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Tianma modulates proteins with various neuro-regenerative modalities in differentiated human neuronal SH-SY5Y cells

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ABSTRACT

Tianma (*Rhizoma gastrodiae*) is the dried rhizome of the plant *Gastrodia elata Blume* (*Orchidaceae* family). As a medicinal herb in traditional Chinese medicine (TCM) its functions are to control convulsions, pain, headache, dizziness, vertigo, seizure, epilepsy and others. In addition, tianma is frequently used for the treatment of neurodegenerative disorders though the mechanism of action is widely unknown. Accordingly, this study was designed to examine the effects of tianma on the proteome metabolism in differentiated human neuronal SH-SY5Y cells to explore its specific effects on neuronal signaling pathways. Using an iTRAQ (isobaric tags for relative and absolute quantitation)-based proteomics research approach, we identified 2390 modulated proteins, out of which 406 were found to be altered by tianma in differentiated human neuronal SH-SY5Y cells. Based on the observed data, we hypothesize that tianma promotes neuro-regenerative signaling cascades by controlling chaperone/proteasomal degradation pathways (e.g. CALR, FKBP3/4, HSP70/90) and mobilizing neuro-protective genes (such as AIP5) as well as modulating other proteins (RTN1/4, NCAM, PACSIN2, and PDLIM1/5) with various regenerative modalities and capacities related to neuro-synaptic plasticity.

1. Introduction

Despite the many cell- and gene-based therapeutic efforts made for the treatment of neurological disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and others, these diseases are still fatal and a continuous threat to our aging societies as they result in increasing loss of cognitive and physical functions. Since currently no effective therapy is in place for the treatment of such diseases, recent data predict that the number of people affected by AD and aging-related dementia will continue to grow at an epidemic pace (Both et al., 2011; Chiu and Rao, 2011; Heese et al., 2006; Karran et al., 2011). Increasing attention has thus led to enforced efforts in search of suitable novel medicinal herbs for therapeutic applications (Schachter, 2009; Sucher, 2006; Yuan and Lin, 2000), in particular for the treatment of dementia (Rafii et al., 2011).

Orchids and their derivatives have been shown to improve neural functions in clinical studies but the underlying mechanisms are largely unknown which severely hampered the more extensive application of such potential drugs as well as the potential industrial exploration of it (Bulpitt, 2005; Bulpitt et al., 2007; Hew and Yong, 2007). *Gastrodia elata Blume*, known as tianma in Chinese, is a plant of the orchidaceae family, the tuber of which can be dried and used as a medicinal material. In traditional Chinese medicine theory, tianma has the functions of pacifying the liver and calming wind. Besides, pharmacological studies indicate that tianma has the effects of anti-convulsion, sedation, analgesia, memory improvement, anti-aging, improvement of microcirculation, lowering of blood pressure, improvement of blood vessel functions and anti-inflammatory immunostimulation. In addition, it is often used for the treatment of cardio-, cerebro- and neurovascular diseases including migraine, depression and epilepsy (Bulpitt et al., 2007; Hew and Yong, 2007; Hsieh et al., 1997; Kim et al., 2003a,b; Kim et al., 2001; Ong et al., 2007). Previously, we demonstrated *in vivo* the potential neuro-protective action of tianma and its capacity to enhance cognitive functions in mice (Mishra et al., 2011b).

Recently, we have successfully applied the two dimensional (2D) liquid chromatography coupled with tandem mass spectrometry-based isobaric tag for relative and absolute quantification (2D-LC-MS/MS-iTRAQ) strategy in the area of neuro-degenerative diseases (Datta et al., 2010,2011). Here we used this proteomics approach in our *in vitro* human neural SH-SY5Y cell model for quantitative profiling of tianma-regulated genes. In quest of the

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metabolic changes in the entire proteome, the iTRAQ-based proteomics-bioinformatics platform was applied to generate a list of proteins comprising the regulated proteins from differentiated human neuronal SH-SY5Y cells stimulated by tianma. Finally, some of the regulated proteins were validated at the protein levels by western blot analyzes to delve into their functional activities in neurons (Fig. 1). Our *in vitro* results show for the first time the effect of tianma on human neural proteome changes and its potential implication for possible therapeutic neuro-regenerative applications. Since tianma is a novel potential neuro-protective herb with many unidentified features, our present investigations could further contribute to its operational assignment on human neurons to unravel the mysteries behind the neuro-protective activities of tianma.

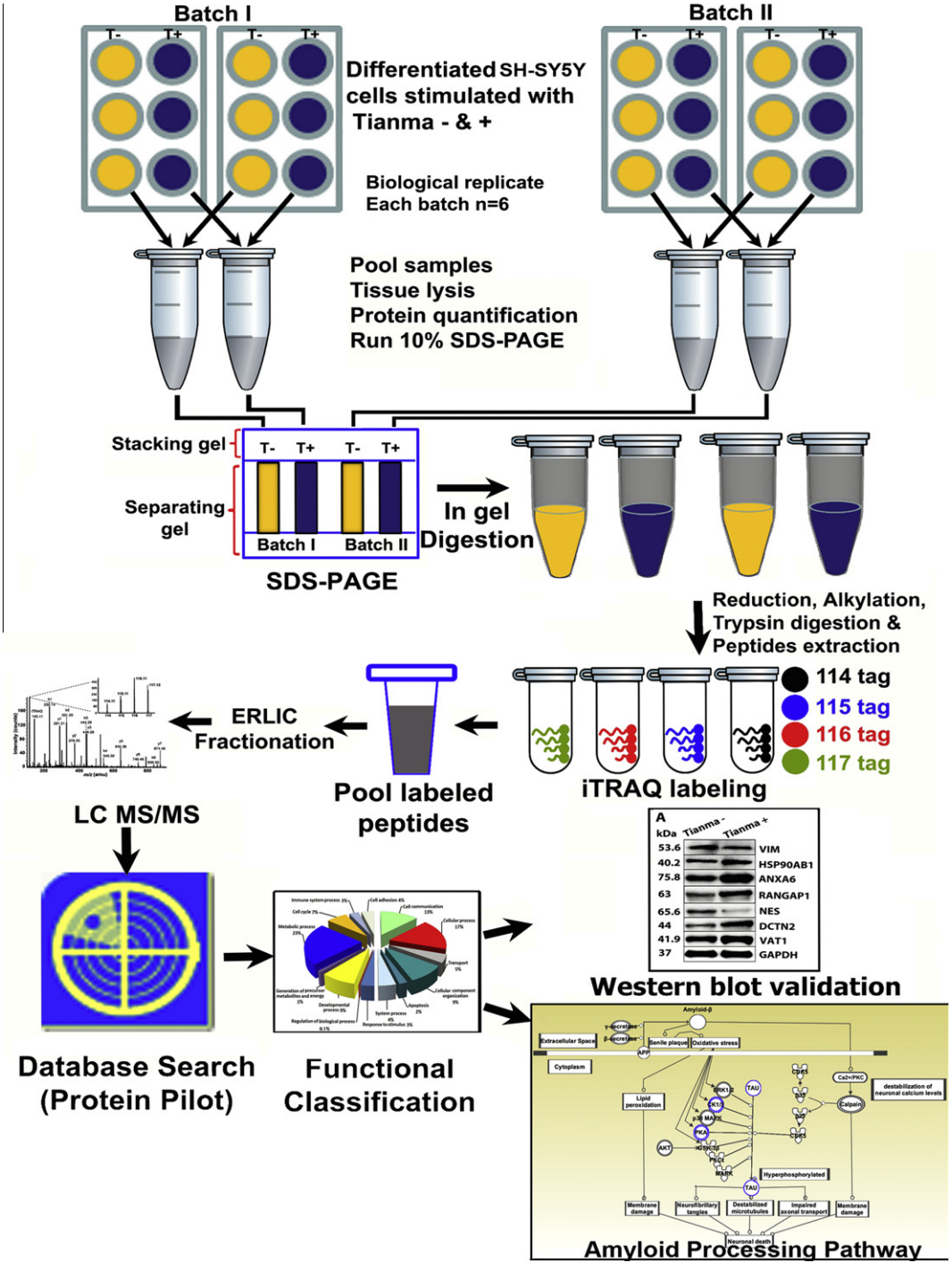

Fig. 1. Schematic representation of the experimental design showing biological and technical replicates. Following SH-SY5Y cell lysis, protein extracts were acetone precipitated and quantified. These were then run in SDS–PAGE and subsequently digested. The quantitative proteomics analyzes of differentiated human neuronal SH-SY5Y cell lysates were performed by labeling with multi-plex isobaric tags (114, 115, 116 and 117) for relative and absolute quantification (iTRAQ) reagents followed by Electrostatic Repulsion-Hydrophilic Interaction Chromatography (ERLIC)-based fractionation, and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)-based multidimensional protein identification technology. The obtained data was analyzed using ProteinPilot software and validated by quantitative Western blots. Finally, proteins were functionally classified into various subgroups.

2. Material and methods

2.1. Reagents

Unless indicated, all reagents used for biochemical methods were purchased from Sigma–Aldrich (St. Louis, MO, USA). Recombinant human brain-derived neurotrophic factor (BDNF) was obtained from iDNA Biotechnology Pte. Ltd. (Singapore). Materials and reagents for SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) were from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). The iTRAQ reagent multi-plex kit, containing the iTRAQ reagents, was bought commercially (Applied Biosystems, Foster City, CA, USA).

2.2. Antibodies

Anti-ANXA6 (annexin A6, 1:800, rabbit polyclonal; ProSci Inc., Poway, CA, USA), anti-DCTN2 (dynactin 2, 1:4000, mouse monoclonal (E-7); Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, 1:3000, mouse monoclonal; Santa Cruz), anti-HSP90AB1 (heat shock protein HSP90-beta, 1:1000, mouse monoclonal; Invitrogen, Carlsbad, CA, USA), anti-RANGAP1 (ran GTPase activating protein 1, 1:1000, goat polyclonal; Abcam, Cambridge, UK), anti-NES (nestin, 1:2000, mouse monoclonal; Millipore (Chemicon), Temecula, CA, USA), anti-VIM (vimentin, 1:1000, mouse monoclonal (V9); Abcam), anti-VAT1 (vesicle amine transport protein 1 homolog, 1:800, rabbit polyclonal; Abcam).

2.3. Tianma preparation

The rhizome of *G. elata* (tianma), grown under standardized conditions (Zhang and Yang, 2007), was collected from Zhaotong City, China and was provided by Dr. Jun Zhou (Kunming Institute of Botany, Chinese Academy of Science, Yunnan, People's Republic of China). The species was identified and chemically analyzed as reported previously (Li et al., 2007; Mishra et al., 2011b). A voucher specimen (0249742) was deposited in the herbarium of the Kunming Institute of Botany, (Chinese Academy of Science, Yunnan, P.R. China). Tianma was dissolved in deionized water to yield a final stock concentration of 100 mg/ml (0.36 g of powdered tianma in 3.6 ml deionized water). The mixture was boiled and at regular intervals mixed using the thermomixer comfort (Eppendorf, Hamburg, Germany) for 1 h. Following this, the mixture was centrifuged at 16,000*g* at 25 °C for 10 min. The supernatant was collected and filtered through a syringe-filter with a pore size of 0.25 μm (Acrodisc® membrane filter, Pall Corporation, Singapore) (Mishra et al., 2011b).

2.4. Cell culture

Human neural SH-SY5Y cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were propagated at 37 °C in humidified 5% CO_2/95% air, in Dulbecco's Modified Eagle's Medium (DMEM, GlutaMax™; Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), non-essential amino acids (Invitrogen), and antibiotic–antimycotic (Invitrogen).

2.5. Tianma stimulation

SH-SY5Y cells were seeded in Poly-D-Lysine (PDL)-coated six-well plates (Becton Dickinson, San Jose, CA, USA) at about 20% confluency per well. Experiments were performed twice, with each set repeated six times. Controls were treated with the solvent only. The cells were allowed to attach and divide for 20 h, after which 10 μM RA (retinoic acid) and 100 ng/ml BDNF were added to 10% FBS-containing media in all experimental plates to promote neuronal differentiation for seven days (Encinas et al., 2000). The media was changed once every three days and images were captured via an inverted microscope (Nikon Eclipse TE2000, Chiyoda-ku, Tokyo, Japan) every 24 h for seven days. On the start of the eighth (8th) day post differentiation in culture, SH-SY5Y cells were stimulated with tianma (without FBS/RA/BDNF) to a final concentration of 1 mg/ml per well, according to previous reports (Mishra et al., 2011b; Sundaramurthi et al., 2012; Teong et al., 2011), for 30 h (control cells received mock-treatment) and subsequently, images were captured before proceeding with cell lysis.

2.6. Cell lysate preparation

All steps were performed on ice. Cell lysis buffer (ice-cold) was prepared with 2% SDS, 0.5 M Triethyl ammonium bicarbonate buffer (TEAB), 1 Complete™ protease inhibitor cocktail tablet (Roche, Mannheim, Germany) and 1 PhosSTOP phosphatase inhibitor cocktail tablet (Roche). The wells were washed with ice-cold phosphate buffered saline (PBS) twice to remove debris and dead cells. 100 μl of cell lysis buffer was added to each well and using a cell scraper (Greiner Bio-One GmbH, Frickenhausen, Germany), the attached cells were collected (six wells with same experimental conditions were pooled). The pooled cell lysate was subjected to a quick spin and sonicated for 1 min (Vibra Cell™ ultrasonic processor, Jencon, Leighton Buzzard, Bedfordshire, UK) at an amplitude of 30 Watt and a pulse (3 s on and 6 s off). The cell lysate samples were centrifuged at 16,000*g* at 4 °C for 1 h, supernatant was collected and stored at −20 °C until further use. The protein concentration was quantified by a '2-D Quant' kit (Amersham, Piscataway, NJ, USA) according to the manufacturer's protocol.

2.7. ITRAQ protocol

A detailed description of the 2D-LC-MS/MS-iTRAQ procedures (Datta et al., 2010,2011; Mishra et al., 2012; Sundaramurthi et al., 2012), including post-proteomic data verification by SDS–PAGE - Western blot analysis (Islam et al., 2009; Mishra et al., 2011a; Shen et al., 2010), can be found in the Supplementary content document as described previously.

2.8. Statistical analysis

The data obtained in the western blot analyzes in this investigation are illustrated as mean ± SD. Differences between the groups were established using an unpaired Student's *t*-test while within-group comparisons were performed using the paired Student's *t*-test. For the iTRAQ analysis ProteinPilot Software 3.0 was used as described in the experimental procedures.

3. Results

All experiments were performed twelve times (subdivided into two sets, batches B-I and B-II; Fig. 1) with each set repeated six times (six controls and six tianma-treated cells for B-I and B-II, respectively). We used four samples to perform iTRAQ (two controls (B-I + B-II) and two tianma-activated (B-I + B-II) samples) as six pooled biological replicates (all done twice: B-I and B-II). This was to insure high confidence in the detection of tianma-regulated proteins in differentiated neuronal SH-SY5Y cells. The quality of the dataset and instrumental reproducibility was then confirmed by comparing and combining three technical replicates (Datta et al., 2010,2011) after labelling the samples with 114, 115, 116 and 117 isobaric tags and processed in LC-MS/MS.

3.1. The morphological effect of tianma on differentiated neuronal SH-SY5Y cells

Upon tianma stimulation of differentiated SH-SY5Y cells, no significant changes in the neurite outgrowth was visibly observed when compared to control differentiated neuronal cells (Supplementary Fig. S1).

3.2. Identification of proteins in differentiated SH-SY5Y cells activated by tianma

Through iTRAQ, we identified a total of 6097 proteins, whereby 2390 proteins were quantified (with a strict cutoff of unused ProtScore $\geqslant 2$ as the qualification criteria, which corresponds to a peptide confidence level of 99% and an applied FDR of 0.33% (<1.0%)), out of which 406 showed an altered protein expression level in both experimental sets B-I and B-II (Fig. 1, Supplementary Table 1).

In order to verify that the protein samples were indeed from the whole cell proteome, the identified protein names were uploaded into JVirGel, a database software that creates a virtual 2D gel picture (Hiller et al., 2003). The proteins were categorized based on their isoelectric point and molecular weight (Supplementary Fig. S2). The virtual 2D gel image confirmed that the samples collected originated from the whole SH-SY5Y cell proteome as the spots in the gel were well distributed.

While analyzing the identified proteins we found a total of 406 proteins that were de-regulated by tianma (Supplementary Table 1). Essentially, 288 of these proteins were up-regulated and the remaining 118 proteins were down-regulated, with the cut-off for up- and down-regulation pre-defined at 1.3 and 0.83, respectively.

3.3. Classification of proteins altered by tianma in differentiated SH-SY5Y cells

We proceeded to use online databases (Panther, UniProt, and NCBI) to identify the functions of these 406 proteins. During the classification process, our objective was also to identify the proteins' sub-cellular localization and action (Fig. 2 and Supplementary Table 1). It is of interest to note that the regulated proteins had a relative equal 'process'-distribution (Fig. 2a) and also a reasonable equal 'function'-distribution (Fig. 2b) indicating that tianma generally activated the cellular metabolism including gene transcription, mRNA processing, protein translation and post-translational modifications. However, a closer look to the sub-categorized groups illustrated in Supplementary Table 1 revealed that, surprisingly, a larger part of the affected proteins belonged to the chaperone/chaperonin and ubiquitin proteasome pathways.

In addition, proteins with potential neuro-protective roles (e.g. CALR, FKBP3/4, PDIA3, HSP70/90, NCAM, TRIM28, ATP5, TOMM70 and API5), that are equally important for neuronal survival and synaptic plasticity during neuro-regenerative processes in the brain, were up-regulated, while proteins that are rather known for their inhibitory effects on neuro-regenerative processes, such as RTN1/4, and others, e.g. LAMP1, SOD2, CRABP2 and ARF4, were down-regulated (Supplementary Table 1).

3.4. Validation of tianma-regulated proteins

Following the database search and classification of proteins, western blots were performed on randomly selected proteins (using the pooled samples from batches B-I or B-II) to verify the iTRAQ values. Eight randomly selected proteins from supplementary Table 1 (viz. ANXA6, DCTN2, HSP90AB1, NES, RANGAP1, VAT1, VIM and GAPDH) were used for iTRAQ data validation (Fig. 3). GAPDH was used as an internal control to insure equal loading of samples as its level was unchanged in the iTRAQ analysis.

Notably, the Western blot images correlated very well and thus confirmed the iTRAQ values obtained.

3.5. STRING protein–protein interaction analysis of tianma-activated proteins

STRING (Search Tool for the Retrieval of Interacting Genes) is a database resource dedicated to protein–protein interactions, including both physical and functional interactions. It weights and integrates information from numerous sources, including experimental repositories, computational prediction methods and public text collections, thus acting as a meta-database that maps all interaction evidence onto a common set of genomes and proteins (Szklarczyk et al., 2011). Thus, this analysis (default settings were used) provided us with essential neural systems-level understanding of cellular events activated by tianma, in a functional differentiated human neuron. Functional partnerships between proteins were at the core of complex cellular phenotypes, and the networks formed by interacting proteins provided us with crucial scaffolds for modeling and data reduction to get insight into the complex interactive mechanisms involved in tianma-activated neural functions. For our current study, the functional link among chaperone proteins, such as HSP70/90, FKBP3/4, CALR, ATP5/6 etc., and their potential link to other metabolically modulated proteins in differentiated SH-SY5Y cells were revealed (Supplementary Fig. S3).

3.6. IPA signaling pathway analysis of tianma-activated proteins

Further bio-computational network analysis of the proteins identified in tianma-stimulated differentiated SH-SY5Y neurons using the Ingenuity Pathways Analysis (IPA) offered us additional

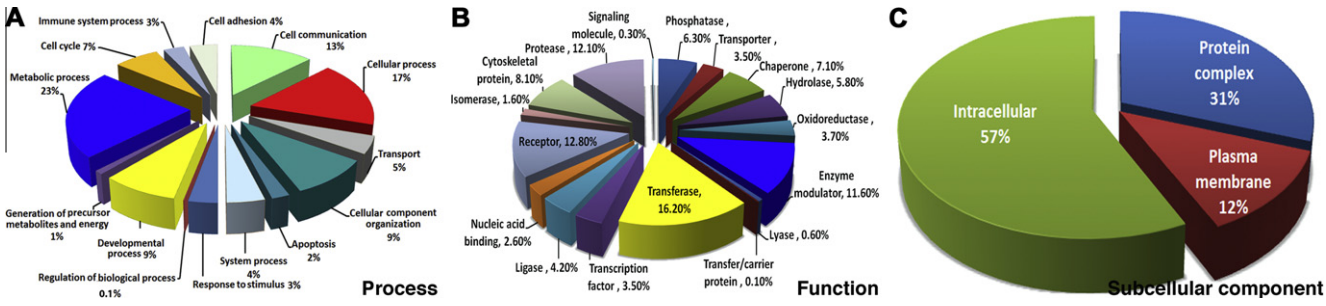

Fig. 2. Pie chart depicting the identified proteins characterized by iTRAQ within the molecular function gene ontology (GO) category. Subcellular and functional processing categories (A–C) were based on the annotations of GO using the human genome informatics GO_Slim chart tool. Representations of proteins based on the whole proteome quantified by iTRAQ.

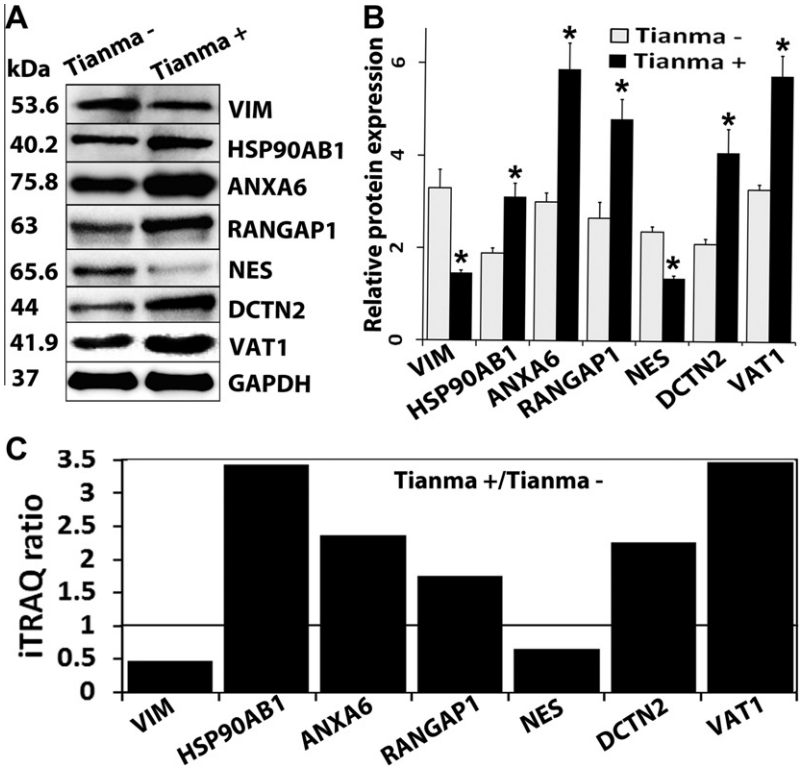

Fig. 3. Western blot validation of iTRAQ results. Randomly selected proteins significantly regulated in tianma-activated differentiated human neuronal SH-SY5Y cells compared with controls. (A) HSP90AB1, RANGAP1, DCTN2, ANXA6 and VAT1 protein levels were increased and NES and VIM levels were all reduced while GAPDH was unchanged. The western blots correlated with the iTRAQ values obtained. GAPDH was used as internal control. Molecular weight of the various proteins is indicated in kDa. (B) Quantitative analyzes of the western blots shown in a. Western blot experiments were performed at least three times for statistical quantification and analyzes ($n = 3$). Values (= relative protein expression) represent the ratio of densitometric scores for the respective western blot products and statistical error was indicated as mean ± SD (*$P < 0.05$, compared with controls) using the GAPDH bands as reference. (C) The histogram indicates a similar close relationship between iTRAQ and western blot expression ratios of tianma-stimulated and control differentiated neuronal SH-SY5Y cells. iTRAQ expression ratios from selected proteins were consistent with the Western blot results and thus verified a strong agreement in the expression data.

valuable clues about the complex interactive link of the various identified proteins within their commonly known interactive protein networks also obtained from other cellular metabolic information (Fig. 4, Supplementary Table 2).

In addition, neural-specific IPA analysis could demonstrate the involvement of the iTRAQ-based analysis-identified proteins and their metabolic interactive pathways within a neuronal network associated with specific neuronal diseases, especially important for the proper functions of active neurons in the central nervous system (CNS) during neuro-regenerative processes such as neural plasticity (Supplementary Fig. S4). In particular, it could demonstrate the network among proteins (e.g. PKA, PKC, PLC, RHOA or 14–3-3-protein) from various intracellular localizations with important roles in cell survival and neuro-regeneration (Supplementary Fig. S5).

4. Discussion

The therapeutic potential of orchids and their derivatives for the treatment of various neuronal disorders has been discussed frequently (Bulpitt et al., 2007; Hew and Yong, 2007; Huang et al., 2007). In particular, increasing attention has been made on tianma due to its wide action also within the CNS (Sun et al., 2004; Tao, 2008). Recent studies could further demonstrate the neuroprotective action of tianma *in vitro* (Huang et al., 2011; Tsai et al., 2011) and its *in vivo* effects on cognitive functions in mice and rats, respectively (Chen et al., 2011; Mishra et al., 2011b).

Here, we provide an additional interesting insight into the molecular and cellular mechanisms of herbal medicine by disclosing the effect of tianma on the full proteome changes upon stimulation of differentiated human neuronal SH-SY5Y cells. In the following sections we briefly discuss some aspects of the identified proteins that were found to be altered upon neuronal tianma stimulation.

4.1. Tianma modulates proteins related to neurodegenerative diseases in differentiated human neuronal SH-SY5Y cells – the ubiquitin proteasome system (UPS)

It is of particular interest to note that tianma modulates those proteins that are involved in the intracellular protein/vesicle transport associated with the microtubule/tau system such as actin, tubulin, or synaptic vesicle membrane proteins as well as other structural and non-motor actin-binding proteins (Supplementary Table 1). In various neurodegenerative diseases transport defects may arise from failures in molecular motors, microtubule abnormalities, and the chaperone/proteasomal degradation pathway leading to aggresomal-lysosomal accumulations. These defects represent important steps in the neurodegenerative cascade. Rod-shaped actin bundles (rods) inhibit the transport system and are sites of amyloid precursor protein (APP) accumulation. These rods have also been shown to play a role in Hirano body formations, which contain actin, cofilin, tubulin, tau protein, proteins of the MAP family and APP, which are involved in the degeneration of neurons. Such deregulated actin filament bundles may finally lead to neurofibrillary tangles (NFTs) formation, characteristic of tau pathology in AD, tauopathies and other neurodegenerative diseases such as HD (Bamburg et al., 2010; Maloney and Bamburg, 2007; Ostrowski et al., 2005). In addition, tianma's effect on actin-binding proteins such as PDLIM1 or PDLIM5 (ENH1) and on AKAP12 may affect pivotal intracellular signaling cascades, such

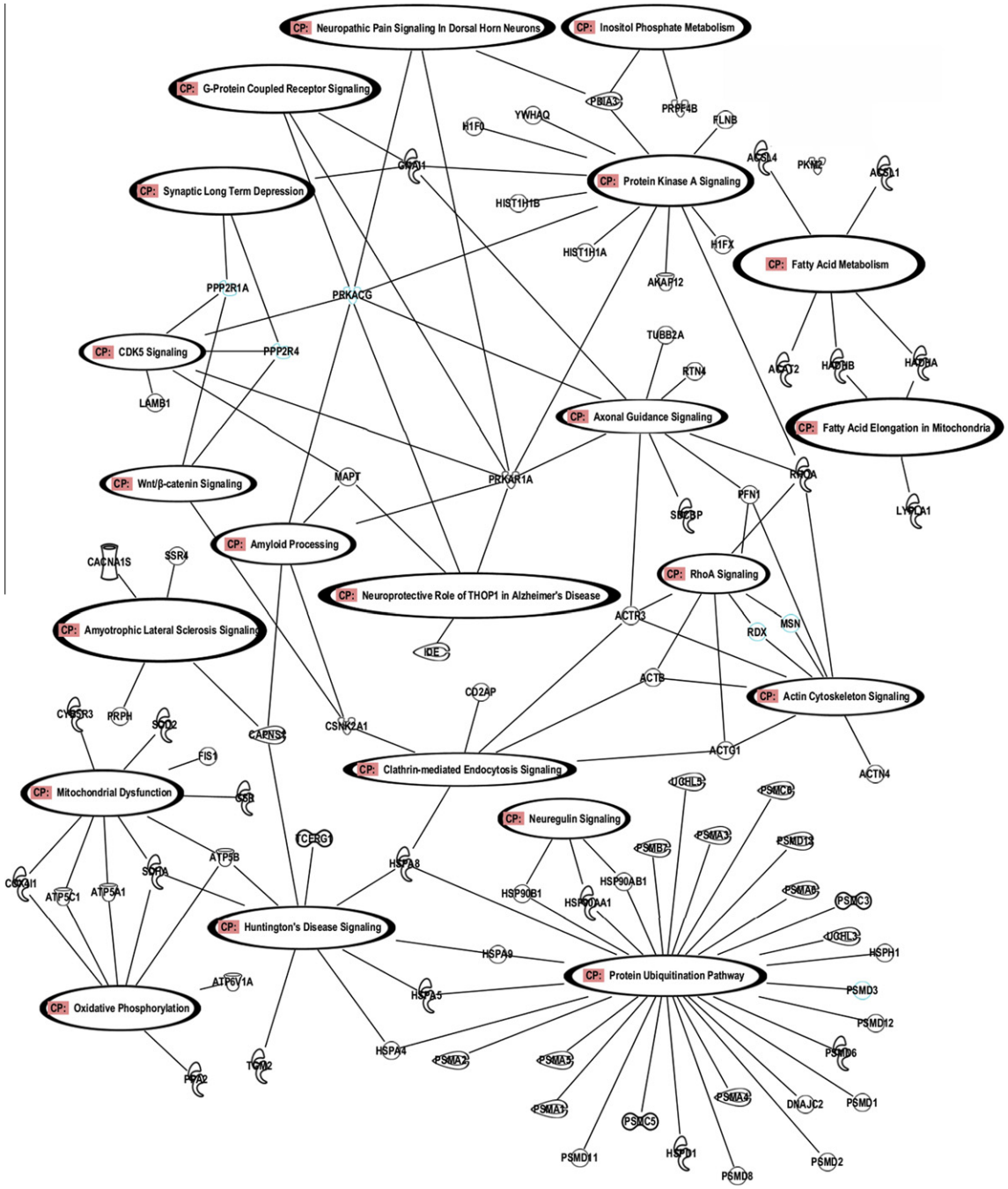

Fig. 4. Neuronal-specific network analysis of iTRAQ-based proteomic metabolism in tianma-activated differentiated human neuronal SH-SY5Y cells using IPA. The 406 tianma-regulated proteins that were quantified by iTRAQ were imported into the IPA, which classifies the proteins into several conical pathways (CP). IPA analysis was performed for the understanding of how the identified proteins (Supplementary Tables 1 and 2) work together by protein–protein interactions within the context of nervous-system- and CNS-diseases-related metabolic signaling pathways that affect cellular processes in the nervous system induced by neural tianma stimulation. IPA conical pathway analysis was carried out based on the iTRAQ data of proteins expressed in tianma-activated differentiated neuronal SH-SY5Y cells. Tianma-regulated proteins that were sub-categorized into other conical signaling pathways were excluded from this scheme.

as PKC and PKA pathways, that are essential for neuronal functions and survival and have been shown to be linked to neuro-psychiatric diseases (Supplementary Fig. S6) (Gardezi et al., 2009; Liu et al., 2008; Maturana et al., 2011; Shi et al., 2008 ; Wang et al., 2012; Zhao et al., 2009).

Besides, tianma also affects proteins of the proteasomal degradation system. It is obvious that most proteins of the UPS are consistently up-regulated by tianma, thus indicating an enhanced UPS-guided control system avoiding abnormal intracellular protein aggregation. Abnormal protein degradation by malfunction of the UPS is considered to contribute to the neuropathogenesis in AD, PD, HD and others (Cali et al., 2011; Nijholt et al., 2011; Riederer et al., 2011). In fact, a common characteristic feature of these neurodegenerative diseases is the accumulation of protein aggregates, which reflects a severe disturbance of protein homeostasis controlled by the two major proteolytic machineries, the UPS and the autophagy/lysosomal system. The UPS is critical for reducing the levels of soluble abnormal proteins, while autophagy plays the major role in clearing of cells from protein aggregates. Since AD is one of the most important age-related neurodegenerative

diseases and aggresome formation is also observed in several other neurodegenerative diseases, this suggests that an activation of similar mechanisms must occur in neurodegeneration as a basic phenomenon. It is important to further investigate in the future, the significance of our findings about the modulatory role of tianma on the UPS and axonal protein/vesicle transport systems (Huang and Figueiredo-Pereira, 2010; Rogers et al., 2010).

4.2. Tianma modulates (ER-resident) molecular chaperones and mt-ATP synthases

In line with the abovementioned observation, it is further noteworthy that tianma also caused a consistent increase in (co-) chaperone protein levels. Chaperones are a group of proteins that play a variety of roles from refolding of misfolded proteins to the degradation and prevention of protein aggregation. Consequently, the aggregation of misfolded proteins, impairment of the UPS and suppression of the heat shock response inhibits the major cell defense systems that play a pivotal role in the progress of neurodegenerative disorders (Almeida et al., 2011; Hinault et al., 2006; Meriin and Sherman, 2005; Winklhofer and Tatzelt, 2006). The heat shock protein (HSP) family has long been associated with a generalized cellular stress response, particularly in terms of recognizing and chaperoning misfolded proteins. HSPs are induced in response to many injuries including stroke, epilepsy, and various other neurodegenerative diseases (Arawaka et al., 2010; Kalia et al., 2010; Nagai et al., 2010; Sajjad et al., 2010). It is worth mentioning that some of these HSPs can not only block protein aggregation in the early stages, but also have a significant impact on attenuating the formation of fibrils (Luo and Le, 2010). Though HSPs have multifaceted roles in neurons, they basically serve a protective role in several different nervous system injury incidents (Aridon et al., 2011; Patury et al., 2009). For instance, HSP70 functions as a chaperone and protects neurons from protein aggregation and toxicity (in PD, AD, polyglutamine diseases, and amyotrophic lateral sclerosis), protects cells from apoptosis (PD), is a stress marker (temporal lobe epilepsy), and also protects cells from cerebral ischemic injury. In particular, it has been shown very recently that HSP70 can suppress AD phenotypes in mice (Hoshino et al., 2011). As another example, the main function of HSP90 complexes is to maintain quality control of proteins and to assist in protein degradation via proteasomal and autophagic-lysosomal pathways. As such, it also plays a major neuroprotective role in the pathology of AD where it is crucially involved (with co-chaperones such as immunophilins of the FKBP protein family) in the control of aberrant phosphorylated tau protein (Salminen et al., 2011). In addition, HSP90 regulates the activity of the transcription factor heat shock factor-1 (HSF-1), the master regulator of the heat shock response mechanism that cells use for protection when exposed to conditions of stress (Fig. 5) (Luo et al., 2010).

CALR is another important molecular chaperone involved in quality control within secretory pathways. As a calcium-binding chaperone of the endoplasmic reticulum (ER) this protein regulates intracellular Ca^{2+} homeostasis and the ER Ca^{2+} storage capacity. As such, it is also involved in the folding of newly synthesized proteins and glycoproteins and, together with calnexin (an integral ER membrane chaperone) and PDIA3 (ERp57, an ER protein of 57 kDa; a PDI (protein disulfide-isomerase)-like ER-resident protein), it constitutes the 'calreticulin/calnexin cycle that is responsible for folding and quality control of newly synthesized glycoproteins. In fact, during recent years, CALR has been implicated to play a pivotal role in many biological systems, including functions inside and outside of the ER, indicating that the protein is a multi-process molecule (Gelebart et al., 2005; Gold et al., 2010; Michalak et al., 2009) that might be involved as an ER-resident chaperone in AD and PD (Kudo et al., 2008; Lai et al., 2009; Wilhelmus et al., 2011). PDIA3 itself is an ER-resident thiol-disulfide oxidoreductase which is modulating STAT (signal transducer and activator of transcription, also affected upon tianma treatment) signaling from the lumen of the ER together with CALR (Chichiarelli et al., 2010; Coe et al., 2010) that might be affected by PD (Kim-Han and O'Malley, 2007).

Mt-ATP synthase catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane of mitochondria during oxidative phosphorylation. It seems obvious that even intermittent and minor impairment of this highly important enzyme could deprive the brain tissue of energy at crucial times, which may predispose or contribute to (age-related) neurological diseases (Boveris and Navarro, 2008; Johnson and Ogbi, 2011). Since tianma seems to have a general positive effect on HSPs, chaperones and ATP synthase activities, its application could eventually counteract aging related dysfunctions (Supplementary Fig. S7) (Dencher et al., 2007; Mishra et al., 2011b).

4.3. Tianma modulates GTPase activity/G-proteins

Intracellular molecules such as RHOA and its effector RHO kinase (ROCK) have been shown to play important roles during neuroregenerative processes after nerve injury and in processes related to neuro-synaptic plasticity that are important for learning, memory and related cognitive functions (Supplementary Fig. S8) (Kleppisch and Feil, 2009; Kubo et al., 2007; Kubo and Yamashita, 2007). Since tianma affects, in addition to RHOA, significantly the SUMOylation (small ubiquitin-related modifier SUMO posttranslationally modifies many proteins with roles in diverse processes including regulation of transcription, chromatin structure, and DNA repair) substrate RANGAP1 that is associated with neuronal intranuclear inclusion disease (NIID), which is a rare neurodegenerative disorder characterized pathologically by the presence of ubiquitinated NII in neuronal cells (Takahashi-Fujigasaki et al., 2006), it gives a further glimpse on the complex linked effects of tianma on neuronal cells. In particular, histone deacetylase 4 (HDAC4) a major component of NIIs, suggesting that a complex crosstalk between acetylation and SUMOylation is important for tianma-mediated neuronal gene regulation (Gill, 2005). This in turn may give us an explanation to why tianma modulates a wide range of proteins involved in gene transcription, mRNA splicing, protein translation and the more general cellular protein metabolism systems as shown in supplementary Table 1.

4.4. Tianma supports synaptic plasticity by modulating cell surface receptors and membrane trafficking regulatory proteins

Tianma's supportive influence on neuro-protective and synaptic plasticity-related mechanisms, that are important for neuro-regenerative processes, are further supported by (*i*) its inhibitory effect on RTN1 and RTN4 (otherwise known as NOGO) (McDonald et al., 2011; Sironen et al., 2004; Wang et al., 2006), that are key players in axonal regeneration processes, and by (ii) tianma's positive impact on cell adhesion molecules, such as NCAM (Gascon et al., 2007; Lavdas et al., 2011), on PKC-signaling proteins (e.g. PACSIN2, PDLIM1/5) (Kessels and Qualmann, 2004; Maturana et al., 2011) and on AHNAK1 that controls pre-synaptic Ca^{2+} mobilization and neurotransmitter release (Supplementary Fig. S9) (Alli and Gower, 2009; Carlson et al., 2010). Moreover, tianma stimulates anti-apoptotic (API5) mechanisms (Morris et al., 2006).

Remarkably, our current study with human neurons is in line with our recent results showing that tianma also modulates proteins involved in neuro-regenerative processes and the chaperone/proteasomal degradation pathways (e.g.: NCAM, HSP90AA1, ATP5A1/5B, SERPINH1, PSMD8, PSMC3 and CCT3) in rat neurons (Sundaramurthi et al., 2012).

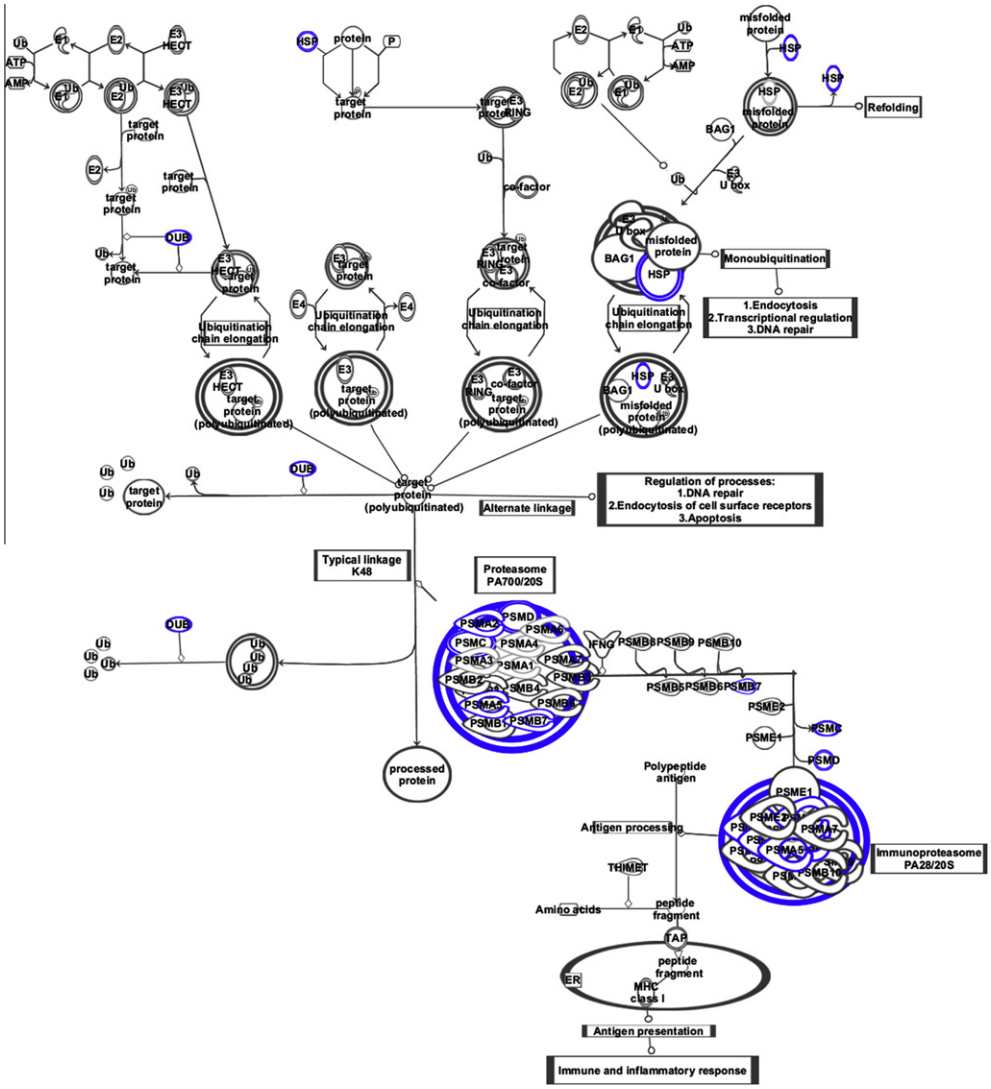

Fig. 5. Tianma's general influence on the HSP- chaperone/proteasomal degradation pathways may have a wide and general positive impact on the entire organism. In the CNS misfolded proteins could be avoided by the stimulation of the proteasomal pathway while in other parts of the body, tianma application may promote the processing of misfolded proteins for antigen presentation and stimulation of the proper immune response. The 406 tianma-regulated proteins that were quantified by iTRAQ were imported into the IPA, which classifies the proteins into several conical pathways. The 'Protein Ubiquitination Pathway' was selected and more than 30 proteins, involved in this signaling pathway, were regulated by tianma: e.g.: DNAJC2, HSP90AA1, HSP90AB1, HSP90B1, HSPA4, HSPA5, HSPA8, HSPA9, HSPD1, HSPH1, PSMA1, PSMA2, PSMA3, PSMA4, PSMA5, PSMA6, PSMB7, PSMC3, PSMC5, PSMC6, PSMD1, PSMD2, PSMD3, PSMD6, PSMD8, PSMD11, PSMD12, PSMD13, UCHL3, UCHL5. Tianma-regulated proteins that were sub-categorized into other conical signaling pathways were excluded from this scheme. Ub: ubiquitin; DUB: deubiquitinating enzyme; E2, E3, E4: ubiquitin conjugating enzyme.

5. Conclusions

Our comprehensive cellular, molecular and quantitative bio-computational analyzes have revealed the modulatory effect of tianma on the entire neuronal proteome of differentiated human neuronal SH-SY5Y cells. The data has shed new insights on the neuronal action of tianma. However, further systemic functional *in/ex vivo* biological studies are required to unscramble the individual bioactive components that are responsible for its potential neuro-regenerative effects in order to develop a novel tianma-based remedy for the treatment of neurodegenerative diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuint.2012.03.012.

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