown at 37 °C in an atmosphere of 5% CO<sub>2</sub> and 95% relative humidity. The cells were grown in 50% Minimal Essential Medium and 50% Ham's F12 supplemented with 100 μg/mL streptomycin, 100 μg/mL penicillin G, 13 mM sodium bicarbonate, 10% platelet poor horse serum,

0.5% endothelial cell growth supplement (ECGS), and adjusted to pH 7.4 with 10 mM HEPES.

### 2.2. Maintenance of HUVECs

Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and cultured in Dulbecco's modified Eagle's media containing 10% fetal bovine serum, 100 units/mL of penicillin G sodium salt, 100 μg/mL of streptomycin sulfate, 2.0 g/L NaHCO<sub>3</sub>, 1.42 g/L HEPES-Na, 1% glutamine, and 1% nonessential amino acids. Cell culture media was replaced every other day after seeding until cells had grown to confluency. Cells were grown at 37 °C under 5% CO<sub>2</sub> and 95% relative humidity.

### 2.3. Rhodamine 123 uptake assay

BMECs were seeded onto 24-well plates and culture media was replaced every other day until cells formed a confluent monolayer. At the start of the experiment, BBMECs were rinsed with warm PBSA (PBS containing CaCl<sub>2</sub>, MgSO<sub>4</sub>, glucose and L-ascorbic acid) and allowed to acclimate to the warm PBSA for 10 min. The PBSA was removed and solutions of TCP derivatives (10 μM) were added to the wells and allowed to incubate for 30 min at 37 °C. Rhodamine 123 was then added to all wells at a concentration of 5 μM and cells were incubated for an additional 2 h. After the incubation, cells were quickly rinsed with ice cold PBS several times. Lysis buffer (0.5%, v/v Triton X-100 in 0.2N NaOH) was then added to all wells and allowed to solubilize cells for at least 30 min. Aliquots (200 μL) were taken from all wells to determine rhodamine 123 concentration and 10 μL aliquots were taken from all wells to determine protein concentration. Rhodamine 123 concentrations were determined on a Biotek FL600 Microplate Fluorescence Reader (excitationλ: 485 nm, emissionλ: 530 nm). Protein determination was performed using a Pierce BCA Protein Assay Kit (Pierce, Rockford, IL) and samples were read on an EIA reader at 540 nm.

### 2.4. BBMEC permeability assay

BBMECs were grown on 0.4 μm Nucleopore<sup>®</sup> polycarbonate membranes in a 100 mm culture dish coated with rat-tail collagen and fibronectin. Once cells had formed a confluent monolayer as determined by light microscopy, the membranes were transferred to Side-by-Side<sup>™</sup> diffusion chambers as previously described by Audus et al. [15,16]. Briefly, each chamber was filled with 3 mL of PBSA and the luminal donor chamber contained 10 μM of the TCP derivative. The temperature was maintained at 37 °C within the chamber with an external circulating water bath and chamber contents were stirred with Teflon<sup>®</sup> coated magnetic stir bars driven by an external console. At the various time points up to 90 min, 200 μL aliquots were removed from the receiver side and 20 μL aliquots of the donor solution were taken at time zero. For the TCP-FA4 derivative, permeability was extremely rapid, so in order to monitor permeability within a linear range, earlier time points had to be taken. For all studies with TCP-FA4, time points were instead taken at 2, 5, 10, 15, 20 and 30 min. All samples were analyzed for concentration using LC/MS/MS analysis. The integrity of the cell monolayer was tested post-experiment by monitoring the permeability of [<sup>14</sup>C]-sucrose, a low permeability paracellular marker that should not readily cross the cell monolayer. All sucrose samples were analyzed for concentration by liquid scintillation counting.

Additionally, this same experimental setup was used to investigate whether there were any directional differences in transport due to membrane transporters. In these bi-directional

permeability studies, compound was added to the abluminal side and the luminal side was monitored over time.

For temperature dependent permeability studies, the same experimental setup was used and permeation of TCP-FA4 was calculated at a range of temperatures from 4 to 37 °C in order to determine an activation energy.

### 2.5. TCP-FA4 permeability inhibition studies

Inhibition studies were performed to determine if permeation of TCP-FA4 was an active process. Inhibitors used included: 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) an anion-exchange inhibitor, ouabain, a sodium pump blocker (Na-K-ATPase), sodium azide, a metabolic inhibitor capable of depleting ATP and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), an ionophore inhibitor. Inhibitor concentrations and treatment conditions were decided upon based on prior studies performed in the laboratory [17]. Permeability studies in the presence of inhibitors were conducted as described in the previous section.

### 2.6. Neuronal cell cultures

Dissociated cortical cell cultures were established from embryonic day 18 rat fetuses recovered from pregnant Sprague–Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, IN) as described previously [18]. After the final precipitation step, neurons were suspended in fresh Dulbecco's modified Eagle's medium/F-12 with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and plated at a density of  $2.5 \times 10^5$  cells in 35 mm glass bottom microwell dishes (MatTek Co., Ashland, MA), coated with poly-D-lysine. Serum-containing medium was removed after 24 h, and the cells were maintained in serum-free Dulbecco's modified Eagle's medium/F-12 containing the N2 supplements. Cultures were grown at 37 °C in 5% CO<sub>2</sub> and 97% humidity as previously described [18].

### 2.7. Measurement of cell viability

The effects of the  $\beta$ -amyloid peptide (A $\beta$ <sub>25–35</sub>) (10  $\mu$ M) and TCP and TCP-FA4 (0.1–200 nM concentrations) were primarily determined by monitoring neuronal cell survival using the Live/Dead assay as previously described [19]. The A $\beta$ <sub>25–35</sub> was synthesized and purified in the Biochemical Research Services Lab at The University of Kansas. We used A $\beta$ <sub>25–35</sub> at a concentration of 10  $\mu$ M in the present studies based on earlier results. In previous studies we have shown that at this concentration we see A $\beta$  induced loss of cortical cells by approximately 50% [20]. This level thus enables us to test drug effects that would not be possible if high A $\beta$  concentration was used to destroy all cells. Following exposure to the peptides and/or test compounds, cells were labeled with 20  $\mu$ M propidium iodide (PI) and 150 nM calcein acetoxy-methylester (Molecular Probes, Eugene, OR) for 30 min at 37 °C. After incubation with the dyes, the dishes were rinsed with phosphate-buffered saline (PBS) and placed on the stage of a Nikon inverted microscope (Nikon Eclipse TE200; Nikon, Tokyo, Japan) with filters for fluorescein isothiocyanate and Texas Red. Digital images were captured and the number of viable (green) and dead (red) neurons was determined by counting the cells in 6–12 microscopic fields per culture dish in duplicate dishes for each treatment. All experimental treatments were carried out on at least two separate embryonic neuronal preparations with approximately 1500 neurons scored under each treatment condition. The fraction of viable cells in each field was calculated based on the total number of cells counted in each field. Raw data from each experiment were combined and the significance of differences between cultures exposed to various treatments was determined using Student's *t* test.

### 2.8. Measurement of BDNF with enzyme-linked immunosorbent assay (ELISA)

To determine if TCP-FA4 can increase the production of brain derived neurotrophic factor (BDNF), analysis of cell culture media post-treatment with TCP-FA4 was performed. Initially, BBMECs were used for these studies; however, there was no existing literature confirming that BBMECs grown in culture synthesize or secrete BDNF. Our studies showed that in fact BBMECs do not secrete detectable levels of BDNF (detection limit: 15.6 pg/mL). Therefore, another cell culture system had to be used and based on studies by Nakahashi et al. [21] it was seen that human umbilical vein endothelial cells (HUVECs) synthesize and secrete BDNF [21]. Ideally a brain-derived cell line would have been used for these studies, but it has been shown that neurotrophin derived from peripheral tissues is available to some peripheral neurons [12]. HUVECs were grown to confluency in a 6-well tissue culture plate (approximately 5 days) then washed three times with phosphate-buffered saline warmed to 37 °C. Solutions of 5  $\mu$ M TCP and 5  $\mu$ M TCP-FA4 were prepared in culture media and added to the wells (*n* = 2). A lower concentration of TCP and TCP-FA4 was used relative to the BBMEC permeability studies to more closely reflect *in vivo* concentrations. Therapeutic plasma concentrations of tranylcypromine are reported to be approximately 1  $\mu$ M [22]. A slightly higher concentration was used to ensure we could detect synthesized BDNF. The remaining two wells contained blank media and served as a control. Aliquots of 200  $\mu$ L were taken from the wells at 0, 24, 31 and 48 h and stored at –20 °C until analysis. Prior to analysis samples were centrifuged at 4 °C at  $1500 \times g$  to remove any particulates. Concentrations of BDNF were measured using a commercial ELISA kit (Promega, Madison, WI). For optimal results, all samples were diluted 1:1 with the provided Block & Sample Buffer. A standard curve was created and linearity ( $R^2 = 0.99$ ) was observed from 7 to 250 pg/mL. According to the manufacturer's brochure, the assay provides specific detection of BDNF with typically less than 3% cross-reactivity with other related neurotrophic factors and provides a lower detection limit of 15.6 pg/mL.

### 2.9. Sample analysis of TCP and derivatives by LC/MS/MS

Samples generated by the permeability studies using BBMECs were analyzed by LC/MS/MS. The chromatography system consisted of a Waters 2690 system and a Phenomenex Onyx monolithic C18 column (50 mm  $\times$  4.6 mm). After chromatographic separation, samples were directed into the mass spectrometer (Micromass<sup>TM</sup> triple quadrupole) and detected by tandem mass spectrometry in positive ion mode using electrospray ionization. There was no evidence of any degradation of TCP nor its derivatives upon permeation across the BBMEC monolayer.

An isocratic HPLC method using a mobile phase of acetonitrile/5 mM acetic acid, pH 3.9 with ammonia was used. The ratio of aqueous to organic was modified slightly based on the lipophilicity of the derivative and ranged from 10:90 (v/v) acetonitrile:5 mM acetic acid to 50:50 (v/v) acetonitrile:5 mM acetic acid. A diversion of the HPLC eluent between 0 and 2 min reduced the exposure of the mass spectrometer to possible salts and cellular contaminants in the sample. Standards of all derivatives showed good linearity over a calibration range of 10–1000 ng/mL. Standard curves for TCP-FA4 were extended to include concentrations up to 5000 ng/mL, and good linearity was still maintained.

### 2.10. Assay for monoamine oxidase inhibition

Inhibition of monoamine oxidase was assessed by addition of TCP-FA4 (in DMSO) to the assay for MAO. MAO A was purified and

assayed spectrophotometrically as in past work [23] in 50 mM potassium phosphate pH 7.4 containing 0.05% Triton X-100 and kynuramine (Sigma–Aldrich, UK) (varied, Km 0.15 mM). Data were analyzed in Prism, using global analysis to fit the data to the equation for competitive inhibition. Purified MAO B was assayed with 0.6 mM benzylamine ( $2 \times K_m$ ) either spectrophotometrically or polarographically because of the high absorbance of TCP-FA4 at 250 nm. All assays were performed at 30 °C. Pre-incubations with inhibitor to assess the time-dependence of inhibition and spectral collections were performed on ice or at 30 °C.

### 3. Results

#### 3.1. Rhodamine 123 uptake assay

Although TCP is not known to be a substrate for any efflux transporters expressed at the brain endothelium, the TCP derivatives were tested for potential interaction with P-glycoprotein (P-gp), one of the most prevalent of the efflux transporters that is responsible for limiting drug entry into the brain [24]. Since these molecules have increased lipophilicity, there is a chance they could be substrates, as P-gp substrates are often lipophilic in nature [25]. The rhodamine 123 assay is a rather facile and reliable way to predict potential P-gp interaction [26]. The effect of the test compound on rhodamine 123 is determined by monitoring intracellular fluorescence. If the test compound is a substrate for P-gp, then addition of the compound will increase rhodamine 123 uptake relative to the negative control. Results from the rhodamine 123 uptake assay suggest that none of the derivatives interact with P-gp (data not shown). All compounds were tested at a concentration of 10  $\mu$ M and only the positive control cyclosporin A showed any effect on intracellular rhodamine 123 uptake.

#### 3.2. BBMEC permeability studies

The permeability of TCP and its derivatives was measured using BBMEC monolayers mounted in Side-bi-Side™ chambers. The apparent permeability values were calculated using the following equation:

$$P_{app} = \frac{\Delta Q / \Delta t}{A \times C_0}$$

where  $\Delta Q / \Delta t$  is the linear appearance of the test compound in the receiver chamber,  $A$  is the cross-sectional area of the cell monolayer (0.636 cm<sup>2</sup>) and  $C_0$  is the initial concentration of the test compound in the donor chamber at  $t = 0$ . TCP had a permeability of approximately  $2.9 \times 10^{-4}$  cm/s and TCP-FA4, a derivative containing a butanoic side chain, showed an improved permeability of  $3.8 \times 10^{-4}$  cm/s. The derivatives containing LAA moieties did not show an improvement in permeability relative to TCP and those derivatives with the longest FA and LAA groups (TCP-FA12 and TCP-LAA12) were below the limit of detection (Fig. 2). Sucrose values for these studies ranged from  $2.0 \times 10^{-5}$  to  $7 \times 10^{-5}$  cm/s.

#### 3.3. Bi-directional permeability of TCP-FA4

To determine if the improved permeability of TCP-FA4 was due to an uptake transporter, the percentage of TCP-FA4 transported over time across a BBMEC monolayer was monitored in both the luminal to abluminal and abluminal to luminal directions. Studies indicated that there was a slight decrease in permeability going from the abluminal to luminal side (Fig. 3). The apparent permeability of TCP-FA4 in the luminal to abluminal direction had an average  $P_{app} = 5.47 \times 10^{-4}$  cm/s (std dev:  $2.4 \times 10^{-5}$  cm/s) and in the abluminal to luminal direction a  $P_{app} = 4.38 \times 10^{-4}$  cm/s

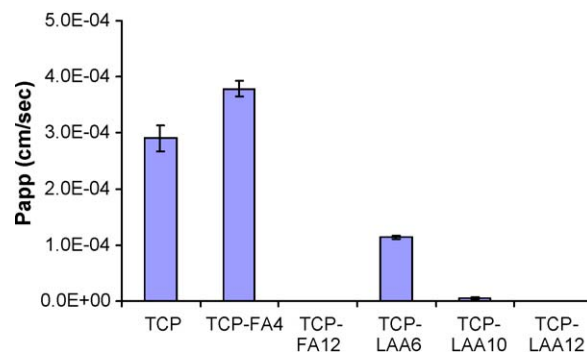


Fig. 2. Permeability of TCP and derivatives in BBMECs (\*\* $p < 0.01$  compared to TCP as determined by ANOVA; data  $\pm$  SD, TCP and TCP-FA4  $n = 8$ , TCP-LAA6 and TCP-LAA10  $n = 4$ ).

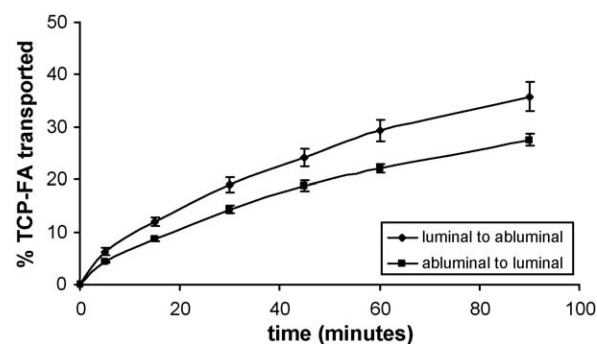


Fig. 3. Percentage of TCP-FA4 transported across BBMEC monolayers over time in the luminal to abluminal and abluminal to luminal directions (data  $\pm$  SD,  $n = 8$ ).

(std dev:  $1.4 \times 10^{-5}$  cm/s). To determine if this difference was in fact due to some sort of uptake or facilitative process, additional temperature dependent and inhibition studies were performed.

#### 3.4. Temperature dependent permeability studies of TCP-FA4

The permeability of TCP-FA4 was monitored in a range of temperatures and an activation energy of transport was calculated. Studies were performed using BBMEC monolayers mounted in Side-bi-Side™ chambers. The activation energy calculated from the slope of the Arrhenius plot was 28.0 kJ/mol (Fig. 4) which is suggestive of a passive process since carrier-mediated processes typically fall within a range of 29–105 kJ/mol [27].

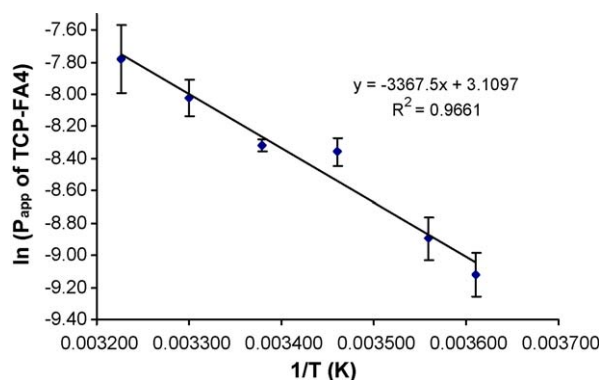


Fig. 4. Arrhenius plot generated by the temperature dependent permeability of TCP-FA4 in BBMECs. Permeability values were calculated at a variety of temperatures ranging from 4 to 37 °C (data  $\pm$  SD,  $n = 4$ , except 4 and 37 °C,  $n = 8$ ).



### 3.5. TCP-FA4: inhibition studies

The effect of various inhibitors on the permeability of TCP-FA4 was investigated (data not shown). For all inhibition studies, BBMECs were pretreated with agents for 15 min prior to performing the permeability study. Ouabain octahydrate, a sodium pump blocker, was used to determine if the permeability of TCP-FA4 was a sodium coupled process. Permeability of TCP-FA4 was monitored in the presence of 100  $\mu$ M ouabain, but no significant changes in permeability were noted. Sodium azide, a respiratory chain inhibitor, was used as a metabolic inhibitor to investigate if the transport of TCP-FA4 was via a process that required cell-dependent energy expenditure. However, the permeability of TCP-FA4 did not decrease in the presence of 10 mM sodium azide. FCCP, a protonophore, was used to determine if the permeability of TCP-FA4 would be altered upon disruption of the proton gradient, but at a final concentration of 50  $\mu$ M FCCP no changes in TCP-FA4 permeability were noted. Furthermore, in the presence of 50  $\mu$ M DIDS, an anion-exchange inhibitor, no changes in TCP-FA4 permeability were observed. These studies suggest that the permeation of TCP-FA4 is most likely a passive process and is neither an active nor facilitative process.

### 3.6. BDNF production by HUVECs in the presence of TCP-FA4

The effect of TCP-FA4 on the production of BDNF in HUVECs was investigated. HUVECs were treated with 5  $\mu$ M TCP-FA4 to determine if the derivative was capable of increasing production of BDNF relative to TCP. Results showed an increase in BDNF production relative to the control (blank media) as well as TCP. Levels of secreted BDNF increased over time and were highest when treated with TCP-FA4. At 48 h post-treatment with TCP-FA4, BDNF was detected at  $122.6 \pm 1.9$  pg/mL, compared to  $101.8 \pm 4.6$  and  $92.3 \pm 2.1$  pg/mL for TCP and blank media, respectively ( $n = 4$ ) (Fig. 5).

### 3.7. Inhibition of MAO by TCP-FA4

The inhibition of MAO A and MAO B by TCP-FA4 was assessed *in vitro*. TCP-FA4 (10  $\mu$ M) gave only 10% inhibition when added to an assay with 0.75 mM substrate ( $5 \times \text{Km}$ ) but the inhibition increased to 70% after pre-incubation with the enzyme for 10 min at 30 °C. This inhibition was fully reversible on dilution. Without pre-incubation, the inhibition was competitive with a  $K_i$  value of  $2.0 \pm 0.4$   $\mu$ M. Thus TCP-FA4 is a reversible inhibitor of MAO A. When

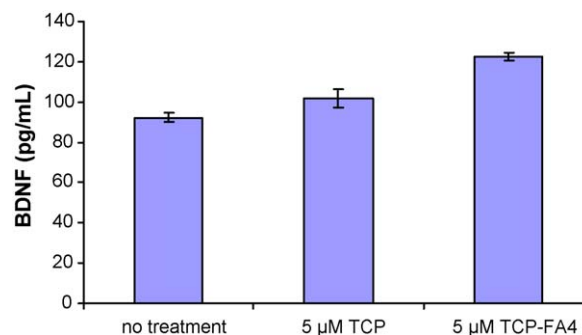


Fig. 5. BDNF secretion by cultured HUVECs in the presence of TCP and TCP-FA4 (\*\* $p < 0.01$  compared to control as determined by ANOVA, data  $\pm$  SD,  $n = 4$ ).

TCP-FA4 was added to purified MAO A there was a very small spectral change indicating binding but no reduction of the enzyme was observed when MAO A and TCP-FA4 (30  $\mu$ M) were incubated together at 20 °C overnight in contrast to the full reduction expected with the parent compound. The spectrum of TCP-FA4 also did not change (data not shown). Thus TCP-FA4 does not act as a suicide substrate of MAO A. No inhibition of human MAO B was observed, even at 100  $\mu$ M.

## 4. Discussion

Derivatives of TCP containing FA or LAA moieties of varying side alkyl chain length were synthesized in an attempt to improve BBB permeability, by increasing the lipophilicity as well as amphipathic character of the drug molecule. We previously investigated the modified lipophilicity of these derivatives by calculating the log  $P$  as well as the partition coefficient between blood and brain (log BB) by means of the software packages ACD logP 5.15, Pallas 3.0, Osiris Property Explorer and KOWIN 1.57 [9]. Results suggested that LAA promoiety containing medium to long side alkyl chains would be most useful in improving the permeability and membrane interaction of these compounds [9]. Additionally, experimental data confirmed that conjugation of TCP to LAA led to a deeper and more complex interaction with biomembrane models consisting of dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles and monolayers [9]. However, when using BBMECs as a model for the BBB, the FA derivative TCP-FA4 showed the greatest improvement in permeability. This derivative contains a four carbon alkyl chain and has an approximate twofold increase in lipophilicity

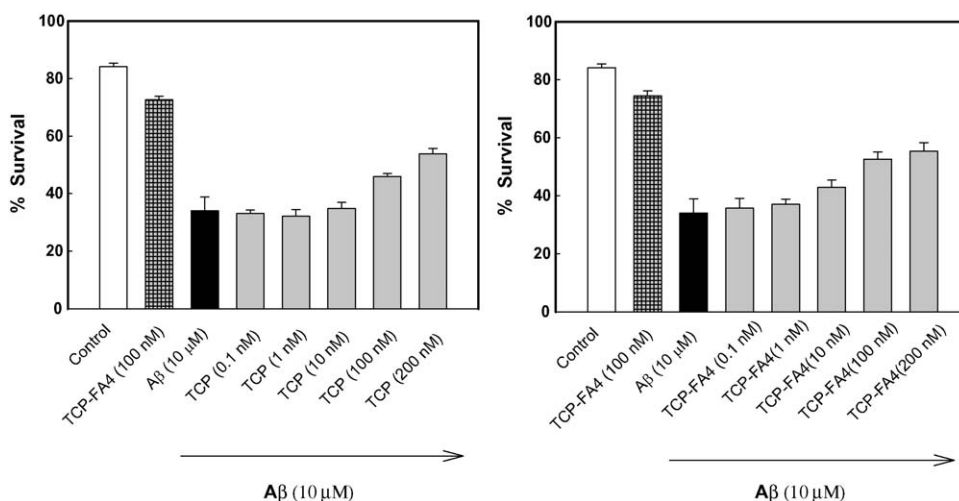


Fig. 6. Effects of A $\beta_{25-35}$ , TCP and TCP-FA4 on neuronal survival. Neuronal cells were treated with vehicle only, TCP, and TCP-FA4. The indicated concentrations of the compound were added 2 h before A $\beta$ . Cell viability was determined 48 h later. Data represent mean survival  $\pm$  SE for three separate experiments with  $\sim 1500$  cells per treatment condition.

compared to the parent drug [9,10]. Computational studies predicted that the large increase in lipophilicity for compounds like TCP-LAA10 and TCP-LAA12 would improve permeability, however in reality in an *in vitro* system, these compounds most likely partition nicely into the cell monolayer and never actually permeate across. They are caught up within the cell but are not released, which therefore results in a low experimental permeability. Additionally, this increase in lipophilicity decreased the compounds aqueous solubility, which inherently affects permeability. The permeability results for TCP-FA4 indicate that it has good passive permeability and is not a substrate for any active transport systems expressed in BBMECs. Furthermore, the utility as a pharmaceutical agent of a TCP derivative possessing improved permeability was investigated. Although it was found that TCP-FA4 does exhibit inhibitory activity against MAO A enzyme, its affinity was reduced compared to the parent compound, TCP [28] and the inhibition was reversible. However, our studies indicate that TCP-FA4 may possess neuroprotective properties. In a  $\beta$ -amyloid-induced neurodegeneration assay, neurons exposed to  $\beta$ -amyloid in the presence of 200 nM TCP-FA4 showed a 20% improvement in survival rate (Fig. 6). Also TCP and TCP-FA4 showed no toxicity to cortical neurons at concentrations up to 200 nM. Although the results of our experiments taken together suggest that TCP-A4 has neuroprotective activities, the cellular mechanisms underlying enhanced neuronal survival have yet to be delineated, primarily due to the fact that the molecular basis for  $\beta$ -amyloid toxicity has still not been fully elucidated. Additionally, using HUVECs grown in culture, increased levels of the neurotrophic factor BDNF were observed in the presence of TCP-FA4. Since BDNF is instrumental in the survival and differentiation of neurons and BDNF expression is altered in many brain disorders, the discovery of small molecules capable of regulating BDNF may be beneficial.

## Acknowledgments

Support for Dr. K.E.D. travel to the University of Catania for a BBB permeation expertise transfer to the forthcoming spinoff ETNALEAD (Scientific Director: S. Guccione) and research in the laboratories of the Dipartimento di Scienze Farmaceutiche has been provided by Dr. Marco Pintore (CEO BioChemicals Consulting, SAS, 111Bld.Duhameldu Monceau, 45166 Olivet cedex, France and ETNALEAD).

HUVEC cells were a gracious gift from the laboratory of Dr. Teruna Siahaan at the University of Kansas, Department of Pharmaceutical Chemistry. Technical assistance was provided by Dr. Sumit Majumdar.

Professor Venerando Pistarà, at the Department of Chemical Sciences, University of Catania, is gratefully acknowledged for the NMR analysis.

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