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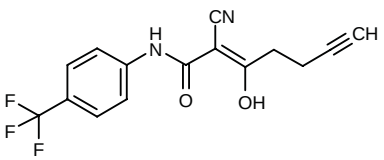
FK-778

Treatment of Transplant Rejection
Dihydroorotate Dehydrogenase Inhibitor

HMR-1715
MNA-715
X-920715

2-Cyano-3-hydroxy-*N*-[4-(trifluoromethyl)phenyl]hepta-2-en-6-ynamide



$C_{15}H_{11}F_3N_2O_2$
Mol wt: 308.2579
CAS: 152418-67-2
EN: 201758

Abstract

The malononitrilamide FK-778 is a leflunomide derivative that inhibits both the proliferation of T- and B-cells and the production of IgG and IgM. Preclinical studies have shown that FK-778 induces strong immunosuppressive effects in several models of autoimmune diseases, including graft-versus-host disease, experimental allergic encephalomyelitis and rheumatoid arthritis. FK-778 may also inhibit allograft and xenograft rejection, and its effects are increased when combined with other immunosuppressants such as tacrolimus. FK-778 seems to have great potential as a new drug in the management of autoimmune diseases and transplants, but its promising preclinical properties need to be confirmed in future trials with humans.

Synthesis

FK-778 is obtained by selective alkylation of leflunomide (I) (1) at the methyl group with propargyl iodide (II) by means of butyl lithium in THF at –78 °C to give *N*-(4-trifluoromethylphenyl)-5-(3-butynyl)isoxazole-4-carboxamide (III), which is finally treated with water at 0 °C (2). Scheme 1.

Introduction

Leflunomide (Arava™) (5-methyl-*N*-[4-(trifuoromethyl)phenyl]isoxazole-4-carboxamide) is a drug currently being used in the treatment of rheumatoid arthritis. It inhibits T-cell and B-cell functions and proliferation, suppresses the synthesis of anti-donor antibodies in transplantation models and also prevents the formation of neointimal hyperplasia (*i.e.*, proliferation of smooth muscle cells in the blood vessel wall). However, the long half-life of leflunomide (15-18 days in humans) makes it suitable for treating autoimmune diseases but cannot be used in transplantation. This has prompted the search for derivatives that are as strong immunosuppressants as leflunomide but that remain active in the recipient for shorter periods of time.

Malononitrilamides are low-molecular-weight derivatives of A-771726, the active metabolite of leflunomide. These compounds bind to and inhibit the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH). This enzyme is responsible for the conversion of dihydroorotate to orotate, and blocking this step leads to inhibition of *de novo* pyrimidine biosynthesis and arrest of cell cycle progression mainly between the G_1 and early S phases. These effects are similar to those found with the specific DHODH inhibitor brequinar (3, 4). Malononitrilamides retain strong immunosuppressive properties: they prevent acute rejection of rat skin and cardiac allografts, prolong xenograft survival, suppress intimal thickening in arterial allografts and inhibit the cellular and humoral immune responses by blocking B-cell and T-cell proliferation and suppressing IgM and IgG production. One of these compounds, FK-778 (MNA-715, HMR-1715, X-920715), has been extensively studied as a potential therapeutic option in the treatment of autoimmune diseases and in the management of transplant recipients.

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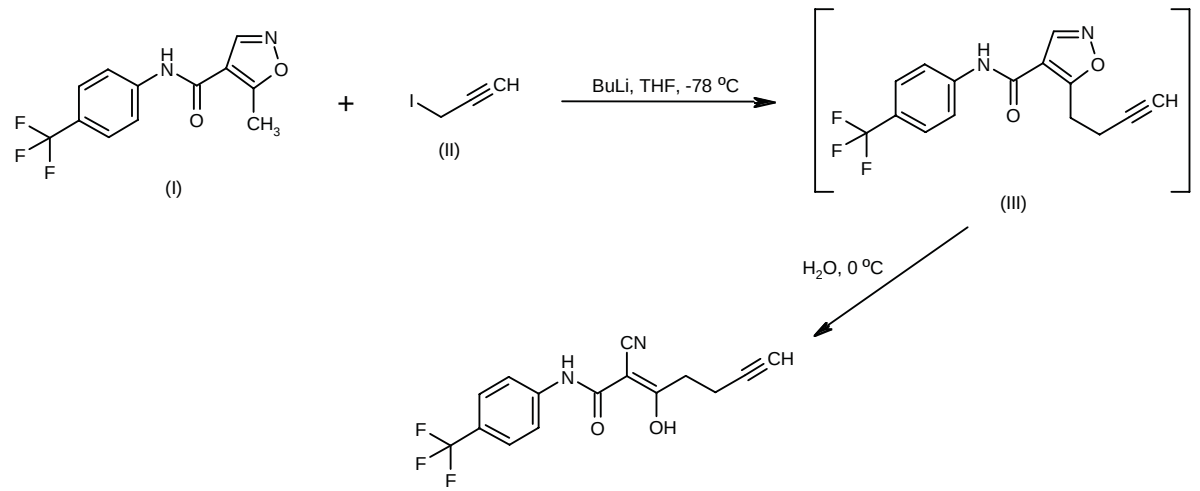
Scheme 1: Synthesis of FK-778



In vitro studies

The direct effect of FK-778 on T- and B-cells was studied using primary lymphocytes isolated from humans, mice and rats. *In vitro* experiments with concentrations of FK-778 ranging from 0.01-10 μmol/l revealed that FK-778 inhibited the proliferation of T-cells previously stimulated with a mitogen (phytohemagglutinin) or with specific monoclonal antibodies against TCR and CD28. A concentration of 5 μmol/l of FK-778 also inhibited the synthesis of IgG and IgM and the proliferation of activated B-cells; these effects were unrelated to the simultaneous presence of T-cells in the culture media (5). The IC_{50} estimated for FK-778 in this model was below 10 μmol/l, although different IC_{50} values have been reported in other models; for example, inhibition of lymphocyte proliferation by FK-778 showed an IC_{50} of 100 mmol/l when white blood cell samples were used and an IC_{50} of 91.2 mmol/l when administered to Lewis rats (6). More recently, a cell culture proliferation technique has revealed that different dose combinations of FK-778 plus tacrolimus have additive or synergistic effects in the inhibition of B-cell proliferation (7).

The antiproliferative effects of FK-778 on the mesenchymal cells in the transplantation donor organ were assessed using vascular smooth muscle cells. FK-778 inhibited the proliferation of the A10 cell line (embryonic thoracic aorta smooth muscle cells from rats) in a dose-dependent manner (IC_{50} = 171 μmol/l). Proliferation of murine A10 cells stimulated with 0.4 μg/ml of PDGF was also inhibited (IC_{50} = 4.2 μmol/l). Again, treatment with 1 mmol/l uridine almost completely inhibited the antiproliferative effects of FK-778 (8, 9).

The specificity of FK-778 varies in different species. The dose response curves for the inhibition of T-cell proliferation by FK-778 were identical in T-cells from humans and chimpanzees and similar in T-cells from baboons and cynomolgus monkeys. Mice and rats show very homogeneous inhibitory effects for FK-778 and no strain effect has been described. T-cells from large animals such as dogs, cats, sheep and pigs showed lower sensitivity to the immunosuppressive effects of FK-778, although a higher sensitivity was reported by Gregory *et al.* for T-and B-cells from dogs (10, 11).

As is the case with other malononitrilamides, FK-778 mediates its immunosuppressive effects through inhibition of the mitochondrial enzyme DHODH. Incubation of kidney, liver and intestine tissue sections from normal mice with 120 μmol/l of FK-778 resulted in complete inhibition of DHODH activity (10). The immunosuppressant effects of FK-778 were reversed by administration of uridine, which replenished the nucleotide pool and allowed the cells to proliferate. *In vivo*, coadministration of uridine (3 g/kg t.i.d.) with FK-778 (20 mg/kg) decreased graft survival compared with FK-778 monotherapy (12).

In vivo studies

Graft-versus-host disease

FK-778 is effective against different forms of graft-*versus*-host disease (GvHD). Schorlemmer *et al.* found that administration of FK-778 for 3 days at doses ranging from 7.5-30 mg/kg/day strongly inhibited the development of local GvHD reaction in the form of lymph node hyperplasia induced by injection of allogeneic cells in rats. Better effects were found with multiple administration of FK-778 than with a single oral dose (13, 14). These effects were caused by inhibition of the DHODH enzyme,

as administration of exogenous uridine reversed the antiproliferative effects in this assay (15). In a mouse model of acute lethal GvHD, treatment of transplanted mice with 2.5-20 mg/kg/day of FK-778 shortly after induction of the disease increased survival and decreased splenomegaly in a dose-dependent manner (16). Similarly, symptoms of chronic GvHD reaction in hybrid mice (*e.g.*, higher serum IgE and IgG_1 levels, mesenteric lymphadenopathy, splenomegaly or high levels of serum autoantibodies against double-stranded DNA) decreased after administration of 30 mg/kg/day of FK-778 by oral gavage on days 6 and 33 after disease induction (3, 16, 17).

Autoimmune diseases

FK-778 has also been tested in experimentally induced autoimmune diseases, such as rheumatoid arthritis in rats. Adjuvant arthritis was induced in rats by subplantar injection of cFA and *Mycobacterium butyricum*; both the injected paw and the contralateral paw began to swell 13 days after induction in rats that received no treatment for arthritis. Oral treatment with 5-20 mg/kg of FK-778 during induction of the disease prevented spreading to the noninjected paw in a dose-dependent manner. FK-778 also improved the survival rate and clinical signs in MRL/lpr mice, which showed an autoimmune disease very similar to human lupus erythematosus, largely through inhibition of circulating rheumatoid factors and autoantibodies (18, 19).

FK-778 is also an attractive therapeutic option for the treatment of human inflammatory and autoimmune diseases affecting the central nervous system, such as multiple sclerosis. In Lewis rats suffering from acute experimental allergic encephalomyelitis (EAE), treatment with FK-778 did not completely inhibit the development of the disease but did allow a complete recovery of the animals, preventing mortality in a dose-dependent manner. FK-778 was also effective in the treatment of rats with chronic EAE, a model particularly similar to human multiple sclerosis, where it reduced the severity of the symptoms and the number of relapses and also prevented mortality of diseased animals (20, 21).

Allograft rejection

Different allograft rejection models have been used to assess the immunosuppressive effects of FK-778. Using the nonvascularized rat tail skin transplant model, treatment of transplanted rats with oral gavage doses of 2.5-20 mg/kg/day of FK-778 given for 15 days immediately after transplantation resulted in a dose-dependent prolongation of graft survival. This effect was found with different donor-receptor combinations and rejection responses (22). Interestingly, administration of FK-778 alone at 7 days after transplantation reversed the rejection reaction in a manner similar to that found when FK-778 was administered immediately after transplantation. In contrast, treatment with ciclosporin at 7 days after transplantation failed to reverse rejection and prolong graft survival (23). Treatment with 20 mg/kg/day of FK-778 given by oral gavage either simultaneously with presensitization or 2 days before the second skin graft prevented hyperacute skin graft rejection and increased the mean graft survival time for more than 16 days. In agreement with this finding, FK-778 but not ciclosporin decreased the levels of donor-specific IgM and IgG antibodies in skin graft recipients (24).

The rat tail skin transplant model was used to assess the effects of FK-778 on the modulation of mononuclear phagocyte function. Preliminary *in vitro* experiments with human, rat and mouse macrophages revealed that incubation with different concentrations of FK-778 dose-dependently inhibited oxidative metabolism without inducing detectable cytotoxic effects. Additional studies showed that FK-778 also inhibited oxygen radical generation in rat and mouse macrophages *in vivo* and in rats transplanted with skin allografts; this inhibition was correlated with skin graft survival (25, 26).

The efficacy of FK-778 has also been evaluated in the prevention and reversal of rejection in heterotopic heart transplantation. In transplanted rats that received no immunosuppressive therapy, the cardiac allograft showed a median graft survival time of 7.5 days. Treatment with 20 mg/kg/day of FK-778 or 10 mg/kg/day of ciclosporin during days 0-9 after transplantation increased the median survival time to 20 and 18.5 days, respectively. Likewise, both FK-778 and ciclosporin administered between days 4 and 13 after transplantation reversed rejection and resulted in median graft survival times of 21.5 and 24 days, respectively. These effects were correlated with lower allospecific IgM and IgG titers compared to nonimmunosuppressed controls (27). FK-778 was also shown to be better than tacrolimus alone in the prevention of graft rejection and induction of long-term cardiac allograft survival in mice (28).

Xenograft rejection

The potential use of FK-778 in the prevention of xenograft rejection has also been assessed. One model used skin grafts from BALB/c mice transplanted to Lewis rats. Recipient rats showed a dose-dependent improvement in xenograft survival after administration of a combination of 10 mg/kg/day ciclosporin plus 10 or 20 mg/kg/day F-778 immediately after transplantation (6.4 ± 0.9 days with ciclosporin alone and up to 27.9 ± 1.5 days with the larger FK-778 dose). Similar results were obtained when the same combination was given at 5 days after transplantation in order to reverse the expected xenograft rejection (up to 30.0 ± 0.8 days). In all cases, no toxic effects were observed in the treated animals (29). These results correlated with inhibition of donor-specific IgM and IgG xenoantibodies *in vivo* (30, 31). When combined with tacrolimus, FK-778 showed similar syner-

gistic effects in the same mouse-to-rat xenograft model as with ciclosporin (32).

The possible use of FK-778 in preventing dysfunction of experimental islet xenografts was examined using two different models. In a pig-to-rat transplantation model, treatment with FK-778 alone or combined with ciclosporin had no effect on xenograft rejection and was associated with severe toxicity and high mortality (33). However, spontaneously diabetic NOD mice that received a rat islet xenograft responded synergistically to a combination therapy of FK-778 (20 mg/kg/day) and ciclosporin (10 mg/kg/day), with longer survival times than when either drug was administered alone. The exact characterization of early graft failure following xenotransplantation will contribute to the establishment of adequate immunosuppressive therapies for preventing xenograft rejection (34).

Combination with other immunosuppressants

Some studies have established the synergistic effects of the combination of FK-778 and other drugs. In the rat skin allograft model, simultaneous administration of ciclosporin and FK-778 at subtherapeutic doses of 5 mg/kg/day for 25 days induced a significant increase in graft survival compared to treatment with each drug alone (18.0 ± 0.9 days compared to 16 days with FK-778 alone) (22). Similarly, the combination of ciclosporin and FK-778 improved cardiac graft survival compared to FK-778 alone and compensated the rejection-promoting effect of the immunomodulator linomide after one or two allograft transplantations(27, 35, 36). Other authors reported that the combination of 5 mg/kg/day ciclosporin and 10 mg/kg/day FK-778 induced long-term survival of rat cardiac allografts (37). A recent study revealed that the combination of FK-778 with ciclosporin was highly effective in preventing rejection of rat fetal pancreatic allografts and allowed the development of the endocrine element of the grafts. No mononuclear cell infiltration was found in the combination-treated allografts for 30 days, and weight loss was the side effect associated with this therapy during the first 14 days of treatment (38). A combination of ciclosporin and FK-778 has also proven effective in prolonging renal allograft survival in mismatched dogs (39). After heterotopic renal transplantation, the dogs were treated with either ciclosporin 10 mg/kg b.i.d alone or with ciclosporin 10 mg/kg b.i.d. plus 2 mg/kg FK-778. Four dogs treated with ciclosporin and FK-778 and three dogs treated with ciclosporin alone survived to 100 days after transplantation with normal plasma creatinine concentrations. Overall, dogs treated with the combination therapy survived for a longer period of time and showed less inflammatory cell infiltration in the allograft than dogs treated with ciclosporin monotherapy (40).

Synergistic immunosuppressive effects have also been described for the combination of FK-778 and tacrolimus in different models. In a model of acute skin allograft rejection in rats, administration of a suboptimal dose of 0.1 mg/kg/day of tacrolimus on days 0-9 after transplantation followed by 20 mg/kg/day of FK-778 on days 5-9 significantly increased graft survival (39.2 ± 0.5 days compared to 27.1 ± 0.6 with FK-778 or 16.7 ± 0.8 days with tacrolimus). The combined treatment also resulted in long-term allograft survival and reversal of acute allograft rejection without any signs of toxic effects (41). These same researchers found that transplantation of a second skin graft from the same donor strain on those animals showing long-term graft survival did not induce rejection of the second graft without further drug treatment. This confirmed that the combination treatment of tacrolimus and FK-778 had induced tolerance in this model (42). Other studies found that this combination improved cardiac allograft survival and prevented graft rejection in rats (43).

In a rat model of chronic renal allograft rejection, oral administration of 10 mg/kg/day of FK-778 alone, 20 mg/kg/day of FK-778 alone or a combination of 3 mg/kg/day of FK-778 and 1 mg/kg/day of tacrolimus allowed maintenance of normal serum creatinine levels after 90 days of treatment. Treatment with FK-778 induced a significant, dose-dependent decrease in chronic histological changes, including decreased intragraft CD8 T-cell, NK cell and macrophage infiltration, thus confirming prevention of chronic renal allograft rejection (44). In a similar model, the combination of tacrolimus and FK-778 delayed renal graft rejection and increased the mean survival time: 16.8 ± 3.1 days with 10 mg/kg/day FK-778, 10.5 ± 1.4 days with 1.0 mg/kg/day tacrolimus, 25.5 ± 5.9 days with simultaneous administration of tacrolimus and FK-778, and 74.9 ± 14.8 days when FK-778 administration was delayed for 7 days after transplantation (7). Using a Vervet monkey kidney allograft model, researchers found that untreated control animals showed graft rejection after 7.8 ± 2.5 days. Treatment with 1.0 mg/kg/day of tacrolimus or with 2.5 mg/kg/day of FK-778 increased the mean survival time of the graft to 15.5 ± 3.0 days and 9.8 ± 3.0 days, respectively. Combination therapy with these two drugs at the same doses further increased the mean survival time to 18.8 ± 10.6 days when the drugs were administered right after transplantation, and to 36.6 ± 16.6 days when FK-778 therapy was delayed until 7 days after transplantation (45).

Pharmacokinetics

Evidence of the short half-life of FK-778 was found after treatment of cynomolgus monkeys with a daily oral dose of 10 mg/kg, which decreased lymphocyte proliferation to 23% of baseline values and gave steady-state trough plasma FK-778 levels at 48 h. With daily doses of FK-778 of less than 10 mg/kg, the drug did not reach steady-state levels and these dropped to zero after 2-4 days. A single loading dose of 20 mg/kg followed by 5 mg/kg once daily induced a gradual decrease of FK-778 trough levels in plasma, and lymphocyte proliferation was 11.4 ± 8.6% of pretreatment baseline values (46).

Conclusions

Malononitrilamides are new immunosuppressive agents with a unique mechanism of action that sets them apart from all other immunosuppressants. One of these compounds, FK-778, has been proven effective in several preclinical models of autoimmune diseases, such as graft-versus-host disease, EAE and rheumatoid arthritis, and also in the management of xenograft and allograft transplantation. By inhibiting T-cell and B-cell proliferation, FK-778 improved the clinical signs of autoimmune diseases and prevented graft rejection to an apparently greater extent than another malononitrilamide, MNA-279, with which it has been compared in many of the studies reported here. Treatment with FK-778 also seemed to be relatively safe; however, further studies on the toxicity of FK-778 in humans are needed, as the parent drug leflunomide is more toxic in dogs and rodents than in humans (27). Despite the fact that clinical trials need to be conducted in humans before realizing the full potential of FK-778 as an immunosuppressant, the preclinical data reported here suggest good clinical potential for FK-778, either as monotherapy or combined with other immunosuppressants, in the management of autoimmune disorders and graft rejection.

Source

Discovered at Aventis Pharma SA (FR); licensed to Fujisawa Pharmaceutical Co., Ltd. (JP).

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