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Iodinated 1,2-diacylhydrazines, benzohydrazide-hydrazones and their analogues as dual antimicrobial and cytotoxic agents

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ABSTRACT

Hydrazide-hydrazones have been described as a scaffold with antimicrobial and cytotoxic activities as well as iodinated compounds. A resistance rate of bacterial and fungal pathogens has increased considerably. That is why we synthesized and screened twenty-two iodinated hydrazide-hydrazones **1** and **2**, ten 1,2-diacylhydrazines **3** and their three reduced analogues **4** for their antibacterial, antifungal, and cytotoxic properties. Hydrazide-hydrazones were prepared by condensation of 4-substituted benzohydrazides with 2-/4-hydroxy-3,5-diiodobenzaldehydes, diacylhydrazines from identical benzohydrazides and 3,5-diiodosalicylic acid *via* its chloride. These compounds were investigated *in vitro* against eight bacterial and eight fungal strains. The derivatives were found potent antibacterial agents against Gram-positive cocci including methicillin-resistant *Staphylococcus aureus* with the lowest values of minimum inhibitory concentrations (MIC) of 7.81 μM. Four compounds inhibited also human pathogenic fungi (MIC of ≥1.95 μM). The derivatives had different degrees of cytotoxicity for HepG2 and HK-2 cell lines (IC_{50} values from 11.72 and 26.80 μM, respectively). Importantly, normal human cells exhibited lower sensitivity. The apoptotic effect was also investigated. In general, the presence of 3,5-diiodosalicylidene scaffold (compounds **1**) is translated into enhanced both antimicrobial and cytotoxic properties whereas its 4-hydroxy isomers **2** share a low biological activity. *N*′-Benzoyl-2-hydroxy-3,5-diiodobenzohydrazides **3** have a non-homogeneous activity profile. Focusing on 4-substituted benzohydrazide part, the presence of an electron-withdrawing group (F, Cl, CF_3, NO_2) was found to be beneficial.

1. Introduction

Hydrazide-hydrazones containing azomethine group (–NH–N=CH–) connected with carbonyl group (i.e., with the following moiety, –C(=O)–NH–N=CH–) have gained a great importance due to their diverse biological properties and wide spectrum of pharmaceutical applications.[1] Among the biological properties of this class of compounds, antimicrobial activity is the most frequently encountered in scientific literature.[2–5] Hydrazide-hydrazones of 3-methoxybenzoic acid and 4-*tert*-butylbenzoic acid possessed high bactericidal activity against *Bacillus subtilis* (minimum inhibitory concentrations, MIC, of 1.95–31.25 mg/L).[6] Conjugates containing hybrids of bis-pyrazole scaffolds joined through a hydrazone-hydrazide linker were synthesized and evaluated for their antimicrobial activity against a panel of Gram-positive and Gram-negative bacteria. Some of them showed also excellent anti-*Candida* activity with MIC values of 3.9 mg/L.[7] Hydrazide-hydrazone pharmacophore approach for synthesis of new antibacterial agents was also used in designing of quinoxaline hybrids having incorporated *N*-propionic and *O*-propionic hydrazide moieties.[8]

The hydrazide-hydrazones have attracted large number of researchers also because of their anticancer activity.[9–12] Pyridine-based bis(hydrazide-hydrazones) were screened for their antibacterial activities as well as anticancer activities. Some of them showed significant activity against human colon cancer as well Ishikawa human endometrial cancer cell lines (IC_{50} of 6.78–8.88 μM).[13]

1,2-(or *N*,*N*′-)Diacylhydrazine scaffold (–CO–NH–NH–CO–) have also been employed in medicinal chemistry, although less frequently than the hydrazide-hydrazones. 1,2-Diacylhydrazines are often used for the construction of 1,3,4-oxadiazole ring. This approach includes dehydration followed by simultaneous cyclization of diacylhydrazines

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under the action of various dehydrating reagents – thionyl chloride, polyphosphoric acid, phosphorus pentoxide, trichloride or oxychloride, tosyl chloride, acetic anhydride, sulfuric acid etc. Another approach is oxidative cyclization of hydrazide-hydrazones.[14] Diacylhydrazines are present in naturally occurring bioactive compounds and they have been investigated as antioxidant, cytotoxic, antifungal, antimycobacterial and antiviral agents.[15–17]

Halogenation represents an important approach in optimization for drug development and about half of the molecules used in high-throughput screening are halogenated.[18] Iodine (I_2) is known antiseptic and disinfectant that acts against bacteria, fungi, and viruses at millimolar concentrations.[19] Its aqueous solutions with potassium iodide, called Lugol's solution was recently tested on long-term preservation of marine plankton.[20] The biological significance of iodine in the group of 2-(aminomethyl)-4-ethynyl-6-iodophenols has been described for anticancer agents developed as p53-Y220C stabilizers.[21] Iodinated phenolic products were prepared from, e.g., iodovanillin, acetovanillone or methyl vanillate by laccase catalysis at an excess of potassium iodide. The iodinated derivatives had significant growth inhibitory effects on several wood degrading fungal species.[22] Iodobenzoboroxoles were screened *in vitro* for antimicrobial activity against Gram-positive bacteria, *Micrococcus luteus* and *Bacillus cereus*, and two Gram-negative strains, *Escherichia coli* and *Serratia marcescens*. The results indicated that 4-iodo-6-methoxybenzo[*c*][1,2]oxaborol-1(3*H*)-ol possesses antibacterial activity against Gram-positive strains with MIC of 16 and 13 mg/L for *M. luteus* and *B. cereus*, respectively.[23] Hydrazide-hydrazones of 5-bromo-2-iodobenzoic acid were synthesized and identified as mild antimicrobial agents together with a significant cytotoxicity.[24] Iodinated 4-aryloxymethylcoumarins were evaluated against two cancer cell lines (MDA-MB human adenocarcinoma mammary gland and A-549 human lung carcinoma) and two mycobacterial strains; they were found to exhibited a dual anticancer-antimycobacterial action.[18]

In our previous search for antimicrobial agents, we have identified Schiff bases derived from 2-hydroxy-3,5-diiodobenzaldehyde as promising derivatives to combat Gram-positive cocci including methicillin-resistant *Staphylococcus aureus* (MRSA). Some of them also exhibited a substantial cytotoxicity for human cell lines (Fig. 1).[25–27]

According to the World Health Organization, antimicrobial resistance is a global health threat requiring an urgent multidisciplinary action. New antimicrobials are urgently needed unequivocally. An inappropriate use of antimicrobial agents is a major driver in the development of acquired drug resistance. For common infections, high rates of resistance against antibiotics frequently used to treat them have been observed worldwide. Especially alarming is the rapid global spread of multi- and pan-resistant bacteria causing untreatable infections. Focusing on *Staphylococcus aureus*, a common cause of both community and nosocomial infections, people infected with methicillin-resistant *Staphylococcus aureus* (MRSA) infections are 64% more likely to die than people with methicillin-susceptible infections. Moreover, the antibacterial and antifungal development pipeline is almost empty.[28]

Giving together here mentioned findings, we evaluated *N′*-(2-/4-hydroxy-3,5-diiodobenzylidene) 4-substituted hydrazides (i.e., hydrazide-hydrazones) and 1,2-diacylhydrazines as potential antimicrobial and cytotoxic agents.

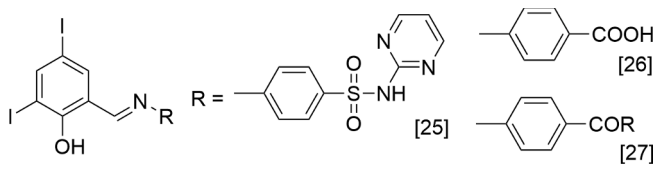


Figure 1. Antibacterial 3,5-diiodosalicylidene-imines identified by our group.[25,26,27]

2. Material and methods

2.1. Chemistry

2.1.1. General

All the reagents and solvents were purchased from Sigma-Aldrich (Darmstadt, Germany), Penta Chemicals (Prague, Czech Republic), and Lach-Ner (Neratovice, Czech Republic). They were used as received without any purification. The reactions and the purity of the products were monitored by thin-layer chromatography (TLC) using a mixture with a ratio of dichloromethane to methanol of 4:1 (*v/v*) as the eluent. Plates were coated with 0.2 mm Merck 60 F254 silica gel (Merck Millipore, Darmstadt, Germany) and were visualized by UV irradiation (254 nm). The melting points were determined on a Melting Point B-540 apparatus (BÜCHI, Flawil, Switzerland) using open capillaries. The reported values are uncorrected. Infrared spectra were recorded on a FT-IR spectrometer using ATR-Ge method (Nicolet 6700 FT-IR, Thermo Fisher Scientific, Waltham, MA, USA) in the range 600–4000 cm^{-1}. The NMR spectra were measured in dimethyl sulfoxide (DMSO)-d_6, or tetrahydrofuran (THF)-d_8 at ambient temperature using a Varian V NMR S500 instrument (500 MHz for ^1H and 126 MHz for ^{13}C; Varian Corp., Palo Alto, CA, USA). The chemical shifts δ are given in ppm and were referred indirectly to tetramethylsilane *via* signals of DMSO-d_6 (2.50 for ^1H and 39.51 for ^{13}C spectra) or THF-d_8 (1.72 and 3.58 for ^1H, 25.31 and 67.21 for ^{13}C spectra). The coupling constants (*J*) are reported in Hz. Elemental analysis (C, H, N) was performed on an automatic microanalyser CHNS-O CE instrument (FISONS EA 1110, Milano, Italy).

2.1.2. Synthesis

The synthesis and full characterization of 4-iodobenzohydrazide, 3,5-diiodosalicylidenehydrazides **1**, 4-hydroxy-3,5-diiodobenzylidenehydrazides **2**, *N′*-(4-fluorobenzoyl)-2-hydroxy-3,5-diiodobenzohydrazide **3f** and reduced analogues **4a** and **4b** were published previously by our group.[29]

*2.1.2.1. Synthesis of hydrazide-hydrazones **1** and **2**.* Briefly, 4-substituted benzohydrazides (1 mmol) were treated with 2- or 4-hydroxy-3,5-diiodobenzaldehyde (for hydrazones **1** and **2**, respectively; 1.1 mmol; 411.3 mg) in boiling methanol for 2 h. The hydrazide-hydrazones spontaneously precipitated were crystallized from MeOH if necessary.[29]

*2.1.2.2. Synthesis of 1,2-diacylhydrazines **3**.* 1,2-Diacylhydrazines **3** were prepared by a two-step procedure from appropriate 4-substituted benzohydrazides and 3,5-diiodosalicylic acid.[29] 3,5-Diiodosalicylic acid (389.9 mg, 0.001 mol) was dissolved in thionyl chloride (7 mL) and after cooling to 0 °C, 3 drops of *N*,*N*-dimethylformamide (DMF) were added. The mixture was to let stir without external cooling and then heated to 60 °C under nitrogen atmosphere. After six hours, it was evaporated to dryness under reduced pressure. Crude 3,5-diiodosalicyloyl chloride was used further without any purification. It was added into dry tetrahydrofuran (THF; 7 mL) under nitrogen atmosphere, and under vigorous stirring, a solution composed of 1 mmol of 4-substituted benzohydrazide, 4 mmol of triethylamine (558 μL) and 7 mL of THF was added dropwise. Alternatively (**3j**), the hydrazide was added as a solid together with 2 equivalents of potassium carbonate. The mixture was stirred for 24 h at room temperature. Then, it was evaporated to dryness and using ethyl acetate, water and 0.1 M hydrochloric acid, it was transferred into a separation funnel. The organic phase was washed with 0.1 M aqueous hydrochloric acid, 5% aqueous sodium bicarbonate, 0.1 M hydrochloric acid again, followed by saturated brine. The organic phase was dried over anhydrous sodium sulfate. The filtrate was concentrated under reduced pressure and *n*-hexane was added to start precipitation. After 24 h at +4 °C, the suspension was filtered off to give required derivatives **3** that were purified using crystallization from ethyl

acetate, a mixture of THF/acetonitrile or column chromatography with the mixture of toluene with ethyl acetate 4:0.3 (*v/v*).

2.1.2.2.1. N′-(Benzoyl)-2-hydroxy-3,5-diiodobenzohydrazide ***3a.*** White solid; yield 48%; mp 219–222 °C. IR (ATR): 3225 (N—H), 1658 (CO-NH) cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6): δ 13.16 (1H, s, OH), 11.15 (1H, s, NH), 10.73 (1H, s, NH), 8.33 (1H, d, $J = 2.0$ Hz, H6′), 8.26 (2H, d, $J = 1.9$ Hz, H4′), 7.94–7.90 (2H, m, H2, H6), 7.64–7.60 (1H, m, H4), 7.56–7.51 (2H, m, H3, H5). ^{13}C NMR (126 MHz, THF): δ 168.89, 166.65, 161.51, 151.63, 136.10, 133.78, 132.61, 129.16, 128.43, 116.11, 88.55, 80.60. Anal. Calcd for $C_{14}H_{10}I_2N_2O_3$ (508.05): C 33.10, H 1.98, N 5.51, found: C 33.23, H 1.98, N 5.80.

2.1.2.2.2. 2-Hydroxy-3,5-diiodo-N′-(4-methylbenzoyl)benzohydrazide ***3b.*** White solid; yield 43%; mp 193–196 °C. IR (ATR): 3287 (N—H), 1659 (CO-NH) cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6): δ 13.20 (1H, s, OH), 10.87 (1H, s, NH), 10.38 (1H, s, NH), 8.32 (1H, d, $J = 2.1$ Hz, H6′), 8.02 (1H, d, $J = 2.1$ Hz, H4′), 7.84–7.80 (2H, m, H2, H6), 7.38–7.35 (2H, m, H3, H5), 2.46 (3H, s, CH_3). ^{13}C NMR (126 MHz, DMSO): δ 165.45, 155.23, 150.65, 142.59, 135.94, 129.25, 128.99, 127.91, 127.36, 118.15, 87.54, 86.09, 21.25. Anal. Calcd for $C_{15}H_{12}I_2N_2O_3$ (522.08): C 34.51, H 2.32, N 5.37, found: C 34.39, H 2.10, N 5.44.

2.1.2.2.3. 2-Hydroxy-3,5-diiodo-N′-(4-methoxybenzoyl)benzohydrazide ***3c.*** Greyish solid; yield 53%; mp 264–267 °C. IR (ATR): 3232 (N—H), 1652 (CO-NH) cm^{-1}. ^1H NMR (500 MHz, THF-d_8): δ 13.17 (1H, s, OH), 11.05 (1H, s, NH), 10.55 (1H, s, NH), 8.30 (1H, d, $J = 2.0$ Hz, H6′), 8.23 (1H, d, $J = 1.9$ Hz, H4′), 7.88 (2H, d, $J = 8.7$ Hz, H2, H6), 7.03 (2H, d, $J = 8.8$ Hz, H3, H5), 3.80 (3H, s, CH_3). ^{13}C NMR (126 MHz, THF): δ 168.02, 165.38, 162.43, 159.71, 150.23, 135.49, 129.63, 124.26, 115.18, 114.02, 89.19, 81.97, 55.63. Anal. Calcd for $C_{15}H_{12}I_2N_2O_4$ (538.08): C 33.48, H 2.25, N 5.21, found: C 33.36, H 2.31, N 5.59.

2.1.2.2.4. N′-[(4-Tert-butyl)benzoyl]-2-hydroxy-3,5-diiodobenzohydrazide ***3d.*** Greyish solid; yield 61%; mp 173–176 °C. IR (ATR): 3283 (N—H), 1644 (CO-NH) cm^{-1}. ^1H NMR (500 MHz, THF-d_8): δ 10.84 (1H, s, OH), 9.97 (1H, s, NH), 9.74 (1H, s, NH), 8.27 (1H, d, $J = 2.1$ Hz, H6′), 8.15 (1H, d, $J = 2.2$ Hz, H4′), 7.87 (2H, d, $J = 8.3$ Hz, H2, H6), 7.50 (2H, d, $J = 8.3$ Hz, H3, H5), 1.36 (9H, s, CH_3). ^{13}C NMR (126 MHz, THF): δ 165.42, 155.46, 155.24, 150.65, 146.40, 135.98, 129.03, 127.76, 125.52, 118.15, 87.57, 86.11, 34.93, 31.05. Anal. Calcd for $C_{18}H_{18}I_2N_2O_3$ (564.16): C 38.32, H 3.22, N 4.97, found: C 37.49, H 2.96, N 5.14.

2.1.2.2.5. N′-(4-Chlorobenzoyl)-2-hydroxy-3,5-diiodobenzohydrazide ***3g.*** Yellowish solid; yield 46%; mp 246–249 °C. IR (ATR): 3233 (N—H), 1658 (CO-NH) cm^{-1}. ^1H NMR (500 MHz, THF-d_8): δ 12.11 (1H, s, OH), 10.11 (1H, s, NH), 9.94 (1H, s, NH), 8.28 (1H, d, $J = 2.0$ Hz, H6′), 8.14 (1H, d, $J = 2.0$ Hz, H4′), 7.93–7.85 (2H, m, H2, H6), 7.52–7.46 (2H, m, H3, H5). ^{13}C NMR (126 MHz, THF): δ 165.77, 161.00, 156.59, 151.80, 137.81, 130.23, 130.07, 129.40, 129.35, 119.39, 86.40, 84.95. Anal. Calcd for $C_{14}H_9ClI_2N_2O_3$ (542.50): C 31.00, H 1.67, N 5.16, found: C 30.79, H 1.81, N 5.22.

2.1.2.2.6. N′-(4-Bromobenzoyl)-2-hydroxy-3,5-diiodobenzohydrazide ***3h.*** Beige solid; yield 55%; mp 235–238 °C. IR (ATR): 3545 (O—H), 3241 (N—H), 1656 (CO-NH) cm^{-1}. ^1H NMR (500 MHz, THF-d_8): δ 12.99 (1H, s, OH), 10.41 (1H, s, NH), 10.04 (1H, s, NH), 8.24 (1H, d, $J = 2.0$ Hz, H6′), 8.11 (1H, d, $J = 2.0$ Hz, H4′), 7.84 (2H, d, $J = 8.1$ Hz, H2, H6), 7.68 (2H, d, $J = 8.1$ Hz, H3, H5). ^{13}C NMR (126 MHz, THF): δ 168.85, 165.81, 161.47, 151.69, 136.08, 133.47, 132.49, 132.37, 130.26, 127.19, 88.57, 80.63. Anal. Calcd for $C_{14}H_9BrI_2N_2O_3$ (586.94): C 28.65, H 1.55, N 4.77, found: C 28.47, H 1.70, N 5.09.

2.1.2.2.7. 2-Hydroxy-3,5-diiodo-N′-(4-iodobenzoyl)benzohydrazide ***3i.*** Greyish solid; yield 64%; mp 214–217 °C. IR (ATR): 3582 (O—H), 3298 (N—H), 1647 (CO-NH) cm^{-1}. ^1H NMR (500 MHz, THF-d_8): δ 12.86 (1H, s, OH), 10.39 (1H, s, NH), 10.08 (1H, s, NH), 8.20 (1H, d, $J = 2.0$ Hz, H6′), 8.07 (2H, d, $J = 2.1$ Hz, H4′), 8.02–7.91 (4H, m, H2, H3, H5, H6). ^{13}C NMR (126 MHz, DMSO): δ 165.68, 163.67, 148.64, 146.51, 138.23, 137.67, 132.97, 129.88, 119.45, 99.16, 87.61, 86.11. Anal. Calcd for $C_{14}H_9I_3N_2O_3$ (633.94): C 26.52, H 1.43, N 4.42, found: C 26.19, H 1.30, N 4.87.

2.1.2.2.8. 2-Hydroxy-3,5-diiodo-N′-[4-(trifluoromethyl)benzoyl]benzohydrazide ***3j.*** White solid; yield 72%; mp 251–254 °C (decomp.). IR (ATR): 3566 (O—H), 3361 (N—H), 1598 (CO-NH) cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6): δ 14.10 (1H, s, OH), 10.85 (1H, s, NH), 8.10 (2H, d, $J = 8.0$ Hz, H3, H5), 7.90–7.85 (3H, m, H2, H6, H4′), 7.72 (1H, d, $J = 2.1$ Hz, H6′). ^{13}C NMR (126 MHz, DMSO): δ 165.25, 163.16, 148.15, 146.70, 138.12, 137.29, 131.96 (q, $J = 31.8$ Hz), 128.88, 125.82 (q, $J = 3.8$ Hz), 123.64 (q, $J = 272.3$ Hz), 119.26, 88.06, 84.67. Anal. Calcd for $C_{15}H_9F_3I_2N_2O_3$ (576.05): C 31.28, H 1.57, N 4.86, found: C 31.21, H 1.59, N 4.83.

2.1.2.2.9. 2-Hydroxy-3,5-diiodo-N′-(4-nitrobenzoyl)benzohydrazide ***3k.*** Yellow solid; yield 47%; mp 221–224 °C. IR (ATR): 3559 (O—H), 3361 (N—H), 1661 (CO-NH) cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6): δ 13.23 (1H, s, OH), 10.92 (1H, s, NH), 10.50 (1H, s, NH), 8.31 (2H, d, $J = 8.6$ Hz, H3, H5), 8.12 (2H, d, $J = 8.7$ Hz, H2, H6), 7.85 (1H, d, $J = 2.6$ Hz, H6′), 7.69 (1H, d, $J = 2.6$ Hz, H4′). ^{13}C NMR (126 MHz, DMSO): δ 167.46, 164.73, 162.20, 149.21, 146.29, 139.19, 137.89, 129.16, 123.70, 119.16, 87.88, 84.53. Anal. Calcd for $C_{14}H_9I_2N_3O_5$ (553.05): C 30.40, H 1.64, N 7.60, found: C 30.61, H 1.45, N 7.73.

2.1.3. Synthesis of reduced analogues ***4***

The reduced analogue **4a** was prepared from the hydrazide-hydrazone **1f** by treatment with lithium aluminium hydride in THF under nitrogen atmosphere.[29]

2.1.4. Reductive amination[29]

An appropriate 4-halogenobenzohydrazide (1 mmol) was dissolved in 7 mL of a mixture of THF with MeOH (1:1, *v/v*), followed by addition of 3,5-diiodosalicylaldehyde (411.3 mg, 1.1 mmol). After a complete dissolution, sodium cyanoborohydride (100 mg; 1.6 mmol) and glacial acetic acid (100 μL) were added. The mixture was heated under reflux for 4 h and then let stir for 48 h at room temperature. After this time, the reaction mixture was diluted with distilled water and let stir for 30 min. Then, the mixture was evaporated to dryness and suspended in a small volume of MeOH. After additional 2 h, the resulted precipitate was filtered off and dried to provide pure product.

4-Chloro-*N′*-(2-hydroxy-3,5-diiodobenzyl)benzohydrazide **4c**. White solid; yield 68%; mp 256.5–259 °C. IR (ATR): 3227 (N—H), 1656 (CO-NH) cm^{-1}. ^1H NMR (500 MHz, THF-d_8): δ 11.41 (1H, s, OH), 8.33 (1H, s, CONH), 8.04 (1H, d, $J = 2.0$ Hz, H4′), 7.91 (2H, d, $J = 8.1$ Hz, H2, H6), 7.63 (1H, d, $J = 2.1$ Hz, H6′), 7.52 (2H, d, $J = 8.2$ Hz, H3, H5), 4.12 (1H, s, NH), 2.59 (2H, s, CH_2). ^{13}C NMR (126 MHz, THF): δ 162.79, 158.66, 148.33, 148.01, 139.91, 130.14, 129.81, 129.55, 121.12, 87.51, 80.69, 51.93. Anal. Calcd for $C_{14}H_{11}ClI_2N_2O_2$ (528.51): C 31.82, H 2.10, N 5.30, found: C 31.88, H 2.12, N 5.25.

2.2. Biology

2.2.1. Antibacterial activity

Antibacterial activity was evaluated against four Gram-positive and four Gram-negative bacterial strains of a clinical importance, namely: *Staphylococcus aureus* ATCC (American Type Culture Collection) 29213, CCM (Czech Collection of Microorganisms) 4223 (methicillin-susceptible strain, MSSA), methicillin-resistant *Staphylococcus aureus* ATCC 43300, CCM 4750 (MRSA strain), *Staphylococcus epidermidis*, ATCC 12228, CCM 4418; *Enterococcus faecalis* ATCC 29212, CCM 4224; *Escherichia coli* ATCC 25922, CCM 3954, *Klebsiella pneumoniae* ATCC 10031, CCM 4415, *Serratia marcescens*, clinical isolate, laboratory ID No. 62–2016, and *Pseudomonas aeruginosa* ATCC 27853, CCM 3955. These strains were obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic), one strain (*Serratia marcescens*) is a clinical isolate kindly provided from the Department of Clinical Microbiology, University Hospital in Hradec Králové, Czech Republic.

The microdilution broth method was performed according to EUCAST (The European Committee on Antimicrobial Susceptibility Testing) instructions[30] with slight modifications starting from the

concentration of 0.49 μM. Briefly, the cultivation was done in Cation-adjusted Mueller-Hinton broth (CAMHB, M−H 2 Broth, Sigma-Aldrich, St. Louis, MO, USA) at 35 ± 2 °C without agitation. Tested compounds were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) to produce stock solutions. The final concentration of DMSO in the testing medium did not exceed 1% (*v/v*) of the total solution composition and did not affect the growth of bacteria. Positive (microbe solely), negative (cultivation medium and DMSO) controls and internal quality standards were involved in each assay. Antibacterial activity is expressed as minimum inhibitory concentration (MIC, reported in μM) after 24 and 48 h of static incubation in dark and humidified atmosphere at 35 ± 2 °C. The experiments were performed in duplicates. For the results to be valid, the difference in MIC determined from two parallel measurements must not be greater than one step on the dilution scale. MIC was determined by naked eye in the well with the lowest drug concentration, where no visible growth of microbial agent was detected. MIC determination scale started from 0.49 μM.

Broad-spectrum beta-lactam antibiotic piperacillin was used as reference compound; its results were read after 24 h. Standard antibiotics (ciprofloxacin, gentamicin; data not shown) and internal quality control/reference strains are routinely included in parallel testing-basic screening of antibacterial activity.

2.2.2. Antifungal activity

Antifungal activity was evaluated against four yeast strains, namely: *Candida albicans* ATCC 24443, CCM 8320, *Candida krusei* ATCC 6258, CCM 8271, *Candida parapsilosis* ATCC 22019, CCM 8260, *Candida tropicalis* ATCC 750, CCM 8264; and four strains of filamentous fungi, namely: *Aspergillus fumigatus* ATCC 204305, *Aspergillus flavus* CCM 8363, *Lichtheimia corymbifera* CCM 8077, and *Trichophyton interdigitale* ATCC 9533, CCM 8377. A microdilution broth method was performed according to EUCAST instructions (EUCAST 7.3.1. and 9.3.1)[31,32] with slight modifications. Briefly, tested compounds were dissolved in DMSO and diluted in a twofold manner with RPMI-1640 medium with L-glutamine, supplemented with 2% glucose (*w/v*) and buffered to pH 7.0 with 3-(*N*-morpholino)propane-1-sulfonic acid (MOPS). All these components were purchased from Sigma-Aldrich, St. Louis, MO, USA. The final concentration of DMSO in the tested medium did not exceed 1% (*v/v*) of the total solution composition, and it was confirmed that this concentration did not inhibit the fungal growth. Static incubation was performed in dark and humidified atmosphere, at 35 ± 2 °C for 24 and 48 h (72 and 120 h for *Trichophyton interdigitale*). Positive controls consisted of tested fungus solely, while negative controls consisted of medium and DMSO. The experiments were conducted in duplicates. For the results to be valid, the difference in MIC determined from two parallel measurements must not be greater than one step on the dilution scale. MIC was determined by naked eye in the well with the lowest drug concentration, where no visible growth of microbial agent was detected. MIC determination scale started from 0.49 μM.

Triazole antimycotic drug fluconazole was involved as reference compound for a comparison. MIC values of fluconazole mean MIC_{50} values, i.e., the lowest drug concentration giving growth inhibition of 50% of that of the drug-free control. Results were read after 24 h (yeasts) or 48 h (moulds) microdilution plates cultivation without agitation at 35 ± 2 °C in humidified atmosphere. The results were read with a microdilution plate reader (SynergyTM HTX, BioTek Instruments, Inc., VT, USA) at wavelength 530 nm. Internal quality control was included too. Standard antimycotics (voriconazole, amphotericin B; data not shown) are routinely included in parallel testing-basic screening of antibacterial activity.

2.2.3. Cytotoxicity determination

The human hepatocellular liver carcinoma cell line HepG2 (passage 18–19) purchased from Health Protection Agency Culture Collections (ECACC, Salisbury, UK) was cultured in Minimum Essentials Eagle Medium (MEEM) supplemented with 10% foetal bovine serum, 1% L-glutamine solution and non-essential amino acid solution in a humidified atmosphere containing 5% CO_2 at 37 °C. For subculturing, the cells were harvested after trypsin/EDTA treatment at 37 °C. The human kidney cells HK-2 (passage 12–14; American Type Culture Collection; USA) were cultured in DMEM (Dulbecco's and Eagle's Modified Medium) under the same conditions. To determine cytotoxicity of the compounds, the cells treated with the tested substances were used as experimental groups whereas untreated cells served as control groups. All used chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The cells were seeded in a density of 10 000 cells per well in a 96-well plate. The next day the cells were treated with each of the tested substances at a broad range of concentrations (based on their solubility) in triplicates. The compounds were dissolved in DMSO (maximal incubation concentration of DMSO was 1% *v/v*). The controls representing 100% cell viability, 0% cell viability (treated with 10% DMSO), no cell control and vehiculum control were incubated in parallel also in triplicates. After 24 h of incubation in a humidified atmosphere containing 5% CO_2 at 37 °C, the reagent from the kit CellTiter 96 AQueous One Solution Cell Proliferation Assay (CellTiter 96; PROMEGA, Fitchburg, WI, USA) was added. After 2 h of incubation at 37 °C, absorbance of samples was recorded at 490 nm (TECAN, Infinita M200, Grödig, Austria). A standard toxicological parameter IC_{50} was calculated by nonlinear regression from a semilogarithmic plot of incubation concentration versus percentage of viability relative to untreated controls using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results of the experiments are presented as inhibitory concentrations that reduce viability of the cell population to 50% from the maximal viability (IC_{50}). Known cytotoxic compounds tamoxifen (as tamoxifen citrate) and cisplatin were involved as reference compounds.

2.2.4. Measurement of caspase activities

To determine effect of the tested compounds on cellular apoptosis, activation of caspase 3/7 in HepG2 cells was evaluated for five the most interesting compounds using a standard luminescent kit (Caspase-Glo 3/7® Assay, PROMEGA, Fitchburg, USA). The cells were seeded in 96-well plates at a concentration of 15 000 cells per well. The compounds were dissolved in complete MEEM and 0.1 mL of the diluted toxicant in medium per well was added to cells cultured in a 96-well plate. Control untreated cells were incubated in the vehicle in parallel and only complete medium was added. Following incubation for 24 h in a humidified atmosphere containing 5% CO_2 at 37 °C, the caspase activity was determined according to the manufacturer's instructions. Reagent from the Caspase-Glo® 3/7 Assay was added. The plate was incubated in a shaker at 350 rpm and room temperature for 60 min in the dark. The addition of the Caspase-Glo reagent resulted in cell lysis, followed by caspase cleavage of the substrate and generation of a luminescent signal produced by luciferase. Luminescence is proportional to the amount of caspase present. Luminescence was measured and recorded using the plate reader Synergy 2 (BioTek Instruments, Winooski, Inc., VT, USA). Luminescence data were expressed as fold from untreated controls. Tamoxifen was involved a positive control. In all experiments, cells were incubated in triplicates.

3. Results and discussion

3.1. Chemistry

Based on the biological activity of hydrazide-hydrazones and iodinated compounds, we used 4-substituted hydrazides and 2-/4-hydroxy-3,5-diiodobenzaldehydes in drug design to investigate their mutual hydrazide-hydrazones as potential antimicrobial and cytotoxic agents. Remaining derivatives, i.e., 1,2-diacylhydrazines **3** and reduced forms of the most active hydrazones, compounds **4**, were designed as their analogues based on the biological activity identified initially in this study.

Regarding parent hydrazides, we have involved various substitution at position 4 of the phenyl. The substituents were representatives of functional groups with different electron and steric effects: hydrogen (neutral), alkyls (+I, small methyl and bulky *tert*-butyl), OH and CH_3O (-I and stronger + M), as well as electron-withdrawing moieties: halogens from fluorine to iodine (negative inductive and weak positive mesomeric effect), NO_2 (strong -I and -M effects) and CF_3 (strong -I effect).

Among eleven hydrazides, only 4-iodobenzohydrazide, precursor of the derivatives **i**, was synthesized in-house by a two-step procedure from 4 to iodobenzoic acid *via* Fischer esterification followed by hydrazinolysis.[29]

The hydrazides (1 eq.) were treated with 3,5-diiodosalicylaldehyde (compounds **1**) or its isomeric 4-hydroxy-3,5-diiodobenzaldehyde (derivatives **2**) in a mild excess (1.1 of equivalents) in boiling methanol for 2 h (Fig. 2). The yields of hydrazide-hydrazones **1** and **2** were 61–94% and 44–88%, respectively.[29]

Based on promising antimicrobial and cytotoxic assessment of these two series, we decided to prepare also similar 1,2-diacylhydrazines **3** and reduced analogues of the most potent halogenohydrazides **1f** and **1g**. The derivatives **3** were prepared in two steps from 3,5-diiodosalicylic acid that was converted to 3,5-diiodosalicyloyl chloride using an excess of thionyl chloride and DMF as a catalyst. After evaporation of volatile components, solution of the hydrazide (1 eq.) and triethylamine (4 of equivalents) in THF was added to this crude chloride (Fig. 3). The moderate yields were consistent within the range of 43–72 %. 4-Hydroxybenzohydrazide-based derivative **3e** was not obtained due to formation of a complex mixture of particular products.

To parallel CH=N and C=O bonds reduction, we used lithium aluminium hydride to obtain racemic secondary alcohol **4a** (83%; Fig. 4). The reduced analogues, i.e., *N′*-substituted hydrazides **4b** and **4c** with reduced imine bond were obtained by reductive amination using sodium cyanoborohydride (Fig. 5) with yields of 64 and 68%, respectively.

3.2. Biology

3.2.1. Antibacterial activity

Initially, we investigated antimicrobial activity of the synthesized compounds **1**–**4**. MIC values were determined by the microdilution broth method against four Gram-positive strains (*Staphylococcus aureus* ATCC 29213; MRSA, i.e., methicillin-resistant *Staphylococcus aureus* ATCC 43300; *Staphylococcus epidermidis* ATCC 12228; *Enterococcus faecalis* ATCC 29212) and four Gram-negative strains (*Escherichia coli* ATCC 25922; *Klebsiella pneumoniae* ATCC 10031; *Serratia marcescens*, clinical isolate laboratory ID No 62–2016; *Pseudomonas aeruginosa* ATCC 27853). A broad-spectrum beta-lactam antibiotic piperacillin (PIP) was used for comparison of MIC (Table 1). From chemical point of view, it is an ureidopenicillin, from pharmacological perspective, it is highly active against Gram-negative bacteria including *Pseudomonas aeruginosa* and Gram-positive pathogens that do not produce penicillinase and are susceptible to methicillin.

At the beginning, we evaluated hydrazones **1** prepared from 3,5-diiodosalicylaldehyde (Table 1). They abolished growth of all Gram-positive bacteria predominantly with MIC values from 15.62 μM (7.97 mg/L). The hydrazones **1b**-**1d** and **1i** derived from 4-(O)CH_3, 4-*tert*-butyl and 4-I-benzohydrazides were inactive, thus disfavouring lipophilic electron-donating substituents and the heaviest iodine atom. Contrarily, 4-fluorine, 4-chlorine and 4-CF_3-benzohydrazides provided highly active hydrazones (**1f**, **1g**, and **1j**, respectively; MIC of 15.62–62.5 μM), followed closely by nitro and hydroxyl groups (**1k** and **1e**). Unsubstituted hydrazide and 4-brominated analogue (**1a** and **1h**) were moderately active with slightly higher MIC for *E. faecalis*. Interestingly, MIC values for MSSA and MRSA are identical, i.e., the mechanism of action is not related to beta-lactam antibiotics.

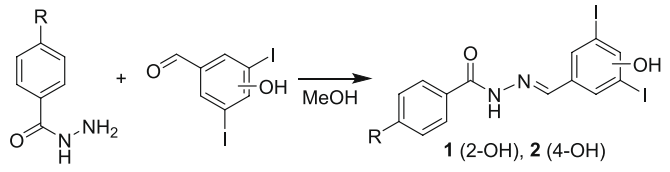


Figure 2. Synthesis of the hydrazide-hydrazones **1** and **2** (R = H **a**, CH_3 **b**, OCH_3 **c**, *t*-Bu **d**, OH **e**, F **f**, Cl **g**, Br **h**, I **i**, CF_3 **j**, NO_2 **k**).

Having these promising results of **1** in hands, we prepared and screened also their analogues: isomeric **2**, diacylhydrazines **3** and reduced analogues **4**. However, the series **2** and **3** share only negligible antibacterial activity generally when compared to the hydrazide-hydrazones **1**. MIC values of the hydrazide-hydrazones **2** started from 125 μM against Gram-positives. In contrast to **1**, when 4-halogen, hydroxy, nitro or 4-CF_3-benzohydrazide was used as a parent compound, the resulting hydrazones **2** share no or limited activity. Contrarily, 4-hydroxy-3,5-diiodobenzylidenehydrazones obtained from 4-alkyl/alkoxyl hydrazides (**2b**-**2d**) exhibited better activity than their isomers **1b**-**1d**, although with a comparatively high MIC values (≥125 μM) with the *tert*-butyl derivative **2d** superiority.

Focusing on the hydrazides **3**, we were not able to determine MIC of several of them (**3a**, **3e**, **3h**, **3j**, and **3k**) due to their insufficient solubility in the testing medium or a massive precipitation during processing. Three of them exhibited poor inhibition of bacteria (MIC of ≥500 μM; **3c**, **3d**, **3f**), the remaining ones showed an improved activity, especially those derived from 4-chlorobenzohydrazide and 4-iodobenzohydrazide **3g** and **3i** with MIC values from 31.25 and 62.5 μM, respectively. *N′*-(4-Chlorobenzoyl)-2-hydroxy-3,5-diiodobenzohydrazide **3g** was found generally similarly active as the salicylidene derivative **1g**.

In fact, the last modification consisting in a reduction of one or two double bond(s) present in the most active halogenated hydrazones **1f** and **1g** was the most successful approach. Among 4-fluorinated compounds, the reduction of both imine and carboxamide bonds provided the secondary alcohol **4a** with a retained activity against MSSA, MRSA and even improved in the case of *S. epidermidis* and *E. faecalis* (up to four times). Reduction of only C=N bond (**4b**) led to the molecule with identical inhibition (i.e., ±one dilution) of *E. faecalis* and *S. epidermidis* but higher MIC for *S. aureus*. For the chlorinated hydrazide-hydrazone **1g** is the reduction of imine bond also beneficial since it retains activity against both *S. aureus* strains but boosts suppression of *E. faecalis* and *S. epidermidis* (up to four times). In sum, these reduced analogues **4a** and **4c** belong to the most effective agents to combat MSSA, *S. epidermidis* and *E. faecalis* with MIC values from 7.81 μM (i.e., 4.01 mg/L).

Drawing a comparison to piperacillin, the derivatives **1**–**4** were able to supress PIP-resistant *S. epidermidis* and MRSA strains. The most active hydrazine derivatives **4a** and **4c** exhibited similar *in vitro* efficacy for *E. faecalis* in both molar and weight concentration scales (±one dilution). On the other hand, our derivatives were less active against beta-lactams-susceptible *S. aureus* and Gram-negative bacteria that are main therapeutical areas of PIP, predominantly used in a fixed combination with beta-lactamase inhibitor tazobactam.[33]

In general, the derivatives **1**–**4** have antibacterial properties only against all Gram-positive cocci at comparable concentrations whereas they are *de facto* inactive towards Gram-negative species. Only the 4-hydroxybenzohydrazide derivative **1e** inhibited *E. coli*, remaining pathogens showed a complete resistance (*P. aeruginosa*, *K. pneumoniae*, *S. marcescens*). Importantly, there are no differences of MIC values for MSSA and MRSA that means no cross-resistance to beta-lactams, fortunately. The optimal structural requirements are as follows: (1) benzohydrazide substituted by fluorine, chlorine, trifluoromethyl or nitro group at the position 4; (2) substituted by 2-hydroxy-3,5-diiodobenzylidene at *N′*, this imine double bond can be reduced individually or together with carboxamide moiety.

Among other salicylidene hydrazides, 3,5-diiodosalicylidene hydrazides have also been screened for their inhibition activity on type III

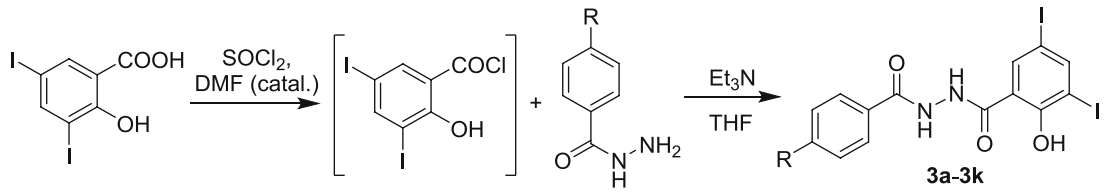


Figure 3. Synthesis of 1,2-diacylhydrazines **3** (DMF: *N*,*N*-dimethylformamide, THF: tetrahydrofuran; R = H **a**, CH_3 **b**, OCH_3 **c**, *t*-Bu **d**, F **f**, Cl **g**, Br **h**, I **i**, CF_3 **j**, NO_2 **k**).

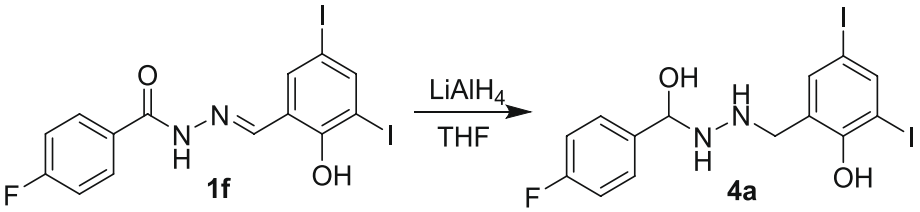


Figure 4. Reduction of **1f** (THF: tetrahydrofuran).

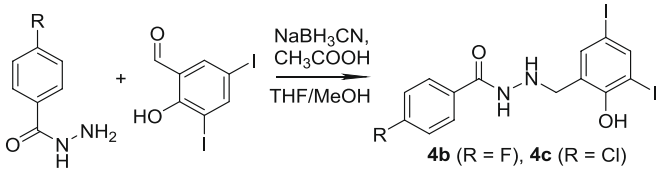


Figure 5. Reductive amination (THF: tetrahydrofuran).

secretion in Gram-negative pathogen *Yersinia pseudotuberculosis*. Type III secretion is a virulence system constituting a potential drug target discovered in a wide range of clinically important Gram-negative pathogens. However, three investigated derivatives showed only a mild inhibition.[34] To the best of our knowledge, this is the first report of iodinated salicylidene benzohydrazide-hydrazones as potent antibacterial agents inhibiting Gram-positive cocci so far.

3.2.2. Antifungal activity

We screened 1,2-disubstituted hydrazine derivatives **1**–**4** also against eight strains of human fungal pathogens. The panel of microorganisms covers four yeasts (*Candida albicans* ATCC 24443, *Candida krusei* ATCC 6258, *Candida parapsilosis* ATCC 22019 and *Candida tropicalis* ATCC 750) and four moulds (*Aspergillus fumigatus* ATCC 204305, *Aspergillus flavus* CCM 8363, *Lichtheimia* (*Absidia*) *corymbifera* CCM 8077, and *Trichophyton interdigitale* ATCC 9533). Triazole fungistatic drug fluconazole (FLU) was used for comparison of MIC values (Table 2). MIC of the investigated compounds means a complete inhibition of fungal growth determined by naked eye, whereas MIC of FLU mean IC_{50} values determined spectrophotometrically.

Among the iodinated derivatives, only four of them exhibited significant antifungal properties (Table 2; **1f**, **1k**, **4a**, and **4b**), MIC of remaining ones were greater than 125 μM. Obviously, for antifungal action, 4-fluoro- and 4-nitrobenzohydrazide scaffold are optimal choice in combination with 2-hydroxy-3,5-diiodobenzylidene substitution (**1f**, **1k**). 4-Hydroxy-3,5-diiodobenzylidenes **2** and 1,2-diacylhydrazines **3** did not offer any antifungal properties. 4-Nitrobenzohydrazide-based hydrazone **1k** exhibited the most potent antimycotic action with MIC from 1.95 μM (1.05 mg/L) inhibiting four strains from total eight (*C. albicans*, *C. krusei*, *L. corymbifera* and *T. interdigitale*). 4-Fluorinated hydrazide-hydrazone **1f** showed inhibition of five fungi, but at higher concentrations than **1k**. Focusing on fluorinated analogues, the reduction of imine bond is partly tolerated (**4b**, that has lower MIC for *L. corymbifera* but higher for yeasts); on the other hand, an additional reduction of carboxamide double bond brought a drop in the activity (**4a** vs. **1f** and **4b**).

T. interdigitale was the most susceptible strain. Contrarily, *C. tropicalis* and both *Aspergillus* strains tolerated all the derivatives **1**–**4**. Importantly, our derivatives exhibited comparable or superior activity than clinically used drug fluconazole against *C. albicans* (**1k**), *C. krusei* (**1f**, **1k**), *L. corymbifera* (**1f**, **1k**, **4b**) and *T. interdigitale* (all four active compounds).

Previously, iodinated salicylidene benzohydrazides (free or in a combination with copper salts) have been reported as effective agents against phytopathogenic fungi,[35] but here presented derivatives are active against human fungal pathogens at low concentrations.

3.2.3. Cytotoxicity

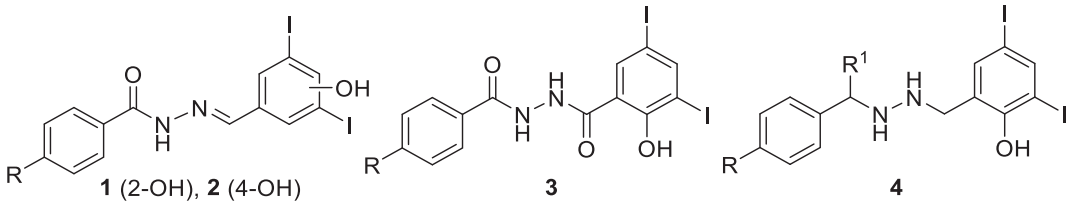
Cytotoxicity of the tested compounds **1**–**4** was measured using two types of cells, a cancer cell line HepG2 and a HK-2 cells derived from normal kidney tissue. The used CellTiter 96 assay is based on the reduction of tetrazolium of MTS dye ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)–2*H*-tetrazolium]) in living cells to formazan, which is then determined colorimetrically. The reduction of the reagent is related to availability of NADH or NADPH. Their drop causes lowered production of formazan indicating decreased cell viability.

Results of the experiments are presented as inhibitory concentration which reduces viability of the cell population to 50% from the maximal viability (IC_{50}). This IC_{50} parameter was used as a quantitative measure of cytotoxicity, which allows the quantitative comparison of the toxicity among tested compounds through three series of hydrazine derivatives.

We were able to determine IC_{50} of the majority of the investigated substances. However, IC_{50} of seven hydrazide-hydrazones (**1a**, **1b**, **2b**, **2c**, **2g**-**2i**) couldn't be determined due to the precipitation in the culture medium at higher concentrations than reported in Table 3. However, they have exhibited no signs of cytotoxicity among soluble concentrations. The effective cytotoxic concentrations of the compounds **1**–**4** are listed in Table 3. Considerable differences in cytotoxicity of the tested compounds were found also in HK-2 cells as treatment led to different rate of damage. However, the found IC_{50} values of the five selected compounds (based on activity against HepG2) did not have so broad dispersion (Table 4). For this cell line, the toxic concentrations were possible only to be assessed in three agents due to limited solubility of **2d** and **3i**.

Focusing on cancer HepG2 cells, the IC_{50} values among the hydrazine derivatives **1**–**4** compounds differ in two orders of magnitude (from 11.72 to 190.90 μM). We were able to identify relatively non-toxic compounds for HepG2 cells (IC_{50} greater than 100 μM: **2a-2c**, **2e-2g**, **3c**, **3j**, and **3k**). On the other hand, cytotoxicity of the most toxic

Table 1
Structures and antibacterial activity of hydrazines **1**–**4.**



Code	R	MIC [μM] (mg/L)									
		SA		MRSA		SE		EF		EC	
		24 h	48 h	24 h	48 h	24 h	24 h	24 h	48 h	24 h	48 h
1a	H	62.5	125	62.5	125	62.5	125	250	500	>500	>500
2a	H	250	250	500	>500	>500	>500	>500	>500	>500	>500
3a	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1b	CH_3	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
2b	CH_3	250	500	>500	>500	>500	>500	>500	>500	>500	>500
3b	CH_3	125	>125	>125	>125	125	>125	>125	>125	>125	>125
1c	OCH_3	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
2c	OCH_3	500	500	>500	>500	>500	>500	>500	>500	>500	>500
3c	OCH_3	500	>500	500	>500	500	>500	500	>500	>500	>500
1d	*t*-Bu	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
2d	*t*-Bu	125	125	125	250	125	250	250	500	>500	>500
3d	*t*-Bu	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
1e	OH	**31.25**	62.5	62.5	62.5	62.5	125	250	250	**250**	**250**
2e	OH	500	500	>500	>500	>500	>500	>500	>500	>500	>500
1f	F	**15.62** (**7.97**)	**31.25**	**15.62** (**7.97**)	**31.25**	31.25	31.25	**31.25**	62.5	>500	>500
2f	F	125	500	500	>500	>500	>500	>500	>500	>500	>500
3f	F	500	500	500	500	500	500	500	500	>500	>500
1g	Cl	**31.25**	**31.25**	**31.25**	62.5	31.25	62.5	62.5	62.5	>500	>500
2g	Cl	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
3g	Cl	62.5	62.5	62.5	62.5	31.25	62.5	125	125	>500	>500
1h	Br	62.5	62.5	62.5	125	62.5	125	250	250	>250	>250
2h	Br	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
3h	Br	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1i	I	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
2i	I	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
3i	I	250	250	125	250	62.5	125	125	250	>250	>250
1j	CF_3	**31.25**	**31.25**	**31.25**	**31.25**	31.25	31.25	**31.25**	**31.25**	>250	>250
2j	CF_3	125	500	500	500	500	500	500	500	>500	>500
3j	CF_3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1k	NO_2	**31.25**	62.5	**31.25**	62.5	31.25	62.5	62.5	125	>125	>125
2k	NO_2	250	250	500	>500	>500	>500	>500	>500	>500	>500
3k	NO_2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4a	F; R^1: –OH	**15.62** (**8.03**)	**15.62** (**8.03**)	**31.25**	62.5	**7.81** (**4.01**)	**7.81** (**4.01**)	**15.62** (**8.03**)	**15.62** (**8.03**)	>500	>500
4b	F; R^1: =O	62.5	125	62.5	125	62.5	62.5	**31.25**	62.5	>500	>500
4c	Cl; R^1: =O	**31.25**	62.5	**31.25**	62.5	**15.62**	**15.62**	**15.62** (**8.26**)	**15.62** (**8.26**)	>500	>500
PIP	–	1.85 (1)		14.83 (8)		>59.31 (>32)		7.41 (4)		3.71 (2)	

PIP = piperacillin sodium salt. ND: not determined due to solubility issues. SA: *Staphylococcus aureus* ATCC 29213; MRSA: methicillin-resistant *Staphylococcus aureus* ATCC 43300; SE: *Staphylococcus epidermidis* ATCC 12228; EF: *Enterococcus faecalis* ATCC 29212; EC: *Escherichia coli* ATCC 25922. ND: not determined due to solubility issues.
The lowest MIC values for each strain are given in bold. MIC in mg/L were calculated for the lowest MIC identified in μM.

Table 2
Antifungal activity of hydrazine derivatives **1** and **4.**

Code	R	MIC [μM]									
		CA		CK		CP		LC		TI	
		24 h	48 h	24 h	24 h	24 h	48 h	24 h	48 h	72 h	120 h
1f	F	62.5	125	**125**	**125**	**62.5**	**125**	125	125	**15.62**	62.5
1k	NO_2	**1.95**	**7.81**	**62.5**	>125	>125	>125	**31.25**	62.5	**3.9**	**15.62**
4a		>500	>500	>500	>500	>500	>500	>500	>500	62.5	62.5
4b		125	>125	>125	>125	>125	>125	**15.62**	**31.25**	**15.62**	31.25
FLU*		6.5	6.5	>104.5	>104.5	3.3	3.3	>104.5	>104.5	52.2	52.2

FLU = fluconazole. *: MIC means IC_{50} values. CA: *Candida albicans* ATCC 24443; CK: *Candida krusei* ATCC 6258; CP: *Candida parapsilosis* ATCC 22019; AC: *Lichtheimia corymbifera* CCM 8077; TI: *Trichophyton interdigitale* ATCC 9533.
One or two of the best MIC value(s) for each strain are shown in bold.

Table 3
Toxicity of the hydrazine derivatives **1**–**4** in HepG2 cells.

Code		IC_{50} [μM]	Range of concentrations tested	Code		IC_{50} [μM]	Range of concentrations tested
1a	H	>50*	1–1000	**1g**	Cl	14.75	1–500
2a	H	190.90	1–250	**2g**	Cl	>100*	1–100
3a	H	98.03	1–100	**3g**	Cl	48.65	1–50
1b	CH_3	>25*	1–1000	**1h**	Br	22.27	1–500
2b	CH_3	>100*	1–100	**2h**	Br	>50*	1–50
3b	CH_3	86.42	1–250	**3h**	Br	62.79**	1–50
1c	OCH_3	45.15	1–1000	**1i**	I	31.81	1–500
2c	OCH_3	>100*	1–100	**2i**	I	>50*	1–50
3c	OCH_3	132.40**	1–50	**3i**	I	25.05	1–100
1d	*t*-Bu	41.59	1–1000	**1j**	CF_3	11.72	1–250
2d	*t*-Bu	45.92	1–250	**2j**	CF_3	57.43	1–250
3d	*t*-Bu	87.32	1–100	**3j**	CF_3	167.00	1–100
1e	OH	19.17	1–1000	**1k**	NO_2	24.65	1–1000
2e	OH	110.40	1–500	**2k**	NO_2	92.08	1–500
1f	F	12.07	1–500	**3k**	NO_2	158.80**	1–100
2f	F	116.70	1–500	**4a**	F	41.91	1–1000
3f	F	86.99	1–100	**4b**	F	52.20	1–1000
tamoxifen		19.6	1–500	**4c**	Cl	52.22	1–1000
cisplatin		21.3	1–500				

* Higher concentrations precipitated in the culture medium.
** Estimated value based on the curve.

Table 4
Toxicity of the selected hydrazine derivatives in HK-2 cells.

Code	IC_{50} [μM]	Range of concentrations tested [μM]
2d	˃250	1–250
1f	55.61	1–500
3i	˃100	1–100
1j	49.85	1–250
1k	26.80	1–1000

derivatives was comparable or higher in comparison with anticancer drugs tamoxifen and cisplatin (19.6 and 21.3 μM, respectively): **1e**-**1k**, and **3i**. Focusing on remaining derivatives, they have toxicities between these values. The small substituents with a moderate rate of lipophilicity and electron-donating or neutral properties (hydrogen **a**, methyl **b**, methoxy **c**) are associated with a lower toxicity for eukaryotic cancer cells.

In general, the presence of 2-hydroxy-3,5-diiodobenzylidenehydrazide (compounds **1**) is associated with an escalated toxicity especially when combined with halogens and strong electron-withdrawing substituents (CF_3, NO_2). The 4-CF_3-benzohydrazide-derived hydrazone **1j** (IC_{50} of 11.72 μM) was the most cytotoxic compound within this study. Contrarily, their positional isomers **2** are predominantly the least toxic analogues (up to ten-times less, as demonstrated in the pair **1f** and **2f**). Also, the replacement of imine bond by second carboxamide (hydrazide-hydrazones **1** → 1,2-diacylhydrazines **3**) led to a substantial lower toxicity. The switch of 2-hydroxy-3,5-diiodobenzylidene substituent for 4-fluoro- and 4-chlorobenzohydrazide to 2-hydroxy-3,5-diiodobenzyl (**1f** and **1g** to **4b** and **4c**, respectively), i.e., reduction of imine double bond, resulted in a decreased toxicity (up to 4.3 times), thus improving selectivity for microbes.

Keeping in hands these results, we investigated the most active compounds also against normal human cell line to compare their inhibition of cancer and healthy cells. We used HK-2 line, epithelial kidney cortex/proximal tubule-derived cells. Following representatives were chosen: the most cytotoxic 2-hydroxy-3,5-diiodobenzylidene hydrazones **1f**, **1j**, and **1k**; 4-hydroxy-3,5-diiodobenzylidene hydrazone **2d** and 1,2-diacylhydrazine **3i**. IC_{50} data are reported in Table 4.

Importantly, four hydrazines showed lower toxicity for normal than for cancer cells, only the nitro compound **1k** was an exception with identical IC_{50} values (26.80 and 24.65 μM, respectively). HK-2 line was inhibited at at least four times higher concentrations than HepG2 (4.5× and 4.3× for fluorine substituted molecules **1f** and **1j**, respectively). Also, the most cytotoxic *tert*-butylated 4-hydroxybenzylidene derivative **2d** showed a lesser inhibition of HK-2 (at least 5.4× due to its limited solubility in DMEM) as well as triiodinated diacylhydrazine **3i** (more than 4 times). These results indicate a higher sensitivity of cancer cells, fortunately. The found differences in cytotoxicity between HepG2 and HK-2 cells can be explained by different origin and character of the employed cell lines.

To describe potential mechanism of the antiproliferative effect of the tested compounds, we evaluated selected ones (hydrazide-hydrazones **1f**, **1j**, **1k**, **2d**, and 1,2-diacylhydrazine **3i**) for changes in caspase 3/7 activities in HepG2 cells (Fig. 6). Tamoxifen was involved as a comparator. Caspase activity was demonstrated in cells treated with this drug at concentrations of 25 and 50 μM (1.69- and 1.81-fold control, respectively). The performed standard test did not show a significant proapoptotic effect in most of the agents. A small increment in caspase 3/7 activity was found in two fluorinated hydrazide-hydrazones **1f** and **1j**. The CF_3-derivative **1j** at the concentration of 100 μM led to 1.37-fold control, while **1f** increased caspase 3/7 activity at two concentrations of 1 μM and 100 μM (1.36- and 1.33-fold control, respectively). However, the apoptotic effect is not a class-effect. This finding means that inhibitory effect of the compounds towards the cells is likely based on impairment of another metabolic process than apoptosis.

The selectivity to particular biological activities can be quantified using selectivity indexes (SI). In analogy to therapeutic index, SI is calculated as a ratio of the IC_{50} and MIC values. Its value above a threshold of 10 means a selective action. The nitro hydrazide-hydrazone **1k** provides selective activity against *C. albicans* (SI of 13.7), other compounds share a balanced dual antibacterial (for **1f** and **1k**; **4a** and **4b** antifungal as well) and cytotoxic action, for some compounds with a more pronounced toxicity, for others with a stronger antimicrobial action.

Previously, some iodinated organic compounds have been reported and investigated as potential cytotoxic/anticancer agents.[24,26,36–38] Based on IC_{50}, the most cytotoxic here presented derivatives are at least comparable to these molecules, thus opening a new field for their possible application.

The coincidence of cytotoxic and antibacterial/antifungal properties in one molecular entity can be also beneficial since anticancer drugs with an adjuvant antimicrobial action are of a special interest.[39] For some compounds clinically used as antimicrobials, a potent and promising anticancer action has been described later. Although used for treatment of bacteria and protozoa-caused diseases widely, they may be useful for oncologic patients not only with a cancer-associated infection

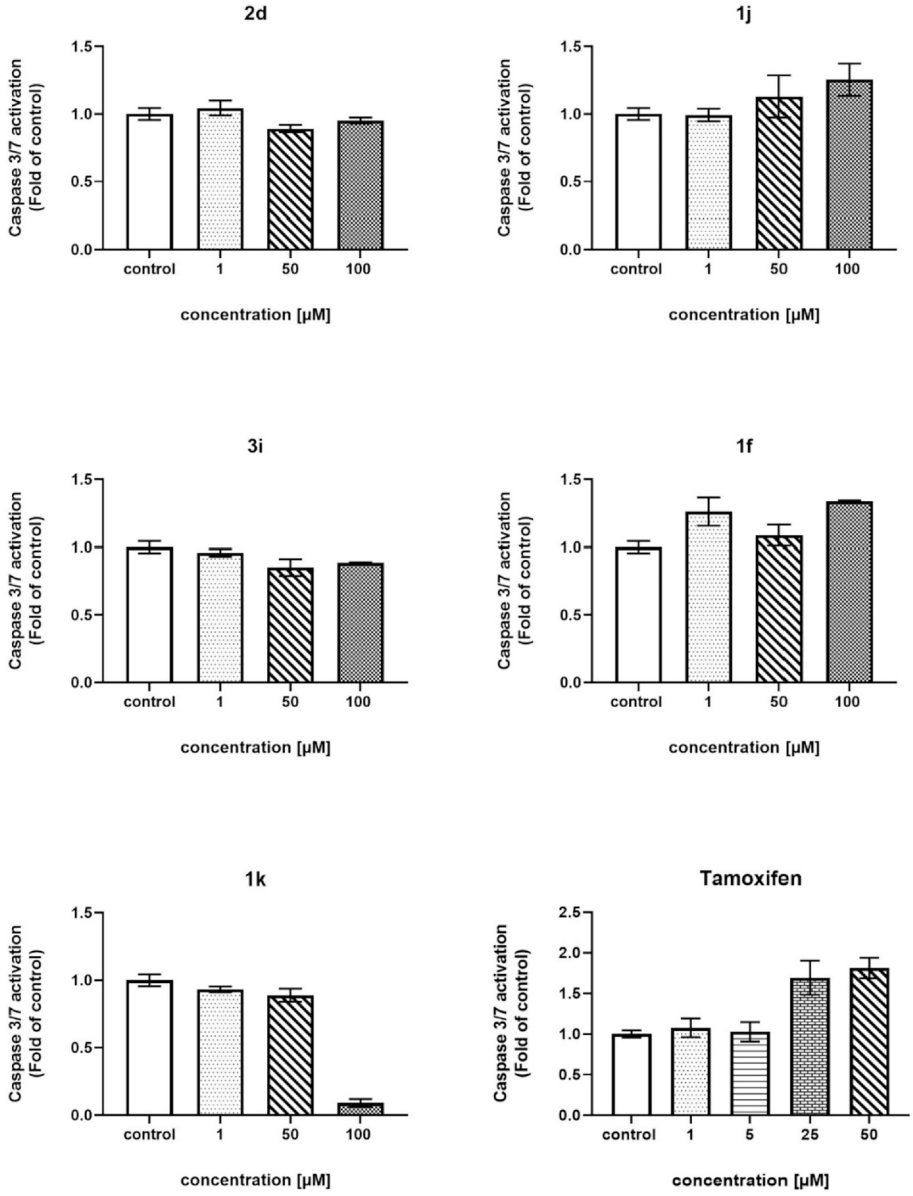


Figure 6. Caspase 3/7 activities in HepG2 cell treated with selected tested compounds (mean ± SD; n = 6). Cytostatic drug tamoxifen was used as a positive control. The lower value at the highest concentration of **1k** indicates cellular necrosis.

due to their direct cytostatic and cytotoxic activities (both *in vitro* and *in vivo*), e.g., salicylic derivative niclosamide,[40] fluoroquinolones, 8-hydroxyquinolines, thiazole antibiotics etc.[39,41] In addition to various anticancer effects, antimicrobial agents can benefit cancer patients by killing oncogenic-related microorganisms and protecting from immunosuppression-induced opportunistic infections.[39] From another point of view, also "classical" anticancer drugs have shown an interesting inhibition of various microbes, e.g., anthracycline antibiotics,[39–41] 5-fluorouracil, busulfan, mitomycin C, methotrexate[42] or tamoxifen.[42–44] This repurposing represents a viable concept for introducing "novel" drugs.

4. Conclusions

In this study, we evaluated twenty-two iodinated hydrazide-hydrazones and two their reduced analogues as potential antimicrobial and cytotoxic agents. Based on their promising results, we designed and synthesized eleven additional analogues, iodinated 1,2-diacylhydrazines and one reduced molecule. Advantageously, the targeted compounds are easily synthetically available.

The 4-substituted benzohydrazide modification using 2-/4-hydroxy-3,5-diiodobenzaldehydes and 3,5-diiodosalicylic acid offers compounds that are active against Gram-positive bacteria including MRSA strain and some of them are also antifungal agents with low MIC values from 1.95 μM. The cytotoxicity ranged from low micromolar concentrations to hundreds of μM. Structure-activity analysis found following relationships: (1) the presence of 3,5-diiodosalicylidene moiety is required for potent bioactivity; (2) the imine bond can be also reduced; (3) 4-hydroxy-3,5-diiodobenzylidene and 3,5-diiodosalicyloyl scaffolds led to lower antimicrobial action, but they contribute to cytotoxicity; (4) for substitution of benzohydrazide, electron-withdrawing atoms/groups are optimal (smaller halogens, CF_3, NO_2). Several compounds provided "pure" cytotoxic action, while some members of the series share a dual antimicrobial-cytotoxic action that can be promising, too. Two fluorinated hydrazide-hydrazones induce an increase in caspase-3/7 activity, i.e., they have proapoptotic effect.

CRediT authorship contribution statement

Martin Krátký: Conceptualization, Investigation, Writing - original draft, Methodology, Supervision. **Klára Konečná:** Investigation, Writing - original draft. **Michaela Brablíková:** Investigation, Supervision. **Jiří Janoušek:** Investigation. **Václav Pflégr:** Investigation. **Jana Maixnerová:** Investigation. **František Trejtnar:** Methodology, Writing - review & editing, Supervision. **Jarmila Vinšová:** Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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