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Synthesis and biological evaluation of 1,3-diaryl pyrazole derivatives as potential antibacterial and anti-inflammatory agents

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ABSTRACT

Three series of 1,3-diaryl pyrazole derivatives bearing aminoguanidine or furan-2-carbohydrazide moieties have been synthesized, characterized and evaluated for antibacterial and anti-inflammatory activities. Most of the synthesized compounds showed potent inhibition of several Gram-positive bacterial strains (including multidrug-resistant clinical isolates) and Gram-negative bacterial strains with minimum inhibitory concentration values in the range of 1–64 μg/mL. Compounds **6g**, **6l** and **7l** presented the most potent inhibitory activity against Gram-positive bacteria (e.g. *Staphylococcus aureus* 4220), Gram-negative bacteria (e.g. *Escherichia coli* 1924) and the fungus, *Candida albicans* 7535, with minimum inhibitory concentration values of 1 or 2 μg/mL. Compared with previous studies, these compounds exhibited a broad spectrum of inhibitory activity. Furthermore, compound **7l** showed the greatest anti-inflammatory activity (93.59% inhibition, 30 min after intraperitoneal administration), which was more potent than the reference drugs ibuprofen and indomethacin.

The incidence of bacterial infection has increased rapidly during the past two decades.[1] Furthermore, many drug-resistant pathogens have emerged in recent years because of the increasing use or abuse of antibacterial agents for all kinds of human infectious diseases.[1–4] In addition to bacterial infection, many factors can lead to inflammation, such as biological agents, physical agents, chemical injuries and allergic reactions. Such conditions may lead to bacteremia, toxemia, septicemia and pyemia. As a result, the development of novel antibacterial and anti-inflammatory agents is crucial for ongoing effective therapeutic intervention.[5–8]

Pyrazoles occupy a distinct niche in heterocyclic chemistry and represent a key motif in medicinal chemistry because of their capability to exhibit an array of bioactivities such as antimicrobial,[9,10] anticancer,[11] anti-inflammatory,[12] antidepressant,[13] anticonvulsant,[14] antipyretic[15] and selective enzyme inhibitory[16] activities. In recent years, the guanidinium derivatives have been infrequently investigated as pharmaceutical antimicrobial agents.[17] In our previous work, we found that several pyrazole derivatives showed moderate to good activity against Gram-positive strains (including multidrug-resistant clinical isolates).[5,9] One of these derivatives, compound **A**, exhibited inhibitory activity with a minimum inhibitory concentration (MIC) value of 4 μg/mL (Fig. 1).[5] Unfortunately, however, none of these derivatives displayed any activity against Gram-negative bacteria, even at 64 μg/mL. Dibama et al. reported that compound **B** showed potent antibacterial activity against *Escherichia coli* with an MIC value of 32 μg/mL (Fig. 1).[18] In this work, as part of our ongoing studies toward the development of novel antibacterial agents, we designed new hybrid compounds in which the rhodanine moiety of **A** was replaced by a guanidine moiety or its isostere furan-2-carbohydrazide, simultaneously changing the substituents on the phenyl ring of the pyrazole, to investigate their effects on activity. Inspired from existing antibacterial agents bearing nitro groups, such as chloramphenicol that operates via inhibition of bacterial protein synthesis after the nitro group is metabolized to a hydroxyamino group, two nitro groups were introduced into the phenyl ring on the N^1 position of the pyrazole. Thus, three novel series of 1,3-diaryl pyrazole derivatives bearing aminoguanidine or furan-2-carbohydrazide moieties were designed, synthesized, and evaluated for their antimicrobial activities. Meanwhile, in light of the fact that some drugs contain a pyrazole moiety, such as phenazone and metamizole,[19] these compounds were also evaluated for their anti-inflammatory activity.

The synthesis of the target compounds is presented in Scheme 1.[22] Hydrazone derivatives (compounds **2** and **4**) were prepared by reacting substituted acetophenones with phenylhydrazine (or 2,4-dinitrophenylhydrazine) in the presence of glacial

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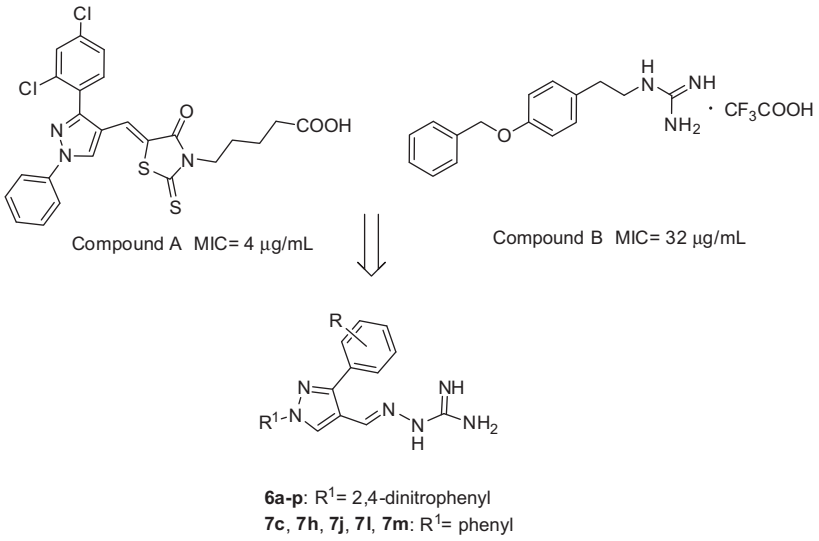
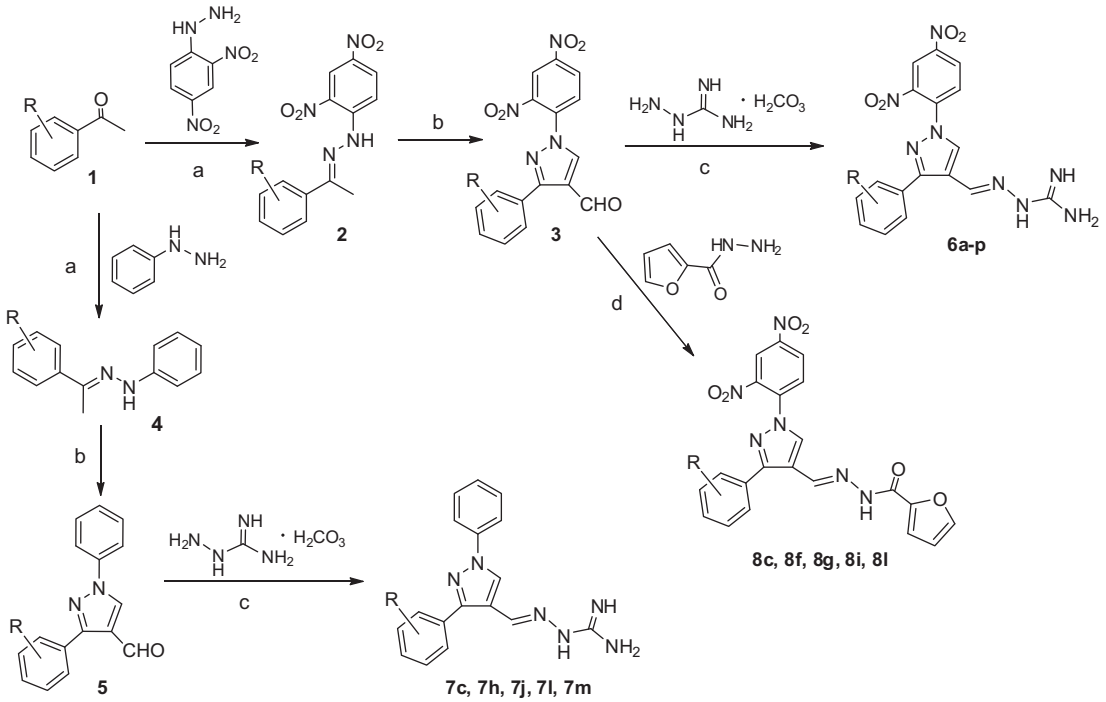


Figure 1. Previously reported compound **A**, **B** and the structure-based design of the target compounds.



Scheme 1. Synthetic scheme for the synthesis of compounds **6**–**8**. Reagents and conditions: (a) EtOH, AcOH or AcONa, reflux 5–7 h, (b) $POCl_3$, DMF, 0 °C 0.5 h, 80 °C 6 h, (c) EtOH, HCl, Reflux 20 h. (d) EtOH, reflux 3 h.

acetic acid in ethanol. Compound **2** (or compound **4**) reacted under Vilsmeier–Haack (DMF–$POCl_3$) conditions and afforded corresponding 4-carboxaldehyde functionalized pyrazole derivatives **3** (or **5**). In this reaction, four equivalents of the reagent, instead of three as described by Pascal Rathelot,[20] were necessary to obtain compounds **3** and **5** in good yield. Compounds **3** or **5** were then reacted with aminoguanidine bicarbonate in the presence of catalytic amounts of hydrochloric acid in ethanol to provide compounds in series **6** and **7**. Compounds in series **8** were prepared by reacting compounds **3** with furan-2-carbohydrazide in refluxing ethanol. The structures of the compounds synthesized were confirmed by IR, ^{1}H NMR, ^{13}C NMR, and mass spectroscopy.

In vitro anti-bacterial activities of the synthesized compounds were evaluated using a 96-well microtiter plate and a serial dilution method to obtain the Minimum Inhibitory Concentration (MIC) values for different strains (including multidrug-resistant clinical isolates). Four Gram-positive strains (*Staphylococcus aureus RN* 4220, *S. aureus KCTC* 209, *S. aureus KCTC* 503, *Streptococcus mutans* 3065), five Gram-negative strains (*Escherichia coli KCTC* 1924, *Escherichia coli CCARM* 1356, *Pseudomonas aeruginosa* 2742,

Pseudomonas aeruginosa 2004, *Salmonella typhimurium* 2421) and one fungus (*Candida albicans* 7535) were used for the biological assay. Gatifloxacin, moxifloxacin, norfloxacin, and oxacillin were used for the antibacterial activity, fluconazole and itraconazole for the antifungal activity as positive controls. In vivo inflammatory inhibition assay was carried out using xylene-induced ear edema[21] model in mice. Indomethacin and ibuprofen were used as positive controls.

As indicated in Table 1,[23] most of the tested compounds except for the compounds in series **8** showed potent antibacterial and antifungal activity against different strains including Gram-positive strains (*Staphylococcus aureus* and *Streptococcus mutans*), Gram-negative strains (*E. coli, Salmonella typhimurium* and *Pseudomonas aeruginosa*) and one fungus (*Candida albicans* 7535), with MIC values in the range of 1–64 μg/mL. As a whole, the compounds in series **6** exhibited more potent anti-bacterial activities against all microorganisms compared with series **7**. Compounds **6c**, **6d**, **6g**, **6h**, **6l**, **6m**, **6n** and **7l** showed the strongest activity with MIC values of 2 μg/mL, equivalent to those of the standard drug moxifloxacin (MIC = 2 μg/mL) and gatifloxacin (MIC = 2 μg/mL) against *S. aureus KCTC* 209 and 503. Compounds **6g**, **6h**, **6l** and **7l** displayed the same inhibitory effect as fluconazole with MIC values of 1 μg/mL against *C. albicans* 7535. In particular, compound **7l** (MIC = 1 μg/mL) exhibited more potent activity than the positive control moxifloxacin (MIC = 2 μg/mL) and gatifloxacin (MIC = 2 μg/mL) against *E. coli* 1924. Compounds **6c**, **6g**, **6h** and **6l** represented a 4-fold increase in potency relative to the standard drug gatifloxacin (MIC = 16 μg/mL) with MIC values of 4 μg/mL, and **6a**, **6b**, **6d**, **6e**, **6j**, **6m**, **6n** and **7c** displayed a 16-fold increase in potency relative to the standard drug moxifloxacin (MIC = 128 μg/mL) with MIC values of 8 μg/mL against *E. coli* 1356. For the *P. aeruginosa* 2742 and 2004 strains, however, only compound **7c** exhibited good activity with MIC values of 32 μg/mL and 16 μg/mL, respectively.

As indicated in Table 2,[23] all of the synthesized compounds were also tested for their inhibitory activities against the clinical isolates of several different multidrug-resistant Gram-positive bacterial strains (*MRSA CCARM* 3167 and 3506, *QRSA CCARM* 3505 and 3519). The MIC values of the compounds differed greatly, ranging from 1 to 32 mg/mL. Compounds in series **6** and **7** exhibited more potent activity than the compounds in series **8**. Compounds **6g** and **7l** presented the highest activity with MIC values of 1 μg/mL against *MRSA CCARM* 3167, and compounds **6g**, **6h**, **6l** and **7l** showed the highest activity with MIC values of 1 μg/mL against

Table 1
Inhibitory activity (MIC, μg/mL) of compounds **6a-p**, **7c**, **7h**, **7j**, **7l**, **7m**, **8c**, **8f**, **8g**, **8i** and **8l** against strains of Gram-positive (*Staphylococcus aureus* and *Streptococcus mutans*) bacteria, Gram-negative (*Escherichia coli, Salmonela typhinurium* and *Pseudomonas aeruginosa*) bacteria and *Candida albicans* 7535

Compound	R	Gram-positive strains				Gram-negative strains					Fungus
		S. aureus			*S. Mutans*	*E. Coli*		*P. aeruginosa*		*S. typhimurium*	*C. albicans*
		4220[a]	209[b]	503[c]	3065[d]	1924[e]	1356[f]	2742[g]	2004[h]	2421[i]	7535[j]
6a	4-OCH_3	4	4	4	8	4	8	>64	>64	8	2
6b	3-OCH_3	4	4	4	8	4	8	>64	>64	8	2
6c	4-CH_3	2	2	2	4	2	4	>64	>64	4	2
6d	3-CH_3	2	2	2	4	2	8	>64	>64	8	2
6e	2,4-(CH3)2	2	4	4	4	2	8	>64	>64	8	2
6f	2-Br	4	8	4	16	4	16	>64	>64	16	2
6g	3-Br	1	2	2	4	2	4	>64	>64	4	1
6h	4-Br	2	2	2	2	2	4	>64	>64	4	1
6i	2-F	8	8	8	16	8	16	>64	>64	16	4
6j	4-F	4	4	4	8	4	8	>64	>64	8	2
6k	2-Cl	4	8	4	16	4	16	>64	>64	8	2
6l	3-Cl	1	2	2	4	2	4	>64	>64	4	1
6m	4-Cl	2	2	2	4	2	8	>64	>64	4	2
6n	2,4-(Cl)2	2	2	2	4	2	8	>64	>64	8	2
6o	H	8	8	8	16	8	16	>64	>64	16	4
6p	2-NO2	16	32	16	32	16	32	>64	>64	32	8
7c	4-CH_3	2	4	4	8	2	8	32	16	8	2
7h	4-Br	4	8	4	>64	8	>64	>64	>64	>64	4
7j	4-F	8	8	16	>64	8	>64	>64	>64	>64	8
7l	3-Cl	2	2	2	8	1	16	>64	>64	8	1
7m	4-Cl	16	>64	>64	>64	>64	>64	>64	>64	>64	>64
8c	4-CH_3	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
8f	2-Br	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
8g	3-Br	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
8i	2-F	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
8l	3-Cl	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
Gatifloxacin	—	0.25	2	4	0.50	2	16	1	1	0.50	0.50
Moxifloxacin	—	0.25	2	2	0.25	2	128	1	2	0.50	0.50
Fluconazole	—	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1
Itraconazole	—	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.06

n.d.: not determined.
S. aureus RN 4220: A genotype of *S. aureus*. *KCTC* (Korean Collection for Type Cultures). *CCARM* (Culture Collection Antimicrobial Resistant Microbes).
[a] *Staphylococcus aureus RN* 4220.
[b] *Staphylococcus aureus* 209.
[c] *Staphylococcus aureus* 503.
[d] *Streptococcus mutans* 3065.
[e] *Escherichia coli KCTC* 1924.
[f] *Escherichia coli CCARM* 1356.
[g] *Pseudomonas aeruginosa* 2742.
[h] *Pseudomonas aeruginosa* 2004.
[i] *Salmonella typhimurium* 2421.
[j] *Candida albicans* 7535.

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Table 2
Inhibitory activity (MIC, μg/mL) of compounds **6a–p**, **7c**, **7h**, **7j**, **7l**, **7m**, **8c**, **8f**, **8g**, **8i** and **8l** against clinical isolates of multidrug-resistant Gram-positive strains

Compound	R	Multidrug-resistant Gram-positive strains			
		MRSA		QRSA	
		3167[a]	3506[b]	3505[c]	3519[d]
6a	4-OCH_3	4	2	4	4
6b	3-OCH_3	4	2	4	4
6c	4-CH_3	2	2	2	2
6d	3-CH_3	2	2	2	4
6e	2,4-(CH3)2	2	2	2	4
6f	2-Br	4	4	4	8
6g	3-Br	1	1	2	2
6h	4-Br	2	1	2	2
6i	2-F	8	4	8	8
6j	4-F	4	4	4	4
6k	2-Cl	4	4	4	8
6l	3-Cl	2	1	2	2
6m	4-Cl	2	2	2	2
6n	2,4-(Cl)2	2	2	2	2
6o	H	8	8	8	8
6p	2-NO2	16	8	16	32
7c	4-CH_3	2	2	4	4
7h	4-Br	8	4	16	16
7j	4-F	8	8	16	16
7l	3-Cl	1	1	2	2
7m	4-Cl	>64	>64	>64	>64
8c	4-CH_3	>64	>64	>64	>64
8f	2-Br	>64	>64	>64	>64
8g	3-Br	>64	>64	>64	>64
8i	2-F	>64	>64	>64	>64
8l	3-Cl	>64	>64	>64	>64
Gatifloxacin	—	2	2	8	4
Moxifloxacin	—	1	1	4	4
Norfloxacin	—	8	4	>64	>64
Oxacillin	—	>64	>64	1	1

[a] *Methicillin-resistant S. Aureus CCARM* 3167.
[b] *Methicillin-resistant S. Aureus CCARM* 3506.
[c] *Quinolone-resistant S. Aureus CCARM* 3505.
[d] *Quinolone-resistant S. Aureus CCARM* 3519.

Table 3
Anti-inflammatory activity of compounds **6a–p**, **7c**, **7h**, **7j**, **7l**, **7m**, **8c**, **8f**, **8g**, **8i** and **8l** administrated ip

	R	Dose (mg/kg)	Number of mice	Edema mean ± SD (mg)	Inhibition rate (%)
DMSO	—	—	10	12.99 ± 0.71	—
Indometacin	—	50	10	7.11 ± 0.50***	45.23
Ibuprofen	—	50	10	9.15 ± 0.58*	29.56
6a	4-OCH3	50	10	1.75 ± 0.43***	86.53
6b	3-OCH_3	50	10	4.25 ± 1.95***	67.28
6c	4-CH_3	50	10	1.63 ± 0.30***	87.43
6d	3-CH_3	50	10	3.00 ± 0.74***	76.90
6e	2,4-(CH3)2	50	10	2.65 ± 0.65***	79.60
6f	2-Br	50	10	2.85 ± 0.47***	78.06
6g	3-Br	50	10	2.38 ± 1.04***	81.65
6h	4-Br	50	10	3.95 ± 0.67***	69.59
6i	2-F	50	10	1.67 ± 0.60***	87.17
6j	4-F	50	10	4.95 ± 1.62***	61.89
6k	2-Cl	50	10	2.00 ± 0.32***	84.60
6l	3-Cl	50	10	2.28 ± 0.36***	82.42
6m	4-Cl	50	10	3.70 ± 0.69***	71.52
6n	2,4-$(Cl)_2$	50	10	4.95 ± 2.11***	61.89
6o	H	50	10	1.70 ± 0.48***	86.91
6p	2-NO_2	50	10	5.42 ± 0.55***	58.30
7c	4-CH_3	50	10	1.75 ± 0.61***	86.53
7h	4-Br	50	10	7.43 ± 1.11***	42.78
7j	4-F	50	10	5.17 ± 1.15***	61.38
7l	3-Cl	50	10	0.83 ± 0.26***	93.59
7m	4-Cl	50	10	7.02 ± 0.83***	45.96
8c	4-CH_3	50	10	8.32 ± 1.85*	35.95
8f	2-Br	50	10	10.72 ± 0.97	17.47
8g	3-Br	50	10	9.70 ± 1.45	25.32
8i	2-F	50	10	9.35 ± 1.44	28.02
8l	3-Cl	50	10	9.58 ± 1.04	26.23

*: $0.01 < p < 0.05$, ***: $p < 0.001$ compared with vehicle group.
—: no anti-inflammatory activity.

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Table 4
Anti-inflammatory activity of compounds **7l** administered orally at different times before xylene application

Time(h)	Dose (mg/kg)	Inhibition (%)	
		7l	Indometacin
1	50	29.69	23.02
2	50	9.93	23.60
3	50	50.86***	41.43***
4	50	4.39	34.89**
5	50	35.18	26.87
24	50	—	—

**: $0.001 < p < 0.01$, ***$p < 0.001$ compared with vehicle group.
—: no anti-inflammatory activity.

MRSA CCARM 3506, showed the same potency as moxifloxacin (MIC = 1 μg/mL), 4 to 8-fold more potent activity relative to the standard drug norfloxacin (MIC = 4–8 μg/mL), and 64-fold more potent activity relative to oxacillin (MIC > 64 μg/mL). Compounds **6c**, **6g**, **6h**, **6l**-**n** and **7l** exhibited 2 to 4-fold more potent activity relative to gatifloxacin and moxifloxacin (MIC = 4–8 μg/mL) with MIC values of 2 μg/mL against *QRSA CCARM* 3505 and 3519, but weaker than that of oxacillin (MIC = 1 μg/mL). Regretfully, compounds in series **8** did not exhibit any inhibitory activity against *MRSA CCARM* (3167 and 3506) and *QRSA CCARM* (3505 and 3519) strains.

Analysis of these results revealed several structure–activity relationships. First, a comparison of the activities across the three different series of compounds revealed a general order of antibacterial activity of **6** > **7** > **8**, which indicated that the aminoguanidine moiety was critical for the anti-bacterial activity of pyrazole derivatives. Second, none of the pyrazole derivatives previously reported by our group showed any activity against the Gram-negative bacteria *E. coli* 1924 and 1356, and *P. aeruginosa* 2742 and 2004.[5,9] However, the introduction of an aminoguanidine moiety resulted in moderate to good levels of inhibition against the Gram-negative strains. In series **7**, only the compound **7l** exhibited an equal potency to those compounds (most of which exhibited strong activity against all of the microorganisms tested) in series **6**, indicating that the 2,4-dinitrophenyl moiety is beneficial for the activity and the chloro substituent at the 3-position might be critical for the activity. Third, no clear pattern was found for the structure/activity relationship between the anti-bacterial activity and the position and physicochemical properties of different substituents on the phenyl ring, indicating that the electronic effect of the substituent on the benzene ring is not critical. As a result, derivatives **6g**, **6l** and **7l** are promising target compounds to be investigated further.

All of the synthesized compounds were also evaluated for their anti-inflammatory activity and the results are shown in Table 3.[24] In the primary screening, we used dimethyl sulfoxide as the vehicle and ibuprofen and indomethacin as the reference drugs. In this test, compounds were screened in a xylene-induced ear-edema test in mice, in which anti-inflammatory activity was assessed by the ability of the test compounds to prevent edema. The data revealed that most of the test compounds showed significant anti-inflammatory effects at 50 mg/kg administered via the intraperitoneal route, with inhibition ranging from 58.30% to 93.59%. It is worth noting that compound **7l** exhibited the strongest inflammatory inhibition at 93.59%, higher than ibuprofen (29.56%) and indomethacin (45.23%). Compounds **7h**, **7m** and **8c** displayed slightly improved activity compared with the reference drugs. Compounds **8f**, **8g**, **8i** and **8l**, however, did not exhibit any anti-inflammatory activity at the same dosage, indicating that the aminoguanidine moiety was more beneficial to biological activity than the furan-2-carbohydrazide moiety.

Table 5
Anti-inflammatory activity of compound **7l** administered orally at different doses

Time (h)	Dose (mg/kg)	Inhibition (%)	
		7l	Indometacin
3	50	53.66***	52.69***
3	25	39.28***	53.89***
3	12.5	56.67***	56.27***

***: $p < 0.001$ compared with vehicle group.

In view of its considerable anti-inflammatory activity, compound **7l** was chosen for further evaluation. A dose of 50 mg/kg was administered via the oral route at different intervals (1, 2, 3, 4, 5 and 24 h) for xylene application. As shown in Table 4,[24] the activity profile of compound **7l** was distinct from that of indomethacin, for which the activity showed no regularity as the interval lengthened. The activity of compound **7l** peaked at 3 h and exhibited more potent activity than indomethacin. In addition, the effect of dosage on the activity of compound **7l** was also tested at concentrations of 12.5, 25 and 50 mg/kg, 3 h after oral administration, and showed a maximal effect with an ear inflammation inhibition rate of 56.67% at 12.5 mg/kg. Whereas lower activity was observed at 25 mg/kg administered 3 h after xylene application (Table 5).[24]

In conclusion, based on our previous work, three novel series of 1,3-diaryl pyrazole derivatives bearing aminoguanidine or furan-2-carbohydrazide moieties were synthesized, characterized, and evaluated for antibacterial (including Gram-positive and Gram-negative bacterial strains) and anti-inflammatory activities. The results showed that most compounds exhibited moderate to good levels of antibacterial and anti-inflammatory activities. In particular, compounds **6g**, **6l** and **7l** exhibited the greatest antibacterial activities against Gram-positive bacterial strains (*S. aureus RN* 4220, *S. aureus KCTC* 209, *S. aureus KCTC* 503, *S. mutans* 3065, *MRSA CCARM* 3167 and 3506, *QRSA CCARM* 3505 and 3519), and Gram-negative strains (*E. coli KCTC* 1924, *E. coli CCARM* 1356, *S. typhimurium* 2421), and antifungal activity (*C. albicans* 7535) with MIC values of 1–8 μg/mL, higher than those observed for the positive controls. Compound **7c** exhibited a good level of broad antimicrobial inhibition against all of the strains (including *P. aeruginosa* 2742 and 2004). Regarding anti-inflammatory activity, compound **7l** showed the most potent ear inflammation inhibition rate (93.59%), which was higher than that of ibuprofen (29.56%) and indomethacin (45.23%) at 50 mg/kg (ip). These results suggested that introduction of aminoguanidine into the pyrazole ring was beneficial to antibacterial activity, providing valuable information for the design of Gram-negative bacteria inhibitors. The mechanism of action of these compounds remains unknown and further investigations are currently underway in our laboratories.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.10.028.

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22. Preparation of **6l**: First, compound **3l** (1 mmol) was dissolved in absolute ethanol (10–15 mL). Second, aminoguanidine bicarbonate (1.1 equiv) and catalytic amounts of hydrochloric acid were added. Then, the reaction mixtures were refluxed for 20 h. Finally, the solvent was evaporated under reduced pressure and the crude product was purified by silica gel column chromatography (dichloromethane/methanol, 15:1) to afford the desired compounds as yellow solid. Yield 81.59%; mp 236–238 °C. IR (KBr) cm^{-1}: 3456, 3238, 3120 (NH_2, NH), 1676 (C=N), 1548, 1354 (NO_2). ^1H NMR (DMSO-d_6, 300 MHz, ppm): δ 7.59–7.66 (m, 4H, Ar–H), 7.81 (br s, 3H, NH), 8.25 (d, 1H, J = 9 Hz, Ar–H), 8.28 (s, 1H, pyrazole-H), 8.74 (d, 1H, J = 9 Hz, Ar-H), 8.96 (d, 1H, J = 3 Hz, Ar-H), 9.28 (s, 1H, CH=N), 11.89 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz, ppm): δ 155.67, 152.10, 146.46, 143.14, 138.60, 135.56, 134.13, 133.40, 132.32, 131.40, 129.66, 128.75, 128.04, 127.30, 125.96, 121.86, 118.71. MS m/z 429 (M+1).
23. *Anti-bacterial activity assay:* The micro-organisms used in the present study were *S. aureus* (*S. aureus RN* 4220, *S. aureus KCTC* 503 and *S. aureus KCTC* 209), *S. mutans KCTC* 3065, *Escherichia coli* (*E. coli KCTC* 1924 and *E. coli CCARM* 1356), *Salmonella typhimurium* 2421, *P. aeruginosa* 2742 and 2004, *C. albicans* 7535. The strains of multidrug-resistant clinical isolates were multidrug-resistant *Staphylococcus aureus* (*MRSA CCARM* 3167 and *MRSA CCARM* 3506) and quinolone-resistant *Staphylococcus aureus* (*QRSA CCARM* 3505 and *QRSA CCARM* 3519). Clinical isolates were collected from various patients hospitalized in several clinics. A two-fold serial dilution technique was used to obtain final concentrations of 64-0.5 μg/mL. Test bacteria were grown to mid-log phase in Mueller-Hinton broth (MHB) and diluted 1000-fold in the same medium. The bacteria of 10^5 CFU/mL were inoculated into MHB and dispensed at 0.2 mL/well in a 96-well microtiter plate. As positive controls, oxacillin, norfloxacin, gatifloxacin, moxifloxacin, fluconazole and itraconazole were used. Test compounds were prepared in DMSO, the final concentration of which did not exceed 0.05%. The MIC was defined as the concentration of a test compound that completely inhibited bacteria growth during 24 h incubation at 37 °C. Bacteria growth was determined by measuring the absorption at 650 nm using a microtiter enzyme-linked immunosorbent assay (ELISA) reader. All experiments were carried out three times.
24. *Anti-inflammatory assay:*
Assay in the xylene-induced ear edema method via the intraperitoneal route: The anti-inflammatory activity was evaluated by an in vivo inhibition assay by monitoring xylene-induced ear edema in mice. In the primary screening, all tested compounds, ibuprofen and indomethacin were freshly prepared (dissolved with DMSO) prior to administered ip at a dose of 50 mg/kg to mice and at a concentration of 0.05 mL/20 g body weight. Control mice received the vehicle only (DMSO, 0.05 mL/20 g of body weight). Thirty minutes after administration ip, animals were used in the xylene-induced ear edema test, 20 μL xylene was applied to the surface of the right ear of each mouse by a micropipette. After keeping them from struggling for 30 min, a cylindrical plug (diameter, 7 mm) was excised from each of the treated and untreated ears. Edema was quantified by the difference in weight between the two plugs. Anti-inflammatory activity was expressed as percent reduction in edema compared with the DMSO-administered control group. The NSAID ibuprofen and indomethacin were tested in parallel as reference.
Assay in the xylene-induced ear edema method via the oral route: In the latter evaluation, tested compounds and indometacin were homogenized with 0.5% sodium carboxymethylcellulose (CMC-Na) and administered via the oral route to mice at a concentration of 0.4 mL/20 g mice weight. Control mice received the vehicle only (0.5% CMC-Na, 0.4 mL/20 g). To explore the peak activity of the compound, edema was quantified at different intervals (1, 2, 3, 4, 5, and 24 h). Compounds **7l** and indomethacin homogenized with 0.5% CMC-Na were administered orally to mice (lower doses of 25 mg/kg and 12.5 mg/kg and 0.4 mL/20 g mice body weight). Control mice received 0.5% CMC-Na (0.4 mL/20 g body weight) and edema quantified at the peak interval of 3 h.
Edema was quantified by the difference in weight between the two plugs. Edema values, expressed as mean standard deviation, were compared statistically using one-way-ANOVA followed by Dunnet's test. A level of $p < 0.05$ was adopted as the test of significance.