Pharmacological Inhibition of Transforming Growth Factor β Signaling Decreases Infection and Prevents Heart Damage in Acute Chagas’ Disease

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Chagas’ disease induced by Trypanosoma cruzi infection is a widely distributed debilitating parasite Trypanosoma cruzi, is a widely distributed debilitating human illness affecting 15 million people in Central and South America that is an important cause of mortality and morbidity (13, 23). One-third of T. cruzi-infected individuals living in areas where Chagas’ disease is endemic will eventually develop Chagas’ disease cardiomyopathy, while the majority will remain asymptomatic. Present available therapies are inadequate and insufficient (7). Nifurtimox and benznidazole, the only two trypanocidic drugs available, have toxic side effects and are not effective for all parasite strains. Moreover, no therapeutic approach targeting Chagas’ disease heart pathology is presently available. Chronic Chagas’ disease patients are treated symptomatically depending on the grade of cardiovascular and/or intestinal system lesions (30).

Transforming growth factor β (TGF-β) is the prototypic member of a family of polypeptide growth and differentiation factors that play a great variety of biological roles in such diverse processes as inflammation, fibrosis, immunosuppression, cell proliferation, cell differentiation, and cell death (16, 25). TGF-β is also involved in many direct and indirect interactions between infectious agents and their hosts (24). Several studies have demonstrated that TGF-β plays a major role in the establishment and pathogenesis of T. cruzi infection (reviewed in reference 2). TGF-β plays a crucial role in three important processes associated with Chagas’ disease: (i) stimulation of fibrosis, as demonstrated in Chagas’ disease patients and experimental animal models (1, 31); (ii) parasite cellular invasion and proliferation (10, 18, 32, 34); (iii) downregulation of cellular and immune mechanisms of parasite control (15, 27, 28). Moreover, significantly higher circulating levels of TGF-β1 have been observed in patients with Chagas’ disease cardiomyopathy (1).

TGF-β interacts with specific transmembrane receptors possessing intracellular serine/threonine kinase activity, present at the cell surface and known as TGF-β receptors I and II (TβRI and TβRII, respectively) (16). Upon ligand binding, TβRII phosphorylates and stimulates the serine/threonine kinase activity of TβRI, also known as activin receptor-like kinase 5 (ALK5). Upon activation, ALK5 phosphorylates the cytoplasmic signaling proteins Smad-2 and Smad-3, which then associate with Smad-4, translocate into the nucleus as a multiprotein complex, and stimulate the transcription of TGF-β-responsive genes, thereby inducing specific biological responses.

Our recent in vitro studies established that the small chemical inhibitor of ALK-5 activity, 4-(5-benzo[1,3]dioxol-5-yl-4-pyridin-2-yl-1H-imidazol-2-yl)-benzamide (SB-431542) (Fig. 1)
(5), reduces the infection of cardiomyocytes by T. cruzi, inhibits intracellular parasite differentiation, induces parasite apoptosis, and inhibits tripeptidyl peptidase II release (33). In the present study, we performed preclinical in vivo assays to evaluate protective effects of SB-431542 on the acute phase of experimental Chagas’ disease as determined by clinical, parasitological, and biochemical parameters. We found that this compound reduced mortality, decreased parasitemia, and prevented heart damage due to acute Chagas’ disease.

MATERIALS AND METHODS

Parasites. Bloodstream trypomastigotes of the Y strain were used and harvested by heart puncture from T. cruzi-infected Swiss mice at the peak of parasitemia as described previously (19).

In vivo infection. Male Swiss mice (age 6 to 8 weeks, weight 15 to 20 g) were obtained from the animal facilities of CEAL (FIOCRUZ, Rio de Janeiro, Brazil). Mice were housed for at least 1 week before parasite infection at the Animal Experimentation Section at Cell Biology Laboratory-IOC/FIOCRUZ under environmental factors and sanitation according to the Guide for the Care and Use of Laboratory Animals (31). Infection was performed by intraperitoneal (i.p.) injection of 10^5 bloodstream trypomastigotes. Age-matched noninfected mice were maintained under identical conditions. This project was approved by the FIOCRUZ Committee of Ethics in Research (protocol number 009/01).

Experimental groups. The animals were divided into the following groups: not infected, not treated; infected, treated with 0 mg of SB-431542 per kg of body weight (to control toxicity), infected and not treated, and infected and treated with 10 mg/kg of SB-431542. Eight to 10 mice from each group were used for analysis at each different postinfection (dpi), and four independent experiments were performed.

Drug and treatment. The compound SB-431542 (Tocris Bioscience, Bristol, United Kingdom) or vehicle dimethyl sulfoxide (DMSO) was used. A stock solution (20 mg/ml) of SB-431542 was prepared in DMSO, and mice received single 0.1-ml i.p. injections of 5, 10, or 20 mg/kg/mouse at 3 dpi for preliminary dose concentration studies. The control group received injection of vehicle on the same treatment schedule. Parasitological evaluation indicated that 10 mg/kg/mouse was the best drug concentration.

Mortality, parasitemia, and body weight. The mortality of the mice was checked daily until 30 dpi and expressed as a percentage of cumulative mortality. Parasitemia was individually checked by direct microscopic counting of parasites in 5 μl of blood as described previously (19). At 0, 8, and 14 dpi, body weight was determined.

Biochemistry. Blood samples (32 μl) were collected from the tips of the tails of mice in all experimental groups at 14 dpi and immediately analyzed for the determination of creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and urea levels with Reflotron Plus (Roche) according to the manufacturer’s recommendations. ALT and AST activities were used to evaluate hepatic dysfunction, and the results were expressed as enzyme concentration in units/liter. ALT and AST belong to the group of transaminases that catalyze the conversion of amino acids into the corresponding α-keto acids and vice versa by transferring amine groups. Urea was measured to evaluate renal function, and the results were expressed as a concentration (in milligrams/deciliter). Briefly, urea is hydrolyzed into ammonium carbonate, and ammonium is released by alkaline buffer. This reaction partially alters the color of an indicator to green/blue, and the intensity of the color is proportional to the urea concentration in the tested sample. Creatine kinase was measured to evaluate muscle lesions, and the results were expressed as enzyme concentration (in units/liter).

RESULTS

The aim of the present study was to evaluate whether SB-431542 (Fig. 1), which has been shown to inhibit cardiomyocyte infection by T. cruzi in vitro (33), could also have a beneficial effect in vivo in an experimental model of mouse infection by T. cruzi and whether it can protect infected mice from parasite-induced alterations of cardiac functions. The inhibitor SB-431542 was i.p. injected 3 dpi into male Swiss mice previously infected with 10^5 bloodstream trypomastigotes of the Y strain.

SB-431542 decreases mortality and reduces parasitemia in T. cruzi-infected mice. We first tested different doses (5, 10, and 20 mg/kg) of SB-431542 and observed that 20 mg/kg was effective at reducing parasitemia but induced a higher mortality rate, indicating a possible toxic effect (data not shown). Therefore, we chose a maximal dose of 10 mg/kg in the following experiments. SB-431542 i.p. injection significantly reduced mortality (Fig. 2A). In Fig. 2A, the untreated infected group and the SB-431542-treated infected group presented 86% and 56% mortality at 22 dpi, respectively. This result was confirmed in four independent experiments.

Parasitemia peaked at 8 dpi in T. cruzi-infected mice (Fig. 2B). We observed a decrease in parasitemia after SB-431542 treatment, although this was not statistically significant (Fig. 2B). This is probably due to a large variation that is common in the T. cruzi infection models. As expected, the infection induced a loss of body weight at 14 dpi (Fig. 2C) compared to noninfected animals (24.3 ± 1.4 g and 35.4 ± 2.1 g, respectively); SB-431542 injection had no effect on the weight of control or infected mice (33.7 ± 2.5 and 26.9 ± 3 g, respectively) (Fig. 2C).

SB-431542 prevents heart damage in T. cruzi-infected mice. In order to understand how SB-431542 treatment decreases mortality and to study the possible toxicity of this drug, we measured different circulating markers that reflect kidney, liver, and muscle status. Urea levels, reflecting kidney status, were not significantly different among the groups studied (Fig. 2D).

Statistical analysis. Differences were considered statistically significant when P was <0.05 or P was <0.01 as determined by the nonparametric Mann-Whitney test.
In contrast, creatine kinase, a well-known marker of muscle damage, was increased 10-fold by T. cruzi infection (Fig. 3B), and SB-431542 treatment significantly reduced this increase. The noninfected, SB-431542-treated mice did not show evidence of increased levels of CK, indicating that SB-431542 alone did not cause muscle injury (Fig. 3B). Measurement of the levels of aspartate aminotransferase and alanine aminotransferase in serum showed that infection by T. cruzi significantly increased liver markers (10- and 5-fold, respectively; Fig. 3C and D). In the presence of SB-431542, serum levels of AST in infected animals were significantly lower than the levels in untreated mice, whereas the level of ALT was not reduced.

**SB-431542 reduces inflammatory infiltrates and parasite load in the myocardium of infected mice.** To investigate whether SB-431542 treatment during the acute phase of T. cruzi infection would affect the myocardial parasitism and influx of inflammatory cells, we analyzed sections of infected hearts collected at 14 dpi using histochemical techniques. Uninfected animals (SB-431542 treated or sham treated) showed no inflammatory infiltration in the myocardium (Fig. 4A and B). Myocardial sections from the T. cruzi-infected and sham-treated (injected with DMSO) group had numerous inflammatory foci that were frequently associated with necrotic areas (Fig. 4C) and numerous amastigote nests (Fig. 4E). Infected and SB-431542-treated mice showed significantly fewer and smaller inflammatory foci (Fig. 4D and G) than the sham-treated group (Fig. 4C and G), although mononuclear cells were more diffuse in the myocardium (Fig. 4D and F). The decrease in the number of cardiac amastigote nests in SB-431542-treated, infected mice (Fig. 4H) was in accordance with the decrease in blood parasitemia (Fig. 2B). Amastigote nests were also larger in the hearts of sham-treated, infected mice (Fig. 4E) than in SB-431542-treated mice (Fig. 4F).

**SB-431542 prevents bradycardia and AVB in T. cruzi-infected mice.** We next analyzed electrocardiograms of the different groups of mice (Fig. 5 and Table 1). At 14 dpi, the ECG of infected mice demonstrated significantly higher PR intervals compared to uninfected mice (54.0 versus 29.4 ms, respectively) (Fig. 5A and C and Table 1). PR intervals larger than 40 ms suggest slower transmission of the electrical impulses and atrioventricular block (AVB), which is characteristic of acute T. cruzi infection (21). SB-431542 administration (Fig. 5D) significantly prevented this AVB, as PR intervals were decreased to 34.5 ms. In addition, we observed a clear sinus bradycardia following infection in infected mice relative to the control group (548 and 730 bpm, respectively), and SB-431452 bradycardia following infection in infected mice relative to the control group (548 and 730 bpm, respectively), and SB-431452 treatment also diminished this process, with a mean heart rate of 619 bpm (Table 1). QT and QRS intervals did not show significant alterations in the animals studied. These results demonstrated that a single SB-431542 administration was effective to prevent the important alterations of the cardiac electrical conduction system during acute experimental T. cruzi infection.

**DISCUSSION**

In the present work, we show for the first time that in vivo treatment of T. cruzi-infected mice with an inhibitor of the TGF-β type I receptor kinase (ALK5), SB-431542, reduces the severity of infection and tissue lesions, leading to a significant decrease in mortality. This result demonstrates that drugs blocking TGF-β signaling could be valuable tools in the treatment of Chagas’ disease patients. These data are consistent with our predictions from previous in vitro studies in which we demonstrated that SB-431542 decreases T. cruzi invasion of cardiomyocytes, inhibits intracellular parasite differentiation, and induces parasite apoptosis and thereby greatly decreases trypomastigote release (33).

Swiss mice infected with the Y strain of T. cruzi usually die
between 18 and 21 days postinfection, as a result of a complex host-parasite interplay involving inflammation, systemic activation of the natural and acquired immune responses, progressive renal and heart dysfunctions, and eventually systemic shock (20). We found that pharmacological treatment of \( T.\) \( cruzi \)-infected mice with a single dose of 10 mg/kg SB-431542 given on day 3 postinfection led to improved survival rates compared to untreated animals and that this difference was significant at 20 dpi. Interestingly, no toxicity was found with this drug at the concentration employed. We and others have previously found that TGF-\( \beta \) is involved in host cell invasion and parasite growth (10, 18, 32, 33). Therefore, an important first step where TGF-\( \beta \) could be effective is parasitemia. Indeed, we found that SB-431542 treatment could decrease both tissue and circulating parasite loads. Besides parasitism, inflammation is another important component of the pathological mechanisms of Chagas’ disease that can be controlled through inhibition of TGF-\( \beta \) signaling. In the early acute phase, activated macrophages secrete inflammatory cytokines, especially tumor necrosis factor alpha and interleukin-12, which, in turn, stimulates the secretion of gamma interferon (IFN-\( \gamma \)) by NK cells and by CD4 and CD8 T cells (26). The prevention of acute inflammation in nonlymphoid tissues (heart and liver) and of the resultant tissue damage is believed to be based on a subsequent wave of anti-inflammatory cytokines, such as TGF-\( \beta \) and interleukin-10. It is possible that when SB-431542 impairs the activity of TGF-\( \beta \), secretion of inflammatory cytokines is stimulated, thereby favoring parasite destruction by activated macrophages. We found fewer large inflammatory foci but a higher number of diffuse mononuclear cells in the myocardium of SB-431542-treated mice (Fig. 4). This is suggestive of such an immune activation without compromising cardiac functions, which large foci of inflammatory infiltrates might be expected to cause. Future studies should address this hypothesis by immunological characterization of the cytokine response including, in particular, the IFN-\( \gamma \) pathway. Silva et al. showed that when anti-TGF-\( \beta \) monoclonal antibodies are injected into \( T.\) \( cruzi \)-infected mice, a higher IFN-\( \gamma \) response takes place, which increases resistance during the acute phase (27).

In \( T.\) \( cruzi \) infection, the main lesions occur in the heart, not in the liver or kidney. This is confirmed here. The concentration of urea did not change, indicating that the kidney was preserved. The infection increased liver markers (AST and ALT), but liver lesions are considered only when ALT and AST are 10- to 15-fold higher than the normal levels. Here we show that ALT levels increased five times in infected mice, indicating that liver lesions were not prominent in this model. On the other hand, AST levels increased 10 times, but AST reflects both hepatic and heart lesions. The level of CK, a well-known marker of muscle damage, also increased 10-fold. Interestingly, we observed that SB-431542 treatment significantly reduced AST and CK levels in infected mice, demonstrating that SB-431542 treatment decreased muscle damage. The dramatic change in the heart affected by \( T.\) \( cruzi \) infection is also detected by important changes in ECG parameters in infected animals. After 14 dpi, the ECGs of infected mice reveal higher PR intervals, resulting from atrioventricular block and slower transmission of the electrical impulses, and a significant sinus bradycardia (Fig. 5). TGF-\( \beta \) is a key mediator in infectious diseases that affects cardiac function, since it is implicated in heart homeostasis as a regulator of cell proliferation, cell death, extracellular matrix remodelling, and angiogenesis (2). In canine models of heart failure, atrial TGF-\( \beta \)
FIG. 4. Heart histopathology of untreated and SB-431542-treated \textit{T. cruzi}-infected mice. Male Swiss mice were injected i.p. with $10^4$ bloodstream trypomastigotes or not injected with trypomastigotes. At 14 dpi, the mice were sacrificed, and their hearts were collected, fixed, and embedded in paraffin. Sections (3 \textmu m) stained by hematoxylin-eosin were analyzed by light microscopy. (A) Uninfected nontreated mice. (B) Uninfected treated mice. (C and E) Untreated \textit{T. cruzi}-infected mice. (D and E) Treated \textit{T. cruzi}-infected mice. Tissue shown in panels A and B showed the same histological features. Untreated \textit{T. cruzi}-infected mice showed large inflammatory infiltrates (filled arrow and higher-magnification inset in panel C) and amastigote nests (open arrow and higher-magnification inset in panel E). Infected mice treated with 10 mg/kg SB-431542 had smaller inflammatory infiltrates (filled arrow in panel D) and amastigote nests (open arrow in panel F). (G) Mean number of inflammatory infiltrates (more than 10 mononuclear cells) in 30 fields. (H) Mean number of amastigote nests in 30 fields. Values for the group treated with SB-431542 (SB) that were significantly different from the value for the DMSO control group (**, $P < 0.01$). Bars, 50 \textmu m.
expression increases, and inhibition of this expression prevents atrial fibrosis and the development of atrial fibrillation (4). In chronic Chagas’ disease cardiac pathology, one of the main complications is heart failure due to extensive fibrosis (11) and arrhythmia (6). TGF-β regulates connexin 43 (Cx43) expression and thus affects gap junction intercellular communication (12). The disturbance of gap junction signaling could lead to slower impulse propagation between cardiomyocytes and to ventricular arrhythmogenesis in myopathic heart (21). Our ECG results show that animals treated with SB-431542 have better-preserved cardiac electrical conduction systems, with a low incidence of AVB and a more normal heart rate. Beta-blockers, which are extremely useful in other types of heart disease (14), have been avoided for the treatment of Chagas’ disease because of bradyarrhythmia and atrioventricular conduction dysfunction and because of the high incidence of thromboembolism in this disease. Pharmacological inhibition of TGF-β signaling could represent a new strategy to be assessed for the treatment of Chagas’ disease using alternative drug schedules, such as successive injections after the first and second week postinfection in order to help decrease the parasite load and conduction effects of high TGF-β levels.

Several ALK5 inhibitors (SD-208, GW788388, and GW66004) have been tested in different murine models without toxicity.

FIG. 5. Electrocardiographic parameters during the acute phase of T. cruzi infection. Male Swiss mice were injected i.p. with $10^4$ bloodstream trypomastigotes or not injected with trypomastigotes. SB-431542 (10 mg/kg/mouse) or DMSO was then injected i.p. at 3 dpi. Representative electrocardiographic tracings of the four groups, group NI (not infected and not treated), group NI + SB (not infected and treated with 10 mg/kg SB-431542), group Tc (infected with T. cruzi and not treated), and group Tc + SB (infected with T. cruzi and treated with 10 mg/kg SB-431542) at 14 dpi, are shown. Note the normal patterns in uninfected mice and the variations in the heart rate (traced lines) for infected but untreated animals, which were partially recovered in the treated group. The arrow indicates arrhythmia. Broken lines indicate systolic time intervals.

TABLE 1. Electrocardiograph parameters of four groups of mice treated with SB-431542 (10 mg/kg) or not treated with SB-431542

<table>
<thead>
<tr>
<th>Group</th>
<th>ECG parameter (mean ± SEM)*</th>
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<tbody>
<tr>
<td></td>
<td>Heart rate (bpm)</td>
</tr>
<tr>
<td>Noninfected, treated with DMSO</td>
<td>730 ± 75.6</td>
</tr>
<tr>
<td>Noninfected, treated with SB-431542</td>
<td>732 ± 54.5</td>
</tr>
<tr>
<td>Infected with T. cruzi, treated with DMSO</td>
<td>548 ± 45.4^b</td>
</tr>
<tr>
<td>Infected with T. cruzi, treated with SB-431542</td>
<td>619.3 ± 37.4^b</td>
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* ECG parameters were evaluated in the acute phase of infection at 14 dpi, using the following standard criteria: (i) heart rate (monitored by beats/minute), and (ii) the variation of the P wave and PR, QRS, and QT intervals, all measured in milliseconds.

^b Significant differences ($P < 0.05$) between the values for SB-431542-treated and non-SB-431542-treated (DMSO) groups of infected mice.
These inhibitors were clearly beneficial in several fibrosis models (lung, liver, and kidney) (3, 8, 22). Anti-TGF-

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creatic and mammary tumors) (9, 17, 29). Our present work is the first demonstration of a beneficial effect of anti-TGF-

also found to improve several tumoral models (glioma and pan-

agents should be considered in association with trypanocidal com-

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