Activities of TMC207, Rifampin, and Pyrazinamide against *Mycobacterium tuberculosis* Infection in Guinea Pigs

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Received 16 July 2010/Returned for modification 10 August 2010/Accepted 30 September 2010

The experimental compound TMC207 is showing promise against infections caused by *Mycobacterium tuberculosis* both in a variety of animal studies and in the field. In this study, we used the guinea pig model, a species that shows several similarities to human tuberculosis, including the hallmark of primary granuloma necrosis, to determine the efficacy of a combination regimen combining TMC207 with rifampin and pyrazinamide. This drug regimen rapidly reduced the bacterial load in the lungs to undetectable levels by 8 weeks of treatment. This reduction was associated with a substantial improvement in lung pathology, but despite this effect areas of residual necrosis still remained. In the draining lymph nodes, however, tissue damage was rapid and not significantly reversed by the drug treatment. Approximately 10 to 11 months after the treatment had ended, the animals began to trigger a Karnovsky scale indicating bacterial regrowth and potential relapse, an event confirmed by the new development of both pulmonary and extrapulmonary granulomatous lesions. Interestingly, a similar rate of relapse was also seen in animals receiving 24 weeks of rifampin, pyrazinamide, and isoniazid standard chemotherapy. These data indicate that TMC207 could be a useful addition to current treatment regimens for tuberculosis.

The increasing incidence of drug-resistant forms of *Mycobacterium tuberculosis*, now thought to exceed half a million new cases a year (8, 10, 11, 26), further complicates the critical need to discover new antituberculosis drugs. Equally troubling, newly emerging evidence suggests that a significant percentage of new clinical isolates of *M. tuberculosis* are of extremely high virulence (8). Within the current drug discovery pipeline (12), a particularly attractive new candidate is the diarylquinolone TMC207 (2). This investigational drug potently inhibits the mycobacterial enzyme complex ATP synthase, thus interfering with energy production and homeostasis. As a consequence, TMC207 is highly active against both drug-sensitive and drug-resistant isolates of *M. tuberculosis*.

In animal studies, the majority of data has been obtained from the mouse model, where TMC207 has activity equivalent to, and synergizes with, standard rifampin, isoniazid, and pyrazinamide therapy (RHZ) (3, 13, 14, 17, 18, 30). However, although the mouse is an excellent and cost-effective screening tool (15), it lacks elements of the disease pathogenesis in humans, including granulomatous lymphadenitis, lymphangitis, and the development of both pulmonary and extrapulmonary lesions exhibiting caseous necrosis with dystrophic calcification (15). In contrast, the guinea pig model of tuberculosis mimics multiple elements of the pathogenesis of the disease in humans (4, 6, 7).

Virtually all studies evaluating drug efficacy in animal models utilize the laboratory strains *M. tuberculosis* H37Rv or Erdman. However, it is becoming increasingly apparent that newly emerging isolates are of very high virulence, including the WBeiijing family of *M. tuberculosis*, which is now globally distributed (8) and documented as a cause of major outbreaks of infection worldwide that often involve multidrug-resistant organisms. Because such high virulence now appears not to be unusual but rather the norm, we performed the studies here with the highly virulent Erdman K01 strain. Moreover, our recent studies with this and other virulent strains indicated that these infections are potent inducers of Foxp3+ regulatory T cells (23), a finding that further illustrates the need to find new drug regimens given the potential that this T-cell subset has the potential to subvert vaccine-induced immunity.

In an earlier study (16) we showed that TMC207 is highly active in the guinea pig model of tuberculosis, with low (~50) numbers of bacilli detectable by culture in the lungs after 6 weeks of therapy. We noted, however, both in this case and in guinea pigs given much longer RHZ treatment (23), that remaining bacteria were extracellular within primary lesions with residual lesion necrosis. Preliminary work in our laboratory has allowed us to hypothesize that these remaining bacteria form extracellular biofilm-like communities which may explain their tolerance to chemotherapy, a possibility recently further supported by *in vitro* studies (20).

Moreover, regions containing persisting bacteria do not seem to develop rapidly but become apparent as the centers of primary lesions become necrotic, a parameter that seems to be driven by the induction of inflammation and the virulence of the *M. tuberculosis* isolate (24, 25). This raises the possibility that rapidly effective regimens could potentially sterilize these lesions before necrosis fully develops, thus preventing such...
biofilm-like communities from forming. The results of the current study show that a combination regimen of rifampin, pyrazinamide, and TMC207 (RZ/TMC207) appeared to rapidly render lungs culture-negative in just 8 weeks of therapy. Despite this, however, we observed that the RZ/TMC207 regimen failed to completely resolve primary lesions, leaving residual areas of necrosis and dystrophic calcification which we believe could potentially serve as the sites of disease relapse, an event that first became observable 10 to 11 months after the drug therapy had ceased. These data support the notion that TMC207 containing regimens have the potential to dramatically reduce the duration of treatment of tuberculosis, but relapse still is likely to occur.

MATERIALS AND METHODS

Guinea pigs. Female outbred Hartley guinea pigs (approximately 500 g in weight) were purchased from the Charles River Laboratories (North Wilmington, MA) and held under barrier conditions in a biosafety level III animal laboratory. The specific-pathogen-free nature of the guinea pig colonies was demonstrated by testing sentinel animals. All experimental protocols were approved by the Animal Care and Usage Committee of Colorado State University. Guinea pigs were infected using a Madison chamber aerosol generation device, which delivered \(20 \times 10^{4} M. tuberculosis\) strain Erdman K01 bacilli into the lungs.

Course of infections. Bacterial loads in the organs of guinea pigs (\(n = 5\)) were determined prior to the start of drug treatment on day 20 and then after 2, 4, and 6 weeks of treatment. As previously described (23), the bacterial loads in the organs of guinea pigs at each time point of the study were determined by plating serial dilutions of homogenates of lungs (right cranial lobe), spleens, and mediastinal lymph nodes on nutrient 7H11 agar and counting the CFU after 6 weeks incubation at 37°C. The bacterial load for each organ was calculated and converted to logarithms. The data were expressed as mean log\(_{10}\) CFU plus the standard error of the mean (SEM) for each group.

Drug treatments. On day 20 of the infection, four guinea pigs were euthanized to determine the bacterial load prior to the start of the treatment. The remaining animals were randomly assigned to three groups: an untreated control group, a group receiving the RZ/TMC207 treatment regimen, and additional animals set aside for relapse studies. Treatment with chemotherapy was administered 5 days a week for 8 weeks. Because of the challenging animal husbandry issues associated with drug treatment, described in detail elsewhere (23), animals were given 1-mL formulations containing 40% sucrose (wt/vol), 20% pumpkin (wt/vol) (Libby’s 100% pure pumpkin) mixture supplemented with vitamin C (50 mg/kg) and commercial Lactobacillus (BD Lactinex) (all purchased from Walmart, Fort Collins, CO). Rifampin (50 mg/kg), pyrazinamide (100 mg/kg of mean body weight), and TMC207 (15 mg/kg) were incorporated into this sucrose/probiotic vehicle. Animals were treated on a microtome. Tissue sections were stained with hematoxylin and eosin. The lung and lesion area was determined on representative hematoxylin and eosin stained sections evaluated at a x200 magnification. A total of 8 to 12 fields were selected randomly from the肺 sections and a counting frame (2,000 μm\(^2\)) containing probe points with a grid spacing of 200 μm was used to define the areas of interest (lesion and lung). The data are expressed as the mean ratio of lesion area to lung area of all of the animals within a treatment group. Lesions represented by photomicrographs represent the section that is closest to the mean value in each group.

Flow cytometry. To prepare single-cell suspensions for flow cytometry, the lungs were perfused with 20.0 mL of a solution containing PBS and heparin (50 U/mL Sigma-Aldrich, St. Louis, MO) through the pulmonary artery, and the caudal lobe and portions of the lymph nodes were aseptically removed from the pulmonary cavity, weighed, placed in medium, and dissected as described before (22). The data acquisition and analysis was performed by using a FACSCalibur (BD Biosciences, Mountain View, CA) and CellQuest software (BD Biosciences, San Jose, CA). Analyses were performed with an acquisition of at least 100,000 total cellular events.

Histometric analysis of cell surface markers. Single-cell suspensions from the lungs were prepared as recently described (23). Thereafter, cell suspensions from each individual guinea pig were incubated first with Serotec antibodies to CD4, CD8, pan-T-cell, CD45, M14, B-cell, macrophage, and class II antibodies at 4°C for 30 min in the dark and after washing the cells with PBS containing 0.1% sodium azide (Sigma-Aldrich). In addition, membrane permeabilization using Leucomer (Serotec, Inc., Raleigh, NC) was completed according to the instructions prior to staining with macrophages and major histocompatibility complex (MHC) class II antibodies. The data acquisition and analysis were done by using a FACSCalibur and CellQuest software. Compensation of the spectral overlap for each fluorochrome was done using RZ/TMC207 (RZ/TMC207 regimens failed to completely resolve primary lesions, leaving residual areas of necrosis and dystrophic calcification which we believe could potentially serve as the sites of disease relapse, an event that first became observable 10 to 11 months after the drug therapy had ceased. These data support the notion that TMC207 containing regimens have the potential to dramatically reduce the duration of treatment of tuberculosis, but relapse still is likely to occur.

RESULTS

Changes in bacterial load after chemotherapy. The effects of the combination RZ/TMC207 therapy in guinea pigs infected with M. tuberculosis are shown in Fig. 1. After 4 weeks of treatment, the bacterial loads in the lungs, lymph nodes, and spleens had dropped to barely detectable levels. No colonies could be detected in the lungs after 6 weeks, but a few colonies were noted in the draining lymph nodes and spleen at 4 and 6 weeks when homogenates were replated on 5% bovine serum albumin (BSA)/7H11.

Influence of chemotherapy on lung and lymph node histopathology. Representative photomicrographs of lungs and lymph node from guinea pigs treated for 2, 4, and 6 weeks of combination drug therapy with RZ/TMC207 are shown in Fig. 2. The images in Fig. 2A to C are representative images of lungs and lymph nodes from guinea pigs prior to the initiation of drug therapy. Images from animals treated with RZ/TMC207 for 2 weeks (Fig. 2D to F), 4 weeks (Fig. 2G to I), and...
FIG. 1. Bacterial burden in the lungs, lymph nodes, and spleens in infected guinea pigs treated with RZ/TMC207. Bacterial counts in the lungs (A), lymph nodes (B), and spleens (C) of untreated control and RZ/TMC207 treatment animals which began at day 20, and the effects of drug treatment measured 2, 4, and 6 weeks later. The results are expressed as the average (n = 5) of the bacterial load in each group expressed as the log10 CFU ± the SEM for untreated control animals (○) and animals receiving RZ/TMC207 (□). Student t test: *, P < 0.050.

6 weeks (Fig. 2J to L) are also shown. The images in Fig. 2M to O are from untreated control guinea pigs that received the sucrose carrier only, which were euthanized on day 76 after aerosol infection. Combination RZ/TMC207 therapy from 2 to 6 weeks decreased the percentage of lung affected by foci of granulomatous inflammation (Fig. 2D, G, and J), but lesions with central necrosis had a progressive increase in dystrophic calcification over the time of treatment (Fig. 2B, E, H, and K). Drug therapy with RZ/TMC207 had no benefit in reducing the lesion burden and the percentage of lesion with necrosis in lymph nodes (Fig. 2C, F, I, L, and O). There was almost complete effacement of the lymph node architecture as early as 20 days of infection (Fig. 2C), which remained unchanged in extent throughout the infection. There was marked progression of granulomatous inflammation in the lung (Fig. 2M and N) and lymph node (Fig. 2O) in untreated control animals receiving the sucrose carrier alone.

Analysis of lung lesion burden. The tuberculosis that develops in the guinea pig after aerosol exposure to M. tuberculosis can be divided into acute, subacute, and chronic stages of infection based on the pattern of bacterial growth and dissemination, as well as patterns of pulmonary and extrapulmonary pathology. During acute infection, an ~2-week period of rapid bacterial proliferation occurs in the lung and draining lymph nodes and is characterized by progression of granulomatous inflammation and necrosis in the primary lesion complex of the lung and draining mediastinal lymph nodes. In the subacute stage, infection is established in multiple extrapulmonary sites, such as the spleen and liver, by hematogenous dissemination of bacilli. Concurrent with bacillemia and exponential bacterial growth in extrapulmonary sites, there is reinfection of the lung by the hematogenous route, creating a non-necrotic secondary lesion (22).

Treatment of infected guinea pigs with combination therapy consisting of RZ/TMC207 decreased the lung lesion burden but had no significant benefit in the draining lymph nodes. The data in Fig. 3 represent the mean percent lung area affected by granulomatous inflammation, as well as the percentage of lesion affected by necrosis and the percentage of necrotic foci that had dystrophic calcification in guinea pigs treated for 6 weeks with RZ/TMC207 combination therapy. Six weeks of RZ/TMC207 combination drug therapy reduced the area of lungs with lesions and the percentage of lesions with necrosis. Lesions with dystrophic calcification were first evident at 2 weeks of RZ/TMC207 therapy and were maximal at 4 weeks of therapy consistent with progressive healing of necrotic lesions. Treatment of guinea pigs out to 6 weeks had no additional benefit at reducing lesion burden and even showed a slight increase in lesion necrosis compared to 4 weeks of therapy.

Changes in cellular influx in the lungs after chemotherapy. In untreated control animals, we observed a steady increase in the numbers of activated CD4+ T cells expressing CD45+ (Fig. 4A), as well as CD4 and CD8 T cells expressing the homing receptor CT4+ (Fig. 4B and C). There were large numbers of MR-1+ macrophages brightly expressing MHC class II+ (Fig. 4D), as well as MIL4+ neutrophils (Fig. 4E) accumulating in the lungs over the course of the infection, but this influx was significantly reduced by drug treatment.

Relapse after chemotherapy. The remaining guinea pigs were monitored after completing either 8 weeks of RZ/TMC207 treatment, in parallel with a group of animals remaining from an earlier study that had been given 24 weeks of RHZ treatment. Bacterial quantification yielded culture-negative lungs after 6 weeks of RZ/TMC207 treatment and 24 weeks of RHZ treatment. After about 11 months, we started to see signs of distress in various individual animals, triggering our Karnovsky scale and hence warranting euthanasia. In the first animal to succumb, we noted considerable extrapulmonary dissemination, including multiple lesions visible in the lungs and around within the pericardium (data not shown). As more animals began to die, we consistently noted lung lesions, consisting of mixtures of granulomas that were relatively small and non-necrotic, and lesions showing considerable necrosis, with death resulting from lung consolidation. Overall, 23% (3 of 13)
of these animals were euthanized, starting around 11 months after cessation of RZ/TMC207 therapy. We compared this rate to relapse in a parallel group of RHZ-treated guinea pigs (Fig. 5) and found that while relapse in the latter was higher (6 of 15 [40%]), this was not statistically different ($P = 0.3$).

**DISCUSSION**

There is increasing interest in using the guinea pig as a model to test drug regimens (1, 23), given the similarity of the disease process to that seen in humans (4). The results of the present study show that treatment of guinea pigs with a combination of RZ with TMC207 rapidly reduced the bacterial load in the lungs after infection of these animals by a low-dose aerosol exposure with the highly virulent Erdman K01 strain of *M. tuberculosis*. A 3-log drop was seen in the lungs after 2 weeks of treatment, and after 4 weeks only a few bacilli could be detected, even when using 7H11–5% BSA or LJ plates to account for protein binding by TMC207 and carryover dilution issues. These results are obviously superior to our previous results (23), in which we demonstrated that RHZ therapy of guinea pigs infected with the K01 strain was initially highly effective, reducing the bacterial load in the lungs > 3-log in just 14 days. This clearance rate then rapidly declined, and it took drug therapy for another 125 days before we could no longer detect bacilli in the lungs of these animals.

In the present study RZ/TMC207 caused rapid clearance of the secondary lesions that arise as a result of hematogenous dissemination (4) but failed to completely resolve primary lesions in which necrosis had already developed. More importantly, RZ/TMC207 failed to significantly reduce the progression of disease in the draining lymph nodes which, as in humans, is an important part of the primary lesion complex in guinea pigs following aerosol exposure (6). The significance of these observations is that we now have increasing evidence that these residual pulmonary and extrapulmonary lesions with necrosis harbor extracellular, drug-tolerant bacilli that serve as the source of disease reactivation after drug therapy is discontinued. These data further support our working hypothesis that the microenvironment created by the necrosis of host cells...
(particularly neutrophils) favors the persistence of drug tolerant biofilm-like communities in vivo.

Severe lung consolidation caused by cellular influx and granuloma formation is a central aspect of progressive disease in this animal model. Consistent with the histopathology data, analysis of the influx of various cellular phenotypes using flow cytometry revealed a substantial reduction in the numbers of activated cells accumulating in the lungs. It has to be conceded that at this time such analyses are very limited due to the lack of reagents, but some information can be gleaned by measuring the expression of CD45 and the homing receptor CT4. Here, whereas the numbers of activated T cells expressing these markers continued to increase in untreated controls, the numbers were reduced in animals receiving treatment. A similar pattern was observed in the case of activated, MHC class II-expressing macrophages accumulating in the lungs, and also for numbers of MIL4-positive granulocytes. The latter observation is very important, given the central involvement of these cells in the process of necrosis development.

To expand on this latter issue further, guinea pig neutrophils (“heterophils”) are important components of the early development of the granuloma in the lungs (29), and we have earlier postulated that degranulation of these short-lived cells may contribute to the development of lesion necrosis (28, 29). In addition, neutrophils are important early sources of oxygen free radicals, which may be the root cause of local vascular damage, further promoting necrosis and lesion hypoxia (4). These areas of necrosis harbor small clusters of bacilli (16), which we speculate may represent biofilm-like communities. This concept is further supported by in vitro models (20) and by preliminary data from our laboratory showing that cell debris generated by the death of these neutrophils can provide a key attachment matrix for extracellular bacilli and may even provide a trigger or signal for initial biofilm development.

Given the rapid clearance of the infection, it was somewhat surprising therefore to see evidence of potential relapse in the RZ/TMC207-treated animals, starting about 10 to 11 months posttreatment. For comparison purposes, we had kept aside a similar number of animals from a parallel study using RHZ; these animals also began to show evidence of relapse at much the same time. Relapse frequencies were a little higher in the RHZ group, but this was not statistically different. In the animals we were able to necropsy we consistently observed reactivation of extrapulmonary lesions. These observations in the guinea pig reflect what is seen in humans, in which relapse occurs up to 2 years after treatment. Since we think that reactivation in the RZ/TMC207-treated animals is arising from within primary lesion residual necrosis, this implies that even if necrosis is minimal it may still be sufficient to serve as a source of disease relapse.

It has been noted (17) that the bactericidal effects of TMC207 seem to increase over a few weeks of treatment. This bactericidal effect is suggested by PK studies in humans (27), suggesting that drug effects targets bacilli that undergo physiologic adaptations that favor extracellular survival in response to the stress conditions generated by host immunity. In our mode of extracellular persistent communities within necrotic lesions, these bacteria survive drug therapy even though the rate of replication is low. This microenvironment contains concentrated micronutrients such as ferric and ferrous iron that may serve as a source of nutrition for extracellular bacilli similar to what has been described for intracellular bacilli in...
infected macrophages (5). All such processes would require a certain level of energy generation, which TMC207 specifically interferes with. Furthermore, in the present study TMC207 was given for 6 weeks, and it would be interesting to see whether prolonging this duration of therapy might yield reduced relapse.

In addition to this limitation to the study design, we should acknowledge other limitations as well, all predicated by the extreme cost of these extensive studies. First, in addition to extending the treatment duration (the reader will note that neither the draining lymph nodes or spleen were completely sterilized at day 62), it would be interesting to see the degree to which RZ alone would have reduced the bacterial load, given the well-known antagonism between rifampin and isoniazid. Drug carryover in plating is also an issue in these types of studies, and so treatment stopped 2 days prior to the assays, and we only observed trace numbers of bacteria at day 62 when we plated on agar supplemented with BSA to try to account for protein binding. Another issue was related to the use of a Karnovsky scale to detect potential relapse. In classical studies, animals dying in any treatment group could then be assessed by microbiological assays to verify that they had active infection. In the current climate, however, animal usage regulations preclude this, and so we automatically euthanize animals triggering the scale. This arose from our vaccine studies over the past decade, and it is highly predictive of active tuberculosis. In fact, because of our confidence in this, we can use the harvested organs to answer other questions, such as the precise site or origin of regrowth. In the present study, we observed new primary lesions both in the lungs of relapsing guinea pigs, and

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FIG. 4. Combination drug therapy with RZ/TMC207 significantly reduced the accumulation of activated T cells, macrophages expressing MHC class II, and neutrophils into the infected lungs. The numbers of CD4+ CD45+ (A), CD4+ CT4+ (B), CD8+ CT4+ (C), macrophage MHC class IIhi (D), and heteroneutrophil (E) cells accumulating in the lungs of untreated controls (○) and RZ/TMC207(□) treatment which began on day 20 were determined, and the effects of drug treatment measured 2, 4, and 6 weeks later in the guinea pigs were charted. The results are expressed as the average total number of cells (10^7) expressing the indicated phenotypes (n = 4) per 1.0 g of tissue (± SEM). Student t test: *, P < 0.050.

FIG. 5. Survival curve of guinea pigs treated for 8 weeks with RZ/TMC207. The long-term survival curve of guinea pigs treated for 6 weeks with RZ/TMC207 (□) compared to a second set of animals that received standard RHZ (■) therapy for 6 months was determined. Although there was less relapse in the RZ/TMC207-treated group, Kaplan-Meier analysis did not reveal any statistical difference (P = 0.38).
in extra-pulmonary sites including the pericardium (data not shown). Thus, while the lack of culture data in the relapsing animals is a limitation, other interesting information can be obtained.

As has been noted here, most other animal studies to date have provided encouraging results using TMC207 as part of treatment regimens. Recent studies in mice, using high-dose intravenous infection, have looked at various regimens. These studies have included a comparison between RZ/TMC207 and RHZ, in which the results were comparable (14), although relapse rates were rather high (4 months after therapy). In a similar study (30), it was found that monotherapy with TMC207 gave better results than the experimental rifampin-like drug rifapentine and that RZ/TMC207 given just once weekly was better than RHZ daily. Taken in concert with our own results reported here, this suggests that RZ/TMC207 could potentially be used as intermittent therapy, reducing the cost of treatment even further, as recently suggested (19). The effectiveness of TMC207 has also been shown in the guinea pig model, both as monotherapy (16) and in combination with RZ in the studies described here.

The previous observation (18) that TMC207 also works well in combination with second-line drugs, coupled with good in vitro activity against drug-resistant strains (2), indicates that this drug may prove very useful in treating multidrug-resistant (MDR) TB. In this regard, a recent study (9) examined the effect of adding TMC207 to standard therapy (pyrazinamide plus kanamycin, ofloxacin, ethionamide, and cycloserine or terizidone) for MDR TB in a clinical trial in South Africa. The results were encouraging, with a reduction in time to sputum negativity in newly diagnosed MDR patients and increased effectiveness of TMC207 has also been shown in the guinea pig model of tuberculosis. Am. J. Respir. Crit. Care Med. 169:1011–1015.


