In vitro efficacy of acetohydroxyacid synthase inhibitors against clinical strains of *Mycobacterium tuberculosis* isolated from a hospital in Beijing, China

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ABSTRACT

الأهداف: تقييم فعالية مثبطات حمض الأسيتوهيدروكسي في مقاومة البكتيريا المسببة للسل في الصين مثل سلالات السل المقاوم للأودية المتعددة، والسل الشديد المقاومة للأدوية.

الطريقة: أُجريت هذه الدراسة في قسم المختبرات السريرية التابع لمستشفى حرية الشعب 309 واستمرت خلال الفترة من يناير 2010م إلى نوفمبر 2010م. لقد قمنا باستخدام طريقة تخفيف الأنبوب وطريقة وسط الأجار ميدل بروك 7H10 من أجل وصف فعالية مثبطات حمض الأسيتوهيدروكسي (سولفوميتورن ميثيل، مونوسولفورون، ومونوسولفورون إيستر) في مقاومة البكتيريا المسببة للسل في الصين مثل سلالات السل المقاوم للأودية المتعددة، والسل الشديد المقاومة للأدوية والتي تم جمعها من نفس المستشفى، وقد شملت 26 عزلة سريرية بالإضافة إلى H37Rv. وقد تم أيضاً تقييم سمية الخلايا لهذه المركبات.

النتائج: أشارت نتائج الدراسة إلى فعالية مثبطات حمض الأسيتوهيدروكسي في مقاومة سلالات البكتيريا المسببة للسل التي تم اختبارها في المختبر وهي: سولفميتورن ميثيل (8-16 ملغ/ل)، ومونوسولفورون إيستر (8-16 ملغ/ل)، ومونوسولفورون (16-64 ملغ/ل). كما أظهرت هذه المثبطات درجة عالية في مقاومة سلالات السل المقاومة للأودية المتعددة، والسل الشديد المقاومة للأدوية بالمقارنة مع مقاومتها للسلالات الحساسة. وقد كانت سمية هذه المركبات للخلايا متدنية، كما كانت مقاومتها للخلايا الظهارية (HBE) أعلى من 300 ملغ/ل.

خاتمة: أظهرت هذه الدراسة أن حمض الأسيتوهيدروكسي يعمل كبروتين يستهدف مقاومة السلالات البكتيرية المسببة للسل في الصين.

Objectives: To assess the efficacy of acetohydroxyacid synthase (AHAS) inhibitors against *Mycobacterium tuberculosis* from China, including multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) strains.

Methods: In this study, the tube dilution method and Middlebrook 7H10 agar media were used to describe the in vitro efficacy of 3 AHAS inhibitors (sulfometuron methyl, monosulfuron, and monosulfuron-ester) against H37Rv and 26 clinical isolates, which include MDR-TB and XDR-TB strains, from the 309th Hospital of Chinese People's Liberation Army (PLA 309), Beijing, China. Cytotoxity of these compounds were then evaluated using the 3-[4,5-dimethylthiazol-2yl]-2,5-dipheny tetrazolium bromide assay with HBE cells. All the experiments were performed from January 2010 to November 2010 in the Department of Clinical Laboratory of the PLA 309 hospital.

Results: Sulfometuron methyl (minimum inhibitory concentration [MIC] range, 8-16 mg/L), monosulfuron-ester (MIC range, 8-16 mg/L), and monosulfuron (MIC range, 16-64 mg/L) showed significant activity against all *Mycobacterium tuberculosis* strains tested in this study in vitro, and they exhibited the same degree of activity against MDR and XDR isolates with that shown against the susceptible strains. All 3 compounds showed little cytotoxicity, with an IC_{50} against HBE cells greater than 300 mg/L.

Conclusion: The results suggest that AHAS could serve as a target protein for the development of novel anti-TB therapeutics in China.

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Multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) are international public health concerns. In 2008, an estimated 390,000-510,000 cases of MDR-TB were reported worldwide. Globally, an estimated 3.6% (95% confidence interval [CI]: 3.0-4.4) of all TB cases were MDR-TB, and nearly 50% of MDR-TB cases were estimated to occur in China and India.[1] Extensively drug-resistant patterns is MDR-plus resistance to at least one of the 3 injectable second-line drugs (amikacin, kanamycin, or capreomycin and a fluoroquinolone), and has been found in up to 60 countries to date, with high rates in India, Russian Federation, South Africa, and some areas of China.[1,2] Multidrug-resistant tuberculosis and XDR-TB are more formidable forms of the disease, for which few treatment strategies have proved effective. Therefore, the discovery and development of new anti-TB drugs are urgently needed to treat MDR-TB and XDR-TB cases, especially in regions with high risk of MDR-TB and XDR-TB, such as China. Acetohydroxyacid synthase (AHAS) catalyzes the conversion of 2 molecules of pyruvate to 2-acetolactate and CO_2, which is a critical step in the biosynthetic pathway of branched-chain amino acids (leucine, isoleucine, and valine) in higher plants, algae, fungi, and bacteria but not in animals. Because of this, AHAS is believed to be a target specific to bacteria and plants, and little mammalian toxicity is expected to occur.[3,4] Some sulfonylurea compounds, such as sulfometuron methyl, which are widely and safely used as herbicides, are effective inhibitors of AHAS.[5] Previous studies have examined the bacteriostatic ability of these sulfonylurea compounds against TB strains, and the results indicated their potent activity both in vitro and in vivo.[6,7] However, data documenting the efficacy of these inhibitors against clinical isolates from China, including MDR-TB and XDR-TB strains, is yet to be reported. Our team has isolated a number of clinical TB strains from the 309th Hospital of Chinese People's Liberation Army (PLA 309), Beijing, China, and determined the drug resistance patterns of these isolates to first-line and second-line anti-TB drugs by performing drug-susceptibility testing (DST).[8] In this work, we tested 3 inhibitors of AHAS (sulfometuron methyl, monosulfuron, and monosulfuron-ester) for their anti-TB activity by using H37Rv and clinical isolates.

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Methods. Twenty-six clinical isolates, which were isolated from the patients of Chinese PLA 309 hospital, including 3 XDR strains, along with H37Rv[8] were included in this study. All the experiments were performed from January 2010 to November 2010 in the Department of Clinical Laboratory of Chinese PLA 309, Beijing, China. All the experiments were carried out according to the principles of Helsinki Declaration. The Ethics Committee of PLA 309 hospital approved the study.

Minimum inhibitory concentration (MIC) determination. All strains were cultured on Lowenstein-Jensen (LJ) media before testing. Sulfometuron methyl was obtained from Chem Service (Part Number: PS1074), and monosulfuron and monosulfuron-ester (**Figure 1**) were kindly provided by Professor ZhengMing Li (National Pesticide Engineering Research Center, Nankai University, China). All compounds were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mg/mL. Minimum inhibitory concentration was defined as the lowest antimicrobial concentration that inhibited more than 99% of bacterial growth, and the MIC was determined by serially diluting each compound into 2-fold on Middlebrook 7H10 agar media supplemented with oleic acid, albumin, dextrose, and catalase (OADC) according to the Clinical Laboratory Standards Institute (CLSI).[9]

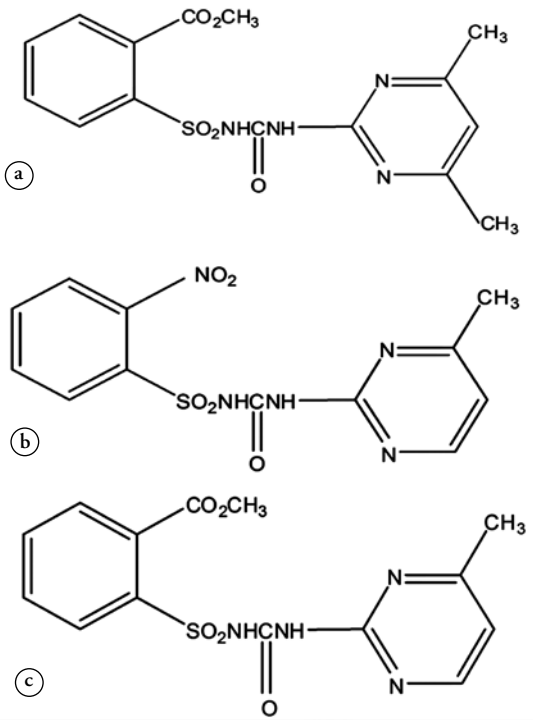

Figure 1 - Chemical structure of the sulfonylurea compounds tested in this study **a)** sulfometuron methyl, **b)** monosulfuron, and **c)** monosulfuron-ester.

An inoculum of 10^4 colony-forming units (CFUs) of each *Mycobacterium tuberculosis (M. tuberculosis)* strain was added to each tube. The tubes were incubated at 36.5ºC; bacterial growth was examined using CFUs counting, and it was recorded 3 weeks later.

Cytotoxicity assay. The 3-[4,5-dimethylthiazol-2yl]-2,5-dipheny tetrazolium bromide (MTT) assay[10,11] which test for cell proliferation and survival was used in this study to assay the cytotoxity of the compounds. Mammalian lung cell HBE were incubated in Dulbecco's modified Eagle medium (DMEM) plus 8% calf serum in 6 cm plate at 37°C, and 5% CO_2 before use. The MTT reduction by cultured cells was performed in 96-well plates containing 100 µL of medium per well. When the cells were subcultured to 50-60% concentrations, compounds were added at different concentrations and the cells were incubated at 37°C, 5% CO_2 and 95% relative humidity. Forty-eight hours later, a total of 50 µL MTT solution was added to each well and incubated at 37°C temperature for 4 hours. After incubation, MTT was aspirated and 150 µL per well of DMSO was added to each well to dissolve the foramazan precipitate. Subsequently, ELISA Reader read the optical densities of the plates at 570 nm.

Statistical analysis was performed using the Chi square test or Fisher's exact test. A p value of less than 0.05 was considered statistically significant.

Table 1 - Resistance phenotype of 26 clinical isolates from the 309th Hospital of Chinese People's Liberation Army, Beijing, China.

Isolates	No. of isolates	Resistance phenotype
H37Rv	1	Susceptible
All-susceptible	11	Susceptible
MDR (n=12)		
	3	RFP, INH
	3	RFP, INH, SM
	4	RFP, INH, EMB
	2	RFP, INH, EMB, SM
XDR (n=3)		
	1	RFP, INH, KN, OLF
	2	RFP, INH, AM, OLF

RFP - rifampin, INH - isoniazid, SM - streptomycin, EMB - ethambutol, OLF - ofloxacin, KN - kanamycin, AM - amikacin, MDR - Multidrug-resistant, XDR - extensively drug-resistant

Results. The drug-resistant phenotype of isolates is shown in **Table 1**. Of the 26 strains chosen for this study, 11 were susceptible to every drug assessed by DST. The number of MDR (but not XDR) and XDR strains were 12 and 3, respectively. Of the MDR-TB isolates, 3 were resistant to only isoniazid (INH) and rifampin (RFP), 7 were resistant to one additional drug, and 2 were resistant to INF, RFP, ethambutol (EMB), and streptomycin (SM). All 3 XDR isolates were resistant to INH, RFP, and ofloxacin (OLF), and one of these was resistant to kanamycin, while the other 2 were resistant to amikacin.

Activity of the 3 sulfonylurea compounds against TB. The activity of the 3 sulfonylurea compounds against all the strains tested is summarized in **Table 2**. The MIC of sulfometuron methyl against H37Rv was 8 mg/L, which is a little higher than that in previous report. The MICs for the clinical isolates ranged from 8-16 mg/L. Monosulfuron-ester, with a MIC range of 8-16 mg/L, was slightly less effective against the clinical isolates than sulfometuron methyl was (p<0.05). Monosulfuron was the least effective of the 3 compounds tested (MIC range, 16-64 mg/L). As expected, these compounds exhibited the same degree of activity against MDR and XDR isolates with that shown against the susceptible strains. As positive controls, MICs of INH and RFP were shown in **Table 2**.

Cytotoxity assay. In order to determine whether sulfometuron methyl, monosulfuron, and monosulfuron-ester have deleterious effects in mammalian cells, we evaluated their cytotoxic effects in mammalian HBE cell line using a MTT kit. All 3 compounds showed

Table 2 - The antimycobacterial activity of 3 sulfonylurea compounds against various kinds of *Mycobacterium tuberculosis* isolates in the 309th Hospital of Chinese People's Liberation Army, Beijing, China.

Isolates	INH MIC (mg/L)	RFP MIC (mg/L)	Sulfometuron methyl MIC (mg/L)			Monosulfuron MIC (mg/L)			Monosulfuronester MIC (mg/L)		
			MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
H37Rv	0.2	0.4	8	-	-	32	-	-	16	-	-
All-susceptible	≤0.2	≤0.4	8-16	8	8	32-64	32	64	8-16	16	16
MDR	≥8	≥8	8-16	8	8	16-64	32	64	8-16	16	16
XDR	≥8	≥8	8	8	8	32-64	64	64	8-16	8	16

RFP - rifampin, INH - isoniazid, MIC_{50} minimum inhibitory concentration for 50% isolates, MIC_{90} - minimum inhibitory concentration for 90% isolates.

little cytotoxicity, with an IC_{50} against HBE cells greater than 300 mg/L (sulfometuron methyl: 337.356mg/L; monosulfuron: 323.408mg/L; monosulfuron-ester: 508.735mg/L) tested 48 hours after compounds were added, which is much higher than the MIC for the *M. tuberculosis* strains.

Discussion. Over the past 20 years, there has been a decreasing trend in the morbidity rate of TB across the world. However, the disease is still considered one of the most fatal infectious diseases in developing countries. It is recommended treatment using no less than 4 drugs known to be effective,[12] but the soaring incidence of drug resistance has caused increasing difficulty in the treatment, leading to higher cost and a longer treatment cycle. China has the second largest number of TB cases in the world, and the number of MDR-TB cases reported in China is second only to the number of cases reported in India.[1] Given that second-line drugs have been widely used in China for many years, it is safe to assume that XDR-TB is also prevalent here. Resistance to anti-TB drugs is a significant problem in China. New anti-TB agents that act via novel mechanisms are urgently needed to present an effective choice for the treatment of TB in China. Until recently, AHAS was not considered an anti-TB target because the infecting organisms may obtain AHAS-related amino acids from their hosts. However, recent investigations indicated an exciting results regarding the targeting of AHAS for the treatment of TB. Some studies evaluated the activity of sulfometuron methyl and some sulfonylurea derivatives against MDR-TB strains in vitro and ex vivo, and concluded that AHAS is an attractive target for the development of novel antibacterial classes with potent anti-TB activity.[7] In this study, we evaluated the antimicrobial activity of 3 AHAS inhibitors against clinical TB strains isolated from PLA 309 hospital in China. The MIC of different isolates varied. Sulfometuron methyl was very effective against most of the strains with a MIC_{90} of 8 mg/L, thereby suggesting that anti-TB agents based on sulfometuron methyl would be effective for the clinical isolates in China, even MDR or XDR strains. Additionally, low-cytotoxicity of all the 3 compounds demonstrated in this study ensures that, if they were to be developed as antibiotics, there would be few adverse effects in humans. The general features of the sulfonylureas are a central sulfonylurea bridge with an o-substituted aromatic ring attached to the sulfur atom and a heterocyclic ring attached to the nitrogen atom. Sohn et al[7] discussed the relationship between the structure of sulfonylureas and anti-TB activity, and it was presumed that the aromatic backbone and the heterocyclic ring tail structure of these compounds determine the differences in anti-TB activity. However, the heterocyclic ring tail was substituted in both meta-positions in all the compounds tested by them. Wang et al[5] showed that some sulfonylurea compounds with only one substituent at the meta-position also exhibited significant AHAS inhibitory activity, but no data was collected for their anti-TB activity.[13] In the present work, monosulfuron and monosulfuron-ester (both with 1 substituent) had observable activity against *M. tuberculosis*; however, they appeared to be weaker than sulfometuron methyl. The data also indicated that the activity of monosulfuron was lower than that of monosulfuron-ester, which implies that the substituent on the aromatic backbone plays an important part in the anti-TB activity of these inhibitors (**Figure 1**). This study was limited in the compound that we evaluated as there were not efficient enough for drug development. The MIC values suggested that even the most potent compound (SM) demonstrated much lower efficacy than INH and RFP. However, our findings showed that these inhibitors are all effective against *M. tuberculosis* isolated in China, even for MDR and XDR cases. It suggested that these inhibitors are promising leading compounds for the development of novel antimycobacterial agents for the treatment of *M. tuberculosis* infections in China.

There are some limitations to this study. For example, this is only a hospital-based survey and the results from this work were not representative of China as a whole and the in vivo efficacy of these agents was not examined.

Further studies should be conducted for structural modification of these compounds to find more efficacy anti-TB agents. Furthermore, evaluation of the in vivo efficacy of these agents for the treatment of MDR- and XDR-TB is needed.

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