In vitro investigation of nuclear and extranuclear effects of pharmacologic PARP inhibition on different tumor cell models, with special attention to the mitochondrial structural and functional changes

PhD thesis

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1. Introduction

1.1. The Poly (ADP-ribose) polymerase enzyme and its nuclear and extranuclear effects

The 116kDa PARP-1 protein is the most famous member of the PARP superfamily [1]. Upon its activation, it recognizes its target-molecules through the DNA-binding module and begins the synthesis of 50-200 ADP-ribose groups on them, a process called poly(ADP-ribose) polymerization (PARilation) [2] [1].

Numerous repair molecules have PAR-binding motif, including the base-excision repair (BER), nucleotide excision repair (NER) and the non-homologous end joining repair (NHEJ) proteins, which suggests that PARP-1 has a crucial role in the coordination of repair functions upon the deoxyribonucleic acid (DNA) damage [3] [4] [5] [4, 6]. Several *in vitro* and *in vivo* experiments shed light on the PARP-1's role in repair independent physiologic and pathophysiologic processes [3] [7]. For example with the PARilation of transcription factors, PARP-1 modulates inflammatory proteins, the members of the electron transport chain and the process of mitochondrial transcription [8]. Furthermore PARP-1 apparently influences important signaling pathways, such as the protein kinase B (AKT/PKB) or MAPK (mitogen-activated protein kinase signaling [9] [10] [11] [12].

1.1. The therapeutic importance of PARP inhibition

In several tumors, PARP inhibition treatment with DNA-damaging agents leads to chromosome instability, cell-cycle arrest and apoptosis induction [13-15]. In support of PARP-1's extranuclear effects, PARP inhibition showed cellular protection in cerebral ischemic processes, *Diabetes mellitus*, myocardial ischemia-reperfusion and septic-shock model [16-18] [19]. The effect of PARP inhibition on different mitochondrial mechanisms is an important and interesting data. Indeed, in a previous research we found that by activation of different signaling

pathways, PARP inhibition protects the mitochondrial membrane potential upon oxidative stress [10, 20, 21].

Several in vivo studies reported encouraging results related to PARP inhibitors, while others showed controverting data. In a Phase-1 study on BRCA mutation carrier patients with ovarium and prostate cancer, the PARP inhibitor olaparib showed significant anti-tumor effect, at the same time, in another study on melanoma patients, there was no observed synergism between olaparib and dakarbazin [22, 23]. Similarly, controversial results were observed in a Phase II study, where the olaparib was effective in patients with ovarium carcinoma but it was ineffective in breast carcinoma, independently of the presence of the patients' BRCA status [24].

The observation that PARP-1, apart from its repair functions, modulates several signaling pathways and independently from the DNA damage response it bounds the chromatin and may contribute to several gen's induction, shed light on a new dimension of PARP-mediated functions, furthermore it may explained the therapeutic failures.

2. Aims

Based on its role in DNA repair the PARP-1 may be an important oncological therapeutic target, however it also influences several repair-independent mechanisms. Thus, logically, we may not observe a standard cell-type and host-organism independent cellular response upon PARP inhibition. We aimed to examine the effect of PARP inhibition on MAPK cascade, AKT signalization and other extranuclear mechanisms, in different tumor cell lines. Thereafter we investigated the effect of PARP inhibition on cell toxicity, and on the mitochondrial structure and function. Our aims were the followings:

- Investigate the effect of HO3089 and PJ34 PARP inhibitors on the colony formation capacity of different cell lines alone or in combination with cytostatic agents or irradiation.

- Investigate the effect of PARP inhibition on the different cell signaling pathways, MAP kinases and pro-and antiapoptotic factors.
- Investigate the effect of PARP inhibition on the mitochondrial structure and function alone and in combination with cytostatic agents

3. Materials and methods

3.1. Cells, cell cultures and reagents

As experimental models we used B16F10 mouse melanoma, human A549 lung cancer, human U251 glioblastoma and murine 4T1 breast cancer cell lines, which were obtained from American Type Culture Collection (Manassas, VA, USA). The used agents and concentrations were the following: PARP inhibitor PJ34 10 μM, a temozolomide 25 μM, a cisplatin 25 μM, PARP inhibitor HO3089 10 μM, PD98059 MAPKK inhibitor 4 μM, SB203580 p38 inhibitor 1.2 μM, LY294002 PI3K (phosphatidylinositol 3-kinases) inhibitor 10 μM and the JNK inhibitor II 1 μM. . The used reagents were ordered from Sigma-Aldrich (S.r.l., Milan, Italy), apart from the HO3089 PARP inhibitor which was a generous gift from Prof. Kálmán Hideg, the PD98059, SB203580 p38, LY294002 and JNK II inhibitors which were ordered from Calbiochem (Darmstadt, Germany) and the pPARPGFPC1/N3 construct, which was a generous gift from Dr. Balázs Debreceni. The dsRED plasmid contains a mitochondrial target sequence on its 5' end, which allows the investigation of the mitochondrial structural changes. The pPARPGFPC1/N3 construct functions as a dominant negative PARP enzyme by binding the PARP inhibitory locuses and expressing a PARP-DNA binding module (DBD), without performing PARilation activity [25] [20]. A telecobalt external irradiation equipment (Teratron 780C, average photon energy of 1.25 MeV) was applied for irradiation of the cells with a dose of 2.0 or 4.0 Grays.

3.2. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay

We used the MTT test in order to examine the cells' mitochondrial NADPH reductase activity. Cells were seeded in flat-bottom 96-well plates at the 2.5 × 104 per well density and cultured overnight before the assay. Following treatments, medium was replaced to a fresh one containing 0.5% 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) tetrazolium substrate and incubated for 3 hours. The water-insoluble violet formazan precipitate was solubilized in 100 ml 20% sodium dodecyl sulfate solution and optical densities were measured by an Infinite 200 Pro plate reader (Tecan Italia S.r.l., Milan, Italy) at 570 nm.

3.3. Colony formation assay

In order to examine their survival and proliferative capacity, B16F10, U251 and 4T1 cells were plated in 6-well plates at 300 cells/well density, cultured overnight before treatments and incubated for 10 days post-treatment. Cells were washed with 1 × PBS and stained with 0.1% Coomassie blue (Bio-Rad Laboratories S.r.l., Milan, Italy). Plates were scanned and the number of colonies was determined using the ImageJ software.

3.4. Analysis of cell death

In order to examine the cell death induction upon different treatments, we performed Flow cytometric analysis. B16F10 cells were seeded and treated, then the samples were stained with FITC labeled Annexin-V and Propidium iodide (eBioscience, Life Technologies, Milan, Italy) according to the manufacturer's protocol. Cell death was measured by flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson Italia S.r.l., Milan, Italy), and the data were analyzed by CellQuest Pro software.

3.5. Comet Assay

In order to examine the DNA fragmentation upon the treatments, we applied Comet assay. B16F10 cells were seeded into 6-well plates. After treatment, cells were harvested, centrifuged,

and mixed with low melting point agarose in PBS. The suspension was pipetted onto the pre-coated slides which were electrophoresed. Slides were stained with Hoechst 33342 and visualized by a Nikon Eclipse Ti-U fluorescent microscope equipped with a Spot RT3 camera using a 60× objective lens. Comet attributes were analyzed using ImageJ 1.43f software.

3.6. Analysis of nuclear fragmentation

In order to investigate the apoptosis upon the different treatments we quantified the presence of the apoptotic nuclei. The B16F10 cells were seeded in 96-well plates, treated and stained with Hoechst 33342. Nuclei were visualized by a Nikon Eclipse Ti-U fluorescence microscope equipped with a Spot RT3 camera using 4× and 20× objective lenses. Nuclei having condensed or fragmented apoptotic characteristics were quantified using the ImageJ software (NIH).

3.7. Analysis of mitochondrial morphology

In order to examine the mitochondrial morphological changes upon the different treatments we transfected the B16F10 cells with pDsRed2-Mito and/or pPARPGFPC1/N3 construct. Confocal Z-stacks were acquired by confocal microscopy (IMIC Andromeda system, Fondis Electronic), with a 60× objective. Length of mitochondria was determined by measuring 10 mitochondria per cell manually, using the ImageJ software (NIH). In each sample, at least 20 cells were analyzed.

3.8. Tetramethyl-rhodamine methyl ester (TMRM) time-laps fluorescent imaging

To determine the mitochondrial membrane potential changes upon the different treatments we performed TMRM time-laps fluorescent imaging, as previously described [26]. Cells were incubated in 10 nM TMRM [dissolved in Hank's Balanced Salt Solution (HBSS)] supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), in the presence of 1 mM P-glycoprotein inhibitor cyclosporine H in 5% CO2 atmosphere at 37◦C for 30 min. Sequential images of TMRM fluorescence were acquired every 60 seconds using the aforementioned IMIC Andromeda system for 30 minutes. To compromise the respiratory

chain 2 mM oligomycin was added at 5 minutes post-treatment. As a depolarization control 2.5 mM carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone (FCCP) was added at 25 minutes post-treatment. Analyses of the TMRM fluorescence of the mitochondrial regions of interest were carried out using the ImageJ software (NIH).

3.9. Immunoblot analysis

We examined the protein expressional changes upon the different treatments by immunoblot analysis. B16F10 cells were harvested in cold RIPA lysis buffer complemented with 1% protease inhibitor cocktail and 10% Phos-stop phosphatase inhibitor mixture (Roche, Sigma-Aldrich S.r.l., Milan, Italy). Proteins (20 mg/lane) were separated on Tris-acetate 3–8% or Bis-Tris 4–12% (NuPAGE, Life Technologies, Milan, Italy) polyacrylamide gels (Life Technologies, Milan, Italy) and transferred to PVDF membranes (Merck, Sigma-Aldrich S.r.l., Milan, Italy) that were blocked in 5% Bovine Serum Albumin (BSA) diluted in 0.1% Tween-20 containing tris-buffered saline (TBST) at room temperature for 1 hour. Primary antibodies against OPA1 (Becton Dickinson Italia S.r.l., Milan, Italy, 1:1000), b-Actin (Sigma-Aldrich S.r.l. Milan, Italy 1:10 000) and PAR (Santa Cruz Biotechnology, 1:500) were incubated in 5% BSA containing TBST. Horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-rat (Bio-Rad Laboratories S.r.l., Milan, Italy) secondary antibodies were diluted in 1:3000 in 5% milk containing TBST and membranes were incubated at room temperature for 1 hour. Peroxidase labeling was visualized using enhanced chemiluminescence substrate (Life Technologies, Pierce, Milan, Italy) and detected by an Image Quant mini Luminescent Image Analyzer 4000 (GE Healthcare Italia S.r.l., Milan, Italy).

In case of U251 and 4T1 cells, cells were seeded and cultured overnight before chemicals were added to the medium in a concentration as indicated earlier. After the 24 hours incubation period, the cells were harvested in cold lysis buffer (0.5 mM sodium metavanadate, 1 mM ethylenediaminetetraacetic acid [EDTA] and a protease inhibitor cocktail in phosphate-buffered

saline, pH: 7.4). After cell disruption in a Teflon/glass homogenizer, the homogenate was pelleted, and protein content of the supernatant was measured by bicinchoninic acid reagent and equalized for 1 mg/ml protein content in Laemmli sample buffer. Proteins (50 μg/lane) were separated in 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDSPAGE) gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% low-fat milk for 1 hour at room temperature, then exposed to anti-Akt-1, phospho-specifc anti-Akt-1 Ser473, anti-Bax, anti-Bcl-2, phospho-specific anti-Erk1/2 Tr202/Tyr204, phospho-specific anti-p38 MAPK Tr180/Tyr182 (Cell Signaling Technology, Danvers, MA, USA), anti-PARP-1, anti-PAR (Santa Cruz Biotechnology, Heidelberg, Germany), phospho-specific anti-SAPK/JNK Tr183/ Tyr185 (RandD Systems, Abingdon, UK), phospho-specific anti-p53 Ser15 (PromoKine, Heidelberg, Germany), anti-caspase-3, phospho-specific anti-Raf1 Ser338, anti-p21 (Thermo Scientific, Runcorn, UK) or anti-β actin (Sigma Aldrich Co, Budapest, Hungary) at 4°C overnight, in a dilution of 1:1000 in 5% bovine serum albumin, 1 tris(hydroxymethyl) aminomethane buffered saline and 0.1% Tween20 solution. Appropriate horseradish peroxidase-conjugated anti-rabbit (1:3000, Bio-Rad, Budapest, Hungary), anti-mouse (1:5000, Sigma Aldrich Co, Budapest, Hungary) or anti-rat (1:5000, Enzo Life Sciences, Lörrach, Germany) secondary antibodies were applied for 1 hour at room temperature. Peroxidase labeling was visualized by enhanced chemiluminescence (ECL) using ECL Western blot detection system (GE Healthcare, Freiburg, Germany). After the scanning, pixel densities of bands were quantified by means of NIH ImageJ program. Pixel densities of bands of the same film were normalized to that of the loading control and expressed as a percentage of the respective untreated, unirradiated control samples.

3.10. Statistical analysis

Data were analyzed by one-way ANOVA with Tukey *post hoc* comparison tests with alpha = 0.05; $n \geq 3$. Significance was expressed as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. In case of

U251 and 4T1 cells statistical analysis was performed by using two factor analysis of variance with replication for correlations, and unpaired Student's *t*-test for group comparisons. Differences with *p* values below 0.05 were considered as significant. All experiments were performed at least three times.

4. Results

4.1. Effect of PARP inhibition and irradiation on colony formation in A549 cells

HO3089 pretreatment of A549 cells prior irradiation resulted in significant decrease in colony formation: compared to untreated control group, irradiation by 2 Grays and 4 Grays without and with simultaneous HO3089 exposition showed a median of 60.98%, 20.73%, 25.61% and 4.88% colony count decrease, respectively.

4.2. Effect of PARP inhibition and irradiation on MAP kinases and pro- and antiapoptotic factors in A549 cell line

We examined the protein expression and activation changes upon 24 hours treatment in order to understand the molecular background behind the HO3089s irradiation sensitizing effect. Significantly increased amount of the cleaved proapoptotic caspase-3 was observed in all cases compared to the control. AKT phosphorylation was elevated in irradiated cells, independently of the cotreatment with HO3089. Definite p53 activation was observed as a result of the PARP inhibitor exposition, which was even more obvious in case of co-treatments with irradiation, as the irradiation fraction doses were increased. Parallel with the results of caspase-3 activation, an elevated level of p21 phosphorylation appeared in irradiated cells irrespective of the fact whether they had been treated or not with PARP inhibitor. The proapoptotic signal transduction member, Bax, was found to be expressed in the same extent in all samples examined. On the other hand, antiapoptotic Bcl-2 level decreased in all circumstances compared to control, apart from HO3089 single treatment. In A549 cell line, apart from the control group, Raf-1

phosphorylation – activation – was detected in all other cases. We observed Erk1/2 activation upon HO3089 treatment – similarly to its upstream regulator Raf-1 – as well as upon treatment with irradiation alone. JNK/SAPK activation decreased in a dose-dependent manner in case of irradiation had been applied. In parallel, increased activation of p38 MAPK was detected, following irradiation.

4.3. Effect of PARP inhibition and irradiation on MAP kinases and pro- and antiapoptotic factors in 4T1 cell line.

The phospho p53 level was not affected by the PARP enzyme inhibitor HO3089 in itself, however, following irradiation we observed its activation. There was no significant elevation in the Bcl-2 expression upon HO3089 single treatment. However, its significant dose-dependent reduction was observed following irradiation (32% and 54% reduction as a fraction of the control value). Single exposition by HO3089 did not influence the Bax expression, although significant increase was evident following irradiation (median elevation of 68%, 76%, 72%, and 75%, respectively). Erk1/2 presented significant increase only when HO3089 pretreated cells were irradiated (69% and 81% median change). JNK activation was a significant consequence of PARP inhibition or irradiation as well (respective medians of increase are of 30%, 36% and 63%). Phospho-p38 MAPK level significantly increased both after single agent HO3089 treatment and irradiation, irrespectively of the dose applied (62% and 107% median increase).

4.4. Effect of MAPK inhibitors on PARP inhibitor-induced radiosensitizing in A549, 4T1 and U251 cells.

By using the selective inhibitors, we investigated their interactions with HO3089 in all three cell lines. HO3089 pretreatment showed inverse effect on A549 and 4T1 cell proliferation, while it did not affect the U251 cell line. While Erk1/2 inhibition with HO3089 resulted in

significant improvement in cell viability in A549 and U251 cells, it had a contrary effect in 4T1 cells. Meanwhile, p38 MAPK inhibition achieved considerable improvement in 4T1 cells' viability along with HO3089 exposition. It was not observed in the other cell lines. JNK inhibitor significantly counteracted the PARP inhibitor-related cell survival deterioration or benefit in A549 and 4T1 cells, while it did not influence the colony formation of U251 cells.

After determining the interaction between the PARP- and the MAPK inhibitors, we utilized the inhibitors to verify the role of the respective kinase signaling pathways in mediating the radiosensitizing effect of the PARP inhibitor in the cell lines. Application of irradiation resulted in decreased colony formation ability in all cell lines. This effect was dramatically antagonized by Erk1/2 inhibitor in the case of A549 and U251 cells, compared to the appropriate irradiated groups with no Erk1/2 inhibition. Similarly, significantly improved proliferation was observed in the groups above in case cells were coexposed to JNK inhibitor and irradiation. In 4T1 cells, we observe either no or slight colony formation decrease, in a dose dependent manner. The p38 MAPK inhibitor increased the number of colonies in 4T1 irradiated cells, while it did not influence the other two cell lines' colony formation capacity. HO3089 administration to the culture medium before irradiation significantly decreased proliferation in all cell lines examined.

The combinatory treatment together with Erk1/2 inhibitor enhanced colony formation ability of A549 and U251 cells. Significant improvement in cell viability also occurred upon JNK/SAPK inhibition. This effect was evident even in case of low fraction dose treatment in A549 cells, but U251 and 4T1 cell lines showed the same effect only when high fraction dose was applied. Proliferation of 4T1cells was strikingly improved by p38 MAPK inhibitor exposition. Significant increase in colony numbers was found even in comparison to the 2 Gy-irradiated HO3089 pretreated 4T1 cell sample but results seen with high fraction dose in p38 MAPK-inhibited 4T1 cells are even more dramatic.

The activated cell signaling pathways detailed previously may mean a possible connection between the nuclear and extranuclear effects of PARP inhibitors, thus they could also be the possible mediators of the mitochondrial changes, observed upon PARP inhibition. The numerous data which demonstrated the PARP-1's protective effect on mitochondrial metabolism, by maintaining the mitochondrial membrane potential, decreased oxygen/glucose consumption or decreased ROS concentration, inspired us to observe the effect of PARP inhibition on mitochondrial structure and function [27]. Several studies showed the PARP inhibitors potentiating effect on the cisplatin and temozolomide induced cell toxicity [28, 29], thus we decided to investigate the effect of cisplatin and temozolomide alone or in combination with PJ34 on the viability and clonogenic capacity of B16F10 cells.

4.5. Effect of PJ34 and cisplatin or temozolomide on viability and colony formation capacity of B16F10 melanoma cells

In order to investigate the putative combined effects of PJ34 and cisplatin or temozolomide on cellular viability, we performed flow cytometry using Annexin-VFITC and propidium iodide labelling in B16F10 melanoma cells. We found that neither the alkylating agents nor the PJ34 showed cytotoxicity in B16F10 cultures within the first 24 hours of treatment. At 48 hours post-treatment, PJ34 developed a mild, although statistically not significant cytotoxicity. This effect was further increased at 72 hours post-treatment. Cisplatin also significantly decreased the viability at 72 hours post-treatment. This effect, became more pronounced when cisplatin was used in combination with PJ34 for 48 and 72 hours. In contrast to cisplatin, temozolomide did not induce cytotoxicity in B16F10 cultures.

After 48 hours, co-treatment with PJ34 and cisplatin caused significant increase in the amount of Annexin V-positive cells. 72 hours post-treatment, all substances but temozolomide increased apoptosis significantly; in combination in a more pronounced way than alone. We did not observe any significant change in necrosis.

In order to evaluate the effects of PJ34, cisplatin and temozolomide on B16F10 melanoma cells' proliferation capacity, we performed colony formation assays. We found that both PJ34 and cisplatin decreased colony formation in B16F10 cultures, while temozolomide did not show the same effect.

4.6. A Effect of PJ34 and cisplatin or temozolomide on apoptosis and nuclear fragmentation of B16F10 melanoma cells

In order to study the potential underlying mechanisms, we analyzed the DNA-damaging effects of the compounds used. We exposed the B16F10 cells to cisplatin, temozolomide or PJ34 for 4 and 24 hours treatment in different combinations and we performed alkaline single cell gel electrophoresis assay. We found that all compounds examined triggered DNA fragmentation. Temozolomide-treated cells accumulated DNA breaks in comparable extent to that of PJ34 but they were less effective than cisplatin alone. At the same time, combination of the pharmacologic PARP inhibition with cisplatin or temozolomide, led to increased number of persisting DNA-breaks. This potentiating effect of PJ34 was more pronounced in combination with temozolomide.

In order to confirm the presence of elevated apoptosis, we quantified the number of apoptotic nuclei in B16F10 cells treated with cisplatin, temozolomide either alone or in combination with PJ34. We found that cisplatin triggered apoptotic nuclear morphology in B16F10 cells. Surprisingly, this effect was not influenced by PJ34 during the first 24 hours of the treatments. Combinatorial treatment with PJ34 and temozolomide resulted in significantly more apoptotic nuclei than that of the temozolomide treatment alone. After 48 hours, PARP inhibition increased the number of apoptotic nuclei. This effect was more pronounced in combination with cisplatin.

Overall, flow cytometry and nuclear morphology data indicate distinct kinetic of the cisplatin- and temozolomide-induced cytotoxicity in B16F10 cells: cisplatin provoke a prominent early

apoptotic response, while the cytotoxic effect of temozolomide unfolds gradually. PJ34-mediated pharmacologic inhibition of PAPR-1 enhances the cytotoxic effect of both cisplatin and temozolomide and this effect was accompanied by hallmarks of apoptosis including elevated Annexin V binding and apoptotic nuclear morphology.

4.7. Effect of PJ34 and cisplatin or temozolomide on mitochondrial fragmentation in B16F10 Cells

In order to investigate whether mitochondrial events are involved in the cytotoxic and cytostatic effects of PJ34, cisplatin, and temozolomide in the B16F10 melanoma model, we treated the B16F10 cells expressing a mitochondria-directed red fluorescent protein (B16F10mtRFP) with cisplatin, temozolomide, PJ34 and their combinations and we examined the structure of the mitochondrial network with confocal microscopy. Elevated mitochondrial fragmentation was observed in B16F10 cells treated with cisplatin or temozolomide 4 hours post-treatment. PJ34 attenuated the mitochondrial effects of cisplatin and temozolomide, maintaining the fused mitochondrial phenotype when it was used in combination with the cytostatic compounds: this effect was persistent for at least 48 h post-treatment.

In order to confirm if the observed mitochondrial effects of PJ34 are mediated by the blockade of PARylation activity, B16F10mtRFP cells were transfected with a GFP-tagged peptide spanning the N-terminal DNA-binding domain of PARP-1 ($PARP^{DN}$). $PARP^{DN}$ attenuated the mitochondrial fragmentation triggered by both cisplatin and temozolomide, supporting the concept that the mitochondrial effects of PJ34 are mediated by the reduction of endogenous poly(ADP-ribosyl)ation.

4.8. The effect of PJ34, cisplatin and temozolomide on the short-versus long isoforms of OPA1

In order to investigate the potential underlying mechanisms of the PJ34 protective effects on mitochondrial morphology, we studied the ratio of the short and long isoforms of the Dynamin-like 120 kDa mitochondrial protein OPA1 (S-OPA1, L-OPA1), one of the key regulators of mitochondrial dynamics. We found no differential expression of either the short or the L-OPA1 in first 24 hours post-treatment. In contrast, an increase of S-OPA1 was detected in melanoma cells treated with PJ34 in combination with cisplatin for 48 hours. Considering the fact that the S-OPA1 accumulation does not correlate with the observed morphological deterioration of the mitochondrial network, we suppose that the cisplatin-, temozolomide- and PJ34-related morphological mitochondrial effects are not mediated by OPA1.

4.9. Effect of PJ34 and Cisplatin or Temozolomide on Mitochondrial Membrane Potential (ψ_m)

In order to investigate whether the cisplatin, temozolomide and PJ34-mediated effects on mitochondrial morphology are associated with functional alterations, we observed the functionality of mitochondrial respiratory chain by measuring the mitochondrial membrane potential upon the different treatments. Neither cisplatin nor temozolomide affected the ψ_m and PJ34 did not influence the mitochondrial membrane potential either. Interestingly, when PJ34 was used in combination with the cytostatic agents, we did not detect the collapse of the mitochondrial membrane potential. Instead, a slight, although statistically not significant, trend of membrane hyperpolarization was seen. This trend was more obvious in the presence of temozolomide compared to cisplatin. In order to confirm the PARylation dependency of the observed mitochondrial membrane hyperpolarization, we repeated the above experiments using cells transfected with a dominant negative mutant of PARP-1 ($PARP^{DN}$). Robustly elevated

mitochondrial membrane potential was measured in the PARPDN expressing B16F10 cells treated with cisplatin or temozolomide. These data suggest that the cytotoxic effect observed in B16F10 cells exposed to both PJ34 and the cytostatic agents is not mediated by the collapse of the mitochondrial membrane potential.

To further investigate the potential interplay between the PJ34-mediated enhancement of the alkylating agents cytotoxic effects, we also tested functionality of the mitochondrial NADPH reducing system, performing MTT assays in cells treated with combinations of PJ34, cisplatin, and temozolomide. In accordance with our observations on the mitochondrial membrane potential, we did not find any difference in the activity of the mitochondrial NADPH reductases in the presence of PJ34.

5. Discussion and conclusions

Several papers reported on the significance of PI3K-PTENAkt-mTOR, Ras-Raf-MEK-Erk1/2 or even JNK/SAPK and p38 MAPK pathway activation in the development and promotion of cancer cells [30]. In order to investigate the activation and significance of these pathways in the processes related to PARP inhibition, we examined the signaling protein activation upon PARP inhibition and irradiation and the changes in the cells' clonogenic capacity upon MAPK inhibition.

In the A549 cell line, application of irradiation and HO3089 resulted in decreased colony formation ability, but this effect was antagonized by Erk1/2 inhibitor. In 4T1 cells treated with HO3089, however, ERK inhibition attenuated the colony formation ability, while in U251 it improved it. The JNK inhibitor antagonized the deteriorated colony formation capacity of irradiated U251 and A549 cells while it did not influence the 4T1 cell colonies. JNK inhibition also ameliorated the colony formation capacity of A549 but further compromised the colony formation capacity of 4T1 cells, previously treated with HO3089. p38 inhibition antagonized

the compromised colony formation capacity of 4T1 cells both with irradiation and HO3089 single treatments but it did not influence the U251 and A549 cells' clonogenity (Figure 1).

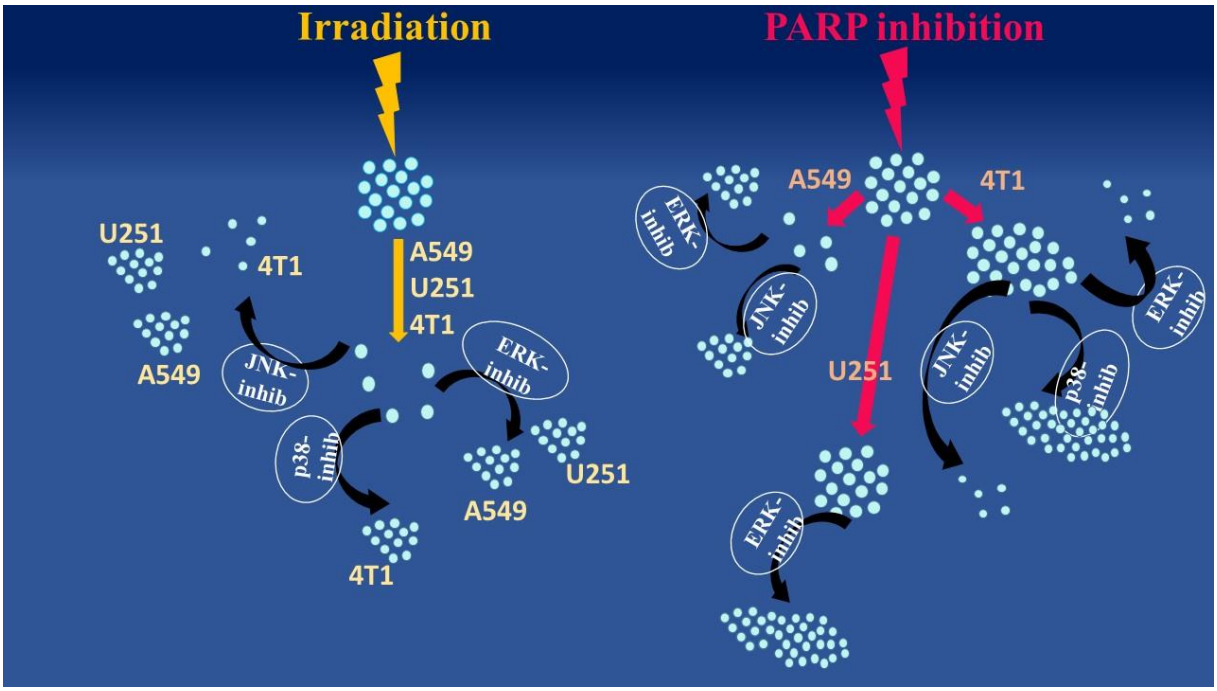

Figure 1. The effect of irradiation and PARP inhibition on the human A549 lung adenocarcinoma, human U251 glioblastoma and mouse 4T1 breast adenocarcinoma cells' colony formations capacity upon treatment with different MAPK inhibitors.
Irradiation deteriorated the colony formation capacity of all the three cell lines. JNK inhibitor in 4T1 cells further compromised their colony formation capacity, while it ameliorated the growth of U251 and A549 cells
p38 inhibitor cotreatment with irradiation ameliorated the colony formation capacity in 4T1 cells, and ERK inhibition ameliorated the growth of A549 and U251 cells.
Upon PARP inhibition we observed deteriorated growth in A549 cells. It did not influence the colony formation capacity of U251cells and it ameliorated the 4T1 cells' clonogenity. ERK inhibition ameliorated the clonogenity of A549 and U251, and deteriorated the 4T1 cells' growth. Upon JNK inhibition we observed augmented clonogenity in A549 cells but deteriorated colony formation capacity in 4T1 cells compared to HO3089 single treatment. p38 inhibition ameliorated the clonogenity of 4T1 cells compared to HO3089 single treatment.

In accordance with previous literature data, we found extremely compromised colony formation capacity in all the cell lines tested, in case they were both irradiated and treated with HO3089 [31] [32].

Taken together, our data reports that while the ERK1/2 pathway activation seems to be important for cell death processes in HO3089 treated and irradiated A549 cells, p38 activation is important for the same reason in 4T1 cells.

p53 is responsible for the mitochondrial outer membrane permeabilisation and for the release of several proapoptotic factors in the cytoplasm, thus upon DNA damage, p53 activation is a generally observed cellular signaling response which is connected to the intrinsic apoptotic pathway [33]. As a consequence, the presence of a wild-type p53 is a crucial element for a successful treatment upon irradiation or cytostatic therapy [34] [33] [35]. PARP facilitates the p53 transport from the nucleus which contributes to the cell death activation [36]. While investigating the pro- and antiapoptotic processes in A549 cells we found p53 phosphorylation when these cells were treated both with irradiation and HO3089. Increased p53 activity was a common result of irradiation and PARP inhibition. Furthermore we observed constant Bax expression, while the Bcl-2 expression decreased considerably in A549 cells were treated both with irradiation and HO3089.

MAPK cascade activation following irradiation may contribute to a positive therapeutic effect, conversely, their inhibitors may deteriorate the anti-tumor effect at the same time [37] [38] [39] [40] [41] [42]. The diversified answer upon the combined treatment with MAPK and PARP inhibitors highlight the importance to consider the cell and tissue type, when designing a therapeutic plan. Furthermore it underlines the importance of considering the extranuclear effects of PARP when using its inhibitors in tumor therapy.

The interplay between PARylation and cisplatin has been proposed decades ago on the basis of the central role of PARP-1 in the nuclear DNA repair mechanisms [43]. In the present study, we investigated the effects of two alkylating agents, cisplatin and temozolomide, commonly used in melanoma treatment, and the PARP inhibitor compound PJ34 using the *in vitro* B16F10 melanoma model. We found a marked difference between cisplatin and temozolomide toxicity on B16F10 cells: cisplatin exerted a slowly developing cytotoxicity that was statistically significant 72 hours post-treatment, in contrast, temozolomide was inefficient in provoking significant cell death in B16F10 cultures. PJ34 potentiated the cytotoxicity of both

agents, furthermore it had a potentiating effect in case of nuclear fragmentation, apoptosis induction as well as in case of the colony formation inhibition.

Since mitochondrial swelling, loss of *cristae* and disruption of the outer mitochondrial membrane were all observed in cisplatin-treated cells, later on, intense research on the mechanism of action of cisplatin has proposed mitochondrial targets as alternative effectors of cytotoxicity as well [44] [45]. In accordance with the previous literature data, we have also found extensive disintegration of the mitochondrial network preceding the onset of cytotoxicity in cisplatin-treated B16F10 cells. Interestingly a similar effect was observed in temozolomide-treated cells even though it did not show cytotoxicity in the melanoma cultures examined. At the same time, PJ34 cotreatment with cisplatin and temozolomide protected against the mitochondrial fragmentation.

One regulator of mitochondrial network dynamics is OPA1, a large mitochondrial dynamin like GTPase that facilitates fusion of mitochondria tightly regulated equilibrium of long and short OPA1 isoforms [46] [47]. In B16F10 cells exposed to both PJ34 and the cytostatic agents examined, we detected a disturbed equilibrium of the OPA1 isoforms 48 hours post-treatment. Although this time point correlates to the onset of the cytotoxic effects of the combinatorial treatments, dysregulation of the OPA equilibrium was not observed in corresponding morphological alterations of the mitochondrial network. These data suggest that if OPA1 contributes to the PJ34-augmented cytotoxic effects of cisplatin and temozolomide, it is mediated independently of its function in the regulation of mitochondrial morphology. It is widely accepted that fragmentation of mitochondria is a sign of cellular stress, while fusion is usually present in cells with balanced metabolic homeostasis. In our experiments, the PJ34-mediated inhibition of mitochondrial fission did not rescue cells also from cell death. This observation suggests independent effects of PJ34 on viability and mitochondrial morphology in B16F10 cells.

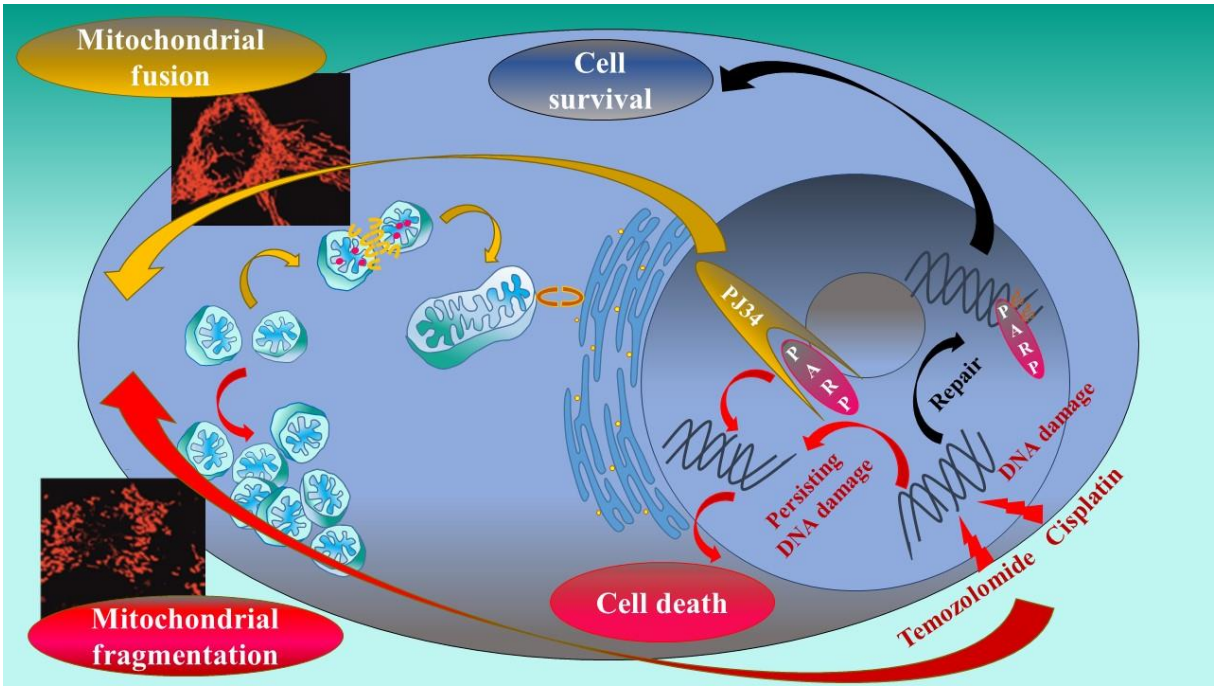

Figure 2. The effect of PARP inhibition on B16F10 cells. The temozolomide and cisplatin caused DNA damaging effect is corrected by the repair mechanisms, thus leading to cell survival. PJ34 blocks the repair, thus it leads to persisting DNA damage and finally cell death. Upon both treatment with temozolomide or cisplatin observed mitochondrial fragmentation, which was counteracted by the PJ34 treatment, maintaining a fused mitochondrial structure. *The figure has been created by the author. It is based on the articles cited in the thesis and on the results of the research work detailed.*

Our data suggest that, when used in combination with alkylating cytostatics, the dominant, long-term effect of PARP inhibition is enhancement of DNA damage, probably, by reducing the repair activities. Our model is in accordance with literature data on the overall effect of PARP modulation that is, seemingly, determined by the intricate relationship of the targeted molecular mechanisms [48]. The diverse, occasionally opposing effects of PARP inhibitors, even within the same model, however, underlines the importance of further, careful evaluation of the use of these compounds in human pathologies.

6. Summary of the new findings presented in the dissertation

- **We performed a comprehensive study in three cell lines, analyzing protein expression, phosphorylation and clonogenity changes upon treatment with 3 MAPK inhibitors, PARP inhibitor and irradiation.** The cell-type dependent clonogenity upon the treatments with different inhibitors may explain also the diversified responses observed upon tumor therapy. Furthermore, we showed that in most cases MAPK inhibitors antagonized the compromised clonogenity caused by irradiation or PARP inhibition. This result, underlines the importance of considering the molecular background of ever tumor before designing a tumor treatment.
- **We demonstrated for the first time that PARP inhibition protects against the mitochondrial fragmentation caused by cisplatin or temozolomide and it facilitates the mitochondrial respiration.** This observation may have an importance in the cases where tumor resistance was observed upon therapy with PARP inhibitors.
- **We performed for the first time a comprehensive study demonstrating that PARP inhibition cause parallel cell-protective and toxic effects within the same model.** We found that in short-term treatment PARP inhibition leads to apparently mitochondrial protective effects against cisplatin and temozolomide, but in parallel, it increases the cellular toxicity of these agents, potentiating also their effect on colony formation blockage. These parallel but controversial mechanisms may explain the diversified and sometimes controversial cellular response observed upon PARP inhibition.

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Articles related to the thesis

1. PARP inhibitor PJ34 protects mitochondria and induces DNA-damage mediated apoptosis in combination with cisplatin or temozolomide in B16F10 melanoma cells

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2. PARP inhibitor attenuated colony formation can be restored by MAP kinase inhibitors in different irradiated cancer cell lines

Hocsak E[1], **Cseh A**, Szabo A, Bellyei S, Pozsgai E, Kalai T, Hideg K, Sumegi B, Boronkai A.

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Scientific publiction: ***International journal of radiation biology 2014,*** **Impact Factor: 1.687**

List of publications and abstracts

1. PARP inhibition protects mitochondria and reduces ROS production via PARP-1-ATF4-MKP-1-MAPK retrograde pathway

Hocsak E[1], Szabo V[2], Kalman N[2], Antus C[2], **Cseh A[2]**, Sumegi K[2], Eros K[2], Hegedus Z[3], Gallyas F Jr[4], Sumegi B[4], Racz B[2]

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2. Poly(adenosine diphosphate-ribose) polymerase as therapeutic target: lessons learned from its inhibitors

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3. PJ34 PARP inhibitor reverses the cisplatin and temozolomide induced early mitochondrial changes in B16F10 melanoma cells

Anna Mária Cseh[1,2], József Orbán[1], Rubén Quintana-Cabrera[2], Stephanie Herkenne[2], Zsolt Fábián[3], Balázs Sümegi[1] and Luca Scorrano[2]

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4. Quercetin increases the efficacy of glioblastoma treatment compared to standard chemoradiotherapy by the suppression of PI-3-kinase-Akt pathway

Pozsgai E1, Bellyei S, **Cseh A**, Boronkai A, Racz B, Szabo A, Sumegi B, Hocsak E.

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