

Am80 induces neuronal differentiation in a human neuroblastoma NH-12 cell line

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Received April 19, 2010; Accepted June 4, 2010

DOI: 10.3892/ijmm_00000478

Abstract. Retinoids including natural vitamin A, its derivatives and synthetic compounds work as transcription factors through the retinoic acid receptors (RAR, RXR). All-trans retinoic acid (ATRA), a family of retinoids, is an internal ligand of RAR and well known as a useful differentiation inducer to treat acute promyelocytic leukemia (APL). ATRA therapy is now established as an initial treatment for APL. Recently, to improve therapeutic potency and reduce adverse effects of ATRA, a novel synthetic selective agonist for RAR α and β , Am80, was developed and applied to APL treatment. In this study, we tested whether Am80 was capable of inducing neuronal differentiation in a human neuroblastoma cell line, NH-12 and compared the differentiation effects between Am80 and ATRA. Morphological studies demonstrated that Am80 induced more potent neurite outgrowth and also proved lesser cell toxicity than ATRA. Am80 up-regulated the expression of tropomyosin-related kinase B as well as ATRA. Moreover, Am80 increased the expression of the neuronal marker, growth-associated protein 43. These findings suggest that Am80 induces neuronal differentiation to a greater extent than ATRA and thus may help establishing therapeutic strategies against neuronal degenerative disorders such as Parkinson's disease.

Introduction

All-trans retinoic acid (ATRA), a natural derivative of vitamin A, is known as a differentiation inducer and has

been utilized for the initial therapy to successfully treat acute promyelocytic leukemia (APL) by itself or in combination with other chemotherapies (1-3). A number of synthetic retinoids have been developed to improve its therapeutic potency. Among them, Am80 (Tamibarotene), which is a retinoic acid receptor α (RAR α)- and RAR β -specific synthetic agonist, exhibits greater potency than ATRA in differentiation assays of tumor cells *in vitro* (4,5) and a clinical trial (6).

ATRA-induced cell differentiation effect is dependent on the RARs and retinoid X receptor (RXR) pathways (7). RARs and RXRs are members of the nuclear hormone receptor superfamily acting as transcription factors and can regulate gene transcription associated with neuronal differentiation (8-10). Therefore, ATRA is commonly used to induce neuronal differentiation (11-14). RAR β pathway has been shown to be involved in neuronal differentiation (15,16). More specifically, ATRA has been shown to induce the expression of tropomyosin-related kinase B (TrkB) and to increase sensitivity against brain-derived neurotrophic factor (BDNF) as well as neuronal markers such as growth-associated protein-43 (GAP43) (17-19). In this study, we investigated whether Am80 could induce neuronal differentiation in NH-12 human neuroblastoma cells. We also compared the effect of Am80 with that of ATRA in morphological and biochemical cell differentiation analyses.

Materials and methods

Cell lines and cell culture. Human neuroblastoma cell line, NH-12, was kindly provided by the Cell Resource Center for Biomedical Research Institute of Development Aging and Cancer in Tohoku University (20). NH-12 was cultured in RPMI-1640 supplemented with non-essential amino acid (100 nM), sodium pyruvate (1 mM) and 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO₂ atmosphere.

Reagents. ATRA was obtained from Sigma (St. Louis, MO, USA). Am80, AC55649 and human recombinant BDNF were purchased from Wako (Osaka, Japan). ATRA and Am80 were dissolved in ethanol at 30 and 100 mM, respectively, and AC55649 was dissolved in dimethyl sulfoxide at 100 mM. These solutions were stored at -80°C until further use.

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Abbreviations: APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; BDNF, brain-derived neurotrophic factor; GAP, growth-associated protein; RAR, retinoic acid receptors; RXR, retinoid X receptor; TrkB, tropomyosin-related kinase B

Key words: all-trans retinoic acid, Am80, tropomyosin-related kinase B, brain-derived neurotrophic factor, retinoic acid receptor

Table I. Primers for RT-PCR.

Gene	Primers sequence	Product size (bp)	Annealing	Cycles
TrkA (26)	Forward: TCTTCACTGAGTTCCTGGAG Reverse: TTCTCCACCGGGTCTCCAGA	247	59°C (30 sec)	40
TrkB (26)	Forward: AGTCCAGACACTCAGGATTTGTAC Reverse: CTCCGTGTGATTGGTAACATG	522	53°C (30 sec)	40
TrkC (26)	Forward: CATCCATGTGGAATACTACC Reverse: TGGGTCACAGTGATAGGAGG	228/204	51°C (30 sec)	40
GAP43 (19)	Forward: CTGTCCTTTCCCACCCACTA Reverse: GAACGGAACATTGCACACAC	267	60°C (1 min)	30
GAPDH	Forward: CCATGGAGAAGGCTGGGG Reverse: CAAAGTTGTCATGGATGACC	195	55°C (1 min)	28

Analysis of cell morphology and neurite outgrowth. NH-12 cells were seeded at 2×10^4 cells/well in 24-well plates (Beckton-Dickinson, Bedford, MA, USA) and cultured for 24 h. The culture medium was subsequently exchanged for fresh medium with or without reagents. The cultured cells were observed under phase-contrast microscopy and photographs were taken at each experimental time-point. Neurite length was analyzed by WinRooF software (Mitani, Fukui, Japan).

RT-PCR analysis. Cells (2×10^4 cells/well in 24-well plates) were treated with each reagent for 3 or 5 days. Total RNA was extracted using TRIzol reagent (Invitrogen) and was quantified by UV absorbance spectroscopy. Reverse transcription was performed using the SuperScript First-Strand Synthesis System (Invitrogen), according to manufacturer's instructions. PCR amplification was performed in 25 μ l reactions (1X Gotaq Flexi Buffer with $MgCl_2$ containing 50 ng cDNA, 0.1 μ M of each primer, 400 μ M deoxynucleotide triphosphates and 1.25 U Gotaq polymerase) (Promega, Madison, WI, USA). $MgCl_2$ concentrations were adjusted to 5 mM (PCRs for TrkA, B, C, GAP43 and GAPDH) according to manufacturer's protocols. The primer pair sequences and other parameters are listed in Table I. The PCR products were electrophoresed in a 1.5%-agarose gel and were stained with ethidium bromide. Product amounts were quantified with an image analyzer (Fluorchem IS8900, AlphaInotech, San Leandro, CA, USA).

Western blotting. Cells (1×10^6 cells/well in a 24-well plate) were treated with each reagent for 3, 5 or 7 days, and lysed in lysis buffer [for $RAR\beta_2$, 20 mM Tris-HCl pH 7.6, 137 mM NaCl, 1% SDS and 1 mM EDTA containing Protease Inhibitor Cocktail (Sigma); for TrkB, 20 mM Tris-HCl pH 7.4, 25 mM Mega-9, 2 mM EDTA, 150 mM NaCl, 5 mM $MgCl_2$, 25 mM KCl, 0.5% NP40 and 0.05 % NaN_3 containing Protease Inhibitor Cocktail]. A total of 40 μ g protein from each sample was electrophoresed by SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA,

USA). The membranes were incubated with 2% ECL Advance blocking agent (Amersham, Buckinghamshire, UK) in TBS-T buffer (20 mM Tris-HCl pH 7.6, 137 mM NaCl, and 0.1% Tween-20) for 1 h at room temperature. The membranes were incubated for 1 h with primary antibodies: anti- $RAR\beta_2$ (1:10000; GeneTex, San Antonio, TX, USA), anti-TrkB (1:2000; Neuromics, Edina, MN, USA) and anti- β -actin (1:25000; Sigma). After washing the membranes, they were incubated for 1 h with secondary antibody (anti-rabbit or anti-mouse HRP-conjugated IgG antibody, 1:25000; Jackson ImmunoResearch, West Grove, PA, USA). Labeled proteins were detected using the ECL advance Western blotting detection kit (Amersham).

Statistical analysis. Statistical significance was determined using one-way ANOVA followed by Dunnett's, Tukey-Kramer and Student's t-test with StatView 5.0 software (Abacus Concept, Berkeley, CA, USA). A value of $p < 0.05$ was considered statistically significant.

Results

Morphological differentiation. As shown in Fig. 1A, when NH-12 cells were treated in culture with 10 μ M ATRA or Am80 for 7 days, the neurite outgrowth as a morphological differentiation was seen (Fig. 1A). In terms of neurite length ratio, ATRA treatment induced 2.12- (3 days), 2.51- (5 days) and 2.71-fold (7 days) extension, and Am80 induced 2.81- (3 days), 3.06- (5 days) and 3.48-fold (7 days) extension compared with controls ($p < 0.01$; Fig. 1B). Both reagents enhanced neurite outgrowth in a dose-dependent manner within the range of 0.1-20 μ M (Fig. 2A). Although a high concentration of ATRA (50 μ M) had cytotoxicity, resulting with decreased outgrowth and cell viability, but not Am80 (Fig. 2 and data not shown).

Expression of neuronal differentiation marker. GAP43, one of the critical molecules for establishing neurite outgrowth during neuronal differentiation (14,19), was subsequently

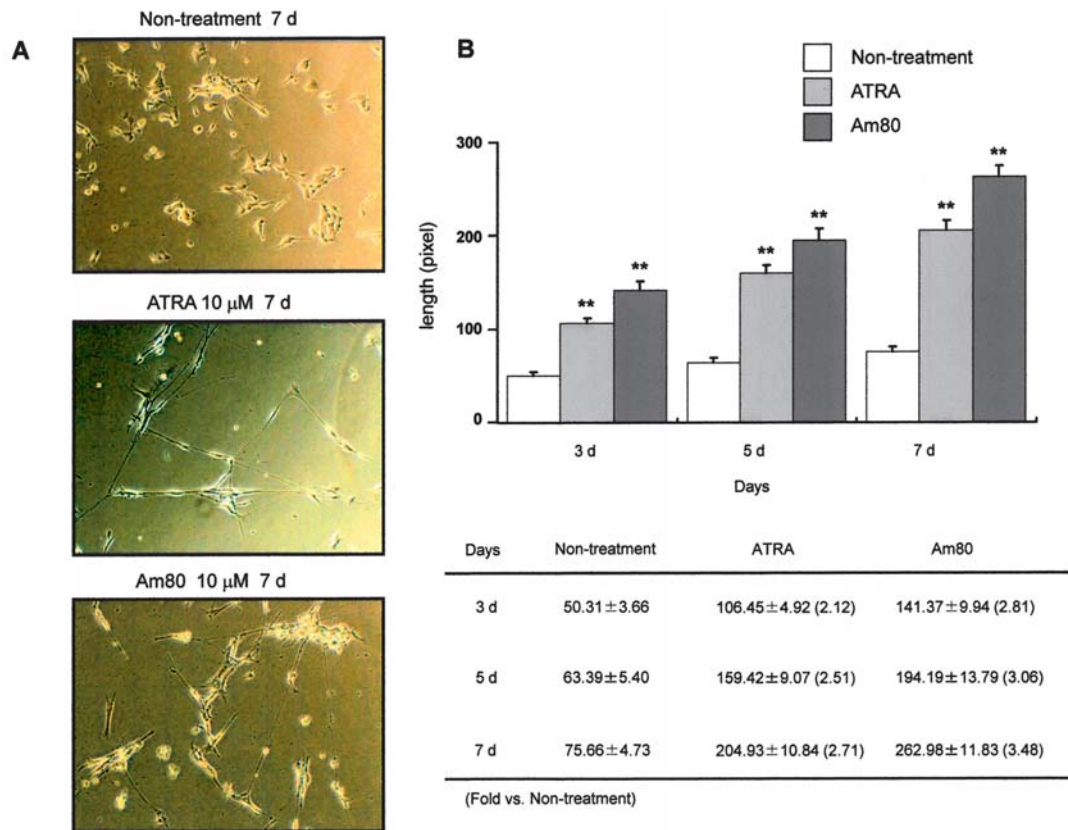


Figure 1. Differential effects of ATRA and Am80 in NH-12 cells. (A) Morphological changes in NH-12 cells. Cells were treated with 10 μ M of each reagent for 7 days. (B) Effects on neurite outgrowth. Cells were treated with 10 μ M of each reagent for the indicated periods. Data are shown as mean \pm SEM (n=60). **p<0.01, significantly different from control (non-treatment).

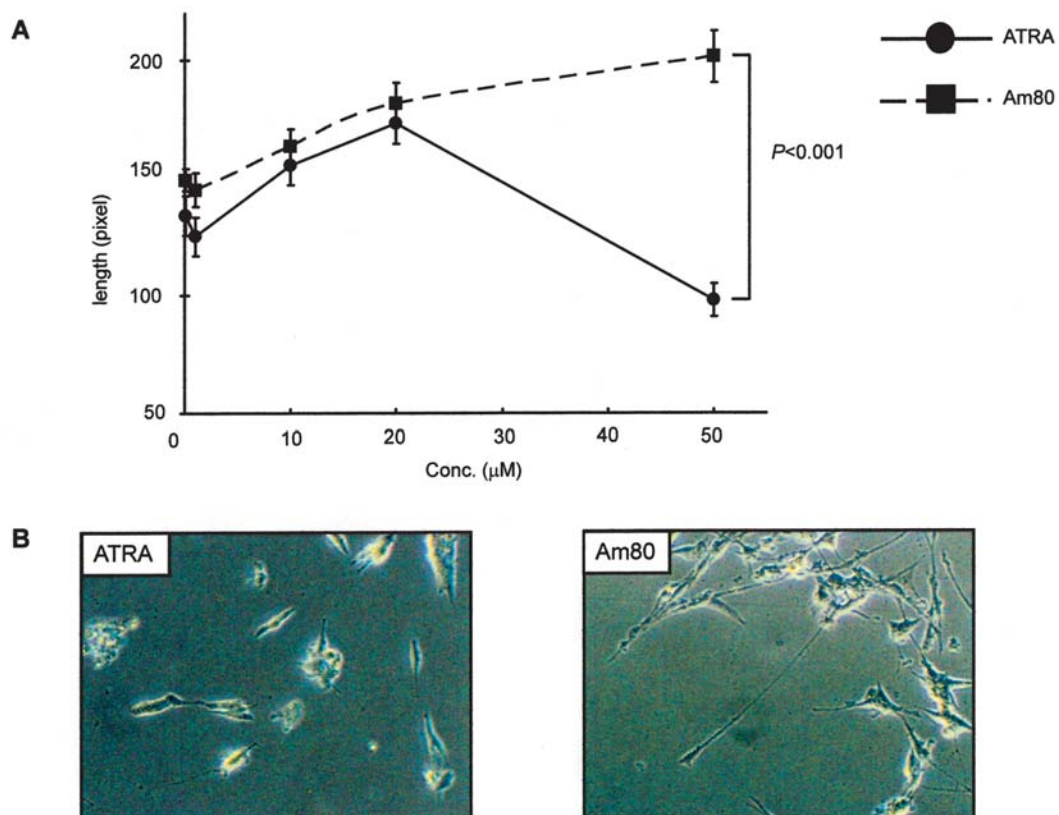


Figure 2. Effects of ATRA and Am80 on neurite outgrowth at various concentrations. (A) Cells were treated with ATRA or Am80 at the concentrations of 50, 20, 10 and 1 μ M, and 100 nM for 7 days. Data are shown as mean \pm SEM (n=60). (B) Morphological changes at 50 μ M.

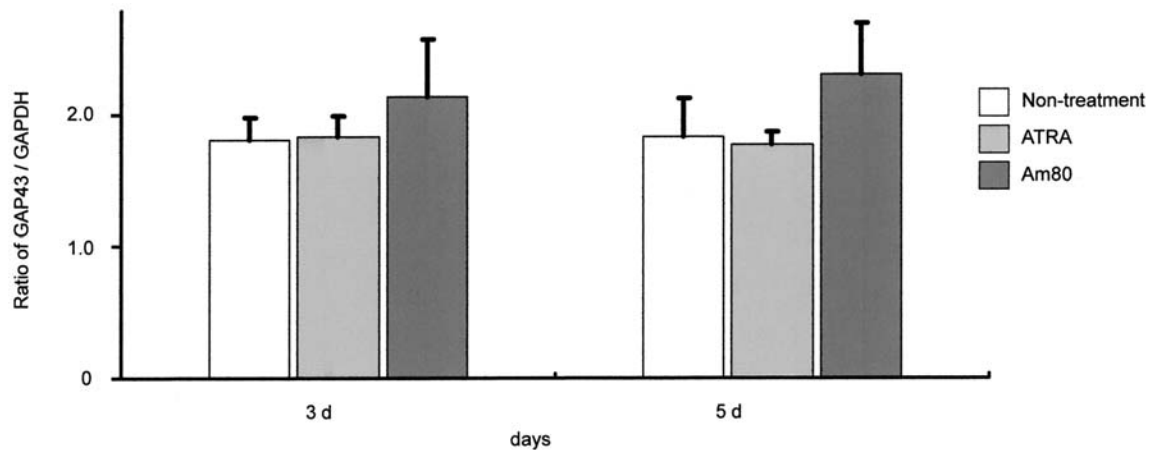


Figure 3. Effects of ATRA and Am80 on GAP43 mRNA expression. GAP43 mRNA expression was measured by RT-PCR. Cells were treated with 10 μ M of ATRA and Am80 for the indicated days. Data were calculated as the ratio of GAP43/GAPDH (mean \pm SEM, n=3).

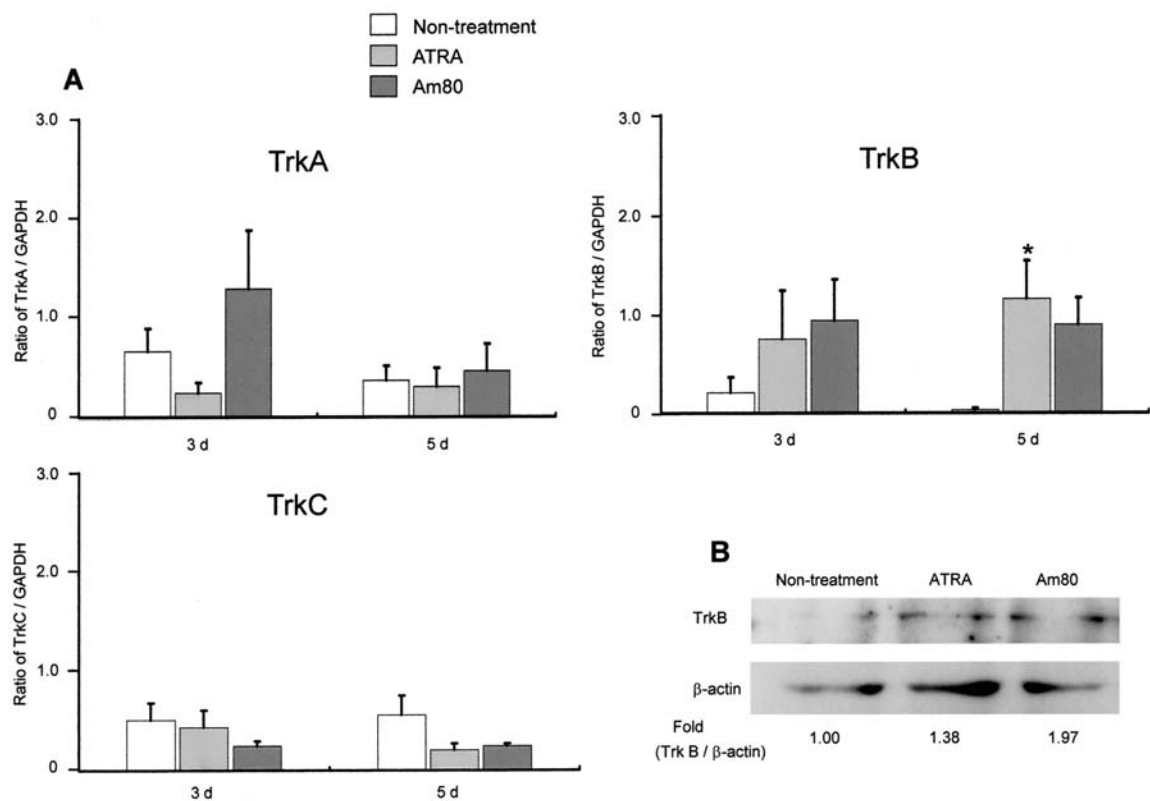


Figure 4. Effects of ATRA and Am80 on Trk family expression. (A) Trk family mRNA expression was measured by RT-PCR. Cells were treated with 10 μ M of ATRA and Am80 for the indicated days. Data were calculated as the ratio of TrkA, B or C/GAPDH (mean \pm SEM, n=3). *p<0.05, significantly different from control (non-treatment). (B) TrkB protein expression was detected by Western blotting. Cells were treated with 10 μ M of ATRA and Am80 for 5 days. A total of 40 μ g protein was subjected to SDS-PAGE and run on a 7.5% gel, followed by immunoblotting using anti-TrkB antibody and β -actin antibody. Quantification and relative abundance were expressed relative to control (non-treatment), which was arbitrarily set to 1.00.

measured during the treatment of ATRA and Am80. As shown in Fig. 3, Am80 treatment increased GAP43 mRNA expression compared with controls at 3 days (1.18-fold) and 5 days (1.26-fold) although no significant difference was statistically shown. But, ATRA treatment did not enhance GAP43 mRNA expression compared with controls.

Induction of TrkB. Previous studies have demonstrated that ATRA induces expression of TrkB and responsiveness to

BDNF in several human neuroblastoma cell lines (18,19). Therefore, we investigated whether Am80 could induce expression of Trk family members. As shown in Fig. 4, Am80 treatment also increased TrkB mRNA expression (4.7-fold at 3 days and 28-fold at 5 days) and ATRA treatment significantly increased expression at 5 days (38.3-fold) compared with controls. However, there were no definite changes in TrkA and TrkC mRNA expression. Additionally, TrkB protein expression was subsequently measured by Western blot

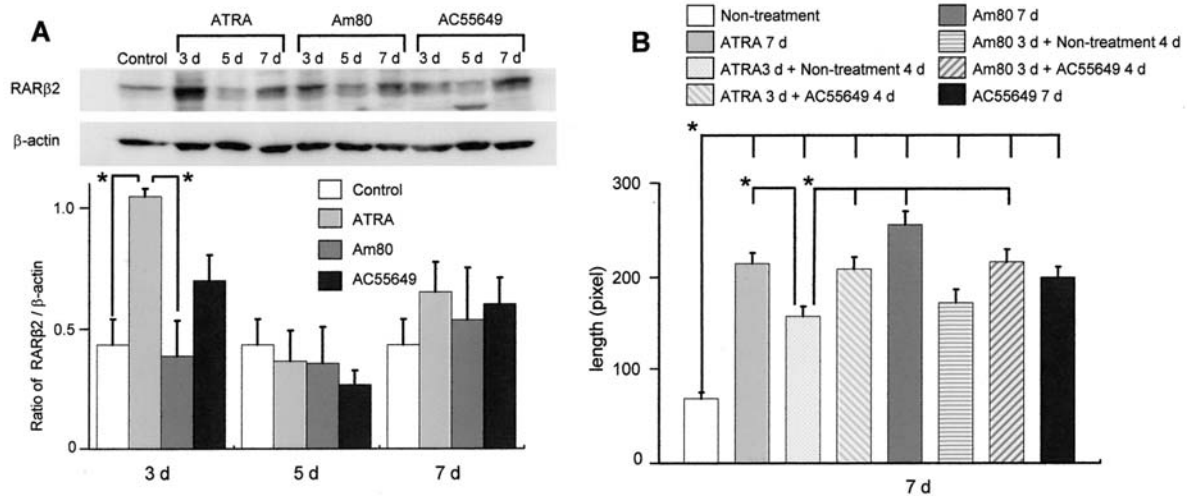


Figure 5. RARβ₂ expression and neurite outgrowth. (A) Effect of ATRA, Am80 and AC55649 on RARβ₂ protein expression. RARβ₂ expression was detected by Western blotting. Cells were treated with 10 μM of ATRA, Am80 and AC55649 for the indicated number of days. Data are calculated as the ratio of RARβ₂/β-actin and are shown as mean ± SEM (n=3). *p<0.05; data were analyzed by ANOVA followed by Tukey-Kramer tests. (B) Effect of co-stimulation with ATRA or Am80 and AC55649 on neurite outgrowth. Cells were treated with 10 μM of ATRA, Am80 and AC55649 for the indicated number of days. Data are expressed as mean ± SEM (n=60). *p<0.05; data were analyzed by ANOVA followed by Tukey-Kramer tests.

Table II. Summary of co-stimulation effect with ATRA or Am80 and AC55649 on neurite outgrowth.

Reagent	Culture days	Length (pixel ± SEM)
Non-treated	7	69.24±7.24
ATRA	7	213.99±11.22
Am80	7	255.48±14.11
ATRA 3 days + non-treated	4	157.28±10.46
Am80 3 days + non-treated	4	171.90±14.54
ATRA 3 days + AC55649	4	208.10±12.54
Am80 3 days + AC55649	4	215.83±12.98
AC55649	7	199.24±11.13

Significantly different, see Fig. 5B.

analysis. After 5 days treatment with 10 μM ATRA and Am80, TrkB protein expression increased by 1.38- and 1.98-fold compared with controls, respectively (Fig. 4B).

Involvement of RARβ₂ in Am80-induced morphological differentiation in NH-12 cells. It has been reported that RARβ₂ activation might be responsible for the initial step in ATRA~BDNF-induced differentiation (15,16). Therefore, we investigated, using AC55649, a RARβ₂ selective agonist (21), whether the RARβ₂ pathway contributed to Am80-induced neuronal differentiation. As shown in Fig. 5A, after 3 days of treatment, ATRA (10 μM) significantly increased RARβ₂ expression (2.44-fold) compared with controls, and AC55649 (10 μM) also increased RARβ₂ expression, but the expression decreased by Am80 treatment (10 μM). Next additional 4 day

culture, medium containing ATRA or Am80 was replaced to medium with or without AC55649 (ATRA-AC55649, Am80-AC55649, ATRA- and Am80-non-treatment groups). Neurite outgrowth was morphologically evaluated (Fig. 5B and Table II). Seven days treatment (10 μM) of ATRA and Am80 resulted in neurite length of 213.99±11.22 and 255.48±14.11 pixels, respectively. In the ATRA- and Am80-non-treatment groups, neurite length was shorter; 157.28±10.46 and 171.90±14.54 pixels, respectively. In the ATRA-AC55649 and Am80-AC55649 groups, neurites length was 208.10±12.54 and 215.83±12.98 pixels, respectively, which was almost similar to ATRA (213.99±11.22 pixels) or AC55649 (199.24±11.13 pixels) treatment for 7 days. Namely, additional AC55649 treatment after 3 days ATRA treatment restored neurite out-growth compared with the ATRA-non-treatment group. By contrast, the Am80-AC55649 group did not induce similar neurite outgrowth behavior compared with 7 days of treatment with Am80.

Discussion

Am80, a RARα- and RARβ-selective synthetic retinoid, has been accepted for the treatment against APL and it has been demonstrated that Am80 has better therapeutic potency than ATRA (4-6). ATRA also has been widely used also in studies of therapies for neurodegenerative diseases such as Parkinson's and Alzheimer's disease because of its ability to induce neuronal differentiation (7,22). However, the behavioral effects of Am80 have been reported, but morphological and biochemical effects of Am80 on neuronal differentiation has not been fully elucidated yet (23). The present study investigated the ability of Am80 in neuronal differentiation by measuring neurite outgrowth length and expression of neuronal differentiation markers.

Neurite outgrowth is one of the typical morphological effects in ATRA-induced neuronal differentiation in culturing

cells (18,19,24). In the NH-12 cell line, Am80 treatment induced neurite outgrowth (Fig. 1A), which was more potent than ATRA treatment at every observed time-point and concentration (Fig. 1B and Fig. 2). In addition, cytotoxic effects were seen against NH-12 cells with 50 μ M ATRA but not with 50 μ M Am80 (Fig. 2A). These findings suggest that Am80 exhibits more potent differentiation effects with less cytotoxicity, compared with ATRA.

It has been reported that the expression of GAP43, a widely utilized neuronal differentiation marker (18,19), is up-regulated by ATRA treatment in various human neuroblastoma cell lines (14,19). In addition, BDNF could induce GAP43 expression (19) and the process of neuronal differentiation has been shown to be dependent on the BDNF-stimulated pathway mediated by TrkB (17-19). In our study, it demonstrated that Am80 treatment increased GAP43 mRNA expression but not ATRA treatment (Fig. 3). Moreover, Am80 induced TrkB protein expression greater than ATRA treatment (Fig. 4). Therefore, Am80 might be useful as a neuronal differentiation reagent.

RAR β_2 is an important molecule in retinoid-induced neuronal differentiation (15,16) and many studies suggest that ATRA-induced neuronal differentiation takes place via the RAR β_2 pathway. The present study investigated the involvement of the RAR β_2 pathway in Am80-induced neurite outgrowth through the use of AC55649. After 3 days treatment with ATRA or Am80, culture medium was replaced to the medium with AC55649 (ATRA-AC55649 and Am80-AC55649 groups) or the medium without any retinoids (ATRA- and Am80-non-treatment groups). In these groups, neurite length decreased compared with the treatment with ATRA or Am80 for 7 days. Especially, the ATRA-non-treatment group showed significant decreases (Fig. 5B). However, the ATRA-AC55649 group displayed similar neurite outgrowth to 7 days treatment with ATRA or AC55649. In contrast, the Am80-AC55649 group did not lead to fully restored neurite length, compared with 7 days of Am80 treatment. According to the present results, RAR β_2 pathway was involved in ATRA-induced neuronal differentiation, which was consistent with previous reports (15,16,21). In contrast, Am80 may not activate RAR β_2 pathway but presented potent of neurite outgrowth effect. These findings also suggest that Am80 may activate another pathway to induce potent neuronal differentiation. However, further experiments are needed for analyzing RAR stimulation mechanism by Am80.

In conclusion, Am80, compared with ATRA, showed greater morphological and biochemical effects for neuronal differentiation, and less cytotoxic effect. These results suggest that Am80 may be a useful candidate for applying to novel therapeutic strategies for neurodegenerative diseases such as Parkinson's disease. Very recently, Takenaga *et al* has reported that Am80 induces TrkB expression and increased BDNF sensitivity in SH-SY5Y human neuroblastoma cells (25). Our findings agree with their data.

Acknowledgements

We thank Dr M. Hosokawa and Mr. K. Machida for their technical assistance.

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