Combination of unsaturated fatty acids and ionizing radiation on human glioma cells: cellular, biochemical and gene expression analysis

Ph.D. dissertation thesis

Author: Otilia Tamara Antal

Supervisor: Dr. László Puskás

Doctoral School of Biology
University of Szeged

BIOLOGICAL RESEARCH CENTRE, SZEGED, CENTRE OF EXCELLENCE OF THE EUROPEAN UNION – LABORATORY OF FUNCTIONAL GENOMICS

2015

List of scientific publications

MTMT identifier: 10047278
The dissertation is based on the following scientific papers:

Otilia Antal, Mária Péter, László Hackler Jr., Imola Mán, Gábor Szebeni, Ferhan Ayaydin, Katalin Hideghéty, László Vígh, Klára Kitajka, Gábor Balogh, László G Puskás (2015): Lipidomic Analysis Reveals a Radiosensitizing Role of Gamma-Linolenic Acid in Glióma Cells. *Biochim Biophys Acta.* 1851(9):1271-1282. (IF:4.66)

Otilia Antal, László Hackler, Junhui Shen, Imola Mán, Katalin Hideghéty, Klára Kitajka, László G Puskás (2014): Combination of unsaturated fatty acids and ionizing radiation on human glióma cells: cellular, biochemical and gene expression analysis. *Lipids in Health and Disease*, 13:142. (IF:2.31).

Further scientific papers:

Rita Gombos, Ede Migh, **Otilia Antal**, Anindita Mukherjee, Andreas Jenny, József Mihály (2015): The Formin DAAM Functions as Molecular Effector of the Planar Cell Polarity Pathway during Axonal Development in Drosophila. *The Journal of Neuroscience*, 35(28):10154-10167. (IF:6.75).

Otilia Antal, Mariann Karisztl-Gácsi, Anna Farkas, Attila Kovács, András Ács, Norbert Törő, Gyula Kiss, Martin L. Saker, János Győri, Gáspár Bánfalvi, Ágnes Vehovszky (2011): Screening the toxic potential of *Cylindrospermopsis raciborskii* strains isolated from Lake Balaton, Hungary, *Toxicon*, 57(6): 831-840. (IF: 2.58)

Mariann Gácsi, **Otilia Antal**, Gábor Vasas, Csaba Mathé, György Borbély, Martin L. Saker, János Győri, Anna Farkas, Ágnes Vehovszky, Gáspár Bánfalvi (2009): Comparative study of cyanotoxins affecting cytoskeletal and chromatin structures in CHO-K1 cells, *Toxicology in Vitro*, 23(4):710-718. (IF: 3.21)

1. Introduction

Glioma is a lethal disease, which, besides chemotherapy, is usually treated with radiotherapy. On many occasions, irradiation is not an effective therapy.

The exact molecular networks which influence the development of glioma are still unknown, although several signaling pathways have been verified. These could be the basis of new therapeutic methods. Numerous genetic alterations that are related to differentiation of glioma cells have been identified and a number of these have direct therapeutic implications (Fan et al., 2010; Soni et al., 2005; Wang et al., 2010).

The polyunsaturated fatty acids (PUFAs) cannot be synthesized in animal tissues and must be obtained from the diet. Besides their essential role in membrane integrity, receptor signaling, inflammation and cellular stress modulation, among others, it was found that they have anticancer properties by inducing lipid peroxidation and oxidative stress, altering key signaling pathways, modulating gene expression and lipid homeostasis (Cowing and Saker, 2001; Kitajka et al., 2004; Puskás et al., 2010; Ntambi and Bené, 2001; Vartak et al., 1997).

It was found that PUFAs also change the radio sensitivity of cancerous cells, and protect normal cells from the harmful effects of radiation (Dupertuis et al., 2007). The radio sensitivity induced by PUFAs may be related to their involvement in prostanoid synthesis; however the underlying mechanism is still unclear (Vartak et al., 1998).

For cancerous cells, the constitutive activation of fatty acid synthesis represents selective advantage. Numerous genes that are related to fatty acid synthesis can be responsible for cancerogenesis. Inhibition of fatty acid synthesis causes

apoptosis of cancerous cells (Igal, 2010). Besides selective fatty acid synthesis inhibitors it was shown that PUFAs also decrease the activity of key lipid biosynthetic genes (Pucer et al., 2013; Vessby et al., 2002).

Gamma linolenic acid (GLA) has been already tested in clinical phase, and found to be effective in glioma treatment (Das, 2004; Das, 2007). Radiation alone seems to be non-effective on lower grade gliomas, unfortunately it does not influence the average survival rate (Sandrone et al., 2014). Therefore, in our studies we investigated the effects of PUFAs as adjuvants to irradiation by using different cellular, biochemical and gene expression analysis methods.

2. Objectives

In the experiments presented in this dissertation we had the following objectives:

1. To select the UFAs (out of the investigated ones: OA, EPA, AA, DHA, and GLA) that act in a synergistic manner with radiation (5 and 10 Gy); with biochemical and kinetic measurements on U-87 MG cell line.
2. To sort out by biochemical and kinetic assays the UFAs and the concentrations, that should be applied in further experiments.
3. To investigate the effect of the selected UFAs on the morphology of U-87 MG cell line.
4. Measure gene expression of PUFAs (some cases just GLA, based on literature analysis) and/or radiation (10 Gy, some cases 5 Gy, too) treated U-87 MG cell line. We were interested in the expression of therapeutic targets, stress-

related genes, genes linked to fatty acid synthesis, lipid binding proteins gene, genes of heat shock proteins.

5. Determine alteration of miRNA expression of PUFA and/or radiation (10 Gy) treated U-87 MG cell line. The investigated miRNAs were chosen by literature analysis.
6. Estimation of lipid droplet accumulation following GLA treatment, radiation (5 Gy and 10 Gy) or combination of both.
7. Complementing gene expression results with FACS (fluorescence activated cell sorting) assay. Determination of the rate of apoptosis on U-87 MG cells after treatment with 5 Gy or 10 Gy, and/or PUFAs.

3. Methods

a. **Cell culture and treatment**

24 hours after plating we treated U-87 MG (glioblastoma cell line with number: ATCC HTB-14^{TM}) cells with arachidonic acid (AA), docosahexaenoic acid (DHA), GLA, eicosapentaenoic acid (EPA) or oleic acid (OA). In an hour, cells were irradiated with 5 Gy or 10 Gy with a Teragam K-01 cobalt unit, and then incubated for 24, 48 or 72 hours.

b. **Kinetic and biochemical assays**

We measured LDH (lactate dehydrogenase) and MTS (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) activity 72 hours after treatment. We compared the difference between the samples with two-sample unequal variance, two-tailed distribution Student's t-Test. We investigated the normalized cell index of cells for 72 hours with Real-Time Cell Electronic Sensing (RT-CES) instrument. The RT-CES instrument measures the impedance in

the wells where cells were plated. It determines normalized cell index, which is an indicator of cell number, proliferation, viability and cell growth (Kürti et al., 2012, Ózsvári et al., 2010). Thus, it is possible to follow the kinetics of the growth of cells treated with different substances.

c. **Evaluation of the rate of apoptosis**

To evaluate the rate of apoptosis we stained the cells with Annexin-V-Alexa Fluor 488 and propidium iodide, then analyzed them with BD FACSCalibur Flow Cytometer. We estimated the difference between the samples with two-sample equal variance, two-tailed distribution Student's t-Test.

d. **Gene expression analysis**

After an incubation interval of 24 and 48 hours we isolated ribonucleic acid (RNA), which we transcribed into complementary deoxyribonucleic acid (cDNA). We investigated the expression of genes chosen by literature analysis with real time quantitative reverse transcription polymerase chain reaction (QRT-PCR). The significance of difference between samples was determined by two-sample unequal variance, two-tailed distribution Student's t-Test.

e. **miRNA expression analysis**

After 48 hours of treatment we isolated microRNA (miRNA). We investigated the expression of seven miRNAs with QRT-PCR. The difference between the samples was determined by two-sample unequal variance, two-tailed distribution Student's t-Test.

f. **Morphological analysis of cells**

24 and 48 hours after treatment (in case of control cells also at the moment of treatment) we made holographic images with HoloMonitor^{TM} M3 of at least three representative frames of the samples. With the software of the apparatus we measured cell number, confluence, and in case of each cell average cell irregularity and average cell thickness. We compared the samples by two-sample, two-tailed distribution Student's t-Test.

g. **Evaluation of lipid droplet accumulation**

The lipid droplets (LD) of U-87 MG cells were stained with AC-202 48 hours after treatment. The lipid droplets were visualized with Olympus Fluoview confocal laser scanning microscope. We interpreted the pictures with ImageJ software and Olympus Fluoview software. The significance of the difference between the samples was determined by two-sample equal variance, two-tailed distribution Student's t-Test. The results were also presented by the internet application created by Spitzer et al. (2014) (http://boxplot.tyerslab.com/).

4. Results

a. **Cellular and biochemical measurements**

According to RT-CES measurements 25-75 μM AA, 25-75 μM DHA, 50 μM GLA, 400 μM OA enhanced the effect of irradiation and diminished in a significant manner the normalized cell index compared to the cells that were only irradiated. The normalized cell index is an indicator of cell proliferation. Treatment with OA had an interesting effect on U-87 MG cells: 100-200 μM increased the cell proliferation compared to the control cells.

In case of LDH activity measurement all doses (5 Gy; 10 Gy) and AA concentration (25 μM; 50 μM; 75 μM AA) and the combination of these had a significant effect compared to control. In case of MTS assay 75 μM AA and 10 Gy had a synergistic effect.

During LDH activity assays when cells were treated with 25 μM DHA and 10 Gy or 50 μM and 5 Gy we could detect synergistic effect. With cell viability measurements we noticed significant effect in case of every dose (5 Gy; 10 Gy) and every concentration (25 μM; 50 μM; 75 μM DHA) and the combination of these.

In case of every dose (5 Gy and 10 Gy) and every GLA concentration (50 μM; 75 μM and 100 μM) and the combination of these a significant difference could be detected between the LDH activity of the treated samples and the control. 50 and 75 μM GLA does not influence the cell viability of U-87 MG cells significantly. Addition of 50-100 μM GLA to cells that were irradiated with 5 Gy and 10 Gy does not influence the cell viability of U-87 MG cells.

In case of LDH activity 100 μM OA and 10 Gy had synergistic effect. 400 μM OA diminished the LDH activity and cell viability below 20%. In case of MTS assay 400 μM OA and 10 Gy had synergistic effect.

During LDH activity measurements we could detect synergistic effect when 75 μM (with 5 Gy) and 100 μM (with 5 Gy and 10 Gy) EPA was applied. In case of cell viability assays we noticed significant synergistic effect for the application of 50 μM EPA and 10 Gy. 100 μM OA and 50-100 μM EPA did not affect the cell viability and LDH activity of U-87 MG cells.

According to our biochemical assays we found that AA, DHA and GLA were the most effective fatty acids, therefore we selected there for further cellular and gene expression analysis.

We applied AA and DHA in 25 μM concentration, while GLA was used at 50 μM concentration.

b. **Morphological assessment after exposure to PUFA and/or irradiation by using holographic imaging**

Holographic imaging allows the evaluation of live cells without utilization of invasive stains. The integrated software permits us to measure more than 40 parameters of the cells, indicating information regarding to cytotoxicity (Madácsi et al., 2013).

PUFA treatment did not alter cell number, confluence, average cell thickness, and average cell irregularity after 24 hour treatment. Co-application of AA and irradiation changed cell number, confluence, average cell thickness and average cell irregularity compared to control cells after 48 hour exposure. After 48 hours the combination of PUFAs and 10 Gy changed all four parameters significantly compared to the cells that were only irradiated, except in case of average cell irregularity parameter, when DHA was applied on 10 Gy irradiated cells.

c. **Measurement of apoptosis after PUFA treatment and/or irradiation by Fluorescence activated cell sorting (FACS) analysis**

In opposition with 5 Gy; a 10 Gy dose increased the rate of apoptotic cells significantly. 25 μM AA and 25 μM DHA did not influence the rate of apoptosis. 50 μM GLA increased significantly the number of apoptotic cells. 25 μM AA and 25 μM DHA increased the number of apoptotic cells significantly when it was combined with 5 Gy. Similar effects could be registered, when 25 μM AA was combined with 10 Gy.

d. **Gene expression analysis of glioma cells in resonse to PUFA and/or irradiation**

After 24h treatment genes related to oxidative stress (*TRXR1*, *GCLM*, *SRXN1*, *GSR*, *GSTO1*, *HMOX1*, *AKR1C1*, *NQO1*, *DDIT3* and *EGR1)* only minor changes could be observed. Therefore, further studies were done 48 h after treatment.

The expression of *SRXN1* was increased by AA and DHA, while *GCLM* expression by DHA. The expression of *HMOX1* increased due to 10 Gy treatment, while the expression of *AKR1C1* and *NQO1* not. AA and DHA treatment increased the *HMOX1* expression, none of the PUFAs affected *AKR1C1*, *NQO1* was affected only by DHA. The expression of the latter mentioned three genes increased when cells were exposed to 10 Gy and PUFA in the same time, compared to the case when they were exposed just to 10 Gy.

Irradiation caused an increase in the expression of the four early response genes (*EGR1*, a *TNF-α,* a *FOSL1* and *C-FOS).*

The expression of *EGR1* increased due to all three PUFAs. Application of AA diminished significantly the grade of increment of *EGR1* expression compared to just irradiated cells, while GLA significantly increased it. In case of all three PUFAs, the combination of irradiation and PUFA caused a significantly higher expression than PUFA treatment alone.

PUFAs do not change the expression of *TNF-α* and *C-FOS.* If we treat the cells with PUFAs and 10 Gy in the same time, the *TNF-α* and *C-FOS* expression increases significantly. The expression of *TNF-α* and *C-FOS* decreases significantly compared to only irradiated cells if 10 Gy is applied together with AA treatment.

The expression of *FOSL1* increased significantly compared to control cells, when PUFA and 10 Gy were applied on the same

sample. AA and GLA increased *FOSL1* expression significantly.

The *NOTCH1* expression increased significantly when cells were treated with GLA, 10 Gy or when 10 Gy was combined with AA or DHA. The addition of GLA to 10 Gy diminished significantly the *NOTCH1* expression compared to cells that were only irradiated.

Treatment with AA, or application of 10 Gy increased *GADD45A* expression significantly. The combination of AA or DHA with 10 Gy also raised *GADD45A* messenger RNA (mRNA) level significantly.

Only the 10 Gy dose increased *TP53* expression significantly, the PUFA treatment alone or in combination with 10 Gy did not cause a change in *TP53* mRNA level.

AA and 10 Gy increased *C-MYC* expression. In case of each PUFA their combination with irradiation also caused a significant increase in *C-MYC* expression.

After 48 hours PUFA treatment, 10 Gy or irradiation combined with PUFA treatment did not alter the expression of *MMP14, SIRT1, TGFBI, TIMP3.*

e. **Evaluation of miRNA expression after exposure to PUFA and/or 10 Gy**

The expression of miR34a, miR96, miR148a, miR148b and miR152 did not change in 48 hour after treatment. The expression of miR146 increased due to DHA treatment and due to combined exposure to 10 Gy and GLA. miR146a expression decreased due to GLA treatment. The level of miR181a increased after DHA treatment.

f. **Detection of expression changes in genes responsible for fatty acid synthesis, *PLIN3* and heat shock proteins after PUFA treatment and/or irradiation**

After 24h treatment 10 Gy and GLA treatment diminished significantly the *SCD1*, *SCD5* expression and increased *FADS1* mRNA level. DHA, GLA, 10 Gy alone, and combined diminished *FADS2* expression significantly. GLA treatment or the combination of 10 Gy and PUFA diminished *FASN* expression in a significant manner.

After 48h treatment the expression of *FASN*, *SCD1*, *SCD5*, *FADS1*, *FADS2* significantly diminished due to GLA treatment. The expression of *FASN* increased after irradiation. The expression of *FADS3*, *ELOVL1*, *ELOVL2*, *ELOVL5*, *HSPB2* did not change nor due to GLA treatment, nor due to irradiation, nor due to the combination of GLA treatment and irradiation. The expression of *PLIN3* increased due to irradiation. GLA treatment increased *HSP90AA1* mRNA level significantly. 10 Gy, GLA, 5 Gy and GLA or 10 Gy and GLA increased the expression of *HSPB1* significantly.

g. **Lipid droplet accumulation after GLA treatment and/or irradiation**

Irradiation reduced significantly the LD content of glioma cells. GLA treatment increased significantly the lipid droplet accumulation of irradiated U-87 MG cells.

5. Conclusions, new scientific results

1. Until now, no data are available on LDH and MTS assays combined with kinetic measurments on glioma cells; in order to assess the effect of UFAs and/or irradiation (5 Gy and 10 Gy). In case of all investigated UFAs (AA, DHA, GLA, OA and

EPA) we could detect synergistic effects with 10 Gy by LDH or MTS measurments.
2. Based on our LDH, MTS and kinetic measurments 25 μM AA, 25 μM DHA, 50 μM GLA seemed appropriate for further experiments.
3. The combined effect of PUFAs and radiation has not yet been investigated on U-87 MG cell morphology. Application of PUFA treatment with radiation (10 Gy) influenced remarkably the cell morphology after 48 hours treatment.
4. Based on our result the changes in gene expression are PUFA and irradiation specific. According to the results and literature, the effects that were detected are tissue specific. The effects of application of PUFAs as adjuvants to radiation, on gene expression of glioma cells, had not been determined yet. The incubation time is an important influencing factor in case of gene expression analysis. AA, DHA and GLA influences gene expression in a specific manner. PUFAs altered the effect of radiation (10 Gy) – enhanced or modified it, in a favorable way.
5. The effect of co-application of PUFAs with radiation on miRNA expression of glioma cell line is a novel information. We could observe small change in expression only in case of miR146a. In case of miR181a only the expression of non-irradiated DHA treated sample was altered. In case of miR34a, miR96, miR148a, miR148b and miR152 the applied treatments were not effective.
6. GLA treatment did not induce LD accumulation. According to our knowledge this is a novel finding; the consequence of GLA treatment of U-87 MG cells on LD accumulation was not yet studied. Radiation (5 and 10 Gy) diminished LD quantity in U-87 MG cells, which has not yet been described. The accumulation of LDs, has not been investigated on GLA treated and radiated glioma cell line yet. In case of application of GLA

as adjuvant to radiation (5 Gy and 10 Gy), the LD quantity was significantly higher, than in case of cells that were exposed only to radiation.
7. Detailed description can be found in scientific literature about the cytotoxicity of PUFA-s and/or radiation on cancerous cells. Nonetheless, it was advisable to complement the gene and miRNA expression measurements with information about the apoptotic effect, on the same model system, under the same circumstances, as the estimation of transript alteration was performed. PUFAs alone can be effective as agents, that act against cancer. 25 μM AA and 25 μM DHA does not increase the apoptotic index, while 50 μM GLA does. A synergistic effect could be detected between AA or DHA and radiation (5 Gy) regarding the degree of apoptosis.

To understand the intracellular effect of the application of PUFAs alone, or as adjuvants to radiation further experiments are necessary.

6. References

Cowing BE, Saker KE (2001): Polyunsaturated fatty acids and epidermal growth factor receptor/mitogen-activated protein kinase signaling in mammary cancer. *J. Nutr* 131(4):1125-8.

Das UN (2004): From bench to the clinic: gamma-linolenic acid therapy of human gliomas. Prostaglandins Leukot Essent Fatty Acids. 70(6):539-52.

Das UN (2007): Gamma-linolenic acid therapy of human glioma-a review of in vitro, in vivo, and clinical studies. Med Sci Monit. 13:7.

Dupertuis YM, Mequid MM, Pichard C (2007): Colon cancer therapy: new perspectives of nutritional manipulations using polyunsaturated fatty acids. Curr Opin Clin Nutr Metab Care 10(4):427-32.

Fan X, Khaki L, Zhu TS, Soules ME, Talsma CE, Gul N, Kuoh C, Zhang J, Li YM, Maiaczyk J, Nikkhah G, Dimeco F, Piccirillo S, Vescovi Al, Eberhart CG (2010): NOTCH pathway blockade depletes CD133-positive glioblastoma cells and inhibits growth of tumor neurospheres and xenografts. Stem Cells 28(1):5-16.

Igal RA (2010): Stearoyl-CoA desaturase-1: a novel key player in the mechanisms of cell proliferation, programmed cell death and transformation to cancer. Carcinogenesis 31(9):1509-15.

Kitajka K, Sinclair AJ, Weisinger RS, Weisinger HS, Mathai M, Jayasooriya AP, Halver JE, Puskás LG (2004): Effects of dietary omega-3 polyunsaturated fatty acids on brain gene expression. Proc Natl Acad Sci USA 2004, 101(30):10931-6.

Kürti L, Veszelka S, Bocsik A, Dung NT, Ozsvári B, Puskás LG, Kittel A, Szabó-Révész P, Deli MA (2012): The effect of sucrose esters on a culture model of the nasal barrier. Toxicol In Vitro 26(3):445-54.

Madácsi R, Kanizsai I, Fehér LZ, Gyuris M, Ozsvári B, Erdélyi A, Wölfling J, Puskás LG (2013): Aromatic sulfonamides containing a condensed piperidine moiety as potential oxidative stress-inducing anticancer agents. Med Chem 9:7.

Ntambi JM, Bené H (2001): Polyunsaturated fatty acid regulation of gene expression. J Mol Neurosci 16(2-3):273-8.

Ózsvári B, Puskás LG, Nagy LI, Kanizsai I, Gyuris M, Madácsi R, Fehér LZ, Gerö D, Szabó C (2010): A cell-microelectronic sensing technique for the screening of cytoprotective compounds. Int J Mol Med 25:4.

Pucer A, Brglez V, Payré C, Pungerčar J, Lameau G, Petan T (2013): Group X secreted phospholipase A2 induces lipid droplet formation and prolongs breast cancer cell survival. Mol Cancer 12(1):111

Puskás LG, Fehér LZ, Vizler C, Ayaydin F, Rásó E, Molnár E, Magyary I, Kanizsai I, Gyuris M, Madácsi R, Fábián G, Farkas K, Hegyi P. Baska F, Ozsvári B, Kitajka K (2010): Polyunsaturated fatty acids synergize

with lipid droplet binding thalidomide analogs to induce oxidative stress in cancer cells**.** Lipids Health Dis. 9:56.

Sandrone SS, Repossi G, Candolfi M, Eynard AR (2014): Polyunsaturated fatty acids and gliomas: A critical review of experimental, clinical, and epidemiologic data.Nutrition, S0899–9007:00075–00076.

Soni D, King JA, Kaye AH, Hovens CM (2005): Genetics of glioblastoma multiforme: mitogenic signaling and cell cycle pathways converge. J Clin Neurosci. 12(1):1-5.

Spitzer M, Wildenhain J, Rappsilber J, Tyers M (2014): BoxPlotR: a web tool for generation of box plots. Nat Methods. 11(2):121-2

Vartak S, Robbins ME, Spector AA (1997): Polyunsaturated fatty acids increase the sensitivity of 36B10 rat astrocytoma cells to radiation-induced cell kill. Lipids. 32(3):283-92.

Vartak S, McCaw R, Davis CS, Robbins ME, Spector AA (1998): Gamma-linolenic acid (GLA) is cytotoxic to 36B10 malignant rat astrocytoma cells but not to 'normal' rat astrocytes. Br J Cancer 77(10):1612-20.

Vessby B, Gustafsson IB, Tengblad S, Boberg M, Andersson A (2002): Desaturation and elongation of fatty acids and insulin action. Ann N Y Acad Sci 967:183-95.

Wang J, Wakeman TP, Lathia JD, Hjelmeland AB, Wang XF, White RR, Rich JN, Sullenger BA (2010): Notch promotes radioresistance of glioma stem cells. Stem Cells. 28(1):17-28.