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A Cysteine Protease Inhibitor Cures Chagas’ Disease in an Immunodeficient-Mouse Model of Infection

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Chagas’ disease, caused by the parasite Trypanosoma cruzi, remains the leading cause of cardiopathy in Latin America, with about 12 million people infected. Classic clinical manifestations derive from infection of muscle cells leading to progressive cardiomyopathy, while some patients develop megacolon or megaeosophagus. A very aggressive clinical course including fulminant meningoencephalitis has been reported in patients who contract Chagas’ disease in the background of immunodeficiency. This includes patients with human immunodeficiency virus infection as well as patients receiving immunosuppressive therapy for organ transplant. Currently, only two drugs are approved for the treatment of Chagas’ disease, nifurtimox and benznidazole. Both have significant limitations due to common and serious side effects as well as limited availability. A promising group of new drug leads for Chagas’ disease is cysteine protease inhibitors targeting cruzain, the major protease of T. cruzi. The inhibitor N-methyl-Pip-F-homoF-vinyl sulfonyl phenyl (N-methyl-Pip-F-hF-VS) is in late-stage preclinical development. Therefore, the question arose as to whether protease inhibitors targeting cruzain would have efficacy in Chagas’ disease occurring in the background of immunodeficiency. To address this question, we studied the course of infection in recombinase-deficient (Rag1−/−) and normal mice infected with T. cruzi. Infections localized to heart and skeletal muscle in untreated normal animals, while untreated Rag1−/− mice showed severe infection in all organs and predominantly in liver and spleen. Treatment with the dipeptide N-methyl-Pip-F-hF-VS rescued immunodeficient animals from lethal Chagas’ infection. The majority (60 to 100%) of inhibitor-treated Rag1−/− mice had increased survival, negative PCR, and normal tissues by histopathological examination.

Chagas’ disease remains the leading cause of heart disease in Latin America, with 10 to 12 million people estimated to be infected and 100 million people at risk. Chagas’ disease is caused by the protozoan parasite Trypanosoma cruzi which gains entrance into the human host most commonly through an insect vector or blood transfusion (8, 33). Classic clinical manifestations derive from parasite infection of cardiac muscle, leading to progressive cardiac myocyte loss and cardiomyopathy. Some patients, particularly in regions of Brazil, develop megacolon or megaeosophagus due to infection of muscle cells and nerve ganglia of the myenteric plexus (9).

A very aggressive clinical course has been reported in patients who contract or reactivate Chagas’ disease in the background of immunodeficiency. This includes patients with human immunodeficiency virus infection and low T-cell counts, as well as patients receiving immunosuppressive therapy for organ transplant and for heart transplant due to Chagasic myocardopathy. A fulminant meningoencephalitis, with a high mortality rate, has been reported in AIDS patients with Trypanosoma cruzi infection (5, 7, 10).

Only two drugs have been approved worldwide for the treatment of Chagas’ disease, nifurtimox and benznidazole. Both have significant limitations due to common and serious side effects (6, 27), as well as limited efficacy (2, 11, 18, 21). Drug resistance is quite common in the course of human infection (11, 21; P. S. Doyle et al., unpublished data).

A promising group of new drug leads for Chagas’ disease is cysteine protease inhibitors targeting cruzain, the major protease of Trypanosoma cruzi (13, 16; C. D. Emal, A. Alvarez-Hernandez, Y. Truong, E. Hansell, P. S. Doyle, J. H. McKerrow, and W. R. Roush, submitted for publication). Cruzain has various functions during the life cycle of Trypanosoma cruzi (3, 31), including a role in immune evasion in the mammalian host (P. S. Doyle, Y. M. Zhou, I. Hsieh, D. Greenbaum, M. Bogyo, J. C. Engel, and J. H. McKerrow, unpublished data). In view of the lethality of T. cruzi infection in immunocompromised patients, the question arose as to whether protease inhibitors targeting cruzain would have efficacy in Chagas’ disease occurring in the background of immunodeficiency. To address this question, we studied the course of infection in recombinase-deficient Rag1−/− mice infected with a myotropic strain of T. cruzi, an animal model for Chagas’ disease in immunodeficiency. The vinyl sulfone cysteine protease inhibitor N-methyl-Pip-F-homoF-vinyl sulfonyl phenyl (N-methyl-Pip-F-hF-VS) cured T. cruzi infection, even in the absence of a functioning adaptive immune response.

MATERIALS AND METHODS

Cells. CA-I/72 T. cruzi parasites were isolated from a chronic Chagasic patient, cloned, and maintained as previously described (14). T. cruzi was also maintained in bovine embryo skeletal muscle (BESM) cells (12). Infectious trypomastigotes were collected from BESM culture supernatants 5 days postinfection (12).

Animal trials. Four to six-week-old female, recombinase-deficient (Rag1−/−) mice on a B6 background (B6: 129S7-Rag 1/J) and wild-type C57BL/6J controls were from The Jackson Laboratory (Jax). Rag1−/− animals were lodged in
groups of three to five per cage for 24 h prior to infection with 100 tissue culture-derived trypomastigotes of the myotropic T. cruzi CA-I/72 cloned stock. Animals were treated with 50 mg N-methyl-Pip-F-hF-VS\textsuperscript{28} (also known as K11777) kg of body weight in 100 l of solution (30% dimethyl sulfoxide:70% sterile distilled water) intraperitoneally twice a day (15, 16). Treatment was initiated within 1 h postinfection and continued for a total of 27 days. Appropriate untreated controls were included in each experimental trial. Three independent experiments were performed.

For comparison of tissue tropism, parental (C57BL/6J) and C3HeB/Fej (Jax) immunocompetent animals were infected with 10\textsuperscript{6} CA-I/72 trypomastigotes intraperitoneally, separated in identical lots, and a group treated as above.

**PCR.** PCR was performed as described by Avila et al. (4). Briefly, DNA was extracted from tail blood specimens (20 to 50 l) with a DNeasy tissue and blood kit (QIAGEN, CA) and amplified with T. cruzi kinetoplast (k)-specific probes (4) using a PTC-100 programmable thermal controller (MJ Research, Inc.). For PCR with tissue DNA, we used the DNeasy protocol for paraffin-embedded tissues with the only exception that extraction with xylene was overnight.

**Histology.** All untreated Rag1\textsuperscript{−/−} animals died of acute Chagas’ disease 30 to 40 days postinfection and were immediately necropsied. The majority of N-methyl-Pip-F-hF-VS\textsubscript{28}-treated Rag1\textsuperscript{−/−} mice survived infection, and they were euthanized at 16 to 24 weeks postinfection. Animals were necropsied, and selected tissues were processed for histopathology, i.e., heart, skeletal muscle, liver, spleen, and colon. Fixed tissues were stained with hematoxylin eosin and examined by light microscopy using Openlab software.

**RESULTS**

The treatment regimen was selected based on protocols that rescue immunocompetent mice from acute infection (16; P. S. Doyle, J. C. Engel, and J. H. McKerrow, unpublished data). Treatment with the vinyl sulfone cysteine protease inhibitor N-methyl-Pip-F-hF-VS\textsubscript{28} (K11777) (26) for 27 days rescued 100% of immunocompetent animals from lethal acute Chagas’ infection with CA-I/72 T. cruzi. All untreated control mice developed massive ascites indicative of severe heart failure and paralysis of the hind legs by 10 to 20 days postinfection and died by 30 to 40 days postinfection with CA-I/72 T. cruzi, as reported previously for the Y strain (16, 17). Heart and skeletal muscle were the preferential sites of infection in untreated, immunocompetent mice challenged with the myotropic CA-I/72 T. cruzi clone (Fig. 1A and B). Other tissues were free of parasites.

Infected, untreated immunodeficient Rag1\textsuperscript{−/−} mice showed T. cruzi infection in all organs examined, i.e., heart, skeletal muscle, liver, spleen, and colon (Fig. 2). Tissue tropism and level of infection differed markedly in Rag1\textsuperscript{−/−} mice compared to wild-type animals. Massive infection of hepatic Kupffer cells and spleen macrophages of Rag1\textsuperscript{−/−} mice was noted (Fig. 2B and D), while the heart muscle contained only rare amastigote nests and negligible inflammation.

All untreated Rag1\textsuperscript{−/−} mice challenged with T. cruzi died within 40 days (Fig. 3). In contrast, 60 to 100% of Rag 1 knockout mice treated with 50 mg N-methyl-Pip-F-hF-VS\textsubscript{28}/kg weight twice a day for 27 days survived the lethal infection for up to 150 to 162 days (20 to 24 weeks postinfection) (Fig. 3, 2T
Surviving mice showed no trace of infection by histopathological examination and all tissues examined were normal (Fig. 4). To confirm that the cure was drug related, a single N-methyl-Pip-F-hF-VS6-treated Rag1−/− mouse was rechallenged at 112 days with 100 trypomastigotes of CA-I/72 T. cruzi as described above (Table 1). This untreated animal died at 32 days of acute Chagas’ disease (not shown).

All untreated Rag1−/− mice (Fig. 3) had a positive T.
cruzi-specific PCR (Table 1). Representative results are shown in Fig. 5. PCRs with blood specimens extracted 27 days after treatment were still positive in several Rag 1–/– animals treated with N-methyl-Pip-F-hF-VS6 (Fig. 5A; Table 1). Kinetoplast DNA is very stable and can be detected with Avila and colleagues’ method for up to 40 days posttreatment of T. cruzi-infected BESM cultures with benznidazole or N-methyl-Pip-F-hF-VS6 (P. S. Doyle, unpublished data). Subsequent PCRs (performed between 80 to 162 days postinfection) were negative in three independent treatment experiments (Table 1; Fig. 5B). PCRs with liver, spleen, and heart tissues from two animals treated with N-methyl-Pip-F-hF-VS6 were negative versus positive PCRs for the same tissues from an untreated control animal (Fig. 6). Significant prolonged survival without gross signs of Chagas’ disease compared to untreated controls, negative PCR, and normal tissues by histopathological examination were considered evidence of cure of inhibitor-treated Rag 1–/– animals that survived the acute T. cruzi infection (Fig. 3; Table 1).

DISCUSSION

Reactivation of Chagas’ disease leading to meningoencephalitis and/or acute myocarditis occurs in immunosuppressed patients with AIDS or following heart and organ (e.g., kidney, bone marrow, or liver) transplantation and certain cancer treatments (5, 7, 10). Fatal T. cruzi infection has recently been reported in the United States from organ transplants from infected organ donors (7, 28). Cardiac transplant in patients with chronic chagasic cardiopathy is now a common procedure, but reactivation of the trypanosomiasis occurs, with clinical manifestations characterized by fever, acute myocarditis, erythematous cutaneous lesions, and abundant T. cruzi-infected macrophages (20). Treatment with nifurtimox or benznidazole can lead to temporary remission and decreased mortality in these patients, though T. cruzi infection persists (20).

The only drug now available in Latin America for treatment of Chagas’ disease is benznidazole. Nifurtimox is the only drug approved for treatment in the United States. Both compounds have significant toxicity (27) leading to severe adverse effects that require medical supervision. Benznidazole may cause skin rash, peripheral neuropathy, granulocytopenia, generalized edema, fever, lymphoadenopathy, articular and muscular pain, and thrombocytopenic purpura. Nifurtimox also has serious side effects including weight loss, peripheral neuropathy, skin rash, psychosis, nausea and vomiting, and leukopenia. Toxicity studies with nifurtimox noted neurotoxicity, testicular and ovarian damage, and deleterious effects in adrenal, colon, esophagus, and mammary tissues (6). Both drugs are potentially mutagenic and carcinogenic (23–25). Chemotherapy for Chagas’ disease was traditionally recommended during the acute and indeterminate stages of infection (2, 11, 19), though recent treatment of chronically infected patients resulted in improved cardiac status (32). Retrospective studies in Latin
America revealed that current chemotherapy does not clear parasitemia and drug resistance is common (11, 21).

Clearly new therapy for Chagas’ disease is a critical need. One promising lead series is protease inhibitors targeting cruzain (also known as cruzipain), the major T. cruzi protease (15). A member of this series, N-methyl-Pip-F-hF-VSΦ, with a good safety profile and pharmacokinetics/pharmacodynamics (1, 16) was used in this study. Rag1−/− animals lack functional recombinase leading to defects in the initial phases of variable, diversity, and joining recom-
bination of immunoglobulin and T-cell receptor genes. Their primary immune defects include B- and T-cell deficiency, B- and T-cell arrest, and deficits in CD3+ cells and T-cell receptor αβ+ cells that render them susceptible to pathogens (22). Indeed, Rag 1−/− mice were extremely susceptible to T. cruzi infection and died within 10 days when challenged with an infectious dose that kills immunocompetent animals in 30 to 40 days (not shown). The infectious titer was decreased in these experiments to more closely resemble the natural course of disease. As anticipated, 100% of untreated Rag 1−/− mice challenged with T. cruzi died of acute Chagas’ disease. In contrast, 60 to 100% of Rag 1−/− mice treated with N-methyl-Pip-F-hF-VS for 27 days survived until sacrificed at 16 to 24 weeks (112 to 162

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a Pos, positive; Neg, negative; Hi, histopathology; Tc, T. cruzi amastigotes and inflammation; N, normal tissues; ND, not done.
b Two of three mice were sacrificed; one mouse out of three was rechallenged with T. cruzi and died of acute Chagas’ disease 32 days postinfection.
c Sacrificed due to T. cruzi infection.
d Animals incorrectly euthanized by animal facility personnel at 50 and 140 days postinfection and not available for further studies.
e Four of five mice had positive PCRs at day 27 postinfection, and two of five mice available for testing at 150 days postinfection had negative PCRs.

FIG. 5. (A) T. cruzi-specific PCR in Rag1−/− mice at 27 days postinfection. Representative results shown are from tests 2C and 2T (Table 1; Fig. 3). Blood DNA specimens collected 27 days postinfection were subjected to PCR with a specific T. cruzi probe. With the exception of one animal (lower gel, lane 6), all untreated controls (upper gel, lanes 4 to 6) and most inhibitor-treated mice (lower gel, lanes 2 to 5) had positive PCRs 27 days postinfection (arrow; 330-bp band). Upper gel: lane 1, molecular weight ladder; lane 2, negative control (no DNA); lane 3, noninfected mouse blood; lanes 4 to 6, blood from untreated, T. cruzi-infected Rag1 mice; lane 7, not applicable; lane 8, positive control, T. cruzi DNA. Lower gel: lane 1, DNA ladder; lanes 2 to 6, blood from N-methyl-Pip-F-hF-VSΦ-treated and T. cruzi-infected Rag1 mice no. 1 through 5, respectively. (B) Negative PCR at 5 months for Rag1−/− animals treated with N-methyl-Pip-F-hF-VSΦ. Five months after treatment with the inhibitor, PCRs with blood collected from the same surviving animals were negative (330-bp T. cruzi DNA band absent; arrow). Lane 1, DNA ladder; lanes 2 to 3, N-methyl-Pip-F-hF-VSΦ-treated infected Rag1−/− mouse no. 4; lane 4, not applicable; lanes 5 to 6, N-methyl-Pip-F-hF-VSΦ-treated infected Rag1−/− mouse no. 5; lane 6, not applicable; lane 7, positive control, T. cruzi DNA.
transplant patients (P. S. Doyle and J. Koehler, unpublished data). Because of differences in kDNA detection ranging from 2 days to 3 weeks reported by other authors (29, 30, 34), we compared amplification of kDNA with the Avila PCR method in muscle cells treated with benznidazole and N-methyl-Pip-F-hF-VS or not treated. We detected kDNA amplification for up to 40 days after infection of treated cells (P. S. Doyle, data not shown). These results probably reflect not only differences in the PCR methods used by other investigators but also the period required by the drugs to eradicate intracellular T. cruzi infection and the stability of organized intracellular kinetoplasts that remain visible for days in the cytoplasm of cured stained cells (16; P. S. Doyle, unpublished observations). Injected denatured kDNA may be more readily processed by inflammatory cells recruited to the site.

A shift in CA-1/72 T. cruzi tissue tropism was noted in Rag 1–/– mice. Massive T. cruzi infection was apparent in Rag 1–/– hepatic and spleen macrophages versus heart and skeletal muscle cells in wild-type animals. Similar observations have been reported in Brucella infections of immunodeficient experimental animals (22). Rag 1–/– phagocytes recruited by both pathogen and host factors were unable to kill pathogens effectively. Cytokines provided by NK cells in the absence of signals from specifically sensitized T cells appear to be insufficient to activate macrophages for effective pathogen killing (22). These experimental observations correlate well with clinical reports of abundant T. cruzi amastigotes within macrophages of immunosuppressed transplant patients (20).

Even in the absence of a functioning immune system, the cysteine protease inhibitor N-methyl-Pip-F-hF-VS rescued mice from an acute, lethal T. cruzi infection. As this compound is in late preclinical development, future clinical trials can now be considered that include immunodeficient or immunosuppressed patient populations.

ACKNOWLEDGMENTS

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FIG. 6. Negative PCR with tissues of Rag1–/– animals treated with N-methyl-Pip-F-hF-VS. Upper gel: lane 1, DNA ladder; lane 2, no DNA (negative control); lane 3, macrophage DNA (negative control); lane 4, liver of K11777-treated mouse 1 from experiment 1T; lane 5, spleen of K11777-treated mouse 1 from experiment 1T; lane 6, heart of K11777-treated mouse 1 from experiment 1T; lane 7, liver of K11777-treated mouse 2 from experiment 1T; lane 8, spleen of K11777-treated mouse 2 from experiment 1T; lane 9, heart of K11777-treated mouse 2 from experiment 1T; lane 10, T. cruzi DNA (positive control). Lower gel: Lane 1, DNA ladder; lane 2, no DNA (negative control); lane 3, macrophage DNA (negative control); lane 4, liver of untreated control mouse 1C; lane 5, spleen of untreated control mouse 1C; lane 6, heart of untreated control mouse 1C; lane 7, empty; lane 8, T. cruzi DNA (positive control); lanes 9 and 10, empty. Amplified T. cruzi kDNA is indicated with an arrow; lower molecular weight bands are primers.

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