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A novel orexin antagonist from a natural plant was discovered using zebrafish behavioural analysis

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Abstract. – OBJECTIVE: Phenotypic screening is one of the most practical approaches to the identification of mediators of behaviour, since it is difficult to model brain function *in vitro*, at a cellular level. We used a zebrafish *(Danio rerio)* behavioural assay to discover novel, natural, neuroactive compounds.

MATERIALS AND METHODS: A zebrafish behavioural assay was performed for seven natural compounds, obtained from plants. The behavioural profiles were compared to those of known psychoactive drugs. We characterised a natural compound exhibiting a behaviour profile similar to that of suvorexant, using *in silico, in vitro* and microarray expression analysis.

RESULTS: The behavioural analysis performed in this study classified central nervous system drugs according to their mechanism. Zebrafish treated with a natural compound, 8β-(4'-Hydroxytigloyloxy) costunolide (8β), showed behaviour profiles similar to those of zebrafish treated with suvorexant, a known orexin antagonist. This behavioural assay was validated using *in silico* and *in vitro* assays, which revealed that the new compound was a dual orexin receptor antagonist. In addition, transcriptome analysis suggested that 8β might regulate the nuclear factor-κB (NF-κB) related pathway.

CONCLUSIONS: We conclude that zebrafish phenotypic screening, combined with *in silico* assays and gene expression profiling, is a useful strategy to discover and characterize novel therapeutic compounds, including natural products.

Key Words:
Zebrafish, Behaviour, Phenotype-based screening, Sleep, Orexin.

Introduction

Many successful therapeutics have been discovered using cell-based and biochemical screening methods. However, these methods do not focus on the interactions of the whole organism's activity, resulting in difficulty to ensure success at an organismal level. To overcome these difficulties and to increase the efficiency of screening, pharmacological researchers have adopted a phenotype-based screening method. Many first-in-class drugs have been discovered by the phenotype-based approach, rather than the target-based approach[1].

Phenotypic screening is one of the most practical approaches to the identification of mediators of behaviour, since it is difficult to model brain function *in vitro*, at a cellular level. During the last decade, zebrafish behaviours have become one of the major screening systems used to identify neuroactive compounds, through behaviour-based chemical screening[2]. Zebrafish are vertebrates, and the structure and function of their organs and tissues are similar to those of humans[3,4]. The receptors and neurotransmitters are highly conserved between humans and zebrafish[5].

In this study, we attempted to discover novel, natural, neuroactive compounds by phenotypic screening, using zebrafish behavioural analysis. As a test case, we focused on a natural compound exhibiting a behaviour profile similar to that of suvorexant, a well-known orexin receptor antagonist.

Orexins are neuropeptides involved in the regulation of the sleep-wake cycle, feeding pattern, energy balance, and stress in mammals[6,7]. Dysregulation or loss of orexin signalling has been linked to narcolepsy[8-10]. Several dual orexin receptor antagonists have been investigated as potential treatments for insomnia, and one of them, suvorexant, received approval by the US Food and Drug Administration (FDA), in 2014, for the treatment of such a condition[11]. Although these drugs have an outstanding safety profile, there are concerns regarding the use of these drugs, due

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to potential side-effects and lack of evaluation of long-term effects. Therefore, natural compounds could be promising agents, because of their multi-functional health effects and limited toxicity.

In this study we characterised a natural compound exhibiting a suvorexant-like profile, based on zebrafish behavioural analysis, and determined whether phenotype-based screening using zebrafish could serve as a screening model for natural compounds with the desired mechanisms of action.

Materials and Methods

Ethical Approval

The investigation conformed to the Ethical Guidelines established by the Institutional Animal Care and Use Committee of Mie University, Japan.

Compounds

Haloperidol, Chlorpromazine hydrochloride, and Imipramine hydrochloride were purchased from Tokyo chemical industry (TCI) (Tokyo, Japan). Diazepam and Clonazepam were purchased from Sigma-Aldrich (St. Louis, MO, USA). Suvorexant was purchased from Toronto research chemicals (North York, ON, Canada). These compounds were dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) to make stock solutions.

Zebrafish Husbandry

In this study, the nacre/rose/fli1: egfp zebrafish was obtained by cross-breeding nacre (−/−)/rose (−/−) mutants and Tg (fli1: egfp) transgenic zebrafish as described previously[12]. The zebrafish were incubated at 28°C under a 14 h light: 10 h dark cycle and, in environmental quality water, according to The Zebrafish Book[13]. Embryos were obtained via natural mating and cultured in 0.3× Danieau's solution [19.3 mM NaCl, 0.23 mM KCl, 0.13 mM $MgSO_4$, 0.2 mM Ca $(NO3)_2$, 1.7 mM HEPES, pH 7.2] until 8 days post-fertilization (dpf).

Behavioural Analysis

The behavioural assay was performed on 48 zebrafish at 8 dpf. Each zebrafish was placed into a well on a round 48-well plate (10 mm diameter, 300 µl of 0.3× Danieau's solution) at 12 pm. The 48-well plates were then placed in an incubator set at 28°C with constant light (255 lx) from 12 pm to 3:30 pm. After incubation, 300 µl of 0.3× Danieau's solution with or without the sample compounds were added to each 48-well plates. The plates were then placed in the Daniovision system (Noldus, Wageningen, The Netherlands), wherein they were blocked from daylight and illuminated from below with white light (255 lx) from 4 pm to 7 pm and from 5 am to 4 pm. The behaviour of zebrafish in each well was monitored using the Daniovision system with a resolution of 1280 × 960 pixels at 25 frames per seconds. Eight larvae were assigned to examine the effect of each concentration of the compound. Two independent experiments were performed for each compound.

The recorded videos and images were subjected to Ethovision XT11 (Noldus) to analyse the behaviour of zebrafish in each well. The mean velocity, total distance moved, distance to centre zone (2 mm radius circle) of the well, frequency entering the centre zone, turn angle, and mobility were measured. The parameters used in this behavioural analysis are shown in Figure 1-a. Mobility was calculated by taking every pixel identified as the subject and comparing it with the current image and the previous one. If all the pixels were the same, this would indicate zero mobility. If all the pixels were different, this would indicate 100% mobility. In this study, we defined 5%-35%, 35%-65%, and 65%-95% as low, medium, and high mobility, respectively[14].

Behavioural endpoints were measured: the distance moved total (DMT), distance to the zone in the centre of the well (DTZ), in zone frequency (IZF), and turn angle (TA) in each mobility category for each period (L1-time, the first light time period for 3 h; D-time, dark time period for 10 h; L2-time, second light time period for 11 h), resulting in 36 measured endpoints. Overview of the behavioural analysis used in this study is shown in Figure 1-b. The means obtained were compared by analysis of variance using Prism 7 (GraphPad, La Jolla, CA, USA). Alpha was set at 0.05 and Dunnett's multiple comparisons test was used for post hoc analyses when significant differences were observed.

For hierarchical clustering, the data for each behavioural endpoint were normalized according to the controls. Hierarchical clustering was performed using MultiExperiment viewer.

Natural Product Library for in-silico Assay

To perform an *in silico* assay, a natural product library was constructed, consisting of 9334 plants-derived compounds, including 8β. The products were collected from AnalytiCon (Potsdam, Germany), App Tec (Shanghai, China),

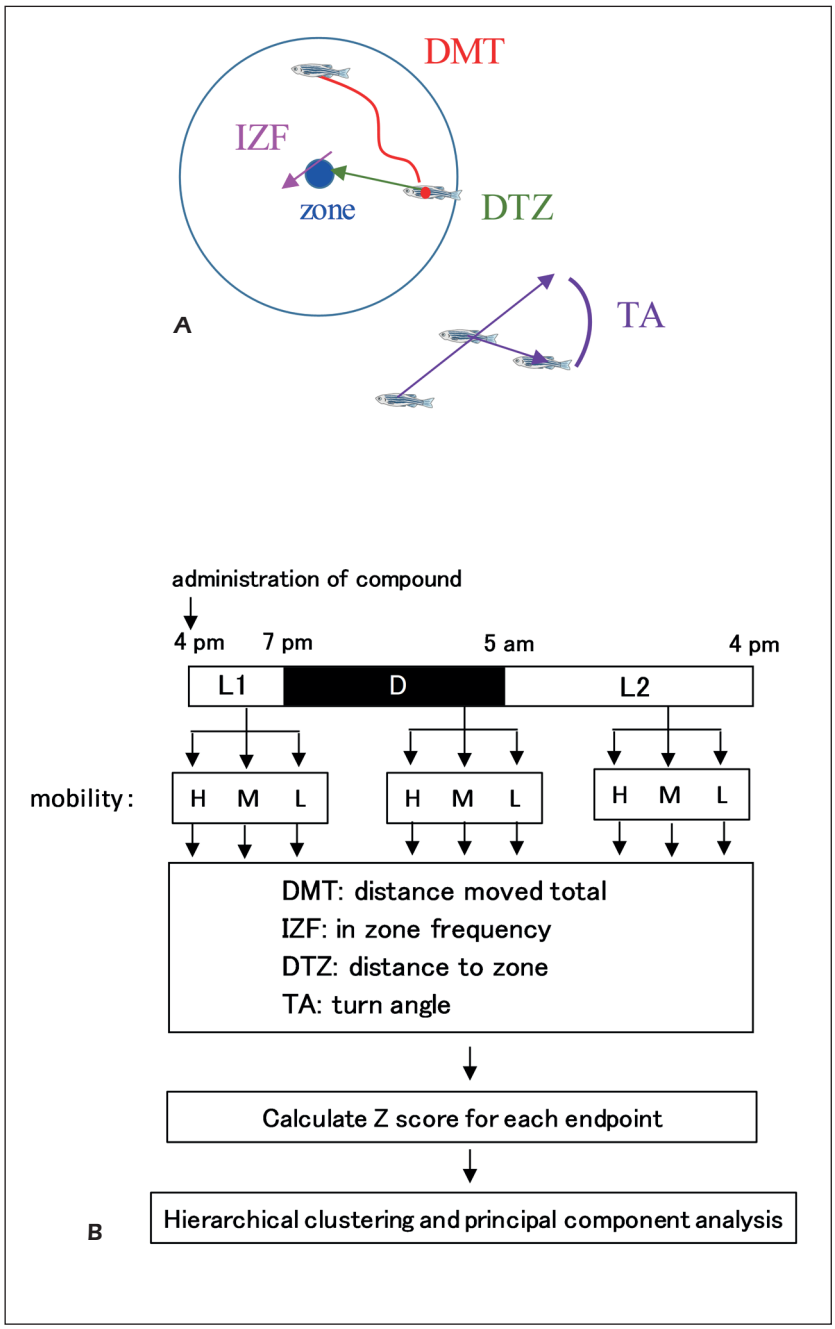

Figure 1. Larval zebrafish behavioural assay was scored based on the following parameters: **A**, Illustration of the behavioural analysis parameters; **B**, Overview of the behavioural analysis used in this study.

BioBioPha (Yunnan, China), Biopurify (Sichuan, China), Chemfaces (Hubei, China), and Pharmeks (Moscow, Russia).

Collection of Known Antagonists for OX1 and OX2 Receptors

We collected known antagonists of OX1 and OX2 receptors (**Supplementary Figure 1**). OX1 antagonists consist of suvorexant[15], almorexant[16], SB-649868[17], SB-334867[18], SB-408124 and SB-674042[19], and GSK-1059865[20]. OX2 antagonists consist of suvorexant, almorexant, SB-649868, TCS-OX2-29[21], and MK-3697[22]. Suvorexant, almorexant, and SB-649868 exhibit antagonist activities towards both OX1 and OX2 receptors.

In silico Assay Using Pharmacophore-Search and Docking Simulation

Pharmacophores of OX1 antagonists were constructed and pharmacophore searches were performed using the natural product library in the Ligandscout software[23,24]. After 3D conformations

of the antagonists were generated, clusters were created and categorized into two classes: (a) suvorexant, almorexant, SB-649868, and SB-674042, (b) SB-334867 and SB-408124. A pharmacophore model was constructed for each class (**Supplementary Figure 1-a, 1-b**). Finally, we performed a pharmacophore search from the natural product library using both the pharmacophore models.

The same methods were employed to search for OX2 antagonists. The known OX2 antagonists were categorized into two groups: (c) suvorexant and SB-649868, (d) almorexant and TCS-OX2-29. A pharmacophore model was constructed for each group (**Supplementary Figure 1-c, 1-d**), which was then followed by the pharmacophore search. Crystal structures of the OX1 (PDBID: 4ZJ8) and OX2 (PDBID: 4S0V) receptors forming a complex with suvorexant were obtained from the Protein Data Bank (PDB) database[25-27]. After the bound ligand was removed, the structures of the disordered loops and flexible side chains were modelled, and the dominant protonation state at pH 7.0 was assigned for titratable residues using the structure preparation module in the Molecular Operating Environment (MOE, Chemical Computing Group, Montreal, Canada), version 2013.08. This was followed by molecular docking of high-scoring compounds obtained from the pharmacophore search. The compound binding site in the OX1 or OX2 receptor was defined to include all atoms within 10 Å from bound suvorexant. Each of the high-scoring compounds was docked into this site using the rDock software[28], and the top-ranked docking pose was extracted.

Cell-Based Reporter Assay

HEK293T (human embryonic kidney 293T) cells were transfected with human OX1 or OX2 receptors using the Orexin 1 or Orexin 2 Receptor Assay Kit (Cayman #600240 or #600250, respectively), according to the manufacturer's instruction. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Nacalai Tesque, Kyoto, Japan) media supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) at 37°C with 5% CO_2. Cells were seeded into the 96-well plates at a density of 50,000 cells/well and cultivated at 37°C for 16 h in the culture medium. The following day, solutions of approximately 50 µl, containing DMSO (3%), suvorexant (3 µM) and 8β (8.7 µM), were added to the cultured cells in the 96-well plates to obtain a final volume of 150 µl, resulting in 1 µM of suvorexant and 2.9 µM of 8β. The cultured cells were then incubated for approximately 1 h at 23°C. To evaluate the antagonist effect, solutions containing 50 µl of orexin A were added to the cultured cells in the 96-well plates to obtain final culture medium volume of 200 µl. Final concentration of orexin A in the culture medium was 50 nM. After 24 h, 5 µl of cell supernatant were transferred from each well to a fresh 96-well plate. After incubation at 65°C for 30 min, 50 µl of substrate were added to each well; the wells were mixed and subsequently incubated at 23°C for 30 min. The luminescence intensity of the secreted SEAP (Secreted Alkaline Phosphatase) was measured using the SpectraMax i3 platform (Molecular Devices, San Jose, CA, USA).

Orexin activity rate was calculated using the following formula:

$$\text{Activity ratio} = (C\text{-}C_{posi})/ (C_{neg}\text{-}C_{posi})$$

Where C was reported in rectified linear unit (RLU) in the presence of a test antagonist plus orexin A. C_{posi} was reported in RLU in the presence of suvorexant (as positive control) plus orexin A, and C_{neg} was reported in RLU in the presence of orexin A only. In this assay, suvorexant was added at a concentration of 1 µM. Experiments were carried out in triplicate. Data were analysed using the Student's *t*-test. A *p*-value less than 0.05 was considered statistically significant.

RNA Extraction

The zebrafish were placed, individually, into wells in round, 48-well plates (10 mm diameter, 300 µl of 0.3× Danieau's solution) after 8 dpf. Three hundred microliters of the sample solutions (8β, suvorexant or imipramine) were added to the 48-well plates, which were then placed in an incubator, maintained at 28°C with constant light (255 lx). After 3 h, the fishes were anesthetized with 0.05% (v/v) ethylene glycol monophenyl ether (Nacalai Tesque, Kyoto, Japan). The zebrafish brains were extracted under a stereoscopic microscope (Nikon SMZ800) and immediately fixed in RNA*later*™ (Applied Biosystems, Foster City, CA, USA) at 4°C for 1 day. The dissected brains were then stored at −80°C until RNA extraction took place. The total RNA was extracted from ten brain samples, using a RNeasy Micro kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Microarray Analysis

Microarray experiments were performed by an Agilent-certified microarray service provider (TaKaRa Bio, Otsu, Shiga, Japan). Quantification

and quality analysis of RNA were confirmed using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

Microarray experiments were carried out on three independent trials. Total RNA (100 ng) was converted into labelled cRNA using the Low Input Quick Amp Labelling Kit (Agilent, Santa Clara, CA, USA). The Cy3-labelled cRNA was hybridized onto Agilent Zebrafish Oligo DNA Microarrays ver.3.0 according to the manufacturer's protocol. Microarrays were scanned using the Agilent SureScan Microarray Scanner (G2600D) and analysed using the Agilent Feature Extraction Software 12.0.3.1. Data were normalized using the Agilent GeneSpring 14.9.1 software package. Identification of gene sets differentially expressed in microarray analysis was carried out by one-way ANOVA ($p<0.05$). The data were deposited to the Gene Expression Omnibus (GEO) database (GEO accession number GSE129812) run by the National Center for Biotechnology Information (NCBI).

Results

Behavioural Profiling of Zebrafish

Changes in zebrafish behaviour were analysed for seven natural compounds, obtained from plants, and were compared to those of known psychoactive drugs. The behavioural activities of the larvae were calculated, based on the Z-score, as described in Materials and Methods, and examined for 36 behavioural endpoints. One of the compounds, 8β, significantly affected zebrafish behaviour. As shown in Figure 2, 8β significantly

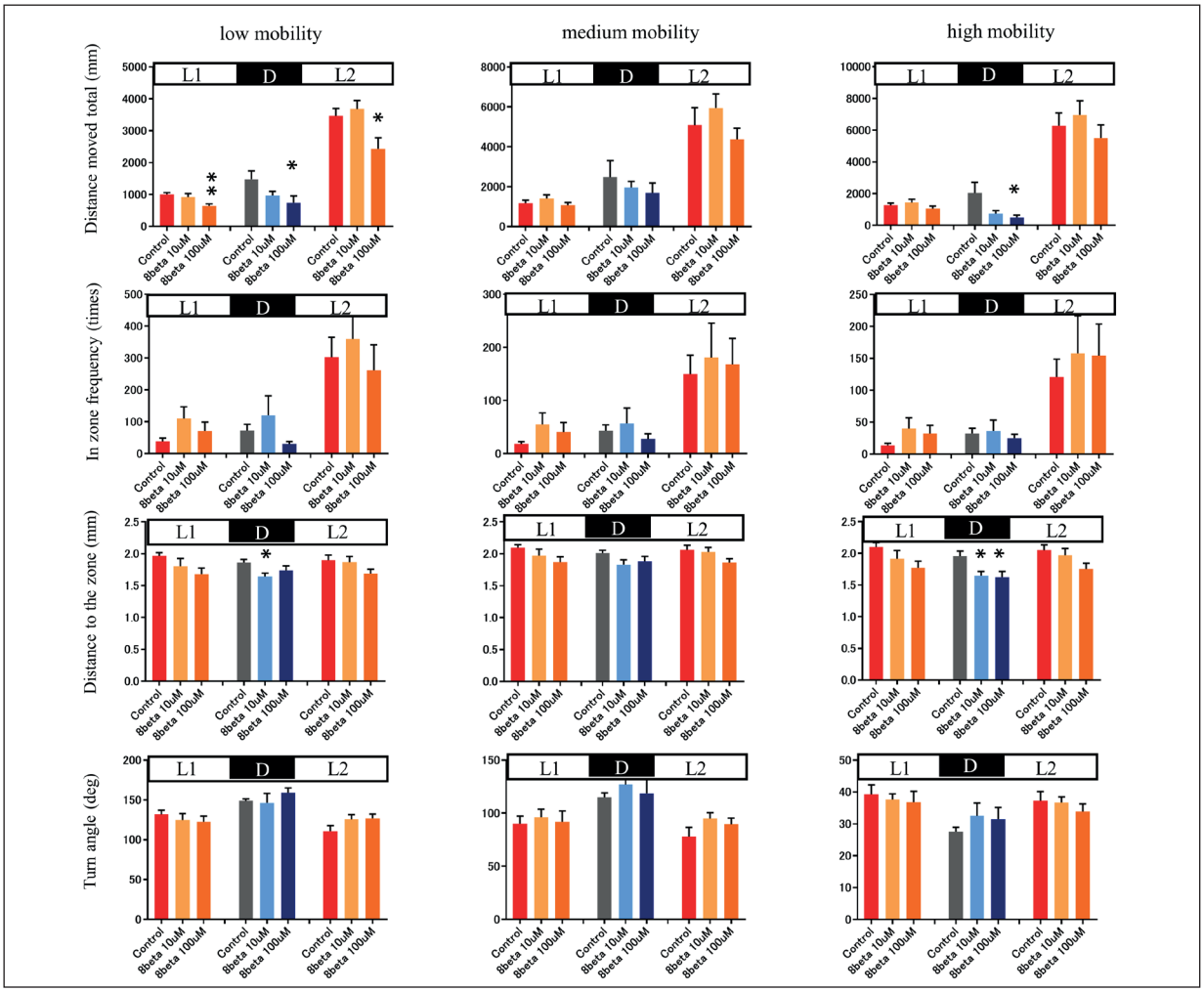

Figure 2. Natural compound 8β-(4'-Hydroxytigloyloxy) costunolide affected zebrafish behaviour. The behaviour of zebrafish treated with 8β was classified into three groups based on their mobility. Total distance moved (DMT), in zone frequency (IZF), distance to zone (DTZ), and turn angle (TA) at L1-time, D-time, and L2-time periods are shown for each mobility classification. The data are represented as mean with the standard error of mean. * < 0.05, ** < 0.01 *vs.* control by Dunnett's multiple comparisons test.

decreased the distance moved total (DMT) scores at high and low mobility during the dark time period (D-time), at a concentration of 100 μM. The zone in the centre of the well (DTZ) scores also significantly decreased in the presence of 8β during the D-time. Hierarchical clustering analyses were performed, to test the functional similarity of 8β to the psychoactive drugs, using the quantitative data matrix from the 36 behavioural endpoints (Z-score). As shown in Figure 3, hierarchical clustering categorized the psychoactive drugs according to their mechanism of action. Thus, psychoactive drugs with a similar mechanism of action had a similar behavioural profile in this assay. Clustering analysis showed that the behavioural profiles by 8β were most similar to those treated with suvorexant, a well-known orexin receptor antagonist, suggesting that 8β has a mechanism similar to that of suvorexant. In addition, the next best fit with 8β, in behavioural profile, was that of imipramine, indicating that 8β might act on multiple targets.

In Silico Assay of Orexin Antagonists

The antagonist binding sites were compared, between human and zebrafish OX receptors, by analysing their amino acid sequences. Based on the crystal structures of the OX1 and OX2 receptors, 21 amino acids located within 4 Å from the bound suvorexant were defined as pocket-forming residues (Figure 4-a, b). Multiple alignment of the three amino acid sequences of human and zebrafish OX receptors using MAFFT (Multiple Alignment using Fast Fourier Transform)[29], showed

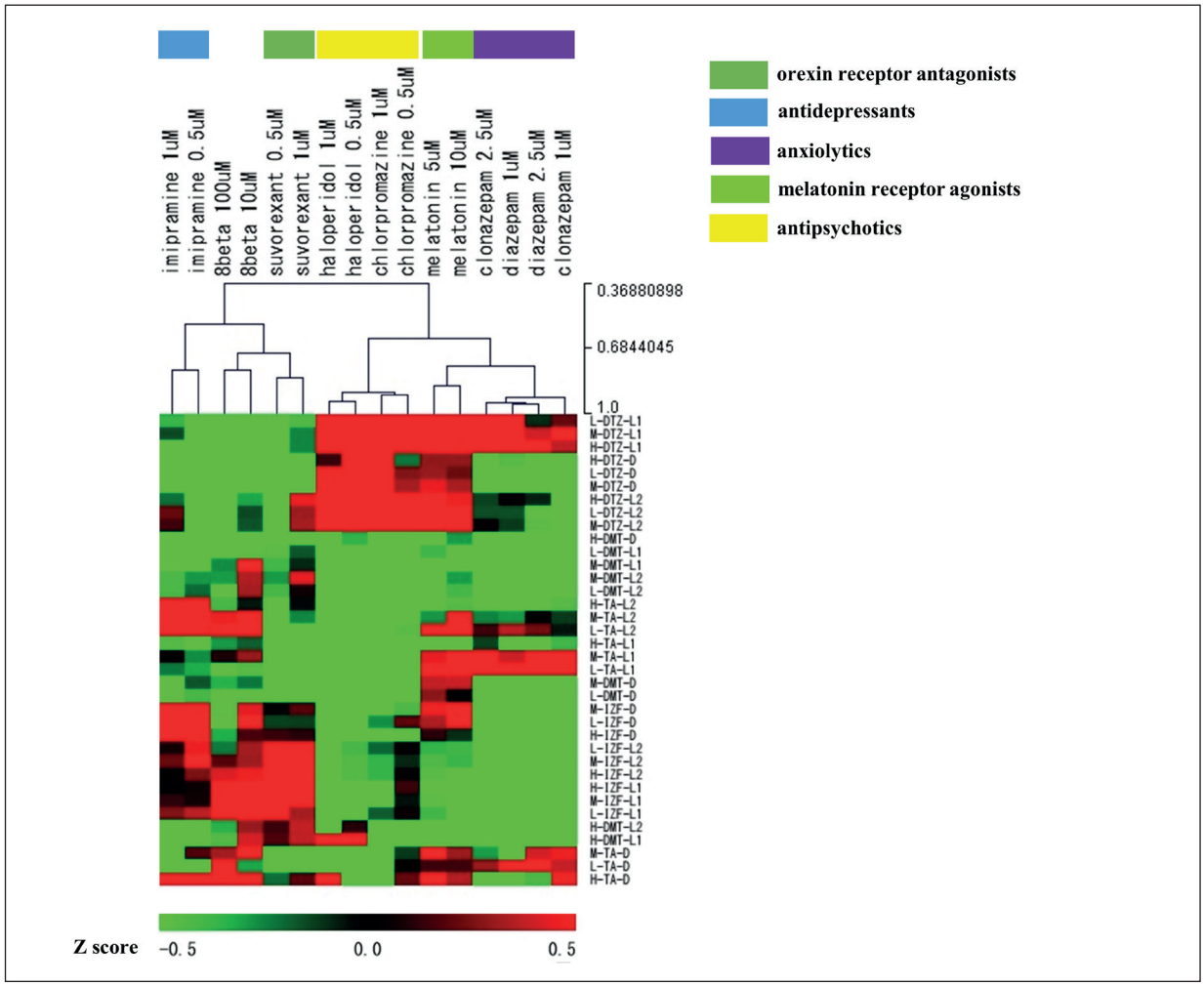

Figure 3. Hierarchical clustering of the behaviour of zebrafish treated with psychoactive drugs. The Z scores of each behavioural endpoint were subjected to hierarchical clustering using Pearson correlation with single linkage. Colours shown in the top of hierarchical clustering tree indicate drug class (green, orexin receptor antagonists; light blue, antidepressants; purple, anxiolytics; yellow green, melatonin receptor agonists; yellow, antipsychotics).

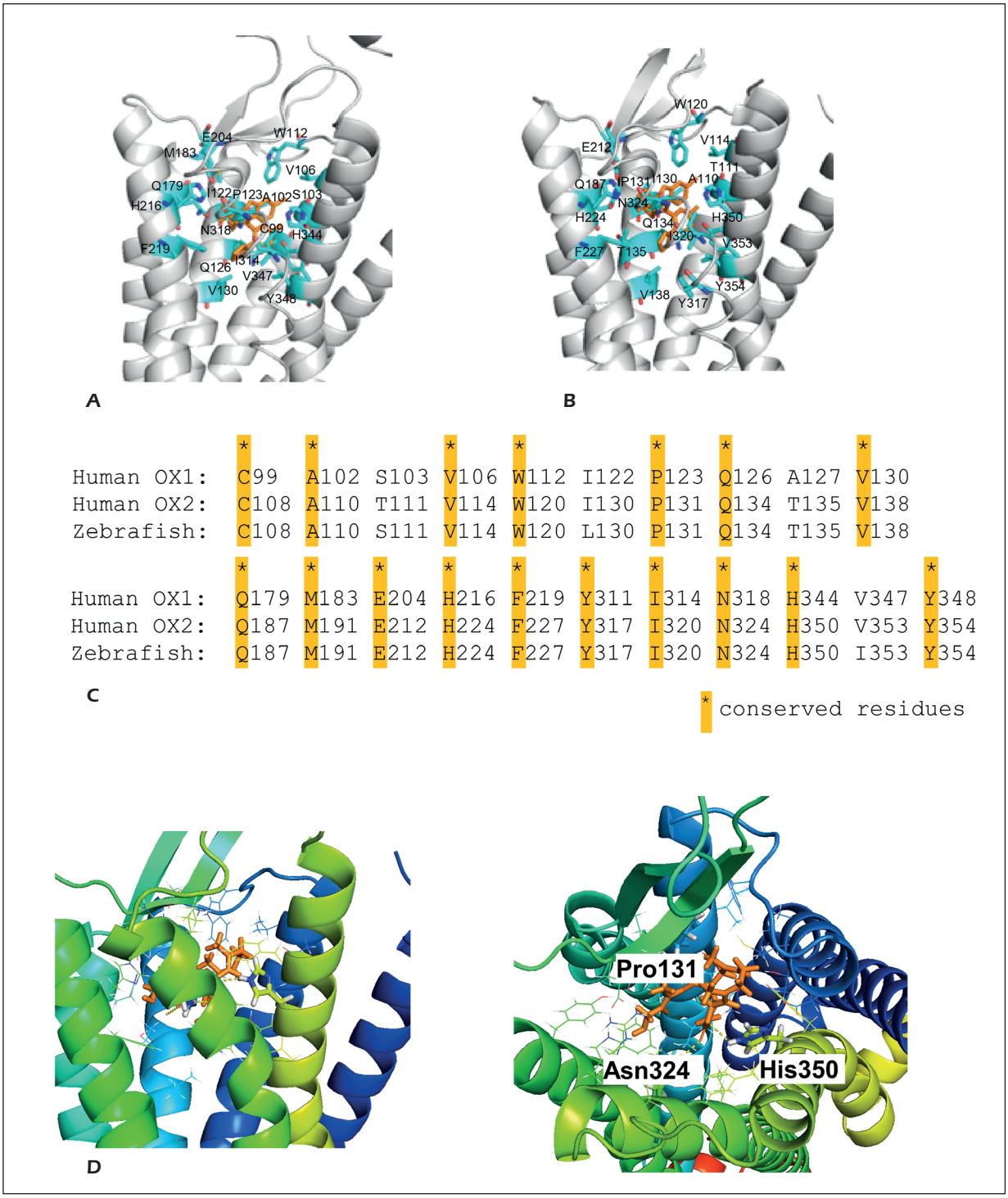

Figure 4. Comparison of the antagonist binding site between human and zebrafish OX receptors. **A**-**B**, Crystal structure of the (**A**) OX1 (PDBID: 4ZJ8) and (**B**) OX2 (PDBID: 4S0V) receptors (grey cartoon) bound to suvorexant (orange sticks). For each structure, 19 residues located within 4 Å from suvorexant are depicted with cyan sticks. **C**, Binding pose between the OX2 receptor and 8β-(4'-Hydroxytigloyloxy) costunolide. Side view (left) and top view (right) from the extracellular side of the OX2 receptor. 8β-(4'-Hydroxytigloyloxy) costunolide shows orange sticks. Yellow broken lines show hydrogen bonds between 8β and Pro131, Asn324, and His350 of the OX2 receptor. These figures were generated using the Pymol software[35]. **D**, Alignment of amino acid residues forming the antagonist binding site in the human and zebrafish OX receptors. Of the 21 residues defined as binding sites in OX1 or OX2 receptors, 17 are conserved among these three receptors.

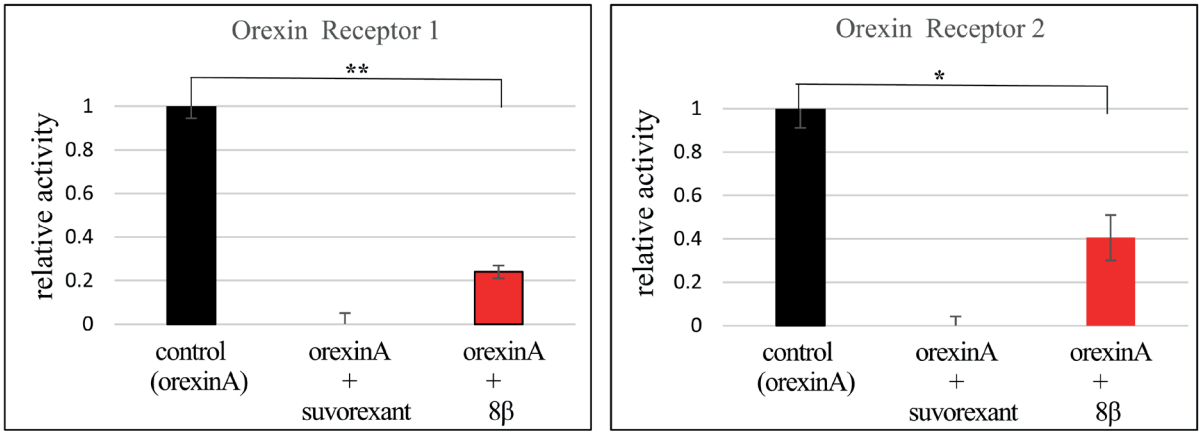

Figure 5. *In vitro* assay suggests 8β-(4'-Hydroxytigloyloxy) costunolide as a dual orexin receptor antagonist. HEK293T (human embryonic kidney 293T) cells were seeded into 12-well plates (50,000 cells/well) and treated with orexin A (50 nM), suvorexant (0.75 μM) plus orexin A (50 nM) or 8β 2.2 μM) plus orexin A (50 nM). Cell supernatants were collected after 24 h and activity of SEAP in culture media was examined. Error bars represent the standard error of mean SE. * < 0.05, ** < 0.01 *vs.* control by the Student's *t*-test.

that 17 out of the 21 amino acids were conserved among the three receptors (Figure 4-c), suggesting similarity in the shape of the binding pockets.

Among the natural compounds used for behavioural analysis, 8β had the highest ranking, in silico. Figure 4-d shows binding pose of the OX2 receptor with 8β. 8β formed hydrogen bonds with Pro131, Asn324, and His 350 of the OX2 receptor. These amino acids were conserved between human and zebrafish OX2 receptors (Figure 4-c). These analyses resulted in the identification of 8β as a candidate for an OX1 and OX2 receptor antagonist.

In vitro Reporter Assay with 8β-(4'-Hydroxytigloyloxy) Costunolide

An *in vitro* reporter assay was conducted to determine whether 8β has an antagonistic activity towards OX1 and OX2 receptors. The antagonistic function of 8β towards orexin A was evaluated on a recombinant expressed OX1 or OX2 receptor in HEK293T. Results of the assay showed that 8β significantly reduced the reporter expression of both OX1 and OX2 receptors, while suvorexant reduced the reporter expression to a much higher degree (Figure 5). This result suggests that 8β has the capacity to bind to OX1 and OX2 receptors.

Microarray Expression Analysis Using 8β-(4'-Hydroxytigloyloxy) Costunolide

To reveal the molecular mechanism of 8β, the gene expression change was analysed by microarray analysis. The brain tissues of larvae at 8 dpf were treated with 8β, imipramine, or suvorexant for 3 h. Through this analysis, 377 genes were identified using 10 μM of 8β, 1328 genes were identified using 100 μM of 8β, 50 genes were identified using 1 μM of suvorexant, and 254 genes were identified using 1 μM of imipramine that were differentially up- or down-regulated compared with the DMSO-treated larvae ($p < 0.05$, fold change ≥ 2). As shown in Figure 6, hierarchical analysis using microarray data indicated that 10 μM of 8β was most closely clustered with suvorexant, and second most closely clustered with imipramine.

Integration of Behavioural Profiling and Microarray Analysis

To determine whether changes in the behaviour pattern are correlated with changes in gene expression, the correlation coefficient between the log2 fold change of gene expression and Z-score of the behaviour profile, when treated with 10 μM of 8β was calculated. We then calculated the percentage of the number of gene symbols (NGS) whose absolute values of correlation coefficient were above 0.85, to determine which behavioural parameters are correlated with changes in gene expression. The total number of gene symbols was 1340. We used the following formula:

$$100 \times \frac{\text{(NGS whose absolute values of correlation coefficient were above 0.85)}}{1340}$$

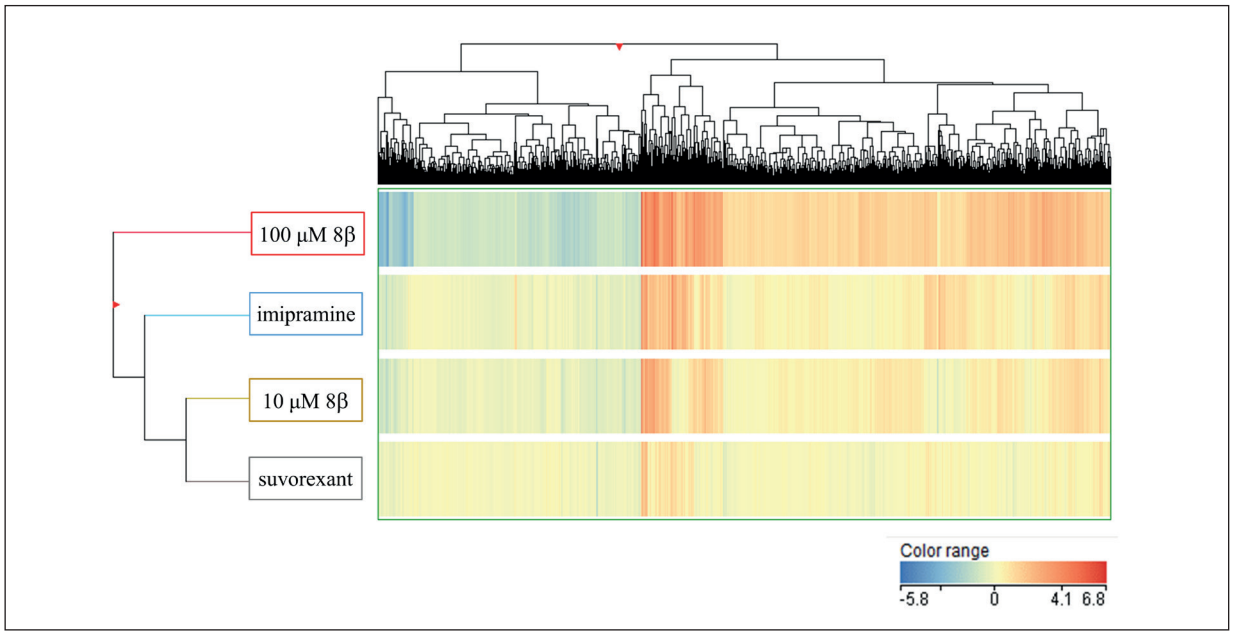

Figure 6. Hierarchical clustering of gene expression profile shows that 10 μM of 8β-(4'-Hydroxytigloyloxy) costunolide was closely clustered with suvorexant. Hierarchical clustering was performed using Ward's method and Chebyshev distance metric.

We found that only two parameters had calculated values above 15%: total distance of movement with low mobility during the dark time (L-DMT D) and turn angle with medium mobility during the dark time (M-TA D). The cases of increase in both the log2 fold-change and the Z-score, or of decrease in both the log2 fold-change and the Z-score, were referred to as a "correlation." Conversely, if the log2 fold change or Z-score showed opposite changes, it was referred to as an "inverse correlation." A clustered correlation analysis showed that L-DMT and M-TA had significant correlation or inverse correlation to gene expression changes (Figure 7). As shown in Figure 2, 8β significantly decreased the DMT scores at low mobility, during the D-time. Therefore, a list of the top 30 probes, showing the most significant positive or inverse correlations in L-DMT, was compiled to construct functional networks, using ingenuity pathway analysis (IPA, Ingenuity Systems, CA, USA). Through this, it was found that NF-κB is a significant hub molecule involved in the gene networks of L-DMT treated with 10 μM of 8β (Figure 8).

Discussion

A novel, natural compound, capable of modulating the sleep/wake behaviour, probably via the orexin receptors, was identified by phenotypic screening, using zebrafish behavioural analysis. The behavioural analysis performed in this study classified the central nervous system drugs based on their mechanism of action. Zebrafish treated with 8β showed behaviour profiles similar to those of zebrafish treated with suvorexant, a known orexin antagonist. In addition, the next best fit with 8β, in behavioural profile, was that of imipramine, indicating that 8β might act on multiple targets. 8β is contained in herbal medicines, from *Eupatorium lindleyanum* and *Inula helenium*. Extracts from these plants have been used in traditional medicines for a long time and are thought to have positive effects on the respiratory and nervous systems.

Through an *in silico* assay, 8β was confirmed to be a novel, high-potency antagonist for orexin receptors. *In silico* assaying is a powerful tool for high-throughput screening in the target-based approach[30]. However, limitations should be considered when interpreting the organism's activity as a whole. We believe that integration of zebrafish phenotypic screening, combined with *in silico* assaying, shows considerable potential, in drug discovery, to utilize the advantages of both methods.

In vitro screening is a well-established technique used in drug discovery. The demonstration of antagonistic function by 8β to OX1 and OX2 receptors was supported by the *in vitro* assay. In

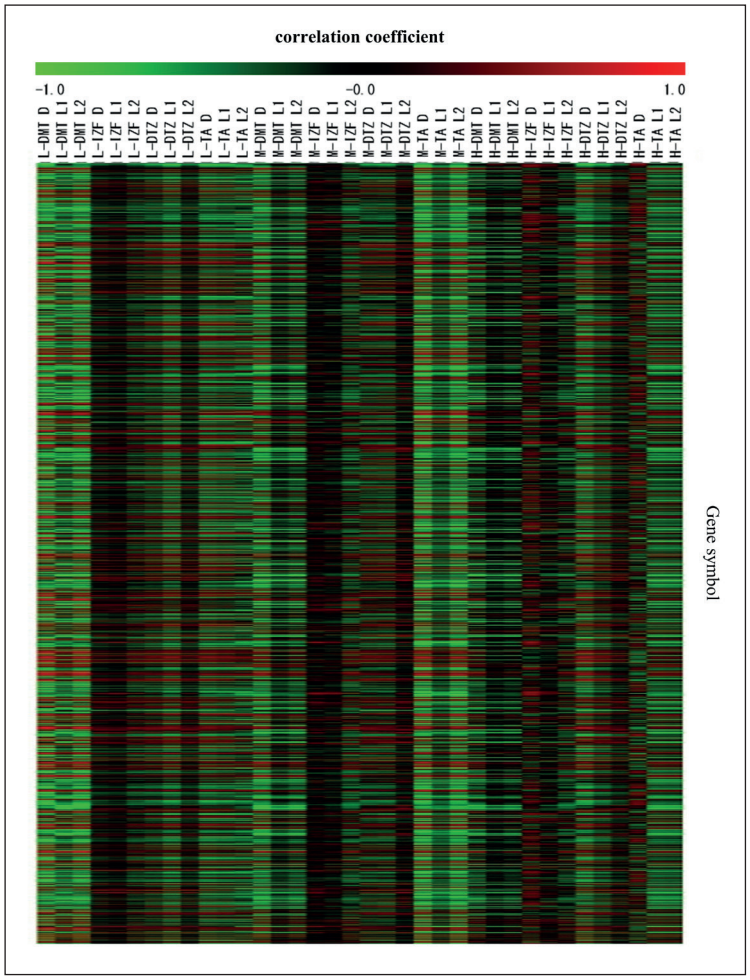

Figure 7. A clustered correlation analysis identifying the behavioural analysis parameters correlated to gene expression changes. Each square of the clustergram represents the correlation coefficient between log2 fold change of gene expression and Z score of behaviour profile.

addition, the gene expression profiles of brain tissue, from 10 μM 8β-treated larvae, showed closest similarity to those treated with suvorexant, and second closest similarity with those treated with imipramine, the same as that described by their behaviour profiles. Although the gene expression profiles by 100 μM 8β-treated larva did not show similarity with those treated with suvorexant, it may be due to nonselective binding to other receptors, at high concentrations.

Zebrafish exhibit various behavioural patterns, which appear to be equivalent to human behaviour, at a functional level. Moreover, drug screening using zebrafish larvae allows high-throughput analysis of behavioural phenotypes, so that large sets of data can be obtained. Data obtained using transcriptome and behavioural analysis were used to identify the molecular mechanism underlying specific types of behaviour phenotypes in zebrafish. Application of this approach to 10 μM 8β-treated larva showed that total distance of movement with low mobility (L-DMT) and turning angle (TA) correlate closely with gene expression changes in brain tissues. Since the exposure to central nervous system depressants such as triazolam and zolpidem causes the reduction of DMT scores and the increase of TA scores in zebrafish[14], the effect of 8β may be due to its molecular mechanism partially relative to the effects of hypnotic drugs. Transcriptome data from 8β-treated larva were all obtained in a narrow time period, just prior to changing from L1-time to D-time, and not at other times. To understand the relationship between the change in gene expression caused by 8β and the behaviour pattern shown during daily light cycle, it would be necessary to collect data sets at multiple time points.

Based on our analysis, NF-κB, a hub molecule, was proposed to be involved in the decrease of DMT scores in the presence of 10 μM of 8β. It has been reported that inflammatory cytokines, such

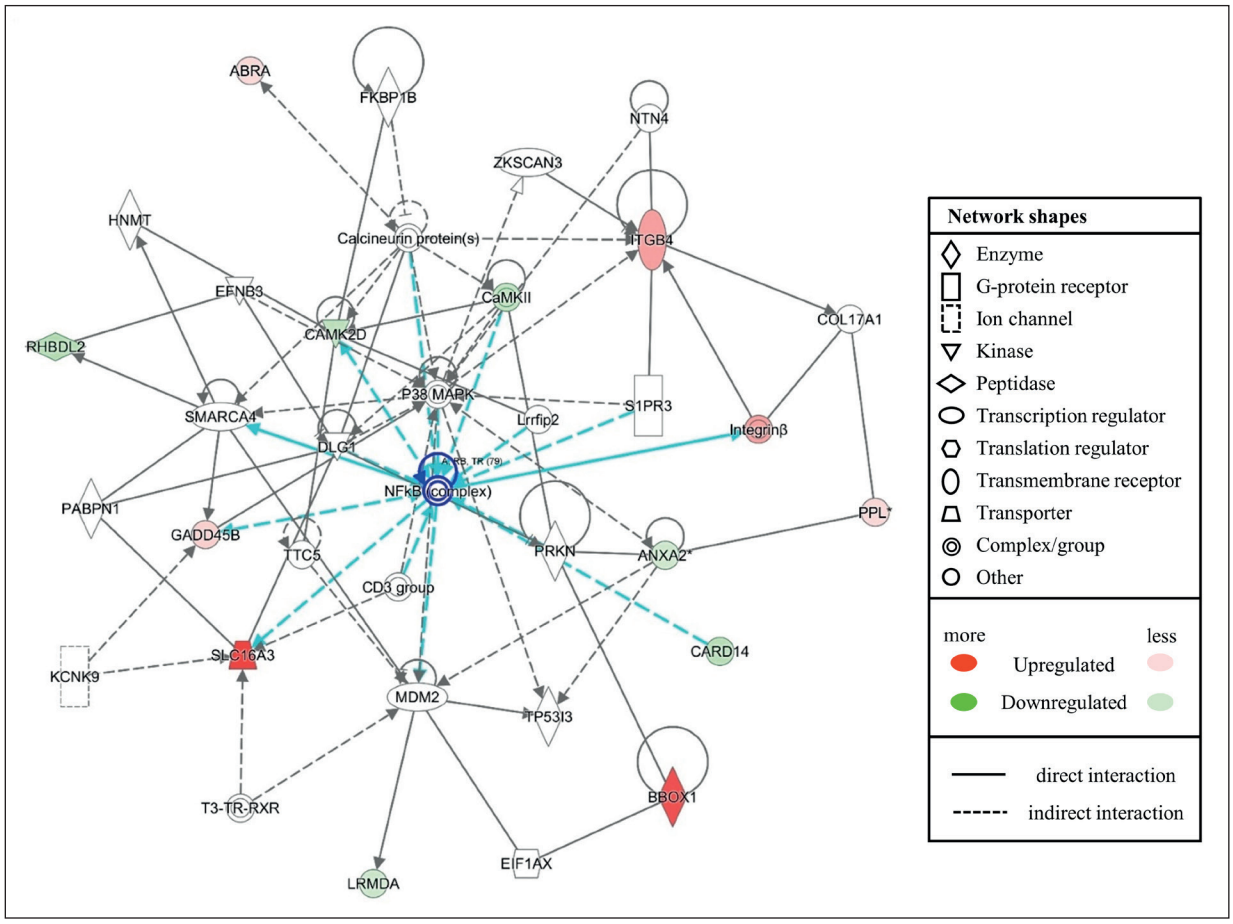

Figure 8. Pathway analysis related to total distance of movement with low mobility (L-DMT) shows that 8β-(4'-Hydroxytigloyloxy) costunolide may be involved in inflammatory immune responses. Direct interaction is in solid line, whereas indirect interaction is indicated by a dotted line. The relationship related to NF-κB is highlighted in light blue. The genes in green were down-regulated and the genes in red were up-regulated. The intensity of the node colour correlates with the degree of gene upregulation. IPA network legend is on the right side.

as tumour necrosis factor (TNF) and interleukin (IL)-1β, can facilitate sleep via the NF-κB pathway[31-33]. In fact, the activity of orexin-expressing neurones is inhibited by TNF[34].

Conclusions

From this study, zebrafish phenotypic screening, which can be combined with an *in silico* assay and gene expression profiling, provides a feasible strategy for discovering and characterizing novel therapeutic compounds, including natural products.

The strategy described here could be applied to identify new compounds and their molecular mechanism, not only for sleep-wake regulation but also for other types of behaviours, such as depression/motivation and learning. To expand this strategy to other behavioural assays, there are several issues that need to be addressed, such as extrapolation of zebrafish data to humans. We expect the strategy described here to be a stepping stone in the development of new compounds for treatment of conditions of the nervous system.

Author Contributions

MY conceived the study, analysed the data, and wrote the manuscript. KM, HI, MA, and YO performed the *in silico* assay. MO and JK performed the experiments. TT conceived the study and wrote the manuscript.

Conflict of Interests

The author(s) declare that they have no conflict of interests.

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