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Simultaneous estimation of pharmacokinetic properties in mice of three anti-tubercular ethambutol analogs obtained from combinatorial lead optimization

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Abstract

Integrating combinatorial lead optimization of [1,2]-diamine core structure based on ethambutol with high-throughput screening has led us to focus on three promising analogs (SQ37, SQ59 and SQ109) as potential anti-tubercular drug candidates from thousands of synthesized diamine analogs for further characterization of their biopharmaceutical and pharmacokinetic properties by using liquid chromatography/tandem mass spectrometry (LC/MS/MS) and cassette dosing for pharmacokinetic screening. Simultaneous separation of the three analogs was achieved on reversed phase HPLC using a gradient mobile phase composed of MeOH/CH_3COONH_4 (5 mM)/trifluoroacetic acid: 80/20/0.1 (v/v/v). After extraction with acetonitrile from biomatrices, samples were analyzed on the LC/MS/MS system in the positive mode using an electrospray ion source. The retention time for the analogs ranged from 3.70 to 4.48 min. Incubation of SQ37 with plasma at 37 °C for 6 h resulted in its degradation in human and rat plasma (20–35%), but no significant degradation was observed in mouse and dog plasma. SQ59 was relatively stable in the plasma of the four species. SQ109 was degraded in human and dog plasma (30–40%), but stable in mouse and rat plasma during the 6 h incubation. A rapid multiple pharmacokinetic screening was taken by cassette dosing of the three analogs to mice and simultaneous analysis of their plasma concentrations. The analogs showed large Vd_{ss} ranging from 11,300 (SQ37), 12,800 (SQ109) to 63,900 ml/kg (SQ59). The clearance ranged from 3240 (SQ109), 3530 (SQ37) and 8043 ml/kg/h (SQ59). The elimination $t_{1/2}$ ranged from 4.4 to 21.1 h dependent on the routes. The oral bioavailability was 5.1 (SQ59), 20.1 (SQ37) and 7.8% (SQ109), respectively. Both SQ37 and SQ109 possess good pharmacokinetic properties.
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Keywords: Anti-tubercular drug; Ethambutol; Pharmacokinetic screening; Cassette dosing

1. Introduction

Originally described in 1961, ethambutol is one of the first-line drugs used for treatment of tuberculosis [1]. Nearly all strains of *M. tuberculosis* and *M. Kansasii* as well as a number of strains of *M. avium* complex are sensitive to ethambutol. Ethambutol has no effect on other bacteria. Ethambutol has been used with notable success in the therapy of tuberculosis of various forms when given concurrently with isoniazid. The drug has a lower incidence of toxic effects and better acceptance by patients [2]. Ethambutol suppresses the growth of most isoniazid- and streptomycin-resistant tubercle bacilli. Although resistance to ethambutol develops very slowly in vitro, ethambutol is always given in combination with other tuberculosis drugs because resistance emerges rapidly in vivo among mycobacteria when the drug is used alone. Recent studies suggest that the enzyme target of ethambutol is likely to be an integral membrane arabinosyltransferase [3]. Since its discovery, very little optimization has been undertaken and

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little regard has been given to ethambutol because the biochemical tools for new anti-tubercular drug discovery were not as sophisticated as they are at present [4]. And our current understanding of biology of the *M. tuberculosis* from genomic and proteomic perspectives is much better than ever before [5,6].

To develop "a better ethambutol" by improving its potency and reducing the toxicity and to possibly identify new scaffolds with anti-tubercular activity, a diverse library of 63,238 ethambutol analogs with 1,2-diamine pharmacophore was synthesized and tested against *M. tuberculosis* using high-throughput screening [3]. About 2796 mostly lypophilic compounds were found to be active, and 26 of the compounds had anti-tubercular activity in vitro equal to or better than that of ethambutol. Among them three analogs were identified as the most promising in the series (Fig. 1). They are *N*-(2,2-diphenylethyl)-*N*′-(-)-*cis*-myrtanylethyl-1,2-diamine (SQ37; MW 376.2), *N*-(cyclooctyl)-*N*′-(1*R*, 2*R*, 3*R*, 5*S*)-(-)-isopinocampheylethane-1,2-diamine (SQ59; MW 306.4) and *N*-geranyl-*N*′-(2-adamantyl)ethane-1,2-diamine (SQ109; MW 330.2) with the minimal inhibitory concentration of 1.25, 12.5 and 0.63 μg/ml, respectively.

Without biopharmaceutical and pharmacokinetic information about the three analogs, however, it was impossible to determine which of the three had the potential to be clinically more effective in treating tuberculosis since effects on absorption, distribution, metabolism, and elimination may make highly active molecules appear less active. To facilitate preclinical screening of the three analogs, we developed a highly sensitive and selective LC/MS/MS method enabling the detection and independent quantitation of the three analogs in biomatrices, which usually cannot be chromatographically resolved and quantitated. Using this method we evaluated the biopharmaceutical stability of the three analogs and determined their pharmacokinetic profiles after simultaneous cassette dosing to individual mice, a procedure for higher-throughput pharmacokinetic screening [7]. The results of the studies are reported herein.

2. Materials and methods

2.1. Separation method

Simultaneous separation of each of ethambutol analog from plasma was achieved on a Beta Basic C_{18} analytical column (150 mm × 2 mm, 5 μm) preceded by a Beta Basic C_{18} guard column (4 mm × 2 mm; Keystone Scientific, Bellefonte) at room temperature. Ethambutol analogs and terfenadine (internal standard) were eluted using a mobile phase composed of solvent A (5 mM CH_3COONH_4 with 0.1% trifluoroacetic acid, pH 6.8) and solvent B (MeOH with 0.1% trifluoroacetic acid) according to the following gradient program: 50% buffer A and 50% buffer B were held for 0.5 min, and then the buffer A was linearly decreased to 20% over 3 min and remained constant for 1 min when these analytes were eluted, followed by re-equilibration to initial condition via a step gradient for 3 min. The flow rate was 0.6 ml/min.

2.2. Instrumentation

A PE Sciex API 3000 triple quadrupole mass spectrometer equipped with a Turbo Ion spray source as LC/MS interface was used for analysis of the analogs in biological matrix. Samples were analyzed in both positive and negative ion modes using an electrospray ion source. Ionization temperature was set at 450 °C with hydrocarbon-free air used as both the auxiliary and nebulizer gas, and nitrogen as the collision gas. The mass selective detector was operated in scan mode for the qualitative analyses of the analogs (*m*/*z* from 50 to 500) and in selected ion monitoring mode for quantitative studies (*m*/*z* = 377 for SQ37, *m*/*z* = 307 for SQ59, and *m*/*z* = 331 for SQ109). The mass spectrometer was programmed to transmit the protonated molecules $[M+H]^+$ through the first quadrupole and following collision induced dissociation in Q2. For all three analogues the collision gas used was nitrogen at a pressure of 3.3×10^{-5} Torr achieved with a collisionally activated dissociation gas setting of five from MassChrom (Version 1.1) software.

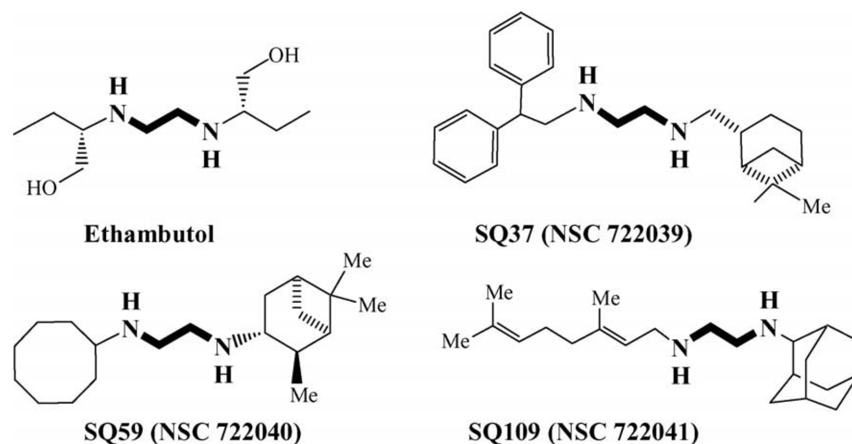

Fig. 1. Chemical structures of ethambutol and its three analogs.

The collision energy was optimized at −32 eV for SQ37, −28 eV for SQ59 and −29 eV for SQ109 with respect to the fragment ion intensity. The appropriate (predominant) product ions selected for monitoring and quantitating in Q3 were $m/z = 224$ for SQ37, $m/z = 154$ for SQ59, $m/z = 178$ for SQ109, and $m/z = 436$ for the internal standard, respectively.

Peak area ratios obtained by multiple reaction monitoring detection of parent to product ion transitions for ethambutol analogs and the internal standard were used for the construction of calibration curves via weighted (reciprocal of concentration) linear least square regression of the compound concentrations and the measured area ratios. Data were acquired using the MassChrom and processed using TurboQuan (Version 1.0) of the PE Sciex software program.

2.3. Sample preparation

Plasma samples for the standard curves were prepared by spiking 200 μl of human, dog, rat and mouse plasma with various concentrations of SQ37, SQ59 and SQ109 ranging from 0.98 to 500 ng/ml and a constant volume (10 μl) of the internal standard terfenadine (10 μg/ml in methanol). To each tested plasma samples (200 μl), 10 μl of the same concentration of terfenadine was added. The plasma was then mixed with 2 ml of acetonitrile. After vortexing and centrifugation at $1000 \times g$ for 5 min, the supernatant was removed and evaporated to dryness at 50 °C with a gentle stream of dry nitrogen. The residue was reconstituted in 200 μl of the mobile phase. The supernatant was transferred to a microcentrifuge tube, centrifuged at $10{,}000 \times g$ for 5 min. The supernatant was transferred to autosampler vials, and only 10 μl of the supernatant was injected into the LC/MS/MS system for analysis. The amount of each analog in the tested plasma samples was back calculated using the standard curves.

2.4. Method validation

The intra-batch precision and accuracy were determined on the same day by analyzing three replicates of spiked samples containing each analog at 3.9, 31.3, and 250 ng/ml. The inter-batch precision and accuracy were also carried out by analyzing three replicates of spiked samples at two concentrations on different days. The precision of the analysis was determined by the relative standard deviation (R.S.D.) or coefficients of variation (%C.V.), and the accuracy of the analysis was determined by comparing the nominal concentrations with the corresponding calculated concentrations. The recovery of each analog from plasma was calculated by comparing concentrations of the analog extracted from plasma with those of the unextracted pairs in solvent samples. The specificity of the analysis was determined by simultaneously monitoring more than one major product ions derived from parent analog ions in blank plasma. The limit of quantitation (LOQ) was defined by the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. For this method the criteria used was a signal to noise ratio of at least 5:1 with a precision of 20% and accuracy of 80–120%.

2.5. Stability in mouse, rat, dog and human plasma

Mouse, rat, dog and human plasma was spiked with all three analogs, respectively, at a concentration of either 1 or 15 μg/ml. Individual plasma mixtures were then incubated at 37 °C. At 1, 2, 3 and 6 h, aliquots of the plasma mixtures were removed, and added to acetonitrile for extracting the analytes for quantitative analysis pertain to plasma stability.

2.6. Cassette dosing and simultaneous pharmacokinetic analysis

A dosing solution containing each of the three analogs was prepared in 0.9% saline. For oral cassette dosing, the concentration and dose of each of the analogs were 1.5 mg/ml and 25 mg/kg, respectively. For intravenous cassette dosing, the concentration and dose of each of the analogs were 0.3 mg/ml and 3 mg/kg, respectively. For intraperitoneal cassette dosing, the concentration and dose of each of the analog were 0.6 mg/ml and 6 mg/kg, respectively. After administration of the dosing solution, four male CD2F1 mice (23–27 g) were anesthetized with isoflurane and blood was collected from the brachial region of each animal at the following time points: 3, 6, 10, 15 and 30 min and 1, 3, 6, 10 and 24 h after a single i.v. dosing; 5, 15 and 30 min and 1, 2, 4, 6, 10 and 24 h after a single p.o. or i.p. dosing. Each blood sample was collected into a tube containing EDTA and centrifuged ($2000 \times g$, 10 min) to separate plasma and red blood cells. To each 200 μl of plasma sample, 10 μl of internal standard solution (10 μg/ml) of terfenadine was added. The analytes were separated and then analyzed simultaneously according to the previously described procedures. The peak area ratio of each individual analog to the internal standard was calculated and the unknown concentration of a given analyte in plasma samples was determined by interpolation from the appropriate standard curve. Pharmacokinetic parameters were calculated using the computer program WinNonlin (Pharsight Co., Mountain View, CA). Bioavailability was calculated as $((AUC_{\text{p.o. or i.p.}}/AUC_{\text{i.v.}}) \times (\text{dose}_{\text{i.v.}}/\text{dose}_{\text{p.o. or i.p.}})) \times 100$.

3. Results

3.1. Separation and specificity

To demonstrate specificity of the method, typical chromatograms of extracts of mouse plasma are shown in Fig. 2, which illustrates the peaks of each analog and the internal standard separately. Each compound was eluted at distinctive retention time of less than 5 min without significant interference from other compounds. The retention time for SQ59,

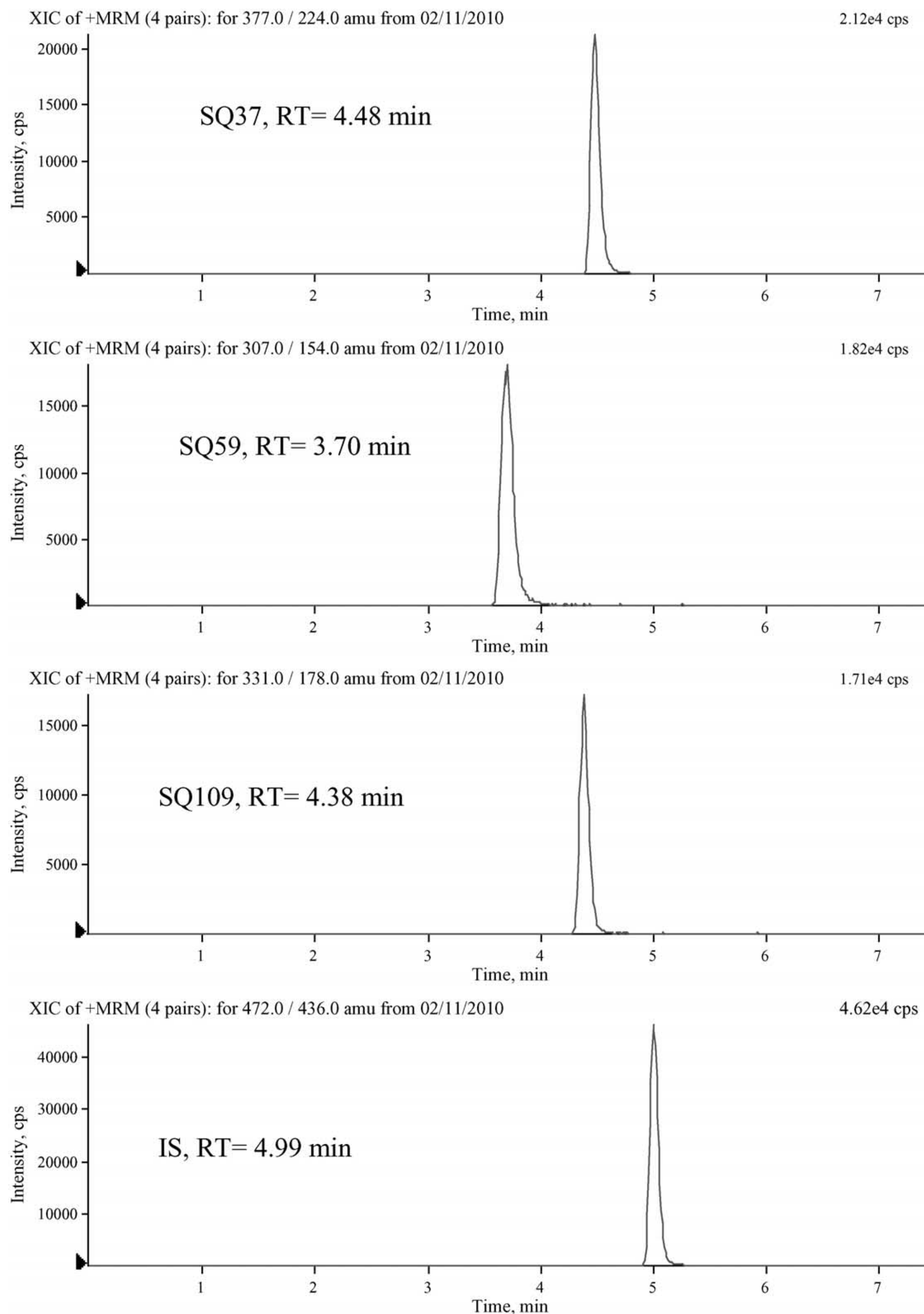

Fig. 2. Selected reaction monitoring chromatograms of an extract of mouse plasma. The representative chromatograms show that the analytes are simultaneously eluted at their corresponding retention time (RT) illustrated from up to low panels: 4.48 min (SQ37), 3.70 min (SQ59), 4.38 min (SQ109) and 4.99 min (internal standard).

SQ37, SQ109 and the internal standard was 3.70, 4.48, 4.38 and 4.99 min, respectively.

3.2. Method validation

The intra-batch precision and accuracy generally fell in the range of 2.06–8.48 and 92.3–111%, respectively, verified by using three concentrations (3.9, 31.3 and 250 ng/ml) of each analyte in three replicates. Inter-batch precision and accuracy, compared at two concentrations on four separate occasions, exhibited 2.1–8.69 and 91.3–114%. The recovery of SQ59 at 3.9, 31.3 and 250 ng/ml from human, dog, rat and mouse plasma ranged from 71.3 to 91.2%. The recovery of SQ37 from human, dog, rat and mouse plasma at the three concentrations ranged from 78.1 to 87.8%. The recovery of SQ109 from human, dog, rat and mouse plasma at the three concentrations ranged from 73.1 to 90.6%. The limit of quantitation of each analog in plasma was determined to be 1.95 ng/ml with correlation coefficients of greater than 0.99.

3.3. Stability in plasma

Fig. 3 shows the stability profile of the three analogs incubated at 37 °C with different species of plasma at 1 μg/ml—the plasma concentration that could be practically achievable after administration of the analogs to mice. High plasma concentration of the analogs (15 μg/ml) exhibited similar kinetics. SQ37 underwent approximately 20% degradation in human plasma and 40% degradation in rat plasma, respectively, after 6 h incubation. No significant degradation of the analog was observed in dog and mouse plasma under the same conditions. SQ59 appeared to be stable in mouse, rat, dog and human plasma for at least 6 h when incubated at 37 °C. SQ109 underwent approximately 30 and 40% degradation in human and dog plasma, respectively, after the 6 h incubation. SQ109 seemed to be stable in mouse and rat plasma at 37 °C (Fig. 3.) All three analogs at concentrations of 1 and 15 μg/ml were very stable at 4 °C for at least 24 h in mouse, rat, dog or human plasma (data not shown).

3.4. Pharmacokinetics of ethambutol analogs

Fig. 4 shows the plasma concentration-time course of the three analogs after p.o., i.p. and i.v. administration to mice. As shown in Fig. 4, each analog was detectable in plasma at 24 h after dosing. For instance, the mouse plasma concentrations of SQ 37, SQ59 and SQ109 were 6.3 ± 4.0, 4.6 ± 1.6 and 5.0 ± 2.3 ng/ml (mean ± S.D.), respectively, at 24 h after oral dosing. The relevant pharmacokinetic parameters are summarized in Table 1. Based on the goodness-of-fit criteria set by the WinNonlin program, the plasma concentration-time data of intravenous SQ59 were best fitted to a three compartmental model, while the plasma concentration-time data of intravenous SQ37 and SQ109 were best fitted to a two compartmental model.

The pharmacokinetics of simultaneously administered SQ37 and SQ109 were very similar following i.v., p.o. and i.p. administration. Independent of the route of administration, the terminal slopes of SQ37 plasma concentration versus time curves declined parallel to the corresponding curves for SQ109. Hence, comparison of the half-lives between the two analogs did not reveal significant differences in pharmacokinetics related to their distribution and elimination.

SQ59 showed poor pharmacokinetics as evidenced by its overall low AUC, C_{max} and bioavailability as well as broader

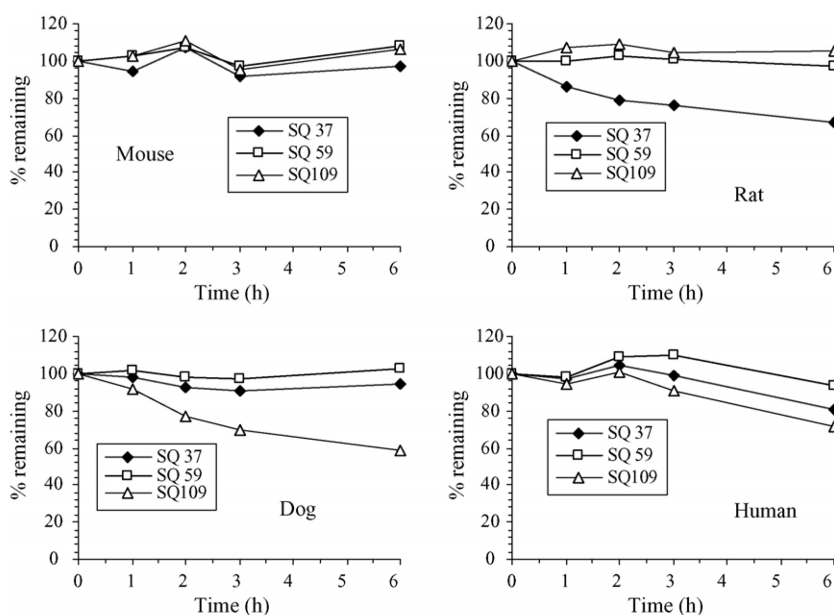

Fig. 3. Stability profile of the three analogs (1 μg/ml) incubated at 37 °C in plasma of different species. The percentage of the analog remaining intact was calculated by integration of chromatographic peak area. Each point represents three tests.

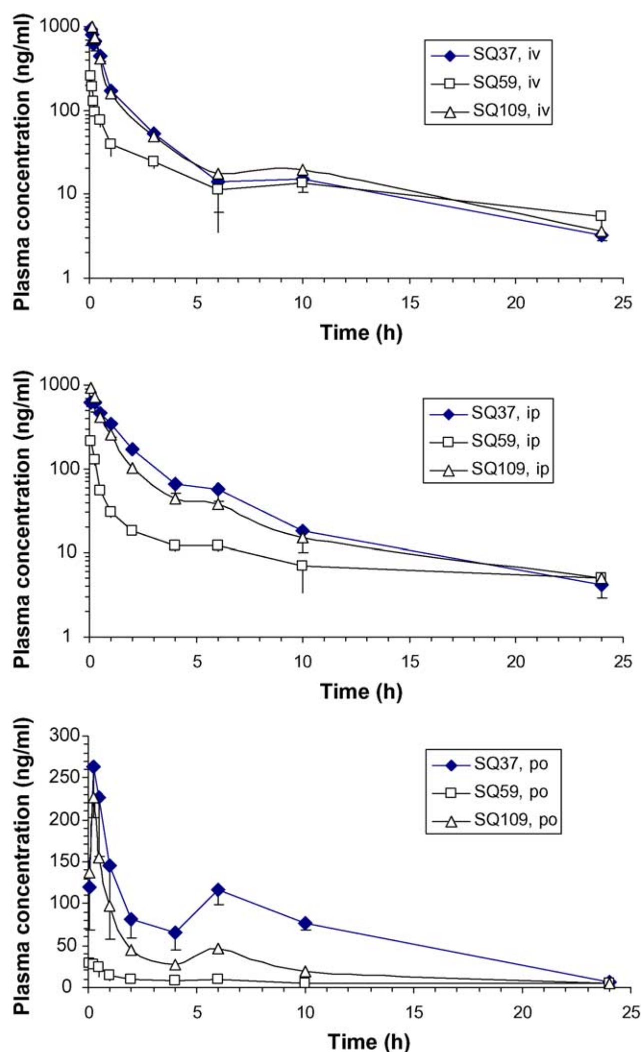

Fig. 4. Plasma concentration-time course of the three analogs after cassette dosing through different routes to mice: i.v., 3 mg/kg; i.p., 6 mg/kg; p.o., 25 mg/kg. Each point represents the mean ± S.D. of four to five mice.

Vd_{ss} in comparison to pharmacokinetics of SQ37 and SQ109 (Table 1). Therefore, it would not be chosen for further development based on the result from the cassette dosing pharmacokinetic screening.

The three analogs showed the Vd_{ss} significantly larger than other compounds that we had studied [8–11]. The large Vd_{ss} may be attributed to the large hydrophobic moieties and the diamine that the three analogs commonly possess. These structures are found to be necessary for the isoprenoid binding site of the arabinosyltransferase target of ethambutol [3].

4. Discussion

The plasma stability study is one of the most important secondary screening assays in drug development, largely because it determines whether or not the in vivo clearance is due to enzymatic degradation and eliminates unstable candidate drugs from further development. Since the three analogs are relatively stable when incubated with plasma, concern is greatly reduced that the primary metabolism may occur in the circulation.

Of greater interest found in the present study is that the three analogs possess a large Vd_{ss}. Vd_{ss} corresponds to the equivalent plasma volume in which a drug is distributed into the body. In fact, Vd_{ss} would equal the plasma volume in addition to the sum of each tissue: plasma partition coefficient multiplied by its respective tissue volume. Vd_{ss} under in vivo conditions is commonly determined by the product of clearance and mean residence time after a single intravenous dose of the drug [12]. Vd_{ss} is mainly governed by two determinants: drug lipophilicity (partitioning into lipids and water) and plasma protein binding (e.g., a reversible binding to common proteins such as albumin, globulins and lipoproteins present in plasma and interstitial space). For most drugs muscle and fat may empirically be two main determinants of Vd_{ss} [13]. The magnitude of Vd_{ss} is a useful indicator for the amount of drug outside the central compartment or in the peripheral tissues and organs. The larger the Vd_{ss}, the greater the amount of the drug resides in the extravascular compartment. Therefore, these analogs may have favorable tissue kinetics, i.e., rapid penetration into the extravascular compartment, and particularly, lung tissue. Indeed, we have observed that the lung concentration of SQ109 after i.v. administration to mice was at least 180-fold higher than in plasma over 8 h observation [14].

Table 1
Comparison of pharmacokinetic parameters of three ethambutol analogs after cassette dosing to mice

	Route								
	Intravenous			Intraperitoneal			Oral		
Dose (mg/kg)	3			6			25		
Analogs	SQ59	SQ37	SQ109	SQ59	SQ37	SQ109	SQ59	SQ37	SQ109
$AUC_{0-24\,h}$ (ng h/ml)	384	954	1006	272	1372	1099	169	1602	655
C_{max} (ng/ml)	343	1079	1190	217	630	935	29	263	227
T_{max} (min)				5	5	5	5	15	15
$T_{1/2el}$ (h)	9.2	7.7	6.1	9.7	4.9	4.4	21.1	5.7	7.0
CL (ml/kg/h)[a]	8043	3530	3240						
Vd_{ss} (l/kg)	63.9	11.3	12.8						
Bioavailability (%)				35	72	55	5.3	20.1	7.8

[a] Total body clearance.

It is indisputable that cassette dosing pharmacokinetics and the use of LC/MS/MS have greatly enhanced our ability to profile many drug candidates simultaneously in a single sample [15,16]. Cassette dosing is generally used to screen drug candidate for systemic clearance (i.v. dosing) and for oral bioavailability (p.o. dosing). Compared with conventional pharmacokinetic studies, cassette dosing pharmacokinetics has the advantage of speed, because the slow steps of animal dosing, blood collection, and sample analysis are minimized by using this method. Another advantage is that animal usage is greatly reduced, which is particularly important when dog or monkey is the test species [17]. Since there are concerns over the potential for the occurrence of drug–drug interactions to compromise the results, we conducted a separate conventional single-compound dosing (SQ109 only) complementary to the above described cassette pharmacokinetic studies, and demonstrated that the pharmacokinetic parameters of SQ109 obtained from the conventional single-compound dosing were similar to those from the present cassette dosing [14]. Small size of cassette dosing has not significantly affected entire pharmacokinetic profiling of each individual analog in comparison with the conventional single dosing of SQ109 although sporadic changes in pharmacokinetic parameters were observed. The cassette dosing pharmacokinetic screening has enabled us to make early and critical development decisions based on the pharmacokinetic criteria although other physicochemical and efficacious properties of the drug candidates are also considered important in the decision-making. The trade-off for using this screening is that we will spend less time and resources on the drug candidates with poor pharmacokinetic profiles.

Optimization of ethambutol analogs to enhance efficacy of the parent compound seems to be an intellectual strategy to improve tubercular treatment and make the therapeutic implementation easier. In in vitro experiment, the minimal inhibitory concentrations of SQ37, SQ59 and SQ109 required to produce 90% growth inhibition of the *M. tuberculosis* H37Rv were found 1.25, 12.5 and 0.63 μg/ml, respectively. SQ109 was the most potent compound in this series of analogs. The peak plasma concentrations and peak lung concentrations of SQ109 after i.v. and p.o. administration were above the MIC level for the susceptible *M. tuberculosis* microbes [14], providing a sufficient therapeutic margin and a comfortable safety margin during anti-tubercular therapy, assuming that C_{max}/MIC is the indicative therapeutic parameter. The SQ109 concentrations in the respiratory tract remained above the MIC for more than 10 h after oral dosing. Hence, an oral dosing regimen seems to provide an excellent safety margin for the treatment of respiratory tract infections caused by the tubercular bacteria.

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