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Activity of Nitazoxanide and Tizoxanide against *Mycobacterium tuberculosis* in vitro and in whole blood culture

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Abstract

Nitazoxanide (NTZ) and its metabolite tizoxanide (TIZ) were studied as antimycobacterial agents *in vitro* (in mycobacterial growth indicator tube [MGIT] cultures) and in a whole blood bactericidal assay. Both NTZ and TIZ show high protein binding. In MGIT cultures (albumin concentration=78 µM), inhibition of *Mycobacterium tuberculosis* growth occurred at total drug concentrations of 16 µg/ml, whereas in whole blood cultures (albumin concentration=350 µM), 128 µg/ml was required. Free drug fractions at these two conditions were estimated to be 69% and 2%, respectively. Co-incubation of NTZ and TIZ in human plasma for 72 hours nearly completely eliminated their ability to inhibit mycobacterial growth in MGIT. Interactions with plasma proteins may limit the potential of NTZ and TIZ as drugs for human tuberculosis.

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Keywords

Mycobacterium tuberculosis; nitazoxanide; tizoxanide; Mycobacteria Growth Indicator Tube; BCG; whole blood assay

1.0 Introduction

The high prevalence and mortality of tuberculosis is largely due to a lack of easy to deliver, effective treatment. Better medications are needed. Nitazoxanide (NTZ) is a synthetic nitrothiazolyl-salicylamide derivative approved for the treatment of intestinal protozoal infections. It is inexpensive, orally available, well tolerated, and safe [1]. The mechanisms of action of NTZ against *Mycobacterium tuberculosis* (MTB), disruption of membrane potential and pH homeostasis, differ from those against intestinal pathogens [2]. In addition to targeting MTB directly, NTZ may enhance the host's immune response by stimulating macrophage autophagy, and MTB appears to have a high threshold for developing resistance to NTZ [3–5].

NTZ is rapidly and completely hydrolyzed (half-life of 6 min) by plasma esterases into its active metabolite tizoxanide (TIZ), the only form found in the circulation [6]. This conversion to TIZ is unchanged even when plasma is diluted 10 fold [6–7]. NTZ also hydrolyzes spontaneously to TIZ in aqueous media alone, with a half-life of 3 hours under the conditions in this study [6]. TIZ is 99% protein bound at concentrations reached in plasma after oral dosing [8]. However, the impact of protein binding on the bioactivity of TIZ has not been previously reported.

Although further assessment of NTZ's anti-TB properties is warranted, studies in mice have not proven useful, as NTZ is glucuronidated to an inactive form in that species [9]. An alternative method for evaluating the intracellular activity of NTZ and TIZ against MTB is the whole blood bactericidal assay (WBA). In this assay, a small number of mycobacteria are inoculated into whole blood diluted with an equal volume of tissue culture medium. Bacilli are phagocytosed by leukocytes and allowed to grow for a 72 hour incubation period [10]. This assay requires a sensitive mycobacterial growth detection method, such as the mycobacterial growth indicator tube system (MGIT) [11]. After 72 hours, phagocytes are lysed and remaining mycobacteria are pelleted, and then cultured in MGIT cultures. Differences in time to positivity (TTP) of the original inoculum and experimental results reflect a change in the number of viable mycobacteria [4, 10–13]. If no mycobacterial growth or killing occurred, TTP of mycobacteria at the end of the WBA equals that of the original inoculum. Increased TTP correlates with lower residual mycobacteria and indicates intracellular killing during the incubation in whole blood due to drug effects and/or host defenses [10]. NTZ has not previously been evaluated in the WBA, although it has anti-MTB activity in macrophage studies [2, 3, 9, 14]. This study evaluated the activity of NTZ and TIZ against MTB *in vitro* and by WBA.

2.0 Materials and Methods

2.1 Preparation of NTZ and TIZ

NTZ and TIZ were received from the Infectious Disease Research Institute on behalf of the Lilly MDR-TB Partnership Drug Discovery Initiative as lyophilized powder. Drugs were reconstituted in 100% DMSO to a concentration of 32mg/ml, aliquoted and stored at -70°C .

2.2 Preparation of MTB, *M. bovis* BCG and use of MGIT system

M. tuberculosis-H37Rv (NR-13648) (MTB) was obtained from BEI Resources (Manassas, VA), grown in MGIT growth medium, aliquoted, and stored at -70°C . MGIT growth medium consisted of Middlebrook 7H9 broth with OADC (oleic acid, bovine albumin, dextrose and catalase) growth supplement with an antibiotic mixture (800 μL PANTA: polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin). *M. bovis* BCG (35745) (BCG) was obtained from ATCC (Manassas, VA), grown in 7H9 supplement with ADC, glycerol and Tween80; aliquoted and stored in 7H9 supplement at -70°C . BCG was used in one experiment when lab safety prohibited the use of MTB. Each experiment used 10^6 CFU of MTB or BCG and used the automated BACTEC MGIT 960 system (Becton Dickinson, Sparks, MD) to detect mycobacterial growth as TTP [4, 10–13].

2.3 Preparation of direct inoculation experiments

NTZ and TIZ stocks were diluted in RPMI (a standard cell growth medium supplemented with amino acids and vitamins) for final drug concentrations of 2 to 128 $\mu\text{g/ml}$ and added to MTB before being inoculated directly into MGIT tubes. Depending on the experimental volume of drug, mycobacteria (and/or human plasma, as used in section 2.4 plasma experiments), MGIT broth was added for a final volume of 500 μL before inoculating MGIT tubes.

2.4 Plasma experiments

To evaluate plasma protein binding/inactivation of NTZ and TIZ, both drugs (8–128 $\mu\text{g/ml}$) were combined with 250 μL of increasing concentrations of human plasma (0, 5, 10, 25 and 50% vol/vol) and BCG or MTB for 30 minutes before inoculation into MGIT cultures. The maximum volume of plasma carried over into the MGIT cultures was 250 μL .

To evaluate the effect of 72 hour exposure to plasma on NTZ and TIZ, drugs (16–128 $\mu\text{g/ml}$) were incubated in 5, 10, 25 or 50% human plasma with BCG for 72 hours (the duration of WBA incubation) before inoculating MGIT culture tubes. Except for the use of plasma instead of whole blood, the processing methods were the same as described below in section 2.5 for the WBA.

2.5 Whole blood assay

Whole blood was collected by venipuncture into heparinized tubes from healthy consenting adults who were tuberculin skin test and/or Quantiferon test negative by history (study protocol approved by the Institutional Review Board for Human Investigations at University Hospitals Case Medical Center, Cleveland, USA). 500 μL of blood was added to the MTB inoculum in 2.0 mL microcentrifuge tubes and incubated at ambient temperature for 30

minutes to allow phagocytosis of MTB before adding drug. NTZ and TIZ were diluted in RPMI and added to the tubes at final drug concentrations of 2, 4, 16, 32, 64 or 128 µg/ml. Tubes containing drug, whole blood, and MTB were incubated at 37°C on a rotator for 72 hours. After incubation, tubes were centrifuged to pellet cells, supernatant removed, and blood cells lysed with sterile water. MTB were pelleted by centrifugation and re-suspended in 500 µl of MGIT broth, inoculated into MGIT tubes and incubated in the MGIT system; TTP was recorded. Serial dilutions of MTB stock were inoculated directly into MGIT to create a standard curve. Log change in viability during whole blood culture was calculated as $\log(\text{final}) - \log(\text{initial})$, where initial and final are the apparent volumes of the inoculum and the completed whole blood culture based on the standard curve. This method is more sensitive, reproducible, safe and time efficient than counting CFU on solid media. Rifampin (RIF) (10 µg/ml) served as positive and RPMI alone (no drug) as negative control.

2.6 Estimation of protein binding

Estimated free and bound TIZ concentrations were calculated using the equation $K_d = (A \cdot B) / AB$ where A and B are free molar concentrations of TIZ and albumin, respectively, and AB is bound concentration. Binding at a 1:1 molar ratio was assumed. K_d was estimated at 6 based on the findings of Zhao et al [8]. The equation was solved iteratively using the Solver module of Excel. The resulting predicted interactions of total drug and albumin concentrations on free drug concentration are shown in supplemental Fig. S1.

2.7 Statistical Analysis

Data was analyzed using SPSS Statistics [IBM, version 22, 2013]. Comparison of normally distributed means between groups was done using independent T-test and the one-way ANOVA. Data that was not normally distributed was analyzed using the Mann-Whitney U test or the Kruskal Wallis H test. A level of significance of $p < 0.05$ was used to reject the null hypothesis in all analyses.

3.0 Results

3.1 Direct effect of NTZ and TIZ on MTB growth in MGIT

NTZ and TIZ directly inoculated into MGIT tubes began to inhibit MTB growth, as shown by a significantly greater TTP than “no drug”, at 16 µg/ml ($p = 0.002$ and 0.006 , for NTZ and TIZ, respectively), with almost complete inhibition of growth at 64 µg/ml (Figure 1A). TTP was also significantly longer at 32 µg/ml when compared to 16 µg/ml and at 64 µg/ml when compared to 32 µg/ml ($p = 0.002$ and 0.001 for NTZ and $p = 0.004$ and < 0.0005 for TIZ, respectively). The concentration of bovine albumin in OADC-supplemented MGIT tubes is 78 µM. If binding similar to human serum albumin is assumed, the free fraction of TIZ ranged from 18% (at 16 µg/ml) to 69% (at 64 µg/ml). RIF served as positive control in these experiments.

3.2 Effect of plasma on NTZ and TIZ's ability to inhibit mycobacterial growth

Data for TIZ is given, for simplicity, since it is the active metabolite, and did not differ significantly from NTZ. When BCG was added to NTZ or TIZ (at varying concentrations)

and increasing concentrations of human plasma (added together for 30 min before inoculating MGIT culture tubes), TTP across a given drug concentration did not vary as plasma concentrations increased ($p=0.756$). For this reason, for a given drug concentration, data for the different plasma concentrations were combined as shown in Fig. 1B. Each drug concentration had a significantly greater TTP than no drug, and significantly greater TTP than the preceding lower drug concentration (except for 32–64 $\mu\text{g/ml}$ and 64–128 $\mu\text{g/ml}$). There was almost complete inhibition at 64 $\mu\text{g/ml}$ (Fig. 1B). There was no significant effect on TTP when the combination of NTZ (at 128 $\mu\text{g/ml}$), MTB and 50% plasma was compared to NTZ (at 128 $\mu\text{g/ml}$) and MTB without plasma (mean of 33.2 and 42 days, respectively, ($p=0.7$) or when this experiment was run with TIZ (all values were 42 days) (data not shown). However, when NTZ or TIZ were incubated in 5%–50% plasma with BCG for 72 hours (the duration of the WBA incubation) no drug effect was seen at all drug concentrations tested and across all plasma concentrations ($p=0.517$) (Fig. 1B). Post hoc analysis revealed that the adjusted p value (for mean ranks) was not significant between any drug concentration and “no drug”.

3.3 WBA of NTZ and TIZ

Preliminary experiments with THP-1 macrophage cells and BCG confirmed inhibition of mycobacterial growth at NTZ and TIZ total concentrations of 3.1 $\mu\text{g/ml}$ to 6.1 $\mu\text{g/ml}$, consistent with studies reporting MICs of NTZ for MTB from 3.1 $\mu\text{g/ml}$ to 16 $\mu\text{g/ml}$ [3, 14]. DMSO, at concentrations of <1% vol/vol used in our experiments, did not affect cell viability (data not shown). However, these cultures also contained 10% human serum, yielding an albumin concentration (70 μM) similar to MGIT cultures. At 8 $\mu\text{g/ml}$ TIZ, a free drug concentration of 1 $\mu\text{g/ml}$ is expected.

Whole blood cultures without added drug showed MGIT TTPs of approximately 4 days, corresponding to MTB growth of 0.4 log/day of whole blood culture. Growth was strongly inhibited by RIF, but only weakly inhibited by NTZ or TIZ, for which concentrations of 128 $\mu\text{g/ml}$ were required to approach bacteriostasis ($p < 0.0005$ for both NTZ and TIZ). These data are shown in Fig. 2A as prolongation of TTP, and in Fig. 2B as corresponding log change in bacillary number (based on TTP and the known volume of inoculum). No tested concentration resulted in bactericidal activity, which would be represented as negative values in Fig. 2B. In contrast to tissue cultures, whole blood cultures contain albumin at half the concentration in plasma (350 μM). At this albumin concentration, a TIZ concentration found effective in macrophage cultures (8 $\mu\text{g/ml}$) yields a free drug concentration of only 0.1 $\mu\text{g/ml}$, one-tenth that in cell culture.

4.0 Discussion

Peak plasma TIZ concentrations in healthy human volunteers after 7 days of treatment with 500 mg of NTZ every 12 hours (the recommended dose) reached 10 $\mu\text{g/ml}$; dosing at 1 g every 12 h produced 22 $\mu\text{g/ml}$ [15]. Single doses of 4 grams yielded C_{max} values of 17.5 $\mu\text{g/ml}$, with an upper range of 26.5 $\mu\text{g/ml}$ [6]. Our study demonstrates that NTZ and TIZ begin to have activity against MTB when directly inoculated into MGIT tubes at 16 $\mu\text{g/ml}$, although the magnitude of the response appears relatively small until 32 $\mu\text{g/ml}$, a supra-

therapeutic concentration. NTZ and TIZ had less convincing MTB killing in the WBA, with limited activity at 128 µg/ml. The reduced effects in whole blood culture are consistent with reduced bioavailability due to increased protein binding. Albumin concentrations in vivo are twice those in whole blood culture, further reducing bioavailability. These observations bring into question the potential role of NTZ and TIZ as anti-mycobacterial drugs [15].

Due to the relative inactivity of NTZ and TIZ in the WBA, plasma experiments were performed to investigate the effects of protein binding/inactivation on drug activity. When MTB/BCG and drug, along with varying plasma concentrations, were directly inoculated into the MGIT culture tubes (with only 30 minutes of incubation), TTP was not affected by plasma exposure. However, 72 hour co-incubation (both in plasma and whole blood) resulted in marked loss of anti-mycobacterial activity, either due to inactivation or tight protein binding of NTZ and TIZ. Dog plasma studies by the manufacturer indicated that TIZ (both TIZ as the original compound and NTZ metabolized to TIZ) were stable at 37°C for 72 hours, arguing against further metabolism. Assuming 1:1 binding of TIZ to albumin, then free drug in plasma would be 2–3% of that present in our experiments using MGIT plus OADC or RPMI with 10% serum.

Several studies have demonstrated good activity of NTZ against MTB, but none of these have been *in vivo* studies [2, 3, 9, 14]. The WBA is a novel method to evaluate anti-MTB activity of drugs. The WBA combines the effects of host immune function and *in vivo* drug concentration on mycobacterial growth and has been shown to correlate with sputum bactericidal activity and culture conversion [10, 11]. WBA predicts the bactericidal activity of anti-MTB drugs, including rifampin, isoniazid, pyrazinamide, ethambutol, levofloxacin, moxifloxacin, linezolid and PNU-100480 [9, 15]. However, with clofazimine, which has intracellular bactericidal activity against MTB, the WBA indicated MTB killing only at supra-therapeutic concentrations [11]. Therefore, the lack of effect of NTZ and TIZ in the WBA at therapeutically achievable concentrations does not eliminate their prospects as drugs for TB treatment. However, other approaches are required to determine what, if any, role NTZ and its primary metabolite TIZ will have as TB drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Stockis A, Deroubaix X, Lins R, Jeanbaptiste B, Calderon P, Rossignol JF. Pharmacokinetics of nitazoxanide after single oral dose administration in 6 healthy volunteers. *Int J Clin Pharmacol Ther*. 1996; 34(8):349–351. [PubMed: 8864798]

2. De Carvalho LP, Darby CM, Rhee KY, Nathan C. Nitazoxanide disrupts membrane potential and intrabacterial pH homeostasis of *Mycobacterium tuberculosis*. *ACS Med Chem Lett*. 2011; 2(11): 849–854. [PubMed: 22096616]
3. Lam KK, Zheng X, Forestieri R, Balgi AD, Nodwell M, Vollett S, Anderson HJ, Andersen RJ, Av-Gay Y, Roberge M. Nitazoxanide stimulates autophagy and inhibits mTORC1 signaling and intracellular proliferation of *Mycobacterium tuberculosis*. *PLoS Pathog*. 2012; 8(5):e1002691. [PubMed: 22589723]
4. Wallis RS, Jakubiec W, Mitton-Fry M, Ladutko L, Campbell S, Paige D, Silvia A, Miller PF. Rapid evaluation in whole blood culture of regimens for XDR-TB containing PNU-100480 (sutezolid), TMC207, PA-824, SQ109, and pyrazinamide. *PLoS One*. 2012; 7(1):e30479. [PubMed: 22279595]
5. Wallis RS, Jakubiec WM, Kumar V, Silvia AM, Paige D, Dimitrova D, Li X, Ladutko L, Campbell S, Friedland G, Mitton-Fry M, Miller PF. Pharmacokinetics and whole-blood bactericidal activity against *Mycobacterium tuberculosis* of single doses of PNU-100480 in healthy volunteers. *J Infect Dis*. 2010; 202(5):745–751. [PubMed: 20629533]
6. Broekhuysen J, Stockis A, Lins RL, De Graeve J, Rossignol JF. Nitazoxanide: pharmacokinetics and metabolism in man. *Int J Clin Pharmacol Ther*. 2000; 38(8):387–394. [PubMed: 10984012]
7. Stockis A, Allemon AM, De Bruyn S, Gengler C. Nitazoxanide pharmacokinetics and tolerability in man using single ascending oral doses. *Int J Clin Pharmacol Ther*. 2002; 40(5):213–220. [PubMed: 12051573]
8. Zhao Z, Xue F, Zhang L, Zhang K, Fei C, Zheng W, Wang X, Wang M, Zhao Z, Meng X. The pharmacokinetics of nitazoxanide active metabolite (tizoxanide) in goats and its protein binding ability in vitro. *J Vet Pharmacol Ther*. 2010; 33(2):147–153. [PubMed: 20444039]
9. Shigyo K, Ocheretina O, Merveille YM, Johnson WD, Pape JW, Nathan CF, Fitzgerald DW. Efficacy of nitazoxanide against clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2013; 57(6):2834–2837. [PubMed: 23507275]
10. Wallis RS, Vinhas SA, Johnson JL, Ribeiro FC, Palaci M, Peres RL, Sá RT, Dietze R, Chiunda A, Eisenach K, Ellner JJ. Whole Blood Bacterial Activity during treatment for Pulmonary Tuberculosis. *JID*. 2003; 187:270–278. [PubMed: 12552451]
11. Wallis RS, Palaci M, Vinhas S, Hise AG, Ribeiro FC, Landen K, Cheon SH, Song HY, Phillips M, Dietze R, Ellner JJ. A Whole Blood Bactericidal Assay for Tuberculosis. *JID*. 2001; 183:1300–1303. [PubMed: 11262217]
12. Janulionis E, Sofer C, Song HY, Wallis RS. Lack of activity of orally administered clofazimine against intracellular *Mycobacterium tuberculosis* in whole-blood culture. *Antimicrob Agents Chemother*. 2004; 48(8):3133–3135. [PubMed: 15273133]
13. Janulionis E, Sofer C, Schwander SK, Nevels D, Kreiswirth B, Shashkina E, Wallis RS. Survival and replication of clinical *Mycobacterium tuberculosis* isolates in the context of human innate immunity. *Infect Immun*. 2005; 73(5):2595–2601. [PubMed: 15845461]
14. de Carvalho LP, Lin G, Jiang X, Nathan C. Nitazoxanide kills replicating and nonreplicating *Mycobacterium tuberculosis* and evades resistance. *J Med Chem*. 2009; 52(19):5789–5792. [PubMed: 19736929]
15. Stockis A, De Bruyn S, Gengler C, Rosillon D. Nitazoxanide pharmacokinetics and tolerability in man during 7 days dosing with 0.5 g and 1 g b.i.d. *Int J Clin Pharmacol Ther*. 2002; 40:221–227. [PubMed: 12051574]

Figure 1. Effect of nitazoxanide (NTZ) and tizoxanide (TIZ) on mycobacterial growth in MGIT
 (a) Activity of NTZ and TIZ against *M. tuberculosis* (MTB) when inoculated directly with MTB into MGIT. Changes in MTB growth were measured as Time to Positivity (TTP). Results represent mean $\pm$ S.D. of 3 experiments. (b) The effect of plasma on TIZ's activity against *M. bovis*-BCG (BCG) in MGIT. BCG and TIZ were inoculated into MGIT either 30 min after TIZ had been mixed with increasing concentrations of plasma (0–50% vol/vol) (**direct inoculation** bars), or after 72 h of incubation with increasing concentrations of plasma (5–50% vol/vol) (**incubated** bars). Results represent the mean $\pm$ S.D. of combined

data for plasma levels. For **direct inoculation** plasma levels of 0, 5, 10, 25 and 50% vol/vol were combined; for **incubated** data plasma levels of 5, 10, 25 and 50% were combined. Results represent the mean of 10 and 8 experiments, respectively. For **direct inoculation** experiments, TTP for all drug concentrations was significantly greater than “no drug”. For **incubation** experiments there was no statistical difference between “no drug” and any drug concentration. After 42 days cultures were considered to have no mycobacterial growth. Rifampin (10 µg/ml) and no drug were positive and negative controls.

Figure 2. Effect of nitazoxanide (NTZ) and tizoxanide (TIZ) on *M. tuberculosis* (MTB) growth in whole blood assay (WBA)

(a) Drug effects on time to positivity. (b) Corresponding effects expressed as log change in viability (Δ log CFU per day of whole blood culture). Positive numbers on the vertical axis indicate growth; zero indicates bacteriostasis. Rifampin (10 µg/ml) and no drug served as positive and negative controls respectively. Results represent mean $\pm$ S.D. for WBA with blood from 11 healthy adults. Growth of MTB was significantly inhibited at 128 µg/ml only.