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7,8-dihydroxyflavone, a selective tyrosine kinase receptor B agonist and BDNF mimic, promotes angiogenesis.

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By

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Abstract

7,8-dihydroxyflavone (7,8-DHF), which is a member of the flavonoid family, is a selective tyrosine kinase receptor B (TrkB) agonist that has neurotrophic effects in various neurological diseases such as ischemic stroke and Parkinson's disease [3]. In this study, we assessed the angiogeneic effect of 7,8-DHF in endothelial cells derived from resistance vessel of the brain. Angiogenesis by 7,8-DHF is an important factor that helps prevent and treat various ischemic diseases. In this study, we found that rat RV cells used in the experiment possess the TrkB receptor. Our data also demonstrates that 7,8-DHF is able to stimulate cell proliferation in RV cells, suggesting that 7,8-DHF is capable of inducing angiogenesis. The 7,8-DHF activates the TrkB receptor which then leads to cell proliferation. In our study we also showed the effects of 7,8-DHF in the presence of the TrkB inhibitor cyclotraxin-B. Addition of cyclotraxin-B blocked the TrkB receptor and counteracted the effects of 7,8-DHF. Cell proliferation occurs in RV cells with the addition of 7,8-DHF, but this proliferation is inhibited by cyclotraxin-B .

Introductions

Flavonoids are part of a large family of polyphenolic compounds that are found in high concentrations in both fruits and vegetables [4]. A variety of studies have clearly demonstrated that flavonoids such as 3,6-dihydroxyflavone, 3,7-dihydroxyflavone, 5,6,7-dihydroxyflavone and 3,5,7-dihydroxyflavone shield against neuronal cell death, augment existing neuronal function, and promote neuronal regeneration [11]. 7,8-dihydroxyflavone (7,8-DHF) is an example of one of these flavanoids that has neurotrophic activities in various neurological diseases such as stroke and Parkinson's disease [6]. In a recent study, 7,8-DHF was shown to be a mimic of brain-derived neurotrophic factor (BDNF) as well as a selective tyrosine kinase B (TrkB) receptor agonist. BDNF is a cognate ligand of the TrkB receptor; BDNF-induced activation of these receptors results in neuronal survival, neuronal differentiation, and neurogenesis in a variety of disorders including stroke, amyotrophic lateral sclerosis, Parkinson’s disease, and Alzheimer’s disease [10].

Ischemic cardiovascular and cerebrovascular disorders resulted from atherosclerosis-associated arterial infarction are leading causes of death in Western society. The functional recovery of the ischemic tissues and organs is dependent on re-establishing collateral networks that sufficiently supply hyper-oxygenated blood to specialized cell populations. In response to ischemic insults, most tissues in the body have extraordinary capabilities to compensate low levels of oxygen by mechanisms of vasodilation, angiogenesis, arteriogenesis, vascular remodeling and hematopoiesis [3]. This study primarily focuses on the benefits and applications of angiogenesis, the formation of new vessels that is caused by the sprouting of endothelial cells from preexisting vessels.

The process of angiogenesis occurs after cerebral ischemia, or brain injury from severe

reduction of blood supply to the brain [7]. Ischemia occurs in a wide range of pathologic processes and is the focus of much research involving strokes and other diseases. The role of ischemia in pathophysiology is different in each case, and stimulating angiogenesis can help treat disease in some cases [5]. The main purpose of this study is to determine whether 7,8-DHF can stimulate angiogenesis, which would be beneficial to stroke patients.

Specifically, we examined whether 7,8-DHF is able to stimulate cell proliferation and calculated a dose-response curve. We also conducted a study using an inhibitor of 7,8-DHF and an inhibitor of BDNF. This study used RV cells, which are rat brain derived endothelial cells. In our study, we hypothesized that 7,8-DHF can induce cell proliferation in RV cells.

Methods and Materials

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2(4-sulfophenyl)2-H-tetrazolium, inner salt (MTS) came from Promega Corporation (Madison, WI, USA). 7,8-dihydroxyfalvone was purchased from TCI America (Portland, OR). Dulbecco's modified Eagle's Medium (DMEM), fetal bovine serum, trypsin, and Penicillin-Streptomycin Solution were all obtained from Gibco Company. TrkB antibody was purchased from BD Biosciences. Beta-tubulin antibody was purchased from Cell Signaling Technology Inc. Enhanced chemiluminescence system was purchased from Thermo Corporation. AlphaEase Image Analysis Software was obtained from Alpha Innotech Corp.

The RV Cell Line

Rat brain derived endothelial cells (a generous gift from Dr. Diglio Clement, Department of Pathology, Wayne State University) were maintained in DMEM containing 10% fetal bovine

serum (FBS) and were grown on tissue culture dishes or plates.

Cell Viability Assay

Cell viability was determined by the ability of the viable cells to metabolize 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2(4-sulfophenyl)2-H-tetrazolium, inner salt (MTS), which was described previously [13]. Prior to this, the RV cells were plated in 96-well plates, left to attach for 5 hours, and then the experimental doses of 7,8-DHF were added. Following the indicated experimental treatments, the RV cells were cultured overnight and 10 μL MTS solution was added to the cells the next day. After 3 hour incubation at 37° C, absorbance at 490 nm was then measured by a Thermo plate reader. The cells that were left untreated were considered as the controls, and the growth medium devoid of cells in the presence of MTS solution was used as the solution background. Cell viability was presented as a percentage of the untreated controls. For the MTS assay, there were eight samples in each group.

Inhibitor cyclotraxin-B

Cyclotraxin-B is the first highly potent and selective TrkB inhibitor that displays anxiolytic properties in mice. Cyclotraxin-B allosterically alters the conformation of TrkB, which leads to the inhibition of both the BDNF-dependent and –independent (basal) activities. Studies have shown that cyclotraxin-B might not only be a potent tool in investigate the role of BDNF and TrkB in pathology and physiology, but it also represents a lead compound for the development of new therapeutic approaches to treat brain disorders [2].

Inhibitor Study

Another study was run to determine the effects of cyclotraxin-B on the experimental doses of DHF and BDNF. The experimental doses that were used in this research were the control, 5μM cyclotraxin-B, 4 nM BDNF, 4 nM BDNF + 5μM cyclotraxin-B, 100 nM DHF, and 100 nM DHF + 5μM cyclotraxin-B. The doses were added and the RV cells were cultured overnight and 10μL MTS solution was added to the cells the next day. After 3 hour incubation at 37°C, absorbance at 490nm was then measured by a Thermo plate reader. The cells that were left untreated were considered the controls, and the growth medium devoid of cells in the presence of MTS solution was used as the solution background. Cell viability with the presence of cyclotraxin-B was compared with the viability of the cells with the experimental dose and expressed in the form of a percentage. For the MTS assay, there were eight samples in each group.

Western Blot Analysis

Proteins were subjected to electrophoresis in Tris-glycine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10%) and electrotransferred to nitrocellulose membrane. The blots were then incubated with primary antibody against TrkB at 4°C overnight. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour. Blots were developed with the enhanced chemiluminescence system and were captured on Kodak autoradiographic films. Beta-tubulin was also detected as a loading control.

Statistical evaluation

All of the values that were reported were expressed as mean ± SEM. Statistical differences

among groups were tested by one-way ANOVA, followed by Bartlett's test for homogeneity of variances. The value $p < 0.05$ was considered statistically significant.

RESULTS

7,8-DHF Induces Cell Proliferation

in a 96 well plate of RV cells containing 2000 cells. The cells were first maintained in DMEM containing 10% FBS and were grown on tissue culture plates. The cells were allowed to grow and attach for 5 hours. The experimental doses of 7,8-DHF were then added. The cell culture dish that was the least confluent was used in the experiment. The remaining surface medium was vacuumed out of the dish, the cells were washed with DPBS solution, and then 2 ml of TrypLE Express (trypsin) was added to remove the attached cells from the surface of the dish. Once the cells became unattached, 8 ml of DMEM containing 10% FBS was added to cease the trypsin's effect. The cells were then quantified via light microscope and the correct concentration of both the cells and medium were determined and then combined into a preparatory dish. The cells were then plated into a 96 well plate containing 2000 of the RV cells. Once the cells had been allowed to incubate for 5 hours, the experimental doses of 7,8-DHF were added to every well except the controls. The concentrations of 7,8-DHF started at 5.0 nM and progressed up all the way up to 1.0 mM. The cells were then allowed to incubate over night (24 hours) and were then quantified the next day.

To investigate if 7,8-DHF would induce cell proliferation, we conducted a dose-response study of 7,8-DHF as described above. Cell viability was measured by the MTS assay. 7,8-DHF induced cell proliferation at every concentration except 5.0 nM and 50 nM, with the higher doses

having the greatest effect (Figure 1). There were two distinct peaks of cell proliferation, one at 1.0 and the other at 0.5 mM. The results shown in this dose-response study clearly demonstrate that 7,8-DHF has a positive effect on the proliferation of RV cells.

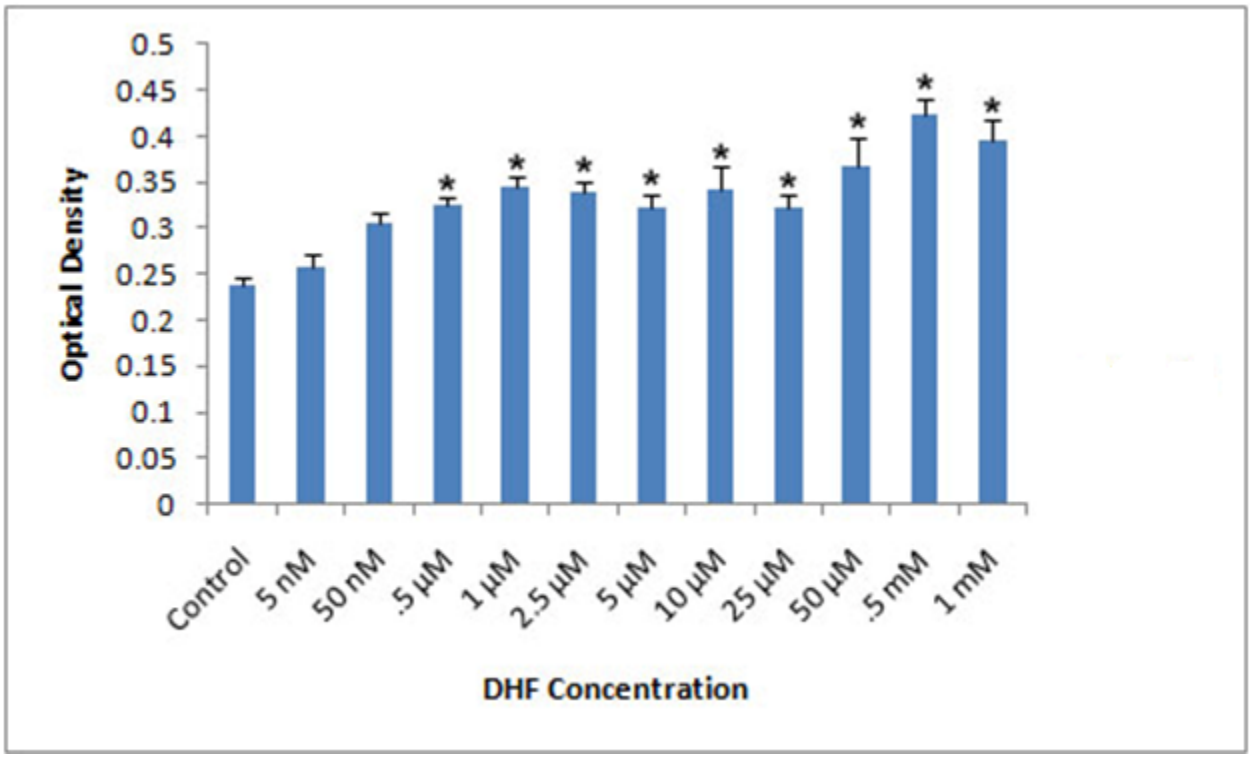

Figure 1. The cell proliferation effects of 7,8-DHF on RV cells. The cells were plated in a 96-well plate and all were treated with experimental doses of 7,8-DHF except for the control. The results were obtained via the cell viability assay using MTS. The increasing concentrations are used in order to determine the effects of the dose size on cell proliferation, thus creating a dose curve. *p< 0.01, versus control group.

Both BDNF and 7,8-DHF are Inhibited by Cyclotraxin-B

In our inhibitor study, the control group contained untreated RV cells and was compared against other cells that were treated with specific experimental doses along with cells that were just treated with 5 µM of the inhibitor cyclotraxin-B. One set of cells was treated with 4 nM of BDNF and compared against another set that contained the original 4 nM of BDNF as well as 5 µM of cyclotraxin-B. In the other set of cells that were compared against one another, one group contained 100 nM of DHF and was compared against another that group that contained both 100 nM DHF and 5 µM of cyclotraxin-B.

In the inhibitor study, the BDNF and the 7,8-DHF groups were compared to the control group; both groups showed cell proliferation. BDNF and 7,8-DHF cells that were treated with cyclotraxin-B did not show this cell proliferation (Figure 2).

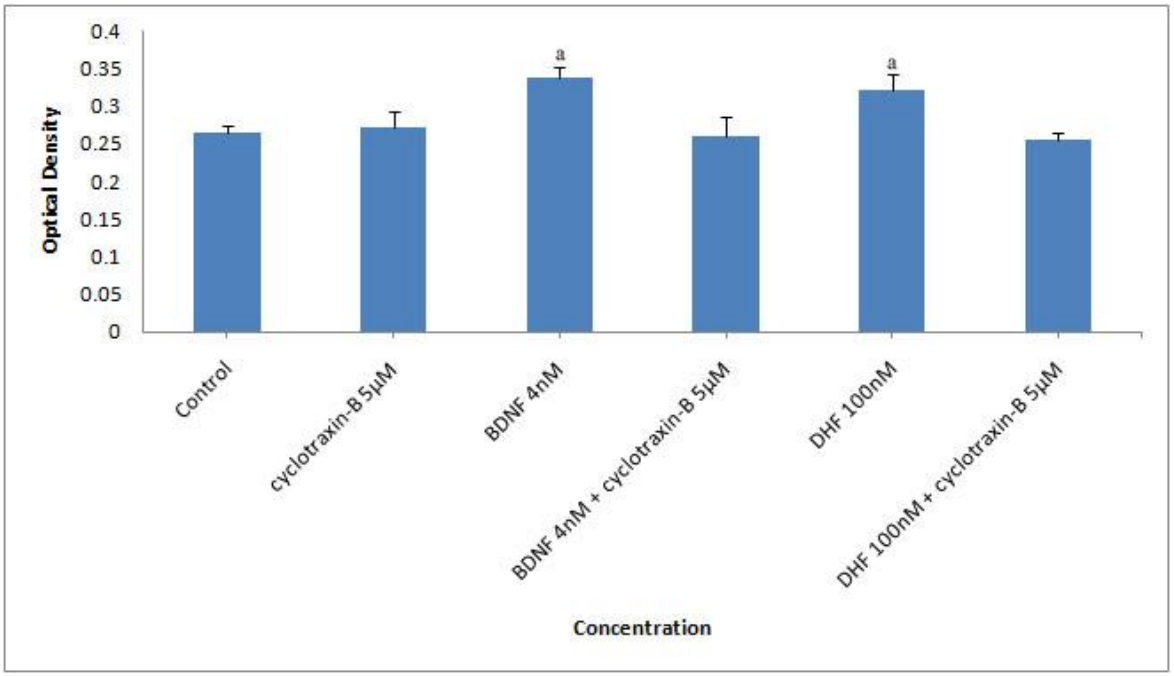

Figure 2. The inhibitive effects of cyclotraxin-B on both BDNF and DHF. The cells were plated in a 96-well plate and were treated with different experimental doses. The control was left untreated, the BDNF and the BDNF+cyclotroxin-B were compared, and the 7,8-DHF and the 7,8-DHF+cyclotraxin-B were compared. The results were obtained via the cell viability assay using MTS. This figure clearly demonstrated the differences between the control and BDNF and 7,8-DHF, along with the visible differences between the 7,8-DHF and its inhibited counterpart, and the BDNF and its inhibited counterpart. $^{a}p< 0.01$, versus control group.

Western Blot Analysis Confirms the Presence of TrkB Receptor in RV Cells

Western blot analysis confirmed the presence of the TrkB receptor in RV cells (Figure 3). This suggests that the 7,8-DHF activates the receptor, which then leads to the cell proliferation. It also suggests that cyclotraxin-B inhibits the receptor is inhibited, which prevents the cell proliferation.

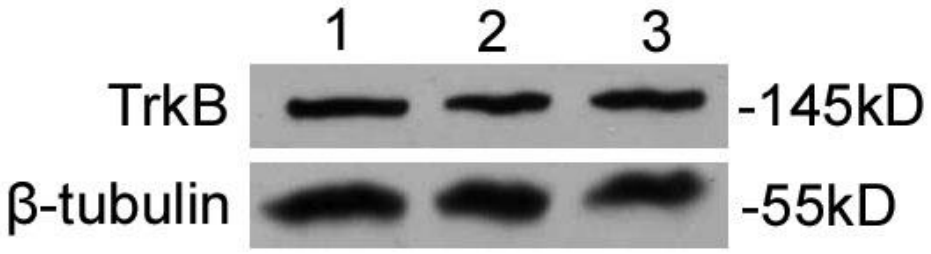

Figure 3. Western blot showing the presence of TrkB in the RV cells. The β-tublin is the loading control and the TrkB is the receptor that is shown to be found in the rat brain derived endothelial cells.

Discussion

Researchers have recently been focusing on counteracting the adverse effects of ischemia by inducing angiogenesis. In this study, we demonstrated that 7,8-DHF can induce angiogenesis. Past studies have shown that 7,8-DHF is a selective TrkB agonist and BDNF mimic that may provide neuroprotection in different diseases models via anti-oxidative activity and TrkB agonist activity [5]. TrkB, which is the most abundant Trk receptor that is expressed in the central nervous system, is mainly activated by BDNF and NT4/5 [3]. In this study, we showed that it is not the antioxidant activity of 7,8-DHF that causes cell proliferation, but rather its ability to mimic BDNF and activate the TrkB receptor. This study as a whole suggests that the use of the 7,8-DHF as a mimic to the protein BDNF can lead to TrkB activation and cell proliferation.

There are two adjacent hydroxyl (-OH) groups in the structure of 7, 8-DHF and hydroxyl groups are thought to confer antioxidant activity. As such the structure of 7, 8-DHF itself suggested the possibility that 7,8-DHF has anti-oxidant activity [3] (Figure 4). This being said, we do not believe that the antioxidant activity is the factor that causes angiogenesis due to the TrkB receptor activation by 7,8-DHF. The results of this study confirm this possibility.

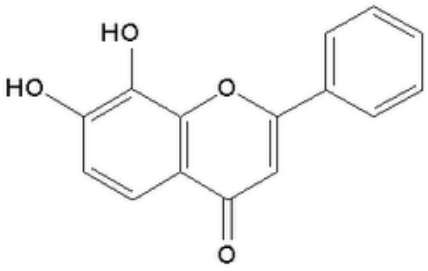

Figure 4. Structure of 7,8-dihydroxyflavone. This figure demonstrates the organic structure of 7,8-DHF. The two adjacent hydroxyl (-OH) groups in the structure are thought to confer the antioxidant activity.

Flavonoids belong to a group of natural substances with variable phenolic structures and are found in fruit, vegetables, grains, bark, roots, stems, flowers, tea, and wine [8]. These natural products were known for their beneficial effects on health long before flavoniods were isolated as the effective compounds. More than 4000 different varieties of flavonoids have been identified, many of which are responsible for the attractive colors of flowers, fruit, and leaves. The research on flavonoids received an added impulse with the discovery of the French paradox, i.e., the low cardiovascular mortality rate observed in Mediterranean populations in association with red wine consumption and a high saturated fat intake. Furthermore, epidemiologic studies suggest a protective role of dietary flavonoids against coronary heart disease [9].

Multiple studies have shown that flavanoids can protect against brain damage and help prevent and treat ischemic diseases. Flavanoids protect neurons against injury induced by neurotoxins, suppress neuroinflammation, and promote memory, learning and cognitive function[13]. This study shows that another beneficial effectse in flavanoids like 7,8-DHF is angiogenesis. This angiogeneic effect suggests that 7,8-DHF could be a potentially promising therapeutic intervention for the clinical treatment of neurological diseases such as ischemic

stroke, Parkinson’s disease and Alzheimer's disease [1].

Conclusion

In summary, we showed that 7,8-DHF stimulates angiogenesis and provided evidence that this angiogenic effect is mediated the ability of 7,8-DHF to mimic BDNF and activate the TrkB receptor. Clinically, inducing angiogenesis could be a promising therapeutic approach to the treatment of various neurological diseases. Many challenges remain, however several large trials indicated that delivery of pro-angiogenic factors to patients suffering from myocardial infarction and leg ischemia did not confer significant benefits. Despite the success shown in animal models, much research needs to be done before pro-angiogenic agents are used clinically [1].

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References

[1] Y. Cao, Therapeutic angiogenesis for ischenic disorders: What is missing for clinical benefits?, Retrieved from http://www.discoverymedicine.com/Yihai-Cao/2010/03/03/therapeutic-angiogenesis-for-ischemic-disorders-what-is-missing-for-clinical-benefits/ (2010).

[2] M. Cazorla, A.Jouvenceau, C. Rose, J-P. Guilloux, C. Pilon, A. Dranovsky, J. Prémont, Cyclotraxin-B, the first highly potent and selective TrkB inhibitor, has anxioloytic properties in mice, PLoS ONE 5(3): e9777. doi:10.1371/journal.pone.0009777 (2010)

[3] J. Chen, K.W. Chua, C.C. Chua, H. Yu, A. Pei, B.H. Chua, R.C. Hamdy, X. Xu, C.F. Liu, Antioxidant Activity of 7,8-dihydroxyflavone provides neuroprotection against glutamate-induced toxicity, Neurosci Let 499 (2011) 181-185

[4] J.B. Harborne, C.A. Williams, Advances in flavonoid research since 1992, Phytochemistry 55 (2000) 481-504.

[5] T. Hayashi, N. Noshita, T. Sugawara, P.H. Chan, Temporal Profile of Angiogenesis and Expression of Related Genes in the Brain After Ischemia, Journal of Cerebral Blood Flow and Metabolism 23 (2003) 166-180.

[6] S.W. Jang, X. Liu, M. Yepes, K.R. Shepherd, G.W. Miller, Y. Liu, W.D. Wilson, G. Xiao, B. Blanchi, Y.E. Sun, K. Ye, A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone, Proc Natl Acad Sci U S A 107 (2010) 2687-2692.

[7] T.N. Lin, S.W. Sun, W.M. Cheung, F. Li, C. Chang, Dynamic Changes in Cerebral Blood Flow and Angiogenesis After Transient Focal Cerebral Ischemia in Rats, Stroke 33 (2002) 2985-2991.

[8] E.J. Middleton, Effect of plant flavoniods on immune and inflammatory cell function. Adv Ecp Med Biol 439 (1998) 175-182

[9] R.J. Nijveldt, E. van Nood, D.E.C. van Hoorn, P.G. Boelens, K. van Norren, P.A.M. van Leeuwen, Flavonoids: a review of probable mechanisms of action and potential applications, Am J Clin Nutr 74 (2001) 418-485

[10] G.J. Siegel, N.B. Chauhan, Neurotrophic factors in Alzheimer's and Parkinson's disease brain, Brain Res Brain Res Rev 33 (2000) 199-227.

[11] J.P. Spencer, Food for thought: the role of dietary flavonoids in enhancing human memory, learning and neuro-cognitive performance, Proc Nutr Soc 67 (2008) 238-252.

[12] D. Vauzour, K. Vafeiadou, A. Rodriguez-Mateos, C. Rendeiro, J.P. Spencer, The neuroprotective potential of flavonoids: a multiplicity of effects, Genes Nutr 3 (2008) 115-126.

[13] X. Xu, C.C. Chua, J. Kong, R.M. Kostrzewa, U. Kumaraguru, R.C. Hamdy, B.H. Chua, Necrostatin-1 protects against glutamate-induced glutathione depletion and caspase-independent cell death in HT-22 cells, J Neurochem 103 (2007) 2004-2014.