

mTOR is a selective effector of the radiation therapy response in androgen receptor-positive prostate cancer

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Abstract

Ionizing radiation (IR) is used frequently in the management of multiple tumor types, including both organ-confined and locally advanced prostate cancer (PCa). Enhancing tumor radiosensitivity could both reduce the amount of radiation required for definitive treatment and improve clinical outcome. Androgen suppression therapy improves clinical outcomes when combined with radiation therapy but is associated with significant acute and chronic toxicities; hence, there is a clear need for alternative means to increase the therapeutic window of radiotherapy. Herein, it is demonstrated that the mammalian target of rapamycin (mTOR) inhibitors rapamycin (sirolimus) and temsirolimus limit both hormone therapy (HT)-sensitive and castration-resistant PCa (CRPC) cell proliferation as single agents and have a profound radiosensitization effect when used in combination with IR. Importantly, the observed radiosensitization was influenced by the treatment schedule, in which adjuvant administration of mTOR inhibitors was most effective in limiting PCa cell population doubling. This schedule-dependent influence on *in vitro* treatment outcome was determined to be the result of relative effects on the cell cycle kinetics. Finally, adjuvant administration of either mTOR inhibitor tested after IR significantly decreased clonogenic cell survival of both HT-sensitive and CRPC cells compared with IR alone. Taken together, these data demonstrate that inhibition of mTOR confers a radiosensitization phenotype that is dependent on relative cell cycle kinetics and provide a foundation for clinical assessment.

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Introduction

Prostate cancer (PCa) is the most frequently diagnosed non-cutaneous malignancy and the second leading cause of death due to cancer in men in the United States (Jemal *et al.* 2010). Treatment options for localized disease include watchful waiting, surgery, and radiotherapy (RT; Klein *et al.* 2009). In the context of definitive treatment, adjuvant therapy after radical prostatectomy, and in some cases metastatic disease, RT is becoming of increasing significance for successful management of PCa (Kwok & Yovino 2010).

Androgens and the cognate receptor (androgen receptor (AR)) have a well-described function in all stages of PCa.

If disseminated at the time of diagnosis, first-line therapy is targeted against the AR signaling axis. Suppression of AR activity is achieved by using GnRH agonists that induce ligand depletion (chemical castration) and is sometimes used in combination with direct AR antagonists (such as bicalutamide; Klotz 2006, Loblaw *et al.* 2007, Taplin 2007, Chen *et al.* 2008, Knudsen & Scher 2009). For locally advanced or high-risk disease, RT is frequently used, thus underscoring the need to delineate the impact of combination therapy. AR-directed therapeutics is initially effective due to the dependence of this tumor type on AR signaling; however, after a median time of 2–3 years, tumors recur and are deemed ‘castration resistant’

(castration-resistant PCa (CRPC)). CRPC development is highly attributed to inappropriate resurgence of AR activity, which occurs despite the absence of circulating serum androgens and administration of direct AR antagonists (Knudsen & Scher 2009, Yuan & Balk 2009). Strikingly, few therapeutic options have shown efficacy against this stage of the disease, and a major goal of current translational research is to develop means for preventing or delaying progression to CRPC. One means by which PCa cells bypass AR-directed therapeutics involves upregulation of rapamycin (Rapa)-sensitive signaling (Mousses et al. 2001), and that combining mammalian target of rapamycin (mTOR) inhibition with AR-directed therapies prolongs hormone sensitivity in xenograft models of PCa (Schayowitz et al. 2010). Moreover, AR is known to promote mTOR activity (Xu et al. 2006b), thus suggesting that combining mTOR- and AR-directed therapeutics may cooperate to improve cellular and clinical responses to therapy.

Given the poor outcomes associated with resurgent AR activity and CRPC development, it is imperative to develop new means for enhancing therapeutic efficacy and thus to prevent the transition to CRPC. In patients with locally advanced PCa treated with RT alone, the 5-year disease-free survival rate is 40% (Bolla et al. 2002). Therefore, improving the overall efficacy of RT could be of significant clinical benefit. Several potential mechanisms lead to RT failure, including altered proliferative and pro-survival potential, both of which are frequently observed in PCa.

A frequent genetic lesion that leads to both events is loss of PTEN function. Sixty percent of PCa demonstrate loss of heterozygosity at the *PTEN* locus (Cairns et al. 1997, McMenamin et al. 1999). Decreased expression of PTEN has been detected in 85% of primary PCa tumors compared to normal prostatic tissue of the same patient (Kremer et al. 2006), and patients with tumors harboring mutant PTEN have decreased survival, higher metastatic frequency, and higher prostate-specific antigen (PSA) levels, suggesting higher AR activity (Pourmand et al. 2007); therefore, PTEN is one of the most frequently altered genes in human PCa and is associated with lethal tumor phenotypes. The PTEN phosphatase serves at the molecular level to counteract the functions of phosphoinositide 3-kinase, which promotes proliferation and cell survival, in part through activation of mTOR (Sansal & Sellers 2004). Akt serves as an intermediate signaling molecule for mTOR, which is a serine/threonine kinase that mediates cell growth, proliferation, survival, protein translation, and other oncogenic functions.

mTOR activity is often deregulated in Pca (Kremer et al. 2006), in part due to the prevalence of PTEN

dysfunction. Genomic deletion of PTEN is associated with both increased Akt activation and AR activity (Sircar et al. 2009). mTOR mediates proliferation in PCa cells, at least in part, due to androgen-induced upregulation of D-type cyclin translation (Gao et al. 2003, Xu et al. 2006b). This event is suggested to, therefore, promote cell cycle progression. In addition, mRNA translation events that are dependent on mTOR are rapidly activated in response to ionizing radiation (IR), resulting in DNA repair and survival (Braunstein et al. 2009). As such, the mTOR signaling pathway is a potential target for enhancing RT efficacy and improving therapeutic intervention in PCa.

Pharmacological mTOR inhibition has been demonstrated to block the induction of the proliferative, pro-survival, and oncogenic functions of mTOR (Hidalgo & Rowinsky 2000), with remarkable effects in PTEN-deficient tumors. mTOR inhibitors (e.g. everolimus) have been approved by the FDA for treatment of renal cell carcinoma based on a successful phase III clinical trial (Motzer et al. 2008); thus, mTOR is an established therapeutic target and mTOR inhibitors appear to be reasonably well tolerated. At the cellular level, mTOR inhibitors have been shown to sensitize multiple tumor types to DNA damage-inducing agents, including IR, using both *in vitro* and *in vivo* models of human disease (Beuvink et al. 2005, Wu et al. 2005, Cao et al. 2006, Aissat et al. 2008, Morgan et al. 2008, Ekshyyan et al. 2009, Fung et al. 2009, Matsuzaki et al. 2009, Murphy et al. 2009, Saunders et al. 2010). Moreover, mTOR signaling has been implicated as a determinant of cell survival in response to DNA damage (Shen et al. 2007).

This study assessed the impact of mTOR inhibition in clinically relevant models of hormone therapy (HT)-sensitive PCa and CRPC tumor cells both alone and in combination with RT. Survival analyses revealed that mTOR inhibitors sensitized both HT-sensitive PCa and CRPC cells to IR at clinically attainable doses. The impact of sequence of mTOR inhibition as a radiosensitizer was also assessed, where it was observed that the radiosensitization events were influenced by the scheduling. Strikingly, mTOR inhibitors were most effective at conferring radiosensitization effects when administered in the adjuvant setting. Schedule dependence was determined to be due to cell cycle kinetics, in which neoadjuvant use of mTOR inhibitors limited entry of the cells into a state of active DNA replication. On combining these studies, it is demonstrated that mTOR inhibitors radiosensitize AR-positive PCa cells dependent on treatment schedule and relative cell cycle inhibition and provide evidence of a viable combinatorial treatment strategy.

Materials and methods

Cell culture and reagents

LNCaP, C4-2, and LAPC4 cells were cultured under standard conditions at 37 °C and 5% CO₂ as described previously (Sharma *et al.* 2010). Rapa was obtained from Calbiochem (San Diego, CA, USA) and dissolved in DMSO. Temsirolimus (Tem) was obtained from LC Laboratories (Woburn, MA, USA) and dissolved in ethanol.

Ionizing radiation

A Panatek orthovoltage X-ray irradiator was used to deliver IR. The irradiator was calibrated daily using a Victoreen dosimeter.

Cell counting/survival

To monitor cell number over time, indicated cells were seeded on poly-L-lysine-coated dishes at equal densities and subjected to treatment/schedules described. At the time of harvest, cells were trypsinized and counted using Trypan Blue exclusion and a hemacytometer. Total cell number was determined from at least three independent experiments of three biological replicates.

Cell cycle analysis/bivariate FACS

To monitor bromodeoxyuridine (BrdU) incorporation/DNA content, LNCaP cells were seeded on poly-L-lysine-coated dishes at equal densities and subjected to treatment/schedules described. Two hours prior to harvest, cells were incubated with BrdU (1:1000, Amersham Cell Proliferation Labeling Reagent, GE Healthcare, Buckinghamshire, UK). After labeling, cells were trypsinized and harvested, washed with PBS, and then re-suspended in PBS. Cells were then fixed with cold 100% ethanol, pelleted, then re-suspended in 2 M HCl, and incubated for 20 min at ambient temperature. HCl was neutralized with 0.1 M sodium tetraborate, washed with IFA buffer, followed by a wash with IFA buffer supplemented with 0.5% Tween 20, then re-suspended in IFA buffer containing 6% FITC-conjugated anti-BrdU anti-sera (BD Biosciences, San Diego, CA, USA), and incubated for 45 min. Cells were then washed with IFA buffer supplemented with 0.5% Tween 20, stained with propidium iodide (0.2 g/ml), and subjected to flow cytometry. Samples were quantified on a Coulter Epics XL-MCL using XL System II Software (Beckman Coulter, Brea, CA, USA) and analyzed using FlowJo Software (Tree Star, Inc., Ashland, OR, USA). To monitor only the DNA content,

LNCaP cells were seeded on poly-L-lysine-coated dishes at equal densities and subjected to treatment/schedules described. Cells were trypsinized and harvested, washed with PBS, and then re-suspended in PBS. Cells were then fixed with cold 100% ethanol, pelleted, stained with propidium iodide (0.2 g/ml), and subjected to flow cytometry. Samples were quantified on a Coulter Epics XL-MCL using XL System II Software (Beckman Coulter) and analyzed using FlowJo Software (Tree Star, Inc.).

Clonogenic cell survival

Exponentially growing cells were trypsinized and counted using Trypan Blue exclusion. Cells were diluted serially to appropriate concentrations and plated into 50 ml tissue culture flasks in triplicate for 24 h. Then, cells were treated with increasing doses of IR (0, 2, 4, 6, and 8 Gy). After 24 h, cells were treated with Rapa (10 nM), Tem (10 nM), or nothing. After 14 days of incubation, the colonies were fixed and stained with 4% formaldehyde in PBS containing 0.05% crystal violet. Colonies containing >50 cells were counted. Surviving fraction was calculated as (mean colony counts)/(cells inoculated) × (plating efficiency), in which plating efficiency was defined as (mean colony counts)/(cells inoculated for un-irradiated controls).

Results

Single-agent mTOR inhibitors or IR limit HT-sensitive PCa cell growth

mTOR activity has been observed to be increased in PCa through various mechanisms and upstream signaling defects. To challenge the consequence of mTOR inhibition in PCa, HT-sensitive cells were treated with increasing doses of two pharmacological inhibitors of mTOR activity, Rapa and Tem. It has been demonstrated that in this cell type, androgens induce mTOR signaling that culminates in cell cycle progression via an increased translation of cyclin D1 (Xu *et al.* 2006b), which is part of the molecular machinery responsible for the G₁-S phase transition (Baldin *et al.* 1993). Consistent with previous reports, mTOR inhibition resulted in decreased cell number after 72 h of treatment (Fig. 1A; van der Poel *et al.* 2003). As demonstrated, there was no significant difference between either of the mTOR inhibitors tested with regard to response at any of the doses tested. As IR is used as definitive treatment for localized, HT-sensitive PCa, the effect of IR on HT-sensitive cells was assessed. These results demonstrate a dose-dependent decrease in population cell doubling after exposure to IR (Fig. 1B).

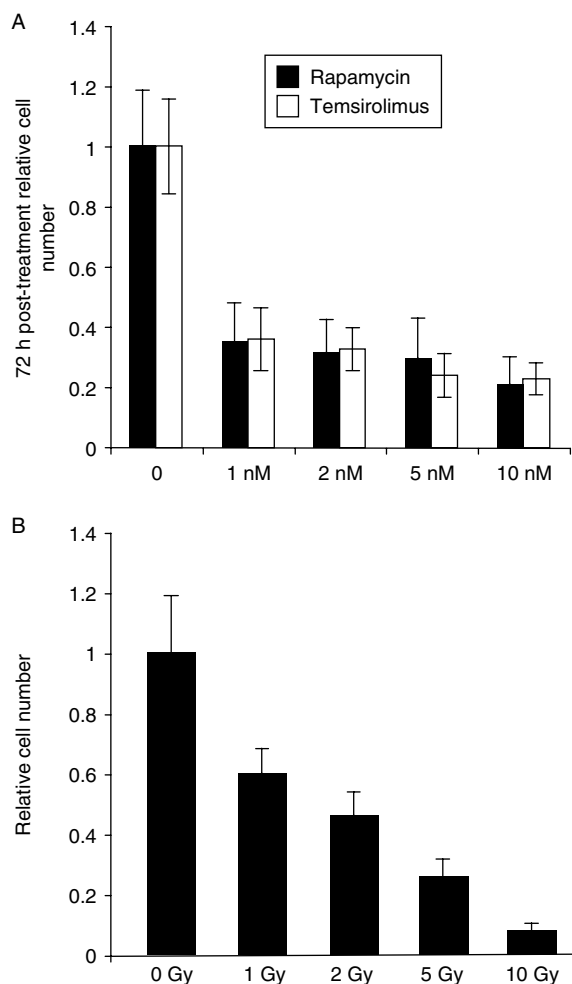


Figure 1 mTOR inhibitors and ionizing radiation (IR) are sufficient to limit hormone therapy-sensitive prostate cancer (PCa) cell doubling as single agents (A). LNCaP cells were treated with indicated doses of rapamycin, temsirolimus, or vehicle control. 72 h after treatment, cell number was assessed via Trypan Blue exclusion using a hemacytometer. Cell number in the vehicle controls was set to '1'. Averages of three independent experiments and s.d. are shown. (B) LNCaP cells were exposed to the indicated doses of IR. 168 h after treatment, cell number was assessed via Trypan Blue exclusion using a hemacytometer. Cell number in un-irradiated controls was set to '1'. Averages of three independent experiments and s.d. are shown.

Taken together, these data demonstrate that single-agent mTOR inhibitors and IR affect HT-sensitive cells. Additionally, there was no observable difference in the efficacy of Rapa and Tem in this context.

Combining mTOR inhibition and IR is more effective than single agent in limiting HT-sensitive PCa cell number

While IR is a frequently used treatment modality for locally advanced disease, there is a 10–60% recurrence

rate (Allen *et al.* 2007), suggesting that means to improve the efficacy of RT is a significant clinical need. Based on this premise, and the observation that mTOR signaling is both involved in PCa cell cycle progression/survival (Gao *et al.* 2003, Xu *et al.* 2006b) and induced by IR (Tirado *et al.* 2003, Shen *et al.* 2007), the impact of mTOR inhibition on the response to IR was determined in HT-sensitive cells. To determine whether scheduling of the treatment affected outcome, a strategy was used to test concurrent (Schedule I), neo-adjuvant (Schedule II), and adjuvant (Schedule III) mTOR inhibitor administration. The time from final treatment to assessment of outcome was identical for all schedules tested. Cells were sensitized to IR when mTOR inhibition was co-administered (Fig. 2A; compare IR, Rapa, and Tem alone to Rapa + IR and Tem + IR). To assess impact on the neo-adjuvant context, mTOR inhibitors were administered 48 h prior to IR treatment; as shown in Fig. 2B, there was a significant decrease in cell number following this treatment schedule (compare single agents vs combination). Finally, adjuvant mTOR inhibition conferred radiosensitization effects (Fig. 2C). Notably, Schedule III was most effective in limiting cell doubling (~15% of control) when compared with Schedule I (~20%) or Schedule II (~23%), suggesting that scheduling of treatment should be considered in therapeutic design. The impact of schedule was likely attributed to relative effects on cell cycle progression and was conserved in another HT-sensitive PCa cell model (LAPC4; Supplementary Figure 1, see section on supplementary data given at the end of this article). The LAPC4 model maintains wild-type PTEN (Whang *et al.* 1998) and harbors a mutant p53 allele (van Bokhoven *et al.* 2003), suggesting that neither PTEN or p53 status alters the radiosensitization effect of mTOR inhibitors. Although the contribution of PTEN status to mTOR inhibitor sensitivity has been documented, data herein demonstrate that both PTEN-proficient and PTEN-deficient cells can be radiosensitized by mTOR inhibition. Of note, the PTEN-proficient cell line LAPC4 may be intrinsically more radioresistant compared to the other model systems used. This is not without precedent, as it has been demonstrated that this cell line is relatively insensitive to chemotherapy (Xu *et al.* 2006a, Qian *et al.* 2010). However, mTOR inhibition still renders this cell type more sensitive to radiation. Taken together, these data demonstrate that combining mTOR inhibitors with IR is effective in limiting PCa cell number over time regardless of scheduling; however, adjuvant use of mTOR inhibitors may be most efficacious.

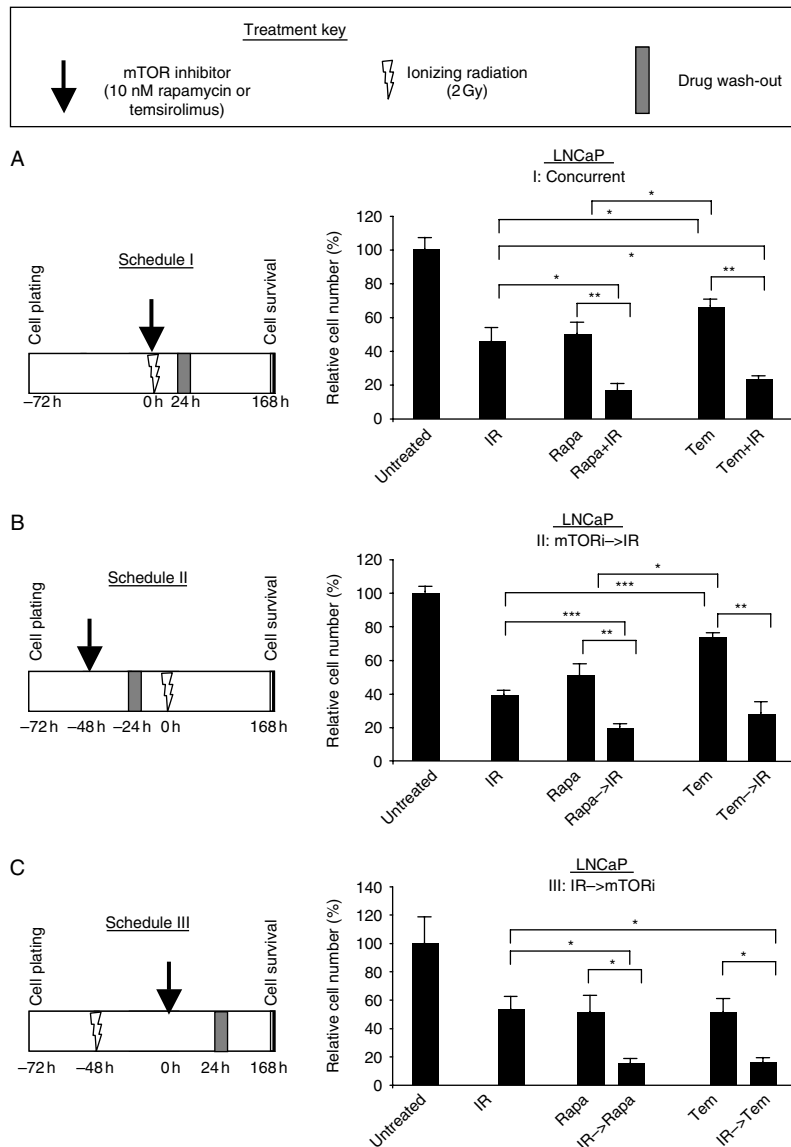


Figure 2 Schedule-specific radiosensitization of PCa cells by mTOR inhibition (A) Left panel: schematic of Schedule I treatment strategy (concurrent administration). As depicted, cells were seeded 72 h prior to final treatment, mTOR inhibitors, IR, or combination thereof were administered concurrently (Schedule I; set as time '0'), drug was washed out 24 h later, and cell number was assessed 168 h after treatment. Right panel: LNCaP cells were treated with 10 nM rapamycin (Rapa), 10 nM temsirolimus (Tem), 2 Gy IR (IR), combination of rapamycin and IR (Rapa + IR), combination of temsirolimus and IR (Tem + IR), or vehicle control (untreated). Cell survival in the untreated control was set to 100%; averages of three independent experiments and s.d. are shown. (B) Left panel: schematic of Schedule II (mTOR inhibitors as neoadjuvant). As depicted, cells were seeded 72 h prior to final treatment, administered 10 nM of either mTOR inhibitor, which was washed out of culture media 24 h later, then 24 h after wash, which at time 0 was exposure to 2 Gy IR, and cell number was assessed 168 h post-IR. Right panel: same as in (A), but with neoadjuvant mTOR inhibitor administration. (C) Left panel: schematic of Schedule III (mTOR inhibitors as an adjuvant post-IR). Cells were seeded 72 h prior to final treatment, administered 2 Gy IR, and then treated 48 h prior to final treatment (time 0), in this case it was either 10 nM rapamycin or temsirolimus. Right panel: same as in (A) and (B), but with adjuvant mTOR inhibitor administration. Statistical analysis of the indicated averages was performed using Student's *t*-test where **P*<0.05, ***P*<0.01, and ****P*<0.001.

mTOR inhibition radiosensitizes CRPC cells

In addition to being used as a therapy for localized disease, IR is also used for local recurrence and metastases, when the cells have frequently become resistant to HT (CRPC cells). In the presence of androgens, mTOR inhibition sensitizes CRPC cells to IR (Fig. 3A). While there was less dependence on scheduling in this cellular context, Schedule III (adjuvant) remained the most effective in limiting cell doubling. To assess whether mTOR inhibition sensitizes CRPC cells to IR in a castrate environment, parallel studies were performed in steroid-depleted conditions. mTOR inhibitors retained the capacity to radiosensitize CRPC cells in the castrate condition (Fig. 3B), albeit to a lesser extent than observed in the presence of androgens

(compared to Fig. 3A). Regardless, Schedule III remained the most effective, which suggests that there is a potential cell cycle component involved in the efficacy of the combination treatment. Since mTOR inhibitors alone can suppress AR-dependent cyclin D1 accumulation and cell cycle progression, it was hypothesized that these cytostatic effects underlie the scheduling effects of mTOR inhibitors.

Relative cell cycle inhibition in combination treatment is inversely correlated to efficacy of inhibiting population doubling

To examine the relative cell cycle distribution of cells in each of the treatment schedules prior to irradiation, the amount of DNA in cell populations was determined

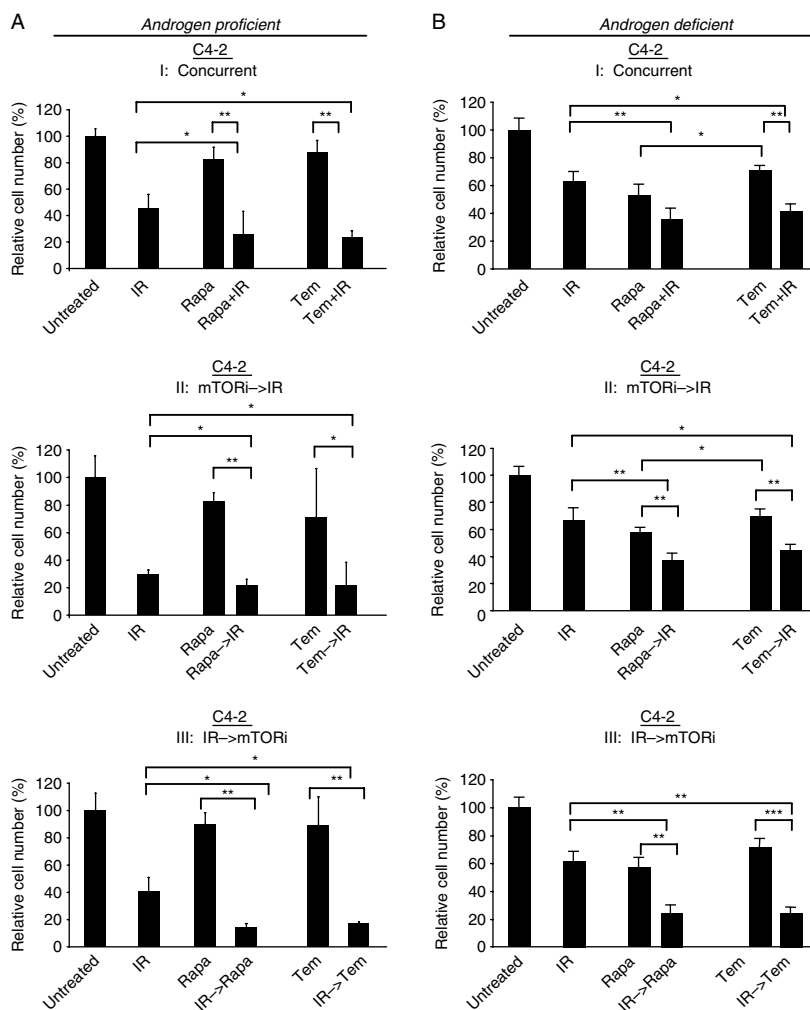


Figure 3 mTOR inhibitors sensitize CRPC cells to the effects of irradiation (A) C4-2 cells were treated as in Fig. 2. Top: mTOR inhibitor administration concurrent with IR (Schedule I). Middle: mTOR inhibitors administered as neoadjuvant to IR (Schedule II). Bottom: mTOR inhibitors administered as adjuvant to IR (Schedule III). (B) C4-2 cells were cultured in steroid-depleted media and treated as in (A). Cell survival in the untreated control is set to 100%, averages of three independent experiments and s.d. are shown. Statistical analysis of the indicated averages was performed using Student's *t*-test where **P*<0.05, ***P*<0.01, and ****P*<0.001.

by flow cytometry. There was a significant increase in G_1 enrichment in both the concurrent and the neoadjuvant schedules compared with the adjuvant, with a concomitant decrease in G_2/M enrichment (Fig. 4A). This observed alteration in cell cycle distribution resulted in increased cells in a relatively radioresistant portion of the cell cycle (G_1 ; Yau *et al.* 1980) and a decrease in the number of cell in a relatively more radiosensitive portion of the cell cycle (G_2/M ; Sinclair & Morton 1966) when mTOR inhibition was administered either concurrently or as a neoadjuvant. This same observation held true for CRPC cells as shown in Supplementary Figure 2A, see section on supplementary data given at the end of this article. In order to test the hypothesis that administration of mTOR inhibitors prior to the DNA-damaging insult of IR resulted in cytostatic effects that limited progression of cells to the radiosensitive cell cycle window, the relative change in S-phase progression was assessed for all treatment in the three schedules. There was significant inhibition of BrdU incorporation in both single-treatment mTOR inhibitor and IR in all

schedules tested (Fig. 4A). The observed inhibition of S-phase progression was enhanced by combining mTOR inhibition and IR, but to a lesser extent in Schedule III (Fig. 4B). Representative PI/BrdU traces are provided in Supplementary Figure 2B, see section on supplementary data given at the end of this article. When these data were compared to the relative impact on cell number in Fig. 2 (Schedule III being the most effective in limiting population doubling), there was an apparent inverse correlation between relative cell cycle inhibition and inhibition of cell number. Therefore, while combinatorial treatment in Schedule III was least effective in limiting BrdU incorporation, it was this regimen that proved most effective in limiting cell number over time. To formally assess the impact of inhibition of cell cycle progression on relative treatment efficacy, cells were arrested with the DNA polymerase inhibitor aphidicolin (or not) prior to combination of mTOR inhibitors and IR (Schedule III). When the cell cycle was inhibited prior to administration of the Schedule III regimen, there was a significant alteration of the efficacy of combination

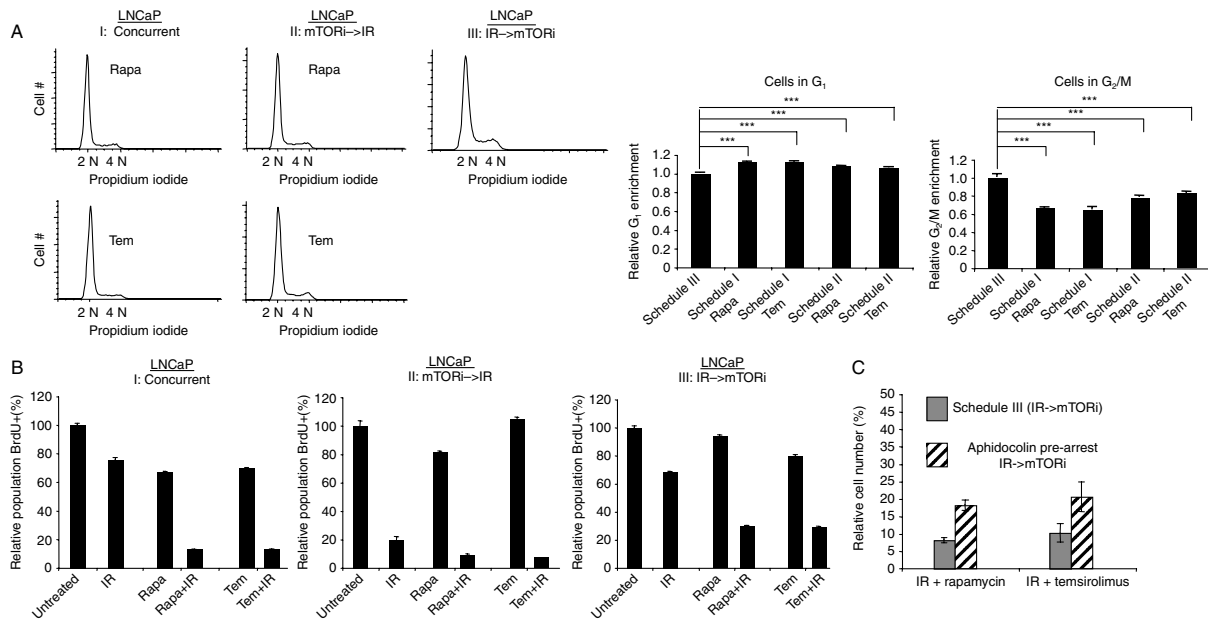


Figure 4 mTOR inhibitor-induced radiosensitization is a function of relative cell cycle inhibitory effect based on scheduling (A) Left panels: representative flow cytometry traces for each treatment Schedule that LNCaP cells were subjected to as depicted in Fig. 2 and harvested just prior to when IR would have been administered, fixed, and prepped for FACS analysis of DNA content as described in the Materials and methods section. Right panels: quantitation of the above. Data represented as relative G_1 (left) and G_2/M (right) enrichment averages and s.d. of at least three independent experiments. Schedule III (adjuvant) is set to 1. (B) LNCaP cells were treated according to the schemata depicted in Fig. 2, then 24 h post treatment, cells were harvested, fixed, and prepped for FACS analysis of BrdU incorporation and DNA content as described in the Materials and methods section. The data shown depicts the averages and s.d. of at least three independent experiments analyzing the percent of the cell population that is BrdU positive compared to untreated control, which is set to 100%. (C) LNCaP cells were either pre-arrested with vehicle control (gray bars) or aphidicolin (hashed bars) and then subjected to the mTOR inhibitors administered as an adjuvant to IR (Schedule III). Cell number was assessed 168 h after the last treatment by Trypan Blue exclusion and hemacytometer. Graph represents averages and s.d. of at least three independent experiments, with survival of untreated cells set to 100%. *** $P < 0.001$.

treatment (Fig. 4C). Taken together, these data demonstrate that limiting cell cycle progression, either in the context of the scheduling or with another compound, prior to IR is less effective than using mTOR inhibitors in an adjuvant context. These data suggest that the anti-proliferative effect of mTOR inhibition prior to treatment is likely a detriment to therapeutic outcome, as the effects of IR may be greater in cells that are actively cycling, while the anti-survival effect of these compounds after IR may be of therapeutic benefit.

mTOR inhibition combined with IR hinders clonogenic PCa cell survival

To determine whether the observed mTOR inhibitor-mediated radiosensitization translated in long-term assays to significantly decrease in cell survival/clonogenicity, the Schedule III regimen was used in a clonogenic cell survival assay. Both Rapa and Tem when used in combination with IR significantly decrease clonogenic cell survival in HT-sensitive (Fig. 5A) and castration-resistant (Fig. 5B) cell models. These results, in a system that is a validated predictor of therapeutic response, indicate that adjuvant administration of mTOR inhibitors decreases PCa cell survival and replicative capacity.

Discussion

This study identifies mTOR inhibition as a therapeutic approach that, when combined with IR, suppresses cancer cell growth. While both IR and the two mTOR inhibitors tested (Rapa and Tem) showed single-agent efficacy in limiting PCa cell doubling at clinically relevant doses (Fig. 1), the data presented in this study provide evidence that when the combination of IR and mTOR inhibition is used, there is an additive effect in limiting both HT-sensitive PCa and CRPC cell doubling (Fig. 2). This cooperative effect was observed to be dependent on the scheduling of the treatment in that treatment of PCa cells with mTOR inhibitor(s) after IR treatment (adjuvant, Schedule III) resulted in the most additive effect as determined by both cell number and BrdU incorporation (Figs 2 and 4). Further, this observation was supported by the finding that arresting the cell cycle prior to administration of the most effective schedule reduced the efficacy of this treatment regimen (Fig. 4B). Finally, using clonogenic cell survival modeling, which is a predictor of *in vivo* efficacy (Wilson *et al.* 1984, Hirabayashi *et al.* 1987, Yung 1989), it was observed that adjuvant administration of either of the mTOR inhibitors tested resulted

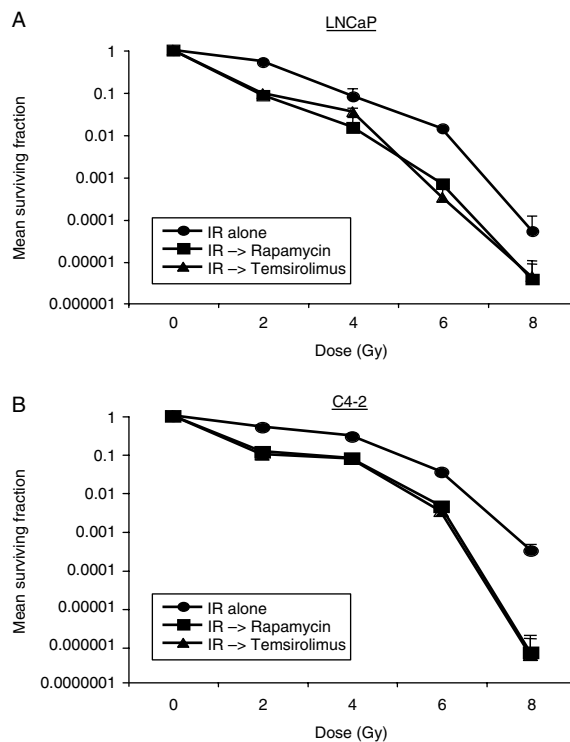


Figure 5 Clonogenic cell survival of both hormone therapy-sensitive and CRPC cells is reduced with mTOR inhibition following IR (A) LNCaP cells were serially diluted to appropriate concentrations and seeded in 50 ml tissue culture flasks. 24 h later, cells were exposed to indicated doses of IR. After 24 h, cells were treated with 10 nM rapamycin, 10 nM temsirolimus, or vehicle control. Cells were incubated for 14 days, fixed, stained with crystal violet, and colonies were counted. Colonies containing > 50 cells were considered in the analysis. The data are represented on a semi-log scale, where the x-axis represents the dose of IR and the y-axis represents the mean surviving fraction and s.d. (B) C4-2 cells were seeded, treated, processed, counted, and analyzed as in (a).

in decreased replicative capacity of both HT-sensitive PCa and CRPC cells (Fig. 5). Only the schedule that proved to be most effective with respect to radiosensitization was utilized in the clonogenic assay. This was to ensure that any observed effects on sensitivity were not due to relative baseline plating efficiency to prior mTOR inhibitor administration. Together, these studies demonstrate that mTOR inhibition can radiosensitize PCa cells, and scheduling of the treatment alters the ultimate outcome as determined by both monitoring population doubling and clonogenic cell survival.

Despite the approved use of mTOR inhibitors for the treatment of renal clear cell carcinoma (RCC), there are few data regarding the impact of mTOR as a therapeutic target in PCa. However, a recent pharmacodynamic study (Armstrong *et al.* 2010) demonstrated that an mTOR inhibitor (Rapa) could be administered to men with localized PCa, attaining high intra-prostatic

levels of the compound with minimal adverse effects and effectively limiting mTOR signaling as determined by S6 kinase phosphorylation, which is a downstream effector of mTOR activity involved in protein translation. While there was little significant biological effect in these tumors with regard to cellular outcomes (as determined by the assessment of proliferative and apoptotic indices), this may have been a result of the short course of treatment (14 days). Nonetheless, these clinical data demonstrate the feasibility of targeting mTOR in PCa cells, thus revealing a potentially fruitful platform for combination therapy. There are currently a number of clinical trials at various stages, some using mTOR inhibitors as single agents and others in combination with agents such as docetaxel or AR-directed strategies (as reviewed in [Morgan et al. \(2009\)](#)); however, none of these trials are investigating the combinatorial use of IR and mTOR inhibition in PCa. While this study indicates some modest impact of mTOR inhibitors as single agents, the most significant anti-tumor activity was observed in combination with IR. Therefore, the data presented herein demonstrating the radiosensitization of both HT-sensitive and CRPC cells in the clonogenic cell survival assay emphasize the importance of considering treatment schedule and provide the basis for clinical investigations. These data present a substantive advance, as there are no clinical agents currently approved, which confer sensitization to RT aside from androgen deprivation therapy (ADT). Of note, in CRPC cells cultured in conditions mimicking ADT, mTOR inhibition served as a radiosensitizer as well.

A critical finding herein was that the efficacy of mTOR inhibitors as a means to radiosensitize was significantly influenced by treatment schedule in both HT-sensitive PCa and CRPC cells. The evidence shown suggests that the observed schedule dependence can be attributed to the impact of mTOR inhibitors on cell cycle progression. The G₁ arrest induced by mTOR inhibitors prior to IR protected against radiation-induced cellular outcomes, whereas mTOR inhibitors in the adjuvant setting resulted in a more robust decrease in cell doubling. Interestingly, the effect of mTOR inhibitors was not influenced by PTEN status, as both PTEN-proficient (LAPC4) and PTEN-deficient (LNCaP) cells exhibited similar response to schedule-dependent combination therapy. Moreover, the impact of mTOR inhibition on radiosensitization was independent of p53 status as, in contrast to LNCaP cells, the LAPC4 model system lacks functional p53 ([van Bokhoven et al. 2003](#)). Schedule-dependent sensitization to DNA damaging therapies by mTOR inhibition is not without precedent.

It has been demonstrated that co-treatment of doxorubicin with an mTOR inhibitor was synergistic in T-cell lymphoma *in vitro* ([Huang et al. 2010](#)), as was adjuvant administration of mTOR inhibitor, compared to neo-adjuvant mTOR inhibition with these agents, which resulted in no synergistic effect on cellular outcomes. Additionally, it has been demonstrated that Tem administered to HT-sensitive PCa cells after docetaxel was more effective in limiting clonogenic cell survival, compared with concomitant treatment ([Fung et al. 2009](#)). These collective observations underscore the importance of assessing the impact of sequencing when combining mTOR inhibitors with genotoxic agents, especially with regard to the relative impact of these agents to alter cell cycle inhibition. As demonstrated herein, administration of mTOR inhibitors prior to radiation results in larger proportions of the cell populations being in relatively radioresistant portions of the cell cycle (G₁) and fewer cells in radiosensitive portions (S and G₂/M).

As demonstrated herein, clinically relevant doses of both Rapa and Tem exhibit single-agent cytostatic and cytotoxic effects in PCa cells and conferred schedule-dependent radiosensitization. The underlying mechanism(s) by which adjuvant administration of mTOR inhibition sensitizes cells to RT is the focus of ongoing investigation. Recently, it was demonstrated that mTOR is directly involved in the repair of DNA damage with respect to double-strand breaks, which occur frequently in cells exposed to IR ([Chen et al. 2010](#)), and these effects could therefore contribute to the radiosensitization observed in this study. Consonantly, it has been demonstrated that mTOR inhibition confers radiosensitization phenotypes in multiple tumor types ([Ekshyyan et al. 2009](#), [Nagata et al. 2010](#), [Saunders et al. 2010](#)), and that mTOR inhibition radiosensitizes soft tissue sarcoma and tumor vasculature ([Murphy et al. 2009](#)), which could have a similar impact on the response to RT. mTOR inhibitors also show cooperative effects with RT-independent DNA damaging agents, including doxorubicin (in T-cell lymphoma ([Huang et al. 2010](#))), 5-fluorouracil and/or docetaxel (in gastric cancer ([Matsuzaki et al. 2009](#))), carboplatin and paclitaxel (in head and neck cancer ([Aissat et al. 2008](#))), and cisplatin (in hepatocellular carcinoma ([Aissat et al. 2008](#))). In PCa cells, limited evidence suggests that mTOR inhibition can confer sensitization to doxorubicin ([Grunwald et al. 2002](#)), and combining mTOR inhibitors with docetaxel has been shown to be effective in limiting PCa cell growth *in vitro* and *in vivo* in a schedule-dependent manner ([Fung et al. 2009](#)). While mTOR inhibitors have been shown to

cooperate with DNA damage in AR-negative PCa cells both *in vitro* and *in vivo* (Wu et al. 2005, Cao et al. 2006), the relevance of these models to the majority of human tumors, which retain AR, remains uncertain. One study has demonstrated that mTOR inhibition and docetaxel administration is an effective combination in an intra-tibial AR-positive model of PCa (Morgan et al. 2008), while the other has shown that combining mTOR inhibition and AR antagonistic therapy results in PCa cell apoptosis and delayed progression to castration resistance (Schayowitz et al. 2010). As such, mTOR inhibitors appear to harbor the capacity to improve responses to RT and selected DNA damage-inducing therapeutics, as well as AR-directed strategies.

In summary, the studies presented herein demonstrate that mTOR inhibitors exhibit schedule-dependent effects on the RT response in PCa cells and confer significant radiosensitization effects when used in the adjuvant setting. Remarkably, the effects of mTOR inhibition as a means to achieve radiosensitization was conserved in both the HT-sensitive PCa and the CRPC settings, thus indicating that mTOR inhibitors may be an effective means to improve response to DNA damage-inducing therapeutic regimens in advanced disease. Combining these data herein provide the foundation for clinical investigation and illuminate new means by which PCa treatment may be improved.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-11-0072>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M J S, M A A, Y R L, A P D, and K E K conceived and designed the experiments. M J S, R D, D T H, and M A A performed the experiments. M J S, R D, D T H, Y R L, A P D, and K E K analyzed the data. K E K contributed reagents or analysis tools. M J S and K E K wrote the paper.

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