The New Antitubercular Drugs SQ109 and TMC207 Act Synergistically In Vitro to Kill *M. tuberculosis*

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BACKGROUND

A number of initiatives to discover and develop new therapeutic agents that not only cure TB but also shorten the duration of treatment are ongoing (5, 13). SQ109 and TMC207 are among new drugs being developed for therapy of TB, and both are currently in human clinical trials. SQ109 is a N-Geranyl-N'-(2-adamantyl) ethane-1,2-diamine discovered as best-in-class of a 63,000 combinatorial chemistry library of diamine antimicrobial agents (8,10). It is most active against *M. tuberculosis* (MTB) complex bacteria, and is as active on multi-drug resistant (MDR) MTB as on drug sensitive strains. SQ109 inhibits cell wall biosynthesis, although the exact site of action is not yet known (7). TMC207 (also known as R207910) is a derivative of the diarylquinoline class of antimicrobial drugs. TMC207 is highly specific for *Mycobacteria*, and is equally effective against susceptible and MDR MTB (1). Both SQ109 and TMC207 are bactericidal. In combination drug studies with existing front-line antituberculars, SQ109 acts synergistically with isoniazid (INH) and rifampin (RIF) (3), whereas TMC207 is synergistic with pyrazinamide (PZA) (6). In this study we investigated, *in vitro* and in macrophages, the efficacy of combinations of TMC207 and SQ109 against MTB.

PURPOSE AND HYPOTHESIS

To determine the *in vitro* interaction of SQ109 and TM207, with and without RIF, for killing of MTB.

MATERIALS AND METHODS

Antimicrobial agents: TMC207 was obtained from Tibotec Pharmaceuticals, LTD. cGMP SQ109 was synthesized and purified on contract for ongoing human clinical trials by the National Cancer Institute, NIH, and Bethesda, MD.
Mycobacteria: *M. tuberculosis* (MTB)-H37Rv, MTB-H37Rv luciferase reporter strain (pSMT1), drug-sensitive clinical isolates of MTB (8330 and 8642) and *M. smegmatis* (MSMG-MC^{2}155). Mycobacteria were grown in 7H9 broth supplemented with 10% albumin-dextrose-catalase (ADC) and 0.05% Tween 80.
Determination of Minimal Inhibitory Concentration (MIC): MIC of SQ109, TMC207, and RIF was determined by microdilution and BACTEC radiometric methods (4,11,14).
Determination of synergistic activity: Synergistic activity was determined by checkerboard titration by microdilution assay (11). Fractional inhibitory concentrations (FIC) were calculated to determine synergistic activity using the formula: FIC = MIC in combination/MIC alone, and fractional inhibitory index (ΣFIC)= $FIC_A + FIC_B$ [ΣFIC = ≤0.5 indicates synergy, ≥4.0 antagonistic, values in between were additive] (4, 11).
Rate of killing: The viable number of MTB, initial and at different times of incubation thereafter, was determined by three methods: counting the Relative Light Units (RLU) in a luminometer, Growth Index (GI) in BACTEC TB-460 reader and Colony-Forming Units (CFU) on 7H11 agar plates. Rate of killing was determined by estimating time that the drug or drug-combination killed ≥90% of the initial inoculum.

Post antibiotic effect (PAE): PAE was determined as described (2). PAE was calculated from the formula PAE = T- C, where T is the time required for the drug-exposed organisms to reach a cumulative GI of 100 and C is the time required for the controls to reach a cumulative GI of 100.

Determination of drug intracellular activity: Intracellular activity of compounds was determined using J774A.1 mouse macrophage (MΦ) cell line. MΦ were infected with Psmt1 strain of MTB an MOI of 10:1. MTB-infected MΦ were incubated with drugs for 1 or 4 days and then culture fluid was replaced with drug-free DMEM and incubated up to 7 days. At day 0 and at day 7, MΦ in triplicate wells were lysed by adding 1 ml of 0.1 % Triton X-100 in distilled water and the RLU of lysates were determined as described (12).

CONCLUSIONS

1. In these *in vitro* studies of two- and three-drug combinations using SQ109, TMC207 and RIF, the combination SQ109+TMC207 was synergistic against the drug susceptible laboratory strain of MTB (H37Rv) or additive against the two clinical isolates of MTB. On the other hand, the combination TMC207+RIF combination was additive against both. Addition of RIF to the SQ109+TMC207 combination did not further improve efficacy of the two drugs.

2. TMC207+SQ109 combination killed MTB faster as determined by both RLU and GI estimation, and both methods were more sensitive than CFU determination. The apparent increased rate of killing by TMC207 in bioluminescence assay could be due to depletion of ATP by the drug. Since RLU depends on the amount of ATP within the bacteria, minor changes in the ATP levels in the bacteria reflect the RLU levels in the assay.

3. Increased rate of killing by the SQ109+TMC207 combination was also evident in BACTEC, which is the gold standard for susceptibility testing in MTB.

4. SQ109 combined with TMC207 enhanced the PAE of TMC207.

5. In macrophages, TMC207 was more effective at killing MTB than SQ109 or RIF. In combination, SQ109+TMC207 was superior to either TMC207+RIF or SQ109+RIF. It will be of interest to determine whether SQ109+TMC207 combination shows improved activity *in vivo*.

RESULTS

MIC against MTB (H37Rv):
By microdilution, the MIC of SQ109, TMC207 and RIF against H37Rv were 0.5, 0.25 and 0.0078 µg/ml, respectively. By BACTEC method the MIC of these drugs against H37Rv were 0.25, 0.062 and 0.062 µg/ml, respectively. The MIC of SQ109 and TMC207 were one or two dilutions lower by BACTEC compared to microdilution method; the reverse was true for RIF.
***In vitro* synergy between SQ109, TMC207, and RIF:**
Two drug combinations: Combination of SQ109 with TMC207 was synergistic with H37Rv with ΣFIC of 0.375 in one experiment and 0.5 in the second. Against two clinical isolates of MTB, SQ109+TMC207 combination was additive displaying better activity against MTB8330 than MTB8642 with ΣFIC 0.625 and 2.0, respectively. SQ109+TMC207 combination displayed additive effect against MSMG (ΣFIC=1.0). SQ109+RIF combination, on the other hand, was synergistic against MTB (H37Rv) as well as MSMG with ΣFIC of 0.094 and 0.132, respectively. In contrast TMC207+RIF combination had additive effects against H37Rv as well as two other clinical isolates of MTB with ΣFIC of 2.0 against all the isolates. None of the combinations was antagonistic (**Table 1**).
Three drug combinations tested for interactions on MTB-H37Rv: The two drugs (TMC207 and SQ109) were titrated in checkerboard titration in microtiter plates containing different concentrations of RIF: 0.5, 0.2, 0.1 and 0.05 x MIC. This study was designed to determine the lowest concentration (sub-MIC) of RIF that interacts with TMC207 and SQ109 to induce the greatest synergistic activity. **Table 2** shows three drug combination data from one of the two experiments. RIF at 0.5 and 0.2 MIC caused 2 fold decrease in MIC of SQ109 (Plate 1 & 2), but had no effect at 0.1 and 0.05 MIC (Plates 3 & 4). RIF did not contribute to already existing synergy between SQ109 and TMC207 in combination (Plate 5).
Rate of killing of MTB: Rate of killing is the time required to kill ≥90% of initial organisms in the original culture at a given concentration of drug. By RLU, the drugs individually at 2x MIC ranked SQ109>RIF>>TMC207, but the drugs at 4x and 8x MIC ranked as follows: TMC207>>SQ109>RIF (Table 3). In drug combinations, SQ109+TMC207 was more effective, followed by TMC207+RIF and SQ109+RIF. Combinations of RIF with either of the drugs failed to enhance the rate of killing significantly over the individual drugs.

By BACTEC method (**Table 4**), the order of efficacy (≥90% kill) of drug combinations at 2x MIC was SQ109+RIF>>SQ109+TMC207>TMC207+RIF, and at higher concentrations, SQ109+RIF=SQ109+TMC207>TMC207+RIF.

The order of efficacy for reducing CFU count to ≤10^6 CFU (≥90% kill) for individual drugs was SQ109>RIF>TMC207 (**Table 5**); the order of drug combinations varied depending upon the day at which data was collected. At 4 days, the order was SQ109+TMC207=TMC207+RIF> SQ109+RIF; by 15 days, however, the SQ109-containing regimens were clearly better, and the order was SQ109+TMC207=SQ109+RIF>TMC207+RIF. In fact, however, the rate of kill of all the combinations was quite similar.

PAE of the drugs alone and in combination: The mean cumulative GI of drug free control and each drug (exposed for 2 hr) was plotted against time (**Figure 1**). Two hr exposure to SQ109 did not induce any PAE; PAE was 9 hr following 2 hr exposure to TMC207. INH, the positive control in this experiment, had a PAE of over 15 hr, which has been reported elsewhere (11). The PAE of the SQ109+TMC207 combination was 13.6 hr, in the same range as INH.

Determination of intracellular killing activity of the drug, alone or in combination.
When infected MΦ were exposed to drugs 4 days, TMC207 was clearly the best at elimination of intracellular bacteria, even at its MIC. When MΦ were exposed to a single day of drugs, all three drugs, SQ109, TMC207, and RIF showed dose responses, with the order of activity TMC207>SQ109>>RIF (**Table 6**). Drug combinations had better activity than single drugs, SQ109+TMC207 was by far the best, and TMC207+RIF had the same activity as TMC207 by itself. Again, the combination of SQ109+RIF was substantially better than either drug alone (**Table 7**). When tested in combination at and below their MIC (**Table 8**) the order of activity was SQ109-1+TMC207-1 > TMC207-1 > SQ109-0.5+TMC207-0.5 > SQ109-0.25+TMC207-0.25.

Table 1. Assessment of synergistic interactions (*) between SQ109 and TMC209 or RIF

Organism	Drug combination	MIC (µg/ml) alone	MIC (µg/ml) combination	ΣFIC
MTB (H37Rv)	SQ109	0.5	0.125	0.375*
	TMC207	0.25	0.031	
	SQ109	0.5	0.125	0.5*
	TMC207	0.25	0.062	
	TMC207	0.5	0.5	2.0
	RIF	0.0078	0.0078	
	SQ109	0.5	0.031	0.094*
	RIF	0.015	0.00048	
MTB8330	SQ109	0.5	0.25	0.625
	TMC207	0.062	0.0078	
	TMC207	0.062	0.062	2
	RIF	0.0019	0.0019	
MTB8642	SQ109	0.125	0.125	2
	TMC207	0.062	0.062	
	TMC207	0.062	0.062	2
	RIF	0.0009	0.0009	
MSMG	SQ109	2.0	1.0	1.0
	TMC207	0.031	0.0156	
	SQ109	4.0	0.5	0.132*
	RIF	8.0	0.062	

Figure 1. PAE of drugs and drug combination against MTB H37Rv

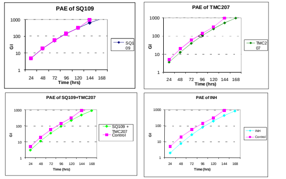

Table 2. Interactions between SQ109, TMC207 and RIF in 3-drug combinations

Plate 1 — RIF 0.5 MIC (0.0039 µg/ml)

SQ109 (µg/ml) \ TMC (µg/ml)	0	0.0019	0.0039	0.0078	0.0156	0.031	0.062	0.125	0.25	0.5
0	+	+	+	+	+	+	+	+	-	-
0.007	+	+	+	+	+	+	+	+	-	-
0.015	+	+	+	+	+	+	+	+	-	-
0.031	+	+	+	+	+	+	+	+	-	-
0.062	+	+	+	+	+	+	+	+	-	-
0.125	+	+	+	+	+	+	+	+	-	-
0.25	-	-	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	-	-	-	-	-

Plate 2 — RIF 0.2 MIC (0.00156 µg/ml)

SQ109 (µg/ml) \ TMC (µg/ml)	0	0.0019	0.0039	0.0078	0.0156	0.031	0.062	0.125	0.25	0.5
0	+	+	+	+	+	+	+	+	-	-
0.007	+	+	+	+	+	+	+	+	-	-
0.015	+	+	+	+	+	+	+	+	-	-
0.031	+	+	+	+	+	+	+	+	-	-
0.062	+	+	+	+	+	+	+	+	-	-
0.125	+	+	+	+	+	+	+	+	-	-
0.25	-	-	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	-	-	-	-	-

Plate 3 — RIF 0.1 MIC (0.00078 µg/ml)

SQ109 (µg/ml) \ TMC (µg/ml)	0	0.0019	0.0039	0.0078	0.0156	0.031	0.062	0.125	0.25	0.5
0	+	+	+	+	+	+	+	+	-	-
0.007	+	+	+	+	+	+	+	+	-	-
0.015	+	+	+	+	+	+	+	+	-	-
0.031	+	+	+	+	+	+	+	+	-	-
0.062	+	+	+	+	+	+	+	+	-	-
0.125	+	+	+	+	+	+	+	+	-	-
0.25	+	+	+	+	+	+	-	-	-	-
0.5	-	-	-	-	-	-	-	-	-	-

Plate 4 — RIF 0.05 MIC (0.00039 µg/ml)

SQ109 (µg/ml) \ TMC (µg/ml)	0	0.0019	0.0039	0.0078	0.0156	0.031	0.062	0.125	0.25	0.5
0	+	+	+	+	+	+	+	+	-	-
0.007	+	+	+	+	+	+	+	+	-	-
0.015	+	+	+	+	+	+	+	+	-	-
0.031	+	+	+	+	+	+	+	+	-	-
0.062	+	+	+	+	+	+	+	+	-	-
0.125	+	+	+	+	+	+	+	+	-	-
0.25	+	+	+	+	+	+	-	-	-	-
0.5	-	-	-	-	-	-	-	-	-	-

Plate 5 — No RIF (two drug control)

SQ109 (µg/ml) \ TMC (µg/ml)	0	0.0019	0.0039	0.0078	0.0156	0.031	0.062	0.125	0.25	0.5
0	+	+	+	+	+	+	+	+	-	-
0.007	+	+	+	+	+	+	+	+	-	-
0.015	+	+	+	+	+	+	+	+	-	-
0.031	+	+	+	+	+	+	+	+	-	-
0.062	+	+	+	+	+	+	+	+	-	-
0.125	+	+	+	+	+	+	+	+	-	-
0.25	+	+	+	+	+	+	-	-	-	-
0.5	-	-	-	-	-	-	-	-	-	-

Table 3. Rate of killing of MTB-pSMT1 by SQ109, TMC207, or RIF, alone and combination (Exp 1)

Drug-MIC	Conc. µg/ml	Mean RLU at day 0	1	2	4	7	12	16
SQ109-2	0.5	1337	1557	1384	339	18	9	186
SQ109-4	1	1337	1416	1124	197	11	5	1
SQ109-8	2	1337	1078	759	161	7	3	1
TMC207-2	0.25	1337	141	210	391	319	416	1935
TMC207-4	0.5	1337	46	33	54	52	40	24
TMC207-8	1	1337	23	4	3	3	3	1
RIF-2	0.062	1337	1780	1620	875	197	25	4
RIF-4	0.125	1337	1766	1589	778	182	25	3
RIF-8	0.25	1337	1855	1391	706	139	16	2
SQ109-2 + TMC207-2	0.5 + 0.25	1337	11	3	8	12	3	3
SQ109-4 + TMC207-4	1.0 + 0.5	1337	12	1	1	3	1	3
SQ109-8 + TMC207-8	2 + 1	1337	7	5	11	38	8	2
TMC207-2 + RIF	0.25 +0.062	1337	161	133	85	19	2	1
TMC207-4 + RIF-4	0.5 + 0.125	1337	78	33	28	9	1	0
TMC207-8 + RIF-8	1 + 0.25	1337	53	10	5	1	1	1
SQ109-2 + RIF-2	0.5 + 0.062	1337	1766	1462	664	149	15	2
SQ109-4 + RIF-4	1.0 + 0.125	1337	1728	1316	558	107	12	2
SQ109-8 + RIF-8	2 + 0.25	1337	1605	1098	293	43	3	2
Control	0	1337	4038	8632	53146	140089	36588	96767

Table 4. Rate of killing of MTB by SQ109, TMC207, and RIF, alone and combination: BACTEC method.

Dugs – MIC	Conc. µg/ml	Mean Growth index at day 1	2	3	4	5	6	7	8
SQ109-2	0.5	218	262	256	237	216	194	193	171
SQ109-4	1	167	205	205	202	201	166	209	195
SQ109-8	2	140	170	174	180	182	178	188	182
TMC207-2	0.125	185	286	262	212	162	122	100	74
TMC207-4	0.25	160	223	202	166	124	86	65	48
TMC207-8	0.5	138	151	140	118	88	66	52	38
RIF-2	0.125	266	270	224	179	149	128	108	94
RIF-4	0.25	250	224	168	122	94	78	66	54
RIF-8	0.5	174	148	105	80	58	44	32	26
SQ109-2 + TMC207-2	0.5 + 0.125	141	136	116	104	82	70	65	56
SQ109-4 + TMC207-4	1 + 0.25	116	68	36	26	20	16	14	12
SQ109-8 + TMC207-8	2 + 0.5	108	62	32	23	16	13	10	8
TMC207-2 + RIF-2	0.125 + 0.125	200	230	190	161	130	102	85	68
TMC207-4 + RIF-4	0.25 + 0.25	154	153	112	82	62	42	32	24
TMC207-8 + RIF-8	0.5 + 0.5	142	120	78	51	35	24	17	12
SQ109-2 + RIF-2	0.5 + 0.125	117	102	74	56	42	23	23	17
SQ109-4 + RIF-4	1 + 0.25	98	83	60	41	30	19	14	10
SQ109-8 + RIF-8	2 + 0.5	84	70	46	26	18	10	6	3
Control	0	559	977	999	999	999	999	999	999

Table 5. Rate of killing of MTB Determined by CFU Counts (log_{10} CFU).

Group	Conc. µg/ml	CFU at day 0	4	8	15
SQ109-1	0.5	7.02	6.68	8.81	9.55
SQ109-2	1	7.02	5.07	2.70	3.67
SQ109-4	2	7.02	3.90	2.60	1.00
TMC207-1	0.25	7.02	7.09	7.76	9.21
TMC207-2	0.5	7.02	6.77	6.71	7.71
TMC207-4	1	7.02	6.43	6.00	5.98
RIF-1	0.062	7.02	6.04	5.15	3.68
RIF-2	0.125	7.02	5.79	5.08	3.43
RIF-4	0.25	7.02	5.74	5.08	3.20
SQ109-1 + TMC207-1	0.5 + 0.25	7.02	5.98	5.49	4.68
SQ109-2 + TMC207-2	1.0 + 0.5	7.02	5.71	4.76	3.35
SQ109-4 + TMC207-4	2.0 + 1.0	7.02	5.83	4.10	1.90
SQ109-1 + RIF-1	0.5 + 0.062	7.02	6.74	5.10	3.41
SQ109-2 + RIF-2	1.0 + 0.125	7.02	5.54	5.02	3.08
SQ109-4 + RIF-4	2.0 + 0.25	7.02	5.48	4.12	1.95
TMC207-1 + RIF-1	0.25 + 0.062	7.02	5.95	4.82	3.06
TMC207-2 + RIF-2	0.5 + 0.125	7.02	5.85	4.37	2.54
TMC207-4 + RIF-4	1.0 + 0.25	7.02	5.70	4.46	2.30
Control	**0**	**7.02**	**8.63**	**10.58**	**11.07**

Table 6. Intracellular activities of anti-TB drugs against MTB in J774 MΦ

4 day drug exposure

Drug	Conc. µg/ml	Mean RLU at day 0	7
SQ109-1	0.5	249	184
SQ109-2	1	249	153
SQ109-4	2	249	177
TMC207-1	0.25	249	18
TMC207-2	0.5	249	5
TMC207-4	1	249	3
RIF-1	0.062	249	3019
RIF-2	0.125	249	1812
RIF-4	0.25	249	103
Control	0	249	1524

1 day drug exposure

Drug	Conc. µg/ml	Mean RLU at day 0	7
SQ109-1	0.5	85	48
SQ109-2	1	85	11
SQ109-4	2	85	5
TMC207-1	0.25	85	18
TMC207-2	0.5	85	5
TMC207-4	1	85	1
RIF-1	0.062	85	259
RIF-2	0.125	85	111
RIF-4	0.25	85	34
Control	0	85	1148

Table 7. Intracellular activities of drug alone and combination.

Drug – x MIC	Conc. µg/ml	Mean RLU at day 0	7
SQ109-1	0.5	271	100
TMC207-1	0.25	271	14
RIF-1	0.062	271	171
SQ109 + TMC207	0.5 + 0.25	271	6
TMC207 + RIF	0.25 + 0.062	271	15
SQ109 + RIF	0.5 + 0.062	271	29
Control	0	271	3084

Table 8. Intracellular activities of drug alone and combination.

Drug – x MIC	Conc. µg/ml	Mean RLU at day 0	7
SQ109-1	0.25	113	732
SQ109 -0.5	0.125	113	1275
SQ109 -0.25	0.0625	113	1160
TMC207 -1	0.125	113	70
TMC207 -0.5	0.062	113	351
TMC207 -0.25	0.031	113	954
SQ109 -1 + TMC207 -1	0.25 + 0.125	113	48
SQ109 -0.5 + TMC207 -0.5	0.125 + 0.062	113	212
SQ109 0.25 + TMC207 0.25	0.062 + 0.031	113	420
Control	0	113	1334

BIBLIOGRAPHY

1. Andries, K., P. Verhasselt, et al. (2005). "A diarylquinoline drug active on the ATP synthase of Mycobacterium tuberculosis." Science **307**(5707): 223-7.
2. Chan, C. Y., C. Au-Yeang, et al. (2001). "Postantibiotic effects of antituberculosis agents alone and in combination." Antimicrob Agents Chemother **45**(12): 3631-4.
3. Chen, P., J. Gearhart, et al. (2006). "Synergistic interactions of SQ109, a new ethylene diamine, with front-line antitubercular drugs in vitro." J Antimicrob Chemother **58**(2): 332-7.
4. Heifets, L. B., Ed. (1991). Drug susceptibility in the chemotherapy of mycobacterial infections, CRC Press.
5. Hudson, A., Imamura, T., Gutteridge, W., Kanyok, T., Nunn, P. (2003). The current anti-TB drug research and development pipeline, WHO: 1-44.
6. Ibrahim, M., K. Andries, et al. (2007). "Synergistic activity of R207910 combined with pyrazinamide against murine tuberculosis." Antimicrob Agents Chemother **51**(3): 1011-5.
7. Jia, L., L. Coward, et al. (2005). "Pharmacoproteomic effects of isoniazid, ethambutol, and N-geranyl-N'-(2-adamantyl)ethane-1,2-diamine (SQ109) on Mycobacterium tuberculosis H37Rv." J Pharmacol Exp Ther **315**(2): 905-11.
8. Lee, R. E., M. Protopopova, et al. (2003). "Combinatorial lead optimization of [1,2]-diamines based on ethambutol as potential antituberculosis preclinical candidates." J Comb Chem **5**(2): 172-87.
9. Nikonenko, B. V., M. Protopopova, et al. (2007). "Drug therapy of experimental tuberculosis (TB): improved outcome by combining SQ109, a new diamine antibiotic, with existing TB drugs." Antimicrob Agents Chemother **51**(4): 1563-
10. Protopopova, M., C. Hanrahan, et al. (2005). "Identification of a new antitubercular drug candidate, SQ109, from a combinatorial library of 1,2-ethylenediamines." J Antimicrob Chemother **56**(5): 968-74.
11. Reddy, V. M., L. Einck, et al. (2008). "In vitro antimycobacterial activities of capuramycin analogues." Antimicrob Agents Chemother **52**(2): 719-21.
12. Snewin, V. A., M. P. Gares, et al. (1999). "Assessment of immunity to mycobacterial infection with luciferase reporter constructs." Infect Immun **67**(9): 4586-93.
13. Spigelman, M. K. (2007). "New tuberculosis therapeutics: A growing pipeline." J Infect Dis **196**: S28.
14. Wallace, R. J., Jr., D. R. Nash, et al. (1986). "Susceptibility testing of slowly growing mycobacteria by a microdilution MIC method with 7H9 broth." J Clin Microbiol **24**(6): 976-81.