CANCER CELL-SPECIFIC INDUCTION OF ENDOPLASMIC RETICULUM STRESS BY SMALL MOLECULES FOUND IN THE SERUM

DOCTORAL (Ph.D.) THESIS

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1. Summary

The main goal of this Ph.D. project was to provide a mechanism for apoptosis induction elicited by a mixture containing amino acids, monosaccharides, nucleobases, etc.

We demonstrated that a defined mixture of small molecules selectively induces endoplasmic reticulum stress and activates the unfolded protein response (UPR) signaling cascade in cancer cells, which leads to the activation of a pro-apoptotic transcription program.

We have revealed a novel miRNA mediated feedback mechanism of the transcriptional upregulation of certain UPR signaling components.

In addition, we have identified several new molecules found in the serum capable of enhancing the effect of the AM ("active mixture") which have substantial cancer cell growth inhibitory effect when applied in a mixture.

2. Introduction

In our earlier studies we have experimentally selected some molecules present in the serum whose mixture (AM) produced a selective *in vitro* and *in vivo* toxic effect on various tumor cell lines, but not on normal cells. The AM is composed of certain essential amino acids, vitamins, nucleobases and metabolic intermediates: L-arginine, L-histidine, L-methionine, L-phenylalanine, L-tryptophan, L-tyrosine, L-ascorbic acid, D-biotin, pyridoxine, (−)-riboflavin, adenine, 2-deoxy-D-ribose, hippuric acid, L-(−)-malic acid, D-(+)-mannose, and orotic acid. We have demonstrated that the AM selectively induces apoptosis of cancer cells *in vitro*. Furthermore, we have provided evidence that the treatment with AM has a significant tumor inhibitory effect *in vivo*. In addition to our results Bonfili et al. reported that mixtures of essential amino acids also induce apoptosis in a cancer specific manner, and showed that proteasome inhibition and induction of autophagy plays a role in this process. Our earlier mechanistic studies of the AM identified the mitochondrial pathway of apoptosis induction, which was accompanied by the upregulation of genes contributing to apoptosis induction and cell cycle arrest, however the signaling events leading to the initiation of apoptosis were not known.

2.1. Endoplasmic reticulum stress and unfolded protein response

The endoplasmic reticulum (ER) is responsible for correct modification, folding and assembly of secretory and membrane-bound proteins prior to transport to the Golgi apparatus.

The lumen of the ER is an oxidizing environment allowing formation of disulphide bonds and it contains the highest concentration of calcium within the cells, which are required for proper protein folding and protein chaperone functions.

Many disturbances, including alteration in redox state, depletion of ER calcium, perturbations in posttranslational modification or viral infections reduce the protein folding capacity of the ER, which results in the accumulation and aggregation of unfolded proteins leading to the so-called ER stress.

Under ER stress conditions, the cells activate a pro-survival response to reduce the accumulation of unfolded proteins termed the unfolded protein response (UPR). The UPR attenuates the translation of mRNAs to decrease the protein influx, induces the expression of genes that are involved in protein folding and degradation, and activates the process of ER-associated protein degradation (ERAD) to remove the unfolded proteins.

The UPR is primarily a pro-survival response, however if the ER dysfunction is severe or prolonged and the UPR fails to restore homeostasis, apoptotic cell death ensues. Apoptosis triggered by ER stress has been implicated in the pathogenesis of several human diseases, including diabetes, cancer, neurodegenerative and cardiovascular diseases.

2.2. UPR signaling pathways

In mammals, there are three main UPR signaling pathways initiated by ER transmembrane receptors: activating transcription factor-6 (ATF6), inositol-requiring transmembrane kinase/endoribonuclease 1 (IRE1), and protein kinase R (PKR)-like ER kinase (PERK). In unstressed condition, the ER chaperone GRP78 (78-kDa glucose regulated protein), also referred to as BiP (Binding immunoglobulin protein) binds to all three ER stress receptors and keeps them in an inactive state. In response to the accumulation of unfolded proteins, GRP78 dissociates from the three receptors, leading to their activation and launching the UPR.

2.2.1. The ATF6 pathway

ATF6 is a basic leucine zipper protein (bZIP)-containing transcription factor and a type II ER transmembrane protein, with its C-terminal domain present in the ER lumen and its N-terminal DNA-binding domain facing the cytosol. In mammals, two homologous proteins, ATF6α and ATF6β exist. In response to stress, BiP dissociates from ATF6, and in turn ATF6 translocates from the ER to the Golgi apparatus where it is cleaved by proteases. In turn, the

cleaved bZIP containing domain translocates to the nucleus and induces genes, such as ER chaperone proteins (e.g. GRP78, GRP94) and X-box-binding protein 1 (XBP1).

2.2.2. The IRE1 pathway

IRE1 is a type I transmembrane protein that has both serine-threonine protein kinase domain and an endoribonuclease domain. Two mammalian IRE1 protein homologues, IRE1α and IRE1β, were identified. IRE1α is ubiquitously expressed, whereas IRE1β is expressed in epithelial cells of the gastrointestinal tract. Dissociation of GRP78 triggers oligomerization and autophosphorylation of the kinase domain and leads to the activation of the RNase domain. Following activation, the endoribonuclease activity of IRE1 cleaves a 26-nucleotide intron from XBP1 mRNA to yield a stable, active bZIP-family transcription factor, spliced XBP1 (XBP1s). sXBP1 upregulates transcription of different UPR target genes to restore homeostasis such as ER chaperones (GRP78, ERDj4, HEDJ, and PDI-P5), ERAD components (EDEM, $p58^{IPK}$).

Furthermore, the RNase activity of IRE1α mediates the selective degradation of mRNAs and microRNAs, in a process called as regulated IRE1α-dependent decay (RIDD). RIDD signaling has been associated with both pro-survival and pro-apoptotic roles depending on the duration and degree of the ER stress.

2.2.3. The PERK pathway

PERK is a serine-threonine protein kinase and dissociation of GRP78 from PERK upon ER stress, leads to its activation by dimerization and autophosphorylation. Activated PERK phosphorylates the eukaryotic initiation factor 2α (eIF2α), thereby causing general translational attenuation and reducing further protein load on the ER. However, certain set of mRNAs containing short open reading frames within the 5' untranslated region e.g. ATF4, DDIT3, can bypass the eIF2-dependent translational block, and are translationally induced.

ATF4 is a bZIP transcription factor family member which activates multiple genes that determine cell fate. ATF4 regulates the expression of ER chaperone proteins (GRP78 and GRP94), genes involved in amino acid biosynthesis, redox reactions, protein secretion, autophagy and apoptosis. One of the pro-death target gene of ATF4 is the transcription factor DDIT3, also known as C/EBP homologous protein (CHOP). The overexpression of DDIT3 has been reported to cause cell cycle arrest and/or cell death. ATF4 and DDIT3 were shown to interact and transcriptionally activate a number of additional UPR genes including activating transcription factor 3 (*ATF3*), and tribbles pseudokinase 3 (*TRIB3*). Additionally, ATF4 and

ATF3 are able to induce glutathione-specific gamma-glutamylcyclotransferase 1 (*CHAC1*), which has been demonstrated to be part of the UPR cascade and possess pro-apoptotic activity mediated by its capability to degrade glutathione.

The phosphorylation of eIF2α serves as a point of convergence for ER stress independent signaling mechanisms mediated by EIF2AK4 (also known as GCN2), EIF2AK2 (also known as PKR) or EIF2AK1 (also known as HRI), which are activated by amino acid starvation, viral infection, or heme deprivation, respectively. Therefore, the phosphorylation of eIF2α and its downstream events of are also referred as the integrated stress response (ISR).

3. Aims of the study

- Investigate the effect of AM in different cancer cell lines compared to HRE (primary human renal epithelial cells) normal cells at both transcriptional and translational level.
- Determine the extent to which ER stress contributes to the anticancer activity of the AM.
- Investigate the relative contribution of the individual components of AM to the activation of the ER stress.
- Knockdown of ATF3 and growth differentiation factor 15 (GDF15) to examine whether they are essential for the cell growth inhibitory effect of the AM and investigate the role of mIR-3189-3p, intronic miRNA of GDF15, in the anticancer activity of AM and in the regulation of ATF3 and DDIT3 during ER stress.
- Reveal the contribution of apoptosis, autophagy, ferroptosis and necroptosis to the effect of AM.
- Study how the apoptosis mediators, BBC3 and PMAIP1 modulate the effect of AM.
- Identification of further compounds capable of enhancing the cancer growth inhibitory effect of the active mixture.

4. Materials and methods

4.1. Cell culture

HeLa (human cervix adenocarcinoma), MCF-7 (human breast adenocarcinoma), PC-3 (human prostate adenocarcinoma), Caco-2 (human colorectal adenocarcinoma), A549 (human lung adenocarcinoma), HT-29 (human colorectal adenocarcinoma), HepG2 (human hepatocellular carcinoma) and 4T1 (mouse mammary carcinoma) cells were cultured in MEM supplemented with 10% (v/v) FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin. HRE (human renal epithelial cells, pooled from donors with different sex) cells were cultured in Renal Epithelial Cell Basal Medium supplemented with hEGF, Hydrocortisone, Epinephrine, Insulin, Triiodothyronine, Transferrin, GA-1000, and 0.5% FBS. Cells were incubated at 37 °C in a humidified atmosphere at 5% CO_2.

4.2. Active mixture

The selection of the components of the active mixture and the control mixture has been described previously. Throughout the investigation of the mechanism of the active mixture effect we used a mixture of sixteen selected small molecules (AM16). The composition of AM16 was the following: 0.2 mM adenine, 0.5 mM L-tryptophan, 0.5 mM pyridoxine hydrochloride, 0.75 mM L-methionine, 0.5 mM biotin, 1 mM orotic acid monohydrate, 2.5 mM 2-deoxy-D-ribose, 2 mM L-tyrosine disodium salt hydrate, 2.5 mM L-histidine, 2.5 mM L-phenylalanine, 2.5 mM L-arginine, 5 mM L-(−)-malic acid, 5 mM sodium hippurate hydrate, 5 mM D-(+)-mannose, 0.0025 mM (−)-riboflavin, 0.15 mM L-ascorbic acid, and 8.95 mM sodium bicarbonate. In some experiments the components of AM16 were divided into subgroups (AM1, AM2, AM3, and AM10). The composition of AM1 was the following: 0.5 mM L-tryptophan, 0.75 mM L-methionine, 2 mM L-tyrosine disodium salt hydrate, 2.5 mM L-histidine, 2.5 mM L-phenylalanine, 2.5 mM L-arginine, and the pH was set to 7.4 with 1N hydrogen-chloride. The composition of AM2 was the following: 0.2 mM adenine, 1 mM orotic acid monohydrate, 2.5 mM 2-deoxy-D-ribose, 5 mM L-(−)-malic acid, 5 mM sodium hippurate hydrate, 5 mM D-(+)-mannose, and 10.8 mM sodium bicarbonate. The composition of AM3 was the following: 0.5 mM pyridoxine hydrochloride, 0.5 mM biotin, 0.0025 mM (−)-riboflavin, 0.15 mM L-ascorbic acid, and 0.65 mM sodium bicarbonate. The composition of AM10 was the following: 0.5 mM L-tryptophan, 0.75 mM L-methionine, 2 mM L-tyrosine disodium salt hydrate, 2.5 mM L-histidine, 2.5 mM L-phenylalanine, 2.5 mM L-arginine, 0.2

mM adenine, 5 mM L-(−)-malic acid, 5 mM sodium hippurate hydrate, 5 mM D-(+)-mannose, and 7.3 mM sodium bicarbonate.

The composition of the 100% AM during the selection process of the new compounds was the following: 4 mM L-methionine, 0.75 mM L-tryptophan, 0.08 mM L-tyrosine disodium salt, 5 mM L-phenylalanine, 5 mM L-arginine, 4 mM L-histidine, 5 mM N-benzoyl glycine, 2 mM D-biotin, 1 mM pyridoxine hydrochloride, 0.006 mM riboflavin-5-phosphate sodium salt, 0.3 mM L-ascorbic acid, 1 mM lipoic acid, 0.16 mM orotic acid, 1 mM adenine hydrochloride, 7.5 mM 2-deoxy-D-ribose, 5 mM D-mannose, 0.5 mM D-glucosamine hydrochloride, 7.5 mM malic acid, 5 mM oxaloacetic acid, 0.05 mM adenosine triphosphate disodium salt, and 23.46 mM sodium hydrogen carbonate. All chemicals were purchased from Sigma Aldrich.

4.3. Control mixture

The CM had the following composition: 0.2 mM hypoxanthine, 0.5 mM L-proline, 0.5 mM nicotinic acid, 0.75 mM glycine, 0.5 mM thiamine hydrochloride, 1 mM uracil, 2.5 mM D-(−)-ribose, 2 mM L-alanine, 2.5 mM L-serine, 2.5 mM L-valine, 2.5 mM L-asparagine, 5 mM sodium succinate dibasic hexahydrate, 5 mM betaine, 5 mM D-(+)-glucose, 0.0025 mM D-pantotenic acid hemicalcium salt, 0.15 mM folic acid. All chemicals were purchased from Sigma Aldrich.

4.4. New mixture

The composition of the 100% new mixture (NM) was the following: 2 mM D-phenylalanine, 1 mM D-tryptophan, 1 mM D-arginine, 0.5 mM 5-hydroxi-L-tryptophan, 0.05 mM melatonin, 5 mM mandelic acid, 2.5 mM 3,4-dihydroxymandelic acid, 0.8 mM p-coumaric acid, 0.8 mM trans-cinnamic acid, 1 mM indole-3-acetic acid, 1 mM phenylacetic acid, 0.1 mM 3,4-dihydroxyphenylacetic acid, 0.25 mM indole-3-pyruvic acid, 2.5 mM phenylpyruvic acid sodium salt, 1 mM 4-hydroxy-phenylpyruvic acid, 1 mM 3-phenyllactic acid, 2.5 mM D-glyceric acid calcium salt, 0.25 mM glyceraldehyde, 3 mM 3-methyl-2-oxobutyric acid sodium salt, 5 mM 4-guanidinobutyric acid, 2.5 mM 3-methyl-2-oxovaleric acid sodium salt, 3 mM 4-methyl-2-oxovaleric acid sodium salt, 5 mM 3-hydroxy-3-methyl-glutaric acid, 1 mM gentisic acid sodium salt, 5 mM urocanic acid, 2.5 mM homovanillic acid, 2.5 mM xanthurenic acid, 5 mM levulinic acid calcium salt, 5 mM 4-hydroxy-benzoic acid, 5 mM pyrrole-2-carboxylic acid, 0.25 mM adenosine, 0.1 mM agmatine-sulphate, 0.5 mM cysteamine, 5 mM creatinin, 38.45 mM sodium-hydrogencarbonate. All chemicals were purchased from Sigma Aldrich.

4.5. Microarray analysis

HeLa cells were treated with AM16 for 3 hours, 6 hours and 24 hours in triplicates. Total RNA was isolated with RNeasy Plus mini kit (QIAGEN). RNA quality was assessed with agarose gel electrophoresis. Microarray hybridization and initial data processing were performed by Personmed Ltd. (Turku, Finland) as contract research. In brief, triplicate samples for each time point were converted into biotin-labeled cRNA and were hybridized to a Human HT-12 v.4 Expression BeadChip (Illumina) using standard protocols. Average probe intensities were computed with Genome Studio (Illumina) and analyzed with the following Bioconductor packages: affy, limma, gplots, beadarray, lattice, amap, simpleaffy, xtable, scatterplot3d, ade4 and made4. Data were quantile normalized and differentially expressed genes were identified with 2-sided t-test and fold change. Genes with >1.3 fold change and p value < 0.05 were considered differentially expressed.

4.6. Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) was performed with the java GSEA Desktop Application version 2.2.3. The gene ontology biological process gene set collection (GO BP) version 5.2 was used for the enrichment analysis. Upregulated pathways were defined by a normalized enrichment score (NES) > 3, downregulated pathways were identified by a NES < -3. Pathways with a false discovery rate (FDR) p value < 0.25 were considered significantly enriched.

4.7. Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated with PureLink RNA Mini Kit (ThermoFisher Scientific) and was treated with DNase I (Sigma Aldrich). cDNA was prepared with High Capacity RNA-to-cDNA Kit (ThermoFisher Scientific). PCR primers used for real-time quantitative amplification of the human housekeeping genes *B2M*, *GAPDH*, *HPRT1*, *RPL32*, and *PPIA* were described previously. PCR primers for human *APAF1*, *BAX*, *BCL2L1*, *BIRC2*, *BIRC3*, *CASP3*, *CDKN1A*, *CDKN2A*, *IKBKG*, *NFKBIA*, *NFKB1* were also described previously. Total XBP1, spliced XPB1, and unspliced XBP1 transcripts were quantified with primers described previously. All other PCR primers were designed by Primer Express Software. The expression levels of *BBC3*, *PMAIP1*, *RPL32* were measured with TaqMan gene expression assays. RPL32 was used for normalization. For miRNA analysis, small RNA fractions were isolated using the miRVana miRNA isolation kit (ThermoFisher Scientific). The expression of miR-3189-3p and U6

snRNA were measured using TaqMan microRNA assays (ThermoFisher Scientific) following the manufacturer's instructions. U6 snRNA was used for normalization. PCR reactions were run in triplicates using PowerUp SYBR Green Master Mix (ThermoFisher Scientific) or Taqman gene expression master mix II, no UNG (ThermoFisher Scientific) on an ABI StepOne Real Time PCR System. The stability of the expression level of the housekeeping genes was analyzed in preliminary experiments and RPL32 was chosen for normalization of target gene expression. Fold change values were calculated by dividing the normalized target gene expression measured in the treated samples by that of the untreated control samples.

4.8. Western blot

Cells were seeded onto 150 mm Petri dishes at a density of $1.6x10^6$ or onto 6-well plates at a density of $1.7x10^5$/well. Following the indicated treatments cells were lysed in ice cold 1X RIPA buffer (Abcam) containing Protease and Phosphatase Inhibitor Cocktail (Abcam). Protein concentrations were measured with the DC protein assay (Bio-Rad). 30-100 μg proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk (NFDM) (Sigma Aldrich) or in the case of antibodies against phosphoproteins with 5% bovine serum albumin (BSA) (Sigma Aldrich) in Tris-buffered saline (150mM NaCl, 20mM Tris-base pH 7.6, 0.1% Tween 20) (TBS-T) for 1 hour at room temperature. Primary antibodies diluted in 5% NFDM/TBS-T or 5% BSA/ TBST-T were applied at 4°C overnight. HRP-conjugated anti-rabbit antibody diluted in 5% NFDM/TBS-T was applied for 1 hour at room temperature. Membranes were developed with LumiGLO chemiluminescent substrate (Cell Signaling Technology) and exposed to x-ray films.

4.9. Reverse transfection

HeLa cells were reverse transfected in 96-well plates at a density of 2.5×10^3 cells per well or in 6-well plate at a density of 1.7×10^5 using DharmaFECT 1 (Dharmacon) according to the manufacturer's instructions. ON-TARGETplus Human GDF15 (9518) SMARTpool siRNA (Dharmacon), ON-TARGETplus Human ATF3 (467) SMARTpool siRNA (Dharmacon) and ON-TARGETplus Non-targeting Pool (Dharmacon) siRNAs were used at a final concentration of 50 or 100 nM as indicated. The miRIDIAN microRNA hsa-miR-3189-3p Hairpin Inhibitor (Dharmcon) and miRIDIAN microRNA Hairpin Inhibitor Negative Control (Dharmacon) miRNAs were transfected at a final concentration of 50 and 200 nM, respectively, as indicated. The miRIDIAN microRNA hsa-miR-3189-3p Mimic (Dhamacon) and miRIDIAN microRNA

Mimic Negative Control (Dharmacon) miRNAs were transfected at a final concentration of 10 nM. The BBC3 and PMAIP1 silencer select pre-designed siRNAs (ThermoFisher Scientific) were used at a final concentration of 10 nM. At 24 hours post-transfection, the transfection medium was removed, and treatment was initiated. At 48 hours post-transfection, cells were either fixed and labeled for cell counting or were harvested and analyzed by qRT-PCR.

4.10. Cell counting and immunocytochemistry

HeLa cells were plated and transfected at 2.5×10^3 per well in 96-well black-walled, glass bottom plates, then treated as indicated. The cells were fixed with 4 % paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes, then were permeabilized with Triton X-100 for 10 minutes, followed by blocking with 5 % goat serum in PBS for 1 hour at room temperature. The antibody against ATF4 diluted in PBS containing 1 % BSA and 0.05 % Triton-X 100 was applied at 4 °C overnight. After washing three times with PBS for 5 minutes, the cells were incubated with Alexa Fluor 555 Anti-Rabbit IgG diluted as above, in the dark for 1 hour at room temperature. Cells were washed once with PBS for 5 minutes, and then the nuclei were counterstained with DAPI for 5 minutes. For experiments involving only cell counting, the fixation of the cells was directly followed by DAPI staining and imaging. Cells were imaged using an automated, high-content screening station (Olympus IX83ZDC2 equipped with scan^R software platform, v2.5.0).

4.11. Live/dead staining, EdU and TUNEL labeling

HeLa cells were plated at 2.5×10^3 per well in 96-well plates and treated as indicated. For live/dead staining cytocalcein violet 450 and 7-Aminoactinomycin D (7-AAD) were used from the Apoptosis/Necrosis detection kit (Abcam) following the manufacturer's instructions. For EdU (5-ethynyl-2'-deoxyuridine) labeling EdU was added at a final concentration of 10 μM at treatment initiation. The incorporated EdU was detected with the Click-iT Plus EdU Alexa Fluor 555 imaging kit (ThermoFisher Scientific) following the manufacturer's instructions. TUNEL (terminal deoxynucleotidyl transferase-dUTP nick end labeling) was performed with the use of Click-iT TUNEL Alexa Fluor 594 imaging kit (ThermoFisher Scientific). For EdU and TUNEL nuclei were labeled with Hoechst 33342 (5 μg/ml). Image acquisition and analysis was performed as described for immunocytochemistry.

4.12. Determination of combination indexes

Cells were plated in 96-well plates and treated with three point, two-fold dilution series of the inhibitors, dilution series of AM16 alone (40–100%), or with the combination of the two higher concentration of inhibitors and 60 %, 80 %, 100 % AM16 for 24 hours. Cells were counted as described for cell counting and the combination indexes were calculated with the Compusyn software. The following inhibitors were tested (starting concentration of the dilution series in brackets): deferoxamine (Abcam) (100 μM), ferrostatin-1 (Sigma Aldrich) (20 μM), Trolox (Sigma Aldrich) (300 μM), 2-mercaptoethanol (Sigma Aldrich) (20 μM), U0126 (Sigma Aldrich) (20 μM), necrostatin-1 (Abcam) (10 μM), necrosulfonamide (Abcam) (5 μM), Z-VAD(OMe)-FMK (Abcam) (80 μM), E64d (Aloxistatin) (Abcam) (50 μM), ALLN (Abcam) (500 nM), cyclosporine A (Abcam) (10 μM), 3-methyladenine (Abcam) (4 mM), bafilomycin A1 (Abcam) (1 μM), chloroquine diphosphate (Abcam) (50 μM). Erastin (Sigma Aldrich) was used at 2.5–10 μM concentrations. Necroptosis was induced with 20 ng/ml recombinant human TNF alpha protein (Abcam) + 20 μM z-VAD-fmk + 500 nM BV6 (Smac mimetic) (Sigma Aldrich).

4.13. Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics 22. Normal distribution of the data was evaluated with Shapiro-Wilk test. Homogeneity of variances was assessed with Levene-test. For normally distributed variables with equal variances p values were calculated with one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. For normally distributed variables with unequal variances p values were calculated with Welch test followed by Games-Howell test. P values less than 0.05 were considered statistically significant.

5. Results

5.1. Gene expression analysis of AM16 treated cancer cells

Microarray analysis showed striking changes in gene expression in HeLa cells after 3 hours, 6 hours, and 24 hours treatment with AM16. Gene set enrichment analysis (GSEA) of the upregulated transcripts revealed the enrichment of gene sets corresponding to RNA splicing and translational initiation at 3 hours, ER stress induced apoptosis and cell cycle regulation at 6 hours, and apoptosis and TGF-β signaling 24 hours. Among the gene sets enriched in all three time points "response to ER stress" had the highest normalized enrichment score (NES).

In order to validate these results, we have analyzed one hundred genes with qRT-PCR. We also applied a control mixture (CM) which had the same osmolality as AM16 and contained ineffective small molecules with chemically or physiologically similar properties as components of AM16. HeLa and HRE normal cells were treated with AM16 or CM for 24 hours. We have found upregulation of genes contributing to the UPR (*CHAC1, GDF15, TRIB3, ATF3, DDIT3, PPP1R15A, ATF4, XPB1*), to autophagy (*SESN2*, *ULK1*, *DRAM161*), and to apoptosis (*BIRC3, GADD45A, PMAIP1, BBC3*) only in HeLa cells treated with AM16 for 24 hours, but not in HeLa cells treated with CM or HRE treated with AM16 or CM.

Genes that showed specific and significant gene expression differences in AM16 treated HeLa cells were further analyzed in MCF-7, PC-3 and Caco-2 cells. We have found that ATF3, *DDIT3, PPP1R15A, PMAIP1* and *GDF15* were specifically upregulated in all of the four AM16 treated cell lines, while the upregulation of *CHAC1* could only be shown in AM16 treated HeLa and PC-3 cells.

These results indicate that the AM16 specifically induces gene expression changes characteristic for ER stress in cancer cells.

5.2. Detection of proteins contributing to the ISR in AM16 treated cancer cells

The integrated stress response causes global attenuation of protein synthesis while allowing the preferential translation of certain upstream open reading frame containing mRNAs e.g. *ATF4, DDIT3*. Therefore, we examined the expression of ATF4, ATF3, DDIT3, CHAC1 and GDF15 proteins in AM16 or CM treated HeLa, PC-3, Caco-2, MCF-7, and HRE cells by western blot.

We observed a time-dependent induction of ATF4, ATF3, DDIT3, CHAC1, GDF15 in AM16 treated HeLa cells. We detected the accumulation of ATF4, ATF3 and GDF15 in PC-3, Caco-2 and MCF-7 cells treated with AM16 for 24 hours, but not in CM treated cells. We have

found a slight increase of CHAC1 and GDF15 in AM16 treated HRE cells. Furthermore, we examined the sub-cellular localization of ATF4 with immunocytochemistry, and we found that ATF4 is accumulated in the nuclei of HeLa cells after 24 hours treatment with AM16. These results confirm the activation of the ISR pathway.

Next we investigated the phosphorylation of eIF2α, the core event in the ISR pathway, which mediates the global translational shutdown and the simultaneous preferential translation of certain mRNAs. We found a time dependent phosphorylation of eIF2α in AM16 treated HeLa cells.

Next we investigated the phosphorylation of the ER stress responsive eIF2α kinase PERK and the phosphorylation two ER stress independent kinases, GCN2 and PKR in HeLa cells. We found a time dependent phosphorylation of PERK, however GCN2 and PKR were not phosphorylated upon treatment with AM16. Since PERK was activated upon treatment, we also investigated two further ER stress sensors IRE1α and ATF6, and the accumulation of BiP, a chaperone playing a crucial role in ER stress. The treatment with AM16 induced a time dependent phosphorylation of IRE1α, and a time dependent increase in the amount of ATF6 and BIP in HeLa cells. The activation of ATF6 was confirmed by the detection of the cleaved fragment of ATF6, the 50kDa transcription factor domain, whereas the activation of IRE1α was demonstrated with the elevated splicing of *XBP1* mRNA upon treatment with AM16.

All together these results show that the phosphorylation of eIF2α induced upon treatment with AM16 is caused by the activation of PERK and not by other ER stress independent kinases, and that AM16 activates all three branches of the UPR.

5.3. Effects of ER stress inhibitors in AM16 treated cells

Several ER stress inhibitors were applied to determine the extent to which ER stress contributes to the anticancer activity of the AM16. We investigated the effect of the ISR inhibitor ISRIB - a molecule which renders cells resistant to the effect of eIF2α phosphorylation -, salubrinal - an inhibitor of eIF2α dephosphorylation and ER stress induced apoptosis -, and 4-phenylbutyrate (4-PBA) – a chemical chaperone able to reduce the amount of misfolded proteins in the ER -, on the AM16 treated cells. ISRIB completely inhibited the upregulation of *ATF4*, *CHAC1*, *DDIT3* and *GDF15* mRNA level induced by 6 hours AM16 treatment, however the induction of *ATF3* was only reduced. In addition, ISRIB was able to attenuate the cell growth inhibitory effect of the AM16. Salubrinal and 4-PBA had no effect on the anticancer activity of AM16. Furthermore, we also tested two additional IRE1α inhibitors GSK2850163

and STF-083010, but these inhibitors also failed to block the AM16 induced cell growth inhibition.

Taken together these results show that the UPR plays a significant role in the AM16 cell growth inhibitory effect, and suggest that eIF2α phosphorylation is the major arm of the UPR activated by AM16.

5.4. Relative contribution of the individual components of AM16 to the ER stress induction

To determine whether all of the sixteen small molecules of the AM16 are required to the induction of ER stress, first we formed three groups from the compounds of the AM16 and tested the effect of them alone or in combination. AM1 contained the amino acid components, AM2 contained adenine, L-(-)-malic acid, 2-deoxy-D-ribose, orotic acid, D-(+)-mannose and hippuric acid, AM3 contained the vitamin components. We have found that the combination of AM1 and AM2 was able to significantly reduce the cell number, and induced the expression of *ATF3*, *ATF4*, *CHAC1*, *DDIT3*, *GDF15* transcripts, while the other combinations or the per se application of AM1, AM2, AM3 had no effect. Important to note, that the growth inhibitory effect of AM16 was still significantly higher than the effect of AM1+AM2.

Next we combined randomly paired components of AM1 with AM2, or vice versa randomly paired components of AM2 with AM1 and measured expression of *ATF3*, *ATF4*, *CHAC1*, *DDIT3*, *GDF15*. On the basis of our results we identified 10 (amino acids, adenine, L-(-)-malic acid, D-(+)-mannose and hippuric acid) compounds out of the sixteen, those which were able to activate the ER stress genes to levels comparable with AM1+AM2. The effect of this ten component mixture (AM10) was further analyzed. We have found that AM10 induced the expression of ER stress genes to levels comparable with AM16, and ISRIB completely inhibited the increase of *ATF4*, *CHAC1*, *DDIT3* transcripts after 24 hours AM10 or AM16 treatment, but was not able to completely block the induction of *ATF3* and *GDF15*. The cell growth inhibitory effect of AM10 was also attenuated but not completely blocked by ISRIB. The AM10 induced changes in gene expression and the cell growth inhibitory effect could also be demonstrated in PC-3, MCF-7 and Caco-2 cells.

Taken together, the components of AM10 are sufficient to induce ER stress, while the other six components are able to enhance the cell growth inhibitory effect.

5.5. Role of ATF3, GDF15 and mIR-3189-3p on the effect of AM16

Since *ATF3* and *GDF15* expression could not be completely blocked by ISRIB upon AM16 treatment, the effect of the knockdown of these proteins alone, or in combination with ISRIB was analyzed on AM16 induced cell growth inhibition. We found that the knockdown of *ATF3* and *GDF15* had no effect on cell number decrease caused by 24 hours treatment with AM16, even in the presence of ISRIB.

Next, we focused on an intronic miRNA of *GDF15*, the mIR-3189-3p, which is co-expressed with *GDF15* and was demonstrated to have pro-apoptotic activity. We measured the induction of mIR-3189-3p upon treatment with AM16, and we found a 3-fold increase in the expression of mIR-3189-3p after 24-hours treatment with AM16 in HeLa cells, CM had no effect. Tunicamycin, a known inducer of ER stress, also produced a 4-fold increase in mIR-3189-3p level. To test whether mIR-3189-3p has a role in the cell growth inhibition caused by AM16, we applied a miRNA inhibitor, however, despite the efficient knockdown of mIR-3189-3p, we could not prevent the decrease of the cell number.

The role of mIR-3189-3p was further investigated, and we found that mIR-3189-3p mimic was able to significantly increase the levels of *ATF3*, *DDIT3*, and *GDF15* transcripts, which was completely blocked by miR-3189-3p inhibitor, but not by negative control miRNA inhibitor.

The transcription factor JDP2 suppresses the transcription of *ATF3* and *DDIT3*, and multiple histone deacetylase family (HDACs) members are associated with JDP2 at the promoters of *ATF3* and *DDIT3*. Hence we measured the mRNA levels of *HDAC1-6*, *JDP2* and two additional JDP2 targets *GSG1* and *PCDH7* after transfection with mIR-3189-3p mimic. As a result, it was observed that the levels of *HDAC1*, *HDAC3*, *JDP2* transcripts were decreased, and the levels of *GSG1*, *PCDH7* transcripts were significantly increased. The upregulation of *GSG1* and *PCDH7* further verifies the downregulation of *JDP2*.

Taken together these results indicate that ATF3 and GDF15 are not essential for the cell growth inhibitory effect of the AM16, whereas the mIR-3189-3p, upregulated by treatment with AM16, is probably able to increase the expression of *ATF3* and *DDIT3* through the downregulation of *JDP2*, *HDAC1* and *HDAC3*.

5.6. Investigation of cell fate outcomes- cell cycle arrest and cell death- caused by AM16 treatment in cancer cells

Since we were not able to completely inhibit the effect of AM16, we further investigated whether the effect of AM16 is mainly due to cytotoxic, growth arresting or apoptosis inducing activity. The number of necrotic/late apoptotic cells was increased in HeLa cells upon treatment with AM16 as demonstrated with cytocalcein/7-AAD staining. In addition, treatment of HeLa cells with AM16 caused cell growth arrest measured with EdU incorporation and significantly increased the number of apoptotic cells measured with TUNEL assay.

Next we investigated the contribution of apoptosis, autophagy, ferroptosis and necroptosis to the effect of AM16 using different inhibitors of these cell death pathways. AM16 was used alone or in combination with the inhibitors, and we measured the combination index (CI) using cell counting. The CI is the quantitative measure of interaction between the effect of inhibitors and AM16 (CI > 1.1 indicates antagonism, CI < 0.9 indicates synergism).

Then we selected the optimal cell line for investigating the role of ferroptosis. Erastin, a known inducer of ferroptosis, was tested on HeLa, MCF-7, PC-3, Caco-2, HT-29 and A549 cells, in combination with the inhibitors of ferroptosis: the iron chelator deferoxamine and the lipid peroxidation inhibitor ferrostatin-1. Effect of erastin could be only inhibited in A549 cells, thus we have chosen the HeLa and A549 cell lines to test the modulatory effect of ferroptosis inhibitors: deferoxamine, ferrostatin-1, trolox, the 2-mercaptoethanol and U0126 on the effect of AM16. We demonstrated a dose dependent decrease in cell number of HeLa and A549 cells upon treatment with AM16, however the inhibitors of ferroptosis could not prevent the effect of AM16 in HeLa and A549 cells.

To determine whether AM16 treatment causes necroptosis we tested the necrostatin-1 and the necrosulfonamine on HT-29 cells because HeLa, PC-3, MCF-7 and Caco-2 cells are missing components of the necroptosis signaling cascade and not responding to the TNF-α + z-VAD-fmk + Smac mimetic necroptosis inducing stimulus. The inhibitors of necroptosis failed to prevent the dose dependent cell number decrease of AM16 in HT-29 cells.

Next we examined the effect of inhibitors of caspase, cathepsin or calpain proteases (z-VAD-fmk, E64d, ALLN), cyclophilin D (cyclosporine A), and autophagy/lysosomal function (bafalomycin A1, 3-methyladenine, and chloroquine) in HeLa, A549, and HT-29 cells. We have found that z-VAD-fmk partially antagonized the effect of AM16 in A549 and HT-29 (CI: 1.14-1.42), but not in HeLa cells, whereas E64d, ALLN, cyclosporine A partially antagonized the

effect of AM16 in all three cell lines (CI: 1.2-1.67). The autophagy inhibitors bafalomycin A1 and 3-methyladenine enhanced the effect of the AM16 in all three cell lines (CI: 0.32-0.86).

These results show that ferroptosis and necroptosis are not participated in AM16 induced cell death, while apoptosis has an important role in the effect of AM16.

5.7. Role of BBC3 and PMAIP1 on the effect of AM16

Since we were not able to inhibit totally the effect of AM16, we searched for additional mediators of AM16 induced apoptosis. We found that the transcript levels of the apoptotic mediators, *BBC3* and *PMAIP1* were elevated upon treatment with AM16 in HeLa, MCF-7, PC-3 and Caco-2 cells. In addition, we tested how the knockdown of these mediators could influence the effect of AM16. Interestingly, the combination of double knockdown and ISRIB completely inhibited the effect of AM16.

5.8. Identification of additional compounds capable to enhance the cell growth inhibitory effect of AM

Our next aim was to investigate additional molecules found in the serum whether other compounds are able to enhance the effect of the previously identified components of the active mixture. We examined 130 new compounds in a per se non-toxic concentration in combination with AM. We identified 34 new compounds which are able to enhance the effect of AM, though the extent of enhancement differed between the compounds. The newly identified compounds are the following: D-phenylalanine, D-tryptophan, D-arginine, 5-hydroxi-L-tryptophan, melatonin, mandelic acid, 3,4-dihydroxymandelic acid, p-coumaric acid, trans-cinnamic acid, indole-3- acetic acid, phenylacetic acid, 3,4-dihydroxyphenylacetic acid, indole-3-pyruvic acid, phenylpyruvic acid, 4-hydroxy-phenylpyruvic acid, 3-phenyllactic acid, D-glyceric acid, glyceraldehyde, 3-methyl-2-oxobutyric acid, 4-guanidinobutyric acid, 3-methyl- 2-oxovaleric acid, 4-methyl-2-oxovaleric acid, 3-hydroxy-3-methyl-glutaric acid, gentisic acid, urocanic acid, homovanillic acid, xanthurenic acid, levulinic acid, 4-hydroxy-benzoic acid, pyrrole-2-carboxylic acid, adenosine, agmatine, cysteamine, creatinine.

5.9. Effect of the new mixture on cancer cells

Next we examined the effect of the mixture containing the newly identified 34 compounds, called the new mixture (NM), compared to AM on HELA, PC-3, MCF-7, Caco-2, HepG-2, 4T1 cancer cells and on HRE normal cells. We have found that NM caused a significantly greater

cell growth inhibition in different cancer cells than AM. We also tested the combination of AM and NM. The combined treatment (AM+NM) produced a more efficient cell growth inhibition compared to AM or NM alone. The proliferation of HRE cells was not decreased by any of the mixtures.

To further investigate the effect of NM, we measured the expression of certain pro- and anti-apoptotic genes, as well as genes regulating the cell cycle by qPCR. HeLa cells were treated with 30% AM, 20% NM or the combination of 30% AM and 20% NM. The combined treatment with 30% AM and 20% NM significantly induced the expression of the pro-apoptotic genes *BIM*, *BBC3*, *PMAIP1*, *DR4* (*TNFRSF10A*), *DR5* (*TNFRSF10B*) and the anti-apoptotic genes *BCL-2*, *cIAP*, *cIAP2* compared to the untreated control. The level of cell cycle inhibitor, *CDKN1A* was also substantially induced. 20% NM mixture applied alone also induced changes in the expression of pro- and anti-apoptotic genes but to a lesser extent than AM+NM. Treatment with 30% AM had a slight or no effect on the expression of genes investigated compared to the control.

All together these results show that the mixture of new substances has a specific inhibitory effect on cancer cells greater than the AM, and the combination of AM and NM induces gene expression changes related to apoptosis and cell cycle arrest.

6. Discussion

In this work, we demonstrated that a defined mixture of amino acids, vitamins and other small molecules found in the serum (AM) selectively induce endoplasmic reticulum stress and activates the unfolded protein response in cancer cells. The treatment with AM first induces the expression of ER stress related genes (*ATF3, ATF4, DDIT3, XBP1*), followed by the upregulation of genes playing role in apoptosis and cell cycle regulation (*BBC3, PMAIP1, TNFRSF10B, CDKN1A*). These results, together with the temporally sustained induction of ATF4, ATF3, and DDIT3 proteins, point toward that AM induces ER stress mediated induction of apoptosis.

Further analysis revealed that AM activates all three arms of the UPR signaling pathway (PERK, ATF6, IRE1α), however the results of our inhibition experiments suggest that the activation of PERK and the consequential eIF2α phosphorylation, accompanied by the preferential translation of the upstream open reading frame containing mRNA of *ATF4* are the dominant ER stress contributors in the effect of AM.

ATF4 is considered to be the master transcription factor in UPR which can form heterodimers with various basic leucine zipper transcription factors, including its own target DDIT3. The induction of ATF4 precedes the appearance of DDIT3 indicates, together with the finding that when the accumulation of ATF4 is blocked by ISRIB the increase in *DDIT3* transcript is diminished, that AM elicited induction of DDIT3 is mediated by ATF4. The heterodimer formed by ATF4 and DDIT3 transcriptionally activates a number of additional UPR genes including *ATF3*, and *TRIB3*. This is in agreement with our results which also show the induction of these genes upon treatment with AM.

ATF4 together with ATF3 is able to induce *CHAC1*, which has been demonstrated to be part of the UPR cascade and possess pro-apoptotic activity mediated by its capability to degrade glutathione. In agreement with this, our results demonstrate that AM induces CHAC1 in an ATF4 dependent manner, and raise the possibility that CHAC1 plays an important role in the apoptosis inducing effect of AM.

GDF15 is a member of the TGF-β superfamily with a diverse and controversial role in cancer development and progression. Our results demonstrate that treatment with AM induces GDF15 in all cancer cell lines tested.

The *GDF15* locus contains an intronic miRNA (mIR-3189), the 3p product of which (mIR-3189-3p) has been shown to transcriptionally co-regulated with *GDF15* and demonstrated to have potent *in vitro* pro-apoptotic and *in vivo* anti-tumorigenic activity in colon cancer cells. In addition, the tumor growth inhibitory effect of mIR-3189-3p on glioblastoma xenografts and the downregulation of mIR-3189-3p in glioblastoma clinical samples have been also reported. Our finding that both AM and the known ER stress inducer tunicamycin increase the amount of mIR-3189-3p points toward the possible role of this miRNA in the UPR and the consequential induction of apoptosis. In fact, we have found that mIR-3189-3p specifically increased the amounts of *ATF3*, *DDIT3*, and *GDF15* transcripts. The transcription factor JDP2 has been shown to repress *ATF3* and *DDIT3* transcription, and various members of the histone deacetylase family (HDACs) were demonstrated to be associated with JDP2 at the promoters of *ATF3* and *DDIT3*. Our results demonstrate a specific downregulation of *JDP2*, *HDAC1* and *HDAC3* transcripts by mIR-3189-3p, indicating that these mRNAs are targets of mIR-3189-3p. The downregulation of JDP2 was further confirmed by the increase in the transcript levels of *GSG1* and *PCDH7*, which are known targets of the repressor JDP2. Based on the data above, we speculate that mIR-3189-3p enhances the expression of *ATF3* and *DDIT3* most probably through the downregulation of *JDP2*, *HDAC1* and *HDAC3* thereby lifting the repression form the promoters of *ATF3* and *DDIT3*. The increased amount of ATF3 and DDIT3 in turn could

potentially stimulate the transcription of *GDF15* or stabilize the *GDF15* transcript, thus providing the basis for the mIR-3189-3p feedback loop.

However, the fact that the individual inhibition of *ATF3*, *GDF15*, or mIR-3189-3p induction did not influenced the cell number reducing effect of AM indicate that these components are not essential for the anti-cancer activity of AM.

The relative contribution of individual AM components to the activation of the UPR revealed that the amino acid components (L-arginine, L-tyrosine, L-histidine, L-tryptophan, L-methionine, and L-phenylalanine) if applied in conjunction with adenine, L-(-)-malic acid, D-(+)-mannose and hippuric acid are necessary and sufficient to induce ER stress. To the best of our knowledge there are no reports implicating these substances in the activation of the UPR except L-arginine and D-(+)-mannose.

Our investigation revealed that the cell proliferation inhibition and the induction of apoptosis are the two main mechanisms involved in the effect of AM. In addition, our result link AM induced ER stress to the inhibition of the cell proliferation and to the BBC3 and PMAIP1 mediated induction of apoptosis, which is in agreement with the role of ER stress in cell fate control.

In addition, we have identified 34 new compounds which were able to significantly enhance the effect of the AM on different cancer cells when applied in a per se non-toxic concentration. These compounds are mainly L-amino-acid metabolites, phenolic acids, D-amino acids and keto acids. Treatment with any given mixtures, new mixture alone or in combination with AM, caused no toxic effect on HRE normal cells. These results revealed that the mixture of selected substances has a specific inhibitory effect on cancer cells. The upregulation of *BBC3*, *PMAIP1* and *CDKN1* transcripts upon treatment with the combination of AM and NM points toward the induction of apoptosis and cell cycle arrest, which is also in agreement with our results.

7. List of publications

List of publications related to this thesis:

Scheffer D, Kulcsár G, Czömpöly T. Identification of Further Components of an Anticancer Defense System Composed of Small Molecules Present in the Serum. Cancer Biother Radiopharm. 2019 Apr; 34(3):160-170. **Impact factor: 1.894**

Scheffer D, Kulcsár G, Nagyéri G, Kiss-Merki M, Rékási Z, Maloy M, Czömpöly T. Active mixture of serum-circulating small molecules selectively inhibits proliferation and triggers apoptosis in cancer cells via induction of ER stress. Cell Signal. 2020 Jan; 65:109426. **Impact factor: 3.388**

Posters related to this thesis:

Dalma Scheffer, Tamás Czömpöly, Gyula Kulcsár. Effects of a mixture of amino acids and other small molecules on gene expression profile in human cervical cancer cells (HeLa). 45. Membrane Transport Congress, Sümeg, Hungary, 2015.

Dalma Scheffer, Tamás Czömpöly, Gyula Kulcsár. A mixture of amino acids and other small molecules induces endoplasmic reticulum stress in different cancer cell lines. 46. Membrane Transport Congress, Sümeg, Hungary, 2016.

Other posters:

Viktor Sándor, **Dalma Scheffer**, Ágnes Dörnyei, Anikó Kilár, Béla Kocsis, Ferenc Kilár. Electrophoretic and mass spectrometric analysis of the lipid A part of bacterial lipopolysaccharides. 8th International Interdisciplinary Meeting on Bioanalysis. Brno, Czech Republic, 2011.

Árpád Czéh, Tamás Czömpöly, Gyula Kulcsár, Erika Lantos, György Nagyéri, Viktória Németh, **Dalma Scheffer**, Marianna Merki, György Lustyik. A novel approach for in vitro characterization of anticancer effects of experimentally selected mixtures of amino acids and other small molecules. CYTO Congress, Glasgow, Scotland, 2015.

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