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Graphical Abstract

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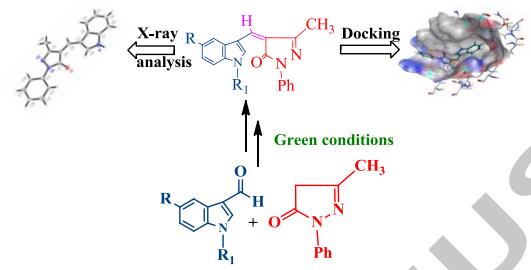

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Design, synthesis & biological evaluation of indolylidinepyrazolones as potential anti-bacterial agents

*A.Indrasena**[1], Sd.Riyaz[†,#,1], Prema L.Mallipeddi[†,#,2], P.Padmaja[1], B.Sridhar[3], P.K.Dubey[1]*

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ABSTRACT

A series of indolylidinepyrazolones were synthesized using a simple, green & effective route and evaluated as anti-bacterial agents. The compounds were further studied via structure-guided docking study. One of the compounds exhibiting H-bonding interactions with conserved residue Arg144 turned out to be the most potent compound of the series. The minimum inhibitory concentration values ranged from 50 to 25 ug/mL against ***Staphylococcus aureus*** in their anti microbial evaluation.

Pyrazolone is a key structure in numerous compounds of therapeutic importance.[1] Pyrazolones are gaining importance especially in drug discovery studies against cerebralischaemia[2] and cardiovascular diseases.[3] Due to its diverse pharmacological properties, the chemistry of pyrazolones is gaining attention, and there have been numerous methodologies reported recently.[4] These reports prompted us to synthesize new compounds containing pyrazolone as one of the constituent units.

The importance of indoles is also well recognized[5] by synthetic as well as biological chemists. In continuation of our earlier work on Knoevenagel condensation of heterylaldehydes with active methylene compounds,[6] it was considered worthwhile to couple both the moieties *i.e.* pyrazolone and the indole ring system. The condensation of indole-3-carboxaldehyde with pyrazolone was reported by Suzaldev[7] *et.al* using piperidine as catalyst in tertiary butanol under refluxing to obtain the product in 90% yield and by Azev[8] *et.al* in ethanol under refluxing to obtain the product in 75% yield. Herein we report a, water mediated and uncatalyzed Knoevenagel condensation of indole-3-carboxaldehydes with pyrazolone at 100 ^{o}C for about 30 min in 90-92 % yield and the alkylation studies in aq. NaOH at room temperature.

DNA gyrase is a heterodimer (A2B2). DNA gyraseA subunit is involved in breakage and reunion of DNA double strand. The B subunit has ATPase activity and provides energy for DNA supercoiling.[9] There are two classes of DNA gyrase inhibitors – Coumarins target DNA gyrase B and quinolones target DNA gyraseA. Notable among the DNA gyraseA inhibitors is the ciprofloxacin, which has been successfully used as broad spectrum antibiotic. Novobiocin was licensed for clinical use against *Staphylococcus aureus.*[10,11] However, both the drugs exhibit resistance and other limitations such as toxicity.[12,13] Among novel DNA gyraseA inhibitors, the 6-fluoroquinolone classes of DNA gyrase inhibitors are in clinical practice.[14] DNA GyrB which has the ATP binding site is a potential site for novel antibacterial and anti-cancer therapeutics.[15] High-resolution inhibitor crystal structures with courmarins as well as other inhibitor classes are excellent sources for structure guided drug discovery.[16,17,18]

Here we aim to identify a novel class of antibacterial agents potentially targeting DNA gyrase inhibitors via structure based drug design method. A small virtual library of pyrazolones was developed and docked against the Staphylococcus GyrB crystal structure. Preliminary analysis revealed potential for optimization. Analogs of compounds were virtually designed and docked. Compounds exhibiting favorable interactions were subsequently synthesized. Biological results validate crucial interactions predicted via docking studies. Current study provides novel chemical class of DNA gyrase inhibitors with scope for further optimization.

Docking Studies: In order to validate our docking methodology, we docked crystal ligand against Staphylococcus aureus GyrB (PDB ID: 3G7B) active site. The top ranked pose replicated crystal pose with an RMSD of 1.08 Å. Next, we docked the first compound of the series –i.e. compound **3a** (Table-2). Analysis of the top ranked docked pose revealed the indole moiety fits in a hydrophobic pocket formed by residues Ile102, Leu103, Ser128, Ser129 (Fig. 1).

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[#] PLM and SR contributed equally.

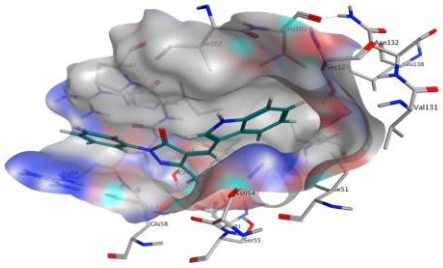


Fig. 1: Docked pose of Compound **3a** reveals indole ring occupies hydrophobic pocket formed by residues Ile102, Leu103, Ser138 and Ser129

The carbamate of inhibitor in the co-crystal structure points towards this hydrophobic region.[17] Indeed, this hydrophobic region was proposed to render specificity for S. aureus inhibitors versus E. coli. Addition of alkyl chain extending further into hydrophobic pocket might contribute to favorable interactions with Ile51 while rendering specificity. Docking studies reveal the butyl chain optimally fills in hydrophobic pocket. Accordingly, N-alkyl indole analogs were synthesized and the compounds (Compounds **4a-c** Table-2) exhibited improved activity. Substituting a more bulky phenyl group in place of alkyl groups resulted in loss of activity (compound **4d** Table-2), as there is not enough space for such a bulky group to occupy the hydrophobic region formed by Ile51. Compound **4e** was synthesized by substituting a sulfonyl group in order to capture H-bonding interaction with Asn54 in addition to hydrophobic interactions with Ile51. Hydrogen-bonding interactions of the sulfonyl group contributed to favorable binding resulting in compound **4e** exhibiting activity vs. compound **4d,** which is inactive. Thus, the binding pose predicted by docking is consistent with biological results.

The predicted binding pose of compound **3a** (**Fig 1**) also shows potential for hydrogen or halogen bonding interaction with Ser129. Halogen bonding has been established as a promising tool in drug design.[19] Thus, a series of bromine substituted indolydinepyrazolone analogs were synthesized and biologically tested. However, the series did not demonstrate significant enhancement in activity. Hence, further exploration of halogen has been withheld**.**

With the limited success gained from the bromine series, a series of nitro substituted indolydinepyrazolone analogs were synthesized to capture hydrogen-bonding interactions. Addition of a nitro group resulted in most potent compound of the series (compound **4i**, Table 2, Fig. 2). Docking of the nitro series reveals alternative binding pose for the indolylidinepyrazolone series. The nitro group of the top ranked pose of the most active compound 4i exhibited H-bonding interactions with Arg144 and is close to Arg84. Arg136 (E. coli numbering, equivalent to Arg144) is a crucial residue, interactions with which contribute to tight binding for coumarin inhibitors including novobiocin.[18,20,21] Additionally mutational studies of Arg136 resulted in loss of antibacterial activity

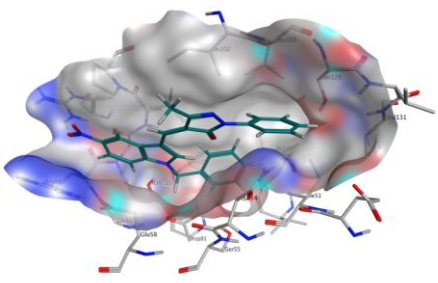


Fig. 2: Docked pose of compound **4i** reveals H-bonding interaction with Arg144. N-methylphenyl exhibits Vander Waals interactions with Ile86 while phenyl on the pyrazole ring interacts with Ile102.

further confirming the importance this residue.[22,23] The N-methylphenyl moiety of compound **4i** (Table-2, Fig. 2), exhibits hydrophobic interactions with Ile86. The phenyl on the pyrazole ring compound **4i** (Table-2, Fig. 2) exhibits hydrophobic interactions with Ile102. Among the nitro series, N-alkylated analogues did not have major impact on biological activity unlike N-alkyl analogs compound (**4a-d**). Docking analysis of the N-alkylated compounds (**4f-h**) reveals the substitution results in a conformation where the hydrophobic groups (N-alkylated or N-sulfonyl phenyl) points away from the hydrophobic residue Ile86. The binding pose however exhibits H-bonding interactions with Arg144. Co-crystal structure of Staphylococcus aureusGyrB with a pyrazolo-thiazole compound as well as coumarins reveal crucial interactions with an Asp and a conserved crystal water.[18,20] Addition of a sulfonyl (compound **4j**), in order to capture H-bonding interactions with Asp81, shifted the bulky rings away from the hydrophobic pockets rending the compound inactive.

There is substantial potential to further explore the SAR of the indolylidinepyrazolone analogs as discussed below. Co-crystal structures of coumarins reveal H-bonding interaction with Asp73 and a water mediated interaction with Thr165.[18,20] Addition of a functional group in place of the crystal water to the indole ring might help in capturing these crucial interactions as well as additional interactions with Glu58 and Arg84. Mutation of Ile175 conferred resistance to coumermycin, known to bind to DNA gyrase B and inhibit supercoiling activity.[24] Thus, the N-methyl phenyl analog (compound **4i**) can further be optimized by extending the methyl chain length to tightly fit into the hydrophobic pocket formed by residues Ile175, Ile51 and L103 (**Fig. 2**).

As shown in Scheme 1, indole-3-carboxaldehyde **(1a)** was condensed with pyrazolone **(2)** in water at 100 ^{o}C for about 30 min. The progress of the reaction was monitored by TLC, after completion of the reaction, the separated product **3a** was filtered and dried. The melting point of **3a** was matched with the literature report[8] (mp found 238-240^{o}C, Lit mp 238-240^{o}C). However, the structure of the product was assigned as **3a** on the basis of its IR, ^{1}H-NMR and Mass spectral data. The structure of the product **3a** was further confirmed by the X-ray crystallography (**Fig. 3**).[25]

The Knoevenagel condensation of indole-3-carboxaldehyde with pyrazolone was carried out in various solvents such as DMF, DMSO, acetic acid and toluene at 100 ^{o}C and in methanol, ethanol, and acetonitrile at their refluxing temperature (Table-1). Among these solvents, water was found to be the best solvent for the reaction in terms of yield and reaction time. The above reaction was found to be general and extended to other substituted indole-3-carboxaldehydes, i.e. **1b**&**1c**.[26] In this methodology, the indolylidinepyrazolones are isolated by simple filtration and as a result of which yield losses are avoided. The reactions were completed in a shorter time and under milder reaction conditions. By considering these aspects, our method shows the following advantages: cleaner synthesis, shorter time, higher selectivity, catalyst free, good yields and eco-friendly.

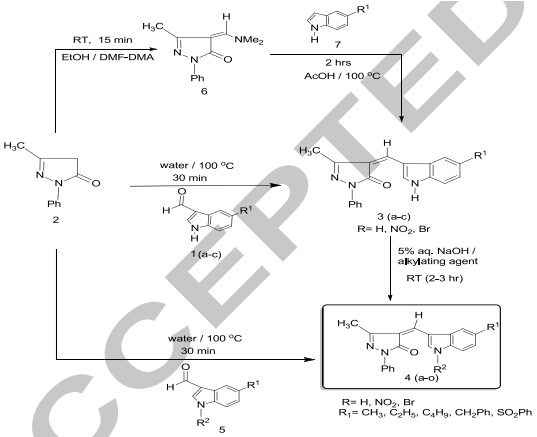
Treatment of **3a-3c** with one equivalent of alkylating agent / arylating agent (DMS, DES, n-Butyl-Cl, $PhCH_2Cl$ and $PhSO_2Cl$) in aqueous NaOH (5%) solution at RT gave N-substituted alkyl derivatives **4a-4o,** in good yields.[27] The structures of the products were assigned on the basis of IR, NMR & Mass spectral data. The absence of peak at 3525 cm^{-1} in IR and

the absence of signal at δ 12.63 in ^{1}H-NMR, supports that the alkylation's takes place on the –NH of indole. Alternatively **4a-o** could be synthesized by condensing N-substitutedindole-3-aldehydes **(5)** which were prepared by literature method,[28] with pyrazolone (**2)** in distilled water at 100 ^{o}C about 30 min. The reaction was completed by obtaining an orange coloured solid as the product.[29] The products obtained were identical in mp, mmp and Co-TLC with the corresponding derivatives prepared in the route **1→3→4**.

3 could also be prepared alternatively by the following synthetic route shown in Scheme 2. Thus, treatment of pyrazolone **2** with DMF-DMA in ethanol at RT yielded an intermediate 4-dimethylaminomethylene-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one **6,** which was isolated and characterized by spectral methods.[30] **6** on subsequent treatment with respective indoles **7** in AcOH at 100 ^{o}C gave products **3**,[31] which were found to be identical (in M.P, M.M.P & TLC) with those prepared through Scheme 1**.**

Table 1: Condensation of **1a** with **2** in different solvents

S.No	Solvent	Yield (%) of **3a**	Reaction time	Temparature
1	***Water***	***92***	***30 min***	100 ^{o}C
2	DMF	84	50 min	100 ^{o}C
3	DMSO	82	50 min	100 ^{o}C
4	AcOH	85	2 hrs	100 ^{o}C
5	Toluene	46	1 hrs	100 ^{o}C
6	MeOH	70	2 hrs	Reflux
7	EtOH	68	2 hrs	Reflux
8	CH_3CN	60	5 hrs	Reflux



Scheme 1

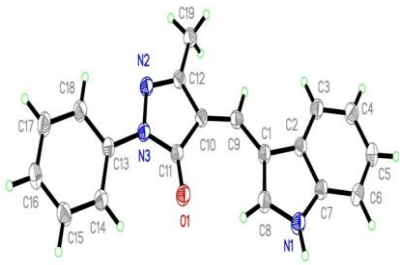
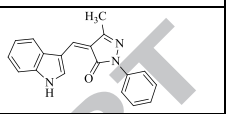
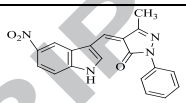
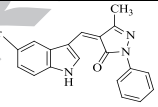
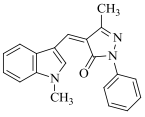
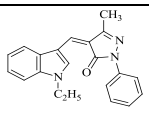
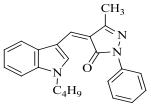
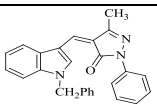
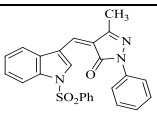
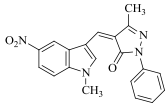
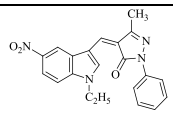
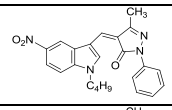
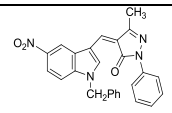
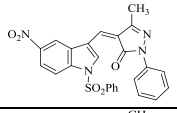
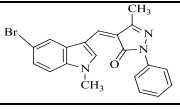
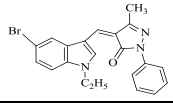


Fig. 3: X-ray crystal structure of **3a**

Determination of Minimum Inhibitory Concentration (MIC) for against S.aureus.

The test samples **4b** and **4i** showed good inhibition with MIC value of 25 and 25 µg/ml respectively. **4a**, **4c**, **4e** and **4g** showed moderate inhibition. Remaining samples showed inhibition with an MIC range of 50-100 µg/ml. Standard Ciprofloxacin showed MIC value at15.6 µg/ml.

Table 2: Anti-bacterial activity of synthesized compounds **3a-c, 4a-o** tested against *Staphylococcus aureus*.

RR No.	MIC value (µg/ml)	Structure
3a	50	
3b	50	
3c	50	
4a	35	
4b	25	
4c	31.25	
4d	250	
4e	30	
4f	50	
4g	40	
4h	50	
4i	25	
4j	100	
4k	50	
4l	40	

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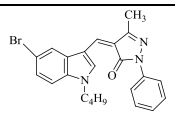
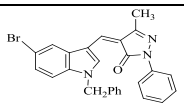
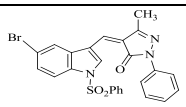
# PLM and SR contributed equally.

4m	40	
4n	40	
4o	250	

In conclusion, two therapeutically important moieties, pyrazolone and indole were coupled to identify a novel class of antibacterial agents potentially targeting DNA gyrase. An eco-friendly route was developed to synthesize the indolylidinepyrazolone analogs resulting in good yields. Compounds synthesized exhibited antimicrobial activities against *Staphycoccous aureus*. Docking studies revealed hydrogen-bonding interaction with Arg144 as a major contributing factor for favorable binding. Docking analysis also suggests that there remains considerable SAR that remains to be explored, as discussed. Nevertheless, it is encouraging that the initial set of leads designed based on DNA gyrase B exhibited antimicrobial activities.

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25. CCDC985703 contains supplementary crystallographic data for the structure **3a.**
26. Preparation of **3a-c**: A mixture of **1a-c** (5.0 mmol), **2** (0.87 gm 5.0 mmol) in 30 ml of distilled water refluxed at 100 ^{O}C for about 30 min. At the end of this period, the product was separated as an orange colour solid which was confirmed by TLC. The reaction mixture was, filtered, washed with ethanol and dried to obtain crude **3a-c.** The latter on recrystallization from ethyl acetate gave pure **3a-c.**
27. General procedure for preparation of **4a-o**: A mixture of **3a-c** (2 mmol), appropriate alkylating agent (2.2 mmol) (DMS, DES, n-Bu-Cl, $PhCH_2Cl$ or $PhSO_2Cl$) was stirred in 5 % aqueous NaOH solution (15 ml) at RT for 2-3 h. At the end of this period, the mixture was poured in to ice cold water (30 ml) and neutralized with HCl (15 ml). The separated solid was filtered, washed with ethanol (10 ml) and dried to obtain crude **4a-o,** which on recrystallization from suitable solvent to gave pure **4a-o.**
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29. Preparation of **4a-o** from **5** & **2** (General Procedure): A mixture of **5** (2.0 mmol) which were prepared by literature method,[28] pyrazolone **2** (0.35 gm 2.0 mmol), 30 ml of distilled water refluxed at 100 ^{O}C for about 30 min. At the end of this period, the product was separated as an orange colour solid. The reaction mass was, filtered, washed with 10 ml of ethanol and dried to obtain crude **4**, which on recrystallization from suitable solvent to gave pure **4.**
30. Preparation of **6** from **2**: A mixture of **2** (0.83 g, 5 mmol), DMF-DMA (0.15 ml, 5 mmol) and ethanol (10 ml) was stirred at RT for 15 min. At the end of this period, the separated solid was filtered, washed with ethanol (10 ml) and dried to obtain **6** (yield 0.67 g, 80%)**,** which on recrystallization from ethylacetate gave pure **6**.
31. Preparation of **3** from **6**: A mixture of **6** (1 mmol), respective indole **7** (1 mmol) and AcOH (10 ml) was heated at 100 ^{o}C for about 2 h on a steam bath. At the end of this period, the mixture was cooled to RT, the separated product was filtered, washed with cold acetic acid (10 ml) and dried to yield crude **3**, which on recrystallization from ethyl acetate gave pure **3.**