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## **Solubilization of Sirolimus in Aqueous Solution Through Complexation with Metal Ions**

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## Abstract

Sirolimus is one of the most successful immunosuppressant drugs available but it has an extremely low solubility in water that dramatically limits its oral bioavailability to approximately 20%. This study is aimed to increase solubility of Sirolimus in aqueous solutions through complexation with a metal ion. Using computer modeling and UV absorbance spectroscopy, it was demonstrated that 1) Sirolimus interacts with  $\text{Al}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ; 2) this interaction decreases UV light absorbance of Sirolimus measured at  $\lambda=280$  nm; 3) a previously reported value of solubility of Sirolimus in water is underestimated; 4) Sirolimus can exist in aqueous solutions in different soluble physical forms, including multimers; 5) complexation of Sirolimus with  $\text{Al}^{3+}$  increases its aqueous solubility that potentially can improve its oral bioavailability. Similar approach, i.e. complexation with a metal ion, can be used to increase aqueous solubility of other compounds containing ligands with unshared electron pairs, for example, cyclic macrolides like Tacrolimus, derivatives of Tacrolimus and Sirolimus, as well as peptide drugs such as Cyclosporin A.

MONTCLAIR STATE UNIVERSITY

Solubilization Of Sirolimus In Aqueous Solution Through Complexation With Metal

Ions.

by

Ralph A. Falen

A Master's Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

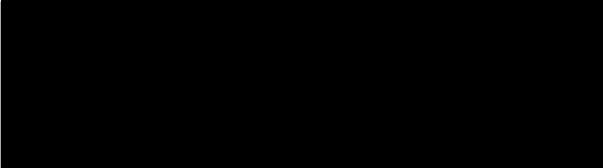
Master of Science, Molecular Biology

August 2013

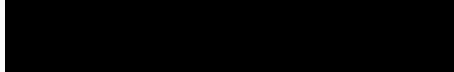
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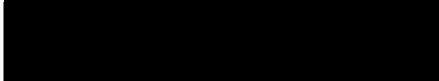
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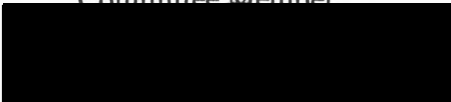
  
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Solubilization Of Sirolimus In Aqueous Solution Through Complexation With Metal  
Ions.

A Thesis

Submitted in partial fulfillment of the requirements  
For the Degree of Master of Science, Molecular Biology

by

Ralph A. Falen  
Montclair State University  
Montclair, NJ  
August 2013

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## Abbreviations

Alum  $\text{AlK}(\text{SO}_4)_2$

CsA Cyclosporin A

IL-2 Interleukin-2

mTOR mammalian Target Of Rapamycin

MOA Mechanism Of Action

NFAT Nuclear Factor of Activated T-cells

SEM Square Error of the Mean

## **Introduction**

### **Goal**

To improve the aqueous solubility of Sirolimus through construction of a drug-metal ion complex.

### **History of Sirolimus**

Organ transplantation is a life saving medical procedure worldwide. However, the procedure does have its drawbacks, mainly being the insufficient quantity of donor organs and organ rejection. As of May 2013, approximately 128,000 patients in the United States were on the waiting list for organ transplantation, and actual transplantations that were performed in 2012, were approximately 28,000 (Organ Procurement and Transplantation Network (OPTN), 2013. OPTN: View data reports). Once the organ is transplanted, the recipient's immune system identifies the foreign antigens of the donor organ and rejects the organ essentially destroying the organ. In order to avoid graft rejection, both donor and recipient are matched as closely as possible in regards to their antigens[1]. Recipients are still required to be placed on a lifelong regiment of immunosuppressive drugs.

Sirolimus (Rapamune, Rapamycin) (Fig. 1A) is an example of a successful immunosuppressive drug that is prescribed post kidney transplantation. Sirolimus was first isolated from a soil sample obtained from Easter Island in 1975[2]. Sirolimus is produced by the bacterium *Streptomyces hygroscopicus* and its initial properties were identified as having antimicrobial activity[3]. The progression of Sirolimus initial discovery to the approval usage as an immunosuppressive drug took approximately 26



years (Fig. 2). Later research demonstrated that the drug also had immunosuppressive properties and in 1999 Sirolimus was approved by the U.S. FDA as an immunosuppressive drug that could be prescribed for the prevention of rejection in renal transplant patients[3].

Sirolimus is also used in medical devices, such as the Cypher stent, which is used for the expansion of blocked coronary arteries preventing restenosis. Sirolimus is gradually released within the artery wall and lowers the amount of normal cell growth in order to prevent re-blockage of the artery (U.S. Food and Drug Administration (FDA), 2012. FDA: Medical devices, CYPHER™ Sirolimus-eluting Coronary Stent - P020026).

### **Mechanism of action (MOA)**

The mechanism of action (MOA) of Sirolimus differs from its counterparts, Cyclosporine (Fig. 1B) and Tacrolimus (Fig. 1C). The ability of T-cells to recognize proteins located on the antigen-presenting cell allows for cellular signaling to take place *via* the T-cell receptor, which activates the calcineurin phosphate that dephosphorylates the nuclear factor of activated T-cells (NFAT). The dephosphorylation of NFAT allows it to enter the nucleus and act as a transcription factor, regulating Interleukin-2 (IL-2) production. The regulation of IL-2 promotes T-cell activation and proliferation after the interaction of the IL-2 receptor aided by the co-stimulatory signals of CD28[5].

Cyclosporine and Tacrolimus both inhibit the calcineurin phosphate by binding with their corresponding protein resulting in a complex formation. The formation of the drug complex allows for calcineurin to be inhibited and stops IL-2 production that stops T-cell activation. Sirolimus does bind to its corresponding immunophilin in the same manner as Cyclosporine and Tacrolimus (Fig 3); however, the MOA is distinct for

Sirolimus. Once bound to its binding protein, Sirolimus binds to the mammalian target of Rapamycin (mTOR) complex of T-cells. This binding results in the disruption of the IL-2 receptor and inhibits T-cell proliferation by cell cycle arrest[5].

In addition, Sirolimus has been shown in recent research to have the ability to promote autophagy in mice that have suffered spinal cord injury[4]. The study indicates that the reason for autophagy promotion has to do with the inhibition of mTOR, which, in return, allows for a higher concentration of the autophagy proteins LC3 and Beclin 1 in the injured spinal cord of the mice[4].

### **Solubility of Sirolimus**

Sirolimus has a molecular formula  $C_{51}H_{79}NO_{13}$  (molecular weight 914.2), is a cyclic macrolide, does not have ionizable groups, is freely soluble in DMSO, and has a low solubility in aqueous solutions that dramatically limits its bioavailability to approximately 20%[6]. According to the Product Information from Product Number R0395 (Sigma-Aldrich), Sirolimus is soluble in DMSO at 25 mg/mL (27.3 mM) with reference to Sigma-Aldrich Production/Quality Control (Reference 2 in the Product Information sheet). The Product Information sheet states that Sirolimus is “substantially insoluble in water” with reference to The Merck Index. Monograph 8114 of The Merck Index 14<sup>th</sup> makes the only statement about solubility of Sirolimus in water: “Substantially insol in water”. Studies on solubility of Sirolimus in water report values of 2.6 µg/mL (2.8 µM)[6].

### **Cost of Sirolimus**

In addition to Sirolimus having a low bioavailability that is caused by its low solubility in aqueous solutions, it also is an expensive drug treatment for patients. An annual treatment of Sirolimus cost approximately US \$7,000.00 for patients who have had kidney transplantations[7]. This study is aimed to increase solubility of Sirolimus in aqueous solutions through the complexation of charged metal ions in order to potentially increase its bioavailability.

### **Complexation with metal ions changes drug properties**

Metal complexation is known to affect drug properties. Thus, Cu(II) and Zn(II) significantly improve the antimicrobial activity of cephalexin[8], bacitracin is formulated together with Zinc due to its higher stability (Merck Index, 14<sup>th</sup>, Monograph Number 936). As a free acid, aceglutamide is a nootropic drug, and in complex with aluminum it is used as an antiulcerative (The Merck Index 14<sup>th</sup>, Monograph 25). Aloxiprin, a complex of aspirin and aluminum, has better pharmacokinetic properties at acidic pH than aspirin (The Merck Index 14<sup>th</sup>, Monograph 309). On the other hand, the antimicrobial activity of 1,4-naphthoquinones was decreased by metal complexation[9], tetracyclines lose their antimicrobial activity when coordinated with metals[10], Mg<sup>2+</sup> complexed with fluoroquinolones results in a decrease in oral bioavailability[11], metal complexation of the fluoroquinolone norfloxacin decreases oral bioavailability of this antimicrobial agent[12].

### **Hypothesis: “Complexation of Sirolimus with a metal ion can result in a charged complex and thus increase its water solubility”**

The use of certain immunosuppressive drugs, for example cyclosporines, Tacrolimus and Sirolimus and their derivatives, is hampered by their low aqueous

solubility, and thus, low bioavailability. The same applies to drugs like Cyclosporin A (CsA) (Fig. 1B), which is a peptide. With different biosynthesis and not chemically related to Tacrolimus (Fig. 1C) and Sirolimus (Fig. 1A), which are macrocyclic lactones, CsA has one striking similarity among to them: the presence of 11 carbonyl and one hydroxyl oxygens in CsA (Fig. 1B), 4 carbonyl, 3 hydroxyl, 3 methoxyl, 1 lactone, and 1 epoxy oxygens in Tacrolimus (Fig. 1C), and 5 carbonyl, 3 hydroxyl, 3 methoxyl, 1 lactone, and 1 epoxy oxygens shown here in Sirolimus (Fig. 1A). These oxygens are strong Lewis bases and form donor-acceptor bonds with metal ions, especially d-metal ions, the interaction being stronger when the number of dents increases and the ligand has the cyclic chemical structure. Thus, if the above-mentioned compounds, their analogs and derivatives, would be able to bind metal ions, these drug-metal complexes should have increased solubility in water.

In order to improve the aqueous solubility of Sirolimus, it might be possible to form a stable complex of Sirolimus with a metal ion. The complexation of Sirolimus with a charged metal ion would result in a charged complex and consequently increase its water solubility, and improved water solubility of Sirolimus may lead to an improvement of its bioavailability.

### **A choice of metal ions**

The first candidates considered were  $\text{Fe}^{3+}$  and  $\text{Al}^{3+}$  ions since they are strong Lewis acids and interact with such hard Lewis base as oxygen, and, importantly, have lower toxicity than other high charged metal ions. Though  $\text{Fe}^{3+}$  is a stronger acid than  $\text{Al}^{3+}$ ,  $\text{Al}^{3+}$  is a better candidate to start with since  $\text{Fe}^{3+}$  ion intensely absorbs in that UV

part of the spectrum where Sirolimus can be detected by its absorbance, and  $\text{Al}^{3+}$  has a smaller atom, which may fit better in the central pore of Sirolimus.

## **Materials and Methods**

### **Computer Modeling of Sirolimus with metal ions**

ChemBio3D Ultra 12.0 software (CambridgeSoft Corporation, Cambridge, MA) was used to model Sirolimus-metal ion interaction. MMFF94 algorithm was used to minimize energy.

### **Preparation of Sirolimus**

1 mg of Sirolimus (Sigma-Aldrich R0395) was diluted in 0.109 mL of freshly opened DMSO (Sigma-Aldrich D2650) to make final concentration of 10 mM, aliquoted by 5  $\mu$ L aliquots and stored at -20°C. Using a precise Hamilton syringe, 1  $\mu$ L of the 10 mM stock was dissolved in 1 mL of solvent or buffer to make 10  $\mu$ M of the final concentration of Sirolimus for spectrophotometric measurements. For all experiments 18.2 M $\Omega$  water (Milli-Q, Millipore) was used. All chemicals were purchased from Sigma-Aldrich Co.

### **Absorbance measurements**

Spectra of Sirolimus were measured with a Nanodrop ND-1000 spectrophotometer. Absorbance of Sirolimus at particular wavelengths was obtained from the spectrum measured with the Nanodrop ND-1000 spectrophotometer. Additionally, absorbance of Sirolimus at  $\lambda=280$  nm was measured in a quartz cuvette with the spectrophotometer attachment of the Hitachi F-7000 spectrofluorometer. All absorbance units are reported for 1.0 cm light path unless noted otherwise. All measurements were done at room temperature. The advantage of using a spectrofluorometer in the transmission mode is that both the incident light and transmitted light passes through

their own monochromators that avoids the fluorescence artifact if substances under investigation are fluorescent. The incident light monochromator was set to  $\lambda=280$  nm with 5 nm slit, and the transmitted light monochromator was set to  $\lambda=280$  nm with 20 nm slit. The PMT voltage was set to 400 V.

### **Preparation of Sirolimus- $\text{Al}^{3+}$ (SirAl) complex**

1  $\mu\text{L}$  of 10 mM Sirolimus in DMSO was mixed with 2  $\mu\text{L}$  of 100 mM  $\text{AlCl}_3$  solution in ethanol and evaporated in an Eppendorf Vacufuge vacuum centrifuge at  $30^\circ\text{C}$  for 15 min. The pellet was reconstituted in buffer containing KCl (150 mM), HEPES (10 mM), and pH=7.0 (NaOH) to model the intracellular ionic environment and precipitate excess of free  $\text{Al}^{3+}$  as  $\text{Al}(\text{OH})_3$ . For control, the second sample was prepared with 2  $\mu\text{L}$  of ethanol and no  $\text{AlCl}_3$ . The samples were centrifuged for 1 min with a Mini Centrifuge to sediment  $\text{Al}(\text{OH})_3$  and other insoluble matter, and the supernatant was analyzed with the Nanodrop ND-1000 spectrophotometer. The spectra were taken in 4-6 replicas in each independent trial and averaged.

## Results

### Computer Modeling of Sirolimus with metal ions

Energy minimization of free Sirolimus with MMFF94 algorithm revealed a central pore (Fig. 4A). One generic dipositive metal ion ( $M^{2+}$ ) was manually placed next to the structure of Sirolimus and minimization was done thereafter. The procedure was repeated several times when  $M^{2+}$  was placed at different location next to Sirolimus. The attempts to minimize energy with one  $M^{2+}$  ion starting from different locations surprisingly revealed two different binding sites, the first of which is at two carbonyl oxygens at C26 and C32 and one hydroxyl oxygen at C28 (Fig. 1A & 4B). Upon  $M^{2+}$  binding at this site, the molecule of Sirolimus did not change significantly its maximum linear dimension and the central pore opened even further (Fig. 4A and 4B, planar views). The second binding site was found at three carbonyl oxygens at C8, C9, C26, one hydroxyl oxygen at C28, and one methoxyl oxygen at C27 (Fig. 1A & 4C). Upon  $M^{2+}$  binding at this site, the molecule of Sirolimus decreased its maximum linear dimension and the central pore closed. For the first binding site, energy minimization resulted in a decrease from 255.9 kcal/mol to 100.6 kcal/mol, and at the second binding site that resulted in a decrease from 255.9 kcal/mol to 99.2 kcal/mol. It is important that modeling predicts two different interactions resulting in either no change in the maximum linear dimension (Fig. 4B) that should not significantly change light absorbance, or a decrease in the maximum linear dimension that can potentially decrease light absorbance (Fig. 4C). A second  $M^{2+}$  ion was added next to Sirolimus and minimization was done thereafter. Modeling with two  $M^{2+}$  ions predicted no change in the maximum linear dimension and even further opening of the pore in the center (Fig. 4A, 4D) probably due



to the electrostatic repulsion of positively charged  $M^{2+}$ s (Fig. 4D), and energy minimization resulted in a decrease from 255.9 kcal/mol to 155.6 kcal/mol. Introduction of the third  $M^{2+}$  ion resulted in an unstable behavior of the algorithm but binding three  $M^{2+}$ s may also be possible.

### **Interaction of Sirolimus with metal ions**

After addition of 100  $\mu$ M of  $AlK(SO_4)_2$ , the absorbance of 10  $\mu$ M Sirolimus in water decreased from  $0.370 \pm 0.018$  to  $0.333 \pm 0.016$  ( $n=10$ ,  $p < 0.001$ , paired t-test) (Fig. 5A, 5C). The absorbance of 100  $\mu$ M of  $AlK(SO_4)_2$  was negligible ( $0.004 \pm 0.003$ ,  $n=4$ ) (Fig. 5B, 5C). Fig. 5 also reports similar experiments with 100  $\mu$ M of other selected metal ions and EDTA. Control absorbances of 100  $\mu$ M of corresponding metal ions and EDTA were taken in separate experiments (Fig. 5B, 5C). Surprisingly, EDTA that was also tested to chelate possible contaminating metals in Sirolimus caused a similar decrease in absorbance (Sigma-Aldrich does not have information on trace metal analysis of Sirolimus, R0395). All effects of metal ions and EDTA in Fig. 5 were significant ( $P < 0.05$ ) and corrected for metal ion absorbances that were important only in a case of  $Fe^{3+}$ .

### **Dilution of Sirolimus**

1  $\mu$ L of 10 mM stock of Sirolimus was added to 1 mL of DMSO and quickly mixed. Dilution of Sirolimus in DMSO from the initial concentration of 10  $\mu$ M to 0 in 1  $\mu$ M steps results in a linear decrease of absorbance (Fig. 6A, squares). In four experiments shown,  $R^2$  of the linear fit were 0.9997, 0.9998, 0.9999, and 0.9993 (Fig. 6A, squares). Dilution of Sirolimus in water from the initial concentration of 10  $\mu$ M to 0 in

1  $\mu$ M steps resulted in non-linear curves with  $R^2$  values of 0.981, 0.986, 0.976, and 0.987 that indicated significant deviation from linearity (Fig. 6A, circles). Interestingly, that between 10  $\mu$ M and 5  $\mu$ M, the water dilution curves were smoothly bending and asymptotically approaching a linear region between 5 and 0  $\mu$ M ( $R^2=0.989, 0.997, 0.996,$  and 0.995). Mean $\pm$ SEM values of experiments in Fig. 6A are plotted in Fig. 6B. The line in Fig. 6B represents the best linear fit of the points between 0 and 5  $\mu$ M. Fig. 6C summarizes  $R^2$  values for the linear fit of the indicated ranges of Sirolimus concentrations.

### **Dilution of Sirolimus in the presence of Alum**

The dilution experiment repeated in water in the presence of 100  $\mu$ M Alum (Fig. 6D, triangles) demonstrated that  $Al^{3+}$  ion significantly decreases absorbance of Sirolimus compared to free Sirolimus in water (Fig. 6D, circles).

### **UV Spectrum of Sirolimus**

Water solution of Sirolimus was prepared at 10  $\mu$ M concentration in a 1 mL vial, and its spectrum was immediately taken with the Nanodrop ND-1000 spectrophotometer (Fig. 7A), and approximately 20 min later from the same vial (Fig. 7B). Absorbance spectra of Sirolimus (10  $\mu$ M) in water demonstrated that there was no loss of absorbance at least for 20 min (Fig. 7).

### **SirAl**

In the control group with no  $AlCl_3$  (Fig. 8, No Al) there was only background absorbance  $-0.016\pm 0.021$  ( $n=6$ ), while with  $AlCl_3$  (SirAl) the spectra were similar to the ones of Sirolimus (Fig. 7) and absorbance was  $0.468\pm 0.039$  ( $n=8$ ), significantly different of NoAl ( $P<0.001$ ). Control experiments demonstrated that  $AlCl_3$  alone did not contribute

significantly to absorbance ( $-0.002 \pm 0.006$ ,  $n=3$ ). The same approach was used to make and detect Sirolimus- $\text{Fe}^{3+}$  complex (Ferrolimus) but due to intense absorbance of  $\text{Fe}^{3+}$  ion at  $\lambda=280$  nm, the UV absorbance data are difficult to interpret, and Ferrolimus data are not included in this manuscript.

## Discussion

### Computer Modeling of Sirolimus with metal ions

Computational modeling of Sirolimus with metal ions revealed the possibility of two distinct conformational changes that could take place in Sirolimus upon metal ion binding (Fig. 4B and 4C). Because the shape of Sirolimus changes upon metal ion binding and absorbance is proportional to the longest linear dimension of a molecule, this change can potentially affect light absorbance, a decrease in Fig. 4C case. Though energy minimization is similar for states B and C with a small favor of state C *in silico*, *in situ* state C is expected to be more favorable because, in this state, coordination to 5 oxygens is predicted compared to only 3 oxygens in state B (Fig. 4).

### Solubility of Sirolimus in water

Though it is reported that solubility of Sirolimus in water is limited to 2.6  $\mu\text{g/mL}$  (2.8  $\mu\text{M}$ )[6], both the time scans of 10  $\mu\text{M}$  of Sirolimus absorbance at  $\lambda=280$  nm (Fig. 5A) and spectra of 10  $\mu\text{M}$  of Sirolimus taken immediately after dilution and 20 min after (Fig. 7) demonstrate that there is no loss of absorbance at least for 20 min. At 10  $\mu\text{M}$  concentration Sirolimus is soluble in water but may exist as Absorbance of Sirolimus in the concentration range between 10 and 0  $\mu\text{M}$  in DMSO follows Beer-Lambert law (Fig. 4A squares) suggesting that only one form of Sirolimus is likely to be present in DMSO, most likely a monomer. In water, however, the linear part of the dilution curve falls in the range up to 5  $\mu\text{M}$ , a value that is as twice as higher than reported previously for the solubility of Sirolimus in water obtained by the HPLC method[6]. At concentrations higher than 5  $\mu\text{M}$  the dilution curve is deviating from linearity that can be seen in no change or a decrease in  $R^2$  values with increasing number of data points (Fig. 5). Taking

into account that there is no loss in absorbance of Sirolimus at 10  $\mu\text{M}$  concentration (Fig. 6), we speculate that Sirolimus is soluble at 10  $\mu\text{M}$  concentration in water, however at this concentration it may form a mixture of monomers and multimers, while approaching 5  $\mu\text{M}$  concentration the multimers dissociate and Sirolimus exists as a monomer. Hypothetically, the hydrophobic chains between C17 and C25 (Fig. 1A) can interact decreasing their surface of exposure to water, thereby increasing water solubility of the dimer.

### **Interaction of Sirolimus with metal ions**

There are several lines of evidence that Sirolimus interacts with metal ions. First, computer modeling predicts this with a slight favor of a conformational change that decreases light absorbance (Fig. 4C). Second, a significant decrease in absorbance was detected upon binding of  $\text{Al}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$  (Fig. 5) that favors a transition from either state A or state B, or their combination, to state C (Fig. 4C) but not D (Fig. 4D). Third, the dilution experiment with  $\text{Al}^{3+}$  (Fig. 6D) even further supports a decrease of absorbance of Sirolimus in the presence of metal ions.

### **SirAl**

In eight trials UV absorbance measurements of SirAl (two representative experiments are shown in Fig.8) showed the signature UV profile of the Sirolimus peak (Fig. 7), while in the control trials no significant absorbance was observed indicating that SirAl has significantly higher aqueous solubility than Sirolimus. It is impossible at this moment to tell what exact concentration of SirAl in water was. For reference, 10  $\mu\text{M}$  of Sirolimus added as 1  $\mu\text{L}$  of 10 mM stock in DMSO to 1 mL of water without the

centrifugation procedures used for making SirAl results in absorbance between 0.347 and 0.395 at  $\lambda=280$  nm (Fig. 5C). The confounding parameters here are that 5  $\mu\text{M}$  is the edge of Lambert-Beer range of Sirolimus in water (Fig. 6B), and Sirolimus decreases its absorbance when in complex with  $\text{Al}^{3+}$  (Fig. 5A, 5C, 6D). It may seem confusing that in control (NoAl, Fig. 8) no characteristic UV spectrum of Sirolimus was detected though the target concentration of Sirolimus was 10  $\mu\text{M}$ . In contrast, 10  $\mu\text{M}$  of Sirolimus resulted in absorbance in the range between 0.347 and 0.395 in Fig. 5A, 5C, 6B, and 7. The difference was that dry Sirolimus was attempted to be solubilized in an aqueous solution for NoAl control, while in the rest of the figures Sirolimus was solubilized in water from 10 mM stock in DMSO that served as a carrier and helped to solubilize Sirolimus in water. This even further suggests that  $\text{Al}^{3+}$  serves as carrier for dilution of Sirolimus in water. SirAl in water is more promising to partition in an aqueous phase compared to Sirolimus carried by DMSO because  $\text{Al}^{3+}$  ion is highly charged compared to DMSO; thus, SirAl can potentially increase bioavailability of Sirolimus. It still remains a question if bioavailability of SirAl and Ferrolimus is higher than that of Sirolimus, and if the complexes retain immunosuppressive properties of Sirolimus.

## Conclusions

By demonstrating that interaction of Sirolimus with a metal ion,  $\text{Al}^{3+}$  in particular, increases its aqueous solubility, the original goal was achieved. Similar approach can be used to improve aqueous solubility of compounds containing unshared electron pairs, for example, cyclic macrolides like Tacrolimus, derivatives of Tacrolimus and Sirolimus, as well as peptide drugs such as Cyclosporin A, to increase their solubility through complexation with metal ions. It has also been demonstrated that 1) interaction with

metal ions decreases the size of Sirolimus and its absorbance, 2) a previously reported value of solubility of Sirolimus in water is underestimated, 3) Sirolimus can exist in aqueous solutions in different soluble physical forms, including multimers. A number of questions, however, remain unanswered, such as the paradoxical effect of EDTA on absorbance of Sirolimus, the analytical identification/quantification of SirAl and Ferrolimus via UPLC, determination of the dissociation constants of Sirolimus with metal ions. These questions will be the subject of further studies as well as testing the bioavailability of SirAl and Ferrolimus, and *in vitro* experimentations using T-cells in order to observe if SirAl and Ferrolimus retain the immunosuppressive properties of Sirolimus.

## Figures

Figure 1A. Structure of Sirolimus.

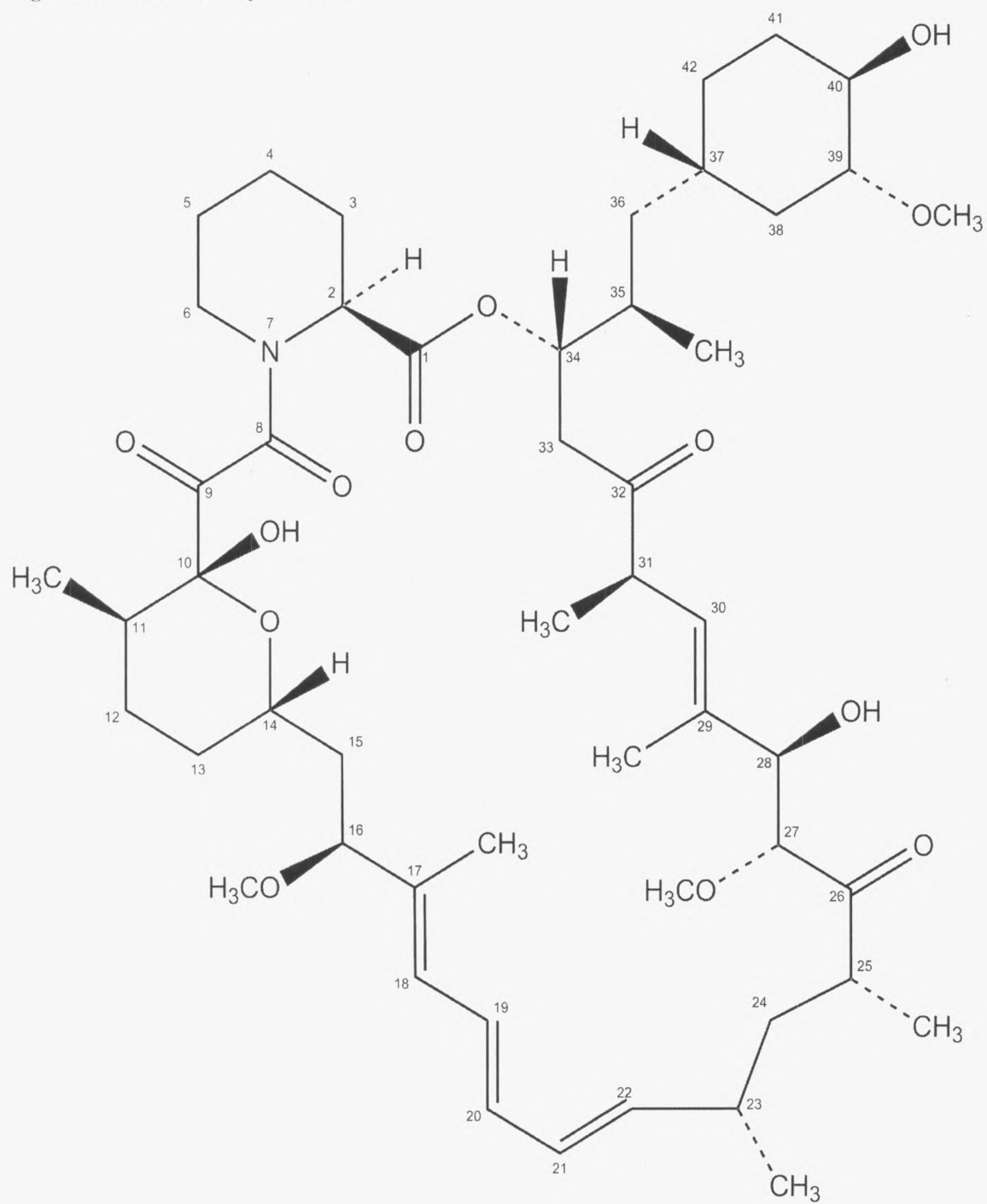
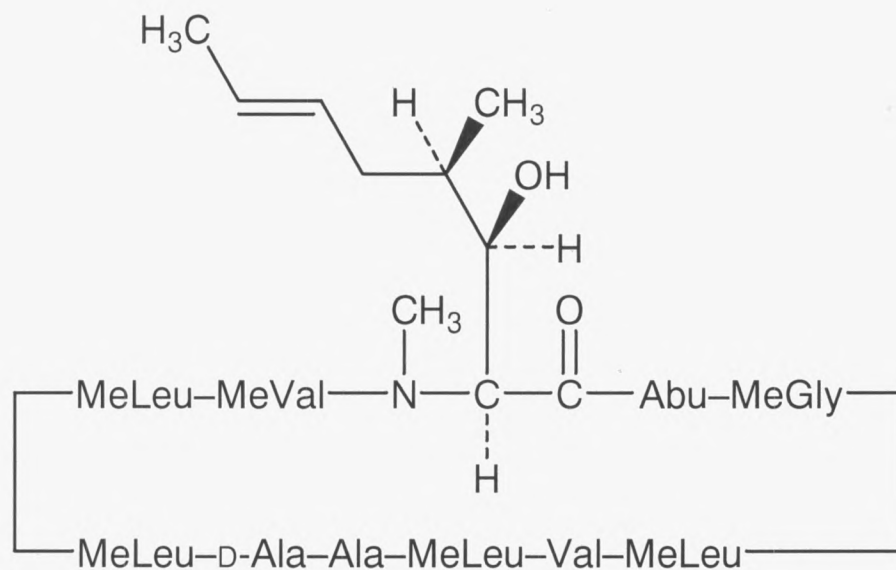




Figure 1B. Structure of Cyclosporin A.

Top panel is shown as in the Merck index, 14<sup>th</sup> edition, Monograph 2752. Bottom panel is shown as in PubChem (Substance ID 134339249).



Cyclosporin A

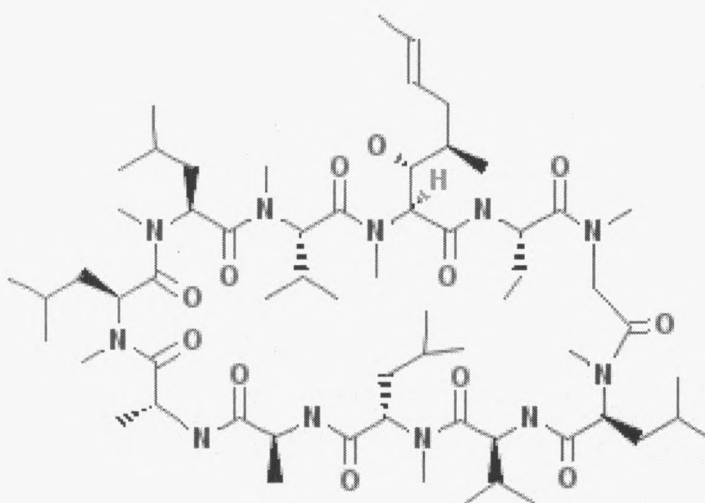
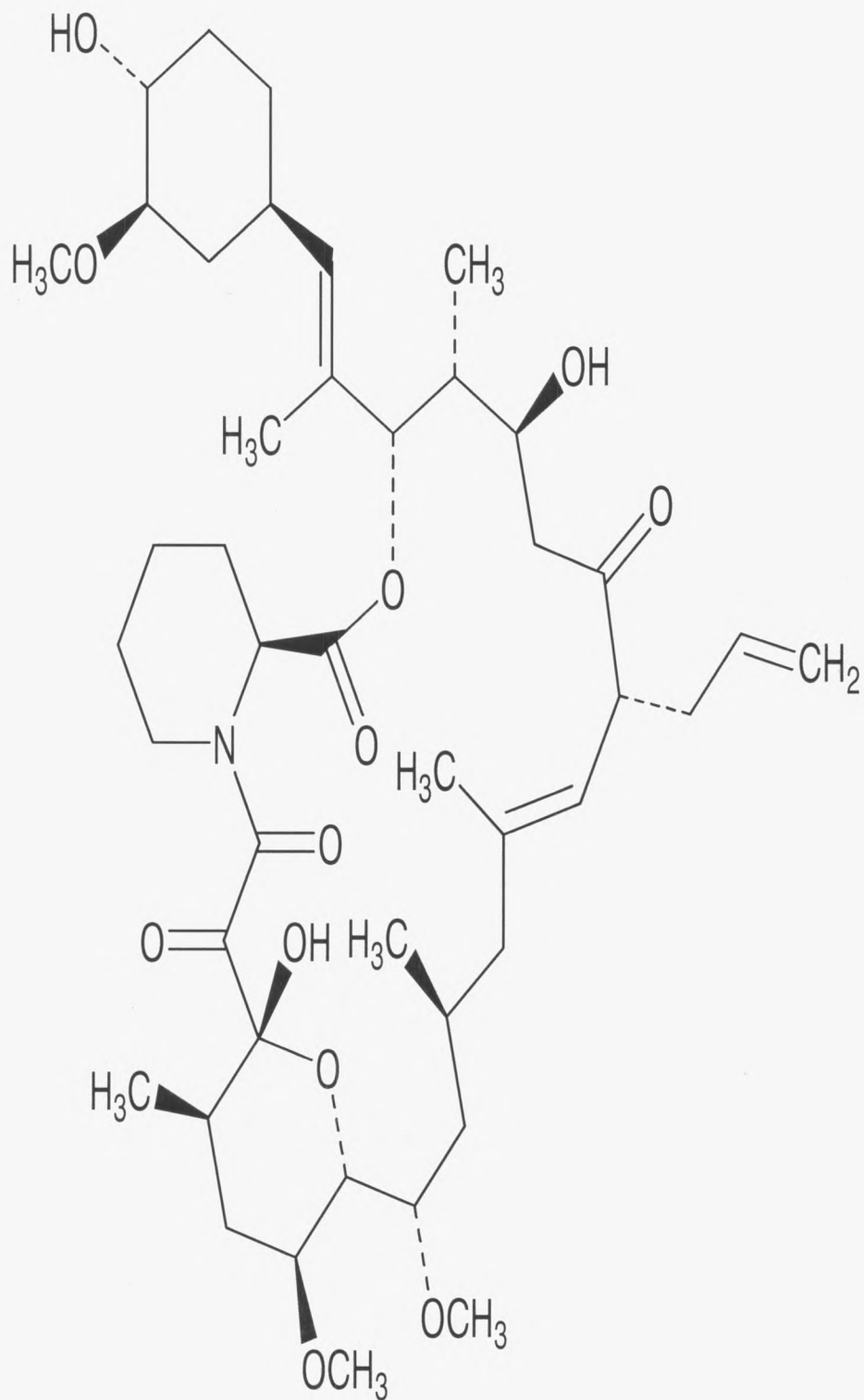


Figure 1C. Structure of Tacrolimus (FK-506).



*Figure 2. History of Rapamycin (adopted from [3]).*

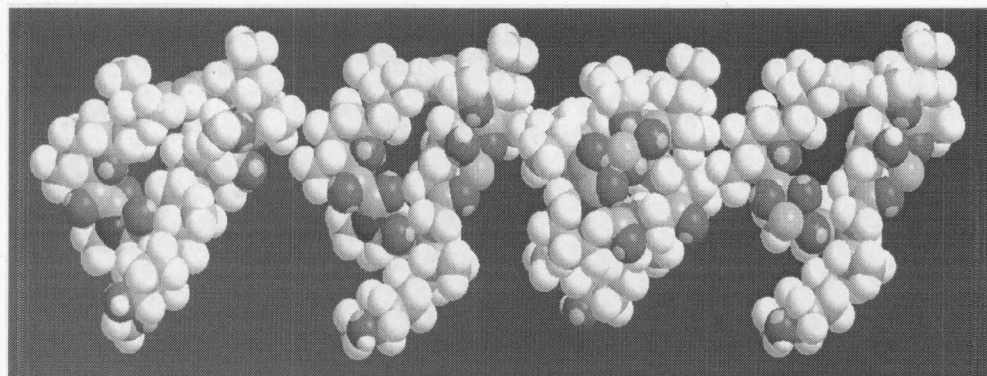
- 1973 Isolation from Easter Island soil sample and characterization of antimicrobial activity.
- 1974 Wyeth scientists observe that rapamycin is immunosuppressive. It inhibits the development of adjuvant arthritis and experimental allergic encephalities in rodents.
- 1975 Rapamycin is demonstrated to have antitumor activity.
- 1980 Elucidation of chemical structure as a macrocyclic lactone.
- 1988 Demonstration that rapamycin prevents allograft rejection in stringent animal models of organ transplantation.
- 1990 Preclinical work completed to support IND filing for rapamycin in transplantation.
- 1991 First IND submission for rapamycin intravenous formulation. Oral formulation follows soon thereafter.
- 1994 First Phase II trial in de novo recipients of renal allografts in combination with cyclosporine. Rapamycin tablet formulations are tested in human subjects.
- 1995 Phase II study in de novo recipients of renal allografts with rapamycin as the base therapy without cyclosporin.
- 1996 Phase III randomized, controlled clinical trials are initiated in North America, Europe, and Australia to achieve approval of rapamycin in combination with cyclosporine.
- 1997 Clinical program initiated in oncology based on extensive work in animal models, discussions with National Cancer Institute, and support from new head of oncology at Wyeth.  
Work is initiated with Cordis to develop a rapamycin-coated intracoronary stent based on results in rodents and pigs showing prevention of restenosis with rapamycin.
- 1998 Randomized Phase III clinical trials for cyclosporine withdrawal are initiated. Fifty-five studies of rapamycin for prevention of renal allograft rejection are submitted to the US FDA and the European Medicines Evaluation Agency 2 Weeks ahead of schedule.
- 1999 US FDA approves rapamycin in combination with cyclosporine and corticosteroids for prevention of rejection in renal transplant recipients less than 2 months after unanimous vote of FDA advisory committee.  
NDA is submitted for approval of the 1-mg rapamycin tablet based on pharmacokinetic studies and one large randomized controlled trial showing the equivalent clinical activity of the tablet and liquid formulations.
- 2001 European CPMP on appeal approves rapamycin as maintenance therapy for patients in whom cyclosporine can be eliminated.
- 2002 FDA device advisory committee votes unanimously to approve rapamycin coated stent for prevention of restenosis in coronary angioplasty.  
Response to FDA approvable letter for Rapamune Maintenance Regimen is submitted.



*Figure 4. Computer simulation of Sirolimus-Metal Ion interaction.*

Carbons, hydrogens, oxygens, nitrogens, metal ions are colored grey, white, red, blue, green, respectively.

**Planar views.**



**Side views.**

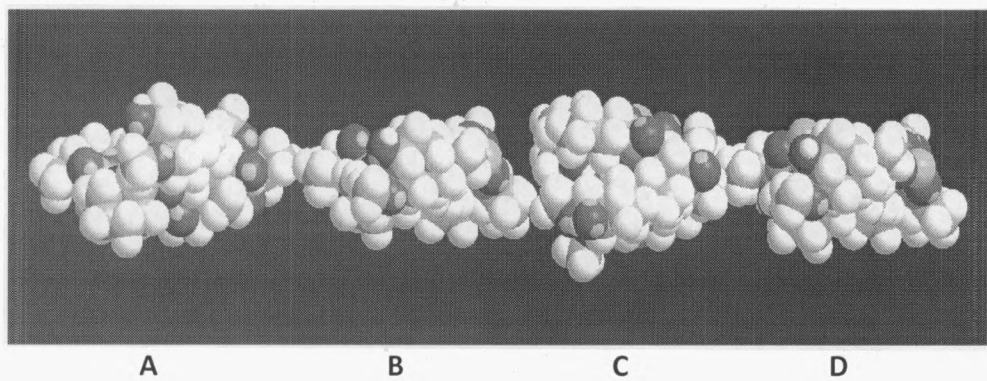
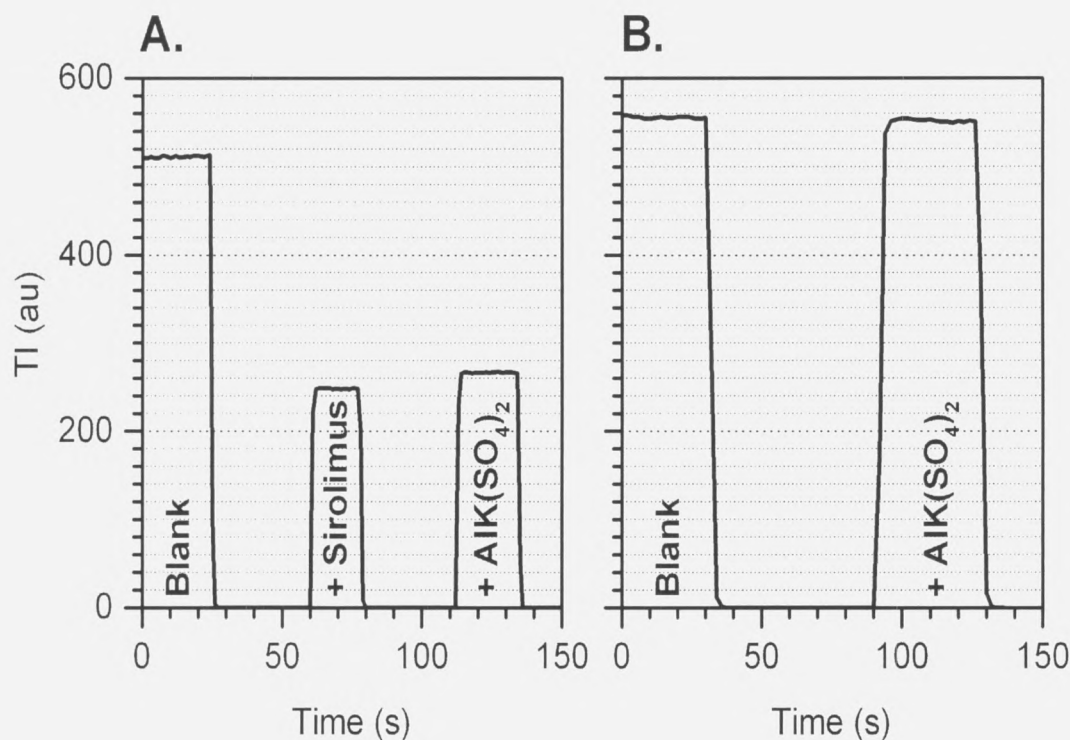


Figure 5. Selected metal ions and EDTA decrease absorbance of Sirolimus.



A. Absorbance of Sirolimus+Metal Ion (a representative experiment).

Absorbance was measured with the Hitachi F-7000 in the time scanning mode. After taking the blank measurement (Blank) containing 2 mL of water in a 1 cm quartz cuvette, 2  $\mu$ L of 10 mM Sirolimus in DMSO was added to the cuvette at approximately 60 seconds, and measured for about 20 seconds. Then, 2  $\mu$ L of 100 mM AlK(SO<sub>4</sub>)<sub>2</sub> was added at approximately 110 seconds and measured for about 20 seconds. Similar protocol was used with CuSO<sub>4</sub>, Fe(NO<sub>3</sub>)<sub>3</sub>, MnCl<sub>2</sub>, ZnSO<sub>4</sub>, and EDTA. In this experiment, the Blank intensity was 511.2, 248.0 after addition of Sirolimus, and 267.0 after addition of Alum. This corresponds to Sirolimus absorbance of  $\lg(511.2/248.0)=0.314$ , and

$\lg(511.2/267.0)=0.282$  after addition of Alum, a decrease in  $0.314-0.282=0.032$

absorbance units. TI, transmission intensity.

*B. Absorbance of metal ions (a representative experiment).*

Control experiment for A. Absorbance was measured with the Hitachi F-7000 in the time scanning mode. After taking the blank measurement (Blank) containing 2 mL of water in a 1 cm quartz cuvette, 2  $\mu$ L of 100 mM  $\text{AlK}(\text{SO}_4)_2$  was added at approximately 90 seconds and measured for about 20 seconds. Similar protocol was used with  $\text{CuSO}_4$ ,  $\text{Fe}(\text{NO}_3)_3$ ,  $\text{MnCl}_2$ ,  $\text{ZnSO}_4$ , and EDTA. In this experiment, the Blank intensity was 556.0, and 552.0 after addition of Alum. This corresponds to Alum absorbance of  $\lg(556.0/552.0)=0.003$ . TI, transmission intensity.

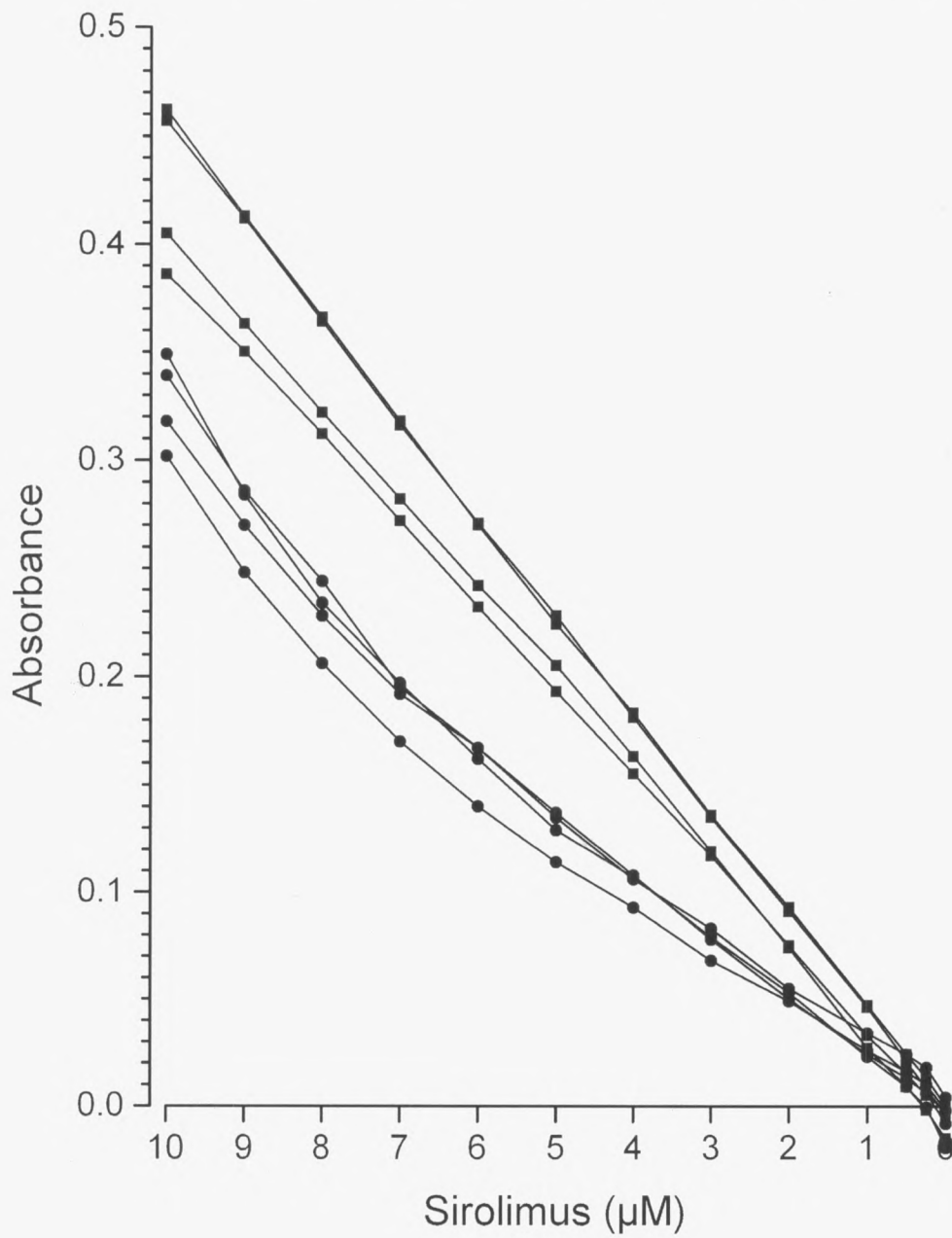
*C. Summary Table.*

The table summarizes the effects of other selected metal ions and EDTA on absorbance of Sirolimus obtained similarly to the panel A (Sirolimus, Sirolimus+Metal) and the panel B (Metal). n, number of experiments; SD, standard deviation; *P*, Student *t*-test *p* value. The last but one column shows the corrected changes resulted from interaction of Sirolimus and metal ion.

| Metal                      | n  | Sirolimus | SD    | Sirolimus+<br>Metal | SD    | Metal (Mean $\pm$ SEM)   | (Sirolimus+Metal)-<br>(Sirolimus)-(Metal) | <i>p</i> |
|----------------------------|----|-----------|-------|---------------------|-------|--------------------------|---|----------|
| Alum                       | 10 | 0.370     | 0.057 | 0.333               | 0.052 | 0.004 $\pm$ 0.003 (n=4)  | -0.041                                    | <0.      |
| $\text{CuSO}_4$            | 4  | 0.387     | 0.032 | 0.356               | 0.022 | -0.003 $\pm$ 0.001 (n=4) | -0.029                                    | <0.      |
| $\text{Fe}(\text{NO}_3)_3$ | 4  | 0.395     | 0.026 | 0.578               | 0.022 | 0.219 $\pm$ 0.011 (n=9)  | -0.037                                    | <0.      |
| $\text{MnCl}_2$            | 4  | 0.370     | 0.021 | 0.342               | 0.025 | -0.002 $\pm$ 0.001 (n=4) | -0.026                                    | <0.      |
| $\text{ZnSO}_4$            | 11 | 0.347     | 0.062 | 0.314               | 0.061 | 0.002 $\pm$ 0.003 (n=4)  | -0.035                                    | <0.      |
| EDTA                       | 4  | 0.369     | 0.067 | 0.324               | 0.063 | -0.001 $\pm$ 0.001 (n=4) | -0.045                                    | <0.      |

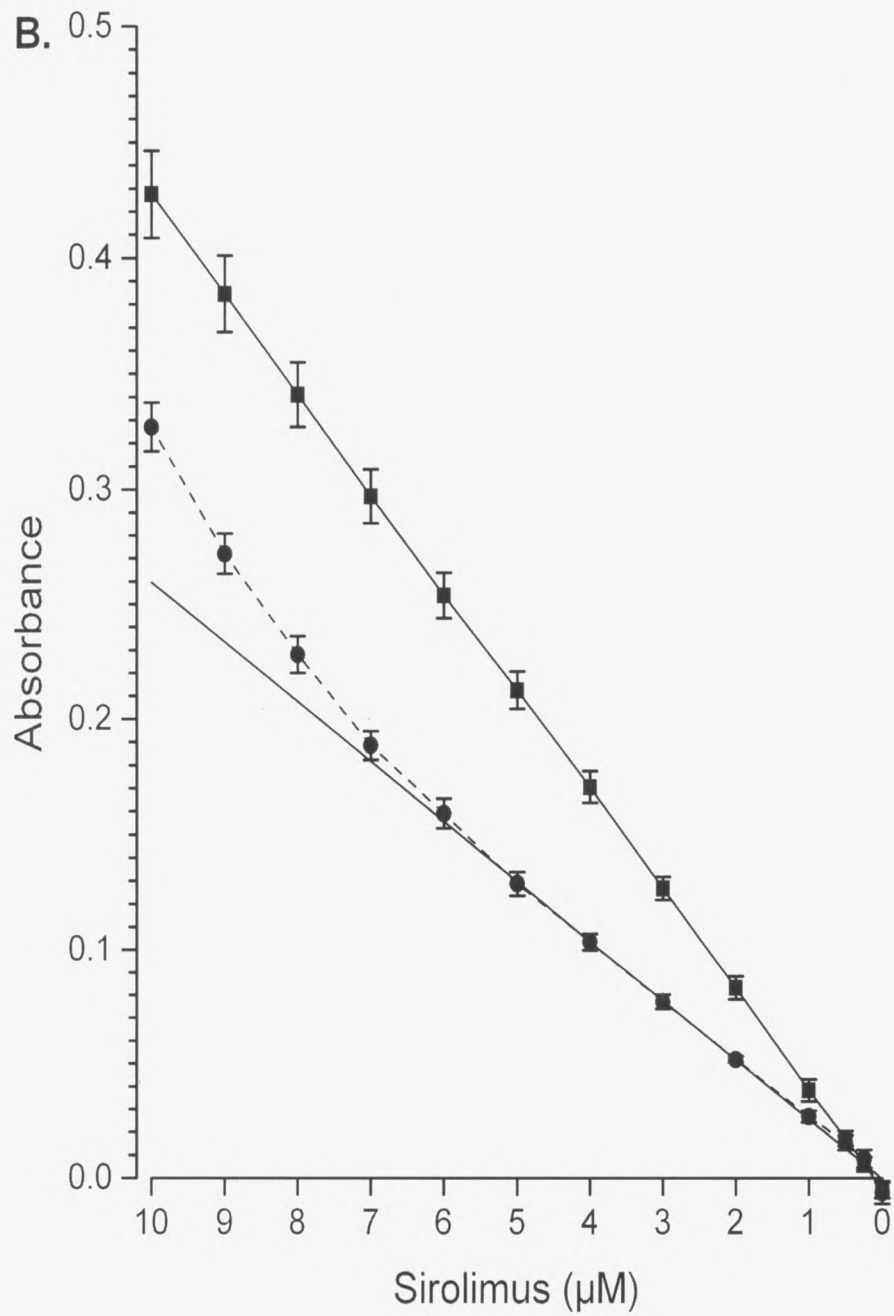
Figure 6. Dilution of Sirolimus.

A. Individual runs of Sirolimus absorbance in DMSO (squares), and water (circles).





B. Means $\pm$ SEM of Sirolimus absorbance in the panel A.



C.  $R^2$  of the linear fit for indicated ranges of Sirolimus concentrations.

| Sirolimus ( $\mu\text{M}$ ) | $R^2$ |
|-----------------------------|-------|
| 0-10                        | 0.984 |
| 0-9                         | 0.991 |
| 0-8                         | 0.996 |
| 0-7                         | 0.998 |
| 0-6                         | 0.998 |
| 0-5                         | 0.996 |
| 0-4                         | 0.994 |
| 0-3                         | 0.987 |

D. Dilutions in DMSO (squares), water (circles), and in water in the presence of Alum (triangles).

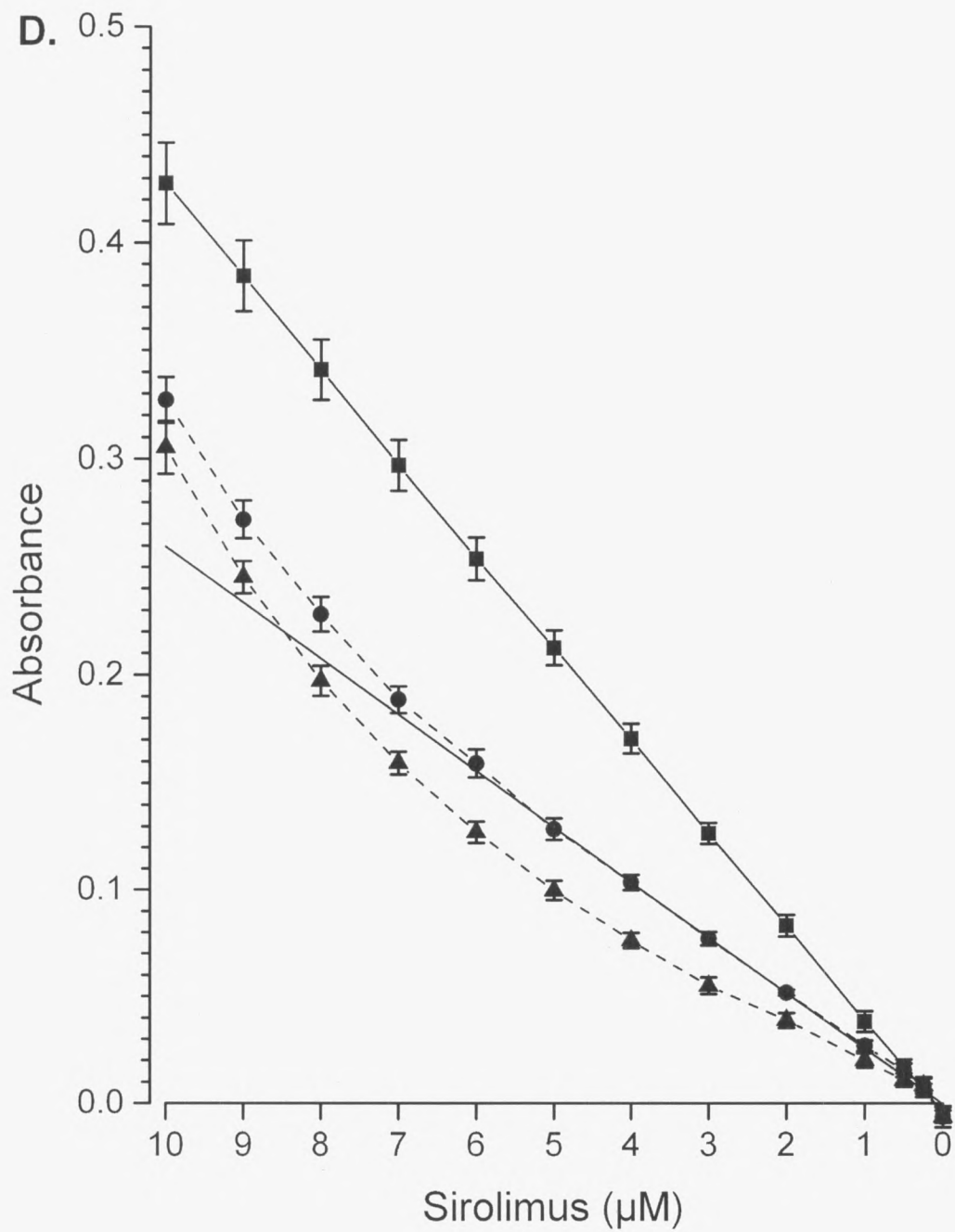
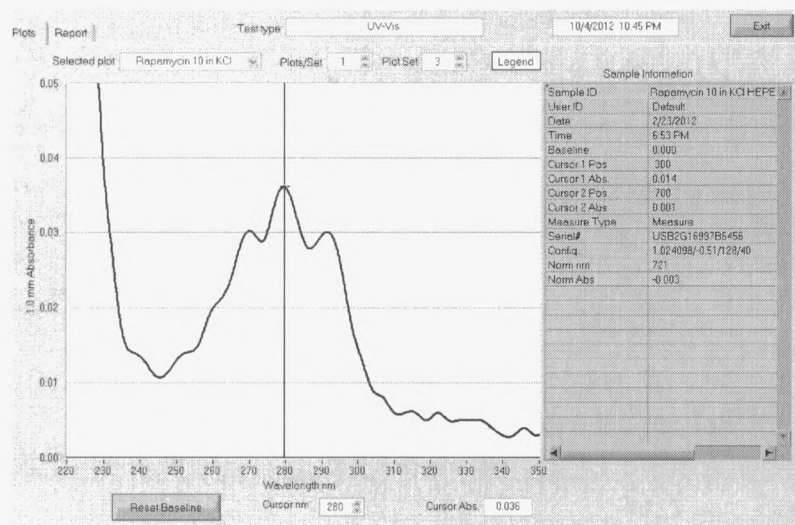


Figure 7. UV spectra of Sirolimus.

Note that the absorbance units in this figure is reported for the 1.0 mm light path, and should be multiplied by 10 to be converted to the 1.0 cm light path.

### A. Initial Reading



### B. 20 Minute Reading

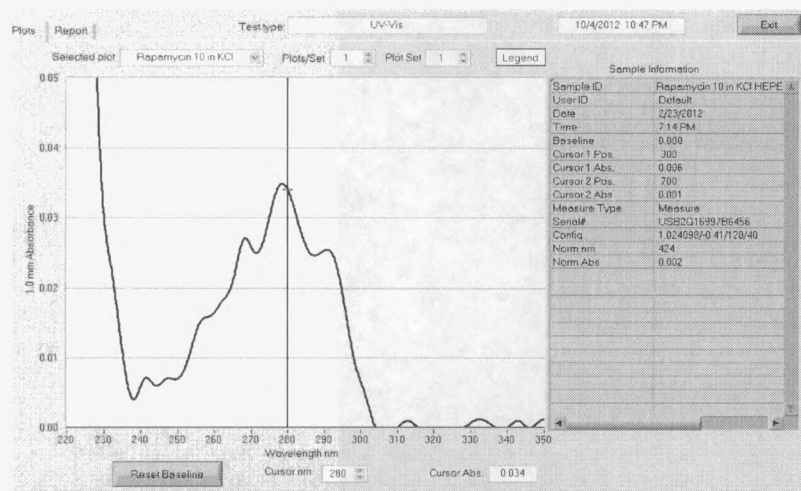
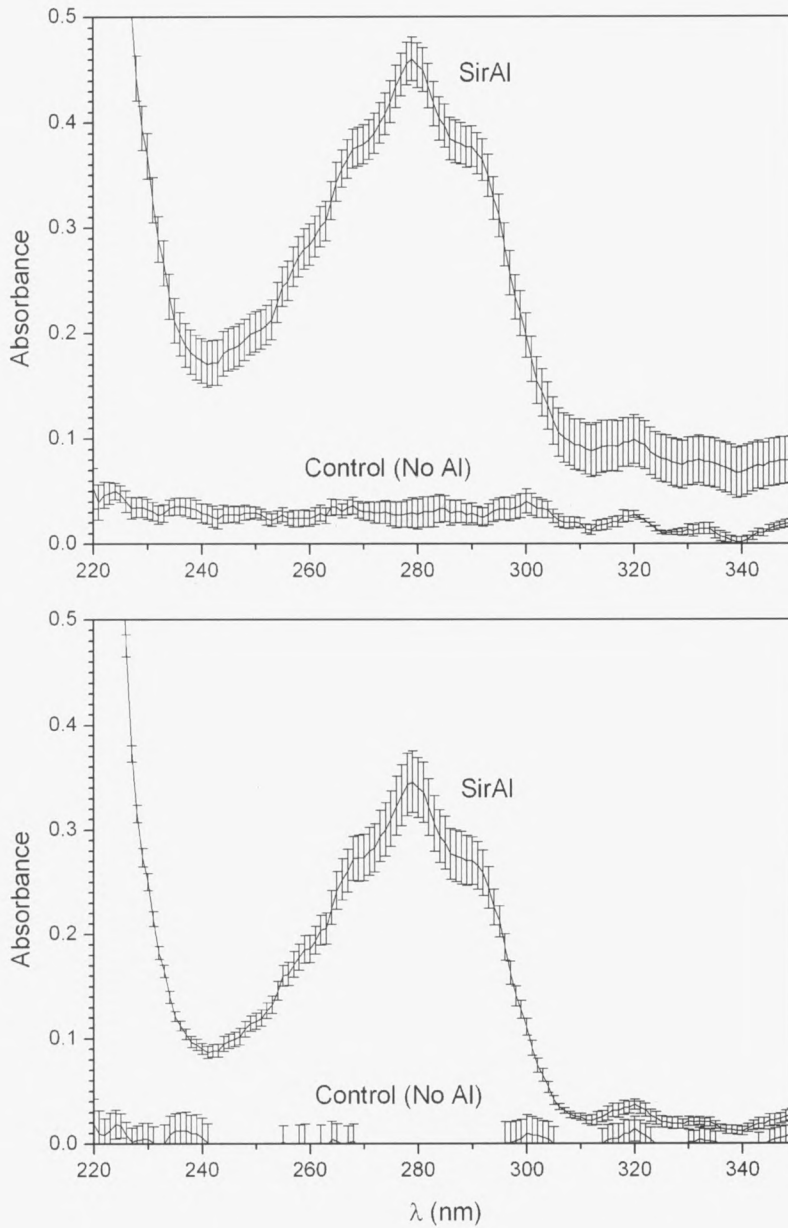


Figure 8. UV spectra of Sirolimus-Aluminum complex (SirAl).

Two representative experiments of eight are depicted.

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