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Abstract

In the present study, fluorophenyl retinoic acid triazole, a retinoic acid derivative having more bioavailability compared to the parent retinoic acid was investigated against cell growth and proliferation. The results indicated that fluorophenyl retinoic acid triazole leads to a significant expression of TRK mRNA in time a dependent manner in NB1643 cells. There were about 10-, 14-, and 21-fold induction of expression after 1, 2, and 3 days of treatment, respectively. The results revealed that TRK and TRKB were induced at 6 and 12 hours after the treatment respectively and the expression of TRKB was maximum at day 3. Fluorophenyl retinoic acid triazole exerts neurite outgrowth activity by inducing TRKB expression, which is a receptor for brain-derived neurotrophic factor and a protein-tyrosine kinase. Fluorophenyl retinoic acid triazole accumulation generates many small vesicles in the cell which assembles at the neck of each small prominence and lead to significant morphological changes after 24 hours of treatment.

Introduction

Binding of neurotrophins to membrane receptors of neurons initiates a cascade of intracellular processes which enhance survival and trophic effects. Neurotrophins are specific in binding to the receptors of tyrosine kinase receptor family (nerve growth factor binds to TrkA, brain-derived neurotrophic factor, brain-derived neurotrophic factor and neurotrophin-4, NT-4 bind to TrkB and neurotrophin-3, NT-3 binds to TrkC receptors with high affinity).

Down-regulation of brain-derived neurotrophic factor in dentate gyrus and hippocampus of chronic stress model of rats indicated potential involvement of it in depression (Smith et al., 1995). Latter brain-derived neurotrophic factor was found to be main agent in the depression and suicidal behaviours (Dwivedi, 2009; Lee and Kim, 2010). The postmortem brain samples from depressed humans (Tripp et al., 2012) and lymphocytes

of depressed patients were found to be deficient of brain-derived neurotrophic factor, brain-derived neurotrophic factor-regulated genes, and the receptor TrkB (Pandey et al., 2010). The brain-derived neurotrophic factor was found to be deficient in serum of MDD patients (Karege et al., 2002; Kim et al., 2007) and the extent of its polymorphism indicated chronicity level of the disease (Lee et al., 2013).

The antidepressant treatments including electroconvulsive therapy and repetitive transcranial magnetic stimulation enhanced the expression of brain-derived neurotrophic factor (Chen et al., 2001; Altar et al., 2003; Müller et al., 2000, Gonul et al., 2005; Lang et al., 2006) and produced antidepressant effect on behavioral models of depression (Siuciak et al., 1996; Shirayama et al., 2002) deficient of TrkB receptor (Saarelainen et al., 2003). All these reports suggest a potential involvement of brain-derived neurotrophic factor in the genesis of MDD.



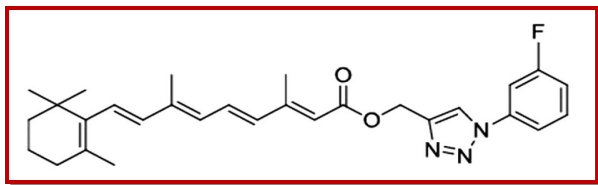


Figure 1: Structure of fluorophenyl retinoic acid triazole

The role of all-trans retinoic acid as an additive for *in vitro* neuronal differentiation of stem cells is well known (Jiang et al., 20012; Nojehdehian et al., 2009; Guan et al., 2001; Xu et al., 2012; Christie et al., 2010). Retinoic acid regulates a cascade of changes during the development of central nervous system which result in neuronal differentiation (Maden, 2007; Maden, 2002). Neurodegenerative diseases like Parkinson's disease (Krezel et al., 1998), schizophrenia (Goodman, 1998), motor neuron diseases (Corcoran et al., 2002), and even the decline of mental abilities with age also involve deficiency of retinoids and retinoid signalling loss. Development of neurons and maintenance of synaptic signal transmission also involve a potential role of brain-derived neurotrophic factor (Huang et al., 2001; Waterhouse and Xu, 2009; Cohen-Cory, 2010). It is reported that *in vitro* brain-derived neurotrophic factor supplementation mediates differentiation in NSC (Ahmed et al., 1995). It also mediates survival and maturation of neurons (Vutskits et al., 2001; Kirschenbaum et al., 1995). However, *in vivo* administration of brain-derived neurotrophic factor leads to differentiation of NSCs into new neurons (Pencea et al., 2001; Zigova et al., 1998). In the present study, we studied the role of fluorophenyl retinoic acid triazole (Figure 1) in differentiation and proliferation of NB1643 cells for therapeutic purposes.

Materials and Methods

Reagents and cell culture

Fluorophenyl retinoic acid triazole (Sigma) stocks were prepared by dissolving it in ethanol. Human brain-derived neurotrophic factor and NGF were purchased from Santa Cruz Biotechnology, USA. NB1643 cells were grown in 10% FBS/RPMI (RPMI 1640 medium containing 2 mM glutamine (BioWhittaker) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and 50 units/mL penicillin, 50 mg/mL streptomycin) (Life Technologies, Inc.) in 5% CO₂ atmosphere at 37°C with tissue culture plates from Costar. For counting and splitting, cells were trypsinized in trypsin versene mixture (BioWhittaker). Nuclei were prepared using the method of Butler (Butler, 1984) and counted using a Z2 counter equipped with a 256 channelizer (Coulter) for both cell plating and for growth and survival assays. The tissue culture plates were coated with poly-lysine by incubating plates for 14 hours with 1 mL of 50 mg/mL poly-lysine in H₂O. The plates after

washing with H₂O were air dried under UV light, and subsequently cross-linked using UV light supplied by a Stratlinker (Stratagene).

Differentiation of NB1643 cells

2.5 × 10⁵ NB1643 cells were plated per well in 6-well tissue culture dishes with or without 5 μM fluorophenyl retinoic acid triazole in 10% FBS/RPMI medium. The concentration of ethanol was 0.1% in all wells. Photographs at a magnification of x225 were taken at day 3 following the treatment.

Cell viability assays

Inmedium (10% FBS/RPMI) containing 5 μM fluorophenyl retinoic acid triazole on poly-lysine-coated 96-well plates NB1643 cells at a density of 2.5 × 10⁴ cells per well were distributed. After 24 hours of fluorophenyl retinoic acid triazole treatment, the fresh medium containing brain-derived neurotrophic factor (50 ng/mL) and varying concentrations of FBS (0, 0.1, 0.2, and 0.5%) and 5 μM fluorophenyl retinoic acid triazole was added. After 24 hours, wells were aspirated and 1 μM Calcein AM (Molecular Probes) in Hanks' solution (Life Technologies, Inc.) was added for 50 min at 37°C. Quantization of fluorescence using a cytofluor fluorescent plate reader (Millipore) was performed. A linear fluorescent signal with respect to viable cell number was obtained. To establish linearity of detection, 5, 10, 25, 50, and 75 × 10³ cells were plated in 96-well plates and cultured overnight. Quadruplicate wells were aspirated, and 1 μM Calcein AM (Molecular Probes) in Hanks' solution (Life Technologies, Inc.) for 1 hour at 37°C was added for Calcein AM detection. Quantitated of fluorescence using a cytofluorescent plate reader was done. Triplicate wells were trypsinized, and nuclei were prepared and counted for direct assessment of cell number in 3.5-cm tissue culture wells (Costar) coated with poly-lysine with 5 μM fluorophenyl retinoic acid triazole in 10% FBS/RPMI. Final ethanol concentration was 0.1% in all wells. Medium was changed 24 hours later to medium with or without 50 ng/mL BDNF and 5 μM fluorophenyl retinoic acid triazole in 0.1% FBS/RPMI. Triplicate wells were trypsinized, and nuclei were counted as described above at various times subsequent to brain-derived neurotrophic factor addition.

Growth assays

In 6-well tissue culture dishes (3.5 cm diameter wells) were plated 5 × 10⁵ NB1643 cells per well. After 12 hours medium was changed to medium with or without 5 μM fluorophenyl retinoic acid triazole. 2.5 × 10⁵ NB1643 cells were plated per well in 6-well tissue culture dishes. For the brain-derived neurotrophic factor growth rate experiments, 2.5 × 10⁵ NB1643 cells were plated per well in 6-well tissue culture dishes (3.5 cm diameter wells) (Costar). Medium was changed 14 hours later to medium with or without 5 μM FPRT.

After 24 hours, brain-derived neurotrophic factor was added (100 ng/mL). Final ethanol concentration was 0.1% in all wells. Triplicate wells were harvested by trypsinization and nuclei were counted as described above. Linear regression and statistical analysis to determine if the dependence of growth rate (slopes) of NB1643 cells differs significantly were performed using the method described by Zar.

Immunoblotting analysis of TRK and TRKB

The fluorophenyl retinoic acid triazole induced expression of TRK and TRKB was analysed by plating 4×10^6 NB1643 cells in dishes followed by treatment with $5 \mu\text{M}$ fluorophenyl retinoic acid triazole after 2 days or no treatment. Cell lysis in each dish was achieved in 1 mL of RIPA with fresh $1 \mu\text{M}$ phenylmethylsulfonyl fluoride at 4°C . The lysates were cleared by centrifugation at $10,000 \times g$ for 45 min. The supernatants were incubated with 10 mL of TRK C-14 antisera (Santa Cruz) followed by incubation on rotation with 20 mL of protein A-Sepharose (Repligen) at 4°C . The immune complexes were again suspended in 1 mL of RIPA, vortexed, and pelleted in a microcentrifuge. After 3-washes, RIPA buffer was removed. The samples were subjected to electrophoresis in 30 mL of 23 SDS-polyacrylamide. The gels were transferred to immobilon-P (Millipore) using the milliblot semidry apparatus (Millipore). Detection of TRK and TRKB was carried out by using immunoblot analysis. The procedure involves membrane blockage for 1 hour in 5% milk in TBST (10 μM Tris-HCl, pH 8.0, 150 μM NaCl, 0.05% Tween-20). Washing of blot first for 20 min and then 10 min twice was followed by incubation with TRK C-14 (1:200) in 1% milk/1% bovine serum albumin in TBST for 1 hour. Washing membrane as above was followed by incubation with anti-rabbit horse radish peroxidase in 1% milk/1% bovine serum albumin in TBST for 1 hour. The membrane was washed for 20 min and then four times for 10 min in TBST. Visualization of TRK and TRKB bands was achieved using chemiluminescent detection with the ECL kit (Amersham Pharmacia Biotech) and XAR 5 film (Eastman Kodak Co.). For analysis of TRK- or TRKB-specific expression, 2×10^6 NB1643 cells were plated with either 0 or $5 \mu\text{M}$ fluorophenyl retinoic acid triazole for 4 days. TRK was immunoprecipitated using 1 mg of TRK-specific antibodies (catalog No. 9142, New England Biolabs) followed by immunoblotting with TRK C-14 antisera using the methods described above. For TRKB expression, TRKBs were immunoprecipitated with TRK C-14 antisera followed by immunoblotting with TRKB-specific antiserum 5050 (1:400) and TRKB antibodies (1:200) (Santa Cruz).

Results

The results demonstrated that fluorophenyl retinoic

acid triazole induces significant expression of TRK mRNA in time dependent manner in NB1643 cells (Figure 2A). There were about 10-, 14-, and 21-fold induction of expression after 1, 2, and 3 days of fluorophenyl retinoic acid triazole treatment, respectively (Figure 2B). We used antibodies against the C-terminal 14 amino acids of TRK to determine the expression time interval of TRK and TRKB (Figure 2C) on fluorophenyl retinoic acid triazole treatment. The results revealed that TRK and TRKB were induced at 6 and 12 hour after fluorophenyl retinoic acid triazole treatment respectively and the expression of TRKB was maximum at day 3. The expression of FPRT induced-TRK was further confirmed by immunoprecipitation with a TRK-specific antibody and then immunoblotting with the C-14 TRK antibodies (Figure 2D). The confirmation of fluorophenyl retinoic acid triazole induced-TRKB was performed by immunoprecipitation with a TRK C-14 antibodies followed by immunoblotting with TRKB-specific antibodies (Figure 2E). Therefore, fluorophenyl retinoic acid triazole induces TRKB expression, which is a receptor for brain-derived neurotrophic factor and a protein-tyrosine kinase. It seemed likely that NB cell differentiation may be comparable to NGF-induced differentiation of rat PC12 cells, because the origins of these cell lines are similar.

We observed a decrease in brain-derived neurotrophic factor mRNA levels in NB1643 cells on fluorophenyl retinoic acid triazole treatment (Figure 3). The results demonstrated a correlation between intervals of expression of TRKB with the time duration of phenotypic differentiation. This supports the notion that differentiation may be the result of an autocrine mechanism involving brain-derived neurotrophic factor and TRKB.

Our results showed that fluorophenyl retinoic acid triazole significantly promote NGF (10 ng/mL)-mediated neurite outgrowth (Figure 4).

In order to elucidate the mechanism for the neurite outgrowth activity of fluorophenyl retinoic acid triazole, the NB1643 cells were incubated with fluorophenyl retinoic acid triazole (15 $\mu\text{g}/\text{mL}$) in the presence of NGF (10 ng/mL) (Figure 5). We observed no alteration in cell morphology during the initial 6 hours period. Fluorophenyl retinoic acid triazole molecule dispersed uniformly into the cell to generate many small vesicles (Figure 5, left picture). After 12 hours, short prominences appeared at the outer edge of the cell. Intriguingly, the fluorescent vesicles assembled at the neck of each small prominence (Figure 5, middle picture). Significant morphological changes were observed after 24 hours. Elongation of the prominences, where the fluorophenyl retinoic acid triazole molecules accumulated, became particularly noticeable (Figure 5, right picture). These results show the fluorophenyl retinoic acid triazole accumulate in the prominences.

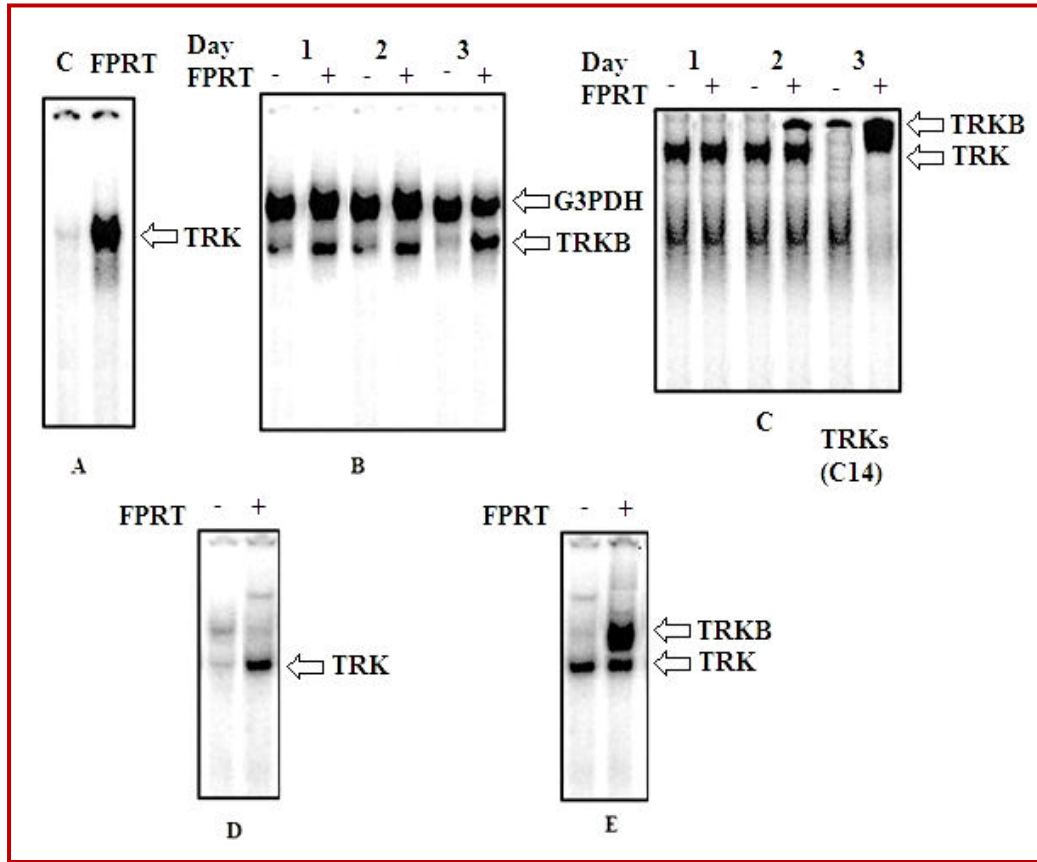


Figure 2: ATRA induces expression of *TRK* mRNA

A. Northern analysis on 20 mg of total RNA isolated from NB1643 cell lines grown in 0 or 5 mM FPRT for 3 days. B. Total RNA was isolated from NB1643 cells grown in 0 or 5 mM ATRA for 1, 2, and 3 days. B. RT-PCR analysis for expression of *TRKB* and *G3PDH* are shown. Arrows indicate the predicted 371-bp *TRKB* and 983-bp *G3PDH* PCR products. C. Immunoprecipitation of TRKs followed by immunoblotting analysis is depicted at 1, 2, and 3 days after ATRA treatment. D. TRK was immunoprecipitated with TRK-specific antiserum followed by immunoblotting with TRK C-14 antiserum. E. TRKs were immunoprecipitated with C-14 TRK antiserum followed by immunoblotting using TRKB-specific antibodies (794 and 5050) and generic TRK C-14 antibodies

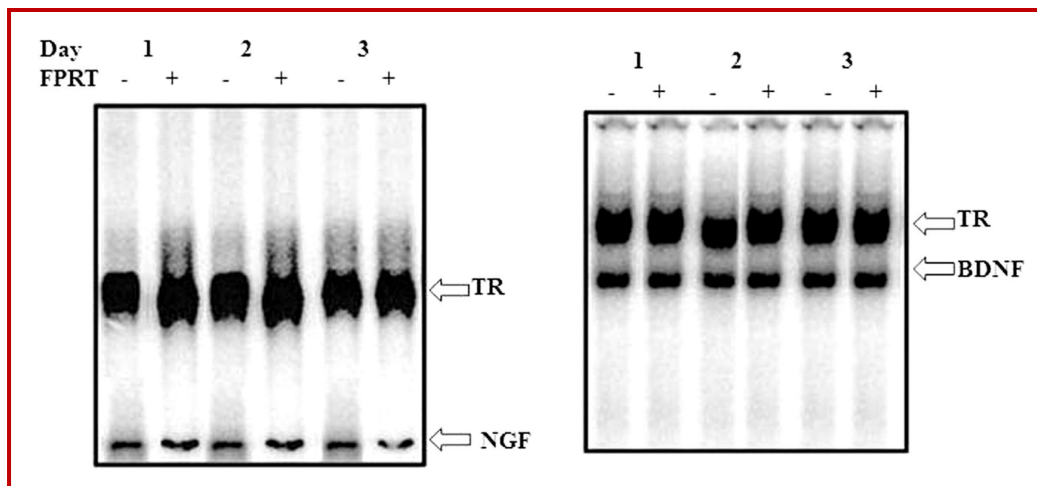


Figure 3: FPRT induces expression of TRKB, and BDNF and NGF in NB1643 cells

Total RNA was isolated from NB1643 cells grown in 0 or 5 mM FPRT for 1, 2, and 3 days. RT-PCR analysis for expression of BDNF, NGF, and *G3PDH* are shown. NB1643 cells were treated with 0 or 5 mM ATRA. Arrows indicate the predicted 492-bp BDNF, 432-bp NGF, and 1347-bp transferrin receptor PCR products. ³²P-Labeled PCR products were analyzed using a PhosphorImager

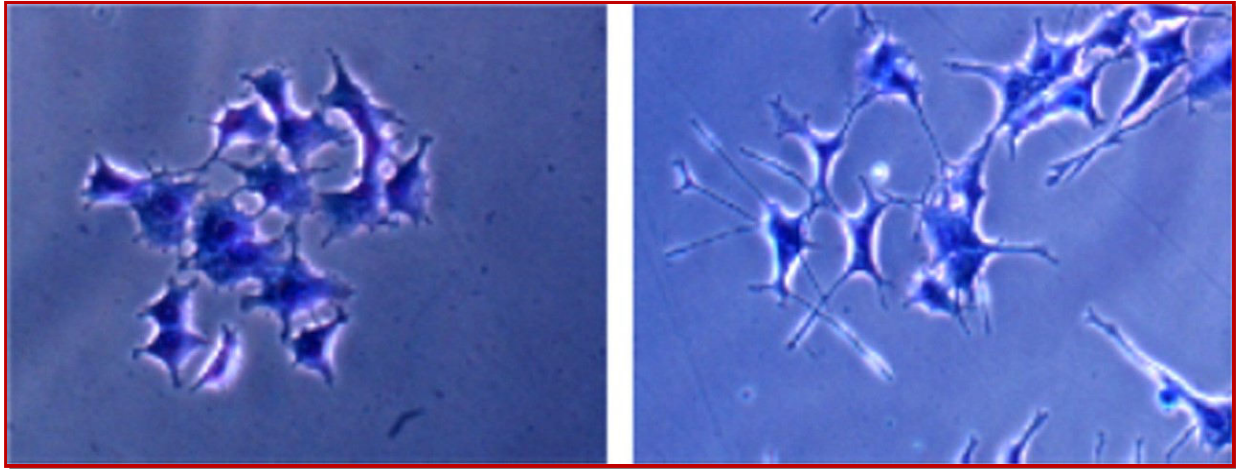


Figure 4: Result of the neurite outgrowth activity assay for FPRT

NB1643 cells cultured in DMEM/2% HS + 1% FBS and treated for 96 hours, Left picture: NB1643 cells under 10 ng/mL of NGF as control. No remarkable morphological change was observed. Right picture: significant neurite growth was observed in the presence of 15 μ g FPRT and 10 ng/mL of NGF

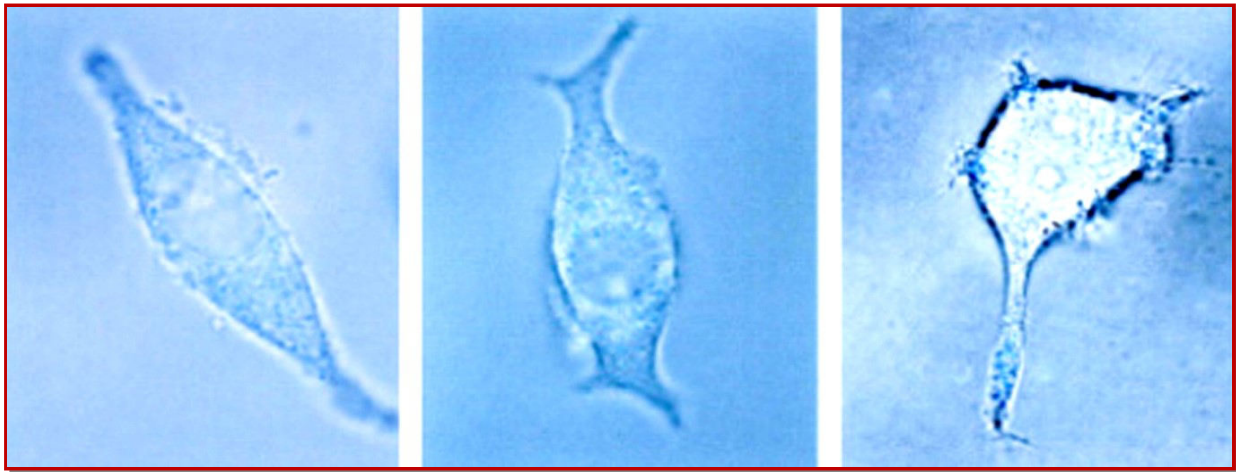


Figure 5: An analysis of the localization of FPRT inside a NB1643 cell

Left picture: NB1643 cell after 6 hours of the culture with 15 μ g FPRT and 10 ng/mL NGF. FPRT was identified as small-blue particles. Middle picture: NB1643 cell after 12 hours. Right picture: NB1643 cell after 24 hours. Note that the FPRT (blue particles) has accumulated inside each prominence

Discussion

The results indicated that fluorophenyl retinoic acid triazole induces expression of TRK, brain-derived neurotrophic factor, NGF and causes an increase in neurite outgrowth in NB1643 cells.

Fluorophenyl retinoic acid triazole treatment led to a significant expression of TRK mRNA in time dependent manner in NB1643 cells. There were about 10-, 14-, and 21-fold induction of expression after 1, 2, and 3 days of fluorophenyl retinoic acid triazole treatment, respectively. The results revealed that TRK and TRKB were induced at 6 and 12 hours after fluorophenyl retinoic acid triazole treatment respectively and the expression of TRKB was maximum at day 3. Therefore, fluorophenyl retinoic acid triazole induces TRKB expression, which is a receptor for brain-derived neurotrophic factor and a protein-tyrosine kinase. There was a

decrease in brain-derived neurotrophic factor mRNA level in NB1643 cells on fluorophenyl retinoic acid triazole treatment. Our results showed that fluorophenyl retinoic acid triazole significantly promote NGF (20 ng/mL)-mediated neurite outgrowth. In order to elucidate the mechanism for the neurite outgrowth activity of fluorophenyl retinoic acid triazole, the NB1643 cells were incubated with fluorophenyl retinoic acid triazole (20 μ g/mL) in the presence of NGF (10 ng/mL). Initially no alteration in cell morphology was observed for 6 hours period. During this period, fluorophenyl retinoic acid triazole molecule dispersed uniformly into the cell to generate many small vesicles. After 24 hours, short prominences appeared at the outer edge of the cell. Fluorophenyl retinoic acid triazole molecules then assembled at the neck of each small prominence which caused significant morphological changes after 24 hours.

The results of the study demonstrate that fluorophenyl retinoic acid triazole has a potent neurite outgrowth activity. Fluorophenyl retinoic acid triazole exerts neurite outgrowth activity by inducing TRKB expression, which is a receptor for brain-derived neurotrophic factor and a protein-tyrosine kinase. Fluorophenyl retinoic acid triazole accumulation generates many small vesicles in the cell which assembles at the neck of each small prominence and lead to significant morphological changes after 24 hours.

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