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Proteomic analysis of gamma-irradiated human leukemic cells

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Abstract of the dissertation

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Introductory page

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Summary (CZE)

Název dizertační práce: Proteomická analýza leukemických buněčných linií po ozáření

V této dizertační práci jsme se zaměřili na objasnění molekulárních mechanismů radiosensibilizace leukemické buněčné linie MOLT-4 specifickou inhibicí kináz z rodiny fosfatidylinositol-3-kináza příbuzných kináz (PIKKs). Byly testovány dva vysoce účinné a selektivní inhibitory VE-821 (inhibitor ATR) a KU55933 (inhibitor ATM), pro jejich účinky na proliferaci, viabilitu a buněčný cyklus neozářených a ozářených buněk MOLT4. Aplikace obou inhibitorů způsobila radiosensibilizaci MOLT-4 buněk a 10 μM VE-821 navíc působil jako silné antiproliferativní agens i v neozářených MOLT-4 buňkách.

K dalšímu popisu mechanismů, které jsou zodpovědné za radiosensibilizaci MOLT-4 buněk VE-821 inhibitorem byly použity hmotnostně spektrometrické metody. Pomocí metod kvantitativní proteomiky jsme identifikovali a kvantifikovali změny v proteomu a fosfoproteomu (tj. změny na úrovni fosforylace proteinů) buněk, které byly způsobeny účinkem inhibitoru v ozářených buňkách. Protože detekce a kvantifikace fosforylovaných peptidů v komplexních vzorcích je komplikována mimo jiné jejich relativně nízkým zastoupením, zaměřili jsme se nejprve na výběr optimální metody pro jejich selektivní izolaci ze směsi s nemodifikovanými peptidy. Optimalizovaný protokol byl pak dále využit ke studiu změn v buňkách radiosensibilizovaných VE-821. Dle očekávání inhibitor nevyvolal žádné změny na úrovni proteomu hodinu po ozáření. Při studiu fosfoproteomu jsme ale nalezli 623 signifikantně změněných fosforylačních míst, z nichž většina (431) byla upregulována. Pomocí bioinformatických nástrojů jsme zjistili změny signálních drah a aktivity kináz přímo odpovídajících na poškození DNA, ale také signálních drah a kináz primárně souvisejících s regulací buněčného metabolismu. Detekovali jsme snížení v aktivitě mTOR kinázy, které bylo nejspíše způsobené nespecifickým působením inhibitoru v 10 μM koncentraci. Tato snížená aktivita může pravděpodobně přispívat k zástavě proliferace buněk po podání vysoké dávky inhibitoru.

Vliv VE-821 na metabolismus ozářených buněk byl dále zkoumán pomocí cílené metabolomické analýzy. V této analýze bylo kvantifikováno 206 intermediárních metabolitů. Při následné analýze dat bylo zjištěno, že VE-821 potencuje rozvrat energetického metabolismu a může ovlivňovat odpověď na oxidační stres způsobený ozářením. Dále jsme ukázali, že obnova poškozených deoxynukleotidů by mohla být negativně regulována VE-821.

V této práci jsme tedy komplexně popsali, jaké signální a metabolické dráhy by mohly být závislé na ATR nebo spuštěny ATR inhibicí VE-821 v ozářených MOLT-4 buňkách. Výsledky této práce mohou být dále použity jako zdroj informací pro další navazující studie.

Summary (ENG)

Title of the dissertation: Proteomic analysis of gamma-irradiated human leukemic cells

In the presented doctoral thesis, we aimed to elucidate molecular mechanisms underlying radiosensitization of MOLT-4 cell line (T-ALL) by specific inhibition of kinases from the phosphatidylinositol-3 kinase-related kinases (PIKKs) family. We tested two highly potent inhibitors of ATR and ATM, VE-821 and KU55933, respectively, for their effects on proliferation, viability, and cell cycle of sham-irradiated and irradiated MOLT-4 cells. Both inhibitors proved to radiosensitize MOLT-4 cells and furthermore, 10 μM VE-821 was shown to act as a strong antiproliferative agent in sham-irradiated MOLT-4 cells.

To further describe cellular mechanisms underlying the VE-821-mediated radiosensitization of MOLT-4 cells, we employed high-resolution mass spectrometry to identify and quantify changes in proteome and phosphoproteome of irradiated VE-821-treated cells. As the detection and quantification of phosphorylated peptides in complex biological samples is challenging due to their low stoichiometry, we first compiled and optimized protocol for their enrichment. The protocol was then applied to study changes in radiosensitized MOLT-4 cells. In concordance with our expectations, VE-821 did not cause any significant changes on the proteome level one hour after irradiation. However, we detected 623 differentially regulated phosphorylation sites; most of them (431) were upregulated in response to VE-821 treatment. Using bioinformatic tools, we revealed changes in DDR related pathways and kinases, but also pathways and kinases involved in maintaining cellular metabolism. Notably, we found downregulation of mTOR, the main regulator of cellular metabolism, which was most likely caused by an off-target effect of the inhibitor, and we proposed that mTOR inhibition could be one of the factors contributing to the phenotype observed after treating MOLT-4 cells with 10 μM VE-821.

To investigate the potential modulation of cellular metabolism, we performed a targeted metabolomic analysis of irradiated MOLT-4 cells pre-treated by 10 μM VE-821. In this analysis, 206 intermediary metabolites were quantified. Subsequent data analysis showed that VE-821 potentiated metabolic disruption induced by IR and affected response to IR-induced oxidative stress. Our data indicated that upon IR, recovery of damaged deoxynucleotide triphosphates might be affected by VE-821, hampering DNA repair by their insufficiency.

Thus, in this thesis we described a complex scenario of cellular events that might be dependent on ATR or triggered by ATR inhibition by VE-821 in irradiated MOLT-4 cells. Importantly, data presented in this work might serve as a resource for follow-up studies and provide a platform for future work with other kinase inhibitors.

1. Introduction

1.1. Characterization of DNA damage induced by ionizing radiation

The term **ionizing radiation (IR)** describes a radiation that has enough energy to liberate an atomic particle from an originally electrically neutral atom or molecule, ionizing it. The ionization can occur either directly or indirectly. When cells are exposed to IR, cellular structures can be damaged by ionization directly by deposition of energy (this mechanism dominates in cells with a low water content), but also indirectly by ionization of water molecules in cells with a high water content – by a mechanism called **water radiolysis**. Such ionization leads to generation of highly **reactive oxygen species (ROS)**, which secondary attack DNA and other biomolecules (reviewed in (1,2)).

There is a wide range of DNA lesions typically occurring in response to IR; however, DNA double stranded breaks (DSB) are assumed to be the most lethal class of DNA damage. A failure to repair just a single DSB can result in cell death (3) implying the importance of DSB repair for cell viability. Moreover, both repair and misrepair of DSBs can lead to mutations and chromosomal rearrangements, potentially resulting in cancer development. Since genetic alterations have such a significant impact on cell survival and viability, cells have evolved robust, but finely regulated molecular mechanisms that include detection of DNA lesions, signalling of their presence, promoting their repair, and activation of cell cycle checkpoints. Altogether, these mechanisms are summarized under the term **DNA damage response (DDR).** The key components of DDR triggering cellular response to detected DNA lesions, are protein kinases that belong to **phosphatidylinositol 3-kinase (PI3K) family class IV**, better known as **phosphatidylinositol-3 kinase-related kinases** (**PIKKs**; reviewed in (4)).

1.2. Phosphatidylinositol-3 kinase-related kinase family

The PIKK family comprises 6 proteins with most of the members possessing serine/threonine kinase activity: ataxia telangiectasia mutated kinase (**ATM**), ataxia telangiectasia and Rad3 related kinase (**ATR**), DNA-dependent protein kinase catalytic subunit (**DNA-PKcs**), mammalian target of rapamycin (**mTOR**), suppressor with morphological effect on genitalia family member (**SMG-1**), and transactivation/transformation-domain-associated protein (**TRRAP**). Among the PIKKs, **ATM, ATR, and DNA-PK** act as the main regulators of cellular response to DNA damage and DNA replication stress.

1.3. **ATM-Rad3 related kinase (ATR)**

ATR is the central kinase responding to replication stress, which cells undergo during scheduled DNA replication and after exposure to genotoxic events. DNA damage induced by IR, UV, chemotherapeutic drugs, or inhibitors of DNA replication causes DNA-polymerases to stall at DNA lesions while the replicative helicases continue to unwind the DNA helix ahead of the replication fork leading to generation of long ssDNA stretches. Additionally, ssDNA structures can be formed at DSB sites after 5' to 3' nucleolytic degradation of one of the chains, a step required for DSBs repair by homologous recombination (reviewed in (5,6)). ATR is an essential protein; homozygous mutation of ATR caused peri-implantation embryonic lethality in mice (7,8), and no living human completely lacking ATR has been identified. Nevertheless, a hypomorphic ATR mutation is responsible for the **Seckel syndrome** – a recessive autosomal hereditary disorder characterized by microcephaly and mental- and growth- retardation (9). **Checkpoint kinase 1** (**Chk1)** is often described as the primary target and main downstream effector of activated ATR. However, it has been repeatedly reported that ATR- and Chk1-inhibition provided different outcomes when combined with genotoxic stress in cancer cells (10,11) suggesting that despite being the main effector, Chk1 is not responsible for all downstream signalling of activated ATR.

1.4. **Targeting DDR as a promising strategy in oncology**

DNA damage induction by either radio- or chemo- therapy has been the most widely used approach in oncology exploiting one of the hallmarks of cancer: genomic instability. However, such treatment is very unspecific and often accompanied by collateral damage to healthy tissues. In recent years, much effort has been put on discovery and development of tumour specific treatment, which would only specifically target cancer cells and not affect the normal tissues. A promising approach that has been developed recently is to take advantage of the tumour specific abnormalities in DDR. Two promising examples of such a strategy are **targeting the S and G2/M DNA damage checkpoints in G1/S DNA damage checkpoint deficient cells** (12) and **targeting proteins and protein kinases involved in replication stress response** (13). Inhibition of the ATR/Chk1 pathway has been shown to be synthetically lethal in both examples. In several studies, it has been shown that inhibiting this pathway is selectively toxic in cells with high levels of oncogene-induced replication stress (13–19). On the other hand, there have been several papers published, in which the authors emphasized the efficiency of ATR inhibition in combination with genotoxic therapy in p53- or ATM- deficient cells (6,20–23). Taken together, selective targeting of the ATR/Chk1 pathway offers a promising

therapeutic approach for cancer treatment in a broad range of tumours in both monotherapy and selective sensitization of cancer cells to current genotoxic treatment.

1.5. The preclinical and clinical development of ATR inhibitors

Given their importance in DDR, small molecule kinase inhibitors have been synthesised for each one of the kinases from the PIKKs family to investigate their potential for development of a cancer-specific treatment. The first potent and selective inhibitors of ATR that may offer a great promise in cancer treatment were presented by Vertex Pharmaceuticals in 2011. Charrier et al. reported the discovery of selective ATR inhibitors from the series of **3-amino-6-arylpyrazines** (24). In the following studies, two compounds from this series were further evaluated – **VE-821** and **VE-822 (**or **VX-970)**.

VE-821 (3-amino-6-(4-(methyl-sulfonyl)phenyl)-N-phenylpyrazine-2-carboxamide; IC50(ATR): 26 nM, Ki(ATR): 13 nM, Ki(mTOR) > 1 μM, Ki(DNA-PK): 2.2 μM, Ki(ATM): 16 μM) has been shown to be able to selectively induce irreversible growth arrest in several cancer cell lines that were either p53-deficient or p53-wt, but with dysfunctional ATM/Chk2/p53 pathway, while inducing only reversible growth arrest in normal cells. Moreover, VE-821 showed synergy with different genotoxic agents including cross-linking drugs, antimetabolites, topoisomerase I and II poisons, and ionizing radiation. The dependence on the functionality of ATM signalling for the sensitivity to ATR inhibition was confirmed in experiments with A-T primary skin fibroblasts, in normal cells treated with ATM inhibitor KU-55933 or with knockdown of p53 expression (22). These results were confirmed in 2012 by Pires et al., who mostly focused on the radiosensitizing effect of ATR inhibition. In this study, the radiosensitization using VE-821 was proven in a variety of cell lines from several different tumour types suggesting that this effect is independent of tumour type. Moreover, VE-821 was shown to increase radiation-induced DNA damage and loss of viability of cancer cells under hypoxic conditions (25), which is of particular interest, as hypoxic tumours have been reported to be more resistant to radiotherapy (26). The ability of VE-821 to sensitize pancreatic cancer cell lines to radiotherapy or antimetabolites-based chemotherapy under both normoxic and hypoxic conditions was further confirmed by Prevo et al. (27). Since then, VE-821 has been consistently shown in several studies to sensitize a variety of different tumour types to different genotoxic events (11,28–34).

1.6. Protein phosphorylation and its role in eukaryotic cells

Several hundred of post-translational modifications (PTMs) of proteins have been described so far; among them, protein phosphorylation is one of the most studied as it is one of

the most important PTMs in nature. Protein phosphorylation is a transient, reversible PTM, and this dynamic nature of phosphorylation enables being the major driving force of most of the cellular processes which require rapid and tightly regulated signalling. These processes include cellular signalling and communication, proliferation, differentiation, metabolism, transcriptional and translational regulation, degradation of proteins, and cell survival. The presence of phosphorylation in specific regions of a protein molecule is believed to induce conformational changes in the target protein, which influence its behaviour within the cell. Such conformational changes can modulate activity of an enzyme (such as phosphorylation in an activation loop of a protein kinase), subcellular localization of a protein, or its stability (35).

1.7. Overview of fractionation and enrichments methods used for the phospho-enrichment on peptide level

Multiple methods for the bottom-up phosphoproteomics have been developed so far. These methods either exploit the presence of a phosphate group in a peptide to chemically introduce an affinity tag (*i.e.* ***chemical derivatization methods***), the affinity of a phosphate group for Lewis' acids such as metal ions and their compounds (*i.e.* ***affinity enrichment methods***), or physicochemical properties of phosphorylated peptides that can be used to more or less specifically fractionate their mixture with unmodified peptides (*i.e.* ***chromatographic fractionation methods***). Nevertheless, none of them is able to yield comprehensive information about the phosphoproteome of a complex biological sample. Therefore, the approaches are often combined to obtain complete information about the phosphopeptide pool in analysed samples. The affinity enrichment and separation methods include **IMAC** (Immobilized metal affinity chromatography), **MOAC** (Metal oxide affinity chromatography), **HAP** (Hydroxyapatite chromatography), **SCX** (Strong cation-exchange chromatography) and **SAX** (Strong anion-exchange chromatography), **HILIC** (Hydrophilic interaction chromatography), and **ERLIC** (Electrostatic repulsion hydrophilic interaction chromatography). Currently, the most widely used methods for the enrichment of phosphorylated peptides are IMAC and MOAC.

1.8. Detection of phosphorylation

Mass spectrometry is a current method of choice to detect dynamic changes in protein phosphorylation. However, a direct MS analysis of phosphorylation from unfractionated peptide samples is still not feasible because of relatively low abundance of phosphorylated proteins in eukaryotic cells. It is of a great importance to choose an appropriate "phosphate-friendly" fragmentation method to preserve phosphorylation during peptide fragmentation. It

has been shown that during the fragmentation using the most common fragmentation method in proteomics, collision-induced dissociation (**CID**) operated in positive-ion mode, the phospho-amino acid containing peptides will typically undergo β-elimination of phosphoester bond resulting in a loss of the phosphate group and limited fragmentation across the peptide backbone. Application of an alternative fragmentation method such as high-energy CID (**HCD**; (36)), electron capture dissociation (**ECD**; (37)), or electron transfer dissociation (**ETD**; (38)) leads to more efficient fragmentation without the cleavage of the phosphoester bond and thus allows more confident peptide identification and localization of the modification.

1.9. Quantification techniques for phosphoproteomics

Quantitation in proteomics has become very robust in recent years; there are multiple well established quantitation techniques that provide very precise and accurate quantification of proteins in samples and allow comparison of samples coming from different origin. Quantification of PTMs has also become relatively feasible – but it is important to consider that the quantification of modifications is more difficult than and not as straightforward as quantification of whole proteins. The quantification of PTMs usually rely on one modified peptide, contrary to proteins with more peptides available (and usually required). Additionally, a modified peptide can bear multiple modifications with different degree of regulation further complicating the analysis.

Label-free quantification is the "simplest" quantification method that does not require any stable-isotopic labelling step in the experimental workflow and only relies on spectral counting or MS^1 intensity of the quantified feature. The unnecessity to use any isotopic label provides this method several attractive benefits: the implementation costs are low; there are no additional steps in the workflow that may introduce undesirable biases into the analysis, and the number of treatment conditions and replicates is basically unrestrained allowing relative flexibility in the experimental designs. On the other hand, the absence of multiplexing increases the total number of samples to be measured, and thus longer acquisition time costs must be considered when choosing this strategy.

Contrary to the label-free method for peptide quantification, multiple quantification methods have been developed based on introduction of an isotopic label into the studied system. Generally, there are two groups of label-based approaches – metabolic and chemical labelling. In **metabolic labelling**, the labels are incorporated into every protein during cell growth and division. Consequently, the isotopic label is introduced in the earliest possible step of the experiment, thereby eliminating systematic errors that could arise from sample handling. The

most widely used metabolic labelling method in both proteomics and phosphoproteomics – stable isotope labelling with amino acids in cell culture (SILAC) was introduced in 2002 (39). In a typical **chemical labelling** approach, the isotopic labels are incorporated into the proteins later in the experimental workflow – either on the protein level (possible, but not widely used), or much more often on the peptide level. In principle, any reactive group within the amino acid residues might be used to chemically attach a label to a peptide; however, most of the current labelling techniques take advantage of the reactivity of primary amines and target either peptide N-terminus or ε-amino group of lysine. Based on the label, the relative (or absolute) quantification using the chemical labelling approach can be conducted either on the MS^1 level by comparing intensities of the differentially labelled peptides or on the MS^2 level via comparing differentially isotope encoded reporter ions in the peptide fragmentation spectra.

2. Aims of the Study:

2.1. **Optimization of experimental workflow for the enrichment, detection, and quantification of phosphorylated peptides using mass spectrometry:**

- Selection of a chromatographic material and protocol for enrichment of phosphorylated peptides using standard peptide mixtures
- Application of the best performing protocols to the enrichment of phosphorylated peptides from a real complex sample and to the analysis of the selected model system (irradiated SILAC-labelled MOLT-4 cells)

2.2. **Characterization of cellular mechanisms underlying the VE-821-mediated radiosensitization of MOLT-4 cells:**

- Characterization of the response of MOLT-4 cells to VE-821 and its combination with IR using cell biology methods
- Application of the optimized phospho-enrichment workflow to analyse phosphorylation response of irradiated MOLT-4 cells and its modulation by VE-821
    - Detection of phosphorylation sites responsive to VE-821 treatment
    - Functional annotation of regulated phosphorylation sites and identification of potentially perturbed signalling pathways and biological processes
    - Analysis of phosphorylation motifs surrounding detected phosphorylation sites and identification of activated/inhibited protein kinases

- Description of VE-821-induced metabolome alterations in irradiated MOLT-4 cells using targeted metabolome analysis
  - Identification of metabolites with abundance changes induced by VE-821 treatment in irradiated MOLT-4 cells
  - Functional characterization of regulated metabolites, detection of modulated metabolic pathways
- Integration of the acquired data and validation using database and literature search

3. Materials and Methods

3.1. Model proteins and peptide mixtures

α-casein, asialofetuin, BSA, and myoglobin were dissolved in 50 mM ABC buffer (pH 7.8), reduced with 10 mM DTT, alkylated with 20 mM IAA, and subjected to trypsin digestion at an enzyme to protein ratio 1:50 at 37 °C overnight. Digested samples were desalted using Oasis® HLB SPE cartridges.

3.2. Cell culture and cell culture conditions

MOLT-4 cells were cultured in IMDM containing 20% foetal bovine serum, 2 mM glutamine, 100 UI/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C under controlled 5% CO_2 and humidified atmosphere.

3.3. Phosphopeptide enrichment protocol and HILIC fractionation

Titansphere® TiO_2 particles (5 µm diameter; GL Sciences, Torrance, CA) were used for phosphopeptide enrichment using a "microtube approach". The sample was dissolved in 200 µl of LB and incubated with TiO_2 particles (peptide-to-TiO_2 ratio 1:8; mass/mass) on a shaker for 20 minutes. TiO_2 beads were then washed with 200 µl of LB, 200 µl of WB 1, and twice with 100 µl of WB 2. Phosphopeptides were eluted with 50 µl of EB and the eluted fraction was acidified with a mixture of 100% FA and 10% TFA (to final pH < 2). Eluted fractions were concentrated and desalted prior to MS analysis.

Dried peptide samples were fractionated by HILIC according to a protocol that has been published previously (40) using the 4.6 × 25 cm TSKgel® Amide-80 HR 5 µm particle column with the TSKgel® Amide-80 HR 5 µm 4.6 × 1 cm guard column operated with Waters Separations Module 2695 at 0.5 mL/min.

3.4. Chromatography and mass spectrometry

MALDI-TOF-MS/MS analysis was performed using an ABI 4800 mass spectrometer. **UV-VIS chromatography**: Peptides were separated by reversed phase chromatography using Dionex Ultimate 3000 and detected using Dionex Ultimate 3000 Variable Wavelength detector. **RPLC-ESI-MS/MS analysis** of the complex samples (HeLa cells lysate, SILAC labelled irradiated MOLT-4 cells) was performed on Thermo Scientific Dionex Ultimate™ 3000 RSLCnano system coupled through Nanospray Flex ion source with Q Exactive mass spectrometer.

3.5. Electrophoresis and western blotting

Whole cell extracts were prepared by lysis in 500 μL of lysis buffer per $1x10^7$ cells (137 mM NaCl; 10% glycerol; 50 mM NaF; 20 mM Tris-HCl, pH = 8; 1% n-octyl-β-glucopyranoside; 1 tablet of protease inhibitors Complete™ Mini/ 10 ml, 1:100 Phosphatase inhibitor cocktail 2 and 3). The lysates containing equal amount of protein (30 μg) were loaded onto a 12% SDS polyacrylamide gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane and hybridized with an appropriate antibody.

3.6. Cell proliferation/viability WST-1 assay

Proliferation of MOLT-4 cells was evaluated by WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate) colorimetric assay (Roche Diagnostics, Mannheim, Germany).

3.7. Cell cycle analysis and apoptosis detection by flow cytometry

For cell cycle analysis, DNA was stained using Vindelov's solution (10 mM Tris-HCl pH=7.6, 0.6 mg/ml NaCl, 0.01 mg/ml Ribonuclease A, 0.05 mg/ml PI). For apoptosis detection, a Apoptest-FITC kit was used. Flow cytometric analysis was performed on a FACS analyser CyAn DakoCytomation.

3.8. Mass spectrometry data processing and bioinformatic analysis

Raw data files acquired by LC-MS/MS were processed with MaxQuant *v1.5.2.8* (41). Peak lists were searched against the human SwissProt database (November 2015) using Andromeda search engine (42). Global rank test (GRT) was used to find differentially regulated phosphorylation sites. Only phosphorylation sites quantified in all three replicates were subjected to GRT, and FDR was estimated non-parametrically as described by Zhou et al. (43). The significance cut-off for differentially regulated sites was set to FDR < 0.005. Gene

ontology (GO) and signalling pathways over-representation analyses were performed using ConsensusPathDB over-representation analysis web tool (44,45). To analyse and visualize sequence motifs surrounding phosphorylation sites identified and quantified in our study, we employed iceLogo tool (46) and motif-x algorithm (47). Class I phosphorylation sites quantified in all three biological replicates were annotated with previously known kinase-substrate relationships downloaded from the PhosphoSitePlus database (48). To increase the number of annotated phosphorylation sites, two kinase predictors were employed: NetworKIN 3 (49) and iGPS *v1.0* (50). The significance of global changes in kinase activities were evaluated using "1D annotation enrichment" tool available in Perseus software *v1.5.2.6* (51).

3.9. **Targeted metabolomic analysis**

For the quantitative targeted metabolomic analysis, MOLT-4 cells were pipetted into five sample volumes of a pre-cooled (-40 °C) quenching solution (60% methanol, 0.85% ABC, pH 7.4) as described previously (52). For separation of the extracted metabolites, an LC system UltiMate 3000 RS (Dionex, Sunnyvale, CA, USA) was used. Detection was performed using a Triple Quad 6500 tandem mass spectrometer (AB Sciex, Foster City, CA, USA) fitted with electrospray ionisation in both positive and negative mode. The instrument was controlled using the Analyst v1.6.2 software. The analytes were detected and identified according to the multiple reaction monitoring transitions and retention times in the MultiQuant *v3.0* software (AB Sciex, Foster City, CA, USA). The peak areas were extracted, and the corresponding peak areas were taken to create the final dataset.

4. Results

4.1. **Optimization of metal oxide affinity enrichment of phosphopeptides from a standard tryptic peptide mixture**

The optimization of conditions for phosphopeptide enrichment was evaluated using MALDI-TOF/TOF mass spectrometry (MS). Standard tryptic peptide mixtures were subjected to phosphopeptide enrichment via different enrichment methods and analysed by MS. The evaluation of enrichment efficiency for phosphorylated peptides of different chromatographic resins under different buffer conditions was performed by comparing the maximal numbers of detected phosphopeptides and repeatability of the detection in three independent experiments. In this analysis, Titansphere® particles and NuTips® clearly outperformed the TopTips®. All

three kinds of TopTips® tested in our experiments provided lower number of detected phosphopeptides than Titansphere® particles and NuTips® columns. Next, the binding selectivity for phosphorylated peptides was evaluated by comparing relative intensities of phosphorylated peptides with those of nonphosphorylated peptides in spectra recorded by MALDI-TOF mass spectrometry. The two most intensive nonphosphorylated peptides were **1749.66** (ECCHGDLLECADDR) and **2458.18** (DAIPENLPPLTADFAEDKDVCK) from BSA. The intensity of these two unmodified peptides decreased in the following order: **5% TFA < 1 M LA < 0.1 M Glu < 350 mg/ml DHB**, and thus the phosphoenrichment was the most selective when DHB was added into the loading buffer. Two best performing protocols (NuTips® and Titansphere® particles combined with 0.1 M glutamic as a loading buffer additive) were tested in a real complex sample analysis. The samples were prepared by digesting 1 mg of HeLa cells lysate using trypsin and enriched for phosphopeptides using either NuTips® or Titansphere® particles. In these experiments, we repeatedly observed that NuTips® were not an adequate enrichment media for such a high amount of a complex mixture since the amount of material in the eluates was extremely low in comparison to the fractions obtained from Titansphere® enrichment.

4.2. **Radiosensitization of MOLT-4 cells by selective ATR and ATM inhibitors VE-821 and KU55933**

Two potent and specific inhibitors of ATM and ATR, KU55933 and VE-821, respectively, for their effects on proliferation, viability, and cell cycle of sham-irradiated and irradiated MOLT-4 cells. The inhibition of ATM by KU55933 was well tolerated in concentrations up to 10 μM. On the other hand, inhibitor of ATR, VE-821, inhibited the growth and viability of MOLT-4 cells in a dose dependent manner already at lower concentrations than KU55933 a significant growth inhibition was achieved by application of 1 μM VE-821. In WST-1 cell viability assays, the irradiation of MOLT-4 cells by a dose of 1 Gy led to growth inhibition which resulted in markedly decreased number of viable cells 72 and 144 hours after irradiation. The addition of VE-821 in both 1 μM and 2 μM concentrations further significantly enhanced the antiproliferative effect of IR. Additionally, in a cell counting-based proliferation assay, VE-821 significantly sensitized cells to IR in 2 μM and 10 μM concentrations even when the inhibitor was present in cell culture media only transiently, for the first 24 hours of the treatment. In contrast to continuous treatment, VE-821 2 μM did not significantly affect the proliferation of sham-irradiated cells when washed out after 24 hours; however, it still increased the sensitivity of cells to ionizing radiation (1.5 Gy).

Chk1 Ser 345 and Chk2 Thr 68 phosphorylation were assessed using immunoblotting one hour after 3 Gy of IR to detect activation of both ATM and ATR kinases upon irradiation and their specific inhibition by the inhibitors. Chk2 Thr 68 phosphorylation (marker of activated ATM) was not detected in sham-irradiated cells; however, it was strongly induced by irradiation. The treatment with KU55933 markedly decreased Chk2 phosphorylation in irradiated cells. Chk1 Ser 345 phosphorylation (marker of activated ATR) was also detected in sham-irradiated cells, and upon irradiation, it was slightly upregulated. VE-821 abrogated this phosphorylation in a concentration dependent manner.

We further examined cell cycle effects of ATM and ATR inhibition. DNA content analysis was performed using propidium iodide (PI) staining and flow cytometry. In sham-irradiated cells, none of the conditions induced significant cell cycle perturbation except for VE-821 10 μM, which caused a significant increase in number of G1 cells ($p < 0.01$). The irradiation by a dose of 3 Gy led to a significant G2/M arrest in MOLT-4 cells. The pre-incubation with VE-821 in both concentrations and its combinations with KU55933 disrupted the IR induced G2/M checkpoint.

To assess the impact of different inhibitor concentrations on viability of MOLT-4 cells and IR induced cell death we applied flow-cytometric detection of nonviable cells using the Apoptest-FITC kit. 10 μM VE-821 significantly affected viability of MOLT-4 cells 24 hours after the addition of the treatment and/or irradiation. Viability was not significantly affected in any of the KU55933- and combination- treated groups. 72 hours after irradiation, KU55933 increased the number of non-viable cells in irradiated cells, and further accumulation of nonviable cells was detected in VE-821 treated groups. Thus, both inhibitors increased IR-induced cell death.

4.3. **Proteomic and phosphoproteomic analysis of VE-821 treated MOLT-4 cells**

In summary, on a site false discovery rate level of 0.01, we identified 9285 phosphorylation sites from 3090 protein groups, among them 4504 were quantified in all three biological replicates (nearly 63 % of the data set). Only those sites quantified in all replicates were subjected to a non-parametric version of a global rank test (43) to identify sites with significantly up- or down- regulated phosphorylation consistently regulated in all three biological replicates. In GRT, we identified 623 regulated phosphorylation sites (from 455 phosphoproteins); most of them were upregulated (431). The identified and quantified phosphorylation sites were further subjected to bioinformatic analysis. The results of these analyses are presented in the discussion section.

4.4. **Targeted metabolomic analysis of VE-821 treated MOLT-4 cells**

Metabolite profiling was performed by a label-free targeted analysis of cellular extracts collected 6 and 12 hours after the treatment starting point. A method combining high-performance liquid chromatography and detection of the metabolites by Triple Quad 6500 tandem mass spectrometer enabled a quantitative analysis of 206 intermediary metabolites. The identified metabolites were further mapped on KEGG pathways to identify metabolic pathways that were potentially affected by VE-821 treatment. The metabolomic analysis was performed in collaboration with Hana Janečková and David Friedecký from the Laboratory for Inherited Metabolic Disorders at the University Hospital Olomouc.

5. Discussion

5.1. **Selection of the protocol for phosphopeptide enrichment from standard and a complex biological samples**

The evaluation of enrichment efficiency revealed that in our experiments, Titansphere® particles and NuTips® clearly outperformed the TopTips® in terms of the total and average numbers of phosphopeptides detectable by MALDI-TOF MS in the eluted fractions. Further analysis of the nature of peptides detected by different protocols showed that the main differences in numbers of phosphorylated sites detected between the two well performing products and TopTips® were caused by lower detectability of multiply-phosphorylated peptides while the enrichment efficiency for monophosphorylated peptides was comparable across the resins and buffer conditions. Doubly- phosphorylated peptide 1927.69 $[M+H]^+$ from α-S1-casein was observed in most of the enrichments; however, multiply phosphorylated peptides were more challenging to detect. These peptides were typically observed as low intensity peptide ions in the higher m/z range of the MALDI-TOF MS spectra, and were only detected in the low complex mixture A using the most specific enrichment protocols. TopTips® enrichment usually yielded a high number of nonspecifically bound non-phosphorylated peptides even under the most efficient buffer conditions tested, which might explain the low detectability of multiply phosphorylated peptides in the spectra as their ionization/signals were suppressed by the high amount of the nonphosphorylated peptides. Hence, the binding specifity was monitored by relative intensity of the two most intensive non-phosphorylated peptides originating from BSA. Both nonspecifically enriched peptides contained a relatively high number of acidic amino acids (as underscored in the sequences), which is in concordance with

previously reported fact that especially nonphosphorylated peptides containing greater proportions of aspartic and glutamic acid bind nonspecifically to phosphopeptide enrichment resins (53).

In 2005, Larsen and his co-workers investigated the effect of different aromatic carboxylic acids and aliphatic carboxylic acids added into loading buffer for phosphopeptide enrichment. DHB and other substituted aromatic carboxylic acids (salicylic acid, phthalic acid) showed the best efficacy in inhibition of adsorption of nonphosphorylated peptides (54). DHB was also found to be the most potent additive in another study (55). In the fourth protocol, ***buffer conditions 4***, **DHB** was added to the loading buffer in a concentration of **350 mg/ml** (close to a saturated solution) for complex mixtures of peptides according to the recommendation in Larsen's protocol. We observed that DHB was the most potent excluder for nonphosphorylated peptides when added during phosphopeptide enrichment using Titansphere® particles and TopTips® since the relative intensities of nonphosphorylated peptides were very low. Additionally, multiply phosphorylated peptides were more clearly detected than in other MALDI-TOF MS spectra obtained by different phosphopeptide enrichment protocols probably because of lowered ion suppression, which is normally affecting the signals of multiply phosphorylated peptides during MALDI-TOF MS. On the other hand, monophosphorylated peptides were slightly less reproducibly enriched when DHB was used, which might indicate that weaker bound monophosphorylated peptides could be also displaced from metal oxide surface by this potent "excluder". Thus, the non-phosphopeptide excluding efficiency of the additives increased in the following order: **5% TFA < 1 M LA < 0.1 M Glu < 350 mg/ml DHB**.

Even though DHB was shown to provide the highest non-phosphopeptide exclusion efficiency among the additives tested, it was actually 0.1 M Glu that enabled the detection of the most phosphorylated peptides. Although the signals of multiply phosphorylated peptides were not as clear as in the case of the DHB protocol, glutamic acid MOAC chromatography seemed to have the highest enrichment efficiency within the four protocols tested. Additionally, the protocol that included Titansphere® particles and glutamic acid reached the best repeatability among all the protocols tested with 15 phosphopeptides repeatedly detected in all three independent enrichments. The lower yields of phosphopeptides detected in DHB enriched eluates were mostly caused by aforementioned lower recovery of monophosphorylated peptides in the presence of 350 mg/ml DHB. In addition to the negative effect on the detection of mono-phosphorylated peptides, we also observed a high level of noise in the low m/z region of

MALDI-TOF MS spectra recorded from the samples enriched using NuTips® and DHB as an additive. This observation has been already reported in a previous study that also evaluated the performance of NuTips® for phosphopeptide enrichment (56). Moreover, it has been reported previously (57) that DHB added to loading buffer decreased the number of nonspecifically bound nonphosphorylated peptides but also the number of identified phosphopeptides because it caused problems in the RPLC-ESI-MS/MS system used in the study. Therefore, it might be risky to use DHB in cases when the samples are supposed to be injected into an LC-MS/MS system. On the contrary, Glu is a compound that does not have such a strong affinity for hydrophobic reversed phase, and thus its use in such system is more feasible. Therefore, we selected the glutamic acid including protocol for further studies.

5.2. **Radiosensitization of MOLT-4 cells by selective ATR and ATM inhibitors, VE-821 and KU55933**

To confirm the inhibitory effect of VE-821 and KU55933 on ATR and ATM kinases, respectively, we assessed phosphorylation of two downstream effector kinases: **Checkpoint kinase-1 (Chk1)** Ser 345 and **Checkpoint kinase-2 (Chk2)** Thr 68. As expected, both phosphorylation sites were upregulated upon irradiation (3 Gy, one hour after irradiation). Chk2 phosphorylation, which is a marker of DNA DSBs-activated ATM, was markedly decreased with increasing concentration of KU55933. Accordingly, the status of Chk1 Ser 345 phosphorylation site, which is a widely-accepted marker of ATR activation, was proportionally inhibited by increasing concentration of VE-821. Therefore, we confirmed that in our cell line model the pre-incubation with VE-821 and KU55933 specifically inhibited ATR and ATM kinase, respectively, without any off-target effect towards the other kinase.

KU55933 was the first potent and selective ATM inhibitor with IC50 of 13 nM (58). As well as its more recent analogues, it has been shown to sensitize cells to IR and DSB-inducing drugs without sensitizing cells derived from A-T patients (58–60). Radiosensitization using KU55933 and its derivatives is not specifically targeting cancer cells; however, ATM inhibition alone has been shown to be nontoxic for normal tissues outside the radiation field (60). In the presented data, KU55933 was well tolerated by the p53-wt expressing MOLT-4 cells in concentrations up to 10 μM. Since MOLT-4 cells express a wild-type p53 and possess a functional ATM/p53 pathway (61–63), and cellular response to DNA DSBs is to a great extent ATM/p53 pathway dependent, we expected that ATM inhibition would sensitize MOLT-4 cells to IR. In concordance with our expectation, 10 μM KU55933 caused a significant decrease in MOLT-4 cells proliferation when combined with IR and detected 6 days after irradiation.

While ATM mainly responds to severe DNA lesions, ATR is an indispensable regulator of cellular proliferation as it responds to stress that cells undergo during normal replication of DNA as well as replicative stress caused by exposure to genotoxic agents. It has been shown to be essential for viability; homozygous mutation of ATR caused peri-implantation embryonic lethality in mice (7,8). However, transient inhibition of ATR by VE-821 has been proven to cause mere reversible growth arrest in normal cells, which was abrogated when the inhibitor was removed from the cultivation media (22). Importantly, the newest highly potent and specific ATR inhibitors have been shown to directly eradicate or sensitize cancer cells to a variety of genotoxic agents without affecting normal, non-tumour, cells (20,22,23,64,65). In the presented study, VE-821 significantly inhibited proliferation of p53-wt MOLT-4 cells already at 1 μM concentration although such concentration caused only a partial ATR inhibition when detected as a CHK1 Ser 345 phosphorylation using western blotting. This effect was further accented in correlation with increasing inhibitor concentration. In combination with IR, VE-821 significantly abated the number of viable cells in both irradiated groups already 72 hours after irradiation. Additionally, we also observed that VE-821 in 2 μM and 10 μM concentrations modulated the proliferation of irradiated MOLT-4 cells when the inhibitor was present in cell culture media only transiently, for the first 24 hours of the treatment, and then it was washed out. In contrast to continuous treatment, 2 μM VE-821 did not significantly affect the proliferation of sham-irradiated cells when washed out after 24 hours; however, it still enhanced the antiproliferative effects of IR. Taken together, these data showed that VE-821 strongly affected proliferation of p53-wt MOLT-4 cells, and in combination with IR, the proliferation was influenced even when the inhibitor was present only transiently.

As one of the proposed mechanism of radiosensitization using ATR inhibitors is the disruption of the G2/M checkpoint in G1 checkpoint-deficient cells (22), we investigated modulation of the cell cycle by ATR and ATM inhibition. In concordance with our previous results (66), irradiation by the dose of 3 Gy led to a significant G2/M arrest in viable MOLT-4 cells 24 hours after irradiation. The pre-incubation with VE-821 and with the inhibitor combinations led to a significant disruption of G2/M checkpoint in the viable fraction of cells. On the other hand, the inhibition of ATM by KU55933 further increased the proportion of cells in G2/M together with corresponding significant decrease in the number of cells in G1.

5.3. **Phosphoproteomic analysis of VE-821 treated MOLT-4 cells**

To describe cellular mechanisms underlying the VE-821-mediated radiosensitization of MOLT-4 cells, we employed high-resolution MS to identify and quantify changes in proteome,

phosphoproteome, and metabolome of irradiated VE-821-treated cells. The quantification on both proteomic levels was based on SILAC (67)). To specifically enrich for peptides modified by phosphorylation, desalted tryptic peptide samples were fractionated using HILIC followed by phosphopeptide enrichment using titanium dioxide chromatography (54), RPLC-MS/MS detection, and peptide identification and quantification using MaxQuant *v.1.5.2.8* (41). Using this approach, we identified 9285 phosphorylation sites from 3090 protein groups at a site FDR level of 0.01, among them 4504 were quantified in all three biological replicates (nearly 63 % of the data set). In GRT, we identified 623 regulated phosphorylation sites (455 phosphoproteins); among them the majority were upregulated (431). To functionally classify phosphoproteins identified and quantified in our study and statistically evaluate enriched categories, we performed a functional annotation and over-representation analysis using ConsensusPathDB over-representation analysis web tool (44,45). By this tool, proteins were annotated using GO terms level 4 and the over-representation was evaluated using hypergeometric testing. In this analysis, we identified over-represented biological processes, cellular compartments, and molecular functions. Regulated phosphoproteins were over-represented in nucleus, specifically in chromosomes, mitotic spindle, and replication fork and involved in chromatin organization, DNA repair and metabolism, cell cycle, and regulation of transcription factors.

Using the ConsensusPathDB tool, we also mapped regulated phosphoproteins to signalling pathways from three different pathways databases: KEGG (68,69), REACTOME (70), and PID (71) and pathway coverage was calculated for each pathway. The list of pathways containing proteins with VE-821-regulated phosphorylation sites, and thus pathways potentially modulated by VE-821 treatment contained pathways involved in **DNA repair**, **replication**, and **telomeres synthesis**, **apoptosis**, **regulation of mitosis**, **transcription factors regulation**, **chromatin regulation via histones modification**, but also pathways primarily related to **cellular metabolism**.

Next, we analysed and visualized sequence motifs using iceLogo tool (46) and motif-x algorithm (47). In the upregulated dataset, proline-directed motifs followed by basic amino acids were significantly over-represented, which well corresponded to the known sequence logo of CDK 1 and 2. On the other hand, from the sequences surrounding downregulated sites, SQ motif was extracted using motif-x providing a confirmation of the downregulation of DNA repair kinases-mediated phosphorylation as this motif is typical for PI3K-related kinases ATM, ATR, and DNA-PK.

In the DNA content analysis, we showed that VE-821 disrupted IR-induced G2/M arrest in MOLT-4 cells. Cells treated with ATR inhibitor were not able to activate the G2/M checkpoint, which in turn led to faster progression into mitosis with unrepaired IR-induced DNA damage. Such progression has been shown to cause mitotic catastrophe and apoptotic cell death in haematological malignant cell lines (33,72,73). Using phosphoproteomics, we aimed to elucidate, which kinases were dysregulated by ATR inhibition and thus may contribute to the G2/M checkpoint disruption. Our analysis revealed a possible upregulation of G2/M checkpoint controlling **CDK1** kinase, mitotic kinases **Aurora A** and **B**, and kinases from the NEK family, particularly **Serine/threonine protein kinase NEK2 (NEK2)** kinase. These results indicated that ATR inhibition induced dysregulation of the main mitotic kinases. The results are in concordance with previous data, which were obtained using similar treatment in a different leukemic cell line HL-60 (32), in which dysregulation of CDK1, PLK1, and NEK2 was observed. Data mining in published studies confirmed that many of the substrates assigned to each one of these mitotic kinases have already been described as the essential players in cell cycle and mitosis control. Moreover, we found multiple regulated phosphorylation sites on these proteins, which might be worth functional validation in further studies.

Furthermore, we found a significant downregulation of the **Serine/threonine protein kinase mTOR (mTOR)**. The protein kinase **mTOR** is the principle regulator of cellular metabolism promoting anabolic processes and inhibiting catabolic processes such as autophagy (reviewed in (74)). In total, we found seven known direct mTOR targets downregulated in our study; most of them involved in translation regulation. In addition to proteins involved in translation regulation, we also identified significantly changed phosphorylation of two phosphoproteins linked to autophagy. Using immunoblotting, we showed that mTOR is inhibited by 10 μM VE-821 in both control and irradiated cells, suggesting that most likely the mTOR inhibition was achieved by an off-target effect of the inhibitor. The mTOR inhibition by 10 μM VE-821 and not 2 μM treatment correlated with the results of our proliferation and DNA analysis experiments and explain the difference we observed between the 2 μM and 10 μM VE-821 treated groups. The mTOR inhibition might also explain the high sensitivity of MOLT-4 cell line to VE-821 treatment. It has been shown that *PTEN*-deficient tumours were more likely to be sensitive to mTOR inhibition (75,76), and according to the COSMIC database, MOLT-4 cells suffer from homozygous deletion of *PTEN*.

In addition to the downregulation of the direct downstream phosphorylation targets of mTOR, we also directly observed a significant downregulation of the p70S6K (or RPS6KB1)

in all kinase activity analyses. P70S6K is an important kinase that acts downstream of mTOR to promote protein synthesis and cellular proliferation, providing further evidence for translation inhibition caused by inhibitor treatment. On the known/predicted p70S6K substrates list detected in our study, we did not find phosphoproteins involved solely in protein synthesis. **Dihydroorotase (CAD)** is an enzyme required for the first steps of *de novo* pyrimidine synthesis. Phosphorylation on Ser 1859 by p70S6K downstream of mTOR stimulates dihydroorotase activity of CAD to induce pyrimidine synthesis (77). Ser 1859 was detected to be downregulated in our study, suggesting that VE-821 treatment might also hamper synthesis of nucleotides, which are necessary for both cellular proliferation and DNA repair after DNA damage induction.

5.4. **Metabolomic analysis of VE-821 treated MOLT-4 cells**

Using the phosphoproteomic analysis of VE-821 treated irradiated cells, changes in the mTOR activity were detected via kinases activity analysis and further confirmed by western blotting. As mTOR is the principal regulator of cellular metabolism (reviewed in (74)), this finding raised an intriguing question – whether the inhibitor treatment modulates cellular metabolism after irradiation. The metabolic responses of different cell lines to various doses of IR have been investigated in several studies (78–80). However, to our knowledge, no study has been published yet investigating the modulation of this response by small molecular kinase inhibitor of any of the PIKKs family.

When cells are exposed to IR, cellular structures can be damaged by ionization directly by deposition of energy, but also indirectly by water radiolysis and stimulation of nitric oxide synthases, which leads to generation of highly reactive oxygen and nitrogen species (ROS/RNS) secondarily attacking nucleic acids and other biomolecules such as proteins and lipids contained in membranes (reviewed in (81). Reduced glutathione (GSH) is an important cellular antioxidant and the reduced/oxidized glutathione ratio (GSH/GSSG) is a sensitive biomarker of oxidative stress (82). In our study, we observed a decrease in GSH over incubation time and this decrease was amplified by inhibitor exposure. Additionally, increased level of allantoin was observed. Allantoin can be formed in the presence of ROS by non-enzymatic reactions, and similarly to GSH, it can be used as a marker of oxidative stress (83). In addition to the changes in cellular redox system, direct effect on proteins and membranes was observed. Increased levels of several amino acids (phenylalanine, proline, tryptophan, tyrosine, valine, isoleucine, and methionine) might indicate increased turnover of proteins which were damaged

by ROS/RNS exposure. Elevated levels of docosahexaenoic acid and choline were also detected; this finding might reflect degradation of membranes.

IR has been shown to disturb energetic metabolism demonstrated by a rapid decrease of cellular ATP levels in irradiated cells (78,84) and serum ATP levels in irradiated mice (85). In our study, we found decreased levels of nucleotide triphosphates (NTPs; ATP, GTP, CTP, and UTP), diphosphates (NDPs; ADP and UDP), and their derivatives (UDP-hexoses, GDP-hexoses, and UDP-N-acetylglucosamine) and on the contrary, increased levels of ribonucleosides (adenosine, guanosine, and pseudouridine) and bases (uracil). Decreased levels of high-energetic phosphorylated compounds might indicate disruption in energy metabolism due to exposure to IR; VE-821 amplified the energy depletion caused by IR.

Significant changes were observed in glycolysis and citric acid cycle. Increased glucose and decreased intermediates of glycolysis can probably reflect inhibition of hexokinase - the first step of glycolysis – probably because of ATP insufficiency. Markedly lower levels of aconitate, malate, and citrate/isocitrate were found in VE-821 treated cells. On the contrary, succinate and fumarate were slightly increased in VE-821 treated cells. We assume that these elevated intermediates could have been replenished by anaplerotic reaction from the amino acids obtained by protein degradation.

Decreased levels of deoxynucleotide diphosphates (dNDPs; dADP and dTDP) and triphosphates (dNTPs; dATP, dGTP, and dTTP) were found in VE-821 treated irradiated cells and on the other hand, several purine and pyrimidine bases and ribosides (xanthine, inosine, cytidine, uridine, and thymidine) were elevated in VE-821 treated cells. Several mechanisms could contribute to dNTPs depletion upon IR in VE-821 treated cells including the inhibition of deoxycytidine kinase (dCK) by ATR inhibition and thus downregulation of deoxynucleotide salvage pathways. Further validation experiments would be necessary to reveal the exact mechanism.

Observed changes in the acylcarnitine profile might indicate mitochondrial disturbances enhanced by VE-821. Acylcarnitine changes have been already observed in a study investigating metabolic effects of low dose (20 mGy) X-irradiation (80) and in a targeted lipidomic study investigating changes in livers of irradiated mice (86). Our observation of the lipid changes in mitochondrial membrane might be explained by lipid peroxidation induced by increased ROS generation in VE-821 treated cells.

6. Conclusion

DNA damage induction by either radio- or chemo-therapy has been the most widely used approach in oncology exploiting one of the hallmarks of cancer: genomic instability. However, such treatment is very unspecific and often accompanied by collateral damage to healthy tissues. In recent years, much effort has been focused on discovery and development of tumour specific treatment, which would only specifically target cancer cells and not affect the normal tissues. A promising concept that has been developed recently is to take advantage of the tumour specific abnormalities in DDR, and target the DDR kinases from the PIKKs family - ATM, ATR, and DNA-PK.

In the presented doctoral thesis, we aimed to elucidate molecular mechanisms underlying radiosensitization of MOLT-4 cell line (T-ALL) by PIKKs inhibitors. To do so, we tested two highly potent inhibitors of two kinases from the PIKKs family - ATR and ATM, VE-821 and KU55933, respectively for their effects on proliferation, viability, and cell cycle of sham-irradiated and irradiated MOLT-4 cells. In these initial tests, both inhibitors proved to radiosensitize MOLT-4 cells and furthermore, 10 μM VE-821 was shown to act as a strong antiproliferative agent in sham-irradiated MOLT-4 cells.

To further describe cellular mechanisms underlying the VE-821-mediated radiosensitization of MOLT-4 cells, we employed high-resolution MS to identify and quantify changes in **proteome, phosphoproteome**, and **metabolome** of irradiated VE-821-treated cells. As the detection and quantification of phosphorylated peptides in complex biological samples is challenging due to their low stoichiometry, first of all we optimized protocol for the enrichment of phosphorylated peptides from their mixture with their nonphosphorylated counterparts. Several commercially available chromatographic materials and published protocols were selected and tested using MALDI-TOF MS detection of phosphorylated peptides enriched from standard tryptic peptides mixtures. The most successful protocols were further evaluated using complex samples, such as HeLa cells lysate and SILAC-labelled MOLT-4 cells lysate. Based on the optimization part, a protocol including the **Titansphere® particles** and loading buffer containing **200 mM glutamic acid** as a specificity and efficiency enhancer was compiled for further experiments.

The optimized protocol was then successfully applied to study VE-821-dependent changes in irradiated MOLT-4 cells. In concordance with our expectations, VE-821 **did not cause any significant changes on the proteome level**. However, we detected **623**

differentially regulated phosphorylation sites; most of them (431) were upregulated in response to the inhibitor treatment. Using bioinformatic tools, we described, which protein kinases and signalling pathways might be affected by the inhibitor treatment upon irradiation. These analyses revealed changes **in DDR related pathways and kinases**, but also pathways and kinases involved in **maintaining cellular metabolism**. Notably, we found a **downregulation of mTOR**, the main regulator of cellular metabolism, which was also confirmed by western blotting. Although there are multiple signalling pathways connecting IR-induced DDR and mTOR regulation that might be potentially regulated by ATR inhibition, we assumed that in our case the downregulation was most likely caused by an **off-target effect of VE-821 at 10 μM concentration**. We further concluded that mTOR inhibition could be one of the factors contributing to the phenotype we observed after treating MOLT-4 cells with 10 μM VE-821, which was different from a lower, mTOR non-inhibitory, concentration.

To our best knowledge, no study has been published yet investigating the modulation of cellular metabolism by small molecular kinase inhibitor of any of the PIKKs family kinases. Therefore, we performed a targeted metabolomic analysis of irradiated MOLT-4 cells, whose response to IR was modulated by 10 μM VE-821. In this analysis, **206 intermediary metabolites were quantified**. Subsequent data analysis showed that VE-821 potentiated the **metabolic disruption** induced by IR and increased the IR induced **oxidative stress**. Our data also indicated that in response to IR, **recovery of dNTPs** might be affected by VE-821 possibly hampering the DNA repair by dNTPs insufficiency.

Thus, in this thesis we described a complex scenario of cellular events that might be dependent on ATR or triggered by ATR inhibition in irradiated MOLT-4 cells. Data presented in this work might serve as a resource for follow-up studies and provide a platform for future work with other (more potent or specific) inhibitors. Taken together, we conclude that using ATR inhibitors in order to radiosensitize cancer cell seems to be an effective anti-tumour strategy. Nevertheless, even using a highly specific inhibitor might lead to a complex response and similar MS-based studies are suitable to reveal additional information on off-target effects and provide insights into other possibly non-reported regulatory mechanisms.

7. References

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8. Overview of publications

Original papers with IF

Šalovská B, Fabrik I, Ďurišová K, Link M, Vávrová J, Řezáčová M, Tichý A. Radiosensitization of human leukemic HL-60 cells by ATR kinase inhibitor (VE-821): phosphoproteomic analysis. Int J Mol Sci. 2014;15(7):12007–26. **IF = 2.862**

Salovska B, Tichy A, Fabrik I, Rezacova M, Vavrova J. Comparison of Resins for Metal Oxide Affinity Chromatography with Mass Spectrometry Detection for the Determination of Phosphopeptides. Analytical Letters. 2013 Jul 3;46(10):1505–24. **IF = 0.982**

Tichy A, Durisova K, **Salovska B**, Pejchal J, Zarybnicka L, Vavrova J, Dye NA, Sinkorova Z. Radio-sensitization of human leukaemic MOLT-4 cells by DNA-dependent protein kinase inhibitor, NU7441. Radiation and Environmental Biophysics. 2014 Mar;53(1):83–92. **IF = 1.528**

Review papers with IF

Salovska B, Tichy A, Rezacova M, Vavrova J, Novotna E. Enrichment strategies for phosphoproteomics: state-of-the-art. Reviews in Analytical Chemistry. 2012 Mar;31(1):29–41. **IF = 0.436**

Durisova K, **Salovska B**, Pejchal J, Tichy A. Chemical inhibition of DNA repair kinases as a promising tool in oncology. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2015 Oct 21; **IF = 0.924**

Tichy A, **Salovska B**, Rehulka P, Klimentova J, Vavrova J, Stulik J, Hernychova L. Phosphoproteomics: Searching for a needle in a haystack. Journal of Proteomics. 2011 Nov 18;74(12):2786–97. **IF = 4.878**

Novotna E, Tichy A, Pejchal J, Lukasova E, **Salovska B**, Vavrova J. DNA-dependent protein kinase and its inhibition in support of radiotherapy. International Journal of Radiation Biology. 2013 Jun;89(6):416–23. **IF = 1.837**

Original papers without IF

Tichý A, Novotná E, Durisová K, **Salovská B**, Sedlaríková R, Pejchal J, Zárybnická L, Vávrová J, Sinkorová Z, Rezácová M. Radio-sensitization of human leukaemic molt-4 cells by DNA-dependent protein kinase inhibitor, NU7026. Acta Medica (Hradec Kralove). 2012;55(2):66–73.

Oral presentations

Šalovská B.: Radio-sensitization of human leukemic cells HL-60 by ATR-kinase inhibitor (VE-821): Phosphoproteomic analysis. 9. Fakultní konference studentů doktorských studijních programů, October 21 2013, Hradec Králové

Šalovská B.: Radiosenzibilizace leukemických buněk HL-60 pomocí inhibitoru ATR-kinázy (VE-821): Fosfoproteomická analýza. 3. Neformální proteomické setkání, November 21-22 2013, Hradec Králové

Šalovská B.: Radio-sensitization of human leukemic cells HL-60 by ATR-kinase inhibitor (VE-821): Phosphoproteomic analysis. 10th International Medical Postgraduate Conference, November 21-22 2013, Hradec Králové

Šalovská B.: Radiosenzibilizace leukemických buněk HL-60 pomocí inhibitoru ATR-kinázy (VE-821): Fosfoproteomická analýza. Výroční zasedání Ústavu molekulární patologie FVZ 2013, November 28-29 2013, Velká Úpa, ústní prezentace

Šalovská B.: Radiosenzibilizace leukemických buněk HL-60 pomocí inhibitoru ATR-kinázy (VE-821): Fosfoproteomická analýza. Seminář Katedry radiobiologie FVZ 2013, December 11 2013, Hradec Králové

Šalovská B.: Radiosensitizing effect of DNA-repair inhibition in MOLT-4 and SAOS-2 cell lines: phosphoproteomic analysis of irradiated cancer cells. XVIII. vědecká konference LFHK a FNHK, January 22 2014, Hradec Králové

Poster presentations

Salovska B., Tichy A., Fabrik I., Rezacova M., Vavrova J.: Phosphoproteomic analysis of irradiated human leukemic cells: radiosensitization of HL-60 cells by ATR kinase inhibitor (VE-821). HUPO 12th Annual World Congress, September 14 – 18 2013, Yokohama, Japan

Salovska B., Tichy A., Fabrik I., Rezacova M., Vavrova J.: Radio-sensitization of human leukemic cells HL-60 by ATR-kinase inhibitor (VE-821): Phosphoproteomic analysis. 3. konference České společnosti pro hmotnostní spektrometrii, October 16 -18 2013, Hradec Králové

Barbora Salovska, Ariel Bensimon, Ales Tichy, Ruedi Aebersold: Interactome analysis of key protein complexes responsive to DNA damage and replication stress, HUPO 14th Annual World Congress, September 27 – 30 2015, Vancouver, Canada