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Differential Potential of Pharmacological PARP Inhibitors for Inhibiting Cell Proliferation and Inducing Apoptosis in Human Breast Cancer Cells

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ABSTRACT

BRCA1/2-mutant cells are hypersensitive to inactivation of poly(ADP-ribose) polymerase 1 (PARP-1). We recently showed that inhibition of PARP-1 by NU1025 is strongly cytotoxic for *BRCA1*-positive BT-20 cells, but not *BRCA1*-deficient SKBr-3 cells. These results raised the possibility that other PARP-1 inhibitors, particularly those tested in clinical trials, may be more efficacious against *BRCA1*-deficient SKBr-3 breast cancer cells than NU1025. Thus, in the presented study the cytotoxicity of four PARP inhibitors under clinical evaluation (olaparib, rucaparib, iniparib and AZD2461) was examined and compared to that of NU1025. The sensitivity of breast cancer cells to the PARP-1 inhibition strongly varied. Remarkably, *BRCA-1*-deficient SKBr-3 cells were almost completely insensitive to NU1025, olaparib and rucaparib, whereas BRCA1-expressing BT-20 cells were strongly affected by NU1025 even at low doses. In contrast, iniparib and AZD2461 were cytotoxic for both BT-20 and SKBr-3 cells. Of the four tested PARP-1 inhibitors only AZD2461 strongly affected cell cycle progression. Interestingly, the anti-proliferative and pro-apoptotic potential of the tested PARP-1 inhibitors clearly correlated with their capacity to damage DNA. Further analyses revealed that proteomic signatures of the two studied breast cancer cell lines strongly differ, and a set of 197 proteins was differentially expressed in NU1025-treated BT-20 cancer cells. These results indicate that BT-20 cells may harbor an unknown defect in DNA repair pathway(s) rendering them sensitive to PARP-1 inhibition. They also imply that therapeutic applicability of PARP-1 inhibitors is not limited to *BRCA* mutation carriers but can be extended to patients harboring deficiencies in other components of the pathway(s). J. Cell. Biochem. 116: 2824–2839, 2015.

KEY WORDS: APOPTOSIS; CASPASE-3 ACTIVATION; CELL CYCLE; DNA REPAIR; DNA DAMAGE; PARP-1 SYNTHETIC LETHALITY

Cancer is a major public health problem in both developed countries and many others [Siegel et al., 2014]. Breast cancer is the most frequent malignancy in women and the second most common cancer-linked cause of death among women worldwide [DeSantis et al., 2014]. In 2008 more than 1.3 million new cases were registered among women globally [Bray et al., 2013]. Breast cancer is an extremely heterogeneous disease, encompassing numerous disorders affecting the same organ [Bertos and Park, 2011]. Thus, tumors arising in the breast have various disease courses and clinical characteristics that are critical determinants of optimal treatments [Cortes et al., 2014]. Numerous studies have shown that various genetic defects accumulate during the development and progression of human tumors, including the amplification and overexpression of certain cell cycle genes and oncogenes, and mutations or deletions of tumor suppressor genes [Perou et al., 2000], [Sorlie et al., 2001], [Stephens et al., 2012], [Ellis and Perou, 2013].

Some genetic changes, e.g., germline mutations in *BRCA1* and *BRCA2* (tumor suppressor genes that play key roles in

Abbreviations: ATM, Ataxia telangiectasia mutated; BER, base excision repair; BRCA1, breast cancer susceptibility protein type 1; HR, homologous recombination; NER, nucleotide excision repair; NHEJ, non-homologous end joining; PARP-1, poly(ADP-ribose)polymerase-1; PD, Petri dish.

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maintenance of genomic stability), are characteristic of human breast cancer and play important roles in specific subsets of breast cancer [Miki et al., 1994], [Wooster et al., 1995], [Huen et al., 2010], [Roy et al., 2012]. Proteins encoded by both genes are significantly involved in the repair of double DNA strand breaks (DSBs) by homologous recombination (HR), a high fidelity process [Huen et al., 2010], [Roy et al., 2012]. Interestingly, inhibition of poly(ADP-ribose) polymerase 1 (PARP-1), a very sensitive detector of single strand breaks and a nuclear enzyme involved in their repair by base excision repair (BER) [for a review, see [Schreiber et al., 2006], is highly cytotoxic for cancer cells lacking *BRCA1/BRCA2* genes or harboring mutations in them [Farmer et al., 2005], [Bryant et al., 2005]. Thus, PARP-1 inhibition has been recently identified as a personalized therapy target, to exploit intrinsic defects in cancer cells, and shown to be selectively cytotoxic in breast cancer patients harboring germline mutations in *BRCA1* and *BRCA2* [Rouleau et al., 2010]. This is because physiologically-related SSBs remain unrepaired and progress to DSBs, which accumulate exclusively in malignant cells due to the failure of HR [Venkitaraman, 2009].

To implement this new therapeutic strategy selective and potent PARP-1 inhibitors were needed. Shortly after these findings several pharmacological inhibitors of PARP-1 were developed, including olaparib [Menear et al., 2008], rucaparib [Thomas et al., 2007] and iniparib [Patel and Kaufmann, 2010], which rapidly entered clinical trials. Several studies have shown that pharmacological interference with PARP-1 activity can act synergistically with anti-cancer drugs, such as topoisomerase I poisons, or radiotherapy [Rouleau et al., 2010]. However, in addition to predicted results of the new therapeutic strategy some unexpected outcomes have been observed. Contrary to predictions, some human *BRCA1/2* mutant breast cancer cells have not responded to the tested PARP-1 inhibitors [Wesierska-Gadek et al., 2012] or become resistant to pharmacological interference with PARP-1 activity [Lawlor et al., 2014]. However, PARP-1 inhibition proved to be cytotoxic for *BRCA1*-proficient breast cancer cells [Wesierska-Gadek et al., 2012], suggesting that dysfunctions in other components of the DNA damage response (DDR) machinery may limit *BRCA1*-proficient cancer cells' ability to repair DSBs and thus be synthetically lethal with PARP-1 inhibitors [for a review, see [Bouwman and Jonkers, 2012]].

We recently showed that NU1025-mediated inhibition of PARP-1 activity is strongly cytotoxic for *BRCA1*-positive BT-20 cells, but not *BRCA1*-deficient SKBr-3 cells [Wesierska-Gadek et al., 2012]. These results raised the possibility that other PARP-1 inhibitors, particularly those tested in clinical trials, may be more efficacious against *BRCA1*-deficient SKBr-3 breast cancer cells than NU1025. Thus, in the study presented here the cytotoxic properties of four small-molecule inhibitors of PARP-1 already under clinical evaluation (iniparib, olaparib, rucaparib, and AZD2461) were examined and compared to those of NU1025. Proteomic responses of the NU1025-sensitive and resistant breast cancer lines to the PARP-1 inhibition were also examined. The results show that the sensitivity of the tested breast cancer cell lines to the PARP-1 inhibition, and accompanying changes in proteomic profiles, strongly differed.

MATERIALS AND METHODS

DRUGS AND CHEMICALS

The PARP-1 inhibitors (NU1025, AZD2461, iniparib, olaparib, and rucaparib) [Bowman et al., 2001], [Menear et al., 2008], [Thomas et al., 2007], [Patel and Kaufmann, 2010] were obtained from AXON Medchem BV (Groningen, Netherlands), prepared as stock solutions in DMSO and stored at −20°C until use (Fig. 1).

CELLS AND TREATMENT

BT-20 and SKBr-3 human primary breast cancer cell lines [Lasfargues and Ozzello, 1958], [Thompson et al., 2011] were purchased from the American Type Culture Collection (ATCC, Manassas, VA). SKBr-3 cells were cultivated in DMEM medium with 10% FCS and BT-20 in RPMI medium under an atmosphere containing 5% CO_2. Twenty four hours after plating (at 60–70% confluence), the cells were treated with the PARP-1 inhibitors NU1025, AZD2461, iniparib, olaparib, and rucaparib at concentrations ranging from 50 to 200 μM, 5 to 50 μM, 5 to 50 μM, 1 to 10 μM, and 0.3 to 10 μM, respectively, for durations indicated in figures 1–7.

DETECTION OF CHROMATIN CHANGES IN INDIVIDUAL CELLS BY FLUORESCENCE MICROSCOPY

Cells grown in 35 mm Petri dishes were treated with PARP-1 inhibitors (NU1025, AZD2461, olaparib, and rucaparib), again at 60–70% confluence for durations indicated in Figure 2, and washed three times in PBS. The washed cells were immediately fixed in 3.7% paraformaldehyde in PBS, then washed four times in PBS and stained with Hoechst 33258 dissolved in PBS at a final concentration of 1.5 μg/ml [Wesierska-Gadek et al., 2004]. The stained cells were inspected under an Eclipse TE300 inverted fluorescence microscope (Nikon Corporation, Tokyo).

DETERMINATION OF NUMBERS OF LIVING CELLS

Numbers of viable human breast cancer cells and their sensitivities to the tested drugs at various concentrations were determined using CellTiter-Glo™ cell viability assays (Promega Corporation, Madison, WI), which measure luminescent signals that are correlated with cellular ATP levels [Wesierska-Gadek et al., 2009]. Tests were performed at least in quadruplicate, and cell luminescence was measured using an Infinite® M200PRO multilabel, multitask plate counter (Tecan Group Ltd., Männersdorf, Switzerland). Each presented data point represents the mean ± SD (bars) of replicates from at least three independent experiments (Figs. 1 and 5).

DETECTION OF APOPTOTIC CELLS BY SUPRAVITAL HOECHST 33342 STAINING

To track induction of apoptosis in tested human breast cancer cells after the PARP- 1 inhibition we performed live-cell assays using the vital DNA dye Hoechst 33342 (HO342) and the non-vital DNA dye 7-amino-actinomycin D (7-AAD). Due to changes in plasma membrane permeability, early apoptotic cells take up more Hoechst 33342 than live cells, but exclude 7-AAD. (Fig. 3) The non-vital DNA dye 7-AAD is added to distinguish late apoptotic or necrotic cells that have lost membrane integrity

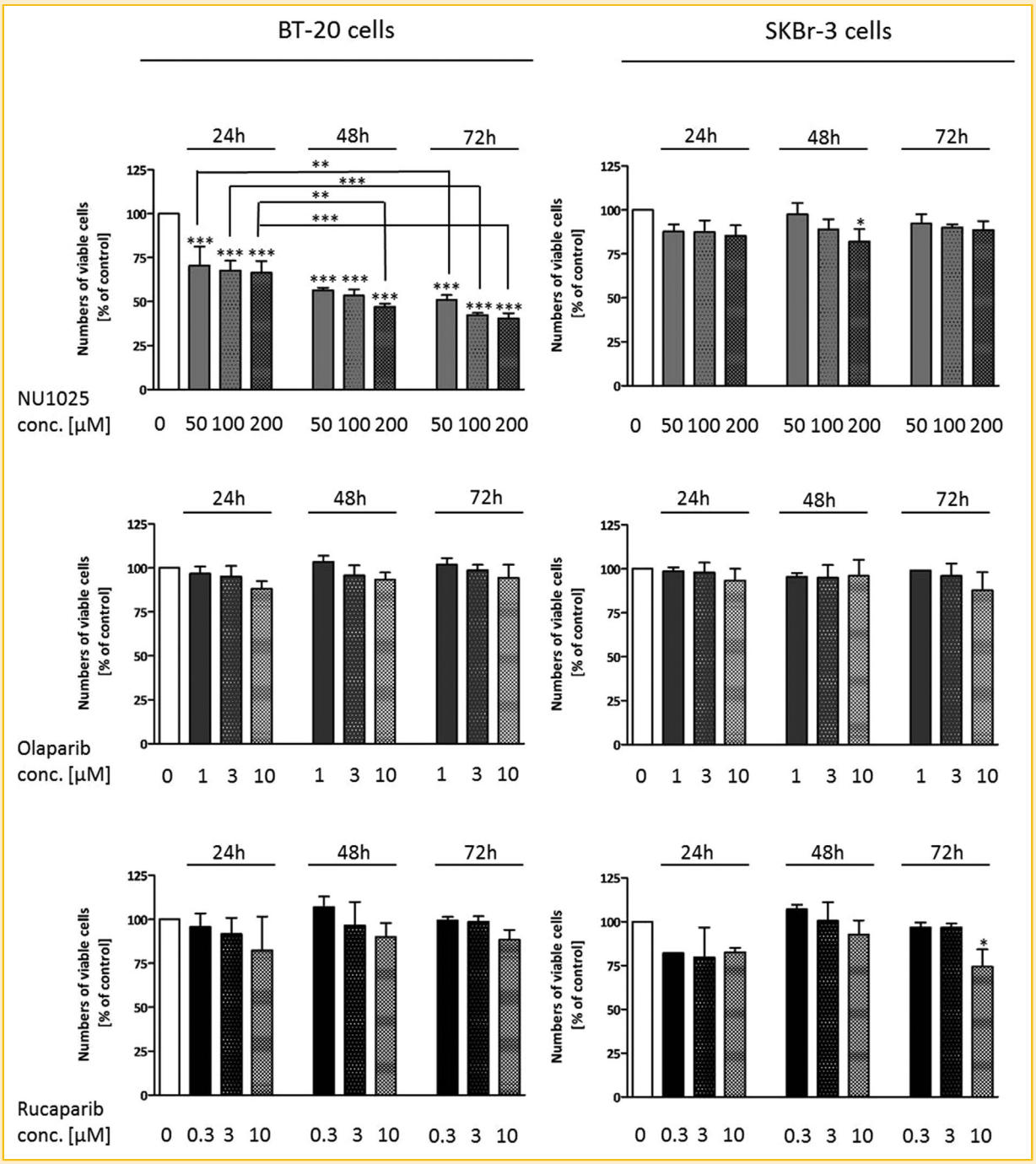

Fig. 1. Effects of pharmacological inactivation of PARP-1 by NU1025, olaparib and rucaparib in human breast cancer cells with different deficits in DNA repair mechanisms. Exponentially growing human BT-20 and SKBr-3 breast cancer cells were plated in 96-well microtiter plates then separately treated 24 h after plating with NU1025, olaparib, and rucaparib at final concentrations of 50–200, 1–10 μM and 0.3–10 μM, respectively, for 48 h or 72 h. Numbers of viable cells were determined directly after each treatment using the CellTiter-GloTM assay system (Promega Corporation, Madison, WI). Presented data points represent means ± SD (bars) for replicates from at least three independent quadruplicate experiments. Results were analyzed using GraphPadPrism software (GraphPad Software, Inc.). The statistical significance of the observed reductions in cell numbers following treatment was calculated using Dunnett and Bonferroni's Multiple Comparison test. Asterisks directly above individual bars denote statistically significant differences between the corresponding treatment and control. Asterisks located above lines connecting two bars denote statistically significant differences between the indicated treatments. Single, double and triple asterisks denote significant differences at the $P<0.05$, $P<0.01$ and $P<0.001$ level, respectively.

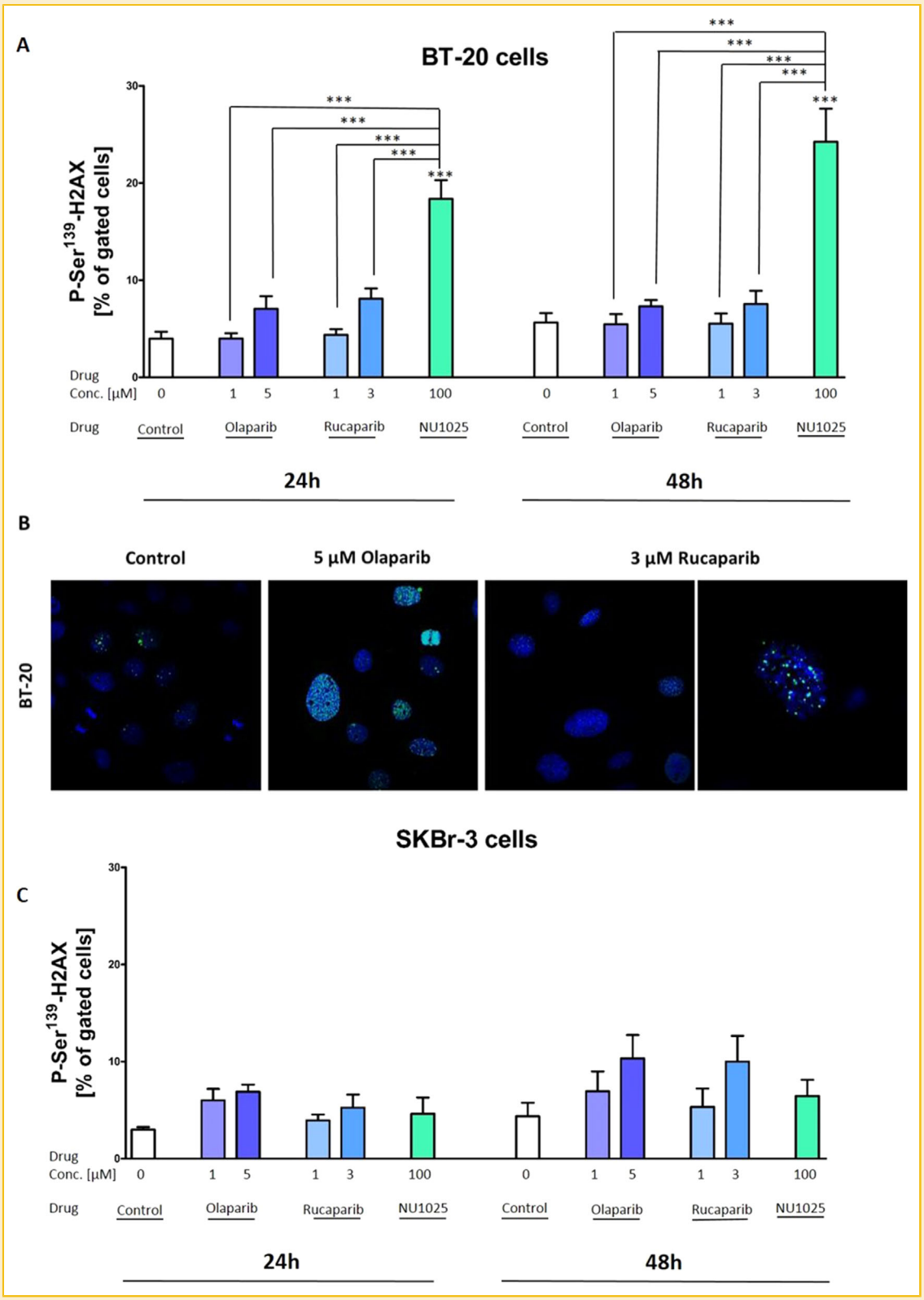

Fig. 2. DNA double strand break accumulation kinetics differ between human NU1025-treated BT-20 and SKBr-3 breast cancer cells. Human BT-20 and SKBr-3 breast cancer cells were exposed to NU1025, olaparib and rucaparib for 24 h and 48 h. Cells were harvested and incubated with antibodies directed against P-S139 H2AX protein. P-S139 H2AX-positive cells were quantified by flow cytometry or inspected under a fluorescent microscope.

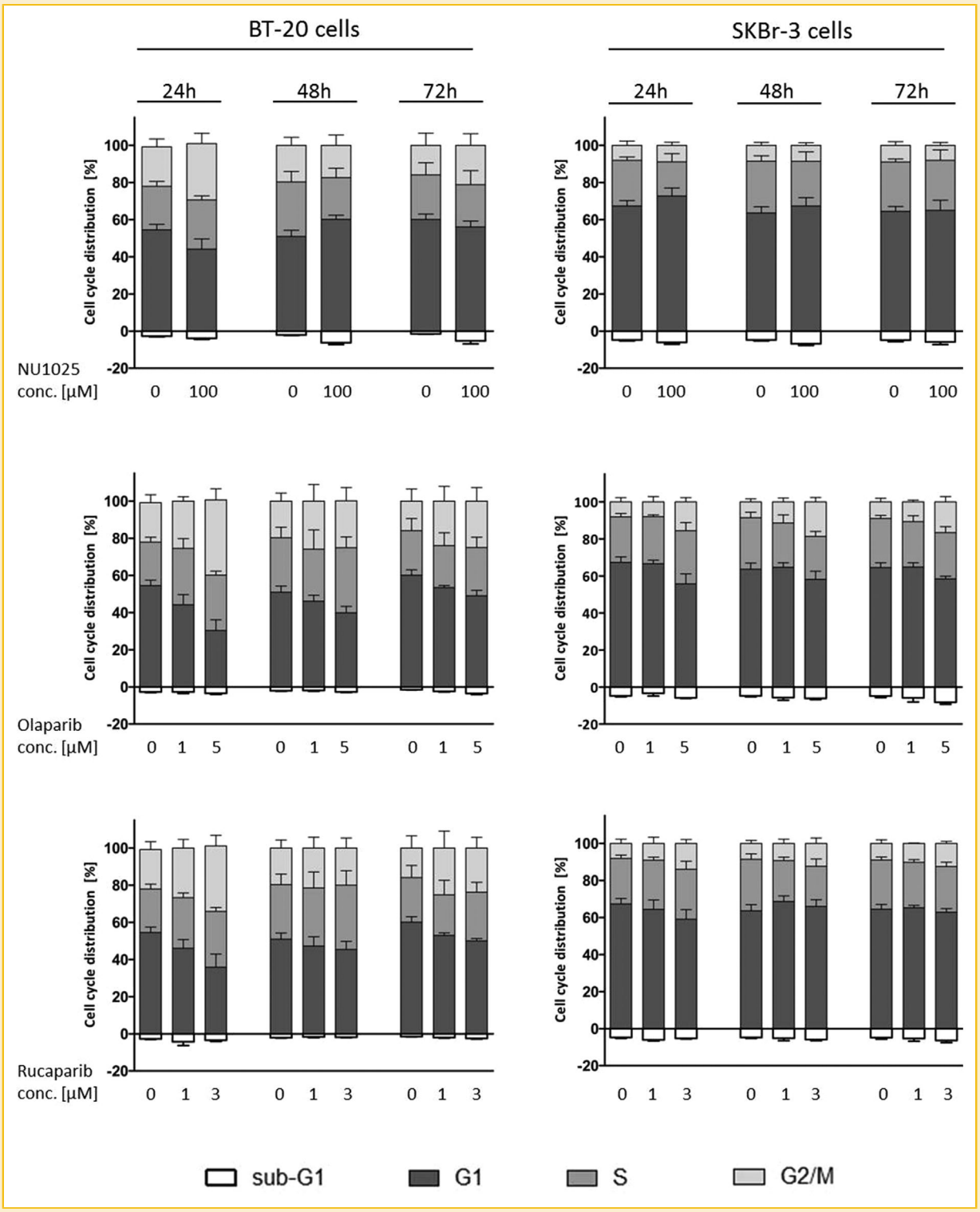

Fig. 3. Inactivation of PARP-1 differentially affects the cell cycle progression in human BT-20 and SKBr-3 breast cancer cells. Exponentially growing BT-20 and SKBr-3 cells were treated with NU1025, olaparib and rucaparib at final concentrations of 100 μM, 1–5 μM and 1–3 μM, respectively, for 24 h, 48 h or 72 h. Cells were harvested immediately after treatment and stained with propidium iodide. DNA content in single cells was measured by flow cytometry. DNA concentrations were evaluated using ModFIT software. The data represent mean values ± SD from three independent experiments.

from early apoptotic cells that still have intact membranes according to dye exclusion tests [Schmid et al., 2007].

Cells grown in Petri dishes were treated with PARP-1 inhibitors (NU1025, olaparib and rucaparib), again at 60-70% confluence for durations indicated in Figure 4.

To visualize apoptotic cells, these tests were performed directly *in situ*, and to quantify apoptosis rates frequencies of Hoechst-stained cells were determined by flow cytometry. Control cells and cells exposed to PARP-1 inhibitors were collected, suspended in 1% BSA/PBS solution, pelleted by centrifugation at 250 x g, resuspended in 1 ml 1% BSA/PBS solution (pre-warmed to 37°C) then HO342 was added to a final concentration of 1 μg/ml. After incubation with HO342 at 37°C for exactly 7 min, the cell suspension was placed on ice, 7-AAD dye was added to a final concentration of 1 μg/ml and the cells were incubated on ice for 10 min. To quantify apoptotic cells, cells excluding both dyes, cells accumulating solely vital dye and cells accumulating both vital and non-vital dyes were then immediately quantified by dual-laser flow cytometry.

DETERMINATION OF CASPASE-3/7 ACTIVITY

Caspase-3/7 activity was determined using the Caspase-3-GLO Assay (Promega Corporation, Madison, WI) with a luminogenic caspase-3/7 substrate harboring the caspase-3/7 DEVD sequence, as previously described [Wesierska-Gadek et al., 2005]. Human BT-20 and SKBr-3 breast cancer cells were plated into 96-well microtiter plates, then treated with PARP-1 inhibitors (NU1025, AZD2461, and iniparib) 24 h later, again at 60–70% confluence for 48 h. BT-20 cells treated for 72 h with camptothecin (CPT) at a final concentration of 1 μM were used as positive controls. After termination of the treatment, equal volumes of the Caspase-3-GLO reagent were added and the probes were incubated at 37°C for varying times to identify the best signal-to-background ratio. The generated luminescence was measured at 30 min intervals. The luminescence, which is directly proportional to the amount of activated caspase-3/7, was measured using an Infinite® M200PRO multilabel, multitask plate counter (Tecan Group Ltd., Männersdorf, Switzerland). The measured caspase-3/7 activity was normalized against the number of living cells as determined by the CellTiter-Glo™ assay and expressed as a percentage of the control value. Each presented data point represents the mean ± SD (bars) obtained from two independent experiments, performed at least in triplicate.

DETERMINATION OF DNA DOUBLE-STRAND BREAKS BY FLOW CYTOMETRIC QUANTIFICATION OF Γ-H2AX-POSITIVE CELLS

DNA double-strand breaks in control and drug-treated human breast cancer cells were quantified by flow cytometric determination of P-Ser^{139}-H2AX-positive cells after staining by specific Alexa Fluor 488-linked antibodies, as follows. Cells were detached from the substratum by limited trypsinization, collected by centrifugation (900 x g, 3 min) and resuspended in 0.5 ml PBS. Next paraformaldehyde was added dropwise to a final concentration of 0.7%. After fixation for 10 min at 37°C, cells were chilled on ice for 1 min and again pelleted to remove fixative and permeabilized by resuspension in ice-cold 90% methanol. Then cells were incubated for 30 min on ice with 0.5% BSA in PBS, incubated with specific antibody against P-Ser^{139}-H2AX for

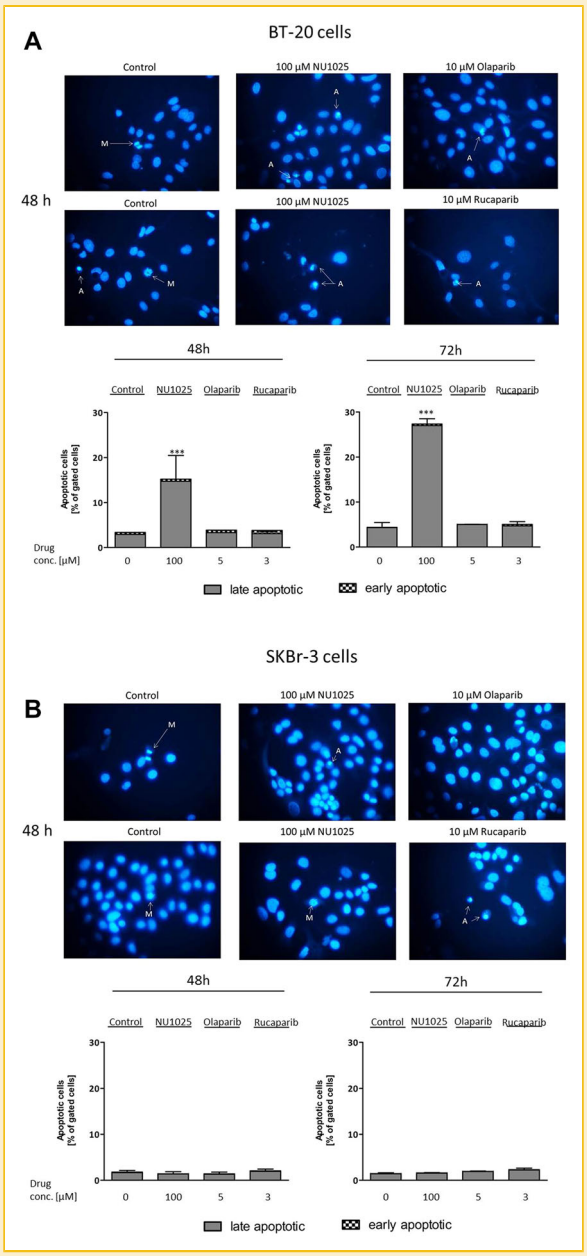

Fig. 4. NU1025 strongly induces apoptosis in BT-20 cells but not in SKBr3 cells. Untreated controls and cells treated with NU1025, olaparib and rucaparib for 48 h or 72 h were harvested and supravitally stained with Hoechst 33258 as described in the section Material and Methods. Results for BT-20 cells are shown in Fig. 4A, for SKBr-3 cells in Fig. 4B. For each type of treatment two images were prepared to show supravitally stained cells at two distant sites in the cell culture. Upper panels. Changes in chromatin structure visualized by Hoechst 33258 were monitored immediately after staining by fluorescence microscopy. Images were taken at higher magnification (40 x objective). White arrows indicate apoptosis (A) and mitosis (M). Lower panels. Quantification of apoptotic cells by dual-laser flow cytometry. Cells excluding both dyes, cells accumulating solely vital dye and cells accumulating both vital and non-vital dyes were determined. Cells accumulating solely vital dye (Hoechst 33258) represent cells in early stage of apoptosis; cells accumulating both vital and non-vital dyes represent cells in late stage of apoptosis.

30 min in the dark and subsequently analyzed by flow cytometry [Wesierska-Gadek et al., 2012].

MEASUREMENT OF DNA CONCENTRATION IN SINGLE CELLS BY FLOW CYTOMETRY

DNA contents of single cells were measured by flow cytometry using the method of Vindelov et al. [Vindelov, 1977], with slight modifications as described elsewhere [Wesierska-Gadek and Schmid, 2000]. Briefly, adherent cells were detached from the substratum by limited trypsinization then all cells were harvested by centrifugation and washed in PBS. Samples of 1×10^6 cells were stained with propidium iodide as previously described and their fluorescence was measured using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) after at least 2 h incubation at +4°C in the dark. The DNA content of the cells was evaluated using ModFIT LT™ cell cycle analysis software (Verity Software House, Topsham, ME) and DNA histograms were generated using the CellQuest™ software package (Becton Dickinson, Franklin Lakes, NJ).

COMPARATIVE ANALYSIS OF PROTEOMES OF BREAST CANCER CELLS EXPOSED TO NU1025

Human BT-20 and SKBr-3 breast cancer cells were cultivated for 48 h in the presence or absence of NU1025 at a final concentration of 100 μM, harvested, washed three times with ice-cold PBS, centrifuged and the resulting pellets were dissolved in SDS-sample buffer. Proteins in the samples were precipitated by a methanol-chloroform mixture [Wessel and Flugge, 1984], dissolved in 50 mM of triethylammonium bicarbonate and their concentration was determined using the Bradford assay. The proteins were then tryptically digested overnight at 37°C using a trypsin/protein ratio of 1:50. Digestion was stopped by acidification with 10 μl of 1% trifluoroacetic acid (TFA) and 20 μl portions of digested peptides were further diluted with 30 μl 0.1% TFA. They were subsequently separated and analyzed by nano-high performance liquid chromatography (nano-HPLC)-tandem mass spectrometry.

The HPLC equipment consisted of an UltiMate Plus nano HPLC system (LC Packings, Amsterdam, The Netherlands), including a Famos autosampler, a Switchos column switching unit, a UltiMate nano pump, a UV detector, an Acclaim C18 trap column (300 μm ID × 5 mm) operated at ambient temperature, and an Acclaim C18 nano separation column (75 μm ID x 250 mm) mounted in the column oven and operated at 45°C. Samples were loaded onto the trap column using 0.1% TFA at 30 μl/min as the initial mobile phase and separated using a ternary gradient (shown in Table I, flow rate 300 nl/min) composed of: 0.1 formic acid (FA) in 5% aqueous acetonitrile (AcN); 0.08% FA in methanol (MeOH):AcN: water (15:15:70, v/v); and 0.08% FA in AcN:MeOH:2, 2, 2-trifluoroethanol (TFE) (60:30:10, v/v). Every sample injection was followed by two blank runs with injections of TFE to remove residues from the preceding run in the injector or on the trap column and prevent carry-over in the separation system [Mitulovic et al., 2009]. A user-defined injection program was used for sample injections, blank injections and trap column washes. Eluting peptides were monitored by the UV detector (at 214 nm) then passed to a coupled LTQVelos IonTrap mass spectrometer (ThermoFisher, Bremen, Germany), selecting the "top 20", i.e., 20 most intense, ions from each MS scan for MS/MS. Single charged ions were excluded from fragmentation and detected ions were excluded from further fragmentation for 3 min after initial MS/MS fragmentation. The acquired data (MS and MS/MS) were used as queries for Mascot 2.4.1 (Matrix Science, London, UK) searches of the most recent version of the SwissProt database, with a mass tolerance of 0.4 Da. Identifications with at least two peptides per protein and a Mascot score of >40 were accepted. All search results were refined and analyzed using Scaffold 3.6.2 (http://www.proteomesoftware.com) with >95% confidence thresholds. All MS experiments were performed in duplicate and the results shown have been merged from two search runs using Scaffold 3.6.2. For quantification we used the label-free approach and applied spectral counting for quantitative analysis as previously described [Haudek-Prinz et al., 2012], [Zhou et al., 2010], [Cooper et al., 2010], [Neilson et al., 2011]. For this purpose, the "Total Spectra Counting" option in Scaffold was used. This is the default option for label-free analysis, and enables quantitation based on the sum of spectra associated with a specific protein (as described, with further details, in the Scaffold Help-File).

Changes in the cellular levels of proteins after treatment with NU 1025 were designated as follows:

A strong effect = higher than 10-fold

A weaker effect = higher than 2-fold and lower than 9-fold

TABLE I. HPLC Gradient Applied for Peptide Separation

Time	Flow (μl/min)	%A	%B	%C
0	0.3	97	3	0
8	0.3	97	3	0
45	0.3	0	100	0
65	0.3	0	0	100
70	0.3	0	0	100
72	0.3	97	3	0
85	0.3	97	3	0

STATISTICAL ANALYSES

GraphPad. Prism software (GraphPad Software, Inc., La Jolla, CA) was used for all statistical analyses and significance levels were evaluated using Bonferroni and Dunnett's Multiple Comparison Tests. Differences between treatments were deemed to be extremely significant, very significant, significant and not significant if their P values (according to these tests) were <0.001, $0.001<P<0.01$, $0.01<P<0.05$ and >0.05, respectively. In the tables and figures such differences are indicated by three asterisks (***), two asterisks (**), one asterisk (*) and no asterisks, respectively.

RESULTS

IN CONTRAST TO NU1025 OLAPARIB AND RUCAPARIB ARE NOT CYTOTOXIC FOR HUMAN BT-20 BREAST CANCER CELLS

We recently observed that human *BRCA1*-deficient SKBr-3 breast cancer cells are completely resistant to PARP-1 inhibition by

NU1025, surprisingly as they are strongly affected by topoisomerase I and II inhibitors. To elucidate the observed resistance we used olaparib and rucaparib, two next-generation PARP inhibitors that are approximately 200-fold more potent than NU1025 and 1,000 times more strongly cytotoxic for *BRCA1/2*-deficient or -mutated cells than *BRCA1/2*-proficient cells [Menear et al., 2008], [Thomas et al., 2007]. Unexpectedly, both examined breast cancer cell lines were completely insensitive to olaparib under the test conditions, while rucaparib had no effects on BT-20 and only reduced numbers of SKBr-3 cells by 25% after 72 h exposure to the highest test concentration, 10 μM (Fig. 1)

PARP-1 INHIBITORS CAUSE DNA DAMAGE WITH DIFFERING SEVERITY AND KINETICS IN BOTH BREAST CANCER CELL LINES

Our next objective was to determine whether inhibiting PARP-1 with olaparib and rucaparib would have similar effects on DNA integrity to NU1025 inhibition. For this purpose, since DNA damage resulting in generation of DSBs induces site-specific phosphorylation of the H2AX protein *via* ATM kinase activation [Rogakou et al., 1998], we incubated breast cancer cells exposed to PARP-1 inhibitors and controls with Alexa Fluor 488-linked antibodies against P-Ser^{139}-H2AX protein. We then quantified the labeled cells by flow cytometry. As shown in Fig. 2A exposure to NU1025 for 24 h induced a 6-fold increase in DSBs in BT-20 cells, relative to controls, but a weaker change in their frequency in SKBr-3 cells. Olaparib and rucaparib affected DNA integrity very weakly in BT-20 cells (Fig. 2A & B). P-Ser^{139}-H2AX-positive foci were found only in a few BT-20 cells (Fig. 2B). Thus, only NU1025 strongly increased the numbers of DSBs in BT-20 cells, and this correlated with the appearance of apoptotic cells (Fig. 4). In contrast, exposing SKBr-3 cells to all three PARP-1 inhibitors moderately increased DNA breakage, after 48 h (Fig. 2C).

INTERFERING WITH PARP-1 ACTIVITY HAS DIFFERENT EFFECTS ON CELL CYCLE PROGRESSION IN BT-20 AND SKBR-3 CELLS

To determine effects of PARP-1 inhibitors on cell cycle progression in human BT-20 and SKBr-3 breast cancer cells, exponentially growing cultures were exposed to PARP-1 inhibitors for 24, 48, and 72 h at final concentrations indicated in Fig. 3. The cultures were then harvested and the DNA concentration of single cells was determined by flow cytometric measurement of their fluorescence intensity following propidium iodide staining. In exponentially growing BT-20 cultures, treatment with the PARP-1 inhibitors for 24 h strongly and slightly increased the abundance of G_2-phase and S-phase cells, respectively, at the expense of the G_1 cell population (Fig. 3). The highest increases in G_2 cell frequencies were induced by olaparib followed by rucaparib and NU10125 (approximately 20, 15, and 10% increases, respectively). However, the changes in cell cycle distributions after interference with PARP-1 were transient; after 24 h or at most 48 h no noteworthy differences were detected between controls and PARP-1i-treated cells. Unlike BT-20, SKBr-3 cells were barely affected by the pharmacological interference with PARP-1 activity.

These results indicate that pharmacological interference with PARP-1 activity has moderate but contrasting effects on cell cycle progression in the two human breast cancer lines considered. Moreover, the three PARP-1 inhibitors used in this experiment show varying potential to induce G_2- and S-phase arrest in BT-20 cells.

NU1025 STRONGLY AND TIME-DEPENDENTLY INDUCES APOPTOSIS IN BT-20 CELLS

To assess the extent to which DNA damage generated by pharmacological PARP-1 inhibitors may initiate apoptosis, we performed live-cell assays [Schmid et al., 2007]. Control BT-20 and SKBr-3 cells and cells exposed to PARP-1 inhibitors were supravitally stained with Hoechst 33342 and immediately inspected under a fluorescence microscope or analyzed by flow cytometry. As shown in Figure 4, mitotic and occasionally apoptotic cells were detected in control BT-20 and SKBr- 3 cells. Upon treatment with PARP-1 inhibitors the mitotic index decreased in both cell lines, i.e., the frequency of detected mitotic cells decreased. Treatment with 100 μM NU1025 induced apoptosis exclusively in BT-20 cells, and the rate of apoptosis increased both significantly and time-dependently (from approximately 15% at 48 h to 30% at 72 h) (Fig. 4A). Unlike NU1025, olaparib and rucaparib did not increase the rate of apoptosis in the studied cell lines. However, NU1025 activated caspase-3 in BT-20 cells, even at a low dose (50 μM), whereas olaparib and rucaparib had no noteworthy effect on the activity of this effector caspase (data not shown). The observed activation of caspase-3 mediated apoptosis in NU1025-treated BT-20 cells is consistent with our previous findings [Wesierska-Gadek et al., 2012].

INIPARIB AND AZD2461 ARE MUCH MORE CYTOTOXIC FOR SKBR-3 CELLS THAN THE OTHER THREE TESTED PARP-1 INHIBITORS

Clinical studies have established that some potent PARP-1 inhibitors such as olaparib or rucaparib generally elicit significant responses in patients with *BRCA*-mutant cancers [Drew and Plummer, 2009]. However, not all trials have had such positive outcomes, partly because some patients do not respond well to the treatment or develop resistance to the inhibitors [Patel et al., 2012]. Therefore, we used iniparib and AZD2461, other potent PARP-1 inhibitors that have been previously tested on a panel of triple-negative and non-triple-negative breast cancer lines and recommended for treating metastatic triple-negative breast cancer patients in combination with chemotherapy [Pierce et al., 2013], [O'Shaughnessy et al., 2011]. AZD2461 was more cytotoxic for BT-20 cells than iniparib (Fig. 5); it induced apoptosis in time- and concentration-dependent manner as evidenced by live-cell assays (data not shown). Surprisingly, in the present study iniparib and especially AZD2461 more strongly affected SKBr-3 cells than olaparib and rucaparib (Fig. 5); exposure to them at a final concentration of 50 μM for 72 h reduced numbers of living SKBr-3 cells by 30% and 50%, respectively. They were also much more toxic for SKBr-3 cells than NU1025 (Fig. 5).

ACCUMULATION OF S- AND G_2-PHASE CELLS AFTER EXPOSURE OF BT-20 CELLS TO AZD2461

Our next objective was to determine whether interfering with PARP-1 activity using iniparib and AZD2461 would affect cell cycle progression and promote pro-apoptotic changes in the studied breast cancer lines. Flow cytometric analyses of DNA content in control

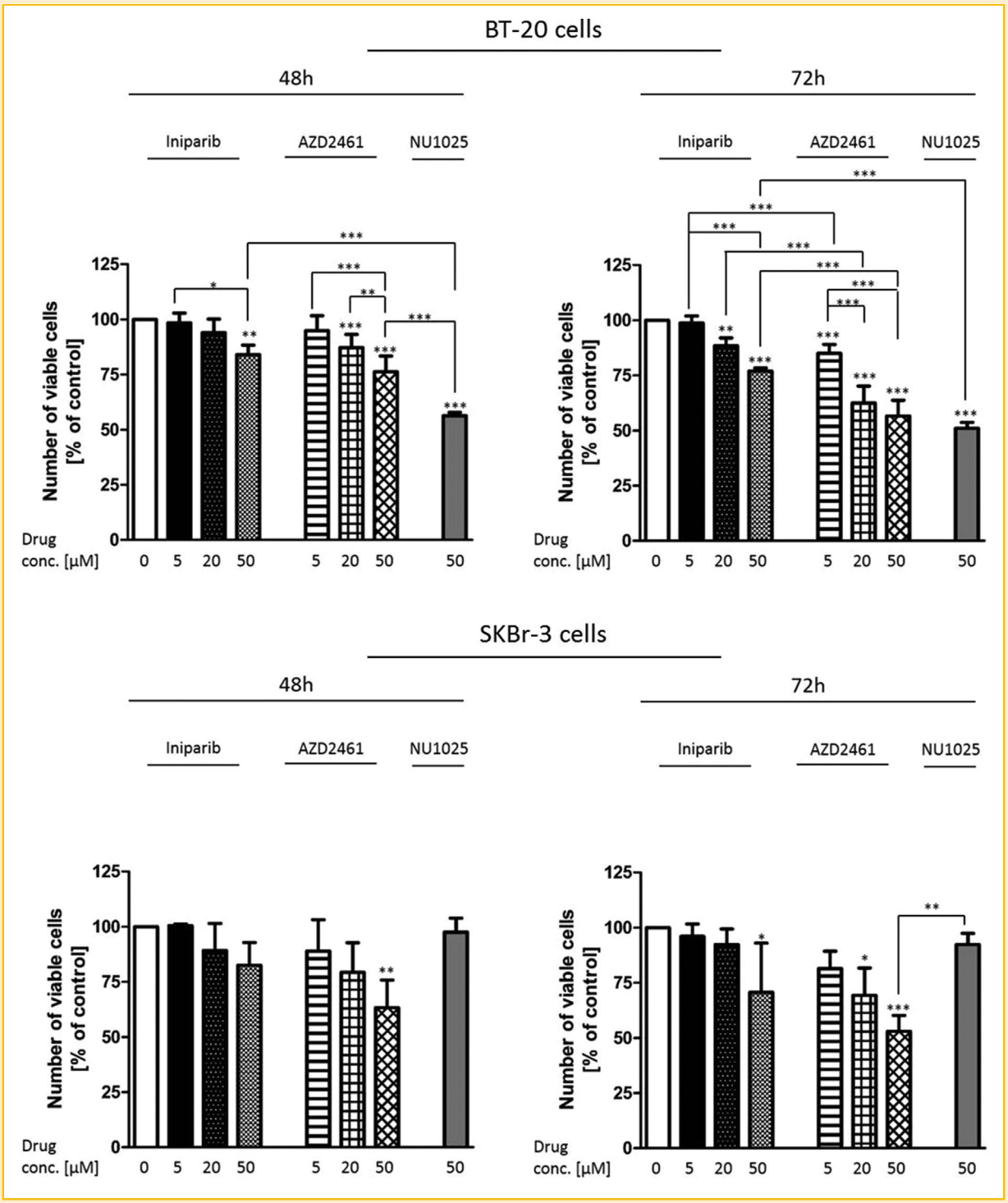

Fig. 5. AZD2461 is more cytotoxic for SKBr-3 cells than NU1025.Human BT-20 and SKBr-3 breast cancer cells were plated as described in the legend of Fig. 1. Cells were treated with iniparib, AZD2461 and NU1025 at indicated concentrations for 48 h and 72 h. The data were analyzed and evaluated as described in detail in Fig. 1.

cells and cells exposed to PARP-1 inhibitors revealed that AZD2461 affected their cell cycle progression more strongly than iniparib (Fig. 6), and that the cell cycle distribution was more strongly affected in BT-20 cultures than in SKBr-3 cultures. After treating BT-20 cells with AZD2461 for 24 h the proportions of S- and G_2-phase cells increased dose-dependently at the expense of G_1 cells, and during the following 24 h G_2 cells further accumulated (Fig. 6A). In contrast, iniparib only weakly changed the distribution of cells in the

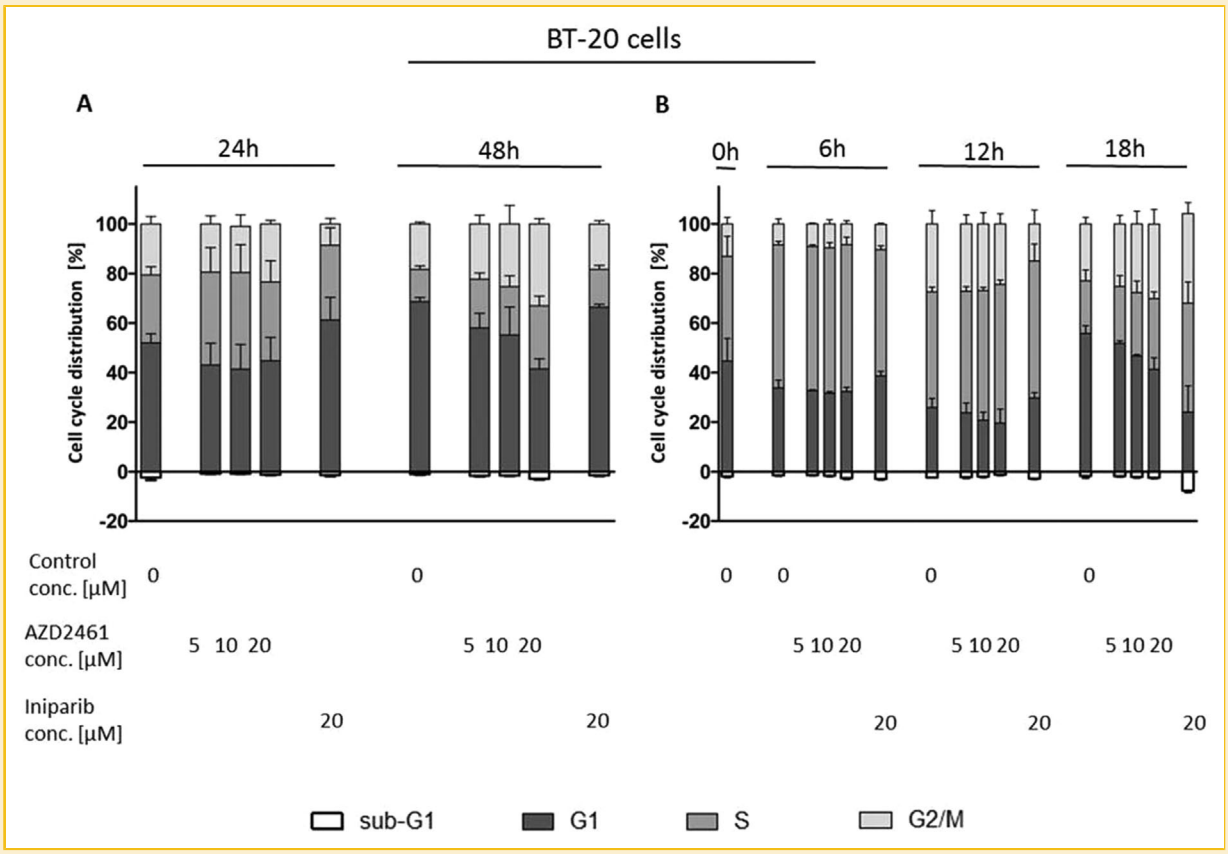

Fig. 6. Interference with PARP-1 activity by AZD2461 affects time-dependently the progression of cell cycle in BT-20 cells. Human BT-20 breast cancer cells were harvested immediately after treatment and stained with propidium iodide. DNA content in single cells was measured by flow cytometry. DNA concentrations were evaluated using ModFIT software. The data represent mean values ± SD from three independent experiments.

cell cycle phases. SKBr-3 cells were much more weakly affected than BT-20 cells by both tested PARP-1 inhibitors (Fig. 7A). As PARP-1 inhibition for 24 h and 48 h only marginally affected the cell cycle progression of SKBr-3 cells we decided to ascertain impact of PARP-1 inhibitors on the changes in of cell cycle distribution in a shorter time frame (between 6 h and 18 h). As shown in Figure 7B, the distribution of SKBr-3 cells in the cell cycle phases was barely affected by the pharmacological interference with PARP-1 activity. In more sensitive BT-20 cells first changes in the cell cycle distribution occurred after PARP-1 inhibition for 18 h (Fig. 6A). These results collectively indicate that inhibition of PARP-1 in human SKBr-3 cells had only marginal effect on the cell cycle and are in concordance with other results showing low cytotoxicity of PARP inhibitors in SKBr-3 cells.

NU1025 INDUCES PROTEOMIC CHANGES IN BT-20 CELLS

Finally, we assessed NU1025's proteomic impact in sensitive (BT-20) and insensitive (SKBr-3) cells by digesting proteins from cells exposed to NU1025 at 100 μM for 48 h then analyzing generated peptides by HPLC-tandem mass spectroscopy.

Comparison of proteins expressed in untreated BT-20 and SKBr-3 control cells revealed that 307 proteins were expressed in both cell lines, 286 only in BT-20 cells and 111 only in SKBr-3 cells (data not shown). After exposure of BT-20 cells to 100 μM NU1025 for 48 h, a subset of proteins (138) was changed (Fig. 8A); in NU1025-treated SKBr-3 cells the abundance of 199 proteins was modulated (Fig. 8B). Interestingly, after exposure to 100 μM NU1025 a common set of 373 proteins was found in both cell lines: Moreover, we detected 197 and 166 proteins that were differentially expressed only in treated BT-20 and SKBr-3 cells, respectively (Fig. 8C).

Careful analysis of cell-specific changes in the abundance of proteins following NU1025 treatment revealed that several nucleolar proteins and proteins involved in transcription regulation were modulated by NU1025 treatment. As shown in Table II following cells exposure to NU1025 for 48 h the abundance of some proteins was reduced and the abundance of others increased. Changes in the cellular levels of three proteins (Msh2, Msh6, and RAD50) were confirmed by immunoblotting supporting results of the proteomics analysis (data not shown). These results clearly show that NU1025-sensitive and -resistant breast cancer cells differentially respond to interference with PARP-1 activity.

DISCUSSION

One of the major challenges in cancer treatment is to develop robust criteria for identifying optimal personalized therapies for

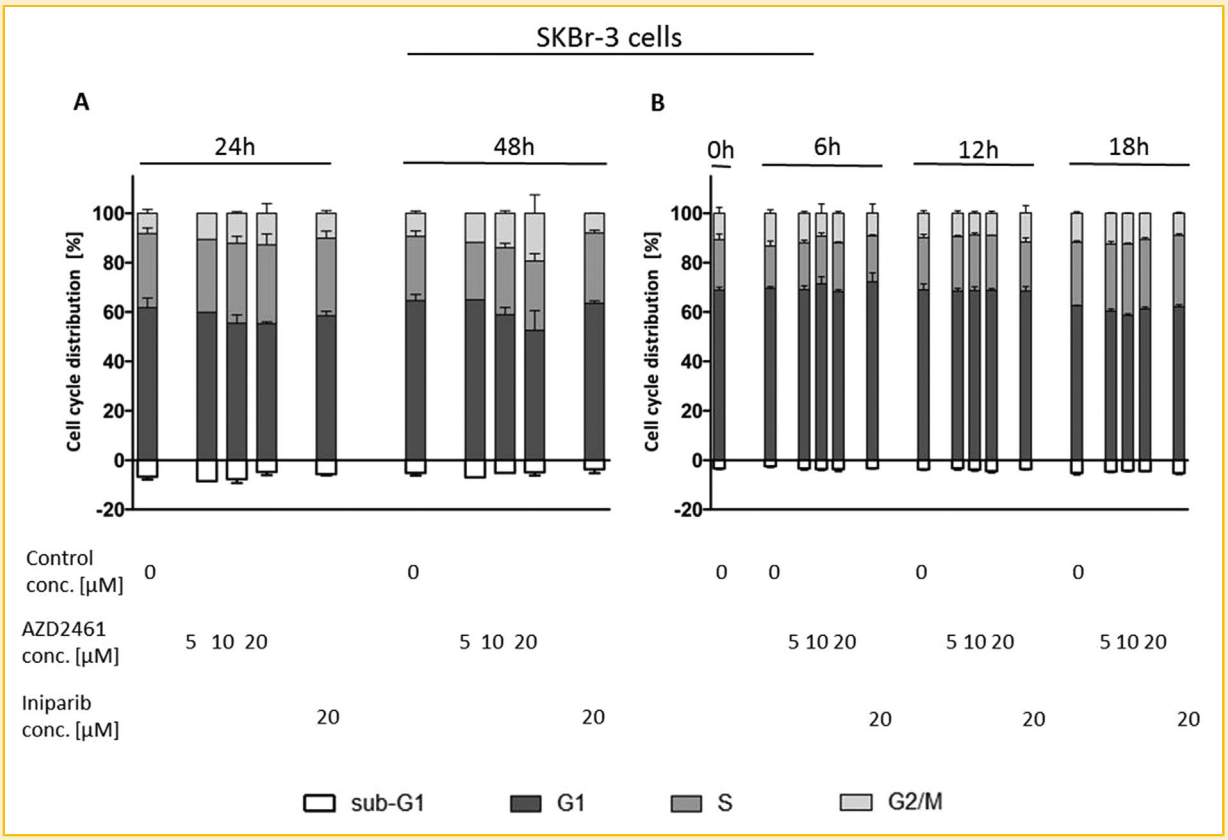

Fig. 7. The progression of cell cycle in SKBr-3 cells is weakly affected by AZD2461. Human SKBr-3 cells were harvested immediately after treatment and stained with propidium iodide. DNA content in single cells was measured by flow cytometry. DNA concentrations were evaluated using ModFIT software. The data represent mean values ± SD from three independent experiments.

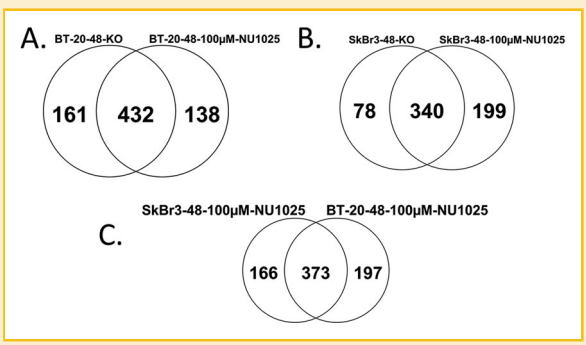

Fig. 8. Comparative proteomic analysis of human BT-20 and SKBr-3 breast cancer cells following PARP-1 inhibition with 100 µM NU1025. Proteins prepared from untreated control cells and cells exposedfor 48 h to 100 µM NU1025 were subjected to limited trypsin proteolysis; then generated peptides were separated by HPLC and analyzed by mass spectrometry. For comparative analyses Venn diagrams were generated. (A) Changes in expression of proteins in BT-20 cells treated with 100 µM NU1025 for 48 h. (B) Changes in expression of proteins in SKBr-3 cells treated with 100 µM NU1025 for 48 h. (C) Comparison of proteins expressed in BT-20 and SKBr-3 cells treated with 100 µM NU1025 for 48 h.

individual patients. To do so tumors must first be rigorously and accurately characterized. The most recent approach for classifying breast cancer is based on molecular, especially transcriptional, profiling [Ellis and Perou, 2013], [Cortes et al., 2014]. The rationale for this is that diverse chemical and physical carcinogenic agents induce mutations in genes involved in cell cycle progression, proliferation and maintenance of genomic stability. Cells harboring such mutations acquire new properties that may give them a competitive edge (e.g., unlimited proliferative potential or independence from growth factors) over surrounding normal cells, leading to cancerous growth. Thus, detailed identification of characteristic changes in breast cancer cells is required to identify optimal personalized therapeutic targets. These include mutations resulting in upregulation of hormone receptors (ER-α and PgR) [Deblois and Giguere, 2013], [Brisken, 2013] and overexpression of HER2/neu [Yan et al., 2014]. The most effective therapies are available for patients with these perturbations, including endocrine therapies such as administration of tamoxifen or aromatase inhibitors for the former [Segal and Dowsett, 2014], and therapeutics such as the humanized monoclonal antibody trastuzumab, which prevents HER2/neu activation and

TABLE II. Changes in the Abundance of the Selected Proteins in NU1025-Treated BT-20 (A) and SKBr-3 (B) Cells

No.	Protein name	Gene name	Change	Function
A				
1.	Adenine phosphoribosyl-transferase	*APRT*	↑	Regulation of vesicular traffic and actin remodelling
2.	ADP-ribosylation factor 1	*ARF1*	↑	Regulation of vesicular traffic and actin remodelling
3.	DNA damage-binding protein 1	*DDB1*	↑	Role in nucleotide excision repair
4.	Nucleolar RNA helicase 2	*DDX21*	↑	rRNA processing
5.	Nucleolar and coiled-body phosphoprotein	*NOLC1*	↑	rRNA transcription, nucleologenesis
6.	Cell division control protein 42 homolog	*CDC42*	↑	A small GTPase; diverse cellular functions
7.	Testin	*TES*	↑	Regulation of cell motility
8.	Fascin	*FSCN1*	↑	Organization of actin filament
9.	RNA binding protein 39	*RBM39*	↑	Regulation of transcription
10.	Mitochondrial import receptor subunit TOM70	*TOMM70*	↑	Receptor accelerating the import of mitochondrial precursor proteins
B				
1.	ADP-ribosylation factor 4	*ARF4*	↑	Regulation of vesicular traffic and actin remodelling
2.	Breast carcinoma-amplified sequence 1	*BCAS1*	↑	A putative oncogene
3.	Proliferating cell nuclear antigen	*PCNA*	↗	Role in both DNA synthesis and DNA repair
4.	U5 small nuclear ribonu-cleoprotein 200 kD helicase	*SNRNP200*	↗	Role in pre-mRNA splicing
5.	Ribonucleotide reductase large subunit	*RRM1*	↑	Catalysis of the biosynthesis of deoxyribonucleotides
6	Cell division control protein 42 homolog	*CDC42*	↑	A small GTPase; diverse cellular functions
7	Stathmin	*STMN1*	↑	Regulation of microtubule dynamics
8	Heterogeneous nuclear ribonucleoprotein A/B	*HNRNPAB*	↑	Role in pre-mRNA processing
9	Heterogeneous nuclear ribonucleoprotein Q	*SYNCRIP*	↑	Role in pre-mRNA processing
10	Mitochondrial import receptor subunit TOM40	*TOMM40*	↑	Regulation of protein transport into mitochondria

↗, a weaker effect; ↑, a strong effect.

stimulation of downstream pathways, for the latter [Rexer and Arteaga, 2013].

However, triple-negative breast cancer (TNBC), a third type of breast cancer, defined by the absence of staining for HER2/neu and both estrogen and progesterone receptors, is insensitive to the most effective therapies available for breast cancer treatment such as those mentioned above. These tumors account for approximately 15% of breast cancer cases [Duffy et al., 2012]. Therefore, new TNBC-specific therapeutic targets must be identified. As tumors are highly heterogeneous it is unlikely that any single targeted therapy will be efficacious in all TNBC patients. However, a subset of TN tumors with *BRCA1/2* mutations have high sensitivity to pharmacological inhibition of PARP-1 [Duffy et al., 2012], [Narod, 2010]. The BRCA1 protein has multiple interactors and participates in several cellular pathways, including DNA repair by HR, chromatin remodeling, transcription regulation, and checkpoint activation [for reviews, see [Huen et al., 2010], [Caestecker and Van de Walle, 2013]. The multi-functionality of BRCA1 is associated with a series of complexes with distinct functions. Among numerous proteins binding to BRCA1 several (including BARD1, PALB2, RAD51, CtIP, MRN, RAP80, and TopBP1) seem to be required for its functions [for a review, see [Lord and Ashworth, 2013], [Caestecker and Van de Walle, 2013]]. In the last decade it has been recognized that defects in HR DNA repair provide promising therapeutic targets [Farmer et al., 2005], [Bryant et al., 2005]. Accordingly, *BRCA1/2*-deficient or mutated breast cancers were initially used for testing a new strategy for elucidating and exploiting cytotoxic mechanisms, designated synthetic lethality, in preclinical model systems. The strategy is based on identifying combinations of mutations that are lethal in combination, but not singly. The therapeutic potential of applying the strategy using combinations of treatments including administration of pharmacological inhibitors of PARP-1 that block the BER process, resulting in selective cancer cell killing, has also been investigated. Several well-tolerated PARP-1 inhibitors including AZD2461, olaparib, rucaparib, veliparib, and iniparib have been developed and tested in clinical trials both as single agents and in combination with other chemotherapeutics, inter alia cisplatin, carboplatin, and temozolomide [Tentori et al., 2014], [Mukhopadhyay et al., 2012], [Rottenberg et al., 2008]. Gradually, this new therapeutic strategy has been extended to other cancer types (e. g., ovarian and prostate cancer) [Cheng et al., 2013], [Banerjee et al., 2010], [Postel-Vinay et al., 2013], [Tentori et al., 2014] not only with BRCA mutations, but also with mutations in other genes involved in HR DNA repair, including *BARD1*,*BRIP1*, *MRE11A, PALB2, RAD51C, and RAD51D* [Lord and Ashworth, 2013].

The data presented here confirm our previous observation that inhibition of PARP-1 by NU1025 is synthetically lethal for *BRCA1*-proficient BT-20 breast cancer cells [Wesierska-Gadek et al., 2012], [Wesierska-Gadek and Heinzl, 2014], but raise further questions, particularly regarding the differing responses of BRCA1-deficient SKBr-3 cells to three PARP-1 inhibitors: NU1025, olaparib and rucaparib. As olaparib is a P-glycoprotein substrate [Lawlor et al., 2014] one might speculate that our SKBr-3 cells developed multidrug resistance. However, SKBr-3 cells are also resistant to NU1025 and rucaparib, which have not been identified as P-gp substrates. More recently, a novel mechanism of resistance to olaparib that cannot be explained by P-gp-mediated drug efflux was reported, based on partial restoration of HR due to loss of p53BP1 protein, in BRCA1-deficient mouse mammary cancer [Jaspers et al., 2013]. However, this was a long-term response, and it is not clear whether it could occur in SKBr-3 cells exposed to olaparib for just 48 or 72 h.

Intriguingly, unlike the other inhibitors tested in our study, AZD2461 was synthetically lethal for SKBr-3 cells, much more cytotoxic for SKBr-3 cells than for BT-20 cells after a short treatment (24 h), and its cytotoxic effect was dose- and time-dependent. Moreover, SKBr-3 cells were stronger affected by iniparib ($C_E = 50$ μM) after 48 h than BT-20 cells. A potential explanation offered by very recent results is that iniparib is not a *bona fide* PARP inhibitor,

although it was originally developed as one, and cell death following iniparib treatment seems to be due to induction of DNA damage. It has also been shown that the nitroso metabolite of iniparib forms adducts with many cysteine-containing proteins [Liu et al., 2012].

Thus, our results confirm the resistance of human BRCA1-negative SKBr-3 cells to the three tested inhibitors. However, our data also show that PARP-1 inhibitors (and iniparib) have differing cytotoxic potential, as evidenced for human BT-20 breast cancer cells. Moreover, the comparative proteomic analyses revealed that treatment with NU1025 for 48 h changed the expression of many more proteins in sensitive than in resistant breast cancer cells.

To gain further insights into the impact of PARP-1 inhibition we examined the functional classes and localizations of proteins whose expression was affected by NU1025 treatment in both examined cell lines (BT-20 and SKBr-3). Interestingly, the analyses revealed that PARP-1 inhibition induced changes in the abundance of several functional protein groups, including, oncogenes, transcriptional regulators, DNA repair proteins, chromatin remodellers, apoptosis factors, and proteins regulating cell adhesion and motility (Table II). Proteins in several subcellular compartments such as nuclei, nucleoli, mitochondria, and cytoplasm were also affected. Importantly, these changes differed between BT-20 and SKBr-3 cells. These results demonstrate that the interference with a single nuclear enzyme like PARP-1 can affect levels of proteins with diverse functions, inter alia roles in chromatin organization, DNA repair, regulation of transcription, cell architecture and intracellular movements.

As SKBr-3 breast cancer cells are defective in homologous recombination (HR) due to a lack of BRCA1 tumor suppressor protein expression their resistance to PARP inhibitors was surprising and difficult to explain. Numerous previous studies have shown that tumors with dysfunctions in HR, e.g., cancers with mutations in *BRCA1/BRCA2* genes, or other DNA repair processes are extremely sensitive to PARP inhibition due to the combination or synthesis of deficiencies in multiple DNA repair pathways resulting in cell death. It is widely accepted that inhibition of PARP induces an increase in single-strand breaks (SSBs), which are converted during replication to irreparable DNA double-strand breaks (DSBs) in DNA repair defective cells [Helleday, 2011]. These observations fostered the concept of synthetic lethality, which provides foundations of novel therapeutic approaches to treat cancer.

As shown in Figure 4, in contrast to BT-20 cells PARP inhibition did not generate DSBs in SKBr-3 cells, possibly because they have defects in components required for key steps in cellular responses to DNA damage, such as signalling of DNA lesions. However, SKBr-3 cells are extremely sensitive to inhibition of topoisomerase II, which is known to induce DNA breaks [Wesierska-Gadek et al., 2012].

A potential explanation is that overexpression of the strong oncogene *ERBB2* (a trait of human SKBr-3 breast cancer cells), and mutated *TP53*, enhance activation of downstream signalling pathways and uncontrolled proliferation [Yarden and Sliwkowski, 2001]. Interestingly, *ERBB2*-positive breast cancers appear to be heterogeneous and display diverse patterns of gene amplifications at 17q12, 8q24, 11q13, and 20q13 chromosomal regions [Collins et al., 1998].

Our proteomic analyses revealed that upon NU1025 treatment cellular levels of breast cancer amplified sequence 1 protein, the *BCAS1* gene product, increased in SKRr-3 cells. The breast cancer *BCAS1* gene maps to 20.q12.3, a region of recurrent amplification in tumors of several types, including breast cancer. In the 20q13.2 breast cancer amplicon six other genes were identified. Two of the identified genes, *ZNF17* and *BCAS1*, have become generally known as potentially strong oncogenes. *BCAS1* is overexpressed in most breast cancer cell lines in which it was amplified [Collins et al., 1998]. Notably, recurrently amplified DNA regions are predicted to encode dominantly acting genes that may play a role in tumor progression and are associated with pure prognosis [Collins et al., 1998]. It is conceivable that increases in the abundance of BCAS1 protein after inhibition of PARP-1 combined with high expression of *ERBB2* conferred human SKBr-3 cells' observed resistance to PARP inhibitors.

As treatment with NU1025 has been found to damage DNA severely in human BT-20 breast cancer cells [Wesierska.Gadek et al., 2012] we examined its effects on cellular levels of proteins involved in the repair of DNA lesions. The abundance of BRCA1 protein remained almost unchanged. However, NU1025 treatment strongly affected cellular levels of some other proteins involved in DNA repair, notably it reduced abundance of DNA damage binding protein 1, the *DDB1* gene product, 10-fold after 24 h in BT-20 cells (Table II). Moreover, cellular levels of other DNA repair proteins (Msh2, Msh6 and RAD50) were moderately reduced (data not shown). These data show that inhibition of PARP-1 in human BT-20 breast cancer cells reduced the abundance of proteins engaged in the regulation of three DNA repair pathways: not only nucleotide excision repair (NER), but also mismatch repair (MMR) and homologous recombination (HR).

Damage-specific DNA damage binding protein 1 (DDB1), which contains a repetitive sequence designated WD40, is a multifunctional protein that forms heterodimers with DDB2 protein or Cockayne Syndrome protein (CSA) that recognize UV- or chemical mutagen-induced DNA lesions and recruit the NER machinery required to repair the damage [for a review, see: Iovine et al., 2011]. In humans, attenuation or loss of NER is associated with genetic disorders such as xeroderma pigmentosum (XP). Recent work has revealed that DDB1 has a wide range of functions, involved in various processes including cell cycling, apoptosis and transcription [Arias and Walter, 2006; Iovine et al., 2011]. In addition to its involvement in DNA repair DDB1 is an integral adaptor protein component of Cul4A-RING ubiquitin E3-ligases (CRL4s) [Hu et al., 2004]. The RAD50 protein also plays a key role, but in the detection and repair of double strand breaks (DSBs) as a component of the Mre11-Rad50-Nbs1 complex (MRN). More specifically, it binds to strands of damaged DNA and holds the broken ends together during the repair process and simultaneously interacts with two other components of the complex [for a review, see Paull and Dephande, 2014]. In contrast to the two proteins discussed above, Msh2 and Msh6 proteins play important roles in mismatch repair (MMR), and are essential for the proper repair of DNA replication mistakes. Mutations in *MMR* genes are known to increase risks for developing cancer [Bellizzi and Frankel, 2009; Jenkins, 2009].

These observations suggest that NU1025-mediated reduction in the abundance of DNA repair proteins in BT-20 breast cancer cells could potentially explain much of the high cytotoxicity of the PARP-1 inhibitor. Clearly, functional DNA repair pathways allow cancer to overcome DNA damage induced by chemotherapy and survive.

Thus the efficacy of anti-cancer drugs is highly influenced by the cellular DNA repair capacity and dysfunctions in DNA repair processes sensitize malignant cells to chemotherapy and help to reduce severe side effects. In the last 20 years germline and somatic mutations in a number of DNA repair genes have been detected, and associated with predisposition to the development of breast, ovarian and other cancers [Barcellos-Hof and Kleinber, 2013; Rustgi, 2014].

Knowledge of the effects and mechanisms of deficits in DNA repair machinery has also recently provided rationales for the establishment of synthetic lethality [Helleday, 2011; Wesierska-Gadek and Skladanowski, 2012]. This new therapeutic strategy, currently being developed and exploited in clinical oncology, is selective because cancer cells are primarily affected by the treatment. Therefore, very recent studies have focused on determining DNA repair gene profiles as prognostic and predictive factors for distinct cancer subtypes [Santarpia et al., 2013].

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