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m-TOR inhibitors: What role in liver transplantation?

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The development of calcineurin inhibitors (CNIs) led to marked improvements in patient and graft survival after liver transplantation (LTx). We have been left, however, with a dependence on immunosuppressive agents with nephrotoxicity, neurotoxicity, adverse impacts on cardiac risk profile, and risk for malignancy. These challenges need to be met against a dominance of hepatitis C virus (HCV) and hepatocellular carcinoma (HCC) as indications for liver transplant. Unmet needs for immunosuppression (IS) in LTx include:

(1) Effective drugs that avoid CNIs toxicities.
(2) Agents without adverse impact on HCV recurrence.
(3) Compounds that minimize risk of HCC recurrence.

New immunosuppressives will need to address the above needs while supporting patient and graft survival equivalent to those achievable with CNIs, ideally without important new toxicities. Two new classes of agents are currently in advanced clinical development: belatacept, and the mammalian target of rapamycin inhibitors (m-TORi). This manuscript will review evidence for a role for m-TORi in LTx in a range of clinical scenarios including patients with CNI nephrotoxicity or neurotoxicity, patients at risk of (or with) HCV recurrence, and patients at risk of HCC recurrence.

Introduction

Liver transplantation (LTx) has become a standard therapy for end stage liver disease, including that due to hepatitis C cirrhosis and non-resectable hepatocellular carcinoma (HCC). The outcomes of LTx have improved with advances in surgical procedures and immunosuppressive drugs, especially calcineurin inhibitors (CNIs). However, chronic kidney disease (CKD) caused by CNIs, recurrence of hepatitis C in the transplanted liver, and recurrence of HCC remain major problems after LTx. Renal insufficiency in LTx is associated with progression to end stage renal disease and a decrease in patient and graft survival [1–3]. CNIs have been associated with a dose-dependent increase in the post-transplant risk of HCC recurrence [4]. Minimizing the nephrotoxicity and exploring for anti-tumor effect of immunosuppressive regimens may help to reduce the number of patients developing CKD and HCC recurrence after LTx.

Sirolimus (SRL) and everolimus (EVRL) inhibit mammalian target of rapamycin (m-TOR). The m-TOR is an evolutionarily conserved PI3-kinase family member that plays a key role in integrating different biochemical and growth factor signals, including amino acids, glucose, ATP, and insulin [5]. m-TOR inhibitors (m-TORi) continue to be explored as immunosuppressive drugs in allogeneic transplantation and as novel anticancer agents. In this review article, we will discuss the impact of m-TORi in LTx, with specific reference to the important areas of kidney function, hepatitis C recurrence, and HCC recurrence, and thereby explore the rationale for selective use of m-TORi in liver transplantation.

The first report on use of m-TORi in LTx achieved a modest 67% 1-year survival in 15 patients, 8 of whom had HCC [6]. Early reports by McAlister, Chang, and Trotter supported the potential for SRL-based immunosuppression to achieve outcomes equal to CNI-based protocols [7–9]. After a report of the second international multicenter trial of SRL in LTx, delivered at the American Transplant Congress in 2002, documenting an increase in rate of graft loss and death and a trend to an increase in HAT in the SRL/Tac arm [10], the Food and Drug Administration (FDA) issued a "black box" warning on use of SRL in LTx. As appropriate under the "black box", subsequent studies with SRL have focused on areas and patients where potential adverse impacts from SRL were felt to be outweighed by likely benefit. These include patients with post-transplant nephrotoxicity or neurotoxicity, and patients with hepatocellular carcinoma (HCC), where the anti-tumor impact of m-TORi may prove beneficial. Review of outcomes with m-TORi in these areas of liver transplantation will form the core of this review.

Mammalian target of rapamycin (m-TOR) inhibitors

Rapamycin, also known in clinical usage as SRL, was isolated from a soil sample obtained in Easter Island (Rapa Nui) and was

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Abbreviations: AFP, alpha fetoprotein; CNIs, calcineurin inhibitors; CKD, chronic kidney disease; CPM, central pontine myelinolysis; CsA, cycrosporine; EVRL, everolimus; FDA, Food and Drug Administration; GFR, glomerular filtration rate; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IS, immunosuppression; LTx, liver transplantation; TLR, Toll-like receptor; m-TORi, mammalian target of rapamycin inhibitors; Tregs, regulatory T cells; MMF, mycophenolate mofetil; SRL, sirolimus; Tac, tacrolims; VEGF, vascular endothelial growth factor.

identified as a potent antifungal metabolite. This macrolide, produced by *Streptomyces hygroscopicus*, inhibited cell proliferation and so produced antitumor and immunosuppressive activity [11]. In 1999, SRL was FDA approved for prevention of kidney allograft rejection [12]. Rapamycin and three analogs modified at C43 to increase solubility and bioavailability have undergone clinical evaluation. The addition of an ester, ether, or phosphonate group yield temsirolimus, EVRL, and deferolimus, respectively. SRL and EVRL are mainly used as immunosuppressive medications in transplantation and are the focus of this review (Fig. 1).

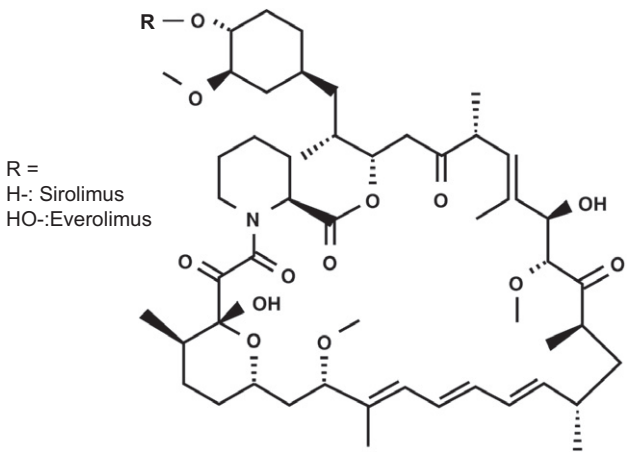


Fig. 1. Structures of sirolimus and everolimus.

The m-TOR signaling pathway

TOR was identified in yeast followed by the discovery of the m-TOR. m-TOR is a key signaling kinase that affects broad aspects of cellular functions, including metabolism, growth, survival, aging, synaptic plasticity, and memory. Rapamycin engages FK506-binding protein 1A, 12 kDa (FKBP12); the complex engages and inhibits TOR but not calcineurin, thereby blocking cell cycle progression at the G1 to S phase, causing inhibition of T cell proliferation [13].

As shown in Fig. 2, the m-TOR pathway is activated by a variety of different classes of stimulations. There are at least two distinct m-TOR complexes, m-TOR complex1 (m-TORC1) and m-TOR complex2 (m-TORC2), that have distinct relationships both to upstream and downstream effectors and to each other [14,15]. Signals from growth factors (insulin or IGF-1), various cytokines, co-stimulatory signals, Toll-like receptor (TLR) ligands, cellular energy levels, hypoxia, cellular stress and DNA damage determine m-TORC1 activity. These signals mediate their effects through the tuberous sclerosis complex 1 (TSC1)-TSC2 complex, which is the main negative regulator of m-TORC1. Activated m-TORC1 promotes mRNA translation by stimulating S6 kinase (S6K1) and inhibiting EIF4EBP1 (eukaryotic translation initiation factor-binding protein 1). m-TORC2 is not inhibited directly by rapamycin, although long-term rapamycin administration disrupts its assembly in some cells. m-TORC2 regulates actin cytoskeletal dynamics through the small GTPase RAS homologue (RHO) and protein kinase C (PKC).

The m-TOR in immunity and mechanism of immunosuppression

m-TORi: impact on innate immunity

In addition to the regulating effects of m-TOR in dividing cells, it has been recently demonstrated that m-TOR affects the innate immunity system [16]. Inhibition of m-TOR promotes pro-inflammatory cytokines such as IL-12 and IL-1β, inhibits the anti-inflammatory cytokines such as IL-10, and boosts MHC antigen presentation via autophagy in monocytes/macrophages and dendritic cells. Moreover, m-TOR regulates type1 interferon production and the expression of chemokine receptors and co-stimulatory molecules [17]. m-TORi blocks progression from G1 to S phase in natural killer (NK) cells but does not affect interferon-γ production in primary NK cell lines; cytotoxicity assays showed modestly decreased NK cell activity against the YAC-1 cell line [17]. *In vivo* study in a rat to hamster skin xenograft model did not show significant effects [17,18].

m-TORi: impact on adaptive immunity

Inhibition of innate immunity by m-TORi affects adaptive immunity via co-stimulatory molecules and cytokine production. m-TORi also marked thymic involution, which is associated with decreased T cell output [17]. By blocking cell cycle progression from G1 into S phase in IL-2 stimulated T cell lymphocytes [18]; rapamycin potently decreases the proliferation of $CD4^+$ T cells, although it does not alter the proportion of $CD4^+$ single positive T cells that upregulate their expression of forkhead box P3 (FOXP3) in the thymus [16,17]. Furthermore, m-TOR-deficient $CD4^+$ T cells efficiently differentiate into $FOXP3^+$ regulatory T cells (Tregs) upon stimulation, compared to wild-type. Differentiation into T-helper (Th) 1, Th2, or Th17 cells was severely inhibited in m-TOR-deficient $CD4^+$ T cells even in the presence of appropriate polarizing cytokines [19]. m-TORi may be permissive to induction of Tregs in organ transplantation [19], another potential mechanism for immune suppression. Detail of T cell mechanisms regulated by m-TOR have been reviewed [21].

Pharmacology of m-TORi

The half-life of SRL and EVRL is approximately 60 h and 30–40 h, respectively, and EVRL has a more rapid time to steady state (4 days versus 6 days for SRL) [20,21]. Both compounds are cleared through the liver via the hepatic cytochrome P450-3A4 microsomal system, which is the same metabolic pathway used by cyclosporine and tacrolimus (Tac). Drugs which inhibit or compete with the activity of cytochrome P450 system may significantly impair the clearance of both SRL and EVRL and lead to significant increase in systemic levels. Common drugs that may cause clinically significant elevations in blood concentrations through inhibition of metabolism include fluconazole, azithromycin, and protease inhibitors.

Clinical experience with m-TORi: adverse events and risks

A rationale for selective substitution of m-TORi for CNIs in LTx depends on evidence for effective immune suppression and a

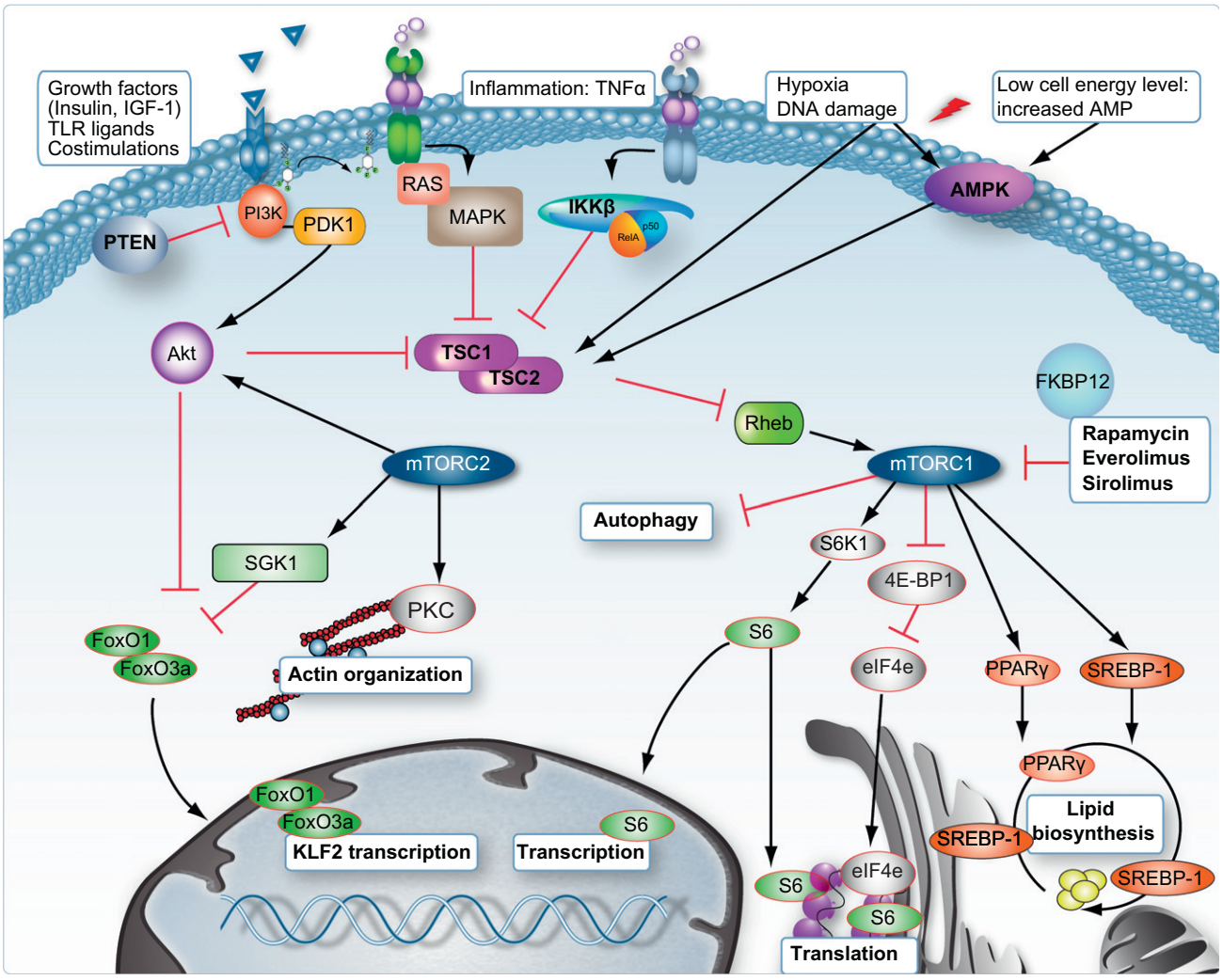


Fig. 2. An overview of the m-TOR signaling pathway and relative activities.

favorable balance in toxicities. After the black box warning of 2002, the FDA, in 2009, notified health care professionals of preliminary data suggesting increased mortality in stable liver transplant patients after conversion from CNIs to SRL.

A retrospective review of SRL-associated adverse events in 175 patients demonstrated bilateral leg edema (57.1%), dyslipidemia (44%), dermatitis (25.3%), oral ulcers (24.2%), joint pain (23%), pleural effusion (16.5%), increased abdominal girth (5.5%), general edema (5.5%), pericardial effusion (5.5%), facial edema (2.2%), and upper extremity edema (1.3%) [22]. Asrani published a systematic review and meta-analysis of SRL in LTx patients with renal insufficiency [23]. The relative risk ratios of death (RR = 1.12), graft failure (RR = 0.8), and rejection (RR = 0.88) were not significantly increased at 1 year after SRL initiation. Proteinuria and poor wound healing were similar in both groups. SRL was associated with a trend to a higher risk ratio (not statistically significant) of renal replacement therapy (RR = 1.71) and need for statin therapy (RR = 2.93) as well as a significantly higher risk of infection (RR = 2.47), rash, edema (RR = 2.49), and oromucosal ulcers (RR = 7.44). Discontinuation due to intolerance was significant in the SRL arm (33–55%, RR = 3.61). Neff reported SRL-related, biopsy confirmed hepatotoxicity requiring discontinuation in 2 out of 10 LTx patients switched for renal insufficiency (n = 6) or chronic rejection [24]. SRL-related acute hepatitis was also reported in a renal transplant recipient [25]. Similar adverse events have been observed with EVRL *vs.* cyclosporine A (CsA) monotherapy. A 78 patient study demonstrated higher cholesterol and a trend to more incisional hernias [26], while a 145 patient study demonstrated higher rash (6.9%), cholesterol (13.9%) and mouth ulceration (26.4%) [27].

A multicenter retrospective analysis in 240 LTx patients demonstrated similar adverse effects with EVRL [28]. Mean white blood count decreased significantly and total cholesterol and triglyceride levels increased significantly. The principal adverse event was dyslipidemia, which was controlled by reducing the dose and adding a statin in LTx with SRL or EVRL [29].

Adverse effects of m-TORi are frequent and may be important. This m-TORi toxicity profile supports CNI as the agents of first choice post liver transplantation. While successful clinical application requires experience and careful observation, m-TORi may be an acceptable immunosuppressive alternative in LTx patients

intolerant of CNIs, or in clinical scenarios with the potential for specific benefits from m-TORi.

Potential clinical advantages of m-TORi: nephrotoxicity

CNIs have been implicated as a principal cause of post-transplant renal dysfunction. Non-renal solid-organ transplant recipients have a shorter lifespan than the general population, and their survival is further compromised when chronic kidney disease (CKD) develops [30]. Ojo *et al.* reported that the relative risk of death after development of CKD in non-renal organ recipients was 4.55 [2]. While present even before the need for dialysis, it was highest for recipients on dialysis [31–33]. CNIs induce reversible vasoconstriction of afferent and efferent glomerular arterioles. Renal hemodynamic studies in CsA-treated patients have revealed decreased glomerular filtration rate (GFR), elevated mean arterial pressure, increasing renal vascular resistance, and increased albumin excretion. With chronic use, these perturbations result in progressive arteriolopathy and glomerular ischemic collapse. Hyperfiltration injury occurs in remaining nephrons, sometimes leading to end stage renal disease [34]. Renal biopsy studies among nonrenal organ transplant recipients with CKD showed interstitial fibrosis with a "striped" appearance, nodular arteriolar hyalinosis, and later, tubular atrophy with glomerulosclerosis and arteriosclerosis [2,35–37]. m-TORi may have a role in minimizing post-transplant CKD; a critical look at m-TORi impact on renal function is required. The published impact of m-TORi on renal function has been variable, including proteinuria with *de novo* SRL [38], while less data is available about EVRL [39]. Results from animal experiments [40,41] and clinical studies [42–45] suggest that pre-existing chronic nephropathy or renal impairment might increase the risk of adverse effects from SRL and that inhibition of vascular endothelial growth factor (VEGF) is important for these effects. To gain a better focus on the relative renal toxicity risks from CNI and m-TORi, we next review renal function outcomes in clinical trials with m-TORi in LTx.

Clinical trials of m-TORi in LTx patients with renal insufficiency

De novo clinical studies of m-TORi in LTx

A placebo-controlled trial randomized 119 patients to EVRL at 0 mg/day, 1 mg/day, 2 mg/day, or 4 mg/day, all with prednisone and oral CsA (trough 150–400 ng/ml) [46]. Creatinine and creatinine clearance remained stable to 36 months, while cholesterol and triglyceride increased in all groups. No differences were observed in creatinine clearance and acute rejection, thrombocytopenia or leucopenia, although patients taking 2 mg/day or 4 mg/day EVRL had the lowest numerical rate of rejection. This study demonstrated that EVRL in combination with CsA in *de novo* liver transplant recipients had an acceptable safety and tolerability profile and was associated with a lower rejection rate (not statistically significant) in comparison with CsA combined with placebo. No evidence for benefit to renal function was seen. Animal studies demonstrated a risk of increased nephrotoxicity with CsA/m-TORi combination therapy [47,48], consistent with the results from renal transplantation with combination of CsA and SRL [49]. Therefore, it is important to investigate use of *de novo* m-TORi without CNIs in liver transplantation. The course of renal function in *de novo* liver transplantation recipients with EVRL therapy has been evaluated in an open-label, randomized multicenter phase III study (Clinical Trials.gov Identifier NCT00378014), although results are not yet available (http://clinicaltrials.gov/show/NCT00378014).

Clinical studies of CNI conversion to m-TORi in LTx

An important question is whether CNIs can be stopped and patients switched to m-TORi to avoid CNI-related CKD. Morard switched 48 patients to SRL a median of 19.4 months after LTx for reasons of renal impairment (78%), CNI-neurotoxicity (13%), or post-transplant cancer (9%) [50]. Nineteen percent presented severe (cGFR 20–40 ml/min) and 45% moderate (40–70 ml/min) renal impairment at switch. Mean cGFR improved from 33 to 48 ml/min in patients with severe and from 56 to 74 ml/min in patients with moderate renal impairment. Patients with a cGFR >70 ml/min did not benefit. Acute rejection occurred in 8 patients (17%) with a mean delay of 4 months; 5 out of 8 patients improved after increasing SRL trough levels to 10–15 μg/L. The authors concluded that conversion from CNI to SRL is safe and is associated with significant renal function (cGFR) improvement, but warned that SRL may worsen nephropathy (some developed severe albuminuria >500 mg/L) if patients have severe hypertension and pre-existing albuminuria. Preconversion albuminuria (>30 mg/L) and high SRL trough levels (>9.5 μg/L) were significant risk factors for SRL treatment withdrawal. Recently, a multicenter study of 240 patients converted to EVRL a mean of 4.9 years after liver transplant was e-published [51]. In the complete cohort, estimated GFR improved from 64.2 ml/min at day 0 to 68.4 ml/min at month 12 after LTx; no control group (CNIs alone) was included in this study. Patients with baseline serum creatinine >130 μmol/L had an improvement in eGFR from 44.3 ± 15.7 ml/min to 53.7 ± 26.0 ml/min after 12 months ($p = 0.003$). Over 60% of patients were maintained free of CNIs at 12 months with a rate of biopsy proven acute rejection of only 1.6% (4 patients) and an acceptable safety profile.

Several groups have reported that significant improvement of renal function was observed in patients with shorter times between LTx and conversion to m-TORi from CNI [52,53]. Two small prospective randomized, single center trials demonstrated that CNI withdrawal was associated with a significant improvement in creatinine clearance at 3 months, but that this improvement was no longer statistically significant at 12 months [54,55]. Watson showed improvement in creatinine clearance at 3 months on SRL (75 ml/min) compared to control (56 ml/min) ($p = 0.012$), with a trend at 12 months (72 ml/min *vs.* 58 ml/min; $p = 0.09$). In a recent multicenter randomized EVRL conversion study, 145 LTx patients at mean >3 years post-transplant with CNI reduction (38%) or discontinuation (62%) showed stable (but not improved) renal function at 6 and 12 months (Fig. 3) with no difference in graft loss or acute rejection [27], again suggesting that conversion several years after transplant may be too late to show benefit.

A prospective trial of early (4 week) conversion in 40 LTx recipients demonstrated that 57% of subjects improved creatinine clearance by conversion from CNIs to EVRL-monotherapy [56]. A second early (30 day) conversion study that was a 12-month, prospective, randomized, single center, open-label, parallel-group

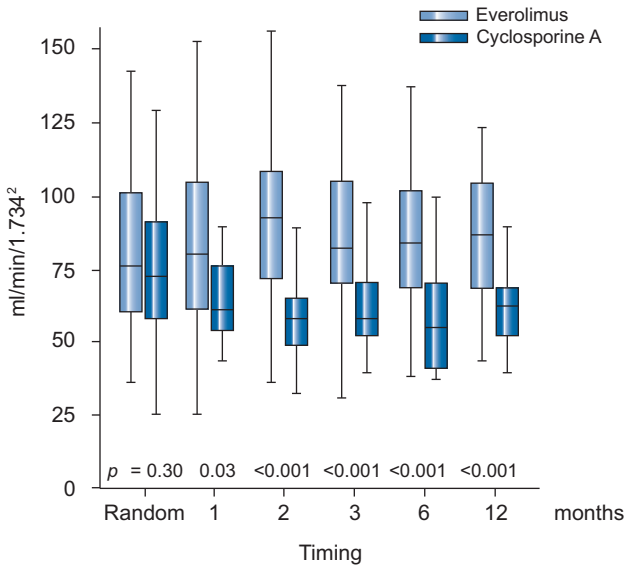
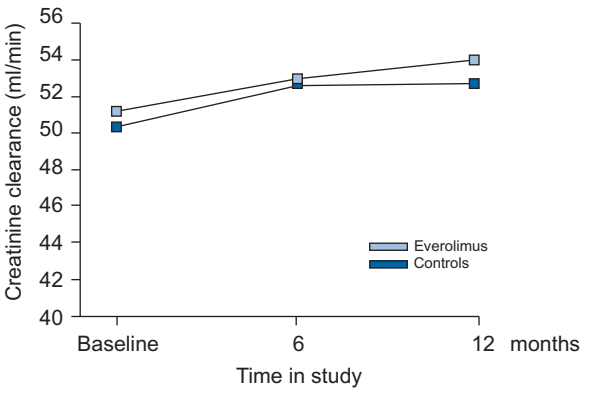


Fig. 3. Glomerular filtration rate (GFR) either with everolimus or cyclosporine A after liver transplantation. Significantly improved GFR was observed in the everolimus group from 1 month after liver transplantation with early withdrawal of cyclosporine A. [28].



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Everolimus	71	65	60
Controls	71	71	68

Fig. 4. Creatinine clearance with everolimus or cyclosporine A after liver transplantation. Stable, but not improved renal function was observed after conversion from a calcineurin inhibitor to everolimus a relatively long mean time post-liver transplant (>3 years) [29].

study demonstrated the effect of early withdrawal of CNI and EVRL monotherapy in LTx in 78 patients (EVRL n = 52, CsA n = 26) [26]. All patients were treated with CsA for the first 10 days, then received EVRL in combination with CsA up to day 30, then were either continued on EVRL-monotherapy or maintained on CsA with/without mycophenolate mofetil (MMF) in case of chronic kidney disease. Significant improvement of GFR was observed at 1, 2, 3, 6, and 12 months after conversion (Fig. 4). Early post transplant CNI-free immunosuppression with EVRL-monotherapy in *de novo* LTx recipients was found to be associated with a significant improvement in renal function, with similar incidences of rejection and major complications. Results from a recently published large SRL conversion study in renal transplantation revealed similar outcomes. Of 830 renal allograft recipients 6–120 months post-transplant, 555 were converted to SRL while 275 were maintained on CNI (Tac or CsA). While no significant difference was seen in patients with GFR <40 ml/min at baseline, on-therapy analysis in the 743 patients within the GFR >40 ml/min stratum revealed significantly higher GFR at 12 and 24 months in the SRL conversion group (66.2 *vs.* 60.1 ml/min, $p = 0.004$ and 63.8 *vs.* 59.0 ml/min, $p = 0.049$) [57]. While the literature is not homogeneous, the balance of results supports a modest benefit in renal function post-LTx when CNIs are switched to m-TORi early and with use of moderate SRL/EVRL trough levels. The data suggest less benefit in patients with severe renal dysfunction or even of adverse impact post conversion in patients with preconversion albuminuria (>30 mg/L).

m-TORi in CNI-induced neurotoxicity after LTx

Neurotoxicity is a relatively common and potentially serious adverse effect of CNIs [58] that occurs in about 25% of patients after LTx. Both CsA and Tac may produce a spectrum of neurological impact that varies from relatively mild tremor and acute confusional state to headache, seizures, status epilepticus, and major speech or language abnormalities that at its most severe may be associated with central pontine myelinolysis (CPM) [59–62]. Neurotoxicity correlates poorly with trough levels. The impact of concomitant electrolyte abnormalities may be important, including the association of hypomagnesemia with seizures and rapid rises in serum sodium with CPM. Appropriate response may vary from dose reduction or switching from Tac to CsA for mild toxicity, to immediate cessation of any CNI for life threatening neurotoxicity. Switch from Tac to CsA has been associated with rates of acute rejection up to 30% [63]. A 202 patient study demonstrated that SRL use was not associated with neurotoxicity in kidney or liver recipients [64], nor in heart transplantation [65]; suggesting that a switch from CNIs to m-TORi is another option for CNI related neurotoxicity. Vivarelli *et al.* reported 3 LTx patients that were switched to SRL because of Tac-related neurotoxicity with improvement of a severe speech disorder in one case and encephalopathy in the other two [66]. A conversion study also demonstrated that 6 LTx patients (13%) were switched from CNIs to SRL due to neurotoxicity; symptoms such as headache and tremor completely disappeared after the switch [50], chronic partial epileptic crisis did not relapse. Another study reported on 3 LTx patients (7.5% of trial total) that were switched from CNIs to EVRL with recovery from peripheral neuropathy in 2 but persistence of headache in the third [56].

Switching from CNIs to m-TORi appears a safe and effective strategy for consideration in patients who develop CNI-related neurotoxicity.

The impact of sirolimus on the clinical course of recurrent hepatitis C (HCV) after LTx

Graft and patient survival after LTx have improved for all indications except HCV-related cirrhosis, where they continue to be 10–15% lower than non-HCV controls [67]. HCV re-infection usually occurs immediately after LTx with a rapid increase in HCV-RNA peaking at 1–3 months; acute lobular hepatitis developing in 60–80% of patients at a median of 4–6 months and

cirrhosis in 20% by 5 years [68,69]. Progression of HCV disease to cirrhosis is affected by several variables: notably high levels of pre-transplant viremia, donor variables (age, steatosis, ischemic/preservation injury), human immunodeficiency virus/cytomegalovirus co-infection, higher levels of immunosuppression (pulse steroids or T cell depleting antibody use – OKT3 or thymoglobulin), abrupt modifications of immune status, and post-LTx diabetes [69,70].

Optimization of immunosuppression is a key in managing recurrent HCV disease. Data regarding the effect of different immunosuppressive agents on HCV replication and/or progression of liver disease is controversial [71]. CNIs exert different *in vitro* and *in vivo* effects on HCV disease. While CsA has a clear *in vitro* antiviral effect in the subgenomic HCV replicon system [72–74], there is little evidence that clinical disease progression, measured by RNA levels or the necroinflammatory score, is different between patients on CsA *vs.* Tac [72,75,76].

Laboratory evidence suggests that m-TORi may affect HCV disease progression by altering the rate of HCV replication or by influencing the necroinflammatory effects of infection on the allograft. SRL has been reported to have potent anti-fibrotic properties in rat models of cirrhosis with significant inhibitory effects on procollagen-alpha 1 and transforming growth factor-beta 1. Decreased activity and fibrosis progression have been reported in patients with recurrent HCV disease [77].

Laboratory evidence for the role of m-TORi on HCV progression is mixed. Cell culture experiments using an Huh7 replicon cell line demonstrate that infected cells are resistant to apoptosis, and stimulation of the pro-survival PI3K-Akt pathway and m-TOR by HCV not only protects cells against apoptosis but may also contribute to the maintenance of steady-state levels of HCV replication [78]. Biochemically, the NS5A protein has been found to enhance HCV virus replication through p70S6K phosphopeptide [79]. SRL inhibits the m-TOR/p70S6K pathway and may reduce *in vivo* phosphorylation of NS5A phosphopeptides and therefore viral replication. This effect, however, may be dose dependent. Studies of non genotype-1 virus in cell culture which used higher doses of rapamycin (100 nM) have shown that m-TORi may increase the production of HCV core protein by inhibiting, the suppressor of cytokine signalling 3 (SOCS3) [80,81].

Clear clinical data on the effect of SRL on HCV recurrence remains scarce. In contrast to early, unpublished reports that SRL may worsen biochemical outcomes in recurrent HCV [82], Wagner *et al.* reported lower viral RNA levels, slower fibrosis progression, and higher survival in the 39 SRL treated patients from a group of 67 patients with post-LTx HCV recurrence [83]. We found the use of SRL in an HCV-positive cohort did not affect timing of recurrence, but markedly slowed the progression of the disease on serial biopsy [84]. Similar findings have been reported in other series. Mckenna *et al.* reported a decrease in RNA levels as well as reversal of established fibrosis on serial protocol liver biopsies in HCV-positive LTx recipients after SRL conversion [85]. Preliminary data from our own center suggests that SRL based immunosuppression is associated with improved response rates to anti-HCV treatment after LTx [86].

The published evidence is mixed in respect to the impact of m-TORi on HCV. The central role of m-TOR in a host of cellular signalling pathways has resulted in suggesting potential beneficial and adverse impacts when different intracellular signaling cascades have been examined. Intriguingly, some of the variation in the literature appears to support dose-dependent variable effects of SRL on HCV proliferation. Clinical evidence is developing for a beneficial role of m-TORi on slowing the progression of HCV disease after recurrence and possibly in enhancing SVR rates. At minimum, we note little evidence for adverse impact of m-TORi therapy on HCV disease post-LTx.

m-TOR inhibitors in LTx for hepatocellular carcinoma (HCC)

Immunosuppression after LTx for HCC must achieve safe control of the immune response. Can it, in parallel, achieve a reduction in tumor recurrence (improved tumor-free survival), and so improved overall survival? We will explore these two questions sequentially in the next section. Control of alloimmunity in LTx does not mandate an extremely low rate of rejection, but requires a manageable rate and severity of acute rejection, with an absence of graft loss due to rejection whether acute or chronic.

We reported a sequential series of 40 patients with HCC (21 beyond Milan criteria) transplanted between 1996 and 2003 on SRL based therapy post-transplant [87]. Eligibility criteria included single tumors to 7.5 cm, and multiple tumors (no limit by number) up to 5 cm. At mean 44 months follow-up, 5 tumors (12.5%) had recurred, with 81% of within Milan and 77% of extended criteria patients alive and free of HCC recurrence. We updated our experience to a sequential series of 70 consecutive HCC patients transplanted to 2007 with 49 months median follow-up [88]. Eight tumors had recurred, 2 out of 34 (6%) within Milan criteria, and 6 out of 36 (17%) in the extended criteria group. Four-year tumor free survivals were 73% (Milan) and 75% (beyond Milan). Acute rejection was seen in 52% of patients with this protocol of aggressive reduction of immunosuppression targeting SRL-monotherapy, but no grafts were lost to acute or chronic rejection. No patient experienced hepatic artery thrombosis. Infection related mortality was 3%. SRL was continued in 88% of patients at 1 year and in 80% at 4 years, while 70% of patients were on the protocol planned SRL-monotherapy at 4 years. These papers support the ability of SRL based immunosuppressive protocols to safely control the alloimmune response and to achieve outcomes equal to CNI based regimens, while achieving survival outcomes acceptable to most centers with patients whose tumor burden are both within and significantly beyond the Milan criteria. While encouraging, such uncontrolled series cannot establish an effective anti-tumor impact of m-TORi on HCC. To date, no adequately powered randomized clinical trials of m-TORi immunosuppression in HCC have been reported. Nevertheless, a wealth of evidence is available from *in vitro* experiments, *in vivo* animal studies, and clinical experience to support further prospective randomized trials of replacement of standard CNI/steroid immunosuppression with m-TORi in patients with HCC. These bodies of published evidence on the impact of both CNI and m-TORi on HCC will now be reviewed.

Hojo *et al.* reported CsA inducing an invasive phenotype in adenocarcinoma cells *in vitro* and leading to increased tumor growth and metastasis by a TGF-beta associated mechanism independent of recipient immune cells [89].

Following liver transplant in rats with HCC, Freise documented that CsA treatment was associated with survival reduction from 47% to 18% at 100 days in parallel with increased numbers of metastases [90].

Early clinical reports of adverse impact of CNI immunosuppression on HCC included a dramatic reduction in tumor doubling

time from 274 to 44 days when comparing HCC recurrences after liver resection to those after liver transplant with CsA [91], and higher recurrence rates in patients maintained on steroid long term [92]. Additional evidence of an adverse impact of CNI on HCC is provided by Vivarelli's report of 70 HCC patients on CsA based immunosuppression with a 10% recurrence rate. Multivariate analysis revealed the sole independent predictor of recurrence was blood CsA level ($p = 0.001$) [93]. *In vitro* reports of SRL impact on tumor cell lines included Schumacher's paper where SRL inhibited while Tac stimulated *in vitro* growth of hepatoma cell lines [94]. Guba *et al.* demonstrated that SRL inhibited tumor growth by an anti-angiogenic mechanism that involved blockage of VEGF signalling to endothelial cells [95]. A subsequent report by Guba demonstrated that SRL induced extensive local microthrombosis of mouse tumor tissue vasculature via tissue factor in the presence of VEGF, without such an impact on adjacent non-tumor tissues [96]. Intravital microscopy of tumors implanted in a dorsal skinfold chamber showed dramatic decrease in tumor vascularity after 3 days treatment with rapamycin at 1.5 mg/kg/day. Tumor specific thrombosis was documented in both orthotopically implanted pancreatic tumors and heterotopically implanted colon carcinomas (mimicking primary and metastatic tumors, respectively). The central role of m-TOR as a regulator of nutrient uptake (impact on amino acid and glucose transporters), cell growth and proliferation, and angiogenesis has been reviewed [15,97,98,101].

Does the beneficial impact of m-TORi on cancer in the lab translate to the clinic?

Clinical research supporting a beneficial impact of m-TORi in malignancy includes studies reporting skin cancers, total malignancies and HCC outcomes in renal and liver transplant populations. Stallone *et al.* documented complete remission of cutaneous Kaposi's sarcoma in 15 out of 15 renal transplant recipients after 3 months therapy with SRL (and CNI discontinuation) [99]. Kaufmann *et al.* reported use of m-TORi was associated with reduction in overall rate of new onset post-transplant malignancy (NOPTM) [100]. In a review of 33, 249 patients from the Scientific Registry of Transplant Recipients (SRTR), 30,424 CNI treated patients had an incidence of NOPTM of 1.81% within 963 days of transplant, and a 1.00% incidence of *de novo* non-skin solid malignancies. Of 504 SRL treated patients (in the absence of CNI), 3 (0.60%) developed NOPTM and none developed *de novo* non-skin solid malignancies. Patients treated with SRL in combination with CNI had an intermediate risk (0.47% *de novo* non-skin solid malignancies and 0.60% NOPTM). Similar outcomes with reduced rates of skin cancers as well as total malignancies were reported by Schena *et al.* in a series of 830 renal allograft recipients 6–120 months post-transplant who were randomized to continue CNI (n = 275), or switch to SRL (n = 555). At 24 months, total malignancies were 21 (3.8%) in the SRL conversion group and 30 (11.0%) in the CNI continuation group ($p < 0.001$) while skin cancers totalled 12 (2.2%) *vs.* 21 (7.7%), respectively ($p < 0.001$) [57]. Salgo *et al.* recently reported a prospective, single center trial of 44 recipients a mean of 229.5 months after kidney transplant who had developed skin lesions and were randomized to a switch to SRL/prednisone or continuation of baseline immunosuppression (predominantly azathioprine/prednisone) [101]. The stop of progression or regression of premalignant lesions was significantly superior in the SRL group at 6 months ($p < 0.0005$) with an increased impact at 12 months ($p < 0.0001$) Assessment by the single treatment-blinded dermatologist (including skin biopsy as indicated) also reported only one non-melanoma skin cancer (NMSC) in the SRL treated patients within the next year and 8 NMSC in the control group ($p = 0.0176$). SRL-based immunosuppression was able to delay or induce regression of premalignant lesions and to decelerate the incidence of new NMSC in renal transplant recipients even when instituted many years after transplantation.

Several reports of association of m-TORi with improved outcomes in HCC patients in the clinic have also been published. Elsharkawi *et al.* reported 3 patients with pulmonary metastases from HCC who achieved complete remission for 18 months after SRL was substituted for CNI [102]. Zimmerman *et al.* reported 97 patients transplanted for HCC: 45 received SRL and low dose CNI while 52 were treated with CNI alone. SRL was administered to all tumor patients in the first 3 years of the series (2000–2002) and to those with creatinine levels above 1.5 mg/dl thereafter. Overall, 12 patients (12.4%) experienced HCC recurrence and there were 10 HCC related deaths. Post-transplant therapy with SRL and low dose CNI was associated with a significantly lower rate of HCC recurrence (6.7% *vs.* 17.3%) and an increased rate of recurrence free survival (79% *vs.* 54%) than standard CNI treatment [103]. While all 3 SRL recurrences occurred in stage 3 cases, 3 out of 9 recurrences in the CNI group occurred in patients with early stage disease. Zhou *et al.* [104] reported data on 73 HCC patients from Shanghai. In patients beyond Milan criteria, 27 patients on SRL therapy achieved higher overall 2 year survival than 46 on Tac (80% *vs.* 59%, $p = 0.001$). Both studies were uncontrolled, retrospective, single center analyses. Vivarelli *et al.* reported 2 groups of 31 patients matched for year of transplant, tumor histology, and alpha fetoprotein (AFP). SRL was administered to patients who demonstrated unfavorable prognostic factors for tumor recurrence or had renal or neurological dysfunction. Despite the unfavorable tumor and survival characteristics, those patients on SRL and low dose Tac (mean 4.6 ng/ml) achieved a >20% survival advantage at 2 years (85% *vs.* 64%, $p = 0.0001$) over patients on Tac only (mean level 8.5 ng/ml) [105].

Chinnakotla *et al.* [106] reported a case-control study of 227 patients transplanted for HCC (1995–2006) and within Milan criteria on imaging. SRL was the preferred immunosuppression for HCC patients subsequent to 2000. Analysis revealed no differences between SRL treated and Tac/MMF treated patient groups for tumor size, number of nodules, proportion within Milan criteria, vascular invasion, tumor grade, Metroticket-estimated 5-year survival (for those beyond Milan criteria), or tumor prognostic score. While 121 SRL treated patients had a 5-year Metroticket predicted score of 52.9%, and 106 Tac/MMF patients had a predicted 52.3% survival, Kaplan–Meier actual survivals for 5 years were 80% and 59%, respectively ($p = 0.0001$). Only 4 SRL treated patients (3.3%) died due to tumor recurrence, while 19 (17.9%) Tac/MMF treated patients succumbed for this reason. While the preference for SRL in the group transplanted after 2000 could bias survival toward this cohort, most (14.6%) of the 21% survival advantage was directly due to a lower incidence of death due to HCC recurrence, not an era sensitive factor.

We recently reported analysis of 2491 patients from the SRTR transplanted for HCC (2002–2009) and continued on stable immunosuppression for a minimum of 6 months after hospital

discharge [107]. Given UNOS guidelines on adherence to Milan criteria in the USA, the cohorts for analysis on differing immunosuppressive agents were remarkably homogenous with only 0.2% beyond a total tumor volume of 115 cm^3, and only 6% with serum AFP above 400 ng/ml, two factors we have reported to predict tumor recurrence in the SRTR dataset. Univariate analysis of immunosuppressive treatment inclusive of on *vs.* off Tac, CsA, SRL, MMF, steroids, anti-CD25 antibody induction therapy, or thymoglobulin induction therapy revealed a survival advantage of 6.4% at 3 years and 14.4% at 5 years for 109 patients treated with ongoing SRL for >6 months (p <0.05) when compared to 2382 patients on non-SRL immunosuppression. Anti-CD25 antibody induction therapy had a smaller (6% at 5 years) but significant survival advantage as well.

In order to clarify if the benefits were specific to HCC patients, we next analyzed 12,167 non HCC SRTR patients from the same time span. While the requirement for a minimum 6 months of stable immunosuppression excluded 25,201 of a total 39,859 patients from the analysis, we believe this qualification for inclusion in the analysis strengthens the validity of the interpretation. In the non-tumor cohort, anti-CD25 antibody therapy was not associated with any difference in outcomes, while SRL treated patients demonstrated a slight trend to poorer outcomes (3.3% at 5 years, $p = 0.14$). On multivariate Cox analysis (corrected for MELD, year of transplant, primary non-HCC liver disease, age at transplant, and when applicable for total tumor volume, AFP, and pre-transplant HCC treatment) independent predictors of survival in HCC patients were: SRL therapy (hazard ratio 0.53, 95% confidence intervals 0.31–0.92, p <0.05), induction therapy with anti-CD 25 antibody (HR 0.64, CI 0.45–0.90, p <0.01), and in non-HCC patients: CsA therapy (HR 1.3, 95% CI 1.0–1.7, p <0.05). The benefit of SRL therapy in the HCC patient group was not seen in the non-tumor patients, strongly supporting a specific benefit of SRL for patients with HCC. Of interest, Tac therapy was associated with a 6.1% survival advantage at 5 years in the non-tumor patients ($p \leqslant 0.001$), but demonstrated no advantage in the HCC patient group.

Conclusions

CNIs have been documented to have adverse impact on cancer in *in vitro* and *in vivo* animal studies and to have dose related adverse impact on HCC recurrence and survival in clinical series. m-TORi have demonstrated multiple mechanisms of anti-tumor activity *in vitro* including angiogenesis inhibition associated with reduced VEGF, proliferation inhibition associated with reduced TGF beta, and tumor specific microvascular thrombosis associated with increased tissue factor production. SRL has been associated with a reduction in HCC recurrence and improved overall and tumor free survival in several single center retrospective reviews of HCC patients undergoing LTx. An SRTR database review has demonstrated a 14.4% survival advantage at 5 years for HCC patients receiving SRL based immunosuppression. While to date, no reports of adequately powered and controlled prospective studies have been published or presented, we believe the outcomes reviewed above justify expanded exploration of m-TORi in HCC patients undergoing LTx and are strong impetus for further active study in this area. Outcomes of an ongoing international multicenter trial of SRL *vs.* non-SRL containing immunosuppressive protocols in patients with HCC (the SiLVER study) are anticipated [108].

An ongoing phase III study of EVRL in LTx will also analyze impact in the subset of patients with HCC and so will add additional valuable insights to this important question – do m-TORi benefit patients undergoing liver transplantation for HCC?

How might m-TORi help meet unmet needs in immunosuppression for liver transplantation?

m-TORi are capable of replacing CNIs in patients with nephrotoxicity, with potential for significant improvement in renal function. Results appear to be best with early conversion; benefits may be limited when patients have advanced renal dysfunction; in patients with significant proteinuria, aggravation of renal dysfunction may occur. m-TORi appear as an acceptable immunosuppressive alternative when neurotoxicity is a significant post-transplant challenge.

There is as yet no clear message on the impact of m-TORi in HCV infected liver transplant patients. *In vitro* and clinical data reporting impact on replication is mixed. Preliminary data supports slower progression of disease (especially fibrosis) and includes a suggestion of improved SVR rates.

There exists a substantial body of *in vitro* data and *in vivo* animal data on beneficial impact of m-TORi (and adverse impact of CNIs) on rates of cancer development and metastasis, inclusive of detailed mechanistic studies. *In vitro* and animal data with HCC models is limited. A growing body of clinical evidence supports SRL based immunosuppression and/or reduction of CNI to be associated with decreased rates of new cancer development and suggests benefit for patients transplanted for HCC. Prospective randomized clinical trials addressing m-TORi immunosuppression in liver transplantation for HCC are ongoing.

Conflict of interest

N.K. has received financial support from Wyeth (Pfizer) for unrelated research involving Sirolimus.

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