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Preclinical Evaluation of a New Immunosuppressive Agent, FTY720

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Introduction

FTY720 is a synthetic analog of the drug Myriocin (ISP-1), which is present in culture filtrates of *Isaria sinclairii* (1), an ascomycete. Ascomycetes are mycelial forms of Fungi Imperfecti, which are characterized by asexual spore phases and are usually parasitic on insects or plants. Although *Isaria sinclairii* has been widely used in Chinese traditional medicine, toxicology studies have shown that ISP-1, which is a structural analog of sphingosine, produces severe digestive disorders, resulting in the death of experimental animals (2). Because of the potent immunosuppressive activity of ISP-1 *in vitro*, synthetic modifications have been performed to generate less toxic and more active compounds (2,3). One of these new compounds is FTY720, 2 amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol hydrochloride (Figure 1), which was developed by Prof. T. Fujita (Taito Co. Ltd.) in collaboration with Yoshitomi Pharmaceuticals Ltd.; hence the name, FTY720.

Immunosuppressive effects

In vitro

In mouse models, ISP-1 and related compounds have been shown to inhibit allogenic mixed lymphocyte reactions (MLR) (2) and interleukin-2 (IL-2)-dependent proliferation (4) of the mouse cell line CTLL-2 in a dose-dependent manner. The drug's potency is 10- to 100-fold greater than that of cyclosporine (CsA). Unlike ISP-1, exogenous addition of FTY720 in doses up to 1000 nM does not inhibit MLR proliferation, or IL-2 production by antigen- or mitogen-stimulated T cells (5).

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In vivo

Autoimmune and inflammatory models

FTY720 administered to mice at doses greater than 0.03 mg/kg/d has been shown to inhibit induction of delayed-type hypersensitivity responses by human albumin in a dose-dependent manner. Using a model of adjuvant arthritis in rats, researchers showed that low doses (0.1 mg/kg) of the drug completely inhibited joint destruction and paw edema. In the same study, FTY720 also effectively ameliorated T cell-mediated autoimmune responses in rat models of collagen-induced arthritis and allergic encephalomyelitis (6).

Transplant models

ISP-1 has been shown to reduce the number of plaque-forming cells generated in response to sheep red blood cells, and to reduce the frequency of alloreactive cytotoxic T lymphocytes (1). It has been shown that FTY720 remarkably prolongs the survival of skin, heart, or liver allografts in MHC-incompatible and -compatible rat strain combinations in dose-dependent fashion over the range of 0.1 to 10 mg/kg (Table 1) (7,8). FTY720 potentiated the immunosuppressive effects of subtherapeutic doses of CsA (5,7,9) and/or rapamycin (10). In addition, administration of FTY720 (5 mg/kg) to rats on days 3 and 4 post-grafting significantly prolonged allograft survival, suggesting that the drug can reverse ongoing rejection (9). In a model of graft-versus-host disease in rats, low doses of FTY720 (0.1 to 0.3 mg/kg) induced long-lasting unresponsiveness (11). In addition, 10 mg/kg doses of FTY720 delayed acute rejection after canine kidney transplantation (9,12,13) and potentiated the effects of subtherapeutic doses of CsA (7,9). Finally, a brief pretransplant 2-day course of FTY720 prolonged rat liver and kidney dog allograft survival (9). Our own preliminary results show that FTY720 doses of 0.3 and 1 mg/kg prolong monkey kidney transplant survival. Remarkably, the drug has shown no toxic effects in

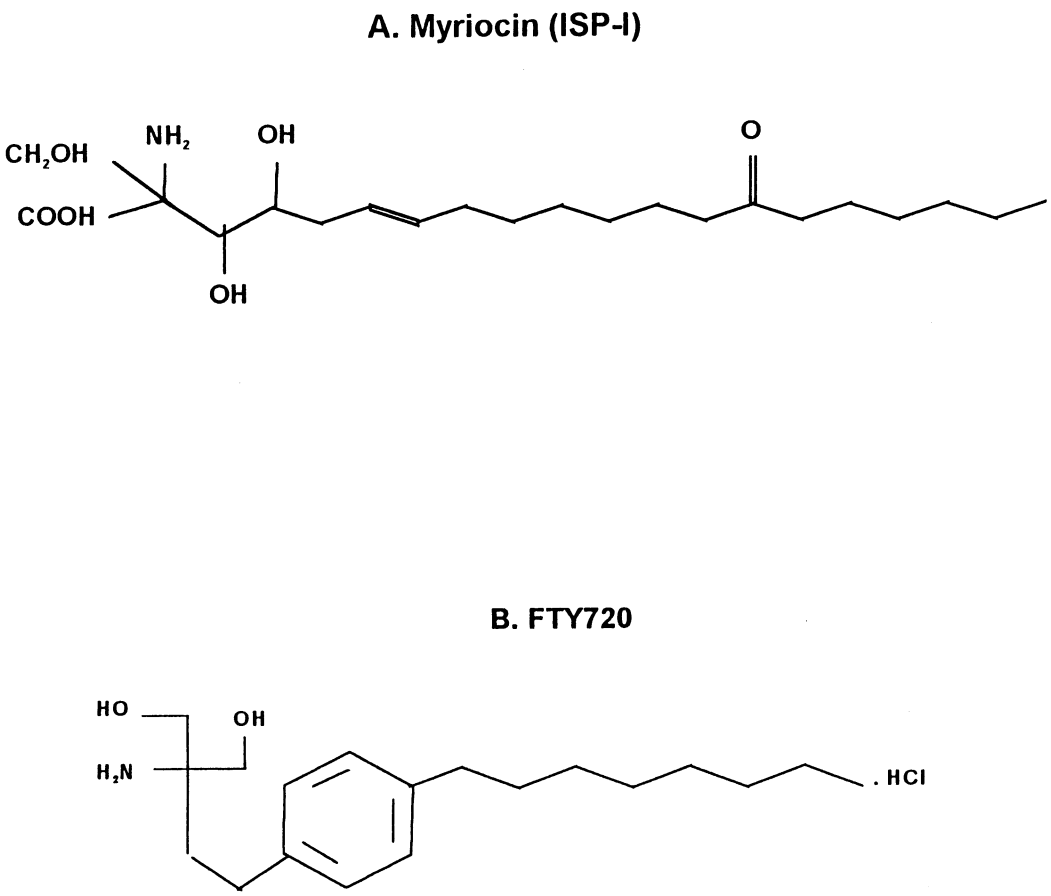

Figure 1 — Molecular structure of Myriocin (ISP-1) and FTY720.

preclinical studies when used either alone or in combination with other immunosuppressive agents.

Preliminary data suggest that trough blood level concentrations of unchanged drug display a linear correlation with dose (manufacturer information). In rats and dogs, FTY720 seems to be metabolized predominantly by oxidation to produce carboxylic acid derivates, primarily by T-oxidization at the terminal position of the side chain. None of the identified metabolites seems to display immunosuppressive activity, suggesting that only the parent compound is active. Metabolites have been identified in the urine and in the feces at ratios of 40 to 50% and 20 to 50%, respectively. Under the rigorous median effect analysis, FTY720 displays a high degree of synergism with CsA and rapamycin administered either separately or in combination (10).

Mechanism of action

In vitro

High doses of FTY720 inhibit the proliferation of human mononuclear cells in response to stimulation with phytohemagglutinin (PHA) or anti-CD3 monoclonal antibody (OKT3) (10). The inhibitory activity of ISP-1 on (CTLL-2) apparently depends upon inhibition of the activity of serine palmitoyl tranferase (4), the enzyme that catalyzes the first step in the biosynthetic pathway of mammalian sphingolipids: namely, the condensation of serine and palmitoyl CoA into ketodihydrosphingosine. The sphingolipid pathway has been associated with various steps in signal transduction, differentiation, and apoptosis (14). In theory, FTY720 might also act on the sphingolipid during the early events of T cell activation. *In vitro*, however, FTY720 has no inhibitory effect on serine palmitoyl tranferase (4). Unlike CsA or tacrolimus, FTY720 has not been shown to inhibit the production of IL-2 or the induction of IL-2 mRNA expression by alloantigen (5). Therefore, FTY720 must act on a distinctive pathway.

Colorimetric assays suggested a dose-dependent reduction in cell viability, and genomic DNA analyses suggested apoptosis when human lymphocytes were incubated with FTY720 (15). Addition of FTY720 (2 to 10 $\times$ 10^6 M) to rat spleen cells produced an increased percentage of stained dead cells. On electron microscopy, these cells displayed features characteristic of apoptosis, including the absence of surface microvilli, condensation of chromatin, and formation of apoptotic bodies (9). Agarose gel electrophoresis revealed fragmentation of chromosomal DNA. All of these phenomena were consistent with apoptotic cell death.

Because FTY720 treatment produces a dose-de-

TABLE 1
Effects of FTY720 on Graft Survival in Different Models of Animal Transplantation

Species	Type of graft	FTY720 (mg/kg/d)	MST ± SD	% Above control	Reference
Rat	Skin	0.1	23 ± 2	155	(3)
		1	44 ± 6	388	
		3	59 ± 10	555	
	Heart	0.1	20.0 ± 4.5	78	(7)
		1	22.3 ± 5.7	83	
		10	72.2 ± 38.2	545	
	Liver	1	57.6 ± 29.8	350	(10)
		2	74.4 ± 35.1	481	
	Small bowel	2	12.2 ± 2.3	15	
		8	23.5 ± 4	122	
Mouse	Heart	2	11.4 ± 2.8	39	
		8	20 ± 3.7	143	
	Islet	2	58.6 ± 28.8	113	(8)
		4	71.7 ± 28.4	406	
Dog	Kidney	10	27.7 ± 7.6	147	(13)
Monkey	Kidney	0.1	70.7 ± 34.5	732	(a)
		0.3	67 ± 15.9	688	
		1	50 ± 25.5	488	

[a] Stepkowski S, unpublished data, 1998.

pendent reduction of cell viability of Fas-negative mutant thymocytes, FTY720-induced apoptosis must occur via a mechanism distinct from Fas-mediated cell death (16). A study suggested that human lymphocytes treated with FTY720 displayed a reduced ratio of Bcl-2 to Bax (15,17), thereby changing the intracellular ratio of the Ced-9 related protein products of a multigene family of cell death regulators. This change favors the generation of apoptotic stimuli leading to cell death (18). In addition, Jurkat lymphoma cells transfected with Bcl-2 genes were shown to be resistant to FTY720.

IN VIVO

Some *in vivo* evidence from our unpublished experiments is not consistent with the hypothesis that an effect of FTY720 to cause apoptotic cell death is the mechanism of prolonged graft survival. First, thymidine deoxynucleotide kinase staining of the lymph node lymphocytes from rats that displayed unresponsiveness toward heterotopic heart transplants showed the same frequency of apoptotic elements as those from normal hosts. Second, the frequency of specific memory cytotoxic T cells on limiting dilution analysis was also the same as in naïve animals; there was no evidence of deletion of alloreactive lymphocyte clones. A similar response was observed in animals rendered tolerant by combined treatment with FTY720 and allochimeric class I MHC antigens (19). In contrast, a study showed that FTY720 therapy was highly effective in inducing lymphocyte apoptosis *in vivo* (16). Finally, high-dose FTY720 treatment did not block the capacity of alloreactive cells to mediate accelerated rejection in pre-sensitized hosts. In order to analyze the *in vivo* effects of FTY720, methodologies must be applied to detect specific apoptotic events induced in lymphocytes and other immune cells by a variety of physiological stimuli, including glucocorticoids, antigen receptor engagement, tumor necrosis factor, or antibodies to the APO-1/Fas surface antigen (20). In order to elucidate the mechanism of *in vivo* action of FTY720, the relative significance of altered lymphocyte circulation compared to that of augmented lymphocyte apoptosis and other mechanisms must be discerned.

Studies have consistently shown that the immunosuppressive effects of FTY720 are related to a decrease in the number of circulating lymphocytes (5,9). *In vivo* administration of FTY720 to normal rats rapidly reduces the number of peripheral blood lymphocytes to less than 3% of the control value during the first 3 days of treatment (21) without altering either the total number or the percentage of lymphocytes of various subpopulations in the thymus, spleen, or lymph nodes (9,21). The number of peripheral blood lymphocytes significantly decreased within 3 to 6 h after oral administration of FTY720, while the number of polymorphonuclear leukocytes is increased. The number of monocytes was not affected. *In vivo*, the number of $CD4^+$ T cells seems to be sensitive to FTY720, while B cells are resistant to its effect (9,21).

It has been suggested that treatment with FTY720 may increase the expression, affinity, or avidity of α_4-integrin (VLA-4) expression based upon the effect of pre-incubation of treated lymphocytes with an anti-VLA-4 monoclonal antibody to prevent the lymphodepletion effect of FTY720. If FTY720 alters VLA-4 expression, it would "home" lymphocytes to high endothelial venules in lymph nodes and Peyer's patches and thus away from the allograft.

Pharmacokinetics

A stable compound of low molecular weight (343.94 daltons), FTY720 ($C_{19}H_{33}NO_2$; HCl) is soluble in water and ethanol. The oral bioavailability of FTY720 exceeds 60% in dogs, 80% in rats, and 40% in subhuman primates. It is more highly distributed in blood cells than in plasma, with ratios in rats or dogs of 7.8 to 17.6 or 4.0 to 8.0, respectively, following oral administration of 3 mg/kg FTY720. The ratios after intravenous administration of 1 mg/kg to rats and dogs are 5.3 to 20.8 and 4.8 to 8.3. The nature and extent of binding of FTY720 to plasma proteins is unclear. After administration of 3 mg/kg FTY720 orally to rats or dogs, the drug reaches a maximum concentration at 8 or 9 hours, respectively, and shows an elimination half-life ($t_{1/2}$) of unchanged drug in the blood of 12 and 29 h, respectively, after oral dosing, and 12 and 25 h, respectively, after intravenous administration (Murakami, unpublished data, 1997).

Concomitant administration of CsA and FTY720 to animals does not appear to affect the blood concentrations of either drug (9).

Toxicity

In rats, the LD_{50} value after a single oral administration is 300 to 600 mg/kg. No deaths were reported in dogs that received doses of up to 200 mg/kg. Vomiting, diarrhea, and anorexia were the most conspicuous pre-morbid symptoms. Autopsies on animals treated with supralethal doses showed gastric ulceration, vacuolation of medullary cells in the adrenal and peripheral nerves, and mononuclear cell infiltration into the brain and around the spinal vessels. Upon chronic administration of FTY720 to animals for 4 to 6 months, the lethal dose was more than 10 mg/kg. No toxic effects were observed at doses of 3 mg/kg in monkeys or 0.3 mg/kg in rats.

Although FTY720 seems to increase fetal mortality in rats, there has not been a study of mutagenicity, fertility, or embryo toxicity. Unlike CsA and tacrolimus, FTY720 does not produce renal, hepatic, pancreatic, or bone marrow toxicities or an increased incidence of neoplasms in animals.

Summary

FTY720 is a synthetic analog of a fungal metabolite that shows potent immunosuppressive activity *in vitro* and *in vivo* with little apparent toxicity. The drug displays marked synergistic effects *in vivo* with CsA and/or rapamycin. Therefore, this drug may improve the therapeutic window of agents that target cytokine synthesis or signal transduction. Because of these promising findings, the agent is likely to be tested in humans as an adjunct to clinical immunosuppressive regimens.

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