Oral activity of a nature-derived cyclic peptide for the treatment of multiple sclerosis

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Abstract

Multiple sclerosis (MS) is the most common autoimmune disease affecting the central nervous system. It is characterized by auto-reactive T cells that induce demyelination and neuronal degradation. Treatment options are still limited and several MS medications need to be administered by parenteral application but are modestly effective. Oral active drugs such as fingolimod have been weighed down by safety concerns. Consequently, there is a demand for novel, especially orally active therapeutics. Nature offers an abundant source of compounds for drug discovery. Recently, the circular plant peptide kalata B1 was shown to silence T-cell proliferation in vitro in an IL-2-dependent mechanism. Owing to this promising effect, we aimed to determine in vivo activity of the cyclotide [T20K]kalata B1 using the MS mouse model experimental autoimmune encephalomyelitis (EAE). Treatment of mice with the cyclotide resulted in a significant delay and diminished symptoms of EAE by oral administration. Cyclotide application substantially impeded disease progression and did not exhibit adverse effects. Inhibition of lymphocyte proliferation and the reduction of proinflammatory cytokines, in particular IL-2, distinguish the cyclotide from other marketed drugs. Considering their stable structural topology and oral activity, cyclotides are candidates as peptide therapeutics for pharmaceutical drug development for treatment of T-cell-mediated disorders. Therefore, cyclotides have become attractive tools in chemical biology and drug development (15), for instance as templates for molecular grafting applications (16) as well as for receptor ligand design (17), because they presumably exhibit activity following oral administration (18).

Cyclotides, in particular [T20K]kalata B1, inhibit T-cell proliferation by down-regulation of IL-2 release as well as IL-2R/CD25 surface expression (13). The cytokine IL-2 physiologically plays an important role in T-lymphocyte activation and acts as an autocrine factor to stimulate T-cell proliferation (19). Enhanced or continuous T-cell activation is a major cause of autoimmune disorders and can lead to persistent inflammation, causing tissue and organ damage (20). Multiple sclerosis (MS) is the most common type of autoimmune disease in young adults, which is characterized by sustained inflammation of the CNS. Autoreactive T lymphocytes of the Tp<sub>17</sub> subset target myelin brain antigens, eliciting inflammatory cell influx into the CNS, demyelination, axonal damage, and neuronal degradation (21, 22). Several therapeutics targeting different aspects to modulate or suppress T-cell signaling are available, but the parenteral administration route of many drugs reduces their attractiveness for chronic treatment (23). Only three marketed compounds that are specific for MS treatment are active via oral administration [i.e., dimethyl fumarate, teriflunomide, and fingolimod (Gilenya)], a sphingosine 1-phosphate receptor modulator that stimulates the sphingosine-1-phosphate receptor-1 (S1P1) on T cells, resulting in the inhibition of lymphocyte egress from the blood vessels (24). Therefore, there is a need to develop new or enhance existing oral active therapeutics for MS treatment.

Significance

Multiple sclerosis (MS) imposes substantial economic burdens on patients, their families, and society. Until now, there are few therapies available, but often unattractive parenteral application or severe side effects are serious issues. This study highlights the use of circular peptides as orally active T-cell-specific immunosuppressive therapeutics against the MS model experimental autoimmune encephalomyelitis, without inducing major adverse effects. Our work provides a proof of principle that nature-derived cyclic peptides serve as oral active therapeutics, utilizing their intrinsic bioactivity and stable three-dimensional structure. Cyclotides are considered a combinatorial peptide library and they can be anticipated to complement the existing collections of natural products that are used in drug discovery.


Conflict of interest statement: C.G. and C.W.G. serve as members of the scientific advisory board of Cyxone AB since January 25, 2016.

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receptor ligand; however, many and severe side effects limit their therapeutic use (24).

Owing to their remarkable stability and hydrophobic surface properties, cyclotides are ideally suited for oral administration. Their immunosuppressive properties have been confirmed on human T cells. In the present study we demonstrate the effect of the cyclotide [T20K]kB1 in the state-of-the-art in vivo model for MS, the murine experimental autoimmune encephalomyelitis (EAE) assay, after oral administration. In particular, we investigated their efficacy to reduce the polarization of pathogenic Th17 cells and the rate of relapse by prophylactic administration of cyclotides before disease induction. Moreover, we analyzed the therapeutic application of cyclotides during disease progression, which potently ameliorated the EAE symptoms. Biodistribution and systemic uptake of the peptide drugs has been monitored using the in vivo imaging system (IVIS) and mass spectrometry, respectively. Our observations suggest cyclotides may be good candidates as MS therapeutics, without causing any adverse effects based on preliminary toxicity studies in mice. The results provide proof of principle for the application of an orally active cyclic peptide drug in the treatment of autoimmune disorders and could inspire pharmacological screening as well as preclinical development of other peptide-based therapeutics of natural origin (25).

Results

Modulation of T-Cell Proliferation by [T20K]kalata B1. Extensive screening efforts for the discovery of novel nature-derived drugs has led to the identification of antiproliferative properties of the cyclotide kB1 toward human activated lymphocytes in vitro (13). Recently, structure-activity screening of cyclotides isolated from the plant Oldenlandia affinis (Rubiacaeae) established [T20K]kB1 as an active lead compound to inhibit T-cell proliferation and, interestingly, replacement of valine to the positively charged lysine in loop 2 resulted in a loss of activity in vitro (13). In the current work, [T20K]kB1 was synthesized following a modified protocol for the generation of thioester peptides and subsequent cyclization by native chemical ligation (Fig. 1 and Fig. S1) (26, 27).

To evaluate the immunosuppressive effects of cyclotides in vivo, the murine T-cell autoimmune-specific EAE was investigated as a model for human MS. In EAE, autoreactive CD4+ T cells of the subtype Th17 are the major cause initiating and provoking demyelination of the CNS that leads to the typical appearance of paralysis symptoms (28). As a preliminary experiment, [T20K]kB1 and [V10K]kB1 were incubated at different concentrations with mouse immune cells ex vivo, which were isolated from spleens of EAE-induced, viz. myelin oligodendrocyte glycoprotein (MOG35–55)-immunized mice, to confirm the immunosuppressive effects previously observed in human T cells (13). The proliferation of T cells, measured by flow cytometry, as well as the autocrine acting proliferation-supporting cytokine IL-2 and the Th1 and Th17 signature cytokines IFN-γ and IL-17A in the supernatant of MOG-restimulated cells (Fig. S2 A–C), were down-regulated by [T20K]kB1 in a concentration-dependent fashion; the inactive control cyclotide [V10K]kB1 had significantly less effect in reduction of cytokine secretion. Cyclosporine A, which leads to IL-2 suppression by modulating calcineurin activity (29), was used as a positive control to confirm the antiproliferative properties of the cyclotide. Down-regulation of cytokine-related mRNAs caused by [T20K]kB1 was confirmed by quantitative PCR (Fig. S2D).

Finally, significant antiproliferative effects and a reduction of inflammatory cytokine release of IL-2, IFN-γ, and IL-17A has been demonstrated by restimulation of splenic T cells isolated from 2D2 MOG–specific TCR transgenic mice following cyclotide [T20K]kB1 treatment (Fig. S2E).

Activity and Therapeutic Effects of Cyclotides to Treat EAE. The cyclotide [T20K]kB1 exhibits concentration-dependent immunosuppressive activity in vitro, which encouraged the investigation of the in vivo activity of the circular plant peptide using the T-cell-specific EAE model. C57Bl/6 mice immunized with MOG and treated in advance (day −7) i.p. with [T20K]kB1 (10 mg/kg) demonstrated a significant delay of the onset and only minor symptoms of the autoimmune encephalomyelitis, whereas the inactive mutant or the untreated control group exhibited no significant effects in disease reduction; these mice experienced severe bilateral paralysis and weight loss (Fig. 2A, Fig. S3A, Table S1, and Movie S1). Survival analysis of EAE experiments, including moribund mice (clinical score ≥4, see SI Materials and Methods), further supported the observation that treatment of EAE mice with the cyclotide [T20K]kB1 exerts long-lasting and protective T-cell antiproliferative properties (Fig. 2B). Using IVIS, a lower chemiluminescent signal based on the release of myeloperoxidase by inflammatory cells was detected in the appropriate regions in cyclotide-treated EAE–induced mice, compared with the untreated control group (Fig. 2C). Splenic T cells of EAE-induced mice enhance inflammatory cytokine expression when incubated with the antigen MOG. Isolation and MOG restimulation of splenocytes from cyclotide-pretreated
cells infiltrating the spinal cord the later the cyclotide injection was performed. Thus, the highest degree of reduction of inflammation and the lowest grade of demyelination of axons was observed in [T20K]kB1- (day −7) treated mice (Fig. S4C). Proliferation-inducing IL-2 and other TH1- and TH17-related inflammatory cytokines were significantly reduced in MOG-restimulated isolated splenic T lymphocytes, influenced by the time point of cyclotide treatment (Fig. S4D). Following these promising protective properties of the cyclotide [T20K]kB1 against autoimmunity, EAE-induced mice were treated therapeutically at a score of 2, meaning at a disease stage of partial paraparesis. A pilot experiment with a single injection of cyclotide (10 mg/kg) resulted in a significant reduction in the clinical score (Figs. S4B and S3C), but effects on cytokine secretion were moderate (Fig. S4E). Nevertheless, treatment with three injections (10 mg/kg) administered every third day (Table S1) seemed to be more promising. The progression of the autoimmune encephalomyelitis was not only substantially blunted, but also the health status of the cyclotide-treated mice was slightly improved (Fig. 2F and Fig. S3D). In addition, autoimmune-related cytokine levels were reduced, suggesting a therapeutic potential of...
the nature-derived peptide [T20K]kB1 (Fig. S4F). These findings have been further verified by histological experiments that depicted an amelioration of the encephalomyelitis due to a lower grade of axonal demyelination in cyclotide-treated animals and decreased numbers of inflammatory cells in the CNS (Fig. 2G). In fact, cyclotide treatment resulted in a reduced number of CD3+ (P < 0.001) and CD4+ (P < 0.01) cells in the CNS and in decreased IL-2 release (P = 0.07) (Fig. S5).

**Oral EAE Treatment Using the Cyclotide [T20K]kalata B1.** Because cyclotides are resistant against enzymatic and chemical degradation, due to their unique 3D structure, [T20K]kB1 was tested in an oral treatment experiment for its T-cell antiproliferative properties. Mice were treated with two different doses: one group of mice was given 10 mg/kg, the same dose as was used for the i.p. injections, and a second group was treated with 20 mg/kg (Table S1). Accordingly, [T20K]kB1 applied orally improved the EAE clinical score in a dose-dependent manner in comparison with the control group (Fig. 3A and Fig. S3E). The cumulative clinical score for the [T20K]kB1-treated mice (20 mg/kg) after oral administration (mean ± SEM: 21.5 ± 9.7) was significantly lower compared with the control group (64.1 ± 17.5), whereas treatment with a lower dose of [T20K]kB1 (10 mg/kg) was not significantly different (52.0 ± 24.2). Therefore, cytokine release of the 20 mg/kg [T20K]kB1 treatment was analyzed poststimulation of splenic T cells after 48–72 h with their natural antigen MOG (Fig. S6a). These findings were substantiated with histology analysis, which revealed a minor inflammatory index and reduced areas of axonal demyelination in orally cyclotide-treated mice, compared with the EAE-induced control mice (Fig. S6b). Following up on those results, [T20K]kB1 was evaluated for its therapeutic application (Table S1). A significant amelioration of the autoimmune encephalomyelitis regarding the clinical score and histological analysis has been demonstrated by oral treatment using MOG-immunized mice (Fig. 3B and Figs. S3F and S6d). Mice treated three times with [T20K]kB1 at a score of 2 yielded a cumulative clinical score of 43.5 ± 12.3 compared with the control group, which exhibited 78.5 ± 10.7.

To analyze the biodistribution of cyclotides, [T20K]kB1 was labeled with the NIR-fluorescent VivoTag. After oral administration of the derivatized cyclotide, an uptake into the gastrointestinal tract, systemic distribution and excretion mainly via the biliary tract. After oral application of 20 mg/kg of [T20K]kB1 in healthy mice, body weight and temperature as well as the liver parameters mentioned above were monitored and confirmed its safety. In addition, the concentration of the lipid parameters triglycerides and cholesterol indicated no difference between [T20K]kB1-treated mice and the control group. Histology of the gastrointestinal tract further confirmed that cyclotides do not cause cellular lesions or noticeable adverse effects after oral administration (Fig. S8).

Furthermore, we carried out a head-to-head comparison of the circular plant peptide and the oral bioavailable drug fingolimod (Fig. 3C). The latter is being used in the clinic to treat MS and is therefore the appropriate reference. A single administration of 20 mg/kg fingolimod a week before (day −7) MOG immunization with 20 mg/kg did not mitigate the course of the disease: Clinical scores of fingolimod-treated and control animals were comparable. In contrast, the cyclotide ameliorated the subsequent course of the disease in a statistically significant manner (P < 0.0001) (Fig. S9A and Table S1). Application of 20 mg/kg three times in 3-d intervals resulted in a reduction in EAE disease progression; the effect was comparable in magnitude for both fingolimod and the cyclotide (Fig. S9B). Daily administration (starting at day 0) of fingolimod at an established dose of 1 mg/kg (30) seemed to be the most effective treatment to halt progression of EAE. Similarly, the cyclotide [T20K]kB1 achieved comparable efficacy if animals were treated with a daily dose of 1 mg/kg (Fig. S9A). In fact, daily cyclotide treatment leads to a substantially reduced level of cytokine secretion (IL-2, IFN-γ, and IL-17A), as well as a significantly lower degree of demyelination and a minor inflammatory index (Fig. S6C). No differences in adverse effects between the cyclotide (20 mg/kg) and fingolimod (20 mg/kg and 1 mg/kg daily) after oral administration can be observed using this animal study as determined by the same rigorous level of tolerability evaluation as described above (Fig. S8). Comparison of the cyclotide [T20K]kB1 to other immunosuppressant drugs revealed that morbidity and mortality (moribund mice) was significantly reduced in [T20K]kB1-, prednisolone-, azathioprine- and cyclosporine A-treated mice (Fig. S9C).

**Discussion**

Although preliminary studies have shown that the cyclotide kalata B1 can inhibit lymphocyte proliferation in vitro (12) the effectiveness of nature-derived cyclotides to prevent or treat autoimmune disorders in vivo after oral administration has hitherto not been reported. Our study demonstrates that the peptide [T20K]kB1 is an orally active therapeutic for treatment of the T-cell-mediated MS model EAE (22) in vivo. Antiproliferative effects of the cyclotide kalata B1 and the mutant [T20K]kB1 have been investigated on human mononuclear cells and purified T cells, highlighting the IL-2–specific inhibitory mechanism in vitro (12, 13). Release of TGFβ and IL-17 signature cytokines were not only inhibited in vitro when incubated with the cyclotide but also after oral administration (Fig. S9D). Moreover, although [T20K]kB1 achieved comparable efficacy if animals were treated with a daily dose of 1 mg/kg (Fig. S9A). In fact, daily cyclotide treatment leads to a substantially reduced level of cytokine secretion (IL-2, IFN-γ, and IL-17A), as well as a significantly lower degree of demyelination and a minor inflammatory index (Fig. S6C). No differences in adverse effects between the cyclotide (20 mg/kg) and fingolimod (20 mg/kg and 1 mg/kg daily) after oral administration can be observed using this animal study as determined by the same rigorous level of tolerability evaluation as described above (Fig. S8). Comparison of the cyclotide [T20K]kB1 to other immunosuppressant drugs revealed that morbidity and mortality (moribund mice) was significantly reduced in [T20K]kB1-, prednisolone-, azathioprine- and cyclosporine A-treated mice (Fig. S9C).

**A**. Clinical EAE score (mean ± SEM) of orally [T20K]kB1-treated (day −7) mice with two different doses of 10 mg/kg (dark red diamond, n = 6) or 20 mg/kg (red sphere, n = 6) and untreated control group (black triangle, dotted line, n = 5). Two-way ANOVA was used to calculate statistical significance between [T20K]kB1-treated mice and the control group. **B**. EAE clinical score (mean ± SEM) after therapeutic oral treatment of mice with [T20K]kB1 (20 mg/kg, three times in 3-d intervals, indicated by the arrows). [T20K]kB1: n = 5; control: n = 6. Statistical significance between cyclotide-treated and untreated EAE mice was analyzed by two-way ANOVA as of the first day of treatment. **P < 0.0001.**
control
control
control

Fig. 4. Biodistribution and uptake of cyclotides after oral administration. (A) Biodistribution of orally applied VivoTag-labeled [T20K]kB1, using two doses of 10 mg/kg and 5 mg/kg, and untreated control mice. Fluorescence intensity (radiant efficiency) was monitored using the IVIS at the indicated time points (5 min, 40 min, and 4, 24, and 72 h) after oral gavage from dorsal and abdominal directions. (B) Organs of orally treated mice with VivoTag-labeled [T20K]kB1 and VivoTag label alone and from untreated control mice were scanned for fluorescence intensity (radiant efficiency) using IVIS 72 h after compound administration. Calculation and quantification was performed using IVIS Living Image software, illustrating radiant efficiency of the kidneys, the liver, and the stomach of indicated animals. Data demonstrate mean ± SEM of two independent experiments. (C) Analysis of native [T20K]kB1 in serum samples after oral administration of 20 mg/kg peptide, 1 h (Left) and 2 h (Inset) postadministration, respectively, using MALDI-TOF mass spectrometry. Control measurements of [T20K]kB1 spiked into fresh blood at 0.2 mg/kg corresponding to 1% of the total administered dose (Right). Peptide peaks are shown as monoisotopic mass [M+H]+.

vivo activity to reduce EAE-associated symptoms of [T20K]kB1 by parenteral application (i.p.). Interestingly, in vivo activity of [T20K]kB1 was sequence-specific because the cyclotide [V10K]kB1 (or the untreated control group) exhibited neither significant effects in disease reduction nor any significant reduction of inflammation or demyelination in the CNS. An effective way to prevent an episode of EAE was administration of cyclotide to mice, the gold-standard animal model of human multiple sclerosis (22). Incidentally, cyclosporine A produced by the fungus Tolypocladium inflatum is also a cyclic (undecapeptide) and it is considered the prototype of a new generation of immunosuppressive drugs (22). Historically, cyclosporine A was instrumental to the development of modern immunology and it is still used today (29, 36). Despite the limitations of such comparisons, we believe that the rich diversity of cyclotides (17, 43) justifies their position as a treasure trove for drug discovery.

Materials and Methods

Detailed materials and methods are given in SI Materials and Methods.
Peptide Synthesis. Peptides were synthesized following a recent protocol for the generation of thioresterpeptides using Fmoc-SPPS and their use in native chemical ligation (26) and its adaptation for amino acid coupling assisted by microwave heating (27).

Animals and Ethics. Eight-wk-old C57BL/6 mice were purchased from Charles River and ZD2 myelin oligodendrocyte glycoprotein MOG35-55-specific Tcer mice on a C57BL/6 background (C57BL/6-Tg[Tcra2D2,Tcrb2D2]1Kuch/J) were purchased from Jackson Laboratories. All experiments were approved according to the European Community rules of animal care with the permission of the Austrian Ministry of Science (BWMF-66.009/0241-I/38/2011).

EAE. C57BL/6 mice were immunized at day 0 according to the protocol described recently in Sahin et al. (44). Progression of EAE was divided into five clinical stages: score 0, no signs; score 1, complete tail paralysis; score 2, partial paraparesis; score 3, severe paraparesis; score 4, tetraparesis; and score 5, moribund condition. Mice were euthanized by deeply anesthetizing them with ketamine reaching a score of 3–4 due to ethical guidelines.

In Vivo Imaging. Cyclotide-treated (10 mg/kg i.p. on day −7) and untreated control EAE mice received Redilight D-Lucifer bioluminescent substrate (PerkinElmer) i.v. on day 12 after MOG immunization. Monitoring with the IVIS (PerkinElmer) was performed on day 13 by measuring chemiluminescence signal. Higher chemiluminescence levels represent enhanced inflammation in the appropriate regions. VivoTag 680 XL (PerkinElmer) labeled [T20K]81 was injected i.v., i.p., or orally (p.o.) into naïve mice. Fluorescence signal (excitation: 665 ± 5 nm, emission: 688 ± 5 nm) was monitored after indicated time points. Organs of euthanized mice were screened for the fluorescence and quantified by using IVIS Living Image software.

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