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In Vitro Activity of 3-Triazeneindoles against *Mycobacterium tuberculosis* and *Mycobacterium avium*

Boris V. Nikonenko,[a,d] Albert Kornienko,[b] Konstantin Majorov,[a] Pavel Ivanov,[b] Tatiana Kondratieva,[a] Maria Korotetskaya,[a] Alexander S. Apt,[a,d] Elena Salina,[c] Valeriya Velezheva[b,d]

Central Institute for Tuberculosis, Moscow, Russia[a]; A. N. Nesmeyanov Institute of Organoelement Compounds of the Russian Academy of Sciences, Moscow, Russia[b]; A. N. Bach Institute of Biochemistry of the Russian Academy of Sciences, Moscow, Russia[c]; TB-Stopper, Skolkovo, Moscow, Russia[d]

Among 230 target-synthesized indole-based compounds, seven 3-triazenoindoles showed MICs of 0.2 to 0.5 μg/ml against *Mycobacterium tuberculosis* strain H37Rv and isoniazid-resistant human isolate CN-40. The TU112 compound was active also against a dormant form of *M. tuberculosis*. Some of these triazenoindoles were active against *Mycobacterium avium*, with MICs of 0.05 to 0.5 μg/ml. The selectivity indices (SI) for *M. tuberculosis* and *M. avium* were significantly higher than 10, making these compounds acceptable for the next testing step.

A series of indole-based compounds possessing antimycobacterial activity were reported during last decade (1–3), including those from our panel of *Mycobacterium*-targeting *N-N*-containing indoles synthesized according the procedure described previously (4). Here, we present a few indole agents containing three lined-up nitrogen atoms representing a series of 3-triazenoindoles and demonstrating the most prominent antimycobacterial capacities. Synthesis of the target 3-triazenoindoles was performed using 2-ethoxycarbonyl-3*H*-diazoindole and appropriate secondary amines (or malononitrile for the compound TU113) by the method reported earlier (5). The characteristics of the compounds are displayed in Table 1.

All reagents and solvents were purchased from commercial sources and were used without further purification. The yields refer to purified products and are not optimized. Melting points were uncorrected. Infrared (IR) spectra were run as KBr disks on an IR Fourier Magna-IR 750 Nicolet spectrometer. ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were performed on a Bruker Avance-300 and Bruker Avance-400 (300 and 400 MHz, respectively), using tetramethylsilane (TMS) as an integral standard and hexadeuterodimethyl sulfoxide (DMSO-d6) and CDC1 as solvents. Splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (br); chemical shifts are expressed as δ ppm, and the coupling constant (*J*) is given in hertz (Hz). Elemental analysis was performed at the laboratory of microanalysis of the A. N. Nesmeyanov Institute of Organoelement Compounds, Moscow, Russia. Mass spectra were recorded on Finnigan PolarisQ mass spectrometer.

Mycobacterium tuberculosis strain H37Rv, a clinical isolate of isoniazid (INH)-resistant *M. tuberculosis* CN-40, and *Mycobacterium avium* strain 724R were obtained from the Department of Immunology of Central Institute for Tuberculosis (Moscow, Russia). The origin, storage conditions, properties, and preparation of bacterial cultures were described earlier (6–8). Initially, the compounds were tested for their capacity to inhibit [^{3}H]uracil incorporation (as preliminary screen) into *M. tuberculosis* H37Rv and CN-40 (4, 6), followed by MIC testing of the most active ones. MICs were determined by a standard microdilution assay using microtubes with Dubos medium containing 0.05% Tween 80. The lowest concentration of a compound resulting in no visible growth of *M. tuberculosis* for 2 weeks was considered the MIC (4, 9, 10). All samples were tested twice in triplicate. In addition, the samples were plated on Dubos agar for CFU counting and determination of the MIC_{99}. We also used a micro method developed in our laboratory based on measurement under a microscope of the volume of growing compact mycobacterial culture in the wells of round-bottom 96-well plates in the presence or absence of a tested compound (K. Majorov, unpublished data). The MICs for *M. avium* were determined in an identical manner, except with a 1-week incubation instead of two.

To assess whether or not the compounds under study were toxic for mammalian cells, the level of macrophage lysis was determined by measuring the enzymatic activity of lactate dehydrogenase (LDH) in culture supernatants using the CytoTox 96 kit (Promega). Specific lysis was calculated according to the formula (A_{490} from experimental well − A_{490} spontaneous release)/(A_{490} maximal release − A_{490} spontaneous release) × 100 (6). The selectivity index (SI) then was calculated by dividing the 50% inhibitory concentration (IC_{50}) by the MIC; an SI of >10 was considered sufficient for a compound's further evaluation (11). The *in vitro* model of *M. tuberculosis* dormancy and resuscitation was described previously (12).

All compounds displayed in Table 2, except TU113, demonstrated a high level of *in vitro* activity against *M. tuberculosis* H37Rv (MIC, 0.25 to 0.5 μg/ml, comparable to that of INH) and against INH-resistant strain CN-40 (MIC, 0.2 to 0.5 μg/ml, comparable to that of rifampin [RIF]). These compounds demonstrated also a high level of activity against the virulent *M. avium* strain 724R, comparable to that of clarithromycin (Table 2). The SIs for compounds TU111, TU112, TU114, and TU152 were

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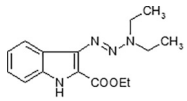
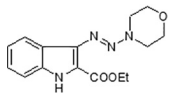
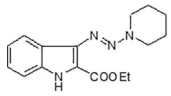
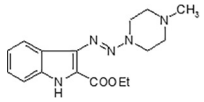
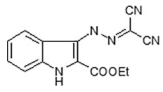
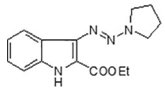
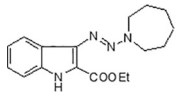
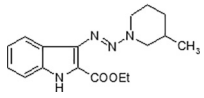
Address correspondence to Boris V. Nikonenko, b.nikonenko@mail.ru.

TABLE 1 Characteristics of 3-triazenoindoles and hydrazone TU113

Sample	Structure	Empirical formula	Molecular mass (kDa)	Melting point (°C)
TU90		$C_{15}H_{20}N_4O_2$	288.35	154–156
TU91		$C_{15}H_{18}N_4O_3$	302.34	142–143
TU111		$C_{16}H_{20}N_4O_2$	300.36	162–164
TU112		$C_{16}H_{21}N_5O_2$	315.38	143–144
TU113		$C_{14}H_{11}N_5O_2$	281.28	204–206
TU114		$C_{15}H_{18}N_4O_2$	286.34	173–174
TU152		$C_{17}H_{22}N_4O_2$	314.39	172–174
TU167		$C_{17}H_{22}N_4O_2$	314.39	169–174

much higher than 10 regarding all three mycobacterial strains, indicating the necessity of their further testing.

In addition, compounds TU111 and TU112 were tested with respect to their activity against nonculturable dormant cells of *M. tuberculosis* H37Rv. Since the compound TU111 demonstrated no activity (data not shown), a more detailed assessment was performed with the compound TU112. Remarkably, whereas RIF and INH were ineffective against metabolically inactive mycobacteria,

TABLE 2 Antimycobacterial testing results of 3-triazenoindoles

Compound[a]	Activity (MIC_{99} [μg/ml]) against[b]:			IC_{50} (μg/ml)[c]	SI for[d]:		
	M. tuberculosis H37Rv	INH^r CN-40 strain	*M. avium*		*M. tuberculosis* H37Rv	INH^r CN-40	*M. avium*
TU90	0.46	0.46	NT	18	39	39	NT
TU91	0.36	0.45	NT	10	28	22	NT
TU111	0.25	0.5	0.1	100	400	200	2,000
TU112	0.25	0.2	0.05	18	72	90	360
TU113	>1	2–10	1	7	<7	<3.5–0.7	7
TU114	0.3	0.1–1	0.25	50	166	50–500	200
TU152	0.1–0.5	≤0.5	<0.1	50	100–500	100	500
TU167	<0.5	<0.5	2	10	>20	>20	5
INH	0.05	20	NT	NT	NT	NT	NT
RIF	0.1	0.1	NT	NT	NT	NT	NT
CLA	NT	NT	0.2	NT	NT	NT	NT

[a] INH, isoniazid; RIF, rifampin; CLA, clarithromycin.
[b] INH^r, INH resistant; NT, not tested.
[c] IC_{50}, concentration killing 50% of host macrophages.
[d] SI, selectivity index.

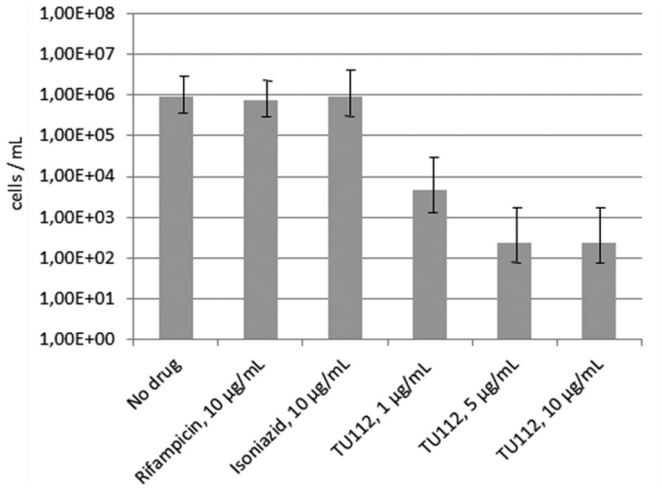


FIG 1 Activity of the compound TU112 against dormant nonculturable *M. tuberculosis*. Mycobacteria were incubated in the presence of different concentrations of the compound TU112 for 7 days at 37°C. Viability of mycobacteria was tested by measuring the numbers of cells recovered from a nonculturable state by most probable number (MPN) assay as described earlier (12). The difference between TU112 groups and the untreated control was significant, with a *P* value of <0.01. Error bars indicate standard deviation.

TU112 killed these cells even at the 10-fold-lower concentration (Fig. 1). The privileged indole core of the compounds tested is directly connected to the line assembly of three nitrogen atoms as a side chain substituent. The compound TU113 with two nitrogen atoms at the side chain and 2-ethoxycarbonylindole without any substituent (data not shown) proved to be, respectively, poorly active and inactive against all three mycobacterial strains. Therefore, the substituents present in the above-mentioned species are relevant for antituberculosis activity and differ in their absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties. The study of these properties is now in progress. Regarding the chemical structure of the compounds tested, the presence of three lined-up nitrogen atoms seems to be a key requirement for the antimycobacterial activity (Table 2). Six compounds presented herein should be considered very promising drug candidates for further testing in other models, including *in vivo* testing. The compound TU167 possesses significant activity against H37Rv and CN-40 strains but is toxic, so we do not consider it a promising candidate. Significant activity of the triazenoindole TU112 with *N*-methyl piperazine substituent against *M. tuberculosis* H37Rv, including dormant cells, and against CN-40 suggests that this substituent controls physicochemical properties within an optimal range (neither too lipophilic, nor too hydrophilic).

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