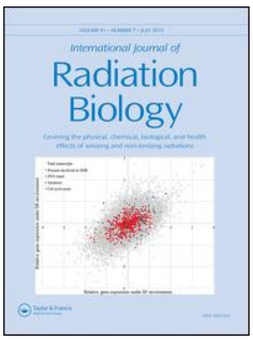

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RESEARCH ARTICLE

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Evaluation of the radiosensitizing potency of chemotherapeutic agents in prostate cancer cells

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ABSTRACT

Purpose: Despite recent advances in the treatment of metastatic prostate cancer, survival rates are low and treatment options are limited to chemotherapy and hormonal therapy. Although ionizing radiation is used to treat localized and metastatic prostate cancer, the most efficient use of radiotherapy is yet to be defined. Our purpose was to determine *in vitro* the potential benefit to be gained by combining radiation treatment with cytotoxic drugs.

Materials and methods: Inhibitors of DNA repair and heat shock protein 90 and an inducer of oxidative stress were evaluated in combination with X-radiation for their capacity to reduce clonogenic survival and delay the growth of multicellular tumor spheroids.

Results: Inhibitors of the PARP DNA repair pathway, olaparib and rucaparib, and the HSP90 inhibitor 17-DMAG, enhanced the clonogenic cell kill and spheroid growth delay induced by X-radiation. However, the oxidative stress-inducing drug elesclomol failed to potentiate the effects of X-radiation. PARP inhibitors arrested cells in the G_2/M phase when administered as single agents or in combination with radiation, whereas elesclomol and 17-DMAG did not affect radiation-induced cell cycle modulation.

Conclusion: These results indicate that radiotherapy of prostate cancer may be optimized by combination with inhibitors of PARP or HSP90, but not elesclomol.

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Hsp90; oxidative stress; PARP; prostate cancer; radiosensitization

Introduction

While external beam radiotherapy is effective for local control and palliation in prostate cancer patients, its use to treat widespread disease is limited (Bonkhoff 2012). Furthermore, intense local irradiation can result in damage to adjacent, non-cancerous tissues, and wide-field radiotherapy is associated with bone marrow toxicity. In order to enhance radiotherapy, there are several options for intervention, including the application of agents directed against DNA repair pathways, redox homeostasis and pathways associated with tumor cell survival (Mairs & Boyd 2011).

The activity of the DNA repair enzyme, poly(ADP-ribose)-polymerase [PARP], is generally greater in cancer cells than in normal cells (Zaremba et al. 2009). Inhibitors of PARP improve the efficiency and selectivity of DNA-damaging agents (Calabrese et al. 2004). The PARP inhibitors olaparib and rucaparib have entered clinical trials for several cancer types, either as monotherapy or in combination with cytotoxic drug therapy (Audeh et al. 2010; Tutt et al. 2010; Plummer et al. 2013; Bendell et al. 2015; Mateo et al. 2015; Drew et al. 2016). Radiosensitization has been demonstrated in human and rodent cell lines and in experimental tumors by treatment with PARP inhibitors (Virag & Szabo 2002; Brock et al. 2004; Calabrese et al. 2004), indicating their potential as components of combination therapy.

Ionizing radiation damages DNA directly or through the production of reactive oxygen and nitrogen species (ROS and RNS). These reactive molecules, particularly hydroxyl radical, induce clustered DNA lesions and lethal double strand breaks and can be derived from radiolysis of water, the mitochondrial electron transport chain or bystander effects. Cancer cells produce ROS in abundance due to aberrant mitochondrial activity and unregulated metabolism (Trachootham et al. 2009). Therefore, they are susceptible to treatments that further disrupt the oxidant/antioxidant balance resulting in an increase in ROS levels which may overwhelm antioxidant capacity and initiate apoptosis (Storz 2005). In contrast, normal cells are less sensitive to ROS generation because their ROS content is lower and their antioxidative metabolism is more efficient. The primary mechanism of action of the drug elesclomol [N-malonyl-bis (N′-methyl-N′-thiobenzoylhydrazide)] is the generation of ROS leading to oxidative stress which induces growth arrest and apoptosis in cancer, but not normal, cells (Kirshner et al. 2000; Qu et al. 2009; Nagai et al. 2012). In preclinical studies, elesclomol sensitized cancer cells to other chemotherapeutic agents (Qu et al. 2009; Blackman et al. 2012) and, when combined with paclitaxel in a phase II clinical trial, was shown to prolong progression-free survival in patients with metastatic melanoma (O'Day et al. 2009). Therefore, drug-induced increase in ROS is a

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potentially effective method to enhance the efficacy of radiotherapy.

Due to the increased stress response in metabolically hyperactive cancer cells, elevated levels of heat shock proteins have been observed in many tumor types (Mahalingam et al. 2009). Heat shock protein 90 (Hsp90) is a molecular chaperone critical for the folding, assembly and activity of multiple proteins. Its client proteins include signalling molecules, such as nuclear hormone receptors and tyrosine kinases (Lu et al. 2012), whose mutation or overexpression promotes the growth and survival of tumor cells. Inhibition of Hsp90 is a mechanism for inhibiting simultaneously multiple signaling pathways that promote cancer cell survival and proliferation. Geldanamycin and its derivatives display potent antitumor activity resulting from high affinity binding to Hsp90 (Tian et al. 2004). One such drug is 17-allylamino-demethoxygeldanamycin (17-AAG, tanespimycin), which is less hepatotoxic than geldanamycin. Preclinical data indicated that 17-AAG synergistically enhanced the cancer killing activity of various cytotoxic agents (Lu et al. 2012). Therefore, therapies which induce further cellular stress on tumor cells, including ionizing radiation, are likely to enhance the effects of Hsp90 inhibitors (Solit & Chiosis 2008). However, the clinical use of 17-AAG has been hampered by poor solubility and lack of oral bioavailability. The water-soluble geldanamycin derivative 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG, alvespimycin) appeared to overcome these problems and exhibited antitumor activity in preclinical models (Hollingshead et al. 2005). Significantly, 17-DMAG sensitized human tumor cells to radiation both *in vitro* and *in vivo* (Bull et al. 2004).

To determine the potential therapeutic benefit to be gained from combined modality treatments, the antitumor potencies of two PARP inhibitors, olaparib and rucaparib, elesclomol and 17-DMAG were evaluated and compared in combination with X-radiation in 2- and 3-dimensional *in vitro* models of prostate cancer cell lines using as endpoints clonogenic survival and growth delay of multicellular tumor spheroids.

Materials and methods

Reagents

All cell culture media and supplements were purchased from Life Technologies (Paisley, UK), unless stated otherwise. Olaparib, rucaparib and elesclomol were purchased from Selleckchem (Suffolk, UK). All other chemicals, including 17-DMAG, were from Sigma-Aldrich (Dorset, UK). Stock solutions of olaparib, 17-DMAG and elesclomol were prepared in dimethyl sulfoxide (DMSO). The maximum DMSO concentration in culture medium was 0.1% (*v/v*).

Tissue culture

Human prostate cancer cell lines, PC3 and LNCaP, were obtained from American Type Culture Collection (Manassas, VA) and were used in this study for less than 6 months after resuscitation. PC3 cells were maintained in F12K medium supplemented with 10% (*v/v*) fetal bovine serum (Autogen Bioclear, Wiltshire, UK), 2 mM L-glutamine, 0.1 mM sodium pyruvate and 50 μg/ml gentamicin. LNCaP cells were maintained in RPMI 1640 medium supplemented with 10% (*v/v*) fetal bovine serum (Hyclone, Fisher Scientific, UK), 1% (*v/v*) HEPES, 1% (*v/v*) D-glucose, 1 mM sodium pyruvate, 4 mM L-glutamine, 50 μg/ml gentamicin.

MTT cell proliferation assay

MTT reduction was performed according to the method of Mosmann (1983). Cells were seeded in 96-well plates, and incubated for 2 days to allow exponential phase growth. Cells were then washed twice with PBS and medium containing drug at the required concentration was added. After 48 h, MTT was added to a final concentration of 0.5 mg/ml and cultures were incubated for 2 h. Cells were then solubilized with DMSO before measuring absorbance at 570 nm.

Clonogenic survival assay

PC3 cells were seeded in 25 cm^2 flasks at 10^5 cells/flask. When cultures were in exponential growth phase, medium was removed and replaced with fresh medium containing drug. Cells were irradiated using an X-Strahl RS225 X-ray irradiator at a dose rate of 1.6 Gy per min. Flasks were then incubated for 24 h at 37 °C in 5% CO_2. After treatment, cells were counted and seeded in triplicate Petri dishes for clonogenic survival assay. Cells were incubated at 37 °C in 5% CO_2 for 10 days. Colonies were fixed in methanol, stained with crystal violet solution and colonies of at least 50 cells were counted.

Multicellular spheroid growth assay

Multicellular tumor spheroids consisting of LNCaP cells were obtained using the liquid overlay technique (Yuhas et al. 1977). Spheroids were initiated by inoculating cells into a plastic flask coated with 1% (*w/v*) agar. After 3 days, aliquots of spheroids were transferred to sterile plastic tubes and centrifuged at 12 *g* for 3 min. Thereafter, spheroids were irradiated or re-suspended in serum-free culture medium containing drug. After treatment, the spheroids were washed twice and those of approximately 100 μm in diameter were transferred individually into agar-coated wells of 24-well plates. Individual spheroid growth was monitored twice per week for 3 weeks using an inverted phase-contrast microscope connected to an image acquisition system. Two perpendicular diameters, d_{max} and d_{min}, were measured using image analysis software (ImageJ) and the volume, V (μm^3), was calculated using the formula: $V = \pi \times d_{max} \times d_{min}^2/6$ (Neshasteh-Riz et al. 1997). The area under the V/V_0 versus time curve (AUC) was calculated for individual spheroids using trapezoidal approximation.

Cell cycle analysis

Following treatment for 6 or 24 h, LNCaP or PC3 cells were trypsinized, then washed twice with PBS. Cells were fixed in

ice cold 70% (v/v) ethanol; then washed twice with PBS and re-suspended in PBS containing propidium iodide (10 μg/ml) and RNase A (200 μg/ml). Cells were stained for 30 min before flow cytometric analysis using a FACSCalibur (BD Biosciences, Mountain View, CA).

Statistical analysis

Data are presented as means ± standard error of the mean (SEM), with the number of independent repetitions provided in the legend to each figure. Statistical significance was determined using Student's *t*-test. A p value <0.05 was considered to be statistically significant and <0.01 highly significant. To test for differences in spheroid growth between experimental therapy groups, the Kruskal-Wallis test was used with post hoc testing by the Mann-Whitney U test with Bonferroni correction. Analysis was carried out using SPSS software.

Results

Drug-induced inhibition of proliferation

All drugs used in this study (olaparib, rucaparib, elesclomol, 17-DMAG) decreased the rate of proliferation of PC3 and LNCaP cells in a concentration-dependent manner, as shown in Figure 1. Rucaparib and elesclomol significantly inhibited proliferation in both cell lines when administered at concentrations $\geq$50 μM, whereas olaparib significantly inhibited proliferation only in LNCaP cells. 17-DMAG decreased proliferation at concentrations $\geq$50 nM.

Enhancement of radiation-induced clonogenic cell kill

The concentration-dependent effect of drugs administered as single agents is shown in Supplementary Figure 1, available online. As shown in Figure 2(A), administration of the PARP inhibitor olaparib (1 and 3 μM) simultaneously with X-radiation increased the kill of PC3 clonogens induced by either agent alone. The IC_{50} values obtained following the exposure of PC3 cells to X-rays alone, or in the presence of 1 and 3 μM olaparib were 1.92 ± 0.14, 1.25 ± 0.05 and 0.96 ± 0.08 Gy, respectively. The surviving fractions following radiation treatment at a dose of 2 Gy (SF2) were 0.50 ± 0.08, 0.21 ± 0.01 and 0.12 ± 0.03 for 0, 1 and 3 μM olaparib, respectively. These observations indicate concentration-dependent radiosensitization by olaparib.

The alternative PARP inhibitor rucaparib caused a similar degree of radiosensitization, as presented in Figure 2(B). The IC_{50} values obtained following the exposure of PC3 cells to X-rays alone, or in the presence of 1 or 3 μM rucaparib were 1.81 ± 0.21, 1.41 ± 0.20 and 1.00 ± 0.04 Gy, respectively. The SF2 values were 0.45 ± 0.01, 0.26 ± 0.05 and 0.13 ± 0.02 for 0, 1 and 3 μM rucaparib, respectively. These observations indicate concentration-dependent radiosensitization by rucaparib with potency similar to that of olaparib.

Simultaneous administration of elesclomol with X-radiation did not enhance the clonogenic cell kill achieved by

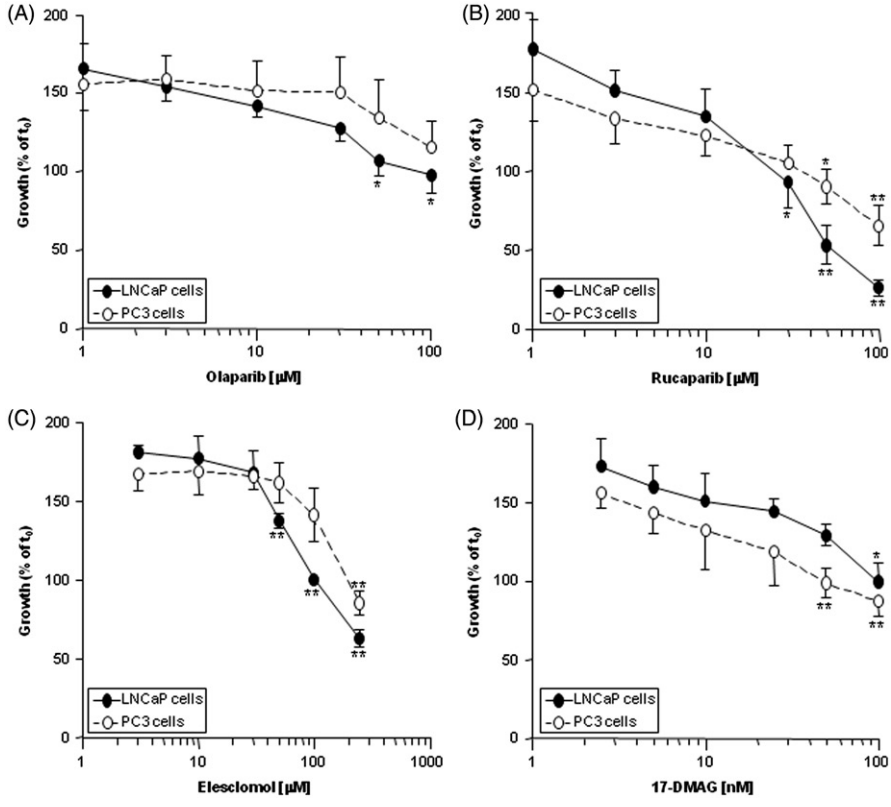

Figure 1. The proliferation of PC3 and LNCaP cells exposed to drugs at a range of concentrations for 48 h was assessed using MTT assay. Cells were exposed to (A) olaparib, (B) rucaparib, (C) elesclomol or (D) 17-DMAG. Results are expressed as percentage of MTT absorbance of untreated cells at 0 h. *$p < .05$ and **$p < .01$ compared to untreated controls. Data are means ± SEM; $n = 4$.

either treatment alone, manifest as overlapping radiation survival curves in Figure 2(C). The IC_{50} values obtained following the exposure of PC3 cells to X-rays alone, or in the presence of 10 and 30 μM elesclomol were 1.69 ± 0.10, 1.63 ± 0.16 and 1.59 ± 0.08 Gy, respectively. The SF2 values were 0.43 ± 0.04, 0.35 ± 0.06 and 0.36 ± 0.04 for 0, 10 and 30 μM elesclomol, respectively. The lack of significant differences in IC_{50} and SF2 values compared to radiation alone suggests that elesclomol did not sensitize these cells to radiation.

Inhibition of Hsp90 by 17-DMAG enhanced the clonogenic killing ability of X-radiation when administered simultaneously, as shown in Figure 2(D). The IC_{50} values obtained following the exposure of PC3 cells to X-rays alone, or in the presence of 5 and 10 nM 17-DMAG were 1.78 ± 0.05, 1.11 ± 0.06 and 1.10 ± 0.09 Gy, respectively. The SF2 values were 0.53 ± 0.02, 0.22 ± 0.02 and 0.23 ± 0.02 for 0, 5 and 10 nM 17-DMAG, respectively. These observations indicate the radiosensitizing activity of 17-DMAG.

Enhancement of radiation-induced spheroid growth delay

As shown in Figure 3, radiation alone caused a reduction in the growth of multicellular spheroids composed of LNCaP cells, indicated by an area under the spheroid growth curve (AUC) of $56.2 \pm 4.3\%$ of untreated control spheroids, reported in Table 1. The ability of the chemotherapeutic agents to enhance this spheroid growth delay was investigated using simultaneously administered combinations.

The PARP inhibitors olaparib and rucaparib had no significant effect on spheroid growth when administered at 30 μM, whereas 100 μM caused a reduction in spheroid growth. Although the radiation-induced decrease in spheroid growth was not significantly enhanced by 30 μM olaparib, simultaneous administration of 100 μM olaparib with radiation resulted in a significantly enhanced spheroid growth delay, shown in Figure 3(A). Rucaparib (100 μM) also significantly enhanced the growth delay induced by radiation treatment, as can be seen in Figure 3(B). As shown in Figure 3(C) and Table 1, although elesclomol inhibited spheroid growth when administered as a single agent, the growth delay resulting from radiation exposure was not significantly enhanced by co-administration of elesclomol at either 30 or 100 μM. Administration of 17-DMAG to spheroids as a single agent decreased their growth rate, as shown in Figure 3(D). The growth delay effect of radiation alone was significantly enhanced by combination with 25 nM 17-DMAG.

Drug-induced alteration of cell cycle distribution

The effects of X-radiation and drug treatment on cell cycle progression was assessed in LNCaP and PC3 cells and are reported in Figure 4. Radiation alone significantly increased the proportion of LNCaP and PC3 cells in the G_2/M phase of the cell cycle 6 h after administration. The G_2/M arrest was reduced in PC3 cells 24 h after irradiation and in LNCaP cells a significant decrease in cells in G_2/M phase was accompanied by an increase in G_1 phase cells. Administration of drugs

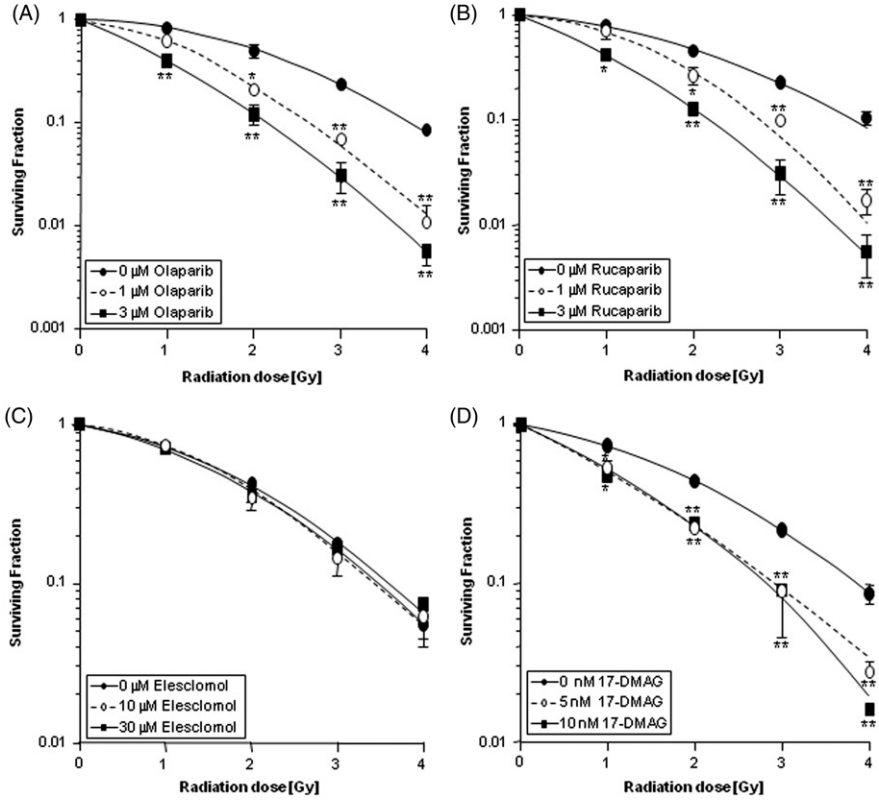

Figure 2. The effect of drugs on radiation-induced clonogenic cell kill. Drugs were administered simultaneously with X-rays and 24 h later clonogenic assay was carried out. PC3 cells were exposed to (A) 1 or 3 μM olaparib, (B) 1 or 3 μM rucaparib, (C) 10 or 30 μM elesclomol or (D) 5 or 10 nM 17-DMAG. $^{*}p < .05$ and $^{**}p < .01$ compared to X-rays alone. Data are means ± SEM; $n = 3$.

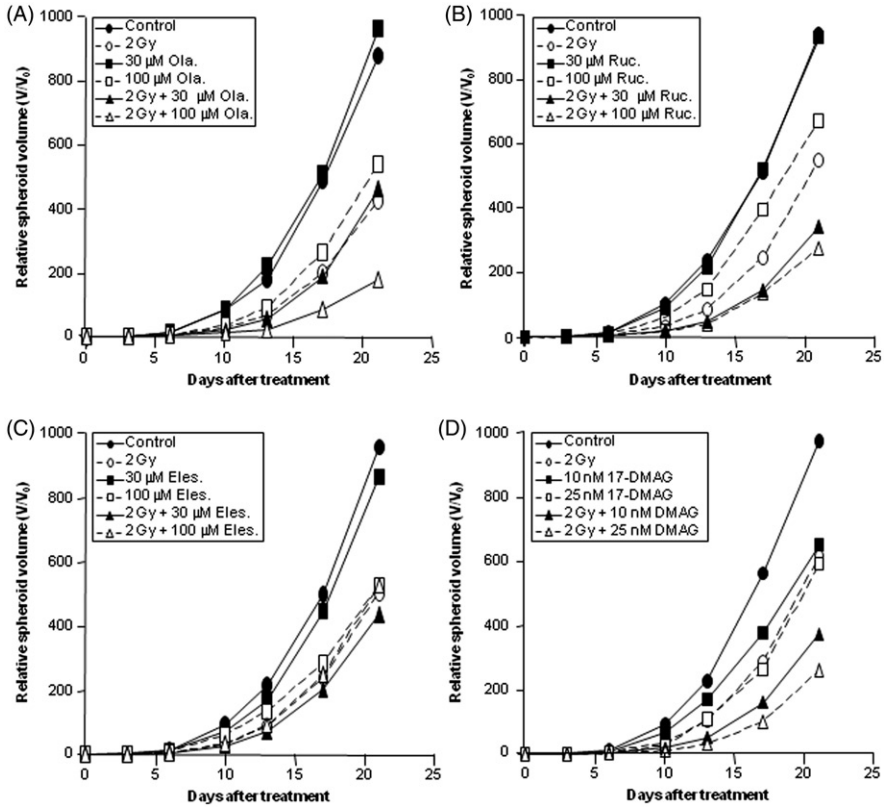

Figure 3. The effect of radiation and drugs, alone or in combination, on the growth of LNCaP spheroids. Spheroids composed of LNCaP cells were exposed to (A) 30 or 100 μM olaparib, (B) 30 or 100 μM rucaparib, (C) 30 or 100 μM elesclomol or (D) 10 or 25 nM 17-DMAG, alone or in simultaneous combination with 2 Gy X-radiation and 24 h later were transferred to agar-coated plates. Each spheroid was then photographed twice per week and change in spheroid volume (V/V_0) was measured up to 21 days. Data are means, $n = 3$. Error bars are omitted for clarity.

Table 1. Comparison of the effect of single-agent treatment with combination treatment on the growth of LNCaP spheroids. Data are expressed as area under the volume-time curve (AUC). Values are means ± SEM of three separate experiments.

Treatment	AUC (% of control)
2 Gy X-ray	56.2 ± 4.3
30 μM olaparib	101.4 ± 10.6
2 Gy X-ray +30 μM olaparib	53.3 ± 9.5*
100 μM olaparib	76.5 ± 12.8
2 Gy X-ray +100 μM olaparib	27.7 ± 12.3*†
30 μM rucaparib	93.8 ± 8.5
2 Gy X-ray +30 μM rucaparib	28.7 ± 5.0*
100 μM rucaparib	72.3 ± 8.6
2 Gy X-ray +100 μM rucaparib	28.4 ± 10.5*†
30 μM elesclomol	88.8 ± 4.4
2 Gy X-ray +30 μM elesclomol	45.5 ± 14.2*
100 μM elesclomol	61.9 ± 13.1
2 Gy X-ray +100 μM elesclomol	51.4 ± 8.7
10 nM 17-DMAG	75.9 ± 3.6
2 Gy X-ray +10 nM 17-DMAG	44.2 ± 14.9*
25 nM 17-DMAG	59.9 ± 13.9
2 Gy X-rays +25 nM 17-DMAG	21.5 ± 5.4*†

*pertains to combinations compared with drug alone; †pertains to combinations compared with X-radiation alone; */† $p < .05$.

for 6 h had no significant effect on cell cycle distribution. Treatment with the PARP inhibitors olaparib or rucaparib for 24 h resulted in significant arrest in G_2/M phase. Combination of PARP inhibitor with X-radiation resulted in an increase in LNCaP and PC3 cells in G_2/M phase compared with radiation alone.

In LNCaP cells, 30 μM elesclomol induced a significant decrease in G_2/M cells 24 h after administration. However, the combination of elesclomol with radiation induced no further decrease in G_2/M arrest compared with radiation alone. In PC3 cells, 30 μM elesclomol induced a small increase in G_2/M ($p < .05$). However, when PC3 cells were treated with a combination of X-radiation and 30 μM elesclomol, no significant difference in the proportion of G_2/M cells was observed compared to untreated cells or cells treated with radiation only. 17-DMAG induced no significant change in cell cycle distribution of LNCaP 24 h after administration, whereas a small increase in G_2/M was observed in PC3 cells. No alteration of the radiation-induced effect was observed in either cell line after simultaneous administration of 17-DMAG.

Discussion

We have assessed the ability of several classes of chemotherapeutic agents to enhance the tumor-killing efficacy of radiation, administered in the form of external beam X-rays. The anti-proliferative activity of the drugs was evaluated as well as their ability to decrease survival of clonogens and growth of multicellular spheroids. In addition, the cell cycle distribution of cells after administration of drugs alone and in combination with radiation was determined. Two prostate cell lines were used in this study: PC3 cells which are androgen-insensitive and form clonogens, but not multicellular spheroids; and LNCaP cells which are androgen-sensitive and

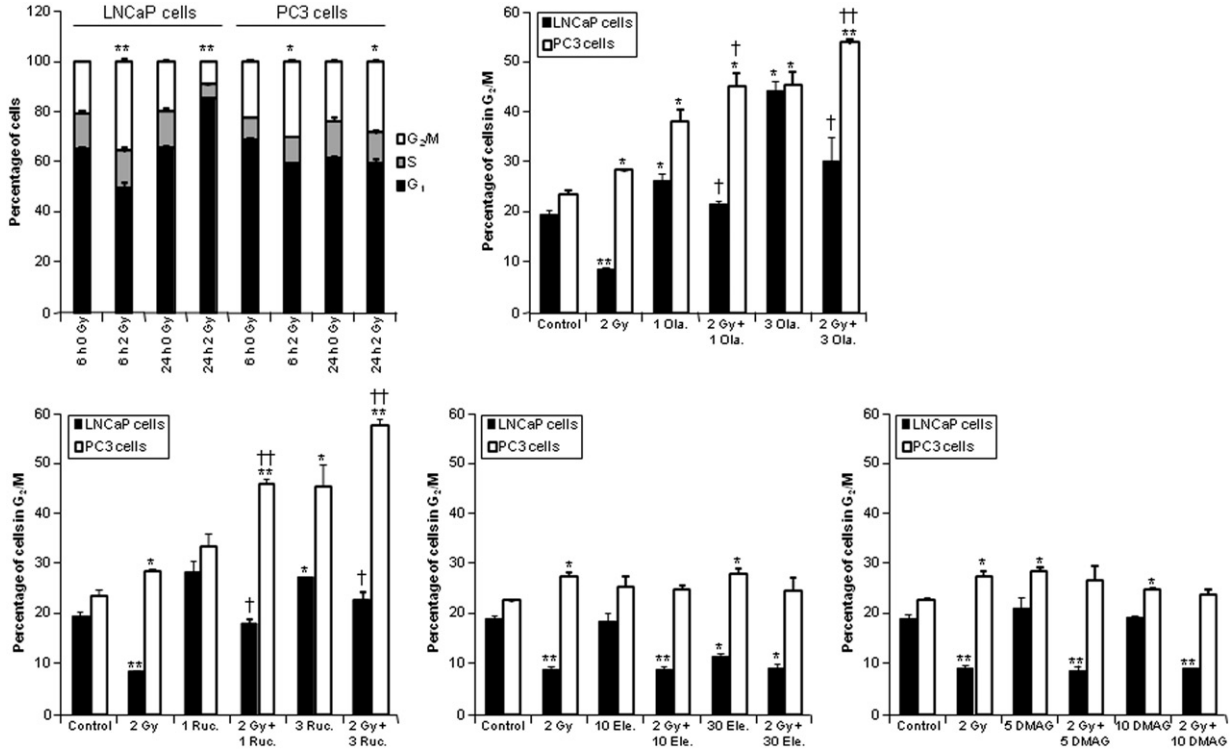

Figure 4. (A) Cell cycle distribution of LNCaP and PC3 cells 6 and 24 h after 2 Gy X-irradiation. LNCaP and PC3 cells in G_2/M phase of the cell cycle 24 h after exposure to (B) 1 or 3 μM olaparib, (C) 1 or 3 μM rucaparib, (D) 10 or 30 μM elesclomol and (E) 5 or 10 nM 17-DMAG. Data are means ± SEM; $n = 3$. $^{*}p < .05$ and $^{**}p < .01$ compared to untreated contol cells. $^{\dagger}p < .05$ and $^{\dagger\dagger}p < .01$ compared to radiation treatment alone.

form spheroids, but did not form clonogens in our hands. The anti-proliferative and cell cycle modulating activity in monolayer cultures of both cell lines did, however, allow some comparisons to be made.

The radiosensitizing potential of drugs was assessed in 2- and 3-dimensional tumor models. The concentrations of drugs required to significantly affect the proliferation and clonogenic survival of monolayer cultures of both LNCaP and PC3 cells were similar, whereas the concentration required to induce significant delay of spheroid growth were greater for all drugs used. Multicellular spheroids are representative of micrometastases in their prevascular stage of development. The relative resistance of multicellular spheroids has previously been observed and is most likely due to differences in drug penetration and the microenvironment of the various layers within the spheroid (Kwok & Twentyman 1985). The results observed here confirm that the greater concentration required for growth delay and radiosensitization of spheroids were not due to differences in the cell response, but most likely due to the 3-dimensional structure of spheroids.

It is possible that the combination of radiation with radiosensitizing drugs increased the induction of apoptosis, similar to the radiosensitizing effect of the fatty acid inhibitor C75 (Rae et al. 2015). The response to drugs or radiation may also be influenced by the p53 status of the cells. PC3 cells are p53 non-functional, whereas LNCaP cells are p53 wild-type (Carroll et al. 1993). As demonstrated here and previously (Rae et al. 2015), the cell cycle distribution differs between PC3 and LNCaP cells after irradiation. Cell cycle arrest in G_2/M phase was observed in both cell lines 6 h after irradiation. In PC3 cells, the cell cycle distribution returns to control proportions 24 h after irradiation, whereas LNCaP cells accumulate in G_1 phase. This may be explained by the inefficiency of DNA damage repair mechanisms reported in LNCaP cells in G_2/M phase (Xie et al. 2010) and consequent reliance of G_1 checkpoints, whereas PC3 cells lack the ability to arrest in G_1 phase.

DNA repair is a potential target pathway for tumors and inhibitors of repair have been shown to be effective radiosensitizers (Brock et al. 2004). Tumors with aberrant DNA repair pathways are particularly sensitive to PARP inhibitors (Farmer et al. 2005; Horton et al. 2014) and olaparib is approved by the FDA for the treatment of patients whose ovarian cancers harbour BRCA mutations. Furthermore, olaparib led to a high response rate in prostate cancer patients with metastatic, castrate-resistant disease characterized by defects in DNA-repair genes (Mateo et al. 2015), suggesting that PARP inhibitors would be a particularly useful addition to the treatment of this cancer.

PARP binds to both single- and double-stranded DNA breaks, partly via the base excision pathway, which plays an important role in repairing single-strand breaks induced by ionizing radiation (Chalmers 2009). Therefore, it is likely that PARP inhibition promotes increased formation of DNA single-strand breaks by ionizing radiation, which are subsequently converted to potentially lethal double-strand breaks during cell replication. Rucaparib has been used as a radiosensitizer in phase I and II clinical trials in head and neck cancers and CNS neoplasm (Verheij et al. 2010; Mangerich & Burkle 2011), indicating its potential in the clinical management of other solid tumours.

Here, we show that both PARP inhibitors, olaparib and rucaparib, had an anti-proliferative effect in both LNCaP and PC3 cells at similar concentrations when cultured as monolayers, with rucaparib being the more potent drug. Crucially, radiosensitization was demonstrated in both LNCaP (androgen-sensitive) and PC3 (androgen-insensitive) cell lines, suggesting that androgen sensitivity did not affect their activity. Furthermore, olaparib and rucaparib increased the proportion of cells in G_2/M phase of the cell cycle in both LNCaP (p53 wild type) and PC3 (p53 non-functional) cell lines, suggesting that p53 status was not a determining factor in their activity. Olaparib and rucaparib enhanced the clonogenic cell killing and spheroid growth delay activity of radiation and accordingly, these PARP inhibitors can be considered as sensitizers of prostate cancer cells to radiation.

The lethality of ionizing radiation and some chemotherapeutic agents is hypothesized to be due to increasing ROS concentrations to cytotoxic levels preferentially in tumors which typically have elevated levels of ROS and impaired antioxidant activity as a consequence of higher rates of proliferation and metabolism (Trachootham et al. 2009). The sensitivity of cancer cells to therapeutic schemes which stimulate cell death through ROS production encourages the use of these drugs in combination with radiotherapy.

Clinical trials using elesclomol have primarily assessed its efficacy in melanoma patients. Due to melanin biosynthesis, melanoma cells contain particularly high levels of ROS (Wittgen & van Kempen 2007), and this may result in this cancer being sensitive to elesclomol-induced cytotoxicity. Advanced prostate cancer is also associated with a state of high oxidative stress (Yossepowitch et al. 2007), suggesting that the prostate cancer cell lines used in this study would be similarly sensitive to elesclomol. A phase I/II study of elesclomol plus docetaxel in patients with metastatic castration refractory prostate cancer has recently been completed (NCT00808418), although the findings are currently unknown. The encouraging results of a phase II clinical trial of elesclomol in combination with paclitaxel (O'Day et al. 2009) were not confirmed in a subsequent phase III trial (O'Day et al. 2013). However, it is possible that a population of patients with normal serum lactate dehydrogenase (LDH) levels may respond well to this therapy, whereas high LDH levels may predict a negative clinical effect (Nagai et al. 2012; O'Day et al. 2013), reflecting the importance of tumor mitochondrial activity.

Concentrations of elesclomol used in this study were sufficient to decrease proliferation, clonogenic survival and spheroid growth. Although the degree of ROS generation was reported to be higher in PC3 cells than in LNCaP cells (Kumar et al. 2008), we demonstrate here that, when administered to monolayer cultures as a single agent, elesclomol affected both cell lines similarly, with growth inhibition and redistribution of the cells cycle observed in response to similar concentrations of elesclomol. Although oxidative stress can directly activate p53 pro-apoptotic signalling, the cytotoxic effect of elesclomol was similar in PC3 and LNCaP cells, suggesting that p53 does not play a major role in the observed effects of elesclomol. Radiation induced an accumulation of LNCaP cells in the G_1 phase of the cell cycle 24 h after administration, which was similar to the effect of elesclomol, and this was not increased by combination treatment. In addition, combination with radiation enhanced neither the radiation-induced clonogenic kill of PC3 cells nor the decreased growth of LNCaP spheroids, confirming that elesclomol did not sensitize these cell lines to X-radiation.

Elesclomol is most active in well-oxygenated cells, whereas hypoxia is likely to reduce activity (O'Day et al. 2013). Although LNCaP spheroids contain a hypoxic core of cells, this occurs only in spheroids $\geq$300 μm in diameter (Ballangrad et al. 1999). We have recently demonstrated the absence of hypoxic regions in newly-initiated spheroids ($<$100 μm diameter) (Tesson et al. 2016). When administered as a single agent at a concentration of 100 μM, elesclomol inhibited spheroid growth. However, no radiosensitization was observed in response to this concentration of elesclomol. Furthermore, no enhancement of radiation-induced clonogenic cell kill was observed following the treatment of 2-dimensional cultures with elesclomol at concentrations which, as a single agent, decreased clonogenic survival. This suggests that the observed lack of radiosensitization by elesclomol was unlikely to be a result of the development of a hypoxic core in the spheroids.

As both radiation and elesclomol can interact with the electron transport chain (ETC) to elevate intracellular ROS levels (Blackman et al. 2012; Yamamori et al. 2012), it is possible that radiation-induced ROS generation is sufficient for lethal DNA damage to occur and that further increasing oxidative stress by co-administration of elesclomol offers no additional cell killing effect. Combinations of elesclomol with drugs which inhibit the ETC complex synergistically enhanced the cytotoxic effects of elesclomol (Blackman et al. 2012), whereas ETC function was upregulated by ionizing radiation (Yamamori et al. 2012). This may explain the lack of enhancement of single modality treatment which was observed in this study and suggests that combination of treatments which act through a shared mechanism of action may not be an effective method for sensitizing tumors to radiotherapy.

Elevated Hsp90 expression in many solid and hematologic tumors correlates with poor prognosis (Den and Lu 2012). Hsp90 promotes tumor growth and survival by chaperoning factors such as HER2 proto-oncogene and molecules such as Akt (involved in cell survival, inhibiting apoptosis), c-Src (promotes tumor growth and survival) and Raf-1 (promotes cell division via MAPK/Erk pathway) (Soo et al. 2008). Hsp90 is also implicated in tumor invasion and metastasis through its interaction with H1F1α, VEGF and MMP-2 (Soo et al. 2008).

Previous *in vitro* studies have indicated that the Hsp90 inhibitor 17-DMAG enhanced radiation-induced kill of cells from a variety of tumors (Bull et al. 2004; Dote et al. 2006; Koll et al. 2008). In agreement with previous reports of the efficacy of treatment with 17-AAG and 17-DMAG of prostate cancer clonogens and spheroids (Enmon et al. 2003; Bull et al. 2004), we have demonstrated here that 17-DMAG sensitized prostate cancer cell lines to radiation in both 2- and 3-dimensional models in a supra-additive manner. We observed that the concentration of 17-DMAG required to enhance the growth delay of LNCaP spheroids was 10 nM. This was significantly lower than the previously reported concentration of 17-AAG (1000 nM) required for synergism with 2 Gy radiation

(Enmon et al. 2003), suggesting that 17-DMAG is a more potent radiosensitizer than 17-AAG. Crucially, the concentration of 17-DMAG, as a single agent, required for radiosensitization, had negligible cytotoxicity, indicating that therapeutic enhancement would be possible with minimal increase in toxicity.

The radiosensitizing effect of Hsp90 inhibition may be caused by modulation of the activity of proteins associated with radioresistance, including Raf-1, ErbB2 and Akt (Russell et al. 2003), leading to alterations in DNA repair ability, diminished NF-κB activity, cell cycle regulation and apoptosis (Dote et al. 2006; Koll et al. 2008). The p53 status of cells has been suggested to play only a minor role in radiosensitization by 17-DMAG (Koll et al. 2008), and this was confirmed by the radiosensitizing effect observed here in both PC3 cells and LNCaP cells. Radiosensitization induced by the Hsp90 inhibitor radicicol in prostate cancer cells has been ascribed to degradation of the androgen receptor, which is an Hsp90 client (Harashima et al. 2005). However, we have shown here a radiosensitizing effect in both androgen-sensitive (LNCaP) and androgen-insensitive (PC3) cell lines, suggesting that 17-DMAG may be clinically useful even during progression of hormone-refractory disease.

Clinical activity of Hsp90 inhibitors has been demonstrated primarily in tumors with known alterations in oncoproteins which are Hsp90 client proteins, such as amplified HER2 in breast cancer, ALK rearrangements in non-small cell lung cancer and mutant BRAF in melanoma (Solit & Chiosis 2008; Butler et al. 2015). Therefore, as with PARP inhibitors, it is likely that the major clinical impact will be obtained in stratified patient groups selected for known biomarkers indicating sensitivity to these agents. A phase I trial of 17-DMAG for advanced solid tumors revealed clinical activity in several tumor types including a complete response in hormone-refractory prostate cancer (Pacey et al. 2011). However, in general, clinical activity of Hsp90 inhibitors as single agents has been modest and it is predicted that the maximum clinical benefit from these agents are likely to be in combination therapies.

This preliminary screening study indicates that it is possible to sensitize prostate cancer cells to radiation using pharmacological inhibitors of DNA repair and stress response proteins, and efforts concentrating on these pathways are likely to lead to improvements in the efficacy of radiotherapy. Although the radiosensitizing potential of PARP inhibitors has been suggested previously, this study compares for the first time two alternative PARP inhibitors currently in clinical trials. The ability of elesclomol to potentiate radiation-induced cancer killing has also not been previously evaluated. In addition, the inclusion of the radiosensitizing Hsp90 inhibitor 17-DMAG in this study allows direct comparison of the potency and mode of action with respect to cell cycle modulation. Further investigation *in vivo* of the efficacy of the radiosensitizing agents in combination with radiotherapy may expedite progress of this therapeutic strategy for the clinical management of metastatic prostate cancer.

Disclosure statement

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