in vivo *19*: 539-546 (2005)

In Vitro and *In Vivo* Antimycobacterial Activity of an Antihypertensive Agent Methyl-L-DOPA

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Abstract. *Methyl-L-DOPA, an antihypertensive agent, has significant in vitro activity against a variety of atypical mycobacteria such as the Mycobacterium avium complex, M. scrofulaceum, M. xenopi and M. marinum, and rare pathogens like M. fortuitum. In the present investigation, the screening of the in vitro activity was further extended by testing the in vitro activity against a total of 53 different strains of mycobacteria, including 34 clinical isolates of both drug-sensitive and drug-resistant Mycobacterium tuberculosis. Most of the strains were inhibited at 10-25 μg/mL concentrations of the drug. When methyl-L-DOPA was injected into male mice at a concentration of 10 μg/g body weight (20 g each), methyl-L-DOPA significantly protected them when challenged with a 50 median lethal dose of M. tuberculosis $H_{37}Rv102$. According to the χ^2test, the in vivo data were highly significant ($p<0.01$).*

Tuberculosis is still a leading cause of death among infections with a single etiology and one-third of the world population is latently infected with *Mycobacterium tuberculosis*. Both the increase in AIDS-associated infections and recent outbreaks of diseases sustained by multidrug-resistant (MDR) strains of *M. tuberculosis* should motivate the development of new, more effective antitubercular drugs for alternative treatments. Several tuberculosis control trials and MDR reversals are aimed at preventing the spread of tuberculosis and MDR-tuberculosis infections. Interestingly, the new microcapsule administration with drug-loaded microspheres or some drug combination therapies have increased the therapeutic efficacy and reduced the toxicity of the administered antimycobacterial drugs. Despite such progress, it is necessary to develop more potent drugs for the MDR reversal treatment of mycobacteriosis. Antimicrobial agents, such as artificial chemicals with diverse pharmacological effects, have also revealed powerful efficacy against a great variety of Gram-positive and Gram-negative genera, such as: (i) antihistamines [diphenhydramine, bromodiphenhydramine (1), methdilazine (2) promethazine (3)], (ii) psychotropics [promazine (4), fluphenazine (5) and trifluoperazine (6)], (iii) antihypertensives [methyl-L-DOPA (7)], (iv) anti-inflammatory drug [diclofenac (8, 9)] and (v) cardiovascular drugs [oxyfedrine (10), amlodipine (11)]. These chemotherapeutic agents have been grouped together and are termed "non-antibiotics" (12). Some non-antibiotics, such as chlorpromazine (13), levopromazine and promethazine (14), promazine and desipramine (15), trifluoperazine (16), methdilazine (17), thioridazine (18) and other phenothiazines (19), are also antitubercular. Based on these findings, methyl-L-DOPA, a non-antibiotic, was tested against different strains of mycobacteria. *In vitro* observations were further confirmed by *in vivo* protection tests in mice against many strains of mycobacteria.

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Key Words: Antihypertensive agent, methyl-L-DOPA, anti-mycobacterial activity.

Materials and Methods

Chemicals. The chemicals were obtained as pure dry powders from their respective manufacturers in India, as follows: methyl-L-DOPA (m-L-DOPA) from Dolphin Laboratories, rifampicin (Rf) from Hindustan Ciba Geigy, streptomycin (Sm) from Sarabhai Chemicals, ethambutol (Eb) from Lyka Laboratories and isonicotinic acid hydrazide (INH) from Glaxo Laboratories. All chemicals were stored at 4˚C.

Bacteria. Fifty-three strains of mycobacteria were tested. The strains and their sources are given in Table I. The strains were identified by radiometric method (BACTEC 460) and biochemical tests (Niacin, Nitrate, Urease, Catalase, Tween 80, Tellurite and 5% NaCl tests).

Table I. *Source of mycobacterial strains tested.*

Mycobacteria	Source
Reference strains	
M. tuberculosis H37 Rv102, H37Ra16	Tuberculosis Research Center, Chennai, ICMR, Govt. of India
M. marinum NCTC 2273, *M. scrofulaceum* 1302, *M. scrofulaceum* 1323, *M. gordonae* 1324, *M. flavescens* 1451, *M. xenopi* 10, *M. xenopi* 160, *M. avium* 724, *M. avium* NCTC 8551, *M. intracellulare* 1406, *M. terrae* 1450, *M. terrae* 1540, *M. trivate* 1453, *M. smegmatis* 798, *M. smegmatis* 1546, *M. fortuitum* 1529, *M. phlei* L1	Central JALMA Institute for Leprosy ICMR, Agra, India
Clinical strains	
M. tuberculosis Bajaj, J15, N23, *M. tuberculosis* 90657, 905358, 905574, 906909, 910708, 911053, 11337, 911447, 911454, 911677, 911831, 911884, 911928, 912042, 912056, 912073, 912234, 912359, 912447	Tuberculosis Research Centre, Chennai, India
M. tuberculosis K1, K2, *M. tuberculosis* BTA 1(10376), BTA 2 (10378), BTA 3 (1811), BTA 4 (1813), BTA 5 (1824), BTA 6 (1831), BTA 7 (1834), BTA 8 (1854), BTA 9 (1856), BTA 10 (1864)	K.S. Roy TB Hospital, Calcutta Bengal Tuberculosis Association (BTA), Calcutta, India

Media. Kirchner's Liquid Medium (KLM) (20) was used to grow and suspend the organisms. The solid medium was Lowenstein Jensen Medium (LJM), prepared as described by the International Union Against Tuberculosis and Lung Diseases (IUATLD), 1955 (21).

Preparation of inocula for susceptibility tests. The bacteria were first grown in KLM. The inoculum was prepared by homogenizing the KLM culture with glass beads, spinning down the larger particles, and matching the supernatant against McFarland's standard (22).

Determination of minimum inhibitory concentration (MIC) of antibiotics / non-antibiotics against different strains of mycobacteria. The MICs of the test strains were determined by tube dilution method (23). In this test, MIC represented the lowest concentration of an antibiotic or a non-antibiotic that completely inhibited the growth of the organism. The MIC of a given antibiotic / non-antibiotic for a test strain was compared with its modal MIC for the control strains, and the resistance ratio was expressed as MIC for the test strain / modal MIC for the control strains. A ratio of 4 or more for a test strain indicated its resistance to a given drug. The drugs such as streptomycin, rifampicin, INH and ethambutol were used at concentrations of 0, 0.25, 0.5, 1, 2, 4 and 8 μg/mL. The resistant strains were further processed for determining the resistant breakpoint by using 8, 16, 32, 64 and 128 μg/mL of the drug in LJM. The following drugs had MIC levels of the primary antitubercular drugs indicating resistance: streptomycin (Sm) ≥32 μg/mL, isoniazid (INH) ≥1 μg/mL, rifampicin (Rf) ≥128 μg/mL and ethambutol (Eb) ≥8 μg/mL. Likewise, the MIC of methyl-L-DOPA with respect to the different strains of mycobacteria was determined by using 0, 5, 10, 12.5, 15, 20, 25 and 50 μg/mL concentrations. The amount of inoculum used to inoculate each tube was 0.01 mL. Incubation was at 37˚C for 10-20 days as required. The *in vitro* screening was

Table II. *Antibiogram patterns of different strains of mycobacteria.*

Mycobateria	Sensitivity of primary antituberculous drugs			
	INH	Rf	Sm	Eb
M. tuberculosis (24)	S	S	S	S
M. tuberculosis (12)	R	R	S	S
M. tuberculosis (4)	R	S	S	S
M. tuberculosis (1)	S	R	S	S
M. tuberculosis (1)	R	S	R	S
M. tuberculosis (1)	R	S	S	R
M. tuberculosis (1)	R	R	R	S
M. tuberculosis (1)	R	R	S	R
M. tuberculosis (1)	R	S	R	R
M. tuberculosis (7)	R	R	R	R

S, sensitive; R, resistant; INH, isoniazid; Rf, rifampicin; Sm, streptomycin; Eb, ethambutol.

Table III. *Antimycobacterial profile of methyl-L-DOPA.*

Bacterial strain	MIC (μg/mL)	Mean±SD
Sensitive strains *M. fortuitum* 1529, *M. scrofulaceum* 1323, *M. scrofulaceum* 1302, *M. flavescens* 1541, *M. trivate* 1453, *M. phlei* L1	10	10±0.82
M. smegmatis 798, *M. smegmatis* 1546, *M. xenopi* 160, *M. avium* 724, *M. avium* NCTC 8551, *M. intracellulare* 1406, *M. marinum* NCTC 2273	12.5	12.5±0.41
Mycobacterium tuberculosis H37 Rv102, *M. marinum* 50, *M. gordonae* 1324, *M. terrae* 1450, *M. terrae* 1540, *M. tuberculosis* Bajaj, J15, N23, H37Ra16, K1, 911928, 912447, 912234, 912359, 906909	15	15±1.63
Multidrug-resistant *M. tuberculosis* BTA1, BTA2, BTA3, BTA4, BTA5, BTA6, BTA7, BTA8, BTA9, BTA10, 995574, 905358, 910657	20	20±2.45
Polydrug-resistant *M. tuberculosis* 911454, 912042, 911831, 911447, 911677, 910708, 911884, 912056, 911053, 911337, 912073	25	25±1.41

carried out in triplicate for most of the organisms, and five times for the potent ones, and the mean values of the MIC were indicated. For some selected strains, the drug was tested in concentrations ±2 of its MIC value, in order to find out its mean ± standard deviation values with respect to these organisms.

Animal experiments. Inbred Swiss Albino male mice (*ca*. 18-20 g) were maintained in the animal house under standard conditions at 21±1˚C and 50-60% relative humidity, with a photoperiod of 14:10 hours of light-darkness. Water and a dry pellet diet were given *ad libitum*. *M. tuberculosis* H_{37}Rv102 was used as the test bacterium, which was naturally virulent to mice. The median lethal dose (MLD / LD_{50}) of the strain (after repeated passage through mice) was determined by using graded challenges in batches of mice and recording the mortality up to 30 days. The LD_{50} was not affected by the freeze-drying and reconstitution. Reproducibility of the challenge dose was ensured by standardizing its optical density at 640 nm in a Klett-Summerson colorimeter to obtain the desired colony forming units (cfu) on KLM.

Systemic infections were produced in 20 mice. Each mouse was administered intraperitoneally (*i.p.*) 0.05 mL of a suspension (containing 0.5 mg homogenized KLM culture deposit, representing < 9 x 10^9 cfu) (24); of the 20 animals, 10 mice were administered m-L-DOPA (dose 10 μg/g body weight/day x 6 weeks), while the other 10 mice did not receive any drug and served as the control. The viscera from the animals autopsied 6 weeks after infection were obtained, taking strict precautions regarding sterility, and examined for macroscopic lesions of systemic infections, *e.g.*, tubercles and caseation, both for the treated and untreated groups (25). Portions of each organ were processed for histological study of the lesions, while the remainders were homogenized aseptically in sterile glass homogenizers in saline, examined under the microscope as stained smears (Hematoxylin and Eosin, as well as Ziehl-Neelsen stains) for the presence of acid fast bacilli (AFB)/ and contaminants, and inoculated onto nutrient/blood agar plates to determine rapid growth, if any. Sterile specimens (as well as contaminated specimens after adequate decontamination by Petrov's method) were plated out on LJM in 0.1 mL amounts and examined for growth of the infecting *M. tuberculosis*. The growth was confirmed by radiometric method.

Results

Minimum inhibitory concentration (MIC) of INH, Sm, Rf, Eb and m-L-DOPA against different strains of mycobacteria. Nineteen reference strains including *Mycobacterium tuberculosis* H_{37}Rv102, H_{37}Ra16, *M. phlei* L1, *M. avium* complex, *M. scrofulaceum*, *M. xenopi*, *M. marinum* and *M. kansasii,* and rare pathogens like *M. fortuitum,* were sensitive to all four conventional antimycobacterial drugs. Among 34 clinical strains of mycobacteria, 5 strains were sensitive to all these 4 drugs, 12 strains showed resistance to both INH and Rf, 4 strains were found to be resistant to INH and one strain to Rf. Resistance to both Sm and INH, or INH and Eb was noticed in one strain each. Similarly, resistance to 3 drugs, either INH, Rf and Eb, or

Table IV. *Evidence of protection by methyl-L-DOPA[a] against M. tuberculosis H37 Rv 102[b] infection in mice.*

Treatment	Liver	Spleen	Peritoneum / Intestine	Lung	Cumulative value	Recovery of H_{37}Rv102 in smears	culture
Untreated (10)							
Smears AFB (+)	10/10	10/10	10/10	10/10	40/40		
Tubercles	8	5	9	5	27	10[c]	5
Caseation	3	1	1	0	5		
Treated (10)							
Smears AFB (+)	0/10	2/10	2/10	0/10	4/40		
Tubercles	2	4	3	0	9	4[d]	1
Caseation	0	0	0	0	0		

Untreated: did not receive methyl-L-DOPA.
Treated: received methyl-L-DOPA.
[a] 10 μg/g body weight/day.
[b] 4.5 x 10^9 CFU/mouse intraperitoneally.
[c] from at least one viscera of 10 animals, all viscera did not yield (+) culture; the viable counts (CFU) varied from 10^3 to 10^6/mL.
[d] only from 4 animals, recovery from even a single organ being counted as positive, other organs did not yield any growth;CFU 10^1-10^3/mL in the positive samples.

INH, Rf and Sm, or INH, Sm and Eb, was noted in one strain each. Finally, 7 strains were resistant to all the 4 drugs (Table II). The MIC of m-L-DOPA with respect to 53 strains of mycobacteria tested is given in Table III. The MIC of m-L-DOPA was much higher than the MIC of the conventional antimycobacterial drugs (Sm, Rf, INH, Eb). Among 53 strains of mycobacteria tested, 6 strains (*M. fortuitum* 1529, *M. scrofulaceum* 1323, *M. scrofulaceum* 1302, *M. flavescens* 1541, *M. trivate* 1453, *M. phlei* L1) were inhibited by m-L-DOPA at 10 μg/ml, while 7 strains (*M. smegmatis* 798, *M. smegmatis* 1546, *M. xenopi* 160, *M. avium* 724, *M. avium* NCTC 8551, *M. intracellulare* 1406 and *M. marinum* NCTC 2273) were inhibited at 12.5 μg/mL of m-L-DOPA. These 13 strains were highly sensitive with respect to conventional antitubercular drugs. Sixteen strains (*M. marinum* 50, *M. gordonae* 1324, *M. terrae* 1450, *M. terrae* 1540, *M. tuberculosis* Bajaj, J15, N23, H_{37} Rv102, H_{37}Ra16, K1, K2, 911928, 912447, 912234, 912359 and 906909) were inhibited at 15 μg/mL of m-L-DOPA. Thirteen strains (*M. tuberculosis* BTA1, BTA2, BTA3, BTA4, BTA5, BTA6, BTA7, BTA8, BTA9, BTA 10, 905574, 905358 and 910657) were found to be MDR. They were inhibited by m-L-DOPA at 20 μg/mL. Finally, *M. tuberculosis* 912042, 911831, 911447, 911677, 911454, 910708, 911884, 912056, 911053, 911337 and 912073 were inhibited by m-L-DOPA at 25 μg/mL. These strains were polydrug-resistant. The susceptible strains such as *M. tuberculosis* H_{37}Rv102 were inhibited at lower doses of conventional antitubercular agents (0.5 to 2 μg/mL), while the single-, poly- and multidrug-resistant clinical isolates (*M. tuberculosis* 906909, *M. tuberculosis* 911454, *M. tuberculosis* BTA8 and others) were inhibited at much higher concentrations, and some were even resistant. The MIC of m-L-DOPA against *M. tuberculosis* H_{37}Rv102 was 15 μg/mL, while it was 25 μg/mL for the drug-resistant strains. The MIC values of m-L-DOPA in terms of mean±standard deviation with respect to 5 strains (*M. fortuitum* 1529, *M. smegmatis* 798, *M. tuberculosis* H_{37}Rv102, *M. tuberculosis* BTA1 and *M. tuberculosis* 911454) are given in Table III. It was noticed that even the multidrug-resistant strains were susceptible to m-L-DOPA, although at a higher concentration (25 μg/mL).

In vivo assessment. An analysis of the results given in Table IV showed that, in the untreated group, out of a total of 10 animals, 8 developed minute tubercles in the liver, 5 in the spleen, 5 in the lungs and 9 in the peritoneum and intestines; in the liver of 3 animals and in the spleen, peritoneum and intestines each in one animal, microscopic necrosis was detected, which suggested caseation. From centrifuged deposits (for 100 fields) of tissue homogenates, smears for acid-fast bacilli (AFB) were prepared by Z-N stain; such smears showed all 10 mice to be smear-positive at the time of autopsy, suggestive of successful infection in these animals. However, in macroscopic examination of the treated group (10 animals), minute tubercles were seen

to be present in 2 liver specimens, in 4 samples of spleen, and in the peritoneum, as well as in the intestine of each of 3 animals, but no tubercle was detected in the lungs. AFB was present only in 4 cases, as revealed by Z-N-stained smears. *M. tuberculosis* H_{37}Rv102 could actually be recovered on subculture (as confirmed by BACTEC test) in only one animal of the treated group, in contrast to 5 animals of the untreated group, which was statistically significant ($p<0.01$). There was a considerable decrease in the number of infiltrations in infected mice treated with methyl-L-DOPA, as compared to the untreated animals (controls), as observed in the histopathological sections of the lungs (Figure 1a, 1b, 1c).

Discussion

Methyl-L-DOPA, both an antihypertensive and Parkinson's disease drug, was found to possess conspicuous antibacterial activity on testing against 405 strains of bacteria including a large number of Gram-positive and Gram-negative genera. The MIC of m-L-DOPA ranged from 10-200 μg/mL. This bacteriostatic agent could also offer significant protection to mice when challenged with a virulent bacterium (7).

In the present study, m-L-DOPA showed a noteworthy antitubercular activity against diverse mycobacteria. The MIC of m-L-DOPA ranged from concentrations of 10-25 μg/mL with respect to most strains tested. Many of the antimycobacterial non-antibiotics reported so far have shown *in vitro* MIC values ranging from 10 to 25 μg/mL, which seems to be in accordance with that of m-L-DOPA. Phenothiazines, such as chlorpromazine (13), promethazine (14) and thioridazine (18), have been shown to have *in vitro* activity against clinical strains of *M. tuberculosis*. This activity required concentrations that are beyond those that are clinically achievable (*i.e.* 1 μg/mL). Thus, a clinically acceptable administration dosage for a tuberculosis patient might contribute to an inhibitory effect when the *in vitro* dosages are close to the *in situ* intracellular doses. This suggests that MDR reversal drugs such as m-L-DOPA could be clinically used as very effective adjuvants in a new regimen for the management of freshly diagnosed tuberculosis.

In the animal tests with *M. tuberculosis* H_{37}Rv102 in mice, several minute tubercles were observed in the liver, spleen, lungs, peritoneum and intestines of infected mice. However, m-L-DOPA administration significantly protected the mice (Table IV).

In spite of the toxic effect of m-L-DOPA, it was well tolerated and could protect the mice for the entire period of 6 weeks of daily *i.p.* administration at the dose of 10 μg/g body weight. Because m-L-DOPA is a precursor, which is metabolized in the brain to the active form, m-L-DOPA might play a primary role after the precursor has been metabolized and activated. When administered orally, methyl-L-DOPA is absorbed by an active amino acid transporter. The peak effective plasma concentration was reached after 2 to 3 hours. Methyl-L-DOPA is distributed in a relatively small apparent volume (0.4 L/kg) and is eliminated with a half-life of about 2 hours. The transport of m-L-DOPA into the central nervous system (CNS) is apparently also an active process. Methyl-L-DOPA is excreted in the same way as other different kinds of metabolites such as methyldopamine, methylnorepinephrine and o-methylated products of these catecholamines. The rate of de-esterification of m-L-DOPA is variable among patients, and an intravenous m-L-DOPA administration might deliver less m-L-DOPA to the circulation when compared to an oral administration (26).

The above results of preliminary screening showed that m-L-DOPA could be used as a starting material for further new and safe drugs with increased antimycobacterial and reduced antihypertensive activity.

Acknowledgements

We are grateful to the All India Council for Technical Education (AICTE), Goverment of India, for providing financial support for this work.

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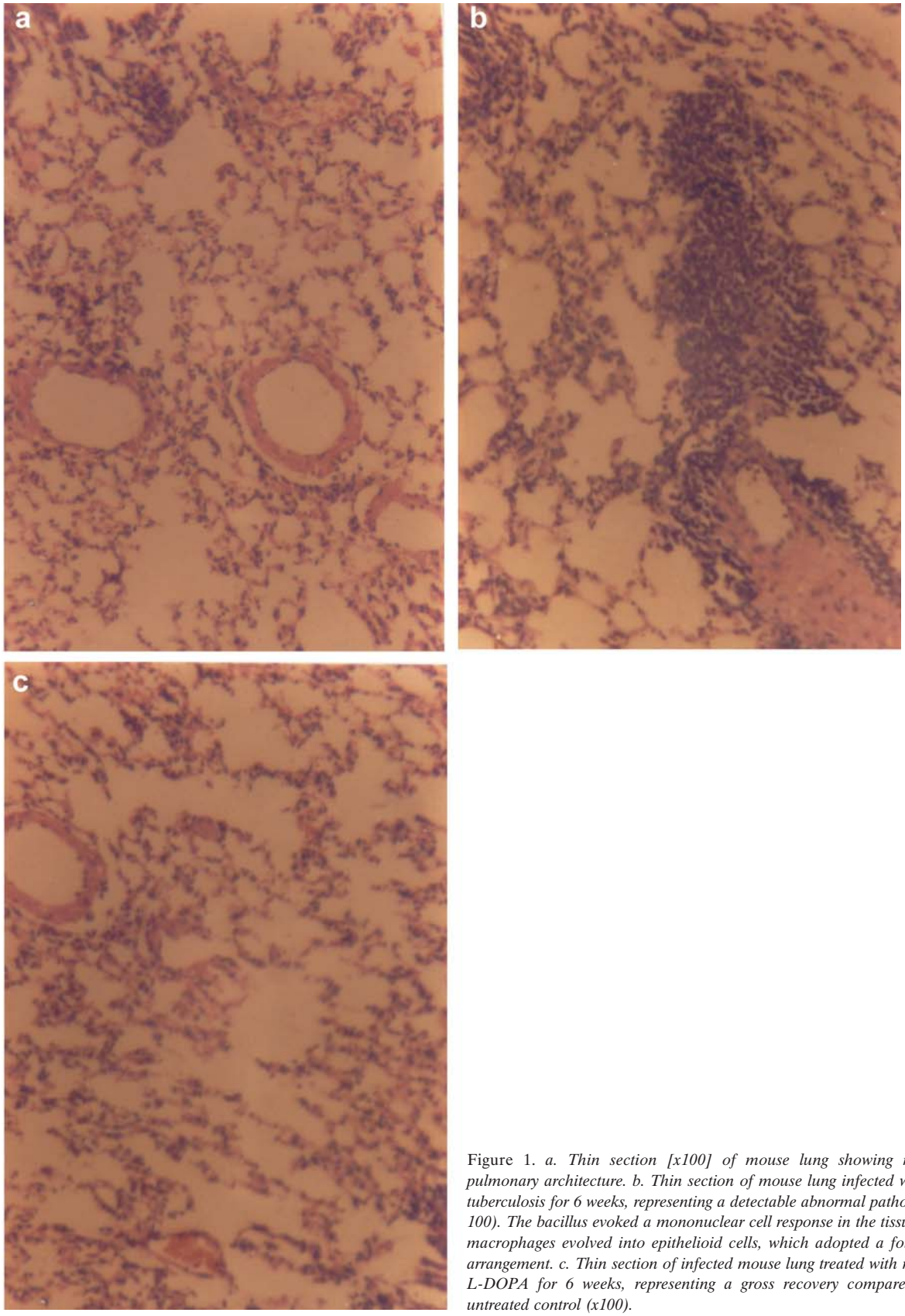

Figure 1. *a. Thin section [x100] of mouse lung showing normal pulmonary architecture. b. Thin section of mouse lung infected with M. tuberculosis for 6 weeks, representing a detectable abnormal pathology (x 100). The bacillus evoked a mononuclear cell response in the tissue. The macrophages evolved into epithelioid cells, which adopted a follicular arrangement. c. Thin section of infected mouse lung treated with methyl-L-DOPA for 6 weeks, representing a gross recovery compared with untreated control (x100).*

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Received September 13, 2004
Revised February 2, 2005
Accepted March 4, 2005