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Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation

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Many systemically effective drugs such as cyclosporin A are ineffective topically because of their poor penetration into skin. To surmount this problem, we conjugated a heptamer of arginine to cyclosporin A through a pH-sensitive linker to produce R7–CsA. In contrast to unmodified cyclosporin A, which fails to penetrate skin, topically applied R7–CsA was efficiently transported into cells in mouse and human skin. R7–CsA reached dermal T lymphocytes and inhibited cutaneous inflammation. These data establish a general strategy for enhancing delivery of poorly absorbed drugs across tissue barriers and provide a new topical approach to the treatment of inflammatory skin disorders.

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The skin offers a formidable barrier to the delivery of large and small molecular therapeutic agents[1–4]. The basis for this barrier has been studied extensively and has been found to reside within the outer layers of the stratified epithelium of the epidermis[1,5]. The complex lipid and cellular structure within the cornified layer of intrafollicular epidermis is composed of ceramides, cholesterol and free fatty acids[6] and provides a tight barrier to microbial invasion and desiccation[7,8]. At the same time, this epidermal permeability barrier also poses a substantial obstacle to the delivery of promising therapeutics for the treatment of systemic or primary skin diseases.

Many drugs available for the treatment of primary cutaneous disease are effective when given systemically but are ineffective topically because of poor absorption. A well-studied example of such a drug is cyclosporin A (CsA). Orally administered CsA is effective against a broad range of inflammatory skin diseases, including widespread conditions such as psoriasis and atopic dermatitis[9–14]; however, systemic administration is limited by considerable side effects, including nephrotoxicity and many drug interactions[15,16]. Attempts at topical application of CsA in a range of inflammatory skin disorders have proven ineffective[17–22], and this lack of effectiveness has been attributed to poor topical absorption[23,24]. This shortcoming is particularly relevant because the effectiveness of other potent anti-inflammatory agents such as high-potency topical glucocorticoids is compromised by a host of problems that can include cutaneous atrophy, acneiform changes, hypothalamic–pituitary axis suppression, exacerbation of diabetes mellitus and tachyphylaxis[25–28]. The development of a means of enhancing topical absorption of systemically effective but poorly absorbed drugs such as CsA is thus urgently needed.

Short oligomers of arginine efficiently cross biological membranes and can be used to facilitate the uptake of a variety of small molecules to which they are attached (D. Mitchell *et al.*, in the press and P.A.W. *et al.*, manuscript submitted). Arginine oligomers are far more efficient than peptides from HIV tat (ref. 29,30) and the third helix of Drosophila antennapedia[31], both of which have been used to enhance the uptake of proteins across cell and tissue barriers. The transport mechanism responsible for this cellular uptake is energy dependent and seems to be very conserved through prokaryotes[32]. However, the precise mechanism by which transport of molecules across cell membranes and across tissue barriers is facilitated is unknown. To determine whether short oligomers of arginine could enhance drug delivery into and through the skin, we coupled CsA to a heptamer of arginine through a linker designed to release the active drug at physiologic pH within tissue. This modification facilitatedpenetration of CsA into skin with functional inhibition of cutaneous inflammation. This delivery technology has broad potential for enhancing the penetration of a wide range of therapeutic agents into and across skin and other tissue barriers.

Peptide penetration of skin

To test whether oligomers of arginine were able to cross the stratum corneum and enter the epidermis, we mixed a 1-mM solution of biotinylated hepta-D-arginine (biotin r7) dissolved in PBS with an equal volume of lotion vehicle. We applied biotin alone and the biotin r7 conjugate to separate locations on the back of a nude mouse. Two hours later, we obtained skin biopsies and stained tissue cryosections with fluorescein or peroxidase labeled streptavidin. Skin treated with biotin alone showed no evidence of uptake (Fig. 1). In contrast, almost every keratinocyte in the epidermis and a high percentage of the cells in the dermis showed high levels of uptake of the biotin r7 conjugate (Fig. 1). Dual-color fluorescence microscopy using antibodies to cell surface proteins expressed on macrophages, endothelial cells and fibroblasts showed that all of these cell types took up this material within intact skin, without apparent preference (data not shown). In all cases, the strong dermal signals were cells or clusters of cells; in some cases these signals were so strong they extended into the surrounding area and gave the appearance of larger structures, an artifact that was distinct in images obtained with shorter exposures. The depth of penetration and the intensity of staining were dependent on the length of application and the concentration used (data not shown).

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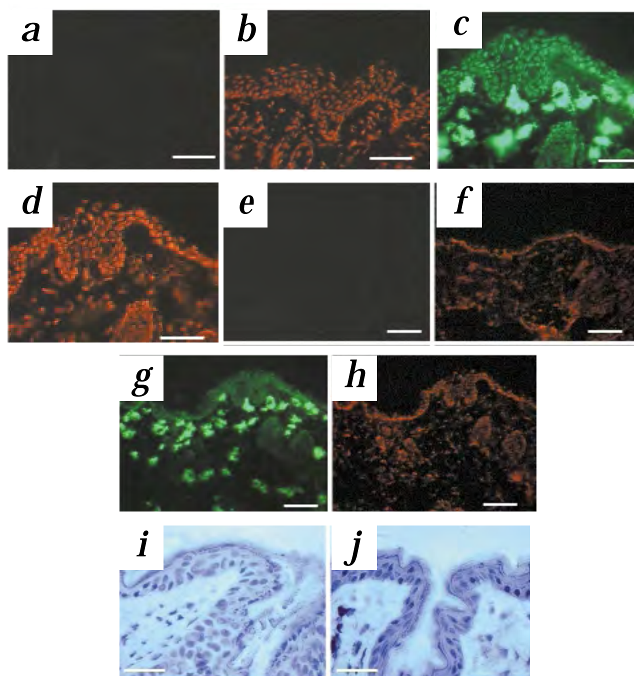

Fig. 1 Heptamers of D-arginine penetrate the skin barrier. ***a, c, e*** and ***g***, Fluorescein detection of biotinylated peptide or biotin. ***b, d, f*** and ***h***, Propidium iodide counterstaining of the same tissue sections in ***a, c, e*** and ***g***, respectively, to demonstrate tissue architecture. ***i*** and ***j***, Immunoperoxidase detection using streptavidin conjugated to horseradish peroxidase. Scale bars represent 20 mm (a–d, i and j) and 50 mm (e and h).

Entry of CsA–peptide conjugates into the epidermis and dermis

Having established that oligomers of arginine enhance penetration of biotin through the cutaneous barrier, we examined their ability to enhance topical delivery of drugs. We used CsA because of its oral efficacy in the treatment of inflammatory skin diseases and its lack of efficacy when applied topically. We synthesized a non-releasable chemical conjugate of a short oligomer of biotinylated arginine and CsA (Fig. 2*o*). The resultant biotin r7–CsA conjugate (Fig. 2*o*, sequence 3) was both very water-soluble and very stable in physiological conditions. Biotinylated CsA (Fig. 2*o*, sequence 4) was dissolved in 1 mM ethanol and 1 mM biotin r7–CsA in PBS. Aliquots of each compound and vehicle alone were applied to the skin of a nude mouse. There was no detectable CsA delivery in skin treated with either vehicle alone or the biotin CsA conjugate (Fig. 2). In contrast, the biotin r7–CsA conjugate penetrated into all skin tissue layers. Almost every keratinocyte in the epidermis and many cells in the dermis were heavily stained (Fig. 2).

Human and mouse skin differ considerably in many ways, with human epidermis being considerably thicker. To determine if the r7–CsA conjugate could also penetrate human skin, we applied biotin r7–CsA to full-thickness human skin grafted onto the back of a severe combined immunodeficiency mouse. As in mouse skin, conjugated CsA penetrated human epidermis and dermis (Fig. 2). Fluorescence was present mainly in the nuclei of cells in tissue exposed to biotinylated peptides alone, but in sections stained with biotin r7–CsA, most fluorescence was cytosolic (Fig. 2), consistent with binding of r7–CsA to CsA's known cytoplasmic targets[33].

CsA–transporter conjugates enter T cells in the dermis

To determine whether the r7–CsA conjugate reached infiltrating T cells within inflamed skin *in vivo*, we applied biotin r7–CsA to the site of inflammation on the back of a mouse with experimentally induced contact dermatitis. We stained inflamed skin with rhodamine-labeled goat antibody against mouse CD3 to localize T cells and with fluorescein-labeled streptavidin to localize the biotin r7–CsA. Biotin r7–CsA was present in all $CD3^+$ T cells in the tissue in addition to being present in a variety of other cells, including other inflamma-

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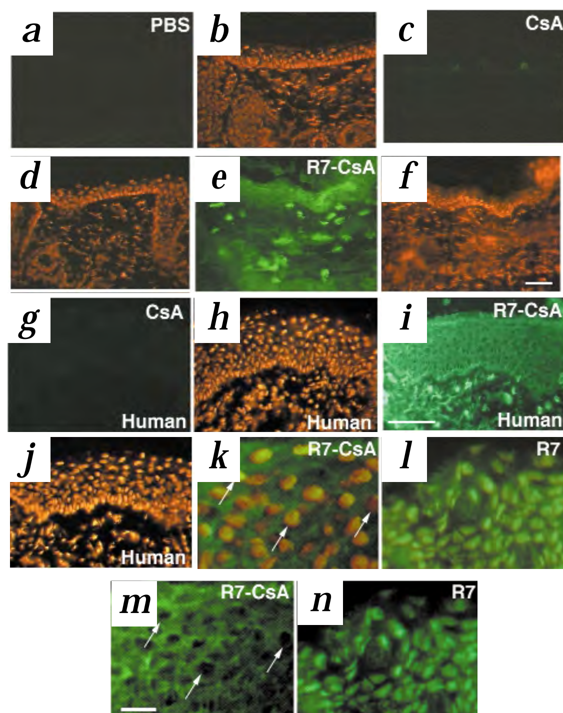

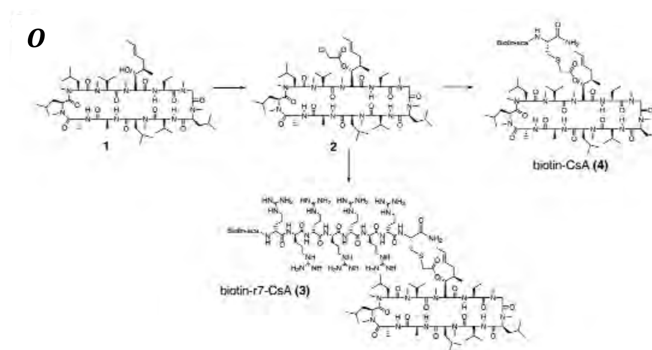

Fig. 2 Biotinylated r7–CsA conjugates enter the epidermis and dermis of mouse and human skin. ***a, c, e, g*** and ***i***, Streptavidin–fluorescein staining. ***b, d, f, h*** and ***j***, Propidium iodide staining of fields corresponding to those in a, c, e, g and I, respectively, to 'highlight' tissue architecture. ***k–n***, Higher magnification shows preferential cellular localization. k and m, R7–CsA, for cytoplasm. l and n, R7 peptide alone, for the nucleus. k and l, double-stained with propidium iodide (orange) to emphasize cellular nuclei (arrows indicate the same three cell nuclei in each field); these are higher magnifications of tissue from Figs. 2*i* and 1*c*, respectively. Scale bars represent 50 mm (a–f), 100 mm (g–j) and 10 mm (k–n). ***o***, Synthetic scheme for non-releasable chemical conjugate between a biotinylated heptamer of D-arginine and cyclosporin A.

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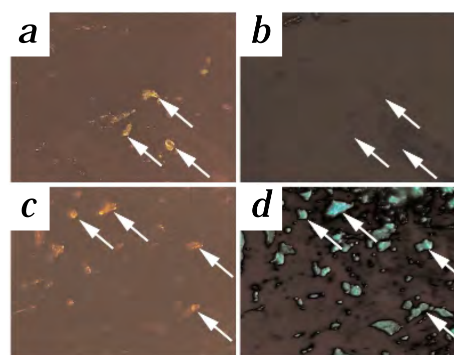

Fig. 3 Biotinylated r7-CsA enters dermal T cells in inflamed skin. ***a,b,*** Biotin CsA or ***c,d,*** biotin r7-CsA were applied to inflamed mouse skin. a,c, detection of CD3+ T cells (orange) and b,d, detection of CsA (green). Note uptake of CsA in all T cells in the field and in additional dermal cells in skin treated with biotin r7-CsA.

tory cells such as macrophages as well as resident fibroblasts (Fig. 3 and data not shown). These data indicate that biotin r7–CsA penetrated inflamed skin to reach essential target T lymphocytes.

Synthesis of a releasable arginine-CsA conjugate

Modification of the secondary alcohol of CsA results in considerable loss of its biological activity[34]. Consequently, to ensure release of free CsA from its conjugate after transport into cells, we conjugated CsA to an 'oligo-arginine' transporter through a pH-sensitive linker (Fig. 4). The resultant conjugate was stable at acidic pH but at pH levels greater than 7 it underwent an intramolecular cyclization involving addition of the free amine to the carbonyl adjacent to CsA (Fig. 4), which resulted in the release of unmodified CsA. Another modification in the design of the releasable conjugate was the use of L-arginine (R) and not D-arginine (r) in the transporter. Whereas we used the 'oligo-D-arginine' transporters for the histological experiments to ensure maximal stability of the conjugate and therefore accuracy in determining its location through fluorescence, we incorporated oligomers of L-arginine into the design of the releasable conjugate to minimize its biological half-life. Consistent with its design, the resultant releasable conjugate was stable at acidic pH, but labile at physiological pH in the absence of serum. The half-life of this releasable CsA conjugate in PBS at a pH of 7.4 was 90 minutes (data not shown).

In vitro and *in vivo* activity of R7–CsA

The releasable R7 conjugate of CsA was biologically active, as shown by its inhibition of interleukin (IL)-2 secretion by the human T-cell line Jurkat stimulated with phorbol 12-myristate 13-acetate and ionomycin *in vitro*[35] (Fig. 5*a*). The conjugate was added 12 hours before the addition of phorbol 12-myristate 13-acetate and ionomycin, and there was dose-dependent inhibition by the releasable R7–CsA conjugate. This inhibition did not occur with a non-releasable analog that differed from the releasable conjugate by retention of the tert-butyloxycarbonyl protecting group, which prevented cyclization and resultant release of the active drug. The median effective concentration of the releasable R7 cyclosporin conjugate was approximately half that of CsA dissolved in alcohol and added at the same time as the releasable conjugate.

We assessed the releasable R7–CsA conjugate *in vivo* for functional activity using a mouse model of contact dermatitis. We applied R7–CsA topically in concentrations ranging from 0.01% to 1.0% to span the range of most topical therapeutics now in use. Treatment with the 1% releasable R7–CsA conjugate resulted in a reduction in ear inflammation of 73.9 ± 4.0% (Fig. 5*b*). In mice treated on one ear with R7–CsA, there was no reduction in inflammation in the untreated ear, indicating that the action of R7–CsA was local and not systemic. In support of this, assay of serum from mice after receiving a course of topical 1% R7–CsA showed no detectable CsA in the circulation (<1ng/mL). There was decreasing inhibition in the ears of mice treated topically with 0.1% and 0.01% R7–CsA (64.8 ± 4.0% and 40.9 ± 3.3%, respectively), demonstrating that the effect was titratable. Treatment with the fluorinated corticosteroid positive control resulted in reduction in ear swelling (34.1 ± 6.3%), but this was substantially less than that with 0.1% releasable R7–CsA (Fig. 5*b*). There was no reduction of inflammation in any of the mice treated with unmodified CsA, vehicle alone, R7 or non-releasable R7–CsA, and there was no evidence of adverse consequences of R7 peptide application clinically or histologically during the studies.

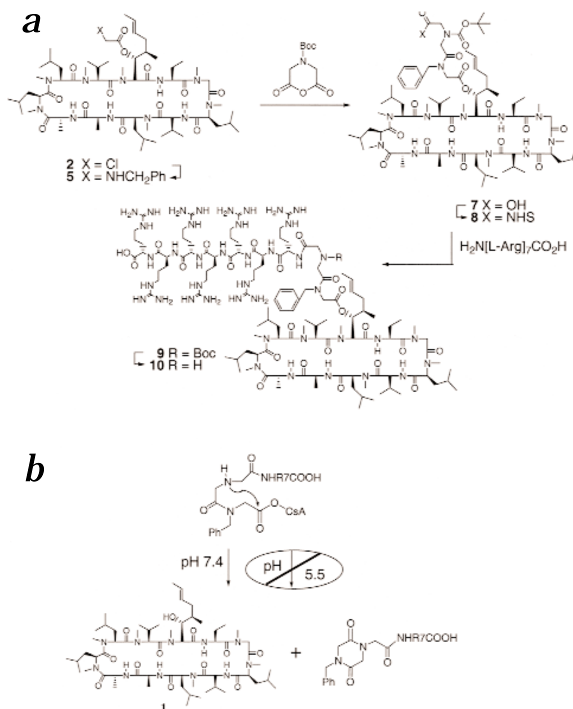

Fig. 4 Conjugate synthesis and chemical release. ***a***, Synthetic scheme for a chemical conjugate between a heptamer of L-arginine and cyclosporin A. ***b***, pH-dependent chemical release of conjugate.

Discussion

Here we have shown that short oligomers of arginine facilitated transport across the cutaneous barrier when applied topically to either mouse or human skin. In addition, the conjugate of 'oligo-arginine' and cyclosporin was much more water soluble (tens of milligrams per milliliter) than cyclosporin itself (micrograms per milliliter). Even though the 'oligo-arginine'–cyclosporin conju-

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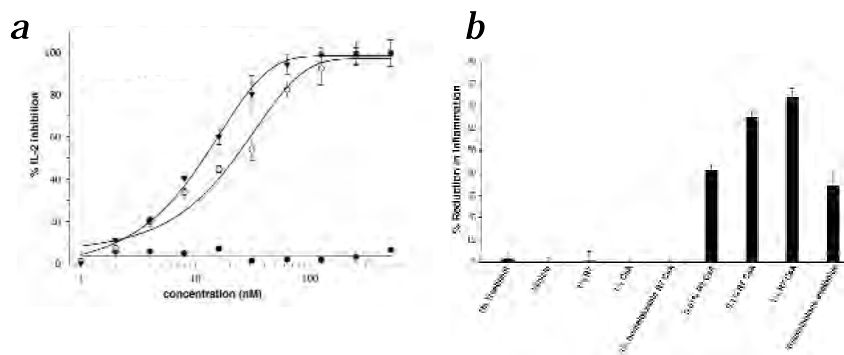

Fig. 5 Functional activity of releasable R7-CsA. ***a***, Inhibition of IL-2 secretion by the releasable R7–CsA conjugate. IL-2 inhibition as a function of the concentration of non-releasable (●), releasable (○) R7–CsA conjugate or CsA (▼) in Jurkat cells. ***b***, Releasable R7–CsA inhibits inflammation in mouse contact dermatitis. Reduction of inflammation due to allergic contact sensitization by vehicle alone, 1% R7 peptide alone, 1% CsA, 1% non-releasable R7–CsA, 0.01%/0.1%/1.0% releasable R7–CsA, or the fluorinated steroid positive control 0.1% triamcinolone acetonide.

gate was much more polar, and theoretically less likely to cross the stratum corneum than the unmodified drug, it penetrated the skin as well as the arginine peptide did. This result demonstrated that the properties of the transporter were not adversely affected by the addition of the drug subunit. These experiments showed that short oligomers of arginine can be used to transport therapeutic drugs through the stratum corneum and distribute them throughout the epidermis and dermis. The pattern of intracellular staining with the peptide differed from that with the peptide drug conjugate, indicating that both the drug and the transporter influenced cellular localization. There was considerable staining of both the cytosol and nucleus in sections of skin exposed to the biotinylated peptide; in contrast, most of the biotinylated drug conjugate was in the cytosol of cells with little nuclear localization. The cyclosporin–arginine conjugate used in the histological experiments was designed to be stable in the assay conditions, so the resultant fluorescent pattern reflected the distribution of the conjugate and not just the biotinylated peptide alone. The lack of nuclear staining is consistent with the preferential binding of the modified cyclosporin conjugate to its cytosolic target cyclophilins, rather than its localization in the nucleus. The r7–drug conjugate not only penetrated into the dermis, but also entered target tissue T cells. The amount of conjugate delivered and the free drug released was therapeutically effective, as shown with a well-established animal model of contact dermatitis in which preparations containing 1, 0.1 and 0.01% of a chemically releasable cyclosporin conjugate reduced ear inflammation.

The mechanism of transport by 'oligo-arginine' conjugates is being investigated. Because the barrier function of human skin xenografts on immune-deficient mice is maintained[36], our data indicate that these conjugates penetrate the intact cutaneous barrier, a biological boundary that is different from the plasma membrane. Although cells in the stratum corneum are non-viable, this environment is metabolically active, with an array of enzymatic activities, and this active metabolism may contribute to transport. In cells, the peptide and the drug conjugates seem to directly enter the cytosol by active transport across the lipid rich cell membrane[32,37]. Lipid components are also central elements in the cutaneous barrier. Protein transduction through guanidino head groups may permit transport across lipid barriers in general, including the plasma membrane as well as tissue barriers such as that seen in epidermis. As in earlier studies (D. Mitchell *et al.*, manuscript submitted), there was no evidence of fluorescein enclosed in endocytotic vesicles. Both the peptide and the peptide–drug conjugate seemed to enter a layer of cells, saturate it and enter the adjacent layer forming a gradient from the outer to the inner layers of cells. This would require a mechanism for exiting cells. Fluorescently labeled transporters have been shown to exit suspension cells at observable rates (J.B.R. *et al.*, unpublished results), consistent with this possible mechanism. Alternatively, the transporters could have penetrated multiple layers by going between and not through cells. Although possible, we did not find an inordinate amount of material between or on the surface of cells. Although mechanistic studies continue, the rapid rate of uptake, the therapeutically useful amount of drug transported and the depth of penetration make this technology very attractive. Short oligomers of arginine also can be used to enhance topical uptake of a wide variety of therapeutic drugs into and through the skin, such as paclitaxol, tacrolimus and hydrocortisone (J.B.R. *et al.*, unpublished results), indicating the potential broad utility of this approach in drug delivery.

Methods

Synthesis of reagents. Peptides were synthesized using solid phase techniques and commercially available fluorenylmethoxycarbonyl (Fastmoc) amino acids, resins and reagents (PE Biosystems, Foster City California and Bachem Torrence, Califonia) on a Applied Biosystems 433 peptide synthesizer. Fastmoc cycles were used with O-(7-azabenzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexfluorophosphate (HATU). The peptides were cleaved from the resin using 96% trifluoroacetic acid, 2% triisopropyl silane and 2% phenol for 12 h. The longer reaction times were necessary to completely remove the 2,2,4,6,7-Pentamethyldihydrobenzofurane-5-sulfonyl (Pbf)-protecting groups from the polymers of arginine. The peptides subsequently were filtered from the resin, precipitated using diethyl ether, purified using high-performance liquid chromatography reverse-phase columns (Alltech Altima, Chicago, Illinois) and characterized using either electrospray mass spectrometry (PE Biosystems, Foster City, California). For synthesis of non-releasable r7–CsA, CsA was allowed to react with chloroacetic anhydride to provide the expected α-chloroacetyl product. Attachment of the transporter was realized by reaction with a hepta-D-arginine transporter that was labeled with biotin at one end and had a cysteine residue on the other. For synthesis of releasable CsA, the α-chloro ester was treated with benzylamine in the presence of sodium iodide to effect substitution, giving the secondary amine. This was treated with anhydride, and the resultant crude acid was converted to its corresponding N-hydroxy succinimide (NHS) ester. The resulting ester was then coupled with the amino terminus of hepta-L-arginine, giving the Nitrogen-butyloxycarbonyl-protected CsA conjugate. Finally, removal of the butyloxycarbonyl protecting group with formic acid afforded the conjugate as its octatrifluoroacetate salt after high-performance liquid chromatography purification.

IL-2 inhibition assay. Jurkat cells (5×10^4) were incubated with varying concentrations of either CsA or R7–CsA prodrug conjugates overnight at 37 °C to allow for uptake and release of the active form of CsA. The next day, T cells were stimulated to produce IL-2 by the addition of 10 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 1 μM ionomycin (CalBiochem, San Diego,

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Califonia). Cultures were incubated overnight at 37 °C, and the next day supernatants were collected and IL-2 was measured using fluorescent enzyme-linked immonoassay. Plates were coated with 4 ng/ml antibody against human IL-2 (23191D; PharMingen, San Diego, California), blocked for 1 h at room temperature with PBS containing 10% FBS and washed, and supernatants were added and incubated for 1 h. Media was removed and 1.6 ng/ml biotinylated antibody against human IL-2 (23112D; PharMingen) was added for 1 h. The plates were washed, and then 0.04 ng/ml europium-labeled streptavidin (1244-360; Delfia, Turku, Finland) was added for 1 h. After another wash enhancement solution (1244-105; Delfia) was added and the resulting fluorescence was measured using a Wallac plate reader (Wallac, Turku, Finland).

Animal studies. The abdomens of BALB/c mice 6–7 weeks of age ($n = 20$ mice per group) were painted with 100 µl of a 0.7% solution of dinitrofluorobenzene in acetone olive oil (95:5). Then, 3 d later, the mice were re-stimulated by having both ears painted with 0.5% solution of dinitrofluorobenzene dissolved in acetone. The amount of ear inflammation was measured 24 h later with a spring-loaded caliper, by investigators 'blinded' to mouse identity. The right ear of each mouse was treated with reagents 1, 5 and 20 h after re-stimulation. Normal ear thickness was 0.005 ± 0.001 inches, expanding to 0.015 ± 0.003 inches after stimulation with dinitrofluorobenzene. The percent reduction of inflammation was determined using the following formula: (t–n)/u–n), where t represents thickness of the treated ear, n represents the thickness of a normal untreated ear and u represents thickness of an inflamed ear without any treatment. Twenty mice were in each group. No systemic effects of the treatment were seen by reduction of the left, stimulated, but untreated ear of each mouse. Serum CsA was measured by high-performance liquid chromatography followed by tandem mass spectroscopy[38]. Whole blood (100 µl) obtained at the time of ear measurement was vortexed with 2 ml ethyl ether. After centrifugation, the upper layer was isolated and dried, and the residue was 'taken up' in 500 µl water:acetonitrile (70:30). Then, 20 µl were injected into the liquid chromatography/tandem mass spectrometry system. The analytical column was a C18 Alltima column (Alltech, Deerfield, Illinois), and sample elution progressed through a linear gradient from 50% to 100% acetonitrile. Mass spectrometry used a PE-Sciex API III triple quadrupole mass spectrometer equipped with an ion spray interface (P-E Sciex, Toronto, Canada). The precursor–product mass pairs (m/z) focused for CsA and the internal standard CsC as described[38].

Histochemical analysis. Tissue cryosections 5 µm in thickness were fixed in 100% acetone for 15 min at room temperature, then air-dried and blocked with 10% goat serum in PBS for 30 min. For biotin-labeled conjugates, blocking was followed by incubation for 30 min with 30 µg/ml streptavidin conjugated to fluorescein isothiocyanate (Vector, Burlingame, California). This was followed by a 30-minute wash in PBS and mounting (Vectashield; Vector, Burlingame, California). In some cases, counterstaining was also done with 1 µg/ml propidum iodide antibodies (Sigma) to 'highlight' cellular nuclei. For immunohistochemical staining, blocked cryosections were incubated with antibodies at a dilution of 1:50 in PBS. Antibodies used recognized cell surface proteins expressed on T lymphocytes (CD3; Santa Cruz Biotechnology, Santa Cruz, California), macrophages (F4/80; Serotec, Oxford, UK), endothelial cells (factor VIII; Dako, Carpenteria, California) and fibroblasts (α5 integrin; a gift from J. Lie, Stanford, California). Secondary antibodies labeled with tetramethylrhodamine isothiocyanate (Sigma) were used at a dilution of 1:500 in PBS. Sections were then mounted and visualized using dual-color fluorescence microscopy.

RECEIVED 22 MAY; ACCEPTED 19 SEPTEMBER 2000

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