Poly (ADP-ribose) polymerase inhibitor efficacy in head and neck cancer

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ABSTRACT

Objectives: Poly (ADP-ribose) polymerase inhibitors (PARPi) have shown single agent activity against tumors with deficiencies in the DNA repair mechanism homologous recombination including, but not limited to those harboring BRCA mutations. We hypothesized that, in the context of homologous recombination deficiency (HRD), PARPi could have an effect in head and neck cancer (HNC).

Materials and Methods: We evaluated TCGA data for evidence of HRD using a copy number data signature established for breast cancer. The comparative potency of three PARPi was evaluated using cell viability assays in a panel of HNC cell lines and response was compared to BRCA-deficient breast cancer cell lines. The change in foci formation of γH2AX and RAD51 was assessed with immunofluorescent staining after exposure to a PARPi. Baseline gene expression was analyzed using microarray data.

Results: We found a subgroup in the TCGA HNC cohort harboring genomic aberrations consistent with HRD in breast cancer. Rucaparib activity was superior to olaparib and veliparib and showed single agent activity in a subset of HNC cell lines that was comparable to BRCA-deficient breast cancer cell lines. Rucaparib-sensitive and rucaparib-resistant groups showed significant differences in γH2AX and RAD51 foci formation after rucaparib exposure. Expression of genes involved in chromosome structure was strongly associated with rucaparib resistance.

Conclusion: We demonstrate that PARPi are effective in a subset of HNC cell lines and propose that HRD may be present in HNC in vivo suggesting that these compounds could play a role in the treatment of HNC.

Keywords: Head and neck cancer; Poly (ADP-ribose) polymerase; Rucaparib; DNA repair; Homologous recombination

BACKGROUND

Head and neck cancer

Despite the progress made in identifying and addressing molecular targets in head and neck cancer (HNC), the overall survival rates remain modest and prognosis for metastatic disease is poor [1]. DNA damaging therapies continue to be a mainstay of therapy including radiation and chemotherapy e.g. using platinating agents. There is an urgent need for more effective therapeutic approaches.

PARP inhibitors

One of the hallmarks of cancer is genome instability and progressive accumulation of mutations/genetic aberrations [2]. While genetic aberrations are essential for carcinogenesis, survival of cancer cells also depends on their ability to continually repair their own DNA. Most DNA single strand breaks (SSB) are restored by baseline expression. Poly (ADP-ribose) polymerases (PARP) are critical for the induction of this mechanism [3].

If PARP is suppressed by PARP inhibitors (PARPi), SSB accumulate, increasing the number of SSB that develop into double strand breaks (DSB) during replication. DSB are repaired mainly by non-homologous-end-joining or homologous recombination (HR), the latter being more accurate. Important proteins involved in HR are BRCA1/2 and RAD51 [4]. PARP inhibition can be compensated for by HR in normal cells, however with deleterious mutations in HR genes (e.g. BRCA genes in breast cancer), DSB accumulate and lead to cell death. This is a prime example of “synthetic lethality” [5,6], a concept that has been validated for PARPi by in vitro studies, as
well as clinical trials in breast cancer patients with BRCA mutations [7–10].

Deficiencies in the HR pathway

Recently, the effects of PARPi have been postulated to also play a role in tumors with HR defects in general, and not solely in those attributed to germline BRCA mutations [11]. “HR deficiency” (HRD, also known as “BRCAness”) describes a set of phenotypic characteristics that some germline cancers share with tumors containing germline BRCA mutations [12]. Several mechanisms have been found to contribute to the HRD phenotype, such as silencing of BRCA2 by EMTS2 [13], downregulation of BRCA1 by the transcription factor ETS-1 [14], or hypermethylation of the BRCA1 promoter [15]. Beyond BRCA, defects in the Fanconi anemia pathway, hypermethylation of RAD51C and mutations in the DNA-damage recognizing genes ATM and ATR or PTEN, which regulates RAD51 transcription, have been suggested to contribute to a HRD phenotype [16]. Altogether, any defect in the HR machinery may lead to HRD and to PARPi susceptibility.

Aim of this study

We therefore hypothesized, that these agents could affect a larger group of head and neck tumors beyond uncommon somatic BRCA mutations. To test this hypothesis for in vivo samples, we applied a HRD/BRCAness copy number signature developed by Joosse et al. [17] to HNC data from The Cancer Genome Atlas (TCGA). Subsequently, we evaluated the comparative potency in HNC cell lines of veliparib (ABT-888), olaparib (AZD2281, KU-0059436) and rucaparib (CO-338, AG-014699, PF-01367338) and investigated correlative markers of activity related to DNA repair. Our study shows that rucaparib in particular has promising potency in HNC and is not dependent on BRCA mutations.

Materials and methods

Detailed description of the standard resazurin assay, colony-forming assay, Syto60® assay and immunoblot as well as ImageJ analysis pathways, previously performed targeted sequencing protocols and cell line details can be reviewed in the Supplementary Methods.

Cell culture

All cell lines were maintained in 10 mm dishes at 37 °C in a humidified atmosphere with 5% CO2. The BRCA1-mutated (5382insC) breast cancer cell line HCC1937 [18] and the BRCA1-methylated breast cancer cell line UACC-3199 [19] were kindly provided by Prof. Olufunmilayo Olopade (University of Chicago). All cell lines were tested negative for mycoplasma infection (Lonza-kit #LT-07118). All experiments were carried out within ten passages. Drugs were purchased from Selleck Chemicals (Houston, TX; veliparib #S1004; olaparib #S1060; rucaparib #S1098).

Prediction of BRCA1-association

We utilized a previously published method to predict BRCA1-association in the TCGA HNC cohort [17]. The two most different copy number aberrations between BRCA1-associated samples and sporadic samples in breast carcinomas, 3q22–27 (gain) and 5q12–14 (loss), were used to hierarchically cluster the HNC samples. The branch with both of those alterations was predicted to have a HRD/BRCAness phenotype.

Immunofluorescent staining

In an effort to study the HR competency of HNC cell lines and to investigate possible prediction methods, we employed a HR assay for ovarian cancer cell lines introduced by Mukhopadhyay et al. [20]. The group reported a positive predictive value of 93% and a negative predictive value of 100% for rucaparib sensitivity using post-treatment immunofluorescent staining of the HR marker RAD51. We applied this assay to three sensitive and three resistant HNC cell lines. Modifications included the addition of a 2 Gy ionizing radiation (IR) treatment regimen as a positive control (Fasitron® X-ray; 43855 Series), the addition of a permeabilization step (0.1% Triton X-100) and a counterstaining step with Hoechst-Dye (Life Technologies™).

Images were taken with a Leica TCS SP2 confocal microscope, 100X magnification and 2X optical zoom for all cell lines but SCC-61. Random pictures were acquired from three cover slips allowing analysis for at least 45 cells per treatment regimen. Secondary antibody controls were prepared for every cell line. Lasers were set at the 2 Gy treatment samples as positive control and were not changed for the other treatments. Foci-counting was performed with the ImageJ software and a Macro was created to accelerate the process, which can be reviewed in the Supplementary Methods.

Each experiment was repeated independently at least three times.

Gene expression analysis

We previously generated a gene expression microarray dataset (Agilent Technologies 4 × 44 k and 4 × 44 k v2, Santa Clara, CA) of our HNC cell lines (manuscript in review, GEO accession number GSE52088). We tested for gene-drug associations using Pearson’s correlation tests of gene expression levels against log-transformed rucaparib IC50 values. We controlled for the family wise error rate using Bonferroni correction and the false discovery rate using the Benjamini and Hochberg (BH) method. Gene set analysis of Gene Ontology (GO) terms was performed using the DAVID software [21].

Statistical analysis

For PARPi comparison, repeated measures ANOVA was performed using R. The clear outlier (SCC-61) in the rucaparib-treated group was omitted (see Fig. 2). Analyses of the foci-counts from immunofluorescence experiments were performed in Microsoft Excel 2010 and R. For comparison of foci formation changes within the cell lines, raw count data was analyzed using a generalized linear model (the glm() function in R) with a Poisson error-distribution. While count data is often log-transformed to satisfy parametric test assumptions, it has been shown that this generalized linear model approach is more accurate [22]. For the comparison of foci formation of rucaparib-sensitive and rucaparib-resistant groups, we used median log-values of the change in average number of foci per cell using one-tailed t-tests. In both analyses technical replicates were summarized by their median as this is more robust to outliers given the small number of samples.

Results

Estimated fraction of HRD in the TCGA HNC cohort

In order to approximately estimate the occurrence of HRD in HNC in vivo, we utilized a previously described method to detect BRCA1-association in tumors without BRCA mutations [17]. Joosse et al. reported a molecular phenotype based on genomic copy number aberrations that are significantly different in BRCA1-deficient tumors compared to sporadic breast cancer. Their approach
yielded 88% sensitivity and 94% specificity and was able to detect BRCA1 promoter methylation samples in the validation set in the absence of BRCA mutations. The aberrations 3q (gain) and 5q (loss) showed the highest level of statistical significance in the above-mentioned study and were also reported as important discriminatory aberrations elsewhere [23,24]. We applied this genomic phenotype to the TCGA HNC cohort of 424 samples and found 50 HNC samples (11.8%) exhibiting this phenotype (Fig. 1, see Supplementary Table S1 for sample names). Our findings lend support to the hypothesis that there may be a subset of >10% of head and neck tumors deficient in HR leading to our investigation of PARPi in this type of malignancy.

**BRCA alterations in HNC**

Somatic BRCA variants were identified in HNC tumor samples in the TCGA cohort, for approximately 3% of cases for both BRCA1 and BRCA2, although the functional impact on HRD has not been investigated in HNC (Supplementary Fig. 1) [28,29]. Furthermore, we investigated variation in the BRCA genes of our HNC cell lines. We detected SNPs/mutations in four of the used 10 cell lines and compared the variations to mutations already described on the COSMIC database [30] (Supplementary Table S2). Only the BRCA2 variation c.53G > A of the HNC cell line SAS had already been described as a missense mutation in malignancies of the central nervous system, although classified as non-pathogenic and of no clinical relevance [31,32].

**Comparison of three PARPi**

To determine the comparative potency of PARPi in HNC, we performed viability assays on six HNC cell lines evaluating the compounds veliparib, olaparib and rucaparib. Consistent with prior reports, the standard three-day assay did not allow enough time for the PARPi to unfold their full effect and induce cell death permitting the calculation of robust IC50 values [33] (All survival curves of six HNC cell lines for the three PARPi are shown in Supplementary Fig. 2). Therefore, we compared the area under the curve (AUC) for the three compounds (Fig. 2).

Veliparib, a non-locking PARPi [34], showed only minimal effects on viability. By contrast, olaparib and rucaparib showed a significantly greater effect (P = 0.02 and P = 9.8 × 10−5) compared to veliparib. Rucaparib impaired cell viability significantly more than olaparib (P = 0.02). There was also a highly significant difference in PARPi potency when all three drugs were compared (P = 6.3 × 10−5 from repeated measures ANOVA model comparing the mean of the three groups).

**Establishment of robust IC50 values for rucaparib**

Hence, we examined the potency of rucaparib in 10 HNC cell lines by performing a more stringent method to establish robust IC50 values. The colony-forming assay was chosen, because a longer time period (six to eight days as opposed to three days in the resazurin assay) is allowed for cells to suffer endogenous or exogenous DNA damage and therefore for the DNA repair targeting PARPi to unfold its effect. We found very large variability in drug susceptibility among the 10 tested cell lines and categorized them as “sensitive” (IC50 < 2 μM, similar to prior reports [35]) or “resistant” (Fig. 3(a)).

**Comparison to BRCA-deficient breast cancer**

Because PARPi have proven to be beneficial for patients with BRCA-mutated breast cancer [9], we compared our sensitive HNC cell lines to the breast cancer (BC) cell lines HCC1937, that harbors a BRCA mutation and UACC-3199, which is BRCA-deficient through epigenetic silencing. Due to the poor colony-forming abilities of the BC cell lines, we performed the viability assay Syto60® to compare rucaparib-susceptibility (Fig. 3(b)). As this is a DNA based rapid viability assay, the IC50 values differed from those established with colony-forming assays and resazurin staining, yet all assays were consistent regarding the ranking of cell lines. Strikingly, the sensitive HNC cell lines (BB-49, Detroit562 and HN4) were notably more sensitive (IC50 values: 4.4 μM, 7.0 μM and 9.1 μM, respectively) to rucaparib than the BRCA-mutated BC cell line HCC1937 (IC50 value: 13.1 μM). The response of UACC-3199, a cell line that has recently been found to be sensitive to rucaparib [36], was comparable to the response

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Fig. 1. Prediction of BRCA1-association in TCGA HNC cohort. The two most different copy number aberrations between BRCA1-associated samples and sporadic samples in breast carcinomas, 3q22–27 (gain) and 5q12–14 (loss), were used to predict the BRCA1-association for TCGA head and neck cancers. The TCGA samples were hierarchically clustered using the copy number data of 27 chromosome bands from these two regions. The branch with both 3q22–27 (gain) and 5q12–14 (loss) was considered BRCA1-associated (11.8%) and highlighted with a blue box (names of these samples are shown in Supplementary Table S1).
of sensitive HNC cell lines (IC50 value: 6.4 μM). This strongly suggests that there is a subset of HNC cell lines susceptible to PARPi, possibly due to HRD.

Estimating HR competency by RAD51 foci formation assay

RAD51 foci formation

Given the findings of Mukhopadhyay et al. [20], we hypothesized that we would see no increase in RAD51 foci formation in rucaparib-sensitive cell lines and an increase in rucaparib-resistant cell lines. For treatment with positive control (2 Gy IR), we found this to be the case. In sensitive cell lines, the median foci formation decreased and conversely, RAD51 foci formation in resistant cell lines increased after exposure to 2 Gy (Fig. 4(b)). The difference between sensitive and resistant cell lines was significant \( (P = 0.02) \), which strongly suggests that HR is defective in sensitive cell lines.

We next examined the change in RAD51 foci formation after treatment with rucaparib. In some cell lines, treatment with rucaparib induced the RAD51 foci formation, suggesting an intact HR-process. In others, the number of RAD51 foci remained constant or decreased as compared to the DMSO control. In these cases, one can assume a defective HR pathway and Mukhopadhyay et al. accurately predicted the susceptibility to rucaparib of cell lines showing this pattern. However, the ability of HNC cells to form RAD51 foci after rucaparib-treatment was not associated with sensitivity to rucaparib in our experiment (Fig. 4(c)). The difference between the groups was not significant \( (P = 0.47) \).

Because we were unable to reproduce the assay proposed by Mukhopadhyay et al., we validated our findings by performing Western Blots comparing RAD51 protein level before and after rucaparib treatment. Again, RAD51 level change did not coincide with sensitivity, consistent with the results generated by the immunofluorescence assay (Supplementary Fig. 3).

DNA double strand breaks (γH2AX foci)

As anticipated, the treatment with 2 Gy IR resulted in a significant increase of γH2AX-marked DSB [37] across all cell lines (Fig. 4(a) and (b)) BB-49: \( P = 0.03 \), Detroit562: \( P = 1.0 \times 10^{-3} \), HN4: \( P = 1.8 \times 10^{-7} \), HN5: \( P = 0.02 \), SAS: \( P = 0.03 \), SCC-61 \( P = 2.74 \times 10^{-5} \). Of note, the rucaparib-sensitive cell lines showed a higher increase of DNA DSB after exposure to 2 Gy IR than resistant cell lines (Fig. 4(b) \( P = 0.05 \)).

We hypothesized that the treatment of cells with rucaparib would lead to an increase of γH2AX foci formation designating DNA DSB in sensitive cell lines and to a constant level of γH2AX foci in resistant cell lines. Indeed, in sensitive cell lines the average percentage gain of median number of foci per nucleus ranged between 11.7% and 70.0% compared to DMSO control. We observed a significant increase of γH2AX foci formation in two rucaparib-sensitive cell lines (Detroit562: \( P = 0.01 \), HN4: \( P = 0.03 \) Fig. 4(c)). In resistant cell lines, γH2AX foci formation response was stable in two cell lines and decreased in the other (Fig. 4(c)). Interestingly, the difference in DNA DSB formation between sensitive and resistant groups after rucaparib exposure was significant \( (P = 0.03) \).

Overall, we were able to show that exposure of cells to 2 Gy IR lead to significant increases in γH2AX foci formation across all cell lines. In rucaparib-sensitive cell lines, the number of γH2AX foci increased after exposure to rucaparib as well. Conversely, in rucaparib-resistant cell lines, the foci formation after rucaparib-treatment by trend remained constant. Importantly, the rucaparib-sensitive and -resistant groups showed significant differences in γH2AX foci formation both after treatment with rucaparib and 2 Gy IR. This points towards their differing ability to repair DNA DSB and therefore their susceptibility to PARPi. We investigated...
this further by gene expression analysis of these cell lines using microarrays.

Expression analysis

We investigated the association of baseline expression of a number of genes thought to be important for HR (e.g. BRCA, EMSY, PTEN) with rucaparib sensitivity. Of these, only RAD51 showed evidence of association, with a nominal $p$-value of 0.04, although this value was not significant after correction for multiple testing. However, we observed a positive correlation ($r_p = 0.58$) between RAD51 baseline expression and rucaparib log IC50 (Fig. 5).

We performed correlation tests of rucaparib IC50 against 9138 genes measured by microarray (see Methods). This analysis revealed only one gene of borderline significance genome-wide (IL-18) after correction for multiple testing ($P = 9.9 \times 10^{-6}$ $P_{adj} = 0.09$). However, correlation for the top 10 genes was high ($r_p = 0.95$) suggesting that this analysis was underpowered (Supplementary Table S3). The low level of enrichment is unsurprising given the small number of samples, thus, we hypothesized that a gene set analysis may improve power to detect genuine biological signal. Hence, we tested for enrichment of Gene Ontology (GO) terms among the 100 genes most positively or inversely correlated with rucaparib sensitivity (see Methods and Supplementary Table S4). Interestingly, we found that GO terms associated with chromosomal structure were highly significantly enriched among genes that were inversely correlated with rucaparib sensitivity (Table 1 & Supplementary Table S5). Encouragingly, no additional gene sets were identified and this result is strongly consistent with our immunofluorescence data, where we showed that rucaparib-resistant cell lines show less DNA DSB after exposure to IR and rucaparib compared to rucaparib-sensitive cell lines (Fig. 4). This suggests that there are differences in chromosomal stability among HNC cell lines (possibly due to HRD) that may cause PARPi sensitivity.

Undoubtedly, reliable and accurate methods to predict sensitivity to new targeted agents like rucaparib are needed. We applied several established methods to construct a gene signature, for example, PAM, lasso, ridge and ElasticNet regression, but none of these methods were significant in leave-one-out cross validation (LOOCV) testing [38–40], although our limited sample size was likely a factor. However, the strong enrichment of GO terms related to chromosome structure suggests that these genes may play an important role and provides evidence that it may be possible to predict PARPi sensitivity from baseline gene expression levels given a better-powered dataset.

![Image 1](https://example.com/image1.png)

![Image 2](https://example.com/image2.png)

![Image 3](https://example.com/image3.png)
values for rucaparib. We found a subset of HNC lines that appear to be well tolerated and should be further investigated effective as a single agent (without additional cytotoxic agents), [43–45]. Together with our results, this suggests that rucaparib is 360 mg for veliparib, olaparib and rucaparib, respectively compounds used in current clinical trials are 60 mg, 400 mg and lines. Interestingly, the determined maximal tolerated dose of the results suggest that rucaparib has the largest effect on HNC cell potency of the evaluated PARPi differed significantly and our process.

IC50 We therefore chose the colony-forming assay to establish robust for DNA damage to accumulate, thus yielding unreliable results. The cancer genome project recently screened a panel of almost 700 cell lines for sensitivity to 138 compounds, including rucaparib in particular for HNC. Identification of more robust prediction assay proposed by Mukhopadhyay et al for the above-mentioned assay [48]. However, the prediction accuracy of this assay using post-treatment RAD51 foci formation was not recapitulated in HNC. While these results do not unequivocally imply that RAD51 is unrelated to variability in rucaparib response (Fig. 5), it strongly suggests that other biomarkers will be necessary to robustly predict drug response in HNC. Interestingly, we were able to show significant differences in γH2AX foci formation both, after exposure to IR and to rucaparib between sensitive and resistant cell lines, suggesting that there are differences in the tendency of chromosomes to accumulate DSB. This was further supported by strong enrichment of genes involved in chromosome structure among the set of genes most highly correlated with rucaparib resistance, lending support to the hypothesis that these genes may be potential candidate biomarkers for rucaparib response in HNC.

Conclusions

In conclusion, we demonstrated that HRD is present in HNC, both in patient samples and HNC cell lines, which were susceptible to the PARPi rucaparib to the same extent as BRCA-deficient BC cell lines (and independent of BRCA mutations). These results have important implications for future investigation of PARPi in general and rucaparib in particular for HNC. Identification of more robust predictive biomarkers will be essential and ongoing efforts should be applied to head and neck squamous cell tumors [25–27].

Conflict of interest statement

All authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.oraloncology.2014.06.004.

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