Original Research Article

The HIV protease and PI3K/Akt inhibitor nelfinavir does not improve the curative effect of fractionated irradiation in PC-3 prostate cancer *in vitro* and *in vivo*

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ABSTRACT

Background: Radiotherapy has a high curative potential in localized prostate cancer, however, there are still patients with locally advanced tumours who face a considerable risk of recurrence. Radiosensitization using molecular targeted drugs could help to optimize treatment for this high-risk group. The PI3K/Akt pathway is overexpressed in many prostate cancers and is correlated to radioresistance. Nelfinavir, an HIV protease inhibitor (HPI), was found to block this pathway and to radiosensitize cancer cells of different origin. This is the first study examining the effect of nelfinavir in combination with irradiation on prostate cancer cell survival *in vitro* as well as on growth time and local tumour control *in vivo*.
Methods: The *in vitro* effect of nelfinavir on radioresponse of PC-3 was tested by colony formation assay with 10 μM nelfinavir. *In vivo*, the effect of nelfinavir alone and in combination with irradiation was tested in nude mice carrying PC-3 xenografts. For evaluating tumour growth time, mice were treated with 80 mg nelfinavir/kg body weight, daily at 5 days per week over 6 weeks. Simultaneous irradiation with 30 fractions and total doses between 30 and 120 Gy was applied to calculate local tumour control for day 180 after treatment.
Results: Nelfinavir inhibited Akt phosphorylation at Ser473 and showed a minor but significant effect on clonogenic cell survival *in vitro* with slightly higher cell survival rates after combined treatment. The treatment of PC-3 xenografts with nelfinavir alone led to no significant increase of tumour growth time and no improvement of local tumour control.
Conclusions: Despite promising growth delay effects of nelfinavir in other tumour models and first clinical applications of this drug as anti-cancer agent, PC-3 prostate cancer cells express no or only minor sensitivity to nelfinavir treatment alone and no radiosensitizing effect *in vitro* or *in vivo*.

Introduction

Radiotherapy is one of the two curative standard treatment options for prostate cancer. The rates of biochemical recurrence free survival or local tumour control are high, however, there is still a patient group with locally advanced tumours which is undertreated and face a considerable risk of recurrence [1]. Molecular targeting approaches for radiosensitization bear the potential of treatment optimization in such high-risk patients. Promising tar-

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gets include the PI3K/Akt pathway which prevents apoptosis and mediates cell survival (reviewed in [2] and [3]). This pathway is often upregulated in prostate carcinoma (reviewed in [4]) and constitutively active Akt is known to lead to radioresistance in cell lines of different tumour entities including breast [5–7], lung [8], genitale squamous cell carcinoma [4], bladder and colon [5], and prostate cancer [6,7]. HIV protease inhibitors (HPIs) were found to inhibit the PI3K/Akt pathway and thus viability and proliferation of tumour cells. One of these drugs is nelfinavir, which appears to be the most potent when applied alone in different prostate cancer cell lines [8,9]. Nelfinavir has been shown previously to have a higher radiosensitizing effect than other HPIs at clinically relevant doses for example in head and neck, bladder, glioblastoma, and pancreatic cancer *in vitro* and *in vivo*, which was attributed to Akt inhibition [10–13]. Clinical phase I studies using nelfinavir in combination with radiotherapy or chemoradiotherapy in patients with pancreatic, rectal or non-small cell lung cancer revealed acceptable toxicity and promising response rates [14–17]. Also on prostate carcinoma cell lines (among others, PC-3) nelfinavir was found to have antiproliferative and apoptotic effects [9,18]. However, in contrary, Mathur et al. did not detect a significant decrease of cell survival in PC-3 after treatment with nelfinavir [19]. There are currently no preclinical or clinical studies available on a potential radiosensitizing effect of nelfinavir on prostate cancer. Also, a potential improvement of local tumour control after combined radiotherapy and nelfinavir has not been shown in any of the tumour entities.

In the present study, we tested the effect of nelfinavir in combination with ionizing radiation in prostate cancer cells *in vitro* and, based on clinical treatment regimens, *in vivo*. For our investigations, we have chosen the androgen receptor negative metastatic prostate cancer cell line PC-3 due to its phosphatase and tensin homologue (PTEN)-null status, with a resulting constitutively activated PI3K/Akt pathway [20,21].

Material and methods

Cell lines and culture conditions

The origin and stability of the human metastatic prostate cancer cell line PC-3 (ATCC, Wesel, Germany), was routinely monitored by short tandem repeat analysis (microsatellites) and the cells were tested for mycoplasma. The cells were cultured in RPMI 1640 with L-glutamine (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (Sigma–Aldrich, Taufkirchen, Germany) at 37 °C, 95% humidity, and 5% CO_2.

Western blotting

Cells were lysed with RIPA buffer (Sigma–Aldrich, Taufkirchen, Germany) containing Complete™ protease inhibitor cocktail (Thermo Fisher Scientific, Dreieich, Germany), HALT™ protease inhibitor and HALT™ phosphatase inhibitor (Thermo Fisher Scientific, Dreieich, Germany). Western blot was performed with proteins from whole cell lysates. Primary antibodies: anti-Akt and anti-pAkt (Ser473) (Cell Signaling, Frankfurt a. M., Germany); anti-β-actin (Abcam, Cambridge, UK). Secondary antibodies: goat anti-rabbit and goat anti-mouse (Santa Cruz, Heidelberg, Germany). For detection, Western blotting Luminol Reagent (Santa Cruz, Heidelberg, Germany), and Amersham Hyperfilm™ ECL (GE Healthcare Limited, Buckinghamshire, UK) were used. Densitometry was done using ImageJ 1.48v (Wayne Rasband (National Institutes of Health), MD, USA).

Colony formation assay

PC-3 cells were seeded in 6-well plates (Falcon, Wiesbaden, Germany) with cell numbers of 300/1000/3000/5000 for 0/2/4/6 Gy. After 24 h, 10 μM nelfinavir (Viracept®, nelfinavir mesylate, Agouron, Durham, NC, USA) diluted in DMSO (dimethyl sulphoxide, Roth, Karlsruhe, Germany) was added. 1 h later, media was exchanged for nelfinavir-free media and cells were irradiated. The drug concentration and incubation time were chosen based on Pore et al. [22]. The incubation time for forming colonies was 10 days. Thereafter, the cells were fixed with 80% ethanol and stained with Coomassie G250. Colonies with ⩾50 cells were scored. The surviving fractions (*SF*) and the standard error of the mean (SEM) were calculated. *PE* (Plating Efficiency) = counted colonies/seeded cell number * 100%; $SF = PE_{xGy}/PE_{0Gy}$. The colony formation assay was performed in triplicate and was done three-fold independently.

Animals and tumour models

The experiments were performed using 7–15 week old male NMRI (nu/nu) mice obtained from our in-house pathogen-free animal breeding facility. For further immunosuppression, animals were whole body irradiated with 4 Gy 2–5 days before tumour transplantation. 500,000 PC-3 cells suspended in matrigel (BD matrigel matrix growth factor reduced, BD, Heidelberg, Germany) / PBS (Biochrom, Berlin, Germany) (1:2) were transplanted subcutaneously into the right hind leg of anaesthetized mice (120 mg/kg body weight ketamine and 16 mg/kg xylazine, intraperitoneal). Constancy of the tumour model was checked by evaluation of the volume doubling time of each 5 untreated animals per transplantation cohort and by evaluation of short tandem repeats (microsatellite analysis) within the donor cell line of the cryostock. The median volume doubling time of untreated tumours was 10.3 d (range 6.2–14.5 d) with no significant differences between the transplantation cohorts (Supp. Table 2). This time was consistent to other *in vivo* studies with PC-3 xenografts [23,24]. The animal facility and experiments were approved according to the institutional guidelines and the German animal welfare regulations.

Experimental design

When tumours reached a size of 6–8 mm in diameter, animals were entered into the experiment. Two endpoints were investigated: tumour growth time and local tumour control.

For growth time, mice were treated with 80 mg nelfinavir/kg body weight (active component of Viracept®, Agouron, Durham, NC, USA) suspended in water (aqua ad iniectabilia Braun, B. Braun, Melsungen, Germany) daily at 5 days per week over 6 weeks. The drug concentration was chosen based on Pore et al. [22]. The control group received the appropriate amount of water. The drug or water was administered orally using a gavage (nelfinavir: n = 17; water: n = 18).

For evaluation of local tumour control, animals were treated with nelfinavir or water in the same manner as for growth time. Irradiation (IR) was applied simultaneously 4 h after each administration of the drug (6 weeks, 30 fractions). Total irradiation doses of 30/40/50/60/72.5/85/100/120 Gy were used to evaluate the dose–response relationship (nelfinavir + IR: n = 87; water + IR: n = 92). The time interval of 4 h between drug application and irradiation was chosen due to a high transport activity into diverse organs in mice potentially leading to lowering of the plasma concentration after this time [25].

Follow-up and evaluation of tumour growth time and local tumour control

The tumour size was measured with a sliding calliper twice per week. Animals were sacrificed if they appeared to suffer, when a recurrent tumour reached a diameter of 15 mm or after reaching a follow-up time of 270 days after treatment. The long follow-up was used to ensure that no further recurrences would occur after the evaluation timepoint for local tumour control (day 180). The tumour volume was calculated as $V = \pi/6 \cdot a \cdot b^2$, where a is the longest and b is the perpendicular shorter tumour diameter.

Growth time was evaluated by calculating the median time needed to reach a 2- to 5-fold of the starting tumour volume.

Local tumour control rates were evaluated for each irradiation dose group for day 180 after end of treatment. Tumour control probabilities and tumour control dose 50% (TCD$_{50}$) values with 95% confidence intervals were calculated using a binary logistic model as described previously [26]. A tumour recurrence was scored, when the tumour volume increased at three measurements after shrinkage or when the tumour continued to grow without shrinkage. Recurrences later than 180 days after treatment were considered as locally controlled for evaluation of TCD$_{50}$ after 180 days (nelfinavir: $n = 3$; water: $n = 2$). Including these values into the analysis did not alter the results. Animals were censored when they died without recurrence later then day 20 after end of treatment. Animals that died earlier then day 20 after end of treatment without recurrence were excluded from analysis.

Irradiation (IR)

Irradiation of cells, hind legs of mice and whole body irradiation was performed at room temperature with 200 kV X-rays (0.5 mm Cu added filtration) using a Yxlon Y.TU 320-D03 (Yxlon International GmbH, Hamburg, Germany), at a dose rate of approx. 1.3 Gy/min. Dosimetry was checked daily.

Statistical analysis

Data analyses for *in vitro* experiments and tumour growth time was performed with GraphPad Prism 5, San Diego, CA, USA: Colony formation assay was analyzed using the LQ model: $S = \exp(-\alpha d - \beta d^2)$. Curve fits were compared using *F*-test. Plating efficiencies were compared using the *t*-test. Medians of growth time were compared with Mann–Whitney *U* test.

Statistical analysis and comparison of local tumour control data were performed using STATA/SE 11.2 (StataCorp LP, College Station, TX, USA). A *p*-value $\leqslant 0.05$ was considered statistically significant.

Results

Clonogenic survival after nelfinavir treatment

In order to define the necessary exposition time of PC-3 cells to nelfinavir, the cells were incubated with 10 μM nelfinavir for 1/2/4/8/24 h. While 10 min after irradiation no change of AKT phosphorylation occurred (Supp. Fig. 1), western blot experiments revealed a reduction of Akt phosphorylation at Ser473 already 1 h after nelfinavir treatment (Fig. 1A). Consequently, the following colony formation assay was performed with 1 h nelfinavir incubation. The media was exchanged for nelfinavir-free media directly before irradiation to exclude any side effects of nelfinavir that could manipulate the result of the colony formation assay while a block of Akt phosphorylation at the timepoint of irradiation was ensured.

Nelfinavir appeared to have little effect on clonogenic survival of PC-3 cells with slightly higher cell survival after combined treatment. Despite this minor effect, the results for combined irradiation and nelfinavir treatment compared to irradiation alone were significantly different ($p = 0.02$, *F*-test) (Fig. 1B, Supp. Table 1). Nelfinavir treatment did not lead to a significant change (*t*-test) in plating efficiency at 0 Gy indicating no relevant independent cytotoxic effect *in vitro* (Fig. 1C, Supp. Table 1).

Effect of nelfinavir on growth time and local tumour control

As contradictory *in vitro* and *in vivo* results for combined irradiation and nelfinavir treatment have been described earlier for head and neck squamous cell carcinoma and lung carcinoma cells, with small effects on clonogenic cell survival *in vitro* but a distinct higher effect of the combined treatment *in vivo* [22], we performed additional *in vivo* experiments despite the negative *in vitro* results.

First, we tested if nelfinavir affects proliferation of PC-3 cells *in vivo* by evaluation of tumour growth time. Therefore the median time needed to reach a 2- to 5-fold of the starting tumour volume was calculated. The slight prolongation of tumour growth times shown in Fig. 2A was statistically not significant (*p*-values for time to 2- to 5-fold of the starting volume: 0.2–0.4, Mann–Whitney *U* test) (Supp. Table 3).

Local tumour control did not show a difference between nelfinavir treatment combined with fractionated irradiation and fractionated irradiation alone (Fig. 2B, Supp. Table 4). TCD$_{50}$ was 68.8 Gy (95% CI: 50.8; 91.9) for nelfinavir combined with irradiation; and 64.6 Gy (95% CI: 54.8; 83.4) for irradiation alone ($p = 0.9$).

Discussion

After promising data on combined radiotherapy and Akt inhibition with nelfinavir treatment, showing radiosensitization in different tumour models *in vitro* and growth delay *in vivo* as well as clinical applicability of the combined approach in rectal cancer [10–13,16], the aim of the present study was to evaluate the combined treatment in prostate cancer.

In PC-3 with its constitutively active PI3K/Akt pathway, we have shown a decrease in Akt phosphorylation at Ser473 after nelfinavir treatment. However, no radiosensitization could be observed *in vitro*. Nelfinavir led to a minor but statistically significant effect on clonogenic cell survival *in vitro* with slightly higher radioresistance after combined treatment. This is in contrast to published data on different tumour cell lines, where the inhibition of Akt by HPIs leads to radiosensitization [10–13]. Herein head & neck and bladder cancer cell lines were treated with 5 μM nelfinavir and glioblastoma cancer cell lines with 10 μM and 20 μM 1 h prior irradiation [10–12]; pancreas cancer cell lines were treated with 1 μM nelfinavir 26 h prior and 2 h after irradiation [13]. However, there are also data showing that although nelfinavir induced Akt inhibition, the anti-tumour effect could also be ascribed to other mechanisms triggered by nelfinavir [8,9,27], which are known to modify the radiosensitivity of cells [28–31]. These mechanisms are mentioned at the end of this section in more detail.

In vivo evaluation of the drug alone revealed no significant prolongation of growth time. The latter findings on drug treatment alone are consistent with Mathur et al. who also showed a non-significant reduction of cell survival after nelfinavir administration *in vitro* in the prostate cancer cell lines PC-3 and LNCaP C42B [19]. Further, using a treatment schedule differing from ours, they found no significant anti-tumour effects of nelfinavir alone or in combination with chemotherapy in C4-2B xenografts *in vivo* [29]. On the other hand, a study on the prostate cancer cell line LNCaP in
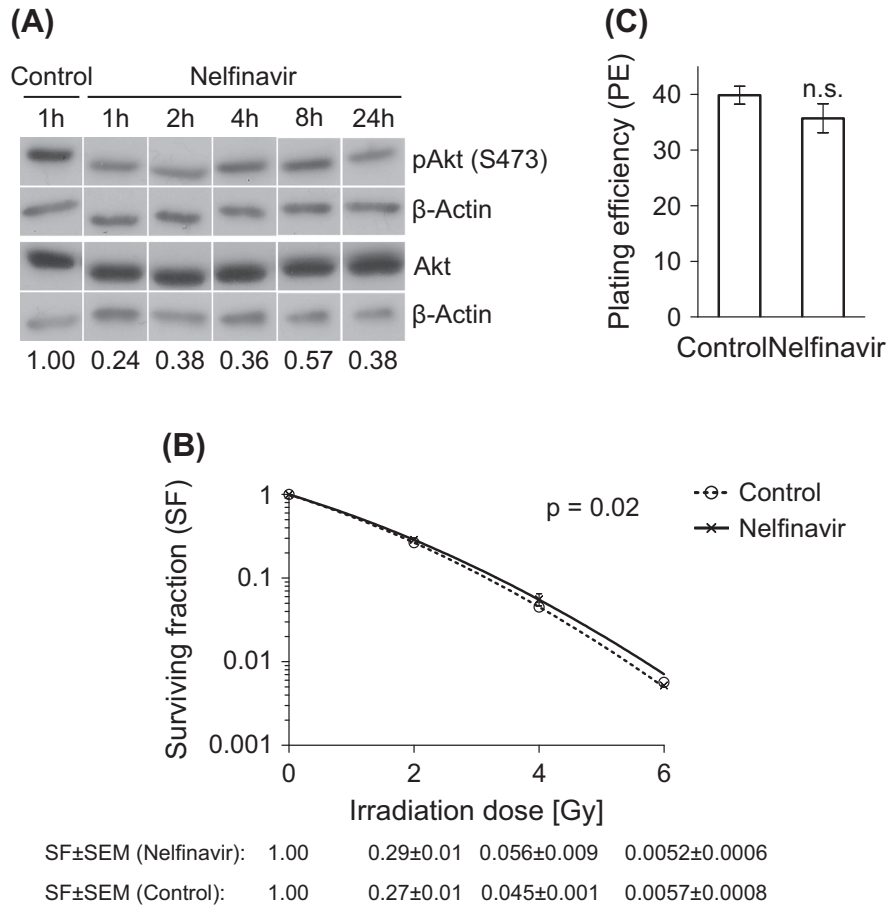
Fig. 1. *In vitro* effect of nelfinavir on PC-3: (A) A western blot for pAkt (S473) and ß-Actin and a western blot for Akt and β-Actin after incubation with nelfinavir for different incubation times are shown. The densitometry quantitations were evaluated by calculating pAkt:Akt, at which each value was set into relation with its corresponding ß-Actin value. The calculated ratios were normalized to control (DMSO) and listed below the western blots. This experiment was performed only once, as a test if the drug concentration and incubation time used by Pore et al. [22] can be adopted for our experiment (B) Clonogenic cell survival after different irradiation doses with or without nelfinavir incubation. All data were normalised to a surviving fraction of 1 at 0 Gy irradiation dose. The colony formation assay was performed after treatment of PC-3 with 10 μM nelfinavir or its solvent DMSO for 1 h. The medium was exchanged before irradiation. Three independent experiments were performed. Means with SEM are indicated. The curve fitting was done using the linear-quadratic model. Curve fits were compared using *F*-test. (C) Corresponding to (B) the plating efficiency at 0 Gy is shown without normalisation. Plating efficiencies were compared using the *t*-test.

xenografts could demonstrate a significant growth delay when treated with nelfinavir without irradiation even in a lower concentration and shorter period of drug administration compared to the data presented here [9]. Within the same dataset, Yang et al. [9] evaluated that nelfinavir still inhibited the proliferation of DU145 *in vitro* – a prostate cancer cell line whose Akt is not constitutively activated (in contrast, to LNCaP and PC-3 which have constitutively activated Akt [20]). However, the effect was less pronounced compared to PC-3 and LNCaP, the latter showing the highest response [9].

Due to the fact that nelfinavir has been described before with distinct higher effects *in vivo* than *in vitro* [22], we performed an *in vivo* study with irradiation and simultaneous nelfinavir administration using a clinically relevant radiotherapy fractionation schedule and the clinically relevant endpoint local tumour control. Such local tumour control experiments have not been performed by others in any tumour model before. The results show that nelfinavir did not improve the curative effect of radiotherapy, suggesting that nelfinavir application may not be useful in tumours solely selected by their PI3K/Akt pathway and by effects in Akt phosphorylation.

Due to the negative functional data in our experiments with PC-3 cells we did not perform further animal experiments for mechanistic clarification of the *in vivo* results. However, Nelfinavir also

inhibits e.g. STAT3 [9,32], ERK1/2 [32], the androgen receptor [9], and BCL-2 [27], which otherwise contribute to radioresistance of cells [28,33], [34], [29], and [30], respectively. Further, nelfinavir down-regulates VEGF and HIF-1α (which may be secondary to Akt inhibition) that consequently lead to radiosensitization [22]. Another effect of nelfinavir is the inhibition of HSP90 [35] which is involved in a large number of pathways leading to cancer cell survival and proliferation (reviewed in [36]) like for example the activation of HIF-1α [37]. Nelfinavir also induces endoplasmic reticulum stress (ER-stress) known to lead to radiosensitization [31]. The ER-stress induces apoptosis [8,18,38,39] countered by induced pro-survival autophagy [8,18,38,40]. Together with our negative results in PC-3 tumours, these data suggest that the evaluation of nelfinavir effects with irradiation might be more promising in prostate cancer cells possessing a particular profile of nelfinavir targets beyond PI3K/Akt overexpression.

Conclusions

In summary, we could show that Akt inhibition by nelfinavir led to a minor but significant effect of PC-3 cells *in vitro* with slightly higher clonogenic cell survival and to no significant effect on tumour growth time without irradiation or on local tumour control when combined with fractionated irradiation *in vivo*. This result
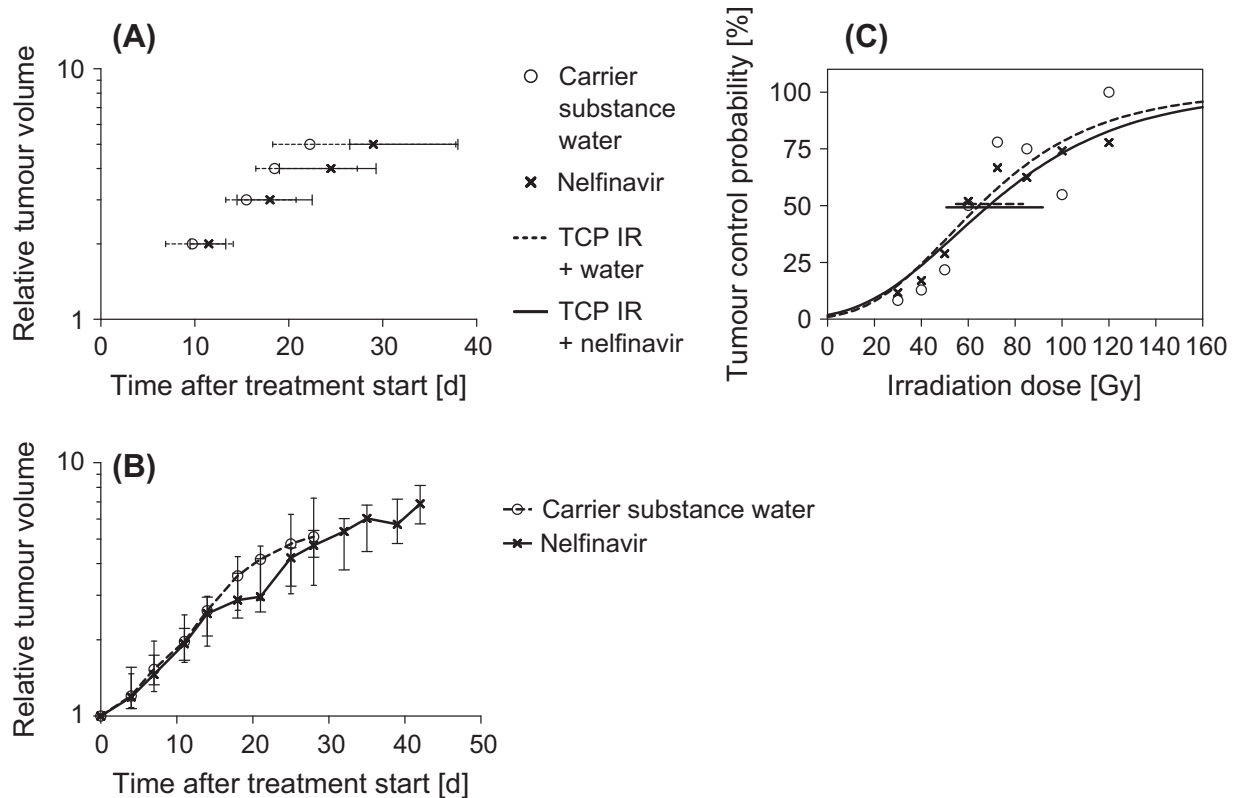
Fig. 2. *In vivo* effect of nelfinavir on PC-3: (A) Relative tumour volumes of PC-3 xenografts after treatment with nelfinavir (80 mg/kg body weight) or its carrier substance water (5 times weekly, 6 weeks) are depicted. Medians with quartiles are displayed. (B) Corresponding to (A), the data were plotted as growth curves. (C) The observed local tumour control rates (symbols) and calculated tumour control probabilities (TCP) after treatment with nelfinavir (80 mg/kg body weight) or its carrier substance water and irradiation with 30 fractions within 6 weeks are shown. The error bars represent the 95% confidence intervals of TCD_{50}.

does not exclude radiation dose-modifying effects in other tumour models. A potentially interesting approach might be to evaluate whether tumour cells with a particular profile of nelfinavir targets might be better responders.

List of abbreviations

Akt	protein kinase B
CI	confidence interval
DMSO	dimethyl sulfoxide
HPI	HIV protease inhibitor
HIV	human immunodeficiency virus
PE	plating efficiency
PI3K	phosphatidylinositol 3-kinase
PTEN	phosphatase and tensin homologue
SEM	standard error of the mean
SF	surviving fraction
TCD_{50}	tumour control dose 50%

Ethics approval

The animal experiments were approved according to the German animal welfare regulations.

Competing interests

All authors declare no conflict of interest.

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Author's contributions

SL: performance of the experiments, evaluation of data, writing of the manuscript
LK: statistical analysis, *in vivo* data evaluation
SL: statistical analyses
MHM: supervision of the project, reviewing of the manuscript
MK: conception and supervision of the project, reviewing of the manuscript

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ctro.2016.12.002.

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