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Natural inhibitors of indoleamine 3,5-dioxygenase induced by interferon-gamma in human neural stem cells

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ABSTRACT

Indoleamine dioxygenase (IDO) is a heme- containing enzyme that catalyzes the oxidation of tryptophan to *N*-formylkynurenine, kynurenine and the downstream quinolinic acid. Though IDO is physiologically important in maintaining tissue integrity, aberrant IDO expression represses T cell function and promotes regulatory T cells (Treg) in cancer. It additionally exacerbates Alzheimer, depression, Huntington and Parkinson diseases via quinolinic acid. Inhibition of IDO has thus been recently proposed as a strategy for treating cancer and neuronal disorders. In the present study, we have developed a cell-based assay to evaluate the suppressive effect of anti-inflammatory phytochemicals on the enzyme. When stimulated by INF-γ, profound high expressions of IDO-1 mRNA as well as the protein were detected in human neural stem cells (hNSC) and verified by real-time retro-transcribed PCR and western blot analysis, respectively. The protein activity was measured by kynurenine concentration and the assay was validated by dose-responsive inhibition of IDO-1 antagonists including 1-methyltryptaphan, indomethacin and acetylsalicylic acid. Among the tested compounds, apigenin, baicalein, chrysin, and wogonin exhibit a potent repressive activity with IC_{50s} comparable to that of indomethacin. The inhibition was further found to be independent of gene expression and protein translation because of the unaltered levels of mRNA and protein expression. Although curcumin displayed a potent inhibitory activity to the enzyme, it appeared to be cytotoxic to hNSCs. Morphological examination of hNSC revealed that baicalein and wogonin at the inhibitory concentrations induced neurite outgrowth. In conclusion, our data shows that certain phytochemicals with 2-phenyl-1-benzopyran-4-one backbone (flavones) attenuate significantly the IDO-1 protein activity without harming hNSCs. The inhibitory activity might have partially contributed to the anti-cancer and neuro-protective property of the compounds.

1. Introduction

The heme- containing enzyme indoleamine 2,3-dioxygenase (IDO) oxidizes the pyrrole moiety of L-tryptophan (Trp) and represents the initial and rate-limiting step of the kynurenine (Kyn) pathway [1]. Constitutive IDO is expressed in various cell types, including fibroblasts, macrophages, dendritic cells, trophoblasts, and epithelial cells [2,3]. Induction of IDO and metabolism of Trp are implicated in a variety of physiological and pathophysiological processes, including maternal tolerance towards allogeneic concept, anti-microbial, anti-tumour defence, neuropathology and immune-regulation [4–6].

Up-regulation of IDO can be induced by various inflammatory stimuli such as INF-γ and TNF-α [7–9]. In cancer, secretion of IDO from cancer cell directly or from surrounding tissues such as dendritic cells and macrophages, is partially responsible for the immune-escape property of cancers [7]. It creates a tumour immunosuppressive microenvironment by inhibiting T lymphocytes and activating Treg (Regulatory T cells) at lymph nodes [10–12]. Chronically hyperactive IDO is correlated with diverse tumour progression and shorter patient survival rate in ovarian, endometrial, prostate and colorectal carcinoma [13,14]. Preclinical data indicate that inhibition of IDO can delay and suppress tumour growth and can synergize with chemotherapy [6,14].

High level of IDO protein in brain has also been shown to be linked to neurological disorder such as Alzheimer, depression, Huntington and Parkinson diseases. The disorder is partially caused by the up regulation of the toxic metabolite quinolinic acid in the kynureneine pathway [15–17]. Repression of IDO appears to improve the condition of these diseases.

Abbreviations: hNSCs, human neural stem cells; IDO, indoleamine 2,3-dioxygenase; Trp, L-tryptophan; Treg, regulatory T cell.

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This study was set out to investigate the relationship between IDO activity and natural anti-inflammatory agents. A group of common phytochemicals which represent the chemical structure of flavonoids were tested in the study. As the second metabolites in plants, these phytochemicals are present in high concentration in vegetables, fruits and medicinal plants. For example, apigenin is found in parsley, thyme and celery heart. It activates monoamine transporters and inhibits monoamine oxidase [18,19]. Baicalein, chrysin and wogonin are the principal active compounds in the Chinese anti-microbial plant, *Scutellaria baicalensis*. They have been reported as neuroprotective agents in preventing brain cells injury and suppressing brain tumour [20,21]. Curcumin is a broad spectrum anti-inflammatory compound from turmeric (ginger family) used in Indian Ayurvedic medicine for chemoprevention and cancer treatment [22].

In order to evaluate IDO enzymatic activity, we have developed a cell based assay. When human neural stem cells (hNSCs) are treated with human IFN-γ, they dramatically up regulate IDO-1 mRNA and protein expression. In the assay, the enzyme activity was measured by the concentration of secreted kynureic acid (Kyn) in the cell culture media. We tested each of the phytochemicals in this assay. Detail of our findings is reported below.

2. Material and methods

2.1. Reagents

Chemicals of apigenin, acetylsalicylic acid, baicalein, chrysin, curcumin, indomethacin, wogonin and l-methyl-tryptophan were purchased from Aldrich and Sigma. All chemicals were dissolved in DMSO and diluted in cell culture media at least 2500 fold before use.

2.2. hNSC

hNSC cells were derived from a clonal, conditionally immortalized cell line and cultured as previously described [23]. Briefly, hNSC cells between passage number 21–34 were routinely maintained on mouse laminin coated tissue culture flasks (10 μg/ml in DMEM: F12 for 1 h at 37 °C) in a reduced media formulation (DMEM: F12 supplemented with HSA (0.03%) (Grifols); L-glutamine (2 mM) (Gibco); human transferrin (5 μg/ml), putrescine dihydrochloride (16.2 μg/ml), human insulin (5 μg/ml); and progesterone (60 ng/ml), sodium selenite (selenium) (40 ng/ml), bFGF (10 ng/ml) and EGF (20 ng/ml), all from Sigma when not specified. Cells were seeded at approximately 50,000/cm^2 in media supplemented with 4-OHT and cultured at 37 °C in 5% CO_2, with a media change every 2–3 days. When approximately 70–90% confluent the cells were passaged using TrypZean/EDTA (Lonza) and a defined trypsin inhibitor then reseeded for expansion or experimental use.

2.3. Real-time PCR analysis

hNSC total RNA was isolated using RNAeasy (Qiagen), according to the manufacturer's protocol. Two micrograms of total RNA was reverse-transcribed into first-strand cDNA using random primer. Reverse transcription was performed with Superscript II reverse transcriptase (Invitrogen) for 1 h at 42 °C, inactivated for 15 min at 70 °C and cooled to 4 °C. 2.5 μl of cDNA were used in a PCR reaction containing 2× Roche master mix, 0.1 μg of human universal probe library (UPL, Roche), and 0.4 μM primers. The cDNA was amplified using 35 PCR cycles in LC480 Roche. RT-PCR results were expressed as relative quantification based on the ΔΔct method. The primers used in the real time RT-PCR are listed in Table 1. The fold change was calculated according to the $2^{-\Delta\Delta ct}$ method. ATP5B and YWHAZ were used as housekeeping genes.

2.4. Assay of IDO-1 enzymatic activity

IDO-1 activity was determined by measuring the concentration of the tryptophan metabolite L-kynurenine [24]. Hundred microliter of conditioned media from hNSC cells, 24 h after stimulation with INF-γ (10 ng/ml), was mixed with 30% trichloroacetic acid and centrifuged at 12,000 rpm for 10 min. Fifty microliter of the supernatant was then added to an equal volume of freshly prepared Ehrlich solution (2% w/v *p*-dimethylbenzaldehyde in glacial acetic acid) in a 96 well plate and the optical density of each well obtained at 490 nm. Alongside the unknowns, standard concentrations of kynurenine (Kyn) (0–20 μg) were assayed in the same way to produce a standard curve.

2.5. hNSC cell cytotoxicity assay

The cytotoxicity of the polyphenol compounds to hNSC was assessed using the commercially available cell proliferation assay CyQUANT (Invitrogen, UK). Briefly, proliferating cultures of hNSC cells were seeded into laminin coated 96 well plates, and allowed to become 70–80% confluent (2–3 days). The cells were then treated (in triplicate) with 5 and 10 ug/ml of each compound diluted in normal growth medium alongside an untreated control and incubated for a further 24 h under normal culture conditions (5% CO_2, 37 °C). The next day the medium was removed, the entire plate frozen at −20 °C and the CyQUANT assay performed.

2.6. Western blot analysis

Western blot analysis for IDO-1 protein was carried out using whole-cell lysates prepared after 24 h of treatment with IFN-γ (10 ng/ml) in the presence and absence of baicalein/wogonin (10 μg/ml). hNSC cell monolayer in T75 flasks were rinsed with cold PBS (4 °C) after treatment, and lysed with 750 all 1 × SDS buffer and dithiothreitol (DTT) reducing agent (Invitrogen, UK). Samples were then heated at 70 °C for 10 min before being separated on a 4–20% NuPAGE Bis-Tris mini gel (Invitrogen, UK), at 20 μl per well. The protein was transferred onto nitrocellulose and non-specific binding blocked using 5% milk powder in PBS for 1 h at room temperature. Immuno-detection was performed using a mouse monoclonal antibody against human IDO (ABCAM, UK at 2.5 ug/ml) over night at 4 °C and an anti-mouse IgG horseradish peroxidise–conjugated antibody (1:1000) for 2 h at room temperature. The nitrocellulose membrane was then processed using chemiluminescence detection reagents (Thermo scientific) and imaged using a BioRad FluorS Imaging Unit.

2.7. Morphological examination

Immunocytochemistry was carried out on hNSC cells fixed in 96-well plates (BD) using 4% PFA/4% sucrose in PBS for 15 min after 24 h of treatment with either baicalein/wogonin (5 or 10 ug/ml). The cells were treated with 0.1% Triton X100 for 15 min at room temperature after washed with PBS. Non-specific binding was blocked using 10% normal goat serum (NGS) in PBS for 1 h prior to the cells being probed with primary antibodies raised against nestin (1:1000, Chemicon), and GFAP (DAKO 1:10000) diluted in 1% NGS, overnight at room temperature. Positive staining was visualized using goat anti-mouse (1:1000, Molecular Probes) and goat anti-rabbit (1:2500, Molecular Probes) antibodies conjugated to Alexa 488 and Alexa 568, respectively, for 2 h at room temperature. All wells were then washed with PBS and counter stained with

Table 1
Primers used in the real time RT-PCR assay.

Access	Gene	F	R	Probe
NM_002164.4	IDO-1	catttcgtgatggagactgc	ggaattactttgattgcagaagc	31
NM_005651.2	TDO-2	aagaggaattcataaggattcagg	agcacctctttttgcttctga	83
NM_194294	IDO-2	gaaatgaagcttgacacttcacc	tctgtggggctccattattt	49

1 μM Hoechst 33342 (Sigma) for 2 min and visualized on an Olympus IX70 fluorescent microscope.

3. Results

3.1. Effects of phytochemicals on mRNA expression of IDO and TDO-2 in hNSC cells stimulated by INF-γ

Three enzymes are involved in tryptophan degradation, IDO-1, IDO-2 and TDO-2 (Tryptophan Dioxygenase-2). IDO-1 and IDO-2 are isozymes and TDO-2 is expressed mostly in liver. In order to confirm IDO-1 is the only enzyme involved in our INF-γ treated cell based assay, we conducted RT-PCR measurements to evaluate their mRNA expression. Fig. 1A summarizes the time course analysis of IDO-1 mRNA expression stimulated by INF-γ and treated by phytochemicals. There are 1000–5000 fold changes in the gene expression. Similar analysis was also performed for IDO-2 and TDO-2 genes. As seen in Fig. 1B, the average cross point (cp) level per reaction for both IDO-2, and TDO-2 was above cp 30 at all-time points. A cp level of 30 indicates a very low or absent expression of the gene. These results demonstrate that (1) Only IDO-1 gene is highly expressed in the hNSCs when stimulated by INF-γ; (2) Addition of phytochemicals did not significantly modulate IDO-1 mRNA expression.

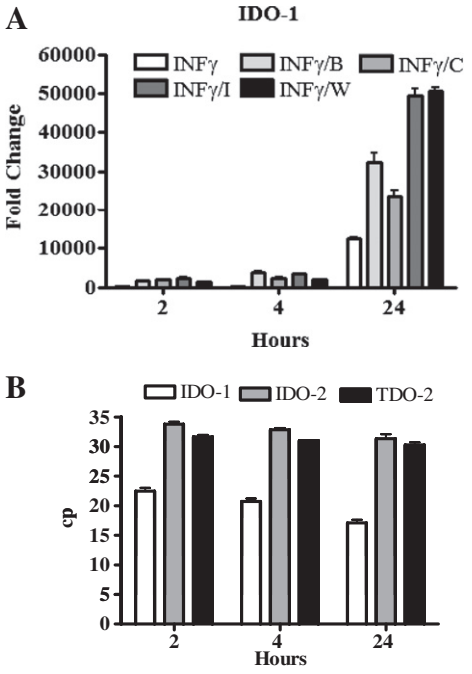

Fig. 1. Effects of phytochemicals on IDO-1, IDO-2, and TDO-2 mRNA expressions. (A) Time course analysis of up-regulation of IDO-1 mRNA following treatment with INF-γ and phytochemicals measured by qRT-PCR, I: indomethacin was used as a reference, B: baicalein, C: chrysin, W: wogonin; (B) RT-PCT time course analysis on average cp values of IDO-1, IDO-2, and TDO-2 expression in INF-γ and phytochemicals treated samples.

3.2. Western blot (WB) analysis on the effect of baicalein/wogonin on IDO-1 protein expression stimulated by INF-γ

In order to quantify IDO-1 protein induced by INF-γ stimulation, WB was carried out in hNSC lysate in the absence and presence of baicalein and wogonin, respectively. Fig. 2 shows that similar level of IDO-1 protein was present in all INF-γ treated or untreated samples. The result suggests none of the phytochemicals inhibited IDO-1 protein translation.

3.3. Establishment of IDO-1 activity assay

Following the confirmation of IDO-1 protein secretion, Kyn production was then measured. Fig. 3A demonstrates a dose-responsive correlation of Kyn and INF-γ. At 1 ng/ml of INF-γ, Kyn production reached maximum. Fig. 3B displays the Kyn standard curve. Moreover, several IDO-1 inhibitors were tested to validate the assay. Fig. 3C confirms the inhibition of enzyme by l-methyl-tryptophan (l-MTP) at several concentrations.

3.4. Effects of phytochemicals on IDO-1 enzymatic activity in hNSC cells stimulated by INF-γ

The above established protocol was used to evaluate the phytochemical inhibitory activities. Fig. 3D displays dose-repressive activities of apigenin, baicalein, chrysin and wogonin. The activities are further compared with that of the other known IDO inhibitors indomethacin and acetylsalicylic acid. As expected, indomethacin potently suppresses IDO activity while the weak inhibitor acetysalycilic acid has no effect at micromolar concentration. It is seen that the inhibitory activities of the phytochemicals are comparable to

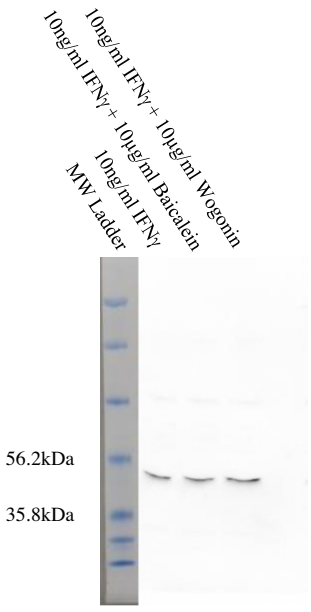

Fig. 2. Western blot analysis on the effect of baicalein and wogonin on IDO-1 protein expression.

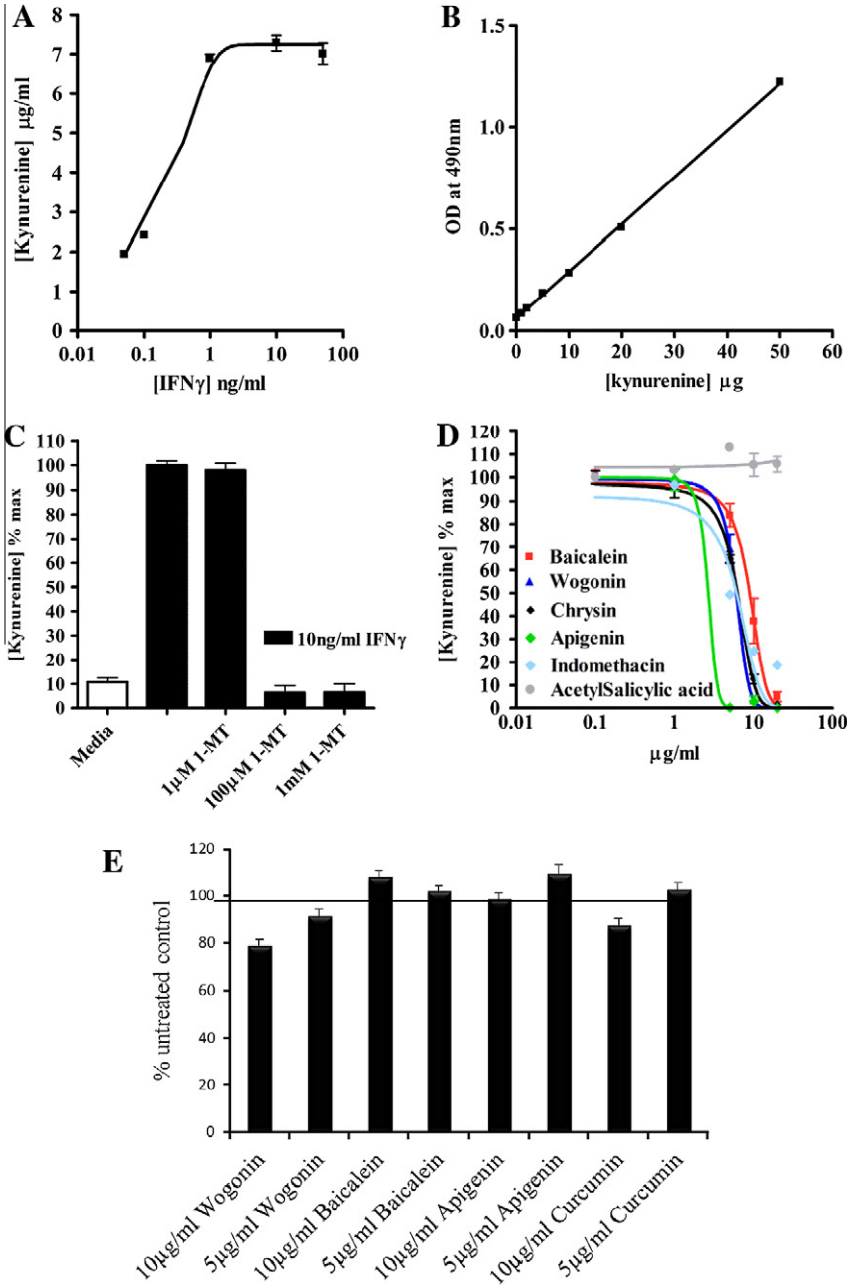

Fig. 3. (A) Dose-responsive secretion of kynureneine (Kyn) metabolite from HNSC cells stimulated by INF-γ. (B) Standard curve of Kyn concentration. (C) Inhibition of IDO-1 protein by 1-L-methyltryptophan at several concentrations. (D) Comparison of Apiegenin, baicalein, chrysin and wogonin with known IDO inhibitors of indomethacin and acetylsalicylic acid. (E) Cell viability assay of hNSCs at 5 and 10 μg/ml of phytochemicals. concentrations.

that of indomethacin. Among them, Apigenin is the most potent inhibitor. The IC50 for each compound was thus calculated and presented in Table 2.

Table 2
IC50 (μM) of antiinflammtory phytochemicals: apigenin, baicalein, chrysin, and wogonin.

Compounds	IC_{50} μg/ml (μM)
Apigenin	2.7 (10 μM)
Baicalein	8.9 (33 μM)
Chrysin	6.2 (24.4 μM)
Wogonin	6.0 (21.1 μM)

3.5. Evaluation of cytoxic effect of phytochemicals on hNSCs

During the enzymatic activity assays, it was observed that curcumin inhibited IDO-1 activity but also induced cell detachment at the same time. In order to further investigate the cytotoxicity of curcumin, cell viability assay was conducted. Fig. 3E demonstrates the viability of hNSC in the absence and presence of phytochemicals. A slight decrease in hNSC population in the presence of wogonin or curcumin was observed. Since no cell detachment nor apoptosis was observed when treated by wogonin, a decrease in cell population might be attributed to certain degree of cell differentiation as suggested by neurite outgrowth. In contrast, treatment of curcumin significantly induced cell detachment. At 20 μM of curcumin, majority of cells were detached.

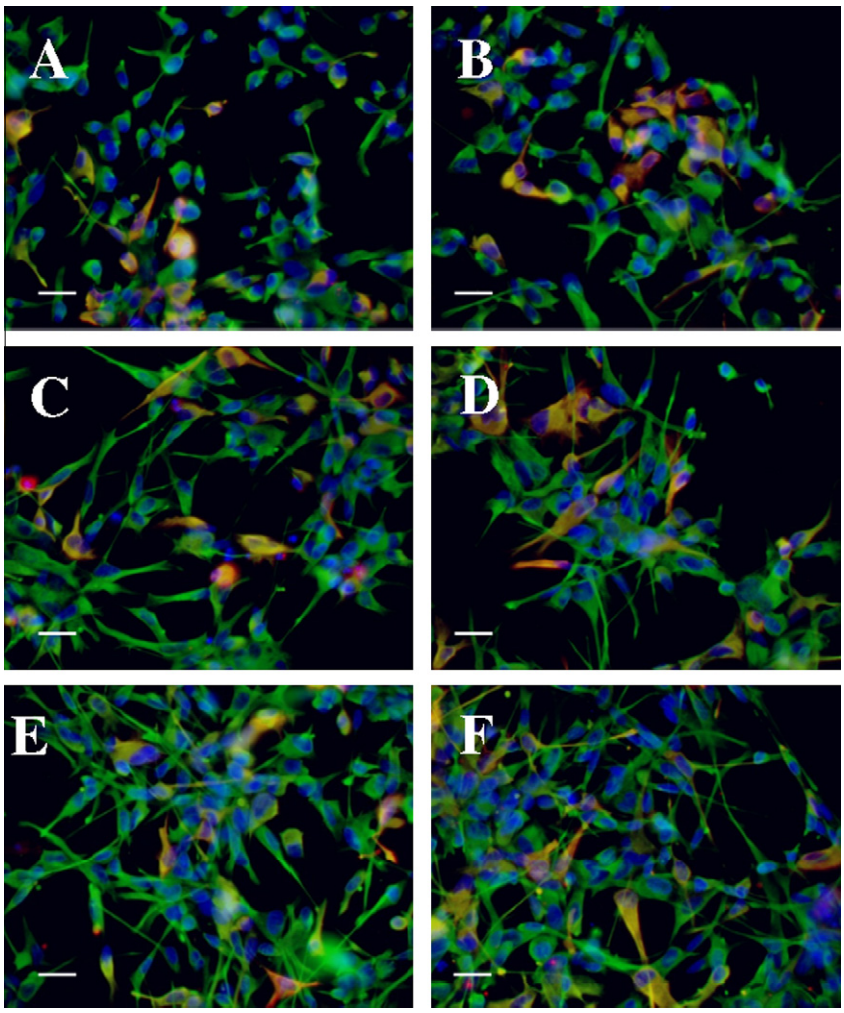

Fig. 4. Morphological changes of hNSCs induced by phytochemicals. Cells were labeled using specific antibodies against the stem cell marker Nestin (green), the neuronal marker Doublecortin (red) and counterstained using the nuclear stain Hoechst 33342 (blue). The scale bar is 100 μm. (A) hNSCs in the absence of baicalein; not to many (green color) neurite (B) hNSCs in the absence of wogonin; not to many (green color) neurite (C) Neurite outgrowth at 5 μg/ml baiclein, long green extensions out of cell surface. (D) Neurite outgrowth at 5 μg/ml wogonin, long green extensions out of cell surface. (E) Neurite outgrowth at 10 μg/ml baicalein, increase in green extensions. (F) Neurite outgrowth at 10 μg/ml wogonin, increase in green extensions.

3.6. Alteration of hNSC morphology in the presence of phytochemicals

As seen from Fig. 4A–F, no apoptosis of hNSC cells was observed in the micrographs when cells were treated by baicalein or wogonin. However, alteration in cell morphology was observed. The micrographs show that baicalein or wogonin had induced cell neurite outgrowth. The outgrowth was clearly identifiable at two different concentrations of baicalein (5 μg/ml in Fig. 4C and 10 μg/ml in Fig. 4E) and wogonin (5 μg/ml in Fig. 4D and 10 μg/ml in Fig. 4F) when compared with the control cells (Fig. 4A and B).

4. Discussion

The relationship of IDO and inflammation is complex. Recent studies show that IDO-1 might act on the downstream COX-2 (cyclooxygenase-2) [25,26]. In our study, wogonin as a known COX-2 inhibitor was found to suppress IDO-1 protein activity as well. Since COX-2 mRNA was not expressed in normal cells, we don't know whether there is a direct correlation between between the two enzymes.

Among the compounds tested, apiegenin, baicalein, chrysin and wogonin are the most potent inhibitors which exhibited null toxicity toward hNSC cells (Fig. 3D and E). Furthermore, each of these compounds exhibits a comparable IC50 in the μM range. In contrast to the above compounds, curcumin induced hNSC cell death during enzymatic activity assay. This observation was unexpected. In order to investigate the cytotoxicity, we conducted a cell viability assay (CyQuant) on some of the tested compounds (Fig. 4E). The result confirmed the microscopic finding that incubation of curcumin, with hNSC cells resulted in cell death.

Flavonoids in general are known anti-inflammatory and antioxidant agents [27]. However, the potency is dependent on chemical structures. Apigenin, baicalein, chrysin and wogonin share the same basic chemical structure of flavonone. Moreover, baicalein has two adjacent OH groups (catechol group) at 3,4 positions at B ring while wogonin has a methoxy group at 8-position. Because of the catechol group, baicalein is the only flavonoids exhibiting scavenging activity to superoxide, hydroxyl free radicals and hydrogen peroxide simultaneously [28]. It is also a potent reagent in Fenton reaction [29]. As such, the two OH groups in baicaein had been shown to form a very tight complex with transition metal ion including Fe^{2+} [29]. Regardless of the minor difference in OH group position, the above four compounds exhibited a similar inhibitory activity to IDO-1 protein. The only common moiety in the four compounds is the 4-OH group at B-ring and carbonyl group (C═O) at A ring. This moiety was suggested as a weak iron

complexing site and can also result in inhibition of hydroxyl free radical production via Fenton reaction and thus contribute to antioxidant properties [30]. Whether this common moiety had contributed to inhibition of IDO-1 activity awaits to be investigated.

In addition to enzyme repression, baicalein and wogonin were found to induce neurite out-growth in hNSC cells and shifted the stem cells toward a more differentiated morphology (Fig. 4A–F). Wogonin had recently been shown by other researchers to induce neurite outgrowth as well as differentiation in neuronal precursor cells *in vitro* and *in vivo* [31]. A recent study also demonstrated a similar differentiation activity of wogonin in rat cortical and neuroblastoma cells [32]. Moreover, an early study showed that the pharmacological action of wogonin in central nervous system was to attenuate the H_2O_2-induced oxidative stress in human neuroblastoma SH-SY5Y cells and inhibit the neuronal damage induced in primary cultured rat cortical cells [33].

In contrast to wogonin, there was no study reporting neurite outgrowth induced by baicalein. Instead, baicalein was shown to be neuroprotective in several neural cells via reduction in hydrogen peroxide-mediated oxidative stress in degeneration of dopaminergic neurons [20,34,35]. Based on our data, we propose that partial neuroprotective effects of baicalein and wogonin might be attributed to induction of stem cell neurite outgrowth and inhibition of IDO-1 protein activity.

Further to neuroprotection, apiegenin, baicalein and wogonin were previously shown to exhibit anti-cancer activities in leukemia, ovarian, prostate, colon and lung cell lines and in mouse models [36–38]. The anti-cancer mechanism was thought to be related to antioxidant activities and to suppressive effects on tumour metastasis. Here we suggest that the anti-tumour mechanism of these compounds might partly via suppression of IDO-1 activity, enhancing T cell response and taming immunosuppressive microenvironment. In several clinical observations, expression of IDO in tumour cells has been associated with an impaired prognosis [13,14]. To further evaluate the role of IDO in cancer progression, several IDO inhibitors including 1-methyl tryptophan are currently under clinical investigations [39].

Conflict of Interest

The authors declare no conflict of interest.

Authorship

All authors participated in concept proposal, experimental design, data acquisition, analysis, interpretation, manuscript drafts as well as revisions.

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