Poly (ADP-ribose) polymerase inhibition enhances trastuzumab antitumour activity in HER2 overexpressing breast cancer

Jetzabel García-Parra, Alba Dalmases, Beatriz Morancho, Oriol Arpí, Silvia Menendez, Mohammad A Sabbaghi, Sandra Zazo, Cristina Chamizo, Juan Madoz, Pilar Eroles, Sonia Servitja, Ignasi Tusquets, Jose Yelamos, Ana Lluch, Joaquín Arribas, Federico Rojo, Ana Rovira, Joan Albanell

Aim: Poly (ADP-ribose) polymerase (PARP) inhibitors have shown promising results in Breast Cancer (BRCA) deficient breast cancer, but not in molecularly unselected patient populations. Two lines of research in this field are needed: the identification of novel subsets of patients that could potentially benefit from PARP inhibitors and the discovery of suitable targeted therapies for combination strategies.

Methods: We tested PARP inhibition, alone or combined with the anti-HER2 antibody trastuzumab on HER2+ breast cancer.
olaparib and rucaparib, as well as genetic downmodulation of PARP-1 for in vitro studies. DNA damage was studied by the formation of γH2AX foci and comet assay. Finally, the in vivo anti-tumour effect of olaparib and trastuzumab was examined in nude mice subcutaneously implanted with BT474 cells.

**Results:** In a panel of four HER2 overexpressing breast cancer cell lines, both olaparib and rucaparib significantly decreased cell growth and enhanced anti-tumour effects of trastuzumab. Cells exposed to olaparib and trastuzumab had greater DNA damage than cells exposed to each agent alone. Mechanistic exploratory assays showed that trastuzumab downmodulated the homologous recombination protein proliferating cell nuclear antigen (PCNA). Combination treatment in the BT474 xenograft model resulted in enhanced growth inhibition, reduced tumour cell proliferation, and increased DNA damage and apoptosis.

**Conclusion:** Taken together, our results show that PARP inhibition has antitumour effects and increases trastuzumab activity in HER2 overexpressing breast cancer. These findings make this novel combination a promising strategy for clinical development.

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### 1. Introduction

Poly (ADP-ribose) polymerase (PARP) inhibitors target poly (ADP-ribose) polymerase (PARP) family members, most notably PARP-1 and PARP-2. These two enzymes regulate gene transcription, genome stability, apoptosis, angiogenesis and DNA damage repair. This latter role has been the basis for studying PARP inhibitors in tumours with defective DNA repair mechanisms [1]. Clinical studies with the PARP inhibitor olaparib have reported antitumour activity only in patients with Breast Cancer (BRCA) mutant tumours [2]. Therefore, identification of additional groups of patients that might benefit from PARP inhibitors is needed [3]. One strategy is to combine PARP inhibitors with traditional cytotoxic agents, but these combinations are toxic [3]. In contrast, the combination of PARP inhibitors with other targeted agents may be better tolerated than with chemotherapy. Along this line, two studies show that blockade of PI3K impairs homologous recombination (HR) and sensitises triple negative breast cancer to PARP inhibitors [4,5].

Here, we hypothesised that PARP inhibition could enhance the antitumour activity of the anti-HER2 anti-body trastuzumab in HER2+ breast cancer. Trastuzumab is used in the treatment of the vast majority of HER2+ patients, albeit resistance inevitably occurs in the metastatic setting. Therefore finding novel drug partners may help to circumvent, at least temporarily, this problem. Two observations supported the testing of a combination of trastuzumab plus PARP inhibitors in HER2+ breast cancer. First, trastuzumab causes DNA damage [6,7] and modulates the transcription of genes involved in DNA repair [8,9]. Second, recent reports indicate that PARP inhibition has antitumour effects in HER2+ breast cancer cells [10-13], albeit one study had discordant results [14]. To further address this issue and provide novel data with potential clinical impact, we performed a study of trastuzumab combined with PARP inhibition in HER2+ breast cancer.

### 2. Material and methods

#### 2.1. Cell lines and reagents

Breast cancer cell lines BT474, SKBR3, AU565, MCF-7 and EFM129A were obtained from the American Type Culture Collection. Olaparib and rucaparib were from Selleck Chemicals and trastuzumab were from Selleck Chemicals and trastuzumab from Hospital del Mar pharmacy.

#### 2.2. Western blot

Western blots were performed according to standard protocols. The antibodies used were: HER2 (Biogenex), poly ADP-ribose (PAR) (BD Pharmingen), γH2AX (Ser139), proliferating cell nuclear antigen (PCNA), IKK-α, phospho-p65 (Ser536), and IκB-α (all from Cell Signalling) β-actin and β-tubulin (Sigma) and PARP-1 (Clone A6.4.12) [15].

#### 2.3. Real-time quantitative polymerase chain reaction (PCR)

Total RNA was isolated using RNeasy mini Kit (Qiagen). Primers were designed using DNAsStar Primer design (DNASTAR, Inc.) and the NCBI database for PCNA (NM_002592.2) Fw: 5′-GGAGAACTTGGAAAC-3′; Rv: 5′-CTGGTTCATTTCTCTATGG-3′; Ki67 (NM_002417.4) Fw: 5′-CAAGGAACTGGATTCAGGAGAAG-3′; Rv: 5′-GCTGTITTTGCTGATTCTGTG-3′ and RPLP0 (NM_001002.3) Fw: 5′-GCAGGTGTTCGACAATGGC-3′; Rv: 5′-CTGGCAAGTCCCGCAGACAC-3′; housekeeping gene. RNA was reversely transcribed to cDNA (High Capacity cDNA Reverse Transcription kit, Applied Biosystems).
Amplification was done in a Lightcycler480 R-PCR-System at 45 cycles.

2.4. PARP-1 knockdown

Stable PARP-1 knockdown (shPARP-1) and scrambled control (shCT) cell lines were generated using MISESION® short hairpin RNA (shRNA) lentiviral construct for PARP-1TRCN0000007931 (Sigma). Retroviruses was prepared by transfecting the shRNA transfer vector together with viral vectors using Polyethylenimine (PEI) (PolyScience) [16]. After puromycin selection, resistant cells were pooled.

2.5. Proliferation assays

For anchorage dependent clonogenic assays, cells were platted at clonal density in triplicates. Twenty-four hours later, olaparib, rucaparib, trastuzumab or solvent were added. Medium and drugs were refreshed every three days. Cell colonies were stained with crystal violet. Blue intensity was measured in arbitrary units using ImageJ. Treatments were performed in duplicate, and experiments were repeated three times. When indicated, cell viability was measured by automated Scepter counting.

2.6. TUNEL assay

Apoptosis was assayed using the In Situ Death Detection Kit (Roche) according to the manufacturer’s instructions. Cells were seeded on chamber slides and the following day they were treated with olaparib (5 μM) and/or trastuzumab (20 μg/mL) for 96 h. Each condition was assessed in duplicate in three independent experiments.

2.7. Immunofluorescence (IF) detection of γH2AX-foci

Cells were washed and fixed according to standard procedures and stained with anti-γH2AX antibody for 1 h followed by Alexa555 anti-rabbit (Invitrogen) for 30 min.Slides were counterstained with 4’,6-diamidino-2-phenylindole (DAPI). Each treatment condition was assessed in duplicate and 300 nuclei were evaluated for γH2AX-foci presence. Data were obtained from three independent experiments.

2.8. Comet assay

The alkaline comet assay (Comet Assay kit, Trevigen) was carried out as per suppliers manual. At least 40 non-overlapping randomly selected comets were analysed. Each experiment was repeated three times.

2.9. In vivo xenografts

All animal work was conducted as per the PRBB Institutional Animal Care and Scientific Committee guidelines. Briefly, 46-week old female Balb/C nude mice were subcutaneously inoculated in their flank with 10 x 10^6 BT474 cells mixed with Matrigel as previously described [17]. Tumour growth was measured twice a week. Mice bearing subcutaneous 150–250 mm^3 tumours were distributed homogenously into four groups of 10 mice each. In the first group, olaparib (50 mg/kg) was inoculated intraperitoneally (i.p.) daily [18]. In the second group, trastuzumab (0.3 mg/kg) was inoculated i.p. every four days [19]. The third group received the combination of both drugs at the same doses. Trastuzumab and olaparib vehicle solvents (IgG1 Kappa 0.3 mg/kg and 10% 2-hydroxy-propyl-β-cyclodextrine/phosphate buffered saline (PBS), respectively) were also inoculated in the olaparib and trastuzumab groups, respectively. The last group received vehicle solvents with no active drugs. At the end of the experiment, tumours were harvested and formalin-fixed.

2.10. Immunohistochemistry

Three micrometer tumour tissue sections were immunostained [20,21] using the same antibodies employed for western blot, followed by incubation with an anti-rabbit Ig dextran polymer (Flex+, Dako) and 3,3’diaminobenzidine as chromogen in a Dako Link platform. Percentage of positive tumour cells was calculated by two investigators (SZ and FR).

2.11. Statistics

Statistical analysis was carried out with SPSS version 13.0 (SPSS, Inc.). One-way analysis of variance (ANOVA) was used to assess differences among treatment conditions and Tukey’s Post Hoc pairwise comparison was calculated. All statistical tests were conducted at the two-sided 0.05 level of significance.

3. Results

3.1. PARP inhibitors reduced growth, increased apoptosis and enhanced anti-tumour effects of trastuzumab in HER2+ breast cancer cell lines

We tested the drugs olaparib and rucaparib, which target PARP-1 and PARP-2. We decided to test two different PARP inhibitors to reasonably study whether effects on cell growth were class-dependent instead of drug dependent. We confirmed that both drugs inhibited PARP activity, as assayed by western blot of its product (poly ADP-ribose (PAR) levels), at starting concentrations of 300 nM of olaparib and 500 nM of rucaparib (Fig. 1A). The susceptibility of HER2+ cells to the PARP inhibitors was tested by anchorage dependent colony formation assays. This is a highly sensitive assay widely used to ascertain the potential sensitivity to
PARP inhibitors [12,14,22–24]. The final readout was done at two weeks by crystal violet followed by calculating the area of the colonies in each dish [25]. Olaparib significantly reduced clonogenic growth and PAR production at similar concentrations, and the effect was greater at higher concentrations (Fig. 1B). Of note, pharmacokinetic data from a phase I trial suggest that these are clinically achievable plasma concentrations [2]. We obtained similar results with rucaparib (Fig. 1B) [22,24]. However, we studied olaparib in greater detail since it was the drug furthest advanced in clinical development. Interestingly, recent data suggest that olaparib is the most specific PARP inhibitor among a panel of PARP inhibitors, that include among others rucaparib and veliparib [26].

We then tested the effects of these PARP inhibitors, alone or in combination with trastuzumab, in BT474, SKBR3, AU565 and EFM129A HER2+ breast cancer cell lines. Both PARP inhibitors reduced cell growth in the four lines and significantly increased the inhibitory effects of trastuzumab (Fig. 1C) in three of the four cell lines. In the non-HER2+ luminal breast cancer MCF7 cells, olaparib modestly decreased cell growth, but did not sensitise cells to trastuzumab (Fig. 1C, right panel). In BT474 cells, long-term trastuzumab exposure caused a profound reduction of cell viability and the addition of
olaparib did not enhance trastuzumab effects. Notably, short-term viability studies at 96 h showed enhanced antiproliferative effects with concomitant treatments both in BT474 and SK-BR3 cells (Fig. 1D). In addition, there was a significant increase in apoptosis when both drugs were combined (Fig. 1E). These experiments were performed at higher drug concentrations than the clonogenic assays since the readout was performed after a shorter time-exposure [14,27–29].

3.2. Genetic PARP-1 inhibition sensitised HER2+ cells to trastuzumab

We transfected a lentivirus carrying control or shRNA sequences targeting PARP-1 to BT474 and SKBR3 cells [16]. Both transduced HER2-overexpressing cells showed a profound reduction in PARP-1 protein compared with control cells, while PAR levels, a surrogate marker of PARP activity, were only slightly modified, perhaps due to the compensatory activity of other PARP enzymes (Fig. 2A). We observed a slight decrease in HER2 in PARP-1 depleted cells. This result was in line with the modulation of HER2 by PARP-1 in synovial cells [30]. Albeit PARP-1 inhibition slightly reduced HER2 levels, it appears unlikely that this is a key contributor to the enhanced effects of trastuzumab, since HER2 downmodulation might be reasonably excluded as a mediator of trastuzumab effects [31]. When we treated shPARP-1 or control cells (Fig. 2B) with trastuzumab, PARP-1 depleted cells were significantly more sensitive to trastuzumab than control cells.

3.3. The combination of trastuzumab and olaparib increased DNA damage

Trastuzumab partially inhibits DNA repair after chemotherapeutic and radiation treatments through down-regulation of different repair genes leading to accumulation of DNA strand breaks [6,7]. Moreover, HER2 depletion downregulates DNA repair mechanisms [30]. We assayed DNA damage in HER2+ cells exposed to olaparib or trastuzumab alone or in combination by determining percentage of cells exhibiting γH2AX foci, a biomarker of DNA double-strand breaks. The combination treatment significantly increased the number of γH2AX foci, in both BT474 and SKBR3 cells (Fig. 3A) [20]. Similar results were observed by assaying γH2AX by western blot (Fig. 3B). We also tested DNA damage by using comet assay. Combined treatment significantly increased comet head and tail morphology compared with each drug alone (Fig. 3C) [32].

The mechanisms of this potentiation are unknown. We explored two possibilities based on prior

Fig. 2. Poly (ADP-ribose) polymerase-1 (PARP-1) inhibition sensitised HER2 overexpressing cells to trastuzumab. (A) Total cell lysates from stable PARP-1 knockdown and scrambled control (shCT) cells were subjected to western blot analysis for PARP-1, PAR, and HER2 levels. β-tubulin was used as loading control. (B) Parental and genetically modified SKBR3 and BT474 cells were seeded in triplicate in 12-well plates at a density of 8,000 cells per well and allowed to adhere. Next day cells were treated with or without 15 μg/mL trastuzumab for 0, 3, 5, and 7 days. Cells were then harvested by trypsinisation and automatically counted using a Specter cell counter (Millipore) at the indicated time-points (days). Growth curves represent the average of three independent experiments. In Graph plots cell number at day 7 is represented. One-way analysis of variance (ANOVA) was used to assess differences between combined treatment conditions and Tukey’s Post Hoc pairwise comparison was calculated. In each case, the graph shows standard deviations and statistical p values among trastuzumab and trastuzumab plus olaparib groups.

n.s.: non-significant, *p < 0.05, **p < 0.01, ***p < 0.001.
Fig. 3. Combination of olaparib and trastuzumab increased DNA damage as assessed by phosphorylated H2AX (γH2AX) assays and Comet assay. Cells were treated with olaparib (5 μM) and/or trastuzumab (10 μg/mL) for 96 h. (A) Immunofluorescence (IF) detection of γH2AX-foci. Representative images of IF staining for γH2AX-foci (upper panel) and DAPI (lower panel). Scale bar, 50 μM. Those nuclei with ≥ 4 γH2AX-foci were considered as positive DNA-damaged cells. Each bar represents the mean ± S.D. of percentage of DNA-damaged cells from three independent experiments. (B) DNA-damage by western blot of γH2AX expression. Representative images from three different experiments are shown. Protein levels were quantified by densitometry. Data were normalised to β-tubulin and expressed as fold induction versus control arbitrarily set at 1. (C) Alkaline comet assay of BT-474 and SK-BR3 treated with trastuzumab (10 μg/mL) and olaparib (5 μM) for 48 h. Representative IF images from each condition are shown; IF (Top), images processed with ImageJ (Bottom) determining the amount of fluorescence (DNA) in tails versus nuclei. Scale bar, 50 μM. Data are presented in arbitrary units and each bar represents the mean ± S.D. amount of DNA in tails in each experimental condition. Each experiment was repeated three times. One-way analysis of variance (ANOVA) was used to assess differences between combined treatment conditions and Tukey's Post Hoc pairwise comparison was calculated. *p < 0.05, **p < 0.01, ***p < 0.001.
publications with these types of agents. First, it has been
described that trastuzumab down-modulates the expres-
sion of the proliferating cell nuclear antigen (PCNA) [9]
involved in homologous recombination (HR) [33]. We
observed PCNA downmodulation by trastuzumab at
mRNA and protein levels both in BT474 and SKBR3
cells (Fig. 4A and B). The decrease in PCNA occurred
at relatively early time points, preceding any change in
proliferation assayed by Ki67 (Fig. 4A). Olaparib did
not affect PCNA expression and the combination did
not further decrease PCNA compared to trastuzumab
alone. This effect of trastuzumab on PCNA is consistent
with a sensitisation effect of olaparib in HER2+ tumour
cells.

Another possibility was related to olaparib effects on
NF-κB, in line with a prior report with veliparib [12].
However, olaparib treatment did not modify the expres-
sion levels of the inhibitor IκBα, and there was a non-
significant trend towards decreased p-p65 and IKK-α
(Fig. 4), which are surrogate markers of NF-κB activa-
tion (Fig. 4C and data not shown). The lack of a potent
effect of olaparib on NF-κB is in contrast with a clear
effect reported with veliparib [12]. However, as men-
tioned earlier, olaparib is more selective that veliparib
[26] and this may explain this difference, at least in part.
As expected trastuzumab [34] did not affect NF-κB.
Thus based on these results, it appears unlikely that
NF-κB plays an important role, if any, in the observed
potentiation of olaparib and trastuzumab.

3.4. Olaparib enhanced trastuzumab anti-tumour effects
in vivo

We used BT474 xenografts to evaluate in vivo the
activity of trastuzumab plus olaparib compared to
each agent given alone (Fig. 5A). Results were consis-
tent with our in vitro data. At study termination, the
control group had a tumour size (mean ± SD) of

![Graphs showing PCNA expression](image)

**Fig. 4.** Proliferating cell nuclear antigen (PCNA) is downmodulated by trastuzumab and NF-κB signalling by olaparib. (A) Cells were treated with trastuzumab (10 l-g/ml) for 24 h. Left panel, PCNA mRNA was evaluated by real-time quantitative polymerase chain reaction (RT-PCR) analysis. Relative gene expression was calculated by cycle threshold (Ct) method. Graphs indicate the results obtained compared to control condition. Data are mean ± S.D. Right panel, time course analysis for Ki67 mRNA expression after trastuzumab treatment assessed by RT-PCR. (B) Cells were treated with olaparib (1 μM) and/or trastuzumab (10 l-g/mL) for 72 h. PCNA protein from cell lysates was evaluated by Western Blot. Actin was
used as loading control. (C) Cells were treated with olaparib (1 μM) and/or trastuzumab (10 μg/mL) for 24 h. Total cell lysates were subjected to western blot analysis for IKK-α, p-p65, and IκBα. Actin was used as loading control. Representative images from three different experiments are
shown. Protein levels were quantified by densitometry. Data were normalised to actin and expressed as fold induction versus control arbitrarily set
at 1. *p < 0.05, **p < 0.01, ***p < 0.001.
437 ± 256 mm$^3$, the trastuzumab group of 249 ± 198 mm$^3$, and the group of mice that received olaparib plus trastuzumab had a tumour size of 108 ± 92 mm$^3$. The difference was statistically significant when the combination was compared to trastuzumab alone ($p = 0.046$) or olaparib alone ($p = 0.001$). Furthermore, the combined treatment resulted in regressions of established tumour xenografts.

Tumour specimens collected at the end of the experiments were analysed by immunohistochemistry (Fig. 5B). The olaparib–trastuzumab combination significantly reduced proliferation (phosphorylated H3), enhanced apoptosis (cleaved caspase 3) and increased...
DNA damage in tumour cells (γH2AX-foci) compared with trastuzumab alone.

4. Discussion

Here we show that PARP inhibition has antitumour effects in HER2+ breast cancer and significantly augments the efficacy of trastuzumab both in vitro and in vivo. These results provide novel evidence on the potential benefit of combined treatment with trastuzumab and PARP inhibitors that merits further development.

The clinical use of PARP inhibitors is a subject of ongoing debate [35] but three lines of research are active: improving the identification of novel patient subsets that can benefit [3,36]; early incorporation of pharmacodynamic assays and discovery of suitable targeted therapies to be combined with PARP inhibitors [10,24,37,38]. Additionally, high PARP-1 expression has been associated to poor prognosis in breast cancer [15,39].

Our results show for the first time that trastuzumab potentiates the antitumour effects of PARP inhibitors, and also confirm and extend recent studies showing that HER2+ cells are sensitive to PARP inhibitors in vitro [13]. In one report, the PARP inhibitors veliparib and olaparib exhibited anti-proliferative effects [12]. In that study, veliparib effects were mediated, at least in part, by inhibition of NF-κB signalling, and augmented the in vitro cytotoxicity of lapatinib, a dual EGFR/HER2 tyrosine kinase inhibitor [12]. It has also been shown that PARP activity is essential for NF-κB activation following ionising radiation [40]. Other works reported that exposure of BT474 cells to olaparib resulted in dose-dependent cell death [11] or slightly increased apoptosis in a dose-dependent fashion [10]. In contrast, another study in a large panel of breast cancer cell lines reported that BT474 and SKBR3 cells were resistant to olaparib [14]. The reasons for this latter study showing discrepant results are not clear. However, taking into consideration all the studies reported to date [10–14] and our data, the evidence collectively supports that PARP inhibitors are able to affect growth of HER2+ breast cancer.

Trastuzumab downmodulates the expression of repair genes [9] and HER2 depletion also downregulates DNA repair mechanisms [30]. In agreement with these reports, we show that trastuzumab induces DNA damage and downmodulates PCNA, as previously reported [9]. PCNA downmodulation may impair HR [33] which in turn may result in synthetic lethality in the presence of a PARP inhibitor [1]. We also observed that olaparib was able to induce some extent of DNA damage in HER2+, BRCA-proficient, breast cancer cells and the combined treatment lead to a significant increase in DNA damage. A similar mechanism appears to explain the efficacy of the anti-EGFR antibody cetuximab plus a PARP inhibitor in head and neck cancer cells [37].

In the BT474 xenograft model the results were consistent with the in vitro data. Olaparib plus trastuzumab was significantly better than either drug alone and induced regressions of established xenografts, reduced tumour proliferation, and enhanced apoptosis and DNA damage markers.

To summarise, we showed that PARP inhibition augments the efficacy of the anti-HER2 antibody trastuzumab in vitro and in vivo in HER2+ breast cancer cells. Despite ups and downs in their clinical development, the results from early clinical trials of PARP inhibitors strongly suggest a role in the treatment of patients with germline mutations in the BRCA genes [1]. In addition, preclinical data of PARP inhibitors with other agents, such as PI3K or AKT inhibitors, histone deacetylases, nicotinamide dephosphoribosyl transferase [23] or anti-angiogenic drugs are promising. We feel our results provide novel data to support further testing of the combination of PARP inhibitors and trastuzumab for HER2+ breast cancer.

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Conflict of interest statement

None declared.

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