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Isoxyl particles for pulmonary delivery: *In vitro* cytotoxicity and potency

Chenchen Wang, Anthony J. Hickey*

Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Division of Molecular Pharmaceutics, Campus Box #7571, 1310 Kerr Hall, Chapel Hill, NC 27599, United States

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ABSTRACT

Pulmonary delivery of isoxyl may increase drug efficacy by targeting alveolar macrophages which are host cells for Mycobacteria. Isoxyl microparticles (1–2 μm) were obtained by antisolvent precipitation and simultaneous spray drying method. The controls were made by mixing isoxyl solution in DMSO with cell culture media. Depending on the drug concentration, either isoxyl solution or nanosuspension was obtained in these controls. In the study, MTT (methylthiazol tetrazolium) and LDH (lactose dehydrogenase) assays were utilized to test cytotoxicity of these particle suspensions or solutions toward macrophages. Isoxyl microparticles and controls in concentrations up to 100 μg/ml were not toxic to macrophages. Both isoxyl microparticle suspensions and controls showed bactericidal activity, as estimated by death of mycobacteria inside the macrophages, at a concentration of 5 μg/ml.

1. Introduction

The successful modern chemotherapy of TB started in 1944 when streptomycin (SM) was administered to the first patient (Bryskier and Grosset, 2005). The subsequent use of the first- and second-line anti-tuberculosis drugs lead to a steep decline in TB incidence. However, the absence of novel effective TB therapy remains a significant public health threat worldwide due to rapid development of multi-drug resistant strains (MDR) and extremely-drug resistance strains (XDR). Development of potent chemotherapeutic alternatives is crucial to preventing future epidemics of this insidious form of the disease. Discovery and marketing of a new drug usually takes billions of dollars and up to 15 years (Malik, 2008). Therefore, screening optimized formulations and employing alternative routes of administration for anti-tuberculosis drugs, abandoned because of their poor pharmaceutical properties, may be a more rapid and effective approach to new therapies.

Isoxyl (ISO, thiocarlide, 4,4′-diisoamythio-carbanilide) was synthesized in 1953 by Buu-Hoi and Xuong (Bartmann, 1988), and began to be used clinically to treat tuberculosis in the 1960s. Two mechanisms of action of isoxyl have been proposed: (1) it inhibits short-chain fatty acid biosynthesis distinct from the mechanisms of isoniazid (INH) and ethionamide (ETH); and (2) similar to INH and ETH, it inhibits the biosynthesis of mycolic acid which is hallmark of mycobacterial cell wall (Phetsuksiri et al., 1999; Schroeder et al., 2002). The unique mechanism of action makes isoxyl an excellent drug candidate. The *in vitro* studies showed that isoxyl is a drug to treat various multi-drug resistant (MDR) strains with MIC: 1–10 μg/ml (Phetsuksiri et al., 1999).

However, isoxyl efficacy has been questioned due to failure of some clinical trials in which isoxyl was administered to patients orally in the 1960s (Leading Article, 1965; Tousek, 1970). Isoxyl is almost completely insoluble in water. This leads to poor dissolution of isoxyl in the gastrointestinal tract and consequently low *in vivo* drug exposure following oral administration. Consequently, oral administration delivers a limited amount of the drug is to the lungs, a major site of *Mycobacterium tuberculosis* (MTB) infection. Moreover, only a small fraction of the local dose in the lungs can penetrate into granulomas and lesions with poor vascular systems. Therefore, it is plausible that oral malabsorption resulted in isoxyl concentration far below the therapeutic range at the main site of infection.

Pulmonary delivery of isoxyl may revive this old drug as a new and effective therapeutic agent most notably through increasing local activity. Isoxyl particles with aerodynamic diameters between 1 and 3 μm can penetrate to the deep lungs which are patrolled by

* Corresponding author at: Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Division of Molecular Pharmaceutics, Campus Box #7571, 1310 Kerr Hall, Chapel Hill, NC 27599, United States. Tel.: +1 919 962 0223; fax: +1 919 966 0197.
E-mail address: ahickey@unc.edu (A.J. Hickey).

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an average of 50–100 macrophages per alveolus (Jones et al., 2002). It has been reported that particles with diameters of 500 nm to 3 μm are efficiently phagocytized when these particles encounter alveolar macrophages (Makino et al., 2004; Ahsan et al., 2002; Tam et al., 2008). Phagocytosis of these particles by macrophages may result in accumulation of drug in phagosomes and, simultaneously, enhance killing of intracellular mycobacteria (Barrow et al., 1998; Anisimova et al., 2000).

Isoxyl has been reported to be non-toxic when it is administered exclusively by the oral route. When the drug was given to rats at an oral dose up to 14 g/kg daily, much higher than therapeutic dose (50–100 mg/kg), it showed no effects on food consumption, reproduction potential, growth, renal and hepatic function, and hematopoietic systems, and it is not teratogenic, embryotoxic, and carcinogenic. The high dose only caused a slight increase in the weight of liver, level of alkaline phosphatases, and transitory increase in the weight of the thyroid gland, but no biological dysfunctions have been detected (Stahle, 1964; Lambelin and Parmentier, 1966).

Due to extremely low oral bioavailability of isoxyl, the previous *in vivo* efficacy and toxicity studies of the drug deserve further investigation using *in vitro* cell models, which allows for higher and more accurate dosing. However, the study of efficacy and cytotoxicity of isoxyl using macrophages is rare. Phetsuksiri et al. reported that *in vitro* efficacy study of isoxyl using murine bone marrow macrophages showed dose-dependent bactericidal activities and killed intracellular bacilli with a 4-log-unit reduction at a concentration of 2.5 μg/ml. The Alamar Blue assay showed that isoxyl was not toxic at up to 2.0 μg/ml in isolated murine bone marrow macrophages (Phetsuksiri et al., 1999). Further investigation is needed to illustrate the cytotoxicity of the drug at higher concentration. After a large dose of isoxyl is directly delivered to the lungs the therapeutic window of the drug delivered as an aerosol may need to be redefined.

The human acute monocytic leukemia cell (THP-1) was selected as a model cell line in the present study, because they can be differentiated by PMA to macrophage-like cells which have been used widely to mimic *in situ* association of *M. tuberculosis* with alveolar macrophages (Fontan et al., 2008; Riendeau and Kornfeld, 2003; Yadav and Misra, 2007). In the present study, macrophages were exposed to particle suspensions at high concentrations and dose and particle size dependent effects on drug efficacy and toxicity were evaluated.

2. Materials and methods

2.1. Materials

Isoxyl was purchased from Cayman Chemical Co. (Ann Arbor, Michigan). Difco™ Middlebrook 7H10 agar and Difco™ Middlebrook 7H9 were from BD. DMSO (Dimethyl Sulfoxide), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and PMA (phorbol myristrate acetate) were from Sigma. Triton X-100 was obtained from Fluka. Formalin (10%, certified buffer), anhydrous isopropanol and the 8-well Lab-Tek™ II Chamber Slide™ Systems were from Fisher Scientific. 10X ADS was made from 5% BSA, 0.2% dextrose and 0.81% NaCl. 7AGT media were prepared by 0.47% Difco™ Middlebrook 7H9, 0.5% Glycerol, 0.05% Tween 80 and 1X ADS.

2.2. Cell culture and bacteria strains

THP-1 cells were purchased from American Type Culture Collection (ATCC) and grown in complete RPMI 1640 media (Cellgro) supplemented with 10% heat inactivated fetal bovine serum (FBS, Atlanta), 10 mM HEPES (cellgro), 1.0 mM sodium pyruvate (Gibco), 0.05 mM 2-mercaptoethanol (Gibco), 1X MEM non-Essential Amino Acids (Gibco). THP-1 cells were seeded on 98-well plate and differentiated into macrophage-like cells by treating with 50 ng/ml of PMA for 48 h, followed by washing with incomplete RPMI media (without FBS) three times to remove PMA. *Mycobacterium bovis* Calmette-Guerin (BCG) was from Organon Teknika Corp., Durham, NC. BCG was grown in 7AGT media until $OD_{600\,nm}$ reached log phase.

2.3. Preparation of isoxyl suspensions and solutions

Isoxyl microparticles were prepared by antisolvent precipitation and simultaneous spray drying. Water (antisolvent) and isoxyl solution in isopropanol (5 mg/ml) were mixed by a 3-fluid nozzle and sprayed into a BUCHI B-191 mini spray-drier (Buchi Labortechnik AG, Flawil, Switzerland) under the following conditions: water feed rate 0.7 ml/min, organic feed rate 0.7 ml/min, nitrogen flow rate 600 l/min, atomization pressure 3 bar, inlet temperature 100 °C and 90% of maximum flow (approximately 35 $m^3\ h^{-1}$). Microparticle size distribution and morphology were determined by a Laser Diffraction (LD, Series 2600C Malvern Instruments, Malvern, UK) and SEM (JSM 6300V scanning electron microscopy, JEOL USA, Peabody, NY, USA), reported elsewhere (Wang and Hickey, 2010). Isoxyl microparticles were accurately weighed and then suspended in complete RPMI media to make a concentration of 100 μg/ml. Concentrations of 0.5, 1, 2.5, 5, 10, 50 μg/ml were prepared by a serial dilution with complete RPMI 1640 media. As controls, isoxyl was dissolved in DMSO at concentrations of 0.05, 0.1, 0.25, 0.5, 1, 5, 10 mg/ml and then diluted by 100-fold with RPMI 1640 media. This resulted in the same mass drug concentrations as those of microparticles and DMSO solution with a final concentration of 1%. The spray-dried isoxyl microparticles suspended in 1% DMSO and isoxyl in 0.5% and 2% DMSO in cell culture media were also made as described above to study the effect of DMSO on drug penetration. Isoxyl precipitate particles sizes, from mixing DMSO with media in the controls, were measured by Dynamic Light Scattering (DLS, Nicomp particle sizing systems, Autodilute^{PAT} Model 370, Santa Barbara, CA).

2.4. Minimum inhibitory concentration (MIC) determination

The agar plate dilution method was used to determine MIC of isoxyl, which was defined as the lowest drug concentration that inhibited colony growth on the agar plates. Agar plates were prepared with Difco™ middlebrook 7H10 containing 1X ADS, 0.5% glycerol and a series of concentrations of isoxyl (0, 0.1, 0.5, 1, 2.5, 5, 10 μg/ml). A BCG suspension (2.2×10^5 cells/ml) was prepared. Three 10-fold dilutions of the suspension (10^{-1}, 10^{-2}, 10^{-3}) of 100 μl were plated on isoxyl-containing and isoxyl-free agar plates. The colony-forming units (CFUs) were counted 4 weeks after incubation of agar plates at 37 °C.

2.5. Cell viability assays

After the THP-1 cells were exposed to isoxyl microparticle suspensions or molecule/nanoparticle controls, the viability of these cells was assessed by their ability to reduce yellow MTT to purple formazan or level of LDH (lactose dehydrogenase) released to the cell culture media. Negative controls consisted of cells without exposure to isoxyl. The cell supernatants were collected and used to measure activities of LDH by using the commercially available kit from Sigma. Fresh media without phenol red and FBS of 100 μl and MTT (5 g/ml) of 10 μl were added to each well and the plates were incubated for 3.5 h. MTT solubilization solution (10% triton X-100, 0.1 N HCl in anhydrous isopropanol) of 100 μl was added to each

well. The plates were then stored overnight in 37 °C incubator to completely dissolve formazan crystals. Absorbance was measured by using Bio-Rad Model 3550 microplate reader (Bio-Rad Laboratories, Hercules, CA) at a wavelength of 570 nm and background absorbance at 690 nm.

2.6. Preliminary efficacy studies

The PMA-differentiated THP-1 cells were cultured in the 8-well Lab-Tek™ II Chamber Slide™ Systems at a concentration of 2×10^5 cells/well. BCG, which reached mid-exponential growth phase, was diluted in complete RPMI media and then added to the cell monolayer at a Multiplicity of Infection (MOI) of 1. After incubation at 37 °C for 4 h, the supernatants were discarded and the cells were washed with RPMI media three times to remove the intercellular bacteria. One slide was used to determine the initial infection at day 0. Three wells of the slide were lysed with buffer (0.05% sodium dodecyl sulfate in PBS). Cell lysates were diluted three times (10^{-1}, 10^{-2}, 10^{-3}) and 111 μl of the dilutions were spread on drug-free M7H10 agar plates. For the rest slides, the BCG-infected THP-1 cells were exposed to a series of concentrations of isoxyl microparticles suspensions or molecule/nanoparticle controls or 1% DMSO (vehicle control) or blank media (untreated control). After 5-days of incubation at 37 °C, three wells of each slide were washed once by RPMI media, lysed and then plated. The colony-forming units (CFUs) of BCG were counted 4 weeks after incubation.

3. Results

3.1. Characterization of isoxyl particles in the suspensions

Microparticles produced by antisolvent precipitation and spray drying were approximately spherical, 1.77 μm in count median diameter and 1.48 in geometric standard deviation (GSD), determined by microscopy (Fig. 1) (Wang and Hickey, 2010). For the controls, nanoparticles with an intensity-weighted mean diameter of 100–200 nm, from DLS, were produced when isoxyl, at concentrations of 5 and 10 mg/ml, was mixed with cell culture media at a ratio of 1:100 (Table 1). This produced isoxyl nanosuspension of final concentrations of 50 and 100 μg/ml. The solubility of isoxyl in cell media containing 1% DMSO was ~2 μg/ml [Unpublished data]. Therefore, for isoxyl in 1% DMSO of 5 and 10 μg/ml, nanoparticles may be produced but the concentrations were too low to be detected by DLS. The forms of isoxyl available in 1% DMSO in cell culture media at various concentrations were listed in Table 2.

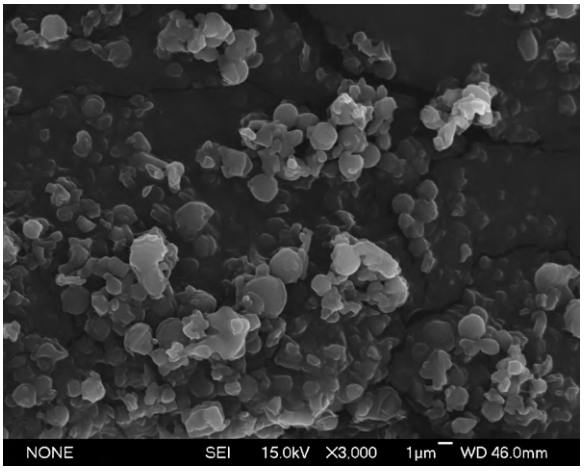

Fig. 1. SEM micrograph of the spray-dried isoxyl microparticles. The micrograph was taken at magnification 3000×.

Table 1
Particle size distributions of the isoxyl nanoparticles.

Concentration (μg/ml)	Mean (nm)	Std. deviation (nm)
100	151.8	70.07
50	147.5	66.49

Table 2
The form of isoxyl available in 1% DMSO at various concentrations.

Conc. (μg/ml)	100	50	10	5	2.5	1	0.5
Dominant form	NP	NP	ND	ND	M	M	M

NP: nanoparticles which were detected by DLS, ND: not determined, M: molecule.

3.2. MIC determination

No visible colony was detected when the isoxyl concentration was ≥2.5 μg/ml. The CFUs (colony-forming units) of the plate containing 1 μg/ml isoxyl in 1% DMSO were comparable to the untreated control containing 1% DMSO (Table 3).

3.3. Cytotoxicity of isoxyl microparticles and nanoparticles

Isoxyl microparticles did not show any cytotoxic effect on the THP-1 cells one (Fig. 2A) or five (Fig. 3A) days following exposure. More than 90% of the cells were still viable when ≤500 μg/ml of isoxyl microparticles was tested. In comparison, the cells exposed to isoxyl molecule/nanoparticle controls at high concentrations (10–100 μg/ml) exhibited larger effects on cell viability, as estimated by MTT assay, 1 day following exposure, which were 50–70% of control (Figs. 2A and 3A). However, the mitochondrial dehydrogenase activity of the THP-1 cells recovered to around 100% of control 2 days later (Fig. 3A). Positive controls lost most of their mitochondrial dehydrogenase activity (1.4–2.5% of control). DMSO is a well-known penetration enhancer and can play a role in the drug solution effects at day 1. Several controls, the spray-dried isoxyl microparticles suspended in 1% DMSO and isoxyl in 0.5–2% DMSO in cell culture media (isoxyl can be in the form of nanoparticles or molecules depending on the drug concentrations in these controls), were included to assess this possibility. Regardless of DMSO concentration it appeared to have no effect from which it was inferred that DMSO has no effect on penetration of the drug into the cells but isoxyl forms, either microparticles or molecule/nanoparticles, were important for uptake (Fig. 2C). DPPC, a lung surfactant, was employed to mimic the *in vivo* environment and to aid in microparticle dispersion. As expected, DPPC at 0.05% did not affect viability of THP-1 cells, but seemed to activate the metabolic state of THP-1 cells as indicated by higher mitochondrial dehydrogenase activities than the controls (around 120%) (Fig. 2A and B).

Levels of LDH leakage were determined as an indication of challenges to the integrity of the cells leading to cytoplasmic enzyme release. All the treatments including molecule/nanoparticle controls did not release significantly greater LDH then the untreated controls for the period of the study (Fig. 3B). However, the positive control resulted in sudden release of LDH (Fig. 3B).

3.4. Preliminary efficacy studies

The bacterial burden expressed in colony-forming units (CFUs) of the untreated control and 1% DMSO treated cells increased 2.6-

Table 3
Colony-forming units (CFUs) on plates inoculated with BCG cells.

Isoxyl conc. (μg/ml)	0	1.0	2.5	5	10
CFUs	127 ± 38	119 ± 33	0	0	0

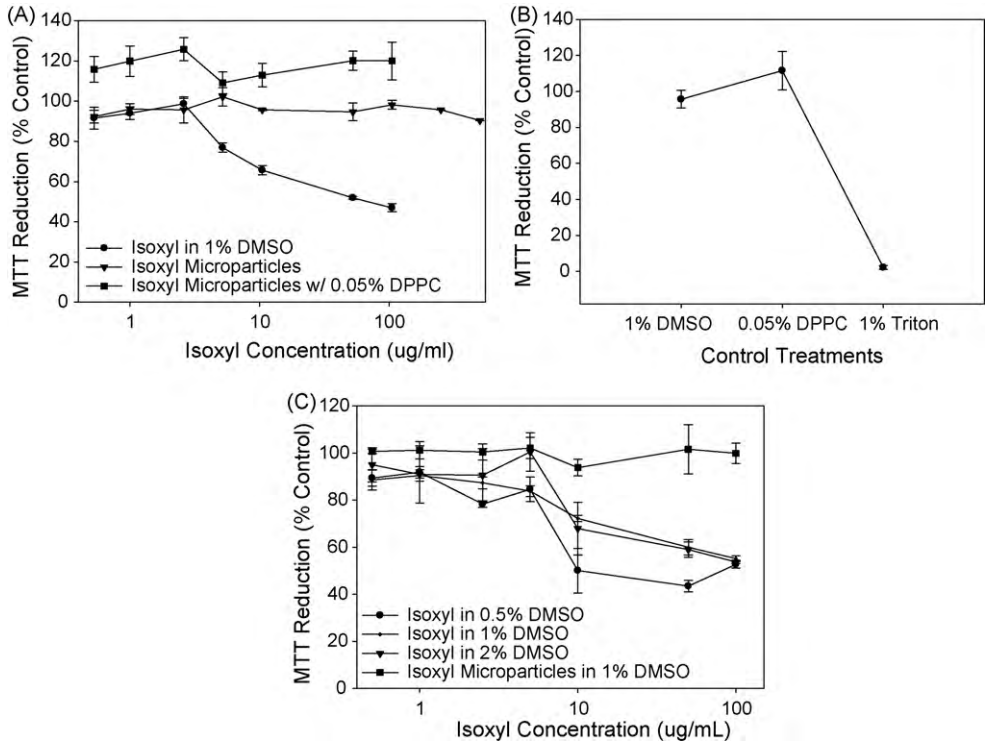

Fig. 2. MTT reductions of THP-1 cells after they were exposed to isoxyl for 24 h. (A) Isoxyl in 1% DMSO, isoxyl microparticles, and isoxyl microparticles with 0.05% DPPC. (B) Controls of 1% DMSO, 0.05% DPPC and 1% Triton. (C) Isoxyl in 0.5%, 1%, 2% DMSO and isoxyl microparticles in 1% DMSO.

and 3.3-fold 5 days after incubation, but those of isoxyl at concentrations of 0.5–2.5 μg/ml in 1% DMSO (molecule/nanoparticle controls) were unchanged. This may be explained by isoxyl being bacteriostatic at concentration between 0.5 and 2.5 μg/ml. When the drug concentration increased to 5 μg/ml, a 3.9-fold reduction in bacterial count was observed in comparison to day 0 and a 10.3-fold reduction in comparison to untreated control (Fig. 4A). For isoxyl microparticles suspended in cell culture media, concentrations ≥5 μg/ml showed bacteriocidal activity. When isoxyl microparticle concentration was equal to 5 μg/ml, bacterial count reduced by 2.8-fold in comparison to day 0 (Fig. 4B).

4. Discussion

The poor solubility of isoxyl limits its use as a therapeutic agent for treatment of MDR strains of tuberculosis following oral route of administration. Isoxyl microparticles, 1–2 μm in median diameter, which may be delivered as aerosols from a dry power inhaler (DPI) directly to the lungs of infected patients, can be prepared by antisolvent precipitation and simultaneous spray drying (Wang and Hickey, 2010). It was expected that direct delivery of these microparticles to the lungs will not only result in high local concentrations but may also target alveolar macrophages, the host cells of TB bacilli. For controls, isoxyl was spiked into DMSO and then diluted with cell culture media due to extremely low aqueous solubility of isoxyl. However, when isoxyl concentrations were ≥50 μg/ml, the drug formed nanoparticles of 100–200 nm in diameter detected by DLS. Nanoparticles <500 nm are expected to be phagocytized by macrophages in a less extent in comparison to microparticles (Oberdoerster, 2005; Muttil et al., 2009; Tsapis et al., 2002). It was reported that phagocytosis of hydrophobic nanoparticles decreases with decreasing size (Chen et al., 1997). Therefore, these controls, either nanoparticles or molecules depending on final drug concentrations in the cell culture media, may dispose

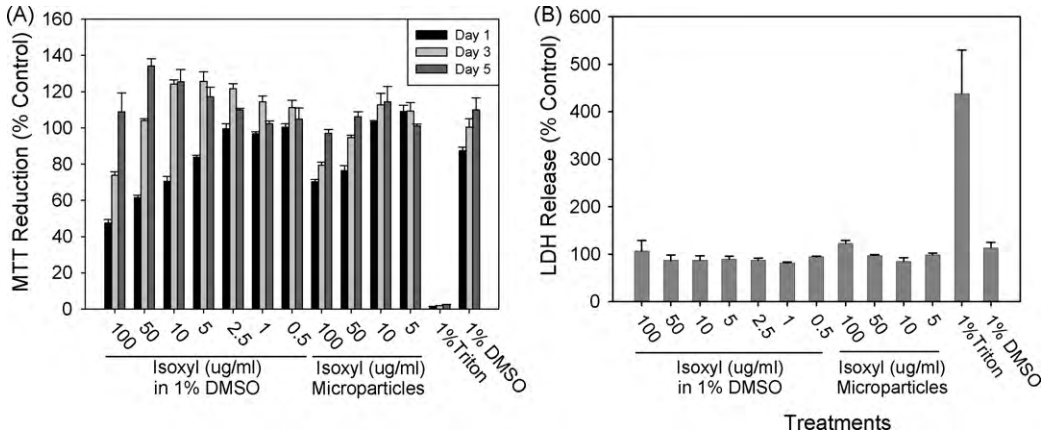

Fig. 3. (A) The effect of isoxyl in 1% DMSO or isoxyl microparticles at a series of concentrations on THP-1 cells determined by (A) MTT assays and (B) LDH assays.

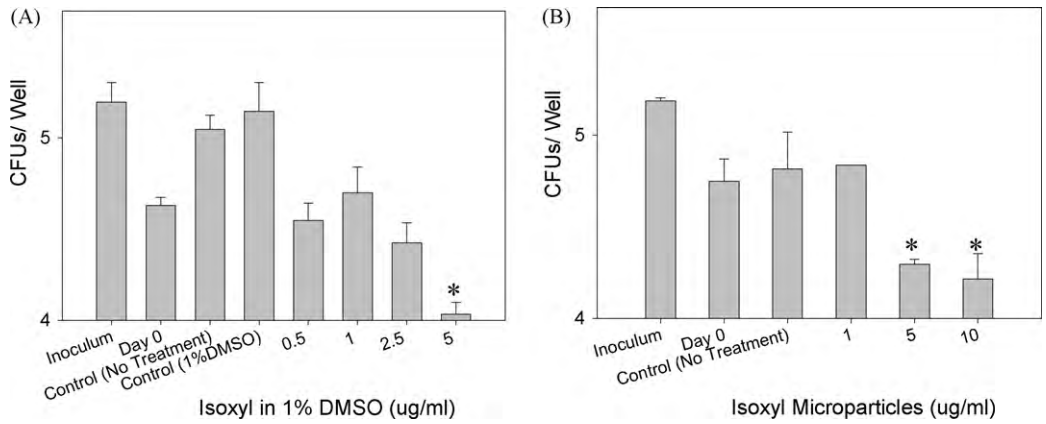

Fig. 4. *In vitro* efficacy of isoxyl in 1% DMSO (A) and isoxyl microparticles (B) determined by CFUs. *Bacterial count of the treatment was statistically different from that of day 0 ($P < 0.05$, Dunnett's *t*-test was performed).

differently in comparison to the microparticles. The present study evaluated these different forms of isoxyl in terms of *in vitro* drug cytotoxicity and efficacy.

Two cytotoxicity studies were performed. MTT assays measured the mitochondrial dehydrogenase activity of viable cells. Interference with the enzyme or cell death decreases the activity of the dehydrogenase as indicated by reduction of methylthiazol tetrazolium (MTT) to colored formazan. This phenomenon was seen after THP-1 cells were exposed to isoxyl molecule/nanoparticle controls which were produced by mixing the drug solution in DMSO with cell culture media for 1 day. However, the enzyme activity recovered after the cells exposed to these controls for an extended period of time. This implied that these isoxyl molecules or nanoparticles interfered with activity of the mitochondrial dehydrogenase, but the effect was tolerated by the viable cells subsequently. Lactose dehydrogenase (LDH) bioassays also showed that isoxyl, in any form, did not kill the cells. LDH is a stable cytoplasmic enzyme. For a certain culture conditions, the LDH release per cell is constant, but lysis of the cells increases LDH leakage. When the cells were treated with isoxyl molecule/nanoparticle controls and microparticles at the concentrations $\leq 100\,\mu g/ml$, LDH level were equivalent to untreated control. In contrast, positive controls had 4-fold increase in LDH leakage in comparison to the untreated controls. The isoxyl in either form did not impair the integrity of the cells as estimated by LDH release. This is further indication that isoxyl microparticles and molecule/nanoparticle controls were not cytotoxic.

The results of the MTT assay suggest that isoxyl interferes with the enzyme to a greater extent when delivered as molecules/nanoparticles at concentrations $\geq 10\,\mu g/ml$ compared to microparticles but it is not clear that this results in loss of viability. Nanoparticles were detected by DLS in the controls at concentrations $\geq 50\,\mu g/ml$. Though the concentration of 10 μg/ml isoxyl in 1% DMSO could not reach the detection limit of DLS, nanoparticles will form because this concentration was much higher than the known solubility of the drug (~2 μg/ml). This suggested that nanoparticles appear to be more effectively phagocytized by macrophages than microparticles, which seems to conflict with the previous reports. However, this is not surprising, because phagocytosis efficiency is associated with not only primary particle size but also other particle characteristics including particle aggregation, shape, charge, crystalline structure, surface and dispersion properties. Isoxyl microparticles are spherical and contain crystal form II (Wang and Hickey, 2010). The shape and polymorphism of the isoxyl nanoparticles precipitated from DMSO requires investigation. It is known that isoxyl can occur in crystal form I if it is precipitated from ethanol (Caira et al., 2005). Also, dispersion of particles in cell culture media can play an important role. Treatment of 50 and 100 μg/ml isoxyl microparticles provided ratio of particles to cells about 6:1 and 12:1 respectively. However, microscopic examination demonstrated that the isoxyl microparticles formed aggregates in the media. These aggregates can constrain uptake to a limited number of cells. Large aggregates may avoid macrophage uptake altogether. However, the nanoparticles were made freshly in cell culture, so they may attach to macrophages immediately as individual particles or form aggregates in microparticle sizes suitable for rapid uptake. Consequently, the cells were exposed to higher number concentration of the isoxyl nanoparticles than the microparticles when the mass doses were the same. Therefore, care should be taken when it comes to a conclusion that primary particle size is a key factor for macrophage phagocytosis before studying particle behavior leading to secondary structures in cell culture media.

Higher intracellular drug levels delivered by the nanoparticles compared to microparticles would be expected to lead to improved efficacy, in treating infected cells. However, this was not the case. The preliminary *in vitro* studies showed that both isoxyl microparticles and isoxyl molecule/nanoparticle controls had bactericidal activity against intracellular BCG at a concentration of 5 μg/ml. These particles will be employed in future to carry out more *in vitro* efficacy studies. Future studies will focus on formulation optimization of microparticles and nanoparticles which will be delivered to tuberculosis infected guinea pigs (Garcia-Contreras et al., 2006).

5. Conclusions

TB remains a threat in human health due to rapid development of resistant strains. Isoxyl was shown to be an effective agent to treat MDR strains *in vitro*, but poor physicochemical properties of the drug resulted in failure of some clinical trials performed four decades ago. Targeting macrophages in the lungs may revive this drug by increasing drug concentration at the main site of action. In the study, the *in vitro* cytotoxicity and efficacy of isoxyl microparticles in comparison to nanoparticle/solution controls were studied. Isoxyl $\geq 2.5\,\mu g/ml$ inhibited growth of BCG completely on agar plates and $\geq 5\,\mu g/ml$ showed bactericidal activity to intracellular BCG. Isoxyl nanoparticles precipitated from mixing DMSO with cell culture media may result in the transport of drug more effectively into the THP-1 phagocytic cells than microparticles as indicated, indirectly, by MTT assays, but this did not produce higher cytotoxicity or efficacy in the infected cell model. Further *in vitro* work is required prior to conducting efficacy studies in animal model of tuberculosis.

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References

Leading Article, 1965. Isoxyl. Tubercle 46, 298–300.

Ahsan, F., Rivas, I.P., Khan, M.A., Torres Suarez, A.I., 2002. Targeting to macrophages: role of physicochemical properties of particulate carriers—liposomes and microspheres—on the phagocytosis by macrophages. J. Control Release 79, 29–40.

Anisimova, Y.V., Gelperina, S.E., Peloquin, C.A., Heifets, L.B., 2000. Nanoparticles as antituberculosis drugs carriers: effect on activity against M. tuberculosis in human monocyte-derived macrophages. J. Nanoparticle Res. 2, 165–171.

Barrow, E.L., Winchester, G.A., Staas, J.K., Quenelle, D.C., Barrow, W.W., 1998. Use of microsphere technology for targeted delivery of rifampin to Mycobacterium tuberculosis-infected macrophages. Antimicrob. Agents Chemother. 42, 2682–2689.

Bartmann, K., 1988. Antituberculosis Drugs. Springer-Verlag, New York.

Bryskier, A., Grosset, J., 2005. Antimicrobial Agents: Antibacterials and Antifungals. ASM Press, Washington, DC.

Caira, M., Crider, M., De Villers, M., Liebenberg, W., 2005. New synthesis and physicochemical properties of two crystal forms of the antitubercular agent isoxyl. AAPS Annu. Meet. Exposition.

Chen, H., Langer, R., Edwards, D.A., 1997. A film tension theory of phagocytosis. J. Colloid Interface Sci. 190, 118–133.

Fontan, P., Aris, V., Ghanny, S., Soteropoulos, P., Smith, I., 2008. Global transcriptional profile of Mycobacterium tuberculosis during THP-1 human macrophage infection. Infect. Immun. 76, 717–725.

Garcia-Contreras, L., Sethuraman, V., Kazantseva, M., Godfrey, V., Hickey, A.J., 2006. Evaluation of dosing regimen of respirable rifampicin biodegradable microspheres in the treatment of tuberculosis in the guinea pig. J. Antimicrob. Chemother. 58, 980–986.

Jones, B.G., Dickinson, P.A., Gumbleton, M., Kellaway, I.W., 2002. Lung surfactant phospholipids inhibit the uptake of respirable microspheres by the alveolar macrophage NR8383. J. Pharm. Pharmacol. 54, 1065–1072.

Lambelin, G., Parmentier, R., 1966. Study of the toxicity of isoxyl. Research on the pathological effects of isoxyl in the rat undergoing chronic experimentation. Arzneimittelforschung 16, 881–886.

Makino, K., Nakajima, T., Shikamura, M., Ito, F., Ando, S., Kochi, C., Inagawa, H., Soma, G., Terada, H., 2004. Efficient intracellular delivery of rifampicin to alveolar macrophages using rifampicin-loaded PLGA microspheres: effects of molecular weight and composition of PLGA on release of rifampicin. Colloids Surf. B Biointerfaces 36, 35–42.

Malik, N.N., 2008. Drug discovery: past, present and future. Drug Discov. Today 13, 909–912.

Muttil, P., Wang, C., Hickey, A.J., 2009. Inhaled drug delivery for tuberculosis therapy. Pharm. Res. 26, 2401–2416.

Oberdoerster, G., 2005. Effects and fate of inhaled ultrafine particles. In: ACS symposium series (nanotechnology and the environment), pp. 117–136.

Phetsuksiri, B., Baulard, A.R., Cooper, A.M., Minnikin, D.E., Douglas, J.D., Besra, G.S., Brennan, P.J., 1999. Antimycobacterial activities of isoxyl and new derivatives through the inhibition of mycolic acid synthesis. Antimicrob. Agents Chemother. 43, 1042–1051.

Riendeau, C.J., Kornfeld, H., 2003. THP-1 cell apoptosis in response to Mycobacterial infection. Infect. Immun. 71, 254–259.

Schroeder, E.K., De Souza, N., Santos, D.S., Blanchard, J.S., Basso, L.A., 2002. Drugs that inhibit mycolic acid biosynthesis in Mycobacterium tuberculosis. Curr. Pharm. Biotechnol. 3, 197–225.

Stahle, I., 1964. Monotherapy with 4-4′diisoamyloxythio-carbanilide (isoxyl). Acta Tuberc. Pneumol. Scand 44, 327–334.

Tam, J.M., Mcconville, J.T., Williams 3rd, R.O., Johnston, K.P., 2008. Amorphous cyclosporin nanodispersions for enhanced pulmonary deposition and dissolution. J. Pharm. Sci. 97, 4915–4933.

Tousek, J., 1970. On the clinical effectiveness of Isoxyl. Antibiot. Chemother. 16, 149–155.

Tsapis, N., Bennett, D., Jackson, B., Weitz, D.A., Edwards, D.A., 2002. Trojan particles: large porous carriers of nanoparticles for drug delivery. Proc. Natl. Acad. Sci. U.S.A. 99, 12001–12005.

Wang, C., Hickey, A.J., 2010. Isoxyl aerosols for tuberculosis treatment: preparation and characterization of particles. PharmSciTech. 11 (2), 538–549.

Yadav, A.B., Misra, A., 2007. Enhancement of apoptosis of THP-1 cells infected with Mycobacterium tuberculosis by inhalable microparticles and relevance to bactericidal activity. Antimicrob. Agents Chemother. 51, 3740–3742.