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Inhibition of PP2A by hesperetin contributes to Akt and ERK1/2 activation in primary cortical neurons

David Vauzour ¹, * Simone Corsini¹, Michael Müller¹ and Jeremy P.E Spencer ²

¹ Norwich Medical School, University of East Anglia, Norwich NR4 7UQ, UK
² Hugh Sinclair Unit for Human Nutrition, School of Chemistry, Food and Pharmacy, University of Reading, Reading RG2 6AP, UK

* To whom correspondence should be addressed: Dr David Vauzour, Norwich Medical School, University of East Anglia, Norwich NR4 7UQ, UK. D.Vauzour@uea.ac.uk ; Tel.: +441603 591732
ABSTRACT

Flavonoids and their metabolites are well reported to modulate the activation/phosphorylation of various cellular kinases, such as ERK1/2 and JNK, although the mechanism by which they do so is unclear. In this study, we investigated the impact of flavanones on the activation of PI3K/Akt and ERK1/2 and determine whether this is mediated, in part, by the inhibition of phosphatases. Primary cortical neurons were exposed to physiological concentrations of hesperetin and the phosphorylation status of the kinases PI3K/Akt and ERK1/2 and the phosphatases PP2A and PTEN were assessed by immunoblotting after 30 min. Exposure to 100-300 nM hesperetin led to significant increases in the phosphorylation of ERK1/2 and Akt and significant decreases in PP2A levels and enzyme activity. Using in silico docking, hesperetin was found to fit into the active site of PP2A interacting within the hydrophobic cage of the catalytic unit. These data suggest a potential mechanism by which flavanones may lead to increased activation of ERK1/2 and Akt, commonly observed in cell models. Their interaction with and inhibition of cellular phosphatases counteract normal physiological regulation of signaling pathway activation thus facilitating and/or maintaining the activation status of ERK1/2 and Akt, important regulators of brain functions.

KEYWORDS: Flavonoids, brain, signaling pathways, phosphatase, MAP Kinase
1. BACKGROUND

Accumulating evidence suggests that diet and lifestyle can play an important role in delaying the onset or halting the progression of age-related neurodegenerative diseases. A growing number of dietary intervention studies in humans and animals, in particular those using flavonoid-rich diets have been proposed to exert a multiplicity of neuroprotective actions within the brain, including a preservation of cognitive performance with ageing [1-3], a delay in the onset of Alzheimer’s disease [4, 5] and a reduction in the risk of developing Parkinson’s disease [6, 7]. Their neuroprotective potential has been shown in both oxidative stress- [8, 9] and Aβ-induced neuronal death models [10]. Evidence also supports the beneficial and neuromodulatory effects of flavonoid-rich blueberry and grape extracts, particularly in connection with age-related cognitive decline and Alzheimer’s disease [11, 12].

Flavonoids are well reported to cross the blood-brain-barrier (BBB) and have been detected in areas of the brain such as hippocampus, cerebral cortex, cerebellum and striatum [13-16], which are important for learning and memory formation and are also adversely affected by aging and neurodegenerative disorders [17, 18]. The flavanone hesperetin is one of the most lipophilic flavonoids and has previously been shown to have high apparent permeability (Papp) across the in vitro BBB model [19]. The precise cellular mechanisms underlying the actions of flavonoids are still unknown but are thought to involve, in addition to their free radicals scavenging activity, the modulation of protein and lipid kinase signaling pathways [20, 21]. Previous investigations have highlighted that modulations of MAP kinase and PI3K/Akt signaling are central to mediating the cellular effects of flavonoids [20-22]. For example, we previously reported that hesperetin was effective at preventing neuronal apoptosis via a mechanism involving the activation/phosphorylation of both Akt/protein kinase B and extracellular signal-regulated kinase 1 and 2 (ERK1/2) at physiological concentrations [23]. Furthermore, the flavanone naringenin was able to inhibit p38 mitogen-activated protein kinase (MAPK) phosphorylation and downstream signal transducer and activator of transcription-1 (STAT-1) in LPS/IFN-gamma stimulated primary mixed glial cells [24]. In addition to MAPK signaling, recent evidence is also suggestive that flavonoids are able to modulate reversible phosphorylation through protein phosphatase activity, an important mechanism of signal transduction in eukaryotic cells [25]. For example, the flavanol EGCG was previously reported to strongly inhibit...
protein phosphatase-1 (PP1) while showing a ten-fold lower activity toward the close homologue PP2A [26]. More recently, procyanidins were reported to negatively affect the activity of the PP1 and PRL phosphatases in regenerating livers [27]. These data not only indicate that flavonoids may exert cellular effects via direct interactions with MAPK and PI3K/Akt signaling proteins but may also be capable of cellular actions through protein phosphatases modulation. However, data related to the impact of physiologically relevant flavonoids on neuronal protein phosphatases remain to be investigated.

In this study we investigated the ability of the flavanone hesperetin to modulate neuronal signaling pathways at physiologically relevant concentrations, known in humans to be in the nanomolar to low micromolar range. We show that this dietary-derived compound has the capacity to activate both ERK and Akt signaling pathways, and that this kinase activation may be modulated through the inhibition of the protein phosphatase PP2A. In support of this, we show the molecular docking of hesperetin with the PP2A protein within the phosphatase active site of the catalytic unit. These results offer new avenues to the modulatory effect of flavonoids on neuronal signaling.

2. MATERIALS AND METHODS

Reagents
Reagents were from Sigma Chemicals Co. (Poole, Dorset, UK) unless otherwise stated. Hesperetin was obtained from Extrasynthese (Genay, France). Antibodies used were: anti-ACTIVE MAPK (ERK1/2), anti-pAkt (Ser-473), ERK1/2, Akt were from New England Biolabs (Hitchin, Hertfordshire, UK). PP2A and PTEN antibodies were from Insight Biotechnology Ltd (Wembley, UK). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma, Poole, UK), ECL reagent and Hyperfilm-ECL were purchased from Amersham Biosciences (Amersham, UK). Ultrapure water (18.2 MΩ.cm) passed through a purification system (Purite Ltd., Oxon, UK) was used for all purposes.

Neuronal culture and treatments
Primary cultures of mouse cortical neurons were prepared as described previously [23, 28]. Neurons were plated onto 6-well Nunc multi-well plates that had been pre-coated overnight with poly-L-ornithine and then with 10% heat inactivated fetal bovine serum (Gibco-BRL, Paisley, UK) for 2h. Following removal of the final coating solution cells were plated (10^6/ml) in a serum-free medium composed of a mixture of DMEM and F-12 nutrient (1:1 v/v) supplemented with glucose (33 mM), glutamine (2 mM), sodium bicarbonate (6.5 mM), HEPES (pH 7.4, 5 mM), streptomycin (100 µg/ml) and penicillin (60 UI/ml) (all Gibco-BRL). A mixture of hormones and salts composed of insulin (25 µg/ml), transferrin (100 µg/ml), putrescine (60 µg/ml), progesterone (20 nM) and sodium selenate (30 nM) (all from Sigma) was also added to the culture medium. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO2 and after 5 to 7 days the vast majority of cells were neuronal (> 98%) with < 2% astrocytes as determined by -tubulin and GFAP immunocytochemistry respectively [29]. To investigate the effect of the flavanone on signaling pathways, neurons were incubated for 30 minutes with hesperetin (0-0.3 µM). This time point was selected based on our previous investigation [23].

**Phosphatase assay**

Phosphatase activity of mouse cortical neuronal cells was quantitated using the DuoSet® IC Active PP2A assay kits (R&D Systems, DYC3309) by measuring the dephosphorylation of a synthetic phospho-peptide, DLDVIPGRFDERRVS(PO3)VAAE by active phosphatase PP2A. Briefly, cortical neurons plated in 6-cm Petri dishes were rinsed twice in TBS and lysed in the phosphatase buffer (50 mM HEPES, 0.5% NP-40 (pH 7.5), 0.1 mM EGTA, 0.1 mM EDTA, 120 mM NaCl) containing protease inhibitors (25 µg/mL Leupeptin, 25 µg/mL Pepstatin, 2 µg/mL Aprotinin, 1 mM PMSF). Proteins were extracted on ice with periodic vortexing for 15 min. Cells were thoroughly solubilised by passing the lysates through a 19-gauge needle 20 times and cleared by centrifugation at 12,000 x g for 5 min at 4°C. Diluted supernatants were then incubated with 200 µM of synthetic phosphopeptide DLDVIPGRFDERRVS(PO3)VAAE on a rocking platform at 30 rpm for 30 minutes at 37°C in a buffer consisting of 50 mM Tris (pH 7.5), 10 mM MgCl2, 0.02% Brij-35, 1 mg/mL BSA without or with 20 nM okadaic acid. Following incubation, Malachite Green was added to the mixture and incubated for 20 min at room temperature to allow color development. Absorbance was measured at 620 nm using the Genios Tecan microplate reader. Phosphate released was determined by comparing absorbance
To a standard phosphate curve. To calculate PP2A specific activity, total PP2A levels were also quantified using the DuoSet® IC Total PP2A assay kits (R&D Systems, DYC1653).

**Immunoblotting**

Following exposures, neurons were washed with ice-cold PBS (+ EGTA 200 mM) and lysed on ice using Tris (50mM), Triton X-100 (0.1%), NaCl (150mM) and EGTA/EDTA (2 mM), containing mammalian protease inhibitor cocktail (1:100 dilution), sodium pyrophosphate (1 mM), PMSF (10 mg/ml), sodium vanadate (1 mM) and sodium fluoride (50 mM). Lysed cells were scraped and left on ice to solubilise for 45 min. Lysates were centrifuged at 1,000xg for 5 min at 4°C to remove unbroken cell debris and nuclei. Protein concentration in the supernatants was determined by the bicinchoninic acid (BCA) Protein Assay (Thermo-Fisher Scientific, Loughborough, UK). Samples were incubated for 5 min at 95°C in boiling buffer (final conc. 62.5mM Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.0025% bromophenol blue). Boiled samples (20-40 µg/lane) were run on 9 -12 % SDS-polyacrylamide gels and proteins were transferred to nitrocellulose membranes (Hybond-ECL; Amersham) by semi-dry electroblotting (1.5 mA/cm²). The nitrocellulose membrane was then incubated in a blocking buffer (20mM Tris, pH 7.5, 150 mM NaCl; TBS) containing 4% (w/v) skimmed milk powder for 45 min at room temperature followed by 2 x 5 min washes in TBS supplemented with 0.05% (v/v) Tween 20 (TTBS). Blots were then incubated with either anti-ACTIVE MAPK (ERK1/2) pAb (1:1000), anti-phospho-Akt (Ser 473) pAb (1:1000), anti-ERK1/2 (1:1000), anti Akt (1:1000), anti-PTEN pAb (1:1000) or anti-PP2A pAb (1:1000) in TTBS containing 1% (w/v) skimmed milk powder (antibody buffer) overnight at room temperature on a three dimensional rocking table. The blots were washed 2 x 10 min in TTBS and incubated with goat anti-rabbit IgG conjugated to HRP (1:1000 dilution) for 60 min. Finally, blots were washed 2 x 10 min in TTBS rinsed in TBS and exposed to ECL-reagent for 1-2 min and developed. Bands were analyzed using Bio-Rad Quantity One 1-D Analysis software. Molecular weights of the bands were calculated from comparison with pre-stained molecular weight markers (MW 27,000 – 180,000 and MW 6,500 - 45,000, Sigma) that were run in parallel with the samples. The equal loading and efficient transfer of proteins was confirmed by staining the nitrocellulose with Ponceau Red (Sigma).
In Silico Docking

Docking calculations were conducted with SwissDock, a protein-small molecule docking web service [30]. SwissDock is based on the docking software EADock DSS, whose algorithm consists of the following steps: i) many binding modes are generated either in a box (local docking) or in the vicinity of all target cavities (blind docking); ii) simultaneously, their CHARMM energies are estimated on a grid; iii) the binding modes with the most favorable energies are evaluated with FACTS, and clustered; iv) the most favorable clusters can be visualized online and downloaded on a personal computer (http://swissdock.vital-it.ch/). UCSF Chimera (a visualization system for exploratory research and analysis) was used for molecular structure viewing [31]. The crystal structure was obtained from the PDB with the accession code 2NYM_C [32]. Hesperetin 3D structure was obtained by the ZINC 15 Database [33, 34] (code 39092).

Statistical analysis

All results are expressed as means ± SEM of three separate experiments unless otherwise stated. The statistical evaluation of the results was performed by one-way analysis of variance (ANOVA) followed by a Bonferroni’s multiple comparison test using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). Significance was defined as p < 0.05.

3. RESULTS

3.1. Hesperetin stimulates Akt and ERK phosphorylation in cortical neurons

To investigate the phosphorylation state of ERK1/2 and Akt/PKB, phospho-specific antibodies were employed that recognize the dually phosphorylated motif pTEpY within activated ERK1/2, and the phosphorylated Ser473 of Akt/PKB respectively. Exposure of cortical neurons to hesperetin (0.3 µM; 30 min) resulted in a significant increase in Akt phosphorylation at Ser473 [F (2, 3) = 15.45; P = 0.0364], an event known to be essential for full activation of the kinase (Figure 1A). No significant changes were observed at smaller concentrations. We also determined the effects of hesperetin on the ERK1/2 pathway, by immunoblotting protein samples from neurons that had been exposed to increasing concentrations of the flavanone (0-0.3 µM; 30 min). Exposure of neurons to hesperetin
resulted in a marked increase in ERK phosphorylation relative to basal levels, as demonstrated by a robust increase in the relative intensity of the immuno-detectable bands relating to phospho-ERK1 (44 kDa) and phospho-ERK2 (42 kDa). Similar to Akt/PKB phosphorylation state, we observed the largest increase in ERK activation at 0.3 µM \[F (2, 6) = 7.319; P = 0.0007\], although ERK1/2 phosphorylation was also significantly increased in neurons exposed to 0.1 µM \[F (2, 6) = 7.219; P = 0.0306\] (Figure 1B). Parallel blots were run and probed with antibodies that detected total levels of ERK1/2 and total levels of Akt/PKB, demonstrating no modification in the total amount of proteins and an equal amount of loading (Figure 1A and 1B). Assessment of the other mitogen activated protein kinases (i.e. JNK and p38) did not reveal any modification in their phosphorylation state under these experimental conditions (data not shown).

3.2. Hesperetin decreases PP2A activity in cortical neurons

Since phosphatases can modulate the activities of several kinases in vitro and in vivo, such as ERK and Akt (reviewed in [35]), we investigated the impact of hesperetin on PP2A and PTEN, by immunoblotting protein samples from neurons that had been exposed to increasing concentrations of the flavanone (0-0.3 µM; 30 min). Exposure of cortical neurons to hesperetin (0.3 µM; 30 min) resulted in a significant decrease in PP2A levels relative to vehicle treated cells \[F (2, 3) = 9.210; P = 0.0470\] (Figure 2A). Although we observed a small increase in PTEN immunoreactivity following hesperetin incubation, this effect didn’t reach significance \[F (2, 6) = 4.351; P = 0.2471\].

To further investigate the impact of hesperetin on PP2A, we quantified the phosphatase activity using both the Active and Total PP2A DuoSet IC activity assays. Incubating cortical neurons with hesperetin (0-0.3µM; 30 min) led to a robust decrease in phosphate released (Figure 3A), indicating strong inhibition of PP2A activity \[F (2, 9) = 13.32; P = 0.0020\]. To determine non-specific activity, cortical neurons were also incubated with Okadaic acid (OA; 20 nM), a known PP2A inhibitor. Following incubation with OA, PP2A activity was significantly decreased \[F (1, 9) = 67.40; P < 0.0001\] suggesting specific inhibition of the enzyme. These results are consistent with previous reports demonstrating specific inhibition of PP2A activity by OA [36]. Next, we determined PP2A specific activity by calculating the ratio of phosphate released per minute to total PP2A protein. Although there
was a loss of total PP2A protein with hesperetin treatment (data not shown), a significant decrease in the specific activity of the enzyme was observed [F (2, 6) = 20.17; P = 0.0022] (Figure 3B). These data correlate well with the relative total amounts of PP2A detected by western blot, showing a significant decrease in PP2A immunoreactive bands.

### 3.3. Molecular docking of Hesperetin on PP2A

The PP2A core enzyme consists of a 36 kDa catalytic subunit, or C subunit, and a 65 kDa scaffolding protein, known as the A or PR65 subunit [37]. Previous investigations looking into OA binding to PP2A revealed a binding pocket located in the active site of PP2A catalytic unit. On one end of the binding pocket, four amino acids in the catalytic subunit of PP2A, Gln122, Ile123, His191, and Trp200, form a hydrophobic cage, which accommodates the hydrophobic end of OA. On the other end of the binding pocket, Leu243, Tyr265, Cys266, Arg268, and Cys269 make multiple van der Waals interactions with the hydrophobic portion of the bound toxin [38].

In order to gain insight into the putative binding mode of hesperetin with PP2A catalytic subunit, it was docked with a crystallographic structure of human PP2A using SwissDock. The crystallographic structure was obtained from the Protein Data Bank (PDB), accession code 2NYM_C [32]. The docking model predicted by SwissDock indicates that hesperetin is able to fit into the active site of PP2A (Fig 4A and B). In particular, we observed interaction within the hydrophobic cage at two main sites (site A and B) representing the overall binding pocket of the catalytic unit. In particular, hesperetin interacts with residues of site A (Pro190) and B (Leu243 and Hsd252; Fig 4C) where it creates hydrogen bonds. In addition, hesperetin also established hydrophobic links with the ligand rings through interaction with the site B surface residues Val244, Asn249 and Trp250 (Fig 4D). Predicted binding energies for hesperetin–PP2A complex are summarized in Table 1.

### 4. DISCUSSION

Flavonoids have been proposed to exert beneficial effects in a multitude of diseases, including cancer, cardiovascular disease and neurodegenerative disorders [39-41]. Increasingly they have been observed to exert their cellular effects via the modulation of cell signaling pathways, such as the
mitogen activated protein kinase (MAPK) and the PI3K pathways, two essential signaling cascades that regulate multiple processes in the central nervous system including proliferation, survival, metabolism and cell migration [42]. For example, flavanols have been demonstrated to inhibit stress activated extracellular signal-regulated kinase 1 and 2 (ERK1/2) and c-Jun N terminal kinase (JNK) signaling pathways resulting in the protection of neurons from apoptosis [43]. Our current data indicate that exposure of primary cortical neurons to hesperetin resulted in a significant increase in the phosphorylation of ERK1/2 and Akt at Ser473. Those data are in agreement with our previous findings demonstrating that hesperetin was effective at modulating both Akt/protein kinase B and extracellular signal-regulated kinase 1 and 2 (ERK1/2) in neurons [24]. Nanomolar concentrations of flavonoids have previously been reported in the brain in animal models following oral ingestion [14, 15, 44] and hesperetin itself has been shown to have high apparent permeability (Papp) across the in vitro BBB model, mainly due to its lipophilic nature [19] and was observed in the brain tissue at nanomolar concentrations [45, 46]. The concentrations used in our study reflect those observed in the brain in vivo. This is very important, as flavonoids have been shown to act as pro-apoptotic stimuli at higher concentrations. Indeed, we have previously shown that quercetin and its metabolites 3’-O-methyl quercetin and 4’-O-methyl quercetin inhibit protein kinase B/Akt (Akt) and ERK1/2 phosphorylation, an action underlying their pro-apoptotic action towards cortical neurons [47].

Reversible phosphorylation is a very important mechanism of signal transduction and is mediated by a series of kinases and phosphatases [25]. Amongst phosphatases, protein phosphatase 2A (PP2A) is one of the most conserved proteins and together with PP1, is responsible for up to 90% of all serine/threonine activity in a cell [48]. As a phosphatase, PP2A functions in many of the major cell signaling pathways including those that regulate the cell cycle, cell metabolism, cell migration and cell survival [49]. Numerous observations in vitro suggest that PP2A plays a major role in the down-regulation of the ERK and Akt/PKB pathways, and that PP2A may be active at several levels of the signaling cascade [35]. Our results show that concomitant to ERK and Akt activation/phosphorylation, PP2A was significantly decreased by hesperetin treatment.

The balanced and highly dynamic interplay between kinases and phosphatases is critical for the control of intracellular signalling and consequently aberrant regulation of either protein kinases and/or phosphatases may play a causative role in diseases such as diabetes and neurological disorders [50].
As such both kinases and phosphatases may act as therapeutic in the aetiology of many diseases [51-56]. With respect to phosphatases, their regulation and/or activation status may lead to a variety of outcomes. For example, protein tyrosine phosphatase (PTP) 1B has been reported to negatively regulate insulin and leptin signalling making it a prime target for enhancing insulin sensitivity and controlling body mass [53]. In support of this, PTP1B-deficient mice show an enhanced insulin sensitivity, improved glycemic control, and resistance to high fat diet induced obesity [57, 58]. As such, if flavonoids such as hesperetin are capable of inhibiting PTP1B they may counteract insulin resistance via a mechanism that facilitates insulin-induced autophosphorylation of its receptor, thereby sustaining downstream kinase cascades that induce synthesis of the short-term energy storage glycogen and synthesis of fatty acids and proteins. Clearly the success of such inhibitors will depend on the effective absorption and selectivity of the flavonoid or its metabolites towards the phosphatase, however as indicated flavanones such as hesperetin are amongst the most brain bioavailable flavonoids and thus their activity in animal models is justified.

Many human clinical trials have indicated that flavonoid intervention, including that of citrus flavanones can induce reductions in blood pressure in hypertensive patients and increased blood flow in normotensive individuals (add various refs here). These changes have been speculated to involve eNOS activation and nitric oxide synthesis. On the one hand, activation of Akt, as observed here, is known to regulate the activation of eNOS phosphorylation/activation at serine-1176, and thus maintaining Akt phosphorylation, in part through inhibition of PP2A, is predicted to increase nitric oxide bioavailability and thus promote optimum blood flow/pressure. Additionally, removal of the phosphate group from S1176 is also catalyzed by PP2A, thus its inhibition by flavanones may also lead to sustained activation of eNOS and nitric oxide generation in the vascular endothelium, which may underpin observations of increased flow-mediated dilatation and reductions in blood pressure in human clinical trials.

In addition to their role in modulating key intracellular signalling pathways involved in various aspects of physiology, phosphatases are thought to play a key role in neuronal function, including synaptic plasticity, apoptotic cell death [59] and neuroinflammation [60]. PP2A is speculated to be a potential biological target for the drug treatment of neurodegenerative diseases such as Parkinson's disease and Alzheimer’s disease [59, 61]. Here, they may contribute to the abnormal phosphorylation of
proteins, such as tau in the neurofibrillary tangles of Alzheimer’s disease [62] or α-synuclein in the Lewy bodies of PD [63]. The abnormal hyperphosphorylation of such proteins is generally believed to lead to their mis-folding and aggregation [64]. Thus, effective inhibition of phosphatases such as PP2A may attenuate the progression of these neurodegenerative diseases. However, it remains unclear whether activation or inhibition of such phosphatases would be most therapeutic and also whether their inhibition may promote carcinogenesis. While it seems counterintuitive to inhibit a tumour suppressor such as PP2A, inhibitor such as the water-soluble small molecule LB-100 are known to sensitize tumours to chemotherapy and radiotherapy by blocking the removal of phosphate groups from proteins essential for cell cycle progression. Thus, when used with radio- or chemotherapy treatments, malignant cells will progress through the cell cycle without having their damaged DNA repaired thus shunting them to undergo tumour cell apoptosis. With respect to the ability of flavanones to inhibit other phosphatases, it is known that the function of protein-tyrosine phosphatases depends on a critical cysteine residue at the catalytic site, which forms a thiol-phosphate intermediate during catalysis [65, 66]. Interaction with this thiol residue may therefore disrupt enzyme active. Whilst it is currently unknown whether flavanones may interact within this essential cysteine, under cellular conditions some flavonoids are capable of autoxidising and covalently binding to cellular thiols [67].

In summary, our findings suggest that observations relating to increases in kinases following exposure to flavonoids, including flavanones such as hesperetin may result in part due to the inhibition of phosphatases such as PP2A. Although the precise mechanism is yet to be established, and importantly the activity of phase II conjugated metabolites, such as glucuronides and sulphates, inhibition of phosphatases by flavanone aglycones may represent one mechanism by which flavonoids influence physiological function at the cellular level.

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AUTHOR CONTRIBUTIONS: DV and JPES conceived and designed the experiments. DV performed the neuronal culture, phosphatase assays and the immunoblotting. SC and MM contributed to the
molecular docking. DV and JPES wrote the paper. All authors gave final approval of the version to be published. All authors read and approved the final manuscript.

**CONFLICTS OF INTEREST:** The authors have no conflict of interest to declare.
**Table 1.** Docking results of Hesperetin onto PP2A catalytic unit.

<table>
<thead>
<tr>
<th>Site</th>
<th>Ligand</th>
<th>Full Fitness</th>
<th>Binding energy, ΔG (kcal/mol)</th>
</tr>
</thead>
<tbody>
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<td>A</td>
<td>Hesperetin</td>
<td>-1568.00</td>
<td>-6.808</td>
</tr>
<tr>
<td>B</td>
<td>Hesperetin</td>
<td>-1562.99</td>
<td>-6.539</td>
</tr>
</tbody>
</table>
FIGURES CAPTION

**Figure 1:** Hesperetin stimulates Akt and ERK1/2 phosphorylation in a concentration-dependent manner. Cortical neurons were exposed to increasing concentrations of hesperetin (0.1 µM-0.3µM) for 30 min and the levels of phosphorylated Akt (Ser473), the dually phosphorylated ERK1/2 (pTEpY), total Akt and total ERK1/2 were measured by immunoblotting. (A) Representative immunoblots from a single experiment showing phosphorylated pAkt (Ser473) and total Akt; (B) phosphorylated ERK1/2 and total ERK1/2. Band intensities were determined by densitometric analysis using BioRad Quantity One 1-D Analysis. *** p < 0.001; * p < 0.05: indicate significant increases in phosphorylation of Akt or ERK1/2 relative to vehicle treated cells, n = 3.

**Figure 2:** Hesperetin decreases PP2A levels in a concentration-dependent manner. Cortical neurons were exposed to increasing concentrations of hesperetin (0.1 µM-0.3µM) for 30 min and the levels of PP2A, PTEN and beta actin were measured by immunoblotting. Representative immunoblots from a single experiment showing PP2A, PTEN and beta actin. Band intensities were determined by densitometric analysis using BioRad Quantity One 1-D Analysis and normalized against beta actin levels. * p < 0.05: indicate significant decrease in PP2A levels relative to vehicle treated cells, n = 3.

**Figure 3:** Hesperetin decreases PP2A activity in cortical neurons. Cortical neurons were exposed to increasing concentrations of hesperetin (0.1 µM-0.3µM) for 30 min and PP2A phosphatase activity of was quantitated using the DuoSet® IC Active and Total PP2A assay kits. (A) Phosphate released in cortical neurons lysates following 30-minutes incubation with increased concentration of hesperetin (-OA). To determine non-specific activity, 20 nM Okadaic acid (+ OA) was added to some of the samples and incubated for 15 minutes at room temperature. (B) Specific PP2A activity was calculated as the ratio of phosphate released to the amount of Total PP2A protein. *** p < 0.001; ** p < 0.01; * p < 0.05: indicate significant decreases in phosphatase activity relative to vehicle treated cells, n = 3.

**Figure 4:** Molecular docking of hesperetin on PP2A. Panel (A), Hesperetin docked into the site A and B of PP2A catalytic subunit. Panels (B) and (C), the binding mode and the hydrogen bond network established by Hesperetin and neighboring residues in sites A and B (black lines in panel B):
Hesperetin is hydrogen-bonded with the side chains of Leu243 and Hsd252 (green lines in panel (C)).

Panel (D), interactions of Hesperetin rings with Val244, Asn249 and Trp250. Images are depicted using the Chimera software.
REFERENCES


Figure 1

A

60 KDa → p-Akt (Ser473)
60 KDa → Akt

B

44 KDa → p-ERK1/2
42 KDa → ERK1/2

Hesperetin (μM)
Figure 2
Figure 3

A

Phosphate released (pmol/min/10^6 cells)

- OA
+ OA

Hesperetin (µM)

0 0.1 0.3

B

PP2A specific activity (nmol/min/ng)

0 0.1 0.3

Hesperetin (µM)
Figure 4