

## Some flavonoids and DHEA-S prevent the *cis*-effect of expanded CTG repeats in a stable PC12 cell transformant

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### Abstract

Expanded CUG triplet repeats carrying mRNA seem to be responsible for myotonic dystrophy type 1 (DM1). To study the pathogenesis of DM1, we constructed a DM1 cell culture model using a PC12 neuronal cell line and screened flavonoids that ameliorate this mRNA gain of function. The expanded 250 CTG repeat was subcloned into the 3'-untranslated region of the luciferase gene yielding a stable transformant of PC12 (CTG-250). The cytotoxicity of CTG-250 was evaluated by intracellular LDH activity, and the *cis*-effect by luciferase activity. To find agents that alter CTG-250 toxic effects, 235 bioflavonoids were screened. An increased *cis*-effect and cytotoxicity were found when CTG-250 was treated with nerve growth factor to induce differentiation. Western blotting with anti-caspase-3 antibody suggested that cell death was caused by apoptosis. Screening analysis confirmed that a flavone (toringin), an isoflavones (genistein and formononetin), a flavanone (isosakuranetin), and DHEA-S prevent both the cytotoxicity and *cis*-effect of CTG-250 and that a flavanone (naringenin), isoflavone (ononin), and xanthylatin strongly inhibit the *cis*-effect of CTG repeats. In conclusion, we found that this neuronal cell line, which expresses the CUG repeat-bearing mRNA, showed *cis*-effects through the reporter gene and neuronal death after cell differentiation in vitro. However, some flavonoids and DHEA-S inhibit both the *cis*-effect and cytotoxicity, indicating that their chemical structures work to ameliorate both these toxic effects. This system makes it easy to evaluate the toxic effects of expanded CTG repeats and therefore should be useful for screening other DM1 treatments for their efficacies.

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**Keywords:** Myotonic dystrophy type 1; Trinucleotide repeats; PC12; Flavonoid; RNA gain of function; Tauopathy

### 1. Introduction

Myotonic dystrophy, type 1 [MIM 160900; dystrophia myotonica 1 (DM1)] is an autosomal dominant form of

muscular dystrophy. Although its common features are myotonia and progressive type 1 dominant muscle fiber atrophy, other frequent defects are cardiopathy, cataracts, insulin resistance, frontal balding, mild dementia, and other systemic involvements [1]. To date, two mutations (DM1 and DM2 [MIM 602668; dystrophia myotonica 2 (DM2)]), at unlinked loci, are known to be associated with myotonic dystrophy.

The DM1 mutation has been identified as an expanded CTG trinucleotide repeat tract in the 3'-untranslated region (3'-UTR) of the DM1 protein kinase (DMPK) gene [2]. In DM1 patients, the mRNA for DM1 protein kinase (DMPK)

**Abbreviations:** DM1, myotonic dystrophy type1; DM2, myotonic dystrophy type2; DMPK, DM1 protein kinase; 3'-UTR, 3'-untranslated region; RT-PCR, reverse transcription polymerase chain reaction; MW, molecular weight; kDa, kilo-Dalton; EtBr, ethidium bromide; conc., concentration; nt, nucleotides length; DHEA-S, dehydroepiandrosterone sulfate; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; CNS, central nervous system

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has from 50 to more than 2000 CTG repeats in its 3'-untranslated region (3'-UTR) [3]. Although this DM1 mutation accounts for 98% of DM cases, it is not known exactly how it causes the disease [4]. Several mechanisms, which indicate altered DMPK and/or isoforms, and mis-expression of self or neighboring genes at the DM1 locus (*cis*-effect), and/or at *trans*-dominant mutant DMPK mRNA (*trans*-effect), have been proposed [5].

Recently, a CCTG repeat expansion disease (DM2), in which the CCTG repeat is in intron 1 of the *zinc finger protein 9* (ZNF9) gene, has been reported [6,7]. Like the CUG-bearing RNA in DM1, the CCUG-bearing RNA in DM2 accumulates at a small number of foci in the nucleus. The expression of RNA carrying either a CUG or CCUG expanded repeat, rather than a deficiency of the DMPK, SIX5 or ZNF9 proteins, therefore is thought to account for the disease features common to DM1 and DM2 [4]. Mutant transcripts are retained in the nuclei of mouse C2C12 myoblast cells and aggregate into distinct foci [8,9]. Moreover, there is increasing evidence that mutant DMPK mRNA acts in *trans*- to disrupt cellular RNA metabolism (the toxic RNA theory of DM1 or RNA gain of function theory) [10–12]. Further, we have shown that CTG repeats interfere with the expression of the reporter gene in *Xenopus* oocytes but not in embryos (fertilized eggs). Early embryonic cells undergo extensive division, whereas the oocyte nucleus never divides and does not liberate nuclear materials into the cytoplasm. CTG repeats therefore are thought to interfere with the nucleus–cytoplasm transport of mRNA, especially at the nuclear membrane, rather than transcription of the gene [13].

To explain how RNA gain of function causes DM1, the protein sequestration hypothesis has been proposed [10–12,14]. According to this hypothesis, the DM1 expansion leads to sequestration of (CUG)<sub>n</sub> binding proteins on mutant DMPK RNAs and depletion from other transcripts that require these proteins for normal gene expression. Several (CUG)<sub>n</sub> binding proteins have been subcloned, and proteins in the MBNL (muscleblind-like) family bind to expanded CUG repeats in vitro and colocalize with mutant DM transcripts in vivo [11,15,16]. Expression of CUG and CCUG expansion RNAs induces MBNL recruitment into nuclear RNA foci, and disruption of the mouse Mbnl1 gene leads to muscle, eye, central nervous system (CNS) and RNA splicing abnormalities that are characteristic of DM1 [11,17,18].

The human CNS has tau proteins that consist of six isoforms which differ by the presence or absence of alternatively spliced exons 2, 3 and 10. The main pattern of aggregated tau isoforms in DM1 brain lesions is the shortest human tau isoform. Reduced expression of tau isoforms containing exon 2 occurs at both the mRNA and protein levels [19]. The same tau isoform pattern occurs in the CNS of a mouse transgenic model for DM1 [20]. These findings indicate that normal neuronal functions are disturbed by the CTG repeat expansions, resulting in abnor-

mal phosphorylation of tau protein, which has recently been called tauopathy. Further, the expanded CTG<sub>90</sub> repeats is reported to inhibit neuronal differentiation in a PC12 rat neuronal cell line [21].

We established a PC12 cell line that stably expresses expanded 250 CUG repeat-bearing mRNA. In this system, mitosis occurs, and the nuclear membrane disappears during every mitotic period until cell differentiation starts, but once differentiation starts, mitosis never occurs [22]. We hypothesized that this system shows the toxicity of CUG repeat-expanded mRNA after cell differentiation much more effectively than do cell lines with frequent mitosis. Since tau plays a significant role in the cytoskeletal changes that occur during apoptosis in the PC12 cell line [23], for example, in the histopathology of Alzheimer's disease patients or tauopathy, and because the removal of serum from the culture medium causes oxidative stress and triggers cell injury characterized by signs of apoptosis [24,25], we first examined the abnormal phosphorylation of tau protein resulting from the different alternative splicings of the tau gene using this system. We confirmed the cytotoxicity and *cis*-effect of the expanded repeat under the oxidative stress after neuronal cell differentiation in vitro.

Then, we screened chemicals that ameliorate the cytotoxicity and *cis*-effect of toxic RNA with expanded CUG repeats. For the screening, panels of bio-flavonoids were the candidates for treatment because (1) flavonoids are ubiquitous polyphenolic compounds in the human diet, (2) the basic flavonoid structure is very simple, but there are hundreds of derivatives, and (3) some flavonoids have a variety of biological activities that include beneficial effects on cancer or for coronary heart disease prevention [26]. We found that some bioflavonoids inhibit the cytotoxicity and/or *cis*-effect of expanded CTG repeats carrying mRNA.

## 2. Materials and methods

### 2.1. Plasmid constructions

A CTG repeat-bearing DNA fragment was isolated from genomic DNA extracted from leukocytes obtained from a mildly affected 47-year-old male with DM1 after obtaining his informed consent. When this genomic DNA was subjected to Southern blot analysis, it was found to carry a 250 CTG repeat expansion. The amplified DNA fragment with the repeats was digested with *MspI* and subcloned into the luciferase expression vector pGL3 (Promega) at the blunt-ended *XbaI* sites. The pGL3 vector carrying the luciferase gene and 250 CTG repeats was digested with *HindIII* and *BamHI*, blunt-ended and then subcloned into the pCAGGSneo vector [27] at the blunt-ended *EcoRI* site. Subcloned plasmids were sequenced by the dideoxy sequencing method with a Dye Deoxy Terminator Kit (Perkin-Elmer Applied Biosystems).

## 2.2. Cell culture and transfection

PC12 cells were maintained as described elsewhere [28]. Cells plated on collagen-coated dishes (Sigma) were transfected with reporter plasmids by means of the Lipofectamin Plus Reagent (Gibco-BRL). Stable transfectants were isolated after extended culture in growth medium containing 0.4 mg/ml Neomycin (Gibco-BRL). Surviving cells were re-cultured, and luciferase activity was measured to screen for colonies with a high level of expression. A RT-PCR done on the mRNA extracted from these stable cells was performed to analyze whether the clones had 250 CTG repeats. A selected PC12 cell line carrying 250 CTG repeats and efficiently expressing the luciferase gene was named CTG-250. A cell line that expresses the luciferase reporter gene only was named LUC and used as the control. To induce differentiation, cells were treated with 100 ng/ml nerve growth factor (NGF; Gibco-BRL) for up to 7 days. Serum was removed by replating the differentiated PC12 cells onto new collagen-coated dishes as described elsewhere [29], and cells were maintained in 100 ng/ml NGF with serum-free medium for the appropriate period under oxidative stress [24,25]. For immunohistochemical analysis, cells were reseeded onto glass coverslips in 24-well plates coated with 12  $\mu$ g/ml poly D-lysine (Sigma).

## 2.3. Immune fluorescence microscopy

For immuno-histochemical analysis, cells were reseeded onto glass coverslips in 24-well plates coated with 12  $\mu$ g/ml poly D-lysine (Sigma) at a density of  $2.5 \times 10^4$  cells/well. Two to 6 days after reseeding with NGF in serum-free medium, cultured the PC12 cells placed cultured on coverslips were fixed with 4% paraformaldehyde-phosphate-buffered saline (PBS) for 15 min. After residual formaldehyde had been quenched with 50 mM NH<sub>4</sub>Cl-PBS for 10 min, cells were permeabilized in 0.2% Triton X-100-PBS for 10 min and incubated with 10% fetal bovine serum in PBS for 30 min to block nonspecific antibody binding. Cytoplasmic neurofilaments were detected with an anti-neurofilament 200 kDa monoclonal antibody (clone NE14, Boehringer Mannheim Biochemica) in PBS (0.5 U/ml) for 1 h. Cells on coverslips were mounted in 90% glycerol containing 0.1% *p*-phenylenediamine dihydrochloride in PBS and examined by the method described elsewhere (Fig. 1c) [30]. All the steps were done at room temperature, and the cells were rinsed with PBS between steps.

## 2.4. RNA extraction and reverse transcription

Total RNA was extracted from the cultured cells with RNeasy Mini (Qiagen), and the concentration measured by means of a NanoDrop (NanoDrop Tech) then used for Northern blotting and the RT-PCR. One microgram of total RNA was reverse transcribed with a First-Strand cDNA

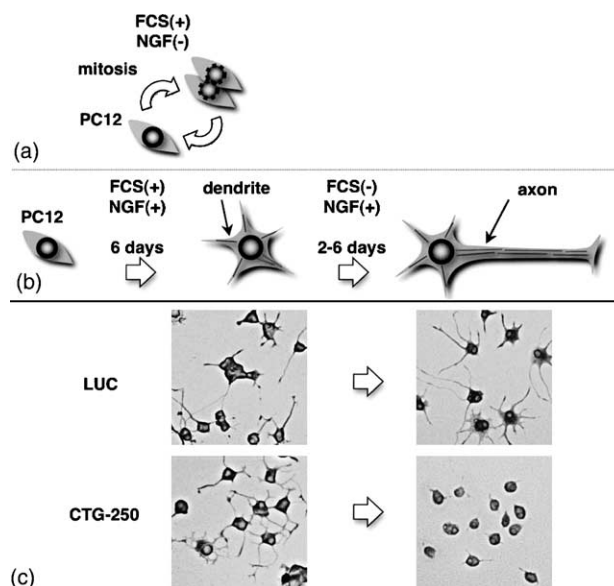


Fig. 1. Schematic presentation of the PC12 cell cycle without differentiation (a), with differentiation (b), and the effect of CTG repeat expression on neurite outgrowth in NGF-treated stably transfected PC12 cell lines under oxidative stress (c). Immunohistochemical staining of the LUC control and CTG-250 neuronal cell lines is shown (neurofilament (NF) staining, 200 $\times$ ). Photos of cells are placed under the corresponding cell figure (c). The nuclear membrane of PC12 cells disappears during every mitotic event (broken line) (a). After cellular differentiation by NGF during 6 days of incubation with fetal calf serum (FCS), PC12 cell mitosis stops, the nuclear membrane is maintained and dendrites and axons are formed (FCS(+), NGF(+)) (b). Note deterioration of the axon and dendrite processes in CTG-250 cells cultured without FCS, but with NGF (FCS(-), NGF(+)) for 2–6 days (c).

Synthesis Kit (Pharmacia Biotech) using random hexamers. After incubation, the reaction mixture was diluted to 30  $\mu$ l with water, and 1  $\mu$ l of the dilution was used for the RT-PCR analysis.

## 2.5. Northern blot hybridization

Five micrograms of the total RNA from each cell was electrophoresed in an agarose gel and then transferred to a Hybond N<sup>+</sup> membrane (Amersham Inc.). These membranes were hybridized with a <sup>32</sup>P-labeled luciferase cDNA probe, labeled by the Random Primer labeling system using [<sup>32</sup>P]dCTP as the radioactive nucleotide. Blots were exposed on imaging plates, and the radioactivity of the bands quantified with the BAS2000 System (Fuji film Inc.). The radioactivity of the mRNA bands from each cultured cell obtained at each time point (24–48 h) was calculated as the ratio of the value obtained for the undifferentiated CTG-250 cell line. Hybridized blot analysis was performed three times (Fig. 2).

## 2.6. RT-PCR

PCR amplification of the tau transcripts was done with rat tau primers that recognize differences in the sizes of tau gene products. No DNA amplification was found in the

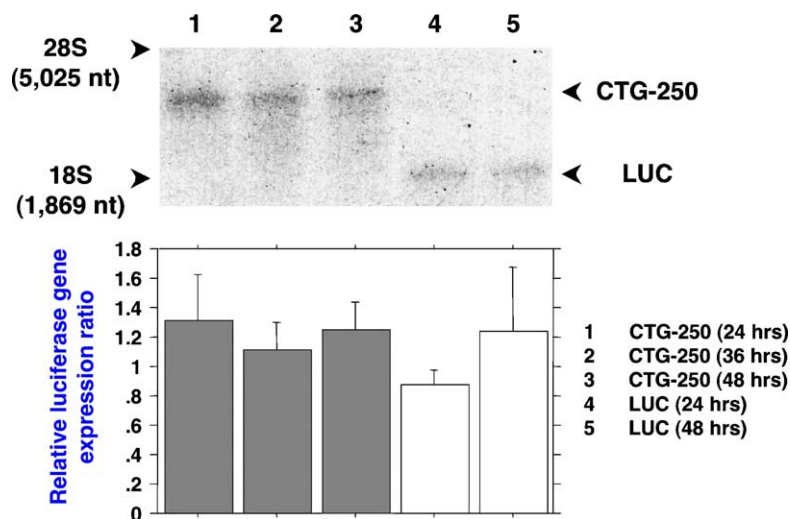


Fig. 2. Quantitative transcriptional analysis in expanded (CTG-250) and non-expanded (LUC) reporter genes by RNA blotting. Bands were scanned into Image-J. Three independent experiments were done. Results are shown as ratios of the undifferentiated CTG-250 cell line. Note the absence of a significant difference in reporter gene transcription between CTG-250 and LUC with and without differentiation.

controls. The forward and reverse primer sequences used were M-TauE1.FWD 5'-GGC TTA AAA GAG TCT CCC CCA C-3'; M-TauE1\_2.FWD 5'-GGC TTA AAA GAG TCT CCC CCA C-3'; M-TauE4.RV 5'-GGT GTC TCC GAT GCG TGC TTC TTC-3'; M-TauE9.FWD 5'-CCA GAC CTA AAG AAG GTC AGG-3'; M-TauE11.RV 5'-GGA CAT GGG TGA TGT TAT CC-3'; R-GAPDH.FWD 5'-AAT GGC ACA GTC AAG GCT GAG-3'; R-GAPDH.RV 5'-ACC AGT GGA TGC AGG GAT GAT-3' (Fig. 3a). PCR amplification was done at a final volume of 20  $\mu$ l containing 10 mM Tris/HCl, pH 9.0, 50 mM KCl, 2 mM  $MgCl_2$ , 0.2 mM of each dNTP, 10% DMSO, 20 pmol of each primer set and 0.2 IU HS-Taq polymerase (Takara). The PCR program used a "hot start" PCR protocol, an initial step of 93 °C for 5 min and 72 °C for 2 min (hot start) followed by 42 cycles of 30 s each at 93 °C for DNA denaturation, 30 s each at 55 °C for the annealing of primers, and 4 min at 72 °C for primer extension. After the addition of 10  $\mu$ l of 10 $\times$  loading buffer, the RT-PCR products were electrophoresed on 2% NuSieve GTG agarose (Cambrex Co.) and a 0.7% agarose mixture gel in 1 $\times$  TAE buffer followed by staining with 0.2  $\mu$ g/ml EtBr. Each gel image was recorded with a CCD camera (MiniVisionary, Fotodyne Inc.), and the intensity of each band measured. Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was the internal standard amplified with a pair of primers (R-GAPDH.FWD and R-GAPDH.RV) and a separate aliquot of the reaction mixture in the same PCR machine (GeneAmp PCR system 9700, Applied Biosystems Inc.).

### 2.7. Semi-quantitative analysis of tau isoforms mRNA

For each pair of gene-specific primers, semi log plots of the fragment intensity as a function of cycle number were

used to determine the cycle number range over which linear amplification occurred. The number of PCR cycles was kept within this range. The range was linear from 33 to 39 amplification cycles (data not shown). The amount of the tau gene product was determined as a ratio for the intensity of the GAPDH band. Because there is an abundance of GAPDH mRNA in neuronal cell lines, we added a set of primers at the 11th amplification cycle after the first 10 cycles of idle running of PCR to get a linear amplification between 33 and 39 amplification cycles. Based on these preliminary amplification experiments, we measured the intensity of bands at 36 amplification cycles (data not shown). Tau gene products were quantified by comparing the intensity of each band with the GAPDH band intensity. Experiments were repeated three times independently, and the data used for the statistical analysis (Fig. 3b–d).

### 2.8. Immunoblotting analysis of the tau gene and caspase-3 products

Cell homogenates were prepared for immunoblotting analysis as described previously [31]. Ten micrograms of protein was separated by electrophoresis on a 7.5% SDS-polyacrylamide gel then transferred to an Immobilon-P membrane (Millipore Co.). The membrane was blocked with 3% nonfat dry milk (Morinaga Co.) then incubated with AT-8 (Pierce Biotechnology, Inc.), rat whole-tau antibody (graciously supplied by Dr. M. Miyazono and Professor T. Iwaki) or phosphorylated tau pS<sup>396</sup> antibody (BioSource International) as the primary antibody and with caspase-3 (Cell Signaling Technology) for the analysis of apoptosis (Fig. 10). After incubation with horseradish peroxidase-conjugated secondary antibody, immunoblots were developed by an Enhanced



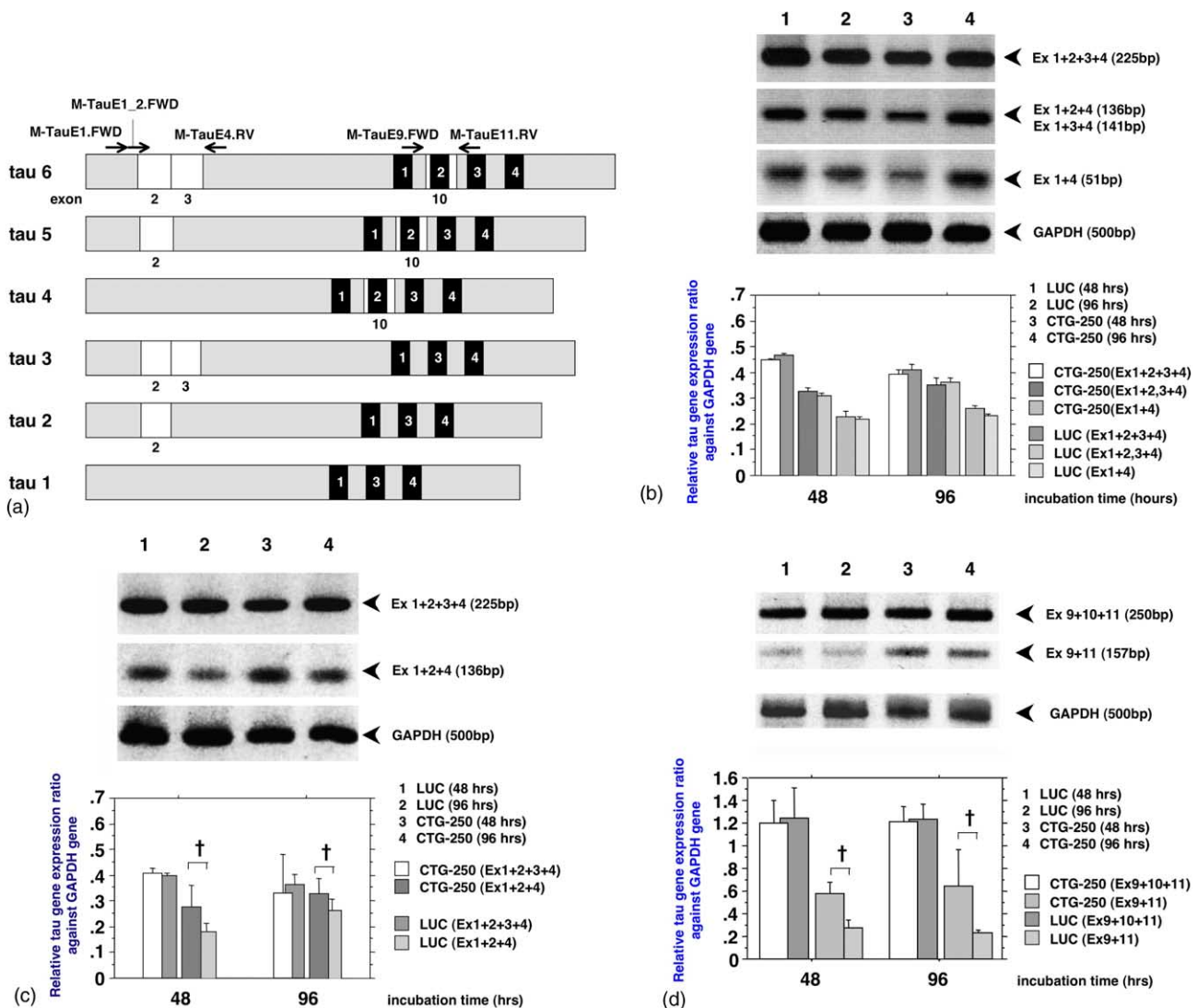


Fig. 3. (a) Diagram of tau isoform structures [34]. Alternatively spliced exons are shown as white boxes. Regions common to all isoforms are shaded. Microtubule (MT) binding repeats (three or four) are indicated as numbered black boxes. The diagram is not drawn to scale. Names and locations of the primers used for the RT-PCR are shown as relative positions at the top. The six tau isoforms, taus 1–6, were derived by alternative splicing of exons 2, 3, and 10, three with and three without exon 10, that encode for the additional MT-binding repeat. (b) Expression profile of rat tau mRNA isoforms. RT-PCR products corresponding to the various isoforms were obtained with two sets of primers. Primers M-tauE1.FWD and M-tauE4.RV (b) or M-tauE1\_2.FWD and M-tauE9.FWD (c) were also used to differentiate tau mRNA isoforms that have or lack exons 2, 3 or both (a). Tau mRNA isoforms, that have or lack exon 10 also were identified with primers M-TauE9.FWD and M-TauE11.RV (d). LUC control cells after 48 h (lane 1), and 96 h (lane 2) incubations with differentiation; CTG-250 cells after 48 h (lane 3), 96 h (lane 4) incubations with differentiation (b–d). GAPDH was the internal standard. Relative tau expression ratios are shown in the lower berth of each figure. Data are expressed as transcribed tau gene products normalized to GAPDH gene expression, mean  $\pm$  S.E.M. ( $n = 3$  in separate experiments).  $^{\dagger}P < 0.05$  compared with PC12 cells without expanded CTG repeats (LUC). Note the decrease in tau mRNA isoforms that lack exons 3 and 10 after differentiation for 48–96 h.

Chemiluminescence System (ECL system) (Amersham Pharmacia Biotech).

### 2.9. Luciferase assay and LDH cytotoxicity detection assay

To evaluate the transcription and translation of the CTG repeat-bearing reporter gene, luciferase activity was assayed with a Luciferase Assay System according to the manufacturer's protocol (Promega Co.). After CTG-250 and LUC cell differentiation with NGF, PC12 cells

were plated in 96-well plates at  $1.3 \times 10^4$  cells/well. After 2–6 days incubation period with a flavonoid under oxidative stress, the cells were washed with PBS and assayed (Figs. 4a, 6a, 7a, 8a, and 9a). The amount of protein in each sample was determined by the Lowry–Folin assay [32]. Luciferase activity was expressed on a per-protein basis.

To determine the cytotoxic effect of the expanded CTG repeat, lactate dehydrogenase (LDH) activity in the cell was measured as a gauge of the total number of living cells with a kit from Takara (LDH Cytotoxicity Detection Kit).

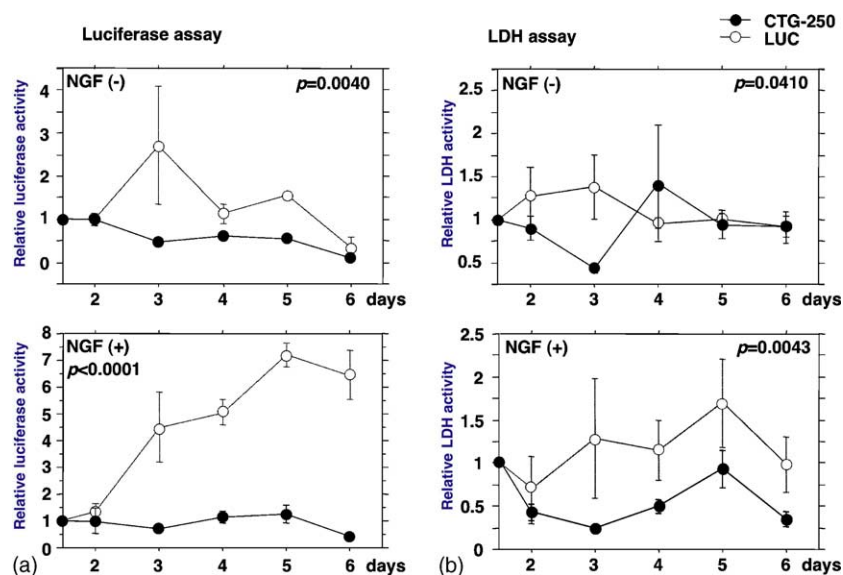


Fig. 4. In vitro luciferase (a) and LDH (b) assay of cultures without (upper graphs) and with (lower graphs) NGF and the incubation periods. The graphed points represent the means  $\pm$  standard errors of the LUC ( $\circ$ ) and CTG-250 ( $\bullet$ ) cells. Values shown are ratios to luciferase or LDH activity without incubation. Note that the luciferase activity of LUC cells with NGF is significantly increased ( $P < 0.0001$ ) vs. that of CTG-250 cells. as incubation progresses. The LDH activity of CTG-250 decreased slightly ( $P = 0.0043$ ) when incubated with NGF.

Results are shown as intracellular LDH activity on a per-protein basis (Figs. 4b, 6b, 7b, 8b and 9b).

#### 2.10. First screening of bioflavonoids and other chemicals for RNA gain of function prevention

In the cell treatment experiment, 235 purified bioflavonoids and other chemicals that structurally resemble bioflavonoids were screened initially. All bioflavonoids were supplied from by the Laboratory of Pharmacognosy, Toyama Medical and Pharmaceutical University. They were originally obtained by isolation from higher plants growing in Japan. Most of them were identified by spectral analyses. Some compounds were obtained by chemical modification of the isolated compounds. The purity of each compound was checked by thin layer chromatography, NMR spectrum, and elemental analysis. The bioflavonoids and other chemicals were dissolved in dimethyl sulfoxide (DMSO) at a maximum DMSO concentration of 0.5%. After differentiation, PC12 cells (LUC and CTG-250) were re-plated in collagen-coated 96-well plates at  $1.3 \times 10^4$  cells/well in serum-free medium containing 100 ng/ml NGF and 10  $\mu$ g/ml of each flavonoid for cell treatment. After 2 days of incubation, the cells were washed with PBS, lysed in Cell Lysate Buffer (Promega Co.), and used in the luciferase assay. Luciferase activity data were compared with the data for untreated cells. Flavonoids and other related chemicals that produced luciferase activity more than three times that of the untreated controls were selected and prepared for second and third screenings. DHEA-S was used for in the second screening directly because its effectiveness has already reported [33].

#### 2.11. Second and third screenings of flavonoids and other chemicals

After differentiation, PC12 cells (LUC and CTG-250) were re-plated in collagen-coated 96-well plates at  $1.3 \times 10^4$  cells/well in serum-free medium containing 100 ng/ml NGF. Various concentrations of flavonoids for cell treatment (10, 20, 40, and 80  $\mu$ g/ml in the second screening, and 5, 10, 20, 80, and 160  $\mu$ g/ml in the third) were added on the second and fourth days of culture. Luciferase activities of the CTG-250 and LUC cell lines were compared over the time course. Several flavonoids and other chemicals had statistically significant effectiveness in preventing the *cis*-effect, cytotoxicity of expanded CTG repeats or both (Figs. 6–9 and Table 1). The chemical structures of those flavonoids are presented in Fig. 5.

#### 2.12. Data analysis and statistics

The expression of the luciferase gene in Northern blotting and of the tau protein in RT-PCR were determined by use of the National Institutes of Health Image-J (V1.31b), Java-based image processing program in a Macintosh OS X based computer system. Data were analyzed statistically by the Student's *t*-test and repeated measures ANOVA.

Data obtained from the experiment that detected RNA gain of function with or without differentiation were expressed as ratios of their respective control values obtained on the first day of sample re-plating, which was not under differentiated conditions (FCS(+), NGF(-), in Fig. 1a) (Fig. 4). In the cell treatment experiment with bioflavonoids and other chemicals, the data are expressed as ratios of the respective control values for the untreated

Table 1  
Inhibitory effect of flavonoids on expanded 250 CTG repeats transcribing PC12 neuronal cell (CTG-250)

Chemical name (MW)	Conc. ( $\mu\text{g}/\mu\text{l}$ )	Conc. ( $\mu\text{M}$ )	<i>Cis</i> -effect	Cytotoxicity
<b>Flavone</b>				
Baicalein (280)	5	17.9	+	–
	10	35.7	+	–
	20	71.4	+	–
Toringin (416)	5	12.0	–	–
	10	24.0	–	–
	20	48.0	+	+
Cosmosiin (432)	5	11.6	–	–
	10	23.1	–	–
	20	46.3	–	–
Acacetin (284)	5	17.6	–	–
	10	35.2	–	–
	20	70.4	–	–
Apigenin (270)	5	18.5	+	–
	10	37.0	+	–
	20	74.0	+	–
Irisolone (312)	5	16.0	+	–
	10	32.0	–	–
	20	64.1	–	–
Genkwanin (284)	5	17.6	+	–
	10	35.2	+	–
	20	70.4	–	–
<b>Isoflavone</b>				
Genistein (270)	5	18.5	–	–
	10	37.0	+	+
	20	74.0	–	–
Formononetin (268)	5	18.7	++	–
	10	37.3	++	–
	20	74.6	–	–
Ononin (430)	5	11.6	++	–
	10	23.2	+	–
	20	46.5	–	–
Daidzein (254)	5	19.7	–	–
	10	39.4	–	–
	20	78.7	–	–
<b>Flavanone</b>				
Isosakuranetin (286)	5	17.4	–	–
	10	34.9	+	–
	20	69.9	++	++
Naringenin (272)	5	18.4	–	–
	10	36.8	+	–
	20	73.5	++	–
<b>Coumarin</b>				
Xanthylatin (228)	5	21.9	–	–
	10	43.9	++	–
	20	87.7	++	–
<b>Steroid</b>				
DHEA-S (368)	5	13.6	+	–
	10	27.2	+	+
	20	54.3	–	–

(+) effective for inhibition ( $P < 0.05$ ); (++) highly effective for inhibition ( $P < 0.0001$ ); (–) not effective for inhibition; MW, molecular weight; DHEA-S, dehydroepiandrosterone-sulfate.

cells (FCS(–), NGF(+), Fig. 1b) (Figs. 6–9). Data are expressed as means  $\pm$  S.D. A repeated measures ANOVA was used for the statistical analysis between CTG-250 and LUC cells based on the time courses in all the experiments related to detecting *cis*-effect and cytotoxicity (Figs. 4 and

6–9, Table 1). Three separate experiments were performed. A  $P$  value  $< 0.05$  was considered significant.

The results of in vitro luciferase and LDH assay cultures without and with NGF were analyzed according to the time course (Fig. 4). Thus, the result is displayed only as a  $P$  value. The data on inhibition of the *cis*-effect and cytotoxicity by bioflavonoids (Figs. 6–9) was analyzed with ANOVA according to the time course (two and four incubation days) between CTG-250 and LUC cells, and the results are shown in the graph at various bioflavonoid concentrations (0, 5, 10 and 20  $\mu\text{g}/\text{ml}$ ). Thus, the results of statistical analyses are shown by symbols on the graph.

### 3. Results

#### 3.1. Establishment of stable PC12 clones

Luciferase was used as the reporter gene to determine the *cis*-effect of the CUG repeat expansion in the 3'-UTR region of the RNA. Reporter plasmids that carried the 3'-UTR region, with or without expanded CTG repeats, were transfected into PC12 cells, producing several stable clones that were selected by the neomycin resistance of each construct. PC12 cells have been widely used as a model for studying the characteristics of neuronal cells because they respond to NGF and differentiate into cells that are morphologically and biologically similar to neurons (Fig. 1a and b). A PCR done on the genomic DNA extracted from these stable cells, performed to analyze whether the clones had CTG repeats in their genomes and expressed CUG repeats carrying RNA in vitro, confirmed the presence of the expanded CTG repeat (data not shown). Northern blotting with luciferase cDNA as the probe also showed expanded 250 CUG repeats and the same luciferase mRNA intensities in CTG-250 and LUC, evidence of the successful transcription of the luciferase gene in this system (Fig. 2).

#### 3.2. mRNA carrying expanded CUG repeats produce different patterns of alternative splicing of the tau gene but not abnormal phosphorylation

Because differences in the alternative splicing of the tau gene and the abnormal phosphorylation of tau protein have been reported in brains of DM1 patients and in a DM1 mouse model [19,20,34], we examined splicing and phosphorylation in our PC12-based cell system. When primer sets detecting tau1-6 isoforms (M-TauE1.FWD and M-TauE4.RV) (Fig. 3a) were used, no difference in the expression patterns of tau isoforms was detected (Fig. 3b). However, when a primer set that specifically encodes tau 2, 3, 5, 6 were used (M-TauE1\_2.FWD and M-TauE4.RV) (Fig. 3a), decreased expression of the exon 3 spliced-out isoform was confirmed (Fig. 3c). These results indicate that in the CTG-250 PC12 cell line, tau 2 or tau 5 isoforms, rather than tau 3 or tau 6 isoforms, are increased.

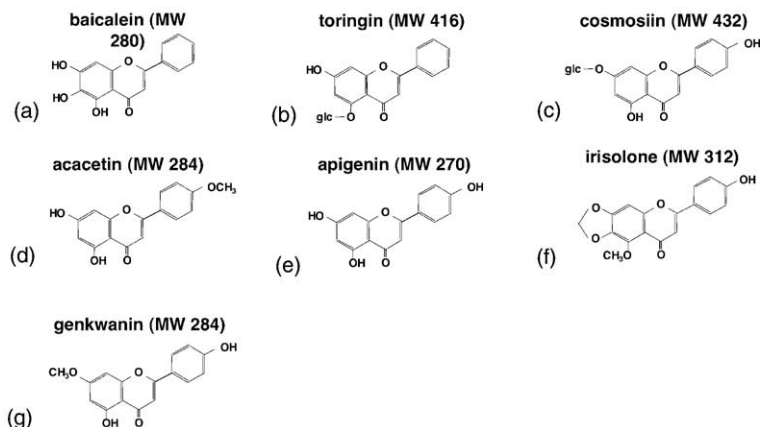
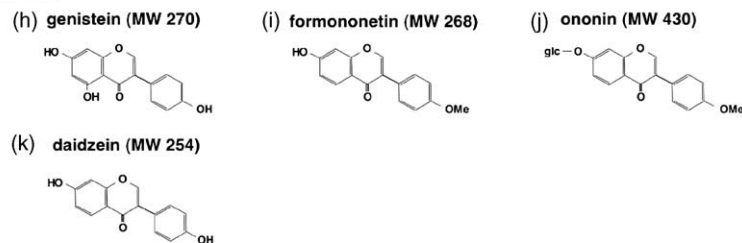
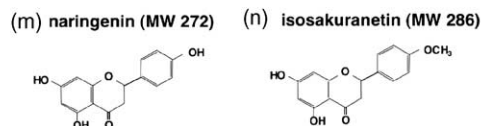
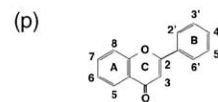
**flavone****isoflavone****flavanone****basic structure of flavonoids**

Fig. 5. Structures of flavonoids and the other test chemicals of (1) the flavone family, (2) the isoflavone, flavanone family and the basic structure of flavonoids, MW: molecular weight.

These findings are compatible with previous reports [19,20], but there is a different tau isoform expression pattern, a splice out of exon 10 of the tau gene, also was present in our CTG-250 cell line (Fig. 3d). A different

abnormal phosphorylation pattern also has been reported in DM1 patients and in the DM1 mouse model [19,20], but no significant difference in the phosphorylation pattern was found for the stable, repeat-expressing PC12

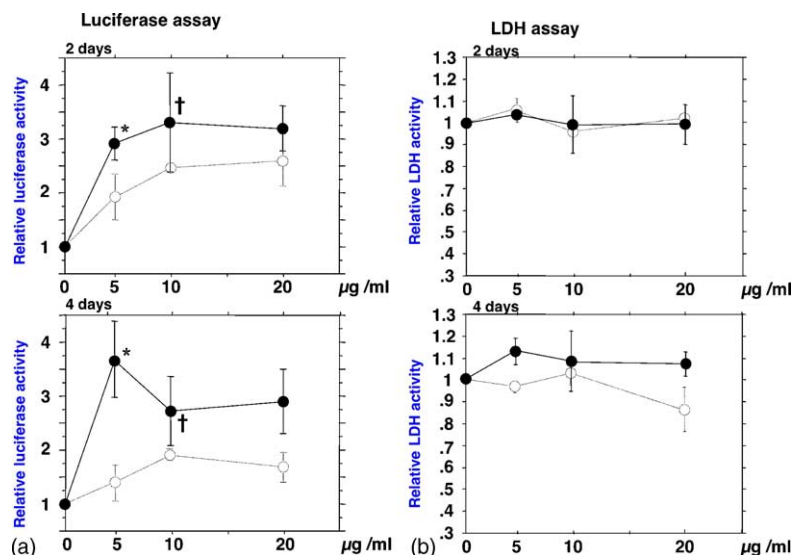


Fig. 6. Inhibition by ononin (isoflavone) of the *cis*-effect (a) and cytotoxicity (b) caused by expanded CTG repeats. The points represent means  $\pm$  standard errors of LUC (○) and CTG-250 (●) cells. Values are ratios for luciferase (a) or LDH activity (b) for each untreated cell. The upper boxes shown findings after 2 days of incubation, the lower ones after 4 days of incubation (FCS(–), NGF(+)). Statistical significance also is shown (\* $P < 0.0001$ ; † $P < 0.05$ ).



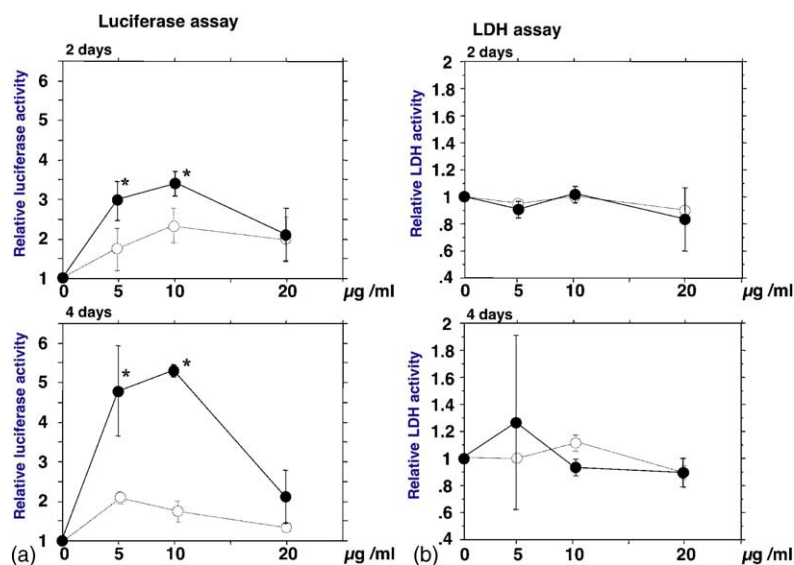


Fig. 7. Inhibition by formononetin (isoflavone) of the *cis*-effect (a) and cytotoxicity (b) caused by expanded CTG repeats (data and abbreviations are the same as in Fig. 6).

(CTG-250) and control cell lines (LUC) (data not shown).

### 3.3. mRNA carrying expanded CUG repeats shows cytotoxicity

To investigate the effect of expanded CTG repeat transcription on the neuronal differentiation and cytotoxicity of PC12 cells, morphological changes in the stable clones were examined during NGF-induced differentiation (Fig. 1c). After 6 days of NGF treatment under oxidative stress, the changes in the characteristics of the CTG-250 cell line were swelling of the cell body and very short stubby processes that failed to elongate into dendrites and

axons, whereas the LUC cell line did not show any marked change (Fig. 1c).

To evaluate the extent of those defects in the CTG-250 cell line, cell function was analyzed with LDH and the luciferase assay. Comparison of the luciferase activity of the CTG-250 cell line, which had cells that expressed expanded CTG repeats, with that of repeat-free clone (LUC) cells carrying repeat-free luciferase showed significantly low translation of luciferase, especially with NGF ( $P < 0.0001$ ) (Fig. 4a).

Results of the LDH assay, which reflects the cytotoxicity of cultured cells, appeared to be equivalent to those of the repeat-free cell lines. They did not significantly differ between CTG-250 and LUC cells without NGF

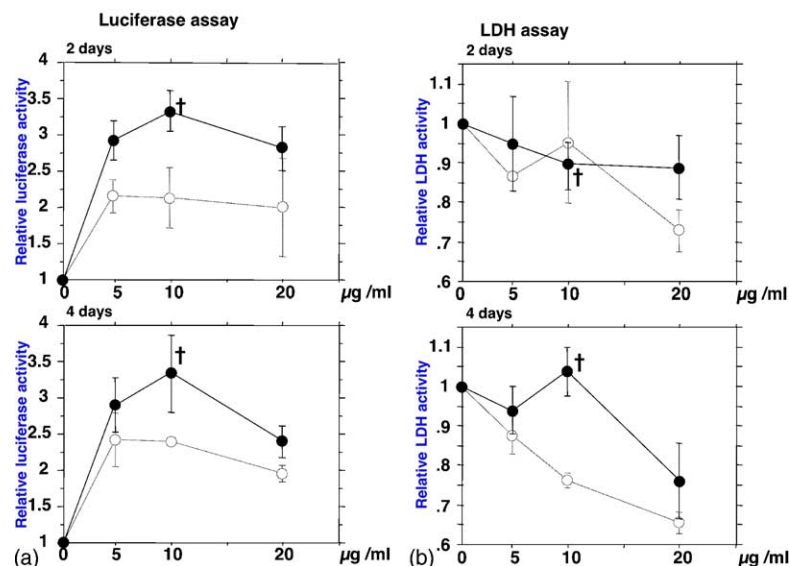


Fig. 8. Inhibition by genistein (isoflavone) of the *cis*-effect (a) and cytotoxicity (b) caused by expanded CTG repeats (data and abbreviations are the same as in Fig. 6).

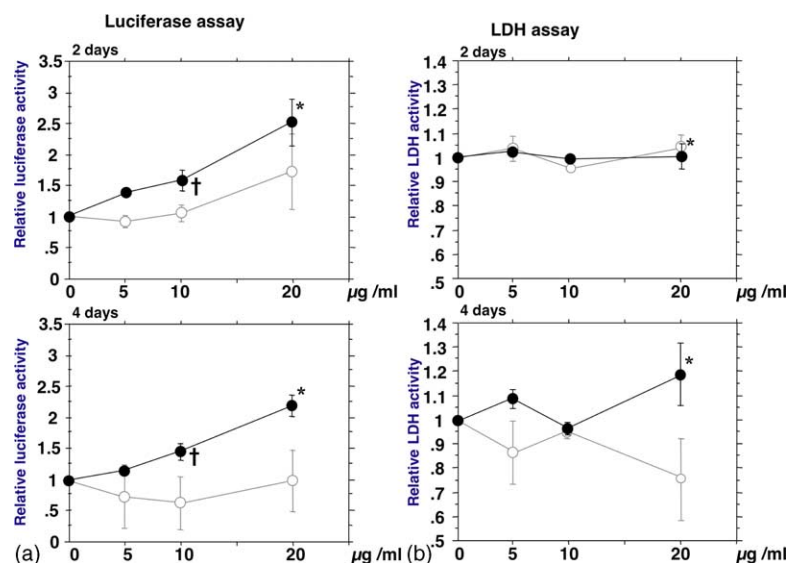


Fig. 9. Inhibition by isosakuranetin (flavanone) of the *cis*-effect (a) and cytotoxicity (b) caused by expanded CTG repeats (data and abbreviations are the same as in Fig. 6).

( $P = 0.041$ ), but with NGF, the difference is significant ( $P = 0.0043$ ) (Fig. 4b). Therefore, expression of the expanded 3'-UTR RNA, affects the NGF-based signaling pathway in particular. These results are evidence that the expression of RNA that carries the 250 CTG repeat expansion in the 3'-UTR region disrupted cell function, especially under oxidative stress. These results also indicate a *cis*-effect on the inhibition of protein production. These findings are consistent with those of a previous report on a muscle cell model [5,35].

### 3.4. Protective effects of some flavonoids against oxidative stress

When it became clear that the CTG-250 cell to some extent simulates the pathological mechanisms of the neu-

ronal cell of DM1, we screened bioflavonoids and related chemicals for their abilities to inhibit the RNA gain of function seen in this cell system. The structurally related bioflavonoids examined in the third screening are shown in Fig. 5, and their effects on CTG-250 and LUC cell line proliferations are presented in Figs. 6–9. These flavonoids decreased the differences in luciferase activity, cytotoxicity, or both, between the CTG-250 and LUC cell lines. The addition of 5–20  $\mu\text{g/ml}$  or any of them inhibited cell cytotoxicity, lessened the *cis*-effect of CTG repeats, or both. Ononin accelerated luciferase activity that resulted from the reduced *cis*-effect of the CTG repeats (Figs. 6a and 7a, Table 1). Genistein, formononetin and isosakuranetin decreased both the *cis*-effect and cytotoxicity of the CTG repeats significantly (Figs. 8a and b and 9a and b, Tables 1 and 2).

Table 2

Inhibitory effect of flavonoids on different expanded 250 CTG repeats transcribing PC12 neuronal cell lines (CTG250\_1, CTG250\_2)

Chemical name (MW)	Conc. (μg/μl)	Conc. (μM)	Cis-effect		Cytotoxicity	
			CTG250_1	CTG250_12	CTG250_1	CTG250_12
Isoflavone						
Genistein (270)	10	37.0	+	+	+	+
Formononetin (268)	5	18.7	+	+	+	++
	10	37.3	—	—	—	—
Daidzein (254)	10	39.4	—	—	—	—
Flavanone						
Isosakuranetin (286)	10	34.9	+	+	+	+
	20	69.9	+	—	+	—
Coumarin						
Xanthylatin (228)	10	43.9	+	++	+	—
Steroid						
DHEA-S (368)	10	27.2	+	+	+	+

(+) effective for inhibition ( $P < 0.05$ ); (++) highly effective for inhibition ( $P < 0.0001$ ); (–) not effective for inhibition; MW, molecular weight; DHEA-S, dehydroepiandrosterone-sulfate.

Dose–response experiments showed that exposure to ononin (11.6  $\mu$ M) (Fig. 6, Table 1); formononetin (18.7–37.3  $\mu$ M) (Fig. 7, Tables 1 and 2); naringenin (73.5  $\mu$ M) (Table 1); or xanthylatin (43.9–87.7  $\mu$ M) (Tables 1 and 2) significantly decreased the *cis*-effect of toxic RNA, whereas no decrease in cytotoxicity was found during the 2- to 4-day incubation periods. The use of 10–20  $\mu$ g/ml of genistein (37.0–74.1  $\mu$ M) (Fig. 8, Table 1), isosakuranetin (34.9–69.9  $\mu$ M) (Fig. 9, Table 1), or toringini (48.0  $\mu$ M) (Table 1), however, progressively decreased not only the *cis*-effect of the expanded CTG repeats but cytotoxicity as well. Interestingly, dehydroepiandrosterone sulfate (DHEA-S) (27.2  $\mu$ M), which is reported to improve the symptoms of DM1 patients was efficacious against cytotoxicity and the *cis*-effect in our system (Table 1) [33,36].

We have independently constructed two other stable clones containing expanded CTG repeats in the same way (Table 2, CTG250\_1, CTG250\_12). Using these clones, we confirmed the protective effect of these flavonoid; all of them were revealed to be effective in the same conditions as the previous experiment at a suitable concentration (Table 1). Using these clones, protective effects were also observed with isosakuranetin, formononetin, xanthylatin and DHEA-S, which is almost the same result as that of the CTG-250 and LUC cell lines, except that

formononetin showed a protective effect for both *cis*-effect and cytotoxicity (Table 2).

### 3.5. Protective effect of some flavonoids against the *cis*-effect and cytotoxicity

The underlying cellular mechanisms by which these flavonoids give neuroprotection in this cellular model have yet to be determined, but a significant number of neuronal deaths in a previous C2C12 myoblast cells study done with a transient expression system, showed features of apoptosis with prominent caspase-3 activation [35,37]. We therefore considered whether flavonoid treatment blocks expanded CTG repeat-induced caspase-3 activation in the CTG-250 cell line. First, we used Western blotting to search for formation of the activated form of caspase-3, the p17 form, as well as by cleavage of known caspase-3 substrates [38] and found an increase in the active form of caspase-3 in CTG-250 cells cultured without a flavonoid (Fig. 10a). In cell lysates derived from samples treated at the appropriate concentration with isosakuranetin, genistein, formononetin, or ononin, however, no cleavage of caspase-3 or its substrates was found in the Western blotting (Fig. 10b). Because caspase-3 works during the last stage of apoptotic pathways, it is not clear whether flavonoids directly block this apoptotic cascade, but our findings suggest that inhibition of the *cis*-effect of the CTG repeat expansions also may inhibit apoptotic neuronal death.

## 4. Discussion

We showed that a PC12 rat neuronal cell line which expresses expanded CTG repeats that carry reporter mRNA can be characterized simply by measuring its gene translational product in order to evaluate the *cis*-effect of expanded CTG repeats. Under oxidative stress, our neuronal cell line underwent neuronal death just after cell differentiation and showed different alternative splicing patterns of the tau gene. Of course, this system is not completely identical to that of the neuronal cells of DM1 patients or the DM1 mouse model because the gene expression pattern of the tau isoforms differs slightly from that of DM1, and no different pattern of abnormal phosphorylation has yet been observed. These differences may be owing to a difference in the species or organs used, the absence of a neuron–glia cell relationship, or the short culture period used in this system. Its simplicity and ease of handling, however, make the system useful for detecting the mechanisms of altered alternative splicing or apoptosis caused by toxic RNA. Further, in this system, although the nuclear membrane disappears at every mitosis before differentiation, it is preserved after the induction of differentiation by NGF. This means the effects of the retention of the expanded CUG repeats carrying mRNA can be determined at any time in detail (Fig. 1a and b). With this

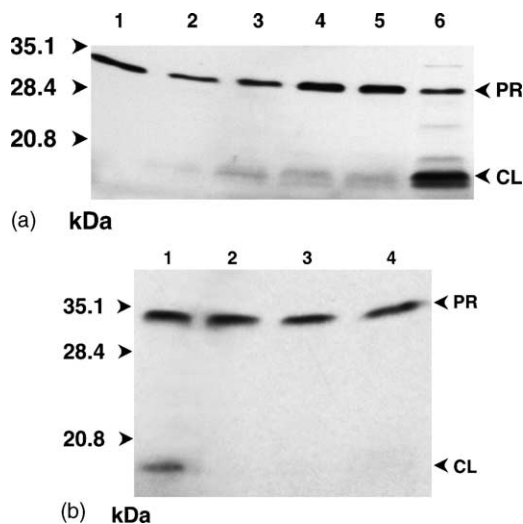


Fig. 10. Oxidative stress increases the active form of caspase-3 (CL) in CTG-250 cells (a), but the increase is inhibited by flavonoid treatment (b). (a) Long incubation of CTG-250 cells in serum-free media with NGF increases cleaved caspase-3 (active form of caspase-3) (CL) more than uncleaved caspase-3 (inactive form) (PR). LUC cells incubated after differentiation (FCS(–), NGF(+)), for 48 h (lane 1), 96 h (lane 2) and 144 h (lane 3), and CTG-250 cells incubated after differentiation (FCS(–), NGF(+)), for 48 h (lane 4), 96 h (lane 5) and 144 h (lane 6). (b) Increase in the active caspase-3 form (CL) in CTG-250 cells is inhibited by flavonoid treatment. Lane 1: no flavonoid, lane 2: ononin (5  $\mu$ g/ml), lane 3: formononetin (10  $\mu$ g/ml), lane 4: isosakuranetin (20  $\mu$ g/ml), PR: inactive form of caspase-3 (procaspase-3), CL: active form of caspase-3 (cleaved caspase-3). Positions to which molecular markers (kDa) migrate are indicated on the left.

system, we also demonstrated that some flavanones and isoflavones, in particular, isosakuranetin, inhibit the *cis*-effect and cytotoxicity caused by CTG repeat expansions.

Retention of nuclear–cytoplasm transportation is disturbed in cultured DM1 fibroblasts and myoblasts, and increased numbers of probe signals have been confirmed by the FISH method with CUG-repeat or DMPK-specific probes [8,39], resulting in decreased expression of DMPK gene products [40,41]. These phenomena, referred to as the decreased *cis*-effect and reduced cytotoxicity, which are caused by expanded CTG repeats in 3'-UTR may correspond to improvement of the *trans*-effect [5]. The nuclear membrane may have an important role in that situation [13]. Increased luciferase activity in this system, unlike in repeat-free control cells, means that the *cis*-effect of expanded CTG repeats in 3'-UTR was reduced after treatment with several flavonoids. Results of RNA blotting showed that in this system, the amount of gene translation did not differ greatly between CTG-250 and LUC cells. Therefore, the difference in luciferase activity in this system is considered to be a result of translation rather than gene transcription and is generated by repeats that produce RNA intoxication from a disturbance in nucleus-to-cytoplasm transportation in the nuclei [12].

Our findings provide evidence that the expression of expanded 250 CTG repeats in the 3'-UTR region disrupts cellular functions under oxidative stress after differentiation. Quintero-Mora et al. also reported that an expanded 90-CTG repeat in a stable cell line suppressed NGF-induced neuronal differentiation during 6 days of culture [21]. Our system showed not so strong inhibition of NGF-induced neuronal differentiation under oxidative stress, but fragility followed by apoptosis. Because gene expression efficiency differs a bit in every stable cell lines, if the repeat size is beyond the normal range, the repeat size may not necessarily correlate exactly with the toxic effect [42]. So, both 90-CTG repeats carrying a CAT reporter gene and our 250 CTG repeats with a luciferase reporter gene system are suitable for in vitro research on the effects of toxic RNA in the nucleus or tauopathy in the CNS [4,19].

Our findings also indicate that exposure to isosakuranetin, toringin, genistein, or DHEA-S protects and rescues PC12 neuronal cells that transcribe expanded CTG repeats in the 3'-UTR region of the luciferase gene from apoptotic death under the condition of oxidative stress, acting principally via its flavonoid constitution (Table 1). This is the first report of the efficaciousness of flavonoids for ameliorating the RNA gain of function caused by expanded CTG repeats. Flavonoids are reported to have various biological activities and beneficial actions against cancers, coronary heart disease [26], oxidative stress-induced toxicity [43,44,45], and  $\beta$ -amyloid-induced toxicity [46,47], among other pathologies.

Because no chemical ameliorates only cytotoxicity, flavonoids may reduce the *cis*-effect of toxic RNA, causing

a reduction in cytotoxicity. Not all the flavonoids examined were equally effective against the *cis*-effect of mRNA and cytotoxicity. The greatest inhibition was produced by isosakuranetin (Fig. 9, Table 1), toringin, genistein, and DHEA-S being less effective (Table 1). Little is known about isosakuranetin, which is present in *Poncirus trifoliata* or *Chromolaena odorata* (Eupolin), and is reported to enhance the proliferation of fibroblasts and endothelial cells [48]. Genistein, a naturally occurring isoflavonoid in soybean, is a well-known anti-carcinogen that is commonly sold as a nutrient. It is reported that the genistein lowers the risk of breast, pancreatic, and prostatic cancers [49–51], although there is a study that did not find it beneficial in vivo [52]. Some steroid derivatives, such as DHEA-S, are reported to be useful for the improvement of the clinical symptoms of muscular dystrophy [33,36]. The effectiveness of DHEA-S in treating DM1 also was confirmed in our model cell system, as was the usefulness of the system for screening various other chemicals.

A comparison of the chemical structures of isoflavones and flavanone may help us to understand how they inhibit the *cis*-effect and/or cytotoxicity. In the isoflavone group, the only difference between genistein and daidzein is the existence of the hydroxyl group at C-5 (Fig. 5h and k). Replacement of the hydroxyl group at the C-4' of daidzein with a methoxy group may increase the compound's ability to protect cells (Fig. 5i and k). However, this effect differs slightly in each cell line (CTG-250, CTG250\_1 and CTG250\_12, Tables 1 and 2). Further, if the phenolic group at C-3 in genistein is moved to C-2, the effect against cytotoxicity is lost (Fig. 5e and h), and if the hydroxyl group at C-4' in apigenin is replaced with a methyl group (Fig. 5d and e), both effects are lost. In the flavanone group, naringenin possesses a hydroxyl group at the C-5 and C-4' positions, and it prevents the *cis*-effect only (Fig. 5m). On the other hand, isosakuranetin possesses a hydroxyl group at C-5 and a methyl group at C-4' (Fig. 5n), and it prevents both the *cis*-effect and cytotoxicity constitutively. Although the only constructive difference among isosakuranetin and acacetin is the existence of a double bond between C-2 and C-3 (Fig. 5d and n), the preventative effect differs greatly.

Based on these findings, the presence of a hydroxyl group either at C-4' and C-5 in an isoflavone may inhibit cytotoxicity. Similarly, either the hydroxyl group at C-5 or the methyl group at C-4' in flavanones may inhibit cytotoxicity. It is not known how these flavonoids ameliorate cytotoxicity and the *cis*-effect, but a previous report showed that genistein and daidzein, which are the same natural soybean extract, have the same effect [51]. In our study, genistein was effective, whereas, daidzein was not. These results suggest that the basic flavonoids mechanism against neoplastic and CTG-250 PC12 cells differs in vitro.

Because repeat binding proteins such as CUG-binding protein or muscleblind protein (MBNL1/EXP) are reported to play an important role in nuclear–cytoplasm transporta-



tion and gene expression [11,15,16,18], these flavonoids may intervene in the increment or decrement of the affinity between the repeat-expanded mRNA and repeat-specific binding protein. Likewise, it is not clear whether they will be as effective in the DM1 mouse model and/or treatment of DM1 patients in this PC12-based model cell. Therefore, before starting bioflavonoid treatment, there are various factors that must be clarified by detailed examination: (1) the effectiveness of these flavonoids for DM1 model myoblast cells, (2) the permeability of the blood brain barrier to these flavonoids, and (3) the toxicity of these flavonoids to neuronal and glial cells during long-term culture. Detailed studies with this cell model of the effects of these flavonoids on repeat-mediated toxicity are needed.

In summary, we constructed a neuronal cell model that simulates the pathogenesis of DM1 and discovered that several bioflavonoids ameliorate repeat-mediated *cis*-effects, cytotoxicity, or both, that are produced by expanded CTG repeats. These bioflavonoids may have strong potential as agents against the repeat-mediated *cis*-effect, cytotoxicity or both, caused by expanded CTG DM1 or DM2 repeats. Based on the findings presented, effects mainly are attributable to polyvalent actions of the flavonoid constituents of isosakuranetin, including the ability to modulate apoptotic pathways. The protective/rescuing properties of those flavonoids underlie the potential beneficial effects against gain of function of expanded repeats carrying mRNA and other related pathologies likely to be associated with the deleterious effects of increased production of expanded CUG repeat containing RNA.

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