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Original article

New α-(*N*)-heterocyclichydrazones: evaluation of anticancer, anti-HIV and antimicrobial activity

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Dedicated to the memory of Professor Cesare Pellerano

Abstract

A series of 3- and 5-methylthiophene-2-carboxaldehyde α-(*N*)-heterocyclichydrazones were synthesized and submitted to an in vitro investigation of their anticancer, anti-HIV and antimicrobial activities. Some of the newly synthesized compounds were found to possess antiproliferative properties, whereas no anti-HIV activity was seen; the most active of the series was the derivative **2i**, which exhibited tumour growth inhibition activity against all cell lines displaying GI_{50} values between 1.63 and 26.5 μM. The title compounds were generally ineffective against Gram-positive and Gram-negative bacteria, while showed a moderate antifungal activity against *C. albicans* and *A. fumigatus*.

Keywords: α-(*N*)-Heterocyclichydrazones; Thiophenecarboxaldehyde; Antitumoral activity; Antimicrobial activity

1. Introduction

In the last few years, there has been considerable interest in the development of chelating agents and their metal chelates as potential antineoplastic agents [1]. Quinolylhydrazones constitute an important class of compounds. The interest of these compounds arises due to their biological properties as well as their chelating properties towards metal ions [2–4].

It is well known that several compounds containing an N*–N*–S* tridentate ligand system, e.g. α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones (α-HCATSs) and 2,2′-dipyridyl-6-carbothiamide derivatives [5–11], exhibit tumour inhibitory activity, which is usually attributed to their ability to function as tridentate ligands, therefore, it has been showed that complexation of these compounds results in derivatives that are potent cytotoxic agents [12].

Our previous studies described the synthesis of 2-quinolylhydrazones and *bis*-2-quinolylhydrazones, containing, respectively, the N*–N*–N*, N*–N*–O* tridentate systems and the N*–N*–N*–N* tetradentate system, which were endowed with antitumoral activity [4,13].

Therefore, on account of the above, as further development of our researches, in an attempt to obtain antitumoral agents, we have been prompted to prepare the new compounds **1** and **2**, with an N*–N*–S* tridentate ligand system, that might be of further interest as potential antitumoral and chelating agents.

The title compounds were evaluated for anticancer and anti-HIV activities; on account of biological profile of quinolylhydrazones previously synthesized [14–20], the new derivatives **1** and **2** were tested in vitro for antimicrobial activity in order to evaluate their antibacterial and antifungal properties.

2. Chemistry

The heterocyclichydrazones **1** and **2** were prepared through the synthetic pathway shown in Scheme 1, according to previously described procedure [3] by reacting equimolar

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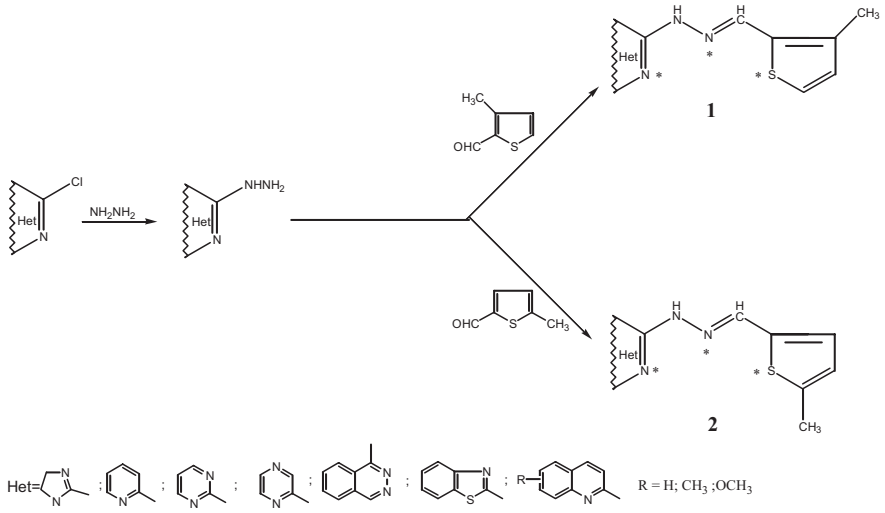


Scheme 1. Synthetic pathway of heterocyclichydrazones **1** and **2**

amounts of properly α-(*N*)-heterocyclichydrazine and 3- or 5-methylthiophene-2-carboxaldehyde in EtOH.

All the synthesized compounds were isolated in satisfactory yields (60–80%) and their chemical structures were consistent with both analytical and spectroscopic data (IR and ^{1}H NMR). The assignment of chemical shifts to corresponding protons was exactly attributed by using NOE and NOESY experiments.

In the IR spectra of hydrazones **1** and **2**, a weak band between 3200 and 3340 cm^{-1} due to the stretching vibration of the NH group was observed. The ^{1}H NMR showed, among others, a singlet between 2.29 and 2.52 ppm (δ) attributable to the methyl group of thiophene ring and two distinct doublets at the range 6.73–6.98 and 6.92–7.09 ppm (δ) attributable to 4,5 thiophene H (cps **1**) and to 3,4 thiophene H (cps **2**), respectively.

Physicochemical properties are summarized in Table 1; IR and ^{1}H NMR of heterocyclichydrazones are reported in Table 2.

3. Results and discussion

3.1. Anticancer activity

The results of in vitro anticancer evaluation are illustrated in Tables 3 and 4, where for each compound the growth inhibitory power (GI_{50}) and the mean graph midpoints (MG-MID) values are considered, obtained by averaging the individual values for each cell line.

Generally, the susceptible tumours were inhibited by 10^{-5} M concentration of the tested compounds and in some cases also by micromolar concentrations.

From the reported data, it appears that a number of the title compounds possess antiproliferative activity. The most potent were the 5-methylthiophene-2-carboxaldehyde hydrazones **2**, among these compounds **2f–k** exhibited inhibitory activity against most subpanel cell lines. The most active compound of the series was the hydrazone **2i**, which proved to be significantly effective against the majority of the cell lines displaying GI_{50} values between 1.63 and 26.5 μM (Table 3). Furthermore, **2i** resulted more potent (MG-MID 7.24 μM) than 5-fluorouracil, which is used in treating colorectal cancer [21], and melphalan, an established drug against leukaemia [22], whose MG-MID values towards all cell lines were 27.3 and 19.1 μM, respectively [23]. Compound **2g** showed the best activity against breast cancer MDA-MB-231/ATCC, leukaemic cell line MOLT-4, ovarian cancer OVCAR-3 with GI_{50} 0.439, 1.42 and 2.65 μM, respectively. Concerning compound **2h**, the most sensitive cell lines resulted in leukaemia, non-small cell lung, colon, renal cancer and melanoma, against which the title compound showed GI_{50} in the range 3.61–19.7, 3.26–15.70, 3.51–6.09, 3.36–17.10 and 4.52–19.0 μM, respectively.

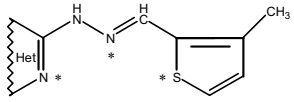
Among the 3-methylthiophene-2-carboxaldehyde hydrazones, compounds **1f,g,j,k** were cytotoxic in many of the subpanel cell lines. It is interesting to note that **1g**, similar to **2g**, was highly cytotoxic against MDA-MB-231/ATCC (breast cancer) (GI_{50} 0.636 μM). Compounds **1b** and **1c** showed GI_{50} 1.71 and 0.74 μM towards breast cancer MCF7 cell line, respectively.

The most sensitive cell line were MCF7 (breast cancer) and IGROVI (ovarian cancer), against which the compounds **1a–l** displayed GI_{50} in the range 0.741–55.5 and 1.36–40.1 μM, respectively.

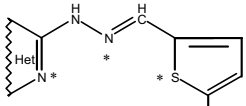
If we take into consideration the MG-MID, whose average values against various cancer types were given (Tables 3 and 4), also the compounds **1g,j,k** with MG-MID 11.0, 16.2, and 10.5 μM and **2f–h,j,k** with MG-MID 20.9, 12.3, 7.76, 18.2, 11.5 μM values, respectively, proved to be of interest.

These results allow us to make some observations about structure/activity relationships. Derivatives **2**, carrying a 5-methyl on the thiophene nucleus resulted more active than derivatives **1**, with a 3-methyl on the thiophene. Greater

Table 1
Physicochemical data and molecular formula of derivatives **1** and **2**



1



2

Compound	Heterocycle	Mp (°C)	Cryst. solvent	Molecular formula	Anal.
1a	2-Imidazolinyl	198-200	EtOH-H_2O	$C_9H_{12}N_4S$	C,H,N
1b	2-Pyridyl	132-135	EtOH-petr.et.[a]	$C_{11}H_{11}N_3S$	C,H,N
1c	2-Pyrimidyl	208-211	EtOH-H_2O	$C_{10}H_{10}N_4S$	C,H,N
1d	2-Pyrazinyl	183-185	EtOH-H_2O	$C_{10}H_{10}N_4S$	C,H,N
1e	2-Benzothiazolyl	210-212	EtOH	$C_{13}H_{11}N_3S_2$	C,H,N
1f	2-Quinolyl	139-141	Acetone-H_2O	$C_{15}H_{13}N_3S$	C,H,N
1g	4-Methyl-2-quinolyl	160-161	Acetone-H_2O	$C_{16}H_{15}N_3S$	C,H,N
1h	4,6-Dimethyl-2-quinolyl	217-218	Acetone	$C_{17}H_{17}N_3S$	C,H,N
1i	4,8-Dimethyl-2-quinolyl	208-209	EtOH	$C_{17}H_{17}N_3S$	C,H,N
1j	4-Methyl-6-methoxy-2-quinolyl	195-197	EtOH	$C_{17}H_{17}N_3OS$	C,H,N
1k	4-Methyl-7-methoxy-2-quinolyl	161-162	EtOH-H_2O	$C_{17}H_{17}N_3OS$	C,H,N
1l	1-Phthalazinyl	153-156	EtOH	$C_{14}H_{12}N_4S$	C,H,N
2a	2-Imidazolinyl	167-168	EtOH	$C_9H_{12}N_4S$	C,H,N
2b	2-Pyridyl	170-173	EtOH	$C_{11}H_{11}N_3S$	C,H,N
2c	2-Pyrimidyl	191-193	EtOH-H_2O	$C_{10}H_{10}N_4S$	C,H,N
2d	2-Pyrazinyl	198-200	EtOH-H_2O	$C_{10}H_{10}N_4S$	C,H,N
2e	2-Benzothiazolyl	195-197	EtOH	$C_{13}H_{11}N_3S_2$	C,H,N
2f	2-Quinolyl	166-167	EtOH	$C_{15}H_{13}N_3S$	C,H,N
2g	4-Methyl-2-quinolyl	167–168	Acetone-H_2O	$C_{16}H_{13}N_3S$	C,H,N
2h	4,6-Dimethyl-2-quinolyl	160-162	EtOH	$C_{17}H_{17}N_3S$	C,H,N
2i	4,8-Dimethyl-2-quinolyl	149-150	Acetone-H_2O	$C_{17}H_{17}N_3S$	C,H,N
2j	4-Methyl-6-methoxy-2-quinolyl	137-139	EtOH-H_2O	$C_{17}H_{17}N_3OS$	C,H,N
2k	4-Methyl-7-methoxy-2-quinolyl	190-192	EtOH-H_2O	$C_{17}H_{17}N_3OS$	C,H,N
2l	1-Phthalazinyl	203-204	EtOH	$C_{14}H_{12}N_4S$	C,H,N

[a] Petroleum ether, bp 60-80 (°C).

effectiveness was also observed for quinoline derivatives, especially those containing methyl and methoxy substituents on quinoline nucleus.

Several chelating agents have the enzyme ribonucleotide reductase (RR) as the main intracellular target. RR reduces ribonucleotides to provide deoxyribonucleotides which are building blocks for the synthesis of DNA. By analogy with the thiosemicarbazones, it might be expected that our hydrazones are directed at the non-heme iron subunit of RR destroying the tyrosil free radical, instead of inhibit RR by sequestering the iron (as Desferal does) [24–27].

None of the compounds exhibited anti-HIV activity.

In conclusion, between the tested hydrazone derivatives the methylthiophene-2-carboxaldheyde hydrazones of quinoline demonstrated antiproliferative properties that they warrant further investigation as potential anticancer agents.

The cytotoxic profile suggests that they have potential against the growth of a number of cancer cell lines where effective agents are currently sought.

Further studies are in progress to define the exact mechanism of action and to evaluate the chelating properties of the above derivatives.

3.2. Antimicrobial activity

Heterocyclichydrazones **1** and **2** were tested in vitro for antibacterial and antifungal activity. The results obtained showed that the majority of these compounds did not show any activity against the reference strains at the highest concentration tested (1000 μg/ml). The most susceptible microorganisms were found to be the two Gram positives *Staphylococcus epidermidis* and *Bacillus cereus*, the yeasts *Saccharomyces cerevisiae* and *Candida albicans* and the fungus *Aspergillus fumigatus*. All Gram negatives were resistant to all compounds at the highest concentration tested. The following compounds only possess some activity: **2h** with a MIC of 100 μg/ml against *B. cereus, C. albicans*, and 250 μg/ml against *S. epidermidis*, followed by compounds **1g** and **1k** with a MIC of 250 μg/ml against *B. cereus* and *C. albicans*.

Moderate antifungal properties, particularly in inhibiting *A. fumigatus* growth, were exhibited by some of the heterocyclichydrazones (Table 5). At the concentration of 100 μg/ml, certain compounds (**1f,g,h,j,k** and **2f,g,h**) completely inhibited the growth of *A. fumigatus*. Compound **2g** was the most effective, being able to partially inhibit (66%) the growth of the fungus.

Table 2
^{1}H NMR in ppm and IR spectral data of compound **1** and **2**

Compound	^{1}H NMR (δ)	IR (cm^{-1})
1a	($CDCl_3$): 2.29 (s, 3H, CH_3); 3.58 (s, 4H, 2 × CH_2 imidazoline); 5.16 and 5.64 (2br s, 2H, 2 × NH, D_2O exchangeable); 6.79 (d, 1H, H-4 thiophene, J = 5.1); 7.11 (d, 1H, H-5 thiophene, J = 5.1); 8.32 (s, 1H, CH=N)	3430 (NH)
1b	($CDCl_3$): 2.33 (s, 3H, CH_3); 6.73–6.83 (m, 2H, H-4 thiophene and 1H pyridine); 7.17 (d, 1H, H-5 thiophene, J = 5.0); 7.33 (d, 1H, H-3 pyridine, J = 8.0); 7.60 (td, 1H, H-4 pyridine, J = 7.0, J = 1.8); 7.98 (s, 1H, CH=N); 8.13 (d, 1H, H-6 pyridine, J = 4.32); 9.39 (br s, 1H, NH, D_2O exchangeable)	3220 (NH)
1c	($CDCl_3$): 2.32 (s, 3H, CH_3); 6.73 (t, 1H, H-5 pyrimidine, J = 4.9); 6.80 (d, 1H, H-4 thiophene, J = 5.0), 7.22 (d, 1H, H-5 thiophene, J = 5.0); 8.19 (s, 1H, CH=N); 8.45 (d, 2H, H-(4,6) pyrimidine, J = 4.9); 9.05 (br s, 1H, NH, D_2O exchangeable)	3230 (NH)
1d	($CDCl_3$): 2.37 (s, 3H, CH_3); 6.86 (d, 1H, H-4 thiophene, J = 5.0), 7.25 (d, 1H, H-5 thiophene, J = 5.0); 7.98–8.06 (m, 3H, CH=N and H-(5,6) pyrazine); 8.49 (br s, 1H, NH, D_2O exchangeable); 8.75 (s, 1H, H-3 pyrazine)	3200 (NH)
1e	(DMSO-d_6): 2.36 (s, 3H, CH_3); 6.98 (d, 1H, H-4 thiophene, J = 5.0); 7.08–7.34 (m, 2H, H-(6,5) benzothiazole); 7.41 (d, 1H, H-4 benzothiazole, J = 7.7); 7.54 (d, 1H, H-5 thiophene, J = 5.0); 7.76 (d, 1H, H-7 benzothiazole, J = 7.7); 8.38 (s, 1H, CH=N); 12.05 (br s, 1H, NH, D_2O exchangeable)	3220 (NH)
1f	(DMSO-d_6): 2.33 (s, 3H, CH_3); 6.96 (d, 1H, H-4 thiophene, J = 4.9); 7.30 (td, 1H, H-6 quinoline, J = 7.0, J = 1.8); 7.45–7.67 (m, 4H, H-5 thiophene and H-(3,4,7) quinoline); 7.78 (d, 1H, H-5 quinoline, J = 7.8); 8.18 (d, 1H, H-8 quinoline, J = 9.0); 8.37 (s, 1H, CH=N); 11.24 (br s, 1H, NH, D_2O exchangeable)	3195 (NH)
1g	(DMSO-d_6): 2.34 (s, 3H, CH_3 thiophene); 2.67 (s, 3H, CH_3 quinoline); 6.97 (d, 1H, H-4 thiophene, J = 5.0); 7.34–7.37 (m, 2H, H-(3,6) quinoline); 7.49 (d, 1H, H-5 thiophene, J = 5.0); 7.61–7.64 (m, 2H, H-(7,8) quinoline); 7.92 (d, 1H, H-5 quinoline, J = 8.1); 8.37 (s, 1H, CH=N); 11.16 (s, 1H, NH, D_2O exchangeable)	3190 (NH)
1h	(DMSO-d_6): 2.32 (s, 3H, CH_3 thiophene); 2.47 (s, 3H, 6-CH_3 quinoline); 2.62 (s, 3H, 4-CH_3 quinoline); 6.94 (d, 1H, H-4 thiophene, J = 4.9); 7.31 (s, 1H, H-3 quinoline); 7.40–7.46 (m, 2H, H-5 thiophene and H-7 quinoline); 7.54 (d, 1H, H-8 quinoline, J = 8.3); 7.68 (u d, 1H, H-5 quinoline); 8.33 (s, 1H, CH=N); 11.04 (s, 1H, NH, D_2O exchangeable)	3200 (NH)
1i	(DMSO-d_6): 2.32 (s, 3H, CH_3 thiophene); 2.61 (s, 3H, 8-CH_3 quinoline); 2.64 (s, 3H, 4-CH_3 quinoline); 6.95 (d, 1H, H-4 thiophene, J = 5.0); 7.21 (t, 1H, H-6 quinoline, J = 7.5); 7.36 (s, 1H, H-3 quinoline); 7.44–7.48 (m, 2H, H-5 thiophene and H-7 quinoline); 7.75 (d, 1H, H-5 quinoline, J = 8.2); 8.47 (s, 1H, CH=N); 11.00 (s, 1H, NH, D_2O exchangeable)	3210 (NH)
1j	(DMSO-d_6): 2.31 (s, 3H, CH_3 thiophene); 2.63 (s, 3H, CH_3 quinoline); 3.89 (s, 3H, OCH_3); 6.93 (d, 1H, H-4 thiophene, J = 5.1); 7.24–7.32 (m, 3H, H-(3,5,7) quinoline); 7.44 (d, 1H, H-5 thiophene, J = 5.1); 7.57 (d, 1H, H-8 quinoline, J = 8.5); 8.31 (s, 1H, CH=N); 10.96 (s, 1H, NH, D_2O exchangeable)	3195 (NH)
1k	(DMSO-d_6): 2.32 (s, 3H, CH_3 thiophene); 2.60 (s, 3H, CH_3 quinoline); 3.88 (s, 3H, OCH_3); 6.95 (d, 1H, H-4 thiophene, J = 5.0); 6.98–7.03 (m, 2H, H-(3,6) quinoline); 7.17 (u d, 1H, H-8 quinoline); 7.46 (d, 1H, H-5 thiophene, J = 5.0); 7.79 (d, 1H, H-5 quinoline, J = 8.9); 8.35 (s, 1H, CH=N); 11.00 (s, 1H, NH, D_2O exchangeable)	3200 (NH)
1l	($CDCl_3$): 2.39 (s, 3H, CH_3); 6.86 (d, 1H, H-4 thiophene, J = 5.0); 7.26 (d, 1H, H-5 thiophene, J = 5.0); 7.46–7.51 and 7.53–7.66 (2m, 3H phthalazine); 7.83 (s, 1H, H-4 phthalazine); 8.36 (u td, 1H, H-(6,7) phthalazine, J = 4.5); 8.69 (s, 1H, CH=N); 10.56 (br s, 1H, NH, D_2O exchangeable)	3340 (NH)
2a	($CDCl_3$): 2.48 (s, 3H, CH_3); 3.59 (s, 4H, 2 × CH_2 imidazoline); 4.46 and 5.61 (2br s, 2H, 2 × NH, D_2O exchangeable); 6.68 (d, 1H, H-4 thiophene, J = 3.8); 7.10 (d, 1H, H-3 thiophene, J = 3.8); 7.57 (s, 1H, CH=N)	3200 (NH)
2b	($CDCl_3$): 2.49 (s, 3H, CH_3); 6.67 (d, 1H, H-4 thiophene, J = 3.0); 6.76 (t, 1H pyridine, J = 6.0); 6.92 (d, 1H, H-3 thiophene, J = 3.0); 7.31 (d, 1H, H-3 pyridine, J = 8.4); 7.60 (td, 1H pyridine, J = 7.2, J = 1.3); 7.86 (s, 1H, CH=N); 8.12 (d, 1H, H-6 pyridine, J = 4.7); 9.29 (br s, 1H, NH, D_2O exchangeable)	3210 (NH)
2c	($CDCl_3$): 2.48 (s, 3H, CH_3); 6.67 (d, 1H, H-4 thiophene, J = 3.7); 6.73 (t, 1H, H-5 pyrimidine, J = 4.7); 7.02 (d, 1H, H-3 thiophene, J = 3.7); 8.04 (s, 1H, CH=N); 8.45 (d, 2H, H-(4,6) pyrimidine, J = 4.7); 8.90 (br s, 1H, NH, D_2O exchangeable)	3240 (NH)
2d	($CDCl_3$): 2.50 (s, 3H, CH_3); 6.69 (d, 1H, H-4 thiophene, J = 3.7); 6.98 (d, 1H, H-3 thiophene, J = 3.7); 7.85 (s, 1H, CH=N); 8.00–8.04 (m, 2H, H-(5,6) pyrazine); 8.29 (br s, 1H, NH, D_2O exchangeable); 8.71 (s, 1H, H-3 pyrazine)	3200 (NH)
2e	(DMSO-d_6): 2.50 (s, 3H, CH_3); 6.84 (d, 1H H-4 thiophene, J = 3.6); 7.11 (t, 1H, H-6 benzothiazole, J = 7.7); 7.22 (d, H, H-3 thiophene, J = 3.6); 7.27–7.47 (m, 2H, H-(5,4) benzothiazole,); 7.76 (dd, 1H, H-7 benzothiazole, J = 7.7, J = 1.8); 8.26 (s, 1H, CH=N); 12.08 (br s, 1H, NH, D_2O exchangeable)	3220 (NH)
2f	(DMSO-d_6): 2.49 (s, 3H, CH_3); 6.81 (d, 1H, H-4 thiophene, J = 3.4); 7.12 (d, 1H, H-3 thiophene, J = 3.4); 7.26–7.34 (m, 1H, H-6 quinoline); 7.48 (d, 1H, H-3 quinoline, J = 9.0); 7.59–7.66 (m, 2H, H-(7,8) quinoline); 7.78 (d, 1H, H-5 quinoline, J = 7.9); 8.19 (d, 2H, H-4 quinoline and CH=N); 11.28 (s, 1H, NH, D_2O exchangeable)	3190 (NH)
2g	(DMSO-d_6): 2.51 (s, 3H, CH_3 thiophene); 2.67 (s, 3H, CH_3 quinoline); 6.82 (d, 1H, H-4 thiophene, J = 3.6); 7.13 (d, 1H, H-3 thiophene, J = 3.6); 7.34–7.36 (m, 2H, H-(3,6) quinoline); 7.61–7.63 (m, 2H, H-(7,8) quinoline); 7.92 (br d, 1H, H-5 quinoline, J = 8.1); 8.21 (s, 1H, CH=N); 11.19 (br s, 1H, NH, D_2O exchangeable)	3200 (NH)
2h	(DMSO-d_6): 2.47 (s, 3H, CH_3 thiophene); 2.48 (s, 3H, 6-CH_3 quinoline); 2.63 (s, 3H, 4-CH_3 quinoline); 6.80 (d, 1H, H-4 thiophene, J = 4.8); 7.09 (d, 1H, H-3 thiophene, J = 4.8); 7.29 (s, 1H, H-3 quinoline); 7.40–7.54 (m, 2H, H-(7,8) quinoline); 7.68 (u d, 1H, H-5 quinoline); 8.16 (s, 1H, CH=N); 11.07 (s, 1H, NH, D_2O exchangeable)	3195 (NH)
2i	(DMSO-d_6): 2.48 (s, 3H, CH_3 thiophene); 2.61 (s, 3H, 8-CH_3 quinoline); 2.64 (s, 3H, 4-CH_3 quinoline); 7.09 (d, 1H, H-4 thiophene, J = 3.8); 7.20 (t, 1H, H-6 quinoline, J = 7.4); 7.34 (s, 1H, H-3 quinoline); 7.46 (d, 1H, H-7 quinoline J=6.9); 7.69 (d, 1H, H-3 thiophene, J = 3.8); 7.74 (d, 1H, H-5 quinoline, J=7.9); 8.26 (s, 1H, CH=N); 11.05 (br s, 1H, NH, D_2O exchangeable)	3200 (NH)

(continued on next page)

Table 2
(continued)

Compound	^{1}H NMR (δ)	IR (cm^{-1})
2j	(DMSO-d_6): 2.48 (s, 3H, CH_3 thiophene); 2.64 (s, 3H, CH_3 quinoline); 3.89 (s, 3H, OCH_3 quinoline); 6.79 (d, 1H, H-4 thiophene, J = 3.0); 7.08 (d, 1H, H-3 thiophene, J = 3.0); 7.24–7.31 (m, 3H, H-(3,5,7) quinoline); 7.56 (d, 1H, H-8 quinoline, J = 9.1); 8.15 (s, 1H, CH=N); 11.00 (s, 1H, NH, D_2O exchangeable)	3195 (NH)
2k	(DMSO-d_6): 2.49 (s, 3H, CH_3 thiophene); 2.60 (s, 3H, CH_3 quinoline); 3.89 (s, 3H, OCH_3); 6.80 (d, 1H, H-4 thiophene, J = 3.5); 6.95 (dd, 1H, H-6 quinoline, J = 8.9, J = 2.3); 7.02 (u d, 1H, H-8 quinoline); 7.11 (d, 1H, H-3 thiophene, J = 3.5); 7.18 (s, 1H, H-3 quinoline); 7.79 (d, 1H, H-5 quinoline, J = 8.9); 8.21 (s, 1H, CH=N); 11.05 (br s, 1H, NH, D_2O exchangeable)	3230 (NH)
2l	($CDCl_3$): 2.52 (s, 3H, CH_3); 6.73 (d, 1H, H-4 thiophene, J = 3.6); 7.10 (d, 1H, H-3 thiophene, J = 3.6); 7.50 (u td, 1H phthalazine, J = 4.3); 7.61–7.66 (m, 2H phthalazine); 7.82 (s, 1H, H-4 phthalazine); 8.35 (u td, 1H phthalazine, J = 4.5); 8.53 (s, 1H, CH=N); 10.50 (br s, 1H, NH, D_2O exchangeable)	3340 (NH)

The results indicated that the above compounds **1** and **2** are generally weakly active or completely inactive against Gram-negative and Gram-positive bacteria, whereas they showed a moderate antifungal activity against *C. albicans* and *A. fumigatus* (MIC 100 μg/ml). The most interesting antimicrobial properties were also displayed by the quinoline derivatives, confirming the favourable influence of this moiety to the activity of this class of compounds.

4. Experimental protocols

4.1. Chemistry

Melting points were determined on a Köfler blok or on a Büchi 510 apparatus and are uncorrected. Elemental analyses were performed on a Perkin–Elmer 240 C Elemental Analyzer by Laboratories of Dipartimento Farmaco Chimico Tecnologico, Università di Siena (Italy) and the data for C, H, and N are within ±0.40% of the theoretical values. IR spectra were determined in nujol mull on a Perkin–Elmer FT-IR 1600 spectrophotometer. ^{1}H NMR spectra were recorded on a Varian VRX 300 or AC 200 Bruker instruments. The chemical shifts (δ) are relative to Me_4Si used as internal standard; the following abbreviations were used: s, singlet; d, doublet; t, triplet; dd, double doublet; td, triplet of doublets; m, multiplet; br, broad; u, unresolved. The coupling constants J were in Hertz. TLC on silica gel plates (Merck, 60, F_{254}) was used for purity check and reaction monitoring. Column chromatography on silica gel (Merck, 70–230 mesh and 230–400 mesh ASTH for flash chromatography) was applied when necessary to isolate and purify the reactions products.

2-Hydrazinopyrimidine and 2-hydrazinopyrazine required for the preparation of compounds **1c,d** and **2c,d** were resynthesized and the physical and spectral properties are consistent with literature values [28,29]. The 2-quinolylhydrazines employed in the preparation of derivatives **1g–k** and **2g–k** were prepared from the corresponding 2-chloroquinoline and hydrazine hydrate as previously described by us [30]. The other heterocyclichydrazines and the carbonilic compounds were of the best available commercial quality and were used without further purification.

*4.1.1. General procedure for the preparation of 3- and 5-methylthiophene-2-carboxaldehyde α-(N)-heterocyclichydrazones **1** and **2***

The title compounds were prepared by refluxing the appropriate α-(*N*)-heterocyclichydrazine and 3- or 5-methylthiophene-2-carboxaldehyde in EtOH for 1–2 h. After cooling and diluting with H_2O the heterocyclichydrazones **1** and **2** precipitated, generally as yellow solids.

The products were filtered off and recrystallized from a suitable solvent (Table 1) (60–80% yields). When the suitable heterocyclichydrazine hydrochloride was employed, an equimolar amount of NaOAc was added to the mixture to liberate the free base for reaction.

4.2. Biological evaluation

4.2.1. In vitro anticancer and anti-HIV activity

The synthesized compounds **1** and **2** were evaluated for antitumour and anti-HIV activity at the National Cancer Institute (NCI) of Bethesda, MD, USA, following the known in vitro disease-oriented antitumour screening program against a panel of ~60 human tumour cell lines derived from various cancer types (lung, colon, melanoma, brain, prostate, renal, ovarian and leukaemia) and the anti-HIV drug testing system [31,32]. The percentage growth was evaluated spectrophotometrically versus controls non-treated with test agents. A 48 h continuous exposure protocol was followed and a sulphorhodamine B (SRB) protein assay was used to estimate cell viability of growth. The activity was deduced from the dose–response curve on the basis of the data provided by NCI.

4.2.2. Antimicrobial assay

All compounds **1** and **2** were tested in vitro for antibacterial activity against Gram-positive, Gram-negative bacteria and fungi. The reference strains used in all antimicrobial assays were: *Escherichia coli* ATCC 25922, *E. coli* O157:H7 ATCC 35150, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Yersinia enterocolitica* ATCC 9610, *B. cereus* ATCC 11778, *Listeria monocytogenes* ATCC 7644, *C. albicans* ATCC 10231, *S. cerevisiae* ATCC 9763 and *A. fumigatus* ATCC 1022.

Table 3
Inhibition of in vitro tumour cell growth by heterocyclichydrazones **1a–l**

Cell line	Cytotoxicity (GI_{50} in μM) [a,b,c]											
	1a	**1b**	**1c**	**1d**	**1e**	**1f**	**1g**	**1h**	**1i**	**1j**	**1k**	**1l**
Leukaemia												
CCRF-CEM	>100	40.5	>100	>100	15.4	27.6	17.2	>100	ND	19.5	39.8	53.2
HL-60 (TB)	ND	ND	>100	35.9	ND	11.8	12.2	28.4	3.78	18.3	46.8	ND
K-562	>100	43.1	>100	>100	14.7	59.9	9.05	>100	ND	38.0	ND	>100
MOLT-4	>100	32.8	>100	78.8	16.2	8.03	9.22	>100	ND	18.7	13.5	44.8
RPMI-8226	>100	19.3	>100	>100	19.4	48.8	3.28	ND	ND	9.37	5.69	81.9
SR	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	22.8	ND
Non-small cell lung cancer												
A549/ATCC	>100	7.40	>100	>100	16.3	7.43	9.56	84.0	>100	20.5	13.3	96.3
EKVX	>100	15.2	>100	63.5	19.3	20.3	6.82	>100	>100	18.3	ND	>100
HOP-62	>100	>100	>100	>100	8.99	19.8	22.3	29.3	>100	16.4	15.2	>100
HOP-92	>100	49.3	>100	>100	13.4	18.0	13.7	35.0	>100	6.07	16.1	>100
NCI-H226	>100	>100	>100	>100	19.4	35.9	16.0	>100	>100	18.8	10.0	>100
NCI-H23	>100	70.6	>100	>100	31.8	51.1	11.2	>100	>100	10.1	14.7	>100
NCI-H322M	>100	>100	>100	>100	21.8	23.3	18.1	66.5	>100	20.6	0.217	>100
NCI-H460	ND	ND	ND	>100	ND	ND	ND	ND	ND	13.2	ND	ND
NCI-H522	ND	ND	ND	>100	ND	ND	ND	ND	ND	ND	10.2	ND
Colon cancer												
COLO 205	>100	71.2	>100	>100	35.1	27.7	5.91	>100	>100	13.6	3.91	>100
HCC-2998	>100	99.6	>100	>100	63.2	24.1	14.0	>100	>100	17.5	12.6	>100
HCT-116	>100	43.8	>100	>100	11.2	28.1	12.1	42.5	ND	11.6	4.09	>100
HCT-15	>100	60.1	>100	>100	50.5	24.8	11.4	>100	ND	14.1	6.03	>100
HT29	>100	32.8	>100	76.9	34.6	12.5	3.61	5.49	4.14	8.66	2.81	>100
KM12	>100	35.7	32.3	99.5	24.2	32.1	15.6	>100	>100	22.5	8.06	>100
SW-620	>100	74.2	>100	>100	25.0	85.2	17.2	>100	>100	20.8	21.6	>100
CNS cancer												
SF-268	>100	>100	>100	>100	21.4	32.1	17.6	>100	>100	16.5	17.4	93.4
SF-295	55.4	>100	>100	>100	10.6	11.1	3.04	>100	9.34	7.23	14.7	>100
SF-539	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	14.3	ND
SNB-19	>100	>100	>100	>100	12.8	39.3	16.6	48.2	>100	29.0	21.7	>100
SNB-75	>100	50.2	>100	>100	20.5	32.9	13.8	38.7	>100	15.0	12.1	>100
U251	>100	76.6	>100	>100	13.5	49.0	14.0	92.7	>100	15.4	17.9	58.3
Melanoma												
LOX IMVI	>100	37.2	>100	76.4	23.9	24.1	12.0	>100	ND	32.9	7.59	97.0
MALME-3M	>100	34.9	>100	ND	ND	ND	ND	ND	ND	31.3	6.95	ND
M14	>100	54.0	>100	>100	28.0	29.1	8.62	>100	>100	25.3	11.8	>100
SK-MEL-2	>100	34.3	>100	>100	26.2	25.2	17.9	>100	>100	63.1	15.6	14.1
SK-MEL-28	>100	98.6	>100	>100	25.1	80.4	14.5	>100	>100	50.5	14.8	>100
SK-MEL-5	>100	25.9	>100	>100	21.0	3.39	2.17	28.8	ND	25.5	6.23	96.4
UACC-257	>100	54.3	>100	>100	20.4	6.13	6.78	>100	>100	32.3	10.1	>100
UACC-62	>100	73.9	>100	>100	27.8	25.1	12.5	>100	>100	27.1	15.9	>100
Ovarian cancer												
IGROVI	>100	16.9	>100	40.1	11.0	8.27	1.36	37.2	1.60	32.4	5.82	15.9
OVCAR-3	>100	47.5	>100	>100	12.9	10.4	3.94	>100	>100	45.8	2.98	>100
OVCAR-4	>100	76.2	>100	>100	19.7	29.7	14.6	34.9	>100	99.5	20.9	>100
OVCAR-5	>100	>100	>100	94.0	40.9	>100	24.3	>100	>100	61.3	13.5	>100
OVCAR-8	>100	66.1	>100	27.3	23.0	41.4	14.7	>100	>100	>100	13.4	>100
SK-OV-3	>100	>100	>100	>100	12.5	16.5	17.3	21.1	84.4	32.1	17.1	70.7

(continued on next page)

Table 3
(continued)

Cell line	Cytotoxicity (GI_{50} in μM) [a,b,c]											
	1a	**1b**	**1c**	**1d**	**1e**	**1f**	**1g**	**1h**	**1i**	**1j**	**1k**	**1l**
Renal cancer												
786-0	>100	68.6	>100	>100	12.3	30.7	16.2	29.1	24.4	27.6	ND	90.1
A498	>100	55.0	>100	62.3	2.35	7.76	10.4	>100	>100	26.9	ND	>100
ACHN	>100	74.7	>100	>100	14.0	15.1	12.9	>100	>100	56.2	21.7	>100
CAKI-1	>100	>100	>100	>100	15.5	17.4	16.5	>100	>100	>100	ND	>100
RXF 393	>100	>100	>100	>100	6.27	30.6	20.8	61.8	ND	62.6	14.9	15.8
SN12C	>100	>100	>100	>100	13.3	>100	15.2	>100	>100	48.2	7.12	>100
TK-10	>100	57.3	>100	>100	13.9	19.9	14.6	29.1	>100	28.1	17.1	>100
UO-31	>100	71.4	>100	>100	20.8	26.7	10.4	>100	ND	65.1	17.1	21.9
Prostate cancer												
PC-3	>100	39.5	>100	>100	14.3	19.6	14.7	35.0	4.89	38.7	12.9	34.3
DU-145	>100	55.6	>100	>100	26.8	48.8	12.6	>100	>100	44.5	6.68	>100
Breast cancer												
MCF7	>100	1.71	0.741	3.53	11.5	11.4	1.56	25.3	2.75	55.5	11.4	37.0
NCI/ADR-RES	>100	45.3	>100	>100	21.4	30.5	13.9	>100	>100	55.1	10.0	>100
MDA-MB-231/ATCC	>100	50.0	>100	>100	12.8	ND	0.636	14.7	ND	16.1	1.43	>100
HS 578T	>100	>100	>100	>100	23.2	37.6	16.7	83.2	>100	55.3	12.5	>100
MDA-MB-435	>100	50.6	>100	>100	70.2	56.5	14.0	>100	>100	43.9	16.7	>100
MDA-N	>100	50.6	>100	>100	34.8	43.3	14.5	>100	>100	39.4	14.3	>100
BT-549	>100	>100	>100	>100	71.4	34.4	21.0	>100	>100	32.2	13.1	>100
T-47D	>100	60.7	>100	>100	48.8	>100	17.8	>100	>100	>100	7.77	>100
MG-MID [d]	>100	52.5	89.1	87.1	19.5	19.5	11.0	66.1	61.7	16.2	10.5	79.4

[a] Data obtained from NCI's in vitro disease-oriented tumour cells screen.
[b] GI_{50} is the molar concentration causing 50% growth inhibition of tumour cells. Compounds with GI_{50}>100 μM are considered inactive.
[c] ND, not determined.
[d] Mean panel values (mean graph midpoints MG-MID) of the response parameter were obtained by averaging the individual values for each cell line.

Bacterial strains were grown in nutrient broth (NB) and incubated at 37 °C. Yeasts were grown in sabouraud broth (SB) and incubated at 25 °C. Moulds were grown in potato dextrose broth (PDB) and incubated at 25 °C. Minimum inhibitory concentrations (MIC) against bacteria and yeasts were determined using a broth microdilution method according to Carson and Riley [33].

Stock solutions of each compound were prepared in dimethylsulphoxide (DMSO) and serial doubling dilutions were performed in a 96-well microtitre plate (Nunc, Copenhagen, Denmark) over the range of 1000–15.62 μg/ml.

Overnight broth cultures were prepared in NB or SB and adjusted so that the final concentration in each well following inoculation was approximately 5.0×10^5 cfu/ml. The concentration of each inoculum was confirmed using viable counts on tryptic soy agar (TSA) plates for bacteria and sabouraud dextrose agar (SDA) for yeasts.

The plates were incubated aerobically at 37 °C for 24 h and the MICs determined. Microbial growth was indicated by the presence of turbidity and a "pellet" on the well bottom. MICs were determined presumptively as the first well, in ascending order, which did not produce a pellet. To confirm MICs, 10 μl of broth was removed from each well and inoculated on TSA or SDA plates. After aerobic incubation at 37 °C overnight, the number of surviving organisms was determined. The MIC was the lowest concentration which resulted in a significant decrease in inoculum viability (>90%). The antifungal assay was carried out according to Thompson [34].

Stock solutions of each compound were prepared in DMSO then diluted into PDB to reach final concentrations of 50 and 100 μg/ml. *A. fumigatus* spore suspension was adjusted to approximately 5×10^6 conidia per ml as determined by direct microscopic count.

Mycelia growth inhibition percentages were determined according to the formula of Ahmand and Branen [35]:

$$100 - \frac{\text{Mycelium in test medium (wt)}}{\text{Mycelium in control medium (wt)}} \times 100$$

Positive and negative growth controls were included in every test. A microbial susceptibility control test was performed with gentamicine for bacteria, with ketoconazole for yeasts and with amphotericin B for moulds.

All tests were conducted in triplicate and with three replication, and the modal MIC values were selected.

Table 4
Inhibition of in vitro tumour cell growth by heterocyclichydrazones **2a–l**

Cell line	Cytotoxicity (GI_{50} in μM) [a,b,c]											
	2a	**2b**	**2c**	**2d**	**2e**	**2f**	**2g**	**2h**	**2i**	**2j**	**2k**	**2l**
Leukaemia												
CCRF-CEM	ND	>100	>100	>100	>100	>100	13.1	5.11	4.42	28.0	4.69	>100
HL-60 (TB)	>100	59.0	ND	>100	>100	41.5	33.2	4.71	3.28	27.2	6.74	>100
K-562	41.0	>100	ND	65.1	31.5	16.9	6.99	3.99	3.46	14.0	4.38	>100
MOLT-4	>100	>100	ND	65.1	>100	9.55	1.42	5.76	3.21	12.9	8.45	>100
RPMI-8226	>100	>100	>100	>100	>100	14.2	3.91	3.61	6.66	11.2	4.32	>100
SR	>100	>100	ND	ND	ND	22.2	6.38	19.7	2.71	25.4	5.17	>100
Non-small cell lung cancer												
A549/ATCC	>100	>100	>100	>100	>100	14.3	16.5	6.78	6.31	>100	ND	ND
EKVX	>100	>100	>100	80.5	50.7	4.74	4.84	5.71	13.5	26.4	6.90	>100
HOP-62	>100	>100	>100	>100	26.3	20.8	15.7	15.7	11.8	20.1	16.5	>100
HOP-92	>100	>100	>100	>100	>100	19.0	27.2	4.44	6.40	13.9	10.8	>100
NCI-H226	79.5	>100	>100	38.7	>100	80.8	14.0	10.1	9.02	14.8	15.3	48.0
NCI-H23	>100	>100	>100	>100	>100	18.6	17.5	7.97	12.2	13.5	11.0	70.2
NCI-H322M	>100	>100	>100	>100	>100	14.6	7.11	3.26	1.76	12.5	14.2	94.8
NCI-H460	ND	>100	ND	ND	>100	ND	ND	ND	ND	ND	5.28	>100
NCI-H522	>100	>100	>100	83.0	>100	15.7	4.07	4.52	2.80	39.7	ND	ND
Colon cancer												
COLO 205	>100	>100	>100	>100	>100	12.9	4.71	3.79	6.98	19.4	3.72	>100
HCC-2998	>100	>100	>100	>100	55.6	15.3	15.3	4.69	6.38	16.0	7.70	53.0
HCT-116	>100	>100	>100	>100	>100	7.79	3.85	3.69	2.82	4.64	10.0	51.5
HCT-15	>100	>100	>100	>100	19.4	12.0	7.36	4.52	3.73	7.97	4.35	>100
HT29	>100	>100	>100	47.1	40.9	7.47	3.86	3.51	2.87	6.09	3.85	>100
KM12	>100	>100	>100	>100	49.1	32.3	16.4	4.76	7.31	19.7	5.39	21.4
SW-620	>100	>100	>100	>100	>100	23.2	21.7	6.09	8.15	25.1	11.2	>100
CNS cancer												
SF-268	>100	>100	>100	>100	49.8	60.1	26.1	11.6	6.74	20.1	38.4	>100
SF-295	>100	>100	>100	>100	40.9	16.4	15.1	7.94	7.10	16.9	12.6	45.5
SF-539	>100	>100	>100	>100	91.9	83.4	21.3	11.0	13.9	16.5	14.8	46.0
SNB-19	>100	>100	>100	>100	51.9	21.3	41.6	12.9	26.5	17.3	21.0	>100
SNB-75	>100	ND	>100	>100	46.4	>100	31.6	ND	11.9	24.3	30.8	>100
U251	>100	>100	>100	>100	81.2	21.1	16.8	9.50	10.6	21.1	11.1	53.5
Melanoma												
LOX IMVI	>100	>100	>100	>100	>100	17.1	10.3	4.52	1.63	16.3	6.25	37.5
MALME-3M	>100	>100	>100	>100	>100	14.9	5.75	12.8	15.1	30.9	16.0	57.5
M14	>100	>100	>100	>100	>100	16.8	13.7	10.2	4.32	16.6	11.7	45.5
SK-MEL-2	>100	>100	>100	>100	>100	>100	34.1	19.0	16.0	21.7	17.8	>100
SK-MEL-28	>100	>100	>100	>100	>100	17.2	20.3	6.76	22.4	26.5	ND	ND
SK-MEL-5	>100	>100	>100	>100	38.9	9.40	8.30	4.56	9.85	15.5	ND	ND
UACC-257	>100	>100	>100	>100	>100	6.01	7.54	5.56	18.7	17.2	14.8	>100
UACC-62	>100	>100	>100	>100	74.6	34.8	23.4	10.6	17.9	16.8	14.1	27.2
Ovarian cancer												
IGROVI	>100	>100	>100	>100	18.8	16.3	10.2	6.87	2.61	26.2	14.2	>100
OVCAR-3	>100	>100	>100	>100	35.0	5.59	2.65	6.23	8.02	19.6	4.00	>100
OVCAR-4	>100	>100	>100	>100	34.4	20.8	20.0	10.3	13.9	13.9	15.1	30.4
OVCAR-5	>100	>100	>100	>100	>100	27.3	15.6	10.1	9.92	15.7	17.1	>100
OVCAR-8	>100	>100	>100	>100	>100	26.7	23.2	12.9	10.4	19.5	14.7	82.2
SK-OV-3	>100	>100	>100	>100	58.8	30.2	19.3	18.3	16.4	22.9	20.3	70.7

(continued on next page)

Table 4
(continued)

Cell line	Cytotoxicity (GI_{50} in μM) [a,b,c]											
	2a	**2b**	**2c**	**2d**	**2e**	**2f**	**2g**	**2h**	**2i**	**2j**	**2k**	**2l**
Renal cancer												
786-0	>100	>100	>100	>100	66.5	18.8	18.8	3.36	2.73	13.0	11.2	>100
A498	>100	>100	>100	>100	ND	ND	ND	ND	ND	ND	19.4	>100
ACHN	>100	>100	>100	>100	>100	17.3	20.1	11.7	16.5	17.7	14.2	>100
CAKI-1	>100	>100	>100	>100	79.3	>100	11.4	17.1	15.8	23.0	15.8	>100
RXF 393	>100	>100	>100	>100	82.6	37.7	23.3	13.3	9.87	19.2	15.8	>100
SN12C	>100	>100	>100	42.5	12.6	13.0	13.1	12.7	9.07	17.9	13.8	>100
TK-10	>100	>100	>100	>100	69.3	22.1	30.8	8.97	14.7	21.6	14.2	27.1
UO-31	44.9	>100	>100	>100	51.7	>100	>100	9.81	5.80	17.7	12.8	>100
Prostate cancer												
PC-3	>100	>100	>100	>100	27.8	13.2	14.0	11.8	8.20	17.1	15.6	93.9
DU-145	>100	>100	ND	ND	>100	ND	ND	11.0	10.5	17.7	12.1	>100
Breast cancer												
MCF7	>100	60.8	>100	17.4	5.54	17.5	12.0	6.80	4.11	18.0	11.6	30.9
NCI/ADR-RES	>100	>100	>100	>100	>100	35.5	11.1	12.3	2.99	19.7	18.9	77.6
MDA-MB-231/ATCC	>100	>100	>100	96.2	24.7	15.5	0.439	16.1	ND	21.5	13.0	57.2
HS 578T	>100	>100	>100	>100	66.0	25.3	31.5	15.2	13.9	22.1	55.7	37.9
MDA-MB-435	>100	>100	>100	>100	>100	15.8	21.2	7.31	7.14	19.0	13.5	94.2
MDA-N	>100	>100	>100	99.3	>100	13.4	13.8	7.80	8.90	16.3	17.6	>100
BT-549	23.4	82.2	>100	>100	ND	18.1	21.3	ND	5.45	14.9	22.6	31.1
T-47D	>100	>100	>100	>100	4.05	8.28	5.49	7.91	4.15	17.1	11.7	>100
MG-MID [d]	93.3	97.7	100	91.2	60.3	20.9	12.3	7.76	7.24	18.2	11.5	74.1

[a] Data obtained from NCI's in vitro disease-oriented tumour cells screen.
[b] GI_{50} is the molar concentration causing 50% growth inhibition of tumour cells. Compounds with GI_{50} >100 μM are considered inactive.
[c] ND, not determined.
[d] Mean panel values (mean graph midpoints MG-MID) of the response parameter were obtained by averaging the individual values for each cell line.

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Table 5
Growth inhibition (%) for the most active heterocyclichydrazones **1** and **2** against *A. fumigatus*

Compound	Concentration	
	50 μg/ml	100 μg/ml
1c	50	50
1d	42.9	50
1e	33	33
1f	33.3	100
1g	50	100
1h	50	100
1j	25	100
1k	50	100
1l	50	57
2f	33.3	100
2g	66.6	100
2h	50	100
2i	33.3	75
2k	33.3	50

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