REGULATED CELL DEATH: APOPTOSIS AND NECROPTOSIS IN U937 CELL LINE

Ph.D thesis

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1. Introduction

Kerr, Wyllie and Currie proposed a controlled cell elimination process, which acts complementary but opposite to cell division, to keep tissue homeostasis. That was suggested to be an active and programmed process which can be initiated or inhibited by various physiological or pathological stimuli. Since then, apoptosis as they termed, became a widely investigated cell physiological process. Later Horvitz *et al.* described the molecular genetic pathway responsible for apoptosis that leads to genetically determined cell elimination during the development of the model organism *Caenorhabditis elegans*. Apoptosis has become a widely used term and is often considered to be synonymous with programmed cell death (PCD), while necrosis remained a cell death type lacking the morphological signs of apoptosis. In the last decades accumulating evidences imply that necrotic cell death can also be a genetically regulated event and can be classified as programmed cell death in line with apoptosis. However, contrary to the fairly well characterized pathways of apoptosis the molecular constituents of necrotic pathway(s) are hardly known.

Apoptosis, secondary necrosis and necroptosis

Conventional knowledge considers apoptosis as a caspase-dependent, programmed, non-immunogenic process, characterized by cellular shrinkage, membrane blebbing, chromatin condensation and DNA degradation. During apoptosis dying cell looses its contacts to the neighbouring cells and finally is fragmented into compact membrane-enclosed structures, called apoptotic bodies. Under normal physiological circumstances apoptotic bodies are engulfed by macrophages and are removed from the tissue without activating immune response.

It is accepted that the main, but not obligate, hallmarks of apoptosis are the activation of caspases resulting in cleavage of a selective pool of proteins, the loss of phospholipid asymmetry of the plasma membrane, cell shrinkage and the early oligonucleosomal DNA fragmentation.

In absence of corpse clearing mechanism the apoptotic process is terminated in an autolytic necrotic outcome, with loss of plasma membrane integrity. This phenomenon was called as secondary necrosis by Wyllie *et al.* with the intention to better distinguish this mode of cell elimination from “cellular necrosis occurring *ab initio*”, which should be called “primary necrosis”.

Originally the word ne

crosis was used as a pathological term which describes the morphology of dead cells observed in many human diseases such as neurodegenerative diseases as pancreas adiponecrosis, trauma, ischemia-reperfusion in myocardial infarction or in cerebral infarction, bacterial infection, tumor

malignancies and not as description characterising the way how cells dye. Morphologically, necrosis is marked by oncosis accompanied by early loss of the integrity of the plasma membrane and intracellular compartments.

Accumulating evidences have confirmed that necrotic cell death can also be a regulated event and therefore be classified as programmed cell death in line with apoptosis.

Necroptosis

A novel, necrotic-like, caspase-independent cell death form has been recently described and termed as necroptosis. It was demonstrated that stimulation of the extrinsic apoptotic pathway by tumor necrosis factor-alpha (TNFα) or Fas ligand (FASL) under caspase-compromised conditions in certain cell types resulted in a necrotic-like cell death process. This pathway can be hampered by a small molecular weight inhibitor called necrostatin-1 (Nec), which acts by inhibiting the kinase activity of receptor-interacting protein kinase 1 (RIPK1) and by necrosulfonamide (NSA), an inhibitor of mixed lineage kinase domain-like protein (MLKL), a substrate of receptor-interacting protein kinase 3 (RIPK3). It was shown that autophagy can be activated as downstream consequence of necroptosis. Moreover the role of poly(ADP-ribose) polimerase (PARP) was documented in RIPK-mediated necrosis. Here we use the term necroptosis a type of programmed necrosis which requires the kinase activity of RIPK1 and RIPK3 (receptor-interacting protein kinase 1 and 3) and fulfills under caspase-compromised conditions.

Former results

Previously we studied the nature of the switch mechanism between apoptosis and necrosis and investigated the intrinsic apoptotic pathway in staurosporine (STS)-treated U937 cells. STS is a generally accepted inducer of the intrinsic apoptotic pathway and it is a wide spectrum inhibitor of protein kinases. STS initiates apoptosis by enhancing mitochondrial permeability transition. In the absence of clearing mechanism, the apoptotic cell death process is followed by secondary necrosis, an uncontrolled disruption of the plasma membrane. Earlier, we and others found that STS could provoke necrosis in caspase-compromised cancer cells. We were interested to examine the role of necroptosis in the same necrotic process, in spite of the fact that necroptosis is generally defined as a result of a death receptor-triggered cell death pathway.

2. Objectives

Our aim was to characterize the molecular components of the STS-induced necrosis in U937 cell line. We were curious about the role of RIPK1, MLKL, PARP-1 and the effect of lysosomal enzyme inhibitor CA-074-OMe in STS-triggered necrosis to differentiate secondary necrosis and

necroptosis. We concomitantly established a TRAIL-induced necroptotic type of cell death model with U937 cells as a reference, death receptor-mediated necrosis. We intended to compare these two inducer (STS and TRAIL) evoked cell death mechanisms by using a panel of pharmacological inhibitors: caspase inhibitor: z-Val-DL-Asp-fluoromethylketone (zVD), PARP inhibitor: PJ-34, HSP90 inhibitor: geldanamycin (GA), PI3K (type III) inhibitor: 3-methyladenine (MA), cystein cathepsin inhibitor: CA-074-OMe (CA), RIP1K inhibitor: necrostatin-1 (Nec) and MLKL inhibitor: necrosulfonamide (NSA).

3. Methods

3. 1. Cell culture

U937 promonocytic cells were cultured in RPMI 1640 medium, supplemented with 10 % heat inactivated fetal bovine serum, 2 mM L-glutamine and 100 μg/mL penicillin and 100 units/ml streptomycin at 37 ^{o}C in a 5% CO_2 containing, humidified incubator. For treatments, cells were distributed into 48-well suspension plates at a cell density of 3 x 10^5 cells/mL or 5 x 10^5 cells/mL 60 minutes before drug treatment. Then zVD (10 μM) was added with or without together other drugs as described above started 1 hr (Nec, CA, NSA, PJ) or 4 hrs (GA) before STS (1 μM) or TRAIL (50 ng/mL) addition. Duration of drug treatments varied between 4-24 hours.

3. 2. Detection of the cell death-associated functional changes by flow cytometry

Data collection was carried out by using FACScan or FACScalibur flow cytometers and analysis of the results was performed by WINLIST software.

3. 2. 1. Assay of propidium iodide (PI) uptake of native cells representing the damage of plasma membrane

Treated cells were stained directly by adding 0.5 mL PBS containing 5 mM glucose and 10 μg/mL PI to each well. Cells were analyzed on FL2H log scale histograms.

3. 2. 2. Characterization of phosphatidylserine distribution in the plasma membrane by flow cytometric analysis of Annexin V-FITC and PI double-labeled cells

Cells were stained with PBS containing 5 mM glucose and 10 μg/mL PI for 10 minutes at 37 ^{o}C. After centrifugation, cells were suspended in binding buffer and stained with AnnexinV-FITC. Thereafter an additional 400 μL of binding buffer, containing 1 μg/mL PI was add to the samples and FACS analysis was carried out immediately.

3. 2. 3. Changes of mitochondrial transmembrane potential were characterized by the $DiOC_6(3)$ uptake method

Treated cells were stained with PBS containing 5 mM glucose, 10 nM $DiOC_6(3)$ and 10 µg/mL PI and incubated further for 15 minutes at 37 ^oC and analyzed without further washing steps by FACS.

3. 2. 4. Functioning lysosomal compartments are characterized by the red fluorescence of acridine orange (AO) emitted in the acidic environment of lysosomes

Treated cells were stained with PBS containing 5 mM glucose and 5 µg/mL AO for 15 minutes at 37 ^oC. The pelleted cells were resuspended in 1 mL of 5 mM glucose containing PBS and analyzed by FACS immediately.

3. 2. 5. Determination of oligonucleosomal DNA fragmentation by the measurement of sub-G1 population of cells

Treated cells were pelleted and suspended in 70% ethanol. Then the ethanol-fixed cells were sedimented again and their oligonucleosomal sized DNA content were extracted by extraction buffer. The DNA remaining inside the permeabilized cells were stained with PI before FACS analysis.

3. 2. 6. Representation of side scatter change and DNA fragmentation

Treated cells were fixed, stained with PI and gated according to the sub-G1 technique. The gated populations were analyzed on the (SSC, FL2H) diagrams.

3. 3. Agarose gel electrophoresis

Samples were phenol-chloroform extracted once and their DNA content were precipitated in ethanol, pelleted and redissolved in TE buffer. DNA was electrophoresed in 1.5% agarose gel in TBA buffer, stained with ethidium bromide and DNA was detected under UV light.

3. 4. Light microscopic studies

Cytospin preparations were fixed in methanol, stained with hematoxylin-eosin, were dehydrated with ethanol, acetone and xilol. The morphological changes of apoptosis were studied by light microscopy at 400x magnification.

3. 5. Fluorescent microscopic studies

Treated U937 cells were pelleted and were stained with Hoechst dye and PI to distinguish between apoptotic and necrotic cells. After incubation cells were immediately photographed under a fluorescence microscope at 400x magnification.

3. 6. Western blot representation of PARP-1 and RIPK1 cleavage

Cells were washed and resuspended in Laemmli buffer. After boiling, the samples were electrophoresed in a 10% SDS-PAGE gel and blotted onto nitrocellulose. After blocking membranes, specific antibodies against PARP-1, RIPK1 or GAPDH were added, incubated and membranes were washed. Blots were incubated with HPO-labeled second antibodies. Proteins were visualized by ECL.

3. 7. Caspase activity assay

Aliquots of cells were centrifuged and washed with PBS and finally were suspended in caspase buffer. After transferring samples into wells of 96-well plates, Triton X-100 was added and cell lysis was completed. Caspase substrate z-DEVD-AMC was admixed, and fluorescence intensities of the liberated amc were recorded in fluorescence plate reader. Excitation wavelength was 380 nm and emission was measured at 445 nm.

3. 8. Statistics

Statistical analysis and significance of differences in comparable values were calculated by applying Student's t-probe (two tailed, two sample unequal variance).

4. Results

4. 1. TRAIL induces necroptosis in U937 cell line in the presence of a caspase inhibitor

We found U937 cells sensitive to TRAIL cytokine in 20 hrs. TRAIL treatment generated apoptotic nuclear DNA condensation together with nucleosomal DNA fragmentation. These changes resulted in apoptotic PARP-1 fragmentation due to caspase-3 activation. Finally, signs of secondary necrosis occurred when cells turn permeable for PI.

When cells were pre-treated with the pan-caspase inhibitor zVD, the executioner caspase activation was prevented, therefore PARP-1 processing was inhibited. Apoptotic type oligonucleosomal DNA fragmentation diminished, however, the high molecular weight fragmentation of DNA appeared resembling necrosis and DNA condensation was evaded. Instead of apoptotic morphology, dying cells were swelling and showing a "ghost-like" morphology resembling necrosis. Plasma membrane rupture detected and quantified by PI uptake revealed that the number of PI positive cells in caspase inhibitor-sensitized U937 cells upon TRAIL treatment were increasing compared to cells treated with TRAIL alone. In the presence of RIPK1 inhibitor Nec, PI uptake was reduced only marginally in caspase-competent (secondary necrotic) cells, while it was completely reduced in caspase-compromised cells after TRAIL treatment.

To further confirm the role of RIPK1 activity in TRAIL-induced necroptotic or secondary necrotic cell death processes we studied the effect of geldanamycin (GA). GA, an inhibitor of the heat shock protein 90 kDa (HSP90) was known to downregulate the protein level of RIPK1 and therefore was expected to halt necroptosis. Indeed, GA pre-treatment significantly reduced the extent of TRAIL-induced necroptosis detected by PI uptake.

These results indicate that in the presence of caspase inhibitor, TRAIL-exposed U937 cells undergo necroptosis instead of apoptosis. Moreover TRAIL-triggered necroptosis can be suspended both by Nec and GA.

4. 2. STS induces primary necrosis in the presence of a caspase inhibitor

To study the STS-evoked necrosis we applied the caspase inhibitor zVD-fmk. We found that in the presence of zVD, DEVDase activity was completely halted and STS triggered mainly plasma membrane damage after shorter incubation time (8-12 hrs) meanwhile we could detect both sub-G1 and PI-positive population after prolonged treatment (20 hrs).

Next we tested the effect of the RIPK1 inhibitor under caspase-compromised conditions. Nec concentration dependently abrogated the necrosis confirmed by the reduced ratio of PI positive cells. Nec and GA arrested the necrosis after 8, 12 hrs of incubation time confirmed by flow cytometry and by fluorescent microscopic studies using Hoechst dye and PI double staining technique.

After prolonged (20 hrs) treatment, the STS and zVD-triggered plasma membrane rupture was diminished only partially by Nec (~50%). Furthermore, Nec unaffected the caspase-mediated DNA fragmentation and condensation either after shorter (8, 12 hrs) or after longer incubation time (20 hrs). The STS-induced secondary necrosis, the final stage of cell death, was affected by neither Nec nor GA.

Concerning the apoptotic parameters, STS and zVD-exposed samples showed high molecular weight DNA fragments confirmed by agarose gel electrophoresis, accompanied with low molecular weight DNA fragments that are primarily smear-like, opposite to apoptotic DNA fragmentation and typical to necrosis. STS treatment resulted in nucleosomal DNA ladder fragmentation trimmed possibly by postmortem DNase activity.

These results indicate that STS-induced apoptosis in U937 cells ensued by secondary necrosis, while under caspase-compromised conditions STS induced primary necrosis that is partially inhibitable by Nec and GA, two drugs affecting RIPK1 activity.

4. 3. STS and TRAIL induce RIPK1 and MLKL-dependent necroptosis

To confirm the role of RIPK1 in the STS-induced necroptosis, we tested the proteolytic degradation of RIPK1 in U937 cell line by Western blot analysis. STS-triggered caspase activation led to RIPK1 processing. Full length RIPK1 was proteolytically cleaved by caspase-8 during STS-induced apoptosis. Processing of RIPK1 was highly inhibited in the presence of the caspase inhibitor zVD.

To further study the molecular components involved in STS-triggered necroptotic pathway under caspase-compromised conditions we investigated the role of MLKL. Cells were treated with various concentrations of necrosulfonamide (NSA), an inhibitor of MLKL. NSA reduced the ratio of PI positive cells in a concentration-dependent fashion independently of the way of cell death trigger (either TRAIL or STS) under caspase-compromised condition. NSA arrested both the TRAIL and STS-triggered necrosis, confirmed by PI uptake measurements and also by Hoechst/PI double staining method. Meanwhile NSA failed to reverse the STS-evoked DNA fragmentation or prevent secondary necrosis.

These results confirmed the crucial role of RIPK1 and MLKL in STS and TRAIL-induced necrosis under caspase-compromised conditions. Therefore we can consider the observed necrosis in U937 cells as necroptosis.

4. 4. MA reduces STS-induced necroptosis

To test the contribution of autophagy to the STS-induced necroptotic process we studied the effect of 3-methyladenine (MA). MA is known to inhibit autophagy by blocking autophagosome formation via inhibition of type III phosphatidylinositol 3-kinases. We could detect only partial inhibition of MA either at 10 mM final concentration on STS+zVD-induced necroptosis, meanwhile MA was failed to adjust STS-triggered apoptotic pathway. In comparison to the effect of Nec, we also detected a partial protection of plasma membrane integrity under our standard assay conditions when MA was applied. The combination of Nec with MA resulted in an additive protection and no synergism was observed indicating that necroptosis and autophagic cell death are presumably two independent cell death pathways when cell death is induced by STS+zVD in U937 cells.

4. 5. CA inhibits both the TRAIL and STS-induced necroptosis in the presence of a caspase inhibitor

Previously we found that CA, an inhibitor of cysteine cathepsins, rescued the caspase-independent necrotic form of cell death of promyelocytic leukemia cells treated by STS. In this

study we tested whether CA treatment might promote the survival of U937 cells dying under necroptotic conditions.

Interestingly CA almost completely hampered the TRAIL-induced necroptotic cell death measured by plasma membrane rupture. Moreover, CA was comprehensive inhibitor of STS-triggered necroptosis too. To further characterize the effect of CA on the necroptotic pathway and to compare to the inhibitory activities of Nec and GA, its action on different cellular compartments were investigated. Time course detection of mitochondrial membrane depolarization was carried out by flow cytometric analysis of $DiOC_6(3)$ stained STS+zVD-treated U937 cells. Our results confirmed that a continuous, time-dependent reduction of mitochondrial membrane integrity happens in the cell population. While Nec prevented partially the cells to loose their mitochondrial membrane potential for STS-treatment, CA provided nearly complete protection under caspase activity arrested conditions.

In case of STS treatment, the effect of CA treatment on the reduction of phosphatidylserine translocation and plasma membrane rupture was complete. Similarly, the complete arrest of CA on the rupture of plasma membrane was detected by PI uptake and by Hoechst/PI double staining techniques for STS or TRAIL treated U937 cells in the presence of zVD. Moreover, the loss of lysosomal acidity induced either by STS or TRAIL treatment under necroptosis inducing conditions was remarkably withheld by CA pretreatment. Additionally CA could significantly prevent the loss of mitochondrial membrane depolarization in both cases of STS and TRAIL treatment in the presence of the caspase inhibitor zVD.

The observed apoptotic parameters: the invariable percentage of the sub-G1 cell and the presence of ladder type DNA degradation observed in agarose gels proved that CA had no effect on caspase-dependent apoptosis or on the ensuing secondary necrosis in TRAIL or STS-exposed cells. Moreover, the absence of proteolytic degradation of RIPK1 also confirmed the avoidance of apoptosis.

From these data we surmised that CA, similarly to NSA is also an effective inhibitor of the necroptotic pathway induced by STS in the presence of caspase inhibitor. While Nec and GA withheld only the early phase of STS-induced necroptosis in U937 cells.

4. 6. PJ-34 does not arrest either the TRAIL or STS-induced necroptosis in the presence of a caspase inhibitor

Participation of PARP-1 enzyme is well documented at several forms of cell death processes. Applying PARP inhibitor reduces the rate of PARP activation and slows down the exhaustion of

NAD and ATP pools, therefore generally shifts the cell death process towards apoptosis and prevents necrosis.

In our experimental system we tested the effect of PARP-1 enzyme inhibitor PJ-34 on the necroptotic cell death pathway. Major differences in the necroptotic process were not observed in the presence of the inhibitor. In STS or in TRAIL-treated and caspase-inhibited U937 cells PJ-34 did not influence the changes observed in mitochondrial transmembrane potentials, in PI permeability of cells, in the acidity of lysosomes or in the PS distribution in the plasma membrane. Furthermore, neither the caspase-dependent nor the caspase-independent STS or TRAIL-induced apoptotic DNA fragmentation was abolished by the applied PARP-1 inhibitor.

At the same time the secondary necrotic cell death process was postponed by PJ-34 in a concentration-dependent manner. This effect was confirmed by the observed intact plasma membrane integrity both under STS and TRAIL-induced conditions where the enzyme activity of PARP-1 was increased in the absence of the inhibitor. In addition, the loss of lysosomal acidity was notably reduced by PJ-34 during prolonged treatment of U937 cells with STS and this effect, although with much less extent was observed after TRAIL treatment as well.

Contrary to the necrotic process, in cases of STS or TRAIL-induced apoptosis PJ-34 showed only marginal effect on the cell death process. The hallmarks of apoptosis including phosphatidylserine distribution, loss of mitochondrial transmembrane and the DNA fragmentation were not influenced by PJ-34. These results indicate that PJ-34 could only delay the secondary necrosis, but was unable to prevent the caspase-dependent apoptosis. Our results show that the enzymatic activity of PARP-1 directly or indirectly influences the process of secondary necrosis but is dispensable in necroptosis at least in TRAIL and STS-treated U937 cells.

5. Conclusions

1.) TRAIL, a death receptor ligand cytokine, which generally induces apoptotic cell death through the extrinsic pathway, under caspase-compromised conditions triggers necroptosis in U937 cells inhibitable by necrostatin, geldanamycin and necrosulfonamide.

2.) Staurosporine, a kinase inhibitor compound which generally induces apoptosis through the intrinsic pathway can also induce necroptosis under caspase-deprived conditions inhibitable by necrostatin, geldanamycin and necrosulfonamide.

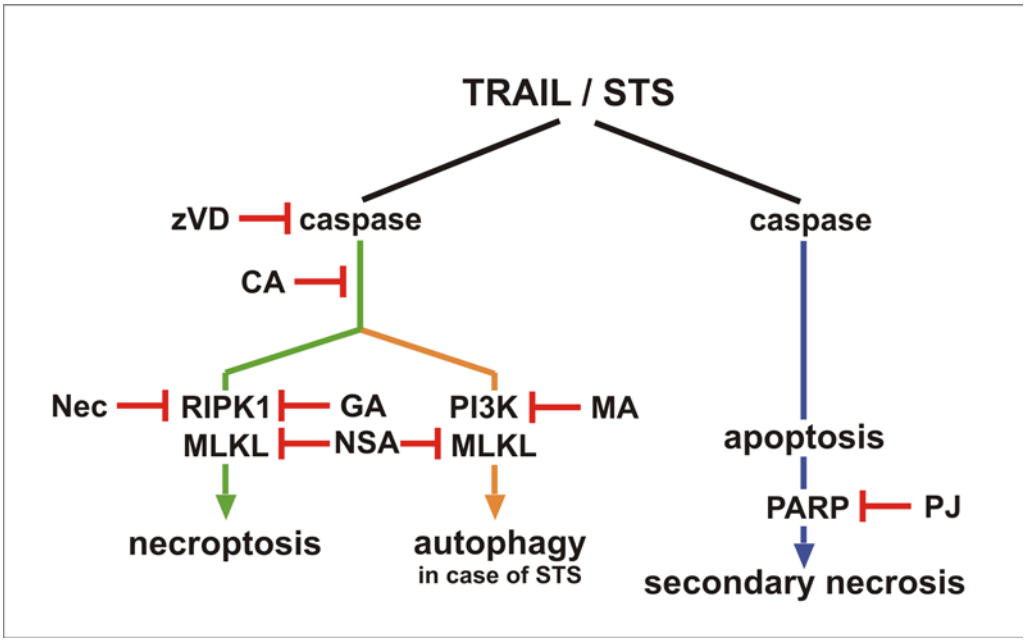
3.) In parallel with the staurosporine-induced RIP-mediated necroptotic process, an autophagy-like process is also activated, and shown to be partially blocked by the PI3K inhibitor MA. The

combination of necrostatin with 3-methyladenin resulted in an additive protection of cell death and no synergism was observed indicating that RIP-mediated necroptosis and autophagic-like cell death are presumably two parallel processes in U937 cells.

5.) CA inhibits the necroptotic cell death in U937 cells induced by either TRAIL or staurosporine. Note that while the inhibitory potential of necrostatin and geldanamycin is only partial, CA-074-OMe treatment of cells provide complete protection upon staurosporine treatment. *(These results point out that a non-cathepsin B, off-target of CA-074-OMe can be an initiator position of necroptosis.)*

6.) In U937 cells neither apoptosis nor necroptosis was influenced by PJ-34. On the other hand, while the secondary necrosis of U937 cells was significantly postponed by PJ-34, it was not affected by necrostatin, geldanamycin, necrosulfonamide or CA-074-OMe at all. *(If these results will be proved in other cancer cell lines, clinical application of PARP inhibitors might control the tumor lysis syndrome).*

The flow chart shown here summarizes the hypothetic places of actions, where the drugs used in the current study inhibit the staurosporine or TRAIL-induced cell death pathways in U937 cells. We hope that the presented results will contribute to the better understanding of the molecular background of necroptosis.



Schematic diagram of the action of inhibitors on TRAIL and staurosporine-induced cell death pathways in U937 cell line.

6. Publications

6. 1. Publications related to the thesis

1. **Dunai ZA**, Imre G, Barna G, Korcsmaros T, Petak I, Bauer PI, Mihalik R. Staurosporine induces necroptotic cell death under caspase-compromised conditions in U937 cells. PLoS One 2012;7(7):e41945. Epub 2012 Jul 31. **IF: 4,092**

2. **Dunai ZA**, Bauer PI, Mihalik R Necroptosis: Biochemical, physiological and pathological aspects. Pathol Oncol Res. 2011 Dec;17(4):791-800. **IF: 1,366**

3. Imre G, **Dunai ZA**, Petak I, Mihalik R Cystein cathepsin and Hsp90 activities determine the balance between apoptotic and necrotic cell death pathways in caspase-compromised U937 cells. Biochim Biophys Acta. 2007 Oct;1773(10):1546-57. **IF: 4.374**

6. 2. Publications not directly related to the thesis

4. Stemmer U, **Dunai ZA**, Koller D, Purstinger G, Zenzmaier E, Deigner HP, Aflaki E, Kratky D, Hermetter A. Toxicity of oxidized phospholipids in cultured macrophages. Lipids Health Dis. 2012 Sep 7;11(1):110. **IF: 2,17**

5. Barna G, Sebestyen A, **Dunai ZA**, Csernus B, Mihalik R Heparin can liberate high molecular weight DNA from secondary necrotic cells. Cell Biol Int. 2012;36(12):1281-6. **IF: 1,482**

6. Fekete A, Kenesi E, Hunyadi-Gulyas E, Durgo H, Berko B, **Dunai ZA**, Bauer PI. The guanine-quadruplex structure in the human c-myc gene's promoter is converted into B-DNA form by the human poly(ADP-ribose)polymerase-1. PLoS One. 2012;7(8):e42690. **IF: 4,092**

7. Kun E, Mendeleyev J, Kirsten E, Hakam A, Kun AM, Fekete A, Bauer PI, **Dunai ZA**, Mihalik R. Regulation of malignant phenotype and bioenergetics by a π-electron donor-inducible mitochondrial MgATPase. Int J Mol Med. 2011 Feb;27(2):181-6. **IF: 1,573**