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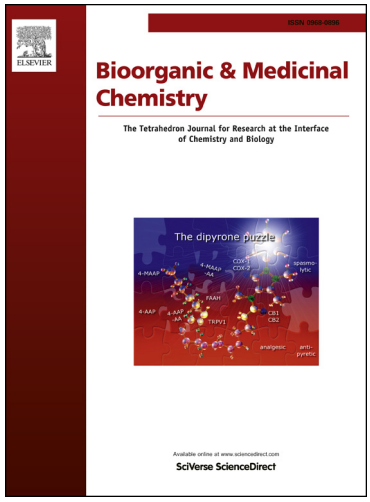

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Synthesis and evaluation of thiophene-based guanylhydrazones (iminoguanidines) efficient against panel of voriconazole-resistant fungal isolates

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ABSTRACT

A series of new thiophene-based guanylhydrazones (iminoguanidines) were synthesized in high yields using a straightforward two-step procedure. The antifungal activity of compounds was evaluated against a wide range of medicaly important fungal strains including yeasts, molds, and dermatophytes in comparison to clinically used drug voriconazole. Cytotoxic properties of compounds were also determined using human lung fibroblast cell line and hemolysis assay. All guanylhydrazones showed significant activity against broad spectrum of clinically important species of *Candida spp.*, *Aspergillus fumigatus*, *Fusarium oxysporum*, *Microsporum canis* and *Trichophyton mentagrophytes*, which was in some cases comparable or better than activity of voriconazole. More importantly, compounds **10**, **11**, **13**, **14**, **18** and **21** exhibited excellent activity against voriconazole-resistant *C. albicans* CA5 with very low minimal inhibitory concentration (MIC) values <2 μg mL^{-1}. Derivative **14**, bearing bromine on the phenyl ring, was the most effective compound with MICs ranging from 0.25 to 6.25 μg mL^{-1}. However, bis-guanylhydrazone **18** showed better selectivity in terms of therapeutic index values. *In-vivo* embryotoxicity on zebrafish (*Danio rerio*) showed improved toxicity profile of **11**,**14** and **18** in comparison to that of voriconazole. Most guanylhydrazones also inhibited *C. albicans* yeast to hyphal transition, essential for its biofilm formation, while **11** and **18** were able to disperse preformed *Candida* biofilms. All guanylhydrazones showed the equal potential to interact with genomic DNA of *C. albicans* in vitro, thus indicating a possible mechanism of their action, as well as possible mechanism of observed cytotoxic effects. Tested compounds did not have significant hemolytic effect and caused low liposome leakage, which excluded the cell membrane as a primary target. On the basis of computational docking experiments using both human and cytochrome P450 from *Candida* it was concluded that the most active guanylhydrazones had minimal structural prerequisites to interact with the cytochrome P450 14α-demethylase (CYP51). Promising guanylhydrazone derivatives also showed satisfactory pharmacokinetic profile based on molecular calculations.

1. Introduction

Fungal infections have received less attention over past decades, however mortality and morbidity rate of opportunistic fungal infections is exponentially increasing and the number of fatal incidence due to fungi is becoming comparable with that of tuberculosis and malaria.[1] Pathogenic fungi, particularly *Candida* and *Aspergillus* species are one of the most common diagnosed opportunistic infections and are the causes of significant mortality in immunocompromised patients.[2] On the other side, less threatening fungal skin infections were in the top 10 most prevalent diseases worldwide in 2010, affecting 984 million people[3] and posing enormous health burden globally. Given that the majority of infections caused by *C. albicans* are biofilm-related, and that this form is usually more resistant to traditional antifungal treatment it adds to recurrence and chronicity of the disease.[4,5]

Antifungal agents are mainly divided into four classes: polyenes, pyrimidines, echinocandins and azoles (Fig. 1).[6] The

polyene amphotericin B was developed in 1953, and by far is the most potent antifungal agent for almost all invasive fungal infections. It binds to ergosterol, which is the major sterol in fungal cell membranes, and thereby forming membrane-spanning channels that lead to the leakage of intracellular constituents and finally fungal cell death. The most frequent side effects associated with amphotericin B are infusional toxicity and nephrotoxicity. Flucytosine, pyrimidine-base antifungal was synthesized in 1957 as a potential antitumor drug but later on it was discovered that it had significant fungistatic activity. Flucytosine itself has no antifungal activity but it is converted to 5-fluorouracil within fungal cell, which then inhibits DNA and RNA synthesis. Usually, it is used in combination therapy with amphotericin B. The echinocandin derivative caspofungin inhibits the synthesis of β-(1,3)-d-glucan, an important component of the fungal cell wall. The major disadvantages associated with caspofungin are narrow spectrum of activity (only active against *Candida* spp. and *Aspergillus* spp.) and poor oral bioavailability. Voriconazole is a member of clinically important triazoles that are widely used for serious invasive fungal infections, as well as for dermal mycoses.[7] Voriconazole has fungistatic activity against *Candida* spp. and *Fusarium* spp. and with fungicidal activity against *Aspergillus* spp. The mechanism of action of voriconazole is inhibition of fungal cytochrome P450-mediated (14α-lanosterol demethylation, an essential step in the synthesis of ergosterol. This eventually causes the depletion of ergosterol and disrupts the integrity of fungal cell membranes, eventually leading to cell lysis.[8,9] The most common side effects of voriconazole therapy include visual disturbances, skin rashes, elevation of hepatic enzyme levels, headache, and hallucinations.[10-12]

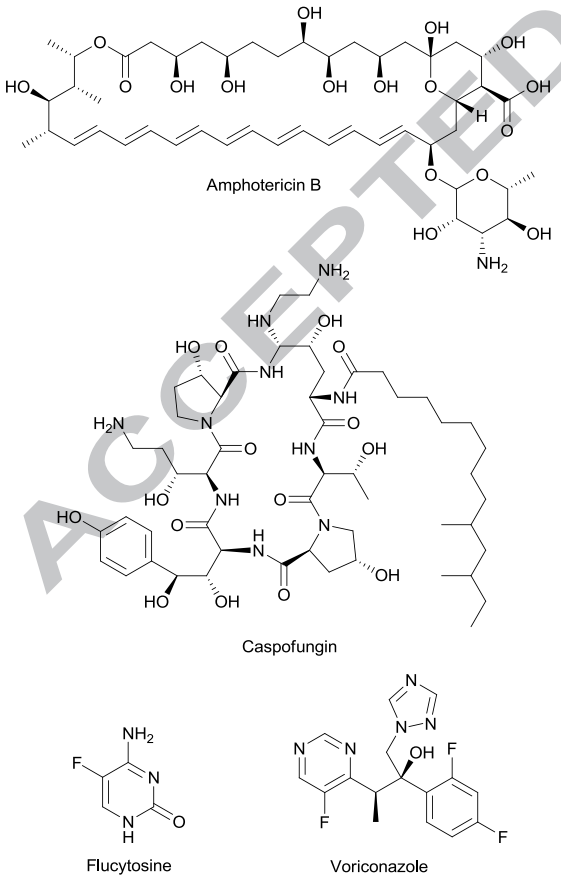


Figure 1. Clinically used antifungals.

The increasing number of reports of fungal infections among immunocompromised patients and additional problems associated with toxicity and resistance to standard antifungal drugs cause an urgent need for the development of novel safe and effective antifungals.[13] Apart from lower toxicity and higher selectivity, panfungal (broad spectrum) activity is also very desirable property that novel antifungals should have.

Compounds containing imonoguanidine moiety have been known for a while and explored for their wide variety of biological activities. Antiviral,[14-16] antiparasitic,[17-19] and antibacterial[20-23] activities of this versatile group of compounds have been described. However, their antifungal potential has not been extensively explored.[24] More importantly, to the best of our knowledge, they have not been evaluated against human fungal pathogens.

In a view of the general lack of novel antifungal agents and in a continuation of our studies towards the discovery of biologically active heterocyclic molecules, herein we report the synthesis and structural characterization of novel guanylhydrazones with their subsequent in vitro biological evaluation for antifungal activity including activity against fungal biofilms, their cytotoxic properties, in-vitro DNA interaction ability and in-silico analysis. *In-vivo* embryotoxicity on zebrafish (*Danio rerio*) of the most promising derivatives has been determined. The druglikeness of studied compounds was also established using Lipinski's "rule of five".[25,26]

2. Materials and methods

2.1. Instrumentation

Melting points were determined on a Boetius PMHK apparatus and were not corrected. IR spectra were recorded on a Thermo-Scientific Nicolet 6700 FT-IR Diamond Crystal. NMR: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Ultrashield Advance III spectrometer (at 500 and 125 MHz, respectively) using tetramethylsilane (TMS) as the internal standard. The NMR solvents are specified individually for each compound. Chemical shifts are expressed in parts per million (ppm) on the (δ) scale. Chemical shifts were calibrated relative to those of the solvents. ESI MS spectra of the synthesized compounds were recorded on an Agilent Technologies 6210 Time-of-Flight LC/MS instrument in positive ion mode using CH_3CN/H_2O = 1:11:1 with 0.2 % HCOOH as the carrying solvent solution. The samples were dissolved in pure acetonitrile (HPLC grade). The selected values were as follows: capillary voltage = 4 kV; gas temperature = 350 °C; drying gas = 12.1 min^{-1}; nebulizer pressure = 45 psig; fragmentator voltage = 70 V. All yields reported refer to isolated yields. Compounds were analyzed for purity using: Agilent 1200 HPLC system equipped with Quat Pump (G1311B), Injector (G1329B) 1260 ALS, TCC 1260 (G1316A) and Detector 1260 DAD VL+ (G1315C), and Waters 1525 HPLC dual pump system equipped with an Alltech Select degasser system, and a dual λ 2487 UV-VIS detector. All compounds were > 95% pure. Spectroscopic data of compounds are listed below (^{1}H and ^{13}C NMR spectra are in the Supplementary material).

2.2. General procedure for Suzuki coupling reactions

5-phenylthiophene-2-carbaldehyde (3) [27]: To a dry glass flask purged with argon were added $Pd(OAc)_2$ (3.4 mg, 0.015 mmol), PPh_3 (16 mg, 0.06 mmol) and dry DME (2 mL). The resultant solution was stirred at room temperature for 10 min, and 5-bromo-2-thiophenecarboxaldehyde (77 μL, 0.65 mmol) and Na_2CO_3 (aq) (2 M, 0.65 mL, 1.3 mmol) were added. After 5 min a solution of phenylboronic acid (99 mg, 0.82 mmol) in ethanol (1 mL) was added and reaction mixture was purged with argon and refluxed for 2 h under argon. The solution was cooled to room temperature and filtered through a pad of Celite, washed with CH_2Cl_2 and dried with anh. Na_2SO_4. The organic solvent

was removed under reduced pressure and the crude product was purified by dry-flash chromatography (SiO_2: hexane/EtOAc = 19:1) to afford the title compound **3** (110 mg, 90%) as an pale-yellow amorphous powder; mp = 88-90 °C. IR (ATR): 3281w, 3099w, 1637s, 1527w, 1436s, 1388m, 1329w, 1227s, 1060m, 993w, 905w, 814m cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 9.89 (s, 1H), 7.74 (d, 1H, *J* = 3.5 Hz), 7.70-7.65 (m, 2H), 7.47-7.37 (m, 4H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.78, 154.30, 142.48, 137.36, 133.05, 129.44, 129.20, 126.44, 124.08 ppm. GC/MS (*m/z* (%)): 187.0 ($[M]^+$, 100), 115.1 (55).

5-(4-methylphenyl)thiophene-2-carbaldehyde (4)[27]: The general Suzuki coupling procedure was followed. The reaction mixture was refluxed for 18 h. The crude product was purified by dry-flash chromatography (SiO_2: hexane/EtOAc = 19:1) to afford the title compound **4** (112 mg, 85%) as an pale-yellow amorphous powder; mp = 88-91 °C. IR (ATR): 3351w, 3291w, 3095w, 3026w, 2970w, 2921w, 2850w, 2818w, 2751w, 1657s, 1614w, 1581m, 1535w, 1490w, 1449s, 1383m, 1333w, 1286w, 1226s, 1141w, 1123w, 1084w, 1056m, 1038w, 957w, 901w, 854w, 802s cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 9.88 (s, 1H), 7,72 (d, 1H, *J* = 4.0 Hz), 7.57 (d, 2H, *J* = 8.5 Hz), 7.36 (d, 1H, *J* = 4.0 Hz), 7.24 (d, 2H, *J* = 8.0 Hz), 2.39 (s, 3H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.73, 154.64, 141.98, 139.74, 137.47, 130.30, 129.87, 126.32, 123.57, 21.32 ppm. GC/MS (*m/z* (%)): 202.1 ($[M]^+$, 100), 173.1 (8), 129.1 (28), 115.1 (6).

5-(4-methoxyphenyl)thiophene-2-carbaldehyde (5)[27]: The general Suzuki coupling procedure was followed. The reaction mixture was refluxed for 2 h. The crude product was purified by dry-flash chromatography (SiO_2: hexane/EtOAc = 1:1) to afford the title compound **5** (175 mg, 82%) as an pale-yellow amorphous powder; mp = 120-121 °C. IR (ATR): 3029w, 1693w, 1644s, 1600s, 1568m, 1530m, 1507m, 1453s, 1331w, 1308w, 1287s, 1254s, 1220s, 1178s, 1112m, 1054s, 1022s, 831s, 799s cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 9.86 (s, 1H), 7.70 (d, 1H, *J* = 4.0 Hz), 7.61 (d, 2H, *J* = 9.0 Hz), 7.29 (d, 1H, *J* = 4.0 Hz), 6.94 (d, 2H, *J* = 9.0 Hz), 3.85 (s, 3H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.64, 160.71, 154.52, 141.52, 137.65, 127.80, 125.78, 122.98, 114.58, 55.42 ppm. GC/MS (*m/z* (%)): 218.0 (100), 203.0 (38), 175.0 (12), 147.0 (14).

5-(4-fluorophenyl)thiophene-2-carbaldehyde (6)[27]: The general Suzuki coupling procedure was followed. The reaction mixture was refluxed for 2 h. The crude product was purified by dry-flash chromatography (SiO_2: hexane/EtOAc = 4:1) to afford the title compound **6** (145 mg, 72%) as an pale-brown amorphous powder; mp = 114-116 °C. IR (ATR): 3092m, 2855w, 1633s, 1594s, 1528m, 1502m, 1441s, 1413s, 1384m, 1327m, 1306m, 1231s, 1158s, 1100s, 1058s, 959m, 832s, 803s cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 9.89 (s, 1H), 7.73 (d, 1H, *J* = 4.0 Hz), 7.68-7.61 (m, 2H), 7.34 (d, 1H, *J* = 4.0 Hz), 7.17-7.10 (m, 2H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.71, 163.40 (d, *J* = 248.2 Hz), 153.03, 142.53, 137.38, 129.28 (d, *J* = 3.5 Hz), 128.29 (d, *J* = 8.1 Hz), 124.06, 116.31 (d, *J* = 21.8 Hz) ppm. GC/MS (*m/z* (%)): 206.0 (100), 178.0 (10), 133.0 (80), 120.0 (10), 107.0 (10).

5-(4-bromophenyl)thiophene-2-carbaldehyde (7) The general Suzuki coupling procedure was followed. The reaction mixture was refluxed for 18 h. The crude product was purified by dry-flash chromatography (SiO_2: hexane/EtOAc = 9:1) to afford the title compound **7** (125 mg, 46%) as an pale-yellow amorphous powder; mp = 94-97 °C. IR (ATR): 3309w, 3103w, 2829w, 2802w, 2742w, 1662s, 1585m, 1530m, 1491w, 1448s, 1399m, 1324w, 1269w, 1226s, 1117w, 1079w, 1055m, 1004w, 959w, 823s, 804s cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 9.90 (s, 1H), 7.73 (d, 1H, *J* = 4.0 Hz), 7.60-7.51 (m, 4H), 7.39 (d, 1H, *J* = 4.0 Hz) ppm. ^{13}C NMR (125 MHz, CD_3CD): δ 182.68, 152.71, 142.84, 137.26, 132.38, 132.01, 127.83, 124.39, 123.62 ppm. GC/MS (*m/z* (%)): 267.9 (100), 192.9 (40), 158.0 (20), 115.0 (30).

4-(5-formylthiophen-2-yl)benzonitrile (8)[27]: The general Suzuki coupling procedure was followed. The reaction mixture was refluxed for 18 h. The crude product was purified by dry-flash chromatography (SiO_2: hexane/EtOAc = 3:7) to afford the title compound **8** (131 mg, 73%) as an pale-yellow amorphous powder; mp = 166-176 °C. IR (ATR): 3304w, 3099w, 3052w, 2857w, 2826w, 2762w, 2221s, 1692s, 1673s, 1604m, 1532w, 1450s, 1414m, 1332w, 1285w, 1231s, 1181w, 1129w, 1065w, 837w, 802m cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 9.94 (s, 1H), 7.81-7.75 (m, 3H), 7.75-7.69 (m, 2H), 7.50 (d, 1H, *J* = 4.0 Hz) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.70, 150.98, 144.15, 137.23, 137.02, 132.97, 126.82, 125.82, 118.26, 112,66 ppm. GC/MS (*m/z* (%)): 212.0 ($[M]^+$, 100), 140.1 (45).

5-phenylfuran-2-carbaldehyde (9)[27]: The general Suzuki coupling procedure was followed. The reaction mixture was refluxed for 2 h. The crude product was purified by dry-flash chromatography (SiO_2: hexane/EtOAc = 9:1) to afford the title compound **9** (145 mg, 87%) as an yellow oil; IR (ATR): 3114w, 2814w, 1669s, 1520m, 1474m, 1449m, 1391m, 1255m, 1028m, 967m, 920w, 802m, 765s cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 9.65 (s, 1H), 7.85-7.80 (m, 2H), 7.47-7.42 (m, 2H), 7.41-7.37 (m, 1H), 7.32 (d, 1H, *J* = 3.5 Hz), 6.84 (d, 1H, *J* = 4.0 Hz) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.20, 159.40, 152.01, 129.65, 128.92, 125.27, 123.44, 107.66 ppm. GC/MS (*m/z* (%)): 172.1 ($[M]^+$, 100), 144.1 (15), 115.1 (100), 102.1 (5), 89.1 (20), 77,1 (7), 63.1 (15), 51.1 (10).

*2.3. General procedure for reparation of guanylhydrazones **10-18** and **21***

2-[(5-phenylthiophen-2-yl)methylidene]hydrazine carboximidamide hydrochloride (10) To a solution of aldehyde (65 mg, 0.35 mmol) in absolute ethanol (12 mL) was added aminoguanidine hydrochloride (38 mg, 0.35 mmol). The resultant solution was stirred at room temperature for 5 min, and solution of concentrated HCl in absolute EtOH (39 µL, 1:25 v/v) was added. The reaction mixture was heated to reflux for 18 h and allowed to cool to room temperature. The solvent was removed under reduced pressure, and the crude product was washed with CH_2Cl_2 and then crystallized from EtOH/hexane (9:1) to provide the title compound **10** (98 mg, 100%) as a pale-yellow solid; mp = 184-187 °C. IR (ATR): 3818w, 3418m, 3307w, 3220w, 3133s, 3086m, 2848w, 1676m, 1643s, 1608m, 1462w, 1433w, 1253w, 1154w, 1108w, 1068w, 1005w, 925w, 802w, 757m, 688m cm^{-1}. ^1H NMR (500 MHz, CD_3OD): δ 8.26 (s, 1H), 7.72-7.67 (m, 2H), 7.45-7.38 (m, 4H), 7.37-7.32 (m, 1H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 156.97, 149.62, 144.35, 138.20, 135.09, 134.24, 130.35, 129.79, 127.09, 125.04 ppm. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 245.08616 (error 2.52 ppm). HPLC purity: Method A: RT 6.274, area 99.28%; method B: RT 7.407, area 99.88%.

2-{[5-(4-methylphenyl)thiophen-2-yl]methylidene} hydrazinecarboximidamide hydrochloride (11) Following the general procedure for guanylhydrazone formation, **11** was obtained as a pale-yellow solid (85 mg, 59%). Mp = 244-245 °C. IR (ATR): 3938w, 3428m, 3364m, 3320m, 3220m, 3093s, 2979m, 2916m, 2833m, 2773m, 1672s, 1617s, 1539w, 1511w, 1463m, 1432w, 1311w, 1239w, 1185w, 1123m, 1059w, 928m, 819w, 795m, 712w, 662w, 581w cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 8.25 (s, 1H), 7.57 (d, 2H, *J* = 8.5 Hz), 7.39 (d, 1H, *J* = 3.5 Hz), 7.35 (d, 1H, *J* = 4.0 Hz), 7.23 (d, 2H, *J* = 7,5 Hz), 2.36 (s, 3H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 159.94, 149.90, 144.42, 140.08, 137.62, 134.29, 132.33, 130.93, 127.01, 124.47,

21.29 ppm. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 259.10187 (error 2.59 ppm). HPLC purity: Method A: RT 6.377, area 97.25%; method B: RT 7.543, area 99.87%.

2-{[5-(4-methoxyphenyl)thiophen-2-yl]methylidene} hydrazinecarboximidamide hydrochloride (12) Following the general procedure for guanylhydrazone formation, **12** was obtained as a pale-yellow solid (140 mg, 72%). Mp = 260-264 ^oC. IR (ATR): 3392s, 3312m, 3285m, 3222m, 3136s, 3130s, 3000m, 2970m, 2939m, 2916m, 2848m, 1668s, 1639s, 1603s, 1563m, 1541w, 1507m, 1465m, 1431m, 1351m, 1289m, 1250s,1180m, 1158m, 1119w, 1025m, 922w, 829w, 796m, 629w cm^{-1}. ^1H NMR (500 MHz, *d*-DMSO): δ 8.34 (s, 1H), 7.76-7.55 (m, 6H), 7.50 (d, 1H, *J* = 4.0 Hz), 7.43 (d, 1H, *J* = 4.0 Hz), 7.06-6.97 (m, 2H), 3.80 (s, 3H) ppm. ^{13}C NMR (125 MHz, *d*-DMSO): δ 159.99, 155.10, 147.14, 142.88, 135.87, 133.72, 127.46, 126.10, 123.49, 115.11, 55.70 ppm. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 275.09530 (error -2.95 ppm). HPLC purity: Method A: RT 6.302, area 99.10%; method B: RT 7.392, area 99.91%.

2-{[5-(4-fluorophenyl)thiophen-2-yl]methylidene} hydrazinecarboximidamide hydrochloride (13) Following the general procedure for guanylhydrazone formation, **13** was obtained as a pale-yellow solid (145 mg, 100%). Mp = 195-200 ^oC. IR (ATR): 3387s, 3278s, 3221s, 3167s, 2989m, 2855m, 2362w, 1690s, 1619s, 1538m, 1505s, 1463w, 1434w, 1350w, 1317w, 1230m, 1154m, 1103m, 1058m, 1009m, 916m, 830m, 791m, 658m, 578m cm^{-1}. NMR (500 MHz, CD_3OD): δ 8.26 (s, 1H), 7.75-7.69 (m, 2H), 7.41 (d, 1H, *J* = 4.0 Hz), 7.37 (d, 1H, *J* = 4.0 Hz), 7.20-7.13 (m, 2H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 164.45 (d, *J* = 246.4 Hz), 156.98, 148.35, 144.27, 138.30, 134.26, 131.60 (d, *J* = 2.6 Hz), 129.12 (d, *J* = 8.1 Hz), 125.17, 117.18 (d, *J* = 21.8 Hz) ppm. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 263.07710 (error 3.72 ppm). HPLC purity: Method A: RT 6.293, area 98.89%; method B: RT 7.413, area 99.59%.

2-{[5-(4-bromophenyl)thiophen-2-yl]methylidene} hydrazinecarboximidamide hydrochloride (14) Following the general procedure for guanylhydrazone formation, **14** was obtained as a pale-yellow solid (72 mg, 99%). Mp = 236-240 ^oC. IR (ATR): 3466s, 3423m, 3277s, 3139s, 2862m, 1678s, 1611s, 1526m, 1491w, 1463w, 1440w, 1398w, 1347w, 1314w, 1243w, 1153w, 1111w, 1079w, 1007w, 954w, 928w, 826w, 803m cm^{-1}. ^1H NMR (500 MHz, CD_3CD): δ 8.27 (s, 1H), 7.63-7.54 (m, 4H), 7.44-7.39 (m, 2H) ppm. ^{13}C NMR (125 MHz, CD_3CD): δ 156.93, 147.93, 144.14, 138.73, 134.23, 134.18, 133.44, 128.70, 125.62, 123.43 ppm. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 322.99565 (error -1.27 ppm). HPLC purity: Method A: RT 6.406, area 95.31%; method B: RT 7.581, area 96.53%.

2-{[5-(4-cyanophenyl)thiophen-2-yl]methylidene} hydrazinecarboximidamide hydrochloride (15) Following the general procedure for guanylhydrazone formation, **15** was obtained as a pale-yellow solid (68 mg, 65%). Mp = 190-195 ^oC. IR (ATR): 3936w, 3313s, 3182s, 2364w, 2233m, 1679s, 1637s, 1599s, 1504m, 1466m, 1412w, 1318m, 1271w, 1247m, 1177m, 1154m, 1013w, 832w, 800w cm^{-1}. ^1H NMR (500 MHz, CD_3OD): δ 8.29 (s, 1H), 7.88-7.84 (m, 2H), 7.79-7.74 (m, 2H), 7.59 (d, 1H, *J* = 4.0 Hz), 7.47 (d, 1H, *J* = 4.0 Hz) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 157.00, 146.65, 143.86, 140.43, 139.42, 134.21, 134.18, 127.59, 127.35, 119.64, 112.66 ppm. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 270.08137 (error 2.12 ppm). HPLC purity: Method A: RT 6.216, area 98.83%; method B: RT 7.217, area 98.44%.

2-[(5-phenylfuran-2-yl)methylidene]hydrazine carboximidamide hydrochloride (16) Following the general procedure for guanylhydrazone formation, **16** was obtained as a yellowish solid (180 mg, 100%). Mp = 66-69 ^oC. IR (ATR): 3901w, 3882w, 3621w, 3361s, 3148s, 2962s, 2861s, 2786m, 1676s, 1633s, 1545m, 1479m, 1448m, 1336w, 1278w, 1220w, 1147m, 1074w, 1624w, 975w, 924w, 793w, 754m, 689w, 665w, 638w cm^{-1}. ^1H NMR (500 MHz, CD_3CD): δ 8.04 (s, 1H), 7.84-7.78 (m, 2H), 7.43-7.40 (m, 2H), 7.37-7.31 (m, 1H), 7.03 (d, 1H, *J* = 3.5 Hz), 6.94 (d, 1H, *J* = 3.5 Hz) ppm. ^{13}C NMR (125 MHz, CD_3CD): δ 158.12, 157.07, 149.62, 138.83, 131.26, 130.12, 129.78, 125.61, 118,.44, 108.85 ppm. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 229.10821 (error -0.76 ppm). HPLC purity: Method A: RT 6.238, area 99.40%; method B: RT 7.318, area 98.69%.

5-(pyridin-4-yl)thiophene-2-carbaldehyde[27] The general Suzuki coupling procedure was followed. The reaction mixture was refluxed for 18 h. The crude product was purified by dry-flash chromatography (SiO_2: hexane/EtOAc = 1:1) to afford 5-(pyridin-4-yl)thiophene-2-carbaldehyde (125 mg, 50%) as an pale-brown amorphous powder; mp = 107-110 ^oC. IR (ATR): 3445s, 2361m, 1646s, 1543m, 1447m, 1415m, 1228m, 805m, 754w, 704m cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 9.95 (s, 1H), 8.68 (d, 2H, *J* = 6.0 Hz), 7.80 (d, 1H, *J* = 4.0 Hz), 7.58 (d, 1H, *J* = 4.0 Hz), 7.54 (d, 2H, *J* = 6.0 Hz) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.73, 150.74, 150.14, 144.22, 140.03, 136.86, 126.01, 120.23 ppm. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 190.03231 (error, +1.04 ppm).

2-{[5-(pyridin-4-yl)thiophen-2-yl]methylidene}hydrazine carboximidamide dihydrochloride (17) Following the general procedure for guanylhydrazone formation, **17** was obtained as a pale-yellow solid (27 mg, 32%). Mp = 272-276 ^oC. IR (ATR): 3311s, 3090s, 2992s, 2846s, 1678m, 1631s, 1535m, 1560s, 1462w, 1376w, 1326m, 1241w, 1202w, 1145w, 1698w, 1004w, 799w, 626w, 581w, 551w cm^{-1}. ^1H NMR (500 MHz, D_2O): δ 8.59-8.55 (m, 2H), 8.11-8.09 (m, 1H), 8.09-8.07 (m, 2H), 7.82 (d, 1H, *J* = 4.5 Hz), 7.39 (d, 1H, *J* = 4.0 Hz) ppm. ^{13}C NMR (125 MHz, D_2O): δ 154.66, 149.52, 143.66, 141.73, 141.26, 139.39, 133.22, 131.54, 122.06 ppm. (+)ESI-HRMS (*m/z*): $[M + 2H]^{2+}$ 123.54429 (error 2.09 ppm). HPLC purity: Method A: RT 6.184, area 99.51%; method B: RT 6.273, area 96.37%.

5-(4-formylphenyl)thiophene-2-carbaldehyde To a dry glass flask purged with argon were added $Pd(OAc)_2$ (2.9 mg, 0.013 mmol), PPh_3 (14 mg, 0.050 mmol) and dry DME (2 mL). The resultant solution was stirred at room temperature for 10 min, and 5-bromo-2-thiophenecarboxaldehyde (30 µL, 0.26 mmol) and Na_2CO_3 (aq) (2 M, 0.3 mL, 0.6 mmol) were added. After 5 min a 4-formylphenylboronic acid (49 mg, 0.33 mmol) was added and reaction mixture was purged with argon and refluxed for 3 h under argon. The solution was cooled to room temperature and filtered through a pad of Celite, washed with CH_2Cl_2 and dried with anh. Na_2SO_4. The organic solvent was removed under reduced pressure and the crude product was purified by dry-flash chromatography (SiO_2: hexane/EtOAc = 7:3) to afford 5-(4-formylphenyl)thiophene-2-carbaldehyde (54 mg, 96%) as an pale-yellow amorphous powder; mp = 130-132 ^oC. IR (ATR): 3078w, 3047w, 2844w, 2757w, 1693s, 1658s, 1601s, 1566m, 1506w, 1447s, 1396m, 1318w, 1291w, 1224s, 1183m, 1059w, 961w, 840m, 806m cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 10.06 (s, 1H), 9.95 (s, 1H), 7.99-7.94 (m, 2H), 7.88-7.83 (m, 2H), 7.80 (d, 1H, *J* = 4.0 Hz), 7.55 (d, 1H, *J* =4.0 Hz) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 191.20, 182.75, 151.85, 143.95, 138.52, 137.08, 136.48, 130.52, 126.84, 125.72 ppm. GC/MS (*m/z* (%)): 214.9 ($[M]^+$, 100).

2-[(5-{4-[(2-carbamimidoylhydrazinylidene)methyl] phenyl}thiophen-2-yl)methylidene]hydrazinecarboximidamide dihydrochloride (18) Following the general procedure for guanylhydrazone formation, **18** was obtained as a pale-yellow

solid (37 mg, 100%). Mp = 248-250 °C. IR (ATR): 3352s, 3275s, 3153s, 2872m, 1668s, 1612s, 1536m, 1437m, 1350w, 1237m, 1181w, 1141m, 1011w, 829w cm $^{-1}$. ^{1}H NMR (500 MHz, DO_3OD): δ 8.30 (s, 1H), 8.15 (s, 1H), 7.86 (d, 2H, *J* = 8.0 Hz), 7.78 (d, 2H, *J* = 8.0 Hz), 7.52 (d, 1H, *J* = 4.0 Hz), 7.45 (d, 1H, *J* = 4.0 Hz) ppm. ^{13}C NMR (125 MHz, DO_3OD): δ 157.26, 156.96, 148.75, 148.28, 144.14, 139.14, 137.25, 134.81, 134.29, 129.67, 127.32, 126.04 ppm. (+) ESI-HRMS (*m/z*): $[M + H]^+$ 329.12823 (error: -2,77 ppm). HPLC purity: Method C: RT 6.322, area 99.52%; method D: RT 2.543, area 96.86%.

4,4'-thiene-2,5-diyldibenzaldehyde (20)[28]: A reaction tube containing a stirrer bar was evacuated and backfilled with argon. The tube was then charged with $Pd(OAc)_2$ (16 mg, 0.070 mmol), PPh_3 (73 mg, 0.28 mmol) and dry DME (7 mL) under argon. The resultant solution was stirred at room temperature for 10 min, and 2,5-dibromothiophene (150 mg, 0.620 mmol) and Na_2CO_3 (aq) (2 M, 1.2 mL, 2.4 mmol) were added. After 5 min, 4-cyanophenylboronic acid (225 mg, 1.54 mmol) was added, the tube was returned under argon and capped. The reaction mixture was heated with stirring for 2 h at 80 °C in a MW reactor. The solvent was then removed under reduced pressure, and the reaction mixture was suspended in CH_2Cl_2, transferred to a separation funnel, and washed well with saturated Na_2CO_3 solution (2×25 mL) containing 5 mL NH_3. The organic layer was collected, dried with anh. Na_2SO_4, and the solvent was removed under reduced pressure. The crude product was purified by dry-flash chromatography (SiO_2: CH_2Cl_2) to afford the 4,4'-thiene-2,5-diyldibenzonitrile (160 mg, 90%) as an pale-yellow amorphous powder; mp = 282-284 °C. IR (KBr): 3432m, 2222s, 1599s, 1491w, 1410w, 1278w, 1176w, 838m cm $^{-1}$. ^{1}H NMR (500 MHz, DMSO): δ 7.97-7.87 (m, 8H), 7.84 (s, 2H) ppm.

To a stirred suspension of di-nitrile (20 mg, 0.070 mmol) in PhMe (2.5 mL) was added DIBAH (0.45 mL, 1 M in toluene) at 0 °C. After stirring at 0 °C under Ar atmosphere for 1 h the reaction was quenched with 5% H_2SO_4 (0.60 mL), and stirring was continued for 1 h at r.t. The reaction mixture was transferred into a separation funnel as an emulsion in EtOAc (50 mL). The organic layer was separated, and the aqueous layer was additionally extracted with CH_2Cl_2 (3×25 mL). The combined organic layers were washed with brine (15 mL) and dried over anh. Na_2SO_4. The organic solvent was removed under reduced pressure and the crude product **20** (20 mg, 98%) was obtained as a pale-yellow solid and was used for the next reactions without further purification. Mp = 149-151 °C. IR (ATR): 3349w, 3198w, 3069m, 2923m, 2854m, 2764w, 1694s, 1599s, 1568m, 1498w, 1451w, 1423w, 1397w, 1346w, 1310w, 1280m, 1216m, 1167m, 1116w, 838m cm $^{-1}$. ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.03 (s, 2H), 7.93 (d, 4H, *J* = 8.5 Hz), 7.80 (d, 4H, *J* = 8.5 Hz), 7.50 (s, 2H) ppm.

(2,2'-{thiene-2,5-diylbis[benzene-4,1-diylmethylylidene]} dihydrazinecarboximidamide dihydrochloride (21) Following the general procedure for guanylhydrazone formation, **21** was obtained as a yellow solid (20 mg, 48%). Mp = 266 °C. IR (ATR): 3362m, 3332m, 3160m, 2917m, 2850m, 1737m, 1673s, 1619s, 1539m, 1511m, 1456w, 1148w cm $^{-1}$. ^{1}H NMR (500 MHz, DO_3OD): δ 8.12 (s, 2H), 7.88-7.84 (m, 4H), 7.80-7.76 (m, 4H), 7.54 (s, 2H) ppm. ^{13}C NMR (125 MHz, DMSO): δ 155.39, 146.25, 142.60, 135.11, 132.82, 128.44, 126.26, 125.36 ppm. (+) ESI-HRMS (*m/z*): $[M + H]^+$ 405.16006 (error: -0,94ppm). HPLC purity: Method C: RT 6.342, area 99.06%; method D: RT 2.761, area 95.73%.

2.4. Broth dilution method for antifungal susceptibility testing

The minimum inhibitory concentration (MIC) of guanylhydrazones to six human clinical fungal strains was determined using a reference method for testing antimicrobial agents for yeasts and moulds in a 96-well microtitre plate assay (EUCAST 2012; EUCAST 2014).[29,30] All tested compounds were initially dissolved in DMSO in stock concentration of 50 mg mL^{-1}. Further dilutions were made in RPMI 1640 medium (Roswell Park Memorial Institute 1640) with 2% (w/v) glucose in the concentration range of 250-0.02 μg mL^{-1}. Additionally, MIC of commercial antifungal voriconazole was determined for each isolate (concentration range 16-0.001 μg mL^{-1}; Sigma Aldrich, Munich, Germany). Time-kill dependence was monitored spectrophotometrically at 600 nm over 12 h period at 37 °C for selected guanylhydrazones (**11**, **14** and **18**) and voriconazole using sub-MIC concentrations (70% of MIC value determined for the planktonic growth) on Tekan Infinite 200 Pro multiplate reader (Tecan Group Ltd., Männedorf, Switzerland).

Pathogenic strains and preparation of inocula: Human clinical fungal strains were obtained from collection of National Reference Medical Mycology Laboratory (Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade, Belgrade) and included: yeasts isolated from blood (*Candida albicans* CA5 and *C. parapsilosis* CA27); non-dermatophyte mould isolated from upper respiratory tract (*Fusarium oxysporum* AB18); and dermatophyte moulds isolated from skin derivates (*Trichophyton mentagrophytes* DMT2 and *Microsporum canis* DMT4). The reference strains *C. albicans* ATCC10231, *C. krusei* ATCC34135 and *Aspergillus fumigatus* ATCC13073 were obtained from American Type Culture Collection (ATCC). Stock culture of each isolate was maintained on Potato dextrose agar (PDA; Hi Media Laboratories Mumbai, India) at 4 °C. Prior to testing, working cultures were obtained by preculturing stock cultures onto PDA in Petri dishes and incubating for 7 days at 30 °C. The inocula were prepared by resuspending fungal conidia from PDA working cultures in 4 mL of sterile distilled water (for yeasts) or in 0.9% (w/v) NaCl with 0.1% (v/v) Tween 20 (for moulds). Conidial suspensions were adjusted to 2-5 $\times 10^6$ CFU mL^{-1} by using Neubauer haemocytometer and diluted in RPMI 1640 medium with 2% glucose in a ratio of 1:10 (2-5 $\times$ 10^5 CFU mL^{-1}).

Volume of 100 μL of prepared inocula was incubated together with 100 μL of different concentrations of guanylhydrazones and voriconazole at 37 °C (for yeasts) or at 30 °C (for moulds) and the growth inhibition was assessed over time (24 h for *Candida* spp.; 48 h for *A. fumigatus* and *F. oxysporum* and 96 h for *M. canis and T. mentagrophytes*). Controls containing solvents were carried out in each assay. MIC was defined as the lowest concentration of compound at which no evident fungal growth was observed. The assay was repeated two times in triplicates.

2.5. Microscopic studies

C. albicans ATCC10231 overnight culture was diluted to OD600=1 and incubated with MIC of compounds **11** and **18**. At time points 0 min and 2 h the cell viability and membrane integrity were assessed after staining with 1 μg mL^{-1} 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, Sigma Aldrich), 2.5 μM propidium iodide (PI, Sigma Aldrich) and 50 μg mL^{-1} Concanavalin A conjugated with FITC (Sigma Aldrich) in PBS using fluorescence microscope (Olympus BX51, Applied Imaging Corp., San Jose, USA) at 40× magnification.

2.6. Aniproliferative assay on human fibroblast cell line

Antiproliferative activity was tested by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

assay on human lung fibroblasts (MRC5; ATCC collection). The cells were incubated in the media containing test compounds at concentrations ranging from 0.1 to 250 μg mL^{-1} and the cell viability was measured after 48 h. MRC5 cell line was cultured in RPMI-1640 medium supplemented with 100 μg mL^{-1} streptomycin, 100 U mL^{-1} penicillin and 10% (v/v) fetal bovine serum (FBS) (all from Sigma, Munich, Germany). Cells were maintained as a monolayer (1 x 10^4 cells per well) in RPMI-1640 and grown in humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

The extent of MTT reduction was measured spectrophotometrically at 540 nm using Tekan Infinite 200 Pro multiplate reader (Tecan Group Ltd., Männedorf, Switzerland) and the cell survival was expressed as percentage of the control (untreated cells). The percentage viability values were plotted against the log of concentration and a sigmoidal dose response curve was calculated by non-linear regression analysis using Graphpad Prism software version 5.0 for Windows (Graphpad Software, CA, USA). Cytotoxicity is expressed as the concentration of the compound inhibiting growth by 50% (IC_{50}).

2.7. Hemolytic assay

Sheep red blood cells in PBS (1% v/v, Torlak, Belgrade, Serbia) were treated for 1 h with concentration of compounds that corresponded to determined MIC and IC50 values at 37°C. Hemoglobin absorbance was measured at 405 nm (Tekan Infinite 200 Pro multiplate reader (Tecan Group Ltd., Männedorf, Switzerland). The hemolysis percentage was calculated using the following equation: hemolysis (%) = 100[(Abs_{405nm} (treated) - Abs_{405nm} (non treated) / (Abs_{405nm} (0.1% Triton X-100 lysed) - Abs_{405nm} (non treated)].

2.8. Liposomes leakage assay

Mixtures containing L-α-phosphatidylcholine (PC, from egg yolk) (Sigma Aldrich, Munich, Germany) and L-a-phosphatidylserine (PS, from bovine brain) (Sigma Aldrich, Munich, Germany) (PC/PS=8:2), or PC, PS and ergosterol (E)(Sigma Aldrich, Munich, Germany) (PC/PS/E=11/4/5) were dissolved in chloroform and dried under a stream of argone followed by vacuum drying. Large unilamellar vesicles (LUVs) with a diameter of 0.4 mm containing 50 mM calcein (Molecular Probes, Invitrogen) were prepared using an extruder (Avestin Europe GmbH., Mannheim, Germany) according to Hope et al.[31] The non-encapsulated dye was removed from the liposome suspension by ultracentrifugation. The osmolarity of the solution (calcein and buffer) was measured with an Osmomat 030, Gonotec GmbH (Berlin, Germany).

Liposome leakage assay was performed in 96-well plate with 50 nmol LUV suspension added per well and calcein release was recorded by measuring the fluorescence at excitation and emission wavelengths of 485 and 518 nm, respectively at room temperature using Tekan Infinite 200 Pro multiplate reader (Tecan Group Ltd., Männedorf, Switzerland). The percentage of fluorescence F_t at time t is defined as:

$$\% F_t=(I_t-I_0/I_f-I_0)x100;$$

where I_0 was the initial fluorescence obtained after the dilution of the vesicles in the appropriate buffer, I_f was the total fluorescence observed after addition of Triton X-100, and I_t was the fluorescence at time t corrected for the dilution.

2.9. In vitro DNA binding by gel electrophoresis assay

Genomic DNA (gDNA) from *C. albicans* was purified with a DNeasy tissue kit (Qiagen, Hilden, Germany). The quality and the concentration of DNA were estimated by measuring UV absorbance with a NanoVue Plus spectrophotometer (GE Healthcare, Freiburg, Germany). The ability of guanylhydrazones to bind gDNA from *C. albicans* was examined by using agarose gel electrophoresis.[32] Briefly, gDNA (500 ng) was treated with the compounds (25 μg mL-1) in phosphate buffer (pH 7.4), and the contents were incubated for 12 h at 37 °C, then subjected to gel electrophoresis on 0.8% (w/v) agarose gel containing 0.1 μg/mL of ethidium bromide in TAE buffer (40 mM Tris acetate/1 mM EDTA, pH 7.4) buffer at 60 V for 2 h. Gels were visualized and analyzed using the Gel Doc EZ system (Bio-Rad, Life Sciences, Hercules, USA), equipped with the Image Lab™ Software.

2.10. The effect of guanylhydrazones on C. albicans yeast to hyphe transition using Spider medium

Morphological changes of *C. albicans* in the presence and absence of guanylhydrazones and voriconazole in subinhibitory concentrations ($MIC_{70;}$ 70% of MIC value determined for the planktonic growth) was observed upon *C. albicans* growth on Spider medium as previously described.[33]

2.11. The effect of guanylhydrazones on Candida biofilm formation and disruption

The biofilm disruption ability of guanylhydrazones was tested by determining minimal biofilm eradication concentration (MBEC), defined as the lowest concentration of an antimicrobial agent required to completely eradicate biofilm. *Candida* biofilms were developed in flat bottom 96-well clear microtitre plates as described previously.[34] Briefly, harvested from the overnight grown liquid *C. albicans* ATCC10231, *C. krusei* ATCC34135 and *C. parapsilosis* CA27 cultures were washed twice and collected by centrifugation (3000 × g) in sterile PBS, resuspended in RPMI-1640 with 2% glucose and adjusted to cell density of 1 × 10^6 cells mL^{-1} using Neubauer haemocytometer. Biofilms were formed by pipetting 100 μL of the prepared cell suspension into wells of a microtiter plate and incubated for 48 h at 37°C. The established biofilms were rinsed with PBS to remove non-adherent cells and subsequently treated with two-fold serial dilutions of guanylhydrazones (concentration range 1000-25 μg mL^{-1}). The resultant biofilm biomass was quantified using the CV assay and was compared with untreated controls. The bioassay was performed in six replicates of each strain.

To study the effect of selected guanylhydrazones (**11** and **18**) on *C. albicans* biofilm formation, overnight fungal culture grown in RPMI medium supplemented with 2 % glucose and 10 % FCS was diluted to optical density OD600 of 0.1 and the biofilms were grown on plastic cover slips in the presence of MIC of guanylhydrazones or DMSO. After 24 h growth at 37 °C, biofilms were washed with PBS, stained with 2.5 μM propidium iodide (PI, Sigma Aldrich) and 50 μg mL^{-1} Concanavaline A conjugated with Fluorescence isothiocyanate (FITC; Sigma Aldrich) in PBS, and observed by microscopy.

To examine the biofilm disruption activity of the same compounds, 48 – 72 h pre-formed *C. albicans* biofilms were washed with PBS and then incubated with MIC of **11** and **18** or DMSO in RPMI for 24 h followed by DAPI, PI and Concanavaline A staining as above. Biofilms and cells were examined using fluorescence microscope (Olympus BX51, Applied Imaging Corp., San Jose, USA) at 20× magnification.

2.12. Molecular docking studies

Glide module incorporated in the Schrödinger Molecular Modeling Package (Maestro Version 10.1.013, free trial) was

used for performing molecular docking studies. For estimating protein-ligand binding affinities Glide Extra-Precision (XP) scoring function was adopted in the presented study. In this scoring function force field-based parameters like solvation and repulsive interactions, lipophilic, hydrogen bonding interactions, metal-ligand interactions as well as contributions from coulombic and van der Waals interaction energies are incorporated in the empirical energy functions calculations. Up to this day there is no reported X-ray structure of sterol 14α-demethylase (CYP51) from *C. albicans*. For this reason homology model was used for docking study.[35] The X-ray structure of human CYP51 (PDB code: 3JUV) was obtained from the RCSB Protein Data Bank (PDB), http://www.rcsb.org/pdb. Protein preparation wizard with the application of the OPLS-2005 force field was applied for preparing protein-inhibitor complexes. Hydrogen atoms were added to the structure corresponding to pH 7.0 with the consideration of the appropriate ionization states for both the acidic and basic amino acid residues and any crystallographic water, if present was eliminated. Further, prepared structure was subjected to energy minimization until the average root meansquare deviation (r.m.s.d.) reached 0.3 Å. The 3D structures of the studied molecules were built in the Maestro Suite of the module and subsequently optimized using the LigPrep module in the Schrödinger Suite. Partial atomic charges were computed using the OPLS-2005 force-field and possible ionization states were generated at a pH of 7.0. The ligand structures thus obtained were further optimized by energy minimization using the LBFGS method until a gradient of 0.01 kcal $mol^{-1}Å^{-1}$ was achieved.

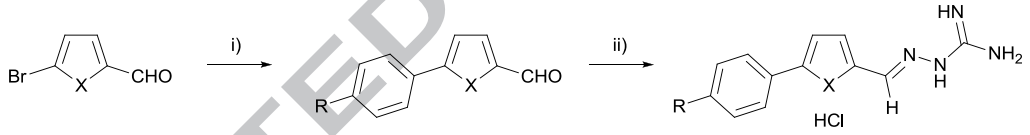
Molinspiration tool (Molinspiration Cheminformatics-2013) was used for calculating physic-chemical properties of investigated guanylhydrazones. For calculating of logP fragment-based contributions and correlation factors were used.

2.13. Zebrafish embryotoxicity of selected guanylhydrazones

The assessment of toxicity (lethality and teratogenicity) of selected guanylhydrazones (**11**, **14** and **18**) and voriconazole on zebrafish embryos was performed following general rules of the OECD Guidelines for the Testing of Chemicals.[36] All experiments involving zebrafish were performed in compliance with the European directive 86/609/EEC and the ethical guidelines of Guide for Care and Use of Laboratory Animals of Institute for Molecular Genetics and Genetic Engineering, University of Belgrade.

Adult zebrafish (*Danio rerio*, wild type) were maintained in the fish medium (2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.7 mM $NaHCO_3$, 0.07 mM KCl) at 27 ± 1°C and 14 h light/10 h dark cycle, and regularly fed twice daily with commercially dry flake food supplemented with *Artemia nauplii* (TetraMin™ flakes; Tetra Melle, Germany). Eggs at 6 hours post fertilization (hpf) were treated with five different concentrations (2.5, 5, 10, 25, and 50 μg mL^{-1}) and 0.15% DMSO as negative control. Embryos were then individually transferred into 24-well plates containing 1000 μl test solution, 10 embryos per well, and incubated at 28°C. Experiments were repeated three times, using 30 embryos per concentration.

Apical endopoints (Table S1) for toxicity evaluation were recorded at 24, 48, 72, and 96 hpf using an inverted microscope (CKX41; Olympus, Tokyo, Japan. At 96 hpf, the embryos were anesthetized by addition of 0.1% (w/v) tricaine solution (Sigma-Aldrich, St. Louis, MO), photographed and killed by freezing at −20°C for ≥ 24 h.

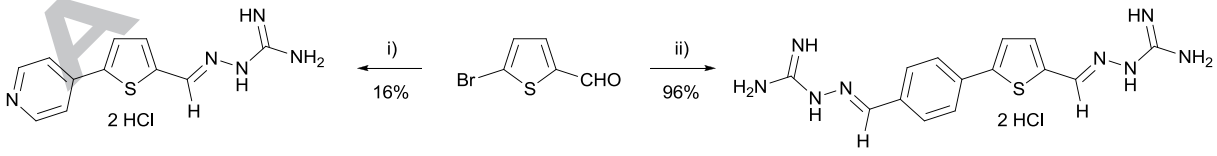


1: X = S
2: X = O

3: X = S, R = H, 90%
4: X = S, R = CH_3, 85%
5: X = S, R = OCH_3, 82%
6: X = S, R = F, 72%
7: X = S, R = Br, 46%
8: X = S, R = CN, 73%
9: X = O, R = H, 87%

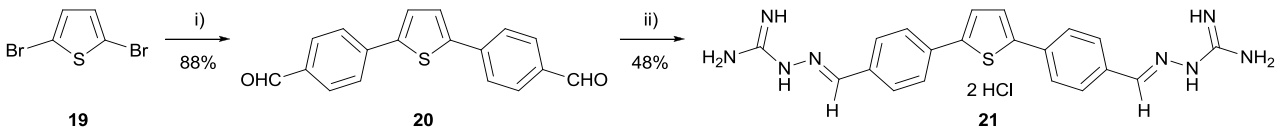
10: X = S, R = H, 100%
11: X = S, R = CH_3, 59%
12: X = S, R = OCH_3, 72%
13: X = S, R = F, 100%
14: X = S, R = Br, 99%
15: X = S, R = CN, 65%
16: X = O, R = H, 100%

Scheme 1 Synthesis of the target compounds **10-16**. Conditions: (i) $ArB(OH)_2$, $Pd(OAc)_2$, PPh_3, DME, Na_2CO_3 aq, EtOH, 90 °C; (ii) $H_2NNHC(NH)NH_2$·HCl, EtOH, HCl, 90 °C.



17 **1** **18**

Scheme 2 Synthesis of the target compounds **17** and **18**. Conditions: (i) (a) $ArB(OH)_2$, $Pd(OAc)_2$, PPh_3, DME, Na_2CO_3 aq, EtOH, 90 °C; (b) $H_2NNHC(NH)NH_2$·HCl, EtOH, HCl, 90 °C; (ii) (a) $ArB(OH)_2$, $Pd(OAc)_2$, PPh_3, DME, Na_2CO_3 aq, 90 °C; (b) $H_2NNHC(NH)NH_2$·HCl, EtOH, HCl, 90 °C



19 **20** **21**

Scheme 3 Synthesis of the target compound **21**. Conditions: (i) (a) $ArB(OH)_2$, $Pd(OAc)_2$, PPh_3, DME, Na_2CO_3 aq, 80 °C, μW; (b) DIBAL, PhMe, 0 °C; (ii) $H_2NNHC(NH)NH_2$·HCl, EtOH, HCl, 90 °C

3. Results and discussion

3.1. Chemistry

The synthesis of guanylhydrazone derivatives **10-16** was accomplished using a simple two-step procedure described in Scheme 1. The first step involved Suzuki cross-coupling reaction between commercially available thiophene **1** and furan **2** derivatives and arylboronic acids.[27] Subsequent condensation of aldehydes **3-9** with aminoguanidine hydrochloride afforded compounds **10-16** in moderate to excellent yields.

Following the similar two-step procedure guanylhydrazone derivative **17** and bis-guanylhydrazone **18** were obtained (Scheme 2).

Preparation of the bis-guanylhydrazone **21** was accomplished by reaction of dialdehyde **20**[28] with aminoguanidine hydrochloride (Scheme 3).

3.2. Antifungal activity and cytotoxic properties of guanylhydrazones

The aldehyde **3** and guanylhydrazones **10-18** and **21** were evaluated for their in vitro antifungal activity against *Candida albicans* ATCC10231, *Candida albicans* CA5, *Candida krusei* ATCC34135, *Candida parapsilosis* C27, *Aspergillus fumigatus* ATCC13073, *Fusarium oxysporum* AB18, *Microsporum canis* DMT2 and *Trichophyton mentagrophytes* DMT4, where voriconazole was used as reference drug. The antifungal activity on well characterized clinical isolates (*C. albicans* CA5, *C. parapsilosis* C27, *F. oxysporum* AB18, *M. canis* DMT2 and *T. mentagrophytes* DMT4) was carried out at National Reference Medical Mycology Laboratory, Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade, Serbia. Clinical isolates (*C. albicans* CA5, *C. parapsilosis* C27 and *F. oxysporum* AB18) exhibited increased resistance towards standard antifungal compounds, including resistance to voriconazole. The results of in vitro antifungal screening (MIC values: minimal inhibitory concentrations that causes fungicidal effect) and cytotoxicity (IC_{50} values) of the test compounds are given in Table 1.

All synthesized guanylhydrazones showed considerable antifungal activity against type strains, as well as human clinical isolates (Table 1). Aldehyde **3** did not show significant antifungal activity with MIC values of 250 μg mL^{-1} and 125 μg mL^{-1} against *A. fumigatus*, which indicated that the guanylhydrazone group was essential for the antifungal activity of the tested compounds. Thiophene-based guanylhydrazone **10** showed higher inhibitory activity than corresponding furan analog **16** against all screened fungi, so all further examined derivatives contained thiophene substructure.

Nine out of ten guanylhydrazones exhibited promising activity against *C. albicans* type strain with MICs ranging from 0.9 to 7.8 μg mL^{-1}, with derivative **11** containing methyl group in the *para*-position of the phenyl ring being the most active one. It is noteworthy that seven out of ten derivatives showed excellent activity against voriconazole resistant *C. albicans* CA5 with MICs ranging from 0.25 to 3.9 μg mL^{-1}. In the case of this clinical isolate, bis-guanylhydrazone **21** exhibited the best activity with MIC in submicromolar range (MIC = 0.25 μg mL^{-1}). Additionally, desirable high selectivity towards *C. albicans* CA5 strain in comparison to human lung fibroblasts (SI = 20) was observed for compound **21** (Table 1). Similarly, 20-fold ratio was observed for another bis-guanylhydrazone derivative **18** in the case of *C. albicans* CA5 strain, suggesting lower cytotoxicity of bis-derivatives.

Table 1 Antifungal activity and cytotoxic properties of guanylhydrazones **10-21** in comparison to aldehyde **3** and voriconazole (VOR)

Compd	Minimal inhibitory concentrations (MICs, μg mL^{-1})								Cytotox. (μg mL^{-1})[a]
	Candida albicans ATCC10231	*Candida albicans* CA5	*Candida krusei* ATCC34135	*Candida parapsilosis* C27	*Aspergillus fumigatus* ATCC 13073	*Fusarium oxysporum* AB18	*Microsporum canis* DMT2	*Trichophyton mentagrophytes* DMT4	MRC5[b]
3	250	250	>250	>250	125	250	250	250	50
10	1.95	1.95	6.25	6.25	25	62.5	1.56	3.12	5
11	0.90	0.50	3.12	3.90	6.25	7.8	1.56	1.56	2.5
12	7.80	3.9	6.25	6.25	25	>250	0.97	3.12	5
13	3.90	1.95	6.25	6.25	25	>250	0.97	1.56	4
14	3.12	1.56	1.56	3.90	6.25	3.9	0.25	0.97	2
15	3.90	7.80	25	12.5	25	>250	0.97	6.25	5
16	7.80	15.6	50	50	125	125	1.56	6.25	20
17	15.6	125	>250	>250	250	>250	125	25	25
18	3.90	0.50	25	50	25	>250	0.97	3.90	10
21	3.90	0.25	50	50	25	>250	0.25	0.25	5
VOR[c]	0.50	R[d]	0.06	R	0.06	R	0.50	0.25	40

[a] IC_{50}; SD values ±2-5%. [b] human lung fibroblasts. [c] Control drug: VOR as a voriconazole. [d] Resistance.

Guanylhydrazones **11** and **14** with methyl- and bromo-substituent in *para*-position on the phenyl ring, respectively, showed broad spectrum activity. Of particularly interest was their activity against voriconazole resistant *C. parapsilosis* C27 (MIC = 3.9 μg mL^{-1}) and *F. oxysporum* AB18 (**11**: MIC = 7.8 μg mL^{-1}; **14**: MIC = 3.9 μg mL^{-1}) strains. However, they both exhibited potent in vitro antirpoliferative effect on human fibroblasts (Table 1). Nevertheless, in the case of **11**, MIC concentrations were 5 and 2.7 times lower in comparison to IC_{50} value for *C. albicans* CA5 and ATCC10231 strains, respectively. With respect to dermatophyte *M. canis*, compounds **14** and **21** showed improved activity (MICs = 0.25 μg mL^{-1}) in comparison to the standard drug voriconazole (MIC 0.5 = μg mL^{-1}). The MIC values for novel guanylhydrazones were comparable or better to recently reported novel triazole derivatives containing the 1,2,3-triazole group against *Candida* strains.[37]

Antifungal activity of new guanylhydrazones **10-17** was not apparently dependent on the electronic effect of substituent on phenyl ring. However, based on the activity of the compounds **11** and **14,** hydrophobic effect could be important for the pronounced antifungal activity. Interestingly, although deemed essential for antifungal activity, an additional guanylhydrazone substructure did not have an additive effect on the antifungal activity of compound **18** in comparison to parent compound **10** against most of the tested strains. On the other side, bis-guanylhydrazone **18** was 4 times more potent than **10** against voriconazole resistant *C. albicans* CA5 human clinical isolate. Based on the obtained results, compounds **11**, **14** and **18** were identified as promising candidates and were further evaluated. Antifungal properties and time-kill dependence of selected guanylhydrazones were examined and followed further using subinhibitory concentrations of **11**, **14** and **18** and compared to activity of voriconazole (Fig. 2A).

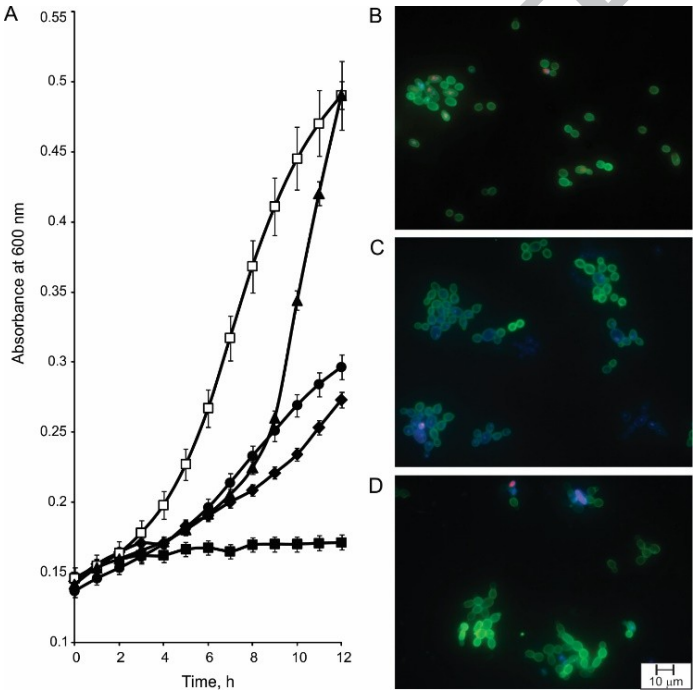


Figure 2. Antifungal effect of selected guanylhydrazones on *C. albicans* growth. A) Time-kill effect of subinhibitory concentrations of **11** (diamond), **14** (circle), **18** (black square), and voriconazole (triangle) on *C. albicans* growth measured by optical density in comparison to DMSO treated contol (white square). Effect of MIC concentrations of **11** (C) and **18** (D) on culture of *C. albicans* (OD600=1) after 2 h in comparison to DMSO treated culture (B) revealed by fluorescent microscopy using Concavaline-FITC/PI/DAPI staining.

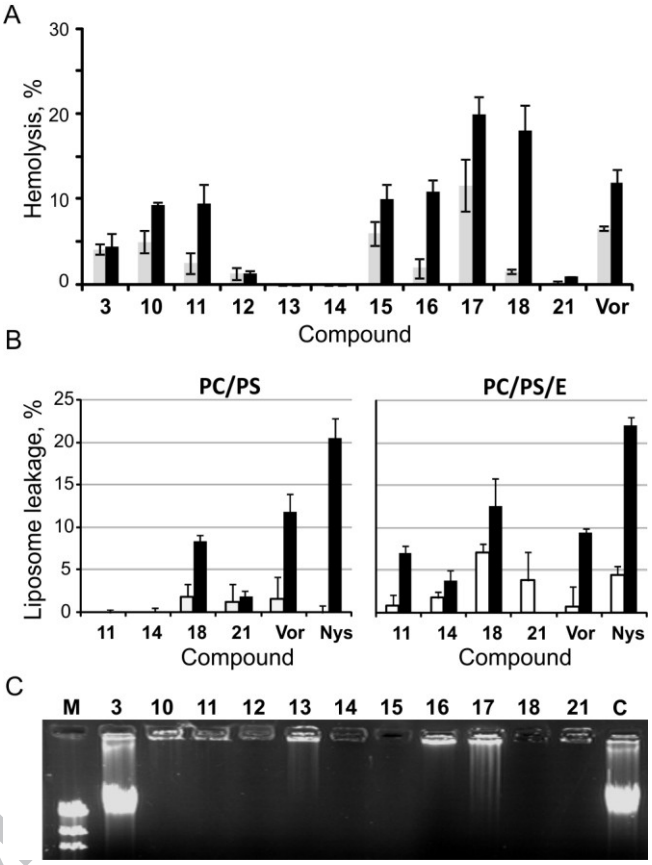


Figure 3. A) Hemolytic effect of guanylhydrazones using MIC (black bars) and IC_{50} (light grey bars) concentrations. B) Release of calcein from PC/PS and PC/PS/E liposomes in the presence of selected guanylhydrazones and voriconazole (VOR) and nystatin (NYS) was monitored for 30 min at 518 nm. C) In-vitro DNA interaction ability of guanylhydrazones with *C.albicans* DNA (M-molecular marker λ DNA/HinDIII digest; C-DMSO treatment).

Within 12 h of incubation, voriconazole-treatred cells reached the growth of the control that was treated with DMSO, while **11** and **14** treated cells reached 50% of the control growth. On the other side, compound **18** exhibited pronounced fungistatic effect even after 12 h, not allowing the growth of *C. albicans* culture. However, within 24 h all cultures reached the full growth (results not shown). It can be concluded, that under conditions tested, **11** and **14** showed better fungistatic effect then voriconazole, but not as good as **18**. Next, we examined the effect of **11** and **18** on *C. albicans* membranes by the combined concavaline/PI/DAPI cell staining assay. PI is membrane impermeable dye and binds to nucleic acid only in the dead cells yielding fluorescence in the red wavelength region. DAPI easily passes the membrane and strongly binds to DNA of both living and dead cells (giving blue color). *C. albicans* was treated with **11** and **18** MIC concentrations for 2 h and showed that treatment with compound **18** induced the membrane damage and the cell death in only about 20 % cells according to red PI staining (Fig. 2 D). The cell death was not observed in *Candida* sample treated with **11**, as PI staining of these cells (Fig. 2C) was comparable to non-treated control (Fig. 2B). None of the treatments induced apoptosis, since the DAPI staining appeared light blue and homogenous in all samples indicating the absence of nuclei condensation (Fig. 2C and D).

We also studied the effect of compounds on the membrane integrity of sheep red blood cells (RBC) to determine their ability to cause hemolysis and to examine their effect on cell membrane (Fig. 3A). We have monitored the release of haemoglobin from RBCs treated with concentrations of compounds corresponding

to the lowest MIC against fungal strains and IC_{50} values (Table 1) and showed that generated guanylhydrazones mostly did not exhibit membrane disturbances (up to 10% hemolysis), except for **17** and **18** at 25 μg mL^{-1} and 10 μg mL^{-1} respectively, causing about 20% hemolysis (Fig. 3A).

Given that polyenes, such as clinically used nystatin, with known membrane disturbance mode of action,[38,39] caused 35-40% hemolysis at 40 μg mL^{-1} (results not shown) under same conditions, we concluded that membrane may not be the primary target of novel guanylhydrazones. In addition, the ability of selected guanylhydrazones (**11**, **14**, **18** and **21**) to disrupt unilamellar liposomes was assessed (Fig. 3B). Two type of liposomes were included in the leakage assay: ones containing L-α-phosphatidylcholine and L- α -phosphatidylserine (PC:PS) and the ones mimicking fungal membrane consisting of L-α-phosphatidylcholine, L- α -phosphatidylserine and ergosterol (PC:PS:E) in the ratio 11:4:5. At MIC concentrations, only **18** induced 8% PC:PS liposome leakage in comparison to that of **12** and **21** induced by voriconazole and nystatin, respectively. However, low leakage of liposomes containing ergosterol and mimicking fungal membrane of 7-12% was induced by all selected guanylhydrazones at MIC concentrations (Fig. 3B). Therefore, membrane disruption was excluded as primary mode of action of these compounds.

We also examined *in-vitro* interaction of these molecules with *C. albicans* genomic DNA using gel electrophoresis assay after prolonged incubation (12 h) of compounds (25 μg/mL) with purified DNA. This study was prompted by ability of the guanylhydrazone groups to bind to DNA.[40] The basicity of the guanylhydrazone groups open possibilities that this functionality bind to the phosphate groups of the DNA molecule. Furthermore, intercalating properties might be also envisaged due to possible near planar geometry of guanylhydrazone group and aromatic ring.

Under tested conditions, all guanylhydrazones, apart from aldehyde **3,** showed the excellent and comparable ability to competitively intercalate double stranded fungal genomic DNA, which resulted in the inability of ethidum bromide to intercalate and emit under UV exposure (Fig. 3C). Ethidium bromide is one of the most sensitive fluorescence probes for DNA binding: intercalation of a substrate into DNA leads to a decrease in the fluorescence intensity of the ethidium bromide-DNA complex. Significant and comparable DNA interaction properties of all tested guanylhydrazones lead to the conclusion that this may be a primary cause of their observed cytotoxicity, as well as one of the possible mechanisms of antifungal action (Table 1).

3.3. Activity of guanylhydrazones against Candida biofilms

C. albicans can grow in three different morphologies: yeast, pseudohyphae and hyphae. In particular, the hyphal form plays an important role during infection process and virulence[41] and it is as critical in biofilm formation. Biofilms represent complex community of microorganisms surrounded by extracellular polymeric matrix that are attached to a surface, such as plastics and catheters.[42,43] Biofilms protect microorganisms from host human immune system and antimicrobial agents.[44] *Candida* spp. biofilms are difficult to eradicate because they are resistant to most antifungal drugs, including the azoles and amphotericin B, but are susceptible to the echinocandins.[45] Therefore, we have examined the effect of guanylhydrazones on *Candida* yeast to hypae transition, and biofilm formation and dispersion.

Compounds **10-13**, **15**, **17** and **18**, as well as voriconazole at sub-MIC concentrations (70% of MIC value determined for the

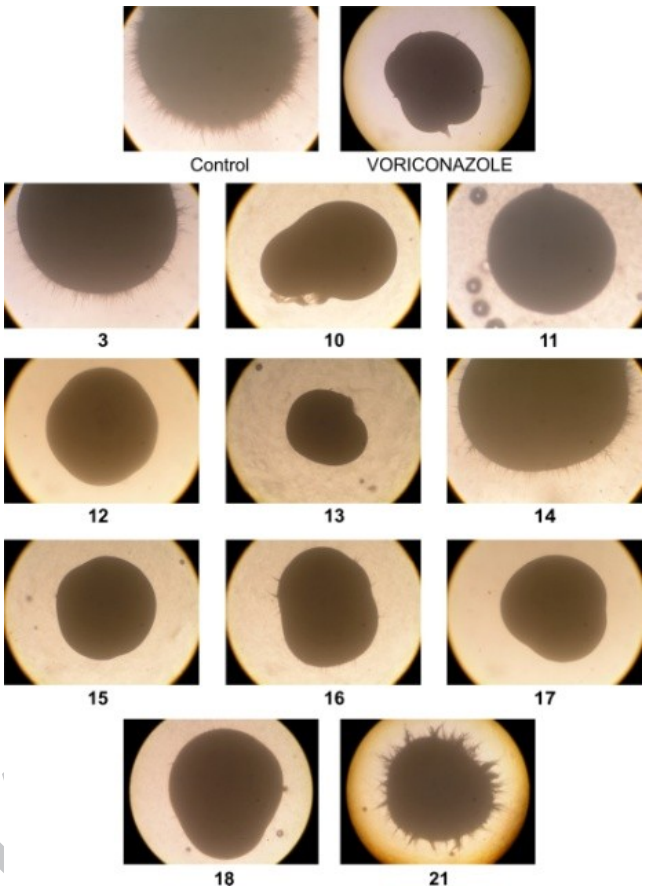


Figure 4. Effect of subinhibitory amounts (MIC_{70}) of guanylhydrazones derivatives on *C. albicans* hyphal growth.

planktonic growth; MIC_{70}) showed complete inhibition of *C. albicans* filamentous growth on the hyphal inducing Spider medium (Fig. 4). Guanylhydrazone **16** almost completely inhibited the formation of hyphe, while aldehyde **3** and compound **14** showed the inhibition to lesser extent in comparison to DMSO control. On the other hand, **21** that showed excellent activity against *C. albicans* CA5, apparently induced higher level of hyphal formation in comparison to the control (Fig. 4).

Combined results for compounds **11** and **18** supported further screening for their ability to inhibit *C. albicans* biofilm formation and to disrupt pre-formed mature biofilms. *C. albicans* was cultured in the absence or in the presence of MIC concentrations of compounds **11** and **18**, and biofilms were allowed to form on plastic coverslips for 24 h. Fluorescence isothiocyanate conjugated Concanavalin A-FITC staining of *Candida* cells demonstrated that, comparing to non-treated control, both compounds completely prevented the hyphal growth which is an essential feature for biofilm formation (Fig. 5A). Observed result was in accordance to obtained effects on the Spider medium (Fig. 4) and showed that both compounds have an outstanding antifungal properties in terms of preventing *Candida* biofilms formation. Obtained result is very important, having in mind that *C. albicans* usually very readily forms biofilms on indwelling medical devices and mucosal tissues, which then serve as infectious reservoir that is difficult to eradicate and influence drug response.[46]

When mature *Candida* biofilms were pre-formed for 48 h and then exposed to MIC concentrations of the same compounds, Concanavalin A-FITC staining showed that **11** was more effective in biofilm disruption (Fig. 5B) versus **18**. However, minimal biofilm eradication concentration (MBEC) determined

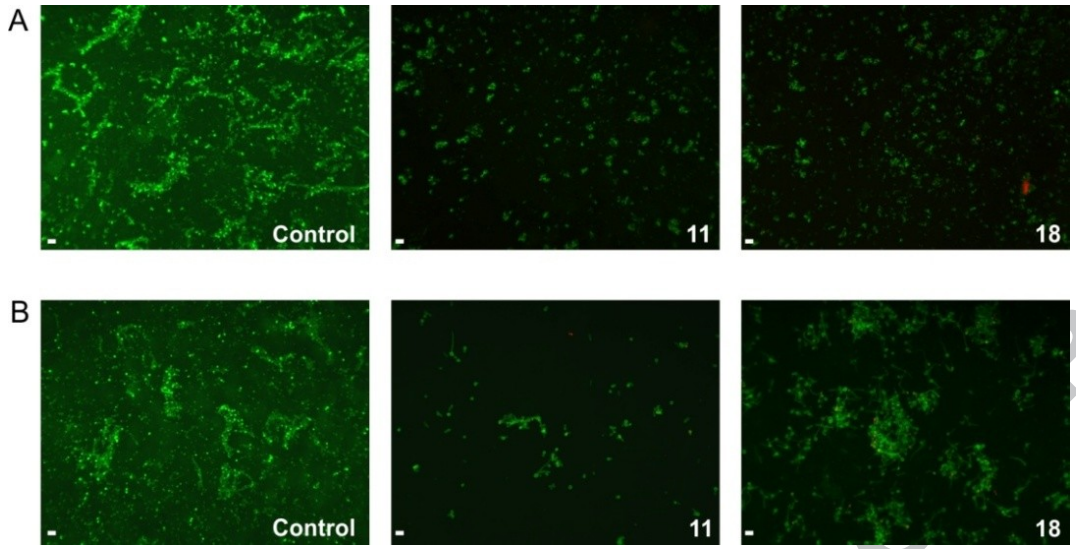


Figure 5. Activity of **11** and **18** on *C. albicans* biofilms. A) inhibition of biofilms formation and B) disruption of preformed *Candida* biofilms (Scale bar=10 µm)

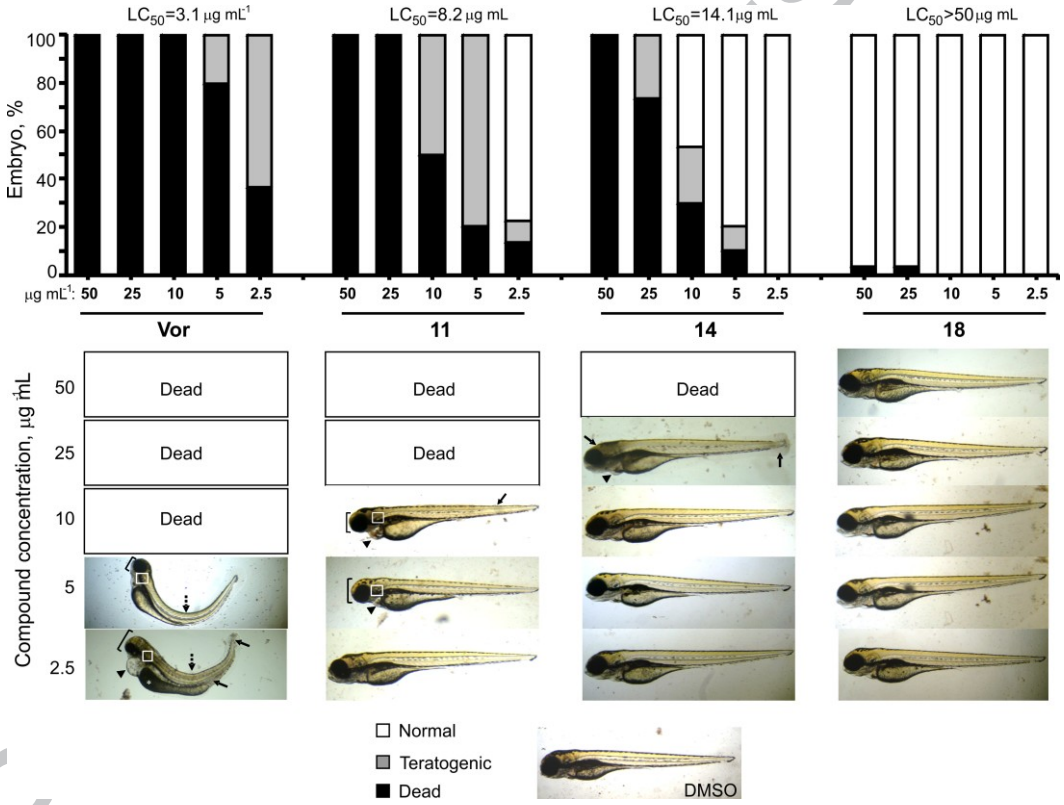


Figure 6. Effects of **11, 14** and **18** on development of zebrafish embryos in comparison to **Vor** and DMSO treated control at 96 hpf. LC_{50} represent compound concentration estimated to cause lethal effect in 50% of embryos. This data is from three repeat experiments. 30 embryos were tested for each treatment. Developmental embryos' malformation such as pericardial edema (arrowhead), unresorbed egg yolk (asterisk), lordosys (dashed arrow), tail tissue disintegration (arrow), small head (bracket) and reduced otoliths (boxed) are denoted.

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against mature biofilms of three strains *C. albicans* ATCC10231, *C. krusei* ATCC34135 and *C. parapsilosis* CA27 were all much higher than MIC values and in the range above 125 µg mL^{-1} (Table S2) suggesting that these compounds could not be used for efficient biofilm eradication.

3.4. In-vivo embryotoxicity assessment

The zebrafish allows high-quality *in-vivo* validation of drug leads early in drug discovery, well before clinical trials serving as a very useful bioassay platform for toxicology studies.[47] Therefore, the effects of the most potent guanylhydrazones **11**, **14** and **18** and those of voriconazole on the development of *Danio rerio* embryos were examined at concentrations close to MIC and IC_{50} values determined in antifungal and cytotoxicity assessment (Table 1). Strikingly, **18** showed no lethal or adverse developmental effects up to 50 µg mL^{-1}, while LC_{50} values of **11** and **14** were comparable and 2.6- and 4.5-fold higher in comparison to that of **Vor**, respectively (Fig 6). None of tested guanylhydrazones, applied at concentration of 2.5 µg mL^{-1}, showed lethal or adverse developmental effects on treated embryos until 96 hpf (Fig. 6). Survived embryos upon **Vor** treatment, found only at 2.5 and 5 µg mL^{-1}, were reduced in growth and exhibited a serious skeletal deformities, whereas at 5 µg mL-1 embryos suffered of cardiac dysfunctions such as

pericardial edemas, low heart beating and impaired caudal circulation (Fig. 6). Embryos treated with compounds **11** and **14** did not developed scoliosis like those upon **Vor** treatment, and suffered only of relatively small pericardial edemas (Fig. 6). At the highest concentration of **11** (10 μg mL^{-1}) and **14** (25 μg mL^{-1}) where alive embryos were still detected, some tissue disintegration in tail region was observed. At 5 and μg mL^{-1} of **11**, embryos were reduced in growth, had smaller head and otoliths reduced in size (Fig. 6).

Embryotoxicity data showed that at tested concentrations **11**, **14** and **18** were less embryotoxic and teratogenic than voriconazole. To the best of our knowledge there are no studies that evaluated voriconazole in zebrafish embryos model, however toxic side effects of this triazole drug have been recently reported such as visual disturbances through inhibition of TRPM1 and TRPM3 cation channels or the effect on renal function.[48,49]

3.5. Molecular docking and evaluation

In order to further evaluate possible mode of antifungal action of studied compounds, we have decided to examine one of the most studied molecular targets of antifungal compounds, namely lanosterol 14α-demethylase (CYP51) from the ergosterol biosynthetic pathway, by *in silico* molecular docking. CYP51 is a member of the cytochrome P450 super family and is implicated in the biosynthesis of fungal ergosterol, by catalyzing the oxidative removal of the 14α-methyl group of lanosterol to give the $\Delta^{14,15}$- unsaturated key intermediates.[50] Given that up to date the crystal structure of CYP51 enzyme for *Candida* spp. has not been obtained we have used appropriate homology model for computation analysis. It is reported that typical CYP51 inhibitors fit in the putative active site of CYP51 by a combination of heme co-ordination, hydrogen bonding, π-π stacking and hydrophobic interactions.[51,52] These interactions at the active site of CYP51, provide the basis for the design of novel and potential antifungal agents with a broad antifungal spectrum and possibly with less potential to develop drug resistance. On the another hand, most commonly used antifungal drugs show undesirable side effect by reacting with human cytochrome P450 super family enzymes.[53] Therefore, we have carried out comparative binding study of synthesized guanylhydrazones with CYP51 and human cytochrome P450 to examine the possibility if synthesized guanylhydrazones have the minimal structural prerequisites to interact with these targets.

The docking scores of all studied compounds were calculated and top four studied compounds for both investigated enzymes according to Docking Score are presented in Table 2. In the case of *C. albicans* CYP51, the highest interaction was calculated for bis-guanylhydrazone **18**, followed by **16**, **12** and **3**. In the case of human CYP51, compound **18** still had the highest calculated score, followed by **16**, **17**, and **13**. The analysis of the Glide docking scores showed that these compounds docked to the active site of the cytochrome P450 sterol 14α-demethylase, so the antifungal activity of studied compounds could be reflected by their interaction with the heme cofactor as well as amino acids in the CYP51 active site, where the guanylhydrazones side chain plays very important role (Fig. 7).

Furthermore, the interaction of these compounds at the active site of CYP51 may also be influenced by the heterocyclic ring fused to the main scaffold along with the substituents borne by it. It has to be noted that in presented study the real interaction profile for the synthesized compounds discussed herein has not been establish experimentally. However, it is interesting to discuss the predicted interactions for the best docking poses, since two and three compounds, respectively to studied enzyme, showed better activity in comparison to that of voriconazole. Surprisingly, one of the most active compounds **11**, did not rank amongst the first five in docking analysis while inactive aldehyde **3** did (Table 2).

Docking mode of the best compounds poses, according to docking score, in the active site of CACYP51 is presented in Figure 6. There are many amino acid residues in the active site of cytochrome P450 sterol 14α-demethylase that can participate in close van der Waals and coulombic interactions with the inhibitor, when bound to it. For this reason the active site of cytochrome P450 sterol 14α-demethylase can be considered as highly conserved. Another important interaction of ligand with enzyme active site is the formation of hydrogen bond with amino acids. The amino acid THR-311 is most prominently involved in the interaction in the case of most ligands. Comparison of docking scores and other studied functions presented at Table 2 revealed that compound **18** (Fig. 7A) possibly has better affinity for CACYP51 in comparison to human enzyme. Overall, good correlation between docking scores and antifungal activity was observed (Table 1, Table 2).

Beside bis-guanylhydrazone **18**, guanylhydrazones **11** and **14** also showed pronounced antifungal activity (Table 1) and it was important to examine these two molecules closer through molecular docking study. Both compounds **11** and **14** have comparable binding energy (-37.549 kcal mol^{-1} and -42.449 kcal

Table 2. Molecular docking data

Compd	Docking Score	Glide evdw (kcal mol^{-1})	Glide ecoul (kcal mol^{-1})	Glide Energy (kcal mol^{-1})	Glide Emodel (kcal mol^{-1})	Hbond (kcal mol^{-1})
C. albicans CYP51						
18	-6.953	-46.632	-2.688	-49.32	-73.003	-1.765
16	-5.958	-33.459	-5.388	-38.846	-47.43	-1.304
12	-5.431	-30.357	-2.822	-33.179	-50.543	0
3	-5.38	-23.664	-2.992	-26.656	-35.746	-0.7
Human CYP51						
18	-6.44	-39.031	-17.407	-56.438	-92.105	-2.308
16	-6.166	-36.826	-9.226	-46.052	-67.781	-1.87
17	-6.076	-30.717	-15.666	-46.384	-63.977	-2.525
13	-5.617	-33.981	-9.564	-43.545	63.41	-0.991

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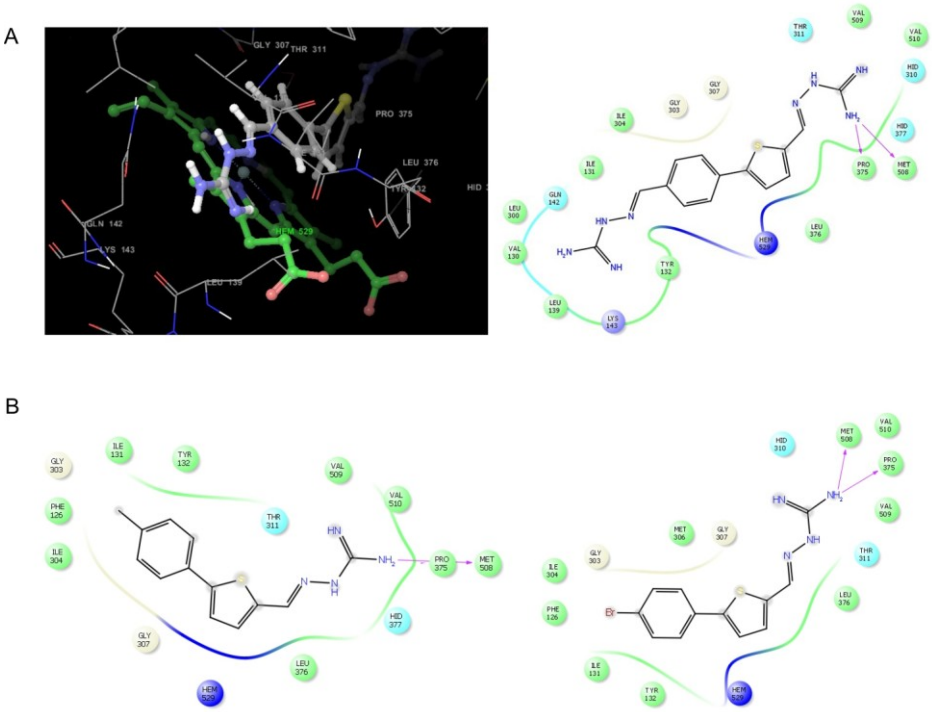

Figure 7. Docking mode of A) **18** (top panel) and B) **11** (left) and **14** (right) in the active site of CACYP51.

mol^{-1}, respectively) and glide emodel was similar between two of them (-47.964 kcal mol^{-1} and -55.012 kcal mol^{-1}). These two parameters for aldehyde **3** were much lower (Table 2 and Table S3). Docking mode of compounds **11** and **14** inside the active site of CACYP51 revealed strong interactions with amino acids PRO-375 and MET-508 (Fig. 7B), which could indicate their importance in enzyme inhibition and can be correlated with their experimentally establish activity.

Docking studies of compounds **11** and **14** with human CYP51 revealed that among all studied compounds they have the lowest energy interactions with enzyme represented as docking score (-4.609 for compound **11** and -4.295 for compound **14**), and low values of binding energy (-44.330 kcal mol^{-1} and -28.262 kcal mol^{-1}, respectively; -48.185 kcal mol^{-1} and -45.407 kcal mol^{-1} for glide emodel, respectively; Table S3), suggesting lower affinity towards human CYP51. Nevertheless, calculated low affinity towards human cytochrome P450 was not mirrored in antiproliferative cytotoxic experiments (Table 1). Overall, in silico analysis and docking study using CYP51s supported the hypothesis that inhibition of CACYP51 could be one of the mechanisms of antifungal activity of guanylhydrazone compounds. However, discrepancies between experimentally obtained data and predicted parameters from in silico analysis indicate that other modes of action could also be involved.

Many problems in the drug development are the result of pharmacokinetic disadvantages such as poor absorption, first pass effect, a high degree of binding to the protein, therefore, establishing druglikeness is still a very important step in the search for new potential drug candidates. Druglikeness allows the assessment of the pharmacokinetic profile of the tested molecules based on the prediction of their absorption and distribution. Based on Lipinski's "Rule of five", the critical limit for acceptable drug-likeness is that no more than one violation of the rule exists in molecule.[25,26]

Molinspiration tool (Molinspiration Cheminformatics-2013) was used for calculating physicochemical properties of investigated guanylhydrazones. For calculating of logP fragment-based contributions and correlation factors were used. Calculated physicochemical properties of investigated compounds are shown in Table S4. Data indicate that all guanylhydrazones except **18** and **21** do not violate the "Rule of five". For all investigated compounds clogP are below 4 and therefore they have favourable physicochemical profiles for oral bioavailability. Topological polar surface area (TPSA) is a good descriptor for the drug transport properties, drug absorption, including intestinal absorption, bioavailability, Caco-2 permeability. TPSA can be defined as a sum of the surface areas occupied by the oxygen and nitrogen atoms and the hydrogen atoms attached to them and represent the hydrogen bonding capacity of the molecules. Molecules with TPSA < 140 Å^2 have good intestinal absorption, while those with TPSA < 60 Å^2 show good blood–brain barrier penetration. All investigated compounds except **18** and **21** satisfied the criterion for good intestinal absorption (Table S4).

The number of H-bond donors and acceptors can be used for establishing hydrogen bonding capacity of the investigated compounds. An important factor for oral bioavailability, as well as for the efficient bonding to receptors and channels, is the conformational flexibility of the molecules described by the number of rotatable bonds. Sufficient oral bioavailability is expected for molecules with 10 rotatable bonds or fewer. In all investigated compounds there is at least one rotatable bond, but no more than 8 (compound **21**).

Summarizing the physicochemical properties of investigated compounds, conclusion can be made that they all meet criteria for good solubility and permeability and can be considered as potential drug candidates.

4. Conclusions

A new therapeutic options and better understanding of their mechanisms of action is essential to improving the outcome of fungal infections. A novel thiophene-based guanylhydrazones (iminoguanidines) were obtained in high yields using a simple

two-step procedure. In vitro antifungal activity assays showed their potent, antifungal effect against yeasts, molds and dermaptophytes, the most common human and animal clinical isolates. Compounds **11**, **18** and **21** exhibited excellent activity against voriconazole resistant *C. albicans* CA5 with MIC <1 μg mL^{-1} having selectivity index in comparison to human fibroblasts from 5 to 20. Additionally, bis-guanylhydrazone **18** showed better embryotoxicity profile, as well as better fungistatic effect in comparison to clinically used voriconazole and very efficiently inhibited *Candida* biofilm formation. Interaction with genomic DNA of *C. albicans* in vitro, indicated a possible mechanism of their activity. From the hemolysis experiments cell membrane was excluded as the primary target of these compounds, whereas in silico analysis and docking study using CYP51s supported the hypothesis that inhibition of CACYP51 could be one, but not the only mechanism of antifungal activity. For all compounds it has been calculated to have satisfying pharmacokinetic profile based on Lipinski's "rule of five". This study provides strong evidence that selected guanylhydrazone derivatives are good structural platform for further derivatization and possible application in antifungal therapy.

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Supplementary Material

Supplementary data related to this article is available including: ^{1}H NMR, ^{13}C NMR and HPLC analyses of gua

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Synthesis and evaluation of thiophene-based guanylhydrazones (iminoguanidines) efficient against panel of voriconazole-resistant fungal isolates

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