



mTOR inhibitors show promising *in vitro* activity in bladder cancer and head and neck squamous cell carcinoma

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Abstract. Bladder cancer and head and neck squamous cell carcinoma (HNSCC) are frequent but lack efficient therapies especially in advanced disease. Almost no studies on mTOR function and inhibition in these tumor entities have been reported. We examined the gene and protein expression levels of mTOR and its activated form (pmTOR) in three human bladder carcinoma cell lines (RT-4, T24, EJ28) and three HNSCC cell lines (PCI-1, PCI-13, BHY). Furthermore, the consequences of mTOR inhibition by mTOR-specific siRNAs and the mTOR inhibitor temsirolimus were analysed *in vitro* using immunohistochemical Ki-67 staining, mTOR and pmTOR Western blot analysis, MTT assay, as well as cell cycle analysis with flow cytometry. Especially pmTOR protein expression levels showed marked differences between cell lines. siRNA transfection was associated with dose-dependent target protein reduction but not proliferation inhibition or apoptosis. On the contrary, temsirolimus significantly reduced cell viability and induced apoptosis and cell cycle arrest. According to these data, bladder cancer and HNSCC are promising tumor entities for mTOR inhibition with temsirolimus.

Introduction

Bladder cancers and head and neck squamous cell cancers are frequent tumors. Bladder cancers are the second most

common urologic malignancy in the Western world with estimated 70,980 new cases diagnosed each year in the United States (1) and 85,880 in Europe (2). The gold standard for muscle invasive (>T1) urothelial cancer of the bladder is radical cystectomy with radiochemotherapy as an alternative. While 5-year overall survival rates for localized muscle-invasive bladder cancer after cystectomy are approximately 80%, 5-year survival rates are already significantly poorer after cystectomy in T3a tumors (20-43%) and especially in lymph node positive patients (17-35%) (3). Patients with metastatic disease have a mean survival of 3-6 months. The currently best option in these patients is cisplatin-based chemotherapy which results in 10-50% complete remissions. However, the survival benefit is less than 4 months.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common tumor occurring almost exclusively among middle-aged tobacco and alcohol abusers (4). Tumors of the oral cavity and pharynx were estimated with 35,720 new cases diagnosed each year in the United States (1) and 74,440 in Europe (2). During the past decades, treatments for HNSCC have changed dramatically due to novel approaches such as combined modality therapy and improvements in surgical and radiotherapeutic techniques, resulting in improved function and quality of life of patients with this disease. Nevertheless, despite innovative surgery, new radiation strategies, and the development of novel chemotherapeutic drugs, the overall 5-year survival rate remained poor and nearly unchanged. Recurrent and/or metastatic HNSCC carries a dismal prognosis with a median survival of about 6 months. The overall response rate with single agent cytotoxics ranges between 15 and 30%. Higher response rates are reported with combination regimens, but better survival has not yet been demonstrated (5).

Especially cells of head and neck cancer are known to develop molecular strategies to escape efficient antitumor immune responses. It is supposed that tumor production of various immunosuppressive mediators contributes to suppressed immune functions. The molecular mechanisms responsible for these malignant transformation processes and the development of the immunosuppressive HNSCC micro-environment remain mostly unknown (5-11).

Due to these poor therapeutic results, preclinical evaluation of torisel alone or in combination with other agents in these tumors seems promising.

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Interestingly, except for initial NIH cell line testing, only one report on preclinical or clinical evaluation of mTOR inhibition has been published for bladder cancer and only three studies have been published including preclinical data on temsirolimus in squamous cell carcinoma of the head and neck. However, the success of current treatment regimens in these tumors is highly limited in cases with locally advanced or metastatic disease. Our aim was to evaluate mTOR inhibition with temsirolimus and specific siRNAs in these tumor entities.

Materials and methods

Cell culture. Three human bladder carcinoma cell lines (RT4, T24 and EJ28) and three human HNSCC-cell lines (PCI-1, PCI-13 and BHY) were used. The cell lines RT4 and T24 were grown in RPMI-1640 supplemented with 10% FCS, 1% sodium pyruvate and penicillin-streptomycin. EJ28, PCI-1, PCI-13 and BHY were cultured in DMEM containing 10% FCS, 1% MEM (non-essential amino acids), 1% HEPES and penicillin-streptomycin. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. The mTOR-inhibitor temsirolimus (CCI-779) (Torisel®), kindly provided by Wyeth (Wyeth Pharma, Münster, Germany), was stored at 4°C and suspended in DMSO the day of use.

Immunohistochemistry/fluorescence microscopy. Immunohistochemical detection of mTOR, phospho-mTOR and Ki-67 was performed on cytospin preparations using the following antibodies: anti-mTOR pS2448 (rabbit) by Rockland (Hamburg, Germany), anti-mTOR (rabbit) by Millipore (MA, USA) anti-human Ki-67 antigen (mouse, clone: MIB-1) by Dako (Hamburg, Germany) and DAPI (4',6-Diamidino-2'-phenylindole dihydrochloride) by Roche (Mannheim, Germany). Secondary antibodies were a FITC-labelled goat anti-Rabbit IgG (ZyMax™ Grade) by Zymed (San Francisco, CA, USA) and a Cy3-labelled goat anti-rabbit antibody (Dianova GmbH, Hamburg, Germany). Replacement of primary antibody with PBS was used as the control.

The photographs were taken with AxioCam MRm Digital Camera (Zeiss, Göttingen, Germany) of the fluorescence microscope Zeiss Axiovert 200M and edited by AxioVision 4.7. For evaluation of antibody-positive fractions the software Image J 1.42q was used; on average a number of 266 cells per slide were counted.

Western blot analysis. Cells were washed with phosphate-buffered saline (PBS), scraped using Laemmli-buffer and lysed by QIAshredder (Qiagen, Hilden, Germany). The protein contents of the samples were determined by Pierce BCA Protein Assay Kit according to the manufacturer's protocol. Cell lysates were separated by electrophoresis through a 7.5% SDS-PAGE gel for the detection of mTOR and phosphorylated mTOR (pmTOR) respectively a 12.5% SDS-PAGE for β -actin control and transferred to a nitrocellulose membrane. The membranes were incubated with mTOR Anti-mTOR (rabbit) (by Millipore, USA) and pmTOR pS2448 antibodies (by Rockland, USA) 1:2500, subsequently incubated with secondary anti-rabbit IgG-coupled HRP antibody 1:50000 (Sigma, A0545, Munich, Germany). Blots

were visualized by adding Pierce ECL Western Blotting substrate.

RT-PCR. Transfection was performed with appropriate mTOR siRNA (concentration: 1, 10, 100 nM). Cells were harvested and total RNA was prepared by use of the Quiashredder/Rneasy Mini Kit (Qiagen). DNA was digested using Dnase I according to the Gibco/Invitrogen protocol (18). For cDNA synthesis 500 ng of total RNA was reverse-transcribed according to the Gibco/Invitrogen protocol real-time PCR was performed with the iCycler (Bio-Rad) PCR Mastermix protocol (qPCR Core Kit for SYBR-Green I, Eurogentec, Belgium).

The 5'-3' primer sequences are: GAPDH: forward, 5'-GAC AGT CAG CCG CAT CTT CTT-3', and reverse, 5'-TCC GTT GAC TCC GAC CTT C-3' (Metabion, Martinsried, Germany); mTOR: forward, 5'-TGC CAA CTA TCT TCG GAA CC-3', reverse, 5'-GCT CGC TTC ACC TCA AAT TC-3' (Metabion).

After an initial activation for 2 min at 95°C the 40 amplification reaction cycles consisted of 95°C for 45 sec, 60°C for 45 sec. Quantification of gene expression was performed by measuring $\Delta\Delta C_t$ -method. All values represent the mean of duplicates for each concentration.

MTT assay. Cells were seeded in 96-well plates at density of 2×10^4 /well. After 24 h, cells were treated with CCI-779, cisplatin or mTOR siRNA at given concentrations for 24 or 48 h. At the end of the experiment, 5 μ l MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, Munich) in solution with 95 μ l RPMI respectively DMEM was added to each well, followed by incubation at 37°C for 4 h. The medium was carefully aspirated and 100 μ l dimethyl sulfoxide (DMSO) was then added to dissolve blue formazan crystals formed in the live cells. Optical density was measured with a spectrometer at 550 nm.

Flow cytometry. Flow cytometric analysis was performed with a FACSCanto™ flow cytometer using BD FACS Diva software™ (Becton-Dickinson, Heidelberg, Germany).

siRNA sequences and transfection. Three different small interfering RNAs (siRNAs) targeting the human mTOR were used. The sequences were as follows: siRNA mTOR1: sense 5'-GGC CUA UGG UCG AGA UUU A dTdT-3', anti-sense 5'-UAA AUC UCG ACC AUA GGC C dTdT-3'; siRNA mTOR2 sense 5'-CCU GCU GGA UGC UGA AUU A dTdT-3' anti-sense 5'-UAA UUC AGC AUC CAG CAG G dTdT-3'. siRNA mTOR3 sense 5'-GGA GUC UAC UCG CUU CUA U dTdT-3' anti-sense 5'-AUA GAA GCG AGU AGA CUC C dTdT-3'.

siRNA sequences were purchased from Eurogentec (Cologne, Germany). As a control for transfection efficiency FITC-labelled non-silencing siRNA (Qiagen) was used and fluorescence measured as described (flow cytometry).

Cells (3.5×10^5) were transfected with 0, 1, 10 and 100 nM of mTOR siRNA using Lipofectamine 2000 by Invitrogen according to the manufacturer's protocol. Cells were harvested at 24 and 48 h.

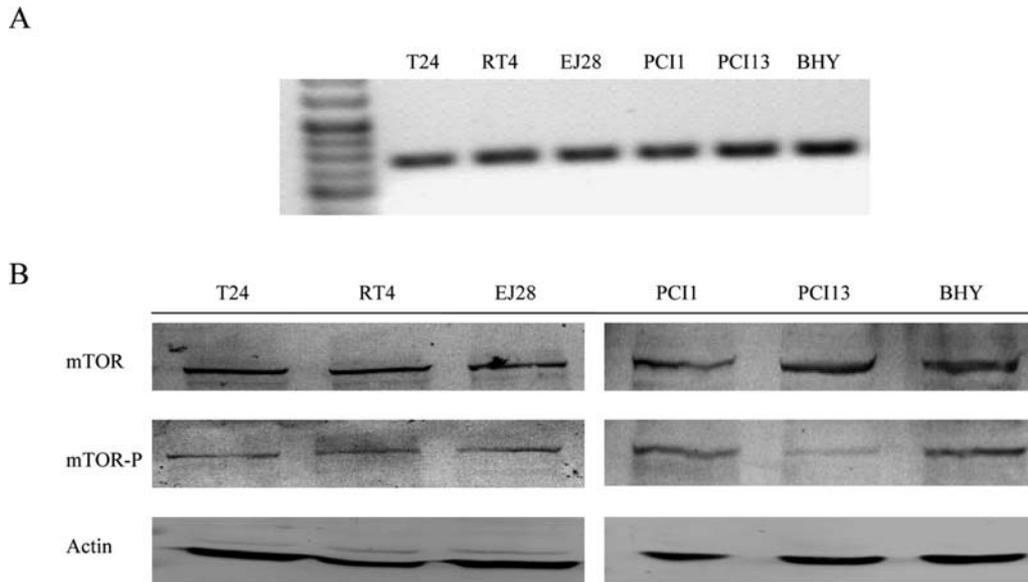


Figure 1. mTOR expression levels in bladder cancer and HNSCC. Expression levels of mTOR were analyzed in different permanent cell lines from bladder cancer (T24, RT4, EJ28) and HNSCC (PCI-1, PCI-13, BHY) on the mRNA (A) and on the protein level (B). (A) Constitutive mTOR mRNA expression was analysed in untreated cells of different cancer cell lines. Gel electrophoresis revealed no marked differences between cell lines. One representative gel is shown. (B) Western blot analysis confirmed the presence of the protein products of both the unphosphorylated as well as phosphorylated form of mTOR in all analyzed cell lines. Actin was used as an internal loading control.

Results

mTOR expression levels in bladder cancer and HNSCC. We examined the expression of mTOR and its phosphorylated (active) counterpart in various permanent cell lines of bladder cancer (T24, RT4, EJ28) and HNSCC (PCI1, PCI13, BHY). Reverse transcriptase-polymerase chain reaction showed that mTOR is constitutively expressed in all analyzed cell lines with no significant deviations (Fig. 1A). Furthermore, Western blot analysis confirmed the presence of the protein products of both the unphosphorylated as well as phosphorylated form of mTOR in all analyzed cell lines (Fig. 1B). Actin was used as an internal loading control and the cell lines of both tumor entities revealed strong expression levels of phosphorylated mTOR with the exception of HNSCC cell line PCI13 in which lower levels were observed (Fig. 1B). The results suggest that mTOR is highly active in bladder cancer and HNSCC under normal conditions.

siRNA mediates down-regulation of mTOR protein. Three different siRNA constructs against mTOR were used, from which two were newly designed and one was published before (12). Transfection of tumor cells resulted in a dose-dependent protein down-regulation shown in Western blot analysis. Reduced mTOR protein levels in response to siRNA transfection were evaluated in four different cell lines of the two analyzed tumor entities (Bladder cancer: RT-4, T24/HNSCC: PCI-1, PCI-13). Fig. 2 illustrates the protein expression of mTOR and its phosphorylated counterpart.

Best results were achieved using siRNA concentration of 100 nM with the most effective construct range between 39 and 76% for mTOR, and between 34 and 62% for pmTOR. Only RT-4 cells were characterized by low inhibition with <30%. Despite of reduced mTOR translation levels our data

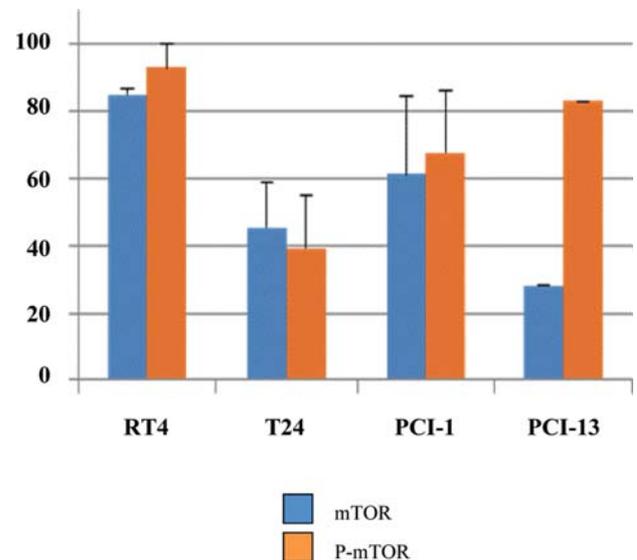


Figure 2. siRNA mediates down-regulation of mTOR and pmTOR protein. Reduced mTOR protein levels in response to siRNA transfection were evaluated in four different cell lines of the two analyzed tumor entities (bladder cancer, RT-4; T24/HNSCC: PCI-1, PCI-13). The relative protein expression levels compared to untreated cells are shown (100%).

revealed hardly any reduction in levels of active mTOR in the majority of the analyzed cell lines, suggesting a cellular counteraction via increased mTOR phosphorylation (Fig. 2).

Reduced cell proliferation in response to temsirolimus. Cell viability and proliferation of different permanent cell lines of bladder cancer and HNSCC were analyzed in response to mTOR siRNA transfection and temsirolimus treatment, respectively.

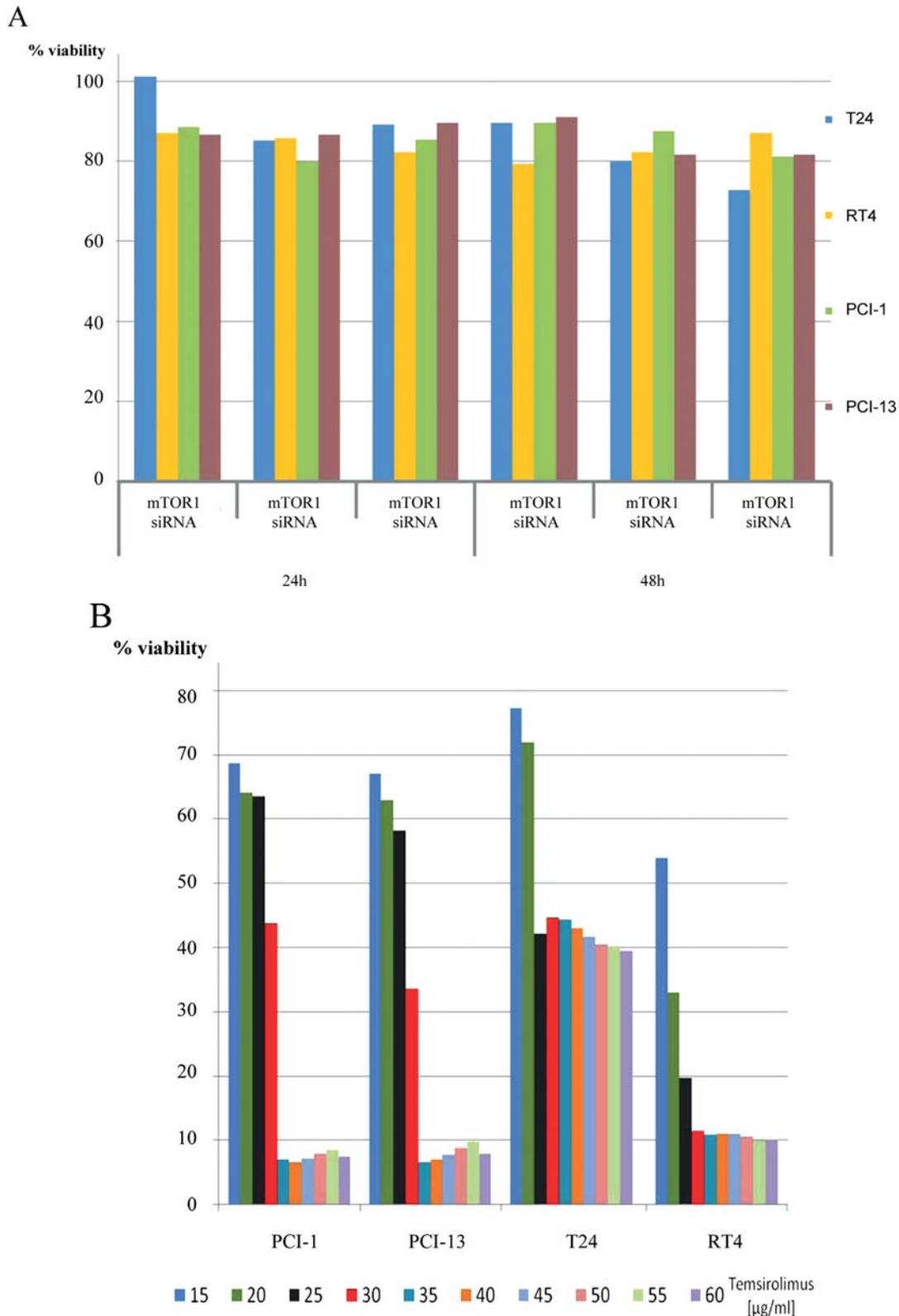


Figure 3. Reduced cell proliferation in response to Temeirolimus. (A) MTT assays revealed no significant influence of the different siRNA constructs on cell growth and viability with any differences between bladder cancer and HNSCC. Four cell lines (T24, RT-4, PCI-1, PCI-13) were examined. (B) In contrast, temsirolimus treatment resulted in strongly reduced cell viability and cell proliferation in all analyzed cell lines. These effects were clearly dose-dependent with a maximum impact between 25 and 60 μg temsirolimus per ml. The four cell lines T24, RT-4, PCI-1 and PCI-13 were analysed.

MTT assays revealed no significant influence of the different siRNA constructs on cell growth and viability with any differences between bladder cancer and HNSCC (Fig. 3A). In contrast, temsirolimus treatment resulted in

strongly reduced cell viability and cell proliferation in all analyzed cell lines. These effects were clearly dose-dependent with a maximum impact between 25 and 60 μg temsirolimus per ml (Fig. 3B).

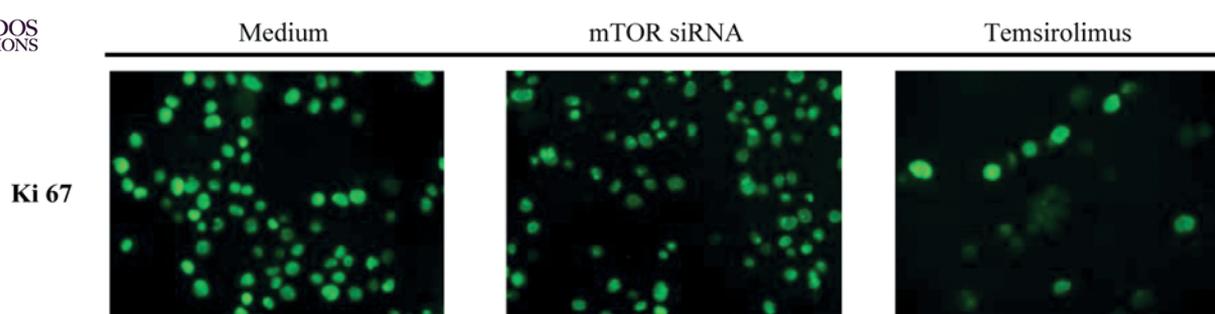


Figure 4. Reduced cell proliferation in response to temsirolimus. In addition, cell proliferation was investigated using immunofluorescence staining of Ki-67. The proliferation status represented by Ki-67 positivity was strongly reduced in response to temsirolimus and not in response to siRNA transfection. PCI-13 cells are shown representatively.

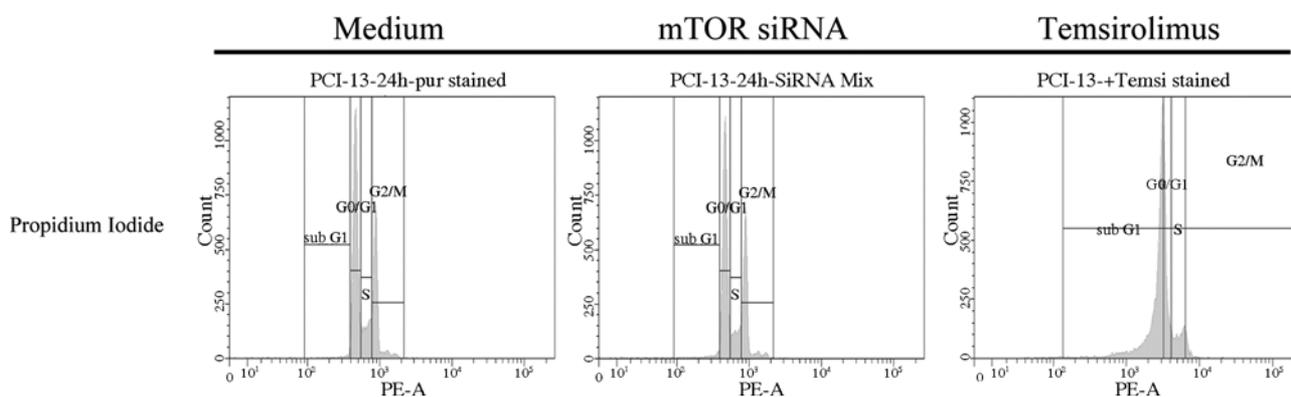


Figure 5. Cell cycle inhibition in response to temsirolimus. Flow cytometric measurements of histograms illustrate that the transfected siRNA constructs have no influence on the cell cycle behavior, whereas temsirolimus clearly leads to apoptosis induction and a G2/M to G0/G1 shift. PCI-13 cells are shown representatively.

In addition, cell proliferation was investigated using immunofluorescence staining of Ki-67. The rate of cell proliferation represented by Ki-67 positivity was strongly reduced in response to temsirolimus and not in response to siRNA transfection (Fig. 4). Temsirolimus reduced cell proliferation on average by 36% with PCI-13 being the most sensitive cell line (reduction by 51%) and T24 the least sensitive (reduction by 20%). These data underline the potential of temsirolimus as a promising agent against tumor progression.

Cell cycle inhibition in response to temsirolimus. Flow cytometric measurements with propidium iodide as a DNA selective stain were used to analyze DNA content and cell cycle distribution in response to siRNA transfection and temsirolimus treatment.

The resulting histograms illustrate that the transfected siRNA constructs have no influence on the cell cycle behavior, whereas temsirolimus clearly leads to apoptosis induction and a G2/M to G0/G1 shift (Fig. 5).

Discussion

Temsirolimus, a novel inhibitor of mammalian target of rapamycin (mTOR), is clinically used in several tumor entities. It has, for instance, demonstrated prolonged overall

survival and progression-free survival compared with interferon α (IFN) in patients with advanced renal cell carcinoma (RCC) (13). mTOR is a central element in an evolutionary conserved signalling pathway that regulates proliferation, cell growth, and survival, orchestrating signals originating from growth factors, nutrients or particular stress stimuli. Two important modulators of mTOR activity are the AKT and ERK/MAPK signalling pathways (14). Many studies have shown that mTOR plays an important role in the biology of malignant cells, including deregulation of the cell cycle, inactivation of apoptotic machinery and resistance to chemotherapeutic agents. The development of several mTOR inhibitors, in addition to rapamycin, has facilitated studies of the role of mTOR in cancer, and verified the anti-tumour effect of mTOR inhibition in many types of neoplasm.

Interestingly, except for initial NIH cell line testing, very few reports on preclinical or clinical evaluation of mTOR inhibition have been published for bladder cancer and for squamous cell carcinoma of the head and neck. However, the success of current treatment regimens in these tumors is highly limited in cases with locally advanced or metastatic disease.

We have found marked mTOR mRNA and mTOR and pmTOR protein levels in several bladder cancer and HNSCC cell lines when compared with RCC cell lines. siRNA inhibition of mTOR was successfully established and the

effect of mTOR inhibition with temsirolimus and with mTOR siRNAs was examined.

Numerous studies have examined the effect of rapamycin analogues in different tumor entities. An inhibition of proliferation after treatment has repeatedly been described. This could be confirmed in bladder cancer and HNSCC cell lines. Temsirolimus treatment reduced cell viability and cell numbers (data not shown) in MTT tests and in cell growth curves to a much larger extent than mTOR-specific siRNAs. Inhibition of the proliferation fraction demonstrated by Ki-67 staining was found after temsirolimus treatment but not after siRNA treatment.

Furthermore, we have shown in all cell lines a G2/M to G0/G1 shift and an increase of apoptotic cells characterized by the enhanced sub-G1 fraction in histograms.

The first study on inhibition of mTOR as a therapeutic strategy with rapamycin analogues in bladder cancer has recently been published (15). The authors found after treatment of 9 different bladder cancer cell lines with RAD001 (Everolimus) a significant inhibition of proliferation and a cell cycle arrest in the G0/G1 phase without induction of apoptosis. Vega *et al* have shown that mTOR inhibition with siRNA and rapamycin induced cell cycle arrest in G1 phase with reduction of S-phase fraction and apoptosis in anaplastic lymphoma (16). Peponi *et al* found cell cycle arrest in G1 after silencing of mTOR with specific siRNAs in mantle cell lymphoma (12). Liu *et al* examined the level of mTOR expression during cell cycle in poorly differentiated oral squamous carcinoma and in HeLa cells (17). In HNSCC cells mTOR mRNA and protein levels increased in M phase. In HeLa cells the level of mTOR mRNA and protein did not change during the cell cycle. Activity of mTOR was highest in G2 and M phase. While an early cell cycle arrest after mTOR inhibition has repeatedly been described, data on apoptosis induction is highly heterogeneous. Obviously, effects are very much dependent on tumor entity and potentially on cell differentiation. This will be a central issue of our further investigations.

The results of this study indicate that mTOR inhibition with temsirolimus may be a promising therapeutic approach for bladder cancer and HNSCC.

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