Development of Potential Orphan Drug Therapy of Intravesical Liposomal Tacrolimus for Hemorrhagic Cystitis Due to Increased Local Drug Exposure

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Purpose: The potent immunosuppressive effect of systemic tacrolimus is limited by the high incidence of severe adverse effects, including nephrotoxicity and hypertension. Intravesical application of tacrolimus is hindered by its poor aqueous solubility, justifying the search for novel delivery platforms such as liposomes. We evaluated the pharmacokinetics of tacrolimus encapsulated in liposomes (lipo-tacrolimus), which is being developed as a potential orphan drug indication for hemorrhagic cystitis.

Materials and Methods: A single dose of lipo-tacrolimus was instilled in the bladder with the rat under anesthesia. Also, tacrolimus was instilled intravesically or injected intraperitoneally in other rat groups. The tacrolimus dose was constant in all formulations at 200 μg/ml. At different times blood, urine and bladder samples were collected and stored at −80C until analysis. Tacrolimus levels in samples were analyzed using microparticle enzyme immunoassay II.

Results: The AUC of lipo-tacrolimus in serum at 0 to 24 hours was significantly lower than that of tacrolimus instillation or injection. Noncompartmental pharmacokinetic data analysis revealed maximum concentration of lipo-tacrolimus and tacrolimus in serum and urine at 1 and at 2 hours, respectively. Urine $AUC_{(0-24)}$ after intravesical administration was significantly higher than in the intraperitoneal group ($p < 0.05$). Bladder tacrolimus $AUC_{(0-24)}$ did not differ significantly between the groups.

Conclusions: Single dose pharmacokinetics revealed that bladder instillation of liposome encapsulated tacrolimus significantly decreased systemic exposure to instilled tacrolimus as well as vehicle related toxicity. Intravesical liposomal tacrolimus may be a promising approach as an orphan drug indication for hemorrhagic cystitis.

Key Words: urinary bladder, cystitis, tacrolimus, liposomes, pharmacokinetics

Abbreviations and Acronyms

LP = liposome

Tmax = time to peak concentration

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TACROLIMUS (FK506) is a potent hydrophobic immunosuppressive agent that hinders interleukin-2 dependent T-cell activation by inhibiting calcineurin phosphatase.[1] Tacrolimus has a direct inhibitory effect on cell mediated immunity but systemic administration is limited by the high incidence of severe adverse effects, including nephrotoxicity and hypertension.[2,3] Site specific tacrolimus treatment was efficacious as an ointment or

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lotion formulation for inflammatory skin conditions without systemic side effects.[4]

The restriction of immune response mechanisms to the targeted site or organ[5] by topical therapy using potent immunosuppressive drugs prompted us to investigate bladder instillation of tacrolimus for immunogenic cystitis or sterile hemorrhagic cystitis. There is a reported 5.35% incidence of hemorrhagic cystitis in patients treated with cyclophosphamide and ifosfamide, and a lower incidence of hemorrhagic cystitis due to radiation requiring therapy. The estimated prevalence of hemorrhagic cystitis is less than 80,000 cases in the United States.[6]

However, tacrolimus delivery in the bladder is hindered due to its poor aqueous solubility. LPs were used in the past as pharmaceutical nanocarriers to deliver poorly water soluble drugs.[7,8] LPs are vesicles composed of concentric phospholipid bilayers separated by aqueous compartments.[9] LPs can serve as vehicles for drug and gene delivery because they adsorb to cell surfaces and fuse with cells.[10,11] The ability of LPs to form a molecular film on cell surfaces led to their use in healing wounds and injured uroepithelium.[11–13]

An earlier report from our laboratory demonstrated local immunosuppression produced by intravesical delivery of tacrolimus using LPs in a rat model of sterile hemorrhagic cystitis.[5] From the therapeutic point of view it is important to quantify the drug concentration in urine and drug penetration in the site of action. In the current study we hypothesized that the improved therapeutic efficiency of liposomal tacrolimus is due to a sustained therapeutic concentration in urine and bladder tissue, and decreased systemic absorption. We tested the hypothesis by evaluating the single dose pharmacokinetics of lipo-tacrolimus after intravesical administration compared with the pharmacokinetics of a plain tacrolimus formulation administered intravesically and systemically.

MATERIALS AND METHODS

Preparation of LPs, Lipo-Tacrolimus and Plain Tacrolimus

A 5 mg/ml stock solution of tacrolimus (Sigma®) was prepared in methanol (Thermo Fisher Scientific, Pittsburgh, Pennsylvania). Dehydrated LPs (1.8 mg in 1 ml) were dispersed in physiological saline, in which dispersion is in liposomal form. LPs encapsulating tacrolimus (lipo-tacrolimus) were prepared by loading a dried film of 200 μg tacrolimus (Sigma) into the LP dispersion. Briefly, a stock methanolic solution of tacrolimus (80 μl) was dried by nitrogen in a glass tube to remove methanol. After the tube was dry with tacrolimus sticking to the inside wall, 3.6 mg lyophilized lipid powder were added to the tube. Deionized water (2 ml) was then added. The tube was vortexed vigorously for 15 minutes, frozen at −20C for 1 hour and vortexed again to achieve a turbid LP suspension. For the plain tacrolimus formulation the stock solution of tacrolimus in methanol was diluted 25-fold in normal saline to 200 μg/ml and used in animal experiments.

Drug Administration

All experimental procedures were reviewed and approved by the Kaohsiung Chang Gung Memorial Hospital institutional animal care and use committee. Rats were divided into group 1—6 treated with intravesical lipo-tacrolimus, group 2—5 treated with intravesical tacrolimus and group 3—4 treated with intraperitoneal tacrolimus. An intravesical catheter was tied in place by a ligature around the urethral orifice with the rat under isoflurane anesthesia. The bladder was emptied of urine and instilled with 1 ml lipo-tacrolimus or tacrolimus (containing 200 μg tacrolimus) for 1 hour via the catheter. The intraperitoneally injected group received the same tacrolimus dose (200 μg) while under isoflurane anesthesia. The tacrolimus dose was uniform in all groups at 200 μg.

Serum, Urine and Bladder Pharmacokinetics

Baseline urine and blood samples were collected from all experimental rats. After drug administration, the rats were sacrificed at 1, 2, 4, 8, 12 and 24 hours, respectively, to collect venous blood. Urine samples were collected from awake rats kept in a metabolic cage and at sacrifice by a urethral catheter. For tissue harvesting the bladder was emptied and excised at 1, 2, 8, 12 and 24 hours, respectively. The rats were deeply anesthetized using pentobarbital and sacrificed by transcardiac perfusion with Krebs buffer, followed by 4% paraformaldehyde fixative. The rats were then dissected to harvest the bladder.[5] To estimate tacrolimus levels, samples were stored at −80C until analysis.

Tacrolimus Assay

Serum and urine samples, and the bladders of rats instilled intravesically with lipo-tacrolimus or tacrolimus and rats injected intraperitoneally with tacrolimus were maintained at −80C until analysis. The microparticle enzyme immunoassay II for tacrolimus was performed on an IMX Analyzer (Abbott Laboratories, Abbott Park, Illinois) according to manufacturer instructions.[5]

Histopathology

The bladder was fixed in 10% buffered formaldehyde for 24 to 48 hours and embedded in paraffin. Bladder tissue for histology was cut in 3 μm pieces and stained with hematoxylin and eosin.

Statistical Analysis

AUC was calculated using the linear trapezoidal rule. Quantitative data are shown as the mean ± SEM. Statistical analysis was performed using the Student t test with $p < 0.05$ considered significant using the Bonferroni correction for multigroup comparison.

RESULTS

Pharmacokinetics

Serum. Serum levels were detectable at all time points until 24 hours after the initiation of treat-

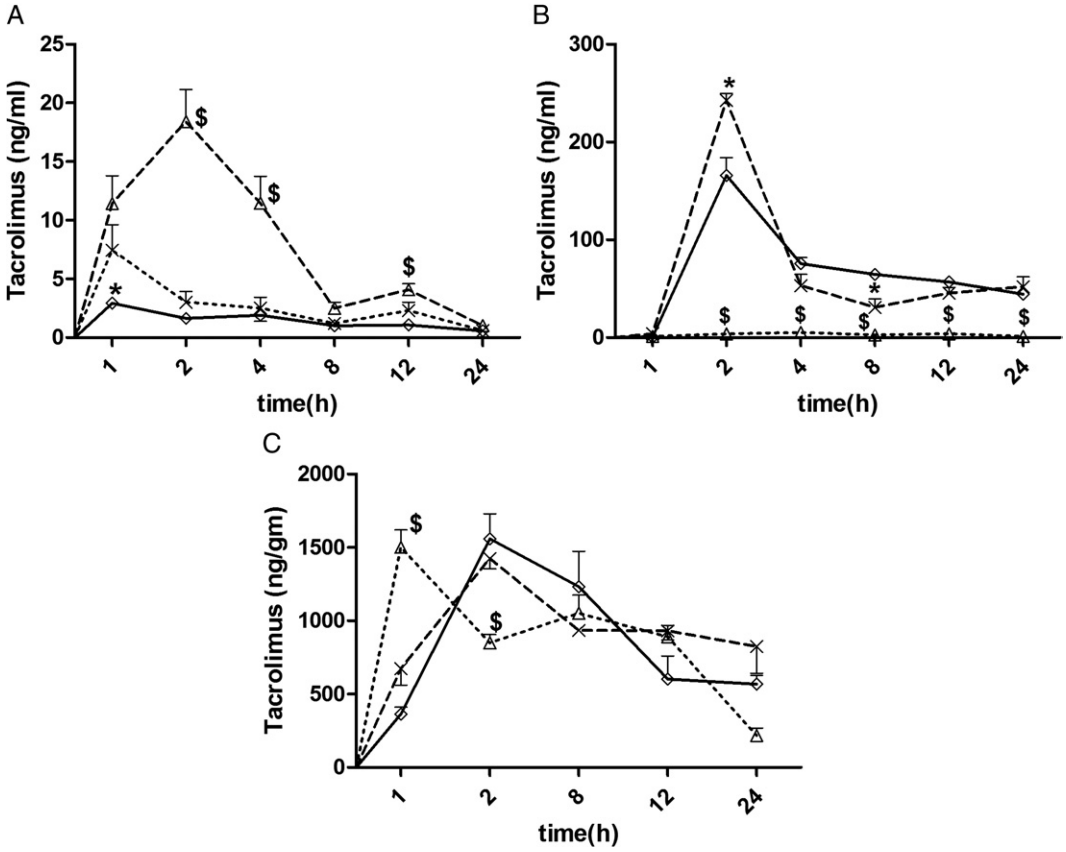

Figure 1. Concentration-time profiles in rats in serum (*A*), urine (*B*) and bladder tissue (*C*) measured after administration of intravesical lipo-tacrolimus (solid line, diamonds) and tacrolimus (dashed line, ×), and intraperitoneal (dashed line, triangles) tacrolimus. Data points represent mean ± SEM.*h*, hours. Asterisk indicates intravesical lipo-tacrolimus vs intravesical tacrolimus p ≤0.05. Dollar sign indicates intravesical vs intraperitoneal tacrolimus p ≤0.05.

ment in rats administered lipo-tacrolimus, and intravesical and intraperitoneal tacrolimus. The time vs concentration graph of tacrolimus showed that the serum tacrolimus concentration after a single intraperitoneal administration was significantly higher at 2, 4 and 12 hours than that in rats instilled intravesically with tacrolimus or lipo-tacrolimus (p <0.05, fig. 1, *A*). The table shows the AUC of serum tacrolimus in the groups. The intravesical tacrolimus AUC was 4.5-fold lower than the intraperitoneal tacrolimus AUC. Lipo-tacrolimus showed lower concentration at all points and the level was significantly lower at 1 hour than that of intravesical tacrolimus (p ≤0.05). For lipo-tacrolimus and intravesical tacrolimus T_{max} was attained at 1 hour, while for intraperitoneal tacrolimus it was attained at 2 hours.

Tacrolimus $AUC_{(0-24)}$

	Mean ± SEM $AUC_{(0-24)}$ (ng/ml/gm/hr)*		
	Serum	Urine	Bladder
Intravesical lipo-tacrolimus	25.72 ± 4.02	384.87 ± 16.91	20,033.81 ± 2,621.6
Tacrolimus:			
Intravesical	42.55 ± 4.26	399.76 ± 19.77	22,381.67 ± 792.4
Intraperitoneal	192.57 ± 11.75	114.27 ± 3.09	17,412.86 ± 1,233.0

* Serum lipo-tacrolimus vs intravesical and intraperitoneal tacrolimus, serum intravesical vs intraperitoneal tacrolimus, urine lipo-tacrolimus vs intraperitoneal tacrolimus, urine intravesical vs intraperitoneal tacrolimus and bladder intravesical vs intraperitoneal tacrolimus p ≤0.05.

Urine. Figure 1, *B* shows urine tacrolimus levels in the groups. After intraperitoneal injection, a significantly lower tacrolimus concentration was achieved in urine than in the groups treated intravesically with tacrolimus and lipo-tacrolimus (p ≤0.05). The AUC in urine for intraperitoneal injection was 3.5-fold less than in the groups treated with instillation. At the 2-hour point comparison of the intravesical instillation groups revealed a significantly lower urine drug concentration in the lipo-tacrolimus group than in the tacrolimus group (p ≤0.05). However, at later time points levels in the lipo-tacrolimus group were higher, including significantly higher at 8 hours (p ≤0.05), suggesting sustained drug delivery. The table shows that the AUC of the intravesical lipo-tacrolimus and tacrolimus groups

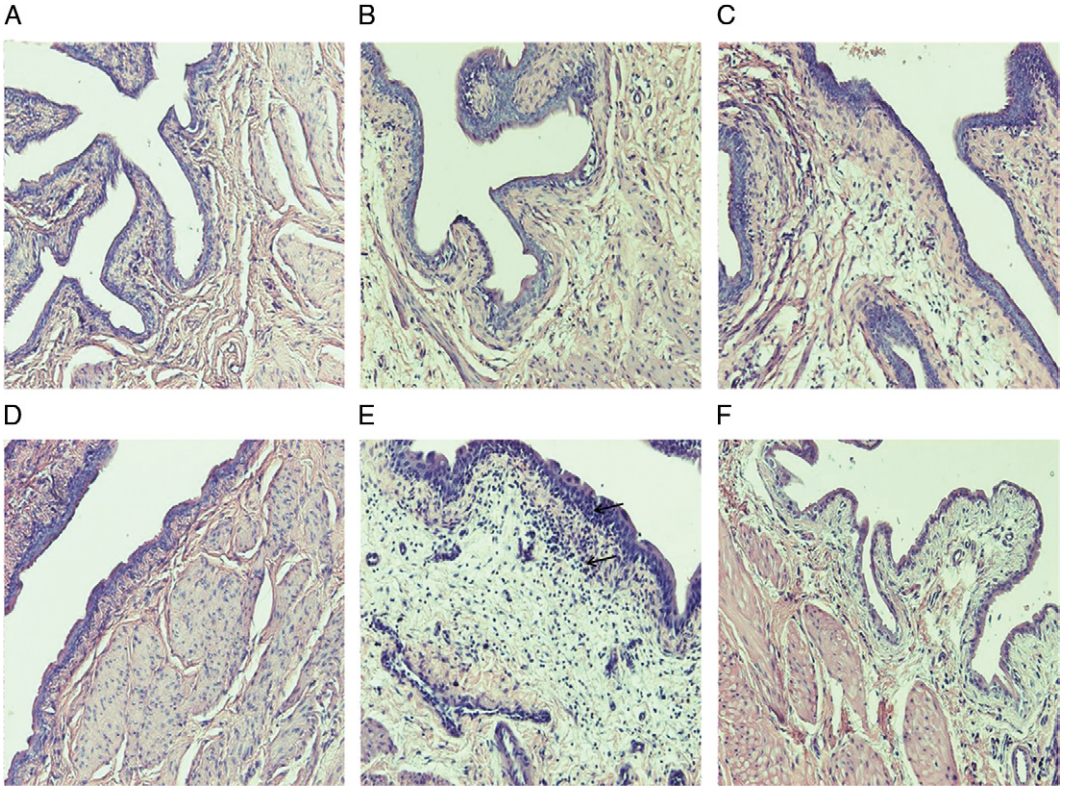

Figure 2. Histopathology in rats after intraperitoneal injection (*A* and *D*) and instillation (*B* and *E*) of tacrolimus, and lipo-tacrolimus instillation (*C* and *F*) in rats sacrificed 1 (*A* to *C*) and 24 (*D* to *F*) hours after instillation. Infiltration of large numbers of mononuclear inflammatory cells suggested that bolus delivery of tacrolimus dose with alcohol induced bladder inflammation (*E* and *F*). Photographs taken at 24 hours in other 2 groups were histologically indistinguishable from rat bladder harvested at 1 hour. Reduced from ×100.

was significantly higher than the AUC of the intraperitoneal tacrolimus group ($p \leq 0.05$), although no significant difference was found between the intravesical lipo-tacrolimus and tacrolimus groups.

Bladder. There was no significant difference in the tissue distribution profile in the intravesical lipo-tacrolimus and tacrolimus groups. The bladder distribution of tacrolimus in the intraperitoneally injected group showed rapid absorption at 1 hour, significantly greater than in the intravesical group ($p \leq 0.05$, fig. 1, *C*). From hour 2 it showed a clearance phase, which continued until the 24-hour time point. AUC calculations revealed no significant difference between the groups except for intravesical vs intraperitoneal tacrolimus (see table).

Histopathology

The safety of tacrolimus instilled locally in the bladder using different formulations was assessed by histopathology. No visible differences in histopathology across the groups were noted in rats sacrificed at 1 hour but differences were identified in the bladders of rats sacrificed at 24 hours (fig. 2). Infiltration of a large number of mononuclear inflammatory cells was noted between the tacrolimus and lipo-tacrolimus groups (fig. 2, *E* and *F*), suggesting that bolus delivery of the tacrolimus dose using alcohol as the carrier induced inflammation. Photographs taken at 24 hours in other 2 groups were histologically indistinguishable from rat bladders harvested at 1 hour. Since the tacrolimus dose was constant in all formulations, improved histopathology emphasized the safety and biocompatibility of LPs as a tacrolimus delivery platform (fig. 2, *F*).

DISCUSSION

Tacrolimus is a clinically effective immunosuppressive agent for inflammatory disorders.[1,4] However, its efficacy after systemic administration is limited by drug related side effects, such as hypertension, renal failure and neurotoxicity.[2,3] Previous studies showed that topical administration of tacrolimus did not compromise its anti-inflammatory efficacy, while decreasing its systemic side effects.[4,14]

Such encouraging reports prompted us to develop the local administration of LP encapsulated tacrolimus as a potential treatment for immunogenic cystitis or hemorrhagic cystitis.[5] Sterile hemorrhagic cystitis is a serious condition with significant unmet

medical needs. It is found in patients who received 1 or each of 2 specific chemotherapy agents (cyclophosphamide and ifosfamide) or pelvic radiation. In a previous study our group observed that intravesical lipo-tacrolimus attenuated hemorrhage, inflammatory reaction and an overactive micturition pattern in cyclophosphamide induced hemorrhagic cystitis.[5] In the current study we investigated the mechanism of improved efficacy of a liposomal formulation of tacrolimus by studying its pharmacokinetics in serum, bladder tissue and urine. In addition, we evaluated lipo-tacrolimus local tissue toxicity and biocompatibility in normal rats by histopathology.

Although tacrolimus after bladder instillation showed higher drug exposure in serum, liposomal tacrolimus inverted this phenomenon in favor of an increased drug concentration in urine and bladder tissue. Decreased systemic exposure caused by LP encapsulation was also evident in the 2.5-fold lower serum levels at 1 hour for lipo-tacrolimus relative to tacrolimus (mean 2.93 ± 0.25 vs 7.44 ± 2.18 ng/ml, fig. 1, *A*). Furthermore, the AUC of lipo-tacrolimus was 1.7-fold lower than that of tacrolimus (mean 42.55 ± 4.26 ng/ml per hour). Lower serum tacrolimus due to LP formulation is expected to cause lesser drug related adverse effects (neurotoxicity and neuropathy).[2,3] Decreased systemic exposure of tacrolimus can be partly explained by higher drug exposure in bladder tissue. Serum T_{max} in the intravesical groups was attained at 1 hour, while T_{max} in tissue and urine was attained at 2 hours in the same groups. In contrast, serum T_{max} in the systemic group was achieved at 2 hours. The different timing of T_{max} across the groups probably indicates differences in the absorption mechanisms of the 2 routes investigated (fig. 1, *A*).

Since the drug concentration in urine was also available for bladder uptake, we then determined urine pharmacokinetics in all groups (fig. 1, *B*). A higher tacrolimus concentration in the groups instilled with lipo-tacrolimus persisted up to 12 hours relative to that in the group instilled with tacrolimus. The prolonged higher urinary concentration of tacrolimus in the lipo-tacrolimus group was consistent with the slow drug release provided by LP encapsulation, as previously reported in penile skin.[15]

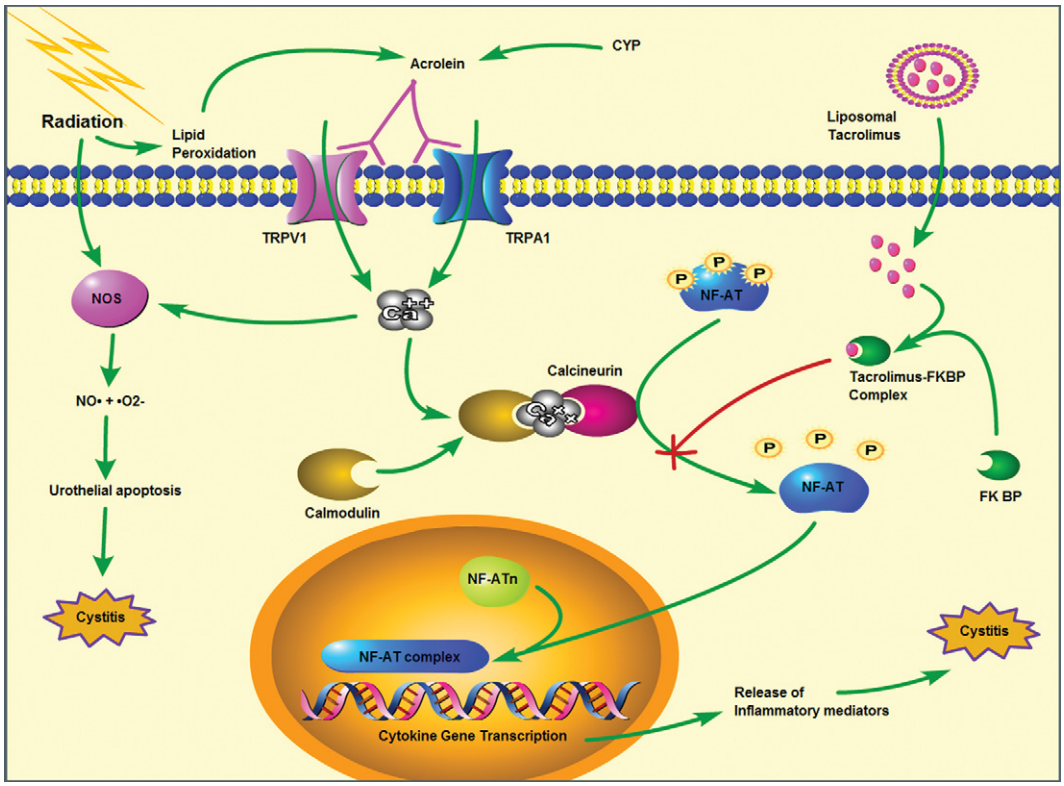

Figure 3. Possible mechanisms of action of radiation and cyclophosphamide (*CYP*) induced inflammation leading to hemorrhagic cystitis and possible role of tacrolimus liposomal formulation. Chemotherapy agent cyclophosphamide is converted to acrolein, which activates urothelial transient receptor potential cation channel (*TRPV1* and *TRPA1*) receptors, causing calcium influx and cytokine gene transcription via calcineurin dependent nuclear factor of activated T cells (*NF-AT*) pathway. Radiation can have similar effect by causing membrane lipid peroxidation, leading to acrolein formation and activating inflammatory pathways. Radiation can induce cytoplasmic free radical formation, leading to oxidative damage and inflammation. Tacrolimus-FK506 binding protein (*FKBP*) complex inhibits dephosphorylation of nuclear factor of activated T cells (*NF-AT*), preventing formation of nuclear factor of activated T cells complex and suppressing transcription of several cytokine genes. *NOS*, nitric oxide synthase. *NO*• + •*O2*−, free radicals. *P*, phosphate.

The higher AUC in serum (192.57 ± 11.75 ng/ml per hour) was complemented by the lowest AUC in urine (114.2 ± 3.09 ng/ml per hour) in rats with systemic injection of tacrolimus. Serum protein binding of tacrolimus may also explain the lower levels in urine.

Tissue pharmacokinetics revealed that tacrolimus was retained in bladder tissue up to 24 hours in all groups without a significant difference in the AUC (fig. 1, *C*). However, there was a significant increase at 1 hour in the intraperitoneal injection group, which may have been caused by the high concentration in serum from which the drug was absorbed through the systemic circulation. Higher tissue levels of tacrolimus in the lipo-tacrolimus group may also be related to the protection of tacrolimus in the LPs from the efflux caused by P-glycoprotein expression in bladder tissue.[16] This protective effect of LPs against P-glycoprotein may explain the supplementary increase in the drug concentration in bladder tissue at the 2 and 8-hour points. The slight increase in the bladder tacrolimus concentration after intravesical plain tacrolimus administration may have been caused by disruption of the epithelial barrier due to the alcohol in the formulation.

Decreased infiltration of mononuclear inflammatory cells on histopathology in normal rats after lipo-tacrolimus instillation further revealed the superior tissue safety provided by LPs over an alcohol solution of tacrolimus (fig. 2, *F*). In contrast to alcohol,[17] LPs do not irritate the epithelial surfaces or induce an inflammatory reaction. The lack of a difference between the groups in histopathology images obtained at 1 hour show that 1 hour is too brief to cause inflammatory cell infiltration.

CONCLUSIONS

Based on these serum, urine and bladder pharmacokinetics data we propose that a single intravesical lipo-tacrolimus administration can yield pharmacologically active drug levels in the bladder with significantly decreased systemic levels that are maintained for at least 24 hours. Intravesical lipo-tacrolimus has the desirable attributes of higher residence in the bladder and retention in urine as well as significantly decreased systemic exposure of instilled tacrolimus and its resultant toxicity. Further studies are warranted to study pharmacokinetics in rats with induced hemorrhagic cystitis. Intravesical tacrolimus formulated in LPs may hold promise as an orphan drug therapy for hemorrhagic cystitis induced by radiation and chemotherapy (fig. 3).

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REFERENCES

1. Migita K and Eguchi K: FK 506-mediated T-cell apoptosis induction. Transplant Proc 2001; **33:** 2292.
2. Naesens M, Kuypers DR and Sarwal M: Calcineurin inhibitor nephrotoxicity. Clin J Am Soc Nephrol 2009; **4:** 481.
3. Akar Y, Yucel G, Durukan A et al: Systemic toxicity of tacrolimus given by various routes and the response to dose reduction. Clin Experiment Ophthalmol 2005; **33:** 53.
4. Ebert AK, Rosch WH and Vogt T: Safety and tolerability of adjuvant topical tacrolimus treatment in boys with lichen sclerosus: a prospective phase 2 study. Eur Urol 2008; **54:** 932.
5. Chuang YC, Tyagi P, Huang HY et al: Intravesical immune suppression by liposomal tacrolimus in cyclophosphamide-induced inflammatory cystitis. Neurourol Urodyn 2011; **30:** 421.
6. Perez-Brayfield MP and Kirsch AJ: Hemorrhagic Cystitis. Emedicine 2009. Available at http://emedicine.medscape.com. Updated September 18, 2009.
7. Fahr A, Van-Hoogevest P, Kuntsche J et al: Lipophilic drug transfer between liposomal and biological membranes: what does it mean for parenteral and oral drug delivery? J Liposome Res 2006; **16:** 281.
8. Fahr A and Liu X: Drug delivery strategies for poorly water-soluble drugs. Expert Opin Drug Deliv 2007; **4:** 403.
9. Reimer K, Fleischer W, Brogmann B et al: Povidone-iodine liposomes——an overview. Dermatology 1997; **195:** 93.
10. Giannantoni A, Di Stasi SM, Chancellor MB et al: New frontiers in intravesical therapies and drug delivery. Eur Urol 2006; **50:** 1183.
11. Tyagi P, Wu PC, Chancellor M et al: Recent advances in intravesical drug/gene delivery. Mol Pharm 2006; **3:** 369.
12. Fraser MO, Chuang YC, Tyagi P et al: Intravesical liposome administration—a novel treatment for hyperactive bladder in the rat. Urology 2003; **61:** 656.
13. Chuang YC, Lee WC and Chiang PH: Intravesical liposome versus oral pentosan polysulfate for interstitial cystitis/painful bladder syndrome. J Urol 2009; **182:** 1393.
14. Erdogan M, Wright JR and McAlister VC: Liposomal tacrolimus lotion as a novel topical agent for treatment of immune-mediated skin disorders: experimental studies in a murine model. Br J Dermatol 2002; **146:** 964.
15. Foldvari M, Oguejiofor C, Afridi S et al: Liposome encapsulated prostaglandin E1 in erectile dysfunction: correlation between in vitro delivery through foreskin and efficacy in patients. Urology 1998; **52:** 838.
16. Hoffmann AC, Wild P, Leicht C et al: MDR1 and ERCC1 expression predict outcome of patients with locally advanced bladder cancer receiving adjuvant chemotherapy. Neoplasia 2010; **12:** 628.
17. Trevisani M, Gazzieri D, Benvenuti F et al: Ethanol causes inflammation in the airways by a neurogenic and TRPV1-dependent mechanism. J Pharmacol Exp Ther 2004; **309:** 1167.