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Inhibitors of Ca^{2+} and K^+ Transport Enhance Intracellular Killing of *M. tuberculosis* by Non-killing Macrophages

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Abstract. *Background: Human monocyte-derived macrophages that have little killing activity of their own kill intracellular Staphylococcus aureus when cultured in the presence of inhibitors of calcium and potassium efflux pumps. The aim of this study was to evaluate the effect of inhibitors such as ouabain, reserpine and verapamil in the killing activity of macrophages infected with Mycobacterium tuberculosis. Materials and Methods: Macrophages obtained from peripheral blood were infected with M. tuberculosis ATCC27294 H37Rv and treated with reserpine, ouabain and verapamil. Results: After three days post-infection, macrophages treated with the inhibitors demonstrated an enhancement of the killing activity destroying the internalized bacteria. Conclusion: Whereas drugs that target the bacterium are predicted to lose effectiveness due to mutation of the bacterial target, drugs that enhance killing by macrophages that normally do not kill mycobacteria may yield a more effective form of infections therapy caused by multidrug resistant M. tuberculosis.*

Pulmonary tuberculosis (TB) caused by *Mycobacterium tuberculosis* is an intracellular infection of the pulmonary macrophage that has little killing activity of its own (1). Consequently, the therapy of this infection is successful only if the antibiotic is able to penetrate the macrophage and have activity against the offending bacterium at the site where it resides, namely, the phagosome of the pulmonary macrophage (2). TB caused by organisms that are susceptible to the two most effective anti-TB agents, isoniazid (INH) and rifampin (RIF), is not difficult to treat if proper management of the patient ensures that the agents are taken as prescribed and that non-compliance is avoided (3). When these two aspects of therapy are not conducted properly, spontaneous mutations of one antibiotic target will be selected and the infection will be monoresistant – usually resistant to INH alone. Resistance to both INH and RIF, commonly known as multidrug resistance (MDRTB), results from serial replication or transmission of monoresistant bacteria in patients that are poorly managed with INH and RIF (4). MDRTB produces high mortality in immunocompetent patients (5) and is essentially lethal within a matter of months if the patient has AIDS (6). As of this writing, no agents have been made available for the therapy of MDRTB and because of the terminal nature of this infection among a growing population of individuals with HIV/AIDS (7), there is an urgent need for an agent that can either prolong or improve the quality of life and, even better, afford a cure even if the patient is immuno-incompetent.

Our *in vitro* (8), *ex vivo* (9-11) and *in vivo* (12) studies support the use of existing phenothiazines for the therapy of MDRTB infections (13). Although much academic attention has been given to these studies (14-18), these compounds and their derivatives are of little interest to the pharmaceutical industry due to a number of reasons: namely, patent protection is not possible and high rates of MDRTB exist primarily in countries that are economically disadvantaged and cannot even afford conventional antibiotics for the therapy of antibiotic susceptible infections. The waiting period for new agents that are effective against MDRTB is anticipated to be long.

The anti-tubercular nature of phenothiazines, although known since the advent of the first effective neuroleptic chlorpromazine (19), has until recently not been understood with respect to its mechanism of action. Pioneering studies conducted by Crowle and his group (20) and those of Amaral and his group (8, 10-12) suggested that the enhanced killing activity of macrophages against intracellular mycobacteria could be the result of the macrophage concentrating the agent to intracellular levels equivalent to those that kill the mycobacterium *in vitro* (9).

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This assumption has never been proven although it has been cogently supported by the fact that phenothiazines are concentrated by cells that are rich in lysosomes (21, 22).

Recent evidence suggests that killing of intracellular bacteria is dependent upon the availability of calcium (Ca^{2+}) (23) and potassium (K^+) (24), both of which are regulated by active transport processes that depend upon Ca^{2+}-dependent ATPases and hence are inhibited by phenothiazines (25). We had previously examined the possibility that enhanced killing of intracellular *Staphylococcus aureus* by non-killing human macrophages could be brought about by agents that are known to inhibit the transport of K^+ either directly or indirectly, inasmuch as K^+ plays the major role for the acidification of the lysosome (26) and subsequent activation of hydrolases responsible for intracellular killing of the bacteria (27). Preliminary experiments showed that ouabain, a direct inhibitor of K^+ transport (28, 29), and verapamil and reserpine, indirect inhibitors of K^+ transport by limiting access of Ca^{2+} to Ca^{2+}-dependent ATPases (5, 27, 30), caused the killing of intracellular *S. aureus* within a few hours after its phagocytosis (26). Because this *ex vivo* model was successfully employed for rapidly establishing conditions for the study of enhanced killing of intracellular mycobacteria by phenothiazines, in the study described herein, we examined whether ouabain, reserpine and verapamil can similarly enhance the killing of mycobacteria by non-killing human macrophages.

Materials and Methods

Bacterial strains. M. tuberculosis H37Rv ATCC27294 was grown in 7H11 medium containing 0.06% of Tween-80 until it reached an optical density between 0.5 and 0.6 at 600 nm. After this optical density was reached, cells were washed with phosphate-buffered saline (PBS) and the pellet re-suspended in PBS containing 15% glycerol. Aliquots were kept at –80˚C and used as the source of mycobacteria (31).

Materials. Thioridazine (TZ), reserpine, ouabain, verapamil, Trypan blue, RPMI medium, Hank's balanced salt solution, PBS, Tween-80 and sodium dodecyl sulphate (SDS) were purchased from Sigma Aldrich Química SA. (Madrid, Spain). TZ derivatives were synthesized by Professor Dr. G. Hajös, Institute for Chemistry of the Hungarian Academy of Science (11, 12). 7H11 Mycobacteria medium and OADC supplement were purchased from Difco (Detroit, MI, USA). Reagents were prepared in distilled, sterile water on the day of the experiment.

Evaluation of susceptibility of M. tuberculosis H37Rv ATCC27294 to the compounds employed in this study. Minimum inhibitory concentration (MIC) of TZ, TZ derivatives, reserpine, ouabain and verapamil against *M. tuberculosis* H37Rv ATCC27294 were conducted as described elsewhere (11, 12). Briefly, *M. tuberculosis* H37Rv ATCC27294 was grown in BACTEC 460 medium (Quilaban, Lisbon, Portugal) until it reached a maximum growth index. After this, 0.1 mL aliquots of an adjusted inoculum suspension (0.5 of McFarland standard) (BioMérieux, Portugal) was transferred to BACTEC 460 vials (Quilaban, Lisbon, Portugal) containing concentrations of each compound that ranged from 0.0 to 200 mg/L. In addition, an absolute control containing 1/100 of the culture, as required by the method for the determination of an MIC, was conducted (32). All cultures were maintained at 37˚C and growth was assayed by daily readings in the BACTEC 460TB until the absolute control (no drug) reached the maximum value (growth index =999).

Human monocyte-derived macrophages. Twenty millilitres of intravenous blood from healthy voluntary donors were transferred into 50 U of preservative-free sodium heparin (Monoparin; CP Pharmaceuticals Ltd, Wrexham, UK). Human monocytes were obtained from buffy coats by Ficoll-hypaque density gradient centrifugation and then cultured as described elsewhere (1). Briefly, cells were cultured for 5 days at a density of $1x10^6$ cells/mL in RPMI medium containing 2 mM L-glutamine (Sigma, Madrid, Spain) and supplemented with 10% of autologous serum at 37˚C and 5.5% CO_2. Of the total cells that adhere to the wells of the plate, 10% are monocyte-derived macrophages. After 3 days of culture, the medium was removed, the cells washed 3 times with Hank's balanced salt solution and new medium was distributed to the wells of the 24-well tissue culture plate. The cells were incubated for 2 more days and after this interval, monocyte-derived macrophages were infected with *M. tuberculosis*. The infection process was performed adding 0.1 mL aliquots of a bacterial suspension containing $1x10^6$ *M. tuberculosis*/mL to the monocyte-derived macrophages cultures. All blood samples were processed on the same day of the collection. Blood was obtained from donors who provided written informed consent.

Cytotoxicity of TZ, TZ derivatives, reserpine, ouabain and verapamil. Determination of the cytotoxic effects of TZ, TZ derivatives, reserpine, ouabain and verapamil on human lymphocytes was determined by the use of the Trypan blue exclusion method as described elsewhere (11, 12). Briefly, different concentrations of each compound were added to wells containing isolated human lymphocytes and the cells were incubated for up to 3 days. After this interval, an aliquot of 0.01 mL was retrieved from each well, mixed with 0.09 ml of 0.9% Trypan blue-balanced salt solution and placed on a haemocytometer in order for cell counting to be performed. Cellular toxicity is defined when more than 10% of the cells exhibit Trypan blue-stained cytoplasm.

Determination of the effects of TZ derivatives, reserpine, ouabain and verapamil on the killing of intracellular M. tuberculosis. Human monocyte-derived macrophages obtained from peripheral blood ($1.0x10^6$ cells per ml) were cultured in RPMI containing 2 mM L-glutamine (Sigma) and 10% of autologous serum for 5 days. Bacterial suspensions adjusted to $1.0x10^6$ cells were added to monolayer cultures of monocyte-derived macrophages (1 macrophage: 10 bacteria) and incubated at 37˚C, with 5.5% CO_2 for 1 hour (phagocytosis). After this time, non-phagocytosed bacteria were removed by 3 consecutive washes with Hank's balanced salt solution. Aliquots of the compounds (0.1 mL) were then added to macrophage cultures at a concentration of 0.1 mg/L (for TZ and derivatives) or 80 mg/L (for the other efflux pumps inhibitors) and the adhered cells-phagocytosed bacteria cultures were incubated at 37˚C and 5.5% CO_2 for 0, 1, 2 and 3 days.

Table I. *Minimum inhibitory concentration (MIC) of TZ, derivatives and other efflux pumps inhibitors against M. tuberculosis.*

	M. tuberculosis H37Rv ATCC27294 MIC (mg/L)
Phenothiazines	
TZ	15
#1867; #1875; #1926	5
#1470; #1870; #1876	10
Other efflux pump inhibitors	
Reserpine	100
Ouabain	>200
Verapamil	128

Table II. *Toxicity of TZ, TZ derivatives, reserpine, ouabain and verapamil on human lymphocytes.*

	Toxicity (%)		
Phenothiazines	0.1 mg/L	3 mg/L	10 mg/L
TZ	0	50	100
#1867; #1875; #1926; #1470; #1870; #1876	0	1.6	5.3
Other efflux pump inhibitors	2 mg/L	50 mg/L	100 mg/L
Reserpine	0	0.54	1.1
Ouabain	0	5.5	10[a]
Verapamil	0	2.4	4.8

[a]Concentration of 100 mg/L of ouabain produces marginal toxicity.

At the end of each culture period, the supernatant was removed from each well (saved for later determination of extracellular mycobacteria that may have been caused by the lysis of the macrophage due to the agents that had been added). SDS (1.0 mL, 0.01%) was added to each well to cause the lysis of the adhered macrophages thereby releasing the trapped mycobacteria. Aliquots of 0.1 mL were removed and serially diluted in PBS and plated on 7H11 medium. Aliquots of 0.1 mL of the supernatants removed from each of the wells prior to the addition of the lysing agent were also plated on 7H11 medium. The plates were then incubated for 3 to 4 weeks at 37˚C and the number of viable bacterial cells present in the supernatant of the lysed cultures was determined by colony-forming unit (CFU) counts, as described elsewhere (1). The data presented are the average of at least 3 separate experiments each conducted in triplicate.

Results

The evaluation of thioridazine (TZ), derivatives of TZ, ouabain, reserpine and verapamil for direct *in vitro* activity against *M. tuberculosis* H37Rv ATCC27294 by the broth dilution method that identifies the MIC for each agent is summarised in Table I. Briefly, whereas TZ and its derivatives had an MIC of 15 and 5 to 10 mg/L, respectively, a concentration of ouabain, reserpine or verapamil as high as 80 mg/L had no significant effect on the replication of the mycobacterium. The evaluation of the toxicity of TZ, derivatives of TZ, ouabain, reserpine and verapamil on lymphocytes cultured for up to 3 days is summarised in Table II. Briefly, whereas TZ was cytotoxic at concentrations that exceeded 0.5 mg/L, concentrations of ouabain, reserpine and verapamil as high as 100 mg/L did not produce significant toxicity, *i.e.* the percentage of lymphocytes that exhibited trypan blue staining of their cytoplasm did not exceed 10%. As shown by Figure 1, macrophages derived from human blood did not appreciably kill phagocytosed *M. tuberculosis* during a period of 3 days' incubation. These results are consistent with those previously presented (1, 11). In contrast, cultures containing the phenothiazine TZ at a concentration of 0.1 mg/L yielded only a few live mycobacteria after 1 day of culture, still fewer after 2 days, and none after 3 days.

Because previous studies have shown that some derivatives of TZ can enhance the killing of intracellular mycobacteria (11), we evaluated 6 other non-toxic derivatives for similar activity. Among these 6 non-toxic TZ derivatives (#1867, #1875, #1926, #1470, #1870, #1876), each of which demonstrated greater *in vitro* activity against *M. tuberculosis* than its parental molecule (see Table I), derivatives #1875, #1926 and #1876 enhanced the killing of mycobacteria at a concentration of 0.1 mg/L. Derivative #1875 was especially active and more effective than TZ since after 2 days post-infection no colony forming units (CFU) were retrieved from the lysed supernatants.

In order to determine whether the enhanced killing could be due to the inhibition of transport of Ca^{2+} and K^+, we examined whether common inhibitors of Ca^{2+} and K^+ transport can enhance the killing of intracellular bacteria at concentrations of agents which are non-toxic. As can be seen in Figure 2, these efflux pump inhibitors enhanced the killing activity of infected macrophages, however, at concentrations much higher (80 mg/L) than those used for TZ (0.1 mg/L). Verapamil demonstrated a greater activity than TZ. The maximum concentration of the K^+ inhibitors examined for ability to enhance the killing of intracellular *M. tuberculosis* was 80 mg/L inasmuch as this concentration was devoid of any significant toxicity against human lymphocytes (see Table II).

Discussion

Phenothiazines inhibit the replication of *S. aureus* (26, 33, 34), and *M. tuberculosis* (1, 11, 32) and are bactericidal at concentrations that are multiples of their MIC (8). Because phenothiazines are concentrated as much as 100-fold by cells that are rich in lysosomes (2, 8, 10, 12, 21, 22), the

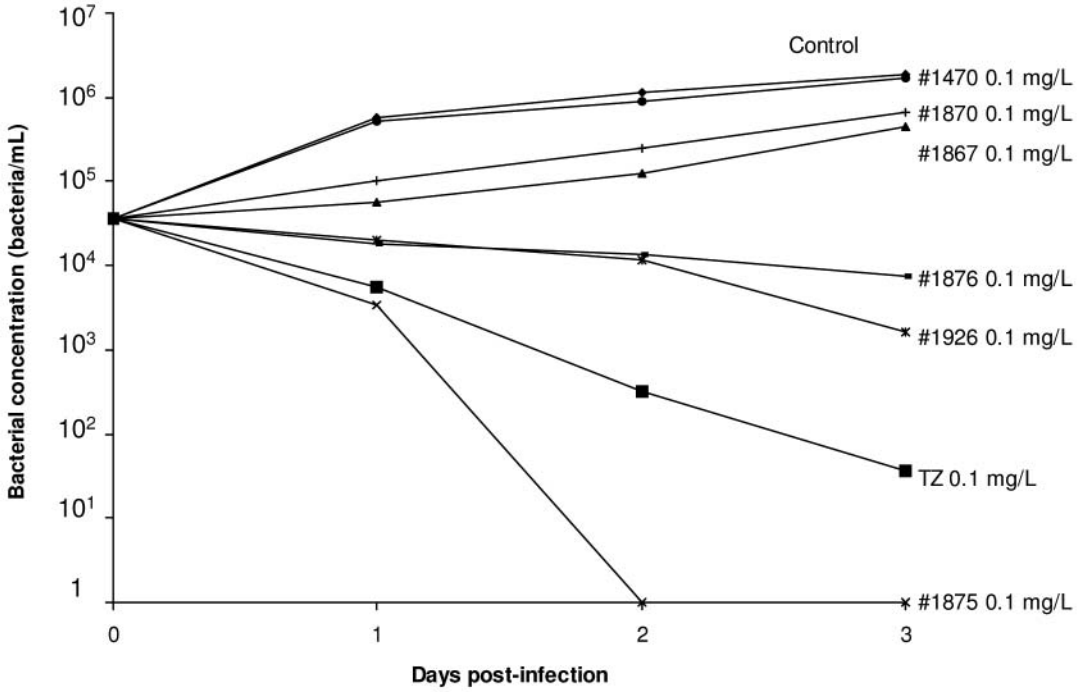

Figure 1. *Effect of TZ and its derivatives in the killing activity of macrophages infected with M. tuberculosis H37Rv ATCC27294. Human macrophages (1x10^5 cells/mL) were infected with M. tuberculosis at a ratio of 1:10 (1 macrophage:10 bacteria). After 1 hour of phagocytosis, cells were washed to remove non-phagocytosed bacteria. Aliquots of the test compounds were added to the corresponding wells and cells incubated for 0, 1, 2 and 3 days. After incubation, supernatants of the cells were removed and plated in order to determine whether the addition of the agents cause the lysis of the macrophage and subsequent release of the bacterium. Adhered cells were lysed with SDS 0.01% in order to release all the intracellular bacteria and aliquots of the lysed cells were plated in 7H11 plates and incubated at 37˚C. After 3 to 4 weeks, CFU were counted and bacterial concentrations calculated. The data presented are averages from three independent experiments each of which was conducted in triplicate.*

enhanced intracellular killing produced by TZ and its derivatives could be the result of concentrations that are comparable to those that are bactericidal (1, 32). Because previous studies have shown that concentrations of verapamil and reserpine that are considerably higher than those employed in our study do not significantly affect the growth of bacteria (31, 33), enhanced killing is probably not due to any antimicrobial activity that results from their being concentrated by the macrophage.

Recent studies have shown that the killing activity of the neutrophils is dependent upon the transport of Ca^{2+} (23), K^+ and hydrolysis of ATP (24). Because these processes are inhibited by phenothiazines (12, 34, 35) enhanced killing may be due to the effect that the phenothiazine has on the transport of Ca^{2+} and K^+.

This raises the question of how these inhibitors of Ca^{2+} and K^+ transport enhance the killing of intracellular mycobacteria. After phagocytosis, the bacterium is contained in a phagosome that was formed from the invagination of the plasma membrane of the macrophage. The plasma membrane is rich in pumps that transport Ca^{2+} and K^+ into the cell, and due to invagination, would now pump these ions that leak into the phagolysosome to the cytoplasm of the cell. When fusion of the phagosome with a lysosome takes place, the absence or low concentration of Ca^{2+} and K^+ prevents the acidification of the phagolysosome and hence the bacterium is not killed. The unification of a vacuole that contains the inhibitor of Ca^{2+} and K^+ transport with the phagolysosome inhibits the transport of these ions to the cytoplasm and they begin to accumulate within the phagolysosome. This would soon be followed by the activation of the V-ATPase system, which is probably ouabain insensitive (probably also phenothiazine insensitive); K^+ is extruded and hydrogen ions flood into the phagolysosome resulting in the acidification of the phagolysosome and activation of hydrolytic enzymes. The killing of the bacterium subsequently takes place. However, the possibility that a direct action of the agent on the phagosome-trapped bacterium due to the agent being concentrated to levels equivalent to those that kill the bacterium *in vitro* cannot be ruled out. The question of whether the results obtained in this study will further contribute to the therapy of MDRTB is intriguing.

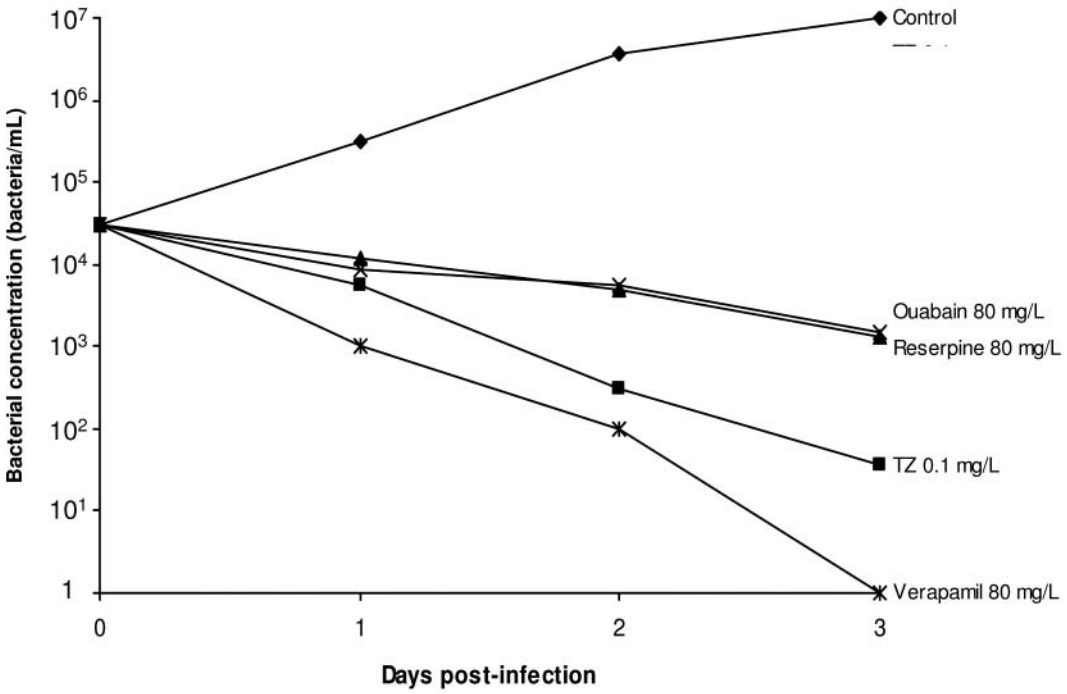

Figure 2. *Effect of TZ and other efflux pumps inhibitors in the killing activity of macrophages infected with M. tuberculosis. Cultures of isolated human macrophages (1x10^5 cells/mL) were infected with M. tuberculosis at a ratio of 1:10 (1 macrophage:10 bacteria). After 1 hour of phagocytosis, cells were washed to remove non-phagocytosed bacteria. Aliquots of the tested compounds were added to the wells and cells incubated for 0, 1, 2 and 3 days. After incubation, supernatants of the wells were removed and kept for CFU study in order to determine whether the addition of the agents cause the lysis of the macrophage and subsequent release of the bacterium. Adhered cells were lysed with SDS 0.01% in order to release all the intracellular bacteria and aliquots of the lysed cells were plated on 7H11 medium. Plates were incubated at 37˚C for 3 to 4 weeks. After the incubation period, CFU were counted and bacterial concentrations calculated. The data presented are averages from three independent experiments each of which was conducted in triplicate.*

Firstly, therapy of mice infected with *M. tuberculosis* with phenothiazine derivatives has proven successful (12, 36, 37). Secondly, these positive results are consistent with the predictions provided by *ex vivo* studies (1, 11, 12, 26, 34). Thirdly, with the advent of the first neuroleptic chlorpromazine (CPZ), anecdotal clinical observations suggested that this agent had anti-tubercular properties (12). Although CPZ produces high frequencies of severe side-effects, TZ, equivalent to CPZ as a neuroleptic, has far fewer (8). Nevertheless, regardless of over 35 years of usage experience, TZ can produce arrhythmia and even torsade de pointes, albeit at an extremely low frequency (1 sudden death per 10,000 man years of used) (2, 8). Because of the negative prognosis of an MDRTB infection, we have repeatedly suggested the use of TZ for its therapy especially for compassionate reasons (8). This recommendation remains.

The results of the current study suggest an entirely different approach for the therapy of TB, and especially, MDRTB. This approach involves the design of agents which target the macrophage and transform it into an effective bacterial killer. Whereas agents which target the bacterium are predicted to become ineffective due to mutations of the bacterial target, agents that enhance the killing activity of the macrophage will not suffer from this limitation. Moreover, this approach is also viable for the therapy of other intracellular infections, such as malaria, leishmaniasis, trypanosomiasis, inasmuch as phenothiazines have already been shown to be effective (10, 13, 38-40). Perhaps the search for effective agents for the therapy of intracellular infections should be extended to include all agents that are known to inhibit Ca^{2+} and K^+ transport processes.

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References

1 Ordway D, Viveiros M, Leandro C, Bettencourt R, Almeida J, Martins M, Kristiansen JE, Molnar J and Amaral L: Clinical concentrations of thioridazine kill intracellular multi-drug resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother *47*: 917-922, 2003.

2 Amaral L, Kristiansen JE, Viveiros M and Atouguia J: Activity of phenothiazines against antibiotic-resistant *Mycobacterium tuberculosis*: a review supporting further studies that may elucidate the potential use of thioridazine as anti-tuberculosis therapy. J Antimicrob Chemother *47*: 505-511, 2001.

3 Raviglione MC, Sudre P, Rieder HL, Spinaci S and Kochi A: Secular trends of tuberculosis in Western Europe. Bull World Health Organ *71*: 297-306, 1993.

4 Centres for Diseases Control and Prevention: National Plan for Reliable Tuberculosis Laboratory Services Using a Systems Approach: Recommendations from CDC and the Association of Public Health Laboratories Task Force on Tuberculosis Laboratory Services. MMWR *54*: 1-12, 2005.

5 Rossau R, Traore H, De Beenhouwer H, Mijs W, Jannes G, De Rijk P and Portaels F: Evaluation of the INNO-LiPA Rif. TB assay, a reverse hybridization assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampin. Antimicrob Agents Chemother *41*: 2093-2098, 1997.

6 Viveiros M, Martins M, Couto I, Kristiansen JE, Molnar J and Amaral L: The *in vitro* activity of phenothiazines against *Mycobacterium avium*: potential of thioridazine for therapy of the co-infected AIDS patient. In Vivo *19*: 733-736, 2005.

7 Sethi S, Mandal J, Kumar P, Kumar S, Meharwal S, Jindal SK and Sharma M: Susceptibility testing of *Mycobacterium tuberculosis* by broth microdilution method: a rapid alternative method. Diagn Microbiol Inf Dis *57*: 447-449, 2007.

8 Amaral L and Kristiansen JE: Phenothiazines: an alternative to conventional therapy for the initial management of suspected multidrug resistant tuberculosis. A call for studies. Int J Antimicrob Agents *14*: 173-176, 2000.

9 Amaral L and Kristiansen JE: Phenothiazines: potential management of Creutzfeldt-Jacob disease and its variants. Int J Antimicrob Agents *18*: 411-417, 2001.

10 Amaral L, Viveiros M and Kristiansen JE: Non-antibiotics: Alternative therapy for the management of MDRTB and MRSA in economically disadvantaged countries. Curr Drug Targets *7*: 887-891, 2006.

11 Martins M, Schelz Z, Martins A, Molnar J, Hajos G, Riedl Z, Viveiros M, Yalcin I, Aki-Sener E and Amaral L: The *in vitro* and *ex vivo* activity of thioridazine derivatives against *Mycobacterium tuberculosis*. Int J Antimicrob Agents *29*: 338-340, 2007.

12 Amaral L, Martins M and Viveiros M: Enhanced killing of intracellular multidrug-resistant *Mycobacterium tuberculosis* by compounds that affect the activity of efflux pumps. J Antimicrob Chemother *59*: 1237-1246, 2007.

13 Amaral L, Viveiros M and Kristiansen JE: Phenothiazines: potential alternatives for the management of antibiotic resistant infections of tuberculosis and malaria in developing countries. Trop Med Int Health *6*: 1016-1022, 2001.

14 Chan YY, Ong YM and Chua ML: Synergistic interaction between phenothiazines and antimicrobial agents against *Burkholderia pseudomallei*. Antimicrob Agents Chemother *51*: 623-630, 2007.

15 Gumbo T, Louie A, Liu W, Ambrose PG, Bhavnani SM, Brown D and Drusano GL: Isoniazid's bactericidal activity ceases because of the emergence of resistance, not depletion of *Mycobacterium tuberculosis* in the log phase of growth. J Infect Dis *195*: 194-201, 2007.

16 Marquez B: Bacterial efflux systems and efflux pumps inhibitors. Biochimie *87*: 1137-1147, 2005.

17 Ramon-Garcia S, Martin C, Ainsa JA and De Rossi E: Characterization of tetracycline resistance mediated by the efflux pump Tap from *Mycobacterium fortuitum*. J Antimicrob Chemother *57*: 252-259, 2006.

18 Tomioka H: Current status of some antituberculosis drugs and the development of new antituberculous agents with special reference to their *in vitro* and *in vivo* antimicrobial activities. Curr Pharmac Design *31*: 4047-4070, 2006.

19 Kristiansen JE and Vergmann B: The antibacterial effect of selected phenothiazines and thioxanthenes on slow-growing mycobacteria. Acta Pathol Microbiol Immunol Scand [B] *94*: 393-398, 1986.

20 Crowle AJ, Douvas GS and May MH: Chlorpromazine: a drug potentially useful for treating mycobacterial infections. Chemother *38*: 410-419, 1992.

21 Daniel WA and Wojcikowski J: Contribution of lysosomal trapping to the total tissue uptake of psychotropic drugs. Pharmacol Toxicol *80*: 62-68, 1997.

22 Daniel WA and Wojcikowski J: The role of lysosomes in the cellular distribution of thioridazine and potential drug interactions. Toxicol Appl Pharmacol *158*: 115-124, 1999.

23 Ahluwalia J, Tinker A, Clapp LH, Duchen MR, Abramov AY, Pope S, Nobles M and Segal AW: The large-conductance Ca^{2+}-activated K^+ channel is essential for innate immunity. Nature *427*: 853-858, 2004.

24 Reeves EP, Lu H, Jacobs HL, Messina CG, Bolsover S, Gabella G, Potma EO, Warley A, Roes J and Segal AW: Killing activity of neutrophils is mediated through activation of proteases by K^+ flux. Nature *416*: 291-297, 2002.

25 Ratnakar P, Rao SP, Sriramarao P and Murthy PS: Structure-antitubercular activity relationship of phenothiazine-type calmodulin antagonists. Int Clin Psychopharmacol *10*: 39-43, 1995.

26 Martins M, Viveiros M, Ordway D, Kristiansen JE, Molnar J and Amaral L: Reserpine, ouabain and the calcium channel blocker verapamil, cause intracellular killing of *Staphylococcus aureus*. Res J Microbiol *1*: 203-209, 2006.

27 Pillay CS, Elliott E and Dennison C: Endolysosomal proteolysis and its regulation. Biochem J *363*: 417-429, 2002.

28 Contreras RG, Flores-Maldonado C, Lazaro A, Shoshani L, Flores-Benitez D, Larre I and Cereijido M: Ouabain binding to Na^+, K^+-ATPase relaxes cell attachment and sends a specific signal (NACos) to the nucleus. J Memb Biol *198*: 147-158, 2004.

29 Orlov SN, Thorin-Trescases N, Pchejetski D, Taurin S, Farhat N, Tremblay J, Thorin E and Hamet P: Na^+/K^+ pump and endothelial cell survival: $[Na^+]i/[K^+]i$-independent necrosis triggered by ouabain, and protection against apoptosis mediated by elevation of $[Na^+]i$. Cell Mol Physiol *448*: 335-345, 2004.

30 Gao Z, Lau CP, Chiu SW and Li GR: Inhibition of ultra-rapid delayed rectifier K^+ current by verapamil in human atrial myocytes. J Mol Cell Cardiol *36*: 257-263, 2004.

31 Viveiros M, Portugal I, Bettencourt R, Victor TC, Jordaan AM, Leandro C, Ordway D and Amaral L: Isoniazid-induced transient high-level resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother *46*: 2804-2810, 2002.
32 Bettencourt MV, Bosne-David S and Amaral L: Comparative *in vitro* activity of phenothiazines against multidrug-resistant *Mycobacterium tuberculosis*. Int J Antimicrob Agents *16*: 69-71, 2000.
33 Kristiansen MM, Leandro C, Ordway D, Martins M, Viveiros M, Pacheco T, Molnar J, Kristiansen JE and Amaral L: Thioridazine reduces resistance of methicillin resistant *Staphylococcus aureus* by inhibiting a reserpine and CCCP sensitive putative efflux pumps. In Vivo *20*: 361-366, 2006.
34 Ordway D, Viveiros M, Leandro C, Jorge Arroz M, Molnar J, Kristiansen JE and Amaral L: Chlorpromazine has intracellular killing activity against phagocytosed *Staphylococcus aureus* at clinical concentrations. J Infect Chemother *8*: 227-231, 2002.
35 Amaral L, Viveiros M and Molnar J: Antimicrobial activity of phenothiazines. In Vivo *18*: 725-731, 2004.
36 Weinstein EA, Yano T, Li LS, Avarbock D, Avarbock A, Helm D, McColm AA, Duncan K, Lonsdale JT and Rubin H: Inhibitors of type II NADH: menaquinone oxidoreductase represents a class of antitubercular drugs. PNAS *102*: 4548-4553, 2005.
37 Yano T, Li LS, Weinstein E, Teh JS and Rubin H: Steady-state kinetics and inhibitory action of antitubercular phenothiazines on *Mycobacterium tuberculosis* type-II NADH-menaquinone oxidoreductase (NDH-2). J Biol Chem *281*: 11456-11463, 2006.
38 Grácio MA, Grácio AJ, Viveiros M and Amaral L: Since phenothiazines alter antibiotic susceptibility of microorganisms by inhibiting efflux pumps, are these agents useful for evaluating similar pumps in phenothiazine-sensitive parasites? Int J Antimicrob Agents *22*: 347-351, 2003.
39 Happi CT, Gbotosho GO, Folarin OA, Bolaji OM, Sowunmi A, Kyle DE, Milhous W, Wirth DF and Oduola AM: Association between mutations in *Plasmodium falciparum* chloroquine resistance transporter and *P. falciparum* multidrug resistance 1 genes and *in vivo* amodiaquine resistance in *P. falciparum* malaria-infected children in Nigeria. Am J Trop Med Hyg *75*: 155-161, 2006.
40 Wainwright M and Amaral L: The phenothiazinium chromophore and the evolution of antimalarial drugs. Trop Med Int Health *10*: 501-511, 2005.

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