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Research paper

Design, synthesis and evaluation of diarylpiperazine derivatives as potent anti-tubercular agents

Ashok Penta [a], Scott Franzblau [b], Baojie Wan [b], Sankaranarayanan Murugesan [a, *]

[a] Medicinal Chemistry Research Laboratory, Department of Pharmacy, Birla Institute of Technology & Science, Pilani, 333031, India
[b] Institute for Tuberculosis Research, MC-964 College of Pharmacy, University of Illino's at Chicago, 833 S. Wood St, Chicago, IL, 60621-7231, USA

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ABSTRACT

Molecular hybridization is an emerging approach to design novel ligands by combination of two or more pharmacophoric subunits of known bioactive compounds. In the present study, we have designed a novel series of diarylpiperazine analogues, synthesized, characterized using FTIR, ^{1}H NMR, Mass, Elemental analysis and evaluated their *in-vitro* anti-tubercular activity. Among the reported sixteen diarylpiperazines, eleven analogues exhibited significant anti-tubercular activity against *Mycobacterium tuberculosis* H37Rv strain with MIC values below 6.25 μg/mL and good selectivity index. Structure activity relationship studies concluded that, ortho-para directing group (except para chloro) substitution on ortho and para position of piperazine attached phenyl ring favored anti-tubercular activity.

1. Introduction

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis,* one of the leading causes of death worldwide [1–3]. According to the statistics from WHO, 9.0 million new infections and 1.5 million TB deaths were reported in the year 2013 [4]. It is projected that, if control measures are not strengthened further, over 125 million people will get sick with more than one billion new infections and 30 million people will die with TB by 2020 [5]. Tuberculosis has become more significant in the recent years with increased co-infection with HIV and emergence of drug resistance [multiple drug-resistant *M. tuberculosis* (MDR-TB) and extremely drug resistant *M. tuberculosis* (XDR-TB)] [6,7]. Unfortunately, first line treatment regimen of TB mainly consist of decade old drugs [8] and some of the strains have become resistant to most of the currently available anti-tubercular drugs, known as totally drug resistant *M. tuberculosis* (TDR-TB) [9]. Hence, there is an urgent need to develop novel anti-tubercular agents active against both sensitive and drug resistant strains. In the year 2012, U.S. Food and Drug Administration (US-FDA) approved new anti-tubercular drug Bedaquiline for the treatment of MDR-TB patients in combination with other drugs [10].

Nature remains one of the important source to develop anti-infective agents [11]. Identification of natural products and development of synthetic derivatives of natural products with potent activity are always promising way in drug discovery especially for anti-infective agents. β-carboline represents a tricyclic pyrido[3,4-*b*]indole ring system present in large number of natural products isolated from various sources like territorial plants [12], marine sponge [13], fast food [14] and humans [15]. Natural as well as synthetic β-carboline derivatives displayed biological activities like anti-cancer, anti-thrombotic, anti-microbial, anti-malarial, anti-leishmanial, anti-tubercular and anti-viral activity [16,17]. Manzamines are a unique group of β-carboline alkaloids with an unusual polycyclic system, present in different species of marine sponges found in the Indian and Pacific Ocean. Large number of manzamine alkaloids and their synthetic derivatives displayed significant anti-tubercular activity. Manzamine A is the first compound of this group isolated from Okinawa sponge in 1986 and exhibited potent anti-tubercular activity (MIC 1.53 μg/mL) [17,18] (Fig. 1). Nosto-carboline (Fig. 1), a new quaternary β-carboline alkaloid isolated from the freshwater cyanobacterium *Nostoc* 78-12A [19] and its synthetic derivatives exhibited significant anti-tubercular activity [20]. Along with these natural β-carboline alkaloids, semi-synthetic and synthetic β-carboline derivatives also exhibited significant to moderate anti-tubercular activity [20]. Piperazine moiety has the privileged position in the medicinal chemistry and is the second most frequent ring present in all FDA approved drugs till 2013 [21]. In addition to this, piperazine containing compounds displayed wide range of biological activities such as anti-microbial [22,23],

* Corresponding author.
E-mail address: murugesan@pilani.bits-pilani.ac.in (S. Murugesan).

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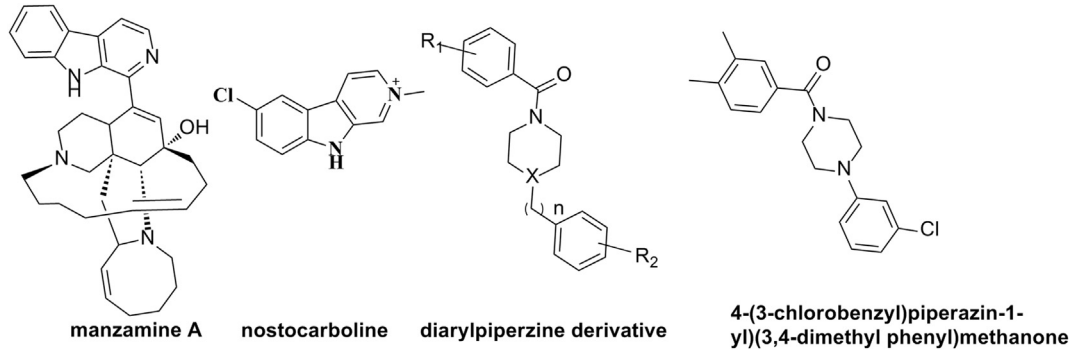


Fig. 1. Structure of some β-carboline and diarylpiperazine derivatives reported as anti-tubercular agents.

anti-protozoal [24,25], anti-leishmanial [26], anti-cancer [27], anti-tubercular [27,28] (Fig. 1) and anti-viral activity [29]. He et al., 2007, reported anti-tubercular activity of simple di-arylpiperazine derivatives as enoyl acyl carrier protein reductase InhA inhibitors. These analogues displayed significant to moderate anti-tubercular activity, among these analogues, (4-(3-chlorobenzyl)piperazin-1-yl) (3,4-dimethyl phenyl)methanone (Fig. 1) showed most potent anti-tubercular activity (IC_{50} 0.99 μM) [30].

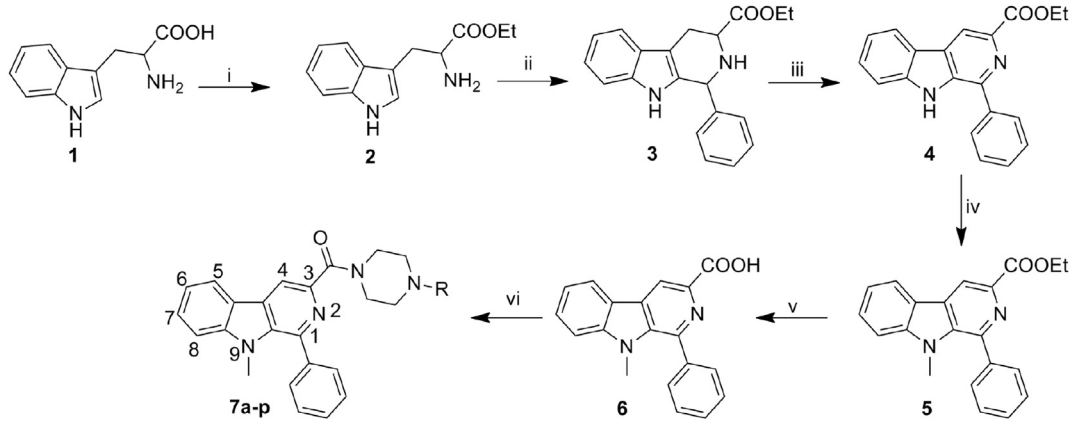
With our continuous interest to develop novel β-carboline derivatives as anti-tubercular agents, in the present study, we have designed a series of novel diarylpiperazine derivatives based on molecular hybridization technique. Molecular hybridization is a rational approach to design new ligands by combination of pharmacophoric sub-units of two or more known bioactive derivatives (β-carboline and piperazine scaffold). In the present study, we have reported design, synthesis, *in-vitro* anti-tubercular evaluation and structure activity relationship study of novel diarylpiperazine analogues.

2. Results and discussion

2.1. Chemistry

The synthetic protocol of the titled diarylpiperazine analogues is illustrated in Scheme 1. The compounds were synthesized from the starting material, DL-Tryptophan **(1)** in a sequence of reactions. Initial esterification of DL-Tryptophan **(1)** using thionylchloride to obtain the ethyl ester of tryptophan **(2)** was followed by pictet–spengler reaction in the presence of trifluoroacetic acid to afford tricyclic ethyl 2,3,4,9-tetrahydro-1-phenyl-1*H*-pyrido[3,4-*b*] indole-3-carboxylate **(3).** Upon oxidation with potassium permanganate, ethyl-1-phenyl-9*H*-pyrido[3,4-*b*]indole-3-carboxylate **(4)** was obtained [31,32], continued by 9-*N* methylation with methyliodide in presence of potassium hydroxide to obtain ethyl-9-methyl-1-phenyl-9*H*-pyrido[3,4-*b*]indole-3-carboxylate **(5)** [33], followed by alkaline ester hydrolysis to afford 9-methyl-1-phenyl-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid **(6)** as key intermediate. The carboxylic acid group containing key intermediate **(6)** was further treated with appropriate amines (aryl-substituted piperazines) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and hydroxybenzotriazole (HOBt) to obtain the desired products **(7a-p)** in good to excellent yields [34].

All the synthesized compounds were characterized by IR, NMR, Mass and Elemental analysis. IR spectra of the reported compounds showed *C═O* stretching at 1652 to 1614 cm^{-1}, aromatic *C–H* stretching at 3069 to 3043 cm^{-1}, aromatic *C═C* stretching at 1597 to 1516 cm^{-1}, *C–O* stretching (methoxy) at 1253 to 1240 cm^{-1}, *N–O* asymmetric stretching at 1385 cm^{-1} and *C–Cl* absorption band at 1068 to 1054 cm^{-1}. The ^1H NMR spectrum of the compounds showed, characteristic singlet around δ ~8.50 due to position-4 proton of β-carboline ring, eight piperazine protons appeared as two multiplets around δ value 4.19 to 4.02 ($O{=}CN(CH_2)_2$) and 3.64 to 2.62 ($CN(CH_2)_2$). Piperazine protons were shifted to down field (δ ~4.19 and 3.64) on nitro substitution whereas, shifted up field in benzyl derivatives (δ ~3.91 and 2.62). Methoxy protons appeared as



Scheme 1. Reagents and conditions: i) thionylchloride, ethanol, reflux, 30 min, 76%; ii) benzaldehyde, trifluoro aceticacid, DCM, rt, 3 h, 82%; iii) $KMnO_4$, THF, rt, 24 h, 68%; iv) methyliodide, KOH, DMSO, rt, 30 min, 72%; v) 50% aq. NaOH, reflux, 30 min, 78%; vi) EDCl, HOBt, THF, piperazines, 0 °C-rt 6 h, 62–82%.

singlet at δ ~3.80, aromatic methyl protons as singlet at δ ~2.30 and $N-CH_3$ protons as singlet at δ ~3.50. In mass spectral analysis, M + 1 peak appeared as parent ion peak. Analytical purity of the compounds was determined using Elemental analysis and values were found to be within the acceptable limits.

2.2. Biological evaluation

All the synthesized titled compounds were evaluated for their inhibitory potency against *M. tuberculosis* strain H37Rv using Microplate Alamar Blue Assay (MABA) [35]. The minimum inhibitory concentration (MIC) is the minimum concentration of the inhibitor required to complete inhibition of bacterial growth. The MIC values (μg/mL and μM) were determined (Table 1) and compared with standard anti-tubercular drug rifampicin. Among the synthesized compounds, several compounds displayed significant anti-tubercular activity. Among these reported 16 compounds, eleven compounds such as 7b, 7c, 7d, 7e, 7g, 7j, 7l, 7m, 7n, 7° and 7p exhibited significant activity against *M. tuberculosis* strain H37Rv with MIC value < 6.25 μg/mL.

In the present report we have studied, the effect of different substitutions on phenyl ring and replacement of phenyl ring attached to piperazine moiety on their anti-tubercular potency. Among these synthesized diarylpiperazine derivatives, prototype compound containing un-substituted phenyl ring showed weak anti-tubercular activity. Substitution of electron donating groups on *ortho* and *para* position of the phenyl ring favored anti-tubercular activity, especially substitution on *ortho* position (compound 7g) resulted in six times enhancement in potency and substitution on *para* position (compound 7c) increased the potency by three times, whereas electron donating group substitution on *meta* position (compound 7f) completely abolished the activity. Electron withdrawing chlorine group substitution on *meta* (7i) and *para* position (7h) led to decrease in potency, while on *ortho* position (7j) boosted the anti-tubercular potency. Interestingly, 2,3-dichloro substitution (7n) on phenyl ring produced eight times increase in potency and showed most potent anti-tubercular activity with good selectivity index. Fluorine substitution on *ortho* (7m) and *para* (7l) position resulted in increase in potency (nearly five times), whereas strong electron withdrawing nitro group on *para* position (7k) led to complete loss of activity. Replacement of phenyl ring with benzyl (7b) and pyridine ring (7°, 7p) produced three times increment in anti-tubercular activity. From the SAR study, we observed that, position and nature of the substituent on phenyl group attached to piperazine moiety has significant impact on the anti-tubercular activity and replacement of the phenyl group with pyridyl moiety improves the anti-tubercular activity three folds.

2.3. Cytotoxicity evaluation

Compounds displayed significant anti-tubercular activity were evaluated for their cytotoxicity using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay against vero cell lines [36]. Initially compounds were screened at 50 μg/mL concentration. All the tested compounds were non-cytotoxic to vero cell line at tested concentration, except compound 7° which showed cytotoxicity with CC_{50} and selectivity index values of 18.02 μg/ml and 4.5 respectively.

3. Conclusion

In summary, a new series of diarylpiperazines were successfully designed through molecular hybridization approach. All the designed compounds were synthesized, characterized, evaluated for their *in-vitro* anti-tubercular activity against *M. tuberculosis* H37Rv and cytotoxicity against vero cell lines. Among the synthesized analogues, compounds 7b, 7c, 7d, 7e, 7g, 7j, 7l, 7m, 7n, 7° and 7p displayed significant anti-tubercular activity with MIC values below 6.25 μg/mL and good selectivity towards *M. tuberculosis*. Structure activity relationship studies suggested that, ortho as well as para substitution on phenyl ring attached to piperazine with ortho-para directing groups favored anti-tubercular activity (except para chloro substitution) of these novel diarylpiperazine analogues.

4. Experimental protocols

4.1. Chemistry

4.1.1. All solvents and reagents purchased from Sigma or Merck companies were used as received without further purification

Solvent system used throughout the experimental work for running Thin Layer Chromatography (TLC) was Ethyl acetate and Hexane Mixture (6:4) in order to monitor the reaction. Column chromatography was performed using silica gel (100–200 mesh, SRL, India) as stationary phase, mixture of ethyl acetate and hexane as mobile phase. Melting point was determined in open capillary

Table 1
In-vitro anti-tubercular activity of the synthesized compounds.

Comp. Code	R	CC_{50} (μg/mL) vero cell	MIC(μg/ml)	MIC μM	Selectivity Index[b]
7a	$-C_6H_5$	>50	12.2	27.4	>4.1
7b	$-CH_2C_6H_5$	>50	4.1	8.9	>12.2
7c	$-4-CH_3C_6H_4$	>50	4.3	9.3	>11.6
7d	$-2-CH_3C_6H_4$	>50	2.4	5.2	>20.8
7e	$-4-OCH_3C_6H_4$	>50	5.8	10.1	>8.6
7f	$-3-OCH_3C_6H_4$	NT[a]	>50	–	–
7g	$-2-OCH_3C_6H_4$	>50	2.0	3.5	>25
7h	$-4-ClC_6H_4$	>50	40.0	83.3	>1.3
7i	$-3-ClC_6H_4$	>50	38.2	79.6	>1.3
7j	$-2-ClC_6H_4$	>50	2.4	5.0	>20.8
7k	$-4-NO_2C_6H_4$	NT[a]	>50	–	–
7l	$-4-FC_6H_4$	>50	2.6	5.6	>19.2
7m	$-2-FC_6H_4$	>50	2.7	5.8	>18.5
7n	$-2,3-di-ClC_6H_3$	>50	1.5	2.9	>33.3
7°	-4-Pyridyl	18.0	4.4	9.8	4.5
7p	-2-Pyridyl	>50	4.4	9.8	>11.4
Rifampicin	–	>150	0.11	0.13	>1363.4

[a] Not Tested.
[b] CC_{50}/MIC (both values in μg/mL).

tube on a Precision Buchi B530 (Flawil, Switzerland) melting point apparatus containing silicon oil and are uncorrected. IR spectra of the synthesized compounds were recorded using FTIR spectrophotometer (Shimadzu IR Prestige 21, India). ^{1}H NMR spectra was recorded on a Bruker DPX-400 spectrometer (Bruker India Scientific Pvt. Ltd., Mumbai) using TMS as an internal standard (chemical shifts in δ). Elemental analysis was performed on Vario EL III M/s Elementar C, H, N and S analyzer (Elementar Analysensysteme GmbH, Germany). The ESMS was recorded on MICROMASS Quattro-II LCMS system (Waters Corporation, Milford, USA).

*4.1.2. General procedure for the synthesis of **2***

To a solution of DL-tryptophan (**1**) (5 g, 0.0245 mol) in 50 mL of ethanol, $SOCl_2$ (5.32 ml, 0.073 mol) was added drops wise at 0 °C. Subsequently, the reaction mixture was refluxed for 1 h. After completion of reaction as monitored by TLC, solvent was evaporated in vacuo and the residue obtained was dissolved in ethyl acetate. Organic layer was washed twice with $NaHCO_3$ solution. The separated organic layer was dried (Na_2SO_4) and concentrated under vacuum to get intermediate **2** as a white solid, yield 76%.

*4.1.3. General procedure for the synthesis of **3***

To the mixture of **2** (5 g, 0.022 mol) and benzaldehyde (2.2 mL, 0.022 mol) in 100 mL of dichloromethane, 2.5 mL of trifluoroacetic acid was added drops wise at 0 °C for 10 min. Then, reaction mixture was stirred at room temperature for 3 h. After completion of reaction as monitored by TLC, the reaction mixture was poured into 100 mL of saturated $NaHCO_3$ solution. Organic layer was separated and washed twice with brine solution. Organic layer was dried using anhydrous Na_2SO_4 and concentrated under vacuum to get **3** as a white solid, yield 82%.

*4.1.4. General procedure for the synthesis of **4***

To a solution of **3** (4 g, 0.013 mol) in 200 mL of dry THF, $KMnO_4$ (6.15 g, 0.039 mol) was added and reaction mixture was stirred at room temperature for 24 h. After completion of reaction as monitored by TLC, the reaction mixture was passed through celite bed to filter off $KMnO_4$. Then, solvent was evaporated in vacuo, the residue was dissolved in ethyl acetate and washed twice with 50 mL of distilled water. The separated organic layer was dried using anhydrous Na_2SO_4 and concentrated under vacuum to get **4** as a white solid, yield 68%.

*4.1.5. General procedure for the synthesis of **5***

To the mixture of **4** (3 g, 0.01 mol) and KOH (1.66 g, 0.03 mol) in DMSO, methyl iodide (0.015 mol) was added at 0 °C. Reaction mixture was allowed to come to room temperature and stirred for 30 min. After completion of reaction as monitored by TLC, 50 mL of ice cold water was poured in to reaction mixture. The resulting solid was collected by filtration and dried over P_2O_5 in a desiccator under reduced pressure to obtain **5** as a white solid, yield 72%.

*4.1.6. General procedure for the synthesis of **6***

To a solution of **5** (3 g, 0.009 mol) in ethanol water (50:50) mixture, NaOH (1.13 g, 0.028 mol) was added and refluxed for 30 min. After completion of reaction as monitored by TLC, ethanol from the reaction mixture was removed under vacuum and neutralized with dil. HCl. Then, the reaction mixture was extracted with ethyl acetate (2 x 30 mL), collected organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum to get **6**, as white solid, yield 78%.

*4.1.7. General procedure for the preparation of **7***

To the stirred solution of **6** (0.29 g, 0.001 mol) in dry THF, HOBt (0.16 g, 0.012 mol) and EDCI. HCl (0.23 g, 0.012 mol) were added and continued stirring for 30 min. To the reaction mixture, substituted phenylpiperazine (0.001 mol) was added under ice cold temperature and the reaction mixture was further stirred at room temperature for 6 h. After completion of reaction as monitored by TLC, solvent was evaporated under vacuum. Reaction mixture was extracted with ethyl acetate (2 x 20 mL), collected organic layer was dried over anhydrous Na_2SO_4, concentrated under vacuum and passed through small bed of silica gel (60–120) using mobile phase (ethyl acetate:hexane; 3:7) to obtain analytically pure final product **7**.

*4.1.7.1. (9-Methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl) (4-phenylpiperazin-1-yl)methanone (**7a**).* White solid; Yield 76%; mp 232–234 °C; R_f 0.45 (Hexane: EtOAc = 6:4); IR νmax/cm^{-1}(KBr): 3046 (C–H, aromatic stretching), 1636 (C=O, stretching), 1564 (C=C, aromatic stretching), 1468 (C–H, bending); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (s, 1H, aromatic), 8.21 (d, *J = 7.8 Hz*, 1H, aromatic), 7.70-7.61 (m, 3H, aromatic), 7.58-7.44 (m, 4H, aromatic), 7.36 (t, *J = 7.2 Hz*, 1H, aromatic), 7.30-7.28 (m, 2H, aromatic), 6.96-6.90 (m, 3H, aromatic), 4.04 (brs, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.52 (s, 3H, *N*-methyl), 3.33-3.23 (m, 4H, piperazinyl (CN(CH_2)$_2$)); ESI-MS: *m/z* 447 (M + 1$^+$, 100%); Anal. Calcd for $C_{29}H_{26}N_4O$: C, 78.00; H, 5.87; N, 12.55, Found: C, 78.02; H, 5.85; N, 12.51.

*4.1.7.2. (4-Benzylpiperazin-1-yl) (9-methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (**7b**).* White solid; Yield 78%; mp 158–160 °C; R_f 0.40 (Hexane: EtOAc = 6:4); IR νmax/cm^{-1}(KBr): 3055 (C–H, aromatic stretching), 1614 (C=O, stretching), 1552 (C=C, aromatic stretching), 1438 (C–H, bending); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (s, 1H, aromatic), 8.22 (d, *J = 7.7 Hz*, 1H, aromatic), 7.66-7.62 (m, 3H, aromatic), 7.56-7.52 (m, 3H, aromatic), 7.47 (d, *J = 8.3 Hz*, 1H, aromatic), 7.39-7.34 (m, 5H, aromatic), 7.30-7.28 (m, 1H, aromatic), 3.91-3.87 (m, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.57 (s, 2H, benzyl), 3.52 (s, 3H, *N*-methyl), 2.62-2.48 (m, 4H, piperazinyl (CN(CH_2)$_2$)); ESI-MS *m/z* 461 (M + 1$^+$, 100%); Anal. Calcd for $C_{30}H_{28}N_4O$: C, 78.23; H, 6.13; N, 12.16, Found: C, 78.26; H, 6.11; N, 12.19.

*4.1.7.3. (9-Methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl) (4-p-tolylpiperazin-1-yl)methanone (**7c**).* White solid; Yield 72%; mp 188–190 °C; R_f 0.40 (Hexane: EtOAc = 6:4); IR νmax/cm^{-1}(KBr): 3059 (C–H, aromatic stretching), 1652 (C=O, stretching), 1558 (C=C, aromatic stretching), 1448 (C–H, bending); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.55 (s, 1H, aromatic), 8.23 (d, *J = 7.8 Hz*, 1H, aromatic), 7.68-7.64 (m, 3H, aromatic), 7.58-7.53 (m, 3H, aromatic), 7.48 (d, *J = 8.3 Hz*, 1H, aromatic), 7.38 (t, *J = 7.5 Hz*, 1H, aromatic), 7.11 (d, *J = 8.2 Hz*, 2H, aromatic), 6.88 (d, *J = 8.5 Hz*, 2H, aromatic), 4.06-4.04 (m, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.54 (s, 3H, *N*-methyl), 3.29-3.18 (m, 4H, piperazinyl (CN(CH_2)$_2$)), 2.30 (s, 3H, methyl); ESI-MS *m/z* 461 (M + 1$^+$, 100%); Anal. Calcd for $C_{30}H_{28}N_4O$: C, 78.23; H, 6.13; N, 12.16, Found: C, 78.22; H, 6.16; N, 12.14.

*4.1.7.4. (9-Methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl) (4-o-tolylpiperazin-1-yl)methanone (**7d**).* White solid; Yield 64%; mp 148–150 °C; R_f 0.43 (Hexane: EtOAc = 6:4); IR νmax/cm^{-1}(KBr): 3053 (C–H, aromatic stretching), 1636 (C=O, stretching), 1546 (C=C, aromatic stretching), 1419 (C–H, bending); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (s, 1H, aromatic), 8.21 (d, *J = 7.7 Hz*, 1H, aromatic), 7.65-7.62 (m, 3H, aromatic), 7.55-7.50 (m, 3H, aromatic), 7.45 (d, J = *8.2 Hz*, 1H, aromatic), 7.36 (t, *J = 7.5 Hz*, 1H, aromatic), 7.20-7.15 (m, 2H, aromatic), 7.01-6.99 (m, 2H, aromatic), 4.02-4.00 (m, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.51 (s, 3H, *N*-methyl), 3.06-2.94 (m, 4H, piperazinyl (CN(CH_2)$_2$)), 2.35 (s, 3H, methyl); ^{13}C NMR ($CDCl_3$, 100 Hz) d (ppm): 17.50, 35.88, 42.40, 47.54, 51.51, 51.97, 110.59, 114.44, 119.04, 120.22, 120.55, 121.89, 123.14, 126.52, 128.01, 128.46,

128.85, 129.72, 129.88, 130.78, 131.93, 134.24, 138.99, 141.65, 142.41, 142.78, 150.98, 167.51; ESI-MS *m/z* 461 (M + 1^+, 100%); Anal. Calcd for $C_{30}H_{28}N_4O$: C, 78.23; H, 6.13; N, 12.16, Found: C, 78.20; H, 6.12; N, 12.18.

*4.1.7.5. (4-(4-methoxyphenyl)piperazin-1-yl) (9-methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (**7e**).* White solid; Yield 82%; mp 170–172 °C; R_f 0.30 (Hexane: EtOAc = 6:4); IR νmax/cm^{-1}(KBr): 3064 (C–H, aromatic stretching), 1651 (C=O, stretching), 1516 (C=C, aromatic stretching), 1454 (C–H, bending), 1255 (C–O, stretching); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.53 (s, 1H, aromatic), 8.21 (d, *J = 7.8 Hz*, 1H, aromatic), 7.66-7.62 (m, 4H, aromatic), 7.54-7.50 (m, 4H, aromatic), 7.45 (d, *J = 8.3 Hz*, 1H, aromatic), 7.36 (t, *J = 7.5 Hz*, 1H, aromatic), 6.86 (d, *J = 8.8 Hz*, 2H, aromatic), 4.10 (brs, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.77 (s, 3H, methoxy), 3.51 (s, 3H, *N*-methyl), 3.25-3.17 (m, 4H, piperazinyl (CN(CH_2)$_2$)); ESI-MS *m/z* 477 (M + 1^+, 100%); Anal. Calcd for $C_{30}H_{28}N_4O_2$: C, 75.61; H, 5.92; N, 11.76, Found: C, 75.65; H, 5.88; N, 11.71.

*4.1.7.6. (4-(3-methoxyphenyl)piperazin-1-yl) (9-methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (**7f**).* White solid; Yield 74%; mp 196–198 °C; R_f 0.35 (Hexane: EtOAc = 6:4); IR νmax/cm^{-1}(KBr): 3057 (C–H, aromatic stretching), 1625 (C=O, stretching), 1556 (C=C, aromatic stretching), 1454 (C–H, bending), 1253 (C–O, stretching); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (s, 1H, aromatic), 8.21 (d, *J = 7.8 Hz*, 1H, aromatic), 7.66-7.62 (m, 3H, aromatic), 7.56-7.50 (m, 3H, aromatic), 7.46 (d, *J = 8.3 Hz*, 1H, aromatic), 7.36 (t, *J = 7.5 Hz*, 1H, aromatic), 7.20 (t, *J = 8.0 Hz*, 1H, aromatic), 6.62-6.48 (m, 3H, aromatic), 4.13-4.07 (m, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.80 (s, 3H, methoxy), 3.52 (s, 3H, *N*-methyl), 3.36-3.26 (m, 4H, piperazinyl (CN(CH_2)$_2$)); ESI-MS *m/z* 477 (M + 1^+, 100%); Anal. Calcd for $C_{30}H_{28}N_4O_2$: C, 75.61; H, 5.92; N, 11.76, Found: C, 75.58; H, 5.94; N, 11.79.

*4.1.7.7. (4-(2-methoxyphenyl)piperazin-1-yl) (9-methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (**7g**).* White solid; Yield 62%; mp 168–170 °C; R_f 0.35 (Hexane: EtOAc = 6:4); IR νmax/cm^{-1}(KBr): 3058 (C–H, aromatic stretching), 1643 (C=O, stretching), 1536 (C=C, aromatic stretching), 1496 (C–H, bending), 1240 (C–O, stretching); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (s, 1H, aromatic), 8.23 (d, *J = 7.8 Hz*, 1H, aromatic), 7.68-7.64 (m, 3H, aromatic), 7.58-7.52 (m, 3H, aromatic), 7.48 (d, *J = 8.3 Hz*, 1H, aromatic), 7.38 (t, *J = 7.5 Hz*, 1H, aromatic), 7.06-7.04 (m, 1H, aromatic), 6.96-6.90 (m, 3H, aromatic), 4.08 (brs, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.90 (s, 3H, methoxy), 3.54 (s, 3H, *N*-methyl), 3.22-3.12 (m, 4H, piperazinyl (CN(CH_2)$_2$)); ESI-MS *m/z* 477 (M + 1^+, 100%); Anal. Calcd for $C_{30}H_{28}N_4O_2$: C, 75.61; H, 5.92; N, 11.76, Found: C, 75.63; H, 5.97; N, 11.73.

*4.1.7.8. (4-(4-chlorophenyl)piperazin-1-yl) (9-methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (**7h**).* White solid; Yield 76%; mp 160–162 °C; R_f 0.40 (Hexane: EtOAc = 6:4); IR νmax/cm^{-1}(KBr): 3055 (C–H, aromatic stretching), 1633 (C=O, stretching), 1542 (C=C, aromatic stretching), 1471 (C–H, bending), 1062 (C–Cl, stretching); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (s, 1H, aromatic), 8.24 (d, *J = 7.8 Hz*, 1H, aromatic), 7.68-7.64 (m, 3H, aromatic), 7.56-7.53 (m, 3H, aromatic), 7.50-7.48 (m, 1H, aromatic), 7.41-7.37 (m, 1H, aromatic), 7.26 (d, *J = 8.9 Hz*, 2H, aromatic), 7.02-6.95 (m, 2H, aromatic), 4.10 (brs, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.54 (s, 3H, *N*-methyl), 3.34-3.25 (m, 4H, piperazinyl (CN(CH_2)$_2$)); ESI-MS *m/z* 481 (M + 1^+, 100%), 483 (M + 3^+, 33%); Anal. Calcd for $C_{29}H_{25}ClN_4O$: C, 72.42; H, 5.24; N, 11.65, Found: C, 72.39; H, 5.26; N, 11.63.

*4.1.7.9. (4-(3-chlorophenyl)piperazin-1-yl) (9-methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (**7i**).* White solid; Yield 70%; mp 130–132 °C; R_f 0.42 (Hexane: EtOAc = 6:4); IR νmax/cm^{-1}(KBr): 3069 (C–H, aromatic stretching), 1614 (C=O, stretching), 1534 (C=C, aromatic stretching), 1487 (C–H, bending), 1054 (C–Cl, stretching); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.95 (s, 1H, aromatic), 8.50 (s, 1H, aromatic), 8.27-8.19 (m, 2H, aromatic), 7.69-7.60 (m, 5H, aromatic), 7.59-7.56 (m, 1H, aromatic), 7.51-7.48 (m, 1H, aromatic), 7.46-7.44 (m, 1H, aromatic), 7.42-6.86 (m, 2H, aromatic), 4.03 (m, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.54 (s, 3H, *N*-methyl), 3.38-3.25 (m, 4H, piperazinyl (CN(CH_2)$_2$)); ESI-MS *m/z* 481 (M + 1^+, 100%), 483 (M + 3^+, 33%); Anal. Calcd for $C_{29}H_{25}ClN_4O$: C, 72.42; H, 5.24; N, 11.65, Found: C, 72.46; H, 5.27; N, 11.68.

*4.1.7.10. (4-(2-chlorophenyl)piperazin-1-yl) (9-methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (**7j**).* White solid; Yield 66%; mp 152–154 °C; R_f 0.40 (Hexane: EtOAc = 6:4); IR νmax/cm^{-1}(KBr): 3043 (C–H, aromatic stretching), 1629 (C=O, stretching), 1556 (C=C, aromatic stretching), 1435 (C–H, bending), 1068 (C–Cl, stretching); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (s, 1H, aromatic), 8.21 (d, *J = 7.8 Hz*, 1H, aromatic), 7.65-7.62 (m, 3H, aromatic), 7.56-7.44 (m, 4H, aromatic), 7.46-7.44 (m, 2H, aromatic), 7.24-7.20 (m, 1H, aromatic), 7.05-6.97 (m, 2H, aromatic), 4.04 (brs, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.51 (s, 3H, *N*-methyl), 3.19-3.08 (m, 4H, piperazinyl (CN(CH_2)$_2$)); ESI-MS *m/z* 481 (M + 1^+, 100%), 483 (M + 3^+, 33%); Anal. Calcd for $C_{29}H_{25}ClN_4O$: C, 72.42; H, 5.24; N, 11.65, Found: C, 72.40; H, 5.20; N, 11.64.

*4.1.7.11. (9-Methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl) (4-(4-nitrophenyl)piperazin-1-yl)methanone (**7k**).* Yellow solid; Yield 70%; mp 200–202 °C; R_f 0.30 (Hexane: EtOAc = 6:4); IR νmax/cm^{-1}(KBr): 3053 (C–H, aromatic stretching), 1633 (C=O, stretching), 1597 (C=C, aromatic stretching), 1471 (C–H, bending), 1385 (N–O, stretching); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.61 (s, 1H, aromatic), 8.24 (d, *J* = 7.8 Hz, 1H, aromatic), 8.15 (d, *J* = 9.3 Hz, 2H, aromatic), 7.70-7.63 (m, 3H, aromatic), 7.61-7.53 (m, 3H, aromatic), 7.49 (d, *J* = 8.4 Hz, 1H, aromatic), 7.39 (t, *J* = 7.5 Hz, 1H, aromatic), 6.83 (d, *J* = 9.4 Hz, 2H, aromatic), 4.16-4.06 (m, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.62-3.54 (m, 7H, piperazinyl (CN(CH_2)$_2$), *N*-methyl); ESI-MS *m/z* 492 (M + 1^+, 100%); Anal. Calcd for $C_{29}H_{25}N_5O_3$: C, 70.86; H, 5.13; N, 14.25, Found: C, 70.82; H, 5.12; N, 14.28.

*4.1.7.12. (4-(4-fluorophenyl)piperazin-1-yl) (9-methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (**7l**).* White solid; Yield 76%; mp 188–190 °C; R_f 0.40 (Hexane: EtOAc = 6:4); IR νmax/cm^{-1}(KBr): 3057 (C–H, aromatic stretching), 1614 (C=O, stretching), 1550 (C=C, aromatic stretching), 1487 (C–H, bending), 1036 (C–F, stretching); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (s, 1H, aromatic), 8.23 (d, *J = 7.7 Hz*, 1H, aromatic), 7.68-7.65 (m, 3H, aromatic), 7.58-7.53 (m, 3H, aromatic), 7.48 (d, *J = 8.4 Hz*, 1H, aromatic), 7.38 (td, *J = 7.6, 0.8 Hz*, 1H, aromatic), 7.06-6.96 (m, 2H, aromatic), 6.96-6.88 (m, 2H, aromatic), 4.06-4.05 (m, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.54 (s, 3H, *N*-methyl), 3.26-3.16 (m, 4H, piperazinyl (CN(CH_2)$_2$)); ESI-MS *m/z* 465 (M + 1^+, 100%); Anal. Calcd for $C_{29}H_{25}FN_4O$: C, 74.98; H, 5.42; N, 12.06, Found: C, 74.94; H, 5.45; N, 12.03.

*4.1.7.13. (4-(2-fluorophenyl)piperazin-1-yl) (9-methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (**7m**).* White solid; Yield 68%; mp 168–170 °C; R_f 0.45 (Hexane: EtOAc = 6:4); IR νmax/cm^{-1}(KBr): 3055 (C–H, aromatic stretching), 1633 (C=O, stretching), 1544 (C=C, aromatic stretching), 1435 (C–H, bending), 1026 (C–F, stretching); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.53 (s, 1H, aromatic), 8.21 (d, *J = 7.8 Hz*, 1H, aromatic), 7.65-7.62 (m, 3H, aromatic), 7.55-7.50 (m, 3H, aromatic), 7.45 (d, *J = 8.3 Hz*, 1H, aromatic), 7.36 (t, *J = 7.5 Hz*, 1H, aromatic), 7.07-6.93 (m, 4H, aromatic), 4.05-4.04 (m, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.51 (s, 3H, *N*-methyl), 3.23-3.13 (m, 4H, piperazinyl (CN(CH_2)$_2$)); ESI-MS *m/z* 465 (M + 1^+, 100%);

Anal. Calcd for $C_{29}H_{25}FN_4O$: C, 74.98; H, 5.42; N, 12.06, Found: C, 74.96; H, 5.39; N, 12.10.

*4.1.7.14. (4-(2,3-dichlorophenyl)piperazin-1-yl) (9-methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (**7n**).* White solid; Yield 74%; mp 166–168 °C; R_f 0.32 (Hexane: EtOAc = 6:4); IR νmax/cm^{-1}(KBr): 3059 (C–H, aromatic stretching), 1614 (C=O, stretching), 1524 (C=C, aromatic stretching), 1438 (C–H, bending), 1056 (C–Cl, stretching); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (s, 1H, aromatic), 8.21 (d, *J* = *7.8 Hz*, 1H, aromatic), 7.67-7.62 (m, 3H, aromatic), 7.58-7.48 (m, 3H, aromatic), 7.46 (d, *J* = *8.3 Hz*, 1H, aromatic), 7.36 (t, *J* = *7.5 Hz*, 1H, aromatic), 7.20-7.11 (m, 2H, aromatic), 6.95 (dd, *J* = *7.6, 1.9 Hz*, 1H, aromatic), 4.04 (brs, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.51 (s, 3H, *N*-methyl), 3.18-3.08 (m, 4H, piperazinyl (CN(CH_2)$_2$)); ESI-MS *m*/*z* 516 (M + 1$^+$, 100%), 517 (M + 2$^+$), 518 (M + 3$^+$); Anal. Calcd for $C_{29}H_{24}Cl_2N_4O$: C, 67.58; H, 4.69; N, 10.87, Found: C, 67.62; H, 4.64; N, 10.91.

*4.1.7.15. (9-Methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl) (4-(pyridin-4-yl)piperazin-1-yl)methanone (**7o**).* Yellowish white solid; Yield 68%; mp 198–200 °C; R_f 0.35 (Hexane: EtOAc = 2:8); IR νmax/cm^{-1} (KBr): 3045 (C–H, aromatic stretching), 1620 (C=O, stretching), 1558 (C=C, aromatic stretching), 1456 (C–H, bending); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (s, 1H, aromatic), 8.31 (d, *J* = *5.4 Hz*, 2H, aromatic), 8.24 (d, *J* = *7.8 Hz*, 1H, aromatic), 7.68-7.62 (m, 3H, aromatic), 7.58-7.55 (m, 3H, aromatic), 7.50 (d, *J* = *8.4 Hz*, 1H, aromatic), 7.38 (t, *J* = *7.5 Hz*, 1H, aromatic), 6.68 (d, *J* = *6.0 Hz*, 2H, aromatic), 4.10-4.03 (m, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.51 (s, 3H, *N*-methyl), 3.53-3.42 (m, 4H, piperazinyl (CN(CH_2)$_2$)); ESI-MS *m*/*z* 448 (M + 1$^+$, 100%); Anal. Calcd for $C_{28}H_{25}N_5O$: C, 75.15; H, 5.63; N, 15.65, Found: C, 75.19; H, 5.68; N, 15.68.

*4.1.7.16. (9-Methyl-1-phenyl-9H-pyrido[3,4-b]indol-3-yl) (4-(pyridin-2-yl)piperazin-1-yl)methanone (**7p**).* Yellowish white solid; Yield 62%; mp 174–176 °C; R_f 0.30 (Hexane: EtOAc = 2:8); IR νmax/cm^{-1} (KBr): 3053 (C–H, aromatic stretching), 1614 (C=O, stretching), 1538 (C=C, aromatic stretching), 1471 (C–H, bending); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (s, 1H, aromatic), 8.24-8.21 (m, 2H, aromatic), 7.69-7.65 (m, 3H, aromatic), 7.58-7.47 (m, 5H, aromatic), 7.38 (t, *J* = *7.5 Hz*, 1H, aromatic), 6.69-6.66 (m, 2H, aromatic), 4.02 (brs, 4H, piperazinyl (O=CN(CH_2)$_2$)), 3.71-3.69 (m, 4H, piperazinyl (CN(CH_2)$_2$)), 3.55 (s, 3H, *N*-methyl); ESI-MS *m*/*z* 448 (M + 1$^+$, 100%); Anal. Calcd for $C_{28}H_{25}N_5O$: C, 75.15; H, 5.63; N, 15.65, Found: C, 75.12; H, 5.61; N, 15.62.

4.2. Biological evaluation

Anti-microbial susceptibility testing was performed in black, clear-bottomed, 96-well microplates (black view plates; Packard Instrument Company, Meriden, Conn.) in order to minimize background fluorescence. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Initial drug dilutions were prepared in either dimethyl sulfoxide or distilled deionized water and subsequent two fold dilutions were performed in 0.1 ml of 7H9GC (no Tween 80) in the microplates. BACTEC 12B-passaged inocula were initially diluted 1:2 in 7H9GC and 0.1 ml was added to wells. Subsequent determination of bacterial titers yielded 1×10^6, 2.5×10^6 and 3.25×10^5 CFU/ml in plate wells for H37Rv. Frozen inocula were initially diluted 1:20 in BACTEC 12B medium followed by a 1:50 dilution in 7H9GC. Addition of 1/10 ml to wells resulted in final bacterial titers of 2.0×10^5 and 5×10^4 CFU/ml for H37Rv. Wells containing drug only were used to detect auto fluorescence of compounds. Additional control wells consisted of bacteria only (B) and medium only (M). Plates were incubated at 37 °C. Starting at day 4 of incubation, 20 μl of 10X alamar blue solution (Alamar Biosciences/Accumed, Westlake, Ohio) and 12.5 μl of 20% Tween 80 were added to one B well and one M well and plates were re-incubated at 37 °C. Wells were observed at 12 and 24 h for a color change from blue to pink and for a reading of ≥50,000 fluorescence units (FU). Fluorescence was measured in a Cytofluor II microplate fluorometer (PerSeptive Biosystems, Framingham, Mass.) in bottom-reading mode with excitation at 530 nm and emission at 590 nm. If the B wells became pink by 24 h, reagent was added to the entire plate. If the well remained blue or ≤50,000 FU was measured, additional M and B wells were tested daily until a color change occurred, at which time reagents were added to all remaining wells. Plates were then incubated at 37 °C and results were recorded at 24 h post-reagent addition. Visual MICs were defined as the lowest concentration of drug that prevented a color change. For fluorometric MICs, a background subtraction was performed on all wells with a mean of triplicate M wells. Percent inhibition was defined as 1 - (test well FU/mean FU of triplicate B wells) × 100. The lowest drug concentration effecting an inhibition of ≥90% was considered as MIC.

4.3. Cytotoxicity evaluation

Vero cells (ATCC CRL-1586) were cultured in 10% fetal bovine serum (FBS) in minimum essential medium Eagle. J774A.1 cells were cultured in 10% FBS in Dulbecco's Modified Eagle's Medium (DMEM). The cells were incubated at 37 °C under 5% CO_2 until confluent and then diluted with phosphate-buffered saline to 10^6 cells/mL. In a transparent 96-well plate (Falcon Microtest 96), threefold serial dilutions of the macrolide stock solutions resulted in final concentrations of 102.4 to 0.42 μM in a final volume of 200 μl. After incubation at 37 °C for 72 h, medium was removed and monolayers were washed twice with 100 μl of warm Hanks' Balanced Salt Solution (HBSS). One hundred microliters of warm medium and 20 μl of freshly made MTS-PMS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium and phenylmethasulfazone] (100:20) (Promega) were added to each well, plates were incubated for 3 h and absorbance was determined at 490 nm.

Competing interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.10.024

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