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A Novel Molecule with Notable Activity against Multi-Drug Resistant Tuberculosis

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Abstract

Multi-drug resistant tuberculosis (MDR-TB) is emerging as a serious global health problem, which has been elevated through co-infection involving HIV and MDR-*Mtb*. The discovery of new compounds with anti-MDR TB efficacy and favorable metabolism profiles is an important scientific challenge. Using computational biology and ligand docking data, we have conceived a multifunctional molecule, **2**, as a potential anti-MDR TB agent. This compound was produced through a multi-step synthesis. It exhibited significant *in vitro* activity against MDR-TB (MIC 1.56 µg/mL) and its half-life ($t_{1/2}$) in human liver microsomes was 14.4 h. The metabolic profiles of compound **2** with respect to human cytochrome P450 (CYP) and uridine 5'-diphospho-glucuronosyltransferase (UGT) isozymes were favorable. Compound **2** also had relatively low *in vitro* cytotoxicity in uninfected macrophages. It displayed synergistic behavior against MDR-TB in combination with PA-824. Interestingly, compound **2** also displayed *in vitro* anti-HIV activity.

Keywords

Drug discovery; Anti-MDR TB; Synergism; CYP; UGT profiles

Almost a third of the world's population (2 billion people) are infected with the gram-positive bacterium, *Mycobacterium tuberculosis* (*Mtb*), the etiological agent of the disease known as tuberculosis (TB). Of those people with latent infection, some 5–10% may become sick or may be infectious during their lifetimes. People with HIV are much more likely to develop TB. The World Health Organization (WHO) suggest that about 2 million people die from TB each year.^{1–3} Also, multidrug-resistant tuberculosis, MDR-TB, is spreading.^{4–8} However, the existing pipeline for approved and investigational drugs against MDR-TB is slim.^{9–12} Co-infection of HIV and MDR-TB is a potentially fatal liaison.^{13–15} Thus, the discovery of new drugs with efficacy against MDR-TB remains a critical scientific challenge.

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Our search for a HIV-1 integrase inhibitor^{16–19} that would possess significant anti-HIV efficacy and a favorable profile with respect to resistance and drug-drug interactions, led us to structure-activity studies on inhibitors of the strand transfer step of HIV-1 integrase.²⁰ A novel anti-HIV active integrase inhibitor **1** (Fig. 1) emerged following a more focused advancement of these studies.²¹ Compound **1** displayed significant *in vitro* activity against a broad set of HIV-1 subtypes, as well as against HIV-2 and SIV, and with very low cell cytotoxicity.²¹ In addition, this compound exhibited a positive drug susceptibility profile against some key clinically-relevant integrase mutations. The *in vivo* pharmacokinetic and toxicokinetic data were also compelling.²²

In examining the nature of the binding of compound **1** to the active site of HIV-1 integrase intasome (pdb code 3OYA), including key interactions with the active site magnesium ions and aspartic acid residues, and comparing this to the active site composition of the MDR-TB DNA-dependent RNA polymerase, the possibility for a novel application occurred to us. Would the retention of key functional groups in compound **1** and the introduction of other appropriate functional fragments suggested from available crystallographic data and computational biology on MDR-TB DNA-dependent RNA polymerase lead to the design of new compounds that would exhibit anti-TB activity? This strategy,²³ was aided by ligand functional/conformational sampling docking scores [Surflex dock in Sybyl-X 1.3 (winnt. os5x, created April, 2011)]. The docking scores led to the design of compounds with an appropriately substituted cyclic amide moiety attached to the enolic-keto functionality, a pyridinone scaffold adorned with appropriately substituted lipophilic benzyl groups and a piperazine ring of the cyclic amide in which the second nitrogen is substituted with a heteroaromatic ring (Fig. 2).

Of the series of compounds synthesized with the structural features indicated in Figure 2, the most interesting compound overall in terms of anti-MDR TB activity and *in vitro* metabolic and drug interaction profile, was 1-(4-(4-(1H-pyrrol-1-yl)phenyl)piperazin-1-yl)-4-(5-(3,5-difluorobenzyl)-1-(2-fluorobenzyl)-2-oxo-1,2-dihydropyridin-3-yl)-2-hydroxybut-2-ene-1,4-dione **2** (Fig. 3). The focus of this communication will be on compound **2**, which has the following structural properties: a high cLogP (6.3) for cell wall and cell membrane entry; a suitable 3D polar surface area (84 Å²); appropriate numbers of hydrogen bond accepting and donating groups (7 and 1, respectively) for interaction with amino acid residues in the ligand binding site; a pKa of 9, which is in the range of some key anti-MDR TB compounds; a high number of rotatable bonds (10); the presence of an enolic-keto functional group capable of binding to Mg²⁺; and a heterocyclic scaffold discovered earlier by us to be critical for anti-retroviral activity.^{21,22}

Compound **2** was synthesized from its immediate precursor **8** (Scheme 1), by treatment of **8** with 1-hydroxy-benzotriazole (HOBt) in DMF followed by addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI.HCl) at 0°C. The mixture was stirred at 0°C for 20 minutes, treated with the amine **9**, stirred for 2 h at 0–5°C and then quenched with water. The resulting yellow solid was filtered and purified by column chromatography on silica gel to afford compound **2**. Synthesis of the precursor **8** from commercially available 5-bromo-2-methoxypyridine (**3**) involved the following steps: *in situ* generated aromatic nucleophile addition to aldehyde; demethylation/deoxygenation; radical

bromination; benzylation; palladium-catalyzed cross-coupling; Claisen condensation; and acid or base-catalyzed hydrolysis.²⁴ Yields for the individual steps are indicated in brackets in Scheme 1. Compound **2** was produced in >99.8% purity.²⁵

The MIC (minimum inhibitory concentration, i.e., lowest concentration of test compound that completely inhibits the growth of the organism) against MDR-TB was determined using the dilution method with Middlebrook 7H10 Agar solid growth medium (Gold standard for MIC determinations). The MIC for compound **2** was 1.56 µg/mL, but higher for congeners and analogs of **2** [e.g., without fluorines on benzyl groups (MIC 3.125 µg/mL), with increased number of fluorines on benzyl groups (MIC 3.125 µg/mL), and with imidazole and pyrazole rings in place of pyrrole (MIC 6.25 µg/mL and 3.125 µg/mL, respectively)]. Thus, pyrrole substitution at the para-position on the phenyl ring was preferred over the corresponding imidazole and pyrazole substitutions. Also, the compound with no substitution on the piperazine phenyl ring had lower activity (MIC 6.25 µg/mL). The positive control was streptomycin and the negative control was the untreated organism.²⁶ Serial two-fold dilutions (25 µg/mL to 0.012 µg/mL) were used in the assays.

Further evidence for the activity of compound **2** against MDR-TB was investigated through drug combination studies (see Table 1) involving the *in vitro* interactions of compound **2** with PA-824, a mycolic acid synthesis inhibitor, which also exhibits anti-MDR TB activity.²⁷ The combination of compound **2** and PA-824 was clearly synergistic resulting in a Σ FIC value of 0.37. A value of Σ FIC of 0.5 is indicative of synergy.²⁸ The combination resulted in reduction of CFU (colony forming unit) by >99% compared to untreated plate CFU. The combination improved the MIC of compound **2** by 8-fold and that of PA-824 by 4-fold.

A drug cytotoxicity control plate assay (MTT proliferation assay) was conducted for 7 days in uninfected macrophages to determine if the compounds displayed significant cytotoxicity towards macrophages. This study showed that after 7 days at 4 x MIC of compound **2** (6.25 µg/mL or 9.6 µM), the percent viability of the macrophages was > 85%. The percent viability of the macrophages with the positive control compound, streptomycin, was close to 90% for 4 x MIC.

Because of the importance of establishing early, the behavior of a potentially interesting new anti-MDR TB compound in phase I metabolism, our next studies involved the active compound **2** and its *in vitro* microsome stability as well as its cytochrome P450 (CYP) profile.^{29–31} The microsome stability studies were performed through incubations using pooled, mixed-gender human liver microsomes.³² Analyses were performed by HPLC separation combined with UV detection and supporting HRMS bioanalytical analyses. The results showed that compound **2** was relatively stable in pooled mixed-gender human liver microsomes (Fig. 4), exhibiting a half-life ($t_{1/2}$) of 14.4 ± 1.3 hr.

The cytochrome P450 (CYP) isozyme profile study also utilized pooled mixed-gender human liver microsomes and two substrates for CYP 3A4 (testosterone and triazolam) and one substrate for CYP 2D6 (dextromethorphan).³³ These two CYP isozymes account for the metabolism of about 80% of drugs that are metabolized in phase I metabolism by CYP

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isozymes.²⁹ The data indicate only relatively small changes in the level of substrate activity compared to the control study in each case at concentrations of **2** below 25 μM for CYP3A4. The IC_{50} values for CYP3A4 inhibition for hydroxylation of both substrates, testosterone and triazolam, were $>50 \mu\text{M}$ and $>85 \mu\text{M}$, respectively. The IC_{50} for CYP2D6 inhibition, with demethylation of substrate dextromethorphan, was $>185 \mu\text{M}$. Thus, compound **2** exhibits only very weak inhibition of these key CYP isozymes. Significant activation was not observed. Thus, the *in vitro* CYP profile is very favorable.

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UDP-glucuronosyltransferases (UGTs) are a superfamily of human phase II metabolizing isozymes, which are involved in the glucuronidation and subsequent clearance through bile or urine of a significant number of drugs.^{34–36} Thus, in this investigation, the UGT profile of compound **2** was considered to be of significance, particularly in terms of co-infection therapeutics.^{5,13,35} Glucuronidation studies of compound **2** (Figure 5) were determined in pooled, mixed-gender human liver microsomes verified to contain UGT 1A1, 1A4, 1A6, 1A9 and 2B7.³⁷ Compound **2** was not a substrate for these key UGTs. Furthermore, in the kinetic studies in human liver microsomes, there was no indication of the activation of UGT isozymes. The positive control compound, raltegravir, was a substrate for UGT (Fig. 5), which is consistent with previously reported data.³⁸ We also examined the possible competitive inhibition of UGTs by compound **2** using 4-methylumbelliferone (4-MU), a substrate for multiple isoforms of UGT. However, no evidence for significant competitive inhibition of the key UGT isozymes 1A1, 1A6, 1A9 and 2B7 was found ($\text{IC}_{50} >300 \mu\text{M}$). In addition, compound **2** was not a substrate or inhibitor of another key UGT isozyme, namely UGT1A4. While some activation of UGT1A4 was noted, these occurred only at higher concentrations ($>100 \mu\text{M}$).

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A suggested mechanism for the observed activity of compound **2** may be through inhibition of TB DNA-dependent RNA polymerase (RNAP) activity.^{39–41} The binding target appears to be the β' -subunit of the polymerase,³⁹ some 13–18 Å away from the binding site of rifampin in the β -subunit [Fig. 6, Surflex dock in Sybyl-X 1.3 (winnt. os5x)]. Compound **2** appears to seek out the Mg^{2+} ion in the β' -subunit for chelating and it also binds to amino acid residues, many of which are similar to those involved in the binding of inhibitor **1** to the active site of HIV-1 integrase.²¹ Further studies are in progress to lend support to this proposed mechanism of action. Interestingly, compound **2** also displayed activity against HIV-1 in MAGI-R5 cells with an EC_{50} of $50 \pm 10 \text{ nM}$, suggesting the potential for dual therapeutic applications of this compound.

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In summary, we have designed and synthesized a novel multifunctional molecule, **2**, that exhibits notable activity against MDR-TB. Compound **2** possessed a relatively long half-life in pooled, mixed-gender human liver microsomes. It displayed favorable drug interaction profiles with respect to key phase I and II isozymes of CYP and UGT. Evaluation of toxicity in uninfected macrophages revealed relatively low *in vitro* cytotoxicity. Compound **2** also displayed synergistic behavior against MDR-TB in combination with PA-824, a mycolic acid synthesis inhibitor. Further studies on this interesting compound are in progress.

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25. Data for compound 2. UV (methanol): 376 nm (ϵ 15,065), 269 nm (ϵ 31,601). $^1\text{H-NMR}$ (CDCl_3 , 500MHz): δ 8.15 (d, 1H, $J = 2.5$ Hz), 7.58 (s, 1H), 7.50–7.55 (m, 2H), 7.32–7.36 (m, 3H), 7.10–7.20 (m, 2H), 7.00–7.05 (m, 4H), 6.68–6.78 (m, 3H), 6.36 (t, 2H, $J = 1.75$ Hz), 5.22 (s, 2H), 3.88 (bs, 2H), 3.80 (s, 2H), 3.77 (bs, 2H), 3.25–3.29 (m, 4H). HRMS: calculated mass 653.2376 for $\text{C}_{37}\text{H}_{32}\text{F}_3\text{N}_4\text{O}_4$ $[\text{M}+\text{H}]^+$; found 653.2357.
26. Middlebrook 7H10 agar medium was prepared from a dehydrated base by mixing with purified water containing glycerol and heated to completely dissolve. This solution was subsequently autoclaved at 121°C for 10 minutes and oleic albumin dextrose Catalase (OADC) enrichment was added to medium when cooled. Test compounds were solubilized in DMSO at a concentration of 1 mg/mL, filter sterilized and subsequently diluted using phosphate buffered saline (PBS) to the desired concentration range. Each test concentration was mixed with media contained within 250mL media flasks, immediately poured into sterile petri dishes (plates) and allowed to solidify. Plates were inoculated with approximately 2×10^3 CFU of multi-drug resistant (isoniazid and rifampin resistant) *Mtb* and incubated for a maximum of 21 days at 37°C . After incubation, bacterial colonies were enumerated and the minimum inhibitory concentration (MIC) reported. Testing was conducted in triplicate and the following controls were used: (i) streptomycin (positive control, MIC 0.39 $\mu\text{g/mL}$); (ii) organism only (negative control).
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32. Incubations contained 0.5 mg/mL protein, 50 μM compound 2, 2 mM NADPH, 0.5 U/mL G-6-P-DH, 5 mM MgCl_2 , and 5 mM G-6-P in a final volume of 400 μL in 100 mM potassium phosphate buffer (pH 7.4). The reaction mixture was pre-incubated for 3 min at 37°C before initializing by addition of NADPH solution, followed by incubation for 60 min. A solution of 60 μL was withdrawn at 0, 30, 60, 120 and 240 min and quenched with 60 μL of ice-cold acetonitrile. The precipitated proteins were removed by centrifugation at $5,000 \times g$ for 5 min at room temperature before analysis of the supernatant by HPLC. Analysis was done using a reversed-phase HPLC column on a Beckman Coulter Gold 127 solvent system and Gold 166 UV analytical detector. The data were collected and processed using Gold software. The elution process involved: analytical column Discovery® C18 (5 μm , 250 x 4.6 mm) with a mobile phase of (A)10 mM potassium phosphate buffer (pH 6.5) and (B) acetonitrile, which were used as follows: 0–2 min, 30 % B, 2–8 min, 30–60 % B, 8–18 min, 60 % B. The flow rate was kept at 1.5 mL/min, with UV detection at 360 nm. Retention time for compound 2 was 13.0 minutes.
33. Cytochrome P450 Studies. Incubation mixtures contained potassium phosphate buffer (100 mM, pH 7.4), MgCl_2 (5 mM), the requisite substrate dissolved in an appropriate solvent, compound 2 (range of concentrations 0 to 200 μM) and pooled human liver microsomes. The reaction mixture (final volume 100 μL) was pre-incubated for 3 min at 37°C before initiation of the reaction by the addition of NADPH (final concentration 2 mM). In each incubation mixture, the organic solvent concentration was kept at or below 1.5% by volume. Each reaction was terminated by adding ice-cold acetonitrile (100 μL). Proteins were precipitated from the samples by centrifugation at $5000 \times g$ for 10 min at room temperature in a microcentrifuge tube. The supernatant was analyzed by HPLC-UV for detection of substrates and metabolites. Reversed-phase HPLC analyses were performed on a Beckman Coulter Gold 127 solvent system with a Gold 166 UV analytical detector. The data were collected and processed using a Gold software package. CYP HPLC analytical assays: Testosterone and 6 β -hydroxytestosterone analysis involved analytical column Discovery® C18 (5 μm , 250 x 4.6 mm) eluting with (A)10 mM potassium phosphate buffer (pH

4.5) and (B) acetonitrile, in a gradient mixture starting with 25% (B) at time 0 min to 100% (B) at time 23 min. at a flow rate of 1.2 mL/min, with detection wavelength set at 254 nm. Testosterone and 6 β -hydroxytestosterone were detected at 13.38 and 8.50 min RT, respectively. The analytical column Novapak C18 (4 μ m, 150 mm $\times$ 3.9 mm, 60 $\text{\AA}$) mounted on the above HPLC system was used in the analysis of triazolam and 4-hydroxytriazolam as well as dextromethorphan and dextrorphan respectively. The elution solvent for both assay system was similar to testosterone above with variation in gradient system. Triazolam and hydroxytriazolam were separated with a gradient solvent mixture of 30 % (B) at 0 min to 100 % B at 16 min, at a flow rate of 1.0mL/min while detecting at 260 nm wavelength. The RT for triazolam and hydroxytriazolam were 9.63 and 7.60 min, respectively. Dextromethorphan and dextrorphan were similarly separated with gradient solvent elution system of 28% (B) at 0 min to 100 % (B) after 16 min, at a flow rate of 1.0 mL/min, with UV detection set at 278 nm. RT for dextromethorphan and dextrorphan were 11.80 and 8.72 min, respectively.

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37. Incubation mixtures (final volume of 500 μ L) contained human liver microsomes (1.0 mg/mL proteins), compound 2 or raltegravir (50 μ M in DMSO, <1% of final mixture), UDPGA (4 mM), alamethicin (0.024 mg/mg protein), D-saccharic acid 1,4-lactone (10 mM) in potassium phosphate buffer (100 mM, pH 7.4) containing MgCl_2 (5 mM). Initially, the mixture of human liver microsomes and alamethicin in the buffer containing MgCl_2 was kept in ice (0 $^{\circ}$ C) for 15 min. D-saccharic acid-1,4-lactone and the test compound were then added. This mixture was preincubated at 37 $^{\circ}$ C for 3 minutes and UDPGA (4 mM) was then added to initiate the reaction. An aliquot (60 μ L) was removed each sampling time, quenched with acetonitrile (60 μ L), centrifuged and the supernatant analyzed by HPLC and HRMS.
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Fig. 1.
Structure of compound **1** in the crystalline state.

Fig. 2.

Design of anti-MDR TB compounds using computational biology and molecular modeling data.

Fig. 3.
Structure of lead designed compound **2** (left) and its calculated minimum- energy conformation (right).

Fig. 4. Stability of compound **2** in pooled, mixed-gender human liver microsomes. Data bars indicate the mean from three determinations. The error bars represent standard deviations from the mean.

Fig. 5. UGT-catalyzed glucuronidation study of compound **2** (blue) and positive control compound, raltegravir (red).

Fig. 6.

Depiction of the binding of compound **2** (green) in the β' -subunit of DNA-dependent RNA polymerase (RNAP), mutated for resistance to rifampin (pdb ID: 1i6v). The RNAP is shown in the ribbon rendition with secondary structures in different colors (β -sheets - blue, α -helices - red, connecting strands - yellow). The Mg^{2+} ion is shown in purple and is located behind the compound. Active site amino acid residues interacting with compound **2** include C/Glu445, C/Gly446, C/Arg557, C/Met560, C/Lys838, C/Lys846, C/His999, C/Lys1004, C/Arg1031, D/Arg622, D/Arg628, D/Asp739, D/Asp741, D/Asp743, D/Gln744, D/Arg1087, D/Mg1600 (purple ball).

Scheme 1. Methodology for the total synthesis of compound **2**.

Table 1
Synergy between compound **2** and PA-824 in a two drug combination study against MDR-TB *in vitro*.^{*}

Organism	Drugs Alone or in Combination	MIC µg/mL Alone	MIC µg/mL Combination	FIC#	ΣFIC#
MDR-TB	Compound 2 (Drug A)	1.563	0.195	0.125	-
MDR-TB	PA-824 (Drug B)	0.126	0.031	0.246	-
MDR-TB	Combination of Drug A and B	-	-	-	ΣFIC = 0.371

^{*} Agar dilution drug susceptibility assays.

[#] Fractional Inhibitory Concentration (FIC) = MIC combination/MIC alone. ΣFIC FIC of first drug + FIC of second drug. ΣFIC 0.5 indicates synergy. ΣFIC 4.0 indicates antagonism activity.²⁸
Negative Control: organism only. Positive Control: Streptomycin.