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ORIGINAL RESEARCH ARTICLE

A very promising antibiofilm activity against *Candida albicans* from an in vitro screening for antimicrobial, antibiofilm and antiproliferative activity of new synthesized 4-cinnamamido- and 2-phenoxyacedamido-1H-pyrazol-5-yl)benzamides

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Abstract

Several new synthesized 4-cinnamamido- and 2-phenoxyacedamido-(1H-pyrazol-5-yl)benzamides were obtained by two multi step different synthetic routes in order to maximize their yield. The new derivatives were screened to determine the antiproliferative, antimicrobial and antibiofilm activity. The biological results showed how, respect to the antiproliferative and antimicrobial activities, the compounds have a low to missing activity. Different are the results obtained concerning the antibiofilm activity, especially towards *Candida albicans*. Most of the synthesized compounds showed a good percentage inhibition of biofilm formation ranging from 60 to 73% with a Biofilm Inhibition Concentration 50% (BIC_{50}) from 0.13 to 0.01 μM. Among the synthesized compounds the ethyl 5-(4-(2-(4-chlorophenoxy)acetamido)benzamido)-1-methyl-1H-pyrazole-4-carboxylate (**27c**) resulted the most active molecule with a BIC_{50} of 0.01 μM. According to the results obtained, such compound could be considered a lead subject of further studies to obtain novel and more effective antibiofilm agents against *C. albicans*.

Keywords 4-cinnamamido-(1H-pyrazol-5-yl)benzamides · 2-phenoxyacedamido-(1H-pyrazol-5-yl)benzamides · Antiproliferative activity · Antimicrobial activity · Antibiofilm activity · Biofilm associated infections *Candida albicans*

Introduction

Acetamidobenzamides are well represented in literature as antimicrobial, antibiofilm and antiproliferative agents. Fusaribenzamide A **1** [1], fusarithioamide B **2** [2], and N-(1-Adamantylcarbamothioyl)benzamides **3** [3] are examples of antimicrobial acetamidobenzamides (Fig. 1). Also, the antibiofilm activity is well represented among acetamidobenzamides such as cationic lipo-benzamides **4** [4] and N-(oxazolylmethyl)-thiazolidinediones **5** [5] (Fig. 1).

For such kind of compounds, the antiproliferative activity is also reported. Examples of antiproliferative acetamidobenzamides are Fusarithioamide B **2** [2] which showed cytotoxic effect against BT-549, MCF-7, SKOV-3, and HCT-116 cell lines, the N-(2-aminophenyl)benzamide acridines **6** [6] endowed with HDAC inhibitory activity and the 3-2-(1H-benzo[d]imidazol-2-ylthio)acetamido)-N-(4-methoxyphenyl)benzamide **7** which showed an IC_{50} of 4.12 μM against the HCT116 cells [7] (Fig. 1). Our research group has long been interested in the chemistry and pharmacology of this class of molecules aiming to study their biological activity [8–11]. In Fig. 2, compounds **8–11**, the most active among our previously synthesized benzamides against MDA-MB231 breast cancer cells, are reported.

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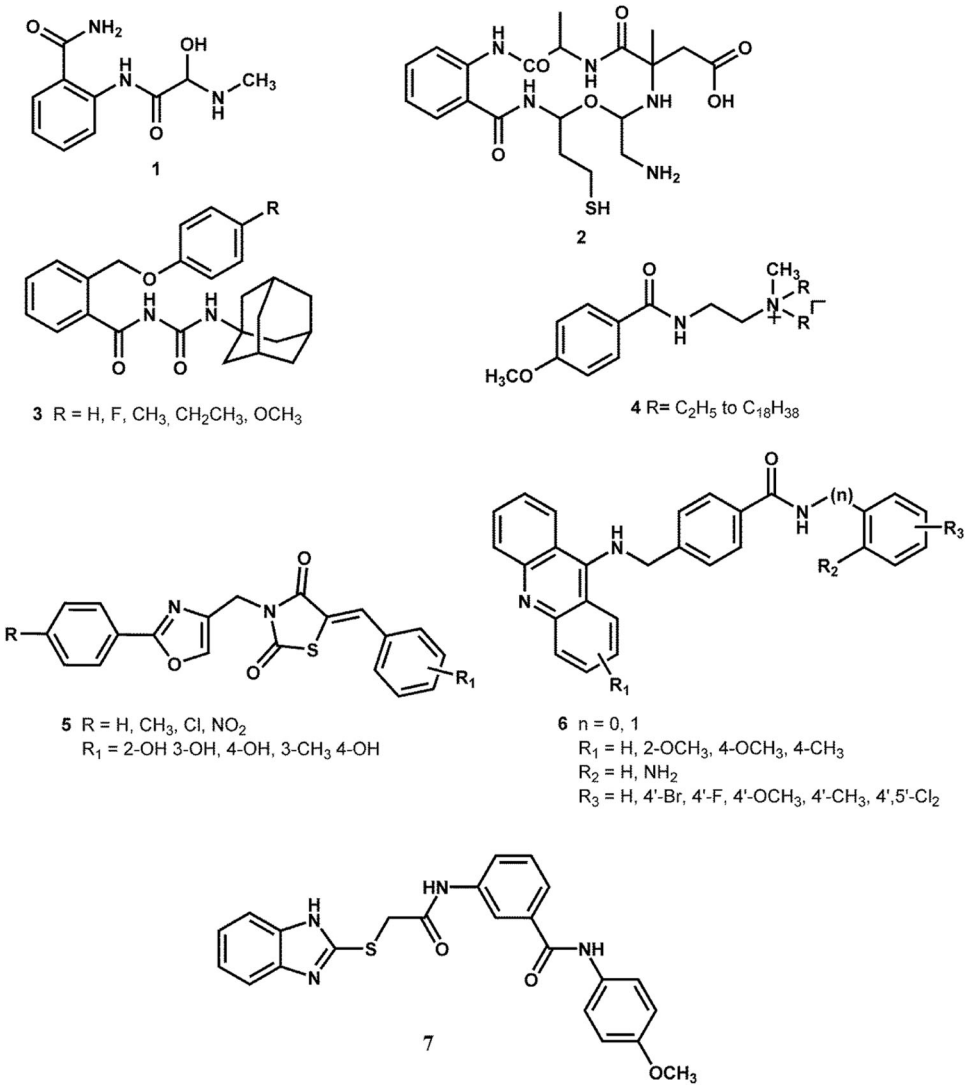


Fig. 1 Examples of antimicrobial, antibiofilm and antiproliferative acetamidobenzamides [1–7]

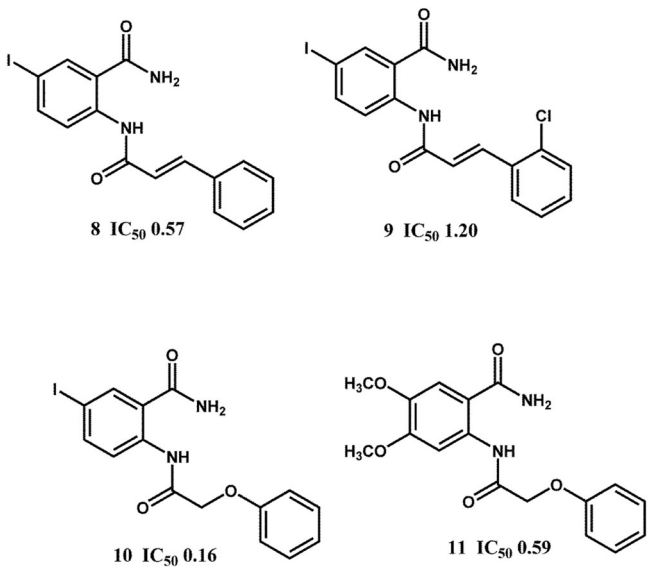


Fig. 2 The most active benzamides against MDA-MB231 breast cancer cells previously synthesized by the authors of the present paper [8–11]

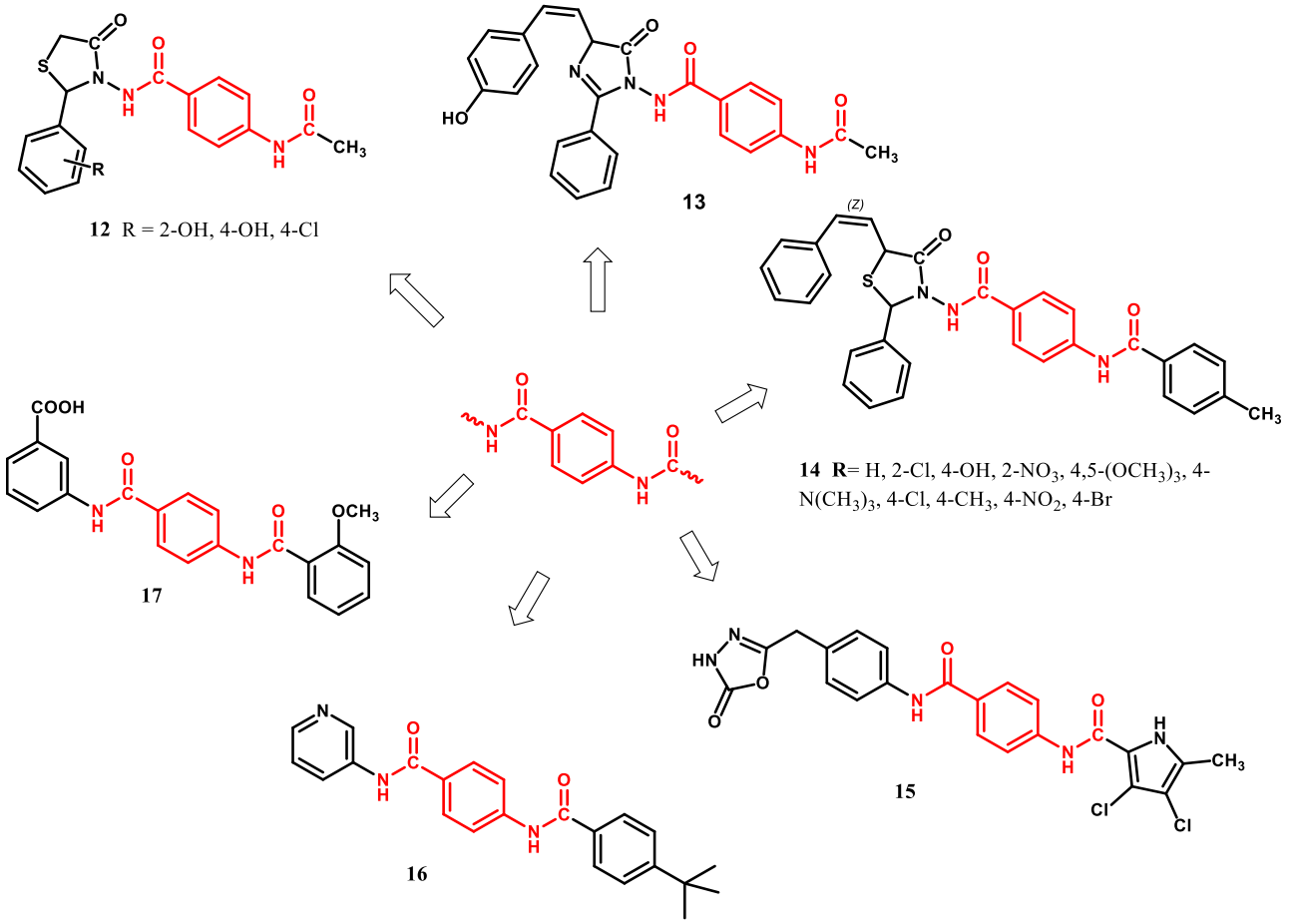


Fig. 3 Examples of some antimicrobial and antiproliferative agents bearing the 4-acetamido-N-methylbenzamido scaffold [12–17]

Furthermore, among this class of derivatives, the 4-acetamido-N-methylbenzamido is the most common scaffold found in many antimicrobial and antiproliferative agents. Particularly, antimicrobial activity for 4-acetamido thiazolidin-4-ones **12** [12], 4-acetylamino 1H-imidazolylbenzamide **13** [13], 4-benzamido-N-(4-oxo-2-phenylthiazolidin-3-yl) benzamides **14** [14], and 1H-pyrrole-2-carboxamide **15** [15] (Fig. 3) has been reported. Also, the antiproliferative activity has been observed for 3-aminopyridine-derived amides **16** [16] and 3-(4-(2-methoxybenzamido)benzamido) benzoic acid 17 (Fig. 3) [17].

Previously reported data demonstrated that 2-(2-phenoxyacetamido) benzamides caused growth inhibition against many tumor cell lines. In particular, compounds bearing a phenoxyacetamido scaffold reduced the proliferation of leukemic K562 cells by arresting the cell in G1 phase of cell cycle [10]. On the basis of our previous research [8–11] and considering the biological activity of 4-acetamido-N-methylbenzamides and the antibiofilm activity already reported for pyrazole-4-carboxamides [18], formerly synthesized benzamides **8–11** (Fig. 2) have been modified as reported in Fig. 4 to enhance the biological activity. In particular, in the present work the carboxamide group has been moved from the 2 to 4 position to obtain the 4-acetamido-N-methylbenzamido structure and the molecule has been stretched by adding a pyrazole nucleus. The antimicrobial, antibiofilm as well as the antiproliferative activities were evaluated for the new synthesized derivatives.

Results and discussion

Chemistry

4-Nitrobenzoyl chloride **18** from commercial furnisher has been used. Crude 2-phenoxyacetyl chlorides **24b–d, 33a–d** and cinnamoyl chlorides **23a–c** were obtained by refluxing the appropriate acids **22b–d**, **32a–d** and **21a, c** with thionyl chloride (Schemes 1 and 2).

Compounds **25a, e, 26a, e, f, 27b–d, 28a, e, f, 29e** and **34a–d**, were obtained by two different synthetic routes in order to maximize their yield as reported in Schemes 1 and 2. However, despite all attempts to obtain complete

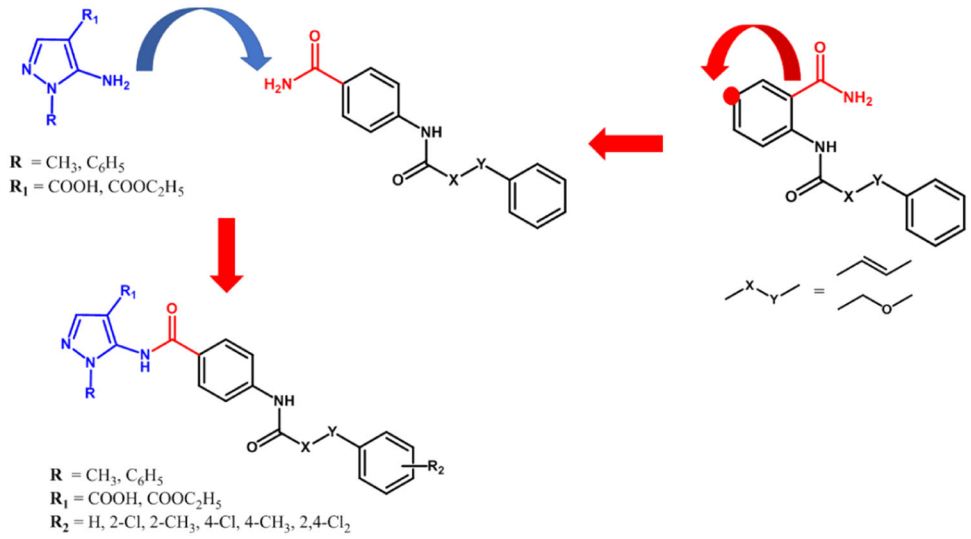
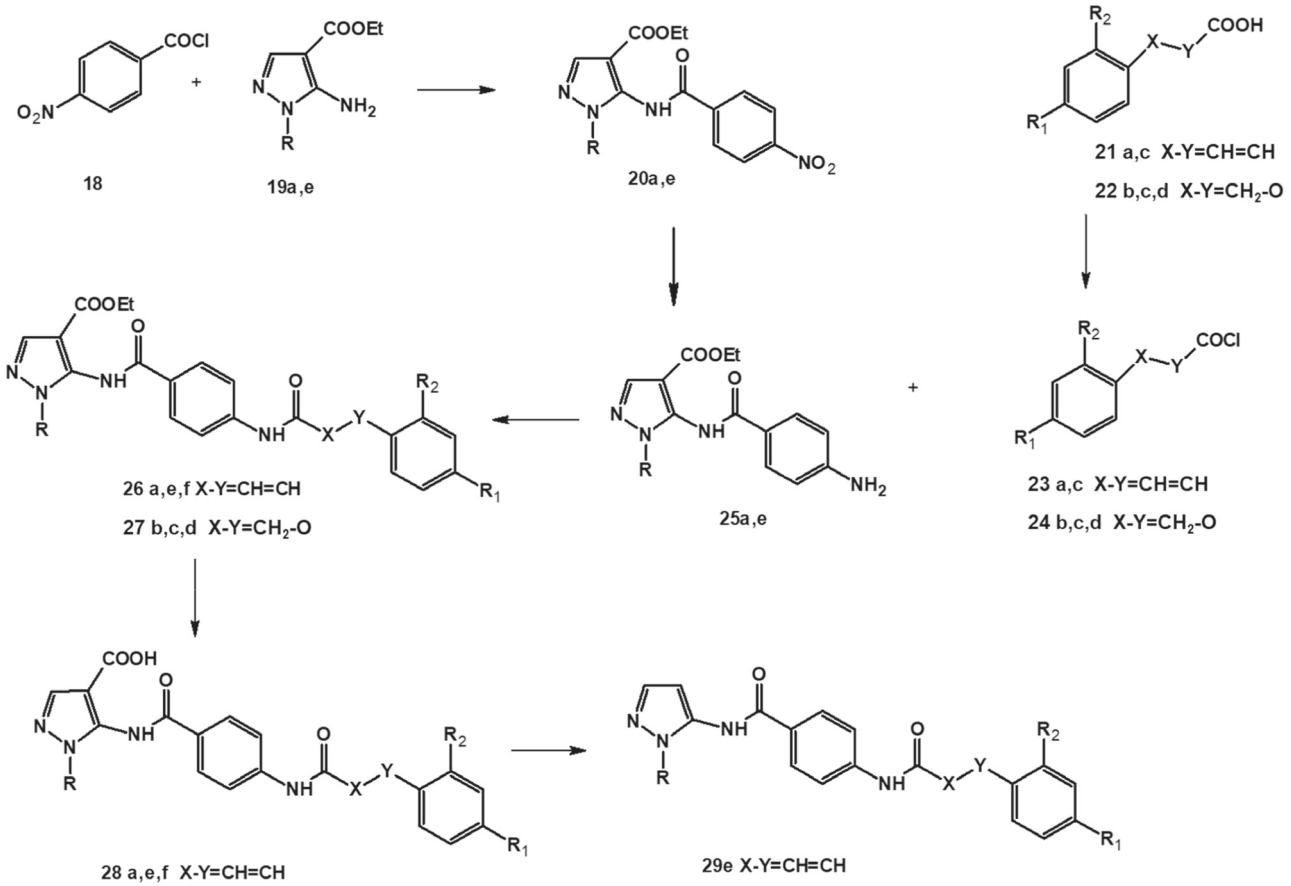
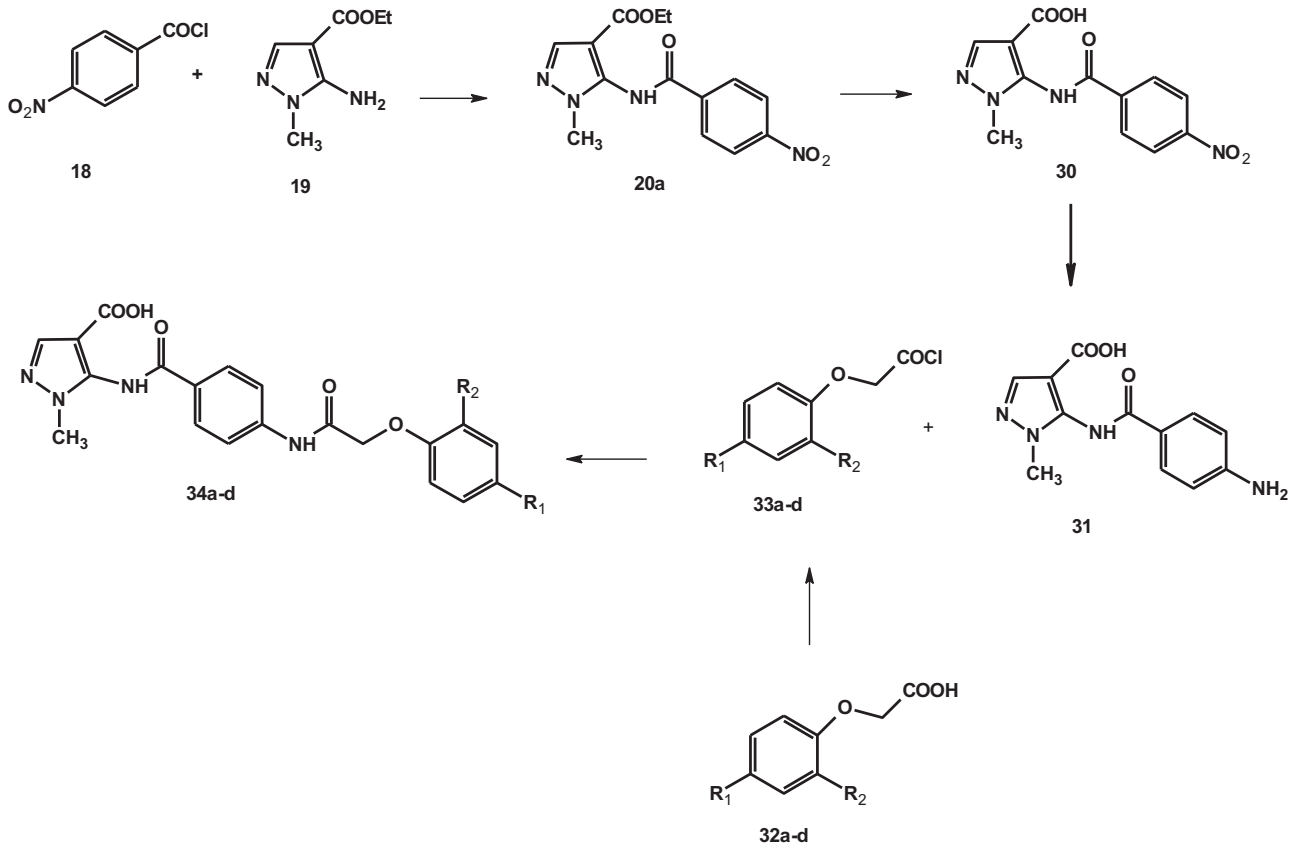


Fig. 4 Structural modifications applied to previously synthesized benzamides



	a	b	c	d	e	f
R	CH_3	CH_3	CH_3	CH_3	C_6H_5	C_6H_5
R_1	H	H	Cl	Cl	H	Cl
R_2	H	CH_3	H	Cl	H	H

Scheme 1 Synthetic pathway for the formation of derivatives **26**–**29**



	a	b	c	d
R_1	Cl	Cl	H	CH_3
R_2	H	Cl	CH_3	H

Scheme 2 Synthetic pathway for the formation of derivatives **34a–d**

series of derivatives by varying the experimental conditions, we have not been able to isolate some of the foreseen compounds. In particular, 4-cinnamamidobenzamido-1H-pyrazoles **25a, e, 26a, e, f, 27b–d** and **29e** were obtained according to Scheme 1. By refluxing for 8 h in acetonitrile 4-nitrobenzoyl chloride **18** with the appropriate ethyl 5-amino-1-R-1H-pyrazole-4-carboxylate **19a, e**, the ethyl 1-R-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylates **20a, e** were obtained. By reduction with hydrogen and 10% Pd-C in a Parr apparatus, the parent nitro derivatives **20a, e** were transformed in the corresponding key intermediates **25a, e**.

By refluxing for 8 h in acetonitrile the opportune cinnamoyl or phenoxychloride **23a, e** and **24b–d** with the intermediates **25a, e**, ethyl 5-(4-cinnamamidobenzamido)-1-methyl-1H-pyrazole-4-carboxylates **26a, e, f** and ethyl 1-methyl-5-(4-(2-phenoxyacetamido)benzamido)-1H-pyrazole-4-carboxylate **27b–d** were obtained. Furthermore, hydrolysis of derivatives **26a, e, f** with a mixture formed by equal volumes of 4% aqueous solution of sodium hydroxide and ethanol, produced the 5-(4-cinnamamidobenzamido)-1-methyl-1H-pyrazole-4-carboxylic acid **28a, e, f**. Finally, by fusion of 5-(4-cinnamamidobenzamido)-1-methyl-1H-pyrazole-4-carboxylic acid **28a** it was possible to obtain 4-cinnamamido-N-(1-methyl-1H-pyrazol-5-yl)benzamide **29e**.

Finally, regarding the 1-methyl-5-(4-(2-phenoxyacetamido)benzamido)-1H-pyrazole-4-carboxylic acids **34a–d**, any attempt to obtain them by the same procedure (Scheme 1), failed. Derivatives **34** were therefore obtained by a different synthetic route, according to the Scheme 2.

By refluxing for 8 h in acetonitrile 4-nitrobenzoyl chloride **18** with the ethyl 5-amino-1-methyl-1H-pyrazole-4-carboxylate **19**, the ethyl 1-methyl-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylate **20a** was obtained. This last was hydrolyzed with a mixture formed by equal volumes of 8% aqueous solution of sodium hydroxide and ethanol to give

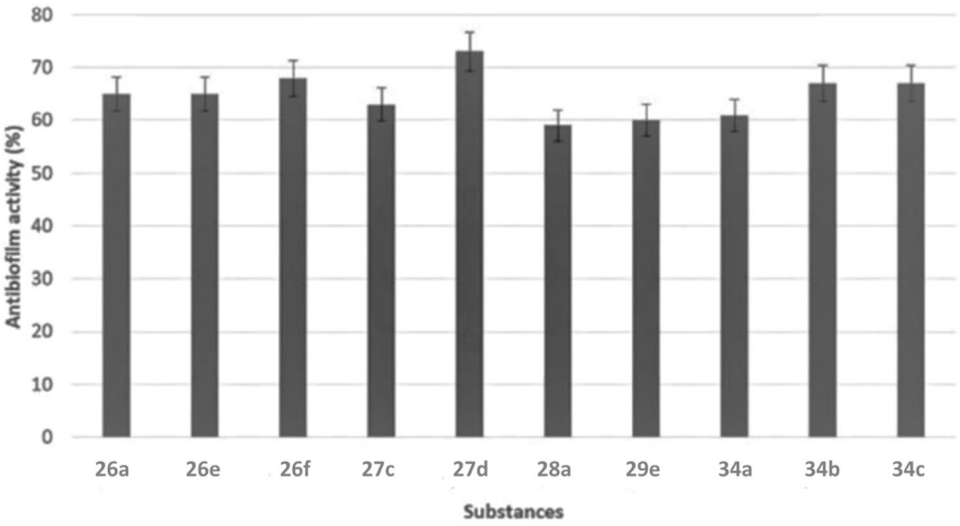


Fig. 5 Inhibition of biofilm formation, data are the mean ± SD of three independent experiments, each performed at least in quadruplicate, and expressed as inhibition percentage respect to the growth control. Data were considered significant at $P < 0.05$

1-methyl-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylic acid **30**. The reduction of the nitro derivative **30** with hydrogen and 10% Pd-C in a Parr apparatus provided the corresponding 5-(4-aminobenzamido)-1-methyl-1H-pyrazole-4-carboxylic acid **31**. Finally, by reacting compound **31** with the opportune 2-phenoxyacetyl chlorides **33a–d**, the desired 1-methyl-5-(4-(2-phenoxyacetamido)benzamido)-1H-pyrazole-4-carboxylic acids **34a–d** were synthesized.

Biology

Antimicrobial and antibiofilm activity of substances

All the compounds were tested against four relevant bacteria pathogens, Gram-positive (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212) or Gram-negative (*Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* ATCC 25922), and against the yeast *Candida albicans* ATCC 10231. The results representing the antimicrobial activity were expressed in terms of minimal inhibitory concentration (MIC) in μg/mL. No antibacterial or antifungal activities were detected at the maximum tested concentration of 100 μg/mL against strains in planktonic (free living) form tested.

In consideration of the increasing importance of the role of biofilms in chronic and polymicrobial infections, we also evaluated the antibiofilm properties of the compounds in terms of interference with biofilm formation. The inhibition of biofilm formation was tested against bacterial and fungal strains using a screening concentration of substances equal to 100 μg/mL. All samples showed moderate or weak bacterial biofilm inhibition activities against pathogens, *S. aureus* ATCC *25923*, *E. faecalis* ATCC *29212*, *P. aeruginosa* ATCC *15442, E.coli* ATCC *25922*, ranging from 44 to 10% inhibition percentages. Instead, we observed a much more relevant activity of inhibition of biofilm formation of *C.albicans*, the results, in terms of inhibition percentages, at the maximum tested concentration (100 μg/mL) of all compounds are reported in Fig. 5.

Table 1 Values of concentrations of substances at which the percentage of inhibition of biofilm formation is equal to 50% (BIC_{50})

BIC_{50} (μg/mL)		
C. albicans ATCC 10231		
	μg/mL	μM
26a	32	0.08
26e	19	0.04
26f	13	0.02
27c	6	0.01
27d	28	0.06
28a	18	0.05
29e	53	0.13
34a	18	0.04
34b	29	0.06
34c	29	0.07

As shown in the Fig. 5, all the tested compounds are endowed of a good activity higher than 50% against the biofilm formation of *C. albicans*. Particularly, the best efficacy in inhibiting the biofilm formation was showed by sample **27d** with an activity value of 73%. We also evaluated the biofilm inhibition concentration 50% (BIC_{50}), that is the concentration at which the percentage of inhibition of biofilm formation is equal to 50%. As reported in Table 1, all the tested compounds showed an inhibition in the micro or sub-micromolar concentrations. Among the tested compounds, the sample **27e** with a BIC_{50} of 0.01 μM (6 μg/mL)

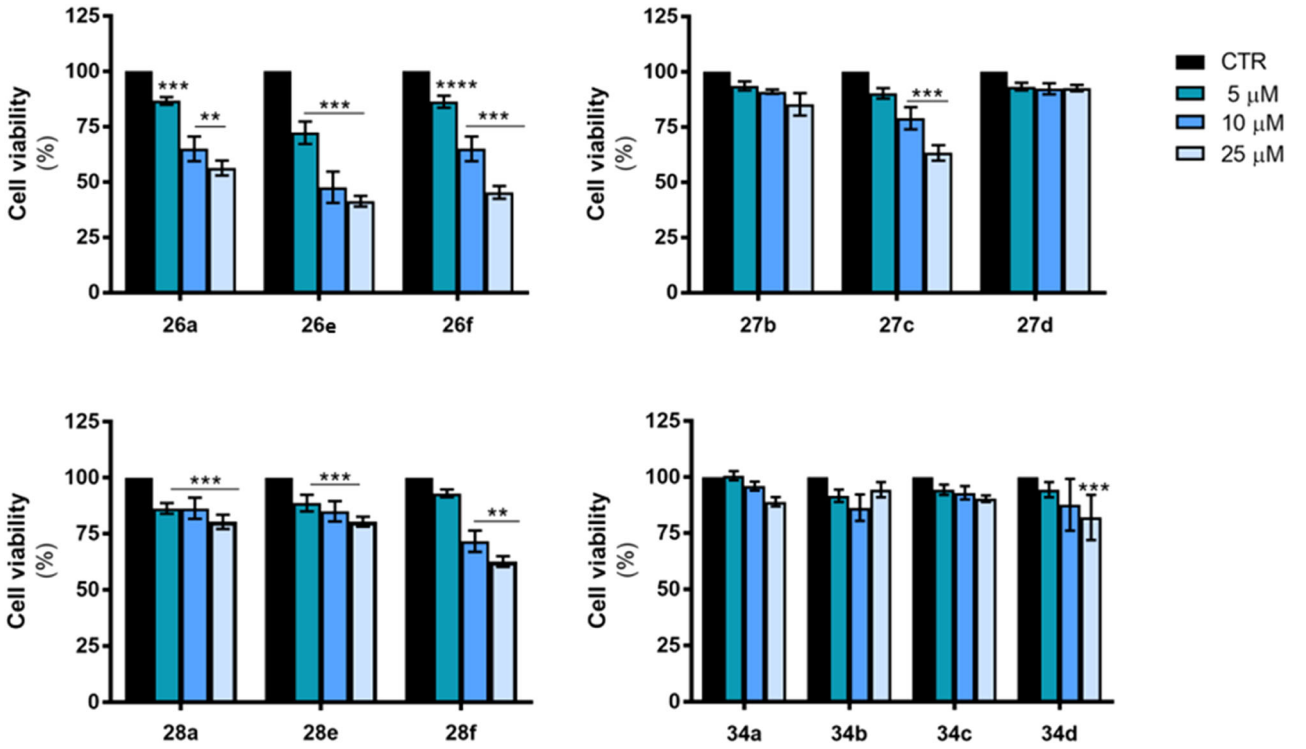


Fig. 6 Cytotoxic effects of the synthesized benzamido derivatives on MDA-MB231 breast cancer cells. Dose dependence effect of compounds on cell viability. MDA-MB231 cells (8×10^3) were incubated with various concentrations of compounds (5–25 μM) for 48 h. Then, cell viability was evaluated by using MTT assay as reported in methods. Values are reported as the mean ± SE. $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ vs. the control (CTR)

was the most effective in inhibiting biofilm formation of *C. albicans*.

Cytotoxic effects

Synthesized benzamido derivatives **26a, e, f, 27b–d 28a, e, f**, and **34a–d** were preliminarily tested in vitro for their anti-tumor activity against the human triple-negative breast cancer MDA-MB231 cells (Fig. 6). Triple-negative breast cancer (TNBC) accounts for about 10–15% of all breast cancers. This form of breast cancer has few treatment options because these cancer cells do not express estrogen receptor, progesterone receptor, or human epidermal growth factor receptor 2 (HER-2) to make hormone therapy or targeted HER2 drugs [R19]. Among the tested compounds only **26e** and **26f** had a weak inhibitory activity against the MDA-MB231 cells. In fact after 48 h of treatment with 10 μM, the viability of MDA-MB231 cells was reduced by 51% and 30% with **26e** and **26f**, respectively.

Conclusion

Based on our previous research, considering the biological activity of 4-acetamido-N-methylbenzamides and the antibiofilm activity described by us for pyrazole-4-carboxamides, compounds **26a, e, f, 27b–d, 28a, e, f, 29e** and **34a–d** were synthesized and evaluated for their antimicrobial and antibiofilm activity as well as for their cytotoxic effects. No antibacterial or antifungal activities were detected at the maximum tested concentration of 100 μg/mL against tested strains in planktonic (free living) form. The synthesized compounds resulted also inactive as anti-tumor agents being only derivatives **26e** and **26f** endowed of a weak inhibitory activity against the breast cancer MDA-MB231 cells.

The synthesized compounds resulted in active interference with biofilm formation, even if in a moderate or weak way, against bacterial pathogens, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 15442, *E. coli* ATCC 25922. Good activity higher than 50% in inhibiting biofilm formation was instead shown against *C. albicans* being compound **27c** the most active. These observations are very encouraging. *C. albicans* is a very versatile fungal pathogen and its clinical relevance very often depends on its ability to develop as a multilayered community (biofilm) on natural (host tissues) or artificial surfaces (embedded medical devices, including catheters, prostheses, etc.). The biofilms of *C. albicans* are naturally resistant to conventional antifungal therapies, and new

antifungal agents capable of interfering with the growth as biofilms are needed [20]. Based on obtained results, compound **27c** can be considered a good candidate as lead compound, useful for further developments of agent that interfere with biofilm formation of a relevant nosocomial fungal pathogen.

Experimental

Chemistry

General

Reaction progress was monitored by TLC on silica gel plates (Merck 60, F254, 0.2 mm). Organic solutions were dried over Na_2SO_4. Evaporation refers to the removal of solvent on a rotary evaporator under reduced pressure. All melting points were determined on a Büchi 530 capillary melting point apparatus and are uncorrected. IR spectra were recorded with a Perkin Elmer Spectrum RXI FT-IR System spectrophotometer, with compound as a solid in a KBr disc or nujol. 1H NMR (300 MHz) and APT (75 MHz) spectra were recorded with a Bruker AC-E spectrometer at room temperature; chemical shifts (δ) are expressed as ppm values. Microanalyses data (C, H, N) were obtained by an Elemental Vario EL III apparatus and were within ±0.4% of the theoretical values. Yields refer to purified products and are not optimized. The names of the compounds were obtained using Chem Draw Ultra 12.0 software (CambridgeSoft).

General procedure for preparation of benzoyl chlorides 18, 23a, e, 24b–d and 33a, d [21]

4-Nitrobenzoyl chloride **18** was commercially available. Substituted benzoyl chlorides **23a, c, 24b–d** and **33a, d** were obtained by refluxing for 5 h the appropriate acid derivatives **21a, c, 22b–d** and **32a, d** (0.01 mole) with thionyl chloride (7.25 mL). After evaporation under reduced pressure, the crude liquid residue was used for subsequent reactions without purification.

General procedure for preparation of compounds 20a, e [22, 23]

A solution of ethyl 1-R-5-amino-1H-pyrazole-4-carboxylate **19a, e** (0.01 mole) in acetonitrile (50 mL) was heated under reflux with the 4-nitrobenzoyl chloride **18** (0.01 mole) for 7 h. The solid which separated was collected then recrystallized from ethanol to give compounds **20a, e** that were identical in all respect (mp, mixed mp, Rf, IR, 1H-NMR) with an authentic specimen of ethyl 1-methyl-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylate **20a** [22] and ethyl 5-(4-nitrobenzamido)-1-phenyl-1H-pyrazole-4-carboxylate **20e** [23].

General procedure for preparation of ethyl compounds 25a, e [23, 24] and 31

To a solution of ethyl 1-R-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylate **20a, e** (0.013 moles) or 1-methyl-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylic acid **31** (0.017 moles) in warm ethanol (200 mL) 300 mg of 10% Pd-C as catalyst was added. The mixture was left under hydrogenation in a Parr apparatus at 50 psi for 24 h. The suspension was filtered, and the filtrate was concentrated to a small volume affording a compound which was identical in all respect (mp, mixed mp, Rf, IR, 1H-NMR) with an authentic specimen of ethyl 5-(4-aminobenzamido)-1-methyl-1H-pyrazole-4-carboxylate **25a** [24] and ethyl 5-(4-aminobenzamido)-1-phenyl-1H-pyrazole-4-carboxylate **25e** [23]. Compound **31** was isolated as white crystalline product.

5-(4-aminobenzamido)-1-methyl-1H-pyrazole-4-carboxylic acid (31): yields: 68%; mp 248–250 °C. I.R. (cm^{-1}): 3470–2593 (NH_2, NH, OH); 1680 (CO); 1672 (CO). 1H NMR (DMSO-d6) (δ): 3.63 (3H, s, CH_3); 5.89 (2H, s, exchangeable, NH2); 6.60–7.81 (5H, set of signals, C_6H_4 and pyrazole H-3); 9.85 (1H, s, exchangeable, NH); 12.27 (1H, broad, exchangeable, OH); 13C NMR (DMSO-d6) (δ): 36.54, 108.26, 113.01, 119.50, 130.30, 140.09, 140.20, 153.28, 163.91, 166.10. Anal. Calc. for $C_{12}H_{12}N_4O_3$: C, 55.38%; H, 4.65%; N, 21.53%. Found: C, 55.40%; H, 4.67%; N, 21.40.

General procedure for preparation of 1-Methyl-5-(4-(3-phenylpropanamido)benzamido)-1H-pyrazole-4-carboxylate 26a, e, f and ethyl 5-(4-(2-(2-R_2-4-R_1-phenoxy) acetamido)benzamido)-1-methyl-1H-pyrazole-4-carboxylate 27b, e, d

A solution of 5-(4-aminobenzamido)-1-R-1H-pyrazole-4-carboxylate **25a, e** (4 mmol) and the appropriate cinnamoyl chlorides **23a, c** (4 mmol) in acetonitrile (20 mL) was refluxed for 8 h. The solvent was partially evaporated under reduced pressure until a product precipitates. The residue was collected and recrystallized from ethanol to give pure **26a, e, f**. Compounds **27b–d** were obtained with the same procedure using 5-(4-aminobenzamido)-1-R-1H-pyrazole-4-carboxylate **25a, e** (1.74 mmol) and the appropriate 2-phenoxyacetyl chlorides **24b–d** (4 mmol) in acetonitrile (20 mL).

Ethyl 5-(4-cinnamamidobenzamido)-1-methyl-1H-pyrazole-4-carboxylate 26a: yields 82%, mp 195–200 °C; I.R (Nujol) cm^{-1} 3389–3273 (NH), 1694 (CO) 1660 (CO), 1H NMR ($CHCl_3$) δ: 1.32 (t, 3H, CH_3); 3.88 (s, 3H, CH_3);

4.276 (q, 2H, CH_2); 6.597 (d, 1H, J = 15.9 Hz, olefinic CH); 7.26–7.99 (m, 15H, ArH and olefinic CH); 8.23 (s, 1H, pyrazole H3); 9.29 (s, 1H, exchangeable, NH). 13 C NMR(δ) ($CDCl_3$) 14.35, 38.56 60,46, 104.40, 119.56, 120.35, 127.71, 128.08, 128.97, 129.11, 130.32, 134.32, 139.49, 140.66, 142.46, 143.31, 164.08, 164.37, 165.07. Anal. Calc. for $C_{23}H_{22}N_4O_4$: C, 66.02%; H, 5.30%; N, 13.39%. Found: C, 65.68%; H, 5.01%; N, 13.05.

Ethyl 5-(4-(3-(4-chlorophenyl)acrylamido)benzamido)-1-methyl-1H-pyrazole-4-carboxylate 26c: yields 103,5%, mp 205–207 °C; I.R (Nujol) cm^{-1} 3558–3254 (NH), 1708-1891-1655 (CO), 1H NMR (DMSO) δ: 1.14 (s, 3H, CH_3); 4.13 (q, 2H, CH_2); 6.87 (d, 1H, J = 15.9 Hz, olefinic CH); 7.51–7.90 (m, 15H, ArH and olefinic CH); 8.01 (s, 1H, pyrazole H3); 10.33 (s, 1H, exchangeable, NH); 10.56 (s, 1H, exchangeable, NH). 13 C NMR(δ) (CDCl3) 14.59, 36.46, 39.17, 39.45, 39.73., 40.01, 40.29, 40.56, 40.84, 108.09, 119.04, 123.18, 127.86, 129.53, 129.58, 130.00, 134.02, 134.89, 139.42, 140.03, 143.27, 162.19, 164.23, 165.85. Anal. Calc. for $C_{23}H_{21}ClN_4O_4$: C, 61.00%; H, 4.67%; N, 12.37%. Found: C, 60.69%; H, 4.70%; N, 12.32.

Ethyl 5-(4-(3-(4-chlorophenyl)acrylamido)benzamido)-1-phenyl-1H-pyrazole-4-carboxylate 26f: yields 60%, mp 250–255 °C; I.R (Nujol) cm^{-1} 3583-3356-3212 (NH), 1894 (CO) 1698 (CO), 1H NMR (DMSO) δ: 1.14 (s, 3H, CH_3); 4.18 (q, 2H, CH_2); 6.88 (d, 1H, J = 15.9 Hz, olefinic CH); 7.40–7.94 (m, 15H, ArH and olefinic CH); 8.19 (s, 1H, pyrazole H3); 10.41 (s, 1H, exchangeable, NH); 10.56 (s, 1H, exchangeable, NH). 13 C NMR(δ) (DMSO) 14.56, 39.16, 39.44, 39.71, 39.99., 40.27, 40.55, 40.83, 110.63, 119.09, 123.16, 124.21, 127.88, 128.88, 129.37, 129.57, 129.73, 129.99, 134.01, 134.90, 138.41, 139.19, 140.03, 141.77, 143.27,162.04, 164.22, 166.44. Anal. Calc. for $C_{28}H_{23}ClN_4O_4$: C, 65.31%; H, 4.50%; N, 10.88%. Found: C, 65.55%; H, 4.84%; N, 11.22.

Ethyl 1-methyl-5-(4-(2-(o-tolyloxy)acetamido)benzamido)-1H-pyrazole-4-carboxylate 27b: yields 40%; mp. 183–85 °C. I.R. (cm^{-1}): 3330 (NH); 3210 (NH); 1715(CO); 1664 (CO). 1 H NMR (DMSO-d_6) (δ): 1.20 (3H, t, CH_3); 2.18 (3H, s, CH_3); 3.67 (3H, s, CH_3); 4.20 (2H, q, CH_2); 4.70 (2H, s, CH_2); 6.85–8.01 (m, 9H, 2 x C_6H_4 and pyrazole H3); 10.30 (1H, s, exchangeable, NH); 10.38 (1H, s, exchangeable, NH). Anal. Calc. for $C_{23}H_{24}N_4O_5$: C, 63.29%; H, 5.54%; N, 12.84%. Found: C, 63.09%; H, 5.15%; N, 12.57.

Ethyl 5-(4-(2-(4-chlorophenoxy)acetamido)benzamido)-1-methyl-1H-pyrazole-4-carboxylate 27c: yields 44%; mp 168–70 °C. I.R. (cm^{-1}): 3341 (NH); 3228 (NH); 1716 (CO); 1666 (CO). 1H NMR (DMSO-d_6) (δ): 1.13 (3H, t, CH_3); 3.69 (3H, s, CH_3); 4.12 (2H, q, CH_2); 4.77 (2H, s, CH_2); 7.03–8.02 (9 H, m, 2 x C_6H_4 and pyrazole H3); 10.33 (1H, s, exchangeable, NH); 10.43 (1H, s, exchangeable, NH). Anal. Calc. for $C_{22}H_{21}ClN_4O_5$: C, 57.84%; H, 4.36%; N, 12.26%. Found: C, 57.66%; H, 4.98%; N, 12.52.

Ethyl 5-(4-(2-(2,4-dichlorophenoxy)acetamido)benzamido)-1-methyl-1H-pyrazole-4-carboxylate 27d: yields 37%; mp 188–90 °C. I.R. (cm^{-1}): 3386 (NH); 1712 (CO); 1681 (CO). 1 H NMR (DMSO-d_6) (δ): 1.12 (3H, t, CH_3); 3.68 (3H, s, CH_3); 4.12 (2H, q, CH_2); 4.92 (2H, s, CH_2); 7.12–8.01 (8 H, m, C_6H_3, C_6H_4 and pyrazole H3); 10.33 (1H, s, exchangeable, NH); 10.51 (1H, s, exchangeable, NH). 13 C NMR(δ) (DMSO) 14.59, 36.46, 59.92, 68.21, 108.10, 115.83, 119.17, 122.96, 125.56, 128.17, 128.52, 129.52, 129.89, 139.39, 140.05, 142.45, 153.11, 162.19, 165.81, 166.76. Anal. Calc. for $C_{22}H_{20}Cl_2N_4O_5$: C, 53.78%; H, 4.10%; N, 11.40%. Found: C, 54.09%; H, 3.81%; N, 11.02.

General procedure for preparation of 1-R-5-(4-(3-phenylpropanamido)benzamido)-1H-pyrazole-4-carboxylic acid 28a, e, f

To a solution of ethyl 1-R-1H-pyrazole-4-carboxylates **26a, e, f** (3.2 mmoles) in ethanol (18.75 ml), a solution aqueous 4% of NaOH (22.5 ml) was added. The reaction mixture is heated under reflux for 15 ‘, then left at room temperature for 12 h.

After this time, the ethanol was removed under reduced pressure and the remaining aqueous solution was acidified with 1 M HCl until complete precipitation of the acids. Finally, the precipitate was filtered and crystallized with ethanol to give compounds **28a, e, f**.

5-(4-cinnamamidobenzamido)-1-methyl-1H-pyrazole-4-carboxylic acid (28a): yields 80% mp 228–232 °C; I.R (Nujol) cm^{-1} 3254 (NH) 1698 (CO); 1H NMR (DMSO) δ: 3.68 (d, 1H, J = 15.9 Hz, olefinic CH); 7.05–7.94 (m, 15H, ArH and olefinic CH); 8.05 (s, 1H, pyrazole H3); 10.35 (s, 1H, exchangeable, NH); 10.38 (s, 1H, exchangeable, NH); 12.35 (s, 1H, broad, exchangeable, OH). 13C NMR(δ) (DMSO) 36.50, 108.77, 118.91, 122.72, 127.64, 128.28, 129.50, 130.39, 135.14, 139.37, 140.32, 141.01,143.57, 163.71, 164.56, 165.84. Anal. Calc. for $C_{21}H_{18}N_4O_4$: C, 64.61%; H, 4.65%; N, 14.35%. Found: C, 64.74%; H, 4.45%; N, 14.43.

5-(4-cinnamamidobenzamido)-1-phenyl-1H-pyrazole-4-carboxylic acid (28e): yields 80% mp 230–232 °C; I.R (Nujol) cm^{-1} 3308 (NH) 1764, 1681, 1655 (CO); 1H NMR (DMSO) δ: 3.68 (d, 1H, J = 15.9 Hz, olefinic CH); 7.41–7.91 (m, 15H, ArH and olefinic CH); 8.15(s, 1H, pyrazole H3); 10.37 (s, 1H, NH); 10.54 (s, 1H, NH); 12.56 (s, 1H, broad, exchangeable, OH). 13C NMR (δ) ($CDCl_3$) 39.14, 39.42, 39.70, 39.98, 40.26, 40.53, 40.81, 111.36,

119.08, 122.34, 124.15, 127.87, 128.31, 128.74, 129.37, 129.53, 129.67, 130.47, 135.04, 138.60, 139.08, 141.46, 142.09, 143.31, 163.57, 164.42, 166.33. Anal. Calc. for $C_{26}H_{20}N_4O_4$: C, 69.02%; H, 4.46%; N, 12.38%. Found: C, C, 68.79%; H, 4.27%; N, 12.60%.

5-(4-(3-(4-chlorophenyl)acrylamido)benzamido)-1-phenyl-1H-pyrazole-4-carboxylic acid 28f: yields 70% mp 155–160 °C; I.R (Nujol) cm^{-1} 3579, 3185 (NH) 1693, 1625 (CO); 1H NMR (DMSO) δ: 3.90 (d, 1H, J = 15.9 Hz, olefinic CH); 7.40–7.92 (m, 15H, ArH e olefinic CH); 8.14 (s, 1H, pyrazole H3); 10.35 (s, 1H, NH); 10.59 (s, 1H, NH); 12.51 (s, 1H, NH), 12.46 (s, 1H, broad, exchangeable, OH). 13C NMR(δ) (DMSO) 119.05, 122.41, 124.14, 125.76, 127.86, 128.30, 128.71, 129.34, 129.52, 129.66, 130.44, 130.92, 135.06, 138.60, 139.09, 141.37, 142.06, 142.32, 163.55, 164.42, 166.32. Anal. Calc. for $C_{26}H_{19}ClN_4O_4$: C, 64.14%; H, 3.93%; N, 11.51%. Found: C, 64.25%; H, 3.60%; N, 11.73.

General procedure for preparation of 4-cinnamamido-N-(1-methyl-1H-pyrazol-5-yl)benzamide 29e

The acid 5-(4-cinnamamido)-1phenyl-1H-pyrazole-4-carboxylic **28e** has been decarboxylated by melting to obtain the corresponding compound **29e** that has been purified by crystallization.

4-cinnamamido-N-(1-phenyl-1H-pyrazol-5-yl)benzamide 29e: yields 70% mp 195–197 °C; I.R (Nujol) cm^{-1} 3585, 3254 (NH), 1741 (CO), 1679 (CO); 1H NMR (DMSO) δ: 6.48 (s, 1H, pyrazole H3); 6.86 (d, 1H, J = 15.9 Hz, olefinic CH); 7.35–7.89 (m, 15H, ArH e olefinic CH, pyrazole H4); 10.29 (s, 1H, NH); 10.53 (s, 1H, exchangeable, NH). 13C NMR(δ) (DMSO) 104.72, 119.01, 122.28, 132.72, 127.78, 128.05, 128.32, 129.28, 129.55, 129.58, 130.49, 135.01, 136.53, 139.39, 140.21, 141.44, 143.14, 164.39, 165.92. Anal. Calc. for $C_{25}H_{20}N_4O_4$: C, 73.51%; H, 4.94%; N, 13.72%. Found: C, 73.73%; H, 5.03%; N, 13.35.

General procedure for preparation of 1-methyl-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylic acid 30

Compound **30** is known [25] but was prepared in a different way. To a solution of ethyl 1-methyl-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylate **20a** (12.3 mmoles) in ethanol (24 ml), a solution aqueous 8% of NaOH (24 ml) was added. The reaction mixture was left at room temperature for 12 h.

After this time, the ethanol was removed under reduced pressure and the remaining aqueous solution was acidified with 1 M HCl until complete precipitation of the acids. Finally, the precipitate was filtered and crystallized with ethanol to give a compound which was identical in all respect (mp, mixed mp, Rf, IR, 1H-NMR) with an authentic specimen of 1-methyl-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylic acid **30** [25].

General procedure for preparation of 5-(4-(3-(2-R2-4-R1-phenyl)acrylamido)benzamido)-1-methyl-1H-pyrazole-4-carboxylic acids 34a–d

A suspension of 5-(4-aminobenzamido)-1-methyl-1H-pyrazole-4-carboxylic acid **31** (1.73 mmol) and the appropriate 2-phenoxyacetyl chloride **33a–d** (1.73 mmol) in acetonitrile (60 mL) was refluxed for 8 h. The reaction mixture was filtered, then the solvent was partially evaporated under reduced pressure until a product precipitates. The residue was collected and recrystallized from ethanol to give pure **34a, c, d**. Compound **34b**, which separated directly from the reaction mixture, was directly crystallized from ethanol.

5-{4-[2-(4-chlorophenoxy)acetamido]benzamido}-1-methyl-1H-pyrazole-4-carboxylic acid (34a): yield 14%; mp 230–34 °C. I.R. (cm^{-1}): 3259–2605 multiple bands (NH, OH); 1685 (CO); 1655 (CO). 1H-NMR (DMSO) (δ): 3.67(3H, s, CH_3); 4.78 (2H, s, CH_2); 7.03–8.02 (9 H, 2 x C6H4 e pyrazole H-3); 10.36 (1H, s, exchangeable NH); 10.44 (1H, s, exchangeable NH); 12.40 (1H, broad, exchangeable OH). 13C-NMR (DMSO-d6) (δ): 36.39, 67.54, 108.68, 116.93, 119.49, 125.50, 128.15, 129.45, 129.77, 139.12, 140.37, 142.21, 157.00, 163.65, 166.01, 167.38. Anal. Calc. for $C_{20}H_{17}ClN_4O_5$: C, 56.02%; H, 4.00%; N, 13.07%. Found: C, 55.75%; H, 4.34%; N, 12.76.

5-{4-[2-(2,4-dichlorophenoxy)acetamido]benzamido}-1-methyl-1H-pyrazole-4-carboxylic acid (34b): yields 34%; mp 245–47 °C. I.R. (cm^{-1}): 3388–2671 (multiple bands, NH, OH); 1701 (broad, CO);. 1H NMR (DMSO-d6) (δ): 3.66 (3H, s, CH_3); 4.92 (2H, s, CH_2); 7.12–8.02(8 H, m, C_6H_3, C_6H_4 e pyrazole H3); 10.30 (1H, s, exchangeable, NH); 10.51 (1H, s, exchangeable NH); 12.25 (s, 1H, broad, exchangeable, OH). Anal. Calc. for $C_{20}H_{16}Cl_2N_4O_5$: C, 51.85%; H, 3.48%; N, 12.09%. Found: C, 52.03%; H, 3.86%; N, 12.00.

1-methyl-5-{4-[2-(2-methylphenoxy)acetamido]benzamido}-1H-pyrazole-4-carboxylic acid (34c): yields 16%; mp 235–37 °C. I.R. (cm^{-1}): 3220–2507 (multiple bands, NH, OH); 1677 (CO); 1659 (CO). 1H NMR (DMSO-d6) (δ): 2.23 (3H, s, CH_3); 3.66 (3H, s, CH_3); 4.70 (2H, s, CH_2); 6.89–8.02 (9 H, m, 2 x C_6H_4 and pyrazole H3); 10.30 (1H, s, exchangeable, NH); 10.38 (1H, s, exchangeable, NH); 12.31 (s, 1H, broad, exchangeable, OH). Anal. Calc. for $C_{21}H_{20}N_4O_5$: C, 61.76%; H, 4.94%; N, 13.72%. Found: C, 61.82%; H, 4.91%; N, 13.41.

1-methyl-5-{4-[2-(4-methylphenoxy)acetamido]benzamido}-1H-pyrazole-4-carboxylic acid (34d): yields 13%; mp 233–35 °C. I.R. (cm^{-1}): 3223–2507 (multiple bands NH, OH); 1712 (CO); 1697 (CO). 1H NMR (DMSO-d6)

(δ): 2.24 (3H, s, CH_3); 3.70 (3H, s, CH_3); 4.75 (2H, s, CH_2); 6.87–8.04 (9 H, m, 2xC_6H_4 and pyrazole H3); 10.31 (1H, s, exchangeable NH); 10.39 (1H, s, exchangeable NH); 12.29 (s, 1H, broad, exchangeable, OH). Anal. Calc. for $C_{21}H_{20}N_4O_5$: C, 61.76%; H, 4.94%; N, 13.72%. Found: C, 61.68%; H, 4.56%; N, 14.04.

Biology

Cell lines and culture conditions

Triple negative breast cancer MDA-MB231 cells, obtained from Istituto Scientifico Tumori (Genoa, Italy), were grown as monolayers in DMEM medium. supplemented with 10% (v/v) fetal bovine serum (FCS), 2 mM glutamine and 1% non-essential amino acids. The cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 as previously reported [26]. For the experiments, cells were plated on 96-well plates, then were allowed to adhere overnight in culture medium before the treatment with chemicals or vehicle only. Stock solutions of the synthesized benzamido derivatives were prepared in DMSO and stored at −20 °C. In each experiment, the compounds were diluted to their final concentrations in the culture medium. The final concentration of DMSO never exceeded 0.04%, a concentration which had no discernible effects MDA-MB231 cells in comparison with the control.

Cell viability assay

For the evaluation of the effects of benzamides derivatives on cell viability, MDA-MB231 breast cancer cells were plated in 96-well plate (8×10^3/well) in the presence of different concentrations of the compounds (5–25 µM). After 48 h cell viability was determined by a colorimetric assay incubating the cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), MTT reagent (11 mg/mL in PBS, 20 µL) was added to each well and incubated for another 2 h at 37 °C. Then, the colored crystal of produced formazan was dissolved in 100 µL of lysis buffer (20% sodium dodecyl sulphate in 50% N,N-dimethylformamide, pH 4.0). The absorbance was measured by a microplate reader (OPSYS MR, Dynex Technologies, Chantilly, VA, USA) at 540 nm with a reference wavelength of 630 nm. Cell viability was measured as the percentage of the optical density (OD) values of treated cells compared with untreated cells as control. Each experiment was performed in triplicate. We reported in Table 2 the absorbances of three experiments and the mean value used for the Fig. 6.

For these experiments, MDA-MB231 cells were plated in 96-well plate (8×10^3/well) in the presence of different concentrations of the compounds. After 48 h cell viability

Table 2 Values of absorbances (ABS) at 540 nm and mean values measured by a microplate reader (OPSYS MR, Dynex Technologies, Chantilly, VA, USA) with a reference wavelength of 630 nm

	ABS of three experiments	Mean of ABS of three different experiments	Cell viability (% of control)
CTR	0.502	0.503	**100**
	0.504		
	0.503		
5 µM 26a	0.441	0. 440	**86**
	0.442		
	0.438		
10 µM 26a	0.360	0.353	**70**
	0.350		
	0.349		
5 µM 26a	0.323	0.320	**63**
	0.319		
	0.320		
CTR	0.503	0.505	**100**
	0.504		
	0.507		
5 µM 26e	0.370	0.368	**73**
	0.365		
	0.369		
10 µM 26e	0.248	0.245	**49**
	0.243		
	0.246		
25 µM 26e	0.230	0.234	**46**
	0.238		
	0.236		
CTR	0.560	0.557	**100**
	0.555		
	0. 558		
5 µM 26f	0.493	0.491	**88**
	0.489		
	0.490		
10 µM 26f	0.394	0.392	**70**
	0.390		
	0.393		
25 µM 26f	0.289	0.286	**51**
	0.292		
	0.276		
CTR	0.578	0.575	**100**
	0.575		
	0. 574		
5 µM 27b	0.561	0.558	**97**
	0.554		
	0. 560		
10 µM 27b	0.530	0.525	**91**
	0.525		
	0. 522		
25 µM 27b	0.489	0.480	**83**
	0.477		
	0.472		
CTR	0.510	0.515	**100**
	0.514		
	0. 520		

Table 2 (continued)

	ABS of three experiments	Mean of ABS of three different experiments	Cell viability (% of control)
5 μM 27c	0.472	0.470	**91**
	0.476		
	0.464		
10 μM 27c	0.410	0.408	**79**
	0.399		
	0.414		
25 μM 27c	0.367	0.363	**70**
	0.358		
	0.365		
CTR	0.672	0.669	**100**
	0.668		
	0.666		
5 μM 27d	0.638	0.642	**96**
	0.645		
	0.643		
10 μM 27d	0.635	0.638	**95**
	0.640		
	0.639		
25 μM 27d	0.627	0.624	**93**
	0.621		
	0.625		
CTR		0.600	**100**
5 μM 28a	0.551	0.546	**91**
	0.543		
	0. 545		
10 μM 28a	0.546	0.543	**90**
	0.541		
	0. 543		
25 μM 28a	0.514	0.516	**86**
	0.518		
	0. 515		
CTR	0.674	0.669	**100**
	0.667		
	0.666		
5 μM 28e	0.639	0.644	**96**
	0.650		
	0.642		
10 μM 28e	0.605	0.607	**90**
	0.601		
	0.614		
25 μM 28e	0.558	0.552	**82**
	0.554		
	0. 546		
CTR	0.582	0.584	**100**
	0.590		
	0. 579		
5 μM 28f	0.538	0.542	**93**
	0.542		
	0. 545		
10 μM 28f	0.449	0.452	**77**
	0.451		
	0.455		

Table 2 (continued)

	ABS of three experiments	Mean of ABS of three different experiments	Cell viability (% of control)
25 μM 28f	0.391	0.385	**66**
	0.387		
	0.378		
CTR	0.673	0.669	**100**
	0.668		
	0.666		
5 μM 34a	0.677	0.675	**101**
	0.669		
	0.678		
10 μM 34a	0.658	0.660	**98**
	0.662		
	0.659		
25 μM 34a	0.639	0.638	**95**
	0.640		
	0.636		
CTR	0.604	0.600	**100**
	0.599		
	0.597		
5 μM 34b	0.586	0.584	**97**
	0.590		
	0. 576		
10 μM 34b	0.554	0.559	**93**
	0.548		
	0. 575		
25 μM 34b	0.605	0.607	**101**
	0.602		
	0.613		
CTR	0.671	0.669	**100**
	0.670		
	0.666		
5 μM 34c	0.635	0.636	**95**
	0.640		
	0.634		
10 μM 34c	0.620	0.627	**93**
	0.631		
	0.629		
25 μM 34c	0.621	0.628	**93**
	0.633		
	0.629		
CTR	0.604	0.600	**100**
	0.600		
	0.596		
5 μM 34d	0.582	0.582	**97**
	0.588		
	0. 577		
10 μM 34d	0.575	0.570	**95**
	0.568		
	0. 566g		
25 μM 34d	0.507	0.510	**85**
	0.516		
	0. 508		

Cell viability measured as the percentage of the optical density (OD) for different concentrations of compounds

was determined by a colorimetric assay incubating the cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as reported [10, 11]. MTT is yellow tetrazolium salt that can be reduced to purple formazan by mitochondrial enzymes of living cells. The absorbance of the formazan was measured by a microplate reader (OPSYS MR, Dynex Technologies, Chantilly, VA, USA) at 540 nm with a reference wavelength of 630 nm and cell viability was quantified as the percentage of the optical density (OD) values of treated cells compared with that of untreated control cells. Each experiment was performed in triplicate.

Microbial strains

The reference strains *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 15442, *Escherichia co*li ATCC 25922 and *Candida albicans* ATCC 10231 were used in the determination of Minimum Inhibitory Concentrations (MICs), and Inhibition of Biofilm Formation (IBF) tests. The bacterial strains were cultured aerobically in Muller-Hinton broth (MHB) or tryptic soy agar (TSA) [27]. Fungal *C. albicans* strain was cultured aerobically on Sabouraud (BS) broth or agar medium [28].

Determination of Minimum Inhibitory Concentrations (MICs)

MICs were determined by a microdilution method. Briefly, a series of solutions were prepared with a range of concentrations from 100 to 1.5 µg/mL (obtained by two-fold serial dilution). The serial dilutions were made in Mueller–Hinton broth (MH) (Sigma Aldrich) in a 96-wells plate, starting from a stock solution of 100 µg/mL in MH [29]. To each well, 10 µL of a bacterial suspension from a culture grown at 37 °C for 24 h on Tryptic Soy Agar (TSA), containing ~10^6 cfu/mL was added. A growth control and negative control, consisting respectively of bacterial strains in the medium without tested substances, and the medium without both substance and inoculum were also included in the 96-wells plate [30]. A substance control, consisting only of the substance solution in the medium without bacterial inoculum were added to evaluate the absorbance of substance at the tested concentrations. The plate was incubated at 37 °C for 24 h, the MICs were determined by a microplate reader (Glomax Multidetection System TM297 Promega, Milano Italy) as the lowest concentration of compound whose OD, read at 570 nm, was comparable with the negative control wells (broth only, without inoculum) [27]. Antifungal activity against *C. albicans* ATCC 10231 was evaluated by using a micro-method described above, using Sabouraud broth (BS) (Sigma-Aldrich) as growth medium.

Inhibition of biofilm formation (crystal violet method)

Compounds 26a, c, f, 27b–d, 28a, e, f, 29a and 34a–d were tested for their ability to interfere with biofilm formation of *C. albicans* ATCC 10231 and above mentioned bacterial strains. The yeast was grown in Sabouraud broth (BS) containing 2% (w/v) glucose overnight at 37 °C. After the incubation time, 2.5 µL of fungal suspension (containing ~10^6 cfu/mL) was placed into each well of a sterile flat-bottom 96-well loaded with 200 µL of BS with 2% glucose, supplemented with a screening concentration of 100 µg/mL of each substance [31]. The plates were incubated at 37 °C for 24 h; after this incubation time, the medium was removed, the plates were washed twice with sterile NaCl 0.9%, air-dried and then each well was filled with 100 µL of crystal violet solution (0.1%) for 15 min. The plate was then washed three times with water, and the crystal violet was dissolved in 200 µl of ethanol by pipetting up and down. Each assay was performed in triplicate and repeated at least twice. The plate was read at 570 nm using a microplate reader (Glomax Multi-detection System TM297 Promega, Milano, Italy). Inhibition percentages at screening concentration (or at lower concentrations in the case of activity higher than 50% of each sample) were obtained by comparing the OD of control wells with that of the sample wells, by using the following formula:

$$\text{Inhibition}(\%) = (\text{OD growth control} - \text{OD sample})/\text{OD growth control}) \times 100.$$

BIC_{50} (the concentration at which the percentage of inhibition of biofilm formation is equal to 50%) was calculated using AAT Bioquest, Inc. Quest Graph™ IC50 Calculator (v.1), retrieved from https://www.aatbio.com/tools/ic50-calculator-v1.

Inhibition of bacterial biofilms was determined by using the method described above, using Tryptose broth (TS) (Sigma-Aldrich) enriched with 2% w/v of glucose as growth and test medium [32].

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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