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

Kathrin Thelli, Roland Hellinger, Emine Sahin, Paul Michenthaler, Markus Gold-Binder, Thomas Haider, Mario Kuttke, Zita Liutkevičiūtė, Ulf Göransson, Carsten Gründemann, Gernot Schabbauer, and Christian W. Gruber

*Center for Physiology and Pharmacology, Medical University of Vienna, 1090 Vienna, Austria; **University Clinic for Trauma Surgery, Medical University of Vienna, 1090 Vienna, Austria; †School of Biomedical Sciences, The University of Queensland, Brisbane, QLD 4072, Australia; ‡Division of Pharmacochemistry, Department of Medicinal Chemistry, Biomedical Centre, Uppsala University, 75123 Uppsala, Sweden; and *Center for Complementary Medicine, Department of Environmental Health Sciences and Hospital Infection Control, Medical Center-University of Freiburg, 79106 Freiburg, Germany

Edited by Jerrold Meinwald, Cornell University, Ithaca, NY, and approved February 23, 2016 (received for review October 12, 2015)

Multiple sclerosis (MS) is the most common autoimmune disease affecting the central nervous system. It is characterized by autoreactive 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 mostly 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 abundance 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]kB1 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.

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 immuno-suppressive 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.

This article is a PNAS Direct Submission. Freely available online through the PNAS open access option.

To whom correspondence may be addressed. Email: gernot.schabbauer@medunivien.ac.at or christian.w.gruber@medunivien.ac.at.

This article contains supporting information online at www.pnas.org/cgi/doi/10.1073/pnas.1519960113/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1519960113

PNAS Early Edition | 1 of 6
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 (Rubiaceae) 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 (MOG)-specific MOG-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. S2A–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

![Fig. 1. Synthesis of cyclotides. Cyclotides were assembled as linear precursors using FMOC chemistry, and cyclized using native chemical ligation. (1) Dawson’s resin containing di-Fmoc-3,4-diaminobenzoic acid (Dbz) as linker is the starting point. (2) Couplings are performed using microwave-assisted FMOC synthesis (asterisk marks the first amino acid; the last amino acid is a 4-Nitrophenylchloroformate) (5c) oxidativelfoldin gyield (5c) oxidative folding to yield cyclotides with the native fold. Ribbon representation of a cyclotide (kalata B1, PDB ID code 1NB1) and sequence of [T20K]kalata B1 are shown. Cysteines, disulfide bonds (yellow), and intercysteine loops are indicated.

![Diagram of cyclotide synthesis](image-url)
mice exhibited a significant reduction of IL-2, IFN-γ, and IL-17A levels compared with both the [V10K]kB1-treated and untreated control group (Fig. 2D). Furthermore, no significant perivascular infiltration of mononuclear cells and an intact myelin sheath of the spinal cord of cyclotide-treated mice were observed using H&E and Klüver Barrera Luxol Fast Blue (LFB) stains. This explains the significant reduction of inflammation and lower grade of demyelination in the CNS (Fig. 2E). Taken together, [T20K]kB1 cyclotide treatment not only leads to halt of disease progression, but also induces long-lasting effects supporting survival and hindering restimulation of splenic T cells. Consequently, we evaluated the effects of a therapeutic treatment for reversion of autoimmune disease progression. C57BL/6 mice were treated with 10 mg/kg [T20K]kB1 1 wk before (day −7) and 1 wk after (day 7) (Table S1). Mice treated with the cyclotide revealed an inhibition or a delay in the progression of the autoimmune encephalomyelitis reliant on the time point of the treatment (Figs. S4A and S3B). This was confirmed by histological H&E and LFB stains, which showed increased inflammation and advanced demyelination induced by mononuclear 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-induced IL-2 and other T17- and T177-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) administered 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 < 0.0001) and CD4+ (P < 0.001) 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 postrestimulation 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. The latter is being used in the treatment of 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 TGFβ1 and TGFβ1 signature cytokines were not only inhibited in vitro when incubated with the cyclotide, but also after ex vivo restimulation of EAE-induced [T20K]kB1–pretreated splenocytes with their natural antigen MOG. In particular, the reduction in the disease-relevant T-cell cytokine IL-17A supports the observed clinical and histological reversion of disease progression upon cyclotide treatment. Initially various treatment regimens confirmed in
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 (radant efficiency) was monitored using the IVIS at the indicated time points (5 min, 20 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 given as monoisotopic mass [M+H]+.

Thell et al. PNAS Early Edition | 5 of 6

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 healthy mice 1 wk before disease induction. This could be an advantage in the relapsing-remitting form of MS, one of the most common types (31). After the decline of the first symptoms, cyclotide treatment could potentially interfere with the recurrence of more profound disabilities. In addition, daily treatment with lower doses seemed to be very efficient to prevent disease progression. However, [T20K]kB1 was also effective to treat mice at a disease stage of paraparesis, which impeded progression of EAE substantially.

To take advantage of the structural stability of cyclotides, the oral treatment experiments describe for the first time to our knowledge cyclotides used as oral active therapeutics for EAE. Administration of cyclotides at therapeutic doses did not induce adverse effects; it not only elicited a short-lived effect on the secretion of signature cytokines (IL-2, IFN-γ, and IL-17A) but also maintained the health status of the cyclotide-treated mice. Application of cyclotide leads to reversion of disease progression and induces long-lasting effects supporting survival and hindering restimulation of splenic T cells. The observed inhibition on proliferation of splenocytes isolated from the genetically modified 2D2 mouse model further supports a strong beneficial effect on disease development.

Oral treatment required a higher dose compared with parenteral administration likely due to low bioavailability of [T20K]kB1, as commonly observed for peptide therapeutics (3). In fact, grafted cysteine-knot peptides are thought to have limited bioavailability of less than 1%. Nevertheless, it was possible to detect traces of [T20K]kB1 in serum by MALDI-TOF mass spectrometry up to 2 h following oral administration. The gastrointestinal uptake and systemic biodistribution of a derivatized cyclotide ([T20K-VivoTag]kB1) has been confirmed by IVIS. Although the VivoTag-labeled cyclotide may exhibit different physicochemical properties, IVIS seemed to be a valid model system to monitor the trajectory of the cyclotide following oral administration based on the known stability of cyclotides in proteolytic environment and at acidic conditions (32).

The long-term effect induced by the cyclotide [T20K]kB1 was comparable to known immunosuppressive drugs, such as cyclosporine A, prednisolone, and azathiopeine, but their clinical use in MS therapy is limited due to low activity, lack of oral bioavailability, or high incidence of systemic side effects (33–35). In a head-to-head comparison of [T20K]kB1 and fingolimod, a recently approved orally bioavailable drug to prevent MS progression, the cyclotide seemed to be an advantageous therapeutic option when administered at a single dose; fingolimod was only effective with multiple administrations, and best at daily administration of its therapeutic dose. Consequently, cyclotides exhibiting oral activity are very promising acquisitions in drug discovery, not only regarding the treatment of MS but as a potential option for oral treatment of other autoimmune-related diseases (25, 36). Successful advances in chemical and recombinant synthesis (37, 38) as well as enzymatic folding (39) and cyclization (40) may yield affordable production of cyclotides at clinical scale in the near future.

At the more general level, our work provides a proof of principle for the concept that nature-derived cyclic peptides serve as oral active therapeutics using their intrinsic bioactivity and stable 3D structure. In fact, cyclotides have been recently used as scaffolds to improve the stability of peptides that have interesting pharmaceutical activities. This grafting introduced peptide sequences into cyclotide loops and resulted in a chimeric molecule that was orally active as bradykinin B1 receptor antagonist in the treatment of chronic inflammatory pain (18). Cyclotides represent a natural combinatorial peptide library and they probe a chemical space that is difficult to target by using small organic molecules (17). Thus, at the very least, they can be anticipated to complement the existing collections of natural products or synthetic molecules that are used in drug discovery. In particular, building on the unique topology of cyclotides (16) may complement efforts in rational design of bioavailable cyclic peptide therapeutics (41).

To the best of our knowledge, this is the first report that documents that a ribosomally synthesized, plant-derived cyclic peptide is effective after oral administration to prevent and to treat EAE in mice, the gold-standard animal model of human multiple sclerosis (22). Incidentally, cyclosporine A produced by the fungus Tolypocladium inflatum is also a cyclic (undeca)peptide and it is considered the prototype of a new generation of immunosuppressive drugs (42). Historically, cyclosporine A was instrumental to the development of modern immunopharmacology and it is still in clinical use 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 thioesterpeptides 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 2D2 myelin oligodendrocyte glycoprotein MOG35-55-specific TCR transgenic (tg) mice on a C57BL/6 background (C57BL/6-Tg[Tra2D2, Tcrb2D2]1KuchJ) 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 (BMVF-66.009.0241-0/3B/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 Rediinject D-Luciferin bioluminescent substrate (PerkinElmer) i.v. on day 12 after MOG immunization. Monitoring with the IVS (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 [20k]B1 was injected i.v., i.p., or orally (p.o.) into naive mice. Fluorescence signal (excitation: 665 ± 5 nm, emission: 688 ± 5 nm) was monitored after indicated time points. Organs of euthanized mice for the fluorescence and quantification were used by IVS Living Image software.

ACKNOWLEDGMENTS. We thank David Craik (University of Queensland) and Bachem AG for supplying peptides for this research, Günter Lametschwandtner and Hannes Mühlens (Apeiron Biologics AG) and Richard Clark (University of Queensland) for technical support, and Kjell Stenberg (Acequua AB) for comments on the manuscript. This research was supported by Austrian Science Fund Grant FWF-P24743, Austria Wirtschaftsservice GmbH Prize-P1308423, and Australian Research Council Future Fellowship FT14007030 (to C.W.G.). C.G. was financially supported by the Software AG Foundation and DAMUS-DONATA e.V.