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Anti-Mycobacterial Activity of Tamoxifen Against Drug-Resistant and Intra-Macrophage *Mycobacterium tuberculosis*

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Recently, it has become a struggle to treat tuberculosis with the current commercial anti-tuberculosis drugs because of the increasing emergence of multidrug-resistant (MDR) *tuberculosis* and extensively drug-resistant (XDR) *tuberculosis*. We evaluated here the anti-mycobacterial activity of tamoxifen, known as a synthetic anti-estrogen, against eight drug-sensitive or resistant strains of *Mycobacterium tuberculosis* (TB), and the active intracellular killing of tamoxifen on TB in macrophages. The results showed that tamoxifen had anti-tuberculosis activity against drug-sensitive strains (MIC, 3.125–6.25 μg/ml) as well as drug-resistant strains (MIC, 6.25 to 12.5 μg/ml). In addition, tamoxifen profoundly decreased the number of intracellular TB in macrophages in a dose-dependent manner.

Keywords: *Mycobacterium tuberculosis*, tamoxifen, intracellular killing, MDR, XDR

Tuberculosis, caused by the bacterium *Mycobacterium tuberculosis* (TB), is one of the world's major health problems [11]. Although successful treatment of TB was achieved for several decades using a combination of antibiotics, the combination has shown limited efficacy in recent years, and is failing to prevent spread of the disease owing to the emergence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) TB [13, 16]. A synthetic anti-estrogen, tamoxifen (TAM), used in the chemotherapy of breast cancer, has been reported to also have the ability to kill bacteria, fungi, and parasites, besides breast cancer cells [1, 6, 12]. Recently, TAM was found to have anti-tuberculosis activity against TB H37Ra (drug-sensitive strain) using the computationally predicted TB drugome [4]. To further clarify the activity of TAM against TB, the anti-TB activities of TAM against six clinically isolated drug-resistant TB strains (including MDR and XDR) were evaluated herein, along with the intracellular killing effect of TAM on macrophages infected with TB.

For determination of the anti-TB activities of TAM (Sigma and KBNMB (Korea Bioactive Natural Material Bank)) against drug-resistant TB, clinically isolated, drug-resistant TB strains from the Korea *Mycobacterium* Resource Center (KMRC) in Korea were employed. Isoniazid (INH; Sigma) and Rifampicin (RIF; Sigma) were used as reference standard drugs. The anti-mycobacterial activities of TAM, INH, and RIF were tested using the resazurin assay, as described by Palomino *et al.* [14]. In brief, 50 μl of bacterial suspension (2.5×10^5 cells) was added to 96-well plates containing 50 μl of the test drug suspension in 7H9 broth (Difco) with OADC (Oleic Albumin Dextrose Catalase) growth supplement at a predetermined concentration. After incubation of the plates at 37°C for 7 days, 30 μl of resazurin solution (0.02%) was added to each well and the plates were incubated for two more days. MICs were expressed as an interval (*a* to *b*), where *a* is the highest concentration tested at which TB cells are still growing and *b* is the lowest concentration that causes complete growth inhibition. Table 1 shows the MIC ranges for TAM and the control drugs (INH and RIF) against the two reference TB strains (H37Ra and H37Rv, drug-sensitive strains) and six drug-resistant TB strains. In contrast to INH and RIF, TAM retained its anti-TB activity against the drug-resistant strains, as in the sensitive TB strains H37Ra and H37Rv.

Table 1. MICs of TAM and two control drugs against TB H37Ra, H37 Rv, and drug-resistant TB.

Samples	MICs (μg/ml)							
	H37Ra	H37Rv	MDR	XDR	INH-r	RIF-r	Pyr -r	Strep-r
TAM	3.125–6.25	3.125–6.25	6.25–12.5	6.25–12.5	6.25–12.5	6.25–12.5	6.25–12.5	6.25–12.5
INH	0.0062–0.012	0.0062–0.012	25–50	>100	>100	50–100	50–100	50–100
RIF	0.05–0.1	0.1–0.195	12.5–25	>100	0.05–0.1	50–100	0.1–0.195	0.05–0.1

MDR: multidrug-resistant TB (KMRC 00116); XDR: Extensively drug-resistant TB (KMRC 00203); INH-r: isoniazid-resistant TB (KMRC 00120); RIF-r: rifampin-resistant TB (KMRC 00121); Pyr-r: pyrazinamide-resistant TB (KMRC 00130); Strep-r: Streptomycin-resistant TB (KMRC 00122).

For all tested strains, the MICs of TAM were in the range of 3.125–6.25~6.25–12.5 μg/ml. The anti-TB activity of TAM was superior to that of RIF against the MDR, XDR, and RIF-r strains, and stronger than that of INH against all the drug-resistant TB strains tested. Next, the effects of combinations of TAM with INH or RIF against the two drug-sensitive TB strains and five drug-resistant TB strains were evaluated as previously described method [2]. The results of the combination of TAM with INH or RIF against TB H37Ra, H37Rv, and the clinical isolates are listed in Table 2. Among the seven strains tested, the combination of TAM with RIF showed favorable synergistic anti-TB effects against all the strains except RIF-r strain, whereas the combination with INH did not. Specifically, TAM showed the strongest synergistic effect with RIF against Pyr-r (FICI: 0.1875) compared with the other strains.

TB is a known intracellular pathogen and usually resides inside macrophages [8]. A recent study showed that *M. bovis* BCG infects 50–60% of macrophages and 30–40% of dendritic cells in the lungs after aerosol infection [15], indicating that macrophages are primarily infected by TB [5]. Therefore, we examined the effectivity of TAM on TB-infected macrophage *in vitro*. In this study, an intracellular killing assay using strain GFP-TB was employed followed by *in vitro* cytotoxicity assay. Briefly, subcultured macrophages (Raw 264.7, KTCC AC-28116) were harvested and seeded in 96-well plates at 1×10^4 cells/ml. After 24 h of incubation in a 37°C incubator with 5% CO_2 and humidity, the cells were treated with predetermined TAM concentrations and incubated for an additional 24 and 48 h. Cell viability was determined at this indicated time point by simply adding EZ-Cytox Cell Viability Assay solution WST-1 (Daeil Lab Service, Jong-No, Korea) and measured after 3 h by a multireader instrument at 460 nm (Perkin Elmer 2030

Table 2. Fractional inhibitory concentration (FIC) of combinations of TAM with two first-line anti-TB drugs.

Strains	Compound 1	Compound 2	FIC_a/FIC_b[1]	FICI[2]	Outcome
	Name	Name			
TB H37Ra	TAM	INH	1/1	2	Indifference
		RIF	0.125/0.125	0.25	**Synergistic**
H37Rv	TAM	INH	1/1	2	Indifference
		RIF	0.25/0.004	0.254	**Synergistic**
MDR	TAM	INH	2/1	3	Indifference
		RIF	0.25/0.25	0.5	**Synergistic**
INH-r	TAM	INH	1/-	-	-
		RIF	0.125/0.25	0.375	**Synergistic**
RIF-r	TAM	INH	2/2	4	Indifference
		RIF	0.5/1	1.5	Indifference
Pyr-r	TAM	INH	2/1	3	Indifference
		RIF	0.0625/0.125	0.1875	**Synergistic**
Strep-r	TAM	INH	2/1	3	Indifference
		RIF	0.125/0.125	0.25	**Synergistic**

[1]FIC_a = MIC of the combination/MIC_a alone; FIC_b = MIC of the combination/MIC_b alone; a = TAM; b = INH or RIF. Interpretation of the data was achieved by calculating the fractional inhibitory concentration index (FICI), as follows: [2]FICI = ($MIC_{a\ combination}/MIC_{a\ alone}$) + ($MIC_{b\ combination}/MIC_{b\ alone}$). The FICI was used to interpret the test results as follows: FICI ≤ 0.5, synergy; FICI = 0.5–4, indifference; FICI > 4.0, antagonism.

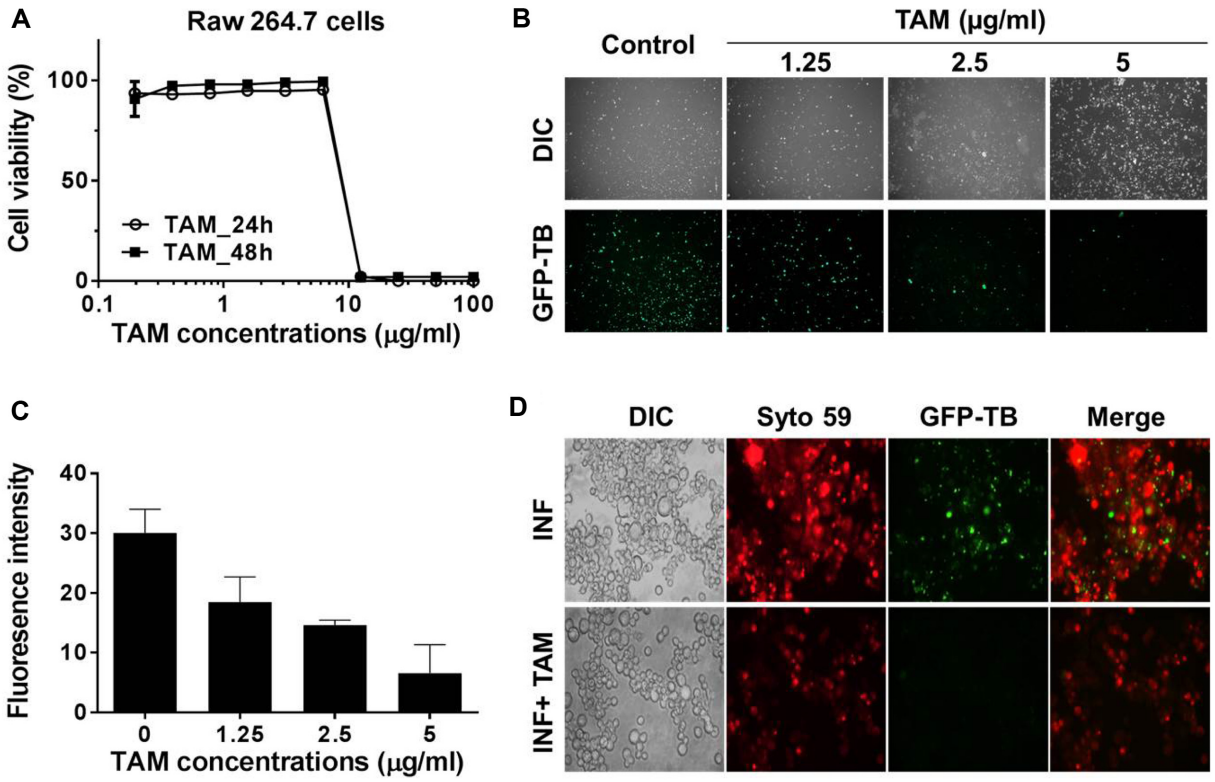

Fig. 1. Intracellular killing activity of TAM.

(**A**) The cytotoxicity of TAM against cells was measured at 460 nm using a Perkin Elmer 2030 Reader (Victor X3). Triton X-100 (1%) was used as the control for 100%, and 0.01% DMSO was used as the drug-free control. Cell viability (%) = (A_{460} of sample - A_{460} of peptide-free control)/(A_{460} of 100% control - A_{460} of drug-free control) × 100. (**B**) TB H37Ra-GFP-infected Raw 264.7 cells were incubated with TAM at predetermined concentrations (1.25, 2.5, and 5 μg/ml) for 5 days and visualized by fluorescence microscopy (Zeiss, Japan). Representative pictures of the infected macrophages treated with TAM and DMSO are provided at day 5. (**C**) The average cytosolic intensity of TB H37Ra-GFP at each concentration of TAM was graphed in the cell population (~2,500 cells) using the image J program. (**D**) TAM (5 μg/ml) and non-treated cells infected with TB were stained with Syto59. INF: infected.

Reader, Victor X3). The cytotoxicity assay of TAM (both 24 and 48 h incubation) showed a CC_{50} (50% cytotoxicity) value of 9.375 μg/ml against murine macrophages (Fig. 1A). Thus, the concentration of TAM for use in the intracellular killing assay was determined to be below 6.25 μg/ml. The intracellular killing activity of TAM was assessed according to the technique previously reported [5]. Briefly, TB H37Ra-GFP was constructed by following the methods described by Changsen *et al.* [3]. pFPCA1 plasmid was kindly provided by Dr. Palittapongarnpim, and electroporation and selection of transformants were performed as described previously [3]. GFP-associated fluorescence was determined by a Perkin Elmer 2030 Reader (Victor X3) with excitation at 485 nm and emission at 535 nm. Raw 264.7 cells (1.56×10^6 cells) were infected with H37Ra-GFP in suspension at the MOI of 1:10 in RPMI 1640 supplemented with 10% heat-inactivated FCS for 2 h at 37°C, 5% CO_2. After washing twice by centrifugation, the extracellular TB cells remaining in the suspension with infected macrophages were killed by treatment with amikacin (20 μM) for 1 h. After washing twice, cells (2×10^4 cells) were treated with TAM at predetermined concentrations and incubated at cell culture conditions for 5 days. Finally, image acquisition was performed *via* fluorescence microscopy (Zeiss, Japan). Fig. 1B shows pictures of the TB-infected macrophages treated with TAM at the concentrations of 0, 1.25, 2.5, and 5 μg/ml. The GFP pixel intensity was normalized to macrophage numbers (~ 2,500 cells) and graphed using the Prism program (Fig. 1C). Treatment with TAM decreased the number of intracellular mycobacteria at 5 days after infection compared with the control in a dose-dependent manner (Figs. 1B and 1C). Next, non-treated and TAM (5 μg/ml)-treated macrophages infected with GFP-TB were stained with Syto59 (5 μM; Invitrogen) for 30 min at 37°C

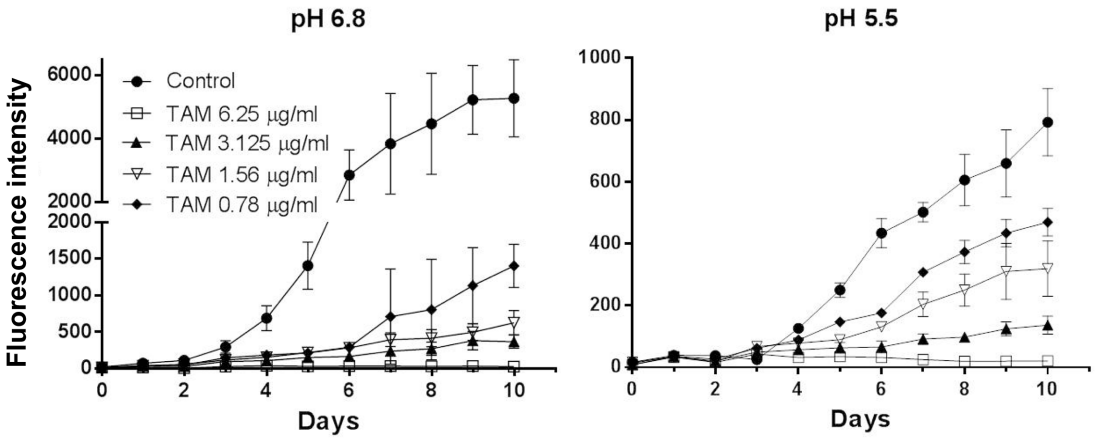

Fig. 2. Effect of pH on anti-TB activity of TAM against GFP-TB H37Ra.
The effect of TAM against GFP-TB H37Ra was tested at pH 6.8 and 5.5 in Middlebrook 7H9 broth. Values represent means ± standard errors of the means of triplicates.

and visualized separately under two different fluorescence channels, green and red, for macrophages and GFP-TB, respectively. The two images were then merged for visualization of the intracellular killing efficacy of TAM against the GFP-TB infected macrophages. Merged images showed only red signal with very weak green signal in the TAM-treated macrophages infected with GFP-TB, whereas many of the GFP-TB colonies overlapped the macrophages in the non-treated sample infected with GFP-TB (Fig. 1D). This indicated that TAM is able to exert its anti-TB effects on intracellular TB after translocation into the macrophages.

Further clarification of the intracellular killing effect of TAM was obtained by testing pH effects on TAM activity against GFP-TB H37Ra. Briefly, 7H9 broth (pH 6.8) was adjusted to pH 5.5 using HCl. TAM was added to 7H9 broth (pH 6.8 and 5.5) at four concentrations (0.78, 1.56, 3.125, and 6.25 μg/ml). Controls were kept without TAM at both pH 6.8 and 5.5. Growth of GFP-TB in the two media in the presence and absence of TAM was monitored by measuring increased fluorescence intensities using a Perkin Elmer 2030 Reader (Victor X3). Fig. 2 shows that TAM could maintain its activity (MIC 3.125–6.25 μg/ml) at pH 5.5 as well as pH 6.8, even though the TB culture at pH 5.5 was not able to proliferate well. Indeed, after inhalation of TB into the lungs, it is engulfed by macrophages into phagosomes [9]. Then, the phagosomes fuse with lysosomes (resulting in pH from 4.5~4.8), which are involved in the digestion and clearance of invading microorganisms [17]. However, TB is able to resist the acidic environment of the arrested phagosome or mature phagolysosome [18]. Therefore, the drug should exert activity at a low pH for being effective in TB-infected macrophages. Although we did not show the activity of TAM against TB at pH 4.5, because TB cells did not grow below pH 5.5, these results suggested that TAM could retain activity in phagosomes or phagolysosomes.

The antimicrobial action mechanism of TAM is not fully understood. However, it was reported that TAM completely inhibited *Bacillus stearothermophilus* cell growth by interacting with lipid and proteins in the membranes of *B. stearothermophilus* [10]. Moreover, a previous study showed that TAM caused significant efflux of K^+ and Na^+ from the bacterial cells [7]. Such efflux caused loss of transmembrane potential and ultimately cell death [7]. Thus, the membrane of the bacillus TB cell might be a potential target of TAM to delineate its inhibitory action. However, further studies on this hypothesis should be carried out to understand the action mechanism of TAM against TB.

In this study, we showed that TAM is worthy of further investigation as a template for the development of novel anti-mycobacterial compounds, since it showed activity against drug-resistant TB strains, although the anti-TB activity of TAM was less than that of INH or RIF against drug-sensitive TB. Thus, we are currently examining the biological activities of TAM-derived compounds for the development of an ideal TAM with stronger activity and less cytotoxicity.

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