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Duale Inhibition von PARP und dem intra-S/G2-Zellzykluskontrollpunkt als neue Strategie zur hocheffizienten Strahlensensitivierung HPV-positiver HNSCC

Dissertation

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Dual Inhibition of PARP and the Intra-S/G2 Cell Cycle Checkpoints Results in Highly Effective Radiosensitization of HPV-Positive HNSCC Cells

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In head and neck squamous cell carcinoma (HNSCC), tumors positive for human papillomavirus (HPV) represent a distinct biological entity with favorable prognosis. An enhanced radiation sensitivity of these tumors is evident in the clinic and on the cellular level when comparing HPV-positive and HPV-negative HNSCC cell lines. We could show that the underlying mechanism is a defect in DNA double-strand break repair associated with a profound and sustained G2 arrest. This defect can be exploited by molecular targeting approaches additionally compromising the DNA damage response to further enhance their radiation sensitivity, which may offer new opportunities in the setting of future de-intensified regimes. Against this background, we tested combined targeting of PARP and the DNA damage-induced intra-S/G2 cell cycle checkpoints to achieve effective radiosensitization. Enhancing CDK1/2 activity through the Wee1 inhibitor adavosertib or a combination of Wee1 and Chk1 inhibition resulted in an abrogation of the radiation-induced G2 cell cycle arrest and induction of replication stress as assessed by γ -H2AX and chromatin-bound RPA levels in S phase cells. Addition of the PARP inhibitor olaparib had little influence on these endpoints, irrespective of checkpoint inhibition. Combined PARP/Wee1 targeting did not result in an enhancement in the absolute number of residual, radiation induced 53BP1 foci as markers of DNA double-strand breaks but it induced a shift in foci numbers from S/G2 to G1 phase cells. Most importantly, while sole checkpoint or PARP inhibition induced moderate radiosensitization, their combination was clearly more effective, while exerting little effect in p53/G1 arrest proficient normal human fibroblasts, thus indicating tumor specificity. We conclude that the combined inhibition of PARP and the intra-S/G2 checkpoint is a highly effective approach for the radiosensitization of HPV-positive HNSCC cells and may represent a viable alternative for the current standard of concomitant cisplatin-based chemotherapy. *In vivo* studies to further evaluate the translational potential are highly warranted.

Keywords: head and neck cancer, human papillomavirus (HPV), molecular targeting, radiotherapy, radiosensitization, PARP, Wee1, Chk1

INTRODUCTION

In locally advanced squamous cell carcinoma of the head and neck (HNSCC), positivity for human papillomavirus (HPV) confers a favorable prognosis, especially for patients with tumors located in the oropharynx (OPSCC) (1, 2). Standard treatment of locally advanced disease is cisplatin-based chemoradiation, either in the primary setting or as adjuvant treatment after surgery. The combination of high cure rates but often dramatic toxicity under these regimes has resulted in the development of various clinical trials testing de-intensification approaches, and some early phase trials have reported promising results (3–7). Two phase 3 trials, however, which together recruited more than 1,000 patients, concordantly reported inferiority of the rather cautious de-intensification concept of exchanging cisplatin for the also approved anti-EGFR antibody cetuximab under maintenance of the full radiation dose (8, 9). In line with these negative clinical results, we had previously shown that cetuximab completely fails to radiosensitize HPV-positive HNSCC cells *in vitro* (10). This clearly urges caution and speaks in favor of careful preclinical evaluation of novel agents and concepts.

A way to very directly induce radiosensitization is the molecular targeting of proteins involved in the DNA damage response (DDR) and DNA repair. Poly(ADP-ribose) polymerase 1 (PARP1) is responsible for poly(ADP-ribose) polymerization at the sites of DNA damage, which marks the lesion and recruits further DNA repair factors. PARP1 is involved in single-strand break repair but also in double-strand break (DSB) repair *via* the alternative end-joining (alt-EJ) backup DSB repair pathway (11, 12). Sole PARP inhibition is especially effective in tumors with a severe deficiency in homologous recombination (HR). Following the well-known concept of synthetic lethality, PARP inhibition increases the need for effective HR by interfering with the repair of intrinsic single-strand lesions and PARP-trapping at the break sites. Upon collision with replication forks, these structures can lead to the formation of one-ended DSBs, the repair of which requires HR (13, 14). Ionizing radiation induces both single- and double-strand breaks, and PARP-inhibitors are well known radiosensitizers (15).

Cell cycle checkpoints constitute another important factor in the response towards irradiation, providing more time for DNA repair before entering S-phase or mitosis in order to avoid mutations and especially mitotic cell death (16). In HNSCC, the majority of HPV-positive and -negative tumors are functionally deficient for p53 and subsequently also for the G1-S cell cycle checkpoint, increasing the dependence on the G2-M checkpoint. Reduction of the radiation-induced G2 arrest can be achieved by inhibition of the ATR/Chk1/Wee1 axis, as the inhibition of any of these kinases finally counteracts Wee1-mediated inhibitory phosphorylation of cyclin dependent kinase 1 (CDK1), which, in its active state will continue to drive G2-M transition (16, 17). Premature mitotic entry and induction of severe replication stress are further therapeutic effects resulting from enhanced CDK1 and CDK2 activity upon inhibition of the ATR/Chk1/Wee1 axis also without irradiation (18–20).

We and others have demonstrated that PARP inhibition as well as inhibition of radiation induced cell cycle checkpoints *via* targeting of Chk1, ATR, or Wee1 can radiosensitize HPV-

positive HNSCC cells (10, 21–25). Different mechanisms may account for the observed sensitization. HPV-positive HNSCC cells are described to rely on PARP-dependent alt-EJ (26, 27) and to be defective in homologous recombination (HR) (27–31). Due to an ineffective DSB repair, these cells further rely on an especially profound and long lasting radiation-induced G2 arrest for the repair of radiation-induced DSBs before the critical passage through mitosis (21, 22, 32, 33). Apart from interfering with G2 arrest, the inhibition of Wee1, Chk1, or ATR can directly compromise the ability to perform HR (34–36) and the induction of replication stress, which is to a large extent caused by nucleotide shortage due to unrestrained CDK activity and enhanced origin firing (18), that may create an unfavorable environment for DNA repair in S phase. Given these potential S/G2 phase-based mechanisms, it is easily imaginable that the combined inhibition of PARP and the S/G2 cell cycle checkpoints could be an especially effective treatment option for HPV-positive HNSCC cells, and its radiosensitizing effect has already been demonstrated in preclinical studies in a number of other cancer entities (37, 38). Against this background, we tested the combined inhibition of PARP and the S/G2 cell cycle checkpoint in intrinsically DSB repair-compromised HPV-positive HNSCC cells using clinically relevant inhibitors, all of which are already being tested in combination with radiotherapy in clinical trials in HNSCC.

MATERIAL AND METHODS

Cells and Cell Culture

All cell lines were grown in RPMI (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany) at 37°C, 5% CO₂ and 100% humidification. HPV-positive HNSCC cells UD-SCC-2, UM-SCC-47 and UPCI-SCC-154, UPCI-SCC-90, 93VU-147T, UT-SCC-45, and normal human fibroblasts F184 were described previously (21, 33, 39). Tumor cell lines were identified by a short tandem repeat multiplex assay (Applied Biosystems, Waltham, MD, USA). PARP inhibition was performed using 1 μM olaparib (MyBiosource, San Diego, CA, USA). Wee1 inhibition was performed using 240 nM adavosertib (Selleckchem, Houston, TX, USA) and combined Wee1/Chk1 inhibition was performed at a dose of 60 nM adavosertib and 1 nM prexasertib (MedChemExpress, Monmouth Junction, NJ, USA) unless stated otherwise. Supplementation with nucleosides (EmbryoMax 100×, Sigma-Aldrich, St. Louis, MO, USA) was performed at a final dilution of 1/12.5.

Cell Proliferation

For cell proliferation analysis, cells were seeded into T25 cell culture flasks and after 4 h treated with inhibitors. The numbers of resulting cells were assessed after 5 days using a Coulter counter (Beckmann-Coulter, Brea, CA, USA).

Cell Cycle Assessment

Cells were harvested, fixed with 70% ethanol, briefly washed with PBS/0.2% Triton X-100, and subsequently incubated with PBS/

1% BSA/0.2% Triton X-100/DAPI (4',6-Diamidin-2-phenylindol, 1 µg/ml) for 30 min at room temperature in the dark. Cells were washed once with PBS/0.2% Triton X-100, and flow cytometric analysis was performed using a MACSQuant10 with MACSQuantify Software (Miltenyi Biotec, Bergisch Gladbach, Germany). The proportion of cells in the respective cell cycle phases was calculated using ModFit LT™ software (Verity Software House, Topsham, ME, USA).

X-Irradiation

Cells were irradiated at room temperature with 200 kV X-rays (Gulmay RS225, Gulmay Medical Ltd., Suwanee, GA, USA; 200 kV, 15 mA, 0.8 mm Be + 0.5 mm Cu filtering; dose rate of 1.2 Gy/min).

DSB Reporter Gene Assay

Exponentially growing HNSCC cells containing stably integrated copies of the previously described GFP-based HR or NHEJ reporter plasmids pGC or pEJ (40) were transfected with an I-SceI expression vector for targeted DSB induction using Fugene HD (Promega, Fitchburg, WI, USA). Six hours post transfection, the medium was exchanged and supplemented with inhibitors or solvent (DMSO) as indicated, followed by another exchange plus supplementation 24 h post transfection. At 48 h post transfection, the cells were harvested and assessed for GFP expression by flow cytometry using a FACS Canto with FACS Diva software (Becton Dickinson, Franklin Lakes, NJ, USA). The gating of GFP-positive cells was set according to the negative control (Fugene HD + empty vector). Rates of DSB repair (% GFP-positive cells) were normalized to the respective transfection efficiency of the individual experiment as determined by parallel transfection with a GFP-expression vector (pEGFP-N1).

Immunofluorescence

Cells grown on glass cover slips were fixed with PBS/4% formaldehyde for 10 min, and permeabilized/blocked for 1 h or overnight with PBS/1% BSA/0.2% Triton X-100. The cells were subsequently incubated for 1 h at room temperature with the primary antibodies [mouse anti-53BP1 (clone BP13, Millipore, Billerica, MA, USA); rabbit anti-geminin (#10802-1-AP, Proteintech, Manchester, UK)] in blocking solution, washed four times with PBS/0.5% BSA/0.1% Triton X-100 before incubation with the secondary antibodies plus DAPI (1 µg/ml) and were then washed again four times before mounting with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Cells were inspected using an AxioObserver Z1 fluorescence microscope with ApoTome and Axiovision Software (Zeiss, Oberkochen, Germany). 53BP1 foci per nucleus were manually counted using stack images in maximum intensity projection. Nuclei with ≥ 20 foci were scored as "20".

Flow Cytometric Protein Quantification

Flow cytometric measurement of relative protein staining intensity per cell in relation to the cell cycle phase was performed on either a FACS Canto with FACS Diva Software

(Becton Dickinson, Franklin Lakes, NJ, USA) using FxCycle FarRed (Molecular Probes, Eugene, OR, USA) as nuclear counterstain or on a MACSQuant10 with MACSQuantify and Flowlogic Software (Miltenyi Biotec, Bergisch Gladbach, Germany & Inivai, Mentone Victoria, Australia) using DAPI as nuclear counter stain. In brief, cells were harvested, fixed with PBS/4% formaldehyde for 10 min, and then permeabilized and blocked with PBS/1% BSA/0.2% Triton X-100 for a minimum of 1 h. The cells were subsequently incubated (1 h; room temperature) with the primary antibody [rabbit anti-P-Histone3 (#06-570, Millipore, Billerica, MA, USA), mouse-anti- γ H2AX antibody (clone JBW301, Millipore, Billerica, MA, USA), and mouse anti-RPA32 (clone ME34, Santa Cruz, Santa Cruz, CA, USA)] in blocking solution, washed three times with PBS/0.5% BSA/0.1% Triton X-100 before incubation (1 h; room temperature) with the second antibody and were then washed again three times. DNA counterstaining was either performed with DAPI added to the secondary antibody or with FxCycle FarRed (Molecular Probes, Eugene, OR, USA) plus 300 ng/ml RNase A and 0.2% Triton X-100 for 30 min at room temperature in the dark following the last washing step.

In case of RPA staining, the cells were pre-extracted after trypsinization by gentle resuspension (wide bore tips) of the harvested cell pellet in 500 µl ice cold PBS/0.1% Triton X-100/1 mM DTT followed by gentle shaking in horizontally placed reaction tubes on ice for 10 min. Afterwards, 1 ml cold PBS/1% BSA/1mM DTT was added, tubes were inverted several times, and the pre-extracted cells were collected in a pre-cooled centrifuge (5 min, 400 g). After discarding the supernatant, the pre-extracted cells were resuspended (wide bore tips) in PBS/4% formaldehyde and fixed for 10 min at room temperature before regular subsequent staining procedures as described above.

Colony Formation Assay

Radiosensitization was determined using delayed plating colony formation assay. Exponentially growing cells were treated with inhibitor and irradiated after 2 h of incubation. Twenty-four hours post irradiation the cells were seeded in defined numbers into T25 cell culture flasks without addition of inhibitors. Incubation time until colony formation varied between cell lines from 2 to 4 weeks; irradiated samples of HPV-positive cell lines were allowed to grow for an extended period of time, as colony formation was apparently delayed. The number of colonies containing more than 50 cells was assessed. In the case of UM-SCC-47, the cell number was adjusted to 5000 by addition of feeder cells (UM-SCC-47; 20 Gy) to support plating efficiency, and for UPCI-SCC-154 and F184 the medium was changed to a 1:1 mixture of RPMI/10% FBS and Amniomax C-100 medium/7.5% Amniomax Supplement (both Gibco, Thermo Fisher Scientific, Waltham, MA, USA)/7.5% FBS one (F184) or three (UPCI-SCC-154) weeks after seeding to facilitate colony formation.

Data Evaluation

Data analyses were performed using Excel (Microsoft, Redmond, WA, USA) and GraphPad Prism 6 (GraphPadSoftware, San Diego, CA, USA). All experiments were performed at least

three times, and single experiments always contained the full set of substances and radiation doses as indicated. Values presented are mean \pm SD unless indicated otherwise. Two-tailed Student's t-test was used to assess statistically significant differences using GraphPad Prism 6.

RESULTS

To assess whether the dual inhibition of PARP and Wee1 may exert some additive or synergistic effects in HPV-positive HNSCC cells, we tested a combination of the PARP inhibitor olaparib and the Wee1 inhibitor adavosertib (MK-1775/AZD-1775) with regard to cell proliferation and cell cycle distribution. To this end we used individual inhibitor doses that previously demonstrated moderate effects on their own with regard to the respective cell lines and endpoints or a maximum concentration of 1 μ M olaparib in the cell cycle analyses, which was previously proven sufficient to completely suppress the poly(ADP)-ribosylation of HPV-positive HNSCC cells upon H₂O₂ treatment (10, 22). Regarding proliferation we observed several statistically significant differences and the generally strongest reduction under combined inhibition but without a clear hint for a meaningful synergistic effect (Figure 1A). Regarding cell cycle distribution, adavosertib induced an accumulation of cells in the S-phase, indicative of replication stress, while olaparib had little effect on its own or when added to Wee1 inhibition (Figure 1B).

Radiation-Induced Cell Cycle Arrest

While the previous results did not indicate prominent synergistic effects, we further tested dual PARP and S/G2 checkpoint inhibition combined with ionizing irradiation. To assess a direct effect on the radiation-induced G2 arrest, we quantified the amount of phospho-histone H3 positive mitotic cells 5 h after 6 Gy \pm inhibitor treatment (Figure 2A). Sole adavosertib treatment (240 nM) increased the rate of mitotic cells in two cell lines, indicating unscheduled mitotic entry upon Wee1 inhibition as previously described (41). Irradiation largely blocked mitotic entry in all strains irrespective of olaparib treatment (1 μ M). Adavosertib completely suppressed this G2 arrest, except for UD-SCC-2 cells, where it could only partially override checkpoint execution (Figure 2B). Additionally testing a later time point of 8 h post irradiation, adavosertib treatment \pm olaparib further relieved UD-SCC-2 cells from the radiation-induced G2 checkpoint (Figure 2C). We had previously shown that Wee1 inhibition activates Chk1, which could in part compensate the reduction in Wee1 activity and, indeed, dual inhibition was effective at profoundly reduced doses (22). As low dose dual Wee1/Chk1 inhibition may potentially offer a clinical alternative to high dose single inhibitor treatment, we also included a combination using especially low concentrations of 60 nM adavosertib and 1 nM of the Chk1/2 inhibitor prexasertib, which showed limited effectiveness on their own (Supplementary Figure S1). This dual checkpoint inhibition resulted in checkpoint abrogation comparable to the higher dose (240 nm) of sole adavosertib treatment irrespective of the addition of olaparib in all strains (Figure 2B).

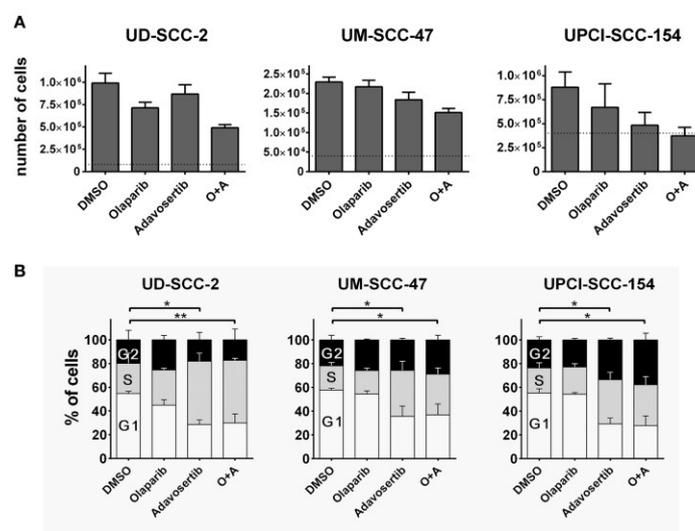
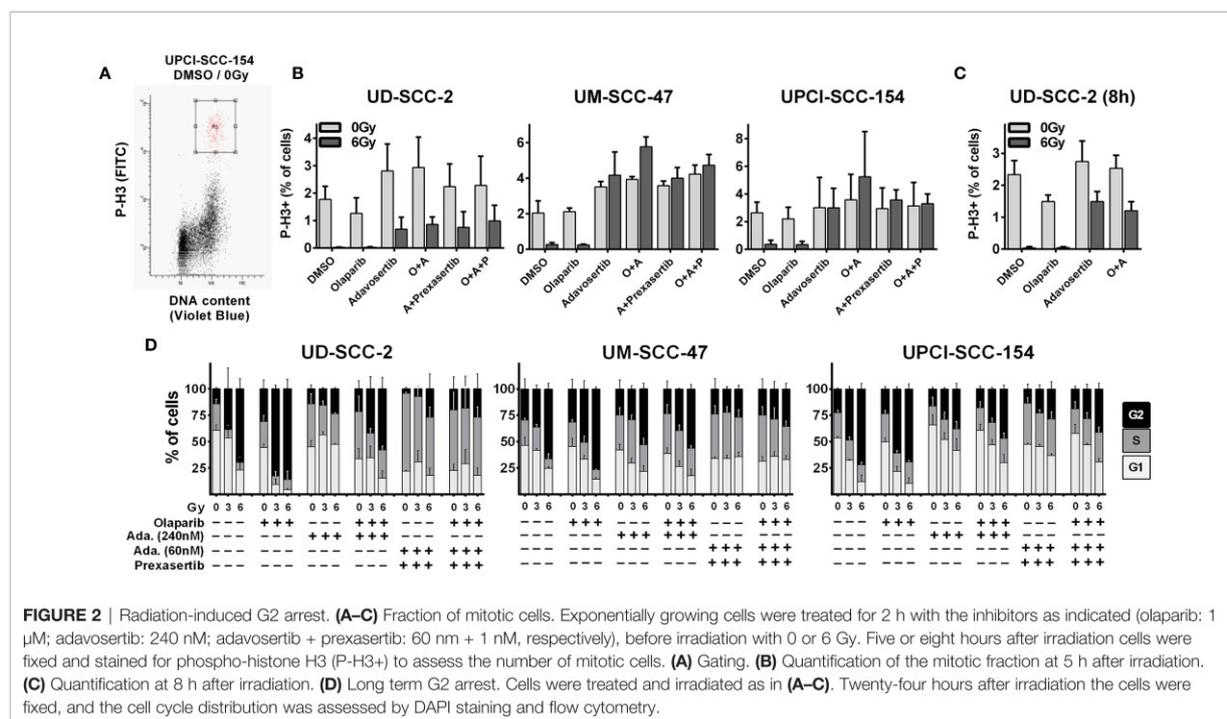


FIGURE 1 | Interactions of PARP and Wee1 inhibition. **(A)** Proliferation. Cells were seeded and after 4 h treatment with inhibitors as indicated. Five days later the respective numbers of cells were assessed. Dotted lines indicate the number of cells seeded. Adavosertib: UD-SCC-2 & UM-SCC-47, 120 nM; UPCI-SCC-154, 60 nM. Olaparib: all strains 500 nM. **(B)** Cell cycle. Cells were seeded and on the next day treated with the respective inhibitors. After 24 h, the cells were fixed and subjected to DAPI staining and flow cytometric assessment of cell cycle distribution. Adavosertib: UD-SCC-2 & UPCI-SCC-154, 480 nM; UM-SCC-47, 960 nM. Olaparib: all strains 1 μ M. Statistical evaluation was performed for changes in the S-phase population; addition of olaparib did not induce any significant changes. Asterisks depict significant differences with * and ** indicating $p < 0.05$ and $p < 0.01$, respectively (two-tailed Student's t-test).



As HPV-positive HNSCC cells show prolonged G2-checkpoint responses due to an inefficient DNA DSB repair (33), we further assessed cell cycle distribution at a later time point of 24 h after irradiation where all cell lines demonstrated profound radiation-induced G2 arrest (**Figure 2D**). In line with the short term experiments described above, adavosertib treatment reduced the amount of radiation-induced G2 arrest also at 24 h after irradiation but not to the full extent. The combination of adavosertib and prexasertib also reduced G2 arrest and partly increased the amount of S phase cells, suggesting severe replication stress. Addition of olaparib to adavosertib \pm prexasertib did not induce any further accumulation in S-phase irrespective of radiation. In UD-SCC-2 cells, sole olaparib treatment resulted in a clear increase of cells in G2, especially after irradiation but also at baseline. In UM-SCC-47 and UPCI-SCC-154 the increase was subtle but highly reproducible, which is in line with enhanced DNA damage levels after PARP inhibition as frequently reported (**Supplementary Figure S2A**) (42–44). Enhanced damage levels are further supported by higher intensity of the DNA damage marker γ H2AX in cells residing in radiation-induced G2-arrest after olaparib treatment in all three cell lines (**Supplementary Figure S2B**).

For all the following experiments, we continued with concentrations of 1 μ M olaparib and 240 nM adavosertib or, alternatively, the reduced concentration of 60 nM adavosertib combined with 1 nM prexasertib, which demonstrated similar G2 checkpoint abrogation in these assays.

Replication Stress

Unscheduled activation of dormant origins and subsequent nucleotide depletion is described as a mechanism of antitumor

activity through Wee1 and/or Chk1 inhibition (18, 20). This leads to replication stress and, if severe, S-phase arrest as partially observed for the combined Wee1/Chk1 inhibition described above. Chk1 is further described as a replication fork protection factor (45) and PARP1, apart from its functions in DNA repair, was reported to be involved in the restart of stalled replication forks and Chk1-dependent S-phase checkpoint activation and fork protection (46–49).

In S-phase, cell stretches of single-strand DNA (ssDNA) upon replication fork stalling as well as DSBs upon replication fork collapse are recognized through the related ATR and ATM kinases, and such areas are subsequently decorated by γ H2AX. In line with these mechanisms, the inhibition of Wee1 as well as the combined inhibition of Wee1/Chk1 resulted in a strong increase in γ H2AX signal intensity in S and partly G2 phase cells. However, neither olaparib alone nor the addition of olaparib to Wee1 or to Wee1/Chk1 inhibition resulted in any substantial increase in γ H2AX levels with the exception of sole addition in UD-SCC-2 cells. Here, a considerable number of cells demonstrated higher γ H2AX levels, but the rise in signal intensity was very modest (**Figures 3A, B** and **Supplementary Figure S3A**). Although less uniform than the γ H2AX staining, the results were in principle confirmed when assessing the amounts of chromatin-bound RPA, which, as the primary ssDNA binding and protection factor, represents a very direct and robust marker for replication stress (50) (**Figures 3C, D**). Notably here, in UD-SCC-2 and UPCI-SCC-154, sole Wee1 inhibition resulted in a more moderate induction of RPA signal intensity compared to combined Wee1/Chk1 inhibition, in line with the stronger

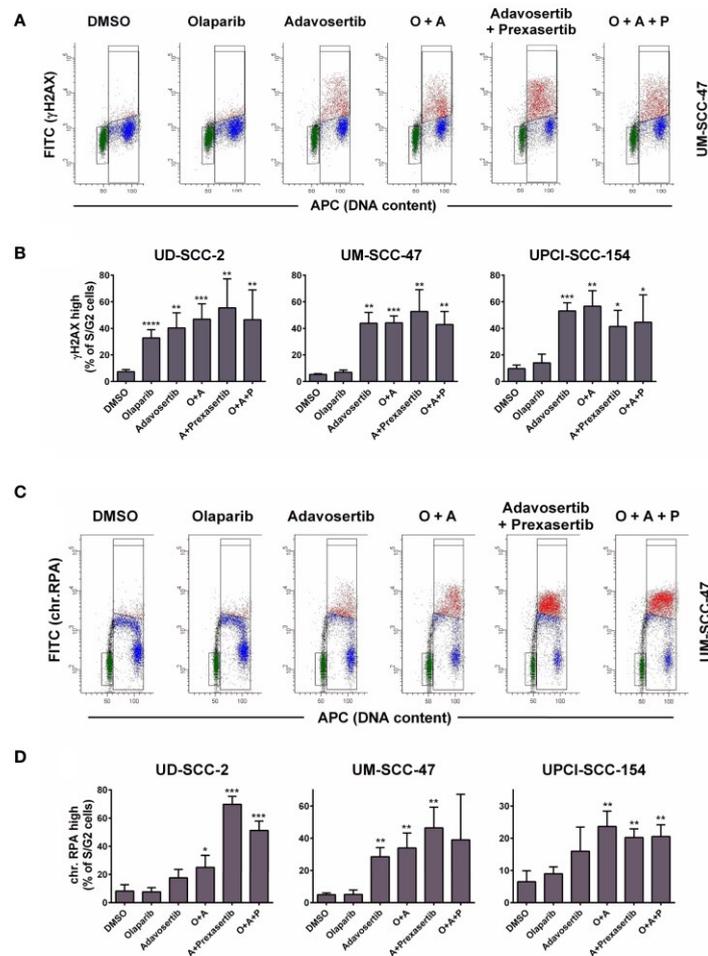


FIGURE 3 | Effect of PARP and intra-S/G2 checkpoint inhibition on γ H2AX and chromatin-bound RPA staining intensity. Cells were treated with inhibitors as indicated for 24 h before fixation, staining, and flow cytometric measurements. In case of RPA staining the cells were pre-extracted before fixation. **(A)** Examples of the flow cytometric measurement of γ H2AX. Gates are set to select cells in G1 (green), in S/G2 (red & blue) or cells in S/G2 with enhanced γ H2AX levels (red). **(B)** Fraction of S/G2 phase cells that demonstrate enhanced γ H2AX levels. **(C)** Examples of the flow cytometric measurement of chromatin-bound RPA, which is highest in the replicative S-phase. Gates are set to select cells in G1 (green), in S/G2 (red and blue) or cells in S with enhanced RPA levels (red). **(D)** Fraction of S/G2 phase cells that demonstrate enhanced RPA staining levels. Asterisks depict significant differences to solvent (DMSO) treatment with *, **, ***, and **** indicating $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively (two-tailed Student's t-test).

accumulation in the S-phase described above (**Supplementary Figure S3B**). Adding the PARP inhibitor did not prominently change the amount of cells positive for γ H2AX or chromatin-bound RPA.

Together these results demonstrate that under Wee1 and especially Wee1/Chk1 inhibition S phase cells will have to repair radiation induced DNA damage under conditions of replication stress and with a severely reduced ability to halt the cell cycle in G2 and therefore without extra time for DNA repair before the critical passage through mitosis. Additional inhibition of PARP did not prominently impact on replication stress or inhibition of G2 arrest according to the endpoints measured.

DSB Repair

The reduced DNA DSB repair capacity of HPV-positive HNSCC cells has been frequently ascribed to a defect in the DNA repair pathway homologous recombination (HR) (28–31) and also a switch towards the error prone alt-EJ pathway has been reported (26, 27). As PARP1 is a key component of the latter (12) and Wee1 has been described as a relevant HR factor (35), we tested the influence of PARP- and Wee1 inhibition on NHEJ and HR using established GFP-based reporter gene constructs stably integrated in HPV-positive UD-SCC-2 and UPCI-SCC-154 cells (51) (**Supplementary Figures S4A, B**). Although the pEJ construct can interrogate classical NHEJ and alt-EJ repair (52),

PARP inhibition did not reduce the rate of measurable NHEJ in either cell line (**Supplementary Figure S4C**). Despite the reported HR defect of HPV-positive HNSCC cells, we had also been able to establish UD-SCC-2 and UPCI-SCC-154 HR reporter cells. Unexpectedly, Wee1 inhibition did not reduce the rate of HR repair as assessed through the pGC reporter construct and the combination with PARP inhibition even increased the rate of GFP-positive cells (**Supplementary Figure S4D**).

In line with the reporter gene assay results, we also did not observe an enhancement of residual 53BP1 nuclear foci as markers of unrepaired DSBs at 24 h after irradiation with 2 Gy under combined PARP/Wee1 inhibition in UD-SCC-2 and UPCI-SCC-154 and only a slight, non-significant increase in UM-SCC-47 (**Figure 4A** and **Supplementary Figure S5A**). We did, however, observe a common phenotype regarding the distribution of foci with respect to the cell cycle phase as determined by geminin co-staining, which marks cells in S and G2 phases (**Figure 4B**). In all cell lines, the average foci number in G1 increased significantly upon combined PARP/Wee1 inhibition, whereas foci in S/G2 phase cells decreased (**Figure 4C** and **Supplementary Figure S5B**). In line with the respective cell cycle data (**Figure 2**), this underscores that under combined inhibition cells with unrepaired DSBs exit G2 arrest and take the critical passage through mitosis despite the

enhanced risk of acute and delayed mitotic cell death. In general, cells with low numbers of residual radiation-induced DSBs are the ones most likely to survive and the fraction of such potentially surviving cells after 2 Gy was decreased in all strains upon dual PARP/Wee1 inhibition, albeit in UD-SCC-2 slightly missing significance ($p = 0.0777$) (**Figure 4D**). Regarding cell cycle, this reduction was observed in the G1-phase in all strains, again underpinning premature mitotic passage (**Figure 4E**). Surprisingly, in UD-SCC-2 the fraction of cells with few residual foci was also significantly reduced in S/G2 phase cells upon dual inhibition, despite the overall decrease in average foci numbers in this fraction (**Figure 4C**).

Radiosensitization

So far while we did not observe clear hints pointing towards enhanced cytotoxicity when adding a PARP inhibitor to intra-S/G2 checkpoint inhibition, radiosensitization through PARP inhibition is clearly established owing to an enhanced induction of replication-induced one-ended DSBs, the inhibition of alt-EJ and further mechanisms (53). Moreover, we had previously observed highly effective radiosensitization in HPV-positive HNSCC cells when combining olaparib with the Chk1 inhibitor PF-00477736 (10). In line with these results, a significant reduction of colonies indicating radiosensitization was now observed upon combined PARP/Wee1 inhibition as

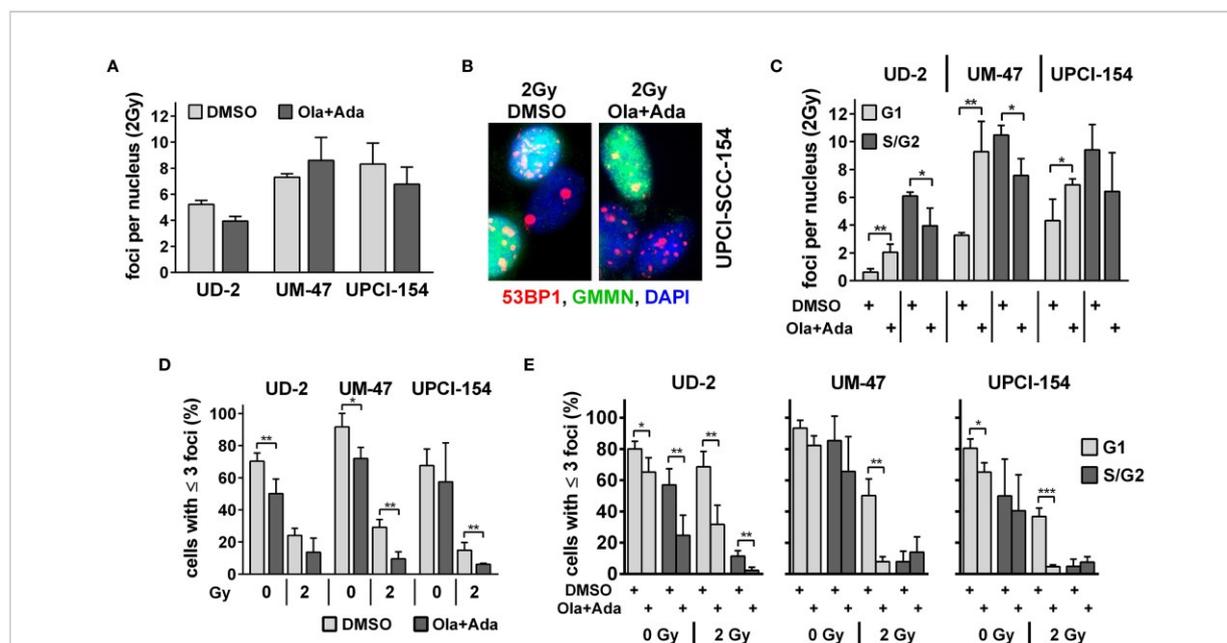


FIGURE 4 | Effect of PARP and Wee1 targeting on DSB repair. **(A)** Quantification of radiation-induced nuclear 53BP1 foci at 24 h after 2 Gy irradiation. Counts were normalized to the DNA content of the respective cell lines as assessed previously (33), foci numbers in non-irradiated controls were subtracted. **(B)** Example of immunofluorescence co-staining of 53BP1 and the S/G2 phase marker geminin (GMMN). **(C)** Quantification of radiation-induced nuclear 53BP1 foci with respect to the cell cycle phase as determined by geminin co-staining. Foci numbers in non-irradiated controls were subtracted. **(D)** Fraction of cells with ≤ 3 53BP1 nuclear foci. **(E)** Fraction of cells with ≤ 3 53BP1 nuclear foci with respect to cell cycle phase as determined by geminin co-staining. Significant changes are indicated with *, **, and *** indicating $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively (two-tailed Student's t-test).

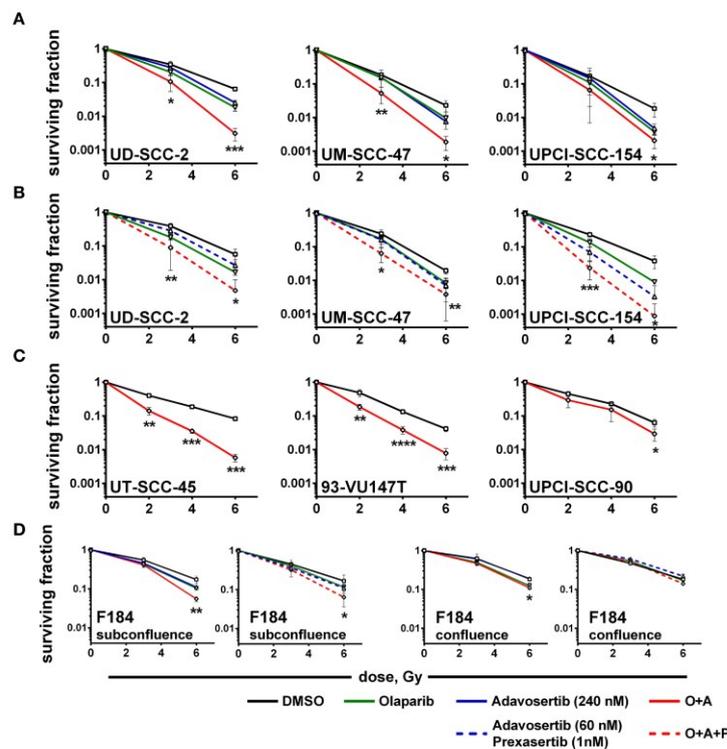


FIGURE 5 | Radiosensitization. Exponentially growing cells were seeded and on the next day treated with inhibitors as indicated and irradiated 2 h thereafter; 24 h later, irradiated cells were seeded in low, defined numbers for colony formation. **(A)** Radiosensitization of HPV-positive HNSCC cells using dual PARP/Wee1 inhibition or **(B)** combined PARP/Wee1/Chk1 inhibition. **(C)** Validation of radiosensitization through combined PARP/Wee1 inhibition using three additional HPV-positive HNSCC cell lines. **(D)** Effect on normal human fibroblasts as an example of normal tissue cells. Significance was assessed for solvent control vs. combined PARP + S/G2 checkpoint inhibition. In case of a statistically significant difference the respective dose points are marked with asterisks with *, **, *** and **** indicating $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, and $p \leq 0.0001$, respectively (two-tailed Student's *t*-test).

compared to single inhibitor usage (Figure 5A). Highly similar results were obtained when replacing the 240 nM adavosertib treatment with 60 nM adavosertib/1 nM prexasertib (Figure 5B). To further estimate whether radiosensitization occurs in a majority of HPV-positive HNSCC cells, we tested dual PARP/Wee1 targeting in three additional strains, all of which were also sensitized, two very effectively and UPCI-SCC-90 to less extent (Figure 5C). To assess tumor specificity, we further tested dual targeting in p53/G1 arrest proficient normal human fibroblasts. In a proliferative state, fibroblasts were radiosensitized by combined inhibition but to a lesser extent than five of the six HPV-positive tumor cell lines. In confluent cultures, the effect of intra-S/G2 checkpoint targeting was completely lost, and radiosensitization was marginal or absent (Figure 5D and Supplementary Table S1). A comparison of the plating efficiency rates of the non-irradiated controls did not reveal a clear differential effect of the dual vs. the triple inhibition approach in HPV-positive HNSCC cells and virtually no reduction of survival in the normal fibroblasts (Supplementary Figure S6).

Nucleoside Supplementation Counteracts Radiosensitization Through Wee1 but Not PARP/Wee1 Inhibition

We finally wanted to estimate to what extent the induction of replication stress may contribute to the profound radiosensitization upon combined treatment. As a shortage in nucleotides contributes to replication stress upon intra-S/G2 checkpoint inhibition, it can partly be compensated by external addition of nucleosides (18, 54). To test the effect in our cells, we analyzed γ H2AX levels in S-phase cells at 4 h after combined PARP/Wee1 inhibition, a time point corresponding to 2 h post irradiation in the colony formation assays when DSB repair would be highly active. We found γ H2AX levels to be induced by combined inhibition in S phase cells and partly suppressed by nucleoside supplementation. A substantial degree of induction and normalization was observed in UD-SCC-2 and UPCI-SCC-154 cells (Figures 6A, B). Despite these similarities, nucleoside supplementation did not influence radiation sensitivity in UPCI-SCC-154 but in UD-SCC-2 induced a quite clear trend towards radioresistance in the PARP/Wee1-inhibited samples (6 Gy: $p = 0.0862$). Unexpectedly, resistance was induced in the

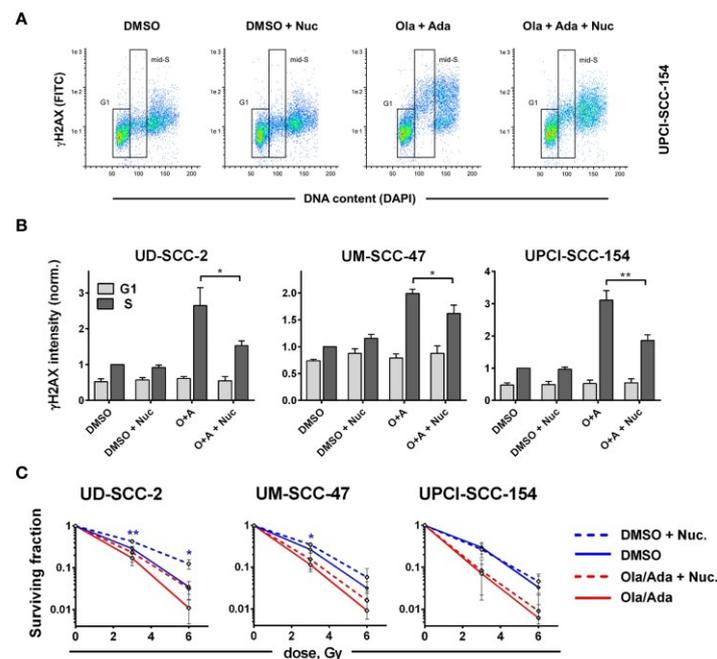


FIGURE 6 | Cell line-dependent induction of radioresistance through nucleoside supplementation. Exponentially growing cells were treated with or without the combination of olaparib and adavosertib and with or without external nucleosides as indicated. **(A)** Example of gating for γ H2AX intensity measurement in G1 and mid-S phase cells as assessed by DAPI co-staining. The cells were fixed after 4 h of treatment and analyzed for γ H2AX induction by flow cytometry. **(B)** Bars depict the average median γ H2AX staining intensity of cells in G1 and mid-S phase of the respective experiments. Asterisks mark statistically significant differences upon nucleoside supplementation. Values were normalized to the intensity of DMSO-treated mid-S phase cells of the respective experiments. Asterisks mark statistically significant differences upon nucleoside supplementation. **(C)** Two hours after addition of inhibitors \pm nucleosides the cells were irradiated and after further 24 h seeded for colony formation without addition of inhibitors. Asterisks mark statistically significant differences in survival upon nucleoside supplementation, color indicates solvent controls or inhibitor treatment. Differences between DMSO treatment and dual inhibition without nucleoside supplementation (solid lines) were significant for all cell lines (not indicated). Significant changes are indicated with * and ** indicating $p \leq 0.05$ and $p \leq 0.01$, respectively (two-tailed Student's t-test).

solvent-treated controls to a very similar extent reaching significance for the 6 Gy dose point (**Figure 6C**). In comparison, sole Wee1 inhibition induced a similar increase in γ H2AX levels, and nucleoside supplementation resulted in a pertinent normalization. In contrast to the situation under combined targeting, nucleoside supplementation counteracted adavosertib-mediated radiosensitization in UM-SCC-47 and UPCI-SCC-154, with no or little effect in the respective solvent-treated controls. Solely in UD-SCC-2, nucleoside supplementation exerted a similar effect on adavosertib and control treated cells (**Supplementary Figure S7**). So while these data strongly suggest that replication stress caused by nucleotide shortage can play a prominent role in the radiosensitization under sole Wee1 inhibition, they question a meaningful role for the radiosensitization under combined PARP/Wee1 inhibition in our cells.

The cause for radioresistance under nucleoside supplementation in solvent treated UD-SCC-2 cells currently remains elusive. In a set of pilot experiments, nucleosides increased the fraction of G1 at the cost of S phase cells in UD-SCC-2 and reduced their proliferation speed (**Supplementary Figures S8A, B**). Also especially in UD-SCC-2, the radiation-induced G2 arrest was diminished upon

nucleoside supplementation, suggesting that fewer residual DSBs were present to trigger the G2 cell cycle checkpoint (**Supplementary Figure S8A**). Finally, analyses of residual DSBs under nucleoside supplementation *via* 53BP1 nuclear foci in UD-SCC-2 cells demonstrated an increase in the fraction of cells with few (≤ 3) foci after irradiation, in line with radioresistance induction. The effect was present and significant in both cells that were or were not actively replicating at the time of irradiation (**Supplementary Figure S8C**). Further analyses will be necessary to clarify this intriguing finding of radioresistance through nucleoside supplementation in otherwise unperturbed cells.

DISCUSSION

Inhibition of Wee1 by adavosertib was recently described as a highly effective single-agent treatment for HPV-positive HNSCC dependent on FOXM1 activation (55) and single agent radio- and chemosensitization through PARP, as well as through intra-S/G2 checkpoint inhibition, which was repeatedly demonstrated in HPV-positive HNSCC models (10, 21–23, 25, 30, 31, 56, 57).

In this study we demonstrate a highly effective radiosensitization of HPV-positive HNSCC cells using dual inhibition of PARP and the S/G2 cell cycle checkpoint in five and moderate radiosensitization in one out of six cell lines tested. A similar result has recently been independently described for the HPV-positive strain UPCI-SCC-154 (24). Here it was suggested that the combination of PARP plus Chk1 inhibition is more effective in HPV-positive HNSCC cells, whereas the combination of PARP plus Wee1 inhibition is more effective in HPV-negative ones but the estimation was based on only one cell line per group. For this particular HPV-positive strain, we have indeed also observed an exceptionally strong radiosensitization when including a Chk1 inhibitor (Figure 5). The data are also in line with previous findings of strong radiosensitization using sole Chk1 and combined Chk1/Wee1 inhibition, but again the effect was only specific for UPCI-SCC-154 rather than for HPV-positive cells in general (22). Of note, this strain was also an outlier in the response to the particular Chk1 inhibitor PF-004776, but here demonstrated non-responsiveness for various endpoints, which further suggests irregularities (21). Effective radiosensitization through combined inhibition of PARP and the intra-S/G2 cell cycle checkpoint has also been described for other entities and for different approaches of checkpoint targeting, such as Chk1 or ATR inhibition (10, 58–60). The combination of PARP/Wee1 inhibition was previously tested in lung and pancreatic cancer cells with similarities but also some differences to our findings in HPV-positive HNSCC cells (61, 62). Contrasting these studies we did not observe inhibition of HR upon Wee1 inhibition in plasmid reconstruction assays and we neither observed a reduction of NHEJ upon PARP inhibition despite the reported enhanced usage of alt-EJ in HPV-positive HNSCC (26, 27). Furthermore, while replication stress was clearly evident upon intra-S/G2 checkpoint inhibition, we could not confirm an important role for the radiosensitization under combined inhibition, since for example in UD-SCC-2 targeting the intra-S/G2 checkpoint by combined Wee1/Chk1 inhibition induced replication stress more effectively than sole Wee1 inhibition but radiosensitization was highly similar (Figures 2, 3, 5). And while external nucleoside supplementation succeeded in partly relieving replication stress, it either failed to reduce radiosensitization (UPCI-SCC-154) or induced radioresistance in the solvent-treated controls to a similar extent as under combined PARP/Wee1 inhibition (UD-SCC-2) (Figure 6). In contrast, nucleoside supplementation demonstrated a pertinent reduction in replication stress and effectively counteracted radiosensitization upon sole Wee1 inhibition in two out of three cell lines tested (Supplementary Figure S7), suggesting additional mechanisms and a more robust radiosensitization upon combined inhibition. These findings are actually in line with previous reports, where the addition of nucleosides also counteracted radiosensitization under sole Wee1 (62, 63) but not under combined Wee1/PARP inhibition (62). Interestingly, nucleoside supplementation had also induced radioresistance in solvent treated samples in one of three (hepatocellular) carcinoma cell lines tested, while in NSCLC cells no results for the solvent treated controls were presented

(62, 63). While clearly not the focus of this manuscript, our observation of profoundly enhanced radioresistance upon nucleoside supplementation in solvent-treated UD-SCC-2 cells is interesting and warrants future mechanistic investigations.

A puzzling finding of our study is the slight reduction in the overall number of 53BP1 foci upon combined treatment (Figure 4). In general, an enhancement in DNA damage in S/G2 phase upon PARP inhibition is very well established (13, 14, 64) and, accordingly, we observed an increase in G2 arrested cells and enhanced γ H2AX levels in G2 phase cells upon PARP inhibition and moderate radiosensitization here and previously (Figures 2, 5 and Supplementary Figure S2) (10). A possible explanation, in line with the cell cycle data and the shift in foci number from G2 to G1 phase cells (Figures 2, 4) may be that overriding the otherwise long lasting G2 checkpoint can result in immediate mitotic catastrophe and cell elimination, preferentially of those cells with high damage and foci levels that would otherwise reside long enough in G2 to be scored. In line with this theory, the proportion of irradiated G2 phase cells with ≥ 20 53BP1 foci decreased in UD-SCC-2 and UPCI-SCC-154 upon combined inhibition (data not shown). Importantly, the fraction of cells with very low foci numbers was reduced upon dual inhibition in all cell lines tested. Overall, our results point towards a mechanism for radiosensitization driven by the abrogation of the, in HPV-positive HNSCC cells extensive, G2 cell cycle arrest in combination with the induction of additional DNA damage in S/G2 through PARP inhibition. While differences may exist in detail, the described effectiveness in different entities and by application of various checkpoint inhibitors clearly point towards a very robust radiosensitization of proliferating tumor cells by this combinatorial approach (37, 38). In contrast, normal fibroblasts, representing p53-proficient normal tissue cells, were only modestly affected in our study (Figure 5D), which indicates a fair degree of tumor specificity, especially given that many normal tissues do not or only slowly proliferate.

From the translational view, HPV-positive HNSCC may represent an especially promising entity for radiosensitization through molecular targeting. Patients possess a favorable prognosis and therefore targeting agents may not be added to concomitant chemotherapy (CT) but could rather replace CT and this should reduce, instead of increase, the risk of severe systemic side effects. Safe de-intensification of treatment is already the common goal in clinical trials for HPV-positive HNSCC. A major drawback, however, was the reported inferiority of cetuximab compared to cisplatin despite maintaining full dose radiotherapy in two phase 3 trials (65). These studies clearly highlight the need for effectiveness and thorough preclinical evaluation of molecular targeting approaches despite the overall favorable prognosis. In the frame of recent clinical data on de-intensification, promising initial results were obtained for reducing radiation dose in definitive chemoradiation and after induction chemotherapy (ICT) (3, 5, 7). In the frame of the latter, effective targeting may also be an alternative to adjuvant chemotherapy after ICT in the frame of risk-adapted, de-intensified radiotherapy and may evade potential chemoresistance mechanisms selected for or acquired during ICT. All inhibitors used in this study are

already being tested in clinical trials in combination with radiotherapy in HNSCC (66, 67) (NCT02555644, NCT01758731, NCT02308072, NCT02585973). Olaparib is clinically approved in other entities, and the combination of adavosertib and radiotherapy (plus gemcitabine) was recently reported to yield promising results in pancreatic cancer (68). Moreover, combined treatment with olaparib and adavosertib as well as with prexasertib is also being clinically tested in a number of entities (NCT02576444, NCT02511795, NCT03579316, NCT03330847), albeit so far not in combination with radiotherapy. From our point of view, the clinical stage of the inhibitors available and the preclinical evidence provided in this study clearly warrant subsequent *in vivo* experiments as a next step towards a possible clinical exploration of the described approaches in the frame of de-intensification trials in HPV-positive HNSCC.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

REFERENCES

- Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tân PF, et al. Human Papillomavirus and Survival of Patients With Oropharyngeal Cancer. *N Engl J Med* (2010) 363(1):24–35. doi: 10.1056/NEJMoa0912217
- Sedghizadeh PP, Billington WD, Paxton D, Ebeed R, Mahabady S, Clark GT, et al. Is P16-Positive Oropharyngeal Squamous Cell Carcinoma Associated With Favorable Prognosis? A Systematic Review and Meta-Analysis. *Oral Oncol* (2016) 54:15–27. doi: 10.1016/j.oraloncology.2016.01.002
- Chen AM, Felix C, Wang PC, Hsu S, Basehart V, Garst J, et al. Reduced-Dose Radiotherapy for Human Papillomavirus-Associated Squamous-Cell Carcinoma of the Oropharynx: A Single-Arm, Phase 2 Study. *Lancet Oncol* (2017) 18(6):803–11. doi: 10.1016/S1470-2045(17)30246-2
- Chera BS, Amdur RJ, Tepper JE, Tan X, Weiss J, Grilley-Olson JE, et al. Mature Results of a Prospective Study of Deintensified Chemoradiotherapy for Low-Risk Human Papillomavirus-Associated Oropharyngeal Squamous Cell Carcinoma. *Cancer* (2018) 124(11):2347–54. doi: 10.1002/cncr.31338
- Patel RR, Ludmir EB, Augustyn A, Zaorsky NG, Lehrer EJ, Ryali R, et al. De-Intensification of Therapy in Human Papillomavirus Associated Oropharyngeal Cancer: A Systematic Review of Prospective Trials. *Oral Oncol* (2020) 103:104608. doi: 10.1016/j.oraloncology.2020.104608
- Takemoto N, Seo Y, Nakahara S, Yoshioka Y, Teshima T, Fujii T, et al. Radiation Therapy Alone for Human Papillomavirus-Related Squamous Cell Carcinoma of the Oropharynx: A Single-Arm, Phase 2 Study. *Int J Radiat Oncol Biol Phys* (2020) 110(2):403–11. doi: 10.1016/j.ijrobp.2020.12.025
- Yom SS, Torres-Saavedra P, Caudell JJ, Waldron JN, Gillison ML, Xia P, et al. Reduced-Dose Radiation Therapy for HPV-Associated Oropharyngeal Carcinoma (NRG Oncology Hn002). *J Clin Oncol* (2021) 39(9):956–65. doi: 10.1200/JCO.20.03128
- Gillison ML, Trotti AM, Harris J, Eisbruch A, Harari PM, Adelstein DJ, et al. Radiotherapy Plus Cetuximab or Cisplatin in Human Papillomavirus-Positive Oropharyngeal Cancer (NRG Oncology RTOG 1016): A Randomised, Multicentre, non-Inferiority Trial. *Lancet* (2019) 393(10166):40–50. doi: 10.1016/S0140-6736(18)32779-X
- Mehanna H, Robinson M, Hartley A, Kong A, Foran B, Fulton-Lieuw T, et al. Radiotherapy Plus Cisplatin or Cetuximab in Low-Risk Human Papillomavirus-Positive Oropharyngeal Cancer (De-ESCaLaTE HPV): An

AUTHOR CONTRIBUTIONS

KH, TB, AO, SC, and FG conducted experiments under the supervision of TR. KH, TB, AO, SC, FG, and TR analyzed the data. MK, KR, CP, CB, NS and TR contributed conception and design of the study; AO, NS, KR, and TR wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2021.683688/full#supplementary-material>

- Open-Label Randomised Controlled Phase 3 Trial. *Lancet* (2019) 393(10166):51–60. doi: 10.1016/S0140-6736(18)32752-1
- Guster JD, Weissleder SV, Busch CJ, Kriegs M, Petersen C, Knecht R, et al. The Inhibition of PARP But Not EGFR Results in the Radiosensitization of HPV/p16-Positive HNSCC Cell Lines. *Radiother Oncol* (2014) 113(3):345–51. doi: 10.1016/j.radonc.2014.10.011
- Satoh MS, Lindahl T. Role of Poly(ADP-Ribose) Formation in DNA Repair. *Nature* (1992) 356(6367):356–8. doi: 10.1038/356356a0
- Wang M, Wu W, Wu W, Rosidi B, Zhang L, Wang H, et al. PARP-1 and Ku Compete for Repair of DNA Double Strand Breaks by Distinct NHEJ Pathways. *Nucleic Acids Res* (2006) 34(21):6170–82. doi: 10.1093/nar/gkl840
- Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, et al. Specific Killing of BRCA2-Deficient Tumours With Inhibitors of Poly(ADP-Ribose) Polymerase. *Nature* (2005) 434(7035):913–7. doi: 10.1038/nature03443
- Mateo J, Lord CJ, Serra V, Tutt A, Balmaña J, Castroviejo-Bermejo M, et al. A Decade of Clinical Development of PARP Inhibitors in Perspective. *Ann Oncol* (2019) 30(9):1437–47. doi: 10.1093/annonc/mdz192
- Jannetti SA, Zeglis BM, Zalutsky MR, Reiner T. Poly(ADP-Ribose) Polymerase (PARP) Inhibitors and Radiation Therapy. *Front Pharmacol* (2020) 11:170. doi: 10.3389/fphar.2020.00170
- Hauge S, Eek Mariampillai A, Rørdland GE, Bay LTE, Landsverk HB, Syljuåsen RG. Expanding Roles of Cell Cycle Checkpoint Inhibitors in Radiation Oncology. *Int J Radiat Biol* (2021) p:1–10. doi: 10.1080/09553002.2021.1913529
- Parker LL, Piwnicka-Worms H. Inactivation of the P34cdc2-Cyclin B Complex by the Human WEE1 Tyrosine Kinase. *Science* (1992) 257(5078):1955–7. doi: 10.1126/science.1384126
- Beck H, Nähse-Kumpf V, Larsen MS, O'Hanlon KA, Patzke S, Holmberg C, et al. Cyclin-Dependent Kinase Suppression by WEE1 Kinase Protects the Genome Through Control of Replication Initiation and Nucleotide Consumption. *Mol Cell Biol* (2012) 32(20):4226–36. doi: 10.1128/MCB.00412-12
- Brangan TB, Kozono D, Schade AE, Deraska P, Rivas HG, Sambel L, et al. MMB-FOXMI-Driven Premature Mitosis is Required for CHK1 Inhibitor Sensitivity. *Cell Rep* (2021) 34(9):108808. doi: 10.1016/j.celrep.2021.108808
- Sorensen CS, Syljuåsen RG. Safeguarding Genome Integrity: The Checkpoint Kinases ATR, CHK1 and WEE1 Restrain CDK Activity During Normal DNA Replication. *Nucleic Acids Res* (2012) 40(2):477–86. doi: 10.1093/nar/gkr697

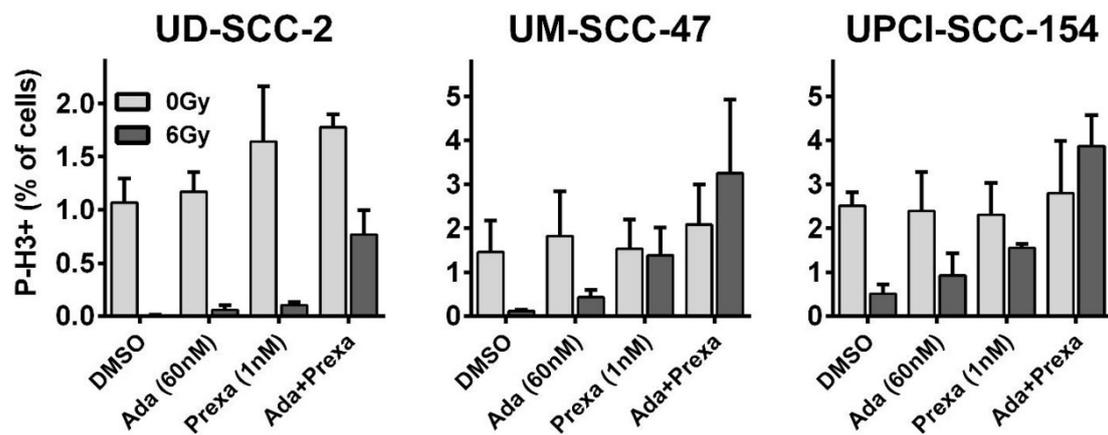
21. Busch CJ, Kriegs M, Laban S, Tribius S, Knecht R, Petersen C, et al. HPV-Positive HNSCC Cell Lines But Not Primary Human Fibroblasts are Radiosensitized by the Inhibition of Chk1. *Radiother Oncol* (2013) 108 (3):495–9. doi: 10.1016/j.radonc.2013.06.035
22. Busch CJ, Kröger MS, Jensen J, Kriegs M, Gatzemeier F, Petersen C, et al. G2-Checkpoint Targeting and Radiosensitization of HPV/p16-Positive HNSCC Cells Through the Inhibition of Chk1 and Wee1. *Radiother Oncol* (2016) 122 (2):260–6. doi: 10.1016/j.radonc.2016.11.017
23. Dok R, Glorieux M, Bamps M, Nuyts S. Effect of ATR Inhibition in RT Response of HPV-Negative and HPV-Positive Head and Neck Cancers. *Int J Mol Sci* (2021) 22(4). doi: 10.3390/ijms22041504
24. Molkentine JM, Molkentine DP, Bridges KA, Xie T, Yang L, Sheth A, et al. Targeting DNA Damage Response in Head and Neck Cancers Through Abrogation of Cell Cycle Checkpoints. *Int J Radiat Biol* (2020) p:1–8. doi: 10.1080/09553002.2020.1730014
25. Nickson CM, Moori P, Carter RJ, Rubbi CP, Parsons JL. Misregulation of DNA Damage Repair Pathways in HPV-Positive Head and Neck Squamous Cell Carcinoma Contributes to Cellular Radiosensitivity. *Oncotarget* (2017) 8 (18):29963–75. doi: 10.18632/oncotarget.16265
26. Leeman JE, Li Y, Bell A, Hussain SS, Majumdar R, Rong-Mullins X, et al. Human Papillomavirus 16 Promotes Microhomology-Mediated End-Joining. *Proc Natl Acad Sci USA* (2019) 116(43):21573–9. doi: 10.1073/pnas.1906120116
27. Liu Q, Ma L, Jones T, Palomero L, Pujana MA, Martinez-Ruiz H. Subjugation of TGFbeta Signaling by Human Papilloma Virus in Head and Neck Squamous Cell Carcinoma Shifts DNA Repair From Homologous Recombination to Alternative End Joining. *Clin Cancer Res* (2018) 24 (23):6001–14. doi: 10.1158/1078-0432.CCR-18-1346
28. Dok R, Kalev P, Van Limbergen EJ, Asbagh LA, Vázquez I, Hauben E, et al. P16ink4a Impairs Homologous Recombination-Mediated DNA Repair in Human Papillomavirus-Positive Head and Neck Tumors. *Cancer Res* (2014) 74(6):1739–51. doi: 10.1158/0008-5472.CAN-13-2479
29. Wang L, Zhang P, Molkentine DP, Chen C, Molkentine JM, Piao H, et al. TRIP12 as a Mediator of Human Papillomavirus/P16-Related Radiation Enhancement Effects. *Oncogene* (2016). doi: 10.1158/1538-7445.AM2016-1661
30. Weaver AN, Cooper TS, Rodriguez M, Trummell HQ, Bonner JA, Rosenthal EL, et al. DNA Double Strand Break Repair Defect and Sensitivity to Poly ADP-Ribose Polymerase (PARP) Inhibition in Human Papillomavirus 16-Positive Head and Neck Squamous Cell Carcinoma. *Oncotarget* (2015) 6 (29):26995–7007. doi: 10.18632/oncotarget.4863
31. Ziemann F, Seltzams S, Dreflke K, Preising S, Arenz A, Subtil FSB, et al. Roscovitine Strongly Enhances the Effect of Olaparib on Radiosensitivity for HPV Neg. But Not for HPV Pos. HNSCC Cell Lines. *Oncotarget* (2017) 8 (62):105170–83. doi: 10.18632/oncotarget.22005
32. Kimple RJ, Smith MA, Blitzer GC, Torres AD, Martin JA, Yang RZ, et al. Enhanced Radiation Sensitivity in HPV-Positive Head and Neck Cancer. *Cancer Res* (2013) 73 (15):4791–800. doi: 10.1158/0008-5472.CAN-13-0587
33. Rieckmann T, Tribius S, Grob TJ, Meyer F, Busch CJ, Petersen C, et al. HNSCC Cell Lines Positive for HPV and P16 Possess Higher Cellular Radiosensitivity Due to an Impaired DSB Repair Capacity. *Radiother Oncol* (2013) 107(2):242–6. doi: 10.1016/j.radonc.2013.03.013
34. Buisson R, Niraj J, Rodrigue A, Ho CK, Kreuzer J, Foo TK, et al. Coupling of Homologous Recombination and the Checkpoint by ATR. *Mol Cell* (2017) 65 (2):336–46. doi: 10.1016/j.molcel.2016.12.007
35. Krajewska M, Heijink AM, Bisselink YJ, Seinstra RI, Silljé HH, de Vries EG, et al. Forced Activation of Cdk1 via Wee1 Inhibition Impairs Homologous Recombination. *Oncogene* (2013) 32(24):3001–8. doi: 10.1038/onc.2012.296
36. Sorensen CS, Hansen LT, Dziegielewska J, Syljuåsen RG, Lundin C, Bartek J, et al. The Cell-Cycle Checkpoint Kinase Chk1 is Required for Mammalian Homologous Recombination Repair. *Nat Cell Biol* (2005) 7(2):195–201. doi: 10.1038/ncb1212
37. Hintelmann K, Kriegs M, Rothkamm K, Rieckmann T. Improving the Efficacy of Tumor Radiosensitization Through Combined Molecular Targeting. *Front Oncol* (2020) 10:1260. doi: 10.3389/fonc.2020.01260
38. Parsels LA, Engelke CG, Parsels J, Flanagan SA, Zhang Q, Tanska D, et al. Combinatorial Efficacy of Olaparib With Radiation and ATR Inhibitor Requires PARP1 Protein in Homologous Recombination-Proficient Pancreatic Cancer. *Mol Cancer Ther* (2021) 20(2):263–73. doi: 10.1158/1535-7163.MCT-20-0365
39. Busch CJ, Becker B, Kriegs M, Gatzemeier F, Krüger K, Möckelmann N, et al. Similar Cisplatin Sensitivity of HPV-Positive and -Negative HNSCC Cell Lines. *Oncotarget* (2016) 7(24):35832–42. doi: 10.18632/oncotarget.9028
40. Mansour WY, Schumacher S, Roskopf R, Rhein T, Schmidt-Petersen F, Gatzemeier F, et al. Hierarchy of Nonhomologous End-Joining, Single-Strand Annealing and Gene Conversion at Site-Directed DNA Double-Strand Breaks. *Nucleic Acids Res* (2008) 36(12):4088–98. doi: 10.1093/nar/gkn347
41. Tominaga Y, Li C, Wang RH, Deng CX. Murine Wee1 Plays a Critical Role in Cell Cycle Regulation and Pre-Implantation Stages of Embryonic Development. *Int J Biol Sci* (2006) 2(4):161–70. doi: 10.7150/ijbs.2.161
42. De Vos M, Schreiber V, Dantzer F. The Diverse Roles and Clinical Relevance of PARPs in DNA Damage Repair: Current State of the Art. *Biochem Pharmacol* (2012) 84(2):137–46. doi: 10.1016/j.bcp.2012.03.018
43. Murai J, Pommier Y. PARP Trapping Beyond Homologous Recombination and Platinum Sensitivity in Cancers. *Annu Rev Cancer Biol* (2019) 3:131–50. doi: 10.1146/annurev-cancerbio-030518-055914
44. Ray Chaudhuri A, Nussenzweig A. The Multifaceted Roles of PARP1 in DNA Repair and Chromatin Remodelling. *Nat Rev Mol Cell Biol* (2017) 18(10):610–21. doi: 10.1038/nrm.2017.53
45. Forment JV, Blasius M, Guerini I, Jackson SP. Structure-Specific DNA Endonuclease Mus81/Eme1 Generates DNA Damage Caused by Chk1 Inactivation. *PLoS One* (2011) 6(8):e23517. doi: 10.1371/journal.pone.0023517
46. Bryant HE, Petermann E, Schultz N, Jemth AS, Loseva O, Issaeva N, et al. PARP is Activated at Stalled Forks to Mediate Mre11-Dependent Replication Restart and Recombination. *EMBO J* (2009) 28(17):2601–15. doi: 10.1038/emboj.2009.206
47. Ying S, Hamdy FC, Helleday T. Mre11-Dependent Degradation of Stalled DNA Replication Forks is Prevented by BRCA2 and PARP1. *Cancer Res* (2012) 72(11):2814–21. doi: 10.1158/0008-5472.CAN-11-3417
48. Haynes B, Murai J, Lee JM. Restored Replication Fork Stabilization, a Mechanism of PARP Inhibitor Resistance, can be Overcome by Cell Cycle Checkpoint Inhibition. *Cancer Treat Rev* (2018) 71:1–7. doi: 10.1016/j.ctrv.2018.09.003
49. Min W, Bruhn C, Grigaravicius P, Zhou ZW, Li F, Krüger A, et al. Poly(ADP-Ribose) Binding to Chk1 at Stalled Replication Forks is Required for S-Phase Checkpoint Activation. *Nat Commun* (2013) 4:2993. doi: 10.1038/ncomms3993
50. Toledo LI, Altmeyer M, Rask MB, Lukas C, Larsen DH, Povlsen LK, et al. ATR Prohibits Replication Catastrophe by Preventing Global Exhaustion of RPA. *Cell* (2013) 155(5):1088–103. doi: 10.1016/j.cell.2013.10.043
51. Schotz U, Balzer V, Brandt FW, Ziemann F, Subtil FSB, Rieckmann T, et al. Dual PI3K/mTOR Inhibitor NVP-BE225 Enhances Radiosensitivity of Head and Neck Squamous Cell Carcinoma (HNSCC) Cell Lines Due to Suppressed Double-Strand Break (DSB) Repair by Non-Homologous End Joining. *Cancers (Basel)* (2020) 12(2). doi: 10.3390/cancers12020467
52. Mansour WY, Rhein T, Dahm-Daphi J. The Alternative End-Joining Pathway for Repair of DNA Double-Strand Breaks Requires PARP1 But is Not Dependent Upon Microhomologies. *Nucleic Acids Res* (2010) 38(18):6065–77. doi: 10.1093/nar/gkq387
53. Lesueur P, Chevalier F, Austry JB, Waissi W, Burckel H, Noël G, et al. Poly(ADP-Ribose)-Polymerase Inhibitors as Radiosensitizers: A Systematic Review of Pre-Clinical and Clinical Human Studies. *Oncotarget* (2017) 8 (40):69105–24. doi: 10.18632/oncotarget.19079
54. Parsels LA, Karnak D, Parsels JD, Zhang Q, Vélez-Padilla J, Reichert ZR, et al. PARP1 Trapping and DNA Replication Stress Enhance Radiosensitization With Combined WEE1 and PARP Inhibitors. *Mol Cancer Res* (2018) 16 (2):222–32. doi: 10.1158/1541-7786.MCR-17-0455
55. Diab A, Gem H, Swanger J, Kim HY, Smith K, Zou G, et al. FOXM1 Drives HPV+ HNSCC Sensitivity to WEE1 Inhibition. *Proc Natl Acad Sci USA* (2020) 117 (45):28287–96. doi: 10.1073/pnas.2013921117
56. Tanaka N, Patel AA, Wang J, Frederick MJ, Kalu NN, Zhao M, et al. Wee-1 Kinase Inhibition Sensitizes High-Risk HPV+ HNSCC to Apoptosis Accompanied by Downregulation of MCL-1 and XIAP Antiapoptotic Proteins. *Clin Cancer Res* (2015) 21(21):4831–44. doi: 10.1158/1078-0432.CCR-15-0279
57. Zeng L, Nikolaev A, Xing C, Della Manna DL, Yang ES. CHK1/2 Inhibitor Prexasertib Suppresses NOTCH Signaling and Enhances Cytotoxicity of Cisplatin and Radiation in Head and Neck Squamous Cell Carcinoma. *Mol Cancer Ther* (2020) 19(6):1279–88. doi: 10.1158/1535-7163.MCT-19-0946

58. Ahmed SU, Carruthers R, Gilmour L, Yildirim S, Watts C, Chalmers AJ. Selective Inhibition of Parallel DNA Damage Response Pathways Optimizes Radiosensitization of Glioblastoma Stem-Like Cells. *Cancer Res* (2015) 75 (20):4416–28. doi: 10.1158/0008-5472.CAN-14-3790
59. Carruthers RD, Ahmed SU, Ramachandran S, Strathdee K, Kurian KM, Hedley A, et al. Replication Stress Drives Constitutive Activation of the DNA Damage Response and Radioresistance in Glioblastoma Stem-Like Cells. *Cancer Res* (2018) 78(17):5060–71. doi: 10.1158/0008-5472.CAN-18-0569
60. Vance S, Liu E, Zhao L, Parsels JD, Parsels LA, Brown JL, et al. Selective Radiosensitization of P53 Mutant Pancreatic Cancer Cells by Combined Inhibition of Chk1 and PARP1. *Cell Cycle* (2011) 10(24):4321–9. doi: 10.4161/cc.10.24.18661
61. Karnak D, Engelke CG, Parsels LA, Kausar T, Wei D, Robertson JR, et al. Combined Inhibition of Wee1 and PARP1/2 for Radiosensitization in Pancreatic Cancer. *Clin Cancer Res* (2014) 20(19):5085–96. doi: 10.1158/1078-0432.CCR-14-1038
62. Parsels LA, Karnak D, Parsels JD, Zhang Q, Vélez-Padilla J, Reichert ZR, et al. The Contribution of DNA Replication Stress Marked by High-Intensity, Pan-Nuclear GammaH2ax Staining to Chemosensitization by CHK1 and WEE1 Inhibitors. *Cell Cycle* (2018) 17(9):1076–86. doi: 10.1080/15384101.2018.1475827
63. Cuneo KC, Morgan MA, Davis MA, Parcels LA, Parcels J, Karnak D, et al. Wee1 Kinase Inhibitor AZD1775 Radiosensitizes Hepatocellular Carcinoma Regardless of TP53 Mutational Status Through Induction of Replication Stress. *Int J Radiat Oncol Biol Phys* (2016) 95(2):782–90. doi: 10.1016/j.ijrobp.2016.01.028
64. Wu W, Wang M, Wu W, Singh SK, Mussfeldt T, Iliakis G. Repair of Radiation Induced DNA Double Strand Breaks by Backup NHEJ is Enhanced in G2. *DNA Repair (Amst)* (2008) 7(2):329–38. doi: 10.1016/j.dnarep.2007.11.008
65. Rieckmann T, Kriegs M. The Failure of Cetuximab-Based De-Intensified Regimes for HPV-Positive OPSCC: A Radiobiologists Perspective. *Clin Transl Radiat Oncol* (2019) 17:47–50. doi: 10.1016/j.ctro.2019.05.003
66. Kong A, Good J, Kirkham A, Savage J, Mant R, Llewellyn L, et al. Phase I Trial of WEE1 Inhibition With Chemotherapy and Radiotherapy as Adjuvant Treatment, and a Window of Opportunity Trial With Cisplatin in Patients With Head and Neck Cancer: The WISTERIA Trial Protocol. *BMJ Open* (2020) 10(3):e033009. doi: 10.1136/bmjopen-2019-033009
67. Yang ES, Deutsch E, Mehmet A, Fayette J, Tao Y, Nabell L, et al. A Phase 1b Trial of Prexasertib in Combination With Chemoradiation in Patients With Locally Advanced Head and Neck Squamous Cell Carcinoma. *Radiation Oncol* (2021) 157:203–9. doi: 10.1016/j.radonc.2021.01.032
68. Cuneo KC, Morgan MA, Sahai V, Schipper MJ, Parsels LA, Parsels JD, et al. Dose Escalation Trial of the Wee1 Inhibitor Adavosertib (AZD1775) in Combination With Gemcitabine and Radiation for Patients With Locally Advanced Pancreatic Cancer. *J Clin Oncol* (2019) 37(29):2643–50. doi: 10.1200/JCO.19.00730

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

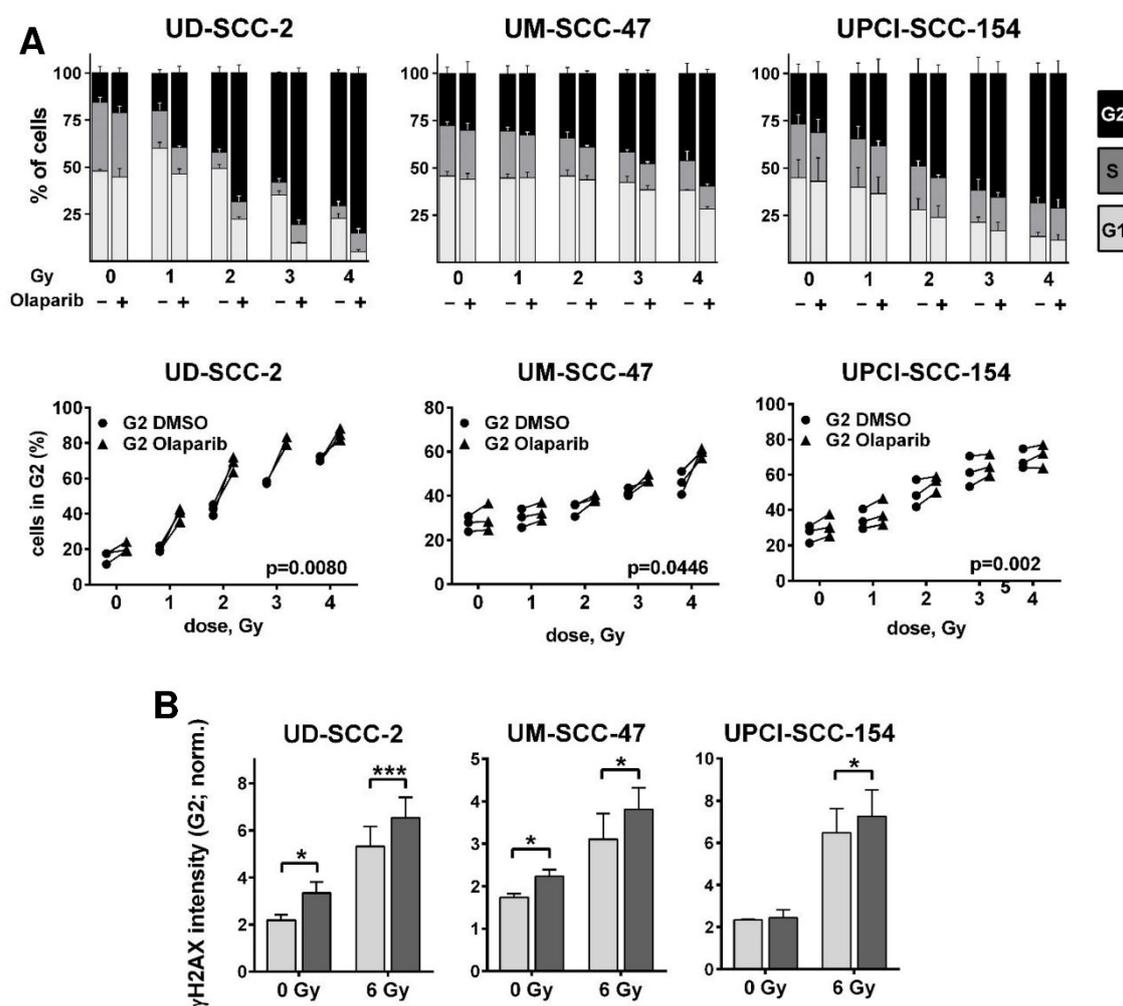
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Supplementary Material

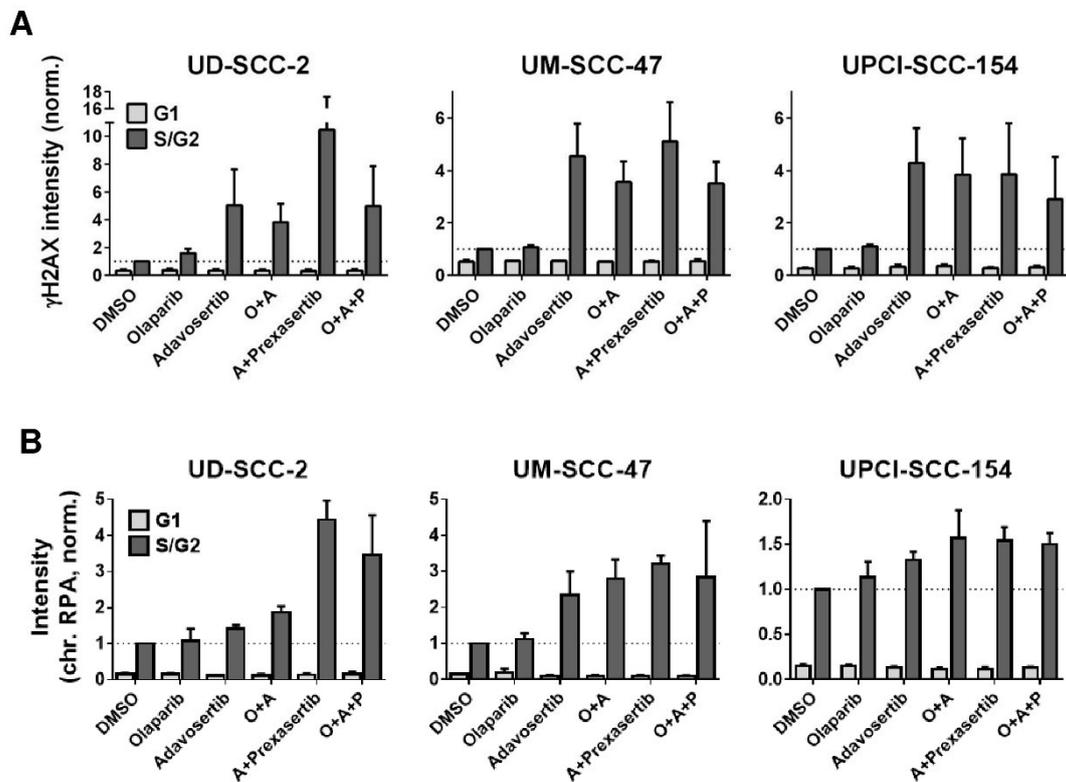


Supplementary Figure S1. Effectiveness of G2 arrest agrogation by low dose Wee1 and Chk1 or combined inhibition. Fraction of mitotic cells. Exponentially growing cells were treated for 2 hours with the inhibitors as indicated, before irradiation with 0 or 6 Gy. Eight h after irradiation cells were fixed and stained for the mitosis marker phospho-histone H3 (P-H3) to assess the number of mitotic cells. Enhanced mitotic fractions upon dual treatment and irradiation may be explained by aberrant mitotic divisions of cells entering mitosis with high levels of DNA damage.

Supplementary Material

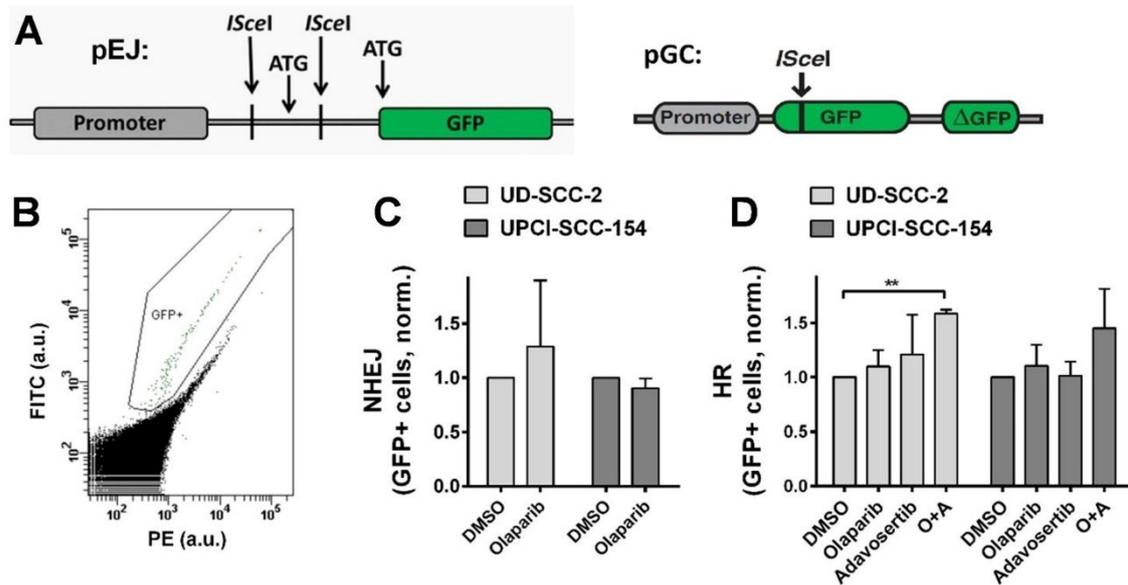


Supplementary Figure S2. Enhanced fractions of G2 phase cells and enhanced γ H2AX levels in G2 arrested cells after PARP inhibition. (A) Top: Exponentially growing cells were treated and irradiated as indicated. Twenty-four h after irradiation the cells were fixed and the cell cycle distribution assessed by DAPI staining and flow cytometry. Bottom: While the increase of G2 phase cells upon olaparib treatment in UM-SCC-47 and UPCI-SCC-154 was subtle, the difference was seen in virtually every experiment and reached significance when including all doses (paired two-tailed Student's t-test). (B) Exponentially growing cells were treated with olaparib and after 2 h irradiated \pm 6 Gy. Twenty-four h later, when the majority of irradiated cells is arrested in G2, the cells were fixed and DNA damage levels assessed by flow cytometric measurement of γ H2AX levels plus DNA content (DAPI). Olaparib induced a significant increase in DNA damage levels in all strains in G2 arrested cells 24 h after 6 Gy irradiation and in 2/3 cell lines without irradiation (paired two-tailed Student's t-test). Depicted are the average values of the median γ H2AX intensities of G2 phase cells, individual values were normalized to the γ H2AX level of the non-irradiated G1 phase populations of the respective experiments.



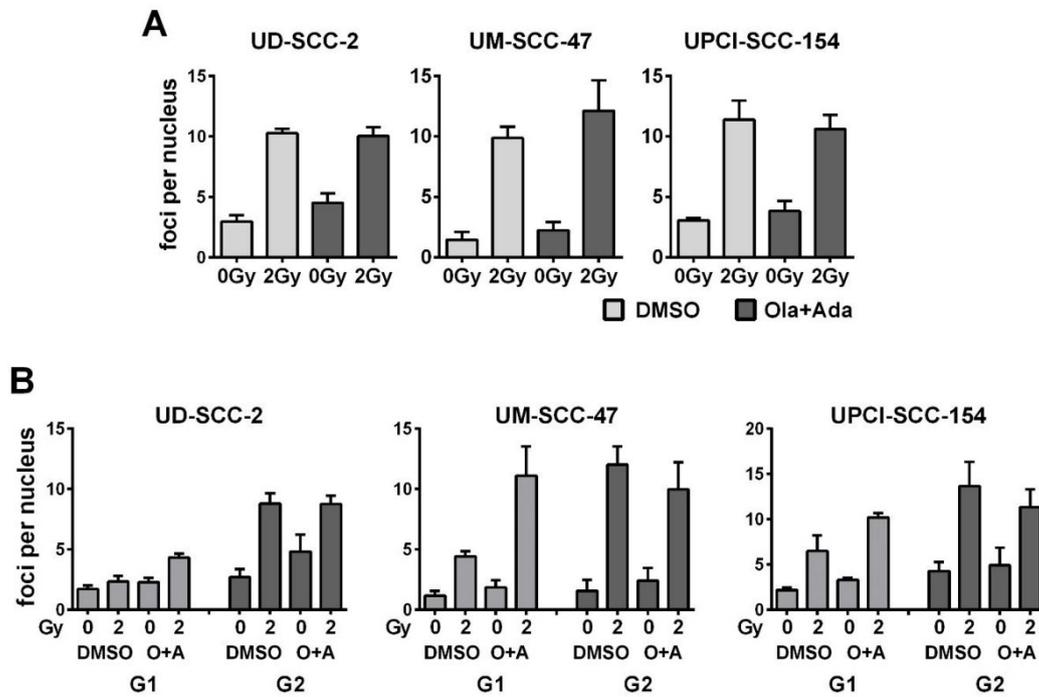
Supplementary Figure S3. Effect of PARP- and intra-S/G2 checkpoint inhibition on γ H2AX and chromatin-bound RPA staining intensity. Data are based on the same experiments as for Figure 3. Graphs depict the staining intensities of G1 and S/G2-phase cells (green or blue + red populations in Figure 3A,C). (A) γ H2AX. (B) Chromatin-bound RPA. Values were normalized to the solvent control (DMSO) value of the S/G2 population in each experiment.

Supplementary Material



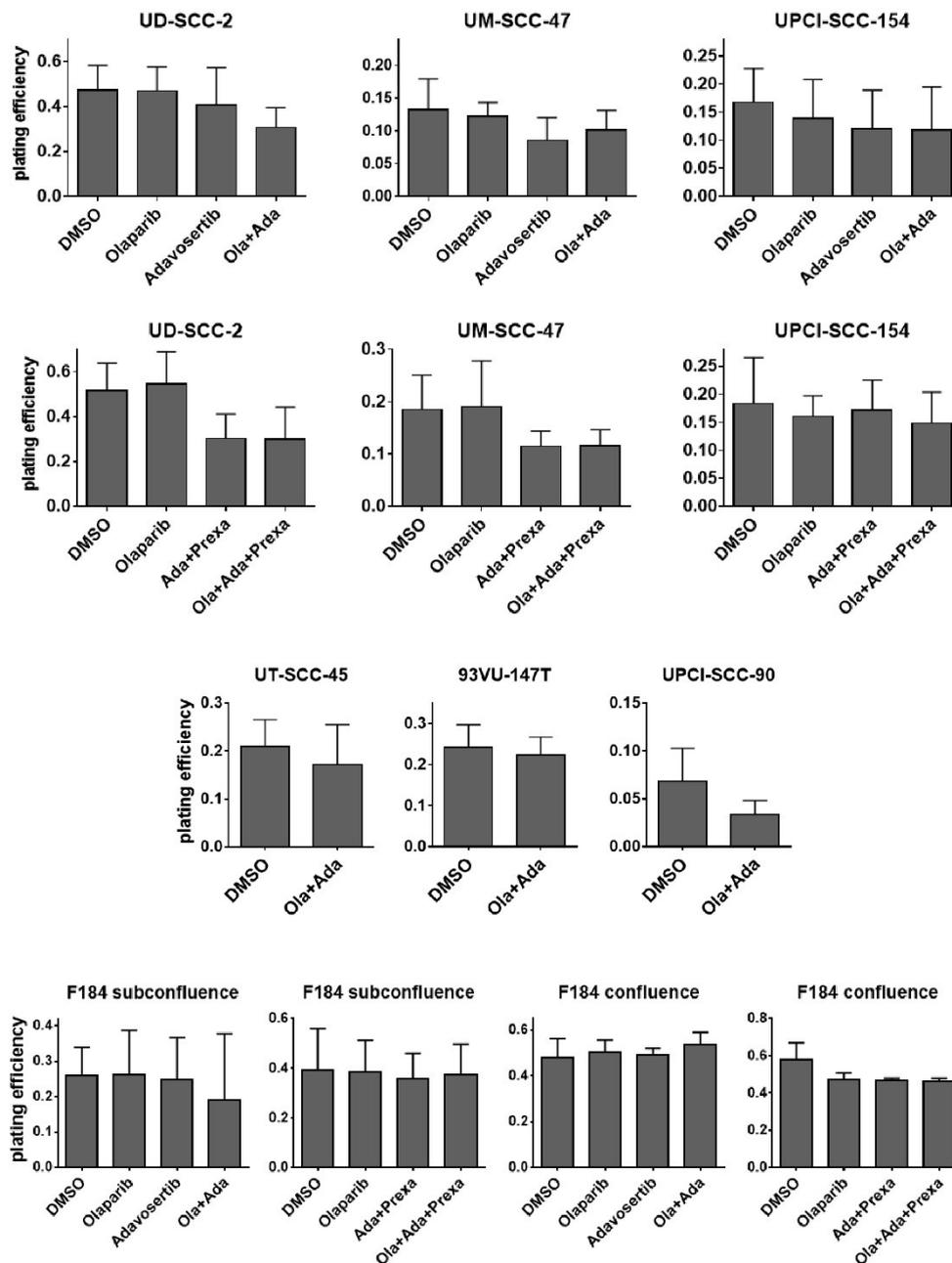
Supplementary Figure S4. Effect of PARP and Wee1 targeting on DSB repair pathways as assessed by GFP-based reporter gene assays. Reporter strains with stable integration of the respective repair constructs were transfected with an I-SceI expression vector. Six hours and once more 24 h after transfection the medium was exchanged and inhibitors added; 48h after transfection the fraction of GFP-positive cells was assessed by flow cytometry. **(A)** Scheme of the respective NHEJ and HR reporter constructs. **(B)** Example of flow cytometric assessment. **(C)** Effect of PARP inhibition on NHEJ efficacy and **(D)** of PARP and Wee1 inhibition on HR efficacy. Results were generally normalized to the respective transfection efficiencies and DMSO controls of the individual experiments.

Significant changes are indicated with ** indicating $p < 0.01$, respectively (two-tailed Student's t-test).

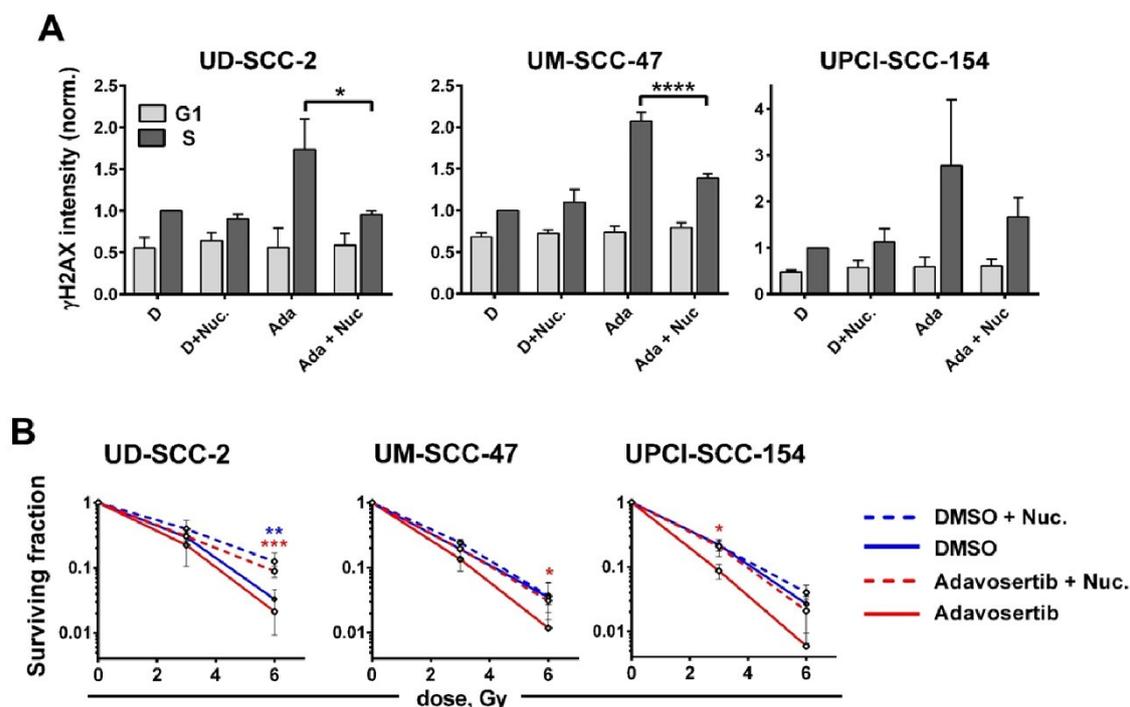


Supplementary Figure S5. Radiation-induced 53BP1 foci. Quantification of nuclear 53BP1 foci at 24 h after 0 & 2 Gy irradiation. Graphs are based on the same experiments as presented in Figure 4, for which the non-irradiated background levels of each experiment (0 Gy) had been subtracted. (A) Radiation-induced 53BP1 foci in all cell cycle phases. (B) Radiation-induced 53BP1 foci in G1 vs. S/G2 phase as assessed by geminin co-staining.

Supplementary Material

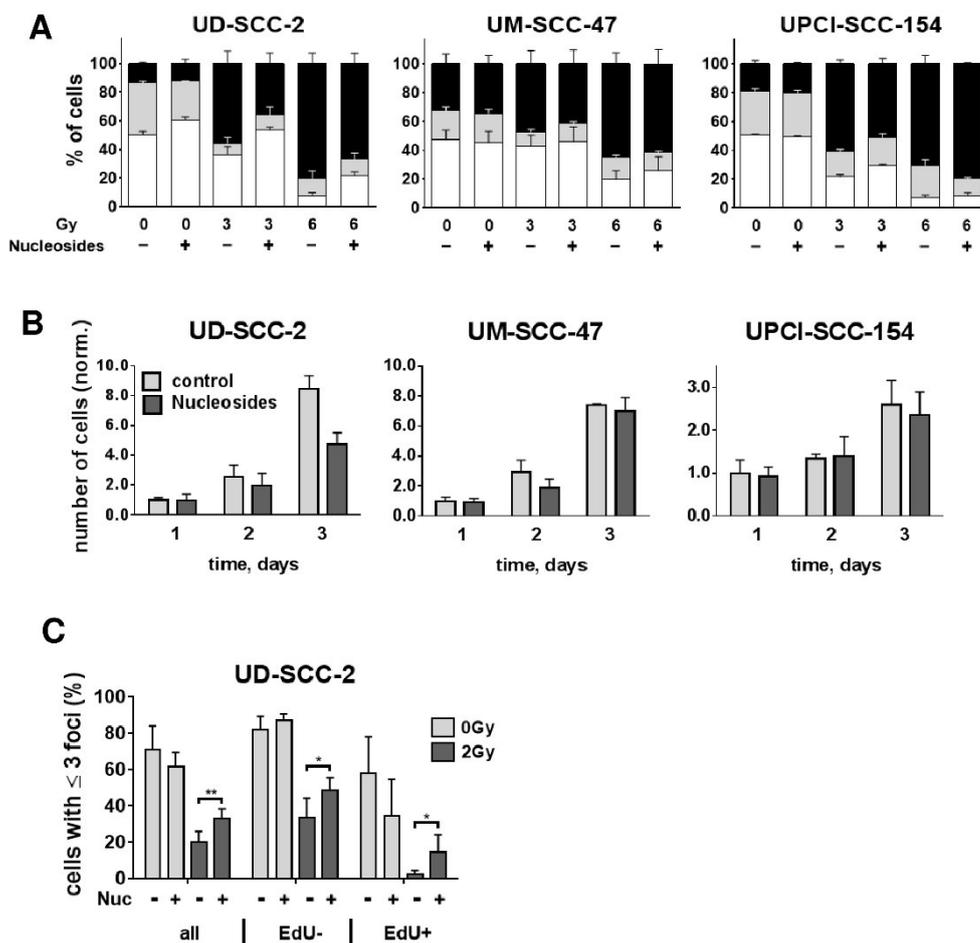


Supplementary Figure S6. Plating efficiencies of colony formation assays. Exponentially growing cells were treated with inhibitors as indicated and after 26 h seeded in defined, low numbers without inhibitors for colony formation. Graphs represent the non-normalized surviving fractions of the 0 Gy samples from the experiments shown in Figure 5.



Supplementary Figure S7. Effect of nucleoside supplementation on radiosensitization through sole Wee1 inhibition. (A) Cells were treated with adavosertib \pm nucleoside supplementation as indicated. After 4 h of treatment the cells were fixed and analyzed for γ H2AX induction by flow cytometry. Bars depict the average median γ H2AX staining intensity of cells in G1 and mid-S phase as assessed by DAPI co-staining. Values were normalized to the intensity of DMSO treated mid-S phase cells of the respective experiments. Asterisks mark statistically significant differences upon nucleoside supplementation. (B) Cells were irradiated 2 h after addition of adavosertib \pm nucleosides and after further 24 h seeded for colony formation without addition of inhibitor or nucleosides. Asterisks mark statistically significant differences in survival upon nucleoside supplementation, color indicates solvent controls or inhibitor treatment. Differences between DMSO and adavosertib treatment reached significance only in UPCI-SCC-154 cells (not indicated). Significant changes are indicated with *, ** and *** indicating $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively (two-tailed Student's t-test).

Supplementary Material

**Supplementary Figure S8. Induction of radioresistance through nucleoside supplementation.**

(A) Exponentially growing cells were treated with nucleosides and inhibitors as indicated and were irradiated after 2 h. Twenty-four hours later the cells were fixed and the cell cycle distribution assessed. (B) Exponentially growing cells were seeded and after 4 h treated with or without nucleosides and the resulting numbers of control treated cells were assessed at the indicated time points. Data are normalized to the resulting cell number after 24 h (day 1). (C) Exponentially growing cells were pulse labelled with EdU for 30 min, washed 4 times, supplemented with nucleosides and after further 30 min irradiated as indicated. Cells were finally fixed 24 h after irradiation and stained for 53BP1, DAPI and EdU. Cells showing few foci (≤ 3) are rare in the fraction of UD-SCC-2 cells irradiated in/immediately after S phase (EdU+). Nucleoside supplementation increases the proportion of cells with few foci in the fraction of cells irradiated in (EdU+) but also of those cells irradiated outside (EdU-) of S phase. Asterisks in (C) depict significant differences upon nucleoside supplementation with * and ** indicating $p < 0.05$, $p < 0.01$, respectively (two-tailed Student's t-test).

Cell line /treatment	DEF (25%)	CKEF (3Gy)
UD-SCC-2 / Ola+Ada	1.92	3.23
UM-SCC-47 / Ola+Ada	1.64	3.50
UPCI-SCC-154 / Ola+Ada	1.50	2.62
UT-SCC-45 / Ola+Ada	2.24	3.92
93VU-147T / Ola+Ada	1.85	3.01
UPCI-SCC-90 / Ola+Ada	1.51	1.5
F184 /Ola+Ada, subconf.	1.29	1.34
F184 /Ola+Ada, conf.	1.22	1.35
UD-SCC-2 / Ola+Ada+Prexa	2.15	4.35
UM-SCC-47 / Ola+Ada+Prexa	1.95	3.85
UPCI-SCC-154 / Ola+Ada+Prexa	2.54	9.85
F184 /Ola+Ada+Prexa, subconfl.	1.38	1.41
F184 /Ola+Ada+Prexa, confl.	1.06	0.89

Supplementary Table S1. Dose enhancement at 25% survival and cell kill enhancement at 3 Gy.

The Dose Enhancement Factor (DEF) at 25% survival was directly assessed from the dose response curves depicted in Figure 5 using GraphPad Prism. The Cell Kill Enhancement Factor (CKEF) at 3 Gy was either calculated directly from the 3 Gy dose points of the dose response curves (see Figure 5) or, in case a 3 Gy dose point was lacking (UT-SCC-45, 93VU-147T and UPCI-SCC-90), was assessed by interpolation using GraphPad Prism.

Zusammenfassende Darstellung der Publikation

Einleitung

Maligne Neoplasien des Kopf-Hals-Bereichs umfassen Lokalisationen wie die Mundhöhle und Lippen, die Speicheldrüsen, den Nasopharynx sowie Nase und Nasennebenhöhlen, Oropharynx, Hypopharynx sowie Larynx. Überwiegend handelt es sich dabei um Plattenepithelkarzinome, im Englischen *head and neck squamous-cell carcinoma*, kurz HNSCC. Sie gehören zu den sechst häufigsten Krebserkrankungen weltweit (Ferlay et al. 2019).

Als wichtigste Risikofaktoren sind Tabak- und Alkoholabusus, sowie chronische Infektionen mit dem Humanen Papillomvirus (HPV) zu nennen (Wienecke and Kraywinkel 2019). Letzteres konnte insbesondere für die Gruppe der Oropharynxkarzinome (*oropharyngeal squamous cell carcinomas/ OPSCC*) der Tonsillen von Gaumen und Zungengrund, gezeigt werden. Epidemiologische Studien zeigen einen Rückgang des Tabakkonsums und der klassischen tabak- und alkoholassoziierten HNSCC, wie Mundhöhlen-, Larynx- und Hypopharynxkarzinome. Bei einem zeitgleichen Anstieg der OPSCC wird daher ein kausaler Zusammenhang zwischen dem zeitlichen Trend in dieser Subgruppe und dem HPV angenommen (Simard et al. 2014, Wittekindt et al. 2019).

HPV ist ein DNA-Virus mit mehr als 200 bekannten Subtypen, der Epithelien der Haut und Schleimhaut befällt. Bei einem Großteil der HPV-positiven HNSCC findet sich eine Infektion mit dem *high-risk* Subtyp 16 (Michaud et al. 2014). Es wird angenommen, dass die Onkoproteine dieser Viren, insbesondere die Genprodukte von E6 und E7, über Veränderungen in der Zellzyklusregulation, der Apoptose und Proliferation zur Kanzerogenese führen. E6 führt unter anderem zur Degradation des Tumorsuppressors p53, wodurch HPV-positive HNSCC, in denen p53 meist in wildtyp-Sequenz vorliegt, funktional trotzdem p53 defizient sind. E7 interferiert, neben zahlreichen weiteren Interaktionen, mit dem Tumorsuppressor Retinoblastom-Protein (pRb) und führt ebenfalls zu seiner Degradation (Tumban 2019, Pan et al. 2018, Gonzalez et al. 2001).

Patienten mit HPV-positiven OPSCC besitzen eine bessere Prognose als die HPV-negativen, was mit einem besseren Therapieansprechen auf Radio- und Radiochemotherapie in Verbindung gebracht wird (Albers et al. 2017, Wagner et al. 2017, Wang et al. 2015). Um dies zu berücksichtigen, wurde in der achten Version der TNM-Klassifikation der UICC für HPV-positive OPSCC eine eigene Klassifikation eingeführt. Für den Nachweis einer biologisch aktiven HPV-Infektion wird dabei der Expressionsstatus des HPV-Surrogatmarkers P16^{INK4a} (p16, auch CDK-Inhibitor 2A) herangezogen (Bonner et al. 2017, Lassen et al. 2009). P16 ist ein Protein der zelluläre Stressantwort, das den Übergang in die S-Phase reguliert über Inhibition der Cyclin-abhängigen Kinase 4/6, welche wiederum das RB-Protein phosphoryliert. Durch die Inaktivierung von RB durch E7 kommt wiederum zur Überexpression von p16 in HPV-positiven HNSCC (Rahimi 2020). Hierbei sei jedoch erwähnt, dass eine p16 Überexpression kein direkter Nachweis einer HPV-Infektion ist und die Notwendigkeit direkter Nachweisverfahren wie PCR oder *in-situ* Hybridisierung Bestandteil offener Diskussionen sind (Augustin et al. 2020).

In der Therapie der HNSCC ist die Radio- oder Radiochemotherapie ein wichtiger Bestandteil, insbesondere bei inoperablen oder lokal fortgeschrittenen Tumoren. Besteht die Indikation für eine simultane Chemotherapie, ist diese meist platinbasiert (Ott et al. 2018). Die Nebenwirkungen und Spätfolgen der aggressiven, multimodalen Therapie sind erheblich, weshalb eine Übertherapie der Patienten mit guter Prognose vermieden werden muss.

Neben Deeskalationsstrategien, die sich mit einer Verringerung der Strahlendosis bei

ausgewählten Patienten befassen, sowie risikoadaptierter adjuvanter Therapie nach einer Induktionstherapie oder minimalinvasiver Chirurgie, ist ein weiterer Ansatzpunkt das molekulare Targeting (Tribius et al. 2011, Rosenberg and Vokes 2021). Aufgrund jedoch nicht abschließend geklärter Evidenz bezüglich der Identifizierung der geeigneten Subgruppe und Umsetzung der Therapiedeeskalation, finden alle genannten Deeskalationsstrategien bisher nur im Rahmen klinischer Studien statt und konnten noch kein Bestandteil der Leitlinien werden.

Einzig etablierte Zielstruktur in Bezug auf molekulares Targeting ist in diesem Kontext der *epidermal growth factor receptor* (EGFR), mit dem überwiegend eingesetzten Cetuximab als monoklonaler anti-EGFR-Antikörper (Adelstein et al. 2019). Allerdings stehen HPV-positive HNSCC aufgrund ihrer erheblich durch die Onkoproteine E6/E7 getriebenen Onkogenese weniger in einer Abhängigkeit von aberranten Wachstumsfaktorkaskaden und exprimieren weniger EGFR als HPV-negative HNSCC (Hong et al. 2010, Markovic and Chung 2012). Tatsächlich zeigte sich die simultane Gabe von Cetuximab zur Radiotherapie bei lokal fortgeschrittenen HPV-positiven OPSCC als der Standardtherapie mit Hochdosis-Cisplatin signifikant unterlegen und es konnte auch keine verringerte Langzeittoxizität gezeigt werden (RTOG1016, De-ESCALaTE, TROG 12.01) (Gillison et al. 2019, Mehanna et al. 2019). Eine spezifische Ausrichtung des Targetings auf molekulare Charakteristika der Zielentität erscheint somit als notwendig, um aus dem wachsenden Spektrum an Substanzen vielversprechende Ansätze zu identifizieren.

Vorarbeiten, unter anderem des Labors für Strahlenbiologie des UKE, haben als eine mögliche zentrale Schwachstelle HPV-positiver HNSCC eine erhöhte zelluläre Strahlensensitivität basierend auf einer reduzierten DNA-Doppelstrangbruch (DSB)-Reparaturkapazität gezeigt. Dies ist zudem assoziiert mit einem ausgeprägten und besonders langen G2-Arrest (Rieckmann et al. 2013, Kimple et al. 2013) (Abbildung 1).

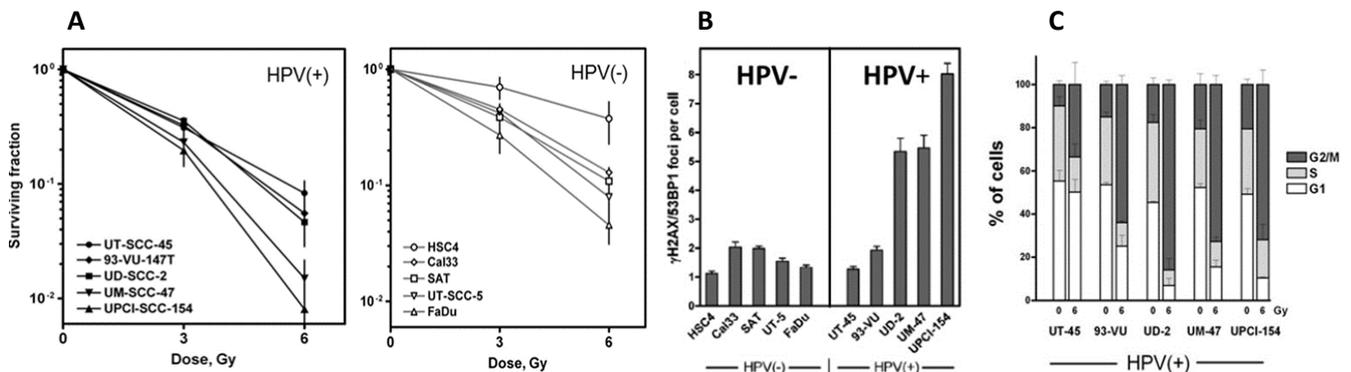


Abbildung 1. Strahlenbiologie HPV-positiver HNSCC Zelllinien (Rieckmann et al. 2013).

(A) Zelluläre Strahlensensibilität im Koloniebildungsassay fünf HPV/p16-positiver und fünf HPV-negativer Zelllinien nach Bestrahlung. (B) Nach Bestrahlung (2Gy) zeigt sich eine hohe Anzahl an residuellen DSBs (24h) in den HPV+ Zelllinien. (C) Bei der Untersuchung des Zellzyklus konnte ein verlängerter strahleninduzierter G2-Arrest festgestellt werden.

Inhibition des strahleninduzierten G2-Arrestes

Nach Bestrahlung sind die Reparatur- und p53 defizienten HPV-positiven HNSCC Zellen auf einen suffizienten G2-Arrest angewiesen, um DNA-Schäden vor dem Eintritt in die Mitose zu beheben. Eine Schlüsselrolle spielt dabei die Cyclin-abhängige Kinase 1 (Cdk1), welche im aktiven Zustand die G2/M-Transition ermöglicht. Die Kinase Cdc25 aktiviert die Cdk1 durch Dephosphorylierung inhibitorischer Phosphatgruppen. Wird ein DNA-Schaden u.a. durch die ATR-Kinase

registriert, aktiviert diese die Checkpointkinase1 (Chk1), welche wiederum Cdc25 inhibiert und somit den Übergang in die Mitose stoppt und der Zelle Zeit zur DNA-Reparatur verschafft (van Harten et al. 2019, Goto et al. 2012) (Abbildung 2).

In Arbeiten von Busch et al. im Labor für Strahlenbiologie des UKE konnte gezeigt werden, dass durch Inhibition von Chk1 dieser durch DNA-Schäden vermittelte Zellzyklusarrest unterdrückt wird (Busch et al. 2013, Busch et al. 2017).

Die Kinase Wee1 inaktiviert Cdk1 durch Phosphorylierung, womit Chk1 und Wee1 negative Regulatoren der G2/M-Transition darstellen. Studien deuten darauf hin, dass Wee1 weiteren Funktionen innen hat, wie zum Beispiel regulatorische Funktionen bei der DNA-Replikation in der S-Phase, bei der Stabilisierung von Replikationsgabeln, sowie bei der Homologen Rekombination (Matheson et al. 2016). Unter anderem wurde gezeigt, dass die Inhibition von Wee1, neben einer Inhibition des G2-Arrestes, durch unkontrolliertes Feuern von Replikationsursprüngen zu Replikationsstress durch Nukleotiddepletion führt (Beck et al. 2012, Elbæk et al. 2020).

Da jedoch auch gezeigt wurde, dass die Inhibition von Wee1 zu einer Aktivierung von Chk1 führt, die der Wee1-Inhibition potentiell entgegenwirkt, wurde von Busch et al. außerdem eine kombinierte Inhibition beider Targets untersucht, welche bereits bei sehr niedrigen Einzelkonzentrationen eine Radiosensibilisierung bewirkte (Busch et al. 2017). Der synergistische Effekt einer kombinierten Wee1 und Chk1 Inhibition ohne Bestrahlung konnte zudem bereits in mehreren Entitäten in einer ganzen Reihe von Veröffentlichungen gezeigt werden (Carrassa et al. 2012, Chaudhuri et al. 2014, Chila et al. 2015, Davies et al. 2011, Guertin et al. 2012, Qi et al. 2014, Russell et al. 2013, Magnussen et al. 2015, Mak et al. 2015).

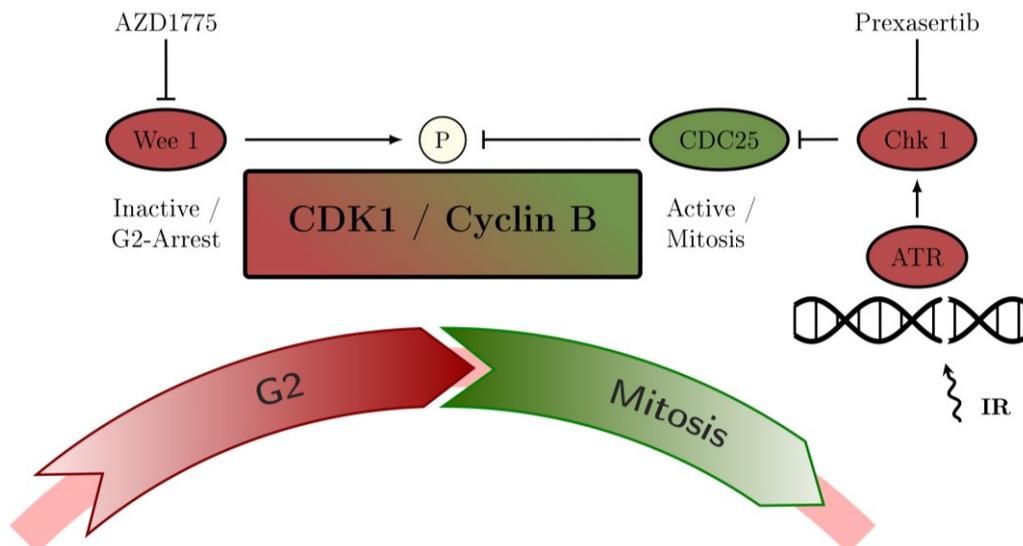


Abbildung 2.

Regulation der G2/M-Transition durch Wee1 und Chk1 über den Phosphorylierungsstatus des CDK1/Cyclin B-Komplex. Adavosertib (AZD1775) und Prexasertib begünstigen über Wee1, bzw. Chk1-Inhibition die unphosphorylierte, aktive Form der CDK1 und somit den G2-M-Übergang.

Die Mitotische Katastrophe ist ein Vorgang, der zur Inaktivierung (Verlust der langfristigen Teilungsfähigkeit), Zelltod oder Seneszenz von Zellen führt und die Folge von nicht oder falsch reparierten DNA-DSBs und daraus resultierenden letalen Chromosomenaberrationen nach

genotoxischer Schädigung, zum Beispiel nach Bestrahlung, ist. Zum Teil durchlaufen die Zellen noch einige Zellteilungen, bevor der Verlust des genetischen Materials aus der aberranten Mitose zur Inaktivierung bzw. zum Zelltod führt (Galluzzi et al. 2018, Vakifahmetoglu et al. 2008, Koch et al. 2021). Ob eine Zelle nach der Bestrahlung mit unreparierten DSBs in die Mitose eintritt, hängt somit stark vom G2-Arrest ab, welcher der Zelle Zeit für die Reparatur gibt. Bei zu vielen Schäden oder zu geringer DSB-Reparatureffizienz ist dieser Zeitgewinn oftmals nicht ausreichend, um die Zellen vor Inaktivierung/Zelltod zu bewahren. Insbesondere ist zu beachten, dass p53-profiziente Normalgewebezellen auch in der G1-Phase arretieren können und somit weniger auf den G2-Arrest angewiesen sind.

Inhibition von PARP1

Ein gut etablierter Ansatz mit den zellulären DNA-Reparaturmechanismen zu interferieren, ist die Inhibition der Poly(ADP-ribose)-Polymerase1 (PARP1) welche die ADP-Ribosylierung von Chromatinproteinen katalysiert. PARP1 ist entscheidend an den Reparaturwegen „Basenexzisionsreparatur (BER)“ (Ronson et al. 2018) und „alternatives Endjoining (altEJ)“ (Audebert et al. 2004, Wang et al. 2019) beteiligt, so dass PARP-Inhibition replikationsabhängige und unabhängige DSB begünstigt. Neben der Inhibition der PARP-abhängigen Reparatur ist ein wichtiger Faktor das sog. *PARP-trapping*, die Unfähigkeit sich von geschädigter DNA durch Eigenparilyierung wieder zu lösen, so dass durch an der Bruchstelle gebundenes PARP zusätzliche Hindernisse für Transkription und vor allem Replikation entstehen (Murai et al. 2012, Hopkins et al. 2015).

Es ist zu vermuten, dass die Reparatur-defizienten HPV-positiven HNSCC weniger Möglichkeiten haben die PARP-Inhibition auszugleichen. Dabei ist zu beachten, dass ionisierende Bestrahlung neben einigen DSBs auch viele Basen- und Einzelstrangschäden verursacht (Nickson et al. 2017). Weiter sind in Anlehnung an das Prinzip der synthetic lethality insbesondere Zellen mit einer defizienten Homologen Rekombination (HR) vulnerabel für eine PARP1-Inhibition (Bryant et al. 2005) und es gibt Studien, die eine HR-Defizienz für HPV-positive HNSCC beschreiben (Dok et al. 2014, Liu et al. 2018).

Im Labor für Strahlenbiologie des UKE beobachtete Guster et al. nach Olaparib Behandlung einen verlängerten strahleninduzierten G2-Arrest bei den HPV-positiven Zelllinien, was auf einen erhöhten Schadenslevel hindeutet. In allen untersuchten (5/5) HPV-positiven Zelllinien konnte dazu passend eine Radiosensibilisierung durch Olaparib gezeigt werden (Guster et al. 2014).

Die kombinierte Inhibition von PARP und dem intra-S/G2-Zellzykluskontrollpunkt

In der hier vorgestellten Arbeit wurde die kombinierte Inhibition von PARP mit Olaparib und des intra-S/G2-Zellzykluskontrollpunktes mit klinisch relevanten Substanzen zur Wee1- und Chk1-Inhibition als Targetingansatz zur effektiven Radiosensibilisierung getestet. Die Rationale ist, dass die Inhibition der Targets Wee1/Chk1 die Zellen nach Bestrahlung in die Mitotische Katastrophe führt, insbesondere nach zusätzlicher Beeinträchtigung der DNA-Reparaturfähigkeit der HPV-positiven HNSCC Zellen durch PARP-Inhibition. Einen weiteren möglichen Faktor zur Radiosensibilisierung könnte der Replikationsstress infolge der Wee1- und/oder Chk1-Inhibition darstellen. Eine mögliche Verstärkung des Effektes durch PARP-Inhibition ist vorstellbar, war bis dato aber noch nicht beschrieben. Auf Grund dieser Annahmen ergibt sich ein potentiell

synergistischer und tumorspezifischer Ansatz zur Radiosensibilisierung der HPV-positiven Tumorzellen.

Material und Methoden

Im Folgenden werden die Materialien und Methoden für die von mir selber durchgeführten experimentellen Arbeiten aufgeführt. Für die weiteren Experimente sind die entsprechenden Informationen der Veröffentlichung zu entnehmen.

Zellen und Zellkultur

Verwendet wurden die drei HPV-positiven HNSCC Zelllinien UD-SCC-2, UM-SCC-47 und UPCI-SCC-154. Als Wachstumsmedium verwendet wurde RPMI (Sigma-Aldrich, St. Louis, MO) mit 10% FCS (Biocrom AG, Berlin, Germany) bei 37°C, 5% CO₂ und 100% Luftfeuchtigkeit.

PARP-Inhibition erfolgte mit Olaparib (MyBiosource, San Diego, CA, USA) in einer Konzentration von 1 µM. Wee1-Inhibition erfolgte mit Adavosertib (Selleckchem, Houston, TX, USA) in einer Konzentration von 240 nM und in der kombinierten Wee1/Chk1-Inhibition mit 60 nM Adavosertib und zusätzlich 1 nM Prexasertib (MedChemExpress, Monmouth Junction, NJ, USA). Abweichende Konzentrationen in den beiden initialen Experimenten werden im Ergebnissteil entsprechend aufgeführt.

Bestrahlung

Die Bestrahlung erfolgte mit 200 kV Röntgenstrahlung in einer Dosisrate von 1,2 Gy pro Minute (Gulmay RS225, Gulmay Medical Ltd., Suwanee, GA, USA, 200 kV, 15 mA, 0,8 mm Be und 0,5 mm Cu Filter)

Zellproliferation

4 h nach Aussaat der Zellen in T25er Zellkulturflaschen wurden die Zellen mit den Inhibitoren behandelt. Nach 5 Tagen wurde die Zellzahl mithilfe eines *Coulter Counter* (Beckmann-Coulter, Brea, CA, USA) bestimmt.

Zellzyklusverteilung

Nach dem Ablösen mittels Trypsins wurden die Zellen mit 70%igen Ethanol fixiert und anschließend mit einer PBS/0,2%-Triton X-100 gewaschen. Die quantitative Färbung der DNA wurde durch 30-minütige Inkubation in 1% BSA/0,2% Triton X-100/1 µg/ml DAPI bei Raumtemperatur im Dunkeln erreicht. Nach erneutem Waschen erfolgte die flusszytometrische Messung mit einem MACSQuant10 und der MACSQuantify Software (Miltenyi Biotec, Bergisch Gladbach, Germany). Die Auswertung der Zellzyklusverteilung erfolgte mithilfe der ModFit LT Software (Verity Software House, Tosham, ME, USA).

Quantifizierung des Proteins γ H2AX

Zur Zellzyklus-spezifischen Messung der Intensität des an Serin 139 phosphorylierten Histons H2AX ($=\gamma$ H2AX) wurde das Durchflusszytometer FACS Canto und die Software FACS Diva (Beckton Dickinson, Franklin Lakes, NJ, USA) genutzt. Die mittels Trypsins abgelösten Zellen wurden mit PBS/4%Formaldehyd für 10 Minuten fixiert und mit PBS/1% BSA/0,2% Triton X-100 für mindestens eine Stunde geblockt und permeabilisiert. Die Inkubation mit dem primären Maus-anti- γ H2AX Antikörper (clone JBW301, Millipore, Billerica, MA, USA) erfolgte für eine Stunde bei Raumtemperatur. Hiernach wurden die Zellen drei Mal mit PBS/0,5% BSA/ 0,1% Triton X-100 gewaschen und dann eine weitere Stunde mit dem sekundären Antikörper (anti-Maus Dy-Light488, Jackson ImmunoResearch, West Grove, PA, USA) inkubiert und drei weitere Male wie zuvor beschrieben gewaschen. Die DNA-Färbung erfolgte mit FxCycle FarRed (Molekular

Probes, Eugene, OR, USA) unter Zugabe von 300 ng/ml RNase und 0,2% Triton X-100 für 30 Minuten im Dunkeln.

Koloniebildungsassay

Exponentiell wachsende Zellen wurden zwei Stunden nach der Behandlung mit den Inhibitoren bestrahlt. Weitere 24 Stunden später wurden definierte, geringe Zellzahlen in Medium ohne Inhibitor in T25er Zellkulturflaschen zur Koloniebildung ausgesät. Bei der Zelllinie UM-SCC-47 wurde die bekannte schlechtere Effizienz bei Aussähen (*plating efficiency*) durch Zugabe sog. *feeder cells* (UM-SCC-47; 20 Gy) bis zu einer Gesamtzellzahl von 5000 unterstützt. Bei der Zelllinie UPCI-SCC-154 wurde jeweils drei Wochen nach der Aussaat das Medium ausgetauscht durch 50% RPMI/10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) und 50% Amniomax C-100/7,5% Amniomax Supplement (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) /7,5%FBS um das Wachstum zu unterstützen. Die Zeit zur Koloniebildung variierte zwischen zwei und vier Wochen, wobei insbesondere die bestrahlten Zellen aufgrund von Wachstumsverzögerungen mehr Zeit zur Koloniebildung benötigten. Gewertet wurden Kolonien ab 50 Zellen.

Auswertung der Daten

Alle Versuche wurden mindestens drei Mal durchgeführt, immer mit der vollständigen Anzahl an Dosispunkten sowie Inhibitoransätzen und Kontrollen. Statistische Signifikanz wurde mittels *two-tailed Student's t-test* getestet. Zur Datenauswertung wurden Excel (Microsoft, Redmond, WA, USA) und GraphPad Prism 6 (GraphPadSoftware, San Diego, CA, USA) verwendet.

Ergebnisse

Die von mir durchgeführten Experimente für diese Publikation stehen in der zeitlichen Chronologie der Versuche am Anfang. In der vorgelegten Publikation wurden zudem Daten von den weiteren Co-Autoren veröffentlicht und im Gesamtbild interpretiert. Diese werden hier kurz, aber weniger detailliert und ohne Abbildungen beschrieben. Die Daten zum Zellzyklusarrestes nach Bestrahlung (Abbildung 4) wurde gemeinsam von mir und den Co-Autoren erarbeitet.

Untersuchung möglicher Synergien ohne Bestrahlung

Zunächst wurde der Einfluss einer kombinierten Inhibition von Wee1 und PARP ohne Bestrahlung auf Proliferation und Zellzyklusverteilung untersucht.

Dafür wurden Konzentrationen des Wee1-Inhibitors Adavosertib (AZD1775) und des PARP-Inhibitors Olaparib gewählt, welche in vorherigen Arbeiten des Labors moderate Effekte gezeigt hatten, bzw. eine maximale Konzentration von 1 μ M Olaparib, welche eine effektive Unterdrückung der ADP-Ribosylierung nach H₂O₂ Behandlung zeigte (Guster 2014, Busch 2017).

Im Proliferationsassay zeigte sich nach der Einzelbehandlung mit Olaparib oder Adavosertib eine heterogene Wachstumsinhibition, die bei der kombinierten Inhibition in allen Linien am stärksten ausgeprägt war, jedoch ohne einen klaren Hinweis auf eine synergistische Wirkung (Abbildung 3A). Eine Wee1-Inhibition mittels Adavosertib resultierte in einer Zunahme des Anteils der Zellen in der S/G2-Phase, was auf die Induktion von Replikationsstress hindeutet. Eine Inhibition von PARP mit Olaparib alleine oder in der Zugabe zu Adavosertib führt hingegen zu keiner veränderten Zellzyklusverteilung (Abbildung 3B).

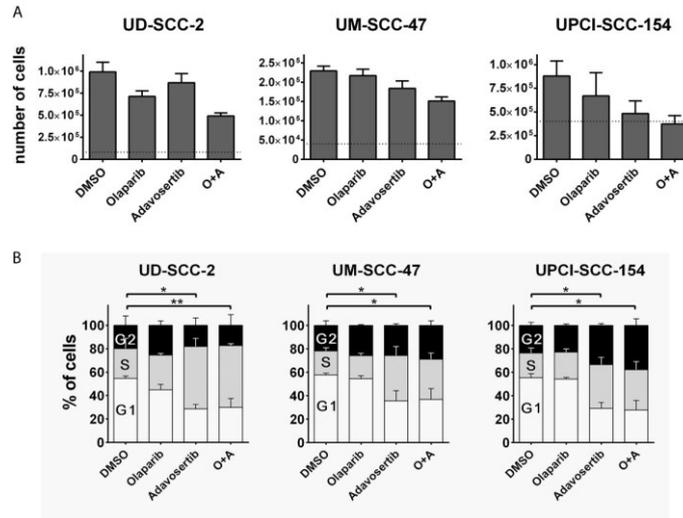


Abbildung 3. Kombinierten PARP- und Wee1-Inhibition ohne Bestrahlung.

(A) Proliferation. Die Zellen wurden 4h nach der Aussaat mit den Inhibitoren behandelt. Olaparib: 500 nM. Adavosertib: UPCI-SCC-154 60 nM; UD-SCC-2 und UM-SCC-47 120 nM. Nach 5 Tagen wurde die resultierende Anzahl der Zellen ausgewertet. (B) Zellzyklus. Einen Tag nach der Aussaat wurden die Inhibitoren zugegeben und nach weiteren 24 h die Zellen fixiert und nach einer DAPI-Färbung mittel fluoreszenzbasierter Durchflusszytometrie in Bezug auf die Zellzyklusphasen ausgewertet. Olaparib: 1 μ M. Adavosertib: UD-SCC-2 und UPCI-SCC-154 480 nm; UM-SCC-47 960 nM.

Untersuchung des Zellzyklusarrestes nach Bestrahlung

In der Kombination mit Bestrahlung wurde zuerst die Effektivität der dualen Inhibition auf die Unterdrückung des strahleninduzierten G2-Arrestes untersucht. Aufgrund der Vorarbeiten des Labors (Busch 2017) wurde in diesem und den folgenden Versuchen alternativ zu 240 nM Adavosertib auch eine niedrig konzentrierte Kombination aus 60 nM Adavosertib und 1 nM des Chk1/2-Inhibitors Prexasertib eingesetzt.

Es zeigte sich der zuvor beschriebene ausgeprägte G2-Arrest nach Bestrahlung. Stimmig zu den Ergebnissen von Guster et al. (Guster et al. 2014), nahm der Arrest nach einer der Bestrahlung vorausgegangenem Behandlung mit Olaparib weiter zu (Ausnahme UPCI-SCC-154 - 6Gy). Bei Behandlung mit Adavosertib vor der Bestrahlung, reduzierte sich der Anteil der Zellen im G2-Arrest deutlich, jedoch nicht vollständig. In der dosisreduzierten Kombination aus Adavosertib und Prexasertib zeigte sich je nach Zelllinie eine moderate bis deutliche Zunahme von Zellen in der S-Phase unabhängig von Bestrahlung, die weitere Hinzunahme von Olaparib verstärkte diesen Effekt nicht (Abbildung 4).

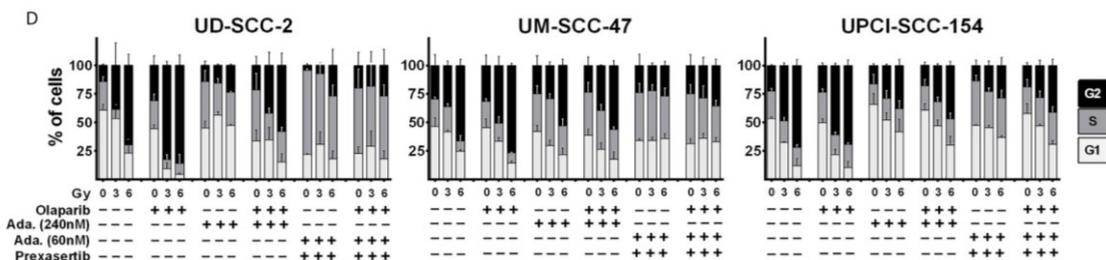


Abbildung 4. Bestrahlungsinduzierter G2-Arrest.

Analyse der Verteilung der Zellen im Zellzyklus 24 h nach Bestrahlung (0, 3 und 6 Gy) und Inhibitorbehandlung mittels DAPI-Färbung und Durchflusszytometrie. Olaparib: 1 μ M; Adavosertib: 240 nM;

Kombination aus Adavosertib und Prexasertib mit 60 nM und 1 nM. Weitere Versuche, wenn nicht anders angegeben, ebenfalls mit diesen Inhibitorkonzentrationen.

Untersuchung des Effektes auf den Replikationsstress

Die antitumorale Wirkung von Wee1- und Chk1-Inhibition wird unter anderem auf den resultierenden Replikationsstress zurückgeführt (Beck et al. 2012, Sørensen and Syljuåsen 2012).

Ein Marker für Replikationsstress (sowie auch für DNA-DSBs) ist die phosphorylierte Form des Histons 2AX (γ -H2AX). Dieses wird unter anderem von den Proteinkinasen ATM und ATR nach der Detektion eines DNA-Doppelstrangbruchs oder auch einer blockierten Replikationsgabel phosphoryliert, was effiziente Reparaturkaskaden einleitet (Kinner et al. 2008, Kuo and Yang 2008).

Die Inhibition von Wee1, sowie die kombinierte Inhibition von Wee1 und Chk1, führte zu einer deutlichen Zunahme des detektierbaren γ -H2AX-Signals im Durchflusszytometer, insbesondere in S-Phase Zellen (detektiert anhand des DNA-Gehaltes), wobei sich Zellen in der späten S- und G2-Phase nicht eindeutig unterscheiden lassen. Die alleinige PARP-Inhibition führt zu keinem bzw. in den UD-SCC-2 Zellen zu einem leichten Anstieg der γ H2AX-Signalintensität (Abbildung 5B, siehe auch Supplementary Figure 3A). Auch die Zugabe von Olaparib zu den Inhibitoren des intra-S/G2-Checkpoints bewirkte keine eindeutige γ H2AX Zunahme.

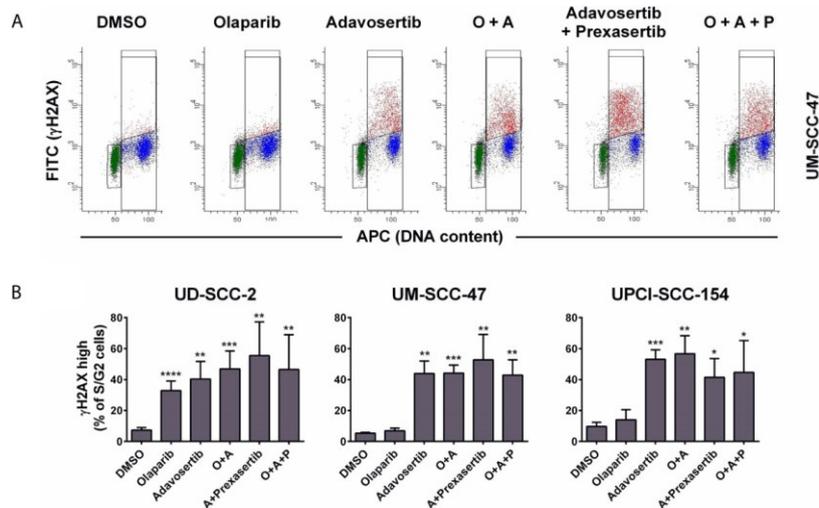


Abbildung 5. Effekte der PARP-, sowie der Wee1- und Chk1-Inhibition auf das γ H2AX-Level.

Analyse mittels Durchflusszytometrie 24 h nach Inhibitorbehandlung. **(A)** Gating nach DNA-Gehalt. G1 (grün), S/G2 (blau). Erhöhtes γ -H2AX-Level in S/G2-Phase (rot). **(B)** Anteil der Zellen mit erhöhten γ H2AX-Level in S/G2-Phase.

Radiosensibilisierung im Kolonieassay

In den zuletzt von mir durchgeführten Experimenten wurde die Strahlensensibilisierung der HPV-positiven HNSCC durch die duale Inhibition von PARP und dem intra-S/G2-Zellzykluskontrollpunkt direkt in Kolonieassays untersucht. Alle drei Zelllinien zeigten nach kombinierter Inhibition von PARP und dem intra-S/G2-Zellzykluskontrollpunkt eine besonders ausgeprägte Radiosensibilisierung, während alleinige Inhibitorgabe jeweils eine moderate Radiosensibilisierung induzierte (Abbildung 6A, B).

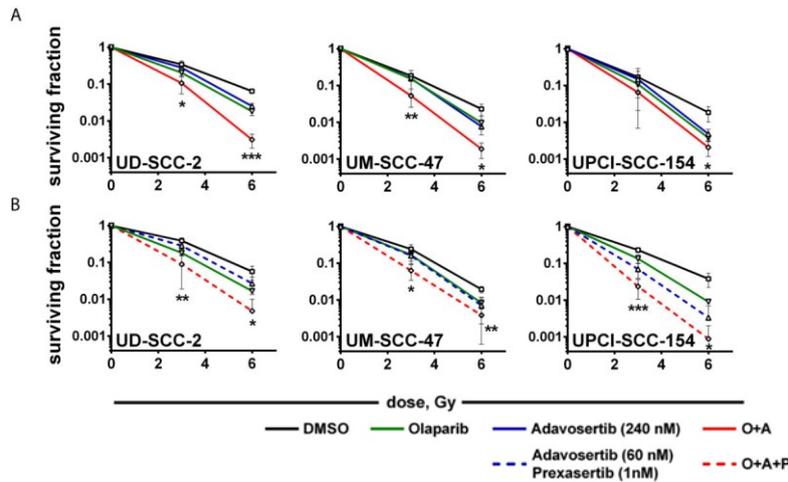


Abbildung 6. Radiosensibilisierung.

Exponentiell wachsende Zellen wurden am Tag nach der Aussaat mit den Inhibitoren behandelt und zwei Stunden später bestrahlt. Weitere 24 h später wurden die Zellen in Medium ohne Inhibitoren in einer definierten, niedrigen Zahl ausgesät und bis zur Koloniebildung inkubiert (2-4 Wochen).

Die hier beschriebenen Versuche bildeten die Grundlage der oben dargestellten Publikation. Insbesondere die effektive Radiosensibilisierung war ein Schlüsselexperiment, da es die Wirksamkeit der kombinierten Inhibition am besten abbildet. In weiteren Experimenten nach Abschluss der Versuche zu dieser Dissertation ist die Radiosensibilisierung und die Wirkung der kombinierten Inhibition nochmals weitergehend mechanistisch untersucht worden. Einige der Ergebnisse werden hier im Folgenden nochmals in aller Kürze dargestellt.

Versuche der Co-Autoren

DNA-Schädigung durch PARP-Inhibition: Die in der Literatur bereits gut beschriebene Wirkungsweise der PARP-Inhibitoren über DNA-Schädigung (Bryant et al. 2005, Mateo et al. 2019) wurde hier für unsere Zelllinien nochmals anhand eines verstärkten G2-Arrestes und γ H2AX im FACS verifiziert (siehe Supplementary Figure 2).

Strahleninduzierter G2-Arrest: Die Daten zum strahlungsinduzierten G2-Arrest wurden durch den Nachweis phosphorylierter Histone H3 (Ser10) als Mitosemarker nach kürzerem Zeitintervall ergänzt. Eine Behandlung mit Adavosertib alleine oder kombiniert mit Prexasetib unterdrückte den G2-Arrest teilweise (UD-SCC-2) oder komplett (UM-SCC-47, UPCI-SCC-154). PARP-Inhibition zeigte keinen bedeutenden Einfluss (siehe Publikation Figure 2 A-C, Supplementary Figure S1)

Replikationsstress: Auch in Bezug auf den Replikationsstress konnten durch weitere Versuche der Co-Autoren bestätigende Ergebnisse erzielt werden. Replication Protein A (RPA) bindet mit hoher Affinität einzelsträngige DNA und schützt diese. Erhöhte Mengen an Chromatin-gebundenem RPA in der S-Phase sind daher ein noch direkterer Marker für Replikationsstress als γ H2AX. Vor allem in UD-SCC-2 zeigten die Zellen nach der kombinierten Wee1/Chk1-Inhibition die deutlichste Zunahme an RPA, passend zu γ H2AX und der Zellzyklusverteilung. PARP-Inhibition hatte keinen deutlichen Einfluss (siehe Publikation Figure 3 C,D, Supplementary Figure S3B).

Radiosensibilisierung im Kolonieassay: Die effektive Radiosensibilisierung wurde zusätzlich bestätigt durch die Testung weiterer HPV-positiver Zelllinien (UT-SCC-45, 93-VU-147T, UPCI-SCC-90). Zwei Linien zeigten eine deutliche Radiosensibilisierung und eine Linie eine moderate (siehe Publikation Figure 5 C, D). Um die These der Tumorspezifität zu testen, wurden weitere Kolonieassays in p53-wildtyp Fibroblasten mit unbeeinträchtigtem G1-Arrest durchgeführt. Unter Proliferation zeigten diese eine deutlich geringere Radiosensibilisierung als 5 der 6 HPV-positiven HNSCC-Zelllinien. In nicht proliferierenden, konfluenten Fibroblastenkulturen war nur eine sehr geringe Radiosensibilisierung durch PARP-Inhibition feststellbar und kein Effekt durch die Inhibition des intra-S/G2-Zellzykluskontrollpunkts.

DSB-Reparaturmarker in Abhängigkeit des Zellzyklus: Das p53 Binding Protein 1 (53BP1) wird bei DNA-Doppelstrangbrüchen rekrutiert und eignet sich neben γ H2AX als Marker für nicht reparierte DSBs (Mirza-Aghazadeh-Attari et al. 2019, Marini et al. 2019). Die Bildung nukleärer Foci wird aber erheblich weniger durch Replikationsstress induziert. Es zeigte sich nach der Auswertung der residualen nukleären 53BP1-Foci 24 h nach Bestrahlung und kombinierter PARP/Wee1-Inhibition zusammengefasst keine generelle Zunahme der DSBs, jedoch eine Zunahme der Foci in der G1-Phase bei Abnahme der Foci in der S/G2-Phase. Dies unterstützt die Zellzyklusdaten, wonach die Zellen nach kombinierter Inhibition mit unreparierten DSBs die G2-Phase verlassen und die Mitose durchlaufen. Der Anteil der Zellen mit besonders wenigen Foci (≤ 3) nach Bestrahlung, der die größte Wahrscheinlichkeit auf Langzeitüberleben hat, ließ sich durch kombinierte PARP/Wee1-Inhibition verringern. Dieser Effekt zeigte sich in allen Zelllinien in der G1-Phase (Siehe Publikation Figure 4 und Supplementary Figure 5).

Einfluss des Replikationsstress: Als letzter Schritt wurden Versuchen durchgeführt, um den Einfluss des Replikationsstresses auf die Radiosensibilisierung durch kombinierter PARP/Wee1-Inhibition, auf die Zellen noch genauer zu beleuchten. Wie bereits zuvor erwähnt, führt die Wee1 und Wee1/Chk1-Inhibition mit und ohne PARP-Inhibition zu Replikationsstress. Als Ursache beschrieben ist ein erhöhtes Feuern von Replikationsursprüngen und damit einhergehend ein hoher Nukleotidverbrauch durch zu viele laufende Replikationsgabeln. Externe Zugabe von Nukleosiden kann dieses teilweise kompensieren (Beck 2012, Parsels, 2018). Tatsächlich wurde so eine tlw. Reduktion des Replikationsstresses, gemessen mittels γ H2AX, erreicht (Publikation Figure 6A,B; Supplementary Figure S7A). Bei kombinierter PARP/Wee1-Inhibition konnte, anders als bei alleiniger Wee1-Inhibition, allerdings kein spezifischer Einfluss des Replikationsstresses nachgewiesen werden, da die Nukleosidzugabe entweder keinen Effekt hatte oder in der DMSO-Kontrolle in ähnlichem Maße Radioresistenz induzierte. Im Gegensatz dazu wurde unter alleiniger Wee1-Inhibition in 2 Zelllinien, jedoch nicht in der DMSO-Probe, Radioresistenz erzeugt (siehe Publikation Figure 6C und Supplementary Figure 7B und 8).

Diskussion

In dem von mir bearbeiteten Teil der Publikation konnte ich die hocheffektive Radiosensibilisierung HPV-positiver HNSCC durch den Ansatz der dualen Inhibition von PARP und dem intra-S/G2- Zellzykluskontrollpunkt im Zellkulturmodellen zeigen. Ein zentrales Ergebnis war die Radiosensibilisierung der Tumorzellen im Koloniebildungsassay. Durch die bessere Prognose der

Patienten mit HPV-positiven Tumoren gegenüber den HPV-negativen HNSCC gibt es viele Langzeitüberlebende, für die neben den akuten auch die späten und irreversiblen Nebenwirkungen der Therapie hochrelevant sind.

Durch eine tumorspezifische Radiosensibilisierung wäre es denkbar, dass ggf. die Gesamt-Strahlendosis reduziert werden kann ohne eine Verschlechterung des Outcomes oder die nebenwirkungsreiche konkomitante Chemotherapie mit Cisplatin durch die zielgerichteten Substanzen ersetzt werden kann. In Bezug auf die Bestrahlungsdosis ist zum Beispiel ein klarer Zusammenhang zwischen der Dosis auf die krikopharyngeale Muskulatur, pharyngeale Konstriktoren und Larynx mit einer chronischen Dysphagie belegt (Mazzola et al. 2014, Mortensen et al. 2013). Cisplatin kann unter anderen zu dauerhaften Nierenschäden oder einer Minderung des Hörvermögens führen (Crona et al. 2017, Dasari and Tchounwou 2014).

Es sollte jedoch bedacht werden, dass sowohl Cisplatin, als auch der hier vorgestellte Targetingansatz, über Schädigung der DNA Wirkung zeigen und somit bei einer Hinzunahme zur platinbasierten Radiochemotherapie mit einer Verstärkung der Cisplatinwirkung und Nebenwirkungen zu rechnen ist. Vorstellbar wäre jedoch ein sequentielles Schema mit einer platinbasierten Induktionschemotherapie, um zum einen ein gut ansprechendes Kollektiv für eine mögliche Deintensivierung zu identifizieren und zum anderen die Fernmetastasierung zu kontrollieren, gefolgt von einer Radiatio in Kombination mit den molekular zielgerichteten Radiosensitizern. Es muss natürlich angemerkt werden, dass auch zielgerichtete Therapien Nebenwirkungen haben und diese bei mehreren Substanzen in der Kombination schwerer vorhersehbar sind. Die Ergebnisse mit den p53-profizienten Fibroblasten (Publikation Abbildung 5D und Supplementary Figure S6) deuten auf eine gewisse Tumorspezifität hin und zeigen bei einer guten *plating efficiency* keinen Hinweis auf eine erhöhte Toxizität oder Radiosensibilisierung. Es ist jedoch im Allgemeinen eine besondere Problematik der Radioonkologie, dass wenige klinische Studien zu den Wechselwirkungen von neuen Systemtherapien und Bestrahlung vorliegen, da die Substanzen zunächst meist in Studien ohne simultane Radiatio eingesetzt werden.

Der hier untersuchte Ansatz der dualen Inhibition von PARP und dem intra-S/G2-Zellzykluskontrollpunkt stellt nach unseren Daten eine vielversprechende Option zur Radiosensibilisierung dar mit den vorangehend skizzierten Anwendungsmöglichkeiten. Durch PARP-Inhibition werden zusätzliche DNA-Schäden verursacht und weiter durch Wee1/Chk1-Inhibition der Zelle die Zeit zur Reparatur vor dem Eintritt in die Mitose genommen. Die Induktion von Replikationsstress durch erhöhten Nukleotidverbrauch scheint nach unseren Daten für die kombinierte PARP und S/G2-Arrest-Inhibition keine zentrale Rolle für die Radiosensibilisierung zu spielen.

Generell stellt duales molekulares Targeting eine vielversprechende und hochinteressante Strategie zur entitätsspezifischen oder personalisierten Radiosensibilisierung dar. Valide präklinische Daten der verschiedenen Kombinationen sind eine Voraussetzung, diese erfolgreich in die klinische Anwendung zu bringen. Ein Großteil der Publikationen zum dualen Targeting bezieht sich auf die klassischen kinasevermittelten Signaltransduktionswege von EGFR, MAPK oder PI3K/AKT/mTOR (Hintelmann et al. 2020). Der Vorteil des DNA-Reparatur-Targetings liegt darin,

dass die direkten und essenziellen Schutzmechanismen der Zellen vor ionisierender Bestrahlung angegriffen werden.

Die Inhibition von Wee1 durch Adavosertib als hoch effektive Monotherapie (Diab et al. 2020, Tanaka et al. 2015) und auch die Effektivität von alleiniger PARP-Inhibition (Guster et al. 2014, Weaver et al. 2015), sowie durch Inhibition des intra-S/G2 Zellzykluskontrollpunktes (Busch et al. 2013, Busch et al. 2017, Dok et al. 2021, Ziemann et al. 2017, Zeng et al. 2020), konnte mittlerweile in einer Vielzahl von HPV-positiven HNSCC Zellmodellen gezeigt werden. Die kombinierte PARP und intra-S/G2-Zellzykluskontrollpunkt-Inhibition ist zudem für andere Entitäten und unterschiedliche Ansätze des Checkpoint-Targetings untersucht. Ein weiteres gut untersuchtes Target stellt hierbei das zuvor erwähnte ATR-Protein dar (siehe Abbildung 3) (Guster et al. 2014, Ahmed et al. 2015, Carruthers et al. 2018, Vance et al. 2011, Ziemann et al. 2017, Karnak et al. 2014, Parsels et al. 2018).

Der PARP-Inhibitor Olaparib wird neben seiner Zulassung durch die EMA in bestimmten Situationen beim Ovarial-, Mamma-, Pankreas- und Prostatakarzinomen im Rahmen von Klinischen Studien in Kombination mit Bestrahlung angewendet, u.a auch in HNSCC (Monotherapie + RT: NCT02229656; Kombinationstherapie mit Cetuximab + RT: NCT01758731; Kombinationstherapie mit Cisplatin NCT02308072).

Wee1-Inhibition in Kombination mit Bestrahlung wird ebenfalls zurzeit in klinischen Phase 1 Studien in HNSCC untersucht (NCT02585973 mit Cisplatin, NCT03028766) (Kong et al. 2020) sowie Prexasertib bei HNSCC (NCT02555644) (Yang et al. 2021). Die kombinierte Inhibition vom Chk1 und PARP bei lokal fortgeschrittenen soliden Tumoren wird ebenfalls in einer Phase 1 Studie untersucht (NCT03057145) sowie die kombinierte Inhibition von Wee 1 und PARP in Phase 1 und 2 (NCT04197713, NCT03579316, NCT03330847, NCT02576444, NCT02511795), beide letztgenannten Kombinationen allerdings bisher nur ohne Bestrahlung.

Die hier gezeigte präklinische Evidenz sowie die weiteren präklinischen und klinischen Daten zu den eingesetzten Inhibitoren sprechen klar für *in vivo* Versuche, um die hier gezeigte hocheffektive Radiosensibilisierung in HPV-positiven HNSCC weiter zu untermauern. Diese wurden daher vom Labor für Strahlenbiologie des UKE bereits gestartet (persönliche Kommunikation PD Dr. T. Rieckmann). So kann der Weg zu weiteren klinischen Untersuchungen geebnet werden, um neue Therapiestrategien im Rahmen der Deintensivierung dieser klinisch und biologisch distinkten Tumorsubgruppen zu erarbeiten.

Zusammenfassung

HPV-positive HNSCC stellen eine klinisch und biologisch unterscheidbare Subgruppe gegenüber der HPV-negativen HNSCC dar und zeichnen sich durch eine bessere Prognose, bedingt durch ein gutes Therapieansprechen insbesondere auf ionisierende Bestrahlung, aus. Als zugrunde liegender Mechanismus konnte eine eingeschränkte Reparaturkapazität bei DNA-Doppelstrangbrüchen, einhergehend mit einem ausgeprägten G2-Arrest, gezeigt werden. Hieraus ergibt sich die Rationale, durch eine Inhibition der DNA-Reparatur und der Zellzykluskontrolle durch molekulares Targeting eine Radiosensibilisierung zu erreichen. Getestet wurde der PARP-Inhibitor Olaparib, sowie die Inhibitoren des intra-S/G2-Checkpoints Adavosertib als Wee1-Inhibitor und Prexasertib als Chk1-Inhibitor. Die Inhibition des intra-S/G2-Checkpoints konnte den bestrahlungsinduzierten G2-Arrest deutlich unterdrücken und führte zu Replikationsstress, detektierbar durch eine Ansammlung der Zellen in der S-Phase und ein in dieser Zellzyklushase ebenfalls deutlich erhöhtes Level an γ H2AX, als Marker für Replikationsstress und DSB. Dieser Effekt schien durch die Hinzunahme des PARP-Inhibitors nicht beeinflusst, jedoch zeigte sich eine starke Radiosensibilisierung der HPV-positiven HNSCC durch die Kombination und insgesamt ein geringer Effekt auf die Normalgewebsfibroblasten. HPV-positive Tumorzellen werden als p53-defizient angesehen und somit insbesondere abhängig von einem zuverlässigen G2-Arrest. Dies und die bereits erwähnte eingeschränkte DNA-Reparaturkapazität tragen zu der Tumorspezifität des hier gezeigten Ansatzes bei. Nach kombinierter Inhibition treten die Zellen mit unreparierten DSB nach Bestrahlung und durch Bedingungen des Replikationsstresses aus der G2-Phase in die Mitose ein mit einem hohen Risiko für den mitotischen Zelltod.

HPV-positive HNSCC can be considered as a clinical and biological distinct entity compared to the HPV-negative HNSCC, with a favourable prognosis due to a good response, in particular to ionizing irradiation. A decreased DNA double-strand break repair associated with a pronounced G2 arrest can be shown in this context. This leads to the rationale of a radiosensitisation through inhibition of the DNA repair and the intra-S/G2 cell cycle checkpoint with molecular targeting. We used the PARP inhibitor Olaparib in combination with the inhibitors of the S/G2 cell cycle checkpoint, the Wee1 inhibitor Adavosertib and the Chk1 inhibitor Prexasertib. The inhibition of the intra-S/G2 cell cycle checkpoint resulted in abrogation of the radiation induced G2 arrest and leads to replication stress, detectable by an accumulation of cells in the S phase and an increased level of the replication stress and DSB marker γ H2AX in this phase of the cell cycle. This was not affected by the PARP inhibition, but we could show a highly effective radiosensitization of the HPV-positive HNSCC cells and little effect on normal human fibroblasts, using the combined inhibition. HPV-positive tumor cells can be considered p53 deficient and therefore rely on a sound G2 arrest. This attribute and the mentioned impairment of the DNA repair implicate the tumor specificity of the presented approach. Combined inhibition of PARP and the intra-S/G2 cell cycle checkpoint with simultaneous irradiation leads to unrepaired DSB under conditions of severe replication stress, with cells entering mitosis with a relevant risk for mitotic catastrophe

Abkürzungsverzeichnis

<i>ATR</i>	Ataxia teleangiectasia and Rad3 related
<i>altNHEJ</i>	alternatives nicht-homologes Endjoining
<i>BER</i>	Basenexzisionsreparatur
<i>Cdk-1</i>	cyclin dependent kinase 1
<i>Chk1</i>	Checkpointkinase 1
<i>DSB</i>	DNA-Doppelstrangbruch
<i>EGFR</i>	epidermal growth factor receptor
<i>HNSCC</i>	head and neck squamous-cell carcinoma
<i>HPV</i>	Humane papilloma Viren
<i>HR</i>	Homologe Rekombination
<i>OPSCC</i>	oropharyngeal squamous-cell carcinoma
<i>PARP</i>	Poly(ADP-ribose)-Polymerase
<i>pRB</i>	Retinoblastoma Protein
<i>P16^{INK4a} /CDK-Inhibitor 2A</i>	Cyclin dependent kinase inhibitor 2A
<i>RPA</i>	Replication Protein A
<i>53BP1</i>	p53 Binding Protein 1

Reference List

- Adelstein, D. J., Ismaila, N., Ku, J. A., Burtneß, B., Swiecicki, P. L., Mell, L., Beitler, J. J., Gross, N., Jones, C. U., Kaufman, M., Le, Q.-T., Semrad, T. J., Siu, L. L. & Ridge, J. A. 2019. Role of Treatment Deintensification in the Management of p16+ Oropharyngeal Cancer: ASCO Provisional Clinical Opinion. *Journal of Clinical Oncology*, 37, 1578-1589.
- Ahmed, S. U., Carruthers, R., Gilmour, L., Yildirim, S., Watts, C. & Chalmers, A. J. 2015. Selective Inhibition of Parallel DNA Damage Response Pathways Optimizes Radiosensitization of Glioblastoma Stem-like Cells. *Cancer Res*, 75, 4416-28.
- Albers, A. E., Qian, X., Kaufmann, A. M. & Coordest, A. 2017. Meta analysis: HPV and p16 pattern determines survival in patients with HNSCC and identifies potential new biologic subtype. *Scientific Reports*, 7, 16715.
- Audebert, M., Salles, B. & Calsou, P. 2004. Involvement of poly(ADP-ribose) polymerase-1 and XRCC1/DNA ligase III in an alternative route for DNA double-strand breaks rejoining. *J Biol Chem*, 279, 55117-26.
- Augustin, J. G., Lepine, C., Morini, A., Brunet, A., Veyer, D., Brochard, C., Mirghani, H., Péré, H. & Badoual, C. 2020. HPV detection in head and neck squamous cell carcinomas: what is the issue? *Frontiers in Oncology*, 10.
- Beck, H., Nähse-Kumpf, V., Larsen, M. S., O'hanlon, K. A., Patzke, S., Holmberg, C., Mejlvang, J., Groth, A., Nielsen, O., Syljuåsen, R. G. & Sørensen, C. S. 2012. Cyclin-dependent kinase suppression by WEE1 kinase protects the genome through control of replication initiation and nucleotide consumption. *Mol Cell Biol*, 32, 4226-36.
- Bonner, J. A., Mesia, R., Giral, J., Psyrr, A., Keilholz, U., Rosenthal, D. I., Beier, F., Schulten, J. & Vermorken, J. B. 2017. p16, HPV, and Cetuximab: What Is the Evidence? *Oncologist*, 22, 811-822.
- Bryant, H. E., Schultz, N., Thomas, H. D., Parker, K. M., Flower, D., Lopez, E., Kyle, S., Meuth, M., Curtin, N. J. & Helleday, T. 2005. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature*, 434, 913-7.
- Busch, C. J., Kriegs, M., Laban, S., Tribius, S., Knecht, R., Petersen, C., Dikomey, E. & Rieckmann, T. 2013. HPV-positive HNSCC cell lines but not primary human fibroblasts are radiosensitized by the inhibition of Chk1. *Radiother Oncol*, 108, 495-9.
- Busch, C. J., Kroger, M. S., Jensen, J., Kriegs, M., Gatzemeier, F., Petersen, C., Munscher, A., Rothkamm, K. & Rieckmann, T. 2017. G2-checkpoint targeting and radiosensitization of HPV/p16-positive HNSCC cells through the inhibition of Chk1 and Wee1. *Radiother Oncol*, 122, 260-266.
- Carrassa, L., Chila, R., Lupi, M., Ricci, F., Celenza, C., Mazzoletti, M., Brogini, M. & Damia, G. 2012. Combined inhibition of Chk1 and Wee1: in vitro synergistic effect translates to tumor growth inhibition in vivo. *Cell Cycle*, 11, 2507-17.
- Carruthers, R. D., Ahmed, S. U., Ramachandran, S., Strathdee, K., Kurian, K. M., Hedley, A., Gomez-Roman, N., Kalna, G., Neilson, M., Gilmour, L., Stevenson, K. H., Hammond, E. M. & Chalmers, A. J. 2018. Replication Stress Drives Constitutive Activation of the DNA Damage Response and Radioresistance in Glioblastoma Stem-like Cells. *Cancer Res*, 78, 5060-5071.
- Chaudhuri, L., Vincelette, N. D., Koh, B. D., Naylor, R. M., Flatten, K. S., Peterson, K. L., McNally, A., Gojo, I., Karp, J. E., Mesa, R. A., Sproat, L. O., Bogenberger, J. M., Kaufmann, S. H. & Tibes, R. 2014. CHK1 and WEE1 inhibition combine synergistically to enhance therapeutic efficacy in acute myeloid leukemia ex vivo. *Haematologica*, 99, 688-96.
- Chila, R., Basana, A., Lupi, M., Guffanti, F., Gaudio, E., Rinaldi, A., Cascione, L., Restelli, V., Tarantelli, C., Bertoni, F., Damia, G. & Carrassa, L. 2015. Combined inhibition of Chk1 and Wee1 as a new therapeutic strategy for mantle cell lymphoma. *Oncotarget*, 6, 3394-408.
- Crona, D. J., Faso, A., Nishijima, T. F., McGraw, K. A., Galsky, M. D. & Milowsky, M. I. 2017. A Systematic Review of Strategies to Prevent Cisplatin-Induced Nephrotoxicity. *Oncologist*, 22, 609-619.
- Dasari, S. & Tchounwou, P. B. 2014. Cisplatin in cancer therapy: molecular mechanisms of action. *Eur J Pharmacol*, 740, 364-78.
- Davies, K. D., Cable, P. L., Garrus, J. E., Sullivan, F. X., Von Carlowitz, I., Huerou, Y. L., Wallace, E., Woessner, R. D. & Gross, S. 2011. Chk1 inhibition and Wee1 inhibition combine synergistically to impede cellular proliferation. *Cancer Biol Ther*, 12, 788-96.

- Diab, A., Gem, H., Swanger, J., Kim, H. Y., Smith, K., Zou, G., Raju, S., Kao, M., Fitzgibbon, M., Loeb, K. R., Rodriguez, C. P., Méndez, E., Galloway, D. A., Sidorova, J. M. & Clurman, B. E. 2020. FOXM1 drives HPV+ HNSCC sensitivity to WEE1 inhibition. *Proc Natl Acad Sci U S A*, 117, 28287-28296.
- Dok, R., Glorieux, M., Bamps, M. & Nuyts, S. 2021. Effect of ATR Inhibition in RT Response of HPV-Negative and HPV-Positive Head and Neck Cancers. *Int J Mol Sci*, 22.
- Dok, R., Kalev, P., Van Limbergen, E., Asbagh, L., Vázquez, I., Hauben, E., Sablina, A. & Nuyts, S. 2014. p16INK4a Impairs Homologous Recombination-Mediated DNA Repair in Human Papillomavirus-Positive Head and Neck Tumors. *Cancer research*, 74.
- Elbæk, C. R., Petrosius, V. & Sørensen, C. S. 2020. WEE1 kinase limits CDK activities to safeguard DNA replication and mitotic entry. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 819-820, 111694.
- Ferlay, J., Colombet, M., Soerjomataram, I., Mathers, C., Parkin, D. M., Piñeros, M., Znaor, A. & Bray, F. 2019. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *Int J Cancer*, 144, 1941-1953.
- Galluzzi, L., Vitale, I., Aaronson, S. A., Abrams, J. M., Adam, D., Agostinis, P., Alnemri, E. S., Altucci, L., Amelio, I., Andrews, D. W., Annicchiarico-Petruzzelli, M., Antonov, A. V., Arama, E., Baehrecke, E. H., Barlev, N. A., Bazan, N. G., Bernassola, F., Bertrand, M. J. M., Bianchi, K., Blagosklonny, M. V., Blomgren, K., Borner, C., Boya, P., Brenner, C., Campanella, M., Candi, E., Carmona-Gutierrez, D., Cecconi, F., Chan, F. K., Chandel, N. S., Cheng, E. H., Chipuk, J. E., Cidlowski, J. A., Ciechanover, A., Cohen, G. M., Conrad, M., Cubillos-Ruiz, J. R., Czabotar, P. E., D'angioloella, V., Dawson, T. M., Dawson, V. L., De Laurenzi, V., De Maria, R., Debatin, K. M., Deberardinis, R. J., Deshmukh, M., Di Daniele, N., Di Virgilio, F., Dixit, V. M., Dixon, S. J., Duckett, C. S., Dynlacht, B. D., El-Deiry, W. S., Elrod, J. W., Fimia, G. M., Fulda, S., García-Sáez, A. J., Garg, A. D., Garrido, C., Gavathiotis, E., Golstein, P., Gottlieb, E., Green, D. R., Greene, L. A., Gronemeyer, H., Gross, A., Hajnoczky, G., Hardwick, J. M., Harris, I. S., Hengartner, M. O., Hetz, C., Ichijo, H., Jäättelä, M., Joseph, B., Jost, P. J., Juin, P. P., Kaiser, W. J., Karin, M., Kaufmann, T., Kepp, O., Kimchi, A., Kitsis, R. N., Klionsky, D. J., Knight, R. A., Kumar, S., Lee, S. W., Lemasters, J. J., Levine, B., Linkermann, A., Lipton, S. A., Lockshin, R. A., López-Otín, C., Lowe, S. W., Luedde, T., Lugli, E., Macfarlane, M., Madeo, F., Malewicz, M., Malorni, W., Manic, G., et al. 2018. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ*, 25, 486-541.
- Gillison, M. L., Trotti, A. M., Harris, J., Eisbruch, A., Harari, P. M., Adelstein, D. J., Jordan, R. C. K., Zhao, W., Sturgis, E. M., Burtness, B., Ridge, J. A., Ringash, J., Galvin, J., Yao, M., Koefman, S. A., Blakaj, D. M., Razaq, M. A., Colevas, A. D., Beitler, J. J., Jones, C. U., Dunlap, N. E., Seaward, S. A., Spencer, S., Galloway, T. J., Phan, J., Dignam, J. J. & Le, Q. T. 2019. Radiotherapy plus cetuximab or cisplatin in human papillomavirus-positive oropharyngeal cancer (NRG Oncology RTOG 1016): a randomised, multicentre, non-inferiority trial. *Lancet*, 393, 40-50.
- Gonzalez, S. L., Stremlau, M., He, X., Basile, J. R. & Münger, K. 2001. Degradation of the retinoblastoma tumor suppressor by the human papillomavirus type 16 E7 oncoprotein is important for functional inactivation and is separable from proteasomal degradation of E7. *J Virol*, 75, 7583-91.
- Goto, H., Izawa, I., Li, P. & Inagaki, M. 2012. Novel regulation of checkpoint kinase 1: Is checkpoint kinase 1 a good candidate for anti-cancer therapy? *Cancer Sci*, 103, 1195-200.
- Guertin, A. D., Martin, M. M., Roberts, B., Hurd, M., Qu, X., Miselis, N. R., Liu, Y., Li, J., Feldman, I., Benita, Y., Bloecher, A., Toniatti, C. & Shumway, S. D. 2012. Unique functions of CHK1 and WEE1 underlie synergistic anti-tumor activity upon pharmacologic inhibition. *Cancer Cell Int*, 12, 45.
- Guster, J. D., Weissleder, S. V., Busch, C. J., Kriegs, M., Petersen, C., Knecht, R., Dikomey, E. & Rieckmann, T. 2014. The inhibition of PARP but not EGFR results in the radiosensitization of HPV/p16-positive HNSCC cell lines. *Radiother Oncol*, 113, 345-51.
- Hintelmann, K., Kriegs, M., Rothkamm, K. & Rieckmann, T. 2020. Improving the Efficacy of Tumor Radiosensitization Through Combined Molecular Targeting. *Front Oncol*, 10, 1260.
- Hong, A., Dobbins, T., Lee, C. S., Jones, D., Jackson, E., Clark, J., Armstrong, B., Harnett, G., Milross, C., O'Brien, C. & Rose, B. 2010. Relationships between epidermal growth factor receptor expression and human papillomavirus status as markers of prognosis in oropharyngeal cancer. *Eur J Cancer*, 46, 2088-96.

- Hopkins, T. A., Shi, Y., Rodriguez, L. E., Solomon, L. R., Donawho, C. K., Digiammarino, E. L., Panchal, S. C., Wilsbacher, J. L., Gao, W., Olson, A. M., Stolarik, D. F., Osterling, D. J., Johnson, E. F. & Maag, D. 2015. Mechanistic Dissection of PARP1 Trapping and the Impact on In Vivo Tolerability and Efficacy of PARP Inhibitors. *Mol Cancer Res*, 13, 1465-77.
- Karnak, D., Engelke, C. G., Parsels, L. A., Kausar, T., Wei, D., Robertson, J. R., Marsh, K. B., Davis, M. A., Zhao, L., Maybaum, J., Lawrence, T. S. & Morgan, M. A. 2014. Combined inhibition of Wee1 and PARP1/2 for radiosensitization in pancreatic cancer. *Clin Cancer Res*, 20, 5085-96.
- Kimple, R. J., Smith, M. A., Blitzer, G. C., Torres, A. D., Martin, J. A., Yang, R. Z., Peet, C. R., Lorenz, L. D., Nickel, K. P., Klingelhutz, A. J., Lambert, P. F. & Harari, P. M. 2013. Enhanced radiation sensitivity in HPV-positive head and neck cancer. *Cancer Res*, 73, 4791-800.
- Kinner, A., Wu, W., Staudt, C. & Iliakis, G. 2008. Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic Acids Res*, 36, 5678-94.
- Koch, R. A., Harmel, C., Alber, M. & Bahn, E. 2021. A framework for automated time-resolved analysis of cell colony growth after irradiation. *Physics in Medicine & Biology*, 66, 035017.
- Kong, A., Good, J., Kirkham, A., Savage, J., Mant, R., Llewellyn, L., Parish, J., Spruce, R., Forster, M., Schipani, S., Harrington, K., Sacco, J., Murray, P., Middleton, G., Yap, C. & Mehanna, H. 2020. Phase I trial of WEE1 inhibition with chemotherapy and radiotherapy as adjuvant treatment, and a window of opportunity trial with cisplatin in patients with head and neck cancer: the WISTERIA trial protocol. *BMJ Open*, 10, e033009.
- Kuo, L. J. & Yang, L. X. 2008. Gamma-H2AX - a novel biomarker for DNA double-strand breaks. *In Vivo*, 22, 305-9.
- Lassen, P., Eriksen, J. G., Hamilton-Dutoit, S., Tramm, T., Alsner, J. & Overgaard, J. 2009. Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. *J Clin Oncol*, 27, 1992-8.
- Liu, Q., Ma, L., Jones, T., Palomero, L., Pujana, M. A., Martinez-Ruiz, H., Ha, P. K., Murnane, J., Cuartas, I., Seoane, J., Baumann, M., Linge, A. & Barcellos-Hoff, M. H. 2018. Subjugation of TGF β Signaling by Human Papilloma Virus in Head and Neck Squamous Cell Carcinoma Shifts DNA Repair from Homologous Recombination to Alternative End Joining. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 24, 6001-6014.
- Magnussen, G. I., Emilsen, E., Giller Fleten, K., Engesaeter, B., Nahse-Kumpf, V., Fjaer, R., Slipicevic, A. & Florenes, V. A. 2015. Combined inhibition of the cell cycle related proteins Wee1 and Chk1/2 induces synergistic anti-cancer effect in melanoma. *BMC Cancer*, 15, 462.
- Mak, J. P., Man, W. Y., Chow, J. P., Ma, H. T. & Poon, R. Y. 2015. Pharmacological inactivation of CHK1 and WEE1 induces mitotic catastrophe in nasopharyngeal carcinoma cells. *Oncotarget*, 6, 21074-84.
- Marini, F., Rawal, C. C., Liberi, G. & Pelliccioli, A. 2019. Regulation of DNA Double Strand Breaks Processing: Focus on Barriers. *Front Mol Biosci*, 6, 55.
- Markovic, A. & Chung, C. H. 2012. Current role of EGF receptor monoclonal antibodies and tyrosine kinase inhibitors in the management of head and neck squamous cell carcinoma. *Expert review of anticancer therapy*, 12, 1149-1159.
- Mateo, J., Lord, C. J., Serra, V., Tutt, A., Balmaña, J., Castroviejo-Bermejo, M., Cruz, C., Oaknin, A., Kaye, S. B. & De Bono, J. S. 2019. A decade of clinical development of PARP inhibitors in perspective. *Ann Oncol*, 30, 1437-1447.
- Matheson, C. J., Backos, D. S. & Reigan, P. 2016. Targeting WEE1 Kinase in Cancer. *Trends Pharmacol Sci*, 37, 872-881.
- Mazzola, R., Ricchetti, F., Fiorentino, A., Fersino, S., Giaj Levra, N., Naccarato, S., Sicignano, G., Albanese, S., Di Paola, G., Alterio, D., Ruggieri, R. & Alongi, F. 2014. Dose-volume-related dysphagia after constrictor muscles definition in head and neck cancer intensity-modulated radiation treatment. *Br J Radiol*, 87, 20140543.
- Mehanna, H., Robinson, M., Hartley, A., Kong, A., Foran, B., Fulton-Lieuw, T., Dalby, M., Mistry, P., Sen, M., O'toole, L., Al Booz, H., Dyker, K., Moleron, R., Whitaker, S., Brennan, S., Cook, A., Griffin, M., Aynsley, E., Rolles, M., De Winton, E., Chan, A., Srinivasan, D., Nixon, I., Grumett, J., Leemans, C. R., Buter, J., Henderson, J., Harrington, K., Mcconkey, C., Gray, A. & Dunn, J. 2019. Radiotherapy plus cisplatin or cetuximab in

- low-risk human papillomavirus-positive oropharyngeal cancer (De-ESCALaTE HPV): an open-label randomised controlled phase 3 trial. *Lancet*, 393, 51-60.
- Michaud, D. S., Langevin, S. M., Eliot, M., Nelson, H. H., Pawlita, M., Mcclean, M. D. & Kelsey, K. T. 2014. High-risk HPV types and head and neck cancer. *International journal of cancer*, 135, 1653-1661.
- Mirza-Aghazadeh-Attari, M., Mohammadzadeh, A., Yousefi, B., Mihanfar, A., Karimian, A. & Majidinia, M. 2019. 53BP1: A key player of DNA damage response with critical functions in cancer. *DNA Repair (Amst)*, 73, 110-119.
- Mortensen, H. R., Jensen, K., Aksglæde, K., Behrens, M. & Grau, C. 2013. Late dysphagia after IMRT for head and neck cancer and correlation with dose-volume parameters. *Radiother Oncol*, 107, 288-94.
- Murai, J., Huang, S. Y., Das, B. B., Renaud, A., Zhang, Y., Doroshow, J. H., Ji, J., Takeda, S. & Pommier, Y. 2012. Trapping of PARP1 and PARP2 by Clinical PARP Inhibitors. *Cancer Res*, 72, 5588-99.
- Nickson, C. M., Moori, P., Carter, R. J., Rubbi, C. P. & Parsons, J. L. 2017. Misregulation of DNA damage repair pathways in HPV-positive head and neck squamous cell carcinoma contributes to cellular radiosensitivity. *Oncotarget*, 8, 29963-29975.
- Ott, S., Wiegel, T., Laban, S., Hoffmann, T. K., Petersen, C. & Tribius, S. 2018. [Radiotherapeutic studies of head and neck cancer-highlights of the 2018 ASCO Annual Meeting]. *Hno*, 66, 901-906.
- Pan, C., Issaeva, N. & Yarbrough, W. G. 2018. HPV-driven oropharyngeal cancer: current knowledge of molecular biology and mechanisms of carcinogenesis. *Cancers Head Neck*, 3, 12.
- Parsels, L. A., Karnak, D., Parsels, J. D., Zhang, Q., Velez-Padilla, J., Reichert, Z. R., Wahl, D. R., Maybaum, J., O'connor, M. J., Lawrence, T. S. & Morgan, M. A. 2018. PARP1 Trapping and DNA Replication Stress Enhance Radiosensitization with Combined WEE1 and PARP Inhibitors. *Mol Cancer Res*, 16, 222-232.
- Qi, W., Xie, C., Li, C., Caldwell, J. T., Edwards, H., Taub, J. W., Wang, Y., Lin, H. & Ge, Y. 2014. CHK1 plays a critical role in the anti-leukemic activity of the wee1 inhibitor MK-1775 in acute myeloid leukemia cells. *J Hematol Oncol*, 7, 53.
- Rahimi, S. 2020. HPV-related squamous cell carcinoma of oropharynx: a review. *J Clin Pathol*, 73, 624-629.
- Rieckmann, T., Tribius, S., Grob, T. J., Meyer, F., Busch, C. J., Petersen, C., Dikomey, E. & Kriegs, M. 2013. HNSCC cell lines positive for HPV and p16 possess higher cellular radiosensitivity due to an impaired DSB repair capacity. *Radiother Oncol*, 107, 242-6.
- Ronson, G. E., Piberger, A. L., Higgs, M. R., Olsen, A. L., Stewart, G. S., Mchugh, P. J., Petermann, E. & Lakin, N. D. 2018. PARP1 and PARP2 stabilise replication forks at base excision repair intermediates through Fbh1-dependent Rad51 regulation. *Nat Commun*, 9, 746.
- Rosenberg, A. J. & Vokes, E. E. 2021. Optimizing Treatment De-Escalation in Head and Neck Cancer: Current and Future Perspectives. *Oncologist*, 26, 40-48.
- Russell, M. R., Levin, K., Rader, J., Belcastro, L., Li, Y., Martinez, D., Pawel, B., Shumway, S. D., Maris, J. M. & Cole, K. A. 2013. Combination therapy targeting the Chk1 and Wee1 kinases shows therapeutic efficacy in neuroblastoma. *Cancer Res*, 73, 776-84.
- Simard, E. P., Torre, L. A. & Jemal, A. 2014. International trends in head and neck cancer incidence rates: Differences by country, sex and anatomic site. *Oral Oncology*, 50, 387-403.
- Sørensen, C. S. & Syljuåsen, R. G. 2012. Safeguarding genome integrity: the checkpoint kinases ATR, CHK1 and WEE1 restrain CDK activity during normal DNA replication. *Nucleic Acids Res*, 40, 477-86.
- Tanaka, N., Patel, A. A., Wang, J., Frederick, M. J., Kalu, N. N., Zhao, M., Fitzgerald, A. L., Xie, T. X., Silver, N. L., Caulin, C., Zhou, G., Skinner, H. D., Johnson, F. M., Myers, J. N. & Osman, A. A. 2015. Wee-1 Kinase Inhibition Sensitizes High-Risk HPV+ HNSCC to Apoptosis Accompanied by Downregulation of Mcl-1 and XIAP Antiapoptotic Proteins. *Clin Cancer Res*, 21, 4831-44.
- Tribius, S., Ihloff, A. S., Rieckmann, T., Petersen, C. & Hoffmann, M. 2011. Impact of HPV status on treatment of squamous cell cancer of the oropharynx: what we know and what we need to know. *Cancer Lett*, 304, 71-9.
- Tumban, E. 2019. A Current Update on Human Papillomavirus-Associated Head and Neck Cancers. *Viruses*, 11.
- Vakifahmetoglu, H., Olsson, M. & Zhivotovsky, B. 2008. Death through a tragedy: mitotic catastrophe. *Cell Death Differ*, 15, 1153-62.

- Van Harten, A. M., Buijze, M., Van Der Mast, R., Rooimans, M. A., Martens-De Kemp, S. R., Bachas, C., Brink, A., Stigter-Van Walsum, M., Wolthuis, R. M. F. & Brakenhoff, R. H. 2019. Targeting the cell cycle in head and neck cancer by Chk1 inhibition: a novel concept of bimodal cell death. *Oncogenesis*, 8, 38.
- Vance, S., Liu, E., Zhao, L., Parsels, J. D., Parsels, L. A., Brown, J. L., Maybaum, J., Lawrence, T. S. & Morgan, M. A. 2011. Selective radiosensitization of p53 mutant pancreatic cancer cells by combined inhibition of Chk1 and PARP1. *Cell Cycle*, 10, 4321-9.
- Wagner, S., Wittekindt, C., Sharma, S. J., Wuerdemann, N., Jüttner, T., Reuschenbach, M., Prigge, E.-S., Von Knebel Doeberitz, M., Gattenlöhner, S., Burkhardt, E., Pons-Kühnemann, J. & Klussmann, J. P. 2017. Human papillomavirus association is the most important predictor for surgically treated patients with oropharyngeal cancer. *British Journal of Cancer*, 116, 1604-1611.
- Wang, M. B., Liu, I. Y., Gornbein, J. A. & Nguyen, C. T. 2015. HPV-Positive Oropharyngeal Carcinoma: A Systematic Review of Treatment and Prognosis. *Otolaryngol Head Neck Surg*, 153, 758-69.
- Wang, Y., Luo, W. & Wang, Y. 2019. PARP-1 and its associated nucleases in DNA damage response. *DNA Repair (Amst)*, 81, 102651.
- Weaver, A. N., Cooper, T. S., Rodriguez, M., Trummell, H. Q., Bonner, J. A., Rosenthal, E. L. & Yang, E. S. 2015. DNA double strand break repair defect and sensitivity to poly ADP-ribose polymerase (PARP) inhibition in human papillomavirus 16-positive head and neck squamous cell carcinoma. *Oncotarget*, 6, 26995-7007.
- Wienecke, A. & Kraywinkel, K. 2019. Epidemiologie von Kopf-Hals-Tumoren in Deutschland. *Der Onkologe*, 25, 190-200.
- Wittekindt, C., Wagner, S., Bushnak, A., Prigge, E. S., Von Knebel Doeberitz, M., Würdemann, N., Bernhardt, K., Pons-Kühnemann, J., Maulbecker-Armstrong, C. & Klussmann, J. P. 2019. Increasing Incidence rates of Oropharyngeal Squamous Cell Carcinoma in Germany and Significance of Disease Burden Attributed to Human Papillomavirus. *Cancer Prev Res (Phila)*, 12, 375-382.
- Yang, E. S., Deutsch, E., Mehmet, A., Fayette, J., Tao, Y., Nabell, L., Spencer, S. A., Wang, X. A., Spoljoric, E. A., Zhang, W., Hynes, S. M., Decker, R. L., Lin, A. K. B. & William, W. N., Jr. 2021. A Phase 1b trial of prexasertib in combination with chemoradiation in patients with locally advanced head and neck squamous cell carcinoma. *Radiother Oncol*, 157, 203-209.
- Zeng, L., Nikolaev, A., Xing, C., Della Manna, D. L. & Yang, E. S. 2020. CHK1/2 Inhibitor Prexasertib Suppresses NOTCH Signaling and Enhances Cytotoxicity of Cisplatin and Radiation in Head and Neck Squamous Cell Carcinoma. *Mol Cancer Ther*, 19, 1279-1288.
- Ziemann, F., Seltzsaam, S., Dreffke, K., Preising, S., Arenz, A., Subtil, F. S. B., Rieckmann, T., Engenhart-Cabillic, R., Dikomey, E. & Wittig, A. 2017. Roscovitine strongly enhances the effect of olaparib on radiosensitivity for HPV neg. but not for HPV pos. HNSCC cell lines. *Oncotarget*, 8, 105170-105183.

Erklärung des Eigenanteils

Die hier vorgestellten und in der Publikation veröffentlichten experimentellen Arbeiten wurden in einem umfassenden Projekt im Labor für Strahlentherapie & Experimentelle Radioonkologie des Universitätsklinikums Hamburg Eppendorf erarbeitet. Die von mir durchgeführte experimentelle Arbeit steht in der zeitlichen Chronologie am Anfang. Aufgrund der vielversprechenden Daten wurde sich dazu entschlossen, das Projekt fortzuführen und es wurden weitere Versuche durch die Co-Autoren durchgeführt um die genannten Hypothesen zu stützen.

Von mir durchgeführt wurden die experimentellen Arbeiten zu möglichen Synergien der Inhibitoren ohne Bestrahlung. Diese sind in der Publikation zu finden unter Figure 1A und B. Außerdem habe ich gemeinsam mit den Co-Autoren die Versuche zum Zellzyklusarrestes nach Bestrahlung durchgeführt, welche in der Publikation in der Figure 2D dargestellt werden. Weiter habe ich die Ergebnisse den Replikationsstress betreffend erarbeitet, welche in der Publikation unter Figure 3 A und B zusammengefasst sind. Die Koloniebildungsassays, zu denen die Ergebnisse in der Publikation in der Figure 5A und B zu finden sind habe ich ebenfalls durchgeführt.

Meinen Anteil umfasste bei den Versuchen die Planung, die Durchführung, die Auswertung sowie die Interpretation der Ergebnisse. Die Daten aus den von mir durchgeführten Experimenten wurden von mir außerdem in einem Vortrage auf 27. Symposium "Experimentelle Strahlentherapie und Klinische Strahlenbiologie" vorgestellt. Außerdem war ich unterstützend tätig bei der Verfassung des Manuskriptes durch die Letztautoren mit einem besonderen Augenmerk auf die Abschnitte die von mir durchgeführten Arbeiten betreffend.

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Curriculum vitae

Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt.

Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.



Unterschrift: