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Review Article

Inhibitors of mammalian target of rapamycin

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CONTENTS

Summary 1179
Introduction 1179
Role of the mammalian target of rapamycin (mTOR) in T cell signaling 1179
Chemistry and mechanism of action of sirolimus and everolimus 1180
Preclinical animal studies 1182
Clinical studies 1182
Pharmacokinetics and pharmacodynamics 1182
Safety 1183
Efficacy 1184
Prospects 1185
References 1186

Summary

The first generation of immunosuppressants such as azathioprine, steroids and anti-lymphocyte-globulins did not provide long-term graft survival in many transplant recipients. After the introduction of calcineurin inhibitors, solid organ transplantation became a standard therapy for many end-stage organ failures and immunosuppressive therapy was later refined with the introduction of mycophenolate mofetil and monoclonal antibodies. Today, inhibitors of the mammalian target of rapamycin are a new class of immunosuppressants. In contrast to other macrolides, like tacrolimus and ciclosporin, they do not inhibit calcineurin and thus signal I of T cell activation. By inhibiting signal III, the mechanism of action and side effects of sirolimus and the derivative everolimus are distinct from other immunosuppressants. This review will summarize thus far published preclinical and clinical results and discuss the future value of sirolimus and everolimus for clinical transplantation.

Introduction

In 1969 sirolimus (Rapamune®; Wyeth-Ayerst Pharmaceuticals), also known as rapamycin or AY-022989, was isolated from a soil (*Streptomyces hygroscopicus)* collected at the Vai Atore region on Easter Island. Early investigations focused on its potent antifungal and antitumor activities (1). Later its antiproliferative activity was demonstrated in animal models of autoimmune diseases and adjuvant arthritis (2) and in 1988 its potent immunosuppressive activity was reported for the first time (3, 4). It took three more years to understand the mechanism of action of this macrolide and before this new immunosuppressant helped to unveil another pathway of T cell signaling (5) and enhanced our understanding of transplantation immunology. Today, sirolimus is FDA approved for maintenance immunosuppression in kidney allograft recipients and the derivative everolimus (Certican®, Novartis) is being investigated in phase III clinical trials.

Role of the mammalian target of rapamycin (mTOR) in T cell signaling

Resting the cells between the G_1 and the S phase of the cell cycle, sirolimus initiated numerous studies investigating steps of T cell activation and proliferation and led to the discovery of the TOR genes. Genetic screens for yeast mutations resistant against the antiproliferative effect of sirolimus uncovered two novel genes, designated TOR1 and TOR2 (6, 7). The analogue kinase in mammals (mTOR) (8), also called FRAP (FKBP12-rapamycin associated protein) (9), RAFT1 (rapamycin and FKBP12 target-1) (10, 11) or RAPT1 (rapamycin phosphatidylinositol 3-kinase) (12), shows similarities to the catalytic domains of phosphoinositide 3-kinases (PI_3-K) and sits at a branchpoint of the signal 3 pathway in activated T cells (7, 13). Once activated, TOR transduces signals which initiate synthesis of ribosomal proteins, translation of a specific subset of mRNA transcripts and generation of cyclin-dependent kinases promoting the progression of the cell cycle and resulting in T cell proliferation and activation, B cell proliferation, activation and antibody production and also proliferation of nonimmune cells like fibroblasts, endothelial cells, hepatocytes and smooth muscle cells (6-8, 10, 13-15).

After signal I (Ca^{2+}-dependent pathway) T cell activation, the nuclear factor of activated T cells (NF-AT) is in the presence of a valid signal II (Ca^{2+}-independent pathway) calcineurin-dependent dephosphorylated and following transition to the nucleus, it promotes cytokine and growth factor production (transcription). In an autocrine or paracrine manner, the growth factors (*e.g.*, IL-2, IL-4,

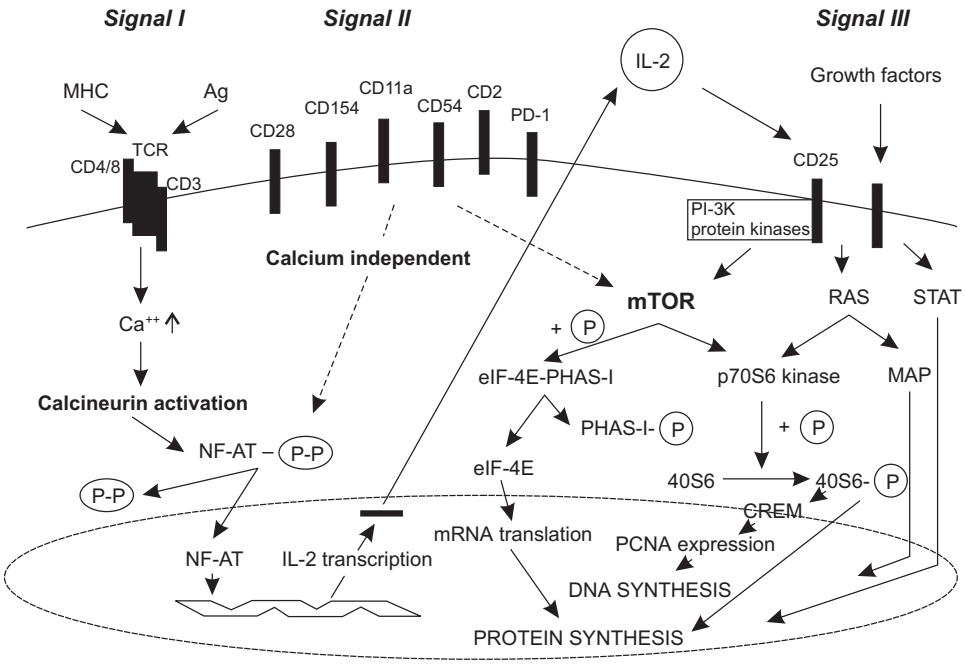

Fig. 1. T cell signaling pathways. After alloantigen activation of the T cell receptor in the presence of a valid signal II, calcineurin activation leads to NF-AT translocation to the nucleus and therby to gene activation (*e.g.*, IL-2). In an autocrine or paracrine manor Il-2 and growth factors bind to their specific receptors which leads to signal III transduction. mTOR is located at a branch point of the signal III pathway. By binding to mTOR sirolimus or everolimus inhibit the activation of $p70^{S6}$ kinase and phosphorylation of PHAS-I resulting in inhibition of cell cycle progression. (From Neuhaus, P., Klupp, J., Langrehr, J.M. *mTOR-Inhibitors, an overview.* Liver Transplantation 2001, 7: 473-484, reproduced with permission.)

IL-7, IL-12, IL-15, IFN-γ, platelet-derived growth factor [PDGF], basic fibroblastic growth factor [bFGF], endothelial cell growth factor [ECGF], transforming growth factor-β [TGFβ], insulin) bind to their specific receptors on the cell surface to deliver signal III. Resulting kinase cascades lead to TOR activation by autophosphorylation. Additionally, T cell activation through CD28 is sensitive to sirolimus, suggesting that TOR activation is also possible through signal II.

Thus far, TOR is known to have two main functions (Fig. 1), the activation of the $p70^{S6}$ kinase and the activation of the eIF-4E-PHAS-I pathway. No direct interaction between TOR and $p70^{S6}$ kinase can be detected in a cell-free system, proving that downstream to TOR and upstream to $p70^{S6}$ kinases and/or phosphatases are still unidentified (7, 9, 16). The only known substrate for $p70^{S6}$ kinase is S6, a 40S ribosomal protein which is phosphorylated on multiple sites after mitogen stimuli. It is believed that these modifications favor the recruitment of the 40S subunit into actively translating polysomes, resulting in protein synthesis and mRNA translation (7, 9, 17). Furthermore, $p70^{S6}$ kinase activates CREMπ, a member of the cAMP response element binding family of transcription factors increasing the expression of proliferating nuclear antigen (PCNA) and thereby promoting DNA replication (16). However, the signaling pathways involved in $p70^{S6}$ kinase activation are multiplex and also include TOR-independent signals downstream to PI_3-K, like the PI_3-K-dependent kinase 1 (PDK-1), which is a direct effector of $p70^{S6}$ kinase phosphorylation (18).

The second known downstream effect of TOR is the phosphorylation of PHAS-I (phosphorylatable heat stable protein I), which releases eIF-4E (eukaryotic initiation factor 4E) and results in the assembly of the eIF-4F complex at the 5'-cap site of the mRNA template and thereby in translation and protein synthesis. These findings indicate that mTOR acts as a terminal kinase in the signaling pathway that links growth factor receptor occupancy to an increase in eIF-4E-dependent protein synthesis in G_1 phase cells (7).

In addition to these TOR-dependent pathways, growth hormones and IL-2 trigger signal III pathways, which are not inhibited by sirolimus or everolimus. RAS and MAP kinase-dependent, stress-induced signals also contribute to the stimulation of eIF-4E function and $p70^{S6}$ kinase activation and deliver antiapoptotic signals from activated cytokine receptors.

More detailed reviews regarding growth factor-induced translation control are available (7, 9, 16, 19, 20).

Chemistry and mechanism of action of sirolimus and everolimus

The lipophilic macrolide sirolimus, a natural fermentation product of *S. hygroscopicus,* has the chemical

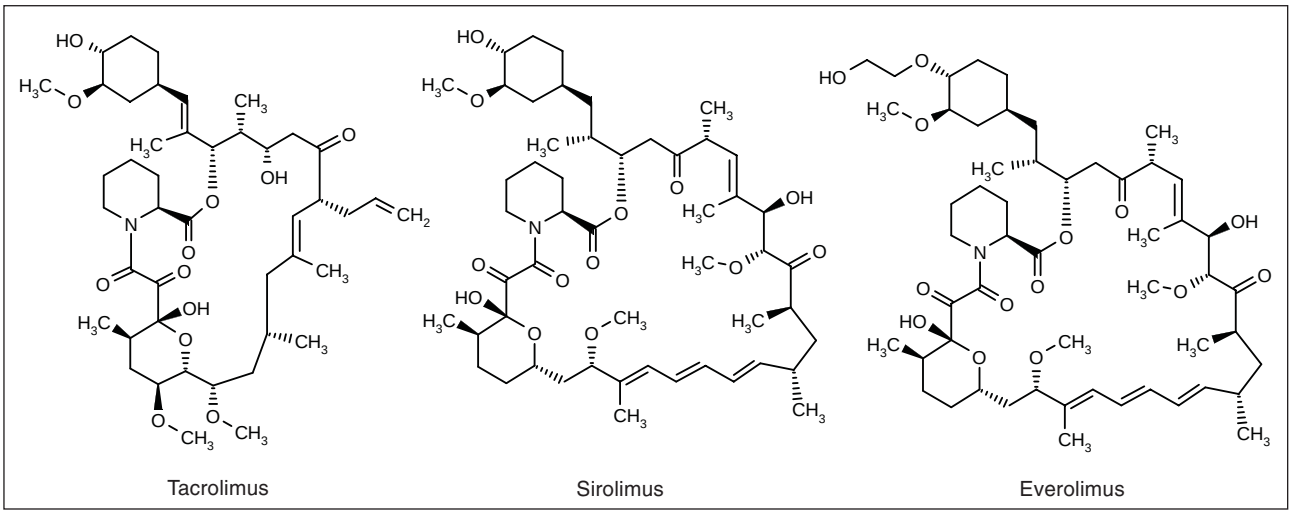

Fig. 2. Due to their structural similarities, tacrolimus, sirolimus and everolimus bind to the FK-binding proteins FKBP12 and FKBP12.6. Due to stereometric differences tacrolimus-FKBP-complexes are able to inhibit calcineurin, whereas sirolimus- and everolimus-FKBP-complexes bind and inhibit the target of rapamycin (mTOR).

formula $C_{51}H_{79}NO_{13}$ and a molecular weight of 914.2. The 40-*O*-(2-hydroxyethyl)-sirolimus derivative everolimus ($C_{53}H_{83}NO_{14}$; MW 958.25) was developed by chemical derivation of sirolimus to improve oral pharmacokinetics while maintaining the pharmacological benefits of sirolimus (21-23). Sirolimus, everolimus and tacrolimus (Fig. 2) bind to the same family of intracellular receptors, named FK506 binding proteins (FKBPs), and thus adding tacrolimus in a millimolar range to an *in vitro* proliferation assay antagonizes the effects of TOR inhibitors. Two isoforms, FKBP12 and FKBP12.6, have been shown to bind TOR in the presence of sirolimus. Sirolimus shows an affinity to FKBP12, which is twice as high as for tacrolimus and 3.3 times higher than everolimus (21, 24). In contrast to tacrolimus, which undergoes conformational change upon binding with the FKBP12, the three-dimensional structure of sirolimus-FKBP12 is almost identical to its conformation in the free crystalline state (13). While the tacrolimus-FKBP12 complex inhibits the Ca^{2+}-dependent serine-threonine phosphatase calcineurin and thereby the signal I pathway of T cell activation (G_0-G_1 transition, transcription, cytokine and growth factor gene activation), the sirolimus-FKBP12 complex binds to and inhibits TOR and thereby signal III (G_1-S transition, translation, cytokine driven T cell proliferation). Although not formally proven that everolimus-FKBP complex also binds to TOR, it is believed that everolimus acts in the same way as sirolimus, since the downstream effects on signal III inhibition (*e.g.*, $p70^{S6}$ kinase inhibition) are identical.

In vitro, immunosuppressive activity of everolimus is about 2-fold lower compared to sirolimus in a two-way mixed lymphocyte reaction (MLR) with mouse spleen cells (IC_{50}: 0.2-1.6 nM *vs.* 0.06-0.9 nM, $p < 0.05$) (21), but the *in vivo* activity of both compounds appears to be similar (14, 21, 25).

Derived from the described functions of TOR, sirolimus and everolimus block Ca^{2+}-independent T cell activation and proliferation after cytokine stimulation and costimulatory interaction. Indirectly, TOR inhibitors inhibit effector functions of $CD4^+$ helper cells, $CD8^+$ cytotoxic cells, activation of monocytes and macrophages, and of other proinflammatory leukocytes (13, 26, 27). B cell activation, proliferation and differentiation into antibody producing cells and antibody release are also inhibited by sirolimus (13, 16, 26) and everolimus (21). Besides the effects on immune cells, TOR inhibitors also block mesenchymal cell response to growth factors and smooth muscle cell proliferation and intimal thickening in experimental models, which is not affected by calcineurin inhibitors (13, 21, 28-31). Furthermore, sirolimus inhibits extracellular matrix deposition in rat models of fibrogenesis and decreases PDGF-induced proliferation of hepatic stellate cells (32).

Cytokine-induced apoptosis downregulates the immune response by clonal deletion of alloactivated cells and is believed to be an essential process for the induction of tolerance (33). Unlike calcineurin inhibitors, sirolimus preserves apoptosis in T cells, because apoptosis is independent of TOR activity (7). At doses of anti-CD3 of 0.5 μg/ml or lower, sirolimus dramatically promoted anti-CD3-induced apoptosis in a T cell hybridoma cell line (34). This rationale is the background for many studies evaluating the combined use of signal II inhibition and sirolimus to receive long-term allograft acceptance without maintenance immunosuppression (35-39).

In combination with ciclosporin, sirolimus showed a synergistic antiproliferative effect on phytohemagglutinin-stimulated T cells and an additive effect on T cell activation (40). Measuring ^{3}H-thymidine incorporation in a two-way mouse MLR, everolimus showed an IC_{70} of 0.3 nM alone compared to an IC_{70} of ciclosporin of 21 nM and an overadditive effect in combination (41).

Preclinical animal studies

Morris showed in 1989 that sirolimus-treated mice (6 mg/kg) heart allograft recipients had a significant longer survival time (14 ± 1.9 days) than untreated controls (10.6 ± 0.2 days) (3). Furthermore, allografts lived longer than in recipients treated with 12 mg/kg ciclosporin (14 ± 1.9 *vs*. 12 ± 1.5; $p = 0.01$). In rats (3 mg/kg) mean survival was prolonged to 24 ± 7.9 days *versus* 7 ± 0.35 days in untreated controls. Later these findings were confirmed (4) and further studies showed not only strong synergism with ciclosporin (42) in rat heart allograft recipients, but also – and in contrast to previous *in vitro* studies – with tacrolimus. In a model of reversal of ongoing acute rejection, two combinations of low doses of tacrolimus and sirolimus demonstrated significantly longer graft survival than each immunosuppressant alone ($p < 0.05$) (43).

In studies of rat kidney allograft recipients, similar results were seen when sirolimus was coadministered with ciclosporin (42). Sirolimus was also effective in preventing acute allograft rejection in pancreas and small bowel rat transplant models (44-47) and showed efficacy in ongoing heart, kidney and pancreas rejection in rats (48, 49). Later on, sirolimus showed efficacy in different large animal models (50-56) and in transplant models in nonhuman primates (57-59). Like sirolimus, everolimus also showed high efficacy in preventing and treating allograft rejections in rat heart (41), kidney (60) and lung transplant models (61-63) and in transplant models in nonhuman primates (64-66).

According to the *in vitro* effects on mesenchymal cells, sirolimus was able to prevent and suppress graft intimal thickening not only in small animal models (29, 30, 67) but also in a model in nonhuman primates. Sirolimus was able to halt already established (58, 59) and prevent (68) graft vascular disease after aorta transplantation between MLR mismatched cynomolgus monkeys. Also, everolimus was able to reduce vascular remodeling and intima thickening in response to cold ischemia in rats (69, 70) and reduced the pace of chronic renal allograft rejection (71). Both sirolimus (29) and everolimus (72) were able to block and reduce bronchial obliteration after lung transplantation in rats and pigs, respectively.

In contrast to calcineurin inhibitors, everolimus showed a significant inhibitory effect on different PTLD-like Epstein-Barr virus and lymphoblastoid B cell lines *in vitro* and marked inhibition of tumor growth was seen in mice (73). These results suggest that mTOR inhibitors may have a beneficial effect in patients with posttransplant lymphoproliferative diseases.

The effect of sirolimus on nephrologic side effects was compared with calcineurin inhibitors in animal studies. One study (74) compared renal blood flow and glomerular filtration rate in rats under treatment with sirolimus (3 mg/kg/day p.o.), ciclosporin (15 mg/kg/day s.c.) and tacrolimus (5 mg/kg/day p.o.). There was no difference compared to vehicle treatment, but significantly better renal function was observed with sirolimus compared to ciclosporin and tacrolimus. Another study (75) measured creatinine clearance in spontaneously hypertensive rats, which was not altered by continuous infusion of sirolimus (0.01 mg/kg) but was reduced by 20% after infusion of ciclosporin (5 mg/kg).

These preclinical studies proved that sirolimus and everolimus not only are highly effective in preventing and treating acute allograft rejections in different transplant models but also are promising agents for the treatment and prevention of chronic rejections. Due to the distinct mechanism of action, these drugs have a different spectrum of side effects, lack nephrotoxicity and show synergism not only with ciclosporin but also with tacrolimus *in vivo*.

Clinical studies

Pharmacokinetics and pharmacodynamics

Sirolimus is rapidly absorbed intestinally, with a median t_{max} after less than 1 h (76). The absorption is modulated by P-glycoproteins, which explains the drug-drug interactions discussed later in this review and the effect of high-fat meals (77, 78). The terminal half-life is 62 h in stable ciclosporin-treated renal transplant recipients and thus, steady state is usually reached within 7-14 days, enabling once-daily dosing (76). Sirolimus and everolimus are metabolized by liver and intestinal cytochrome P450 enzyme CYP3A4 (23, 77) and metabolites are eliminated by the gastrointestinal tract (91%) and by the kidneys (2%) (76). In contrast to ciclosporin ($r^2 = 0.19$), sirolimus trough concentrations are stable over time and strongly correlated ($r^2 = 0.96$) with 24-h exposure ($AUC_{0\text{-}24h}$) (79, 80). Furthermore, sirolimus' whole blood concentrations are linear and dose proportional for doses between 3 and 12 mg/m^2 (76). There is no significant gender, age or ethnic effect on C_{max} or AUC (77). However, ciclosporin microemulsion increased sirolimus bioavailability by 240% when administered simultaneously and only by 80% when administered 4 h apart (81, 82). Also coadministration of diltiazem (+60%), ketoconazole (+990%) and rifampicin (–82%) altered the AUC of sirolimus. Due to its increased bioavailability, everolimus shows a slightly higher AUC than sirolimus, but its half-life (30 h) is much shorter than that of sirolimus (21, 25). Furthermore, the correlation between AUC and dose was stronger for everolimus than for sirolimus (83). In the American phase III trial in 583 *de novo* renal transplant recipients, everolimus showed a dose proportional and stable exposure over the first 6 months after transplantation (84). The pharmacokinetic parameters of everolimus were less affected by ciclosporin than those of sirolimus. Thus it is expected that everolimus can be administered simultaneously with ciclosporin. However, in combination therapy, higher everolimus tissue concentrations were found when coadministered with ciclosporin after lung transplantation in cynomolgus monkeys (85) and first clinical trials indicate that a clinically relevant (2- to 3-fold) decrease in everolimus exposure is expected if

ciclosporin is removed from an everolimus-ciclosporin combination therapy (86).

Currently still under discussion is the necessity of therapeutic drug monitoring for sirolimus and everolimus. There are indications that trough concentrations above 15 ng/ml have a higher risk of thrombocytopenia and hyperlipidemia and that trough concentrations below 6 ng/ml are associated with an increased incidence of acute rejection (79, 87-90). Due to the long half-life of the drug, trough levels obtained 5-7 days after dose adjustments would be sufficient (79).

Assays for pharmacodynamic monitoring were developed by measuring $p70^{S6}$ kinase activity (91), ^{3}H-thymidine incorporation (92, 93) and by detecting PCNA/SG_2M coexpression and expression of T cell surface activation molecules by flow cytometry (94). However, there is no data so far to show that changes in these pharmacodynamic assays reflect *in vivo* efficacy of sirolimus for suppression of rejection (95).

Safety

As predicted from *in vitro* experiments and preclinical animal studies, tolerability of sirolimus and everolimus was very good and distinct from calcineurin inhibitors. Early trials with sirolimus (96) and everolimus (97), both double-blind, placebo-controlled in combination with ciclosporin and corticosteroids in stable renal transplant recipients, showed no nephrotoxicity or neurotoxicity. There was no effect on glomerular filtration rate or serum creatinine levels. Furthermore, there was no change in blood pressure and in AST or ALT levels. As observed in animal studies, main side effects of TOR inhibitors were decreased platelet and white blood cell (WBC) counts and elevated triglyceride and cholesterol levels. Dose-dependent and reversible within 2 weeks after discontinuation of the drug, sirolimus decreased platelet counts (p <0.05). The decrease in WBCs was not entirely dose related, but significant compared to the controls (p = 0.03) (96). No effects were observed at 0.75 mg and 2.5 mg doses of everolimus, but significant differences from baseline were reached after multiple doses of 7.5 mg everolimus (97). During sirolimus or everolimus therapy, serum cholesterol levels increased significantly. However, in sirolimus-treated patients there was no significant elevation of triglyceride serum levels and everolimus increased triglyceride levels by 459% in the 2.5 mg dose group and by 159% in the 7.5 mg dose group. In another study (83) evaluating single doses up to 25 mg of everolimus in stable renal transplant recipients, safety profiles of everolimus and placebo were similar. Only mild or moderate side effects were seen after heart or heart/lung transplantation (98). The most common side effects were headache (4/20), hypertriglyceridemia (5/20) and hypercholesterolemia (1/20).

In a phase II dose escalating study (99) the safety profile of sirolimus remained unchanged in combination with ciclosporin and prednisolone in renal allograft recipients after a follow-up of up to 47 months. There were no differences between the study groups and the historic controls regarding rates of viral and bacterial infections and occurrence of posttransplant malignant diseases. There seemed to be a greater incidence of gastrointestinal side effects of diarrhea, nausea and vomiting compared to the ciclosporin-prednisone historic control (99). In doses up to 7 mg/m^2/day of sirolimus, platelet (POD 365: 232 *vs.* 291/nl, p = 0.01) and WBC counts (POD 365: 6.3 *vs.* 10.7/nl, p <0.0001) were significantly lower than in the control group. Also, myelosuppression was a common side effect of sirolimus treatment and was closely dose related and correlated highly (r = 0.89) with sirolimus trough levels (100). Generally these side effects resolved spontaneously after dose reduction after a median of 7 days. None of the patients required permanent withdrawal of the drug (100).

In a European multicenter phase II trial (101), comparing sirolimus- with ciclosporin-based immunosuppression in combination with azathioprine and steroids, mean serum creatinine levels were consistently lower after 3 and 4 months after renal transplantation in the sirolimus group (p <0.05). Myelodepression and elevated triglyceride and cholesterol levels were more frequent in the sirolimus group and infections were comparable in both groups, except for a higher incidence of pneumonia in the sirolimus group. However, no patient died in the sirolimus group. In combination with mycophenolate mofetil (MMF) (102) the adverse events reported more frequently with sirolimus were thrombocytopenia (45% *vs.* 8%) and diarrhea (38% *vs.* 11%) and with ciclosporin increased creatinine (18% *vs.* 39%), hyperuricemia (3% *vs.* 18%), CMV infection (5% *vs.* 21%) and tremor (5% *vs.* 21%). Reports of sirolimus in combination with tacrolimus are still rare. In a series of 39 transplant recipients after liver, kidney or pancreas transplantation (103), very low rates of renal dysfunction, hypertension and diabetes were observed. Also, the rate of opportunistic infections was low.

In the phase III U.S. double-blind multicenter trial (n = 719) comparing 2 mg and 5 mg sirolimus with azathioprine, in combination with ciclosporin and steroids, survival and causes of death did not differ between the groups in the first year after kidney transplantation (104). All rates of infections were not statistically different between the groups, except for a higher herpes simplex incidence in the 5 mg sirolimus group. Malignant disorders were rare in all groups. The sirolimus 2 mg/day group showed a higher incidence of acne and hypertension, and the sirolimus 5 mg/day group a higher incidence of epistaxis, diarrhea and headache, hirsuitism, hypercholesterolemia, hyperlipidemia, lymphocele and thrombocytopenia. Many patients required concomitant therapy with HMG-CoA reductase inhibitors (105). At 6 months and 12 months the mean serum creatinine concentrations were significantly higher in patients in the two sirolimus groups than in those treated with azathioprine (p <0.001) and mean creatinine clearance was lower (2 mg sirolimus, p <0.01; 5 mg sirolimus, p <0.001). The daily treatment with 5 mg sirolimus seemed to especially

exacerbate ciclosporin-induced toxic effects (104). It is under discussion why the favorable safety profiles from the phase I and phase II trials of the sirolimus and ciclosporin combination were lost in this study. However, the incidence of side effects of this combination has not been higher than those of other phase III trials in the past. In fact, recent trials showed that sirolimus itself is not nephrotoxic and that after ciclosporin withdrawal, sirolimus-treated patients improve in renal function (106).

Comparable results were shown with the American phase III trial with everolimus (107). Everolimus caused mainly reversible hematological changes and moderate increase in lipids (108).

Efficacy

In the first multicenter phase II trial evaluating the combination of sirolimus, ciclosporin and steroids (109), 149 recipients of mismatched cadaveric or living donor recipients of renal allografts were randomized into 6 groups: 3 groups received placebo or 1 or 3 mg/m^2/day sirolimus as well as steroids and full-dose ciclosporin (C_0 target: 200-350 ng/ml) and 3 other groups received steroids, 1, 3 or 5 mg/m^2/day sirolimus and a reduced dose of ciclosporin (C_0 target: 100-175 ng/ml). A significant reduction of biopsy-proven acute rejection episodes was observed at 6 months in the groups receiving full-dose ciclosporin and either 1 or 3 mg/m^2/day sirolimus (8.5%) compared with the group receiving full-dose ciclosporin without sirolimus (32%, p = 0.018). Also, the incidence of acute rejection episodes at 6 months was lower in the reduced-dose ciclosporin groups in combination with either 1, 3 or 5 mg/m^2/day sirolimus (19.5%, p = n.s.). Analysis of renal function data suggested that reduced ciclosporin exposure yielded better renal function. Remarkably, in African-American patients the rate of acute rejections was significantly higher in the reduced ciclosporin groups compared to Caucasian recipients (39.1 and 10.7%, respectively; p <0.009) showing that this immunosuppressive regimen was not beneficial for African-Americans.

In a European phase II study (101), sirolimus-based immunosuppression was compared with ciclosporin-based immunosuppression after primary cadaveric kidney transplantation. Both drugs, in combination with steroids and azathioprine, showed similar efficacy with 12-month graft survivals of 98% and 90% in the sirolimus and ciclosporin groups, respectively, and the incidence of biopsy-proven acute rejections of 41% *vs*. 38%. Another European study (102) compared sirolimus with ciclosporin in combination with steroids and MMF (2 g/day). In this study, graft survival (92.5% *vs*. 89.5%), patient survival (97.5% *vs*. 94.7%) and the incidence of acute rejections (27.5% *vs*. 18.4%) were not statistically different for sirolimus *versus* ciclosporin.

In a phase II everolimus multicenter, open-label trial (110), 111 patients were randomized into one of two treatment groups: 3 mg/day everolimus with either full-dose ciclosporin (targeted trough 150-300 ng/ml through day 60, 125-250 ng/ml thereafter) or reduced-dose ciclosporin (targeted trough 75-125 ng/ml through day 60, 50-100 ng/ml thereafter). All patients received concomitant administration of basiliximab (20 mg on day 0 and 4) and corticosteroids. At 12 months the incidence of biopsy-proven acute rejection, graft loss or patient death was statistically (p = 0.013) significantly lower in the reduced-dose ciclosporin group (8.8% *vs*. 27.8%). Ten patients experienced graft loss, death or lost to follow-up: 7 patients (13%) in the full-dose group and 3 patients (5%) in the reduced-dose group. The inclusion of everolimus in ciclosporin-based immunosuppression regimens allowed for reduction in the dose of ciclosporin, resulting in significant improvements in renal function (measured as GFR) at 12 months in the reduced-dose ciclosporin group (111).

In the U.S. phase II trial (109) oral dose trough concentrations for 1 and 3 mg/m^2 sirolimus correlated with fixed doses of 2 and 6 mg, leading to the use of two fixed oral doses in the phase III trials. Safety and efficacy of sirolimus for the prevention of organ rejection following renal transplantation was evaluated in 1295 patients in two studies. Study 301 (U.S. study) (104, 112) enrolled 719 patients in 38 centers and study 302 (global study) (112) enrolled 576 patients in 34 centers between June 1996 and September 1997. Sirolimus (2 or 5 mg daily) was compared with 2-3 mg/kg/day azathioprine (U.S. study) or with placebo (global study). All groups included ciclosporin microemulsion formulation (target trough: 200-350 ng/ml in the first 3 months and 150-250 ng/ml from month 3 on) administered at least 4 h before the sirolimus dose and steroids (tapered to reach 30 mg/day on day 6 to 5-10 mg/day from month 6 on). Antibody induction therapy was prohibited. Primary endpoint of these studies was composite graft failure (biopsy confirmed acute rejection, graft loss or death) in the first 6 months after transplantation and secondary endpoints were 1-year patient and graft survival.

In the U.S. study, the incidence of efficacy failure at 6 months was 16.8% in the sirolimus 5 mg/day group, 18.7% in the sirolimus 2 mg/day group and 32.3% in the azathioprine group (p = 0.001 and p = 0.002, respectively). In the global study, composite graft failure occurred in 25.6% with sirolimus 5 mg/day, 30.0% with sirolimus 2 mg/day and 47.7% with placebo (p = 0.001 and p = 0.002, respectively) (112). The 6-month incidence of biopsy-proven rejection was significantly lower among patients receiving sirolimus in a dose-response manner. Also, the incidence of Banff (113) grade II-III rejections was significantly lower among patients in the sirolimus 5 mg/day groups in both trials. Furthermore, sirolimus reduced the use of antibody therapies to treat biopsy-proven acute rejections in both trials and prolonged the time to the first acute rejection episode significantly in the U.S. study. Analyzing subgroups in both trials, results suggested that especially for high-risk patients (African-American, 2nd or 3rd transplant, high PRA [> 50%], high degree [> 2] of HLA mismatch, cadaveric donor organ) 5 mg/day sirolimus provides an incremental benefit for the patients

(112). Secondary endpoints were not different in both trials: graft survival reached 88-95% and patient survival reached 95-98% in all groups.

A phase III multicenter, double-blind, randomized trial (114) evaluated the safety and efficacy of everolimus *versus* MMF in combination with ciclosporin and steroids in *de novo* renal transplant recipients. A total of 583 patients were randomized into one of three treatment groups: 1.5 mg/day everolimus, 3.0 mg/day everolimus or 2 g/day MMF. Biopsy-proven acute rejection rates were 19, 22 and 24% in the everolimus 1.5 mg, 3.0 mg and MMF groups, respectively. There was no statistically significant difference in incidence of graft loss or death among everolimus (10.9 and 7.7%) and MMF (6.6%) treatment groups. One-year patient survival was excellent in all groups (> 96%) with no statistical difference between everolimus- and MMF-treated patients. Acute rejection episodes requiring antibody therapy were statistically less frequent in 1.5 mg/day everolimus-treated patients compared to MMF.

Recent studies showed that sirolimus conveys clear therapeutic benefits to reduce incidence of acute and chronic rejections (115). Especially, the progressive elimination of ciclosporin proved to be a safe and effective alternative to a long-term, cislosporin-based regimen (115). Early ciclosporin withdrawal resulted in improved renal function and lower blood pressure (116).

Experience with sirolimus after liver transplantation is still limited. In a pilot study evaluating the value of sirolimus after liver transplantation (117), 15 patients were assigned to 3 different immunosuppressive protocols. Protocol A (n = 4) included sirolimus (no loading dose, 4 mg/m^2), ciclosporin (early target trough 200 ng/ml) and steroids, protocol B (n = 7) included sirolimus (loading dose of 12 mg/m^2 on day 0, 1 and 4 mg/m^2 thereafter) and ciclosporin (early target trough 100 ng/ml) and protocol C (n = 4) was sirolimus monotherapy with the same dose as in protocol B. All protocols aimed to achieve sirolimus monotherapy by 3 months after transplantation. Five out of 15 patients died in less than 8 months after transplantation, 3 of them due to septic complications. Side effects were moderate and sirolimus was only discontinued in 2 patients due to high lipids and *Pneumocystis carinii* infection, respectively. However, number of patients per group and heterogeneity of the immunosuppressive regimens do not allow more than the conclusion that sirolimus maintenance therapy is effective.

Two other groups reported independently about their experience with sirolimus as rescue therapy. Kneteman *et al*. (118) presented results of a study in 29 patients who received sirolimus for severe side effects of conventional immunosuppressants; 4/6 patients with neurotoxicity and 10/12 patients with nephrotoxicity improved after conversion to sirolimus. Nishida *et al*. (119) reported that 8/16 patients with chronic liver allograft rejection responded to therapy. In these patients mean bilirubin levels dropped from 10.1 ± 3.2 mg/dl to 0.7 ± 0.19 mg/dl after conversion to sirolimus (target trough 10-15 ng/ml). In another retrospective study (71), sirolimus was able to improve biochemical function and histological scoring in 7/21 patients.

Another study (103) reported results for 25 liver transplants recipients who were treated with sirolimus (target trough 5-12 ng/ml) in combination with low-dose tacrolimus (target trough 3-7 ng/ml) and steroids (withdrawn within 3 months after transplantation). Except for 1 recipient who stopped all medication by her own volition, none of the other patients experienced an acute rejection during a mean follow-up of 230 days (43-450 days). Although 5/25 patients had been comatose and on mechanical ventilation before transplantation, 23 patients (92%) were alive at the end of the follow-up. Of the remaining 2 patients, one died of subarachnoid hemorrhage and the other from a hepatic-artery hemorrhage caused by a liver abscess. Additionally, only 1 patient had an infection (cytomegalovirus) and 1 patient was treated for hyperlipidemia. Kidney function was near normal in all recipients. In summary, this report showed an effective combination therapy of sirolimus and tacrolimus with very low rates of renal dysfunction, hypertension, diabetes and a low rate of opportunistic infections.

A phase II study in 119 liver allograft recipients (120) suggested that everolimus in combination with ciclosporin provides effective immunosuppression with excellent patient and graft survival in *de novo* liver transplant recipients. Patients were randomized to 1 of 4 groups (everolimus 0.5 mg b.i.d. p.o.; everolimus 1.0 mg b.i.d. p.o.; everolimus 2 mg b.i.d. p.o. or placebo) and additionally received ciclosporin to achieve a target trough level of 250-400 ng/ml and prednisone. Overall patient survival (88%) and graft survival (95%) were excellent. The incidence of treated rejection was less in the everolimus groups than in the placebo group (placebo [36.7%]; everolimus 1 mg/day [28.6%]; 2 mg/day [23.3%]; 4 mg/day [25.8%]; p = n.s.).

More phase II and phase III trials are needed to investigate the overall value of sirolimus and everolimus after liver transplantation.

Prospects

Inhibitors of the mammalian target of rapamycin, sirolimus and everolimus are a new class of immunosuppressants. In this review, we demonstrated that sirolimus and everolimus are efficient in preventing and treating acute rejections after renal, liver and probably also after pancreas, heart and lung transplantation in humans. These two new compounds show synergism with ciclosporin, tacrolimus and other immunosuppressants such as mycophenolate mofetil and, thereby, offer totally new combinations of immunosuppressive agents. Different combinations with sirolimus or everolimus will enrich the variety of immunosuppressive regimens. According to the individual risk profile of the recipient, it will be possible to tailor immunosuppressive treatment. Combinations with sirolimus as primary

immunosuppressive agent alone or in combination with MMF or steroids will be possible as well as sirolimus as an adjuvant immunosuppressant in combination with calcineurin inhibitors. With the different toxicity profiles of all these agents one should be able to reduce the adverse events, *i.e.*, less nephrotoxicity and neurotoxicity, less hypertension due to the sparing of calcineurin inhibitors and less diabetes due to the sparing of tacrolimus and steroids.

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