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Simultaneous PLK1 inhibition improves local tumour control after fractionated irradiation

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ABSTRACT

Purpose: Polo-like kinase 1 (PLK1) plays an important role in mitotic progression, is frequently overexpressed and associated with a poor prognosis of cancer patients, thus providing a promising target in anticancer treatment. Aim of the current project was to evaluate the effect of the novel PLK1 inhibitor BI 6727 in combination with irradiation.

Material and methods: In vitro proliferation and radiation cell survival assays as well as in vivo local tumour control assays after single treatment and combined radiation and drug application were carried out using the squamous cell carcinoma models A431 and FaDu. In addition, cell cycle phases were monitored in vitro and in vivo.

Results: BI 6727 showed a dose-dependent antiproliferative effect and an increase in the mitotic fraction. BI 6727 alone reduced clonogenic cell survival, while radiosensitivity in vitro (SF2) and in vivo (single-dose TCD_{50} under clamped hypoxia) was not affected. In contrast, local tumour control was significantly improved after application of BI 6727 simultaneously to fractionated irradiation (A431: TCD_{50} = 60.5 Gy [95% C.I. 57; 63] after IR alone and <30 Gy after combined treatment; FaDu: 49.5 Gy [43; 56 Gy] versus 32.9 Gy [26; 40]).

Conclusions: Despite the lack of direct cellular radiosensitisation, PLK1 inhibition with BI 6727 during fractionated irradiation significantly improves local tumour control when compared to irradiation alone. This result is likely explained by a considerable effect on cell cycle and an independent cytotoxic potential of BI 6727.

PLK1 is a member of the PLK family, consisting of five kinases (PLK1–5). Due to its overexpression in many human tumours and its association with a poor prognosis [9,34], PLK1 is a promising target for anti-cancer treatment. PLK1 plays an important role in mitotic progression, while PLK1 inhibition results in mitotic arrest and death of many cells due to mitotic catastrophe [20,33]. Preclinical data suggest that normal tissue cells are more likely to survive PLK1 depletion than tumour cells [7,21]. Specific inhibition of PLK1 by the small molecule BI 2536 has been shown to block proliferation of human cancer cell lines in vitro and to inhibit tumour growth in xenograft models [33]. BI 6727 is another small molecule compound, which in preclinical studies in human colon xenografts showed a higher effect on tumour growth and lower toxicity as compared to the classic anti-mitotic agent Docetaxel [28].

In combination with irradiation, knockdown of PLK1 by small interfering RNA (siRNA) resulted in a higher cytotoxic effect of irradiation in FaDu [11] or colon cancer cells in vitro [27]. In vivo, the combination of irradiation with PLK1 knockdown had a stronger inhibitory effect on tumour growth than either treatment alone [11]. In patients, pathologic response after neoadjuvant radiochemotherapy correlates with low PLK1 expression [27]. So far no data are available for combination of small molecule PLK1 inhibitors with radiotherapy. Radiation-induced DNA damage inhibits PLK1 in G2/M phase, leading to a temporary G2/M arrest that allows DNA repair before cell division [26,32,35]. A constitutively active mutant of PLK1 has been shown to override radiation-induced inhibition of centrosome-separation [43]. On the other hand, mitotic cells (as well as cells in late G2 phase) are generally very radiosensitive. In light of this knowledge, specific PLK1 inhibitors theoretically may interact with the effects of irradiation on several levels and thereby might radiosensitise tumour cells and improve the potential of both modalities.

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Aim of the current project was to evaluate the efficacy of the PLK1 inhibitor BI 6727 in combination with irradiation.

Material and methods

Bi 6727

The dihydropterdinone derivate BI 6727 was provided by Boehringer Ingelheim RCV GmbH & Co. KG. The drug competitively inhibits the ATP binding site of the PLK1, thereby reducing its enzymatic activity [34]. Selectivity has been shown by an IC50 (inhibitory concentration 50%) of 0.87 nM for PLK 1, 5 nM for PLK2 and 56 nM for PLK3, whereas in a panel of >50 other kinases no inhibitory activity in concentrations up to 10 μM could be shown [28]. For the experiments, carrier solution was prepared from sterile NaCl with 0.1% 0.1 M sterile HCl. BI 6727 was added to a final concentration of 4 mg/ml (pH = 4).

Cell line and tumour model

A431, squamous cell carcinoma (SCC) of the female genitals, was purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), established as in vivo tumour model and kept as cryostocks. FaDu is a subline of the original cell line available in the American Type Culture Collection (ATCC) [10]. The origin and stability of the xenografts or cells were routinely monitored by short tandem repeat analysis (microsatellites), histology, volume doubling time (VDT) evaluations and lactate dehydrogenase (LDH) gel electrophoresis [22]. The median VDT of 5.6 days [range 2.5–9 d] and 5.8 days [3; 9 d] for the single-dose and the fractionated experiments in A431 and 3.1 days [range 2; 9 d] for FaDu was comparable with other experiments (A431: unpublished; FaDu: [2,13,14,16–18,24,38,39,42]). A significant immunogenic effect of the models was excluded in earlier experiments [4,44] (Gurtner et al., in preparation).

In vitro growth and clonogenic cell survival

Cells were prepared from exponential monolayer culture by trypsinisation. The growth inhibitory effect of Bi 6727 was examined following replicate plating of 3.5×10^5 cells in 25-cm^2 tissue flasks (Nunc, Roskilde, Denmark) containing 5 ml Dulbecco's modified Eagle's medium supplemented with stabile glutamine, 10% fetal calf serum, 1% sodium pyruvate, 1% (v/v) non-essential amino acids, 2% HEPES, and 1% (v/v) penicillin–streptomycin (all from Biochrom, Berlin, Germany). 24 h later, 5 ml medium was added with BI 6727 at different concentrations. For each determination duplicates were prepared. After incubation at 37 °C (5% CO_2, 95% humidity), cells were trypsinised and centrifuged (300×g; 5 min). In 3 independent experiments, cell numbers were determined at different time points, using a CASY1 cell analyser (Roche Applied Science, Mannheim, Germany).

To determine the impact of BI 6727 on clonogenic survival, 5×10^5 A431 cells or 3.5×10^5 FaDu cells were seeded in 25-cm^2 tissue culture flasks. After 24 h, control medium or BI 6727 was added for 3 days. Cultures were then irradiated with 2 Gy (200-kV X-rays, 0.5 mm Cu, ~1 Gy min^{-1}), trypsinised and counted. Single-cell suspensions were seeded in culture dishes, incubated for 14 days, fixed and stained with crystal violet. Colonies with $\geqslant$50 cells were scored. The mean surviving fractions (surviving fraction at 2 Gy, SF2) and their standard errors (SE) were determined for each treatment group. Comparisons between groups were performed using ANOVA test for multiple comparisons. For FaDu, a complete cell survival curve was established using similar procedures with increasing radiation doses. Curves were fitted using the multi-target-model and fits were compared using F-test.

In vitro cell cycle distribution

Exponentially growing cells were incubated in 25-cm^2 tissue culture flasks for 3 days with different concentrations of BI 6727 and harvested by trypsinisation. After centrifugation, cells were washed, suspended in PBS/EDTA, fixed on ice with ethanol at a final concentration of 70% EtOH and stored at −20 °C. For flow cytometry, fixed cells were washed again with PBS/EDTA, centrifuged, resuspended and stained with propidium iodide (10 μg ml^{-1}; Molecular Probes; Eugen, OR, USA) supplemented with RNase (1 mg/ml; Roche Diagnostics, Mannheim, Germany). Cells were measured using a FACSCanto II (Becton Dickinson, San Jose, CA, USA). A minimum of 20,000 events and single cells, respectively were collected and analysed with the *DNA* histogram *analysis software* program MultiCycleAV (Phoenix Flow Systems, USA). At least three independent experiments were performed for each cell line and treatment regime, cell cycle phase distributions were analysed and data were averaged. Means (±SD) were compared statistically using ANOVA and Bonferroni post-test (GraphPad Prism 5 Software, Inc., San Diego, CA, USA).

Animals and follow-up

7- to 14-week-old female and male NMRI (nu/nu) mice were used (specific pathogen-free breeding facility, Experimental Center, Medical Faculty C.G. Carus, Technische Universität Dresden, Germany). Experiments were approved in accordance with institutional guidelines and the German animal welfare regulations. Animals were kept as described previously [15]. In contrast to other experiments [1,13,14,16–18,31,41], we applied no whole body irradiation before tumour transplantation, because of an overlapping toxicity of this procedure with the drug (unpublished). As a relevant immunogenicity of the tumour model has been excluded (vide supra), an impact on the results of the experiments can be excluded.

Application of BI 6727 and tumour irradiation in vivo

BI 6727 or carrier was applied in a dose of 20 mg/kg body weight (b.w.) per intraveneous injection (tail vein). An interval between injection of the drug and irradiation of 10 h was established in pilot experiments (A431) as the time, where the number of mitotic cells reached a maximum (unpublished).

All irradiations were applied using 200 kV X-rays, 0.5 mm Cu, at a dose rate of ~1 Gy/min. For irradiations under homogeneous hypoxia, a heavy clamp was placed proximal to the tumour of anaesthetised mice (ketamine 120 mg/kg b.w. and xylazine 16 mg/kg b.w. intraperitoneally). Treatment protocols were started two times per week (median tumour volume A431 single dose: 144 mm^3 [10–90% percentiles 113–268 mm^3], A431 fractionated: 123 mm^3 [10–90% percentiles 95; 236 mm^3], FaDu: 123 mm^3 [10–90% percentiles 95; 192 mm^3]). The animals were randomised over the experimental matrix in groups of four to five, aiming for about 8 evaluable animals (range 6–10 evaluable animals in the single dose and 4–16 in the fractionated irradiation experiments) for each irradiation dose level in the tumour control experiment. Animals were excluded from analysis if the uncertainty of irradiation or drug dose application, e.g. by machine breakdown or paraveneous injection, was >10%.

Design of the in vivo experiments

For determination of local tumour control after single dose irradiation, BI 6727 or carrier was applied on 2 consecutive days, followed by single dose irradiation under clamped hypoxia 10 h after the second drug application. Seven irradiation dose groups