Use of Irinotecan for Treatment of Small Cell Carcinoma of the Prostate

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BACKGROUND. Prostatic small cell carcinoma (SCC) is a rare variant of prostate cancer. It is extremely aggressive and resistant to available therapies with a median survival range of 5–17 months. No standard chemotherapeutic regimen has been established for its treatment. In search of a new therapeutic approach, we examined the response of patient-derived prostatic SCC *tissue* xenografts to irinotecan, a topoisomerase I inhibitor.
METHODS. A tumor tissue line was established from a patient's prostatic SCC by subrenal capsule grafting using NOD-SCID mice. Mice carrying subcutaneous transplants of the tumor line were then treated for 2 weeks with irinotecan alone and in combination with cisplatin. The effect on tumor volume, histopathology, and apoptosis were determined.
RESULTS. The prostatic SCC tissue line resembled the donor tissue in morphologic and immunohistochemical features. Irinotecan (20 mg/kg/day; days 1–3, 8–10) completely arrested xenograft growth with a small reduction in tumor volume and only minor weight loss of the hosts (7%); irinotecan (12 mg/kg; same schedule) + cisplatin (2.5 mg/kg/day; days 1 and 8) had a similar effect, but with lower weight loss. While the growth inhibition involved apoptosis, it was also associated with a marked increase in autophagy.
CONCLUSIONS. Tumor tissue lines established via subrenal capsule xenografting provide models with clinical relevance and the present study suggests that irinotecan could be useful for therapy of refractory prostatic SCC, in particular in combination with cisplatin. *Prostate*

KEY WORDS: **small cell carcinoma; prostate; irinotecan; subrenal capsule xenografts; autophagy**

INTRODUCTION

Small cell carcinoma (SCC) of the prostate is a rare form of prostate cancer comprising 0.5–2% of all prostate carcinomas [1]; it was first described by Wenk et al. [2]. Prostatic SCC can occur as an initial event at the time of first diagnosis and is then thought to originate from neuroendocrine cells present in the epithelium of prostatic ducts/acini and in the endothelium of prostatic urethra. It may also develop in prostate cancer during progression to castration resistance induced by androgen ablation. The most widely accepted view is that prostatic SCCs arise from totipotential stem cells of the prostate that have the ability to differentiate into either epithelial or neuroendocrine type carcinomas. The latter type is characterized by absence of expression of androgen receptors (AR) and prostate-specific antigen (PSA) and by

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positive expression of the synaptophysin (SYN) neuroendocrine marker [3,4]. Prostatic SCC is an aggressive disease, often described as universally fatal [5]. It is typically diagnosed at an advanced clinical stage with rapid progression and insensitivity to hormonal therapy; patients have a median survival of 5–17 months [6,7]. Prostatic SCC shows similarity to small cell lung carcinoma (SCLC) in morphologic features [6] and in the expression pattern of some genes [8]. In view of this, the commonly used treatment of prostatic SCC, that is, platinum-based chemotherapy and irradiation, has been adopted from SCLC therapeutic modalities. Various anti-cancer agents have been used in combination with cisplatin, including etoposide, cyclophosphamide, doxorubicin, and vincristine; other combinations used are carboplatin plus etoposide or taxanes [9–12]. Cisplatin-based chemotherapy plus radiotherapy has, despite initial positive responses, failed to be effective and a standard therapeutic regimen for prostatic SCC has not yet been adopted since this disease is extremely rare [3]. At present, chemotherapy using cisplatin in combination with etoposide is the most common treatment for prostatic SCC.

In the field of preclinical cancer therapy there is a critical need for models which are relevant to the human disease. Use of mouse xenograft models to represent clinical cancers has become widespread, as they are reasonably inexpensive, able to provide rapid data, largely free of regulatory constraints and can be used with many different tumor types. Although *cell line* xenograft models, generated by injecting cultured human cancer cells, are frequently used, they have profound disadvantages [13]. Typically, the cancer cells used are derived from advanced, highly aggressive or poorly differentiated neoplasms [14] and the developing tumors lack their original microenvironment, including tumor-associated stroma shown to be important in tumor development and progression [15]. Such cancer cell line xenograft models in general show limited ability for predicting clinical efficacy of anticancer agents [16]. To address these limitations, mice can be used carrying xenografts of patient-derived *tumor tissue*. Such models are more representative of the clinical situation since they tend to maintain the anatomical architecture and function of the original tumors. While tumor tissue xenografts are generally based on subcutaneous (s.c.) implantation of cancer tissue, we have adopted subrenal capsule grafting of cancer tissues into NOD-SCID mice to allow optimal establishment of cancer tissue lines. The subrenal capsule graft site allows very high tumor engraftment rates of various human cancers, including prostatic carcinoma. It permits high tissue perfusion and hence promotes viability and growth of the xenografts with retention of tumor heterogeneity, that is, retention of major histopathologic and genetic characteristics of the original cancers, making them useful for drug response evaluation [17–20]. Once established, tumor tissue lines can be used to develop s.c. tumors whose volumes can be readily measured with calipers.

Irinotecan is a topoisomerase I inhibitor which prevents DNA from unwinding. It is a prodrug which is converted to a biologically active metabolite, 7-ethyl-10-hydroxy-camptothecin (SN-38), by a carboxylesterase-converting enzyme [21]. It is mainly used for treatment of colorectal cancer, but also for advanced SCLC. Importantly, in a randomized phase III trial of the latter, cisplatin plus irinotecan showed a better survival rate for patients with good performance status than cisplatin plus etoposide [22,23]. In the present study, we have focused on irinotecan as a potential drug for treating prostatic SCC using xenografts of a tumor tissue line obtained via subrenal capsule grafting of human prostatic SCC in NOD-SCID mice. Evidence was obtained that irinotecan, particularly in combination with cisplatin, is potentially useful for treatment of prostatic SCC.

MATERIALS AND METHODS

Materials and Animals

Chemicals, solvents and solutions were obtained from Sigma–Aldrich (Oakville, ON, Canada) unless otherwise indicated. Six- to 8-week-old NOD-SCID mice were bred in the BC Cancer Research Centre Animal Resource Centre, BC Cancer Agency, Vancouver, Canada. Animal care and experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

Development of a Prostatic SCC Tissue Line for Drug Evaluation

A patient with prostatic adenocarcinoma [Gleason score of 9 (5 + 4)] underwent radical cystoprostatectomy and androgen ablation therapy at the Vancouver General Hospital (Vancouver, Canada). Three months later, because of deteriorating obstructive voiding symptoms, he underwent additional surgery to remove enlarged urethra in which prostatic SCC was identified. A piece of the SCC specimen was obtained with his informed consent following a protocol approved by the University of British Columbia Clinical Research Ethics Board/BC Cancer Agency. A portion of the fresh tumor tissue was frozen at -80°C, some of the tissue was fixed in 10% neutral buffered formalin and embedded in paraffin; the majority of the tissue was used to develop a tumor tissue line using subrenal capsule grafting technology and serial transplantations, as previously

described [18,24]. Following establishment of the tumor line (after three serial transplantations), pieces of xenograft tissue ($3 \times 3 \times 1$ mm) were grafted subcutaneously into mice (four grafts/mouse). After 5 weeks (to allow establishments of the xenografts) the mice were randomly distributed into groups with at least five mice per group and treated for 2 weeks with irinotecan (Sandoz Inc, Princeton, NJ; 20 mg/kg/day), days 1–3, 8–10, and with a combination of irinotecan and cisplatin (Mayne Pharma, Paramus, NJ): irinotecan (12 mg/kg/day), days 1–3, 8–10 and cisplatin (2.5 mg/kg/day), days 1 and 8, administered as i.p. injections; control animals were injected with similar volumes of saline. Drug dosages and treatment schedules were obtained from published studies [25]. Tumor sizes were measured with calipers (mm) on days 1 and 14; all xenografts were harvested on day 14.

Histopathology and Immunohistochemistry

The donor specimens and xenograft tissues were fixed in 10% neutral buffered formalin, processed through graded alcohols and embedded in paraffin. Sections were cut at a thickness of 4 μm, mounted on glass slides and dewaxed in Histoclear (National Diagnostic, Atlanta, GA), and hydrated in graded alcohol solutions and distilled water. For histopathology, routine H&E staining was carried out. For immunohistochemistry, tissue sections were treated with 0.5% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity, followed by washing in PBS (pH 7.4) and incubation with a blocking solution (ImmunoVision Technology, Springdale, AR) for 30 min. The sections were then incubated with the primary antibodies overnight at 4°C or with non-immune mouse IgG (Zymed Corp., San Francisco, CA), used as negative controls, at the same concentration as the primary antibodies. Primary antibodies used: mouse anti-AR (AR; Santa Cruz Biotechnology, Santa Cruz, CA; 1:50), mouse anti-PSA (human PSA; DAKO, Carpinteria, CA; 1:50), rabbit anti-SYN (SYN; Abcam Inc, Cambridge, MA), mouse anti-Ki-67 (DAKO; 1:50), rabbit anti-caspase-3 (Cell Signaling, Danvers, MA; 1:100), and rabbit anti-LC3B (microtubule-associated protein light chain 3, LC3B; Abcam; 1:500). Following incubation, the tissue sections were washed, incubated with either a biotinylated anti-rabbit or anti-mouse antibody (Amersham International, Arlington Heights, IL), and then incubated with avidin–biotin complex (Vector Laboratories, Burlingame, CA) at room temperature. Immunoreactivity was visualized using 3,3-diaminobenzidine in PBS and 0.03% H_2O_2. All sections used for immunohistochemistry were lightly counterstained with 5% (w/v) Harris hematoxylin and dehydrated in graded alcohols.

Analysis and Statistics

For quantification of Ki-67 and caspase-3 immunostaining of cells, five randomly selected high-power (×400) images from each graft were captured using an AxioCam HRCCD mounted on an Axioplan 2 microscope, using Axiovision 3.1 software (Carl Zeiss). The percentages of Ki-67- or caspase-3-positive cells (index) were calculated using the formula: Index = number of positive cells × 100/number of total cells. Viable tumor areas of the treated and control groups were averaged and presented as means ± SD. *P*-values were calculated using a permutation test of the means. Ki-67 and caspase-3 indices are presented as means ± SD and analyzed by the Student's *t*-test. Results with *P*-values <0.05 are considered statistically significant.

RESULTS

Establishment of a Transplantable Prostatic SCC Tissue Line

Subrenal capsule xenografting of the patient's prostatic SCC tissue in NOD-SCID mice (as described in Materials and Methods Section) was successful with an overall engraftment rate >95%. Within 8 months, a transplantable tumor tissue line was established that was non-metastatic when grafted into the subrenal capsule site. The established tumor line (designated LTL-352) grew readily following transplantation into the s.c. graft site in NOD-SCID mice with an approximate doubling time of 11 days. LTL-352 tissue of the established line and of earlier generations was stored frozen in liquid nitrogen to allow checking/use of the tumor tissue at a later date. As shown in Figure 1, comparisons of donor and xenograft tissues showed no major differences with regard to characteristic morphologic and immunohistochemical features, such as growing patterns (Fig. 1a,b), absence of AR expression (Fig. 1c,d), absence of PSA expression (Fig. 1e,f) and positive expression of SYN neuroendocrine marker (Fig. 1g,h). Both donor and xenograft cells showed patterns of cells in sheets and nests, with ribbons, pallisading along fibrous bands; rosette-like structures were occasionally noted. Polygonal, round, or spindled tumor cells had scanty cytoplasm with hyperchromatic nuclei. Taken together, the results show that the human prostatic SCC xenografts in general retained the histopathology and differentiation status of the donor tissues. The data indicate that during the early generations of transplantation (e.g., the fourth generation) the human prostatic SCC line did not show morphological changes in the mouse host and thus was phenotypically quite stable. Furthermore, mice carrying xenografts of the tissue line could be used as models with clinical relevance.

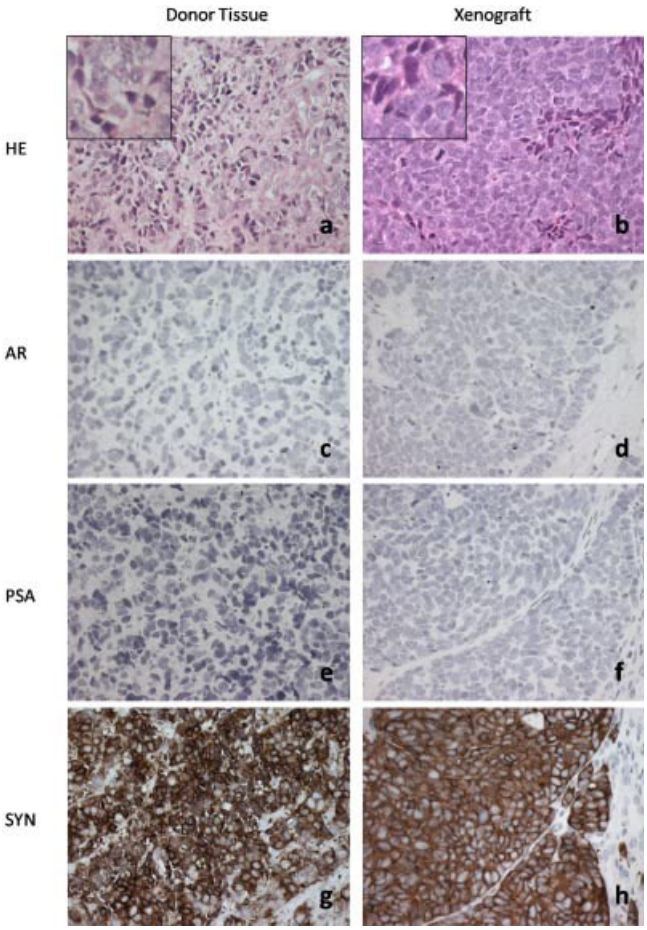

Fig. 1. Histopathology of representative prostatic SCC donor and xenograft tissues. Tissues were stained with H&E (HE; **a**,**b**), and using antibodies against androgen receptor (AR; **c**,**d**), prostate-specific antigen (PSA; **e**,**f**), or synaptophysin (SYN; **g**,**h**). Insets, high-power images (×400).

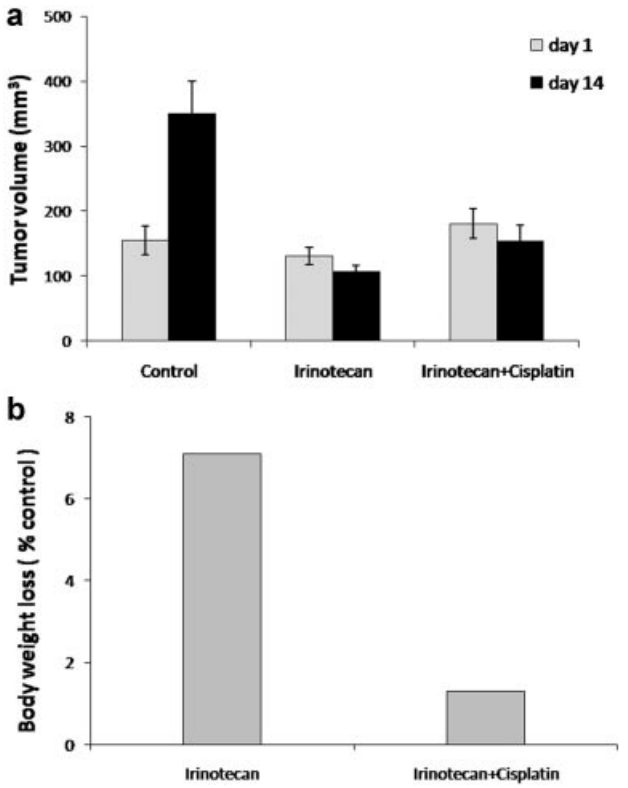

Fig. 2. Effect of treatment with irinotecan and irinotecan + cisplatin on prostatic SCC LTL-352 subcutaneous xenografts in NOD-SCID mice. Injections (i.p.) were given of saline (control), irinotecan (20 mg/kg/day; days 1–3, 8–10) or a combination of irinotecan (12 mg/kg/day; days 1–3, 8–10) and cisplatin (2.5 mg/kg/day; days 1 and 8) over a 14-day period. **a**: Effect on tumor volumes on days 1 and 14; data are expressed as mean ± SD. **b**: Body weight loss on day 14 as compared to controls (%).

Response of Prostatic SCC Xenografts to Chemotherapeutic Regimens

The anti-cancer activities of irinotecan, and a combination of irinotecan plus cisplatin, were determined using mice bearing s.c. LTL-352 prostatic SCC xenografts. The drugs were administered intraperitoneally after the average volume of the tumors had reached 100–200 mm^3. As shown in Figure 2a, the growth of the tumors was markedly inhibited both by irinotecan as a single agent ($P < 0.01$) and by irinotecan + cisplatin ($P < 0.01$). In the control group, the average tumor volume increased over the 2-week period from $154.7 \pm 22.3\,mm^3$ to $349.8 \pm 50.4\,mm^3$ (126.1%); in contrast, the tumor volume was reduced in the irinotecan-treated group from $130.9 \pm 12.8\,mm^3$ to $106.3 \pm 9.9\,mm^3$ (−19%), and from $180.7 \pm 22.9\,mm^3$ to $153.4 \pm 25.4\,mm^3$ (−15%) when cisplatin was used in addition to irinotecan (Fig. 2a). The treated/control value (T/C) was −12.6% for the mice treated with irinotecan alone (dosage, 20 mg/kg/day) and −14.0% for the mice treated with irinotecan + cisplatin (at 12 and 2.5 mg/kg/day, respectively). The regimens did not cause any deaths nor significant toxic side-effects. Compared to control animals, weight loss was 7.1% for the mice treated with irinotecan alone and 1.3% for the mice treated with irinotecan + cisplatin (Fig. 2b, $P > 0.05$). The results indicate that the treatment with irinotecan + cisplatin was more effective and less toxic than the treatment with irinotecan as a single agent.

Histopathological analysis was used to determine the effect of the drugs at the cellular level. A comparison with the control tissues shows that the tissues of both irinotecan- and irinotecan + cisplatin-treated tumors contained more apoptotic bodies, in particular the irinotecan + cisplatin-treated tumors (Fig. 3a–c). A marked increase was found for the staining of caspase-3 (a marker of apoptosis) for both treated groups (Fig. 3d–f), especially in the irinotecan + cisplatin-treated tumors (Fig. 3f, $P < 0.01$), as also

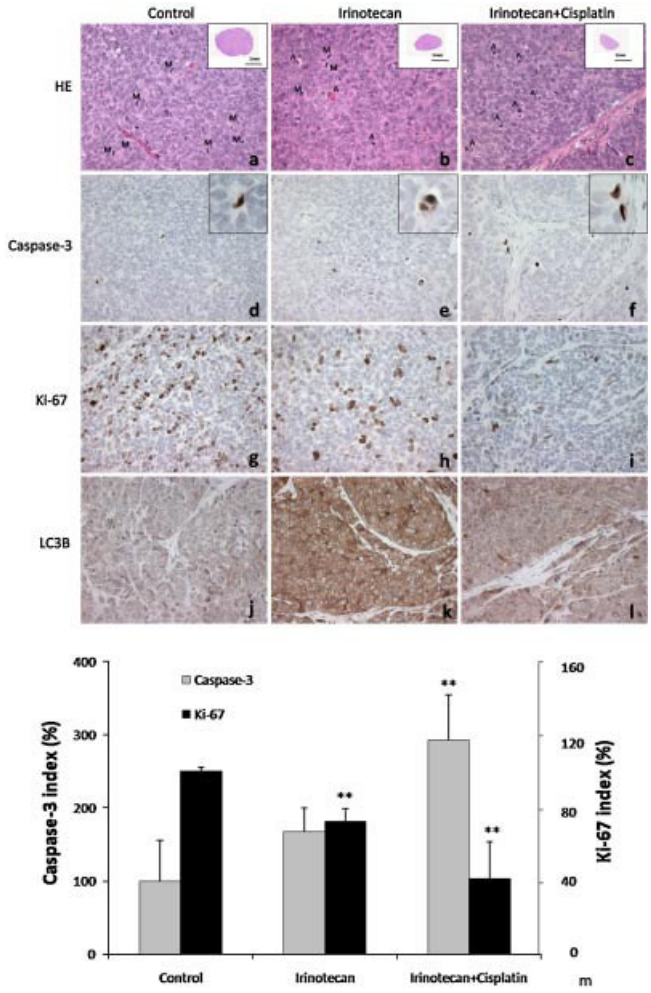

Fig. 3. Immunohistochemical analysis of prostatic SCC LTL-352 tissues from control (untreated), irinotecan-treated and irinotecan + cisplatin-treated xenografts. **a**–**c**: Apoptotic bodies (M, mitotic activity; A, apoptosis); **d**–**f**: caspase-3 expression; **g**–**i**: Ki-67 expression; **j**–**l**: L3CB expression; insets, ×400 magnification; m, caspase-3 and Ki-67 indices compared to controls for irinotecan- and irinotecan + cisplatin-treated xenografts. Asterisks indicate that $P < 0.01$.

indicated in Figure 3m. Ki-67 activity (a marker of cell proliferation) was reduced especially in the irinotecan + cisplatin-treated tumors (Fig. 3i, $12.1 \pm 5.6\%$, $P < 0.01$) compared to the mice treated with irinotecan alone (Fig. 3h, $19.4 \pm 1.9\%$, $P < 0.01$, the proliferative index for the control group was $26.7 \pm 2.8\%$; Fig. 3g,m). Both treated groups showed an increase in expression of LC3B (a marker of autophagy) compared to the control (Fig. 3j), in particular in the tumors treated with irinotecan alone (Fig. 3k). The results indicate that the anti-cancer activity of irinotecan and cisplatin involved apoptosis and that irinotecan may have a role in autophagy.

DISCUSSION

Prostatic SCC is a rare type of prostate cancer. However, it is of clinical importance since it is one of the most aggressive prostatic malignancies with very poor prognosis. Cisplatin-based chemotherapy plus radiotherapy, the most commonly applied regimen in the clinic, is only marginally effective, despite initial positive tumor responses, and new therapeutic approaches are critically required [3]. In the present preclinical study, we focused on use of irinotecan as this drug is a component of a first-line treatment of extensive SCLC [23], a disease that has a number of features in common with prostatic SCC [6,8]. Mice carrying xenografts of the patient-derived LTL-352 prostatic SCC tumor tissue line, showing retention of histopathological and immunohistochemical features of the original cancer (Fig. 1), provided a model with clinical relevance. The finding that irinotecan completely arrested the growth of the xenografts, with a small reduction in tumor volume (Fig. 2) and minor toxicity as indicated by a body weight loss of only ~7%, indicates that this drug is potentially useful for clinical therapy of prostatic SCC. In fact, a combination of cisplatin with irinotecan at a lower dosage may be even more useful, since such a combination led to similar growth arrest of the xenografts with negligible body weight loss (Fig. 2). A greater effectiveness of irinotecan + cisplatin, compared to irinotecan as a single agent, is also indicated by the lower T/C value (−14% vs. −12%), lower Ki-67 values, and increased caspase-3 expression (Fig. 3m). It may be noted that, in a case study, combined chemotherapy using carboplatin plus irinotecan has shown favorable efficacy in a patient with relapsed prostatic SCC complicated with meningeal carcinomatosis [26].

It is of interest that irinotecan has been reported to reduce cellular levels of glutathione by interference with cellular cystine uptake [27]. Reductions in cellular glutathione levels can lead to sensitization of cells to anticancer agents [28,29]. In view of this, irinotecan could, as a reducer of intracellular glutathione levels, be a valuable component in combination chemotherapy of refractory cancers such as prostatic SCC.

Growth-inhibitory activity of irinotecan in cancer cell lines is thought to be mediated by apoptosis induced by SN-38, the active form of irinotecan [30]. The increase in apoptotic bodies and caspase-3 staining observed in cells of the tumors whose growth was arrested by irinotecan (Fig. 3a–f) indicates that the growth inhibition, as shown by tumor volume (Fig. 2) and Ki-67 labeling measurements (Fig. 3m), was also due to induction of apoptosis. Of further interest is the increase in autophagy of the treated tumors as indicated by an increase in LC3B marker expression, in

particular in the tumors treated with irinotecan alone (Fig. 3j–l). The increase in autophagy appears to be due to irinotecan as distinct from cisplatin, as the reduced LC3B expression could be explained by the 40% reduction of irinotecan dosage in the irinotecan + cisplatin regimen. The role of autophagy in cancer is complex, since there is evidence that autophagy supports both promotion and suppression of cancer growth. There is an increasing amount of evidence that autophagy can act as a survival mechanism of cells in response to a wide range of stresses, including treatment with anti-cancer agents [31,32]. To our knowledge, this is the first report that activity of irinotecan may be coupled to autophagy.

CONCLUSIONS

A transplantable tumor tissue line has been established from a patient's prostatic SCC, via subrenal capsule xenografting in NOD-SCID mice, which provides a clinically relevant model for this disease. Using the model it was found that irinotecan can be useful for therapy of prostatic SCC, in particular in combination with cisplatin.

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