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RESEARCH PAPER

Pharmacokinetics and Biodistribution of Tacrolimus after Topical Administration: Implications for Vascularized Composite Allotransplantation

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ABSTRACT

Aim The high doses of oral tacrolimus (TAC) (1,2) necessary to prevent acute rejection (AR) after vascularized composite allotransplantation (VCA) are associated with systemic adverse effects. The skin is the most antigenic tissue in VCA and the primary target of AR. However, the short-term use of topical TAC (Protopic®), as an off-label adjunct to oral TAC, to treat AR episodes pro re nata (PRN), has yielded inconsistent results. There is lack of data on the pharmacokinetics and tissue distribution of topical TAC in VCA, that hampers our understanding of the reasons for unreliable efficacy. Toward this goal, we evaluated the ability of topical TAC to achieve high local tissue concentrations at the site of application with low systemic concentrations.

Materials and Methods We assessed the pharmacokinetics and tissue distribution of topical TAC (Protopic®, 0.03%) after single or repeated topical application in comparison to those after systemic delivery in rats. Animals received a single topical application of TAC ointment (Group 1) or an intravenous (IV) injection of TAC (Group 2) at a dose of 0.5 mg/kg. In another experiment, animals received daily topical application of TAC ointment (Group 3), or daily intraperitoneal (IP) injection of TAC (Group 4) at a dose of 0.5 mg/kg for 7 days. TAC concentrations in blood and tissues were analyzed by Liquid Chromatography–Mass Spectrometry (LC/MS-MS).

Results Following single topical administration, TAC was absorbed slowly with a Tmax of 4 h and an absolute bioavailability of 11%. The concentrations of TAC in skin and muscle were several folds higher than whole blood concentrations. Systemic levels remained subtherapeutic (< 3 ng/ml) with repeated once daily applications.

Conclusion Topical application of TAC ointment (Protopic®, 0.03%) at a dose of 0.5 mg/kg/day provided high concentrations in the local tissues with low systemic exposure. Repeated topical administration of TAC is well tolerated with no local or systemic adverse effects. This study confirms the feasibility of topical

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application of TAC for site specific graft immunosuppression and enables future applications in VCA.

KEY WORDS pharmacokinetics · tacrolimus · tissue distribution · topical delivery · vascularized composite allotransplantation

INTRODUCTION

The calcineurin inhibitor, tacrolimus (TAC, Prograf®, Astellas) is the primary immunosuppressive drug used in the prevention and treatment of acute rejection (AR) in solid organ transplant (SOT) patients as well as those receiving vascularized composite allografts (VCA) (1–3). Oral formulation of TAC has been used in dual or triple drug therapy regimens in combination with mycophenolate mofetil (MMF, CellCept ®, Roche), sirolimus (Rapamune ®, Pfizer) or prednisone in SOT (4–6) as well in VCA (7–12).

The oral bioavailability of TAC is only 25–30% due primarily to extensive intestinal and hepatic metabolism (13,14). When given orally in VCA patients, TAC whole blood concentrations are variable, with much of the drug concentrating into red blood cells instead of the lymphocytes (15) or the allograft tissues (16,17). Further, chronic use of oral TAC is associated with serious metabolic side effects, such as infections, and malignancy (5,18–22).

The skin represents a highly immunogenic, visible and accessible component of many VCA. Hand or face transplants are examples of transplants comprised of skin and/or mucosal components, offering unique opportunities for topical or graft-embedded treatments (site-specific therapies) as well as real-time graft surveillance with clinical observation and guided biopsy (23,24). Topical administration of TAC, via the skin or mucosa in VCA could improve effectiveness of the drug by predominantly concentrating the drug in the graft particularly in the skin, decreasing the systemic exposure, and consequentially off-target side effects. (25), including hand and face transplants. When used pro re nata (PRN), TAC ointment b.i.d (Protopic®, 0.1%) and clobetasol cream b.i.d (Temovate ®, 0.05%) were effective in reversing AR in both hand and face transplants, which are the most commonly performed VCA (26–28). In many VCA patients, Banff Grade 1–2 AR could be treated with topical TAC and clobetasol without increasing the dose of systemic immunosuppression (11,12).

The topical formulation of TAC (Protopic ®, Astellas) is available in two concentrations 0.1%, 0.03%, and has been proven as a safe and effective therapy in ophthalmology (corneal graft rejection) (29) and for immune dermatoses such as atopic dermatitis, contact allergic dermatitis, and psoriasis (30–35). Studies in pediatric and adult patients with atopic dermatitis demonstrate that systemic exposure (area under curve [AUC]) of TAC from Protopic ® ointment (both 0.03% and 0.1% concentration) is extremely low and is approximately 0.5% of values observed with oral doses in kidney and liver transplant patients (36,37). This makes topical TAC an attractive choice for treating AR in skin-containing VCA transplants as they share the same targets for therapy. After topical application, TAC inhibits expression of MHC-II antigens and prevents maturation of epidermal dendritic cells, which are key mediators of skin rejection after VCA (38,39). It also inhibits expression of co-stimulatory molecules (40) and causes apoptosis-induced depletion of T cells (41).

We hypothesized that topical application of TAC will facilitate higher concentrations in the local tissues while limiting systemic exposure. In this proof of concept study, TAC was administered topically to rat hind limbs to recapitulate local tissue vs. systemic whole blood drug exposure in rodents. We chose the rodent hind limb model as it the most commonly used in VCA immunosuppression studies evaluating therapeutic efficacy or toxicity (42–45). The efficacy of topical delivery of TAC has been investigated in experimental skin allograft and VCA models by our lab and other groups (23,46). However, there is scant data on the pharmacokinetics and biodistribution of TAC after single or repeated topical application. The objective of this study was to assess the pharmacokinetics and biodistribution (local tissue vs. blood) distribution of TAC after single or repeated topical application (Protopic®, 0.03%) in comparison to systemic delivery of TAC at a dose of 0.5 mg/kg body weight /day in rats.

MATERIALS, ANIMALS, AND METHODS

Chemicals and Reagents

TAC powder, Cremophor EL (Kolliphor®), and ethanol were obtained from Sigma–Aldrich (St. Louis, MO, USA). TAC was prepared in a vehicle of 0.8% ethanol, 0.2% Cremophor EL (Kolliphor®), and saline solution (Sodium chloride, 0.9% *w/v*, USP) for IV or intraperitoneal (IP) administration at a final concentration of 1 mg of TAC/ml. For systemic delivery, TAC solution was administered at a volume of 0.15 ml for a dose of 0.5 mg/kg body weight of rats (average body weight of 300 g). The amount of TAC ointment (Protopic®, 0.03%, 0.3 mg/g, Astellas, NJ) was calculated to be 0.5 g of ointment/rat for a dose of 0.5 mg of drug/kg body weight. This amount was estimated to be around 1 finger-tip unit [FTU] for a tube of Protopic ® with a 5 mm nozzle). (47–49) The ointment was applied evenly via a massaging motion on the hind limb of rat.

Animals

Experiments were performed in accordance with a protocol approved by the University of Pittsburgh Institutional Animal

Care and Use Committee (IACUC). Male Lewis [Lew]) rats aged 8 to 10 weeks and an average weight of 300 g were housed in a specific pathogen-free barrier facility and maintained in accordance with IACUC guidelines. All procedures were in a compliance with American Association for the Accreditation of Laboratory Animal Care (AALAC) recommendations and the principles set forth in the National Institute of Health Publication 80–23, Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966, as amended. Animals were housed individually, and plastic Elizabethan collars were used to prevent oral ingestion of the topical formulations and to prevent animal access to the application site.

Pharmacokinetics and Tissue Distribution after Single Topical Application of TAC Ointment

Lewis rats were assigned to two groups. Animals received a single topical application of TAC ointment (Protopic®, 0.03%) (Group 1), or an IV bolus injection (Group 2) at a dose of 0.5 mg/kg body weight. TAC ointment was applied on the right "application" hind limb and the left "contralateral" hind limb was used as internal control. Blood samples were collected by tail vein bleeding at 0, 2, 4, 6, 8, 12, and 24 h after drug administration and were analyzed for systemic TAC levels by Liquid Chromatography-Tandem Mass Spectrometry **(**LC-MS/MS) (50,51). Twenty-four hours after administrating topical TAC application, animals were sacrificed and skin, muscle, and draining lymph nodes (DLNs) were collected from the application limb and the contralateral limb for measurement of TAC concentration.

Pharmacokinetics and Tissue Distribution after Repeated Topical Application of TAC Ointment

In another experiment, the drug exposure (drug accumulation) in local tissues and blood after repeated application (once daily for 7 days) was evaluated. Animals were allocated to two groups. Animals received a daily topical dose of TAC ointment (Protopic®, 0.03%) (Group 3), or daily IP injection (Group 4) for 7 days. The administered dose of TAC in both groups was similar (0.5 mg/kg body weight). Blood samples were collected daily before administration of the next dose for measurement of TAC trough levels. Twenty-four hours after administration of the last dose, animals were sacrificed and tissues (skin, muscle, and DLNs) were collected from the application limb and the contralateral limb for measurement of TAC concentrations. The skin at the application site was visually examined for any signs of hair loss, clinical irritation, skin thinning, atrophy or breakdown.

Local Drug Distribution in the Skin and Muscle after Single Topical Application of TAC Ointment

In another experiment, the local drug distribution in skin and muscle over 24 h after topical application of TAC ointment was evaluated. Animals ($n = 4$) received a single topical dose of TAC ointment (Protopic®, 0.03%) applied at a dose of 0.5 mg/kg body weight. Skin and muscle samples were collected at 2, 6, 12, and 24 h post dose administration for measurement of TAC concentration. Before obtaining tissue samples, the skin was thoroughly wiped down with ethanol-soaked gauze to remove any traces of TAC on the skin surface.

Quantification of TAC in Blood by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Blood concentrations of TAC were analyzed by the LC-MS/MS method which is the standard assay used in human transplant patients and validated in our clinical laboratory. Fifty microliters of blood containing an unknown concentration of TAC was added to a conical centrifugation tube, followed by two hundred microliters of a solution of zinc sulfate heptahydrate ($ZnSO4 \cdot 7H2O$) to precipitate blood proteins. Five hundred microliter of acetonitrile containing an internal standard (ascomycin) at a concentration of 15 ng/ml was then added and the mixture was vortexed for two minutes. Samples were centrifuged in a benchtop microcentrifuge (Thermofisher, Rockford, IL) at a relative centrifugal force (RCF) of 13,226 x g for 3 min. The supernatant was collected into LCMS vials for analysis. Analysis was performed using a validated, reverse phased method for the detection of TAC in blood on a Waters micromass Quattro micro API mass spectrometer operated in a positive electrospray ionization mode, utilizing multiple reaction monitoring, after injection of 20 μL of sample. The Waters 2795 Alliance Separations Module was equipped with a nova-pack® C18 column, 2.1 × 10 mm cartridge (Waters # 186003523) heated to 55°C. Analytes were effectively separated using a gradient elution consisting of an aqueous mobile phase (95% $H2O$ / 5% MeOH) and an organic mobile phase (100% MeOH), at a flow rate of 0.6 mL per minute. Mobile phases also contained 0.1% formic acid ($CH2O2$) and 2 mM ammonium acetate. Monitored parent to product mass transitions for TAC and ascomycin were $821.63 \rightarrow 768.33$ and $809 \rightarrow 756$ m/z, respectively. TAC had a retention time of 1.2 min. The standard curve was linear for concentrations ranging from the limit of quantification (LoQ) value of 2 ng/ml up to concentration values as high as 40 ng/mL with an R2 value of 0.9996 (With the lower limit for R2 acceptability being defined as 0.99). Limit of detection (LoD) was 0.1 ng/ml. Both intra- and inter-day precision were acceptable (C.V. <10%, $n = 3$) at concentrations of 4.3, 15.7, and 24.6 ng/mL (52).

Quantification of TAC Concentration in Tissues

The methods used for measuring tissue concentration of TAC were developed and validated in our laboratory in different models of local immunosuppression (1,2,53) The skin sites for tissue sampling were wiped down three times with ethanol-soaked gauze to remove residual ointment on the surface of the skin. Skin and muscle were frozen with liquid nitrogen and pulverized in a pestle and mortar to fragment the frozen tissues samples into fine pieces. Frozen tissues were weighed and homogenized with cold methanol (1 ml) in homogenization tubes using Mini-BeadBeater-1 (Cole-Parmer North America) for cell disruption. The homogenate was sonicated for 1 h at 25°C and then kept overnight at 4°C to allow for the complete extraction of the drug from the tissues. The homogenate was transferred to an appropriately labeled microcentrifuge tube and samples were centrifuged in a benchtop microcentrifuge (Thermofisher, Rockford, IL) at an RCF of 313 x g for 10 min. The supernatant was transferred to a labeled glass vial and evaporated by sample concentrator and the drug residue was reconstituted with blood (1 ml). Tissue drug concentrations are expressed as ng/g of tissue weight. Extraction recovery of TAC from skin and muscle were 87% and 89%. To control for residual ointment on the skin, ointment was applied on limbs ($n = 4$) and immediately cleaned with ethanol-soaked gauze. Biopsies from skin were collected and analyzed for TAC concentration. The highest TAC concentrations from residual ointment that remained on the skin after wiping down with ethanol-soaked gauze were minimal (19 ± 9 ng/g) compared to the actual tissue concentrations.

Pharmacokinetic Analysis

Descriptive pharmacokinetic parameters for TAC after topical and/or systemic administration were estimated by non-compartmental analysis using Phoenix WinNonlin® 6.1 (Certara, St. Louis, MO). The following pharmacokinetic/exposure parameters were obtained directly from the concentration-time profiles: Observed maximum blood level (Cmax), and observed trough blood level (Ctrough), area under the blood concentration-time curve extrapolated to time infinity, AUC0-∞ (calculated as AUC0-t + Ct/λz, where Ct is concentration at time t and λz is terminal elimination rate constant). The bioavailability of TAC after topical administration was obtained as F = (AUC0-∞ topical). (i.v. dose)/(AUC0-∞ i.v.).(Topical dose). Tissue to blood concentration ratios were obtained as tissue concentration/blood concentration. The accumulation ratio R(ac) was obtained as AUC0-t, ss after repeated once-daily doses/AUC0-t, after first dose. The cut off values for non-, weak, moderate, and strong accumulation can be set at $R(ac) < 1.2$, $1.2 \leq R(ac) < 2$, $2 \leq R(ac) < 5$, and $R(ac) \geq 5$, respectively (54).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 statistical software for windows (GraphPad Software, La Jolla, CA, USA). Data sets were checked for normality. Student t test, Mann Whitney test, or Wilcoxon Matched pairs test was used for two groups, and analysis of variance (ANOVA) was used when one independent variable with greater than two conditions or treatments and outcomes was evaluated and compared. Post hoc test (Tukey) was used to do multiple comparisons. All experimental results were expressed as the mean ± standard deviation. A p value <0.05 was considered as statistically significant difference. Statistically significant data were presented as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; and $****P < 0.0001$. Statistical tests are specifically indicated under each figure.

RESULTS

Single Topical Application of TAC (0.03% Ointment, 0.5 Mg/Kg) Results in Significantly Lower Systemic Exposure, as Compared to Systemic Administration of the Same Dose

Pharmacokinetic studies were performed to evaluate the systemic exposure to TAC when the product was administered topically. The mean blood concentration-time profile of TAC after single topical application (0.03% ointment, 0.5 mg/kg) or IV bolus injection (0.5 mg/kg) is shown in Fig. 1. Following a IV bolus dose, TAC concentrations were high initially (40.6 ± 4.3 ng/ml), with concentrations declining quickly thereafter over time to reach low values (2.5 ± 0.4 ng/ml) at 24 h. Following a topical dose, peak TAC concentrations (2 ± 0.5 ng/ml) were reached between 2 and 4 h. Concentrations were low, and the lowest values (0.3 ± 0.1 ng/ml) were achieved at 24 h. The comparative non-compartmental pharmacokinetic exposure parameters of TAC derived from the blood concentrations-time data after topical and/or systemic administration including AUC0-∞, Cmax, Ctrough, and F after single topical application of TAC are presented in Table I. The AUC0-∞, Cmax, and Ctrough of TAC after topical administration were markedly lower than the values obtained after IV bolus of the same dose ($p < 0.0001$). The bioavailability of TAC after topical administration was 11%.

Single Topical Application of TAC (0.03% Ointment, 0.5 Mg/Kg) Results in Significantly Higher Local Tissue Concentrations, as Compared to Systemic Administration of the Same Dose

TAC concentrations in skin, muscle, and (DLNs) at 24 h after single application of TAC (0.03% ointment,

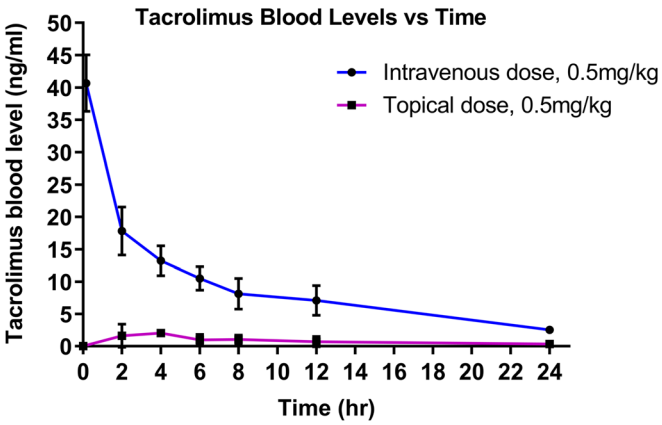

Fig. 1 TAC blood concentration–time profiles following single topical application of TAC (0.03% ointment, 0.5 mg/kg) or IV bolus (0.5 mg/kg). Data shown as mean + SD, $n = 6$/Systemic TAC group, and 5/Topical TAC group.

0.5 mg/kg) or IV bolus (0.5 mg/kg) is presented in Fig. 2. TAC concentrations in skin and muscle after single application of TAC ointment were significantly higher than the values obtained after IV administration of the same dose ($p < 0.05$).

Single Topical Application of TAC (0.03% Ointment, 0.5 Mg/Kg) Results in Significantly Higher Local Tissue Concentrations, as Compared to the Concentrations at the Contralateral Sites

TAC concentrations in the skin, muscle, and DLNs collected from the application limbs and contralateral limbs following a single application of TAC (0.03% ointment, 0.5 mg/kg) are presented in Fig. 3. TAC concentrations in skin, muscle, and DLNs collected from the application limbs are significantly higher than TAC concentrations in the skin, muscle, and DLNs collected from the other contralateral limbs ($p < 0.05$). The high tissue to blood concentration ratios indicates the direct permeation and accumulation of TAC into the local tissues including skin, muscle, and DLNs at the site of application after topical delivery. The tissue (skin, muscle, and DLNs) to blood ratios after topical delivery were significantly higher than the values observed after IV dose (303, 73, and 48 vs. 2, 4.6, and 3.2).

Time Course of TAC Concentrations in Skin and Muscle over 24 H after Single Topical Application of TAC (0.03% Ointment, 0.5 Mg/Kg)

The time course of TAC concentrations in the skin and muscle at 2, 6, 12, and 24 h following single application of TAC (0.03% ointment, 0.5 mg/kg) is shown in Fig. 4. Pharmacokinetic parameters of TAC in the skin and muscle following a single application of TAC (0.03% ointment, 0.5 mg/kg) are presented in Table III. Peak concentrations of TAC in skin and muscle were reached 2 h post topical dose administration (661 ± 141 and 69 ± 10 ng/g respectively), then TAC concentration gradually declined over time to reach low concentrations at 24 h post topical dose administration (171 ± 51 and 26 ± 9 ng/g respectively). Peak concentrations of TAC in blood were reached 4 h post-topical dose administration (2 ± 0.4 ng/ml), thn concentrations gradually declined over time to reach low concentration at 24 h post-topical dose administration (0.3 ± 0.1 ng/ml). Drug exposure (AUC0-α) in the skin and muscle was significantly higher than the values observed in the blood (**$p = 0.0075$, ***$p = 0.0004$). Drug exposure (AUC0-α) in the skin was significantly higher than the values observed in the muscle (****$p < 0.0001$). TAC concentrations in the blood was ≤2 ng/ml during this time period.

Table I Comparative non-compartmental pharmacokinetic exposure parameters (mean + s.d.) following single application of TAC (0.03% ointment, 0.5 mg/kg) or IV bolus of TAC (0.5 mg/kg)

Pharmacokinetic Parameters	Unit	IV administration	Topical administration
C_{max}	ng/ml	40.6 ± 4.3	2 ± 0.5
T_{max}	hr	0.16	4
C_{trough}	ng/ml	3 ± 0.4	0.3 ± 0.1
$AUC_{0\text{-}24h}$	ng.hr./ml	220 ± 29	21 ± 8
$AUC_{0\text{-}\infty}$	ng.hr./ml	254 ± 34	29 ± 9
F	%	100	11 ± 3

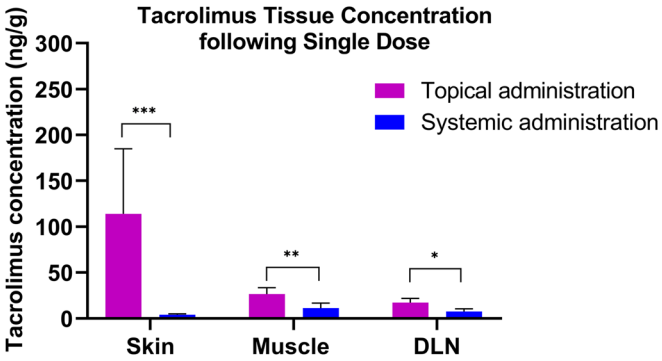

Fig. 2 TAC concentrations (ng/g) in skin, muscle, and DLNs at 24 h after single application of TAC (0.03% ointment, 0.5 mg/kg) or IV bolus (0.5 mg/kg). Asterisks indicate statistical significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$ significantly different from IV administration. Data presented as mean ± SD, $n = 6$. *P* values were calculated by Mann Whitney test.

Daily Topical Application of TAC (0.03% Ointment, 0.5 Mg/Kg) for 7 Days Results in Significantly Higher Local Tissue Concentrations and Lower Blood Levels as Compared to the Concentrations after Daily Systemic Administration of Same Dose

As shown in Fig. 5, the average TAC concentrations in blood, skin, muscle, and DLNs collected from the application leg at 24 h after the last (7th) topical application of TAC (0.03% ointment, 0.5 mg/kg) were significantly higher than the values observed after systemic administration of the same dose ($p < 0.05$), while average TAC concentrations in the blood were significantly lower than the values observed after systemic administration (1.5 ± 0.6 vs. 5.6 ± 0.6 ng/ml, $p < 0.001$). This indicates low systemic drug accumulation and exposure after repeated topical applications of TAC. Tissue to blood concentration ratios at 24 h after the last (7th) topical application of TAC ointment (0.03%, 0.5 mg/kg) or IP injection (0.5 mg/kg) following daily topical applications or IP injections of TAC for 7 days is presented in Table II. The high tissue to blood concentration ratios indicates the accumulation of TAC into the local tissues including skin, muscle, and DLNs after repeated topical application. The tissue to blood concentration ratios were significantly higher than the values observed after IP doses.

Daily Topical Application of TAC (0.03% Ointment, 0.5 Mg/Kg) for 7 Days Results in Significantly Higher Local Tissues Concentrations, as Compared to the Concentrations in the Contralateral Sites

TAC concentrations in skin, muscle, and DLNs collected from both the application limbs and the contralateral limbs at 24 h after the last (7th) topical application of TAC ointment (0.03%, 0.5 mg/kg) are presented in Fig. 6. TAC concentrations in skin, muscle, and DLNs collected from the application limb is much higher than TAC concentrations in the skin, muscle, and DLNs collected from the contralateral limb ($p < 0.05$).

Repeated Once Daily Topical Application of TAC (0.03% Ointment, 0.5 Mg/Kg) for 7 Days Results in Significantly Higher Local Tissues Concentrations, as Compared to the Concentrations Observed after Single Topical Application

TAC concentrations in skin, muscle, and DLNs collected from both the application limbs at 24 h after the first (1st) topical application of TAC (0.03% ointment, 0.5 mg/kg) or the seventh (7th) topical application of TAC ointment are shown in Fig. 7. As results show, from day 1 (1st topical dose) to day 7 (7th topical dose), there was significant increase in the TAC concentrations in the skin, muscle, and DLNs from 114 ±

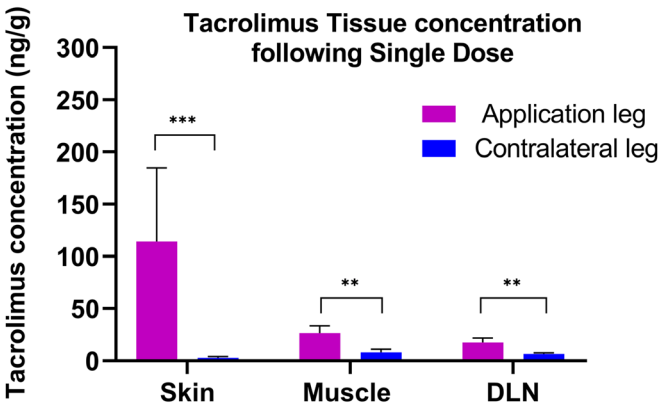

Fig. 3 TAC concentrations (ng/g) in skin, muscle, and DLNs collected from the application leg and contralateral leg at 24 h following single application of TAC (0.03% ointment, 0.5 mg/kg). Asterisks indicate statistical significance. **p < 0.01, ***$p < 0.001$, significantly different from the contralateral leg. Data is presented as mean ± SD, n = 6. P values were calculated by Wilcoxon Matched pairs test.

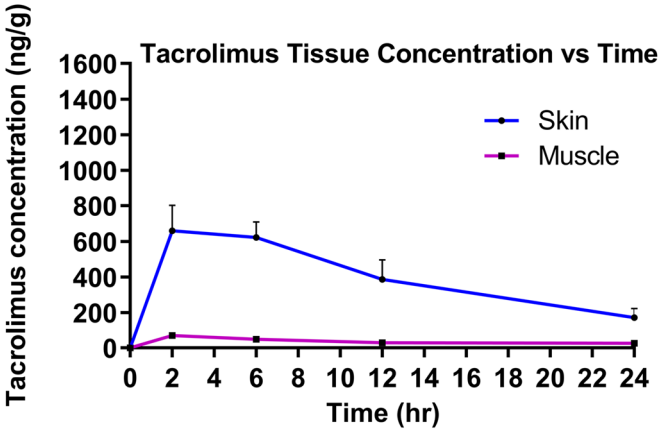

Fig. 4 Time course of TAC concentrations in skin and muscle following a single application of TAC ointment (0.03%, 0.5 mg/kg). Data presented as mean ± SD, $n = 3$.

70 ng/g, 26 ± 7 ng/g, and 17 ± 4 ng/g to 275 ± 58 ng/g, 95 ± 33 ng/g, and 51 ± 17 ng/g respectively, $^{**}p < 0.01$.

Average TAC Concentrations in the Blood over 24 H Following Single Topical Application of TAC (0.03% Ointment, 0.5 Mg/Kg) or Repeated Once Daily Topical Applications

Average TAC concentrations in the blood over 24 h following the first (1st) topical application of TAC (0.03% ointment, 0.5 mg/kg) or the seventh (7th) topical application of TAC ointment are shown in Fig. 8. The comparative non-compartmental pharmacokinetic exposure parameters of TAC in the blood following single topical dose or multiple once daily topical dose (0.5 mg/kg) for 7 days of TAC are presented in Table III. From day 1 to day 7, TAC accumulated to a moderate extent in blood. The mean trough concentration of TAC following the first topical dose on day 1 was 0.3 ± 0.1 ng/ml, after which the mean trough levels increased to 1.6 ± 0.2 ng/ml on day 7 (daily topical doses). However, these levels are lower than the values observed after the first systemic dose (3 ± 0.4 ng/ml). Despite that the mean AUC0-24 h increased from 21 ± 8 ng·h/ml on day 1 to 51 ± 7 ng·h/ml on day 7, the difference was not statistically significant ($p > 0.05$). The estimated ratio of accumulation from day 1 to day 7 was 2.4. However, these values are significantly lower than the values observed after single systemic dose (220 ± 29 ng·h/ml).

Effect of Daily Topical Application of TAC (0.03% Ointment, 0.5 Mg/Kg) on the Body Weight

The percent change in body weights of the control group (untreated animals), systemic TAC group (0.5 mg/kg/day), and topical TAC group (0.03% ointment, 0.5 mg/kg/day) as compared with the initial body weights is evaluated. There were no signs of systemic toxicity in any of the animals that received TAC. Animals in all groups (naïve, systemic TAC, topical TAC) showed significant body weight increase (%) during the treatment periods as compared to the initial body weights (1.4 ± 0.4 vs. 6.4 ± 2, 0.7 ± 1 vs. 5 ± 2, and 1.2 ± 0.7 vs. 6 ± 1.6 g, respectively, $p < 0.05$). Body weight increase was similar for topical TAC group and control group during the different treatment periods ($p > 0.05$). Body weight increase was smaller in the systemic TAC group as compared with the other groups during the treatment periods. However, the differences were found to be statistically non-significant ($p > 0.05$).

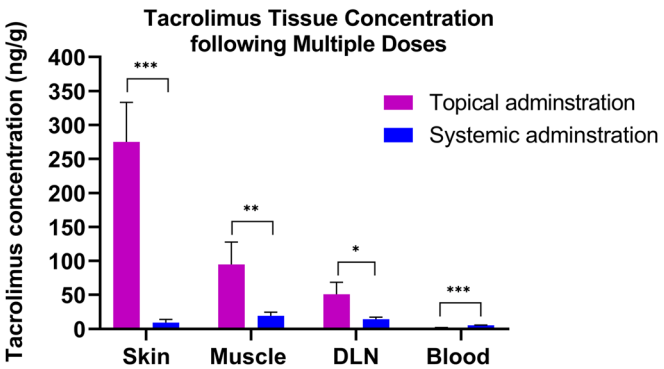

Fig. 5 Average TAC concentrations (ng/g or ng/ml) in skin, muscle, DLN, and blood at 24 h after the last (7th) topical application of TAC ointment (0.03%, 0.5 mg/kg) or IP injection (0.5 mg/kg). Asterisks indicate statistical significance. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, significantly different from systemic administration. Data presented as mean ± SD, $n = 3$. P values were calculated by Student t test.

Table II Comparative non-compartmental pharmacokinetic exposure parameters of TAC (mean + s.d.) in the skin, muscle, and blood following single application of TAC ointment (0.03%, 0.5 mg/kg) (n = 3)

	$AUC_{0-\alpha}$ (ng.hr/ml)	C_{max} (ng/g or ng/ml)	C_{trough} (ng/g or ng/ml)
Skin	12,497 ± 2930	661 ± 141	171 ± 51
Muscle	1567 ± 449	69 ± 10	26 ± 9
Blood	29 ± 9	2 ± 0.4	0.3 ± 0.1
Skin-blood ratio	520	1249	428
Muscle-blood ratio	65	129	60

DISCUSSION

Skin is the most immunogenic tissue in the VCA allograft and the primary target of rejection (25,55). Earlier studies that confirm therapeutic efficacy with topical administration of TAC lack serial blood and tissue level measurements (23,56). Our proof of concept study aims to provide objective evidence for the graft survival benefits of topical TAC (Protopic® 0.03%) by precisely monitoring the dynamics of blood and the local tissue concentrations of the drug.

We used the rat hind limb model to assess the pharmacokinetics and tissue distribution of topical TAC after single or repeated administration as this model is widely used in VCA studies (42–45). Furthermore, studies comparing calcineurin inhibitor (eg. cyclosporine) pharmacokinetics across species (such as humans and rats), reveal a direct proportional correlation between clearance and volume of distribution in relation to body weight (57).

Naïve rats with intact healthy skin were used to evaluate the local effects and tissue/systemic distribution of TAC after topical delivery. The doses used were relatively low and no saturable processes were expected to contribute to the observations. The use of naïve rats allows the study of the topical TAC formulation without the effect of other confounding factors that may change the permeability of TAC, systemic absorption, and exposure (58). Topical TAC (Protopic® 0.03%) when used once daily at a dose of 0.5 mg/kg, resulted in a high tissue to blood concentration ratio, indicating predominant affinity of the drug to local tissues, particularly the skin and muscle with minimal systemic diffusion. This is desirable because skin is the target tissue for rejection in VCA. TAC has large molecular weight (804.018 g/mol) and high lipophilicity (log $P = 3.96 \pm 0.83$) (59) which limits the drug's ability to pass across the skin layers and mainly retained in the lipid-rich layer 'stratum corneum' (60). TAC was also measurable in DLNs, and this may be related to the lipophilicity of TAC (61).

Average TAC concentration in skin, muscle, and DLNs collected from the application site was also significantly higher than drug concentrations in the tissues collected from the contralateral site. This indicates that TAC mainly localizes to the site of topical application with limited distribution to other sites remote from the application site. This supports the possibility of targeting drugs to local tissues by topical administration without high systemic levels.

It is important to determine whether the concentrations of TAC in the local tissues particularly skin are sufficient to exert a therapeutic effect. The observed blood and tissues concentrations should be compared with the minimal concentrations of TAC that have been reported to be effective. Studies have shown that trough blood TAC levels needed to prevent AR are between 5 and 10 ng/ml (62,63), while lower trough blood levels (<5 ng/ml) could result in allograft rejection and thus considered "sub-therapeutic" (63). Studies reported that doses between 0.5–1 mg/kg (systemic TAC) are sufficient to achieve the therapeutic blood levels of TAC and has shown efficacy in preventing rejection in experimental VCA limb transplant models (43).

In rats, systemic administration of TAC has been associated with global toxicity including metabolic complications (64). Systemic TAC decreases the body weight gain rate over time (65). Here, we evaluated the impact of topical application of TAC (Protopic® 0.03%) once daily at a dose of 0.5 mg/kg on body weight change from baseline. Animals that received topical TAC showed significant body weight increase during the treatment periods as compared to the initial body weights. Body weight increase was similar for topical TAC group and control group indicating that systemic exposure to TAC after topical application is minimal with low co-morbidity. Body weight increase was smaller in the systemic TAC group (0.5 mg/kg) during the treatment periods as compared with topical TAC group suggesting that the difference can be attributed to increased systemic exposure to TAC after systemic

Table III Comparative non-compartmental pharmacokinetic exposure parameters of TAC (mean + s.d.) in the blood following single IV dose (0.5 mg/kg), single topical dose, and daily topical dose (0.5 mg/kg) for 7 days of TAC (Data shown as mean + SD, n = 3

Pharmacokinetic Parameters	Unit	1st systemic dose	1st topical dose	7th topical dose
C_{trough}	ng/ml	3 ± 0.4	0.3 ± 0.1	1.6 ± 0.2
AUC_{0-24h}	ng.hr./ml	220 ± 29	21 ± 8	51 ± 7
Accumulation ratio R(ac)			2.4	

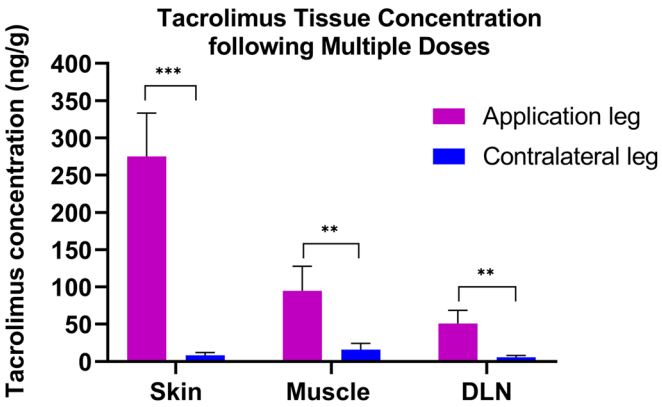

Fig. 6 TAC concentrations (ng/g) in skin, muscle, and DLNs collected from the application leg and contralateral leg at 24 h after the last (7th) topical application of TAC ointment (0.03%, 0.5 mg/kg). Asterisks indicate statistical significance. *p < 0.05, ***p < 0.001 significantly different from the contralateral leg. Data presented as mean ± SD, n = 3. P values were calculated by paired t test.

administration. TAC ointment (Protopic® 0.03%) is well tolerated for local delivery of TAC in VCA. This is consistent with the results of other studies where topical TAC 0.03% mainly partitioned in the skin, with minimal systemic absorption in patients with atopic dermatitis. (30–35)

We addressed potential limitations of the current study as follows: (1). It is possible that rats could ingest some of the topical drug via oral ingestion (licking), which may lead to increased systemic blood levels. The use of Elizabethan Collars and isolated housing prevented oral ingestion of topically applied drugs by the experimental animals or by other animals in the same cage. (2). It is possible that different parts of the limb may get different exposure to the applied drug resulting in variable tissue levels. We minimized this by homogenous application of the topical formulation (by massaging) and by keeping the location and size of tissue sampling consistent across animals.

Our results revealed that single topical application of TAC (Protopic® 0.03%) once daily at a dose of 0.5 mg/kg results in lower concentrations of TAC in the blood and higher concentrations in the skin, muscle, and DLNs, when compared to the concentrations observed after systemic delivery of the same dose (0.5 mg/kg/day). Systemic exposure to TAC following repeated topical application as measured by Ctrough and AUC0-∞, was higher than the values obtained after single topical application (2-fold higher) indicating moderate systemic accumulation of TAC. Average TAC concentrations in the local tissues after repeated administration were significantly higher than the values obtained after single topical delivery, indicating the local accumulation of TAC after repeated topical applications. No clinical evidence of local TAC toxicity (inflammation, itchiness, redness, swelling, cracking, weeping, crusting, scaling or hair loss) was observed in animals receiving multiple applications. Further studies are ongoing to evaluate the efficacy of TAC (Protopic® 0.03%, 0.5 mg/kg/day) in preventing skin rejection in rat and swine models of VCA.

CONCLUSION

To the best of our knowledge this is the first study to show that topical application of TAC ointment (Protopic®, 0.03%) once daily in a dose of 0.5 mg/kg body weight provides high concentrations in the skin, muscle, and DLNs near the site of application. Localization of TAC in the skin is desirable as it is the primary target tissue for rejection in VCA. There were no topical therapy related side effects. This preliminary, pilot study confirms the feasibility of targeting specific tissues by topical delivery of TAC with minimal systemic exposure. Efficacy studies are required in small/large animal VCA models and transplant patients to determine the effective

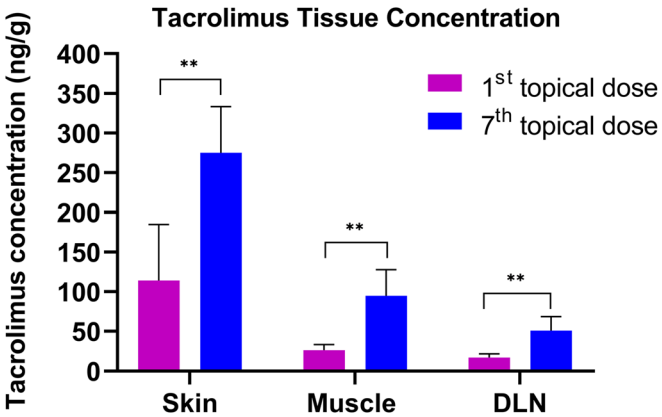

Fig. 7 TAC concentrations (ng/g) in skin, muscle, and DLNs collected from the application leg at 24 h following the first topical dose or the seventh topical dose. Asterisks indicate statistical significance. **p < 0.001 significantly different from the single topical application. Data presented as mean ± SD, n = 3. P values were calculated by Mann Whitney test.

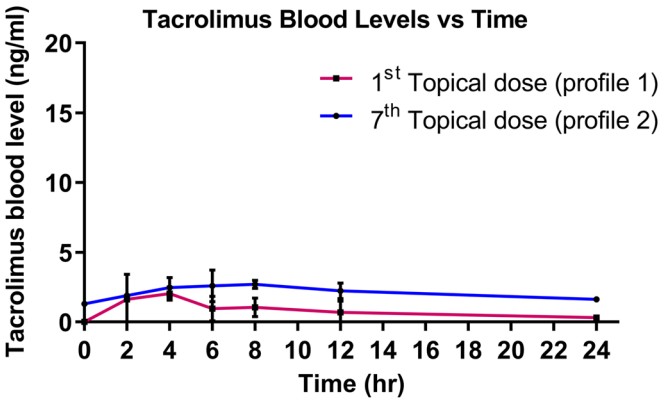

Fig. 8 Average TAC concentrations in the blood over 24 h following the first topical application of TAC (0.03% ointment, 0.5 mg/kg) (Profile 1) or the seventh topical applications of TAC (Profile 2). Data presented as mean ± SD, n = 3.

therapeutic concentrations in target tissues required to prevent rejection.

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AUTHOR'S CONTRIBUTIONS

Overall research idea (VG); conceptual approach, hypothesis (VG, RV, FF); experimental design (FF, VG, RV, MS); protocol development (FF, RV, VG); In vivo experiments and collection of data (FF, JS, PF, VE, SO, HS and LD); Tissue processing for drug level measurement (FF); Manuscript drafting (FF), Extensive manuscript editing (RV, VG), Additional input (MS, AS, JP, JU); Analysis of data and interpretation of results (RV, VG, FF, MS);. All authors read and approved the manuscript.

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