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Malononitrilamides synergistically prevent acute and treat ongoing skin allograft rejection with cyclosporine

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Abstract The low molecular weight malononitrilamides (MNAs), a new class of immunosuppressive agents, belong to the derivatives of leflunomide's active metabolite, A771726. They have been shown to bind specifically to dehydroorotate dehydrogenase and inhibit de novo pyrimidine biosynthesis, thereby blocking T- and B-cell proliferation and strongly suppressing IgM and IgG antibody production. Here we evaluated their efficacy together with cyclosporine (CyA) in rat skin allotransplantation models, using different strain combinations. Monotherapy of transplanted animals in these models with the MNAs HMR 1279 and HMR 1715 resulted in a significant and dose-dependent prolongation of the graft survival time. Even a short-term application showed efficacy in the prevention of acute rejection. The MNAs were also effective when treatment was started at the time of expected rejection crisis, demonstrating strong therapeutic activity to reverse ongoing acute rejection, whereas CyA was ineffective for the treatment of ongoing allograft rejection episodes. Combination therapy of MNAs with CyA proved to be very effective for the prevention of acute skin graft rejection. Interestingly, whereas CyA alone was unable to treat ongoing acute rejection episodes, comedication of MNAs and CyA, even after a short-term application, was synergistically effective and significantly suppressed ongoing allogeneic skin graft rejection. These results demonstrate that MNAs are potent and well tolerated immunosuppressants with a potential comparable to that of CyA, but they are superior to CyA in their ability to reverse acute rejection episodes. They represent powerful rescue drugs and demonstrate synergistic activity with CyA to prevent acute and treat ongoing skin allograft rejection.

Key words Skinallotransplantation · Immunosuppression · Malononitrilamides · Combination therapy · Cyclosporine

Introduction

The novel immunosuppressive malononitrilamides (MNAs) are chemically unrelated and distinct in their mode of action from any other immunosuppressant known so far. HMR 1279 and HMR 1715 are derivatives of leflunomide's active metabolite, A771726, and have been developed for being evaluated in preclinical models of transplantation because they have a shorter half-life in animals and would make dose adjustment easier in transplant patients. They have potent immunosuppressive activity against a variety of experimental autoimmune diseases in rodents [6, 7, 9, 12]. The MNAs inhibit both cellular and humoral immune responses and they mediate their immunosuppressive effects by binding specifically to dehydroorotate dehydrogenase and

inhibiting de novo pyrimidine biosynthesis [15]. Thus they block T- and B-cell proliferation and strongly suppress IgM and IgG antibody production [1, 2]. Both HMR 1279 and HMR 1715 effectively control graft-versus-host diseases in rats and mice [10, 12, 13] and prolong xenograft survival in various models [3, 5]. They also were shown to prevent and reverse acute heart and skin allograft rejection, while they were well tolerated in these rodent transplantation studies [3, 4, 11].

Due to the unique pharmacological ability of the MNAs to interact directly with B- and T-cell functions and to inhibit cellular and humoral immune responses, we were particularly interested to evaluate the efficacy of HMR 1279 [2-cyano-*N*-(4-cyanophenyl)-3-cyclopropyl-3-oxo-propan-amide] and HMR 1715 [2-cyano-3-hydroxy-*N*-[4-(trifluoromethyl)-phenyl]-6-heptynamide] either alone or in combination with cyclosporine (CyA) in rat skin allotransplantation models, using various donor-recipient strain combinations.

Materials and methods

Animals

The inbred rat strains Dark Agouti (DA), Fischer (F344), and Lewis (LEW), with a body weight of about 170 g, were commercially obtained from Charles River (Wiga Sulzfeld, Germany). All animals were fed a standard rat diet and drinking water ad libitum; they were housed in our own animal unit in temperature and light controlled (12 h/day) rooms.

Chemicals

Both MNA 279 (HMR 1279) and MNA 715 (HMR 1715) were supplied in pure form as a white powder (Hoechst Marion Roussel, Werk Kalle-Albert, Wiesbaden, Germany) and were prepared daily as a homogeneous suspension in 1% carboxymethylcellulose (CMC) at varying concentrations. CyA (Sandimmune or Neoral; Sandoz, Basel, Switzerland) was dissolved in wheat germ oil. All drugs were given by oral gavage. Animals were treated with the drugs at the concentrations and schedules given in the text. Control animals received the vehicle solution (1% CMC) only. Drug treatment normally started either on the same day as the transplantation or shortly before the expected rejection crisis.

Skin transplantation

Rat tail skin transplantation was carried out as described previously [8]. The donor skin was cut into square pieces of 1 × 1 cm and transplanted to the tails of the recipient rats. Rejection was defined when the skin graft turned red-brown and became hard. Two rat strain combinations leading either to delayed rejection [LEW ($RTI^{a}l$) to F344 ($RTL^{a}lvl$)] or to fast rejection [DA ($RTI^{a}avl$)to LEW ($RTI^{a}l$)] were used for transplantation studies.

Table 1 Prevention of acute skin allograft rejection by monotherapy with HMR 1279 (MNA 279) in the Dark Agouti (DA)-Lewis (LEW) rat model (*CMC* carboxymethylcellulose, *MNA* malononitrilamide)

Drug application	Days of individual skin graft rejection	Mean survival time in days (± SD)
Control (1% CMC)	7, 8, 8, 8, 8 9, 9, 9, 9, 10	8.5 ± 0.9
Neoral (15 mg/kg) days 0–9	22, 22, 23, 23, 23, 24, 24, 24, 25, 25	23.5 ± 1.1
MNA 279 (20 mg/kg) days 0–9	25, 25, 25, 25, 26 26, 26, 27, 27, 28	26.0 ± 1.1
MNA 279 (30 mg/kg) days 0–7	21, 21, 21, 22, 22 22, 23, 23, 23, 24	22.2 ± 1.0
MNA 279 (30 mg/kg) days 0/4/9	22, 22, 22, 22, 22 22, 23, 23, 24, 24	22.6 ± 0.8
MNA 279 (50 mg/kg) day 0	20, 20, 21, 21, 21 22, 22, 22, 23, 23	21.5 ± 1.1
MNA 279 (50 mg/kg) day 7	21, 21, 22, 22, 23 23, 23, 24, 24, 24,	22.7 ± 1.2
MNA 279 (50 mg/kg) days 0 + 8	22, 22, 22, 22, 23 23, 24, 24, 25, 25	23.2 ± 1.2

Results

MNA monotherapy is able to prevent and reverse acute ongoing skin allograft rejection

Both MNAs (HMR 1279 and HMR 1715) effectively prevented acute rejection following an induction treatment. Even short-term applications (< 5 days) with higher concentrations (30 and 50 mg/kg) of the MNAs were still effective in delaying acute skin allograft rejection. The effects seen for prolongation of skin graft survival by only one or two applications of MNA HMR 1279 or HMR 1715 were comparable to continuous treatment with 15 mg/kg of Neoral (Tables 1, 2).

The delayed administration of the MNAs shortly before the expected rejection crisis on days 7–16 post-transplantation even prolonged skin graft survival (Table 3), which contrasted with the inability of CyA to promote a similar effect. The graft survival times (27.4 ± 1.2 days and 27.5 ± 1.4 days) produced by HMR 1279 and HMR 1715, respectively, were comparable to those of the groups in which treatment was started together with the transplantation on day 0 (prevention). Also, a short-term application regimen on days 7–11 with the MNAs was still therapeutically active in prolonging skin allograft survival up to 25 days. When CyA (20 mg/kg) was administered as a single drug as late as days 7–16, skin allograft survival failed to improve compared to the controls.

It was of particular interest to characterize the effects of these derivatives of leflunomide's active metabolite

Table 2 Prevention of acute skin allograft rejection by monotherapy with HMR 1715 (MNA 715) in the LEW-Fischer (F 344) rat model

Drug application	Days of individual skin graft rejection	Mean survival time in days (± SD)
Control (1% CMC)	14, 14, 15, 15, 15 15, 15, 16, 16, 16	15.1 ± 0.7
Neoral (15 mg/kg) days 0–14	27, 27, 28, 28, 28 28, 29, 29, 30, 30	28.4 ± 1.1
MNA 715 (20 mg/kg) days 0–14	28, 28, 28, 29, 29 30, 30, 30, 31, 31	29.4 ± 1.2
MNA 715 (30 mg/kg) days 0–10	26, 27, 27, 28, 28 28, 28, 28, 29, 30	27.9 ± 1.1
MNA 715 (30 mg/kg) days 0/6/14	27, 27, 27, 28, 29 29, 29, 29, 30, 31	28.16 ± 1.4
MNA 715 (50 mg/kg) day 0	24, 24, 24, 25, 25, 26, 26, 27, 28, 28	25.7 ± 1.6
MNA 715 (50 mg/kg) day 10	27, 27, 27, 27, 27 28, 28, 29, 29, 30	27.9 ± 1.1
MNA 715 (50 mg/kg) days 0–10	28, 28, 29, 29, 29 30, 31, 31, 31, 32	29.8 ± 1.4

Table 3 Treatment of ongoing acute skin allograft rejection with HMR 1279 and HMR 1715 using the DA-LEW rat model

Drug application (20 mg/kg per day)	Days of individual skin graft rejection	Mean survival time in days (± SD)
Control (1% CMC)	8, 8, 8, 9, 9 9, 9, 9, 10, 10	8.9 ± 0.7
Cyclosporine A (days 0–9)	26, 26, 26, 26, 27 27, 27, 27, 28, 29	26.9 ± 1.0
Cyclosporine A (days 0–16)	8, 8, 8, 9, 9 9, 10, 10, 10, 10	9.1 ± 0.9
HMR 1279 (days 7–16)	26, 26, 26, 27, 27 28, 28, 28, 29, 29	27.4 ± 1.2
HMR 1279 (days 7–11)	23, 23, 24, 24, 25 25, 26, 26, 26, 27	24.9 ± 1.4
HMR 1715 (days 7–16)	26, 26, 26, 27, 27 28, 29, 29, 29, 30	27.5 ± 1.4
HMR 1715 (days 7–11)	23, 24, 24, 24, 25 25, 25, 26, 27, 27	25.0 ± 1.3

on the development of alloantibody titers in vivo. When treatment was initiated on the day of transplantation and the isotype composition of the alloreactive antibody titers was studied, both HMR 1279 and HMR 1715 significantly inhibited the development of alloantibody production in vivo (Table 4). The levels of allospecific antibodies, which were predominantly of the IgM isotype, were reduced to almost background levels, but also the generation of an allospecific IgG response was inhibited.

Using a rat skin graft model of hyperacute accelerated rejection in presensitized animals, treatment with

Table 4 Effects of HMR 1279 or HMR 1715 on alloantibody (IgM/IgG) production in a rat skin transplant model (DA-LEW)

Days post-transplantation	Drug treatment (10 × 20 mg/kg postoperatively, days 0–9)			
	Control	Cyclosporine	HMR 1279	HMR 1715
IgM alloantibody titers (channel fluorescence ± SEM)				
– 1	296 ± 21	296 ± 21	296 ± 21	296 ± 21
5	310 ± 21	350 ± 19	319 ± 16	309 ± 20
13	488 ± 26	445 ± 37	313 ± 26	316 ± 33
19	499 ± 34	507 ± 28	334 ± 37	363 ± 46
26	501 ± 15	499 ± 16	378 ± 62	405 ± 35
IgG alloantibody titers (channel fluorescence ± SEM)				
– 1	155 ± 4	155 ± 4	155 ± 4	155 ± 4
5	152 ± 4	154 ± 7	148 ± 4	152 ± 4
13	301 ± 71	279 ± 55	155 ± 8	153 ± 4
19	314 ± 74	346 ± 65	173 ± 30	169 ± 6
25	374 ± 89	380 ± 66	210 ± 21	258 ± 38

Table 5 Control of hyperacute accelerated skin allograft rejection with HMR 1279 and HMR 1715 using DA-presensitized LEW rats

Drug application (20 mg/kg per day)	Days of individual skin graft rejection	Mean survival time in days (± SD)
Control (1% CMC)	4, 4, 5, 6, 6 6, 6, 7, 7, 7	5.8 ± 1.1
Neoral (15 mg/kg) days – 10 to – 1	4, 4, 5, 5, 5 6, 6, 6, 6, 6	5.3 ± 0.8
HMR 1279 days – 10 to – 1	17, 17, 18, 18, 18 18, 19, 19, 19, 19	18.2 ± 0.8
HMR 1279 days – 2 to – 7	15, 16, 16, 16, 16 17, 17, 17, 17, 18	16.5 ± 0.9
HMR 1715 days – 10 to – 1	17, 17, 17, 17, 18 18, 18, 18, 19, 19	17.8 ± 0.8
HMR 1715 days – 2 to 7	15, 15, 15, 16, 16 17, 17, 17, 17, 18	16.3 ± 1.1

CyA (15 mg/kg per day) could not prevent the hyperacute rejection. The graft survival in this group was as fast (5.3 ± 0.8 days) and indistinguishable from the controls. Animals presensitized by a first skin graft on day – 10 rejected their second sets (given on day 0) on day 5.6 ± 0.8. Also, the alloantibody levels (IgM and IgG) remained unchanged and high in the control group (451 ± 37 and 292 ± 47) and the CyA-treated animals (482 ± 45 and 335 ± 59), as their skin grafts were hyperacutely rejected. Treatment with HMR 1279 or HMR 1715 (20 mg/kg per day) given either on days – 10 to – 1 together with the presensitization, or only 2 days prior to the second skin graft on days – 2 to 7, prevented hyperacute skin graft rejection and prolonged the mean graft survival time for more than 16 days (Table 5). When the isotype composition of the alloreactive antibody titers was studied, treatment of the presensitized (LEW) rats with the MNAs (HMR 1279

Table 6 Combination therapy of HMR 1279 and HMR 1715 with cyclosporine *(CyA)* to prevent acute skin allograft rejection using the DA-LEW rat model

Drug application (MNAs 15 mg/kg)	Days of individual skin graft rejection	Mean survival time in days (± SD)
Control (1% CMC)	8, 8, 8, 8, 9, 9, 9, 9, 10, 11	8.9 ± 1.0
CyA (5 mg/kg) days 0–9	8, 8, 9, 9, 9 9, 10, 10, 11	9.2 ± 1.0
MNA 279 days 0–9	23, 23, 23, 23, 24 24, 25, 25, 26	24.0 ± 1.1
MNA 715 days 0–9	21, 23, 23, 24, 25 25, 26, 27	24.3 ± 1.9
CyA + MNA 279 days 0–9	26, 27, 28, 29, 29, 30, 31, 32	29.0 ± 2.0
CyA + MNA 279 days 0–4	26, 26, 26, 26, 27 27, 28, 28, 28, 29	27.1 ± 1.1
CyA + MNA 715 days 0–9	26, 28, 30, 30, 30 31, 31, 32	29.8 ± 1.9
CyA + MNA 715 days 0–4	26, 26, 27, 27, 27, 28, 28, 28, 29, 29	27.5 ± 1.1

Table 7 Combination therapy of the malononitrilamides *(MNAs)* HMR 1279 and HMR 1715 with cyclosporine to prevent acute skin allograft rejection using the LEW-F344 rat model

Drug application (MNAs 15 mg/kg)	Days of individual skin graft rejection	Mean survival time in days (± SD)
Control (1 % CMC)	13, 13, 14, 14, 14, 15, 16, 16, 17, 18	15.0 ± 1.7
CyA (5 mg/kg) days 0–14	14, 14, 14, 14, 15 16, 16, 16, 16, 18	15.3 ± 1.3
MNA 279 days 0–14	22, 23, 23, 23, 25 25, 27, 27, 29, 29	25.3 ± 2.6
MNA 715 days 0–14	23, 23, 24, 24, 25 25, 28, 28, 30, 31	26.1 ± 2.9
CyA + MNA 279 days 0–14	31, 31, 31, 32, 32 33, 36, 39, 39	33.8 ± 3.4
CyA + MNA 279 days 0–4	27, 28, 30, 31, 31 31, 32, 33, 34, 35	31.2 ± 2.5
CyA + MNA 715 days 0–14	30, 31, 31, 33, 33 34, 34, 35, 36, 37	33.4 ± 2.3
CyA + MNA 715 days 0–4	29, 29, 30, 30, 31 31, 31, 33, 34, 35	31.3 ± 2.1

or HMR 1715) resulted in a substantial reduction of anti-DA IgM (284 ± 24 or 303 ± 37) and IgG (162 ± 5 or 160 ± 4) antibodies. The MNAs even suppressed hyperacute rejection when they were administered to animals with preexisting antidonor antibodies, whereas CyA failed to suppress both.

Combination therapy of MNAs and CyA has synergistic effects on skin allograft survival

Combining subtherapeutic doses (5 mg/kg) of CyA and the MNAs caused a significant increase in graft survival compared with graft survival in animals treated with either drug alone. While the skin allograft recipients treated with 5 mg/kg of CyA showed no significant graft prolongation, the combination with HMR 1279 or HMR 1715 improved graft survival to 29.6 ± 3.5 days and 30.6 ± 4.1 days, respectively. Similar results were found when we applied the combinations of an effective dose of the MNAs (15 mg/kg) with an ineffective dose of CyA (5 mg/kg) to transplanted LEW or F344 rats. These improvements reflect a synergistic effect since CyA was given at an individually ineffective dosage. Also, a short-term application on days 0–4 of both MNAs in combination with an ineffective dose of CyA was still synergistically effective in prevention of acute skin allograft rejection. When treatment was started therapeutically at the time of the expected rejection crisis either on days 7–16 or days 10–24 (dependent on the rat strain combinations used), even then both MNAs demonstrated synergistic effects with an ineffective dose of CyA and proved to be very effective in reversing ongoing skin allograft rejection (Tables 6, 7).

Discussion

It has been shown previously in the non-vascularized skin transplantation model that HMR 1279 and HMR 1715 are able to delay the onset of acute rejection and prolong skin allograft survival in a dose-dependent manner [11]. Both MNAs demonstrated that at comparable doses they and CyA were equally effective in preventing skin graft rejection. The minimal effective dose (2.5 mg/kg) prolonged the mean graft survival time for approximately 5 days, whereas with the higher concentrations after stopping drug treatment, the graft survival time was more than double that of control animals. Even a delayed administration of the MNAs shortly before the expected rejection crisis prolonged skin graft survival. When CyA administration started as late as on day 7 as a single drug therapy, skin allograft survival failed to improve compared with the controls. This observation is consistent with previous transplant studies using CyA for single-drug rescue therapy [8, 14]. In animals allowed to undergo acute rejection, both MNAs are superior to CyA in their ability to reverse such a reaction and to produce significant allograft survival. In the skin allotransplantation model described, both MNAs (HMR 1279 and HMR 1715) also inhibited significantly the development of alloantibody production in vivo. The levels of allospecific antibodies of the IgM and IgG isotype were reduced to almost background le-

vels during MNA therapy. Upon cessation of drug treatment, the alloantibody titers slowly and gradually increased. In contrast, CyA given from day 0 did not reduce alloantibody production. The IgM and IgG antibody titer curves were very similar to those of the control group, even though CyA significantly prolonged skin allograft survival. Also, in a rat skin graft model of hyperacute accelerated rejection in presensitized animals, the alloantibody levels of the IgM and IgG isotype were strongly suppressed in MNA-treated animals. Even when the MNAs were administered to presensitized rats with preexisting antidonor antibodies they were still able to inhibit hyperacute skin allograft rejection.

Several investigators [8, 14] previously observed that leflunomide's active metabolite, A771726, the parent drug of the MNAs, in combination with CyA always induced better immunosuppression and graft survival than either drug alone. Also, in the combination regimens of MNAs with CyA studied so far using different organ transplantation models and various strain combinations, all investigators always obtained additive or synergistic effects on graft survival by combining different doses of HMR 1279 or HMR 1715 and CyA [3, 10, 11]. Synergism with CyA and MNAs was always seen for prevention and therapy, and the new immunosuppressants represent powerful rescue drugs. When assessed by body weight, no toxic side effects could be observed in these drug combination experiments with CyA using concentrations up to 20 mg/kg for both drugs. Both MNAs (HMR 1279 and HMR 1715) are potent, well tolerated immunosuppressive agents that are synergistic in their activity with CyA.

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