BCPT Basic & Clinical Pharmacology & Toxicology

Basic & Clinical Pharmacology & Toxicology, 2013, **112**, 63–69 Doi: 10.1111/j.1742-7843.2012.00923.x

Comparative Study of Rapamycin and Temsirolimus Demonstrates Superimposable Anti-Tumour Potency on Prostate Cancer Cells

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(Received 26 March 2012; Accepted 27 June 2012)

Abstract: Rapamycin is a macrocyclic lactone currently used for the treatment of cancer and for the prevention of transplant rejection. The primary pharmacological mode of action of rapamycin occurs through the inhibition (blocking) of the mammalian target of rapamycin (mTOR). By doing so, rapamycin interferes with the phosphoinositide 3-kinase (PI3K)-Akt-mTOR axis that controls several cellular functions involving cell growth, proliferation and angiogenesis. The frequent activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway in advanced prostate cancer has provided a rationale for the use of mTOR inhibitors in this setting. We carried out a comparative study on the effects of rapamycin and temsirolimus on the *in vitro* and *in vivo* growth of the prostate cancer cell lines, LnCap and PC3. Our results demonstrate that rapamycin and temsirolimus exert similar *in vitro* and *in vivo* anti-proliferative effects against prostate cancer cells.

Rapamycin is a macrocyclic lactone currently used for the treatment of cancer and for the prevention of transplant rejection [1, 2]. Rapamycin primarily acts through the inhibition of the mammalian target of rapamycin (mTOR). By doing so, rapamycin also interferes with the phosphoinositide 3-kinase (PI3K)-AKT-mTOR axis that is key to several cellular functions involving cell growth, proliferation and angiogenesis [3–6].

The importance of the mTOR pathway in oncogenesis is also consistent with the key role played in the control of tumour growth by the tumour suppressor gene, phosphatase and tensin homologue deleted on chromosome ten (PTEN). PTEN acts as a lipid phosphatase on intracellular phosphoinositide signalling molecules such as phosphati-trisphosphate that regulate the downstream AKT and subsequently mTOR kinases. PTEN has been found to be mutated in tumours of the bladder, lung, colon and lymphatic system as well as advanced prostate cancers [7].

Rapamycin analogues (rapalogues) sdylinositol 3,4,5uch as temsirolimus, everolimus and ridaforolimus (also known as deforolimus) are different chemical entities with favourable pharmacokinetic profiles derived from the structure of rapamycin. They all have substitution of the C40 hydroxyl group of rapamycin [8]. In particular, temsirolimus is a blocked ester of rapamycin developed for intravenous administration that has been approved by the FDA for the treatment of advanced renal cell carcinoma, and it is currently being investigated in many other cancer settings [9–15].

Although some pharmacokinetic differences have been reported between rapalogues and rapamycin, these analogues appear essentially similar to rapamycin with respect to potency, inhibition of mTOR signalling and overall tolerability [16].

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However, different potencies of rapamycin and temsirolimus have recently been reported on the tumour growth in a rodent model of tuberous sclerosis complex (TSC, which is downstream of AKT but upstream of mTOR), with rapamycin being most effective in reducing tumour growth and improving survival of nude mice bearing tumours [17]. These findings question the concept that rapamycin and temsirolimus possess similar pharmacodynamic effects [16] and suggest that, at least in some specific tumours, rapamycin may be more effective than its analogues. This point appears of particular relevance in the setting of prostate cancer as several studies evaluating combination therapy or single agent treatment with various mTOR inhibitors including rapamycin [13,18] (NCT00311623; NCT01054313), temsirolimus [19] (NCT01083368; NCT01174199; NCT01155258; NCT01020305; NCT00512668; NCT01026623; NCT00887640; NCT01206036), everolimus [20] and ridaforolimus (NCT00777959, NCT00110188) have been initiated and/or recently completed. These observations prompted us to perform a head-to-head study of rapamycin and temsirolimus aimed at evaluating their inhibitory effects on the *in vitro* and *in vivo* growth of both androgen-dependent and androgen-independent LnCaP [21] and androgen-independent PC3 prostate cancer cell lines. Our results demonstrate that both compounds possess a superimposable pharmacological profile on these prostate cell lines.

Materials and Methods

Reagents and cells. Fetal calf serum (FCS) and RPMI-1640 were purchased from Invitrogen (Milan, Italy). Phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), propidium iodide (PI) and colchicine powder were obtained from Sigma-Aldrich (Milano, Italy). DNase-free RNase was obtained from Applichem (Darmstadt, Germany). Annexin V-APC was obtained from Immunotools

(Fryesotye, Germany). Temsirolimus and rapamycin were obtained from LC Laboratories (Woburn, MA, USA).

The human prostate carcinoma LnCaP and PC3 cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were routinely maintained in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine and antibiotics (penicillin-streptomycin) at 37°C in a humidified atmosphere with 5% CO_2. After standard trypsinization, cells were seeded at 2×10^4/well for proliferation assays, 5×10^4/well for cell cycle analysis, apoptosis analysis and Carboxyfluorescein diacetate succinimidyl ester (CFSE) staining in 24-well plates and after the indicated amount of time exposed to the respective compounds as described below.

Animals. Six- to eight-week-old male nude mice, weighing 25–28 g, were purchased from Harlan-Nossan (San Pietro al Natisone, Udine, Italy). The mice were kept under standard laboratory conditions (non-specific pathogen-free) with free access to food and water. The animal studies were carried out in accordance with local guidelines and approved by the local Institutional Animal Care and Use Committee (IACUC).

Determination of cell proliferation. Cell proliferation was evaluated using a flow cytometrical counting method, as previously described [22–26]. Briefly, after standard trypsinization, cells were seeded at 2×10^4/well into 24-well plates and grown for 36 hr into standard medium. Thereafter, increasing drug concentrations from 0.01 to 800 nM were added to the wells for a total volume of 2 mL/well. Basal control wells were trypsinized at time zero with 100 uL of trypsin solution/well; 400 uL of standard medium, with 0.05 ug of PI to exclude dead cells, was added into each well and the obtained suspension was counted under a standard rate for a constant amount of time in a FACS Calibur flow cytometer. After 72 hr of drug exposure, the other wells were trypsinized and the cells were counted under identical conditions as the control wells. Growth inhibition was calculated using the following formula: (T72-T0)/(K72-T0), where T72 is the cell count after 72 hr, T0 is the cell count of the control well at time zero and K72 is the cell count of the control well (medium) after 72 hr. Control values were arbitrarily set to 1.

In selected experiments, the cells were exposed for only 1 hr to the compounds (100 nM concentration), and thereafter grown for 72 hr in complete medium for determination of growth inhibition after drug withdrawal.

Cell cycle distribution analysis. Cell cycle analysis was performed as previously described with a PI-based method [27, 28]. Briefly, the cells were incubated in the presence or absence of increasing drug concentration for 20 hr, and after that they were trypsinized and collected. To assess cell cycle distribution, the cells were fixed in 70% ethanol at 4°C for 30 min., washed twice in PBS and then incubated with RNase (50 ug/mL) for 10 min., and then PI was added at final concentration of (50 μg/mL) for 1 hr at 4°C in the dark. Red fluorescence was analysed with FACSCalibur flow cytometer (BD, Heidelberg, Germany). The proportion of cells in the different cell cycle phases was determined by CellQuest software (Becton Dickinson, Buccinasco, Milan, Italy).

Apoptosis detection. For detection of apoptosis, Annexin V-APC/PI double staining was performed as previously described [29, 30]. Briefly, the cells were incubated in the presence or absence of 100 nM of either compound for 20 hr, and after that they were trypsinized and stained with AnnV-APC/PI in Annexin V staining solution for 20 min. at 4°C [25]. Relative distribution of cells in the PI+, Annexin V+/PI− and Annexin V+/PI− compartments was analysed with a FACSCalibur workstation equipped with CellQuest pro Software (Becton Dickinson, Buccinasco, Milan, Italy).

Carboxyfluorescein diacetate succinimidyl ester staining for detection of cell division. Covalently bound CFSE is divided equally between daughter cells during cell division, allowing discrimination of successive rounds of cell division [31]. Detection of the cells that have undergone cell division was performed by using CFSE staining, as previously described [32–34]. Briefly, the cells were detached and stained with CFSE (1,25 μM) for 8 min. at 37°C, washed twice with complete medium and then seeded into the wells. Subsequently, the cells were exposed to 100 nM of temsirolimus or rapamycin. Positive control wells were exposed to 50 nM colchicine in 0.1% FCS medium. After 48 hr, the cells were detached, washed and the mean green fluorescence intensity of the cell population was analysed by FACSCalibur flow cytometer.

Invasion assay. Cell invasion was evaluated using a 24-well plate invasion chamber fitted with 8 μm pore-sized cell culture inserts coated with Matrigel basement membrane matrix (BD Matrigel™; BD, Milan, Italy). The lower chamber was filled with RPMI supplemented with 10% FBS added as chemoattractant. In the upper chamber, 2.5×10^4 cells were seeded in serum-free medium for 12 hr. The total number of cells that had migrated to the underside of the membranes was counted under a light microscope (×100) after fixation in methanol and staining with Giemsa.

Induction of LnCaP xenografts and experimental treatment. Tumours were induced in athymic nude mice by subcutaneous implantation of LnCaP cells. The cells were dispersed by trypsin, washed (twice) in serum-free medium RPMI-1640 (10 min. centrifugation, $200 \times g$), resuspended at the concentration of 2.5×10^7 cells/mL in Matrigel (BD Basement Membrane Matrix) diluted 1:1 in medium and injected (0.2 mL) subcutaneously in the flank of each mouse using a 0.6-mm needle. Tumour growth was observed daily and measured with callipers (2 perpendicular diameters), and the tumour volume was calculated using the formula $0.52 \times a \times b^2$, where a is the longest and b is the shortest diameter.

Treatment with rapamycin, temsirolimus, cisplatin or vehicle started when the tumours were already palpable with a range volume of 60–70 mm^3. Rapamycin and temsirolimus were prepared immediately before treatment and were injected intraperitoneally (i.p.) at a dose of 3 mg/kg for 18 consecutive days. A group of mice was treated with the vehicle (DMSO 20%), and another group with cisplatin at a dose of 3 mg/kg as positive control. The final volumes of injection for each compound or the vehicle ranged between 100 and 200 μL. The mice were randomly assigned to each experimental group. Post-randomization analysis revealed no significant differences in tumour volumes at the beginning of the treatment among the different groups. The animals were observed for 14 days after the interruption of the treatment.

Statistical analysis. The results are presented as mean ± S.D. of triplicate observations from one representative of at least three experiments, unless indicated otherwise. Student's *t*-test was used to determine statistical significance. Values of $p < 0.05$ were considered to be statistically significant.

Results

Temsirolimus and rapamycin strongly inhibited cell proliferation in a similar manner.

To evaluate the sensitivity of LnCaP and PC3 cell proliferation to temsirolimus and rapamycin, the cells were exposed to a wide range of concentration of both drugs for 72 hr and the cell proliferation assessed by flow cytometric counting.

Both rapamycin and temsirolimus powerfully inhibited cell proliferation showing a maximal effect at as low as 10 nM concentration (fig. 1A,B). An almost identical inhibition of proliferation was observed at all the doses tested for both compounds (fig. 1A,B).

To evaluate whether rapamycin and temsirolimus exerted different effects on the inhibition of cell proliferation after the discontinuation of the treatment, the cells were treated with 100 nM of either drugs for 1 hr and after drug removal, cultivated for 72 hr in the absence of the compounds. Fig. 1C,D show that both treatments are equally effective in inducing a strong and long-term inhibition of cell proliferation in these cell lines.

To determine whether the inhibition of cell proliferation could be ascribed to the induction of apoptosis, after 20 hr of incubation in the presence of the drugs, we performed the Annexin V/PI double staining to detect early apoptotic cells with translocated phosphatidylserine. As expected, none of the compounds induced significant apoptosis, as <5% of the cell population was Annexin V positive (fig. 2A).

Rapamycin and temsirolimus induced a cytostatic effect.

Carboxyfluorescein diacetate succinimidyl ester proliferation assay showed that the anti-proliferative effects of rapamycin and temsirolimus on both LnCap and PC3 cells were related to cytostatic effects of the drugs. Indeed, similar mean fluorescent intensities, which were significantly higher than those of the controls, were obtained with test compounds for both LnCap and PC3 cells (fig. 2B,C). This is consistent with the fact that mTOR blockade induces a G1 cytostatic arrest of proliferating cells. To evaluate whether rapamycin and temsirolimus induced a different block in cell cycle progression, we exposed LnCaP cells to either compounds for 20 hr and therefore assessed the cell cycle distribution of the cell population. As expected and consistently with the results of proliferation analysis, both compounds induced a similar cell cycle arrest at the G0/G1 phase that was almost maximal at 10 nM (fig. 3A). In parallel, the percentage of cells in the S phase decreased in a specular manner (fig. 3B).

Rapamycin and temsirolimus decreased PC3 invasiveness to a similar extent.

To evaluate the comparative ability of rapamycin and temsirolimus to inhibit the motility of PC3 cells, a Matrigel invasion assay was performed. The invasion assay revealed a similar inhibition of the migratory capacity of the PC3 cells upon treatment with either rapamycin or temsirolimus given at the concentration of 100 nM (fig. 4).

Rapamycin and temsirolimus abrogated the in vivo *growth of LnCaP xenografts to a similar extent.*

The mice were treated i.p. with either rapamycin or temsirolimus at the dose of 3 mg/mouse/day for 18 consecutive days, or vehicle starting 14 days after xenograft. The tumour

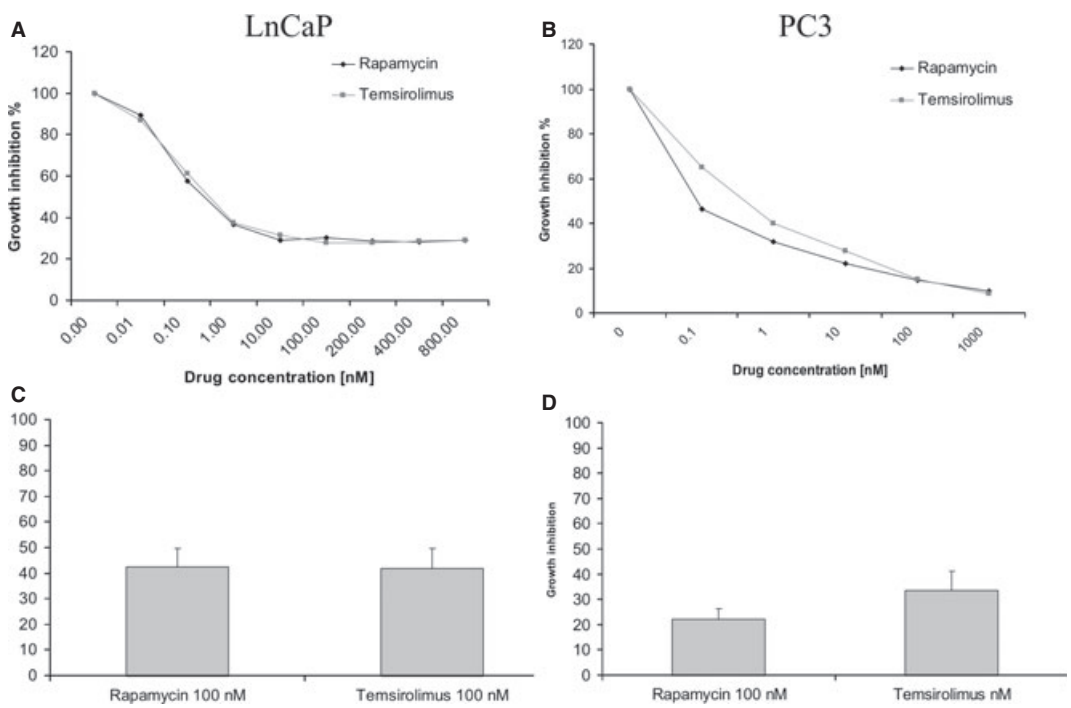

Fig. 1. The effects of rapamycin and temsirolimus on LnCaP and PC3 cells *in vitro*. LnCaP (A) and PC3 (B) cells were exposed to increasing concentrations of rapamycin or temsirolimus for 72 hr. Control values were arbitrarily set to 1; LnCaP (C) and PC3 (D) cells were exposed to 100 nM of rapamycin or temsirolimus for 1 hr. Afterwards, the medium was removed, wells were washed three times with pre-warmed RPMI 1640 and 2 mL of complete medium was added to each well. Cells were left undisturbed for an additional 72 hr. Data represent mean ± S.D. of triplicate observations from three independent experiments with similar results. Control values were arbitrarily set to 1.

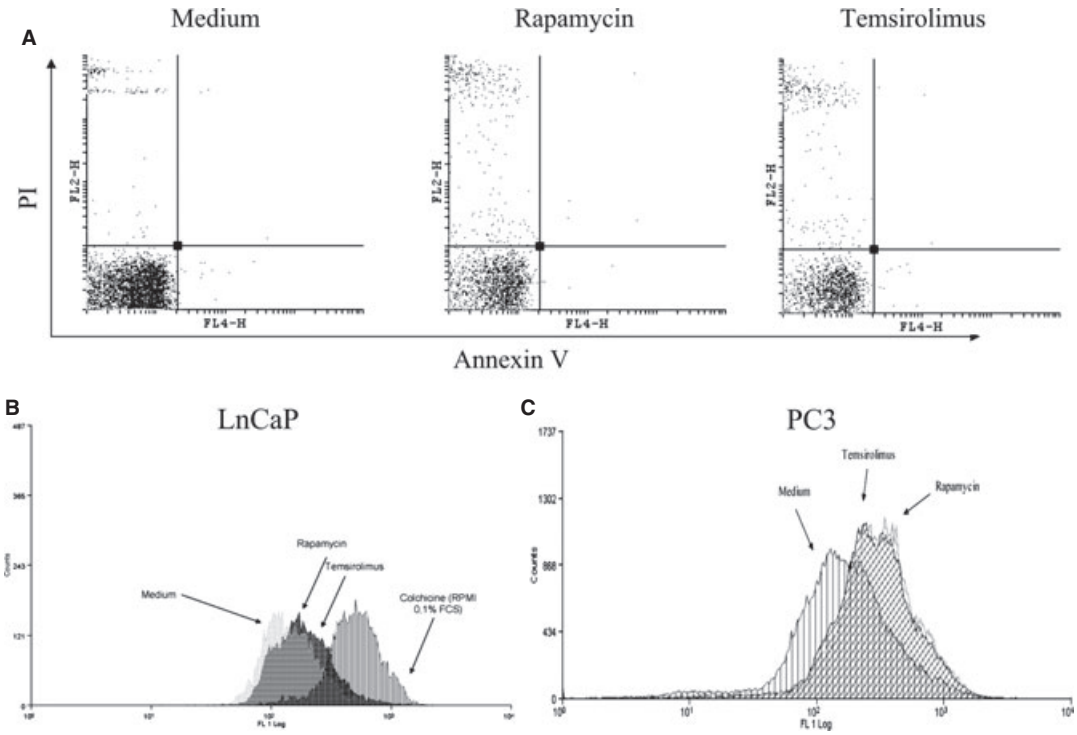

Fig. 2. Effects of rapamycin and temsirolimus *in vitro*. (A) Representative pictures of Annexin V/PI staining of LnCaP cells treated with either rapamycin temsirolimus or vehicle; LnCaP (B) and PC3 (C) cells that were previously loaded with 1.25 uM of Carboxyfluorescein diacetate succinimidyl ester (CFSE) were exposed to 100 nM of either rapamycin or temsirolimus for 48 hr. The figure shows the green fluorescence intensity of representative samples.

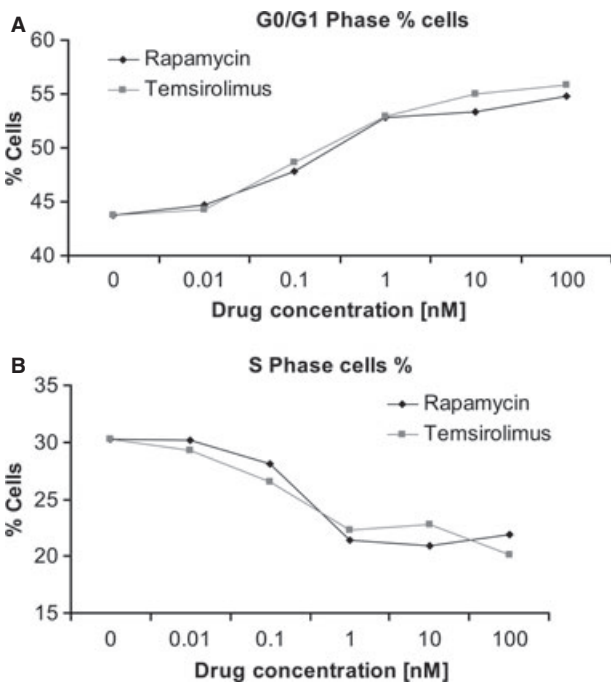

Fig. 3. The effects of continuous exposure to rapamycin and temsirolimus on the *in vitro* cell cycle distribution of LnCaP cells. LnCaP cells were exposed to increasing concentrations of rapamycin or temsirolimus for 20 hr. The figure shows the percentage of cells within the (A) G0/G1 phase or (B) the S phase. Data represent mean of triplicate observations from three independent experiments with similar results.

volume of the mice treated with rapamycin was significantly smaller than those treated with the vehicle ($p < 0.05$) already starting 7 days after the beginning of the treatment (day 21) and continued so until the end of the observational period. The administration of temsirolimus exerted an inhibitory effect on *in vivo* tumour growth comparable to that exerted by rapamycin (fig. 5). Of note, the inhibitory effect on tumour growth exerted by both compounds was similar to that exerted by cisplatin, used as positive control drug (fig. 5).

Discussion

In the present study, we have compared the *in vitro* effects of rapamycin and temsirolimus on different functional parameters of the PTEN-mutant and androgen-dependent LnCaP prostate cancer cell line and of the androgen-independent PC3 cell line, which represents validated pre-clinical models of human prostate cancer. Our results show that the compounds exhibit similar effects on the growth of these cells, inducing a comparably strong and long-lasting inhibition of the proliferation of LnCaP and PC3 cells.

We believe that our present data of particular relevance as limited comparative data are available concerning the anti-cancer efficacy of rapamycin and its analogues such as temsirolimus. Indeed, a different *in vivo* potency between rapamycin and temsirolimus in inhibiting cancer cell growth in a rodent model of tuberous sclerosis has been recently correlated to the blood

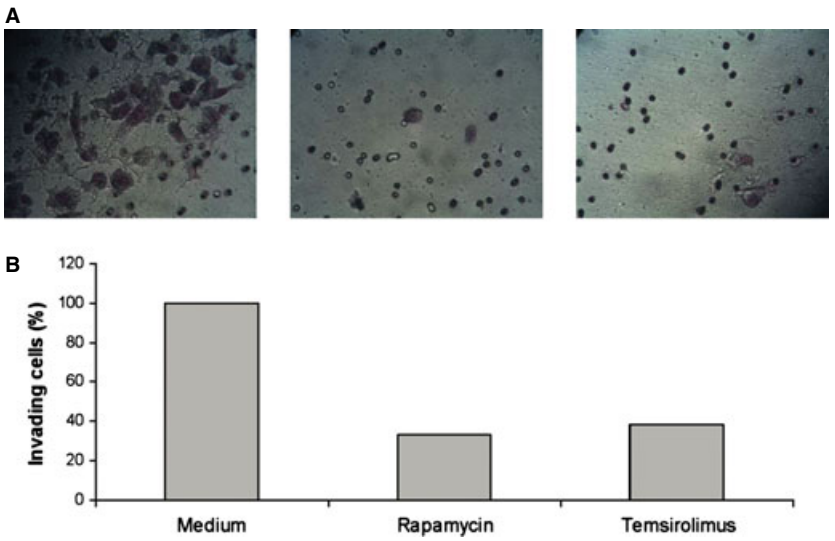

Fig. 4. The effects of rapamycin and temsirolimus on PC3 cell invasion *in vitro*. Cells were cultured in the presence of rapamycin, temsirolimus or vehicle in an invasion chamber. (A) Representative microphotographs of cells in cell invasion assay. (B) Quantitative analysis of cell invasion assay. The results are expressed as the percentage of invading cells with respect to control.

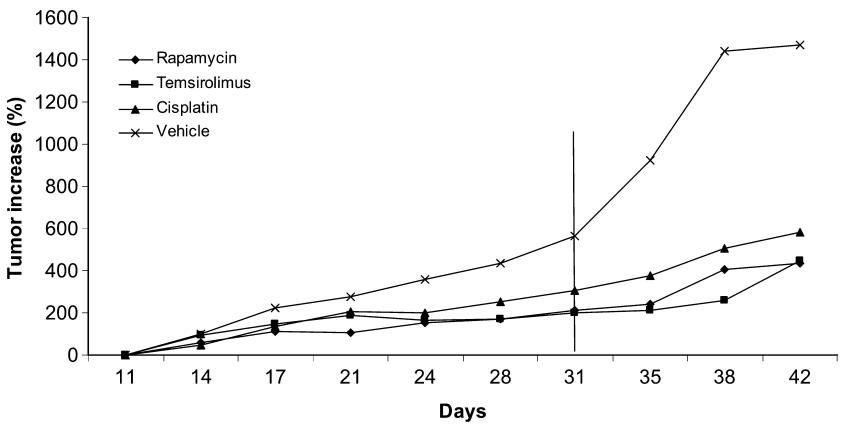

Fig. 5. The effects of rapamycin and temsirolimus on the *in vivo* tumour growth of LnCaP xenografts. Tumours were induced by subcutaneous implantation of 5×10^6 LnCaP cells, and rapamycin, temsirolimus, cisplatin or vehicle was administered for 18 consecutive days starting on day 14 after tumour implantation. Tumour volumes were calculated three times a week until the 45th day after tumour implantation.

and tissue concentration of rapamycin that was higher when the animals were treated with the same agent [17]. In fact, it is known that temsirolimus is converted to rapamycin after injection [35–38], and therefore, clinically relevant pharmacokinetic exposure to temsirolimus may be considered to be a composite of both temsirolimus and rapamycin, with the latter presumably accounting for most of the anti-cancer activities [5, 19, 38, 39]. In addition, a recent study reported that microsomal metabolism of temsirolimus may generate compounds that strongly decrease the anti-neoplastic activity [19].

These pharmacokinetic considerations that may translate into higher efficacy and potency of rapamycin *versus* temsirolimus in different cancer settings would require careful head-to-head comparisons of the former *versus* the latter in relevant *in vitro* and *in vivo* pre-clinical models. This is particularly so in the light of the great attention recently gained from the use of mTOR inhibitors in the treatment of different malignancies including prostate cancer. It should also be noted that rapalogues are also being used in this and other clinical settings [15].

The presently observed superimposable chemotherapeutic capacities of rapamycin and temsirolimus on both cell lines represent a valuable proof of concept that validates the use of these compounds either alone or in association with other chemotherapeutic agents in different clinical settings of prostate cancer. However, the fact that rapamycin possesses a pre-clinical anti-cancer action indistinguishable from that of temsirolimus on the growth of prostate cancer cells may raise an important issue related to the more favourable economic sustainability of using rapamycin rather than temsirolimus, alone or in association with other chemotherapeutic regimens or radiotherapy, for patients with prostate cancer. It was previously calculated that at currently used doses, a 1-month treatment with rapamycin would cost approximately $1000 whereas a 1-month treatment with

temsirolimus at the approved dose for renal cell carcinoma would cost around $5800 [40]. Moreover, rapamycin costs would be further reduced upon the expiration of its patent (US patent #5,100,899) that is expected to occur in 2013.

Along this line of reasoning, we believe that extensive head-to-head comparative studies of rapamycin *versus* rapalogues would be warranted in appropriate pre-clinical models of cancer to demonstrate specific advantages of rapalogues over rapamycin that justifies the inclusion of the more expensive patent-protected rapalogues instead of rapamycin in different cancer settings.

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