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Improved pharmacodynamic (PD) assessment of low dose PARP inhibitor PD activity for radiotherapy and chemotherapy combination trials

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ABSTRACT

Background: PARP inhibitors are currently evaluated in combination with radiotherapy and/or chemotherapy. As sensitizers, PARP inhibitors are active at very low concentrations therefore requiring highly sensitive pharmacodynamic (PD) assays. Current clinical PD-assays partly fail to provide such sensitivities. The aim of our study was to enable sensitive PD evaluation of PARP inhibitors for clinical sensitizer development.

Material and methods: PBMCs of healthy individuals and of olaparib and radiotherapy treated lung cancer patients were collected for ELISA-based PD-assays.

Results: PAR-signal amplification by *ex vivo* irradiation enabled an extended quantification range for PARP inhibitory activities after *ex vivo* treatment with inhibitors. This "radiation-enhanced-PAR" (REP) assay provided accurate IC50 values thereby also revealing differences among healthy individuals. Implemented in clinical radiotherapy combination Phase I trials, the REP-assay showed sensitive detection of PARP inhibition in patients treated with olaparib and establishes strong PARP inhibitory activities at low daily doses.

Conclusions: Combination trials of radiotherapy and novel targeted agent(s) often require different and more sensitive PD assessments than in the monotherapy setting. This study shows the benefit and relevance of sensitive and adapted PD-assays for such combination purposes and provides proof of clinically relevant cellular PARP inhibitory activities at low daily olaparib doses.

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Pharmacodynamic (PD) assays that measure the effect(s) of a drug on its specific biological target(s) are essential for clinical drug development. PD data support clinical decision making concerning the optimal dose and duration of a drug therapy and, in case of combination treatment strategies, the optimal dosing and sequence of the different components [1,2]. Furthermore, it may allow individualized treatment selection or adaptation if drug responses vary among patients.

Next to established monotherapy indications in certain cancers, PARP (poly(ADP-ribose) polymerase) inhibitors are promising anticancer drugs to be combined with chemotherapy and/or radiotherapy. A range of different PARP inhibitors is currently under clinical evaluation, of which olaparib (Lynparza™) is the first FDA and EMA approved drug. PARP is an enzyme involved in the repair of DNA lesions such as single-strand breaks. Upon its activation, PARP produces poly(ADP-ribose)-polymers (PAR) at the expense of nicotinamide adenine dinucleotide (NAD). PARP inhibition by pharmaceutical drugs decreases PAR levels in cells. Several PD assays that measure PARP activity in tumour cells and in peripheral blood mononuclear cells (PBMCs) have been used in clinical studies [3–12].

Currently, there is only one clinically validated PD assay that quantifies basal PAR levels (generated by PARP through endogenously induced DNA damage), both in PBMCs and tumour cells, using an ELISA based method [6–8]. Through extensive NCI (US National Cancer Institute) studies optimizing its clinical application, this assay was able to support the clinical development of PARP inhibitors [13–17]. Especially in PBMCs, however, basal PAR levels can be low with a high day-to-day variation within the same individual [6,7], making sensitive quantification of PAR levels upon

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PARP inhibition difficult. Applicability of this PD assay is therefore limited to patients with sufficiently high PAR levels and it can be difficult to detect clinically relevant reductions in PAR levels. This is particularly important in the development as sensitizer in combination treatments, as PARP inhibitors have been shown to be effective chemo- and radiosensitizers at much lower concentrations and shorter duration [18,19]. Thus, sensitive and robust PD assessments are needed in such combination trials.

The aim of our study was to evaluate PARP inhibition at low drug dose levels for novel combination trials and therefore to achieve highly sensitive quantification of PAR levels and PAR reduction due to PARP inhibition by a clinically applicable PD assay.

Material and methods

Healthy individual and clinical trial patient material

Healthy individual (HI) blood was drawn in the morning after 1 h fasting; informed consent was obtained. Clinical PD data are from non-small cell lung cancer (NSCLC) patients included in a clinical Phase I trial (NCT01562210). Patients were treated with a radical standard of care chemoradiotherapy schedule (66 Gy with daily cisplatin at 6 mg/m^2) combined with olaparib 25 mg bidaily (tablet formulation). The study was performed according to ICH-GCP guidelines after approval by the hospital's ethics committee and national regulatory body. All patients gave written, informed consent prior to undergoing study-related procedures. Patient blood samples were collected prior and during treatment at a steady-state minimal concentration (C-min) level of olaparib and after completion.

Radiation and reagents

PBMCs were exposed to radiation using the Gammacell®40-Exactor (Best Theratronics Ltd., Ottawa, Canada). Olaparib for the *ex vivo* analyses in HI was purchased from Sequoia Research Products (Pangbourne, UK). Niraparib was kindly provided by the Slotervaart-Pharmacy (Amsterdam, The Netherlands). Stock-solutions were prepared in dimethylsulphoxide at a concentration of 5 mM (olaparib) and 0.31 mM (niraparib).

PBMC lysate preparation

Cell lysates were prepared following the NCI advised protocol for clinical use [8] with some minor adaptations as listed in Sup. Table S1. Samples prepared in the 'REP-assay' (Radiation Enhanced Assay) were irradiated with 8 Gy on ice and incubated for 1 h on ice. Intact PBMCs in plasma of healthy (and untreated) individuals were incubated *ex vivo* with olaparib and niraparib at 37° C 1 h before irradiation. Olaparib plasma levels and protein-binding after *ex vivo* incubation were comparable to the clinical situation, as determined by HPLC-MS/MS (Sup. Fig. S1).

PAR assay

Cellular PAR levels were measured by using the HT-PARP *in vivo* Pharmacodynamic Assay II, following the NCI protocol [8] using a Tecan-Infinite-200-Pro. Plates and kits were provided by Trevigen®.

Data analysis and statistics

PAR levels (presented in pg/1E7 PBMCs) were calculated from the linear fits of the PAR standard curves. All further quality control steps and criteria were followed as recommended in the NCI protocol [8] and are specified in Sup. Table 1 together with lower limit of quantification (LLOQ) definition and IC50 (half maximal inhibitory concentration) and E-max (maximal effect) calculations [20]. One-way ANOVA tests and Tukey's multiple comparison test were used and differences were considered significant if P-value < 0.05.

Results

Basal PAR levels in PBMCs

PAR levels in peripheral blood mononuclear cells (PBMCs) of 10 healthy individuals (HIs, Sup. Table S2) were quantified following the clinically validated NCI developed method [8], herein termed NCI-protocol. Consistent with literature [6,16], using this NCI-protocol (i.e. without an *ex vivo* irradiation step) we found a wide range of PAR levels among different HIs: 52–145 pg/1E7 cells with a median of 107 pg/1E7. Basal PAR levels were quantifiable in all. It has been suggested that a minimum of 90% inhibition is required for efficient monotherapy activity [3]. Assuming efficient inhibition, a simulated 90% reduction of PAR levels would only be quantifiable in one out of ten HIs. Our data suggest that basal PAR levels in PBMCs are often too low and too close to background levels to quantify PAR reduction accurately.

PAR amplification after ex vivo irradiation

To amplify the low basal PAR signal, which is based on low levels of endogenous DNA damage, we induced DNA damage by *ex vivo* irradiation. PAR levels increased after irradiation and rapidly decreased with increasing incubation times (Fig. 1A). After incubation on ice, however, PAR level values increased and stabilized after 1 h (Fig. 1B). Radiation increased PAR levels in a dose-dependent and linear manner up to 32 Gy (Fig. 1C). *Ex vivo* irradiation of PBMCs did not negatively influence the stability of PAR levels in cell lysates (Sup. Fig. S2). For subsequent analyses with this assay, termed REP (Radiation-Enhanced-PAR) assay, we chose a radiation dose of 8 Gy followed by an 1 h incubation on ice that provides sufficient PAR signal amplification within a reasonable radiation time-frame. We conclude that this strongly amplifies PAR signal in a strictly linear manner in PBMCs and could therefore enable the sensitive quantification of changes in PAR levels by inhibitors.

Development of the REP-assay

We next compared PAR levels induced by *ex vivo* radiation in a panel of HIs and in different blood samples of the same HI. We found a strong induction of PAR levels in all HIs (Fig. 2A and B). PAR induction rates varied among HIs (range 114–308 pg PAR/1E7 cells per Gy, Sup. Fig. S3). Nevertheless, the day-to-day variation in PAR levels was smaller in all but one HI in the REP-assay than when following the NCI-protocol (Sup. Fig. S4). PAR reduction of 90% would be quantifiable in all HI and of 99% in some HI (Fig. 2C). From this we predict that *ex vivo* irradiation enables accurate quantification of PAR levels in PBMCs even upon efficient PARP inhibition in almost all individuals.

PARP inhibition dynamics after ex vivo irradiation

To explore this further, we incubated intact PBMCs from HIs *ex vivo* with the PARP inhibitor olaparib at different concentrations. Olaparib inhibits radiation-induced PAR formation at all tested radiation doses (Fig. 3A). The relative inhibition values are similar in non-irradiated and irradiated PBMCs. There is also no apparent difference in the inhibition values at different radiation doses (Fig. 3B). Hence, there is no apparent interaction between radiation dose and PARP inhibitor activity.

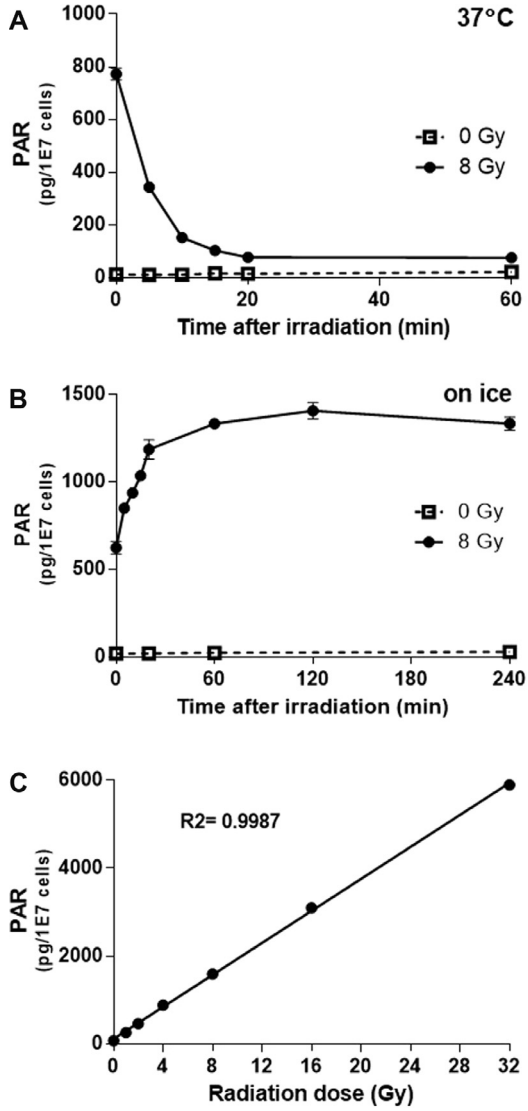

Fig. 1. PAR level kinetics after *ex vivo* irradiation and incubation, and linear radiation dose dependent induction of PAR. (A and B) PAR levels at indicated incubation times at 37 °C and on ice after 0 Gy and 8 Gy *ex vivo* irradiation. (C) PAR levels after different radiation doses and 1 h incubation on ice (linear regression $P < 0.0001$). Data represent mean ± SD of triplicate ELISA measurements.

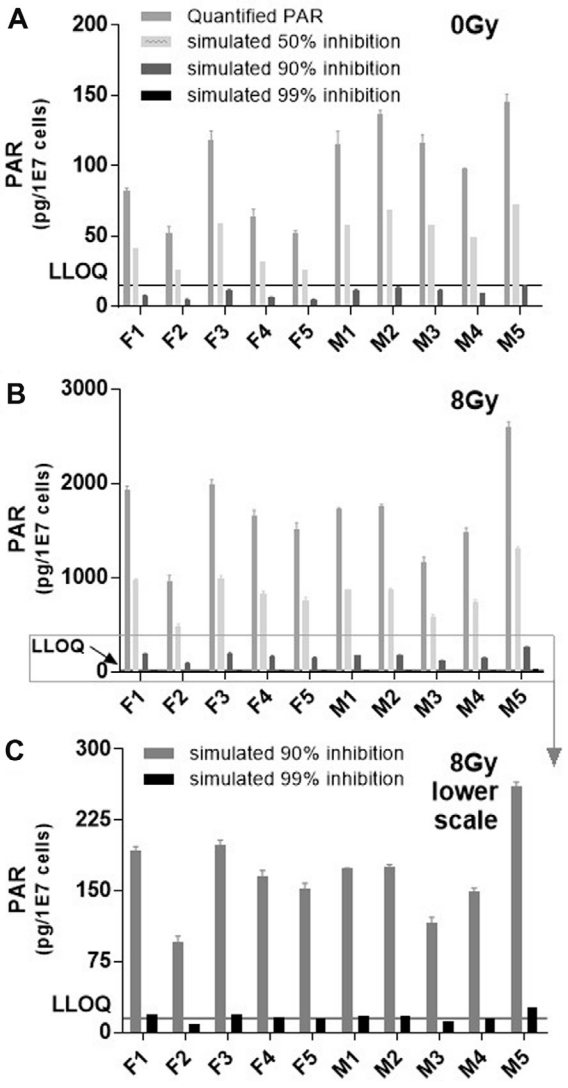

Fig. 2. PAR signal amplification by irradiation enables better the quantification of its inhibition. (A) PAR levels in PBMCs are often too low to quantify a reduction by PARP inhibitor treatment. Quantified PAR levels in PBMCs of 10 HI (F = female, M = male). Simulated 50%, 90% and 99% inhibition and the lower limit of quantification (LLOQ) are also depicted. All 99% inhibition values fall under the LLOQ. (B) The REP-assay was tested in parallel to the NCI-protocol in PBMCs isolated from the same blood samples of the same ten HIs as shown in A. Quantified PAR levels in PBMCs after 8 Gy (i.e. according to the REP-assay) varied among the HIs (median: 1696, range 962–2607 pg/1E7 cells). Simulated inhibition values of 90% and 99% can be detected and quantified in all HIs and 8 and 10 HIs respectively. (C) Reduction of *Y*-axis scale with data from (B) to visualize the lower limit of quantification (LLOQ). Using the REP-assay, PAR reduction of 90% and 99% would be quantifiable (i.e. above the LLOQ) in all HIs and in eight out of the ten HIs, respectively. Data represent mean ± SD of triplicate ELISA measurements.

Maximal PARP inhibition by olaparib

As calculated above, following the NCI-protocol, determination of inhibition levels above 90% are not possible in most individuals. To assess the maximal inhibitory effect of olaparib, we treated PBMCs *ex vivo* with up to 10 μM olaparib (comparable to the maximum concentration at monotherapy doses, [5]) at maximal PAR induction (32 Gy). We found that we could still quantify remaining PAR-levels in all HIs, which were reduced to 0.7% (Fig. 3C). Overall, cellular PARP inhibition was high at this concentration for all HI (>99%), therefore also suggesting consistently strong activities at olaparib concentrations that are clinically relevant for monotherapy settings.

Olaparib activities among HI

The previous data (Fig. 3), suggested some difference in the PD response in different individuals. To investigate this further, we next determined the olaparib concentration dependent PAR response in a group of HIs while directly comparing the performance of the REP-assay with the NCI-protocol. In all HIs, olaparib reduced PAR levels in a concentration dependent manner (Fig. 4A–D). We further determined absolute half maximal inhibitory concentrations (IC50s; Sup. Table S3, Fig. 4E and F). We found that IC50 determinations are inaccurate or impossible when omitting the *ex vivo* irradiation (Sup. Table S3), thereby demonstrating the improvement over the NCI protocol. The day-to-day variation in the IC50 values of each HI was also lower in the REP-assay.

Importantly, the REP-assay revealed significant differences in IC50s between individuals. This remained undetected using the NCI-protocol. Sensitivity analysis, e.g. calculation of a relative IC50 instead of an absolute IC50 [20], confirms this (Sup. Fig. S5) and shows that by optimizing the assay we were able to accurately determine olaparib responses in all HIs and to detect individual differences.

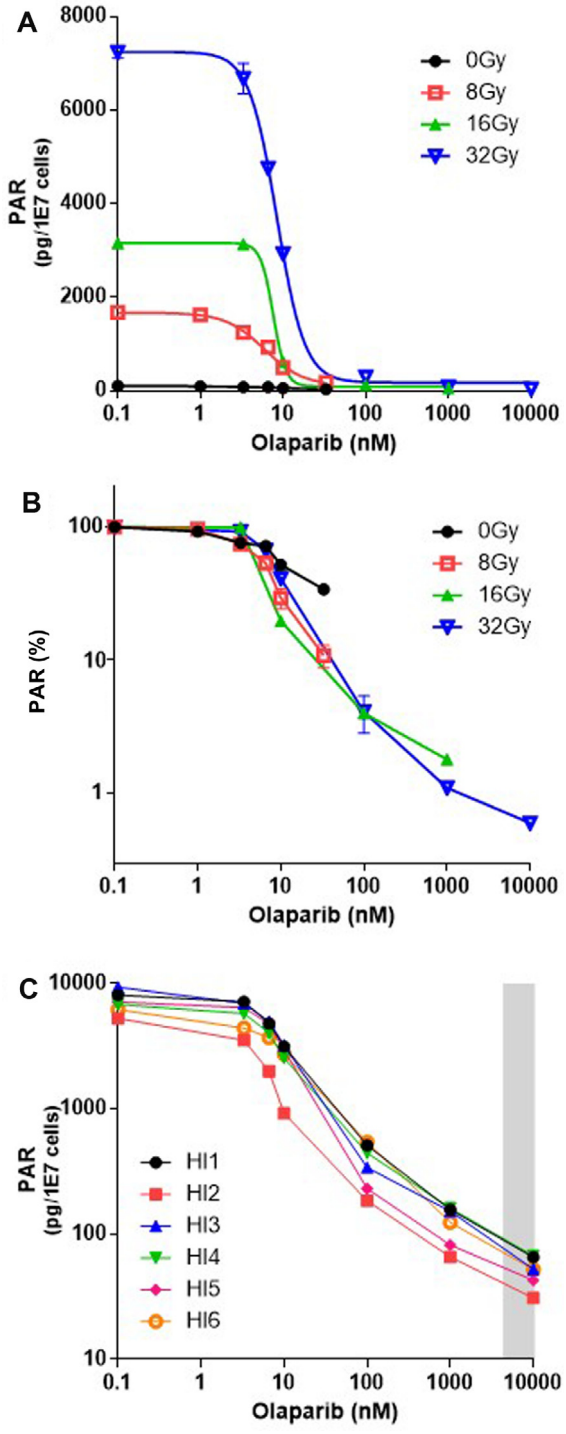

Fig. 3. Determination of the maximal inhibitory effect of olaparib. (A) PAR levels determined in PBMCs of a HI after 1 h *ex vivo* pre-incubation with olaparib at indicated concentrations, *ex vivo* irradiation with 0, 8, 16 and 32 Gy and 1 h incubation on ice. (B). PAR levels as of (A), normalized to the PAR levels of the control treated samples (0 nM olaparib). At 33 nM, PAR levels were reduced to 34% in non-irradiated and to 11% in irradiated PBMCs with similar relative inhibition levels at higher radiation doses. (C) To enable the quantification of maximal inhibitory effect of olaparib and such extensively reduced PAR levels by PARP inhibition, PBMCs were irradiated *ex vivo* with 32 Gy for maximal PAR induction. PAR levels of six HIs determined after 1 h *ex vivo* pre-incubation with olaparib at different concentrations, *ex vivo* irradiation with 32 Gy and 1 h incubation on ice are shown. The grey bar indicates the plasma concentration range of olaparib that was found in plasma of patients that were treated at monotherapy doses in clinical trials [5]. PAR levels were reduced to 0.7% (range 0.6–1.0%) at 10 μM olaparib. Data represent mean ± SD of triplicate ELISA measurements. *Note*: PAR levels of control-treated (0 nM olaparib) samples are plotted at 0.1 nM.

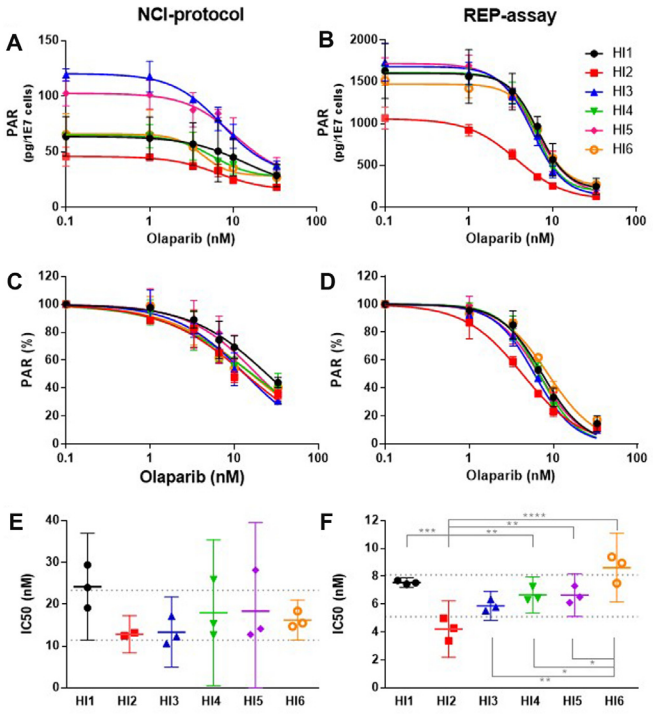

Fig. 4. PARP inhibitor activity is largely consistent but the effectiveness can differ twofold among healthy individuals. (A and B) PBMCs were isolated from 6 HIs at three different days and treated *ex vivo* at increasing olaparib concentrations. PAR levels after *ex vivo* incubation with olaparib 1 h before (sham-)irradiation determined with the NCI-protocol and the REP-assay are shown. Data represent mean ± SD of three independent blood samples. (C and D) To compare relative olaparib responses PAR levels were normalized. PAR levels of figures A and B minus the relative E-max of that individual HI and normalized to control treated samples (0 nM olaparib) are shown. (E and F) IC50 as calculated from figures C and D. The REP-assay revealed up to twofold significant differences in absolute IC50s between individuals (range 4.2–8.6 nM). Dotted lines indicate mean ± SD of IC50s of all HIs. Level of significance as tested by one-way ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Data represent mean ± SD (A–D) or mean ± 95%CI (E and F) of three independent blood samples. *Note*: PAR levels of 0 nM olaparib treated samples are set at 0.00001 nM to calculate fits, however, are plotted at 0.1 nM.

Comparison of different PARP inhibitors

This prompted us to test whether this was drug/compound-dependent. To compare within the same individual, we added different concentrations of olaparib and niraparib *ex vivo* to the PBMCs and chose the HIs with the highest and lowest olaparib IC50s. Consistent with the prior results only the REP-assay yielded accurate IC50 determinations (Sup. Tables S3). We found that the concentration dependent response of olaparib and niraparib was similar (Fig. 5 and Sup. Table S3) and confirmed a significant difference in IC50 between the two HIs (1.8/1.7-fold for olaparib/niraparib) thereby showing a yet unidentified significant variable response to PARP inhibition between different individuals.

REP-assay in patients treated with olaparib

Confirmation of efficient cellular PARP inhibition has been in particular challenging at low drug dose levels such as administrated in Phase I trials and tested in combination treatments. We therefore implemented the REP-assay in a Phase I trial (NCT01562210) for PD analyses on blood samples from NSCLC patients at the lowest dose level of olaparib (i.e. 25 mg bidaily) and treated with concurrent chemoradiation (Sup. Table S4).

Standard NCI-protocol PD revealed a reduction in PAR during 25 mg bidaily olaparib treatment (Fig. 6). The same blood samples assayed according to the REP-assay, however, show strong

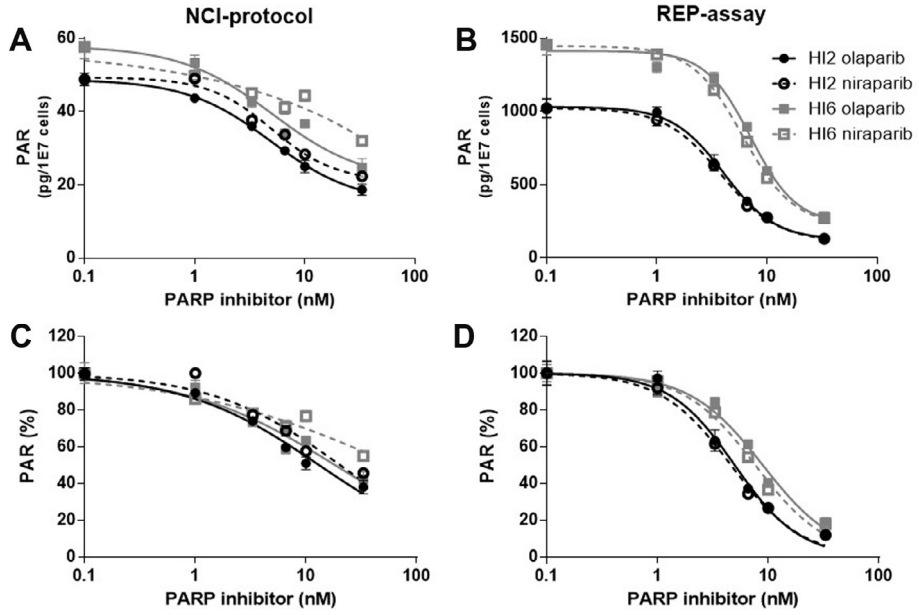

Fig. 5. Comparison of individual *ex vivo* responses to different PARP inhibitors incubation. (A and B) PAR levels in PBMCs of two HIs after *ex vivo* incubation with the PARP inhibitors olaparib and niraparib at different concentrations 1 h before (sham-)irradiation, as determined with the NCI-protocol and REP-assay. All samples for both, olaparib and niraparib treatment, were taken from the same blood sample. (C and D) PAR levels of figures A and B minus the relative E-max of that individual HI normalized to control treated samples (0 nM olaparib). Data represent mean ± SD of triplicate ELISA measurements. *Note*: PAR levels of 0 nM olaparib treated samples are set at 0.00001 nM to calculate fits, however, are plotted at 0.1 nM.

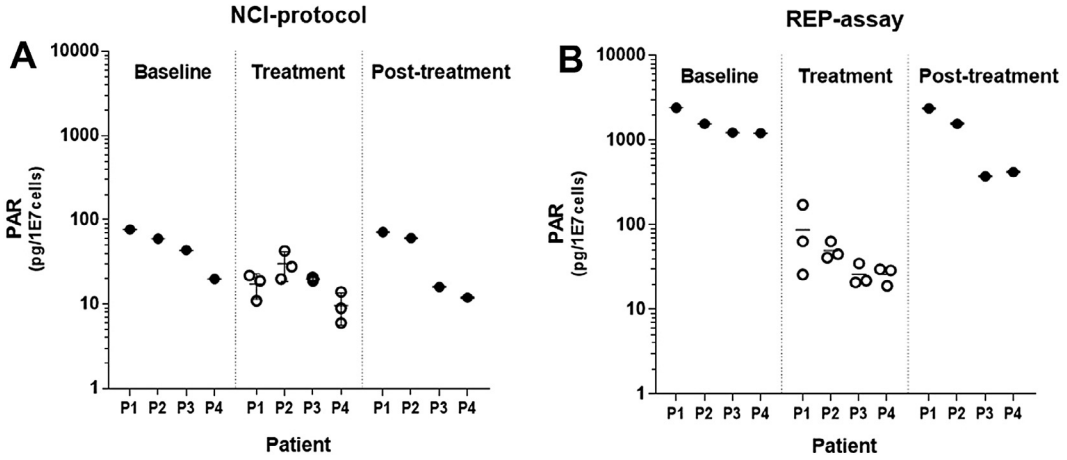

Fig. 6. REP-assay detects strong inhibitor activity at low olaparib doses in NSCLC patients treated with olaparib combined with concurrent chemoradiation in clinic. Blood was drawn from four NSCLC trial patients and assayed in parallel according to the NCI-protocol (A) or REP-assay (B) that applies 8 Gy irradiation as indicated. PAR levels of three blood samples from patients 1–4 under 25 mg bidaily olaparib treatment (open circles) compared to two baseline values (closed circles) are shown. Similar as in the healthy individuals, baseline PAR levels in patients varied (20–77 pg/1E7 cells in the NCI-protocol) and were strongly induced in all patients (1214–2426 pg PAR/1E7 cells per Gy) in the REP-assay, with an average PAR-induction of 195 pg PAR/1E7 cells per Gy. The REP-assay was able to detect strong inhibitory activity in this patient cohort with a 25 mg bidaily dose.

reductions in all patients. The REP-assay revealed a strong median inhibition capacity of 97% (range 96–98%) compared to 53% (range 49–77%) by the NCI-protocol. Using the REP-assay we are able to affirm 25 mg olaparib as biologically effective dose (Fig. 6). Together these data, enabled by the REP-assay, demonstrate a strong cellular inhibition of PARP at low doses in the clinic.

Discussion

We developed and validated a clinical pharmacodynamic assay for the sensitive quantification of PAR levels and PAR reduction upon PARP inhibition in peripheral blood mononuclear cells (PBMCs). As radiation induces DNA damage which consequently increases PAR levels [19,21], we hypothesized that *ex vivo* irradiation of PBMCs will amplify the PAR signal allowing sensitive quantification of PAR levels and explored this further. Indeed, *ex vivo* irradiation strongly induced PAR levels in PBMCs and thereby allowed sensitive and otherwise difficult quantifications of olaparib and niraparib responses in healthy individuals (HI) and patients. Due to the increased sensitivity, we were able to detect yet unidentified differences in PARP inhibitor responses among individuals. The REP-assay also extends the applicability to almost all patients. The greatly increased sensitivity enables clinical studies that could correlate individual patient PD values with individual PARP inhibitor drug responses such as toxicity and efficacy. Importantly, the REP-assay provided evidence of high inhibitory

activity at low olaparib dose levels in clinical trials developing chemoradiotherapy combinations.

PAR induction by *ex vivo* irradiation proved to be a crucial element in the assay optimization. We found a consistent linear PAR-induction in all HIs and observed inter- and intra-individual variations in the slope of the linear PAR-induction upon irradiation (median%CV of 23% and 20% respectively). PARP inhibition responses by two different drugs is, however, very similar further confirming the consistent performance of the REP-assay. Minimizing technical variation in our REP-assay while taking into account day-to-day variations, we discovered different responses to PARP inhibition in healthy individuals upon *ex vivo* PARP inhibitor treatment. Possible explanations for such variabilities include: differences in metabolic state [22], differences in PARP-1 protein expression levels [23], single nucleotide polymorphisms affecting PARP activity [24,25] and differences in DNA damage response efficiency or in the expression of the drug efflux pump P-glycoprotein [26]. Consistent with our findings, individual differences have been found in a study assessing olaparib treatment in breast cancer patients. Using an extracellular PARP activation assay and different from assessing PAR formation in intact cells, Bundred et al. [4] reported a considerable variation in PARP pharmacodynamics at similar pharmacokinetic values, suggesting such a differential individual response. It remained unclear whether these differences were due to technical or day-to-day variation or true significant differences in PARP inhibitor response. Notably, these differences in IC50, that we observed here, translate to different inhibitor doses required for full inhibition (Fig. 3C). Used as sensitizer, we have also shown a gradual and potent olaparib concentration dependent potentiation of radiation induced kill before [19], further arguing for personalized treatment.

The REP-assay enabled us to show high drug activity (up to 90%) at low PARP inhibitor concentrations as low as 33 nM that translates to a dose of just 10 mg olaparib in clinic (after a single capsule administration) [5]. Indeed the clinical trial data confirm that an olaparib dose of 25 mg bi-daily (tablet formulation) can reach inhibition levels of over 95% in patients, a dose 10-fold lower than the recommended monotherapy dose [27]. Our findings are of clinical importance as they warrant careful dose escalation in combination trials. Furthermore, this stresses the necessity of highly sensitive PD assays, such as this REP-assay, in the clinical development of combination treatments, in particular, in choosing optimal schedules and doses. Future application opportunities of the REP-assay include the possibility to monitor drug uptake issues that can be caused by food/absorption differences (e.g. due to gastrointestinal toxicities during treatment) and clinical studies that associate individual patient pharmacodynamics with toxicities. The same principle of signal enhancement by *ex vivo* irradiation could be applied to tumour cells, enabling clinical association studies with tumour response.

In summary, due to its increased sensitivity and quantification accuracy the REP-assay revealed individual differences in pharmacodynamic responses of PARP inhibitors and showed high activities at low drug doses in clinic. These results therefore support clinical development of combination treatments with PARP inhibitors at lower doses.

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Conflict of interest statement

J.G. is an employee of Trevigen, Inc. All other authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.radonc.2017.10.017.

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