Nanoscale drug-delivery systems of flavonoids based on supramolecular carriers targeting fibrosis in diabetes complications (DDSFLAVODIA)

PN-III-P4-ID-PCE-2020-1772

SCIENTIFIC REPORT – Phase 1 - 2021

Abstract:

The human Adult Retinal Pigment Epithelial cell line-19 (ARPE-19) and Embryonic rat cardiac H9c2 (2-1) cells were cultured and two days after seeding, cells were split and exposed at 37°C and 5% CO_2 for 9 days to: 5 mM d-glucose (normal glucose, NG); 35 mM d-glucose (Life Technologies A24940-01) (high glucose, HG); 5 mM d-glucose and 30 mM mannitol (Sigma 69-65-8) as negative control (NG+M). The exposition of ARPE or H9c2 cells to high glucose (35 mM, HG) for 9 days significantly altered cell morphology and reduced the percentage of cell viability compared to cells exposed to normal glucose alone or with mannitol 30 mM (NG+M).

A significant increase of AP4S1 levels was evident in HG cells or NG+M treatments. The up-regulation of AP4 was paralleled by a significant increase of Gal-1 positive cells percentage in ARPE and H9c2 cells exposed to HG, which was absent in cells exposed to NG alone or with mannitol. Accordingly, Gal-1 protein levels was significantly increased in HG cells compared to NG alone or with mannitol (Activity A1.1.).

Both of ARPE and cells H9c2 exhibited an up-regulation of TGF-β1, TGF-βR1 and TGF-βR2 mRNA levels compared to NG cells. NG+M cells did not show any alterations of the mRNA levels compared to NG group. Similarly, the stimulation with HG of ARPE or H9c2 cells increased pSMAD2, SMAD2 and SMAD4 levels. These up-regulation was not shown by cells exposed to NG or to NG+M (Activity A.2.1)

ARPE or H9c2 cells exposed to HG showed a significant increase of P-p38 / p38 MAPK ratio, compared to cells exposed to NG and to NG+M. p38 MAPK phosphorylation was paralleled by a higher percentage of positive NF-kB cells and higher Nf-kB protein levels in HG treatment, as well as by significantly increased ROS levels.

A 1.1. In vitro studies of the cellular upregulation of Gal-1 under hyperglycemic conditions.

Materials and Methods

1) Cell cultures

ARPE

The human Adult Retinal Pigment Epithelial cell line-19 (ARPE-19), obtained from the American Type Culture Collection (ATCC), were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium/nutrient mixture F12 (DMEM/F12, Aurogene AU-L0093), with a glucose concentration of 5 mM. The medium was supplemented with 10% heat inactivated fetal bovine serum (FBS) (AU-S181H Aurogene, Italy), 1% penicillin/streptomycin solution (P/S) (Aurogene Au-L0022), 1% L-Glutamine (L-Glu), Hepes 5 mM (Thermo Fisher 15630080) and 7.5% NaHCO3 (Thermo Fischer 25080094). Two days after seeding, cells were split and exposed at 37°C and 5% CO_2 for 9 days [1] to:

- 5 mM d-glucose (normal glucose, NG);
- 35 mM d-glucose (Life Technologies A24940-01) (high glucose, HG);
- 5 mM d-glucose and 30 mM mannitol (Sigma 69-65-8) as negative control (NG+M).

ARPE-19 morphology was daily observed with optic microscope (Leica DMi1, Germany). After the stimulation period, cells and supernatants were collected and preserved for down-stream analysis. For each assay, three independent experiments were performed, each done in triplicate.

H9c2

Embryonic rat cardiac H9c2 (2-1) cells (ECACC, United Kingdom) were cultured in Dulbecco's modified Eagle's medium (DMEM; AU-L0101Aurogene, Italy), containing 5.5 mM d-glucose and supplemented with 10% heat inactivated FBS, 1% L-Glu and 1% P/S, at 37°C under an atmosphere of 5% CO_2. Then, H9c2 cells were exposed at 37°C and 5% CO_2 for 48 hours [2] to:

- 5.5 mM d-glucose (normal glucose, NG);
- 33 mM d-glucose (high glucose, HG);
- 5.5 mM d-glucose + 27.5 mannitol as negative control (NG+M).

H9c2 cell morphology was daily observed with optic microscope (Leica DMi1, Germany). After the stimulation period, cells and supernatants were collected and preserved for down-stream analysis. For each assay, three independent experiments were performed, each done in triplicate.

2) Cell viability assay

Cell viability was measured by 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Particularly, ARPE-19 (6×10^3 cell/well) and H9c2 (5×10^3 cells/well) cells were seeded in 96-well plates and exposed to normal glucose, high glucose or mannitol, as previously described. At the end of the stimulation period, MTT solution (1:10 in culture medium, 300 µl/well) was added to each well, incubated for 4 h at 37°C and then removed. Each well was then washed at dark for 20 min

with isopropanol-HCl 0.2 N. Optical density (OD) values were measured at 570 nm using a 96-well plate reader (iMark, Bio-Rad Laboratories, Italy).

3) Immunocytochemistry

For immunocytochemical analysis, ARPE-19 and H9c2 (both 25×10^4 cell/well) were plated on slides in 24-well plates and exposed to normal glucose, high glucose or mannitol, as previously described. Cells were fixated with 4% paraformaldehyde and washed with phosphate buffered saline (PBS) (AU-L0615 Aurogene, Italy). Then, in order to inhibit non-specific antibody binding, cells were incubated for 60 min in blocking solution with 3% bovine serum albumin (BSA) (Sigma A7906) and 0.3% Triton X-100 (Sigma 93443) in PBS. Primary antibodiy was diluted in PBS blocking buffer (1% BSA, 0.1% Triton X-100) and slides were incubated overnight at 4°C in primary antibodies to human/rat Galectin 1 (Gal-1) (Cell Signaling 13888; dilution 1:200, to rabbit).

Fluorescent-labeled anti-rabbit (Invitrogen 11008; dilution 1:1000) secondary antibody was used to locate the specific antigens in each slide. Cells were counterstained and mounted with VECTASHIELD Antifade Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) (Novus Biologicals H-1200-NB). Fluorescently-labeled slides were viewed with a fluorescence microscope (Leica, Wetzlar, Germany) and with a fluorescence confocal microscope (LSM 710, Zeiss, Oberkochen, Germany). Immunofluorescence images were analyzed with Leica FW4000 software (Leica, Wetzlar, Germany) and with Zen Zeiss software (Zeiss, Oberkochen, Germany). The percentage of positive cells in each microscope field was calculated by the number of green positive cells of 400 cells in four different microscope fields for each treatment, by considering only DAPI counterstained cells as positive profiles. Data were reported as mean percentage of green positive cells / total cells counted ± standard deviation (S.D.).

4) Western Blotting

After trypsinization, ARPE-19 cells were resuspended in RIPA buffer (Sigma, R0278) containing protease and phosphatase inhibitors. After centrifuging samples at 12,000 rpm for 10 min at 4°C, protein levels in the supernatants were determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, 500-0006). Proteins were then separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then electrotransferred to polyvinylidene difluoride membranes (Merck Millipore, IPFL10100). These were blocked for 1 hr with a 5% non-fat dry milk / Tris Buffered Saline solution (Euroclone, EMR180500; Cell Signaling 12498) before the incubation at 4°C over-night with the following human primary antibodies: anti-Gal-1 (ThermoFisher PA5-95213; 0.3 µg/mL) . Horseradish peroxidase-conjugated secondary anti-rabbit (Santa Cruz, sc-2004; dilution 1:5000) antibody was used to incubate the blots at room temperature for 1 hr. Immunoreactive dots were visualized by an enhanced chemiluminescence system (ThermoFisher, 35055), then quantified with VisionWorks Life Science Image Acquisition and Analysis software (UVP, Upland, CA, USA) and expressed as densitometric units (DU). Gal-1 protein levels were normalized by using β-actin protein levels (Santa Cruz sc-47778, dilution 1:1000).

5) *Statistical analysis*

The results are reported as mean ± S.D. of three independent experiments, each performed in triplicate. Statistical significance was determined using one-way Analysis of Variance (ANOVA)

followed by Tukey's comparison test. A P-value less than 0.05 was considered significant to reject the null hypothesis.

Results

1) ARPE cells

A) Cell viability

The exposition of ARPE cells to high glucose (35 mM, HG) for 9 days significantly altered cell morphology and reduced the percentage of cell viability (48 ± 6, $P < 0.01$ vs NG) compared to cells exposed to normal glucose alone (5 mM, NG; percentage of cell viability: 90 ± 4) or with mannitol 30 mM (NG+M; percentage of cell viability: 87 ± 7).

B) AP4 / Galectin 1 axis

A significant increase of AP4S1 levels was evident in HG cells (1086 ± 130 pg/ml; $P < 0.01$ vs NG) compared to NG (494 ± 49 pg/ml) or NG+M (493 ± 73 pg/ml) treatments. The up-regulation of AP4 was paralleled by a significant increase of Gal-1 positive cells percentage in ARPE exposed to HG (56 ± 10; $P < 0.01$ vs NG), which was absent in ARPE cells exposed to NG alone (13 ± 4 %) or with mannitol (19 ± 7 %). Accordingly, Gal-1 protein levels was significantly increased in HG cells compared to NG alone or with mannitol (fold = 10 ± 2; $P < 0.01$ vs NG).

2) H9c2 cells

A) Cell viability

The exposition of H9c2 cells to high glucose (33 mM, HG) for 2 days significantly altered cell morphology and reduced the percentage of cell viability (45 ± 9, $P < 0.01$ vs NG) compared to cells exposed to normal glucose alone (5.5 mM, NG; percentage of cell viability: 94 ± 3) or with mannitol 27.5 mM (NG+M; percentage of cell viability: 90 ± 5).

B) AP4 / Galectin 1 axis

A significant increase of AP4S1 levels was evident in HG cells (1034 ± 54 pg/ml; $P < 0.01$ vs NG) compared to NG (407 ± 23 pg/ml) or NG+M (402 ± 53 pg/ml) treatments. The up-regulation of AP4 was paralleled by a significant increase of Gal-1 positive cells percentage in H9c2 cells exposed to HG (70 ± 4; $P < 0.01$ vs NG), which was absent in cells exposed to NG alone (20 ± 3 %) or with mannitol (23 ± 4 %).

A 1.2. In vitro studies of the TGFβ1 signalling upregulation under hyperglycemic conditions.

Materials and Methods

1) Cell cultures

ARPE

The human Adult Retinal Pigment Epithelial cell line-19 (ARPE-19), obtained from the American Type Culture Collection (ATCC), were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium/nutrient mixture F12 (DMEM/F12, Aurogene AU-L0093), with a glucose concentration of 5 mM. The medium was supplemented with 10% heat inactivated fetal bovine serum (FBS) (AU-S181H Aurogene, Italy), 1% penicillin/streptomycin solution (P/S) (Aurogene Au-L0022), 1% L-Glutamine (L-Glu), Hepes 5 mM (Thermo Fisher 15630080) and 7.5% NaHCO3 (Thermo Fischer 25080094). Two days after seeding, cells were split and exposed at 37°C and 5% CO_2 for 9 days [1] to:

- 5 mM d-glucose (normal glucose, NG);
- 35 mM d-glucose (Life Technologies A24940-01) (high glucose, HG);
- 5 mM d-glucose and 30 mM mannitol (Sigma 69-65-8) as negative control (NG+M).

ARPE-19 morphology was daily observed with optic microscope (Leica DMi1, Germany). After the stimulation period, cells and supernatants were collected and preserved for down-stream analysis. For each assay, three independent experiments were performed, each done in triplicate.

H9c2

Embryonic rat cardiac H9c2 (2-1) cells (ECACC, United Kingdom) were cultured in Dulbecco's modified Eagle's medium (DMEM; AU-L0101Aurogene, Italy), containing 5.5 mM d-glucose and supplemented with 10% heat inactivated FBS, 1% L-Glu and 1% P/S, at 37°C under an atmosphere of 5% CO_2. Then, H9c2 cells were exposed at 37°C and 5% CO_2 for 48 hours [2] to:

- 5.5 mM d-glucose (normal glucose, NG);
- 33 mM d-glucose (high glucose, HG);
- 5.5 mM d-glucose + 27.5 mannitol as negative control (NG+M).

H9c2 cell morphology was daily observed with optic microscope (Leica DMi1, Germany). After the stimulation period, cells and supernatants were collected and preserved for down-stream analysis. For each assay, three independent experiments were performed, each done in triplicate.

2)Reactive Oxygen Species (ROS) assessment

ROS levels were detected by the conversion of the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to highly fluorescent dichlorofluorescein (DFC) diacetate within cells by ROS.

ARPE-19 and H9c2 cells were seeded in 6 well plates (both 7 x 10^4 cells/well) and exposed to normal glucose, high glucose or mannitol, as previously described. At the end of the stimulation period, cells were loaded with 20 µM DCFH-DA in medium with 5% FBS at 37°C for 30 min, then were trypsinized. Total intracellular ROS production was measured with a fluorometric plate reader at an excitation of 485 nm and an emission of 530 nm. Both cell types were exposed to medium 5% FBS withouth DCFH-DA as negative control (CTR-) or incubated with H_2O_2 (100 µM) 30 min before trypsinization as a positive control (CTR+).

3)Immunocytochemistry

For immunocytochemical analysis, ARPE-19 and H9c2 (both 25×10^4 cell/well) were plated on slides in 24-well plates and exposed to normal glucose, high glucose or mannitol, as previously described. Cells were fixated with 4% paraformaldehyde and washed with phosphate buffered saline (PBS) (AU-L0615 Aurogene, Italy). Then, in order to inhibit non-specific antibody binding, cells were incubated for 60 min in blocking solution with 3% bovine serum albumin (BSA) (Sigma A7906) and 0.3% Triton X-100 (Sigma 93443) in PBS. Primary antibodies were diluted in PBS blocking buffer (1% BSA, 0.1% Triton X-100) and slides were incubated overnight at 4°C in primary antibodies to human/rat nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (Cell Signaling 6956; dilution 1:500, to mouse).

Fluorescent-labeled anti-mouse (Life Techonologies A21202; dilution 1:1000) secondary antibodies were used to locate the specific antigens in each slide. Cells were counterstained and mounted with VECTASHIELD Antifade Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) (Novus Biologicals H-1200-NB). Fluorescently-labeled slides were viewed with a fluorescence microscope (Leica, Wetzlar, Germany) and with a fluorescence confocal microscope (LSM 710, Zeiss, Oberkochen, Germany). Immunofluorescence images were analyzed with Leica FW4000 software (Leica, Wetzlar, Germany) and with Zen Zeiss software (Zeiss, Oberkochen, Germany). The percentage of positive cells in each microscope field was calculated by the number of green positive cells of 400 cells in four different microscope fields for each treatment, by considering only DAPI counterstained cells as positive profiles. Data were reported as mean percentage of green positive cells / total cells counted ± standard deviation (S.D.).

4)Enzyme-Linked Immunosorbent Assay (ELISA)

Cell-biased ELISA assays were performed for both ARPE-19 and H9c2 cells to analyse the cellular levels of p38 Mitogen-activated protein kinase (MAPK, phosphorylated/total) (RayBiotech, CBEL-P38-1), SMAD Family Member 2 (SMAD2, phosphorylated/total) (LSBio, LS-F1057-1) and SMAD Family Member 4 (SMAD4, total) (LSBio, LS-F2315-1). Competitive ELISA tests were used to quantify cellular Adaptor Related Protein Complex 4 Subunit Sigma 1 (AP4S1, total) (My Biosource, MBS7206462 for ARPE-19 and ARP, E02A1578 for H9c2).

5)Western Blotting

After trypsinization, ARPE-19 cells were resuspended in RIPA buffer (Sigma, R0278) containing protease and phosphatase inhibitors. After centrifuging samples at 12,000 rpm for 10 min at 4°C, protein levels in the supernatants were determined by Bio-Rad Protein Assay (Bio-Rad Laboratories,

500-0006). Proteins were then separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then electrotransferred to polyvinylidene difluoride membranes (Merck Millipore, IPFL10100). These were blocked for 1 hr with a 5% non-fat dry milk / Tris Buffered Saline solution (Euroclone, EMR180500; Cell Signaling 12498) before the incubation at 4°C over-night with the following human primary antibody anti-Nf-kB (Cell Signaling 6956S; dilution 1:1000). Horseradish peroxidase-conjugated secondary anti-mouse (Santa Cruz, sc-2005; dilution 1:5000) antibodies were used to incubate the blots at room temperature for 1 hr. Immunoreactive dots were visualized by an enhanced chemiluminescence system (ThermoFisher, 35055), then quantified with VisionWorks Life Science Image Acquisition and Analysis software (UVP, Upland, CA, USA) and expressed as densitometric units (DU). Gal-1, NFkB and IkBβ protein levels were normalized by using β-actin protein levels (Santa Cruz sc-47778, dilution 1:1000).

6)Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA was isolated from ARPE-19 and H9c2 lysates following the miRNeasy Mini kit (Qiagen, 217004). RNA concentration and purity was determined by using the NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific). Genomic DNA (gDNA) contaminations were eliminated from RNA samples before the Reverse Trascription (RT) step, carried out on the Gene AMP PCR System 9700 (Applied Biosystems) by using the QuantiTect Reverse Transcription kit (205311, Qiagen), according to the protocol "Reverse Transcription with Elimination of Genomic DNA for Quantitative, Real-Time PCR". The final step for Real Time PCR (qPCR) analysis was carried out in triplicate on the CFX96 Real-time System C1000 Touch Thermal Cycler (BIORAD). This was performed according to the protocol "Two-Step RT-PCR (Standard Protocol)", by using the QuantiTect SYBR Green PCR Kit (204143, Qiagen) and specific QuantiTect Primer Assays (249900, Qiagen) for each gene tested [Transforming Growth Factor β1 (TGF-β1): QT00000728 and QT00187796; Transforming Growth Factor β receptor 1 (TGF-βR1): QT00083412 and QT00190953; Transforming Growth Factor β receptor 2 (TGF-βR2): QT00014350 and QT01800596). Relative quantization of gene expression was performed by using the $2^{\wedge-\Delta\Delta Ct}$ method, by using GAPDH (QT00079247 and QT01082004) as housekeeping control gene.

7)Statistical analysis

The results are reported as mean ± S.D. of three independent experiments, each performed in triplicate. Statistical significance was determined using one-way Analysis of Variance (ANOVA) followed by Tukey's comparison test. A P-value less than 0.05 was considered significant to reject the null hypothesis.

Results

ARPE cells

A).TGFβ / SMAD signaling

ARPE cells exhibited an up-regulation of TGF-β1 ($2^{\wedge-\Delta\Delta Ct}$: 4.8 ± 0.9; P < 0.01 vs NG), TGF-βR1 ($2^{\wedge-\Delta\Delta Ct}$: 2.1 ± 0.6; P < 0.05 vs NG) and TGF-βR2 ($2^{\wedge-\Delta\Delta Ct}$: 2.8 ± 0.3; P < 0.01 vs NG) mRNA levels compared to NG cells ($2^{\wedge-\Delta\Delta Ct}$ for TGF-β1: 1.9 ± 0.4; $2^{\wedge-\Delta\Delta Ct}$ for TGF-βR1: 1.1 ± 0.2; $2^{\wedge-\Delta\Delta Ct}$ for TGF-βR2: 1.2 ± 0.2). NG+M cells did not show any alterations of the mRNA levels compared to NG group ($2^{\wedge-\Delta\Delta Ct}$ for TGF-β1: 2.0 ± 0.4; $2^{\wedge-\Delta\Delta Ct}$ for TGF-βR1: 1.2 ± 0.2; $2^{\wedge-\Delta\Delta Ct}$ for TGF-βR2: 1.1 ± 0.2).

Similarly, the stimulation with HG of ARPE cells increased pSMAD2 (1.9 ± 0.5 O.D.; P < 0.01 vs NG), SMAD2 (1.3 ± 0.2 O.D.; P < 0.01 vs NG) and SMAD4 (2.0 ± 0.1 O.D.; P < 0.01 vs NG) levels.

These up-regulation was not shown by cells exposed to NG (pSMAD2: 0.5 ± 0.2; SMAD2: 0.5 ± 0.1; SMAD4: 0.6 ± 0.3 O.D.) or to NG+M (pSMAD2: 0.6 ± 0.3; SMAD2: 0.5 ± 0.3; SMAD4: 0.7 ± 0.2 O.D.).

H9c2 cells

A).p38 MAPK / NF-kB / ROS pathway

ARPE cells exposed to HG showed a significant increase of P-p38 / p38 MAPK ratio (3.4 ± 0.6; P < 0.01 vs NG), compared to cells exposed to NG (1.5 ± 0.4) and to NG+M (1.6 ± 0.3). p38 MAPK phosphorylation was paralleled by a higher percentage of positive NF-kB cells (69 ± 11; P < 0.01 vs NG) and higher Nf-kB protein levels (fold = 100 ± 17) in HG treatment, as well as by significantly increased ROS levels (% of DFCH-DA: 66 ± 6; P < 0.01 vs NG).

B).TGFβ / SMAD signaling

HG cells exhibited an up-regulation of TGF-β1 ($2^{-\Delta\Delta Ct}$: 3.9 ± 0.3; P < 0.01 vs NG), TGF-βR1 ($2^{-\Delta\Delta Ct}$: 2.1 ± 0.3; P < 0.05 vs NG) and TGF-βR2 ($2^{-\Delta\Delta Ct}$: 1.7 ± 0.3; P < 0.01 vs NG) mRNA levels compared to NG cells ($2^{-\Delta\Delta Ct}$ for TGF-β1: 1.6 ± 0.4; $2^{-\Delta\Delta Ct}$ for TGF-βR1: 1.0 ± 0.4; $2^{-\Delta\Delta Ct}$ for TGF-βR2: 0.7 ± 0.2). NG+M cells did not show any alterations of the mRNA levels compared to NG group ($2^{-\Delta\Delta Ct}$ for TGF-β1: 1.7 ± 0.6; $2^{-\Delta\Delta Ct}$ for TGF-βR1: 1.1 ± 0.5; $2^{-\Delta\Delta Ct}$ for TGF-βR2: 0.8 ± 0.3).

Similarly, the stimulation with HG of H9c2 cells increased pSMAD2 (2.4 ± 0.3 O.D.; P < 0.01 vs NG), SMAD2 (1.9 ± 0.1 O.D.; P < 0.01 vs NG) and SMAD4 (2.3 ± 0.3 O.D.; P < 0.01 vs NG) levels. These up-regulation was not shown by cells exposed to NG (pSMAD2: 0.8 ± 0.3; SMAD2: 0.9 ± 0.2; SMAD4: 1.0 ± 0.2 O.D.) or to NG+M (pSMAD2: 0.9 ± 0.1; SMAD2: 0.5 ± 0.3; SMAD4: 1.0 ± 0.2 O.D.).

C) p38 MAPK / NF-kB / ROS pathway

H9c2 cells exposed to HG showed a significant increase of P-p38 / p38 MAPK ratio (2.6 ± 0.3; P < 0.01 vs NG), compared to cells exposed to NG (1.0 ± 0.2) and to NG+M (1.0 ± 0.2). p38 MAPK phosphorylation was paralleled by a higher percentage of positive NF-kB cells in HG treatment (73 ± 4; P < 0.01 vs NG), as well as by significantly increased ROS levels (% of DFCH-DA: 65 ± 11; P < 0.01 vs NG). These was lower in cells stimulated with NG (% of NF-kB positive cells: 11 ± 3; % of DFCH-DA: 11 ± 3) and NG+M (% of NF-kB positive cells: 14 ± 3; (% of DFCH-DA: 13 ± 3).

References

1. Maisto R.; Oltra M.; Vidal-Gil L.; Martínez-Gil N.; Sancho-Pellúz J.; Di Filippo C.; Rossi S.; D´Amico M.; Barcia J.M.; Romero F.J. ARPE-19-derived VEGF-containing exosomes promote neovascularization in HUVEC: the role of the melanocortin receptor 5. Cell Cycle, 2019, 18(4): 413-24, doi: 10.1080/15384101.2019.1568745.

2. Trotta M.C.; Maisto R.; Alessio N.; Hermenean A.; D'Amico M.; Di Filippo C. The melanocortin MC5R as a new target for treatment of high glucose-induced hypertrophy of the cardiac H9c2 cells, Front Physiol., 2018, 9:1475, doi: 10.3389/fphys.2018.01475.

A 2.1. Obtaining and characterization of the new supramolecular complex.

Materials and Methods

1. Materials

Calixarene 0118 (OTX008) was purchased from Selleck Chemicals GmbH. Sulfobutylated β-cyclodextrin sodium salt (SBECD) (DS~4) and soluble sulfobutylated beta-cyclodextrin polymer (SBECDPolymer) were the product of Cyclolab Ltd. (Budapest, Hungary). Chrysin (5,7-Dihydroxyflavone) was purchased from Alfa Aesar (by ThermoFisher Scientific, Kandel, Germany), and all other reagents are from Sigma.

2. Methods

2.1. Solubilization studies

2.1.1. OTX008 solulbilization with SBECD and SBECDPolymer

At first 7.3 m/m% SBECD and 9.6 m/m% SBECDPolymer solutions were prepared using ultrapure water (Millipore Direct-Q 5UV system, Merck Millipore, Burlington, MA, USA), then OTX008 was dissolved in the cyclodextrin solutions at a 0.75 mg/ml final concentration to get OTX-SBECD and OTX-SBECDPolymer solutions.

2.1.2. Chrysin solubilization in OTX008-cyclodextrin solutions

Chrysin was added in excess to the OTX-SBECD and OTX-SBECDPolymer solutions prepared by the method 2.1.1. and shaken for 72 hours at room temperature in closed vials. After incubation the samples were centrifuged at 11000 rpm for 10 minutes, the clear supernatant was removed and the dissolved chrysin concentration was measured by UV spectrophotometer (Shimadzu UV-1900) at 270 nm. Chrysisn solubilization was also performed in 7.3 m/m% SBECD and 9.6 m/m% SBEβCDPolymer solutions without OTX008 with the same incubation and sample preparation.

The clear chrysin solutions were frozen at −110 °C, and the samples were lyophilized using a ScanVac CoolSafe freeze dryer (Labogene, Allerød, Denmark). The complexes were stored at −20 °C until used in further experiments.

2.1.3. Phase-solubility study

7.3 m/m% SBECD solution, 9.6 m/m% SBECDPolymer solution, OTX-SBECD and OTX-SBECDPolymer solutions were prepared and diluted in 96-well plates with ultrapure water. Excess amount of chrysin was added to each well, the plate was sealed with plate sealer and was shaken for 72 hours at room temperature. The samples were filtered with a MultiScreen Solvinert 96 Well Filter Plate (pore size 0.45 µm, PTFE, Merck Millipore Ltd. Tullagreen, Ireland) using a MultiScreen Resist

vacuum manifold (EMD Millipore corporation, Burlington MA, USA) and the clear supernatants were placed on a Greiner UV-Star® 96 well plate. Samples were measured at 270 nm by a Thermo Fisher Multiskan Go (Thermo Fisher, Waltham, MA, USA) microplate reader.

2.1.4. Size distribution measurement of the cyclodextrin complexes with dynamic light scattering (DLS)

The size distribution of particles in 7.3 m/m% SBECD solution, 9.6 m/m% SBECDPolymer solution, OTX-SBECD and OTX-SBECDPolymer solutions with the complexed chrysin after the phase solubility test or without chrysin was determined by a Malvern Nano-ZS Zetasizer (Malvern Instruments, Malvern, UK).

2.1.5. pH-dependent chrysin solubility determination

7.3 m/m% SBECD solutions were prepared in sodium acetate - acetic acid buffer with different pH values (pH= 3.65; 5.75; 7.45; 9.95) and placed into a 96 plate. Excess amount of chrysin was added to the wells, the plate was sealed and the chrysin solubility was determined as described in 2.1.3.

In another experimental setup the pH of the 7.3 m/m% SBECD and OTX-SBECD solutions were set to 8.64 and 3.65 with 0.2 M sodium acetate solution and 0.1 M HCl respectively and chrysin solubilization test was performed as described earlier.

A 1.4. Dissemination

International conferences:
Ferenc Fenyvesi, Judit Váradi, Hildegard Herman, Cornel Balta, Anca Hermenean, Application of cyclodextrins and calixarenes in the nanoformulations of bioactive compounds , 42nd Anniversary Symposium Of The Institute Of Cellular Biology And Pathology “Nicolae Simionescu” and 38th Annual Scientific Session Of The Romanian Society For Cell Biology. Virtual. Book of abstracts, p. 53 **Prezentare orală**

Fenyvesi Ferenc, Váradi Judit, Bácskay Ildikó, Vecsernyés Miklós, Anca Hermenean, Simultaneous application of calixarene and cyclodextrin for solubility improvement, Gyógyszerkémiai és Gyógyszertechnológiai Szimpózium ’21, Herceghalom 2021. szeptember 20-21 **Prezentare orală**.