Dear Author,

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Mucopolysaccharidoses (MPSs) are inherited metabolic diseases caused by mutations resulting in deficiency of one of enzymes involved in degradation of glycosaminoglycans (GAGs). These compounds accumulate in cells causing their dysfunctions. Genistein is a molecule previously found to both modify GAG metabolism and modulate cell cycle. Therefore, we investigated whether the cell cycle is affected in MPS cells and if genistein can influence this process. Fibroblasts derived from patients suffering from MPS types I, II, IIIA and IIIB, as well as normal human fibroblasts (the HDFa cell line) were investigated. MTT assay was used for determination of cell proliferation, and the cell cycle was analyzed by using the MUSE® Cell Analyzer. While effects of genistein on cell proliferation were similar in both normal and MPS fibroblasts, fractions of cells in the G0/G1 phase were higher, and number of cells entering the S and G2/M phases was considerably lower in MPS II fibroblasts relative to control cells. Somewhat similar tendency, though significantly less pronounced, could be noted in MPS I, but only at longer times of incubation. However, this was not observed in MPS IIIA and MPS IIIB fibroblasts. Genistein ((5, 7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) was found to be able to partially correct the disturbances in the MPS II cell cycle, and to some extent in MPS I, at higher concentrations of this compound. The trend to increase the fractions of cells entering the S and G2/M phases was also observed in MPS IIIB fibroblasts treated with genistein. In conclusion, this is the first report indicating that the cell cycle can be impaired in MPS cells. The finding that genistein can improve the MPS II (and to some extent also MPS I) cell cycle provides an input to our knowledge on the molecular mechanisms of action of this compound.

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down-regulation of the signal transduction, which normally leads to
stimulation of expression of genes coding for enzymes involved in
GAG synthesis (Jakobkiewicz-Banecka et al., 2009). Despite different
laboratories reported various effects of genistein on GAG production
and accumulation in different cell lines (Piotrowska et al., 2006;
Jakobkiewicz-Banecka et al., 2009; Affi et al., 2010; Kloska et al., 2011;
Otomo et al., 2012; Kingma et al., 2014), very recent studies, employing
microarray analyses followed by real-time quantitative RT-PCR identi-
fied particular genes coding for enzymes necessary for GAG synthesis
which were inhibited by genistein, while most of genes for GAG
lysosomal hydrolases were stimulated by this isoavone (Moskot et
al., 2014, 2015a). This stimulation was apparently due to positive
regulation of the transcription factor EB (TfEB), a master regulator for
lysosomal biogenesis and function (Moskot et al., 2015a).

Interestingly, similar studies with global analyses of gene expression
indicated that genistein can modulate expression of genes coding for
proteins involved in DNA replication and cell cycle regulation, including
MCM2-7, CDKN1A, CDKN1C, CDKN2A, CDKN2B, CDKN2C and GADD45A
(Moskot et al., 2015b). In this light, we aimed to investigate whether cell
cycle is affected in MPS fibroblasts and if genistein can influence this
process in these cells.

2. Materials and methods

2.1. Cell lines, culture media, supplements and genistein solutions

Human Dermal Fibroblasts, adult (HDFa) (Cascade Biologics,
Portland, USA) and MPS fibroblasts (types I, II, IIA and IIIB) (Children’s
Memorial Health Institute, Warsaw, Poland) were cultured in
Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich,
Steinheim, Germany) supplemented with 10% fetal bovine serum
(FBS) and 1% antibiotic/antimycotic solution (Sigma-Aldrich, Steinheim,
Germany) supplemented with 10% fetal bovine serum
(Sigma-Aldrich) supplemented with MTT (1 mg/ml) for another 4 h.
The purple formazan crystals were dissolved in 150 ml DMSO, and
absorbance was determined at 570 nm using Wallac 1420 Multilabel
Counter (Perkin Elmer).

2.2. Cytotoxicity and proliferation assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyterazolium bro-
mide) assay was performed to estimate cell growth and proliferation.
Cells were plated in flat-bottomed 96-well plates and treated with 30,
60, and 100 μM of genistein or 0.05% DMSO as a control for 7 days at
37 °C. After incubation period, medium was replaced with RPMI
(Sigma-Aldrich) supplemented with MTT (1 mg/ml) for another 4 h.
The purple formazan crystals were dissolved in 150 ml DMSO, and
absorbance was determined at 570 nm using Wallac 1420 Multilabel
Counter (Perkin Elmer).

2.3. Cell cycle assay

The effect of genistein on cell cycle was evaluated by seeding HDFa
and MPS fibroblasts into 6-well plates at a density of 1 × 10^5 per well. The
cells were incubated for 24 h, and medium was replaced with a
fresh one supplemented with different concentrations of genistein or
DMSO in control experiments. The cultivation was continued for another
24, 48 or 72 h, and cell cycle phase was determined by MUSE® Cell
Analyzer (Merck Millipore, Germany) using a MUSE® Cell Cycle Assay
Kit (Merck Millipore, Germany) according to the manufacturer's
instructions. An average of at least 10,000 cells was analyzed for each
condition. Triplicate independent experiments were conducted.

3. Results

It was demonstrated previously that genistein inhibits proliferation
of cancer cells (Pavese et al., 2010). However, the effect of this isoava-
one on normal fibroblasts was found to be moderate (Moskot et
al., 2015b). We have tested the influence of genistein on proliferation of
MPS fibroblasts. The effects of this isoavone were dose-dependent,
but similarly to normal fibroblasts (HDFa cell line), moderate slow
down of proliferation was observed for MPS fibroblasts (Table 1).
To test whether cell cycle is affected in MPS cells, we have analyzed
this process, using the MUSE® Cell Analyzer, in MPS I, II, IIA and IIIB
fibroblasts relative to normal fibroblasts (HDFa). Fractions of cells in
G0/G1, S, and G2/M phases were determined after 24, 48 and 72 h of
incubation. We found that the cell cycle was disturbed in MPS II fibro-
blasts in comparison to HDFa cells (Fig. 1). Namely, fractions of cells in
the G0/G1 phase were significantly higher (p < 0.05), while number of
cells entering the S and G2/M phases was lower in MPS II relative to
HDFa (Fig. 1). Similar tendency could be noted also in MPS I fibroblasts
at later times of incubation, however, the differences did not reach sta-
tistical significance (Fig. 1). No such effects were observed in MPS IIA
and MPS IIIB fibroblasts.

Since genistein was found to modulate expression of genes involved
in the regulation of the cell cycle (Moskot et al., 2015b), we asked
whether this isoavone may influence the effects observed in MPS fi-
broblasts. Three concentrations of genistein were used (30, 60 and
100 μM), and the experiments were repeated under conditions de-
scribed above. In these experiments, distributions of fractions of MPS I
and II cells at particular phases of the cell cycle were changed towards
the pattern observed in normal fibroblasts. The G0/G1 fraction de-
creased while the S and G2/M fractions (counted together) increased,
particularly (in MPS II) or exclusively (in MPS I) at longer times of incu-
bation and at higher genistein concentrations (Fig. 1). Although no sig-
ificant changes in the cell cycle were observed in MPS IIA and IIIB
fibroblasts relative to HDFa cells without genistein, in the presence of
this compound, the tendency to increase the S and G2/M fractions was
similar to that found in MPS I and II fibroblasts (Fig. 1).

4. Discussion

Different cellular defects in MPS were reported previously (for re-
views, see (Muenzer, 2011; Cimaz and La Torre, 2014)), however, this
study demonstrates for the first time specific changes in MPS cell
cycle. Less effective progression into S and G2/M phases, observed in
this work (Fig. 1) may be compatible with the phenotypes of patients
which include slower growth and delayed development (Cimaz and
La Torre, 2014; Wraith, 2013). Interestingly, the body growth is not
inhibited in MPS IIA and IIIB (Cimaz and La Torre, 2014), and the cell
cycle did not differ significantly in corresponding fibroblasts, relative
to normal (HDFa) fibroblasts. Since the primary effects of dysfunctions

Table 1

<table>
<thead>
<tr>
<th>Genistein (μM)</th>
<th>Absorbance at 570 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDFa</td>
<td>MPS</td>
</tr>
<tr>
<td>0</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>30</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>60</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td>100</td>
<td>0.6 ± 0.0</td>
</tr>
</tbody>
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4 The values were determined for cultures treated for 7 days with the tested compound at indicated concentrations. Data represent mean ± SD from n ≥ 3.
5 MPS represents collected results from MPS I, MPS II, MPS IIA and MPS IIB fibroblasts.

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of α-L-iduronidase (in MPS I) and iduronate-2-sulfatase (in MPS II) are accumulations of GAGs (DS and HS), the disturbance of the cell cycle in MPS I and II cells is likely a secondary or tertiary effect. In fact, GAGs may interfere with various metabolic pathways, and can disturb basic cellular functions (for a review, see Wegrzyn, 2012). Moreover, abnormalities in basic metabolism may interfere with the cell cycle, as demonstrated recently for glycolysis and the Krebs cycle (Konieczna et al., 2015). Considering differences observed between MPS I/MPS II and MPS III fibroblasts, one might suggest that the DS and HS storage (characteristic for MPS I and II), but not necessary the sole storage of HS (observed in MPS IIIA and IIIB), could disturb (perhaps indirectly) the cell cycle.

In our experiments, the duration of fibroblasts’ treatment was 7 days when cell growth was tested, and up to 3 days when effects of genistein on cell cycle were assessed. These differences were due to experimental requirements, employed in order to obtain interpretable and reliable results. Estimation of effects of a tested compound on cell growth requires a relatively long time (like a weak), particularly if the effects are not strong (as in the case of genistein). On the other hand, to test cell cycle progress, continuation of experiments over 3 days would not.

Fig. 1. Cell cycle analysis in HDFa and MPS fibroblasts, and effects of genistein on cell cycle progression in MPS cells. HDFa, MPS I, MPS II, MPS IIA and MPS IIB fibroblasts were investigated using the MUSE® Cell Analyzer at 24, 48, and 72 h of incubation with either no genistein (empty columns for HDFa, and closed columns for MPS) or 30 (grey columns), 60 (lined columns) or 100 (dotted columns) μM genistein. The indicated data represent mean values ±SD from n ≥ 3 independent experiments. Asterisks indicate p < 0.05 in t-Student test (MPS vs. non-treated HDFa). Results of control experiments (with HDFa cells) are as described previously (Moskot et al., 2015b) (one should note a high reproducibility of results of these experiments).
Genistein was reported to improve GAG storage in MPS cells due to inhibition of GAG synthesis and stimulation of lysosomal biogenesis and function through positive regulation of the TFEB transcription factor (Piotrowska et al., 2006; Jakóbkiewicz-Banecka et al., 2009; Arfi et al., 2010; Kloska et al., 2011; Otomo et al., 2012; Moskot et al., 2014, 2015a). Despite the fact that some other authors could not confirm such effects of genistein in cell culture experiments (Kingma et al., 2014) and in the mouse model of MPS I (Kingma et al., 2015), positive results of studies on other animal models (Malinowska et al., 2009, 2010; Friso et al., 2010) encouraged researchers to perform clinical trials with this isoflavone. Pilot clinical investigations indicated some biochemical improvements in blood and urine samples (Piotrowska et al., 2012; Malinowska et al., 2012; de Ruiter et al., 2012), as well as some functional benefits for patients (Piotrowska et al., 2011). Moreover, it was found that high dose genistein therapy is safe for MPS patients (Kim et al., 2012). Currently, a phase III, double blind, randomized, placebo controlled clinical trial with high dose genistein aglycone administered orally to patients suffering from Sanfilippo syndrome (MPS III) is ongoing (http://public.ukcrn.org.uk/search/StudyDetail.aspx?StudyID=16209). We assume that results reported in this paper may facilitate understanding of genistein effects on MPS cells.

In conclusion, this study demonstrates that genistein can improve disturbances in the cell cycle which are evident in MPS II fibroblasts, and might also appear in MPS I. At the current stage of our knowledge it is not possible to determine whether these effects of genistein are due to a decreased GAG storage or modulation of expression of genes which products are involved in the cell cycle regulation or both. Nevertheless, the partial correction of the cell cycle indicates another kind of cellular processes (apart from previously reported decreased GAG synthesis and storage (Piotrowska et al., 2006; Jakóbkiewicz-Banecka et al., 2009; Arfi et al., 2010; Kloska et al., 2011; Otomo et al., 2012) and enhanced lysosomal biogenesis and function (Moskot et al., 2014)) that can be improved by genistein in MPS cells.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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M. Moskot et al. / Gene xxx (2016) xxx–xxx 5

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